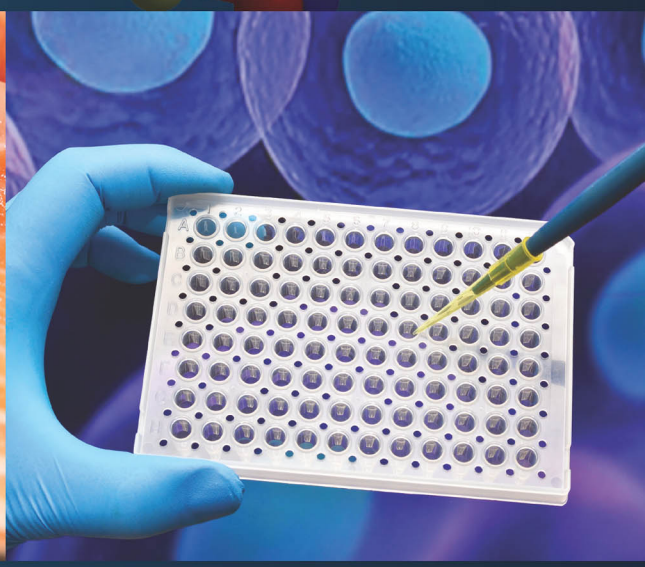
FOURTH EDITION

FOOD LIPIDS

Chemistry, Nutrition, and Biotechnology



Casimir C. Akoh

CRC Press
Taylor & Francis Group

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Preface

The first edition of *Food Lipids* was published in 1998 and the second edition in 2002 by Marcel Dekker, Inc. Taylor & Francis Group, LLC, acquired Marcel Dekker and the rights to publish the third and subsequent editions. I firmly believe that this book has provided those involved in lipid research and instruction with a valuable resource for materials and information. On behalf of my late former coeditor, Professor David B. Min, I thank all those who bought the previous editions and hope that you will find the fourth edition equally or more interesting and helpful. Before describing the content of the current edition, I would like to pay tribute to Professor Min, who worked very hard with me in seeing to the successful recruitment of potential authors and the publication of the previous editions. I miss him dearly and dedicate this fourth edition to his memory and his contributions to our knowledge on lipids, especially, lipid oxidation.

Taylor & Francis Group and I felt the need to update the information in the third edition and include more recent topics of interest to the readers and users of this text/reference book. We continue to believe, based on the sales of the previous editions and the comments of instructors and those who purchased and used the book, that this textbook is and will continue to be suitable for teaching food lipids, nutritional aspects of lipids, and lipid chemistry/biotechnology courses to food science and nutrition majors. The aim of the first, second, third, and fourth editions remains unchanged: to provide a modern, easy-to-read textbook for students and instructors and a reference book for professionals and practitioners with an interest in lipids. The book is also suitable for upper-level undergraduate, graduate, and postgraduate instruction. Scientists and professionals, who have left the university and are engaged in research and development in the industry, government, regulatory, or academics will find this book a useful reference. In this edition, I have deleted some chapters and renamed some chapters and/or had new authors rewrite some of the old chapters with updated references and added many new chapters to reflect current knowledge and interests. In other words, the new edition represents a substantial change from the previous editions. Section I increased from seven to nine chapters and Section II from three to seven, Section III decreased from eight to five, Section IV still has nine chapters but some are new, and Section V increased from five to seven. A great effort was made to recruit and select contributors who are internationally recognized experts. I thank the new authors and the prior authors who updated their chapters for their exceptional attention to detail and timely submission of their manuscripts.

Overall, there are 37 chapters in the fourth edition, and the text has been updated with new and available information. Again, some chapters were removed and new ones added. The new additions or modifications are Chapters 2, 6, 8 through 11, 13 through 17, 20, 22 through 24, 26, 28 through 32, 36, and 37. It is almost impossible to cover all aspects of lipids. I feel that with the added chapters, the book covered most topics that are of interest to our readers. The book is divided into five main sections: chemistry and properties, processing and food applications, oxidation and antioxidants, nutrition, and biotechnology and biochemistry.

I remain grateful to the readers and users of the previous editions and sincerely hope that the much improved and updated fourth edition will meet your satisfaction. Comments on this edition are welcomed. Based on the comments of readers and reviewers of the past editions, I hope that the current edition is an improvement with new chapters and new ways of studying and utilizing lipids to benefit our profession and consumers. I apologize in advance for any errors and urge you to contact me or the publisher if you find mistakes or have suggestions to improve the readability and comprehension of this text.

Special thanks go to our readers and to the editorial staff at Taylor & Francis Group, LLC, for their expertise, suggestions, and completing the publication on time.

Casimir C. Akoh

Editor

Casimir C. Akoh, a certified food scientist (2013), is a distinguished research professor of food science and technology and an adjunct professor of foods and nutrition at the University of Georgia, Athens. He is coeditor of the book *Carbohydrates as Fat Substitutes* (Marcel Dekker, Inc.); coeditor of *Healthful Lipids* (AOCS Press); editor of *Handbook of Functional Lipids* (CRC Press); coeditor of *Food Lipids* (first, second, and third editions); coeditor of *Palm Oil: Production, Processing, Characterization, and Uses* (AOCS Press); author or coauthor of more than 254 referenced SCI publications and more than 40 book chapters; and holder of three U.S. patents. He has made over 275 presentations and given more than 160 invited presentations at national and international conferences.

He is a fellow/WABAB Academician of the International Society of Biocatalysis and Agricultural Biotechnology (2015) and a fellow of the Institute of Food Technologists (2005), American Oil Chemists' Society (2006), the American Chemical Society (2006), a member of the International Society of Biocatalysis and Agricultural Biotechnology, and the Phi Tau Sigma Honorary Society (Honor Society for Food Science). He is currently an associate editor of the *Journal of the American Oil Chemists' Society* and the *Journal of Food Science*, serves on the editorial boards of the *European Journal of Lipid Research and Technology,* the *Journal of Biocatalysis and Agricultural Biotechnology,* and the *Annual Reviews of Food Science and Technology.*

He is a highly cited scientist in agricultural science and has received numerous national and international professional awards for his work on lipids, including the 1996 International Life Sciences Institute of North America Future Leader Award, the 1998 IFT Samuel Cate Prescott Award, 1999 Creative Research Medal Award, 2000 Gamma Sigma Delta Distinguished Senior Faculty Research Award, the 2003 D.W. Brooks Award, the 2004 AOCS Stephen S. Chang Award, the 2004 Distinguished Research Professor, the 2008 IFT Stephen S. Chang Award, the 2008 IFT Research and Development Award, the 2009 AOCS Biotechnology Division Lifetime Achievement Award, the 2012 IFT Nicholas Appert Award, the 2012 AOCS Supelco/Nicholas Pelick Research Award, and the 2015 University of Nigeria, Nsukka, Alumni Achievement Award.

He received his PhD (1988) in food science from Washington State University, Pullman. He holds MS and BS in biochemistry from Washington State University and the University of Nigeria, Nsukka, respectively.

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Section I

Chemistry and Properties

1 Nomenclature and Classification of Lipids

Sean Francis O'Keefe and Paul J. Sarnoski

CONTENTS

I. DEFINITIONS OF LIPIDS

No exact definition of lipids exists. Christie [1] defines lipids as "a wide variety of natural products including fatty acids and their derivatives, steroids, terpenes, carotenoids, and bile acids, which have in common a ready solubility in organic solvents such as diethyl ether, hexane, benzene, chloroform, or methanol."

Kates [2] says that lipids are "those substances which are (a) insoluble in water; (b) soluble in organic solvents such as chloroform, ether or benzene; (c) contain long-chain hydrocarbon groups in their molecules; and (d) are present in or derived from living organisms."

Gurr and James [3] point out that a standard definition describes lipids as "a chemically heterogeneous group of substances, having in common the property of insolubility in water, but solubility in nonpolar solvents such as chloroform, hydrocarbons or alcohols."

Despite its common usage, definitions based on solubility have obvious problems. Some compounds that are considered as lipids, such as C1–C4 very short-chain fatty acids (VSCFAs), are completely miscible with water and insoluble in nonpolar solvents. Some researchers have accepted this solubility definition strictly and exclude C1–C3 fatty acids from the definition of lipids, keeping C4 (butyric acid) only because of its presence in dairy fats. Additionally, some compounds that are considered as lipids, such as some *trans* fatty acids (those not derived from bacterial hydrogenation), are not derived directly from living organisms. The development of synthetic acaloric and reduced calorie lipids such as the sucrose polyester olestra (trade name Olean®) complicates the issue because these lipids may fit into solubility-based definitions but are not derived from living organisms, may be acaloric, and may contain esters of VSCFAs.

The traditional definition of total fat of foods used by the U.S. Food and Drug Administration (FDA) has been "the sum of the components with lipid characteristics that are extracted by Association of Official Analytical Chemists (AOAC) methods or by reliable and appropriate procedures." The FDA has changed from a solubility-based definition to "total lipid fatty acids expressed as triglycerides" [4], with the intent to measure caloric fatty acids. Solubility and size of fatty acids affect their caloric values. This is important for products that take advantage of the calorie-based definition, such as Benefat/Salatrim, so these products would be examined on a case-by-case basis. Food products containing sucrose polyesters would require special methodology to calculate caloric fatty acids. Foods containing vinegar (~4.5% acetic acid) present a problem because they will be considered to have 4.5% fat unless the definition is modified to exclude water-soluble fatty acids or the caloric weighting for acetic acid is lowered [4].

Despite the problems with accepted definitions, a more precise working definition is difficult, given the complexity and heterogeneity of lipids. This chapter introduces the main lipid structures and their nomenclature.

II. LIPID CLASSIFICATIONS

Classification of lipids is possible based on their physical properties at room temperature (oils are liquid and fats are solid), polarity (polar and neutral lipids), essentiality for humans (essential and nonessential fatty acids), or structure (simple or complex). Neutral lipids include fatty acids, alcohols, glycerides, and sterols, whereas polar lipids include glycerophospholipids and glyceroglycolipids. The separation into polarity classes is rather arbitrary, as some short-chain fatty acids are very polar. A classification based on structure is, therefore, preferable.

Based on structure, lipids can be classified as derived, simple, or complex. Derived lipids include free fatty acids and alcohols, which are the building blocks for the simple and complex lipids. Simple lipids, composed of fatty acids and alcohol components, include acylglycerols, ether acylglycerols, sterols, and their esters and wax esters. In general terms, simple lipids can be hydrolyzed to two different components, usually an alcohol and an acid. Complex lipids include glycerophospholipids (phospholipids), glyceroglycolipids (glycolipids), and sphingolipids. These structures yield three or more different compounds on hydrolysis.

The fatty acids constitute the obvious starting point in lipid structures. However, a short review of standard nomenclature is appropriate. Over the years, a large number of different nomenclature systems have been proposed [5]. The resulting confusion has led to a need for nomenclature standardization. The International Union of Pure and Applied Chemists (IUPAC) and International Union of Biochemistry (IUB) collaborative efforts have resulted in comprehensive nomenclature

standards [6], and the nomenclature for lipids has been reported [7–9]. Only the main aspects of the standardized IUPAC nomenclature relating to lipid structures will be presented; greater detail is available elsewhere [7–9].

Standard rules for nomenclature must take into consideration the difficulty in maintaining strict adherence to structure-based nomenclature and elimination of common terminology [5]. For example, the compound known as vitamin K_1 can be described as 2-methyl-3-phytyl-1,4-naphthoquinone. Vitamin K_1 and many other trivial names have been included into standardized nomenclature to avoid confusion arising from long chemical names. Standard nomenclature rules will be discussed in separate sections relating to various lipid compounds.

Fatty acid terminology is complicated by the existence of several different nomenclature systems. The IUPAC nomenclature, common (trivial) names, and shorthand (*n*- or ω) terminology will be discussed. As a lipid class, the fatty acids are often called free fatty acids (FFAs) or nonesterified fatty acids (NEFAs). IUPAC has recommended that fatty acids as a class be called fatty acids and the terms FFA and NEFA eliminated [6].

A. Standard IUPAC Nomenclature of Fatty Acids

In standard IUPAC terminology [6], the fatty acid is named after the parent hydrocarbon. Table 1.1 lists common hydrocarbon names. For example, an 18-carbon carboxylic acid is called octadecanoic acid, derived from octadecane, the 18-carbon aliphatic hydrocarbon. The name octadecanecarboxylic acid may also be used, but it is more cumbersome and less common. Table 1.2 summarizes the rules for hydrocarbon nomenclature.

Double bonds are designated using the Δ configuration, which represents the distance from the carboxyl carbon, naming the carboxyl carbon number 1. A double bond between the 9th and 10th carbons from the carboxylic acid group is a Δ9 bond. The hydrocarbon name changes to indicate the presence of the double bond; an 18-carbon fatty acid with one double bond is called octadecenoic acid, one with two double bonds is named octadecadienoic acid, etc. The double-bond

TABLE 1.1
Systematic Names of Hydrocarbons

Carbon Number	Name	Carbon Number	Name
1	Methane	19	Nonadecane
2	Ethane	20	Eicosane
3	Propane	21	Henicosane
4	Butane	22	Docosane
5	Pentane	23	Tricosane
6	Hexane	24	Tetracosane
7	Heptane	25	Pentacosane
8	Octane	26	Hexacosane
9	Nonane	27	Heptacosane
10	Decane	28	Octacosane
11	Hendecane	29	Nonacosane
12	Dodecane	30	Triacontane
13	Tridecane	40	Tetracontane
14	Tetradecane	50	Pentacontane
15	Pentadecane	60	Hexacontane
16	Hexadecane	70	Heptacontane
17	Heptadecane	80	Octacontane
18	Octadecane		

TABLE 1.2
IUPAC Rules for Hydrocarbon Nomenclature

1. Saturated unbranched acyclic hydrocarbons are named with a numerical prefix and the termination "ane." The first four in this series use trivial prefix names (methane, ethane, propane, and butane), whereas the rest use prefixes that represent the number of carbon atoms.
2. Saturated branched acyclic hydrocarbons are named by prefixing the side chain designation to the name of the longest chain present in the structure.
3. The longest chain is numbered to give the lowest number possible to the side chains, irrespective of the substituents.
4. If more than two side chains are present, they can be cited either in alphabetical order or in order of increasing complexity.
5. If two or more side chains are present in equivalent positions, the one assigned the lowest number is cited first in the name. Order can be based on alphabetical order or complexity.
6. Unsaturated unbranched acyclic hydrocarbons with one double bond have the "ane" replaced with "ene." If there is more than one double bond, the "ane" is replaced with "diene," "triene," "tetraene," etc. The chain is numbered to give the lowest possible number to the double bonds.

Source: IUPAC, *Nomenclature of Organic Chemistry, Sections A, B, C, D, E, F, and H*, Pergamon Press, London, U.K., 1979, p. 182.

positions are designated with numbers before the fatty acid name (Δ9-octadecenoic acid or simply 9-octadecenoic acid). The Δ is assumed and often not placed explicitly in structures.

Double-bond geometry is designated with the *cis–trans* or *E*/*Z* nomenclature systems [6]. The *cis*/*trans* terms are used to describe the positions of atoms or groups connected to doubly bonded atoms. They can also be used to indicate relative positions in ring structures. Atoms/groups are *cis* or *trans* if they lie on same (*cis*) or opposite (*trans*) sides of a reference plane in the molecule. Some examples are shown in Figure 1.1. The prefixes *cis* and *trans* can be abbreviated as *c* and *t* in structural formulas.

The *cis*/*trans* configuration rules are not applicable to double bonds that are terminal in a structure or to double bonds that join rings to chains. For these conditions, a sequence preference ordering must be conducted. Since *cis*/*trans* nomenclature is applicable only in some cases, a new nomenclature system was introduced by the Chemical Abstracts Service (CAS) and subsequently adopted by IUPAC (the *E*/*Z* nomenclature). This system was developed as a more applicable system to describe isomers by using sequence ordering rules, as is done using the *R*/*S* system (rules to decide which ligand has priority). The sequence rule–preferred atom/group attached to one of a pair of doubly bonded carbon atoms is compared with the sequence rule–preferred atom/group of the other of the doubly bonded carbon atoms. If the preferred atom/groups are on the same side of the reference plane, it is the *Z* configuration. If they are on the opposite sides of the plane, it is the *E* configuration. Table 1.3 summarizes some of the rules for sequence preference [10]. Although *cis* and *Z* (or *trans* and *E*) do not always refer to the same configurations, for most fatty acids *E* and *trans* are equivalent, as are *Z* and *cis*.

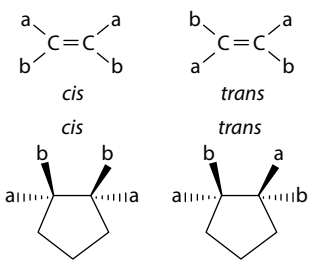


FIGURE 1.1 Examples of *cis*/*trans* nomenclature.

TABLE 1.3
Summary of Sequence Priority Rules for *E/Z* Nomenclature

1. Higher atomic number precedes lower.
2. For isotopes, higher atomic mass precedes lower.
3. If the atoms attached to one of the double-bonded carbons are the same, proceed outward concurrently until a point of difference is reached considering atomic mass and atomic number.
4. Double bonds are treated as if each bonded atom is duplicated.

Source: Streitwieser Jr., A. and Heathcock, C.H., *Introduction to Organic Chemistry*, Macmillan, New York, 1976, p. 111.

B. Common (Trivial) Nomenclature of Fatty Acids

Common names have been introduced throughout the years and, for certain fatty acids, are a great deal more common than standard (IUPAC) terminology. For example, oleic acid is much more common than *cis*-9-octadecenoic acid. Common names for saturated and unsaturated fatty acids are illustrated in Tables 1.4 and 1.5. Many of the common names originate from the first identified

TABLE 1.4
Systematic, Common, and Shorthand Names of Saturated Fatty Acids

Systematic Name	Common Name	Shorthand
Methanoic	Formic	1:0
Ethanoic	Acetic	2:0
Propanoic	Propionic	3:0
Butanoic	Butyric	4:0
Pentanoic	Valeric	5:0
Hexanoic	Caproic	6:0
Heptanoic	Enanthic	7:0
Octanoic	Caprylic	8:0
Nonanoic	Pelargonic	9:0
Decanoic	Capric	10:0
Undecanoic	—	11:0
Dodecanoic	Lauric	12:0
Tridecanoic	—	13:0
Tetradecanoic	Myristic	14:0
Pentadecanoic	—	15:0
Hexadecanoic	Palmitic	16:0
Heptadecanoic	Margaric	17:0
Octadecanoic	Stearic	18:0
Nonadecanoic	—	19:0
Eicosanoic	Arachidic	20:0
Docosanoic	Behenic	22:0
Tetracosanoic	Lignoceric	24:0
Hexacosanoic	Cerotic	26:0
Octacosanoic	Montanic	28:0
Tricontanoic	Melissic	30:0
Dotriacontanoic	Lacceroic	32:0

TABLE 1.5
Systematic, Common, and Shorthand Names of Unsaturated Fatty Acids

Systematic Name	Common Name	Shorthand
c-9-Dodecenoic	Lauroleic	12:1ω3
c-5-Tetradecenoic	Physeteric	14:1ω9
c-9-Tetradecenoic	Myristoleic	14:1ω5
c-9-Hexadecenoic	Palmitoleic	16:1ω7
c-7,*c*-10,*c*-13-Hexadecatrienoic	—	16:3ω3
c-4,*c*-7,*c*-10,*c*-13-Hexadecatetraenoic	—	16:4ω3
c-9-Octadecenoic	Oleic	18:1ω9
c-11-Octadecenoic	*cis*-Vaccenic (Asclepic)	18:1ω7
t-11-Octadecenoic	Vaccenic	[a]
t-9-Octadecenoic	Elaidic	[a]
c-9,*c*-12-Octadecadienoic	Linoleic	18:2ω6
c-9-*t*-11-Octadecadienoic acid	Rumenic[b]	[a]
c-9,*c*-12,*c*-15-Octadecatrienoic	Linolenic	18:3ω3
c-6,*c*-9,*c*-12-Octadecatrienoic	γ-Linolenic	18:3ω6
c-6,*c*-9,*c*-12,*c*-15-Octadecatetraenoic	Stearidonic	18:4ω3
c-11-Eicosenoic	Gondoic	20:1ω9
c-9-Eicosenoic	Gadoleic	20:1ω11
c-8,*c*-11,*c*-14-Eicosatrienoic	Dihomo-γ-linolenic	20:3ω6
c-5,*c*-8,*c*-11-Eicosatrienoic	Mead's	20:3ω9
c-5,*c*-8,*c*-11,*c*-14-Eicosatetraenoic	Arachidonic	20:4ω6
c-5,*c*-8,*c*-11,*c*-14,c-17-Eicosapentaenoic	Eicosapentaenoic	20:5ω3
c-13-Docosenoic	Erucic	22:1ω9
c-11-Docosenoic	Cetoleic	22:1ω11
c-7,*c*-10,*c*-13,*c*-16,*c*-19-Docosapentaenoic	DPA, Clupanodonic	22:5ω3
c-4,*c*-7,*c*-10,*c*-13,*c*-16,*c*-19-Docosahexaenoic	DHA, Cervonic	22:6ω3
c-15-Tetracosenoic	Nervonic (Selacholeic)	24:1ω9

[a] Shorthand nomenclature cannot be used to name *trans* fatty acids.
[b] One of the conjugated linoleic acid (CLA) isomers.

botanical or zoological origins for those fatty acids. Myristic acid is found in seed oils from the Myristicaceae family. Mistakes have been memorialized into fatty acid common names; margaric acid (heptadecanoic acid) was once incorrectly thought to be present in margarine. Some of the common names can pose memorization difficulties, such as the following combinations: caproic, caprylic, and capric; arachidic and arachidonic; linoleic, linolenic, γ-linolenic, and dihomo-γ-linolenic. Even more complicated is the naming of EPA, or eicosapentaenoic acid, usually meant to refer to *c*-5,*c*-8,*c*-11,*c*-14,*c*-17-eicosapentaenoic acid, a fatty acid found in fish oils. However, a different isomer *c*-2,*c*-5,*c*-8,*c*-11,*c*-14-eicosapentaenoic acid is also found in nature. Both can be referred to as eicosapentaenoic acids using standard nomenclature. Nevertheless, in common nomenclature, EPA refers to the *c*-5,*c*-8,*c*-11,*c*-14,*c*-17 isomer. Docosahexaenoic acid (DHA) refers to all-*cis* 4,7,10,13,16,19-docosahexaenoic acid.

C. Shorthand (ω, *n*-) Nomenclature of Fatty Acids

Shorthand (*n*- or ω) identifications of fatty acids are found in common usage. The shorthand designation is the carbon number in the fatty acid chain followed by a colon, then the number of double bonds and the position of the double bond closest to the methyl end of the fatty acid molecule.

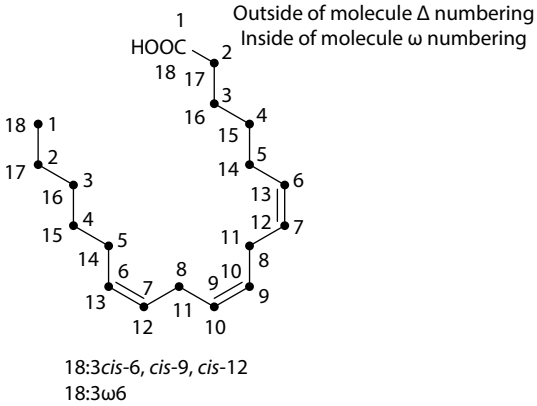


FIGURE 1.2 IUPAC Δ and common ω numbering systems.

The methyl group is number 1 (the last character in the Greek alphabet is ω, hence the end). In shorthand notation, the unsaturated fatty acids are assumed to have *cis* bonding and, if the fatty acid is polyunsaturated, double bonds are in the methylene-interrupted positions (Figure 1.2). In this example, CH_2 (methylene) groups at Δ8 and Δ11 interrupt what would otherwise be a conjugated double-bond system.

Shorthand terminology cannot be used for fatty acids with *trans* or acetylene bonds, for those with additional functional groups (branched, hydroxy, etc.), or for double-bond systems (≥2 double bonds) that are not methylene interrupted (isolated or conjugated). Despite the limitations, shorthand terminology is very popular because of its simplicity and because most of the fatty acids of nutritional importance can be named using this system. Sometimes, ω is replaced by *n*- (18:2*n*-6 instead of 18:2ω6). Although there have been recommendations to eliminate ω and use *n*- exclusively [6], both *n*- and ω are commonly used in the literature and are equivalent.

Shorthand designations for polyunsaturated fatty acids (PUFAs) are sometimes reported without the ω term (18:3). However, this notation is ambiguous, since 18:3 could represent 18:3ω1, 18:3ω3, 18:3ω6, or 18:3ω9 fatty acids, which are completely different in their origins and nutritional significance. Two or more fatty acids with the same carbon and double-bond numbers are possible in many common oils. Therefore, the ω terminology should always be used with the ω term specified.

III. LIPID CLASSES

A. Fatty Acids

1. Saturated Fatty Acids

The saturated fatty acids begin with methanoic (formic) acid. Methanoic, ethanoic, and propanoic acids are uncommon in natural fats and are often omitted from definitions of lipids. However, they are found nonesterified in many food products. Omitting these fatty acids because of their water solubility would make the case for also eliminating butyric acid, which would be difficult given its importance in dairy fats. The simplest solution is to accept the very short-chain carboxylic acids as fatty acids while acknowledging the rarity in natural fats of these water-soluble compounds. The systematic, common, and shorthand designations of some saturated fatty acids are given in Table 1.4.

2. Unsaturated Fatty Acids

By far, the most common monounsaturated fatty acid is oleic acid (18:1ω9), although more than 100 monounsaturated fatty acids have been identified in nature. The most common double-bond

position for monoenes is Δ9. However, certain families of plants have been shown to accumulate what would be considered unusual fatty acid patterns. For example, *Eranthis* seed oil contains Δ5 monoenes and nonmethylene-interrupted PUFAs containing Δ5 bonds [11]. Erucic acid (22:1ω9) is found at high levels (40%–50%) in Cruciferae such as rapeseed and mustard seed. Canola is a rapeseed oil that is low in erucic acid (<2% 22:1ω9) and low in glucosinolates.

PUFAs are best described in terms of families because of the metabolism that allows interconversion within, but not among, families of PUFA. The essentiality of ω6 and ω3 fatty acids has been known since the late 1920s. Signs of ω6 fatty acid deficiency include decreased growth, increased epidermal water loss, impaired wound healing, and impaired reproduction [12,13]. Early studies did not provide clear evidence that ω3 fatty acids are essential. However, since the 1970s, evidence has accumulated illustrating the essentiality of the ω3 PUFA.

Not all PUFAs are essential fatty acids (EFAs). Plants are able to synthesize *de novo* and interconvert ω3 and ω6 fatty acid families via desaturases with specificity in the Δ12 and Δ15 positions. Animals have Δ5, Δ6, and Δ9 desaturase enzymes and are unable to synthesize the ω3 and ω6 PUFAs *de novo*. However, extensive elongation and desaturation of EFA occurs (primarily in the liver). The elongation and desaturation of 18:2ω6 is illustrated in Figure 1.3. The most common of the ω6 fatty acids in our diets is 18:2ω6. Often considered the parent of the ω6 family, 18:2ω6 is first desaturated to 18:3ω6. The rate of this first desaturation is thought to be limiting in premature infants, in the elderly, and under certain disease states. Thus, a great deal of interest has been placed in the few oils that contain 18:3ω6, γ-linolenic acid (GLA). Relatively rich sources of GLA include black currant, evening primrose, and borage oils. GLA is elongated to 20:3ω6, dihomo-γ-linolenic acid (DHGLA). DHGLA is the precursor molecule to the 1-series prostaglandins. DHGLA is further desaturated to 20:4ω6, precursor to the 2-series prostaglandins. Further elongation and desaturation to 22:4ω6 and 22:5ω6 can occur, although the exact function of these fatty acids remains obscure. Relatively high levels of these fatty acids are found in caviar from wild but not cultured sturgeon.

Figure 1.4 illustrates analogous elongation and desaturation of 18:3ω3. The elongation of 20:5ω3 to 22:5ω3 was thought for many years to be via Δ4 desaturase. The inexplicable difficulty in identifying and isolating the putative Δ4 desaturase led to the conclusion that it did not exist, and the pathway from 20:5ω3 to 22:6ω3 was elucidated as a double elongation, desaturation, and β-oxidation.

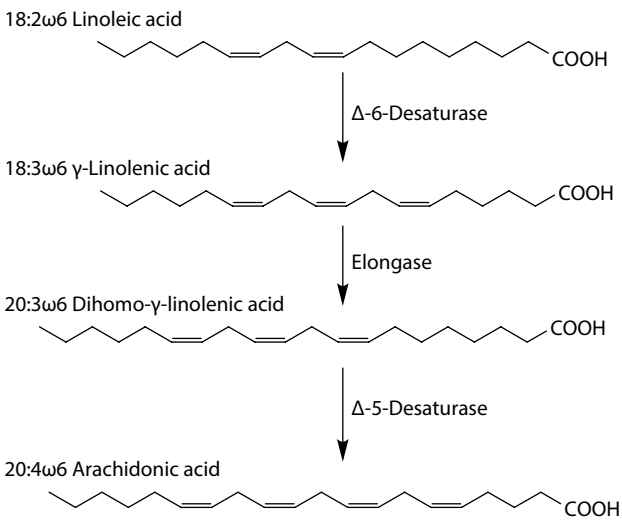


FIGURE 1.3 Pathway of 18:2ω6 metabolism to 20:4ω6.

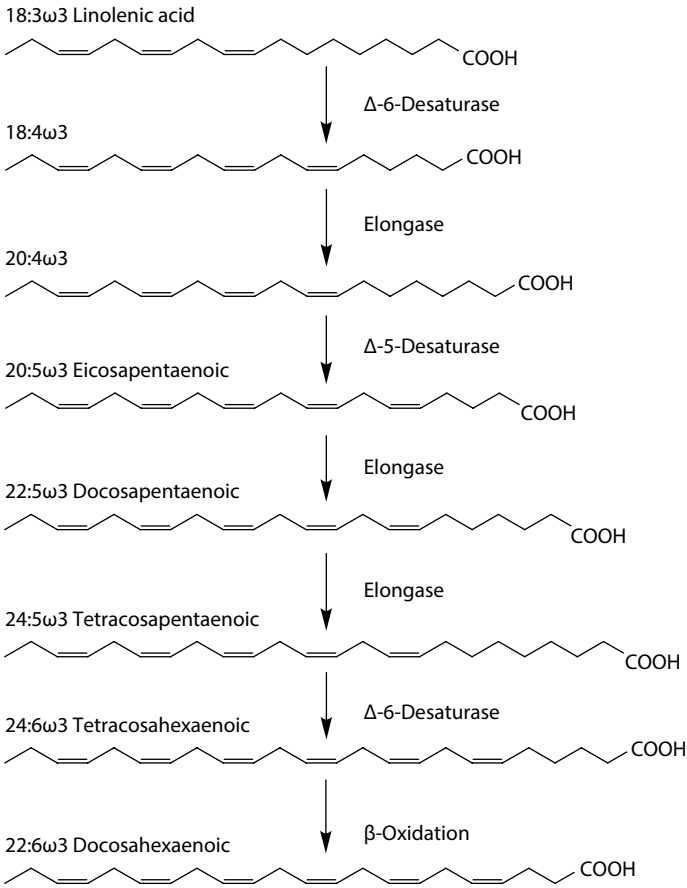


FIGURE 1.4 Pathway of 18:3ω3 metabolism to 22:6ω3.

One of the main functions of the EFAs is their conversion to metabolically active prostaglandins and leukotrienes [14,15]. Examples of some of the possible conversions from 20:4ω6 are shown in Figures 1.5 and 1.6 [15]. The prostaglandins are called eicosanoids as a class and originate from the action of cyclooxygenase on 20:4ω6 to produce PGG_2. The standard nomenclature of prostaglandins allows usage of the names presented in Figure 1.5. For a name such as PGG_2, the PG represents prostaglandin, the next letter (G) refers to its structure (Figure 1.7), and the subscript number refers to the number of double bonds in the molecule.

The parent structure for most of the prostaglandins is prostanoic acid (Figure 1.7) [14]. Thus, the prostaglandins can be named based on this parent structure. In addition, they can be named using standard nomenclature rules. For example, prostaglandin E_2 (PGE_2) is named (5*Z*,11α,13*E*,15*S*)-11,15-dihydroxy-9-oxoprosta-5,13-dienoic acid using the prostanoic acid template. It can also be named using standard nomenclature as 7-[3-hydroxy-2-(3-hydroxy-1-octenyl)-5-oxocyclopentyl]-*cis*-5-heptenoic acid.

The leukotrienes are produced from 20:4ω6 via 5-, 12-, or 15-lipoxygenases to a wide range of metabolically active molecules. The nomenclature is shown in Figure 1.6.

It is important to realize that there are 1-, 2-, and 3-series prostaglandins originating from 20:3ω6, 20:4ω6, and 20:5ω3, respectively. The structures of the 1-, 2-, and 3-prostaglandins differ by the removal or addition of the appropriate double bonds. Leukotrienes of the 3-, 4-, and 5-series are formed via lipoxygenase activity on 20:3ω6, 20:4ω6, and 20:5ω3. A great deal of interest has

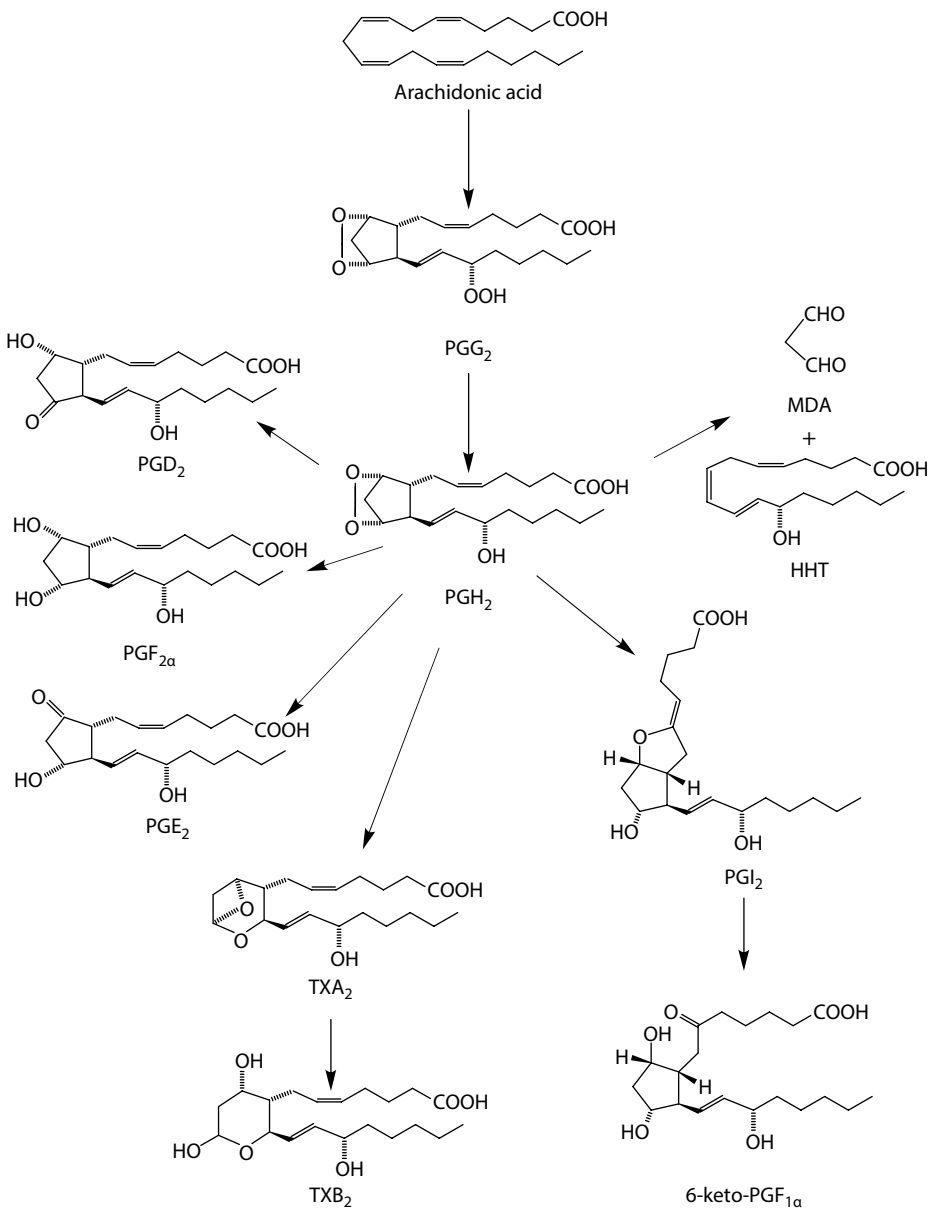


FIGURE 1.5 Prostaglandin metabolites of 20:4ω6.

been focused on changing proportions of the prostaglandins and leukotrienes of the various series by diet to modulate various diseases.

3. Acetylenic Fatty Acids

A number of different fatty acids have been identified having triple bonds [16]. The nomenclature is similar to double bonds, except that the -ane ending of the parent alkane is replaced with -ynoic acid, -diynoic acid, etc.

Shorthand nomenclature uses a lowercase "a" to represent the acetylenic bond; 9*c*,12a-18:2 is an octadecynoic acid with a double bond in position 9 and the triple bond in position 12. Figure 1.8 shows the common names and standard nomenclature for some acetylenic fatty acids. Since the

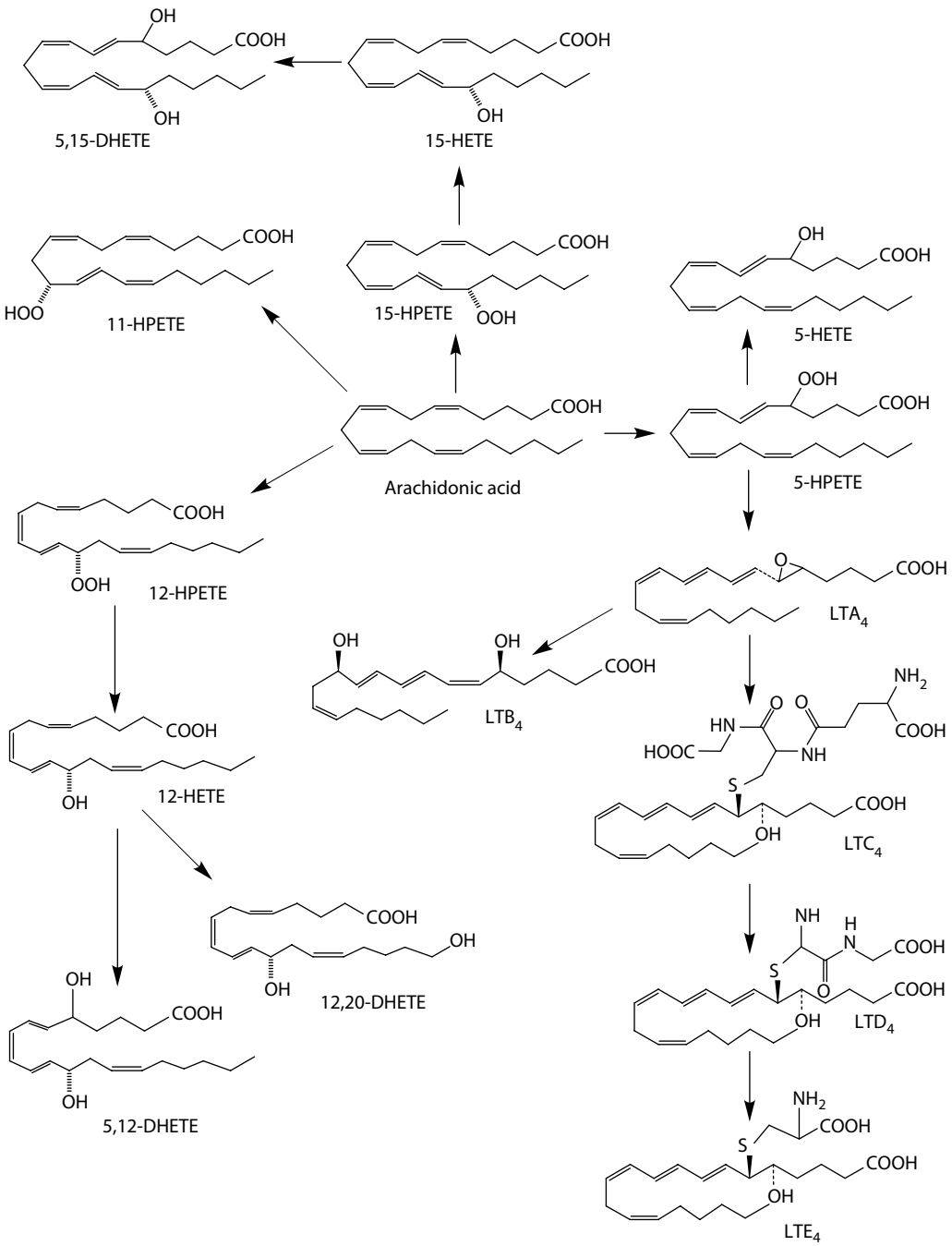


FIGURE 1.6 Leukotriene metabolites of 20:4ω6.

ligands attached to triple-bonded carbons are 180° from one another (the structure through the bond is linear), the second representation in Figure 1.8 is more accurate.

The acetylenic fatty acids found in nature are usually 18-carbon molecules with unsaturation starting at Δ9 consisting of conjugated double–triple bonds [9,16]. Overall, acetylenic fatty acids are rare in nature.

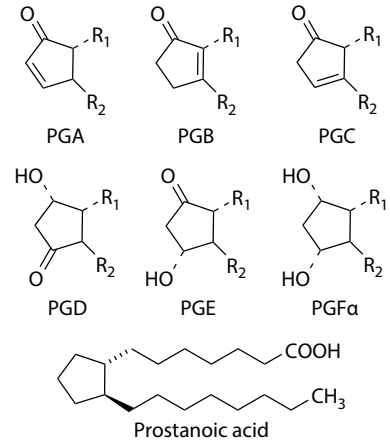


FIGURE 1.7 Prostanoic acid and prostaglandin ring nomenclature.

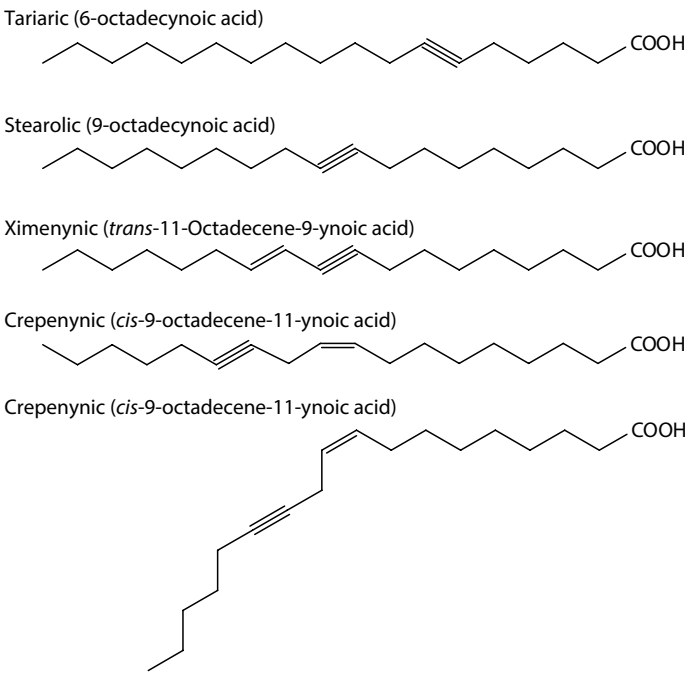


FIGURE 1.8 Some acetylenic acid structures and nomenclature.

4. *Trans* Fatty Acids

Trans fatty acids include any unsaturated fatty acid that contains double-bond geometry in the E (*trans*) configuration. Nomenclature differs from normal *cis* fatty acids only in the configuration of the double bonds.

The three main origins of *trans* fatty acids in our diet are from bacteria, deodorized oils, and partially hydrogenated oils. The preponderance of *trans* fatty acids in our diets is derived from the hydrogenation process.

Hydrogenation is used to stabilize and improve the oxidative stability of oils and to create plastic fats from oils [17]. The isomers that are formed during hydrogenation depend on the nature and

amount of catalyst, the extent of hydrogenation, and other factors. The identification of the exact composition of a partially hydrogenated oil is extremely complicated and time-consuming. The partial hydrogenation process produces a mixture of positional and geometrical isomers. Identification of the fatty acid isomers in a hydrogenated menhaden oil has been described [18]. The 20:1 isomers originally present in the unhydrogenated oil were predominantly *cis*-Δ11 (73% of total 20:1) and *cis*-Δ13 (15% of total 20:1). After hydrogenation from an initial iodine value of 159–96.5, the 20:1 isomers were distributed broadly across the molecules from Δ3 to Δ17 (Figure 1.9). The major *trans* isomers were Δ11 and Δ13, whereas the main *cis* isomers were Δ6, Δ9, and Δ11. Similar broad ranges of isomers are produced in hydrogenated vegetable oils [17].

Geometrical isomers of essential fatty acids linoleic and linolenic were first reported in deodorized rapeseed oils [19]. The geometrical isomers that result from deodorization are found in vegetable oils and products made from vegetable oils (infant formulas) and include 9*c*,12*t*-18:2; 9*t*,12*c*-18:2; and 9*t*,12*t*-18:2, as well as 9*c*,12*c*,15*t*-18:3; 9*t*,12*c*,15*c*-18:3; 9*c*,12*t*,15*c*-18:3; and 9*t*,12*c*,15*t*-18:3 [19–22]. These *trans*-EFA isomers have been shown to have altered biological effects and are incorporated into nervous tissue membranes [23,24], although the importance of these findings has not been elucidated. Geometrical isomers of long-chain v3 fatty acids have been identified in deodorized fish oils.

A mounting body of scientific evidence has linked industrially produced *trans* fats to elevated levels of cholesterol and a major contributor of heart disease [25,26]. The scientific evidence against *trans* fats has led the U.S. FDA to make a decision in June 2015 to remove generally recognized as safe (GRAS) status from industrial *trans* fats. Food companies must remove *trans* fats from their food products by June 2018, although food with less than 0.5 g of *trans* fat per serving can be labeled 0 g of *trans* fat [26]. The ban of *trans* fats extends only to industrially produced *trans* fats as naturally occurring *trans* fats such as ruminant *trans* fatty acids are exempt from the ban.

Trans fatty acids are formed naturally by some bacteria, primarily under anaerobic conditions [27]. It is believed that the formation of *trans* fatty acids in bacterial cell membranes is an adaptation

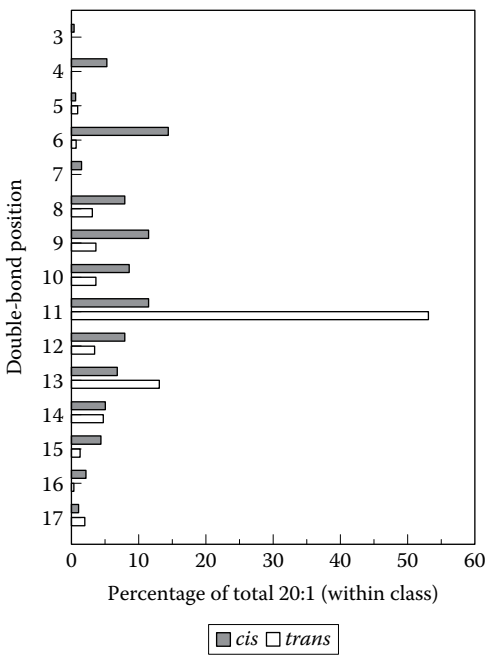


FIGURE 1.9 Eicosanoid isomers in partially hydrogenated menhaden oil. (From Sebedio, J.L. and Ackman, R.G., *J. Am. Oil Chem. Soc.*, 60, 1986, 1983.)

response to decrease membrane fluidity, perhaps as a reaction to elevated temperature or stress from solvents or other lipophilic compounds that affect membrane fluidity.

Not all bacteria produce appreciable levels of *trans* fatty acids. The *trans*-producing bacteria are predominantly gram negative and produce *trans* fatty acids under anaerobic conditions. The predominant formation of *trans* is via double-bond migration and isomerization, although some bacteria appear to be capable of isomerization without bond migration. The action of bacteria in the anaerobic rumen results in biohydrogenation of fatty acids and results in *trans* fatty acid formation in dairy fats (2%–6% of total fatty acids). The double-bond positions of the *trans* acids in dairy fats are predominantly in the Δ11 position, with smaller amounts in Δ9, Δ10, Δ13, and Δ14 positions [28].

5. Branched Fatty Acids

A large number of branched fatty acids have been identified [16]. The fatty acids can be named according to rules for branching in hydrocarbons (Table 1.2). Besides standard nomenclature, several common terms have been retained, including iso-, with a methyl branch on the penultimate (ω2) carbon, and anteiso, with a methyl branch on the antepenultimate (ω3) carbon. The iso and anteiso fatty acids are thought to originate from a modification of the normal *de novo* biosynthesis, with acetate replaced by 2-methyl propanoate and 2-methylbutanoate, respectively [16]. Other branched fatty acids are derived from isoprenoid biosynthesis including pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) and phytanic acid (3,7,11,15-tetramethylhexadecanoic acid).

6. Cyclic Fatty Acids

Some fatty acids that exist in nature contain cyclic carbon rings [29]. Ring structures contain either three (cyclopropyl and cyclopropenyl), five (cyclopentenyl), or six (cyclohexenyl) carbon atoms and may be saturated or unsaturated. Also, cyclic fatty acid structures resulting from heating the vegetable oils have been identified [29–31].

In nomenclature of cyclic fatty acids, the parent fatty acid is the chain from the carboxyl group to the ring structure. The ring structure and additional ligands are considered a substituent of the parent fatty acid. An example is given in Figure 1.10. The parent in this example is nonanoic acid (not pentadecanoic acid, which would result if the chain were extended through the ring structure). The substituted group is a cyclopentyl group with a 2-butyl ligand (2-butylcyclopentyl). Thus, the correct standard nomenclature is 9-(2-butylcyclopentyl)nonanoic acid. The 2 is sometimes

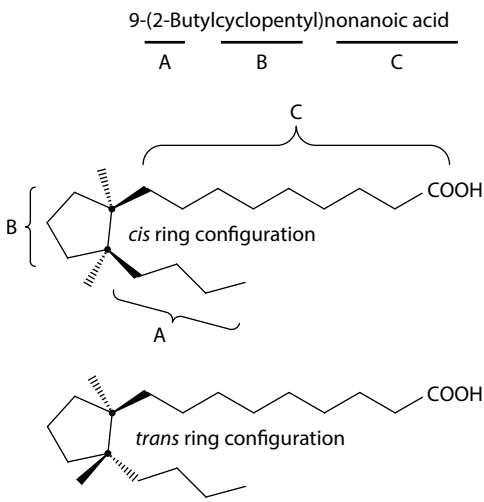
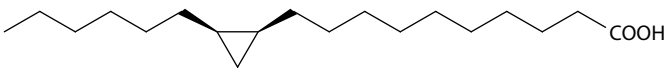


FIGURE 1.10 Nomenclature of cyclic fatty acids.

expressed as 2′ to indicate that the numbering is for the ring and not for the parent chain. The C-1 and C-2 carbons of the cyclopentyl ring are chiral, and two possible configurations are possible. Both the carboxyl and the longest hydrocarbon substituents can be on the same side of the ring, or they can be on opposite sides. These are referred to as *cis* and *trans*, respectively.

The cyclopropene and cyclopropane fatty acids can be named by means of the standard nomenclature noted in the previous example. They are also commonly named using the parent structure that carries through the ring structure. In the example in Figure 1.11, the fatty acid (commonly named lactobacillic acid or phycomonic acid) is named 10-(2-hexylcyclopropyl) decanonic acid in standard nomenclature. An older naming system would refer to this fatty acid as *cis*-11,12-methyleneoctadecanoic acid, where *cis* designates the configuration of the ring structure. If the fatty acid is unsaturated, the term "methylene" is retained but the double-bond position is noted in the parent fatty acid structure (*cis*-11,12-methylene-*cis*-octadec-9-enoic acid) (Figure 1.12).

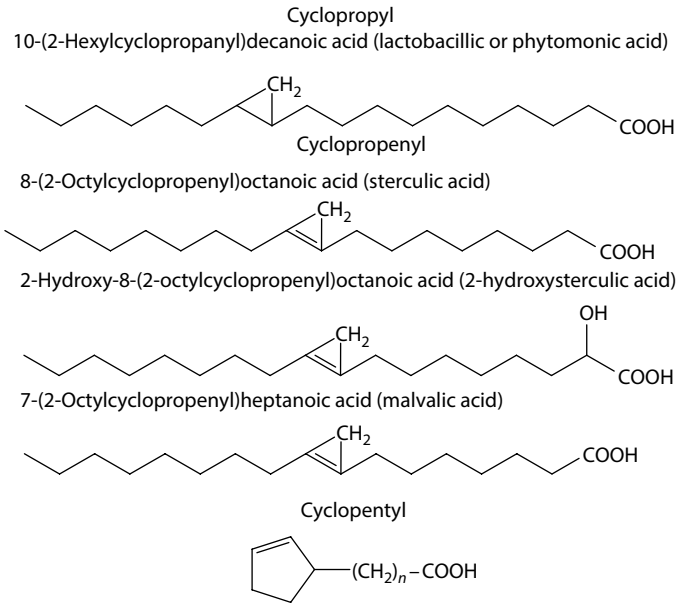


cis-11, 12-Methyleneoctadecanoic acid

cis-10-(2-Hexylcyclopropyl)decanoic acid

Lactobacillic acid / phytomonic acid

FIGURE 1.11 Nomenclature for a cyclopropenoid fatty acid.



n =

2 3-(2-Cyclopentenyl)propanoic acid (aleprolic acid)
4 5-(2-Cyclopentenyl)pentanoic acid (aleprestic acid)
6 7-(2-Cyclopentenyl)heptanoic acid (aleprylic acid)
8 9-(2-Cyclopentenyl)nonanoic acid (alepric acid)
10 11-(2-Cyclopentenyl)undecanoic acid (hyndocarpic acid)
12 13-(2-Cyclopentenyl)tridecanoic acid (chaulmoogric acid)

FIGURE 1.12 Cyclic fatty acid structures and nomenclature.

7. Hydroxy and Epoxy Fatty Acids

Saturated and unsaturated fatty acids containing hydroxy and epoxy functional groups have been identified [1,16]. Hydroxy fatty acids are named by means of the parent fatty acid and the hydroxy group numbered with its Δ location. For example, the fatty acid with the trivial name ricinoleic (Figure 1.13) is named *R*-12-hydroxy-*cis*-9-octadecenoic acid. Ricinoleic acid is found in the seeds of *Ricinus* species and accounts for about 90% of the fatty acids in castor bean oil.

Because the hydroxy group is chiral, stereoisomers are possible. The *R*/*S* system is used to identify the exact structure of the fatty acid. Table 1.6 reviews the rules for *R*/*S* nomenclature. The *R*/*S* system can be used instead of the α/β and *cis*/*trans* nomenclature systems. A fatty acid with a hydroxy substituent in the Δ2 position is commonly called an α-hydroxy acid; fatty acids with hydroxy substituents in the Δ3 and Δ4 positions are called β-hydroxy acids and γ-hydroxy acids, respectively. Some common hydroxy acids are shown in Figure 1.13. Cutins, which are found in the outer layer of fruit skins, are composed of hydroxy acid polymers, which may also contain epoxy groups [16].

Epoxy acids, found in some seed oils, are formed on prolonged storage of seeds [16]. They are named similarly to cyclopropane fatty acids, with the parent acid considered to have a substituted oxirane substituent. An example of epoxy fatty acids and their nomenclature is shown in Figure 1.14. The fatty acid with the common name vernolic acid is named (using standard nomenclature) 11-(3-pentyloxyranyl)-9-undecenoic acid. In older nomenclature, where the carbon chain is carried through the oxirane ring, vernolic acid would be called 12,13-epoxyoleic acid or 12-13-epoxy-9-octadecenoic acid. The configuration of the oxirane ring substituents can be named in the *cis*/*trans*, *E*/*Z*, or *R*/*S* configuration systems.

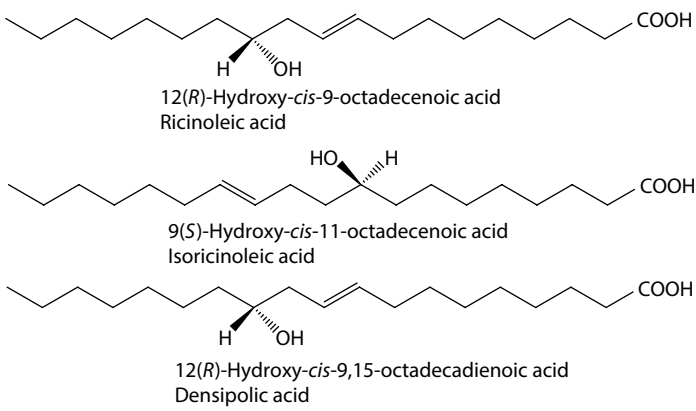
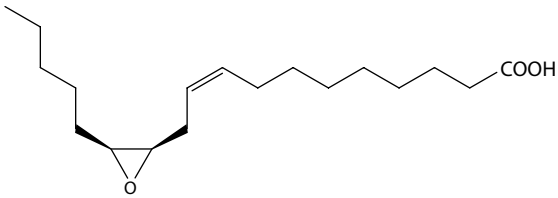


FIGURE 1.13 Hydroxy fatty acid structures and nomenclature.

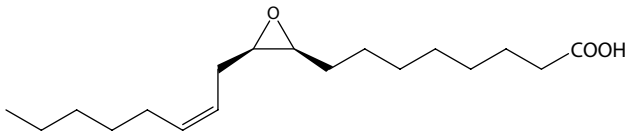
TABLE 1.6
Summary of Rules for *R*/*S* Nomenclature

1. The sequence priority rules (Table 1.3) are used to prioritize the ligands attached to the chiral center ($a > b > c > d$).
2. The molecule is viewed with the *d* substituent facing away from the viewer.
3. The remaining three ligands (*a*, *b*, *c*) will be oriented with the order *a*–*b*–*c* in a clockwise or counterclockwise direction.
4. Clockwise describes the *R* (*rectus*, right) conformation, and counterclockwise describes the *S* (*sinister*, left) conformation.

Source: Streitwieser Jr., A. and Heathcock, C.H., *Introduction to Organic Chemistry*, Macmillan, New York, 1976, p. 111.



11-(3-Pentyloxiranyl)-9-undecanoic acid
cis-12-13-Epoxy-*cis*-9-octadecenoic acid
Vernolic acid
Both the (+) 12*S*, 13*R* and (–) 12*R*, 13*S* forms are found in nature



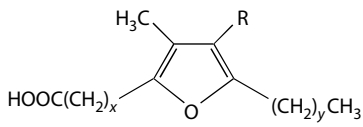
8-(3-*cis*-2′-Nonenyloxiranyl)-octanoic acid
cis-9,10-Epoxy-*cis*-12-octadecenoic acid
Coronaric acid

FIGURE 1.14 Epoxy fatty acid structures and nomenclature.

8. Furanoid Fatty Acids

Some fatty acids contain an unsaturated oxolane heterocyclic group. There are more commonly called furanoid fatty acids because a furan structure (diunsaturated oxolane) is present in the molecule. Furanoid fatty acids have been identified in *Exocarpus* seed oils. They have also been identified in plants, algae, and bacteria and are a major component in triacylglycerols (TAGs) from latex rubber [1,16]. They are important in marine oils and may total several percentage points or more of the total fatty acids in liver and testes [1,32].

Furanoid fatty acids have a general structure as shown in Figure 1.15. A common nomenclature describing the furanoid fatty acids (as F1, F2, etc.) is used [32]. The naming of the fatty acids in this nomenclature is arbitrary and originated from elution order in gas chromatography. A shorthand



Name	*x*	*y*	*R*
F1	8	2	CH_3
F2	8	4	H
F3	8	4	CH_3
F4	10	2	CH_3
F5	10	4	H
F6	10	4	CH_3
F7	12	4	H
F8	12	4	CH_3

FIGURE 1.15 Furanoid fatty acid structure and shorthand nomenclature.

notation that is more descriptive gives the methyl substitution followed by F and then the carbon lengths of the carboxyl and terminal chains in parentheses: MeF(9,5). Standard nomenclature follows the same principles outlined in Section III.A.6. The parent fatty acid chain extends only to the furan structure, which is named as a ligand attached to the parent molecule. For example, the fatty acid named F5 in Figure 1.15 is named 11-(3,4-dimethyl-5-pentyl-2-furyl)undecanoic acid. Shorthand notation for this fatty acid would be F_5 or MeF(11,5). Numbering for the furan ring starts at the oxygen and proceeds clockwise.

B. Acylglycerols

Acylglycerols are the predominant constituent in oils and fats of commercial importance. Glycerol can be esterified with one, two, or three fatty acids, and the individual fatty acids can be located on the three different carbons of glycerol. The terms *monoacylglycerol*, *diacylglycerol*, and *triacylglycerol* (TAG) are preferred for these compounds over the older and confusing names mono-, di-, and triglycerides [6,7].

Fatty acids can be esterified on the primary or secondary hydroxyl groups of glycerol. Although glycerol itself has no chiral center, it becomes chiral if different fatty acids are esterified to the primary hydroxyls or if one of the primary hydroxyls is esterified. Thus, terminology must differentiate between the two possible configurations (Figure 1.16). The most common convention to differentiate these stereoisomers is the *sn* convention of Hirshmann (see Reference 33). In the numbering that describes the hydroxyl groups on the glycerol molecule in Fisher projection, *sn*1, *sn*2, and *sn*3 designations are used for the top (C1), middle (C2), and bottom (C3) OH groups (Figure 1.17). The *sn* term indicates stereospecific numbering [1].

In common nomenclature, esters are named α on primary and β on secondary OH groups. If the two primary-bonded fatty acids are present, the primary carbons are called α and α′. If one or two acyl groups are present, the term "partial glyceride" is sometimes used. Nomenclature of the common partial glycerides is shown in Figure 1.18.

Standard nomenclature allows several different names for each TAG [6]. A TAG with three stearic acid esters can be named as glycerol tristearate, tristearoyl glycerol, or tri-*O*-stearoyl glycerol. The *O* locant can be omitted if the fatty acid is esterified to the hydroxyl group. More commonly,

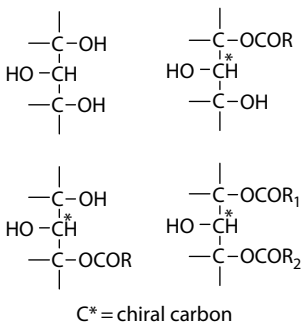


FIGURE 1.16 Chiral carbons in acylglycerols.

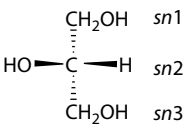


FIGURE 1.17 Stereospecific numbering (*sn*) of triacylglycerols.

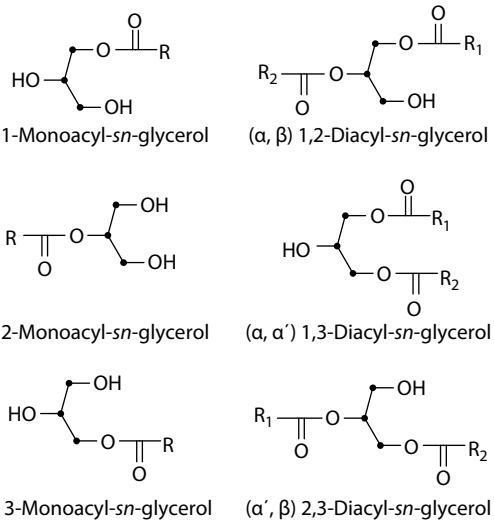


FIGURE 1.18 Mono- and diacylglycerol structures.

TAG nomenclature uses the designation -in to indicate the molecule in a TAG (e.g., tristearin). If different fatty acids are esterified to the TAG—for example, the TAG with *sn*-1 palmitic acid, *sn*-2 oleic acid, and *sn*-3 stearic acid—the name replaces the -ic in the fatty acid name with -oyl, and fatty acids are named in *sn*1, *sn*2, and *sn*3 order (1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol). This TAG can also be named as *sn*-1-palmito-2-oleo-3-stearin or *sn*-glycerol-1-palmitate-2-oleate-3-stearate. If two of the fatty acids are identical, the name incorporates the designation di- (e.g., 1,2-dipalmitoyl-3-oleoyl-*sn*-glycerol, 1-stearoyl-2,3-dilinolenoyl-*sn*-glycerol).

To facilitate TAG descriptions, fatty acids are abbreviated using one or two letters (Table 1.7). The TAGs can be named after the EFAs using shorthand nomenclature. For example, *sn*-POSt

TABLE 1.7
Short Abbreviations for Some Common Fatty Acids

AC	Acetic	Ln	Linolenic
Ad	Arachidic	M	Myristic
An	Arachidonic	N	Nervonic
B	Butyric	O	Oleic
Be	Behenic	Oc	Octanoic
D	Decanoic	P	Palmitic
E	Erucic	Po	Palmitoleic
El	Elaidic	R	Ricinoleic
G	Eicosenoic	S	Saturated (any)
H	Hexanoic	St	Stearic
L	Linoleic	U	Unsaturated (any)
La	Lauric	V	Vaccenic
Lg	Lingnoceric	X	Unknown

Source: Litchfield, C., *Analysis of Triglycerides*, Academic Press, New York, 1972, 355pp.

is shorthand description for the molecule 1-palmitoyl-2-oleoyl-3-stearoyl-*sn*-glycerol. If the *sn*- is omitted, the stereospecific positions of the fatty acids are unknown. POSt could be a mixture of *sn*-POSt, *sn*-StOP, *sn*-PStO, *sn*-OStP, *sn*-OPSt, or *sn*-StPO in any proportion. An equal mixture of both stereoisomers (the racemate) is designated as *rac*. Thus, *rac*-OPP represents equal amounts of sn-OPP and *sn*-PPO. If only the *sn*-2 substituent is known with certainty in a TAG, the designation β- is used. For example, β-POSt is a mixture (unknown amounts) of *sn*-POSt and *sn*-StOP.

TAGs are also sometimes described by means of the ω nomenclature. For example, *sn*-18:0–18:2ω6–16:0 represents 1-stearoyl-2-linoleoyl-3-palmitoyl-*sn*-glycerol.

C. Sterols and Sterol Esters

The steroid class of organic compounds includes sterols of importance in lipid chemistry. Although the term "sterol" is widely used, it has never been formally defined. The following working definition was proposed some years ago: "Any hydroxylated steroid that retains some or all of the carbon atoms of squalene in its side chain and partitions nearly completely into the ether layer when it is shaken with equal volumes of ether and water" [34]. Thus, for this definition, sterols are a subset of steroids and exclude the steroid hormones and bile acids. The importance of bile acids and their intimate origin from cholesterol make this definition difficult. In addition, nonhydroxylated structures such as cholestane, which retain the steroid structure, are sometimes considered sterols.

The sterols may be derived from plant (phytosterols) or animal (zoosterols) sources. They are widely distributed and are important in cell membranes. The predominant zoosterol is cholesterol. Although a few phytosterols predominate, the sterol composition of plants can be very complex. For example, as many as 65 different sterols have been identified in corn (*Zea mays*) [35].

In the standard ring and carbon numbering (Figure 1.19) [35], the actual 3D configuration of the tetra ring structure is almost flat, so the ring substituents are either in the same plane as the rings or in front or behind the rings. If the structure in Figure 1.19 lacks one or more of the carbon atoms, the numbering of the remainder will not be changed.

The methyl group at position 10 is axial and lies in front of the general plane of the molecule. This is the β configuration and is designated by connection using a solid or thickened line. Atoms or groups behind the plane of the molecule are joined to the ring structure by a dotted or broken line and are given the α configuration. If the stereochemical configuration is not known, a wavy line is used and the configuration is referred to as ε. Unfortunately, actual 3D position of the substituents may be in plane, in front of, or behind the plane of the molecule. The difficulties with this nomenclature have been discussed elsewhere [34,35].

The nomenclature of the steroids is based on parent ring structures. Some of the basic steroid structures are presented in Figure 1.20 [6]. Because cholesterol is a derivative of the cholestane structure (with the H at C-5 eliminated because of the double bond), the correct standard nomenclature

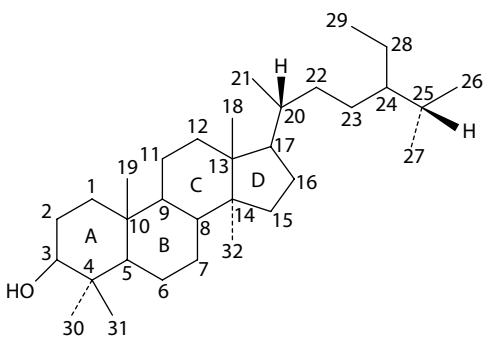


FIGURE 1.19 Carbon numbering in cholesterol structure.

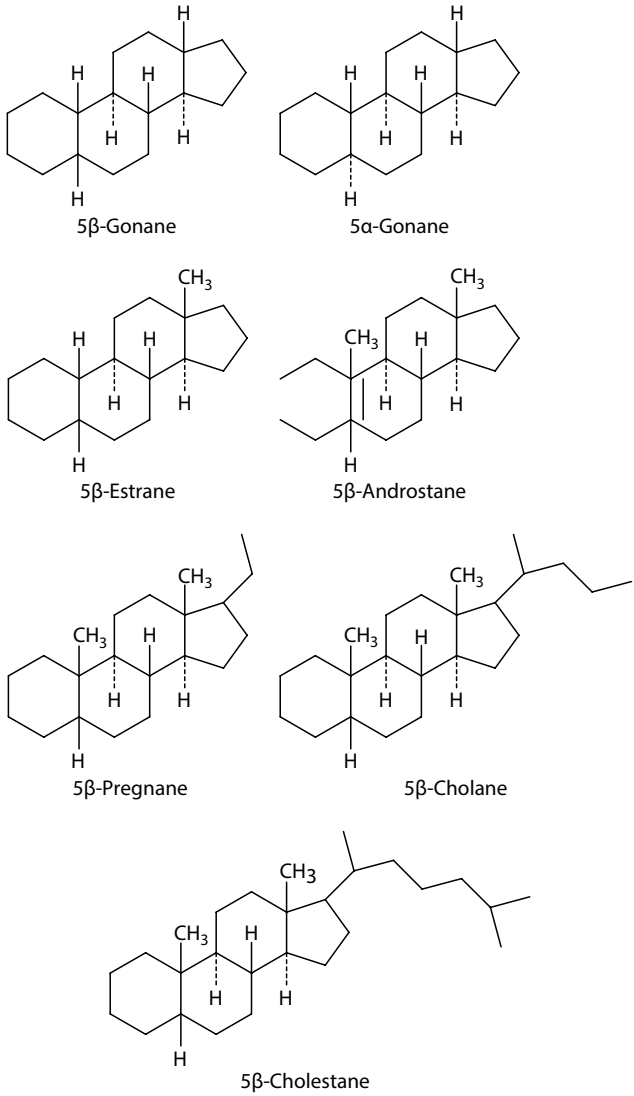


FIGURE 1.20 Steroid nomenclature.

for cholesterol is 3β-cholest-5-en-3-ol. The complexity of standardized nomenclature has led to the retention of trivial names for some of the common structures (e.g., cholesterol). However, when the structure is changed—for example, with the addition of a ketone group to cholesterol at the 7 position—the proper name is 3β-hydroxycholest-5-en-7-one, although this molecule is also called 7-ketocholesterol in common usage.

A number of other sterols of importance in foods are shown in Figure 1.21. The trivial names are retained for these compounds, but based on the nomenclature system discussed for sterols, stigmasterol can be named 3β-hydroxy-24-ethylcholesta-5,22-diene. Studies have suggested that plant sterols and stanols (saturated derivatives of sterols) have cholesterol-lowering properties in humans [36].

Cholesterol has been reported to oxidize *in vivo* and during food processing [37–40]. These cholesterol oxides have come under scrutiny because they have been implicated in the development of atherosclerosis. Some of the more commonly reported oxidation products are shown in Figures 1.22 and 1.23.

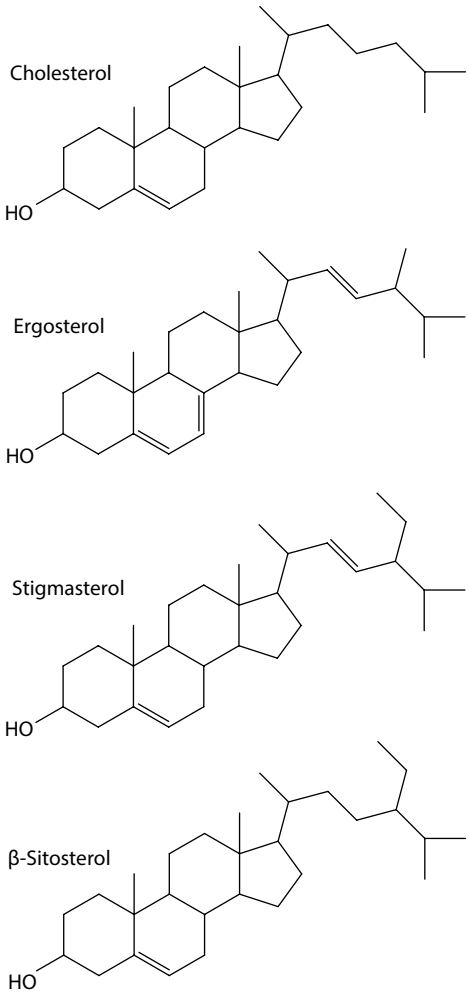


FIGURE 1.21 Common steroid structures.

Nomenclature in common usage in this field often refers to the oxides as derivatives of the cholesterol parent molecule: 7-β-hydroxycholesterol, 7-ketocholesterol, 5,6β-epoxycholesterol, etc. The standard nomenclature follows described rules and is shown in Figures 1.22 and 1.23.

Sterol esters exist commonly and are named using standard rules for esters. For example, the ester of cholesterol with palmitic acid would be named cholesterol palmitate. The standard nomenclature would also allow this molecule to be named 3-*O*-palmitoyl-3β-cholest-5-en-3-ol or 3-palmitoyl-3β-cholest-5-en-3-ol.

D. Waxes

Waxes (commonly called wax esters) are esters of fatty acids and long-chain alcohols. Simple waxes are esters of medium-chain fatty acids (16:0, 18:0, 18:1ω9) and long-chain aliphatic alcohols. The alcohols range in size from C8 to C18. Simple waxes are found on the surfaces of animals, plants, and insects and play a role in prevention of water loss. Complex waxes are formed from diols or from alcohol acids. Di- and triesters as well as acid and alcohol esters have been described.

Simple waxes can be named by removing the -ol from the alcohol and replacing it with -yl, and replacing the -ic from the acid with -oate. For example, the wax ester from hexadecanol and

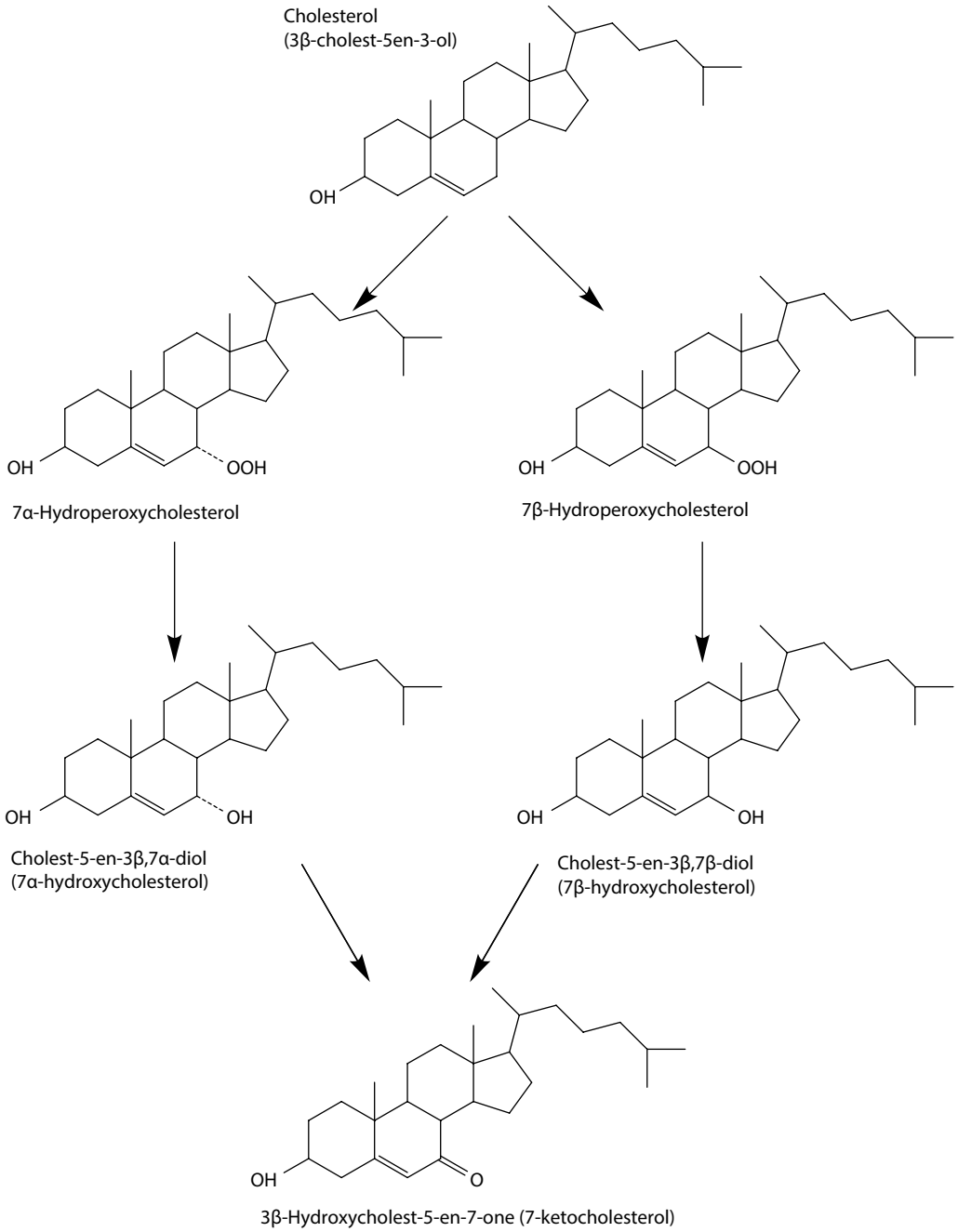


FIGURE 1.22 Cholesterol oxidation products and nomenclature I. (From Smith, L.L., *Chem. Phys. Lipids*, 44, 87, 1987.)

oleic acid would be named hexadecyl oleate or hexadecyl-*cis*-9-octadecenoate. Some of the long-chain alcohols have common names derived from the fatty acid parent (e.g., lauryl alcohol, stearyl alcohol). The C16 alcohol (1-hexadecanol) is commonly called cetyl alcohol. Thus, cetyl oleate is another acceptable name for this compound.

Waxes are found in animal, insect, and plant secretions as protective coatings. Waxes of importance in foods as additives include beeswax, carnauba wax, and candelilla wax.

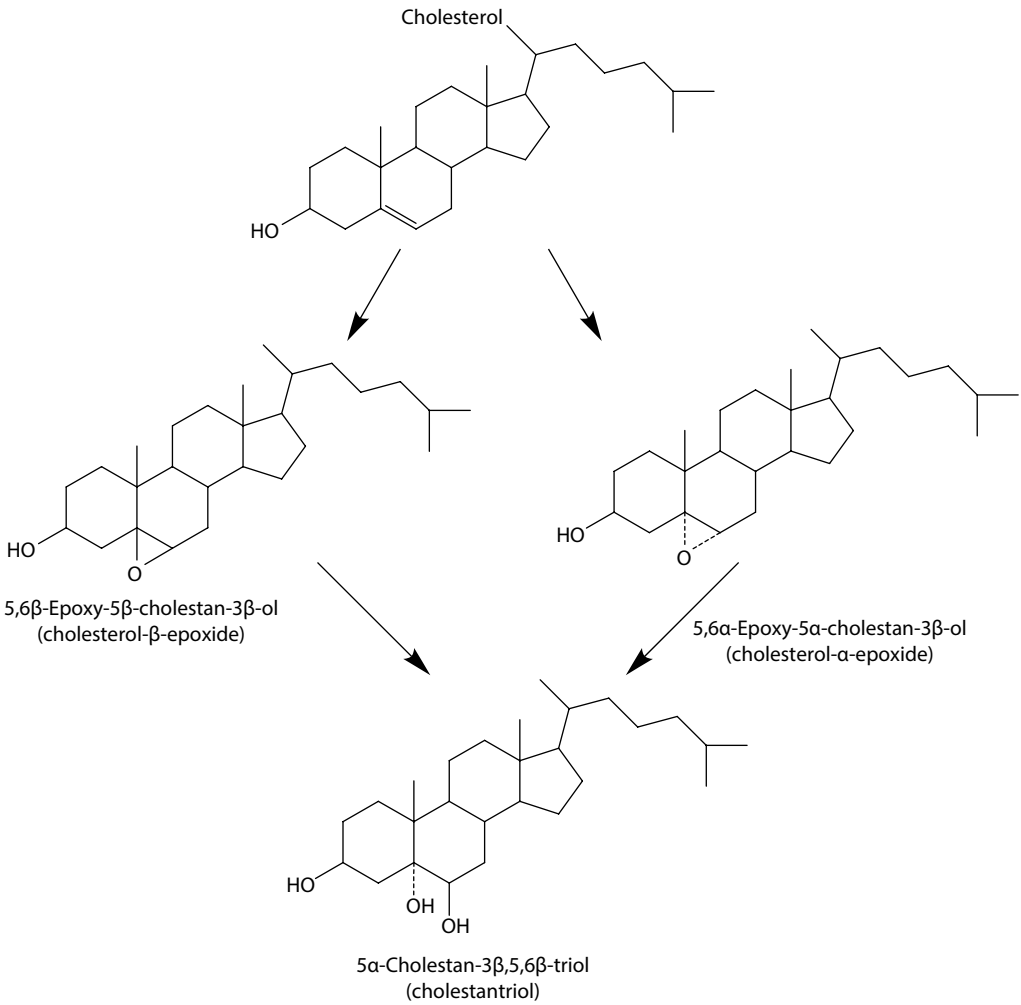


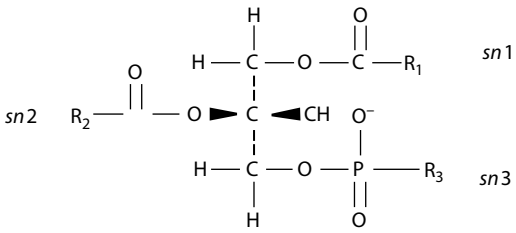
FIGURE 1.23 Cholesterol oxidation products and nomenclature II. (From Smith, L.L., *Chem. Phys. Lipids*, 44, 87, 1987.)

E. Phosphoglycerides (Phospholipids)

Phosphoglycerides (PLs) are composed of glycerol, fatty acids, phosphate, and (usually) an organic base or polyhydroxy compound. The phosphate is almost always linked to the *sn*-3 position of glycerol molecule.

The parent structure of the PLs is phosphatidic acid (*sn*-1,2-diacylglycerol-3-phosphate). The terminology for PLs is analogous to that of acylglycerols except that there is no acyl group at *sn*-3. The prefix lyso-, when used for PLs, indicates that the *sn*-2 position has been hydrolyzed, and a fatty acid is esterified to the *sn*-1 position only.

Some common PL structures and nomenclature are presented in Figure 1.24. Phospholipid classes are denoted using shorthand designation (PC = phosphatidylcholine, etc.). The standard nomenclature is based on the PL type. For example, a PC with an oleic acid on *sn*-1 and linolenic acid on *sn*-2 would be named 1-oleoyl-2-linolenoyl-*sn*-glycerol-3-phosphocholine. The name phosphorylcholine is sometimes used, but it is not recommended [8]. The terms *lecithin* and *cephalin* are sometimes used for PC and PE, respectively, but are also not recommended [8].



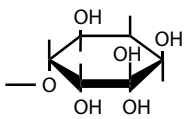
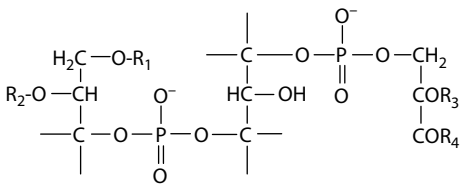
R_3	= Phospholipid class/nomenclature
–OH	Phosphatidic acid (PA)
$–O–CH_2–CH(NH_2)–COOH$	(3-*sn*-phosphatidyl)-L-serine (PS) 1,2-diacyl-*sn*-glycero(3)phospho-L-serine
$–O–CH_2–CH_2–NH_2$	(3-*sn*-phosphatidyl)ethanolamine (PE) 1,2-diacyl-*sn*-glycero(3)phosphoethanolamine
$–O–CH_2–CH_2–N^+(CH_3)_3$	(3-*sn*-phosphatidyl)choline (PC) 1,2-diacyl-*sn*-glycero(3)phosphocholine
	(3-*sn*-phosphatidyl)-L-*myo*-inositol (PI) 1,2-diacyl-*sn*-glycero(3)phospho-L-*myo*-inositol

FIGURE 1.24 Nomenclature for glycerophospholipids.



Cardiolipin
1′, 3′-di-*O*-(3-*sn*-phosphatidyl)-*sn*-glycerol
R1–R4 are fatty acids

FIGURE 1.25 Cardiolipin structure and nomenclature.

Cardiolipin is a PL that is present in heart muscle mitochondria and bacterial membranes. Its structure and nomenclature are shown in Figure 1.25. Some cardiolipins contain the maximum possible number of 18:2ω6 molecules (4 mol/mol).

F. Ether(Phospho)Glycerides (Plasmalogens)

Plasmalogens are formed when a vinyl (1-alkenyl) ether bond is found in a phospholipid or acylglycerol. The 1-alkenyl-2,3-diacylglycerols are termed *neutral plasmalogens*. A 2-acyl-1-(1-alkenyl)-*sn*-glycerophosphocholine is named a plasmalogen or plasmenylcholine. The related 1-alkyl compound is named plasmanylcholine.

G. Glyceroglycolipids (Glycosylglycolipids)

The glyceroglycolipids or glycolipids are formed when a 1,2-diacyl-*sn*-3-glycerol is linked via the *sn*-3 position to a carbohydrate molecule. The carbohydrate is usually a mono- or a disaccharide,

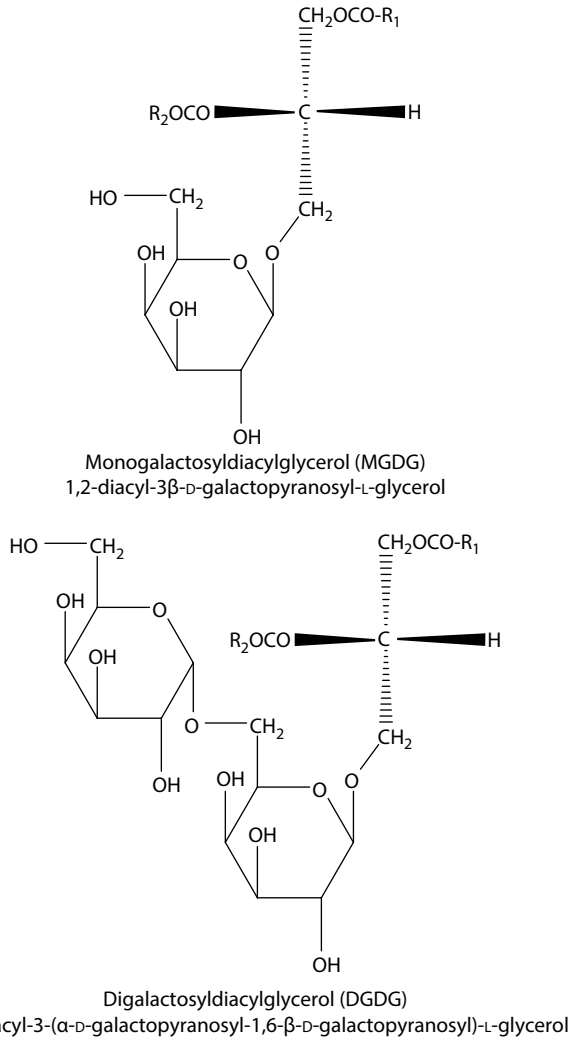


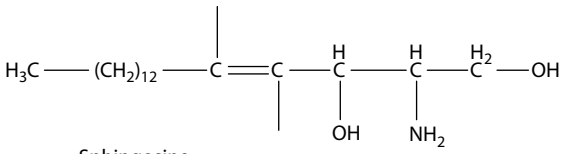
FIGURE 1.26 Glyceroglycolipid structures and nomenclature.

less commonly a tri- or tetrasaccharide. Galactose is the most common carbohydrate molecule in plant glyceroglycolipids.

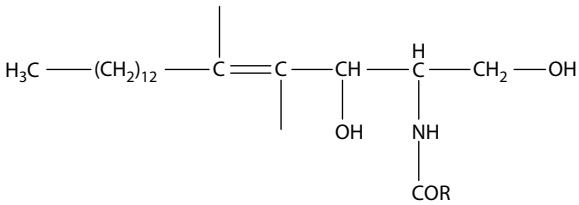
Structures and nomenclature for some glyceroglycolipids are shown in Figure 1.26. The names monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are used in common nomenclature. The standard nomenclature identifies the ring structure and bonding of the carbohydrate groups (Figure 1.26).

H. Sphingolipids

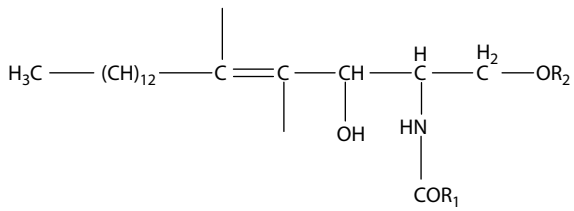
The glycosphingolipids are a class of lipids containing a long-chain base, fatty acids, and various other compounds, such as phosphate and monosaccharides. The base is commonly sphingosine, although more than 50 bases have been identified. The ceramides are composed of sphingosine and a fatty acid (Figure 1.27). Sphingomyelin is one example of a sphingophospholipid. It is a ceramide with a phosphocholine group connected to the primary hydroxyl of sphingosine. The ceramides can also be attached to carbohydrate molecules (sphingoglycolipids or cerebrosides) via the



Sphingosine
D-*erythro*-1,3-dihydroxy-2-amino-*trans*-4-octadecene

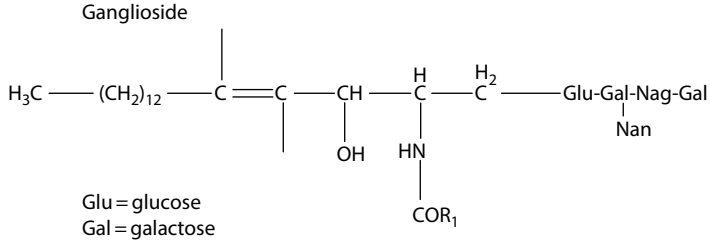


Ceramide
D-*erythro*-1,3-dihydroxy-2(*N*-acyl)-amino-*trans*-4-octadecene
(*N*-acyl-sphingosine)



Cerebroside
1-*O*-β-D-galactopyranosyl-*N*-acyl-sphingosine
R1 = fatty acid
R2 = galactopyranose

Ganglioside



Glu = glucose
Gal = galactose
Nag = *N*-acetylgalactosamine
Nan = *N*-acetylneuraminic acid

FIGURE 1.27 Sphingolipid structures and nomenclature.

primary hydroxyl group of sphingosine. Gangliosides are complex cerebrosides with the ceramide residue connected to a carbohydrate-containing glucose-galactosamine-*N*-acetylneuraminic acid. These lipids are important in cell membranes and the brain, and they act as antigenic sites on cell surfaces. Nomenclature and structures of some cerebrosides are shown in Figure 1.27.

I. Fat-Soluble Vitamins

1. Vitamin A

Vitamin A exists in the diet in many forms (Figure 1.28). The most bioactive form is the all-*trans* retinol, and *cis* forms are created via light-induced isomerization (Table 1.8). The 13-*cis* isomer is the most biopotent of the mono- or di-*cis* isomers. The α- and β-carotenes have biopotencies of

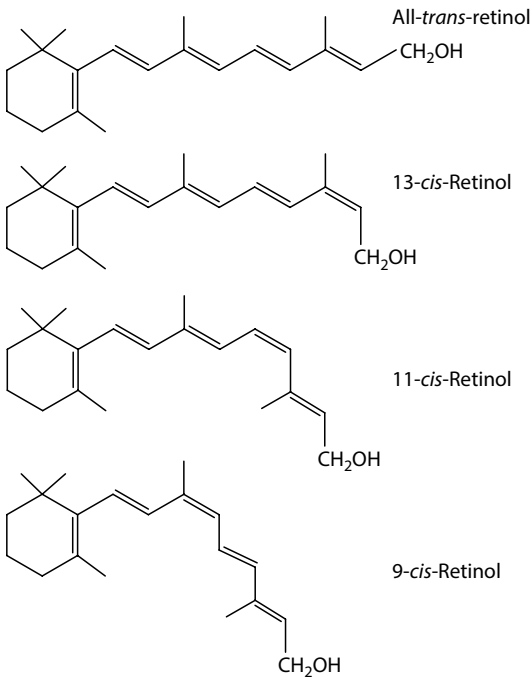


FIGURE 1.28 Structures of some vitamin A compounds.

TABLE 1.8
Approximate Biological Activity Relationships of Vitamin A Compounds

Compound	Activity of All-*trans* Retinol (%)
All-*trans* retinol	100
9-*cis* Retinol	21
11-*cis* Retinol	24
13-*cis* Retinol	75
9,13-Di-*cis* retinol	24
11,13-Di-*cis* retinol	15
α-Carotene	8.4
β-Carotene	16.7

about 8.7% and 16.7% of the all-*trans* retinol activity, respectively. The daily value (DV) for vitamin A is 1000 retinol equivalents (RE), which represents 1000 mg of all-*trans* retinol or 6000 μg of β-carotene. Vitamin A can be toxic when taken in levels exceeding the %DV. Some reports suggest that levels of 15,000 RE/day can be toxic [39].

Toxic symptoms of hypervitaminosis A include drowsiness, headache, vomiting, and muscle pain. Vitamin A can be teratogenic at high doses [41]. Vitamin A deficiency results in night blindness and ultimately total blindness, abnormal bone growth, increased cerebrospinal pressure, reproductive defects, abnormal cornification, loss of mucus secretion cells in the intestine, and decreased growth. The importance of beef liver, an excellent source of vitamin A, in cure of night blindness was known to the ancient Egyptians about 1500 BC [42].

2. Vitamin D

Although as many as five vitamin D compounds have been described (Figure 1.29), only two of these are biologically active: ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Vitamin D_3 can be synthesized in humans from 7-dehydrocholesterol, which occurs naturally in the skin, via light irradiation (Figure 1.30).

The actual hormonal forms of the D vitamins are the hydroxylated derivatives. Vitamin D is converted to 25-OH-D in the kidney and further hydroxylated to 1,25-diOH-D in the liver. The dihydroxy form is the most biologically active form in humans.

3. Vitamin E

Vitamin E compounds include the tocopherols and tocotrienols. Tocotrienols have a conjugated triene double-bond system in the phytyl side chain, whereas tocopherols do not. The basic nomenclature is shown in Figure 1.31. The bioactivity of the various vitamin E compounds is shown in Table 1.9. Methyl substitution affects the bioactivity of vitamin E, as well as its *in vitro* antioxidant activity.

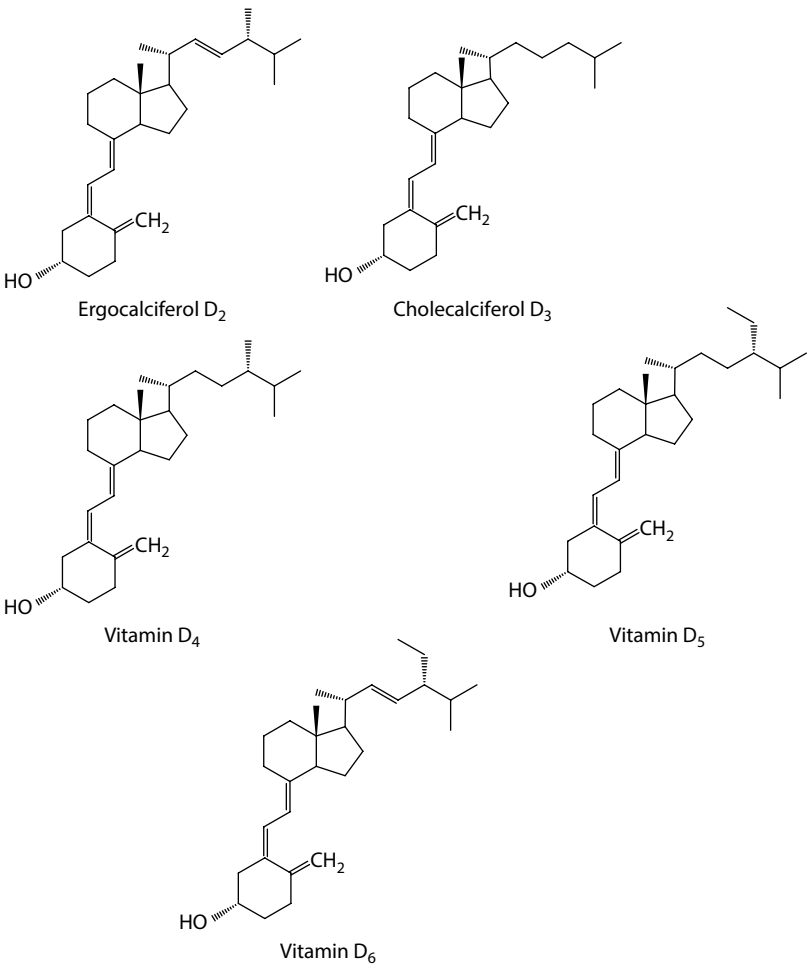


FIGURE 1.29 Structures of some vitamin D compounds.

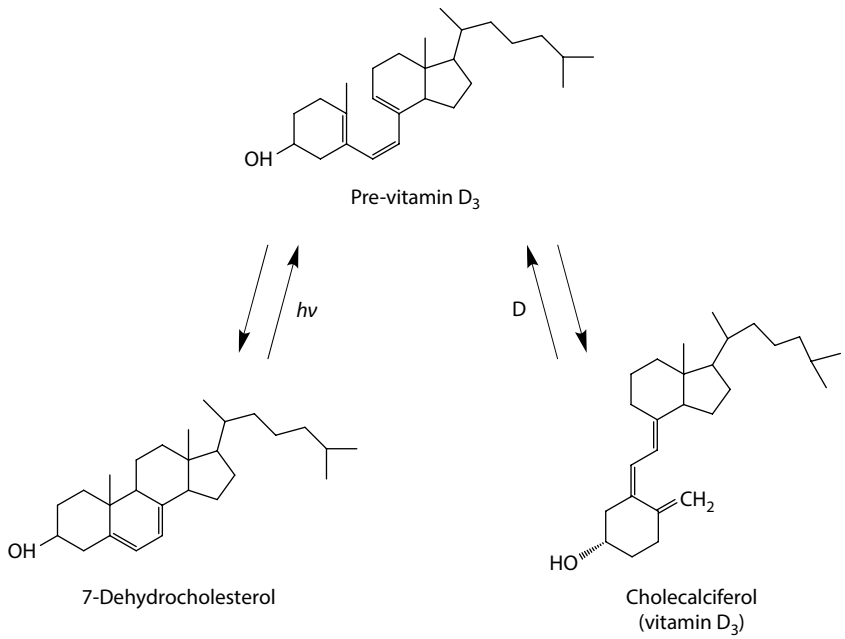
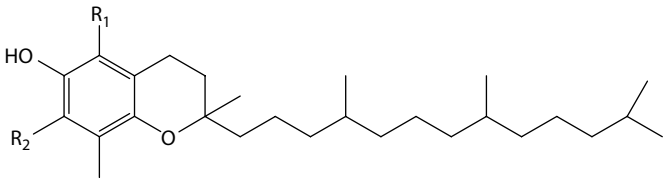
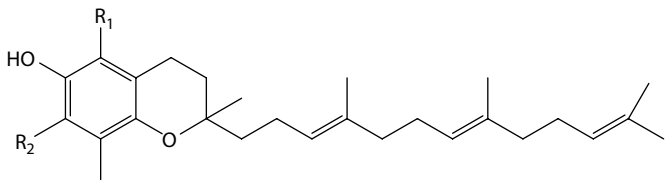


FIGURE 1.30 Formation of vitamin D *in vivo*.



Tocopherol	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H



Tocotrienol	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

FIGURE 1.31 Structures of some vitamin E compounds.

TABLE 1.9
Approximate Biological Activity Relationships of Vitamin E Compounds

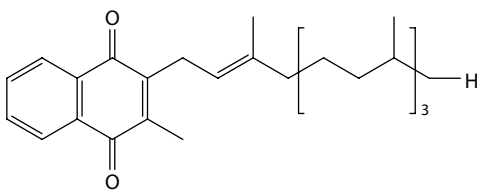
Compound	Activity of D-α-Tocopherol (%)
D-α-Tocopherol	100
L-α-Tocopherol	26
DL-α-Tocopherol	74
DL-α-Tocopheryl acetate	68
D-β-Tocopherol	8
D-γ-Tocopherol	3
D-δ-Tocopherol	—
D-α-Tocotrienol	22
D-β-Tocotrienol	3
D-γ-Tocotrienol	—
D-δ-Tocotrienol	—

4. Vitamin K

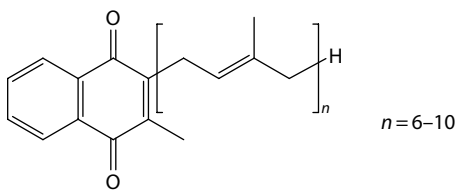
Several forms of vitamin K have been described (Figure 1.32). Vitamin K (phylloquinone) is found in green leaves, and vitamin K_2 (menaquinone) is synthesized by intestinal bacteria. Vitamin K is involved in blood clotting as an essential cofactor in the synthesis of γ-carboxyglutamate necessary for active prothrombin. Vitamin K deficiency is rare due to intestinal microflora synthesis. Warfarin and dicoumerol prevent vitamin K regeneration and may result in fatal hemorrhaging.

J. Hydrocarbons

Hydrocarbons include normal, branched, saturated, and unsaturated compounds of varying chain lengths. The nomenclature for hydrocarbons has already been discussed. The hydrocarbons of most interest to lipid chemists are the isoprenoids and their oxygenated derivatives.



Vitamin K_1
Phylloquinone
2-Methyl-3-phytyl-1,4-naphthoquinone



$n = 6–10$

Vitamin K_2
Menaquinone-*n*
2-Methyl-3-multiprenyl-1,4-naphthoquinone

FIGURE 1.32 Structures of some vitamin K compounds.

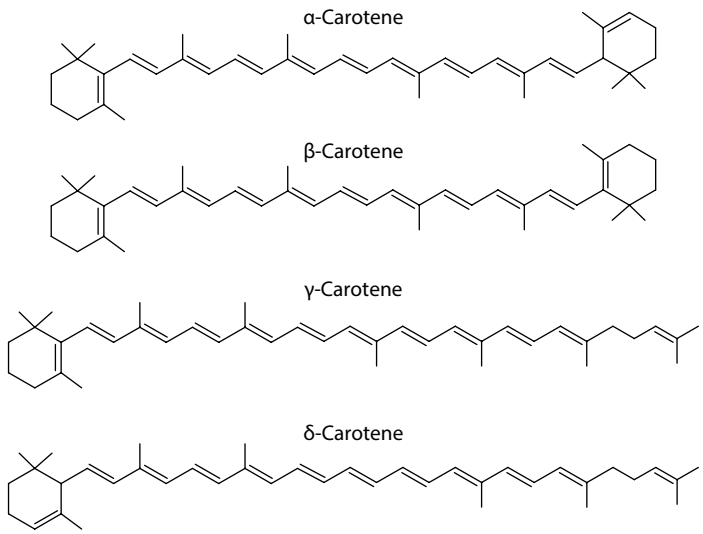


FIGURE 1.33 Structures and nomenclature of carotenoids.

The basic isoprene unit (2-methyl-1,3-butadiene) is the building block for a large number of interesting compounds, including carotenoids (Figure 1.33), oxygenated carotenoids or xanthophylls (Figure 1.34), sterols, and unsaturated and saturated isoprenoids (isopranes). Some 15-carbon and 20-carbon isoprenoids are covalently attached to some proteins and may be involved in control of cell growth [43]. Members of this class of protein-isoprenoid molecules are called prenylated proteins.

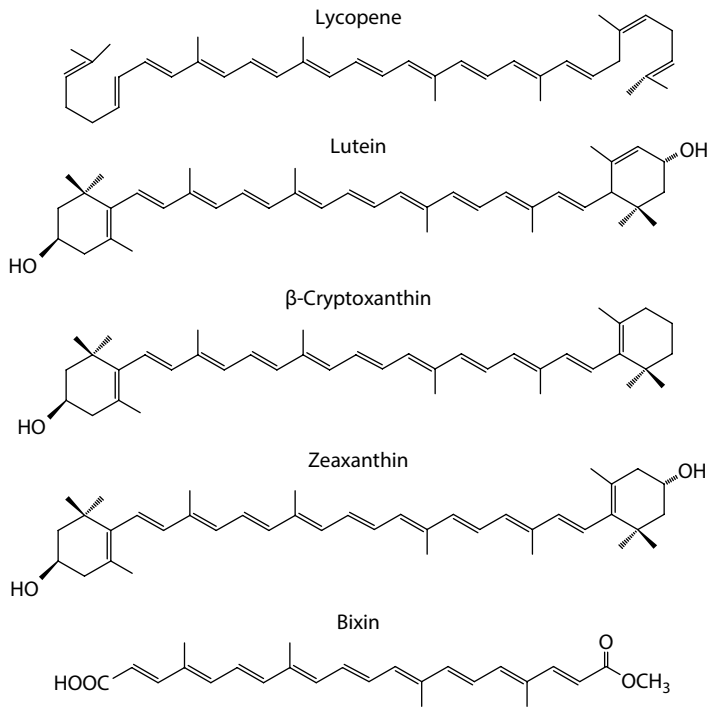


FIGURE 1.34 Structures and nomenclature of some oxygenated carotenoids.

IV. SUMMARY

It would be impossible to describe the structures and nomenclature of all known lipids even in one entire book. The information presented in this chapter is a brief overview of the complex and interesting compounds we call lipids.

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2 Chemistry and Properties of Lipids and Phospholipids

Bianca Pérez, Jingbo Li, and Zheng Guo

CONTENTS

I. INTRODUCTION

Lipids are referred to as a large and diverse group of naturally occurring compounds. The term "lipid" is restricted to long-chain fatty acids, their derivatives, and compounds that are related structurally and functionally to them [1,2]. They are highly soluble in organic solvents (e.g., hexane, diethyl ether, and chloroform) and slightly soluble/nonsoluble in water [3]. Lipids are found ubiquitously in plants, animals, and microorganisms, as well as the human body. They are the major constituents of the cell membrane, which is responsible for holding the cell together [4]. Lipids can be classified as polar and neutral lipids [5]. The International Lipid Classification and Nomenclature Committee developed a more detailed classification scheme (Lipid Classification System LIPID Metabolites and Pathways Strategy; http://www.lipidmaps.org) based on the concept that lipids may result from complete or partial carbanion-based condensations of ketoacyl thioesters or carbocation-based condensations of isoprene units [6]. Accordingly, lipids can be classified into eight groups, namely, fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (Figure 2.1) [7,8]. This classification scheme is applicable to archaea and synthetic lipids, covers eukaryotic and prokaryotic sources, and allows the subdivision of the main categories into subclasses to include the new lipids structures.

Fatty acyls are biosynthesized by the chain elongation of an acetyl-CoA primer with malonyl-CoA or methyl-CoA groups [5]. The two main classes of lipids are (1) glycerolipids, which encompass mainly acylglycerols, and (2) glycerophospholipids, which are characterized by the presence of phosphate. In addition, steroid and prenoid lipids constitute lipids derived from the polymerization of dimethylallyl pyrophosphate/isopentenyl pyrophosphate. Other well-known categories of lipids are sphingolipids, saccharolipids, and polyketides. Sphingolipids contain a long aliphatic amine with two or more hydroxyl groups as their core structure and saccharolipids are lipids that have a fatty acyl linked to a sugar molecule forming structures that are compatible with membrane bilayers. Finally, the last category of lipids is polyketides. These are lipids derived from plants and microbial sources.

The physicochemical properties of lipids depend on their structural features [9]. For instance, the length of the fatty acyl chain, number of unsaturation in the molecules, and size of the polar head determine the physical states, existing as solid, liquid, or liquid crystal. In addition, the structural features provide insight into the oxidative stability of lipids. In relation to the application of lipids in food, the lipid property will to a large extent influence food quality and stability, and thus, in some cases structural modification of lipids is needed to meet the demand of food processing [10].

Modification of lipids has produced a wide variety of fats, lowered production cost, and enabled food products to meet nutritional demands [9,11]. For example, when modifying oils for food applications, producers attempted to meet both nutritional requirements (e.g., increasing concentration of unsaturated fatty acids) and technological requirements to facilitate production and to improve oxidative stability. Although unsaturated fatty acids are preferable over saturated fatty acids from a nutritional point of view [12], they are prone to oxidation. Therefore, to improve their oxidative stability, the produced oils are modified to contain less polyunsaturated fatty acids and more monounsaturated fatty acids such as oleic acid. Thus, modification of fats for the production of higher melting point fats is a strategy to increase food quality as the replacement or redistribution of the fatty acyls in glycerol backbone may alter the melting point, crystallization behavior, and oxidative stability of fats in food processing. Although hydrogenation of vegetable oil has been a widely used approach to generate plastic fat from liquid oil, now this strategy has been increasingly replaced by other procedures such as fatty acyl exchanges through enzymatic modifications of oils and fats because they prevent the production of *trans*-fatty acids that have been associated with coronary diseases [13].

Other examples of physical strategies to modify fats are fractionation of fats at different temperatures and extraction with CO_2 under supercritical conditions [14] or using organic solvents. Furthermore, if the fatty acid composition of different oils is known, they can be combined through physical blending to generate oil with the ratio of fatty acid desired [15]. Other strategies involve the use of genetic

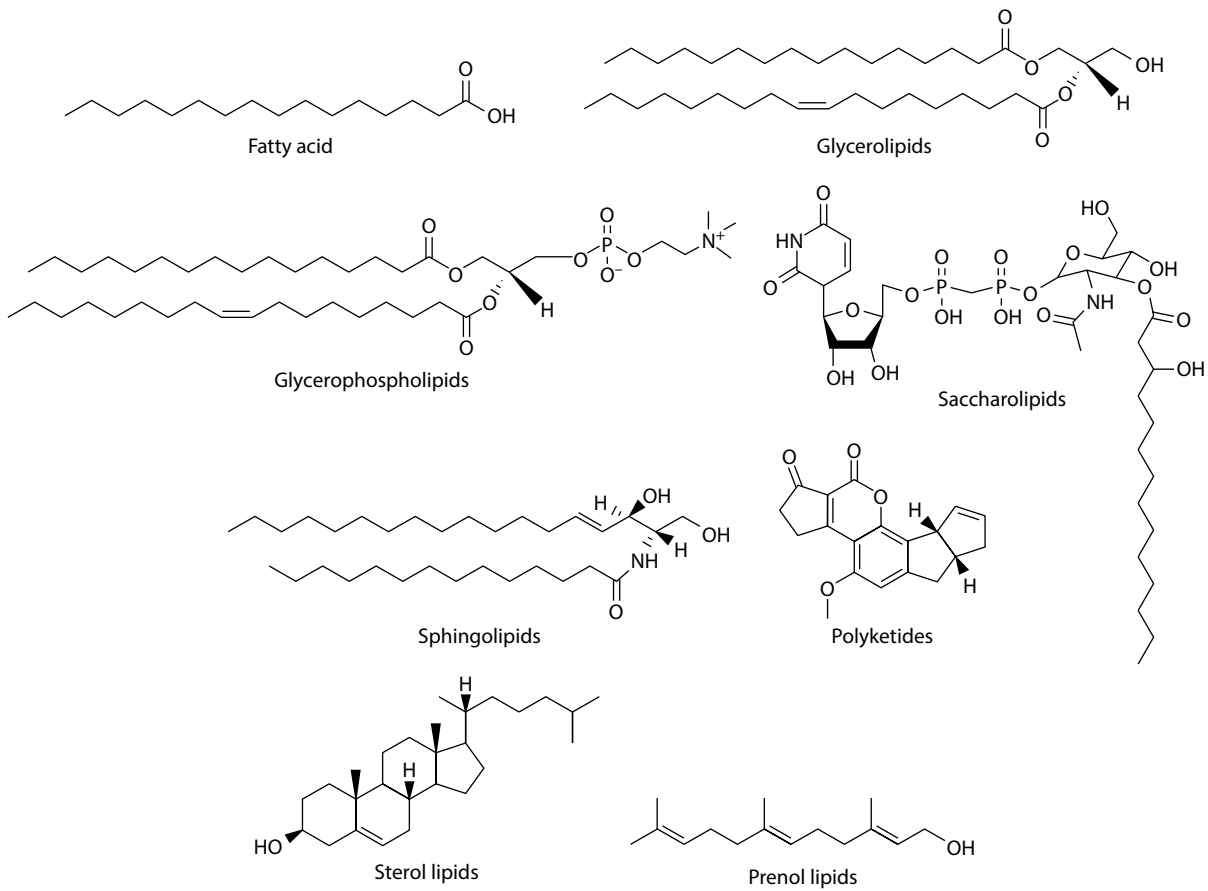


FIGURE 2.1 Representative structures of different classes of lipids. (From Fahy, E. et al., *J. Lipid Res*., 46, 839, 2005.)

engineering to cultivate crops that are highly rich in specific and desired oil [16]. However, ecological safety regarding the cultivation of these types of crops has not been investigated.

On the other hand, examples of chemical synthesis and modification of lipids constituting hydrogenation or chemical esterification or transesterification of oils are still the technology in use today [17,18]. However, chemical approaches are increasingly being replaced by environmentally friendly strategies such as enzymatic synthesis (e.g., enzymatic esterification and transesterification) [19,20]. The latter decreases the amount of hazardous wastes that can be generated in high-scale production of lipids. Nonetheless, enzyme-based production is still expensive and less competitive in price and this is taken into account when designing production line to obtain lipids of relevance for food production.

Analysis and characterization strategies are also important to guarantee the quality of lipids as food ingredients [21–24]. High-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and gas chromatography (GC) are the most common techniques used for the qualitative and quantitative analyses of lipids [25,26]. However, other techniques that are used for structural elucidation of lipids have also proved to be of value for the quantitative analyses of lipids such as Fourier transform infrared spectroscopy [27]. Generally, the use of multiple techniques is necessary to ensure the quality of lipids used for food production. For instance, in terms of physical characterization it is required to combine different techniques such as x-ray diffraction [28], differential scanning calorimetry [29], and nuclear magnetic resonance [30] to understand in detail the physicochemical properties of the lipids in question.

This chapter mainly focuses on the two most abundant classes of lipids in nature: acylglycerol and phospholipids. It describes the physicochemical properties of these lipids and envisages their molecular basis, structural features, and the types of chemical reactions that these classes of lipids undergo. In addition, this chapter reviews the most common strategies used for the synthesis, isolation, analysis, and characterization of lipids. Specifically, the enzymatic and chemical synthesis and modifications of acylglycerols and phospholipids are presented. Accordingly, this chapter provides useful insight for the generation of novel lipids of relevance to food applications.

II. ACYLGLYCEROLS

Acylglycerols are predominant components in oils and fats for food and oleochemical industries [31]. They represent all lipids containing a glycerol backbone covalently linked to fatty acyls. These lipids can be monoacylglycerols, diacylglycerols, and triacylglycerols (Figure 2.2), where the latter represents the main occurring form of acylglycerols for both plants and animals. In nature, acylglycerols also exist in partial glyceride form in relatively small quantity, including monoacylglycerols and diacylglycerols. The presence of hydroxyl group in partial acylglycerols gives them different polarity and properties from triacylglycerols. Natural triglycerides (triacylglycerols) are a mixture of triacylglycerols containing different fatty acids. Depending on the lipids, sources, the types, numbers, and relative contents of

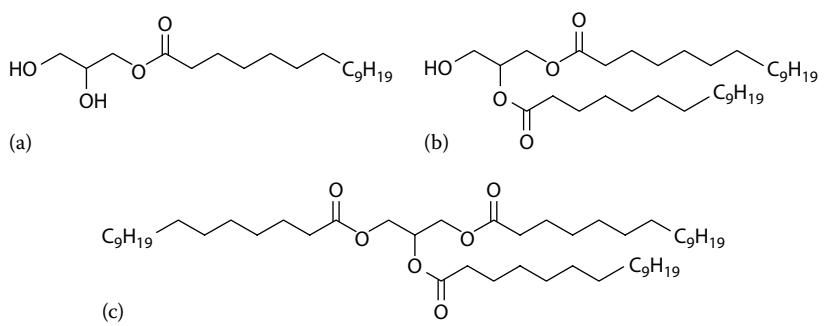


FIGURE 2.2 Representative structures of (a) mono-, (b) di-, and (c) triacylglycerols (MAG, DAG, and TAG, respectively).

fatty acids in triacylglycerol can vary greatly; for example, in milk fat the number of different fatty acids adds up to 400 [32]. Therefore, the total number of triacylglycerol molecules can be very high (400^3). However, for most plant oils they constitute no more than 100 different fatty acids; even so, the number of total triacylglycerol types can also be very high [23]. This complexity in composition of triacylglycerols makes it very difficult to accurately predict their property, while their properties are largely governed by dominating fatty acids. By changing the fatty acyl, the physicochemical properties of triacylglycerols can be altered for specific purpose; for example, by introducing saturated or monounsaturated fatty acids into highly polyunsaturated oils the oxidation stability of the products can be improved.

III. PHYSICAL PROPERTIES OF ACYLGLYCEROLS

The physical properties of acylglycerols depend on the molecular structure of fatty acyls linked to the glycerol backbone. The melting point and intermolecular interaction of lipid is determined by the saturated degree and carbon chain length of fatty acyls. For example, the melting point of saturated fatty acid is governed by the weak dispersion forces or noncovalent interactions known as van der Waals forces as molecules need to overcome these forces in order to be melted. In branched saturated fatty acid chain, these forces are weaker, lowering the energy required for these compounds to be melted [9]. In addition, increasing the number of double bonds and length of the lipid chain decreases the melting point and lowers the organization of lipid chains. For instance, linoleic acid, which contains two double bonds, displayed a lower melting point (–5°C) than oleic acid (m.p. = 13°C) and stearic acid (m.p. = 69°C), which contain 1 and no unsaturation, respectively (Figure 2.3).

In food formulation, the crystallization behavior of triacylglycerols is highly important as crystallization of lipids in food products might influence food structure, taste, and quality. Controlling the crystallization process of triacylglycerols can yield a product with desired physical characteristics [33]. For instance, the tempering process of chocolate determines whether the product will have or not have the desired appearance, snap, and melting properties since the crystallization process can lead to more or less stable crystal forms. During the crystallization process, the main steps that determine the crystal structure of lipids are nucleation and crystal growth. However, it is important to know that the liquid phase prior to crystal forms can retain some degree of ordering. Thus, heating up mixtures of lipids well above the melting point is of importance to erase crystal memory and to obtain the desired crystal structure by controlling the process of nucleation and crystal growth.

A. Nucleation

Nucleation takes place in the formation of crystalline phase from liquid phase. The nucleation rate can determine the number and size of the crystals formed and the resulting polyphorm. Nucleation can

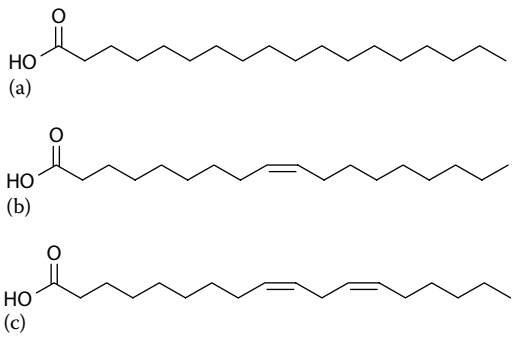


FIGURE 2.3 The effect of the degree of unsaturation on melting point of fatty acids. (a) Stearic acid, m.p. = 69°C; (b) oleic acid, m.p. = 13°C; and (c) linoleic acid, m.p. = –5°C.

occur homogeneously or heterogeneously, or when crystalline elements break from existing crystal and subsequently act as nuclei to promote crystallization [34,35]. Homogeneous nucleation, which is unlikely to occur, takes place without the assistance of a foreign interface. Instead, heterogeneous nucleation is catalyzed by foreign nucleating site such as dust particles or other interfaces that can reduce the free energy required for nucleation to occur. Rapid cooling of lipids in liquid state leads to high nucleation rate and does not allow enough time for the molecules to be organized into lamellae to form well-arranged crystal structures. As a consequence, a rapid cooling process will generate a less stable molecular organization. Other parameters that can alter nucleation are nucleation time, nucleation temperature, and crystal lattice of the nuclei formed.

B. Crystal Growth

Crystal growth occurs when molecules from the liquid phase start to migrate to the crystal surface [33]. For mixed triacylglycerols, the probability of two molecules crystallizing together will depend on the structural similarities such as chain length, degree of saturation, configuration of the double bond, and the position of the fatty acids in the glycerol backbone. For instance, mixed triacylglycerols may crystallize more slowly than pure triacylglycerol forms. Furthermore, cooling and agitation rate, temperature, and composition of lipid phase can also affect the crystal growth. In addition, the presence of some minor components might retard crystal growth.

C. Polymorphism

Polymorphism is the ability of a compound to form more than one crystalline structure [33]. The lipid polymorphism is determined by the coherence force, which results from the exclusion volume, head group interaction, polarity, and van der Waals forces between hydrocarbon chains. The main polymorphic forms of triacylglycerols are alpha (α) form, beta prime (β′) form, and beta (β) form (Figure 2.4). These packing structures differ in their stability, as the most organized form is the most stable crystal. The most densely packed forms constitute the β polymorphs. Accordingly, β form is more stable and closely packed than β′ and α form (Table 2.1). Thus, the β forms have higher melting point and higher heat of fusion than the α forms. Transformation from a least stable polymorph to a more stable form can be achieved by slowly increasing the temperature above the melting point of the least stable form and allowing the liquid to recrystallize at this temperature.

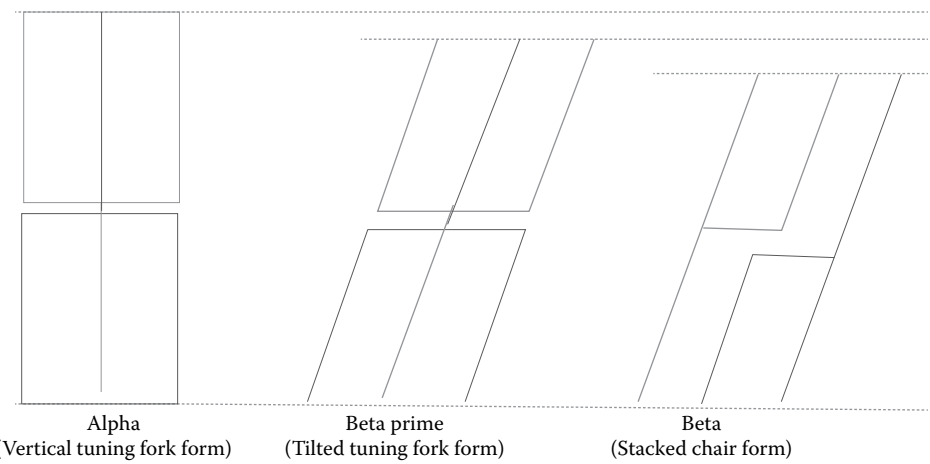


FIGURE 2.4 Triacylglycerols polymorphs.

TABLE 2.1
Physical Features of Triacylglycerols Polymorphs

Alpha	Beta Prime	Beta
Obtained after rapid cooling of liquid fat	Obtained after slow cooling of liquid fat	Obtained after very slow cooling of liquid fat
—	Polymorphic transformation of the α form	Polymorphic transformation of the β′ form
Thermodynamically most unstable	Thermodynamically unstable	Most stable form
Lowest melting point	Intermediate melting point	Highest melting point
Most loosely packed	More closely packed	Most closely packed

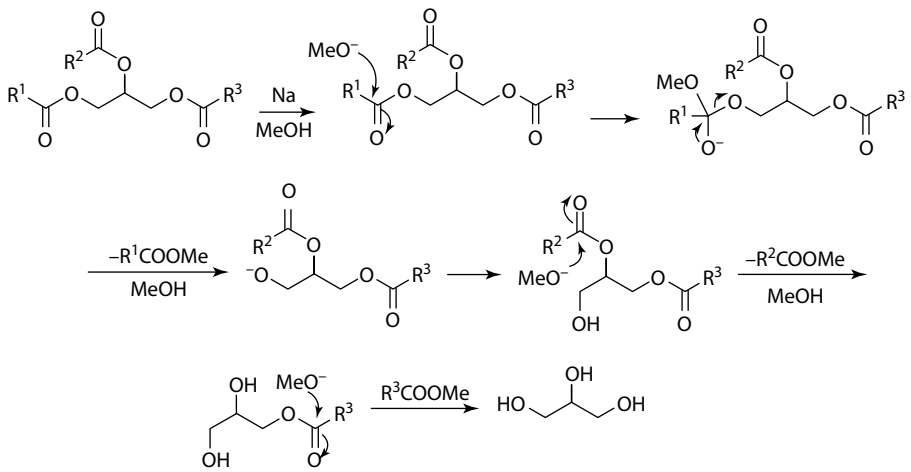
However, this transformation may also take place without melting and in some mixtures of triacylglycerols no β polymorphs may be observed or more than one β′ polymorph could be identified. For example, cocoa butter may display two β′ forms and two β forms [36] that have slightly different melting points but present an x-ray spectra that make them fall into one of the categories [33].

IV. CHEMICAL AND ENZYMATIC SYNTHESIS OF ACYLGLYCEROLS

Acylglycerols can undergo a series of chemical reactions including hydrolysis, esterification, interesterification, hydrogenation, and so forth. The structural modifications of oils and fatty acids can alter the packing behavior of the molecules generating lipids of higher or lower melting point based on the structural modification of the lipid in question [37]. For instance, fully hydrogenated unsaturated oils will yield a product with a higher melting point and more thermodynamically stable.

A. Alcoholysis

Alcoholysis, also known as transesterification, is the reaction between an alcohol and an ester [37]. This normally requires the presence of a catalyst to initiate the reaction. The mechanism of this reaction is displayed in Scheme 2.1. The alcoholysis of triacylglycerols by methanol can generate fatty acid methyl esters and glycerol. Methyl esters are favorable derivatives in oleochemistry (as biodiesels) and gas chromatography analysis because they are less reactive and more volatile than



SCHEME 2.1 Mechanism of triacylglycerols methanolysis.

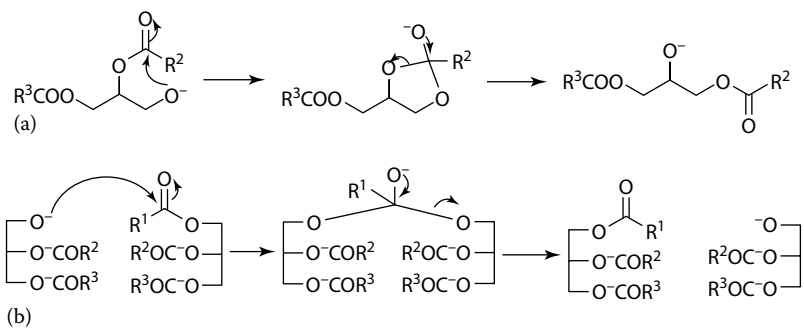
fatty acids or triacylglycerols. Furthermore, the development of the process for the production of methyl esters is of increasing importance due to demand for biodiesels [38]. Alcoholysis of high-purity triacylglycerols under dry condition can be completed in a few minutes with a strong base (such as sodium methoxide) as catalyst. The presence of free fatty acids may retard or completely terminate the reaction if Brønsted or Lewis acid is added as catalyst. Alcoholysis can also be carried out using glycerol as the alcohol donor with lipases or base as catalyst and this is called glycerolysis. Glycerolysis of oils is an important reaction to produce partial glycerides [39]. Monoacylglycerols and diacylglycerols are important food emulsifiers that are produced through glycerolysis reaction of oils and fats, followed by fractionation process.

Recently, Guo's research group reported the enzymatic glycerolysis of sardine oil to produce monoacylglycerols rich in ω-3 polyunsaturated fatty acids (n-3 PUFAs) [40]. In this work, they combined glycerolysis with molecular distillation to yield the desired monoacylglycerols. The reaction conditions used were a mole ratio of 3:1 glycerol:sardine oil, a 5% load of lipase based on the mass of total reactants, 50°C, and 2 h reaction time. In addition, when performing the reaction in larger scale in a 1 L batch reactor under 200 rpm stirring, approximately the same yields were reached. Accordingly, the work generated a scalable experimental procedure to obtain omega-3-enriched monoacylglycerols.

B. Interesterification

Interesterification is the exchanging reaction of two acyl groups between two esters [20,41]. This reversible reaction also requires a catalyst to speed up the reaction to equilibration. Interesterification is generally used to alter the melting profile of fats, crystalline characteristics, solid fat content, and plasticity. For triacylglycerols, interesterification can take place in an intramolecular or intermolecular mode and both modes at the same time (Scheme 2.2). The interesterification in the presence of an alcoholate can also generate monoalcohol esters, as in the methanolysis reaction described earlier (Scheme 2.1). Thus, formed glycerol ion can react with the acyl group in the adjacent carbon in the glycerol molecule to yield an intermediary cyclic species with a negative charge that will promote acyl migration as shown in Scheme 2.2b. However, this can also take place in an intermolecular mode. Lipase-catalyzed interesterification represents a more environmentally friendly way that also offers the advantage of region selectivity; for example, *sn*-1,3-specific lipases such as Lipozyme TL IM (*Thermomyces lanuginosus* lipase) will not, in principle, affect the acyl bond in the *sn*-2 position of glycerol backbone.

Ifeduba et al. [42] recently reported the enzymatic interesterification to generate low saturated, zero-*trans*, interesterified fats with 20% or 30% saturated fatty acids. Accordingly, tristearin or tripalmitin was initially blended with sunflower oil rich in oleic acid at different ratios (0.1:1, 0.3:1,



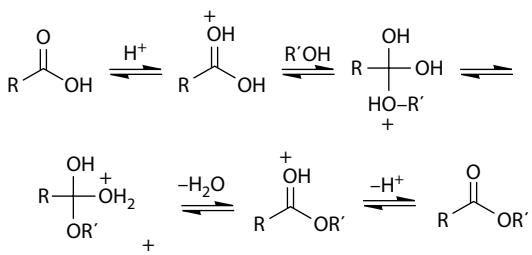
SCHEME 2.2 (a) Intramolecular reaction between a glycerate ion and acylate carbonyl R2; (b) intermolecular reaction between a glycerate ion and the acylate carbonyl R1 of another triacylglycerol molecule.

and 0.5:1 [w/w]). By plotting the total composition of the blended mixture against the molar ratio used, they were able to determine which blended mixture will yield the desired fat. Posteriorly, they carried out the enzymatic interesterification using Lipozyme TL IM. Based on their results, the interesterified fat contained 5%–10% more saturated fatty acids in the *sn*-2 position than the blended mixture and had broader melting profiles.

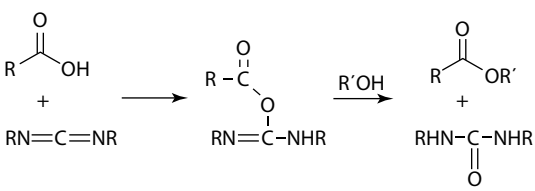
C. Esterification

Esterification is the reaction of an alcohol with a fatty acid; for example, the reaction of one molecule of glycerol with one to three molecules of fatty acids yields acylglycerols [43,44]. This reaction can take place in the presence of a catalyst or by activating fatty acid. For example, esterification can be catalyzed by 1%–5% of a Brønsted acid or by a Lewis acid (e.g., boron trifluoride etherate). The mechanism involves the nucleophilic reaction of an alcohol with a protonated carbonyl, followed by a rapid exchange of protons between oxygen atoms, and the elimination of a water molecule (Scheme 2.3). The direct esterification of alcohol with fatty acid is a reversible reaction and the yield can be improved by increasing the amount of the reactants or by removing the product (e.g., removal of water). This reaction can be performed in an industrial scale where water may be removed by distillation.

Another strategy for esterification constitutes the activation of the carbonyl group of a fatty acid prior to nucleophilic attack by a hydroxyl group [37]. Activating the carbonyl group of a fatty acid can transform the hydroxyl group into a better leaving group. One of the most used synthetic strategies to activate a carbonyl group is the transformation of a fatty acid into an acyl chloride. Other strategies involve performing the reaction in the presence of pyridine, which can trap the generated acid and also act as a cocatalyst by forming an acylium ion, which makes carbonyl more prone to react with alcohols. However, 4-dimethyl aminopyridine is more effective. Carbodiimide is also frequently used as a coupling agent to activate carboxylic acid and generate desired ester bond. Basically, this agent reacts on the diimide to generate an isourea *O*-acyl that can react with alcohols yielding the ester product and dialkyl urea (Scheme 2.4).



SCHEME 2.3 Direct esterification.



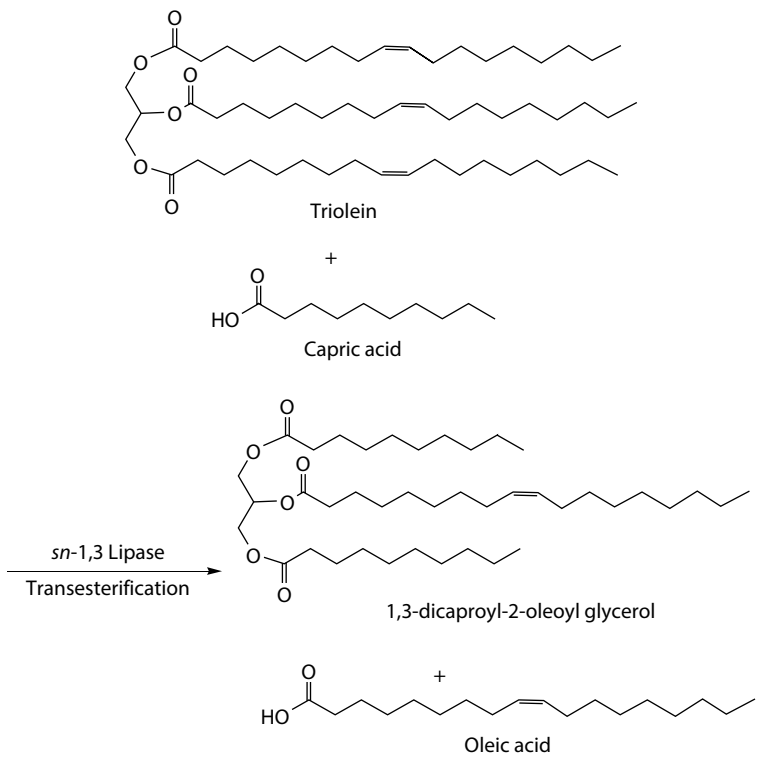
SCHEME 2.4 Carbonyl activation by carbodiimide.

D. Transesterification

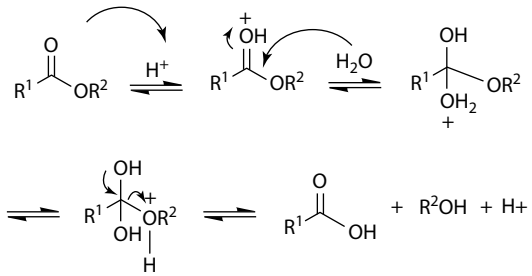
Transesterification refers to the reaction between triacylglycerol and acyl donors (fatty acids or fatty acid short-chain alcohol ester, e.g., fatty acid ethyl esters). The reaction between triacylglycerols and fatty acids is also called acidolysis [45]. As reviewed elsewhere [46], transesterification is an important strategy to produce structured lipids. With *sn*-1,3-specific lipases as biocatalysts, ABA-type structured lipids can be produced. A typical example is illustrated in Scheme 2.5 where the fatty acid exchange takes place at *sn*-1,3 positions of glycerol backbone under catalysis by *sn*-1,3-specific lipase. By applying excessive dosage of external fatty acids, most of the original fatty acyl groups in *sn*-1,3 positions of triacylglycerols will be replaced by external acid. A new type of structured lipids can be produced by the removal of replaced fatty acids and unreacted fatty acids with distillation. This reaction has found a variety of applications in producing milk fat equivalents and low-calorie fats [47].

E. Hydrolysis

Hydrolysis is one of the most used reaction procedures in lipids chemistry. Hydrolysis of triacylglycerols involves the cleavage of an ester bond by a water molecule leading to a fatty acid and a compound containing a hydroxyl group, for example, diacylglycerols, monoacylglycerols, and glycerol [48]. This reaction can take place in acidic, basic, or neutral medium. This reaction is reversible under acidic or neutral conditions but irreversible under basic conditions. The mechanism of the reaction in acidic medium is displayed in Scheme 2.6. The solubility of the reagents can determine the temperature and pressure required for the reaction to proceed. For example, tristearin that is insoluble in water needs high temperature and pressure to be hydrolyzed. Hydrolysis can also take



SCHEME 2.5 Transesterification of triolein and capric acid.



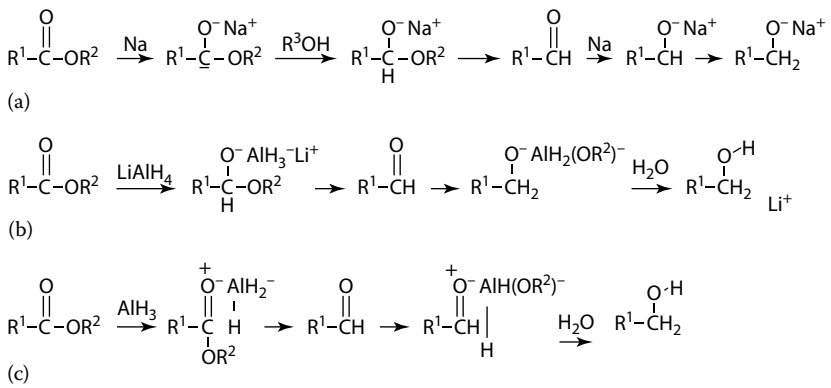
SCHEME 2.6 Mechanism of the hydrolysis of fatty esters.

place through an enzymatic pathway. For instance, lipolytic enzyme and lipase of bacterial and fungal origin can hydrolyze different lipids and fatty acid esters.

A recent example of using enzymatic approach for the hydrolysis of soybean oil was reported by Li et al. [25]. A lipase from *Rhizopus oryzae* (rProROL) produced by recombinant *Pichia pastoris* was used for the hydrolysis of soybean to generate diacylglycerols. They found that pH, temperature, water content, and enzyme concentration could affect reaction progress. The maximal content of diacylglycerols (33.11%) was achieved at a pH of 6.5, 20% water content, enzyme concentration of 30 U/g, 40°C, and 6 h. During the hydrolysis process, the enzyme displayed 1,3-regioselectivity and high hydrolytic activity demonstrating the enzyme potential for industrial processing of oils and fats.

F. Hydrogenolysis

Hydrogenolysis is the reduction of carboxylic acids, esters, ketone, aldehydes, acyl chloride, or unsaturated fatty acid chains into alcohols, aldehydes, or hydrocarbons, depending on the selectivity of the reagents and the solvent used [37]. There are mainly three kinds of reduction agents: alkali metals, hydrides, and hydrogen in the presence of a metal catalyst. For instance, reduction of ester, ketone, and aldehydes can take place in the presence of sodium and an alcohol, where an electron transfer occurs from the metal to the carbonyl as intermediary step for the generation of the product (Scheme 2.7a). Other popular reagents frequently used to reduce esters include anionic (e.g., $LiAlH_4$) or neutral (e.g., AlH_3) hydride. For example, while anionic hydride transfer a hydride through a nucleophilic substitution on an activated carbonyl reducing an aldehyde into an alcoholate, neutral hydride transfers a hydride inside a reducing agent-substrate (Scheme 2.7b and c).



SCHEME 2.7 Multiple mechanisms for the reduction of esters functionalities using Na (a), $LiALH_4$ (b), or AlH_3 (c) as reduction agent.

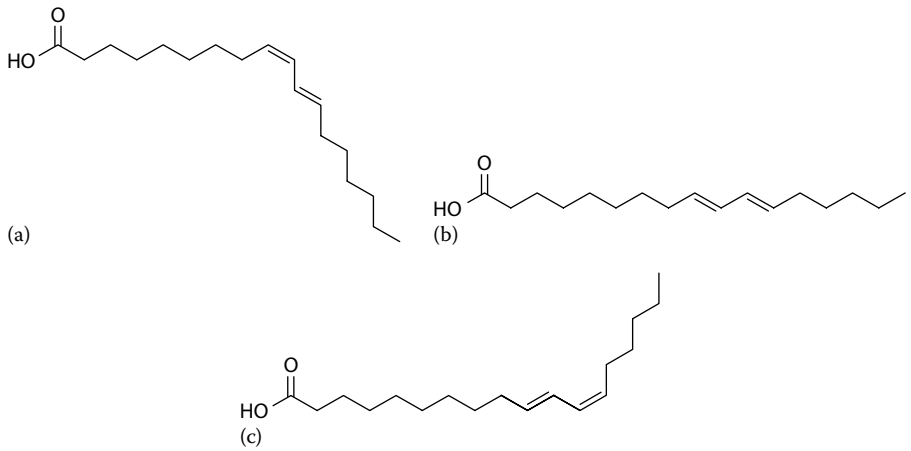
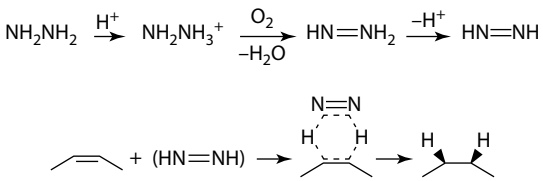


FIGURE 2.5 Structure of three conjugated linoleic acid (CLA) isomers (a) c9,t11; (b) t9,t11; and (c) t10,c12.



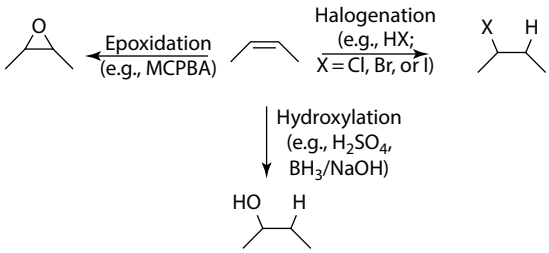
SCHEME 2.8 Noncatalytic hydrogenation using hydrazine.

Hydrogenation of double bonds can be full or partial depending on the catalyst used. This can be carried out in heterogeneous or homogeneous reaction conditions. The former is the most used since it facilitates the removal of the catalyst. Hydrogenation can proceed as partial hydrogenation and full hydrogenation. While full hydrogenation yields a fully saturated product, partial hydrogenation may generate isomerization (e.g., *cis* → *trans*) or migration of double bonds. Isomerization or conjugation reaction for conjugated unsaturated fatty acids/oils is an important reaction to produce biologically active conjugated linoleic acids (CLAs); for example, the beneficial health effects associated with the c9,t11; t9,t11; and t10,c12 CLA isomers (Figure 2.5) [49]. The latter can depend on the catalyst and the operating conditions used. For instance, nickel leads to the formation of more *trans* isomers than copper and the use of low temperature can limit the number of *trans* configuration generated.

Nonetheless, hydrogenation can also take place in a noncatalytic way but with a chemical hydrogen donor conferring a higher stereoselectivity [37]. Examples of reagents that can act as hydrogen donor constitute hydrazine (NH_2–NH_2), azodicarboxylic acid (HOOC–N=N–COOH), tosylhydrazine (CH_3–C_6H_4–SO_2NHNH_2), and hydroxylamine (NH_2–OH). In this type of reaction, the *cis* configuration is not shifted and only the ethylenic and acetylenic bonds are reduced by the hydrogen transfer to sp^2 carbons (Scheme 2.8).

G. Addition Reactions to Double Bond

The most common reaction of double bond is the addition reaction. Different reagents, both inorganic and organic, have proved to add to alkene (Scheme 2.9). This reaction is generally exothermic as the ethylenic pi bond is relatively weak compared to the sigma bond. The most used addition reactions are epoxidation where oxygen is transferred to olefin. Epoxidation of oils and unsaturated fatty acids is used for the generation of oxiranes valuable for industrial application [50]. Other addition reactions include halogenation where halogens are added to the ethylenic bond and hydroxylation



SCHEME 2.9 Examples of addition reactions to double bond.

to generate alcohol. However, halogenation is mostly used for analysis purpose (e.g., to determine iodine values of unsaturated oils and fats) or to form intermediate products in synthesis.

V. PHOSPHOLIPIDS

Phospholipids are the major constituent lipids in biomembranes [51]. Membranes are the barriers that constitute the outer boundary of cell (plasma membrane) and the inner compartment (organelles). They are selectively permeable and control the flow of substances in and out of the cell [52]. Membranes are mainly composed of proteins, sugar, and lipids. The phospholipids are generally organized as a bilayer structure where the protein molecules are totally or partially embedded into the bilayers (Figure 2.6). Noncovalent interactions keep together the lipids and proteins while sugars

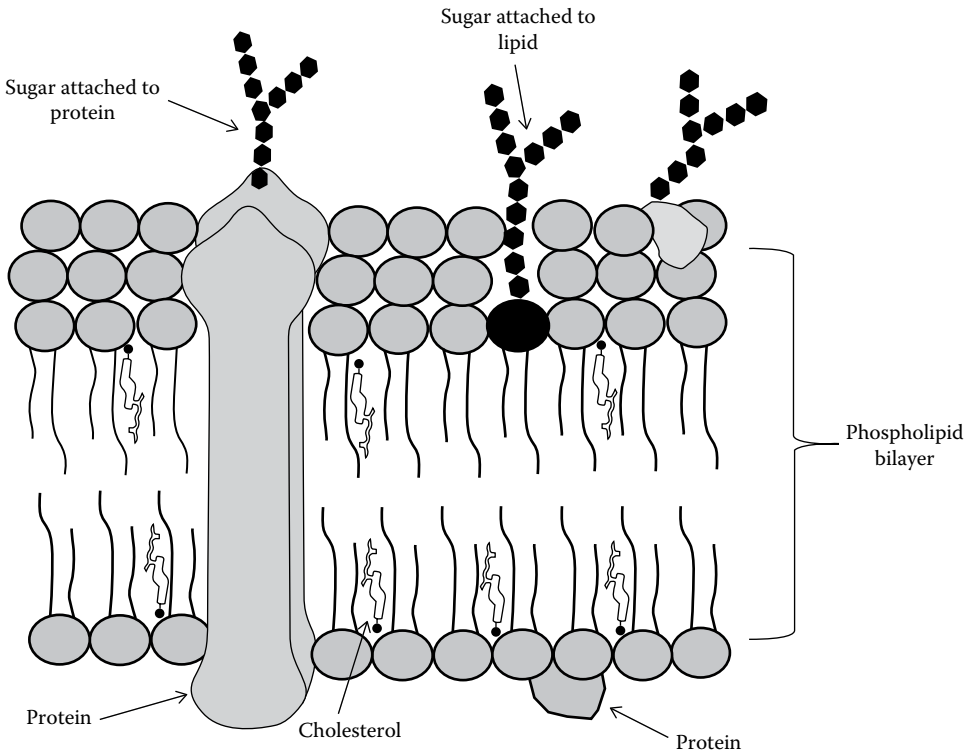


FIGURE 2.6 Graphical representation of a biological membrane. (From Brown, B.S., Biological membranes, The Biochemical Society, http://www.biochemistry.org/portals/0/education/docs/basc08_full.pdf, 1996, accessed October 10, 2016.)

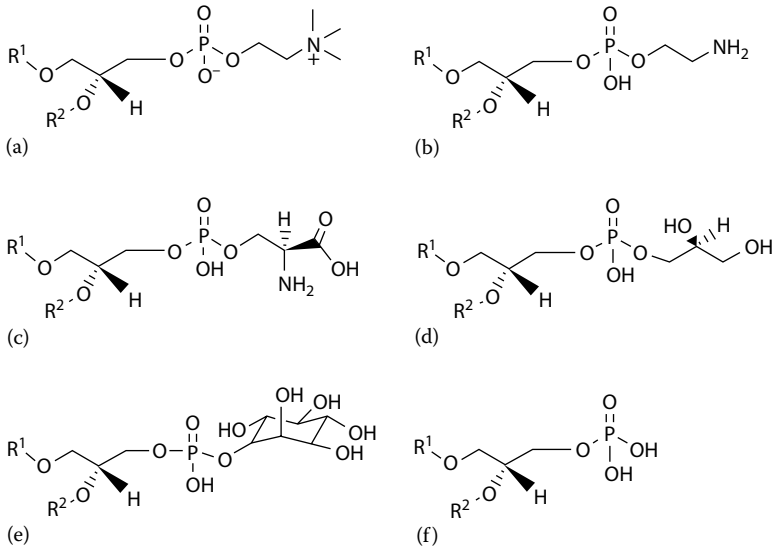


FIGURE 2.7 Structure of glycerophospholipids. (a) PC, (b) PE, (c) PS, (d) PG, (e) PI, and (f) PA. R^1 and R^2 are fatty acids.

are covalently linked to some of the lipids and proteins within the membranes. Different lipids have specific functions in the membrane as their structure will determine the molecular organization influencing membrane fluidity [4].

The most common phospholipids, known as glycerophospholipids, are based on a glycerol backbone. Glycerophospholipids can be subdivided into distinct classes, based on the nature of the polar head group at the *sn*-3 position of the glycerol backbone in eukaryotes and eubacteria or the *sn*-1 position in the case of archaebacteria. Accordingly, the different classes of glycerophospholipids are phosphatidylcholine (PC; Figure 2.7a), phosphatidylethanolamine (PE; Figure 2.7b), phosphatidylserine (PS; Figure 2.7c), phosphatidylglycerol (PG; Figure 2.7d), phosphatidylinositol (PI; Figure 2.7e), and phosphatidic acid (PA; Figure 2.7f). If the *sn*-1 position (R^1 in Figure 2.7) is hydrolyzed, the corresponding lysophospholipid is formed. A second glycerol unit constitutes part of the head group in the glycerophosphoglycerols (PG; Figure 2.7d) and glycerophosphoglycerophosphates (Figure 2.8), whereas for the glycerophosphoglycerophosphoglycerols (cardiolipins; Figure 2.8), a third glycerol unit is typically acylated at the *sn*-1′ and *sn*-2′ positions to create a pseudo-symmetrical molecule.

VI. PHYSICAL PROPERTIES OF PHOSPHOLIPIDS

The packing behavior of lipids is determined by the intermolecular repulsive and attractive forces of the polar head group of the lipid [53]. In phospholipids, the hydrogen bonding between hydrogen bond donor functional group such as −P−OH, −OH, and $-NH_3^+$ and hydrogen bond acceptor including −P−O^- and $-COO^-$ can account for the molecular phase transition to go into a hexagonal phase. If submerged in water, these zwitterionic amphiphilic lipids can assemble to form a bilayer where the polar head group points toward the water and the hydrophobic tails locate in between the hydrophilic heads. This bilayer will form sealed compartments, known as liposomes, to eliminate the lipid tails contact with water (Figure 2.9). Accordingly, liposomes can be absorbed by many cells by interaction with the cell plasma membrane and used to deliver drugs to specific organs in the body.

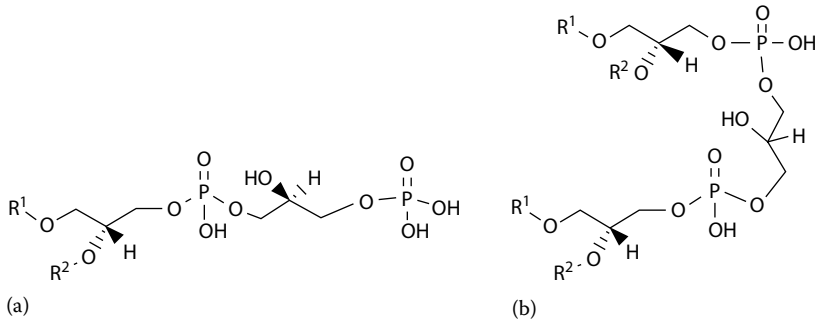


FIGURE 2.8 Structure of (a) glycerophosphoglycerophosphates and (b) glycerophosphoglycerophospho glycerols.

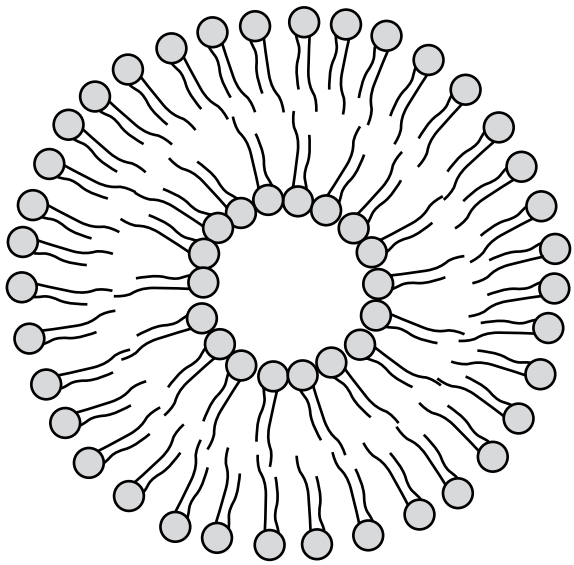


FIGURE 2.9 Representation of a liposome structure.

In membranes, the fluidity of a lipid bilayer depends on temperature, fatty acyl composition, and cholesterol content [54]. At low temperature, the lipid chains are more closely organized. However, as temperature increases the lipid bilayer becomes more fluid, forming a more disordered arrangement. The temperature range at which membranes are more rigid is determined by the length of the fatty acyl chain and the degree of unsaturation. For instance, the temperature at which the membrane becomes fluid is lower if fatty acid chains are shorter or have more double bonds. In addition, the membrane fluidity is also dependent on the cholesterol content. For example, eukaryotic membranes contain one cholesterol molecule per two phospholipid molecules [54]. In membranes, cholesterol locates in between the phospholipid chain with the hydroxyl group pointing toward the polar head of phospholipids and the steroid ring in between the lipid chains.

Phospholipids are zwitterionic surfactants that have GRAS (Generally Recognized as Safe) status, which enables their use in food [55]. For instance, phospholipids can also be used in food nanotechnology. Nanotechnologies can encapsulate, protect, and deliver lipophilic ingredients such as omega-3

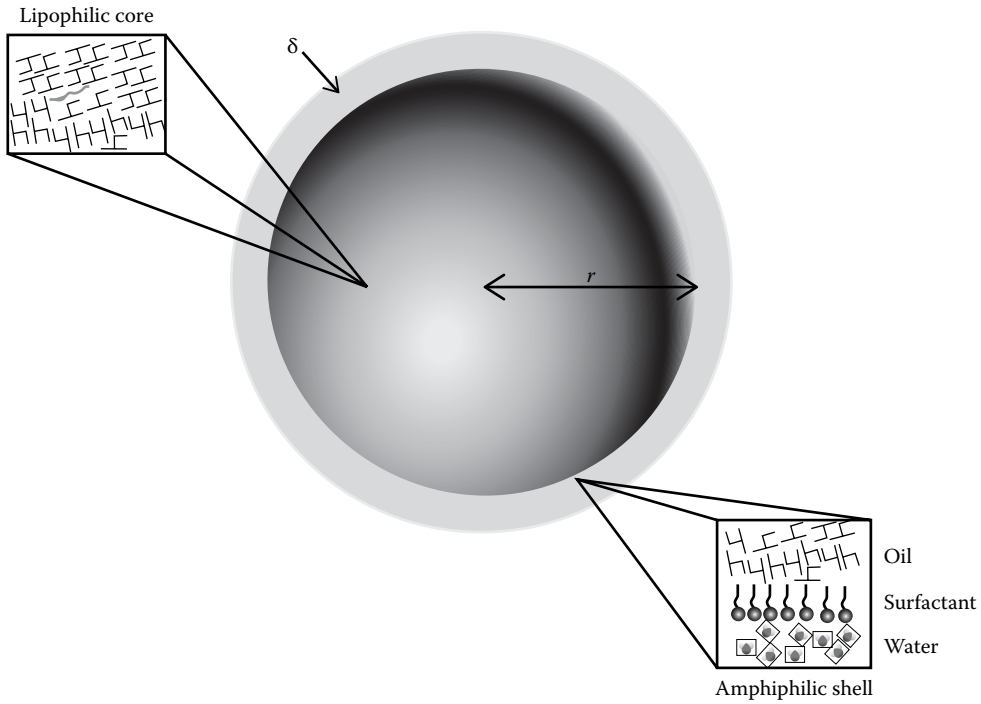


FIGURE 2.10 Schematic representation of nanoemulsions' core shell. (From McClements, D.J. and Rao, J., *Crit. Rev. Food Sci. Nutr.*, 51, 285, 2011.)

fatty acids. In addition, the small particle size of these nanoparticles confers higher stability, optical clarity, and bioavailability. For instance, since nanoparticles such as nanoemulsions poorly scatter light, they can be incorporated into food products such as fortified drinks that are required to be optically clear or only slightly turbid [56]. Nanoemulsions contain (1) an oil phase composed of a nonpolar component such as acylglycerol and free fatty acids, (2) an aqueous continuous phase that mainly consists of water and other water-soluble components, and (3) an interface between oil/water phases, consisting of emulsifiers, stabilizers, or surface-active compounds to improve the long-term stability of the nanoemulsions (Figure 2.10). Emulsifiers adsorb to droplets' surface, prompting droplets' disruption and preventing droplets' aggregation. The stability of nanoemulsions under environmental stresses, including heating, cooling, pH changes, and storage is largely influenced by the property of emulsifiers. In food industry, phospholipids are commonly used as zwitterionic surfactants. However, natural phospholipids usually require the use of a cosurfactant, the compounds containing a hydrocarbon chain and a polar head, to ensure a better stability of nanoemulsions.

Nanoliposome is a type of liposome with the smaller diameter in nano range and is assumed to offer better encapsulation or controlled release of food material so as to ensure better bioavailability and stability and longer shelf-life of oxidation-sensitive food ingredients [55]. Nanoliposomes are formed by the auto-assembly of amphiphilic molecules such as phospholipids when energy is supplied (e.g., heating, sonication). These amphiphilic molecules assemble to shield the hydrophobic chains from the aqueous environment, while the polar heads maintain contact with the water layer and when sufficient energy is provided, a closed organized vesicle (nanoliposomes) could be formed (Figure 2.11). The structural feature of phospholipids responsible for bilayer-forming molecules is their amphiphilicity, whereas the hydrophobic domain between the bilayer of vesicles enables them to entrap and deliver lipophilic, hydrophobic, or amphiphilic compounds. Nanoliposomes have

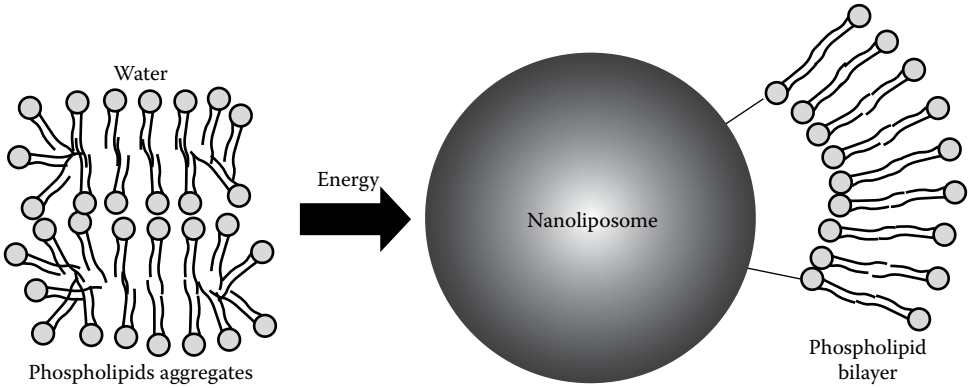


FIGURE 2.11 Simplified representation of the mechanism of nanoliposome formation. (From Mozafari, M.R. et al., *J. Liposome Res*., 18, 309, 2008.)

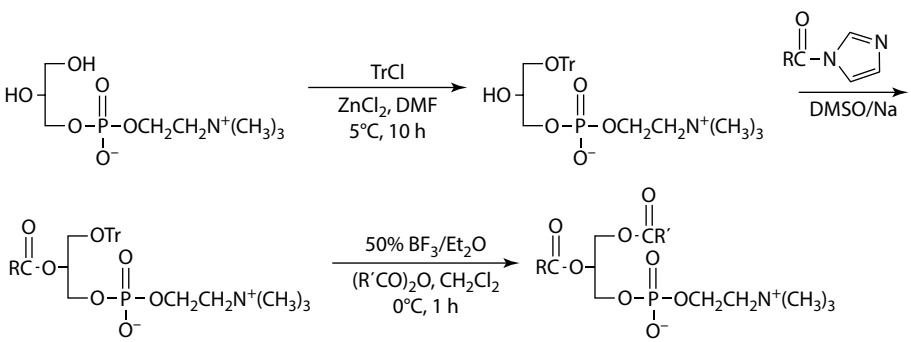
demonstrated its application potential, particularly when used to encapsulate antimicrobials in food products to prevent microbial contamination [55].

VII. CHEMICAL AND ENZYMATIC SYNTHESIS OF PHOSPHOLIPIDS

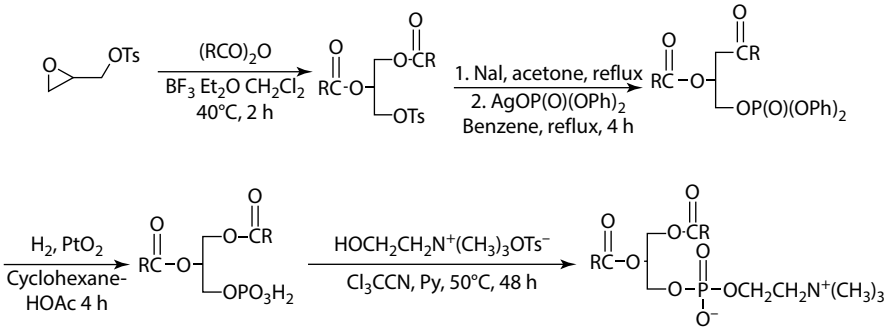
A. Chemical Synthesis of Phospholipids

Introducing specific functional groups in phospholipids has become of interest for the generation of structurally relevant phospholipid derivatives for membrane-associated processes [57]. However, relying on enzymatic process to cleave the acyl bond and later to introduce new functional group through chemical reactions limits the yield obtained for the desired products. Therefore, pure chemical strategies have come into play to address this problem. In this type of synthetic pathway, glycidyl, protected glycerol, and *sn*-glycero-phosphatidylcholine (GPC) are common starting materials used for the synthesis of phospholipids. They can generate mixed chain diacyl phospholipids. For instance, GPC is used to prepare 1-*O*-trityl-*sn*-glycero-3-phosphocholine using trityl chloride and zinc chloride, and 1-*O*-trityl-GPC is posteriorly acylated at *sn*-2 position using oleoyl imidazolide [58]. The resulted product is deprotected using boron trifluoride (BF_3) and acylated with palmitic anhydride (Scheme 2.10).

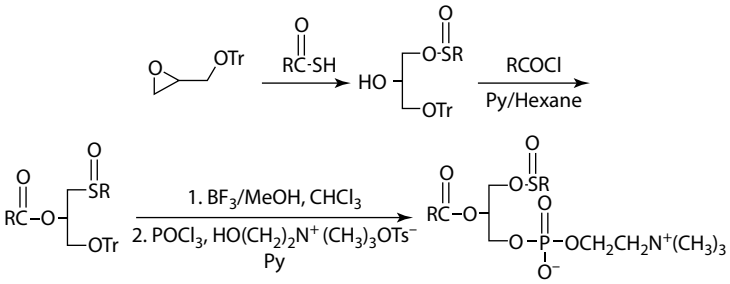
Optically pure diacyl-PCs can be generated using (R)- or (S)-glycidyl tosylate as starting material (Scheme 2.11) [59]. Basically, the arene sulfonate works as a protecting group in the *sn*-3



SCHEME 2.10 Preparation of phospholipids via 1-lyso-2-acyl-*sn*-glycero-3-phosphocholine.



SCHEME 2.11 Preparation of optically pure diacyl-PC from (R)- or (S)-glycidyl tosylate.



SCHEME 2.12 Preparation of 1-thioester-2-ester-PC.

position, thereby preventing acyl migration from *sn*-2 position while a Lewis acid–catalyzed reaction takes place to open and acylate the epoxide functionality using fatty acid anhydride and BF_3. Subsequently, the tosylate group can be converted to phosphate ether using sodium iodide and later silver diphenyl phosphate and the resulting diphenyl phosphate is converted into phosphatidic acid and posteriorly into phosphatidylcholine using choline tosylate in the presence of trichloroacetonitrile. However, chiral glycidyl is expensive, making the synthesis of optically pure diacyl-PCs through this methodology impractical.

Other analogues of phospholipids can also be prepared from protected glycidol [60]. For instance, the 1-thioester-2-ester and 1,2-dithioester-PC can be generated using trityl glycidyl ether as starting material (Scheme 2.12). The ring opening takes place in the presence of thioacid to yield the thioester at *sn*-1 position and later acylation occurs at the *sn*-2 position with acylchloride. After detritylation, the resulted product can be converted into phosphocholine derivatives using phosphorus oxychloride and choline tosylate. Although commercially available, trityloxyglycerols can be prepared from allyl alcohol or from glycidol.

B. Enzymatic Synthesis of Phospholipids

Phospholipids can be structurally modified with the appropriate enzymes (Figure 2.12) [61]. Although phospholipases are found ubiquitously in nature, only a few enzymes have been produced in large scale [62]. Examples of natural enzymes applied for the modification of phospholipids include phospholipases A_1 (EC 3.1.1.32; PLA_1) and A_2 (EC 3.1.1.4, PLA_2) and lipases. These enzymes can be used for acyl modification purposes. *Sn*-1,3 lipases can specifically modify position 1 but not *sn*-2. Only PLA_2 has demonstrated to be valuable for the latter purpose. For instance, PLA_1 hydrolysis yields a 2-acyl lysophospholipid and PLA_2 hydrolysis produces 1-acyl lysophospholipid. Phospholipase D (PLD) can modify polar head and phospholipase C (PLC) can hydrolyze the bond

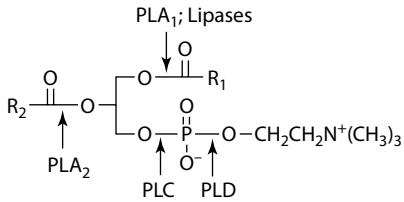


FIGURE 2.12 Enzymatic modification of phospholipids. (From Xu, X. et al., Enzymatic modification of phospholipids and related polar lipids, in: Gunstone, F.D., ed., *Phospholipid Technolology and Applications*, The Oily Press, 2008, pp. 41–82.)

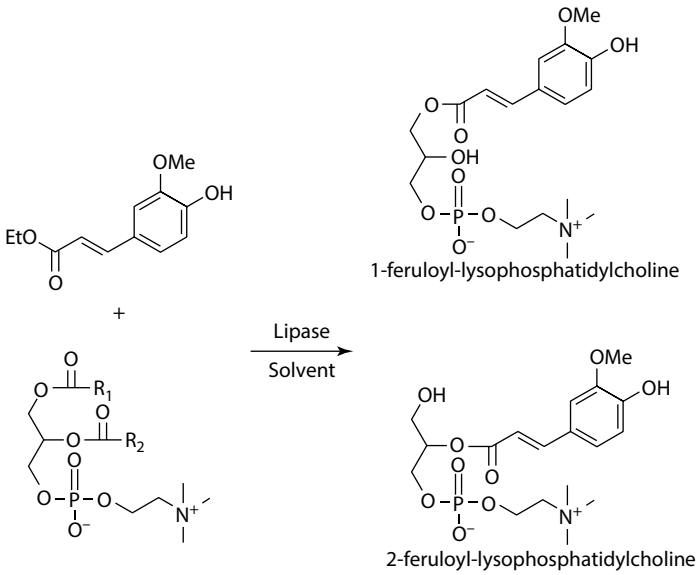
between glycerol OH and the phosphate group. Accordingly, structured phospholipids with specific fatty acid composition and polar head group and with desired chemical and physical properties can be generated by enzyme-catalyzed reactions such as hydrolysis, transesterification, and transphosphatidylation [63].

1. Hydrolysis

PLA_1 has been used for the enzymatic modifications of phospholipids for industrial processes [61]. For instance, PAL_1 is used in the degumming process of edible oils. Removal of one of the acyl chains of phosphatidylcholine generates a more hydrophilic compound known as lyso-PC. This compound possesses higher emulsifying properties since the molecule becomes more hydrophilic and consequently is easy to form oil-in-water emulsions. The enzymatic hydrolysis of naturally occurring lecithin is one of the processes to generate high amounts of lysophospholipids. Both lipases and phospholipases are useful for this process. Different conditions and enzymes have been used to obtain lysophospholipids from low to high yields. Recently, Lim et al. [64] reported an experimental procedure to produce lyso-PC containing high content of unsaturated fatty acids (78% linoleic acid) in relation to the total content of fatty acids. Accordingly, they optimized different reaction parameters such as temperature, water content, and enzyme load to increase yield and minimize acyl migration. Optimal reaction conditions were 10% of water content and 1% of enzyme load based on PC content, 60°C, and 3 h reaction time, under which 84% yield of lyso-PC was obtained [64].

2. Transesterification

Transesterification or replacement of fatty acyl chain by exogenous fatty acids can be performed using PAL_1, PAL_2, and lipases. Several parameters including enzyme dosage, water content, solvents, reaction time, temperature, acyl donor, and PL polar head group can influence the reaction performance [61]. For instance, increasing the fatty acid concentration to perform lipase-catalyzed esterification or transesterification inhibits hydrolysis and increases the yield of the reaction [65]. Although reaction time needed is independent of fatty acid concentration in esterification reaction, the reaction time has to be increased with fatty acid concentration in transesterification. The substrate molar ratio used can influence the cost of removal of the fatty acids from the product. Therefore, a compromise is required to select the substrate mole ratio of the reaction. Water content also plays an important role on enzyme activity. For instance, PAL_2 requires a higher water content than lipases to function and as such yields might be lower since major hydrolysis occurs in parallel [65]. Other strategies are to use water-mimicking molecules such as ethylene glycol or propylene glycol that promote esterification by activating the enzyme but do not contribute to hydrolysis. In addition, if the use of solvent can be avoided, the reaction should be carried out in a solvent-free system, especially, in the case of organic solvents that can generate hazardous wastes. Still organic solvents have demonstrated to increase mass transfer. Accordingly, organic solvents lower the viscosity of the system and allow better accessibility of enzyme to substrate. Nonetheless, the polarity of solvent has strong water solvation effect and thus deactivate enzyme. Therefore, generally used organic solvents have a log $P > 2$.



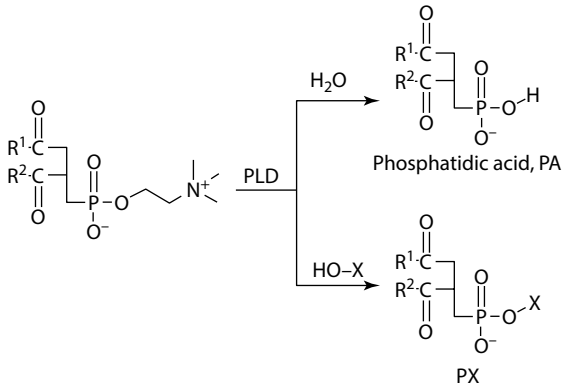
SCHEME 2.13 Lipase-catalyzed synthesis of feruloylated lysophospholipids.

A recent example of the introduction of functional compound into phospholipids to generate a new phospholipid derivative was reported by Yang et al. [66]. The authors reported the enzymatic synthesis of feruloylated lysophospholipids using phosphatidylcholine and ethyl ferulate (EF) as ferulic acid has demonstrated antioxidant properties (Scheme 2.13). Different lipases and solvents have been examined, and Novozym 435 and toluene were found to be good starting point for further optimization. Subsequently, binary solvents were used in combination with toluene and 90:10 (v/v) of toluene: chloroform afforded the optimal enzyme activity. In addition, the optimal conditions for the generation of feruloylated lysophospholipids were 60 mg/mL of enzyme load, 1.5 h of PC's hydrolytic time, and a molar ratio of 5:1 PC:EF.

Recently, Chojnacka et al. [67] reported the lipase-catalyzed transesterification of egg-yolk phosphatidylcholine with pomegranate seed oil (PSO) containing over 77% of conjugated linolenic acid (punicic acid; 18:3, 9c,11t,13c). Three different immobilized lipases were used as biocatalyst and the enzyme load, reaction time, and mole ratio of substrates were optimized. Accordingly, the best results were obtained when using CALB, 20% of enzyme load, 72 h of reaction time, and a mole ratio of PC:PSO, 1:3. In addition, the resulted product contained up to 50% of polyunsaturated fatty acids.

3. Transphosphatidylation

Different from enzymatic modification of fatty acyls in phospholipids, modification of the polar head group of glycerophospholipids can only be carried out using enzymes in the same category as PLD (Scheme 2.14). This modification is generally carried out to increase the content of one particular phospholipid species (e.g., PS, phosphatidylserine) using phospholipids present in lecithin as starting materials [68]. Another type of modification is also performed using alcohol acceptors (HO−X; Figure 2.13) to generate phosphatidyl derivatives of drugs such as genipin, ascorbic acid, and arbutin with different physicochemical properties. In addition, it can also be used to prepare novel derivatives such as alkylphosphate ester of potential pharmaceutical value [61].



SCHEME 2.14 Enzymatic transphosphatidylation of phosphatidylcholine using water or an alcohol acceptor.

VIII. SEPARATION, ANALYSIS, AND CHARACTERIZATION OF LIPIDS AND PHOSPHOLIPIDS

Multiple techniques are used for separation, analysis, and characterization of acylglycerols and phospholipids. The main reasons for lipid analysis and characterization are to ensure food quality, identify and prevent lipid-oxidation products, and optimize processing [69]. Well-known and highly used techniques for lipid analysis are TLC, HPLC, and GC. However, to investigate the structural features and physical properties of lipids, nuclear magnetic resonance spectroscopy (NMR), differential scanning calorimetry (DSC), Fourier transform infrared (FTIR), and x-ray diffraction studies are also required. The combination of multiple techniques enables the isolation and determination of physicochemical features of a lipid. Consequently, the use of multiple techniques helps to disclose the specific functionalities pertaining to acylglycerols or phospholipids.

A. Separation of Acylglycerols and Phospholipids

Several separation techniques are available today to enrich, isolate, or purify lipids and phospholipids. These techniques can be used to modify the properties of lipids. For instance, palm oil, milk fat, and palm-kernel oil can be fractionated by crystallization to generate different functional lipids [70]. The most used techniques for lipid separation in industrial-relevant scale are crystallization, fractionation, and short path distillation (SPD). In the case of SPD, also known as molecular distillation, it is a thermal separation technique that operates at pressure down to 0.001 mbar that allows the separation of heat-sensitive and high-boiling-point products minimizing thermal stress and product decomposition. Figure 2.14 displayed an example of the use of this technique for the separation of different acylglycerol products from the glycerolysis of sardine oil [40]. In laboratory scale, the techniques mainly used are preparative thin-layer chromatography (PTLC) and column chromatography. In both techniques, lipids and phospholipids are isolated based on their polarity. However, PTLC allows the isolation of lipids in milligrams scale. In contrast, column chromatography can be used both from the mg to the industrial scale. However, column chromatography is avoided in food industry due to the use of large quantity of organic solvent that may be needed during the purification process.

B. Analysis of Acylglycerols and Phospholipids

The main techniques used for qualitative and quantitative analyses of lipids in a mixture of lipids are TLC, HPLC, and GC. These techniques separate lipids based on their affinity to a stationary and a mobile phase. However, these techniques rely on the detection technique used. For instance,

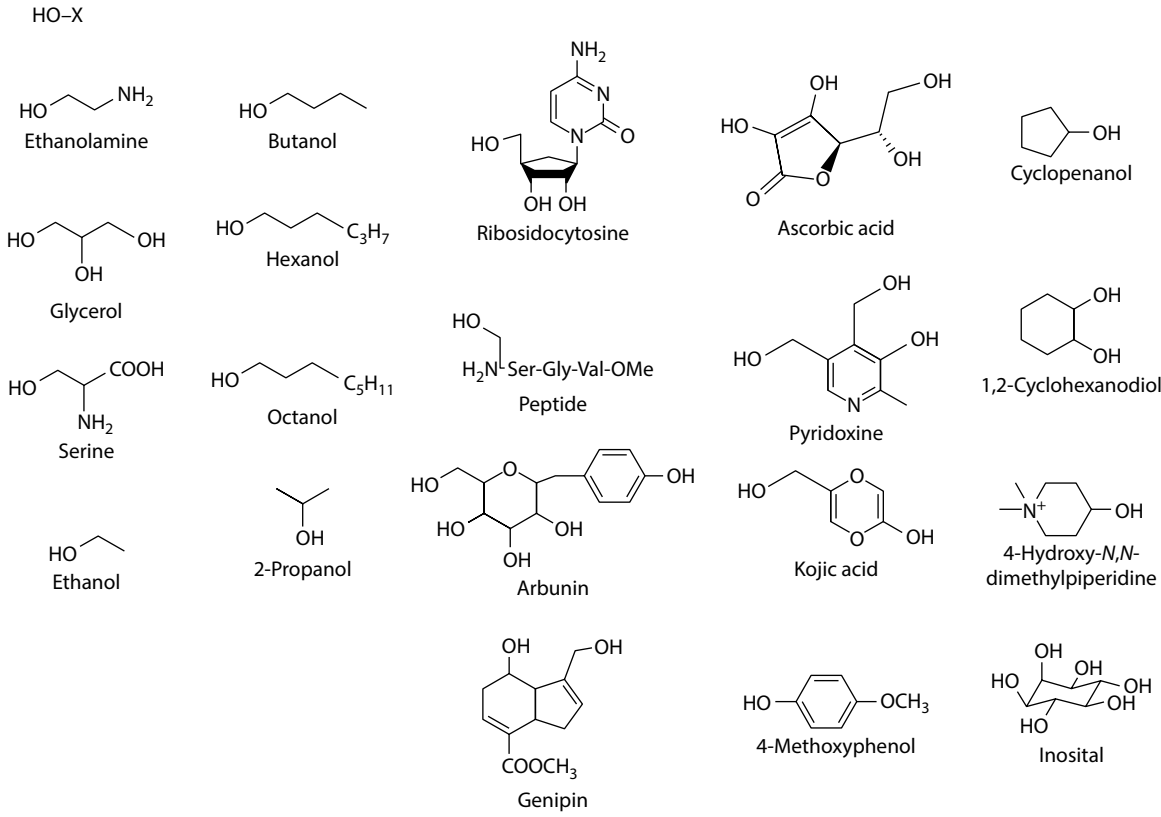


FIGURE 2.13 Alcohol acceptors used in enzymatic transphosphatidylation. (From Xu, X. et al., Enzymatic modification of phospholipids and related polar lipids, in: Gunstone, F.D. ed., *Phospholipid Technolology and Applications*, The Oily Press, 2008, pp. 41–82.)

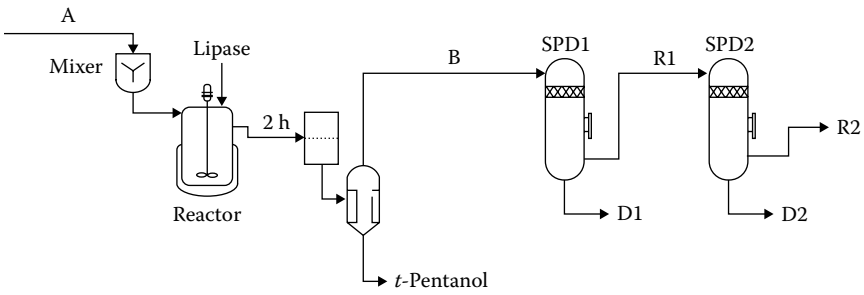


FIGURE 2.14 Representation of the molecular distillation of the product of glycerolysis of sardine oil at selected temperatures, where D and R stand for distillate and residue, respectively. A, Sardine oil + glycerol + *t*-pentanol; B, TAG, DAG, MAG, FFA, glycerol; R1, TAG, DAG, MAG; D1, glycerol, FFA; R2, TAG, DAG; D2, MAG. (From Solaesa, Á.G. et al., *Food Chem*., 190, 960, 2016.)

GC-FID (FID: flame ionization detector) helps measure the concentration of lipids in a sample of interest, while GC-MS (MS: mass spectroscopy) both measures concentrations and provides information regarding the structure of the lipids in the same sample.

1. Thin-Layer Chromatography

TLC is a technique in which compounds are separated based on their affinity to an absorbent material (stationary phase), typically silica gel. Kahovcova and Odavić developed a rapid, simple, and reproducible method for the quantitative determination of phospholipids by TLC [71]. After separation, each phospholipid class was detected by spraying 50% (w/w) sulfuric acid, direct mineralization at high temperature and subsequent determination of the liberated inorganic phosphate with the Hand and Luckhaus reagent. Chloroform–methanol–water (65:25:4, v/v/v) was used as developing solvent. However, in this report only PC, lyso-PC, and PE were involved, meaning that the separation was easier than more complex samples.

For separation of more complex samples, 2D TLC is required. One example was reported by Parsons and Patton [72]. They selected chloroform–methanol–water–28% aqueous ammonia (130:70:8:0.5) in the first dimension development, followed by chloroform–acetone–methanol–acetic acid–water (100:40:20:20:10) in the second. The resultant chromatogram is shown in Figure 2.15a. Another example of the use of 2D-TLC for the determination of phospholipids is the work reported by Rouser et al. [73]. They used chloroform–methanol–28% aqueous ammonia (65:25:5) for first dimension development. Later, the chromatograms were dried for about 10 min in a TLC chamber flushed with nitrogen and then developed in the second dimension with chloroform–acetone–methanol–acetic acid–water (3:4:1:0.5). The resulting chromatograms are shown in Figure 2.15b and c.

TLC is a separation method, meaning it cannot quantify the content directly. Thus, several different quantification methods are developed such as FID (i.e., TLC-FID and high-performance TLC-FID) and colorimetric method to quantify, for instance, phosphorus content of individual spot scraped from the developed plate. The main drawbacks of TLC-based methods are that they are time-consuming and involve multisteps of operation. However, it is an accurate method and is recommended as the AOCS standard method.

2. High-Performance Liquid Chromatography

Similar to TLC, HPLC method also includes two main parts, namely, separation and detection. Both are very critical steps in the analysis of lipids and phospholipids by HPLC. If separation is not good enough, it is not possible to quantify different classes of phospholipids. On the one hand, separation can be carried out using either normal-phase or reversed-phase columns. The latter is capable

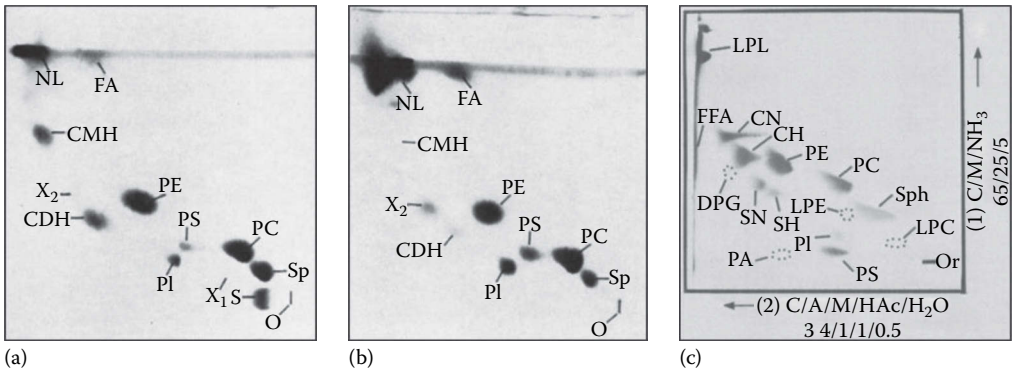


FIGURE 2.15 Two-dimensional thin-layer chromatography (TLC) of bovine milk polar lipids (a) and mammary tissue total lipids (b). The chromatograms were developed from right to left with chloroform–methanol–water–28% aqueous ammonia 130:70:8:0.5 and then in the vertical direction with chloroform–acetone–methanol–acetic acid–water 100:40:20:20:10. O, origin; S, carbohydrate (lactose) and protein; Sp, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; CDH, cerebroside dihexoside; CMH, cerebroside monohexoside; FFA, free fatty acids; NL, neutral lipid; and unknown substances listed as X1 and X2. X2 has been tentatively identified as cardiolipin. (c) Two-dimensional TLC of normal human (23-year-old male) whole brain lipids. LPL, less polar lipid (cholesterol, triacylglycerol, etc.); CN and CH, cerebroside with normal and hydroxy fatty acids; SN and SH, sulfatide with normal and hydroxy fatty acids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; Sph, sphingomyelin; DPG, diphosphatidylglycerol.

of separating different molecular species within the same lipid class. On the other hand, as lipids and phospholipids are eluted out of the column, they can be detected through several types of detectors. There are different HPLC detection techniques. However, they have to be selected based on the mixture of lipids or phospholipids that are analyzed by HPLC. Ultraviolet (UV) detectors working with fixed or variable wavelengths are commonly used in HPLC. However, lipids possess weak UV absorption or none at all. Therefore, some mass detectors, such as evaporative light-scattering detector (ELSD), are generally used instead. In the latter detection mechanism, the eluents from the column pass through a nebulizer, and the formed droplets are evaporated from the nonvolatile analytes, which are detected as particles by a light-scattering device. Any solvents can be used for this detector. Recently, a relatively new detector, charge aerosol detector (CAD) has been proven to show greater sensitivity and better precision than ELSD. Additionally, CAD is quite user-friendly since it does not require any optimization of operating parameters [74].

In general, "normal-phase" chromatography is used when different PL classes have to be separated. A highly common method for the analysis of phospholipid consists of the use of silica normal-phase Zorbax Rx-SIL (Agilent-Technologies) for separation [74,75]. In this method, a linear binary gradient: t_0 min: 0%B, t_{14} 100%B, and finally isocratic conditions (100%B) for 9 min was employed. Total chromatographic run time was 40 min per sample, which consisted of a 23 min analysis, 12 min to restore initial conditions, and 5 min for re-equilibration. Eluent A: $CHCl_3$:MeOH:30% ammonium hydroxide (80:19:0.5) and eluent B: $CHCl_3$:MeOH:30% ammonium hydroxide:H_2O (60:34:0.5:5.5) are used at a flow rate of 1 mL/min. In addition, a Sedex ELSD detector is used for signal detection. For instance, Avalli and Contarini [75] found these conditions to be optimal for the separation of PE, PI, PS, PC, and SM. However, Stith et al. [76] found that these HPLC conditions yield a varying baseline and did not separate certain phospholipids well such as PI or PS from PC. Therefore, they developed another gradient elution strategy to solve the problem. Three eluents were introduced and finally the separation was optimized. The result, indicating well separation of each class of phospholipids, is shown in Figure 2.16.

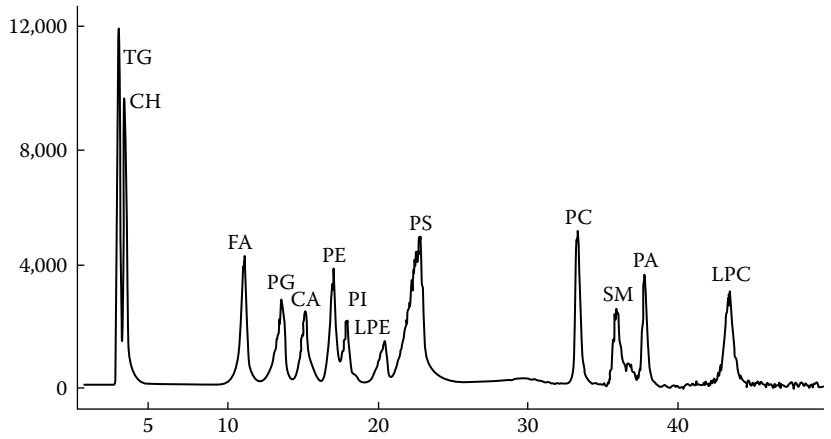


FIGURE 2.16 Separation of lipid standards by the heated diol column and three-pump high-performance liquid chromatography (HPLC) system in the Avanti Polar Lipids analytical laboratories. The abscissa represents time (min), whereas the ordinate presents the evaporative light-scattering detector (ELSD) response (μV). TG, CH, FA, PG, CA, PE, soy PI, LPE, PS, PC, SM, PA, and LPC (20 μg of each standard in a total volume of 20 μL) were injected. Note that, as is commonly found, neutral lipids produce a larger ELSD response than phospholipids or SM.

For the analysis of acylglycerol, reversed-phase HPLC is generally used instead. In this technique, the stationary phase is an *n*-alkyl hydrocarbon chain or aromatic ring immobilized on the chromatography support. Reverse-phase HPLC is a useful technique for the analysis of hydrophobic molecules including lipids, proteins, peptides, and nucleic acids with excellent recovery and resolution [77]. The resolution of the chromatograms may be determined by (1) the hydrophobic interaction of the solute molecules in the mobile phase with the stationary phase and (2) the desorption of the solute from the stationary phase back into the mobile phase. This type of chromatography uses generally gradient elution instead of isocratic elution. For lipid analysis, the lipophilic stationary phase in the column retards and separates lipophilic molecules depending on the combination and structure of the fatty acyl groups. The methylene groups in the chain contribute to an increasing retention time and the double bonds have the opposite effect, decreasing retention time. Accordingly, this technique can also be used for the differentiation of PLs with varying fatty acid composition.

3. Gas Chromatography

GC partitions a lipid mixture between a nonvolatile stationary liquid phase and a mobile gas phase, which is generally nitrogen or helium for packed columns and helium or hydrogen for capillary columns. Through GC technique, it is possible to analyze the fatty acid composition of any given lipids or phospholipids. Analysis of the fatty acid composition of a sample by GC generally requires the transformation of fatty acids into fatty acid methyl esters to yield more volatile compounds. The different fatty acids are first separated on GC columns and later detected by FID. GC-FID determines the fatty acids composition based on the comparison of the retention times displayed in the chromatogram with those obtained by reference samples. Instead, for structure elucidation GC coupled to a mass spectrometer (GC-MS) is the technique required since it provides information regarding molecular structure. However, to determine the distribution of the fatty acid in the glycerol backbone, the use of multiple techniques will be needed. For instance, mixture of *sn*-1 and *sn*-2 monoacylglycerols has to be separated by TLC and later analyzed by GC-FID. Figure 2.17 is an example of a GC chromatogram of seed oil of *Cassia artemisioides* [78].

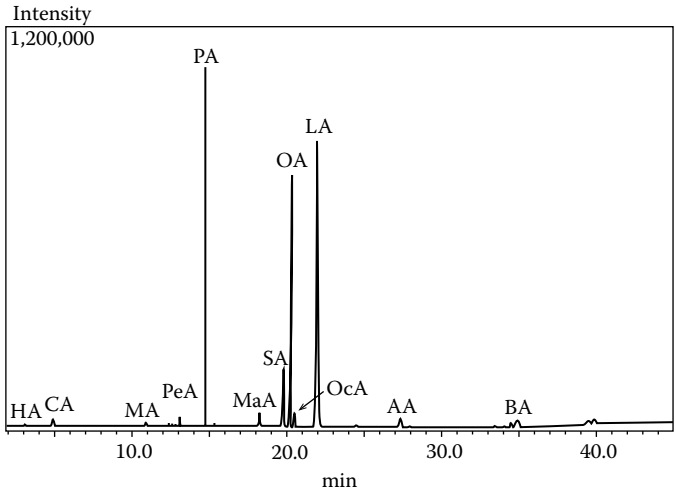


FIGURE 2.17 Gas chromatogram of seed oil of *Cassia artemisioides*. HA, hexanoic acid; CA, caprylic acid; MA, myristic acid; PeA, pentadecanoic acid; PA, palmitic acid; MaA, margaric acid; SA, stearic acid; OA, oleic acid; OcA, octadecenoic acid; LA, linoleic acid (ω-6); AA, arachidic acid; BA, behenic acid. (From Qureshi, M.N. et al., *J. Food Sci. Technol*., 52, 7530, 2015.)

C. Characterization of Lipids and Phospholipids

1. NMR

NMR is definitely a quantitative spectroscopic tool because the intensity of a resonance line is directly proportional to the number of resonant nuclei (spins). This fact enables a precise determination of the quantity of substances in solids as well as in liquids. For example, ^{1}H-NMR is a very useful technique for the structural characterization and quantification of lipids (http://lipidlibrary.aocs.org/nmr/1NMRintr/index.htm). This technique can work for the characterization of mixture of oils without prior isolation of individual components [79]. The chemical shifts obtained are based on the structure of the functional group containing the proton(s) and the intensity of the signals indicates the molar percentage of each component in the studied system. Recently, this technique demonstrated to be novel approach for the determination of glycidyl fatty acid esters, which are contaminants of refined oils and fats that can generate a carcinogenic molecule called glycidol during the digestion process [80]. The quantification formula developed relied on the signal expected for the two epoxy methylene (CH_2) protons at chemical shifts 2.56 and 2.76 ppm. This technique provides a new and very useful approach for the detection of GDE in edible oils (Figure 2.18).

Although ^{1}H-NMR is the most exploited technique due to ^{1}H nucleus abundance in an organic molecule, ^{31}P-NMR and ^{13}C-NMR are of increased interest because they can help solve specific problems in food science. For instance, ^{31}P-NMR can determine the phospholipid content in milk because phospholipids present a polar head group containing a phosphorus atom. Since phosphorus atom can be detected and quantified by ^{31}P-NMR, it becomes possible to quantify the amount and kind of phospholipids in a mixture. Figure 2.19 displays a ^{31}P-NMR spectrum of commercial lecithin in $CHCl_3/CH_3OH/H_2O$–EDTA (10/4/1). Accordingly, the signal corresponding to the phosphorus atom in different phospholipid classes can be distinguished as each displays different chemical shifts. The latter permits the quantification of phospholipids of a sample of interest.

One-dimensional ^{31}P-NMR is no doubt a powerful tool for quantitative and qualitative analyses of some simple phospholipids samples. In this method, standards need to be prepared and run to help assign the peaks to individual phospholipid species. It is of high cost firstly and also some metabolites do not even have a commercial standard. Thus, an automatic method for the

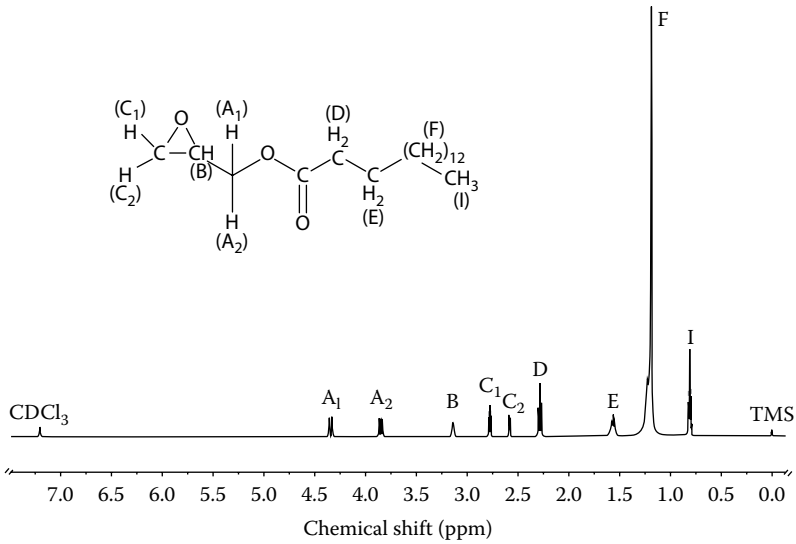


FIGURE 2.18 ^{1}H-NMR of a glycidyl palmitate. (From Song, Z. et al., *Eur. J. Lipid Sci. Technol*., 117, 1, 2015.)

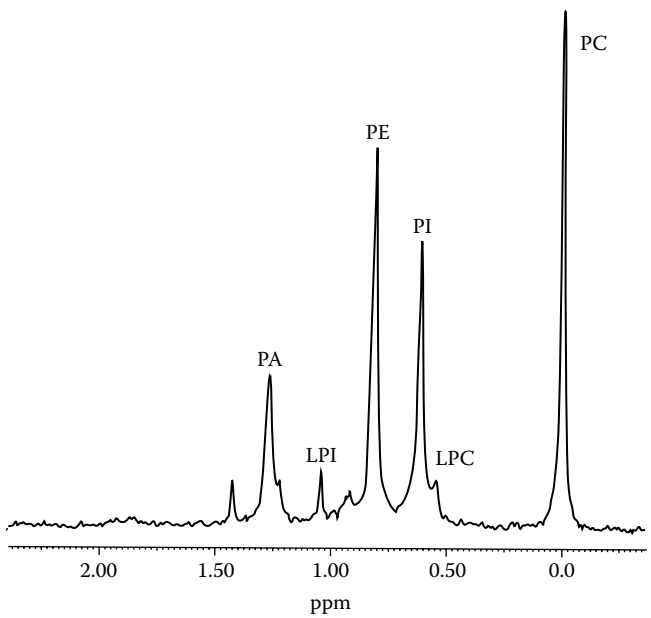


FIGURE 2.19 ^{31}P-NMR (121.5 MHz) of commercial lecithin in $CHCl_3/CH_3OH/H_2O$–EDTA (10/4/1).

identification and quantification of phospholipids in complex lipid mixtures from 2D ^{1}H-^{31}P NMR was developed [81]. In this method, ^{31}P intensities are used to quantify the phospholipid species of interest, while ^{1}H chemical shifts are used to assign the peaks. With the aid of this technique, no standards are needed and a global map of phospholipids can be obtained in a short time and with sufficient accuracy. Figure 2.20 shows the 2D NMR spectra of approximately 10 different ^{31}P signals in the range 0–2 ppm. The corresponding traces in the ^{1}H dimension typically show up to 10

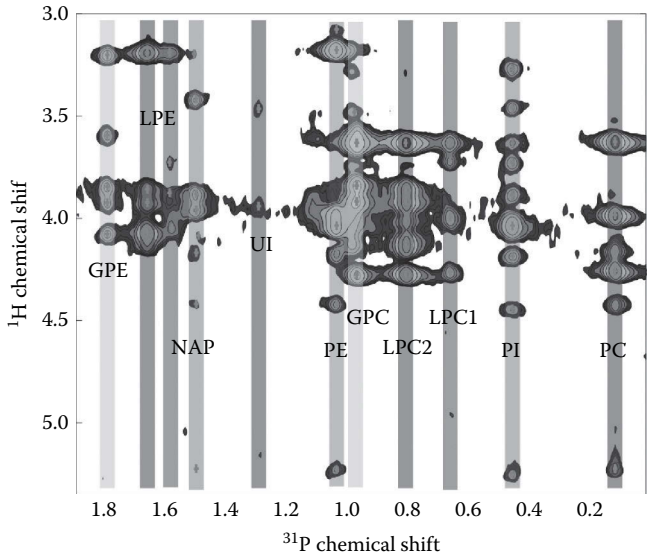


FIGURE 2.20 Contour plot of a 2D ^{1}H-^{31}P TOCSY spectrum of a lipid mixture with assignment of the different signals. The bands represent the lipids identified during the peak picking, and the individual peaks are highlighted by magenta dots. The width of the bands illustrates that the peak positions may fluctuate in the ^{31}P dimension because of their imperfect line shapes or overlap with neighboring peaks. Both the chemical shifts and the intensity distribution in the ^{1}H dimension are important observables for a reliable peak picking algorithm. The band labeled UI (unidentified) represents signals that do not match any entries in the database. LPC1, 1-lysophosphatidylcholine; PI, phosphatidylinositol; LPC2, 2-lysophosphatidylcholine; GPC, glycerophosphorylcholine; PE, phosphatidylethanolamine; NAP, N-acyl phosphatidylethanolamine; LPE, 1-lysophosphatidylethanolamine; GPE, glycerophosphorylethanolamine. (From Balsgart, N.M. et al., *Anal. Chem*., 88, 2170, 2016.)

resonances from the different hydrogen atoms in the vicinity of the phosphorus atoms in the lipids. With combination of all information, the individual phospholipid can be identified and quantified. This modern technique will attract more and more attention in phospholipid analysis.

2. Fourier Transform Infrared Spectroscopy

FTIR is a technique that can be used to elucidate the chemical structure of a lipid. Table 2.2 displays typical signals expected based on chemical structure. In addition, FTIR can be used for quantification. In fact, FTIR demonstrated to be useful to determine the total phospholipid content in vegetable oil. Nzai and Proctor [82] found a linear relationship between the concentration of phospholipids and the area of the band from 1200 to 970 cm^{-1}. Accordingly, they used phospholipid absorption bands between 1200 and 970 cm^{-1} (P−O−C + PO_2 chemical bonds) for quantification.

FTIR is a relatively rapid and reliable method that can potentially provide substantial savings in the fats and oils industry in terms of time and labor. Another advantage of this method is that only a very small amount of solvent or no solvent is needed to run the analysis. However, the main drawback of this method is the vibration due to interference from other nonphospholipid components. Additionally, only total phospholipid content can be obtained by using this method. Recently, Meng et al. [83] quantified the total phospholipid content in edible oils by in-vial solvent extraction coupled with FTIR analysis. They found that the second-derivative differential spectra were better than the differential spectra. Various bands were used to investigate the linear regression, and finally, the asymmetric phosphate diester PO_2^- stretching band at 1243 cm^{-1} in second-derivative spectra was regarded to be sufficient for the accurate measurement of total phospholipids with minimal co-extracted triacylglycerol interferences being encountered.

TABLE 2.2
Mid-Infrared Phospholipid Band Assignments

Wavenumber (cm^{-1})	Assignments
970	Asymmetric C–N stretching of $(CH_3)N^+$
1025	Symmetric ester C–O–P stretch
1068	Asymmetric ester C–O–P stretch in C–O–PO_2^-
1037	Symmetric ester C–O stretch in C=O–O–C
1173	Asymmetric C–O stretch in C=O–O–C
1090	Symmetric phosphate diester stretch PO_2^-
1243	Asymmetric phosphate diester PO_2^- stretch
1376	Symmetric CH_3 bending vibration
1451	δ CH_2 bending vibration
1740	C=O stretch

Source: Meng, X. et al., *J. Agric. Food Chem.*, 62, 3101, 2014.

FTIR can also provide information regarding the molecular organization of lipid chains. This technique can be used to determine the interactions of the head groups and, consequently, yield information regarding the intra- and intermolecular interaction of the study system. FTIR spectra of a lipid sample can determine how well the lipid chains are packed in the system [84]. For instance, if the CH_2 scissoring is split in two bands, a doublet is expected between 1450 and 1500 cm^{-1} (Figure 2.21). The latter suggests the presence of a well-organized system forming orthorhombic packing. However, if no splitting is observed but instead the FTIR spectra display a singlet in the scissoring region, molecules are less organized forming a hexagonal packing. In addition, FTIR can also provide information regarding the stability of a system *versus* temperature. The latter can be measured by collecting the spectra of a specific sample at different temperatures, and when an

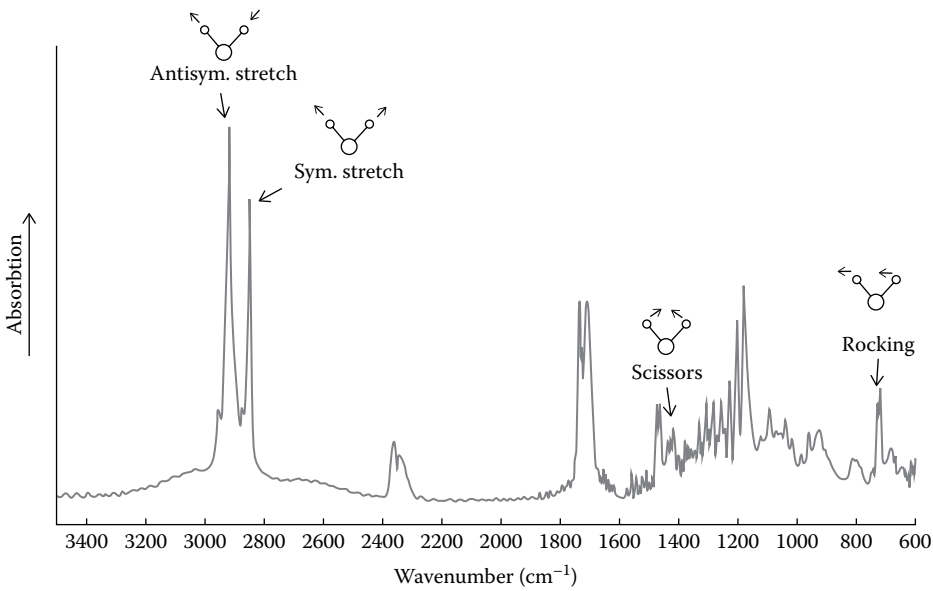


FIGURE 2.21 An example of a Fourier transform infrared (FTIR) spectrum.

increase of the frequency in the region between ~2850 and ~2920 cm^{-1} is registered, a transition from a more organized to a less organized system can be recorded.

3. X-Ray Diffraction

X-ray can be used to characterize the packing and phase behavior of lipids as it can provide information about the interaction of hydrocarbon chains [85]. One of the most used x-ray techniques is wide angle x-ray diffraction (WAXD) [86]. WAXD can detect electron density fluctuations and these fluctuations are related to the characteristic length of the crystalline structure by Bragg's law (Equation 2.1) where d represents the length, θ is the scattering angle, and λ is the wavelength of the beam. Accordingly, the shape and dimensions of the crystalline lattice are determined by the direction of the diffraction beam. This technique is sensitive to small structural changes, nondestructive, and can have high penetration in organic systems. X-ray techniques are of special importance for the characterization of lipids used to generate lipid nanoparticles for food application as the crystalline structure of these lipids will play a role in determining stability and digestibility of these nanosystems.

$$2d \sin \theta = \lambda \tag{2.1}$$

Other x-ray technique constitutes small-angle x-ray scattering (SAXS). The latter can provide information on the structure of complex food materials, for instance, hen-egg yolk and oil-in-water emulsions containing lipids and proteins. Recently, Reinke et al. [87] used microfocus SAXS to investigate the migration pathway of oil in different cocoa matrixes (Figure 2.22) as one of the

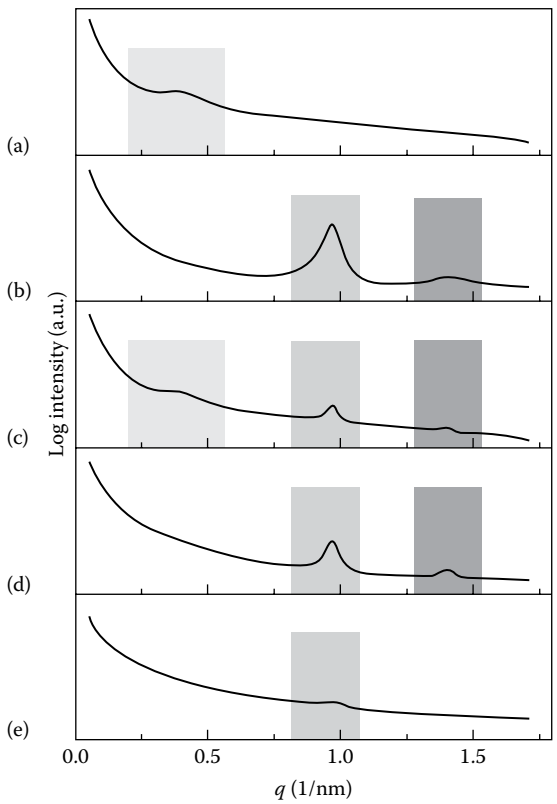


FIGURE 2.22 Microfocus small-angle x-ray scattering (SAXS) curves dry powders before migration of oil started. (a) Skim milk powder; (b) cocoa butter; (c) skim milk powder with cocoa butter; (d) sucrose with cocoa butter; (e) cocoa powder. (From Reinke, S.K. et al., *ACS Appl. Mater. Interfaces*, 7, 9929, 2015.)

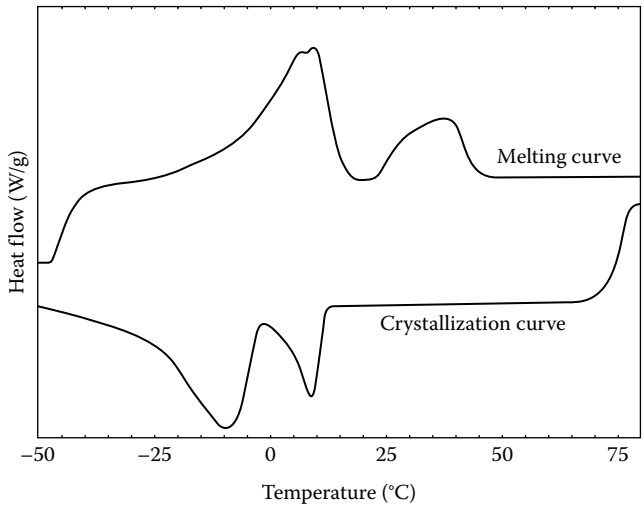


FIGURE 2.23 Differential scanning calorimetry (DSC) melting and crystallization curve of refined–bleached–deodorized palm oil. (From Tan, C.P. and Che Man, Y.B., *Food Chem.*, 76, 89, 2002.)

major problems in chocolate production is the formation of white defects on the chocolate surface, also known as chocolate blooming [87]. Accordingly, the results suggested that the oil first migrates into the pores and crack and later chemical migration takes place through the fat phase prompting softening and partial dissolution of the crystalline cocoa butter. Therefore, this process could be prevented by decreasing porosity and defects in the chocolate matrix.

4. Differential Scanning Calorimetry

DSC provides information regarding lipids' molecular organization and polymorphism by cooling and heating of a lipid sample to break down the lipids' crystalline structure. It measures the thermal behavior of a sample, helping to establish the link between the temperature and the physical properties of a material. DSC can determine phase transitions, melting point, heat of fusion, oxidative stability, percent of crystallinity, and crystallization kinetics. Basically, energy is simultaneously applied to a sample cell (e.g., containing solid sample) and a reference cell (e.g., empty cell) and the temperature increased identically over time. The difference between the energy required for cells to be at the same temperature will be the amount of heat absorbed or released by the sample of interest. Since more energy is required to heat the sample cell to the same temperature as the reference cell, this can translate into heat excess [88]. Accordingly, the conformation of the lipid chains will determine the sharpness, position, and shape in DSC scan curve. Figure 2.23 displays the DSC melting and crystallization curves of refined–bleached–deodorized (RBD) palm oil [89].

IX. SUMMARY

Acylglycerols and phospholipids are molecules encountered in daily life; as such, understanding the chemical and physical features of these compounds is of high relevance to the scientific community. This chapter presented the chemical structure of different classes of lipids and phospholipids, and it discussed the most used chemical and enzymatic reactions to modify and obtain these compounds. It also addressed the role of phospholipids in membranes fluidity and explained the molecular structure of lipids and phospholipids and their self-assembled structures. It described the relevance of this self-assembly structures in food application such as in the case of nanotechnologies, which are generally used to encapsulate ingredients sensitive to oxidation, for example,

some essential polyunsaturated fatty acids. In addition, it presented different enzymatic and chemical approaches for the synthesis of lipids and phospholipids with desired physicochemical properties. Furthermore, the chapter covered the main techniques used for the separation, analysis, and characterization of lipids and phospholipids. Specifically, advantages and disadvantages of each analytical method are compared. For instance, although TLC is relatively inexpensive and straightforward to perform, it is not a good method for separation in large scale. In addition, lipids characterization involves the use of multiple techniques to elucidate the packing behavior, crystal structure, and stability of crystal. For example, though the use of NMR and FTIR provides information regarding the structural features of the lipids and can help to quantify lipid content, DSC and x-ray are the techniques most used to elucidate the packing behavior of these molecules. Overall, this chapter offered well-rounded background for the synthesis and characterization of relevant lipids for food application.

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3 Lipid-Based Emulsions and Emulsifiers

David Julian McClements

CONTENTS

I. INTRODUCTION

Many natural and processed foods exist either partly or wholly as emulsions or have been in an emulsified state at some time during their existence [1–5]. Milk is the most common example of a naturally occurring food emulsion [6]. Mayonnaise, salad dressing, cream, ice cream, butter, and margarine are all examples of manufactured food emulsions. Powdered coffee whiteners, sauces, and many desserts are examples of foods that were emulsions at one stage during their production but subsequently were converted into another form. The bulk physicochemical properties of food emulsions, such as appearance, texture, and stability, depend ultimately on the type of molecules the food contains and their interactions with one another. Food emulsions contain a variety of ingredients, including water, lipids, surfactants, proteins, carbohydrates, minerals, preservatives, colors, and flavors [3]. By a combination of covalent and physical interactions, these ingredients form the individual phases and structural components that give the final product its characteristic physicochemical properties [7]. It is the role of food scientists to untangle the complex relationship between the molecular, structural, and bulk properties of foods so that foods with improved properties can be created in a more systematic fashion.

II. EMULSIONS

An emulsion is a dispersion of droplets of one liquid in another liquid with which it is incompletely miscible [1,8]. In foods, the two immiscible liquids are oil and water. The diameter of the droplets in food emulsions is typically within the 100 nm to 100 μm range [2,3]. A system that consists of oil droplets dispersed in an aqueous phase is called an oil-in-water (O/W) emulsion. A system that consists of water droplets dispersed in an oil phase is called a water-in-oil (W/O) emulsion. The material that makes up the droplets in an emulsion is referred to as the *dispersed* or *internal phase*, whereas the material that makes up the surrounding liquid is called the *continuous* or *external phase*. Multiple emulsions can be prepared that consist of oil droplets contained in larger water droplets, which are themselves dispersed in an oil phase (O/W/O) or vice versa (W/O/W). Multiple emulsions can be used for protecting certain ingredients, for controlling the release of ingredients, or for creating low-fat products [9].

Emulsions are thermodynamically unstable systems because of the positive free energy required to increase the surface area between the oil and water phases [3]. The origin of this energy is the

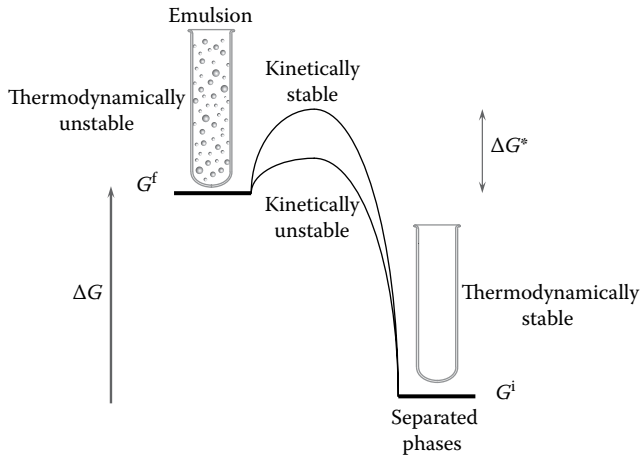

FIGURE 3.1 Emulsions are thermodynamically unstable systems that tend to revert back to the individual oil and water phases with time. To produce an emulsion, energy must be supplied.

unfavorable interaction between oil and water, which exists because water molecules are capable of forming strong hydrogen bonds with other water molecules but not with oil molecules [8,9]. Thus emulsions tend to reduce the surface area between the two immiscible liquids by separating into a system that consists of a layer of oil (lower density) on top of a layer of water (higher density). This is clearly seen if one tries to *homogenize* pure oil and pure water together: initially, an emulsion is formed, but after a few minutes phase separation occurs (Figure 3.1).

Emulsion instability can manifest itself through a variety of physicochemical mechanisms, including creaming, flocculation, coalescence, partial coalescence, Ostwald ripening, and phase inversion (Section VI). To form emulsions that are kinetically stable for a reasonable period (a few weeks, months, or even years), chemical substances known as *emulsifiers* must be added prior to homogenization. Emulsifiers are surface-active molecules that adsorb to the surface of freshly formed droplets during homogenization, forming a protective membrane that prevents the droplets from coming close enough together to aggregate [3]. Most food emulsifiers are *amphiphilic* molecules, that is, they have both polar and nonpolar regions on the same molecule. The most common types used in the food industry are lipid-based emulsifiers (small-molecule surfactants and phospholipids) and amphiphilic biopolymers (proteins and polysaccharides) [2,3]. In addition, some types of small solid particles are also surface active and can act as emulsifiers in foods, for example, granules from egg or mustard.

Most food emulsions are more complex than the simple three-component (oil, water, and emulsifier) system described earlier [3,5,9]. The aqueous phase may contain water-soluble ingredients of many different kinds, including sugars, salts, acids, bases, surfactants, proteins, polysaccharides, flavors, and preservatives [1]. The oil phase may contain a variety of lipid-soluble components, such as triacylglycerols, diacylglycerols, monoacylglycerols, fatty acids, vitamins, cholesterol, and flavors [1]. The interfacial region may be composed of surface-active components of a variety of types, including small-molecule surfactants, phospholipids, polysaccharides, and proteins. It should be noted that the composition of the interfacial region may evolve over time after an emulsion is produced, due to competitive adsorption with other surface-active substances or due to adsorption of oppositely charged substances, for example, polysaccharides [1a]. Some of the ingredients in food emulsions are not located exclusively in one phase but are distributed between the oil, water, and interfacial phases according to their partition coefficients. Despite having low concentrations, many of the minor components present in an emulsion can have a pronounced influence on its bulk physicochemical properties. For example, addition of small amounts (~few mM) of multivalent

mineral ions can destabilize an electrostatically stabilized emulsion [1]. Food emulsions may consist of oil droplets dispersed in an aqueous phase (e.g., mayonnaise, milk, cream, soups), or water droplets dispersed in an oil phase (e.g., margarine, butter, spreads). The droplets and/or the continuous phase may be fluid, gelled, crystalline, or glassy. The size of the droplets may vary from less than a micrometer to a few hundred micrometers, and the droplets themselves may be more or less polydisperse. In addition, many emulsions may contain air bubbles that have a pronounced influence on the sensory and physicochemical properties of the system, for example, ice cream, whipped cream, and desserts [1].

To complicate matters further, the properties of food emulsions are constantly changing with time because of the action of various chemical (e.g., lipid oxidation, biopolymer hydrolysis), physical (e.g., creaming, flocculation, coalescence), and biological (e.g., bacterial growth) processes. In addition, during their processing, storage, transport, and handling, food emulsions are subjected to variations in their temperature (e.g., via sterilization, cooking, chilling, freezing) and to various mechanical forces (e.g., stirring, mixing, whipping, flow through pipes, centrifugation high pressure) that alter their physicochemical properties. Despite the compositional, structural, and dynamic complexity of food emulsions, considerable progress has been made in understanding the major factors that determine their bulk physicochemical properties.

III. LIPID-BASED EMULSIFIERS

A. Molecular Characteristics

The most important types of lipid-based emulsifier used in the food industry are small-molecule surfactants (e.g., Tweens, Spans, monoglycerides, diglycerides, and modified fatty acids) and phospholipids (e.g., lecithin). The principal role of lipid-based emulsifiers in food emulsions is to enhance the formation and stability of the product; however, they may also alter the bulk physicochemical properties by interacting with proteins or polysaccharides or by modifying the structure of fat crystals [1,9]. All lipid-based emulsifiers are amphiphilic molecules that have a hydrophilic "head" group with a high affinity for water and lipophilic "tail" group with a high affinity for oil [1,8,10,11]. These emulsifiers can be represented by the formula RX, where X represents the hydrophilic head and R the lipophilic tail. Lipid-based emulsifiers differ with respect to type of head group and tail group. The head group may be anionic, cationic, zwitterionic, or nonionic. The lipid-based emulsifiers used in the food industry are mainly nonionic (e.g., monoacylglycerols, sucrose esters, Tweens, and Spans), anionic (e.g., fatty acids), or zwitterionic (e.g., lecithin). The tail group usually consists of one or more hydrocarbon chains, having between 10 and 20 carbon atoms per chain. The chains may be saturated or unsaturated, linear or branched, aliphatic and/or aromatic. Most lipid-based emulsifiers used in foods have either one or two linear aliphatic chains, which may be saturated or unsaturated. Each type of emulsifier has unique functional properties that depend on its chemical structure.

Lipid-based emulsifiers aggregate spontaneously in solution to form a variety of thermodynamically stable structures known as *association colloids* (e.g., micelles, bilayers, vesicles, reversed micelles) (Figure 3.2). These structural types are adopted because they minimize the unfavorable contact area between the nonpolar tails of the emulsifier molecules and water [10]. The type of association colloid formed depends principally on the polarity and molecular geometry of the emulsifier molecules (Section III.C.3). The forces holding association colloids together are relatively weak, and so they have highly dynamic and flexible structures [8]. Their size and shape are continually fluctuating, and individual emulsifier molecules rapidly exchange between the micelle and the surrounding liquid. The relative weakness of the forces holding association colloids together also means that their structures are particularly sensitive to changes in environmental conditions, such as temperature, pH, ionic strength, and ion type. Surfactant micelles are the most important type of association colloid formed in many food emulsions, and we focus principally on their properties.

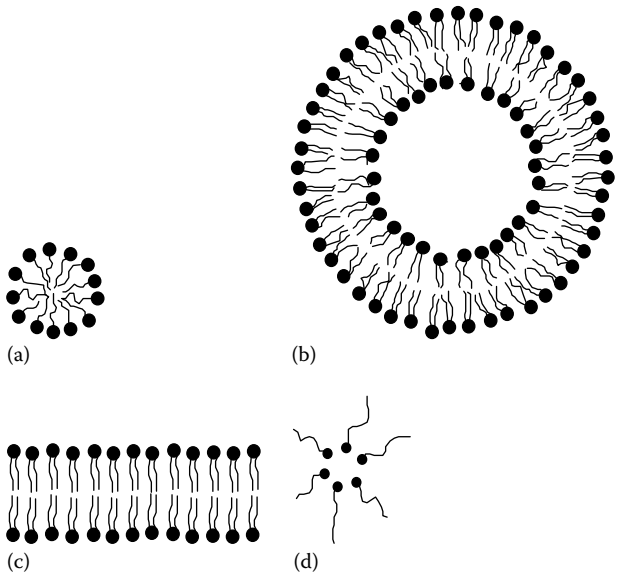

FIGURE 3.2 Association colloids formed by surfactant molecules: (a) micelles; (b) vesicles; (c) bilayers; and (d) reverse micelles.

B. Functional Properties

1. Critical Micelle Concentration

A surfactant forms micelles in an aqueous solution when its concentration exceeds some critical level, known as the *critical micelle concentration* (cmc). Below the cmc, surfactant molecules are dispersed predominantly as monomers, but once the cmc has been exceeded, any additional surfactant molecules form micelles, and the monomer concentration remains constant. Despite the highly dynamic nature of their structure, surfactant micelles do form particles that have a well-defined average size. Thus, when surfactant is added to a solution above the cmc, the number of micelles increases, rather than their size. When the cmc is exceeded, there is an abrupt change in the physicochemical properties of a surfactant solution (e.g., surface tension, electrical conductivity, turbidity, osmotic pressure) [12]. This is because the properties of surfactant molecules dispersed as monomers are different from those in micelles. For example, surfactant monomers are amphiphilic and have a high surface activity, whereas micelles have little surface activity because their surface is covered with hydrophilic head groups. Consequently, the surface tension of a solution decreases with increasing surfactant concentration below the cmc but remains fairly constant above it.

2. Cloud Point

When a surfactant solution is heated above a certain temperature, known as the *cloud point*, it becomes turbid. As the temperature is raised, the hydrophilic head groups become increasingly dehydrated, which causes the emulsifier molecules to aggregate. These aggregates are large enough to scatter light, and so the solution appears turbid. At temperatures above the cloud point, the aggregates grow so large that they sediment under the influence of gravity and form a separate phase. The cloud point increases as the hydrophobicity of a surfactant molecule increases; that is, the length of its hydrocarbon tail increases or the size of its hydrophilic head group decreases [13,14].

3. Solubilization

Nonpolar molecules, which are normally insoluble or only sparingly soluble in water, can be solubilized in an aqueous surfactant solution by incorporation into micelles or other types of

association colloid [9]. The resulting system is thermodynamically stable; however, equilibrium may take an appreciable time to achieve because of the activation energy associated with transferring a nonpolar molecule from a bulk phase to a micelle. Micelles containing solubilized materials are referred to as *swollen micelles* or *microemulsions*, whereas the material solubilized within the micelle is referred to as the *solubilizate*. The ability of micellar solutions to solubilize nonpolar molecules has a number of potentially important applications in the food industry, including selective extraction of nonpolar molecules from oils, controlled ingredient release, incorporation of nonpolar substances into aqueous solutions, transport of nonpolar molecules across aqueous membranes, and modification of chemical reactions [9]. Three important factors determine the functional properties of swollen micellar solutions: the location of the solubilizate within the micelles, the maximum amount of material that can be solubilized per unit mass of surfactant, and the rate at which solubilization proceeds [9].

4. Surface Activity and Droplet Stabilization

Lipid-based emulsifiers are used widely in the food industry to enhance the formation and stability of food emulsions. To do this, they must adsorb to the surface of emulsion droplets during homogenization and form a protective membrane that prevents the droplets from aggregating with each other [1]. Emulsifier molecules adsorb to oil–water interfaces because they can adopt an orientation in which the hydrophilic part of the molecule is located in the water, while the hydrophobic part is located in the oil. This minimizes the unfavorable free energy associated with the contact of hydrophilic and hydrophobic regions and therefore reduces the interfacial tension. This reduction in interfacial tension is important because it facilitates the further disruption of emulsion droplets; that is, less energy is needed to break up a droplet when the interfacial tension is lowered.

Once adsorbed to the surface of a droplet, the emulsifier must provide a repulsive force that is strong enough to prevent the droplet from aggregating with its neighbors. Ionic surfactants provide stability by causing all the emulsion droplets to have the same electric charge, hence to repel each other electrostatically. Nonionic surfactants provide stability by generating a number of short-range repulsive forces (e.g., steric overlap, hydration, thermal fluctuation interactions) that prevent the droplets from getting too close together [1,11]. Some emulsifiers form multilayers (rather than monolayers) at the surface of an emulsion droplet, which greatly enhances the stability of the droplets against aggregation.

In summary, emulsifiers must have three characteristics to be effective. First, they must rapidly adsorb to the surface of the freshly formed emulsion droplets during homogenization. Second, they must reduce the interfacial tension by a significant amount. Third, they must form a membrane that prevents the droplets from aggregating.

C. Ingredient Selection

A large number of different types of lipid-based emulsifier can be used as food ingredients, and a manufacturer must select the one that is most suitable for each particular product. Suitability, in turn, depends on factors such as an emulsifier's legal status as a food ingredient; its cost and availability, the consistency in its properties from batch to batch, its ease of handling and dispersion, its shelf life, its compatibility with other ingredients, and the processing, storage, and handling conditions it will experience, as well as the expected shelf life and physicochemical properties of the final product.

How does a food manufacturer decide which emulsifier is most suitable for a product? There have been various attempts to develop classification systems that can be used to select the most appropriate emulsifier for a particular application. Classification schemes have been developed that are based on an emulsifier's solubility in oil and/or water (Bancroft's rule), its ratio of hydrophilic to lipophilic groups (HLB number) [15,16], and its molecular geometry [17]. Ultimately, all of these properties depend on the chemical structure of the emulsifier, and so all the different classification schemes are closely related.

1. Bancroft's Rule

One of the first empirical rules developed to describe the type of emulsion that could be stabilized by a given emulsifier was proposed by Bancroft. Bancroft's rule states that the phase in which the emulsifier is most soluble will form the continuous phase of an emulsion. Hence, a water-soluble emulsifier will stabilize oil-in-water emulsions, whereas an oil-soluble emulsifier will stabilize water-in-oil emulsions.

2. Hydrophile–Lipophile Balance

The hydrophile–lipophile balance (HLB) concept underlies a semiempirical method for selecting an appropriate emulsifier or combination of emulsifiers to stabilize an emulsion. The HLB is described by a number, which gives an indication of the overall affinity of an emulsifier for the oil and/or aqueous phases [12]. Each emulsifier is assigned an HLB number according to its chemical structure. A molecule with a high HLB number has a high ratio of hydrophilic groups to lipophilic groups and vice versa. The HLB number of an emulsifier can be calculated from a knowledge of the number and type of hydrophilic and lipophilic groups it contains, or it can be estimated from experimental measurements of its cloud point. The HLB numbers of many emulsifiers have been tabulated in the literature [15,16]. A widely used semiempirical method of calculating the HLB number of a lipid-based emulsifier is as follows:

$$\text{HLB} = 7 + \Sigma(\text{Hydrophilic group numbers}) - \Sigma(\text{Lipophilic group numbers}) \tag{3.1}$$

As indicated in Table 3.1 [18], group numbers have been assigned to hydrophilic and lipophilic groups of many types. The sums of the group numbers of all the lipophilic groups and of all the hydrophilic groups are substituted into Equation 3.1, and the HLB number is calculated. The semiempirical equation mentioned earlier has been found to have a firm thermodynamic basis, with the sums corresponding to the free energy changes in the hydrophilic and lipophilic parts of the molecule when micelles are formed.

The HLB number of an emulsifier gives a useful indication of its solubility in the oil and/or water phases, and it can be used to predict the type of emulsion that will be formed. An emulsifier with a low HLB number (4–6) is predominantly hydrophobic, dissolves preferentially in oil, stabilizes water-in-oil emulsions, and forms reversed micelles in oil. An emulsifier with a high HLB number (8–18) is predominantly hydrophilic, dissolves preferentially in water, stabilizes oil-in-water emulsions, and forms micelles in water. An emulsifier with an intermediate HLB number (6–8) has no particular preference for either oil or water. Nonionic molecules with HLB numbers below 4 and above 18 are less surface active and are therefore less likely to preferentially accumulate at an oil–water interface.

Emulsion droplets are particularly prone to coalescence when they are stabilized by emulsifiers that have extreme or intermediate HLB numbers. At very high or very low HLB numbers, a nonionic

TABLE 3.1
Selected HLB Group Numbers

Hydrophilic Group	Group Number	Lipophilic Group	Group Number
—SO_4NA^+	38.7	—CH—	0.475
—COO^-H^+	21.2	—CH_2—	0.475
Tertiary amine	9.4	—CH_3—	0.475
Sorbitan ring	6.8		
—COOH	2.1		
—O—	1.3		

Source: Adapted from Davis, H.T., *Colloids Surf. A*, 91, 9, 1994.

emulsifier has such a low surface activity that it does not accumulate appreciably at the droplet surface and therefore does not provide protection against coalescence. At intermediate HLB numbers (6–8), emulsions are unstable to coalescence because the interfacial tension is so low that very little energy is required to disrupt the membrane. Maximum stability of emulsions is obtained for oil-in-water emulsions using an emulsifier with an HLB number around 10–12 and for water-in-oil emulsions around 3–5. This is because the emulsifiers are sufficiently surface active but do not lower the interfacial tension so much that the droplets are easily disrupted. It is possible to adjust the effective HLB number by using a combination of two or more emulsifiers with different HLB numbers.

One of the major drawbacks of the HLB concept is its failure to account for the significant alterations in the functional properties of an emulsifier molecule that result from changes in temperature or solution conditions, even though the chemical structure of the molecule does not change. Thus, an emulsifier may be capable of stabilizing oil-in-water emulsions at one temperature but water-in-oil emulsions at another temperature. For this reason, a new system referred to as the hydrophile–lipophile deviation (HLD) method has been developed to account for environmental conditions and water-to-oil ratio on surfactant performance.

3. Molecular Geometry and Phase Inversion Temperature

The molecular geometry of an emulsifier molecule is described by a packing parameter p (see Figure 3.3) as follows:

$$p = \frac{\upsilon}{la_0} \tag{3.2}$$

where

υ and l are the volume and length of the hydrophobic tail

a_0 is the cross-sectional area of the hydrophilic head group

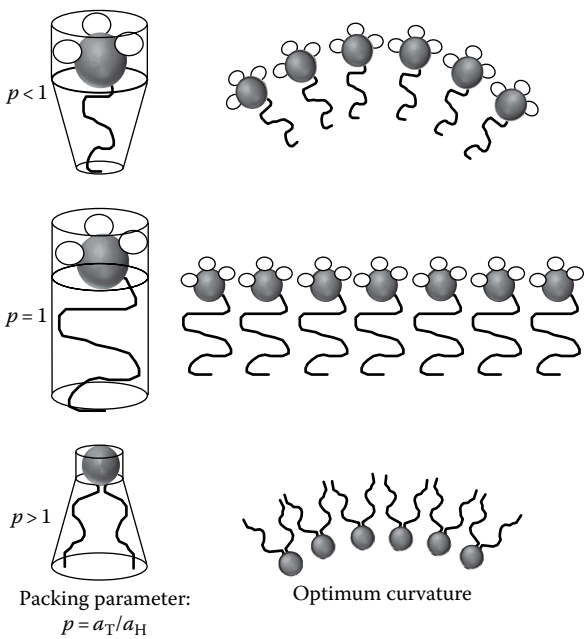

FIGURE 3.3 Relationship between the molecular geometry of surfactant molecules and their optimum curvature.

When surfactant molecules associate with each other, they tend to form monolayers having a curvature that allows the most efficient packing of the molecules. At this *optimum curvature*, the monolayer has its lowest free energy, and any deviation from this curvature requires the expenditure of energy [8,11]. The optimum curvature of a monolayer depends on the packing parameter of the emulsifier: for $p = 1$, monolayers with zero curvature are preferred; for $p < 1$, the optimum curvature is convex; and for $p > 1$, the optimum curvature is concave (Figure 3.3). Simple geometrical considerations indicate that spherical micelles are formed when p is less than 0.33, nonspherical micelles when p is between 0.33 and 0.5, and bilayers when p is between 0.5 and 1 [11]. Above a certain concentration, bilayers join up to form vesicles because energetically unfavorable end effects are eliminated. At values of p greater than 1, reversed micelles are formed, in which the hydrophilic head groups are located in the interior (away from the oil), and the hydrophobic tail groups are located at the exterior (in contact with the oil) (Figure 3.2). The packing parameter therefore gives a useful indication of the type of association colloid that is formed by an emulsifier molecule in solution.

The packing parameter is also useful because it accounts for the temperature dependence of the physicochemical properties of surfactant solutions and emulsions. The temperature at which an emulsifier solution converts from a micellar to a reversed micellar system or an oil-in-water emulsion converts to a water-in-oil emulsion is known as the phase inversion temperature (PIT). Consider what happens when an emulsion that is stabilized by a lipid-based emulsifier is heated (Figure 3.4). At temperatures well below the PIT (≈20°C), the packing parameter is significantly less than unity, and

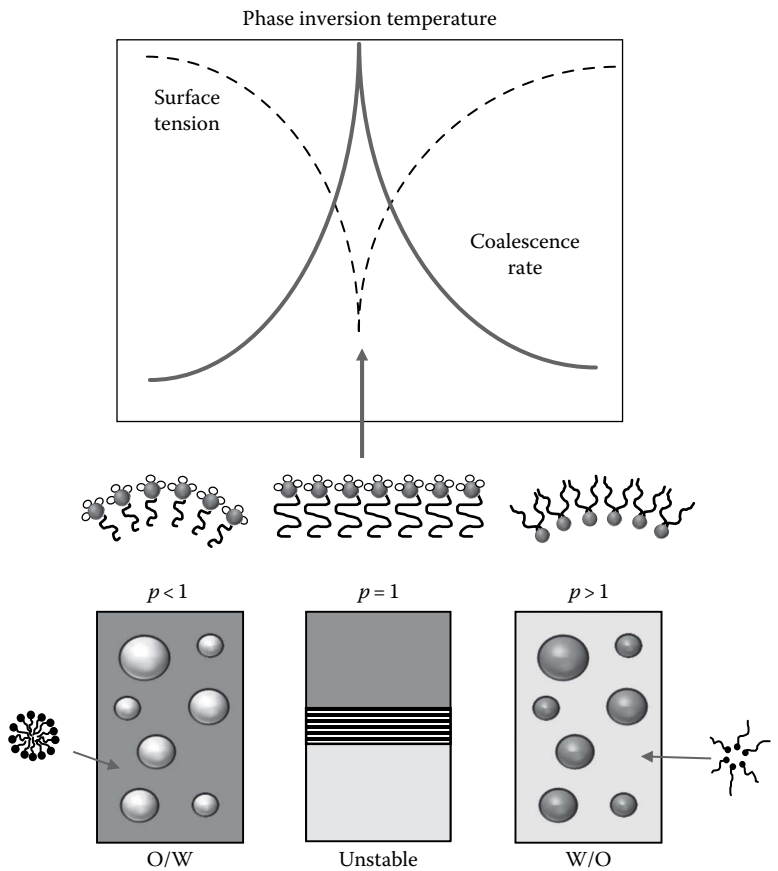

FIGURE 3.4 Phase inversion temperature in emulsions. An oil-in-water emulsion may invert to a water-in-oil emulsion upon heating due to dehydration of the surfactant head groups changing the optimum curvature.

so a system that consists of oil-in-water emulsion in equilibrium with a swollen micellar solution is favored. As the temperature is raised, the hydrophilic head groups of the emulsifier molecules become increasingly dehydrated, which causes p to increase toward unity. Thus, the emulsion droplets become more prone to coalescence and the swollen micelles grow in size. At PIT, $p \approx 1$, and the emulsion breaks down because the droplets have an ultralow interfacial tension and therefore readily coalesce with each other. The resulting system consists of excess oil and excess water (containing some emulsifier monomers), separated by a third phase that contains emulsifier molecules aggregated into bilayer structures. At temperatures sufficiently greater than the PIT, the packing parameter is much larger than unity, and the formation of a system that consists of a water-in-oil emulsion in equilibrium with swollen reversed micelles is favored. A further increase in temperature leads to a decrease in the size of the reversed micelles and in the amount of water solubilized within them. The method of categorizing emulsifier molecules according to their molecular geometry is now widely accepted as the most useful means of determining the types of emulsion they tend to stabilize [17].

4. Other Factors

The classification schemes mentioned earlier provide information about the type of emulsion an emulsifier tends to stabilize (i.e., O/W or W/O), but they do not provide much insight into the size of the droplets that form during homogenization or the stability of the emulsion droplets once formed [1]. In choosing a suitable emulsifier for a particular application, these factors must also be considered. The speed at which an emulsifier adsorbs to the surface of the emulsion droplets produced during homogenization determines the minimum droplet size that can be produced: the faster the adsorption rate, the smaller the size. The magnitude and range of the repulsive forces generated by a membrane, and its viscoelasticity, determine the stability of the droplets to aggregation.

IV. BIOPOLYMERS

Proteins and polysaccharides are the two most important biopolymers used as functional ingredients in food emulsions. These biopolymers are used principally for their ability to stabilize emulsions, enhance viscosity, and form gels.

A. Molecular Characteristics

Molecular characteristics of biopolymers, such as molecular weight, conformation, flexibility, and polarity, ultimately determine the properties of biopolymer solutions. These characteristics are determined by the type, number, and sequence of monomers that make up the polymer. Proteins are polymers of amino acids [19], whereas polysaccharides are polymers of monosaccharides [20]. The 3D structures of biopolymers in aqueous solution can be categorized as globular, fibrous, or random coil (Figure 3.5). Globular biopolymers have fairly rigid compact structures; fibrous

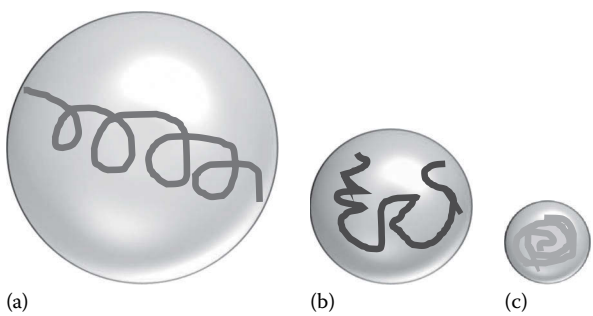

FIGURE 3.5 Typical molecular conformations adopted by biopolymers in aqueous solution: (a) rigid-rod; (b) random coil; (c) globular.

biopolymers have fairly rigid, rodlike structures; and random coil biopolymers have highly dynamic and flexible structures. Biopolymers can also be classified according to the degree of branching of the chain. Most proteins have linear chains, whereas polysaccharides can have either linear (e.g., amylose) or branched (e.g., amylopectin) chains.

The conformation of a biopolymer in solution depends on the relative magnitude of the various types of attractive and repulsive interaction that occur within and between molecules, as well as the configurational entropy of the molecule. Biopolymers that have substantial proportions of nonpolar groups tend to fold into globular structures in which the nonpolar groups are located in the interior (away from the water) and the polar groups are located at the exterior (in contact with the water) because this arrangement minimizes the number of unfavorable contacts between hydrophobic regions and water. However, since stereochemical constraints and the influence of other types of molecular interaction usually make it impossible for all the nonpolar groups to be located in the interior, the surfaces of globular biopolymers have some hydrophobic character. Many kinds of food protein have compact globular structures, including β-lactoglobulin, α-lactalbumin, and bovine serum albumin [6]. Biopolymers that contain a high proportion of polar monomers, distributed fairly evenly along their backbone, often have rodlike conformations with substantial amounts of helical structure stabilized by hydrogen bonding. Such biopolymers (e.g., collagen, cellulose) usually have low water solubilities because they tend to associate strongly with each other rather than with water; consequently, they often have poor functional properties. However, if the chains are branched, the molecules may be prevented from getting close enough together to aggregate, and so they may exist in solution as individual molecules. Predominantly polar biopolymers containing monomers that are incompatible with helix formation (e.g., β-casein) tend to form random coil structures.

In practice, biopolymers may have some regions along their backbone that have one type of conformation and others that have a different conformation. Biopolymers may also exist as isolated molecules or as aggregates in solution, depending on the relative magnitude of the biopolymer–biopolymer, biopolymer–solvent, and solvent–solvent interactions. Biopolymers are also capable of undergoing transitions from one type of conformation to another in response to environmental changes such as alterations in their pH, ionic strength, solvent composition, and temperature. Examples include helix ⇔ random coil and globular ⇔ random coil. In many food biopolymers, this type of transition plays an important role in determining the functional properties (e.g., gelation).

B. Functional Properties

1. Emulsification

Biopolymers that have a high proportion of nonpolar groups tend to be surface active, that is, they can accumulate at oil–water interfaces [1–4]. The major driving force for adsorption is the hydrophobic effect. When the biopolymer is dispersed in an aqueous phase, some of the nonpolar groups are in contact with water, which is a thermodynamically unfavorable condition. By adsorbing to an interface, the biopolymer can adopt a conformation of nonpolar groups in contact with the oil phase (away from the water) and hydrophilic groups located in the aqueous phase (in contact with the water). In addition, adsorption reduces the number of contacts between the oil and water molecules at the interface, thereby reducing the interfacial tension. The conformation a biopolymer adopts at an oil–water interface, and the physicochemical properties of the membrane formed, depend on its molecular structure. Flexible random coil biopolymers adopt an arrangement in which the predominantly nonpolar segments protrude into the oil phase, the predominantly polar segments protrude into the aqueous phase, and the neutral regions lie flat against the interface (Figure 3.6, left). The membranes formed by molecules of these types tend to have relatively open structures, to be relatively thick, and to have low viscoelasticities. Globular biopolymers (usually proteins) adsorb to an interface so that the predominantly nonpolar regions on their surface face the oil phase; thus, they tend to have a definite orientation at an interface (Figure 3.6, right). Once they have adsorbed to an

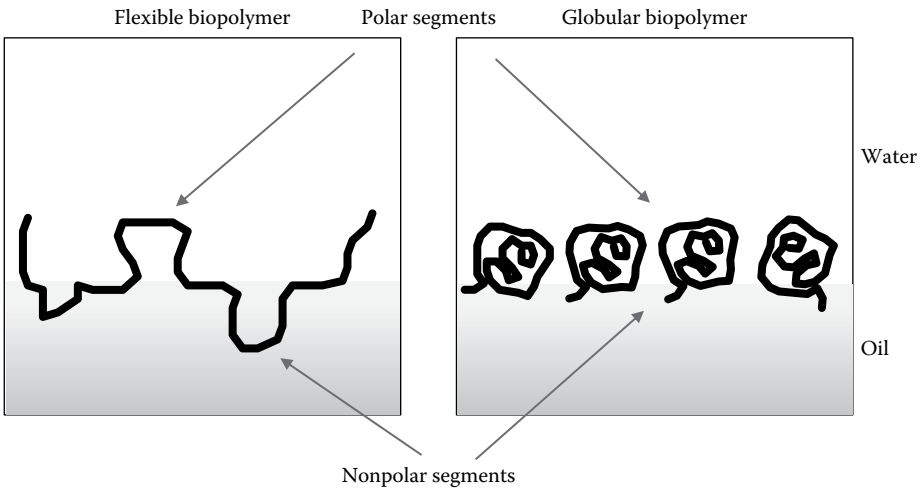

FIGURE 3.6 The conformation of biopolymers at oil–water interfaces depends on their molecular structure and flexibility. Biopolymers adopt a conformation where polar groups extend into water and nonpolar groups extend into oil.

interface, biopolymers often undergo structural rearrangements that permit them to maximize the number of contacts between nonpolar groups and oil [4].

Random coil biopolymers have flexible conformations and therefore rearrange their structures rapidly, whereas globular biopolymers are more rigid and therefore unfold more slowly. The unfolding of a globular protein at an interface often exposes amino acids that were originally located in the hydrophobic interior of the molecule, which can lead to enhanced interactions with neighboring protein molecules through hydrophobic attraction or disulfide bond formation. Consequently, globular proteins tend to form relatively thin and compact membranes, high in viscoelasticity. Thus, membranes formed from globular proteins tend to be more resistant to rupture than those formed from random coil proteins [3].

To be effective emulsifiers, biopolymers must rapidly adsorb to the surface of the emulsion droplets formed during homogenization and provide a membrane that prevents the droplets from aggregating. Biopolymer membranes can stabilize emulsion droplets against aggregation by a number of different physical mechanisms [1]. All biopolymers are capable of providing short-range steric repulsive forces that are usually strong enough to prevent droplets from getting sufficiently close together to coalesce. If the membrane is sufficiently thick, it can also prevent droplets from flocculating. Otherwise, it must be electrically charged so that it can prevent flocculation by electrostatic repulsion. The properties of emulsions stabilized by charged biopolymers are particularly sensitive to the pH and ionic strength of aqueous solutions [1a]. At pH values near the isoelectric point of proteins, or at high ionic strengths, the electrostatic repulsion between droplets may not be large enough to prevent the droplets from aggregating (see Section VI.A.5).

Proteins are commonly used as emulsifiers in foods because many of them naturally have a high proportion of nonpolar groups. Most polysaccharides are so hydrophilic that they are not surface active. However, a small number of naturally occurring polysaccharides have some hydrophobic character (e.g., gum arabic) or have been chemically modified to introduce nonpolar groups (e.g., some hydrophobically modified starches), and these biopolymers can be used as emulsifiers.

2. Thickening and Stabilization

The second major role of biopolymers in food emulsions is to increase the viscosity of the aqueous phase [1a]. This modifies the texture and mouthfeel of the food product ("thickening"), as well as

reducing the rate at which particles sediment or cream ("stabilization"). Both proteins and polysaccharides can be used as thickening agents, but polysaccharides are usually preferred because they can be used at much lower concentrations. The biopolymers used to increase the viscosity of aqueous solutions are usually highly hydrated and extended molecules or molecular aggregates. Their ability to increase the viscosity depends principally on their molecular weight, degree of branching, conformation, and flexibility. The viscosity of a dilute solution of particles increases as the concentration of particles increases [3]:

$$\eta = \eta_0(1 + 2.5\phi) \tag{3.3}$$

where
- η is the viscosity of the solution
- η_0 is the viscosity of the pure solvent
- ϕ is the volume fraction of particles in solution

Biopolymers are able to enhance the viscosity of aqueous solutions at low concentrations because they have an *effective* volume fraction that is much greater than their actual volume fraction [1a]. A biopolymer rapidly rotates in solution because of its thermal energy, and so it sweeps out a spherical volume of water that has a diameter approximately equal to the end-to-end length of the molecule (Figure 3.7). The volume of the biopolymer molecule is only a small fraction of the total volume of the sphere swept out, and so the effective volume fraction of a biopolymer is much greater than its actual volume fraction. Consequently, small concentrations of biopolymer can dramatically increase the viscosity of a solution (Equation 3.3). The effectiveness of a biopolymer at increasing the viscosity increases as the volume fraction it occupies within the sphere it sweeps out decreases. Thus large, highly extended linear biopolymers increase the viscosity more effectively than small compact or branched biopolymers.

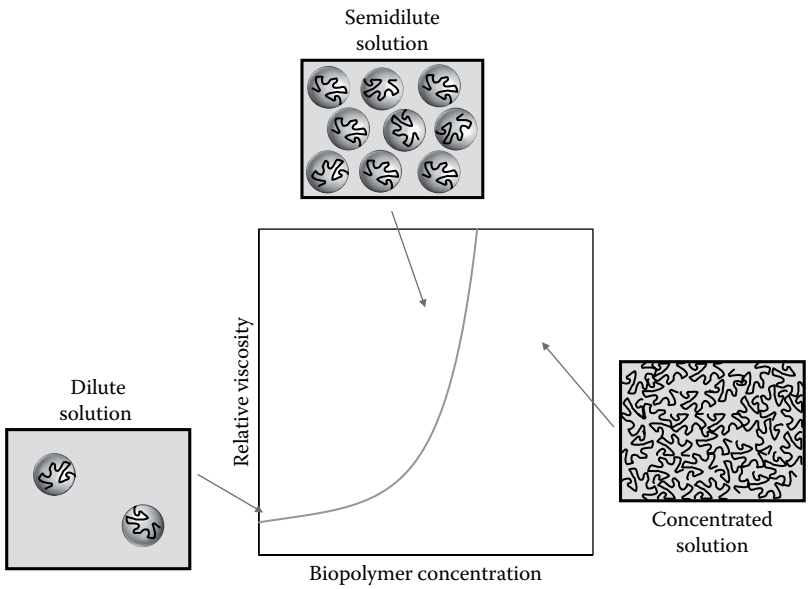

FIGURE 3.7 Extended biopolymers in aqueous solutions sweep out a large volume of water as they rotate, which increases their effective volume fraction and therefore their viscosity. The viscosity of an aqueous biopolymer solution increases with increasing biopolymer concentration.

In a dilute biopolymer solution, the individual molecules (or aggregates) do not interact with each other. When the concentration of biopolymer increases above some critical value c^*, the viscosity increases rapidly because the spheres swept out by the biopolymers overlap with each another. This type of solution is known as a *semidilute* solution, because even though the molecules are interacting with one another, each individual biopolymer is still largely surrounded by solvent molecules. At still higher polymer concentrations, the molecules pack so close together that they become entangled, and the system has more gel-like characteristics. Biopolymers that are used to thicken the aqueous phase of emulsions are often used in the semidilute concentration range [3].

Solutions containing extended biopolymers often exhibit strong shear-thinning behavior; that is, their apparent viscosity decreases with increasing shear stress. Some biopolymer solutions even have a characteristic yield stress. When a stress is applied below the yield stress, the solution acts like an elastic solid, but when it exceeds the yield stress the solution acts like a liquid. Shear thinning tends to occur because the biopolymer molecules become aligned with the shear field, or because the weak physical interactions responsible for biopolymer–biopolymer interactions are disrupted. The characteristic rheological behavior of biopolymer solutions plays an important role in determining their functional properties in food emulsions. For example, a salad dressing must be able to flow when it is poured from a container but must maintain its shape under its own weight after it has been poured onto a salad. The amount and type of biopolymer used must therefore be carefully selected to provide a low viscosity when the salad dressing is poured (high applied stress), but a high viscosity when the salad dressing is allowed to sit under its own weight (low applied stress).

The viscosity of biopolymer solutions is also related to the mouthfeel of a food product. Liquids that do not exhibit extensive shear-thinning behavior at the shear stresses experienced in the mouth are perceived as being "slimy." On the other hand, a certain amount of viscosity is needed to contribute to the "creaminess" of a product.

The shear-thinning behavior of biopolymer solutions is also important for determining the stability of food emulsions to creaming [1a]. As oil droplets move through an emulsion, they exert very small shear stresses on the surrounding liquid. Consequently, they experience a very large viscosity, which greatly slows down the rate at which they cream and therefore enhances stability. Many biopolymer solutions also exhibit thixotropic behavior (i.e., their viscosity decreases with time when they are sheared at a constant rate) as a result of disruption of the weak physical interactions that cause biopolymer molecules to aggregate. A food manufacturer must therefore select an appropriate biopolymer or combination of biopolymers to produce a final product that has a desirable mouthfeel and texture.

3. Gelation

Biopolymers are used as functional ingredients in many food emulsions (e.g., yogurt, cheese, desserts, egg, and meat products) because of their ability to cause the aqueous phase to gel [1a]. Gel formation imparts desirable textural and sensory attributes, as well as preventing the droplets from creaming. A biopolymer gel consists of a 3D network of aggregated or entangled biopolymers that entraps a large volume of water, giving the whole structure some solid-like characteristics. The appearance, texture, water-holding capacity, reversibility, and gelation temperature of biopolymer gels depend on the type, structure, and interactions of the molecules they contain.

Gels may be transparent or opaque, hard or soft, brittle or rubbery, homogeneous or heterogeneous; they may exhibit syneresis or have good water-holding capacity. Gelation may be induced by a variety of different methods, including altering the temperature, pH, ionic strength, or solvent quality; adding enzymes; and increasing the biopolymer concentration. Biopolymers may be cross-linked by covalent and/or noncovalent bonds.

It is convenient to distinguish between two types of gel: particulate and filamentous (Figure 3.8). Particulate gels consist of biopolymer aggregates (particles or clumps) that are assembled together to form a 3D network. This type of gel tends to be formed when there

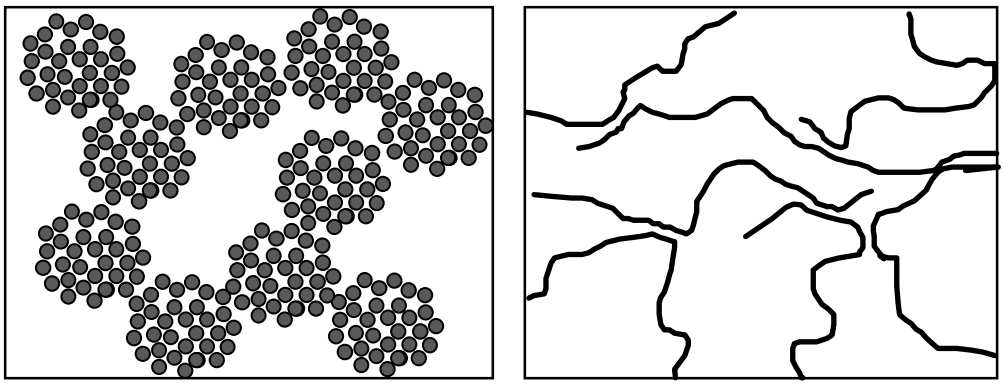

FIGURE 3.8 Biopolymer molecules or aggregates can form various types of gel structure, such as (a) particulate or (b) filamentous.

are strong attractive forces over the whole surface of the individual biopolymer molecules. Particulate gels are optically opaque because the particles scatter light, and they are prone to syneresis because the large interparticle pore sizes means that the water is not held tightly in the gel network by capillary forces. Filamentous gels consist of filaments of individual or aggregated biopolymer molecules that are relatively thin and tend to be formed by biopolymers that can form junction zones only at a limited number of sites on the surface of a molecule, or when the attractive forces between the molecules are so strong that they stick firmly together and do not undergo subsequent rearrangement [3]. Filamentous gels tend to be optically transparent because the filaments are so thin that they do not scatter light significantly, and they tend to have good water-holding capacity because the small pore size of the gel network means that the water molecules are held tightly by capillary forces.

In some foods a gel is formed upon heating (*heat-setting* gels), while in others it is formed upon cooling (*cold-setting* gels). Gels may also be either *thermoreversible* or *thermoirreversible*, depending on whether gelation is or is not reversible. Gelatin is an example of a cold-setting thermoreversible gel: when a solution of gelatin molecules is cooled below a certain temperature, a gel is formed, but when it is reheated the gel melts. Egg white is an example of a heat-setting thermoirreversible gel: when an egg is heated above a temperature at which gelation occurs, a characteristic white gel is formed; when the egg is cooled back to room temperature, however, the gel remains white (i.e., it does not revert back to its earlier liquid form). Whether a gel is reversible or irreversible depends on the changes in the molecular structure and organization of the molecules during gelation. Biopolymer gels that are stabilized by noncovalent interactions and do not involve large changes in the structure of the individual molecules prior to gelation tend to be reversible. On the other hand, gels that are held together by covalent bonds or involve large changes in the structure of the individual molecules prior to gelation tend to form irreversible gels.

The type of force holding the molecules together in gels varies from biopolymer to biopolymer. Some proteins and polysaccharides (e.g., gelatin, starch) form helical junction zones through extensive hydrogen bond formation. This type of junction zone tends to form when a gel is cooled, becoming disrupted when it is heated, and thus, it is responsible for cold-setting gels. Below the gelatin temperature, the attractive hydrogen bonds favor junction zone formation, but above this temperature the configurational entropy favors a random coil type of structure. Biopolymers with extensive nonpolar groups (e.g., caseins, denatured whey proteins) tend to associate via hydrophobic interactions. Electrostatic interactions play an important role in determining the gelation behavior of many biopolymers, and so gelation is particularly sensitive to the pH and ionic strength of the solution containing the biopolymers. For example, at pH values sufficiently far from their isoelectric

point, proteins may be prevented from gelling because of the electrostatic repulsion between the molecules. However, if the pH of the same solution is adjusted near to the isoelectric point, or salt is added, the proteins gel.

The addition of multivalent ions, such as Ca^{2+}, can promote the gelation of charged biopolymer molecules by forming salt bridges between the molecules. Proteins with thiol groups are capable of forming covalent linkages through thiol–disulfide interchanges, which help to strengthen and enhance the stability of gels. The tendency for a biopolymer to form a gel under certain conditions, and the physical properties of the gel formed, depend on a delicate balance of biopolymer–biopolymer, biopolymer–solvent, and solvent–solvent interactions of various kinds.

C. Ingredient Selection

A wide variety of proteins and polysaccharides are available as ingredients in foods, each with its own unique functional properties and optimum range of applications. Food manufacturers must decide which biopolymer is the most suitable for each type of food product. The selection of the most appropriate ingredient is often the key to success of a particular product. The factors a manufacturer must consider include the desired properties of the final product (appearance, rheology, mouthfeel, stability), the composition of the product, and the processing, storage, and handling conditions the food will experience during its lifetime, as well as the cost, availability, consistency from batch to batch, ease of handling, dispersibility, and functional properties of the biopolymer ingredient.

V. EMULSION FORMATION

The formation of an emulsion may involve a single step or a number of consecutive steps, depending on the nature of the starting material, the desired properties of the end product, and the instrument used to create it [1a]. Before separate oil and aqueous phases are converted to an emulsion, it is usually necessary to disperse the various ingredients into the phase in which they are most soluble. Oil-soluble ingredients, such as certain vitamins, coloring agents, antioxidants, and surfactants, are mixed with the oil, while water-soluble ingredients, such as proteins, polysaccharides, sugars, salts, and some vitamins, coloring agents, antioxidants, and surfactants, are mixed with the water. The intensity and duration of the mixing process depend on the time required to solvate and uniformly distribute the ingredients. Adequate solvation is important for the functionality of a number of food components. If the lipid phase contains any crystalline material, it is usually necessary to warm it before homogenization to a temperature at which all the fat melts; otherwise it is difficult, if not impossible, to efficiently create a stable emulsion.

The process of converting two immiscible liquids to an emulsion is known as *homogenization*, and a mechanical device designed to carry out this process is called a *homogenizer.* To distinguish the nature of the starting material, it is convenient to divide homogenization into two categories. The creation of an emulsion directly from two separate liquids will be referred to as *primary* homogenization, whereas the reduction in size of droplets in an existing emulsion will be referred to as *secondary* homogenization (Figure 3.9). The creation of a food emulsion may involve the use of one or the other form of homogenization, or a combination of both. For example, salad dressing is formed by direct homogenization of the aqueous and oil phases and is therefore an example of primary homogenization, whereas homogenized milk is manufactured by reducing the size of the fat globules in natural milk and hence is an example of secondary homogenization.

In many food processing operations and laboratory studies, it is more efficient to prepare an emulsion using two steps. The separate oil and water phases are converted to a coarse emulsion, with fairly large droplets, using one type of homogenizer (e.g., high-speed blender). Then the droplet size is reduced by means of another type of homogenizer (e.g., colloid mill, high-pressure valve homogenizer). In reality, many of the same physical processes that occur during primary

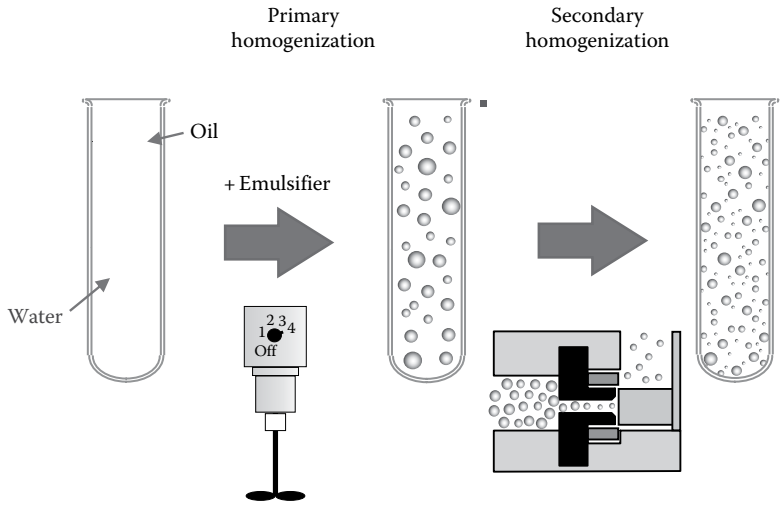

FIGURE 3.9 The homogenization process can be divided into two steps: primary homogenization (creating an emulsion from two separate phases) and secondary homogenization (reducing the size of the droplets in a preexisting emulsion).

homogenization also occur during secondary homogenization, and there is no clear distinction between them. Emulsions that have undergone secondary homogenization usually contain smaller droplets than those that have undergone primary homogenization, although this is not always the case. Some homogenizers (e.g., ultrasound, microfluidizers, membrane homogenizers) are capable of producing emulsions with small droplet sizes directly from separate oil and water phases (see Section V.C).

To highlight the important physical mechanisms that occur during homogenization, it is useful to consider the formation of an emulsion from pure oil and pure water. When the two liquids are placed in a container, they tend to adopt their thermodynamically most stable state, which consists of a layer of oil on top of the water (Figure 3.1). This arrangement is adopted because it minimizes the contact area between the two immiscible liquids and because the oil has a lower density than the water. To create an emulsion, it is necessary to mechanically agitate the system to disrupt and intermingle the oil and water phases. The type of emulsion formed in the absence of an emulsifier depends primarily on the initial concentration of the two liquids. At high oil concentrations a water-in-oil emulsion tends to form, but at low oil concentrations an oil-in-water emulsion tends to form. In this example, it is assumed that the oil concentration is so low that an oil-in-water emulsion is formed. Mechanical agitation can be applied in a variety of ways, the simplest being to vigorously shake the oil and water together in a sealed container. An emulsion is formed immediately after shaking, and it appears optically opaque (because light is scattered from the emulsion droplets). With time, the system rapidly reverts back to its initial state—a layer of oil sitting on top of the water. This is because the droplets formed during the application of the mechanical agitation are constantly moving around and frequently collide and coalesce with neighboring droplets. As this process continues, the large droplets formed rise to the top of the container and merge together to form a separate layer.

To form a stable emulsion, one must prevent the droplets from merging after they have been formed. This is achieved by having a sufficiently high concentration of a surface-active substance, known as an *emulsifier*, present during the homogenization process. The emulsifier rapidly adsorbs to the droplet surfaces during homogenization, forming a protective membrane that prevents the droplets from coming close enough together to coalesce. One of the major objectives of homogenization is to produce droplets as small as possible because this usually increases the shelf life of the

final product. It is therefore important for the food scientist to understand the factors that determine the size of the droplets produced during homogenization. It should be noted that homogenization is only one step in the formation of a food emulsion, and many of the other unit operations (e.g., pasteurization, cooking, drying, freezing, whipping) also affect the final quality of the product.

A. Physical Principles of Emulsion Formation

The size of the emulsion droplets produced by a homogenizer depends on a balance between two opposing mechanisms: *droplet disruption* and *droplet coalescence* (Figure 3.10). The tendency for emulsion droplets to break up during homogenization depends on the strength of the interfacial forces that hold the droplets together, compared to the strength of the disruptive forces in the homogenizer. In the absence of any applied external forces, emulsion droplets tend to be spherical because this shape minimizes the contact area between oil and water phases. Changing the shape of a droplet, or breaking it into smaller droplets, increases this contact area and therefore requires the input of energy. The interfacial force holding a droplet together is given by the *Laplace pressure* (ΔP_1):

$$\Delta P_1 = \frac{2\gamma}{r} \tag{3.4}$$

where
γ is the interfacial tension between oil and water
r is the droplet radius

This equation indicates that it is easier to disrupt large droplets than small ones and that the lower the interfacial tension, the easier it is to disrupt a droplet. The nature of the disruptive forces that act on a droplet during homogenization depends on the flow conditions (i.e., laminar, turbulent, or cavitational) the droplet experiences and therefore on the type of homogenizer used to create the emulsion. To deform and disrupt a droplet during homogenization, it is necessary to generate a stress that is greater than the Laplace pressure and to ensure that this stress is applied to the droplet long enough to enable it to become disrupted [21–23].

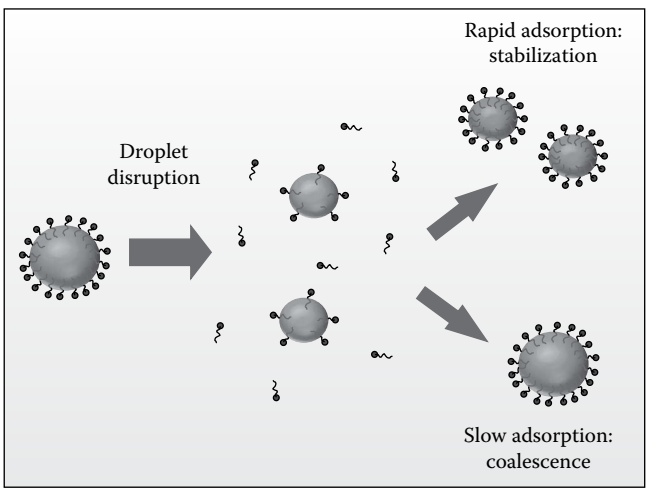

FIGURE 3.10 The size of the droplets produced in an emulsion is a balance between droplet disruption and droplet coalescence events that occur inside a homogenizer.

Emulsions are highly dynamic systems in which the droplets continuously move around and frequently collide with each other. Droplet–droplet collisions are particularly rapid during homogenization because of the intense mechanical agitation of the emulsion. If droplets are not protected by a sufficiently strong emulsifier membrane, they tend to coalesce during collision. Immediately after the disruption of an emulsion droplet during homogenization, there is insufficient emulsifier present to completely cover the newly formed surface, and therefore, the new droplets are more likely to coalesce with their neighbors. To prevent coalescence from occurring, it is necessary to form a sufficiently concentrated emulsifier membrane around a droplet before it has time to collide with its neighbors. The size of droplets produced during homogenization therefore depends on the time taken for the emulsifier to be adsorbed to the surface of the droplets ($\tau_{adsorption}$) compared to the time between droplet–droplet collisions ($\tau_{collision}$). If $\tau_{adsorption} < \tau_{collision}$, the droplets are rapidly coated with emulsifier as soon as they are formed and are stable; but if $\tau_{adsorption} > \tau_{collision}$, the droplets tend to rapidly coalesce because they are not completely coated with emulsifier before colliding with one of their neighbors. The values of these two times depend on the flow profile the droplets experience during homogenization, as well as the physicochemical properties of the bulk phases and the emulsifier [1a,23].

B. Role of Emulsifiers

The preceding discussion has highlighted two of the most important roles of emulsifiers during the homogenization process:

1. Their ability to decrease the interfacial tension between oil and water phases and thus reduce the amount of energy required to deform and disrupt a droplet (Equation 3.4). It has been demonstrated experimentally that when the movement of an emulsifier to the surface of a droplet is not rate limiting ($\tau_{adsorption} \ll \tau_{collision}$), there is a decrease in the droplet size produced during homogenization with a decrease in the equilibrium interfacial tension [24].
2. Their ability to form a protective membrane that prevents droplets from coalescing with their neighbors during a collision.

The effectiveness of emulsifiers at creating emulsions containing small droplets depends on a number of factors: (1) the concentration of emulsifier present relative to the dispersed phase; (2) the time required for the emulsifier to move from the bulk phase to the droplet surface; (3) the probability that an emulsifier molecule will be adsorbed to the surface of a droplet during a droplet–emulsifier encounter (i.e., the adsorption efficiency); (4) the amount by which the emulsifier reduces the interfacial tension; and (5) the effectiveness of the emulsifier membrane at protecting the droplets against coalescence.

It is often assumed that small emulsifier molecules adsorb to the surface of emulsion droplets during homogenization more rapidly than larger ones. This assumption is based on the observation that small molecules diffuse to the interface more rapidly than larger ones under quiescent conditions [3]. It has been demonstrated that under turbulent conditions large surface-active molecules tend to accumulate at the droplet surface during homogenization preferentially to smaller ones [23].

C. Homogenization Devices

There are a wide variety of food emulsions, and each one is created from different ingredients and must have different final characteristic properties. Consequently, a number of homogenization devices have been developed for the chemical production of food emulsions, each with its own particular advantages and disadvantages, and each having a range of foods to which it is most suitably applied [1a]. The choice of a particular homogenizer depends on many factors, including

the equipment available, the site of the process (i.e., a factory or a laboratory), the physicochemical properties of the starting materials and final product, the volume of material to be homogenized, the throughput, the desired droplet size of the final product, and the cost of purchasing and running the equipment. The most important types of homogenizer used in the food industry are discussed in the subsections that follow.

1. High-Speed Blenders

High-speed blenders are the most commonly used means of directly homogenizing bulk oil and aqueous phases. The oil and aqueous phase are placed in a suitable container, which may contain as little as a few milliliters or as much as several liters of liquid, and agitated by a stirrer that rotates at high speeds. The rapid rotation of the blade generates intense velocity gradients that cause disruption of the interface between the oil and water, intermingling of the two immiscible liquids, and breakdown of larger droplets to smaller ones [25]. Baffles are often fixed to the inside of the container to increase the efficiency of the blending process by disrupting the flow profile. High-speed blenders are particularly useful for preparing emulsions with low or intermediate viscosities. Typically, they produce droplets that are between 1 and 10 μm in diameter.

2. Colloid Mills

The separate oil and water phases are usually blended together to form a coarse emulsion premix prior to their introduction into a colloid mill because this increases the efficiency of the homogenization process. The premix is fed into the homogenizer, where it passes between two disks separated by a narrow gap. One of the disks is usually stationary, while the other rotates at a high speed, thus generating intense shear stresses in the premix. These shear stresses are large enough to cause the droplets in the coarse emulsion to be broken down. The efficiency of the homogenization process can be improved by increasing the rotation speed, decreasing the flow rate, decreasing the size of the gap between the disks, and increasing the surface roughness of the disks. Colloid mills are more suitable than most other types of homogenizer for homogenizing intermediate or high viscosity fluids (e.g., peanut butter, fish or meat pastes), and they typically produce emulsions with droplet diameters between 1 and 5 μm.

3. High-Pressure Valve Homogenizers

Like colloid mills, high-pressure valve homogenizers are more efficient at reducing the size of the droplets in a coarse emulsion premix than at directly homogenizing two separate phases [26]. The coarse emulsion premix is forced through a narrow orifice under high pressure, which causes the droplets to be broken down because of the intense disruptive stresses (e.g., impact forces, shear forces, cavitation, turbulence) generated inside the homogenizer [27]. Decreasing the size of the orifice increases the pressure the emulsion experiences, which causes a greater degree of droplet disruption and therefore the production of smaller droplets. Nevertheless, the throughput is reduced and more energy must be expended. A food manufacturer must therefore select the most appropriate homogenization conditions for each particular application, depending on the compromise between droplet size, throughput, and energy expenditure. High-pressure valve homogenizers can be used to homogenize a wide variety of food products, ranging from low viscosity liquids to viscoelastic pastes, and can produce emulsions with droplet sizes as small as 0.1 μm.

4. Ultrasonic Homogenizers

A fourth type of homogenizer utilizes high-intensity ultrasonic waves that generate intense shear and pressure gradients. When applied to a sample containing oil and water, these waves cause the two liquids to intermingle and the large droplets formed to be broken down to smaller ones. There are two types of ultrasonic homogenizer commonly used in the food industry: piezoelectric

transducers and liquid jet generators [28]. Piezoelectric transducers are most commonly found in the small benchtop ultrasonic homogenizers used in many laboratories. They are ideal for preparing small volumes of emulsion (a few milliliters to a few hundred milliliters), a property that is often important in fundamental research when expensive components are used. The ultrasonic transducer consists of a piezoelectric crystal contained in some form of protective metal casing, which is tapered at the end. A high-intensity electrical wave is applied to the transducer, which causes the piezoelectric crystal inside to oscillate and generate an ultrasonic wave. The ultrasonic wave is directed toward the tip of the transducer, where it radiates into the surrounding liquids, generating intense pressure and shear gradients (mainly due to cavitational effects) that cause the liquids to be broken up into smaller fragments and intermingled with one another. It is usually necessary to irradiate a sample with ultrasound for a few seconds to a few minutes to create a stable emulsion. Continuous application of ultrasound to a sample can cause appreciable heating, and so it is often advantageous to apply the ultrasound in a number of short bursts.

Ultrasonic jet homogenizers are used mainly for industrial applications. A stream of fluid is made to impinge on a sharp-edged blade, which causes the blade to rapidly vibrate, thus generating an intense ultrasonic field that breaks up any droplets in its immediate vicinity through a combination of cavitation, shear, and turbulence [28]. This device has three major advantages: it can be used for continuous production of emulsions; it can generate very small droplets; and it is more energy efficient than high-pressure valve homogenizers (since less energy is needed to form droplets of the same size).

5. Microfluidization

Microfluidization is a technique that is capable of creating an emulsion with small droplet sizes directly from the individual oil and aqueous phases [29]. Separate streams of an oil and an aqueous phase are accelerated to a high velocity and then made to simultaneously impinge on a surface, which causes them to be intermingled and leads to effective homogenization. Microfluidizers can be used to produce emulsions that contain droplets as small as 0.1 μm.

6. Membrane Homogenizers

Membrane homogenizers form emulsions by forcing one immiscible liquid into another through a glass membrane that is uniform in pore size. The size of the droplets formed depends on the diameter of the pores in the membrane and on the interfacial tension between the oil and water phases [30]. Membranes can be manufactured with different pore diameters, with the result that emulsions with different droplet sizes can be produced [30]. The membrane technique can be used either as a batch or as a continuous process, depending on the design of the homogenizer. Increasing numbers of applications for membrane homogenizers are being identified, and the technique can now be purchased for preparing emulsions in the laboratory or commercially. These instruments can be used to produce oil-in-water, water-in-oil, and multiple emulsions. Membrane homogenizers have the ability to produce emulsions with very narrow droplet size distributions, and they are highly energy efficient, since there is much less energy loss due to viscous dissipation.

7. Energy Efficiency of Homogenization

The efficiency of the homogenization process can be calculated by comparing the energy required to increase the surface area between the oil and water phases with the actual amount of energy required to create an emulsion. The difference in free energy between the two separate immiscible liquids and an emulsion can be estimated by calculating the amount of energy needed to increase the interfacial area between the oil and aqueous phases ($\Delta G = \gamma \Delta A$). Typically, this is less than 0.1% of the total energy input into the system during the homogenization process because most of the energy supplied to the system is dissipated as heat, owing to frictional losses associated with

the movement of molecules past one another [23]. This heat exchange accounts for the significant increase in temperature of emulsions during homogenization.

8. Choosing a Homogenizer

The choice of a homogenizer for a given application depends on a number of factors, including volume of sample to be homogenized, desired throughput, energy requirements, nature of the sample, final droplet size distribution required, equipment available, and initial and running costs. Even after the most suitable homogenization technique has been chosen, the operator must select the optimum processing conditions, such as temperature, time, flow rate, pressure, valve gaps, rotation rates, and sample composition. If an application does not require that the droplets in an emulsion be particularly small, it is usually easiest to use a high-speed blender. High-speed blenders are also used frequently to produce the coarse emulsion premix that is fed into other devices.

To create an emulsion that contains small droplets (<1 μm), either industrially or in the laboratory, it is necessary to use one of the other methods. Colloid mills are the most efficient type of homogenizer for high-viscosity fluids, whereas high-pressure valve, ultrasonic, or microfluidization homogenizers are more efficient for liquids that are low or intermediate in viscosity. In fundamental studies, one often uses small volumes of sample, and therefore, a number of laboratory homogenizers have been developed that are either scaled-down versions of industrial equipment or instruments specifically designed for use in the laboratory. For studies involving ingredients that are limited in availability or expensive, an ultrasonic piezoelectric transducer can be used because it requires only small sample volumes. When it is important to have monodisperse emulsions, the use of a membrane homogenizer would be advantageous.

D. Factors That Determine Droplet Size

The food manufacturer is often interested in producing emulsion droplets that are as small as possible, using the minimum amount of energy input and the shortest amount of time. The size of the droplets produced in an emulsion depends on many different factors, some of which are summarized here [27–30].

Emulsifier concentration. Up to a certain level, the size of the droplets usually decreases as the emulsifier concentration increases; above this level, droplet size remains constant. When the emulsifier concentration exceeds the critical level, the size of the droplets is governed primarily by the energy input of the homogenization device.

Emulsifier type. At the same concentration, different types of emulsifiers produce differently sized droplets, depending on their surface load, the speed at which they reach the oil–water interface, and the ability of the emulsifier membrane to prevent droplet coalescence.

Homogenization conditions. The size of the emulsion droplets usually decreases as the energy input or homogenization time increases.

Physicochemical properties of bulk liquids. The homogenization efficiency depends on the physicochemical properties of the lipids that comprise an emulsion (e.g., their viscosity, interfacial tension, density, or physical state).

VI. EMULSION STABILITY

Emulsions are thermodynamically unstable systems that tend, with time, to separate back into individual oil and water phases (Figure 3.1). The term "emulsion stability" refers to the ability of an emulsion to resist changes in its properties with time: the greater the emulsion stability, the longer the time taken for the emulsion to alter its properties [1a]. Changes in the properties of emulsions

may be the result of physical processes that cause alterations in the spatial distribution of the ingredients (e.g., creaming, flocculation, coalescence, phase inversion) or chemical processes that cause alterations in the chemical structure of the ingredients (e.g., oxidation, hydrolysis). It is important for food scientists to elucidate the relative importance of each of these mechanisms, the relationship between them, and the factors that affect them so that effective means of controlling the properties of food emulsions can be established.

A. Droplet–Droplet Interactions

The bulk properties of food emulsions are largely determined by the interaction of the droplets with each other. If the droplets exert a strong mutual attraction, they tend to aggregate, but if they are strongly repelled they tend to remain as separate entities. The overall interaction between droplets depends on the magnitude and range of a number of different types of attractive and repulsive interaction. A knowledge of the origin and nature of these interactions is important because it enables food scientists to predict and control the stability and physicochemical properties of food emulsions.

Droplet–droplet interactions are characterized by an interaction potential $\Delta G(s)$, which describes the variation of the free energy with droplet separation. The overall interaction potential between emulsion droplets is the sum of various attractive and repulsive contributions [3]:

$$\Delta G(s) = \Delta G_{\text{VDW}}(s) + \Delta G_{\text{electrostatic}}(s) + \Delta G_{\text{hydrophobic}}(s) + \Delta G_{\text{short range}}(s) \tag{3.5}$$

where ΔG_{VDW}, $\Delta G_{\text{electrostatic}}$, $\Delta G_{\text{hydrophobic}}$, and $\Delta G_{\text{short range}}$ refer to the free energies associated with van der Waals, electrostatic, hydrophobic, and various short-range forces, respectively. In certain systems, there are additional contributions to the overall interaction potential from other types of mechanism, such as depletion or bridging [1a,1b]. The stability of food emulsions to aggregation depends on the shape of the free energy versus separation curve, which is governed by the relative contributions of the different types of interactions [1–3].

1. Van der Waals Interactions

The van der Waals interactions act between emulsion droplets of all types and are always attractive. At close separations, the van der Waals interaction potential between two emulsion droplets of equal radius r separated by a distance s is given by Equation 3.6 [12]:

$$\Delta G_{\text{VDW}}(s) = -\frac{Ar}{12s} \tag{3.6}$$

where A is the Hamaker parameter, which depends on the physical properties of the oil and water phases. This equation provides a useful insight into the nature of the van der Waals interaction. The strength of the interaction decreases with the reciprocal of droplet separation, and so van der Waals interactions are fairly long range compared to other types of interactions. In addition, the strength of the interaction increases as the size of the emulsion droplets increases. In practice, Equation 3.6 tends to overestimate the attractive forces because it ignores the effects of electrostatic screening, radiation, and the presence of the droplet membrane on the Hamaker parameter [11].

2. Electrostatic Interactions

Electrostatic interactions occur only between emulsion droplets that have electrically charged surfaces (e.g., those established by ionic surfactants or biopolymers). The electrostatic interaction between two droplets at close separation is given by the following relationship [5]:

$$\Delta G_{\text{electrostatic}}(s) = 4.3\times10^{-9} r\psi_0^2 \ln(1+e^{-4.5}) \tag{3.7}$$

where

$$\kappa^{-1} = \left(\frac{\varepsilon_0 \varepsilon_r kT}{e^2 \Sigma c_i z_i^2} \right)^{1/2}$$

Here

κ^{-1} is the thickness of the electric double layer
c_i and z_i are the molar concentration and valency of ions of species i
ε_0 is the dielectric constant of a vacuum
ε_r is the relative dielectric constant of the medium surrounding the droplet
e is the electrical charge
ψ_0 is the surface potential
k is the Boltzmann constant
T is the temperature

These equations provide a useful insight into the nature of the electrostatic interactions between emulsion droplets. Usually, all the droplets in food emulsions have the same electrical charge, hence repel each other. Electrostatic interactions are therefore important for preventing droplets from aggregating. The strength of the interactions increases as the magnitude of the surface potential increases; thus, the greater the number of charges per unit area at a surface, the greater the protection against aggregation. The strength of the repulsive interaction decreases as the concentration of valency of ions in the aqueous phase increases because counterions "screen" the charges between droplets, which causes a decrease in the thickness of the electrical double layer. Emulsions stabilized by proteins are particularly sensitive to the pH and ionic strength of the aqueous solution, since altering pH changes ψ_0 and altering ionic strength changes κ^{-1}. The strength of the electrostatic interaction also increases as the size of the emulsion droplets increases.

3. Hydrophobic Interactions

The surfaces of emulsion droplets may not be completely covered by emulsifier molecules, or the droplet membrane may have some nonpolar groups exposed to the aqueous phase [la]. Consequently, there may be attractive hydrophobic interactions between nonpolar groups and water. The interaction potential energy per unit area between two hydrophobic surfaces separated by water is given by

$$\Delta G_{\text{hydrophobic}}(s) = -0.69 \times 10^{-10} r\phi \exp\left(-\frac{s}{\lambda_0} \right) \tag{3.8}$$

where ϕ is the fraction of the droplet surface (which is hydrophobic) and the decay length λ_0 is of the order of 1–2 nm [11]. The hydrophobic attraction between droplets with nonpolar surfaces is fairly strong and relatively long range [11]. Hydrophobic interactions therefore play an important role in determining the stability of a number of food emulsions. Protein-stabilized emulsions often have nonpolar groups on the protein molecules exposed to the aqueous phase, and therefore, hydrophobic interactions are important. They are also important during homogenization because the droplets are not covered by emulsifier molecules.

4. Short-Range Forces

When two emulsion droplets come sufficiently close together, their interfacial layers start to interact. A number of short-range forces result from these interactions, including steric (osmotic and elastic components), hydration, protrusion, and undulation forces [11,12]. Some progress has been made in developing theories to predict the magnitude and range of short-range forces associated with interfacial layers of fairly simple geometry. Nevertheless, both magnitude and range of these

forces are particularly sensitive to the size, shape, conformation, packing, interactions, mobility, and hydration of the molecules in the adsorbed layer, and so it is difficult to predict their contribution to the overall interaction potential with any certainty. Even so, they are usually repulsive and tend to increase strongly as the interfacial layers overlap.

5. Overall Interaction Potential

It is often difficult to accurately calculate the contribution of each type of interaction to the overall interdroplet pair potential because information about the relevant physicochemical properties of the system is lacking. Nevertheless, it is informative to examine the characteristics of certain combinations of interactions that are particularly important in food emulsions, for this provides a valuable insight into the factors that affect the tendency of droplets to aggregate. Consider an emulsion in which the only important types of droplet–droplet interaction are van der Waals attraction, electrostatic repulsion, and steric repulsion (e.g., an emulsion stabilized by a charged biopolymer).

The van der Waals interaction potential is fairly long range and always negative (attractive), the electrostatic interaction potential is fairly long range and always positive (repulsive), while the steric interaction is short range and highly positive (strongly repulsive). The overall interdroplet pair potential has a complex dependence on separation because it is the sum of these three different interactions, and it may be attractive at some separations and repulsive at others. Figure 3.11 shows a typical profile of interdroplet pair potential versus separation for an emulsion stabilized by a charged biopolymer. When the two droplets are separated by a large distance, there is no effective interaction between them. As they move closer together, the van der Waals attraction dominates

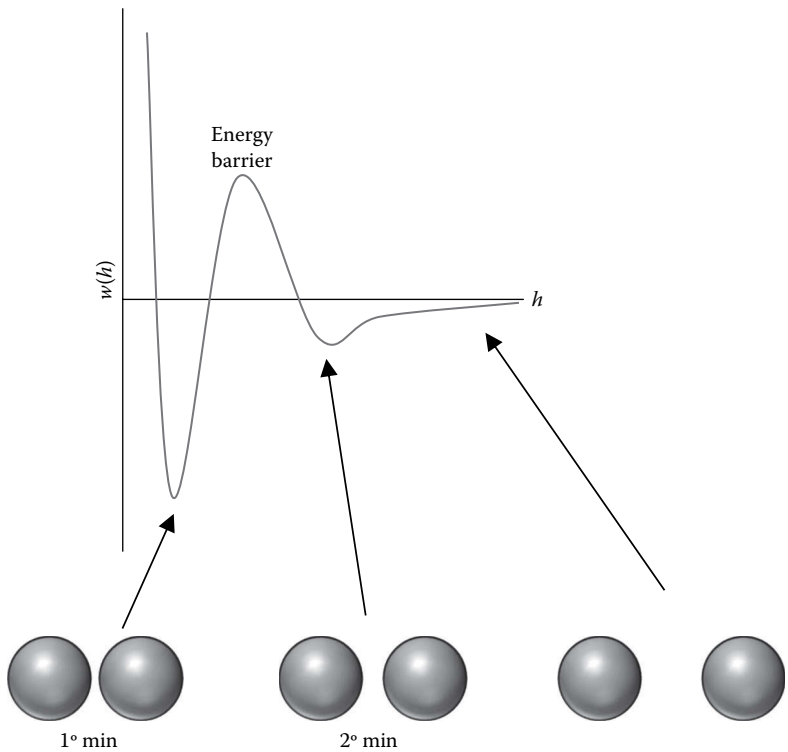

FIGURE 3.11 Typical overall interaction potential for an emulsion stabilized by a charged emulsifier that has a short-range steric repulsion, a medium-range electrostatic repulsion, and a long-range van der Waals attraction.

initially and there is a shallow minimum in the profile, which is referred to as the secondary minimum. If the depth of this minimum is large compared to the thermal energy ($|\Delta G(s_{min})| \gg kT$), the droplets tend to be flocculated. However, if it is small compared to the thermal energy, the droplets tend to remain unaggregated. At closer separations, the repulsive electrostatic interactions dominate, and there is an energy barrier $\Delta G(s_{max})$ that must be overcome before the droplets can come any closer. If this energy barrier is sufficiently large compared to the thermal energy $\Delta G(s_{max}) \gg kT$, it will prevent the droplets from falling into the deep primary minimum at close separations. On the other hand, if it is not large compared to the thermal energy, the droplets will tend to fall into the primary minimum, leading to strong flocculation of the droplets. In this situation, the droplets would be prevented from coalescing because of the domination of the strong steric repulsion at close separations.

Emulsions that are stabilized by repulsive electrostatic interactions are particularly sensitive to the ionic strength and pH of the aqueous phase [1a,1b]. At low ion concentrations, there may be a sufficiently high energy barrier to prevent the droplets from getting close enough together to aggregate into the primary minimum. As the ion concentration is increased, the screening of the electrostatic interactions becomes more effective, which reduces the height of the energy barrier. Above a certain ion concentration, the energy barrier is not high enough to prevent the droplets from falling into the primary minimum, and so the droplets become strongly flocculated. This phenomenon accounts for the tendency of droplets to flocculate when salt is added to emulsions stabilized by ionic emulsifiers. The surface charge density of protein-stabilized emulsions decreases as the pH tends toward the isoelectric point, which reduces the magnitude of the repulsive electrostatic interactions between the droplets and also leads to droplet flocculation.

B. Mechanisms of Emulsion Instability

As mentioned earlier, emulsions are thermodynamically unstable systems that tend with time to revert back to the separate oil and water phases of which they were made. The rate at which this process occurs and the route that is taken depend on the physicochemical properties of the emulsion and the prevailing environmental conditions. The most important mechanisms of physical instability are creaming, flocculation, coalescence, Ostwald ripening, and phase inversion. In practice, all these mechanisms act in concert and can influence one another. However, one mechanism often dominates the others, facilitating the identification of the most effective method of controlling emulsion stability.

The length of time an emulsion must remain stable depends on the nature of the food product. Some food emulsions (e.g., cake batters, ice cream mix, margarine premix) are formed as intermediate steps during a manufacturing process and need remain stable for only a few seconds, minutes, or hours. Other emulsions (e.g., mayonnaise, cream liqueurs) must persist in a stable state for days, months, or even years prior to sale and consumption. Some food processing operations (e.g., the production of butter, margarine, whipped cream, and ice cream) rely on controlled *de*stabilization of an emulsion. We now turn to a discussion of the origin of the major destabilization mechanisms, the factors that influence them, and methods of controlling them. This type of information is useful for food scientists because it facilitates the selection of the most appropriate ingredients and processing conditions required to produce a food emulsion with particular properties.

1. Creaming and Sedimentation

The droplets in an emulsion have a density different from that of the liquid that surrounds them, and so a net gravitational force acts on them [1a,1b]. If the droplets have lower density than the surrounding liquid, they tend to move up, that is, to "cream." Conversely, if they have a higher density they tend to move down, resulting in what is referred to as sedimentation. Most liquid oils have densities lower than that of water, and so there is a tendency for oil to accumulate at the top of an emulsion and water at the bottom. Thus, droplets in an oil-in-water emulsion tend to cream, whereas those in

a water-in-oil emulsion tend to sediment. The creaming rate of a single isolated spherical droplet in a viscous liquid is given by the Stokes equation:

$$\nu = -\frac{2gr^2(\rho_2 - \rho_1)}{9\eta_1} \tag{3.9}$$

where

ν is the creaming rate
g is the acceleration due to gravity
ρ is the density
η is the shear viscosity
The subscripts 1 and 2 refer to the continuous phase and droplet, respectively

The sign of ν determines whether the droplet moves up (+) or down (–).

Equation 3.9 can be used to estimate the stability of an emulsion to creaming. For example, an oil droplet (ρ_2 = 910 kg/m^3) with a radius of 1 μm suspended in water (η_1 = 1 mPa·s, ρ_1 = 1000 kg/m^3) will cream at a rate of about 5 mm/day. Thus, one would not expect an emulsion containing droplets of this size to have a particularly long shelf life. As a useful rule of thumb, an emulsion in which the creaming rate is less than about 1 mm/day can be considered to be stable toward creaming [3].

In the initial stages of creaming (Figure 3.12), the droplets move upward and a droplet-depleted layer is observed at the bottom of the container. When the droplets reach the top of the emulsion, they cannot move up any further and so they pack together to form the "creamed layer." The thickness of the final creamed layer depends on the packing of the droplets in it. Droplets may pack very tightly together, or they may pack loosely, depending on their polydispersity and the magnitude of the forces between them. Close-packed droplets will tend to form a thin creamed layer, whereas loosely packed droplets form a thick creamed layer. The same factors that affect the packing of the droplets in a creamed layer determine the nature of the flocs formed (see Section VI.B.2). If the attractive forces between the droplets are fairly weak, the creamed emulsion can be redispersed by

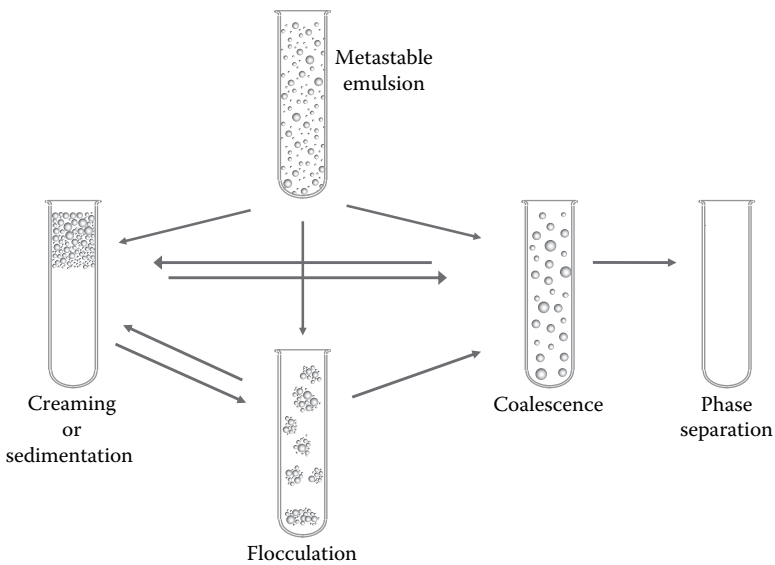

FIGURE 3.12 Common mechanisms of emulsion instability: gravitational separation (creaming or sedimentation), flocculation, coalescence, and phase separation.

lightly agitating the system. On the other hand, if an emulsion is centrifuged, or if the droplets in a creamed layer are allowed to remain in contact for extended periods, significant coalescence of the droplets may occur, with the result that the emulsion droplets can no longer be redispersed by mild agitation.

Creaming of emulsion droplets is usually an undesirable process, which food manufacturers try to avoid. Equation 3.9 indicates that creaming can be retarded by minimizing the density difference ($\rho_2 - \rho_1$) between the droplets and the surrounding liquid, reducing the droplet size, or increasing the viscosity of the continuous phase. The Stokes equation is strictly applicable only to isolated rigid spheres suspended in an infinite viscous liquid. Since these assumptions are not valid for food emulsions, the equation must be modified to take into account hydrodynamic interactions, droplet fluidity, droplet aggregation, non-Newtonian aqueous phases, droplet crystallization, the adsorbed layer, and Brownian motion [1a,2].

2. Flocculation and Coalescence

The droplets in emulsions are in continual motion because of their thermal energy, gravitational forces, or applied mechanical forces, and as they move about they collide with their neighbors. After a collision, emulsion droplets may either move apart or remain aggregated, depending on the relative magnitude of the attractive and repulsive forces between them. If the net force acting between the droplets is strongly attractive, they will aggregate, but if it is strongly repulsive they will remain unaggregated. Two types of aggregation are commonly observed in emulsions: flocculation and coalescence. In flocculations (Figure 3.12), two or more droplets come together to form an aggregate in which the emulsion droplets retain their individual integrity. Coalescence is the process whereby two or more droplets merge together to form a single larger droplet (Figure 3.12). Improvements in the quality of emulsion-based food products largely depend on an understanding of the factors that cause droplets to aggregate. The rate at which droplet aggregation occurs in an emulsion depends on two factors: *collision frequency* and *collision efficiency* [1a,1b].

The collision frequency is the number of encounters between droplets per unit time per unit volume. Any factor that increases the collision frequency is likely to increase the aggregation rate. The frequency of collisions between droplets depends on whether the emulsion is subjected to mechanical agitation. For dilute emulsions containing identical spherical particles, the collision frequency N has been calculated for both quiescent and stirred systems [3]:

$$N = \frac{4kTn_0^2}{3\eta} \tag{3.10}$$

$$N = \frac{16}{3} Gr^3 n_0^2 \tag{3.11}$$

where

n_0 is the initial number of particles per unit volume

G is the shear rate

The collision efficiency, E, is the fraction of encounters between droplets that lead to aggregation. Its value ranges from 0 (no flocculation) to 1 (fast flocculation) and depends on the interaction potential. The equations for the collision frequency must therefore be modified to take into account droplet–droplet interactions:

$$N = \frac{4kTn_0^2}{3\eta} E \tag{3.12}$$

where

$$E = \int_{2r}^{x} \left\{ \exp \left| \frac{\Delta G(x)}{kT} \right| x^{-2} dx \right\}^{-1}$$

with x the distance between the centers of the droplets ($x = 2r + s$) and $\Delta G(x)$ the droplet–droplet interaction potential (Section VI.A). Emulsion droplets may remain unaggregated, or they may aggregate into the primary or secondary minima depending on $\Delta G(x)$.

These equations are applicable only to the initial stages of aggregation in dilute emulsions containing identical spherical particles. In practice, most food emulsions are fairly concentrated systems, and interactions between flocs as well as between individual droplets are important. These equations must therefore be modified to take into account the interactions and properties of flocculated droplets.

The nature of the droplet–droplet interaction potential also determines the structure of the flocs formed and the rheology and stability of the resulting emulsion [1a]. When the attractive force between them is relatively strong, two droplets tend to become "locked" together as soon as they encounter each other. This leads to the formation of flocs that have quite open structures [3]. When the attractive forces are not particularly strong, the droplets may "roll around" each other after a collision, which allows them to pack more efficiently to form denser flocs. These two extremes of floc structure are similar to those formed by filamentous and particulate gels, respectively (Figure 3.8).

The structure of the flocs formed in an emulsion has a pronounced influence on its bulk physicochemical properties. An emulsion containing flocculated droplets has a higher viscosity than one containing unflocculated droplets, since the water trapped between the flocculated droplets increases the effective diameter (and therefore volume fraction) of the particles (Equation 3.3). Flocculated particles also exhibit strong shear-thinning behavior: as the shear rate is increased, the viscosity of the emulsion decreases because the flocs are disrupted and so their effective volume fraction decreases. If flocculation is extensive, a 3D network of aggregated particles extends throughout the system and the emulsion has a yield stress that must be overcome before the system will flow. The creaming rate of droplets is also strongly dependent on flocculation. At low droplet concentrations, flocculation increases the creaming rate because the effective size of the particles is increased (Equation 3.9), but at high droplet concentrations, it retards creaming because the droplets are trapped within the 3D network of aggregated emulsion droplets.

In coalescence (Figure 3.12), two or more liquid droplets collide and merge into a single larger droplet. Extensive coalescence eventually leads to *oiling off*, that is, formation of free oil on the top of an emulsion. Because coalescence involves a decrease in the surface area of oil exposed to the continuous phase, it is one of the principal mechanisms by which an emulsion reverts to its most thermodynamically stable state (Figure 3.1). Coalescence occurs rapidly between droplets that are not protected by emulsifier molecules; for example, if one homogenizes oil and water in the absence of an emulsifier, the droplets readily coalesce. When droplets are stabilized by an emulsifier membrane, the tendency for coalescence to occur is governed by the droplet–droplet interaction potential and the stability of the film to rupture. If there is a strong repulsive force between the droplets at close separations, or if the film is highly resistant to rupture, the droplets will tend not to coalesce. Most food emulsions are stable to coalescence, but they become unstable when subjected to high shear forces that cause the droplets to frequently collide with each other or when the droplets remain in contact with each other for extended periods (e.g., droplets in flocs, creamed layers, or highly concentrated emulsions).

3. Partial Coalescence

Normal coalescence involves the aggregation of two or more liquid droplets to form a single larger spherical droplet, but partial coalescence occurs when two or more partially crystalline droplets

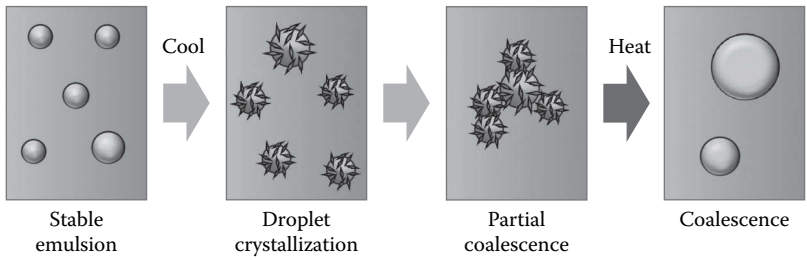

FIGURE 3.13 Partial coalescence occurs when two partly crystalline emulsion droplets collide and aggregate because a crystal in one droplet penetrates the other droplet.

encounter each other and form a single irregularly shaped aggregate (Figure 3.13). The aggregate is irregular in shape because some of the structure of the fat crystal network contained in the original droplets is maintained within it. It has been proposed that partial coalescence occurs when two partially crystalline droplets collide and a crystal from one of them penetrates the intervening membranes and protrudes into the liquid region of the other droplet [1a]. Normally, the crystal would stick out into the aqueous phase, thus becoming surrounded by water; however, when it penetrates another droplet, it is surrounded by oil, and because this arrangement is energetically favorable the droplets remain aggregated. With time the droplets slowly fuse more closely together, with the result that the total surface area of oil exposed to the aqueous phase is reduced. Partial coalescence occurs only when the droplets have a certain ratio of solid fat and liquid oil. If the solid fat content of the droplets is either too low or too high, the droplets will tend not to undergo partial coalescence [5].

Partial coalescence is particularly important in dairy products because milk fat globules are partially crystalline at temperatures commonly found in foods. The application of shear forces or temperature cycling to cream containing partly crystalline milk fat globules can cause extensive aggregation of the droplets, leading to a marked increase in viscosity ("thickening") and subsequent phase separation [9]. Partial coalescence is an essential process in the production of ice cream, whipped toppings, butter, and margarine. Oil-in-water emulsions are cooled to a temperature at which the droplets are partly crystalline, and a shear force is then applied that causes droplet aggregation via partial coalescence. In butter and margarine, aggregation results in phase inversion, whereas in ice cream and whipped cream the aggregated fat droplets form a network that surrounds air cells and provides the mechanical strength needed to produce good stability and texture.

4. Ostwald Ripening

Ostwald ripening is the growth of large droplets at the expense of smaller ones [1a]. This process occurs because the solubility of the material in a spherical droplet increases as the size of the droplet decreases:

$$S(r) = S(\infty)\exp\left(\frac{2\gamma V_m}{RTr}\right) \tag{3.13}$$

Here

V_m is the molar volume of the solute
γ is the interfacial tension
R is the gas constant
$S(\infty)$ is the solubility of the solute in the continuous phase for a droplet with infinite curvature (i.e., a planar interface)
$S(r)$ is the solubility of the solute when contained in a spherical droplet of radius r

The greater solubility of the material in smaller droplets means that there is a higher concentration of solubilized material around a small droplet than around a larger one. Consequently, solubilized molecules move from small droplets to large droplets because of this concentration gradient, which causes the larger droplets to grow at the expense of the smaller ones. Once steady-state conditions have been achieved, the growth in droplet radius with time due to Ostwald ripening is given by

$$\frac{d\langle r\rangle^3}{dt} = \frac{8\gamma V_m S(\infty) D}{9RT} \tag{3.14}$$

where D is the diffusion coefficient of the material through the continuous phase. This equation assumes that the emulsion is dilute and that the rate-limiting step is the diffusion of the solute molecules across the continuous phase. In practice, most food emulsions are concentrated systems, and so the effects of the neighboring droplets on the growth rate have to be considered. Some droplets are surrounded by interfacial membranes that retard the diffusion of solute molecules in and out of droplets, and in such cases, the equation must be modified accordingly. Ostwald ripening is negligible in many foods because triacylglycerols have extremely low water solubilities, and therefore, the mass transport rate is insignificant (Equation 3.14). Nevertheless, in emulsions that contain more water-soluble lipids, such as flavor oils, Ostwald ripening may be important.

5. Phase Inversion

In phase inversion (Figure 3.12), a system changes from an oil-in-water emulsion to a water-in-oil emulsion or vice versa. This process usually occurs as a result of some alteration in the system's composition or environmental conditions, such as dispersed phase volume fraction, emulsifier type, emulsifier concentration, temperature, or application of mechanical forces. Phase inversion is believed to occur by means of a complex mechanism that involves a combination of the processes that occur during flocculation, coalescence, and emulsion formation. At the point where phase inversion occurs, the system may briefly contain regions of oil-in-water emulsion, water-in-oil emulsion, multiple emulsions, and bicontinuous phases, before converting to its final state.

6. Chemical and Biochemical Stability

Chemical and biochemical reactions of various types (e.g., oxidation, reduction, or hydrolysis of lipids, polysaccharides, and proteins) can cause detrimental changes in the quality of food emulsions. Many of these reactions are catalyzed by specific enzymes that may be present in the food. The reactions that are important in a given food emulsion depend on the concentration, type, and distribution of ingredients, and the thermal and shear history of the food. Chemical and biochemical reactions can alter the stability, texture, flavor, odor, color, and toxicity of food emulsions. Thus, it is important to identify the most critical reactions that occur in each type of food so that they can be controlled in a systematic fashion.

VII. CHARACTERIZATION OF EMULSION PROPERTIES

Ultimately, food manufacturers want to produce a high-quality product at the lowest possible cost. To achieve this goal, they must have a good appreciation of the factors that determine the properties of the final product. This knowledge, in turn, is used to formulate and manufacture a product with the desired characteristics (e.g., appearance, texture, mouthfeel, taste, shelf life). These bulk physicochemical and sensory properties are determined by such molecular and colloidal properties of emulsions as dispersed volume fraction, droplet size distribution, droplet–droplet interactions, and interfacial properties. Consequently, a wide variety of experimental techniques have been developed to characterize the molecular, colloidal, microscopic, and macroscopic properties of food

emulsions [1a]. Analytical techniques are needed to characterize the properties of food emulsions in the laboratory, where they are used to improve our understanding of the factors that determine emulsion properties, and in the factory, where they are used to monitor the properties of foods during processing to ensure that the manufacturing process is operating in an appropriate manner [31,32]. The sections that follow highlight some of the most important properties of food emulsions and outline experimental techniques for their measurement.

A. Dispersed Phase Volume Fraction

The dispersed phase volume fraction or ϕ is the volume of emulsion droplets (V_D) divided by the total volume of the emulsion (V_E): $\phi = V_D/V_E$. The dispersed phase volume fraction determines the relative proportion of oil and water in a product, as well as influencing many of the bulk physicochemical and sensory properties of emulsions, such as appearance, rheology, taste, and stability. For example, an emulsion tends to become more turbid and to have a higher viscosity when the concentration of droplets is increased [1a]. Methods for measuring the dispersed phase volume fraction of emulsions are outlined in Table 3.2. Traditional proximate analysis techniques, such as solvent extraction to determine oil content and oven drying to determine moisture content, can be used to analyze the dispersed phase volume fraction of emulsions. Nevertheless, proximate analysis techniques are often destructive and quite time-consuming to carry out and are therefore unsuitable for rapid quality control or online measurements. If the densities of the separate oil and aqueous phases are known, the dispersed phase volume fraction of an emulsion can simply be determined from a measurement of its density:

$$\phi = (\rho_{\text{emulsion}} - \rho_{\text{continuous phase}})(\rho_{\text{droplet}} - \rho_{\text{continuous phase}}) \tag{3.15}$$

The electrical conductivity of an emulsion decreases as the concentration of oil within it increases, and so instruments based on electrical conductivity can also be used to determine ϕ. Light scattering techniques can be used to measure the dispersed phase volume fraction of dilute emulsions ($\phi < 0.001$), whereas NMR and ultrasound spectroscopy can be used to rapidly and nondestructively determine φ of concentrated and optically opaque emulsions. A number of these experimental techniques (e.g., ultrasound, NMR, electrical conductivity, density measurements) are particularly suitable for online determination of the composition of food emulsions during processing.

TABLE 3.2
Experimental Techniques for Characterizing the Physicochemical Properties of Food Emulsions

Dispersed phase volume fraction	Proximate analysis, density, electrical conductivity, light scattering, NMR, ultrasound
Droplet size distribution	Light scattering (static and dynamic), electrical conductivity, optical microscopy, electron microscopy, ultrasound, NMR
Microstructure	Optical microscopy, electron microscopy, atomic force microscopy
Creaming and sedimentation	Light scattering, ultrasound, NMR, visual observation
Droplet charge	Electrokinetic techniques, electroacoustic techniques
Droplet crystallization	Density, NMR, ultrasound, differential scanning calorimetry, polarized optical microscopy
Emulsion rheology	Viscometers, dynamic shear rheometers
Interfacial tension	Interfacial tensiometers (static and dynamic)
Interfacial thickness	Ellipsometry, neutron reflection, neutron scattering, light scattering, surface force apparatus

Source: McClements, D.J., *Food Emulsions: Principles, Practice and Techniques*, 3rd edn., CRC, Boca Raton, FL, 2015.

B. Droplet Size Distribution

The size of the droplets in an emulsion influences many of their sensory and bulk physicochemical properties, including rheology, appearance, mouthfeel, and stability [3,5]. It is therefore important for food manufacturers to carefully control the size of the droplets in a food product and to have analytical techniques to measure droplet size. Typically, the droplets in a food emulsion are somewhere in the size range of 0.1–50 μm in diameter.

Food emulsions always contain droplets that have a range of sizes, and so it is usually important to characterize both the average size and the size distribution of the droplets. The droplet size distribution is usually represented by a plot of droplet frequency (number or volume) versus droplet size (radius or diameter). Some of the most important experimental techniques for measuring droplet size distributions are included in Table 3.2.

Light scattering and electrical conductivity techniques are capable of providing a full particle size distribution of a sample in a few minutes. Since, however, these techniques usually require that the droplet concentration be very low ($\phi < 0.001$), samples must be diluted considerably before analysis. Optical and electron microscopy techniques, which provide the most direct measurement of droplet size distribution, are often time-consuming and laborious to operate, and sample preparation can cause considerable artifacts in the results. In contrast, recently developed techniques based on NMR and ultrasonic spectroscopy can be used to rapidly and nondestructively measure the droplet size distribution of concentrated and optically opaque emulsions [1a]. These techniques are particularly useful for online characterization of emulsion properties.

C. Microstructure

The structural organization and interactions of the droplets in an emulsion often play an important role in determining the properties of a food. For example, two emulsions may have the same droplet concentration and size distribution, but very different properties, because of differences in the degree of droplet flocculation. Various forms of microscopy are available for providing information about the microstructure of food emulsions. The unaided human eye can resolve objects that are farther apart than about 0.1 mm (100 μm). Most of the structural components in food emulsions (e.g., emulsion droplets, surfactant micelles, fat crystals, ice crystals, small air cells, protein aggregates) are much smaller than this lower limit and cannot therefore be observed directly by the eye.

Optical microscopy can be used to study the components of size between about 0.5 and 100 μm. The characteristics of specific components can be highlighted by selectively staining certain ingredients or by using special lenses. Electron microscopy can be used to study components that have sizes down to about 0.5 nm. Atomic force microscopy can be used to provide information about the arrangements and interactions of single atoms or molecules. All these techniques are burdened by sample preparation steps that often are laborious and time-consuming and subject to alter the properties of the material being examined. Nevertheless, when carried out correctly the advanced microscopic techniques provide extremely valuable information about the arrangement and interactions of emulsion droplets with each other and with the other structural entities found in food emulsions.

D. Physical State

The physical state of the components in a food emulsion often has a pronounced influence on its overall properties [1a]. For example, oil-in-water emulsions are particularly prone to partial coalescence when the droplets contain a certain percentage of crystalline fat (Section VI.B). Partial coalescence leads to extensive droplet aggregation, which decreases the stability of emulsions to creaming and greatly increases their viscosity. In water-in-oil emulsions, such as margarine or butter, the formation of a network of aggregated fat crystals provides the characteristic rheological properties. The most important data for food scientists are the temperature at which melting

or crystallization begins, the temperature range over which the phase transition occurs, and the value of the solid fat content at any particular temperature. Phase transitions can be monitored by measuring changes in any property (e.g., density, compressibility, heat capacity, absorption or scattering of radiation) that is altered upon conversion of an ingredient from a solid to a liquid (Table 3.2). The density of a component often changes when it undergoes a phase transition, and so melting or crystallization can be monitored by measuring changes in the density of a sample with temperature or time.

Phase transitions can also be monitored by measuring the amount of heat absorbed or released when a solid melts or a liquid crystallizes, respectively. This type of measurement can be carried out by means of differential thermal analysis or differential scanning calorimetry. These techniques also provide valuable information about the polymorphic form of the fat crystals in an emulsion. More recently, rapid instrumental methods based on NMR and ultrasound have been developed to measure solid fat contents [1a]. These instruments are capable of nondestructively determining the solid fat content of a sample in a few seconds and are extremely valuable analytical tools for rapid quality control and online procedures. Phase transitions can be observed in a more direct manner by means of polarized optical microscopy.

E. Creaming and Sedimentation Profiles

Over the past decade, a number of instruments have been developed to quantify the creaming or sedimentation of the droplets in emulsions. Basically, the same light scattering, NMR, and ultrasound techniques used to measure the dispersed phase volume fraction or droplet size distributions of emulsions are applied to creaming or sedimentation, but the measurements are carried out as a function of sample height to permit the acquisition of a profile of droplet concentrations or sizes. Techniques based on the scattering of light can be used to study creaming and sedimentation in fairly dilute emulsions. A light beam is passed through a sample at a number of different heights, and the reflection and transmission coefficients are measured and related to the droplet concentration and size. By measuring the ultrasonic velocity or attenuation as a function of sample height and time, it is possible to quantify the rate and extent of creaming in concentrated and optically opaque emulsions. This technique can be fully automated and has the two additional advantages: creaming can be detected before it is visible to the eye, and a detailed creaming profile can be determined rather than a single boundary. By measuring the ultrasound properties as a function of frequency, it is possible to determine both the concentration and size of the droplets as a function of sample height. Thus, a detailed analysis of creaming and sedimentation in complex food systems can be monitored noninvasively. Recently developed NMR imaging techniques can also measure the concentration and size of droplets in any region in an emulsion [9]. These ultrasound and NMR techniques will prove particularly useful for understanding the kinetics of creaming and sedimentation in emulsions and for predicting the long-term stability of food emulsions.

F. Emulsion Rheology

The rheology of an emulsion is one of its most important overall physical attributes because it largely determines the mouthfeel, flowability, and stability of emulsions [3]. A variety of experimental techniques are available for measuring the rheological properties of food emulsions. The rheology of emulsions that have low viscosities and act like ideal liquids can be characterized by capillary viscometers. For nonideal liquids or viscoelastic emulsions, more sophisticated instrumental techniques called dynamic shear rheometers are available to measure the relationship between the stress applied to an emulsion and the resulting strain or vice versa. As well as providing valuable information about the bulk physicochemical properties of emulsions (e.g., texture, flow through pipes), rheological measurements can provide information about droplet–droplet interactions and the properties of any flocs formed in an emulsion.

G. Interfacial Properties

Despite comprising only a small fraction of the total volume of an emulsion, the interfacial region that separates the oil from the aqueous phase plays a major role in determining stability, rheology, chemical reactivity, flavor release, and other overall physicochemical properties of emulsions. The most important properties of the interface are the concentration of emulsifier molecules present (the surface load), the packing of the emulsifier molecules, and the thickness, viscoelasticity, electrical charge, and (interfacial) tension of the interface.

A variety of experimental techniques are available for characterizing the properties of oil–water interfaces (Table 3.2). The surface load is determined by measuring the amount of emulsifier that adsorbs per unit area of oil–water interface. The thickness of an interfacial membrane can be determined by light scattering, neutron scattering, neutron reflection, surface force, and ellipsometry techniques. The rheological properties of the interfacial membrane can be determined by means of the 2D analog of normal rheological techniques. The electrical charge of the droplets in an emulsion determines their susceptibility to aggregation. Experimental techniques based on electrokinetic and electroacoustic techniques are available for determining the charge on emulsion droplets. The dynamic or equilibrium interfacial tension of an oil–water interface can be determined by means of a number of interfacial tension meters, including the Wilhelmy plate, Du Nouy ring, maximum bubble pressure, and pendant drop methods.

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4 Chemistry of Waxes and Sterols

Edward J. Parish and Wendell S. Grainger

CONTENTS

I. CHEMISTRY OF WAXES

A. Introduction

The term "waxes" commonly refers to the mixtures of long-chain apolar compounds found on the surface of plants and animals. By a strict chemical definition, a wax is the ester of a long-chain acid and a long-chain alcohol. However, this academic definition is much too narrow both for the wax chemist and for the requirements of the industry. The following description from the German Society for Fat Technology [1] better fits the reality:

> Wax is the collective term for a series of natural or synthetically produced substances that normally possess the following properties: kneadable at 20°C, brittle to solid, coarse to finely crystalline, translucent to opaque, relatively low viscosity even slightly above the melting point, not tending to stinginess, consistency and solubility depending on the temperature and capable of being polished by slight pressure.

The collective properties of wax as just defined clearly distinguish waxes from other articles of commerce. Chemically, waxes constitute a large array of different chemical classes, including

hydrocarbons, wax esters, sterol esters, ketones, aldehydes, alcohols, and sterols. The chain length of these compounds may vary from C_2, as in the acetate of a long-chain ester, to C_{62}, as in the case of some hydrocarbons [2,3].

Waxes can be classified according to their origins as naturally occurring or synthetic. Naturally occurring waxes can be subclassified into animal, vegetable, and mineral waxes. Beeswax, spermaceti, wool grease, and lanolin are important animal waxes. Beeswax, wool grease, and lanolin are by-products of other industries. Vegetable waxes include carnauba wax, the so-called queen of waxes, ouricuri (another palm wax), and candelilla. These three waxes account for the major proportion of the consumption of vegetable waxes. Mineral waxes are further classified into petroleum waxes, ozocerite, and montan. Based on their chemical structure, waxes represent a very broad spectrum of chemical types from polyethylene, polymers of ethylene oxide, derivatives of montan wax, alkyl esters of monocarboxylic acids, alkyl esters of hydroxy acids, polyhydric alcohol esters of hydroxy acids, Fisher–Tropsch waxes, and hydrogenated waxes, to long-chain amide waxes.

We begin with an overview of the diverse class of lipids known as waxes. The discussion presented that follows, which touches on source, structure, function, and biosynthesis, is intended to serve as an entry to the literature, enabling the reader to pursue this topic in greater detail.

B. Properties and Characteristics of Waxes

Ancient Egyptians used beeswax to make writing tablets and models, and waxes are now described as man's first plastic. Indeed, the plastic property of waxes and cold-flow yield values allow manual working at room temperature, corresponding to the practices of the Egyptians. The melting points of waxes usually vary within the range 40°C–120°C.

Waxes dissolve in fat solvents and their solubility is dependent on temperature. They can also wet and disperse pigments and can be emulsified with water, which makes them useful in the furniture, pharmaceutical, and food industries. Their combustibility, associated with low ash content, is important in candle manufacture and solid fuel preparation. Waxes also find application in industry as lubricants and insulators, where their properties as natural plastics, their high flash points, and their high dielectric constants are advantageous.

The physical and technical properties of waxes depend more on molecular structure than on molecular size and chemical constitution. The chemical components of waxes range from hydrocarbons, esters, ketones, aldehydes, and alcohols to acids, mostly as aliphatic long-chain molecules. The hydrocarbons in petroleum waxes are mainly alkanes, though some unsaturated and branched chain compounds are found. The common esters are those of saturated acids with 12–28 carbon atoms combining with saturated alcohols of similar chain length. Primary alcohols, acids, and esters have been characterized and have been found to contain an even straight chain of carbon atoms. By contrast, most ketones, secondary alcohols, and hydrocarbons have odd number of carbon atoms. The chemical constitution of waxes varies in great degree depending on the origin of the material. A high proportion of cholesterol and lanosterol is found in wool wax. Commercial waxes are characterized by a number of properties. These properties are used in wax grading [4].

1. Physical Properties of Waxes

Color and odor are determined by comparison with standard samples in a molten state. In the National Petroleum Association scale, the palest color is rated 0, whereas amber colors are rated 8.

Refined waxes are usually free from taste, this property being especially important in products such as candelilla when it is used in chewing gum. Melting and softening points are important physical properties. The melting points can be determined by the capillary tube method or the drop point method. The softening point of a wax is the temperature at which the solid wax begins to soften. The penetration property measures the depth to which a needle with a definite top load penetrates the wax sample.

Shrinkage and flash point are two frequently measured physical properties of waxes. The flash point is the temperature at which a flash occurs if a small flame is passed over the surface of the sample. In the liquid state, a molten wax shrinks uniformly until the temperature approaches the solidification point. This property is measured as the percentage shrinkage of the volume.

2. Chemical Properties of Waxes

a. Acid Value

The acid value is the number of milligrams of potassium hydroxide required to neutralize a gram of the wax. It is determined by the titration of the wax solution in ethanol–toluene with 0.5 M potassium hydroxide. Phenolphthalein is normally used as the titration indicator.

$$\text{Acid value} = \frac{V_w \times 56.104}{w},$$

where

V_w is the number of milliliters (mL) of potassium hydroxide used in the titration
w is the mass of wax

b. Saponification Number

The saponification number is the number of milligrams of potassium hydroxide required to hydrolyze 1 g of wax:

$$\text{Saponification number} = \frac{(V_b - V_w) \times 56.105}{w},$$

where

w is the weight of wax samples
V_b is the volume (mL) of hydrochloric acid used in the blank
V_w is the volume (mL) of hydrochloric acid used in the actual analysis

The wax (2 g) is dissolved in hot toluene (910 mL). Alcoholic potassium hydroxide (25 mL of 0.5 M KOH) is added, and the solution is refluxed for 2 h. A few drops of phenolphthalein are added and the residual potassium hydroxide is titrated with 0.5 M hydrochloric acid. A blank titration is also performed with 25 mL of 0.5 M alcoholic potassium hydroxide plus toluene.

c. Ester Value

Ester value, the difference between the saponification number and the acid value, shows the amount of potassium hydroxide consumed in the saponification of esters.

d. Iodine Number

The iodine number expresses the amount of iodine that is absorbed by the wax. It is a measure of the degree of unsaturation.

e. Acetyl Number

The acetyl number indicates the milligrams of potassium hydroxide required for the saponification of the acetyl group assimilated in 1 g of wax on acetylation. The difference of this number and the ester value reflects the amount of free hydroxyl groups (or alcohol composition) in a wax. The wax sample is first acetylated by acetic anhydride. A certain amount of acetylated wax (about 2 g) is taken out to be saponified with the standard procedure in the measurement of the saponification number. The acetyl number is the saponification number of the acetylated wax.

3. Properties of Important Naturally Occurring Waxes

a. Beeswax

Beeswax is a hard amorphous solid, usually light yellow to amber depending on the source and manufacturing process. It has a high solubility in warm benzene, toluene, chloroform, and other polar organic solvents. Typically, beeswax has an acid value of 17–36, a saponification number of 90–147, melting point of 60°C–67°C, an ester number of 64–84, a specific gravity of 0.927–0.970, and an iodine number of 7–16. Pure beeswax consists of about 70%–80% of long-chain esters, 12%–15% of free acids, 10%–15% of hydrocarbon, and small amounts of diols and cholesterol esters. Beeswax is one of the most useful and valuable of waxes. Its consumption is not limited to the candle industry, the oldest field of wax consumption. It is also used in electrical insulation and in the food, paper, and rubber industries.

b. Wool Grease and Lanolin

Wool grease is a by-product of the wool industry, and the finest wool grease yields lanolin. Pharmaceutical grade lanolin accounts for about 80% of all wool grease consumption. Wool grease has a melting point of 35°C–42°C, an acid value of 7–15, a saponification value of 100–110, an ester value of 85–100, a specific gravity of 0.932–0.945, and an iodine value of 22–30.

c. Carnauba Wax

Carnauba wax, queen of waxes, is a vegetable wax produced in Brazil. Carnauba wax is hard, amorphous, and tough, with a pleasant smell. It is usually used in cosmetics and by the food industry, in paper coatings, and in making inks. In the food industry, it is a minor component in glazes for candies, gums, and fruit coatings. Carnauba wax is soluble in most polar organic solvents. It contains esters (84%–85%), free acids (3%–3.5%), resins (4%–6%), alcohols (2%–3%), and hydrocarbons (1.5%–3.0%). Typically, carnauba has an acid value of 2.9–9.7, an ester value of 39–55, a saponification value of 79–95, an iodine value of 7–14, and a melting range of 78°C–85°C.

d. Candelilla Wax

Candelilla wax is a vegetable wax produced mainly in Mexico. It is used chiefly in the manufacturing of chewing gum and cosmetics, which represent about 40% of the market. It is also used in furniture polish, in the production of lubricants, and in paper coating. Candelilla wax has a specific gravity of 0.98, an acid value of 12–22, a saponification value of 43–65, a melting point of 66°C–71°C, an ester value of 65–75, and an iodine value of 12–22. The chemical composition of candelilla wax is 28%–29% esters, 50%–51% hydrocarbons, 7%–9% free acids, and small amounts of alcohols and cholesterols.

e. Ozocerite

Ozocerite is a mineral wax found in Galicia, Russia, Iran, and the United States. Most ozocerite consists of hydrocarbons, but the chemical composition varies with the source. Typically, ozocerite has an ester value of 56–66, an acid value of 31–38, a saponification value of 87–104, a melting point of 93°C–89°C, and an iodine value of 14–18. Ozocerite is graded as unbleached (black), single bleached (yellow), and double bleached (white). It is mainly used in making lubricants, lipsticks, polishes, and adhesives.

C. Isolation, Separation, and Analysis of Natural Waxes

Knowledge of the chemical analysis of natural waxes is essential for understanding wax biosynthesis, manufacture, and application. Although the chemical compositions of synthetic waxes are constant and depend on the manufacturing process, the natural waxes are much more complicated in chemical composition. In general, natural waxes are isolated by chemical extraction, separated

by chromatographic methods, and analyzed by means of mass spectrometry (MS); both gas chromatography (GC) and high-performance liquid chromatography (HPLC) techniques are used. The following discussion on chemical analysis is based on an understanding of the general principles of chemical extraction, chromatography, and MS. There are numerous textbooks detailing these principles [5–7].

1. Isolation

Natural waxes are mixtures of long-chain apolar compounds found on the surface of plants and animals. However, internal lipids also exist in most organisms. In earlier times, the plant or animal tissue was dried, whereupon the total lipid material could be extracted with hexane or chloroform by means of a Soxhlet extractor. The time of exposure to the organic solvent, particularly chloroform, is kept short to minimize or avoid the extraction of internal lipids. Because processors are interested in surface waxes, it became routine to harvest them by a dipping procedure. For plants, this was usually done in the cold, but occasionally at the boiling point of light petroleum or by swabbing to remove surface lipids. Chloroform, which has been widely used, is now known to be toxic; dichloromethane can be substituted. After removal of the solvent under vacuum, the residue can be weighed. Alternatively, the efficiency of the extraction can be determined by adding a known quantity of a standard wax component (not present naturally in the sample) and performing a quantification based on this component following column chromatography.

2. Separation

The extract of surface lipids contains hydrocarbons, as well as long-chain alcohols, aldehydes and ketones, short-chain acid esters of the long-chain alcohols, fatty acids, sterols and sterol esters, and oxygenated forms of these compounds. In most cases, it is necessary to separate the lipid extract into lipid classes before the identification of components. Separation of waxes into their component classes is first achieved by column chromatography. The extract residue is redissolved in the least polar solvent possible, usually hexane or light petroleum, and transferred to the chromatographic column. When the residue is not soluble in hexane or light petroleum, a hot solution or a more polar solvent, like chloroform of dichloromethane, may be used to load the column. By gradually increasing the polarity of the eluting solvent, it is possible to obtain hydrocarbons, esters, aldehydes and ketones, triglycerides, alcohols, hydroxydiketones, sterols, and fatty acids separately from the column. Most separations have been achieved on alumina or silica gel. However, Sephadex LH-20 was used to separate the alkanes from Green River Shale. Linde 5Å sieve can remove the n-alkanes to provide concentrated branched and alicyclic hydrocarbons. Additionally, silver nitrate can be impregnated into alumina or silica gel columns or thin-layer chromatography (TLC) plates for separating components according to the degree of unsaturation.

As the means of further identifying lipids become more sophisticated, it is possible to obtain a sufficient quantity of the separated wax components by TLC. One of the major advantages of TLC is that it can be modified very easily, and minor changes to the system have allowed major changes in separation to be achieved. Most components of wax esters can be partially or completely separated by TLC on 25 mm silica gel G plates developed in hexane–diethyl ether or benzene–hexane. The retardation factor (R_f) values of most wax components are listed in Table 4.1 [8].

If TLC is used, the components must be visualized, and the methods employed can be either destructive or nondestructive. The commonly used destructive method is to spray TLC plates with sulfuric or molybdic acid in ethanol and heat them. This technique is very sensitive, but it destroys the compounds and does not work well with free acids. Iodine vapors will cause a colored band to appear, particularly with unsaturated compounds, and are widely used to both locate and quantify the lipids. Since the iodine can evaporate from the plate readily after removal from iodine chamber, the components usually remain unchanged. Iodine vapor is one of the ideal visualization media in the isolation of lipid classes from TLC plates. Commercial TLC plates

TABLE 4.1
TLC Separation of Wax Components on Silica Gel: R_f Values for Common Wax Components

	Solvent Systems							
Component	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Hydrocarbon	0.95	0.96	0.95	0.85	0.83	0.95	0.85	
Squalene							0.80	
Trialkylglyceryl ethers	0.90							
Steryl esters	0.90					0.95	0.57	
Wax esters	0.90	0.82	0.84	0.71	0.65	0.91	0.75	
β-Diketones		0.75	0.54					
Monoketones					0.53			
Fatty acid methyl esters	0.65				0.47	0.75		
Aldehydes	0.55	0.65		0.47				0.66
Triterpenyl acetates							0.53	
Secondary alcohols				0.36				
Triacylglycerols	0.35				0.61	0.37		
Free fatty acids	0.18	0.00	0.00			0.35	0.20	
Triterphenols								0.22
Primary alcohols	0.15	0.14	0.16	0.09	0.15	0.21		0.19
Sterols	0.10				0.16	0.10	0.12	
Hydroxy-β-diketones	0.09	0.04						
Triterpenoid acid							0.05	

Notes: A, petroleum ether (b.p. 60°C–70°C)–diethyl ether–glacial acetic acid (90:10:1, v/v); B, benzene; C, chloroform containing 1% ethanol; D, petroleum ether (b.p. 40°C–60°C)–diethyl ether (80:20, v/v); E, chloroform containing 1% ethanol; F, hexane–heptane–diethyl ether–glacial acetic acid (63:18.5:18.5, v/v) to 2 cm from top, then full development with carbon tetrachloride; G (1) petroleum ether–diethyl ether–glacial acetic acid (80:20:1, v/v); (2) petroleum ether; H, benzene–chloroform (70:30 v/v).

with fluorescent indicators are available as well, and bands can be visualized under UV light. However, if it is necessary to use solvents more polar than diethyl ether to extract polar components from the matrix, the fluorescent indicators may also be extracted, and these additives interfere with subsequent analyses.

To isolate lipid classes from TLC plates after a nondestructive method of visualization, the silica gel can be scraped into a champagne funnel and eluted with an appropriate solvent. Or, the gel can be scraped into a test tube and the apolar lipid extracted with diethyl ether by vortexing, centrifuging, and decanting off the ether. Polar lipids are extracted in the same manner, using a more polar solvent such as chloroform and methanol. HPLC has been used in the separation and analysis of natural waxes, but its application was halted by the lack of a suitable detector, since most wax components have no useful ultraviolet (UV) chromophore. Application of UV detection was limited to wavelength around 210 nm. Some components with isolated double bonds and carbonyl group (e.g., esters, aldehydes, ketones) can be detected in this wavelength. Hamilton and coworkers have examined an alternative detection system, infrared detection at 5.74 mm, which allowed the hydrocarbon components to be detected [9]. Although the sensitivity of this method of detection could not match that of UV detection, it has merit for use in the preparative mode, where it is feasible to allow the whole output from the column to flow through the detector. The third useful mode for HPLC is MS. The coupling of HPLC and MS makes this form of chromatography a very important analytical technique.

3. Analysis

When individual classes of waxes have been isolated, the identity of each must be determined. Due to the complex composition of these materials, combined analytical approaches (e.g., GC–MS) have been used to analyze individual wax classes. MS is a major analytical method for the analysis of this class of compounds. With the electron impact–mass spectrometry, the wax molecules tend to give cleavage fragments rather than parent ions. Thus, soft (chemical) ionization (CI) and fast atom bombardment have been frequently used to give additional information for wax analysis.

In GC–MS analysis, the hydrocarbon fraction and many components of the wax ester fraction can be analyzed directly, whereas long-chain alcohols, the aldehydes, and fatty acids are often analyzed as their acetate esters of alcohols, dimethylhydrazones of aldehydes, and methyl esters of fatty acids. The analysis of wax esters after hydrolysis and derivatization will provide additional information on high molecular weight esters. For example, the chain branching of a certain component might be primarily examined with respect to its unusual retention time on GC analysis then determined by converting to the corresponding hydrocarbon through the reduction of its iodide intermediate with LiAID4 (the functional group end is labeled by the deuterium atom). A similar approach is to convert the alcohol of the target component to an alkyl chloride via methanesulfonyl chloride. This method labels the functional end with a chlorine atom, and its mass spectra are easily interpreted because of the chlorine isotopes. As mentioned earlier, unsaturated hydrocarbons can be separated from saturated hydrocarbons and unsaturated isomers by column chromatography or TLC with silver nitrate silica gel or alumina gel media. The position and number of double bonds affect the volatility of the hydrocarbons, thereby altering their retention in GC and HPLC analysis. The location of a double bond is based on the mass spectra of their derivatives, using either positive or negative CI.

D. Biosynthesis of Natural Waxes

Epicuticular waxes (from the outermost layer of plant and insect cuticles) comprise very long-chain nonpolar lipid molecules that are soluble in organic solvents. In many cases, this lipid layer may contain proteins and pigments, and great variability in molecular architecture is possible, depending on the chemical composition of the wax and on environmental factors [10,11].

A variety of waxes can be found in the cuticle. On the outer surface of plants these intracuticular waxes entrap cutin, which is an insoluble lipid polymer of hydroxy and epoxy fatty acids. In underlying layers, associated with the suberin matrix, another cutin-like lipid polymer containing aliphatic and aromatic components is found [12]. In some instances, internal nonsuberin waxes, which are stored in plant seeds, are the major energy reserves rather than triacylglycerols. In insects, intracuticular waxes are the major constituents of the inner epicuticular layer [13–15].

A variety of aliphatic lipid classes occur in epicuticular waxes. These include hydrocarbons, alcohols, esters, ketones, aldehydes, and free fatty acids of numerous types [16,17]. Frequently, a series of 10 carbon atom homologs occur, while chain lengths of 10–35 carbon atoms are most often found. However, fatty acids and hydrocarbons with fewer than 20 carbon atoms are known, as are esters with more than 60 carbon atoms. Other minor lipids such as terpenoids, flavonoids, and sterols also occur in epicuticular waxes. The composition and quantity of epicuticular wax vary widely from one species to another and from one organ, tissue, or cell type to another [16]. In insects, wax composition depends on the stage of life cycle, age, sex, and external environment [17].

In waxes, the biosynthesis of long-chain carbon skeletons is accomplished by a basic condensation–elongation mechanism. Elongases are enzyme complexes that repetitively condense short activated carbon chains to an activated primer and prepare the growing chain for the next addition. The coordinated action of two such soluble complexes in plastid results in the synthesis of the 16- and 18-carbon acyl chains characterizing plant membranes [18–20]. Each condensation introduces a β-keto group into the elongating chain. This keto group is normally removed by a series of three reactions: a β-keto reduction, a β-hydroxy dehydration, and an enol reduction.

Variations of the foregoing basic biosynthetic mechanism occur, giving rise to compounds classified as polyketides. Their modified acyl chains can be recognized by the presence of keto groups, hydroxyl groups, or double bonds that were not removed before the next condensation took place. It is well established that the very long carbon skeletons of the wax lipids are synthesized by a condensation–elongation mechanism. The primary elongated products in the form of free fatty acids are often minor components of epicuticular waxes. Most of them, however, serve as substrates for the associated enzyme systems discussed. The total length attained during elongation is reflected by the chain lengths of the members of the various wax classes [15–21]. Normal, branched, and unsaturated hydrocarbons and fatty acids are prominent components of plant waxes, whereas insect waxes usually lack long-chain free fatty acids [22–26].

II. CHEMISTRY OF STEROLS

A. Introduction

Sterols are a subclass of steroids. The International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry have defined steroids as, "compounds possessing the skeleton of cyclopenta[α]phenanthrene or a skeleton derived therefrom by one or more bond scissions or ring expansions or contractions" [27]. In summary, most steroids have four fused rings, as shown in Figure 4.1. These four rings, considered the steroid "nucleus," are labeled A through D. IUPAC appoints "steroids carrying a hydroxyl group at C3 and most of the skeleton of cholestane" as sterols [27]. Thus, compounds with a hydroxyl group at the C3 position and the four fused rings seen in Figure 4.1 are classified sterols by the IUPAC.

More recently, sterols were defined in *Chemical Reviews* as "amphipathic compounds that originate in isoprenoid biosynthesis with the main frame composed of a nucleus and a side chain" [28]. This interesting definition divides steroids that lack a side chain, particularly androgens and estrogens, from other sterols. Furthermore, if the term "side chain" is interpreted to mean the C_8H_{17} alkyl group found at the C17 carbon of cholesterol, then progestogens, glucocorticoids, mineral corticoids, most of cholesterol's precursors, plant steroids, and bile acids may be excluded. The latter definition is more amiable with contemporary knowledge of steroids because every steroid appears to have a unique combination of bioactivity and chemical reactivity.

Note that steroidal compounds are named according to the parent structure from which they were derived or most resemble. Some common examples include "lanost-" for lanosterol, "cholest-" for cholesterol, "pregn-" for progestogens, "-cortisol" and "-cortisone" for corticoids, "Andro-" for androgens, and "estr-" for estrogens. Some steroidal compounds have special names such as progesterone and testosterone. All of these steroidal groups share the fused four ring system and may, depending on the definition of sterol, be classified as one.

Sterols have a numbering system to identify carbon atoms [29], as shown in Figure 4.2. (An easy way to remember this numbering system is to think of 1–10 and 11–19 as two circles.) For an example of how to use the numbering system, consider 7-ketocholesterol (Figure 4.3), which has a ketone at the C7 carbon. Note also that sterols have an alpha (α) and a beta (β) face [29]. As Figure 4.2 is seen on the page, the beta side is toward the reader. The alpha side is going away from the reader. This nomenclature can be seen with beta-sitosterol (Figure 4.3), where the ethyl group at C24 is coming toward the reader. The double bonds of steroidal olefins are sometimes noted with a

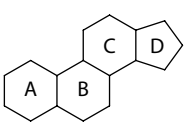


FIGURE 4.1 Cyclopenta[α]phenanthrene; four fused rings of a steroid nucleus.

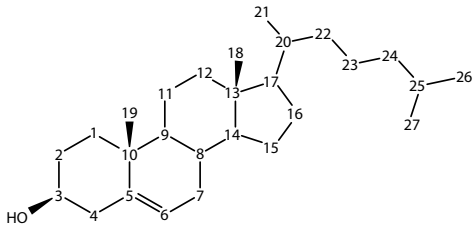


FIGURE 4.2 Cholesterol labeled.

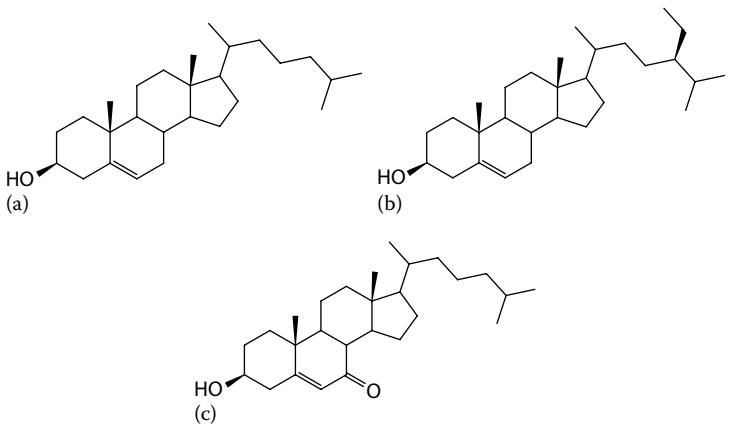


FIGURE 4.3 Examples of sterols. (a) Cholesterol, (b) beta-sitosterol, and (c) 7-ketocholesterol.

"Δ" symbol. Numbers appearing superscripted denote the position of the double bond. For example, cholesterol is a $\Delta^{5(6)}$ steroid or, rather, just Δ^5, meaning that it has a double bond between the C5 and C6 carbons. If the number in parenthesis is sequential in chronological order, then it is not listed. A label with a period between two subscripted numbers such as $\Delta^{5.7}$ indicates a second double bond. In the example, there would be a double bond at C-5 and C-7.

Numerous sterols are known to exist even among the most stringent of sterol definitions and can be further divided into subclasses. For example, oxidized derivatives of sterols are called "oxysterols." An example of an oxysterol would be 7-ketocholesterol, an auto-oxidation product of cholesterol. Plants also have their own unique sterols. They are called "phytosterols." Their structures are similar to that of animal sterols like cholesterol, except they have an additional alkyl group on the side chain or other additional modification. Beta-sitosterol is a well-known phytosterol.

Cholesterol is considered the most important sterol and will thus be focused on greater than other sterols in this chapter. It is the precursor of progestogens, corticoids, androgens, estrogens, bile acids, and vitamin D [29] in addition to many oxysterols and is used in animal cell membranes to maintain a "liquid-ordered" phase [30,31]. The human body must have cholesterol and thus regulates its levels, especially within the brain [32]. (A human brain is the most cholesterol-rich organ in the body!) [32]. If a diet does not supply enough cholesterol, the body will simply upregulate its biosynthesis [33]. It is interesting that a recent meta-analysis reviewing dietary cholesterol intake and cardiovascular disease did not find a conclusive correlation, which they attributed to bodily reduction of cholesterol synthesis to compensate for increased intake [34].

With health concerns regarding cholesterol and cardiovascular deceases, some food producers have included phytosterols in their products, calling these products "functional foods," which are supposed to lower cholesterol [35]. In short, phytosterols compete with dietary cholesterol for absorption into the intestines. Although there are still questions pertaining to dietary cholesterol

absorption inhibition, it is important to note that there are several proposed mechanisms of inhibition and the total inhibition is likely not based on just one mechanism [36–38].

Oxysterols have received much attention due to their biological activities [39], namely, regulatory [40], cytotoxic [41], and possible carcinogenic [42] effects. Thus, food scientists are interested in how much and what kind of these oxysterols are in food [43]. It has been shown that the storage, cooking method, and preparation of meat have been shown to have various effects on steroidal oxidation levels [43]. Currently, the concern is growing for structurally similar oxidized phytosterols as well [44]. (Oxysterols are described in greater detail at a later chapter.)

As an important aside, it should be noted that cholesterol and most sterols are not water soluble. In a water and ether (two phase) mixture, cholesterol will be exclusively present in the ether layer. In the aqueous environment of the human body, lipoproteins are used to transport cholesterol [45,46]. This is how low-density and high-density lipoproteins (LDL and HDL, respectively) are related to cholesterol. HDL is used to return excess cholesterol to the liver while LDL is used as the main lipoprotein to carry cholesterol to peripheral cells [45]. LDL cholesterol is typically called "bad" cholesterol and HDL cholesterol is often called "good" cholesterol [47]. However, in either case, the actual cholesterol molecules in HDL and LDL are identical.

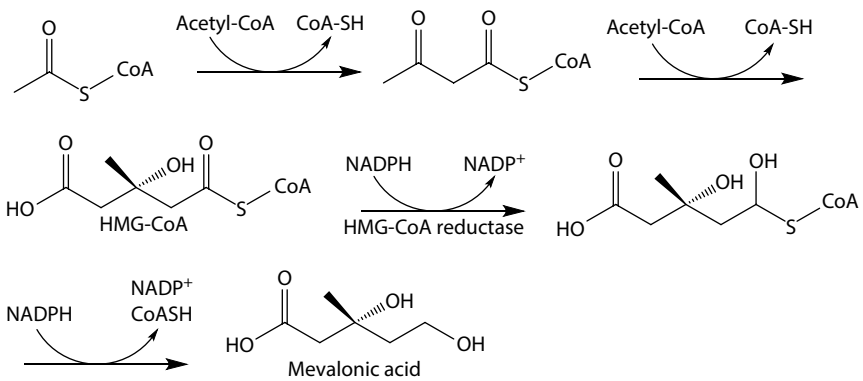
B. Biosynthetic Origins of Sterols

1. Cholesterol Biosynthesis

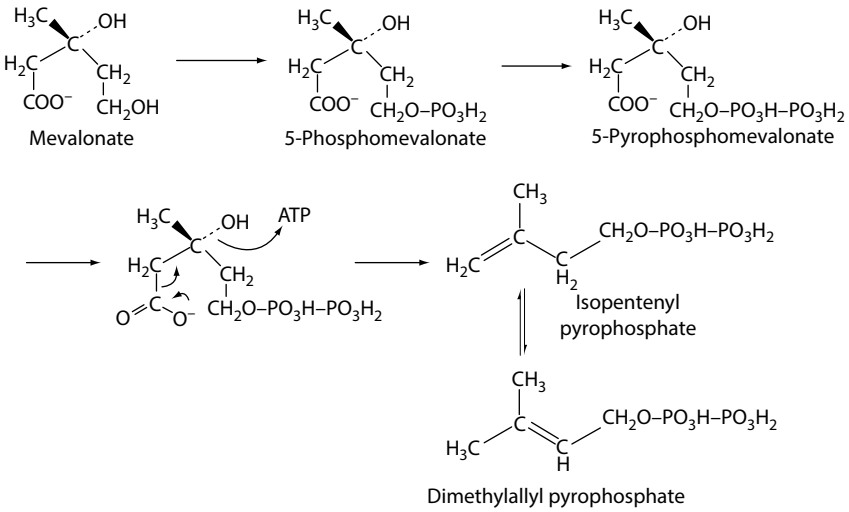
Elucidation of the cholesterol biosynthesis pathway has challenged the ingenuity of chemists for many years. This outstanding scientific endeavor has yielded no less than 14 Nobel Laureates [29]. The cholesterol biosynthetic pathway can be arbitrarily divided into four stages: (1) the formation of mevalonic acid from acetyl CoA, (2) conversion of mevalonic acid to isoprene phosphates to squalene through a series of phosphorylated intermediates, (3) formation of lanosterol from squalene via cyclization of 2,3-epoxysqualene, and (4) the reduction of lanosterol (with 30 carbons, i.e., triterpene) to produce cholesterol (with 27 carbons).

The first stage in the synthesis of cholesterol is the formation of mevalonic acid and isopentyl pyrophosphate from acetyl CoA. The early work of Konrad Bloch in the 1940s showed that cholesterol is synthesized from acetyl coenzyme A (acetyl CoA) [48]. Acetate isotopically labeled in its carbon atoms was prepared and fed to rats. The cholesterol that was synthesized by these rats contained the isotopic label, which demonstrated that acetate is a precursor of cholesterol.

Three molecules of acetyl CoA are combined to produce mevalonic acid, as shown in Scheme 4.1. The first step of this synthesis is catalyzed by a thiolase enzyme and results in the production of acetoacetyl CoA, which is then combined with the third molecule of acetyl CoA to form



SCHEME 4.1 Biosynthesis of mevalonic acid.



SCHEME 4.2 Synthesis of isopentenyl pyrophosphate, the biological isoprene unit, and dimethylallyl pyrophosphate.

3-hydroxy-3-methylglutaryl CoA (HMG-CoA). The reduction of HMG-CoA proceeds in two steps, each requiring NADPH as the reducing reagent.

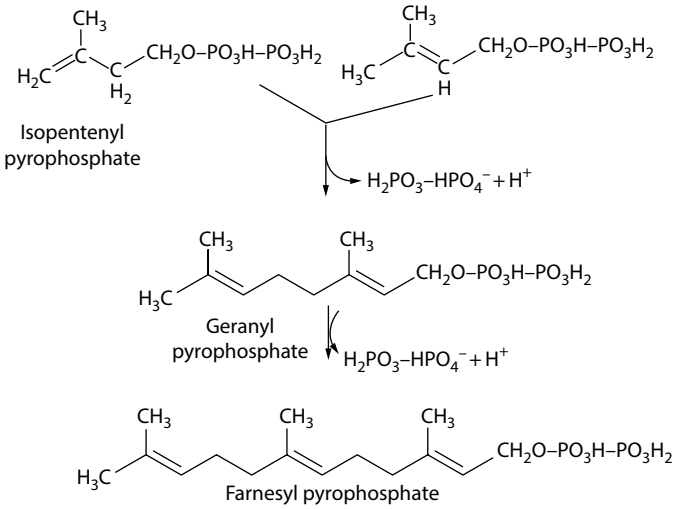
The reduction of HMG-CoA to give mevalonic acid is catalyzed by a microsomal enzyme, HMG-CoA reductase, which is of prime importance in the control of cholesterol biosynthesis. Statin drugs block cholesterol biosynthesis by inhibiting HMG-CoA reductase [49]. Oxysterols have also been shown to inhibit HMG-CoA reductase activity [50,51]. However, it is still questionable as to how much cholesterol and its oxidized derivatives can inhibit cholesterol biosynthesis [52].

Mevalonic acid is phosphorylated by mevalonic kinase to form a 5-phosphomevalonate, which serves as the substrate for the second phosphorylation to form 5-pyrophosphomevalonate (Scheme 4.2). There is then a concerted decarboxylation and loss of a tertiary hydroxyl group from 5-pyrophosphomevalonate to form 3-isopentyl pyrophosphate. In each step, one molecule of ATP is consumed. 3-Isopentyl pyrophosphate is regarded as the basic biological isoprene unit from which all isoprenoid compounds are produced.

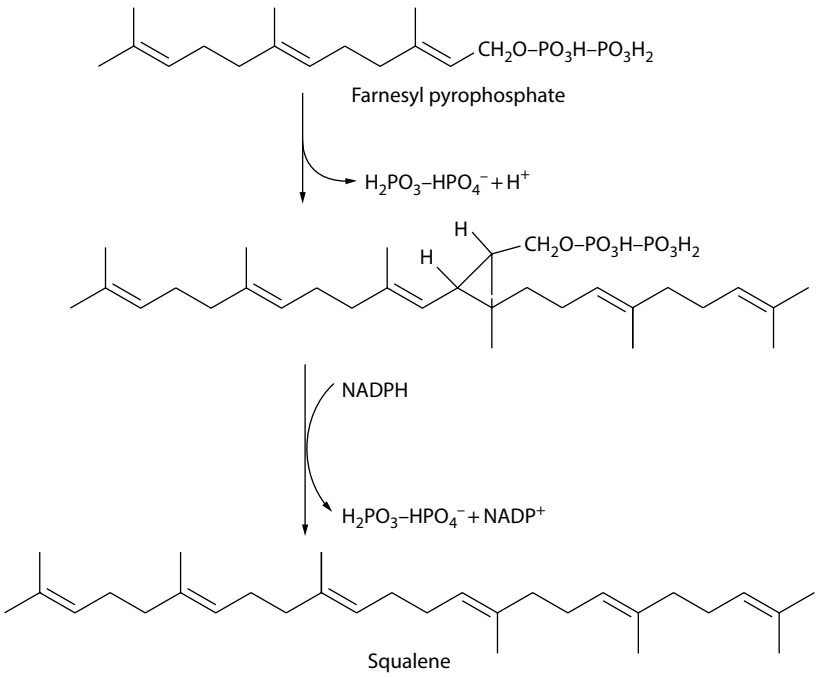
Squalene is then synthesized from isopentyl pyrophosphates by sequential head-to-tail coupling reactions. This begins with the isomerization of isopentyl pyrophosphate to dimethylallyl pyrophosphate. The coupling reaction shown in Scheme 4.3 is initially catalyzed by a soluble sulfydryl enzyme, isopentyl pyrophosphate–dimethylallyl pyrophosphate isomerase. Coupling of these two isomeric C5 units yields geranyl pyrophosphate, which is catalyzed by geranyl pyrophosphate synthetase. Sequentially, geranyl pyrophosphate couples in a similar manner with a third molecule of isopentyl pyrophosphate to produce farnesyl pyrophosphate (C15 structure).

The last step in the second stage, the synthesis of squalene, is a reductive condensation of two molecules of farnesyl pyrophosphate (Scheme 4.4). This step is a two-step sequence, catalyzed by squalene synthetase. In the first reaction, presqualene pyrophosphate is produced by a tail-to-tail coupling of two farnesyl pyrophosphate molecules. Following, the cyclopropane ring of presqualene pyrophosphate is opened with a loss of the pyrophosphate moiety. A molecule of NADPH is required in the second conversion.

The third stage of cholesterol biosynthesis is the cyclization of squalene to lanosterol (Scheme 4.5). Squalene cyclization proceeds in two steps requiring molecular oxygen, NADPH, squalene epoxidase, and 2,3-oxidosqualene–sterol cyclase. The first step is the epoxidation of squalene to form 2,3-oxidosqualene–sterol cyclase. The 2,3-oxidosqualene is oriented as a chair–boat–chair–boat conformation in the enzyme's active center. The acid-catalyzed epoxide ring opening initiates the



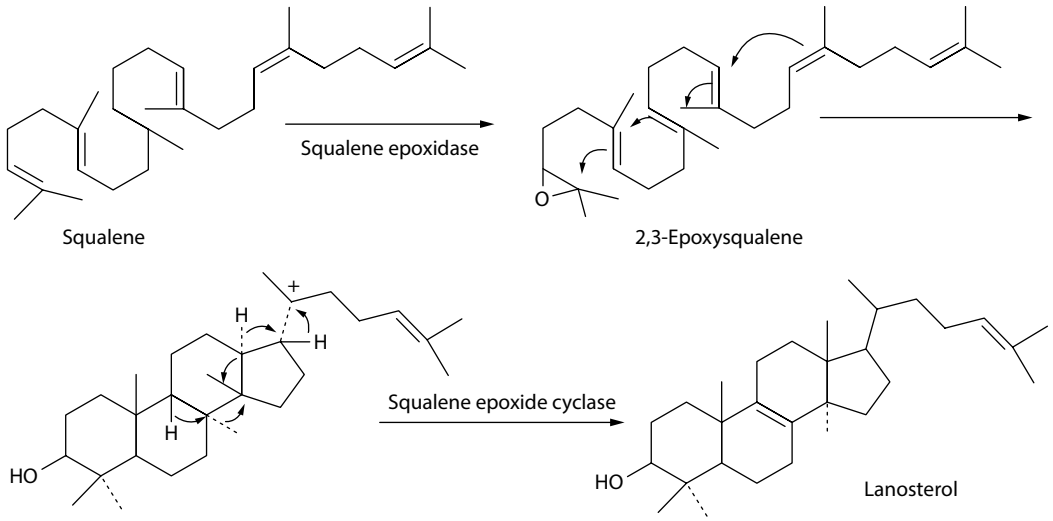
SCHEME 4.3 Synthesis of farnesyl pyrophosphate from the biological isoprenyl unit.



SCHEME 4.4 Synthesis of squalene from the coupling of two molecules of farnesyl pyrophosphate.

cyclization to produce a tetracyclic protosterol cation. This is followed by a series of concerted 1,2-*trans* migrations of hydrogen and methyl groups to produce lanosterol.

Last, cholesterol is produced from the reduction of lanosterol. This process involves approximately 19 steps [53]. Major components of the reduction of lanosterol to cholesterol include the removal of three methyl groups (one at C-14 and two at C-4), the hydrogenation of the double bonds located on the side chain (Δ^{24}) and at Δ^{14} (formed after the removal of the C-14 methyl group), and the migration of the Δ^{8} double bond to the Δ^{5} position. It should be noted that if squalene dioxide (squalene having an epoxide rings formed at the 2,3 and 24,25 positions) is cyclized,



SCHEME 4.5 Cyclization of squalene.

24,25-epoxycholesterol is ultimately formed [54]. It has been proposed that this natural formation of an oxysterol helps regulate cholesterol biosynthesis.

2. Biosynthesis of Plant Sterols

Numerous sterols and triterpenes are found in plants [55]. Sitosterol is usually the major plant sterol present [55]. Note that phytosterol and phytostanol (hydrogenated phytosterol) concentrations vary greatly among food types [56]. Several examples of phytosterols are displayed in Figure 4.4.

Humans and sterol producing plants share the same biosynthetic pathway for the formation of 2,3-oxidosqualene [55]. However, human cells convert 2,3-oxidosqualene to lanosterol, whereas plants convert 2,3-oxidosqualene into cycloartenol (Scheme 4.6), curcurbitadienol for certain plant families [57], and numerous triterpenes [58]. Instead of forming Δ^8 double bond as in humans, a C-9 carbocation intermediate is formed [58]. Following enzymatic deprotonation of C-19, a cyclopropane ring is formed between C-9, C-10, and C-19 to form cycloartenol.

The conversion of cycloartenol into sitosterol can be arbitrarily divided into three categories, which are the alkylation of the side chain at C-24, demethylation of the C-4 and C-14 methyl groups,

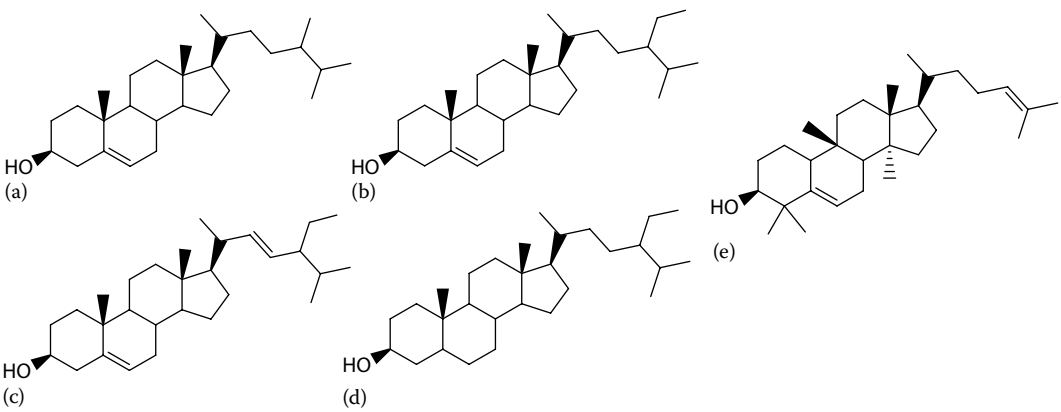
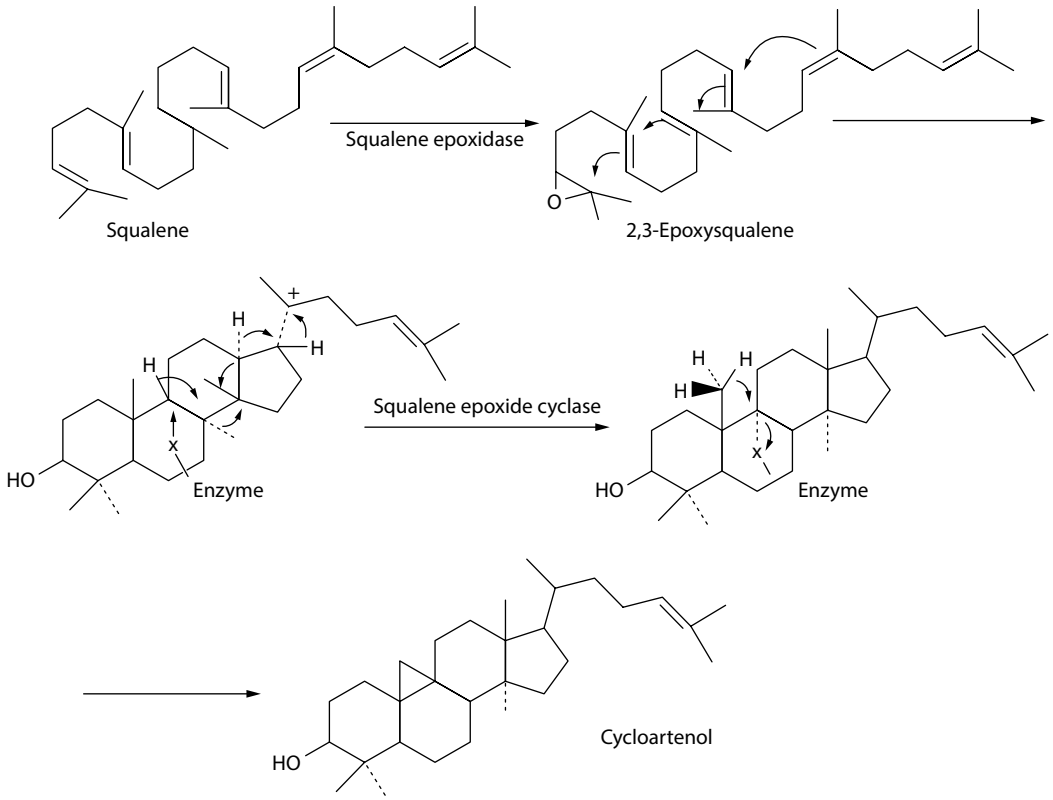


FIGURE 4.4 Examples of plant sterols. (a) Campesterol, (b) sitosterol, (c) stigmasterol, (d) sitostanol, and (e) cucurbetadienol.



SCHEME 4.6 Cyclization of squalene to cycloartenol.

and double-bond manipulation. Although plants do not use the exact same biosynthetic route, these later categories produce similar products to those seen in humans (i.e., no C-4 or C-14 methyl groups and have a Δ^5 double bond). The alkylation of the side chain is a major difference between plant and human sterols, in which the side chain is just hydrogenated. Alkylation in the formation of plant sterols involves methylation at C-24 with the sterol methyltransferase S-adenosylmethionine (SMT1) to produce C28 sterols [59,60]. The further methylation by SMT2, initially described as 24(28)-methylenelophenol methyltransferase, methylene substrate, yields C-24 ethyl sterols [59,60].

C. Cholesterol Homeostasis

As was mentioned earlier in this chapter, the human body regulates the concentration of cholesterol. It must. Cholesterol is necessary for cellular function, yet hypercholesterolemia is a strong risk factor for cardiovascular disease [61]. For the discussion of cholesterol homeostasis, three major pathways for the reduction of cholesterol are described. They are broadly generalized as removal, storage, and the inhibition of cholesterol biosynthesis. (This is not an all-inclusive list.) Because the body can adjust for the decreased or increased cholesterol intake from food, diet will not be considered here as a major factor in cholesterol homeostasis.

Cholesterol biosynthesis is regulated by sterol regulatory element-binding proteins (SREBPs), which control the expression of most genes for cholesterol biosynthetic enzymes [62]. In addition, SREBPs activate transcription of the LDL receptor gene which, in turn, leads to the increased cellular intake of LDL (cholesterol) [63]. SREBPs are synthesized in the endoplasmic reticulum (ER) and are moved to the Golgi apparatus via complexation with SREBP-cleavage activating protein (SCAP). In the Golgi apparatus, SREBP/SCAP complexes are bound to the membrane where two

protease cleavages free the active transcription factor for relocation to the nucleus. SCAP acts as the regulator of this process because without complexing with SCAP, SREBPs remain in the ER and the two necessary protease cleavages never occur. Cholesterol binds to SCAP, which causes a conformational change ultimately blocking a SREBP/SCAP complex and inhibiting the transcription of genes necessary for cholesterol biosynthesis and uptake. Thus, when cholesterol is in excess, the SREBP transport to the Golgi apparatus via SREBP/SCAP complex is inhibited and cholesterol biosynthesis and uptake are also, in turn, inhibited.

Storage of cholesterol happens through acyl-coenzyme A: cholesterol acyltransferase (ACAT). While the mechanism, particularly pertaining to activation, is not completely clear [64,65], it is known that ACAT esterifies cholesterol into less soluble cholesterol esters. This effectively takes cholesterol out of circulation. It has been reported that when cholesterol is well above baseline concentration levels, ACAT is, markedly, switched on [64]. While it seems that removing cholesterol by storage would reduce hypercholesterolemia-associated problems, cholesterol esters have been associated with arterial lesions and there is some effort to inhibit ACAT as a medical treatment [66].

Liver X receptors (LXRs) are related to a multitude of the processes related to cholesterol removal from the body and cells, including cholesterol reverse transport (CRT), conversion to bile acid, and intestinal cholesterol absorption [67,68]. Two LXRs are known, LXRα (found in the liver, kidney, adipose, and adrenal tissue) and LXRβ (ubiquitously expressed) [67,68]. These nuclear receptors are activated by oxysterols rather than cholesterol [69]. When an oxysterol ligand is present, LXR promotes ATP-binding cassette transporter (ABC), and specifically ABCA1 and ABCG1 expression (which mediate efflux of cholesterol from cells to apolipoprotein accepters) [70,71], and inhibits LDL uptake by inducting inducible degrader (s) of LDL receptors (IDOL) [72].

D. Chemistry of Sterols

Enzymatic syntheses give organic chemists a bit of a chuckle when considering that enzymes can do "magic" reactions. Enzymes do very amazing things in the body. In the laboratory setting, sterol chemistry can be an enticing challenge though. Here, a discussion of sterol reactivity is described. Specifically, how are sterols stable and reactive by examining cholesterol. (There are numerous reported steroidal reactions reaching back beyond 1900, and this brief summary barely covers the surface but is a good starting point for understanding.)

Note first that the β side of cholesterol is sterically hindered for reagent approach and group addition because of C-18, C-19, and the side chain at C-17. The α side is relatively vulnerable compared to the β side. Indeed, Breslow's famous remote functionalization reactions involve tethered m-iodobenzene radicals abstracting α facing hydrogen from the α side [73]. For example, C-9 has been halogenated using Breslow's reaction [74]. When observing the cholesterol nucleus from a side perspective (along the nucleus' plane), the greater steric shielding becomes more lucid for β facing carbons such as C-4 or C-8 as compared to α facing carbons such as C-7 and C-9 (Figure 4.5).

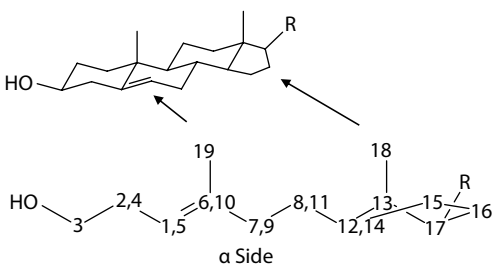


FIGURE 4.5 Side view of a cholesterol nucleus.

Cholesterol's increased stability arises, in part, because of "conformational transmission," [75] essentially a higher energy barrier imparted by the fused rings. While it is obvious that rigid fused rings will resist a ring flip, their resistance to change in hybridization is not as overt. When carbon atoms change from an sp^3 to an sp^2 hybridization, in deprotonation, for example, the geometry of the carbon bonds changes from a tetrahedral to a trigonal planar shape. This causes angular distortion of adjacent carbons and ring strain across the entire fused system. D.H.R. Barton coined this effect as "conformational transmission" [75]. (A simple ball-and-stick model of the cholesterol nucleus can demonstrate this. Torque one carbon and the adjacent rings are also twisted. Keep in mind that the six-membered rings of cholesterol are, for the most part, in stable chair conformations.) This stability of the cholesterol nucleus, due to steric shielding and conformational transmission, is very important considering that the cholesterol nucleus is the essential framework for the biosynthesis of steroids, corticoids, and bile acids.

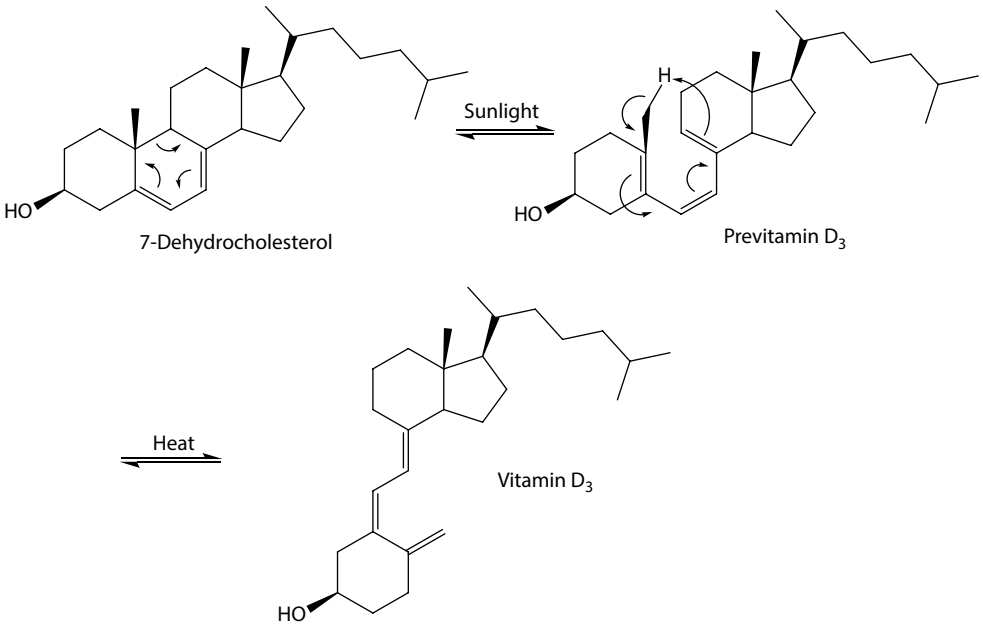
Cholesterol has at least four basic points of reactivity not including multistep exotic synthesis or enzymatic remote functionalization. (1) There is an alcohol at C-3 that can be oxidized by PCC [76] or esterified for protection [77]. (2) The Δ^5 double bond can also be subject to electrophilic addition [78–81] and ring opening [82] but can also be easily protected by halogenation [83]. Autooxidation of cholesterol in the presence of singlet oxygen occurs at the C-5 via ene reaction, but the final product through rearrangement shows oxidation at the C-7 carbon [84]. (3) Allylic oxidation through reactions using a metal or radical initiator and TBHP to form peroxy radicals occur almost exclusively at C-7 rather than C-4 [85–89]. Oxidation of the allylic C-4 carbon rather than C-7 can be accomplished using selenium complexes [90,91]. The selectivity of C-7 over C-4 is due to steric hindrance of the β side [92] and the more stable resonance structure formed by relocation of the radical from C-7 to the tertiary C-5 as opposed to C-4 to the secondary C-6 [93]. (4) C-25 on the side chain is also vulnerable to oxidation by dioxirane [94] and ozone [83] but requires the protection the previously mentioned reactive sites.

Of note, if C-3 is converted into a ketone, acid-catalyzed enol formation and migration of Δ^5–Δ^4 is possible. Migration of Δ^5–Δ^4 is enabled by treatment with oxalic acid [95]. The product of both allylic oxidation at C-7 and migration of Δ^5–Δ^4 leads to α,β-unsaturated ketones, which can be further reacted [96]. Enol formations are subject to conformational transmission in that the enol will form were it is easiest to form (takes less energy) [97]. For example, cholestanone undergoes acid-catalyzed bromination at C-2, whereas coprostanone is brominated at C-4 [98]. (Cholestanone has an A ring in the chair conformation and coprostanone's A ring has a boat conformation.) Also of note is photosynthesis of 7-dehydrocholesterol ($\Delta^{5,7}$), which leads ultimately to Vitamin D_3 [99]. When wavelengths of 290 and 315 nm penetrate human skin, the B-ring is cleaved between C-9 and C-10, as shown in Figure 4.5. The heat of the human body causes a partial change from the *cis*-to-*trans* isomer of the conjugated system by way of an internal [1–7] sigmatropic shift, which then produces Vitamin D_3, also known as cholecalciferol (Scheme 4.7).

E. Analysis of Sterols

This section is perhaps the most difficult to describe in regard to sterols. It is difficult because the technologies pertaining thereto are constantly in flux. Therefore, readers are advised to use this section as a general primer rather than a summary.

(*Extraction*) After the tissue of a plant or animal is broken down, sterols can be extracted using many available methods, including Soxhlet extraction (considered the conventional method), accelerated solvent extraction, cold and hot press, ultrasonic-assisted extraction, subcritical extraction, supercritical CO_2 extraction, mechanical shaking, and microwave-assisted extraction [100–106]. (The references chosen show comparisons between the methods.) The choice of optimal solvent system to extract with depends on the type of sterol desired and the system from which it is being extracted. Chloroform and methanol are popular choices and are often used together to make a



SCHEME 4.7 Synthesis of Vitamin D_3.

binary solvent. Extraction is commonly followed by saponification to free the sterols from their esterified forms [107]. "Direct" saponification (without extraction) has also been reported [108].

(*Isolation*) Sterols are best isolated using some type of column chromatography. In synthetic reactions without many products or purification of stock material, column chromatography is overkill and not needed. These can be isolated simply by washing and recrystallization. For natural products or less selective synthetic reactions, many compounds tend to be present and isolation of the sterols should be done via column chromatography. HPLC and GC [109] are available methods if the intent is merely to analyze the sample. Derivatization may be necessary for some sterols (those that would fall apart at high temperature rather turn into gas or do not ionize efficiently). For collection purposes, an open column having a silica gel stationary phase with a gradient or isocratic (depending on what sterol is being isolated) normal phase elution solvent system can be utilized in a laboratory setting [107].

(*Characterization*) Mass (electron ionization and electrospray ionization), UV, and nuclear magnetic (H and ^{13}C) resonance spectroscopies are all usable in the characterization of sterols. Melting points have also been reported but seem less important in recent decades. An always important method of characterization for sterols is thin-layer chromatography (TLC). In a laboratory setting, TLC is a cheap and quick way to qualitatively determine the presence of targeted sterols. TLC is almost essential for open column chromatography and product elution can easily be elucidated with molybdic acid dye [110]. For GC, flame ionization detection (FID) has been extensively used [109]. Finally, x-ray spectroscopy has been utilized for structural analysis [111].

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5 Extraction and Analysis of Lipids

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CONTENTS

I. INTRODUCTION

Lipids are among the major components of food of plant and animal origin. There is no precise definition available for the term "lipid"; however, it usually includes a broad category of compounds that have some common properties and compositional similarities. Lipids are materials that are sparingly soluble or insoluble in water, but soluble in selected organic solvents such as benzene, chloroform, diethyl ether, hexane, and methanol. Together with carbohydrates and proteins, lipids constitute the principal structural components of tissues. However, the common and unique features of lipids relate to their solubility rather than their structural characteristics [1]. Many classification systems have been proposed for lipids. From the nutrition point of view, according to the National Academy of Sciences' report on nutrition labeling, fats and oils are defined as the complex organic molecules that are formed by combining three fatty acid molecules with one molecule of glycerol [2]. As indicated in Table 5.1 [3–5], lipids are generally classified as simple and compound (complex) or derived lipids according to the Bloor [3] classification.

Foods contain any or all of these lipid compounds; however, triacylglycerols (TAGs) and phospholipids (PLs) are the most abundant and important ones. Liquid TAGs at room temperature are referred to as oils and are generally of plant or marine origin (e.g., vegetable and marine oils). Solid TAGs at room temperature are termed "fats", which are generally of animal origin (e.g., lard and tallow).

Accurate and precise analysis of lipids in foods is important for determining constituting components and nutritive value, standardizing identity and uniformity, preparing nutritional labeling material, and promoting and understanding the effects of fats and oils on food functionality. At the same time, knowledge about the structural characteristics of lipids may allow the development of tailor-made products designed for a particular function or application.

II. EXTRACTION OF LIPIDS FROM FOODS AND BIOLOGICAL MATERIALS

Lipids in nature are associated with other molecules via (1) van der Waals interaction, for example, interaction of several lipid molecules with proteins; (2) electrostatic and hydrogen bonding, mainly between lipids and proteins; and (3) covalent bonding among lipids, carbohydrates, and proteins. Therefore, to separate and isolate lipids from a complex cellular matrix, different chemical and physical treatments must be administered. Water insolubility is the general property used for the separation of lipids from other cellular components. Complete extraction may require longer extraction time or a series or combination of solvents so that lipids can be solubilized from the matrix.

The existing procedures of lipid extraction from animal or plant tissues usually include several steps: (1) pretreatment of the sample, which includes drying, size reduction, or hydrolysis;

TABLE 5.1
General Classification of Lipids

	Simple lipids: Compounds with two types of structural moieties
Glyceryl esters	Esters of glycerol and fatty acids (e.g., triacylglycerols, partial acylglycerols).
Cholesteryl esters	Esters of cholesterol and fatty acids.
Waxes	True waxes are esters of long-chain alcohols and fatty acids; esters of vitamins A and D are also included.
Ceramides	Amides of fatty acids with long-chain di- or trihydroxy bases containing 12–22 carbon atoms in the aliphatic chain (e.g., sphingosine).
	Complex lipids: Compounds with more than two types of structural moieties
Phospholipids	Glycerol esters of fatty acids, phosphoric acid, and other groups containing nitrogen.
Phosphatidic acid	Diacylglycerol esterified to phosphoric acid.
Phosphatidylcholine	Phosphatidic acid linked to choline, known also as lecithin.
Phosphatidylethanolamine	
Phosphatidylserine	
Phosphatidylinositol	
Phosphatidyl acylglycerol	More than one glycerol molecule is esterified to phosphoric acid (e.g., cardiolipin, diphosphatidyl acylglycerol).
Glycoglycerolipids	1,2-Diacylglycerol joined by a glycosidic linkage through position *sn*-3 with a carbohydrate moiety (e.g., monogalactosyl diacylglycerol, digalactosyl monoacylglycerol, sulfoquinovosyl diacylglycerol [monoglycosyl diacylglycerol at position 6 of the disaccharide moiety is linked by carbon–sulfur bond to a sulfonic acid]).
Gangliosides	Glycolipids that are structurally similar to ceramide polyhexoside and also contain 1–3 sialic acid residues; most contain an amino sugar in addition to the other sugars.
Sphingolipids	Derivatives of ceramides.
Sphingomyelin	Ceramide phosphorylcholine.
Cerebroside	Ceramide monohexoside (i.e., ceramide linked to a single sugar moiety at the terminal hydroxyl group of the base).
Ceramide dihexoside	Linked to a disaccharide.
Ceramide polyhexoside	Linked to a tri- or oligosaccharide.
Cerebroside sulfate	Ceramide monohexoside esterified to a sulfate group.

Derived lipids: Compounds that occur as such or released from simple or complex lipids because of hydrolysis (*e.g., fatty acids; fatty alcohols; fat-soluble vitamins A, D, E, and K; hydrocarbons; sterols*)

Sources: Adapted from Bloor, W.R., *Proc. Soc. Exp. Biol. Med.,* 17, 138, 1920; Christie, W.W., *Lipid Analysis*, Pergamon Press, Oxford, U.K., 1982; Pomeranz, Y. and Meloan, C.L., *Food Analysis; Theory and Practice,* 4th edn., AVI, Westport, CT, 1994.

(2) homogenization of the tissue in the presence of a solvent; (3) separation of liquid (organic and aqueous) and solid phases; (4) removal of nonlipid contaminants; and (5) removal of solvent and drying of the extract. Standard methods for lipid extraction have been established by the Association of Official Analytical Chemists [73] International for different types of materials/tissues. However, when it comes to practical situations, each case might require modification of the method.

A. Sample Preparation

As with any form of chemical analysis, proper sampling and storage of the samples are essential for obtaining valid results. According to Pomeranz and Meloan [5], an ideal sample should be identical in all of its intrinsic properties to the bulk of the material from which it is taken. In practice, a sample is satisfactory if its properties under investigation correspond to those of the bulk material within the limits set by the nature of the test. Sample preparation for lipid analysis depends on the

type of food and the nature of its lipids. Effective analysis calls for a knowledge of the structure, chemistry, and occurrence of principal lipid classes and their constituents. Therefore, it is not possible to devise a single standard method for the extraction of all kinds of lipids in different foods.

Extraction of lipids should be performed as soon as possible after the removal of tissues from the living organism so as to minimize any subsequent changes. Immediate extraction is not always possible; however, the samples usually are stored at very low temperatures in sealed containers, under an inert (nitrogen) atmosphere or on dry ice. Yet the freezing process itself may permanently damage the tissues as a result of osmotic shock, which alters the original environment of the tissue lipids and brings them into contact with enzymes from which they are normally protected. Thawing the sample taken from frozen storage before extraction may enhance this deterioration. Therefore, tissue samples should be homogenized and extracted with solvents without being allowed to thaw [4]. Lipolytic enzymes of animal and plant tissues are usually deactivated irreversibly by homogenization with polar solvents. The use of high temperatures should be avoided; it is also advisable, when possible, to maintain an inert atmosphere during sample preparation and extraction, which may minimize oxidation reactions of unsaturated lipids.

B. Pretreatments

1. Drying

Sometimes, nonpolar solvents, such as diethyl ether and hexane, do not easily penetrate the moist tissues (>8% moisture); therefore, effective lipid extraction does not occur. Diethyl ether is hygroscopic and becomes saturated with water and thus inefficient for lipid extraction. Therefore, reducing moisture content of the samples may facilitate lipid extraction. Vacuum oven drying at low temperatures or lyophilization is usually recommended. Predrying facilitates the grinding of the sample, enhances extraction, and may break fat–water emulsions to make fat dissolve easily in the organic solvent and helps to free tissue lipids. Drying the samples at elevated temperatures is undesirable because lipids become bound to proteins and carbohydrates, and such bound lipids are not easily extracted with organic solvents [5].

2. Particle Size Reduction

The extraction efficiency of lipids from a dried sample also depends on the size of the particles. Therefore, particle size reduction increases surface area, allowing more intimate contact of the solvent, and enhances lipid extraction (e.g., grinding of oilseeds before lipid extraction). In some cases, homogenizing the sample together with the extracting solvent (or solvent system) is carried out instead of performing these operations separately. Ultrasonication, together with homogenization in excess amount of extracting solvent, has been successfully used to recover lipids of microalgae [6] and organ tissues [7]. In addition, a method called bead beating has been used in both laboratory and industrial scales. High-speed spinning with fine beads would cause mechanical damage to cells and facilitates oil release [8–11].

Osmotic shock is a less energy–intensive method than bead beating for cell disruption and is considered as a relatively inefficient; thus, it is largely confined to laboratory practices [12]. Osmotic shock is the transfer of water across the outer membrane of the living cells in order to build up the osmotic pressure inside them, resulting in their eventual disruption [12]. Recently, Mandal et al. [13] used osmotic shock as a pretreatment for biodiesel production from microalgae.

3. Acid/Alkali Hydrolysis

To make lipids more available for the extracting solvent, food matrices are often treated with acid or alkali before extraction. Acid or alkali hydrolysis is required to release covalently and ionically bound lipids to proteins and carbohydrates and to break emulsified fats. Digestion of the sample with acid (usually 3–6 M HCl) under reflux conditions converts such bound lipids to an easily

extractable form. Many dairy products, including butter, cheese, milk, and milk-based products, require alkali pretreatment with ammonia to break emulsified fat, neutralize any acid, and solubilize proteins before solvent extraction [14]. Enzymes are also employed to hydrolyze food carbohydrates and proteins (e.g., use of Clarase, a mixture of α-amylase and protease) [2].

C. Lipid Extraction with Solvents

The insolubility of lipids in water makes possible their separation from proteins, carbohydrates, and water in the tissues. Lipids have a wide range of relative hydrophobicities depending on their molecular constituents. In routine food analysis, fat content (sometimes called the ether extract, neutral fat, or crude fat) refers to free lipid constituents that can be extracted into less polar solvents, such as light petroleum ether or diethyl ether. The bound lipid constituents require more polar solvents, such as alkanols, for their extraction. Therefore, the use of a single universal solvent for the extraction of lipids from tissues is not possible. During solvent extraction, van der Waals and electrostatic interactions as well as hydrogen bonds are broken to different extents; however, covalent bonds remain intact.

Neutral lipids are hydrophobically bound and can be extracted from tissues by nonpolar solvents (hexane, cyclohexane, methylene chloride), whereas polar lipids, which are bound predominantly by electrostatic forces and hydrogen bonding, require polar solvents capable of breaking such bonds. However, less polar neutral lipids, such as TAGs and cholesterol esters, may also be extracted incompletely with nonpolar solvents, probably due to inaccessibility of a significant part of these lipids to the solvents. Lipids that are covalently bound to polypeptide and polysaccharide groups will not be extracted at all by organic solvents and will remain in the nonlipid residue. Therefore, a hydrolysis step may be required to release covalently bound lipids to render them fully extractable.

1. Properties of Solvents and Their Mode of Extraction

The type of solvent and the actual method of lipid extraction depend on both the chemical nature of the sample and the type of lipid extract (e.g., total lipids, surface lipids of leaves) desired. The most important characteristic of the ideal solvent for lipid extraction is the high solubility of lipids coupled with low or no solubility of proteins, amino acids, and carbohydrates. The extracting solvent may also prevent enzymatic hydrolysis of lipids, thus ensuring the absence of side reactions. The solvent should readily penetrate sample particles and should have a relatively low boiling point to evaporate readily without leaving any residues when recovering lipids. The solvents mostly used for the isolation of lipids are alcohols (methanol, ethanol, isopropanol, *n*-butanol), acetone, acetonitrile, ethers (diethyl ether, isopropyl ether, dioxane, tetrahydrofuran), halocarbons (chloroform, dichloromethane), hydrocarbons (hexane, benzene, cyclohexane, isooctane), or their mixtures. Although solvents such as benzene are useful in lipid extraction, it is advisable to look for alternative solvents because of the potential carcinogenicity of such products. Flammability and toxicity of the solvent are also important considerations to minimize potential hazards as well as cost and nonhygroscopicity.

Solubility of lipids in organic solvents is dictated by the proportion of the nonpolar hydrocarbon chain of the fatty acids or other aliphatic moieties and polar functional groups, such as phosphate or sugar moieties, in their molecules. Lipids containing no distinguishable polar groups (e.g., TAGs or cholesterol esters) are highly soluble in hydrocarbon solvents such as hexane, benzene, or cyclohexane and in more polar solvents such as chloroform or diethyl ether but remain insoluble in polar solvents such as methanol. The solubility of such lipids in alcoholic solvents increases with the chain length of the hydrocarbon moiety of the alcohol; therefore, they are more soluble in ethanol and completely soluble in *n*-butanol. Similarly, the shorter-chain fatty acid residues in the lipids have greater solubility in more polar solvents (e.g., tributyrin is completely soluble in methanol, whereas tripalmitin is insoluble). Polar lipids are only sparingly soluble in hydrocarbon solvents unless solubilized by association with other lipids; however, they dissolve readily in more polar solvents such as methanol, ethanol, or chloroform [4]. Solvent extraction is a relatively simple and easy method that has been widely used and considered economical for commercial-scale applications [15].

2. Extraction Methods with Single Organic Solvent

Diethyl ether and petroleum ether are the most commonly used solvents for the extraction of lipids. In addition, hexane and sometimes pentane are preferred to obtain lipids from oilseeds. Diethyl ether (bp 34.6°C) has better solvation ability for lipids compared with petroleum ether. Petroleum ether is the low boiling point fraction (bp 35°C–38°C) of petroleum and mainly contains hexanes and pentanes. It is more hydrophobic than diethyl ether and therefore selective for more hydrophobic lipids [5,16]. The main component (>95%) of dietary lipids is TAGs, whereas the remaining lipids are mono- and diacylglycerols, PLs and glycolipids, and sterols. Therefore, nonpolar solvent extractions have been widely employed to extract and determine lipid content of foods. However, oil-soluble flavors, vitamins, and color compounds may also be extracted and determined as lipids when less polar solvents are used. In a recent study, Anthony and Stuart [17] have shown that methylene chloride as being the most effective solvent for the extraction of microalgal oil (*Oocystis* sp.) among the four solvents tested with a total average neutral lipid recovery of 0.25% of dry weight followed by diethyl ether (0.18%), cyclohexane (0.14%), and hexane (0.11%).

In determining total lipid content, several equipment and methods have been developed that utilize single-solvent extraction. Among them, the gravimetric methods are most commonly used for routine analysis purposes. In gravimetric methods, lipids of the sample are extracted with a suitable solvent continuously, semicontinuously, or discontinuously. The fat content is quantified as weight loss of the sample or by weight of the fat removed. The continuous solvent extraction (e.g., Goldfisch and Foss-Let) gives a continuous flow of boiling solvent to flow over the sample (held in a ceramic thimble) for a long period. This gives a faster and more efficient extraction than semicontinuous methods but may result in incomplete extraction due to channeling. In the semicontinuous solvent extraction (e.g., Soxhlet, Soxtec), the solvent accumulates in the extraction chamber (sample is held in a filter paper thimble) for 5–10 min and then siphons back to the boiling flasks. This method requires a longer time than the continuous method, provides a soaking effect for the sample, and does not result in channeling. In the direct or discontinuous solvent extraction, there is no continuous flow of solvent and the sample is extracted with a fixed volume of solvent. After a certain period of time, the solvent layer is recovered, and the dissolved fat is isolated by evaporating the organic solvent. Rose-Gottlieb, modified Mojonnier, and Schmid–Boudzynski–Ratzlaff methods are examples, and these always include acid or base dissolution of proteins to release lipids [14]. Such procedures sometimes employ a combination extraction with diethyl and petroleum ethers to obtain lipids from dairy products. The use of these solvents may allow extraction of mono-, di-, and triacylglycerols, most of the sterols and glycolipids, but may not remove PLs and free fatty acids (FFAs).

3. Methods Using Organic Solvent Combination

A single nonpolar solvent may not extract the polar lipids from tissues under most circumstances. To ensure a complete and quantitative recovery of tissue lipids, a solvent system composed of varying proportions of polar and nonpolar components may be used. Such a mixture extracts total lipids more exhaustively and the extract is suitable for further lipid characterization. The methods of Folch et al. [18] and Bligh and Dyer [19] are most widely used for total lipid extraction. The use of a polar solvent alone may leave nonpolar lipids in the residue; when lipid-free apoproteins are to be isolated, tissues are defatted with polar solvents only [20]. It is also accepted that the water in tissues or water used to wash lipid extracts markedly alters the properties of organic solvents used for lipid extraction.

Commonly, the chloroform–methanol (2:1, v/v) solvent system [18] provides an efficient medium for complete extraction of lipids from animal, plant, or bacterial tissues. The initial solvent system is binary; during the extraction process, it becomes a ternary system consisting of chloroform, methanol, and water in various proportions, depending on the moisture content of the sample [19]. The method of Bligh and Dyer [19] specifically recognizes the importance of water in the extraction of lipids from most tissues and also plays an important role in purifying the resulting lipid extract. A typical procedure by Folch et al. [18] uses a solvent-to-sample ratio of 2:1 (v/w) with a mixture of chloroform and methanol (2:1, v/v) in a two-step extraction. The sample is homogenized with the

solvent and the resultant mixture is filtered to recover the lipid mixture from the residue. Repeated extractions are usually carried out, separated by washings with fresh solvent mixtures of a similar composition. It is usually accepted that about 95% of tissue lipids are extracted during the first step. In this method, if the initial sample contains a significant amount of water, it may be necessary to perform a preliminary extraction with 1:2 (v/v) chloroform–methanol in order to obtain a one-phase solution. This extract is then diluted with water or a salt solution (0.08% KCl, w/v) until the phases separate and the lower phase containing lipids is collected. Bligh and Dyer [19] use 1:1 (v/v) chloroform–methanol for the first step of extraction and the ratio is adjusted to 2:1 (v/v) in the second step of extraction and washing. The original procedure of Folch et al. [18] or of Bligh and Dyer [19] uses large amounts of sample (40–100 g) and solvents; therefore, the amounts may be scaled down when a small amount of sample is present or for routine analysis in the laboratory. Hence, Lee and coworkers [21] described a method that uses the same solvent combination, but in different proportions, based on the anticipated lipid content of the sample. According to this method, chloroform–methanol ratios of 2:1 (v/v) for fatty tissues (>10% lipid), 1:1 (v/v) for medium fat tissue (2%–5%), and 1:2 (v/v) for lean (<2%) tissues of fish are recommended. This method employs a minimum amount of solvent and it may lend itself for small-scale extraction of other samples. A modified Bligh and Dyer [19] extraction that replaces methanol with propan-2-ol and chloroform with cyclohexane has been described by Smedes [22]. This procedure eliminates the use of chlorinated organic solvents. In this extraction, a water–propan-2-ol–cyclohexane mixture (11:8:10, v/v/v) is employed, and subsequent separation step brings all lipids to the upper most layer containing cyclohexane [22].

Extraction by Folch et al. [18] recovers neutral lipids, diacylglycerophospholipids, and most of the sphingolipids. Lysophospholipids are only partly recovered, and more polar acidic PLs and glycolipids may be lost during washing with water. However, both procedures by Folch et al. [18] and Bligh and Dyer [19] may fail to transfer all of the lipids to the organic phase. Lysophospholipids, phosphoinositides, and other highly polar lipid substances are selectively lost. According to Christie [4], tissues rich in phosphoinositides should be stored in such a manner as to minimize their enzymatic degradation, and solvent extraction should be performed initially in the presence of $CaCl_2$. When lysophosphatides are the major component of the tissue extract, it is recommended that acids or inorganic salts be added during extraction with chloroform–methanol or *n*-butanol saturated with water. Therefore, specific applications and modifications of the method are required to ensure complete recovery of tissue lipids.

Due to the potential health hazards of chloroform, solvent mixtures containing alkane–alcohol–water mixtures such as hexane and isopropanol, with or without water, have been successfully used to extract tissue [23,24] and fish meal lipids [25]. Hexane–isopropanol (3:2, v/v) [23,25,26], heptane–ethanol–water–sodium dodecylsulfate (1:1:1, 0.05, v/v/v/w) [27], methylene chloride–methanol (2:1, v/v) [28,29], and hexane–acetone (1:1, v/v) [30] are such solvent combinations employed to extract lipids from biological materials. Azeotropes of isopropanol have also been used to extract lipids from oilseeds as substitutes for hexane [31–33]. Water-saturated *n*-butanol [34] has been most effective in extracting lipids from cereals that are rich in starch. This solvent mixture is used extensively for extracting lipids from starchy foods; however, acid hydrolysis might be needed to release bound lipids or inclusion complexes before their extraction. Recently, Lessig and Fuchs [35] used chloroform–methanol–12 M HCl (2:4:0.1, v/v/v) to extract acidic PLs such as phosphatidylserine and particularly phosphoinositides and found that this solvent system leads to complete hydrolysis of plasmalogens.

Pressurized fluid extraction (PFE), pressurized liquid extraction (PLE), or accelerated solvent extraction (ASE; trade name used by Dionex) techniques have been developed to enhance the capabilities of conventional solvent extraction. These techniques use classical solvent systems to extract lipids, but under varying extraction parameters such as temperature, pressure, and volume. The extraction media are organic solvents or their aqueous mixtures in which lipids are soluble. The system is operated under high pressure and the solvent is kept at much higher temperatures above its atmospheric boiling point. The elevated temperature at which the extraction is conducted increases the capacity of the solvent to solubilize the analyte. Elevated temperature is also known to

weaken the bonds between the analyte and the matrix and thus decreases the viscosity of the solvent with improved penetration into the matrix, resulting in an increased extraction yield. PFE can cut down the solvent consumption by 50% when compared with conventional methods such as Folch extraction procedure [36].

ASE system or ASE is the automated PFE/PLE [37,38]. The ASE process consumes a much lower solvent volume and time as lipid is extracted at temperatures well above the boiling point of the solvent because of the elevated pressure used in the process. This enhances solubilization and diffusion of lipids from samples into the solvent, significantly shortening the extraction time and solvent consumption. The fat could be extracted with no outflow of solvent (static mode) or allowing fresh solvent to flow continuously through the sample (dynamic mode) during extraction. Under elevated temperature and pressure, dissolved lipids diffuse from the core to the surface of the sample particles and then are transferred to the extraction solvent. Compressed gas then purges the solubilized fat into a collection vessel and can then be quantified gravimetrically [16]. According to Shafer [38], the content of fatty acids of the lipids extracted from muscle matrices using ASE (Dionex 200 or 300 system, chloroform–methanol solvent system) was similar or better in comparison with the conventional Folch extraction. The automated solvent extractors contain microwave moisture analyzer to dry the sample before extraction, redry to remove solvent and moisture, and determine the percentage of fat as weight loss due to the extraction process [2]. Possibility of using the ASE system for sequential extraction of lipid classes has been described by Poerschmann and Carlson [39]. Under optimum extraction conditions, *n*-hexane–acetone (9:1, v/v) at 50°C (two cycles, 10 min each) to obtain neutral lipids followed by chloroform–methanol (1:4, v/v) extraction at 110°C (two cycles, 10 min) has been used for phytoplankton lipid extraction with ASE.

Ultrasonic extraction in combination with various solvents has been used in several studies, such as ultrasonic-assisted extraction with ethanol [40], chloroform–methanol [41], *n*-hexane [41,42], and hexane–isopropanol [43]. This method provides a high efficient contact between the solvent and sample matrix [44]. The mechanism of ultrasound sonication extraction is the formation of microbubbles during the negative pressure and compression during the positive pressure. This intermittent change could cause violent collapsing of microbubbles near solid surface of the material and damage the cell walls to facilitate the release of their contents [44]. In addition, few studies have shown microwave extraction of lipids with hexane [42,45] and a pulsed electric field prior to a solid–liquid extraction using ethanol [46].

4. Switchable Polarity Solvent

Recently, various efforts have been made to minimize the use of toxic and polluting organic solvents. Switchable polarity solvents (SPS) provide a new fascinating technique, which would allow the recovery of extracted material from the extracting solvent simply by the incorporation of carbon dioxide. SPS have been shown to exhibit two degrees of polarity, hydrophobic in the nonionic form and hydrophilic in the ionic one, ranging from the polarity of chloroform to that of dimethylformamide [47]. Phan et al. [48] synthesized a SPS, namely, 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU)–alkyl carbonate salts, obtained from the mixtures (1:1) of two neutral liquid components, DBU and an alcohol, which will become more polar when CO_2 is introduced and returns to its original polarity when the CO_2 is removed. Recently, several studies have employed the SPS for lipid extraction, such as DBU–ethanol for soybean oil [48], DBU–octanol, DBU–ethanol for microalgae [47,49], and DBU–*n*-alkyl alcohols [50]. In another study, a switchable hydrophilicity solvent, *N*,*N*-dimethylcyclohexylamine, was used to extract lipids from freeze-dried microalgal samples [51].

5. Methods Using Nonorganic Solvents

a. Microwave-Assisted Extraction

Due to environmental concerns and potential health hazards of organic solvents, nonorganic solvents have become popular. The use of microwave digestion for isolating lipids has recently been reported [52]. It is suggested that microwave energy, by increasing the rotational force on bonds connecting

dipolar moieties to adjacent molecules, reduces the energy required to disrupt hydrophobic associations, hydrogen bonding, and electrostatic forces, thus helping to dissolve all kinds of lipids [52]. A solvent with sufficient dielectric constant is a requirement to absorb microwave energy. Closed-vessel microwave-assisted extraction is performed at high pressure and, therefore, allows extraction at temperatures above boiling point of the solvent. Open-vessel microwave-assisted extraction system works at ambient pressure and refluxing of the solvent. Since extraction time is also a function of the temperature, a high boiling point of the solvent accelerates the extraction. However, evaporation of sample water is a possibility when open-vessel microwave-assisted extraction is used, which facilitates decomposition of cell structure and removal of lipids from their association with cell membrane and lipoprotein. However, low lipid yield is a concern due to increased polarity of solvent when the water content is high; thus, sample drying is recommended [53].

Microwave technology has allowed the development of rapid, safe, and cost-effective methods for extracting lipids and does not require that samples be devoid of water [54]. Performance of microwave lipid extraction was qualitatively (all lipid classes) and quantitatively comparable with that of the conventional Folch method for various biological samples [52].

b. Supercritical Fluid Extraction

When carbon dioxide is compressed at a temperature (31.1°C) and pressure (72.9 atm) above its critical point, it does not liquify but attains a dense gaseous state that behaves like a solvent. Thus, it is called supercritical CO_2 (SC-CO_2). The use of SC-CO_2 for lipid extraction significantly reduces the use of organic solvents, avoids waste disposal problems, eliminates the use of potentially toxic and flammable solvents, and reduces the extraction time. Lipids so extracted are not subjected to high temperatures during the extraction process.

Extraction using SC-CO_2 yields a good recovery of nonpolar lipids including esterified fatty acids, acylglycerols, and unsaponifiable matter. Complex polar lipids are only sparingly soluble in SC-CO_2. The polarity of SC-CO_2 can be varied by using an entrainer or modifier such as methanol, ethanol, or even water to improve the extraction of polar lipids [55–58]. This technique has been used for the extraction of lipids from various matrices, including dehydrated foods [59,60], meats [61–63], oilseeds [64], and fried foods [65]. Particle size also affects lipid recovery because it influences the surface area exposed to SC-CO_2. High moisture content decreases contact between sample and SC-CO_2 as well as the diffusion lipids outside the sample [66]. The extracted lipids from meat or hydrolytic products from the acid hydrolysis step are allowed to absorb onto a solid-phase extraction (SPE) matrix and SC-CO_2 can be used to extract the adsorbed lipids [62]. An increased lipid recovery with decreased moisture content has been demonstrated in wet samples, such as meat [61,67–70]. Therefore, lyophilization is suggested to improve the extraction efficiency of lipids from samples with a high moisture content. The SC-CO_2 extraction is able to recover 97%–100% of lipids when compared with the conventional solvent extraction methods [71,72]; no significant differences between fatty acids extracted were observed. Several researchers have shown that supercritical fluid extraction (SFE) could replace solvent extraction methods in a large variety of samples. In fact, SFE has recently been included in the recommended methods of the AOAC to extract lipids from oilseeds [73]. According to Barthet and Daun [64], for canola and flaxseed, a modifier ethanol (15%, v/v) and multiple extractions are needed to obtain similar values of oil content on exhaustive solvent extraction with petroleum ether. They noted that mustard always rendered 10% lower values when SC-CO_2 extraction was used. The main drawback of SC-CO_2 is equipment cost and the extraction of nonfat materials, such as water [74].

Recently, SFE has been widely used to obtain high-quality fish oil [75,76], due to its moderate temperature and oxygen-free medium, which reduces the oxidation of omega-3 fatty acids during the extraction process [77]. However, the limitation of SFE in the extraction of omega-3 fatty acids is the necessities of freeze-drying of raw material in order to reduce its moisture content below 20% and keep the omega-3 fatty acids and the fish structure unchanged [76]. Recently, Rubio-Rodríguez et al. [77] compared the oils obtained by SFE over freeze-dried fish by-products and other methods

including cold extraction, wet reduction, and enzymatic extraction. Their study showed that SFE prevents lipid oxidation, especially in fish oils rich in omega-3 fatty acids such as salmon oil, and significantly reduces the amount of certain pollutants such as some arsenic species (mainly polar derivatives).

c. Hydrothermal Liquefaction

Hydrothermal liquefaction is a wet biomass–handling process, which utilizes/converts biomass into liquid fuel. This method has been described as a process involving the reaction of biomass in water at subcritical temperatures (<374°C) and high pressure (>vapor pressure of water) for a certain reaction time with or without the use of a catalyst [78]. Direct hydrothermal liquefaction processing of wet algal biomass containing 5%–20% solids has been shown to consume less than 5% of the energy cost associated with the complete removal of moisture by thermal drying [78].

D. Lipid Extraction without Solvents

Lipid extraction methods are mostly wet extraction procedures that do not use solvents, and lipid content is quantified by volumetric means. Such procedures are well utilized in determining the fat content of dairy foods, especially fresh milk, and require the use of specifically designed glasswares and equipment.

1. Acid Digestion Methods

Babcock and Gerber methods are classical examples for acid digestion methods. The basic principle of these methods is destabilization and release of fat from the emulsion with a strong acid (e.g., sulfuric). The less dense fat rises in the calibrated neck of the Babcock bottle, and the centrifugation step helps the separation. Added sulfuric acid digests proteins, generates heat, and releases fat. The content of fat is measured volumetrically and expressed as weight percent. The modified Babcock method uses an acetic–perchloric acid mixture rather than sulfuric acid and is employed to determine essential oil in flavor extracts and products containing sugar and chocolate. The Gerber method uses a principle similar to that of the Babcock method but utilizes sulfuric acid and pentanol. Pentanol prevents charring of sugar, which can occur with the Babcock method; therefore, the Gerber method could be applied to a wide variety of dairy-based foods [2,14].

2. Detergent Method

The detergent method uses a detergent to form a protein–detergent complex to break up emulsion and release fat. For milk, the anionic detergent dioctyl sodium phosphate is added to disperse the protein layer that stabilizes and liberates fat. Then a strong hydrophilic nonionic polyoxyethylene detergent, sorbitan monolaurate, is added to separate the fat from other food components [5].

3. Physical Methods

External compression forces may be used to release tissue contents and extract lipids, especially from the dry matter. Oilseeds (moisture < 5%, oil > 30%) are generally subjected to expeller pressing to obtain lipids without using solvents and, however, may not afford complete recovery of oils.

4. Enzymatic Method

Aqueous enzymatic extraction is considered as an environmentally friendly alternative technology for oil extraction. The use of enzymes such as protease, cellulose, pectinase, and amylase during extraction enhances oil recovery by breaking cell walls and oil bodies [79,80]. Studies have shown that the use of enzymes results in 90%–98% oil recovery from coconut, soybean, corn germ, and sunflower [80–83]. In another study, commercial proteases (Alcalase®, Neutrase®, and Flavourzyme™) were found to be effective for the extraction of oil from marine by-products such as salmon heads [84].

E. Removal of Nonlipid Contaminants from Lipid Extracts and Other Practical Considerations

Removal of nonlipid contaminants from the lipid extract is necessary since most of the solvents employed also dissolve significant amounts of oil-soluble flavors, pigments, sugars, amino acids, short-chain peptides, inorganic salts, and urea. The nonlipid matter must be removed before gravimetric determination of total lipids in order to prevent contamination during subsequent fractionation of the total extract. In chloroform–methanol extract, the commonly used method for removing nonlipid contaminants includes washing with water or a diluted KCl solution (0.88%, w/v). The use of salt solution has the advantage of preventing or minimizing the formation of an intermediate phase. When chloroform–methanol (2:1, v/v) is used for the extraction of the sample, addition of water or diluted salt solution results in the formation of a two-phase system, that is, a lower phase consisting of chloroform–methanol–water (86:14:1, v/v/v) and an upper phase consisting of the same, but in the ratio of 3:48:47 (v/v/v). The lower phase is composed of about two-thirds of the total volume and contains the lipid components, but the upper phase retains the nonlipid contaminants. However, more polar lipids, such as some PLs and glycolipids and all gangliosides, may remain in the upper phase [4,85]. Nonlipid contaminants may also be removed partly or completely by evaporation of the lipid extract to dryness in vacuum or under nitrogen and then reextracted with a nonpolar solvent, such as hexane.

In the Bligh and Dyer [19] method, the sample is homogenized with chloroform and methanol in such proportions that a miscible system is formed with water in the sample. Dilution of the homogenate with chloroform and water separates it into two layers; the chloroform layer contains all the lipids and the methanol–water layer contains all the nonlipid matter. A purified lipid extract could be obtained by isolating the chloroform layer. Traces of moisture can be removed by passing the chloroform extract through a bed of anhydrous sodium sulfate.

Removal of nonlipid contaminants by liquid–liquid partition chromatography on a dextran gel was introduced by Wells and Dittmer [86]. This is done by passing the crude lipid extract through a Sephadex G-25 column (packed in the upper phase of chloroform–methanol–water, 8:4:3, v/v/v, or Folch wash). Lipids free of contaminants would be eluted rapidly from the column by employing the lower phase of the Folch wash. Gangliosides and nonlipids are retained in the column and can be recovered by washing with the upper phase of Folch wash and at the same time regenerating the column [87].

The use of predistilled solvents for lipid extraction is advisable since all solvents contain small amounts of lipid contaminants. The use of plastic containers and non-Teflon apparatus should also be avoided as plasticizers may leach out to the lipid extract. To prevent autoxidation of unsaturated lipids, it is advisable to add an antioxidant (e.g., BHT) to the solvent (at a level of 50–100 mg/L). Furthermore, extraction should be performed under an inert nitrogen atmosphere, and both tissue and tissue extracts should be stored at −20°C under nitrogen, if possible. Most of the methods described earlier are suitable for quantifying the total lipid content of the sample of interest. When high temperatures are involved in extraction, the resulting lipid extract is not suitable for further composition analysis. Method-based extraction by Folch et al. [18] is usually the preferred procedure to obtain total lipids for further analysis. Lipids are recovered from the chloroform layer by the removal of solvent at low temperature under vacuum. Acid hydrolysis also results in decomposition of PLs and possibly TAGs to a certain extent [14].

Quantification of lipids from the extracts is mostly carried out as the weight difference of an aliquot after solvent removal. Removal of the solvent from lipid extracts should be conducted under vacuum in a rotary evaporator at or near room temperature. When a large amount of solvent must be evaporated, the solution must be concentrated and transferred to a small vessel so that the lipids do not dry out as a thin film over a large area of glass. Lipids should be stored immediately in an inert nonalcoholic solvent such as chloroform, rather than being allowed to remain in dry state for long [4].

III. INDIRECT METHODS OF TOTAL LIPID DETERMINATION

Several techniques and instruments have been developed and applied to the indirect and rapid determination of total lipid content of samples. These methods are really not lipid extraction methods, but they are gaining popularity because they are rapid and largely nondestructive. Most of these methods rely on a standard reference procedure and must be calibrated against a methodology to be validated.

A. Density Measurement

It has been reported that the density of flaxseed is highly correlated ($r = 0.96$) with its oil content [88]. Thus, measurement of seed density may be used as a means of screening flax genetic lines for high oil content.

B. Dielectric Method

The dielectric constant of a selected solvent changes when fat is dissolved in it. After an oilseed sample has been ground with a solvent and the dielectric constant of the mixture measured, the lipid content is determined from standard charts that show variation of the dielectric constants of different amounts of lipid in the same solvent [5]. According to Hunt et al. [89], the amount of induced current and oil content determined by solvent extraction of soybean are linearly related ($r = 0.98$).

C. Near-Infrared Spectroscopy

The near-infrared (NIR) reflectance is in the range of 14,300–4,000 cm^{-1} (700–2,500 nm) due to overtone and combination bands of C−H, O−H, and N−H. NIR spectrometry can be used for the determination of contents of oil, protein, and moisture and serves as a very useful tool in the routine analysis of oilseeds.

Rodrigneuz-Otero et al. [90] have used NIR spectroscopy for the measurement of fat, protein, and total solids of cheese. Lee et al. [91] have used short-wavelength (700–1100 nm) NIR with a bifurcated fiber-optic probe to estimate the crude lipid content in the muscle of whole rainbow trout. A very good correlation was observed between fat content determined by chemical analysis and NIR reflectance spectroscopic values obtained for farmed Atlantic salmon fillets [92]. The use of mid-IR spectroscopy to determine the lipid content of milk and dairy products has been described by Biggs [93]. Lipids absorb IR energy at the wavelength of 5730 nm, and the energy absorbed depends on the lipid content of the sample. Quantification is carried out by the standard curve of the IR absorption and lipid content determined by standard analytical methods [94]. Details about the use of IR spectroscopy for lipid analysis are provided in a later section of this chapter.

D. Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) has the unique capability to unequivocally derive molecular formula–measured mass-to-charge ratio for several thousand compounds in each oil [95]. High-resolution electrospray ionization FT-ICR MS has ultrahigh mass resolving power ($m/\Delta m_{50\%} > 350{,}000$, in which $\Delta m_{50\%}$ is the mass spectral peak full width at half-maximum peak height) and mass accuracy (<1 ppm), which allows fast, definite assignments of components having thousands of different elemental compositions in vegetable oil without any prior sample extraction, separation, or chemical derivatization [96]. Coupling of FT-ICR MS to electrospray ionization has been shown to afford the selective ionization of acidic (negative mode) and basic (positive mode) heteroatom containing compounds and thus enables to identify the adulteration of vegetable oils [96]. Sudasinghe et al. [95] have shown detailed compositional characteristics of hydrothermal liquefaction oils derived from two biochemically distinct microalgae, for a range of reaction temperatures measured by high-resolution electrospray ionization FT-ICR MS.

E. Low-Resolution Nuclear Magnetic Resonance Spectroscopy

Time-domain low-resolution nuclear magnetic resonance (NMR) (referred to as wide-line NMR) and frequency-domain NMR could be used to determine the total lipid content of foods. In time-domain NMR (TD-NMR), signals from the hydrogen nuclei (^1H or protons) of different food components are distinguished by their different rates of decay or nuclear relaxation. Protons of solid phases relax (signal disappears) quickly, whereas protons in the liquid phase relax very slowly. Protons of water in the sample relax faster than protons of the lipid. The intensity of the signal is proportional to the number of protons and, therefore, to the hydrogen content. Thus, the intensity of the NMR signal can be converted to the oil content of the sample using calibration curves or tables [97–100]. This method can be used to determine the contents of water, oil, and solid fat and solid-to-liquid ratio of the sample. TD-NMR has been used to analyze the fat content of foods, including butter, margarine, shortening, chocolate, oilseed, meat, milk and milk powder, and cheese [101–103].

Frequency-domain NMR distinguishes food components by resonance frequency (chemical shift) of the peaks in the spectrum. The pattern of oil resonances reflects the degree of unsaturation and other chemical properties [94,101].

F. Turbidimetric/Colorimetric Methods

Haugaard and Pettinati [104] have described a turbidimetric method for rapid determination of lipid in milk. The milk fat is homogenized to obtain uniform globules, and the milk proteins are retained with chelating agents such as EDTA. Light transmission of the sample is measured and then converted to the lipid content with the use of a conversion chart.

The lipid content of milk can also be determined using a colorimetric method [105]. The lipids of milk are allowed to react with an alkaline solution of hydroxamic acid for a specified period. On acidification and addition of ferric chloride, a relatively stable chromophore with a maximal absorbance at 540 nm is formed [106]. A colorimetric method suitable for plasma PL quantitation has been described by Hojjati and Jiang [107]. The first suitable enzymes are employed to release choline from plasma PLs; that is, PC-specific phospholipase D is used for phosphatidylcholine, and sphingomyelinase and alkaline phosphatase combination can be used for sphingomyelin. The resulting choline is directed to generate H_2O_2 in a reaction catalyzed by choline oxidase. Generated H_2O_2 reacts with *N*-ethyl-*n*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS, Trinder's reagent) and 4-aminoantipyrine in a reaction that generates blue chromophore (maximum absorbance at 595 nm) and peroxidase functions as a catalyst [107].

G. Ultrasonic Method

Ultrasonication method involves the application of high-frequency, high-intensity sound waves to the material [108]. Fitzgerald et al. [109] have described an ultrasonic method to determine the amount of fat and nonfat solids of liquid milk. The velocity of sound increases or decreases directly with the lipid content above or below a certain critical temperature. This method of fat determination is based on the speed of sound passing through the milk at various temperatures.

H. X-Ray Absorption

It is known that lean meat absorbs more x-ray than high-fat meat [110]. This fact has been used to determine lipid content in meat and meat products using a standard curve of the relationship between x-ray absorption and the lipid content determined by usual solvent extraction methods [5].

I. Flow Cytometry

Flow cytometry is an indirect measurement of lipid content via the fluorescence of lipid probes such as Nile Red or BODIPY (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-S-indacene). Nile Red is a neutral lipid-staining fluorescent dye, which can be used to image neutral lipid accumulation within cells or to quantify lipid biosynthesis by fluorescence spectroscopy [111,112]. Nile Red is relatively photostable and intensely fluorescent in organic solvents but has a low quantum yield in water [111]. The appropriate choice of excitation and emission wavelengths allows differentiating polar and neutral lipids because Nile Red's emission maximum is blueshifted as the polarity of the surrounding environment decreases [111]. However, Nile Red–based methods are time-consuming and are not as quantitative as chromatographic methods, and there may be variation in the efficiency of Nile Red accumulation into some algal species [113]. In addition, Nile Red does not specifically accumulate within lipid deposits; it could also exhibit nonspecific fluorescence when bound to proteins and other cellular components [114,115]. Due to the limitations with Nile Red, another fluorophore, namely, BODIPY 505/515, has been investigated as an alternative fluorescent label for algal lipid deposits [114,116]. Recently, Bono Jr. et al. [115] measured the lipid accumulation in microalgae using flow cytometry with liquid-state ^{1}H NMR spectroscopy.

IV. ANALYSIS OF LIPID EXTRACTS

Lipid analysis is usually required to determine the composition and structure of the lipid extracted from the sample. Foods must be analyzed to reveal the content and type of saturated and unsaturated lipids as well as their cholesterol content. Such characterization provides information about the caloric value, as well as other properties, including nutritional quality and safety of lipids with respect to their cholesterol and saturated fatty acid contents. In addition, quantification of quality characteristics such as degree of unsaturation, saponification value, refractive index (RI), FFA content, solid fat index (SFI), and oxidative stability is required to determine the market value and potential application of fats and oils. Analysis of individual components of lipids is beyond the scope of this chapter and hence is not discussed here.

A. Bulk Oil Properties

The analysis of bulk properties of lipids is primarily important for defining quality characteristics of oils and fats. Therefore, methods applicable to bulk vegetable oils, confectionary fats (e.g., cocoa butter), and table fats (e.g., butter, margarine) are discussed in the following sections.

1. Degree of Unsaturation

Iodine value (IV) measures the degree of unsaturation of a lipid and is defined as the number of grams of iodine absorbed by 100 g of lipid. The source of iodine (or other halogen, usually Br_2 and Cl_2) for the reaction is Wijs or Hanus reagent; the reaction involved is essentially a volumetric titration.

In a microanalytical method for the determination of the IV of lipids reported by Iskander [117], ethylenic double bonds of the lipid are saturated with bromine vapors, after which the amount of absorbed bromine is determined by neutron activation analysis.

Determination of IV gives a reasonable quantitation of lipid unsaturation if the double bonds are not conjugated with each other or with a carbonyl oxygen. Furthermore, the determination should be carried out in the absence of light for a given period and with an excess of halogen reagent used [118].

The use of hydrogenation methods to determine the degree of unsaturation overcomes the limitations of halogenation methods. Hydrogenation is used to measure the degree of unsaturation

of acetylenic or conjugated double bonds. Such fats do not absorb halogen readily; however, the addition of hydrogen to them is considered to be quantitative. This method is essentially a catalytic reaction of heated lipid; the amount of hydrogen absorbed is determined under standard conditions. The results are expressed on a mole basis or on the basis of IV [5].

At the low-frequency end of the fingerprint region of IR (1500–900 cm^{-1}), a band due to the CH=CH bending absorption of isolated *trans* double bonds would be observed. Beyond the isolated *trans* bond is another group of CH absorption, in this case bending vibrations, including a very strong *cis* absorption. The combination of *cis* and *trans* absorption provides a measure of the total unsaturation or IV [119–121].

2. Free Fatty Acid Content

The presence of FFAs in an oil is an indication of insufficient processing, lipase activity, or other hydrolytic actions. Classically, the acid value, which is defined as the number of milligrams of KOH required to neutralize the free acids in 1 g of sample, is a measure of FFA content. FFAs of oils can be determined colorimetrically by dissolving oil in chloroform (or benzene) and then allowing the FFAs to react with a cupric acetate solution. The organic solvent turns to a blue color because of the FFA–cupric ion complex, which has a maximum absorbance between 640 and 690 nm [122].

As there is a band attributed to the carboxyl group (COOH) in the center region of the mid-IR spectrum, FTIR spectroscopy can be used to determine the content of FFAs [123,124].

3. Oxidative Stability and Oxidation Products

Owing to their degree of unsaturation, lipids are very susceptible to autoxidation. Autoxidation occurs via a self-sustaining free radical mechanism that produces hydroperoxides (primary products), which in turn undergo scission to form various aldehydes, ketones, alcohols, and hydrocarbons (secondary products). The presence of secondary lipid oxidation products influences the overall quality of a lipid. Methods of determination of oxidative stability and oxidation products are discussed in detail in another chapter.

4. Refractive Index

The RI of an oil is defined as the ratio of the speed of light in vacuum (practically in air) to the speed of light in oil at a specified temperature. This ratio also provides a measure of purity of oils and may be used as a means of identifying them. The RI is measured with a refractometer, usually at 20°C–25°C for oils and 40°C for solid fats, which generally liquify at 40°C. The RI declines linearly with decreasing IV; thus, it is also used as an index for reporting the degree of hydrogenation of the oil [5].

5. Saponification Value

The saponification value provides an indication of the average molecular weight of lipids. It is defined as the amount of KOH, in milligrams, required to saponify 1 g of fat, that is, to neutralize the existing FFAs and those liberated from TAG [5].

6. Solid Fat Index

The SFI, an empirical expression of the ratio of liquids in fat at a given temperature, is measured as the change in specific volume with respect to temperature. As the solid fat melts, the volume of the sample increases, and this change is measured by dilatometry. Detection of analysis of phase transformation of fat may also be performed, because lipids expand on melting and contract on polymorphic change to a more stable fat [5]. The use of low-resolution pulse NMR and FTIR [125–129] for the determination of solid fat content has been detailed in the literature.

B. Chromatographic Procedures for Lipid Characterization

Lipid extracts are complex mixtures of individual classes of compounds and require further separation to pure components if needed. Analysis of chemical components of lipid (e.g., lipid classes, fatty acids, *trans* fatty acids, sterols, tocopherols, pigments) primarily involves chromatographic and spectroscopic methods. Usually, a combination of separation techniques is used to achieve a high degree of purity of respective lipid components and this could be analytical (for quantitation) or preparative.

The first step in the analyses involves separation of lipids into their various polarity components. It may simply separate the lipid into its polar and nonpolar fractions or may entail analysis of TAG, FFAs, sterols, steryl esters, glycolipids, and PLs. Traditionally, liquid–liquid extraction, thin-layer chromatography (TLC), or liquid–solid column chromatography has been used for fractionation, cleaning, and concentration of lipid extracts. The most commonly used chromatographic techniques for lipid analysis include column chromatography, gas chromatography (GC), high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), and TLC. Applications of these techniques for the analysis of food lipids are discussed in the following sections.

1. Column Chromatography

Lipid extracts are usually fractionated by column chromatography on a preparative scale before subjecting them to detailed analysis. Solid–liquid (adsorption), liquid–liquid (partition), and ion-exchange chromatography are among the widely used methods of lipid fractionation. In solid–liquid chromatography, separation is based on partitioning and adsorption of the lipid components between solid and liquid (mobile) phases. Elution of the desired lipid class is achieved by varying the polarity and strength of the mobile phase. Common stationary phases for column chromatography are silica, alumina, and ion-exchange resins, whereas the preferred column materials for lipid analysis are silicic acid and florisil (magnesium silicate).

Low-pressure column chromatography using 50–500 mesh adsorbents has been used commonly for the separation of different lipid classes. The main parameters involved in column chromatography include weight of the adsorbent, conditioning of the adsorbent (moisture content), and column size. It is generally accepted that long narrow columns give the best resolution, but large-diameter columns increase sample capacity. For convenience, diameters over 5 cm and heights over 45 cm are not recommended for typical laboratory use [4,130].

In adsorption chromatography, compounds are bound to the solid adsorbent by polar, ionic, and, to a lesser extent, nonpolar or van der Waals forces. Therefore, separation of lipid components takes place according to the relative polarities of the individual components, which are determined by the number and type of nonpolar hydrophobic groups. In general, elution of the column with solvents with increasing polarity separates the lipid mixture according to increasing polarity of its components in the following order: saturated hydrocarbons, unsaturated hydrocarbons, wax esters, steryl esters, long-chain aldehydes, TAGs, long-chain alcohols, FFAs, quinones, sterols, diacylglycerols, monoacylglycerols, cerebrosides, glycosyl diacylglycerols, sulfolipids, acidic glycerophosphatides, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine [131]. The procedure applicable to most lipid mixtures is stepwise elution on a silicic acid column with the solvent sequence of *n*-hexane, acetone, and methanol to separate into neutral lipids, glycolipids, and PLs, respectively [4,131]. The shortcomings of this SPE are incomplete elution from silicic acid; potential contamination of other lipid classes, which requires further purification; and arbitrary assignment of solvents in predefined lipid classes without proof.

Complexing the adsorbent material with silver nitrate enables separation of lipid mixtures according to the number, position, and *cis* and *trans* isomerism of double bonds in unsaturated fatty acids and their derivatives. The use of borate treatment of the column material complexes the compounds containing hydroxyl groups on adjacent carbon atoms and assists the separation of glycolipids [132]. Complexing of adsorbent materials is discussed in Section IV.B.5.

TABLE 5.2
Ion-Exchange Chromatography Materials Used in Lipid Analysis

Ionizing Group	Commercial Classification	Analytical Use
$(CH_2)_2N + H(C_2H_5)_2$ (diethylaminoethyl)	DEAE (anionic exchanger)	Anionic lipids (phospholipids, sulfolipids, sialoglycolipids, fatty acids)
$(CH_2)_2N + (C_2H_5)_3$ (triethylaminoethyl)	TEAE (anionic exchanger)	Anionic lipids
$CH_2COO–$ (carboxymethyl)	CM (cationic exchanger)	Phospholipid mixtures

Source: Adapted from Hemming, F.W. and Hawthrone, J.N., *Lipid Analysis*, BIOS Scientific, Oxford, U.K., 1996.

Nowadays, commercial columns are prepacked with a variety of solid stationary phases, which are available for the separation of lipid classes and may be referred to as SPE columns. SPE requires less time, solvent, and packing material than classical column chromatography [133]. SPE can be used for isolation, concentration, purification, and fractionation of analytes from complex mixtures [134,135]. Aminopropyl-bonded phase has been used for the separation of total lipids in lipid classes obtained from different sources [136–139].

The ion-exchange columns carry ionic groups that bind to the opposite charge of the ionic groups of lipids. Thus, a polymer with fixed cations binds anionic lipids from a mixture, provided that the pH of the solvent mixture allows ionization of the anionic groups. At the same time, the concentration of nonlipid anions in the solvent mixture should not compete for all of the fixed ions [132]. Some of the ion-exchange chromatographic materials commonly used for lipid analysis are given in Table 5.2. Diethylaminoethyl (DEAE) cellulose is used for the separation of lipid classes, and triethylaminoethyl cellulose is useful for the separation of lipids having only ionic carboxyl groups (e.g., fatty acids, bile acids, gangliosides) or phosphatidylethanolamine from ceramide polyhexosides [131]. In polar lipid analysis, DEAE cellulose in the acetate form is the most frequently used anion-exchange material. It is most effective in the pH range 3–6, and often separation of polar lipid is achieved by stepwise elution with ammonium acetate buffer in water–ethanol. The cation exchanger carboxymethylcellulose as its sodium salt has been used occasionally over the same pH range for the separation of PLs [132].

Immunoaffinity column chromatography has been used for isolation and purification of apolipoproteins. Ligand molecules (antibody, antibody to apolipoprotein or antigen, purified apolipoprotein) are immobilized on the solid support (matrix) and bind to corresponding target molecules (antigen or antibody) in a mixture of macromolecules. The bound target molecule (e.g., apolipoprotein) can then be desorbed from the ligands and eluted in the purified form using appropriate buffers [140].

2. Gas Chromatography

The GC (or GLC) analysis of lipids has been much studied in the literature. This method involves partitioning of the components of the lipid mixture in the vapor state between a mobile gas phase and a stationary nonvolatile liquid phase dispersed on an inert support.

Analysis of fatty acid composition by GC usually requires derivatization of fatty acids to increase their volatility. Fatty acid methyl esters (FAMEs) may be prepared by different transmethylation techniques and then separated on GC columns and detected by flame ionization detection (FID). The gas phase for GC is usually nitrogen or helium for packed columns and helium or hydrogen for capillary columns. The identification of chromatographic peaks is based on the comparison of their retention times with those of the authentic samples. GC analysis of TAG of food lipids may also provide information about positional distribution of fatty acids in the molecules. Naturally occurring TAGs that are purified by TLC can then be resolved without derivatization on the basis of their carbon number or molecular weight using capillary GC equipped with 8–15 m long columns coated with methylphenyl-, methyl-, or dimethylsilicone (nonpolar capillary). The use of helium or

hydrogen as a carrier gas for the separation of TAG on such columns requires higher temperatures than those employed for the separation of methyl esters. Mono- and diacylglycerols have to be converted to trimethylsilyl (TMS) or *tert*-butyldimethylsilyl (TBDMS) ethers for complete resolution [141,142]. A combined GC and mass spectrometric technique has been applied for determining molecular species in the glycerol esters. TMS or TBDMS derivatives of glycerol esters separated on GC may be subjected to mass spectrometric analysis in order to obtain information on their molecular structure [143]. Identification of cholesterol oxidation products has great clinical importance. Recently, several authors have described GC separation combined with mass spectroscopic identification of cholesterol oxidation products [144,145].

3. High-Performance Liquid Chromatography

HPLC is a highly efficient form of adsorption, partition, or ion-exchange LC that uses a very uniform, finely divided, microspherical (5–10 mm) support of controlled porosity and degree of hydration. The adsorbent is tightly packed into a stainless steel column (10–30 cm long, 2–4 mm diameter) and requires a high-pressure pump to obtain an adequate and constant flow of solvent through the column. Elution of the column may be carried out either isocratically with a solvent mixture of constant composition or by gradient elution in which the solvent composition may be varied linearly or in a stepwise fashion with both binary and ternary solvent systems. The column eluates are continuously monitored by means of a flow-through detector, which should be insensitive to solvent flow rate, temperature, and composition [131].

Sample derivatization is employed to facilitate the separation and to enhance the limit of detection for the HPLC analysis. Hydrolysis or saponification is done to cleave ester linkages and to obtain FFAs for their subsequent analysis. Although lipids do not possess specific ultraviolet (UV) absorption peaks, they could be detected in the region of 203–210 nm because of the presence of double bonds in the fatty acyl groups, or the functionalities such as carbonyl, carboxyl, phosphate, amino, or quaternary ammonium groups. However, this low UV range greatly restricts the choice of solvent, and it is advisable to avoid chloroform–methanol mixtures because they display a strong absorption below 245 nm. Frequently, diacylglycerols require preparation of UV-absorbing derivatives (e.g., benzoate, dinitrobenzoates, pentafluorobenzoates, and TBDMS ethers) for detection. Fatty acids can be analyzed by forming 9-anthryl-diazomethane (ADAM) derivatives and employing a fluorescence detector. Fatty acids increase their hydrophobicity when ADAM is bound to the carboxyl group; thus, the derivatives are retained longer on a reversed-phase column than fatty acids and good separation is obtained [146]. Fluorescence detectors are specific for detection and quantification of tocopherols and fluorescent derivatives of fatty acids. Evaporative light-scattering detector (ELSD) and FID have been in wide use for detection of all types of lipids following HPLC separation [141,147]. These are sometimes referred to as universal detectors. The principle of ELSD involves evaporation of mobile phase of the separated lipid fraction by a nebulizer (spray the eluent stream with a large volume of nitrogen or air) to obtain droplets of solute (lipids). These solute droplets are directed through a light source (may be a laser light source); the degree of scattering of the light is proportional to the mass of the solute [148]. The RI detection may also be used for lipid analysis [149]. Recently, Jones et al. [150] determined the lipid content of algae using normal-phase HPLC coupled in parallel to an evaporative light-scattering detector (ELSD) and quadrupole mass spectrometer.

Normal-phase HPLC also allows the separation of normal-chain and hydroxy fatty acid–containing TAG. Normal-phase HPLC on silver-ion-loaded anion-exchange columns is currently used to resolve TAG based on their degree of unsaturation [141]. Reversed-phase HPLC (using C18 columns) is also widely applied for separation and quantification of tri-, di-, and monoacylglycerols. Sehat et al. [151] have described that silver-ion HPLC can be employed to separate and identify conjugated linoleic acid isomers.

Since glycerol possesses a prochiral carbon, asymmetrical esterification of the primary position leads to the formation of enantiomers. Although enantiomeric TAGs cannot be resolved by normal HPLC, their diastereomeric naphthylethylurethane derivatives can be separated by HPLC on silica

gel [152,153]. The use of a stationary phase with chiral moieties to separate enantiomers of mono- and diacyl-*sn*-glycerols after derivatization with 3,5-dinitrophenylurethane by HPLC separation of enantiomers has been reported [154–156].

Separation of PLs is very laborious when TLC is used; however, HPLC provides a better means of separation and quantification. At present, the use of gradient (binary or tertiary) elution on silica columns is frequently used for the separation of different classes of PLs. Several polar solvent systems that are suitable for such separations are available [157,158]. UV detection, FID, or ELSD is suitable for PL identification and quantification using silica or normal-phase chromatography [157–159]. Separation of glycolipids can be achieved using a silica column with a binary gradient (hexane–IPA–2.8 mM ammonium acetate) [160] or reversed-phase C18 (ODS) column [148].

Isocratic, normal-phase separation of cholesterol esters, FFAs, and free sterols is widely used. Simultaneous analysis of nonpolar and polar lipids using HPLC silica gel column has also been reported. In addition to normal- and reversed-phase methods, size exclusion HPLC has been used to separate TAG and other nonpolar lipids. This has been specifically employed for the analysis of polymerized lipids such as those generated during deep fat frying [100]. Christopoulou and Perkins [161] have described separation of monomers, dimers, and trimers of fatty acids in oxidized lipids using size exclusion column with an RI detector, whereas Burkov and Henderson [162] have reported the use of a similar column with ELSD to analyze polymers in autoxidized marine oils.

Micro-HPLC columns have the volume of one-hundredth of that of a conventional column and benefit from low consumption of material used as stationary phase, sample and mobile phase, operation at low flow rate, and temperature programming. It can also be easily coupled with a mass spectrometer and an FTIR detector [146].

4. Supercritical Fluid Chromatography

The use of SFC for lipid extraction was discussed in a previous section. When CO_2 is compressed at a temperature and pressure above its critical point, it does not liquify but forms a dense gas; thus, as a mobile-phase SFC is gaseous and solvating. Such a dense gas has a number of properties (e.g., relatively high densities and diffusivities) that makes it attractive for use as a mobile phase for LC. SFC with open tubular columns acts as a substitute for GC, but with the analysis temperature much lower than that employed in GC.

The temperature and pressure required for SC-CO_2 are much lower than that for HPLC. As CO_2 is nonpolar, its SFC can dissolve less polar compounds and is suitable for the analysis of less polar species. To analyze polar components, polar solvents such as methanol or ethanol may be added to the SFC to cover active sites (–Si–OH) on the surface of the supporting material and to increase the dissolving power of the mobile phase. Both packed and capillary columns are used for SFC. Packing materials developed for HPLC are suitable for SFC-packed columns [163]. Similarly, fused silica capillary tubes used for GC are suitable for SFC, and stationary phases may employ dimethylpolysiloxane, methylphenylpolysiloxane, diphenylpolysiloxane, and cyanopropylpolysiloxane [164].

Capillary SFC with FID or UV has been used for the analysis of TAG, FFAs, and their derivatives [165–167]. SFC argentation chromatography has been used for the separation of TAG according to the number of double bonds, chain length, and nature of the double bonds [168,169]. It is necessary to add acetonitrile as a polar modifier to CO_2 to facilitate elution of TAGs. For improved analytical efficiency of lipid having a narrow range of unsaturation, mobile-phase gradient (e.g., temperature, pressure and density, velocity) can be employed. Detectors for ELSD, FID, UV, MS, and FTIR developed for GC and HPLC are applicable to SFC. Combination of SFC with SFE has been successful for analyzing lipids of different food samples. SFE may replace any conventional lipid extraction method, and the quantification of extracted lipids (instead of gravimetric analysis) can be performed with a detector (ELSD) that has been directly connected to the extraction cell. SFE-SFC has also been used to characterize TAG patterns of seed oils [163,170]. SFC is a viable alternative for reducing any solvent use in lipid extraction and analysis and has a great potential for further development.

5. Thin-Layer Chromatography

TLC is one of the main analytical tools used for lipid analysis. TLC can be used for fractionation of complex lipid mixtures, assay of purity, identification, information on the structure, and monitoring extraction and separation of components via preparative column chromatography for routine and experimental purposes. The principles and theory of TLC are based on the difference in the affinity of a component toward a stationary and a mobile phase. The important components of TLC are the stationary phase, mobile phase, detection, and quantification [5]. The adsorbent generally used in TLC for lipid analysis is a very-fine-grade silica gel and may contain calcium sulfate as a binder to ensure adhesion to the plate [4]. Alumina and kieselguhr are also used as stationary phases. These adsorbents can also be modified by impregnation with other substances so as to achieve the desired separations. The most popular impregnations are with boric acid or silver nitrate. Silver nitrate–impregnated (argentation) TLC may be used to separate TAGs or FAMEs according to the number of double bonds and also by virtue of the geometry (e.g., *cis* or *trans*) and position of the double bonds in the alkyl chain. The silver ion forms a reversible complex with the p electrons of the double bond of unsaturated fatty acids, thereby decreasing their mobility [171]. Structural isomers of TAGs (due to their fatty acid constituents) may also be separated on this type of TLC plate [147]. Impregnation of the TLC plates with boric acid (3%, w/v) prevents isomerization of mono- and diacylglycerols while separating neutral lipids [131]. Boric acid complexes with vicinal hydroxyl groups and leads to slower migration of these compounds [171].

Samples of lipid extracts are applied as discrete spots or as narrow streaks, 1.5–2 cm from the bottom of the plate. The plate is then developed in a chamber containing the developing solvent or a solvent mixture. The solvent moves up the plate by capillary action, taking the various components with it at different rates, depending on their polarity and how tightly they might be held by the adsorbent. The plate is removed from the developing chamber when the solvent approaches the top of it and then dried in the air or under a flow of nitrogen. Solvents with low boiling point, viscosity, and toxicity are suitable for TLC application. A low boiling point helps in the quick evaporation of the solvent from the surface layer, and low viscosity facilitates faster movement of the solvent during development. The selection of a suitable solvent is very important for good separation of the lipid classes. Several solvent systems may be used to resolve individual lipid classes, as exemplified in Table 5.3.

TABLE 5.3
Solvent Systems That Could Be Used for Separation of Lipids by TLC

Lipid Component	TLC Adsorbent	Solvent System	References
Complex lipids (animal tissues)	Silica gel G	Chloroform–methanol–water, 25:10:1 (v/v/v)	[172]
	Silica gel H	Chloroform–methanol–acetic acid–water, 25:15:4:2 (v/v/v/v)	[172]
	Silica gel H	First developing system, pyridine–hexane, 3:1 (v/v) and second developing system, chloroform–methanol–pyridine–2 M ammonia, 35:12:65:1 (v/v/v/v)	[172]
Complex lipids (plant tissues)	Silica gel G	Acetone–acetic acid–water, 100:2:1 (v/v/v)	[173] [4]
Simple lipids	Silica gel G	Hexane–diethyl ether–formic acid, 80:20:2 (v/v/v)	[4,174]
	Silica gel G	Benzene–diethyl ether–ethyl acetate–acetic acid, 80:10:10:0.2 (v/v/v/v)	[4,174]
Partial acylglycerol	Silica gel G containing 5% (w/v) boric acid	Chloroform–acetone, 96:4 (v/v)	[4]
Neutral plasmalogens	Silica gel G	Hexane–diethyl ether, 95:5 (v/v), in the first direction; hexane–diethyl ether, 80:20 (v/v), in the second direction	[4]

The location of the corresponding lipid spots on a developed plate has to be detected before their isolation. Detection of the spots may be done using a reagent directly on the plate. This reagent could be specific to certain functional groups of the lipid molecules or may be a nonspecific reagent that renders all lipids visible. There are nondestructive chemical reagents, such as 2′,7′-dichlorofluorescein in 95% methanol (1%, w/v), iodine, rhodamine 6G, and water, which allow recovery of lipids after detection. Lipids exhibit a yellow color and in the presence of rhodamine 6G (0.01%, w/v) produce pink spots under UV light. These chemicals may also be used as a nondestructive spray for preparative TLC. When water is used, separated lipids may appear as white spots in a translucent background and can easily be distinguished. Developed plates may also be subjected to saturated iodine vapor in a chamber and this may produce brown spots because of the reaction of iodine with unsaturated bonds of the lipid molecules. However, unsaturated lipids may form artifacts with iodine, if sufficient time is allowed. The destructive methods include spraying of the plate with sulfuric acid (50%, v/v) and drying at 180°C for 1 h to make lipids visible as black deposits of carbon [4]. Potassium dichromate (5%, w/v) in 40% (v/v) sulfuric acid also works in a similar manner to sulfuric acid spray. Molybdophosphoric acid (5%, w/v) in ethanol turns lipids into blue then black when heated at 120°C for 1 h. Coomassie blue (0.03% in 20% methanol) turns lipid into blue spots on white background. Examples of specific reagents that react selectively with specific functional groups include $FeCl_3$ to detect cholesterol and cholesterol esters, ninhydrin for choline-containing PLs, and orcinol or naphthol/H_2SO_4 for glycolipids [175]. Some lipids contain chromophores and can be visualized directly under UV or visible light without staining.

Lipids detected by nondestructive methods can be recovered by scraping the bands of interest and dissolving them in an appropriate solvent. Complex lipid mixtures cannot always be separated by 1D TLC; however, 2D TLC may resolve such mixtures. The use of high-performance TLC (HP-TLC) plates with spot-focusing slits to minimize spot diffusion on the plate can be used for quantitative determination of analytes. This technique has been demonstrated by Kozutsumi and group [176] for determining indigenous DHA levels of cows' milk after converting to DHA methyl esters. HP-TLC is a fast, relatively inexpensive, and widely used method of separating complex mixtures, which is particularly useful for smaller, apolar compounds and offers some advantages over HPLC [177]. Ettre [178] reported that the most important difference between TLC and HPTLC is the different particle sizes of the stationary phases. Recently, Cieśla and Waksmundzka-Hajnos [179] reviewed the potential application of 2D TLC in the analysis of secondary plant metabolites.

The separation efficiency of TLC plates is affected by the degree of hydration of the adsorbent. Therefore, activation of the adsorbent, which depends on both the time and the temperature as well as the storage conditions of the plates and the relative humidity of the atmosphere, must be considered. The use of authentic lipid standards would allow direct comparison of the resolved lipids in an unknown mixture. HP-TLC plates have high resolving power and speed of separation. They are available commercially as precoated plates with fine (5 mm) and uniform particle size silica gel. However, the amount of sample that can be applied to such HP-TLC plates is very small. TLC is a preferred method of purification and separation of lipid classes before subjecting them to further separation of individual components.

For quantification of TLC-separated lipids, traditional scraping followed by quantitation and in situ determination are available. The separated lipid classes on silica gel can be scraped off, extracted by means of suitable solvents, and quantitated gravimetrically, spectrophotometrically, or by GC. Determination of the phosphorus content of the eluted PLs is a classic example for spectrophotometric quantitation. GC can be employed to quantify separated neutral and PL classes by derivatizing the constituent fatty acids into their methyl esters. Densitometric methods provide an in situ quantification method for lipids. Lipids are sprayed with reagents, and their absorption or fluorescence can be measured under UV or visible light by densitometry.

The spots detected by charring can be measured by scanning photodensitometer areas of peaks, which are proportional to the amount of original lipid present. Scintillation counting is also possible after introducing a correct scintillator (e.g., mixture of 2,5-diphenyloxazole and

1,4-*bis*-2-(5-phenyloxazoyl)benzene, PPO, and POPOP in toluene) into the lipid. Scanning fluorometry allows resolution of lipids on an adsorbent containing a fluorescent dye or by spraying with such reagents.

TLC could be coupled with other methods to facilitate detection, quantitative identification, or quantitation of separated lipids. These include coupling with HPLC (HPLC/TLC), FTIR (TLC/FTIR), NMR (TLC/NMR), and Raman spectroscopy (TLC/RS) [171]. The Iatroscan (Iatron Laboratories of Japan) applies the same principles of TLC to separate lipid mixtures followed by their detection using FID. The TLC medium is silica (7.5 mm thick) sintered onto quartz rods (0.9 mm in ID, 15 cm long, called Chromarods). Chromatographic resolution of lipid classes on Chromarods and the composition of solvent systems used are similar to those employed in classical TLC with modifications. Copper sulfate, silver nitrate, boric acid, or oxalic acid impregnation has been reported to produce better resolution and increased responses in the determination [180]. Parrish and Ackman [181] have shown that stepwise scanning and developing in solvent systems of increasing polarity resolve individual lipid components in neutral lipids of marine origin. FID gives a linear response that is proportional to the number of nonoxidized carbon atoms in the material entering the flame. The Chromarods are reusable up to 100–150 times and require thorough cleaning after each use. The main advantage of TLC-FID is the short analysis time, with the possibility of determining all components in a single analysis. The partial scanning and redevelopment [182–184] that can be done with TLC-FID give a unique advantage over any other lipid analysis technique. TLC-FID system has been used to analyze different types of lipids as detailed by Kaitaranta and Ke [185], Sebedio and Ackman [186], Tanaka et al. [187], Walton et al. [188], Indrasena et al. [189], Kramer et al. [190], Parrish et al. [191], and Pryzbylski and Eskin [192].

C. Spectroscopic Methods of Lipid Analysis

UV and visible spectroscopy are less frequently used but have specific applications for the identification and quantification of lipids. IR spectroscopy was the first spectroscopic method applied for the analysis of lipids. NMR and MS have been widely used for lipid structure determination; however, new applications other than these have been developed. IR, UV, and NMR are nondestructive spectroscopic methodologies.

1. UV–Visible Spectroscopy

UV and visible spectra of organic compounds are attributable to electronic excitations or transitions. Functional groups with high electron density, such as carbonyl and nitro groups with double, triple, or conjugated double bonds, absorb strongly in the UV or visible range at characteristic wavelengths (λ_{max}) and molar extinction coefficients (ε_{max}). Table 5.4 provides some of the diagnostic UV absorption bands for lipid analysis. It should be noted that the λ_{max} for a compound may vary, depending on the solvent used [131,132].

2. Infrared Absorption Spectroscopy

The IR spectrum of an oil provides substantial information on the structure and functional groups of the lipid and also about the impurities associated with it. These information are represented as peaks or shoulders of the spectrum as illustrated in Figure 5.1. At the high-frequency end of the spectrum (3700–3400 cm^{-1}), there is a region where compounds containing hydroxyl groups are absorbed, including water (H−OH) and hydroperoxides (RO−H) and their breakdown products. Some lower frequencies (3025–2850 cm^{-1}) represent the CH stretching region where three bands are visible: a weak *cis* double-bond CH absorption (CH=CH) and strong bonds due to the CH_2 groups of the aliphatic chains of TAGs and the terminal methyl groups. Just beyond this region are secondary oxidation products of lipids such as aldehydes and ketones that absorb energy albeit weakly. Toward the center of the spectrum is a very strong band due to the C=O stretching absorption due

TABLE 5.4
Ultraviolet Absorption Characteristics of Some Chromophoric Groups

Chromophore	Example	λ_{max} (nm)	ε_{max}	Solvent
–C=C–	Octene	177	12,600	Heptane
–C=C–	Octyne	178	10,000	Heptane
		196	2,100	
–C=C–C=C–	Butadiene	217	20,900	Hexane
$-(C=C)_n-$	Conjugated polyenes	217 + 30 (*n*-2)	20,000–100,000	Hexane
C_6H_6	Benzene	184	47,000	Cyclohexane
		202	7,000	
		255	230	
$-(C=C-C=C)_n-$	β-Carotene	452	139,000	Hexane
		478	12,200	
HC=O	Acetaldehyde	290	17	Hexane
C=O	Acetone	275	17	Ethanol
–COOH	Acetic to palmitic acid	208–210	32–50	Ethanol

Source: Adapted from Kates, M., *Techniques of Lipidology*, 2nd edn., Elsevier, New York, 1986.

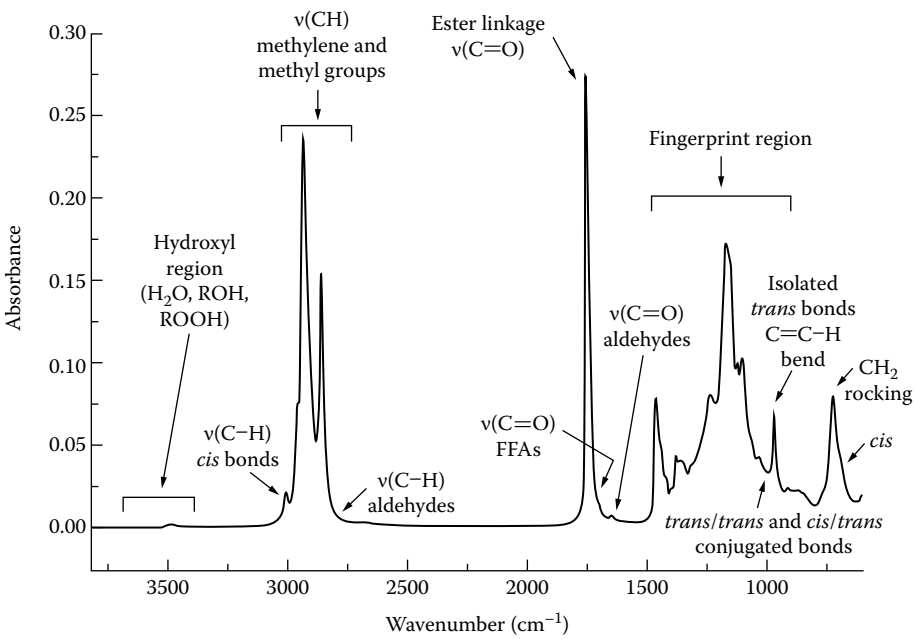

FIGURE 5.1 Mid-infrared spectrum of an edible oil collected on an attenuated total reflectance crystal. Labels indicate absorption bands or regions associated with triacylglycerols or other constituents that may be present in an oil or free fatty acid. (From van de Voort, F.R. and Sedman, J., *Inform*, 11, 614, 2000. With permission.)

to the ester linkage attaching the fatty acids to the glycerol backbone of TAGs. Next to it is a band due to COOH group of FFAs if the lipid is hydrolyzed. In the same region, there would be carbonyl absorption bands of aldehydes (R−CHO) and ketones (R−CO−R) if the oil is oxidized. Continuing to lower frequencies area is the fingerprinting region (1500–900 cm^{-1}) as the pattern of bands in this region is very characteristic of molecular composition of the lipid and could be used to identify different components. At the low-frequency end of the fingerprinting region, a band due to the CH=CH bonding absorption of isolated *trans* double bonds could be seen when *trans*-containing TAGs are present in the oil. The corresponding absorption of conjugated dienes containing *trans–trans* and *cis–trans* double bonds appears at slightly higher frequencies in oxidized polyunsaturated fatty acid (PUFA)-containing oils. Beyond the isolated *trans* bond is another group of CH absorption bending vibrations including a very strong *cis* absorption [193].

The IR absorption signal could be employed to analyze and obtain information about qualitative structural and functional groups of lipids. In principle, since IR band intensities are linearly related to the concentration of the absorbing molecular species, quantitative information about the lipid can also be obtained [193]. IR spectroscopy has been applied to solid lipids to obtain information about polymorphism, crystal structure, conformation, and chain length. In oils, IR is commonly used to determine the presence and the content of *trans* unsaturation. Single-*trans* double bonds show a characteristic absorption band at 968 cm^{-1}, and the frequency does not change for additional double bonds unless these are conjugated. There is no similar diagnostic IR absorption band for *cis* unsaturation; however, Raman spectra show strong absorption bands at 1665 ± 1 cm^{-1} (*cis*-olefin), 1670 ± 1 cm^{-1} (*trans*-olefin), and 2230 ± 1 and 2291 ± 2 cm^{-1} (acetylene) for the type of unsaturation shown [142]. Kates [131] has provided the characteristic IR absorption frequencies that have diagnostic values for the identification of major classes of lipids. It has also been reported that ionic forms of PLs influence the absorption bands associated with phosphate groups that influence the interpretation of the spectra [131]. The FTIR spectrometer finds its uses in measuring IV, saponification value, and FFAs [194]. Oxidative stability of lipids as reflected in the formation of peroxides and secondary oxidation products may also be determined by FTIR [193,195]. Recently, Dean et al. [113] used FTIR in the analysis of lipid accumulation in response to nitrogen in freshwater microalgae.

3. Nuclear Magnetic Resonance Spectroscopy

Low-resolution pulsed 1H NMR spectroscopy is employed to determine the solid fat content of lipids as well as the oil content of seeds, as discussed earlier in this chapter. High-resolution 1H NMR applied to vegetable oils gives several signals with designated chemical shifts, coupling constant, splitting pattern, and area. This information can be used to obtain structural and quantitative information about lipids. Methyl stearate (saturated fatty acid ester) may give five distinct ^{1}H NMR signals as summarized in Table 5.5. Similar signals appear in methyl oleate and linoleate, but methyl oleate also gives signals for olefinic (5.35 ppm, 2H) and allylic (2.05 ppm, 4H) hydrogen atoms, and for linoleate these are at 5.35 (4H), 2.05 (4H, C8, and C14), and 2.77 ppm (2H, C11), respectively. When a double bond gets close to the methyl group, as in α-linolenate and other ω-3 esters, the CH_3 signal is shifted to 0.98 ppm; oils containing other esters (ω-6, ω-9, and saturated) exhibit a triplet at 0.89 ppm. The area associated with these various signals can be used to obtain semiquantitative information in terms of ω-3 fatty acids (α-linolenate) and other polyenic, monoenoic (oleate), and saturated acids [142,196].

Acylglycerols show signals associated with the five hydrogen atoms on the glycerol moiety. There is a one-proton signal at 5.25 ppm (CHOCOR), which overlaps with the olefinic signals and a four-proton signal located between 4.12 and 4.28 ppm (CH_2OCOR). PLs display characteristic signals for phosphatidylcholine and phosphatidylethanolamine [142].

High-resolution ^{13}C NMR spectra are more complex than 1H spectra and provide more structural information than quantitative ones. It is also possible to locate functional groups such as hydroxy, epoxy, acetylenic, and branched chains in the molecules. The application of ^{13}C NMR to TAG allows

TABLE 5.5
Chemical Shift (ΔH) for Methyl Alkanoates Observed for ^{1}H NMR

ΔH (ppm)	Splitting	H	Group[a]
0.90	Triplet	3	CH_3
1.31	Broad	2*n*	$(CH_2)_n$
1.58	Quintet	2	$CH_2CH_2COOCH_3$
2.30	Triplet	2	$CH_2CH_2COOCH_3$
3.65	Singlet	3	$CH_2CH_2COOCH_3$

Source: Adapted from Gunstone, F.D., *Fatty Acid and Lipid Chemistry*, Blackie, London, U.K., 1996.

[a] Assigned hydrogen is designated as H.

determination of the positional distribution of fatty acids on the glycerol backbone [197,198]. The ^{13}C resonance of the carbonyl group of fatty acids in the *sn*-1 and *sn*-3 positions is well resolved from those esterified at the *sn*-2 position. Most unsaturated fatty acids in the *sn*-2 position are nondegenerative and could be easily differentiated. ^{13}C NMR has been successfully applied for the determination of positional distribution of TAG fatty acids in vegetable oils [199–201] and marine oils [202,203].

Merkley and Syvitski [204] used a high-resolution magic angle spinning (HR-MAS) NMR spectroscopy to profile and quantify metabolites of marine microalgae. They suggested that HR-MAS-NMR offers a robust and rapid screening method, which is capable of ascertaining the absolute quantity of each component with minimal sample manipulation. It has been suggested that conventional solution-state NMR spectroscopy, providing poor spectra for semisolid materials due to the anisotropic effects from restricted molecular mobility, causes line broadening [204]. However, in HR-MAS spectroscopy, samples are spun at the "magic angle" (54.7°) to the static magnetic field removing unwanted line broadening effects.

The NMR imaging is based on the manipulation of magnetic field gradients oriented at right angles to each other to provide spacial encoding of signals from an object, which are converted by FT techniques to 3D NMR images [205]. It produces 3D data by selecting 2D cross sections in all directions. Application of NMR imaging or magnetic resonance imaging (MRI) to foods has been of interest as it is a noninvasive technique that can be applied to track the dynamic changes in foods during storage, processing, packaging, and distribution.

Most magnetic resonance images of foods are based on proton resonances from either water or lipids. Simoneau et al. [206] have applied MRI to the study of fat crystallization in bulk or dispersed systems. Halloin et al. [207] described two MRI techniques, spin-echo imaging and chemical shift imagining, for the study of lipid distribution in pecan embryos. Insect- or fungus-damaged embryos gave images that were less intense than those of normal embryos, reflecting lower oil content. When MRI and NMR were employed to determine the oil content of French-style salad dressings, results were within ±2% of expected values and were in agreement with oil content determined by traditional methods [208]. Pilhofer et al. [209] have studied the use of MRI to investigate the formation and stability of oil-in-water emulsions formed with vegetable oil, milk fat, and milk fat fractions. Distribution of lean and fat in retail meat as a means of quality can be measured [210] using MRI and also to visualize oil and water concentration gradient during deep fat frying food [211].

In recent past, high-resolution ^{1}H NMR has been considered as a fast and accurate technique suitable for the analysis of edible oils and fats [212]. Siciliano et al. [213] reported that high-resolution ^{1}H NMR is complementary to chemometric analysis, which has been used as a rapid quality control and authentication tool. Several studies used this technique to identify geographical and botanical

origin of olive oil [214–216]. Siciliano et al. [213] determined the fatty acid chain profile of lipids in pork meat products during ripening using high-resolution ^{1}H NMR spectroscopy.

^{31}P NMR is also widely used in the identification of isomeric lipids, which could only detect lipids containing phosphorus [177]. Fuchs et al. [177] suggested that although this method can be exclusively used in the case of phosphorous containing compounds (or at least only after suitable derivatization), it allows the differentiation and quantitation of all PL classes within a single spectrum according to differences in their head groups. However, Schiller et al. [217] reported that differences in fatty acyl compositions could be monitored if the lengths or the degree of unsaturation of the fatty acyl residues differ significantly.

In addition, TD-NMR has become a widespread interest for its use as a convenient method to analyze lipid. In comparison to ordinary NMR, TD-NMR is faster, easier to use, and less expensive [218]. Todt et al. [219] reported that TD-NMR is based on the different relaxation times of hydrogen nuclei in different phases of the sample being analyzed.

4. Mass Spectrometry

In conventional MS, compounds in their gaseous state are ionized by bombardment with electrons (electron impact [EI]) in an ionization chamber. The resulting mass spectrum consists of a characteristic pattern of peaks representing molecular fragments with different mass-to-charge (*m/z*) ratios. Some of these peaks or patterns of peaks are structurally diagnostic. The parent ion peak that arises from the unfragmented ionized molecule has the highest *m/z* ratio but may not be always present, depending on the volatility and thermal stability of the compound. Thus, lipids containing polar groups, such as PLs, with low thermal stability and volatility and high molecular weight cannot be analyzed by conventional EI MS. Therefore, fast atom bombardment, chemical ionization, field desorption, or secondary ion MS are required for such lipid analysis [143].

MS is very useful in identifying the structural modification of chain length such as branching or the presence of rings for saturated species. In this regard, matrix-assisted laser desorption ionization and time-of-flight mass spectrometry (MALDI-TOF-MS) have several advantages. It does not require prior derivatization of sample to enhance the volatility of the lipids. The extent of fragmentation of MALDI-TOF-MS is low; thus, detection of molecular ion is possible in most cases. Schiller et al. [220] have successfully used a matrix of 2,5-dihydroxybenzoic acid to identify phosphatidylcholine and different PLs as their molecular ions (M + 1). Diacylglycerols were mainly detected as their corresponding sodium or potassium adducts but not as their protonated form. MALDI-TOF-MS can be used for direct investigation of lipid mixtures occurring, for example, in cell membranes because of its high sensitivity (up to picomolar concentrations).

MS in combination with GC and HPLC is also useful in the structural determination of the individual lipid molecules. Le Quere et al. [221] have developed an online hydrogenation method that allows selective hydrogenation of all the unsaturated species after chromatographic separation for deducing structural information such as carbon skeleton and double-bond equivalents. Le Quere [222] has reviewed this methodology and the use of GC-MS and tandem MS for the analysis of structural features of fatty acids. The introduction of delayed ion extraction (DE) to MALDI-TOF-MS has dramatically improved the resolution and accuracy of the mass spectroscopy for molecular identification. Fujiwaki et al. [223] have described DE-coupled MALDI-TOF-MS for precise identification of lysosphingolipids and gangliosides. Recently, pyrolysis-GC-MS coupled with solid-phase microextraction with a particular focus on nitrogen-containing compounds has been used to evaluate the hydrothermal liquefaction of oil from *Desmodesmus* sp. [224].

D. Enzymatic Methods

Higgins [225] has described an enzymatic method for determining the TAG content of samples. This involves reaction of the TAG with lipase in order to obtain glycerol and FFAs. The glycerol so produced is then converted to α-glycerophosphate using glycerol kinase. The α-glycerophosphate

dehydrogenase is then used to reduce nicotinamide adenine dinucleotide (NAD) to NADH. The resultant NADH is then measured by a colorimetric reaction.

Cholesterol oxidase is used for determining cholesterol concentration in blood plasma. PUFAs with *cis*-methylene groups between their double bonds (e.g., linoleic, linolenic, and arachidonic acids) can be quantitatively measured by reading the UV absorbance of conjugated diene hydroperoxides produced via lipoxygenase (lipoxidase)-catalyzed oxidation. The extinction coefficient of diene hydroperoxides at 234 nm is the same for all PUFAs. Fatty acid esters have to be saponified before such analysis. The phosphatidylcholine or lecithin content of foods (e.g., as a measure of the egg content of foods) can be made by catalyzing conversion of lecithin to phosphatidic acid and choline by lecithinase (phospholipase D) [5].

The method of stereospecific analysis of TAG described by Brockerhoff and Yurkowski [226] uses pancreatic lipase that eventually removes fatty acids from the *sn*-1 and *sn*-3 positions of the TAG. This procedure has recently been employed to determine the existing structural differences of fish oils and seal oil [227]. Phospholipase A2 is used to release fatty acids at the *sn*-2 position of the synthesized phosphatide during the analytical procedure. Lands [228] used a different approach to determine steric positions of the fatty acids. TAG is hydrolyzed with lipase, and the products are separated by TLC. The *sn*-3 hydroxyl of glycerol is phosphorylated with diacylglycerol kinase to produce 3-phosphoryl monoacylglycerol. In the following step of analysis, phospholipase A_2 is used to remove the fatty acids only from the *sn*-2 position. The fatty acids in the *sn*-1 position can be released by saponifying the resultant 1-acyl lysophosphatidic acid. The fatty acids in positions 1 and 2 can be identified by GC analysis. The composition of fatty acids in position 3 can be calculated by comparing these results with those from the total fatty acid composition determination of TAG.

E. Immunochemical Methods

Lipids are not generally very immunogenic. However, most glycolipids (except in the pure form) possess antibodies of high activity and specificity. Therefore, glycolipids to be administered to the animal are conjugated by covalent linking to a foreign protein (as a hapten) or using them as a part of the bilayer of a liposome to stimulate the production of specific antibodies [93,97]. Immunochemical methods have also been developed for the assay of PLs and TAGs [93]. The steroid hormones when conjugated with serum albumin are sufficiently immunogenic to stimulate generation of antibodies with high activity, and this allows their detection.

Immunostaining of TLC plates for detection and assay of glycoproteins is widely done. The TLC chromatogram containing separated glycolipids is treated with a radiolabeled specific antibody (usually with ^{125}I) to stain only the glycolipid antigen even in the presence of overlapping glycolipids. Detection of ^{125}I may be achieved using autoradiography, and the chromatographic mobility and antibody staining serve to identify the glycolipid [132,140,229]. To overcome low sensitivity of the immunoradiolabeled detection of glycolipids, enzyme-linked immunosorbent assay (ELISA) was developed. For ELISA, lipid is usually bound to a solid phase and the antibody is measured either by virtue of itself carrying enzyme or by using a second antibody that carries an enzyme [132,229].

V. SUMMARY

Lipids are integral components and building blocks of biological materials. To understand their constituents, chemistry, and biological functions, lipids have to be isolated and studied. Therefore, an extensive knowledge of the extraction and analysis of lipids is essential to carry out studies on lipids. This chapter provided comprehensive information on methods available for the extraction and analysis of lipids from biological materials with examples when necessary. More details of a particular topic could be obtained from the references listed.

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6 Analysis of Fatty Acid Positional Distribution in Triacylglycerols

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CONTENTS

I. INTRODUCTION

Regio- and stereospecific distributions of fatty acids (FAs) in major neutral lipids such as triacylglycerols (TAGs, Figure 6.1) affect the physical and biological properties of fats and oils. Understanding the relationship between the texture of chocolate and the crystal structures of cacao fat, which mainly contains palmitic acid–oleic acid–palmitic acid and stearic acid–oleic acid–stearic acid, is the basis of the cacao fat substitute industry. Another major example of manufactured fats is oleic acid–palmitic acid–oleic acid, which is a mimic of natural milk fat used in infant formula, has revolutionized modern motherhood and reduced the burden on mothers. In milk fat, palmitic acid is generally located at the *sn*-2 position of glycerol to remain in acylglycerol form following its digestion by lipase. This is favored to avoid saponification with calcium ions and to be an efficient energy source required for infants. Another characteristic of milk fat is that it has short-chain FAs at the *sn*-3 position [1–3] and is believed to be digestive for infants.

Regio- and stereospecific analysis of the FA distribution is, of course, essential to clarify the biosynthetic pathway of natural lipids via glycerol-3-phosphate [4,5]. Moreover, diacylglycerols (DAGs), which are digestive intermediates of TAG and phospholipids, have been shown to function as secondary messengers in intracellular signaling by activating protein kinase C [6]. Thus, the analysis of the positional distribution of FA is increasingly important in the understanding of the biochemical function of lipids and lipidomics.

II. CHEMICAL METHOD FOR REGIOSPECIFIC ANALYSIS OF TAG

The first method for the analysis of positional FA distributions in TAG was reported by Brockerhoff in 1965 [7]. In principle, target TAG was partially degraded by pancreatic lipase or by a Grignard reagent, and the resulting partial acylglycerols were fractionated by thin-layer chromatography

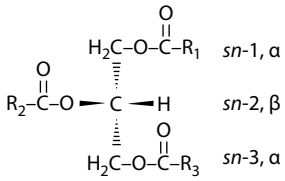

FIGURE 6.1 Fischer projection of triacyl-*sn*-glycerols.

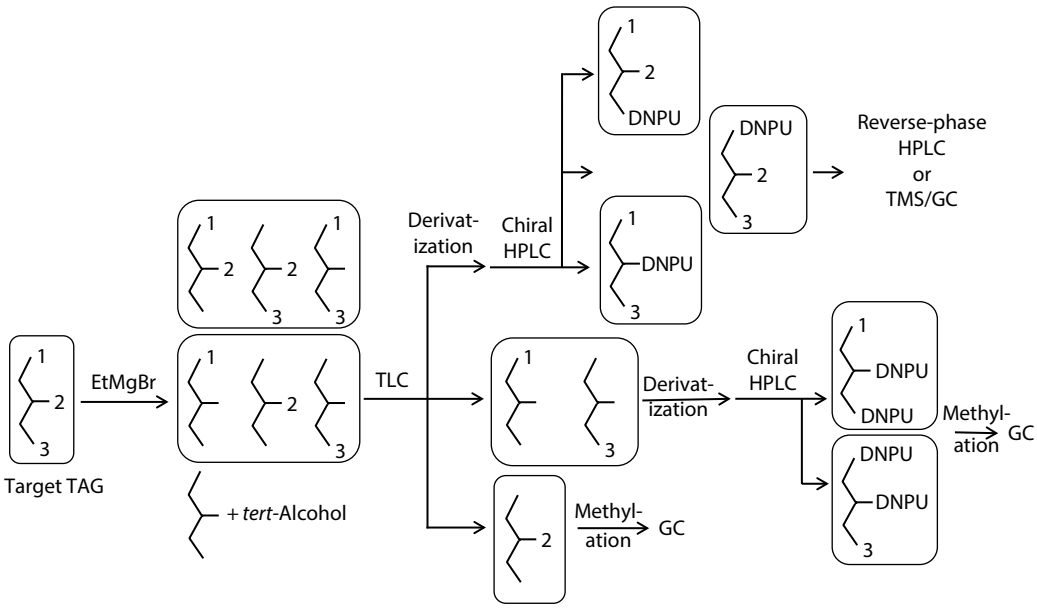

FIGURE 6.2 Overview of the regio- and stereospecific analysis of TAG.

(TLC, Figure 6.2) [7,8]. A detailed review was previously given by Breckenridge [9]. According to the original method, FA distributions are determined on the basis of the FA composition of fractionated *sn*-1(3),2-DAG (α, β-DAG) or *sn*-2 monoacylglycerol (β–MAG). Later, Becker et al. presented that allyl magnesium bromide was superior than ethyl magnesium bromide as a Grignard reagent or to pancreatic lipase in terms of the accuracy and the smaller standard deviations [10]. For the *sn*-2 position, the best estimate was obtained by the direct determination of the FA composition of 2-MAG fraction and the 1(3)-position by the formula of 1.5 × TAG − 0.5 × 2-MAG.

Further on, Turon et al. argued that the regiospecific distribution of FA in TAG was most accurately estimated when the FA composition at the α-position was determined on the basis of the α-MAG directly recovered by the Grignard degradation and at the β-position by calculation according to the formula (3 × TAG) − (2 × α-MAG) [11]. It was discussed that higher accuracy was not achieved because β-MAG was contaminated by FA that migrated from the α-position and because of the technical limitation to precisely fractionate α,β-DAG or β-MAG by TLC. The fractionation of α-MAG was the easiest and precise as it had the lowest R_f than those of other partial acylglycerol species. The protocol was as follows (Figure 6.3).

Step 1: Grignard degradation. Pure TAG (40 mg) was dissolved in 2 mL of dry diethyl ether. Ethyl MgBr solution (0.67 mL) was directly added using a positive displacement pipette and shaken for 15 s. To terminate the reaction, 0.3 mL of acetic acid and 5 mL of 0.4 M boric acid solution were consecutively added. The products were then extracted

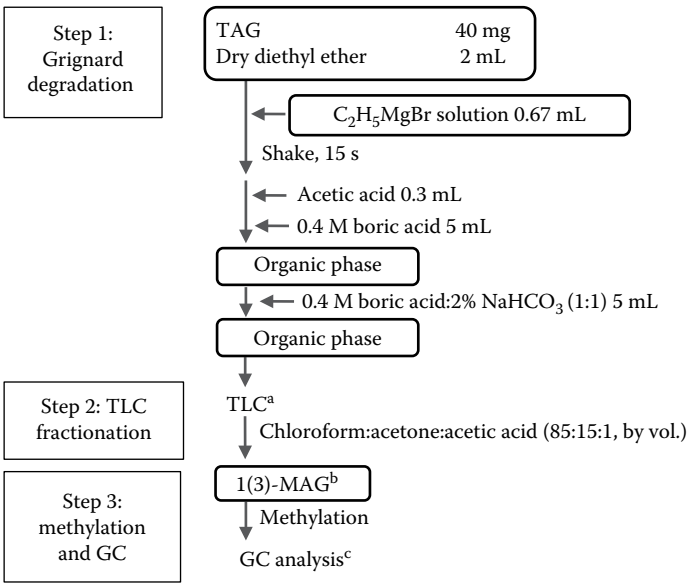

FIGURE 6.3 Regiospecific analysis of TAG based on α-MAG by a chemical method. (From Brockerhoff, H., *J. Lipid Res*., 6, 10, 1965.) *Notes:* [a]Preparative TLC on precoated silicic acid impregnated with 5% boric acid in methanol. Spots were visualized after spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol solution under UV light (366 nm). [b]$R_f = 0.26$. [c]FA composition at the β-position calculated by the formula (3 × TAG) − (2 × α-MAG).

with diethyl ether and washed with a 5 mL mixture of 0.4 M boric acid and 2% $NaHCO_3$ (1:1, by vol.).

Step 2: TLC fractionation and methylation. The resulting products were transferred to TLC to fractionate acylglycerol species, α-MAG, β-MAG, α,β-DAG, α,α′-DAG, tertiary alcohols of deacylated FA, and residual TAG. Silicic acid–precoated preparative TLC, which was impregnated with 5% boric acid solution in methanol, was performed using a mixture of chloroform:acetone:acetic acid (85:15:1, by vol.). Bands were visualized after spraying with 0.2% 2′,7′-dichlorofluorescein in ethanol solution under UV light (366 nm): α-MAG, $R_f = 0.26$; β-MAG, $R_f = 0.38$; α, β-DAG, $R_f = 0.76$; α, α′-DAG, $R_f = 0.85$; and *tert*-alcohol and TAG, $R_f = 0.95$. The required spot was scraped off from the plate and added to a solution of BF_3 in methanol. After heating at 100°C for 1 h, the resulting FA methyl esters (FAMEs) were recovered and analyzed by GC. The FA composition at the β-position is calculated according to the formula (3 × TAG) − (2 × α-MAG). The regiospecific FA distribution in tuna oil determined by the method is given in Table 6.1.

III. CHEMICAL METHOD FOR STEREOSPECIFIC ANALYSIS OF TAG

The stereospecific analysis of TAG is to determine the FA composition at *sn*-1, *sn*-2, and *sn*-3 position separately. According to the original method developed by Brockerhoff [7], TAG was partially hydrolyzed by pancreatic lipase to obtain 1,2-DAG and 2,3-DAG. They were then chemically converted to 1,2-phosphatidylphenol and 2,3-phosphatidylphenol, respectively. 1,2-DAG fraction was then hydrolyzed by phospholipase A_2 selectively at *sn*-2 position. The FA composition at *sn*-2 was determined based on the released FA, whereas that at *sn*-1 was determined based on the resulted 1-acyl-2-hydroxy-phosphatidylphenol. The FA composition at *sn*-3 was estimated by calculation by subtracting those at *sn*-1 and *sn*-2 from the total FA composition of TAG. Later, partial hydrolysis of TAG by pancreatic lipase was substituted by Grignard degradation to avoid the negative effect of FA selectivity of lipase [8].

TABLE 6.1
Regio- and Stereospecific Analysis of Tuna Oil

	FA Composition (mol%)					
	Tuna Oil[a]		Tuna Orbital Oil[b]			
FA	α-Position	β-Position	Total	*sn*-1	*sn*-2	*sn*-3
14:0	4.3 ± 0.6	6.4 ± 0.7	4.1	3.5 ± 0.3	5.8 ± 0.6	2.7 ± 0.4
15:0	1.9 ± 0.5	0.9 ± 0.4	1.1	1.3 ± 0.0	1.2 ± 0.1	0.6 ± 0.1
16:0	18.5 ± 1.8	18.5 ± 1.8	21.4	34.2 ± 0.9	18.1 ± 1.5	11.8 ± 1.1
16:1 n-7	5.7 ± 0.7	5.7 ± 0.9	6.4	8.0 ± 0.2	6.1 ± 0.3	5.0 ± 0.4
16:1 n-5			0.3	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
iso-17:0			0.4	0.4 ± 0.2	0.3 ± 0.0	0.4 ± 0.3
16:2 n-4	1.3 ± 0.2	2.8 ± 0.2	1.9	0.9 ± 0.1	3.1 ± 0.2	1.8 ± 0.1
17:0	1.2 ± 0.2	0.3 ± 0.0	1.1	2.1 ± 0.0	0.5 ± 0.0	0.6 ± 0.0
16:3 n-4			0.9	1.1 ± 0.1	0.6 ± 0.0	0.9 ± 0.1
18:0	7.3 ± 0.1	0.1 ± 0.0	4.4	9.5 ± 0.2	1.2 ± 0.2	2.7 ± 0.1
18:1 n-9	20.2 ± 0.4	5.9 ± 0.4	15.0	19.2 ± 0.5	7.3 ± 0.3	18.8 ± 0.3
18:1 n-7	3.2 ± 0.5	1.0 ± 0.1	2.7	4.3 ± 0.1	1.5 ± 0.0	2.2 ± 0.1
18:1 n-5			0.2	0.3 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
18:2 n-6	1.6 ± 0.1	1.4 ± 0.2	2.3	2.1 ± 0.0	2.4 ± 0.1	2.5 ± 0.1
18:2 n-4			0.6	0.1 ± 0.2	1.5 ± 0.3	0.1 ± 0.2
18:3 n-6	0.5 ± 0.0		0.6	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
18:3 n-3	0.6 ± 0.1	0.5 ± 0.0				
18:4 n-3	0.6 ± 0.1	0.9 ± 0.1	0.8	0.4 ± 0.0	1.2 ± 0.1	0.8 ± 0.0
20:1 n-11, n-13	0.2 ± 0.1	0.3 ± 0.0	0.5	0.4 ± 0.0	0.3 ± 0.0	0.8 ± 0.0
20:1 n-9	2.1 ± 0.2	0.5 ± 0.0	1.0	1.0 ± 0.0	0.4 ± 0.0	1.6 ± 0.1
20:2 n-6	0.5 ± 0.1	0.1 ± 0.0	0.2	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0
20:3 n-6	0.1 ± 0.1	0.2 ± 0.1				
20:4 n-6	1.6 ± 0.2	2.4 ± 0.2	1.6	0.6 ± 0.0	2.0 ± 0.1	2.2 ± 0.1
20:4 n-3			0.4	0.5 ± 0.0	0.2 ± 0.0	0.5 ± 0.0
20:5 n-3	6.3 ± 0.5	8.9 ± 0.9	5.4	2.4 ± 0.1	6.0 ± 0.1	8.1 ± 0.3
22:0	0.4 ± 0.0					
22:1 n-11, n-13	0.6 ± 0.1	0.1 ± 0.0	0.6	0.4 ± 0.0	0.2 ± 0.0	1.1 ± 0.1
22:4 n-6	0.6 ± 0.2	0.3 ± 0.0				
22:5 n-6	1.3 ± 0.3	2.0 ± 0.3	1.4	0.2 ± 0.2	1.8 ± 0.1	2.2 ± 0.2
22:5 n-3	1.5 ± 0.1	1.4 ± 0.3	1.0	0.2 ± 0.2	1.3 ± 0.1	1.6 ± 0.2
22:6 n-3	13.5 ± 1.9	38.4 ± 2.5	21.4	3.9 ± 0.5	33.5 ± 1.7	26.6 ± 1.7
24:1 n-9			0.5	0.4 ± 0.1	0.3 ± 0.2	0.6 ± 0.1
Others	4.4	1.0	2.1	1.6	2.1	2.6

[a] Determined by Grignard degradation followed by fractionation of 1(3)-MAG by silicic acid and boric acid-impregnated TLC. Mean value with *SD* of five replicate analyses [11].

[b] Determined by Grignard degradation followed by fractionation of 1(3)- and 2-MAG by boric acid-impregnated TLC and then by the chiral separation of *sn*-1- and *sn*-3-MAG by HPLC as DNPU derivatives. Mean value with *SD* of triplicate analyses [15].

The recent development in HPLC technique achieved the resolution of MAG and DAG enantiomers. Itabashi et al. reported the chiral HPLC resolution of *sn*-1-MAG and *sn*-3-MAG generated by Grignard degradation, after derivatization by 3,5 dinitrophenylisocyanate [12]. Resulted dinitrophenylurethane (DNPU) derivatives of *sn*-1- and *sn*-3-MAG eluted separately on chiral HPLC, depending solely on the position of acyl groups, but not depending on the FA comprising

each isomer of MAG. Each of *sn*-1- and *sn*-3-MAG fractions was methylated and subjected to GC analysis to determine the stereospecific distribution of FA in TAG [13,14]. Furthermore, the resolution of DNPU derivatives of 1,2-DAG and 2,3-DAG was achieved by chiral HPLC [2]. The overview of the procedure is given in Figure 6.2 and the protocol is described by Ando et al. [15], as outlined as follows.

Step 1: Grignard degradation. TAG (0.1 g) mixed with trinonadecanoylglycerol (5 mg) was dissolved in 3 mL of dry diethyl ether. Following the addition of 0.33 mL of 0.3 M ethyl MgBr solution in dry diethyl ether, the solution was shaken for 1 min. Acetic acid (0.1 mL) and 3 mL of water were added to terminate the reaction. The products were extracted with diethyl ether, rinsed six to seven times with 2% $NaHCO_3$ solution, and then rinsed once with water. The solution was then dried over anhydrous Na_2SO_4.

Step 2: TLC fractionation. The resulting 1(3)- and 2-MAG were fractionated by preparative TLC impregnated with 10% boric acid using a mixture of chloroform:methanol (98:2, by vol.) containing 0.002% butylhydroxytoluene as the developing solvent. Fractionation of 1(3)-MAG and 2-MAG should be conducted prior to the 3,5-DNPU derivatization step to avoid contamination of DNPU derivatives of 2-docosahexaenoylglycerol by DNPU derivatives of 1(3)-MAG during TLC fractionation.

Step 3: Derivatization and chiral HPLC of 1(3)-MAG. Approximately half of the 1(3)-MAG was dissolved in 5 mL of dry toluene, followed by the addition of 15 mg of 3,5 dinitrophenylisocyanate and 0.05 mL dry pyridine, and reacted overnight at room temperature. The resulting *bis*-DNPU derivatives of 1(3)-MAG were purified by preparative TLC using chloroform:acetone (96:4, by vol.) for development. The resolution of 1- and 3-MAG derivatives was improved by HPLC with two connected consecutive columns of Sumichiral OA-4100 (25 cm × 4 mm, 5 μm particles, Sumitomo Chemical Co., Osaka, Japan) using hexane:1,2-dichloroethane:ethanol (40:12:3, by vol.) as the mobile phase at a flow rate of 0.5 mL/min and temperature of −10°C [12,16]. Elution was monitored by a UV detector at 254 nm.

Step 4: Methylation and GC. Each of the fractionated isomers was methylated in a mixture of 1,2-dichloroethane (0.6 mL), methyl acetate (0.025 mL), and 1 M $NaOCH_3$ in methanol (0.025 mL) at room temperature overnight. After the addition of acetic acid (6 μL) and removal of organic solvents, FAME was mixed with hexane and analyzed with GC the FA composition.

Step 5: Derivatization and chiral HPLC of DAG [17,18]. Several milligrams of the DAG obtained in Step 2 was dissolved with dry toluene (1 mL) and added to 3,5 dinitrophenylisocyanate (twofold to threefold to the theoretical amount) and dry pyridine (0.03 mL). The mixture was left for several hours at room temperature. The resulting DAG derivatives were purified by TLC on silicic acid plates containing a fluorescence indicator (F_{254}, 20 × 20 cm) using a mixture of hexane:dichloromethane:ethanol (40:10:3, by vol.), which were also used as the mobile phase for chiral HPLC. The resolution of the enantiomers of the DAG derivatives *sn*-1,2-, *sn*-2,3-, and *sn*-1,3-DAG was achieved by chiral phase HPLC using a YMC-Pack A-K03 column (YMC Inc., Kyoto, Japan) and hexane:dichloromethane:ethanol (40:10:1, by vol.) as the mobile phase. Elution was monitored by UV at 254 nm.

Step 6: Resolution of molecular species by reversed-phase HPLC. The resulting enantiomerically pure *sn*-1,2-DAG or *sn*-2,3-DAG was collected by chiral HPLC and transferred to reversed-phase HPLC connected to a YMC C60 (250 × 4.6 mm) column using acetonitrile:isopropanol (7:3, by vol.) as the mobile phase. Elution was monitored by a UV detector at 254 nm to identify the molecular species. Elution was dependent on the carbon number and the number of unsaturated bonds in the molecule.

TABLE 6.2
Composition of Enantiomeric *sn*-1,2- and *sn*-2,3-DAG Prepared by Partial Grignard Degradation from Perilla Oil TAG

	Original[b]	Enantiomer (mol%)[c]	
Molecular Species[a]	***sn*-1,2(2,3)**	***sn*-1,2**	***sn*-2,3**
18:3–18:3	37.51	29.66	43.83
18:2–18:3	14.12	12.54	16.32
18:2–18:2	4.24	4.11	4.17
18:1–18:3	17.2	15.12	19.68
18:1–18:2	4.91	5.38	4.78
16:0–18:3	7.06	11.28	2.58
16:0–18:2	2.44	3.33	1.03
18:1–18:1	6.58	8.94	4.4
18:0–18:3	1.90	2.71	0.78
16:0–18:1	2.72	4.44	1.57
18:0–18:2	0.51	0.69	0.28
18:1–18:1	0.81	1.12	0.58

Source: Itabashi, Y., *J. Jpn. Oil Chem. Soc*., 47, 971, 1998.

[a] FA combination was distinguished, but not *sn*-positions of FA.

[b] Mixture of *sn*-1,2 and 2,3-DAG prepared by partial Grignard degradation.

[c] Resulted DAGs were derivatized by DNPU derivatization, followed by chiral phase HPLC with YMC C60 (25 cm × 4.6 mm) column and a mixture of acetonitrile/isopropanol (7:3, by vol.), at the flow rate of 0.5 mL/min. Elution was monitored by UV at 254 nm.

Recently, enantiomeric DAG was resolved without derivatization using a Chiralpak IA column (250 × 4.6 mm ID, 5 μm particles, Daicel, Tokyo, Japan) and a mixture of CH_3CN:MeOH (90:10, by vol.) at a flow rate of 0.5 mL/min at 15°C [19]. The elution was monitored by an evaporative light scattering detector. The composition of enantiomeric *sn*-1,2- and *sn*-2,3 DAG analyzed by the method is shown in Table 6.2 [20].

IV. ENZYMATIC METHOD FOR REGIOSPECIFIC ANALYSIS OF TAG

A. *sn*-1(3)-Selective Hydrolysis by Pancreatic or Microbial Lipase

Porcine pancreas lipase was the first to be applied to the enzymatic analysis of the FA distribution of TAG [21,22]. It preferentially hydrolyzes FA at the *sn*-1(3) position in TAG, excluding short-chain FA and polyunsaturated FA (PUFA). An overview of the experimental protocol is schematically described in Figure 6.4 and outlined on the basis of the study of Luddy et al. as follows [21]:

Step 1: Enzymatic hydrolysis. To maximize the yield of MAG and achieve 50% ± 5% hydrolysis, 50 mg of the target TAG, porcine pancreatin in 1 mL of 1 M trishydroxymethylaminomethane (Tris)-buffer (pH 8.0), 0.1 mL of 22% $CaCl_2$ solution, and 0.25 mL of 0.1% bile salts solution were incubated at 40°C for 1.5 ± 0.5 min. The reaction was stopped by adding 0.5 mL of 6 M HCl solution. The reaction scale could be reduced to 1/10.

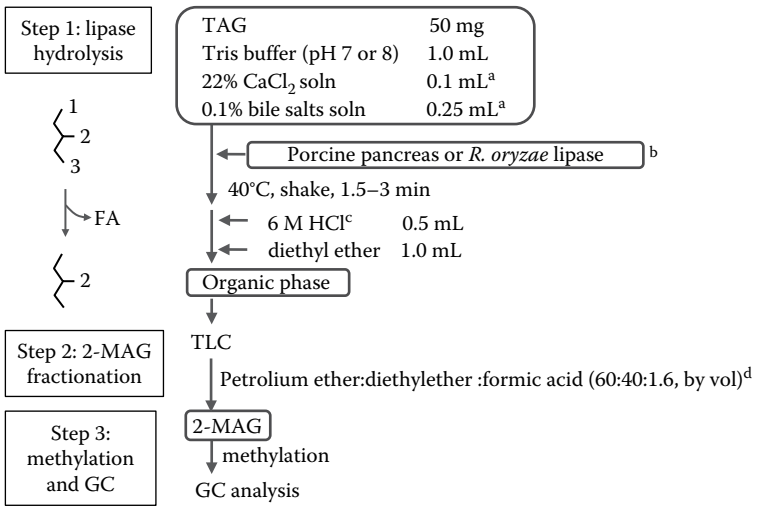

FIGURE 6.4 Regiospecific analysis of TAG without short-chain FAs and PUFAs by enzymatic hydrolysis. *Notes:* [a]Not required for *R. oryzae* lipase hydrolysis. [b]Required amount of pancreas lipase to achieve a 50% degree of hydrolysis in 1.5 min. Required amount of *R. oryzae* lipase to achieve a 30% degree of hydrolysis in 3 min. Reaction scale was doubled for *R. oryzae* lipase. [c]2 M HCl for *R. oryzae* lipase. [d]Petroleum ether:diethyl ether (1:1) could be used.

Step 2: Fractionation of MAG and analysis of FA composition. Acylglycerols and FFA were extracted with diethyl ether. The resulting MAG was fractionated by TLC using a mixture of petroleum ether:diethyl ether (60:40, by vol.) with 1.6% formic acid as the developing solvent. The product was methylated and the FA composition was analyzed by GC.

Similarly, microbial lipases with a preference for *sn*-1(3), such as that from *Rhizopus oryzae* lipase, can be applied. The protocol presented by Kosugi et al. was as follows [23]:

Step 1: Enzymatic hydrolysis. A target TAG of 50 mg, 0.1 mL hexane, 2 mL of 1 M Tris buffer (pH 7.0), and 0.5 mL solution of >12,000 U *R. oryzae* lipase were added to the preincubated solution and vigorously mixed at 40°C for 3 min. The reaction was terminated by adding 1 mL of a 2 M HCl solution. Here, the degree of hydrolysis was reported to be 30% ± 5%, and 10% ± 4% of 2-MAG had accumulated in the reaction mixture. The yield of the acyl migration products 1-MAG and 1,2-DAG was 0.1%–0.8%.

Step 2: Fractionation of MAG and analysis of FA composition. Lipids were extracted with 2 mL of diethyl ether from the reaction mixture and subjected to TLC to fractionate the resulting 2-MAG using a mixture of petroleum ether:diethyl ether:acetic acid (50:50:1, by vol.) as the developing solvent. The lipid components were visualized on a TLC plate after spraying with 0.5% 2′,7′-dichlorofluorescein in ethanol solution. The spots of 2-MAG were scraped off, methylated with a 0.5 M sodium methoxide in methanol solution, and then analyzed by GC.

The hydrolytic methods using pancreatic or microbial lipase have been widely applied to vegetable oils and animal fats. However, due to the FA selectivity of the lipases, it is not suitable for oils with short-chain FA and PUFA such as milk fat and oils of marine sources; the release of short-chain and PUFAs is slower compared to the release of C14–18 FA (Figure 6.5, [24]).

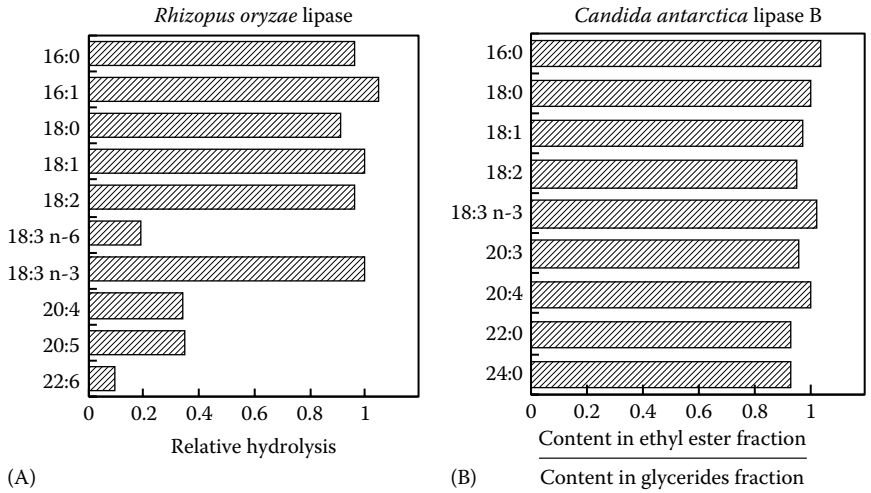

FIGURE 6.5 Fatty acid specificity of *R. oryzae* lipase (A) and *C. antarctica* lipase B (B). (A) The hydrolysis of randomly interesterified oil was conducted at 30°C for 0.5 h by 100 U *R. oryzae* lipase/g oil to reach the hydrolysis degree of 12%. (From Shimada, Y. et al., *J. Am. Oil Chem. Soc*., 74, 1465, 1997.) The hydrolysis degree of each fatty acid was expressed relative to that of oleic acid. (B) The transesterification of randomly interesterified TGA40 oil and ethanol (1:3, w/w) was conducted at 30°C for 1 h by 4 wt% immobilized *C. antarctica* lipase. (From Shimada, Y. et al., *Lipids*, 38, 1281, 2003.) The content of each FA in the fraction of FAEE was expressed relative to that in the fraction of acyl glycerols.

B. *sn*-1(3)-Selective Transesterification by *Candida antarctica* Lipase B, the Principle

Lipases generally prefer FAs located at the *sn*-1(3) position in TAGs. In contrast, lipase from *Pseudozyma* (*Candida) antarctica* (fraction B, CALB) had long been considered to exhibit little selectivity to position [24], releasing FAs at the *sn*-2 position similar to that at the *sn*-1(3) position. Actually, transesterification of TAG in the presence of low amounts of ethanol was not selective toward the *sn*-positions (Figure 6.6, ethanol amount 0.1 and 0.3); CALB produced fatty acid ethyl esters (FAEEs) but little 2-MAG. The residual acylglycerols were mainly in the form of TAG. Irimescu et al. reported that transesterification of TAG and ethanol (1:>3, by wt.) catalyzed by

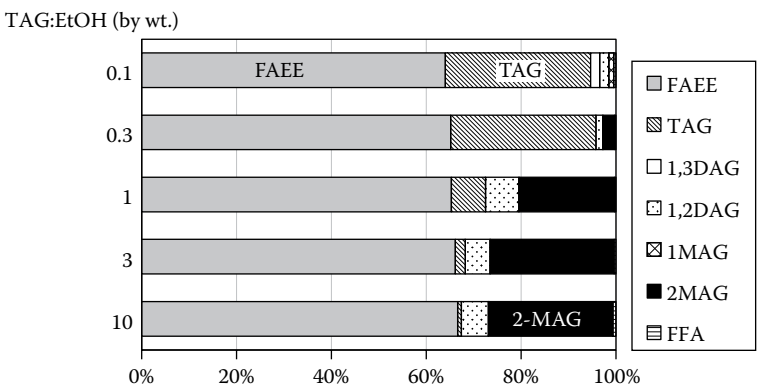

FIGURE 6.6 Effect of ethanol (EtOH) amount on regiospecificity of immobilized *C. antarctica* lipase in transesterification. A mixture consisting of 1 g tuna oil and 0.1–10 g EtOH was shaken at 30°C with 4 wt% immobilized *C. antarctica* lipase to total reaction mixture. The composition of the resulted reaction mixture was expressed in mol FA% to total FA. The reaction time required to produce *ca.* 66 mol% FAEE were 28, 24, 7, 5.5, and 3 h when EtOH amount relative to TAG was 0.1, 0.3, 1, 3, and 10 by wt., respectively.

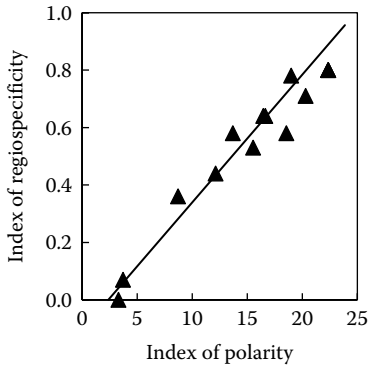

FIGURE 6.7 Effect of polarity on the regiospecificity of CALB. (From Watanabe, Y. et al., *New Biotechnol.*, 26, 23, 2009.) The index of regiospecificity (▲) was defined as the ratio of 2-MAG to the sum of residual TAG, DAG, and MAG in the reaction mixture, when 66 mol% FAEEs were produced. The higher the value, the higher the *sn*-1(3) positional selectivity. The index of the polarity was calculated based on the dielectric constant of each component and its concentration in the original reaction mixture.

CALB at 30°C was highly 1(3)-selective, producing nearly 30% 2-MAG in the reaction product without detectable levels of 1(3)-MAG [24,25]. These observations suggested that the positional selectivity of CALB was dependent on the amount of ethanol in the reaction mixture. Actually, the increase in the amount of ethanol in the transesterification reaction with TAG was confirmed to increase the positional selectivity of CALB as indicated by the ratio of the amount of 2-MAG to that of residual acylglycerols (Figure 6.7). This value is considered as an indicator of the positional selectivity; moreover, the higher the value, the greater the 1(3) positional selectivity. The positional selectivity of CALB was found to be further increased with the polarity of the reaction environment, which can be achieved by decreasing the alkyl chain length of the alcohol as well as by increasing the concentration of the alcohol for CALB-catalyzed transesterification of TAG (Figure 6.7, [24]).

The estimated mode of transesterification by CALB is depicted in Figure 6.8; TAG is converted to DAG, to MAG, and finally to glycerol, producing FAEE at each step (steps a, c, and e). The transesterification of TAG with more than 3 weight parts of ethanol to TAG is highly 1(3)-selective (Figure 6.8A), producing 1(3),2-DAG from TAG (step a), then 2-MAG (step c) from 1(3),2-DAG,

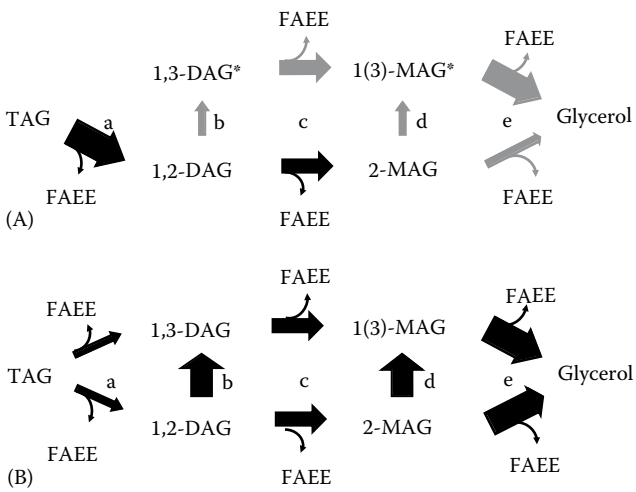

FIGURE 6.8 Estimated regioselective (A) and nonregioselective (B) pathways of TAG transesterification with ethanol by CALB.

and finally producing glycerol from 2-MAG (step e). Since the major intermediate of the reaction is 2-MAG, the order of reaction velocities of steps a, c, and e was estimated to be, from fastest to slowest, a > c > e. Namely, the selectivity of lipase toward acylglycerol species could be in the order of TAG > DAG > MAG. Furthermore, the transfer of the fatty acids from the *sn*-2 position to 1(3) (steps b and d) was estimated to be suppressed under the conditions of excess amount of ethanol, since 1-MAG and 1(3),2-DAG were hardly detected in the reaction mixture (Figure 6.6). Thus, the accumulation of 2-MAG in the reaction mixture could be well explained.

In contrast, nonregioselective transesterification with a small amount of ethanol (Figure 6.6, EtOH amount 0.1 and 0.3) could be explained as schematically shown in Figure 6.8B. Since 1,2-DAGs and 1,3-DAGs were detected as intermediates when the EtOH amount was 0.1 (Figure 6.6), CALB could directly catalyze the transesterification at the *sn*-2 position of TAG producing 1,3-DAG or the acyl transfer from the *sn*-2 position to 1(3) to produce 1,3-DAGs (step b). Since the residual acylglycerols were mainly in the form of TAG, the specificity toward acylglycerols and the velocity of each step of the transesterification are in the order: TAG < DAG < MAG in a low-polarity environment (Figure 6.8A). Moreover, the reaction velocities in the order of MAGs, DAGs, and TAGs are consistent with the observations regarding the transesterification of oil with 3.5 wt% of methanol (TAG:methanol = 1:1, mol/mol) by immobilized CALB [26].

In summary, the polarity of the environment is estimated to change two aspects of CALB properties: catalysis of the acyl transfer and the velocity toward acylglycerol species. The polar environment suppresses acyl transfer and changes the reactivity toward acylglycerol species to TAG > DAG > MAG. Thus, CALB might show strong 1(3)-selectivity to produce 2-MAG from TAG without a detectable amount of 1-MAG. It was further shown that CALB had a preference for the *sn*-3 position over *sn*-1 [27].

C. Analysis of FA Composition at *sn*-2 by CALB Suitable for TAG with Short-Chain and Polyunsaturated FAs

To date, CALB is known to be the least discriminatory in FA selectivity among the known lipases from microbial and other origins. The reactivity toward common FAs is given in Figure 6.5. In the reaction between TAG and ethanol, CALB releases arachidonic acid (C20:4), which is similar to major FAs such as palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1).

Based on the fact that CALB was highly selective to *sn*-1(3)-position during the transesterification reaction and that it is least discriminatory toward FA species, CALB was applicable to the regiospecific analysis of FA in TAG with short-chain FAs and PUFAs. This was performed by isolating the resulting 2-MAG from the transesterification reaction by analyzing its FA composition following the protocol as follows (Figure 6.9, [28,29]). The regiodistributions of FA in sardine oil and in milk fat determined by the method are given in Tables 6.3 and 6.4.

Step 1: Enzymatic transesterification producing 2-MAG. The target TAG (0.5 g), ethanol (5.0 g), and 2200 PLU of immobilized CALB lipase (Novozym 435 0.22 g, Novozymes; Chirazyme L2 C2 0.33 g, Roche Diagnostics) were placed in a screw-capped vial. The vial should be placed on its side and vertically shaken at 30°C for 3 h. For fats with melting points of approximately 40°C, such as milk fat, the vial containing the target oil and ethanol should be preincubated in a 40°C water bath to liquefy the target oil before adding immobilized CALB. For fats with melting points of approximately 50°C, the vial with the target oil and ethanol should be preincubated in a >50°C water bath to liquefy the oil. After the addition of CALB, the reaction was conducted at 50°C for 10 min and then held at 30°C for 2.8 h (total, 3 h). The reaction mixture was recovered by removing immobilized CALB by filtration with absorbent cotton and then stored at −20°C until further analysis.

Step 2: 2-MAG fractionation. Prior to normal-phase chromatography, ethanol in the reaction mixture was removed by evaporation or under a gentle stream of nitrogen gas. The resulting

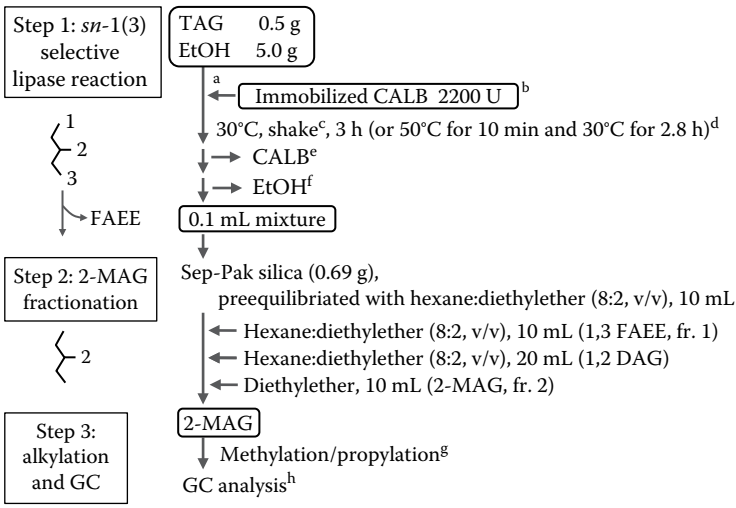

FIGURE 6.9 Regiospecific analysis of TAG including short-chain FAs and PUFAs by CALB. *Notes:* [a]Preincubate the target oil and ethanol mixture near the melting point until TAG liquefies. [b]Novozym 435 (Novozymes), 0.22 g; Chirazyme L2-C4 (Roche Diagnostics), 0.33 g. [c]Set the glass vials sideways on the shaker and shake horizontally. [d]Temperature conditions for TAG with the melting point of approximately 50°C. The reaction mixture is homogeneous after 10 min. [e]The reaction mixture can be filtered by absorbent cotton. The filtered mixture containing EtOH can be stored at −20°C until use. [f]Under vacuum or under a gentle stream of nitrogen gas. This step should be performed immediately before Step 2. [g]Propylation for TAG with short-chain FAs such as milk fat. [h]DB-23 column (J&W Scientific) is most recommended for FA including short-chain FAs. Capillary columns designed for *trans*-FAs are not recommended for FA including short-chain or PUFAs.

oil mixture (0.1 mL) was immediately charged on a Sep-Pak silica column, preequilibrated with a mixture of hexane:diethyl ether (8:2, by vol.). The first fraction, which was eluted with 10 mL of the same mixture, contains FAEE mainly derived from the *sn*-1(3)-position, with approximately 8% from the *sn*-2-position. The Sep-Pak silica column was further eluted with 20 mL of the same mixture to remove DAG. MAG was then recovered by elution with 10 mL of diethyl ether. For the elution of MAG, methanol was found not to be suitable, exhibiting larger variations in the results as determined in a collaborative study evaluating the experimental protocol.

Step 3: Derivatization and GC analysis. After methylation of the MAG fraction and original TAG, the FA composition at the 2-position was determined by GC analysis. The FA composition at the β-position was calculated on a molar basis according to the formula (3 × TAG) − (2 × α-MAG). The mole percentage of each FA was obtained by correcting percent area obtained by GC analysis on the basis of a theoretical flame ionization detector (FID) response factor, (active carbon number)$^{-1}$ × (atomic weight of carbon)$^{-1}$, where the active carbon number is one smaller number than the carbon number of FA alkyl esters.

Propylation or butylation is suitable for TAG containing short-chain FA to avoid loss of hydrophilic short-chain FA during the experimental procedure. The stationary phase of the GC capillary column was also found to affect the FID response of short-chain FA; the theoretical FID response factor was well fitted with the actual FID responses of C4:0–C12:0 FAs when FAs were analyzed as propyl esters (PEs) by a DB-23 column; however, they were not well fitted when analyzed by wax-type columns. Capillary columns designed for *trans*-FA analysis were not suitable for PEs or butyl ester analysis due to the overlap of propanol and butanol with short-chain FA. Columns for *trans*-FA were not suitable for PUFA analysis due to the lower or higher response against PUFA.

TABLE 6.3
Positional Distribution of FA in Sardine Oil Determined by CALB Method

	Composition (mol%)[a]		
FA	**TAG**	***sn*-2[b]**	***sn*-1(3)[c]**
12:0	0.1	0.2	0.1
14:0	7.3	12.0	5.0
14:1	0.3	0.4	0.2
15:0	0.6	0.8	0.5
16:0	17.6	20.5	16.2
16:1	9.4	11.4	8.4
16:2+17:0[d]	1.9	2.3	1.6
16:3	1.5	1.8	1.3
17:1	0.2	0.2	0.2
16:4	2.2	2.7	1.9
18:0	3.6	0.5	5.1
18:1 n-9	8.8	3.9	11.3
18:1 n-7	3.5	1.0	4.8
18:2 n-6	1.3	0.9	1.6
18:3 n-3	0.6	0.6	0.6
18:4 n-3	3.0	2.8	3.1
20:0	0.3	nd[e]	0.4
20:1	1.1	0.3	1.5
20:2 n-6	0.2	nd[e]	0.3
20:3 n-6	0.2	nd[e]	0.3
20:4 n-6	1.1	0.7	1.3
20:4 n-3	0.8	0.4	1.0
20:5 n-3	17.4	9.6	21.3
22:0	0.6	nd[e]	0.9
22:1	0.6	0.4	0.7
22:5 n-6	0.2	0.4	0.2
22:5 n-3	1.9	3.4	1.1
22:6 n-3	9.7	20.4	4.3
Others	3.9	2.3	4.7

[a] Mean value of area% in duplicate GC analyses was corrected to mol% by response factor, (active carbon number)$^{-1}$ × (MW of atomic carbon, 12.01)$^{-1}$.

[b] Determined by CALB method.

[c] *sn*-1(3) = (3 × TAG − *sn*-2)/2 in mol.

[d] The active carbon number was set at 16.5.

[e] Not detected.

Of the columns investigated so far, DB-23 column was most suitable for FA analysis enabling the determination of short-chain FAs and PUFAs.

Meanwhile, the positional distribution of *trans*-fats in ruminant fat and partially hydrogenated oil was successfully analyzed by the CALB method. 2-MAG was prepared by CALB and its FA composition was analyzed as methyl esters by GC connected to the capillary columns designed for *trans*-FA (Figures 6.10 and 6.11, [30]). *Trans*-9-C18:1, which is a major *trans*-C18:1 positional

TABLE 6.4
Positional Distribution of Major FA in Milk Fat Determined by CALB Method in the Collaborative Study of 10 Labs

	FA (Area% of Propyl Esters)										
Sample	**4:0**	**6:0**	**8:0**	**10:0**	**12:0**	**14:0**	**16:0**	**16:1**	**18:0**	**18:1**	**18:2**
Milk fat											
Mean value[a]	3	2	1.3	2.9	4.5	11.2	33.1	1.6	8.8	21.8	2.4
Repeatability standard deviation[b]	0.07	0	0.03	0.05	0.06	0.13	0.18	0.04	0.13	0.14	0.06
Reproducibility standard deviation[c]	0.33	0.18	0.19	0.37	0.47	0.54	0.65	0.05	0.65	1.11	0.16
sn-2 fraction of milk fat											
Mean value[a]	0.3	0.4	1	2.5	5.5	17.3	35	2.2	4	17.7	3
Repeatability standard deviation[b]	0.04	0.05	0.03	0.08	0.04	0.59	0.43	0.06	0.23	0.73	0.15
Reproducibility standard deviation[c]	0.17	0.11	0.19	0.36	0.36	0.61	2.27	0.13	0.4	2.09	0.31

Source: Yoshinaga, K. et al., *J. Oleo Sci*., 65, 291, 2016.

[a] $M = \Sigma(a + b)/2n$, where a and b are area% of duplicate analyses by each lab.

[b] $SDr = (\Sigma\ (a - b)^2/2n)^{1/2}$.

[c] $SDR = (\Sigma L^2 + Sr^2)^{1/2}$.

isomer, was selectively located at the *sn*-2 position of partially hydrogenated canola oil; however, most of *trans*-C18:1 was found at the *sn*-1(3)-position of the TAG of ruminant fat.

The MAG fraction obtained by the CALB method did not contain detectable amounts of phosphorus derived from the added phospholipids. Therefore, this method may be applicable for the determination of crude oil without degumming in addition to TAGs composed of C4–C22 FA and PUFA. Meanwhile, TAG rich in hydroxyl FAs such as castor oil could not be applied to the CALB method. The regiodistribution of FAs in hydroxyl FA-rich oils has been analyzed by HPLC-MS, as discussed in the following section.

V. DIRECT ANALYSIS OF REGIO- AND STEREODISTRIBUTION OF FA IN TAG: HPLC-MS, NMR, AND CHIRAL HPLC

All the methods described earlier require multiple experimental steps including partial degradation of TAG by any means, resolution of the resulting molecular species, and compositional analysis of FAs. In contrast, with some limitations, direct analysis of TAGs is possible with HPLC-MS, NMR, and chiral HPLC.

To the HPLC-MS analysis of TAG, the atmospheric pressure chemical ionization (APCI) method or the electrospray ionization method has widely been applied. In general, TAG is ionized to produce adduct species such as protonated adduct $[M + H]^+$, ammonium adduct $[M + NH_4]^+$, and sodium adduct $[M + Na]^+$. TAG further releases one acyl group at the ion source to form DAG ion $[M\text{-}RCOO]^+$. Here, the acyl groups at *sn*-1,3 positions tended to be easily released. The ratio of TAG regioisomers can be estimated based on the detected ratio of DAG isomer ions [31]. Using HPLC-MS technique, Lin et al. identified most of the molecular species comprising castor and lesquerella oils, which are rich in hydroxyl FA, and clarified the regiodistribution of FA in these oils [32–34].

Compared to HPLC analyses that require complicated operations and long analytical time for each sample, nondestructive analysis by ^{13}C NMR is quick and is a suitable alternative for analyzing

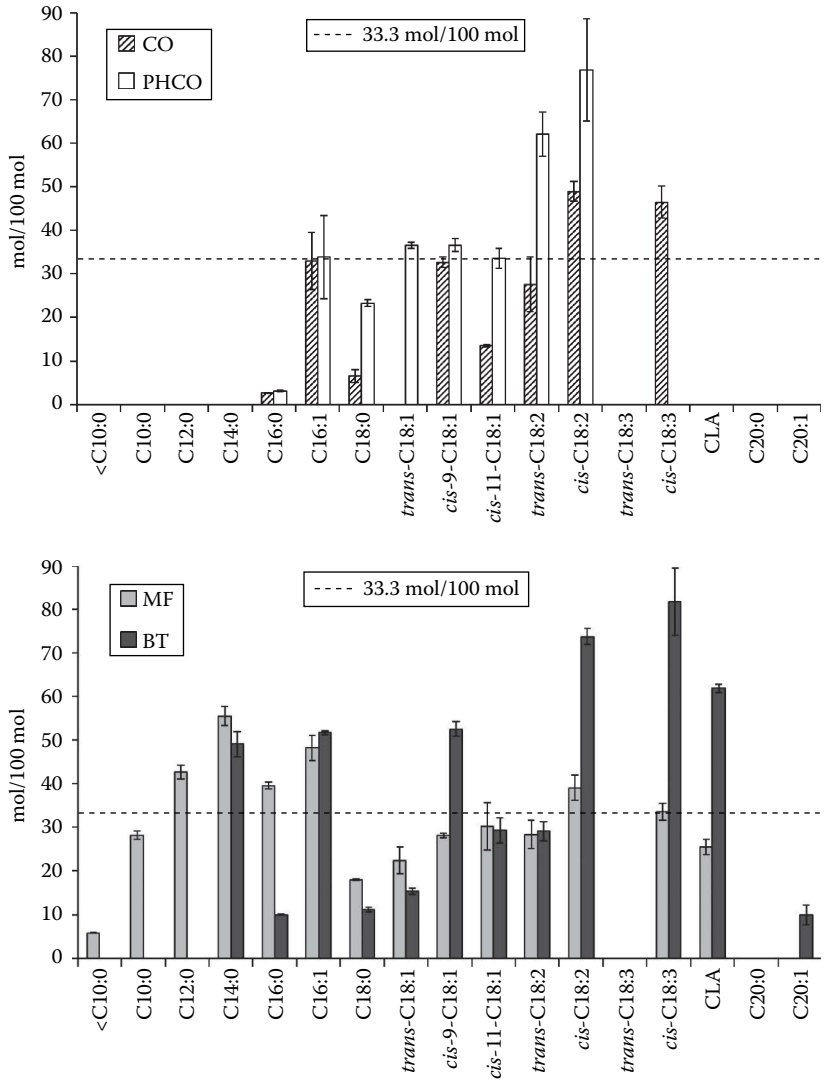

FIGURE 6.10 Regiospecific distribution of FAs at the *sn*-2 position of TAG (mean ± SE) in canola oil (CO), partially hydrogenated canola oil (PHCO), milk fat (MF), and beef tallow (BT). (From Yoshinaga, K. et al., *J. Oleo Sci*., 64, 617, 2015.) The dotted line at 33.3 mol/100 mol indicates the regiospecific value at which the FA is equally distributed. A value higher than 33.3 mol/100 mol indicates the selective distribution at the *sn*-2 position, whereas a lower value indicates the selective distribution at the *sn*-1(3) position.

the regiodistribution of especially PUFA in TAG [35]. On the ^{13}C NMR spectra, the chemical shifts of carbonyl carbons on the glycerol backbone differ depending on the *sn*-1,3 position and *sn*-2 position. In fish oil, for example, signals appear from the high magnetic field side to the low in the following order: docosahexaenoic acid (DHA) at the *sn*-2 position, DHA at the *sn*-1,3 positions, eicosapentaenoic acid (EPA) at the *sn*-2 position, and EPA at the *sn*-1,3 positions. Based on the ratio of the integral values, distribution of DHA or EPA to *sn*-1,3 and to *sn*-2 position can be calculated. Typical spectrum of sardine oil is illustrated in Figure 6.12. The technique is well reviewed and the chemical shifts reported in the literature are compiled in [36].

Until recently, the direct resolution of TAG enantiomers had not been reported and had long remained a challenge for lipid analysts. The first report achieved the resolution of *sn*-DC8C8/*sn*-C8C8D

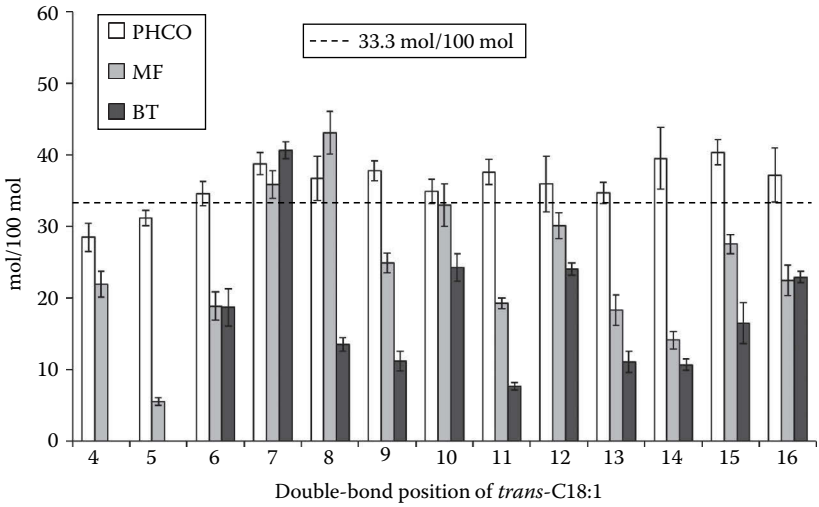

FIGURE 6.11 Regiospecific distribution of *trans*-octadecenoic acid (C18:1) positional isomers at the *sn*-2 position of TAG (mean ± SE) in PHCO, MF, and BT. (From Yoshinaga, K. et al., *J. Oleo Sci*., 64, 617, 2015.) For abbreviations and regiospecific value; see Figure 6.10.

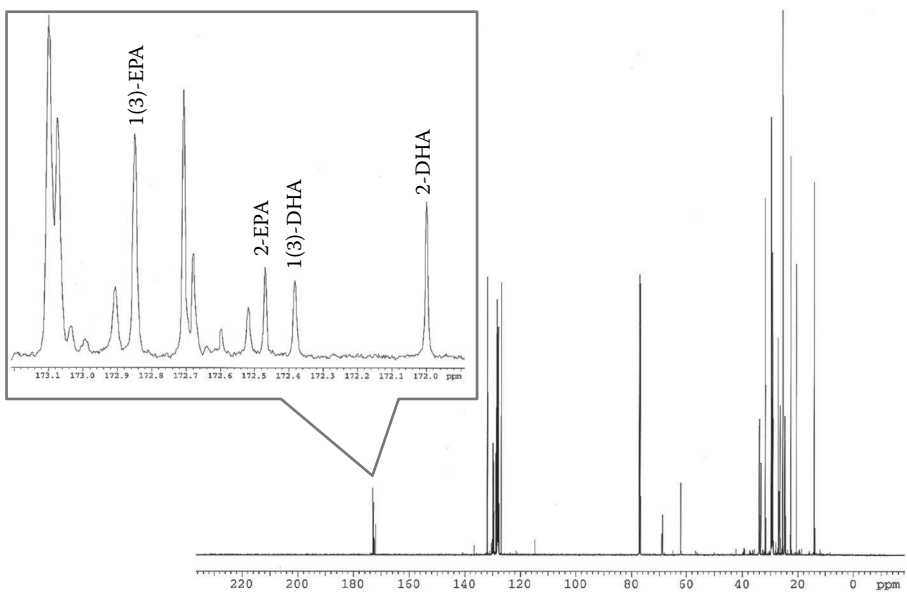

FIGURE 6.12 ^{13}C NMR spectrum of sardine oil, measured by Japan Food Research Laboratories (Tokyo, Japan) using Varian NMR System 500 (Varian, Inc., ^{13}C, 125 MHz). Acquisition time, 3.0 s; pulse degree 45°; dwell time, 6.0 s; accumulation times, 1600; temperature, 25°C.

and *sn*-EC8C8/*sn*-C8C8E by normal-phase chromatography using chiral HPLC column, where C8 means caprylic acid, E means EPA, and D means DHA [37]. The HPLC technique was further improved to achieve the direct resolution of TAG positional isomers and enantiomers in natural oils. Lísa and Holčapek achieved resolution of all TAG enantiomers containing 1–8 double bonds and different fatty acyl chain lengths by chiral HPLC/APCI-MS method, using two cellulose-tris-(3,5-dimethylphenylcarbamate) columns consecutively with a gradient of hexane-2-propanol mobile

phase [38]. By the method, the composition of TAG regioisomers and enantiomers in hazelnut oil and human plasma samples were determined. Nagai et al. achieved resolution of the positional isomers in natural oils by reversed-phase HPLC on an octacosyl (C28) column [3]. Resulted fraction was further treated by chiral HPLC equipped with recycling system to resolve enantiomers of TAG including palm oil, fish oil, and marine mammal oil. In milk fat, for example, short-chain FAs were proven to specifically locate at the *sn*-3 positions (Figure 6.13, [3]).

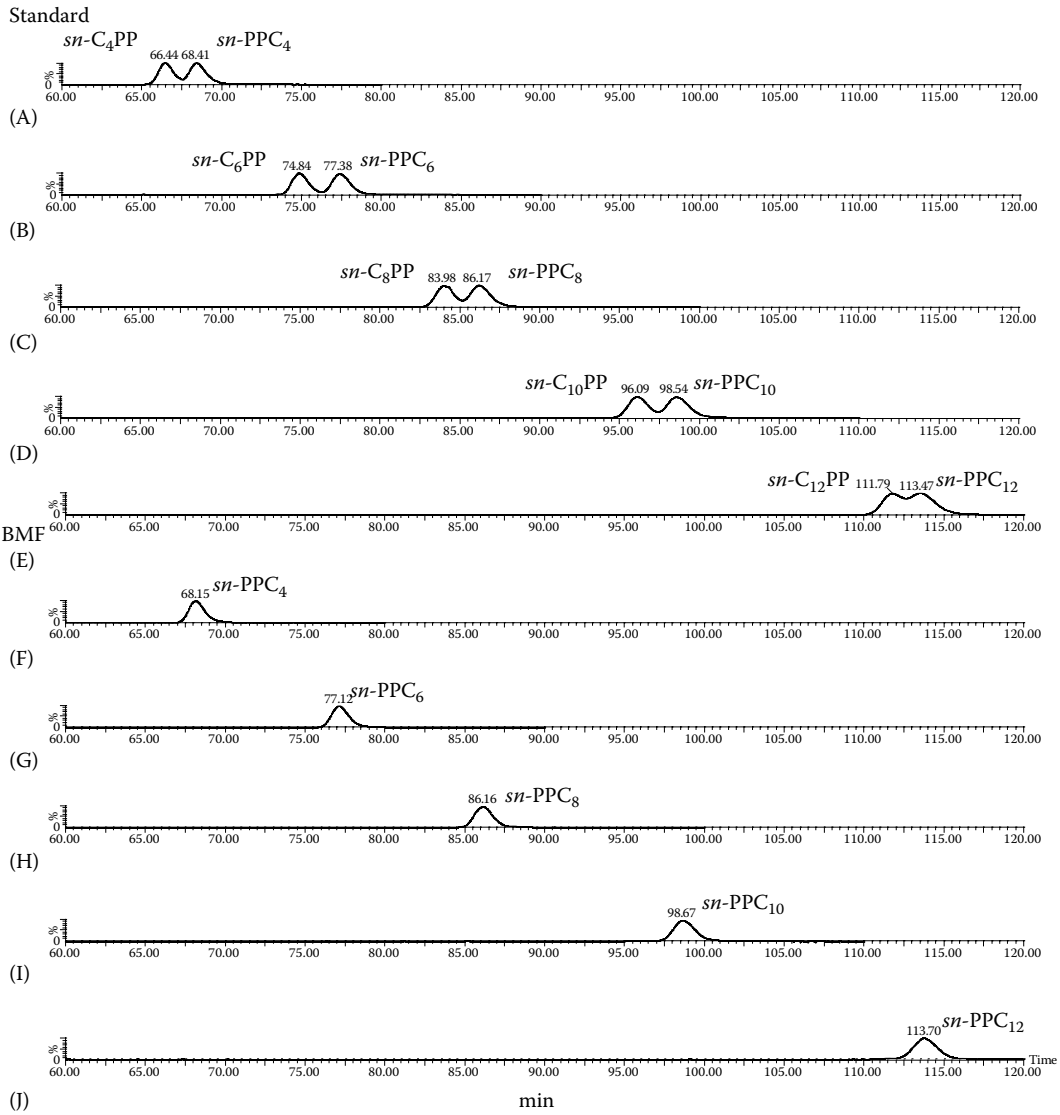

FIGURE 6.13 TAG enantiomer separation of bovine milk fat asymmetric TAGs comprising two palmitic acids and one short- or middle-chain FAs. (From Nagai, T. et al., *J. Oleo Sci*., 64, 943, 2015.) (A) *rac*-PPC_4, (B) *rac*-PPC_6, (C) *rac*-PPC_8, (D) *rac*-PPC_{10}, and (E) *rac*-PPC_{12} are selected reaction monitoring (SRM) chromatograms of the standard solutions, and (F) *sn*-PPC4, (G) *sn*-PPC_6, (H) *sn*-PPC_8, (I) *sn*-PPC_{10}, and (J) *sn*-PPC_{12} are SRM chromatograms of BMS. Column: CHIRALCEL OD-3R (150 × 4.6 mm, 3 μm) with a guard cartridge (CHIRALCEL OD-3R (100 × 4.6 mm, 3 μm), column temperature, 25°C; mobile phase, methanol; flow rate, 0.5 mL/min; the number of passages through the column by the recycle HPLC system, five passes for each TAG; ion source of MS, A APCI positive; SRM transitions, *m/z* 639 → 551 for PPC_4; *m/z* 667 → 551 for PPC_6; *m/z* 695 → 551 for PPC_8; *m/z* 723 → 551 for PPC_{10}; *m/z* 751 → 551 for PPC_{12}.

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7 Methods for *Trans* Fatty Acid Analysis

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CONTENTS

I. INTRODUCTION

Most *trans* fatty acids found in processed food products and dietary supplements come from industrially produced partially hydrogenated oils (PHOs), although *trans* fat content can also be detected in natural sources, such as dairy products. The major source of *trans* fats in the diet used to be margarines; however, the long shelf life and solid consistency of PHOs were found to be extremely useful for food manufacturers and fast food chains. As a result, processed foods, such as snacks and fast foods, became more likely to be the major sources of dietary *trans* fats [1,2]. While *trans* fat levels have been dramatically reduced since 2005, good substitutes for PHOs were difficult to identify, and therefore, PHOs continued to be used in frosting and refrigerated dough products. Nonetheless, based on the available scientific evidence and the findings of expert scientific panels,

the Food and Drug Administration (FDA) made a final determination in June 2015 that there was no longer a consensus among qualified experts that PHOs are *generally recognized as safe* (GRAS) under any condition of use in human food and therefore must be considered food additives [3]. The FDA gave time for food manufacturers until 2018 to remove PHOs from all food products. Although food companies may petition the FDA for food additive use of PHOs as an ingredient, no PHO can be used in human food after June 18, 2018, unless otherwise approved by the FDA.

The nutritional properties of *trans* fatty acids have been debated for many years, particularly with respect to their effects on the amounts of low-density and high-density lipoprotein (LDL, HDL) contained in human serum. Some studies have shown that *trans* fatty acids elevate the levels of serum LDL cholesterol and lower HDL cholesterol, a pattern that has been associated with poorer cardiac outcomes [4–9]. Such results drew a great deal of attention, which eventually led to the mandatory labeling of *trans* fatty acids on food products in the United States, Canada, and many other countries [10–13]. For nutrition labeling purposes, *trans* fats are defined as the sum of all unsaturated fatty acids in a food product that contain one or more isolated, nonconjugated, double bonds with a *trans* double-bond geometric configuration. Importantly, certain conjugated fatty acids, such as conjugated linoleic acid (CLA) isomers, that have a *trans* double bond, are excluded from this definition of *tran*s fats. In the United States, label declaration of *trans* fats is not required for products that contain less than 0.5 g *trans* fat per reference amount and per labeled serving, provided that no claims are made for fat, fatty acids, or cholesterol. In Canada, in order to claim that a product is *trans* fat-free, it must contain less than 0.2 g of *trans* fat per serving and per reference amount [14]. Unlike other countries, Denmark imposed an upper limit on the amount of *trans* fats in foods [15,16], specifying that oils and fats must contain fewer than 2 g of *trans* fatty acids per 100 g of total fat, and *trans* fat–free labels are only allowed if a food product contains less than 1.0 g of *trans* fat per 100 g of that fat or oil.

While consumption of *trans* fat has been reported as a possible risk factor for coronary heart disease [17], some *trans* conjugated fatty acids in ruminant fat have been reported to have several beneficial physiological effects in experimental animals [18]. For example, *trans*-vaccenic acid (*trans*-11–18:1), which is the major *trans* fatty acid isomer present in meat and dairy products from ruminants [19,20], has been shown to be converted to *cis*-9,*trans*-11–18:2, a CLA isomer [21–23], by the action of Δ9 desaturase present in mammalian tissue [24,25]. The relationship of *trans*-18:1 fatty acid isomers and CLA isomers has been a promising and an increasingly active area of research [26–28].

Many countries now have mandatory requirements to declare the amount of *trans* fat present in food products and dietary supplements [11,29], which in turn requires validated official methods that can rapidly, sensitively, and accurately quantify total *trans* fatty acids. As the legally permissible amounts of *trans* fatty acids continue to decline, these methods have needed continual refinements. Analytical procedures used to quantify and identify fatty acids have been reviewed [12,13,30,31], and researchers continue to improve various methodologies for determining the *trans* fatty acid content of various products. This chapter discusses several of these techniques and the latest developments in analytical methodologies, namely, infrared (IR) spectroscopy, near-infrared (NIR) spectroscopy, gas chromatography (GC), silver ion (Ag) chromatography, as well as powerful hyphenated, online GC-IR and GC-mass spectrometry (MS) techniques. The analysis of *trans* fatty acid isomers is extremely challenging and complex. Various combinations of techniques have been shown effective for determining the quantity and confirming the identity of individual *trans* and *cis* isomers. However, improved methods are still needed to accurately and conveniently determine the total *trans* fat content as well as specific *trans* fatty acid isomers in foods and dietary supplements for regulatory compliance.

II. ANALYSIS USING SPECTROSCOPY

First, the traditional IR transmission methods for the determination of total *trans* fat will be presented, including methods based on novel internal reflection approaches.

A. IR Spectroscopy

IR spectroscopy is a widely used technique for determining nonconjugated *trans* unsaturation in both natural and processed fats and oils [12,31]. For increased accuracy, oil samples have traditionally been converted to methyl esters prior to analysis. This eliminates interfering absorptions associated with the carboxyl groups of free fatty acids and the glycerol backbone of triacylglycerols. IR spectroscopy is not applicable to materials having functional groups with absorption bands close to 966 cm^{-1}, which is the strong absorption band arising from the C–H deformation about a *trans* double bond. This absorption band is absent in natural vegetable oils that are composed of saturated fatty acids (SFA) and fatty acids with only *cis*-unsaturated double bonds.

1. Conventional IR Methods

The 1961 Official Method of the American Oil Chemists' Society (AOCS), Cd 14–61, for the determination of *trans* fatty acid concentrations in fats and oils was based on comparisons of the absorption at 966 cm^{-1} for standards and unknowns; the most recently approved version of this method was reapproved in 2009 [32]. Test samples and standards were diluted in carbon disulfide and placed in an absorption cell so that the transmittance or absorbance could be measured using an IR spectrophotometer. The quantitation of the *trans* concentration was based on Beer's law:

$$A = abc \tag{7.1}$$

where

A is the absorbance = log(1/transmittance)
a is the absorptivity
b is the path length
c is the *trans* concentration

The disadvantages of using this method include (1) the need to make methyl ester derivatives at *trans* levels less than 15%, (2) the use of the toxic, volatile solvent carbon disulfide, (3) the high bias found for triacylglycerols, and (4) low accuracy obtained for *trans* levels less than 5%.

To overcome some of the drawbacks of the earlier procedures, a two-component calibration curve was proposed in 1982 [33]. First, a calibration curve was developed by means of different levels of the *trans* monoene, methyl elaidate, and methyl linoleate dissolved in carbon disulfide. Then calibration standards and the test samples in carbon disulfide were scanned against a carbon disulfide background and recorded from 1500 to 900 cm^{-1}. After a baseline had been drawn as a tangent from about 935 and 1020 cm^{-1} at the peak minima, the corrected absorbance of the calibration standards *trans* peaks, at 966 cm^{-1}, could be obtained. The baselines for spectra of the test samples were obtained by overlaying the spectra of the calibration standards at the corresponding concentrations. This method compensated for the low bias of earlier methods [34,35] and eliminated the need for correction factors.

The introduction of Fourier transform IR spectroscopy (FTIR) instruments also facilitated more accurate and rapid determination of *trans* fatty acids. An innovation in 1988 used an absorption band–height ratio procedure with an attenuated total reflection (ATR) cell, which allowed the use of neat samples and eliminated the need for toxic volatile solvents [36]. A further refinement of this method used an FTIR spectrometer equipped with a thin (0.1 mm) transmission flow cell, enabling an automated procedure for calculating the percentage of *trans* in fats and oils [37].

2. "Ratioing" of Single-Beam FTIR Spectra

Figure 7.1 shows that the 966 cm^{-1} *trans* band is only a shoulder at low levels (near 2% of total fat). This is due to the overlap of the *trans* band with other broad bands in the spectrum, producing a highly sloped background that diminishes the accuracy of the *trans* analysis. Many researchers

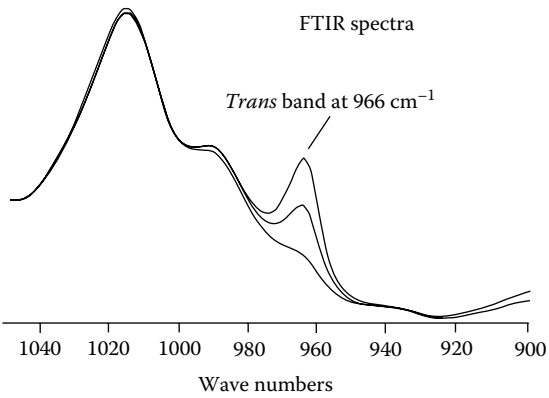

FIGURE 7.1 Observed conventional IR absorption spectra for *trans* FAMEs in carbon disulfide. At low *trans* levels, the *trans* band is reduced to a shoulder.

have proposed changes to the procedures mentioned earlier, ranging from minor refinements to major modifications, all aimed at overcoming some of the limitations. These studies paved the way for procedures that use spectral subtraction to increase accuracy, as well as providing methods for analyzing neat samples in order to eliminate the use of solvents.

Mossoba et al. [38] described a rapid IR method using an FTIR spectrometer equipped with an ATR cell for quantitating *trans* levels in neat fats and oils. This procedure measured the 966 cm^{-1} *trans* band as a symmetric feature on a horizontal background (Figure 7.2). The ATR cell was incorporated into the design to eliminate one potential source of error: the weighing of test portions and their quantitative dilution with the volatile carbon disulfide solvent. The high bias previously found for triacylglycerols was attributed to the overlap of the *trans* infrared IR band at 966 cm^{-1} with ester group absorption bands [33,37]. However, by "ratioing" the FTIR single-beam spectrum of the oil or fat being analyzed against the single-beam spectrum of a reference material (triolein, a mixture of saturated and *cis*-unsaturated triacylglycerols or the corresponding un-hydrogenated oil), the interfering absorption bands could be eliminated, and baseline-resolved *trans* absorption bands at

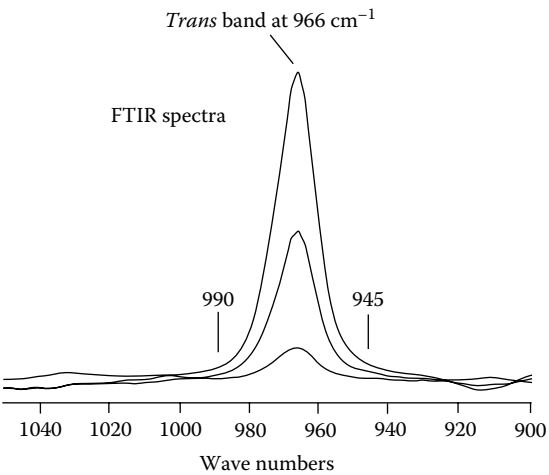

FIGURE 7.2 Observed attenuated total reflection–Fourier transform IR spectroscopy (ATR-FTIR) absorption spectra for neat (without solvent) FAMEs. Symmetric bands were obtained because the spectrum of the *trans*-free fat (methyl oleate) was subtracted from those of different concentrations of *trans* fats (methyl elaidate).

966 cm^{-1} obtained [38]. This same approach has also been applied to methyl esters [38]. Ideally, the reference material should be a *trans-free* oil that is otherwise similar in composition to the test sample being analyzed.

The simplified FTIR method just outlined allowed analyses to be carried out on neat fats or oils that were applied directly to the ATR crystal with little or no sample preparation. This method eliminated the interference of the ester absorptions with the 966 cm^{-1} *trans* band and the uncertainty associated with the location of the baseline. Figure 7.2 shows the symmetric spectral bands that were obtained when different concentrations of methyl elaidate (ME) in methyl oleate (MO) were "ratioed" against methyl oleate. A horizontal baseline was observed, and the 966 cm^{-1} band height and area could be readily measured. The minimum identifiable *trans* level and the lower limit of quantitation were reported to be 0.2% and 1%, respectively, in hydrogenated vegetable oils [38].

Further refinements of the "ratioing" procedure have used single-bounce, horizontally attenuated total reflection (SB-HATR) IR spectroscopy [39]. In this procedure, only 50 μL (about 2–3 Pasteur pipette drops) of neat oil (either triacylglycerols or methyl esters) is placed on the horizontal surface of the zinc selenide element of the SB-HATR IR cell. The absorbance versus concentration values of interest exhibited a linear function. The test portion of the neat oil can easily be cleaned from the IR crystal by wiping with a lint-free tissue before the next neat sample is applied. This method is accurate for *trans* concentrations greater than 1%. The SB-HATR FTIR procedure has been successfully used to determine the *trans* content of 18 food products [39].

This internal reflection FTIR procedure was voted an Official Method AOCS Cd 14d-99 by the AOCS in 1999 [40] and after testing and validation in a 12-laboratory international collaborative study became Official Method 2000.10 for AOAC International in 2000 [41]. Analytical ATR-FTIR results exhibited high accuracy relative to the gravimetrically determined values. Comparison of test materials with similar levels of *trans* fatty acids indicated that the precision of the current ATR-FTIR official method was superior to two previously approved transmission mode IR Official Methods: AOAC 965.34 [42] and AOAC 994.14 [43].

The ATR-FTIR method has also been evaluated for use with matrices of low *trans* fat and/or low total fat contents such as milk [44] and human adipose tissue [45]. Preliminary results indicated that the presence of low levels (<1%) of conjugated *cis*/*trans* dienes in these matrices, with absorbance bands near 985 and 947 cm^{-1}, interfered with the accurate determination of total isolated (nonconjugated) *trans* fatty acids [44,45]. However, attempts to eliminate those interfering absorbance peaks by using spectral subtraction techniques [44,45] were not satisfactory. In order to overcome the effect of interferences, the ATR-FTIR method was modified to include the standard addition technique [46]. This modified method was applied to several food products, namely, dairy products, infant formula, and salad dressing. Using this standard addition, modification successfully minimized the interfering conjugation absorbance bands. For example, the presence of <1% CLA in two butter and two cheese products containing 6.8%, 7.5%, 8.5%, and 10.4% *trans* fatty acids (as a percentage of total fat) would have resulted in errors of 11.6%, 10.4%, 17.6%, and 34.6%, respectively, in *trans* fat measurements using the unmodified method [46].

3. Application of the Negative Second Derivative FTIR Method

Although these ATR-FTIR Official Methods [40,41,47] eliminated the baseline offset and slope, they were only partly successful in improving accuracy. This is because finding a reference fat that is absolutely *trans*-free and whose composition can closely match every unknown test sample is impossible. This fact also had a negative impact on sensitivity.

To improve sensitivity and accuracy, another ATR-FTIR procedure that measures the height of the negative second derivative of the *trans* absorption band, relative to air (Figure 7.3), was tested [48,49]. Reference standards consisting of the *trans* monoene trielaidin (TE, *trans*-18:1) diluted in triolein (TO, *cis*-18:1) were initially used to generate calibration data. The negative second derivative procedure totally eliminated both the baseline offset and slope of the *trans* IR band as well as the

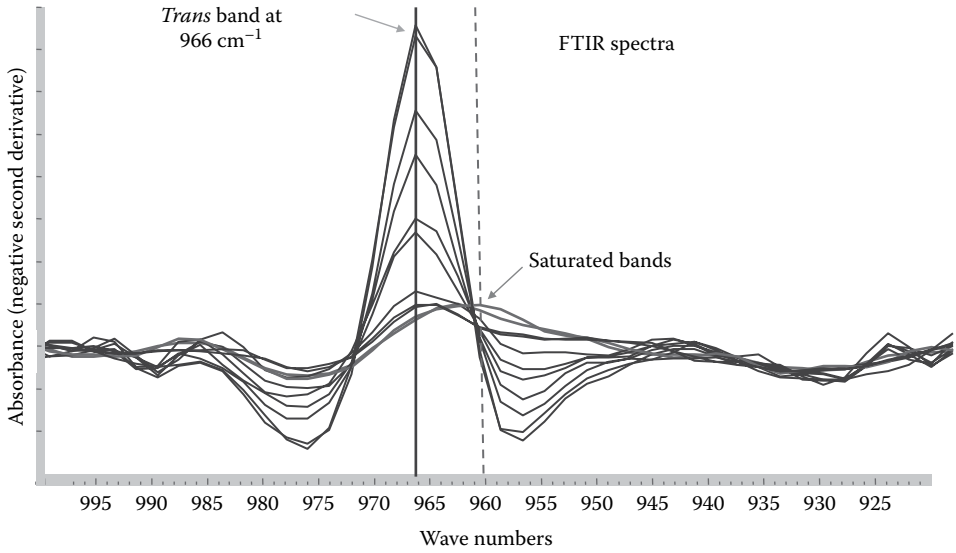

FIGURE 7.3 Negative second derivative for observed ATR-FTIR absorption spectra of neat (without solvent) triacylglycerols. The presence of weak IR bands, attributed to *saturated* fats and oils, could be identified by this procedure. (From Mossoba, M.M. et al., *J. Am. Oil Chem. Soc.*, 84(4), 339, 2007.)

requirement to use a *trans*-free reference fat. Using the second derivative of an absorbance spectrum enhanced the resolution of IR bands and made it possible to notice small shifts in IR band position and the presence of interferences.

The advantages offered by the negative second derivative ATR-FTIR procedure made it possible to improve sensitivity and resolve a discrepancy in accuracy between GC and IR at low *trans* levels [49]: a hydrogenated soybean oil (HSBO) sample had found to have no *trans* fat by GC, but was reported by IR to have a low, yet significant, *trans* level of 1.2% of total fat [48]. The negative second derivative ATR-FTIR spectrum observed for the HSBO test sample [48] indicated that the band position was shifted to 960 cm^{-1} from the expected position of the *trans* band absorption at 966 cm^{-1}. It was subsequently recognized that for this fully hydrogenated soybean oil, the weak band at 960 cm^{-1} should have been attributed to tristearin (TS, 18:0) [49], which is a saturated, rather than a *trans*, fat. This result was confirmed by showing that a reference sample of TS exhibited an identical weak band at 960 cm^{-1}.

Another finding reported in the negative second derivative study [48] was the detection of a 0.5% contaminant, mainly composed of TE, in the commercially available TO (as assessed by GC). It was therefore recommended that commercially available TO should no longer be used to prepare calibration standard mixtures. Instead, several saturated fats, in addition to TS, were screened for possible interferences with the *trans* band at 966 cm^{-1} [49]. The saturated fats trilaurin (TL, 12:0), trimyristin (TM, 14:0), tripalmitin (TP, 16:0), and triarachidin (TA, 20:0) were measured by IR and were all found to exhibit similar weak absorption bands with varying degrees of interferences. TP exhibited a band at 956 cm^{-1}, which was farthest from the *trans* absorption at 966 cm^{-1}. This least interfering saturated fat, TP, also had the lowest absorptivity, and therefore, TP was used as a substitute for TO in preparing calibration standard mixtures of TE in TP as low as approximately 0.5% of total fat [49]. This negative second derivative procedure was subsequently validated in an international collaborative study [50] and adopted as Official Method AOCS Cd 14e-09 in 2009 [51].

For fats and oils containing high levels of saturated fats and only a trace amount (≤0.1% of total fat as determined by GC) of *trans* fat, such as coconut oil and cocoa butter, the weak bands observed at energies slightly lower than 966 cm^{-1} (Figure 7.3) must not be mistaken for, and erroneously

reported as, *trans* fat bands [49]. By recognizing these potential interferences from saturated fats, IR spectra for unknown *trans* fats can be interpreted correctly, thereby improving the accuracy of the IR determinations at low *trans* levels (particularly ≤1% of total fat) and making this relatively sensitive negative second derivative IR procedure suitable for the rapid determination of total *trans* fats and the labeling of food products and dietary supplements.

4. Portable FTIR Devices for Total *Trans* Fat Determination

Portable FTIR devices operating in the transmission or ATR modes have been commercially developed in recent years and successfully used to meet an increasing demand for rapid (<5 min) and accurate determination of the total *trans* fat content of fat and oils as well as lipids extracted from food matrices. The performance of two portable FTIR devices was evaluated and, as described next, found to be as equally satisfactory as that of a benchtop ATR-FTIR spectrometer for the determination of total *trans* fat content.

The first portable FTIR system, equipped with a heated nine-reflection diamond ATR crystal, was evaluated and compared to that of a benchtop single-reflection ATR-FTIR spectrometer in 2012 [52]. This portable FTIR device (footprint 8 by 11 in.; height 5 in.) (Agilent, formerly A2 Technologies, Danbury, CT) had a sample capacity of approximately 10 μL, and its optical bench included a Michelson interferometer with a mechanical bearing moving mirror, a potassium bromide substrate beam splitter, and a deuterated triglycine sulfate (DTGS) detector. To enhance the signal-to-noise ratio, 256 scans were co-added and signal averaged. During analysis, the ATR cell was warmed to 65°C so that all neat fats and oil test samples would remain in a melted state during measurement. By using a nine-bounce diamond ATR crystal, the limit of quantification of *trans* fat, as a percentage of the total fat, was lowered from approximately 2% to 0.34%. The data collected from accurately weighed gravimetric standards and 28 unknown test samples ranging in *trans* fat contents from approximately 0.5% to 54%, as a percentage of the total fat, indicated that applying the Official AOCS Method Cd14e-09 [51] using this nine-bounce portable ATR-FTIR device could lead to a fivefold enhancement in sensitivity, relative to that provided by the single-reflection system. Implementing these changes should facilitate regulatory compliance and verification of fat and oil samples for *trans* fat content.

More recently, the performance of a second portable FTIR device (Cary 630, Agilent), operating in the transmission mode, was evaluated and compared to that of a benchtop ATR-FTIR spectrometer [53]. This FTIR device was equipped with a DialPath™ accessory, factory calibrated to three different fixed path lengths (30, 50, and 100 μm). After conversion to FAME, the concentration of total *trans* FAME in the fat extracted from 19 representative fast foods was rapidly (5 min) quantified in a single measurement. Although the amount of time required for extraction and derivatization was significantly longer than for FTIR spectral data collection, derivatization was necessary to convert the lipid extracts into samples that were clear and free of impurities. For all 19 extracts, the total *trans* FAME concentration varied from approximately 0.5% to 11% of total FAME. The *trans* fat content (mean ± SD), expressed in grams per serving and calculated on the basis of total fat content and FTIR quantification of the total *trans* fat content, was found to be 1.00 ± 0.42 for hamburgers, 0.67 ± 0.78 for chicken tenders, 1.00 ± 1.24 for French fries, and 0.27 ± 0.23 for apple pies. Determinations of total *trans*-unsaturated FAME were consistent with the results obtained by using the ATR-FTIR [51] and GC [32] official methods, indicating that this portable FTIR device, in transmission mode, was suitable for the rapid and routine quantification of total *trans* fat, measured as FAME, prepared from fats extracted from fast foods.

B. FTIR–Partial Least Squares Regression

Quantitative analyses of *trans* unsaturation have also been performed using a transmission FTIR in conjunction with a partial least squares (PLS) procedure [54]. Compared to the conventional GC methods described in the following text, this approach significantly reduced analysis time while effectively assessing raw materials and food products containing a wide range of *trans* values [54].

A heated SB-HATR sampling accessory was used to develop an FTIR-PLS method for the simultaneous determination of iodine value (IV) and *trans* content for neat fats and oils [55]. To develop the calibration models, PLS regression was employed and a set of nine pure triacylglycerol test samples served as the calibration standards. Satisfactory agreement (SD < 0.35) was obtained between the predictions from the PLS calibration model and *trans* determinations by the SB-HATR FTIR method. Similar PLS analyses have been used to establish calibration models for the simultaneous determination of isolated and conjugated *trans* fatty acids in virgin olive oil using chemometric analysis of their FTIR spectra [56]. These methods use the region 1000–650 cm^{-1} where the CH bending bands of the *trans* fatty acids and CH deformation bands of CLA isomers were observed. Two calibration models were generated for *trans* and/or CLA isomers in the ranges 1%–2.5% and 0%–30% in olive oil and were cross validated before these models were then used to determine the *trans* and CLA contents of unknown test samples [56].

In 2014, a rapid procedure to determine *trans* fatty acids at concentrations of <1% in edible fat and oil samples (palm, peanut, soybean, and sunflower) extracted from food products was developed, using ATR-FTIR in conjunction with traditional linear regression and PLS, and used to model the observed FTIR data [57]. The resulting calibration models of edible oils test samples showed a coefficient-of-correlation of >0.982 and a standard error of prediction (SEP) between 0.03% and 0.06%. These ATR-FTIR results of extracted oils and fats were reportedly in good agreement with capillary gas chromatographic data, demonstrating that using ATR-FTIR and chemometrics together can be used to rapidly determine total *trans* fatty acid contents of <1% in oils and fats [57].

C. NIR Spectroscopy

In 2000, a generalized PLS calibration model was developed for the determination of the *trans* content of edible fats and oils using Fourier transform *near*-infrared (FT-NIR) spectroscopy [58]. The *trans* primary reference data, determined by using the *mid*-IR SB-HATR FTIR official method (AOCS Cd 14d-99) [40], were used to develop the generalized FT-NIR calibration. The FT-NIR *trans* predictions obtained using this generalized calibration were in good agreement with the SB-HATR results. The authors concluded that FT-NIR and PLS therefore provided a viable alternative to the SB-HATR-FTIR [58].

Advances in FT-NIR have made it possible to determine not only the total *trans* fatty acid content of a fat or oil but also its fatty acid composition [59,60], without prior derivatization to volatile derivatives as required for GC analysis (see the following text). Quantitative FT-NIR calibration models were developed using accurate GC results (for FAME) as the primary method. The FT-NIR spectra showed unique fingerprints for SFAs, *cis* and *trans* monounsaturated fatty acids (MUFA), and all *n*-6 and *n*-3 polyunsaturated fatty acids (PUFA) within triacylglycerols to permit qualitative and quantitative comparisons of fats and oils. The quantitative calibration models were developed, as mentioned earlier, by incorporating accurate GC fatty acid composition data obtained for different fats and oils with FT-NIR spectral data. Three calibration models were developed: one was used for the analysis of common fats and oils with low levels of *trans* fatty acids (liquid oils, <2% *trans* fatty acids), and the other two were used for semisolid fats and oils with intermediate (<20% *trans* fatty acids) to high (<60% *trans* fatty acids) levels of *trans* fatty acids. This FT-NIR procedure exhibited the potential to rapidly determine fatty acid composition of unknown fats and oils in their neat form and without derivatization [60]. It should be noted that the FT-NIR methodology is matrix dependent [59,60]; thus, it is highly dependent on factors that could influence the NIR absorption spectra. It is therefore expected that external contaminations, such as residual solvent [61], temperature of measurement, or native oil components, such as β-sitosterol, could alter the absorption spectrum and ultimately distort the fatty acid analysis, unless these factors are accounted for and robust calibration models are developed [62].

A pre-calibrated FT-NIR Standard Procedure, AOCS 14f-14 [63], based on the method of Azizian and Kramer [60] was collaboratively studied [64] and adopted by AOCS in 2014.

This procedure may be applied to the rapid (<5 min) determination of the total SFA, MUFA, PUFA, and *trans* fatty acid contents in triacylglycerol-based vegetable oils and fats. This standard procedure involves the measurement of neat test samples in the transmission mode in disposable glass tubes (3 mm path length) or in the transflection mode with a fiber-optic probe (2 mm path length) [63]. PLS1 pre-calibration models (NIR Technologies Inc., Oakville, Ontario, Canada) are then applied without any further calibration effort to determine total SFA, MUFA, PUFA, and *Trans* FA content [65].

III. ANALYSIS USING CHROMATOGRAPHY

The chromatography of lipids involves the separation of individual components of a lipid mixture as they pass through a medium with a stationary matrix. This matrix may be packed or bound to a column, as in GC and high-performance liquid chromatography (HPLC), or bound to a glass plate, as in thin-layer chromatography (TLC). The mobile phase in gas chromatography is usually an inert gas such as helium, hydrogen, or nitrogen. For HPLC or TLC, the mobile phase may be an aqueous or organic solvent. Intact triacylglycerols or fatty acids can be separated, but many chromatographic applications consist of separating the methyl ester or other derivatives of individual fatty acids.

A. Gas Chromatography

The most significant advance in the separation of lipids was the development of gas chromatography, which remains the industry standard. In 1956, FAMEs, including saturates and unsaturates, were first successfully separated using a 4 ft long column packed with Apiezon M vacuum grease [66]. These separations could be performed on the basis of chain length, degree of unsaturation, and geometric configuration (*cis* or *trans*).

1. Separations Using Capillary Columns

The development of flexible fused silica columns in the early 1980s [67] led to the popularity of capillary columns, which dramatically increased the number of effective plates, thus improving column separation efficiencies. The number of effective plates could be raised from 40,000 to 250,000 by increasing the column length from 15 to 100 m.

When long capillary columns became widely available [68–81], offering a variety of diameters and stationary phases, the use of packed columns declined. As Figure 7.4 indicates, monoene and diene FAMEs and their isomers from milk fat could only partially be separated in a single run on a 100 m capillary column [79,80]. The *trans* triene isomers present in partially hydrogenated soybean oil were first separated into four peaks on a 100 m capillary GC column [69]. Fractional crystallization and reversed-phase HPLC, followed by GC analysis of hydrazine reduction products, served to identify and quantify these triene isomers.

Using capillary GC analysis in the separation and identification of positional and geometric isomers of unsaturated fatty acids was well established by 1995 [70]. Today, most laboratories use long capillary columns to quantify individual fatty acid isomers found in PHO. Performing GC analysis on dairy products or partially hydrogenated fish oils is much more complex than analyzing hydrogenated vegetable oils, due to the significantly greater number of geometrical and positional isomers found in those animal-based products. For example, an extensive analytical study of hydrogenated menhaden fish oil using capillary GC showed that at an iodine value of 84.5, the most unsaturated isomers were eliminated, although 13.1% diene, 8.3% triene, and 0.4% tetraene isomers were still present [71,72]. Further analysis of these 20-carbon isomers determined the *cis* and *trans* bond positions along the fatty acid chain: a wide range of monoene, diene, and triene *cis* and *trans* positional isomers were identified at virtually every position of the carbon chain.

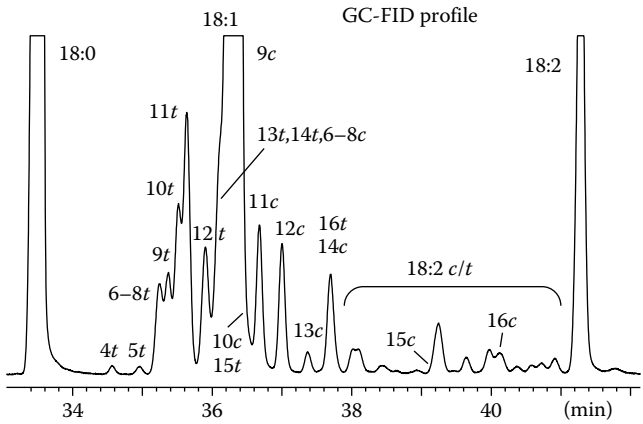

FIGURE 7.4 Partial gas chromatography (GC) chromatogram observed for *trans* and *cis* FAME isomers from a milk fat test sample using a 100 m fused silica capillary column. (From Kramer, J.K.G. et al., *Eur. J. Lipid Sci. Technol*., 103(9), 600, 2001; Kramer, J.K.G. et al., *Lipids*, 37(8), 823, 2002.)

2. Official Capillary GC Methods

Capillary GC has long been the most widely used analytical method to analyze FAME [76–80]. A successful GC determination of total *trans* FAME composition depends on the experimental conditions dictated by the method used, as well as sound judgment by the analyst to correctly identify peaks attributed to *trans* FAME and their positional isomers. The most recent GC official methods to determine *trans* FAME describe separations that require long capillary columns with highly polar stationary phases [82]. Under these conditions, a separation is based on the chain length of the fatty acid, degree of unsaturation, and the geometry and position of double bonds. The expected elution sequence for specific fatty acids with the same chain length on highly polar columns is as follows: saturated, monounsaturated, diunsaturated, etc. *Trans* positional isomers are followed by *cis* positional isomers, but today there is still *extensive* overlap among the geometric isomers.

The improved resolution of the large number of peaks attributed to *trans* and *cis* positional isomers obtained with polar columns has not eliminated the partial or complete overlap of many peaks belonging to the two different groups of geometric isomers [76–81]. This is because the retention time range for late eluting *trans* 18:1 positional isomers, starting at Δ12 (or Δ13 depending on experimental conditions used), is the same as that for the *cis* 18:1 positional isomers, Δ6–Δ14. In addition, *cis*/*trans* 18:2 methylene- and non-methylene-interrupted fatty acid retention time range also overlaps with that of the *cis*-18:1 positional isomers.

Elimination of GC peak overlap usually requires prior separation of the *cis* and *trans* 18:1 geometric isomers by silver ion (Ag)-TLC [76–81] or other methods. GC analysis of the isolated *trans* and *cis* fractions [79,80] clearly demonstrates the extent of overlap (Figure 7.5). Most individual 18:1 isomers can be completely resolved by GC under isothermal conditions at significantly lower temperatures [76–81].

The effect of this GC peak overlap on the accuracy of *trans* FAME determinations for various matrices depends on (1) the nature of the *trans* fat matrix being analyzed (vegetable oil, fish oil, milk fat) as these will exhibit vastly different isomeric distributions; (2) the GC experimental conditions (for instance, the length and age of a column, nature of the carrier gas, and temperature program); (3) the analyst's experience, ability, and skill in optimizing the performance of the gas chromatograph; and subsequently, (4) the correct identification of all the observed GC peaks in the widely different and complex fatty acid profiles.

Since 2006, oils used by the food industry have contained significantly lower *trans* fat contents, necessitating a reevaluation of GC official methods that has been used by the food industry and

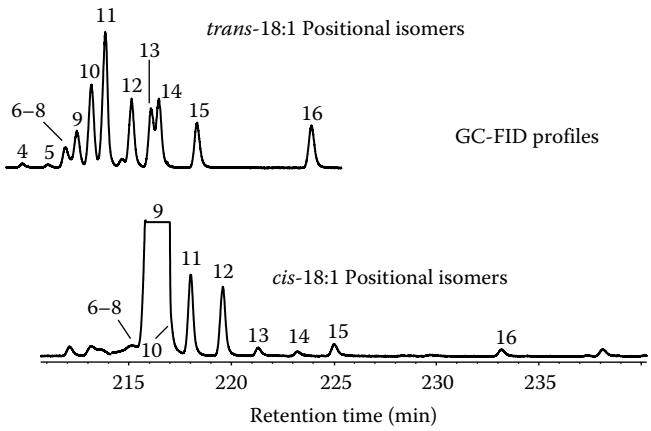

FIGURE 7.5 GC chromatograms demonstrating the overlap between *trans* 18:1 and *cis* 18:1 FAME isomeric fractions from a milk fat test sample using a 100 m fused silica capillary column, after fractionation of the geometric isomers by silver ion TLC. (From Kramer, J.K.G. et al., *Eur. J. Lipid Sci. Technol*., 103(9), 600, 2001; Kramer, J.K.G. et al., *Lipids*, 37(8), 823, 2002.)

regulatory agencies for the determination of total *trans* fat [83]. AOAC Official Method 996.06 is the conventional analytical method used for the determination of the contents of total, saturated, and unsaturated fat in foods [84]. Those chromatographic conditions involve the separation of FAME on a 100 m SP-2560 column (Supelco, Bellefonte, PA, USA) using a ramped temperature program and helium as the carrier gas. Quantification of total *trans* fat is calculated by summing the peak area attributed to *trans* C18:1 FAME that elutes prior to *cis* C18:1 and the area for *trans* C18:2 FAME that elutes between *cis* C18:1 and *cis,cis* C18:2 FAME [84]. However, AOAC Official Method 996.06 is not appropriate for the separation of individual *trans* fatty acid isomers because it results in the coelution of several C18:1 and C18:2 geometric FAME isomers, including *trans*-13/*trans*-14 18:1 and *cis*-9 18:1, which are found in milk fat. The proposed optimization of Rozema et al. [85] resulted in greater separation of individual *cis* and *trans* C18:1 FAME and better resolution of the C18:2 *trans* isomers. Nevertheless, FDA regulations for nutrition labeling as of 2015 require only the declaration of total *trans* fat content and not the content of individual *trans* fatty acid isomers.

AOCS Official Methods Ce 1h-05 [82] and Ce 1j-07 [86] are specifically GC-based methods, recommended for the determination of *trans* fatty acids, among other components, in edible fats and oils and extracted food lipids. These methods recommend the use of 100 m cyanopropyl polysiloxane columns, such as the Supelco SP-2560 or CP-Sil 88 (Chrompack, Middelburg, the Netherlands), and the use of hydrogen as the carrier gas. At similar carrier gas flow rates, the use of hydrogen gas reduces the separation time but maintains the degree of peak resolution that can be obtained with helium gas [87]. AOCS Official Method Ce 1h-05 describes an isothermal separation of FAME at 180°C that is appropriate for analyzing crude, refined, partially hydrogenated, or fully hydrogenated fats and oils from vegetable and nonruminant animal sources [82]. AOCS Official Method Ce 1j-07 is applicable to fats derived from either plants or ruminant animals (but not their mixtures) and uses a temperature program in which the oven is maintained at 180°C for 32 min, then ramped to 215°C and held for 31.25 min [86]. This method was amended in 2013 to include collaborative study data for 22 different food matrices having total *trans* fatty acid contents ranging between 0.0% and 7.3% of total fatty acids.

Validating an analytical method is necessary to establish its performance characteristics and entails demonstrating the accuracy, linear range, limits of detection (LOD) and quantification (LOQ), precision (i.e., repeatability, reproducibility), recovery, and specificity of a given method for

one or more analytes. Specific performance criteria (such as precision) are available for these AOAC or AOCS official methods because these have been validated through multilaboratory collaborative studies. Notably, however, none of these methods identify a specific LOQ for *trans* fat.

To fill this gap, many methods-endorsing organizations now use the Horwitz ratio ("HorRat") as the basis for acceptance of a method's performance [88]. The "HorRat" or Horrat value provides an indication of method performance based on the precision of inter-laboratory validation data and has become the accepted tool to evaluate precision for collaborative studies among many laboratories. The Horrat value is calculated as the ratio of the reproducibility relative standard deviation (RSD_R) to the predicted RSD_R, which is determined from the mass fraction of the analyte. The empirically acceptable range is 0.5–2.0: Horrat values >2.0 indicate a need for further method optimization or analyst training, and values <0.5 indicate better than expected method performance or analyst skill [88]. In collaborative studies, the lowest concentration before which the Horrat value exceeds 2.0 is now often accepted as the LOQ [88].

Table 7.1 illustrates how validation data for the determination of *trans* fat in 10 edible fats and oils, according to AOCS Official Method Ce 1h-05 [82], can be used with HorRat values to assess method precision. For oils with a high *trans* fat content (11.62%–45.01% of total fat), HorRat values ranged from 0.79 to 2.02, indicating that the method performs acceptably with these samples. However, for oils having a low *trans* fat content (0.06%–1.00% of total fat), the HorRat values exceeded 2.65, indicating that the method does not perform well at this low concentration range. It is important to note that the original collaborative study for official method AOCS Ce 1h-05 did not include samples with a *trans* fat content in the range 1.00%–11.62% of total fat, despite the fact that range includes many refined, bleached, and deodorized edible oils [89].

Multilaboratory collaborative data for AOCS Official Method Ce 1h-05 [82] are now available for 22 different food matrices (Table 7.2). The Horrat values for samples of cheese powder, tallow, and chocolate cake mix, with *trans* fatty acid contents varying from 0.90% to 7.27% of total fatty acids, fell within the acceptable range, indicating that this method was appropriate for

TABLE 7.1
Determination of *trans* FA in Edible Fats and Oils: Multilaboratory GC–FID Data for Validating AOCS Official Method Ce1h-05

Sample	Total *trans* FA (% of Total Fat)	RSD_R (%)[a]	Horrat[b]
Vegetable shortening	45.01	4.55	2.02
Canola oil	26.55	2.45	1.00
Canola oil	26.27	2.97	1.21
Margarine oil	11.62	2.18	0.79
Hydrogenated lard	1.00	21.64	5.41
Lard	0.90	21.70	5.34
Sunflower oil	0.17	60.34	11.50
Coconut oil	0.11	14.80	2.65
Coconut oil	0.10	35.88	6.37
Cocoa butter	0.06	69.66	11.40

Source: Tyburczy, C. et al., *Anal. Bioanal. Chem.*, 405(17), 5759, 2013.

Note: Horrat values are calculated from the Horwitz formula as follows: Horrat = RSD_R/$PRSD_R$. The predicted RSD_R ($PRSD_R$) is calculated as follows: $PRSD_R = 2C^{-0.15}$, where C is the mass fraction of the analyte.

[a] RSD_R, reproducibility relative standard deviation.

[b] Horrat values are reported in AOCS Method Ce 1h-05.

TABLE 7.2
Determination of *trans* FA in Extracted Oils: Multilaboratory GC–FID Data for Validating AOCS Method Ce 1j-07

Sample	Total Fat (g/100 g Sample)	Total Trans Fat (% of Total Fat)	RSD_R (%)[a]	Horrat[b]
Cheese powder	28.38	7.27	5.04	1.70
Tallow	95.21	7.14	4.20	1.41
Anhydrous milk fat	88.92	5.11	13.14	4.21
Butter	67.76	2.49	17.29	4.97
Chocolate cake mix	10.34	0.90	7.43	1.83
Encapsulated DHA/EPA	53.66	0.68	33.82	8.00
Whole-egg powder	38.47	0.43	12.99	2.87
Frozen cheese pizza	7.66	0.37	18.70	4.04
Evaporated milk	5.97	0.33	15.89	3.37
Yogurt	5.51	0.32	7.94	1.68
Extruded dog food	21.06	0.31	34.97	7.35
Creamy ranch-dressing	44.16	0.24	65.50	13.25
Potato chips	34.44	0.22	62.69	12.52
DHA/EPA-fortified infant formula	27.58	0.15	78.47	14.79
Peanut butter	51.69	0.06	75.73	12.44
Oatmeal cookie	18.33	0.05	44.84	7.17
Canned cat food	5.44	0.05	49.55	7.92
Full-fat soy flour flakes	22.05	0.02	73.10	10.19
DHA/EPA-fortified orange juice	0.11	0.00	0.00	N/A
Dry cereal fortified with flax	1.82	0.00	0.00	N/A
Horse feed	3.07	0.00	0.00	N/A
Gamebird feed	2.87	0.00	0.00	N/A

Source: Tyburczy, C. et al., *Anal. Bioanal. Chem.*, 405(17), 5759, 2013.

Note: Horrat values were calculated from the Horwitz formula as follows: Horrat = $RSD_R/PRSD_R$. The predicted RSD_R ($PRSD_R$) was calculated as follows: $PRSD_R = 2C^{-0.15}$, where C is the mass fraction of the analyte.

[a] RSD_R, reproducibility relative standard deviation.

[b] RSD_R is reported in AOCS Official Method Ce 1j-07.

analyzing these products. In contrast, Horrat values exceeded 2.0 for samples of cookies, DHA/EPA-fortified infant formula, potato chips, whole-egg powder, butter, anhydrous milk fat, and other samples in the collaborative study—all having *trans* fat contents in the range 0.05%–5.11% of total fat—indicating that Official Method Ce 1h-05 [82] was not suitable for determining the levels of *trans* fat in such products. Because collaborative study data do not include samples of edible fats and oils and foods with *trans* fatty acid contents exceeding 1% of total fatty acids, it appears that official GC method performance limits have not been fully established and may be higher than might be generally believed. These findings emphasize the need for additional validation studies, using more representative samples to cover the critical lower range of *trans* fat contents.

3. Ionic Liquid GC Columns and Two-Dimensional Separation of FAME

The complete and accurate analysis of complex mixtures of fats and oils usually requires multiple GC separations, fractionation by HPLC prior to GC analysis, then identification of fatty acids and confirmation of their identities by applying hyphenated GC-MS, GC-IR spectroscopy, and, finally, comparison to reference standard fatty acids. We will next describe two new approaches to optimize GC separations.

In 2011, a newly available ionic liquid capillary GC column, the SLB-IL111 (Supelco), was shown to provide enhanced separations of unsaturated geometric and positional isomers of FAME, relative to separations achieved using the conventional cyanopropylsiloxane (CPS) columns that had been previously recommended for FAME separations [90]. More recently, a novel 200 m SLB-IL111 ionic liquid column, formed by connecting two 100 m SLB-IL111 columns using a quartz press-fit connector and operated under a combined temperature and eluent flow gradient, was shown to successfully resolve most of the FAME from milk fat FAME in a single GC separation [91]. Separations using the 200 m SLB-IL111 column also provided a complementary elution profile to those obtained by CPS columns, enabling a more comprehensive analysis of total milk fat. These optimized conditions also led to the simultaneous separation of short-chain (starting from 4:0), long-chain PUFA, and most of the unsaturated FAME positional/geometric isomers. Among the MUFA, *t*11–18:1 and *t*10–18:1 FAME, the two most abundant *trans* fatty acids found in most dairy products, were resolved. This is significant since these *trans* FAME isomers reportedly have vastly different biological activities. In addition, the SLB-IL111 column permitted the separation of two CLA isomers commonly found in dairy products, specifically *t*7,*c*9–18:2 from *c*9,*t*11–18:2, which otherwise coelute on CPS columns [90,91].

A study of marine oil omega-3 supplements sold in the United States, published in 2014 [92], illustrates the use of ionic liquid columns. Specifically, the content and composition of fatty acids in 46 commercial marine oil omega-3 supplements, including fish oils, fish oil concentrates, salmon oils, and plant/fish oil blends, was determined by GC using the 200 m SLB-IL111 ionic liquid column. Seventy-three FAMEs were quantified, including *n*-6, *n*-4, *n*-3, and *n*-1 PUFAs. In more than 80% of the products analyzed, the contents of the two main long-chain omega-3 PUFAs, namely, eicosapentaenoic acid (EPA; C20:5*n*-3) and docosahexaenoic acid (DHA; C22:6*n*-3), were consistent with their respective label declarations. Although Srigley and Rader [92] did not evaluate partially hydrogenated fish oils in their study, the presence of low concentrations of the *trans* fatty acid isomers of EPA and DHA, primarily the mono-*trans* isomers, varied from 0.1% to 1.5% of total fat, which agrees with work from Sciotto and Mjøs [93], who examined omega-3 products sold in Europe.

In 2013, a novel protocol was proposed that, for the first time, allowed the separation and identification of all *trans* unsaturated fatty acids and all PUFAs in a single experiment and eliminated the overlap between PUFAs with different chain lengths [94]. The separation of FAME, normally achieved using the 200 m SLB-IL111 ionic liquid capillary column, was further optimized by a novel 2D GC × GC design. This was accomplished by chemically reducing selected analytes prior to separation with a second GC column; specifically, a capillary tube coated with palladium was inserted between the first column and a cryogenic modulator, thus reducing unsaturated FAME to their corresponding saturated forms prior to separation with the second GC column. Then the second separation was achieved by using a short 2.5 m × 0.10 mm SLB-IL111 ionic liquid capillary column, which separated FAME based only on their carbon chain lengths. The chromatographic elution profiles for this 2D separation could be easily interpreted because all the saturated FAME fell on a straight diagonal line bisecting the separation plane, while the FAME having the same carbon skeleton, but a different number, geometric configuration, or position of double bonds, fell on lines parallel to the first dimension's retention time axis [95].

B. Thin-Layer Chromatography and Argentation Methods

TLC has been widely used to separate classes of lipids on layers of silica gel applied to glass plates. This method is simple to use and does not necessarily require sophisticated instrumentation. In most cases, TLC using silica gel does not separate fractions on the basis of number or configuration of double bonds in fatty acid mixtures [96]. The most effective TLC separation of *cis* and *trans* isomers has been achieved by means of argentation: using layers of silica impregnated with silver nitrate.

Argentation is the general term used to describe methods based on the long-known principle that silver ions form complexes with *cis* more strongly than with *trans* double bonds. In argentation chromatography, the separation of *cis* and *trans* isomers depends on the relative interaction strength of the π electrons of double bonds in each isomer with silver ions. The actual interaction of each fatty acid isomer depends upon the geometry, number, and position of double bonds. Since at least 1979, Ag-TLC has been used to separate unsaturated lipids on thin-layer plates [97], and additional argentation methods, including countercurrent distribution (CCD) and silver ion-liquid column chromatography, were in use by 1995 [98]. Several classes of fatty acid isomers can be isolated using preparative argentation TLC. These isolated fractions can then be analyzed further by using ozonolysis [71,72,99–101] and GC. Good resolution can be achieved for a variety of FAMEs using 2D argentation TLC separation and reversed-phase chromatography on a single plate [102].

In studies by Kramer and his coworkers [80,103], Ag-TLC was applied for the separation of saturated, *trans*-monounsaturated, *cis*-monounsaturated, as well as *cis*/*trans* conjugated diene fatty acids. Silica G plates (20 × 20 cm, 0.25 mm thick; Fisher Scientific) were washed with 50:50 methanol/chloroform (v/v), activated at 110°C for 1 h, impregnated with 5% silver nitrate solution in acetonitrile (w/v), and activated again before use. These plates were developed with 90:10 hexane/diethyl ether solution (v/v), and the bands were identified under UV light at 243 nm after spraying with 2′,7′-dichlorofluorescein (2% in methanol, v/v). The bands containing the isolated fatty acids were scraped and collected in test tubes, and the fatty acids were extracted with organic solvent and separated by GC.

As determined by GC, the content of *trans*-C16:1 in human milk usually appears to be higher than expected, due to the overlap with peaks attributed to C17 fatty acids [73]. Using Ag-TLC followed by GC on a highly polar 100 m capillary column was capable of more accuracy, determining that the average content of total *trans*-C16:1 to be 0.15% ± 0.04% from 39 test samples of human milk fat [68]. The C16:1 positional isomers *trans* Δ4, Δ5, Δ6/7, Δ8, Δ9, Δ10, Δ11, Δ12, Δ13, and Δ14 were reportedly quantified in 15 test samples. The mean relative contents were 2.6%, 3.5%, 7.6%, 7.2%, 24.7%, 10.4%, 10.1%, 14.3%, 8.4%, and 11.3% (as a percentage of total *trans*-C16:1), respectively [73]. As Figure 7.5 indicates, the *trans* and *cis* 18:1 fractions from milk fat isolated by Ag-TLC were almost completely separated by GC on a highly polar 100 m capillary column [79,80]. Notably, this analysis also revealed the presence of Δ6–9 *trans* 18:1 positional isomers (unlabeled peaks) in the *cis* fraction.

Wilson et al. [74] combined an Ag-TLC with capillary GC for the quantitative analysis of *trans* monoenes derived from partially hydrogenated fish oil containing *trans* isomers with C-20 and C-22 carbons. This process separated FAMEs into saturates (R-f 0.79), *trans* monoenes (R-f 0.4), *cis* monoenes (R-f 0.27), dienes (R-f 0.10), and PUFAs with three or more double bonds remaining at the origin. These authors reported that direct GC analysis underestimated the *trans* content of margarines by at least 30%. In this study, C-20 and C-22 *trans* monoenes were found in relatively large quantities averaging 13.9% and 7.5%, respectively, in margarines [74].

A similar combination of Ag-TLC and GC was used to analyze more than 2000 test samples of European bovine milk fats to assess the frequency distributions of the CLA isomer *c*9*t*11-C18:2, the isolated or nonconjugated fatty acids *trans*-C18:l, *trans*-C18:2, *t*11-C18:l (vaccenic acid), and *t*11*c*15-C18:2 (major mono-*trans*-C18:2), as well as total *trans* fatty acids [75]. The milk samples used were

obtained under a variety of feeding conditions: barn feeding, pasture feeding, and feeding during the transition periods of spring and late autumn. The average contents were (*c*9*t*11–18:2) 0.76%, (*trans*-C18:l) 3.67%, (*trans*-C18:2) 1.12%, and (total *trans* fatty acids) 4.92%. High correlation coefficients (*r*) were reported between the content of the CLA isomer *c*9*t*11–18:2 and the contents of *trans*-C18:1, *trans*-C18:2, total *trans* fatty acids, and C18:3 of 0.97, 0.91, 0.97, and 0.89, respectively. This analysis also reported a probable metabolic pathway in the biohydrogenation of linolenic acid: *c*9*c*12*c*15 → *c*9*t*11*c*15 → *t*11*c*15 → *t*11 [75,104].

C. Silver Ion Solid-Phase Extraction Chromatography

A silver ion solid-phase extraction (Ag-SPE) cartridge (Supelco, Bellefonte, PA) has become available for the rapid fractionation of FAMEs including *cis*/*trans* geometric isomers. However, its benefits and limitations have yet to be evaluated and compared to established procedures [105].

The application of silver ion solid-phase extraction (Ag-SPE) to the separation of FAME prepared from a partially hydrogenated fish oil with a *trans* fatty acid content of 41% was reported in 2014 [106]. The complex FAME mixture of partially hydrogenated fish oil was resolved into several FAME fractions: SFAs, *trans* MUFAs, *cis* MUFAs, and several PUFA fractions, each consisting of its respective geometric and positional isomers. Collected fractions were subsequently analyzed by GC using a 100 m capillary CPS column, and this method permitted the identification of the *trans* and *cis* MUFAs and other FAME constituents prepared from partially hydrogenated samples [106].

D. High-Performance Liquid Chromatography

For most applications, HPLC does not exhibit the separation efficiencies offered by long capillary GC columns. It does, however, offer several advantages. For example, the mild conditions used in HPLC work enable heat-sensitive components to be separated, a feature that reduces the possibility of isomerization taking place during the analysis of unsaturated fatty acids. Another advantage is that fatty acid fractions can be collected and analyzed further using hyphenated techniques, such as GC-FTIR and GC-MS.

A variety of detectors can be used to identify lipid solutes as they elute from HPLC columns [107]. The most common HPLC detector has been ultraviolet-based equipment that allows one to monitor the column effluent at about 200 nm. The sensitivity of this detection method is limited by the lack of strong absorbing lipid chromophores, unless fatty acid derivatives that absorb strongly in the UV range are prepared. Although refractive index (RI) detectors are also commonly used, these cannot be paired with solvent gradients and are sensitive to temperature fluctuations. Evaporative light-scattering detectors (ELSD) can be used with solvent gradients, are at least as sensitive as the best RI detectors, and can be used for quantitation.

1. Reversed-Phase HPLC

In 2002, a reverse-phase HPLC procedure for the fractionation of *cis*-18:1 and *trans*-18:1 fatty acids was proposed, utilizing two semi-preparative reverse-phase C18 columns in series prior to GC quantitation [108]; however, the observed resolution of the *cis* and *trans* geometric isomers was poor and further optimization was needed [109].

2. Silver Ion HPLC

The many advantages of using argentation HPLC columns over TLC plates include reproducible separation of analytes, column reusability, short run times, and high recoveries. Until the late 1990s, a lack of stable columns with controlled silver levels limited the use of silver ion HPLC separations. The silver ions used to bleed from the silica adsorbent cause unpredictable results, shorten the column life, and hinder the reproducibility of the results. A more successful argentation HPLC

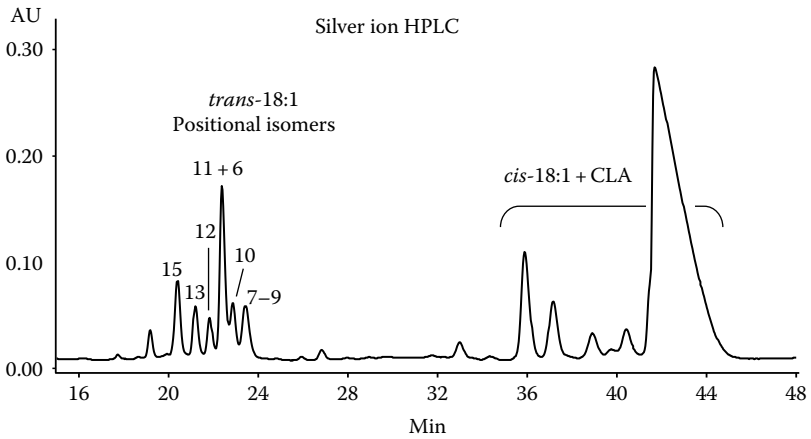

FIGURE 7.6 Silver ion HPLC chromatogram showing the separation between *trans* 18:1 and *cis* 18:1 and CLA FAME positional isomers from a milk fat test sample using three chromosphere five lipid columns in series. (From Delmonte, P. et al., Analysis of *trans*-18:1 fatty acids by silver ion HPLC, in: *Lipid Analysis and Lipidomics*, M.M. Mossoba, J.K. Kramer, J.T. Brenna, and R.E. McDonald, eds., AOCS, Champaign, IL, 2006.)

procedure was developed by linking the silver ions via ionic bonds to a silica–phenylsulfonic acid matrix [110]. This column gave excellent reproducible separations for triacylglycerols, fatty acids, and their positional and geometric isomers [111]. This column was also used for the separation, collection, and quantification of all eight geometric isomers of linolenic acid phenacyl esters, with a mobile phase ranging from 5% methanol in dichloromethane to a 50:50 solvent mixture [112].

Commercial silver ion HPLC columns were introduced approximately 15 years ago and have dramatically increased the use of this technique. A commercially available argentation HPLC column (Agilent) with an acetonitrile–hexane mobile phase was used to separate the *cis*/*trans* fatty acid isomers of methyl oleate, methyl linoleate, methyl linolenate, and 15 of the 16 *cis*/*trans* methyl arachidonate positional isomers [113]. In other research, this column was used with a 0.15% acetonitrile in hexane isocratic mobile phase to obtain four fractions from hydrogenated vegetable oil that were subsequently analyzed by capillary GC [114].

Silver ion HPLC chromatography is a powerful technique for the separation of geometric and positional isomers of fatty acids [115,116] and FAMEs [103,117]. In 2006, Delmonte et al. investigated the silver ion HPLC separation and quantitation of *trans* 18:1 fatty acid positional isomers (from Δ6 to Δ15) (Figure 7.6) as well as their dependence on parameters such as elution temperature [118]. The authors discussed the many limitations of this procedure and the factors that affected the reproducibility of this separation [118].

IV. ANALYSIS USING ONLINE HYPHENATED TECHNIQUES

Fatty acids can be misidentified in gas chromatograms, due to the lack of standards for many fatty acids or their isomers, and problematic coelution of peaks. Work by Mazzola et al. [119] determined that some earlier publications had misidentified *trans* monoene positional isomers [113,120]. Another study determined that the estimated levels of *trans,trans* 18:2 isomers in margarine were an order of magnitude too high (3% instead of 0.3%) due to misidentification of GC peaks [121]. Researchers looking at historical reports should be aware that the published literature contains an incorrect report suggesting that liquid canola shortening was contaminated with fatty acids found in animal fat [122].

To help identify peaks more accurately, a GC column can be interfaced with another instrument such as an IR spectrometer or a mass spectrometer. Hyphenated techniques use *online* detection to

confirm the identity of peaks in a chromatogram. Griffiths [123,124] documented the performance of interfaces between gas, supercritical fluid, and high-performance liquid chromatography and Fourier transform spectrometry (GC-FTIR, SFC-FTIR, and HPLC-FTIR, respectively). Some of these hyphenated techniques were first used to elucidate the structure of unusual fatty acid isomers, including cyclic fatty acid monomers (CFAM) that often contain *trans* double bonds in the hydrocarbon chain and *cis* double bonds in five- or six-membered rings. The formation and the biological effects of these cyclic compounds have been reviewed [125].

A. Gas Chromatography–Fourier Transform Infrared Spectroscopy

For many GC-FTIR instruments, the effluent from the GC column flows continuously through a light pipe (LP) gas cell [123]. LP instruments generally have detection limits of 10–50 ng for unknown complex mixtures of FAMEs. Online IR spectra of CFAM peaks eluting from a gas chromatogram were obtained with an LP GC-FTIR [126]. The results were used to show which of these CFAM contained *cis* and/or *trans* double bonds. Several minor peaks in this mixture could not be identified because of the limited sensitivity of the method.

Although the LP GC-FTIR technique can confirm the identity of intact molecules, its detection limits are unfortunately higher than those of other GC detectors (e.g., flame ionization). In 1984, Bourne et al. [127,128] developed an improved GC-FTIR technique using a matrix isolation (MI) interface that increased the sensitivity of the GC-FTIR determination by an order of magnitude. GC–matrix isolation–FTIR is extremely useful for quantitating peak area, determining peak homogeneity, and obtaining structural information on a compound. Matrix isolation (MI) is a technique in which analytes and an inert gas (argon) are rapidly frozen at cryogenic temperatures (12 K) and then are trapped as a solid matrix on the outer rim of a moving gold-plated disk. The IR spectra of these molecules are free from bands due to intermolecular hydrogen bonding and other band broadening effects. These benefits yielded greater sensitivity that equals, for many applications, that of GC-MS [128].

GC-MI-FTIR has been used to quantitate low levels of saturated, *trans* monoene [129], diene [130,131], triene, and conjugated diene fatty acids and their isomers [131] in hydrogenated vegetable and/or fish oils. Figures 7.7 through 7.10 show IR spectra exhibiting the different stretching and/or out-of-plane deformation absorption bands for *cis* and *trans* double bonds in straight chain fatty acids (Figure 7.7), CFAM (Figure 7.8), as well as for conjugated diene geometric isomers (Figures 7.9 and 7.10). The characteristic absorption bands shown were used to identify functional groups and double-bond configuration. The conjugated *trans,trans* diene isomer had an out-of-plane deformation absorption band at 990 cm^{-1}, whereas, the *cis,trans* isomer had absorption bands at 950 and 986 cm^{-1}. The absorption band for the corresponding methylene-interrupted *trans* diene and the *trans* monoene was at 971 cm^{-1}. Unique absorption bands for *trans* and *cis* diene isomers were also found in the area of the spectrum typical of the carbon–hydrogen stretch vibrations (3035/3005 cm^{-1} and 3018 cm^{-1}, respectively) and in the area typical of the carbon–hydrogen out-of-plane deformation absorption bands (972 and 730 cm^{-1}, respectively) [129–131].

GC-MI-FTIR is also effective in determining the concentration of individual FAMEs without having to consider the relative response of the gas chromatograph's flame ionization detector (FID). This makes it possible to quantitate *trans* isomers even with partial GC peak overlap from *cis* isomers. Quantitation of *trans* diene isomers was based on measurement of the height of the observed C−H out-of-plane deformation band at 971 cm^{-1} for *trans* groups and that of the CH_2 asymmetric stretching band at 2935 cm^{-1} for the 17:0 internal standard [130]. Calibration plots of absorbance versus nanograms injected were generated for the range of 2–33 ng. Recovery (on cryogenic disk) was based on the determination of the internal standard. The amount of analyte present in injected aliquots was calculated from the observed absorbance values and the corresponding calibration plot [130].

When GC-MI-FTIR was used to quantitate the *trans* monoene isomers in margarine, the results showed a high bias relative to that achieved by conventional GC FID response [130,131]. Presumably,

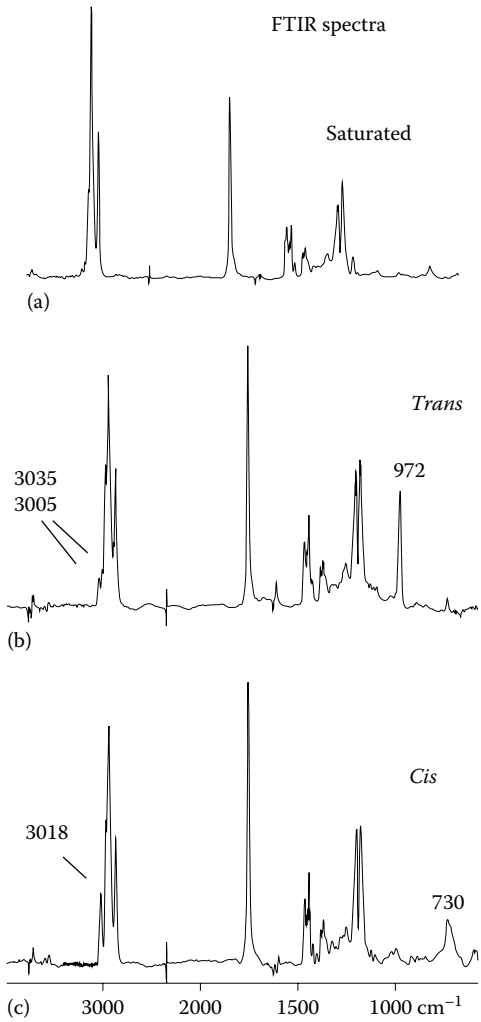

FIGURE 7.7 Sharp matrix isolation-FTIR spectral bands observed at 4 cm^{-1} resolution for (a) 18:0, (b) methylene-interrupted *trans*,*trans*-18:2, and (c) methylene-interrupted *cis*,*cis*-18:2 straight chain FAMEs after separation by capillary GC and cryogenic trapping under vacuum. (From Mossoba, M.M. et al., *J. Agric. Food Chem.*, 38(1), 86, 1990.)

the higher GC-MI-FTIR values resulted from its higher specificity; the MI-FTIR determination is based on a unique discriminatory feature (971 cm^{-1} absorbance band) that is observed only for *trans* species. The intensity of this band is not affected by *cis* isomers even when they chromatographically overlap. These results confirm that relying on GC peak areas for quantification can result in underestimates of *trans* monoenes.

GC-FTIR analysis was used to determine the geometric configuration of FAMEs separated by silver ion HPLC [132]. This was achieved using the more recent direct deposition (DD) interface that condenses GC eluates on a moving zinc selenide window cooled to near the temperature of liquid nitrogen. This DD instrumentation is even more sensitive than the matrix isolation interface [123] because the analytes are condensed on a track that is about 100 μm wide, and microscope objectives are used to collect and focus the IR beam. Unlike GC-MI-FTIR, during GC-DD-FTIR operations, the analytes are not diluted in argon or any other matrix.

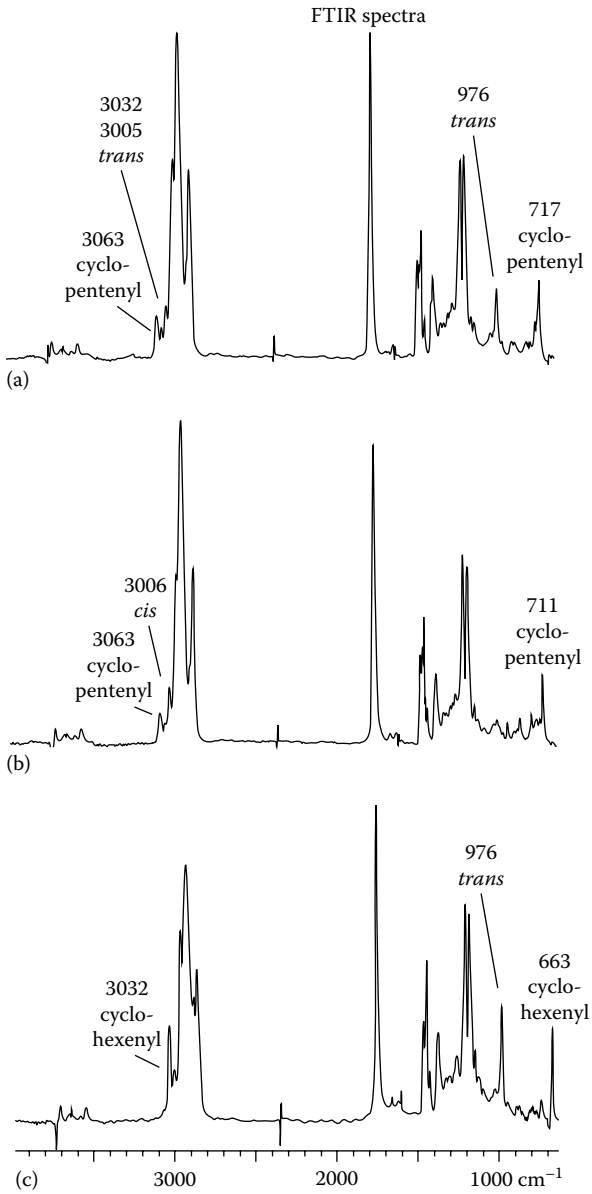

FIGURE 7.8 Sharp matrix isolation-FTIR spectral bands observed at 4 cm^{-1} resolution for methyl ester derivatives of C18 cyclic fatty acid monomers having structures consistent with (a) a cyclopentenyl ring and a *trans* double bond, (b) a cyclopentenyl ring and a *cis* double bond, and (c) a cyclohexenyl ring and a *trans* double bond along the hydrocarbon chain. Data collected after separation by capillary GC and cryogenic trapping under vacuum. (From Mossoba, M.M. et al., *J. Am. Oil Chem. Soc.*, 72(6), 721, 1995.)

B. GC-Electron Impact MS

MS can be a very effective tool when used in combination with GC to determine the location of double bonds in fatty acids and their positional isomers. The major problem of analyzing mass spectra of FAMEs is the tendency of the double bonds to migrate during electron ionization. The mass spectra exhibit low mass ions that do not provide structural information. Two of the methods used to overcome this problem include soft ionization and derivatization [133].

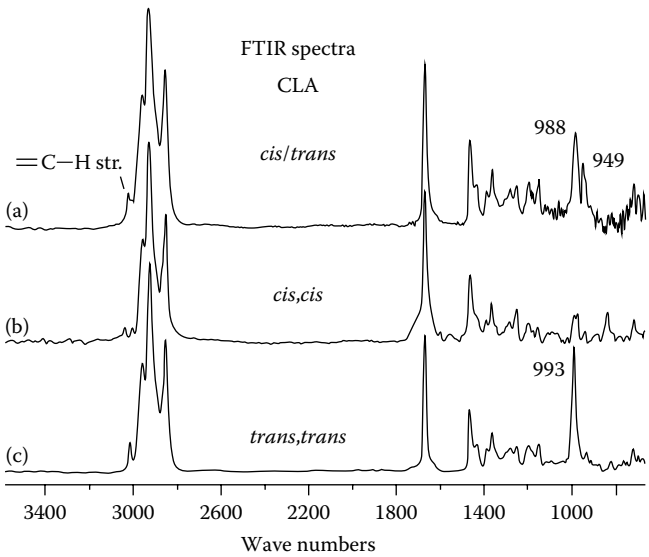

FIGURE 7.9 Direct deposition-FTIR spectral bands observed at 4 cm^{-1} resolution for conjugated (a) *cis/trans* or *trans/cis* 18:2, (b) *cis,cis*-18:2, and (c) *trans,trans*-18:2 FAME CLA isomers after separation by capillary GC and cryogenic trapping under vacuum. (From Mossoba, M.M. et al., *Lipids*, 29(12), 893, 1994.)

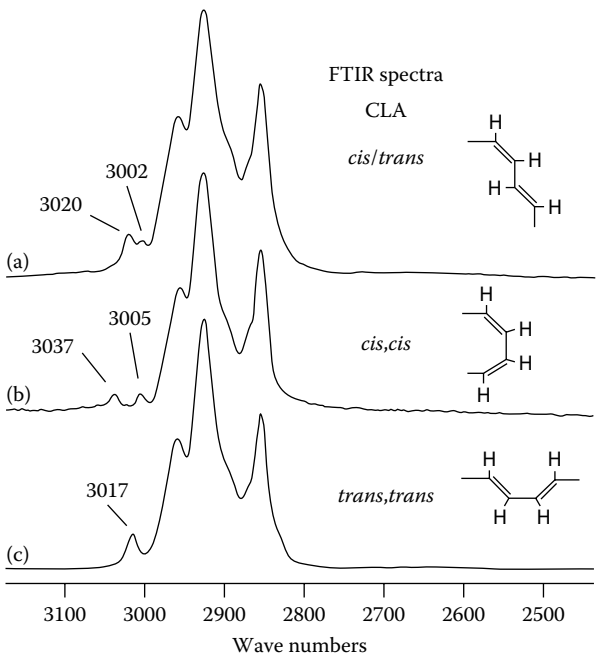

FIGURE 7.10 Direct deposition-FTIR spectral bands observed at 4 cm^{-1} resolution for conjugated (a) *cis/trans* or *trans/cis* 18:2, (b) *cis,cis*-18:2, and (c) *trans,trans*-18:2 FAME CLA isomers after separation by capillary GC and cryogenic trapping under vacuum. Expanded IR spectral range showing C−H stretching bands for conjugated *cis–trans* (top spectrum) and *trans–trans* (bottom spectrum) 18:2 dienes. (From Mossoba, M.M. et al., *Lipids*, 29(12), 893, 1994.)

Chemical ionization (CI) methods for the determination of double- and triple-bond positions were reviewed in 1985 [134]. A CI-MS procedure was used to determine the double-bond positions in fatty acids from marine organisms [135].

One successful approach for elucidating fatty acid structures has been to derivatize the carboxyl group to a nitrogen-containing compound. Common derivatizing agents include pyrrolidide, picolinyl ester, and 4,4-dimethyloxazoline (DMOX). A recent review of these derivatives indicates that the most useful ones by far are the picolinyl ester and DMOX derivatives [136]. With these derivatives, double-bond ionization and migration are minimized. Simple radical-induced cleavage occurs at each C−C bond along the chain. Therefore, for unsaturated fatty acids containing up to several double bonds, there is decreased abundance of low mass ions and an increase in a series of ions resulting from carbon–carbon bond scission. Diagnostic ions occur wherever there is a functional group in the chain that interrupts the pattern of cleavage from C−C bonds. A C=C bond or a five- or six-membered ring might be responsible for such disruptions.

GC-EIMS analysis of picolinyl ester derivatives can identify PUFAs [137,138]. However, the GC resolution of the picolinyl fatty acid esters was not as good as that of other derivatives (such as DMOX). Reversed-phase HPLC fractionation of the picolinyl fatty acid esters, prior to identification by GC-MS, was necessary to obtain acceptable results for hydrogenated samples [139]. A total of 39 fatty acid components in cod liver oil were identified using this method. In a more recent study, silver ion HPLC was used to fractionate CFAMs before converting them to the picolinyl ester derivatives [140]. Some of the GC problems associated with picolinyl esters could be overcome by using high-temperature, low-bleed, cross-linked polar columns [141].

Mossoba et al. [142] reported that 2-alkenyl-4,4-dimethyloxazoline derivatives of diunsaturated CFAMs exhibited distinctive mass spectral fragmentation patterns that could be used to pinpoint the positions of double bonds and of 1,2-disubstituted, unsaturated, five- and six-membered rings along the hydrocarbon chain. One of the advantages of using the DMOX derivatives was the good chromatographic resolution: sometimes higher than that observed for the derivatives of FAMEs [143,144]. Most CFAMs in heated flaxseed oil were identified, and the double-bond configurations (*cis* or *trans*) were unequivocally established by using GC-MI-FTIR (Table 7.3).

TABLE 7.3
Infrared Bands (cm^{-1}) Attributed to Unsaturation Sites in Cyclic Fatty Acid Monomers

GC Peak	Ring Five-Mem	*Chain Trans*	Ring Six-Mem	*Chain Cis*	*Chain Trans*	*Chain Trans*	Ring Six-Mem	Ring Five-Mem	Ring Six-Mem
1	3061	3035			3003	970		719	
2	3061	3035			3003	970		719	
3 + 3′	3061			3005				716	
4	3063	3032			3005	979		716	
6	3063			3006				711	
7	3063			3006				711	
8			3032		3000	976			663
9			3032		3005	972			664
10			3032		3005	972			664
11			3032		3004	975			663
12			3031				723		664
13			3031				725		662
14			3025						
15			3025						

Source: Mossoba, M.M. et al., *J. Am. Oil Chem. Soc.*, 72(6), 721, 1995.

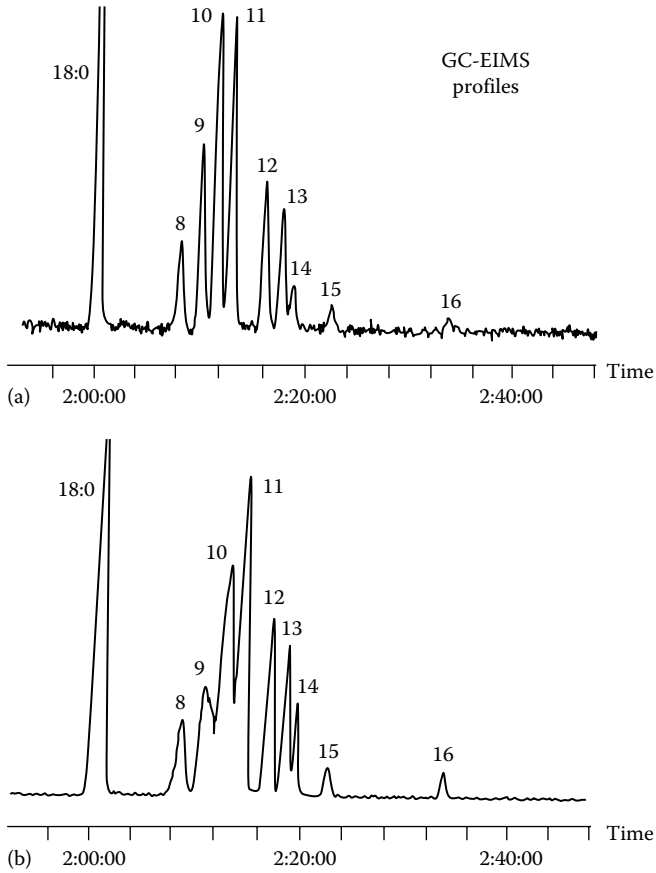

FIGURE 7.11 GC-EIMS chromatographic data for fatty acid DMOX derivatives of *trans* 18:1 positional isomers using an SP-2560 100 m fused silica capillary column at 140°C. Injection volume: (a) 0.4 μL and (b) 1.0 μL. (From Mossoba, M.M. et al., *J. Am. Oil Chem. Soc.*, 74(2), 125, 1997.)

Mossoba et al. [132] confirmed the identity of individual *trans* monoene fatty acid positional isomers in partially hydrogenated soybean oil. FAMEs were fractionated by silver ion HPLC and then analyzed by GC-DD-FTIR to determine geometric configuration and by GC-EIMS on DMOX derivatives to determine double-bond position [132]. GC peak resolution obtained with a 100 m capillary column was higher for the DMOX derivatives than for those of the FAMEs. Figure 7.11 demonstrates the excellent resolution that was obtained for DMOX derivatives of *trans* monoene positional isomers. The bottom GC profile, with about twice the amount of injected sample, shows evidence of overload in the early part of the trace but enhanced response for the Δ13 and Δ14 positional isomers. The double-bond positions for nine individual *trans* monoene positional isomers were confirmed by their unique DMOX mass spectra. Most significantly, this was the first report of the capillary GC separation of the Δ13 and Δ14 *trans* monoene positional isomers in hydrogenated vegetable oil. Figure 7.12 presents the mass spectral data that confirmed their bond position. The identity of the *trans*-Δ13 isomer was further confirmed by comparison with a standard. Hence, the double-bond position and configuration could be readily established for a complex distribution of MUFA isomers found in dietary fat by using the two hyphenated techniques, GC-DD-FTIR and GC-EIMS.

Mass spectral methods that permit direct analysis of FAMEs are highly desirable because GC analysis of FAMEs remains the method of choice for the determination of fatty acid composition.

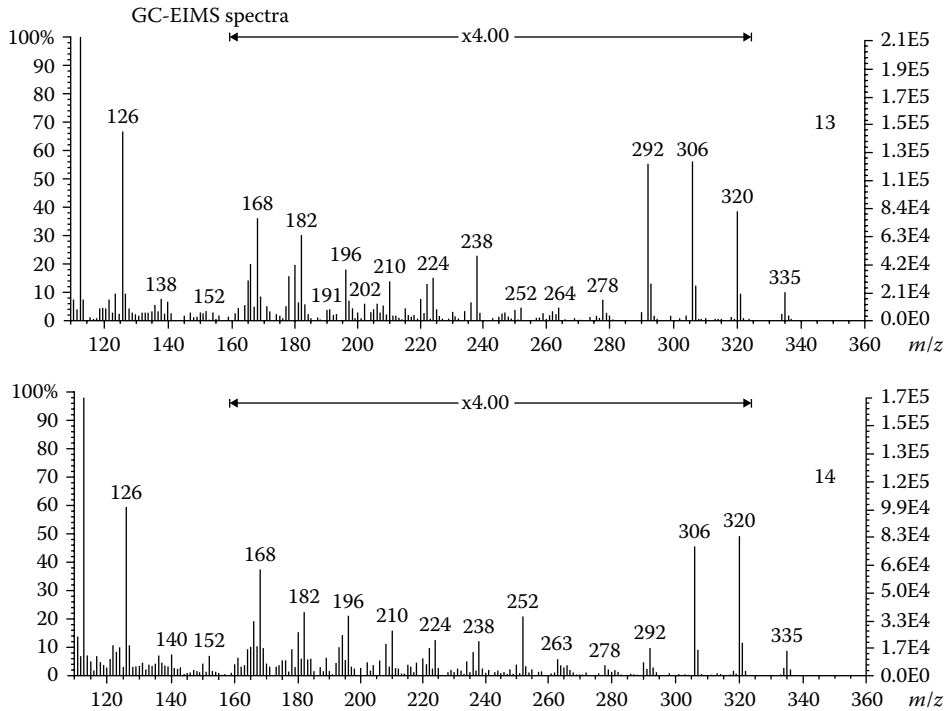

FIGURE 7.12 GC-EIMS spectra for the Δ13 and Δ14 pair of *trans* 18:1 DMOX positional isomers; the ions due to allylic cleavage are at the following positions: Δ13: 238, 293, and 306 *m/z*; Δ14: 252, 306, and 320 *m/z*. (From Mossoba, M.M. et al., *J. Am. Oil Chem. Soc.*, 74(2), 125, 1997.)

Since 1999, Brenna has published a series of papers demonstrating a purely mass spectrometric technique for double-bond localization in FAME isomers that relies on gas-phase derivatization [145–149]. He introduced the acronym CACI, Covalent-Adduct Chemical Ionization, to describe analytical methods that rely upon an ion–molecule reaction followed by tandem mass spectral (MS/MS) analysis. He applied the acetonitrile CACI-MS/MS technique to the analysis of nonconjugated and conjugated FAMEs double-bond localization. Observed highly reproducible mass spectra were readily interpreted and used to identify double-bond position and *cis*/*trans* geometry. In addition, because FAME were analyzed directly, comparable CACI-MS/MS and GC-FID gas chromatograms could be achieved and used to facilitate unknown FAME peak identifications. Subsequent studies with the CACI-MS/MS method demonstrated its utility for quantifying individual *cis* and *trans* MUFA that otherwise coeluted on the GC column [150–152].

C. Supercritical Fluid Extraction–Fourier Transform Infrared Spectroscopy

Capillary SFC is an effective tool for separating nonpolar to moderately polar complex mixtures of natural products having molecular weights of 100–1000 Da. The SFC method uses a gas compressed above its critical temperature and pressure to carry analytes through a chromatographic column. Many applications of SFC to the analysis of lipids were reviewed in the 1990s [153–157]. Among the advantages of SFC are its usefulness in separating certain compounds at lower temperatures than those required for GC and the ability to be simultaneously interfaced with FID and FTIR detectors. Different SFC columns can be used to separate lipids according to carbon number or degree of unsaturation. However, capillary GC and silver ion HPLC columns give better peak resolution when separating fatty acid derivatives from hydrogenated oils.

SFC has also been used to obtain structural information, backed up by MS for detection [158,159]. The main limitation of early applications was the lack of suitable commercial interfaces [160].

Microscale supercritical fluid extraction (SFE) can be directly coupled to a capillary SFC column. This procedure was used to analyze the fatty acid composition of a 1 mg sample of cottonseed kernel [157].

Combining SFC with online FTIR detection (SFC-FTIR) permits elucidation of structural information from fatty acid isomer mixtures. Standards as well as fatty acids were separated from coconut oil, Ivory® soap, soybean oil, and butterfat by means of a packed SFC column [161]. The presence of unsaturated fatty acids was verified by a maximum IR absorption at 3016 cm^{-1}. SFC-IR spectroscopy was used to analyze triacylglycerols and free fatty acids under similar conditions [162]. One study featured an SFC coupled to an FTIR spectrophotometer equipped with an LP flow cell to determine the level of *trans* unsaturation in partially hydrogenated soybean oil [163]. The IR absorption band at 3016 cm^{-1} indicated the presence of linoleic acid, while the 972 cm^{-1} absorption band indicated the presence of *trans* unsaturation.

A packed microcolumn argentation SFC system was used to separate and quantitate triacylglycerols in vegetable, fish, and hydrogenated oils [164]. This system gave excellent separations for these complex samples and was as effective as HPLC.

V. *TRANS* ISOMERS IN COMMERCIAL PRODUCTS

Although the relationship of dietary lipids to human health is a complicated issue that has sparked some controversy, the importance of controlling fat intake to help maintain an active and healthy lifestyle has been recognized for many years. Consumer health concerns about the types and content of dietary fat have resulted in a great deal of research. Much of the negative publicity is due to the effects *trans* fatty acids have on serum cholesterol. As a result, some food products were reformulated to reduce their total fat and *trans* fatty acid content even before the FDA ruling regarding the determination that PHOs are no longer GRAS [3].

A comprehensive survey of approximately 2000 food products known to contain industrially produced *trans* fatty acids, performed during 2009–2010 [165], estimated the cumulative intake of industrially produced *trans* fatty acids to be 1.3 g per person per day (g/p/d) at the mean for the U.S. population, a significant decrease from the previous 2003 estimate of 4.6 g/p/d for adults [165]. This is due to the fact that some food categories (frozen potato products, frozen seafood) have been reformulated to remove PHOs completely, and other categories already had *trans* fat levels below the LOD of 0.1 g/100 g, (e.g., canned soups, cereals, peanut butter, flavored potato chips). Certain products have been successfully reformulated such as baked goods (0–3 g/serving), frozen pizza (0–4.5 g/serving), frozen pies (0–4.5g/serving), and microwave popcorn (0–7 g/serving), by some, but not all, manufacturers. By contrast, some food categories consistently showed *trans* fat levels that had not been reduced, for instance, frosting (1–2.5 g/serving) and refrigerated dough products (1–3.5 g/serving) [165].

Dairy products are another important source of *trans* fats in some countries because milk fat may contain up to 8% *trans* fat (as percent of total fat) [120,166]. The predominant *trans* fatty acid among the *t*-18:1 isomers found in dairy fat is vaccenic acid (11*t*-18:1), and, as stated earlier, that is a precursor of CLA.

VI. CONCLUSIONS

Even though PHOs are no longer considered *GRAS* by the FDA and should be out of the food supply, it is still important to have rapid, accurate, and simple methods to determine the total *trans* content. In fact, precisely because PHOs should not be found in food, it is essential to have rapid, simple, and accurate methods for confirming their presence or absence. As reviewed in this chapter, the validated gas chromatographic and IR methodologies have been, and/or are currently being,

further optimized to increase their accuracy and reliability for measuring low levels of *trans* in food products and dietary supplements for regulatory compliance purposes.

Another important area of study is the nutritional effect(s) of many positional and/or geometric isomers of fatty acids. Any study designed to determine the nutritional properties of various individual *cis* and *trans* geometric and positional isomers with one or more double bonds (nonconjugated or conjugated) must first identify and quantify those individual fatty acid isomers. The complex isomer mixtures in various food matrices inevitably contain overlapping *cis* and *trans* GC peaks that make identification difficult. Capillary gas chromatography on long, highly polar columns will continue to be the industry standard for separating fatty acid isomers. However, as discussed in this chapter, GC alone cannot separate all fatty acid isomers in PHOs or other complex food matrices. Therefore, a combination of off-line, online, or advanced techniques, such as 2D GC separation, should be used to separate and identify individual fatty acid isomers. The commercial availability of reliable Ag-HPLC columns should increase their use, and silver ion chromatography will continue to be an important chromatographic tool that can be combined with other techniques. Complementary mass spectral analyses based on the GC-EIMS DMOX derivatives approach, or the more recent CACI-MS procedures for methyl esters, are also expected to continue to play a critical role in the analysis of both isolated and conjugated *trans* fatty acids.

The accurate determination of the total *trans* content and of individual *trans* isomers in complex mixtures of both natural and partially hydrogenated oils and fats remains a challenging task. The promising role of mid-IR and/or NIR spectroscopies in conjunction with chemometrics for the rapid determination of very low levels (<1%) of total *trans* fat found in foods and refined edible oils will be further pursued in our laboratory.

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8 Physical Characterization of Fats and Oils

Fernanda Peyronel, Edmund D. Co, and Alejandro G. Marangoni

CONTENTS

I. INTRODUCTION

Fats and oils are substances with many material properties. Chief among these are their thermal properties, solid fat content, mechanical properties, and structural features from the molecular size range to the colloidal size range. The physical characterization of fats and oils involves the determination of these material properties, often with a view to their functionality and stability in food systems.

As an example, the spreadability of a shortening is greatly affected by how the solid fat content of the shortening changes over a range of temperatures. Determination of the solid fat content over this temperature range is an indication of how appropriate the shortening would be for manufacturing a specific baked good. Likewise, the melting of chocolate confections in the mouth is dependent on the polymorphic form in which the fat comprising the confection is tempered into as well as the melting point of the fat. Fat and oil processing, in general, is often conducted with an aim of achieving a given material property. For example, interesterification of a hardstock fat with an oil is often conducted to expand the melting range of the blend. A material with such an expanded melting range is more functional than a simple mixture of hardstock and oil.

Fats have been studied for more than 60 years as crystalline materials. Here is a brief description of what a crystalline material is.

A crystal is a material in the solid state where the atomic constituents (referred to as the *asymmetric unit*) are arranged and packed into an orderly and regular fashion in 3D space. The asymmetric unit is arranged relative to a *space lattice*, which is an abstract, 3D, infinite array of lattice points, which serve as the scaffolding for the arrangement of asymmetric units into a crystal. The unique arrangement of asymmetric units around a given lattice point is called the *crystal structure*, which is contained in a fundamental unit called the unit cell.

A crystalline material may be *monocrystalline*, whereby the entirety of the material is a single, continuous, and uninterrupted crystal. Such a material is called a single crystal and is commonly observed in minerals such as rock salt, where the single crystal can achieve dimensions in the millimeter to centimeter range, if not greater. However, molecules with atomic structures and phase behaviors more complicated than those of inorganic salts invariably form polycrystalline materials. *Polycrystalline materials* are materials that consist of several single crystals of different sizes and orientations (called crystallites or grains), which usually have dimensions in the micrometer to nanometer size range, and as such, are often referred to as *microcrystalline materials*. A block of steel, comprising several "grains" of iron and carbon crystals, is such a polycrystalline material. Likewise, a block of fat is a polycrystalline material in the sense that it comprises aggregates of several single crystalline particles (called a crystal nanoplatelet, or CNP) in the nanometer size range. Like many polymers, a block of fat also has the distinction of being a *semicrystalline* material, which is a material that contains both crystalline and amorphous phases. In the case of a block of fat, the amorphous phase is the liquid oil that fills the interstitial space between the crystalline particles.

This chapter discusses several techniques for the determination of the material properties of crystalline fats. These include

1. The x-ray scattering technique with applications into x-ray diffraction (XRD), small-angle x-ray scattering (SAXS), and ultra-small angle x-ray scattering (USAXS) for the determination of the crystal polymorphic form, bilayer dimensions, primary crystal unit size as well as the macromolecular microstructural element sizes
2. Low-resolution pulsed nuclear magnetic resonance (pNMR) for the determination of the solid fat content (SFC) and for the determination of the T_2 relaxation time in fats
3. Differential scanning calorimetry for the determination of the state transition properties of fats, such as melting and crystallization temperatures and the associated melting and crystallization enthalpies
4. Small-deformation and large-deformation rheology for the determination of the viscoelastic and mechanical properties of fats

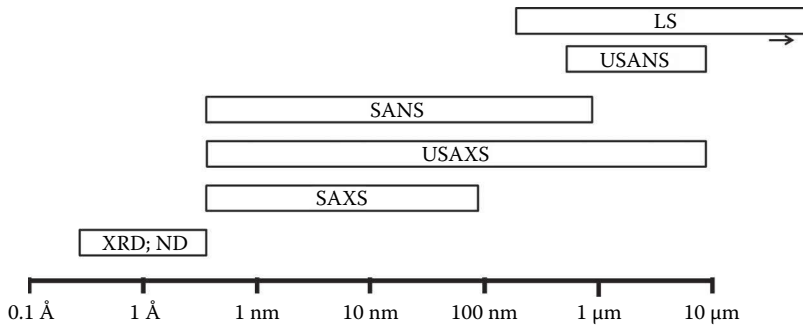

FIGURE 8.1 Comparison of spatial length scales cover by x-rays (ultra-small angle x-ray scattering [USAXS], small-angle x-ray scattering [SAXS], x-ray diffraction [XRD]), neutrons (ultra-small angle neutron scattering [USANS], small-angle neutron scattering [SANS], neutron diffraction [ND]), and light dynamic techniques.

II. SCATTERING METHODS

Scattering is a physical phenomenon wherein a beam characterized by a definite linear momentum is scattered from its original trajectory because of an object in its path. The beams can be constituted of visible light, x-rays, and neutrons. The object that intercepts this beam could be electrons, atoms, molecules, or any nanometer- to micrometer-sized structure.

For example, dynamic light scattering uses a laser in the visible light spectrum and the Brownian motion of suspended particles to determine their sizes. X-ray scattering uses the scattering of an x-ray beam by the electron cloud surrounding the atoms to extract information about the internal structure of the object under study. Neutrons are also used to study the internal structure of materials but in this case, a beam of neutrons is scattered by nuclei.

X-ray and neutron scattering are divided into three categories according to the technique used: diffraction, small-angle scattering, and ultra-small angle scattering. The abbreviations are: x-ray diffraction (XRD); neutron diffraction (ND); small angle x-ray scattering (SAXS); small-angle neutron scattering (SANS); ultra small angle x-ray scattering (USAXS); and ultra-small angle neutron scattering (USANS). The region probed is used to name the technique. X-ray diffraction is mainly used for large angles; hence, wide-angle x-ray scattering (WAXS) is associated with this technique. Each technique is characterized by the optics used before and after the sample as well as the detectors.

Figure 8.1 shows the spatial length scale that is probed by each of the scattering techniques.

The focus of this section is on x-ray scattering. The popularity of x-ray scattering comes from the fact that the sample requires little preparation, allowing an *in situ* characterization.

A. Basics of X-Ray Scattering

An x-ray experiment consists of directing a collimated x-ray beam onto a target or sample and observing the outcoming x-ray beam by means of a detector positioned at the appropriate angle and distance (Figure 8.2) so as to reveal the sample structure at the length scale of interest. An x-ray beam is a collection of photons, which can be described by the wave vector $\mathbf{k}$, which indicates the direction of propagation. The wave number is given by the modulus of the wave vector, $k = |\mathbf{k}| = 2\pi/\lambda$, where λ is the x-ray wavelength. Assuming elastic scattering, the incident $\mathbf{k_i}$ and the scattered $\mathbf{k_s}$ wave vectors have the same magnitude (Als-Nielsen and McMorrow, 2001).

The scattering vector $\mathbf{q}$ is given by

$$\mathbf{q} = \mathbf{k_s} - \mathbf{k_i} \tag{8.1}$$

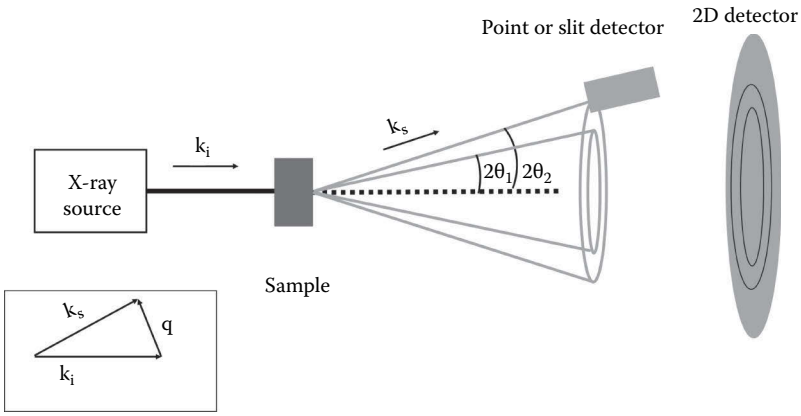

FIGURE 8.2 Basic schematic representation of an x-ray scattering experiment showing the direction of the incident x-ray wave ($\mathbf{k}_i$) coming from the x-ray source and the direction of the scattered wave vector ($\mathbf{k}_s$) after the beam went through the sample. 2θ is the scattered angle according to Bragg's law. Shown are two cartoon representations of detectors. The 2D detector will capture the scattered x-rays that form cones. A slit detector will capture only an arc of the cones and not the whole cone. A point detector detects one point and hence needs to be moved to collect all the necessary information.

This scattering vector identifies the direction and magnitude (inset in Figure 8.2) of what accounts for the angle-dependent phase difference of interfering scattered waves. When an x-ray beam is incident on the sample, the x-rays are scattered in cones.

The scattering wave vector is defined as the difference between the scattered wave vector and the incident wave vector. It can be shown that the magnitude, or modulus, of the scattering q vector is

$$q = \left|\mathbf{k_s} - \mathbf{k_i}\right| = \frac{4\pi \sin\theta}{\lambda} \tag{8.2}$$

This modulus, **q**, has units of length^{-1}; hence, it is referred to as belonging to reciprocal space. q^{-1} represents the length scale, L, of interest in the system under study which can be determined from

$$L = \frac{2\pi}{q} \tag{8.3}$$

For example, a large value of q^{-1} like 2 Å^{-1} indicates atomic distances, while $q^{-1} = 4 \times 10^{-5}$ Å^{-1} indicates distances in the order of 10 μm.

The x-ray scattering regions are typically separated according to the value of the scattering vector q. Table 8.1 shows the scattering vector magnitudes associated with each of the different length scales and techniques.

B. Powder X-Ray Diffraction

The premise of the powder diffraction technique is that the specimen is a collection of randomly oriented crystals. Diffraction experiments are associated with the notion that x-rays scatter in certain preferential directions after their encounter with the scattering object. The length scale of diffraction experiments is in the order of a few nanometers.

Bragg's theory uses the notion of mirror reflections, where the diffraction from a crystalline sample can be explained as a reflection of the incoming x-rays by a series of crystallographic planes, the lattice planes. A regular 3D array of points is considered the basis of a crystal. From these

TABLE 8.1
Spatial Length Scales Covered by the Different X-Ray Techniques

Spatial Scales in the Material	Length Scale or "d" Spacing (Å)	q Range ($Å^{-1}$)	Technique for Observation
Atomic	1–10	6–0.6	WAXS
Molecular	10–100	0.6–0.06	SAXS
Nano	100–5000	0.06–0.007	SAXS/USAXS
Micro	1–20 μm	6×10^{-4}–3×10^{-5}	USAXS

WAXS, wide-angle x-ray scattering; SAXS, small-angle x-ray scattering; USAXS, ultra-small angle x-ray scattering.

points, "crystallographic planes" can be defined according to the position of the atoms in the crystal, considering that all parallel planes contain the same arrangement of atoms. The orientation of the planes in the lattice is defined in terms of the notation introduced by the English crystallographer Miller, the "Miller indices," (hkl), where h, k, l denote integer numbers that identify the position of a plane in relation to the origin (Culity and Stock, 2001). The advantage of using this system is that there exists a family of parallel equidistant planes with a spacing d_{hkl} between them that are parallel to any (hkl) crystallographic plane. In Bragg's approach, each crystallographic plane in the set {hkl} is considered a scattering object. Bragg showed that diffraction from all parallel planes in a set is only possible at certain angles. Only the outgoing diffracted in-phase x-rays will contribute to a constructive interference manifested as a Bragg peak.

Figure 8.2 shows a geometrical description of the x-ray scattering from a set of parallel atomic planes. Since the incident x-rays impinging onto the crystal are in phase, constructive interference among the diffracted x-rays can only occur if the difference in path length that the incident and scattered x-rays travel is a multiple integer of the wavelength. Figure 8.3 shows that constructive interference will be achieved when the path length 2–2′ differs by (δ + δ) from the path length that the x-ray 1–1′ follows. Constructive interference occurs when $n\lambda = 2\delta$, where n is an integer number.

Bragg's law relates the scattered angle (θ) to the wavelength of the incoming x-ray (λ) and the spacing between the atomic planes (d_{hkl}) as shown in Equation 8.4 (Cullity and Stock, 2001; Pecharsky and Zavalij, 2009):

$$n\lambda = 2d_{hkl} \sin\theta \tag{8.4}$$

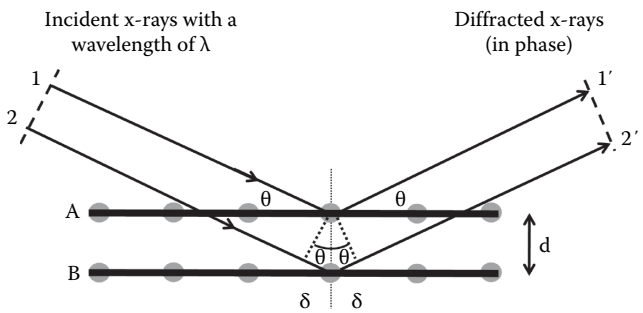

FIGURE 8.3 Diagram representing the geometrical approach to x-ray diffraction. Shown are two incident x-rays that are diffracted by two atomic planes, A and B, separated by a distance d.

Two geometries can be employed in x-ray powder diffractometers: reflection or transmission. In an apparatus configured to use the reflection geometry, x-rays are aimed to the sample at a certain angle of incidence and the diffracted x-ray is collected on a detector positioned at a certain angle. Of course, the phenomenon taking place is not reflection but rather diffraction. X-rays are diffracted off in cones with the opening corresponding to the various Bragg angles. It is important to notice that Equation 8.4 says nothing about orientation. These cones appear because of the nature of the sample: randomly oriented crystals. The incident beam always finds a crystallite in an appropriate direction and the cone seems continuous, but it is generated by different crystallites. Benchtop diffractometers used typically an x-ray tube emitting only at a particular wavelength. If an x-ray copper tube is used, then the wavelength is 0.154 nm. Some instruments are designed in a way that both the x-ray source and the detector are mounted on a goniometer, which may permit the movement of both of them simultaneously. In this reflection geometry, a range of angles are scanned, the exact range being dependent on the structural features to be elucidated. Detectors use this kind of instrument might be either a point or a slit detector. The detector will collect only a section of the scattered cone. The intensity of the diffracted x-ray waves is recorded as a function of the Bragg angle.

When transmission geometry is used, the incident x-ray beam goes through the sample. In this case, the source and the detectors are mounted on fix positions. Instead of "scanning" for each angle as occurs in the reflection setup, all the angles are simultaneously "scanned." This kind of setup typically uses a 2D detector. This detector records the maxima generated by constructive interference as concentric rings (called Debye–Scherrer rings), which are cross sections of the cone of the diffracted x-rays.

XRD can be used to carry out measurements in the WAXS and SAXS regions for fats. Triacylglycerol (TAG) molecules are the main molecules that make up what is known as fat. Thus, the study of fat focuses mainly on understanding the packing of TAG molecules within a crystal. Table 8.2 shows the difference between WAXS and SAXS with regard to the angle of scattering and the information obtained in fats. The hydrocarbon chains are packed side by side in a TAG molecule. The type of lamella formed by longitudinal TAG stacking can be observed in the SAXS region. This is sometimes called polytypism. The lateral atomic packing of TAGs, or the polymorphism, is identified from the WAXS region.

Figure 8.4 shows the WAXS and SAXS regions of fully hydrogenated canola oil. The largest peak in the SAXS region is considered to be the (001) Bragg reflection, while the other significant peak is the (003) reflection. This indicates that $3 \times d_{003} = d_{001}$, where d stands for the interplanar distance as shown in Figure 8.3.

Using the concept of the unit cell, the (001) interplanar distance is identified with the scattering on the TAG lamella denoted by d in Figure 8.5a. The TAG lamella could be formed by the length of two hydrocarbon chains, if the *chair* conformation of the TAGs is followed (Figure 8.5a) or by the length of three hydrocarbon chains, if the *tuning fork* configuration is present.

The subcell concept (Figure 8.5b) has been useful in understanding the x-ray scattering in the WAXS region, as shown in Figure 8.4. The subcell is a subunit of the unit cell, which takes into account at least four hydrocarbon chains and few atoms. Figure 8.5b displays one particular case: the triclinic packing. Three possible sets of crystallographic planes that scatter x-rays are shown,

TABLE 8.2
Information Obtained from the Two Regions, WAXS and SAXS, of X-Ray Diffraction Experiments Studied in Fats

Region Name	Diffracted Angle	Characteristic Size of Scattering Object (Å)	Information Obtained for Fats
WAXS	$6° < \theta < 18°$	2–10	Polymorphism
SAXS	$0.5° < \theta < 6°$	30–300	Polytypism and crystal domain size

Note: θ is the Bragg angle.

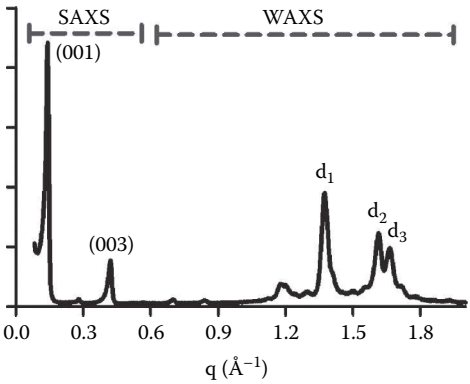

FIGURE 8.4 X-ray pattern in the SAXS and WAXS region for fully hydrogenated canola oil in high oleic sunflower oil.

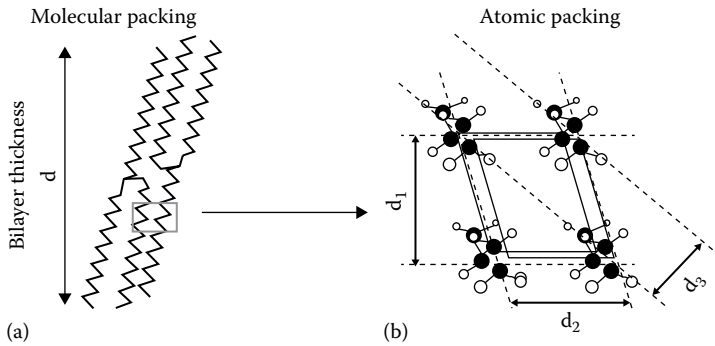

FIGURE 8.5 Hydrocarbon chains denoted as zigzag lines that make up a triacylglycerol molecule. (a) Bilayer formed by the packing of two triacylglycerol (TAG) molecules, showing a chair conformation. (b) The subcell concept is used to explain the crystallographic atomic planes giving rise to the x-ray scattering observed in the WAXS region.

separated by the distances d_1, d_2, and d_3. These three characteristic distances are the one responsible for the three main peaks observed in Figure 8.4 in the WAXS region.

In the case of the fully hydrogenated canola oil in high oleic sunflower oil, d_1, d_2, and d_3 are 4.6, 3.9, and 3.7 Å, respectively, which correspond to the β polymorphic form.

Triacylglycerols display polymorphism, which means that the molecules can pack laterally into different physical structures in the solid state. Three basic polymorphs of mono-acid TAGs have been identified, α, β′, and β, by powder x-ray diffraction, on the basis of their subcell structure (Larsson, 1966). The α form is metastable, with hexagonal packing (H) and $d_1 = 4.15$ Å. The β′ form is also metastable, displaying chains that are packed in an orthorhombic perpendicular fashion (O⊥) and the characteristic $d_1 = 4.2$ and $d_2 = 3.7$, while the β form is stable, with triclinic parallel packing (T//) and $d_1 = 4.6$ Å, $d_2 = 3.9$ Å, and $d_3 = 3.7$ Å. Usually, a rapid cool down from a melt will cause the formation of the metastable α form, which eventually will transform to either the β′ form or the stable β form. The pathway to reach a stable polymorphic form depends on processing conditions such as cooling rate, temperature, shear, pressure, and composition (Sato, 2001).

1. Sample Preparation

A powder diffraction pattern is achieved when there are a sufficiently large number of small randomly oriented particles with dimensions between 10 and 50 μm. If the material is not in the

form of small particles, the particle size should be reduced. If there is no random orientation, the cone made by the scattered x-ray at a particular angle might not be continuous.

Crystal size is not a problem with fats since they are composed of crystallites possessing a suitable size. Randomness might be a problem if the fat was crystallized under laminar shear. In this case, the use of a 2D detector will show the preferential orientation of the crystallites. If a slit detector is used, it might be necessary to manually rotate the sample to be able to detect the orientation. In a 2D detector, the orientation manifests itself with stronger signal at certain points along the circle produced by the cone.

The thermal and processing history of a fat is partially responsible for its crystal structure. These two factors must be taken into account when choosing the accessories to carry out the measurement as well as how to mount the sample. For example, it might be necessary to carry out the x-ray measurements at a particular temperature to prevent polymorphic transformations. In this case, the sample should be mounted on a temperature-controlled sample holder. Another example is a sample that was prepared under fast cooling conditions for which it might be necessary to observe the structure immediately.

The Rigaku multiflex diffractometer uses sample holders with a well not bigger than 20 mm × 20 mm and 0.3 mm in depth. Glass and aluminum are good subtract for the sample holder, with dimensions of 20 × 20 × 1 mm. Filling the cavity with a powderlike material is simple and only requires a spatula. The powder should stay loose in order for the crystals to be in a random order. For samples that are paste-like, it is important to resist the temptation to squeeze down on the sample. Hard fat needs to be converted to powder ensuring that the friction introduced does not affect the structure that one wants to study. Occasionally, it is desired to crystallize directly in the sample holder. In this case, the hot liquid edible fat is poured into the cavity of the sample holder and allowed to crystallize. Care must be taken not to overfill or underfill the sample holder. Figure 8.6 shows the powder pattern histogram of 20% fully hydrogenated soybean oil (FHSO) in soybean oil (SO) when the cavity of the sample holder was overfilled, underfilled, and level.

Notice that in the WAXS region, overfilling samples shifts the peak to higher angles, while underfilling samples shifts the peak to lower angles. In the SAXS region, overfilling also causes the peak to shift to higher angles, while underfilling did not affect peak position.

2. WAXS Example

Polymorphism manifests itself in the WAXS region and is used as a key structural and functional indicator. For example, margarines are typically assumed to possess good spreadability when they are in the β′ polymorphic form (DeMan and Beers, 1987). Figure 8.7 shows two XRD patterns for cocoa butter (CB) in the β_V and in the α form. It is well known that β_V is the desired polymorphic form when manufacturing chocolates.

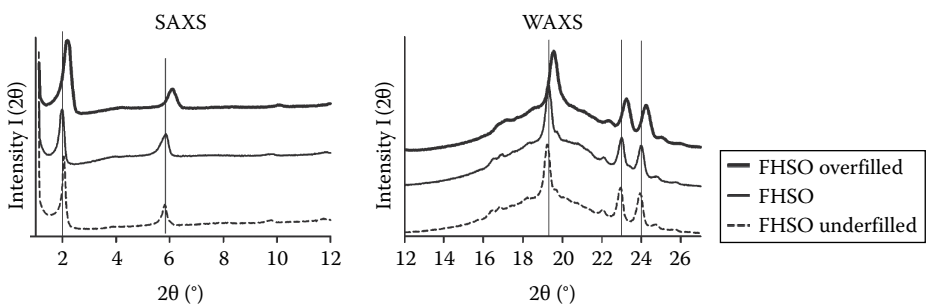

FIGURE 8.6 Powder x-ray diffraction pattern of 20% fully hydrogenated soybean oil (FHSO) in soybean oil (SO) showing a shift in the position of the peaks when the sample is correctly filled, underfilled, or overfilled.

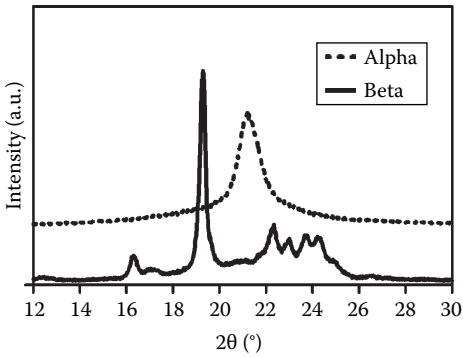

FIGURE 8.7 XRD powder pattern for cocoa butter in the β form (—) and the α (--).

The x-ray patterns shown in Figure 8.7 correspond to the beta and alpha form of cocoa butter. The α form was achieved by a fast cooling rate from 80°C to 20°C. The β form was obtained from the α form by storing the fast-cooled sample at 25°C for 24 hours. This is to emphasize that processing, storage temperature, and time play a crucial role in the final atomic structure of the solid state in fats.

The atomic structure or polymorphism does not tell the story as to which structure is better for which functionality. It is necessary to pair up x-ray experiments to a functional experiment to make this conclusion.

3. SAXS Example

SAXS experiments measure intensities at scattering angles not larger than 2° outside the primary beam. A conventional SAXS machine might be able to cover smaller scattering angles compared with a benchtop diffractometer. SAXS is used to obtain the longitudinal packing of TAGs. A typical SAXS experiment requires a detector placed relatively far away from the sample holder. In countertop instruments, it might be less than 1 m away, while at synchrotron facilities, due to the flux and energies of the x-ray, the detector can be placed as far as 5 m away. In both cases, the flight path of the scattered beam is protected by making it travel inside a tube filled with helium to prevent scattering by air. PinSAXS differs from the usual SAXS experiments with regard to the type and placement of the detector. A PinSAXS point detector is placed tenths of centimeters away from the sample. In order to have a good signal, a PinSAXS data acquisition might require longer accumulation times compared with a regular SAXS experiment (hundredths of seconds instead of few seconds).

An example of powder diffraction results in the SAXS region is displayed in Figure 8.8. The data were collected using a Rigaku multiflex x-ray powder diffractometer, which is neither a PinSAXS nor a true SAXS instrument. The Rigaku multiflex uses a Bragg-Brentano configuration (Pecharsky and Zavalij, 2009). Here, we show two patterns: tristearin (SSS) and tripalmitin (PPP). Three peaks are observed for each sample labeled with the numbers 1, 2, and 3 and a subscript SSS or PPP to identify each sample. Due to limitations in the equipment, values reported from the Rigaku instrument are not absolute values and are not accurate for angles of $2\theta < 3°$. This is why we tend to ignore the reported value of peak 1 and instead use a higher reflection of the (001) to compute the true position of peak 1.

Data points were fitted using Jade 9.0 (MDI, Livermore, CA). Values for the position of the peak's maximum are reported by the software after analysis. The user can choose among four different fitting equations: Gaussian, Pseudo-Voigt, Pearson-VII, and Lorentzian. Table 8.3 shows the results obtained with Jade using the Pseudo-Voigt equation.

The bilayer thickness can be used to determine the longitudinal packing of the TAG molecules, based on their fatty acid (FA) composition. Many bilayers or lamellae stack in the (001) direction

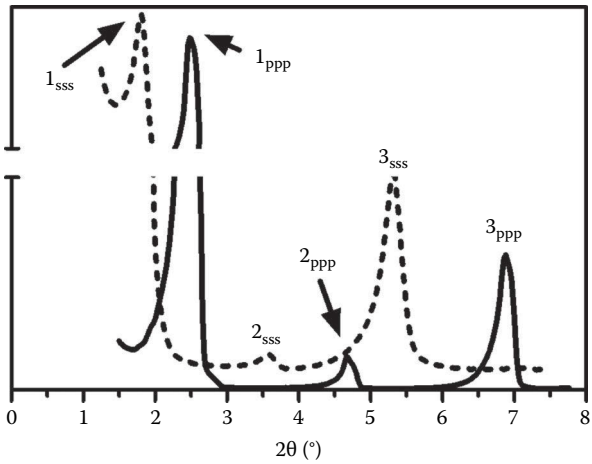

FIGURE 8.8 X-ray patterns in the SAXS region for tristearin (SSS) and tripalmitin (PPP).

TABLE 8.3
Parameters Obtained for the X-Ray Pattern from Figure 8.8 Using Jade and a Cooper Wavelength Value of 1.54 Å

Peak	2θ (°) ± 0.004	d (Å) ± 0.2	n	FWHM (°) ± 0.001	Bilayer Predicted	Crystal Thickness—Domain Computed
1_{PPP}	2.292	38.5	1		16[a] × 2.54[b] Å = 40.64 Å	
2_{PPP}	4.457	19.8	2	0.310		~26 nm
3_{PPP}	6.650	13.3	3			
1_{SSS}	1.780	49.6	1		18[a] × 2.54 Å= 45.72 Å	
2_{SSS}	3.555	24.8	2	0.257		~31 nm
3_{SSS}	5.315	16.6	3			

Notes: 2θ is the scattered angle, d is the particular interatomic distance among crystallographic planes to consider, n is the pertinent reflection (e.g., n = 1 indicates $d_{hkl} = d_{001}$, which is the first reflection), and FWHM is the full width at half maximum for the (002) peak. The predicted bilayer size considers a unit cell of two hydrocarbon chains in a chair configuration. The thickness of the crystal was obtained using Equation 8.6.

[a] Number of carbons in the hydrocarbon chain for this molecule.

[b] Distance between three carbons in the hydrocarbon chain as reported by Small (1966).

to make up the crystal thickness. A 2L longitudinal packing of TAG molecules within a bilayer indicates that the size of the lamellae is given by the length of two fatty acid chains. When the lamellar size corresponds to three fatty acid chains, this longitudinal packing is referred to as 3L.

The Scherrer equation, valid for crystallites with sizes smaller than 100 nm (Klug and Alexander, 1974), has been used to compute the thickness of the crystal (Acevedo and Marangoni, 2010; Mazzanti et al., 2003), referred to as the domain size. Also, due to the limitations of the Rigaku multiflex machine, we use the (002) peak instead of the (001) for this calculation. The position of the Bragg peak given by θ and its full width at half maximum (FWHM), both in radians, were used to compute the thickness of the nanocrystallite or domain size:

$$\text{Crystalline nanoplatelet thickness} = \frac{K\lambda}{\text{FWHM}\cos\theta} \tag{8.5}$$

where

λ is the wavelength (in nm)

K is a constant approximately equal to unity and related to the crystallite shape and to the way that FWHM and the thickness are defined

Scherrer's original derivation was based on the assumptions of a Gaussian line profile and small cubic crystal of uniform size. This gave a value of K = 0.94. The derivation by Klug and Alexander shows a value of K = 0.89 for no particular crystal shape. Here, we use K = 0.9.

This domain is formed by the epitaxial stacking of bilayers of TAGs to create a "crystallite" or nano-crystal now referred to as a CNP. The CNP surfaces were visualized with microscopy using different techniques of oil removal and solid fixation on different edible fats (Heertje et al., 1997; Jewell and Meara, 1970; Poot et al., 1975). In 2010, Acevedo and Marangoni (2010) introduced a new method of cold solvent extraction followed by cryo-TEM, which allowed for the first characterization of CNPs in fully hydrogenated canola oil in high oleic sunflower oil. Other authors have also used this technique in other edible fat systems (Acevedo et al., 2012; Acevedo and Marangoni, 2014a,b, 2015; Maleky, 2012; Maleky et al., 2011; Peyronel et al., 2013; Peyronel and Marangoni, 2014; Ramel et al., 2016) to show that in fact CNPs seem to be the primary crystals in these materials. These authors have shown that the dimensions of the CNPs depend on the TAG composition, the TAG proportions, and the processing conditions.

For the case shown in Figure 8.8 and Table 8.3, the thickness of the CNPs was determined to be ~26 nm for PPP and ~31 nm for SSS.

Using the d_{hkl} values for peak 2 as the bilayer length, one can compute how many bilayers make the thickness of the CNP:

26 nm/(19.8 × 2 × 10 nm) = 6.5 bilayers making the PPP crystal

31 nm/(24.8 × 2 × 10 nm) = 6.3 bilayers making the SSS crystal.

C. Ultra-Small Angle X-Ray Scattering

The technique of USAXS has gained popularity in the last decade thanks to advances in electronics and optics. It can be used to investigate spatial length scales of up to ~15 μm.

The structures observed at the larger spatial length scales are fundamentally different from those observed at atomic scales. USAXS looks at the nano- to microscale where matter is composed of more complex and nonuniform building blocks. USAXS brings about another dimension in the quest of understanding how the fat crystallites form larger structures of microscopic dimensions.

The USAXS technique uses a Bonse–Hart instrument or camera (Ilavsky et al., 2009, 2012). This instrument takes advantage of optics before and after the sample to collimate x-rays. The instrument used at the Advanced Photon Source (APS) in the Argonne National Laboratory uses a photodiode detector that has a dynamic intensity range of nearly nine decades. The Bonse–Hart instrument is paired up with a step-scanning method that uses a slit-smeared geometry. The data collection of USAXS experiments takes less than 2 minutes per scan at the beamline 9ID at APS. One of the unique features of the Bonse–Hart instrument is its absolute intensity calibration.

As with the other x-ray techniques, sample preparation is minimal. Grace-Bio Labs silicon isolators (Bend, OR) sandwiched between two microscope cover slips are excellent sample holders. Thanks to the beamline scientists, collecting data has become a routine and easy procedure. The biggest challenge with the USAXS technique is the analysis of the data. In the case of fats, we have used two analytical models to fit the data: the Unified Fit model (Beaucage, 1995, 1996) and the Guinier–Porod model (Hammouda, 2010). Both models employ a nonlinear regression analysis to find the best parameters. The analysis proceeds by identifying a number of "levels" that can be present at different regions of q. Each level is viewed as a structural level. This is where the challenge arises: How many levels are present? What are the physical interpretations of each of

those levels? Are those levels the result of a hierarchical structure or are they independent from each other?

The parameters of interest obtained from these two models were the slope, the radius of gyration, R_g, and the "s" value that identifies the shape of the scatterer (Peyronel et al., 2013, 2014c). The average size of the scatterer (either the CNP or the microcrystal) was computed from the R_g value. The slope was used to find information about the fractal dimension of the scatterers (Peyronel et al., 2013; Pink et al., 2013). It was concluded that in some cases, CNPs stacked to form thin long rods, the so-called TAGwoods (Pink et al., 2013). TAG mixtures were studied assuming a maximum of three hierarchical levels (Peyronel et al., 2014a,b). Figure 8.4 shows the possible structural levels observed in fats containing no water and less than 20% solids. At the lowest level, or the highest q region, the CNP morphology is given by the value of the slope. The morphology could be smooth, rough, or fuzzy (Peyronel et al., 2014b; Quinn et al., 2014). The level identified as "TAGwood level" is where the CNPs' aggregation into TAGwoods or other structures could be detected. The existence of this level depends not only on the components of the materials but most importantly on processing conditions that can form structures according to diffusion-limited cluster–cluster aggregation (DLCA) or reaction-limited cluster–cluster aggregation (RLCA). The size of these meso- to microstructures is given by the R_g observed at this level.

The inset in Figure 8.9 shows the x-ray pattern of the regions: WAXS, SAXS, and USAXS plotted on a log–log scale. Notice that USAXS covers almost four decades in q compared with less than one for WAXS and for SAXS.

To be able to explain USAXS results, one needs to appeal to a model. Our group looked at the aggregation of CNPs by using a computer model of the CNP (Pink et al., 2013). Computer simulations of the aggregation of CNPs were carried out using the Metropolis Monte Carlo technique. Van der Waals forces as well as a binding energy were the only forces used (Pink, 2012). It was through those simulations that TAGwoods came to life. The simulations and the USAXS experiments showed that in the case of 20% SSS in OOO, CNPs aggregate into long thin cylinders, which were named TAGwoods. These TAGwoods further aggregate to form larger fractal structures via either DLCA or RLCA.

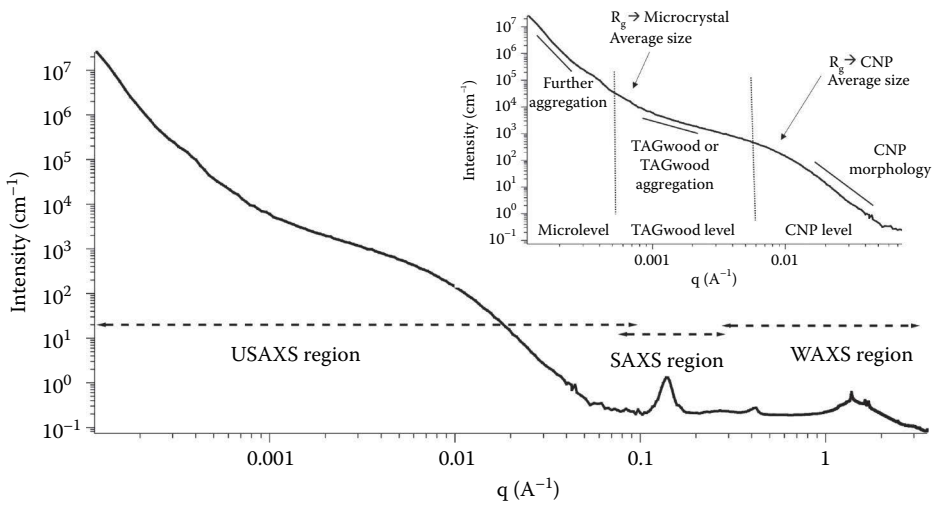

FIGURE 8.9 USAXS pattern of a sample of 20% SSS in OOO showing the three regions. The inset shows the three levels of aggregation obtained using the Unified Fit model that were corroborated by models and computer simulations predictions.

III. LOW-RESOLUTION PULSED NUCLEAR MAGNETIC RESONANCE

Techniques based on nuclear magnetic resonance (NMR) are a class of nondestructive techniques that characterize the physical and chemical properties of a material by exploiting the physical phenomenon known as nuclear magnetic resonance. In such techniques, the material (or more specifically, the NMR-active atoms of the material) is subjected to a static external magnetic field to induce quantization of energy levels and then excited with electromagnetic energy, specifically energy in the radio frequency spectrum. The relaxation of the system generates a signal (the free induction decay, FID), which contains information about the system. Most benchtop NMR techniques used to characterize the physical properties of fats are of the ^{1}H-pNMR variety. These instruments characterize the material by observing the nuclear magnetic resonance of ^{1}H atoms. Also, in such instruments, the system is typically excited by a radio-frequency pulse rather than a continuous application of electromagnetic energy, hence pNMR. Pulsed NMR should be distinguished from continuous-wave NMR (CWNMR). The CWNMR technique uses a radio frequency oscillator to continuously irradiate the sample as a magnetic field is swept through the field region that fits the resonance condition, causing resonance of a nuclear magnetic moment.

pNMR instruments typically work in the time domain, as opposed to the frequency domain, which is frequently used in chemical structure elucidation. NMR measurements made in the time domain occur over a time span of a few seconds to not more than a few minutes. The NMR phenomenon itself occurs in the order of microseconds to milliseconds. As such, NMR has developed into a standard laboratory technique because of its ability to give fast and reproducible measurements. Readers interested in more detailed discussions of NMR are referred to Keeler (2011).

The following section explains the basics of NMR using the Bruker Minispec principles of operation, which is discussed in Section III.E.

A. Basic Principles

The NMR phenomenon can be briefly summarized as follows:

1. The sample is subjected to a permanent external magnetic field, $\mathbf{B}_0$, which affects the spin moments.
2. The spin system responds to $\mathbf{B}_0$, generating a net magnetization $\mathbf{M}_0$ in the equilibrium state.
3. Another oscillating magnetic field $\mathbf{B}_{RF}$ (in the radio frequency spectrum) is applied during a limited time, causing the net magnetization $\mathbf{M}_0$ to change to a nonequilibrium state.
4. $\mathbf{M}_0$ relaxes back to its original equilibrium state. The relaxation process generates a signal, which is monitored over time to obtain information about the system. Additional pulses of radio frequency electromagnetic radiation can be applied during relaxation.

Of the myriad nuclei that exhibit NMR, the one that will be discussed in this section is the most abundant isotope of the hydrogen atom (^{1}H), which contains one proton in its nucleus and no neutrons. The lone proton exhibits a quantum mechanical property called *spin*. The proton is viewed as a spinning positive charge, causing it to have a magnetic dipole moment. This dipole is aligned with, and proportional to, its spin angular momentum, $\mathbf{s}$, such that $\boldsymbol{\mu} = \gamma\mathbf{s}$, where γ is the gyromagnetic ratio. The gyromagnetic ratio is the ratio of the classical magnetic moment to the classical angular momentum. Different NMR-active nuclei will have different gyromagnetic ratios. In particular, $\gamma = 42.567$ MHz/T for the nucleus of the ^{1}H atom.

The net nuclear angular moment $\mathbf{L}$ is the sum of the individual spin angular momenta, $\mathbf{L} = \sum \mathbf{s}$, and the net dipole moment is defined as $\mathbf{P} = \sum \boldsymbol{\mu} = \gamma\mathbf{L}$ of the sample, which is a vector sum of all of the individual dipole moments $\boldsymbol{\mu}_i$. The dipole moment per unit volume is called the *magnetization* and is given the symbol $\mathbf{M}$. Assuming uniformity within a sample of volume V, then $\mathbf{M} = \mathbf{P}/V$.

M is the sum of individual ^{1}H magnetic moments that are randomly oriented. In the absence of an external magnetic field, the net angular momentum will add to zero. If an external magnetic field $\mathbf{B}_0$ is applied, the energy levels that the various nuclear magnetic moments can occupy are quantized. For ^{1}H nuclei, there are two such energy levels: the low energy state (spin-up) and the high energy state (spin-down). At thermal equilibrium, there are more spins in the spin-up state than in the spin-down state. The ratio of the number of nuclear magnetic moments in the higher energy state N^- to the lower energy state N^+ is given as

$$\frac{N^-}{N^+} = e^{\frac{-\Delta E}{kT}} \tag{8.6}$$

where

k is the Boltzmann constant

ΔE is the transition energy between the spin-up and spin-down states

The prevalence of magnetic moments in the spin-up state results in a net equilibrium magnetization $\mathbf{M}_0$ (Figure 8.10) in the direction of $\mathbf{B}_0$, which can be written as $\mathbf{M}_0 = M_0\hat{\mathbf{z}}$ and $\mathbf{M}_0 = \mu(N^+ - N^-)/V$.

It can be shown mathematically (pNMR Manual, 2011) that an oscillating magnetic field can be used to rotate the equilibrium longitudinal magnetization $\mathbf{M}_0$ away from the z-axis so that precessional motion will take place (Figure 8.11). When the $\mathbf{B}_{RF}$ is applied, the magnetic moment of each nucleus $\boldsymbol{\mu}$ exhibits a precession, a type of motion in which the direction of the magnetic moment rotates around the direction of the external magnetic field $\mathbf{B}_0$. This type of motion is analogous to the wobbling of a top under the influence of a gravitational field. The rotation has a characteristic angular speed ω_0, called the *Larmor frequency*. This parameter is dependent on the external magnetic field and gyromagnetic ratio according to the following relation:

$$\omega_0 = \gamma \mathbf{B}_0 \tag{8.7}$$

The Larmor frequency is, in fact, the absorption frequency of a nucleus species under a given external magnetic field. If the spin system being studied belongs to that of ^{1}H and $\mathbf{B}_0 = 0.47$ T (typical for low-resolution pulsed NMR), then the Larmor frequency is 20 MHz.

Due to the precession achieved by using $\mathbf{B}_{RF}$ tune to a particular frequency, $\mathbf{M}_0$ is moved out of its equilibrium position and makes an angle with the direction of $\mathbf{B}_0$. $\mathbf{M}_0$ can now be viewed as having two Cartesian components, the transverse $\mathbf{M}_r$ and the longitudinal magnetization $\mathbf{M}_z$, defined as

$$\mathbf{M}_r = M_x\hat{\mathbf{x}} + M_y\hat{\mathbf{y}} \tag{8.8}$$

$$\mathbf{M}_z = M_z\hat{\mathbf{z}} \tag{8.9}$$

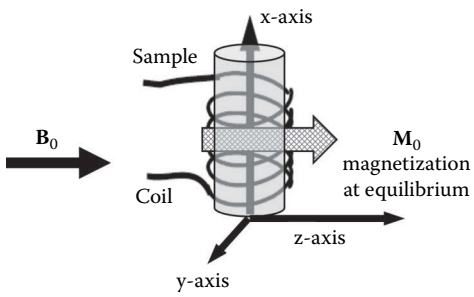

FIGURE 8.10 Explanation of the direction that the magnetization $\mathbf{M}_0$ has in relation to the applied $\mathbf{B}_0$ and the coil position.

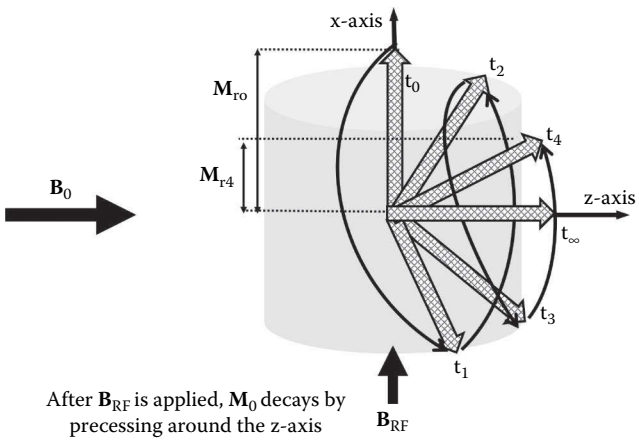

FIGURE 8.11 Explanation of the decay of the magnetization as it precesses around the z-axis. t_i indicates the advance in time, where at t_1 some decay had happened until at $t_\infty \mathbf{M}_0$ is back in its equilibrium position in the z-direction.

The coil (Figure 8.10) records voltage when there is a change in the transverse $\mathbf{M}_r$ component. The signal is independent of $\mathbf{M}_z$ due to the location of the coil. Since the coil can only detect a signal along the x–y plane, the premise of the NMR experiment are to make $\mathbf{M}_0$ precess around the z-axis in order for the signal to be conveniently detected.

When energy corresponding to ΔE is supplied to the system, magnetic moments in the spin-up state transition into the spin-down state in the phenomenon known as *nuclear magnetic resonance*. This ΔE is the energy supplied in the form of a radio frequency by the $\mathbf{B}_{RF}$ pulse. Introducing energy into the spin system causes a transition from the spin-up state to the spin-down state. Changes in the ratio of the spin-up states and spin-down states result in changes to the orientation of the net magnetization vector (the angle θ it forms with the vector describing the direction of the magnetic field $\mathbf{B}_0$), according to $\theta = 2\pi\gamma\tau\mathbf{B}_{RF}$, where γ is the gyromagnetic ratio, $\mathbf{B}_{RF}$ is the strength of the applied oscillating magnetic field, and τ is the pulse width, the duration $\mathbf{B}_{RF}$ was applied.

$\mathbf{B}_{RF}$ can be applied for different durations of time. If $\mathbf{B}_{RF}$ is turned on long enough to bring $\mathbf{M}_0$ to (1) the x–y plane, then it is said that a 90° pulse was applied; but if it brings $\mathbf{M}_0$ to (2) the negative z-axis such that $\mathbf{M}_0$ becomes inverted, then it is said that a 180° pulse was applied. When $\mathbf{B}_{RF}$ is turned off, the net magnetization vector eventually returns to its equilibrium orientation. This process, called relaxation, generates a signal characteristic of the spin system being studied. Typically, the same coil used to generate $\mathbf{B}_{RF}$ is used to detect the signal that $\mathbf{M}_0$ generates during relaxation via electromagnetic induction. The voltage that is induced in the coil is due to variations in the magnetic flux, Φ, generated by $\mathbf{M}_0$ (more precisely, generated by $\mathbf{M}_r$) as it precesses to its equilibrium position. The voltage generated, V, follows Faraday's law of induction, the basic law of electromagnetism where $V = -d\Phi/dt$. Having the coil x-axis oriented perpendicular to $\mathbf{B}_0$ maximizes the change in the magnetic flux, Φ, through the coil.

The relaxation of $\mathbf{M}_z$ to its equilibrium value is called the *longitudinal* or spin–lattice relaxation and is associated with the characteristic time called T_1. T_1 is called the *spin–lattice relaxation time* in reference to NMR in solids where the constituents of the lattice (nuclei and electrons) provide for the exchange of energy. To explain what is happening here, one needs to have in mind that the magnitude of $\mathbf{M}_0$ is obtained by a population of spins in a higher energy state than the other. As soon as $\mathbf{M}_0$ is moved from its equilibrium, these populated states are influenced by the environment, the lattice. The spins can exchange energy with electrons and other nuclei in the sample resulting in a fast approach to the equilibrium state.

The relaxation of the $\mathbf{M}_r$ component is related to the spread of the Larmor frequencies due to the proton's spin moments that are not moving in unison. This is a product of having inhomogeneities in $\mathbf{B}_0$, which causes different moments to precess at different rates. The effect of having spins that "fan out" as they precess is also influenced by random fluctuations of other magnetic fields, like the dipole field of one proton moving past another. The decay of $\mathbf{M}_r$ happens at another time scale compared with $\mathbf{M}_z$. The characteristic time for this process is called T_2, the *spin–spin or transverse* relaxation time.

B. Free Induction Decay

The signal that the coil detects as $\mathbf{M}_0$ goes back to its original equilibrium state is called the FID and is associated with the $\mathbf{M}_r$ component only as this is the only component that can be detected. This is due to the experimental setup.

For example, the FID after a 90° pulse is a voltage signal that oscillates between positive and negative values at the Larmor frequency as seen in Figure 8.11 and Figure 8.12a. The oscillations diminish over time, indicating that $\mathbf{M}_r$ is getting smaller while $\mathbf{M}_z$ grows until the initial value of $\mathbf{M}_0$ is reached, at which point $\mathbf{M}_r = 0$. It is to note that the FID comes only from the transverse magnetization, $\mathbf{M}_r$. The envelope of the FID is typically reported as an exponential decay curve characteristic of the relaxation process (Figure 8.12b).

The FID is in the time domain and, as mentioned before, due to the position of the coil, only $\mathbf{M}_r$ is recorded, which is often modeled by an exponential

$$\mathbf{M}_r(t) = \mathbf{M}_i e^{-t/T_2^*} \tag{8.10}$$

where T_2^* is the transverse relaxation time.

Typically, T_2^* is modeled as

$$\frac{1}{T_2^*} = \frac{1}{T_2} + \frac{1}{T_{2,i}} \tag{8.11}$$

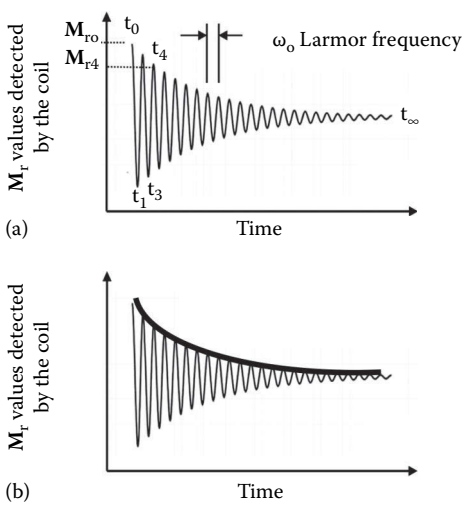

FIGURE 8.12 Nuclear magnetic resonance (NMR) signal showing the free induction decay (FID) obtained as $\mathbf{M}_r$ relaxes (a) NMR signal showing the FID envelope (b).

where

T_2 describes the relaxation of the transverse components $\mathbf{M}_r$

$T_{2,i}$ describes the loss of phase coherence due to field inhomogeneities

$1/T_{2,i} = \gamma\Delta\mathbf{B}_0$, which is equal to a spread in Larmor frequencies $\Delta\omega_0$ due to field inhomogeneities which would cause spin–spin dephasing with a characteristic time of $T_{2,i}$. Other formulations of T_2^* include a third diffusion term, which characterizes the loss of phase coherence due to diffusion. Diffusion is only a concern when the time scales of the experiment are long enough to allow diffusion to occur.

The following two sections explain how the transverse magnetization and the longitudinal magnetization are originated and how the relaxation time for each of them can be measured. This is not a trivial exercise due to how the instrument carries out the measurement.

C. Inversion Recovery: T_1

Recall that the transverse T_1 relaxation time is not measured by the coil as it lays in the z-direction where the coil does not measure. To overcome this, a sequence of pulses is applied in order to obtain T_1. If the initial perturbed longitudinal magnetization is taken as $\mathbf{M}_i$, $\mathbf{M}_z(t)$ would be the exponential

$$\mathbf{M}_z(t) = \mathbf{M}_0 + \left(\mathbf{M}_i - \mathbf{M}_0\right)e^{-t/T_1} \tag{8.12}$$

The sequence of pulses uses first a 180° pulse in order to invert the longitudinal magnetization equilibrium value $\mathbf{M}_0$ so that it ends up pointing in the negative z-axis: $\mathbf{M}_z = -\mathbf{M}_0$. Over time, $\mathbf{M}_z$ will change values going from $-\mathbf{M}_0$ to 0 to $\mathbf{M}_0$. This way of inversion does not generate a signal on the x–y plane, which means that the coil does not pick up any signal, as there is no transverse magnetization to produce an FID. To observe the changing $\mathbf{M}_z(t)$, which is assumed to follow an exponential decay, the magnetization needs to be made to precess around the z-axis. This is achieved by applying a second pulse, this time a 90° pulse, at a desired observation time. The FID will decay with the T_2^* time constant, but the initial amplitude of the signal will be proportional or almost equal to $\mathbf{M}_0$. Typically, the value of T_1 is obtained after making measurements at different observation times tau or τ, called *inversion time*.

This inversion recovery involves the following steps:

$$t_d - 180°\ \text{pulse} - \tau - 90°\ \ \text{pulse} - \text{FID detection} \tag{8.13}$$

t_d is the time needed for recovering the system to its original $\mathbf{M}_0$ value. t_d is used for multiple FID detections. The inversion time τ can be set between 0.04 and 100 μs. At least two measurements need to be made with different inversion times in order to have sufficient equations to solve for the many unknowns ($\mathbf{M}_0$,T_2^*).

When two different values of τ are used, two amplitudes M_1 and M_2 can be obtained. Since relaxation is a first-order process, the decay from M_1 or M_2 is governed by the same characteristic time T_1. Experiments have shown that the value of τ should be $>5 \times T_1$ in order for the system to completely recover.

D. Spin Echo: T_2

The spin echo technique is used to obtain the T_2, the spin–spin relaxation time. The spin echo is a phenomenon that cancels the effect of field inhomogeneities and makes measurements of T_2 possible. There are different methods to measure this time constant.

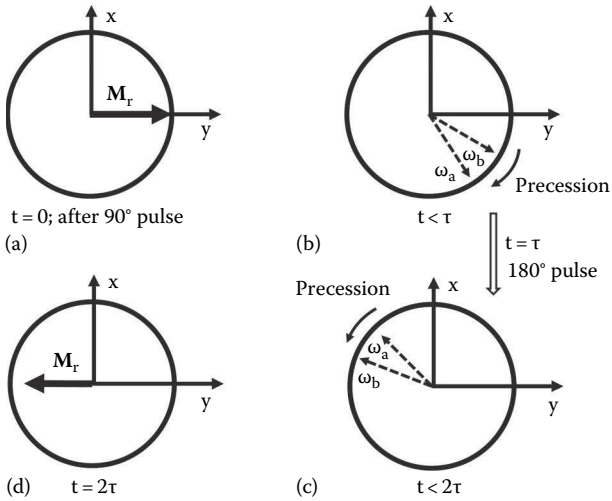

FIGURE 8.13 Hahn spin-echo sequence where the z-axis is coming towards the reader. (a) a 90° pulse rotates $\mathbf{M}_r$ from the z-axis towards the xy plane. (b) de-phasing of $\mathbf{M}_r$ starts to take place (c) after a 180° pulse is applied, the frequencies are inverted 180 degrees and rotate in the opposite direction from which they started (d) the direction of $\mathbf{M}_r$ is now opposite from (a) and it's magnitude smaller. This is the echo.

The simple case is called *Hahn spin-echo sequence.* Where the sequence of event is

$$90^{\circ}_{y} - \text{FID detection} - \tau - 180^{\circ}_{y} - \tau - \text{FID detection} \tag{8.14}$$

After the 90° pulse is applied, the transverse magnetization $\mathbf{M}_r$ appears on the x–y plane (Figure 8.13a). The protons start to precess and the de-phasing of the spins moment starts to take place (Figure 8.13b) where the proton moving with a frequency ω_a is ahead of the one with frequency ω_b.

After time $t = \tau$, which is in the order of ms, a 180° pulse is applied with the effect that the protons on the x–y planes are now moving in a different order. The proton with frequency ω_b is ahead of the one with frequency ω_a. This is to say that the protons that were slow before 180° pulse are now ahead of the previous fast ones (Figure 8.13c). The time duration of the 180° pulse is short (of the order of μs) as compared to the time τ and hence can be neglected but is important when designing the experimental apparatus and when choosing τ. Recall that the same coil is used to generate the pulse and to collect the FID, so a dead time is always present when the electronic is reset.

After the 180° pulse, the protons continue to precess clockwise for another time interval τ, after which the protons are all in phase again. At this time, the magnetization $\mathbf{M}_r$ has been regenerated (Figure 8.13d) and the echo takes place. As no pulse is introduced at this time, the coil can be collecting data with no interruptions.

Figure 8.14 shows the NMR signal detected by the coil under the Hahn spin-echo sequence.

Problems can appear when using a large τ value. For example, the position of the protons before the 180° pulse could be misaligned due to molecular diffusion and a loss of coherent could be the result when trying to regenerate $\mathbf{M}_r$. To compensate for this, a longer sequence of pulses is used.

The *Carr–Purcell–Meiboom–Gill* (*CPMG*) *spin-echo sequence* is used to compensate for the deviations in the time decay associated with the deviation from the required Larmor frequency as well as problems associated with the duration of the $\mathbf{B}_{RF}$. Not using the optimal value for the $\mathbf{B}_{RF}$ pulse can lead to rotations of the individual magnetic moments in less than or greater than the time expected, making the timing of the 180° pulse incorrect. This sequence is as follows:

$$t_d - 90^{\circ}_{y} - (\tau - 180^{\circ}_{y} - \tau - \text{FID detection})_n \tag{8.15}$$

where the sequence $\tau - 180^{\circ}_{y} - \tau$ is applied n numbers of times and the amplitude of the echo is used to generate a T_2 decay.

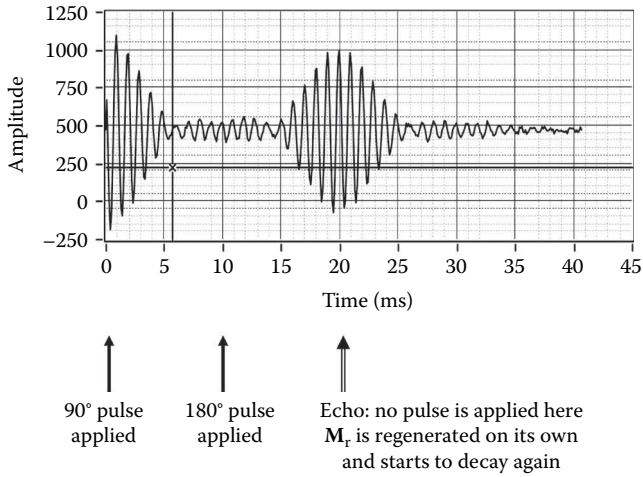

FIGURE 8.14 FIDs obtained after the 90° and the 180° pulse are applied in the Hahn spin-echo sequence. $\tau = 10$ ms. (Adapted from Schicker, R., F61/F62, Manual 2.0: Nuclear Magnetic Resonance, Advanced Physics, Laboratory Experiment, Physics Institute, Heidelberg University, Heidelberg, Germany, 2010.)

The CPMG starts with a 90° pulse to generate a transverse magnetization $\mathbf{M}_r$. When a certain time τ elapses, a 180° pulse is applied. At time $t = 2\tau$, the system will be phase coherent and the echo appears as explained before. As $\mathbf{M}_r$ continues decaying, the systems lose their coherence and a second 180° pulse is applied, at time $t = 3\tau$. This causes the system to be again in a coherent phase at time $t = 4\tau$. This pattern of phase coherence at even multiples of τ and generation of 180° pulses at odd multiples repeat to large times as shown in Figure 8.15.

This way of working with spin echoes makes use only of the FID amplitude at the "echo" point. The number of echoes recorded needs to be large enough to generate a line, which resembles a decay. This is the T_2 decay signal (Figure 8.15b) and is not the FID envelope as before, but rather a collection of individual points that represents the magnitude of the $\mathbf{M}_r$ component measured at the time defined as "echo."

In many experiments, the data are not sampled at each echo, but rather a "dummy echo" is used where no sampling point is collected. The use of an odd number of dummy echoes (e.g., 1, 3, 5, …) in the minispec causes the program to sample at every, for example, second, fourth, and sixth echo. As only a limited number of data points can be acquired, this provides a way of extending the time scale to ensure the entire relaxation event is recorded.

E. The Minispec Bruker Instrument

The focus of this discussion is on the benchtop NMR instruments with the Bruker Minispec MQ20 being used as an example. This instrument operates with a $\mathbf{B}_0$ of 0.47 T such that the proton Larmor frequency is 20 MHz. In the work of the authors, this instrument has been extensively used to measure (1) the SFC and (2) T_2 relaxation times of fats.

The magnet that generates $\mathbf{B}_0$ is strictly kept at 40.0°C to avoid variations in the external magnetic field. A calibration ensures that day-to-day variations in the external magnetic field are kept to a minimum. In practice, the instrument requires a calibration every 24 hours. This calibration routine requires three standard polymer solutions that correspond to certified SFC values. It is during this calibration that the correction factor (F) and digital offset factor (D) are calculated (see the following text).

In order to avoid heat transfer from the probe head chamber to the sample tube, the measurement is usually performed with four scans, resulting in a total measurement time of 6 seconds. As with any reference technique where a calibration is required, the analytical results will only be as good as

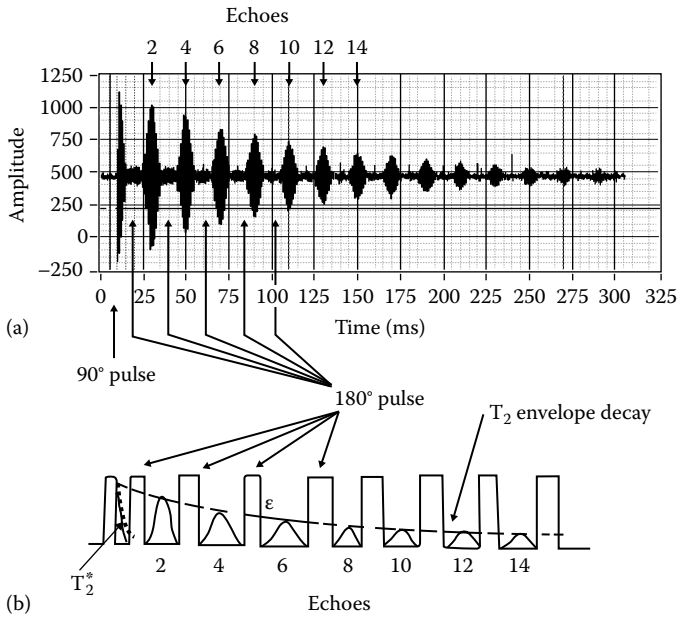

FIGURE 8.15 (a) NMR signal obtained with the Carr–Purcell–Meiboom–Gill (CPMG) sequence for $\tau = 10$ ms showing the pulses application as well as when the echoes appear. (Adapted from Schicker, R., F61/F62, Manual 2.0: Nuclear Magnetic Resonance, Advanced Physics, Laboratory Experiment, Physics Institute, Heidelberg University, Heidelberg, Germany, 2010.) (b) Typical representation of the NMR signal, showing the FID envelope of each $\mathbf{M}_r$ decay between echoes, shown in an exaggerated way, as the pulse is in the order of μs, while the separation between pulses is in ms. Also shown in (b) is the T_2 decay envelope to be fitted. Each point in this envelope comes from the largest $\mathbf{M}_r$ amplitude detected at each echo. Notice that the real T_2^* corresponds to the first FID, right after the 90° pulse.

the calibration they are obtained from. Using up-to-date standards is of prime importance. Another aspect is to understand the "error limits" inherent to the technique. For example, an accuracy of ±0.1% cannot be obtained with a standard for which the value is known to be ±0.5%. Due to the low number of scans set in the SFC measurement, a measurement error of ±0.5% solid fat (absolute) is worldwide accepted.

As soon as the NMR sample tube is inserted into the minispec chamber, the permanent magnetic field $\mathbf{B}_0$, generated by the two large magnets shown in Figure 8.16, quantizes the energy levels accessible to the magnetic dipole moments of all NMR-active nuclei. Notice that $\mathbf{B}_0$ is generated between the two magnets and points horizontally. $\mathbf{B}_{RF}$ is generated perpendicular to $\mathbf{B}_0$ since the coil wraps around the sample holder chamber.

F. Solid Fat Content

1. Direct Method

To obtain SFC using the direct method as given by the American Oil Chemists' Society's official methods (AOCS Cd 16b-93), a 90° $\mathbf{B}_{RF}$ pulse is applied to the fat under study for ~3 μs (van Putte and can den Ende, 1974). The system is allowed to relax and the FID is recorded. The envelope of the FID is taken and particular points from the envelope are used to compute the SFC. Usually, four SFCs are determined from four collected FIDs. The reported SFC is obtained as an average of the four. The delay between successive scans is called the "recycle delay time" and needs to be 5–10 times longer than the longest T_1 expected in the system to ensure the net magnetization vector is in the equilibrium position.

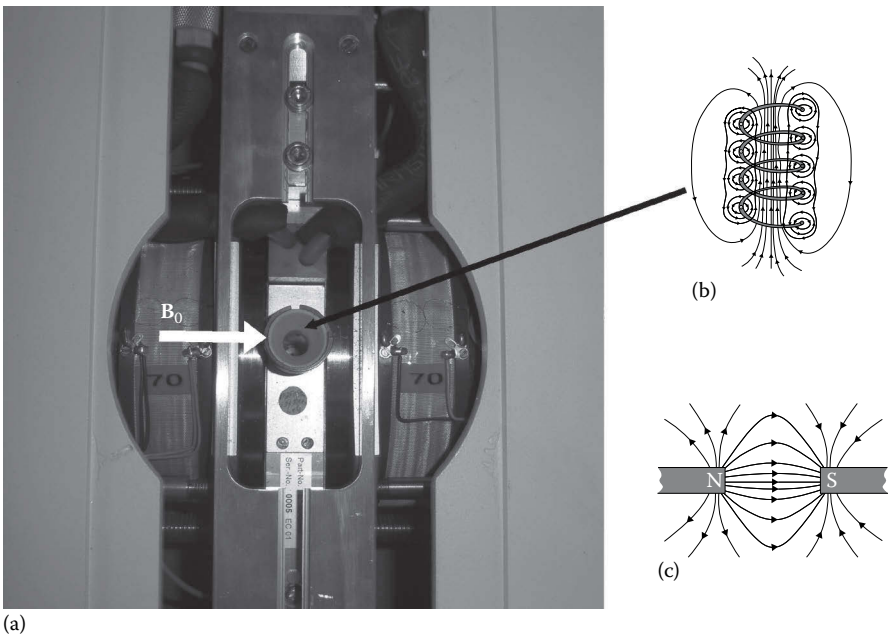

FIGURE 8.16 Minispec sample holder chamber. The two magnets with the numbers 70 generate $\mathbf{B}_0$ (a, c). $\mathbf{B}_{RF}$ is generated by the coil that surrounds the sample as seen from the top view in (a) and displayed on (b).

To calculate the SFC, two points on the envelope are taken: (1) S_{S+SL}, the signal intensity 11 μs after the receiver was turned on, and (2) S_L, the signal intensity 70 μs after the receiver was turned on. The signal intensity obtained at 11 μs corresponds to the total amount of all relaxing protons, those in the solid and liquid state, while the signal intensity at 70 μs corresponds to those protons in the liquid state, assuming the protons in the solid state had already relaxed. The solid/liquid ratio is calculated as

$$\mathrm{SFC} = \frac{(S_{S+SL} - S_L)\,F}{S_L + (S_{S+SL} - S_L)\,F + D} \tag{8.16}$$

where

F is an empirical correction factor (established during calibration) to account for the receiver dead time. This correction factor is dependent on the T_2 value for the proton in the solid fat (van Putte, 1995)

D is the digital offset factor (also established during calibration)

Figure 8.17 shows that the envelope of the FID for a fat is the sum of two decay curves.

2. Indirect Method

The American Oil Chemists' Society's official methods (AOCS Cd 16-81) is used in this case. To obtain the SFC indirectly, only the signal intensity corresponding to the liquid protons (S_L) is utilized. This value is the one measured after 70 μs from the time that the RF pulse ceased. Seventy microseconds was chosen by Bruker as a compromise between the T_2 value and the $\mathbf{B}_0$ inhomogeneities. Only ~0.1% of the initial signal has decayed in 70 μs without interferences from the inhomogeneities that manifest in the order of ms (Van Putte, 1995). SFC is calculated by comparing the signal of a completely melted sample with the signal of the same material after subjecting the sample to a tempering protocol (as described in the AOCS method) to achieve solidification. The tempering

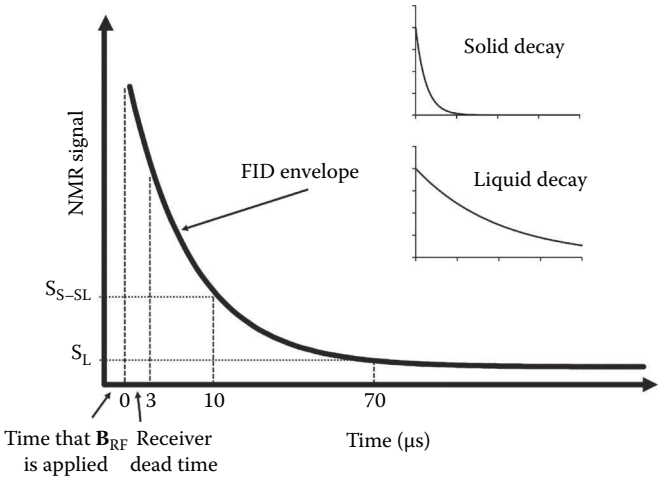

FIGURE 8.17 NMR envelope of FID used for the solid fat content determination. Notice the time at which S_L and S_{S-SL} are measured.

protocol guarantees that all samples are treated the same way. To account for the effect that the temperature might have, the method requires measuring a known standard oil that is completely liquid at both temperatures of interest. The indirect method is more accurate as the instrument offset is removed by using the oil measurement. The equation to use for this case is

$$\%\text{SFC} = 100 - \left(\frac{\text{Oil}_{60}\ \ \text{Sample}_T}{\text{Sample}_{60}\ \ \text{Oil}_T} \right) 100 \tag{8.17}$$

Subscript indicates the temperature at which the FID is obtained, either at 60°C (subscript 60) or the desired temperature T. The user needs to compute the %SFC using Equation 8.18 once the four numbers are measured. This calculation is based on the assumption that both the standard oil and the sample are completely liquid at 60°C and that the standard is completely liquid at the final measurement temperature. Although these assumptions are probably justified in many cases, they may not always be correct, particularly when making measurements at low temperatures. In the case of fats that melt above 60°C, the official method gives no provisions, but common sense indicates that the sample and oil temperature at which the reference measurement is performed (Oil_{60}, Sample_{60}) needs to be adjusted accordingly.

G. Sample Condition and Amount

Details about sample conditioning for the direct or indirect method can be found in either the AOCS or the International Union of Pure and Applied Chemistry (IUPAC) methods. Slight variations in the sample conditioning are necessary when one is dealing with fat materials that require tempering.

The amount of sample introduced in the NMR tube and the position of the tube in the measuring cell are very important, especially when working with relaxation measurements. A sample that is not properly located within the coil will give an erroneous result. See the Bruker Minispec Relaxation Time Manual for exact positioning of the sample (Bruker, 1989).

Care must be taken to center the sample properly within the coil, having in mind that the coil is 1.5 cm in height. The user should check with the manufacturer where the center of the coil is in relation to either the bottom or top of the measuring cell.

Depending on the scope of the experiment, an already crystallized fat or a melted one might need to be deposited inside the NMR tube. Introducing a crystallized fat into the bottom of an NMR tube

is not easy, if not impossible. Different strategies have been used, such as filling up another reservoir that gets dropped into the NMR tube. The back of a glass pipette, a clear glass shell vial, and a short Teflon tube have been successfully used for this purpose. Bruker commercializes small vials with plastic lids, 3 cm in height that easily slide inside the standard glass NMR tubes.

Once the fat is inside the NMR tube and properly positioned in the Bruker measurement cell, specific protocols must be followed before an NMR measurement is performed. The protocol depends on the type of fat under study and the kind of experiment to be performed. The measuring cell in the NMR must stay dry and clean while performing the measurement. Care must be taken to dry the NMR tubes if they were kept in a water bath to prevent hardware damage. Also, rubber or plastic stoppers are recommended to prevent the entry of water, dust, or any other liquid into the NMR tube.

H. SFC Measurement: Application

SFC of fats is usually measured as a function of temperature or as a function of time. Examples of each are shown in Figure 8.18. These figures show the SFC for mixtures of fully hydrogenated soybean oil (FHSO) in soybean oil (SO). Figure 8.18a shows the melting profile of the samples displayed as SFC versus temperature. Samples were conditioned according to the AOCS Cd 16b-93 method. A glass disposable vial insert was used. It was estimated that the extra glass layer introduced by the vial contributes to an additional 0.5%–1.5% to the SFC, which was not corrected for.

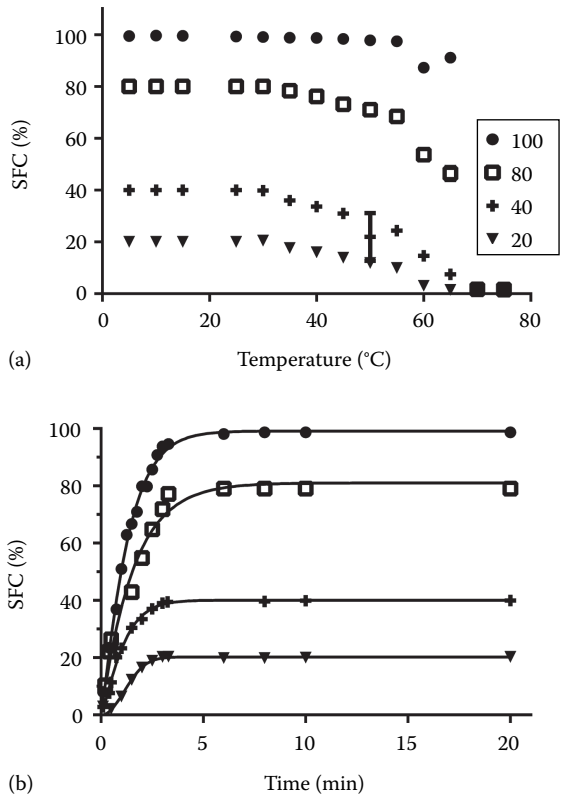

FIGURE 8.18 (a) Melting profile of four samples made with different ratios of FHSO:SO shown as the solid fat content (SFC) as a function of temperature. (b) Crystallization profile of four samples made with different ratios of FHSO:SO shown as the SFC as a function of time.

Figure 8.18b shows the crystallization profile of the SFC as a function of time. Samples inside the vial inserted in the NMR tubes were melted at 80°C for 15 minutes and transported to a water bath kept at 30°C. SFC was measured every 30 seconds for about 6 minutes and a final measurement was taken after 20 minutes.

A kinetic theory of phase changes was developed by Avrami (1939, 1940, 1941). This theory has been modified for fats and the volume converted to solid fat content (Marangoni and Wesdorp, 2012). The crystallization data are typically analyzed using the Avrami model of crystal growth, which includes terms describing the crystal growth rate as well as the growth mechanism:

$$\frac{SFC(t)}{SFC_{max}} = 1 - e^{-kt^n} \tag{8.18}$$

where

SFC(t) is the solid fat content at time t

SFC_{max} is the maximum solid fat content

k is the Avrami constant (units of t^{-n}) and is the growth rate constant

n is the Avrami exponent (dimensionless), which defines the crystal growth mechanism

It is important to note here that this is an isothermal treatment. Table 8.4 explains the possible values of n and their significance.

Using Equation 8.18, values for k and n of the samples shown in Figure 8.18b are displayed in Table 8.5.

A parameter that can be calculated using k and n is the half-time ($t_{1/2}$) of crystallization

$$t_{1/2} = \left(\frac{\ln 2}{k}\right)^{1/n} \tag{8.19}$$

According to Avrami's interpretation of the exponent n, 40%, 80%, and 100% FHSO in SO displayed rodlike growth formed nuclei that were instantaneously formed (Table 8.5). The nucleation for the 20% FHSO sample could be 1D sporadic or 2D instantaneous. The k values indicate that the sample made with 20% FHSO had the slowest crystallization rate, while the half-time crystallization shows that the sample with only 20% had crystallized half of its material in the longest time.

TABLE 8.4
Explanation of the Avrami Exponent n

Avrami Exponent (n)	Various Types of Growth and Nucleation
1 + 0 = 1	1D growth from instantaneous nuclei
1 + 1 = 2	1D growth from sporadic nuclei
2 + 0 = 2	2D growth from instantaneous nuclei
2 + 1 = 3	2D growth from sporadic nuclei
3 + 0 = 3	3D growth from instantaneous nuclei
3 + 1 = 4	3D growth form sporadic nuclei

Source: Adapted from Sharples, A., *Introduction to Polymer Crystallization*, Edward Arnold Ltd., London, U.K., 1966, pp. 44–59.

TABLE 8.5
Avrami Parameters, n, k, and $t_{1/2}$, Obtained Fitting the Avrami Equation to the Data from the Samples Displayed in Figure 8.17

Parameter	20%	40%	80%	100%
k	0.4	0.8	0.6	0.7
n	2.0	1.2	0.9	1.1
$t_{1/2}$	1.3	0.9	1.2	0.9

I. T_2 Relaxation for Peanut Oil with 1% Candelilla Wax

The CPMG sequence (Section III.D) was used to obtain the T_2 relaxation time in a sample made with a commercial peanut oil and 1% candelilla wax. The t2-cp-mb application from Bruker was used, which is the CPMG sequence. "Instrument settings" were acquired with a similar sample to the one to study in order to set the gain and the 90° and 180° pulse correctly. Instrument settings is an application in the software that is used for calibration when dealing with T_2 measurements.

Once the t2-cp-mb application is run, the T_2 decay signal is collected and is ready for analysis (as explained in Section III.D). This T_2 decay signal is the sum of exponential decays given by the different environments in which the protons are found. In order to obtain the decay rate of each exponential, Bruker provides their own software, namely, CONTIN (Provencher, 1982). It carries out an inverse Laplace transform of the *T_2 decay signal* to extract the desired information. Notice that CONTIN (ppt, 2001) does not work on the FID but rather on the T_2 decay signal as explained in Section III.D.

The Laplace transform is typically used for transient signal analysis while the Fourier transform is used for steady-state signal analysis. The inverse Laplace transform takes a function of a complex variable s (often frequency) and yields a function of a real variable t (time).

Both the Laplace and Fourier transforms can be viewed as a continuous summation of a power series of a complex exponential, which are represented with an integral, rather than a sum. The limits of integration for the Laplace integral are 0 and infinity (∞), while for the Fourier integral the limits are −∞ and +∞.

The Laplace equation for a function f(t) is given by

$$F(s) = \int_0^{\infty} e^{st} f(t) \delta t \tag{8.20}$$

where t is time to indicate that f(t) is in the time domain, while the variable s is in, for example, the frequency domain. An inverse Laplace transform will be an equation in which f(t) is represented as an integral of F(s).

The CONTIN software uses the T_2 decay signal. The s value is now $R = 1/T_2$, the decay rate constant. CONTIN models the decay as a sum of exponential decays

$$S(t) = \sum A_i e^{(-tR)} \tag{8.21}$$

or in an integral form as

$$S(t) = \int_0^{\infty} f(R) e^{-tR} dR \tag{8.22}$$

where CONTIN defines (R) = α(R)R. α(R) is described as the fraction of nuclei relaxing with a rate constant between R and R + dR.

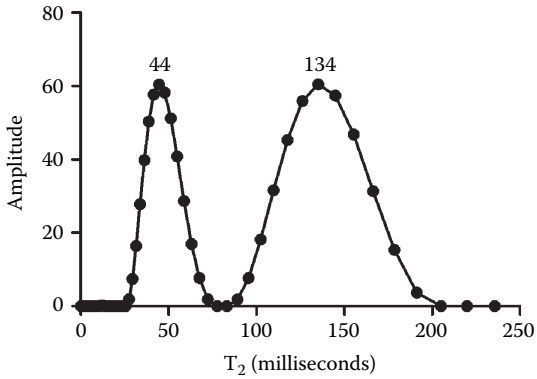

FIGURE 8.19 T_2 values obtained after using CONTIN on a T_2 decay signal of peanut oil with 1% candelilla. The T_2 decay signal was obtained using a CPMG sequence.

In order to find (R), an inverse Laplace transform must be applied.

CONTIN uses the Laplace principle of transforming from one variable to another. Instead of making a transformation from the time domain to the frequency domain, CONTIN transforms from the time domain to the rate constant domain. The result of a CONTIN calculation is a series of peaks, each at one time constant. Each peak is a distribution of the T_2 values that could give rise to the decay. The T_2 associated with the maximum height, h_i, of the Gaussian peak is the most likely value to be used as the time constant T_2 for each decay or species of protons present in the sample. Narrow peaks indicate more accuracy in the obtained T_2 value. The amplitude A_i in Equation 8.21 is proportional to the peak area divided by the time constant T_{2i} (CONTIN ppt, 2001) and given by

$$A_i = h_i \frac{SD}{T_2} \tag{8.23}$$

The FWHM of the peak is the standard deviation of the T_2 value. Figure 8.19 shows the results after applying CONTIN to T_2 decay signal of peanut oil with candelilla wax.

The two peaks in Figure 8.19 indicate that there are two proton species, one which is less mobile than the other. The peak that gives $T_2 \sim 44$ ms is probably due to the protons in the candelilla material, which remains solid at room temperature (~22°C); hence, its protons are less mobile. $T_2 \sim 134$ ms might correspond to the protons belonging to the liquid peanut oil molecules, which are more mobile.

IV. CALORIMETRY

A. Principles of the Technique

Calorimetry, broadly defined, is the study of the transfer of energy to and from a system in the form of heat. The melting of a fat is an endothermic event, wherein the fat sample absorbs energy from the environment in the form of heat, while crystallization is an exothermic process, one in which heat is given off. One of the techniques used to study fats is called differential scanning calorimetry. This technique measures the change in the difference in heat flow (hence differential) between two materials as a function of a time–temperature program (hence scanning). The two materials include the sample material being studied and a reference, which is usually air. One instrument to carry out calorimetric measurements is called differential scanning calorimeter (DSC). DSCs are separated

into two categories: heat-flux DSC and power compensation DSC. These calorimeters differ in the design and measuring principles, but they have in common that the measured signal is proportional to the heat flow rate ϕ.

B. Measuring Cell

A DSC apparatus usually consists of the following components: a cell, a cell controller, an external cooling system, and a purge system. The cell is the heart of the instrument and is where all of the calorimetry is performed. The cell contains furnaces, which heat up the sample and reference pans as well as thermocouples to measure the temperature of the sample and reference pans. An external cooling system provides cooling capacity, while the purge system purges the cell using an inert gas (such as N_2) in order to prevent water condensation or frost formation during the course of an experiment as well as maintain a clean environment. Over the course of a DSC experiment, the sample and reference pans are heated or cooled at a predetermined rate and are thus maintained at identical temperatures.

Pans can be made from materials such as gold, copper, aluminum, graphite, and platinum or alodine-aluminum (TA Instrument, 2001). The pans are closed with lids that can either be hermetic (i.e., provide a complete seal) or be perforated to allow the release of built-up pressure, usually from the evaporation of water at temperatures greater than 100°C. Fat samples have been successfully analyzed using either aluminum pans or alodine-aluminum pans containing no more than 10 mg of sample that are hermetically sealed before the study is carried out.

C. DSC Instruments

There are two general types of DSC instruments: the heat-flux DSC and the power-compensation DSC or heat flow DSC. Most instruments in use today are of the heat-flux variety. However, a brief discussion of power-compensation instruments is included. In a power-compensation (or double-furnace) DSC, the sample and the reference pans are mounted on two identical but independent/separate furnaces housed inside a thermostated chamber. A control circuit increases or decreases the amount of heat supplied by these furnaces. A reduction in the supplied heat allows the temperature to remain unchanged or to decrease when an endothermic reaction is taking place. When a phase transition, such as melting of a fat, occurs over the course of the time–temperature program, a temperature difference between the sample and the reference is developed. As melting is an endothermic process, an amount of heat equivalent to the transition enthalpy (the "latent" heat of melting) must be absorbed in order for the transition to take place. While this transition is taking place, the sample temperature remains constant and as such, a temperature differential, relative to the reference, is developed. Likewise, when the fat is crystallizing, additional heat energy is given off, resulting in an increase of the sample temperature, relative to the reference material.

The temperature difference is measured as the difference between the temperatures of the sample and reference pans as measured by the independent sensors. In a power-compensation DSC, the temperature difference is the measurement signal as well as the input of a second control circuit. The second control circuit compensates for the additional heat being absorbed or liberated by either increasing or reducing, respectively, the heating power supplied to the sample furnace. The sample temperature is thus adjusted (by increasing or decreasing the heat supplied by the furnace to the sample pan) such that its temperature is maintained identical to that of the reference.

In a heat-flux DSC, both the sample and reference pans are mounted on raised thermoelectric platforms on a low-resistance thermoelectric disk (Figure 8.20) using only one furnace for both pans.

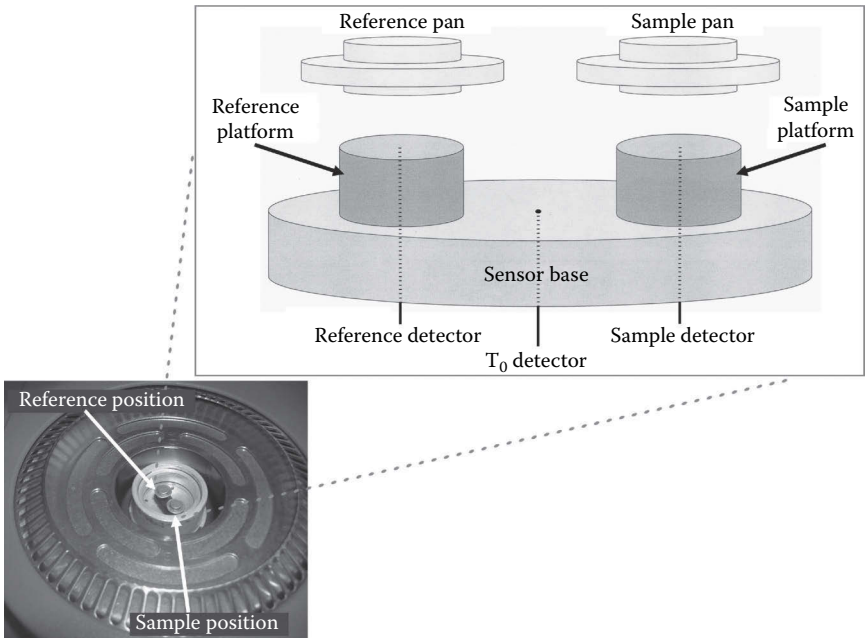

FIGURE 8.20 The constantan sensor showing the two platforms located on the silver base at the bottom of the cell enclosure, holding both the reference and the sample pans. The three detector wires used in the calorimeter are also shown. (From Peyronel, F. et al., Methods used in the study of the physical properties of fats, in: Marangoni, A.G. (ed.), *Structure-Function Analysis of Edible Fats*, AOCS Press, Urbana, IL, 2012, pp. 231–294.)

On these raised platforms are mounted thermocouples (detectors in Figure 8.20) that measure the temperature of the sample and reference pans. As the temperature of the DSC cell changes, heat is transferred from the silver base of the sensor body to the pans. A third set of sensors measures the temperature of the sensor base. The thermoelectric disk, typically made of constantan, is attached to a resistive heating block, typically made of silver. The thermoelectric disk, having high thermal conductivity, ensures that the sample and reference pans are in adequate thermal contact with each other. This is necessary as excess or required heat that must be removed or supplied during an exothermic or endothermic event is done so between the sample and reference pans. A temperature difference between the two pans will result in heat flowing to the sample pan through the constantan disk such that both pans are maintained at identical temperatures.

Interested readers can find more details about the different DSCs in the book *Differential Scanning Calorimetry* (Hohne et al., 2003).

D. Heat Flow

As it was mentioned, the magnitude of this heat flow is proportional to the temperature difference between the sample and the reference. The temperature differential is the primary signal of interest and is used to calculate the "measured" differential heat flow rate Φ_m according to the following equation:

$$\Phi_m = -k' \cdot \Delta T \tag{8.24}$$

where

$-k'$ is a factory-installed calibration

ΔT is the measured temperature difference

In reality, this temperature difference is a voltage difference due to the use of thermocouple sensors in determining the temperature. The heat flow rate depends on the differential heat capacity between the sample pan and the empty reference pan as well as the heating rate. The measured heat flow is composed of three heat flow rates as shown in Equation 8.18 (Danley, 2001, 2003):

$$\Phi_m(T,t) = \Phi_0(T) + \Phi_{cp}(T) + \Phi_r(T,t) \tag{8.25}$$

where

Φ_0 is the heat flow rate due to the temperature difference between the sample and the reference
Φ_{cp} is the heat flow rate due to the heat capacity difference between the sample and the reference
Φ_r is the heat flow rate arising compensations for exothermic and endothermic events

The first and second terms define the baseline while the third term results in the appearance of peaks, which are displayed in the thermogram. In the absence of any exothermic or endothermic phenomena, the difference between the heat flow rate through the sample and reference form the baseline of the thermogram. A change in the difference between the baseline signals (due to a phase transition or the evolution of heat energy from a chemical reaction) leads to the appearance of a peak, the position of which describes the temperature at which the phenomena takes while the magnitude of this peak describes the extent of the phenomena. The area under the peak can be integrated to determine transition properties such as the enthalpy of the transition. In the study of fat materials, the melting/crystallization temperatures, as well as enthalpies, can be determined in this manner.

A DSC trace arising from an endothermic event is called an endotherm while such a DSC trace arising from an exothermic event is called an exotherm. Typical thermograms display the heat flow difference on the y-axis, while the temperature or time is plotted on the x-axis. According to the International Confederation of Thermal Analysis and Calorimetry (ICTAC, 2014), the directions in which the exotherms and endotherms in a thermogram must point will depend on the type of DSC instrument used to obtain the thermogram. In a power-compensation DSC, an exotherm is registered as a negative signal (a dip/trough) as the instrument must reduce the power supplied to the furnace to minimize the temperature difference between the sample and reference pans. Similar logic explains why an endotherm obtained using a power-compensation DSC is registered as a positive signal (a spike/crest). The convention for a power-compensation DSC is thus notated as endo^, to indicate the endotherm points in the upward direction. In a heat-flux DSC, the ICTAC convention states that an exothermic process (such as crystallization) is registered as a positive signal (a spike/crest), while an endothermic process is registered as a negative signal (a dip/trough). The convention for a heat-flux DSC is therefore notated as exo^. Modern thermal analysis software allows the user to choose the direction each signal points to, however, the convention for a heat-flux DSC will be used throughout this section.

Parameters commonly obtained from DSC thermograms of fats include the peak crystallization temperature T_C, the peak melting temperature T_M, the onset crystallization temperatures T_{OC}, the onset melting temperature T_{OM}, the crystallization enthalpy ΔH_C, and the melting enthalpy ΔH_M (Figure 8.21). Peaks in the melting endotherm may also arise from solid–solid polymorphic transformations.

The peak temperatures at which the thermogram displays the highest or lowest rate of heat flow are the temperatures at which at least half of the lipid species have gone through the respective phase transition. Associated with these peaks are onset temperatures (T_{OM} or T_{OC}), defined as the temperature at which the observed transition first takes place. The onset is observed as a deviation from the baseline and is usually determined as the temperature at which a line tangent to the peak of interest intersects the baseline.

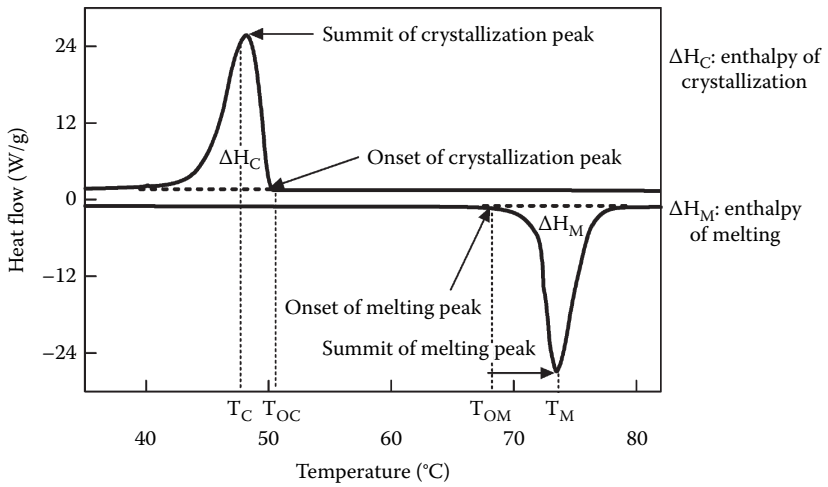

FIGURE 8.21 Schematic diagram of a thermogram, plotting heat flow as a function of temperature, showing the parameters typically measured for fats. (From Peyronel, F. et al., Methods used in the study of the physical properties of fats, in: Marangoni, A.G. (ed.), *Structure-Function Analysis of Edible Fats*, AOCS Press, Urbana, IL, 2012, pp. 231–294.)

The area under the peak corresponds to the enthalpy of the phase transition ΔH. The enthalpy of a system is equal to the energy transferred to the system as heat, only if the system is under constant pressure and when the only work done on the system is due to an expansion caused by heating alone. Under these circumstances, the enthalpy is equal to the heat, Q, supplied by the system so that ΔH = Q. The units are typically Joules (J) or J/g when specific enthalpy is reported.

E. Measuring the Thermal Properties of Fats

The two phase transitions often studied in fats using a DSC include crystallization and melting. The melting properties of a crystallized fat are investigated by heating the sample inside a DSC pan at a controlled rate until the fat is completely melted. The thermal history of the sample will influence the melting profile. These include the crystallization conditions of the fat, the storage temperature, and the storage time. These variables, together with the heating rate, will affect the characteristics of the melting endotherm. In the routine analysis of a fat using DSC, a fat is typically crystallized according to a set time–temperature prior to determining its melting endotherm. To crystallize the fat, the fat is typically melted completely to erase the so-called crystal memory, which is the result of existing fat crystals acting as a nucleation template for crystallizing triglycerides.

An example of a simple crystallization/melting procedure for fats is given in Table 8.6. This procedure first examines the melting transitions of a fat that is crystallized and prepared outside of a DSC. Note that the insertion temperature is 10°C. This is to prevent any melting of the sample before analysis. After melting the fat completely at 80°C, the sample is crystallized under controlled conditions (non-isothermal crystallization to 10°C at a rate of 5°C/min) in the DSC to characterize the crystallization behavior of the fat under these conditions. The experiment ends after this step. Additional steps to measure the melting properties of the fat crystallized under the conditions mentioned earlier can also be introduced. This illustrates the utility and versatility of the DSC apparatus to crystallize fats according to a defined set of crystallization conditions and then studying the melting properties afterward.

TABLE 8.6
Example of a Protocol to Study the Melting Behavior

Step	Software Instruction	Action
1	Initial temperature 10°C	The cell is brought to 10°C, after which the sample pan is introduced into the cell.
2	Ramp 5°C/min to 80°C	The temperature is increased from 10°C (temperature at the previous step) to a final temperature of 80°C at a rate of 5°C/min.
3	Isothermal for 15 minutes	The temperature is maintained at 80°C for 15 minutes.
4	Ramp 5°C/min to 10°C	The temperature is decreased from 80°C to a final temperature of 10°C at a rate of 5°C/min.
5	Isothermal for 15 minutes	The temperature is maintained at 10°C for 15 minutes. This is to ensure complete crystallization.

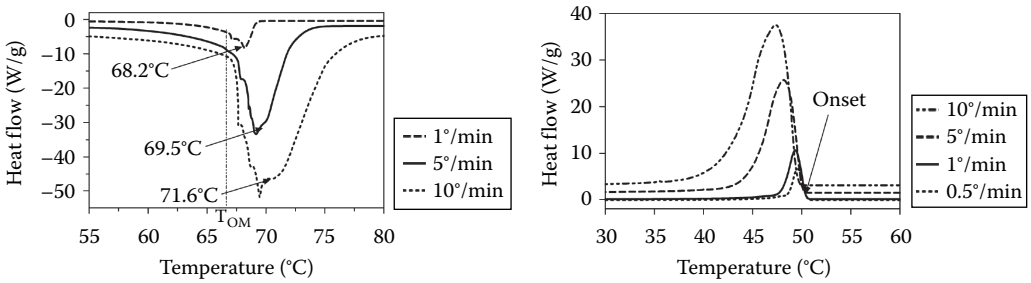

FIGURE 8.22 Melting and crystallization profile of FHSO obtained at three different ramps: 1°C/min, 5°C/min, and 10°C/min. (From Peyronel, F. et al., Methods used in the study of the physical properties of fats, in: Marangoni, A.G. (ed.), *Structure-Function Analysis of Edible Fats*, AOCS Press, Urbana, IL, 2012, pp. 231–294.)

F. Rate of Melting/Crystallization

An important consideration in the operation of a DSC instrument is the melting or crystallization rate. Figure 8.22 shows that the peak melting and crystallization temperatures change when the heating/cooling rate is changed. The higher the heating rate, the higher the peak melting temperature. Some workers prefer to use the onset temperature instead of the peak temperatures as these are not affected by the heating/cooling rate. Simulations of melting behavior (Höhne et al., 2003) have indeed shown that the onset temperatures are relatively independent of experimental parameters.

There are reports (Cassel) that an error of about 1°C–2°C in the position of the crystallization peak is typically incurred, compared to the position of the melting peak (Menczel, 1997; Menczel and Leslie, 1990). This can be avoided by performing a multiple rate calibration. It is important to understand that the maximum of the peak is not necessarily the melting point of the material, that is, the material is not completely melted at the peak temperature. The sample is completely melted only when the baseline is reached again, that is, the endset melting temperature.

G. Polymorphic Transformation

Recrystallization can occur when a fat undergoes a polymorphic transformation with increasing temperatures. It is manifested as three consecutives peaks: an endotherm, followed by an exotherm, followed immediately by a second endotherm. The exothermic peak indicates recrystallization,

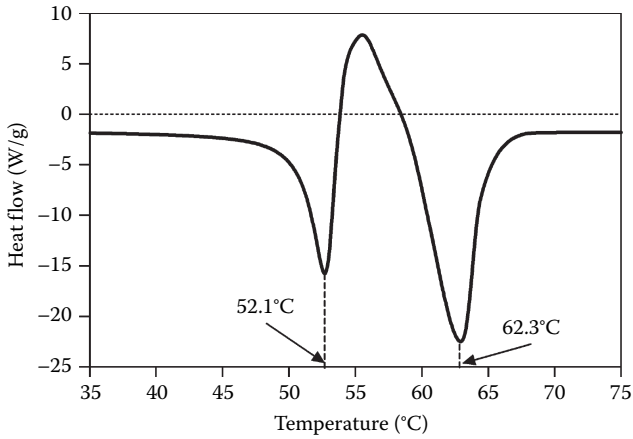

FIGURE 8.23 Melting of FHSO showing a polymorphic transformation. (From Peyronel, F. et al., Methods used in the study of the physical properties of fats, in: Marangoni, A.G. (ed.), *Structure-Function Analysis of Edible Fats*, AOCS Press, Urbana, IL, 2012, pp. 231–294.)

typically due to the formation of a new crystal. For example, the first endothermic peak can indicate the melting of a metastable crystalline form, like the α form. This is only an educated guess, as the polymorph can only be definitively established by x-ray diffraction. After the melting of this α form, the fat recrystallizes into a higher-melting polymorph, such as the β′ form, as evidenced by the exothermic peak. As the temperature continues to increase, this β′ form is melted, as evidenced by the second endothermic peak. The fact that a second endotherm was observed does not imply that this particular polymorph was not initially present.

The presence of two endotherms could also indicate the melting of two different triglyceride species. However, a polymorphic transformation is more likely to be the case when an exotherm is observed between the two endotherms. Figure 8.23 illustrates a typical thermogram for a polymorphic transformation.

V. RHEOLOGY

Rheology is the study of the deformation of materials. Central to the study of rheology is the application of a force on a material and the resulting response (deformation) to the applied force. The force may be applied in different modes, that is, compressive, elongative, or shearing. The applied force is typically normalized per unit area (stress) over which the force is applied while the resulting deformation is likewise normalized over the characteristic dimension(s) over which the deformation develops. As such, stress typically has units of pressure (Pascals) while strains are typically dimensionless and are expressed as a percentage. Edible fats exhibit a wide variety of rheological behaviors. In general, fats behave either as plastic solids or as viscoelastic materials. Both classes of materials can be discussed as iterations of more simple materials such as ideal solids and Newtonian (or non-Newtonian) fluids. Such complex rheological behavior is not surprising in a semicrystalline material such as edible fat, which consists of both a solid phase (which is presumably elastic) and a liquid phase (which is presumably fluid).

What follows discusses the emperical application of rheology for the study of edible fats.

A. Elastic Material or Ideal Solid

A material that exhibits ideal elastic behavior is referred to as a *Hookean* solid. An ideal solid can be visualized as a spring that is fully elastic as given by Hooke's law: $F = k\Delta L$, where F is an applied

force, ΔL is the resulting elongation, and k is a constant of proportionality. An ideal solid is one that stores all of the energy of deformation. This energy is released upon removal of the applied stress. The proportionality constant between the deformation and the force is given by the relation

$$\text{Stress} = \text{Elastic modulus} * \text{Strain}$$

Most elastic materials obey Hooke's law only at small deformations. For an isotropic and homogeneous solid, there are three major types of deformation that are important: simple shear, simple compression (or elongation), and bulk compression. Depending on what stress is applied and how the strain is measured, different moduli can be determined.

The tendency of a sample to be deformed is a measure of its elastic modulus, which is defined as the slope of its strain–stress curve in the linear (elastic) region.

For example, when the shear stress τ is applied, the shear strain γ and the shear modulus G are defined:

$$G = \frac{\tau}{\gamma} \tag{8.26}$$

For tensile and compressive stresses, σ, Young's modulus of elasticity, E, is defined as

$$E = \frac{\sigma}{s} \tag{8.27}$$

where s is the linear deformation.

The bulk modulus, K, is used when there is a change in the volume of the sample upon the application of a 3D stress:

$$K = \frac{\Delta P}{\Delta V/V_0} \tag{8.28}$$

where

ΔP is the change in pressure

$\Delta V/V_0$ is the relative change in volume of the solid

Under all modes of deformation, a solid can maintain ideal rheological behavior only within a certain range of deformations, called the elastic region. The upper limit of this region is the so-called elastic limit. Within this region, the material is elastic, that is, all deformations are fully reversible when the applied stress is removed with the material recovering its original shape. When the material is deformed such that the deformation is above the elastic limit, the material no longer behaves as an ideal solid and may exhibit irreversible deformation, that is, the material does not fully recover its original shape upon removal of the stress. When the deformation is great enough, the material will eventually fail and result in breakage. Solid materials can deform considerably before breakage or may break shortly after exceeding the elastic limit. This is the distinction between plastic solids and brittle solids, which will be discussed later in this section.

B. Ideal Viscous Liquid or Newtonian Liquids

Materials that flow and do not have a constant shape are commonly called fluids. Of the three states of matter, two (gases and liquids) are traditionally considered fluids. A liquid can deform (that is, it flows) upon the application of an external force. However, fluids, by definition, cannot revert to an original shape unlike elastic solids. As such, the relation between an applied stress and strain is meaningless in the discussion of fluids, particularly because a fluid has no shape. More appropriate

to the discussion of fluid deformation is the rate of deformation (as characterized by a shear strain rate or, more simply, the shear rate $\dot{\gamma}$) rather than the extent of actual deformation. Rheological characterization of ideal liquids show that it is the shear rate $\dot{\gamma}$ (rather than the shear strain) that is proportional to the applied shear stress τ.

Fluid flow can be envisioned as a stack of "fluid" cards, which may represent streamlines. The application of a shear stress τ on the top card of this stack of cards results in a dislocation of this top card relative to a fixed bottom card. Due to attractive intermolecular forces between two adjacent cards, the second card immediately below the top card is also dislocated, albeit the deformation is retarded due to frictional forces that resist the tendency of the fluid to flow. The same behavior is likewise repeated between the second card and the third card. Thus, a deformation gradient is developed between the dislocated top card and the immovable bottom card. Considering the time over which this deformation process takes place, a velocity gradient described by the shear rate $\dot{\gamma}$ is developed between the top card and the bottom card. The shear rate can be shown to be related to the magnitude of the applied shear stress σ according to the following formulation:

$$\tau = \eta\dot{\gamma} \tag{8.29}$$

where η is the coefficient of viscosity, a parameter of the fluid that characterizes its resistance to flow. The shear stress τ has units of Pascals (N/m^2) while the shear rate has units of reciprocal time (1/s). As such, viscosity is commonly reported in units of $Pa \cdot s$.

In an ideal, Newtonian fluid, the coefficient of viscosity η is independent of the magnitude of the shear rate $\dot{\gamma}$. Thus, a Newtonian fluid exhibits the same resistance to flow at all values of $\dot{\gamma}$. The plot describing the relation between τ and $\dot{\gamma}$ is therefore linear, with the slope being η. In non-Newtonian fluids, η is dependent on the value of $\dot{\gamma}$. Thus, the resistance to flow in a non-Newtonian fluid varies with the shear rate. In such cases, the viscosity is often referred to as an *apparent viscosity.*

Non-Newtonian fluids are generally categorized into (1) shear-thinning fluids, where the apparent viscosity decreases with increasing shear, or (2) shear-thickening fluids, where the apparent viscosity increases with increasing shear. Shear-thinning fluids are typically called pseudoplastic fluids. When such shear-thinning behavior is observed to be time dependent, the fluid is called a thixotropic fluid. Shear-thickening fluids are typically called dilatant fluids. When such shear-thickening behavior is exhibited over time, the fluid is called a rheopectic fluid.

For these non-Newtonian liquids, the plots describing the relation between τ and $\dot{\gamma}$ are nonlinear and can be modeled using a power law. Hence, these fluids are sometimes called power-law fluids. Power-law behavior is commonly found in biopolymer solutions as well as colloidal suspensions. In liquid oils, such behavior is rarely, if at all, observed and as such, extensive discussion of non-Newtonian fluids is beyond the scope of this section.

C. No Ideal Solids and Liquids: Plastic Materials

Traditionally, fluids are defined to be either liquid or gaseous materials. However, a solid can also be treated as a "fluid" when it undergoes irreversible deformation such that recovery of its original shape is not possible. In such a case, the solid does not store all of the energy imparted by the deformation but rather dissipates some of it in the form of viscous flow. Naturally, such a solid is a nonideal solid and can be more appropriately described using fluid models. An *apparent modulus* can be defined for such nonideal solids. The apparent modulus is simply the modulus for the nonlinear region beyond the limit of elasticity. Even though the material can no longer be characterized as Hookean, it can partially recover its original shape upon removal of the applied stress.

As already discussed, a solid will "flow" when the applied deformation is above the limit of elasticity. The applied stress necessary to exit the elastic region and induce flow in a solid is called the *yield stress.* As such, the yield stress is the minimum amount of stress that needs to be applied

before a solid can flow. A solid that cannot be deformed extensively in the inelastic region before breaking is described as *brittle*. A solid that deforms considerably in the inelastic region prior to failure is called a *plastic* material. The strain at which the material fails is called the *breaking point*.

The flow of a material can be modeled using the Herschel–Bulkley power law:

$$\tau = \tau_0 + K(\dot{\gamma})^n \tag{8.30}$$

where

τ is the applied shear stress
K is the consistency index (which is identical to the apparent viscosity)
$\dot{\gamma}$ is the shear rate
τ_0 is the yield stress
n is the flow behavior index, which characterizes the ideality of the flow behavior

For Newtonian fluids, $\tau_0 = 0$ and $n = 1$. Incidentally, the Herschel–Bulkley power law is also well suited to the description of non-Newtonian fluids. For non-Newtonian fluids, $\tau_0 = 0$ and $n \neq 1$.

The ideal plastic material is referred to as a Bingham and it needs two equations to define the rheological properties—one valid for the region below the yield stress and the second one valid for the region above the yield stress point:

$$\tau = G\gamma \quad \text{for } \tau > \tau_0$$
$$\tau - \tau_0 = \eta \frac{d\gamma}{dt} \quad \text{for } \tau < \tau_0 \tag{8.31}$$

where

G is the shear modulus
η is the viscosity
τ_0 is the yield stress

In effect, a Bingham plastic behaves like an elastic solid below the yield stress and behaves like a Newtonian fluid above the yield stress.

A Casson plastic is similar to a Bingham plastic, except that a Casson plastic behaves as a pseudoplastic (shear-thinning) fluid above the yield stress. A Casson plastic is well suited for describing the deformation of fats above the yield stress. The ease of deforming most fats increases as the shear stress is increased, presumably because the higher rate of deformation results in the destruction of microstructures that impart hardness to a fat. The Casson model is also typically used in the study of chocolate rheology.

D. Viscoelastic Materials

While plastic materials exhibit elastic behavior below the yield stress and viscous behavior above the yield stress, a viscoelastic material exhibits both behaviors simultaneously. Recall that as a shear stress is applied on an ideal solid, it experiences a deformation, storing the energy supplied during the deformation in the material. Upon removal of the stress, the material recovers its original shape completely and dissipates the energy stored in the material. When a shear stress is applied on a Newtonian liquid, the liquid experiences deformation (occurring at a rate described by the shear rate) which is fully maintained even after the stress is removed. The liquid cannot recover its original shape since all of the energy introduced into the material is dissipated as heat. A viscoelastic material is a material that simultaneously behaves as a liquid and as a solid. When a stress is applied on a viscoelastic material, it can simultaneously store and dissipate the energy supplied. Viscoelastic materials, having a viscous component, typically exhibit rheological behavior that is

time dependent. Viscoelastic materials are often characterized using methods where the time scale of the deformation is an important consideration.

The viscoelastic properties of a fat can be characterized using *transient* or *dynamic* methods. Two common transient methods include the creep-recovery experiment and the stress-relaxation experiment. A creep-recovery experiment is conducted under constant stress conditions while a stress-relaxation experiment is conducted using constant strain conditions.

A creep experiment consists of two phases: creep phase and a recovery phase. In the creep phase, a constant stress is applied to the material. The strain response as a function of time (also called the creep) is monitored. After a certain time has elapsed, the applied stress is removed. This is the recovery phase, where the material can partially or wholly recover its original shape. The strain in the recovery phase is measured to determine how quickly the material recovers from the deformation as well as determining the permanent deformation incurred during the experiment. The result of a creep experiment is a creep compliance function, which has units of inverse modulus.

A stress-relaxation experiment is similar to a creep experiment in that the time-dependent deformation and recovery of a material are characterized. In a stress-relaxation procedure, the material is deformed to a constant strain. An initial high stress is required to achieve the set deformation. However, due to the permanent deformation of the viscoelastic material brought about by the viscous component, the stress required to maintain the deformation decreases (or "relaxes") over time. The rate at which this stress relaxes is indicative of the viscoelastic character of the material.

Transient techniques are rarely utilized in studying the viscoelastic properties of fat materials. As such, a more detailed discussion of viscoelastic materials modeled using the Kelvin–Voigt, Maxwell, and Burger models is beyond the scope of this chapter. More commonly, dynamic techniques are utilized to determine the viscoelastic properties of fat. The mode of deformation in dynamic techniques is usually shear deformation. This usually involves testing a disk of material where one face of the disk is sheared in an oscillatory fashion relative to a fixed immovable face. Dynamic techniques have traditionally been in a small-angle regime, in a technique known as small-amplitude oscillatory shear (SAOS). Recently, large-amplitude oscillatory shear (LAOS) has been gaining in popularity.

1. Dynamic Experiments

To measure the viscoelastic properties dynamically, an oscillatory stress or strain input is applied to the test material. Whether a stress or strain is the input would depend on whether the method or instrument is stress controlled or strain controlled. After the input of the oscillatory stress or strain, the resulting oscillatory strain or stress response is then measured. The input stress or strain is usually applied at a certain frequency. The strain or stress response is sinusoidal in nature with the same frequency. The phase lag or phase shift (δ) between the sinusoidal input and the sinusoidal output is used to characterize the viscoelastic nature of the material. A material where the sinusoidal input is in phase with the sinusoidal output is a purely elastic material, whereas a material where the sinusoidal input is out of phase with the sinusoidal output by 90° is a purely viscous material.

Plotting the stress as a function of the strain generates the so-called Lissajous plots, which are elliptic plots of a system of parametric equations that describe complex harmonic motion. The Lissajous plot is circular when the phase shift between the sinusoidal input and the sinusoidal output is $\pm 90°$, as is the case for a fully viscous material. Likewise, the Lissajous plot is linear when the phase shift between the input and the output is 0° or 180°, which is the case for a fully elastic material. A Lissajous plot for a viscoelastic material with a phase shift between 0° and 90° is characterized by an elliptical figure. Figure 8.24 shows such a Lissajous plot.

The amplitude of the input stress or strain must be in the linear viscoelastic region (LVR) for the collected data to be meaningful. Within the LVR, the deformation is reversible and the internal structure of the material that gives rise to its viscoelastic properties is kept intact. If the input stress or strain is such that the LVR is exited, a deformed ellipse is obtained as in the case of Figure 8.25. It is beyond the scope of this section to discuss material behavior outside the LVR.

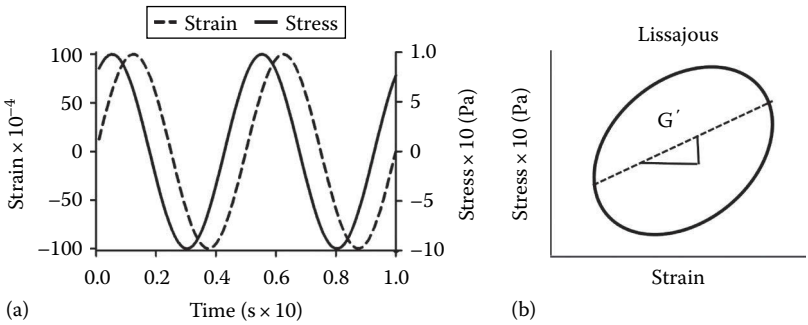

FIGURE 8.24 Applied sinusoidal strain and the corresponding sinusoidal stress response. (a). Stress versus strain Lissajous plot associated with part a (b).

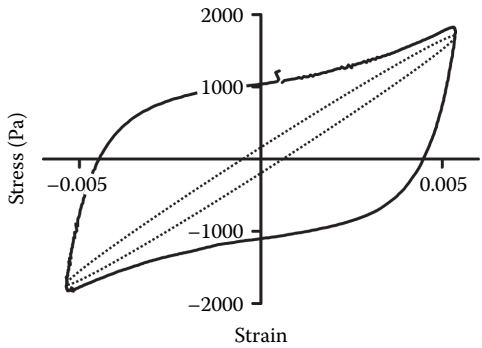

FIGURE 8.25 Deformed ellipse as a consequence of measuring outside of the linear viscoelastic region (LVR).

Extra care must be taken when studying systems outside the LVR region. The test conditions must be set properly to make sure that one is looking at the nonlinear conditions or there could be misinterpretations of results (Ewoldt et al., 2015).

The sinusoidal strain input can be described by the following equation:

$$\gamma = \gamma_0 \sin(\omega t) \tag{8.32}$$

where

γ_0 is the maximum amplitude of the strain
ω is the oscillation frequency expressed in rad/s (in units of Hertz, $\omega/2\pi$ Hz)
t is the time

If nonlinear processes are ignored so that the system is represented by a differential equation involving a simple harmonic oscillator (energy storage) and a frictional (energy dissipation) term that is linear in velocity, then the oscillatory strain produces a shear stress, τ, acting on a surface in contact with the material:

$$\tau = \tau_0 \sin(\omega t + \delta) \tag{8.33}$$

where

τ_0 is the maximum amplitude of the shear stress
δ is the phase shift of the stress response with respect to the strain input

These two equations can be rewritten using trigonometric identities into the following form:

$$\tau = \tau_0 \sin(\omega t)\cos(\delta) + \tau_0 \sin(\delta)\cos(\omega t) \tag{8.34}$$

which can also be rewritten as

$$\tau = \gamma_0 \left[\left(\frac{\tau_0}{\gamma_0} \cos(\delta) \right) \sin(\omega t) + \left(\frac{\tau_0}{\gamma_0} \sin(\delta) \right) \cos(\omega t) \right] \tag{8.35}$$

This equation allows the definition of the storage (G′) and loss (G″) moduli:

$$G' = \frac{\tau_0}{\gamma_0} \cos(\delta) \tag{8.36}$$

$$G'' = \frac{\tau_0}{\gamma_0} \sin(\delta) \tag{8.37}$$

G′ describes the elastic component of the material, while G″ describes the viscous component of the material. This equation can be simplified into

$$\tau = \gamma_0 G' \sin(\omega t) + \gamma_0 G'' \cos(\omega t) \tag{8.38}$$

G′ and G″ are the main parameters determined in a dynamic rheological experiment. A purely elastic material has G″ = 0 for which δ = 0°, while a purely viscous material has G′ = 0 for which δ = 90°.

The complex modulus G* is formulated as follows:

$$G^* = \sqrt{G'^2 + G''^2} = \sqrt{\left(\frac{\tau_o}{\gamma_0} \cos\delta \right)^2 + \left(\frac{\tau_o}{\gamma_0} \sin\delta \right)^2} = \frac{\tau_o}{\gamma_0} \tag{8.39}$$

The ratio of loss modulus G″ to the storage modulus G′ is formulated as tan δ:

$$\tan\delta = \frac{G''}{G'} \tag{8.40}$$

2. LVR Region

The LVR can be determined using a stress or strain sweep, where the viscoelastic storage modulus (G′) is determined for a range of stresses and strains at a constant frequency of oscillation. Arbitrarily, the LVR is considered to be exited if the G′ deviates by more than 10% from a constant plateau value.

After the LVR is determined, the nature of the viscoelastic material is typically characterized using a frequency sweep, which determines the loss and storage moduli at a fixed stress/strain at varying frequencies. The result is the mechanical spectra of the material, which describes the nature of the material. A true solid is one in which the G′ and G″ do not intersect in the mechanical spectra and are thus independent of the frequency throughout the whole range of frequencies sampled. A pseudo-solid is one where the tan δ changes with frequency. This implies that the G′ and G″ change with frequency and intersect at a particular frequency. Typically, the G′ increases and the G″ decreases with increasing frequency, suggesting the material behaves more like an elastic solid the shorter the deformation time scale. Such behavior is commonly observed in some gels and polymer solutions, which exhibit solid-like behavior when the rate of deformation is relatively high. Fats are more typically described as true solids in that the G′ and G″ are invariant with respect to the frequency.

3. SAOS Example

Examples of SAOS studies of fats are given in the following text. Two fat samples were studied: a hardstock consisting entirely of FHSO and a blended fat consisting of a 70:30 mix of FHSO and SO. A stress sweep measurement was carried out from 1 to 10,000 Pa at a frequency of 1 Hz. The frequency was chosen after performing a frequency sweep as shown in Figure 18.27. Technically, any of the frequencies in which G′ shows linearity could be chosen. Results of the stress sweep are displayed in Figure 8.26. The breaking point can be observed as the point where the G′ and G″ intersect at a particular applied stress. At this point, the material has a higher loss component than storage component and can be thought of as "flowing." The mechanical spectra in Figure 8.27 show that the fats behave as solids as the G′ and G″ are invariant with respect to frequency.

Experimentally, the samples were prepared by crystallizing the melt of the respective fats in a PVC mold to make fat "disks" 20 mm in diameter and 3.2 mm in height. The fats were crystallized at 20°C and were measured immediately after solidification (approximately 1 hour). The geometry employed in the measurement was parallel-plate geometry, consisting of a single fixed plate (the bottom plate) and a rotating plate, with the sample introduced in between. The rotating plate shears the sample held between the two plates. The rotating plate was "clamped" on the sample using a normal force of 4 N in order to hold the sample in place. Sandpaper was glued onto the plate surfaces in contact with the fat sample to enhance the grip on the sample and minimize the occurrence

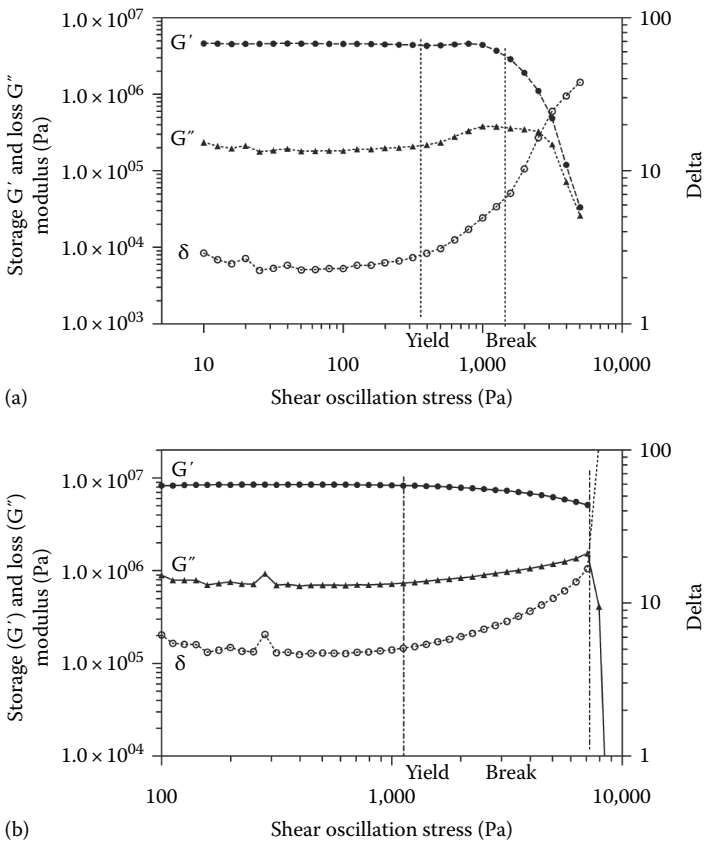

FIGURE 8.26 Behavior of G′, G″, and δ in the LVR region for stress sweeps for two samples of FHSO. (a) 100% FHSO, (b) 70% FHSO and 30% canola oil. (From Peyronel, F. et al., Methods used in the study of the physical properties of fats, in: Marangoni, A.G. (ed.), *Structure-Function Analysis of Edible Fats*, AOCS Press, Urbana, IL, 2012, pp. 231–294.)

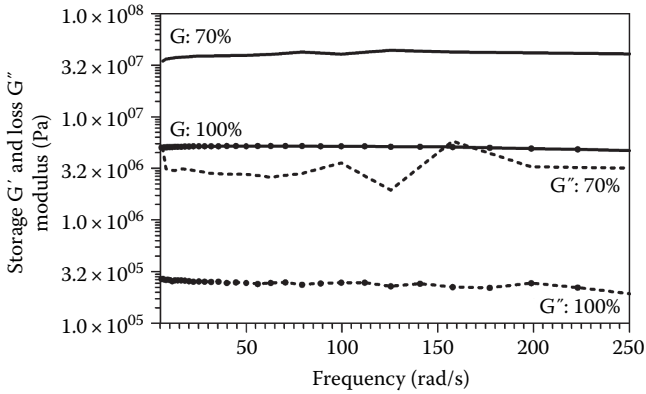

FIGURE 8.27 Results of G′ and G″ for two samples using a controlled-stress rheometer. The samples were 100% FHSO and 70% FHSO mixed with 30% canola oil. (From Peyronel, F. et al., Methods used in the study of the physical properties of fats, in: Marangoni, A.G. (ed.), *Structure-Function Analysis of Edible Fats*, AOCS Press, Urbana, IL, 2012, pp. 231–294.)

of sample slip, whereby the sample disk is dislocated from the gap between the parallel plates by the shearing action. As edible fats have a greasy texture, sample slip is a very common occurrence. The use of serrated parallel plates can also avoid sample slip.

The results are surprising in that the G′ and G″ are higher for the sample consisting of a mix of 70:30 FHSO to SO compared to the one that contains only FHSO. Likewise, the stress at which the 70:30 mixture of FHSO and SO breaks is much higher than that for pure FHSO. The presence of oil should decrease the elastic modulus and breaking point of the sample since this sample contains a lower solids volume fraction. However, powder x-ray diffraction studies showed that 100% FHSO sample is in the alpha polymorphic form while 70% FHSO sample is in the beta polymorphic form, which may explain the differences in the mechanical properties. The fact that 100% FHSO is in the alpha polymorphic form is not surprising given that the material was characterized only one hour after crystallization.

ACKNOWLEDGMENTS

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9 Frying Oil Chemistry

Felix Aladedunye, Usha Thiyam-Hollander, and N.A. Michael Eskin

CONTENTS

I. INTRODUCTION

Deep fat frying is defined as the process of cooking foods by immersing them in edible fat or oil at a temperature above the boiling point of water, typically 150°C–200°C. It is one of the oldest means of preparing food known to man. For instance, Egyptian wall paintings show dough being fried in oil, indicating that Europe and North Africa were using frying as a method of food preparation well before the new era. Although it is an ancient food preparation technique, frying has grown exponentially over the last 50 years, and the consumption of fried food is hardly abated despite various media campaigns against dietary fat consumption. For instance, more than 600,000 institutional and commercial restaurants are involved in deep fat frying in the United States alone and the number continues to grow.

Although the art of deep fat frying is relatively simple, the science is rather complex. The thermally driven interactions involving the food components, the frying oil, and the dissolved oxygen often lead to a progressive breakdown in the oil structure. Although a small amount of oxidative deterioration of the frying oil is needed to develop the golden color and the delicious deep-fried flavor characteristic of fried foods, extensive oil deterioration will compromise the nutritional quality and safety of the fried food. In order to protect frying oils against extensive deterioration, an excellent understanding of the various physical and chemical reactions occurring during the frying process is imperative. This chapter will review the physical and chemical changes in oils during frying, including updated knowledge on available techniques for moderating such reactions. Established methods for measuring oil deterioration will also be discussed in terms of their significance, advantages, and limitations.

II. THE FRYING PROCESS

A. Physical Changes

Fritsch [1] outlined the principal events and the mechanisms of the frying process (Figure 9.1). Basically, heat is transferred from the heat source to the food through the frying oil. Heat transfer from frying oil to the surface of the food involves convective heat transfer while conductive heat transfer

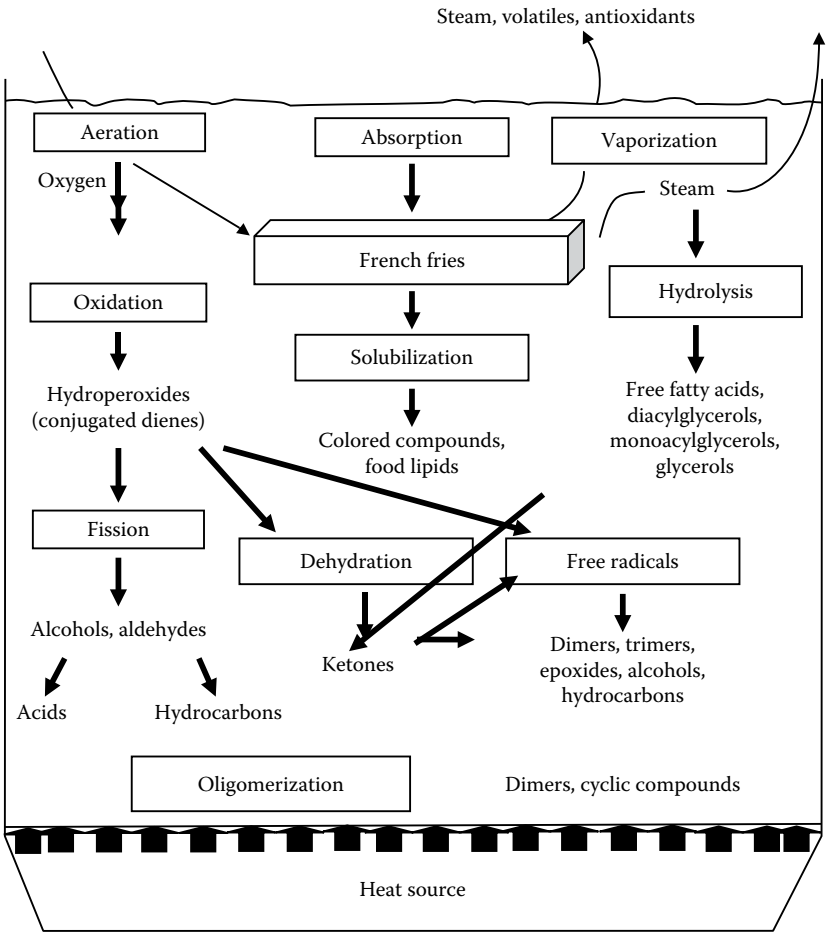

FIGURE 9.1 Principal events occurring during frying. (Adapted from Fritsch, C.W., *J. Am. Oil Chem. Soc.*, 58, 272, 1981.)

occurs within the food being fried [2]. The heat transfer usually instigates extensive mass transfer between the food and the frying oil. Primarily, water in the interior of the food is heated and pumped from the food to the surrounding oil where it is transferred into steam; then, there is extraction and leaching of components from the food to the frying oil; and the frying oil and all the components dissolved in it are also absorbed/adsorbed into the fried food. Ultimately, reactions between the frying oil and the food components are promoted. The leaching of food components into the oil, the breakdown of the oil, and oxygen absorption at the oil–air interface all contribute to changes in the makeup of the oil from almost pure triacylglycerols (>96%) to a mixture of literally hundreds of compounds [3].

Immediately after introducing fresh food into the hot oil, traces of free water at the food surface evaporate quite rapidly resulting in a violent bubbling and drying of the food surface. This initial superficial evaporation and the subsequent in-depth vaporization create a porous, dried, and overheated region generally referred to as the "crust" [4], and depending on the food type, water content, and the frying oil's temperature, the pores can be small voids, molecular interstices, or large caverns filled with water and air [4]. Due to the continuous migration of water from the frying food and the relatively high internal pressure, oil can hardly penetrate into the products during the frying process. Upon removal of the fried products, however, oil that adhered to the surface can penetrate into the pores when the product cools down, highlighting the importance of the dimension and interconnectivity of the pores generated during the frying process to the nutritional quality of the fried products.

The intimate contact between the food and the oil, competently described by the popular Blumenthal's surfactant theory of frying [3], makes frying a more efficient process than the dry oven or wet steam method. Absorption/adsorption of oil, surface dehydration of food with the consequent crust formation, development of surface color, and generation of flavor cumulatively account for the universally desirable taste of fried food [2]. Majority of the physical changes observed during frying (color, flavor, foaming, viscosity/density, and smoking) are consequences of the infinite chemical reactions occurring during the frying process and are discussed next.

B. Chemical Changes

During repeated frying, such as is happening in institutional/restaurant frying operations, the oil is continuously and repeatedly used at elevated temperature, often topped up with fresh oil regularly. The high temperature, continuous exposure to oxygen, coupled with the presence of water from the food result in a series of chemical reactions leading to the degradation of the frying oil and the food components. More than 500 different chemical compounds have been detected as a result of the complex reactions occurring during frying [5].

The major chemical reactions occurring during frying can be primarily classified into three groups, namely, hydrolytic, oxidative, and oligomerization reactions. The various chemical compounds arising from these reactions are responsible for the unique flavor, color, texture, taste, and of course off-flavor of frying oil and food.

1. Hydrolytic Reaction

The main components of edible oils are esters of fatty acids with glycerol. During deep fat frying, water and steam hydrolyze triacylglycerols producing free fatty acids (FFAs), diacylglycerol (DAG), monoacylglycerol (MAG), and eventually glycerol. A typical hydrolytic reaction is shown in Figure 9.2. At the high temperature employed during frying, glycerol and FFAs will partially evaporate and the reaction equilibrium is shifted in favor of other hydrolysis products. Because short and unsaturated fatty acids are more soluble in water than long and saturated fatty acids, they become more accessible to water from food [6]. Consequently, oils with short and unsaturated fatty acids are more susceptible to hydrolytic reactions. It has been reported that frequent top-up of frying oil with fresh oil during frying slows down the prevalence of the hydrolytic reaction [7], while the presence of alkali used for cleaning the fryer increases the hydrolysis of oil [8]. Being an ionic reaction, hydrolysis can also be accelerated by cations and anions present in both the frying oil and the fried food [9].

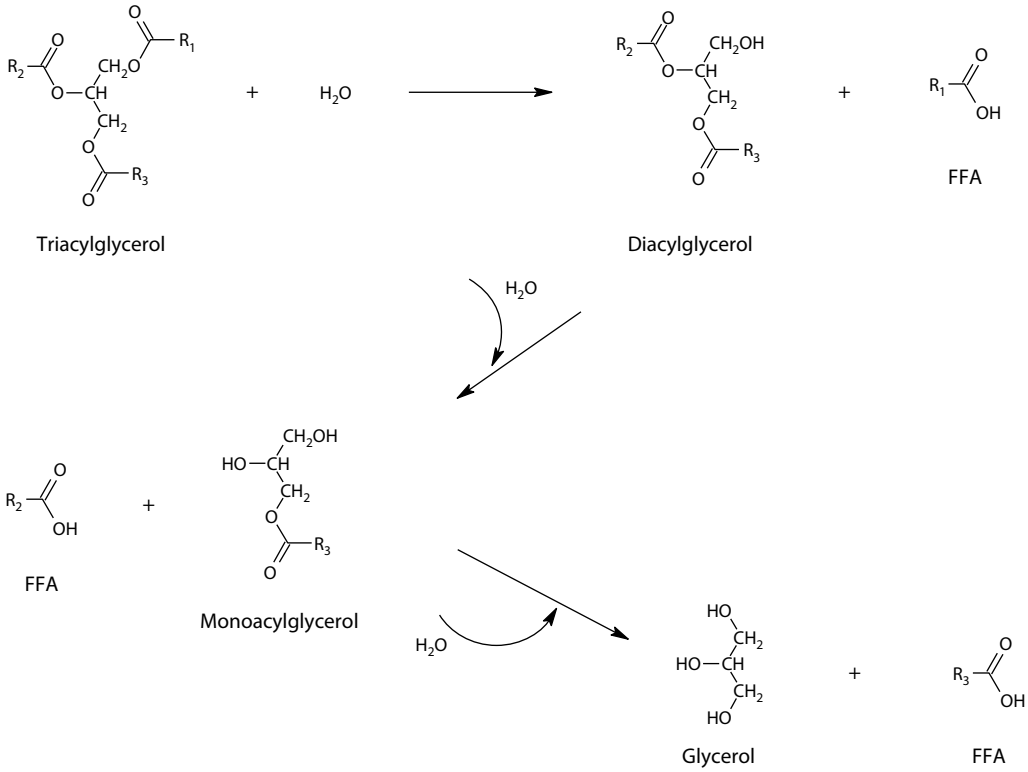

FIGURE 9.2 Hydrolytic reaction and products formed during frying.

Products from hydrolytic reactions seem to have no positive contribution to either the stability of the frying oil or the desirability of the fried food. FFAs and their oxidized compounds produce off-flavor in both the oil and food fried in it. They can catalyze further oxidation of oils by solubilization and activation of metal catalysts [10]. Additionally, as a surface-active substance, FFA lowers the surface tension of the oil thereby increasing oxygen accessibility during frying, promoting oxidative degradation of oil [8,11]. The products from hydrolytic reactions are lower in molecular weight but higher in polarity than the original triacylglycerol [12]. The level of the FFA in the oil is often taken as a measure of the degree of hydrolytic reaction. However, FFAs can also be generated as products of advanced oxidative degradation of frying oils as described in the next section.

From a nutritional or physiological point of view, the products from a hydrolytic reaction are of no consequence as they are also produced in the small intestine by pancreatic lipases prior to absorption [12].

2. Oxidative Reaction

The elevated temperature used during frying accelerates the reactions between atmospheric oxygen and the frying oil resulting in the formation of various degradation products [13,14]. Thermo-oxidation (thermally driven oxidation), like autoxidation (oxidation at ambient temperatures), proceeds by a free radical mechanism, which can be described in terms of initiation, propagation, and termination processes. These processes often consist of a complex series of reactions [10].

At the initiation step, the hydrogen atom from the fatty acid is removed and a lipid alkyl radical is produced. The initiation step is usually catalyzed by an energy carrier, such as temperature and light, and by metal catalysis [15,16]. The energy required to remove hydrogen atoms from fatty acids and initiate radical formation depends on the position of the hydrogen on the molecule. For instance,

the energy required to abstract the *bis*-allylic hydrogen at C11 of linoleic acid is 50 kcal/mol while 75 kcal/mol is required to remove the hydrogen at C8 or C14 of the same fatty acid. On the other hand, the homolytic dissociation energy between a carbon and hydrogen bond on the saturated carbon such as C17 or C18 of linoleic acid is about 100 kcal/mol [17]. Thus, an oil's propensity for oxidative reaction depends on how easy it is to initiate the formation of free radicals.

In the propagation step, a lipid radical reacts with triplet oxygen to produce peroxy radicals, which in turn abstract hydrogen from another lipid molecule to form hydroperoxide and next lipid radical [11]. Thus, a chain reaction occurs at this stage when free radicals are continually produced. Oxidation reaction is terminated when free radicals react to form nonradical products. Lipid hydroperoxides are the primary products of the oxidative degradation. However, due to their instability, lipid hydroperoxides rapidly decompose at frying temperature into secondary products, such as aldehydes, ketones, alcohols, esters, lactones, acids, and hydrocarbons (Figure 9.1). Many of the secondary oxidation products are volatile and evaporate from the oil mainly by steam distillation, while others accumulate in the oil and are absorbed in the fried food. A typical example depicting each of these three steps in frying oil is presented in Figure 9.3.

Volatile oxidation products contribute significantly to the flavor of the oil and the fried food. For example, unsaturated aldehydes, such as 2,4-decadienal, 2,4-nonadienal, 2,4-octadienal, 2-heptenal, or 2-octenal, contribute to the desirable, characteristic deep-fried flavor in oils and fried products [18]. On the other hand, many saturated and unsaturated aldehydes are known to produce distinctive off-flavor in the frying oil. For instance, the grass-like off-flavor in oxidized soybean oil has been attributed to the presence of 2*t*-hexenal while 2*t*,4*c*,7*t*-decatrienal and 1-octen-3-one are responsible for its fish-like off-flavor [19]. A list of common volatile oxidation products and the corresponding characteristic flavor impacted on oxidized frying oil and fried food is presented in Table 9.1 [20].

Besides affecting the flavor, color, and nutritive value of frying oil and fried food, many oxidation products have also been reported to possess detrimental health effects [21–24]. For instance, Shiozawa et al. [25] evaluated the cytotoxicity of several volatile oxidation products against two Chinese hamster cell lines (CHL/IU and CHO-KI) and two human cell lines (HeLa and MCF-7). The volatile oxidation products were added to the culture medium at a final ethanol concentration of 1%, and cytotoxicity was evaluated using the Pre-Mix WST-1 cell proliferation assay system. Although no significant cytotoxic activity was observed for the saturated alcohol and carboxylic acids (pentanol, pentanoic acid, hexanoic acid) and the saturated aldehydes (pentanal, hexanal, octanal, nonanal, decanal), significant cytotoxicities toward all the tested cell lines were reported for the unsaturated aldehydes (2-heptenal, 2-octenal, 2-decenal, 2-undecenal, and 2,4-decadienal). Kimura et al. [26] reported the cytotoxicity of oil fumes against rat hepatocytes. The oil fumes generated during a model frying by a blend of soybean and canola oils were trapped, and the cytotoxicity was evaluated by the MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The researchers further analyzed the composition of the oil fumes using TLC and high-performance liquid chromatography (HPLC) and ascribed the observed cytotoxicity to the volatile carbonyl compounds, primarily 2,4-decadienal.

Recent epidemiological studies revealed a positive relationship between lung cancer and exposure to cooking fumes [27–30]. In 2006, based on available evidence in both humans and experimental animals, emissions from frying were classified as Group 2A carcinogens ("probably carcinogenic to human") by a working committee of the International Agency for Research on Cancer (IARC) [31]. While further studies are required to establish the toxicity and carcinogenicity of volatile oxidation products to humans, the available data indicate that the development of methods to reduce their formation during frying is warranted.

3. Oligomerization Reaction

The initiation and propagation stages of an oxidative reaction can generate several alkyl radicals as previously noted. The alkyl radical, formed during the initiation reaction, the alkylperoxy radicals formed by the addition of oxygen, and the alkoxy radicals formed by the decomposition of hydroperoxides are precursors of dimers and oligomers produced at the termination stage [12].

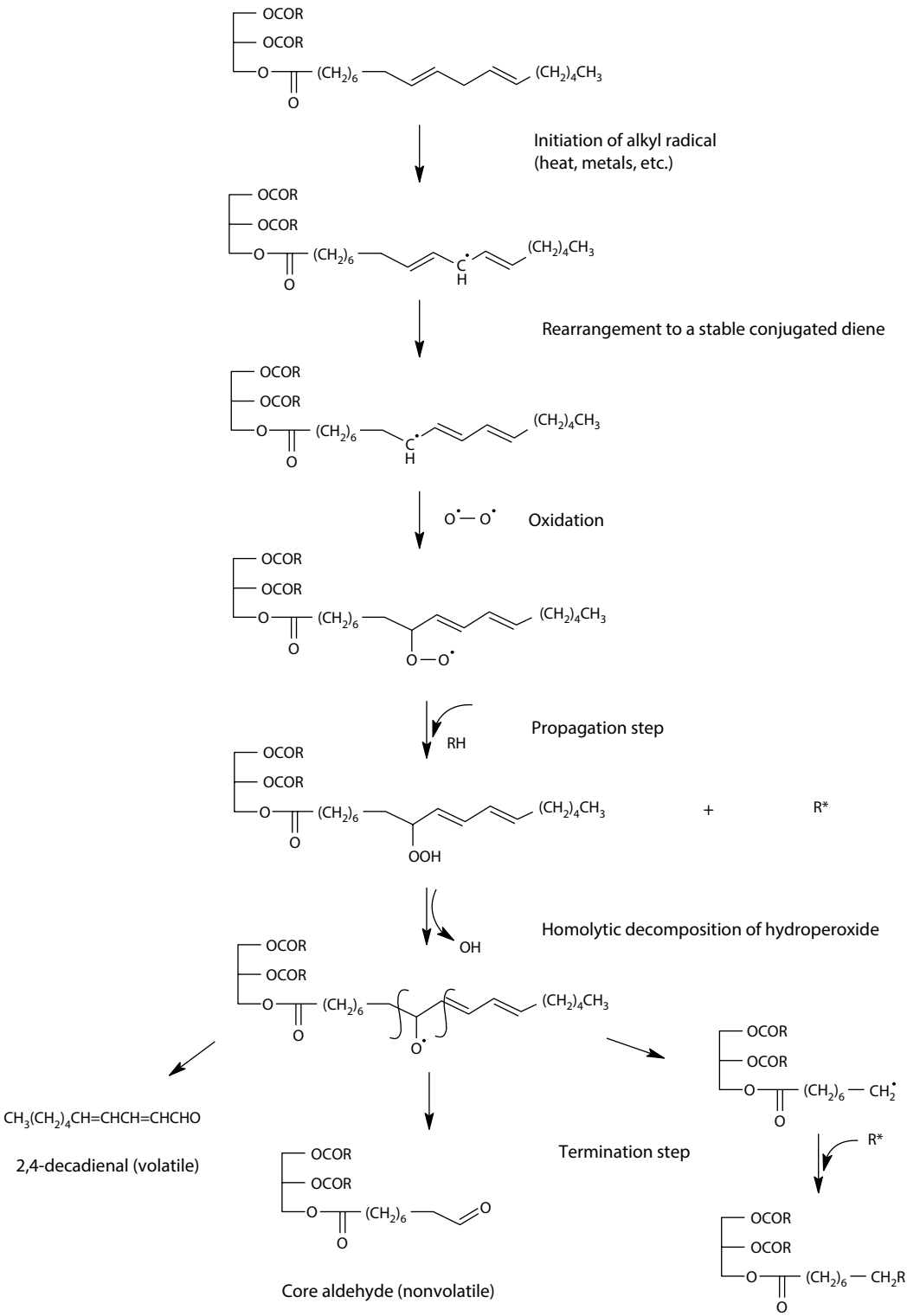

FIGURE 9.3 Oxidative reaction and representative products.

TABLE 9.1
Characteristics of Individual Volatiles Formed from Oxidative Degradation of Oils

Volatile	Reported Odor Threshold in Oil (mg/kg)	Reported Odor Descriptors
Pentane	340	—
Hexane	—	—
Butanal	0.025	—
Pentanal	0.070	Painty, herbal
Hexanal	0.120	Fatty, green, fruity, cut grass, herbal, rancid, painty, crushed weeds
Heptanal	0.055	Weeds, green, sour, sweaty, herbal, painty, rancid
Octanal	1.50	Lime, grassy, citrus, sharp, heavy, candle-like, crushed weeds
Nonanal	1.00	Green, soapy, rubbery, beany
Decanal	—	Fruity, candle-like
2-pentenal	1.00	—
3-Hexenal	0.003	Green, apple-like
2-Heptenal	1.50	—
2-Nonenal	0.15	Green, fatty, tallow
2-Decenal	2.10	Metallic
2,4-Hexadienal	—	—
2,4-Heptadienal	0.04	Fatty, nutty
2,4-Octadienal	2.40	—
2,4-Decadienal	0.135	Waxy, fatty, green

Source: Adapted from Malcolmson, L.J. et al., *J. Am. Oil Chem. Soc*., 73, 1153, 1996.

At the high temperature employed during frying, the solubility of oxygen decreases dramatically. At this reduced amount of oxygen, the initiation reaction becomes more important where the ratio between alkyl radicals ($R^{\bullet}$) and alkylperoxy radicals ($ROO^{\bullet}$) increases. Accordingly, oligomers are formed through reactions involving mainly alkyl and alkoxy radicals [32]. Dimers are formed between two fatty acids, either between or within triacylglycerols, and oligomers with high molecular weight are obtained as these molecules continue to cross-link.

Oligomers can be polar or nonpolar depending on whether the monomers are connected by a –C–C–, –C–O–C or a –C–O–O–C linkage. Formation of polar oligomers during deep frying is primarily a free radical reaction (Figure 9.4). Nonpolar oligomers, on the other hand, can be formed by both a free radical mechanism and Diels–Alder reactions as shown in Figures 9.4 and 9.5 [12,33]. Although Diels–Alder reactions are well-accepted reaction mechanisms for the formation of oligomers in frying oils, a recent study by Hwang et al. [34] did not find any evidence for Diels–Alder reaction products in thermo-oxidized soybean oil by NMR study.

Analysis of dimers isolated from frying fats indicated that they are mainly linked by C–C bond rather than the more polar C–O–C bond, which would be expected to be more abundant if oxidative radical mechanism was the major pathway to oligomerization during frying [35]. Consequently, a nonradical acid–catalyzed reaction mechanism for the dimerization and cyclization of triacylglycerols was proposed [36,37]. As depicted in Figure 9.6, the initial reaction involves the transformation of polyunsaturated fatty acids (PUFAs) to the more reactive conjugated fatty acids [36]. Acid catalysis generates the intermediate cationic reaction products that are effectively stabilized by mesomeric effects to undergo further reactions producing non-oxygen-linked dimers. Although the dimerization of triacylglycerols is expected to take place at temperatures of 220°C and above, acidic components in the frying medium can effectively catalyze these reactions at temperatures as low as 140°C [37].

The amount and type of oligomers formed during frying depends mainly on the type of oil, concentration of oxygen, frying temperature, and number of frying cycles [11]. For instance, oil rich in

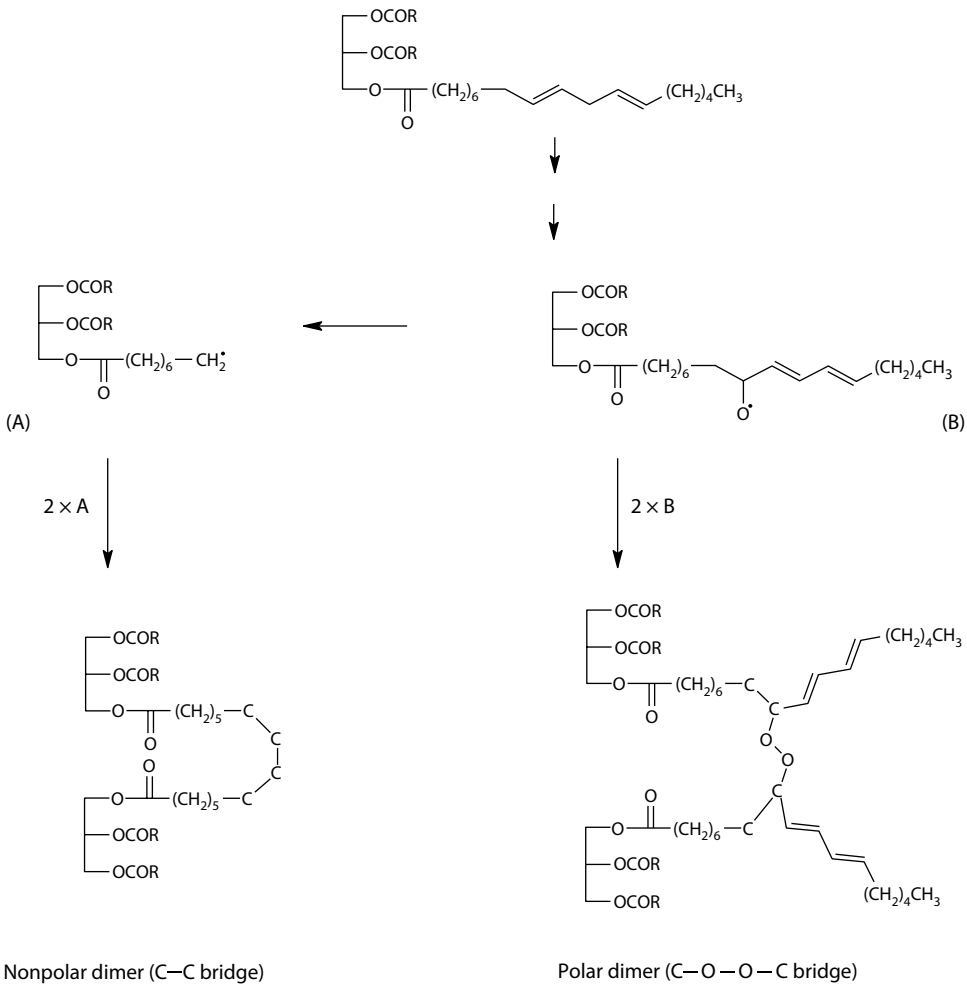

FIGURE 9.4 Formation of polar and nonpolar dimers by free radical mechanism.

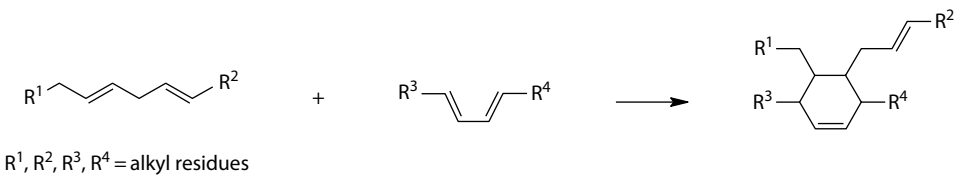

FIGURE 9.5 Formation of nonpolar dimers by Diels–Alder reaction.

linoleic acid oligomerizes more easily during deep frying than oil rich in oleic acid [38–40]. Most of the physical changes observed in the frying oil during prolonged frying are related to the formation and accumulation of oligomers. For instance, increase in the amount of oligomers increases viscosity, darkening, and foaming of frying oil. Since amounts of oligomers steadily increase in the frying oil due to their nonvolatility, they have become reliable indicators of the fat abuse [41].

The nutritional and physiological effects of thermo-oxidized oils have been reviewed by Billek [42] and Dobarganes and Marquez-Ruiz [12]. In a more recent study, Leong et al. [43] associated the elevated blood pressure and impaired vasorelaxation in experimental rats to the consumption of

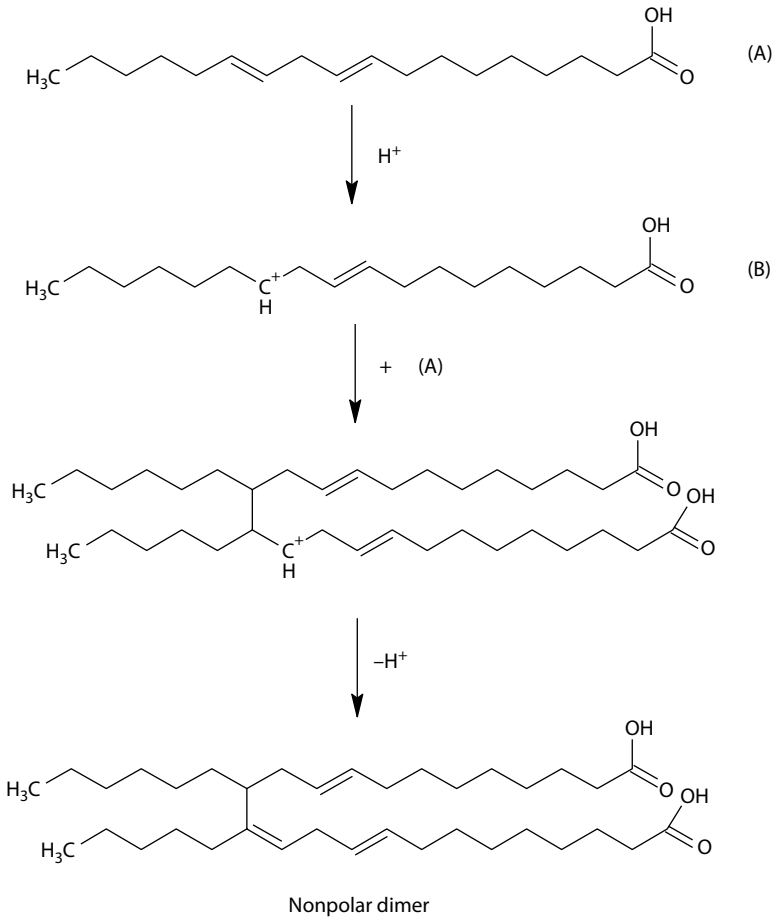

FIGURE 9.6 Formation of nonpolar dimers by cationic mechanism. (Adapted from Brütting, R. and Spiteller, G., *Fat Sci. Technol*., 96, 445, 1994.)

soybean oil heated to 180°C and up to 10 batches of potatoes intermittently fried in it. Sprague-Dawley rats were fed with commercial rat chow supplemented with the thermo-oxidized oil for 6 months. The researchers observed a significant elevation in blood pressure in all the rats fed with the thermo-oxidized oil compared to rats fed with fresh soybean oil. Similar results were reported earlier using heated palm oil [44,45]. A study by David et al. [46] demonstrated that the ingestion of thermally oxidized sunflower oil by rats resulted in a significant increase of intestinal oxidative stress. The observed effect was attributed to the increased amounts of nonvolatile degradation products formed in the oil during frying.

Shuid et al. [47] indicated that the level of toxicity observed in thermo-oxidized oils is dependent on the inherent stability of the oils. Palm oil and soybean oil were subjected to the same frying conditions. A mixture of the thermo-oxidized oils and rat chow (15:100, w/w) were fed to ovariectomized rats for 6 months, and the effects on the bone histomorphometric parameters were assessed. The researchers observed that while no effect was seen in rats fed with thermo-oxidized palm oil, those that consumed the less-stable soybean oil showed significantly deteriorated histomorphometric parameters. Indeed, the susceptibility of polyunsaturated oils to the various thermo-oxidative reactions discussed earlier explains why they are considered unsatisfactory for extended frying operation.

III. FACTORS AFFECTING FRYING STABILITY OF OIL

The stability of frying oil is a measure of its resistance to several of the degradative reactions occurring during frying. The various factors influencing the stability and performance of a frying oil can be categorized into external and internal factors depending on whether they are oil dependent or operator dependent.

A. External Factors

As mentioned earlier, external factors are operator dependent and are independent of the inherent quality of the frying oil. These factors include frying temperature; accessibility to oxygen; duration of frying; size, dimension, and composition of food; design of fryer (shape, size relative to production requirements, surface-to-volume ratio, responsiveness of heating element, and accuracy of temperature controllers); type of frying operations (continuous or discontinuous/intermittent frying); and frying management (debris removal and oil replenishment). These factors are variously discussed in the literature [48–52]; however, the influence of oxygen accessibility, frying temperature, frying time, food preparation, and frying management are quite significant and will be discussed in more depth.

1. Oxygen

Lipid oxidation is arguably the single most important factor affecting the life of edible oil. As previously stated, the alkyl radical formed at the initiation step of oxidation reacts very rapidly with molecular oxygen to form peroxy radical. At oxygen pressures greater than 100 mm Hg, such as usually present in fats and oils at room temperature, the rate of oxidation is independent of the oxygen concentration [10]. However, during deep frying, when the oxygen supply is limited due to its poor solubility at high temperature, and a steam blanketing, the rate of oxidation becomes highly oxygen dependent [10,53].

Oxidation of oil increased with the amount of dissolved oxygen in the oil [54]. Przybylski and Eskin [55] reported that the amount of oxygen dissolved in oil is sufficient to provide a peroxide value of 10 meq/kg. According to Fujisaki et al. [56], the total amount of volatile aldehydes emitted during thermal treatment of high-oleic safflower oil increased with increased oxygen concentration in the oil. Besides oxidative degradation, concentration of oxygen also dictates the nature of degradation products formed during frying. For example, at 2% oxygen, acetaldehyde was reportedly the main volatile carbonyl compound while hexanal and nonanal predominated at 20% oxygen [56]. Because individual volatile carbonyl compounds possess characteristic flavor and threshold values (Table 9.1), the concentration of oxygen during deep frying will invariably affect the flavor of fried food.

Although poor solubility in the frying oil and the release of steam, blanketing the oil's surface, are known to limit oxygen concentration during frying, several factors have also been reported to increase oxygen level in the frying medium. For instance, introduction of fresh food to the frying medium increases this level; thermal agitation accompanying boiling during frying of food breaks the oil surface and enhances accessibility of atmospheric oxygen by enlarging the surface area; and surface-active compound naturally occurring in oil or formed during frying can also enhance access of atmospheric oxygen during frying. According to Mistry and Min [57], FFAs decrease the surface tension of oil, thereby increasing the diffusion rate of oxygen into the oil to accelerate oil oxidation. A frying system with a large surface-to-volume ratio also increases the oxygen availability during frying.

In a recent study by Totani et al. [58], oxygen lost at frying temperature due to poor solubility or direct involvement in thermo-oxidative reactions is slowly restored during cooling and standing until room temperature (between 25°C and 120°C). Thus, the reduced solubility of oxygen at frying temperature may not limit its influence on the performance of the frying oil, especially

during intermittent frying. For this reason, a vital recommendation from the *Seventh International Symposium on Deep-Fat Frying* is to avoid holding oils at temperatures between 60°C and 130°C during downtimes when the oil is not in use for frying [59].

Methods to reduce availability of oxygen during frying have been reported in an effort to protect frying oil from thermo-oxidative degradation. Przybylski and Eskin [55] evaluated the efficacy of nitrogen and carbon dioxide flushing to prevent canola oil oxidation under frying conditions. The oil samples were heated at 195°C in a variety of containers where the flow of nitrogen and carbon dioxide was regulated. The authors observed that oils heated without prior nitrogen or carbon dioxide flushing underwent more rapid oxidation compared to oils with prior nitrogen or carbon dioxide flushing. Based on their findings, they suggested that to prevent thermo-oxidative degradation of frying oils, the following should be considered: (1) flush oil with carbon dioxide rather than nitrogen, (2) the oil should be flushed with nitrogen for 15 min or carbon dioxide for 5 min prior to heating to eliminate any dissolved oxygen, (3) the linear flow of gas in the container should be at 50 cm/min, and (4) the vessel should not be filled to more than 70% of its height. Higher density and greater solubility in oil reportedly accounted for the superior protection offered by carbon dioxide compared to nitrogen in the frying model described by the authors [55]. Although this study indicated that frying under inert gases has potential in limiting thermo-oxidative degradation of oils, their effectiveness during actual deep frying was not verified until two decades later [60].

Shyu et al. [61] reduced the content of oxygen by conducting the frying under vacuum. For 6 consecutive days, carrot slices were fried in palm oil, lard, and soybean oil at 105°C for 20 min each hour in an 8 h shift. The authors concluded that vacuum frying imparted a lower thermo-oxidative degradation on the oils than the typical atmospheric frying. Although the better stability of the oils during vacuum frying was attributed to the lower content of oxygen, the lower frying temperature in the study (105°C) makes comparison with typical frying (temperature ~180°C) unrealistic. According to Aladedunye and Przybylski [62], frying under CO_2 blanket reduced the amount of total polar compounds by 54%, while 76% reduction was observed during vacuum frying compared to standard frying conditions. Similarly, lower oxidative degradation was observed when measured by anisidine value (AV). At the end of frying period, the reduction in unsaturated fatty acid content was 3.8%, 1.9%, and 12.7% when frying under CO_2 blanketing, vacuum, and standard frying conditions, respectively. The rate of tocopherols degradation was 3 and 12 times slower in vacuum frying when compared to CO_2 blanketing and standard frying conditions, respectively. Evidently, oxygen concentration has a huge impact on the performance of oils even at frying temperature, despite the poor solubility.

Silicone, particularly dimethylpolysiloxane (DMPS), is often added to frying oils as an antifoaming agent [63,64]. Although a number of mechanisms have been proposed for DMPS's activity in delaying thermo-oxidative degradation of frying oils, the most prominent is related to its low fat solubility (<1 ppm) and therefore its ability to accumulate at the oil surface where it forms a physical barrier against oxygen [63,65]. Consequently, the minimum concentration of DMPS that exerts a protective action corresponds to that forming a monolayer on the oil surface. This concentration depends on the surface-to-oil-volume ratio and has been reported to be between 0.05 and 0.06 μg/cm^2 oil-to-air surface [63]. Because DMPS's primary mechanism is surface action, a major disturbance to the oil's surface such as during continuous frying of food will be expected to limit its effectiveness; thus, DMPS addition would be of benefit only in discontinuous or intermittent frying operations such as in catering services, fast food outlets, or restaurants where the fryers usually remain without food for a significant period of time [64].

2. Temperature

The various reactions threatening the oxidative stability of oils require some level of energy to proceed. For instance, 50 kcal/mol of energy is required to break the carbon–hydrogen bond on the carbon 11 of linoleic acid and to initiate free radical formation [17]. The oxygen–oxygen bond of alkyl hydroperoxide requires 44 kcal/mol to break it [66]. This energy requirement is more than fulfilled at typical frying temperatures (150°C–200°C). Nawar [67] reported a peroxide value

of 1777 meq/kg when ethyl linolenate was heated at 70°C for 6 h; but only 13.3% of this value remained when the same oil was heated at 180°C for 5 h. At 250°C, significant degradation of hydroperoxide occurred and only 2.5% of the peroxide value was left after 3 h of heating. Therefore, elevated temperature enhances both the initiation step of oxidative degradation and thermal degradation of alkylhydroperoxides, the primary oxidation product. Consequently, oxidative degradation proceeds more rapidly during deep frying than at room temperature [48,68,69].

Increase in frying temperature increases thermal oxidation and oligomerization reactions not only of the fatty acids or triacylglycerol molecules but also of the unsaponifiable minor components. Thus, antioxidant minor components in oil are either thermally inactivated during frying or have their levels severely reduced [70–73]. For instance, while tocopherol in rice bran oil heated to 100°C in the absence of air showed a reduction by 29% at the end of 432 h of heating, its reduction was 100% when the oil was heated at 180°C for 240 h [74]. Oxidation and polymerization of phytosterols, consequent to increase in temperature, have also been studied, and the formation of oligomers arising from thermo-oxidation of phytosterols has been reported [75–77].

The effect of temperature on isomerization reactions has also been reported. Tyagi and Vasishtha [69] reported an increase in *trans* fatty acids from 1.7% to 2.6% in soybean oil when the frying temperature was increased from 170°C to 190°C. Tsuzuki et al. [78] compared to the amounts of *trans* isomers formed when canola oil was heated at different temperatures (160°C, 180°C, and 200°C). They reported a statistically significant increase in the isomerization rate of linolenic acid when the heating temperature was increased from 180°C to 200°C. In a similar experiment, 10 batches of potatoes were fried in canola oil every 10 min, and the amounts of *trans* fatty acids formed at the end of the frying period (100 min) were analyzed by gas chromatography. It was observed that at all tested temperatures, the amounts of *trans* isomers were higher in oils used for deep frying than the heated samples [78]. Aladedunye and Przybylski [62] concluded that "zero-*trans*" claims for fried food could be invalidated by frying at temperatures higher than the optimum, either due to a faulty temperature controller or deliberately, to increase turnover at the peak hours.

3. Frying Period/Time

Although deep frying is a fast method of food preparation and the contact time between food and oil is relatively short, the length of time spent using the frying oil is usually long, especially during continuous and repeated frying such as in industrial and institutional operations. All things being equal, thermo-oxidative degradation increases with increase in length of time for which the oil is used [14,79–81]. In a study by Bansal et al. [82], three different frying oils, palm olein, sunflower oil, and a blend of palm olein, sesame, and peanut oils, were used to prepare French fries. The oils were heated for 6 h daily for 4 days and 10 batches of French fries were fried each day without oil replenishment. The extents of isomerization of the fatty acids were quantified by gas chromatography and infrared spectroscopy. Irrespective of the type of oil and the quantification method, the authors reported a direct relationship between the extent of isomerization and the number of frying cycles.

The contact time between the food and the frying oil, defined as Dwell Time, is also critical and must be balanced with the frying temperature. Generally, the presence of food reduces deterioration of frying oils, compared to heating without food. This can be attributed to the release of water from the fresh food (immediately after the food is added) that lowers the effective frying temperature, coupled with the blanketing effect of the ensuing steam against oxygen during the frying. However, extending the dwell time beyond the optimum can nullify any advantages from steam blanketing and the reduced effective temperature from the fresh food due to (1) the continuous migration and expression of prooxidant metals and decomposition products from the food to the frying oil and (2) the continuous presence of food (after the loss of the initial moisture/steam) that would increase oxygen penetration into the oil by opening the oil surface.

To leverage the effect of frying time, replenishing the oil with fresh oil is a very crucial oil management program; however, this exercise is futile and unhealthy once the oil reaches the "degrading" and "runaway" stages shown in Figure 9.7 [83].

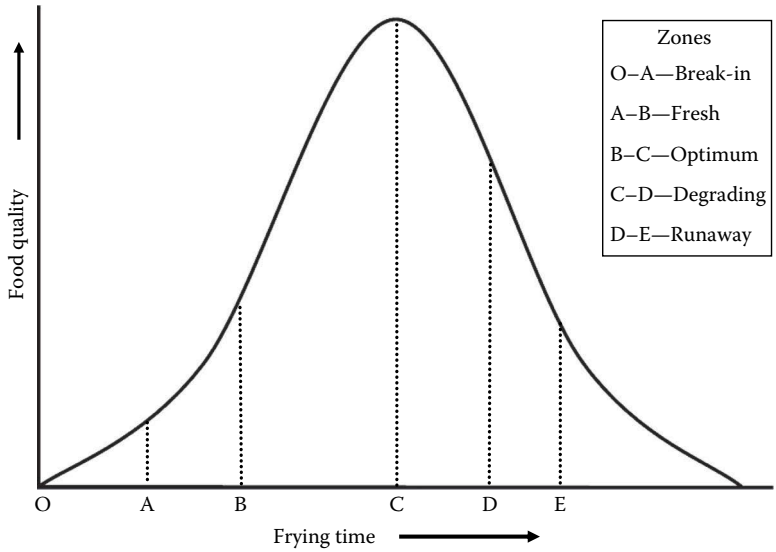

FIGURE 9.7 Blumenthal's frying oil quality curve describing five stages of oil degradation—break-in, fresh, optimum, degrading, and runaway. (Adapted from Stier, R.F., *Eur. J. Lipid Sci. Technol*., 106, 715, 2004.)

4. Food Preparation

It is generally acknowledged that the presence of food slows down thermo-oxidative degradation of oils when compared to heating the oil without any food being fried. Nevertheless, the type, composition, and size of the food can significantly affect the frying stability of the oil. For instance, fatty food items containing highly unsaturated oils could compromise the integrity of the frying oil by increasing the overall level of the intrinsically unstable PUFA in the frying oil through mass exchange between the food and the frying oil [84]. Proteins, peptides, and amino acids in foods can also react with lipid free radicals or lipid degradation products, primarily carbonyl-containing oxidation products, thus inhibiting thermo-oxidation during frying [85]. Starch, on the other hand, reportedly increases thermo-oxidative degradation of frying oils [48]. According to Artz et al. [86,87], transition metals such as iron in meat products can progressively accumulate in the oil during frying, accelerating thermo-oxidative degradation. Iron bonds in myoglobin or hemoglobin are easily released under conditions of frying meat or fish [85]. A direct positive correlation has been reported between the moisture contents of the food being fried and the thermo-hydrolytic and thermo-oxidative degradation in the frying oils [88]. The oxidative status of the food is also important because leaching of preformed hydroperoxides from the food material into the frying oil could result in accelerated degradation of the frying oil. Regardless of the food type and composition, increased cross-sectional area of the frying food (thin-cut strips compared to thick cut, the presence of cracks and rough surfaces compared to smooth surface) often lead to increased thermo-oxidative degradation of the frying oils. This may be related to increased removal of frying oil by the frying food with increased surface area, resulting in reduced volume of the frying oil, thereby increasing oxygen pressure in the fryer. Further, with increased surface area of the frying foods, the opportunity and rate of mass exchange between the food (moisture, prooxidant metals, coloring matters, etc.) and the frying oil is enhanced.

One of the ways to moderate the unwanted mass exchange between foods and the frying oils is by using edible coatings. Various materials have been used for the development of edible coatings, including hydrocolloids based on proteins of animal or plant sources (e.g., whey, soy, corn, legumes), polysaccharides (e.g., cellulose derivatives, alginates, or starches), and even (semi)synthetic polymers (e.g., polyvinyl acetate, hydroxypropylmethylcellulose) [89]. However, interactions between these often multicomponent coatings and the frying oil can result in an enhancement or depression of the stability of the frying oil.

For instance, according to Holownia et al. [90], a decrease in degradation of peanut oil was observed when the fried food (marinated chicken strips) was coated with food grade hydroxypropylmethylcellulose prior to frying. This protection was attributed to the ability of the coating to inhibit moisture migration from the food into the frying oil thereby limiting thermal hydrolytic reaction, and by extension, thermo-oxidative deterioration of the frying oil. On the other hand, Lazarick et al. [91] observed that using whey protein as a base for battering resulted in significant color changes and degradation of the frying oil with the changes being more pronounced in the presence of preformed lipid hydroperoxides. Glucose and glycine are two minor ingredients that also contributed to color formation in oil. Breading materials were prone to cause a more significant amount of oil deterioration when compared to battering ingredients most likely due to excess loose breading particles falling into the oil during frying [91]. In a study by Guerra-Hernandez et al. [92], battering of zucchini with rice or corn flour-based batter significantly increased the amount of acrylamide formed during frying in sunflower oil at 190°C, compared to wheat and chickpea flours. Breaded products showed lower amounts of acrylamide than battered products during the frying process [92]. According to Xue and Ngadi [93], the type of batter system can significantly influence thermal properties of the food products. For example, rice- and corn-based batters require more energy for gelatinization [93], indicating that food coated with batters based on these items may require relatively longer frying period with negative influence on the stability of the frying oil.

5. Oil Management

Oil management involves a comprehensive quality control measures carried out by the frying operators to keep the oil at the peak of its performance. Besides optimizing the processing parameters (temperature, time, surface-to-volume ratio, etc.), critical oil management process involves replenishing the portion of the oil adsorbed/absorbed by the fried food with fresh oil and removal of food debris from the frying medium. Replenishment of frying oil with fresh oil will ensure (1) that the optimum surface-to-volume ratio is maintained thereby limiting inadvertent increase in oxygen pressure and (2) the introduction of fresh endogenous antioxidants into the medium for improved stability. However, as mentioned earlier, oil replenishment is of no use once the oil has reached the "degrading" and "runaway" stages indicated in the Blumenthal's stylized graphics (Figure 9.7).

Food debris, especially when breaded food items are fried, are known to significantly increase the degradation of the frying oil both by increasing the level of dissolved oxygen and through uncontrolled leaching of prooxidative degradation products into the frying oil. Regular oil filtration with the use of passive and active filters can help limit the extent of deteriorations. Passive filters, including metal screens, paper filters, paper cones, plastic cloths, and plate and frame systems with possible augmentation by inert filter aids such as diatomaceous earth, simply remove solid particles from the oil. Active filter, on the other hand, can remove specific oil-soluble degradation compounds from the frying oil, thereby "purifying" the oil for an extended operation. It must be stressed, however, that abused oil cannot be restored to its original status by any filtration system.

B. Internal Factors

Unlike the external factors affecting the stability of frying oils, internal factors arise from the inherent composition of the frying oil. Edible oils are composed of triacylglycerols (>95%) and endogenous minor components (Table 9.2). It is generally agreed that the inherent composition of edible oils exerts significant influence on their frying stability [94–97]. These internal factors are discussed in the following text:

1. Fatty Acid Composition and Distribution

The influence of fatty acid composition of oils on stability has been the subject of studies by many researchers [98–100]. In general, unsaturated oils oxidize more readily than less unsaturated ones. In other words, as the double bonds in the fatty acid increase, both the rate and the amount of

TABLE 9.2
Classes of Minor Components and Examples

Class of Compounds	Examples
Hydrocarbons	Squalene
Phytosterols	β-Sitosterol, stigmasterol
Tocochromanols	α-, β-, γ-, δ-tocopherol/tocotrienols
Ubiquinones	Ubiquinone 9, ubiquinone 10
Phenolic compounds	Phenolic acids, flavonoids, and isoflavonoids
Carotenoids	Carotenes, xanthophylls
Phospholipids	Phosphatidylcholine, phosphatidylethanolamine

Source: Adapted from Shahidi, F., Oxidative stability of edible oil as affected by their fatty acid composition and minor constituents, in: *Freshness and Shelf Life Foods*, ACS Symposium Series 836, American Chemical Society, Washington, DC, 2003.

primary oxidation products at the end of the frying period increase. This observation correlates with the relative rate of the fatty acid alkyl radical formation [10]. On the basis of oxygen uptake, linoleate was 40 times more reactive than oleate, linolenate was 2.4 times more reactive than linoleate, and arachidonate was 2 times more reactive than linolenate [19,101]. The oxidizability of linoleic (18:2), linolenic (18:3), arachidonic (20:4), and cervonic (22:6) acids increased with the number of *bis*-allylic bonds present in the fatty acid ester [102].

Thus, the search for stable oils has led to several modifications to the fatty acid composition of edible oils, such as decreasing the linoleic and linolenic acids and increasing oleic acid [103–105]. Almost all conventional oils now have counterparts with modified fatty acid compositions. Various high-oleic sunflower oils containing from 75% to 90% oleic acid are now on the market. The amount of linolenic acid in conventional canola and soybean oils has been significantly reduced in low linolenic canola and soybean oils. Soybeans containing increased levels of oleic acid with reduced linoleic and linolenic acid levels have been developed or are being developed for commercialization [106]. High-oleic canola oil is currently used extensively in North America for commercial frying and in food manufacturing. Both canola and soybean oils are particularly high in tocopherols that enhance the stability of the corresponding high-oleic oils [107].

Established methods for lowering unsaturation in oils include fractionation, hydrogenation, interesterification (chemical and enzymatic), conventional seed breeding, and genetic engineering. Several reviews covering some aspects of these methods are available [108–113]. The review by Dijkstra [108] covered available modifications to the hydrogenation process that were geared toward reducing the formation of *trans* fat. The oxidative stability of vegetable oils with a modified fatty acid composition by interesterification, technically called structured lipids, compared to conventional oils was reviewed by Martin et al. [113].

Blending of polyunsaturated oils with a more saturated or monounsaturated oils is also being used as a cost-effective way of reducing the amounts of linoleic and linolenic acids [114–120]. This technique is indeed gaining wide acceptance and the literature on the subject is on the increase with applications extending to the nonconventional edible oils [80,116,121–123].

Although frying stabilities of vegetable oil blends are generally reported to be higher than the stabilities of the individual oils used in the blending, the observed increase may not be entirely due to changes in fatty acid composition and endogenous minor components from the constituent oils and other subtle factors may play significant roles. For instance, according to Mezouari and Eichner [124], raising the amount of rice bran oil from 10% to 50% in a sunflower/rice bran oil blend will change the triacylglycerol compositions by increasing OLO, OLP, PPL, OOO, OPO, and decreasing

LLL and LLO contents to a level that can significantly influence thermo-oxidative stability (O, oleic; L, linoleic; P, palmitic). Changes in the triacylglycerol composition have been reported to exert a strong influence on the stability of frying oils [125–127]. Indeed, according to Kim et al. [125], the influence of changes in triacylglycerol composition on foaming of oils during frying exceeded that of the fatty acid composition.

Some of the methods currently used to modify fatty acid compositions of oils are also known to negatively affect the nutritional and functional properties and stability of the oils [128,129]. Hamam and Shahidi [129] reported a significant reduction in tocopherol content during the synthesis of structured lipids by interesterification. Consequently, the modified oils were less stable than their unmodified counterparts despite an increase in the degree of saturation in the products. Their observations agreed with several other studies [130–133].

Apart from reducing the availability of essential fatty acids (linoleic and linolenic), the use of genetic engineering for modifying edible oil is still plagued by suspicious attitudes of consumers toward genetically modified organism (GMO) products [134]. Consequently, researchers are still looking for other ways to improve frying stability without changing the fatty acid composition and distribution in the oils.

2. Endogenous Minor Components

The minor components (e.g., tocopherols, tocotrienols, sterols, carotenes, phenolic acids, waxes, squalene, and oryzanols), also referred to as unsaponifiable matters, are the nontriacylglycerol constituents of the oil and constitute up to 5% of the total lipid composition.

The composition of edible oil minor components is of great importance to the chemistry and stability of the frying oil; however, individual edible oil is naturally endowed with its own unique composition of minor components. For instance, soybean oil is rich in γ- and δ-tocopherol; sunflower oil contains predominantly α-tocopherol; palm oil is rich in tocotrienols and β-carotene; rice bran oil and sesame oil uniquely contain γ-oryzanol (a group of steryl ferulates) and lignans (sesamin and sesamolin), respectively. However, the stability-enhancing capacity of minor components differs from one another. Accordingly, research is being carried out to study the performance of some minor components in oils in which they are naturally absent [135].

The effect of endogenous minor components on oil decomposition and performance during frying has been thoroughly reviewed [73,135–138]. General conclusions can be summarized as follows: (1) endogenous minor components (tocochromanols, phytosterols, γ-oryzanol, and related compounds, sesamins and related compounds, hydroxytyrosol and related compounds, carotenoids, and the phenolics) do exert significant influence on the stability of frying oils; (2) there are optimal concentrations above which some of these endogenous antioxidant compounds become prooxidative, accelerating the decomposition of the frying oil; (3) the effectiveness of antioxidative endogenous minor components also depends on the chemical group and the isomeric composition; (4) depending on the oil and the relative concentrations, there is possibility of both synergistic and antagonistic interactions among these antioxidants and other minor components in the frying oils; and (5) the effect of these components under ambient and storage conditions can be quite different from that during frying, principally because of the more stringent conditions applied during frying. Thus, there may be the need to fortify the endogenous minor components for enhanced performance of the frying oils, and a number of synthetic, natural, and semisynthetic (modified natural) antioxidants have been approved for such application (Table 9.3).

IV. ASSESSING THERMO-OXIDATIVE DETERIORATION OF FRYING OILS

Traditionally, the frying operator employs experience to decide when to halt frying and change oil and often it is based on physical changes such as color, odor, excessive foaming, and smoking. Assessing oil degradation using visual indicators, however, is inadequate and often unreliable due to its subjective nature. A number of reliable analytical methods based on

TABLE 9.3
Approved Antioxidants Most Commonly Used in Food

Antioxidants	E Number[a]
L-Ascorbic acid	E300
Sodium L-ascorbate	E301
Calcium L-ascorbate	E302
Potassium L-ascorbate	E303
Ascorbyl palmitate, ascorbyl stearate	E304
Mixed tocopherol concentrate (natural)	E306
Alpha-tocopherol (synthetic)	E307
Gamma-tocopherol (synthetic)	E308
Delta-tocopherol	E309
Propyl gallate	E310
Octyl gallate	E311
Dodecyl gallate	E312
Erythorbic acid	E315
tert-Butylhydroquinone (TBHQ)	E319
Butylated hydroxyanisole (BHA)	E320
Butylated hydroxytoluene (BHT)	E321
Lecithins	E322
Citric acid	E330
L-Tartaric acid	E334

Source: Adapted from Torres, P. et al., *Open Food Sci. J.*, 2, 1, 2008.

[a] E numbers are number codes for food additives that have been assessed for use within the European Union.

quantification of a specific group of degradation products have been developed to provide objective indication of level of decomposition of frying oils.

Standard methods based on quantification of primary oxidation products such as peroxide value, FFA content, and conjugated dienes are usually unreliable because products measured are thermally unstable with unstructured fluctuations over the course of the frying operation [1,140]. It must be stressed, however, that while the amounts of these unstable primary thermo-oxidation products may not directly correlate with oil abuse or length of use, their levels in the oil (and eventually, the food) can significantly affect sensory acceptance.

Generally, methods based on nonvolatile degradation products are more reliable and dependable indicators of oil degradation than those based on volatile or unstable degradation products. Common analytical methods for assessing the levels of degradation of frying oils including their limitations are discussed next.

A. Total Polar Components

As previously mentioned, chemical reactions occurring during frying generate several groups of compounds with higher polarity than the original triacylglycerols. Polar compounds formed in oils during frying include oxidized and oligomerized triacylglycerols, FFAs, MAGs and DAGs, oxidized and oligomerized sterols, and degradation compounds of antioxidants and other constituents of oil and food. These compounds are nonvolatile and their amounts in oil usually increase as frying progresses.

The analysis of total polar components (TPC) provides the best indicator of frying oil degradation because it measures directly all the degraded products present in the oil. Thus, many European

countries have set a maximum permissible level for TPC in frying oil as a way of regulating the level of abuse in commercial and institutional frying operations [141]. There are differences among the European countries, however, in the discarding level of TPC as follows: Austria at 27%; Belgium, France, and Spain at 25%; and Germany allowing not more than 24% of TPC [141]. According to Gertz [142], the value of 24%–25% of TPC should be taken as borderline in the assessment of the quality of frying oils. Presently, there is no specific regulation in Canada or the United States defining maximum level of TPC.

Official methods for assessing TPC involve a gravimetric procedure that utilizes chromatographic separation of polar and nonpolar components on silica gel with adjusted water content to 5% [143,144]. TPC represents the components remaining on the column after elution of unaltered triacylglycerols and is expressed in weight percent of the starting sample.

Collaborative tests conducted by IUPAC and AOCS showed that the method is exact and reproducible, with a coefficient of variation lower than 5% [143,144]. For rapid analysis and to reduce the consumption of solvents and silica gel, the use of miniaturized columns requiring a small sample size has been proposed [145,146]. However, the level of accuracy and precision is expected to decrease for such small sample amounts, especially in samples with low levels of TPC. Zainal and Isengard [147] suggested the use of an automated accelerated solvent extraction to replace the manual chromatographic step for quicker analysis and to reduce experimental effort.

To eliminate the use of chemicals, the determination of TPC by Fourier transform nuclear magnetic spectroscopy (FT-NMR), Fourier transform infrared spectroscopy (FT-IR), and differential scanning calorimetry (DSC) has been proposed as a substitute for the adsorption chromatographic method [148–151]. Hein et al. [149] evaluated TPC in thermo-oxidized soybean, peanut, and palm oils using a Fourier transform near-infrared (FT-NIR) spectroscopic method. The authors reported correlation coefficients from 0.990 to 0.998 between TPC determined by adsorption chromatography and the values obtained by NIR spectroscopy at the wavenumber range specific for hydroxyl and carbonyl groups (4700–4940 cm^{-1}). Sun and Moreira [148] reported a correlation coefficient of 0.985 for TPC determined by adsorption chromatography and NMR proton relaxation time of thermo-oxidized soybean oils. Similarly, TPC obtained by DSC linearly correlated at $r^2 = 0.956–0.999$ to values obtained using an adsorption chromatographic method for fresh and thermo-oxidized corn oil, palm olein, and soybean oil [150]. Due to its versatility, FT-NIR has become particularly important for a quick, nondestructive analysis of frying oils and FT-NIR-based procedure has been recognized as the German Standard Method, DGF C-VI-21 [152], for the analysis of TPC, polymerized triacylglycerols, and AVs (discussed in subsequent sections). Although instrumental methods offer some advantages, it is unlikely that they will replace the adsorption chromatographic method for routine laboratory analysis of TPC because of its simplicity and the affordability of materials required for adsorption chromatography readily available in most laboratories.

As shown in Table 9.4, several of the observed changes in physical parameters occurring in oils during frying, including increase in conductivity, viscosity, specific heat, and decrease in surface tension and dielectric coefficient are caused by the formation of polar compounds [140]. The direct relationship between these physical changes and the amounts of TPC is explored in a number of the available commercial quick test kits and equipment used for monitoring thermo-oxidative degradation (Table 9.5).

B. Composition of Polar Materials

Although percent of TPC is an excellent indicator of oil degradation, it only determines the total level of polar materials without distinguishing specific compounds. A measure of the specific group of degradation products is, however, important as it provides information about the types and level of reaction occurring in the frying oil and its potential toxicity. For example, although fatty acid MAGs and DAGs (products of thermo-hydrolysis) significantly contribute to the level of TPC, from

TABLE 9.4
Changes in Physical and Chemical Parameters during Deep Frying, Main Causes, and Correlation with Oil Deterioration

Parameter	Changes during Deep Frying	Mainly Causes	Correlation with Oil Deterioration
UV	Increases	Conjugated fatty acids	Yes
Refractive index	Increases	Polar compounds	Yes
Density	Increases	Polymerized TAG	Yes
Dielectric coefficient	Decreases	Polar-oxidized components—affected by FFA and water	Yes
Color	Becomes more intensive and darker	Maillard reaction products of amino acids, protein, sugar, and carbonyl compounds	Yes
Conductivity	Increases	Polar compounds	Yes
Surface tension	Decreases	Polar compounds	Yes
Smoke point	Decreases	Volatile oxidized products	Yes
Specific heat	Increases	Polar compounds	Yes
Viscosity	Increases	Polymerized TAG	Yes
Anisidine value	Increases	Nonvolatile aldehydes	Yes
Iodine value	Decreases	Formation of oxidized fat	Yes
Peroxide value	Fluctuates	Hydroperoxides	No
TPC	Increases	Oxidized and polymerized degradation products	Yes
Polymerized TAG	Increases	Oxidized and not oxidized polymerized TAG	Yes
Free fatty acid/acid value	Fluctuates	Hydrolysis and oxidation products with free carboxyl groups	No
Petroleum ether-insoluble oxidized fatty acids	Increases	Oxidized polymerization products	Yes

Source: Adapted from Gertz, C. and Matthäus, B., *Optimum Deep-Frying*, Recommendations by the German Society for Fat Science (DGF, Deutsche gesellschaft für Fettwissenschaft e.V.), DGF, Deutsche gesellschaft für Fettwissenschaft e.V., Germany, 2008, p. 6.

a nutritional and physiological point of view, they are not as important as polar components arising from thermo-oxidative and oligomerization reactions [12].

High-performance size-exclusion chromatography (HPSEC) is usually used to separate and quantify molecules by their molecular weight [154]. HPSEC method can be applied directly to used oil or the polar fractions isolated from the gravimetric analysis of TPC mentioned earlier. Polar materials are separated into several groups of compounds such as oligomers, oxidized monomeric triacylglycerols, DAGs, and FFAs. HPSEC is an invaluable technique and complements the TPC method. Lending credence to the importance of TPC and CPM analyses, the delegates at the *Third International Symposium on Deep Fat Frying* recommended these analyses as the best indicators of oil degradation [155]. Regulators in European countries required that the total amount of oligomeric triacylglycerols be less than 10% [141].

C. *p*-Anisidine Value (AV)

Thermal decomposition of hydroperoxides during frying generates a number of secondary oxidation products, with carbonyl compounds being the most prominent. Although some of the aldehydes produced are volatile and lost by evaporation during frying, a significant amount remains and is assessed by AV. *p*-Anisidine value is defined by convention as 100 times the optical density

TABLE 9.5
Commercial Test Providing High Correlations with Total Polar Components

Test Kit	Principle	Manufacturers Specifications
Fri-check®	Viscosity	Measurement time: 5 min Operating temperature: 20°C–180°C
Capsens5000 (FOS)	Dielectric constant	Accuracy: ±1.4% Resolution: 1% Range of TPC: 0%–35% Operating temperature: 50°C
Food Oil Monitor (FOM 310)	Dielectric constant	Accuracy: ±2% Resolution: 0.5% Range of TPC: 0%–40% Measurement time: <2 min Operating temperature: 50°C–200°C
Testo 265	Dielectric constant	Accuracy: ±2% Resolution: 0.5% Range of TPC: 0.5%–40% Measurement time: <10 min Operating temperature: 40°C–200°C
TPM Very Fry	Patented gel reacts with the polar compounds to give specific color	Range of TPC: 3%–30% Measurement time: 10–15 s Operating temperature: 65°C

Source: Adapted from Bansal, G. et al., *Crit. Rev. Food Sci. Nutr.*, 50, 503, 2010.

measured at 350 nm in a 1 cm cuvette of a solution containing 1.00 g of the oil in 100 mL of a mixture of solvent and reagent [144]. Official procedures for determining AV utilize the reaction between aldehydes, principally 2-alkenals and 2,4-dienal, and *p*-anisidine reagent in glacial acetic acid solution (ISO 6885; AOCS Cd 18–90; IUPAC 2.502) [156–158]. The resulting Schiff bases possess a characteristic UV absorption at 350 nm, and the absorbance increases with the amount of relevant nonvolatile carbonyl compounds retained in the oil [159]. The contribution of carbonyl compounds is expressed as absorbance units per 1 g of fat and is arbitrary.

The use of FTIR for the determination of AV was described by Dubois et al. [160]. Using a partial least square calibration (PLS) as the chemometric method, the authors reported a strong correlation ($r^2 = 0.998$) between the AV values for thermo-oxidized canola oil determined by FTIR and the official chemical method. Szabó et al. [161] reported a very good correlation ($r^2 = 0.912$) between AV values determined for thermo-oxidized lard by the chemical method and the values obtained by NIR spectroscopy using a wave number range from 4000 to 5000 cm^{-1}. These spectroscopic analytical methods offer the possibility for a rapid, automated, and solvent-free alternative to the current official chemical method.

D. Changes in Fatty Acid Composition

PUFAs such as linoleic and linolenic are the main components affected by the various chemical reactions occurring during frying [18]. The observed changes in the amount and configuration of the fatty acids increase with the temperature and length of frying. Thus, assessment of changes in fatty acid composition can be used to monitor thermo-oxidative degradation occurring during deep frying. Official methods for analyzing fatty acid composition involve transforming the fatty acids into the methyl esters, followed by separation and quantification using gas chromatography

(AOCS methods Ce 1-62, Ce 1f-96, Ce 2-66 [144]; AOAC Method 963.22 [162]; ISO method 5509 [156]; and IUPAC method 2.301 [163]). Methods for preparing the methyl ester derivatives have been reviewed by Liu [164], Eder [165], Seppanen-Laakso et al. [166], and Dijkstra et al. [167].

For routine analysis, the gas chromatogram peak area is normally used to calculate the content of each fatty acid [168]. However, the gas chromatograms must be interpreted with caution since they only refer to standard fatty acids and exclude degradation products such as oxidized and oligomerized fatty acids. In addition, because in GC analysis the total fatty acid content is set by default to 100%, any decrease in the PUFAs by thermo-oxidation is automatically counterbalanced by an increase in the more stable monounsaturated and saturated fatty acids to maintain the sum despite the fact that some materials are not eluted. Thus, the increase in oleic, palmitic, and stearic acid contents often reported by some authors during deep frying gives an erroneous impression that these fatty acids are formed during thermal treatment of the oil. By using an internal standard (e.g., C17:1) the misconception can be overcome, and it becomes evident that the saturated fatty acid content does not increase and that the sum indeed falls below 100%. Also, analysis of fatty acid for monitoring thermo-oxidation is not quite responsive and, depending on the oil, significant changes may not be observed by GC even at significantly high PV, FFA, AV, and TPC.

The observed loss in fatty acids during frying is due to formation of several oxidized and oligomerized degradation products. Hydroxy, keto, and epoxy are the major functional groups identified in oxidized fatty acids of frying oils; GC methods for analyzing them have been reported by Schawrtz et al. [168], Berdeaux et al. [169], Velasco et al. [170], and Marmesat et al. [171]. Generally, methyl ester derivatives of the fatty acids (FAMEs) are obtained by base-catalyzed transmethylation of the triacylglycerols; the isolated FAMEs are then fractionated by adsorption chromatography on silica gel to separate the nonpolar FAMEs from the polar FAMEs containing fatty acids with at least one extra oxygen; polar FAMEs are subsequently subjected to GC analysis. Hydrogenation is often applied to the polar FAMEs in order to achieve a better GC separation of the keto- and hydroxyl-FAME [170]. For the analysis of the various groups of thermo-oxidatively altered fatty acids, Márquez-Ruiz et al. [172] utilized HPSEC to separate the nonpolar FAMEs into monomers and dimers; the polar FAMEs were further separated into oxidized fatty acid polymers, oxidized fatty acid dimers, and the oxidized fatty acid monomers.

E. Color Analysis

In actual practice, the color of the oil becomes unacceptable first, well before the flavor and odor of the oil become objectionable [173]. According to Orthoefer [174], the color of the frying oil is one of the major parameters of acceptance to be evaluated on a daily basis. Indeed, regulations in many countries stipulate that color must be one of the criteria for discarding frying oils [153]. For instance, the "Manufacturing Process Inspection" document, published by the U.S. Department of Agriculture, stipulates that the darkening of oil is evidence of unsuitability of a frying oil and requires rejection of the oil [175].

Many products arising from thermo-oxidative alteration of oil components contribute to color change during frying. The color intensity of a frying oil increases as the amount of polymeric materials increases [68,176]. Leaching of pigments from the food into the frying oil, and the presence of Maillard reaction products, formed during frying by the reaction of carbohydrates and some lipid oxidation products with amines, amino acids, and proteins also affects the color development [177–179]. Furthermore, particles from food being fried can become caramelized and release some fat-soluble pigments into the oil [180]. According to Min et al. [181], products with a molecular weight 300–551 Da and containing double bonds, carboxyl, ester, peroxide, or hydroxyl functions contribute to the darkening of oil during frying.

Wesson (AOCS method Cc 13b-45), Lovibond (AOCS method Cc 13e-92), and spectrophotometric (AOCS method Cc-13c-50) procedures are official methods recognized for the measurement of color in frying oils [144]. The Wesson and Lovibond are colorimetric methods that determine the

color of the oil by comparison with colored glasses of known characteristics. In the spectrophotometric method, the absorbance of the oil is measured at 460, 550, 620, and 670 nm, and the photometric color index (PCI) is computed according to the equation: PCI = $1.29(A_{460}) + 69.7(A_{550}) + 41.2(A_{620}) - 56.4(A_{670})$, where A_{460}, A_{550}, A_{620}, and A_{670} are absorbance at 460, 550, 620, and 670 nm, respectively.

In a study on the spectrophotometric method for the assessment of frying oils, Xu et al. [182] reported that the highest correlation was observed between the absorbance measured at 490 nm and the TPC value (r = 0.953). The results of a recent study by Bansal et al. [82], however, showed that any wavelength in the range of 400–500 nm could be utilized to provide a good correlation between TPC and the spectrophotometric absorbance. Irrespective of the methods used, results obtained on color formation during frying must be interpreted with caution as the rate of color development differs from oil to oil and also depends on the initial color of the oil and the type of the food fried in it [142]. Furthermore, oil components, such as tocotrienols and phenolics, cause faster darkening of oil due to chemical changes in the molecules and by oligomerization.

1. Volatile Carbonyls

A number of excellent reviews on the formation of volatile compounds have been published by Schieberle and Grosch [183], Przybylski and Eskin [184], Frankel [10], Kiritsakis [185], and Valet et al. [186]. It is known that variety and different amounts of volatile compounds are generated during thermo-oxidative degradation of oil components and they relate to the nature and stability of the fatty acids involved and to the frying conditions applied [20,184–185,187,188]. Thus, the amount and rate of formation of volatile compounds during frying can be used as an indicator of an oil's performance. Among the volatile compounds formed during thermo-oxidative degradation of oils, the carbonyls are the major component and account for more than 60% of total volatiles depending on the conditions applied [184].

Gas chromatography is the most common method for analyzing volatile compounds formed during thermal alteration of oils. The volatile samples are often introduced into the gas chromatograph by static headspace (SHS) analysis, dynamic headspace (DHS) analysis, or direct injection. These methods are thoroughly reviewed by Przybylski and Eskin [184] and Frankel [10]. In the direct injection method, the oil is injected directly into a GC injector special glass liner. The injector is maintained at a high temperature (>200°C) and a carrier gas purges the generated volatile compounds to the column. SHS analysis consists of analyzing an aliquot of the vapors in equilibrium with the sample heated in a hermetically sealed vial. Although this method is relatively simple, the sensitivity is rather poor [189]. However, Snyder and Mounts [190] reported improved sensitivity and reproducibility by using a multiple headspace extraction (MHE) technique. The headspace over oil heated at 90°C was sampled three times, and each sample was injected consecutively onto the gas chromatograph. Furthermore, solid-phase microextraction (SPME) also presents a more sensitive and convenient alternative to traditional SHS analysis [191]. However, the SPME method has limitations, including difficulties in interfiber comparisons and diagnosis of fiber performance [192]. DHS analysis has been the most used concentration technique for GC analysis and consists of sweeping the volatile compounds from the headspace sample with an inert gas; the volatile compounds are then trapped on a solid absorbent such as activated charcoal, tenax, poparak, chromosorb, or amberlite [189,192].

The combination of GC and mass spectrometry (GC-MS) is also a versatile technique for analyzing volatile degradation products formed during frying. Snyder et al. [193] evaluated the oxidative and thermal stability of soybean oil by measuring the amounts of volatile compounds with static headspace–gas chromatography–mass spectrometry (SHS-GC-MS). Carbonyl compounds generated during thermal treatment of canola and olive oils at 180°C and 240°C for up to 15 h were adsorbed unto tenax and analyzed by GC-MS after thermal desorption [187]. In a similar study, Katragadda et al. [188] evaluated the variety and the amounts of volatile compounds formed during heating of coconut, extra-virgin olive, safflower, and canola oils at 180°C, 210°C, 240°C, and 270°C

for 6 h by DHS-GC-MS. In a more recent study, Uriarte and Gullén [194] evaluated the formation of volatile alkylbenzenes in extra-virgin olive, sunflower, and virgin linseed oils heated at 190°C for 8 h by SPME-GC-MS. Jeleń et al. [195] evaluated the efficiency of different types of fibers for their capacity to absorb the headspace volatile compounds generated during accelerated storage of canola oil at 60°C for 10 days. Divinylbenzene/carboxene/poly(dimethylsiloxane) (DVB/CAR/PDMS) offered the best performance compared to other tested fibers, namely, polyacrylate (PA), poly(dimethylsiloxane) (PDMS), and carbowax/divinylbenzene (CW/DVB).

As previously mentioned, carbonyls constitute the major group of volatile compounds formed during frying and are the most important, qualitatively [184]. For instance, while the reported odor threshold value of pentane in oil was 340 μg/g, the threshold value for pentanal and the corresponding aldehyde was 0.07 μg/g (Table 9.1). For the analysis of volatile carbonyl compounds, reversed-phase HPLC is a viable alternative to GC analysis. The major advantage of HPLC compared to GC is that it operates at ambient temperatures, thus limiting the risk of artefact formation [196]. Typically, the volatile carbonyl compounds are trapped on a silica cartridge impregnated with 2,4-dinitrophenylhydrazine, after which the 2,4-dinitrophenylhydrazones of carbonyls are eluted and quantified by HPLC-UV at 360 nm [197,198]. In a recent study, Bastos and Pereira [199] utilized HPLC-MS for the quantification of the 2,4-dinitrophenylhydrazones obtained after derivatization of the volatile aldehydes generated during thermal treatment of canola oil at 180°C for 8 h.

2. Changes in Antioxidants

Under thermo-oxidative conditions, endogenous or applied antioxidants are lost, either through direct antioxidant activity, evaporation or by thermal oxidation and polymerization of the antioxidants. It is well known that the observed decrease in antioxidant concentration increases with temperature and time [95,200,201]. Accordingly, monitoring the level of antioxidants during frying can be used as an indicator of frying performance of oils. Analytical methods for assessing major lipid antioxidants have been reviewed by Cert et al. [189], Abidi [202], Ruperez et al. [203], Carrasco-Pancorbo et al. [204], Ladislav et al. [205], Bendini et al. [206], Jensen and Laurisen [207], Liu et al. [208], and Tarascou et al. [209].

Tocochromanols are the most important natural antioxidants in vegetable oils, and the most widely used method for their quantification is the direct normal-phase HPLC analysis of the oil. A fluorescence detection, with an excitation wavelength set at 290 nm and emission wavelength at 330 nm, is the most common approach, being the most sensitive for these compounds [189,210]. Indeed, the AOCS official method requires that a UV detector be used only in the absence of fluorescence detector, and the UV detector should be set at 292 nm [144]. Although the analysis of tocochromanols by reversed-phase HPLC presents the advantage of shorter equilibrium and analysis time, and higher reproducibility than the normal-phase HPLC method, this method is limited by its inability to resolve β- and γ-isomers of both tocopherols and tocotrienols and plastochromanol-8 [189,203]. In addition, because normal-phase HPLC operates with organic solvents in which frying oils are easily soluble, higher loads of samples can be tolerated as they are easy to wash off the column by the nonpolar solvents [203].

There are discrepancies regarding the best column for the normal-phase HPLC analysis of tocochromanols. Kamal-Eldin et al. [211] evaluated six silica, three amino, and one diol columns for the separation of tocochromanols in oat extracts and palm oil. Although the tested columns offered equally good separation depending on the mobile phase used, silica columns were reportedly more stable than diol column. Diol column, on the other hand, has been reported to offer more reproducible and consistent results than silica columns [212–215]. It was also mentioned by Abidi and Mounts [216] that amino columns offer better selectivity than diol-bonded columns. Unlike the diol columns that have no ionizable groups, however, the amino groups of amino-bonded columns ionize with the use of polar organic solvents, resulting in increased retention times and peak broadening [215].

Other natural and synthetic polar phenolic antioxidants are usually analyzed by a reversed-phase HPLC, utilizing either isocratic or gradient elution methods with a UV–vis detector operated at 225, 240, or 280 nm [189,204,206,217,218]. However, some phenolic compounds show several absorption maxima and the use of simultaneous multiple UV detection is recommended for quantification [219–221]. Furthermore, the use of a photodiode array detector allows the spectrum to be obtained at different wavelengths and enables a peak identification and purity test [203]. Due to its versatility, HPLC has been and will remain a vital analytical technique for quantifying lipid antioxidants.

3. Formation of Toxic Components

A number of toxic compounds are produced under the conditions employed during deep frying. If the frying operation is not well controlled and/or the frying oil efficiently stabilized, the toxic compounds can be produced at a concentration that warrants health and safety concerns. Oxygenated α,β-unsaturated aldehydes are the most prominent toxic degradation products formed during frying and include 4-hydroxyhexenal, 4-hydroxyoctenal, 4-hydroxynonenal (HNE), 4-hydroperoxynonenal, 4-oxononenal, and 4,5-epoxynonenal, among others [222]. While formation of other degradation products, such as acrylamide, may depend on the type of food being fried, the formation of oxygenated α,β-unsaturated aldehydes is essentially oil dependent and may be used to assess the frying performance of an oil. With regard to the amounts and the degree of toxicity, HNE is the most important and remains the most studied of the oxygenated α,β-unsaturated aldehydes formed during frying [22]. Analytical methods based on solvent extraction, solid-phase extraction, and chemical derivatization, followed by HPLC or GC, have been developed for the quantification of HNE in frying oil [223–228]. The poor solubility of HNE in water and its loss arising from the elaborate cleanup steps (derivatization, multiple extractions, and purifications) involved in many of the methods described for HNE usually result in inefficient recovery of HNE [228].

F. Probable Carcinogens in Fried Foods

Even though they occur at levels that pose no significant threat to health and safety under proper frying management, a number of chemical compounds found in deep-fried foods have acquired the Group 2A carcinogens status ("probably carcinogenic to human") according to the IARC. These compounds can be broadly categorized into three groups based on their sources, namely, (1) those that are formed by thermo-oxidative degradation of the oil during frying and are subsequently absorbed/adsorbed by the food (e.g., hydroxynonenal and related secondary lipid degradation products; (2) those that are formed predominantly by heat-induced reactions among the chemical components within the food that is being fried, subsequently leaching into the frying oil (e.g., acrylamide and other Maillard reaction products); and (3) those that are predominantly preformed in the raw materials, principally the frying oils, and are then transferred to the fried foods by mass exchange during the frying process. The latter group is aptly represented by 3-monochloropropane-1,2-diol (3-MCPD) and related compounds.

3-Monochloropropane-1,2-diol (3-MCPD) and a number of other chloropropanols (Figure 9.8) are process contaminants formed by the reaction of acylglycerols and a chlorine source (e.g., salt, hydrogen chloride, chlorinated water) at high temperature, typically above 220°C. They were originally discovered in acid-hydrolyzed vegetable protein (acid-HVP) in the late 1970s and subsequently in soy sauces prepared with acid-HVP as an ingredient [229–231]. Recent discovery of 3-MCPD esters, acclaimed as the major precursors of free MCPD in food, in the high μg/g or even low mg/kg range in refined oils/fats and lipid-containing food, has generated serious interest in the subject [232,233]. According to Weisshaar, the values of 3-MCPD esters in refined oils ranged from 0.2 to 20 mg/kg [234].

Unlike other fried food toxic contaminants, such as oxygenated carbonyl compounds and acrylamide that are formed during the frying process, it has been reasonably established that 3-MCPD esters are almost exclusively formed during the deodorization step in the oil refining

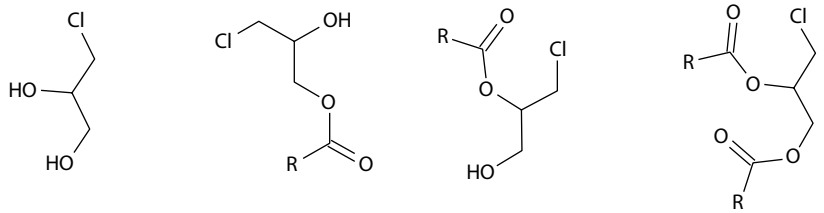

FIGURE 9.8 Representative chloropropanols food contaminants.

process [234,235]. In fact, the level of 3-MCPD in the frying oil decreased during the course of frying, and there is no evidence that significant amounts of 3-MCPD ester are formed under normal frying conditions [236]. The primary factors responsible for the formation of 3-MCPD esters are (1) level of DAGs and MAGs in the oil, with the DAG exerting a stronger influence, (2) the presence of chloride ions, (3) temperature and time and (4) the pH [235]. The presence of sodium hydrogen carbonate and sodium carbonate were found to reduce the amounts of 3-MCPD esters, with sodium hydrogen carbonate being more effective [235,237].

Because the precursors and conditions that favor MCPD esters, formation (partial glycerides, FFAs, chloride ions) are more prevalent in palm oil, the highest concentrations of 3-MCPD esters have been reported in palm oil and palm oil products [234,235]. According to Weisshaar [234], refined vegetable oils and fats can be categorized into three groups according to the level of 3-MCPD ester contents, namely, (1) low level (0.5–1.5 mg/kg), for example, rapeseed, soybean, coconut, and sunflower oil; (2) medium level (1.5–4 mg/kg), for example, safflower, groundnut, corn, olive, cottonseed, and rice bran oil; and (3) high level (>4 mg/kg), for example, hydrogenated fats, palm oil, and palm oil fractions. While there are a number of toxicological animal studies on free 3-MCPD, relatively little is known about the toxicity of 3-MCPD esters; however, due to their structural similarity to DAG and MAG, they are potential substrates for lipases and are easily converted to free 3-MCPD in the gastrointestinal tract [238].

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Section II

Processing and Food Applications

10 Processing and Modification Technologies for Edible Oils and Fats

Selma Türkay and Neşe Şahin-Yeşilçubuk

CONTENTS

I. INTRODUCTION

Edible fats and oils have numerous functions; they add flavor, lubricity, and texture to foods and contribute to the feeling of satiety upon consumption. After extraction and refining, they can be processed into products such as margarine, shortening, salad, and frying oils. Those processed fats and oils are important functional ingredients in food. Supply and consumption of oils and fats are generally described in 17 commodity oils, 4 of which belong to animal origin and the rest are derived from plants [1]. Most vegetable oils are obtained from seeds or beans. Seed extraction is achieved by pressing and/or by extraction with hexane. Palm and olive oils are obtained by pressing out of the soft fruit. Animal tissues are rendered to obtain animal fats.

Most of the vegetable oils, exception being virgin olive oil, require refining in order to remove undesirable materials such as phospholipids, monoacylglycerols (MAGs), diacylglycerols (DAGs), free acids, color and pigments, oxidized materials, flavor compounds, and pollutants [1]. Refined oils consist primarily of triacylglycerols (TAGs) (>99%). They can be modified by hydrogenation, interesterification, or fractionation techniques. Hydrogenation is used to convert liquid oils into

products having different consistencies, melting points, and textures. On the other hand, interesterification produces changes in physical properties by rearrangement or redistribution of fatty acids within and among the TAGs of oils. Fractionation provides a means of producing fats and oils with sharply defined melting characteristics.

II. OIL AND FAT PRODUCTION

Oils and fats can be categorized as vegetable or animal oils and fats according to their sources. Since the production methods depend primarily on the sources, it is necessary to investigate the production processes under two different titles: vegetable and animal oils and fats.

A. Vegetable Oils and Fats Production

Almost all plants contain fats and oils, mainly in their seeds. For a plant to be suitable for oil production on the industrial scale, it must meet the following two criteria [2]:

1. The oil or fat content must reach the minimum for commercially viable exploitation.
2. The plant must be suitable for high acreage cultivation.

The exceptions are the plants that contain oils and fats unique in their composition or with properties that cannot be found elsewhere.

There are two groups of vegetable oils and fats, denominated after their source, namely, pulp or fruit oils and seed oils. Pulp oils, such as palm oil, olive oil, and avocado oil, occur finely dispersed in the fruit's endosperm and have very high water content. They require special treatment, preferably immediately after harvesting. Any mechanical stress that leads to damage of the cell, but also aging, initiates enzymatic reactions that lead to fat splitting or spoilage. For this reason, pulp oils are produced close to the plantation location of the oil fruit. Oilseeds are more suitable for transport. In fact, they are better transported than extracted oil, because the oil is protected in the seed. Seed oils are therefore extracted close to consumer. The main difference between their production steps are the extraction methods of the oils from the oily materials. The pulp oils are extracted by mechanical extraction, while the seed oils can be extracted by both mechanical and solvent extractions, depending on the kind of the seeds and the purposes of usage of the oil produced.

1. Pulp Oil Production

Palm oil and olive oil are the two commercially important edible pulp oils. Palm oil is the major vegetable oil produced in the world, and it is the most consumed edible oil. Olive oil is the most popular gourmet and health-promoting specialty oil. Because of the quick spoilage of the oil and completely different and difficult matrix compared with seed oil, pulp oil production has developed as an independent technology.

a. Palm Oil Production

Palm oil is obtained from the *Elaeis guineensis*, a tree native to Guinea, West Africa. Today palm tree is grown on plantations of the equatorial tropics in Southeast Asia, Africa, and Central and South America [3]. Asian palm fruit contains up to 60% pulp (mesocarp), 10% nuts, and 30% skin and stems. The oil is contained both in the mesocarp (palm oil—rich in palmitic acid and oleic acid) and in the kernel (palm kernel oil—rich in lauric acid), and since their fatty acid compositions are different, extraction procedure must be carried out with care, so as to preserve the majority of the pulp with a minimum breakage of nuts and kernels. The pulp itself contains approximately 40% oil, 40% water, and 20% fiber. The process flow diagram for palm oil is given in Figure 10.1 [4,5].

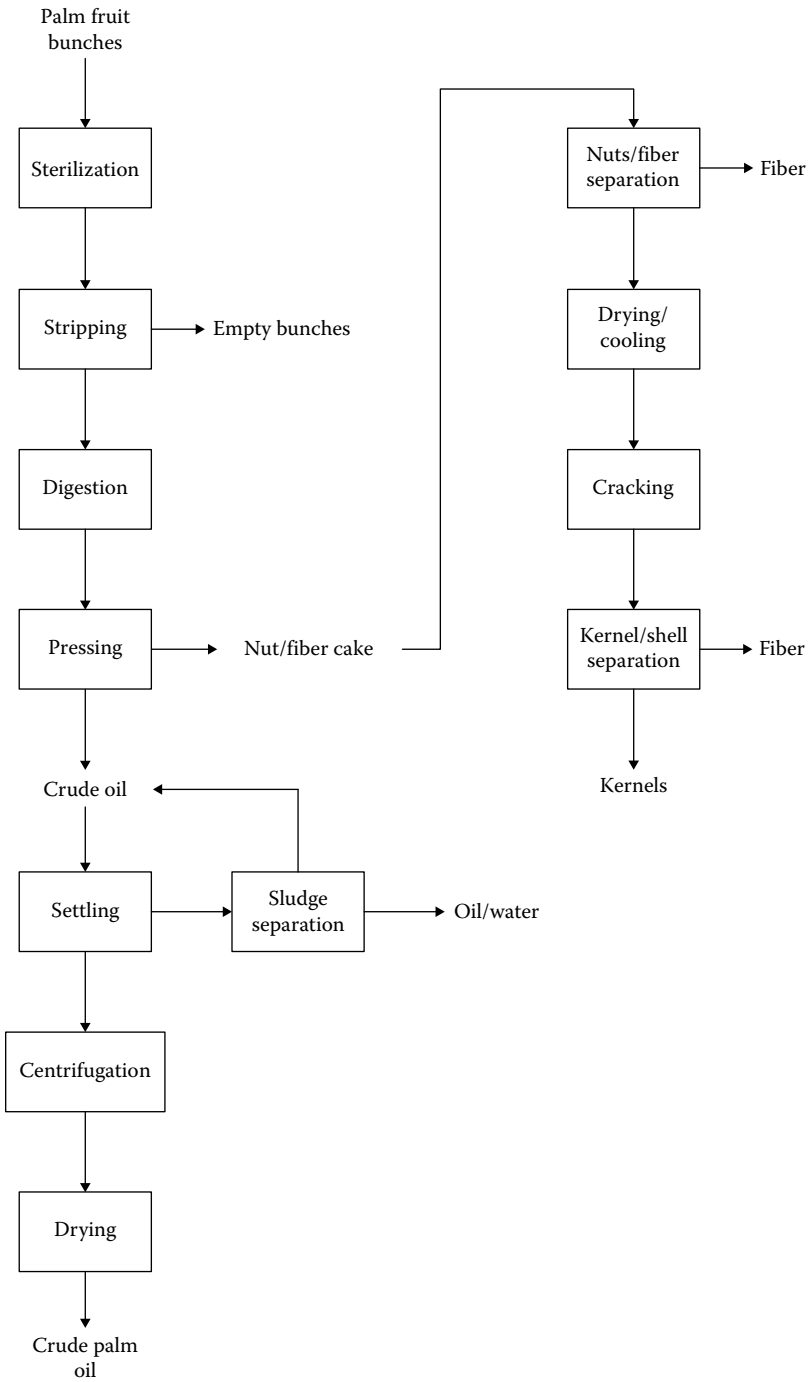

FIGURE 10.1 Palm oil processing steps.

The palm fruit offers a range of products at the various processing steps:

1. *Fruit reception*: The actual processing of palm oil does not start at the mill, but in the plantation itself. In order to obtain good quality oil, it is essential to handle the brunches with great care, preventing the damage of the fruit. At the time of cutting, the level of free fatty acid (FFA) in ripe and unbruised fruit is around 0.3%–0.8%. Improper handling between the tree and the mill causes the acidity to rise rapidly, because in the injured outer part of the fruit, enzymatic hydrolysis reaction commences immediately by contacting very active native lipase of the fruit and the oil.
2. *Sterilization*: This step is a heat treatment process that deactivates lipase enzymes in the fruit in order to prevent further increases in FFA. Sterilization also serves other purposes, such as facilitation of mechanical stripping and preconditioning of the nuts to minimize kernel breakage. Sterilization is carried out in large steam autoclaves. Immediately after arrival at the oil mill, the bunches are processed in the sterilizer at a steam pressure of 2.5–3.0 kg/cm^2 (130°C–135°C) for 45–60 min depending on their size and ripeness.
3. *Stripping* (*threshing*): The objective of stripping is the separation of the fruit from the bunch stalks. The sterilized bunches are fed continuously into a rotary drum thresher or beater arm thresher, which strips and separates the fruit from the brunch stalks. From the thresher, the fruits are conveyed into a digester.
4. *Digestion*: The purpose of digestion is to loosen the pulp from the nuts and to break the oil cell walls by heating and mixing the fruits. This operation is of the utmost importance as it affects the oil yield by pressing. The best digestion conditions are obtained by mixing the fruits at a temperature between 90°C and 100°C for about 20 min. The digester is generally a vertical cylindrical vessel provided with a double steam jacket and a central paddle stirrer.
5. *Pressing*: The mash, consisting of digested pulp and palm kernel nuts, is transferred to the continuous screw presses comprising a perforated horizontal cage in which two screws run. A cone at the discharge end of the cage controls the pressure to ensure a minimum residue oil in the press cake with an acceptable amount of broken nuts. Two products are obtained at the outlet of the press:
   a. A mixture of water, oil, and solid impurities (sand and vegetable residue)
   b. A press cake containing fibers and nuts
6. *Crude oil clarification*: The crude oil from the press has an average composition of 66% oil, 24% water, and 10% solid impurities. Because of the high content of solids, it has to be diluted with water to obtain satisfactory settling. After dilution, crude oil is screened to remove fibrous materials and then pumped to a continuous decantation or settling tank where it separates into oil and sludge parts at high temperature (85°C–90°C). The oil is skimmed off and passed to a centrifugal purifier followed by a vacuum dryer. The lower sludge fraction, which contains some entrapped oil (10%), is centrifuged to recover additional oil, and the oil-rich mixture separated is returned to decantation tank for further separation. The remaining post-centrifuged sludge is a waste stream. Alternatively, three-phase decanters can be used on crude oil or sludge, reducing liquid effluent quantities and maintenance costs.

   In order to increase oil yield in the milling of palm oil, the application of high-frequency ultrasound to the ex-screw press was investigated, and it was demonstrated that this intervention speeds up the gravity separation of the oil, reduces the amount of the oil in the sludge underflow from the clarification process, and improves oil overall recovery [6].
7. *Oil drying and storage*: The oil is dried to below 0.1% moisture. Vacuum dryers are generally used, as the oil is maintained at a low temperature to avoid oxidation.
8. *Nut and fiber separation*: The press cake containing fibers and nuts is fed via a breaking conveyor to a vertical column through which a hot air current is circulated. The fibrous material is sucked into a duct and then separated from the air in a cyclone for use as a boiler fuel. The nuts drop to the bottom of the column.

9. *Nut conditioning*: After separation from the fiber, the fresh nuts are dried to detach the kernels from the shells. The moisture content of the nuts is reduced from 16% to 10%–12%. For ideal nut cracking, it is necessary to dry the nuts sufficiently to loosen the kernel and then cool the nuts to harden the shell before cracking.
10. *Nut cracking and kernel separation*: The properly conditioned nut is cracked in two or more pieces, and the kernel is released. The cracked mixture consists of free kernels, shells, unbroken nuts, partly cracked nuts, and dust. Kernel separation is usually achieved by using a dry separation system (winnowing system), which is used to remove fiber, small pieces of shells, and dust, followed by a clay bath or hydrocyclone. The clay bath separation system is based on the difference in specific gravity between the kernel and the shells. The specific gravity of undried kernel is about 1.06–1.16 and that of shell is about 1.30–1.35. Therefore in a clay water mixture of specific gravity 1.12 (about 24 Twaddell), the kernels will float and the shells will sink. In the hydrocyclones, separation is carried out in a water stream, rotating at high speed inside a cyclone.
11. *Kernel drying*: After separation, the kernels contain about 20% of moisture. If stored in this condition, fresh kernels would soon become moldy. In addition, there would be a rapid increase in the FFA of palm kernel oil. If the moisture content is reduced to about 7%, kernels can be safely stored and transported. Dried palm kernels contain approximately 50% oil. Unlike palm oil, palm kernels are not processed close to the plantation. They are extremely hard and can be transported and stored without risk.

b. Olive Oil Production

The olive, the fruit of the olive tree, has been used for centuries for the production of olive oil. Over 90% of the World olive oil is produced in the Mediterranean region [3]. Being one of the main ingredients of the Mediterranean diet, olive oil was always pointed out as mainly responsible for the health benefits of the nutritional pattern of Mediterranean countries in numerous studies [7]. This is probably the reason for the increased consumption of the olive oil, especially in Northern Europe and the United States, over the last 20 years. The olive tree is an evergreen and one of the oldest known cultivated tree. It has more than 2000 cultivars, which are spread throughout the olive oil producing region. However, a few cultivars are being cultivated for commercial production, due to their higher adaptability and productivity [8].

The whole olive fruit contains pulp in the amount of 65%–80% of the total weight, 15%–30% pit (stone), and 2% skin. The pulp contains 20%–25% oil (40%–60% on dry basis) and 50%–60% water. The pit contains about 80% woody shell and 20% kernel or seed containing 8%–10% oil (12%–17% on dry basis) with a composition that is similar to that of the pulp oil [3,9]. The quality of the olive oil depends on a number of factors, such as the degree of ripening of the fruit, harvesting, and production methods and conditions. Depending on these factors, olive oils of various categories are produced [10] (Figure 10.2).

The first products of the milling process are the virgin olive oils, the pomace, and the solid residue. Virgin olive oil is defined by international regulations as follows: "oils obtained from the fruit of the olive tree solely by mechanical or other physical means under the conditions that do not lead to alterations in the oil." Three categories of virgin olive oil are further defined, based on chemical and sensory quality standards: "extra-virgin olive oil (FFA % $\leq$ 0.8)," "virgin olive oil (FFA % $\leq$ 2.0)," and "lampante olive oil." Only extra-virgin and virgin olive oils are allowed for consumption without being refined.

There are basically two methods for mechanical extraction of the oil from the fruit: pressing, which is the oldest method, and centrifugation. Depending on the harvesting and the extraction methods chosen, pretreatment steps and conditions can be changed [2,3,7].

1. *Cleaning*: The aims of this operation are to remove leaves, small branches, stones, damaged olives, and other foreign materials that may have been collected accidently with olives and to wash dust and soil from the olives.

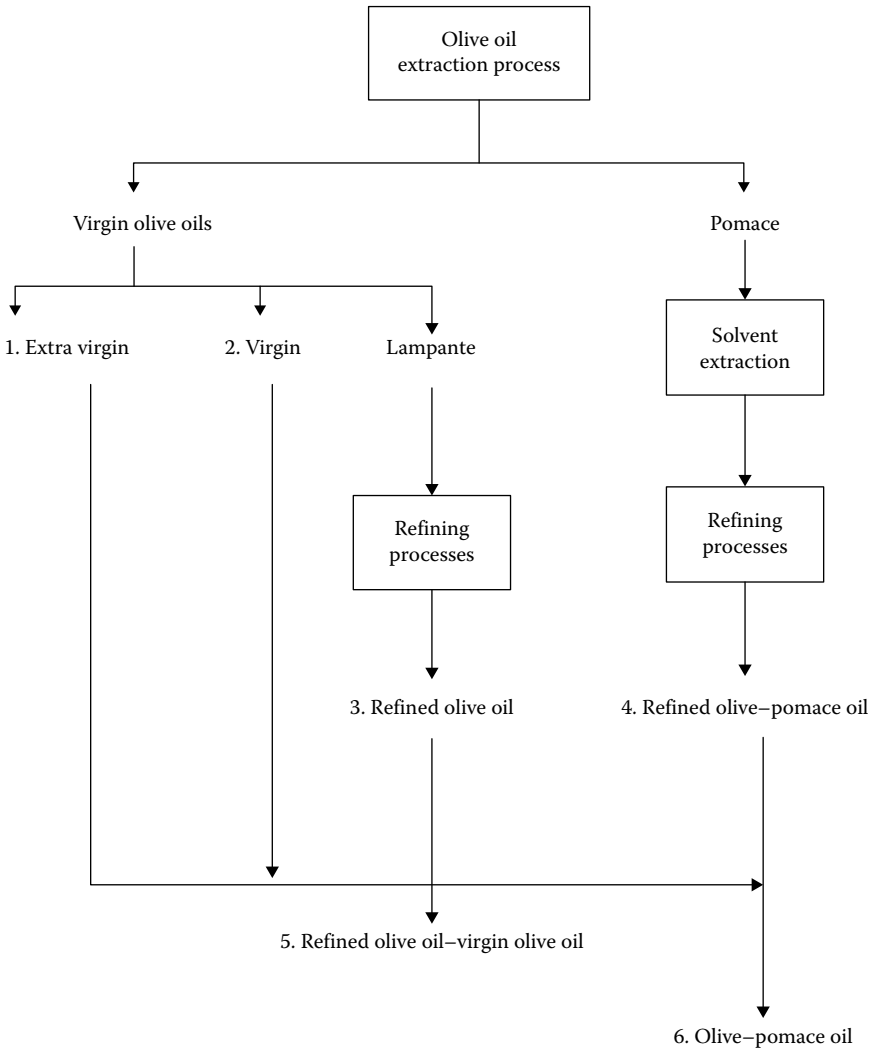

FIGURE 10.2 The classification of olive oils. (From Peri, C., The extra-virgin olive oil chain, in: *The Extra-Virgin Olive Oil Handbook*, Peri, C., Ed., John Wiley & Sons, Inc., New York, 2014.)

2. *Olive crushing*: The aim of crushing is to reduce the olives to a homogeneous paste by breaking pits, skin and pulp cells, and the vacuoles containing tiny droplets of oil [9]. The oil flowing freely from the vacuoles can then be separated from the water and the solid constituents. The olive paste is a semiliquid mixture of two different types of solids (pit fragments and soft pulp and skins) and two types of immiscible liquids (water and oil). The pit fragments make a rigid framework in the olive paste, which facilitates draining and separation of the liquids from solid components. This operation is important for both oil quality and yield. The crushers use stone mills or metal crushers (hammer or disc types) and these contribute to both quality and yield of the oils.
3. *Mixing* (*malaxation*): After crushing, the paste has to be mixed to allow the fine oil droplets to coalesce into large ones. This is done by stirrers that are equipped with blades or that are spiral shaped. The effect of the slow mixing of the olive paste is for not only the coalescence of the oil but also some complex mechanical, physical, and biochemical transformations that affect the extraction yield as well as the nutritional and sensory quality of

the oil [11]. During this operation, the most important parameters that affect the yields and quality of the oil are time (20–60 min) and temperature (25°C–30°C).

4. *Oil extraction with presses*: This traditional process is still used in small plant only. From the mixing step, the paste is spread in layer in a hydraulic press. A single pressing step, lasting 1–1.5 h, is generally applied. The press separates water–oil mixture from the solid residue, (pomace) containing pit fragments and pulp residue. Oil and water are then separated by centrifugation. Pressing is simple and efficient, but it is a labor-intensive, discontinuous process.
5. *Oil extraction with centrifuges*: In the late 1960s, a radical change in the olive oil extraction process was realized with the introduction of horizontal three-phase decanter centrifuge in place of pressing. Centrifugation is a continuous process that separates olive oil from the other liquid and solid materials by Stoke's law. Olive paste, derived from crushing and malaxing operations, is a heterogeneous mixture of three phases. In order of decreasing density, they are [12]:
   a. The insoluble solid phase, consisting of pit fragments and cell wall fragments
   b. The aqueous phase (vegetation water), consisting of water and water soluble components (salts, simple sugars, simple phenolics)
   c. The oil phase

   Separation is carried out inside a decanter, a cylindrical bowl with a similar shaped screw with helical blades that gyrate 3500–3600 rpm. The small difference between the speed at which the blow and the inner screw rotate results in the movements of the solid (olive pomace) to one end of the centrifuge, while the oil and water phases are moved to the other end. For most effective separation, a considerable amount of water is added to the paste, accounting for 60%–100% of the weight of the processed olives. This results in huge quantity of wastewater that has negative environmental effects due to its resistance to biodegradation. This problem has been solved by a new decanter design, the so-called two-phase decanter, which does not need the water addition. The insoluble solids and the aqueous phase are discharged together from the decanter as a semiliquid slurry.

   The oil fractions from both types of decanters undergo a finishing centrifugation step, which is usually carried out in high-speed disc centrifuges to eliminate solid impurities and water droplets. Similarly, the aqueous phase from the three-phase decanter undergoes a finishing centrifugation treatment with separation of the vegetation water as the main product, recovery of a small amount of oil, and discharge of solid impurities.

   The choice of a two- or a three-phase decanter requires a careful evaluation, taking into account various technical, economic, and environmental aspects. Oil yields and quality are similar with the two centrifugation processes. Water content of the pomace from the two-phase decanter is about 60%–65%, compared to 50% from the three-phase decanter and 35% from pressing.
6. *Pomace processing*: The olive pomace contains enough residual oil to make it worth extracting. In order to prevent enzymatic hydrolysis and oxidation reactions, the pomace should be dried as quickly as possible. After drying in horizontal rotary dryers, the pomace contains 6%–8% water, 5%–9% oil, 40%–50% kernel, 9%–10% skins, and 20%–30% crude fiber. The dried pomace is solvent extracted to recover the residual oil. The extracted pomace oil is always refined. The extraction meal is used as fuel or as an additive to fodder or to produce fiberboard after separating kernels.

2. Seed Oil Production

The number of oil-bearing seeds is quite high. However, only a limited number is extracted economically for oil production. Major oil seeds that are exploited and traded worldwide are soybean, rape/canola, sunflower, peanut/groundnut, cottonseed, palm kernel, coconut/copra, corn, sesame, and linseed/flaxseed. The oils produced from these seeds are usually called "commodity oils" with annual production of more than 1 million tons. The oils with an annual production of less than 1 million tons can be considered as "minor oils," such as pumpkin seed oil, wheat germ oil,

hempseed oil, borage oil, and grapeseed oil. Oilseeds are also the main source of protein meal for animal production. For example, extraction meal from soybeans with a protein content of 40%–50% is an excellent feedstock for animal feeding. A prerequisite for the production of good quality oils and meals is to process high-quality oilseeds, which are not damaged during harvesting, transportation, storage, and processing. Therefore, the conditions for the handling of the oilseeds before arrival at the oil production plant should be considered.

a. Seed Drying and Storage

Oilseeds are much more stable mechanically and have lower water content than the oil fruit. It is therefore much better to store the seeds than to store its oils, because the oil is protected in the seed in proper conditions. Safe storage is vital to preserve the quality and the value of the seeds and to maintain the quality. It is very important to lower the water content of the seeds below the critical level to stop all biological and enzymatic activity in the seed. The critical moisture level for safe storage differs from seed to seed. Usually, the higher the oil content, the lower the critical moisture content. Maximum moisture content of soybeans for good storage is around 13%, whereas for sunflower seed and rapeseed, these values are 10.5% and 7.0%, respectively [2]. Storage for extended periods at moisture content exceeding critical moisture level will damage oilseeds, reduce oil and protein yields, and diminish the quality of oil by microbiological attack. Biological activity and respiration are particularly active in newly harvested seeds and moisture induces the appearance and growth of molds or fungi as well as biological respiration and germination that cause the temperature to rise through a series of exothermic reactions. It was observed that during the storage of nonventilated soybeans with a moisture content of 13.2% in a container, the temperature begun to rise steadily after six mounts and reached as high as 93°C after 1 year. Under extreme conditions, the seed may become scorched or even catch on fire.

The maximum allowable storage time depends mainly on temperature and moisture content of the seed. Seed containing more than critical moisture content is immediately processed or dried. Drying can be done at ambient air temperature without any equipment or with the aid of dryers. Drying begins with removing outer moisture from all fresh seeds. Then the inner moisture diffuses to the outer parts, where it is also removed. After some time, an equilibrium between the ambient atmosphere and the seed is reached. This equilibrium depends on the temperature and the relative humidity of the surrounding air. In Table 10.1, the equilibrium moisture content of soybeans depending on relative humidity and temperature is given [3].

TABLE 10.1
Equilibrium Moisture Content of Soybean

	Air Temperature (°C)	
Relative Humidity of Air (%)	**20**	**30**
20	5.4	5.0
40	7.1	6.4
50	8.0	7.17
60	9.5	8.86
70	11.6	10.63
80	15.29	14.5
90	20.86	20.15

Source: Van Doosselaere, P., Production of oils, in: *Edible Oil Processing*, 2nd edn., Hamm, W., Hamilton, R.J., and Calliauw, G., Eds., John Wiley & Sons, Inc., New York, pp. 55–96, 2013.

According to the values given in Table 10.1, in order to keep soybeans below the critical moisture content during storage, the relative humidity of the air should not be above 70% at 20°C–30°C.

Drying can be done at ambient temperature without any equipment as well as with the aid of dryers [2]. Quick drying of very wet material can be usually carried out in horizontal rotary dryers. Other dryers, such as vertical seed dryers, can be used for the seeds of less wet seeds. Drying should be carried out to a level of seed moisture that improves storage stability and does not deteriorate the seed's mechanical stability. Overdrying can cause seed fragility, leading to excessive breakage during handling, storing, and processing. Oils from field- and storage-damaged seeds are of poor quality due to activation of catabolic enzymes.

Oilseeds can be stored in metal or concrete vertical silos or in horizontal warehouse-type silos. The seeds should be well ventilated for storing them for a long time. Aeration is used primarily for cooling, not for drying, and must be used carefully, considering equilibrium moisture of the seeds, particularly in area where the climate is hot and humid. This equilibrium seed moisture is re-established during storage, independent of the moisture level that the seeds had been dried before. A temperature range of 4°C–10°C is preferable, because all enzyme activities slow down dramatically as well as living conditions of insects.

Another aspect that should be considered during storage is the mechanical stress that the seed can withstand. The pressure that is imposed on the seeds depends on the height of the silo and the seed bulk density [2]. If pressure is too great, the lower seed layers can become extremely damaged. The oil in seed is protected by natural compartmentalization of oils and enzymes, such as lipase, lipoxygenase, and phospholipase, within the cells of the seeds. In intact seeds, these enzymes are kept away from the oil; however, damage during harvesting, transporting, conveying, or storage bruises the seed, breaking cell walls and membranes, allowing the oil and enzymes to come into contact, and enzymatic hydrolysis and oxidation reactions, whose rates are dependent on temperature, moisture, and extent of damage, proceed and decrease the quality of the oil [4].

b. Seed Pretreatment

The purpose of the pretreatment process is to properly prepare the seeds for extraction of the oil in order to obtain maximum extraction efficiency, either by solvent or by mechanical methods [13]. Seed endosperm is composed of many cells containing oil and storage protein, which supply energy, nitrogen reserves, and any other metabolites to support the growth of the embryo during germination [4]. The oil inside a cell consists of hundreds of very small bodies each clinging to the inside surface of the cell wall and to the outside surface of the protein bodies [3]. The oil cannot be taken out in an efficient way without changing the shape of the seed and affecting its internal structure. The structure of oil cells must be weakened or broken to the point where the oil can flow out, partially during mechanical pressing, and more fully during solvent extraction. In order to increase the extraction rate, it is necessary to shape the seeds in a way that the solvent can reach the oil through a short capillary path so that the distance over which diffusion occurs is as small as possible. Short capillary path is achieved by fine grinding of the feed material. When the extraction is carried out on the percolation principles, it is important to prepare the seeds in such a way that the solvent can percolate freely. For this reason, most oilseeds are rolled into thin flakes in order to produce a feed material with short capillary paths in one direction and good percolation properties. Some feed materials, such as rice bran and fish meal, cannot be formed in resistant flakes. Pelletizing is the method of choice for the preparation of these materials.

The preparation steps required depend on the kind of seed and on the technology chosen [2,13]. The unit operations usually involved are given in Figure 10.3.

1. *Cleaning*: The first step in processing oilseeds usually involves cleaning the incoming seeds, which may have foreign materials introduced during harvesting, transportation, or storage. These may be plant materials, such as leaves, twigs, or metallic materials from harvesting machines or transports, and other impurities, such as earth, sand, and stones.

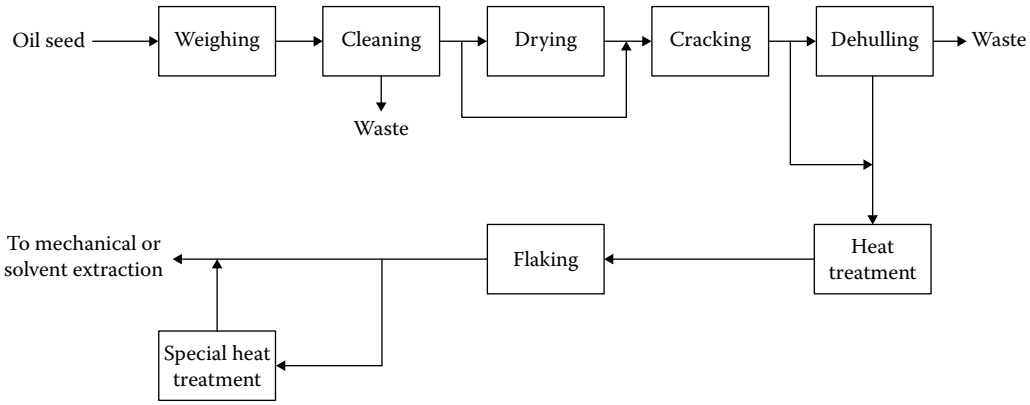

FIGURE 10.3 Pretreatment steps of oilseeds. (From Bockish, M., *Fats and Oils Handbook*, AOCS Press, Champaign, IL, 362pp, 1998; Andersen, D., A primer on oils processing technology, in: *Bailey's Industrial Oil and Fat Products*, Vol. 5, 6th edn., Shahidi, F., Ed., John Wiley & Sons, Inc., New York, 6pp, 2005.)

Cleaning is important to reduce wear and damage to subsequent processing equipments and to improve the quality of the oil and the meal.

When entering the preparation plant, the seeds pass over a magnetic separator, which removes any magnetic metal particles, and then go on to the weighing system to ensure a constant and regular feed to the rest of the plant. From weighing, the seeds are conveyed to the cleaning equipment to separate nonmetallic impurities. There are two ways to separate these kinds of impurities: one is sieving, which works by size difference of the seeds and the impurities, and the other is air separation, which requires the density difference between seeds and impurities. The most used equipment is the multideck screener that is designed to segregate the feed in three fractions by using vibrating screens. These are oversized impurities, the intermediate seed fraction, and the small fraction. The main seed fraction is also subject to aspiration through a current of air to remove light particles.

2. *Dehulling* (*decorticating*): Most of the oilseeds that could be extracted after size reduction are usually processed first to remove the hull or seed coat that surrounds the oilseed. Hulls always contain much less oil and protein than do its kernel or meat. Removing the hull reduces the amount of material that must be handled, extracted, and desolventized, thus increasing downstream capacity. The average hull contents of some oilseeds are given in Table 10.2 [2].

TABLE 10.2
Average Hull Contents of Oilseeds

Oilseed	Percentage
Soybean	7
Cottonseed	31
Sunflower seed	30
Peanuts	47
Rapeseed	15
Safflower seed	48
Palm nuts	55

Source: Bockish, M., *Fats and Oils Handbook*, AOCS Press, Champaign, IL, 1998.

Furthermore, hulls may contain components that would have to be removed from the oil during refining processes. For example, sunflower seed hulls contain waxes that would be transferred to the oil during extraction and become insoluble at cold temperature and make the oil cloudy. Another advantage of dehulling process is to increase the protein content of the meal that can be sold as high protein meal. On the other hand, hulls can be helpful, providing fiber to allow easier pressing or to enhance solvent drainage during extraction. For this reason, a small portion of hulls may leave with the seeds.

Separation of the hulls from the kernel or meat is done after seed or nut cracking (decortication). For this purpose, hammer mills (for nuts), rollers, or disk attrition mills can be used. During cracking, the important point is to ensure that the kernel is not broken into too small particles, which would be difficult to separate from the hull, and thus to minimize oil loss with the hulls. Another method is cracking by pneumatic impact, where the seeds are blown against a wall, and crack. Hulls can be separated from the cracked seeds by screening, air separation, and electroseparation [2].

The steps of the dehulling and the equipments used are chosen depending on the kind of the seeds and on the use of the meal. For example, soybeans are dehulled if high protein, low fiber meal is required. Since the hull sticks to the kernel, soybeans are first dried and then stored for some time. This storage time is called tempering, and during this time, the dry hull loosens itself from the kernel, making the later separation of hulls from kernel easy. Sunflower seed is dehulled because of the high percentage of the hull content and of the wax problem. Rapeseed is rarely dehulled. To extract the oil of nuts, such as palm nuts and coconuts, the nuts have to be dehulled.

3. *Heat treatment*: After dehulling, the seeds are delivered to the conditioner (or cooker) where heat is applied to make the cracked seeds soft and pliable for subsequent flaking operation. Actually, heat treatment of the seeds is important to achieve high oil yields. Cooking (75°C–100°C), roasting (160°C–200°C), and conditioning with moisture (60°C–110°C) are all heat treatment processes [14]. This leads to increases in oil yield by cracking of cell membranes that increases oil extractability, protein coagulation that breaks the intercellular emulsion, and viscosity decrease that makes the oil more fluid.

   Heat treatment also increases the oil quality by enzyme inactivation that results in low FFA content of the oil, sterilization of seeds that leads to a decrease in microorganisms population, and formation of new compounds that may affect oxidation stability, aroma, and taste of the oil in a positive manner. Another advantage of the heat treatment is to increase the digestibility of the meal by decomposition of trace compounds, which negatively influences digestion, thus interfering with the use of the meal as fodder [2].

   Conditioning is always carried out with the adjustment to a certain water content in order to give the seed the right elasticity and to avoid the dry flakes to crumble finely. For direct expelling, no conditioning is necessary. Two types of cooker conditioner are used by the industry, namely, stack cookers (vertical) and drum conditioners (horizontal). Stack cookers are usually coupled with screw press and consist of horizontal steam-heated cooking trays stacked in a vertical vessel. Each tray is equipped with a paddle stirrer, connected to a central rotating shaft. The seeds enter at the top and are swept by stirrers successively over each cooking tray, from top to the bottom. The required residence time of cooking is adjusted by the openings of the trays, which allow the seeds to flow from one stage to the next. Large capacity plants tend to use horizontal rotary drum cookers–conditioners instead of a vertical stack [3]. They consist of a sequence of horizontal drums or tubes heated with steam. The number of drums required depends on the kind of seed and on the capacity. At least two drums are equipped with direct steam injection. The seed is continuously fed to the top drum and gradually progress to the discharge at the opposite end by the action of the rotating drum. The horizontal cookers provide a more constant quality, because more uniform heating and residence time can be obtained compared with the stack cookers.

4. *Flaking*: The seeds leaving the conditioner are finally sent to flaking. The flaking mills are equipped with a pair of smooth rolls that rotate at identical speed. Oil cells are weakened by flaking, and in addition, the shapes of the flakes, with their large surface-to-volume ratio, plus the short distance from the oil cells to the flake surface, facilitate liquid–solid contact and the migration of the oil to the liquid phase. The seed particles are usually flaked to a thickness of 0.20–0.35 mm, depending on the kind of the seeds and on the extraction methods. The flakes are conveyed to the extraction plant.

   In order to increase the percolation performance during the solvent extraction, optional heat treatment processes can be applied, especially for soybean and cottonseeds after flaking: extrusion and the ALCON process [2,3].
5. *Extrusion*: This is a cooking technique that consists of heating the flakes for a few seconds by mixing with live steam and then pushing them through a restriction for structuring the material like sponge. The extruder (expander) consists of a horizontal cylinder through which the material is pushed forward by means of rotating worm assembly. Live steam is introduced inside the extruder and mixed with the material, raising its temperature and moisture, as well as the pressure inside the extruder. Then the material is pushed through the outlet opening, and due to the high pressure reached inside the extruder, an expansion phenomenon of the product and a flash evaporation of the water in the product take place at the outlet, and the product has a sponge-like texture.

   The main advantage of the extrusion is to increase the production capacity of the extraction plant, because it increases the bulk density of the material and percolation rate of the solvent. It also reduces solvent retention of the meal entering the desolventizer.
6. *The ALCON process*: This is also a special heat treatment process of soybean flakes to increase the solvent extraction performance. In an apparatus like stack cooker, the flakes are heated to 95°C–110°C, while the water content increases to 15%–20% by direct injection of steam, and the wetted flakes agglomerate under stirring. The agglomerates are then dried and cooled to the extraction temperature of 60°C.

c. Mechanical Extraction

Even though production of virgin edible oils by pressing goes back more than 8000 years in the history of mankind [15], the first continuous screw press was produced by V.D. Anderson in 1902, and it was called "expeller" [3]. The mechanical extraction using screw press became the commonly used method for oil extraction for various feedstocks until the end of World War II. Then the development of solvent extraction and that of subsequent oil purification by refining allowed supplying enough oil over increasing demand for human nutrition and technical applications [15]. As a result, press extraction was applied only as a prepressing step before solvent extraction, and virgin oils disappeared from the market except virgin olive oil. In the last 20 years, however, gourmet and health-promoting specialty oil market has been largely expanded, and a number of virgin vegetable oils are preferred by consumers due to the beneficial health effects of these oils that are mainly contributed by their fatty acid composition and their bioactive components, each with its specific taste and aroma.

Screw press can be operated continuously, and it is basically a continuous screw auger designed to accept feed material and subject it increasing pressure as it is conveyed through the barrel cage [4]. The barrel is composed of bars surrounding the screw and oriented parallel to the screw axis. The bars are separated by spacers decreasing in size toward the solid discharge end, which allow the oil to drain. Increasing pressure down the length of the barrel is achieved by increasing the root diameter of the screw, decreasing the pitch of the screw flights, and controlling the opening for the discharging cake by means of a choke.

A typical pressing operation involves cooking/conditioning, pressing, cake cooling and finishing, and oil filtration. The operation conditions of these steps usually depend on the purpose of the

production. The pressing extraction can be applied for a full pressing (hard pressing) operation, which means nearly 90% of the oil present in the material is extracted, and during pressing, pressures of up to 400 bar can be reached, causing temperatures up to 115°C–125°C [2]. A residual oil content of the meal as low as 5%–10% can be achieved. Full pressing can be operated as single step or two steps. When the pressing is performed for a prepressing step before solvent extraction, a residual oil content of 15%–20% is targeted, and during pressing, presses reach for 30–40 bar with a temperature of 95°C. These oils can therefore not be termed as "cold-pressed oil," since the Codex Alimentarius does not allow applying heat in the course of any production step of "cold-pressed oils." Thus, cold-pressed oil is also virgin oil, but virgin oil is not necessarily cold-pressed oil. The Codex defines "virgin oils" as "obtained without altering the nature of the oil, by mechanical procedures, for example, expelling or pressing and the application of heat only." Subsequent purification of the oil by washing with water, settling, filtering, and centrifugation is possible. Thus, application of heat in the course of the whole process is allowed. But the limitation is that the nature of the oil must not be changed [16].

The oil from a mechanical pressing operation usually contains fine solids or "foots," which must be removed from the oil. Most of the solids can be simply removed by screening the oil over either a static screen or a vibratory screen. The screened oil is then clarified using a pressure leaf or plate and frame filter. On large prepress plants, centrifugal separators are used. The clarified oil is dried in a vacuum drier and then stored [3].

After the pressing operation, the cake is normally broken and cooled for further utilization of the meal either for solvent extraction or to process the meal as animal feed. The main hazard with full-press cake is spontaneous combustion during storage. In order to reduce this danger, the cake, which may exit a press over 125°C, must be cooled before storage.

d. Solvent Extraction

Solvent extraction is a mass transfer process in which materials are transported from one phase to another for the purpose of separating one or more compounds from mixtures [17]. In the case of oilseed extraction, crude vegetable oil is dissolved in a solvent to separate it from the insoluble meal that is primarily composed of protein, carbohydrates, and fiber.

Solvent extraction can be employed for a variety of oleaginous materials. The residual oil content can be reduced to approximately 1% by weight, making maximization of oil yield which is the economic driver for solvent extraction process [18]. For oilseeds with less than 30% oil by weight, such as soybeans and cottonseed, the seeds are mechanically and thermally prepared and then sent to the solvent extraction process for oil separation (direct solvent extraction). For oilseeds with more than 30% oil by weight, such as rapeseed and sunflower, solvent extraction is combined with a prepressing step, in which the oil content is reduced to 15%–20% by pressing and then 1% by solvent extraction (prepress solvent extraction). Thus, the advantages of both processes, the lower cost for pressing and the good yield of the solvent extraction, are combined [2].

Various solvents have been used in the oilseed solvent extraction process over the years, and today the solvent used in the majority of the plants around the world is commercial hexane, a mixture of hydrocarbons generally boiling at 65°C–69°C. Most commercial hexane contains approximately 65% normal hexane, with the remaining being cyclopentane and hexane isomers. Commercial hexane is preferred due to its wide availability, relatively low cost, excellent diffusivity through oilseed cell walls, high solubility with oils, low solubility with water, low latent heat of vaporization, low specific heat, and moderate boiling range [18]. Hexane vapor is three times heavier than air, and hexane/air mixtures are explosive within the range of 1.2%–7.4% (v/v) hexane. Special care must be taken in constructing and operating hexane extraction processes.

Solvent extraction is an integrated process involving five key unit processes: the solvent extractor, miscella distillation, meal desolventizer, meal dryer/cooler, and solvent recovery. Figure 10.4 illustrates the typical unit operations associated with solvent extraction.

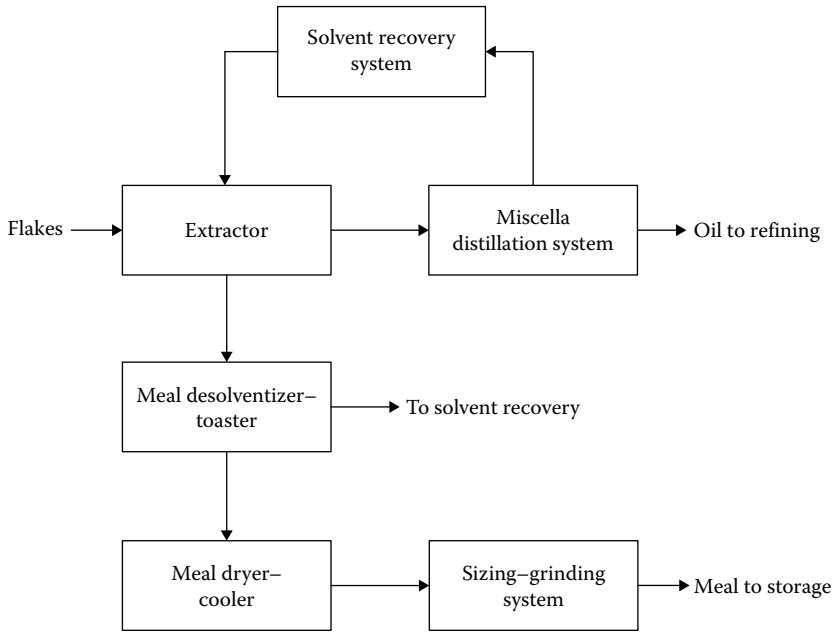

FIGURE 10.4 Oilseed solvent extraction processes.

i. Solvent Extractor The prepared oilseed flakes are conveyed from the preparation process to the solvent extraction process and enter the extractor. In order to understand the extraction process and to determine the parameters affecting the process, it is helpful to investigate it on a microscale using the soybean example. A typical soybean flake has a thickness of 0.25–0.35 mm, and this is approximately 13–18 cells thick. The oil within the cell exits as thousands of spherical oil bodies clinging to the inside surface of the cell walls and to the exterior surface of the protein storage vacuoles [18]. The extraction process starts when the surface of the flake is surrounded by the solvent [19]. After the solvent diffuses inward through the cell wall, it rapidly goes into solution with the oil bodies inside the cell to form miscella within the cell. As miscella continues to enter and go into solution, internal pressure builds within the cell and concentrated miscella diffuses back out of it. This concentrated miscella diffuses through adjacent cell wall and the process of inward diffusion, solution, pressurization and outward diffusion continues and eventually reaches to the solvent surrounding the outside of the flake. It quickly goes into solution with the solvent, incrementally increasing its concentration. This process continues with weak miscella entering the flake and more concentrated miscella leaving the flake. The extraction process is complete when the concentration of the miscella inside the cells comes into equilibrium with the concentration of the miscella surrounding the flake. The time required to reach the equilibrium is different, since the oilseeds have different cell structures. For soybean flakes the equilibrium time is approximately 5 min, while sunflower cakes require 9 min and rapeseed cakes 12 min [18].

Two principal types of extractor have been employed over the years: immersion extractors and percolation extractors. In the immersion process, the seed is completely dipped into the solvent. Today, almost all extraction plants use the percolation extractors. In a percolation extractor, the solvent percolates by gravity through the bed of the flakes, and percolation ensures that locally saturated miscella is permanently replaced by fresh or nonsaturated miscella. In a countercurrent, multistage percolation extractor, miscella flows in successive passes through the bed, while the solvent spray and the bed move in opposite direction to each other as shown in Figure 10.5 [2].

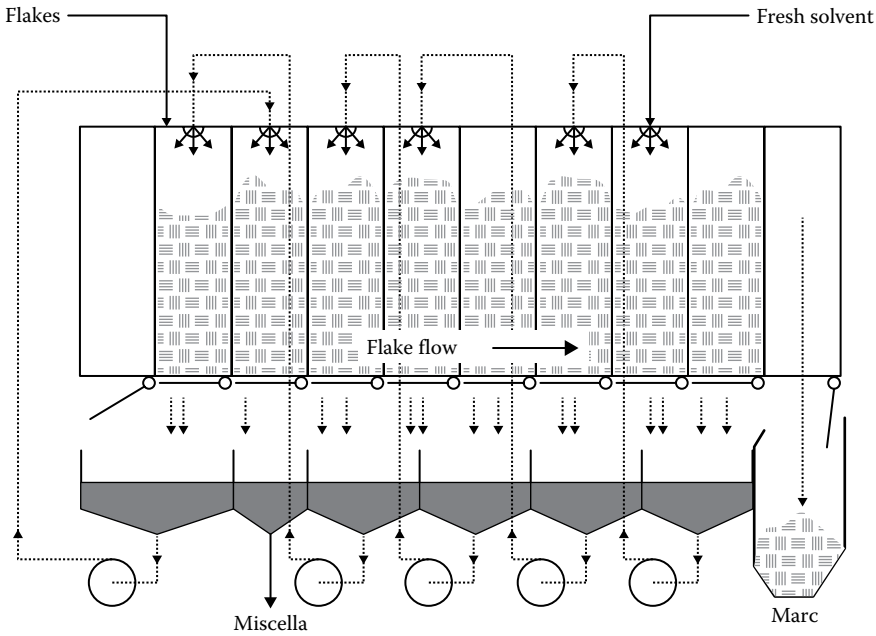

FIGURE 10.5 Principle of countercurrent, multistage percolation extraction. (From Johnson, L.A., Recovery, refining, converting, and stabilizing edible fats and oils, in: *Food Lipids, Chemistry, Nutrition, and Biotechnology*, 3rd edn., Ch. 8, Akoh, C.C. and Min, D.B., Eds., CRC Press, Taylor & Francis Group, Boca Raton, FL, 2008, pp. 205–243.)

The primary parameters affecting the extraction process are extraction temperature, contact time, and flake thickness [17].

As the extraction temperature increases, viscosity of the solvent decreases, and its rate of diffusivity through cell walls and the solubility of the oil in solvent increase. Lower viscosity and higher solubility result in a higher extraction rate. The extraction temperature is held safely below the initial boiling point of the solvent. Most processors operate the extractor at 60°C.

The total time that the material spends in the extractor is its residence time [18]. Contact time is the time that the particle of material is actually in contact with the miscella or solvent. Oil extraction only takes place during contact time. Sufficient contact time is required between the particle and each stage of miscella to allow the miscella within the particle to come into equilibrium with the miscella outside the particle. The contact time depends on the kind of seed, its pretreatment, and the extractor used.

The principal purpose of flaking is to reduce the thickness of the oilseed in order to reduce the distance and the number of cell walls that miscella needs to diffuse through to reach the oil bodies. Also, the more oil cell that is actually ruptured, the more free oil is available to go rapidly into solution with the solvent. If all other extraction parameters remain constant, reduced particles thickness allows a smaller extractor to be used [18]. However, since reducing particles thickness causes an additional electricity cost, the optimum flake thickness is determined according to the economic balance between the initial cost of the extractor and the ongoing electricity costs required for flaking.

ii. Miscella Distillation System Since the miscella leaving the extractor contains up to 1% by weight particles of meal, the first step in miscella distillation is the particles separation by filtration [18,19]. The miscella is then passed through a series of distillation equipments to separate the oil and the solvent. The percolation miscella has an oil content of 20%–30% and a solvent content

of 70%–80%. With the increasing oil content, the vapor pressure of the mixture increases, and thus, the boiling point of the miscella rises. Principally, the solvent is removed in a three-stage operation. In the first step, the miscella is fed to the first-stage evaporator, and approximately 80%–90% of the solvent is evaporated at 300–400 mm Hg absolute pressure and 43°C–48°C, using waste heat from the desolventizer toaster (DT) unit. The composition of the miscella exiting the first evaporator is generally 75%–85% oil and 15%–25% solvent. After heating to 110°C, the concentrated miscella is pumped into the second-stage evaporator, which is similar to the first-stage evaporator with the main difference being that the second stage evaporator is heated with steam. The concentrated miscella exiting this evaporator is generally 95%–98% oil and 2%–5% solvent and approximately at 95°C–110°C. The miscella is then fed into the stripping column, which is operated at 150–300 mm Hg. Live steam injection supports evaporation. The oil exits the stripper with 0.1%–0.3% moisture and 5–200 ppm solvent, at a temperature of 95°C–110°C. In a subsequent step, the oil is dried at 50–80 mm Hg absolute pressure. The oil typically exits the dryer with 0.05%–0.10% moisture and 5–100 ppm solvent, at a temperature of 105°C. The hot oil is cooled in two stages from 105°C to 70°C, and 70°C to 50°C in the heat exchangers using concentrated miscella from first evaporator and cooling water as the cooling medium successively, and the oil is then pumped to storage.

iii. Meal Desolventizer: Toaster (DT) The extracted flakes, containing around 25%–35% by weight solvent, are conveyed to the meal DT at the extraction temperature of 60°C. The desolventization is necessary to recover the solvent, to fulfill legal demands for animal fodder, to reduce pollution of the environment, and to avoid any explosion [2,18,19].

DTs are vertical, cylindrical vessels with multitude of horizontal trays. The meal is mixed above each tray and then conveyed downward from tray to tray, by agitating sweeps anchored to a central rotating shaft. The heat is supplied by steam, introduced directly and indirectly into the meal via the trays. The DT has three different types of tray: predesolventizing trays, countercurrent trays, and toasting–stripping trays. The predesolventizing trays are located in the upper portion of the DT, and 15%–25% of the solvent is removed by heating with indirect steam. The desolventizing trays are located under the predesolventizing trays in the center of the DT, and 75%–85% of the solvent is evaporated by heating with direct and indirect steams. The toasting–stripping trays are at the bottom part of the DT and are heated by both direct and indirect steam. The meal existing the desolventizing trays has the moisture content of 12%–19% and a temperature of 68°C–100°C. The final desolventizing takes place as the ascending steam passing through the meal slowly strips out the final traces of residual solvent, down to 100–500 ppm. The meal temperature increases to 105°C–110°C and the moisture content to 18%–20%. The desolventized meal contains a high amount of urease activity that is detrimental for certain animal feeding purposes. Under the conditions of heat, moisture, and retention time, this enzyme is inactivated and improves the digestibility of the meal.

iv. Meal Dryer: Cooler (DC) After the meal is desolventized, it is conveyed to the dryer–cooler (DC) to reduce the moisture content within trading rule limits and to lower the temperature prior to storage [18,19].

DCs are vertical, cylindrical vessel with a multitude of horizontal trays. The meal enters at the top and is supported by the tray. There are three different trays: steam-drying trays, air-drying trays, and air-cooling trays. The moisture content of the meal is reduced from 18%–20% to 12% and the temperature from 105°C to 110°C to around 60°C by drying with steam and hot air. At the air cooling trays, the meal is cooled using the air from 60°C to nearly 35°C–40°C.

v. Solvent Recovery System Solvent vapors originate from all steps of oil extraction plant, but mainly desolventization, and these vapors are collected from all over the plant. Solvent recovery

system includes solvent and water vapor condensation, solvent–water separation, stripping of solvent from water and air effluent streams, and heating of the solvent prior to reuse in the extractor [2].

B. Animal Fats and Oils Production

Animal fats and oils can be categorized as milk fat (butter), rendered fat, and fish oils (marine oils) [2]. Butter is produced by concentrating cream separated from the milk, and traditionally, the production of butter is a subject of dairy technology. Rendered fats are by-products of meat production and can be classified as lard, tallow, and poultry fat according to their sources. Marine oils are usually by-products of fish to be caught processed as a protein source.

Rendered fat is produced from fatty tissues by the cooking process known as rendering [4]. Raw materials include animal offal, bones, and trimmings from meat processors. Mechanical disintegration helps to break up the structure and to ease fat release from the cells. Heating the raw material also help to break up the cells and to decrease the fat's viscosity. Other positive effects of heating are sterilization, enzyme inactivation, and protein denaturation that helps in breaking emulsion and separating the molten fat.

Disintegration, heating, and separation are the basic steps of rendering process, and it can be performed on a semi-industrial or industrial scale, semicontinuously or continuously. Both wet rendering and dry rendering methods can be used. Wet rendering is the oldest method and involves cooking the broken material (2–3 cm) by direct steam injection for 90–150 min. The water, denatured protein, and other solids settle to the bottom, while fat floats on the top of the liquid. Water is drained off, and the remaining solid–fat mixture goes to a press for fat removal. The high protein solids separating from the press are known as cracklings and are used in livestock feed. The fat discharged from the press is centrifuged and filtered.

In the dry rendering process, the material is cooked in its own fat at 115°C–120°C in agitated, steam jacket vessels for 1.5–4 h, until the moisture has evaporated. The cooked material is first passed across a screen to allow the free fat to drain. The remaining solid–fat mixture is sent to a press, and the remaining steps are the same as for wet rendering.

The processing of fish yields three groups of substances: water, fish oil, and solid, which are mainly protein. Fish as a raw material contains 25%–35% dry matter [2]. The most important point of fish processing is efficient separation of its main components, protein, oil, and water. The raw fish material is prepared by an initial cooking step to coagulate the protein and to open up fat-containing cells. Cooking also helps to pasteurize the fish and inactivate enzymes. Traditional plants use screw presses to separate the liquid (oil/water) from the solid phase. The solids from the press are milled and dried for producing fish meal. Liquid from the press is heated with direct steam and then processed to separate fine solids, water, and oil by using two to three stages of separators. Modern plants are equipped with three-phase decanter together with the press.

III. REFINING VEGETABLE OILS

Generally, crude oils received from mechanical expelling or solvent extraction plant contain several non-TAG components that must be removed. The best-known exception to this general statement is olive oil that is preferred as cold-pressed virgin oils for its characteristic aroma and flavor. The non-TAG components that may be present in crude fats and oils are phosphatides, FFAs, partial acylglycerols (MAGs and DAGs), pigments (such as chlorophyll), odors and flavors (including aliphatic aldehyde and ketone), tocopherols, waxes as well as trace elements, pesticide residues, and polycyclic aromatic hydrocarbons [20]. However, not all of the non-TAG compounds are deleterious. For example, tocopherols are known to be the most important natural antioxidants that protect the oil from oxidation. Moreover, they are also biologically active substances for the body [18,21]. In refining, physical and chemical processes are combined to remove undesirable natural as well as environmental-related components from the crude oil.

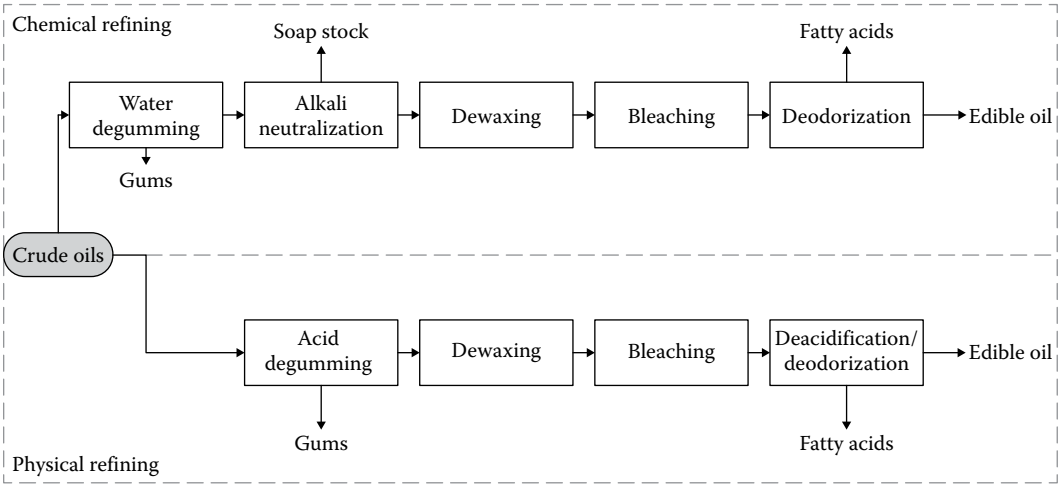

FIGURE 10.6 Chemical and physical refining processes. (From Zeldenrust, R.S., *Alkali Refining, Edible Oil Processing*, AOCS Lipid Library, Champaign, IL, 2012, http://lipidlibrary.aocs.org/OilsFats/content.cfm?ItemNumber=40319.)

There are two major refining processes known as "chemical" and "physical" refining. Generally, the chemical refining process includes degumming, neutralization, bleaching, and deodorization. Physical refining removes FFAs and flavors by distillation and combines the steps of neutralization and deodorization into one operation [4]. Steps in chemical and physical refining are shown in Figure 10.6.

A. Chemical Refining

1. Degumming

Phospholipids are oil-soluble components present in most edible oil sources that may become insoluble upon hydration and form a gummy precipitate referred to as "gums in the edible oil industry." The major phospholipids of oilseeds are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), and phosphatidic acid (PA) [22]. The order of the hydration rate of the different phospholipids is PC > PI > PE > PA, respectively. This means that the nonhydratable phospholipids (NHPs) mainly consist of PA [23].

Phospholipids pose many problems for the storage and processing of the crude oils; therefore, they are removed from oil during refining by a process known as degumming. The purposes of degumming operation are as follows:

- The emulsifying action of phospholipids increases oil losses during alkali refining.
- Gums lead to brown discoloration of oil after heating during deodorization.
- Salts may be formed with cooper, magnesium, calcium, and iron, accelerating oxidative degradation of oil.
- Certain phospholipids, such as lecithin, find widespread industrial application [22].

Different degumming processes such as water degumming, acid degumming, enzymatic degumming, and membrane degumming processes are carried out to remove phosphatides.

In water degumming, a large part of the phosphatides can be hydrated quickly and easily. If the pressed or extracted oil contains a considerable quantity of gums, the oil is subjected to the water degumming process immediately following extraction. In this process, water is added to the oil. After a certain reaction period, the hydrated phosphatides can be separated either by decantation

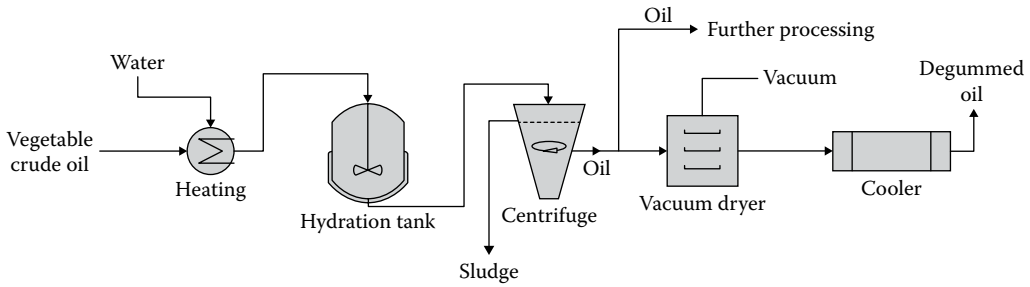

FIGURE 10.7 Water degumming process. (From Xu, L. and Diosady, L.L., Degumming, in: *Nutritionally Enhanced Edible Oil Processing*, Ch. 7, Dunford, N.T. and Dunford, H.B., Eds., AOCS Press, Champaign, IL, 2004, pp. 117–147.)

(settling) or continuously by means of centrifuges. In this process step, a large part of hydratable and even a small proportion of the nonhydratable phosphatides are removed (Figure 10.7). The wet sludge obtained from centrifuge is the raw material for lecithin production for food or can be used as feed or for technical purposes.

Acid degumming is an improvement over conventional degumming described earlier and has become the usual practice in the U.S. soybean industry. A small amount (0.05%–0.2%) of concentrated phosphoric acid (75%) is added to warm oil (70°C) followed by stirring for 5–30 min and degummed as described in connection with conventional degumming. Longer mixing times are often substituted for lower reaction temperatures. Phosphoric acid is added to make the phosphatides more hydratable by binding calcium and magnesium ions before adding water. Phosphoric acid pretreatment also partially removes chlorophyll from the oil [21].

Super degumming processes have also been developed in which more of the phosphatides are rendered hydratable. A strong solution of citric acid is added to warm oil (70°C), and the mixture is stirred and cooled to 25°C to precondition the gums. Then water is added with stirring for an additional 3 h to hydrate the gums. This process causes the phosphatides to form liquid phospholipid crystals, which are easily removed during centrifugation.

Another degumming process called "dry degumming" is occasionally applied to oils containing relatively low levels of phosphatides, such as palm, coconut, and peanut oils. In this process, the oil is treated with concentrated acid to agglomerate the gums. The gums are then separated from bleaching earth during subsequent steps of bleaching and filtering [4].

The enzymatic degumming process was first introduced by the German Lurgi Company as the "Enzy Max process" that used phospholipase enzyme A_2. The enzyme solution (aqueous solution of citric acid, caustic soda, and enzymes) is dispersed into filtered oil at mild temperature, a high-speed rotating mixer used for effective mixing of enzyme and oil. The conversion of NHPs into hydratable phospholipids (HPs) is attained by the effect of enzyme, the enzyme-treated oil is sent to mechanical separation, and the degummed oil received is dried under vacuum and suitable for further process. The main advantage for enzymatic degumming is conversion of the gums to a hydratable form, and the elimination of oil losses as well as losses in lecithin fraction. Furthermore, compared to chemical refining with NaOH, enzymatic degumming process uses lower amounts of chemicals and runs with lower energy consumption that will have a reduced adverse environmental impact [23].

Membrane processing has been applied to remove phospholipids from crude oil/hexane mixtures as well as from crude oil itself without the addition of an organic solvent. The process has been applied to soybean oil and reduced the phospholipids in soybean oil in the range of 85.8%–92.8% with surfactant-aided membrane degumming. HPs and NHPs were removed from the soybean oil. A membrane filter system that is capable of degumming and refining in a single step is expected to produce an oil with a phosphorus level of <2.0 ppm and reduce chlorophyll content by more than half [24]. Compared with the degumming methods in use, membrane degumming has several

advantages such as no chemical use, mild conditions, and lower energy consumption, waste production, and neutral oil loss [22].

2. Neutralization

The primary aim of neutralization (alkali refining) step is the removal of almost all of the FFAs from the crude oil [25].

In the alkali refining process, alkali solution such as caustic soda (sodium hydroxide) reacts with FFAs to produce soap and water. FFAs are converted into soaps that are insoluble and can be separated by settling or centrifugal separation. The feed can be crude oil or may also have been water degummed, acid degummed, or even acid refined or S.O.F.T.® degummed before being alkali refined. If there are phosphatides in the oil, the alkali refining process should remove them. In addition, the process should also remove coloring compounds and/or their precursors so that bleaching the alkali-refined oil requires less bleaching earth and color fixation during subsequent high-temperature treatments is avoided [25]. Refining practices vary between countries and plants due to the number, quality, and kind of source oils processed [24]. Although alkali refining first originated from Europe, it was first used in the United States. The process developed, which is still in use, is referred to as the "Long-Mix" process. It started as a batch process, but with the development of centrifugal separators, their use was recommended in continuous processes [25]. The short-mix process was developed in Europe where a wide range of oils are being processed and the relatively high FFA oils made it necessary to avoid the long contact time and the larger excess of caustic used with the conventional caustic soda refining system used in the United States [24]. Figure 10.8 compares the differences between the long- and short-mix refining processes.

The third industrial process is the Pellerin–Zenith process that is quite different in that its starting material must be phosphatide-free, does not employ a centrifuge, and does not enjoy an economy of scale. It is especially useful for specialty oils. Industrially continuous and batch miscella refining processes are also available. Miscella is the solution or mixture that contains the extracted oil. This type of refining is especially beneficial for cottonseed oil. The advantages of miscella refining can be listed as (1) higher oil yield, (2) lighter color oil without bleaching, (3) elimination of the water wash step, and (4) extraction of the color pigments before solvent stripping [25].

3. Bleaching

The primary purpose of bleaching is the removal of color pigments to improve color. This is achieved by the use of adsorptive clays, synthetic silica, and carbons. Adsorbents commonly used are natural bleaching earth, activated bleaching earths, activated carbon, and synthetic amorphous silica and synthetic silicates [20,24]. During bleaching, other impurities rather than pigments such as soap, trace metals, phosphatides, sulfur compounds, peroxides, and polycyclic aromatic hydrocarbons (if activated carbon is used with bleaching clay) are removed or reduced [20]. Bleaching is carried out at elevated temperatures (90°C–120°C) under a vacuum pressure to exclude oxygen in order to prevent oxidation of oils by bleaching clays and remove residual moisture from the oil [4,20]. Adsorbent (generally 0.2%–2%) is mixed with hot oil for 15–30 min to form a slurry. The pigments are adsorbed onto the surfaces of adsorbents and the solids are removed by filtration. Bleaching clays are generally activated by using a mineral acid such as sulfuric acid to obtain larger volume micropores and smaller particle size. Bleaching may be carried out as batch process; however, semi-continuous or fully continuous bleaching plants are more usual [20]. At the end of bleaching step, color is measured by Lovibond tintometer. Most finished oils are less than 10 yellow and 2.5 red; high-grade shortenings are less than 1.0 red [4].

4. Dewaxing

Waxes are typically the high-melting fatty acid esters of long-chain fatty alcohols with a generally low solubility in oils. These waxes solidify after a period of time resulting in a cloudy appearance, an unsightly thread, or a layer of solidified material [24]. The typical wax content of many

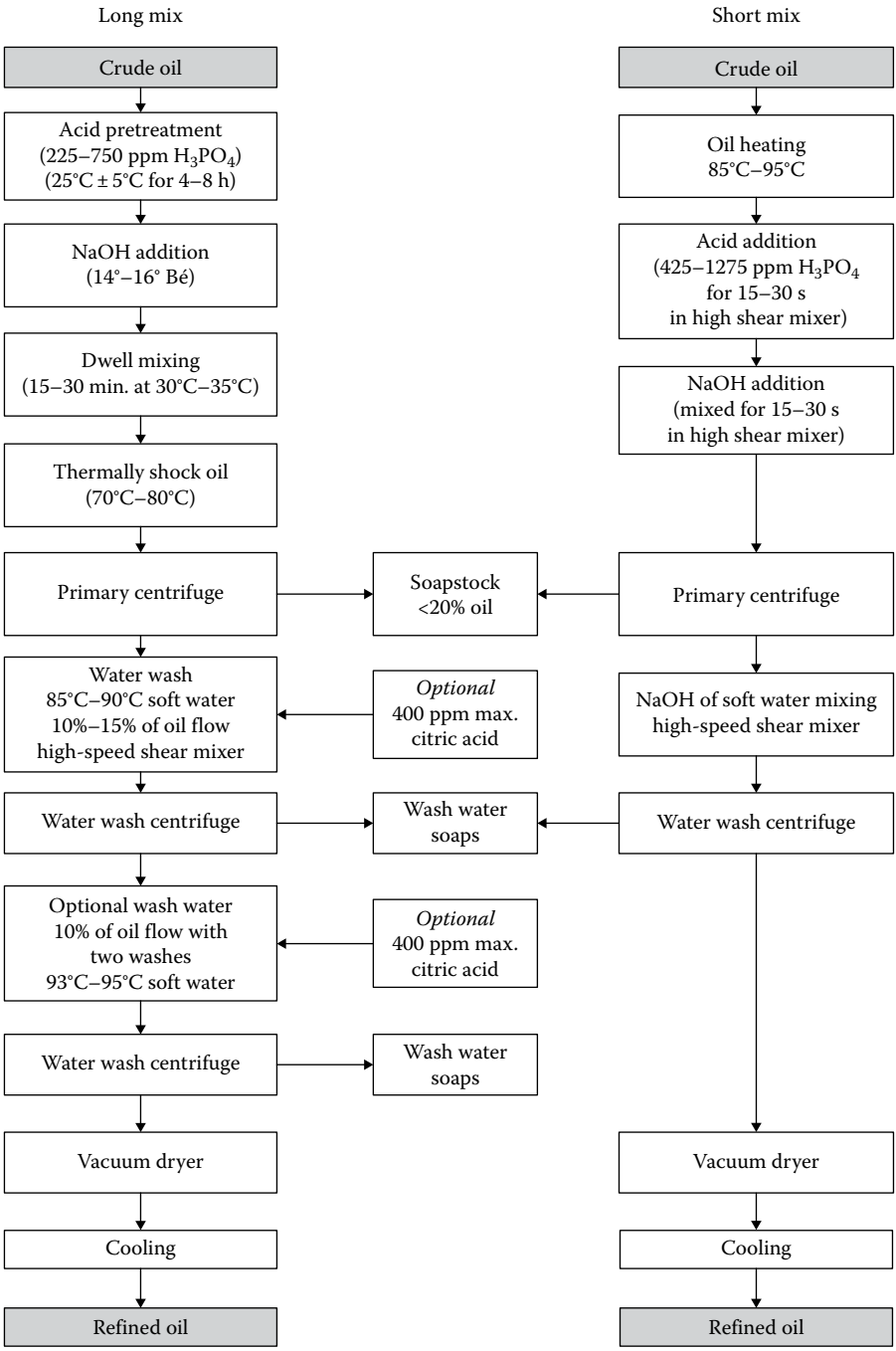

FIGURE 10.8 Comparison of long- and short-mix refining processes. (From O'Brien, R.D., Fats and oils processing, in: *Fats and Oils Formulating and Processing for Applications*, 3rd edn., Ch. 2, O'Brien, R.D., Ed., CRC Press, Taylor & Francis Group LLC, Boca Raton, FL, 2009, pp. 73–196.)

vegetable oils may be only a few hundred ppm, rising to 2000 ppm and above depending on the oil. Peanut, rape, sesame, soy, palm, palm kernel, fish oils, tallow, and lard does not require special dewaxing step in processing. Sunflower and maize oils almost certainly require dewaxing [26]. The wax content must be reduced to less than 10 ppm to prevent cloudy appearance [24]. The classical dewaxing process usually performed after prebleaching and prior to deodorization consists of carefully cooling the oil to crystallize the waxes for removal by filtration. The cooling must be done slowly under controlled conditions. A body feed approximately equal to the wax content of the oils is used to prevent blinding of the filter leaves. Without a body feed, the waxes slime over and blind the screens almost immediately. A continuous dewaxing process that operates efficiently with low-wax oils (500 ppm or less) has the following process flow: The oil is continuously cooled with heat exchangers and a crystallizer to 43°F–46°F (6°C–8°C). A quantity of filter aid equal to the wax content is added to the crystallizer to facilitate crystallization and filtration. Crystallization time is 4 h minimum, followed by a holding period of 6 h to develop the wax crystals. The oil is carefully heated to 64°F (18°C) before filtering to separate the wax crystals from the liquid oil [24].

B. Physical Refining

Physical refining, also known as steam refining, means removal of FFAs from the oil. It was used as a process for the preneutralization of products with high initial FFAs content since 1930, and in 1950s, this process became real with the processing of palm oil. Physical refining combines two steps: deodorization and neutralization (Figure 10.6). There are many advantages of physical refining process such as improvement of oil yield due to minimum neutral oil loss. In addition, physical refining leads to the recovery of higher-grade distilled fatty acids without major pollution problems and eliminates the production of soap stock [4].

Physical refining is applicable to oils that have high FFA content, low phosphorus contents, and low gum amount. Phosphorus content should be <5 ppm because high contents can lead to dark-colored oils. For example, physical refining is appropriate for palm, rice bran, lauric, and coconut oils, and animal fats, whereas soybean, canola, and sunflower oils that contain low levels of FFAs and higher amounts of phosphatides are not suitable for this process [4,24].

IV. OIL MODIFICATION PROCESSES

Modifying the physical and chemical properties as well as nutritional properties of edible oils for obtaining desirable attributes can be possible either through very expensive and genetic modification techniques or through three main modification techniques that are applied at present in the industry. The best-known modification processes are based on chemical, physical, and bio(chemical) reactions like hydrogenation, fractionation, or interesterification [27]. The main purpose of these processes is to change the physicochemical properties of fats or oils through (1) reducing the degree of unsaturation of the acyl groups, (2) redistributing the fatty acid chains, and (3) physical separation of the component TAG by selective crystallization and filtration [28]. Though hydrogenation is still the most common and widely used chemical application, interesterification for producing zero-*trans* fatty acids (TFAs) margarines and fractionation for producing cocoa-butter substitutes, decreasing PUFAs by several methods to increase oxidative stability for frying formulations or combined use of the different modification processes to achieve desired goals, are being applied successfully at industrial scale.

A. Hydrogenation

Hydrogenation is one of the oldest technologies that improve physical and chemical properties of lipids in which unsaturated fatty acids are saturated with hydrogen gas in the presence of a metal catalyst, and a solid or semisolid fat that is used as raw material for margarine, shortening, frying

fats, and other specialty products is produced. For hydrogenation to take place, gaseous hydrogen is bubbled to the liquid oil and the solid catalyst material (usually Ni) in a specially designed reaction vessel under controlled temperature and pressure [29].

The main purposes of hydrogenation are:

1. To impart oxidative stability to the oil
2. To modify the physical state of oils and fats for functionality [27]

Since hydrogenation is a catalytic reaction, it requires the use of catalysts such as nickel, which is the most common catalyst currently used in the hydrogenation of fats and oils [27].

Commercially available nickel catalyst contains 22%–25% active catalyst supported by a totally saturated or completely hydrogenated fat. Other catalyst supports include alumina, kieselguhr, and silica.

For the saturation reactions to take place, oil, gaseous hydrogen, and the catalyst are agitated to introduce hydrogen into the oil and renew the oil at the catalyst surface that is essential for the continuity of the reaction. Research on this subject showed that passage of these compounds to the catalyst surface and their return to liquid medium occurs according to the laws of diffusion [30].

The rate of the reaction is a function of the degree of unsaturation, and the rate and the type of product depend on various process variables such as (1) starting temperature of the oil and the reaction temperature, (2) activity of the catalyst, (3) amount of the catalyst, (4) hydrogen uptake rate, (5) reaction temperature, (6) oil quality and nature, (7) hydrogen purity, and (8) degree of agitation [30]. According to these variables, solid fat index curve, iodine value, and melting point of the product oil may be changed.

Hydrogenation steps include (1) transfer and/or diffusion, (2) adsorption, (3) hydrogenation/isomerization, (4) desorption, and (5) transfer. Transfer and adsorption steps are the critical steps in controlling the degree of isomerization and selectivity of reactions. When the reaction is complete and the endpoint is reached, the batch is cooled and filtered to remove all the catalyst and other impurities. The oil may also be postbleached with filter aid and bleaching clay [30]. In hydrogenation, the central unit is the reactor. There exist two main types of reactors such as dead-end reactor and the gas loop reactor. In the dead end reactor, hydrogen gas is introduced into the oil and remains in the reactor until it is consumed [28]. Hydrogen loss is lowest in this system, and under a hydrogen pressure, effective mechanical stirring is applied resulting in an effective hydrogen solubility in the oil and transfer of the oil to the catalyst surface. Dead-end reactors are made of stainless steel, vertical, closed tank cylinder with a capacity ranging from 5 to 15 tons.

Most unsaturated bonds in vegetable oils naturally occur in *cis* form. However, during partial hydrogenation process, TFAs that are believed to possess some adverse health effects on human health are produced. During the industrial hydrogenation processes, the most important TFA released is the *trans* C18:1 (octadecenoic acid) or elaidic acid. *Trans* isomer of oleic acid has a higher melting point (44°C–45°C) compared with *cis* isomer (13°C–14°C). Geometric structures of *cis* and *trans* isomers of oleic acid are shown in Figure 10.9.

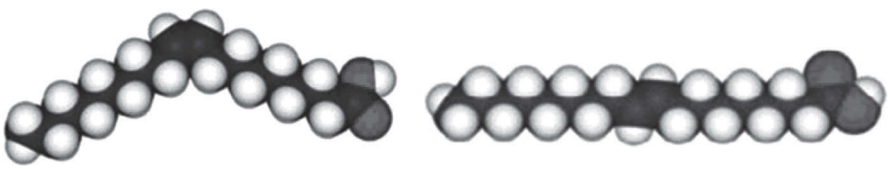

FIGURE 10.9 (a) Oleic acid (9-*cis*-C18:1); (b) elaidic acid (9-*trans*-C18:1).

Intake of high amounts of TFA has been correlated with increased risk of cardiovascular diseases, primarily due to their adverse effects on plasma lipid profile such as increasing low-density lipoprotein cholesterol, total cholesterol, and TAG levels. These adverse effects have heightened health concerns among consumers and regulatory agencies in Europe and the United States. As a result, the U.S. Food and Drug Administration (FDA) issued a final ruling requiring foods containing TFA to be labeled accordingly, effective from January 2006 [31]. The food industry has responded to these concerns and labeling rules by developing processes that will produce foods with zero or reduced trans fat contents. With the aim of eliminating the risk of TFAs on human health, several alternative methods can be used to obtain *trans*-free or low-*trans* fats. The alternative methods developed for the process include the use of chemical or enzymatic interesterification method instead of hydrogenation of fats, tropical fat fractionation, modified hydrogenation process, transgenic oil production, specific structured lipid production, followed by application of emulsification and crystallization techniques. Among hydrogenation, fractionation, and interesterification, the only method for changing the properties of oils without altering the structure and composition of fatty acids originally present is interesterification. Thus, interesterification is clearly distinguished from hydrogenation, in which the level of unsaturation of the fatty acids is reduced, and fractionation, where unmodified TAGs or previously modified TAGs are separated into two or more fractions through crystallization [32]. Structured lipids are synthesized by incorporating high-melting fatty acids into oils or by blending high-melting fractions of natural oils or fully hydrogenated fats with liquid oils. This process therefore increases the solid fat content and oxidative stability of the product and prevents posthardening effects of margarine when stored [31].

B. Interesterification

Interesterification is a catalytic process that consists of fatty acid redistribution on the glycerol backbone of TAG molecules [32]. It also refers to the reaction between different TAGs resulting in the rearrangement of the acyl groups (fatty acids) within and between TAGs. Due to these rearrangements, melting behavior and crystallization or recrystallization properties can be altered for desired applications [27,33]. Furthermore, due to an increasing tendency to produce *trans*-free or low-*trans* products, interesterification technology combined with dry fractionation or full hydrogenation has become popular [32]. Commercially, the interesterification process is utilized for processing edible fats and oils to produce confectionery or coating fats, margarine fats, cooking oils, frying fats, shortenings, and production of structured lipids for specific applications [24].

In the food industry, interesterification can be carried out using a chemical catalyst generally, sodium methoxide or an enzyme generally, lipases. Chemical interesterification is a random reaction that allows random reorganization of the fatty acids. Enzymatic interesterification can be either random or regiospecific [32]. Interesterification involves various types of reactions and may occur by (1) a reaction of an ester with another ester, referred to as interesterification, ester interchange, or sometimes transesterification; (2) reaction of an ester with an acid, known as acidolysis; and (3) reaction of an ester with an alcohol, referred to as alcoholysis or glycerolysis or methanolysis [33]. These reactions are depicted in Figure 10.10.

Enzymatic interesterification processes have gained increasing interest especially for the production of margarines, shortenings, and structured lipids. Enzymatic interesterification reactions are catalyzed by TAG lipases (TAG acylhydrolases, EC 3.1.1.3), which are a group of hydrolase enzymes and can be obtained from different sources such as animal (pancreatic lipase, lingual lipase, pharyngeal lipase, gastric lipase, etc.), plant (oilseed lipases, lipases that are present in germ and the bran part of cereals), and microbial (fungal lipases, bacterial lipases, yeast lipases) sources. Among these lipases, animal and plant lipases are generally less thermostable than microbial lipases. In addition, microbial lipases are less expensive to produce than mammalian enzymes [34]. In the last years, recombinant DNA techniques have been successfully applied for the production of lipases to obtain desired properties [35].

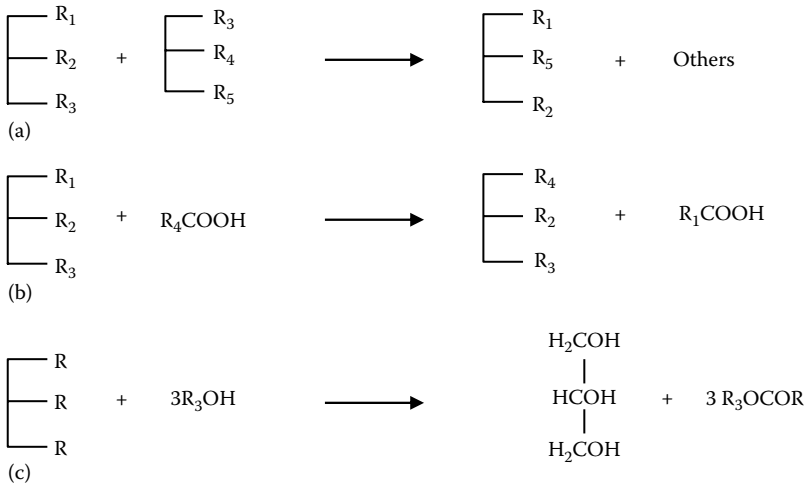

FIGURE 10.10 Types of interesterification reactions: (a) interesterification, (b) acidolysis, and (c) alcoholysis. (From Soumanou, M.M. et al., *Eur. J. Lipid Sci. Technol*., 115, 270, 2013.)

Depending on the reaction system (i.e., aqueous or organic solvent), lipases are able to catalyze various reactions. Lipases catalyze the hydrolysis of a broad range of fats and oils to form DAGs, MAGs, FFAs, and glycerol. Hydrolysis reaction of the TAG substrates—which are insoluble in water—occurs at the lipid/water interface [36]. TAG lipases also catalyze esterification (i.e., reaction of a fatty acid with an alcohol to form an ester), the reverse reaction of hydrolysis in reaction media with low water content.

Moreover, lipases catalyze interesterification and transesterification reactions such as between TAGs or between a TAG and a fatty acid (acidolysis) or an alcohol (alcoholysis) or glycerol (glycerolysis) in media with low water content [37]. Additionally, lipases offer a great potential for lipid modification. However, the greatest advantage of lipases resides in their chemo-, regio-, and also stereoselectivity, which makes them attractive for use in academia and industry [36]. Specificity of lipases for the fatty acids esterified at the *sn*-1, *sn*-2, and *sn*-3 positions of the glycerol backbone varies widely, ranging from nonspecificity for either of the three *sn*-1, *sn*-2, and *sn*-3 positions to strong *sn*-1,3 or *sn*-3 specificity. The substrate specificities and regioselectivities of some common and commercially available lipases are shown in Table 10.3.

Among lipases, microbial lipases are the most used because of their stability under heat. The stability of lipases under high temperatures is an important characteristic for industrial applications. Immobilized lipases can be used at high temperatures especially in microaqueous systems. In enzymatic reactions, two types of lipases are used: *sn*-1,3-specific lipases and lipases that have no position specificity [39].

Enzyme-catalyzed reactions have the advantage of operating effectively under relatively mild conditions when compared to chemical methods. When a reaction occurs with enzymes, it can catalyze the reaction 10^6–10^{15} times faster than chemical methods even at room temperatures [40]. Other benefits of the use of lipases are as follows:

- They catalyze a more directed rearrangement of fatty acids in TAG molecules.
- They have high specificity.
- They have utility in "natural" reaction systems and products.
- They have wide range of sources.
- They reduce environmental pollution, because enzymes are biodegradable.
- They can be improved by genetic engineering [35,41,42].
- Enzymatically interesterified oils are improved as tocopherols and tocotrienols are better retained and oxidative stability is increased [32].

TABLE 10.3
Specificity of Triacylglycerol Lipases

Source of Lipase	Fatty Acid Specificity	Positional Specificity
Microorganisms		
Aspergillus niger	S, M, L	*sn*-1,3 >> *sn*-2
Candida antarctica	S > M, L	*sn*-3
Candida rugosa (syn. *Candida cylindracea*)	S, L > M	*sn*-1,2,3
Chromobacterium viscosum	S, M, L	*sn*-1,2,3
Rhizomucor miehei	S > M, L	*sn*-1,3 >> *sn*-2
Penicillium roqueforti	S, M >> L	*sn*-1,3
Pseudomonas aeruginosa	S, M, L	*sn*-1
Pseudomonas fluorescens	S, L > M	*sn*-1,2,3
Rhizopus delemar	S, M, L	*sn*-1,2,3
Rhizopus oryzae	M, L > S	*sn*-1,3 >> *sn*-2
Plants		
Rapeseed (*Brassica napus*)	S > M, L	*sn*-1,3 > *sn*-2
Papaya (*Carica papaya*) latex		*sn*-3
Animal tissues		
Porcine pancreatic	S > M, L	*sn*-1,3
Rabbit gastric	S, M, L	*sn*-3

Source: Weber, N. and Mukherjee, K.D., Lipid biotechnology, in: *Food Lipids: Chemistry, Nutrition, and Biotechnology*, Akoh, C.C. and Min, D.B., Eds., CRC Press, Boca Raton, FL, pp. 707–765, 2008.

Abbreviations: S, short chain fatty acids; M, medium chain fatty acids; L, long chain fatty acids.

Enzymatic interesterification is now commonly used for the formulation of low-*trans* or *trans*-free margarines and shortening fats and structured lipids such as human milk fat substitutes and cocoa butter substitutes in industrial scale. It is preferred to chemical interesterification as it is a bioprocess, not using chemicals, conducted at relatively low temperature, producing less neutral oil losses (no post-bleaching) and preserving the quality of the interesterified oil [32].

Comparison of chemical interesterification with enzymatic interesterification is given in Table 10.4.

Interesterification is described in much greater detail in Chapters 12 and 34.

TABLE 10.4
Comparison of Chemical Interesterification with Enzymatic Interesterification Methods

Chemical Interesterification	Enzymatic Interesterification
Chemicals such as sodium methoxide are used.	Lipases are used.
Complete positional randomization of the acyl groups in the TAGs.	More specific.
High levels of reaction by-products (MAG, DAG, glycerol).	Low levels of by-products.
Low processing cost (batch reactor).	High processing cost (continuous plug-flow reactor, lipase).
High processing loss (oil saponification).	Minimum processing loss.
Low oxidative stability (tocopherol loss).	No change in oxidative stability.
Flavor reversion problem.	No flavor reversion.
Highly reproducible and easily controlled.	More complex operation and control.

C. Fractionation

In the industrial processing of fats and oils, the term "fractionation" is used to describe fractional crystallization processes of TAGs where the oil is first crystallized and the formed crystals with higher melting point fractions are separated by subsequent filtration [43]. Fractionation processes separate fats and oils into two fractions, solid and liquid often called stearins and oleins, with different melting points in a single-step process or a range of fractions with different melting and crystallization properties can also be produced by sequential fractionation [24,44].

The stages of fractionation consist of three steps:

1. Cooling of the oil to supersaturation to form the nuclei for crystallization
2. Progressive growth of the crystalline and liquid phases
3. Separation of the crystalline and liquid phases [24]

Different factors are important in each stage and the quality of the liquid fraction is affected by the crystallization step, whereas the quality of the hard fraction is affected by both crystallization and separation steps [43]. Quality is defined according to degree of desired TAGs in the separated fraction and is generally assessed by cloud point or solid fat content [43].

The amount and composition of the solid phase is dependent mainly on the crystallization temperature, the cooling rate, the composition of the oil or fat, and the nature of the solvent used. Separation efficiency of the liquid and solid fractions depends primarily on the cooling method, which also determines the crystal form and size. Fats and oils can crystallize in several polymorphic forms such as α, β′, and β. The rate of crystallization for the α form is higher than for the β′ form. It crystallizes quicker than the β form. Rapid cooling causes heavy supersaturation, which forms many small, shapeless, soft crystals of mixed crystal types with poor filtration properties. Gradual cooling of the oil results in stable β and β′ crystals that are easily filtered from the liquid phase. Separation efficiency also is dependent on suspension parameters such as viscosity, particle size and shape, and polymorph as well as on the type of separation unit used. In recent years, pressure filtration has become the most common method used since the separation efficiency is the highest [24,44].

Edible fats and oils are fractionated for the following reasons:

1. Separation of fats and oils into different fractions with different melting points
2. Removal of undesired minor components (e.g., removal of waxes from sunflower oil)
3. Enrichment of a desirable TAG (e.g., POP in palm oil)
4. Separation of a fat or oil into fractions that could be used as functional products from the same original product (e.g., fractionation of beef tallow, palm kernel oil, or palm oil to olein [liquid] and stearin [solid] fractions [24,43])

There are generally three different fractionation processes available today, including dry fractionation (melt fractionation), solvent fractionation and dry fractionation in the presence of a detergent, and supercritical fluid extraction. The most common fractionation techniques include solvent fractionation and dry fractionation [28,44].

1. Dry (Melt) Fractionation

This is the simplest and most economic method. It is one of the most preferred techniques since the technology has been improved by the development of improved separation techniques [24]. It is a two-stage process in which in the first stage the oil is slowly cooled and crystallized without any solvent. In the further stage, remaining liquid is separated from the solid fraction by various processes, such as filtration, centrifugation, hydraulic pressing, rotary drum, or one of the patented processes [24,28]. This type of process is used for the separation of hard stearin and soft olein fractions from

natural products that contain high levels of each, such as coconut oil and palm kernel oil, that form a crystal network rather than isolated crystals on cooling [45].

2. Solvent Fractionation

Solvent fractionation differs from dry crystallization because solvent is used to promote crystallization and enhance separation. The solvents generally used for solvent fractionation applications are acetone, ethanol, hexane, isopropanol, and pentane [44].

Solvent fractionation has several advantages over dry fractionation as efficiency is higher, crystallization is faster, crystallization can be done in single step, and separation efficiency is higher, and the fractions have more distinctive chemical compositions since the fractions are more effectively separated. Beside those advantages, there are some disadvantages related to the use of flammable solvents. Safety precautions should be taken, cooling a dilute solution to lower temperatures as well as separating olein and stearin fractions by distillation increases energy requirements [44,45]. Since the cost is higher, it can be justified only for preparation of value-added, high-quality products such as cocoa butter equivalents, lauric cocoa butter replacers (CBRs), nonlauric CBRs, confectionery products, medium-chain TAGs, and high-stability liquid oils [24].

Commercially, solvent fractionation is carried out by a number of different processes that may be batch, semicontinuous, or continuous [24]. The molten fat is dissolved in a solvent in the ratio of 1:3–5 (oil per solvent) by weight and the solution is cooled to promote crystallization. Crystallization temperatures differ according to the solvent type, concentration of oil in the solution, and the characteristics needed in the final fractions. For lauric oils in acetone solvent, temperature range can be 28°F–68°F (–2°C to 20°C) to obtain stearin with iodine values of 1.8–8.3. After crystallization, crystallized slurry is separated by filtration or by a settling technique. The last stage of solvent fractionation involves removal of solvent from the fractions by several techniques including evaporation, vacuum distillation, or steam distillation [24,44].

3. Detergent Fractionation

This fractionation technology differs from dry fractionation with the use of an aqueous detergent to assist in the separation of olein and stearin fractions. Aqueous detergent solution contains approximately 5% of a detergent such as sodium lauryl sulfate that is used to wet the surface of the crystals by displacing the liquid oil. About 2% of an electrolyte, such as magnesium or aluminum sulfate, is added to the solution to assist in uniting the liquid olein droplets. Separation is then achieved by using a centrifuge. Afterward, the heavier phase is heated to melt the stearin and separate the oil and water with a second centrifuge. Due to high operation costs, potential contamination of the fractions, and the improvements in the dry fractionation technology, the use of detergent fractionation technology has declined in the food industry [24,45].

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11 Crystallization Behavior of Fats
Effects of Processing Conditions

Ashwini Wagh and Silvana Martini

CONTENTS

I. CRYSTALLIZATION: GENERAL PRINCIPLES

Crystallization refers to the formation of a solid from a liquid. During crystallization, randomly oriented molecules of the liquid undergo phase transition to form a solid characterized by a crystalline lattice of highly organized molecules or atoms. The crystallization process consists of four steps: (1) achievement of a thermodynamic driving force for crystallization, (2) nucleation, (3) crystal growth, and (4) recrystallization [1]. The first requirement to form a crystal is the generation of a sufficient thermodynamic driving force for crystallization. Once a sufficient driving force is reached, nuclei will form and, given enough time, these nuclei will grow to form a crystal. Finally, over time, crystals reorganize and recrystallize to reach the most favorable energy state and form a stable crystalline network characterized by specific physical properties. The crystallization process can be affected by several process conditions that affect any of the four steps mentioned earlier. Process conditions of interest include but are not limited to crystallization temperature, agitation, and cooling rate. This chapter will describe the fundamentals of lipid crystallization and will

discuss the effect that processing conditions have on crystallization behavior of lipids and on physical properties of the crystalline network formed such as hardness, elasticity, solid fat content (SFC), crystal morphology and polymorphism, and melting behavior.

Crystallization processes can occur in two ways: from solution, as in the case of a solute/solvent system, or from the melt as in the case of pure compounds that crystallize in the absence of a solvent. Examples of systems that crystallize from solution include inorganic or organic salts and sugars in water where a specific solute and solvent can be identified. Crystallization of water to form ice is an example of crystallization from the melt.

Edible lipids, or fats, consist of a mixture of several hundred triacylglycerols (TAGs), and depending on their chemical composition they can be treated as solutions or as a system that crystallizes from the melt. Crystallization of simple fats composed of only high-melting and low-melting-point TAGs can be treated as a solution where high-melting-point TAGs represent the solute and low-melting-point TAGs act as the solvent. However, when several TAGs with a broad range of melting points are present in the fat, it is likely that TAGs co-crystallize and therefore a single molecular entity that acts as a solvent or solute cannot be identified. In this case, the system is considered to be crystallizing from the melt [2]. Most natural fats such as soybean oil, cocoa butter, and canola oil comprise a wide range of TAGs and therefore are considered to crystallize from the melt. Mechanisms involved in the crystallization from a solution and from the melt will be discussed in this chapter, but greater emphasis will be placed on crystallization from the melt due to its importance in lipids.

A. Thermodynamic Driving Force

The thermodynamic driving force for crystallization is defined as the difference in chemical potential of the solid–liquid transition [2]. Differences in chemical potential in systems that crystallize from solutions or from the melt are driven by different factors as will be described in the following text.

1. Crystallization in Solutions

Crystallization in solution refers to the crystallization of a solute/solvent system. In this case, the thermodynamic driving force is the difference in chemical potential of the supersaturated and saturated solution that can be expressed as

$$\Delta\mu = kT \ln\left(\frac{C}{C_s}\right) \tag{11.1}$$

where

$\Delta\mu$ is the difference in chemical potential
k is the Boltzmann constant per molecule
T is the absolute temperature
C is the solute concentration
C_s is the solute concentration at saturation [3]

Equation 11.1 shows that for crystallization to occur, a concentration greater than the concentration of the saturated solution ($C > C_s$) must exist. The ratio $\beta = C/C_s$ is often referred to as saturation ratio and $\beta - 1$ is called supersaturation. C can be achieved by heating the solvent to dissolve the solute and cooling the system to generate a supersaturated solution. Other methods to generate supersaturation include evaporating the solvent or adding a second solvent in which the solute is insoluble [2]. Equation 11.1 also shows that the higher the supersaturation, the higher the driving force for crystallization.

2. Crystallization from the Melt

When a system crystallizes from the melt, as is in the case of TAGs and fats, the difference in chemical potential for the solid–liquid transition can be expressed by the following equation:

$$\Delta\mu = \Delta H_f \left(\frac{T_f - T}{T_f} \right) \tag{11.2}$$

where

$\Delta\mu$ is the difference in chemical potential between the melt and the crystalline material
ΔH_f and T_f are the enthalpy and temperature of fusion per molecule, respectively
T is the sample's temperature

The difference between T_f and T is usually referred to as supercooling [3]. Equation 11.2 shows that the driving force for crystallization from the melt is driven by the supercooling of the system. The higher the supercooling, the higher the driving force. Therefore, when comparing crystallization behavior of a single fat as affected by processing conditions, it is very common to refer to supercooling as the driving force for crystallization. However, if different polymorphic forms are obtained in the fat and if different fats must be compared, ΔH_f and T_f must be considered to calculate the driving force of crystallization accurately. Polymorphism in fats will be described later in this chapter.

B. Nucleation

Nucleation occurs only after a sufficient thermodynamic driving force is attained. The formation of a nucleus involves diffusion of molecules in the bulk and their rearrangement to form an ordered crystalline lattice. A nucleus is a small crystal formed by accretion of molecules. The formation of a nucleus is a dynamic process where molecules form aggregates that associate and disassociate in the supercooled or supersaturated media. Under the right driving force conditions, the rate of aggregation is greater than the rate of dissociation, and the aggregates eventually grow to form a nucleus. A nucleus is stable when it contains a critical number of molecules and achieves a critical radius that is determined by the thermodynamic driving force. Aggregates that are too small to be considered nuclei are usually referred to as embryos [3].

Mechanisms of nucleation can be classified as either primary nucleation or secondary nucleation. Primary nucleation can be either homogeneous or heterogeneous. Homogeneous nucleation occurs by accretion of two molecules in the absence of foreign particles, while heterogeneous nucleation occurs when impurities in the media promote the formation of a nucleus. Secondary nucleation occurs under metastable conditions where crystallization will not normally occur. In this case, the formation of a nucleus in the metastable solution is induced by the presence of foreign materials such as another crystal or a stirring device [4].

Two energy barriers must be overcome to create a new stable nucleus in a supercooled or supersaturated solution: (1) energy barrier to form a new surface and (2) energy barrier to form the volume or bulk of the crystal. The free energy required to form a new surface is a positive term directly related to the nucleus radius, while the energy required to create the volume or nucleus bulk is a negative term also directly related to the radius of the nucleus. The total energy that must be overcome to form a nucleus is referred to as the activation free energy (ΔG) and is directly related to the radius (r) of the nucleus. This relationship is described by the following equation assuming a spherical nucleus:

$$\Delta G = -\frac{4\pi r^3}{3v}\Delta\mu + 4\pi r^2\gamma \tag{11.3}$$

where

v is the volume of a molecule inside the nucleus
γ is the interfacial free energy

The first term in Equation 11.3 represents the energy required to form the volume of the nucleus and the second term represents the energy required to form its surface. A stable nucleus is obtained when the system reaches equilibrium and when $\delta\Delta G/\delta r = 0$. In this case, the nucleus has achieved a critical size (r_c) and will continue to grow:

$$r_c = \frac{2\gamma v}{\Delta\mu} \tag{11.4}$$

where $\Delta\mu$ is expressed differently depending on whether the system is crystallizing from a solution (Equation 11.1) or from the melt (Equation 11.2). Figure 11.1 shows the ΔG variation as a function of the radius of the nucleus and the critical radius needed to form a stable nucleus [4].

Once a nucleus has been formed, the rate of nucleation can be calculated by the Fisher–Turnbull equation, where the relationship between nucleation rate (J) and activation free energy of nucleation (ΔG_c) can be evaluated:

$$J = \left(\nu n_1\right)\exp\left(-\frac{\Delta G_d}{kT}\right)\exp\left(-\frac{\Delta G_c}{kT}\right) \tag{11.5}$$

This equation is valid for crystallization from the melt or for solutions with high viscosity, where molecular diffusion might be a limiting factor. In these cases, the activation free energy of diffusion (ΔG_d) must be considered in the equation. By replacing Equation 11.4 in Equation 11.3, the critical activation free energy of nucleation can be obtained using the following equation [3]:

$$\Delta G_c = \frac{16\pi v^2\gamma^3}{3\left(\Delta\mu\right)^2} \tag{11.6}$$

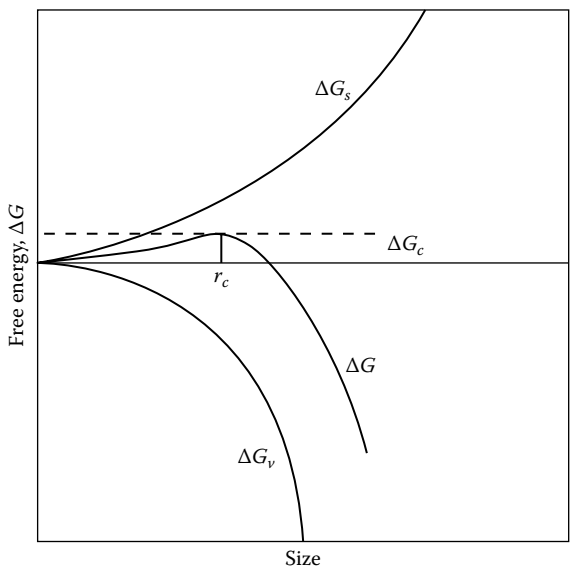

FIGURE 11.1 Free energies that must be overcome to form a nucleus. ΔG_s represents the free energy required to form the surface of the nucleus, ΔG_v represents the free energy required for the volume or bulk of the nucleus, and ΔG_c represents that total free energy required to form a nucleus. (Reprinted with kind permission from Springer Science+Business Media: *Crystallization in Foods, Nucleation*, 2001, pp. 146–191, Hartel, R.W.)

By introducing ΔG_c in Equation 11.5 and considering that the nucleation rate is proportional to the inverse of the induction time of nucleation (τ), activation free energies of nucleation can be calculated for solutions and for melt systems using Equations 11.1 and 11.2. In the case of a solution, ΔG_c can be calculated from the slope (s) of the curve obtained by plotting ln τ versus $1/\ln^2(C/C_s)$ using the following equation [5]:

$$\Delta G_c = \frac{skT}{\ln^2(C/C_s)} \tag{11.7}$$

In the case of a system crystallizing from the melt, ΔG_c can be calculated from the slope of the curve (s) obtained from plotting ln τT versus $1/T(\Delta T^2)$ using the following equation [6]:

$$\Delta G_c = \frac{sk}{(T_m - T_c)^2} \tag{11.8}$$

Calculation of activation free energies of nucleation describes the amount of energy that must be provided to induce nucleation under specific processing conditions.

C. Crystal Growth and Recrystallization

Once a stable nucleus has formed, it grows to form a crystal that continues to grow until the solution is no longer supersaturated. Growth mechanisms for solutions have been described in detail by Boistelle [3] and Hartel [7] and will not be discussed in this chapter. Very few studies have quantified lipid crystal growth in terms of growth rate and growth mechanism. Crystal growth can be evaluated by quantifying crystal size as a function of crystallization time using microscopic techniques such as polarized light microscopy. The thermodynamic driving force for crystallization is an important factor controlling crystal growth rate. In the case of fats, crystal growth rate is directly proportional to the thermodynamic driving force for crystallization, that is, supersaturation or supercooling. That is, the higher the driving force, the faster the growth. High driving forces induce nucleation and generate a high number of small crystals that grow very fast and that in general contain many imperfections. Lower driving forces generate fewer, bigger, and more perfect crystals with incorporation of fewer impurities. Crystal growth rate is not directly related to crystal size. Large crystals might form slowly (low growth rate) and small crystals might form faster (fast growth rate). Crystal growth can also be affected by intrinsic properties of the fat such as viscosity and the presence of impurities and by several processing conditions including agitation, the use of additives, and cooling rate. Some of these factors will be discussed later in this chapter.

After nuclei have formed and crystals have grown to reach a state of equilibrium, further reorganization of molecules might occur within or between crystals. These arrangements result in changes in the size, number, and shape of crystals and often occur during storage over days, weeks, or years. Such recrystallization is usually detrimental to product quality. Processing conditions can be manipulated to avoid or delay recrystallization. Recrystallization can occur by several mechanisms including Ostwald ripening and polymorphic transformation that have been described in detail by Hartel [8]. In lipids, the most common recrystallization process is a change in polymorphism that will be described in detail in the next section.

II. CRYSTALLIZATION AND PHYSICAL PROPERTIES OF FATS

A. Lipid Classification

The Merriam–Webster dictionary defines *lipids* as "any of various substances that are soluble in nonpolar organic solvents (as chloroform and ether), that are usually insoluble in water, that with proteins and carbohydrates constitute the principal structural components of living cells, and that

include fats, waxes, phosphatides, cerebrosides, and related and derived compounds." It is evident from this definition that the word lipids include a wide range of molecular entities that differ in their chemical structure but share solubility properties. However, the term "fat" or "fats and oils" usually refers to those edible lipids that are mainly composed of TAGs.

In general, edible lipids or fats are not composed of a single TAG species but contain several hundred TAGs that provide particular crystallization behavior generating a crystalline network with specific characteristics. Chemically, TAGs have a glycerol backbone with three fatty acids esterified to the hydroxyl groups. The type of fatty acid attached to the glycerol molecule defines the physical properties of the TAG such as melting point. Fatty acids can be divided into saturated and unsaturated fatty acids. Saturated fatty acids are saturated with hydrogen atoms and have no double bonds between individual carbon atoms, while unsaturated fatty acids have one or more double bonds between carbon atoms. Hydrogen atoms in the double bonds of fatty acids usually exist in a *cis* configuration, while only few *trans* fatty acids exist in nature. The number of carbons and type and degree of unsaturation in fatty acids define their melting points. Fatty acids melting points increase as carbon chain length increases and decrease as the degree of unsaturation increases. For example, lauric acid is a fully saturated fatty acid having 12 carbon atoms and has a melting point of 44.8°C, while stearic acid, also a fully saturated fatty acid, with 18 carbon atoms has a melting point of 70.1°C. The addition of one double bond to stearic acid results in oleic acid having a melting point of only 16.0°C. In addition, the configuration of the double bond affects the melting point of the fatty acid. Elaidic acid, the *trans* isomer of oleic acid has a melting point of 44.0°C. Table 11.1 shows the most common fatty acids present in edible lipids and their melting points. The type and proportion of fatty acids attached to the glycerol molecule determine the melting point of the lipid and its physical properties. For example, lipids with high content of long saturated fatty acids are solid at room temperature and are generally harder than those with higher contents of unsaturated fatty acids that are liquid at room temperature.

The melting point (T_f) of lipids is determined by their chemical composition. Since supercooling ($T_f - T$) is directly proportional to the driving force for crystallization in lipids, melting points are one of the main factors that influence lipid crystallization. For example, for a constant temperature (T), a lipid sample with a higher melting point will crystallize faster compared to a sample with a lower melting point. However, lipid crystallization and therefore the physical properties of the crystalline material formed can also be affected by processing conditions. This means that a wide

TABLE 11.1
Common Fatty Acids Found in Edible Fats and Oils and Their Melting Point

Common Name	CN:DB	Melting Point (°C)
Capric acid	10:0	31.6
Lauric acid	12:0	44.8
Myristic acid	14:0	54.4
Palmitic acid	16:0	62.9
Stearic acid	18:0	70.1
Oleic acid	18:1	16
Linoleic acid	18:2	−6.5
Linolenic acid	18:3	−12.8
Arachidic acid	20:0	76.1
Elaidic acid	18:1*t*	44

Abbreviations: CN, carbon number; DB, number of double bonds or unsaturations; *t*, *trans* unsaturation.

range of physical properties can be obtained for a specific lipid using various processing conditions. Physical properties important to the food producer will be described in the following sections and include polymorphism, SFC, melting behavior, hardness, and elasticity.

B. Triacylglycerol Crystal Packing Structure: Polymorphism

The formation of a crystalline network involves an ordered organization of molecules. In the case of fats, these molecules are TAGs. When subjected to a thermodynamic force, TAGs orient themselves to form a crystalline lattice. Depending on chemical composition and on processing conditions, TAGs can crystallize in three structural arrangements: hexagonal, orthorhombic, and triclinic. This ability of TAGs to crystallize in different crystalline lattices is called polymorphism and each crystalline lattice is referred to as a different polymorphic form. Over the years, specific names have been assigned to the different polymorphic forms and their physical characteristics have been quantified. The most common polymorphic forms found in fats are called α, β′, and β in increasing order of stability [15]. Table 11.2 shows a summary of the physical properties and the unit cells of the crystal lattice associated with each polymorphic form. These unit cells represent the cross-sectional packing of the TAG aliphatic chain that is usually referred to as lateral packing. Figure 11.2 shows an excellent representation of typical lateral packing found in TAGs where the

TABLE 11.2
Common Characteristics of Polymorphic Forms in Fats and Oils

Name	Unit Cell	Stability	Density	Melting Point	X-Ray Diffraction Spacing (Å)
α	Hexagonal	Least stable	Lowest	Lowest	4.15
β′	Orthorhombic	Metastable	Intermediate	Intermediate	3.8 and 4.2
β	Triclinic	Most stable	Highest	Highest	4.6

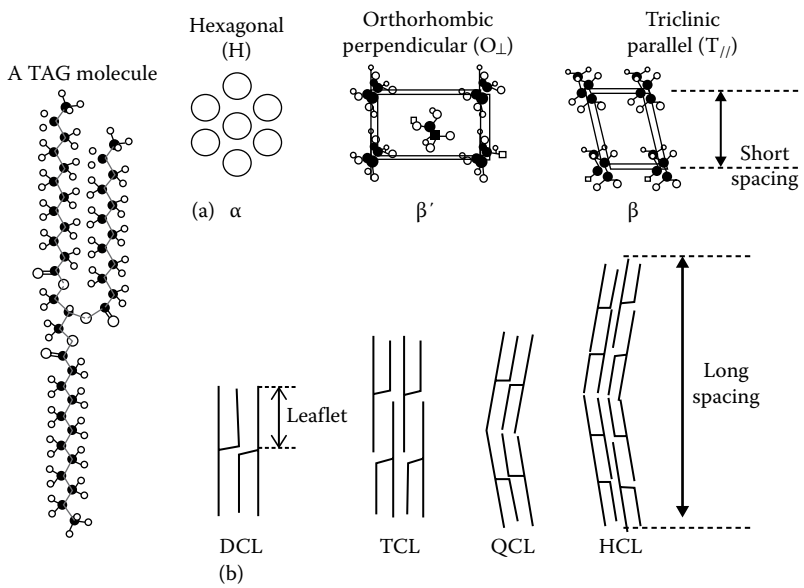

FIGURE 11.2 Lateral (a) and longitudinal (b) packing in TAGs. (Reprinted from *Curr. Opin. Colloid Interface Sci.*, 16, Sato, K. and Ueno, S., Crystallization, transformation and microstructures of polymorphic fats in colloidal dispersion states, 384. Copyright 2011, with permission from Elsevier.)

black circles represent carbon atoms and the white circles represent hydrogen atoms. TAGs can also pack longitudinally to form double, triple, quadruple, or even hexa structures. These structures are also shown in Figure 11.2 [9]. In general, double-chain structures are formed within TAGs with similar fatty acid moieties in the three positions of the glycerol molecule as, for example, in tricaprin [10], while triple-chain structures are formed when different types of fatty acids are esterified in the TAG molecule [9,11]. The type of lateral and longitudinal packing in TAGs can be quantified using x-ray diffraction (XRD). XRD is a nondestructive technique where an x-ray beam is directed toward a crystalline sample and diffracted by atoms or molecules that form the crystalline matrix. Atomic planes or layers in the material generate constructive and destructive interference of the diffracted x-rays as described by Bragg's law:

$$2d \sin \theta = n\lambda \tag{11.9}$$

where

d is the interplanar spacing in the crystal
θ is the Bragg's angle (incidence and reflection angle)
n is the order of interference (n = 1, 2, 3, etc., usually 1)
λ is the x-ray wavelength

In a typical XRD experiment, different Bragg's incidence angles (θ) are scanned through the sample and diffracted x-rays are detected and recorded as a function of the Bragg's angle. The position, intensity, and shape of the peaks in a diffractogram are used to identify specific polymorphic forms. Peaks obtained in the small-angle range (0.07°–5°) are used to identify TAGs' longitudinal packing, while diffraction peaks obtained at wide angles (5°–35°) are used to identify TAGs' lateral packing [12]. Typical wide angles for the α, β', and β polymorphs are shown in Table 11.2. A detailed description of XRD operation was presented by Peyronel and Campos [13].

As previously described, the nucleation step during lipid crystallization plays an important role in the overall crystallization behavior: a specific energy barrier must be overcome to allow for a nucleus to form. Each polymorphic form described earlier has a specific activation free energy of nucleation that must be met for a nucleus to form. Figure 11.3 shows a typical energy diagram for the α, β', and β polymorphs [4]. This diagram shows that the energy barrier to form an α polymorph is lower than that required to form a β' polymorph followed by that needed to form a β polymorph. However, the final energy of each polymorph decreases from α to β' to β polymorphs. This means that even though the β polymorph has the lowest free energy and therefore is the most stable,

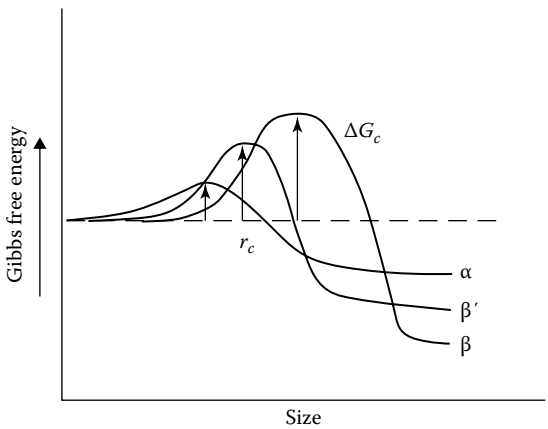

FIGURE 11.3 Activation free energies of nucleation for common polymorphic forms found in fats. (Reprinted with kind permission from Springer Science+Business Media: *Crystallization in Foods, Nucleation*, 2001, pp. 146–191, Hartel, R.W.)

the α polymorph is in general formed first even if the sample has enough thermodynamic driving force to crystallize in the three different polymorphisms. In general, the α polymorph is first formed during crystallization due to the low activation free energy of nucleation (Figure 11.3) and eventually transforms into a more stable polymorphic form (β′ or β) of lower energy.

Depending on their TAG composition, fats are likely to crystallize in specific polymorphic forms. For example, typical β′-forming fats include milk fat, palm oil, cottonseed oil, coconut oil, palm kernel oil, menhaden oil, rapeseed oil, and tallow, while typical β-forming fats include cocoa butter, lard, sunflower oil (SFO), soybean oil, and canola oil, to name a few [14,15]. The type of polymorph formed during crystallization defines the physical and functional properties of the material. Therefore, it is vital to study the polymorphic behavior of fats to optimize product quality and shelf life. For example, β′ crystals are required in production of margarines and spreads, since these crystals are small and provide appropriate smoothness and mouthfeel to the product, whereas large β crystals provide an undesirable grainy texture. However, β crystals are the desired polymorph in cocoa butter and confectionery products since this polymorphic form provides desired properties to the product including gloss, snap, flavor release, and mouthfeel. Cocoa butter, usually present in confectionery products, is unique because it crystallizes in six polymorphic forms, sub-α (or γ), α, β'_2, β'_1, β_2, β_1, in the order of increasing stability. These polymorphic forms are also referred to as forms I, II, III, IV, V, and VI [16]. During chocolate manufacture, crystallization in the most stable polymorphic form, form VI, is desired; however, this form cannot be obtained from the melt and the most stable polymorphic form that can be obtained is form V. If a less stable polymorphic form is obtained, it will gradually transform into a more stable polymorph and result in fat bloom. Crystallization of form V in cocoa butter–containing products is achieved by carefully controlling crystallization conditions using a process usually referred to as "tempering." According to Talbot [16], conventional tempering consists of four steps: (1) complete melting of the crystals at 50°C, (2) cooling to the point of crystallization at 32°C, (3) continuing crystallization at 27°C, and (4) melting unstable polymorphs at 30°C–32°C. Well-tempered cocoa butter crystallizes in form V and melts at 33.8°C just below human body temperature. Over-tempered CB has a melting point above 34°C and feels waxy in the mouth due to residual fat crystals at body temperature [17]. A more detailed description of the tempering process of cocoa butter can be found in Talbot [16].

C. Phase Behavior

As previously mentioned, fats are formed by many TAG molecules that have specific melting points and therefore particular crystallization behavior. This heterogeneous chemical composition results in very complex crystallization behavior. TAGs present in fats and oils can be fully compatible and form solid solutions during crystallization or they can be incompatible and partially co-crystallize. The crystallization behavior of fats and the interaction between TAGs that form them can be evaluated using phase diagrams. Phase diagrams represent temperature and concentration conditions in a binary mixture under which two phases (solid and liquid, in this case) are in equilibrium. Detailed discussion of phase diagrams can be found elsewhere [2,11]. In general, TAGs with similar chain length and degree of saturation form solid solutions. These TAGs include StStSt/EStSt and POSt/StOSt (P, palmitic acid; O, oleic acid; St, stearic acid; E, elaidic acid). Some degree of incompatibility can be observed in certain TAGs and these will crystallize forming eutectics, monotectics, or peritectics. For example, TAGs with similar melting points form eutectics where the melting point of the mixture is lower than the melting point of each pure component. Binary mixtures such as PPP/StStSt, EEE/StOSt, POSt/PStO, PPP/LLL, and PPP/StOSt (L: linoleic acid) fall into this category. When the pure components that form the binary system have differing melting points, they usually show monotectic behavior. Binary blends of TAGs such as StStSt/OOO, StStSt/LLL, PPP/POP, and StStSt/StOSt show this behavior.

Most research studies involving phase diagrams have been conducted with binary mixtures of pure TAGs. Phase equilibrium has also been studied in binary blends of edible lipids consisting of

several hundred TAGs instead of pure TAGs [18,19]. These pseudo-phase diagrams have been used to evaluate the compatibility of confectionery fats such as cocoa butter, milk fat, and cocoa butter replacers (Figure 11.4a). Perhaps, iso-solid diagrams are even more used to study the compatibility of fats. These diagrams show equilibrium temperatures needed to achieve a specific SFC in different blends of fats. They were first used to evaluate the compatibility of milk fat and cocoa butter [18]. Figure 11.4b shows this type of diagram obtained for milk fat and cocoa butter blends. This figure shows an evident softening of the sample when milk is added to cocoa butter. Iso-solid diagrams have been used extensively in the confectionery industry to test compatibility between cocoa butter and different cocoa butter alternatives [20–26]. Similarly, SFC values can be plotted as a function of the composition of the binary blend as reported by Shukla [27]. The combined use of pseudo-phase diagrams, iso-solid, and SFC diagrams are helpful for evaluating the compatibility of fats and determining how mixing two fats can affect the quality of the final product, mainly in terms of texture.

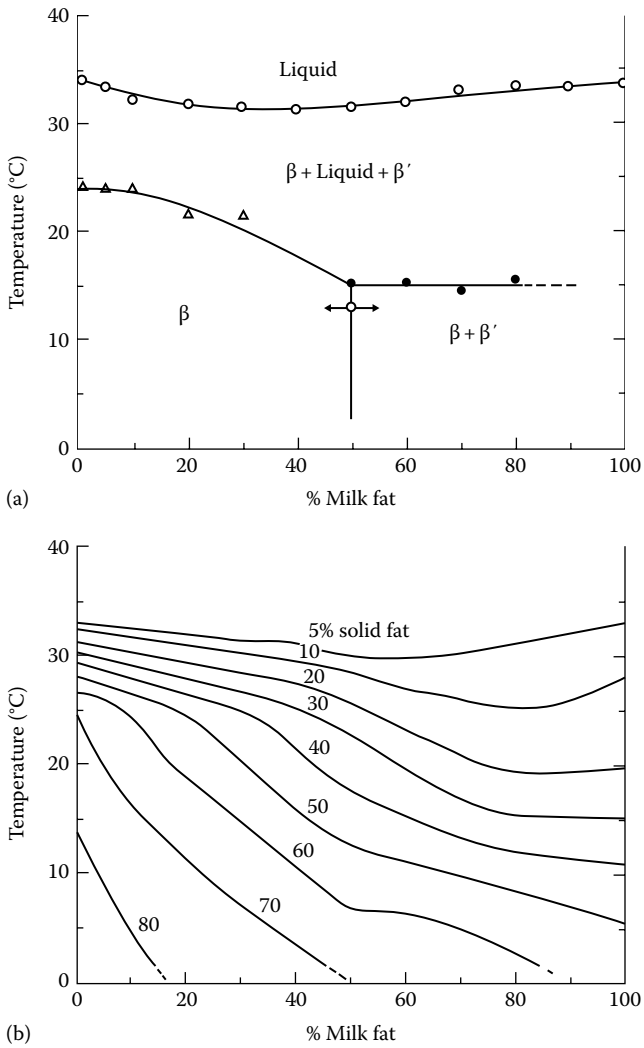

FIGURE 11.4 (a) Pseudo-phase diagram of milk fat and cocoa butter blends. (b) Iso-solid diagrams of milk fat and cocoa butter blends. (Reprinted from *Lebensm. Wiss. Technol.*, 13, Timms, R.E., The phase behavior of mixtures of cocoa butter and milk fat, 61, Copyright 1980, with permission from Elsevier.)

D. Solid Fat Content

When an edible fat is placed at a specific temperature, high-melting-point TAGs crystallize, while low-melting-point TAGs remain in the liquid state. As crystallization temperature decreases, an increasing number of TAGs undergo phase transition and contribute to the solid phase of the system. The amount of solid material obtained therefore depends not only on the crystallization temperature but also on other processing factors that will be described later in this chapter. The amount of solid material is quantified by pulsed nuclear magnetic resonance as the SFC. This parameter measures the ratio of solid to liquid fat in the system. Two methods can be used to measure SFC: (1) the direct method and (2) the indirect method. The *direct method* (AOCS Cd 16b-93) quantifies the relative proportions of hydrogen nuclei in the solid and liquid phase of the material while the *indirect method* (AOCS Cd 16-81) quantifies hydrogen nuclei in the liquid phase of the sample and compares it with the one obtained in the melted sample.

Measurement of SFC is usually included in crystallization experiments since it provides information of the amount of solid material in the sample and it can be correlated with its hardness for a single polymorphic form.

SFC is frequently used to follow the extent of crystallization and quantify crystallization kinetics. The increase in SFC can be fitted with the Avrami equation:

$$\frac{SFC_{(t)}}{SFC_{(\infty)}} = 1 - \exp(-kt^n) \tag{11.10}$$

where
k is the rate constant
$SFC_{(t)}$ is the SFC at a specific time
$SFC_{(\infty)}$ is the SFC at equilibrium
n is the index of the reaction
t is the time

The values of k represent the rate of crystallization and therefore higher k values indicate a faster crystallization. The mechanism of nucleation is quantified by n values. For example, n value of 4 indicates heterogeneous nucleation, 3 is spherulitic growth, 2 is high nucleation rate and platelike growth. Equation 11.10 is usually used to fit SFC when the values increase as a function of time in a single-step sigmoidal manner. Detailed discussion of the use of the Avrami equation and other models to follow lipid crystallization can be found elsewhere [28].

The effect of processing conditions including crystallization temperature, cooling rate, and agitation on SFC values will be discussed later in this chapter.

E. Melting Behavior

Once a crystalline network is formed, it is important to characterize its melting behavior. This physical property is responsible for providing adequate mouthfeel and flavor release during consumption. The melting behavior of a fat can be quantified by two techniques: (1) SFC measurements as a function of temperature and (2) differential scanning calorimetry (DSC). The decrease in SFC values with increasing temperatures can be measured using pulsed nuclear magnetic resonance as discussed earlier. Processing conditions can affect this melting behavior and will be discussed later in this chapter. DSC is a thermal technique that records the heat flow associated with endothermic or exothermic events including phase transitions. The technique is widely used to characterize crystallization and melting behavior of lipids [13,29,30]. Exothermic and endothermic processes are recorded as a change in heat flow in the DSC. Therefore, when a lipid crystalline network is heated or cooled, an endo- or an exotherm, respectively, can be detected as a peak in the heat flow baseline. Peak (T_p) and onset (T_{on}) temperatures can be used to quantify these phase transitions. Onset temperatures are defined as the

temperature at which the sample starts to crystallize or melt, while peak temperatures are defined as temperatures at which the crystallization or melting peak reaches its maximum. In addition, crystallization or melting enthalpy can be quantified. Enthalpy values measure the amount of heat released or absorbed during the phase transition and it is expressed in J/g. Therefore, melting enthalpies can be used to quantify the amount of solid material in a system of defined TAG chemical composition assuming that a single polymorph is present. Enthalpy values can also be used to evaluate the melting behavior of the sample by quantifying the amount of solid remaining at a specific temperature. The melting profile is obtained by plotting percentage of solids obtained from the melting enthalpy values as a function of temperature [25,31–33]. In addition, T_{on} and T_p values can be used to construct phase diagrams and evaluate the degree of compatibility between TAGs.

F. Crystal Morphology

Crystal size and shape are important physical properties of a lipid crystalline network since they affect the quality of the final product. Different crystal sizes are required for specific applications. For example, small crystals are desired for formulating food spreads and margarines, while large crystals are necessary for fractionating edible oils [34]. Crystal size can be modified easily using various processing conditions. In general, fast crystallization rates generate many small crystals, while slow crystallization generates fewer and bigger crystals. Common processing conditions that affect crystal size include crystallization temperature, agitation, cooling rate, acoustic waves, and the use of additives such as emulsifiers.

G. Texture and Viscoelasticity

Many food applications require the use of fats with specific texture or hardness. Fats that remain hard and solid over a wide range of temperatures are called plastic fats and are useful for bakery and roll-in applications, while softer fats are usually preferred in margarines and spreads [14]. Fat hardness is commonly measured using a texture analyzer and it is expressed as the force required to deform the material. The hardness of a fat crystalline network is defined by (1) the amount of solid material, (2) polymorphism, and (3) crystal size. Lipid hardness can be controlled by blending fats from different sources [35] and by changing processing conditions such as cooling rate, crystallization temperature, agitation, and additives.

In many cases, hardness measurements are complemented with viscoelastic measurements where the elastic or storage modulus (G') and the viscous or loss modulus (G'') are used to quantify the solid-like and liquid-like behavior. Such measurements provide information of internal structure of the material since they are performed under small deformation conditions where the material is put under stress without breaking its internal structure. Viscoelastic measurements can be very helpful in measuring the elasticity of soft samples that cannot be measured using a texture analyzer.

III. EFFECT OF PROCESSING CONDITIONS ON CRYSTALLIZATION BEHAVIOR OF FATS

The crystallization behavior of fats and the physical properties of the crystalline network obtained are affected by processing conditions such as crystallization temperature, cooling rate, agitation, and the use of additives. The following sections discuss the effect that these processing conditions have on the crystallization behavior of fats as a single process parameter or in combination.

A. Crystallization Temperature

Crystallization temperature is one of the most important processing parameters that determine the crystallization behavior of lipids. The temperature of crystallization is usually denoted as T_c that

is typically the temperature at which a particular lipid crystallizes. If a lipid sample is placed at a temperature above its melting point (T_f), the fat will not crystallize; however, if the sample is placed at a temperature below T_f, the lipid crystallizes. As mentioned, $T_f - T_c$ is usually referred to as supercooling or ΔT and is directly proportional to the driving force for crystallization. A high ΔT value promotes rapid crystallization while slower crystallization occurs at low ΔT. In addition, samples crystallize sooner when crystallized at high ΔT. The time needed to induce the crystallization of a lipid sample can be quantified by measuring induction times of crystallization. This parameter can also be used to quantify the nucleation rate of the fat crystallized under different conditions as previously described [36]. This parameter is commonly used when the fat is crystallized under isothermal conditions and is defined as the time interval between the moment the sample reaches T_c and the moment when crystals first appear. If this parameter is used to measure non-isothermal crystallization, then the induction time of crystallization is measured from the moment the sample is placed at T_c. This distinction is important since in the latter case the induction time includes a non-isothermal step where the sample's temperature decreases from approximately 50°C–70°C to T_c.

Changes observed in induction times of crystallization caused by different crystallization temperature (T_c) have been reported in several studies [6,37–42]. These studies show that when all other processing conditions remain constant, a high supercooling (low T_c) results in a low induction time, while lower supercoolings (high T_c) result in longer induction times. Figure 11.5 shows induction times of crystallization obtained for a high-melting fraction (HMF) of milk fat and its blends with SFO. It is evident from this figure that the induction times of crystallization increase with temperature. This means that it takes longer for the fat to crystallize at high temperature due to the low supercooling, which is the driving force for crystallization. These authors also showed that induction times increased with the addition of SFO. This is an expected result since T_f decreases with the addition of SFO, and therefore, a lower supercooling is achieved for the same T_c [6]. Crystallization temperature also impacts the number and type of crystals formed in lipids. Many studies have shown that low T_c induces a fast nucleation with the formation of many small crystals [31,32,43–45].

As discussed in a previous section, the crystallization of a lipid system continues during storage where recrystallization occurs until the crystalline network achieves its lowest energy. This recrystallization and molecular reorganization become more important if the temperature fluctuates

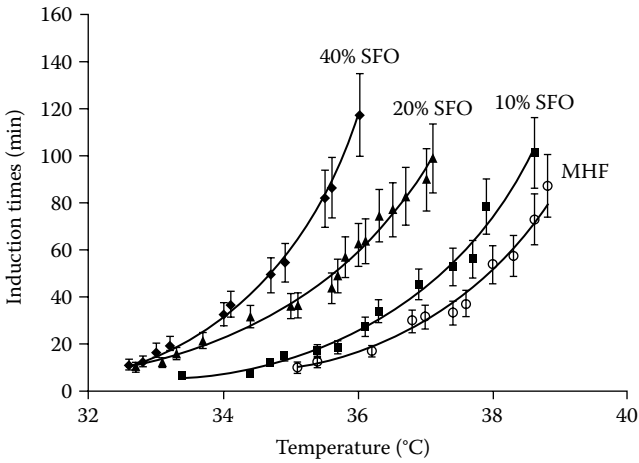

FIGURE 11.5 Induction times of crystallization as affected by crystallization temperature for an HMF of milk fat and its blends with SFO. (Reprinted with permission from Martini, S., Herrera, M.L., and Hartel, R.W., Effect of cooling rate on nucleation behavior of milk fat-sunflower oil blends, *J. Agric. Food Chem.*, 49, 3223. Copyright 2001 American Chemical Society.)

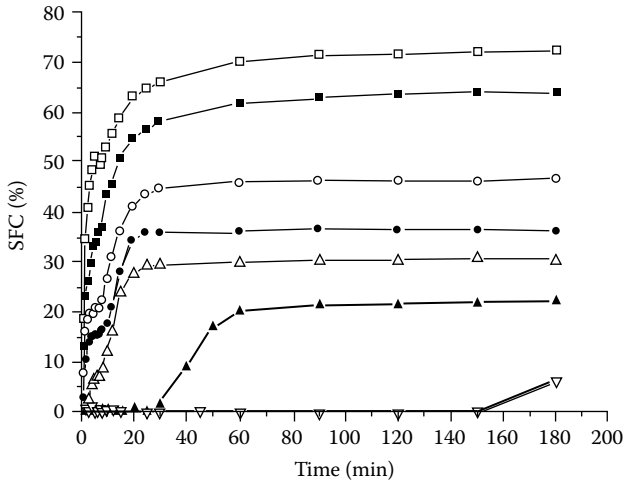

FIGURE 11.6 SFC obtained for an HMF of milk fat at different crystallization temperatures (T_c = 5°C, 10°C, 15°C, 20°C, 25°C, 30°C, and 35°C). (Reprinted with kind permission from Springer Science+Business Media: *J. Am. Oil Chem. Soc.*, Effect of cooling rate on crystallization behavior of milk fat fraction/sunflower oil blends, 79, 2002, 1055, Martini, S., Herrera, M.L., and Hartel, R.W.)

during storage or transportation [46–49]. Recrystallization cannot be underestimated, especially when working with fats that are highly polymorphic.

The effect of T_c on the type of polymorph obtained was discussed earlier. In general, high supercoolings induce the formation of the least stable polymorph (α) that transforms into a more stable polymorph such as β′ and β. Crystallization temperatures must be controlled if a specific polymorphic form must be obtained as in the case of tempering of cocoa butter. Several studies have used similar tempering techniques to obtain specific polymorphic forms in SOS [9] and in stearin fractions of high stearic high oleic SFO [48]. Hence, controlling processing conditions such as T_c is vital to obtain a desired polymorphic form in the crystalline network.

Crystallization temperature also affects SFC and texture. Low T_c results in high SFC values and harder materials. Figure 11.6 shows a typical example of the effect of T_c on SFC values. This study shows how T_c affects not only the final SFC or SFC at equilibrium but also the crystallization kinetics. Higher k values and lower n values were reported for samples crystallized at lower T_c suggesting a fast nucleation rate (low n) and a fast crystallization kinetics (high k) [50].

B. Cooling Rate

The cooling rate used in lipid crystallization experiments can affect the type of crystalline polymorphic form obtained, the crystal size, SFC, and melting behavior. Several studies have reported that slow cooling rates induce the formation of the most stable polymorphic form [51,52]. The effect of cooling rate on polymorphism can be explained by understanding kinetic and thermodynamic phenomena. If the cooling rate is fast, the kinetic energy of the system decreases rapidly and molecules do not have time to align properly and form a nucleus in the most stable polymorphic form. If the cooling rate is slow, the kinetic energy of the system changes slowly and therefore molecules have more time to orient and form a crystalline lattice in the most stable polymorphic form [53].

The effect of cooling rate on crystallization kinetics, crystal size, and melting behavior was studied by Herrera's group [6,43,50] in HMF of milk fat and SFO blends. This study showed faster crystallization for samples crystallized using a slow cooling rate (0.1°C/min) and a lower SFC, especially at low T_c (high supercoolings). In addition, larger crystals were obtained at lower cooling rates. Similar results were reported by Herrera and Hartel [54,55] in mixtures of HMF of milk

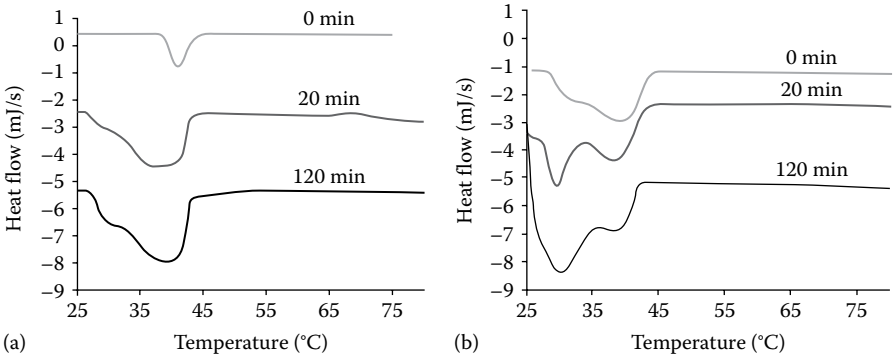

FIGURE 11.7 Melting behavior of an HMF of milk fat crystallized at 25°C under (a) fast (5.5°C/min) and (b) slow (0.1°C/min) cooling rates. Melting behavior was evaluated at different time points during the isothermal crystallization. (Reprinted with kind permission from Springer Science+Business Media: *J. Am. Oil Chem. Soc.*, Effect of cooling rate on crystallization behavior of milk fat fraction/sunflower oil blends, 79, 2002, 1055, Martini, S., Herrera, M.L., and Hartel, R.W.)

fat and low-melting fraction of milk fat. The melting behavior of the crystalline network was also affected by cooling rate. Broader and more fractionated melting peaks were observed for samples crystallized under slow cooling conditions. This difference in the melting behavior can be attributed to the different packing of the TAG during crystallization. Similar to the discussion presented for polymorphism, when samples are crystallized under slow cooling rates, TAGs have more time to arrange themselves in the crystalline lattice and achieve the most stable conformation. Therefore, TAGs with similar structural characteristics such as chain length, degree of unsaturation, and melting point will co-crystallize. This co-crystallization of molecular entities with similar characteristics is represented by a shoulder or a fractionated melting profile in the DSC [50,53–55]. Figure 11.7 shows the melting behavior of fast- and slow-cooled samples [43] at two time points during the crystallization process. Fractionation is clearly observed for the slow-cooled samples.

Cooling rate also affects the hardness and viscoelasticity of the sample. Samples crystallized under slow cooling rates were slightly more elastic than those crystallized at fast cooling rates [56]. The relationship between cooling rate and hardness and viscoelastic properties is not a simple one since these values will be affected by a combination of several other parameters such as SFC, crystal size, and polymorphism.

C. Agitation

Several studies have reported a decrease in crystal size [43,54,55,57–60] and induction time of crystallization [59]; an increase in the number of crystals, crystallization rate, and SFC [57,59]; and a decrease in viscoelasticity [55,56] and texture [57] with agitation. Generation of different polymorphic forms as a function of agitation was also reported. Martini et al. [57] and Shi and Maleky [60] reported more stable polymorphic forms when samples were crystallized under dynamic conditions, while Reyes-Henandez et al. [58] reported that higher shear rates result in less stable polymorphic form and a harder sample. It is very likely that these differences in results are due to the different experimental design (agitation vs. shear). The effect of shear on polymorphic behavior of lipids has been summarized by Sato and Ueno [9] who reported a faster polymorphic transformation in cocoa butter crystallized under shear. Reyes-Hernandez et al. [58] also reported a broader and more fractionated melting peak in samples crystallized under low shear rates.

Some studies suggest that shear affects secondary nucleation [4,61]. Agitation leads to interparticle collisions leading to an increase in crystal growth due to the formation of more secondary nuclei. However, agitation also increases mass transfer and might induce primary nucleation. Sometimes,

an increase in mass transfer is not desired especially during fractionation of fats such as in palm oil fractionation. In this specific application, the crystallization process is controlled to obtain specific fractions. For example, after complete melting of the fats, the sample is cooled slowly with gentle or no agitation to encourage the development of large crystals that leads to separation of fractions with particular physical and chemical compositions [62].

D. Use of Additives

The presence of additives can either promote or inhibit crystallization in a fat sample. The effect that additives have depends on their structure and on the chemical composition of the fat. Smith et al. [63] provide an excellent and detailed review of the effect of additives such as mono- and diacylglycerols, phospholipids, and emulsifiers such as Tween and sucrose esters on fat crystallization. The common characteristic among these additives is their chemical conformation: all these molecules have fatty acid moiety or other hydrophobic residue [63]. In general, it is accepted that the effect of an additive toward changing fat crystallization depends on its structure. If the additive has a similar structure to the TAG in the crystallizing fat, the additive will be incorporated in the crystalline lattice and therefore will delay nucleation and crystal growth. However, if the additive has a very different structure and melting point, it might crystallize before the fat acting as nuclei and inducing nucleation.

Sato's group [64,65] recently showed the promotional effect of new types of additives on fat crystallization including inorganic additives such as talc, carbon nanotube, and graphite and organic additives including theobromine, ellagic acid dihydrate, and terephthalic acid. Similar promotional effects were described by Kerr et al. [41] and Martini and Herrera [46] when using waxes in anhydrous milk fat. The studies by Sato's group [64,65] showed that the additives not only promoted the onset of crystallization but also induced the formation of the most stable polymorphic form. In addition, these authors showed that the crystallization of the fat was initiated at the surface of the additive and that hydrophobic and hydrophilic interactions between the fats and the additives are responsible for this behavior.

Emulsifiers are by far the most commonly used additives and their effect on crystallization behavior has been studied by several researchers. Examples of these studies are summarized in the work by Smith et al. [63]. These authors also provide an excellent summary (Figure 11.8) of the mechanism by which additives, including emulsifiers, might function as a promoter or inhibitor of the crystallization process. In summary, as previously mentioned, the use of additives can affect either nucleation, growth of crystals, or both processes. The effects of additives on crystallization behavior are measured with respect to nucleation time, shift in nucleation temperature, and change in the number of nuclei formed among others. In many cases, the components will not affect the total amount of solid phase when the system has reached equilibrium, however, it can influence the rate of reaching equilibrium [63]. However, it is important to note that the effect of additives might also be affected by other processing conditions such as crystallization temperature [66] and cooling rate [63]. Herrera's group reported an induction in crystallization caused by the sucrose ester S-170 when crystallized at low temperatures and a delay in the crystallization when the same system was crystallized at higher temperatures [66].

E. Other Processing Conditions

In addition to the typical processing conditions described earlier, novel processing tools have been used to influence the crystallization of fats. This section will describe the use of high-intensity ultrasound (HIU) and high pressure.

1. High-Intensity Ultrasound

Recent research has demonstrated that HIU can be used as an additional processing tool to modify the crystallization behavior of fats. Research by Sato's group [67–69] and by Martini et al. [70]

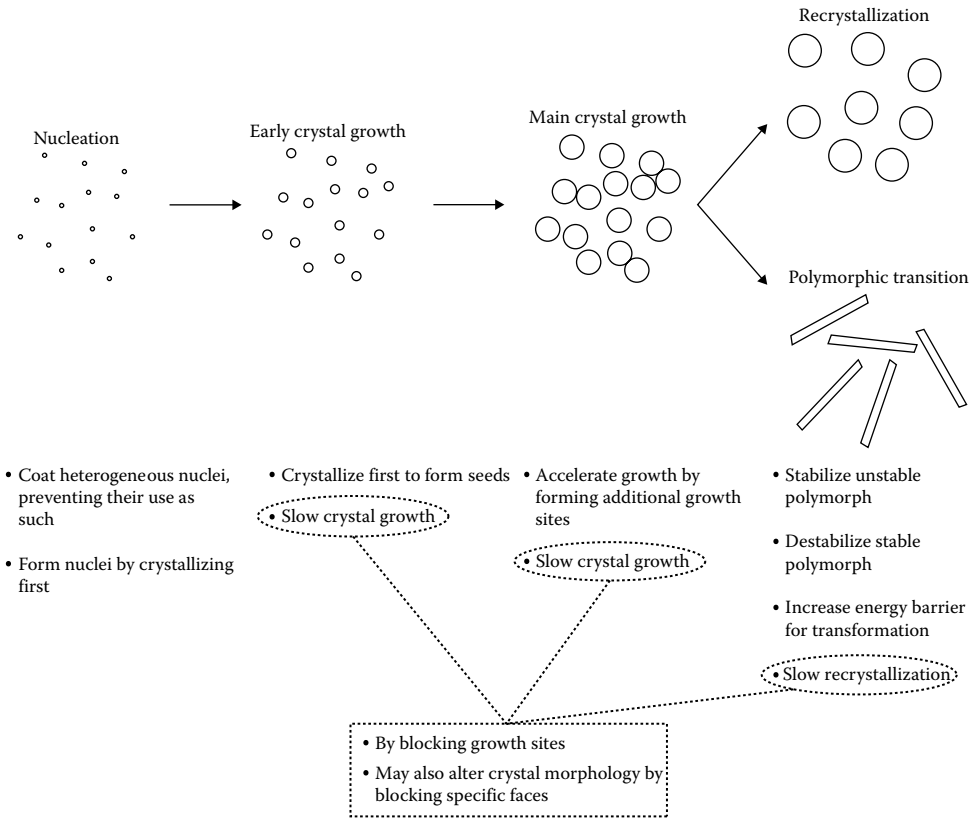

FIGURE 11.8 Mechanism of action of different additives used to change the crystallization behavior of lipids. (Reprinted with kind permission from Springer Science+Business Media: *J. Am. Oil Chem. Soc*., Crystallization of fats: Influence of minor components and additives, 88, 2011, 1085, Smith, K.W., Bhaggan, K., Tablot, G., and Malssen, K.F.)

have shown that HIU can induce crystallization in the most stable polymorphic form. In addition, several studies have reported a reduction in induction times of crystallization [42,71–73] caused by sonication. Perhaps, the most interesting effect of sonication is observed by changes in physical properties of the sonicated fats such as crystal size, hardness, elasticity, and melting behavior. Several studies have reported a significant reduction in crystal size in sonicated samples, a faster crystallization rate, a harder and more elastic material, and a sharper melting profile [31–33,42,47,57,67–74]. The degree of change in crystallization behavior caused by sonication is affected by sonication conditions such as power level and duration of the ultrasound pulse. For example, Chen et al. [73] showed that power level did not affect induction times of crystallization, but they significantly increased the crystallization rate (Figure 11.9). Similarly, longer sonication pulses generated a greater reduction in induction time but did not affect the crystal growth or the kinetics of crystallization. Sonication efficiency is also affected by other processing conditions such as crystallization temperature. In general, a greater effect on crystallization is observed when sonication is used at high crystallization temperatures (or low supercoolings) as reported by Rincón-Cardona et al. [70] and shown in Figure 11.10 [70,73].

The effect that high-intensity ultrasonic waves has on the crystallization behavior of lipids can be attributed to several factors including (1) generation of stable and inertial cavitation, (2) generation of high shear forces, and (3) generation of localized high temperatures and pressures. However, the

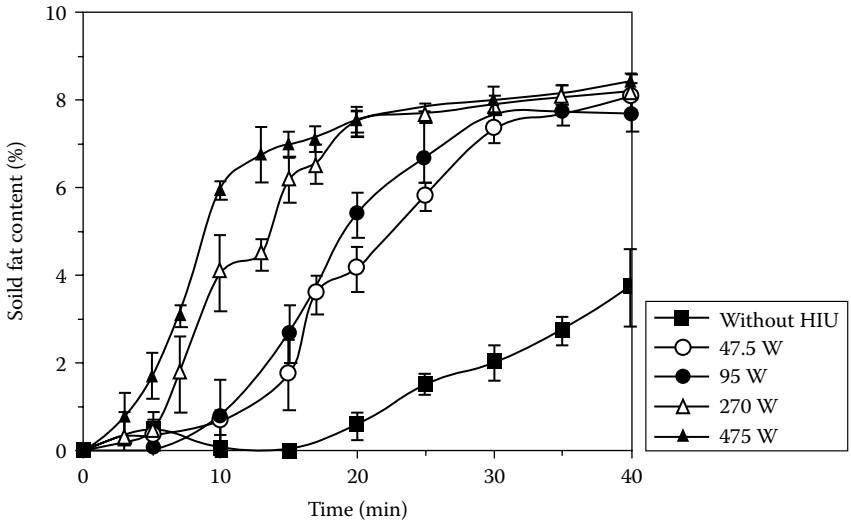

FIGURE 11.9 Effect of HIU power on the crystallization kinetics of palm oil crystallized at 30°C. Sonication was applied for 60 s. (Reprinted with kind permission from Springer Science+Business Media: *J. Am. Oil Chem. Soc.*, Effects of ultrasonic parameters on the crystallization behavior of palm oil, 90, 2013, 941, Chen, F., Zhang, H., Sun, X., Wang, X., and Xu, X.)

exact mechanism responsible for the effects observed during sonocrystallization of lipids is still unknown. Further research must be performed in this area to evaluate the effects of other processing conditions such as agitation and cooling rate on ultrasound-induced crystallization. A detailed summary of the effect of sonication on the crystallization behavior of different lipids can be found in Martini [74].

2. High-Pressure Crystallization

Few studies [75,76] have reported crystallization of lipids at high pressures. These studies show that high pressures induce crystallization, reduce crystal size, and shorten induction time of crystallization while promoting crystal growth. The effects observed are likely due to an increase in the melting temperature of the fat due to the high pressures that will ultimately result in a higher supercooling and higher driving force for crystallization [51]. More research is needed in this area to explore the effects of pressure on other physical properties of the fat such as texture, SFC, and polymorphism.

IV. CONCLUSION

Lipids are important components in foods since they provide mouthfeel and flavor to foods. They are a good source of calories and essential fatty acids. Lipids are used in various food products in the form of shortenings and liquid oils. The type of shortening or oil used depends on the final product and on the physical properties of the lipid source needed to produce a high-quality food. The physical properties of the shortening are defined by the crystallization behavior of the lipid. It is therefore vital to understand the fundamentals of lipid crystallization and how this process is affected by different processing conditions. Chemical composition of the lipid, crystallization temperature, cooling rate, and agitation are some of the most important process parameters that can be controlled to obtain crystalline networks with different physical properties needed by the food industry.

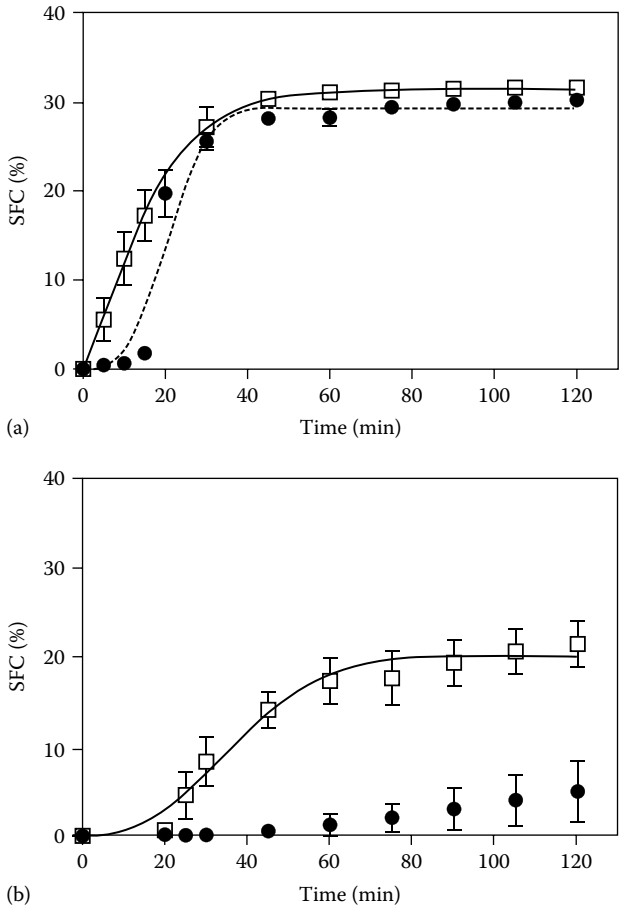

FIGURE 11.10 Effect of HIU on the crystallization behavior of a stearin fraction of high stearic high oleic SFO crystallized at different temperatures, (a) 17°C and (b) 21°C. Open squares represent data obtained with the use of HIU, and closed circles represent data obtained without the use of HIU. (Reprinted with kind permission from Springer Science+Business Media: *J. Am. Oil Chem. Soc.*, Effect of high intensity ultrasound on physical properties of soft stearins of high stearic high oleic sunflower oil, 92, 2015, 473, Rincón-Cardona, J.A., Agudelo-Laverde, L.M., Herrera, M.L., and Martini, S.)

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12 Chemical Interesterification of Food Lipids
Theory and Practice

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CONTENTS

I. INTRODUCTION

Interesterification, hydrogenation, and fractionation are three processes available to food manufacturers to tailor the physical and chemical properties of food lipids [1,2]. Each operation is based on different principles to attain its goal. Fractionation is a physical separation process based on the crystallization behavior of triacylglycerols (TAGs) [4,5]. Hydrogenation, on the other hand, is a chemical process leading to the saturation of double bonds present in fatty acids to harden fats for use as margarine and shortening base stocks. Interesterification, also a chemical process, causes fatty acid redistribution within and among TAG molecules, which can lead to substantial changes in lipid functionality. This chapter discusses the application of the theory of chemical interesterification to the production of edible fats and oils.

II. LIPID COMPOSITION

The chemical composition of a fat partly dictates its physical and functional properties [6]. The chemical nature of lipids is dependent on fatty acid structure and distribution on the glycerol backbone. Fatty acids vary in chain length and in the number, position, and configuration of double bonds [7]. TAGs composed of saturated fatty acids (e.g., lauric, myristic, palmitic, and stearic) have high melting points and are generally solid at ambient temperature, whereas TAGs consisting of unsaturated (monoene, polyene) fatty acids (e.g., oleic, linoleic, and linolenic) are usually liquid at room temperature. Butterfat, for example, contains ~70% saturated fatty acids, whereas many vegetable oils contain almost exclusively unsaturated fatty acids [4,8].

The fatty acid distribution within naturally occurring TAGs is not random [9,10]. The taxonomic patterns of vegetable oils consist of TAGs obeying the 1,3-random-2-random distribution, with saturated fatty acids being located almost exclusively at the 1,3-positions of TAGs [8,11,12]. Conversely, in animal fats (tallow and lard), saturated fatty acids are mostly at the *sn*-2 position [13].

The industrial applicability of a given fat is limited by its nonrandom distribution, which imparts a given set of physical and chemical properties. The objective of modification strategies, such as chemical interesterification, is the creation from natural fats of TAG species with new and desirable physical, chemical, and functional properties [14].

III. A BRIEF HISTORY

Interesterification reactions have been knowingly performed since the mid-1800s. The first published mention was by Pelouze and Gélis [15]. Duffy [16] performed an alcoholysis reaction between tristearin and ethanol. Later, Friedel and Crafts [17] generated an equilibrium interchange between ethyl benzoate and amyl acetate. They found that heating alone was sufficient to reach an equilibrium interchange and distillation of one product could shift the reaction to completion. Glyceride rearrangement was also reported by Grün [18], Van Loon [19,20], and Barsky [21]. The first publication demonstrating the chemical interesterification of edible lipids was presented by Normann [22]. Chemical interesterification has been industrially viable in the food industry since the 1940s to improve the spreadability and baking properties of lard [23,24]. In the 1970s, there was renewed interest in this process, particularly as a hydrogenation replacement for the manufacture of zero-*trans* margarines. Today, it plays a key role in the production of low-calorie fat replacers, such as Proctor and Gamble's Olestra and Nabisco's Salatrim or Benefat [25,26].

IV. THREE FACES OF INTERESTERIFICATION

Excellent reviews in the area of chemical interesterification include Sreenivasan [1], Rozenaal [11], Kaufmann et al. [27], Going [28], Hustedt [29], Marangoni and Rousseau [30], and Dijkstra et al. [31].

Interesterification can be divided into three classes of reactions: acidolysis, alcoholysis, and transesterification [28,32,33].

Acidolysis involves the reaction of a fatty acid and a TAG. The reactions can produce an equilibrium mixture of reactants and products or can be driven to completion by physically removing one of the reaction products. For example, coconut oil (CO) and stearic acid can be reacted to partially replace the short-chain fatty acids of CO with higher-melting stearic acid [28].

Alcoholysis involves the reaction of a TAG and an alcohol and has several commercial applications, primarily the production of monoacylglycerols (MAGs) and diacylglycerols (DAGs). Alcoholysis must be avoided in the interesterification of food lipids, since MAGs and DAGs are undesirable by-products [28]. Glycerolysis is an alcoholysis reaction in which glycerol acts as the alcohol [32].

Transesterification is the most widely used type of interesterification in the food industry. Hence, we concentrate on this reaction. Figure 12.1 shows the effects of interesterification on the fatty acid distribution of a putative TAG (1-stearoyl-2-oleoyl-3-linoleoyl glycerol) (SOL). In sequence, the ester bonds linking fatty acids to the glycerol backbone are split, then the newly liberated fatty acids are randomly shuffled within a fatty acid pool and reesterified onto a new position, onto either the same glycerol (intraesterification) or another glycerol (interesterification) [1]. For reasons involving thermodynamic considerations, intraesterification occurs at a faster rate than interesterification [34]. Once the reaction has reached equilibrium, a complex, random mixture of TAG species is obtained (Figure 12.1).

The extent of the effects of interesterification on the properties of a fat will depend on the fatty acid and TAG variety of the starting material. If a single starting material (e.g., palm stearin [Pst]) is randomized, the effects will not be as great as if a hardstock is randomized with a vegetable oil [35]. Furthermore, if a material has a quasi-random distribution prior to randomization (e.g., tallow), randomization will not lead to notable modifications.

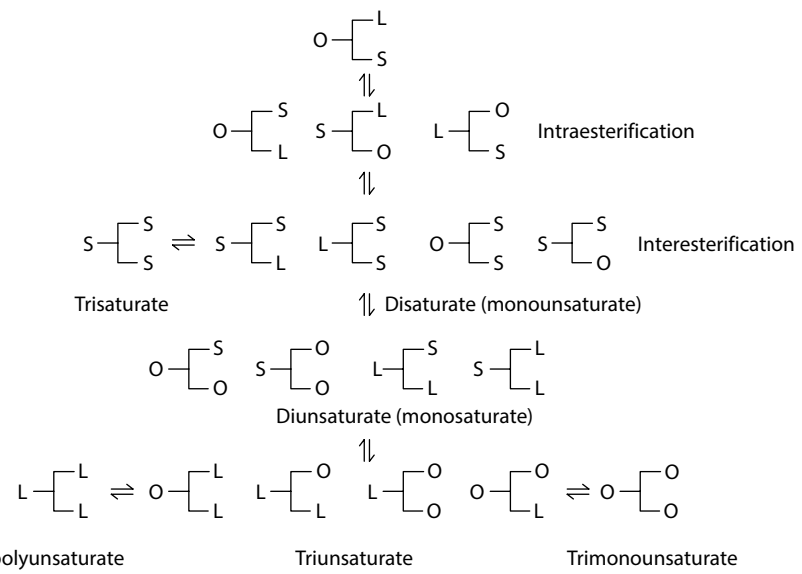

FIGURE 12.1 TAG formation during interesterification. S, stearoyl; O, oleoyl; L, linoleoyl. (Adapted from Sreenivasan, B., *J. Am. Oil Chem. Soc.*, 55, 796, 1978.)

The interesterification reaction consists of three main steps: catalyst activation, ester bond cleavage, and fatty acid interchange. We now examine each subject in detail.

V. INTERESTERIFICATION CATALYSTS

A. Is a Catalyst Necessary for Interesterification?

Interesterification can proceed without a catalyst at high temperatures (~300°C); the desired results are not obtained, however, because equilibrium is slowly attained at such temperatures, and isomerization, polymerization, and decomposition reactions can occur [11,28,36]. In fact, polymerization has been shown to occur at 150°C [37]. Although a higher reaction temperature leads to increased intermolecular ester exchange, intramolecular fatty acid exchange is also promoted at higher temperatures. Willems and Padley [38] described the effect of palm oil physical refining (240°C for 180 min) on the 1,3-dipalmitoyl-2-oleoyl/1,2-dipalmitoyl-3-oleoyl (POP/PPO) TAG ratio. They found that this ratio in palm oil changed from 8 to 6 as a result of intramolecular interesterification, which is undesirable for the production of palm mid fraction.

Addition of a catalyst significantly lowered reaction temperature and duration [39]. Other important considerations include the type and concentration of the catalyst [40].

B. Available Catalysts for Interesterification

There are three groups of catalysts (acids, bases, and their corresponding salts and metals), which can be subdivided into high- and low-temperature groups [27]. High-temperature catalysts include metals salts such as chlorides, carbonates, oxides, nitrates, and acetates of zinc, lead, iron, tin, and cobalt [41]. Others include alkali metal hydroxides of sodium and lithium [42]. Most commonly used are low-temperature catalysts such as alkylates (methylate and ethylate) of sodium and sodium/potassium alloys; however, other bases, acids, and metals are also available [43]. Alkylates of sodium are simple to use and inexpensive, and only small quantities are required. Furthermore, they are active at low temperatures (<50°C). This last characteristic allows their use for directed interesterification [1,44]. Theoretically, addition of 0.2% sodium methoxide leads to the formation of 1.0% soap and 1.0% fatty acid methyl esters (FAMEs) [45].

C. Precautions

Performing a chemical interesterification reaction is a relatively straightforward process. However, a 100% reaction yield is never attainable [46]. Volatile fatty acid alkyl esters, formed in stoichiometric yields with the catalyst during the reaction, must be washed out, and a small amount of partial acylglycerols is always produced [47]. Trace amounts of moisture will inactivate alkylate catalysts by producing the corresponding alcohols. Hence, the fat or oil should contain less than 0.01% (w/w) water [29]. Free fatty acids and peroxides also impair catalyst performance, and levels should be maintained as low as possible, preferably below 0.1% (w/w) and 5 meq/kg oil, respectively. For each 0.1% free fatty acids in oil, an extra 0.02 wt% sodium methoxide should be added to the oil, while for each 5 unit peroxide value, 0.03 wt% extra catalyst should be added to the oil [39,48]. The fat should be well neutralized, dried, and heated (120°C–150°C) under a nitrogen blanket or vacuum before the addition of a catalyst [14]. Finally, sodium alkylates are toxic, highly reactive bases that should be handled with care. Their shelf life is a few months since they are very hygroscopic [11].

With a dry oil devoid of impurities and moisture, only trace amounts of a catalyst [<0.4% (w/w)] are required [14]. Catalyst concentration should be minimized to prevent excessive losses due to saponification [44]. Experience has shown that above 0.4% catalyst, the addition of each additional 0.1% of the catalyst results in the loss of ~1% neutral fat [29]. Konishi et al. [49], however, observed that ester interchange between soybean oil and methyl stearate in hexane was improved by using

10% (w/w) sodium methoxide. It is also necessary to use the catalyst in a form that is easily and completely dispersed [50]. For example, if Na/K catalysts are not finely dispersed in a suitable solvent, a violent reaction with residual moisture may occur at the catalyst surface, followed by splitting of surrounding fat molecules to form a coating of soap. The heat generated by such a reaction is enough to decompose TAGs and cause local charring [28].

D. The "Real" Catalyst

The real catalyst is believed to be a metal derivative of a DAG, and the catalysts mentioned earlier are most likely its precursor [11,44]. Upon catalyst addition to the lipid, a reddish brown color slowly develops (within a few minutes, depending on the application and reaction conditions) in the mixture, indicating the activation of the presumed true catalyst. An alternative mechanism was proposed by Dijkstra et al. [31]. In this mechanism, alpha-hydrogens on carbon centers alpha to the ester bond in fatty acid moieties are abstracted by an alkaline catalyst. The enolate anion that forms acts as the catalytic intermediate. This enolate would abstract a proton from the hydroxyl group of a partial glyceride. This newly formed alcoholate will then attack carbonyl groups of TAGs and start the interesterification reaction.

Some workers time the interesterification reaction from the appearance of the reddish brown color; others simply time the reaction from the moment of catalyst addition. Because it is impossible to predict the reaction onset, it is difficult to obtain partial interesterification. Most reactions are conducted until equilibrium has been reached. Reaction times are longer in industrial settings, because the catalyst must be totally homogenized within the fat [46]. Preactivation is unnecessary if the catalyst is predissolved prior to addition to the substrate [36]. Placek and Holman [51] incorrectly attributed the induction period to the interaction between the catalyst and impurities. Although impurities are sometimes present, the induction period is not strictly due to their presence; rather, it is due to catalyst activation. As stated by Coenen [43] and many others, the activation energy for the catalyst is higher than for the reaction. A preactivation of 15 min has been found to accelerate the reaction itself [49]. Interestingly, Hustedt [29] stated that once the brown intermediate had appeared in the reaction mixture, interesterification was complete. No basis was given for this statement.

E. Reaction Termination

The interesterification reaction is allowed to continue for a predetermined time period and is stopped with addition of water and/or dilute acid. Going [28] described three patents dealing with catalyst removal techniques for minimizing fat loss. Generally, most of the catalyst can be washed out with water to a separate salt, or a soap-rich aqueous phase. Alternatively, a reaction with phosphoric acid results in a solid phosphate salt, which can be filtered out. Both these methods result in substantial fat loss. A technique has been developed that minimizes loss by addition of CO_2 along with water. The system becomes buffered with sodium carbonate at a pH low enough to not split the fat [52]. A new online monitoring patent to control the interesterification process was developed by measuring the color change during the reaction using a fiber-optic spectrometer. By this method, the dosage of sodium methoxide and reaction time could be minimized [53].

VI. REACTION MECHANISMS

The exchange of fatty acids between TAG hydroxyl sites does not occur directly but via a series of alcoholysis reactions involving partial acylglycerols [54]. The proposed mechanisms of chemical interesterification depend on the inherent properties of the TAG ester carbonyl group (C=O). The carbonyl carbon is particularly susceptible to nucleophilic attack because of electronic and steric considerations. The electronegative oxygen pulls electrons away from the carbonyl carbon,

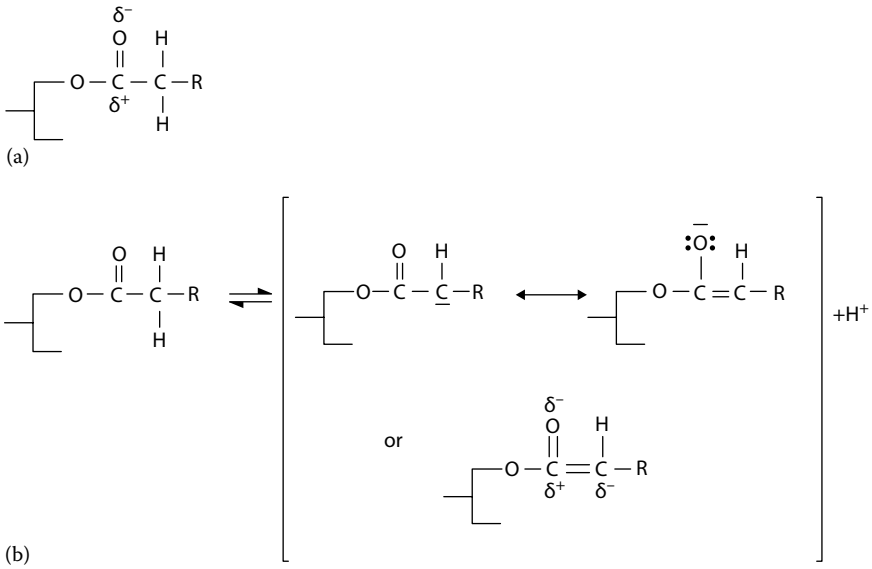

FIGURE 12.2 Carbonyl group properties in TAGs. (a) Increased acidity of the carbon *a* to the carbonyl group due to resonance stabilization of the carbanion. (b) The carbonyl carbon is prone to nucleophilic attack because of the electronegativity of oxygen. (Adapted from Marangoni, A.G. and Rousseau, D., *Trends Food Sci. Technol*., 6, 329, 1995.)

leading to a partial positive charge on the carbon, and also increases the acidity of hydrogens attached to the carbon at a position *a* to the carbonyl group (Figure 12.2).

Steric considerations also come into play. The carbonyl carbon is joined to three other groups by s bonds (sp^2 orbitals); hence, they lie in a flat plane, 120° apart. The remaining p orbital from the carbon overlaps with a p orbital from the oxygen, forming a p bond. This flat plane and the absence of neighboring bulky groups permit easy access for nucleophiles to approach and react with the carbonyl carbon.

The transition state of the reaction is a relatively stable tetrahedral intermediate with a partial negative charge on the oxygen. As the reaction progresses, a group leaves and the structure reverts to the planar carbonyl structure. Strong evidence supports the cleavage of the carbonyl carbon–oxygen bond as the mechanism for the release of the leaving group.

For acid-catalyzed nucleophilic acyl substitution, a hydrogen easily associates with the carbonyl oxygen owing to the polarized nature of the carbonyl function and the presence of free electron pairs on the oxygen, imparting a positive charge to this atom [55]. The carbonyl carbon is then even more susceptible to nucleophilic attack, since oxygen can accept p electrons without gaining a negative charge. Acid-catalyzed interesterification is not discussed further because it is not used for the chemical interesterification of food lipids.

A. Carbonyl Addition Mechanism

In alkaline conditions encountered during interesterification, the catalyst (which is nucleophilic) attacks the slightly positive carbonyl carbon at one of the three fatty acid–glycerol ester bonds and forms a tetrahedral intermediate. The FAME is then released, leaving behind a glycerylate anion (Figure 12.3a). Kinetics of base-catalyzed hydrolysis of esters shows that the reaction is dependent on both ester and base concentration (second-order kinetics). This newly formed glycerylate anion is the nucleophile for subsequent intra- and intermolecular carbonyl carbon attacks, which continue until a thermodynamic equilibrium has been reached (Figure 12.4).

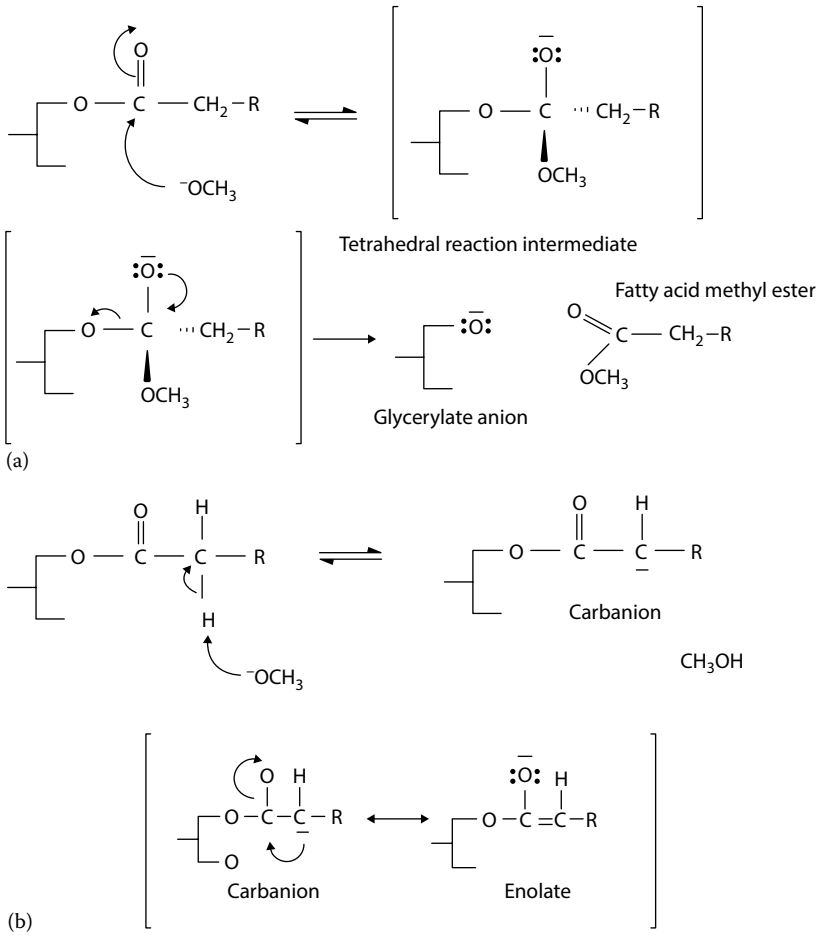

FIGURE 12.3 Proposed reaction mechanisms for chemical interesterification: (a) carbonyl addition and (b) Claisen condensation (enolate formation). (Adapted from Marangoni, A.G. and Rousseau, D., *Trends Food Sci. Technol*., 6, 329, 1995.)

During an attack, a new TAG is not necessarily formed. The transition complex (glycerylate + fatty acid) will decompose, either to regenerate the original species and active catalyst or to form a new TAG and a new active catalyst ion. This process continues until all available fatty acids have exchanged positions and an equilibrium composition of acylglycerol mixture has been achieved [1]. Support for this mechanism was provided by Coenen [43] who presented the kinetics between a simple mixture of S3 and U3. The kinetics were described with six possible reactions between various TAG and DAG anions, with a rate constant 3*k* (Figure 12.5). Not all possible exchanges produced a net change in TAG composition, leading to *2k* and *k* rate constants.

B. Claisen Condensation

In Claisen condensation, sodium methoxide removes an acidic hydrogen from the carbon *a* to the carbonyl carbon, yielding an enolate ester [23]. This reaction produces a carbanion, a powerful nucleophile (Figure 12.3b). This nucleophile will attack carbonyl groups, forming a β-keto ester intermediate and a glycerylate. The glycerylate is now free to attack other carbonyl carbons and exchange esters intra- and intermolecularly (Figure 12.6). Once this carbanion has been created, the same considerations as for the usual carbonyl carbon chemistry apply.

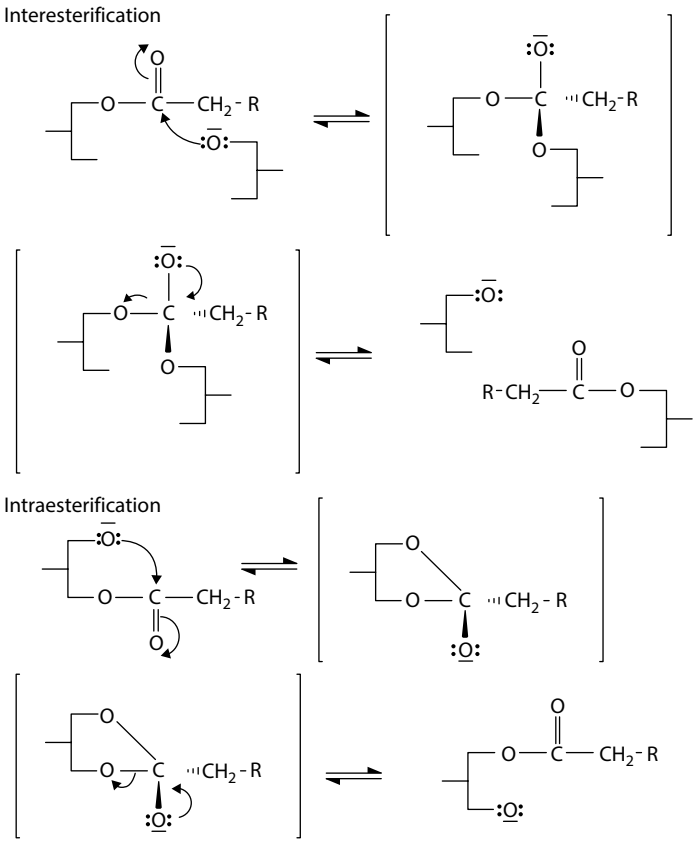

FIGURE 12.4 Reaction mechanisms for the chemical inter- and intraesterification of two triglycerides via the carbonyl addition mechanism. (Adapted from Marangoni, A.G. and Rousseau, D., *Trends Food Sci. Technol*., 6, 329, 1995.)

$$S_3 + U_2ONa \underset{k}{\overset{3k}{\rightleftharpoons}} SU_2 + S_2ONa$$

$$U_3 + S_2ONa \underset{k}{\overset{3k}{\rightleftharpoons}} S_2U + U_2ONa$$

$$SU_2 + U_2ONa \underset{3k}{\overset{2k}{\rightleftharpoons}} U_3 + SUONa$$

$$S_2U + S_2ONa \underset{3k}{\overset{2k}{\rightleftharpoons}} S_3 + SUONa$$

$$S_2U + U_2ONa \underset{k}{\overset{2k}{\rightleftharpoons}} SU_2 + SUONa$$

$$SU_2 + S_2ONa \underset{k}{\overset{2k}{\rightleftharpoons}} S_2U + SUONa$$

FIGURE 12.5 Kinetics of interesterification via the carbonyl reaction mechanism (S, SS, SSS and U, UU, UUU: mono-, di-, and trisaturated and unsaturated, respectively). (Adapted from Coenen, J.W.E., *Rev. Fr. Corps Gras*, 21, 403, 1974.)

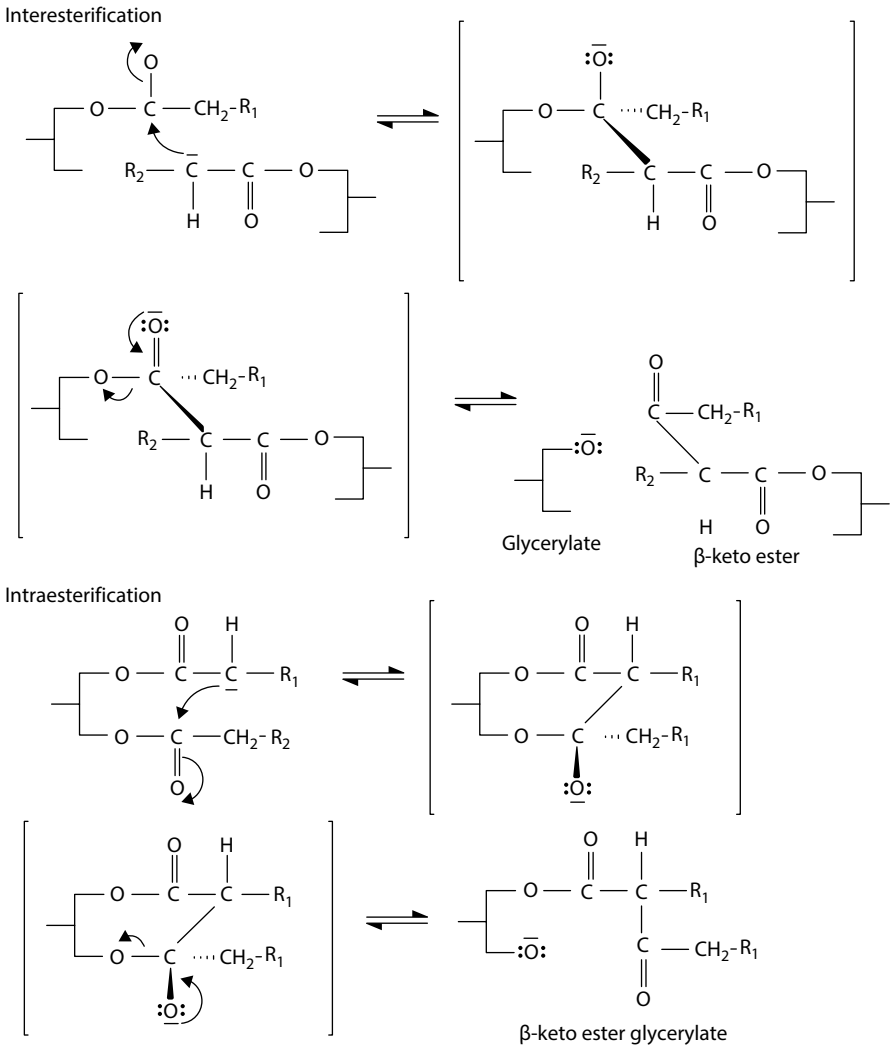

FIGURE 12.6 Reaction mechanism for the chemical inter- and intraesterification of two TAGs via the Claisen condensation mechanism. (Adapted from Marangoni, A.G. and Rousseau, D., *Trends Food Sci. Technol*., 6, 329, 1995.)

VII. RANDOM AND DIRECTED INTERESTERIFICATION

A. Random Interesterification

Interesterification reactions performed at temperatures above the melting point of the highest-melting component in a mixture result in complete randomization of fatty acids among all TAGs according to the laws of probability [28,56].

The energy differences between the various combinations of TAGs are insignificant and do not appear to lead to fatty acid selectivity [57]. Hence, random interesterification is entropically driven (randomization of fatty acids among all possible TAG positions) until an equilibrium is reached [43].

In ester–ester interchange, the fatty acid distribution is theoretically fully randomized, meaning that the resulting TAG structure can be predicted from the overall fatty composition of the mixture (Table 12.1) [11].

TABLE 12.1
Theoretical Triacylglycerol Compositions after Complete Interesterification of *n* Fatty Acids (A, B, C, D, …) with Molar Fractions *a, b, c, d, …*

Type	Quantity	Proportion
Monoacid (AAA, BBB, …)	N	a^3, b^3, …
Diacid (AAB, AAC, …)	$n(n-1)$	$3a^2b$, $3a^2c$, …
Triacid (ABC, DEF, …)	$\frac{n(n-1)(n-2)}{6}$	$6abc$, $6def$, …
Total	$\frac{n(n+1)(n+2)}{6}$	

Source: Adapted from Rozenaal, A., *Inform*, 3, 1232, 1992.

The molar concentrations of fatty acids A, B, and C are shown in Table 12.1 a through c. AAA, AAB, and ABC are TAGs composed of one, two, or three different fatty acids, respectively. For AAB, there are three possible isomers, whereas for ABC there are six. For example, 1-stearoyl-2-oleoyl-3-linoleoyl glycerol results in the following fully randomized equilibrium mixture:

SSS	3.7%
OOO	3.7%
LLL	3.7%
SSO	11.1%
SSL	11.1%
SOO	11.1%
SLL	11.1%
OOL	11.1%
OLL	11.1%
SOL	22.2%

Gavriilidou and Boskou [58] found that a random distribution was obtained after chemical interesterification of olive oil–tristearin blends. They observed that trisaturate and triunsaturate proportions decreased markedly, whereas proportions of SSU and UUS increased (Table 12.2).

TABLE 12.2
Triacylglycerol Makeup for Olive Oil–Tristearin Blends before and after Interesterification

	Olive Oil–Tristearin Blend			
Species[a]	75% Initial	−25% (w/w) Randomized	80% Initial	−20% (w/w) Randomized
SSS	25.1	4.4	20.1	2.8
SSU	3.4	24.1	3.6	19.2
UUS	23.4	44.3	25	44.2
UUU	46.9	27.2	50	33.9

Source: Gavriilidou, V. and Boskou, D., *Int. J. Food Sci. Technol.*, 26, 451, 1991.

[a] S, saturated; U, unsaturated.

Not all workers agree that chemical interesterification is a purely random process. Kuksis et al. [59] found that the TAG composition of rearranged butter and coconut oil approached random distribution but deviated from true random distribution, even when experimental error was accounted for. This result was attributed to differences in the reactivity of the fatty acids and to possibly different esterification rates of the inner and outer hydroxyl sites on the glycerol backbone.

B. Batch Interesterification

Random interesterification can be accomplished in either batch or continuous mode. A typical batch reactor (Figure 12.7) consists of a reaction vessel fitted with an agitator, heating/cooling coils, nitrogen sparger, and vacuum pump [3,14,28]. In a batch process, the raw lipid is heated to 120°C–150°C under vacuum in the reaction vessel to remove any trace of moisture [1,29]. As mentioned, moisture and peroxides deactivate the catalyst. Following the drying step, the mixture is cooled to 70°C–100°C. The catalyst is sucked into the reaction vessel and disperses to form a white slurry. The reaction is allowed to proceed for 30–60 min. When completion has been confirmed by measuring melting point and/or solid fat content (SFC), the catalyst is neutralized in the reaction vessel. Processing losses can be minimized by using as little catalyst as possible and neutralizing with phosphoric acid or CO_2 prior to addition of water. After filtration and soap removal, neutralized catalyst and other impurities are removed from interesterified oil.

C. Continuous Interesterification

During continuous random interesterification, the fat is flash-dried and the catalyst is continuously added. The fat then passes through elongated reactor coils with residence time determined by the coil length and the flow rate of the oil. The catalyst is then neutralized with water, separated from the oil by centrifugation, and dried [14].

Rozenaal [11] mentioned a continuous interesterification process in which a solution of sodium hydroxide and glycerol in water was used as a precatalyst. Heated oil was mixed with the catalyst solution and subsequently spray-dried in a vacuum drier to obtain a fine dispersion

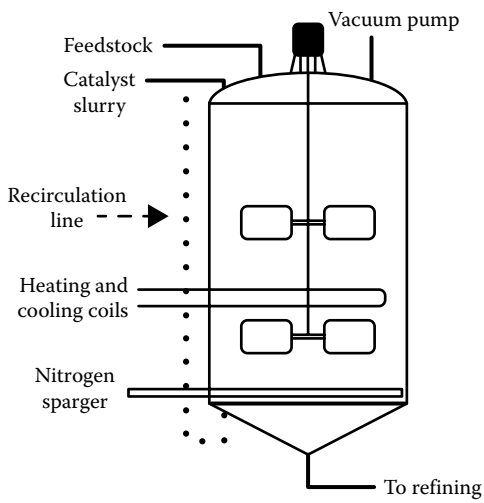

FIGURE 12.7 Batch random interesterification reaction vessel. (Adapted from Haumann, B.F., *Inform*, 5, 668, 1994; Laning, S.J., *J. Am. Oil Chem. Soc*., 62, 400, 1985; Going, L.H., *J. Am. Oil Chem. Soc*., 44, 414A, 1967.)

and to remove the water. The reaction could be carried out in a coil reactor at 130°C. With this setup, the reaction took only a few minutes.

D. Regioselectivity in Interesterification

Elegant work by Konishi et al. [49] demonstrated that chemical interesterification can be regioselective. Sodium methoxide–catalyzed ester interchange between soybean oil and methyl stearate in hexane, at 30°C, revealed that fatty acid interchange at *sn*-1,3 positions progressed 1.7 times faster than at the *sn*-2 position after 24 h of reaction.

Cast et al. [60] demonstrated the regioselectivity of chemical interesterification in the presence of phase transfer catalysts (tetraalkyl ammonium bromides), with trilaurin (LaLaLa) and methyl palmitate in the presence of sodium methoxide. Under normal conditions, interesterification resulted in 17.7% LaLaP and 82.3% unreacted LaLaLa. Using tetrahexyl ammonium bromide, 11.9% LaLaP, 6.2% LaPP, and 81.2% LaLaLa were obtained. Seven quaternary ammonium salts were tested for their effect on the reaction: tetraethyl, tetrapropyl, tetrabutyl, tetrapentyl, tetrahexyl, and tetrahepryl ammonium bromides. The amount of LaLaOH DAG increased as the chain length increased up to tetrahexyl ammonium bromide (Figure 12.8). The amounts of LaPOH and PPOH DAG species increased up to tetraheptyl ammonium bromide. The reactions were performed for 48 h, with a fivefold increase in the amount of LaPOH DAGs compared with the amount after 1 h of reaction.

Most importantly, following lipase hydrolysis and subsequent 2-monoacylglycerol isolation, it was discovered that the reaction between trilaurin and methyl palmitate in the presence of tetrapentyl ammonium bromide contained 49% lauric acid and 51% palmitic acid, which represents an enrichment factor for the 2-position of 1.51 times. Hence, under these conditions, chemical interesterification was not a random process.

E. Directed Interesterification

If the interesterification reaction is carried out at temperatures below the melting point of the highest-melting component (most likely a trisaturated TAG species), the end result will be a mixture enriched in this component. This was first reported by Eckey [36] who, for the interesterification

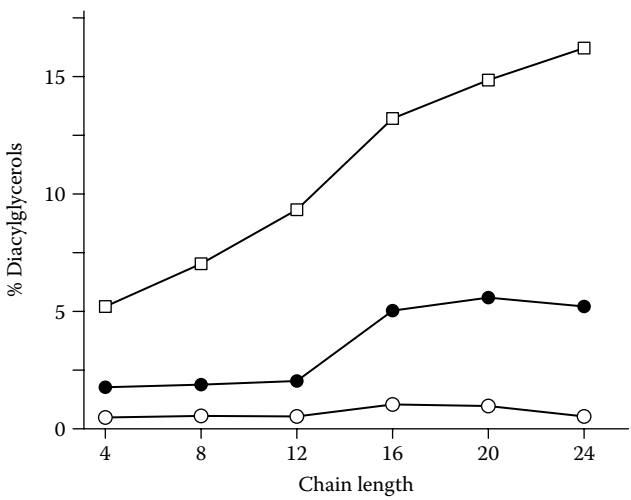

FIGURE 12.8 Composition of DAG content versus phase transfer catalyst chain length for sodium methoxide–catalyzed interesterification of trilaurin and methyl palmitate in the presence of seven quaternary ammonium salts. PPOH (○); LaPOH (●); LaLaOH (□). (Adapted from Cast, J. et al., *Chem. Ind.*, 763, 1991.)

of lard, discovered that certain catalysts were active below the melting point of the fat and that the reaction reached equilibrium within 30 min.

During directed interesterification, two reactions take place simultaneously. As the trisaturate is produced by interesterification, it crystallizes and falls from solution. Then, to regain equilibrium, the reaction equilibrium in the remaining liquid phase is pushed toward increased production of the crystallizing trisaturate [44,51,61]. Crystallization continues until all TAGs capable of crystallizing have been eliminated from the reaction phase [46].

Early developments in the area of directed interesterification showed that the following factors determine the effectiveness of the reaction [51,61]:

Interesterification rate in the liquid phase
Rate of heat removal
Fat crystal nucleation rate
Trisaturate crystallization rate out of liquid phase

The rate of interesterification is an important factor, as the trisaturates will precipitate out of solution as quickly as they are formed.

Fat crystallization generates heat. Removal of this heat is hindered by the poor conductivity of fat and the low convection in viscous or plastic media. Heat removal directly affects the nucleation rate. Rapid cooling to temperatures much below the melting point of trisaturate increases the nucleation rate, hence crystallization. Trisaturate crystallization is also hindered by the viscosity of the lipid phase. Gently yet thorough agitation is helpful in speeding up crystallization.

For directed interesterification, Na/K alloy is the catalyst of choice, given its low-temperature activity compared with that of the metal alone or that of the alkylates [28,51]. Typically, the alloy is continuously metered in by a pump and well dispersed by means of a high shear agitator to provide the proper catalyst particle size, ensuring optimal activity. Initially, the fat is at least partially randomized at temperatures above the melting point of the highest-melting TAG. When the fractional crystallization approach is used, the fat/catalyst slurry is chilled in conventional scraped-wall heat exchangers to specific temperatures in a series of steps designed to maintain the directed fractional crystallization process. Once chilled, the mixture is held under gentle agitation for a period of time so as to achieve the desired degree of crystal formation. Enhancements of the procedure include the stepwise reduction of temperature and the use of temperature cycling [62,63].

Directed interesterification has some benefits compared to random interesterification [48]:

1. Extending plastic range and fat consistency by increasing tri- and disaturated TAGs compared to addition of fully hydrogenated oil after randomization
2. Preparing palm super olein from palm oil by directed interesterification to use as salad oil or high-stability frying oil
3. Direct conversion of liquid oils such as cottonseed oil to hardstock fats

Directed interesterification can be used to increase the SFC without affecting unsaturated fatty acids. Periodic drops in temperature accelerate the reaction since it forms a greater amount of saturated TAGs in a shorter time than obtained by the use of a single temperature throughout the reaction. By periodic drops in temperature during directed interesterification of cottonseed oil starting at 21°C and ending at 4.4°C, about 19% fully saturated TAGs can be produced [64].

Kattenberg [63] applied this knowledge to interesterification of sunflower oil and lard blends and accelerated the reaction by a factor of 3. In another study, various oils, after directed interesterification, were chilled at 15°C for various durations (30–180 min), then subjected to further reaction at 23°C for 12–168 h. These treatments influenced the SFC of the final product [65].

During directed interesterification, controlling temperature is a very important factor to shift the reaction to form specific TAGs. For example, directed interesterification of lard at 20°C–38°C accelerates the formation of S3 TAGs while at 0°C–10°C the formation of S2U TAGs is usually enhanced [31].

The effects of directed interesterification on cottonseed oil were reported by Eckey [36]. Cottonseed oil contains 25% saturated fatty acids. With random interesterification, only 1.5% trisaturates were obtained, whereas directed interesterification led to the production of 19% trisaturates.

A review by Huyghebaert et al. [66] showed that the directed interesterification of an SOL mixture resulted in the following proportions:

Solid	SSS	33.3%
Liquid	OOO	8.3%
	OOL	24.99%
	OLL	24.99%
	LLL	8.3%

The segregation of saturated fatty acids into trisaturated species is necessarily accompanied by a corresponding tendency for unsaturated fatty acids to form triunsaturated species [51].

VIII. KINETICS OF CHEMICAL INTERESTERIFICATION

Random interesterification is usually conducted until equilibrium has been reached. There are many conflicting reports in the literature concerning interesterification reaction rates. Coenen [43] stated that once a sufficient concentration of the catalyst in solution had been reached in the reaction mixture, the actual interesterification reaction was extremely fast, requiring only a few minutes, unless operations had to proceed at very low temperatures. The kinetics were modeled in several ways to support this theory. The first example was a model system consisting of short-chain fatty esters (C8, C10) of ethylene glycol (Figure 12.9). The induction period was long, yet the reaction itself was rapid, even at 32°C. In the second example, interesterification of palm oil was evaluated using SFC determination (Figure 12.10). The reaction rate was faster at higher temperatures. These data confirm that an activation period is indeed required and agree with Weiss et al. [24] and Rozenaal [11], who reported that the catalyst formation phase was longer than the interesterification reaction, since the activation energy was higher for catalyst formation than for the interesterification reaction itself.

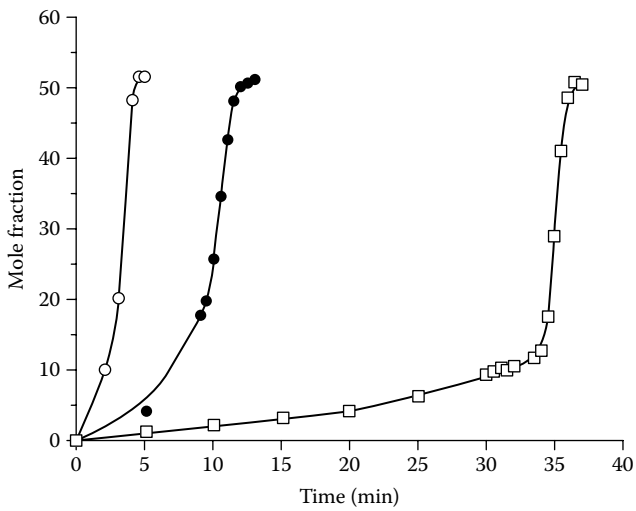

FIGURE 12.9 Theoretical interesterification kinetics of glycol esters of C8 and C10 as a function of time and temperature. 42°C (o), 37°C (●), 32°C (□). (Adapted from Coenen, J.W.E., *Rev. Fr. Corps Gras*, 21, 403, 1974.)

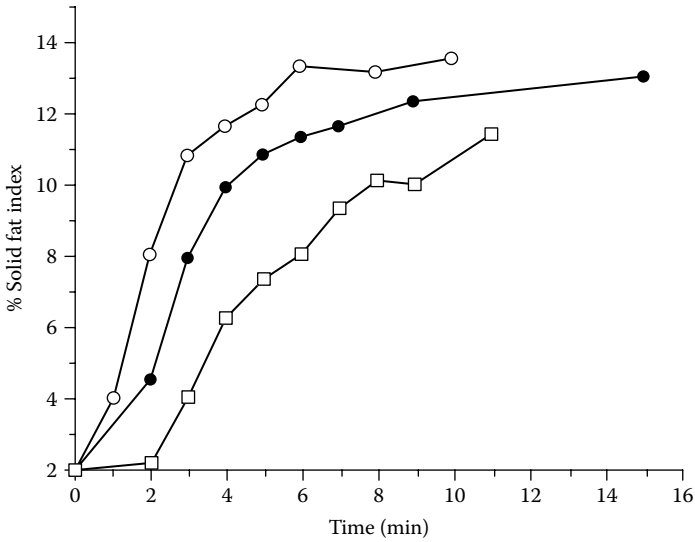

FIGURE 12.10 Practical example of interesterification kinetics with palm oil. The solid fat index (at 40°C) served as a measure of interesterification. Reaction temperatures: 60°C (o), 52°C (●), and 45°C (□). (Adapted from Coenen, J.W.E., *Rev. Fr. Corps Gras*, 21, 403, 1974.)

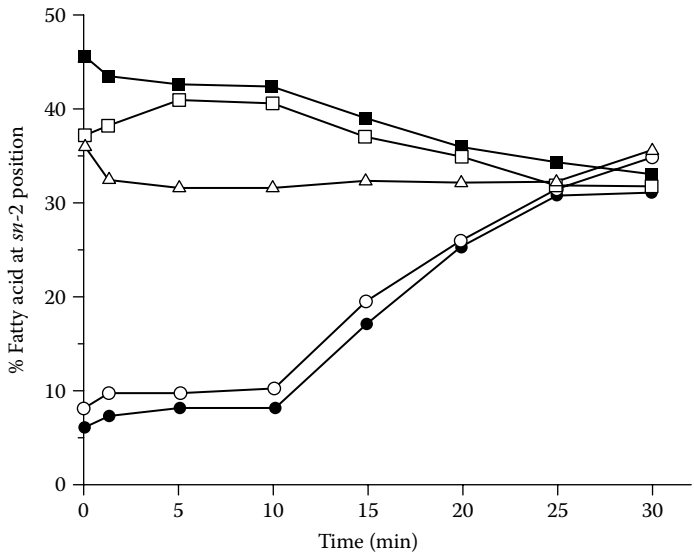

FIGURE 12.11 Changes in the fatty acid distribution at the *sn*-2 position during random interesterification of a 60:40 (% w = w) soybean oil–beef tallow mixture: o, 16:0; ●, 18:0; □, 18:1; ■, 18:2; Δ, 18:3. (Adapted from Lo, Y.C. and Handel, A.D., *J. Am. Oil Chem. Soc.*, 60, 815, 1983.)

Lo and Handel [67] observed that interesterification of soybean oil and beef tallow was complete after 30 min (Figure 12.11). Reaction completion was determined by lipase hydrolysis analysis. The results by Konishi et al. [49] showed that in certain cases the interesterification reaction can progress for as long as 24 h, even with catalyst preactivation. Thus, depending on conditions, randomization can proceed for many hours.

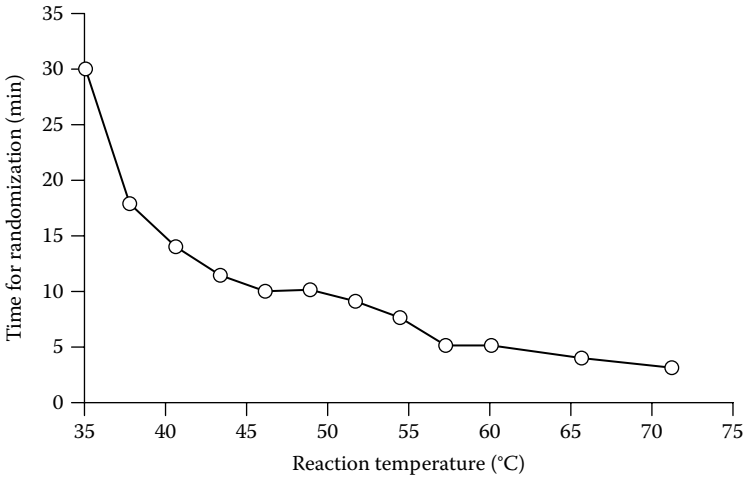

FIGURE 12.12 Influence of temperature on the interesterification reaction rate with glycerol/NaOH catalyst. (Adapted from Laning, S.J., *J. Am. Oil Chem. Soc*., 62, 400, 1985.)

Other factors that may influence the interesterification onset include agitation intensity, catalyst particle size, and temperature. Studies by many, including Konishi et al. [49], Laning [14], and Wiedermann et al. [23], have shown that interesterification kinetics are temperature dependent (Figure 12.12).

Addition of glycerol during directed interesterification is a proper way to accelerate the reaction rate since saturated MAGs and DAGs have less solubility in oil than their corresponding TAGs [31].

IX. ASSESSING THE EFFECTS OF INTERESTERIFICATION ON LIPID PROPERTIES

Fats and oils are usually modified to attain a certain functionality, such as improved spreadability, a specific melting point, or a particular SFC–temperature profile. However, changes in the TAG structure may constitute the purpose of the reaction, as in the synthesis of a particular structure. For that purpose, the fatty acid distribution constitutes the reaction goal. Methods described to assess physical properties include cloud point, Mettler dropping point, pulsed nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC), cone penetrometry, x-ray diffraction, and polarized light microscopy. Chromatographic methods include thin-layer chromatography (TLC), high-pressure liquid chromatography (HPLC), and gas–liquid chromatography (GLC). Other methods not discussed include mass spectroscopy [68], spectroscopy [69], and stereospecific lipase hydrolysis [70,71].

A. Physical Properties

Physical properties can be determined by examining thermal characteristics, rheological characteristics, or crystal habit.

1. Cloud Point

Cloud point, or temperature at which crystallization is induced, producing a crystal cloud, is one of the older indices used to study the physical properties of fats. Eckey [36] monitored the change in cloud point during cottonseed oil interesterification. Generally, randomization increased the cloud point quickly at first and then more slowly until an increase of 13°C–15°C was reached, after which no change was observed, regardless of reaction duration. For Placek and Holman [51], a study of cloud point indicated the extent of interesterification of lard.

TABLE 12.3
Effect of Interesterification on Dropping Point in Palm, Palm Kernel, and Coconut Oil (°C)

Oil	Before Treatment	Random Treatment	Directed Treatment
Palm oil	39.4	42.7	51.1
PKO	28.3	26.9	30.0
CO	25.5	28.2	—
Saturated PKO	45.0	34.4	—
Saturated CO	37.8	31.6	—

Source: Laning, S.J., *J. Am. Oil Chem. Soc*., 62, 400, 1985.

2. Dropping Point

The Mettler dropping point is a simple yet effective method of measuring the effect of interesterification on fats. In this procedure, liquefied fats are crystalized in sample cups and subsequently heated until they begin flowing under their own weight. Kaufmann and Grothues [72] performed a thorough study of the dropping points of hardstock and vegetable oil mixtures as an indicator of catalyst activity. Laning [14] demonstrated the effect of chemical interesterification on palm oil, palm kernel oil (PKO), and CO (Table 12.3). A reduction in dropping point for saturated PKO and saturated CO was due to the lower average molecular weight of the TAG in the randomized fat. The reduction in dropping point reported for randomized PKO was due to an increase of TAG species with intermediate degrees of unsaturation. Cho et al. [73] used the dropping point as an indicator of the measure of reaction equilibrium. A blend of 70% hydrogenated canola oil, 10% Pst, and 20% canola oil had an initial dropping point of 37°C, which dropped to 35°C following 5 min of reaction and to 32°C after 20 min, remaining constant thereafter. List et al. [74] used dropping point as a verification of interesterification completion in the preparation of "zero-trans" soybean oil margarine base stock. Rousseau et al. [35] examined the effect of chemical interesterification and blending on a butterfat–canola oil mixture and found that a linear increase in the proportion of canola oil did not lead to a linear reduction in dropping point.

3. Nuclear Magnetic Resonance

The amount of solid TAGs in a lipid sample at specific temperatures can be determined by means of the pulse-NMR technique. For example in a tub margarine, the SFC at 5°C shows its ease of spreadability at refrigeration temperature, while at 25°C it shows margarine stability against oil separation at room temperature. At 35°C, it indicates its melting at body temperature and flavor release in the mouth [75]. Random interesterification resulted in modest SFC changes, while directed interesterification produced more significant increases, attributable to the increase in trisaturated TAGs. Generally speaking, interesterification results in more linear profiles, owing to the greater variety of TAG species [76].

Blending of butterfat with canola oil produced slight changes in the SFC of butterfat–canola oil blends, as exemplified by a contour profile (Figure 12.13) [35]. No changes greater than ±6% were evident. The biggest increase in SFC produced by interesterification was of butterfat at 25°C; SFC "valleys" were present for the 80% butterfat–20% canola oil blend at 15°C, while the largest decreases were present for the 40% butterfat–60% canola oil blend at 10°C.

4. Differential Scanning Calorimetry

DSC is used to measure the melting or crystallization profile and accompanying changes in the enthalpy of fats. Chemical interesterification can increase, decrease, or even have no effect on the

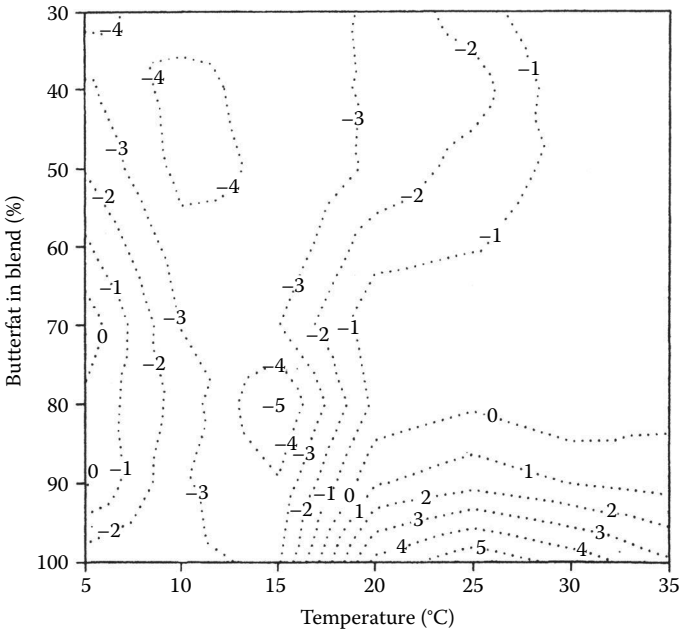

FIGURE 12.13 Contour profile of the effect of chemical interesterification on the SFC of butterfat–canola oil blends. Each line represents a 1% change in SFC. (Adapted from Rousseau, D. et al., *J. Am. Oil Chem. Soc.*, 72, 973, 1996.)

melting point of a fat, since it depends on the average distribution of saturated and unsaturated fatty acids within TAGs after randomization [75]. Rost [77] described the directed interesterification of palm oil. Calorimetry results indicated that the melting thermogram for noninteresterified palm oil consisted of two main peaks centered around 10°C and 19°C. Directed interesterification of palm oil led to a broader melting profile with no distinct peaks. Rossell [78] studied the effects of chemical interesterification on PKO crystallization. Randomization did not alter the shape of the crystallization curve; only peak temperatures were slightly lower. Because of the wide range of TAGs that must be packed into fat crystals, interesterified fats generally show simpler melting curves with less polymorphism upon chemical interesterification [76].

Zeitoun et al. [79], who examined interesterified blends of hydrogenated soybean oil and various vegetable oils (1:1 w/w ratio), found that each oil influenced the melting and crystallization behavior of the interesterified blends differently as a result of initial variations in oil composition. Rousseau et al. [35] found that chemical interesterification of butterfat–canola oil blends also led to simpler, more continuous melting profiles. However, overall changes were minimal.

5. Cone Penetrometry

This is a rapid yet empirical method used in the evaluation of fat texture and rheology [80]. Jakubowski [46] found that interesterification doubled the penetration depth of a blend of 35%–65% tallow–sunflower oil at 15°C. Rousseau et al. [81] reported that interesterification substantially decreased the hardness index of blends of butterfat and canola oil. Other rheological measurements include viscoelasticity measurements [81].

6. X-Ray Diffraction

The polymorphic behavior of fats is important in many food systems (fat spreads, chocolate, etc.) [57]. Fat spread crystals exist as one of the three primary forms: α, β′, and β. The β modification is to be avoided in fat spreads because it results in a sandy texture [79]. The β′ crystals are the most

desirable form. Chemical interesterification alters the crystal morphology and structure of fats. Larsson [82] stated that a greater variety of fatty acids hinders β-crystal formation. Hence, upon interesterification of butterfat, which normally consists of a predominance of β′ crystals and a slight proportion of β crystals, the latter disappeared upon TAG randomization [76,83,84]. Naturally, lard crystals are found in the β polymorphic form, while after chemical interesterification they are found in the β′ form [1].

List et al. [85], while working with margarine oils, found that chemical interesterification and blending of vegetable oils and hydrogenated hardstock of soybean oil or cottonseed oil led to the formation of β′-crystal polymorphs.

Hernqvist et al. [57] interesterified mixtures of tristearin, triolein, and trielaidin. These mixtures were chosen to produce model systems for vegetable oil blends used in margarine. Polymorphic transitions of interesterified blends were studied, and, depending on the blend, two to four polymorphs (sub-α, α, β′, or β) were observed.

7. Polarized Light Microscopy

The morphology of the crystals comprising the 3D fat crystal network is largely responsible for the appearance and texture of a fat and exerts a profound influence on its functional properties. Interesterification leads to noticeable modifications in crystal morphology, which can be examined in great detail by polarized light microscopy [86]. Prior to interesterification, lard consists of large crystals promoting graininess. Following interesterification, tiny delicate crystals, typical of the β′ polymorph, are present [87]. Becker [88] performed an in-depth study on the influence of interesterification on the crystal morphology of binary and ternary mixtures of trilaurin, triolein, and tristearin. He also found that fat crystals following interesterification were smaller before randomization and had different morphologies. A study of butterfat–canola oil blends revealed that gradual addition of canola oil led to gradual spherulitic aggregation of the crystal structure [83]. Ribeiro et al. [75] described the effects of chemical interesterification on palm oil crystal size by polarized light microscopy at 25°C. While the mean crystal diameter in palm oil was 58 μm, it decreased to 14.5 μm after randomization.

B. Chemical Properties

Changes in physical properties provide an arbitrary measure of interesterification structural modifications but give no real information on the compositional changes. Following these changes can be difficult unless simple substances are used [34]. Studies on the molecular rearrangement of TAG species provide a true indication of the chemistry of interesterification. The chemistry of interesterification can be followed with different chromatographic techniques: TLC, HPLC, and GLC.

Freeman [34], who examined the changes in monounsaturated TAGs during the course of interesterification with TLC, found that intraesterification occurred at a faster rate than the general randomization that results from interesterification.

Chobanov and Chobanova [62] made extensive use of TLC to study the alteration in the composition of 10 TAG groups during the monophasic interesterification of mixtures of sunflower oil with lard and tallow.

Parviainen et al. [89] studied the effects of randomization on milk fat TAG; they found an S2U decrease in C36 and C38 species (45% and 52%, respectively) and an increase in trisaturated C44–C50 species. This combination led to a broader crystallization range and higher SFCs at temperatures above 25°C.

Herslöf et al. [90] used reversed-phase HPLC and GC to analyze the interesterification reaction between FAMEs and trilaurin and found that the theoretical and experimental compositions for the interesterified systems matched.

Rossell [78] measured the evolution in TAG species following chemical interesterification of PKO by means of GLC.

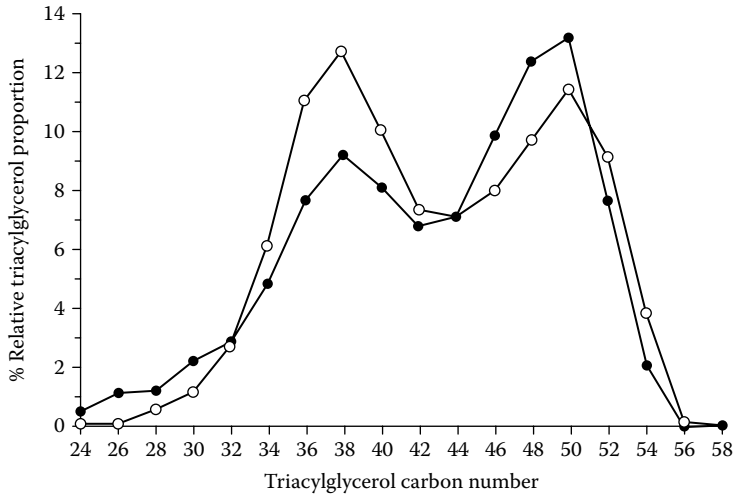

FIGURE 12.14 Effect of interesterification on butterfat TAGs: ○, native butterfat; ●, randomized butterfat. (Adapted from Rousseau, D. et al., *J. Am. Oil Chem. Soc*., 72, 973, 1996.)

TABLE 12.4
Effect of Randomization on Triacylglycerol Distribution of Palm Oil, Its Derivatives, and Palm Kernel Oil

	Palm Oil	Palm Olein	Palm Stearin	Palm Kernel Oil
Before randomization				
S3	7.8	2.1	34.1	69.0
S2U	46.1	48.3	40.7	21.9
SU2	35.1	42.9	21.8	1.4
U3	5.4	6.7	3.4	0.9
After randomization				
S3	12.5	8.4	37.2	60.5
S2U	38.9	32.3	43.6	30.2
SU2	33.9	41.5	17.0	0.4
U3	9.7	17.8	2.2	0.2

Source: Noor Lida, H.M.D. et al., *J. Am. Oil Chem. Soc*., 79, 1137, 2002.
Notes: S3, trisaturated; S2U, disaturated–monounsaturated (SSU/USS + SUS); U2S, monosaturated–diunsaturated (UUS/SUU + USU).

Huyghebaert et al. [66] and Rousseau et al. [35] used GLC to follow the evolution of butterfat TAG species as a result of interesterification. Typical results are shown in Figure 12.14. The effects of randomization on TAG molecular species in palm oil and its derivatives are shown in Table 12.4.

X. APPLYING INTERESTERIFICATION TO FOOD LIPIDS

Chemical interesterification is used industrially to produce fats and oils used in margarines, shortenings, and confectionery fats [66]. Because of legislation and for economic reasons, interesterification is a more common process in Europe than in North America. It is popular for many reasons. For example, little in the way of chemical properties is affected, and the fatty acid distribution is

changed but the fatty acids' inherent properties are not. Moreover, unsaturation levels stay constant and there is no *cis–trans* isomerization [37,46]. Interesterification can improve the physical properties of fats and oils. Similar changes in physical properties may be obtained by means of blending, fractionation, or hydrogenation. Production costs, market prices, or raw material and nutritional concerns will determine the process to be used. Applications described include lard, margarines, palm oil and PKO, milk fat, and fat substitutes.

A. Shortening

Chemical interesterification has been successfully used for decades to improve the physical properties of lard. Natural lard has a grainy appearance, a poor creaming capacity, and a limited plastic range, which is not improved by plasticizing in a scraped-surface heat exchanger [46,51]. Addition of a hardstock plus plasticizing helps in these respects, but the product develops an undesirable graininess during storage [91]. Chemical interesterification halves the SFC of the lard at 20°C, improves the plastic range of the fat considerably, and prevents the development of graininess, which is due to the large proportion (64%) of palmitic acid at the *sn*-2 position [91,92]. This improvement in plasticity and stability is due to alterations in the polymorphic behavior, with interesterified lard crystallizing in a β′-2 form, characteristic of hydrogenated vegetable oil shortenings [91].

Chakrabarty and Talapatra described a combination of randomization at 90°C for 30 min, followed by cooling for 1–3 h at 15°C, followed by directed interesterification at 22°C for 12 and 24 h, on 100% cottonseed oil and a groundnut/cottonseed oil 30:70 (w/w) blend, respectively. They found that this process had potential for the manufacture of plastic fats with a slip melting point of 38°C [66].

Chemically, the beta′-3-tending disaturated OPS (1-oleoyl-2-palmitoyl-3-stearoyl-glycerol) (large crystals responsible for lard graininess) is exchanged for a mixture of disaturated TAGs, with a lower melting point and greater intersolubility; the *sn*-2 palmitic acid concentration drops from 64% to 24% promoting β′ behavior [91]. A detectable morphological change that accompanies these chemical changes is an increase in the relative proportion of small fat crystals [93]. Randomized blends of 25:75 (w/w) fully hydrogenated soybean oil/soybean oil and 40:60 w/w Pst/soybean oil with melting points of 38°C and 41°C, respectively, had the same functionality as confectionery fats (Table 12.5) [45].

Duterte [94] mentioned that the crystalline modifications were observed prior to the theoretical completion of randomization. Production of fine crystals extends lard's plastic range and gives it a smooth appearance [51]. Random interesterification helps to resolve the graininess problem, yet the limited plastic range problem is not fully resolved. Herrera et al. showed that the mean diameter of fat crystals in foods should be lower than 30 μm in order to prevent sandiness in the mouth.

TABLE 12.5
Plastic Fat Production by Chemical Randomization of 20% and/or 40% Palm Stearin and/or Fully Hydrogenated Soybean Oil with Soybean Oil

Sample	SFC (%) at 10°C	SFC (%) at 20°C	SFC (%) at 30°C	Melting Point (°C)
20:80 FHSBO/SBO	12.3	10.0	4.2	31.0
40:60 Pst/SBO	34.6	17.5	8.7	41.0
Tub margarine	11.7	8.1	4.6	32.5
Shortening	N.A.	22.0	11.0	41.0
Confectionary fat	22.7	10.7	6.6	40.6
Stick margarine	44.2	25.4	8.9	36.8

Source: Petrauskaite, V. et al., *J. Am. Oil Chem. Soc*., 75, 489, 1998.

After randomization, a large number of small dispersed fat crystals can provide desirable properties such as good spreadability in shortenings and margarines and stabilize air bubbles during creaming [95]. The S2U TAGs in randomized lard give little plasticity at higher temperature. Directed interesterification resolves the plastic range problem [61]. A chemically interesterified blend of palm oil/cottonseed oil 75:25 (w/w) had the best creaming and cake baking properties [96].

Zeitoun et al. [80] compared the physical properties and functionalities of nine different vegetable oils blended with fully hydrogenated soybean oil 1:1 (w/w) and then chemically interesterified. The blend of CO/fully hydrogenated soybean oil had a melting point of 41°C and the highest proportion of fat crystals in the β′ form (about 75%). The SFCs for this interesterified blend at 10°C, 27°C, 38°C, and 46°C were 89.6%, 65.4%, 28.1%, and 7.5%, respectively, which is in the proper range for laminating shortening [80].

B. Margarines

In the manufacture of margarine, the objective is to produce a fat mixture with a steep SFC curve to obtain a stiff product in the refrigerator that nevertheless spreads easily upon removal and melts quickly in the mouth. It should crystallize as a β′ polymorph [97]. Depending on oil costs and availability, different treatments can be used.

As an alternative to hydrogenation for the production of margarine, Lo and Handel [67] chemically interesterified blends of 60% soybean oil with 40% beef tallow. Final results indicated properties similar to those of commercial tub margarine oil. Yet the interesterified blend contained less polyunsaturated fatty acids and more saturated fatty acids than commercial margarine oil. According to Lida and Ali [98], an SFC at 10°C lower than 32% for a tub margarine guarantees good spreadability at refrigeration temperatures. In order to avoid a waxy feeling in the mouth, tub margarines should have an SFC below 3.5% at 33.3°C.

According to Sonntag [32], short- and medium-chain fatty acids (C6–C14) have good melting properties, whereas long-chain fatty acids (C20–C22) can provide stiffening power in margarine. Acids of these two types can be combined with interesterification to produce TAGs that provide blends with good spreadability, high-temperature stability, and a pleasant taste.

Margarine oil with high proportions of lauric acid has a low melting point and narrow plastic range, which leads to a margarine that is hard in the fridge but partly melts at room temperature [97]. Decreasing the lauric acid concentration can rectify this problem of extremes. For example, CO can be interesterified with an oil such as palm, and 60% of the interesterified mixture is then blended with 40% of oil such as sunflower oil.

In the manufacture of zero-*trans* margarines, chemical interesterification of soybean oil–soy trisaturate using 0.2% (w/w) sodium methoxide at 75°C–80°C for 30 min resulted in a β′-crystallizing fat with good organoleptic properties [71].

List et al. [85] described the preparation of potential margarine and shortening bases by interesterification of vegetable oil and hardstocks (hydrogenated oil or stearin). They found that the interesterified fats possessed plasticity curves similar to those of commercial soft-tub margarine oils prepared by blending hydrogenated hardstocks or commercial all-purpose shortening oils. However, the commercial blends and interesterified blends differed with respect to crystallization behavior.

A trans-free table margarine manufactured by chemical interesterification of a Pst/PKO/soybean oil blend 49:20:31 (w/w) had similar physical properties to a commercial table margarine (Table 12.6) [99].

C. Palm Oil and Palm Kernel Oil

Palm oil has many applications in the food industry. Most often, interesterification of palm oil is combined with hydrogenation and/or fractionation to achieve the most desirable physical and functional properties [14]. Laning [14] described the applications of palm oil in cooking, frying, and

TABLE 12.6
Solid Fat Content of Formulated and Commercial Table Margarines at Different Temperatures

	Solid Fat Content (%) at Different Temperatures (°C)								
Sample	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**	**SMP (°C)**
Formulated table margarine	48.0	44.0	32.0	19.0	13.0	7.0	3.0	1.0	35.5
Commercial table margarine	46.9	45.0	33.7	19.4	11.1	7.1	3.3	1.3	35.0

Source: Fauzi, S.H.M. et al., *Food Chem*., 137, 8, 2013.
Abbreviation: SMP, slip melting point.

TABLE 12.7
Effect of Chemical Interesterification on Solid Fat Content in Palm Oil and Palm Kernel Oil

SFC (%) at	Palm Oil before CIE	Palm Oil after CIE	PKO before CIE	PKO after CIE
10°C	36.0	39.7	61.9	59.1
20°C	13.1	19.5	37.1	26.9
25°C	10.1	16.2	16.8	7.1
30°C	7.0	13.4	0	0
35°C	3.3	7.0		
40°C	1.6	3.8		
45°C	0	1.1		

Source: Grimaldi, R. et al., *Grasas y Aceites*, 52, 349, 2001.
Abbreviation: CIE, chemical interesterification.

salad oils. Corandomization of palm oil with other fats and oils, in combination with fractionation, produced a fluid salad oil.

After randomization of palm oil and palm olein, the amount of trisaturated (S3) TAGs and disaturated-monounsaturated (S2U) TAGs increased, which was the reason for observed increases in SFC and melting point in these oils, while in PKO a significant reduction in S3 content was observed (Table 12.7).

Grimaldi et al. [100] found that chemical interesterification minimized the eutectic effect that was caused by TAG incompatibility between palm oil and PKO and improved plasticity in the final product.

Cocoa butter, used in the production of chocolate, is expensive and not always available, so substitutes are created, such as those that result from the blending of interesterified lauric acid with other fats. According to Sreenivasan [1], PKO is a hard butter that melts at 46°C and produces a waxy feel. By interesterification, the melting point is reduced to 35°C. Furthermore, by blending hydrogenated PKO and the randomized product, a whole series of hard butters with highly desirable melting properties is obtained. The effect of randomization on the melting properties of cocoa butter is shown in Figure 12.15.

D. Milk Fat

Much research has been done on the chemical interesterification of milk fat. Milk fat, like most fats, does not have a random distribution, which conveys a predetermined set of physical properties.

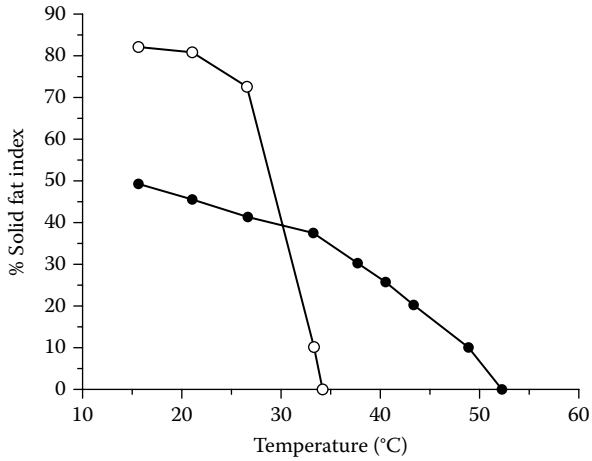

FIGURE 12.15 Solid content index of cocoa butter and randomized cocoa butter measured by dilatometry: ○, native cocoa butter; ●, randomized cocoa butter. (Adapted from Going, L.H., *J. Am. Oil Chem. Soc*., 44, 414A, 1967.)

Butyric and caproic acids, for example, are predominantly located at *sn*-3, while palmitic acid is mostly at *sn*-1 and *sn*-2 [101]. Other fatty acids are not as specific. Interesterification of milk fat can be a powerful means of modifying its functional properties.

Weihe and Greenbank [102] presented the first paper dealing with the chemical interesterification of milk fat, with details appearing in Weihe [103]. These investigators performed randomization of milk fat at 40°C–45°C for 20 min to 6 h with 0.1%–0.3% Na/K alloy. For directed interesterification, xylene or hexane was added before the reaction, which was begun at 25°C–38°C and dropped in three to five steps to 10°C–25°C. Directed interesterification led to more substantial changes than random interesterification (e.g., on solid fat; Figure 12.16). Increases in melting point were greater

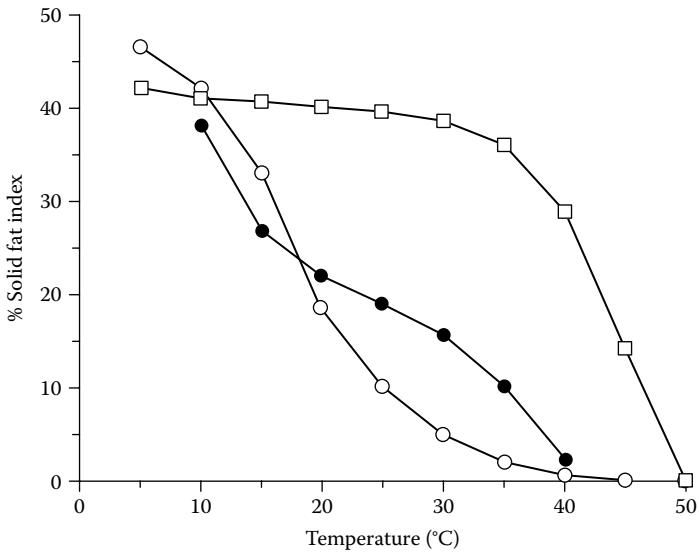

FIGURE 12.16 Proportion of solid fat of native butterfat (○), randomized butterfat (●), and butterfat subjected to direct interesterification (□) measured by dilatometry. (Adapted from Weihe, H.D., *J. Dairy Sci*., 44, 944, 1961.)

in the presence of a solvent than without, and direct interesterification generated larger increases in melting point than random interesterification.

Interesterification increased the softening point of milk fat by 3.7°C–4.2°C, which was explained by the higher proportion (5%–7%) of high-melting TAGs, which translated into a higher hardness. Mickle [104], on the other hand, found that interesterification reduced the hardness of butter and also led to a rancid, metallic flavor. Refining (free fatty acid removal and steam injection under vacuum) removed the undesirable flavor, yet the final product was tasteless. Finally, an in-depth study by Mickle et al. [105] revealed the effects of three interesterification reaction parameters on the hardness of a semisolid resembling butter. All the three parameters—duration (5–55 min), temperature (40°C–90°C), and catalyst concentration (0.5%–5%)—had statistically significant effects ($p < 0.05$), with catalyst concentration (at 1%–2%) having the greatest influence on hardness, which diminished 45%–55%. de Man [106] observed by means of polarized light microscopy that the crystal habit of interesterified milk fat was markedly changed from that of native milk fat. The effects of cooling procedures on consistency, crystal structure, and SFC of milk fat were also examined [107]. Parodi [108] examined the relationship between trisaturates and the softening point of milk fat. Interesterification increased the softening point from ~32.5°C to ~36.5°C. Timms [76] found that milk fat and beef tallow interesterified blends lacked milk fat flavor. Timms and Parekh [109] explored the possibility of incorporating milk fat into chocolate. Interesterified milk fat appeared to be better suited to chocolate than noninteresterified milk fat, but the improvement gained did not compensate for the investment and loss of flavor from interesterification.

E. Fat Substitutes

Other applications of chemical interesterification include the production of low-calorie fat substitutes such as Salatrim and Olestra. Salatrim/Benefat consists of chemically interesterified mixtures of short-chain and long-chain fatty acid TAGs. The short-chain fraction consists of triacetin, tripropionin, or tributyrin, while the long-chain fractions consist of hydrogenated soybean oil [26]. The only adverse effect of Salatrim consumption reported by different studies was gastrointestinal complaints after intake of more than 30 g of Salatrim per day. The relatively high cost of making Salatrim could be another reason limiting its production [48].

Olestra is an acylated sucrose polyester with six to eight fatty acids obtained from vegetable oil (e.g., soybean, corn, sunflower oils). It is prepared by interesterifying sucrose and methyl esters of vegetable oils in the presence of an alkali catalyst at 100°C–140°C [110]. Olestra is nondigestible, hence noncaloric. Its functionality is dependent on the chain length and unsaturation of the esterified fatty acids [111]. It can be exchanged for fats in products such as ice cream, margarine, cheese, and baked goods, and it can be blended with vegetable oil. Before 2003, food products that contained Olestra were required to show on the label the following statement: “olestra may cause abdominal cramping and loose stools.” However, in 2003, the FDA removed the requirement [48].

XI. OXIDATIVE STABILITY

The many advantages of chemical interesterification have been discussed in detail. Many authors have shown, however, that chemical interesterification can negatively influence the oxidative stability of fats and oils. Wang et al. [112] demonstrated that randomized corn oil oxidized three to four times faster than native corn oil. They concluded that the TAG structure probably was implicated, but the mechanisms remained unclear. Lo and Handel [67] showed that interesterified blends of soybean oil and beef tallow were more unstable following interesterification.

Gavriilidou and Boskou [113] examined the effects of chemical interesterification on the autoxidative stability of a blend of 80% olive oil and 20% tristearin. The randomized fats were less stable than the native mixtures (Figure 12.17). Addition of BHT stabilized the fats, resulting in a peroxide value similar to that for commercially processed hydrogenated vegetable oil used in margarine.

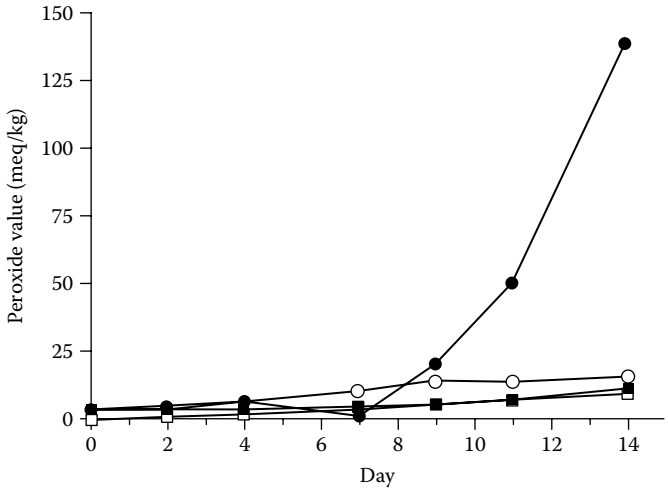

FIGURE 12.17 Change in peroxide value of an 80%:20% olive oil–tristearin blend before and after interesterification: ○, native blend; ●, randomized blend; □, hydrogenated blend; ■, randomized blend + BHT. (Adapted from Gavriilidou, V. and Boskou, D., Effect of chemical interesterification on the autoxidative stability of olive oil-tristearin blends, in: *Food Flavours, Ingredients and Composition*, G. Charalambous, ed., Elsevier Science Publishers, Amsterdam, the Netherlands, 1993, pp. 313–319.)

An important contribution to the literature was made by Zalewski and Gaddis [114], who investigated the effect of transesterification of lard on stability, antioxidant efficiency, and rancidity development. Interesterification of lard did not affect its resistance to oxidation, but changes in oxidative stability due to tocopherol decomposition and the formation of reducing substances were noted. In the absence of antioxidants, both interesterified and native lard had similar peroxide values. Furthermore, because of the position of unsaturated fatty acids at 1,3-positions or randomization toward the 2-position in pork fat TAGs, there was no appreciable effect on initiation of oxidation and autoxidation rates.

Tautorus and McCurdy [115] demonstrated the effects of chemical and enzymatic randomization on the oxidative stability of vegetable oils stored at different temperatures. Noninteresterified and interesterified oils (canola, linseed, soybean, and sunflower) stored at 55°C demonstrated little difference to lipid oxidation, whereas noninteresterified samples were more stable at 28°C. Samples at 55°C underwent much greater oxidation than the samples at 28°C.

Park et al. [116] found that loss of tocopherols accelerated the autoxidation of randomized oils. α-Tocopherol was not detectable following interesterification, while γ-tocopherol and δ-tocopherol decreased 12% and 39%, respectively.

Azadmard-Damirchi and Dutta [117] compared the effect of chemical interesterification at 90°C and 120°C on the stability of natural tocopherols and tocotrienols in olive oil and Pst 1:1 (w/w) blends. After randomization at 120°C, only 3%–4% reduction in total tocopherols was detected, while δ-tocotrienol displayed the largest decrease (35%).

Total tocopherol reduction in 70:30 Pst/soybean oil (w/w) blend was 26.3%, while for total tocotrienols this amount was 29.5% [118].

Konishi et al. [119] found that regioselectively interesterified blends of methyl stearate and soybean oil had increased oxidative stability over both native and randomized blends, as monitored by peroxide value and volatiles analysis. The improved oxidative stability was presumably due to the regioselective incorporation of stearic acid at the *sn*-1(3) carbon sites of the TAG moiety, which stabilized the linoleic acid, predominantly located at the *sn*-2 position.

XII. NUTRITIONAL CONSEQUENCES OF INTERESTERIFICATION

Perhaps, the greatest advantage of chemical interesterification over hydrogenation lies in nutrition, namely, concerns regarding *trans* fatty acids and their links to coronary heart disease identified decades ago [120–122]. Literature data from the 1970s and 1990s indicate that at that time the typical *trans* fatty acid content of margarines was 10%–27% in the United States and 10%–50% in Canada [123,124]. Now, given that customers have become increasingly aware of the fats and oils they consume, there has been a great push for processors to eliminate *trans* fatty acids from foods outright. Robinson et al. [125] studied the acute effects of ingesting high stearic acid interesterified fat on serum TAG, cholesterol, glucose, insulin, and free fatty acids. They found that fatty acid positional distribution in chemically or enzymatically interesterified high stearic fat did not have a significant influence on the postprandial acute metabolic risk factors for diabetes type 2 and cardiovascular disease.

It has been shown that randomization does not influence the nutritional value of unsaturated fatty acids [126]. However, not much is known about the potential importance of stereospecificity in the biological activity of dietary fatty acids [127]. In clinical trials, substitution of randomized butter for natural butter tended to reduce serum TAG and cholesterol concentrations [128]. Human infants absorbed 88% stearic acid when fed lard but only 40% when fed randomized lard. Hence, absorbability and pharmacological properties of fatty acids can be influenced by the molecular form in which they are absorbed [70].

Absorption of oleic acid and polyunsaturated fatty acids did not depend on the fatty acid profile of dietary fat. Kritchevsky [129] found that peanut oil's tendency to produce atherogenicity in rabbits disappeared following chemical interesterification.

It is known that human milk is well absorbed in part because of its proportion of long-chain saturated fatty acids located at the *sn*-2 position. Lien et al. [130] found that mixtures of CO and palm olein were better absorbed by rats if the proportion of long-chain saturated fatty acids at the *sn*-2 position was increased by random chemical interesterification.

Mukherjee and Sengupta [131] found that interesterified soya–butterfat feeding significantly decreased serum cholesterol in humans and rats. The decrease was greater than when noninteresterified blends were fed. The lowering of serum cholesterol paralleled the decrease in concentration of trisaturates and the scattering of myristic acid away from *sn*-2 to *sn*-1 and *sn*-3 positions.

There appears to be some dispute as to the health effects of interesterification. The dietary concerns for avoiding *trans* fatty acids seem well documented, whereas the nutritional effects of fatty acid positional distribution are presently less clear-cut.

XIII. DISTINGUISHING CHEMICAL FROM ENZYMATIC INTERESTERIFICATION

Although great strides have been made with extracellular microbial lipases as catalysts for interesterification, most of the industry still relies on chemical interesterification. Each type of interesterification possesses advantages and disadvantages. Advantages of chemical interesterification over enzymatic transformations primarily involve cost recovery and initial investment. Chemical catalysts are much cheaper than lipases. Even with immobilization procedures, capital investment remains high. Second, chemical interesterification is a tried-and-true approach; it has been around for a long time, and industrial procedures and equipment are available [49].

Costs aside, does treatment by means of chemical or enzymatic interesterification in identical applications result in the same final product? Kalo et al. [132] compared the changes in the TAG composition and the physical properties of butterfat interesterified using either sodium methoxide or a nonspecific lipase from *Candida cylindracea* and found only small differences in both interesterified butterfats. The compositional changes induced by both chemical and enzymatic means were similar, with the trisaturate TAG content being slightly higher in the enzymatically modified product. In terms of physical properties, the chemically interesterified butterfat was

slightly harder than its enzymatically modified counterpart. Hence, for randomization purposes, the methods appeared to yield similar results for the modification of butterfat. However, the product's butter flavor must be taken into account. The harsh process conditions of chemical interesterification result in loss of butter's fine flavor. For purposes where flavor is not a problem, the simpler, tried-and-true chemical process is preferable.

Enzymatic interesterification has many advantages, such as milder processing conditions and the possibility of regiospecificity and fatty acid specificity. This specificity permits structuring not possible by chemical means. For the production of nutritionally superior fats, enzymatic interesterification is ideally suited.

XIV. PERSPECTIVES

Chemical interesterification is likely to remain a force in the food industry for the foreseeable future. With the progressive demise of hydrogenation likely to continue, interesterification (both chemical and enzymatic) will gain greater prominence as a food lipid modification strategy.

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13 Enzymatic Purification and Enrichment of Polyunsaturated Fatty Acids and Conjugated Linoleic Acid Isomers

Yuji Shimada and Toshihiro Nagao

CONTENTS

I. INTRODUCTION

Polyunsaturated fatty acids (PUFAs) and conjugated dienoic and trienoic fatty acids have various physiological activities and have been used widely as pharmaceuticals and nutraceutical lipids. Because these fatty acid (FA) concentrates can have great physiological effects from even a small amount of intake, purification and enrichment of the desired FAs have been studied actively.

In general, physiologically active FAs are unstable to heat and oxidation. This problem is solvable by avoiding contact with oxygen (air) in the process of purification/enrichment. For example,

eicosapentaenoic acid ethyl ester (EPAEE), which is used in the treatment of arteriosclerosis and hyperlipemia, is purified from sardine oil by a combination of chemical ethanolysis under a nitrogen atmosphere, rectification under high vacuum, and urea adduct fractionation [1]. FA with higher degree of unsaturation also combines with large amounts of silver ion. Complex of FA and silver ion is soluble in water; thus, FA with the highest degree of unsaturation can be purified from an FA mixture [2]. Because this procedure is performed under a nitrogen atmosphere, it is effective for the purification of unstable PUFAs. In addition to these methods applicable in an industrial scale, enzyme reactions attract attention recently, which proceed efficiently under mild conditions. Oil containing high concentration of PUFAs, especially docosahexaenoic acid (DHA), is produced industrially using a lipase, which acts on DHA weakly and has been used as a nutraceutical lipid. This chapter deals with the purification and enrichment of useful FAs through lipase-catalyzed reactions, which is applicable even in an industrial scale.

II. A PROCESS COMPRISING ENZYMATIC REACTION AND DISTILLATION

It is well known that enzyme-catalyzed reactions efficiently proceed under mild conditions. The reactions are therefore promising as procedures for the conversion of unstable substances. Meanwhile, distillation is widely used for the fractionation of oil- and fat-related compounds, which have different molecular weights (boiling points). Molecular (short-path) distillation is particularly effective for purifying unstable compounds because the operation is conducted under high vacuum and because the material is heated for only a short time. We thus attempted to develop a new technology comprising enzyme reaction and distillation. If a desired component or contaminants are converted to different molecular forms by selective reaction with a lipase, the purification becomes relatively easy. The principle is schematically depicted in Figure 13.1. As decomposition of contaminants makes difference in the molecular weights of a desired component and contaminants, the desired one can easily be purified by distillation of the reaction mixture (Figure 13.1a). The other example is modification of a desired component. If lipase treatment after addition of a substrate to the raw material induces a change in molecular form of a desired one, the component can easily be purified by distillation (Figure 13.1b). Purification of useful materials according to this principle is described hereafter.

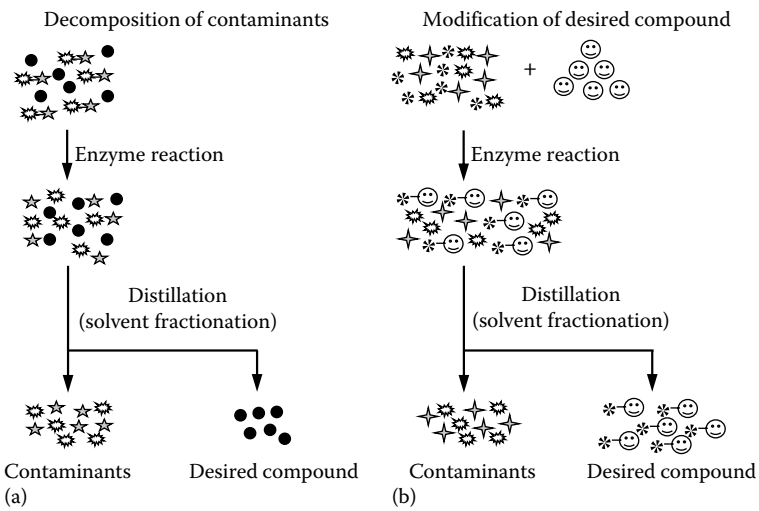

FIGURE 13.1 Purification process comprising enzyme reaction and distillation. (a) Purification process by decomposing contaminants with a lipase. (b) Purification process by converting a desired compound to its different molecular forms with a lipase.

III. ENRICHMENT OF PUFA BY SELECTIVE HYDROLYSIS

A. Strategy for Production of PUFA-Rich Oil

Natural oils and fats are triacylglycerols (TAGs), which consist of various FAs. Lipases hydrolyze ester bond of long-chain FAs and have FA specificity [3,4]. Hydrolysis of oil with a lipase, which acts on a desired FA strongly, releases preferentially the FA from TAGs and enriches it in the free fatty acid (FFA) fraction. The hydrolysis of TAG with a lipase, which acts on the desired FA weakly, results in its enrichment in the unhydrolyzed acylglycerol fraction.

In general, lipases act on PUFAs weakly [3,4], and selective hydrolysis of PUFA-containing oils with the lipases is effective for the production of PUFA-rich oils [5–16]. *Candida rugosa* lipase especially acts very weakly on FAs of which carbon length is 20 or more and distance from the first unsaturated bond to the carboxyl group is 6 or shorter [13,14]. Selective hydrolysis of fish oil with this lipase was useful for the production of oil containing high concentration of DHA [6]. An oil containing 45%–50% DHA has been produced industrially since 1994 in Japan [17] and is used widely as a nutraceutical lipid. This selective hydrolysis with lipase can also be applied to production of oils containing high concentration of γ-linolenic acid (GLA) and arachidonic acid (AA) [7,11,18]. In the next section, a process for producing GLA-rich oil is shown as an example, which can be applicable for its industrial-scale production.

B. Production of GLA-Rich Oil

C. rugosa lipase acts on GLA weakly; thus, selective hydrolysis of borage and evening primrose oils with the lipase produced GLA-rich oil [7,11,19]. A time course of selective hydrolysis of borage oil with *C. rugosa* lipase is shown in Figure 13.2. The contents of palmitic, oleic, and linoleic acids decreased along with the hydrolysis, and the content of GLA increased. The degree of hydrolysis reached 60% after 15 h, and the content of GLA in acylglycerols increased from 22% to 46% (2.1-fold). In this selective hydrolysis, the content of GLA depended on the degree of hydrolysis and was not raised over 46% even if the degree of hydrolysis increased to >60% [7,11,20].

An insufficient content of GLA through a single reaction was due to accumulation of FFAs in the reaction mixture [11]; thus, repeated hydrolysis was effective for further increase in GLA content.

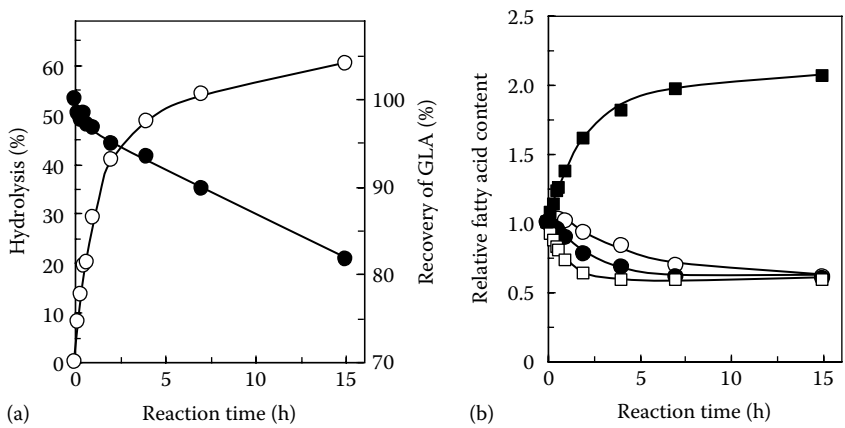

FIGURE 13.2 Selective hydrolysis of borage oil with *Candida rugosa* lipase. Borage oil was hydrolyzed at 35°C in a mixture containing 50% water and 20 U/g of the lipase. (a) Degree of the hydrolysis (○) and recovery of GLA in acylglycerols (●). (b) Content of FA in acylglycerols. The content of each FA was expressed relative to the initial content of FA in borage oil. ○, Palmitic acid (content of the original oil, 9.7%); ●, oleic acid (17.5%); □, linoleic acid (38.3%); ■, GLA (22.2%).

TABLE 13.1
Production of GLA-Rich Oil by Repeated Hydrolysis with *Candida rugosa* Lipase

	Hydrolysis (%)	GLA Content (wt%)	GLA Recovery (%)
Borage oil	—	22.2	100
Single reaction[a]	60.3	45.7	81.7
Repeated reaction[b]			
20 U/g	19.8	54.1	73.3
60 U/g	50.4	58.2	48.7
200 U/g	60.4	59.3	39.6

[a] Borage oil was hydrolyzed at 35°C for 15 h in a mixture of 50% water and 20 U/g of *C. rugosa* lipase.
[b] Acylglycerols obtained by single hydrolysis of borage oil were hydrolyzed at 35°C for 15 h in a mixture of 50% water and 20–200 U/g of *C. rugosa* lipase.

An oil containing 46% GLA was first produced by a single hydrolysis with *C. rugosa* lipase (60% hydrolysis). When the resulting oil was then hydrolyzed again, the GLA content in the acylglycerol fraction rose to 54% at 20% hydrolysis (total degree of hydrolysis, 68%) and to 58% at 50% hydrolysis (total degree of hydrolysis, 80%) (Table 13.1) [11].

Products of hydrolysis of the oil are glycerol, FFAs, and acylglycerols, and the oil layer contains FFAs and acylglycerols. Acylglycerols containing high concentration of GLA can be purified from the oil layer by *n*-hexane fractionation under alkaline conditions: FFAs change to alkali salts (soap) and move from hexane to water layer; acylglycerols are recovered into hexane layer. This *n*-hexane fractionation is useful for an industrial purification of acylglycerols and is especially suitable for a laboratory-scale fractionation of FFAs and acylglycerols.

Short-path distillation is also useful when FFAs and acylglycerols are separated from large amounts of their mixture. The process is summarized in Figure 13.3 [11]. After hydrolysis of

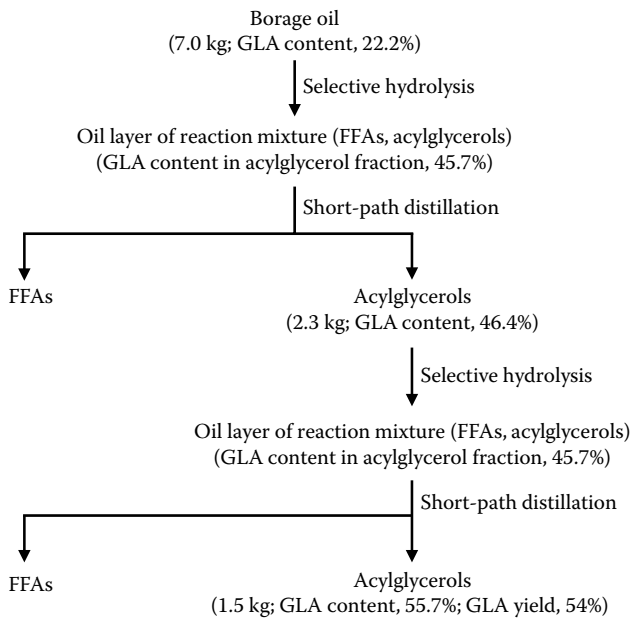

FIGURE 13.3 Large-scale production of GLA-rich oil.

borage oil (7.0 kg) with *C. rugosa* lipase, the reaction mixture was allowed to stand, and the oil layer was recovered. FFAs in the oil layer were removed efficiently by short-path distillation, and acylglycerols were recovered in the distillation residue (the content of GLA, 46%). To further increase the content of GLA, the residue was hydrolyzed with *C. rugosa* lipase again. The oil layer was then subjected to short-path distillation, and acylglycerols were recovered. A series of procedures increased the content of GLA from 22% to 56% in a 54% yield of the initial content, indicating that a process comprising selective hydrolysis and distillation is useful for the production of PUFA-rich oil.

IV. ENZYMATIC SYNTHESIS OF TAGs RICH IN DHA AT *sn*-1,3 POSITIONS

Oils containing 45%–70% DHA have been produced industrially from tuna oil by selective hydrolysis with *C. rugosa* lipase [6,8,10]. This process results in large amounts of FFAs (referred to as by-product FFA). Though by-product FFA includes 20%–45% DHA, it is treated as an industrial waste. Hence, by-product FFA was attempted to be converted to DHA-rich TAGs that can be used as a nutritional supplement.

Immobilized *Pseudozyma antarctica* (reclassified from *Candida antarctica* according to T. Boekhout [21]) lipase effectively converted by-product FFA to TAGs through esterification with glycerol [22,23]. Regiospecific analysis (see Chapter 6; [24–26]) of the resulting TAGs (DHA, 46%) showed that DHA distributed randomly in the TAG molecules (Table 13.2). These results showed that immobilized *P. antarctica* lipase has strong activity against PUFAs and is a positionally nonspecific enzyme in this esterification system [27,28]. In contrast, immobilized *Rhizomucor miehei* lipase, which acted weakly on DHA, showed a unique property [29]. When by-product FFA was esterified with glycerol using immobilized *R. miehei* lipase, the content of DHA at the *sn*-1,3 positions (46.5%) of the resulting TAGs was higher than the content of DHA at the *sn*-2 position (26.2%) (Table 13.2). This esterification proceeded in a one-pot process and may be used to synthesize TAGs via a series of pathways, which is shown in Figure 13.4. First, *sn*-1,3-DAGs, which were mainly esterified with FAs other than DHA (such as palmitic, palmitoleic, stearic, oleic, and linoleic acids), were generated in the early stage of the reaction (Figure 13.4b). As the reaction proceeded, the content of DHA in the FFA fraction became higher (46%–80%, [29]) than its content

TABLE 13.2
Regiospecific Analysis of TAGs Produced by Esterification of By-Product FFAs with Glycerol Using Immobilized *Pseudozyma antarctica* Lipase and Immobilized *Rhizomucor miehei* Lipase

		Fatty Acid Composition (mol%)					
		C16:0	C18:0	C18:1	C20:5	C22:5	C22:6
By-product FFA		7.8	2.2	9.8	7.6	3.7	46.4
P. antarctica lipase	*sn*-1,3	9.0	2.5	10.3	7.7	2.9	43.7
	sn-2	8.3	1.9	9.9	5.3	2.8	46.6
R. miehei lipase	*sn*-1,3	9.8	2.1	10.1	6.2	2.4	46.5
	sn-2	15.3	3.3	18.1	4.0	2.9	26.2
Tuna oil	*sn*-1,3	18.7	5.5	25.3	6.5	2.9	13.9
	sn-2	19.1	1.1	10.2	5.0	2.8	35.8
Seal oil	*sn*-1,3	1.5	0.2	14.1	20.3	8.7	20.9
	sn-2	3.5	0.2	23.2	2.1	0.7	1.7

Source: Data from Yoshida, H. et al., *Biosci. Biotech. Biochem.*, 60, 1293, 1996.

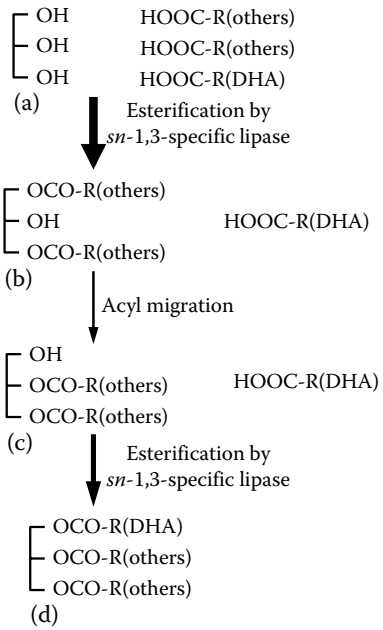

FIGURE 13.4 Proposed pathway for one-pot synthesis of TAGs rich in DHA at the *sn*-1,3-position from by-product FFAs containing DHA with immobilized *Rhizomucor miehei* lipase. (a) Before reaction, (b, c) intermediate, and (d) product.

before the reaction. Then acyl migration of FAs from the *sn*-1(3) position to the *sn*-2 position would occur, and *sn*-2,3(1)-DAGs would be generated (Figure 13.4c). Because this acyl migration was rate-determining step, the resulting *sn*-2,3(1)-DAGs were converted immediately to TAGs; namely, DHA was esterified to the unoccupied *sn*-1(3) position and TAGs rich in DHA at the *sn*-1,3 positions were produced (Figure 13.4d) [29].

It is well known that the content of DHA at the *sn*-2 position of tuna oil is higher than that at the *sn*-1,3 positions [25,30]. In contrast, EPA and DHA in seal oil distribute predominantly at the *sn*-1,3 positions [31,32] (Table 13.2). Harp seal oil was reported to reduce plasma and liver TAGs more effectively than fish oils [32]. Thus, the TAGs rich in DHA at the *sn*-1,3 positions might possess useful physiological activity similar to seal oil.

V. PURIFICATION OF PUFA BY SELECTIVE ESTERIFICATION

The FA specificity of a lipase is stricter in esterification than in hydrolysis and alcoholysis [19,33,34]. Hence, purification of PUFA using selective esterification has been attempted. When an FFA mixture including PUFA was esterified with alcohol using a lipase, which acts on PUFA weakly, PUFA is purified in the FFA fraction. Hills et al. [35] first applied lipase-catalyzed esterification to purification of GLA. They esterified FFAs originating from evening primrose oil with 2 mol of *n*-butanol in *n*-hexane using immobilized *R. miehei* lipase and succeeded in enriching GLA to 85% with a recovery of 64%. Other groups also reported that selective esterification was effective for increasing the content of GLA to near 90% [36–38]. Ju and Chen [39] achieved a process comprising solvent fractionation and selective esterification. FFAs from borage oil were subjected to a two-step solvent crystallization in acetonitrile and acetonitrile/acetone (3:7, v/v), increasing the purity of GLA to 92%. The resulting FFA mixture was esterified with equimolar of *n*-butanol using *R. miehei* lipase in *n*-hexane, resulting in an increase of the purity to 99%. The selective esterification with *n*-butanol is effective, but a large amount of *n*-hexane is required

to attain high purity of the purification. An organic solvent-free reaction system is therefore strongly desirable from an industrial viewpoint.

A. Organic Solvent-Free Reaction System

The reaction mixture after selective esterification contains alcohol, FFAs, and FA esters. Fractionation of the three compounds by short-path distillation is very suitable for industrial-scale purification. Adoption of short-path distillation requires a large difference among the molecular weights (boiling points) of the three compounds. Screening test showed that lauryl alcohol (LauOH) was a suitable alcohol [40]. *Rhizopus oryzae* (reclassified from *Rhizopus delemar*) lipase was found to be a suitable enzyme for the purification of GLA by selective esterification of FFAs originating from borage oil (FFA-borage) with LauOH in an organic solvent-free system. A typical time course of the selective esterification is shown in Figure 13.5. The contents of palmitic, oleic, and linoleic acids in the FFA fraction decreased with the increase in esterification, and the content of GLA reached 76% (3.4-fold) at 16 h with 81% recovery of the initial content [19].

B. Strategy for Purification of GLA

The first step is nonselective hydrolysis of borage oil. The oil can be hydrolyzed by heating with a large amount of ethanol (EtOH) under alkaline conditions. However, this procedure requires a large-scale reactor and carries the risk of isomerization of GLA. Also, the waste water in the chemical process contains EtOH and has a high chemical oxygen demand (COD). Furthermore, pH of the reaction mixture has to be readjusted to acidic conditions to recover the FFAs. On the other hand, when industrial-scale hydrolysis is conducted using a lipase, FFAs can be recovered easily from the reaction mixture by short-path distillation. Hence, enzymatic hydrolysis was adopted. The second step is enrichment of GLA in the FFA fraction by selective esterification of the resulting FFAs with LauOH using *R. oryzae* lipase, which acts on GLA weakly. Because molecular weights of the components (LauOH, FFAs, and FA lauryl esters [FALEs]) in the reaction mixture are largely different, they can be separated easily by short-path distillation. GLA was purified from borage oil according to this strategy.

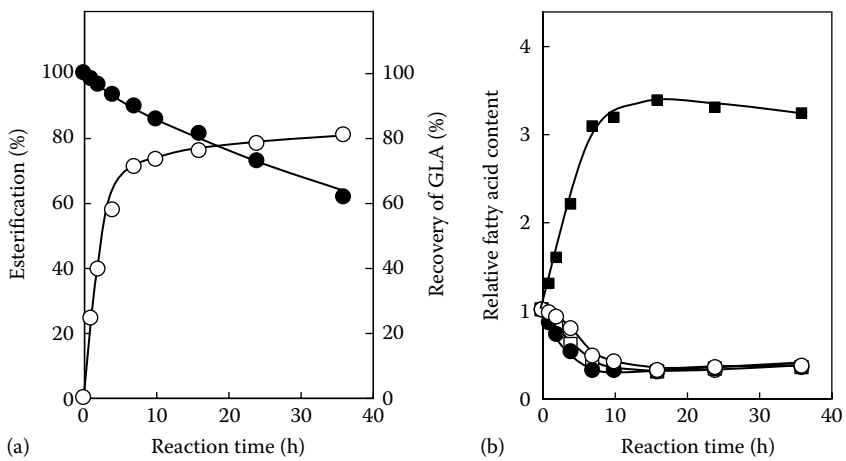

FIGURE 13.5 Time course of selective esterification of FFA-borage with LauOH using *Rhizopus oryzae* lipase. A mixture of FFA-borage/LauOH (1:2, mol/mol), 20% water, and 200 U/g-mixture of *R. oryzae* lipase was incubated at 30°C with stirring. (a) Degree of esterification (○) and recovery of GLA in the FFA fraction (●). (b) Content of FA in the FFA fraction. The content of each FA was expressed relative to the initial content of the FA in FFA-borage. ○, Palmitic acid (the content in FFA-borage, 9.7%); ●, oleic acid (17.2%); □, linoleic acid (37.3%); ■, GLA (22.4%).

C. Large-Scale Purification of GLA

The process is shown in Figure 13.6. An oil (10 kg) containing 45% GLA, which was produced industrially by selective hydrolysis of borage oil as described in Section III.B, was used as a starting material for the purification of GLA. The GLA-rich oil was first hydrolyzed nonselectively with *Burkholderia cepacia* lipase, which acted on GLA as strongly as on the other C_{18} FAs. The FFAs were recovered by short-path distillation and were used for their esterification with LauOH using *R. oryzae* lipase. The selective esterification of the FFAs with LauOH using the lipase increased the content of GLA from 46% to 89%. To further increase the purity, the FFAs were recovered by short-path distillation and were esterified again with LauOH. The repeated esterification raised GLA purity in the FFA fraction to 98%. The FFA fraction recovered from the reaction mixture by distillation contained 14% FALEs and 1% LauOH. The FALEs were completely removed by urea adduct fractionation, but 0.8% LauOH remained. The FFAs with 99% GLA were prepared with a recovery of 49% of the initial content of GLA-rich oil by a series of the purification procedures [41].

D. Purification of Other PUFAs

The process comprising nonselective hydrolysis of natural oil for the preparation of FFAs and selective esterification of the FFAs can also be applied to purification of other PUFAs. DHA was purified

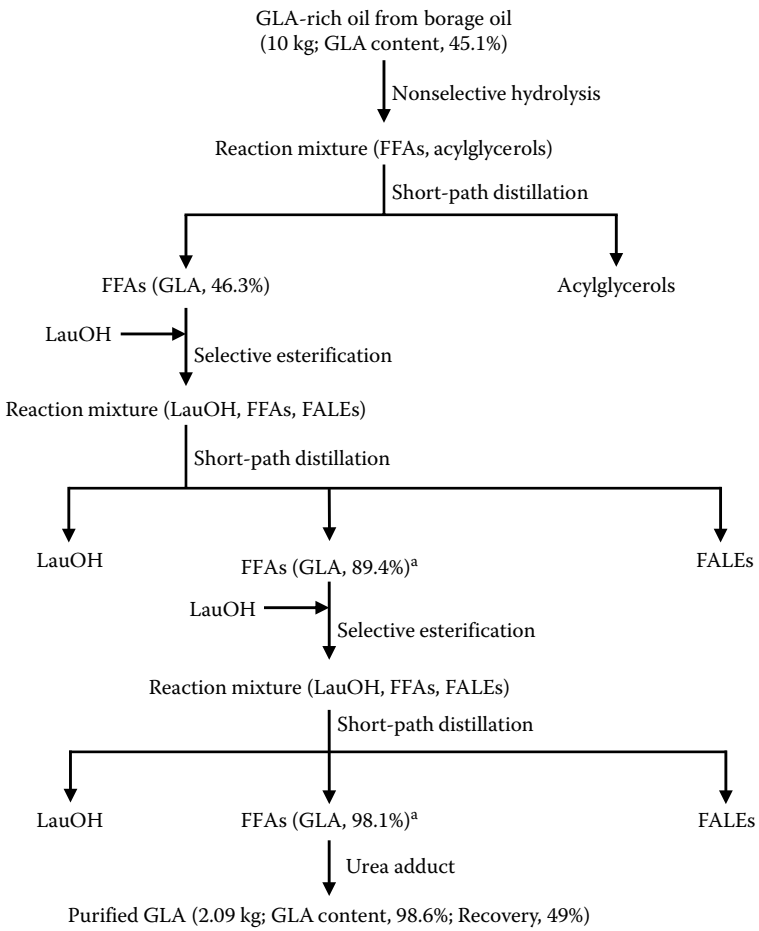

FIGURE 13.6 Large-scale purification of GLA. *Note*: [a]The FFA fraction was contaminated with about 15% FALEs.

from tuna oil to the purity of 91% in a 60% yield [42]. AA was similarly purified from *Mortierella alpina* single-cell oil containing 40% AA. Because *R. oryzae* lipase acted moderately on AA in the selective esterification, *C. rugosa* lipase, which acted on AA weakly, was selected [43]. The selective esterification two times raised the content of AA to 81%. Since the single-cell oil contained 3% GLA and 5% dihomo-GLA (DGLA) and *C. rugosa* lipase acted on these FAs as weakly as on AA, not only AA but also these FAs were enriched in the FFA fraction and the total content of n-6 PUFAs reached 96%. Recoveries of AA and n-6 PUFAs were 53% and 52% of their initial contents in the single-cell oil, respectively [18]. To remove GLA and DGLA from the original FFAs, *B. cepacia* lipase was the most suitable. The lipase esterified AA more weakly than GLA and DGLA and discriminated efficiently between AA and GLA/DGLA. A series of procedure comprising nonselective hydrolysis, urea adduct fractionation, and two-step selective esterification with *B. cepacia* lipase raised the content of AA to 97% with 49% recovery [44]. The selective esterification with lipase also applied to the purification of DGLA from *M. alpina* single-cell oil (DGLA content, 39%). In the purification process, a combination of two-step selective esterification with *C. rugosa* lipase and one-step selective esterification with *Pseudomonas aeruginosa* lipase was suitable, and DGLA was purified to 95% in a yield of 51% [45]. It was also reported that stearidonic and pinolenic acids were purified by similar processes with lipases [46–48].

VI. PURIFICATION OF DHA ETHYL ESTER BY SELECTIVE ALCOHOLYSIS

Development of EPAEE as a pharmaceutical requires an effective purification process of some functional PUFAEEs. Free PUFAs, which were purified by a process involving selective esterification as described in Section V, were converted to their ethyl esters with 96% ethyl esterification using immobilized *P. antarctica* lipase [49]. However, adoption of this process demands three-step enzyme reactions: nonselective hydrolysis of PUFA-containing oil, selective esterification of the FFA mixture with LauOH, and ethyl esterification of the recovered PUFA. If selective alcoholysis of fatty acid ethyl esters (FAEEs) with LauOH is possible, a desired PUFAEE can be enriched through two-step enzyme reactions: nonselective ethanolysis of PUFA-containing oil and selective alcoholysis of the resulting FAEEs. Purification of DHA ethyl ester (DHAEE) was conducted according to this strategy.

A. Ethanolysis of Tuna Oil

The first step is ethanolysis of tuna oil. Ethyl esters of unstable PUFAs can be prepared by chemical ethanolysis of TAG with an alkaline catalyst. However, because heating under alkaline conditions often results in the isomerization of PUFAs, enzyme reaction under mild conditions is preferable.

Breivik et al. [50] first reported that immobilized *P. antarctica* lipase efficiently catalyzed the ethanolysis of TAGs containing DHA and EPA. However, the lipase could not be used repeatedly in their system although it was immobilized. This drawback was clarified to be due to inactivation of the lipase by contact with ethanol (EtOH) that was insoluble in the reaction mixture, and ethanolysis system by stepwise addition of EtOH was attempted [51,52].

Immobilized *P. antarctica* lipase was inactivated irreversibly in the presence of 2/3 molar equivalent of EtOH against the total FAs in tuna oil. To avoid such inactivation, stepwise ethanolysis system was developed. The first step reaction was conducted in a mixture of tuna oil and 1/3 molar equivalent of ethanol with 4% immobilized *P. antarctica* lipase. After ethanol was consumed and 33% of tuna oil was converted to its corresponding ethyl esters, the second step reactions were conducted by adding 1/3 molar equivalent of EtOH. Finally, the third step ethanolysis was conducted by adding EtOH similarly [51]. Typical time courses are shown in Figure 13.7. The stepwise ethanolysis converted more than 95% FAs in tuna oil to their ethyl esters. In addition, recycling of the reactions using the same immobilized lipase showed that the enzyme preparation was used for more than 50 cycles (100 days) without significant decrease of the conversion (half-life of the activity, 65 days).

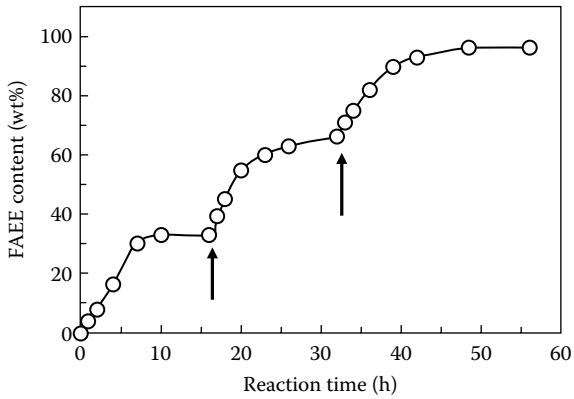

FIGURE 13.7 Three-step ethanolysis of tuna oil using immobilized *Pseudozyma antarctica* lipase. The first reaction was performed at 40°C in a mixture of tuna oil, 1/3 molar equivalent of EtOH against the total FAs in tuna oil, and 4% immobilized *P. antarctica* lipase. After 16 and 32 h, as indicated by 1/3 molar equivalent of EtOH were added, respectively.

The reaction mixture was subjected to short-path distillation. The fractionation enriched DHAEE from 24% to 57% without significant loss of DHAEE. The preparation is named DHAEE57.

B. Selective Alcoholysis of FAEE Originating from Tuna Oil with LauOH

Selective alcoholysis of DHAEE57 with LauOH proceeded efficiently when either immobilized *R. oryzae* or *R. miehei* lipase was used in a nonaqueous system. *R. oryzae* lipase attained high recovery of DHAEE, and *R. miehei* lipase was suitable when high purity was desired [33,53]. In the selective alcoholysis with LauOH using immobilized *R. miehei* lipase, increasing quantities of LauOH improved FA selectivity and raised the purity of DHAEE [54]. DHAEE57 underwent alcoholysis with 7 mol of LauOH using immobilized *R. miehei* lipase, and the time course is shown in Figure 13.8. The degree of alcoholysis reached 60% after 26 h, and the purity of DHAEE increased to 90% (1.6-fold). The time course indicated that FA selectivity of the lipase is in the order of oleic acid > n-3 docosapentaenoic acid (DPA) > EPA > AA > DHA, n-6 DPA (Figure 13.8b). Because the lipase acted on n-6 DPA as weakly as on DHA, the content of n-6 DPA could not be decreased in the selective reaction.

Alcoholysis of DHAEE57 with LauOH using immobilized *R. miehei* lipase was conducted in a batchwise operation and in a continuous flow operation with a fixed-bed bioreactor. The two reactions enriched DHAEE in the FAEE fraction at the same efficiency, and half-life of the lipase activity was 150 days [54].

C. Purification of DHAEE from Tuna Oil

Purification of DHAEE from tuna oil by selective alcoholysis is summarized in Figure 13.9. Ethanolysis of tuna oil (4.5 kg) was conducted first according to Section VI.A, and DHAEE was enriched by short-path distillation. The content of DHA was increased to 57.2% (FAEE, 1.62 kg; yield of DHA, 91%). A mixture of the FAEE fraction and 7 mol of LauOH was introduced to a column packed with immobilized *R. miehei* lipase. Immediately after introducing the mixture, the content of DHAEE in the FAEE fraction increased to 90% with 58% alcoholysis. Even after 150 days, the content of DHAEE remained 87%, although alcoholysis decreased to 48%. The reaction mixture (7.86 kg) flowing from the column was applied to short-path distillation after evaporation of a product (EtOH) for recovering FAEEs. The FAEE fraction (650 g) consisted of 2.4%

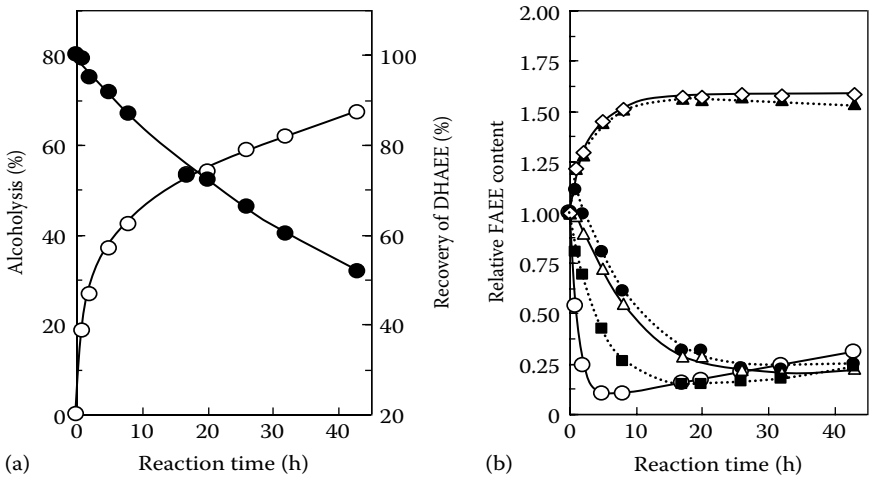

FIGURE 13.8 Selective alcoholysis of DHAEE57 with LauOH using immobilized *R. miehei* lipase. The reaction was performed at 30°C in a mixture of DHAEE57/LauOH (1:7, mol/mol) and 4% immobilized lipase with shaking. (a) Degree of alcoholysis (○) and recovery of DHAEE in the FAEE fraction (●). (b) Content of FAEE in the FAEE fraction. The content of each FAEE was expressed relative to the initial content. ○, Oleic acid ethyl ester (initial content, 3.6%); ■, n-3 DPAEE (2.6%); △, EPAEE (11.3%); ●, AA ethyl ester (AAEE) (2.7%); ▲, n-6 DPA ethyl ester (DPAEE) (4.1%); ◇, DHAEE (57.2%).

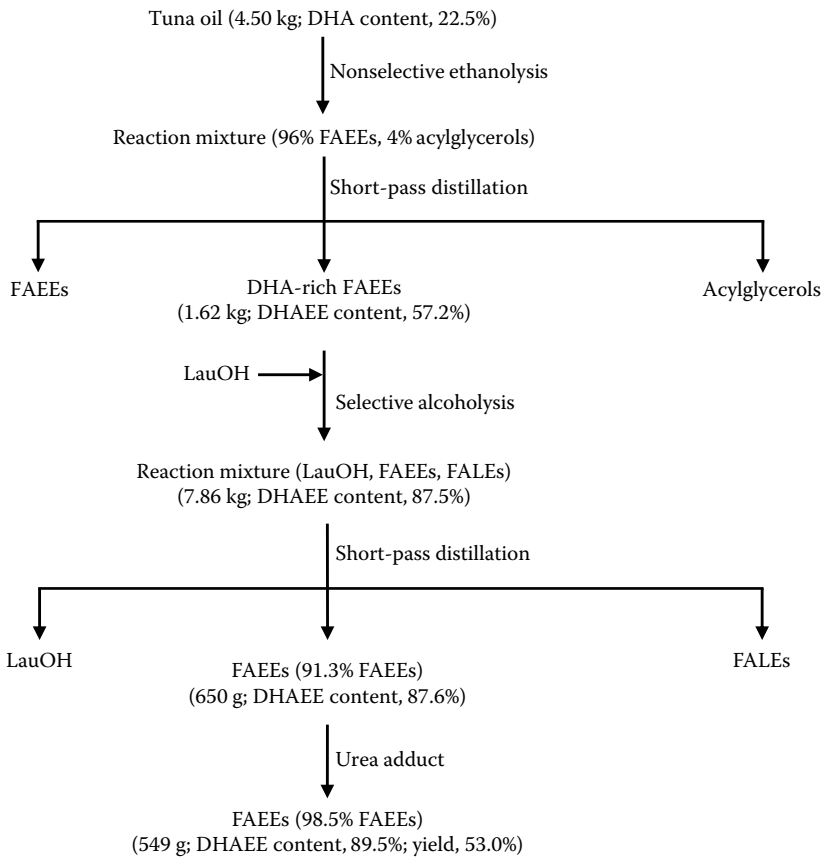

FIGURE 13.9 Purification of DHAEE from tuna oil by a process including selective alcoholysis.

LauOH, 91.3% FAEEs, and 6.3% FALEs. The FALEs could be completely removed by urea adduct fractionation. Through a series of purifications, DHAEE content was raised to 90% in a 53% yield of the initial content in DHAEE57 [54].

VII. ENZYMATIC FRACTIONATION AND ENRICHMENT OF CONJUGATED LINOLEIC ACID ISOMERS

Conjugated linoleic acid (CLA) is a group of C_{18} fatty acids containing a pair of conjugated double bonds in either *cis* or *trans* configuration [55]. It is industrially produced by alkali conjugation of safflower or sunflower oil containing linoleic acid (C18:2), and the resulting FFA mixture (named FFA-CLA) contains almost equal amounts of 9-*cis*,11-*trans* (9*c*,11*t*)-CLA and 10-*trans*,12-*cis* (10*t*,12*c*)-CLA.

FFA-CLA has several physiological activities [55], including decrease in cancer incidence [56–61], beneficial effects on atherosclerosis [62,63], decrease in body fat content [64–66], and improvement in immune functioning [67]. Moreover, each isomer has different physiological activities. 9*c*,11*t*-CLA has anticancer activity [68] and 10*t*,12*c*-CLA acts to decrease body fat content [69–72], increase energy expenditure [73], and suppress development of hypertension [74]. In order to develop nutraceuticals containing each CLA isomer at particular level, a large-scale process for fractionation of each CLA isomer from FFA-CLA is strongly desired.

Many lipases act on CLA isomers to almost the same extent; however, Haas et al. [75] first found that *Geotrichum candidum* lipase recognized 9*c*,11*t*-CLA more strongly than 10*t*,12*c*-CLA. They successfully enriched 9*c*,11*t*-CLA in the methyl ester fraction at early stage of the reaction in which a mixture of CLA isomers was esterified with methanol in an organic solvent system. In addition, 9*c*,11*t*-CLA was enriched in the FFA fraction by hydrolysis of methyl esters of CLA isomers. The purity of CLA isomers can be increased by their procedure, but the recovery is not good. McNeill et al. [76] esterified a mixture of CLA isomers with LauOH using *G. candidum* lipase and separated the reaction mixture into the FFA fraction (10*t*,12*c*-CLA rich) and FALE fraction (9*c*,11*t*-CLA rich) through short-path distillation. The procedure allowed them to fractionate CLA isomers with a good recovery, but the purity was not high. Furthermore, *G. candidum* lipase is commercialized only as a reagent; thus, it is not suitable as a catalyst for oil processing.

Anamorphic yeast *C. rugosa* and fungus *G. candidum* belong to the same order, *Saccharomycetales*, as a microorganism classification. Lipases from the two strains have 45% overall homology and their crystal structures are very similar [77]. Thus, it was strongly assumed that the two lipases possess similar FA specificity: actually, *C. rugosa* lipase also acted on 9*c*,11*t*-CLA more strongly than on 10*t*,12*c*-CLA (Figure 13.10). Since *C. rugosa* lipase has been used for oil processing widely, it was applied to fractionation and enrichment of 9*c*,11*t*- and 10*t*,12*c*-CLAs.

A. Selective Esterification of FFA-CLA with LauOH

Figure 13.11 shows strategy for fractionation and enrichment of 9*c*,11*t*- and 10*t*,12*c*-CLAs with *C. rugosa* lipase. FFA-CLA prepared from safflower oil was used as a starting material, and the process comprised repeated selective esterification, short-pass distillation, urea adduct fractionation, and hydrolysis with NaOH [78]. The first step composed of selective esterification and short-pass distillation was fractionation of FFA-CLA to 10*t*,12*c*-CLA-rich FFAs and 9*c*,11*t*-CLA-rich FALEs. The second step was enrichment of each CLA isomer from the resulting FFAs and FALEs fractions. The enrichment of 10*t*,12*c*-CLA was conducted through selective esterification of 10*t*,12*c*-CLA-rich FFAs, short-pass distillation, and urea adduct fractionation. Meanwhile, the enrichment of 9*c*,11*t*-CLA was conducted by the hydrolysis of 9*c*,11*t*-CLA-rich FALEs, selective esterification, and short-pass distillation; followed by the hydrolysis of the resulting FALEs and

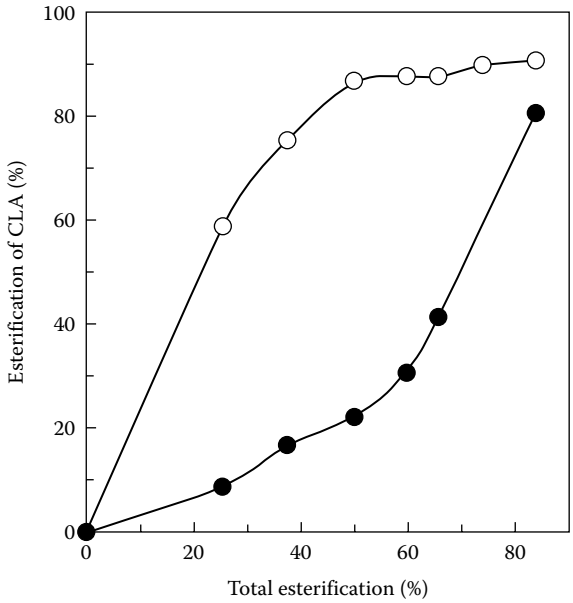

FIGURE 13.10 Selective esterification of FFA-CLA with LauOH using *C. rugosa* lipase. A mixture of 2.4 g FFA, 1.6 g LauOH, and 1.0 g water was stirred at 30°C for 16 h with 4, 8, 20, 40, 80, 120, and 250 U/g-mixture of lipase. Relationships between the degree of esterification of total fatty acids and the degree of esterification of each CLA isomer are shown. ○, 9*c*,11*t*-CLA; ●, 10*t*, 12*c*-CLA.

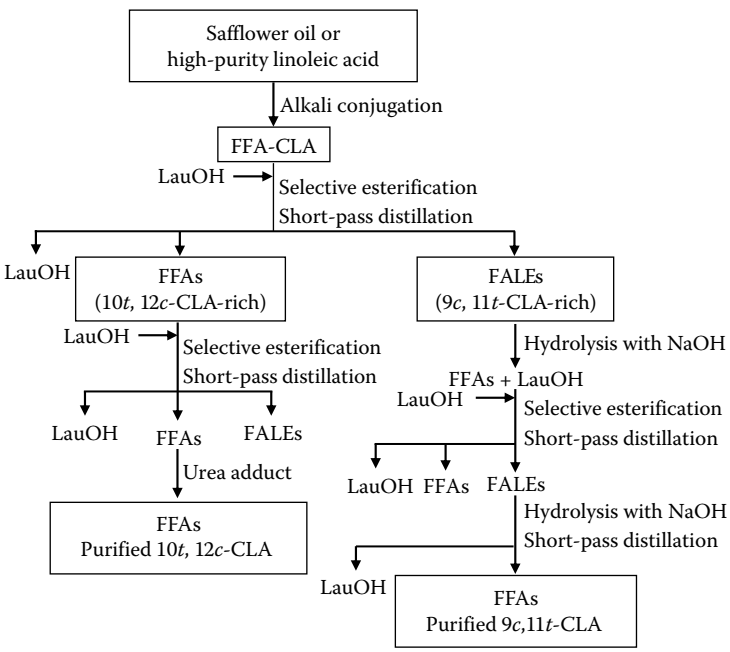

FIGURE 13.11 Fractionation and enrichment of CLA isomers with LauOH and FFA-CLA using *C. rugosa* lipase.

TABLE 13.3
Comparison of Five Methods for Fractionation and Enrichment of CLA Isomers

				CLA Composition (wt%)			Isomer Ratio[a] (%)		Yield (%)
Alcohol (%)									
Origin of FFA-CLA									
Product	16:0	18:0	18:1	9*c*,11*t*	*t*10,*c*12	Others	9*c*,11*t*	10*t*,12*c*	
LauOH[b]									
Safflower oil									
Original	6.7	2.7	17.0	33.1	33.9	4.1	49.4	50.6	
10*t*,12*c*-CLA	ND	ND	2.5	4.2	78.8	9.6		94.9	52
9*c*,11*t*-CLA	ND	ND	9.5	83.5	3.8	1.3	95.6		35
LauOH[b]									
Linoleic acid									
Original	ND	ND	2.0	45.1	46.8	5.3	49.1	50.9	
10*t*,12*c*-CLA	ND	ND	ND	3.0	95.3	1.3		96.9	31
9*c*,11*t*-CLA	ND	ND	1.5	93.1	3.5	0.4	96.4		34
Glycerol[c]									
Safflower oil									
10*t*,12*c*-CLA	4.6	3.7	8.6	13.2	58.3	10.2		81.5	23
9*c*,11*t*-CLA	5.0	1.5	20.1	49.3	18.3	3.4	72.9		27
L-Menthol[b]									
Safflower oil									
10*t*,12*c*-CLA	6.0	5.0	5.1	6.7	66.7	10.4		90.9	40
9*c*,11*t*-CLA	1.7	0.4	18.4	74.2	4.5	0.8	94.3		42
Sterols[b]									
Safflower oil									
10*t*,12*c*-CLA	5.3	3.6	4.7	8.2	65.6	11.6		88.9	47
9*c*,11*t*-CLA	5.3	0.3	12.6	69.8	8.5	1.5	89.1		24

[a] Isomer ratio of 9*c*,11*t*-CLA/(9*c*,11*t*-CLA + 10*t*,12*c*-CLA) × 100 or isomer ratio of 10*t*,12*c*-CLA/(9*c*,11*t*-CLA + 10*t*,12*c*-CLA) × 100.

[b] Selective reaction was esterification with each alcohol.

[c] Selective reaction was hydrolysis of Gly-CLA.

short-pass distillation. Consequently, FA composition of the purified 10*t*,12*c*-CLA preparation was 4.2% 9*c*,11*t*-CLA, 78.8% *t*10,*c*12-CLA, and 9.6% the other CLA isomers (Table 13.3). Isomer ratio of 10*t*,12*c*-CLA (ratio of 10*t*,12*c*-CLA based on the total content of 9*c*,11*t*- and 10*t*,12*c*-CLAs) reached to 94.9%. Meanwhile, FA composition of the purified 9*c*,11*t*-CLA preparation was 83.5% 9*c*,11*t*-CLA, 3.8% 10*t*,12*c*-CLA, and 1.3% the other CLA isomers. Isomer ratio of 9*c*,11*t*-CLA reached to 95.6%.

B. Selective Esterification of FFA-CLA from High Purity of Linoleic Acid with LauOH

When the two CLA isomers were purified from FFA-CLA originated from safflower oil, the 9*c*,11*t*-CLA preparation was contaminated with 9.5% oleic acid (C18:1) and the 10*t*,12*c*-CLA preparation was contaminated with 9.6% the other CLA isomers. Then a decrease in the contents of these contaminants was attempted. The strategy for the preparation of high purity of the two CLA isomers was almost the same as that described in Section VII.A. The differences

were the use of high-purity (97%) linoleic acid instead of safflower oil and the improvement of urea adduct fractionation (Figure 13.11) [79].

The use of high-purity linoleic acid increased the purity of 9*c*,11*t*-CLA to 93.1% and decreased the content of C18:1 to 1.5% (isomer ratio of 9*c*,11*t*-CLA, 96.4%) (Table 13.3). Urea adduct fractionation for eliminating the other CLA isomers was conducted by decreasing the amount of FFAs against urea. Consequently, the purity of 10*t*,12*c*-CLA increased to 95.3% and the content of the other CLA isomers decreased to 1.3% (isomer ratio of 10*t*,12*c*-CLA, 96.9%) (Table 13.3).

C. Selective Hydrolysis of Acylglycerols with CLA

The procedures described in Sections VII.A and VII.B are suitable for the preparation of highly purified CLA isomers, but LauOH and urea cannot be used in the process for the production of food materials. Food material containing high concentration of each CLA isomer was produced according to a strategy shown in Figure 13.12 [80].

At first, acylglycerols were synthesized by nonselective esterification of FFA-CLA from safflower oil with glycerol using immobilized *R. miehei* lipase, which acted similarly on 9*c*,11*t*- and 10*t*,12*c*-CLAs. The resulting acylglycerols (Gly-CLA) were purified through short-path distillation.

Selective hydrolysis of Gly-CLA with *C. rugosa* lipase, which acted on 9*c*,11*t*-CLA more strongly than on 10*t*,12*c*-CLA, enriched 9*c*,11*t*-CLA in the FFAs fraction and 10*t*,12*c*-CLA in the acylglycerols fraction. When the degree of hydrolysis was 30%, isomer ratio of 9*c*,11*t*-CLA in the FFAs fraction reached 72.9% (Table 13.3). Because the isomer ratio of 10*t*,12*c*-CLA in the remaining acylglycerol fraction (1st-Gly) was still low (65.0%), the acylglycerols were hydrolyzed again with *C. rugosa* lipase. Consequently, the purity of 10*t*,12*c*-CLA in the acylglycerol fraction (2nd-Gly) increased to 81.3% at 60% hydrolysis.

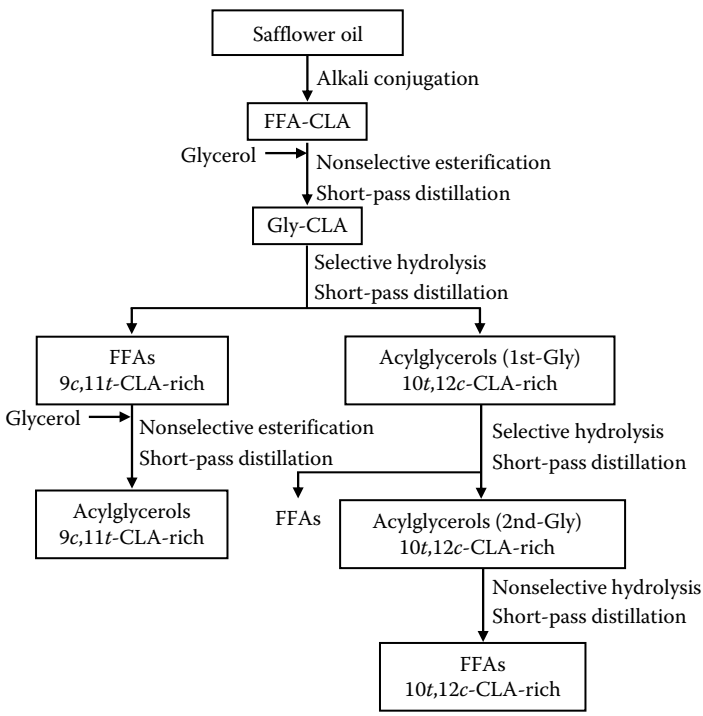

FIGURE 13.12 Fractionation of CLA isomers with glycerol and FFA-CLA using *C. rugosa* lipase.

The 9*c*,11*t*- and 10*t*,12*c*-CLAs concentrates were FFAs and acylglycerols, respectively. The FFAs were converted to acylglycerols by their nonselective esterification with glycerol using *R. miehei* lipase. In addition, the acylglycerols were converted to FFAs without any decrease of the isomer ratio of 10*t*,12*c*-CLA (81.5%, Table 13.3), when the degree of hydrolysis was raised to >80% with a large amount of *C. rugosa* lipase.

D. Selective Esterification of FFA-CLA with L-Menthol

The CLA isomer concentrates prepared by the procedure described earlier can be used as food materials. The isomer ratio of each CLA isomer is, however, still low (Table 13.3), and their recovery cannot satisfy (23%–27%) either. We thus developed an efficient process for the production of purified CLA isomers available as food supplements. L-Menthol has been widely applied for food production, and *C. rugosa* lipase catalyzes the esterification of FFAs with L-menthol efficiently [81–83]; thus, an alternative process with L-menthol was attempted.

The process is composed of repeated selective esterification, short-pass distillation, hydrolysis with NaOH, and *n*-hexane fractionation (Figure 13.13) [84]. The isomer ratio and recovery of each CLA isomer of the resulting products were higher than those produced by the process containing selective hydrolysis of Gly-CLA described in Section VII.C (Table 13.3). However, the fractionation of FFAs and menthol esters by short-pass distillation was not so good. This result was due to a small difference in MWs of menthol ester (*ca*. 418) and FFAs (*ca*. 280). Hence, *n*-hexane fractionation was

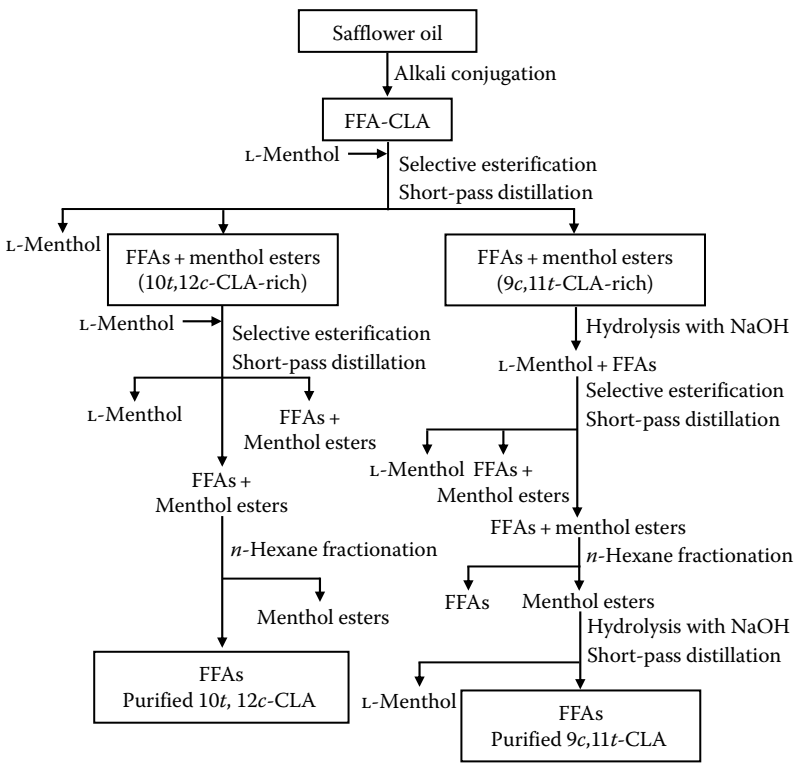

FIGURE 13.13 Fractionation and enrichment of CLA isomers with L-menthol and FFA-CLA using *C. rugosa* lipase.

adopted for the separation of menthol esters and FFAs. As a result, high purity of CLA isomers was produced in a good yield (Table 13.3).

E. Selective Esterification of FFA-CLA with Sterols

Although the process containing selective esterification with L-menthol may be applied to the production of CLA with high isomer ratio, the process was a little complicated. Furthermore, chemical hydrolysis of L-menthol esters with NaOH is not suitable for unstable FFAs. This problem can be solved by developing a system for the hydrolysis of CLA esters with enzyme.

Sterols present in oil seeds are a mixture of campesterol, stigmasterol, β-sitosterol, and so on. The esters of FA with the sterols can be hydrolyzed with lipases, although the degree of hydrolysis is not so high (*ca*. 50%) [85,86]. Hence, the enzymatic hydrolysis may be applied to the conversion of FFA-CLA esters to FFAs instead of chemical hydrolysis. In addition, because *C. rugosa* lipase acts on 9*c*,11*t*-CLA more strongly than 10*t*,12*c*-CLA, the selectivity may be effective for further enrichment of 9*c*,11*t*-CLA. Figure 13.14 shows a process for fractionation and enrichment of CLA isomers using sterols as substrates in selective esterification. The process was composed of repeated selective esterification, short-pass distillation, selective hydrolysis, and *n*-hexane precipitation [87].

The purified 10*t*,12*c*-CLA preparation was enriched to an 88.9% of isomer ratio with a 47% yield after the repeated selective esterification and short-pass distillation (Table 13.3). The purified 9*c*,11*t*-CLA preparation was enriched to an 89.1% of isomer ratio after the selective esterification, short-pass distillation, selective hydrolysis, *n*-hexane precipitation to remove sterols, and short-pass distillation, although the yield of purified 9*c*,11*t*-CLA preparation was low.

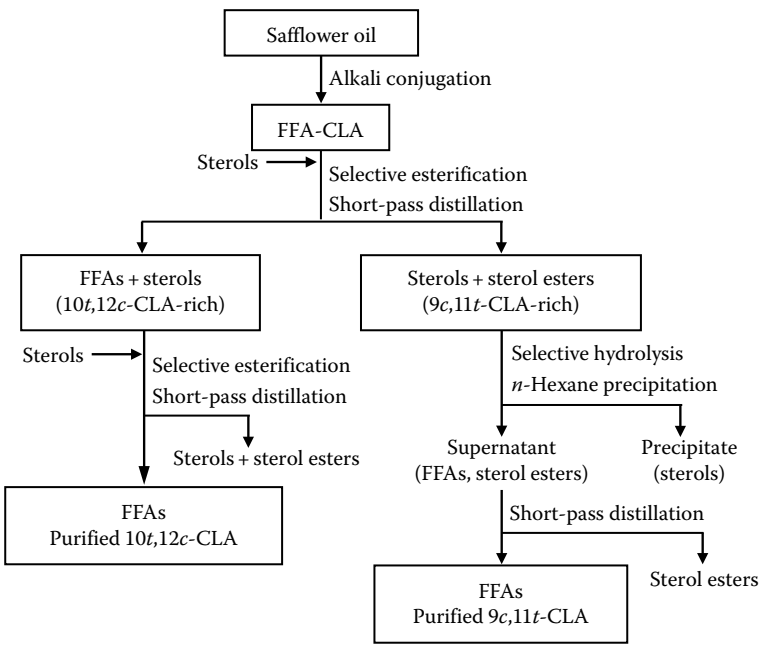

FIGURE 13.14 Fractionation and enrichment of CLA isomers with sterols and FFA-CLA using *C. rugosa* lipase.

F. Comparison of Five Processes for Fractionation and Enrichment of CLA Isomers

Five processes have been described for enzymatic fractionation and enrichment of CLA isomers. The isomer ratios of CLA preparations obtained from these processes decreased in the following order: esterification of FFA-CLA from linoleic acid with LauOH (Section VII.B) > esterification of FFA-CLA from safflower oil with LauOH (Section VII.A) > esterification of FFA-CLA from safflower oil with L-menthol (Section VII.D) > esterification of FFA-CLA from safflower oil with sterols (Section VII.E) > hydrolysis of glycerides of FFA-CLA from safflower oil (Section VII.C) (Table 13.3). These results showed that FA specificity of *C. rugosa* lipase was stricter in esterification than in hydrolysis. In addition, the FA specificity of the lipase became strict in the order of esterification with LauOH > with L-menthol > sterol. Namely, it may suggest that FA specificity of lipases is changeable by devising reaction system.

VIII. CONCLUSION

Lipases have been used as tools for the production of functional lipids or for the improvement of natural oils and fats. The lipase-catalyzed reactions are also very effective for an industrial-scale purification of unstable substances including PUFAs and fractionation of CLA isomers. When a desired compound is purified by a process including enzyme reaction, downstream purification procedure should be decided first, and then an enzyme reaction system suitable for the purification procedure must be constructed. Short-path distillation discussed in this chapter can be used to treat large amounts of reaction mixture for a short time, but not expected to result in precise separation. This drawback is conquerable by constructing the selective reaction system, which enlarges the difference in the molecular weights (boiling points) of a desired compound and contaminants. The strategy, which combines selective enzyme reaction and short-path distillation, is very useful not only for the purification of unstable FAs but also for the purification of a desired compound from unutilized materials. The authors hope that lipases will further be applied in the oil and fat industry.

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14 Microbial Lipid Production

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CONTENTS

I. INTRODUCTION

Microbial lipids, or "single cell oils" (SCOs), can be defined as microbial lipophilic compounds or oils obtained from microorganisms and are considered as alternative sources of edible oils because of their similarity in type and composition to vegetable or animal oils [1]. Lipids are mostly stored as triacylglycerols (TAGs) and small amounts of sterol esters in microorganisms [2]. As it is well known, microorganisms have many advantages over plants and animals such as higher growth rate, higher lipid content, less sensitivity to seasonal or climatic changes, and possibility of production in small areas. Oil yield and composition of the oil can be developed or modified by adjusting cultural conditions. Moreover, they can be improved by genetic modifications much more easily than higher organisms. Therefore, microbial lipids have attracted considerable interest during the past decade as well as other microbial metabolites [2–4].

All microorganisms produce lipids and store them as energy storage molecules and provide fatty acids and sterols for biogenesis and maintenance of cell membranes. Microorganisms accumulating oil higher than 20% of their biomass weight are called "oleaginous microorganisms" [5].

The number of microorganisms that are capable of accumulating oil higher than 20% of their biomass is relatively low compared to the total number of microorganisms. However, in some microorganisms, oil accumulation can exceed 70% of the biomass. Oleaginous microorganisms are mainly yeast and mold, but there are few bacteria that produce high amounts of edible oil. Compared to yeast, other microorganisms and microalgae have a lower lipid accumulation capacity [6]. On the other hand, lipids produced from some algae may contain higher amounts of polyunsaturated fatty acids (PUFAs) similar to fish oil.

One of the most important factors limiting microbial oil production is the cost. Possibility of using several agricultural and industrial wastes and by-products such as glycerol, sweet potato waste, tomato waste, beet molasses, orange-peel extracts, soluble starch, waste rice straw, and whey was investigated in order to reduce the cost [7–16]. Saygün et al. [17] used various oils and oil industry residues (such as linseed oil, borage oil, canola oil, sesame oil, *Echium* oil, trout oil, olive pomace oil, hazelnut oil press cake, and sunflower seed oil cake) as carbon sources for lipid and metabolite production from *Yarrowia lipolytica* YB 423-12.

II. LIPID ACCUMULATION IN OLEAGINOUS MICROORGANISMS

Lipogenesis, that is a process of converting acetyl-CoA to fatty acids is a secondary metabolic activity in living organisms. The main factors affecting lipogenesis are temperature, pH, and oxygen. When hydrophobic substrates are used during lipogenesis, this process is called "ex novo" lipid synthesis that results in the formation of lower quantities of TAGs. In the case where sugar-based substrates (such as glucose, fructose, lactose, sucrose, whey, molasses) are used for microbial growth, this process is called "de novo" lipid synthesis which is an anabolic biochemical process, generating mainly TAGs and also phospholipids, sphingolipids, etc. [18,19]. For "de novo" lipid accumulation, ethanol has been considered as an advantageous substrate since no residual carbon arises from its usage [5,20,21].

Oleaginous microorganisms produce acetyl-CoA, which is the basic unit of fatty acid biosynthesis. Pyruvic acid is produced after glycolysis, which is then converted to acetyl-CoA with pyruvate dehydrogenase. Acetyl-CoA enhances the biosynthesis of cellular fatty acids [5]. Lipid accumulation in microorganisms occurs at stationary phase. Lipid production depends on the type of the strain [10,22].

Lipid accumulation in microorganisms could be increased by excess amounts of carbon in the growth medium and also starving the cells for supply of nitrogen or a nutrient other than carbon [23,24]. Nitrogen limitation is the best method for lipogenesis. At the beginning of growth, nitrogen source is exhausted for the synthesis of proteins and nucleic acids, and after that cell proliferation ceases, excess carbon is assimilated by the cells and converted to storage lipids in oleaginous microorganisms. However, in non-oleaginous species, excess carbon remains unutilized or used for the synthesis of storage polysaccharides including glycogen, glucans, and mannans [25].

For increasing lipid production in oleaginous microorganisms, the growth medium should be formulated with a high C:N ratio, which is suitable for the species.

Genetic properties of microbial species, growth conditions, and composition of the culture medium have an influence on lipid biosynthesis. The C:N ratio, aeration, inorganic salts such as monopotassium phosphate, calcium chloride, disodium hydrogen phosphate, and copper sulfate, pH, and temperature are the most effective factors on lipid production [15].

III. POTENTIAL MICROORGANISMS FOR THE PRODUCTION OF LIPIDS

A. Bacteria

Lipid accumulation in bacterial cells is mostly in the form of phospholipid instead of TAG. Decreasing temperature is an effective method in bacterial cells to increase monounsaturated fatty acids such as palmitoleic (C16:1) and oleic acids (C18:1) [26]. Marine bacteria such as *Shewanella putrefaciens*

and *Flexibacter polymorphus*, *Alteromonas*, *Psychroflexus*, and *Vibrio marinus* are the sources of eicosapentaenoic acid (EPA). *Rhodococcus opacus* and *Clavibacter* sp. can be considered as other lipid-producing bacteria [27].

B. Yeasts

Yeasts have many advantages over bacteria, algae, and fungi for lipid biosynthesis due to their fast lipid-accumulating ability and resemblance of their TAG composition to plant oils [24,28]. Yeasts are single cell microorganisms in fungi group and preferred by many researchers since they are generally recognized as safe (GRAS) [25,29]. They have been used in the fermentation industry to produce bread, alcoholic beverages, etc., for centuries. In recent years, yeasts are utilized in the production of enzymes, antibiotics, steroid hormones, polysaccharides, as well as single cell protein (SCP) and SCO. For example, *Pichia pastoris*, *Candida* spp., and *Y. lipolytica* can be utilized for enzyme, SCP, and SCO production [30]. Some yeast species like *Cryptococcus albidus*, *Cryptococcus curvatus*, *Lipomyces lipofer*, *Lipomyces starkeyi*, *Lipomyces tetrasporus*, *R. opacus*, *Rhodosporidium toruloides*, *Rhodotorula glutinis,Trichosporon pullulans, Trichosporon beigelii*, and *Y. lipolytica* can accumulate TAGs varying from 20% to 80% in dry weight [4,25,31]. Oleaginous yeasts mainly produce myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), linoleic (C18:2), and linolenic (C18:3) acids [6].

C. Molds

In oleaginous fungi, lipid synthesis occurs mainly in the second phase of batch culture growth. In most mold species, decreasing temperature causes an increase in unsaturated fatty acid synthesis as in bacteria. *Mortierella alpina*, *Mortierella elongata*, *Mortierella isabellina*, *Mucor alpina-peyron*, *Rhizopus arrhizus*, *Cunninghamella japonica*, *Pythium ultimum*, *Pythium irregulare*, *Aspergillus terreus*, and *Cladosporium herbarum* are some examples of molds that can accumulate high amounts of lipid [32,33]. Industrial production of microbial oils was performed by several companies (J&E Sturge Ltd, United Kingdom, Idemitsu Ltd, Japan, and Martek Biosciences Corporation [now DSM (Dutch State Mines) NV]) using *Mucor circinelloides*, *M. isabellina*, *M. alpina*, and algae [33].

D. Algae

Microalgae are photosynthetic microorganisms that are capable of producing macromolecules such as oils, polysaccharides, and proteins under light conditions [34]. The faster growth rate and greater lipid content of microalgae compared to that of oilseed crops enabled researchers to develop technologies for algae utilization in the biodiesel production instead of plant oils. Its productivity can be high, up to 127,000 kg/ha year algal biomass [35]. Microbial lipids from microalgae are mostly in the form of TAGs containing especially n-3 PUFAs. There are studies on the production and accumulation of PUFA-rich TAGs using microalgae [36]. *Spirulina maxima*, *Streptomyces platensis*, *Chlorella vulgaris*, *Chlorella minutissima*, *Crypthecodinium cohnii*, *Isochrysis galbana*, *Monodus subterraneus*, *Nannochloropsis oculata*, *Phaeodactylum tricornutum*, and *Porphyridium cruentum* are some examples of microalgae that can accumulate lipids [35]. In microalgae, when nitrogen is limited and light is in excess, growth ceases and photosynthetic fixation of carbon continues. Then the C:N ratio increases and TAGs are produced from excess carbon [36].

IV. FATTY ACIDS OR PRODUCTS OF SPECIAL INTEREST

Lipids play important roles as bioactive compounds in a healthy diet. Studies on microbial lipophilic compounds have been carried out for over 100 years. Microorganisms offer potential for the production of functional oils that have beneficial effects on human health. PUFAs have attracted

more attention among microbial lipids because of the high cost of PUFAs by conventional methods. Therefore, the main target of SCO processes is to produce high-value oils using low-cost substrates or waste products. PUFAs are important targets as dietary supplements, and also numerous clinical and epidemiological studies showed their therapeutic significance. There are two major groups of PUFAs, n-6 and n-3 series, which are synthesized, respectively, from linoleic acid [18:2(n-6)] and α-linolenic acid (ALA) [18:3(n-3)].

PUFAs are obtained from seed plants (linoleic, γ-linolenic acid [GLA], and [ALA]), marine fish (docosahexaenoic acid [DHA], EPA, arachidonic acid [AA]), and certain mammals (AA). To meet the increasing demand of these functional oils, research studies were concentrated on alternative sources like genetic engineering of plants, new crops, and microorganisms. Among microorganisms, some fungi, algae, and bacteria can produce GLA, AA, EPA, and DHA [37]. Especially for fish-based n-3 PUFA, with the risks of decreasing fish reserves and accumulation of heavy metals, alternative sources are being investigated. Considering the increasing human population in addition to the limited natural PUFA sources, much research has been dedicated to PUFA production. Various microbial oils are already commercially produced, most of them containing high amounts of PUFAs such as GLA by the fungus *M. circinelloides* (J&E Sturge), AA by the fungus *M. alpina* (CABIO), and EPA by the yeast *Y. lipolytica* (E.I. Du Pont) [38]. GLA is a valuable fatty acid exerting anticarcinogenic health properties, whereas DHA and AA are found in significant amounts in human milk and have important roles in neural development in infants. DHA, which is the longest-chain unsaturated fatty acid, is essential mainly for brain and eye development [11,39].

Some products such as cocoa butter equivalent (from *Apiotrichum curvatum*), GLA (from *M. isabellina*, *Chlorella*, and *M. circinelloides*), DHA (from *Crypthecodinium* and *Schizochytrium*), and AA (from *Mortierella*, *Porphyridium* spp., and *Pythium*) have been produced commercially [37,40–43].

A. γ-Linolenic Acid

GLA has selective anticancer properties as well as other medicating features such as breast pain and eczema [44]. There are many advantages of microbial production of GLA. GLA could be accumulated in *Cunninghamella echinulata*, *M. isabellina*, *Mortierella ramanniana*, *M. alpina*, and *Mucor rouxii* [30]. This fatty acid attracted attention for its importance to human health. The main sources of GLA belong to Onagraceae and Boraginaceae species. Furthermore, there are commercial products such as Life's GLA™, which is an omega-6 product that contains GLA, derived from evening primrose oil or borage oils, that are available in either highly refined oil or powder formats [45]. Essential microbial sources of GLA are fungi (*Mucor*, *Mortierella*) and algae (*Chlorella* and *Spirulina* spp.). PUFA production was first realized with GLA in the United Kingdom and Japan using *Mucor* spp. [46]. Moreover, the first industrial-scale product as commercial microbial oil rich in GLA (18%, w/w of total fatty acids) was launched in 1985 from *M. circinelloides* by J&E Sturge Company (Selby, North Yorkshire, United Kingdom) [47].

B. Arachidonic Acid

Arachidonic acid [20:4(n-6)] is a long-chain PUFA with 20 carbon atoms and 4 double bonds [48]. The major microbial sources of AA are fungi (*Pythium*, *Mortierella* spp.), algae (*Porphyridium* spp.), and mosses (*Rhytidiadelphus*, *Brachythecium*, *Eurhynchium* spp.) [33]. *Parietochloris incisa* is the main plant source of AA (43% of total fatty acids) [49]. AA is an important PUFA in neural functions, including membrane excitability, gene transcription, apoptosis, cerebral blood flow, spatial learning, and synaptic plasticity [50]. Human milk contains AA and DHA, and there are commercial infant formula applications including microbial AA. There are several companies that produce AA-rich SCO commercially such as Suntory Corp., Japan; Wuhan Alking Bioengineering Co.,

China; DSM Co., Italy; and Belvidere, NJ, United States (for Martek Biosciences Corp.) with the brand name of Life's ARA (formerly ARASCO™) [33].

C. Eicosapentaenoic Acid

Eicosapentaenoic acid [20:5(n-3)] is an important n-3 PUFA that modulates immune function and mediates some human diseases such as rheumatoid arthritis, heart disease, cancers, schizophrenia, and bipolar disorder [51]. The major microbial sources of EPA are fungi (*Halophytophthora spinosa*, *Y. lipolytica, Mortierella, Pythium* spp.), algae (*Chlorella, Monodus, Porphyridium, Nannochloropsis, Cryptopleura, Schizymenia, Navicula* spp., *Tetraselmis* spp.), mosses (*Brachythecium, Eurhynchium, Scleropodium* spp.), and bacteria (*Rhodopseudomonas, Shewanella* spp., *F. polymorphus, Photobacterium*) [33,38,52].

D. Docosahexaenoic Acid

Docosahexaenoic acid [22:6(n-3)] is a long-chain fatty acid that plays an important role in brain and neural development as a key component in cell membranes. The major microbial sources of DHA are fungi (*Thraustochytrium*, *Entomophthora* spp.), algae (*Gonyaulax, Gyrodinium, Crypthecodinium*), and bacteria (*Colwellia, V. marinus*). Life's ARA (formerly ARASCO), Life's DHA (formerly DHASCO™), and Life's OMEGA™, which is a vegetarian source of EPA/DHA omega-3 from algae and also a DHASCO/ARASCO blend as 2:1 ARA:DHA (Formulaid™), are registered trade names of Martek Corp. Inc. (DSM, NV). Examples of infant formulas supplemented with DHA and ARA are Enfamil Lipil® (Mead Johnson Nutritionals, Evansville, IN), Similac Advance® (Ross Products, Columbus, OH), Parents Choice® (Wyeth Nutritionals International, Collegeville, PA), and Good Start® Supreme DHA & ARA (Nestlé, Switzerland) [53].

E. Production of Cocoa Butter Equivalents

Due to the problems that could be faced with cocoa butter production such as uncertainties of cocoa butter production, high and variable prices of cocoa butter, and failure of cocoa-plant protection against harmful insects and infections, chocolate producers have begun to seek alternatives to cocoa butter. Cocoa butter equivalents that have similar composition and properties to cocoa butter are now being produced biotechnologically [54]. Yeasts can be used to produce cocoa butter equivalents with some modifications to increase the stearic acid content of microbial lipid. There exist many approaches to increase the stearate content such as growing the yeast with a deficiency of oxygen, deletion of some genes, and manipulation of fermentation conditions [33].

V. PRODUCTION OF MICROBIAL LIPIDS

Oleaginous microorganisms can be grown in fermenters or in outside ponds. Regardless of the method employed, the subsequent processing would be the same, namely, separation of the biomass from the liquor, drying of the biomass, recovery of the microbial or single cell oil, and the subsequent refining of the oil. The processing steps for making SCOs are shown in Figure 14.1.

A. Extraction of Microbial Lipids

Lipid recovery from fermentation broth involves harvesting microbial cells from the broth, either by drying cell biomass or by forcing cell disruption, and lipid extraction. Cell harvest is expensive when cell density is low in the fermentation broth, which renders high cell density fermentation desirable. Commonly used cell-harvesting methods include centrifugation, filtration, and coagulation or flocculation. Furthermore, numerous methods have been invented, such as high-pressure

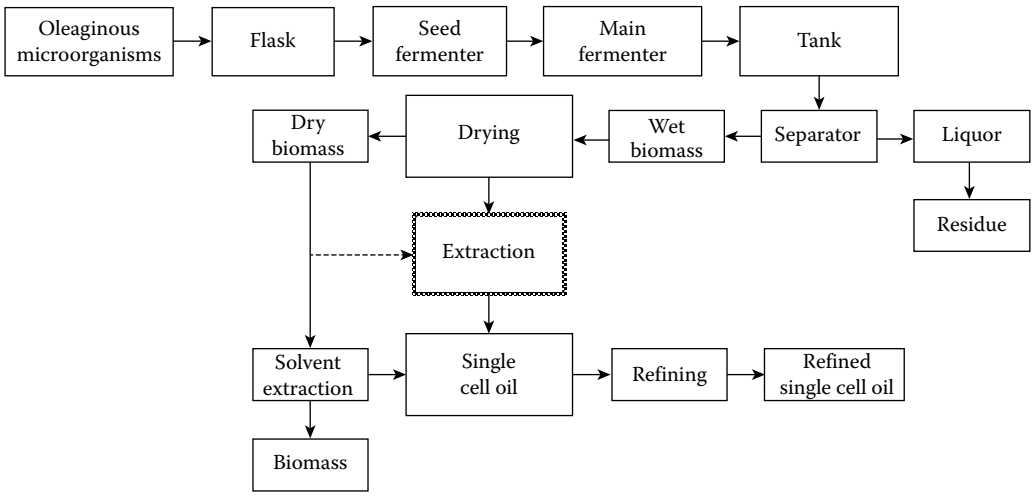

FIGURE 14.1 Production of single cell oils. (Adapted from Wynn, J.P. and Ratledge, C., Microbial production of oils and fats, in: *Food Biotechnology*, Shetty, K., Paliyath, G., Pometto, A., and Levin, R.E. (eds.), CRC Press, New York, 2006, pp. 443–472.)

homogenization, bead beating, ultrasonication, microwave treatment, enzymatic hydrolysis of cell walls, and acid hydrolysis, to efficiently disrupt wet microbial cells so that high lipid yield can also be obtained during extraction [55]. In algae, the oil extraction step includes cell disruption by mechanical, chemical, or biological methods and oil collection by solvent. Major bottlenecks of oil extraction are that the extraction of internal oils is energetically demanding because the cell walls of some species of microalgae are strong and thick; therefore, oil extraction yield is negatively affected in case of a wet biomass. Advanced processes of wet oil extraction have been suggested to overcome these bottlenecks. Extracted microalgal oils are typically converted to biodiesel by transesterification using alcohols and catalysts [3].

B. Strategies to Increase Efficiency of Microbial Lipids Production

The lipid production efficiency of SCO can be properly improved by two means, such as process improvement and strain improvement. Process improvement for the economical production of SCO includes the medium optimization, control of fermentation process, application of low-cost carbon sources, and improvement in downstream process. Recently, genetic manipulation and metabolic engineering techniques are used to improve the oleaginousness of SCO microorganisms.

1. Optimization of Fermentation Conditions

In fermentation processes, optimization is of primary importance since sometimes it may not be obvious to determine which factor is the most important one. First of all, initial screening of important variables and then optimization studies should be conducted to find out optimum levels of these variables [56]. Design of experiment (DOE) is an efficient approach used for an effective optimization with a reduced number of runs. Response surface methodology (RSM) is an important statistical technique for designing experiments, building models, evaluating the effect of the factors, evaluating interactions, and searching for optimal conditions. RSM involves three steps: performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. During the past decades, RSM has been extensively applied in the optimization of medium composition, fermentation conditions

TABLE 14.1
Studies on the Optimization of Lipid Accumulation in Oleaginous Microorganisms

Strain	Subject	Reference
Mortierella alpina	Optimization of AA production by *M. alpina* Wuji-H4 isolate	[58]
Y. lipolytica	Cell growth and lipid accumulation using stearin and oleic rapeseed oil	[54]
Cunninghamella echinulata	Growth and lipid accumulation in glucose and orange-peel medium	[59]
Yarrowia lipolytica	Effects of plant oils like soybean, canola, castor, sesame, wheat bud, sweet almond, bitter almond, olive, walnut, and coconut oils on the growth and metabolite production	[29]
Cryptococcus curvatus	Optimization of oil biosynthesis using beet molasses and corn gluten meal as carbon and nitrogen sources	[10]
Rhodotorula glacialis	Lipid composition and accumulation under different temperatures and carbon:nitrogen sources	[4]
Hansenula polymorpha	Optimization of physical–chemical variables for maximum GLA production. Variables were yeast extract, trace metals, and DOT	[39]
Trichosporon capitatum	Effects of medium components and culture conditions on cell growth and lipid accumulation	[60]
Rhodosporidium toruloides	Lipid production profile in a defined media with various amounts of sulfate supplements	[61]
Rhodotorula glutinis	Effects of acetic acid and furfural in the acid hydrolysate on growth and lipid metabolism	[62]
C. curvatus	Optimal culture conditions for cell density and lipid content	[63]
Trichosporon fermentans	Usage of sulfuric acid–treated sugarcane bagasse hydrolysate for the growth and lipid accumulation	[57]
Zygomycetes	Usage of olive mill wastewater for SCO production	[2]
Chlorella protothecoides	Fatty acid and lipid production from cassava bagasse hydrolysate	[64]
C. protothecoides	Optimization of culture medium and growth conditions for lipid production by utilization of papaya waste	[65]

(such as temperature, pH, incubation time) to increase product yields, and food manufacturing processes. Optimization of fermentation conditions through RSM can improve lipid accumulation [57].

In recent years, optimization studies for improving oil yield from several oleaginous microorganisms were conducted and these studies showed that excess carbon source, dissolved oxygen tension (DOT), ingredient addition such as oils and stearin in growth media can encourage microbial lipid production (Table 14.1).

In these studies, up to 60% lipid yields were achieved. Besides these optimization studies, future studies are mainly focused on lipid regulation metabolism, optimization of growth conditions, lipid accumulation, and understanding of the lipid buildup pathways as well as specific oil production, lipidomic and metabolomic approaches, and genetic and metabolic engineering.

2. Optimization of Culture Medium and Fermentation Process

There have been several reports showing that the medium composition influenced the lipid accumulation rate and the characteristics of lipids in SCO. In most oleaginous strains, lipid accumulation occurred after nitrogen or other mineral sources were depleted from the medium, and the consumption pattern of nitrogen or other mineral sources by an individual microorganism changed [66–70].

The largest amount of microbial biomass and intracellular lipids of *R. toruloides* were obtained from the cultivation using urea as a nitrogen source, and higher lipid production was achieved with ammonium chloride [71]. The significantly higher lipid accumulation was observed when *R. toruloides* was grown on organic nitrogen sources than on inorganic nitrogen sources [72]. Similar results were also shown in *C. albidus* [73]. It was reported that *Rhodotorula gracilis*

produced high lipid content and cell biomass when grown on ammonium sulfate, asparagines, and yeast extract [74].

In *R. toruloides*, lipid accumulation was observed when the phosphate source was exhausted in the medium [75], while bioemulsifier was produced in low amounts with phosphate limitation [76]. The limitation of nitrogen and iron sources affected the lipid production by *C. curvatus* in batch and fed-batch cultures [77].

The ratio of carbon source to nitrogen source, that is, C/N ratio, greatly affected the lipid accumulation in the cell. The extra carbon sources in the culture medium were converted into lipids when cell proliferation stopped due to depletion of nitrogen sources in the medium. Generally, the lipid accumulation was promoted as the C/N ratio increased to a certain extent. *R. glutinis* produced more lipids until the C/N ratio reached 350, and the C/N ratio higher than 350 inhibited the cell growth [25]. The combined use of multiple carbon sources accelerated the lipid production depending on the strains cultivated. The cultivation of *L. starkeyi* on glucose and xylose enhanced lipid accumulation [78], and the use of glucose and glycerol enhanced the lipid production in *Y. lipolytica* [69].

One of the cultivation media containing a high concentration of carbon source is hydrolysates of cellulosic and lignocellulosic materials. Since yeasts cannot hydrolyze cellulosic materials, these materials should be converted into free sugars which yeasts can utilize as carbon sources. The hydrolysis of lignocellulosic materials was carried out chemically and/or enzymatically [79]. The lignocellulosic hydrolysate may be more applicable to produce respiratory sufficient SCO yeasts, which can tolerate the high sugar concentration in the medium. During the hydrolysis of lignocellulosic materials, toxic compounds were produced, and toxic materials should be removed before SCO fermentation [80].

Several cultivation methods such as batch, fed-batch, repeated fed-batch, and continuous cultures have been applied to cultivate oleaginous microorganisms to increase cell biomass and lipid content. In a batch culture of *Y. lipolytica* at 28°C–33°C, 9–12 g biomass/L containing 44%–54% intracellular lipid content was obtained [81]. From 25 days of fed-batch culture of *R. toruloides* Y4, 151.5 g biomass/L containing 48% lipid was obtained [82]. In a repeated fed-batch culture of *R. toruloides* Y4, lipid productivity was 0.55 g/L/h during 358 h fermentation time [83]. A cell biomass of 185 g/L was obtained from fed-batch culture of *Rhodotorula glutinis* using air enriched with oxygen [84], while 153 g/L biomass of *L. starkeyi* with 54% lipid content was produced in 140 h fed-batch culture [85].

From the continuous cultivation of *Rhodotorula glutinis* under nitrogen- and carbon-limited conditions, it was shown that the fatty acid compositions of total lipids were unchanged depending on the growth rates under nitrogen limited conditions, but slight changes were observed under carbon-limited conditions [86]. It was also found that the fatty acid compositions of total lipids changed as the growth rate changed under nitrogen-limited conditions and, therefore, the degrees of fatty acid unsaturation also changed. The continuous culture of *Y. lipolytica* for SCO production was successfully carried out on glycerol with thorough aeration [14]. *A. curvatum* [87] and *Candida curvata* D [88] were cultivated on glycerol. It was commonly observed that lipid accumulation decreased as dilution rate increased, and fatty acid compositions were not largely changed depending upon the changes of dilution rate under nitrogen-limited conditions.

Since most oleaginous yeasts used for SCO production are known as Crabtree negative strains, the aeration influenced the growth and metabolism of strains [89]. The increased rate of oxygen supply to *R. gracilis* induced faster growth and lower lipid content, while the degree of unsaturation of fatty acids increased with the increase of specific oxygen uptake rate [90]. In the continuous culture of *Y. lipolytica* with increased aeration, increased biomass was obtained while lipid production remained almost the same [81]. When a strict aerobe *Rhodotorula glutinis* was grown in continuous culture under nitrogen- and carbon-limited conditions, specific oxygen uptake rate (m mole O_2/g biomass/h) increased as specific growth rate increased [91]. A sudden increase of oxygen demand at the time of nitrogen exhaustion was observed in *Rh. toruloides*,

and immediately oxygen demand decreased [92]. In SCO microorganisms, the oxygen demand changed with the change in lipid productivity [1,93].

In large-scale fermentation with commercial purposes, the acidic pH of a medium is preferred since the growth of most contaminating bacteria was prohibited at acidic pH. Lipid production was successfully achieved at acidic pH with *Rh. toruloides* [92,94], *Y. lipolytica* [81], *Candida bombicola* and *C. curvatus* [95], and *Rhodotorula mucilaginosa* [96]. Generally, no harmful effects of the acidic pH of the culture medium on cell growth and lipid production of SCO yeasts were observed.

The effects of culture temperature on biomass and lipid production of SCO strains varied depending upon the species of an individual microorganism. The cultures of *Y. lipolytica* were grown at different temperatures, between 24°C–33°C, maximum biomass and lipid production were observed at 28°C [81], as well as *R. mucilaginosa* [97] and *M. isabellina* [41] at the same temperature. Psychrophilic SCO yeast *Rhodotorula glacialis* DBVPG 4785 showed growth and lipid accumulation between –3°C and 20°C, and the temperature did not affect the biomass and lipid production, whereas the contents of unsaturated fatty acids in total lipids were increased by lowering the growth temperature [4]. With respect to industrial production of SCO, the cultivations of SCO strains at low temperature are still behind practical application due to the high cost of industrial chilling.

3. Strain Improvement by Genetic Manipulation and Metabolic Engineering

In recent years, with the rapid development of genetic and metabolic engineering techniques, the growth and lipid production of SCO strains were strengthened by the application of these techniques. Principally, genetic and metabolic engineering techniques are based upon the enhancement of fatty acid and TAG biosynthesis, blocking of fatty acid breakdown, and control of bypass pathways related to fatty acid metabolism. Genetic and metabolic engineering techniques are occasionally used in combination for synergistic effects [32,98,99].

The target enzymes for the enhancement of fatty acid biosynthesis through overexpression include acetyl-CoA carboxylase (ACC), glycerol-sn-3-phosphate acyltransferase (GPAT), diacylglycerol acyltransferase (DGAT), glycerol-3-phosphate dehydrogenase (GPDH), acetyl-CoA synthetase (ACS), ATP:citrate lyase (ACL), and glycerol-3-phosphate dehydrogenase (G3PDH) [100,101]. A non-oleaginous *Hansenula polymorpha* cloned with the gene code of ACC from oleaginous *M. rouxii* produced more lipids by 40% [102].

TAGs were synthesized via the Kennedy pathway in living organisms. The key enzymes included in the Kennedy pathway are acylglycerol-P acyltransferase (AGPAT), diacylglycerol acyltransferase (DGAT), diacylglycerol kinase (DGK), DHAPAT, glycerol-P acyltransferase (GPAT), monoacylglycerol acyltransferase (MGAT), and phosphatidic acid phosphohydrolase (PAP) [103]. The first step of TAG synthesis is catalyzed by GPAT. Cloning of GPAT genes increased lipid production of algae [104]. The non-oleaginous *Saccharomyces cerevisiae* overexpressed with DGAT and phospholipid DGAT increased the amount of TAG [105].

The genetically modified *Y. lipolytica* with co-expression of fatty acid and TAG biosynthesis enzymes, such as DGAT and ACC, produced more lipids by 41% than original strain [106].

Several enzymes that are not directly involved in the fatty acid and TAG biosynthetic pathway are also known to play key roles in the control of lipid production steps [99,107]. ACS, ACL, and malic enzyme (ME) are related to the intermediate metabolism in fatty acid biosynthesis [108]. From the culture of *Escherichia coli* with the overexpressed ACS gene, lipid production was increased [109]. It was reported that overexpression of ME in *M. circinelloides* induced the increased lipid production since reducing power for fatty acid synthesis, NADPH, was supplied by ME [110]. In *Rhodotorula glutinis*, however, pentose phosphate pathway was the major source of NADPH [111]. The improved fatty acid productivity was observed in ACL-enhanced *Aspergillus oryzae* [112]. However, in *S. cerevisiae* and *Candida albicans*, ACL enhancement did not affect lipid production [113].

One of the alternative strategies to increase lipid accumulation is to decrease lipid degradation by inhibition of β-oxidation of fatty acid. The deletion of genes related to β-oxidation in

Y. lipolytica enhanced the lipid production, and deletion of genes related to TAG degradation also influenced the lipid production [25]. The inhibition of pathways for the production of phospholipids and starch increased the lipid production since the enzymes in biosynthetic procedures of phospholipids competed with enzymes in the TAG synthetic pathway for a common substrate, phosphatidic acid [114].

The control of multiple genes for enzymes in overexpression of lipid production and deletion of lipid degradation was a useful technique to increase lipid production [106,115]. The control of multiple genes of acyl carrier protein (ACP), 3-ketoacyl-ACP-synthase (KAS), and acyl-ACP thioesterase (FATA) in *Haematococcus pluvialis* resulted in increased fatty acid production [116]. The overexpression of ACC and thioesterase and deletion of ACS induced an increase in fatty acid production by 20 times in *E. coli* [117]. The utilization of multiple genes control would be one of the useful tools for efficient SCO production.

VI. BIODIESEL

Due to the increase of socioeconomical activities in the world, global needs for fossil fuels for transportation and energy generation have been continuously increased in the past decade. The sources of petroleum are sufficient in a limited region in the world, and, therefore, the supply and price of petroleum have occasionally fluctuated. Because of the uncertainty of price and supply of petroleum, nonconventional fuel sources were sought as alternatives to petroleum. In recent years, the emission of carbon dioxide is also highly of concern, and there have been many trials to find out eco-friendly and sustainable fuel sources [118].

Microbial lipids have become a feasible candidate as a substitute for petroleum, and the fuel made from SCO was recognized as biodiesel. Biodiesel is produced from the esterification reaction of TAGs of plant, animal, and microorganisms and methyl and/or ethyl donors. Methyl or ethyl esters of fatty acids can provide similar power of a diesel engine with low emission of toxic air-polluting gases [119].

The production principle of the source material of biodiesel is similar to the production principle of SCO, which contains a high amount of neutral lipids. It is well known that a number of oleaginous microalgae and yeasts produce a substantial amount of TAGs as intracellular storage lipids [120]. In contrast, oleaginous fungi produced more special lipids containing biological functions, and most bacteria are not suitable for SCO production since their cellular lipids are in small amount, and mostly complex lipids that are not suitable for biodiesel production are produced by bacteria [1,121].

A. Microalgae for Biodiesel Production

One of the important criteria in biodiesel production is the cost of the carbon source. Autotrophic microalgae are utilizing unlimited free carbon dioxide from air as a carbon source and abundant free sunlight as an energy source for lipid production metabolism. Several autotrophic microalgae such as *C. vulgaris*, *Navicula pelliculosa*, and *Botryococcus braunii* can accumulate large amounts of intracellular lipids [122]. The lipid production of *Chlorella* spp. was accelerated with nitrogen source limitation and by suitable stress to the microorganism [123].

The fermentation facilities for commercial autotrophic microalgae cultivation were composed of more complex design than those of heterotrophic microalgae. The appropriate intensity of sunlight is prerequisite for microalgal SCO production throughout the fermentation process. For industrial production of microalgal SCO, the raceway ponds and tube-type photobioreactors are widely used. The open raceway pond system is easy to operate, and, therefore, the operating cost is lower than that of a tube-type photobioreactor (Figure 14.2a,b) [124,125]. Closed tube-type photobioreactors are complicated but easier to control the fermentation conditions than the raceway pond system. For mass production of simple microalgal SCO at low cost, the raceway pond system is preferred,

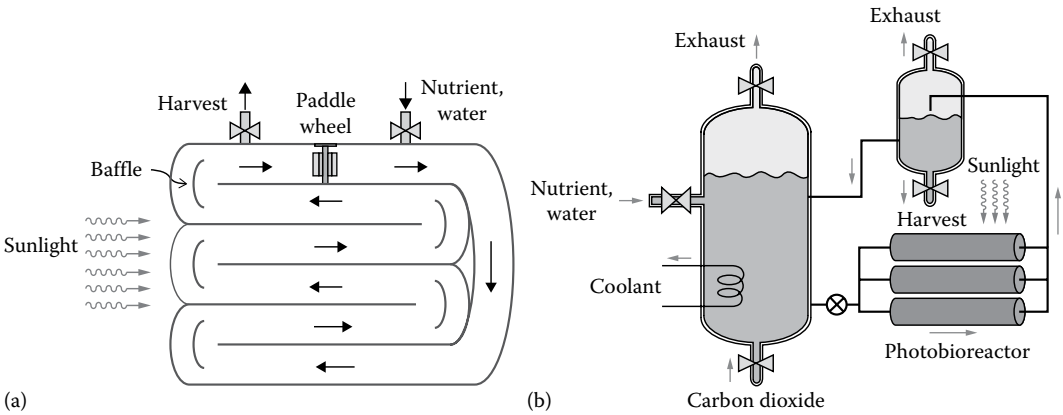

FIGURE 14.2 Outline of raceway pond (a), tube-type photobioreactor (b).

whereas the tubular photobioreactor is used for the production of SCO lipids with consistent quality and specific functionality.

Several microalgae are both autotrophic and heterotrophic. To increase the SCO production efficiency, originally autotrophic *Chlorella protothecoides* was modified into heterotrophic strain via genetic engineering techniques. The lipid production yield by genetically modified heterotrophic strain was higher than that of original autotrophic strain by four times [126,127].

The fermentation process suitable for mircoalgal SCO production is selected by the economical assessment based upon lipid productivity and cost of carbon sources.

B. Yeasts for Biodiesel Production

A number of yeasts, such as *L. starkeyi*, *Rh. toruloides, Rhodotorula glutinis,* and *Y. lipolytica*, are known to be oleaginous microorganisms. The lipid productivity and characteristics of lipids of yeast SCOs are different depending upon the strain used and fermentation conditions [5]. The major fatty acids found in oleaginous yeasts were mostly similar to that of plant oil, which is composed of myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid. These fatty acids comprised the major portion of neutral lipids, and more than 90% of neutral lipids are TAGs. TAGs are converted into methyl or ethyl esters of fatty acids through a chemical or enzymatic esterification process for biodiesel production [128,129].

The kinds of carbon and nitrogen sources, carbon/nitrogen ratio, temperature, pH, trace metal ions, dissolved oxygen concentration in a cultivation medium, and fermentation time influenced the cell biomass production, lipid content, fat coefficient (g lipid/g carbon source), and lipid yield (g lipid/g nitrogen source) of yeast SCO [90,130,131]. Generally, the lipid accumulation started after the nitrogen source is exhausted from the medium. Lipid production yields of *Rhodotorula gracilis* varied depending upon the variety of nitrogen and carbon sources. *R. gracilis* was grown in both inorganic and organic nitrogen sources, while growth and lipid accumulation varied depending upon nitrogen sources. Maximum cell biomass and lipid yield were obtained when *R. gracilis* was grown on glucose. It was reported that inorganic nitrogen sources were found to be suitable for cell growth, but not suitable for lipid production, while organic nitrogen sources such as yeast extract and Bacto-peptone were found to be good substrates for lipid production but not for cell growth [74].

The production cost of heterogeneous yeast SCO is greatly affected by the cost of carbon sources used. Even though glucose is widely and efficiently used for SCO yeast production, there have been continuous trials to replace glucose with cheap carbon sources. The alternatives of glucose included xylose, arabinose, mannose, glycerol, lignocellulose hydrolysates, and agricultural and industrial by-products and wastes [93,132–134].

Since glycerol is produced as a by-product from the esterification process of biodiesel production, it can be used as an efficient carbon source for SCO production. One of the most abundant carbohydrate materials on earth is lignocellulosic materials such as cellulose, hemicellulose, lignin, and pectin. Most SCO yeasts cannot utilize these compounds directly; it is necessary to hydrolyze lignocellulosic materials into utilizable sugars. During hydrolysis processes of lignocellulosic materials, several toxic compounds such as acetic acid and formic acid are being produced, and these toxic compounds should be removed from the hydrolysate for efficient yeast growth [135].

VII. CONCLUSIONS

Microorganisms offer potential since the production of functional oils such as PUFAs is possible by the production of these microbial strains. In industrial applications, specific PUFAs can be produced by manipulating fatty acid biosynthetic pathways or using novel pathways, which is a great achievement in the field.

Some research has been focused on optimization studies to increase lipid accumulation in microbial strains. Besides these optimization studies, future studies are mainly directed toward lipid regulation metabolism, lipid accumulation, and understanding of the lipid buildup pathways as well as specific oil production, lipidomic and metabolomic approaches, and genetic and metabolic engineering.

Since the most important parameter for microbial lipid production is feasibility of the system, the production cost of lipids can be reduced by using industrial waste or by-products as substrates. Furthermore, the food-purposed SCO should be obtained from food grade raw materials only. The usage of SCO lipids for biodiesel production, however, has no limitation to use any raw materials and any impurities present in biodiesel products. Due to sustainability, fast growth, and recycling of biomaterials, SCO is believed to be a potential substrate for biodiesel production in the near future.

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15 Food Applications of Lipids

N.A. Michael Eskin and Gary R. List

CONTENTS

I. FATS AND OILS AVAILABLE FOR FOOD APPLICATIONS

A. Introduction

Worldwide production of edible fats and oils exceeds 190.9 million metric tons (MMT). The USDA tracks the worldwide production of nine major vegetable oils. They include coconut, cottonseed, olive, palm, palm kernel, peanut, rapeseed, soybean, and sunflower oils. The top four vegetable oils are palm/palm kernel, soybeans, canola, and sunflower (Figure 15.1). Current production figures (164.5 MMT) for 2015/2016 show these four oils account for nearly 86% of the total. Palm/palm kernel and soybean oils account for over 64% of the world's production of edible oil. Over the period from 2002/2003 to 2015/2016, production of palm oil has increased from 28 to over 65 MMT and is the largest source of edible oil. Food use over this period increased from 22 to 46.5 MMT. The amount of palm oil used for industrial purposes has grown steadily from 4.5 to 16.5 MMT over the 2003–2015 time frame [1].

The production of animal fats including butter, lard, and edible tallow was estimated at about 26 MMT in 2014. Consumption figures for the United States show that butter, lard, and tallow accounted for 1.51, 0.48, and 1.05 billion pounds, respectively. The emergence of the biodiesel industry over the past decade or so has impacted the food/fuel ratio for fats and oils. Food use/animal feed and biodiesel at one time were about 80/20, but are expected to decrease to 68/32 by the year 2020.

Butter (7.95 MMT) and lard production 8.55 MMT account for nearly 16.4 MMT or 63.1% of animal fat production. India and the EU-28 countries are the major producers of butter while the EU-28 countries and China are the major producers of lard. Exact edible tallow production figures are not readily available but about 10 NNT appears reasonable. Since 2005, the worldwide butter production has increased from 6.68 to 7.94 MMT in 2014 while lard over the same period increased from 7.21 to 8.55 MMT. Clearly, animal fats will be a source of products for food and industrial applications. Removal of *trans* fats from foods is a worldwide phenomenon because of possible links to coronary heart disease (CHD) and strokes [2]. Although animal fats are low in *trans* fat content, they are highly saturated and contain cholesterol, both of which are considered risk factors for heart disease.

An edible fat or oil must satisfy three basic requirements including oxidative stability, functionality, and health/nutrition. Since no fat or oil fully meets these requirements, some modification may be required. Currently, fat modification technologies include catalytic hydrogenation,

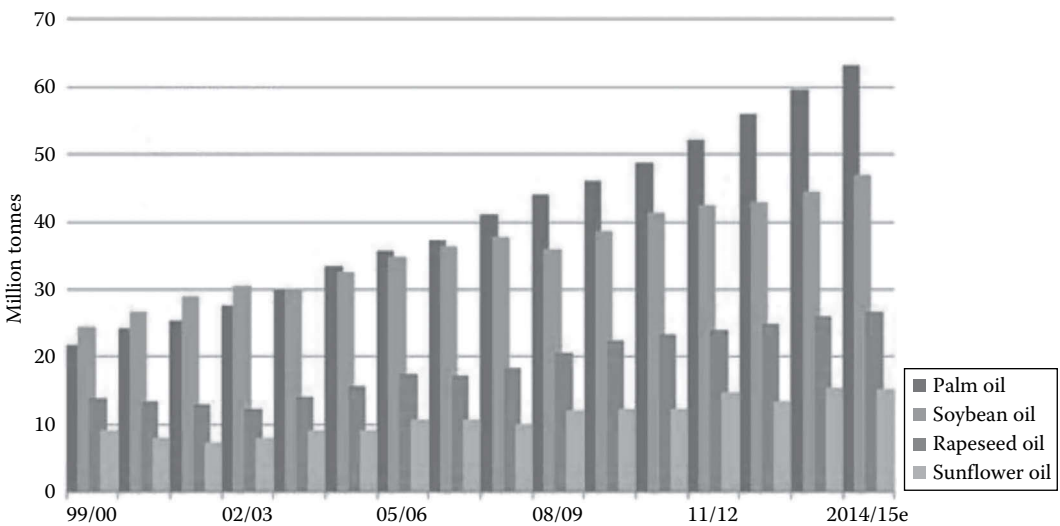

FIGURE 15.1 Global vegetable oil production. *Note*: e, estimated. (From Anon, Oilseeds: World markets and trade, U.S. Department of Agriculture/Foreign Agricultural Service, Washington, DC, November 2015.)

chemical/enzymatic interesterification, fractionation, blending, and trait modification by plant breeding and/or gene insertion. In addition, the formulation of food oils may be accomplished by combinations of these technologies. The reader is referred to a number of reviews of oil processing technologies and fat formulation methods [3–10]. Catalytic hydrogenation has been the backbone of the processing industry for many years. The choice of hydrogenation for food formulation can be attributed to their increased oxidative stability, the abundance of cheap soybean oil, with the *trans* fatty acids present crystallizing quickly in a predictable fashion, and are very flexible. Virtually any shortening or margarine/spread can be formulated from several base stocks including liquid oil and completely hardened vegetable oils. However, under these conditions (selective, high temperatures, low pressure, and slow agitation), significant amounts of *trans* fatty acids are formed. With a one iodine value drop of 0.73%, *trans* fatty acids are formed during the reaction. Thus, partially hydrogenated winterized soybean salad/cooking oil (IV 110) contains about 14% *trans* fatty acids. Shortening base (IV 80) and margarine bases (IV 65) contain about 32%–40% *trans* fatty acids. After blending, the *trans* fatty acid contents of finished products range from 11% to 25% [3]. Since 1990, increasing evidence points to dietary fatty acids as being linked to CHD. Both saturated and *trans* fatty acids raise serum cholesterol in humans and are considered risk factors for CHD [11]. The Nutrition Education Labeling Act (NELA) of 1990 coupled with *trans* fat labeling regulations issued in 2003 have had a major impact on the edible oil industry. In 2003, the number of zero *trans* foods was essentially zero but by 2007 over 1900 foods had been declared zero *trans* (>0.5 g *trans* fat serving). By 2012, further reductions in *trans* fat had occurred with many foods containing 0.2 g/serving [12].

In addition to the United States and Canada, other countries including the EU-27, India, China, Japan, South America, Australia/New Zealand, and Malaysia have all undertaken to remove *trans* fats from the diet [13–20].

B. Fatty Acid Composition of Major Oils

The dominant fatty acids of commodity vegetable oils (Table 15.1) are palmitic (C16:0), oleic (C18:1), and linoleic (C18:2) acids. Commodity oils including soybean, cottonseed, canola, corn, and sunflower are composed of 85%–90% of these three fatty acids. By comparison, stearic acid (C18:0) is a minor component of commodity oils. Linolenic acid (C18:3) is present in soybean, canola, and linseed oils. While a source of omega-3 fatty acids, these oils are relatively unstable to autoxidation, which limits their use in high-temperature applications or when extended shelf life is required.

Coconut and palm kernel are known as lauric oils because of their high content of lauric (C12:0) and myristic (C14:0). Palm oil contains about 50% saturated fatty acids of which palmitic acid (C16:0) predominates. It is important to note that the physical properties of some fats are determined by their glyceride structure as well as their fatty acid composition. For example, mutton tallow and cocoa butter with the same fatty acid composition show vastly different melting profiles. Mutton tallow melts at about 48°C, whereas cocoa butter melts at about 32°C. Mutton tallow is composed of a mixture of symmetrical (SUS) and nonsymmetrical (SSU) triglycerides while cocoa butter consists of entirely symmetrical triglycerides (POP, POS, and SOS). As a result, the highly symmetrical structure provides a sharper melting profile. At temperatures up to about 25°C, cocoa butter is a solid brittle material yet quickly melts at body temperature. On the other hand, mutton tallow melts in the mouth giving a waxy unpleasant sensation.

C. Availability of Fats and Oils in Different Regions of the World

Soybean and palm oil account for about 60% of the world's edible oil supplies. The world's soybean production accounts for a total of 319.6 MMT with the majority grown in the United States, Brazil, Argentina, and China. Nearly 82% the world's supply of soybeans is produced by these four countries. Exports of soybeans amount to 126.5 MMT (88% of production) with the

TABLE 15.1
Fatty Acid Composition of Commodity Vegetable Oils

	Range%					Typical %					Other %				
Fat/Oil	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**C12:0**	**C14:0**	**C20:0**	**C22:0**	**C24:0**
Soybean	8–13.3	2.4–5.4	17.1–26.1	49.8–7.1	5.5–9.5	10.6	4	23.3	53.7	7.6		0.1	0.3	0.3	
Corn	8–19	0.5–4	19–50	34–62	0–2	10.9	2	25.4	59.6	1.2		0.1	0.4	0.1	
Cottonseed	21.4–26.4	2.1–3.3	14.7–21.7	46.7–58.2	0–1	21.6	2.6	18.6	54.4	0.7		0.3	0.3		
Sunflower	5.6–7.6	2.7–6.5	14–39.4	48.3–74	<0.2	7	4.5	18.7	67.5	0.8		0.4	0.4	0.7	0.2–0.3
Peanut	8.3–14	1.9–4.4	36.4–67.1	14–43	<0.1	11.1	2.4	46.7	32	0				2.9	1.5
Olive	7.5–20	0.5–5	55–83	3.5–21	<0.9	0.9	2.7	80.3	6.3	0.7					
Palm	41.8–46.8	4.5–5.1	37.3–40.8	9.1–11	0.4–0.6	44	4.5	39.2	10.1	0.4		1.1	0.4		
Palm kernel	6.5–8.9	1.6–2.6	13.2–16.4	2.2–3.4	0	8.4	2.5	15.3	2.3	0		16.2	0.2		
Coconut	7.7–9.7	2.3–3.2	5.4–7.4	1.3–2.1	0	8.8	2.6	6.2	1.6	0	48.2	18.1	tr		
Canola	2.5–6	0.0–2.1	50–66	18–30	6.1	4.1	1.8	60.9	21	8.8	47.5	0.1	0.7	0.3	0.2

Source: O'Brien, R.D., *Fats and Oils Formulating and Processing for Applications*, CRC Press, Boca Raton, FL, 2008, pp. 1–744.

United States, Brazil, and Argentina being the major exporters. The major importers of soybeans are China and the EU-27 countries that import about 75% of the total produced. The total crush of soybeans is about 272 MMT with China, Brazil, the United States, Argentina, and the EU accounting for 83% of the crush.

Palm/palm kernel is the largest source of edible oil worldwide. In 2014/2015, palm oil production was 61.4 MMT. The major producers are Indonesia (33 MMT) and Malaysia (19 MMT) with both accounting for nearly 85% of total production. Coconut and palm kernel (lauric oils) production is around 10.6 MMT of which 46% (4.9 MMT) is exported.

In 2014/2015, Canada produced 6.05 MMT of soybean oilseeds and 15.55 MMT of canola seed plus small amounts of sunflower seed for a total production of 21.66 MMT. Total crush for oil and meal amounted to 8.7 MMT. Much of the soybean and canola crop is exported. Total exports of soybeans and canola seed were 12.64 MMT or about 58% of production. The major exporters of canola seed are Canada and the EU-27 countries while importing countries include China (3.3 MMT), Japan (2.4 MMT), and the EU-27 countries (2.2 MMT).

India produces 36.8 MMT of oilseeds including cottonseed (12.3 MMT), peanut (4.7 MMT), soybean (11.5 MMT), and canola (7.15 MMT). India, however, does not export oilseeds. Domestic consumption of vegetable oils amounts to 21.66 MMT of which cottonseed, palm, peanut, soybean, and sunflower are the major food oils. Palm oil (9.95 MMT) constitutes 46% of food oil consumption. Total vegetable oil imports are around 14.63 MMT.

China produces 54.8 MMT of oilseeds or 10.4% of the world's total. The major oilseed crops include peanut, canola, soybean, and sunflower. Vegetable oil food uses total 31.7 MMT of which soybean oil accounts for 15.24 MMT. Other seed oils used include peanut, palm, canola, and sunflower. China does not export oilseeds or oil. In order to meet the demand for edible oils, China relies on imports totaling 8.8 MMT of which palm oil totals 5.75 MMT.

The EU-27 countries produce 31.6 MMT of oilseeds, with rapeseed and sunflower seed accounting for nearly 93% of production. Vegetable oil food uses total 13.2 MMT with non-food uses totaling 12 MMT. Significant amounts of imported palm oil (3.1 MMT) serve as feedstocks for the biodiesel industry.

The Middle East Countries produce 2.8 MMT of oilseeds of which cottonseed and sunflower seed account for much of production (83%). These countries rely on imports of vegetable oils to meet domestic food uses. Total vegetable imports are around 5.19 MMT and include palm (2.76 MMT) and sunflower oil (1.82 MMT). Industrial use of vegetable oils in the Middle East is quite small (0.45 MMT).

Southeast Asia produces 24.1 MMT of oilseeds including copra (coconut), palm kernel, and soybeans. These three oilseeds account for nearly 83% of total production in Southeast Asia. Vegetable oil consumption (food/non-food) in the area is split between industrial (12.48 MMT) and food use (11.3 MMT). Palm oil accounts for 17.78 MMT of domestic consumption of which 3.37 MMT is imported.

The minor vegetable oils include peanut cottonseed and olive. Total production in 2014 for peanut was 5.58 MMT, cottonseed was 5.13 MMT, and olive oil was 2.37 MMT. These account for 13.1 MMT or about 7% of the world's vegetable oil production (190.9 MMT).

D. Frying Fats and Oil

Deep fat, pan, and griddle frying are popular methods of food preparation throughout the world. Exact statistics on frying oils are not available outside of the United States. The USDA breaks down edible fats and oils into three categories including baking/frying, margarine, and salad/cooking oils. The efforts to remove *trans* fats from foods have resulted in shifts from high *trans* (up to 40%) baking and frying fats to liquid/lightly hydrogenated salad/cooking oils. Liquid vegetable oils contain very low levels of *trans* fats (1%–2%) arising from bleaching and deodorization. These result from the thermal isomerization of polyunsaturated fatty acids (PUFAs) and are different from

industrially produced *trans* isomers. Linoleic and linolenic acids are isomerized from all *cis* acids to acids containing one of more *trans* bonds. Industrial hydrogenation produces only *trans* monoenoic acids. Lightly hydrogenated oils may contain about 10%–11% *trans* while shortening and margarine base stocks contain 32%–40% *trans* fatty acids. These bases when blended with liquid oil and/or hardstocks result in shortenings with 12%–25% *trans* and margarine/spreads with 10%–20% *trans*. From 1980 to 2003, the United States consumed from 4.18 to 9.62 million pounds of baking/frying fat (3.07 million pounds vegetable oil based, 1.11 million pounds animal fat based in 1980). In 2003, consumption included 9.16 million pounds of vegetable oils and 0.47 million pounds of animal-based baking/frying fats.

The *trans* fat labeling regulations became law in July 2003 [21]. Beginning in 2004, usage of baking/frying fats began to decline. By 2010, vegetable/animal fat–based products consumption shrank to 4.76 billion pounds. On the other hand, consumption of salad/cooking oils over the period from 2003 to 2010 increased from 10.14 to 16.59 million pounds. Clearly, *trans* fat labeling has impacted consumption patterns in the United States.

Frying has been studied by many research groups throughout the world and the reader is referred to a number of books and review articles [22–25]. The open literature reports numerous frying studies made in the laboratory. Most have been short-term studies made on small batches of frying oils. Typically, foods are fried continuously in small batches over a period of several weeks during which time the food and oil are analyzed by chemical tests and sensory evaluation. Oil stability is generally measured by free fatty acids, peroxide value, *p*-anisidine value, and total polar compounds. Test kits are available to measure oil discard points.

A discussion of all the aspects of the frying process is beyond the scope of this chapter but can be found in Chapter 9. The reader is referred to textbooks and review articles describing the chemical changes occurring during frying, the dynamics of frying, sensory evaluation of fried foods, food service and industrial frying, nutrition, and environmental and regulatory issues [25,26].

Worldwide commercially available frying fats are limited to soybean, palm olein, canola, sunflower, corn, cottonseed, coconut, peanut, safflower, and sesame seed oils. However, soybean, palm, canola, and sunflower oils account for about 85% of frying fat production and usage. A number of factors determine the choice of a frying fat. However, oxidative stability, availability, and cost are the most important factors to consider. All frying fats undergo oxidation, which determines when a frying fat should be discarded. Thus, fry life will affect the selection and influences costs to the user and should thus be considered. For example, a cheaper oil with a fry life of 7 days versus a more expensive oil with twice the fry life may or may not be cost-effective. In the United States, availability is not a consideration except for some of the trait-modified soybean, canola, and sunflower oils. Large users wishing to purchase a specific oil desire and demand an adequate supply for their needs. This is confirmed by a case study reported in 2007. A large fast food chain with 5600 chain stores in the United States decided to go *trans* fat–free and, after numerous frying studies and test marketing, selected a low linolenic soybean oil and was able to contract for enough oil to meet their needs. The time required to bring a new oil to commercialization may take 5 years to be able to provide a billion pounds of the oil. Production of high oleic soybean oil is projected to increase from 1 to 4 billion pounds between 2016 and 2020. High oleic canola oil is expected to increase from 5 to more than 6 billion pounds over the same time frame. Exact production figures, however, remain proprietary.

The properties of common frying oils are summarized as follows.

1. Soybean Oil

The reader is directed to a number of reviews covering the properties, composition, processing, and food uses of soybean oil [9,27–29]. Trait-modified soybean oils (low linolenic/high/mid oleic) have been reviewed by Wilkes [30] and Wilkes and Bringe [31]. The advances in plant breeding which brought these oils to commercialization have been reviewed by Burton [32], Hammond [33,34], and

Wilson et al. [35–37]. Other trait-modified oils still in development include high stearic acid lines showing promise as edible food oil applications requiring solid fat [38–40].

Commodity soybean oil is readily available, cheap, and has an excellent fatty acid profile with about 63% of essential PUFAs and as such is considered a healthy oil. The 7%–8% linolenic acid limits its use in heavy duty commercial frying because of a shorter fry life compared to oils free of linolenic acid. Upon prolonged heating, the oil gives off odors described as fishy and painty [41]. The oil is suitable for pan frying in the home but objectionable room odors may result [41]. Prior to *trans* fat labeling, hydrogenated soybean oil was the standard frying fat in the United States and elsewhere. Products available included cubed frying shortenings, fluid opaque frying fats, and clear pourable products. Although all functioned well in deep fat frying the *trans* acid content 20%–40% coupled with the recent Food and Drug Administration (FDA) decision to remove *trans* fats from GRAS status may well make their future doubtful. Pan and griddle frying along with release agents and spray applications may not be affected because unhydrogenated (low *trans*) oil can be used. Currently, low linolenic and mid/high oleic soybean oils are commercially available and perform well in frying applications, which will be reviewed elsewhere in the chapter.

2. Palm Oil/Palm Olein

General reviews of palm oil/palm olein composition, properties, processing, and food uses can be found in References 42–51.

Palm oil is the largest source of edible oil worldwide. Although highly saturated (50%), palm oil and its fractions offer a wide variety of edible products including frying fats. Fractionation of palm oil yields an olein (less saturated) fraction and a more saturated stearin. The palm oil olein fraction is widely used throughout the world for deep fat frying and is considered to be oxidatively stable with an acceptable fry life. Palm and palm kernel oils and their fractions find uses in other edible oil products and will be discussed elsewhere in the chapter.

3. Canola Oil (Rapeseed)

Rapeseed and canola oil composition, properties, processing, and food uses have been reviewed by others and the following papers should be consulted for further information [52–57].

Rapeseed oil has undergone numerous modifications of its fatty acid profile over the years. Originally, the oil was known as high erucic acid rapeseed oil (HEAR). Following reports that erucic acid may have adverse health effects, a concerted effort was made to breed out erucic acid. This oil became known as low erucic acid rapeseed oil (LEAR). Although erucic acid was removed to low levels, LEAR contained about 10% linolenic acid and attempts to remove it by catalytic hydrogenation were only partially successful. The use of rapeseed oil in frying and other high-temperature applications demanded further reduction in linolenic acid. Efforts through plant breeding were successful in achieving this goal. The new rapeseed oil was given the name canola. Several lines of canola have reduced linolenic acid contents and have been readily commercially available. The linolenic acid content of each is about 2%–3%. The oleic acid content ranges from about 60% to 80% so that these oils are known as mid-oleic and high oleic canola oils. Both are highly suitable for heavy duty industrial frying and food service applications. Canola oils with increased oleic acid contents are well suited for blending with other vegetable oils and are currently available commercially. The performance of these oils is discussed elsewhere in the chapter.

4. Cottonseed Oil

The composition, properties, processing, and food uses of cottonseed oil are detailed in a number of reviews [58–60]. Its consumption in the United States is mainly in snack foods.

Cottonseed oil is considered a premium frying oil. It is free of linolenic acid and contains high levels of gamma and delta tocopherols making the oil stable to oxidation. Foods fried in cottonseed oil exhibit a good fried food flavor sometimes described as nutty or buttery. The oil is widely used

in the potato chip industry. Cottonseed oil also contains about 25% saturated acids, which may be a disadvantage in labeling because of possible links between saturated fat and CHD. Markets for cottonseed oil are hindered by limited supplies and a higher cost compared to other commodity oils. From 2004 to 2014, domestic consumption of cottonseed oil has varied from about 510 to 935 million pounds at prices ranging from 28 to 73.5 cents/pound. By comparison, soybean oil was sold for 23–52 cents/pound over the 2004–2014 time period.

5. Corn Oil

The composition, properties, processing, and food uses of corn oil have been reviewed by Strecker [61] and Moreau [62]. Although the vast majority of corn oil is produced by wet milling, dry milled oil and refined, bleached, and deodorized (RBD) oils are available and of superior quality.

Corn oil is considered a premium oil for frying shelf stable snack foods. Foods fried in corn oil have a tendency to have a lower flavor profile initially. However, flavor profiles improve with frying time and are equal to or better than cottonseed oil. Corn oil is rich in tocopherols and ferulic acid derivatives, which protect the oil from oxidation. The oil contains high levels of essential fatty acids and only traces of linolenic acid. Like cottonseed and sunflower oils, corn oil is considered a naturally stable oil because of its low linolenic acid content. Since *trans* fat labeling became law, the use of corn oil has increased considerably. Although worldwide production and consumption statistics are not available, the United States produced 4.49 billion pounds in 2014 of which 3.69 billion pounds were consumed domestically and 800 million pounds were exported.

6. Sunflower Oil

Currently, sunflower oils with three differing fatty acid compositions are available commercially. Conventional sunflower oil has a high amount of linoleic acid (70% or greater). Trait-modified sunflower oils are the mid and high oleic varieties, which through traditional plant breeding contain 65%–80% of oleic acid [63–66]. Conventional sunflower (high linoleic) is unsuitable for heavy duty industrial frying because of its tendency to polymerize. However, the high/mid oleic varieties perform well in industrial frying. Owing to their low levels of tocopherols, sunflower oil is not as stable as other oils with a similar fatty acid composition.

7. Peanut (Groundnut) Oil

Peanut oil is widely used throughout the world for frying. It has a high content of monounsaturated (oleic) acid and coupled with a high content of gamma tocopherol makes the oil stable to oxidation. Peanut oil has an excellent flavor profile described as pleasant and fried foods exhibit excellent shelf life. Although peanuts are known to be allergenic, the oil is not. When fully RBD, peanut oil is highly prized in the United States for the home frying of turkeys.

8. Safflower Oil

Ordinary (high linoleic) safflower oil is unsuitable for heavy duty industrial frying because it readily polymerizes. High oleic safflower oil varieties are available but commercially in limited supply and very expensive.

9. Coconut Oil

Most of the coconut oil is produced in the Philippines and Indonesia. The oil has an excellent flavor profile because fried foods take on a characteristic nutty flavor.

10. Sesame Seed Oil

Sesame seed oil is grown mainly in the Middle East and Asia in small quantities. Nonetheless, sesame seed oil is very stable as a frying fat because of a favorable fatty acid composition and antioxidant content. Fried foods have an excellent flavor profile. The oil is quite expensive, which limits its use as an edible oil.

TABLE 15.2
Fatty Acid Composition of Trait-Modified Vegetable Oils (Non-GMO/Plant Breeding) and Soybean Oils Modified by Plant Breeding and Gene Insertion

	% Fatty Acids				
Oil	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C18:3**
Low Ln soy	11	4	25	57	<3
Mid Ol soy	11	4	50	12–27	<3
Hi Ol soy	6	5	75	15	3
Low Ln canola	4	2	22	64	4
Hi Ol canola	3	2	73.7	14.4	2.9
Mid Ol sun	4	2.7	50–65	27–42	0.4
Hi Ol sun	3.7	5.4	81.3	2.5	<0.3
Hi Ol/low sat soy[a]	3	3	75	15	2.5
Stearidonic enriched[a]	11	4	20	24	10

[a] GMO.

11. Trait-Modified Oils

Trait modification may be defined as altering the fatty acid composition of a commodity oil by traditional plant breeding and/or gene insertion (see Table 15.2). Currently, trait-modified soybean oils include low linolenic, mid oleic, and high oleic lines, which are in commercial production. Trait-modified canola oils include low linolenic, mid oleic, and high oleic. All of these are available commercially. Sunflower oils are mid and high oleic. All of these oils were developed by plant breeding and are non-GMO. Several trait-modified soybean oils are nearing commercialization and are considered genetically modified organism (GMO) and include Vistive Gold, a low saturate, high oleic variety. Stearidonic acid–enriched soybean oil contains a high level of omega-3 fatty acids.

Trait-modified oils are characterized by a decrease in PUFA content and elevated oleic acid content. As such, trait-modified oils are more stable toward oxidation than their parent oil. Sunflower and canola oils are considered low saturate and appeal to health nutrition issues since foods must be labeled for saturated fat content.

12. Animal Fat–Based Frying Fats

Excellent reviews on animal fat–based frying shortenings have been reported by Woerful [67], Kincs [68], and O'Brien [69]. Animal-based frying fats may be used as an all-purpose shortening as well. These products were introduced as early as the 1890s, and several brands were popular well into the 1930s. Typically, they were formulated from 20% beef tallow and 80% liquid cottonseed oil. *Trans* fat labeling has stimulated the use of animal-based baking fats but instead of cottonseed oil, soybean oil is preferred for their manufacture.

Prior to the late 1980s, tallow-based frying fats were very popular in fast food chain outlets. French fried potatoes had a very likable and unique flavor. However, concerns over saturated fats and CHD prompted discontinued use of tallow-based frying fats. Lard is not stable for industrial frying and is highly saturated and contains cholesterol.

Liquid vegetable oils (unhydrogenated) are considered a zero *trans* option for frying and cooking. With the exception of palm, palm kernel, and coconut oils, the other commodity oils are composed of triglycerides having low melting points ranging from –13°C to about 0°C. As such they remain liquid at lower temperatures and are easily handled and pumpable. In the U.S. food service industry, liquid frying fats are often packaged in 35 lb plastic jugs for convenience and disposal. In addition, the jugs are easily handled by inexperienced fast food operators. Large bakeries may employ liquid

shortenings for cakes and bread manufacture. They often purchase large quantities and are stored in tanks. Pumping and metering of liquid fats and oils are distinct advantages in commercial bakeries.

13. Laboratory Frying Studies and Industrial Case Studies

The performance of trait-modified soybean, canola, and sunflower oils in laboratory frying studies has been reported by numerous researchers [70–84]. As final *trans* fat labeling regulations were first announced on July 13, 2003. Fast food chains and restaurants began reformulation of conventional high *trans* frying fats. Of the trait-modified soybean and canola oils, high oleic canola oil was selected for their needs. A study conducted by Texas A and M University compared a number of commercially available zero *trans* frying fats with a high *trans* frying shortening. French fried potatoes were evaluated for sensory performance and acceptability. Chemical tests including total polymer content showed that performance of the zero *trans* oils was equal to or better than that of the control frying oil. In Europe, frying fats must be discarded when total polymers reach 24%. Although the United States does not require a discard point, all of the fats tested were well below the 24% value. A complete summary of the study can be found on the Internet (http.//www.frytest.com.).

14. Griddle, Pan, Spray Frying Fats

In the United States, it is estimated that about 80% of breakfast fast foods are prepared by frying in a pan or griddle. Thus, these fats are important food service items as well as for home usage. In addition, spray and pan release fats are widely used in food service and the home. Prior to *trans* fat labeling, pan and griddle frying oils were formulated from blends of liquid and hydrogenated commodity oils (soybean, palm) and animal fats. Prime considerations in the selection of pan/griddle include *trans*/saturated fatty acids, cholesterol content, cost, stability, and availability. Palm and animal fats are highly saturated and contain cholesterol while hydrogenated oils contain a significant level of *trans* fatty acid. Trait-modified soybean, canola, and sunflower oils have advantages in pan, griddle, and spray applications including a zero *trans*/low saturated fatty acid composition, no cholesterol high oxidative stability, and excellent performance. Disadvantages include higher costs and/or availability. At the retail level, trait-modified oils may sell for over a dollar/pound compared to commodity oils at 70–80 cents/pound.

II. SPREADS: BUTTER, MARGARINE, GHEE, AND VANASPATI

A. Butter

Excellent reviews covering all aspects of butter composition, properties, and manufacture are provided by Hettingia [85], Jerson [86], and Chrysam [87].

The major producers of dairy butter include India 5.04 MMT, the EU-2.72 MMT, the United States 900,000 MMT, New Zealand 5.80 MMT, Russia 2.40 MMT, Mexico 1.95 MMT, and Ukraine 1.10 MMT. Brazil, Canada, Japan, and Argentina produce 3.10 MMT of butter collectively. Most of the butter produced is consumed in the country of origin and little is exported. Butter is generally used as a spread but has been used in baking and frying since ancient times. Butter is a water-in-oil (w/o) emulsion containing 80%–82% fat and an aqueous phase of 18%–22% water and salt. Butter is made from cow's milk (3%–4% fat), which is converted to cream via centrifugation and finally to butter by churning and kneading. Cow's milk is known to contain hundreds (500) of fatty acids with most present in small or trace amounts. Saturated fatty acids range in a chain length from C4 to C18:0. Low levels of branch-chained fatty acids are present. Monounsaturated C18 acids comprise about 30% of the total fatty acids in butter. Butter also contains 4%–8% of *trans* fatty acids that are produced by biohydrogenation in the rumen and are mainly C16 and C18 monoenes of which vaccenic (11t C18:1) is the major acid. Very low levels of polyene acids and oxo and hydroxy acids and lactones are present in trace amounts but are important flavor compounds. Butterfat is largely

composed of triglycerides 97%–98% but also contains phospholipids, free fatty acids, and partial glycerides. Other components include traces of carotenoids, squalene, and vitamins A and D.

As a spread, butter has a solid content too high for good spreadability at refrigerator temperatures (10°C). However, stick margarines are formulated to mimic the solid content of butter. Typical solid fat contents of butter at 10°C, 21.1°C, and 33.3°C are 32, 12, and 2, respectively. Stick margarines have a solid content of 20, 13, and 2 for soft stick and 11, 7, and 2 for soft tub at temperatures of 28°C, 16°C, and 2°C, respectively [87]. The solid content at 10°C determines spreadability, while the solid content at 21.1°C is indicative of holding together at ambient or room temperature (resistance to oil off). Solids at 33.3°C determine melting properties at body temperature. Generally, 10°C and 21.1°C solids may vary from product to product but 33.3°C solids are fairly uniform (1–2) in all product types. Specifications vary among manufacturers depending on the desired organoleptic properties, nutritional claims, marketing displays (refrigerated vs. non-refrigerated), and packaging equipment.

B. Margarine

The number of fat blends used for margarine and spreads is quite extensive and will not be reviewed in detail. The most common methods for fat modification include catalytic hydrogenation (selective and low *trans*) [88], interesterification (chemical and enzymatic) [4], fractionation [90], blending, and trait modification. Controlling the physical and chemical properties of fat blends has been reviewed by Dijkstra [89].

Often combinations of the methods can be used to advantage. For example, catalytic hydrogenation and blending of liquid oils offers a route to soft-tub and stick margarines [3]. Since palm/palm kernel oils are highly saturated, a variety of fats with different physical properties can be made by fractionation. Palm oil yields a less saturated olein and a more saturated stearin fraction. These in turn can be further fractionated to give super oleins and super stearins. Palm kernel oil may be fractionated as well. Palm kernel oil is composed of short-chain fatty acids with physical properties desirable in margarines, spreads, and confectionary fats. An excellent review of fractionated palm oil and food applications is provided by Kheiri [90], Young [91], and Duns [92]. Combinations of hydrogenation and chemical interesterification (random and directed) of palm and palm kernel oils were used to produce plastic fats, zero *trans* margarines, and spreads and were introduced in Europe in 1960 under the acronym BECEL (blood and cholesterol lowering). The fat blend was patented by Unilever in 1967 [93] and consisted of a fully hydrogenated palm hardstock and liquid sunflower oil. For more details, the reader should refer to the following U.S. patents [94–100].

The early objective of the margarine spread industry was focused on increasing essential fatty acids rather than removing *trans* fats. The use of interesterified and completely hydrogenated base stocks accomplished this goal. However, *trans* fat labeling has stimulated interest in enzymatic interesterification and several large suppliers offer *trans* free products based on enzyme technology [101,102].

Margarine has a long history as a spread for foods. Within several years after its discovery in France in 1869, margarine was produced in the United States in 1873. The technological changes in the margarine industry are documented in the following textbooks: Snodgrass [103], Clayton [104], Schwitzer [105], and van Stuyvenberg [106]. Margarine oil formulation and control has been reviewed by Wiedermann [107], Haighton [108], Moustafa [109], Flack [110], and Latondress [111].

Margarine by definition must contain 80% fat, whereas spreads may contain 25%–70% fat. Stick margarines have traditionally been formulated from IV 65 hydrogenated soybean oil and liquid unhyrogenated oil in 50/50 proportions. The same base can be used to formulate soft-tub margarines in a 25/75 ratio. Stick margarines as well as soft-tub products have melting points in the 31°C–32°C range. However, stick products have higher solids at 10°C and 21.1°C because they often require storage at ambient temperatures. Soft-tub products are formulated with lower solid contents at 10°C and 21.1°C to ensure spreadability at 10°C directly out of the refrigerator yet with

enough solid fat to prevent the emulsion from breaking down (oiling off) at ambient temperatures. Both stick and soft margarines have sufficient solids (2%–3%) for sharply melting properties at a body temperature of 33.3°C. Above 2%–3% solids at 33.3°C, waxy or pasty sensations occur in the mouth, whereas 2%–3% solids at 33.3°C, a sharply melting cooling sensation is observed. In order to improve spreadability directly out of the refrigerator, the so-called spreadable sticks were introduced in the 1990s. However, they contained less than 80% fat and did not meet the standard for margarine.

Spreads consist of an emulsion containing less than 80% fat. Most retail spreads contain 35%–60% fat. The spread market developed in response to low-fat low-calorie diets recommended by U.S. dietary guidelines as well as emphasis on heart-healthy foods. Several innovations in the spread market are worth noting. By the mid-1990s, evidence pointed to *trans* fatty acids as a risk factor for CHD and by 2003 a reduction in the *trans* fat content of foods was mandated by U.S. federal law. Thus, spreads were reformulated to meet the less than 0.5 g *trans* fats/serving. Reformulation was accomplished by blending hydrogenated soybean oil with liquid oils or substituting tropical oils for hydrogenated oil for solids. The introduction of cholesterol-lowering spreads in the late 1990s was soon advertised as heart healthy. The cholesterol-lowering properties of phytosterols were employed to make heart-healthy claims. Benecol was launched in the United States in 1999 but has seen limited success primarily because of higher cost compared to other spreads. At the same time, smart balance was introduced but cholesterol-lowering properties were achieved without added phytosterols but rather by blending liquid vegetable oils with palm/palm kernel oils [112]. Recent statistics show that smart balance is the fourth leading brand sold in the United States with annual sales of about US $200 million. Other innovations in the spread industry include products fortified with omega-3 fatty acids with fish oils supplying the desired fatty acids.

Spreads particularly those with low fat content have melting points somewhat lower (26°C) than traditional soft-tub margarines (31°C–32°C) and as such have a tendency to oil off (separation of the oil water emulsion) upon recycling from refrigerator to room temperatures. Spreadability from the refrigerator requires a maximum solid fat content of about 30 at 10°C. Butter generally exceeds this value. In general, the spreadability of plastic fats (butter, margarine, spreads) can be estimated from cone penetrometer measurements from which yield values can be calculated and related to spreadability. A fair correlation was determined between spreadability by sensory panels and cone penetration values as determined by the Official AOCS method [113,114].

The composition and properties of stick margarines made in North America (Canada and United States) were reported by DeMan in 1990 [115]. These workers compared cone penetration, constant speed penetration, and constant speed compression to evaluate texture. Coefficients of variation were 6.6%, 5.3%, and 10.7%, respectively. A study reported by List et al. [116] indicated that the margarine/spread industry had made concerted efforts to reduce the *trans* fat content of their products. Over the period 1992–1999, analysis of seven brands showed a reduction in *trans* fat content ranging from 23% to 82%. In 1992, the average *trans* content was 19.9% and by 1999 had fallen to 8.8% or a nearly 56% reduction over a 7-year period. A complete analysis of common vegetable oils used in margarine/spreads and blended oils is given along with fatty acid composition, solid fat contents, drop melting points, and *trans* acid contents. Thus, by 1999, retail margarines and spreads were well characterized with considerable improvements underway.

Bakery margarines are formulated for different functional properties compared to table spreads [117]. They must contain sufficient solids at temperatures from 10°C to 40°C (a wide plastic range) so that doughs can be worked and machined. Insufficient solids can lead to oil bleeding from the dough. Consequently, they are characterized by higher solid fat contents and melting points. At least five distinct bakery margarines are in common use in the United States. They include table grade baker's margarine, roll in margarines, puff pastry, and specialty rolls. Unlike all-purpose baking shortenings, baker's margarines are emulsified. Baker's margarine can be substituted for butter or all-purpose shortenings in most applications with the exception of doughnut frying. A doughnut frying shortening should have sufficient solids to allow glazes and frostings to adhere to the doughnut.

A number of *trans* free doughnut frying shortenings have been introduced and are available commercially. They are usually formulated from liquid vegetable oils and tropical fats.

Trans fat labeling has brought new baking shortenings to the market place. Many of these are palm oil based. Typical formulations for cakes and pastries include a simple blend of palm stearin (IV 44) and liquid canola oil in a ratio of 60/40. Others include a blend of interesterified palm stearin and soybean oil 70/30 or 100% interesterified palm stearin. Formulas for puff pastry fats include blends of soybean oil, beef stearin, and beef tallow (10/30/60). Others are formulated from blending several palm oils hardened to 42°C melting points (50/50). Soybean oil can be blended with the hydrogenated palm of 42°C melting point and palm oil in ratios of 20/40/40. Palm oil hardened to 45°C melting point can be used as is and accounts for 100% of the fat content [44].

In order to improve the spreadability of 80% fat stick margarines, 60% fat spreadable stick spreads were introduced in the 1990s. They are formulated with less solid fat and as such require refrigeration during storage. Many low-fat spreads are unsuitable for frying because of their higher water content. Spreadable stick products may be used for frying and baking as well.

Margarine manufacture involves preparation of the water-in-fat emulsion, crystallization of the fat phase, and finally plasticizing in scraped surface heat exchangers (votators). Many variations are used in the industry. For example, several votators (A units) may be used in series and/or resting tubes may be placed in the process. As the chilled emulsion passes from the A unit, the crystal structure consists of rather coarse crystals. In order to break up the crystal structure, pin workers (B units) are located after A units. This ensures a smooth textured product. Soft margarines and spreads are smooth because of their high oil content. Stick products are formulated with higher solid contents and tend to lack the shiny smooth texture of the soft products. Stick margarine manufacture generally requires A units and resting tubes, which allows chilling of the emulsion and crystallization to occur. The polymorphic nature of fat blends is important in margarine products. Fats crystallize in three basic forms. The alpha form is unstable and quickly crystallizes into a beta prime structure, or the most thermodynamically stable beta form. Fats that are beta prime tending are preferred for margarine because they form smaller crystals and hence have a smoother texture. In comparison, beta tending fats can be used in pourable liquid shortenings consisting of crystals suspended in liquid oil, which may be hydrogenated or unhydrogenated. Hydrogenated liquid shortenings are more stable toward oxidation but contain high levels of *trans* fats. On the other hand, unhydrogenated liquid shortenings are less stable but free of *trans* fatty acids.

What exactly determines the crystal habit of fats and oils is still not well understood, although a major factor is the triglyceride structure. Beta crystal formers are low in palmitic acid content and are found on the *sn* 1–3 positions of the triglyceride. Thus, the absence at the middle position has been suggested for beta-tending crystal habits. Beta prime fats including cottonseed, palm, tallow, and fish oils are relatively high in palmitic acid with palm and tallow containing some triglycerides with palmitic acid in the 2-position. Palm oil does consist of symmetrical (POP) triglycerides known to crystallize slowly. However, interesterification improves the behavior of palm oil because PPO is formed during the reaction. For further information, Haighton should be consulted [108].

1. Vanaspati and Ghee

Ghee and vanaspati are important foods in India and other eastern countries. A review of these products can be found by Podmore [118].

a. Ghee

Ghee is a product made from cow or buffalo milk butter in which the water has been removed by heating and skimming off the liquids. The texture, color, and taste depend on the quality of the butter, the source of the milk, and the duration of heating. The fatty acids are saturated and monounsaturated. Ghee has a characteristic nutty flavor due to the caramelization of milk solids during prolonged heating. Ghee is an important part of Indian cuisine often served with rice. As a frying oil, ghee is considered ideal because it has a very high smoke point of 250°C, which is well above

that of cooking and frying fats. Ghee made from buffalo milk is preferred for cooking, and the markets are expected to double from US $60 billion to US $115 billion in 2016.

b. Vanaspati

The manufacture, properties, and food uses for vanaspati produced in India have been recently reviewed by Prasad and Yadiov [16]. Like many other countries, *trans* fats have become an important factor in the acceptability of food products.

Vanaspati is used as a cooking fat and for baking in India, Iran, and Northern African countries. At one time, prior to *trans* fat labeling, vanaspati was formulated by blending several hydrogenated (peanut, cottonseed) and liquid vegetable oils. A number of fats and oils are permitted as ingredients for vanaspati manufacture. However, 5% sesame oil must be included as a test for the adulteration of ghee. The standards for vanaspati are set by the government.

III. LIQUID OILS IN FOOD APPLICATIONS OTHER THAN FRYING

Oils are used to make cookies, baked snack crackers, spray oils, pan release agents, liquid shortenings, pretzels, muffins, and tortillas. Since soft cookies require creaming of the fat, all-purpose shortenings (either vegetable or animal based) with added emulsifiers have been the industry standard for years. Typically, the shortening has a short plastic range and high stability. However, hard cookies do not require these attributes and liquid vegetable oils can be used in many recipes. Palm oil is suitable for the baking of short dough cookies that have good texture, flavor, and force to break properties [119]. Several well-known baked snack crackers have been reformulated with liquid nonhydrogenated soybean oil [120]. Trait-modified soybean, canola, and sunflower oils should also be suitable because of their increased stability. In addition to extensive use in the home and the food service sector, spray oils are used in the baking industry. Crackers, croutons, and crisps are sprayed with a thin layer of oil to act as a moisture barrier in order to protect the product from flavor and textural deterioration. Traditionally, either hydrogenated soybean oil (IV, 70) or coconut oil was the preferred spray oil. However, the high *trans* fat and saturate content are a disadvantage. Limited published data indicate that low linolenic soybean, canola, and sunflower oils perform well in spray oil applications. Sensory data after storage of sprayed crackers showed that the soy and mid-oleic sunflower oils were equal to a hydrogenated control [70].

In commercial bakeries, bread is baked in loaf pans that are inverted after exiting the oven. Thus, it is essential that the loaves release readily from the pans [121]. A number of pan release agents have been reported in the patent literature [122–124]. Since they must be easy to apply they are usually in liquid form but solid stick pan lubricants have been patented by Mahler and Doumani [125]. Stick pan lubricants can be formulated from liquid unhydrogenated oil, lecithin, and wax. A typical formulation consists of 50% hydrogenated tallow, 15% double bleached lecithin, and 35% liquid soybean oil. Pretzel dough is formulated from hydrogenated soybean as the fat component. It has been reported that *trans* fats can be replaced with liquid unhydrogenated soybean oil. Normally, about 2% fat is needed for tenderness, mouth feel, and structure/strength. Many home recipes call for vegetable oils in muffins, and corn, soybean, cottonseed, and canola oils all proved to be satisfactory. Flour tortillas are commonly formulated from hydrogenated or animal fats so that improved machinability and reduced dough stickiness result. Replacement of *trans* and saturated fats has stimulated interest in the use of 100% liquid oils despite possible sticking together when stacked in packages. The reader is directed to a recent patent describing the use of liquid oils in tortilla manufacture [126].

A. Liquid Oils in Fluid Shortenings

Fluid shortenings have been used in the baking and food service industry for many years. However, they have never been well received in the retail market place in spite of much research and development by the oil processing and food industries [127–130]. A Procter and Gamble test marketed

a retail fluid shortening (SWIRL) but it was discontinued after a short time [120]. Typically, fluid shortenings have been formulated from either a hydrogenated soybean oil or a completely hardened vegetable oil [131]. The hydrogenated oil usually has an IV of 90–100 while the hardstock (2%–6%) is IV 5 or less. The hardstock is in a beta stable form that allows the fat crystals to remain in suspension with the liquid oil. Food applications include deep fat frying, grilling, toppings, and popping of corn. Frying oils may contain antioxidants and silicones to inhibit oxidation and foaming. Grilling, toppings, and popping oils often contain antioxidants, lecithin, coloring agents, and flavors. Liquid oils have been used in bread and cake baking for many years. The introduction of emulsifiers and surfactants in the 1970s allowed formulation of liquid shortenings free of *trans* fats [132]. For example, IV 130 soybean oil containing partial glycerides and their esters, lecithin, and polysorbates performed well in commercial baking including yeast-raised products. The reader is directed to a number of references detailing the baking performance of liquid shortenings [133–135].

Several reviews cover the use of vegetable oils in salad dressings [70,136], while the formulation of salad dressings is well covered in the patent literature [137–147].

Salad dressings are clearly described in the Federal Code of Regulations as "emulsified semisolid food prepared from vegetable oil(s)." They may contain an optional crystal inhibitor. Salad dressings may not contain less than 30% oil by weight. Dressings may be packaged in an atmosphere in which air is replaced in whole or part by carbon dioxide or nitrogen.

Vegetable oils are a major component of mayonnaise and salad dressings. Although cottonseed was the dominant oil used in mayonnaise, the abundant supply of high-quality soybean oil at a cheaper price soon became the choice for mayonnaise. However, other oils including canola are also used. Although standards of identity allow as little as 65% oil, 80% is preferred. Up until about 1960, cottonseed oil was the oil of choice for mayonnaise.

Salad dressings may take several forms including pourable, spoonable, and multiphase. In addition to oil, water, starch, gums, flavoring components, spices, salt, metal chelators, lecithin, vinegar, and partial glycerides may also be present. A prime consideration for pourable and spoonable dressings is resistance to freeze-thawing during which the emulsion may break. Methods to stabilize pourable and spoonable dressings include the use of non-starch polysaccharides and xanthan gum and shearing of the dressing. Low-fat (30%–70%) spoonable dressings with a resistance to freeze/thaw cycling have been described in which the emulsion contains a *Xanthomonas* colloid and salt. Other approaches for producing low-fat stable dressings include preparation of starch bases from water, vinegar, sugar, salt, spices, and freeze-resistant starch [137–147].

At one time, the presence of waxes and high melting triglycerides in oil components (in unwinterized soybean, cottonseed, corn oils) was thought to be unsuitable for salad dressings because of their tendency to solidify at low temperatures. Partyka [141] reported that blends of hydrogenated soybean and cottonseed oils (IV 82) are suitable for dressings having freeze-resistant properties [139]. Xanthan gum and pectin are both used as stabilizers for pourable dressings including French, Thousand Island, and Italian. A process for preparing emulsified dressings with freeze resistance has been reported in which a cooked starch base is subjected to colloid milling and finally blended with partially hydrogenated soybean oil (IV 85) and then packaged [136]. Other oils suitable include cottonseed oil (IV 99–113), sunflower oil (IV 125–136), safflower oil (IV 140–150), and soybean oil (IV 120–141).

IV. BAKING FATS, DOUGHS, AND SHORTENINGS

A complete review of baking and baking fats is beyond the scope of this chapter. However, the following reviews by Matz [148], Podmore [149], Stauffer [150], and Pyler and Gorton [151] are recommended. Commercial baking margarines were discussed earlier in the chapter and are emulsified with water. Retail and other commercial baking shortenings may not be emulsified with water but may contain emulsifiers when used in aqueous or milk-containing systems. Commonly used emulsifiers for baking shortenings include lecithin, partial glycerides, polyglycerol esters, monoglycerides,

and modified monoglycerides. The list is not complete as Orthoefer [152] presents a detailed list of FDA-approved emulsifiers used in baking. Non-emulsified shortenings including all-purpose are used in puff pastries, pie crusts, cookies, Danish rolls, and doughnut frying. Emulsified shortenings are used in cakes, icing, cake mixes, fillings, and yeast-raised and specialty cakes. Shortening serves to interrupt the gluten strands when added to a dough or batter, which tenderizes the final baked product. Emulsifiers in shortenings serve a number of purposes, including increased shelf life, improved tenderness and flavor release, improved mixing and machinability, better water absorption, improved volume and flour hydration, better texture, and decreased usage of shortening and eggs [152].

Baking represents a major use for fats and oils as breads, cookies, cakes, biscuits, pie crusts, and short and puff pastries are the major baked food products. The functional properties of baking fats include texture, sensory properties (flavor and mouthfeel), aeration, lubricity, oxidative stability, and shelf life. A wide variety of baking fats are available each formulated for specific end uses and product type. Nutritional considerations are also important because of the effects of dietary fatty acids on human health. Both saturated and *trans* acids are known to adversely affect low- and high-density lipoprotein (LDL/HDL) serum cholesterol levels [11].

Liquid unhydrogenated vegetable oils are low in saturated and *trans* fatty acids and perform well in some baking applications. However, many baking applications require solid fat either from hydrogenated vegetable oils (primarily soybean and cottonseed) or tropical oils, including palm, palm kernel oils, and their fractions.

The physical and functional properties of triglycerides can be described by grouping them into four groups based on their melting point ranges [153]. Group 1 consists of primarily tri-unsaturated triglycerides melting from –13°C to 1°C. Examples include UUU, where U = oleic (C18:1), linoleic (C18:2), and linolenic (C18:3). Group 1 triglycerides ensure that a fat or oil will remain liquid and clear at refrigerator temperatures so that the oils may be easily poured, pumped, and handled. Group 1 triglycerides are a rich source of essential fatty acids needed for nutrition and are found in common commodity oils, such as soybean, cottonseed, corn, canola, and peanut.

Group 2 triglycerides have melting points ranging from 6°C to 23°C. Most are UUS (U = unsaturated, with L = linoleic/linolenic; S = saturated, palmitic (P), or stearic (St)) and will remain liquid only at ambient temperatures. Examples include SOL, OOP, and StOO. Group 3 triglycerides melt (27°C–42°C) at near or at body temperatures. They consist primarily of disaturated and monounsaturated triglycerides, including PPL, StStL, PPO, StPO, and StStO. Group 4 triglycerides are high melting (50°C–65°C) and are not found to any great extent in natural vegetable oils or animal fats. Instead they are formed from the complete hydrogenation of commodity oils (corn, soybean, cottonseed, and palm). Examples include StStP, PPP, StStP, and StStSt. Other group 4 triglycerides include those with elaidic acid present in the triglycerides (EEE, ESE, ESS, E = elaidic, St = stearic). These are found in partially hydrogenated oils [154].

A. Hydrogenated Shortenings

The formulation of hydrogenated shortenings has been extensively reviewed by Woerfel [67], O'Brien [9], Latondress [111], and Gohtra [155]. A number of shortenings can be formulated from hydrogenated soybean oil for specific products including pies, cakes, cakes and icings, puff pastries, and all-purpose butter flavor. The physical properties of these products are determined by solid fat content from 10°C to 50°C and their melting points. Typical solid fat contents at various temperatures are as follows: 10°C, 23–40; 21.1°C, 18–30; 26.7°C, 13–28; 33.3°C, 9–22; and 40°C, 2–11. Most have little solid fat at 45°C–50°C. Despite variations in solid fat content, melting points range from 41°C to 48°C. Some baking shortenings are emulsified including cake icing, while all-purpose shortenings are not. *Trans* acid contents range from 11% to 25% [3]. Recently, the FDA proposed that *trans* fatty acids be removed from GRAS status. If implemented, the future of hydrogenation as a fat modification tool is in doubt.

1. Zero *Trans* Shortenings: Palm Oil Based

The manufacture, formulation, and baking performance of palm-based shortenings and margarine have been reported by scientists in Malaysia [154–159] and elsewhere [167–168]. An excellent review by Kirkeby [160] on the formulation and processing of palm-based shortenings and spreads is recommended.

Many hydrogenated baking shortenings have been replaced with palm oil for solid fat. An all-purpose baking shortening can be formulated from 100% RBD palm oil. Crisco shortening has been reformulated to zero *trans* by blending high oleic sunflower oil with completely hydrogenated soybean oil. A zero *trans* shortening was introduced in 2002 based on random interesterification of canola oil and completely hardened soybean oil. In 2003, ADM introduced zero *trans* oils based on the enzymatic interesterification of liquid soybean or cottonseed oil and completely hardened vegetable oil stearins. Oils with different solid fat contents and melting points can be obtained by varying the amount of hardstock in the simple blend. Spreads require about 15% hardstock while about 40% is needed for shortenings [101]. Other major processors have introduced zero *trans* shortenings using enzyme technology [102]. Other approaches to prepare zero/low *trans* shortening oils include modified hydrogenation technologies where low temperatures and phosphoric acid–modified nickel catalysts suppress *trans* acid formation [160,162]. Controlling *trans* isomer content by modified hydrogenation conditions have been reported by various researchers [88,163–167]. Other approaches for *trans* reduction in shortenings include blends of liquid oils and diglycerides [168,169]. Oleogels and structured emulsions have been reported to provide zero *trans* and low saturate options for baking applications [170,171].

2. Performance of *Trans* Fat Replacements in Baking Applications

A number of studies conducted in the laboratory have shown that interesterified zero *trans* shortenings perform well in cookies, pie crusts, cakes, and biscuits. Tests include cookie spread, cake volume, force to break measurements and product volume and height [6,172], and *trans* fat replacement. The results are summarized in a recent review [2]. Palm oil–based baking shortenings perform well, but commercial baking requires special storage and handling. During the summer months, shortenings may become too soft for doughs and vice versa too hard in the winter months. Thus, storage conditions must be adjusted so that working and machining of doughs can be optimized.

Although many of the problems first encountered when using zero *trans* fats such as a drop in solutions for hydrogenated shortenings have probably been overcome as they may behave differently. Standard baking tests are outlined in the Official Methods of the American Cereal Chemists. Some are designed to evaluate flours rather than fats and oils. Cake quality is determined by the use of the baker's hydrogenated emulsified shortening while cookies and pie crusts call for non-emulsified hydrogenated all vegetable shortening for evaluating the finished baked goods. Bakers were required to make adjustments in the baking process and/or the handling/storage of the *trans* fat replacements. These problems and solutions were recently discussed in several reviews [173,174].

V. SALAD OILS, MAYONNAISE AND SALAD CREAM, FRENCH DRESSINGS

The production of mayonnaise and salad creams requires salad oils that are oxidatively stable and free of solids when stored under refrigerator conditions or 4°C. Among the vegetables oils used are soybean and rapeseed/canola. Both these oils are high in linolenic acid and used to be subjected to light hydrogenation to reduce the level of this unsaturated fatty acid. The latter process introduces *trans* fatty acids that are now recognized to be deleterious to human health. Consequently, new low linolenic varieties of both oils have been developed to replace the traditional high linolenic oils. However, oils are generally winterized, a process that removes high melting glycerides that would normally crystallize at refrigerated temperatures. In addition to the high melting glycerides, some solvent-extracted oils are rich in waxes, such as sunflower oil. Unless removed, a haze would form in the oil when cooled so that salad oils must pass what is known as the "cold test," which means the

oil must remain clear for 5.5 h at refrigerated temperatures. Once this is done, the treated vegetable oils can be used for the production of mayonnaise.

Both mayonnaise and salad cream are oil-in-water (o/w) emulsions. While mayonnaise contains between 65% (legal limit) and 80% of oil, salad creams contain only 30%–40% oil. In the case of mayonnaise, the aqueous phase consists of vinegar, citric acid, and egg yolk. The latter is rich in the phospholipid, lecithin, or phosphatidylcholine, an important emulsifying agent. In addition to vegetable oil, vinegar, and egg yolk, a typical mayonnaise will also contain some sugar, salt, mustard, and pepper [39,40]. Low-fat products on the market such as "light" mayonnaise contain much lower levels of oil, between 30% and 40% and 3% and 10% for low-calorie dressings. Unlike mayonnaise and salad cream that are permanent emulsions, French dressings are unstable emulsions made from oil, vinegar, lemon juice, and seasonings that require shaking before using. Addition of egg yolk or other emulsifying agents can be incorporated if a more permanent emulsion is required [13,19,41,42].

VI. CHOCOLATE AND CONFECTIONERY FATS

Chocolate is an important fat-containing food based mainly, but not entirely, on cocoa butter. It can be defined as a suspension of solid particles derived from components such as sugar, milk, and cocoa solids in a continuous fat phase that generates the flavor, aroma, and color of chocolate [175]. The legal definition of chocolate is based on the amount of cocoa butter that limits the use of other fats. Confectionery fats differ from cocoa butter in chocolate but have similar physical and functional properties. Cocoa beans contain 50%–55% fat of which cocoa butter is the main ingredient responsible for chocolate's rheological behavior.

In 2012, the total world production of cocoa beans (*Theobroma cacao*) was 4.1 MMT from which cocoa butter and chocolate are produced. In the country of origin, the harvested cocoa pods are broken open and left on the ground for a week during which time the sugars ferment. After this, the sun-dried beans can be transported and stored. Further processing involves roasting the beans at around 150°C and separating the shells from the cocoa nib, which is then ground to produce a cocoa mass. The cocoa mass is pressed to yield cocoa butter and cocoa powder.

Cocoa butter, a solid fat with a melting point ranging between 32°C and 35°C, is in high demand as it is responsible for the characteristic melting behavior of chocolate [176]. It is hard at ambient temperature giving chocolate its brittleness or snap when it breaks, while the steep melting curve of cocoa butter allows chocolate to melt fast at mouth temperature [177]. The result is a cooling sensation and smooth creamy texture that makes chocolate so desirable. Typical figures for Ghanaian cocoa butter are shown in Table 15.3 in which solid content drops from 45% to 1% between 30°C and 35°C [178]. The chemical composition of cocoa butter is responsible for its unique melting behavior by being rich in palmitic (24%–30%), stearic (30%–36%), and oleic (32%–39%) acids. It also contains linoleic acid as well as small amounts of lauric and myristic acids. Besides Ghana,

TABLE 15.3
Melting Properties of Ghanian Cocoa Butter

Temperature	Solid Content (Pulsed NMR) Tempering at 40 h at 26°C
20°C	76.0
25°C	69.6
30°C	45.0
35°C	1.1

Source: Adapted from Shukla, V.K.S., *Inform*, 8(2), 152, 1997.
Abbreviation: NMR, nuclear magnetic resonance.

TABLE 15.4
Major Fatty Acids (%) in Natural Cocoa Butter Produced from Different Countries

	Fatty Acids			
Country	**Palmitic Acid**	**Stearic Acid**	**Oleic Acid**	**Linoleic Acid**
Ivory Coast	25.8–26.6	36.9	32.9–33	2.6–2.8
Ghana	25.3–25.5	36.7–37.6	32.7–33	2.5–2.8
Indonesia	24.1–25.1	36.9–37.3	33.1–34.3	2.5–2.7
Brazil	25.1–27.9	33.3–33.8	34.5–36.5	3.5–3.6
Ecuador	25.2–25.5	34.6–36	34.6–34.9	2.6–3.0
Malaysia	24.9–26	36–37.4	33.5–34	2.6–3.0

Source: Jahurul, M.H.A. et al. *J. Food Eng*., 117, 467, 2013.

cocoa butter is also produced in Brazil, Indonesia, Ecuador, Malaysia, and the Ivory Coast. The fatty acid composition of cocoa butter varies depending on the location. It is evident from Table 15.4 that Brazilian cocoa butter was higher in linoleic acid and lower in stearic acid compared to that produced in the other countries [179].

Almost 90% of the world's production of cocoa beans comes from the Ivory Coast, Ghana, Indonesia, Cameroon, Nigeria, Brazil, Ecuador, Dominican Republic, and Malaysia. The three major triglycerides in cocoa butter, glycerol-1,3-dipalmitate-2-oleate (POP), glycerol-1-palmitate-2-oleate-3-stearate (POS), and glycerol-1,3-distearate-3-oleate (SOS) account for 92%–96% of the total lipid concentration [180–185]. Of these, POS is the main one accounting for 42.5%–46.4% of the triglycerides, followed by SOS (27.0%–33%) and POP (18.9%–22.6%) [180]. While cocoa butter is high in saturated fatty acids, their presence should not be a concern as stearic acid, for example, is not atherogenic.

The crystal structure of cocoa butter plays a crucial role in the melting properties of chocolate. Six crystalline forms are associated with the solid fat and are designated I–VI. The most preferred crystalline form in chocolate is V because of its good molding characteristics, stable gloss, and favorable snap at room temperature. The preferred V crystal form is produced after extensive tempering to avoid or inhibit conversion to the more stable VI form. Formation of VI is undesirable as it is associated with the appearance of white crystals of fat on the chocolate's surface referred to as "bloom." Fluctuations in temperature during storage can result in this phenomenon due to liquid oil migrating from the nut centers in chocolate. Often mistaken by consumers as microbial contamination, bloom can be inhibited by the small addition of 2-oleo 1,3-dibehenin (BOB) to cocoa butter [186]. Depypere et al. [187] suggested that a chilling treatment of chocolates at –20°C immediately following production might be sufficient to reduce or eliminate visual fat bloom. A recent paper by Tran et al. [188] reported that incorporation of soft and hard SOS-rich fats in plain and hazelnut-based filled dark chocolate retarded oil migration and maintained the quality attributes of the chocolate. More detail about this phenomenon is provided by Padley [189], Smith [190], Lonchampt and Hartel [191], and Green and Rousseau [192].

Simple plain chocolate is composed of sugar and cocoa liquor together with cocoa butter as the sole fat source. Mature cocoa beans contain only small amounts of cocoa butter and are produced in only a few countries [193]. Consequently, cocoa butter is very expensive and often in short supply and unsuitable [194,195]. As a result, the food industry has been searching for fats capable of partially or completely replacing cocoa butter referred to as cocoa butter replacers (CBRs), cocoa butter equivalents (CBEs), and cocoa butter substitutes (CBSs). In Europe, some countries allow

TABLE 15.5
Monounsaturated Triglycerides (%) of Selected Fats and Fractions Suitable for Use in CBEs

Fat Type	POP	POS	SOS
Mango kernel fraction[a]	1	16	59
Kokum butter[a]	Trace	6	72
Sal fraction[a]	Trace	10	60
Shea fraction[a]	1	7	74
Illipe fraction	7	24	45
Cocoa butter	18	17	26

Source: Gunstone, F., *Vegetable Oils in Food Technology, Composition, Properties and Uses*, 2nd edn., Wiley Blackwell, Chichester, U.K., 2011, pp. 291–343.

[a] Stearin (hard/higher melting) fraction.

up to 5% of cocoa butter to be replaced by a fat with a similar fatty acid and triacylglycerol composition. These CBEs include the following tropical fats: palm, illipe, sal, shea, kokum gurgi, and mango [196]. These oils all contain levels of POP, POS, and SOS, the main triglycerides of cocoa butter, and may be used in a fractionated form (Table 15.5).

These CBEs are obtained using chemical or enzymatic fractionation or supercritical carbon dioxide (SC-CO_2) extraction of fats or blends from these various plant sources. Jahurul et al. [179] highlight the production of these cocoa bean alternatives from palm kernel oil, mango seed kernel fats, kokum butter fat, sal fat, shea butter, and illipe fat by blending or modifying them. The overall requirement is that they must have similar physical properties to cocoa butter by being similar in fatty acid and triacylglycerol composition, having an equivalent melting range, producing β-polymorph when processed and tempered, and be compatible with respect to bloom.

VII. ICE CREAM

In 2013, around 1.53 billion gallons (5.78 billion liters) of ice cream and related frozen desserts were produced in the United States with their global production being around twice this amount. Ice cream is a colloidal complex containing fat globules, air, and ice crystals dispersed in an aqueous phase of proteins, salts, sugars, and polysaccharides [198]. Clarke [199] suggested that ice cream was simultaneously an emulsion (fat globules), a sol (ice crystals), and a foam (air bubbles). Fat is an important ingredient in ice cream that accounts for around 5%–12%. It is the main carrier of flavor and contributes to the color, texture, and sensory properties of ice cream [200,201]. Dryness of ice cream, retention of shape, melting in the mouth, and textural creaminess are all properties contributed by the milk fat [202]. Unlike other dairy products that have developed low-fat, low-calorie products, over the past 20 years there has been an upward trend in which premium and superpremium ice creams now contain 12%–16%, respectively. The possibility of replacing part or all of the dairy fat with vegetable fats such as sunflower, groundnut, palm, palm kernel, and coconut varies with the legal requirements for fat in that particular country.

VIII. INCORPORATION OF VEGETABLE OILS INTO DAIRY PRODUCTS

Vegetable oils can serve as replacements for dairy fats. Filled milk is made from skim milk and reconstituted with vegetable oils, including palm, palm kernel, and coconut oils, which are hydrogenated to increase oxidative stability during extended storage. Vegetable oils may be used in cheese manufacture [203–205].

Imitation dairy products have a long history in the market place [206]. A wide variety of products are available to consumers, including whipped toppings, coffee whiteners, infant formulas, dips, and sour dressings. Basically, these products are manufactured in a similar fashion with corn syrup, vegetable fat, and hot water placed in a mixing tank along with emulsifiers, protein stabilizers, colors, and flavors.

Vegetable oil–based toppings may take the form of dry, liquid aerosol or frozen whipped. Dry and liquid toppings contain 36% and 13% fat, respectively. Coffee whiteners also may be solid or liquid. Typically, they contain from 10% to 35% fat. Up until the 1980s, coconut oil was the preferred fat ingredient for many imitation dairy products. However, the issue of saturated fats became a problem and the industry responded by replacing saturated fats with hydrogenated oils [207]. Much of the research leading to whipped toppings was conducted at General Foods that held the early patents on Cool Whip, which remains the top selling brand nearly 40 years later [208–210]. Low-fat whipped toppings are described in the patent literature as well [211–213].

Reconstituted milk was patented by Niemann in 1957 and assigned to the Milnot Company. The popularity of this product was no doubt attributed to the slogan "If cows could give Milnot they would." The process described in the patent allows for the production of condensed whole milk, evaporated milk, and condensed skim milk [214].

IX. EDIBLE COATINGS AND SPRAY PROCESSING

The application of an edible thin layer for coating foods to increase shelf life by minimizing the loss of moisture, oxygen, and carbon dioxide is not new. Such edible protective layers are composed of carbohydrates, proteins, lipids, or a combination of these. Increasing consumer demand for safer, higher quality, and minimally processed foods has intensified in this area [215,216]. The most commonly used lipids are waxes (candelilla, carnauba, or rice bran) plus appropriate triglycerides or acetylated triglycerides [217,218]. Citrus fruits (oranges and lemons), deciduous fruits (apples), vegetables (cucumbers, tomatoes, and potatoes), as well as candies and confectioneries, nuts, raisins, cheeses, and starch-based products (cereals, doughnuts, ice cream cones, and wafers) are among the most frequently coated food products. Specific examples of such edible films include candelilla wax to improve the shelf life and quality of avocados [219] and apples [220]. Using solid lipid nanoparticles (SLNs) prepared with Candeuba®S wax, a mixture of carnauba and candelilla waxes with a melting point of 82°C–86°C, Zambrano-Zaragoza et al. [221] were able to prepare a coating using xanthan gum that increased the shelf life of guavas.

Vegetable oils are used at low levels for spraying on exposed surfaces of food products during roasting, frying, or handling. Such oils have traditionally been soybean or cottonseed oils. They must be liquid at room temperature and oxidatively stable and serve as a moisture barrier, flavor carrier, lubricant, release agent, antidust and anticake agent, and gloss enhancer. While these oils are cheap, they needed elaborate processing including partial hydrogenation and fractionation to meet the required physical state and stability. In light of concerns with *trans* fatty acids, however, high oleic oils are now available. While more expensive, their superior nutritional properties, due to being lower in saturated fatty acids and free of *trans* fatty acids, make them an attractive alternative. Coconut and palm kernel oils, both lauric oils, are used to spray cracker-type biscuits because they provide an attractive appearance, maintain crispness, and enhance eating quality [64,68].

X. EMULSIFYING AGENTS

Emulsifiers are a category of surfactants used primarily to maintain emulsions. They have both hydrophilic and hydrophobic groups that enable them to form dispersions with mixtures of two immiscible fluids such as water and oil. In other words, a heterogeneous system is formed in which one of the immiscible liquids is dispersed as droplets in the other liquid or continuous phase.

The most common or simple emulsion used in the food industry is the o/w emulsion in which the emulsifier forms a thin interfacial protective layer around each oil droplet that prevents them from coming or clumping together [222]. These emulsions are prevalent in such food products as milk, cream, dressings, mayonnaise, sauces, and deserts. Emulsifiers can also stabilize many w/o emulsions in which water is dispersed as droplets in a continuous oil phase such as in margarine and butter.

Whether an emulsifier forms o/w or w/o emulsions is determined by Bancroft's rule, which states that whichever the emulsifier is more soluble in becomes the continuous phase. This is related to the hydrophilic–lipophilic balance (HLB) or hydrophilic–lipophilic difference (HLD) for each emulsifier so that the more water-soluble emulsifiers produce o/w emulsions while emulsifiers more soluble in the oil phase stabilize w/o emulsions. The recent development of structured emulsions is far more complex and includes nanoemulsions, multiple emulsions, multilayer emulsions, solid lipid particles, and filled hydrogel particles [222]. Such systems have tremendous potential for use in foods as well as pharmaceuticals. Bresnard et al. [223] recently reported that multiple emulsions stabilized by a single stimulable polymer did not follow the Bancroft rule.

Molecules with hydrophilic (lipophobic) and lipophilic (hydrophobic) regions are referred to as amphiphilic. Such molecules are able to function as emulsifiers by existing in a physically stable form between the aqueous and fatty substances. Monoacylglycerols (MAGs) and diacylglycerols (DAGs) are examples of widely used nonionic surfactants and emulsifiers with MAGs and DAGs accounting for 75% of the worldwide production of emulsifiers [224]. Industrial-scale production of MAGs currently involves continuous glycerolysis of fats and oils at high temperature (220°C–250°C) in the presence of inorganic alkaline catalysts under a nitrogen atmosphere [225]. This results in a crude mixture of MAGs and DAGs together with a small amount of unreacted triacylglycerol. Because an excess of glycerol is used, dark colored products and undesirable flavors are formed as well as low yields of MAGs (30%–40%) [226]. Molecular distillation is required to produce a highly pure MAG product (>90%) [227]. This is needed by the food industry as highly purified MAG has much better emulsifying properties than a mixture of different acylglycerols [228].

An alternative method to high-temperature glycerolysis is the enzymatic synthesis of MAGs under much milder temperature conditions [228]. Early attempts on the enzymatic production of MAGs included the use of 1,3-regiospecific lipases [229], fatty acid esterification or transesterification with glycerol [230], and the solid-phase enzymatic glycerolysis of beef tallow [231]. Since the cost of the enzyme is prohibitive, Kaewthong et al. [232] examined nine commercial lipase preparations for the production of MAG by glycerolysis of palm olein. One of these, a lipase from *Pseudomonas* sp. immobilized on Accurel EP100 gave the highest hydrolytic activity and thermostability compared to the soluble enzyme. Singh and Mukhopadhyay [233] reported that a commercially immobilized lipase B from *Candida antarctica* on Immobead 50 successfully catalyzed the glycerolysis of olive oil in a solvent-free system. Under optimum operating conditions, a maximum yield of 26% was obtained for MAG and 30% for DAG within 3 h. The low concentration of lipase needed for the production of MAGs and DAGs would favorably reduce the cost of the enzyme. It is evident that the milder enzymatic glycerolysis procedure will soon replace the harsher chemical glycerolysis method for the production of MAGs and DAGs.

Oils commonly used for the production of MAGs and DAGs include lard, tallow, soybean, cottonseed, sunflower, palm, and palm kernel oil—hydrogenated or nonhydrogenated form. One of the most commonly used products is glycerol monostearate (GMS). Depending on the desired specific use, the properties of a MAG can be improved by acylation of one of the free hydroxyl groups by reacting with acid (lactic, citric) or acid anhydride (acetic, succinic, diacetyltartaric).

Emulsifiers used in food have been classified as group A (acceptable) and group B (provisionally acceptable). Among group A are lecithins, MAG and DAG, acetic acid esters of MAG and DAG, polyoxyethylene, sorbitan monostearate, monopalmitate and monostearate and tristearate (Tween 20, 40, 60, 65, and 80), as well as polyglycerol and sucrose esters of fatty acids [234].

XI. NEW DEVELOPMENTS IN STRUCTURED EMULSIONS: OLEOGELS AND BIGELS

One way of converting a liquid oil into a gel-like material is by oleogelation. This approach to edible oil structuring has been studied for many years but has only recently been hailed as a feasible approach for producing gel-like material from oil without the need to chemically modify the oil [235]. These gels or oleogels are very high in liquid oil (≥90%) that is free of *trans* fats, low in saturated fats, but high in unsaturated fats. While in their infancy, oleogels are highly nutritious and have considerable potential for use in food formulations. Patel and Dewettnick [235] categorized oleogelation and oil structuring based on the molecular characteristics of the gelators [236,237], the number of gelators [236,238], and the type of building blocks and structural principle of gelation [239]. Rogers et al. [240] discussed the application of this gel technology for lipids in experimental culinary kitchens around the world.

Recently, Pastel et al. [241] first reported using the structuring properties of fumed silica (hydrophilic colloidal silica particles) to produce triglyceride solvent-based organogels as well as bigels. The latter are a new class of soft matter systems in which both the organic and aqueous phases are distributed as percolating networks [242–244]. The bigels exhibited greater gel strength than the organogels, although the structure recovery behavior was weaker. Nevertheless, the significant influence of the proportions of organic and aqueous phases on the rheological properties of the bigels suggests the potential for fine-tuning.

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16 Encapsulation Technologies for Lipids

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CONTENTS

I. INTRODUCTION

Dietary lipids can be obtained from plant, animal, or microbial sources. They include many broad classes of neutral and partially polar compounds with differing physiological functions. The major components of dietary fats are triacylglycerols (TAGs). Other components with nutritional significance include phospholipids, sterols, fat-soluble vitamins, antioxidants, and other fat-soluble components.

Polyunsaturated fatty acids (PUFAs) are a class of compounds that have a variety of important functions in biological systems. Studies have shown that longer-chain fatty acids, in particular eicosapentaenoic acid (EPA, C20:5n3), arachidonic acid (ARA, C20:4n6), and docosahexaenoic acid (DHA, C22:6n3), have important roles as biosynthetic precursors, as cellular membrane components, and in the protection against oxidative stress.

Consumer food preferences and awareness of the link between health and food have risen sharply over the years. Growing interest due to health benefits of lipid classes and PUFAs has focused attention on the development of functional foods fortified with those lipids. Significant among the growing class of functional foods are foods that are fortified with omega-3 fatty acids.

Most edible oils containing PUFAs are not chemically stable and therefore are susceptible to oxidative deterioration, especially when exposed to oxygen, light, moisture, and temperature. Further effects of oxidative degradation include loss of nutritional quality and development of undesirable flavors, thereby affecting the stability and sensory properties of the oil. This makes the incorporation of these oils and fatty acids a challenge. Therefore, oil encapsulation (i.e., microencapsulation and nanoencapsulation) technology has been increasingly attracting the attention of food ingredient suppliers and food product manufacturers to retard lipid autoxidation and increase the range of applications where otherwise the oil could not be used [1]. Furthermore, this technology has the advantage to mask bad taste or smell and increase the oil bioavailability.

Encapsulation has been employed by the food industry for more than 75 years. Encapsulation can be described as a coating of solid, liquid, or gaseous food components, enzymes, cells and other materials, and microorganisms, with a protein, carbohydrate, and fat- or vegetable oil–based material [2].

In recent years, the food industry concentrated its efforts on the production of functional foods, which includes the addition of functional compounds (e.g., antioxidants, fatty acids, minerals, vitamins, phenolics, phytosterols) and living cells (e.g., probiotics) in products. Since these bioactive food compounds are characterized by rapid deterioration or loss, successful encapsulation of these compounds is therefore important to control their flavor, color, texture, or preservation properties since it slows down the degradation processes (e.g., oxidation or hydrolysis) or prevents degradation until the product is delivered at the desired sites. Thus, the bioactive component would be kept fully functional.

II. TECHNOLOGIES FOR ENCAPSULATION OF LIPIDS

Microencapsulation can be defined as a process of enclosing small particles, liquid or gas within a coating or a matrix for protection, isolation, or controlled release of the enclosed material [3]. The common nomenclature used for defining the ingredient that is to be encapsulated is called an active, core, internal phase, encapsulate, or fill. The material that envelopes the active part is either called the wall material, capsule, membrane, carrier, shell, encapsulant, support phase, or coating [4–6]. Encapsulation that falls within the range of 100–1000 nm is classified as microencapsulation [4,5]. Although most of microcapsules are spherical in shape, microencapsulation technique, composition of the wall material, and physicochemical properties of the material influence the appearance of the microcapsules [7]. Different morphologies given in Figure 16.1 could be obtained: simple microcapsules coated by a uniform thickness, particles containing an irregular shape core, more than one core particles embedded in a continuous matrix of wall material, and several distinctive cores within the same capsule and multiwalled microcapsules [3].

Some of the microcapsule models are shown in Figure 16.1 [8]. Generally, size and shape of the formed microcapsules depend on wall materials and the methods used to prepare them. The reservoir type has a single membrane around the active agent and is commonly referred to as the capsule, single-core, mono-core, or core–shell type. Poly- or multiple-core-type encapsulates also exist. In the matrix type, the active agent found in the form of small droplets is more dispersed over the membrane [9,10].

The main purpose of using microencapsulation in the food industry is to protect the active ingredient, which is the core material from the surrounding environment (e.g., heat, moisture, air, and light) [12].

Furthermore, encapsulation aims to increase the stability of the coated material and to allow controlled release of the microencapsulated material.

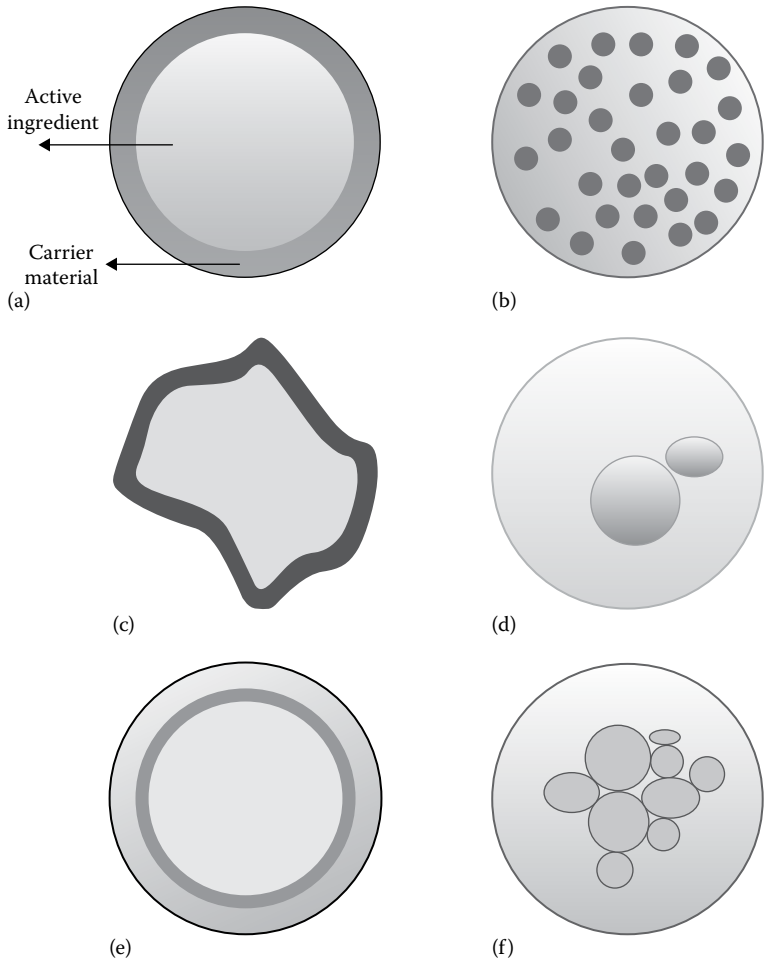

FIGURE 16.1 Morphology of different types of microcapsules: (a) simple microcapsule, (b) matrix, (c) irregular microcapsule, (d) multicore microcapsule, (e) multiwall microsphere, and (f) assembly of microcapsule. (From Zuidam, N.J. and Shimoni, E., Overview of microencapsulates for use in food products or processes and methods to make them, in: Zuidam, N.J. and Nedovic, V., eds., *Encapsulation Technologies for Active Food Ingredients and Food Processing*, Springer, New York, 2010, pp. 3–29.)

The food industry applies encapsulation for a number of reasons:

- The physical characteristics of the original material can be modified and made easier to handle. For example, a liquid component can be converted into solid particles and hygroscopicity can be reduced, and flow and compression properties can be improved.
- The taste and flavor of the active material can be masked.
- It can be used to separate reactive components within a mixture that would otherwise react with one another, thus preventing undesirable interactions with the food matrix.
- It makes possible the application of lipid-based materials in the liquid products that are not normally soluble in water [12].

Interior materials to be coated may be in liquid or solid form [13], and the liquid component may be present as dissolved or dispersed [4]. The inner solid material may be a mixture of active ingredients, stabilizers, and solvents [14]. The wall material is nonreactive with the core.

TABLE 16.1
Materials Used for Microencapsulation of Hydrophobic Materials

Materials	Examples
Polysaccharides	Sugars, starch, octenyl succinate starch, MD, dextrin, cyclodextrin, glucose syrup, cellulose, GA, alginate, carrageenan, pectin
Proteins	Soy, wheat, corn (zein), gelatin, casein, caseinate, whey protein concentrate (WPC), whey protein isolate (WPI), legume proteins
Other polymers	Chitosan, polyethylene glycol, polyvinyl acetate, cellulose, and derivatives such as carboxymethyl cellulose, MC, ethyl cellulose

Sources: Harrington, J. and Schaefer, M., Extrusion-based microencapsulation for the food industry, Chapter 8, in: Gaonkar, A.G. et al., eds., *Microencapsulation in the Food Industry*, Elsevier, 2014, pp. 81–84; Sobel, R. et al., Introduction to microencapsulation and controlled delivery in foods, Chapter 1, in: Gaonkar, A.G. et al., eds., *Microencapsulation in the Food Industry*, Elsevier, 2014, pp. 3–12; Dyvelkov, K.N. and Sloth, J., New advances in spray-drying processes, Chapter 6, in: Gaonkar, A.G., et al., eds., *Microencapsulation in the Food Industry*, Elsevier, 2014, pp. 57–63; Can Karaca, A., Encapsulation of FO using plant proteins, PhD thesis, Department of Food and Bioproduct Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, 2012.

It accounts for 1%–80% of the microcapsule by weight and it must possess the following characteristics [10,13]:

- Should be inert toward the active ingredient
- Should have specific wall width
- Should be compatible with the material to be coated
- Should stabilize the interior material
- May require controlled release under specific conditions
- Should be easily available and cheap
- Should reduce the loss from vaporization

Generally, a single coating material is not expected to meet all these features. It is therefore possible to mix more than one material. In the microencapsulation process, typically sugars, gums, proteins, natural and modified polysaccharides, lipids, waxes, and synthetic polymers can be used [7]. For hydrophobic actives such as fats and oils, a hydrophilic material must be used so that the coating can exist at the surface of the active ingredient. A wide variety of polysaccharides, proteins, and other polymers have been used for encapsulating hydrophobic ingredients as shown in Table 16.1.

There are a number of techniques available for encapsulation of food compounds. Since encapsulating compounds are very often in liquid form, many technologies are based on drying. Microencapsulation techniques can be separated into three classes: physical processes such as spray drying, spray chilling/cooling, freeze-drying, co-crystallization, extrusion, or fluidized bed coating, chemical processes such as molecular inclusion or interfacial polymerization, and physicochemical techniques such as single- or multicore coacervation and liposome encapsulation [1]. The selection of the method to be used for coating the active material with the suitable wall material depends on the material's physical and chemical properties and the application area of the resulting microcapsules [7].

A. Microencapsulation by Spray Drying

Spray drying is one of the oldest and the most extensively applied encapsulation technique in the food industry because it is flexible, continuous, but more importantly it is an economical operation. The prepared particles are small but the size distribution of particles is very wide. The fine powder

prepared by spray drying on a laboratory scale is in the range of 10–50 μm but may be up to 200 μm on an industrial scale [18]. This feature is desired from the standpoint of the sensorial and textural characteristics of final products since these powders can be added into food matrices without significantly changing mouthfeel [18].

Encapsulation is performed by first dissolving, emulsifying, or dispersing the core substance in an aqueous solution of carrier material, which is followed by atomization and spraying of the mixture into a hot chamber [19]. The spray-drying process involves four stages: preparation of a dispersion or emulsion, homogenization of the dispersion, atomization of the feed emulsion into a spray chamber, and dehydration of the atomized particles. Emulsification before the spray-drying process involves the formation of 1–3 μm or small droplets containing the core ingredient in concentrated shell dispersion. Single- or multilayered oil-in-water (o/w) emulsions have been employed to entrap or encapsulate oils [18]. In industrial applications, primary homogenization is applied first and is followed by secondary homogenization such as pressure homogenization or valve homogenization in order to decrease the particle size. Since the particle has an influence on encapsulation efficiency, smaller particle size gives better efficiency.

Figure 16.2 shows the process stages of spray drying that include (1) atomization of liquid feed into a spray chamber, (2) contact between the spray and the drying medium, (3) separation of dried products from the air stream, and (4) cooling the powdered product by fluidized bed coating (depending on the product type).

In contrast to freeze-drying, spray drying is more economical and faster and is a single-step drying method [19]. Although spray drying seems to have high inlet temperatures such as 150°C–250°C, the encapsulated core material does not exceed these high temperatures because of the rapid evaporation of water from the coating material [18].

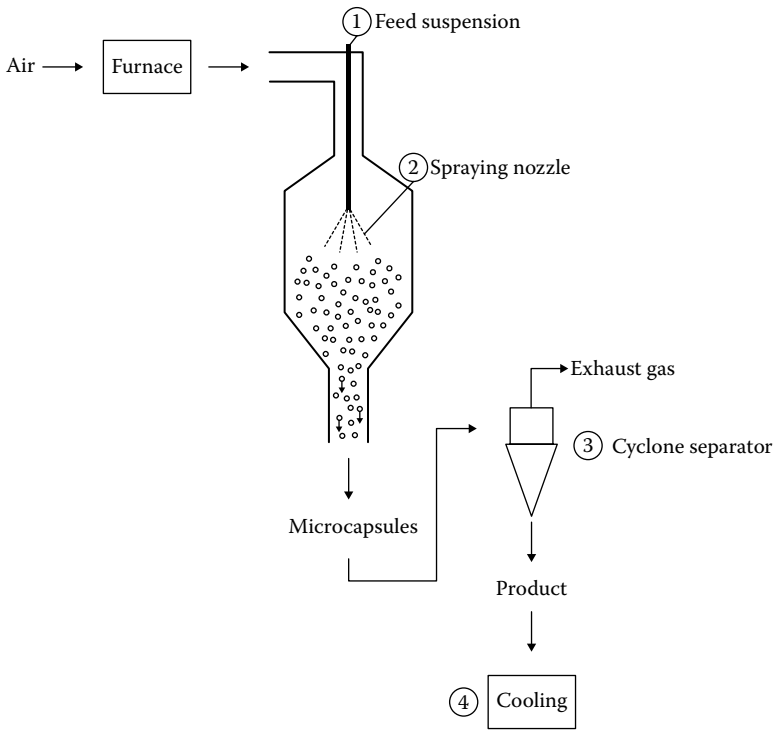

FIGURE 16.2 The process stages of spray-drying microencapsulation: (1) atomization of liquid feed, (2) contact between the spray and the drying medium, (3) separation of dried products from the air stream, and (4) cooling.

Until now, spray drying has been the most widely used technique in the food industry to encapsulate a wide range of food ingredients such as fats and oils, flavors, vitamins, minerals, probiotic cultures, nutraceuticals, and colors in order to protect them from their surrounding environment and to extend shelf life stability during storage [16,19].

Around 80%–90% of encapsulates are the spray-dried ones [18]. Wall or shell materials are numerous, since a large range of carbohydrates (e.g., starch, cyclodextrin, modified starch, maltodextrins [MDs], chitosan), cellulose (e.g., carboxymethylcellulose, methylcellulose, ethylcellulose), gums (e.g., alginate, guar gum, gum arabic, gum acacia, carrageenan), proteins (e.g., gluten, casein, gelatin, albumin, or peptides), and mixtures thereof can be used for encapsulation of lipids [16]. The key factor is that they should be soluble in water, and the viscosity should not be too high for proper atomization during spray drying. Although the use of spray-dryers in the food industry is widespread, there are several disadvantages of this technique such as complexity of the equipment and nonuniform conditions in the drying chamber. Spray drying yields micron size and uniformly spherical particles that offer complete protection of the core material on encapsulation [19]. Although the major concern for spray-drying technology is the control of the particle size, it can be solved by adjusting process parameters and formulations [19]. Moreover, a dusty product is difficult to handle and a high surface area is detrimental to protection against oxidation. The dust issue of spray-dried powder can be resolved using multistage drying technology [20]. Spray drying can be combined with other techniques such as fluid bed coating or agglomeration, granulation or multicore encapsulation, coating or functionalization of the powder [16].

B. Microencapsulation by Freeze-Drying

Freeze-drying or lyophilization is a drying method in which water in a solution or suspension is crystallized at low temperatures and sublimed from the solid state directly into the vapor phase [19]. During freezing of foods, the temperature decreases and water is removed from the food in the form of ice, and the solutes present in the unfrozen products are freeze-concentrated [19].

Usually, it is a required method of microencapsulation for heat-sensitive ingredients such as expensive flavoring agents, omega-3 oils, and phenolics. The quality of freeze-dried products is very high in comparison with that of products dehydrated using other techniques, due to the prevention of heat damage. However, energy intensiveness, long processing time (more than 20 h), and an open porous structure are the main drawbacks of this technology [19]. Since the commercial cost of freeze-drying is much higher than that of spray drying and other methods, it is generally employed for drying high-value ingredients such as probiotic microorganisms and several bioactives [18]. However, spray-freeze-drying may be an effective alternative to conventional freeze-drying in terms of reducing the pore size and drying time [19].

A freeze-drying process provides a product that is stable (in dry form), rapidly soluble (large surface area), and elegant (uniformly colored cake). The freeze-drying process consists of five steps, namely:

1. *Freezing*: A substance is frozen to form crystallized ice. Most food components remain in an amorphous, glassy state (i.e., do not crystallize), but the water component does crystallize.
2. *Primary drying*: Ice is removed by sublimation at low temperature and low pressure. Sublimation occurs at the interface between the frozen and dry material and this starts at the ice surface.
3. *Secondary drying*: Unfrozen water is removed by desorption. This step typically takes one-third of the drying time. The final moisture content for food stuffs is 2%–10% and for biological products is 0.1%–3%.
4. *Final treatment*: The drying chamber is filled with an inert gas (nitrogen for foodstuffs, argon for biological products) for preserving the products after drying [19].
5. Milling to obtain the powdered product.

C. Microencapsulation by Fluidized Bed Coating

Fluidized bed coating, which was developed in the 1950s by D. E. Wurster, is employed by suspending or fluidizing particles of an active material in an upward stream of air and by spraying the coating material to the fluidized particles. Great variations for choosing coating materials exist. Fats, carbohydrates, and proteins are examples of coating systems. They may be used in molten state or dissolved in a suitable and evaporable solvent [2,18].

This process yields encapsulates with particle size higher than 100 μm [2]. This encapsulation method is generally used for coating unstable ingredients such as vitamin C. It is not suitable for primary encapsulation of lipids and fats; however, it can be applied to give an additional coating to primary microencapsulated powdered omega-3 fatty acids [18]. A more recent technology named fluidized bed spray granulation allows particles to have size distributions ranging from 0.2 to 1.2 mm with the possibility of producing uniform particle sizes and shapes [21].

D. Microencapsulation by Extrusion

Extrusion technology is used to produce high-density encapsulated products such as flavor oils, omega-3 fatty acids, and enzymes. Compared to other techniques, it is a relatively new process since it is quite complex and less studied than the other methods [5]. Although the cost of extrusion is twice that of spray drying, it is the second preferred method for encapsulation of flavors, ranking behind spray drying. The process has also been applied to omega-3 oils. The extrusion method consists of mixing of molten carriers with the active material followed by solidification. The core material is dispersed into the hot melt, and thereafter, the hot melt is extruded generally through a single- or twin-screw system to extrude a formed shape of the cooling melt for matrix encapsulation. Subsequent milling of the extruded material results in the formation of matrix particles, as illustrated in Figure 16.3 [2,5]. The major advantage of the extrusion method is that the shelf life of the microcapsules is high due to their glassy state. This is especially important when the microcapsules are applied to lipid-based food products. Disadvantages include less payload compared to other techniques. The active material can be loaded at about 10% maximum. An alternative extrusion technology is coextrusion where annular-jet atomization is applied. In coextrusion, heated aqueous polymer solution is extruded through an outer tube and the active material is extruded through an inner tube into a moving carrier fluid. Core–shell droplets are formed through annular jet and the outer shell droplets then solidify to form a microcapsule shell [22].

In the spinning disk method, an encapsulation matrix passes over a rotating disk operated by centrifugal force. Chilling or drying techniques are employed for solidifying the microparticles [18].

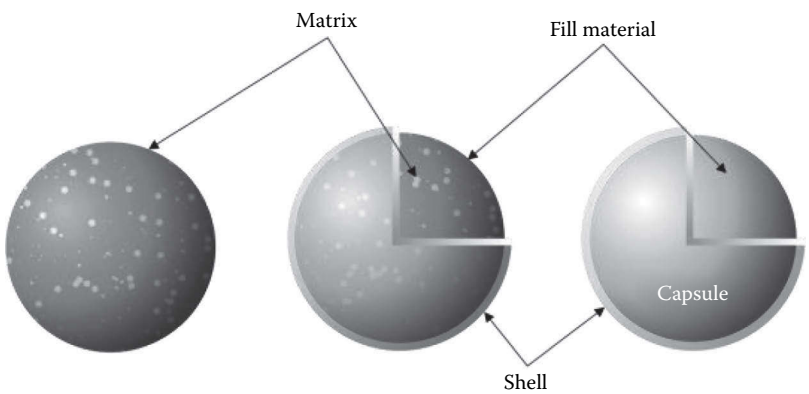

FIGURE 16.3 Morphology of matrix particles formed by extrusion.

The particle sizes of the microcapsules obtained by coextrusion vary between 150 and 2000 μm, whereas with the rotating disk method the particle size range is 30 μm to 2 mm [18]. Coextrusion and spinning disk techniques have potential utilization in the microencapsulation of omega-3 oils if the particle sizes tend to be appropriate for the texture and the mouthfeel of the food to which the microencapsule will be added [18].

E. Emulsion-Based Encapsulation Techniques

Among the microencapsulation techniques, emulsion-based encapsulation techniques such as liposome technique, layer-by-layer emulsions, simple coacervation, complex coacervation, in situ polymerization, interfacial polymerization, micelles, solvent evaporation, in-water drying, solid lipid nanoparticles, and sol-gel chemistry are present. All of these systems are not suitable for use in food but may find applications in relevant areas such as agriculture or food packaging [22].

Recently, a liposome-based microencapsulation technique has gained considerable interest for encapsulation of bioactives. Liposomes are generally made from phospholipid molecules, which have both hydrophilic and hydrophobic parts. The mechanism of liposome formation is based on the unfavorable interactions that occur between amphiphilic compounds (mainly phospholipids) and water molecules, where the polar head groups of phospholipids are subjected to the aqueous phases of the inner and outer media, and the hydrophobic hydrocarbon tails are associated with the bilayer and spherical core shell structures are formed. Therefore, when phospholipids, such as lecithin, are added to water, liposomes form spontaneously because of the interaction of the water-soluble part of the phospholipid with water and the oil-like part of the molecule avoids water [10,21]. A phospholipid bilayer can be formed by orienting oil-like portions toward each other, and this bilayer extends itself in water to form a sheet, which then curls into a liposome as shown in Figure 16.4 [10].

Both the water-soluble and lipid ingredients can be encapsulated at the same time since the interior of liposomes is filled with water and the water-soluble molecules can be encapsulated in the interior, whereas oil-like molecules can be entrapped in the oil-like portion of the phospholipid bilayer [18].

Liposomes have a number of benefits, such as possibility of industrial-scale production using natural ingredients and entrapment of materials with different solubilites, entrapment and release

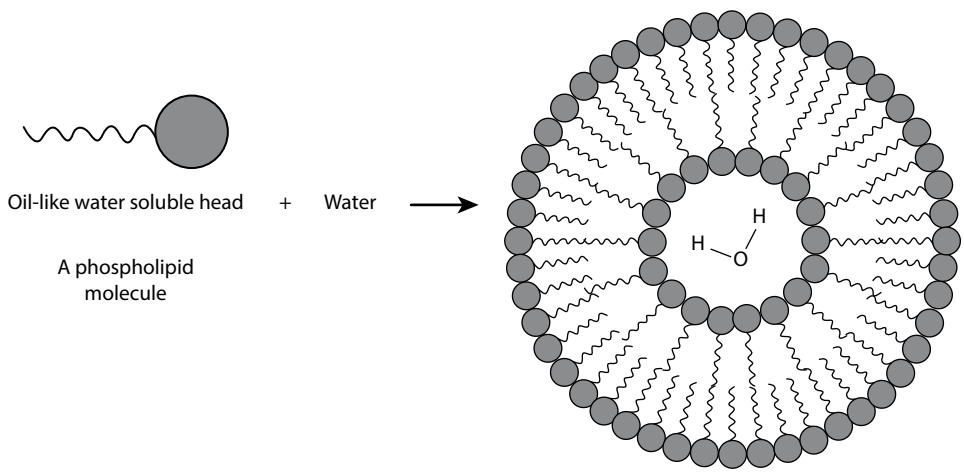

FIGURE 16.4 Formation of liposome structure. (From Mirafzali, Z. et al., Application of liposomes in the food industry, Chapter 13, in: Gaonkar, A.G. et al., eds., *Microencapsulation in the Food Industry*, Elsevier, 2014, pp. 139–150; Barrow, C.J. and Wang, B., Spray drying and encapsulation of omega-3 oils, Chapter 6, in: Jacobsen, C. et al., eds., *Food Enrichment with Omega-3 Fatty Acids*, Woodhead Publishing Limited, 2013, pp. 194–225.)

of water-soluble, lipid-soluble, and amphiphilic materials as well as targetability. Lipid vesicles can be tailored to deliver and release their load in the target site inside and outside the body. It has been shown that the hydrophobic components are better incorporated into the lipid membranes. Furthermore, with liposome systems, DHA-rich oils have been better protected from peroxidation [18]. However, high cost and sometimes low stability due to chemical degradations such as hydrolysis or oxidation, aggregation, and fusion are the main drawbacks of this technology for food or nutraceutical formulation.

F. Microencapsulation by Coacervation

Coacervation is defined as separation into two liquid phases in colloidal systems [3]. The basic idea behind microencapsulation by coacervation is phase separation. Two phases that are present are a rich solvent phase with very small amounts of biopolymer(s) and a rich biopolymer(s) phase [9] and "coacervate" that is more concentrated in the colloid component.

Coacervation is classified into two categories, simple coacervation and complex coacervation. Simple coacervation usually involves one colloid solute and includes the addition of a strong hydrophilic compound to a weaker hydrophilic colloidal dispersion. It generates two layers such as one rich in colloid droplets, and the other one deficient in such droplets. On the other hand, complex coacervation involves more than one colloid and is formed by the interaction of two oppositely charged colloids [8] between a polyanion and a polycation, which are both water soluble [6]. Complex coacervation occurs with the formation of complex microspheres as a result of decreasing solubility of the solution containing two oppositely charged macromolecules with changing ambient conditions [23,24].

Complex coacervation technology was first developed to make carbonless paper for the printing industry several decades ago. The complex coacervation process has been used for encapsulating high-value and labile functional ingredients or volatile compounds, such as flavors, pro-/prebiotics, antioxidants, vitamins, minerals, phytosterols, peptides, lutein, lycopene, and PUFAs, to increase their shelf life under different storage conditions (light, oxygen, moisture, and temperature), to allow alternative food processing (heat, shear, and redox potential), and to mask the taste or to control release of encapsulated components [25].

In order to generate complex coacervation, various macromolecular systems have been used. Polysaccharides are usually used as polyanions. Gum arabic (GA), pectin, alginate, carrageenan, agar, gellan, chitosan, guar gum, and glucan, polyphosphate, and carboxymethylcellulose that are generally recognized as safe are the most widely used molecules. Polycations are mainly proteins, such as gelatin, soy proteins, casein (caseinate), pea proteins, whey proteins, β-lactoglobulin, bovine serum albumin, and egg albumin [25].

The physicochemical properties, thickness, and microstructural properties of the coacervate layer (shell material) are key points to determine the behavior of microcapsules (stability, redispersibility, and microencapsulation efficiency). Hence, choice of the wall material and interaction among its components are other critical issues for successful microencapsulation by coacervation [11].

The complex coacervation process is significantly affected by the pH of the solution, since pH determines the charge density on ampholytes and may even induce structural transitions of proteins and polysaccharides [13]. Furthermore, the nature of shell materials and core ingredients, their composition, and other aqueous conditions, such as pressure, shearing, temperature, and ionic strength, have an influence on the process [25]. Briefly, the complex coacervation process principally consists of three basic steps: emulsification, coacervation, and shell formation and/or hardening [25]. The principle of the complex coacervation method is shown in Figure 16.5.

Since coacervation consists of two extra steps including coacervation and hardening compared with spray drying, it is regarded as a longer, complex, and expensive process [25]. However, microcapsules produced by coacervation were reported to have numerous advantages. They have been reported to have excellent controlled release characteristics since the microcapsules are water

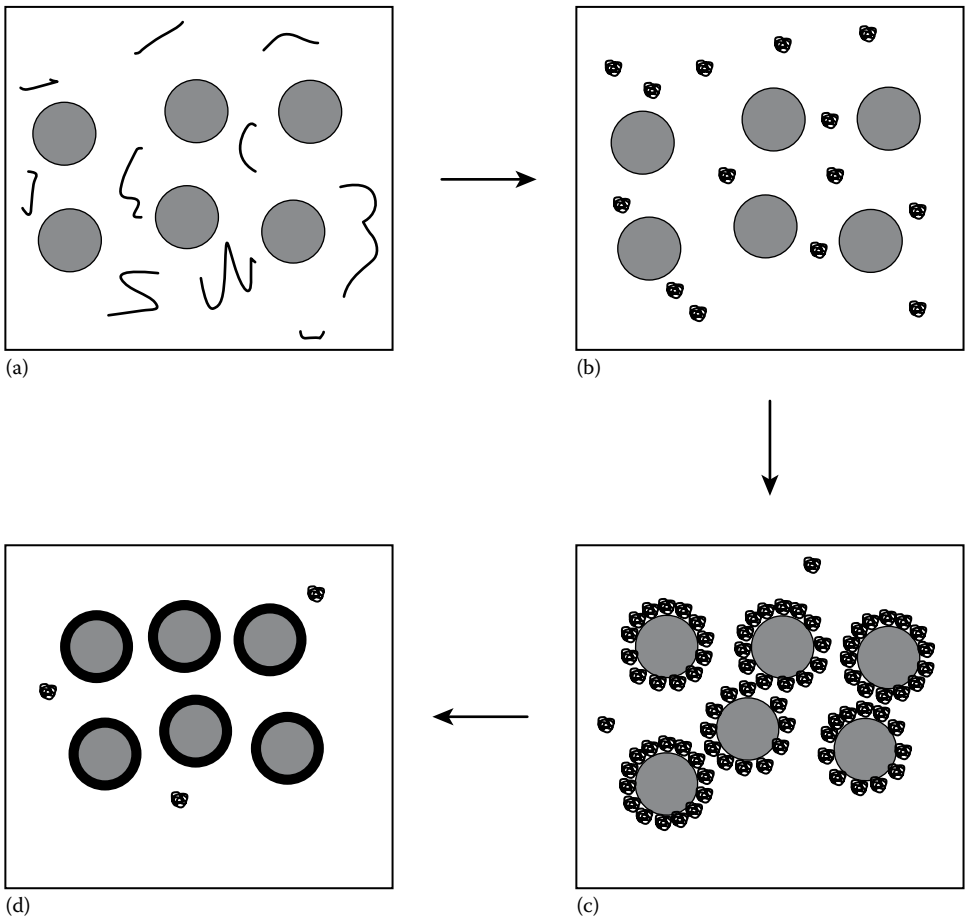

FIGURE 16.5 Example of complex coacervation involving (a) emulsification of oil in an aqueous solution, (b) initial coacervation of polymers after lowering pH, (c) coacervation of polymers on the surface of the oil, and (d) formation of the cross-linked shell by reticulation of the interface by addition of a cross-linking agent. (From Kralovec, J.A. et al., *Food Chem*., 131(2), 639, 2012.)

immiscible and have heat resistance properties depending on the type of wall material and the cross-linking (hardening) step [13]. Moreover, high core loading levels up to 99% are possible with high encapsulation efficiency [25].

The complex coacervation method has been successfully used to microencapsulate omega-3 and omega-6 oils. Two types of complex coacervates depending on the homogenization procedure are used. The first method is named as "single-core complex coacervation" in which the oil droplets are encapsulated within a single shell. And the other method called the "multicore complex coacervation" involves dissolving the two polymers gelatin and polyphosphate in water and further homogenizing and coacervates are formed [18].

A major disadvantage of complex coacervation technology is the limitation in the shell materials. Gelatin has been a widely used material due to its unique gelation properties. However, gelatin has some limitations such as not being vegetarian, and the Kosher versions are relatively expensive. Moreover, gelatin does not always have ideal sensory properties for all applications. Alternative materials to gelatin are needed and being explored. Therefore, in recent years, researchers reported the use of nongelatin materials such as globular proteins with anionic polysaccharides, for example, β-lactoglobulin, bovine serum albumin, egg albumin, soy proteins, pea proteins, and whey proteins

TABLE 16.2
Process Steps for Microencapsulation Techniques

Microencapsulation Technique	Process Steps	Particle Size (μm)
Spray drying	Preparation of a dispersion or emulsion to be processed	10–200
	High-pressure homogenization	
	Atomization	
	Dehydration	
Fluidized bed coating	Preparation of coating liquid	5–5000
	Fluidizing the material to be coated	
	Coating by spraying	
	Cooling	
Extrusion	Blending	300–5000
	Extrusion	
	Cooling	
Coacervation	Emulsion preparation	10–800
	Mixing	
	Drying	

Source: Koç, M. et al., *Pamukkale Üniv. Müh. Bil. Derg*., 16(1), 77, 2009.

with GA, carrageenan, and pectin in complex coacervation systems [1]. Whey protein–GA–fish oil systems are examples of the commercial production of nongelatin vegetarian microcapsules [1].

The process steps of the previously mentioned microencapsulation techniques are summarized in Table 16.2.

G. Microencapsulation Using Sporopollenin Exine Capsule

Recently, a new microencapsulation process was launched by using pollen and plant spore walls found in nature [26]. Spores are generally obtained from a herbaceous plant species *Lycopodium clavatum* (club moss), and the scanning electron micrsocopy (SEM) images belonging to *L. clavatum* are shown in Figure 16.6 [27].

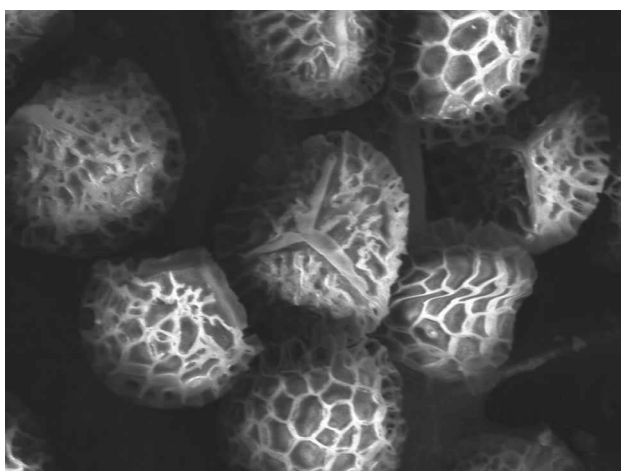

FIGURE 16.6 SEM images of *Lycopodium clavatum* spores.

Spores generally contain lipids, some vitamins, carbohydrates, and proteins in their structure [28]. In the interior of the spore, genetic material exists and they are protected by a complex and robust wall with two principal layers known as "intine" and "exine." Usually, spores have a wall thickness of 1–2 mm [29]. The inner layer is made of cellulose [30]. The intine, which is the innermost of the major layers, underlies the exine and borders the surface of the cytoplasm. Shells containing the exine have an elastic structure and do not deteriorate even when exposed to tons of pressure [26].

Exine is the most important feature of pollens since it consists of a unique polymer in nature known as "sporopollenin," which is one of the most resistant, natural organic materials known [31]. Spore particles that remained intact have been found in ancient sedimentary rocks, which are 500 million years old [28,31].

Sporopollenins have good stability against chemicals and have a stable chemical structure that does not change even when exposed to acids or alkali [26]. Furthermore, at temperatures higher than 250°C, they may even remain stable. In the microencapsulation technology, the most difficult thing is the inability to obtain capsules with a uniform morphological structure, and physical and chemical damages to the capsules during operations are the major problems faced during encapsulation [27].

Such stability and the ease of removal of genetic material by chemical extraction are important for the preparation of microcapsules. Moreover, allergenic proteins, which may be present in the bulk pollen, can be removed easily and cheaply by chemical extraction [26,30,31].

The exine derived from spores has lipophilic character [28]. Hollow exine wall, instead of genetic material, can be filled with oil. The oil particles move slowly inward from the lipophilic walls and the speed can be increased by creating a vacuum (e.g., by shaking) inside the shell [26]. Fish oil microencapsulation technology using exines is believed to have a major role in masking the smell and taste, protecting against oxidation, providing the active as a powder, and minimizing burp-back effect, and is believed to be a new method of interest. Furthermore, it was reported that the bioavailability of EPA is enhanced by encapsulation through the use of pollen exines [32].

III. CHARACTERIZATION OF MICROENCAPSULATED OILS

The physical properties of microencapsulated oils and their stability need to be determined since these properties are important considerations for suppliers and users [2]. Microencapsulated omega-3 and omega-6 oils can be characterized using physical, chemical, or physicochemical properties [33]. Physical properties such as encapsulation efficiency, surface oil and payload, particle size and shape of capsules, flowability, dispersibility, color, moisture content, and water activity are generally analyzed for microencapsulated omega-3 and omega-6 PUFAs [34]. Furthermore, oxidative stability is also measured as a chemical property. Sensory evaluation and shelf life of foods fortified with omega-3 and omega-6 PUFAs are critical and should be measured [34]. Those characteristics are important not only for stabilization of the active material but also for bioavailability and the success of the targeted application [33].

A. Encapsulation Efficiency

The primary aim during microcapsule production is to obtain a high encapsulation efficiency and payload together with high oxidative stability. Therefore, high oil load is desirable [2]. Encapsulation efficiency is defined as "the ratio of the mass of the encapsulated core material to the mass of the active material" in the formulation. Free oil that is not encapsulated is known as "surface oil." Surface oil should be as low as possible since it can oxidize rapidly and may lead to poor stability of capsules. Ideally, it should be smaller than 0.1% (w/w). Homogenization condition can influence the surface oil content. Surface oil can be kept at minimum if effective homogenization can be applied, and thus, the particle size is lowered. Thus, surface oil can be low, whereas encapsulation

efficiency and oxidative stability can be high. Surface oil should be measured by using several methods including extraction methods such as Soxhlet method and quantifying the extracted oil gravimetrically or by using ultraviolet–visible (UV–vis) or infrared (IR) spectroscopy [33].

B. Payload

Payload is defined as "the amount of oil or active material encapsulated within a shell or matrix" [22]. Payload is calculated by taking the ratio of mass of encapsulated oil to the mass of powder [33]. Mass of the encapsulated oil can be determined by quantitative extraction of oil from microcapsules, followed by weighing of extracted oil or by using a more developed technique such as Fourier transform infrared (FTIR) spectroscopy [33].

High payloads are desired during the encapsulation process to make the process economically feasible [33]. Lower payloads require using more encapsulated material to achieve a set concentration of the active material, while for higher payloads a lower amount of powder is required for food applications [22,33]. Even with the advantages of higher payloads, problems such as decreased protection of the encapsulated ingredient may arise with higher loadings and lower amount of a protective matrix. Despite the disadvantages, higher loadings may be required for specific applications such as quick release, granulation, or minimal environmental protection of the core material [22].

Payload amounts may range from less than 1% to 99% depending on the encapsulation process, encapsulation materials, and particle morphology. Emulsion-based processes such as micelles or capsules prepared with layer-by-layer shells offer loadings over 99% [22]. Those high loadings are generally observed during laboratory-scale productions; unfortunately in the large-scale productions, it is not common to reach those very high payloads.

C. Particle Size and Morphology

Particle size has many effects on the mouthfeel, mechanical stability, product homogeneity, and beverage suspension. Therefore, selection of a suitable particle size is crucial for the final application of the encapsulated material [22]. For food applications, the size of microcapsules should be lower than 100 μm [33]. It is well known that physical-based encapsulation methods such as atomization as well as spray coating result in the formation of capsules over 10 μm. Particle size of the capsules is below this range when emulsion-based techniques are used [22]. The size of microcapsules can be measured by dynamic light scattering and electron microscopy such as SEM and transmission electron microscopy [19]. Confocal laser scanning microscopy is also used for investigating the morphology of microcapsules [33]. For surface morphology analysis, atomic force microscopy can be used [19].

D. Oxidative Stability

Encapsulation aims to protect omega-3 or omega-6 PUFAs during processing and storage and to prevent undesirable interactions with the food matrix. PUFAs are protected from oxidation by providing an oxygen barrier with the use of wall materials. Since PUFAs are highly sensitive to environmental processing and/or gastrointestinal (GI) conditions, choice of suitable wall materials is significant. Different wall materials have various oxygen barrier properties between sensitive PUFAs and the environment. Storage conditions such as light, temperature, humidity, and oxygen in the encapsulated oils have a significant influence on oxidation. Stability of the encapsulated materials can be increased by the appropriate selection of wall materials, processing, packaging, and storage conditions.

Oxidation is monitored by measuring primary and secondary oxidation products. Peroxide value (PV) is a measure of primary oxidation products, whereas *p*-anisidine value (AV) is an indicator of secondary oxidation products. Conjugated dienes and trienes as well as thiobarbituric acid

TABLE 16.3
Measurement of Oxidation of Encapsulated Omega-3 Fatty Acids and Recommended Method of Analysis

Analysis	Limit Value	Recommended Method of Analysis
PV	<5 meq/kg	AOCS methods Cd 8–53 and Cd 8b-90
AV	<20	AOCS method Cd 18–90
Conjugated diene and trienes	<2 mmol/kg, N/A	AOCS method Ti 1a-64 [36]
Thiobarbituric acid reactive substances (TBARS)	N/A	[36–38]
Propanal measurement		[37]
Rancimat method		
Sensory panel		
Headspace gas chromatography		[35]

Source: Carnerio, H.C.F. et al., *J. Food Eng*., 115(4), 443, 2013.
Note: AOCS, American Oil Chemists' Society.

reactive substances (TBARS) values have been also measured during oxidation studies [33,34]. Besides, propanal measurement, accelerated storage tests including Rancimat test or oxidative stability index, and Schaal oven test are utilized to determine the oxidative stability of microencapsulated oils. Carnerio et al. [35] used headspace gas chromatography for the stability tests of the microcapsules at time zero (right after drying) and over four weeks of storage [35].

Sensory evaluation with trained sensory panelists is the fastest way to detect the initial oxidative deterioration of encapsulated oils. Panelists can be trained to detect low levels (down to ppb levels) of volatile oxidation products, which is not possible to analyze by traditional methods. The Global Organization for EPA and DHA has set limits for indicators for oxidation. Table 16.3 shows the methods applied for the measurement of oxidation of encapsulated omega-3 oils as well as the desired specifications and the recommended method of analysis.

E. Bioavailability

The use of different matrices and techniques for the encapsulation of oils has varying influences on the bioavailability of their bioactive chemical constituents [39,40]. A great number of factors have been identified to influence the digestion of microencapsulated oils, such as nature and composition of the microcapsule wall, particle size of emulsion droplets, viscosity of the emulsion system, and other components in the bulk phase [41]. On the other hand, an improved understanding of the factors that impact the bioavailability of dietary lipids would enable the food industry to design food to increase, decrease, or control lipid digestion and absorption within the human GI tract. The initial form of the lipid phase within a food may impact its subsequent digestion and absorption. When a lipid is ingested, it may undergo changes in its chemistry and structural organization as it passes through the GI tract [42].

In recent years, *in vivo* feeding methods and *in vitro* digestion models have been used for the assesment of bioavailability of microencapsulated omega-3 and omega-6 PUFAs. *In vivo* studies consisting of feeding trials conducted with volunteers or with cell cultures such as CaCo-2 cells have been reported [43–45]. These cells are derived from a relatively well-differentiated colon adenocarcinoma, with a resemblance to human small intestinal absorptive enterocytes [46]. Cellular activities such as absorption and secretion of nutrients can be studied under conditions that closely mimic the *in vivo* state when cells are grown on filter membranes that allow access to both apical and basolateral sides of the cells [47,48]. *In vitro* studies are carried out in two phases: First, oil microcapsules are exposed to simulated gastric fluid containing pepsin and sodium chloride at low

pH value. After that, an intestinal digestion is simulated by exposing gastric digestion elements to a simulated intestinal fluid containing pancreatin, lipase, and cholic and deoxycholic acids in phosphate-buffered saline buffer [49]. An alternative method for *in vitro* digestion is the use of TNO gastrointestinal model (TIM 1). This system models the human GI tract. The main characteristics of this system are simulation of peristaltic movements, controlled squeezing, absorption of nutrients and water in the respective compartments of the small intestine, and simulations of gastric emptying rates and intestinal transit times. Moreover, gastric, bile, and pancreatic secretions are infused to mimic the pH, body temperature, and concentrations of electrolytes, enzymes, and bile that are observed *in vivo* [50]. This system reproduces with accuracy the main events occurring in the digestive tract that precede absorption through the gut wall, allowing reliable results on the bioaccessibility of nutrients [51]. Gervais et al. [52] evaluated this system for the *in vitro* bioaccessibility of *cis*-9, *trans*-11 18:2 from naturally enriched milk or from synthetic CLA supplements incorporated into milk as free fatty acid (FFA) or as TAG and compared them with the bioaccessibility of other milks.

Specific studies have shown that the bioavailability of foods enriched with microencapsulated omega-3 fatty acids had similar bioavailability values compared with fish oil capsules [33]. Barrow et al. [45] have also showed that the complex coacervation method and whey protein as the wall material increased the bioavailability of omega-3-rich fish oil.

In general, protein shell materials are likely to be digested; therefore, they will have better bioavailability. This is also true for fats and polysaccharides except indigestible chitin where bioavailability studies are especially important [18,45].

IV. FUNCTIONAL FOOD AND BEVERAGE APPLICATIONS OF MICROENCAPSULATED n-3 AND n-6 PUFAs

Recently, attention on foods enriched with omega-3 and omega-6 PUFAs has increased due to the positive health benefits of these fatty acids. Because human diet is lacking in these PUFAs in most of the countries, functional foods enriched with omega-3 and omega-6 PUFAs have become significant for human nutrition. Therefore, an increasing number of foods enriched with PUFAs are entering the marketplace. In the Western diet, concepts related to food are being modified from hunger satisfaction and preventing adverse effects to well-being, improving health, and reducing the risk of diseases [53]. Although there is an increasing demand for PUFA-enriched foods, avoiding lipid oxidation still exists as a major challenge for food producers due to the presence of omega-3 and omega-6 PUFAs [54]. As a consequence of lipid oxidation, lipid hydroxyperoxides as well as volatile oxidation products such as ketones and aldehydes will be formed. From health point of view, these oxidation products will have adverse health effects, and, from product point of view, undesirable rancid and fishy off-flavors will be formed. The most important factors to consider for optimizing the oxidative stability of enriched foods are the quality of the original (fish) oil, composition and quality of the other ingredients, emulsifier, emulsification conditions, type of equipment, and emulsion delivery system [54]. Another important challenge is the sensory properties, which highly affect consumer acceptance of foods.

Commercially encapsulated omega-3 powders are available on the market and are shown in Table 16.4. Detailed study should be performed with these ingredients and should be mandatory prior to product enrichment with the aim of promoting shelf life, protection, sensory properties related to consumer acceptance, masking of the fishy or other flavors related to the oil as well as determining omega-3 and omega-6 bioavailability and functional properties.

As shown in Table 16.4, spray-dried emulsions have been the method of choice for industrial applications. Since high surface oil levels, low oil content, and poor stability have limited the use of the microencapsulated oils, the spray-dried complex coacervation process was launched. This technology has advantages over spray drying, but the bioavailability of the microcapsules has been questioned. Today, enriched foods including dairy products (such as yoghurt, fresh milk,

TABLE 16.4
Commercial Omega-3 and Omega-6 PUFA–Rich Microencapsulated Powders

Carrier System/ Delivery Technology	Key Ingredient/Shell Material	Payloads (mg Omega-3/g Powder)	Shelf Life	Manufacturer/ Producer
Spray-dried emulsion	Hydrolyzed gelatin, soy protein, caseinate, glucose syrup, mannitol	100–215	12 months at 4°C	DSM/legacy Martek
Spray-dried emulsion	Starch coated, caseinate or gelatin, and sucrose	90	12 months at <15°C	DSM
Spray-dried emulsion	MRs with caseinate, various sugars, starches in alternative formulations	69–143	>6 months	Clover/Nu-Mega
Complex coacervation	Gelatin, polyphosphate, or polysaccharides	140–180	18 months at 4°C	Ocean Nutrition Canada (ONC)
Spray-dried emulsion	Modified starch and soy protein	100	6–12 months at 5°C–10°C	National Starch/ Omega Protein
Spray-dried emulsion	Carbohydrate, protein	170–195	24 months at 5°C–10°C	Lipid Nutrition (Loders Croklaan)
Spray-dried emulsion	Caseinate, soy protein, glucose syrup	190	18 months at 5°C–10°C	Kievit
Spray-dried emulsion	Modified starch, GA, glucose syrup	213	N/A	Fuji Chemical
Spray-dried emulsion	Modified starch, corn syrup, whey protein, GA	100–150	6 months at 4°C	Wright Group
Spray-dried emulsion	Caseinate, soy protein, glucose syrup	150–180	18 months at <15°C	Pronova-Polaris
Spray-dried emulsion	Gelatin or caseinate coated with starch granules	68–83	24 months at 8°C–15°C	BASF
Spray-dried emulsion	Calcium salt and surfactant	<100	N/A	Kitii Corp.
Gravity flow dry blending	MD	65–155	2 years	Nutri Pharmaceuticals Research Inc.

Sources: Nickerson, M. et al., Protection and masking of omega-3 and -6 oils via microencapsulation, Chapter 37, in: Gaonkar, A.G. et al., eds., *Microencapsulation in the Food Industry*, Elsevier, 2014, pp. 485–500; Carnerio, H.C.F. et al., *J. Food Eng*., 115(4), 443, 2013.

ice cream/frozen yoghurt), bread, spaghetti, pasta, cereal bars, tortillas, frozen dough, soup, infant formulas and baby food products are available. Nevertheless, the challenge in producing fortified foods has been tremendous.

A. Studies from Literature

The literature was surveyed for years between 2010 and 2016 for the food and beverage applications of microencapsulated omega-3 and omega-6 oils. The most widely used methods for microencapsulation of omega-3 and omega-6 oils are spray drying, freeze-drying, and coacervation with the most common wall materials being GA, gelatin, MD, whey protein, and zein. More recent microencapsulation studies were observed using electrospinning/electrospraying and sporopollenin exine capsules. Vegetable proteins such as chickpea or lentil are being investigated as wall materials. Selected studies are summarized in Table 16.5.

TABLE 16.5
Technologies and Wall Materials for Microencapsulation of Omega-3 and Omega-6 PUFAs

Reference	Oil	Technologies	Wall Materials
[56]	Evening primrose oil	Spray drying	GA, MD, and/or sodium caseinate
[57]	Fish oil	Spray granulation and fluid bed coating	Soybean soluble polysaccharide (SSPS) and MD, hydroxypropyl betacyclodextrin
[58]	Fish oil	Electrospraying	Zein prolamine
[59]	Fish oil	Spray-drying microfluidization	Barley protein
[60]	Fish oil	Spray granulation Spray drying and freeze-drying	SSPS with MD, hydroxypropyl betacyclodextrin, and octenyl succinic anhydride
[61]	FO	Spray drying Freeze-drying	Zein
[62]	Fish oil	Spray drying	Sodium caseinate and casein hydrolysate
[63]	FO	Spray drying	GA, whey protein concentrate, and a modified starch
[64]	Microalgal oil	Complex coacervation	Gelatin, GA with a cross-linking agent (TG)
[35]	FO	Spray drying	MD, GA, whey protein concentrate, tapioca starch, and waxy maize
[65]	Linseed oil	Spray drying	GA and MD
[66]	Linseed oil	Spray drying	GA, MD, MC, WPI
[67]	Sunflower oil	Complex coacervation by membrane emulsification	Fish gelatin and GA
[35]	FO	Spray drying	MD, GA, whey protein concentrate, modified starch
[68]	FO	Spray drying	Legume protein–MD
[69]	FO	Freeze-drying	Chickpea or lentil protein isolate and MD
[70]	Soy oil	Spray drying	Soy protein isolate
[71]	SLs containing DHA, ARA, and GLA	Spray drying	WPI and corn syrup solid
[72]	Fish oil	Complex coacervation	WPI and GA
[73]	Fish oil	Complex coacervation	Gelatin and sodium hexametaphosphate
[74]	Wheat germ oil	Freeze-drying	MD, whey protein concentrate
[75]	Fish oil	Spray drying	Butter milk (BM) or BM with glucose syrup
[76]	CLA	Spray drying	Pea protein isolates or concentrates with different ratios of MD and carboxymethylcellulose
[77]	SDASO	Complex coacervation	Gelatin, GA with a cross-linking agent (TG)

Source: Kaushik, P. et al., *J. Funct. Foods*, 19, 868, 2015.

B. Studies of Food and Beverage Applications

The most important challenges for omega-3 or omega-6 PUFA–enriched new product development using microencapsules are the consumer acceptance due to sensory properties and oxidative stability. Other factors that should be kept in mind are the shelf life of the microcapsules and the products that these microcapsules will be incorporated. It is also important to investigate how the stability, functional properties, and bioavailability of the microencapsulated oils are affected during food processing.

Literature study on food applications of microencapsulated PUFAs revealed different types of products. More recent studies are summarized in the following sections.

1. Bread and Cereals

Bread and cereal products have been studied for enrichment with microencapsulated omega-3 oils for more than 10 years due to their frequent consumption and low cost. Gallardo et al. [66] have tested four formulations, including GA, MD, methyl cellulose (MC), and whey protein isolate (WPI), for linseed oil microencapsulation by spray drying and evaluated their efficiencies and resistance to oxidation. Microcapsules made of 100% GA and ternary mixtures of GA, MD, and WPI presented the highest protection from oxidation, and microencapsulation efficiencies higher than 90% were reported. GA-containing formulation was included in bread manufacturing and it was concluded that fortified bread was similar in appearance to control bread without microcapsules, but α-linolenic acid (ALA) content was reduced significantly after preparation probably due to insufficient oil protection during baking. They have also tested the same formulation in cereal bars. ALA loss was not observed in those cereal bars, and no significant differences in organoleptic scores were mentioned between the control group and those fortified with microencapsulated oils [78].

De Conto et al. [79] evaluated the influence of the addition of 0.00–5.00 g/100 g microencapsulated omega-3 fatty acids (MO), BA35 Plus (*Funcional Mikron* Company [Valinhos, SP, Brazil]) containing 0.000–0.100 g/100 g rosemary extract on the technological characteristics (specific volume, firmness, $L*$ and $C*$) and sensory characteristics (appearance, aroma, and overall acceptance) of white pan breads by central composite rotational design. Increasing MO concentration reduced specific volume and lightness and increased firmness and color saturation. The microencapsulated omega-3 presented good resistance to the baking process temperatures, as evidenced by the lack of EPA and DHA in the lipids extracted from the loaves of bread, being adequate for the bread formulation. In the sensory acceptance test, the bread had good sensory acceptance (scores > 5), even at the maximum dosage of omega-3 microcapsules (5 g/100 g total mass) [79].

Santhanam et al. [80] investigated the effect of sodium caseinate, MD, and soy protein on the oxidative stability of encapsulated fish oil by using spray drying. The oxidative stability of fish oil encapsulates was determined for 32 days at room and refrigerated temperatures. Incorporation of microcapsules into a bakery product like cake was also explored. It was found that fish oil-in-milk emulsions prepared by ultrasonication at different amplitudes were encapsulated by spray drying using different wall materials. Encapsulates prepared from fish-oil-in-milk emulsions by ultrasonication and subsequent spray drying with sodium caseinate as the wall material were found to be chemically stable. The cakes fortified with fish oil encapsulates with sodium caseinate showed a better oxidative stability, desirable color, textural characteristics, and sensory attributes. The analysis of fish-oil-enriched cakes revealed the presence of EPA (1.53%) and DHA (2.32%).

Umesha et al. [81] prepared microencapsulated garden cress seed oil (MGCO) that is rich in ALA using whey protein concentrate with an oil/protein ratio of 0.4, by the spray-drying method. Biscuits were prepared by supplementing MGCO at 20 g/100 g or GCO at 5.0 g/100 g by replacing flour and fat or fat in biscuit formula. ALA content was found to be 1.02 g and 1.05 g/100 g, respectively, in MGCO- and GCO-supplemented biscuits. GCO-supplemented biscuits compared to MGCO biscuits indicated that the encapsulation prevented oxidation of ALA in biscuits. Sensory evaluation results showed that MGCO-supplemented biscuits were acceptable.

2. Baby Foods

Wan et al. [82] compared baby foods containing purified menhaden oil (PMO) and microencapsulated purified menhaden oil (MPMO). PMO had higher total omega-3, total saturated, total monounsaturated, DHA, and EPA contents than MPMO. Emulsion containing PMO (EPMO) exhibited a viscoelastic characteristic and the droplet size of EPMO was around 2–10 μm. The DHA and EPA in the PMO were reduced in MPMO. All baby food samples with added fish oils had similar FFA values and color. DHA and EPA of baby food were significantly increased by adding PMO

and/or MPMO. Total percent values for omega-3 fatty acids of extracted fat from commercial baby food (CB), commercial baby food containing PMO, and commercial baby food containing MPMO were 4.5%, 9.8%, and 10.1%, respectively.

A study carried out by Wan et al. [83] revealed that encapsulated red salmon oil could be added to the CB and increase the percent omega-3 fatty acids of the CB [83].

Nagachinta and Akoh [71] produced structured lipids (SLs) containing long-chain polyunsaturated fatty acids (LCPUFAs) and encapsulated them through spray drying. They used Maillard reaction products (MRPs) obtained from heated WPIs and corn syrup solids solution as a wall material. A high microencapsulation efficiency of 90% was observed. The encapsulated SL powders had low peroxide and thiobarbituric acid–reactive substances values. The results demonstrated suitability of MRPs for the microencapsulation of SLs with a high microencapsulation efficiency for infant formula applications.

3. Dairy Products

Milk and dairy products such as yoghurt and cheese are believed to be good vesicles for the incorporation of omega-3 PUFAs since they are commonly consumed. Rouse et al. [84] enriched processed cheese with DHA and EPA by addition of microencapsulated fish oil (MFO) during the melting stage of processed cheese manufacture. Addition of 25 mg 2:1 DHA/EPA received the highest scores for flavor and overall liking by testers. It was concluded that microencapsulation of fish oils before addition to the food may reduce the rate of oxidation and improve marketability of dairy products fortified with fish oil. *Queso fresco*, cheddar, and mozzarella cheeses were fortified with omega-3 from flaxseed oil (FO) and MFO at specific stages in the cheese-making process. The highest omega-3 retention (8.69 mg/g MFO; 5.08 mg/g FO) was observed in cheddar when added during salting. However, the flavor of cheese with added FO was preferred by panelists more than MFO-fortified cheese [85]. In a study of Bermudez-Aguirre and Barbosa-Canovas [86], it was observed that MFO showed the best retention in the cheese matrix, and, among the nonthermal approaches tested, ultrasound showed favorable results during cheese making with omega-3.

Stratulat et al. [87] enriched cheese with FO, and vitamins A, E, and CoQ10 by the immobilization process of lipophilic bioactive agents in emulsified particles. Emulsions were stabilized with calcium caseinate in the presence or absence of lecithin and used to standardize cheese milk. A good compatibility between bioactive components was observed during co-encapsulation in the cheese matrix. The incorporation of lipophilic ingredients in the cheese matrix in the form of emulsified particles led to their increased retention in the curd, increased the stability and functionality of bioactive components during storage, resistance of lipids to peroxidation, and increased yield of fortified cheese.

Another study found that the encapsulation of vitamin D3 in the cheese in the form of emulsified particles of oil-containing lecithin increased the retention and stability of the vitamin in the curd. The fortification of cheese with vitamin D3 and PUFA had a positive impact on the composition, yield, and chemical stability of the resultant cheese [88].

Barley protein–stabilized fish oil microcapsules (1–5 μm) were successfully prepared by a preemulsifying process followed by a microfluidizer treatment. These barley protein microcapsules possessed a strong ability to protect fish oil against oxidation, and food formulation tests confirmed their successful application in milk and yogurt for their respective shelf lives [59].

Estrada et al. [89] developed a strawberry yogurt containing microencapsulated salmon oil (MSO; 2% wt/vol). Fortification of yoghurt with MSO had no significant effect on yogurt pH or syneresis. Although some slight differences were observed in the color and oxidation of fortified yogurts compared with control, the study demonstrated that yogurt could be fortified with salmon oil.

Tamjidi et al. [90] enriched set yogurt with long-chain omega-3 PUFAs by complex coacervates of gelatin/acacia gum. Acidity, apparent viscosity, and water holding capacity of enriched samples were higher, and gel strength and amount of whey separation were lower during storage. It was suggested that it is possible to manufacture an enriched yogurt containing MFO and flavored for

better sensory properties. Ifeduba and Akoh [77] encapsulated stearidonic acid (18:4n-3) soybean oil (SDASO) by complex coacervation of gelatin (GE)–GA and compared it with that of MRP. A portion of the control microcapsules based on the GE–GA system was cross-linked with transglutaminase (TG). The microcapsules were used to formulate yogurt milk base. They found that the Maillard reaction (MR)-modified microcapsules resulted in the highest thermal stability and the yogurt formulated with MR-modified microcapsules had the best oxidative stability during 14 days' storage at 4°C, demonstrating that their antioxidant components had good carry-through properties during processing of the yogurt.

Perlman [91] developed supplemented milk with omega-3 PUFAs stabilized in vegetable or animal fats or oils such as palm oil, palm kernel oil, and milk fat. Microparticles that were formed by homogenization were resistant to oxidation. It was proposed that combining more than one fat in a blend would provide better oil protection.

4. Other Foods

Rubilar et al. [65] developed a formulation of soup powder enriched with omega-3 fatty acids using microencapsulated linseed oil by spray drying. This study resulted in a healthy soup enriched with omega-3, which was highly acceptable to consumers with high oxidative stability.

Another food enriched with omega-3 fatty acids is the fermented sausage. Dutch-style fermented sausages were manufactured with 15% and 30% pork back-fat substitution by pure or commercial encapsulated fish oil, either added as such or as pre-emulsified mixture with soy protein isolate. It was found that dry fermented sausages with commercial encapsulated fish oil seemed to be the best in retaining overall quality [92].

V. CONCLUSIONS

Enrichment of food products is attracting interest due to consumers' demand for functional foods. There is an increasing demand and consumption of enriched foods with omega-3 and omega-6 PUFAs. The demand resulted in new product launches into the market. It would be an opportunity for the food manufacturers and food scientists or food technologists to design new food products for the delivery of long-chain fatty acids in functional foods by using microencapsulated oils. In order to be successful, it is important to consider the challenges that include lipid oxidation and sensory acceptability. Thus, detailed studies should be performed involving the microencapsulation method, wall materials, characteristics of the microcapsules, application of the capsules in appropriate food products, and proper analysis performed prior to product enrichment.

New technologies such as electrospraying or microencapsulation through the use of yeast cells or sporopollenin exine capsules are attracting attention for the encapsulation of omega-3 and omega-6 oils. The choice of an appropriate microencapsulation technique and wall material depends on the end use of the product and the processing conditions involved.

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Section III

Oxidation and Antioxidants

17 Rethinking Lipid Oxidation

Karen M. Schaich

CONTENTS

I. INTRODUCTION

Of all the chemical reactions that degrade the quality of foods during storage, lipid oxidation unequivocally causes the most pervasive damage and poses the greatest challenge for the food industry to control. During the low-fat/no-fat era when lipids were being taken out of foods, this troublesome series of reactions lost industrial and research attention alike, although some aspects continued to be studied for their medical relevance. Lipid oxidation was definitely not "sexy science" and it compelled little interest. However, that situation is now slowly changing with the resurgence of food formulations that include essential polyunsaturated fatty acids for health. Whether in endogenous triacylglycerols or phospholipids or in oils added as nutraceuticals, polyunsaturated fatty acids are prone to oxidation and not only degrade on their own, but they also broadcast oxidation to other critical molecules by reactions of their intermediates and products, thereby affecting nearly all aspects of food properties and sensory quality. The same reactions occurring physiologically in cells and tissues contribute to aging, cancer, atherosclerosis, dementias, and a wide range of other pathologies. Thus, there are once again strong driving forces to more fully and accurately understand the mechanisms of lipid oxidation, how reaction conditions affect the process, how oxidizing lipids interact with other biologically important molecules, and how the process can be controlled.

Other references have considered in detail the overall process of lipid oxidation, its catalysts and inhibitors, and strategies for control [1–6]. The reader is referred to these for additional background. This chapter takes a new look at reactions of lipid oxidation, focusing on evidence that the free radical chain reaction is more complex than traditionally presented and that a broader range of products, including co-oxidation products, must be monitored to accurately account for the extent of oxidation in foods and biological systems. In essence, this chapter presents a new paradigm in understanding lipid oxidation.

II. LIPID OXIDATION: FUNDAMENTAL REACTIONS

In the 1940s, work of Farmer [7–9] and Bolland [10,11] laid the ground work for most of our current understanding of lipid oxidation. They showed that lipids oxidize by a free radical mechanism in which the first step is creation of an *ab initio* carbon-centered radical ($L^{\bullet}$) by some initiating process (see [1] for a detailed review of initiation processes). Lipid alkyl radicals are relatively unreactive in hydrogen abstractions, but they add oxygen at diffusion-controlled rates ($k > 10^9$ L mol^{-1} s^{-1}) [12] to form lipid peroxyl radicals ($LOO^{\bullet}$), which are much more electrophilic and become the major chain carriers in early oxidation.

$$LH \longrightarrow L^{\bullet} \xrightarrow{O_2} LOO^{\bullet} \tag{17.1}$$

$LOO^{\bullet}$ abstract allylic hydrogens from the neighboring lipid molecules to form hydroperoxides and at the same time leave an unpaired electron (new free radical) on the second lipid (Reaction 17.2). Repetition of these two steps on the new site (Reactions 17.3 and 17.4) propagates the free radical chain reaction and establishes a perpetual process:

$$LOO^{\bullet} + L_2H \longrightarrow LOOH + L_2^{\bullet} \tag{17.2}$$

$$L_2^{\bullet} \xrightarrow{O_2} L_2OO^{\bullet} \tag{17.3}$$

$$L_2OO^{\bullet} + L_3H \longrightarrow L_2OOH + L_3^{\bullet}, \text{ etc.} \tag{17.4}$$

From this sequence, it is easy to see how a single initiating event can affect many lipid molecules up to several hundreds when they are aligned, as in a membrane or mesophase. Also, once the radical chain is started, it is extremely difficult to stop. Together these two characteristics explain much of why lipid oxidation is such a huge problem.

The *ab initio* radical ($L^{\bullet}$, Reaction 17.1) may be formed at any position on the acyl chain, but hydrogen abstractions occur where the C–H bond energies are lowest, that is, allylic carbons next to or between double bonds (74 and 65 kcal/mol, respectively) [13,14]. Thus, there is a hierarchy for ease of H abstraction and preference for reaction at different sites: doubly allylic H's > singly allylic H's next to double bonds >> H's adjacent to the −COOH group > H's on methylene groups further down the acyl chains [15]. The most susceptible positions for one, two, and three double bonds are shown in the following. Heavier arrows indicate a stronger reaction.

$$-CH_2-\overset{\downarrow}{C}H_2-CH=CH-\overset{\downarrow}{C}H_2-CH_2-$$

$$-CH_2-\overset{\downarrow}{C}H_2-CH=CH-\overset{\downarrow}{C}H_2-CH=CH-\overset{\downarrow}{C}H_2-CH_2-$$

$$-CH_2-\overset{\downarrow}{C}H_2-CH=CH-\overset{\downarrow}{C}H_2-CH=CH-\overset{\downarrow}{C}H_2-CH=CH-\overset{\downarrow}{C}H_2-CH_2-$$

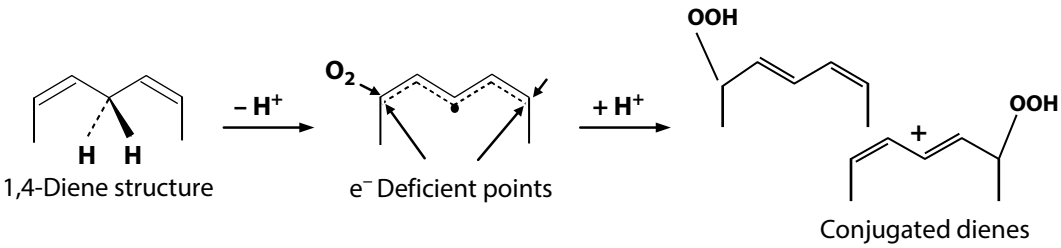
The one exception to this pattern is that in fatty acids with isolated double bonds (most notably oleic acid), hydrogen atoms are abstracted equally at both double bond carbons and both neighboring carbons [16]. Interestingly, abstraction of hydrogen atoms from carbons in double bonds does not affect the bonds themselves. Rather, when radicals are formed here, two of the electrons involved in the double bonds migrate to the next carbon and reform the double bond while inverting it from *cis* to *trans* (Reaction 17.5) [8].

$$RCH_2CH_2\underset{10}{-CH}=\underset{9}{CH}-CH_2(CH_2)_6COOH \longrightarrow RCH_2CH=\underset{10}{CH}-\overset{\bullet}{\underset{9}{C}}H-CH_2(CH_2)_6COOH$$

$$+$$

$$RCH_2CH_2\overset{\bullet}{\underset{10}{C}}H-\underset{9}{CH}=CH(CH_2)_6COOH \tag{17.5}$$

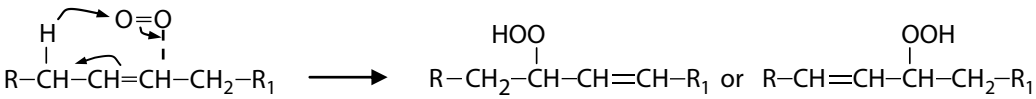
Double bond migration occurs when radicals are formed in polyunsaturated fatty acids as well. In this case, because of the much lower bond energy, a hydrogen atom is abstracted from the carbon between two double bonds, and the free electron left behind becomes distributed across a resonance-stabilized double bond system (shown for linoleic acid in Reaction 17.6).



(17.6)

Electron density concentrates on the center carbon, so the outside positions in the 1,4-diene system become relatively electron deficient and thus provide enhanced targets for oxygen addition and subsequent peroxyl radical formation. In polyunsaturated fatty acids, therefore, dominant sites for formation of hydroperoxides are the external carbons of the extended double bond systems whatever the degree of unsaturation, for example, carbons 9 and 13 in linoleic acid, carbons 9 and 16 in linolenic acid, and carbons 5 and 15 in arachidonic acid [1,17]. When oxygen adds to either of the outside carbons, one pair of the double bond electrons moves over one carbon, reforming a new double bond in *trans* configuration in the new position. This migration generates a conjugated diene (−CH=CH−CH=CH−), the first chemical indicator that oxidation has started.

It is important to note that at this point in the oxidation process, hydroperoxide and conjugated diene concentrations are the same except under two conditions: (1) Internal hydroperoxides are formed at substantial levels only during singlet oxygen ($^1O_2$) oxidation photosensitized by pigments, dyes, or riboflavin [18–22]. The two electrons in singlet oxygen and double bonds are both in the same spin state (electrons paired and in opposite spin), so unlike ground-state triplet oxygen, $^1O_2$ adds directly to double bonds, forming hydroperoxides on either carbon with equal probability and without free radical intermediates (Reaction 17.7).



(17.7)

The double bond migrates in the process, but when the hydroperoxide is on an internal carbon, the migration is outward and the resulting structure is not conjugated. In fact, loss of conjugation while retaining high levels of hydroperoxides is typical of $^1O_2$ photosensitized oxidation. Singlet oxygen reaction with lipids was reviewed in detail in the previous edition of this book [23]. (2) When alternate reactions that compete with hydrogen abstraction are present, conjugated dienes can be lost in transformations to other products independently of hydroperoxides. Thus, comparability of conjugated dienes and hydroperoxides in early oxidation is an indicator of relatively pure hydrogen abstraction (lack of side reactions).

Because hydroperoxides are reasonably stable, they are the product most commonly measured to quantitate lipid oxidation. However, in the presence of heat (Δ), UV light, redox-active metal (M), and other agents, hydroperoxides decompose to radicals (Reactions 17.8 through 17.10) that continue propagating the radical chain and add to complexity of the process (Reaction 17.11):

$$\text{LOOH} \xrightarrow{\Delta,\ \text{UV}} \text{LO}^{\bullet} + {}^{\bullet}\text{OH} \tag{17.8}$$

$$\xrightarrow{M^{n+}} \text{LO}^{\bullet} + {}^{-}\text{OH}\ \text{(fast)} \tag{17.9}$$

$$\xrightarrow{M^{(n+1)+}} \quad LOO^{\bullet} \quad + \quad H^{+} \quad \text{(slow)} \qquad (17.10)$$

$$LOO^{\bullet}, \mathbf{LO^{\bullet}}, {}^{\bullet}OH \quad + \quad L_nH \longrightarrow \quad L_n^{\bullet} \quad + \quad LOOH, \mathbf{LOH}, H_2O \qquad (17.11)$$

Alkoxyl radicals ($LO^{\bullet}$) are bolded in Reaction 17.11 because their formation marks a turning point in the oxidation sequence, moving from primary to secondary stages. Peroxyl radicals that drive the chain reaction in early oxidation are relatively slow ($k = 36–62$ L mol^{-1} s^{-1}) and specific in their reactions [24,25]. In contrast, alkoxyl radicals abstract hydrogen atoms much more rapidly ($k = 10^6–10^7$ L mol^{-1} s^{-1}) and from many more sites on acyl chains, so as LOOH decompose, $LO^{\bullet}$ become the main chain carriers (propagators), oxidation rates increase, and oxidation effects broaden [26,27].

LOH, the H abstraction product of $LO^{\bullet}$, is also bolded because it should be formed in substantial quantities if this propagation path (Reaction 17.11) proceeds as written. Indeed, if the traditional reaction sequence is fully accurate, only hydroperoxides and hydroxylated fatty acids or lipid alcohols should be present at this stage. However, LOH are seldom analyzed as a class of oxidation products, they are too large to be volatile and be detected in gas chromatography analyses, and alcohols, when detected, are present in trace quantities, so there is little direct evidence that the reaction occurs as written. At the same time, routine monitoring of lipid oxidation by gas chromatography detects at least trace levels of other products, particularly aldehydes, concurrently with LOOH decomposition and generations of $LO^{\bullet}$. Either LOH are unstable and rapidly transform or degrade to other products, or H abstraction by $LO^{\bullet}$ is a minor process and alternate reactions of $LO^{\bullet}$ are active and competitive. Alternate reactions will be addressed in the next section.

According to traditional understanding, the progression of lipid oxidation now moves to termination in which free radicals are removed from the propagation stream by some process that converts them to non-radical products. Most conventional depictions give no details of reaction or show only radical recombinations, for example,

$$L_n^{\bullet} \quad + \quad L_n^{\bullet} \qquad (17.12)$$

$$L_nO^{\bullet} \quad + \quad L_nO^{\bullet} \longrightarrow \text{polymers, non-radical monomer products} \qquad (17.13)$$

$$L_nOO^{\bullet} \quad + \quad L_nOO^{\bullet} \qquad (17.14)$$

Studies have verified that products from various combinations of radicals are formed and shown that the dominant combinations vary with reaction conditions, particularly oxygen [28,29]. Some types of products generated from recombinations are shown in Reactions 17.15 through 17.21. Please see [1] for more detailed discussion of these reactions.

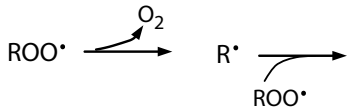
Alkyl radicals:

$$R_1^{\bullet} \quad + \quad R_2^{\bullet} \longrightarrow \quad R_1 - R_2 \qquad \text{alkanes and polymers} \qquad (17.15)$$

Peroxyl radicals [30–33]:

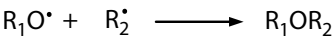
$$2 \quad \begin{matrix} OO^{\bullet} \\ | \\ R_1CHR_2 \end{matrix} \longrightarrow \quad \begin{matrix} R_1 \\ | \\ -C- \\ | \\ R_2 \end{matrix} OOOO \begin{matrix} R_1 \\ | \\ -CH \\ | \\ R_2 \end{matrix} \longrightarrow \quad \begin{matrix} OH \\ | \\ R_1CHR_2 \end{matrix} \quad + \quad \begin{matrix} O \\ \| \\ R_1CR_2 \end{matrix} \quad + \quad O_2 \qquad (17.16)$$

alcohols and ketones



(17.17)

Alkoxyl radicals [27,34,35]:



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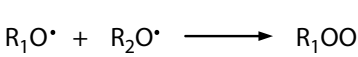

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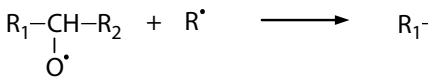

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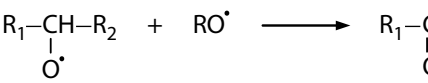
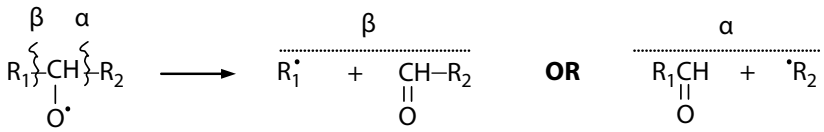


(17.21)

Surprisingly, despite radical recombinations being listed almost universally as the only termination reaction, the ketones, alkanes, peroxides, ethers, and alcohols generated by them are not major lipid oxidation products, so there must be other more active termination pathways. Here, Frankel and Neff [36–43] as well as Grosch [34] and Schieberle [44–46] have added significantly to our understanding of termination reactions with details of alkoxyl radical scissions as a major source of products detected in standard analyses. LO• radicals undergo scission of the C–C bond on either side of the alkoxyl group (α is on the acid end, β is on the distal end) to yield mixtures of aldehydes or oxo-esters plus alkyl free radicals (Reaction 17.22):



(17.22)

The free radicals either anneal into alkane products or add oxygen to form peroxyl radicals and continue the chain reaction. Unsaturated radical fragments oxidize further and then undergo secondary scissions to produce carbonyls and alkanes of shorter chain length. In this way, with combinations of alkoxyl radicals at the different positions on fatty acids, product mixtures accumulating in oxidized lipids can become quite complex.

Typical products arising from alkoxyl radical scissions in oleic, linoleic, and linolenic acids are shown in Table 17.1.

There is no arguing the overall accuracy of this scheme because intermediate radicals have been detected and verified by electron paramagnetic resonance (EPR) [47–49]. However, it is equally clear that the reaction is incomplete as simplistically written. Practical analytical consequences of this scheme are that conjugated dienes must be the first and only product in early oxidation, followed by hydroperoxide formation, then alcohols, and finally unspecified products after hydroperoxides have decomposed. Although some papers have reported this pattern of product development and a graph showing this sequence of product development has been reproduced often in various forms (see for example [1,3,4,29]), it is much more common to find oxidation kinetics and product patterns that are inconsistent with the simplistic radical chain reaction. For example, conjugated diene levels are usually quite different than hydroperoxides, alcohols are seldom detected at more than trace levels even though they are written as a main product, many products form simultaneously with conjugated dienes even if

TABLE 17.1
Primary Alkoxyl Radical Scission Products from Oleic, Linoleic, and Linolenic Acids

Position	α-Scission	β-Scission
8 (O)	Heptanoic acid	8-Oxo-octanoic acid
	2-Undecenal	Decene or decanal
9 (O)	Octanoic acid	9-Oxo-nonanoic acid
	2-Decenal	$CH_3(CH_2)6CH{=}CH$
10 (O)	Nonanal	10-Oxo-8-decenoic acid
	8-Nonenoic acid	Octane or octanal
11 (O)	Octanal	Heptane or heptanal
	9-Decenoic acid	11-Oxo-9-undecenoic acid
9 (L)	Octanoic acid	9-Oxo-nonanoic acid
	2,4-Decadienal	1,3-Nonadienal
13 (L)	Hexanal	Pentane
	12-Oxo-9-dodecenoic acid	13-Oxo-9,11-tridecadienoic acid
9 (Ln)	Octanoic acid	9-Oxo-nonanoic acid
	2,4,7-Decatrienal	1,3,6-Nonatriene
16 (Ln)	Propanal	Ethane
	15-Oxo-9,12-pentadecadienoic acid	16-Oxo-9,12,14-hexadecatrienoic acid

Note: Other oxidation products detected arise either from secondary decompositions of these products or from alternate oxidation pathways.

at different rates, scission products are present at only a small percent of the hydroperoxides, hexanal and decadienal are not present equally or stoichiometrically in oils containing linoleic acid, products such as epoxides cannot be rationalized by the simple chain reaction, and mass balances cannot account for all products normally measured.

These and other inconsistencies led to reevaluation of the literature to find missing reactions and proposal of a reaction scheme that integrates alternate reactions with established hydrogen abstractions [1].

III. ALTERNATE REACTIONS THAT COMPETE WITH HYDROGEN ABSTRACTION

Kochi's extensive body of research on free radicals and their reactions has shown that both LOO$^\bullet$ and LO$^\bullet$ undergo a number of alternate reactions in addition to and in competition with hydrogen abstraction (Table 17.2). Alternate reactions are important chemically because lipid free radicals are very unstable—immediately upon forming they look for a way to pair the odd electron. If abstractable hydrogens are not in close proximity, the free electron finds another avenue. The alternate reactions of LOO$^\bullet$ and LO$^\bullet$ are not just interesting academic exercises—they significantly change the direction, kinetics, and products of lipid oxidation, and their presence explains the inconsistencies noted in earlier text. In some cases, these alternate reactions actually replace hydrogen abstraction and bypass hydroperoxides; in other cases, the alternate reactions supplement hydrogen abstraction and generate a product mix that is different than that expected from traditional oxidation. When the alternate reactions are active, oxidation kinetics and progress determined only by conjugated dienes and hydroperoxides, even perhaps with hexanal added, can seriously underestimate the extent and breadth of lipid oxidation.

TABLE 17.2
Alternate Competing Reactions of Lipid Peroxyl and Alkoxyl Radicals and Their Rate Constants in Nonpolar Organic Solvents

LOO•		
Hydrogen abstraction from LH		<1–400 M^{-1} s^{-1}
From LOOH		600 M^{-1} s^{-1}
Internal rearrangement /cyclization to epidioxides		10^1–10^3 s^{-1}
Addition to double bonds		Not available
Disproportionation		10^6–10^9 L M^{-1} s^{-1}
β-Scission of O_2 to regenerate L•	Oleate	1–8 s^{-1}
	Linoleate	27–430 s^{-1}
LO•		
Hydrogen abstraction from LH		10^4–10^7 M^{-1} s^{-1}
From LOOH		2.5×10^8 M^{-1} s^{-1}
Internal rearrangement/cyclization to epoxides		10^4–10^5 s^{-1}
Addition to double bonds		10^4–10^8 M^{-1} s^{-1}
α- and β-scission (fragmentation)		10^3–10^5 s^{-1}

Source: Excerpted from Schaich, K.M., Lipid oxidation in fats and oils: Theoretical aspects, in: *Bailey's Industrial Fats and Oils*, 6th edn., F. Shahidi, ed., John Wiley, New York, 2005, pp. 2681–2767.

One example of how these alternate reactions may be integrated with hydrogen abstractions of traditional pathways is shown in Figure 17.1 [1]. The traditional reaction sequence involving hydrogen abstractions and hydroperoxides is presented in boxes vertically down the center of the scheme as the core of the oxidation process. Alternate pathways are added as competing reaction cycles that divert peroxyl and alkoxyl radicals and yield different intermediate products and secondary radicals at new sites. These secondary radicals add oxygen and form peroxides which can either reenter the traditional H abstraction flow, designated by the dotted lines, or undergo further reaction outside the core process. Drawn this way, it is clear just how extensively alternate reaction paths increase the complexity of both the kinetics and the product mixes of lipid oxidation.

This integrated scheme is clearly a work in progress. Each reaction has a firm base of experimental evidence, the rate constants in Table 17.2 indicate the reactions should be competitive, and parts of the scheme have been verified in our laboratory [50,51]. However, the overall pathway integration and individual reaction competitions remain to be verified in practical applications. The scheme is presented here to provide a new base for thinking about lipid oxidation, as well as to stimulate readers to look beyond hydroperoxides and hexanal in the analysis of lipid oxidation and to consider other mechanisms that may alter the course, kinetics, and product distribution of lipid oxidation.

Let's look at these alternate reactions in more detail.

A. Alternate Reactions of LOO•

1. β-Scission of O_2 from LOO• to Regenerate L•

Lipid oxidation typically shows an early period, referred to as the induction period, in which no or only low levels of products are detected. Traditional thinking has been that initiation has just not taken hold yet, propagation reactions have been blocked by radical quenchers, or products have just

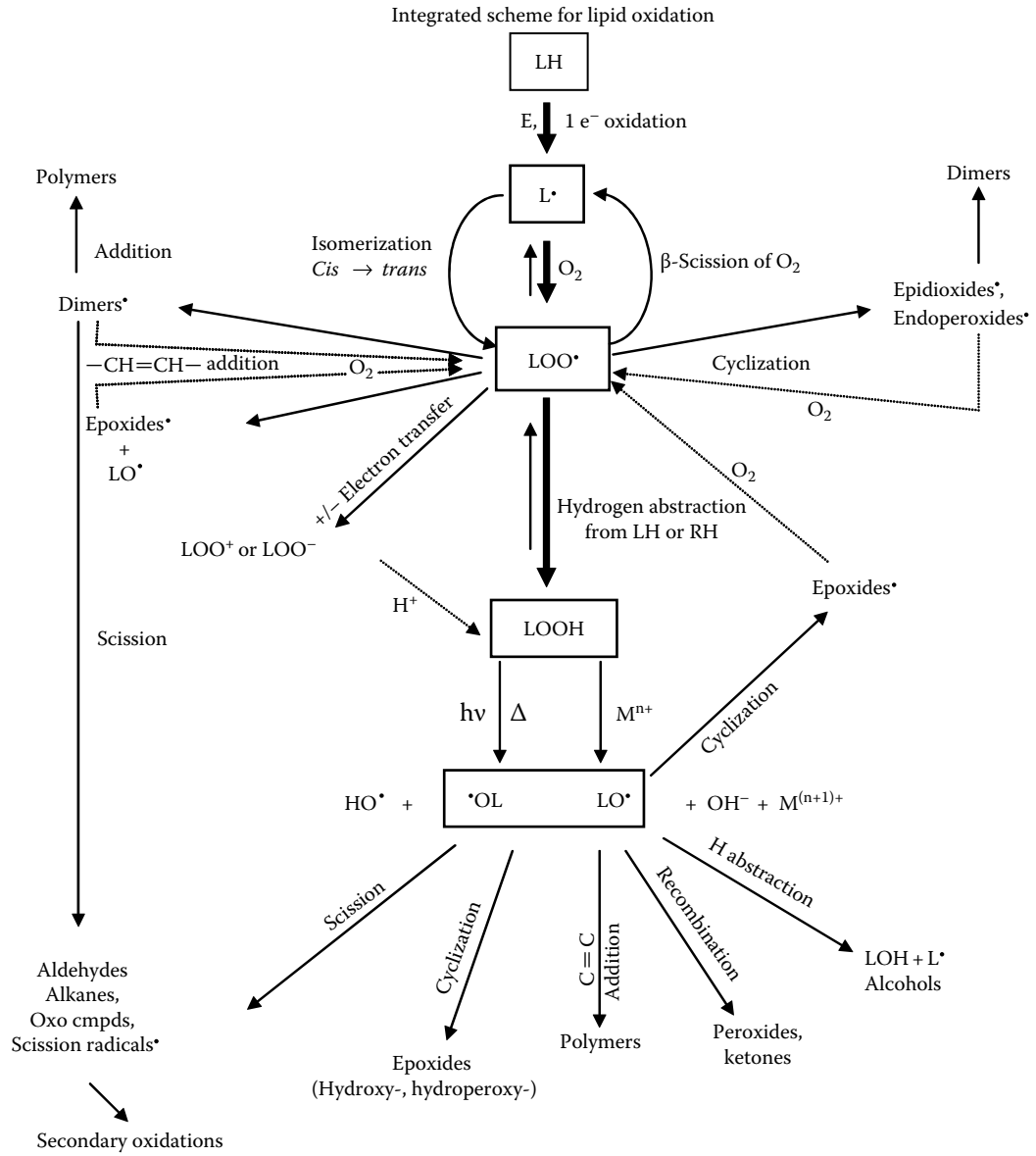


FIGURE 17.1 Proposed scheme for integrating alternate reactions of lipid peroxyl and alkoxyl radicals with traditional hydrogen abstraction, hydroperoxide formation, and subsequent decomposition reactions. (From Schaich, K.M., Lipid oxidation in fats and oils: Theoretical aspects, in: *Bailey's Industrial Fats and Oils*, 6th edn., F. Shahidi, ed., John Wiley, New York, 2005, pp. 2681–2767. With permission.)

not reached detectable levels. However, one alternate reaction—β-scission of O_2 from LOO•—very likely contributes to the induction period and also explains *cis*, *trans* isomerization of double bonds during oxidation. β-Scission in LOO• (also referred to as β-elimination) cleaves the C–OO bond and releases O_2, leaving an alkyl radical behind (Reaction 17.23).

$$\text{LOO}^{\bullet} \longrightarrow \text{L}^{\bullet} + \text{O}_2 \tag{17.23}$$

Without the oxygen, the carbon bond in the acyl chain is able to rotate, and the free radical can migrate within the resonance system of the fatty acid (Reaction 17.6). Hence, when oxygen adds again, chains may be in a different configuration and radicals may be at a new carbon position. Interconversion of peroxyl position and orientation continues indefinitely as long as the radical is in the resonance manifold (Figure 17.2). The process stops with formation of a hydroperoxide and resumes if the LOOH hydrogen is abstracted [52–56].

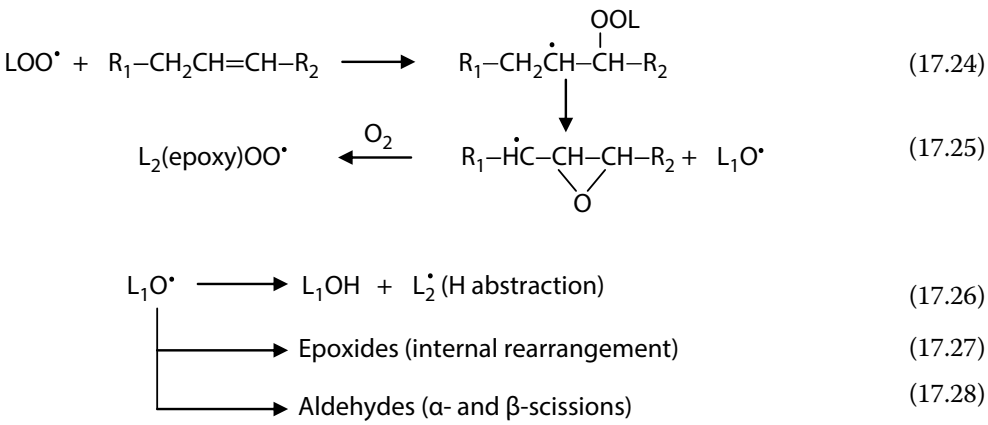
β-Elimination becomes increasingly important when abstractable hydrogens are limited at low lipid concentrations and in aprotic solvents. It is also responsible for shifts in isomer distribution at elevated temperatures, and this in turn alters the ultimate products. For example, higher *trans* levels result, and 13-OOH isomerizes to 9-OOH during heating, and the product mix correspondingly approaches that of 9-OOH [57].

Long induction periods can be misinterpreted as lacking initiators or having sufficient antioxidants when actually β-elimination of oxygen is being allowed by the system conditions. Thus, the possible presence of this alternate reaction must always be considered when analyzing oxidation data. Its presence can be deduced by the presence of high conjugated dienes with very low levels of other products.

Evidence for the reversible addition of oxygen to the pentadienyl system comes from laboratories of Ingold [58], Porter [52–56], and O^{17} studies of Campbell [59] and Chan [60].

2. Addition of LOO· to Double Bonds

Because lipid peroxyl radicals are so electrophilic, they have a strong attraction to double bonds (either *cis* or *trans*) and add to them with relative ease, transferring the unpaired electron to the second carbon of the double bond in the process (Reaction 17.24). This reaction is perhaps one of the most underrated reactions of lipid oxidation. Addition is facilitated by conjugated double bonds (e.g., in oxidized fatty acids) [61–63], so it has been associated mostly with the marked increase in dimers, polymers, and viscosity after prolonged oxidation [64–66]. However, LOO· additions are even more important in early oxidation because the peroxo dimers are not very stable and rapidly decompose to an epoxide with an allylic radical while releasing an alkoxyl radical (Reaction 17.25). The alkoxyl radical then can abstract hydrogen atoms to propagate oxidation at a faster rate (Reaction 17.26), or it can form epoxide and aldehyde products directly (Reactions 17.27 and 17.28).



This process has three important consequences: (1) it effectively accelerates oxidation since two new propagating radicals (LO· and epoxyOO·) with increased reactivities are generated from the initial LOO·, (2) it generates epoxides and aldehydes directly at the very beginning of oxidation without hydroperoxides as intermediates [51], and (3) the epoxide formation provides a source of radicals on C-11 of linoleic acid as well as products derived from that site (Figure 17.2) [50,51]. Reactions at C-11 are currently thought not to occur.

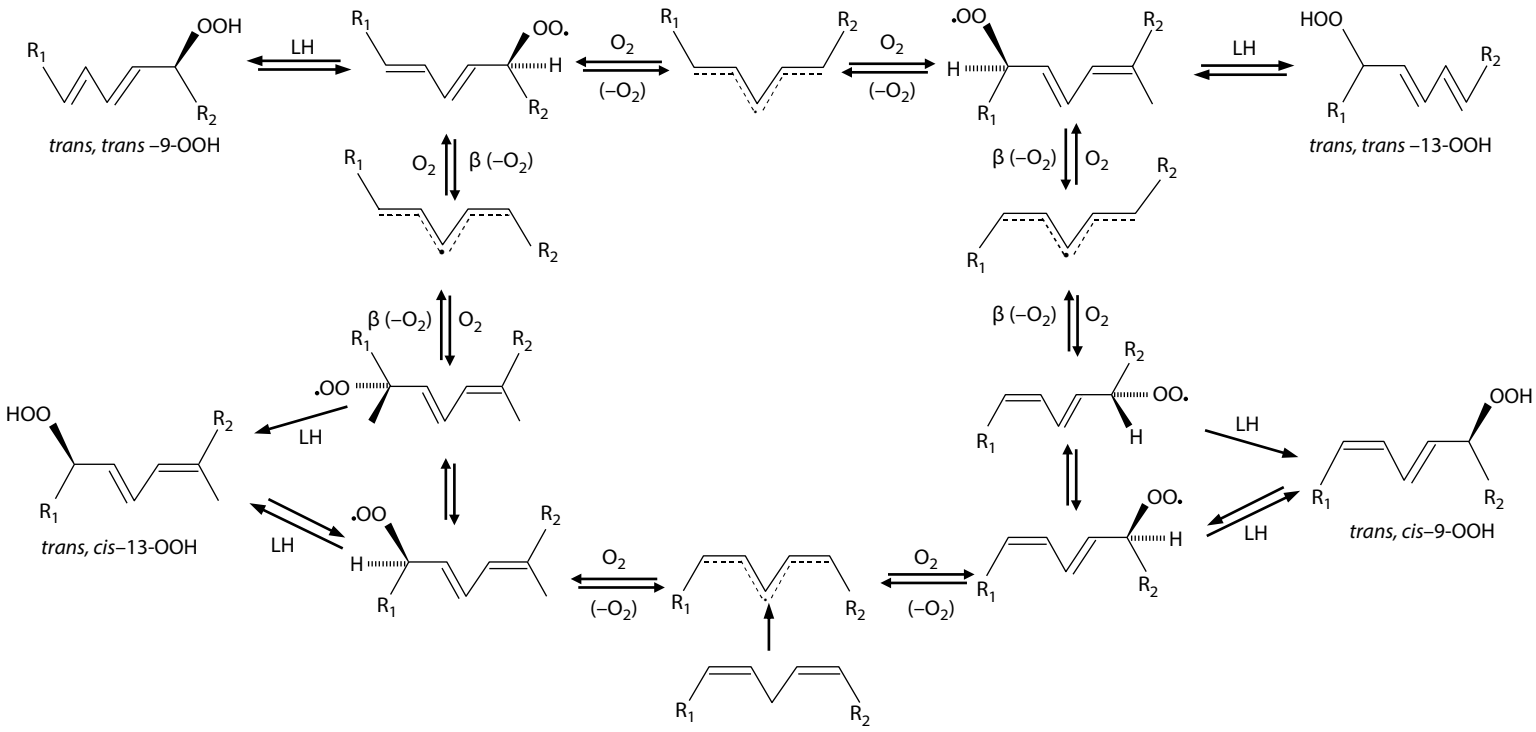
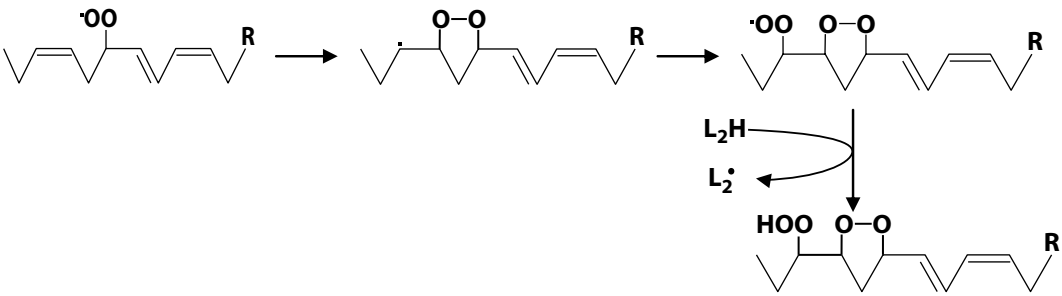


FIGURE 17.2 Reaction manifold for positional isomerization of double bonds and formation of trans, trans-hydroperoxides during oxidation of linoleic acid via reversible β-scission of oxygen. Data from [52–56]. (From Schaich, K.M., Lipid oxidation in fats and oils: Theoretical aspects, in: *Bailey's Industrial Fats and Oils*, 6th edn., F. Shahidi, ed., John Wiley, New York, 2005, pp. 2681–2767. With permission.)

In model systems of neat methyl linoleate, this reaction was responsible for epoxides that formed faster and at higher levels than hydroperoxides [51], and it accounted for the appearance of early major products that could not have resulted from standard hydroperoxide decompositions [50]. We have also observed it in oxidation of margarine-type products based on coconut and sunflower oils (K.M. Schaich and E. Nering, unpublished data). Examination of product lists in many published papers suggests that this reaction is probably quite active but totally overlooked.

3. Internal Rearrangement/Cyclization to Epidioxides

As noted at the beginning of the Alternate Reactions section, free radicals are unstable and immediately upon formation look for a way to pair the odd electron. Another alternative to hydrogen abstraction is for peroxyl radicals to add internally to the first carbon of the β-double bond (two carbons away), in the process generating epidioxides (Reaction 17.29) and leaving a new free radical on the distal carbon of the double bond [67]. Addition of oxygen generates new peroxyl radicals that may abstract hydrogen atoms from neighboring lipid molecules and continue propagating the radical chain, or they may undergo any of the alternate reactions of peroxyl radicals.



(17.29)

Internal rearrangement can occur from any position, but cyclization occurs more readily from internal positions (e.g., C10 and 12 of linoleic acid, C10, 12, 13, and 15 of linolenic acid). Epidioxides are found in the highest concentrations in fatty acids with four or more double bonds and in secondary reactions of photosensitized oxidations following $^1O_2$ addition to internal double bonds [67,68]. Indeed, internal cyclic products are characteristic markers distinguishing autoxidation from photosensitized oxidation.

LOO• cyclization is favored by low oxygen pressures and moderately elevated temperatures (e.g., 40°C) [69], parallel orientation of lipid chains, and aprotic solvents [67]. In oils or pure lipids where abstractable hydrogens are readily available, cyclization competes with H abstraction to generate mixed products whose proportions vary with temperature and extent of oxidation. Cyclization percentages ranging from 30% to 100% in oils or fatty acids have been reported [43,67]. However, these cyclic products are difficult to detect and identify because epidioxide-OO• radicals are particularly prone to dimerization and the dimers often decompose still further [68]. In addition, the epidioxide and its partner hydroperoxide are indistinguishable from standard hydroperoxides in peroxide values assays. Thus, it is often necessary to look for footprints of epidioxides to deduce their contributions to lipid oxidation in foods or biological tissues.

4. Disproportionation

Disproportionation occurs when two peroxyl radicals join to form a tetroxide, which then decomposes to produce two or more different radicals (Reaction 17.30).

$$R_1OO^\bullet + R_2OO^\bullet \longrightarrow [R_1OOOOR_2] \longrightarrow R_1O^\bullet + {}^\bullet OOOR \longrightarrow R_1O^\bullet + O_2 + {}^\bullet OR_2 \quad (17.30)$$

$$[R_1OOOOR_2] \longrightarrow R_1OOR_2 + O_2 \quad (17.31)$$

Since disproportionation requires close radical contact, it occurs when peroxyl radicals accumulate to high concentrations either locally as with high initiation or systemically as in thermal degradations. As with addition, disproportionation also has important consequences. One is that two relatively slow-reacting peroxyl radicals are converted to two alkoxyl radicals that are 1000× more reactive as hydrogen abstractors (Reaction 17.30), so the end result is greatly accelerated propagation and oxidation [33,70]. A second consequence is that at lower temperatures or high viscosity, the tetroxide decomposes to an alkyl peroxide (R_1OOR_2) in a slower termination reaction (Reaction 17.31). This provides a pathway for early formation of peroxides without hydroperoxide involvement, whereas peroxides are usually attributed to alkoxyl radical recombination in late oxidation after hydroperoxide decomposition. Third, both reactions release oxygen back into the headspace or sample to provide fuel to maintain the radical chain. This provides an important means of maintaining the radical chain even when oxygen becomes limiting in samples. At the same time, when lipid oxidation is being followed by oxygen consumption, release of oxygen can make it appear that oxidation has slowed when it is just shifting gears from $LOO^{\bullet}$ to $LO^{\bullet}$ mediation.

It is perhaps useful to note that disproportionation occurs only in lipids oxidizing as pure oils or in aprotic solvents. In polar solvents such as alcohols or aqueous systems such as emulsions, the preferred reaction of $ROO^{\bullet}$ is β-scission with direct release of oxygen ($ROO^{\bullet} \rightarrow R^{\bullet}$) [71,72], and other reactions dramatically increase $ROO^{\bullet}$ decomposition.

5. Hydrogen Abstraction

Even though it is a mainstream rather than "alternate reaction," hydrogen abstraction is included here because it leads to hydroperoxides—the most expected products—so changes in its rates and competition with other reactions as conditions change have a major impact on what we normally "see" as lipid oxidation. Anything that increases [$LOO^{\bullet} + H \rightarrow LOOH$] will appear to enhance lipid oxidation even if it may only shift the balance away from other pathways.

Hydrogen abstraction requires readily available H atoms, so the reaction in lipids is facilitated under conditions providing close contact between lipid chains, that is, in pure lipids, in acyl chains aligned in the lipid interior of membranes, in aprotic solvents, in low-viscosity media that facilitate molecular movement [73], and at elevated temperatures that contribute activation energy [1,12,74]. In solvents, H abstraction is fastest at moderate lipid concentrations where enough substrate is present to supply hydrogens, but at low lipid concentrations other reactions dominate [1]. These preferences are important to keep in mind when monitoring oxidation only with peroxide values. Low peroxides alone do not verify low oxidation.

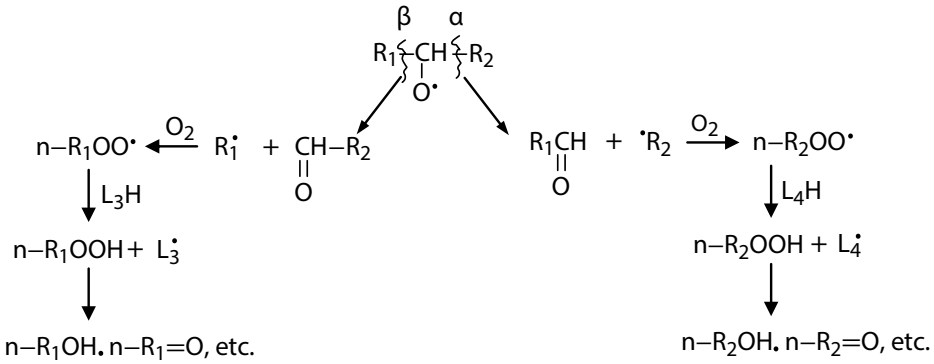
It is important to recognize that hydrogens for $LOO^{\bullet}$ to abstract need not come from lipids alone, so the overall system composition can have dramatic impacts on the directions of lipid oxidation. In fact, high concentrations of H donors such as proteins or phenolic antioxidants can markedly shift the balance of lipid oxidation pathways by favoring H abstraction over alternate pathways or by stabilizing LOOH through hydrogen bonding. For example, in an interesting paradox we recently observed, proteins and tocopherols appeared to increase hydroperoxides and enhance lipid oxidation when in fact they were shifting the most active pathway from $LOO^{\bullet}$ addition and epoxide formation to hydrogen abstraction and LOOH formation (K.M. Schaich, unpublished data). Thus, the potential for alternate hydrogen sources intervening in lipid oxidation must be considered when interpreting lipid oxidation patterns in different food systems.

B. Alternate Reactions of $LO^{\bullet}$

1. α- and β-Scission (Fragmentation)

α- and β-Scission of alkoxyl radicals to yield a mixture of carbonyl products and free radicals has already been described in the context of termination (Reaction 17.22). However, α- and β-scissions are also active and underrecognized propagating reactions that enhance chain branching and

contribute increased complexity to product distributions. Free radicals arising from the scissions can either rearrange internally to non-radical products or add oxygen to form terminal peroxyl radicals (n-ROO•) that then abstract hydrogen atoms to propagate the radical chain (Reaction 17.32) [34,37,38,40,57,75]. Unsaturated fragments, particularly those containing conjugated dienes, are still susceptible to oxidation and their subsequent reactions also contribute to chain branching. Typical products are aldehydes, alkanes, alkenes, and oxo-esters, many of which are commonly used to determine the extent of lipid oxidation.



(17.32)

In oils and phospholipids, half of the LO• scission fragments become free monomer products that are mostly volatile and detectable by gas chromatography, while products from the other scission fragment remain on the parent molecule and are referred to as core aldehydes, epoxides, etc. These core products are not readily volatile and, to be accounted for, must be detected in chemical assays of the oil or extract or by individual product analysis using high-pressure liquid chromatography with mass spectrometry detection. They should not be ignored—the volatile fragments may be largely responsible for "rancid" odors and flavors, but the core products remaining drive polymerizations in the oils as well as interactions with protein, DNA, and other molecules.

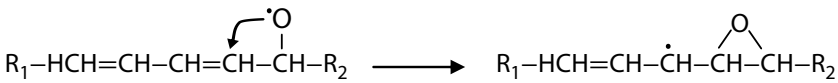
In analyses of lipid oxidation, we generally treat scission products such as hexanal and 2,4-decadienal as if they are generated automatically and quantitatively after decomposition of LOOH and by the same pathways under all conditions. This is certainly not an accurate assumption. Because the scission goes through a charged transition complex, the process changes with the solvent [76]. The scission is rapid in polar protic solvents such as alcohols and is facilitated by water, both of which provide hydrogen bonding to support the transition state [35,73,77–79] as well as H^+ that add immediately to the scission radicals to stabilize products and drive the reaction forward [80]. Even though the scission is slower than H-abstraction (Table 17.1), it still competes effectively under conditions that impede hydrogen abstraction by LO•, for example, polar hydrogen-bonding solvents and low lipid concentrations in nonpolar organic solvents. According to literature reports, the balance between competing pathways shifts increasingly toward LO• scissions as polarity of the medium increases. LO• scission proportions may be as low as 7%–10% in neat fatty acid esters or triacylglycerols, then range to greater than 80% in very polar solvents or in the presence of water or acid [81–84]. Although we have not detected such large shifts in our studies, we feel the solvent effect is important and merits further investigation to determine controlling factors.

In neat oils and in all solvents, LO• scission makes its greatest contribution to propagation and products at elevated temperatures [35] where thermal energy overcomes the large E_a and log A (Arrhenius factor) for the scission [85] and increases competitiveness with other reactions. Combining rapid decomposition of LOOH with enhanced α,β-scission accounts for the disproportionately high levels of aldehydes produced during frying [86]. Shifts in scission products at different temperatures have been reviewed in detail by Grosch [34].

The scission is less favored in neat lipids or aprotic solvents at room temperature where double bonds in unsaturated fatty acids provide the only polarity to drive the scission [73]. Background levels of scission products ranging from about 1% in monoacid triacylglycerols [87] to 10%–20% in free esters of O, L, and Ln [7,83] on a total weight basis have been reported. This difference in LO• scission is critically important when selecting analytical methods and interpreting data, especially when using volatile products to distinguish whether low hydroperoxides result from low oxidation or rapid decomposition. In oils or methyl linoleate model systems where the scission is low, analysis of volatiles as the only adjunct to peroxide values could lead to an erroneous conclusion of low oxidation because other secondary pathways are more active.

2. Internal Rearrangement/Cyclization to Epoxides

As with epidioxide formation by LOO•, internal cyclization of LO• to epoxides (Reaction 17.33) occurs when abstractable H atoms are not immediately available within the reaction radius. The reaction is very fast ($k_{LO•} \sim 10^5\ s^{-1}$) and can even exceed rates of H abstraction under some conditions [88].



(17.33)

Contrary to what is written in the traditional lipid oxidation scheme, cyclization to epoxides exceeds hydrogen abstraction and is the dominant reaction of LO• in aprotic solvents (including neat lipids) [89], when lipids are at low concentration [69] or highly dispersed on a surface ([90,91], at room temperature [81,92,93]), and at low oxygen pressures [62,69]. However, in polar solvents LO• cyclization must compete with α- and β-scissions [88,94] and in aqueous solutions H abstraction is much faster [81]. These solvent effects are quite evident in the change of epoxide yields when oxidation was conducted in different solvents (Table 17.3).

Given the strong tendency of LO• to cyclize and the rapid generation of epoxides by LOO• addition to double bonds, the epoxides should definitely become a standard required assay for tracking lipid oxidation. That being said, detecting epoxides in food oils and foods is not straightforward because epoxides are not stable and they react very rapidly with proteins [95]. Thus, it would be very useful to identify lipid epoxide degradation products in model systems and then analyze those as specific markers or footprints of epoxides in oils and lipid extracts.

TABLE 17.3
Shifts in Alternate Pathways Preference and Resulting Products When Linoleic Acid or Its Methyl Ester Is Oxidized in Different Solvents

Solvent and System	LOH/LOOH[a]	Cyclic[b]	Scission	Other	Unknown	Reference
$CHCl_2$, $FeCl_3$, early after extended rx		100				[89]
Anhydrous MeOH	3–8	75–80	13–15			[95]
Cyclohexane, 7.5 mM	15	68		18[c]		[46]
80% ethanol $FeCl_3$/cysteine	30	11	—	7[d]	7[e]	[96]

Source: Schaich, K.M., Lipid oxidation in fats and oils: Theoretical aspects, in: *Bailey's Industrial Fats and Oils*, 6th edn., F. Shahidi, ed., John Wiley, New York, 2005, pp. 2681–2767. With permission.

[a] Total of all H abstraction products, all isomers.

[b] Total of all products that had any cyclic component.

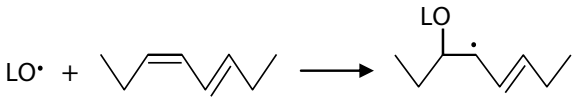
[c] Oxo dienes.

[d] Hydroxyl ethoxylated products from rx with solvent radicals.

[e] Unidentified soluble products and volatile scission products.

3. Addition to Double Bonds

LO• do add to double bonds (Reaction 17.34), but not readily because they are strong hydrogen abstractors and also rapidly cyclize to epoxides. Addition of LO• is favored by the absence of allylic hydrogens and by conjugation, so LO• addition is primarily active in the secondary stages of oxidation. At the same time, LO• adds only to *cis* double bonds [96], so it selects sites away from where oxidation has already occurred and inverted bonds to *trans*.



(17.34)

Propagation by LO• addition is most important in neat lipids and aprotic organic solvents [81]. Although LO• additions do occur in aqueous solvents, they are generally not competitive with scission and rearrangement reactions. LO• additions increase with temperature [97].

IV. WHY ARE ALTERNATE REACTIONS IMPORTANT IN LIPID OXIDATION?

For decades, we have used the simple free radical chain reaction as a road map for working with lipid oxidation, whether designating products for analysis and interpreting results, rationalizing accelerated shelf life testing, or designing food stabilization strategies. However, if one looks beyond hydroperoxides and examines the kinetics and patterns in which products are generated, it is clear that straightforward hydrogen abstraction alone cannot explain the progress and products of lipid oxidation.

The scientific literature provides ample evidence that lipid peroxyl and alkoxyl radicals, and even hydroperoxides, have multiple pathways available and that the balance of pathways active in a given system depends on solvent, fatty acid composition and concentration, initiation mechanisms and catalysts present, temperature, oxygen pressure, and especially on availability of abstractable hydrogens from lipids and other sources. Experimental evidence also shows that shifting among pathways critically affects measured kinetics of oxidation, whether determined by oxygen consumption or appearance of specific products, and can induce large differences in the ultimate mix of products, particularly volatiles.

The presence of alternate reactions competing with hydrogen abstraction has several important implications and consequences:

1. Mixtures of products are formed at all stages, not just at the end of chains after hydroperoxide formation and decomposition.
2. Multiple products must be tracked to accurately determine the extent of lipid oxidation, especially in early stages. If the dominant pathway is not being monitored, an inaccurate picture of the rate, extent, and character of lipid oxidation is generated and oxidation levels are misinterpreted. In particular, use of peroxide values alone under conditions favoring cyclization or scission may miss much of the lipid change altogether.
3. Oxidation reactions are not the same at all temperatures. Accelerated shelf life studies have long been based on the assumption that the rate of lipid oxidation increases with temperature according to Arrhenius kinetics while the general reaction mechanisms do not change. However, increasing evidence verifies that oxidation pathways are not the same at 40°C, 60°C, 100°C, 120°C, and 150°C and that reaction mechanisms and product distributions change in different temperature ranges. Thus, analyzing and interpreting degradation by only a few products will not reflect the differences accurately, may underestimate degradation tremendously, and is not likely to detect reactions and products that cause important damage.
4. Changes in the product distributions by alternate reactions critically alter flavors and odors from lipid oxidation, and also the potential for secondary effects such as nonenzymatic browning and reactions with proteins.

5. Toxicity and safety issues. Off-odors from volatile scission products are the consumer's first clue that foods are rancid and should not be eaten. Shifts from scission to internal rearrangement and addition reactions may reduce some off-odors, but the epoxides thus generated are more toxic and react more rapidly with DNA and proteins, while dimers have reduced digestibility and some evidence of toxicity. More problematically, these products have no sensory warning signals for consumers.
6. Finally, developing the most effective strategies for inhibition of lipid oxidation requires information about dominant and active propagation pathways in each food system under different conditions, and targeting of antioxidants to specific reactions. For example, phenolic antioxidants quench lipid radicals but also shift pathways away from cyclization and addition to LOOH formation. This may seem like a positive effect. However, in polar systems where LOOH decomposition is fast and LO$^{\bullet}$ scission is dominant, the net effect (especially at high antioxidant concentrations) may well be enhanced production of off-flavors and odors. To achieve long-term stability, therefore, antioxidant approaches must be tailored specifically to control all active propagation pathways.

Clearly, these multiple pathways must be considered in determining appropriate analyses for lipid oxidation, interpreting oxidation product data, designing more effective strategies for stabilization of foods where lipid oxidation is a major mode of deterioration, and understanding how lipid oxidation may mediate pathological processes *in vivo*.

The challenge now will be to rethink lipid oxidation in practice, to test the integration of the alternate reactions, and to document how they interact in neat lipids, when lipids are in complex food matrices, and when lipids are compartmentalized in biological tissues.

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18 Polyunsaturated Lipid Oxidation in Aqueous Systems

Kazuo Miyashita

CONTENTS

I. INTRODUCTION

Long-chain polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA; 22:6n-3), eicosapentaenoic acid (EPA; 20:5n-3), and arachidonic acid (AA; 20:4n-6), are known to have significant biochemical and physiological effects that primarily exhibit a positive influence on human nutrition and health. The functions of these PUFAs have attracted consumer's attention, and these compounds are used in functional foods and nutraceuticals. However, these PUFAs are easily oxidized because of the high degree of unsaturation. The lipid oxidation products cause undesirable flavors and lower the nutritional quality and safety of lipid-containing foods. Thus, while investigating the dietary effects of these n-3 and n-6 PUFAs, lipid peroxidation has received considerable attention because of its possible contribution to the flavor deterioration of foods and to the potential damage of biological systems [1–6].

Even at room temperature, PUFAs containing more than two double bonds can react with oxygen to form hydroperoxides, a primary oxidation product. This oxidation is thought to proceed in most cases through a free radical chain reaction consisting of chain initiation, propagation, and termination processes (Figure 18.1) [7–12]. The key event in the initiation is the abstraction of a hydrogen radical ($H^{\bullet}$) from the substrate lipid (LH) to form lipid free radicals ($L^{\bullet}$). The propagation stage begins with the addition of molecular oxygen to the lipid free radicals to form peroxy radicals ($LOO^{\bullet}$). The peroxy radical abstracts a hydrogen atom from another lipid to form a monohydroperoxide (MHP) and a lipid free radical ($L^{\bullet}$). The latter reacts with molecular oxygen in a repetition of the first propagation reaction. The initially formed MHP may subsequently decompose to yield free radicals, such as alkoxy ($LO^{\bullet}$) and hydroxyl ($HO^{\bullet}$) radicals. These radicals serve as initiators for the reactions mentioned earlier.

The rate-limiting step in the reaction is the abstraction of a hydrogen radical ($H^{\bullet}$) from substrate lipids (LHs) to form lipid free radicals ($L^{\bullet}$). Because this hydrogen abstraction occurs at the *bis*-allylic positions ($CH{=}CH{-}CH_2{-}CH{=}CH$) present in PUFAs and because the susceptibility of the PUFA to oxidation depends on the availability of *bis*-allylic hydrogens, the oxidative stability of each PUFA is inversely proportional to the number of *bis*-allylic positions in the molecule or the degree of unsaturation of the PUFA. Thus, when the relative oxidative stabilities of typical PUFAs

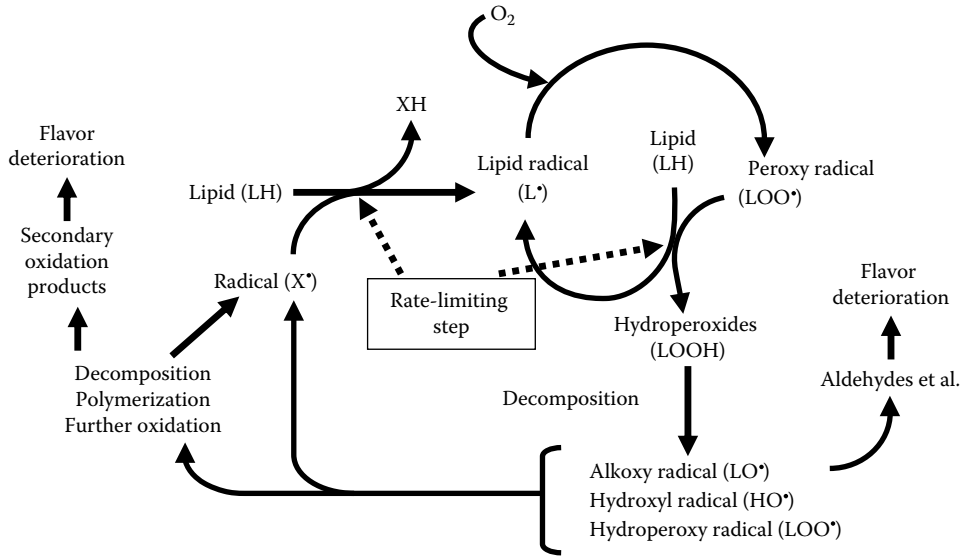

FIGURE 18.1 Oxidation mechanism of polyunsaturated lipids. H•, hydrogen radical; L•, lipid free radicals; LOO•, peroxy radical; LH, substrate.

are compared in air (bulk phase) or in organic solvents, DHA (22:6n-3) is most rapidly oxidized, followed by EPA (20:5n-3), AA (20:4n-6), α-linolenic acid (18:3n-3; α-LN), and linoleic acid (18:2n-6; LA) [13–19]. From these results, it is generally accepted that lipid oxidation of DHA and EPA is a major problem in their utilization to produce food materials, and a high intake of these PUFAs may increase the oxidative stress of biological systems.

It has been reported that DHA ingestion did not increase lipid peroxides to the level expected from the "peroxidizability" index of the tissue total lipids (TLs) [20]. In particular, lipid peroxide levels in the brain and testis decreased when DHA was administered to animals. Ando et al. [21] also examined the effects of fish oil on lipid peroxidation of rat organs and found that the levels of phospholipid hydroperoxides and thiobarbituric acid reactive substances (TBARS) in rats fed on a fish oil diet were similar to those of the safflower-oil diet group. Wander and Du [22] measured plasma lipid peroxidation after supplementation with EPA and DHA from fish oil and tocopherol in postmenopausal women. They found that neither the concentration of plasma TBARS nor protein oxidation changed after fish oil supplementation. These results indicate a difference in the oxidative stability of highly unsaturated fatty acids such as DHA and AA between biological systems and bulk phases.

Although it is clear that any lipid oxidation study should be based on the classical free radical chain reaction, as shown in Figure 18.1, the study of lipid oxidation in foods and biological systems occasionally yields unexpected results that do not always follow the common free radical oxidation theory. This can be due to the complex, multicomponent, and heterogeneous foods and biological systems in which lipids are present with various types of other components in an aqueous medium. It follows that the lipid oxidation in aqueous solutions is important if we are to fully understand the factors that affect lipid oxidation in foods and in biological systems. In emulsions, the membranes surrounding the emulsion droplets consist of surface-active substances, such as emulsifiers and/or proteins. They provide a protective barrier to the penetration and diffusion of metals or radicals that initiate lipid oxidation. The chemical and physical nature of emulsifiers and proteins, therefore, is an important factor for the protection of PUFAs against oxidation in an aqueous phase [12,23–35]. In the emulsions, important factors that affect the lipid oxidation are the relative location of the lipid substrate, antioxidants, and prooxidant, and the partitioning and affinity of these components

to different phases or environments [36–39]. Moreover, in micelles, the relative oxidative stabilities of PUFAs decrease with an increasing degree of unsaturation [40]. This order is the reverse to that found in the bulk phase.

The goal of this study is to highlight the relative order of oxidative stability of lipids having different numbers of unsaturation in aqueous phases.

II. OXIDATIVE STABILITY OF PUFA IN MICELLES

The oxidative stability of PUFA in aqueous micelles is markedly different from that in the bulk phase [40–42]. When the oxidative stabilities of six types of typical PUFAs were compared in micelles (Figure 18.2), LA was the most susceptible to oxidation—as much as 50% of the substrate was lost after only 13 h of oxidation. In contrast, DHA and EPA were highly stable—even after 2000 h of oxidation, more than 80% and 90% of the substrates remained unchanged [40]. In addition, a marked difference in oxidative stability was observed between α-LN and γ-linolenic acid (γ-LN) in micelles, while no difference in the oxidation rate was found between them during oxidation in the bulk phase. Additionally, when a mixture of DHA and LA was oxidized in aqueous micelles, the stability increased as the molar ratio of DHA to LA increased in the mixture [40]. These relationships are the opposite to those found in the bulk phase [17] or in organic solvents [16].

Although the cause for the higher oxidative stability of DHA and EPA in aqueous micelles remains unclear, it is apparent that the physical and stereochemical characteristics of EPA and DHA molecules in micellar conformations are related to the unusual order of the oxidative stability (Figure 18.3). The higher relative oxidative stability of EPA and DHA in the aqueous phase could be explained by the specific inhibition of hydrogen abstraction from the *bis*-allylic positions of both PUFAs. Kato et al. [43] reported that the n-3 double bonds of DHA and EPA reacted with N-bromosuccinimide to convert to the corresponding bromohydrin with 87% and 89% selectivity, respectively. They demonstrated that this high selectivity is due to the coiled configurations of DHA and EPA in an aqueous medium. In the tight packing conformation, the inner *bis*-allylic positions of EPA or DHA will be protected against addition reactions in aqueous micelles. Oxidative attack by free radicals and/or oxygen may also be prevented by this specific confirmation.

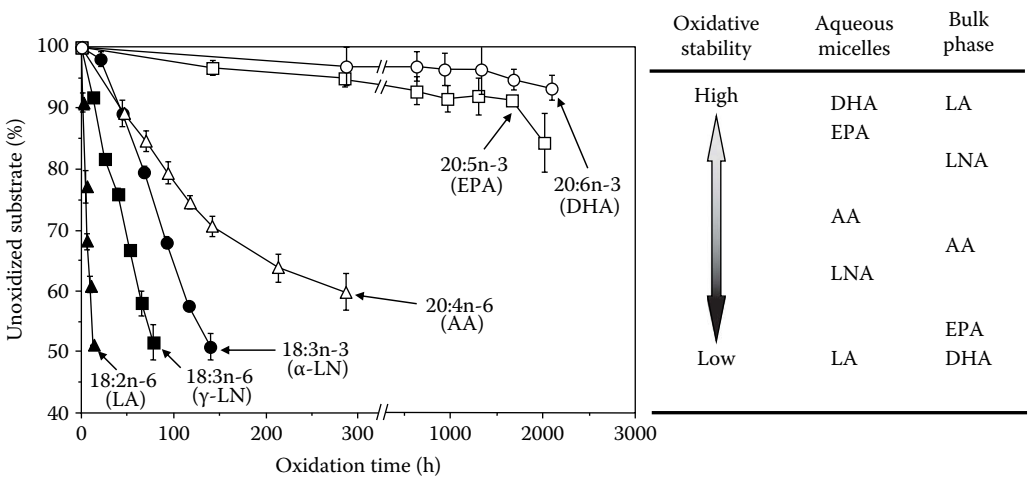

FIGURE 18.2 Oxidative stability of six types of typical PUFA in aqueous micelles. The stability was determined by following the decrease in unoxidized substrate content. Each PUFA (1 mM) was incubated in the dark at 37°C with Fe(II) (1.0 μM) and ascorbic acid (20.0 μM) in 10.05 mL of a phosphate buffer (pH 7.4) containing 1.0% (w/v) of Tween 20. LA, linoleic acid; γ-LN, γ-linolenic acid; α-LN, α-linolenic acid. (From Miyashita, K. et al., *Biosci. Biotechnol. Biochem.*, 57, 1638, 1993. With permission.)

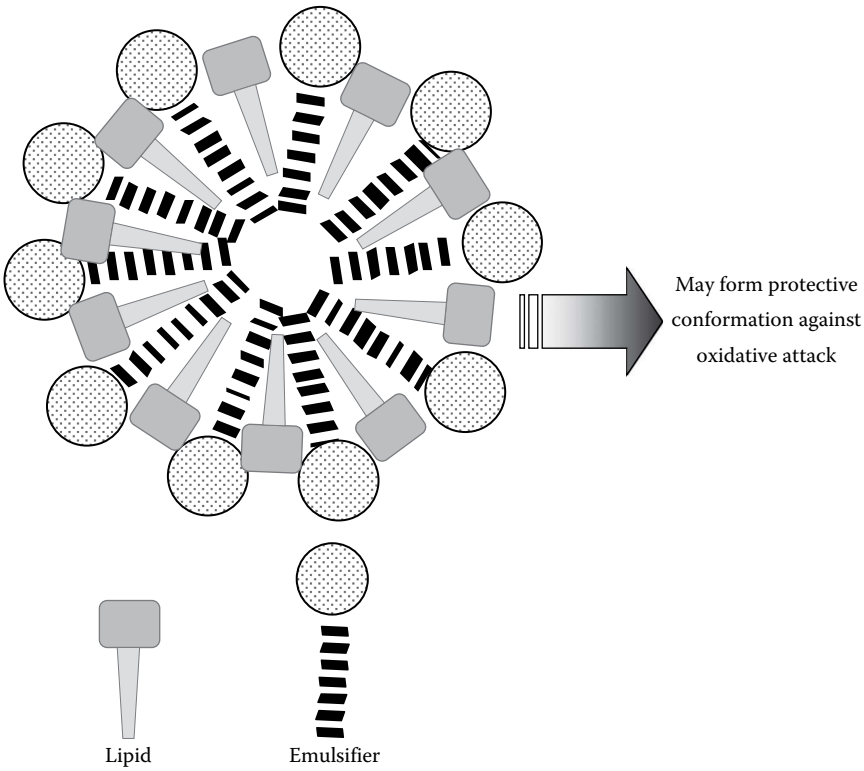

FIGURE 18.3 Structure of micelles.

The conformation and kinetics of a molecule or part of a molecule are reflected in nuclear magnetic resonance (NMR) relaxation times, that is, spin–lattice relaxation (T_1) and spin–spin relaxation (T_2) times. Proton NMR relaxation times of each signal of the PUFAs (Figure 18.4) are shown in Table 18.1 [44]. When comparing the protons in PUFAs forming micelles with those of PUFAs in chloroform solution, two characteristic trends were observed. First, the protons in PUFAs forming micelles had shorter relaxation times than those in chloroform solution, except for methylene adjacent to the methyl terminal (c) of DHA. Second, the T_2 of the protons in micelles was much shorter than their T_1, whereas T_1 and T_2 were nearly the same in chloroform solution. The decrease of T_2 by micelle formation indicates that lipid molecules in micelles are mutually rigidly associated, thus restricting the molecular motion compared with that in non-associated lipid molecules in chloroform solution. The shorter T_2 in micelles compared with T_1 indicates that the aqueous system is closer to a solid state.

Proton T_2 values provide more information about the difference in the molecular conformation of each PUFA. The T_2 of the methyl protons (a) of DHA in micelles had much larger values than those of LA, and those of AA had intermediate values (Table 18.1). The allylic protons (c), olefin protons (d), and *bis*-allylic protons (e) showed a similar tendency to the methyl protons (a). For the proton on the terminal carboxyl (h), LA had a slightly longer T_2 than DHA. Thus, the mobility of the hydrophobic part of the DHA molecule is considerably higher than that of LA when forming micelles. Micelles of AA appear to have an intermediate flexibility between that of DHA and LA; this flexibility may allow water molecules to permeate the micelles. The penetration of water molecules, which allows them to reach acyl moieties in micelles, can inhibit hydrogen abstraction from the *bis*-allylic positions of unoxidized fatty acids using the peroxy radicals of adjacent oxidized fatty acids in the propagation stage of free radical oxidation.

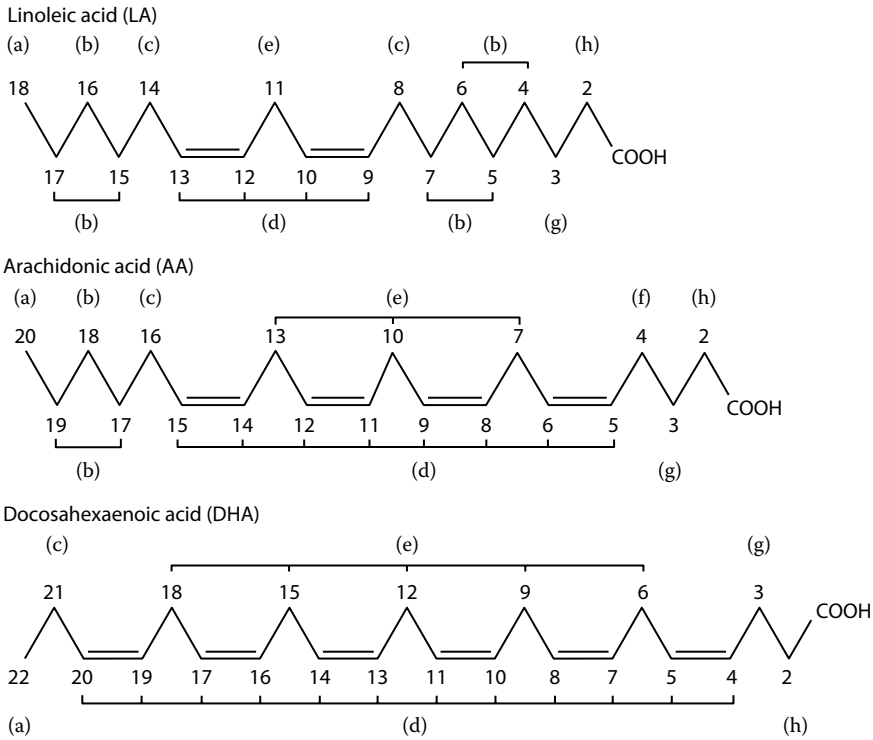

FIGURE 18.4 Molecular structure of three PUFAs. (From Kobayashi, H. et al., *J. Oleo Sci*., 56, 105, 2004. With permission.)

TABLE 18.1
T_1 and T_2 for PUFA

	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)
T_1 chloroform solution								
LA	3.6 ± 0.2	2.0 ± 0.2	2.0 ± 0.3	3.5 ± 0.3	1.9 ± 0.1	2.0 ± 0.3	2.0 ± 0.3	1.8 ± 0.2
AA	3.3 ± 0.2	2.1 ± 0.3	2.2 ± 0.3	3.2 ± 0.5	1.6 ± 0.1	2.2 ± 0.3	1.8 ± 0.2	2.0 ± 0.4
DHA	3.3 ± 0.4	—	1.5 ± 0.1	2.1 ± 0.3	3.5 ± 0.4	—	2.1 ± 0.2	2.1 ± 0.2
Aqueous micelles								
LA	1.6 ± 0.4	1.0 ± 0.3	0.91 ± 0.42	1.2 ± 0.3	0.92 ± 0.10	0.91 ± 0.42	0.82 ± 0.21	0.76 ± 0.11
AA	1.6 ± 0.2	1.3 ± 0.2	1.0 ± 0.6	1.7 ± 0.3	0.95 ± 0.15	1.0 ± 0.2	0.95 ± 0.22	0.97 ± 0.10
DHA	2.3 ± 0.3	—	2.1 ± 0.3	1.0 ± 0.2	1.9 ± 0.3	—	0.89 ± 0.10	1.1 ± 0.2
T_2 chloroform solution								
LA	3.4 ± 0.3	1.8 ± 0.2	1.7 ± 0.3	3.3 ± 0.4	1.8 ± 0.2	1.7 ± 0.3	1.7 ± 0.3	1.6 ± 0.3
AA	3.0 ± 0.3	1.8 ± 0.2	2.1 ± 0.2	3.0 ± 0.4	1.4 ± 0.1	2.0 ± 0.3	1.5 ± 0.2	1.8 ± 0.3
DHA	3.2 ± 0.5	—	1.1 ± 0.1	2.1 ± 0.2	3.4 ± 0.4	—	2.0 ± 0.3	2.0 ± 0.1
Aqueous micelles								
LA	0.25 ± 0.10	0.21 ± 0.10	0.15 ± 0.08	0.39 ± 0.11	0.10 ± 0.05	0.15 ± 0.08	0.19 ± 0.04	0.48 ± 0.12
AA	0.65 ± 0.18	0.64 ± 0.15	0.68 ± 0.24	0.55 ± 0.09	0.49 ± 0.07	0.70 ± 0.18	0.25 ± 0.10	0.19 ± 0.06
DHA	1.4 ± 0.3	—	1.3 ± 0.2	0.86 ± 0.12	1.1 ± 0.2	—	0.28 ± 0.07	0.28 ± 0.09

Source: Kobayashi, H. et al., *J. Oleo Sci*., 56, 105, 2004. With permission.

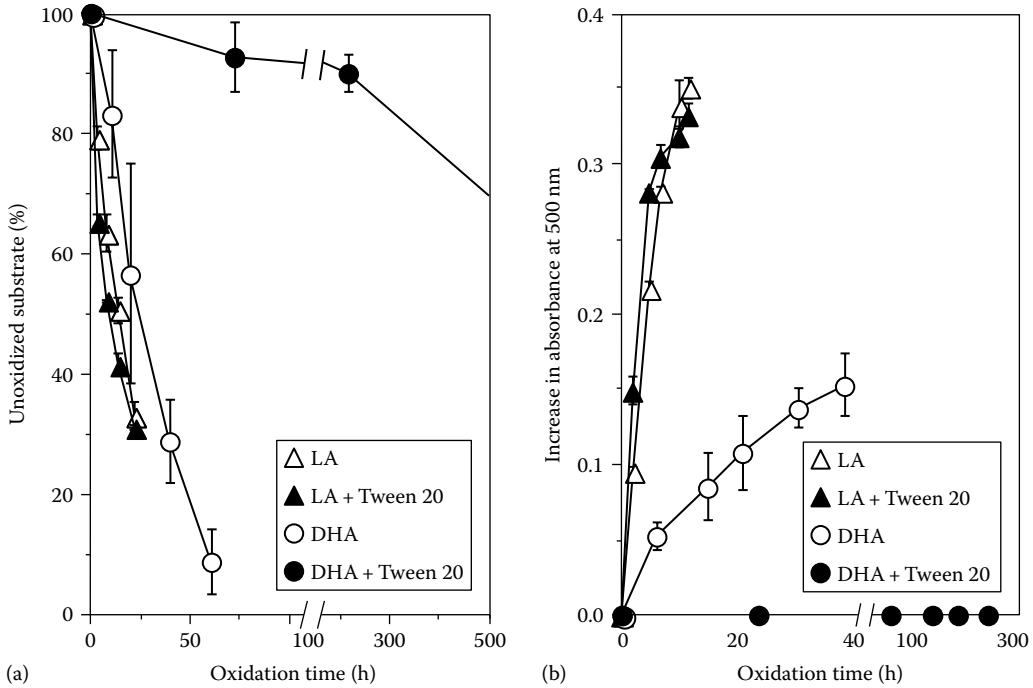

FIGURE 18.5 Effect of Tween 20 on the oxidative stability of LA and DHA in aqueous micelles. Changes in the amounts of unoxidized substrate (a) and development of total peroxides (b) during the oxidation of LA and DHA in a buffer with (solid circle and solid triangle) or without (open circle and open triangle) Tween 20. Each sodium salt (1.0 mM) was incubated at 37°C with $FeSO_4$ (1.0 μM) and ascorbic acid (20.0 μM) in a phosphate buffer (pH 7.4 at 37°C) with or without Tween 20. The unoxidized substrate content was determined by GC as previously described. Total peroxide formation is expressed as the absorbance at 500 nm. (From Miyashita, K. et al., *Biosci. Biotechnol. Biochem.*, 61, 716, 1997. With permission.)

When free fatty acids are dispersed in water, they become arranged in the form of micelles in which the polar head groups lie in the aqueous phase, and nonpolar tails form the oil phase. Emulsifier molecules also arrange themselves with free fatty acids so that the polar head groups are located at the surface, and nonpolar tails are located in the interior (Figure 18.3); therefore, the interaction of the emulsifier and the lipid at the micelles affects the oxidative stability of the lipids. To understand the effect of emulsifiers, a comparison was performed on the oxidative stability of the sodium salts of PUFA, namely, LA and DHA, dispersed in a buffer with and without Tween 20 (polyoxyethylenesorbitan monolaurate) [45]. The oxidative stability was evaluated using different indexes, such as a decrease in unoxidized PUFA (Figure 18.5a) and an increase in peroxides (Figure 18.5b). The change in the unoxidized PUFA was analyzed by gas chromatography after acidifying the reaction solution and extracting and methylating the liberated free fatty acids. Peroxide formation was measured by monitoring the formation of the ferric thiocyanate complex having an absorption maximum at 500 nm. This complex is formed by the reaction between thiocyanate and ferric ion resulting from the oxidation of ferrous ion by lipid hydroperoxides. In the aqueous dispersion, the oxidative stability of DHA was markedly increased by the addition of Tween 20 as an emulsifier. In contrast, the stability of LA with Tween 20 was slightly less than that of LA without Tween 20. This specific effect of Tween 20 to protect DHA against oxidation in aqueous micelles is associated with its different interaction with the two acids. DHA may be protected from attack by free radicals and/or oxygen by forming a packed micellar conformation with Tween 20.

Yazu et al. [46] have also demonstrated the higher oxidative stability of EPA compared with LA in aqueous micelles. They explained the characteristic oxidative stability of EPA in aqueous solution based on the formation of polar peroxy radicals—epidioxy-peroxy radicals—that are easily derived from the peroxy radicals of EPA but not from those of LA. The epidioxy-peroxy radicals easily diffuse from the core to the surface in micelles because of their higher polarity. This localization of peroxy radicals may render the EPA less easily oxidized in aqueous micelles by enhancing the termination reaction rate for peroxy radicals and by reducing the rate of propagation. However, this concept based on the difference in the polarity of peroxy radicals cannot explain the higher oxidative stability of α-LN compared with γ-LN in aqueous micelles and the specific effect of Tween 20 on the oxidation of DHA in aqueous micelles.

III. AQUEOUS OXIDATION OF ETHYL ESTERS

The oxidative stability of the ethyl esters of PUFA in aqueous solution is also different from that in the bulk phase or in organic solvent. When a small amount of ethyl linoleate (ethyl LA), ethyl linolenate (ethyl LN), and ethyl docosahexaenoate (ethyl DHA) was oxidized in an aqueous dispersion with an emulsifier, the solution was clear, indicating that the esters would be solubilized as a microemulsion. In the oxidation of these esters with Fe(II)-ascorbic acid in the microemulsion, the oxidative stability of ethyl DHA was the highest among the three ethyl esters, followed by ethyl LN and ethyl LA, while the reverse order of oxidative stability was obtained when these esters were oxidized in chloroform or ethanol with 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) as an oxidation initiator [47]. The ratio of the interfacial area to the oil phase in the microemulsion is higher than that in an ordinary emulsion so that the oxidative stabilities of the PUFA esters were strongly affected by their stability at the interface where DHA would be oxidatively more stable than LA and LN.

The oxidation products formed from ethyl esters in the aqueous dispersion differ from those in the bulk phase. PUFA containing more than three double bonds can yield hydroperoxy epidioxides as primary oxidation products; they are formed by a rapid 1,3-cyclization and further oxidation from inner monohydroperoxides [8–12]. Reversed-phase high-performance liquid chromatography (HPLC) of oxidized ethyl LN in aqueous micelles showed the formation of hydroperoxy epidioxides, but the ratio of epidioxides to monohydroperoxides was lower than that for ethyl LN oxidized in the bulk phase [47]. Furthermore, no peaks corresponding to epidioxides of oxidized ethyl DHA in the aqueous microemulsion could be detected by HPLC. The lower accumulation of epidioxides in aqueous micelles may result from the inhibitory effect of water penetration to acyl moieties and/or the steric hindrance in the packed conformation of ethyl DHA and the emulsifier.

The high oxidative stability of DHA was found in the oxidation of acylglycerols solubilized as a microemulsion in the aqueous phase. When a small amount of monoacylglycerols was dispersed in aqueous solutions with an emulsifier, monodocosahexaenoin was the most oxidatively stable compound, followed by monoarachidonin, monolinolenin, and monolinolein when each monoacylglycerol (1 mM) was oxidized with Fe(II)-ascorbic acid as an initiator [48]. The same relationship was found in the aqueous oxidation of tridocosahexaenoin and trilinolein [48]. The characteristic oxidative stability of acylglycerols in aqueous solution was also found in triacylglycerols (TAGs) from natural oils [49].

IV. OXIDATIVE STABILITY OF TAG IN EMULSIONS

Knowledge of the characteristic oxidative stability of PUFAs or their esters in an aqueous phase is important for a better understanding of the oxidation of PUFAs in aqueous food systems. However, in these systems, lipids are mainly dispersed as emulsions, and it is important to compare the oxidative stability of different polyunsaturated lipids in an emulsion. Food emulsions are complex

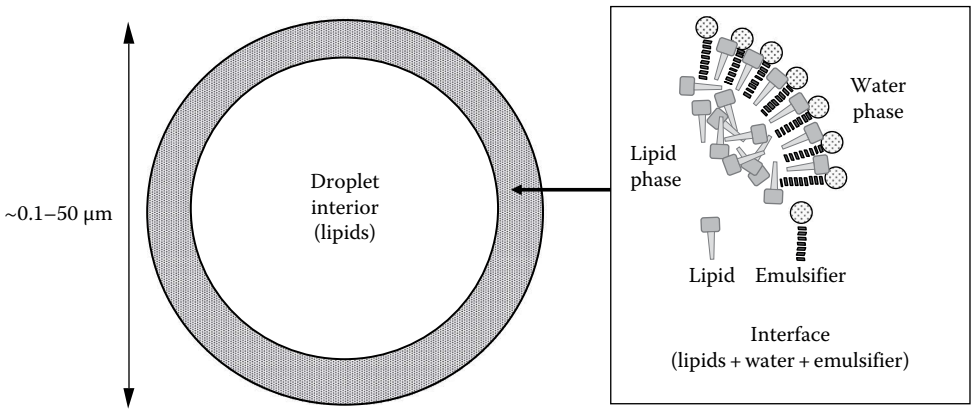

FIGURE 18.6 Aqueous structure of emulsion.

multiphase systems in which different molecular species interact with each other. The various molecules in an emulsion system become distributed according to their polarity and surface activity between different phases, which include the oil phase, the water phase, and the interfacial region. Lipid oxidation in these systems is an interfacial phenomenon that is greatly influenced by the nature of the interface. The aqueous structure of the interface in an emulsion (Figure 18.6) is nearly the same as that of micelles (Figure 18.3). Because the lipid oxidation generally proceeds from the interface to the interior of the oil droplet in oil-in-water emulsions, the oxidation of lipids at the interface is an important factor for predicting the oxidative stability of lipids in an emulsion. In aqueous micelles, DHA or the DHA ester is oxidatively more stable than LA or the LA ester, although DHA is chemically more susceptible to oxidation than LA. This is because DHA itself—or DHA and an emulsifier—would assume a more protective conformation against the oxidative attack of free radicals and/or oxygen.

When the concentrations of the lipid and the emulsifier are the same, the area of the interface increases with decreasing droplet size. The opportunity for attack by an oxidation inducer increases with the area of the interface; therefore, the oxidative stability of lipids in an emulsion generally decreases with decreasing droplet size [50]. However, an opposite relationship was found for DHA esters. Using the monodisperse emulsion of soybean oil TAG rich in LA and fish oil TAG rich in DHA, Azuma et al. [51] found that the stability of fish oil TAG in the emulsions increased with decreasing droplet size, while the reverse result was obtained for the oxidation of soybean oil TAG. The results found for fish oil TAG are in opposition to the general understanding that the oxidative stability of lipids in an emulsion decreases with decreasing droplet size. The oxidative stability of the fish oil TAG emulsion found in this study may be due to the highly protective conformation of the interface against oxidation.

The oxidation of TAG in an aqueous dispersion was also strongly affected by emulsifiers. When oxidative stability was compared among TAGs dispersed with the three types of sucrose esters having the same fatty acyl composition, the stability increased as their hydrophile–lipophile balance (HLB) in both TAGs was increased, although these emulsifiers had no effect on the oxidative stability of SoyTAG and DHATAG in the bulk phase [27]. When the HLB of sucrose ester was the same, the oxidative stability increased as the acyl chain length of the esterified fatty acid increased [27]. These effects of HLB or fatty acyl composition may also be explained by the different packing of the emulsifier at the interface. Polyglycerol esters also affected the oxidative stability of both types of TAG in emulsion, but the magnitude of the effect of polyglycerol esters was less than that of sucrose ester. In addition, the difference in the oxidative stability of TAG dispersed with polyglycerol ester cannot be explained by the relationship with HLB or the acyl composition.

The characteristic oxidation behavior of different polyunsaturated lipids at the interface, as described in this chapter, provides the basic and important information required to fully understand the lipid oxidation in food emulsions and to provide an effective method for the protection of lipids against oxidation in emulsions. This reveals the importance of the chemical and physical properties of the interface to prevent the oxidation of PUFAs. This information will also aid the investigation of the oxidative stability of PUFAs in biological systems because PUFAs are typically present in these systems in an aqueous medium with other components. In phosphatidylcholine (PC) liposomes, using the model systems of biological membranes, the degree of unsaturation of PUFAs had only a slight effect on the oxidative stability, whereas the stability was affected by the positional distribution of PUFAs in the PC molecule [52–56]. This is a fundamental and important result for a better understanding of the oxidation of PUFAs in biological systems. Lipid oxidation in PC liposomes and in cellular model systems is described later.

V. POSITIONAL DISTRIBUTION OF MONOHYDROPEROXIDE ISOMERS

The key event in lipid peroxidation is the formation of a lipid radical by the abstraction of a hydrogen radical from the *bis*-allylic positions. The resulting pentadienyl radical reacts with oxygen at both ends to form two types of conjugated diene MHPs. The specific conformation of PUFAs in aqueous micelles may affect the rate of hydrogen abstraction from the particular *bis*-allylic position and/or the selective attack of oxygen at pentadienyl radicals.

In the free radical oxidation of LA, hydrogen abstraction occurs at the C11 position, which results in the production of a pentadienyl radical between C9 and C13. Then, the radical reacts at either end with oxygen to produce a mixture of 9-MHP and 13-MHP. Because AA and DHA have two or more *bis*-allylic methylene groups, there are several possible positions for hydrogen abstraction: carbons 7, 10, or 13 for AA and carbons 6, 9, 12, 15, or 18 for DHA. Because oxygen can attack carbons at either end of the pentadienyl radical, the resulting MHP isomers were those with hydroperoxide substitution on carbons 5, 9, 8, 12, 11, and 15 for AA, and 4, 8, 7, 11, 10, 14, 13, 17, 16, and 20 for DHA.

MHP isomer distribution from the oxidation of PUFA ethyl esters in chloroform and aqueous solutions is shown in Table 18.2. MHP was determined by gas chromatography–mass spectrometry (GC–MS) after the extraction of ethyl esters from the solution, hydrogenation, and trimethylsilylation [57]. When the distribution of LA ethyl esters was compared in aqueous solution and chloroform solution, an even distribution between the two MHP isomers (9- and 13-MHP) was found in the oxidation of both in chloroform and in aqueous solutions. However, a higher percentage was found for 11- and 15-MHP in the oxidation of AA in aqueous solution than that in chloroform solution (Table 18.2). The difference in the MHP distributions between the oxidation in chloroform and aqueous emulsions was also observed for DHA. As shown in Table 18.2, the proportion of 10- and 16-MHP in aqueous oxidation was much higher than that in chloroform solution. The proportion of 14-MHP in aqueous oxidation was also higher than that in chloroform solution. Conversely, the formation of 7- and 20-MHP in aqueous emulsion was less than that in chloroform solution.

The oxidation of PUFAs containing more than two double bonds, such as AA or DHA, produces a significant amount of hydroperoxy epidioxides as the main oxidation products, other than MHP, at an early stage of oxidation [8–12]. The external MHP (5-MHP and 15-MHP for AA, and 4-MHP and 20-MHP for DHA) formed by hydroperoxidation on the sp^2 carbon of PUFA closest to the methyl terminal or the carbonyl terminal does not result in hydroperoxy epidioxides. Only the internal MHPs, formed by hydroperoxidation on an sp^2 carbon of PUFA adjacent to a *bis*-allylic position, can undergo 1,3-cyclization to form hydroperoxy epidioxides. The formation of hydroperoxy epidioxides affects the positional distribution of MHP as the amount of internal MHP decreases with an increase in the formation of epidioxides. For the oxidation of AA in chloroform solution, the proportion of internal MHPs, 8-, 9-, 11-, and 12-MHP, was lower than that of external MHP isomers, 5- and 15-MHP (Table 18.2). This result could be due to the tendency of the inner-peroxy radicals to undergo rapid 1,3-cyclization. The lower proportion of internal MHPs than external

TABLE 18.2
Isomeric Distribution of MHP Isomers Formed in the Oxidations of PUFA Esters

	MHP Formation		Positional Distribution			
PUFA	Hydrogen Abstraction	Resulted MHP	In Chloroform[a] Solution	In Aqueous[a] Micelles	In PC[b] Liposome	In Cellular[c] PL
LA	C-13	9-MHP	49.3 ± 1.5	48.9 ± 2.3	49.2 ± 3.6	49.7 ± 1.5
		13-MHP	50.7 ± 1.5	51.1 ± 2.3	50.8 ± 3.6	50.3 ± 1.5
AA	C-7	5-MHP	19.4 ± 1.1	17.1 ± 3.3	9.6 ± 0.7	23.7 ± 3.7
		9-MHP	15.3 ± 0.4	15.2 ± 1.5	23.8 ± 2.0	6.6 ± 1.3
	C-10	8-MHP	15.2 ± 0.5	13.9 ± 1.6	5.1 ± 1.1	12.9 ± 2.3
		12-MHP	15.0 ± 1.1	11.7 ± 1.3	26.3 ± 2.4	23.2 ± 3.9
	C-13	11-MHP	15.1 ± 0.5	20.0 ± 0.5	7.4 ± 2.4	3.4 ± 2.3
		15-MHP	19.9 ± 1.3	22.1 ± 1.3	27.9 ± 2.1	30.2 ± 3.4
DHA	C-6	4-MHP	17.7 ± 1.3	21.7 ± 1.1	17.9 ± 2.4	25.7 ± 2.2
		8-MHP	12.9 ± 1.7	9.8 ± 0.6	11.2 ± 1.1	12.8 ± 1.6
	C-9	7-MHP	10.5 ± 0.7	5.2 ± 0.8	6.2 ± 1.1	ND[d]
		11-MHP	7.2 ± 1.0	1.9 ± 1.4	2.1 ± 0.5	ND[d]
	C-12	10-MHP	4.2 ± 0.5	10.1 ± 1.5	13.0 ± 1.1	10.2 ± 1.3
		14-MHP	4.3 ± 0.6	9.5 ± 1.0	4.6 ± 0.8	4.9 ± 4.5
	C-15	13-MHP	3.5 ± 1.0	2.0 ± 0.6	5.5 ± 1.0	ND[d]
		17-MHP	4.5 ± 0.7	3.9 ± 0.3	3.1 ± 0.6	ND[d]
	C-18	16-MHP	8.7 ± 1.4	21.9 ± 2.4	21.3 ± 4.0	24.6 ± 1.5
		20-MHP	26.5 ± 1.1	14.1 ± 1.5	16.4 ± 1.3	21.9 ± 4.3

Source: Kobayashi, H. et al., *Chem. Phys. Lipids*, 126, 111, 2003. With permission.

[a] The ethyl ester of each PUFA was oxidized in chloroform and aqueous solutions. The oxidation was induced by 2,2′-azobis(2,4-dimethyl-valeronitrile) (AMVN) or 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) for chloroform and aqueous oxidation, respectively.

[b] 1-Palmitoyl-2-linoleoyl-phosphatidylcholine (LA-PC), 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (AA-PC), and 1-palmitoyl-2-docosahexaenoyl-phosphatidylcholine (DHA-PC) were oxidized in liposomes. AAPH was used as an oxidation inducer.

[c] Each PUFA was supplemented to the cell (HepG2). Supplementation with PUFA resulted in their incorporation into cellular lipids. Cellular oxidation was accelerated by the addition of H_2O_2.

[d] Not detected.

MHPs was also found in the oxidation of DHA in chloroform solution (Table 18.2). The same tendency was observed in the oxidation of these PUFAs in aqueous solution, except in the case of the methyl-terminal *bis*-allylic position, which gave amounts of 11-MHP for AA and 16-MHP for DHA comparable to those of the corresponding external MHPs (Table 18.2). In the oxidation of DHA in aqueous solution, 10-MHP was also formed at a high proportion. These high proportions of selective internal MHPs would be due to the low rate of 1,3-cyclization of the MHP and/or the high rate of oxygen attack at the positions, which may be caused by the specific conformation of these PUFAs in aqueous solution.

VI. OXIDATION OF PC IN LIPOSOMES

When the three types of synthesized PCs, namely, 1-palmitoyl (PA)-2-linoleoyl (LA)-PC (PA-LA), 1-PA-2-arachidonyl (AA)-PC (PA-AA), and 1-PA-2-DHA-PC (PA-DHA), were oxidized in liposomes, PA-DHA was oxidatively the most stable, followed by PA-AA and PA-LA [58]. In contrast,

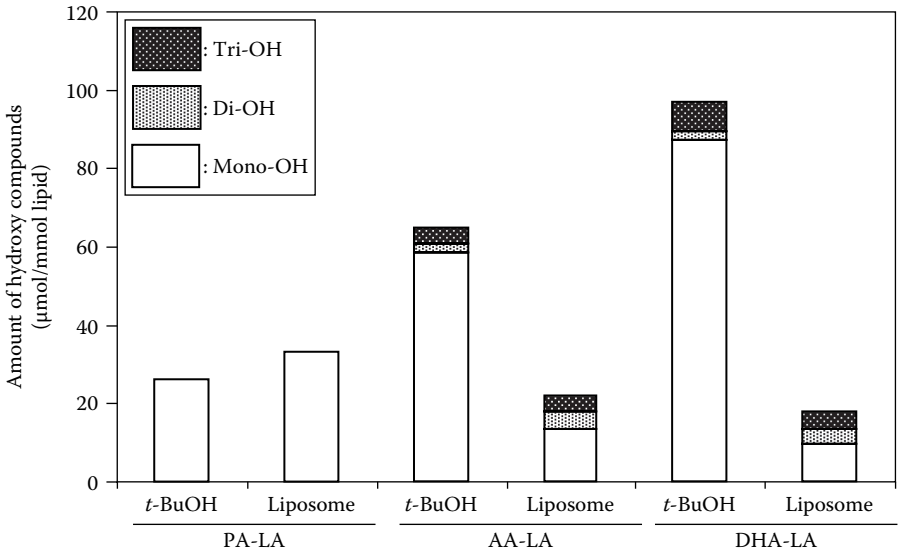

FIGURE 18.7 Amounts of total hydroxy compounds derived from oxidation products of PUFA in PC, formed in *t*-BuOH solution and liposomes. AAPH was used as an oxidation inducer. The oxidation products were extracted with chloroform/methanol, then hydrogenated, transmethylated, trimethylsilylated (TMS), and then subjected to GC–MS analysis for the quantitative comparison of TMS derivatives from mono-, di-, and trihydroxyl compounds. These hydroxyl compounds are derived from monohydroperoxides, hydroperoxy epidioxides, and other oxidation products. (From Kobayashi, H. et al., *Chem. Phys. Lipids*, 126, 111, 2003. With permission.)

the reverse result was obtained in the oxidation of these PCs in the bulk phase and in organic solvents, such as chloroform and *t*-butyl alcohol (*t*-BuOH) [58]. The inverse relationship in the oxidative stability of PUFAs in liposomes and in organic solvents was confirmed via GC–MS analysis of the mono-, di-, and trihydroxyl compounds (Figure 18.7). In this analysis, oxidation products were hydrogenated, trimethylsilylated, and then subjected to GC–MS. Monohydroxyl compounds were mainly derived from MHP by this hydrogenation, while di- or trihydroxyl compounds were derived from secondary oxidation products, such as di-hydroperoxides and hydroperoxy epidioxides. The main hydroxyl compounds obtained in the oxidation of the three types of PCs in *t*-BuOH were mono-OH (Figure 18.7). Conversely, considerable amounts of di- and tri-OH were formed during the oxidation of AA-PC and DHA-PC in liposomes. The comparison of the total amount of hydroxyl compounds showed that DHA-PC was oxidized most rapidly, followed by AA-PC and LA-PC in *t*-BuOH. However, the reverse result was obtained in liposomes (Figure 18.7).

In a study using synthesized PCs, including PA, LA, AA, and DHA in known positions, the stability of a 1:1 mixture of 1,2-dipalmitoyl-PC (diPA-PC) + 1,2-diPUFA-PC (diPUFA-PC) was lower than that of 1-PA-2-PUFA-PC in liposomes, although the degree of unsaturation of each 1-PA-2-PUFA-PC was the same as that of the corresponding mixture of diPA-PC + diPUFA-PC [58]. Liposomes are hollow vesicles composed of single or multiple bilayers, and the packing degree of the PC bilayers may strongly affect the oxidative stability of PUFAs. As shown in Table 18.3, the sizes of 1-PA-2-PUFA-PC were smaller than those of 1,2-diPA-PC + 1,2-diPUFA-PC, suggesting the more tightly packed conformation of 1-PA-2-PUFA-PC than that of 1,2-diPA-PC + 1,2-diPUFA-PC. The higher oxidative stability of 1-PA-2-PUFA-PC than that of 1,2-diPA-PC + 1,2-diPUFA-PC may be attributed to this tight conformation of 1-PA-2-PUFA-PC bilayers. Furthermore, the higher stability of 1-PA-2-PUFA-PC may also be explained by the idea that an intramolecular free radical chain reaction between two PUFAs on 1,2-diPUFA-PC occurs more rapidly than the intermolecular

TABLE 18.3
Mean Vesicle Diameter in PC Liposomes

PC	Mean Diameter (mm)
PA-DHA	2.043 ± 0.012
PA-LA	2.651 ± 0.013
PA-AA	2.057 ± 0.021
diPA+diLA	4.433 ± 0.106
diPA+diAA	4.221 ± 0.313

Source: Araseki, M. et al., *Biosci. Biotechnol. Biochem*., 66, 2573, 2002. With permission.

Note: The values are expressed as the mean ± SD of the results from three separate analyses.

chain reaction of PUFAs between different 1-PA-2-PUFA-PC molecules. A similar effect of the esterified position of PUFAs was observed in the oxidation of TAGs [59]. When the oxidative stability of a 1:2 (mol/mol) mixture of trieicosapentanoylglycerol (EEE) and tripalmitoylglycerol (PPP) was compared with that of synthetic 1,2(or 2,3)-dipalmitoyl-3(or 1) eicosapentaenoylglycerol (PPE), PPE was much more oxidatively stable than PPP + EEE.

The influence of the acyl chain packing arrangements in a cell membrane model has been demonstrated using molecular modeling of DHA containing diacylglycerol (DAG) [60]. In this study, it has been reported that DHA can assume an angle iron shape and a helical structure as the lowest energy conformations. Both structures have nearly straight chain axes formed by methylene carbon alignment. In the angle iron-shaped structure, the carbons of the six double bonds form a line outward from the methylene axis in two nearly perpendicular planes. In the helical structure, the double-bond carbons form a line outward from the axis at nearly 90° intervals. When in these conformations, the DHA hexanes can form tight packing arrangements, especially the angle iron-shaped molecules because of the back-to-back, intermolecular contacts involving these chains. This tight packing conformation has been reported to be strengthened by the presence of different unsaturated fatty acids at the *sn*-2 position of acylglycerols [61,62]. Furthermore, Albrand et al. [63] demonstrated tightly back-folded helical conformations as another packing conformation of DHA. The specific conformations of DHA found in the DAG model are in good agreement with those of the *sn*-2 polyunsaturated phosphoglycerides [64,65].

This theory has been confirmed by quantum mechanical and NMR analyses and molecular dynamics simulations of PCs containing DHA in liposomes. This study indicates a wide variety of DHA conformations, including back-bent, helical, and angle-iron conformations, which occur in aqueous systems [66–69]. The variety of DHA chain conformations can loosen the packing of the membrane at the lipid–water interface and increase water permeability into the lipid bilayers [70,71]. The molecular dynamics simulations also indicated a remarkable overlap of water molecules with the double-bond regions of the DHA chains [68]. Although DHA molecules can assume many different conformations in aqueous systems, such as liposomes, and can show various degrees of flexibility, the tight conformation of the DHA molecule and the presence of water molecules near the DHA molecule are the main preventative mechanisms against hydrogen abstraction at the *bis*-allylic positions that reduce the chain-carrying reaction of lipid oxidation in liposomes.

The characteristic distribution of MHP isomers formed during the oxidation of PA-DHA in liposomes (Table 18.2) suggests steric hindrance toward hydrogen abstraction from DHA molecules [57]. As shown in Table 18.2, in the oxidation of PA-DHA in liposomes, the distributions of DHA-MHP derived from hydrogen abstraction at carbon-18 (16-MHP + 20-MHP) were highest (37.7%),

followed by those at carbon-6 (4-MHP + 8-MHP, 29.1%) and carbon-12 (10-MHP + 14-MHP, 17.6%). The distributions of MHP derived from hydrogen abstraction at carbon-9 (7-MHP + 11-MHP, 8.3%) and those at carbon-15 (13-MHP + 17-MHP, 8.6%) were low. This uneven distribution of MHP in PA-DHA liposomes indicated steric protection against hydrogen abstraction at carbon-9 and carbon-15. In addition, the comparison of two MHP isomer pairs formed by the same hydrogen abstraction, namely, 4- *vs* 8-MHP, 7- *vs* 11-MHP, 10- *vs* 14-MHP, 13- *vs* 17-MHP, and 16- *vs* 20-MHP, showed that the oxygen attack favored the carboxyl terminal side of the pentadienyl radical.

VII. OXIDATION OF PUFA IN HepG2 CELLS

When LA, AA, or DHA were added to the culture medium of HepG2 cells and incubated, a remarkable incorporation of the respective PUFA into cellular TLs was observed [72]. The increase in LA, AA, or DHA was compensated by a decrease in monoenoic fatty acids, such as 18:1n-9 and 18:1n-7. The same change in fatty acid composition was also found in the cellular neutral lipids (NLs) and cellular phospholipids (PLs) [72]. When the peroxidation level in the cells was evaluated by fluorescence analysis [73,74], the incorporation of LA, AA, or DHA enhanced the cellular lipid peroxidation (Figure 18.8) [72]. An increase in membrane phospholipid hydroperoxide after incorporation of LA in HepG2 cells has also been reported by Igarashi and Miyazawa [75]. Conversely, the degree of unsaturation of the added PUFA had only a slight effect on the cellular lipid peroxidation level (Figure 18.8), although the average number of *bis*-allylic positions per 1 mol of fatty acid in cellular PL can be calculated from the fatty acid composition as 0.57 for control cells, 0.64 for LA-supplemented cells, 0.77 for AA-supplemented cells, and 0.81 for DHA-supplemented cells.

Figure 18.8 also shows that the addition of H_2O_2 (0.5 mM) enhanced cellular lipid peroxidation levels in control, LA-, and AA-supplemented cells compared with those without H_2O_2. The enhancement of the lipid peroxidation level by the addition of H_2O_2 has been reported in mouse polymorphonuclear leukocytes [74] and in human monocytic leukemia cells (U-937) [75] over a range of 0.1–1.0 mM of H_2O_2. However, the induction of lipid peroxidation by H_2O_2 was not observed in DHA-supplemented cells. The lower cellular oxidation level in DHA-supplemented cells after H_2O_2 addition was also observed by the analysis of total MHP content in the cellular PL (Table 18.4) [72]. Table 18.4 shows that the main source for MHP was LA in all cases, and a significant amount of

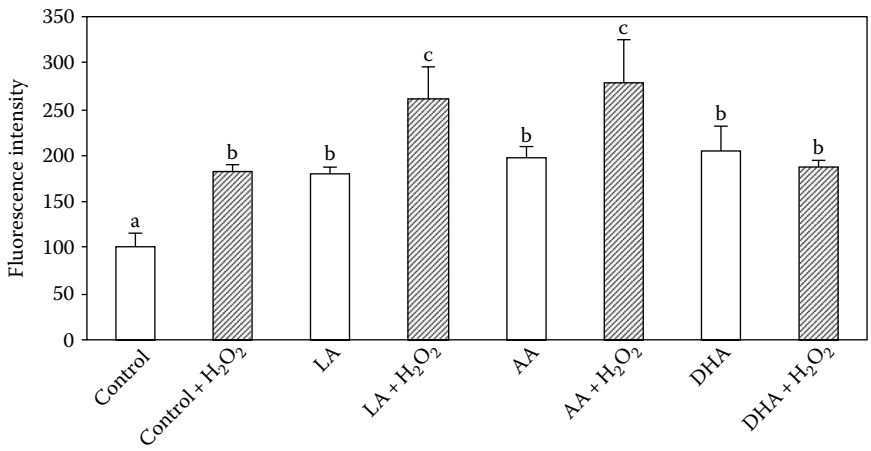

FIGURE 18.8 Formation of MHP in HepG2 cells treated with or without H_2O_2. Values not sharing a common superscript are significantly different at $P < 0.01$. Values are mean ± SD (n-3). (From Araseki, M. et al., *Biosci. Biotechnol. Biochem.*, 69, 483, 2005. With permission.)

TABLE 18.4
Amounts of MHP Formed in the Oxidation of Cellular PL with or without H_2O_2

		Amount of MHP (μmol/mmol Lipid)			
	Cell	**LA-MHP**	**AA-MHP**	**DHA-MHP**	**Total MHP**
Without H_2O_2	Control	1.80 ± 0.43	0.15 ± 0.05	ND	1.95 ± 0.48
	+LA	3.06 ± 0.40	0.17 ± 0.08	ND	3.23 ± 0.35
	+AA	2.17 ± 0.35	1.23 ± 0.11	ND	3.40 ± 0.29
	+DHA	2.37 ± 0.31	0.21 ± 0.06	0.36 ± 0.05	2.93 ± 0.20
With H_2O_2	Control	3.10 ± 0.62	0.32 ± 0.09	ND	3.42 ± 0.71
	+LA	3.50 ± 0.46	0.17 ± 0.06	ND	3.67 ± 0.41
	+AA	2.47 ± 0.47	1.55 ± 0.57	ND	4.02 ± 1.03
	+DHA	2.27 ± 0.47	0.20 ± 0.09	0.33 ± 0.10	2.79 ± 0.65

Source: Araseki, M. et al., *Biosci. Biotechnol. Biochem.*, 69, 483, 2005. With permission.
Note: Data are expressed as mean ± SD (n = 3).
Abbreviation: ND, not detected.

AA-MHP was observed only in AA-supplemented cells. Conversely, a small amount of DHA-MHP was observed in DHA-supplemented cells, but not in other cells. PUFAs containing more than two double bonds, such as AA and DHA, produce further oxidation products from MHP, such as hydroperoxy epidioxides. The relatively lower contribution of AA and DHA to MHP formation found in Table 18.4 might be due to the further oxidation of AA-MHP and DHA-MHP.

A lower cellular oxidation level found in DHA-supplemented cells after H_2O_2 addition may be useful for a better understanding of the *in vivo* lipid oxidation. Several research groups reported that DHA is not always unstable in biological systems. Kubo et al. [20,76,77] found that DHA ingestion did not increase lipid peroxides to the level predicted by the peroxidizability index calculated from the fatty acid composition of the total tissue lipids. This finding has been confirmed by analysis of conjugated diene, chemiluminescence emission, TBARS, and microsomal lipofuscin. Another human study reported no increase in the lipid peroxidation after 9 months of supplementation with 4 g of n-3 PUFA concentrate [78]. Other clinical studies have also reported no significant change in the lipid peroxidation or less oxidative stress following increased consumption of n-3 PUFAs [79–87].

VIII. CONCLUSION

PUFA oxidation in aqueous systems has received significant attention because many lipids coexist with water in foods and biological systems. The aqueous lipid oxidation is often influenced by the interfacial interactions of multiple components, including the lipid substrate, water, emulsifiers, antioxidants, and prooxidants. The research on this interfacial oxidation has shown that multiphase lipid oxidation is affected by not only the chemical reactivity of the substrate lipids but also the physical and/or physicochemical properties of the lipid molecules and other related components.

This chapter shows the characteristic oxidative stability of PUFAs in aqueous systems for a better understanding of the lipid oxidation in foods and in biological systems. In some aqueous systems, such as micelles, microemulsions, and liposomes, the oxidative stability of each PUFA increases as the number of double bonds increases. The order is the reverse of that in the bulk phase and in organic solvents. This can be explained by the specific molecular conformation of PUFA,

especially DHA and EPA, at the lipid–water interface in these aqueous circumstances. The conformation of PUFA is affected by the PUFA structure and the interactions between the PUFA and other molecules. In some cases, such as DHA and EPA in micelles and liposomes, these PUFAs can form a tightly packed conformation with a different ordered arrangement so that the oxidative attack on the PUFA can be prevented by this conformation. The unique conformations and arrangements found in DHA and EPA at interfaces may result in specific interactions between antioxidants and other molecules that further protect against oxidative attack.

Epidemiological, case–control, clinical, and nutrigenetic studies have identified the important cardio-protective effects of EPA and DHA, which are the active forms of omega-3 PUFAs [88–93]. AA has also been known to play an important role in biological systems, such as in the immune response, thrombosis, and brain function [94,95]. In addition, AA and DHA are major constituents of cell membranes and play an important role in the structure of neurons in the central nervous system, where they are present in high concentrations [96]. In contrast, these PUFAs are easily oxidized because of the high degree of unsaturation. Therefore, much attention has been focused on the possible biological systems that protect PUFAs against oxidation. They involve antioxidant systems, such as free radical scavengers, active oxygen scavengers, metal binders, peroxide destroyers, and antioxidant enzymes. Another important factor may be the conformation of PUFAs in biological systems, such as biological membranes. The characteristic oxidative stability of the PUFAs found in aqueous micelles and liposomes suggests that the protection of DHA and EPA against oxidative attack occurs through the specific ordered arrangement of biological membranes.

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19 Methods for Measuring Oxidative Rancidity in Fats and Oils

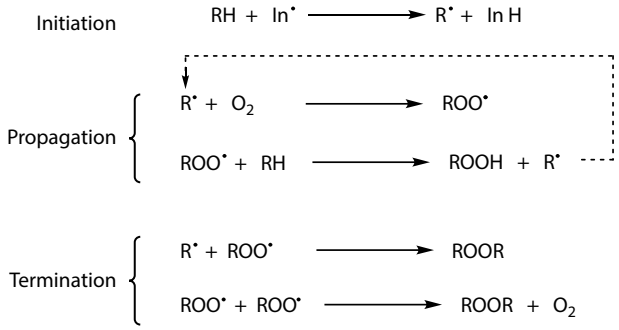
Fereidoon Shahidi, Jiankang Wang, and Udaya N. Wanasundara

CONTENTS

I. INTRODUCTION

Autoxidation is a free radical–induced process that takes place between molecular oxygen and unsaturated fatty acids. Autoxidation of unsaturated fatty acids occurs via a free radical chain mechanism consisting of the basic steps of initiation, propagation, and termination. Initiation starts with the abstraction of a hydrogen atom adjacent to a double bond in a fatty acid (RH) molecule/moiety, and this may be catalyzed by initiators (In·), including light, heat, or metal ions to form a lipid free radical, also named alkyl radical (R·). The resultant alkyl radical reacts with atmospheric oxygen to form an unstable peroxyl radical (ROO·), which may in turn abstract a hydrogen atom from another

unsaturated fatty acid to form a hydroperoxide (ROOH), including conjugated and nonconjugated hydroperoxides, as well as a new alkyl free radical. The new alkyl radical initiates further oxidation and contributes to the chain reaction. The chain reaction (or propagation) may be terminated by the formation of nonradical products resulting from a combination of two radical species.



Transition metals and reactive oxygen species (ROS) are thought to play an important role in initiating free radical autoxidation. Transition metals, such as iron and copper, are capable of initiating lipid oxidation through different mechanisms by either donating an electron or abstracting a hydrogen atom to generate alkyl radicals by reaction with unsaturated fatty acids. Transition metals are also involved indirectly in interacting with triplet oxygen (the normal general ground state of oxygen molecule) to generate a superoxide radical, which leads to the formation of more ROS, such as peroxyl radical anion [1]. In the other pathway, ferrous ions catalyze the conversion of hydrogen peroxide to hydroxyl anion and hydroxyl radical with the production of ferric ion [2]. ROS, such as hydrogen peroxide and hydroxyl radical, also participate in initiating lipid oxidation. Hydrogen peroxide is not capable of reacting directly with unsaturated fatty acids since it is not a strong oxidant, but it can be converted to a hydroxyl radical that attacks unsaturated fatty acids to form alkyl radicals and initiates lipid oxidation [3].

The mechanism of lipid autoxidation has been postulated by Farmer et al. [4], Boland and Gee [5], and Bateman et al. [6]. The propagation step in the autoxidation process includes an induction period when hydroperoxide formation is minimal [7,8]. The rate of oxidation of fatty acids increases in relation to their degree of unsaturation. The relative rate of autoxidation of oleate, linoleate, and linolenate is in the order of 1:40–50:100 on the basis of oxygen uptake and 1:12:25 on the basis of peroxide formation [9]. Therefore, oils that contain relatively high proportions of polyunsaturated fatty acids (PUFAs) may experience stability problems. The breakdown products of hydroperoxides, such as alcohols, aldehydes, ketones, and hydrocarbons, generally possess offensive off-flavors. These compounds may also interact with other food components and change their functional and nutritional properties [10].

II. MEASUREMENT OF OXIDATIVE RANCIDITY

There are various methods available for measurement of lipid oxidation in foods. Changes in chemical, physical, or organoleptic and sensory properties of fats and oils during oxidation may be monitored to assess the extent of lipid oxidation. However, there is no uniform and standard method for detecting all oxidative changes in all food systems. The available methods to monitor lipid oxidation in foods and biological systems may be divided into two groups. The first group measures primary oxidative changes and the second determines secondary changes that occur in each system.

A. Primary Changes

1. Changes in Reactants

Methods that measure primary changes of lipids may be classified as those that quantify loss of reactants (unsaturated fatty acids). Measurement of changes in fatty acid composition is not widely

used in assessing lipid oxidation because it may require total lipid extraction from food and subsequent conversion to derivatives suitable for gas chromatographic analysis. Separation of lipids into neutral, glycolipid, phospholipid, and other classes may also be necessary. However, it has been proven that this method serves as a useful technique to identify different classes of lipids and fatty acids that are involved in the oxidative changes [11,12] and also to assess lipid oxidation induced by different metal complexes that afford a variety of products [13]. On the other hand, changes in fatty acid composition cannot be used in more saturated oils because this indicator reflects only the changes that occur in unsaturated fatty acids during oxidation [14]. Therefore, the oxidative changes in marine oils and highly unsaturated vegetable oils may be monitored using this indicator. Similarly, changes in iodine value due to loss of unsaturation during accelerated oxidation studies may be used as an index of lipid oxidation [15].

2. Weight Gain

It is generally accepted that addition of oxygen to lipids and formation of hydroperoxides are reasonably quantitative during the initial stages of autoxidation. Therefore, the measurement of induction period from weight gain data is theoretically sound. In this method, oil samples (about 2.0 g) are weighed into Petri dishes; then traces of water are removed by placing the samples overnight in a vacuum oven at 35°C and over a desiccant. The samples are then reweighed and stored in an oven at a set temperature. The weight gain of the samples may be recorded at different time intervals.

This method is most suitable for evaluation of marine and vegetable oils that contain high PUFA content [16]. Olcott and Einset [17] reported that marine oils exhibit a fairly sharp increase in their weight at the end of the induction period and are rancid by the time they gain 0.3%–0.5% in weight (at 30°C–60°C). Ke and Ackman [18] reported that this method is simple, has a satisfactory reproducibility, and may be used to compare oxidation of lipids from different parts of fish. Wanasundara and Shahidi [19,20] used this method to compare the storage stability of vegetable (Figure 19.1)

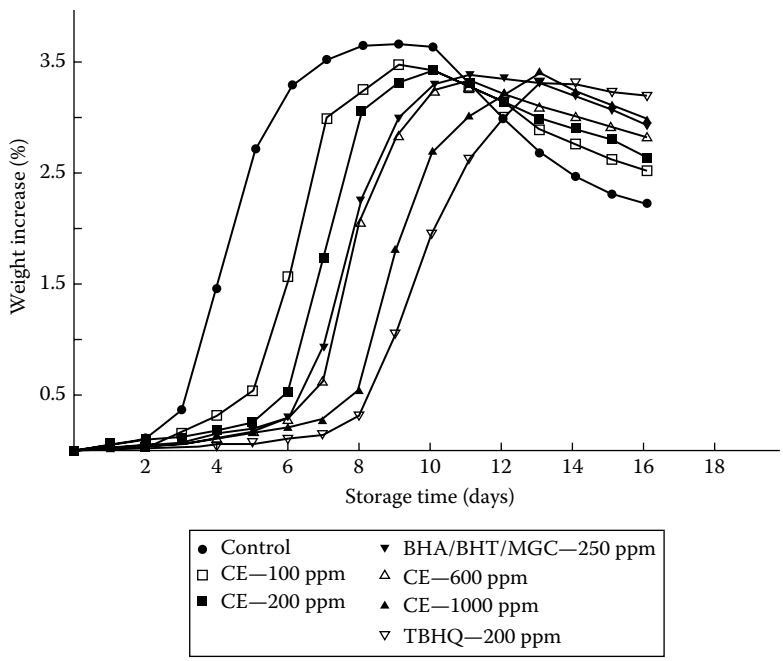


FIGURE 19.1 Effect of canola extracts and commercial antioxidants on the weight gain of canola oil stored at 658°C. BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; MGC, monoacylglycerol citrate; TBHQ, *tert*-butylhydroquinone.

and marine oils as affected by added antioxidants and were able to compare the relative activity of antioxidants employed. However, surface exposure of the sample to air is an important variable in determining the rate of oxidation. Therefore, use of equal-size containers to store samples is essential when carrying out such experiments.

The weight gain method also suffers from certain disadvantages: (1) the weighing frequency hinders monitoring of fast kinetics (a higher frequency would involve nocturnal weighing), and low or moderate temperatures require long analysis times for stable samples; (2) discontinuous heating of the sample (which must be cooled before weighing) may give rise to nonreproducible results, so the heating and cooling intervals must be accurately controlled; (3) the method involves intensive human participation; and (4) the working conditions (sample size, shape of container, and temperature) may influence the results. Extension of this technique to the more sophisticated continuous monitoring of mass and energy changes as in thermogravimetry/differential scanning calorimetry (DSC) has been suggested [21]. Nevertheless, this method offers advantages, such as low instrumentation cost as well as unlimited capacity and speed for sample processing.

3. Hydroperoxides

In the oxidation of fats and oils, the initial rate of formation of hydroperoxides exceeds their rate of decomposition, but this is reversed at later stages [22]. Therefore, monitoring the amount of hydroperoxides as a function of time indicates whether a lipid is in the growth or decay portion of the hydroperoxide concentration curve. This information can be used as a guide for considering the acceptability of a food product with respect to the extent of product deterioration. By monitoring the induction period before the appearance of hydroperoxides, one can assess the effectiveness of added antioxidants on the stability of a food lipid.

4. Peroxide Value

The classical method for quantification of hydroperoxides is the determination of peroxide value (PV). The hydroperoxide content, generally referred to as PV, is determined by an iodometric method [23]. This is based on the reduction of the hydroperoxide group (ROOH) with iodide ion (I^-). The amount of iodine (I_2) liberated is proportional to the concentration of peroxide present. Released I_2 is assessed by titration against a standardized solution of sodium thiosulfate ($Na_2S_2O_3$) using a starch indicator. Chemical reactions involved in PV determination are as follows:

$$ROOH + 2H^+ + 2KI \rightarrow I_2 + ROH + H_2O + 2K^+$$

$$I_2 + 2Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2NaI$$

Potential drawbacks of this method are absorption of iodine at unsaturation sites of fatty acids and liberation of iodine from potassium iodide by oxygen present in the solution to be titrated [24]. The results may also be affected by the structure and reactivity of peroxides as well as reaction temperature and time. The iodometric method for determination of PV is applicable to all normal fats and oils, but it is highly empirical and any variation in procedure may affect the results. This method also fails to adequately measure low PV because of difficulties encountered in determination of the titration end point. Therefore, the iodometric titration procedure for measuring PV has been modified in an attempt to increase the sensitivity for determination of low PV. The modification involves the replacement of the titration step with an electrochemical technique in which the liberated iodine is reduced at a platinum electrode maintained at a constant potential. PV ranging from 0.06 to 20 meq/kg has been determined in this manner, but it is essential to deaerate all solutions to prevent further formation of peroxides. PVs higher than 10 meq/kg are considered unacceptable [25].

Several other chemical methods have also been suggested for monitoring PV. Colorimetric methods based on the oxidation of Fe^{2+} to Fe^{3+} and determination of Fe^{3+} as ferric thiocyanate, and a 2,6-dichlorophenol-indophenol procedure are reported in the literature [26]. The ferrous oxidation

method is less susceptible to oxidation compared to the iodometric method as ferrous ion possesses better tolerance to oxidation than iodide ion [27]. In studies on the oxidation of biological tissues and fluids, measurement of fatty acid hydroperoxides is more common than measurement of their decomposition products. Fatty acid hydroperoxides can be analyzed by high-performance liquid chromatography (HPLC) or their corresponding hydroperoxy acid reduction products may be determined by gas chromatography–mass spectrometry (GC-MS) [28]. Fluorescence methods have also been developed to determine hydroperoxides by allowing them to react with substances such as luminol and dichlorofluorescein, which form fluorescent products [24]. Although determination of PV is common, its usefulness is generally limited to the initial stages of lipid oxidation.

5. Active Oxygen and Oil Stability Index/Rancimat Methods

The active oxygen method (AOM), also referred to as the Swift test of the American Oil Chemists' Society (AOCS), is a common accelerated method used for assessing the oxidative stability of fats and oils (AOCS, 12–57) [23]. This method is based on the principle that aging and rancidification of a fat is greatly accelerated by aeration in a tube held at a constant elevated temperature. In this method, air is bubbled through a heated oil at 98°C–100°C for different time intervals and the PVs are determined. The PVs are then plotted against time, and the induction period determined from the graph. Even though this method has been used extensively over the years, its inherent deficiencies and difficulties have also been identified. These include the following: (1) the end point is determined by the amount of peroxides in the oxidized oil and (2) peroxides are unstable and decompose readily to more stable secondary products. During the rapid oxidation phase, the reaction is extremely susceptible to variations in the oxygen supply. Automated versions of the AOM apparatus, known as the oil stability instrument (OSI), and Rancimat have been developed. The Rancimat method uses a commercial apparatus marketed by Metrohm Ltd. (Herisau, Switzerland). The OSI, a computer-assisted instrument developed by Archer Daniels Midland (ADM), is now produced commercially by Omnion Inc. (Rockland, MA). This method provides the oil stability index value, which is defined as the point of maximal change of oxidation rate [16]. These methods may be considered as automated AOM since both employ the principle of accelerated oxidation. However, the OSI and Rancimat tests measure the changes in conductivity caused by ionic volatile organic acids, mainly formic acid, automatically and continuously, whereas in the AOM PVs are determined. Organic acids are stable oxidation products that are produced when an oil is oxidized by a stream of air bubbled through it. In the OSI and Rancimat methods, oxidation proceeds slowly at first because formic acid is released slowly during the induction period. The end point is selected where the rapid rise in conductance begins. The OSI method has been used as standard industrial practice for assessment of the oxidative stability of oils as well as lipid oxidation inhibition effectiveness of antioxidants. However, it may not be suitable for oils that contain thermally sensitive antioxidants and when volatile antioxidants are used [29].

In addition, oxygen consumption can be measured by directly monitoring the decrease in oxygen pressure, and instruments that monitor the drop in the overhead pressure of an oil during heating might be used. An example of this sort of equipment is the Oxidograph, which is commercially produced by Mikrolab (Aarhus, Denmark). In Oxidograph, a sample of oil or fat is exposed to oxygen or air at elevated temperatures. Heating is done in an aluminum block. As the sample absorbs oxygen, the pressure change in the reaction vessel is measured electronically by means of pressure transducers. The rate of oxygen consumption during the early stages of storage of lipids also provides an ideal parameter for shelf life prediction. Wewala [30] used this method for prediction of shelf life of dried whole milk and found a very good correlation between the headspace oxygen content and storage time (Figure 19.2).

Oxygen consumption can also be measured electrochemically by monitoring the changes in oxygen concentration. A proposed semiautomatic polarographic method that is based on the use of two oxygen meters with microcathode oxygen electrodes, coupled to a computerized data collection and processing unit, can be used to monitor oxygen consumption during lipid oxidation with improved performance [31,32].

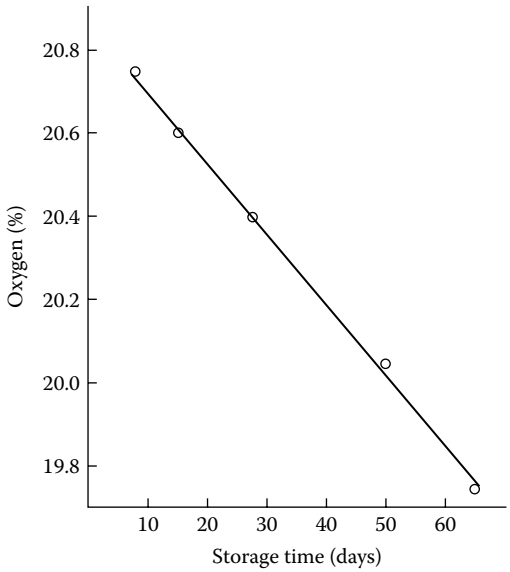


FIGURE 19.2 Change of the headspace oxygen content of stored dried whole-milk samples.

6. Differential Scanning Calorimetry

DSC has been employed to evaluate the oxidative stability of fats and oils as thermally activated reactions are involved during autoxidation [33]. Both isothermal and nonisothermal conditions have been used in the calorimetric techniques to measure the heat released from a particular reaction when oxidation occurs [34]. The heat released by the oxidized oil is recorded as the heat flow signal as a function of temperature [35]. The heat flow signal is maintained the same during the induction period of oxidation, and the length of the induction period is generally an indicator of the oil stability. There is no chemical reaction during the induction period. Shortly after the heat flow signal separates from the baseline, the initiation phase of oxidation begins. The sudden increase in the heat flow signal implies the start of the propagation phase of oxidation. The termination phase is illustrated in the diagram when stable products are formed.

Despite the high-cost, application range limitation by the column capacity of the sample cell, DSC is a simple, fast, solvent-free, and green analytical method that can be used to assess the lipid oxidation reactions [34].

DSC specifically measures the temperature and heat flows associated with lipid oxidation as a function of time and temperature and can provide unique energy profile information [36]. It has been reported that the DSC technique, which is based on thermal release of oxidation reactions, may be used as a nonchemical method for assessing effectiveness of antioxidants on the oxidative stability of fats and oils in their model systems [37]. A microcalorimetry technique has also been developed to provide more versatility than the conventional DSC, which can examine up to three samples at the same time [34].

7. Conjugated Dienes

Oxidation of PUFAs is accompanied by an increase in the ultraviolet (UV) absorption of the product. Lipids containing methylene-interrupted dienes or polyenes show a shift in their double-bond position during oxidation due to isomerization and conjugate formation [38]. The resulting conjugated dienes exhibit an intense absorption at 234 nm; similarly, conjugated trienes absorb at 268 nm [25].

Farmer and Sutton [39] indicated that the absorption increase due to the formation of conjugated dienes and trienes is proportional to the uptake of oxygen and formation of peroxides during the early stages of oxidation. St. Angelo et al. [40] studied the autoxidation of peanut butter by

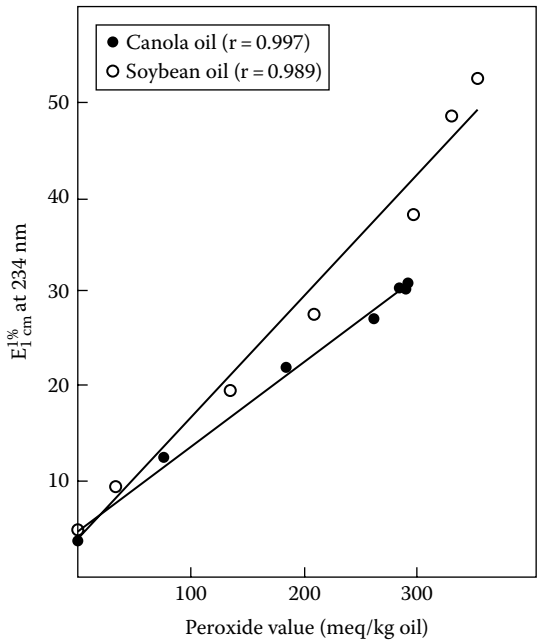


FIGURE 19.3 Relationship between PVs and conjugated diene values of oxidized vegetable oils.

measuring the PV and absorption increase at 234 nm due to the formation of conjugated dienes. Shahidi et al. [41] and Wanasundara et al. [42] found that conjugated dienes and PV of marine and vegetable oils correlate well during their oxidation (Figure 19.3). These authors concluded that the conjugated diene method may be used as an index of stability of lipids in place of, or in addition to, PV. However, carotenoid-containing oils may give high absorbance values at 234–236 nm because of the presence of double bonds in the conjugated structure of carotenoids. The conjugated diene method is faster than PV determination, is much simpler, does not depend on chemical reactions or color development, and requires a smaller sample size. However, the presence of compounds absorbing in the same region may interfere with such determination.

Parr and Swoboda [43] have described an alternate method to determine lipid oxidation of stored oils. In this assay, hydroperoxides of polyenoic fatty acids as well as hydroxy and carbonyl compounds derived from them are converted to more conjugated chromophores by two chemical reaction steps, namely, reduction and then dehydration (Figure 19.4). These yield conjugatable oxidation products (COPs), which are measured and expressed as COP values. The first step of the analytical procedure involves reduction of the carbonyl group by sodium borohydride, which results in the disappearance of the characteristic UV absorption of the carbonyl compounds of oxidized polyenoic fatty acids (oxodienes). The decrease in the absorption at 275 nm is known as oxodiene value. The next step of the COP assay involves changes in the spectrum of the reduced compound to its dehydrated counterpart, which exhibits absorption maxima at 268 and 301 nm. The sum of these absorbance changes at 268 and 301 nm yields the COP value, whereas their relative proportions define the COP ratio. For the calculation of oxodiene and COP results, the concentration of the final lipid solution also has to be taken into account.

B. Secondary Changes

The primary oxidation products (hydroperoxides) of fats and oils are transitionary intermediates that decompose into various secondary products. The decomposition pathway of hydroperoxides is likely to be a homolytic cleavage between oxygen and the oxygen bond, in which alkoxyl and

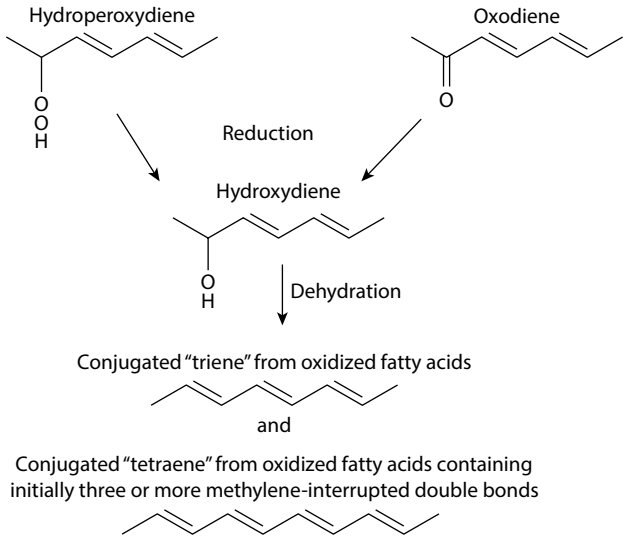


FIGURE 19.4 Chemical reaction steps in the assay of conjugable oxidation products.

hydroxyl radicals are produced [1]. Measurement of secondary oxidation products as indices of lipid oxidation is more appropriate since the secondary products of oxidation are generally odor-active, whereas primary oxidation products are colorless and flavorless. Secondary oxidation products include aldehydes, ketones, hydrocarbons, and alcohols, among others. The following sections describe common methods used for measuring the secondary oxidation products of lipids.

1. 2-Thiobarbituric Acid Value

One of the oldest and most frequently used tests for assessing lipid oxidation in foods and other biological systems is the 2-thiobarbituric acid (TBA) test. The extent of lipid oxidation is reported as the TBA value and is expressed as milligrams of malondialdehyde (MDA) equivalents per kilogram sample or as micromoles of MDA equivalents per gram sample. MDA is a relatively minor product of oxidation of PUFA that reacts with the TBA reagent to produce a pink complex with an absorption maximum at 530–535 nm [16,44]. The adduct is formed by condensation of two molecules of TBA with one molecule of MDA (Figure 19.5). Other products of lipid oxidation, such as 2-alkenals and 2,4-alkadienals, also react with the TBA reagent. However, the exact mechanism of their reaction with the TBA reagent is not well understood. There are several procedures for the determination of TBA values. The TBA test may be performed directly on the sample, its extracts, or distillate. In case of the distillation method, volatile substances are distilled off with steam. Then the distillate is allowed to react with the TBA reagent in an aqueous medium. The advantage of the distillation method is the absence of interfering substances. In the extraction method, TBA-reactive substances (TBARS) are extracted from food material into an aqueous medium (i.e., aqueous trichloroacetic acid) prior to color development with the TBA reagent. The main disadvantages of both of these

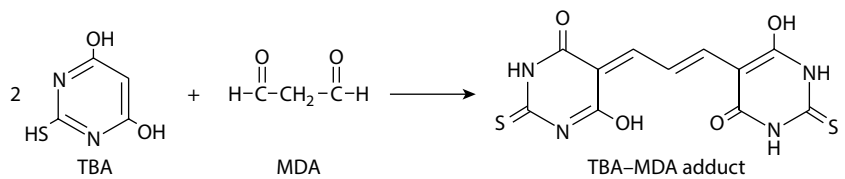


FIGURE 19.5 Reaction of TBA and MDA.

methods are long assay time and possibility of artifact formation. In the direct assay method, lipid sample (oil) reacts with the TBA reagent, and the absorbance of the colored complex so prepared is recorded. The direct assay method is simple and requires less time for sample preparation.

Despite the fact that the TBA test is one of the most commonly used tests for lipid oxidation assessment, there are certain limitations when using the TBA test for evaluation of the oxidative state of foods and biological systems because of their chemical complexity. Dugan [45] reported that sucrose and some compounds in wood smoke react with the TBA reagent to give a red color that interferes with the TBA test. Baumgartner et al. [46] also found that a mixture of acetaldehyde and sucrose when subjected to the TBA test produced a 532 nm absorbing pigment identical to that produced by MDA and TBA. Modifications of the original TBA test have been reported by Marcuse and Johansson [47], Ke and Woyewoda [48], Robbles-Martinez et al. [49], Pokorny et al. [50], Shahidi et al. [51,52], Thomas and Fumes [53], and Schmedes and Holmer [54]. However, it has been suggested that TBARS produce an excellent means for evaluating the relative oxidative state of a system as affected by storage condition or process variables [55]. Nonetheless, it is preferable to quantify the extent of lipid oxidation by a complementary analytical procedure in order to verify the results.

Several attempts have been made to establish a relationship between TBA values and the development of undesirable flavors in fats and oils. It has been shown that flavor threshold values correlate well with the TBA results of vegetable oils, such as those of soybean, cottonseed, corn, safflower [24], and canola [8].

2. Oxirane Value

The oxirane oxygen or epoxide groups are formed during autoxidation of fats and oils. It has been reported that epoxides are formed as a result of the rearrangement of alkoxyl radicals or the intramolecular radical substitution with peroxides, or they are generated from peroxyl radicals directly [56–58]. The epoxide content is determined by titrating the oil sample with hydrobromic acid (HBr) in acetic acid and in the presence of crystal violet, to a bluish-green end point. This method has been standardized by the AOCS in their tentative method (Cd 9–57) [23], but it is not sensitive and lacks specificity. HBr may also attack α, β-unsaturated carbonyls and conjugated dienals, and the reaction is not quantitative with some *trans*-epoxides. Fioriti et al. [59] found that picric acid was the best of several acidic chromophores in its reaction with epoxides. Despite a nonquantitative reaction, the product concentration followed Beer's law. This method has been found to be particularly well suited for the determination of epoxides in heated fats and oils, where the oxirane content is often less than 0.1%.

A quantitative method to determine epoxide concentrations in oxidized oils was developed and validated using proton nuclear magnetic resonance (NMR) [60]. The one-step sample preparation method allows a rapid determination of epoxides at low levels without any addition of external references. Epoxides derived from lipid oxidation gave signals between 2.90 and 3.24 ppm, which are separated from the signals of other lipid oxidation products. When proton NMR signals were compared to the epoxide content determined by the titration method, the results of this method are comparable to those of the titration method [60].

3. *p*-Anisidine Value

p-Anisidine value (*p*-AnV) is defined as 100 times the optical density measured at 350 nm in a 1.0 cm cell of a solution containing 1.0 g of oil in 100 mL of a mixture of solvent and reagent, according to the IUPAC method [61]. This method determines the amount of aldehydes (principally 2-alkenals and 2,4-alkadienals) in animal fats and vegetable oils. Aldehydes in an oil react with the *p*-anisidine reagent under acidic conditions. Later, the AOCS method (Cd 18–90) has been employed as a standard method for anisidine analysis [23]. The reaction of *p*-anisidine with aldehydes affords yellowish products, as shown in Figure 19.6. List et al. [62] reported a highly significant correlation between *p*-AnV and flavor acceptability scores of salad oils processed from undamaged soybeans.

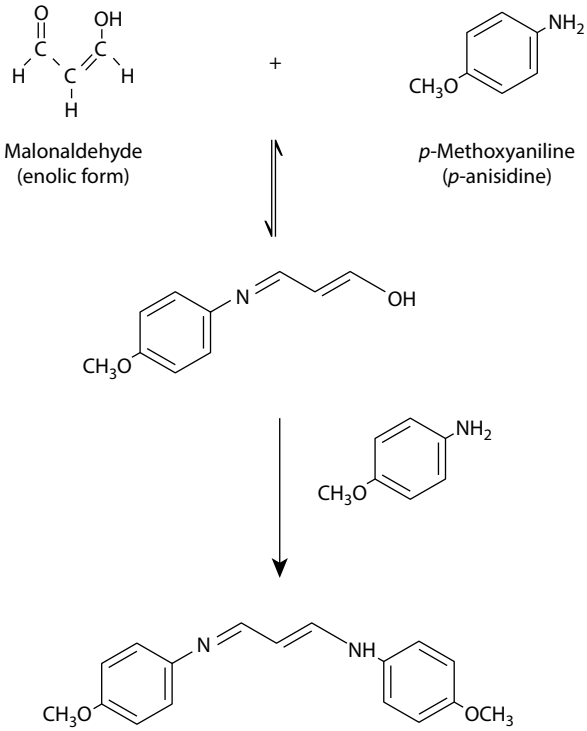


FIGURE 19.6 Possible reactions between *p*-anisidine reagent and MDA.

In addition, a highly significant correlation between *p*-AnV and PV has been reported, but that may depend on storage time, temperature, etc. [62].

The anisidine test can be used as a rough predictor for the future storage stability of freshly processed oils [63]. This test has been reported to be less sensitive to saturated aldehydes in comparison to unsaturated aldehydes as the resultants formed from unsaturated aldehydes have strong absorbance at the chosen wavelength [64].

4. TOTOX Value

The *p*-AnV is often used in the industry in conjunction with PV to calculate the so-called total oxidation or TOTOX value:

$$\text{TOTOX value} = 2\text{PV} + p\text{-AnV}$$

The TOTOX value is often considered to have the advantage of combining evidence about the history of an oil (as reflected in the *p*-AnV) with its present state (as evidenced in the PV). Therefore, determination of TOTOX value has been carried out extensively to estimate oxidative deterioration of food lipids [65]. However, despite its practical advantages, TOTOX value does not have any sound scientific basis because it combines variables with different dimensions. Wanasundara and Shahidi [66] defined $\text{TOTOX}_{\text{TBA}}$ as 2PV + TBA since determination of *p*-AnV may not be always feasible.

5. Octanoate

The test was developed to investigate decomposition products of hydroperoxides, and these products are the residual esters that remained attached to triacylglycerol (TAG) after thermal cleavage of oxidized TAGs [63]. As an important product from the cleavage of hydroperoxides of fatty acid esters,

methyl octanoate may be analyzed by GC after being purified from the oxidation products through high heat-induced decomposition. The octanoate assay offers a measure of the level of the oxidation of crude oils and oils at different stages of processing before deodorization is applied. The method is believed to be a more specific method than the *p*-Anisidine test [63].

6. Carbonyls

An alternative approach for monitoring the extent of lipid oxidation in fats and oils is to measure the total or individual volatile carbonyl compounds formed from degradation of hydroperoxides. One of the more reliable methods for total carbonyl analysis is based on the absorbance of the quinoidal ion, a derivative of aldehydes and ketones. This ion is formed from the reaction of 2,4-dinitrophenylhydrazine (2,4-DNDH) with an aldehyde or ketone, followed by the reaction of the resulting hydrozones with an alkali (Figure 19.7), which is then analyzed spectrophotometrically at a given wavelength. Many variations of this method have been reported [67,68]. Each method offers an alternative solvent, wavelength, or workup to analyze the quinoidal ion. When it is used in the food model system, one of the disadvantages of this method is that carbonyl produced from oxidation of other components such as proteins may also lead to a colorimetric reaction and contribute to higher carbonyl values than expected [16]. In addition, this method is sensitive to high temperatures as some of the short-chain carbonyls can be volatilized and removed by heat; thus, the precision of the assessment may be affected.

The analysis of individual carbonyl compounds is another method that has gained popularity. Hexanal, one of the major secondary products formed during the oxidation of linoleic or other ω-6 fatty acids in lipid-containing foods [69,70], has been used to follow lipid oxidation. Shahidi and Pegg [69] reported a linear relationship between hexanal content, sensory scores, and TBA numbers of cooked ground pork, whereas St. Angelo et al. [71] established a similar correlation for cooked beef. O'Keefe et al. [72] also used hexanal as an indicator to assess the oxidative stability of meat from broiler chickens that were fed fish meal. Supplementation of high amounts of fish meal to the diet increased the hexanal content of the thigh meat during storage. However, other studies have shown that during oxidation of marine oils, which are rich in the PUFA of the ω-3 type, large amounts of propanal are formed and that a good correlation exists between the content of propanal and the amount of TBARS in such samples [73,74]. Therefore, it is essential to use appropriate indicators when assessing the stability of food lipids. We recommend that hexanal be used when oils under investigation are rich in ω-6 fatty acids while propanal would serve as a reliable indicator when oils high in ω-3 fatty acids are being considered.

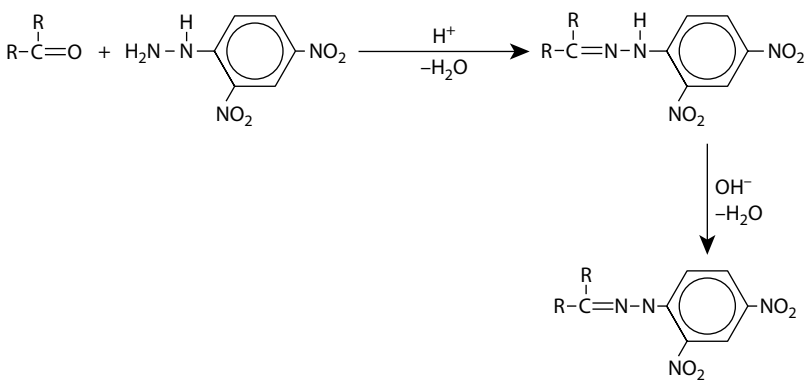


FIGURE 19.7 Reaction steps in the production of hydrozones from carbonyls and 2,4-dinitrophenylhydrazine.

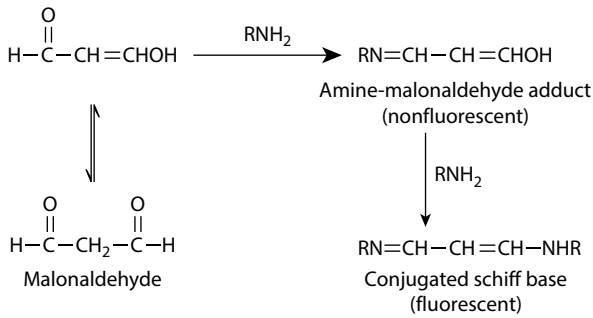


FIGURE 19.8 Production of fluorescent chromophores from the reaction of lipid oxidation products and amines.

7. Hydrocarbons and Fluorescent Products

Studies of oxidized methyl linoleate and soybean oil [75] have revealed that saturated hydrocarbons could be detected when aldehydes are either absent or undetectable. Snyder et al. [76] have reported that ethane, propane, and pentane are predominant short-chain hydrocarbons formed through thermal decomposition of soybean oil. Correlations of flavor acceptability scores and pentane content, determined by GC techniques, have been used to assess rancidity of fats and oils [77]. Significant correlations existed between the amount of pentane produced and the number of rancid descriptions of stored vegetable oils [78]. The correlation of headspace pentane concentrations and sensory scores of stored freeze-dried pork samples was reported by Coxon [11].

Another secondary change that occurs during autoxidation of biological systems is the formation of fluorescent products from the reaction of MDA with amino compounds such as proteins and nucleotides [79]. This method has been used to determine the extent of lipid oxidation in biological tissues. It has been established that fluorescent compounds with a general structure of 1-amino-3-iminopropane may develop through the reaction of an amino group with carbonyl compounds, mainly MDA [80–82] (Figure 19.8).

Kikugawa and Beppu [83] reported that the development of fluorescence depends not only on the formation of condensation products between MDA and free amino groups but also on the nature of the substituents of the latter compounds. Different excitation and emission maxima were observed for different condensation products. Advantages of the fluorescence method as a means of measuring lipid oxidation have been reported by Dillard and Tappel [81]. This method can detect fluorescent compounds at concentrations as low as parts per billion levels and is found to be 10–100 times more sensitive than the TBA assay.

The fluorescence method is a sensitive measure that is suitable for analyzing the oxidative deterioration in fish and other meat products when carbonyl compounds are difficult to be measured in these materials [63].

III. MEASUREMENT OF FRYING FAT DETERIORATION

Deep fat frying is a popular method for food preparation, especially in fast food restaurants. Although vegetable oils are used primarily as a heat exchange medium for cooking, when used for deep frying, they contribute to the quality of fried products. In the process of deep fat frying, a complex series of chemical reactions take place. These reactions are characterized by a decrease in the total unsaturation content of the fat with a concurrent increase in the amount of free fatty acids, cyclic fatty acids (Figure 19.9), foaming, color, viscosity, and formation of polar matter and polymeric compounds. As these reactions proceed, the functional, sensory, and nutritional quality of frying fats change and may reach a point where high-quality foods can no longer be prepared. Therefore, it is essential to determine when the frying fat is no longer usable.

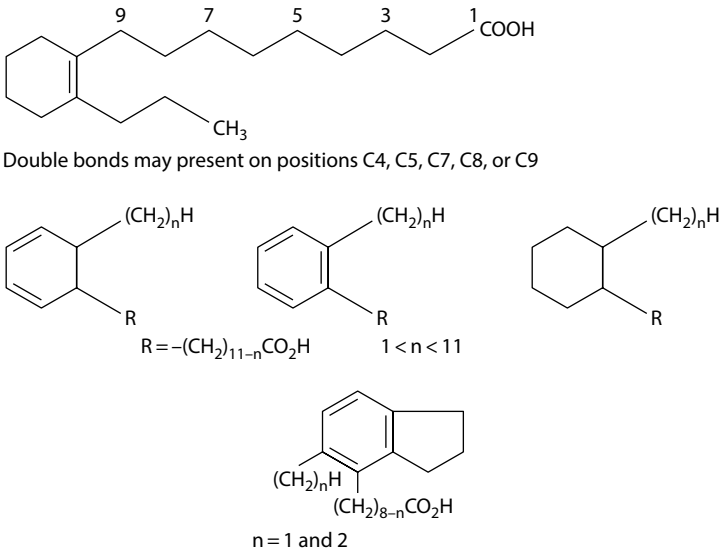


FIGURE 19.9 Structures of cyclic fatty acids formed during frying.

Quality evaluation of frying fats may be carried out in many ways. The first attempt to define a deteriorated frying fat was made by the German Society for Fat Research in 1973. It recommended that "a used frying fat is deteriorated if, without doubt, its odor and taste were unacceptable; or if in case of doubtful sensory assessment, the concentration of petroleum ether–insoluble oxidized fatty acids in it was 0.7% or higher and its smoke point was lower than 170°C; or if the concentration of petroleum ether–insoluble oxidized fatty acids was 1.0% or higher." Although sensory evaluation of foods is the most important quality assessment, taste evaluations are not practical for routine quality control. It is always preferred to have a quantitative method for which rejection point could be established by sensory means. PVs provide an indication of frying fat quality if they are used in a very specific way. However, peroxides generally decompose at about 150°C; hence, at frying temperatures (usually 180°C–190°C), no accumulation of peroxides occurs. Free fatty acids from frying fats can be determined by direct titration with a standardized base in ethanol. Fritsch [84] has shown that in most deep fat frying operations, the amount of free fatty acids produced by hydrolysis is too small to affect the quality of foods. However, industrially, frying oil quality is usually checked by the measurement of color or free fatty acid content in order to tell an operator when a fat is ending its useful life. The foaming characteristics of used fats would also lead one to the same conclusion.

A quick colorimetric test kit is now available for measuring oil quality [85]. Blumenthal et al. [86] developed a spot test to measure free fatty acids in which drops of used fat are placed on a glass covered with silica gel containing a pH indicator in order to give a three-color test scale of blue, green, and yellow. This may indicate the amount of free fatty acids in a sample. Northern States Instrument Corp. (Lino Lakes, MN) has developed an instrument that measures the dielectric constant of insulating liquids. The instrument is a compact unit, relatively inexpensive, simple to operate, and requires only a few drops of oil for each measurement. For evaluation of frying fats, the instrument must be calibrated first with a fresh oil sample prior to its use in frying operations.

Determination of total polar matter in frying fats has emerged as a reliable method for assessing the useful life of fats and oils subjected to frying and is an official method in Europe. Total polar matter is determined by dissolving the fat in a relatively nonpolar solvent, such as toluene or benzene, and running through a silica gel column that adsorbs the polar compounds. After evaporation of the solvent, the nonpolar fat can be weighed, and the total polar matter calculated from the weight difference data or determined directly by their elution from the column with diethyl ether or a

mixture of chloroform and methanol. Sebedio et al. [87] illustrated that polar and nonpolar fractions of fried oils can be quantitatively estimated using Iatroscan thin-layer chromatography–flame ionization detection (TLC-FID) system with Chromarod SII. This method requires a very small sample and is much faster than silicic acid column separation.

IV. RECENT DEVELOPMENTS FOR QUANTITATION OF LIPID OXIDATION

A. Electron Spin Resonance Spectroscopy

Lipid oxidation in foods and biological systems has conventionally been tested by monitoring either primary or secondary oxidation products. Over the last three decades, advances in pulse radiolysis [88] and electron spin resonance (ESR) [89] techniques have facilitated the detection and study of short-lived free radical intermediates. Not only has ESR spectroscopy been widely used to study lipid oxidation in animal tissues and other biological systems, but it is also an important method to evaluate the early stages of lipid oxidation and predict the oxidative stability of fats and oils as well as examination of antioxidant activity [16]. ESR spectroscopy allows selective detection of free radicals. The technique depends on the absorption of microwave energy (which arises from the promotion of an electron to a higher energy level) when a sample is placed in a variable magnetic field. A major limitation in the detection of free radicals by ESR is that the minimum detectable radical concentration should be higher than 10^{-9} M under the optimal conditions [90]. Radical lifetimes in solution are very short (<1 ms), and steady-state concentrations generally remain well below 10^{-7} M. Several approach efforts have been made to overcome this problem, either by enhancing the rate of radical production or by diminishing the rate of its disappearance. These techniques include pulse radiolysis, UV photolysis, continuous flow system rapid freezing, lyophilization, or spin trapping [90,91]. Spin trapping has been considered to be the most effective means as it enhances the detectable concentration of long-lived radicals when an added spin-trapping agent reacts with free radicals to form more stable spin adducts [90,91]. Nitroso compounds and nitrones are the most common spin traps, both leading to nitroxyl-type spin adducts when reacted with free radicals. Although application of ESR spectroscopy as a precise method to study lipid oxidation in animal tissues and other biological systems is commonplace, its application to foods is relatively new.

Yen and Duh [92] and Chen and Ho [93] have reported that inhibition of free radical formation by different antioxidants can be measured using very stable free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH). The mechanism of the reaction of an antioxidant with DPPH radical is as follows:

$$(\text{DPPH}) + \text{HO–R–OH} \rightarrow (\text{DPPH}){:}\ \text{H} + \text{HO–R–O}\cdot$$

$$\text{HO–R–O}\cdot + (\text{DPPH}) \rightarrow (\text{DPPH}){:}\ \text{H} + \text{O=R=O}$$

DPPH radical, with a deep violet color, receives a hydrogen atom from the antioxidant and is converted to a colorless molecule. Using this reagent, the free radical–scavenging ability of the antioxidant can be determined by spectrophotometric methods. The antioxidant activity of different antioxidants or antioxidant extract from the oil can be assessed by examining their radical-scavenging capacity, which then can be used to evaluate the role of antioxidant components in the oxidative stability of oils [94].

B. Infrared Spectroscopy

Infrared (IR) spectroscopy has also been used for measurement of rancidity, and it is of particular value in recognition of unusual functional groups and in studies of fatty acids with *trans* double bonds. Production of hydroperoxides during oxidation of lipids gives rise to an absorption band

at about 2.93 μm, whereas the disappearance of a band at 3.20 μm indicates the replacement of a hydrogen atom on a double bond, or polymerization. It has also been suggested that the appearance of an additional band at 5.72 μm, due to C=O stretching, indicates the formation of aldehydes, ketones, or acids. Furthermore, changes in the absorption bands in the 10–11 μm region indicate *cis–trans* isomerization and probably formation of conjugated bonds. Determination of oxidative deterioration of lipids using the IR method is simple and rapid and requires small amounts of sample (20 mg).

Van de Voort et al. [95] and Sedman et al. [96] investigated the feasibility of employing Fourier transform infrared (FTIR) spectroscopy to assess the oxidative status or to forecast the oxidative stability of oils. These authors constructed a spectral library by recording the FTIR spectra of oils spiked with various compounds representative of common oil oxidation products. Table 19.1 shows that each of the various types of oxidation products gives rise to discernible and characteristic absorptions in the FTIR spectrum. Similar absorption bands were detected in the spectra of oils oxidized under accelerated conditions and monitored in real time by FTIR spectroscopy. On the basis of the results of this study, the authors proposed a quantitative approach whereby the oxidative status of oils could be determined through calibrations developed with oils spiked with appropriate compounds representative of the functional groups associated with typical oxidative end products. These concepts were subsequently put into practice with the development of a calibration for the determination of PV. A similar approach may be used to develop a parallel method for evaluating *p*-anisidine values.

TABLE 19.1
Peak Positions of the Functional Group Absorptions of Reference Compounds Representative of Products Formed in Oxidized Oils

Compounds	Vibration	Frequency (cm^{-1}) at Peak Maximum
Water	*v*OH	3650 and 3550
	δHOH	1625
Hexanol	*v*ROH	3569
tert-Butyl hydroperoxide	*v*ROOH	3447
Hexanal	*v*RHC=O	2810 and 2712
	*v*RHC=O	1727
2-Hexenal[a]	*v*RHC=O	2805 and 2725
	*v*RHC=O	1697
	*v*RC=CH–HC=O	1640
	δRC=CH–HC=O	974
2,4-Decadienal[a]	*v*RHC=O	2805 and 2734
	*v*RHC=O	1689
	*v*RC=CH–HC=O	1642
	δRC=CH–HC=O	987
4-Hexen-3-one[a]	*v*RC(=O)HC=CHR	1703 and 1679
	*v*RC(=O)HC=CHR	1635
	δRC(=O)HC=CHR	972
Oleic acid	*v*RCOOH	3310
	*v*RC(=O)OH	1711

[a] All double bonds in the *trans* form.

Rapid FTIR spectroscopy has been developed for PV evaluation, which is based on the stoichiometric reaction of hydroperoxides with triphenylphosphine (TPP) [97]. In this method, hydroperoxides in the oil react stoichiometrically with TPP and produce triphenylphosphine oxide (TPPO). TPPO presents an intense absorption band at 542 cm^{-1}, in the mid-IP spectrum, and the intensity can be measured and converted to PV. A linear calibration graph with a range of 1–100 PV can be generated by evaluating the band formed at 542 cm^{-1} using *tert*-butylhydroperoxide spiked oil standards [98]. FTIR spectroscopy has also been developed to simultaneously assess conjugated dienes, conjugated trienes, carbonyl compounds, and total polar compounds in canola oil used for frying [99]. As a simple and fast method, FTIR spectroscopy offers an automated, highly efficient, and low-cost path to evaluate the oxidative status of lipids as well as the efficiency of used antioxidants in the model oil systems under accelerated oxidation induced by high temperature [100,101].

Raman spectroscopy has been used as a complementary method to IR to detect fundamental vibrational transitions through an energy scattering [27]. Raman spectroscopy can detect the chemical changes that affect carbon–carbon double bonds and the carbon skeleton of lipid molecules when oxidation occurs, while IR spectroscopy monitors the formation of hydroperoxides and carbonyl compounds [102].

C. Chemiluminescence Spectroscopy

Chemiluminescence (CL) is an electromagnetic wave emission generated from chemical reactions [103]. CL has been used to examine lipid hydroperoxides formed during lipid oxidation [104,105]. In this method, sodium hypochlorite is used to react with hydroperoxides to form singlet oxygen. Light emission occurs when the electron relaxation starts with the singlet oxygen in excitation state. CL offers an economical, fast, and easy technique for investigating hydroperoxides formed during lipid oxidation [106]. CL is a very sensitive method as it is capable of detecting lipid hydroperoxides with a concentration as low as tens of pmol in 100 μL of sample [107].

CL has been reported to assess the oxidative stability of lipids. Peroxyl radicals are generated from the oxidation of unsaturated lipids, double bonds, and these radicals react with each other and form products in the electronic excitation state, which lead to light emission. The amount of light detected can explain the kinetic of lipid oxidation occurred [108].

Burkow et al. [109] reported that hypochlorite-activated CL could provide a useful means for evaluation of antioxidants in edible oils. Because of high sensitivity and the ability to detect small changes in the degree of oxidation of lipids, this method may be employed to evaluate the effects of antioxidants on oils during low-temperature storage (about 35°C) within a 24 h period. CL generally originates from electronically excited stages, such as singlet molecular oxygen in lipid peroxidation [110]. The CL method has been tested for estimating the degree of deterioration of edible oils containing antioxidants [111] as well as for shelf life dating of fish samples [112].

D. Nuclear Magnetic Resonance Spectroscopy

High-resolution NMR spectroscopy makes it possible to determine various types of hydrogen atoms (protons, ^1H) in TAG molecules. This is due to the fact that hydrogen atoms in a strong magnetic field absorb energy, in the radiofrequency range, depending on their molecular environment. During oxidation of food lipids, changes occur in the environment in which protons in an oxidizing TAG molecule are located. These changes may be monitored by employing ^1H NMR spectroscopy [113–116]. For this purpose, the oil is dissolved in $CDCl_3$ and its NMR spectrum is recorded (Figure 19.10). The sharp signal at the extreme right side of the spectrum (high applied field) is due to tetramethylsilane (TMS) added to the solution to serve as an internal standard. The spectrum shows eight groups of signals labeled *a*–*h*. These signals are assigned as follows: *a*, hydrogens directly attached to double-bonded carbons (olefinic protons) and the methine proton in the glyceryl moiety (δ 5.1–5.4); *b*, hydrogens in the two methylene groups in the glyceryl moiety (δ 4.0–4.4);

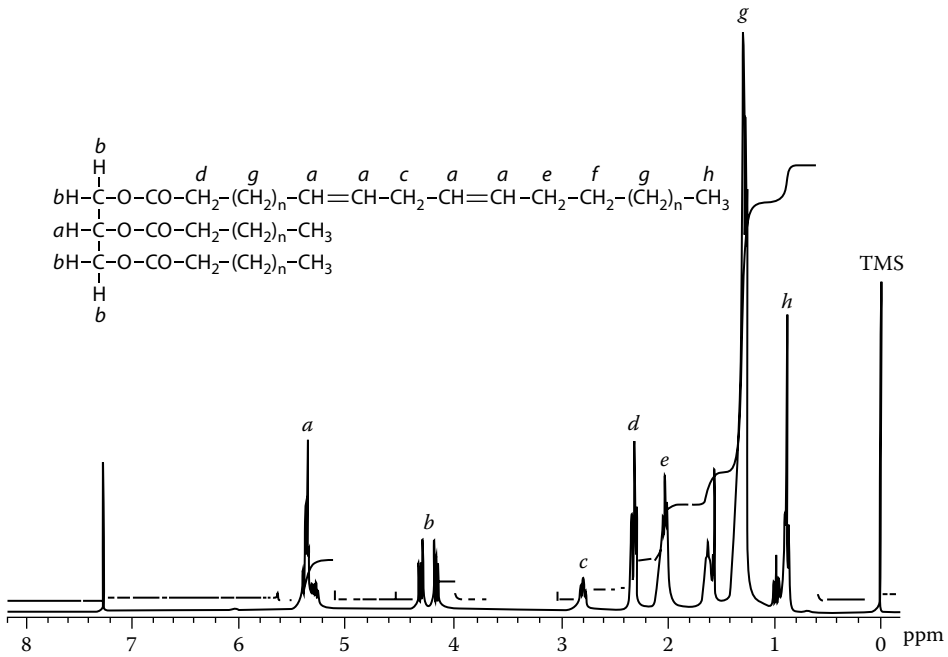


FIGURE 19.10 ^{1}H NMR spectrum of oxidized canola oil (peak at δ 0.00 and 7.26 are for TMS and $CHCl_3$ protons, as impurities in $CDCl_3$, respectively).

c, hydrogens in the CH_2 groups attached to two double-bonded carbon atoms (diallylmethylene protons) (=HC−CH_2−CH=; δ 2.6–2.9); *d*, hydrogens in the three CH_2 groups alpha to the carboxyl groups (α-CH_2; δ 2.2–2.4); *e*, hydrogens in the CH_2 groups attached to saturated carbons and double-bonded carbon atoms (−CH_2−C=; δ 1.8–2.2); *f*, hydrogens in the CH_2 groups attached to the saturated carbon atoms (=C−CH_2=CH_2; δ 1.45–1.8); *g*, hydrogens in the CH_2 groups bonded to two saturated carbon atoms ($[CH_2]_n$; δ 1.1–1.45); and *h*, hydrogens in the three terminal CH_3 groups (δ 0.7–1.0). The relative number of protons in each group is calculated based on the integration of methylene protons of the glyceryl moiety (δ 4.0–4.4) of the TAG (four protons in the two methylene groups of the TAG moiety) molecules. The area per proton is obtained as

$$\text{Area per each proton} = \text{Area of } b\text{-Type protons}/4$$

Since area per proton is known, one may calculate the number of protons belonging to each and every individual signal by dividing the integration number of individual signals by the area per proton. As an example, the total number of diallylmethylene protons equals the area of c-type protons/(the area of b-type protons/4).

The total number of aliphatic, olefinic, and diallylmethylene protons are calculated, from which ratios of aliphatic to olefinic protons (R_{ad}) and aliphatic to diallylmethylene (R_{ad}) protons may be obtained. These ratios increase steadily during the storage and oxidation of oils. Shahidi [115] and Wanasundara and Shahidi [116] have shown that the ratio of olefinic to aliphatic protons, measured by NMR, decreases continuously as long as the oxidation reaction proceeds. They suggested that the NMR technique could be useful for measuring oxidative deterioration of oils containing PUFAs, even at stages beyond the point at which PV profile reaches a maximum. Saito and Udagawa [114] have used this method to evaluate oxidative deterioration of brown fish meal and suggested that NMR methodology is suitable for comparing the storage conditions of the fish meal as well as estimating the effect of antioxidants in both fish meal and fish oil. These authors reported good

correlations between PVs and NMR data. However, Wanasundara and Shahidi [116] found that linear relationships between PVs and NMR data were not as suitable as those between TOTOX values and NMR data. It is obvious that TOTOX values correlate better with R_{ao} and R_{ad} than PVs since both TOTOX and NMR data estimate overall changes that occur in fatty acid profiles as reflected in both primary and secondary oxidation products of lipids. Thus, NMR methodology offers a rapid, nondestructive, and reliable technique for estimating the oxidative state of edible oils during processing and storage [117]. A good correlation has been reported for secondary oxidation products detection between TBA test and ^{13}C NMR [118].

In addition to ^{1}H NMR, ^{13}C NMR is also used for prediction of oxidative stability of oils. For instance, ^{13}C NMR provides direct observation of carbon atoms as it monitors lipid oxidation by examining the changes of carbon chains in fatty acids and TAGs, which can locate the site of oxidative degradation [119,120]. It is worth noting that the selectivity and dispersion of ^{13}C NMR spectra are very high. However, the sensitivity of ^{13}C NMR is normally much lower than that of ^{1}H NMR due to the low abundance of ^{13}C isotope, which is 1.12% of that of ^{12}C [120].

E. Chromatographic Techniques

Different chromatographic techniques have been developed and applied to quantify oxidation products in a variety of substances, including model compounds, oils, and food lipids, subjected to oxidation under very different conditions, from room to frying temperatures. Separation based on reversed-phase or size-exclusion chromatography and detection systems based on UV absorption, IR, refractive index, flame ionization, or evaporative light scattering detection are used for assessment of lipid oxidation products. For quantification of free MDA, reversed-phase HPLC using ion-pairing reagent or size-exclusion separation followed by monitoring the absorbance at 267 nm has been described [121–123]. MDA and 4-hydroxynonenal (4-HNE) can also be derivatized with 2,4-DNDH at room temperature to form dinitrophenylhydrozone (DNP) derivatives. The DNP derivatives could be solubilized in organic solvents and separated on a reversed-phase HPLC and detected at 300–330 nm, depending on the type of hydrozone formed [124–128]. An HPLC–fluorescence method that employs post-column detection can be used for the analysis of hydroperoxide mixtures containing conjugated and nonconjugated diene structures. Lipid hydroperoxides can react with diphenyl-1-1pyrenylphosphine (DPPP) to form DPPP oxides that have excitation at 352 nm and emission at 380 nm [129].

Oils heated at high temperatures (oil used for frying) and TAGs oxidize and form polymeric TAG and hydrolytic products (e.g., diacylglycerols and fatty acids). Solid-phase extraction with silica could be used to separate polar and nonpolar fractions of oxidized oils. The polar fraction can be analyzed by high-performance size-exclusion chromatography (HPSEC) column (highly cross-linked styrene–divinylbenzene copolymer) using a refractive index detector. Polar compounds are separated in an inverse order of their molecular weight: TAG polymers, TAG dimers, oxidized TAG monomers, diacylglycerols, monostearin, and fatty acids [130]. It has been observed that oxidized monomers show a progressive increase during the early stages of oxidation. According to Marquez-Ruiz et al. [131], who used trilinolein (LLL) as the model compound, during the early stages of oxidation, LLL-oxidized monomers increase paralleled that of PV, as primarily hydroperoxides were formed. The peroxides that are labile products readily degrade to a multitude of secondary products, such as oxygenated side products of the same chain length as the parent hydroperoxides. The oxidized TAGs comprise those monomeric TAGs containing at least one oxidized fatty acyl group (e.g., a peroxide group or any other oxygenated function, such as epoxy, keto, hydroxy). Therefore, determination of oxidized TAG monomers may provide a measure of both primary and secondary products of lipid oxidation. HPSEC has also been employed to analyze dimers, trimers, oligomers, partial acylglycerols, and cyclic fatty acids of heated, thermally oxidized lipids such as frying fats [130]. MS coupled with normal- or reversed-phase HPLC permits direct characterization of hydroperoxides and nonvolatile high-molecular-weight secondary oxidation products of TAGs, cholesterol, and phospholipids without pre-column derivatization. Isocratic HPLC-ESI-MS

has been found to be an efficient method to identify and characterize monohydroperoxide, bishydroperoxide, epoxy-epidioxides, and epoxides produced from TAG oxidation [132]. The necessity of removing solvents from labile compounds separated by HPLC is not a requirement when interfaced with chemical ionization or atmospheric pressure chemical ionization. These soft MS techniques are mainly qualitative and allow identification of the molecules. For quantitative analysis, standardization of spectra with authentic reference compounds to interpret fragmentation patterns may be necessary. Lipid oxidation products such as polyunsaturated hydroperoxides are difficult to synthesize and are not readily available.

GC methods to quantify MDA have also been reported. The advantages of GC methods are increased sensitivity, particularly when used with MS detection, and the possibility for simultaneous analysis of several aldehydes. Reduction of MDA to 1,3-propanediol with borane trimethylamine [133] forms a butyldimethylsilyl ether, which can be analyzed by GC-MS with an HP-5 capillary column (25 m long), temperature programmed from 115°C to 165°C, and [$^2H_8$] propanediol as the internal standard. MDA can also be converted to 1-methylpyrozole by reaction with *N*-methylhydrazine at room temperature for a 1 h period; this derivative could be recovered by extraction with dichloromethane and analyzed on a DB-Wax capillary column (30 m long), temperature programmed from 30°C to 200°C, and a nitrogen–phosphorus detector [134,135]. This method is faster and simpler compared to the conventional TBA test.

Oxidized fatty acid methyl esters could also be analyzed with a combination of silica column chromatography and HPSEC separation. The combined chromatographic analysis permits quantitation of groups of compounds (nonpolar fatty acid monomers, dimers, oxidized fatty acid monomers, and fatty acid polymers) differing in the polarity of molecular weight [96]. The HPSEC-separated fractions of these oxidized fatty acids could be further analyzed on GC-MS for detection of their structural identities. The fraction of oxidized fatty acid monomers includes epoxides, ketones, and hydroperoxides as well as polyoxygenated monomeric compounds. Marquez-Ruiz and Dobarganes [136] also described that GC-MS coupled with DB-wax column and AEI-MS was useful in identifying short-chain aldehydes resulting from the breakdown of lipid hydroperoxides.

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20 Antioxidants and Their Mechanisms of Action

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CONTENTS

I. INTRODUCTION

By definition, antioxidants are compounds that serve to inhibit oxidative processes that are generally considered to be deleterious to the quality of lipid foods. The term "antioxidant" is actually a general term that includes a host of compounds that are capable of inhibiting lipid oxidations by various mechanisms. These encompass free radical scavenging, inactivation of peroxides and other reactive oxygen species, chelation or sequestration of transition metal catalysts, and quenching of secondary lipid oxidation products that are associated with oxidative rancidity.

Reactive oxygen species, which include both radical (e.g., hydroxyl radicals) and nonradical (e.g., hydrogen peroxide) species, are generated in biological systems from which all foods are derived and can be generated *via* enzymatic and nonenzymatic reactions. As such, these biological systems typically contain complex and multifaceted endogenous antioxidant systems in order to control oxidative damage, and, under some situations, those antioxidants are preserved when the biological tissues (e.g., plant crops, muscle tissues, dairy products) are converted to foodstuffs. The antioxidant systems found in nature often consist of several distinctively different antioxidants that confer protection against various prooxidative compounds or oxidative insults (e.g., transition metals, heme-containing proteins, photosensitizers, and free radical species). Due to the fact that prooxidants can vary in terms of their polarity and, thus, are soluble in aqueous or organic phases, the endogenous antioxidant systems found in biological systems are usually biphasic. Such multicomponent and biphasic antioxidants represent nature's own hurdle technology antioxidant system.

This chapter is intended to provide an overview of common antioxidants used to preserve lipid foods as well as a discussion of the chemical mechanisms by which those antioxidants function in the complex, heterogeneous matrix we call lipid foods.

II. GENERAL ANTIOXIDANT MECHANISMS

By convention, antioxidants are broadly classified by their mechanism of action and are categorized as either primary or secondary antioxidants, as will be discussed in detail in this section. Some antioxidants exert their protective effect by more than one single mechanism, and, as such, these are often referred to as multiple-function antioxidants.

A. Lipid Oxidation

The chemistry governing the overall lipid oxidation scheme is presented in greater detail in previous chapters; however, for convenience, a brief overview is provided in this section.

The initiation of lipid oxidation occurs when an α-methylenic hydrogen is abstracted from an unsaturated fatty acid (LH) to yield a lipid alkyl radical (L•):

$$LH \rightarrow L^{\bullet} \tag{20.1}$$

This highly reactive alkyl radical (L•) is then capable of quickly reacting with oxygen to form a peroxy radical (LOO•) in a propagation reaction:

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \tag{20.2}$$

It should be noted that triplet-state oxygen ($^3O_2$), itself a biradical species, is incapable of directly reacting with a singlet state species such as a lipid molecule (LH); however, once a lipid is converted to a radical species (L•), its direct reaction with oxygen is no longer spin forbidden and can proceed at fast rates.

During the propagation phase of the general lipid oxidation reaction, lipid peroxyl radicals react with fresh lipid substrate to form a lipid hydroperoxide (LOOH) and a new lipid alkyl radical (L•) (Reaction 20.3). As before, this alkyl radical (L•) will then quickly react with oxygen to produce another peroxy radical (LOO•), resulting in a cyclical, self-propagating oxidative mechanism (Reaction 20.4).

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}, \tag{20.3}$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} + H^{\bullet} \tag{20.4}$$

The lipid hydroperoxides (LOOH) that form as part of reaction (20.3) are relatively unstable under food conditions and can degrade to produce radicals that further accelerate propagation reactions. This degradation (i.e., chemical reduction) of the hydroperoxide can occur through reaction with reduced transition metals (M^{n+}) (Reaction 20.5) that are ubiquitous in foods or by high-energy radiation (e.g., ultraviolet [UV] light) (Reaction 20.6). These reactions yield lipid alkoxyl radicals ($LO^{\bullet}$) that are free to react with fresh lipid substrate (Reaction 20.7).

$$LOOH + M^{n+} \rightarrow LO^{\bullet} + {}^{-}OH + M^{(n+1)+}, \tag{20.5}$$

$$LOOH \rightarrow LO^{\bullet} + {}^{\bullet}OH, \tag{20.6}$$

$$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet}. \tag{20.7}$$

The alkyoxyl radicals ($LO^{\bullet}$) that form as a result of the decomposition of lipid hydroperoxides (LOOH) are themselves unstable, which leads to the breakdown of the molecule. Such reactions, commonly referred to as β-scission reactions, cause cleavage of carbon–carbon bonds on the lipid molecule that result in the production of low-molecule-weight (and therefore relatively volatile) products. These products are easily detectable as off-flavors in lipid foods and, as such, their presence is strongly correlated with consumer perceptions of oxidative rancidity.

B. Primary Antioxidant Mechanisms

Primary antioxidants are occasionally referred to as "type 1" or "chain-breaking" antioxidants, which gives a clue as to their principal mode of action. Indeed, antioxidants in this category work to prevent or inhibit oxidative rancidity by donating an electron to free radical species that would otherwise propagate the overall lipid oxidation reaction.

Primary antioxidants react with lipid alkyl and peroxy radicals and, in so doing, convert them to more stable, nonradical products. Primary antioxidants donate hydrogen atoms to lipid-derived radicals to yield lipid derivatives and antioxidant radicals ($A^{\bullet}$) that are relatively stable and less readily unable to further promote autoxidation. The ability of a primary antioxidant to donate a hydrogen to a free radical can be predicted based on its one-electron reduction potential (Table 20.1). Simply put, any compound that has a reduction potential lower than the reduction potential of a free radical

TABLE 20.1
Standard One-Electron Reduction Potential for Common Free Radical Processes in Foods

Redox Couple	$E^{\circ\prime}$ (mV)
$HO^{\bullet}$, H^{+}/H_2O	2310
$RO^{\bullet}$, H^{+}/ROH	1600
$ROO^{\bullet}$, $H^{+}/ROOH$	1000
$PUFA^{\bullet}$, $H^{+}/PUFA\text{-}H$	600
$Urate^{-\bullet}$, $H^{+}/urate^{-}\text{-}H$	590
Catechol-$O^{\bullet}$, H^{+}/catechol-OH	530
α-Tocopheroxyl$^{\bullet}$, H^{+}/α-tocopherol	500
$Ascorbate^{-\bullet}$, $H^{+}/ascorbate^{-}$	282

Source: Adapted from Buettner, G.R., *Arch. Biochem. Biophys.*, 300(2), 535, 1993.
PUFAs, polyunsaturated fatty acids.

(or oxidized species) is capable of donating a hydrogen to that free radical unless the reaction is kinetically unfavorable. For example, α-tocopherol ($E^{\circ\prime}$ = 500 mV), urate ($E^{\circ\prime}$ = 590 mV), catechol ($E^{\circ\prime}$ = 530 mV), and ascorbate ($E^{\circ\prime}$ = 282 mV) all have reduction potentials below that of the peroxyl radical ($E^{\circ\prime}$ = 1000 mV) and, therefore, are capable of donating a hydrogen to a peroxyl radical to form a peroxide.

Primary antioxidants have higher affinities for peroxy radicals than lipids [1]. Therefore, the peroxy and oxy free radicals formed during the propagation (Reactions 20.2 and 20.4) and branching (Reaction 20.7) steps are quenched by primary antioxidants (Reactions 20.8 and 20.9). The antioxidants may also interact directly with lipid alkyl radicals (Reaction 20.10).

$$\mathrm{LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}}, \tag{20.8}$$

$$\mathrm{LO^{\bullet} + AH \rightarrow LOH + A^{\bullet}}, \tag{20.9}$$

$$\mathrm{L^{\bullet} + AH \rightarrow LH + A^{\bullet}}. \tag{20.10}$$

The antioxidant radical produced by the process described earlier has low relative reactivity with lipids, thereby reducing the rate of propagation. The low reactivity of the antioxidant radical is due to the stability it achieves through delocalization of its unpaired electron, thus forming stable resonance hybrids. Antioxidant radicals are capable of participating in termination reactions with peroxy (Reaction 20.11), oxy (Reaction 20.12), and other antioxidant radicals (Reaction 20.13). The fact that antioxidant dimers are often observed in fats and oils indicates that antioxidant radicals undergo termination reactions.

$$\mathrm{LOO^{\bullet} + A^{\bullet} \rightarrow LOOA}, \tag{20.11}$$

$$\mathrm{LO^{\bullet} + A^{\bullet} \rightarrow LOA}, \tag{20.12}$$

$$\mathrm{A^{\bullet} + A^{\bullet} \rightarrow AA}. \tag{20.13}$$

An induction period that precedes the point where oxidative rancidity is perceived by consumers. This period corresponds to the generation of free radicals and the concomitant consumption of antioxidants that are present and available to the reaction. Therefore, primary antioxidants are most effective if they are added prior to the induction and initiation stages of oxidation the propagation steps have occurred. The addition of antioxidants to lipid systems that already contain substantial amounts of peroxides will quickly result in loss of antioxidant function [2] and is, practically speaking, futile.

In general, primary antioxidants consist of mono- or polyhydroxy phenolic groups that vary in terms of their ring substituents. Substitution with electron-donating groups that are *ortho* and *para* to the hydroxyl group of the phenol increases the antioxidant activity of the compound. These hindered phenolic antioxidants decrease the reactivity of the hydroxyl group by increasing its electron density. Substitution with butyl or ethyl groups *para* to the hydroxyl group enhances overall antioxidant activity. Due to steric hindrance, however, the presence of longer-chain or branched alkyl groups at the *para* positions can decrease overall antioxidant effectiveness. Substitutions of branched alkyl groups at *ortho* positions increase the phenolic antioxidant's ability to form stable resonance structures and further reduce the antioxidant radical's ability to participate in propagation reactions.

The food industry relies heavily on the use of exogenous primary antioxidants. Traditionally, the majority of these antioxidants used were synthetic; however, due to increased consumer skepticism surrounding the health of synthetic antioxidants in foods, there has been a shift toward nonsynthetic ("natural") antioxidants. Common examples from both these categories are discussed at length in the proceeding sections.

C. Secondary Antioxidant Mechanisms

Compounds that confer oxidative stability to lipid foods by mechanisms other than scavenging radical species are referred to as secondary antioxidants, although they are occasionally known as "preventive" or "type 2" antioxidants. Antioxidants belonging to this category slow lipid oxidation rates by a host of various mechanisms, as will be described in this chapter; however, what all secondary antioxidants do have in common is the fact that they do not inhibit oxidation free radical quenching reactions.

Secondary antioxidants work to control lipid oxidation is by chelating prooxidant transition metal catalysts, replenishing or regenerating primary antioxidants, decomposing hydroperoxides to nonradical species (e.g., lipid alcohols), deactivating singlet oxygen, absorbing UV radiation, and/or acting as oxygen scavengers. Some secondary antioxidants are commonly referred to as "synergists" due to the fact that they promote or augment the activity of primary antioxidants; examples of these include citric acid, ascorbic acid, ascorbyl palmitate, lecithin, and tartaric acid.

Secondary antioxidants represent a large and broad category. This chapter will focus exclusively on the more important types of secondary antioxidant mechanisms and will provide examples of compounds that work to inhibit lipid oxidation according to those mechanisms.

1. Chelators

Metals with two or more valence states (Fe, Cu, Mn, Cr, Ni, V, Zn, A1) are known to promote lipid oxidation by catalyzing free radical reactions. These redox-active transition metals, which are pervasive in foods at quantitatively low, but catalytically relevant, concentrations are capable of transferring single electrons to lipid molecules.

Transition metals are thought to promote lipid oxidation by two mechanisms: (1) by reducing lipid hydroperoxides (LOOH) to alkoxyl radicals (LO•) and (2) by reacting directly with unsaturated lipid molecules (LH). According to the latter mechanism, as shown in the following reaction, metals are able to lower the activation energy of the initiation autocatalytic step:

$$M^{(n-1)} + RH \rightarrow M^{n+} + H^{+} + R^{\bullet}. \tag{20.14}$$

However, due to thermodynamic constraints, spin barriers, and extremely slow reaction rates, this direct interaction of metals with unsaturated fatty acid moieties is considered to be a minor and, thus, inconsequential mechanism in foods [3].

Transition metals are known to interact with hydroperoxides in a manner that promotes the overall lipid oxidation scheme. This is thought to occur when a metal–lipid hydroperoxide complex forms and subsequently decomposes to yield lipid radicals. The following two metal–hydroperoxide reactions are possible:

$$M^{(n+1)+} + ROOH \rightarrow M^{n+} + H^{+} + ROO^{\bullet}, \tag{20.15}$$

$$M^{n+} + ROOH \rightarrow M^{(n+1)+} + OH^{-} + RO^{\bullet}. \tag{20.16}$$

Reaction 20.15 is less significant in aqueous solution, as metals in their lower oxidation state accelerate hydroperoxide degradation more than metals in their higher oxidation state [4]. Even trace amounts of these metals promote electron transfer from lipids or hydroperoxides because Reactions 20.15 and 20.16 can be cyclical with regeneration of the lower oxidation state of the metal. Nevertheless, Reaction 20.16, which yields a lipid alkoxyl radical, occurs more slowly than Reaction 20.15.

The chelation of metal catalysts by certain secondary antioxidants mitigates their prooxidative activity by reducing their redox potentials and, thereby, stabilizing the oxidized form of the metal. Chelating compounds may also sterically hinder formation of the metal hydroperoxide complex.

Citric acid (and its lipophilic, monoglyceride ester), phosphoric acid (and its polyphosphate derivatives), and ethylenediaminetetraacetic acid (EDTA) are all capable of performing this chelating function and are discussed in greater detail in subsequent sections. Briefly, EDTA, a common chelator used in lipid foods, is known to form a stable complex with metal ions and is extremely effective in controlling lipid oxidation reactions by slowing lipid hydroperoxide decomposition. The metal-chelating ability of oligophosphates increase with phosphate group number up to six residues. Carboxyl groups of citric acid are also thought to be responsible for binding with metals and forming complexes; malic, tartaric, oxalic, and succinic acids bind metals in the same manner.

In addition to their general antioxidant activity *via* metal chelation, many of the compounds mentioned earlier have other unique functions as food additives. For example, citric acid, malic acid, and tartaric acid are important food acidulants, whereas phosphates are commonly added as buffer components, emulsifiers, acidulants, and water binders. Chelating antioxidants are also referred to as synergists because they can enhance the activity of phenolic antioxidants. This synergism is sometimes referred to as "acid synergism."

2. Oxygen Scavengers and Reducing Agents

Ascorbic acid and its derivatives (e.g., ascorbyl palmitate), erythorbic acid, and sulfites can prevent oxidation by acting as reductants and/or by reducing oxygen concentrations in the surrounding food matrix. This latter activity has often been attributed to "oxygen scavenging," which incorrectly implies that these compounds react directly with triplet state oxygen ($^3O_2$). Rather, the reaction between an "oxygen scavenger" (singlet-state) and oxygen (triplet-state) is most likely mediated by transition metals in foods [5–9], as their direct reaction would otherwise be spin forbidden. According to this reaction sequence, an oxygen scavenger such as ascorbic acid is reduced to an ascorbyl radical [10] as a transition metal catalyst such as Fe^{3+} is reduced to Fe^{2+}, and, in turn, Fe^{2+} is able to reduce oxygen to a peroxy radical in a reaction that regenerates Fe^{3+}. Given that the net result of this overall reaction is oxygen consumption, some might argue that this point is purely academic; however, this may not be the case given the implications that the generation of oxygen (peroxy) radicals [11] might have on an oxidatively labile food system. The antioxidant activity of ascorbic acid and its derivatives is discussed in further detail in the proceeding sections.

3. Singlet Oxygen Quenchers

Singlet oxygen is a high-energy molecule that is responsible for the photooxidation of unsaturated fats and the subsequent generation of hydroperoxides. Singlet oxygen quenchers convert singlet oxygen to lower energy species in a reaction that leads to the dissipation of heat. Carotenoids such as β-carotene, lycopene, and lutein are active singlet oxygen quenchers [12]. The chemistry of singlet oxygen in lipid systems and the singlet oxygen scavenging activity of carotenoids in foods is discussed in greater depth in the subsequent sections of this chapter.

D. Antioxidant Interactions

Biological systems typically contain multicomponent antioxidant systems. A classical and extensively studied example of a multicomponent antioxidant system is the combination of primary antioxidants (i.e., free radical scavengers) and chelators, which have been shown to result in the synergistic inhibition of lipid oxidation [13]. Such synergy is thought to occur by a "sparing" mechanism, whereby the chelator decreases oxidation rates by inhibiting metal-catalyzed oxidation, resulting in net fewer radicals generated in the system. This means that the eventual inactivation of the primary antioxidant through reactions such as termination or autoxidation will be slower, thus increasing its effective concentration at any given time. The combination of chelators and primary antioxidants, therefore, has the overall effect of decreasing free radical generation while simultaneously increasing radical scavenging potential.

Synergistic antioxidant activity can also be observed by the combination of two or more free radical scavengers. This occurs when one scavenger reacts more rapidly with free radicals than the other as a result of differences in bond dissociation energies and/or steric hindrance of the associated radical scavenger–LOO• interactions [13]. Such differences will result in one antioxidant being consumed at a relatively faster rate; however, it may be possible for this radical scavenger to be regenerated by electron transfer to another scavenger. For example, in a system consisting of α-tocopherol and ascorbic acid, α-tocopherol is the primary scavenger due to the fact that it is present in the lipid phase [14]. Ascorbic acid is then capable of regenerating the tocopheroxyl radical (or possibly the tocopheryl quinone) back to α-tocopherol with the concomitant generation of a semihydroascorbyl radical [15], which dismutates to dehydroascorbate [16]. Finally, under biological conditions, dehydroascorbate may be regenerated by enzymes that use NADH or NADPH as reducing equivalents [17].

Due to the fact that multicomponent antioxidant systems are capable of exerting control over lipid oxidation reactions at various stages, the resulting net antioxidant activity can be synergistic. Therefore, the most effective antioxidant systems for foods should contain antioxidants that act according to different mechanisms of action and/or possess different chemical properties (e.g., polarity). Predicting which antioxidants would be most effective for a given system is often challenging, as it depends on an array of factors such as oxidation catalyst type, the physical state of lipid (bulk vs. dispersed lipid), and factors that influence the activity of the antioxidants themselves (e.g., pH, temperature).

III. CHAIN-BREAKING ANTIOXIDANTS

Consumers are increasingly skeptical about the presence of synthetic additives in their diets, which has posed a significant challenge to food processors as they attempt to create chemically stable and palatable foods. Despite the often superior efficacy, relatively lower cost, and higher stability of synthetic antioxidants, the suspicion that these compounds may act to promote carcinogenicity has led to a decrease in their use in foods [18]. A trend toward the use of natural additives in the food industry has been apparent for quite some time. It should be noted that, at present, the term "natural" has no legal or regulatory definition but is generally regarded to mean "nonsynthetic" by consumers. Some natural preservatives are endogenous components of foods, while others can be added to the product or can arise in the course of processing or cooking. Natural food antioxidants (e.g., citric acid, ascorbic acid) are used widely in the food industry, and a large number of recent studies have focused on the identification and isolation of naturally occurring yet efficacious chain-breaking antioxidants [19–22].

A. Phenolics

Phenolic compounds, especially di- and tri-hydroxyl substituted phenolics, are known to exhibit strong radical scavenging (chain-breaking) activity in lipid-based foods. As a group, phenolics encompass a wide spectrum of compounds that can vary based on their electrochemical properties (i.e., redox chemistry), polarity, stability within the food matrix, biological activity (i.e., beneficial health effects or, on the opposite end of the spectrum, toxicity), or provenance (i.e., naturally occurring vs. synthesized). However, the primary mode of action of these compounds with respect to their free radical scavenging activity is essentially the same across the entire category. The mechanisms by which phenolics exert their antioxidant activity will be described in detail in subsequent sections, and examples of commonly used natural and synthetic antioxidants will be discussed.

1. Tocopherols

Tocopherols (Figure 20.1) are perhaps the most well-known chain-breaking antioxidants, whose radical scavenging activity has been widely established, both *in vivo* and in foods [9,23]. Tocopherols are a group of methylated phenolic free radical scavenging isomers that originate in plants and

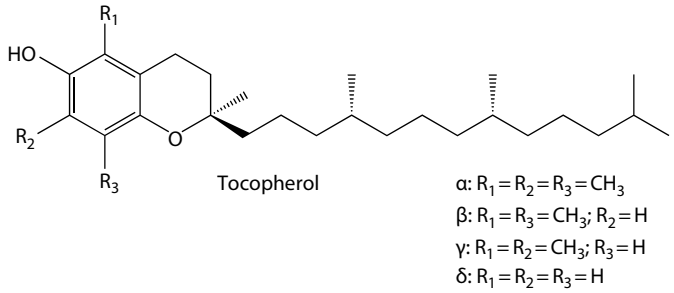

FIGURE 20.1 Tocopherol isomers.

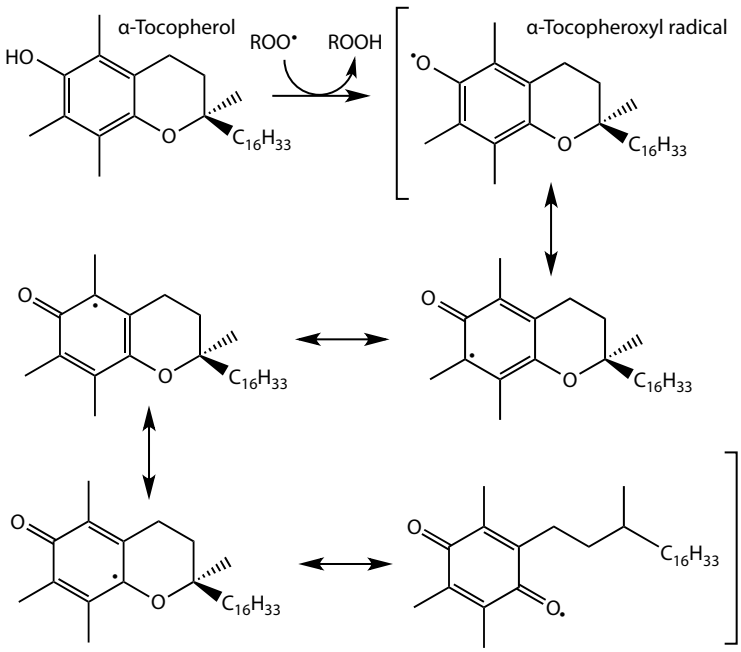

FIGURE 20.2 Tocopherol radical resonance structures.

eventually accumulate in animals through the diet [24]. The interactions between tocopherols and lipid peroxyl radicals lead to the formation of lipid hydroperoxides and several resonance structures of tocopheroxyl radicals (Figure 20.2) [13]. Tocopheroxyl radicals can interact with other compounds, or with each other, to yield a variety of products. The types and concentrations of these products depend on oxidation rates, the nature of the initial radical species, the state of the lipid phase (e.g., bulk vs. membrane lipids), and tocopherol concentration.

Under conditions of low oxidative stress in lipid membrane systems, tocopheroxyl radicals have been shown to primarily convert to tocopheryl quinone, which form upon the interaction of two tocopheroxyl radicals; this disproportionation reaction leads to the formation of tocopheryl quinone and the regeneration of tocopherol (Figure 20.3) [13]. Tocopheryl quinone formation is also thought to occur by the transfer of an electron from a tocopheroxyl radical to a phospholipid peroxyl radical to form a phospholipid peroxyl anion and a tocopherol cation. The tocopherol cation hydrolyzes to 8α-hydroxytocopherone, which rearranges to tocopheryl quinone [25].

Under conditions of higher oxidative stress, high concentrations of peroxyl radicals can favor the formation of tocopherol–peroxyl complexes, which can subsequently hydrolyze to yield

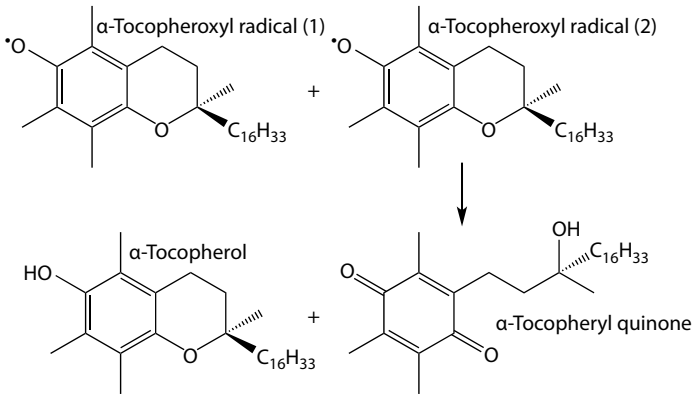

FIGURE 20.3 Formation of α-tocopheryl quinone and regeneration of α-tocopherol from the reaction of two α-tocopheroxyl radicals.

tocopheryl quinones. Of less importance is the interaction between tocopheroxyl and peroxyl radicals, which can lead to an adduct that is positioned *ortho* to the phenoxyl oxygen. The elimination of an alkoxyl radical then proceeds, followed by the addition of oxygen and hydrogen abstraction to form two isomers of epoxy-8α-hydroperoxy tocopherone. Subsequent hydrolysis leads to the formation of epoxyquinones (Figure 20.4) [26,27]. The formation of tocopherol epoxide derivatives represents no net reduction of radicals due to the formation of an alkoxyl radical and the loss of tocopherol from the system, whereas any tocopheryl quinone that is formed can be regenerated to tocopherol in the presence of reducing agents (e.g., ascorbic acid and glutathione [GSH]). An additional reaction that can occur under these conditions is dimerization of two tocopheroxyl radicals [28].

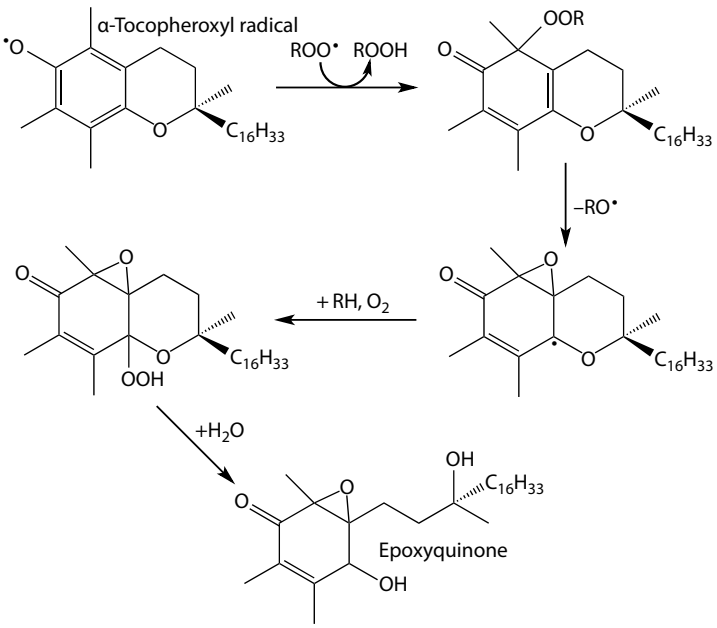

FIGURE 20.4 Formation of an epoxyquinone and an alkoxyl radical from the reaction between an α-tocopheroxyl radical and a peroxyl radical.

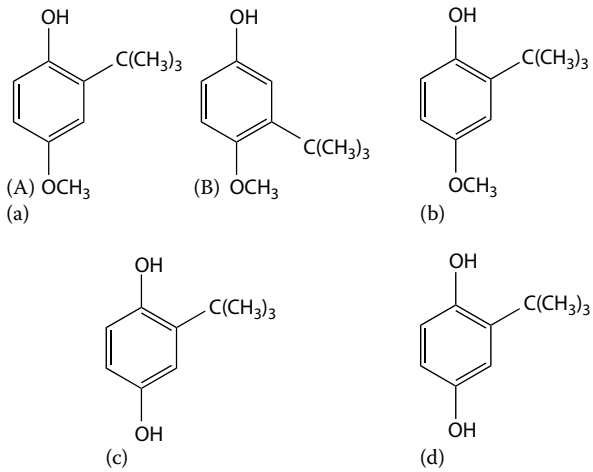

FIGURE 20.5 Chemical structures of some synthetic chain-breaking antioxidants commonly used in lipid foods. (a) BHA, (A) 2-tertiary-4-hydroxyanisole, (B) 3-tertiary-4-hydroxyanisole; (b) BHT, 2,6-ditertiary-butyl-4-methylphenol; (c) TBHQ; (d) gallates—esters of 3,4,5-trihydroxybenzoic acid, R = C_3H_7 (propyl); R = C_8H_{17} (octyl); R = $C_{12}H_{25}$ (dodecyl).

2. Synthetic Phenolics

A relatively small number of synthetic compounds are permitted for use as chain-breaking antioxidants in foods. Among these, the most common include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG) (Figure 20.5). While these compounds all share scavenge radical species by similar mechanisms, they do vary with respect to their polarity (BHT > BHA > TBHQ > PG in terms of hydrophobicity); therefore, the nature of the food matrix (i.e., bulk lipid vs. emulsified lipid) must be carefully considered.

The antioxidant mechanism of synthetic phenolics involves the formation of a resonance-stabilized phenolic radical that neither rapidly oxidizes other molecules nor reacts with oxygen to form antioxidant peroxides that autoxidize [22]. Synthetic phenolic radicals can potentially react with each other by means similar to that of α-tocopherol, as described earlier. These include reactions between two phenolic radicals to form a hydroquinone and a regenerated phenolic (i.e., disproportionation), as well as the formation of phenolic dimers. The phenolic radicals can also react with other peroxyl radicals in termination reactions that result in the formation of phenolic–peroxyl species adducts. In addition, oxidized synthetic phenolics can undergo numerous degradation reactions, which are thoroughly reviewed in Reference 22. Due to the fact that many of these degradation products still contain active hydroxyl groups, the products may retain their antioxidant activity. Therefore, the net antioxidant activity of synthetic phenolics in foods actually represents the activity of the original phenolic in addition to those derived from their various degradation products.

BHA is typically used as a 9:1 mixture of 3-BHA and 2-BHA isomers [23,29], with the 3-isomer displaying greater antioxidant activity compared to the 2-isomer. Sharing some structural similarity to BHA, BHT is another widely used monophenolic antioxidant in foods. BHA and BHT have been shown to act synergistically to confer greater antioxidant activity than either antioxidant alone. The proposed synergistic mechanism of BHA and BHT involves the interactions of BHA with peroxy radicals to produce a BHA phenoxy radical, with this resulting radical then able to abstract a hydrogen from the hydroxyl group of BHT. According to this scheme, BHT effectively acts as a hydrogen replenisher of BHA, allowing BHA to be effectively regenerated. The BHT radical can then react with a peroxy radical and act as a chain terminator.

TBHQ is another highly effective synthetic phenolic antioxidant in lipid systems; however, its use in foods is restricted in many countries and regulatory bodies due to health concerns. TBHQ, a diphenolic molecule, comes as a slightly colored (beige) powder and is commonly used in frying applications when highly unsaturated vegetable oils are used.

As stated earlier, synthetic phenolics are unquestionably efficacious in lipid foods, yet their use in the food industry has markedly declined in recent years due to safety concerns and the industry's push for clean food labels in response to consumer demand.

3. Plant-Derived Phenolics

Plants contain a diverse group of phenolic compounds, including simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives, and flavonoids (Figure 20.6). These compounds are widely distributed in plant foods (e.g., fruits, spices, tea, coffee, seeds, grains) and are typically consumed in relatively high quantities (>1 g/day) in the human diet compared to other antioxidants discussed in this chapter.

All phenolics have the structural features required for free radical scavenging, yet the antioxidant activity of these compounds varies greatly, with some even exhibiting prooxidative activity under some conditions [7,30–32]. Factors influencing the antioxidant activity of plant phenolics include position and degree of hydroxylation, solubility, reduction potential, the stability of the phenolic to various food processing operations, and the stability of the phenolic radical that results from phenolic oxidation. Furthermore, many phenolics contain an acid or ring constituent that may participate in metal complexation or chelation. These metal chelation properties, in addition to high reducing potentials, can accelerate metal-catalyzed oxidative reactions, leading to the prooxidative activity of plant phenolics under certain conditions [33,34].

Herbs and spices are abundant sources of phenolic antioxidants used in foods. In particular, rosemary extracts are the most commercially important source of plant-derived antioxidant ingredients. Carnosic acid, carnosol, and rosmarinic acid are the major antioxidant phenolics in rosemary extracts (Figure 20.7) [35]. Crude rosemary extracts have been found to inhibit lipid oxidation in a wide variety of food products that include muscle foods, bulk oils, and lipid dispersions (e.g., oil-in-water emulsions) [35–37]. Unfortunately, the application of plant-derived extracts, such as rosemary extracts, is often limited due to the presence of highly aroma-active monoterpenes that may negatively affect the flavor of the target food. For example, while consumers might not find the

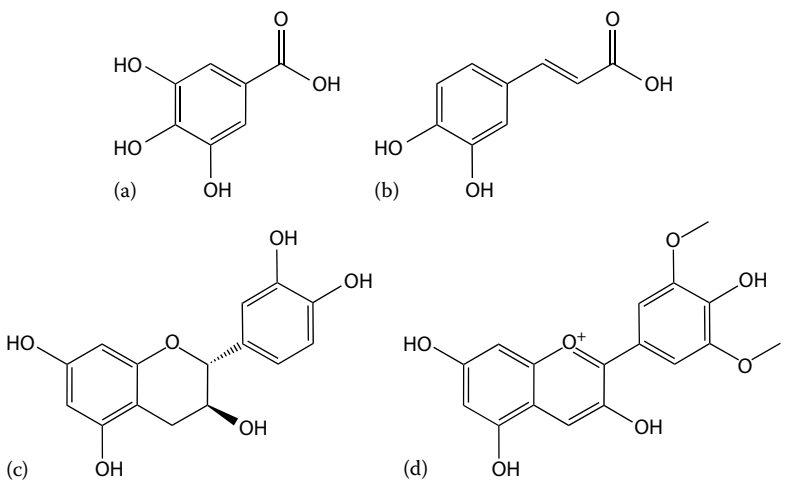

FIGURE 20.6 Chemical structures of some naturally occurring chain-breaking phenolic antioxidants. (a) Gallic acid, (b) caffeic acid, (c) (+)-catechin, (d) malvidin.

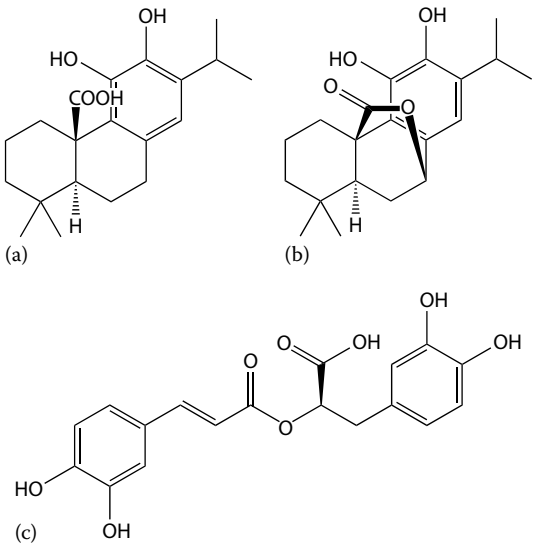

FIGURE 20.7 Chemical structures of (a) carnosic acid, (b) carnosol, and (c) rosmarinic acid.

flavor of rosemary offensive when added as an antioxidant to a meat product, that same flavor might be considered offensive in the context of many nonsavory products. At present, the use of more purified forms of herbal phenolics is restricted by both economic and regulatory hurdles.

4. Ubiquinone

Ubiquinone, or coenzyme Q, consists of a phenolic moiety that is conjugated to an isoprenoid chain and is primarily associated with the mitochondrial membrane [38,39]. Reduced ubiquinone has been shown to inactivate peroxyl radicals, yet its radical scavenging activity is less than that of α-tocopherol [40]. The lower free radical scavenging activity of reduced ubiquinone has been attributed to internal hydrogen bonding, which, in turn, makes hydrogen abstraction more difficult [40]. Despite its lower radical scavenging activity, reduced ubiquinone has been found to inhibit lipid oxidation in liposomes [41] and low-density lipoprotein [42]. Presumably, it could be an important endogenous antioxidant in many foods, and manufacturers may want to consider ways to prevent its loss during the course of food processing operations; however, to date, few studies have specifically addressed this issue.

B. Carotenoids

Carotenoids are a diverse group (>600 compounds) of yellow-to-red colored polyenes that consist of 3–13 double bonds and, in some cases, 6-carbon-hydroxylated ring structures at one or both ends of the molecule [43]. Carotenoids may be important biological antioxidants and are thought to play a role in controlling oxidatively induced diseases (e.g., cancer, atherosclerosis) [44]. The antioxidant properties of carotenoids depend on environmental conditions and the nature of the specific oxidation catalyst or initiator. Carotenoids can be effective antioxidants in the presence of singlet oxygen; however, when peroxyl radicals are the initiating species, the overall antioxidant activity of carotenoids depends on oxygen concentrations [12,45].

β-Carotene (Figure 20.8) is the most extensively studied carotenoid antioxidant, which has been shown to quench lipid peroxyl radicals resulting in the concomitant formation of a carotenoid radical. Burton and Ingold [45] demonstrated that the antioxidant activity of β-carotene is compromised under conditions of high oxygen tension. The authors proposed that by increasing oxygen

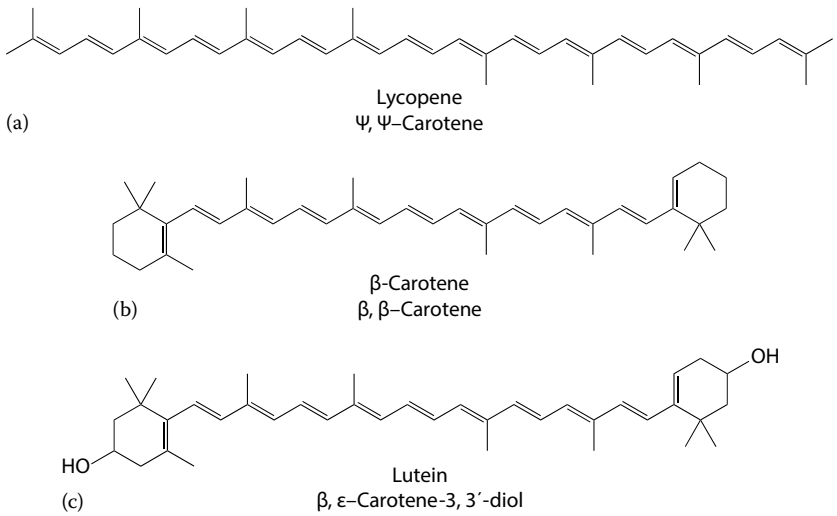

FIGURE 20.8 Chemical structures of the carotenoids (a) lycopene, (b) β-carotene, and (c) lutein.

concentrations, the formation of carotenoid peroxyl radicals increased, which in turn favored autoxidation of β-carotene at the expense of lipid peroxyl radical quenching. In contrast, under conditions of low oxygen tension, the half-life of the carotenoid radical is sufficiently high to allow its reaction with another peroxyl radical, thus allowing for the formation of a nonradical species and effectively inhibiting oxidation by removing radicals from the system.

Incubation of β-carotene with peroxyl radical generators in organic solvents at high (e.g., atmospheric) oxygen tensions leads to addition reactions to form carotenoid peroxyl adducts. The addition of a peroxyl radical to the cyclic end group or the polyene chain, followed by loss of an alkoxyl radical, leads to the formation of 5,6-and 15,15′-epoxides. Elimination of the alkoxyl radical from the 15,15′ positions can also cause cleavage of the polyene chain, resulting in the formation of aldehydes. Since the formation of β-carotene epoxides from the addition of peroxyl radicals results in the formation of an alkoxyl radical, there is no net change in radical number and an antioxidant effect an antioxidant effect is not expected [46].

β-Carotene is capable of donating an electron to peroxyl radicals to produce a β-carotene cation radical and a peroxyl anion. The β-carotene cation radical is resonance stabilized and does not readily react with oxygen to form peroxides. The β-carotene cation radical appears to be sufficiently strong to oxidize other lipophilic hydrogen donors, such as tocopherols and ubiquinone [46]. Additional research is needed to identify the oxidation products that arise from carotenoids under low oxygen partial pressures, as identification of these products may help determine the exact mechanism by which carotenoids act as free radical scavengers under low oxygen conditions. Such insight would make it easier to predict when carotenoids should or should not exhibit antioxidant activity in foods.

C. Ascorbic Acid, Its Salts, and Lipophilized Derivatives

L-Ascorbic acid (Figure 20.9) is ubiquitous in nature as a component of plant tissues and can be produced synthetically in large quantities. Ascorbic acid is attractive as an antioxidant because it has generally regarded as safe (GRAS) status with no usage limits, is a natural or nature-identical product, and is highly recognized as an antioxidant nutrient by the consumer. Ascorbic acid is also used as a flavorant and/or acidulant in many food products. However, in foods that are subjected to thermal processing, ascorbic acid can participate in nonenzymatic browning reactions and may be degraded.

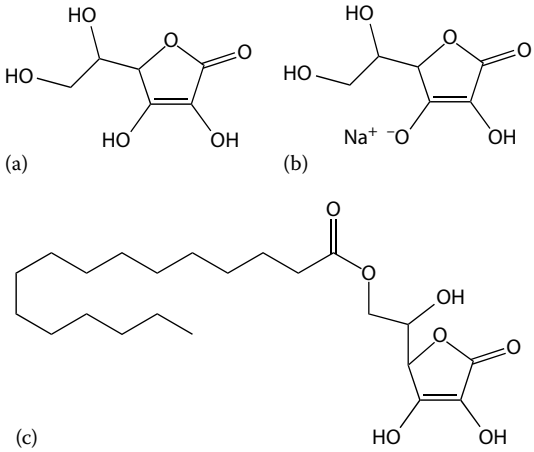

FIGURE 20.9 Chemical structures of (a) ascorbic acid, (b) sodium ascorbate, and (c) ascorbyl palmitate.

Ascorbic acid is thought to act as both a primary and secondary antioxidant. For example, under physiological conditions, ascorbic acid donates hydrogen atoms to inhibit oxidation reactions (i.e., chain-breaking activity) but is also capable of directly converting hydroperoxides to stable products (i.e., preventative activity). Ascorbic acid is an important antioxidant in plant tissues and is essential for the prevention of oxidative cellular damage by hydrogen peroxide [17]. In foods, ascorbic acid is commonly added as a secondary antioxidant with multiple functions. These functions include "oxygen scavenging" (as discussed earlier), shifting the redox potential of food systems to the reducing end of the spectrum, acting synergistically with metal chelators, and regenerating primary antioxidants. With respect to antioxidant regeneration, ascorbic acid has been shown to act synergistically with α-tocopherol. As discussed in preceding sections, the mechanism underlying this synergism is the reduction (i.e., regeneration) of tocopheroxyl radicals to tocopherol by ascorbic acid. This reaction has been extensively studied *in vivo* [16,47–50], as discussed earlier, but more recently in the context of model foods (e.g., micelles, liposomes, emulsions) where some have attempted to show similar antioxidant synergy [51–55]; however, further research in this area is needed.

Ascorbic acid, which is unsubstituted at positions 2 and 3, oxidizes through one- or two-electron transfers that are due to its enediol structure [56]. Oxidation of ascorbic acid occurs in two steps, with monohydroperoxide formed as an intermediate, followed by the formation of dehydroascorbic acid. Ascorbic acid and its salts (e.g., sodium ascorbate, calcium ascorbate) are water soluble and, as such, are not appropriate as antioxidants in bulk lipids.

Ascorbic acid can be made into a lipophilic molecule *via* conjugation to a long-chain aliphatic group. Ascorbyl palmitate and ascorbyl stearate, which are synthetic derivatives of ascorbic acid, are two common examples of such alkylated conjugates. Ascorbyl palmitate is commonly used in lipid foods due to its increased solubility compared to that of ascorbic acid and its salts. For synergistic reasons described earlier, ascorbyl palmitate is usually used in combination with tocopherols. As is the case with ascorbic acid, ascorbyl palmitate has GRAS status, and there is no restriction on usage levels in the United States. Ascorbyl palmitate is hydrolyzed by the digestive system to provide nutritionally available ascorbic acid and palmitic acid, but health claims cannot be made for its vitamin C contribution [56].

The potential prooxidative activity of ascorbic acid is worth noting again. Due to its capacity to act as a reducing agent, ascorbic acid can cause transition metals to speciate to their catalytically active reduced states (e.g., Fe^{2+}, Cu^{+}); these metal ions can then, in turn, reduce hydrogen peroxide and lipid peroxides to form radical species (e.g., hydroxyl and alkoxyl radicals) [57,58]. Ascorbic acid also causes the release of iron from proteins such as ferritin [8], which can in turn promote lipid

oxidation reactions. Finally, the metal-catalyzed oxidation (often referred to as "autoxidation") of ascorbic acid is known to result in reactive oxygen species (e.g., hydrogen peroxide) [59–62].

IV. MANAGEMENT OF OXIDATION PROMOTERS AND PRODUCTS

An effective antioxidant strategy in lipid foods must take into consideration antioxidants that perform their function by non-chain-breaking mechanisms. As discussed earlier in general mechanistic terms, these so-called preventative (or secondary) antioxidants work to inhibit lipid oxidation by preventing radical formation, controlling oxidation catalysts, quenching stable intermediate oxidation products (e.g., peroxides), and/or quenching final oxidation products.

A. Transition Metal Catalysts

Transition metals accelerate lipid oxidation reactions by hydrogen abstraction and peroxide decomposition, resulting in the formation of free radical species [63]. The prooxidative activity of transition metals is influenced by chelators or sequestering agents. Transition metals that are commonly encountered as trace components in foods (e.g., iron, copper) exhibit low solubility at neutral pH values [64]. In food systems, transition metals often exist as chelates to or complexes with other compounds. Many food components are known to form complexes with metals, which often affects the catalytic activity of the metals [31,59]. Some metal chelators increase oxidation reactions by increasing metal solubility and/or altering the redox potential in a way that increases the catalytic activity of the bound metal [65]. Chelators also increase the prooxidative activity of transition metals by decreasing their polarity, thereby increasing their solubility in lipids [66]. Chelators that exhibit antioxidative properties inhibit metal-catalyzed reactions by one or more of the following properties: prevention of metal redox cycling, occupation of all metal coordination sites, formation of insoluble metal complexes, and sterical hindering interactions between metals and lipids or oxidation intermediates (e.g., lipid hydroperoxides). The net prooxidant/antioxidant effect can depend on a host of matrix conditions [32], such as metal and chelator concentrations. For example, EDTA (Figure 20.10) has been shown to be prooxidative when the ratio of EDTA to iron is less than one and antioxidative when that same ratio is greater than one [67].

The most common metal chelators used in foods contain multiple carboxylic acid (e.g., EDTA, citric acid) or phosphate (e.g., polyphosphates, phytate) groups. Chelators are typically water

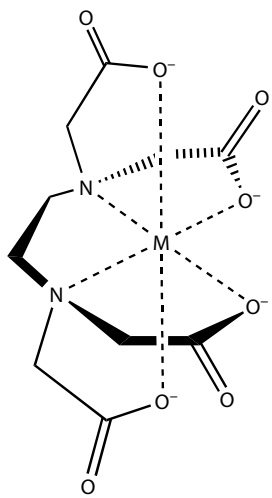

FIGURE 20.10 EDTA–metal complex.

soluble, but some (e.g., citric acid) will exhibit some solubility in lipids, thus allowing the chelator to inactivate metals in the lipid phase [23,68,69]. Chelator activity depends on pH, due to the fact that the chelator must be ionized to be active. Therefore, as the pH approaches the pK_a of the ionizable groups, the overall chelator activity decreases. Chelator activity is also decreased by the presence of other chelatable ions (e.g., calcium), which compete with the target prooxidant metals (e.g., iron) for binding sites.

Although most food-grade chelators are unaffected by food processing operations and subsequent storage, polyphosphates are an exception to this general rule. Polyphosphates are stronger chelators and antioxidants than mono- and diphosphates [70]; however, some foods contain phosphatases, which hydrolyze polyphosphates, thus decreasing their antioxidant activity. This can be observed in muscle foods, where polyphosphates are relatively ineffective in raw meats that contain high levels of phosphatase activity [71] but are highly effective in cooked meats, where the phosphatases have been inactivated [72]. The nutritional implications of this should also be considered when chelators are used as food antioxidants, as chelators affect mineral bioavailability. For example, EDTA is known to enhance iron bioavailability, whereas phytate decreases iron, calcium, and zinc absorption.

Prooxidant metal activity is also controlled in biological systems by proteins. Proteins with strong binding sites include transferrin, ovotransferrin (conalbumin), lactoferrin, and ferritin. Transferrin, ovotransferrin, and lactoferrin are structurally similar proteins consisting of a single polypeptide chain molecular weights (MW) ranging from 76,000 to 80,000. Transferrin and lactoferrin bind two ferric ions each, whereas ovotransferrin has been shown to bind three [73]. Ferritin is a multisubunit protein (MW 450,000) with the capability of storing up to 4,500 ferric ions [74]. Transferrin, ovotransferrin, lactoferrin, and ferritin inhibit iron-catalyzed lipid oxidation by binding iron in its inactive ferric state and, possibly, by sterically hindering metal–peroxide interactions [75]. Reducing agents (e.g., ascorbate, cysteine, superoxide anion) and low pH conditions can cause the release of iron from proteins, resulting in an acceleration of lipid oxidation reactions [76]. Similar to that of iron, copper can also be controlled by binding to proteins. For example, serum albumin binds one cupric ion and ceruloplasmin binds up to six cupric ions [77].

Amino acids and peptides can chelate metals in a manner that decreases their reactivity. Both the chelating and antioxidant activities of the skeletal muscle dipeptide carnosine depend on the metal ion type [78–80]. Carnosine more effectively inhibits the oxidation of phosphatidylcholine liposomes catalyzed by copper than by iron. Decker et al. [80] found that the carnosine can chelate and inhibit the prooxidative activity of copper more effectively than its constituent amino acid histidine. Phosphorylated peptides arising from casein have also been found to be strong iron chelators that can inhibit lipid oxidation [81].

Ceruloplasmin is a copper-containing enzyme that catalyzes the oxidation of ferrous ions to their ferric state. This ferroxidase activity effectively inhibits lipid oxidation by maintaining iron in its oxidized (i.e., less reactive with respect to lipid oxidation) state. As ceruloplasmin is primarily a constituent of blood, one would not expect to find this compound in nonmuscle foods. The addition of ceruloplasmin to muscle foods in a pure form or as part of blood plasma has been found to effectively inhibit lipid oxidation [82].

B. Peroxides

Peroxides are important intermediates of oxidation reactions because they decompose *via* transition metal–mediated reduction, irradiation, and elevated temperatures to yield free radicals (e.g., hydroxyl and lipid alkoxyl radicals). Hydrogen peroxide exists in foods as a result of its direct addition (e.g., aseptic processing operations) and/or formation in biological tissues by mechanisms such as enzymatic superoxide dismutation by SOD, peroxisome activity, and oxidation of phenolic ingredients [31,83]. Hydrogen peroxide is readily decomposed by reduced transition metals (e.g., Fe and Cu) to hydroxyl radicals, which are extremely reactive species capable of oxidizing the most

biological molecules at diffusion-limited reaction rates [30,84,85]. Therefore, removal of hydrogen peroxide from biological materials is critical to the prevention of oxidative damage, especially since the direct scavenging of hydroxyl radicals by chain-breaking antioxidants is difficult due to their high reactivity.

Catalase (CAT) is a heme-containing enzyme that catalyzes the concerted reaction of hydrogen peroxide to water and oxygen, thereby circumventing the formation of intermediate hydroxyl radicals. Hydrogen peroxide in higher plants and algae may be scavenged by ascorbate peroxidase, which inactivates hydrogen peroxide in the cytosol and in chloroplasts by sacrificing ascorbic acid. Two ascorbate peroxidase isozymes, which differ in MW (57,000 vs. 34,000), substrate specificity, optimum pH, and stability, have been described in tea leaves [86]. Finally, many biological tissues contain glutathione peroxidase (GSH-Px) in order to control peroxides. GSH-Px differs from CAT in that it is capable of reacting with both lipid and hydrogen peroxides. GSH-Px is a selenium-containing enzyme that uses reduced GSH to catalyze hydrogen or lipid (LOOH) peroxide reduction. The products of this enzymatic reaction are water and oxidized glutathione (GSSG) or a fatty acid alcohol and GSSG in the case of hydrogen peroxide or lipid hydroperoxide GSH-Px reduction, respectively. Two types of GSH-Px are found in biological tissues, and one shows high specificity for phospholipid hydroperoxides [87].

With respect to nonenzymatic peroxide quenchers, thiodipropionic acid and dilauryl thiodipropionate are capable of decomposing peroxides and peracids, and have been approved as food additives when added at concentrations $\leq$200 ppm; however, they are relatively ineffective antioxidants and are therefore rarely used. Methionine, which has been found to be antioxidative in some lipid systems [88], is thought to decompose peroxides by mechanisms similar to those of thiodipropionic acid and dilauryl thiodipropionate and has been studied as an antioxidant in the context of lipid foods [85,89–91].

C. Singlet Oxygen

Light is capable of activating photosensitizers such as chlorophyll, riboflavin, and heme-containing proteins to their excited state. These light-activated sensitizers can promote lipid oxidation reactions by exciting triplet oxygen ($^3O_2$) to singlet oxygen ($^1O_2$), which can directly oxidize lipid substrate to hydroperoxides, or by transferring an electron to triplet oxygen to form superoxide anion [92]. In general, singlet oxygen quenchers deplete singlet oxygen of its excess energy and dissipate the energy in the form of heat. As discussed earlier, carotenoids (e.g., β-carotene, lycopene, lutein) are common active singlet oxygen quenchers found in plant foods and are known to be effective at low oxygen partial pressure. Carotenoids inactivate photosensitizers by absorbing their energy to yield the excited state of the carotenoid, which then returns to the ground state *via* transfer of energy (e.g., heat) into the surrounding solvent [93].

D. Lipoxygenase

Lipoxygenases are enzymatic lipid oxidation catalysts found in plants and some animal tissues. Lipoxygenase activity can be controlled by thermal inactivation during the course of food processing and by plant breeding programs that result in cultivars containing decreased concentrations of these enzymes. Phenolics have been shown to indirectly inhibit lipoxygenase activity by serving as free radical inactivators and also by reducing the iron in the active site of the enzyme to the catalytically inactive ferrous state [33].

E. Lipid Oxidation Breakdown Products

The oxidation of unsaturated fatty acids results in the inevitable formation of low-molecular-weight breakdown products *via* β-scission reactions, as outlined earlier. These reactions lead to a slew of

oxidation products—some of which are volatile—known as secondary lipid oxidation products, which affect both the sensory characteristics and the functional properties of the food. Aroma-active molecules that are associated with oxidative rancidity arise from the production of these secondary products, particularly those with aldehydes, ketones, and alcohols as functional groups [94]. Secondary lipid oxidation products, and in particular aldehydes, also have a deleterious effect on food quality and nutritional composition by reacting with specific amino acid residues in proteins and various vitamins. Secondary products arising from lipid oxidation are also known to alter the function of proteins, enzymes, biological membranes, lipoproteins, and DNA [95–98].

Due to the fact that aldehydic secondary lipid oxidation products are reactive with many macromolecules, biological systems have evolved mechanisms that control their activity and mitigate their potential damaging effects. For example, sulfur- and amine-containing compounds have the ability to directly interact with (i.e., quench) aldehydes by either Schiff base or 1,4-addition (Michael addition) reactions which may help to explain why many proteins, peptides, amino acids, phospholipids, and nucleotides display antioxidant activity in assays that specifically measure secondary oxidation products (e.g., headspace hexanal, thiobarbituric acid reactive substances). Carnosine and anserine, which can make up over 1% of the wet weight of muscle tissue, are capable of forming complexes with aldehydic lipid oxidation products [99]. Carnosine is more effective with respect to forming adducts with aldehydes than its constituent amino acids, namely, histidine and β-alanine [100,101]. GSH has also been shown to quench aldehydes; however, at concentrations found in muscle foods, carnosine appears to be the dominant aldehyde-binding component [101].

V. EFFECT OF PHYSICAL MATRIX ON LIPID OXIDATION

Lipids are most frequently encountered as dispersions (e.g., emulsions) in foods, and the interfacial region (e.g., oil/water, oil/membrane, oil/air) of these systems is thought to be important with respect to oxidation reactions. This is presumably a result of increased oxygen contact, the presence of aqueous phase free radicals in proximity to the lipid phase, the presence of reactive oxygen generating systems and prooxidative metals, and possibly the migration of relatively polar lipid hydroperoxides out of the hydrophobic lipid core and toward the more polar interface. This topic has been the subject of intense study in recent years and remains an active area of research [69,102–105].

A. Antioxidant Polarity

The effectiveness of phenolic antioxidants is often dependent on their polarity. Porter et al. used the term "antioxidant paradox" to describe how polar antioxidants are generally more effective in bulk lipids, whereas (paradoxically) nonpolar antioxidants are generally more effective in dispersed lipids [106]. In tocopherol-stripped bulk corn oil, Trolox (a water-soluble analog of α-tocopherol) was shown to more effectively inhibit lipid hydroperoxide formation than the highly hydrophobic α-tocopherol. However, when tocopherol-stripped corn oil was emulsified with Tween 20, α-tocopherol inhibited peroxide formation more effectively than Trolox. The observed increase in α-tocopherol antioxidant activity (relative to Trolox) in emulsified oil was attributed to its retention in the oil phases and, possibly, to its ability to concentrate at the oil–water interface due to its surface activity. The authors argued that the lower antioxidant activity of Trolox in emulsions was due to its partitioning into the water phase, where it was not able to inhibit autoxidation of the oil [107]. Similar effects have been observed for the phenolic antioxidants in rosemary extracts, with more polar compounds (e.g., carnosic and rosmarinic acids) shown to be more effective in bulk oils and less polar compounds (e.g., carnosol) shown to be more effective in

emulsified lipids [35]. Similarly, the antioxidant activity of carnosic acid was improved in emulsified corn oil when its hydrophobicity was increased by methylation [107].

B. Electrostatic Interactions

The net charge of dispersed lipid droplets is determined by emulsifier type (anionic, cationic, nonionic), which has been shown to affect lipid oxidation rates, especially in the presence of transition metals. Iron, copper, and other transition metals are ubiquitous in most water systems and are endogenous components of virtually all foods and, as such, are key catalysts in the oxidation of dispersed food lipids. When the net surface charge of dispersed lipids in the form of micelles [108,109] or phospholipid vesicles [110] is negative, iron-catalyzed lipid oxidation rates are markedly higher than when those same interfaces carry a net positive charge. The former effect is thought to be due to electrostatic interactions between cationic (positive) iron present in the aqueous phase and anionic (negative) lipid emulsion droplets, thus resulting in conditions that favor the physical interaction between prooxidant and lipid substrate. Conversely, when the dispersed lipid phase carries a net cationic charge, electrostatic repulsive forces prevent cationic iron species from coming into close proximity of the substrate. While cationic emulsifiers are uncommon in foods, protein emulsifiers can confer a net positive charge to lipid droplets when the pH of the matrix is set below their isoelectric point (pI). For example, oil-in-water emulsions stabilized by soy, whey protein isolate, or casein are more oxidatively stable at low pH (i.e., emulsion droplets are positively charged) than at high pH (i.e., emulsion droplets are negatively charged) [111]. Cationic oil-in-water emulsion droplets produced by absorption of chitosan onto phosphatidylcholine have also been shown to increase the oxidative stability of dispersed lipids [112].

C. Encapsulation

Encapsulation is an effective means of inhibiting lipid oxidation. While this approach has been used to a great extent in the pharmaceutical industry, there are relatively fewer food products that could potentially benefit.

The putative mechanisms by which encapsulation inhibits lipid oxidation include the physical inhibition of oxygen diffusion into the lipid substrate, chemical (e.g., free radical scavenging) and physical (e.g., chelation) antioxidant properties of the encapsulating agents, and the interaction of lipid oxidation products with the encapsulating material. Both protein- and carbohydrate-encapsulating agents have been found to retard oxidation rates. The effectiveness of these encapsulating agents depends on factors such as the concentration of the encapsulating agent [113], the method of encapsulation (which affects the porosity of the encapsulating layer) [114], and the environmental relative humidity under which the encapsulated lipid is stored [115]. Encapsulation technologies for lipids are covered in Chapter 16.

D. Physical State of the Lipid

Lipids in foods often exist as a combination of both liquid and crystalline states, a condition that depends on intrinsic and extrinsic factors such as fatty acid profile and temperature, respectively. The influence of liquid fat concentration on oxidation rates was investigated in liposomes, where arachidonic acid oxidation rates were found to be higher at temperatures below the solid–liquid phase transition temperature of the host lipid [116]. The increase in oxidation rates was attributed to phase separation of the most unsaturated fatty acids, which led to an effective increase in oxidizable substrate concentration within the localized domains [14]. At present, little is known about how transition temperatures influence oxidation rates in food lipids.

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21 Effects and Mechanisms of Minor Compounds in Oil on Lipid Oxidation

Eunok Choe

CONTENTS

I. INTRODUCTION

Lipid oxidation produces low-molecular-weight off-flavor compounds and affects the quality and shelf life of foods [1]. The off-flavor compounds decrease consumer acceptability or industrial use of lipid as a food ingredient. Lipid oxidation destroys essential fatty acids and produces toxic compounds and oxidized polymers [2]. Lipid oxidation is influenced by many factors: energy input such as high temperature or light, food composition, oxygen type, and minor compounds such as free fatty acids, mono- and diacylglycerols, metals, peroxides, thermally oxidized compounds, pigments, and phenolic compounds. Some of them accelerate oil oxidation and others act as antioxidants. The major mechanisms of lipid oxidation during food processing and storage are autoxidation and photosensitized oxidation. The improvement of the oxidative stability of lipid foods can be achieved by a thorough understanding of the chemical mechanisms of lipid oxidation and the functions of some compounds present in oil, naturally or added on purpose, other than triacylglycerols.

II. MECHANISMS OF LIPID AUTOXIDATION

Autoxidation of lipids is a free radical chain reaction, which requires lipids in radical forms. Lipids are normally in a nonradical singlet state, and their reaction with atmospheric triplet oxygen ($^3O_2$) is thermodynamically unfavorable. The atmospheric oxygen has two unpaired electrons, one each in two of $2p\pi^*$ orbitals (Figure 21.1), and hardly reacts with nonradical lipids [3]. Therefore, an occurrence of the autoxidation requires removal of a hydrogen atom in the lipids (RH), with production of alkyl radicals of lipids (lipid radicals, $R^{\bullet}$). Heat, metals, and light accelerate the formation of lipid radicals. The energy required for the removal of the hydrogen atom from lipid molecules

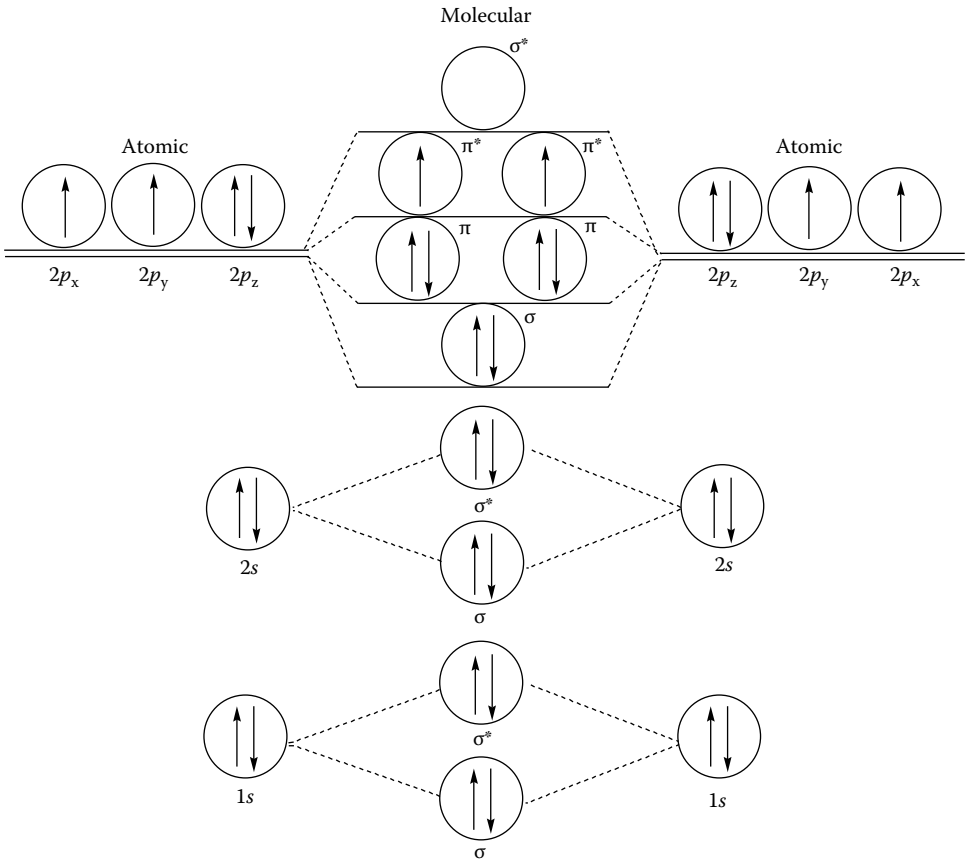

FIGURE 21.1 Molecular orbital of triplet oxygen, $^3O_2$.

is dependent on the position of hydrogen in the molecules. Allylic hydrogen, especially hydrogen bound to the carbon between two double bonds, is easily removed. The energy required for the removal of the hydrogen atom at C8, C11, and C17 of linoleic acid is 314, 209, and 418 kJ/mol, respectively [4]. On formation of lipid radicals by removal of the hydrogen atom, the double bond adjacent to the carbon radical in linoleic and linolenic acids shifts to a more stable next carbon, resulting in conjugated diene and triene, respectively. The double bond shifted mostly takes *trans* form instead of natural *cis* form because of a lower energy level.

The lipid radical reacts with triplet oxygen very quickly at normal oxygen pressure with a rate constant of ca. $2–8 \times 10^9$ M^{-1} s^{-1}, producing peroxy radicals of lipids (lipid peroxy radicals, ROO•), and this reaction is controlled by oxygen migration in unsaturated lipids, whose activation energy is 24 kJ/mol [5]. Lipid peroxy radicals can also react with triplet oxygen. However, the reaction of lipid peroxy radicals with oxygen is slower than the production of lipid peroxy radicals from lipid radicals and oxygen, and thus the concentration of lipid peroxy radicals is usually higher than that of lipid radicals [6]. Lipid peroxy radicals abstract hydrogen atoms from other lipid molecules and produce lipid hydroperoxides (ROOH) and another lipid radical. These diverse radicals catalyze the reaction, and autoxidation is thus called a free radical chain reaction. Figure 21.2 shows the formation of C9- (48%–53%) and C13-hydroperoxide (48%–53%) in the autoxidation of linoleic acid. Oleic acid produces C8- (26%–28%), C9- (22%–25%), C10- (22%–24%), and C11-hydroperoxides (26%–28%), and linolenic acid produces C9- (28%–35%), C12- (8%–13%), C13- (10%–13%), and C16-hydroperoxides (28%–35%) by autoxidation [7].

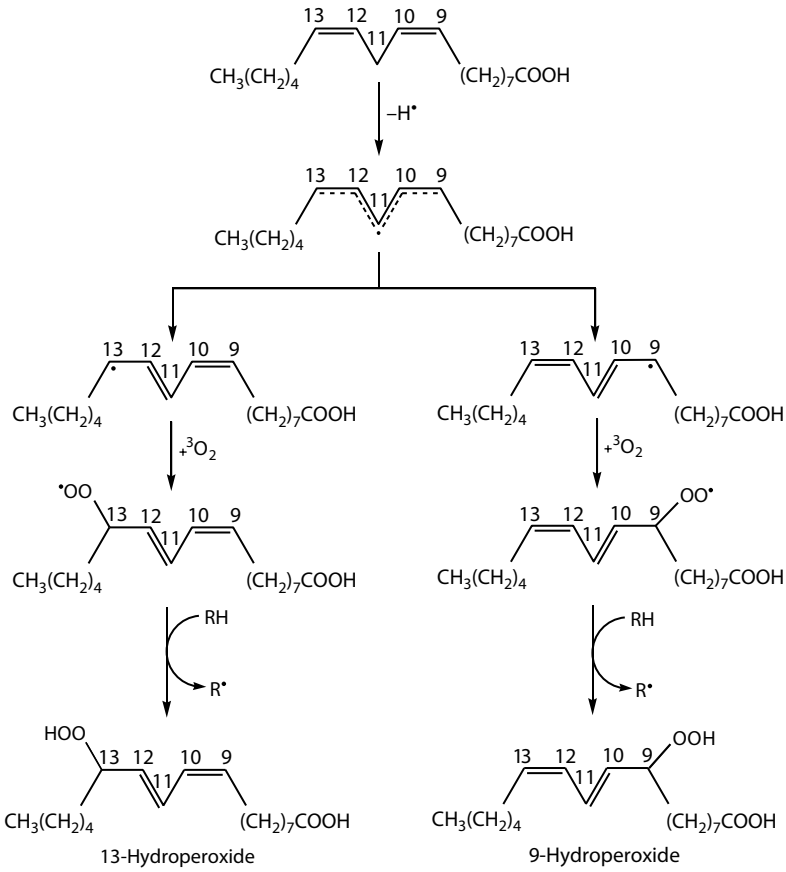

FIGURE 21.2 Hydroperoxide formation in the autoxidation of linoleic acid.

Lipid hydroperoxides, the primary oxidation products, are relatively stable at room temperature. However, in the presence of metals or at high temperature, they are readily decomposed to alkoxy radicals (RO•) via homolytic cleavage in the peroxide bond, requiring 192 kJ/mol [8]. The alkoxy radical is unstable and undergoes homolytic β-scission in the bond between the carbon having an oxygen radical and its adjacent carbon, ultimately producing low-molecular-weight short-chain hydrocarbons and carbonyl compounds such as aldehydes and alcohols (Figure 21.3). Decomposition products of oleic, linoleic, and linolenic acids are listed in Table 21.1. Decomposition of lipid hydroperoxides is largely affected by temperature. The higher the temperature is, the faster the hydroperoxide decomposition is. Crude herring oil showed a higher rate of hydroperoxide decomposition than that of hydroperoxide formation at 50°C in the dark, but the reverse phenomenon was observed in the same oil at 0°C or 20°C [6].

Most decomposition products of lipid hydroperoxides are responsible for the off-flavor in the oxidized oil. No single compound is responsible for the oxidized flavor of oils. Aliphatic carbonyl compounds have lower threshold values and more influence on the oil flavor than hydrocarbons; threshold values for hydrocarbons, alkanals, alk-2-enals, and alka-2,4-dienals are in the range of 90–2150 ppm, 0.04–1 ppm, 0.04–2.5 ppm, and 0.04–0.3 ppm, respectively [7]. *Trans*-2-hex-2-enal gives a grasslike flavor, and a fishlike flavor is related to *trans*, *cis*, *trans*-deca-2,4,7-trienal, and oct-1-en-3-one in oxidized soybean oil [3]. The volatile compounds frequently detected in autoxidized oil are propenal, butanal, pentane, hexanal, hepta-2,4-dienal, oct-1-en-3-ol, dec-2-enal, and deca-2,4-dienal [7,9,10]. Pentane, hexanal, and deca-2,4-dienal are frequently used indicators to determine the extent of oil oxidation [11–13].

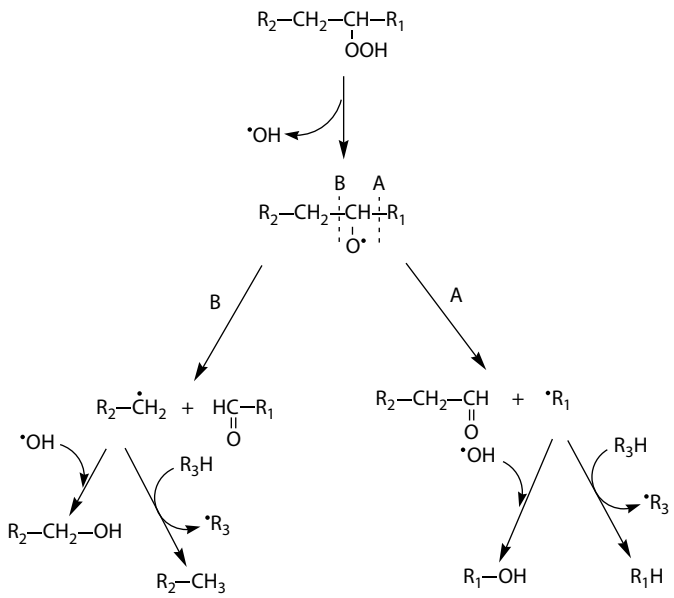

FIGURE 21.3 Mechanisms of C8-hydroperoxide decomposition to form secondary oxidation products during oxidation of oleic acid (R_1,—CH_2—$(CH_2)_5$—COOH; R_2,—CH_2—$(CH_2)_7$—CH_3).

TABLE 21.1
Decomposition Products of Hydroperoxides of Fatty Acid Methyl Esters by Autoxidation

Fatty Acid	Carbonyl Compounds	Hydrocarbons
Oleic acid	1-Heptanol, octanal, methyl heptanoate, nonanal, methyl octanoate, methyl 8-oxooctanoate, decanal, 2-decenal, methyl 9-oxononanoate, methyl 10-oxodecanoate, methyl 10-oxo-8-decenoate, methyl 11-oxo-9-undecenoate	Heptane, octane
Linoleic acid	Pentanal, 1-pentanol, hexanal, 2-octenal, 1-octene-3-ol, methyl heptanoate, 2-nonenal, methyl octanoate, methyl 8-oxooctanoate, 2,4-decadienal, methyl 9-oxononanoate, methyl 10-oxodecanoate	Pentane
Linolenic acid	Propanal, butanal, 2-butenal, 2-pentenal, 2-hexenal, methyl heptanoate, 3,6-nonadienal, methyl octanoate, decatrienal, methyl nonanoate, methyl 9-oxononanoate, methyl 10-oxodecanoate	Ethane, pentane

Source: Frankel, E.N., Chemistry of autoxidation: Mechanism, products, and flavor significance, in: *Flavor Chemistry of Fats and Oils*, Min, D.B. and Smouse, T.H. (eds.), American Oil Chemists' Society, Champaign, IL, 1985, pp. 1–34.

The autoxidation rate depends greatly on the formation of lipid radicals, which depends mainly on the type of lipids. Autoxidation occurs more quickly in more unsaturated oils than in less unsaturated oils [14,15]. Soybean, safflower, or sunflower oil (iodine values > 130) showed a significantly ($p < 0.05$) shorter induction period in the autoxidation at 110°C than coconut or palm kernel oil whose iodine value is less than 20 [16]. The activation energy in the autoxidation of methyl linoleate, linolenic acid, trilinolein, and trilinolenin was 84 ± 8.4 kJ/mol [17], 65 ± 4 kJ/mol [18], 34 ± 8 kJ/mol [5], and 9 ± 2 kJ/mol [5], respectively. The relative autoxidation rate of oleic, linoleic, and linolenic acids was reported as 1:40 to 50:100 on the basis of oxygen uptake [3].

Oxygen concentration affects the rate of lipid oxidation. When oxygen content is low, for example, less than 4% in the headspace, the oxidation rate is dependent on the oxygen concentration but independent of lipid concentration [19,20]. When oxygen is present at sufficiently high concentration,

the rate of oil autoxidation is independent of oxygen concentration but directly dependent on the lipid concentration [21,22]. The rate constant for oxygen disappearance was 0.049, 0.058, 0.126, and 0.162 ppm/h at 2.5, 4.5, 6.5, and 8.5 ppm of dissolved oxygen, respectively, in the autoxidation of soybean oil at 55°C [23]. The content of dissolved oxygen present in edible oil is usually sufficient to oxidize the oil to a peroxide value of ca. 10 meq/kg in the dark [12]. The concentration of dissolved oxygen in soybean oil was reported as 55 ppm at room temperature [20]. Autoxidation of oils increases as temperature increases [24,25]. The effect of oxygen concentration on the autoxidation of oil also increases at high temperature [20]. Oxygen is transported into the oil by diffusion at low temperature because there is little stirring in the oil. However, at high temperature, oxygen can penetrate into the oil from the surface by both diffusion and convection caused by increased molecular movement in the oil, which increases migration of oxygen into the oil.

III. MECHANISMS OF PHOTOSENSITIZED OXIDATION OF LIPIDS

Light increases lipid oxidation, and the presence of photosensitizers further accelerates it. Photosensitizers are normally in a singlet state (^{1}Sen) and become excited in picoseconds upon absorption of light energy [26]. Excited singlet-state sensitizers (^{1}Sen*) can return to their ground state via emission of light ($k = 2 \times 10^8$ s^{-1}) or intersystem crossing ($k = 1–20 \times 10^8$ s^{-1}). Intersystem crossing is a transition process without radiation and results in an excited triplet state of sensitizers (^{3}Sen*). Excited triplet sensitizers may accept a hydrogen atom or an electron from the substrate (RH) and produce radicals (type I), R$^\bullet$ and RH$^{\bullet+}$, as shown in Figure 21.4. Readily oxidizable phenols and amines or reducible quinones are substrates that favor the type I process [27]. Excited triplet sensitizers can also react with triplet oxygen and produce superoxide anion radicals ($O_2^{-\bullet}$) by electron transfer or form singlet oxygen by energy transfer (type II). As a result, the excited triplet sensitizers return to their ground singlet state. Kochevar and Redmond [28] reported that one molecule of the sensitizer may generate 10^3 to 10^5 molecules of singlet oxygen before becoming inactive. Unsaturated hydrocarbons and aromatic compounds are not so readily oxidized or reduced and more often favor the type II process [27]. Chlorophylls are photosensitizers that take type II pathway. Photosensitized oxidation of lipids follows the singlet oxygen oxidation pathway (type II) [29], and Lee and Min [30] suggested that singlet oxygen is involved in the initiation of oil oxidation.

Singlet oxygen has two electrons in one of the $2p\pi^*$ orbitals, and the other $2p\pi^*$ orbital does not have an electron. This completely vacant orbital makes singlet oxygen electrophilic, and singlet oxygen directly reacts with double bonds having high electron density via six-membered ring formation (Figure 21.5). There is no formation of lipid radicals in the oxidation by singlet oxygen [31], and thus the resulting hydroperoxides have nonconjugated double bonds as well as conjugated double bonds. Production of nonconjugated hydroperoxides is not observed in the autoxidation.

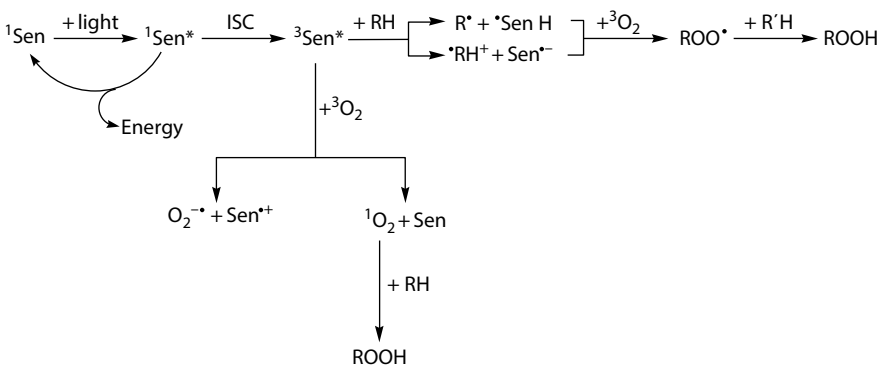

FIGURE 21.4 Photosensitized oxidation (Sen, sensitizer; ISC, intersystem crossing; RH/R′H, substrate).

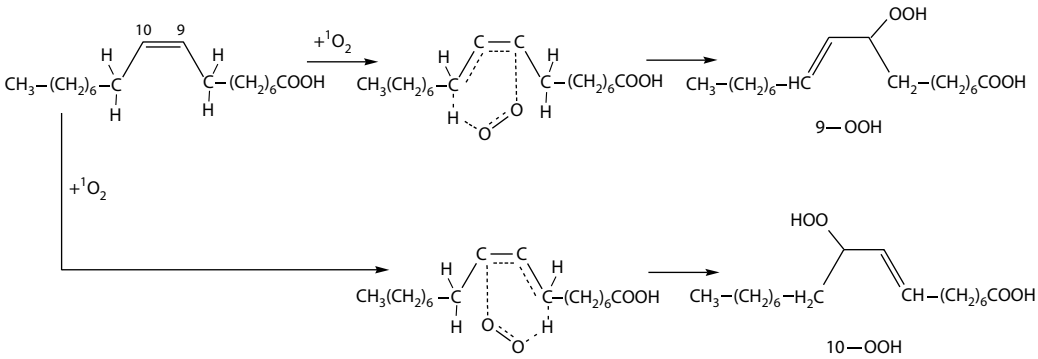

FIGURE 21.5 Hydroperoxide formation in the oxidation of oleic acid by singlet oxygen.

TABLE 21.2
Hydroperoxides of Fatty Acids by Singlet Oxygen Oxidation

Oleic Acid	Linoleic Acid	Linolenic Acid
C9-OOH (48%)[a]	Conjugated	Conjugated
C10-OOH (52%)	C9-OOH (32%)	C9-OOH (23%)
	C13-OOH (34%)	C12-OOH (12%)
	Nonconjugated	C13-OOH (14%)
	C10-OOH (17%)	C16-OOH (25%)
	C12-OOH (17%)	Nonconjugated
		C10-OOH (13%)
		C15-OOH (13%)

Source: Frankel, E.N., Chemistry of autoxidation: Mechanism, products, and flavor significance, in: *Flavor Chemistry of Fats and Oils*, Min, D.B. and Smouse, T.H. (eds.), American Oil Chemists' Society, Champaign, IL, 1985, pp. 1–34.

[a] Relative content.

The oxidation of oleic, linoleic, and linolenic acids by singlet oxygen produces C9- and C10-hydroperoxides; C9-, C10-, C12-, and C13-hydroperoxides; and C9-, C10-, C12-, C13-, C15-, and C16-hydroperoxides, respectively (Table 21.2).

Lipid hydroperoxides formed by singlet oxygen oxidation are decomposed by the same mechanism as the autoxidation; however, kinds and amounts of decomposition products are slightly different. Singlet oxygen oxidation of oleic acid produces higher amounts of dec-2-enal and octane than the autoxidation does, whereas contents of octanal and 10-oxodecanoic acid are higher in autoxidized oleic acid than in singlet oxygen–oxidized oleic acid [7]. Singlet oxygen–oxidized linoleic and linolenic acids produce noticeable amounts of hept-2-enal and but-2-enal, which are negligible in autoxidized lipids [7]. Figure 21.6 shows a formation of but-2-enal by the singlet oxygen oxidation of linolenic acid. Min and others [32] showed that 2-pentylfuran and 2-(2-pentenyl)furan were formed from linoleic and linolenic acids, respectively, in the presence of chlorophyll under light. 2-Pentylfuran and 2-(2-pentenyl)furan cause a beany flavor [33–36].

The reaction rate of singlet oxygen with lipids is much higher than that of triplet oxygen; the reaction rates of singlet oxygen and triplet oxygen with linoleic acid are 1.3×10^5 and 8.9×10^1 $M^{-1}\,s^{-1}$,

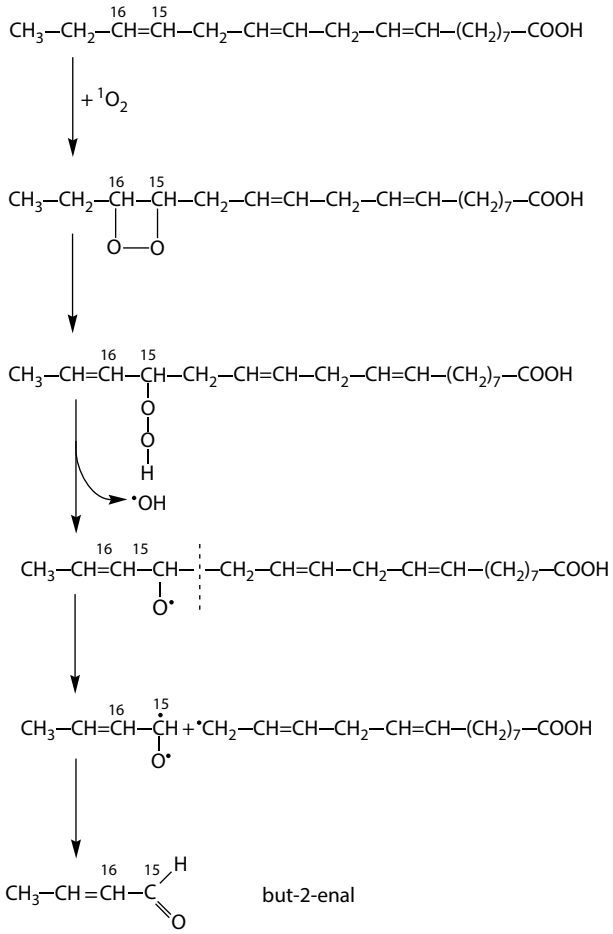

FIGURE 21.6 Formation of but-2-enal from linolenic acid by singlet oxygen.

respectively [29]. The rate of singlet oxygen oxidation depends on the kind of lipids, and differences in the oxidation rates among different lipids are lower in the singlet oxygen oxidation than in the autoxidation. The reaction rate of singlet oxygen with fatty acids is in the order of 10^4 M^{-1} s^{-1}, and the ratio of the rates among stearic, oleic, linoleic, and linolenic acids is 1.2:5.3:7.3:10.0 [37]. Soybean oil reacts with singlet oxygen at the rate of 1.4×10^5 M^{-1} s^{-1} in methylene chloride at 20°C [38]. Conjugation of double bonds in dienes or trienes does not affect the oxidation rate by singlet oxygen [39].

Since the activation energy for the singlet oxygen oxidation is not high (0–25 kJ/mol), temperature has little effect on the singlet oxygen oxidation [39,40] and light is more effective. The light with shorter wavelength results in higher oxidation by singlet oxygen than the light with longer wavelength [41]. The light effect on the lipid oxidation becomes less as temperature increases [25].

IV. EFFECTS OF MINOR COMPOUNDS IN OIL ON THE LIPID OXIDATION

Edible oil consists of mostly triacylglycerols, but it also contains minor compounds such as free fatty acids, mono- and diacylglycerols, metals, phospholipids, thermally oxidized compounds and peroxides, chlorophylls, carotenoids, and tocopherols and other phenolic compounds. Free fatty acids, hydroperoxides, phospholipids, iron, chlorophylls, and tocopherols were present in crude

soybean oil at 0.74%, 2.4 meq/kg, 510, 2.90, 0.30, and 1670 ppm, respectively; however, most of them were removed during refining except tocopherols [42]. The refined, bleached, and deodorized (RBD) soybean oil contained 1670 ppm tocopherols. The minor compounds increase or decrease the lipid oxidation individually or interactively.

A. Free Fatty Acids and Mono- and Diacylglycerols

Free fatty acids, monoacylglycerols, and diacylglycerols are the predominant minor components in bulk oils, derived from the products of oil refining processing, hydrolysis during storage, or the parts from oil seed membranes [43]. Most of the free fatty acids in crude oil are removed by oil refining. Crude and refined soybean oil contains free fatty acids at about 0.7% and 0.02%, respectively [42]. Roasted sesame oil is consumed without refining and contains a higher amount of free fatty acids (0.72%) [44] than other RBD oils. Mono- and diacylglycerols are found in edible oils at 1%–10%; rapeseed, soybean, safflower, olive, and palm oils contain 0.8%, 1.0%, 2.1%, 5.5%, and 5%–11% of diacylglycerols, respectively; and the monoacylglycerol content of soybean oil ranges from 0.07% to 0.11% [45–47].

Free fatty acids are more susceptible to autoxidation than esterified lipids [48]. Rate constants for the autoxidation of linolenic acid and soy lecithin at 100°C are 1.6×10^{-2} s^{-1} and 1.8×10^{-3} s^{-3}, respectively [18]. Free fatty acids present in edible oil accelerate the oxidation of oil [49,50]. The autoxidation rate of soybean oil at 55°C was 2.55×10^{-2} h^{-1} and the presence of stearic acid at 1% increased the rate to 2.91×10^{-2} h^{-1} [51]. The carboxyl group in the free fatty acid molecule does not easily dissolve in the hydrophobic oil, which makes free fatty acids be more concentrated on the surface. Mistry and Min [50] suggested that free fatty acids decreased the surface tension of soybean oil and increased the diffusion rate of oxygen from the headspace into the oil, which accelerates the oil oxidation. Paradiso et al. [52] suggested that free fatty acids are prevailingly involved in accelerating peroxide decomposition more than in their formation, resulting in limited polymerization of polar triacylglycerols. The prooxidant effect of free fatty acids is dependent on the chain length and degree of unsaturation; in a refined peanut oil at 30°C, the shorter the chain length and the higher the unsaturation degree were, the stronger the prooxidant activity was, which is because short-chain hydrocarbons exert less positive inductive effect on the carboxyl group and steric hindrance, but free radical formation is faster in free fatty acids with a high degree of unsaturation [53]. The effect of free fatty acids on oil oxidation is also concentration dependent; the oxidative stability of peanut oil decreased at concentrations lower than 0.5% of free fatty acids, while prooxidant effect gradually decreased at concentrations higher than 1% [54]. Aubourg [53] also reported that the prooxidant activity of free fatty acids increased with increased free fatty acid concentration within the range of 0.01%–1.00%.

Mono- and diacylglycerols increased the soybean oil oxidation at 55°C in the dark [55,56]. They have hydrophilic hydroxy groups and hydrophobic hydrocarbons, which makes them be positioned on the surface of oil. Mono- and diacylglycerols accelerate the oil oxidation by decreasing the surface tension of oil and increasing the oxygen diffusion into the oil. However, different results were shown; diacylglycerols improved the oxidative stability of the oil [57], and Gomes et al. [58] reported an insignificant effect of monoacylglycerols on the oxidation of olive oil. This could be partly due to the concentration, and Chen et al. [43] suggested that the addition of diacylglycerols in small amounts (<2.5 wt%) may not be able to alter the pathway of oil oxidation.

B. Metals

Crude oil contains transition metals such as iron and copper. Crude soybean oil contains 13.2 ppb of copper and 2.80 ppm of iron; however, the quantity decreases by oil refining [59,60], and refined soybean oil contains 2.5 ppb of copper and 0.20 ppm of iron, respectively [61]. Edible oils consumed without refining contain relatively high amounts of copper and iron; virgin olive oils contain 15.5 ppb of copper and 0.03 ppm of iron, respectively [62].

Metals increase the rate of oil oxidation by reducing the activation energy of the initiation step in the lipid autoxidation [63]. Micciche et al. [64] reported that the initiation of the oxidation of methyl linoleate requires the coexistence of ferrous (Fe^{2+}) and ferric ions (Fe^{3+}), and the oxidation is pH dependent. Metals accelerate the lipid oxidation by participating in the production of lipid radicals and some of the reactive oxygen species such as singlet oxygen and hydroxy radicals. Metals directly react with lipids and produce lipid radicals. They also produce superoxide anion radicals ($O_2^{-\bullet}$) by the reaction with triplet oxygen [26]. Superoxide anion radicals can produce hydrogen peroxide by dismutation, and the reaction between hydrogen peroxide and superoxide anion radicals produces hydroxy radicals and singlet oxygen (Haber–Weiss reaction) [65]. The rate of the Haber–Weiss reaction is increased in the presence of transition metals [66]:

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow O_2 + H_2O_2 \text{ (dismutation)}$$

$$H_2O_2 + O_2^{-\bullet} \rightarrow HO^{\bullet} + OH^- + {}^1O_2 \text{ (Haber–Weiss reaction)}$$

The hydroxy radical is also produced from decomposition of hydrogen peroxide in the presence of metals, so-called Fenton reaction [67]. Metals increase the decomposition of lipid hydroperoxide (ROOH) to alkoxy ($RO^{\bullet}$) or lipid peroxy ($ROO^{\bullet}$) radicals, which can accelerate the lipid oxidation [68]:

$$ROOH + Fe^{2+} \rightarrow RO^{\bullet} + Fe^{3+} + OH^-$$

$$ROOH + Fe^{3+} \rightarrow ROO^{\bullet} + Fe^{2+} + H^+$$

Decomposition rate of lipid hydroperoxides by ferrous ion is higher than that by ferric ion [69]. Lactoferrin binds the iron in fish oil or soybean oil and decreases the prooxidant activity of iron in the oil autoxidation at 50°C–120°C [70].

C. Phospholipids

Crude oil contains phospholipids such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, and phosphatidylserine, but most of them are removed by oil processing such as degumming. The oils that are consumed without refining contain higher amounts of phospholipids. Crude soybean oil contains phosphatidylcholine and phosphatidylethanolamine at 501 and 214 ppm, respectively; however, RBD soybean oil contains 0.86 and 0.12 ppm of phosphatidylcholine and phosphatidylethanolamine, respectively [71]. Unroasted sesame oil contains 690 ppm of phospholipids [72]. Extra virgin olive oil contains 34–156 ppm phospholipids and filtration of the oil lowers the contents to 21–124 ppm [73].

Phospholipids act as antioxidants and prooxidants depending on the presence of metals and concentration. Phosphatidylcholine decreased the oxidation of docosahexaenoic acid (DHA) at 25°C–30°C in the dark [74]. The egg yolk phospholipids at 0.031%–0.097% decreased the autoxidation of DHA-rich oil and squalene, and the antioxidant activity of egg yolk phosphatidylethanolamine was higher than that of phosphatidylcholine [75]. The mechanism of antioxidative effects of phospholipids has not yet been elucidated in detail. The polar group in phospholipids such as choline and ethanolamine plays an important role in decreasing the autoxidation of oil under most conditions [76,77]. The nitrogen moieties can donate a hydrogen atom or an electron to antioxidant radicals such as tocopheroxy radicals and regenerate the antioxidants. Phospholipids also chelate prooxidative metals and decrease the lipid oxidation [73]. The antioxidant activity of phosphatidic acid and phosphatidylethanolamine was higher than those of phosphatidylcholine, phosphatidylglycerol, and phosphatidylinositol in the autoxidation of soybean oil in the presence of 1 ppm iron [78].

Soybean oil oxidation was decreased with addition of 5–10 ppm phospholipids, but higher amounts of phospholipids acted as prooxidants; phosphatidylcholine (300 ppm) added to purified

soybean oil increased the oil oxidation [78]. The prooxidant activity of phospholipids was suggested to be related to the copresence of hydrophilic and hydrophobic groups in the structure. Phospholipids decrease the surface tension of oil and increase the diffusion of oxygen from the headspace to the oil, which accelerates the oil oxidation. When 1 ppm iron was copresent in the oil, the prooxidant activity of phospholipids at 300 ppm was not observed, and the phospholipids even decreased the soybean oil oxidation [78]. Phospholipids having polyunsaturated fatty acids can be oxidized in the presence of iron, while the phospholipids having monounsaturated fatty acids are not oxidized. Ferric ion binds to the lipid phosphate, which catalyzes the breakdown of hydroperoxides, and the oxidation of polyunsaturated phospholipids catalyzed by ferric ion occurs selectively adjacent to the specific double bond such as C9 or C11 [79].

The oxidation of phospholipids was also found in the oxidation of extra virgin olive and perilla oil blend under singlet oxygen [80]. There are very limited numbers of researches on the phospholipid effect on the singlet oxygen–related photooxidation of oil. Phosphatidylcholine and phosphatidylethanolamine did not affect the photooxidation of stripped canola oil; however, they significantly increased the oil oxidation under singlet oxygen produced with chlorophyll through protection of chlorophyll from photodegradation [81].

D. Thermally Oxidized Compounds and Hydroperoxides

Since crude oil is usually processed at high temperature, thermally oxidized compounds are produced and retained in edible oils. Dimers and trimers joined through carbon-to-oxygen linkage and hydroxy dimers are examples of oxidized compounds produced during oil processing. The RBD soybean oil contains thermally oxidized compounds at 1.2%, and these oxidized compounds accelerate the oil autoxidation [82]. Lipid hydroperoxides increase the oxidation of soybean oil at 55°C in the dark [83]. The prooxidant activity of oxidized compounds and peroxides is related to their structure. Oxidized compounds have both hydrophilic group and hydrophobic hydrocarbons, which can lower the surface tension of oil and increase oxygen diffusion into the oil to accelerate the oil oxidation [84].

E. Pigments

The most frequently found pigments in edible oils, especially crude oil, are chlorophylls and carotenoids. Chlorophylls are found in virgin olive oil and crude rapeseed oil at 10 and 5–35 ppm, respectively [85]. Crude palm oil and red palm olein contain high amounts of carotenoids of 500–700 ppm [86]. Virgin olive oil contains 1.0–2.7 ppm of β-carotene and 0.9–2.3 ppm of lutein [87]. These pigments are generally removed during the oil processing, especially the bleaching process. Crude canola oil contains 26.2 ppm of chlorophylls; however, only 5% of chlorophylls (1.3 ppm) remained in the bleached oil [88].

Chlorophylls and their degradation products, pheophytins and pheophorbides, act as antioxidants in the autoxidation of lipids [89,90]. Chlorophylls decrease the contents of free radicals in oil possibly by donating hydrogen atoms to free radicals [90], which can break the chain reaction of lipid oxidation. Porphyrin was proposed to be an essential chemical structure for the antioxidant activity of chlorophylls [91]. The antioxidative activity of chlorophylls depends on the derivatives present, the lipid as a substrate, and temperature. Chlorophyll showed a higher antioxidant activity than pheophytin in the autoxidation of rapeseed and soybean oils at 30°C [89]. Pheophytin *a* increased the induction period in the autoxidation of virgin olive oil at 60°C and 80°C, and the antioxidation is concentration dependent [87]. The copresence of pyropheophytin *a* improves the antioxidant activity of pheophytin *a*, and the formation of pyropheophytin from pheophytin is favored at higher temperature [87].

Chlorophylls and their degradation products act as sensitizers in the presence of light and produce singlet oxygen by transferring the energy to atmospheric triplet oxygen, which accelerates the

lipid oxidation [92,93]. Pheophytin has a higher sensitizing activity than chlorophyll, but lower than pheophorbide [39,93].

Carotenoids are tetraterpenoid compounds consisting of isoprenoid units. Double bonds in carotenoids are conjugated and in all *trans* form. β-Carotene is the most frequently found carotenoid in oils and is one of the most studied carotenoids in relation to the oil oxidation. β-Carotene decreases lipid oxidation by scavenging free radicals, filtering out the light, and/or quenching singlet oxygen. A reduction potential of β-carotene ($E^{0\prime}$ = 1.06 V) is not high enough to donate a hydrogen atom to lipid radicals or lipid peroxy radicals [94]. However, β-carotene (CarH) can donate a hydrogen atom to hydroxy radical ($HO^{\bullet}$) whose reduction potential is 2.31 V and become a carotene radical ($Car^{\bullet}$). Carotene radical is more stable than hydroxy radical due to its delocalization of unpaired electron through the conjugated polyene system. Carotene radical may react with lipid peroxy radicals ($ROO^{\bullet}$) at low oxygen concentration and form nonradical carotene peroxides (Car-OOR) [95,96], which can terminate the radical chain reaction:

$$CarH + HO^{\bullet} \rightarrow Car^{\bullet} + H_2O$$

$$Car^{\bullet} + ROO^{\bullet} \rightarrow Car\text{–}OOR$$

β-Carotene added to purified olive oil, which did not contain chlorophylls, decreased hydroperoxide formation and headspace oxygen consumption in the oil under light at 25°C by filtering the light, mainly between 400 and 500 nm [92]. The higher the concentration of β-carotene in purified olive oil was, the lower the oil oxidation under light was [92].

β-Carotene decreases photooxidation of soybean oil by quenching singlet oxygen, too [30]. Singlet oxygen quenching by β-carotene is physically achieved by energy transfer and does not involve the oxidation of β-carotene [97]. The high energy of singlet oxygen (93.6 kJ/mol) is transferred to β-carotene whose energy level (88 kJ/mol) is just below that of singlet oxygen [96], resulting in the production of triplet oxygen having less capability to oxidize other compounds. One mole of β-carotene can quench 250–1000 molecules of singlet oxygen at a rate of 1.3×10^{10} M^{-1} s^{-1} [98].

$$^1O_2 + {}^1CarH \rightarrow {}^3O_2 + {}^3CarH^*$$

$$^3CarH^* \rightarrow CarH + \text{Energy emission}$$

The singlet oxygen quenching activity of carotenoids increases with the number of conjugated double bonds in the structure [96]. The substituents in the β-ionone ring of carotenoids also affect the singlet oxygen quenching activity [99]; β-carotene and lycopene, which have 11 conjugated double bonds, are more effective singlet oxygen quenchers than lutein having 10 conjugated double bonds [100]. The presence of oxo and conjugated keto groups or cyclopentane ring in the carotenoid structure increases the singlet oxygen quenching ability, but β-ionone ring substituted with hydroxy, epoxy, or methoxy group is less effective [100]. The antioxidant activity of carotenoids in the photooxidation of oil is concentration dependent. There was no significant difference in the antioxidant activity between lycopene and β-carotene at 10 ppm, but β-carotene was a better antioxidant than lycopene at 20 ppm, and the reverse phenomenon was observed at 40 ppm [100].

Carotenoids having less than nine conjugated double bonds are not good singlet oxygen quenchers; rather, they act as sensitizer quenchers. Carotenoids absorb energy from excited photosensitizers ($^3Sen^*$) and thus inactivate them. The resulting excited triplet carotenoids return to their singlet state by transferring their energy to the surrounding [101].

$$^1CarH + {}^3Sen^* \rightarrow {}^3CarH^* + Sen^3$$

$$^3CarH^* \rightarrow {}^1CarH + \text{Energy emission}$$

Although carotenoids have been known to decrease the lipid oxidation, their prooxidant activity has also been shown. β-Carotene (200 ppm) increased oxygen consumption in the headspace of RBD soybean oil in the dark at 55°C, and this was suggested to be due to the higher standard one-electron reduction potential of β-carotene (0.6 V) compared to that of the alkyl radicals of polyunsaturated fatty acids [102]. Furthermore, β-carotene may not effectively donate a hydrogen atom to lipid peroxy radicals whose reduction potential (0.77–1.44 V) is very similar to that of β-carotene. Therefore, β-carotene cannot efficiently scavenge alkyl or peroxy radicals of polyunsaturated fatty acids, resulting in little antioxidant activity.

Although it is not easy to donate a hydrogen atom to free radicals, β-carotene still may donate electrons to radicals and become a β-carotene cation radical [103,104]. β-Carotene may undergo the addition reaction to lipid peroxy radicals at high oxygen concentration (e.g., higher than 150 mmHg of oxygen) and produce a carotene peroxy radical (ROO–CarH•) [95]. β-Carotene peroxy radical can react with triplet oxygen and then with lipid molecules (R′H), resulting in peroxy carotene hydroperoxide (ROO–CarH–OOH) and lipid radicals to accelerate the lipid oxidation [105]:

$$\text{CarH} + \text{ROO}^{\bullet} \rightarrow \text{ROO–CarH}^{\bullet}$$

$$\text{ROO–CarH}^{\bullet} + {}^{3}\text{O}_2 \rightarrow \text{ROO–CarH–OO}^{\bullet}$$

$$\text{ROO–CarH–OO}^{\bullet} + \text{R}'\text{H} \rightarrow \text{ROO–CarH–OOH} + \text{R}'^{\bullet}$$

Increased production of hydroperoxides, hydroxy hydroperoxides, and epoxy epodioxides of triacylglycerols (mainly triolein) was reported in refined olive oil by β-carotene addition (300 ppm) during oxidation at 110°C [106].

F. Tocopherols

Tocopherols are the most important natural antioxidants present in edible oils, especially soybean, canola, sunflower, and corn oils, and the contents are affected by the cultivar. Animal lipids contain lower amounts of tocopherols than vegetable oils; most vegetable oils contain more than 500 ppm of tocopherols [107], but beef tallow and lard contain only 34 and 18 ppm [108], respectively. Palm oil contains a low amount of tocopherols (107 ppm), but it has a high concentration of tocotrienols; concentrations of α-, γ-, and δ-tocotrienols in palm oil are 211, 353–372, and 56–67 ppm, respectively [109]. Safflower oil contains tocotrienols at 12–15 ppm in addition to 397–540 ppm of tocopherols [110]. The oil refining, especially deodorization, reduces the contents of tocopherols in oils. Crude, bleached, and deodorized soybean oil contains tocopherols at 1670, 1467, and 1138 ppm, respectively [42]. Crude sunflower oil contains 755 ppm of tocopherols, and bleaching and deodorization reduced the contents of tocopherols to 97% and 75%, respectively [111].

Tocopherols decrease the lipid oxidation by scavenging free radicals. Tocopherols have a reduction potential of 0.5 V and can donate a hydrogen atom to the alkyl, alkoxy, and peroxy radicals of lipids whose reduction potentials are 0.6, 1.6, and 1.06 V, respectively [112]. Tocopherols react with lipid peroxy radicals to produce tocopheroxy radicals and lipid hydroperoxide (ROOH) at a rate of 10^4 to 10^9 M^{-1} s^{-1}, which is higher than the reaction rate between lipid peroxy radicals and lipids (10–60 M^{-1} s^{-1}). This results in a decreased production of lipid radicals and lipid oxidation. Tocopheroxy radicals have a resonance structure as shown in Figure 21.7 and are more stable than alkyl, alkoxy, and peroxy radicals of lipids. Tocopheroxy radicals may scavenge another radical to give a stable nonradical product and react with a reducing agent such as ascorbic acid to regenerate tocopherols. On the other hand, it may also attack lipids to produce new reactive lipid radicals that may initiate a new oxidation chain reaction. These secondary reactions of tocopheroxy radicals influence the total antioxidant capacity of tocopherols [113].

When there are not enough lipid peroxy radicals possibly due to the low degree of lipid oxidation, tocopheroxy radicals (TO•) react with each other instead of reacting with lipid peroxy radicals

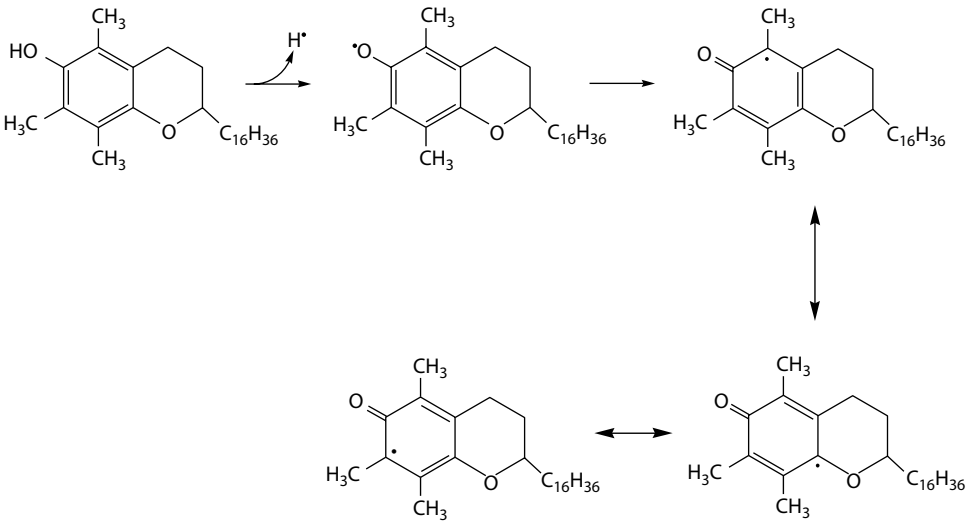

FIGURE 21.7 Resonance stabilization of α-tocopheroxy radical.

and produce tocopheryl quinone and tocopherols (TOH). However, when the lipid oxidation is fast enough, tocopheroxy radicals can react with lipid peroxy radicals and produce tocopherol–lipid peroxy complexes ([TO–OOR]), which are then hydrolyzed to tocopheryl quinone and lipid hydroperoxide [114]:

$$\text{TOH} + \text{ROO}^{\bullet} \rightarrow \text{TO}^{\bullet} + \text{ROOH}$$

$$\text{TO}^{\bullet} + \text{TO}^{\bullet} \rightarrow \text{Tocopheryl quinone} + \text{TOH (under low degree of lipid oxidation)}$$

$$\text{TO}^{\bullet} + \text{ROO}^{\bullet} \rightarrow [\text{TO–OOR}] \rightarrow \text{Tocopheryl quinone} + \text{ROOH (under fast oxidation)}$$

The radical scavenging activity of tocopherols differs among the isomers. δ-Tocopherol generally has the highest free radical scavenging activity followed by γ-, β-, and α-tocopherols [115]. However, Yanishlieva et al. [116] reported that the antioxidant activity of α-tocopherol was higher than that of γ-tocopherol at low concentration (<400–700 ppm) in purified soybean and sunflower oils at 100°C. The optimal concentration of tocopherols as antioxidants is dependent on their oxidative stability; the isomer having lower oxidative stability generally shows lower optimal concentration for the maximal antioxidant activity. α-Tocopherol, the least stable isomer, showed the maximal antioxidant activity at 100 ppm in the autoxidation of soybean oil at 55°C, whereas the optimal concentrations of more stable γ- and δ-tocopherols were 250 and 500 ppm, respectively [117].

Tocopherols, particularly α-tocopherol, act as prooxidants when present at high concentration in oil [118–120]. The prooxidant activity of tocopherols is more obvious when the concentration of lipid peroxy radicals is very low. Addition of 100 ppm α-tocopherol increased the oxidation of purified olive oil at the early stage of autoxidation; however, the same concentration of α-tocopherol added to moderately oxidized purified olive oil or lard (peroxide value = 15 meq/kg) significantly decreased the oil oxidation [121]. Tocopheroxy radical abstracts hydrogen atoms from the lipids with a very low rate and produces lipid radicals, which accelerate lipid oxidation and tocopherol-mediated peroxidation [122]. α-Tocopherol shows the highest prooxidant activity followed by γ- and δ-tocopherols in soybean oil autoxidation at 500–1000 ppm [117]. Ascorbic acid and temperature also affect the prooxidant activity of tocopherols. Ascorbic acid can provide a hydrogen atom to the tocopheroxy radical and thus prevent tocopherol-mediated peroxidation [120]. The prooxidant activity of α-tocopherol decreases as the oxidation temperature increases [123].

In addition to free radical scavenging activity, tocopherols decrease lipid oxidation under light by singlet oxygen quenching [124], but their singlet oxygen quenching activity is 50-fold less than that of carotenoids [99]. Singlet oxygen quenching by tocopherols is physically achieved, mainly by charge transfer. Tocopherols donate an electron to singlet oxygen and then form a singlet-state complex with singlet oxygen ($[^{+}TOH–^{1}O_2]_1$). This complex in a singlet state undergoes intersystem crossing into the triplet state ($[^{+}TOH–^{1}O_2]_3$) and then dissociates to tocopherols and triplet oxygen, which is less reactive than singlet oxygen. Tocopherols can deactivate 40–120 molecules of singlet oxygen before they are destroyed [125]:

$$TOH + {}^{1}O_2 \rightarrow [^{+}TOH–^{1}O_2]_1$$

$$[^{+}TOH–^{1}O_2]_1 \rightarrow [^{+}TOH–^{1}O_2]_3 \text{ (intersystem crossing)}$$

$$[^{+}TOH–^{1}O_2]_3 \rightarrow TOH + {}^{3}O_2$$

The singlet oxygen quenching rate of tocopherols depends on the isomers. The rate of physical quenching of singlet oxygen by α-, β-, γ-, and δ-tocopherols was 4.2×10^7, 2.3×10^7, 1.1×10^7, and 0.5×10^7 M^{-1} s^{-1}, respectively [126]. The difference in the singlet oxygen quenching activity among the tocopherol isomers is affected by their concentration; when tocopherols were present at 1×10^{-3} M, the singlet oxygen quenching activity was in the decreasing order of α-, γ-, and δ-tocopherols; however, there was no significant difference among the tocopherol isomers at 4×10^{-3} M [124].

In addition to physical quenching of singlet oxygen, tocopherols can quench singlet oxygen by reacting with singlet oxygen irreversibly, so-called chemical quenching [4]. Chemical quenching on singlet oxygen by tocopherols is actually an oxidation of tocopherols by singlet oxygen and produces oxidized compounds of tocopherols. α-Tocopherol hydroperoxydienone, tocopheryl quinone, and tocopheryl quinone epoxide were reported as oxidation products of α-tocopherol by singlet oxygen. The rate of chemical quenching on singlet oxygen is much greater for α-tocopherol than for β-carotene [127]. The oxidation rate of tocopherols by singlet oxygen differs among the isomers. α-Tocopherol reacts with singlet oxygen at the highest rate of 2.1×10^8 M^{-1} s^{-1}, followed by β- (1.5×10^8 M^{-1} s^{-1}), γ- (1.4×10^8 M^{-1} s^{-1}), and δ-tocopherols (5.3×10^7 M^{-1} s^{-1}) [128]. The chemical reaction proceeds through an intermediate hydroperoxide that decomposes to tocopheryl quinone and tocopheryl quinone epoxide [125]. The singlet oxygen quenching rate of α-, β-, γ-, and δ-tocotrienols was reported to be 2.16×10^7, 1.99×10^7, 2.05×10^7, and 0.80×10^7 M^{-1} s^{-1}, respectively, in lard under light at 3000 lux in the presence of chlorophyll [129].

G. Other Phenolic Compounds

Phenolic compounds other than tocopherols mostly have large multiples of phenol structural units and thus are called polyphenols. Phenolic compounds are naturally found more in plant-based foods than in animal-based foods. Although polyphenols possess antioxidant activity, the number of polyphenols to contribute to the oxidative stability of edible oils and fats is very limited due to their low solubility in oil [130]. Lignans in flaxseed and sesame oil and tyrosol, hydroxytyrosol, oleuropein, and pinoresinol in olive oil are good examples of polyphenols contributing to the oxidative stability of the oils [131–133]. Flaxseed lignans are mainly secoisolariciresinol and secoisolariciresinol diglucoside, and sesamin, sesamol, sesamolin, sesaminol, and sesamolinol are lignans found in sesame oil. Roasting of sesame seeds causes the hydrolysis of sesamolin to sesamol [132,134] and increases the content of sesamol. Roasted sesame oil contained 36 ppm of sesamol [44], whereas unroasted sesame oil contained less than 7 ppm [135,136]. Phenolic compounds in oils are removed during the refining process; the alkaline refining eliminates hydroxytyrosol, catechol, and luteolin in olive oil (from 2.1, 4.1, and 3.1 ppm to 0.1, 0.6, and 0 ppm, respectively), and tyrosol is removed during deodorization at 240°C [137]. Therefore, polyphenols are especially important in oils consumed without refining such as virgin olive oil and sesame oil.

Polyphenols decrease the lipid oxidation by scavenging free radicals or chelating metals. Polyphenols donate phenolic hydrogen to radicals and produce semiquinone radicals whose stability is comparatively high compared to lipid radicals [138]. The semiquinone radical may scavenge another radical to give a quinone or disproportionate with another semiquinone radical to give the parent compound and quinone [113]. Sesame oil that contains a high amount of unsaturated fatty acids (iodine value = 109) shows a good oxidative stability because of sesame lignans [139]. There was no increase in the peroxide value of the roasted sesame oil up to 9 months at 25°C with a very low degradation rate of lignans, 1.06%/month [140]. The antioxidant activity of sesamol and sesamolin by scavenging radicals was higher than that of sesamin in the autoxidation of sunflower oil or methyl linoleate [141]. Flaxseed lignans also decrease the lipid autoxidation; secoisolariciresinol (300 ppm) increased the induction time from 10.4 to 11.1 h in the autoxidation of canola oil at 23°C and the oxidation decreased as the lignan concentration increased [133].

Virgin olive oil, one of the oils consumed without refining, is very stable to the autoxidation due to free radical scavenging and metal chelating activity of polyphenols [142,143]. The antioxidant role of polyphenols in olive oil is important mainly at the initial stage of autoxidation [144]. Hydroxytyrosol is most effective in decreasing the autoxidation of olive oil [142,145]. In addition to radical scavenging and metal chelating, the singlet oxygen quenching activity of polyphenols has been reported; sesamol acts as a singlet oxygen quencher physically and chemically, with a total rate of 1.9×10^7 M^{-1} s^{-1} at 20°C [146]. The singlet oxygen quenching activity of sesamol in chlorophyll-sensitized photooxidation of soybean oil was lower than that of α-tocopherol and similar to that of δ-tocopherol at the same molar concentration [146].

Osborne and Akoh [147] reported that polar phenolic acids such as quercetin and gallic acid increased the reduction of iron and accelerated the oxidation of canola oil and caprylic acid structured lipid at pH 3.0. Sitosterol, one of the phenolic phytosterols present in sesame and corn oils [136], may compete with lipid radicals at the oil surface and slightly decrease the oil oxidation [148,149], while Yanishlieva and Schiller [150] reported the prooxidant activity of sitosterol by increasing the oxygen solubility in oil.

H. Synergism among Antioxidants

Most oils contain antioxidants more than one kind and show interactions. Synergism is a phenomenon in which a net interactive antioxidant effect is higher than the sum of the individual effects. Synergistic antioxidant activity has been often observed in the copresence of metal chelators and free radical scavengers, because metal chelators mainly act at the initiation step of lipid oxidation and radical scavengers at the propagation step [26]. Tocopherols are the most frequently encountered antioxidants in edible fats and oils, and the synergistic antioxidant activity has been studied mostly with tocopherols.

α-Tocopherol shows synergistic effects with β-carotene to decrease the autoxidation [151] and photosensitized oxidation of soybean oil [152], partly because tocopherols protect β-carotene from degradation. β-Carotene (0.75 M) in oleic acid was sharply degraded from the beginning of the reaction and mostly consumed within 100 h in the absence of tocopherols; however, the copresence of α-tocopherol at 3.8×10^{-3} M increased the time to 1500 h [153].

Phosphatidylethanolamine showed a significant synergism with tocopherols to decrease the oxidation of trilinolein at 37°C by scavenging lipid peroxy radicals directly [154]. In addition, phosphatidylethanolamine donates a hydrogen atom to tocopheroxy radicals, which slows down the oxidation of tocopherols to tocopheryl quinone [78]. On the other hand, phosphatidylcholine that contains a tertiary amine with no hydrogen bound to the nitrogen did not delay the oxidation of tocopherols [155]. Phosphatidylinositol acts as a synergist with tocopherols in decreasing lipid oxidation mainly by forming inactive complexes with prooxidative metals [125]. Kago and Terao [156] proposed that phospholipids form microemulsion in oils and the active phenolic group of

tocopherols can be positioned near the polar region where lipid peroxy radicals are concentrated, resulting in synergistic antioxidative activity with tocopherols.

Ascorbic acid is a well-known synergist with tocopherols in decreasing lipid oxidation by regenerating tocopherols from tocopheroxy radicals or other oxidation products. Polyphenols decrease the autoxidation of olive oil synergistically with tocopherols [25]. Sinapic acid, the main phenolic compound of rapeseed oil, showed synergism with tocopherols in decreasing the oxidation of rapeseed oil at 40°C [157]. The synergistic antioxidant activity of sinapic acid with tocopherols was higher at low concentration of tocopherols (50 μM) than at high concentration (1000 μM). Sesamol and sesaminol show synergistic antioxidant activities with γ-tocopherol in the autoxidation of sunflower oil [136]. Synergistic effects of antioxidants are affected by hydroperoxide concentration in lipids; at low concentration of hydroperoxides (e.g., less than 20 meq/kg), α-tocopherol (100 ppm) decreased the antioxidant activity of 3,4-dihydroxyphenylacetic acid (40 ppm) in purified olive oil at 40°C [121].

In summary, it is strongly recommended to monitor the minor compounds carefully during processing and storage to minimize the lipid oxidation to improve the quality of lipid foods. Metals and oxidized compounds are to be removed and appropriate amounts of antioxidants such as tocopherols and phenolic compounds should be selected. Heat, light, and oxygen should also be excluded as much as possible during handling, processing, and storage of foods containing high amounts of lipids.

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Section IV

Nutrition

22 Digestion, Absorption, and Metabolism of Lipids

Charlotte Lauridsen

CONTENTS

I. INTRODUCTION

Quantitatively, the most important lipid component of the human diet is the triglyceride (TG) fraction, which may amount to 100 g per day. In addition, the diet contains phospholipids, about 5 g per day, and minor contents of cholesterol, cholesteryl esters (CEs), and fat-soluble vitamins. However, the phospholipid and cholesterol present in the digestive tract are not only derived from the diet but also from the bile. The digestion and absorption of TGs are very efficient under normal health circumstances. The composition of the dietary-derived lipids, specifically carbon chain length, distribution of fatty acids (FAs) in TG, and degree of saturation of fat affect lipid metabolism. Medium-chain fatty acids (MCFAs) are saturated 6–12 carbon FAs, which occur naturally as medium-chain triglycerides (MCTGs) in milk fat and some vegetable fat sources such as coconut and palm oils. MCFAs represent immediately available sources of energy that can be supplemented to diets to improve energy supply of premature babies, neonates, or patients to treat diseases such as lipid absorption disorders. Structured lipids are lipids that have been modified from their native form either enzymatically or chemically, and these modifications may result in changes in FA composition and/or FA position in the TG molecule, which may influence the fate of the lipid in the body. These diverse structured TGs may be designed according to special requirements such as enhancing lipid absorption or producing low-calorie fats or fat substitutes. Different types of phospholipids have been shown to be of interest in relation to the potential of inhibiting intestinal cholesterol absorption, because an increased concentration of cholesterol in the blood (i.e., hypercholesterolemia) is

widely recognized as a risk factor for coronary artery disease. The purpose of this chapter is to provide the reader with an insight into digestion, absorption, and metabolism of various lipid components and to explain how dietary fat and modifications can influence lipid digestion, absorption, and metabolism.

II. DIGESTION AND ABSORPTION OF DIETARY FAT

A schematic overview of fat digestion and absorption is given in Figure 22.1, and an excellent review is given by Phan and Tso [1].

This section is mainly concerned with the absorption of FA from dietary TGs, and the predominant phospholipase (PL) in the intestinal lumen phosphatidylcholine (PC), which is the most abundant lipid in the digestive tract after TG [1]. Both lipids are absorbed in the small intestine.

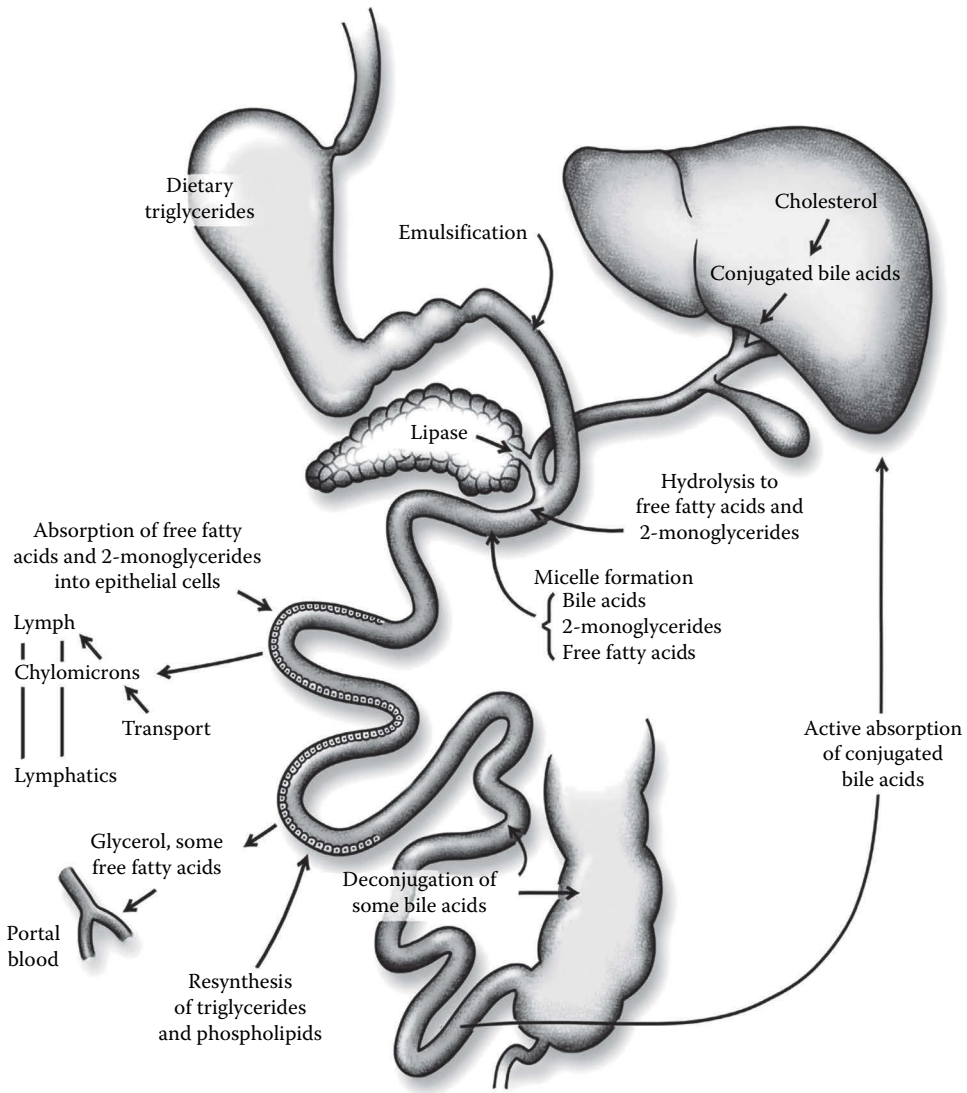

FIGURE 22.1 Schematic overview of fat digestion and absorption. (Modified from Jensen, M.S. et al., *J. Anim. Sci*., 75, 437, 1997, illustration by Mads Salicath.)

In addition, up to 20% of ingested carbohydrates have been estimated to pass to the colon where they are fermented by colonic bacteria to short-chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate, and their absorption in the hindgut has been reviewed by Cummings [2] and will not be further described herein.

A. Digestion

Digestion of dietary lipids begins with emulsification in the stomach in which partial degradation of the TG is performed. The enzymes involved are *lingual lipase*, secreted from the salivary gland, and *gastric lipase*, secreted by gastric mucosa. Both enzymes hydrolyze the FAs in the sn3-position of the TG molecule giving diacylglycerol and FAs (e.g., nonionized long-chain FA), and neither of the enzymes act on PC or cholesterol. In contrast to some animal species, that is, rats and mice, lingual lipase is only present in negligible amount in humans. Human gastric lipase shares many characteristics of lingual lipase, for instance, pH optimum (ranging from 3.0 to 6.0), and it hydrolyzes MCTGs better than long-chain triglycerides (LCTGs). Gastric lipase is responsible for 10%–30% of the hydrolysis of the dietary TG and plays therefore an important role in lipid digestion, particularly in neonates provided with milk fat, which contains a considerable amount of MCFA. Actually, milk fat can be completely digested by gastric lipolysis. Gastric lipase seems to have the unique ability of initiating breakdown of fat droplets in mother's milk. The activity of gastric lipase toward SCFAs, MCFAs, and long-chain FAs is different, that is, the lipolytic activity of pure rabbit gastric lipase toward MCTG was three times higher than that toward LCTG [3].

The major digestion of TG results from hydrolysis with *pancreatic lipase*. Emulsification is an important prerequisite for efficient hydrolysis by pancreatic lipase. The lipid emulsion enters the small intestine as fine lipid droplets, and the combined action of bile and pancreatic juice brings about a marked change in the chemical and physical form of the ingested lipid emulsion. Most of the digestion of TGs is brought about by pancreatic lipase in the upper part of the small intestinal lumen. Pancreatic lipase, which is released from the pancreatic tissue into the duodenum upon hormonal response of cholecystokinin, binds to the surface with colipase as a mediator to overcome the expulsion of the lipase into the water phase caused by the bile acids and digests the TGs. This enzyme (pH optimum of 8) acts mainly on the sn-1 and sn-3 positions of the TG molecule to release 2-monoacylglycerol and free fatty acids (FFAs), but the activity may be lower toward n-3 FAs, in particular 20:5n-3 and C22:6n-3, when located in the sn3-position [4]. The lipase activity implies a general conservation of approximately 75% of the FAs located in the sn2-position [5]. Pancreatic lipase is abundant in pancreatic juice, 2%–3% of the total protein that is present. Its high concentration in pancreatic secretions and its high catalytic efficiency ensure efficient digestion of dietary fat. The lipase is secreted into the duodenum in about 1000-fold excess. Thus, under optimal conditions, 100 kg TG could be hydrolyzed in 24 h instead of the ingested 100 g fat in a normal human diet. Pure pancreatic lipase works extremely inefficiently in a bile salt–lipid mixture, yet lipase present in pancreatic juice hydrolyzes TG extremely efficiently. This is due to the presence of pancreatic colipase, which binds to the lipid/aqueous interface allowing the lipase molecule to bind to the lipid/aqueous interface.

The digestion of PL occurs in the small intestine since lingual and gastric lipases are incapable of hydrolyzing PL. The most common PL in food is PC while other PLs, such as phosphatidylethanolamine (PE), phosphatidylserine, and phosphatidylinositol (PI), are present in much smaller amounts. However, lecithin, which is a commercial name of a mixture of PC, PE, and PI, is widely used as a food additive. In bile, PL (predominantly PC) is found in mixed micelles along with cholesterol and bile salts. In fact, the biliary pathway delivers 10–20 g of PL and approximately 1.5 g of cholesterol to the intestinal lumen per day [6], which is at least two to four times more than that supplied by the diet. Once in the intestinal lumen, the luminal PC will be distributed between the mixed micelles and the TG droplets. PC is then acted upon by pancreatic phospholipase A_2 (PLA_2) at the sn-2 position to yield a FA and a lysophosphatidylcholine (lysoPC). Although the bulk of

intestinal PLA_2 activity is derived from pancreatic juice, there is probably some contribution from intestinal mucosa, where the enzyme is concentrated in the brush border. PLA_2 is activated by trypsin cleavage and its activity requires the presence of bile salts. Sphingomyelin (SM), a phosphorus-containing lipid (i.e., sphingolipid), is ingested at 0.3–0.4 g/day [7], which means that less than 5% of dietary PL is SM. The major enzymes responsible for SM degradation in the intestinal lumen and mucosa are alkaline SMase and neutral ceramidase. These enzymes are located in the surface membrane of mucosal cells with catalytic domains facing the outside of the cell.

Cholesterol in the gut lumen is derived from the diet (approximately 400 mg/day) and the bile (approximately 1 g/day). Most of the dietary cholesterol is present as the free sterol and only 10%–15% as the sterol ester. The solubilization of cholesterol is very poor in the aqueous environment of the gut, and its digestion and absorption are dependent on partitioning into bile micelles (Figure 22.1). CE entering the small intestine has to be hydrolyzed before free cholesterol can be absorbed. The enzyme involved in the hydrolysis process is cholesterol esterase, which is also called carboxylic ester hydrolase (CEH) or sterol hydrolase. Human cholesterol esterase has a broad specificity and can hydrolyze TGs, CEs, and phosphoglycerides and vitamin esters, and its activity is greatly enhanced by the presence of bile salts.

Vitamins A, D, E, and K are the so-called fat-soluble vitamins, and higher animals must obtain vitamin E and A from the diet, whereas vitamin D can be synthesized in the skin upon sun exposure and one of the two naturally existing forms of vitamin K can be synthesized by bacteria. Vitamins E and A in forms of tocopheryl and retinyl ester, respectively, must be completely hydrolyzed within the intestinal lumen to release free tocopherol or retinol before these alcohol forms can be taken up by enterocytes. Hydrolysis requires the catalytic action of pancreatic juice enzymes, and the enzyme that appears to be mainly responsible is CEH.

B. Absorption

The overall scope of the fat digestion is the formation of emulsions, which can be further processed for uptake (absorption) by the enterocytes. The majority of the lipids are absorbed in the proximal part of the small intestine.

1. Importance of Micellar Solubilization

The TGs (mainly of dietary origin) and PLs and other lipolytic products (e.g., FFAs, monoglycerides [MGs], lipid-soluble vitamins) form stable emulsions, and the presence of bile salts forms mixed micelles, which facilitate the transport of the lipids into the enterocytes. Mixed micelles thus contain bile acids; free cholesterol; and PL, together with TG, MG, FA, and lysoPC; and lipid-soluble vitamins. The brush border membrane of enterocytes is separated from the bulk fluid phase in the intestinal lumen by an unstirred water layer, which is a diffusional barrier at the intestinal lumen–enterocyte interface and which is poorly mixed with the bulk phase. To be absorbed into the enterocytes, lipids have to cross the two barriers: the *unstirred water layer* and the *brush border membrane*. The solubility of FA and MG in an aqueous medium is extremely low, and very few molecules will therefore gain access to the brush border membranes. However, the micellar solubilization of MG and FA greatly enhances the number of molecules that is available for uptake by the enterocytes.

2. Permeation into Enterocytes and Transport

It was generally believed that FFAs, MGs, and cholesterol were absorbed by the enterocytes through a simple, energy-independent, passive diffusion process [1]. However, studies indicated the existence of a fatty acid–binding protein associated with the brush border membrane, and that this protein played a role in the uptake of FAs by enterocytes [8]. The general consideration is that FAs are taken up by enterocytes involving protein-mediated and protein-independent processes. The products of the lipid hydrolysis are presented to the apical membranes of enterocytes, and more

recently, several transport proteins that participate in the uptake of various types of lipids have been described in the enterocyte membranes, cytosol, and endoplasmic reticulum membranes, including proteins involved in cholesterol and vitamin absorption [9].

III. METABOLISM

Depending on the chain length of the FAs (Figure 22.1), it may either pass directly from the enterocyte into the portal blood, where it is bound to albumin (FAs with fewer than 12 carbon atoms), or reesterified to TGs within the enterocyte and delivered to the lymph (FAs with more than 12 carbon atoms). Thus, with increasing length of FAs, an increasing proportion of the FAs can be absorbed into the lymph and less into the portal blood. Vitamin E and other fat-soluble vitamins enter the circulation via the lymphatic system. In addition to the chain length, the level of unsaturation of FAs and the structure of the dietary lipids influence whether transport is undertaken via the portal vein or via the lymphatics. In the case where only FFAs are administered, more FAs are transported via the portal system, whereas more FAs are transported via the lymphatics when both FFAs and MGs are administered [5]. When the lipids are given in the form of TG rather than FFA, lesser FAs are transported via the portal vein.

Dietary fat sources containing MCFAs may, on the other hand, be readily accessible as energy due to the transfer into the portal blood and would therefore be useful in high-energy demanding subjects such as patients suffering from impaired fat absorption capability or the lactating sow with a suckling neonatal piglet having a high energy demand and low body fat depots.

A. Resynthesis of TG

The first step in transport of lipid particles through the intestine is reesterification, and the second step is the synthesis of transport particles: the so-called lipoprotein (chylomicron, CM) and very-low-density lipoproteins (VLDLs). The absorbed MG and FFA are reconstituted to TGs on the membranes of the endoplasmic reticulum and Golgi apparatus in the intestinal enterocyte. Actually, the reesterification process can occur in two different ways: either the MG pathway or the glycerol-3-phosphate pathway. The MG is the predominant one when it is available in the enterocyte, whereas, in the absence of MG, the glycerol-phosphate pathway becomes important. Thus, when the diet consists of high amounts of FFAs (i.e., palm oil mix or by-products), the glycerol-phosphate pathway becomes important, whereas, for diets containing a high amount of TGs, the MG is the most important. However, the glycerol-phosphate pathway is preferred when long-chain FAs are absorbed. During resynthesis, rearrangements of the FAs in the TGs occur. Most of the FAs placed in the 2-position of the TG are absorbed as 2-monoacylglyceride and are conserved after resynthesis.

B. Intestinal Lipoproteins

The intestinal synthesis and secretion of lipoproteins is essential for the transportation of dietary lipids in the circulation and delivery of these lipids to other tissues. The major lipoproteins secreted by the intestine are VLDL and CMs. Of these, the CMs are synthesized exclusively in the intestine to transport dietary fat and fat-soluble vitamins into the blood via the thoracic lymphatic duct. These are the largest lipid-carrying particles of the body and have the lowest density. The CMs consist of 86%–95% TG, 4%–13% of phospholipids, 0.7%–1.7% of cholesterol ester, and 1%–1.5% of protein of particle mass with TG, CEs, and fat-soluble vitamins contained in the lipoprotein core, whereas the surface of the CMs contains a monolayer of phospholipids, free cholesterol, and protein. The TG composition of the CM largely reflects the dietary FA and the size but not the number of CMs, which is determined by the flux of TG through the intestinal cell. FAs in phospholipids and cholesterol ester of CMs have little relationship with dietary FA.

VLDL-sized particles are formed when the level of lipids is too low to drive the formation of CMs. VLDL differs from CM in their density, size, lipid content and composition, and protein content. Hence, TG contributes up to 40%, cholesterol 15%, phospholipid 15%, and the total protein 10% of particle mass in VLDL [5].

During fasting, VLDLs are the only lipoproteins produced by the small intestine, and this lipoprotein is thus the major TG-carrying lipoprotein in the lymph. After a meal, the small intestine produces predominantly CMs. The TG-enriched CMs are hydrolyzed rapidly by the lipolytic activities of lipoprotein lipase (LPL), and the action of this enzyme results in FFA, which is taken up by the peripheral tissue for storage or energy production. Furthermore, the hydrolysis of CM TGs by LPL decreases when about 70%–90% of the TG is removed, resulting in remnant particles, which are taken up by the liver and further hydrolyzed by the hepatic lipase; then, remnants are cleared from the blood by the liver mainly via the LDL receptor–related peptide [10].

Apolipoproteins play important roles in lipoprotein clearance and metabolism even though their content in CM and VLDL is low. CMs and VLDLs synthesized in the intestine contain several different apolipoproteins, and the major apolipoproteins made by the small intestine are apo A-1, apo A-2, apo A-IV, and apoBs. Apo A-IV comprised 10%–13% of CM apoproteins and 24%–30% of intestinal VLDLs [5]. ApoBs have a particularly critical role in the assembly of the TG-rich lipoproteins. Apolipoprotein contents in the VLDL particles resemble those of CM [11]. However, even though CM and VLDL both carry TG and have the same apolipoprotein content, VLDL is not a small CM. It is generally believed that the major difference between CM and VLDL in lipoprotein is that CM contains apoB-48, whereas VLDL contains apoB-100 because hepatocytes secrete VLDL-containing apoB-100, which has a hepatic origin. The pathway of intestinal TG biosynthesis seems to have a significant effect on the apolipoprotein mass and, to a lesser extent, on the apolipoprotein and lipid composition of the CM. Because VLDL is present in the lymph during fasting and bile diversion results in depletion of VLDL in the lymph, this lipoprotein is believed to carry lipids of endogenous origin. In addition, the FA composition of the VLDL TG is different from the CM TG suggesting two separate pathways for the VLDL and CM assembly [1,11].

C. Assembly of Intestinal Lipoproteins

The mechanisms for packaging lipids into lipoproteins are still not completely understood. The final site of assembly of lipoproteins is the Golgi apparatus, where the addition of carbohydrate component to the CM occurs. The Golgi apparatus of intestinal epithelial cells participates in the production of two distinct populations of particles, that is, CM and VLDL, and the assembly of intestinal CM and VLDL may occur in two different pathways as mentioned earlier. The complex processes of CM assembly and trafficking and the role of the lipid-binding proteins and lipid-metabolizing enzymes in the enterocytes have been reviewed [10]. In brief, the newly resynthesized TGs are packed into nascent CMs and transferred to the Golgi apparatus. The maturing CMs and VLDL undergo modification of phospholipids and protein contents during transport from the endoplasmic reticulum to the Golgi apparatus. The CMs penetrate through the lamina propria and move further into the lymphatics. Various mechanisms have been shown to regulate the secretion of lipoproteins from the intestine (see review, [9]).

D. Metabolism of Lipoproteins Secreted from the Intestine

Intestinal lipoproteins do not enter the bloodstream directly. Instead, they are secreted into tiny lymph vessels that are found inside each of the intestinal villi and then enter the circulation in the subclavian vein via the thoracic duct and reach the liver for distribution around the body. Once the intestinal lipoproteins are in contact with other plasma lipoproteins, rapid transfer of proteins occurs. The CM TGs are hydrolyzed rapidly by the lipolytic activities of LPL in extrahepatic tissues at or close to the capillary endothelial wall to form the CM remnants, which are rich in cholesterol

esters and removed from the plasma by the liver via LDL receptors. Several factors are expected to influence the overall clearance of CMs from the circulation and thereby the extent of the postprandial lipemia. Apart from the hydrolysis rate by LPL, the remnant uptake rate of the liver, factors like CM size and composition may affect the clearance rate. The liver secretes TG in the form of VLDL, which is then processed by LPL in tissues to release FAs and glycerol. The FAs are taken up by muscle cells for energy or by adipose cells for storage. Once hydrolyzed by LPL, the VLDL becomes a VLDL remnant. A majority of the VLDL remnants are taken up by the liver via the LDL receptor, and the remaining remnant particles become smaller LDL particles, which are either removed directly from the plasma or converted by the action of hepatic TG lipase to LDL. LDL is the major cholesterol-transporting lipoprotein in the plasma and is responsible for the regulated delivery of sterol to cells for growth and hormone of bile salt production. Much of the LDL particles are taken up by LDL receptors in the liver; the remaining LDL is removed by way of scavenger pathways at the cellular level. As LDL is taken up by receptors, free cholesterol is released and accumulates within the cells. Cholesterol can be removed from the tissues and returned by the liver, and high density lipoprotein (HDL) is the key lipoprotein involved in this reverse cholesterol transport and transfer of CEs between lipoproteins. Nascent HDL secreted by the liver and intestine proceeds through a series of conversions (HDL cycle) to attract cholesterol from cell membranes and free cholesterol to the core of the HDL particle.

E. Metabolism of Fatty Acids after Absorption

Mammals lack the Δ12- and Δ15-desaturases for insertion of double bonds at carbon atoms beyond C9 in the FA carbon chain. FAs with double bonds lower than 9 carbon atoms therefore have to be supplied to mammals from the diet. The essential FAs are linoleic (C18:2n-6) and α-linolenic acid (C18:3n-3), which are building blocks of FA of longer chain length in the n-3 and n-6 family through elongation and desaturation (Figure 22.2).

These essential FAs are precursors for prostaglandins, leukotrienes, and thromboxanes. Linoleic acid is desaturated by Δ6-desaturase to γ-linolenic acid (C18:3n-6), which is further converted to dihomo-γ-linolenic acid (C20:3n-6) through elongation, and further desaturation by Δ5-desaturase forms arachidonic acid (C20:4n-6). By the action of cyclooxygenase, C20:3n-6 and C20:4n-6 are converted to 1-series and 2-series prostaglandins, respectively, whereas the action of lipoxygenase converts these FAs into leukotrienes and other oxidative products. The eicosanoid products of the n-6 series have been found to exert clinical efficacy in diseases, including inflammation. The eicosanoids formed through desaturation and elongation of linolenic acid to eicosapentanoic acid have a very similar molecular structure but with a different biological activity. Eicopentanoic acid (C20:5n-3) can be further metabolised to docosapentanoic (C22:5n-3) and further to docosahexanoic acid (C22:6n-3), and both fatty acids serve as precursors for several bioacitive lipid mediators with a range of immune modulating activities. The eicosanoids act as hormonelike regulators of cell function of the immune system and thus influence, for example, lymphocyte proliferation, T cell–mediated cytotoxicity, and inflammatory response. In addition, each fatty acid precursor gives rise to other classes (pro-resolving mediators) and prostaglandin-like compounds (isoprostanes) and hydroxy fatty acids.

F. Lipogenesis

The adipose tissue plays a very important role in the storage and release of energy substrates. Adipocytes are specialized fat cells in which TG, cholesterol, and cholesterol esters are stored. Furthermore, lipids—particularly PL, such as lecithin and SM—are widely distributed in cell membranes and serve an important role in the structural integrity of the cell membranes. The membranes, which consist of PL, cholesterol, and proteins, serve not only as boundaries between individual cells but also as a means to compartmentalize several biochemical processes within the cell.

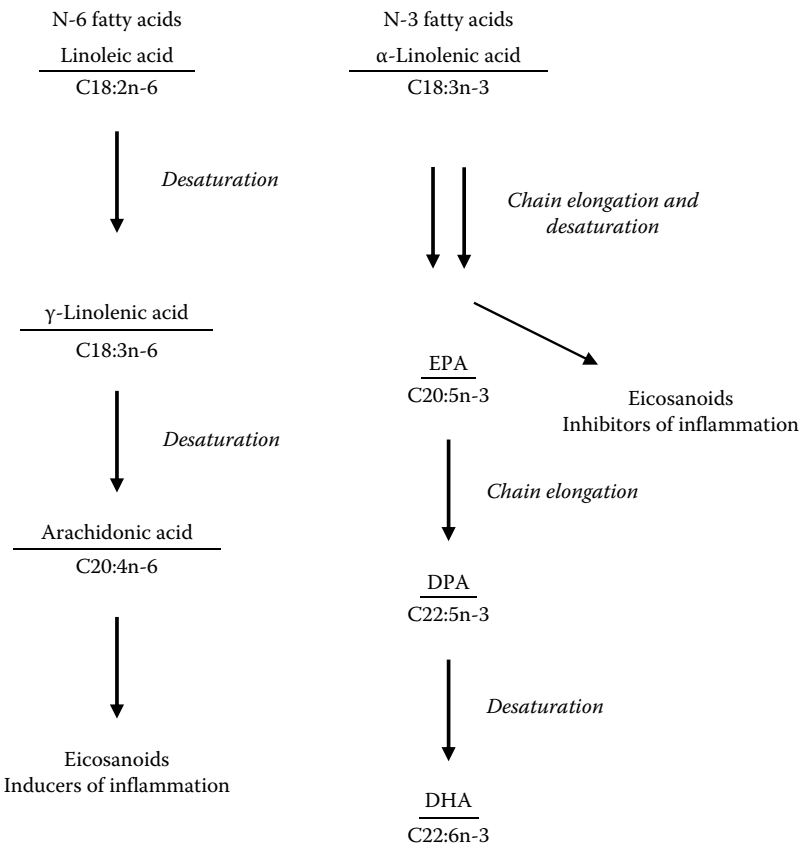

FIGURE 22.2 Metabolism of linoleic and α-linolenic acid to eicosanoids.

Lipogenesis is one of the processes that take place in these fat cells. The substrates for the synthesis of TG in the fat cell originate from TG or cholesterol in the bloodstream, mainly in the CM or VLDL, or by *de novo* synthesis. In order to be stored in the adipocyte, the FAs must be broken down into glycerol and FFA before they enter the cell, and this reaction is catalyzed by LPL synthesized and excreted by fat cells.

In addition to lipogenesis, a second important process that occurs in the adipose tissue cells is lipolysis or degradation/mobilization of the deposited TG. The intensity of the breakdown of TG in these cells is, to a large extent, dependent on the formation of cyclic adenosine monophosphate (cAMP). Briefly described, the degradation of TG is preceded by an entire cascade of reactions beginning from the action of a factor, increasing the amount of cAMP in fat cells, and ending with liberation of glycerol and FFA by cells.

The oxidation of FA as an energy source referred to as β-oxidation occurs in either mitochondrial matrix or peroxisomes, which is dependent on the length of the FA. β-Oxidation is a process, where two-carbon units are removed from the FA. This step is repeated for several times and each step yields energy. Peroxisomal β-oxidation is primarily involved in chain shortening. Partial β-oxidation of very-long-chained FA takes place almost exclusively in the peroxisomes, because mitochondria are unable to oxidize very-long-chain FA. Peroxisomal oxidation is energetically less efficient than mitochondrial oxidation but yields more heat. Long-chain FA with 18 or more carbon atoms are known to be shortened, while FA with 16 or less carbon atoms seems to be completely oxidized once the oxidation has started. The chain-shortened products of the partial β-oxidation in the peroxisomes may move to the mitochondria, where the oxidation process is completed or to

be esterified into the membrane lipids. The amount of energy that may be obtained in the embolic oxidation of FA depends on the length of the carbon chain and its saturation. The longer and more saturated the FA, the more energetic the FA.

De novo FA synthesis involves two key enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN). ACC carboxylates acetyl-CoA to form malonyl-CoA. The malonyl-CoA product is further converted by FASN to long-chain FAs. Most normal human tissues preferentially use dietary (exogenous) lipid for synthesis of new structural lipids, whereas *de novo* (endogenous) FA synthesis is usually suppressed, and FASN expression is maintained at low levels.

IV. DIETARY FAT MODIFICATION AND EFFECT ON UTILIZATION

A. Methods for Investigating Fat Digestibility and Absorption

Evaluation of the nutritional value of different dietary fat sources can be assessed in digestibility experiments, in which *apparent digestibility of fat* is determined as the difference between ingested fat in feed and excreted fat in feces. Collection can be performed either by collection of all feces or by using a marker (for instance, Cr_2O_3), whereby only a fraction of feces needs to be collected. However, the apparent digestibility of fat represents an underestimation of *true digestibility*, because of the occurrence of endogenous or metabolic loss. Hence, the true digestibility of dietary fat is the difference between ingested fat and the sum of excreted fat and endogenous fat loss. Endogenous loss occurs due to loss of bile acids, desquamated cells, structural lipids from mucosa, and cholesterol and may vary not only with dry matter intake but also with the source and level of dietary fat.

Evaluation of the fecal excretion of fat has been used to measure fat absorption. Several mechanisms are involved in the adaptation of the fat absorption to the lipid content of the diet. However, the fecal fat will include a fraction of the secreted bile lipids and bacterial lipids and lipids from excreted intestinal cells. In addition, colon absorption of SCFAs and fermentation in the colon will affect the results. The lipid-mediated adaptability appears to be sufficient to prevent excessive lipid elimination in feces when fat supply is enhanced. Studies of fat absorption using collection of lymph from the thoracic or mesenteric ducts are based on the assumption that the FAs are representative of the ingested fat. This is subject to some limitations unless the studies are using labeled FAs [5].

B. Importance of Structure of Fatty Acids and TG

Fats of animal origin are normally considered as having lower digestibility and hence lower energy value than fats of vegetable origin owing to a higher content of saturated FAs (C16:0 and C18:0), which have lower ileal digestibility than unsaturated FAs (C18:1, C18:2, and C18:3) [12,13]. Dietary minerals have no influence on ileal FA digestibility, while dietary protein level has a slight positive influence on the digestibility of some saturated FAs [12]. In humans, a high content of calcium in the diet has shown reduced digestibility of saturated, as well as unsaturated FA [14]. Another factor that influences fat digestibility is the melting point of dietary fat. From other animal species, it is known that apparent digestibility of fat decreased as melting point increased, irrespective of the fat source. A lower melting point may be caused by either shorter chain length of FAs or increasing content of PUFAs.

Secretion of lipolytic enzymes is positively correlated with the amount of fat consumed by the pig and this is probably also true in humans. In addition, the dietary FA composition and chain length may influence the exocrine secretion. The importance of these factors affecting the secretion of lipolytic enzymes is especially true in situations, when digestive processes are impaired. During weaning of mammals, the activity of the pancreas may initially be low, and the importance of preduodenal lipolysis of fat therefore increases. The pancreatic lipase system is not fully developed in the neonate as demonstrated in the piglet. Jensen et al. [15] showed a relatively low level of pancreatic lipase, which increases with age of the piglet until weaning. At weaning, pancreatic lipase, colipase, and carboxylic esterase decreased, whereas gastric lipase increased before weaning and

remained constant after weaning [15]. The enzymatic changes pre- and postweaning have major importance in the livestock production and have brought many attempts to change the dietary fat to be easily digested by preduodenal lipases during the weaning and first period postweaning. For example, due to the high activity of gastric lipase, the apparent digestibility of fat is high (96%) in suckling pigs; however, at weaning from the sow, the digestibility of fat declines to 65%–80% [16]. After weaning, the piglet is provided fat sources based on other nutritional components other than milk, and a prerequisite of a proper fat digestion is largely dependent on pancreatic enzymes. Fat digestion may also be enhanced by inclusion of emulsifiers in the diet, such as lecithin, which has been demonstrated to increase fat digestibility in piglets after weaning. Likewise, the combined dietary supplementation of MCTGs with different lipases or inclusion of MCTGs in diets for weaned piglets has demonstrated increased weight gains [17]. Hydrolysis of MCTGs occurs rapidly in comparison with that of LCTGs due to higher water solubility without the necessity for emulsification with bile [17]. Overall, studies on energetics in newborn piglets clearly demonstrated a superior energetic exploitation of the MCFAs in comparison with the LCFAs.

MCTGs and MCFAs have been of interest as supplements to diet of not only neonates and young animals to improve their energy supply but also patients. MCFAs are administered in diseases such as lipid absorption disorders, malabsorption syndromes, pancreatic insufficiency, disorders of the gall bladder, gastroenteritis, and diabetes mellitus and as a source of energy in premature babies [18,19]. Fat malabsorption syndromes do not only influence the energy intake but may also lead to deficiency in fat-soluble vitamins. It has been observed that more MCFAs are transported via the portal vein when FFAs are the only kind of administered lipids. Odle [20] has provided a summary on the general contrast of MCTG and LCTG utilization and claimed that the differences in their digestion, absorption, and metabolism stem largely from differences in physical chemistry. To summarize the major differences, hydrolysis of MCTG is more rapid and complete than hydrolysis of LCTG. MCFAs exert less inhibitory effect on pancreatic lipase, and medium-chain-2-MG isomerizes more rapidly than those of the long chain length, thereby facilitating rapid and complete hydrolysis. For LCTG to penetrate mucosal cells, the LCTG must be emulsified and hydrolyzed within the gut lumen, and then the resulting LCFA and 2-MG must be mixed with bile salts to form mixed micelles to be absorbed through the unstirred water layer and into the enterocytes. Furthermore, once inside the mucosal cell, the products of the LCTG digestion are reassimilated and packaged into CM, which enter the lymphatic system. In contrast, the enhanced water solubility of MCFA results in extensive absorption via the portal vein with little incorporation into lipoproteins. The increased flow of blood relative to lymph also contributes to a more rapid entry of MCFA into the circulatory system. Furthermore, the LCFA must be reassimilated into lipoproteins and oxidized within the liver, whereas very little MCFA is reesterified and their major metabolic fate is oxidation.

The structure of the TG is also of importance when considering the nutritional effect of a dietary fat. Ikeda et al. [21] studied the absorption of medium-long-medium (MLM-type) and long-medium-long (LML-type) structured TGs in thoracic duct–cannulated rats after intragastric administration of lipid emulsions and found that the absorption of C18:6n-6 was higher from 10:0/18:2/10:0 than from 18:2/18:2/18:2, probably due to fast hydrolysis of MCFAs. Using mesenteric lymph collection in rats, Christensen et al. [22] determined the absorption of FAs from salmon oil and seal oil, the former having PUFA located mostly in the sn2-position and the latter having PUFA in the sn1,3-positions. The lymphatic transport following intake of fish oil was higher during the first 8 h, which is possibly ascribed to the slow release of n-3 FAs by pancreatic lipase. At 24 h, there was, however, no difference on the accumulated transport of FAs suggesting TG structure is not an important parameter affecting absorption of LCFAs under normal absorption conditions. The resultant CMs reflected the dietary TGs with respect to the location of n-3 PUFA [23]. In the two studies with postweaning piglets, the effects of dietary TG structure on fat digestibility, nitrogen retention, and FA composition of blood and tissues were investigated. The piglets were fed diets for 3 weeks postweaning containing randomized, physical mixture, structured, or control oil. In one study, the oils were produced from rapeseed oil and C10:0 [24] and in another study [25] from rapeseed oil

and coconut oil. Both studies showed that the structured oil improved nitrogen retention and fat digestibility compared to rapeseed oil, indicating a great potential of structured TG for animal feed. Inclusion of dietary fats (coconut oil, rapeseed oil, fish oil, sunflower oil) in lactational diets influences the FA profile of the progeny before and after suckling [26] and influences the performance of the litter during suckling and the FA composition of the milk and blood [27].

SUMMARY

The majority of the dietary fat is in the form of TG. Digestion of TG in the intestine results in the production of 2-MG and FA. PC is hydrolyzed in the lumen to form lysoPC before its absorption. Cholesterol ester is hydrolyzed to form free cholesterol before absorption and likewise with fat-soluble vitamins E and A when supplemented as tocopherol or retinyl esters. These digestion products are solubilized in the presence of bile salts to form mixed micelles and unilamellar vesicles so that the lipid products can be dispensed into an aqueous environment and be transported across the epithelial wall by passive diffusion or FA transporters. Through predominantly monoacyl pathway, MG and FAs are resynthesized to form TG in the endoplasmic reticulum. The lipid droplets, coated with cholesterol, PL, and apolipoproteins, are then further processed in the Golgi apparatus before being released by the enterocytes via exocytosis.

Many factors such as the overall FA profile, the TG structure, and the species composition affect the digestion and absorption of the TGs and will influence the nutritional value of the dietary fat. In addition, the development of several lipolytic enzymes in the neonate may be crucial for optimal hydrolysis of milk fat or other dietary fat sources. Hence, one may speculate on the possibilities of modifying the structure of fats to affect their absorption and the distribution of FAs in the body after digestion and uptake.

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23 Omega-3 Polyunsaturated Fatty Acids and Health

Michelle A. Briggs, Kate J. Bowen, and Penny M. Kris-Etherton

CONTENTS

I. INTRODUCTION

There is mounting evidence that long-chain omega 3 (*n*-3) polyunsaturated fatty acids (PUFAs) have many health benefits. These long-chain *n*-3 PUFAs include alpha-linolenic acid (ALA; 18:3*n*-3 or 18:3 $\Delta^{9,12,15}$), eicosapentaenoic acid (EPA; 20:5*n*-3 or 20:5 $\Delta^{5,8,11,14,17}$), docosapentaenoic acid (DPA; 22:5*n*-3 or 22:5 $\Delta^{7,10,13,16,19}$), and docosahexaenoic acid (DHA; 22:6*n*-3 or 22:6 $\Delta^{4,7,10,13,16,19}$). Their health benefits appear to be associated with several different aspects, including their structure, their interactions with proteins that alter gene expression, and their unique metabolic fates. Structurally, when long-chain *n*-3 PUFAs are incorporated into cell membranes, they increase membrane fluidity, thereby influencing cell function. For example, higher *n*-3 PUFA content in neuronal membranes has been shown to improve neuron function [1]. Besides affecting membrane fluidity, these fatty acids can also influence the structure and function of proteins embedded in the phospholipid bilayer [2]. As mediators of cell sensors and receptors (e.g., G protein–coupled receptor 120 [3]), long-chain *n*-3 PUFAs also have a major impact on gene expression [4]. But perhaps the most studied aspect of long-chain *n*-3 PUFA relates to their metabolic fates. These fatty acids enter biochemical pathways that produce anti-inflammatory compounds (Figure 23.1); in turn, these anti-inflammatory agents decrease the risk of chronic diseases [5].

II. STRUCTURE AND DIETARY SOURCES OF LONG-CHAIN PUFA

Structurally, the *n*-3 PUFAs differ from the *n*-6 PUFAs by virtue of the position of their first double bond. Relative to the methyl ("*n*" or "ω") terminus of the fatty acid chain, the *n*-3 PUFAs have their first *cis* double bond between the third and fourth carbons, whereas the *n*-6 PUFAs have their first *cis* double bond between the sixth and seventh carbons.

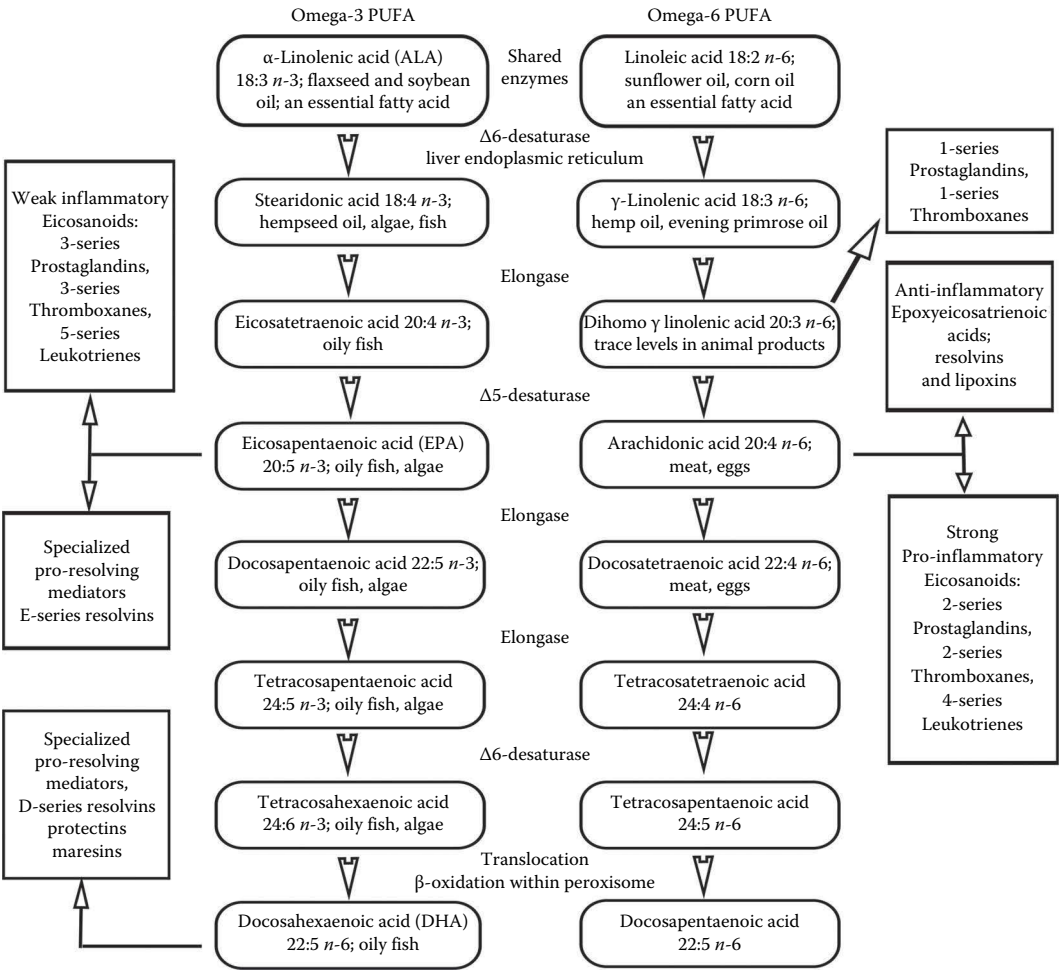

FIGURE 23.1 The enzymatic pathways converting *n*-3 α-linolenic acid and *n*-6 linoleic acid to longer-chain polyunsaturated fatty acids, their common food sources, and the eicosanoids produced from various long-chain polyunsaturated fatty acids. (Adapted from Lenihan-Geels, G. et al., *Nutrients*, 5(4), 1301, 2013; Patterson, E. et al., *J. Nutr. Metab.*, Article ID 539426, 16, 2012; Carlson, S.J. et al., *J. Parenter. Enteral. Nutr.*, 37(1), 15, 2013; Bishop, K.S. et al., *Nutrients*, 7(1), 405, 2015.)

ALA, an *n*-3 with double bonds at the 9th, 12th, and 15th carbons, is an essential fatty acid because humans and all other animals lack delta-12-desaturase and delta-15-desaturase, the enzymes producing double bonds in fatty acids at the 12th and 15th carbons. Green plants, however, contain these desaturases. Thus, ALA is produced in a variety of green vegetables, such as kale and spinach. ALA is available in appreciable amounts from a variety of liquid vegetable oils, including those derived from flaxseeds, walnuts, rapeseeds (canola), and soybeans, as well as the food sources of these oils, that is, walnuts, flaxseeds, etc.

The highest levels of EPA, DHA (see Table 23.1), and DPA are found in fatty fish. Fish and seafood do not inherently produce *n*-3 fatty acids. Rather, they obtain most of their DHA and EPA either directly from sources such as microalgae [6] or indirectly as higher-trophic-level fish feed on smaller fish that consume algae that produce *n*-3 fatty acids [7,8]. While higher plants can synthesize ALA, they lack the ability to produce DHA and EPA unless genetically modified [9]. Genetically modified plants designed to produce EPA and DHA are more sustainable than fish and may help supply the growing global population with sufficient *n*-3 PUFAs. Another sustainable source of EPA

TABLE 23.1
Fish and Seafood Containing Relatively High Amounts of DHA and EPA and Their Known Mercury Concentrations

Fish/Seafood Species and Preparation Method	gDHA per 100 g	gEPA per 100 g	gDHA + EPA per 100 g	EPA:DHA Ratio	Mean Total Mercury Concentration (PPM)
Caviar, black and red, granular	3.800	2.741	6.541	0.72:1	Not reported
Roe, mixed species, cooked, dry heat	1.747	1.26	3.007	0.72:1	Not reported
Shad, American, raw	1.321	1.086	2.407	0.82:1	0.045
Roe, mixed species, raw	1.363	0.983	2.346	0.72:1	Not reported
Herring, Atlantic, kippered	1.179	0.97	2.149	0.82:1	0.084 (herring source not specified)
Salmon, Atlantic, farmed, cooked, dry heat	1.457	0.69	2.147	0.47:1	0.022 (salmon source not specified)
Herring, Pacific, cooked, dry heat	0.883	1.242	2.125	1.41:1	0.084 (herring source not specified)
Anchovy, European, canned in oil, drained solids	1.292	0.763	2.055	0.59:1	0.017 (source not specified)
Herring, Atlantic, cooked, dry heat	1.105	0.909	2.014	0.82:1	0.084 (source not specified)
Mackerel, Pacific and jack, mixed species, cooked, dry heat	1.195	0.653	1.848	0.55:1	Not reported
Salmon, Atlantic, wild, cooked, dry heat	1.429	0.411	1.84	0.29:1	0.022 (salmon source not specified)
Sablefish, smoked	0.945	0.891	1.836	0.94:1	Not reported
Sablefish, cooked, dry heat	0.920	0.867	1.787	0.94:1	0.361
Salmon, Chinook, cooked, dry heat	0.727	1.01	1.737	1.39:1	0.022 (salmon source not specified)
Whitefish, mixed species, cooked, dry heat	1.206	0.406	1.612	1.97:1	0.089
Tuna, fresh, Bluefin, cooked, dry heat	1.141	0.363	1.504	0.32:1	Not reported
Anchovy, European, raw	0.911	0.538	1.449	0.59:1	0.017 (source not specified)
Mollusks, oyster, Pacific, cooked, moist heat	0.500	0.876	1.376	1.75:1	0.012 (source not specified)
Salmon, pink, cooked, dry heat	0.751	0.537	1.288	0.71:1	0.022 (salmon source not specified)
Salmon, Coho, farmed, cooked, dry heat	0.871	0.408	1.279	0.47:1	Not reported
Mackerel, Spanish, cooked, dry heat	0.952	0.294	1.246	0.31:1	0.454 (from Gulf of Mexico) 0.182 (from S. Atlantic)
Salmon, sockeye, cooked, dry heat	0.700	0.53	1.23	0.76:1	Not reported
Mackerel, Atlantic, cooked, dry heat	0.699	0.504	1.203	0.72:1	0.05 (from N. Atlantic)
Halibut, Greenland, cooked, dry heat	0.504	0.674	1.178	1.34:1	0.241 (source not specified)
Salmon, chum, drained solids with bone	0.702	0.473	1.175	0.67:1	Not reported
Trout, rainbow, farmed, cooked, dry heat	0.820	0.334	1.154	0.41:1	Not reported
Salmon, Coho, wild, cooked, dry heat	0.658	0.401	1.059	0.61:1	Not reported
Bluefish, cooked, dry heat	0.665	0.323	0.988	0.49:1	0.368
Trout, rainbow, wild, cooked, dry heat	0.520	0.468	0.988	0.90:1	Not reported
Sardine, Atlantic, canned in oil, drained solids with bone	0.509	0.473	0.982	0.93:1	0.013 (source not specified)
Bass, striped, cooked, dry heat	0.750	0.217	0.967	0.29:1	0.152 (saltwater source)
Trout, mixed species, cooked, dry heat	0.677	0.259	0.936	0.38:1	0.071
Tilefish, cooked, dry heat	0.733	0.172	0.905	0.23:1	1.45 (from Gulf of Mexico) 0.144 (from Atlantic)
Tuna, white, canned in water, drained solids	0.629	0.233	0.862	0.37:1	0.35 (Albacore)
Tuna, light, canned in water, drained solids	0.223	0.047	0.27	0.21:1	0.128

Sources: Modified from USDA Dietary Guidelines, appendix G2 http://www.health.gov/dietaryguidelines/dga2005/report/html/table_g2_adda2.htm, accessed May 22, 2015; Mercury Levels in Commercial Fish and Shellfish (1990–2010) http://www.fda.gov/food/foodborneillnesscontaminants/metals/ucm115644.htm, accessed June 2, 2015.

and DHA is krill [10], small crustaceans feeding on marine algae (phytoplankton) and zooplankton [11]. Compared to oily fish, krill have similar DHA content but higher levels of EPA [12]. In fish, EPA and DHA are mainly incorporated into triglycerides, while in krill these fatty acids are predominantly incorporated into phospholipids [13]. These differences appear to impact availability; krill oil has a greater bioavailability than similar amounts of ingested DHA + EPA from either fish oil or krill meal over a 72 h period [13].

Although fatty fish are the predominant dietary sources of EPA and DHA, many species of fatty fish also contain methyl mercury (see Table 23.1, [14,15]). The toxin is a result of mercury contaminants entering bodies of water where resident bacteria convert mercury to methyl mercury [16]. Methyl mercury travels up the food chain as larger organisms consume smaller organisms, thereby accumulating in the larger fish that are in the ocean for a longer time. Individuals are therefore advised to select fish high in *n*-3 fatty acids and low in methyl mercury to consume sufficient EPA and DHA while avoiding the neurotoxic effects of mercury. This is especially important for pregnant or breastfeeding women and young children who are advised to avoid fish with the highest mercury content and consume a maximum of 6 oz of white albacore tuna per week (a higher mercury fish) [14,16].

The 2015 Dietary Guidelines Advisory Committee (DGAC) reports that farm-raised seafood, such as bass, cod, trout, and salmon, has as much or more EPA and DHA per serving compared to its wild counterpart [14]. The equal or increased *n*-3 fatty acid content of farm-raised seafood is a consequence of the feed provided by the farmer. Farmed fish are fed a mixture of fish oil, fish meal, plant proteins, vegetable oils, minerals, and vitamins, with specifics dependent upon the species [17].

Numerous studies have explored the differences in *n*-3 fatty acid content between wild versus farmed fish, providing an evidence base for the 2015 DGAC statement mentioned above [14]. Low-trophic-level seafood (i.e., catfish and crawfish), irrespective of wild or farmed, contains less EPA and DHA compared to high-trophic-level seafood (i.e., salmon and trout). According to the USDA National Nutrient Database (Release 27), 100 g of raw, wild Atlantic salmon provides 2.539 g of PUFAs, while raw, farmed Atlantic salmon provides 3.886 g of PUFAs; however, the PUFA content is reported as total PUFAs, not individual *n*-3 and *n*-6 fatty acids [18,19]. Cladis et al. [20] obtained 76 finfish species and found the total *n*-3 fatty acid content (ALA + stearidonic acid + eicosatrienoic acid + eicosatetraenoic acid + EPA + DPA + DHA) was highest in Chilean sea bass (3011 mg/100 g), albacore tuna (2631 mg/100 g), farmed Atlantic salmon (2544 mg/100 g), and farmed Chinook salmon (2179 mg/100 g). Of the species with both farmed and wild samples available (cod, striped bass, rainbow trout, white sturgeon, Chinook salmon), the EPA + DHA content was higher, on average, in the farmed samples compared to the wild samples. Weaver et al. [21] determined the *n*-3 fatty acid content of 30 commonly consumed farmed and wild fish collected in 2005 and found the top *n*-3 fatty acid–containing fish (>500 mg fatty acids/100 g fish) were farmed trout, farmed Atlantic salmon, Coho salmon, Toothfish, Copper river salmon, Sockeye salmon, and Bronzini. When comparing the most commonly consumed farmed fish (salmon, tilapia, catfish, and trout), farmed salmon and farmed trout contained the highest total *n*-3 fatty acids compared to farmed catfish and tilapia.

While farm-raised seafood is a viable source to increase dietary intake of *n*-3 fatty acids, providing equal or greater *n*-3 fatty acids compared to wild seafood, the *n*-3 fatty acid content of farm-raised fish has steadily declined and should be monitored. Nichols et al. [22] examined the *n*-3 fatty acid content of farmed Atlantic salmon and farmed barramundi collected from 2010 to 2013 and compared the findings to previously collected data from 2002 or 1998. The farmed Atlantic salmon EPA + DPA + DHA content was highest in 2002 (2010 mg/100 g) and ranged from a maximum in spring 2010 (1770 mg/100 g) to a minimum in spring 2013 (980 mg/100 g). Similarly, the farmed barramundi had a higher EPA + DPA + DHA content in 2002 (1970 mg/100 g) compared to 2010 (790 mg/100 g). However, both the 2002 and 2010 farmed barramundi samples contained greater *n*-3 fatty acids compared to 1998 wild barramundi samples.

Although dietary sources are the preferred option to increase *n*-3 intake, supplementation is an alternative. *n*-3 supplements typically contain EPA and DHA from fish oil, but other marine

sources include krill oil, calamari oil, algal oil, and green-lipped mussel oil. The results are conflicting regarding actual *n*-3 supplement use. NHANES data (2007–2010) were utilized to assess supplement use among Americans from the past 30 days [23]. Of the 11,956 adult respondents, 49% reported supplement usage in the past 30 days; *n*-3 fatty acid/fish oil supplementation was the third most frequently reported supplement (9.8% of supplement users). The Council for Responsible Nutrition also assessed supplement use through annual consumer surveys conducted from 2007 to 2011 (about 2000 individuals surveyed per year [24]). The surveys accounted for regular, occasional, and seasonal use of supplements, rather than the past 30 days in the NHANES survey. The investigators found that 64%–69% of adults use supplements; however, regular supplement use ranged from 48% to 52%, comparable to the reported NHANES findings. The 2011 survey (n = 2015 [24]) specifically assessed supplement types, and the investigators found that *n*-3/fish oil supplements were the second most common supplement used (33% of supplement users from 2011).

In addition to dietary sources and over-the-counter supplements, there also are prescription *n*-3 PUFA formulations. Currently, there are 6 U.S. FDA-approved prescription *n*-3 products for treating hypertriglyceridemia (≥500 mg/dL [25]). These include Lovaza (omega-3-acid ethyl esters; EPA + DHA), Vascepa (icosapent ethyl; EPA), Epanova (omega-3-carboxylic acids; EPA + DHA), Omtryg (omega-3-acid ethyl ester A; EPA + DHA), and 2 generic versions of Lovaza (omega-3-acid ethyl esters; EPA + DHA).

The health benefits and recommendations for consumption have increased consumer demand for long-chain *n*-3 fatty acids. This is reflected in the *n*-3 fatty acid enrichment of foods and beverages. Examples include enrichment of eggs, baked goods, meat products, dairy products, emulsified foods, and infant formulas [26]. The increased demand for long-chain *n*-3 fatty acids is a potential threat to the sustainability of marine sources of *n*-3 fatty acids. Thus, genetically modified organisms that are higher in the desired *n*-3 fatty acids are in the pipeline, including stearidonic acid, *n*-3 soybeans [27], and EPA and DHA production from the oilseed crop *Camelina sativa* [28,29].

A. Dietary Intakes

The Dietary Reference Intakes (DRIs) issued by the Institute of Medicine (IOM) include macronutrient intake recommendations for healthy individuals of various life stages and physiological states. An adequate intake (AI) has been established for the essential *n*-3 fatty acid ALA [30]. The AI is the daily adequate intake level assumed to be sufficient to prevent deficiency symptoms and, in this case, is based on the median intake of ALA by healthy adults in the United States where a deficiency is nonexistent. The AIs for ALA for each life stage and physiological state are as follows:

Infants: 0–12 months 0.50 g/day of *n*-3 PUFAs (*AI for ALA not determined for this life stage)
Children: 1–3 years 0.7 g/day of ALA
4–8 years 0.9 g/day of ALA
Boys: 9–13 years 1.2 g/day of ALA
14–18 years 1.6 g/day of ALA
Girls: 9–13 years 1.0 g/day of ALA
14–18 years 1.1 g/day of ALA
Men: 19 to >70 years 1.6 g/day of ALA
Women: 19 to >70 years 1.1 g/day of ALA
Pregnancy: 14–50 years 1.4 g/day of ALA
Lactation: 14–50 years 1.3 g/day of ALA

AIs have not been established for the nonessential *n*-3 fatty acids (i.e., stearidonic acid, EPA, DPA, and DHA), as they can be derived endogenously from ALA. The IOM [30] also recommends an Acceptable Macronutrient Distribution Range (AMDR) for ALA of 0.6%–1.2% of total energy for healthy individuals aged 1 and older, with up to 10% (0.06%–0.12% of total energy) consumed as EPA and/or DHA.

The Food and Agricultural Organization of the United Nations (FAO) issued an expert consultation report of fat and fatty acids and recommended a greater ALA AMDR compared to the IOM recommendation [31]. Specifically, a minimum of 0.5%–0.6% of energy from ALA is recommended to prevent *n*-3 deficiency, with a total *n*-3 intake range of 0.5%–2% of energy. For the prevention of coronary heart disease (CHD) and potentially degenerative diseases, the FAO panel recommends a minimum of 0.25 g/day of EPA plus DHA for adults and a minimum of 0.3 g/day of EPA plus DHA for pregnant and/or lactating females, with at least 0.2 g/day from DHA. The FAO has set the maximum level of EPA plus DHA at 2 g/day, with recommendations for further research to possibly increase the recommendation in the future. In contrast, the FDA recommends a maximum of 3 g/day of EPA plus DHA, with no greater than 2 g/day from dietary supplements [32].

The 2015–2020 Dietary Guidelines for Americans (DGAs) provides recommendations on healthy eating patterns and emphasizes seafood consumption to increase dietary intake of the long-chain fatty acids EPA and DHA [33]. The DGAs, issued by the U.S. Departments of Agriculture and Health and Human Services, are a set of guidelines for all Americans aged 2 years and older. The guidelines recommend 8 oz/week (less for young children) of a variety of seafood, averaging 0.25 g/day of EPA plus DHA, for reduced risk of cardiovascular disease (CVD). An average of 0.25 g/day provides 1.75 g/week of EPA plus DHA. Two examples of proposed dietary patterns in the DGAs include the U.S.-Style Eating Pattern and the Healthy Mediterranean-Style Eating Pattern. For a 2000-calorie level diet, the U.S.-Style includes 8 oz equivalents of seafood per week and the Mediterranean-Style includes 15 oz equivalents of seafood per week. For pregnant women, the 2015 DGAs recommend at least 8 oz and up to 12 oz of low-methylmercury seafood per week to ensure the health benefits of *n*-3 fatty acids on fetal growth and development. Mozaffarian et al. [34] suggest slightly higher intakes with an average daily range of 0.25–0.4 g of EPA and DHA for primary prevention of CVD. This was based on a prospective study of generally healthy older adults that reported plasma concentrations of individual *n*-3 fatty acids (EPA, DPA, and DHA) and total *n*-3 fatty acids were associated with lower total and CHD mortality.

Other organizations and expert panels have made food-based recommendations to ensure adequate *n*-3 dietary intakes. Examples include the American Heart Association (AHA), the American Heart Association/American College of Cardiology (AHA/ACC), the National Lipid Association (NLA), and the Academy of Nutrition and Dietetics. The AHA recommends at least 2 servings (about 8 oz) of a variety of fish per week, with fatty fish as the preferred marine option to increase EPA and DHA intake and decrease risk of heart disease [15,35]. To increase ALA intake, the AHA recommends plant sources of *n*-3 fatty acids, including soybean, canola, walnut, flaxseed, and their respective oils [35]. The AHA also suggests individuals with documented CVD and/or high triglycerides discuss *n*-3 supplementation with their physicians [15,35]. The 2013 AHA/ACC Lifestyle Management Guidelines advise individuals who would benefit from LDL-C lowering and/or blood pressure lowering to consume a dietary pattern that includes fish, nontropical vegetable oils, and nuts [36]. The strength of the recommendations is graded as strong by the National Heart, Lung, and Blood Institute (NHLBI), indicating there is high certainty that the benefit is substantial based on the evidence. The NLA also recommends ≥2 servings of seafood/fish (preferably oily) per week [37]. The Academy of Nutrition and Dietetics recommends 2 or more servings of fatty fish (preferably oily) per week, providing a minimum of 0.5 g of EPA and DHA per day, with *n*-3 fatty acids accounting for 0.5%–2% of total energy per day [38].

Although daily *n*-3 PUFA intake levels and food-based recommendations have been proposed, Papanikolaou et al. [39] found that Americans, on average, are not meeting the current guidelines. The investigators utilized the 2003–2008 NHANES data of individuals ≥19 years who had complete 24 h dietary recall data (n = 14,338). Mean intake of total fish was 0.61 ± 0.03 oz equivalents/day, and mean usual intake of fish high in *n*-3 fatty acids was 0.15 ± 0.03 oz equivalents/day. Adults ≥51 years had a greater mean intake of total fish and *n*-3 fish compared to individuals 19–50 years. In addition, males of all age groups had greater usual intakes of total fish and *n*-3 fish compared to

their female counterparts. The authors [39] also determined mean intakes of individual *n*-3 fatty acids from food alone; intake of ALA was 1.5 ± 0.01 g/day (males 1.7 ± 0.002 g/day, females 1.3 ± 0.002 g/day), intake of DHA was 63 ± 2 mg/day, and intake of EPA was 23 ± 1 mg/day. Although the mean intake of ALA for both males and females exceeded the IOM AIs for ALA (adult males 1.6 g/day ALA, adult females 1.1 g/day of ALA), the total fish, total *n*-3 fish, DHA, and EPA intakes from food fell short of meeting all recommendations. Consistent findings have been reported in the most recent 2011–2012 NHANES data of nutrient intakes from food and beverages. The mean amount consumed per individual 20 years and over was 1.82 ± 0.025 g/day of ALA, 0.03 ± 0.002 g/day of EPA, and 0.06 ± 0.004 g/day of DHA [40]. Although Americans are meeting the recommendations for ALA intake, they are far below the recommendations for EPA and DHA.

B. Production of Long-Chain PUFA *In Vivo*

In addition to dietary sources of *n*-3 PUFA, *in vivo* conversion of the essential fatty acid ALA to longer-chain *n*-3 PUFA can occur in animals (see Figure 23.1), although this conversion is highly inefficient. While ALA supplements increased plasma levels of both EPA and DPA [41], less than 0.2% of ALA was converted to plasma EPA in adults, and less than 0.1% of ALA was converted to plasma DHA [42]. Another study examining plasma phospholipids after subjects consumed ALA found 7% of the ALA was incorporated into plasma phospholipids, and more than 99% of that amount was converted into EPA. Due to inefficient conversion, only 1% of the EPA plasma phospholipids were converted to DPA, although almost all of the DPA was converted into DHA [43]. These results suggest conversion of ALA to stearidonic acid *via* Δ-6 desaturase is not the rate-limiting step. The authors hypothesized that the limiting factor in conversion of ALA to DHA may be entry of plasma phospholipids into the hepatic phospholipid pool [43].

The extent of ALA conversion to EPA is influenced by many factors. These include gender, with females having a greater conversion rate than males [44] and age, with infants having a greater conversion rate than adults [45]. Hormones other than estrogen can also influence conversion rates; glucagon, adrenaline, glucocorticoids, and thyroxin are known to depress both delta-5-desaturase and delta-6-desaturase activity [46]. An additional source of variation may result from delta-6-desaturase genetic polymorphisms. These polymorphisms are common within populations [47] and may account for much of the observed variation in PUFA levels among individuals ingesting similar amounts of ALA [48]. Davidson [49] hypothesized that these polymorphisms may account for higher levels of inflammatory prostaglandins and leukotrienes in some populations, thus explaining why some ethnic groups may be more prone to hypertension and asthma, or why other populations are more prone to hypertriglyceridemia.

Other *n*-3 PUFAs besides ALA can be converted into longer-chain PUFAs and have the benefit of more efficient conversion rates. For example, stearidonic acid (SDA, 18:4*n*-3) increases plasma and erythrocyte EPA at 30% of the increase seen with EPA supplementation [50]. These researchers also found DPA levels increased after SDA supplementation, although DHA levels were not affected. Compared to the low conversion rate of ALA to EPA [42,43], the greater conversion of SDA to EPA [50] reflects the non-limiting reactions involving elongase and Δ-5-desaturase. In addition to SDA found within algae, fish, and seafood, some seeds containing more than 10% of their seed oil as SDA include hemp (*Cannabis*) and plants within the *Echium*, *Primula*, *Lappula*, and *Lithospermum* genera [51]. A genetically modified soybean was also generated that produces approximately 28% of its total fatty acids as SDA [52].

Ratios of dietary ALA to linoleic acid (18:2*n*-6) may also influence the *in vivo* conversion of ALA to long-chain *n*-3 PUFA due to competition for the enzyme delta-6-desaturase [53; see Figure 23.1]. Although delta-6-desaturase has a higher affinity for ALA than linoleic acid [54], the higher linoleic acid concentrations typically found in individuals consuming a Western diet may out-compete ALA for the enzyme [55], potentially resulting in lower levels of longer chain *n*-3 PUFAs [56].

III. EXCLUSIVE AND COMMON EFFECTS OF EPA AND DHA

Although many studies examine the effects of combined EPA + DHA, there is increasing evidence that individual *n*-3 PUFA can have both unique and overlapping effects. For example, the Cardiovascular Health Study followed more than 2600 older adults who were initially without CHD, heart failure, or a history of stroke [34]. Both individual and combined plasma phospholipid fatty acid levels were examined relative to cause-specific mortality over a period of 16 years. Overall, the highest total plasma phospholipid *n*-3 fatty acids significantly predicted the lowest total mortality risk (27% lower risk). When the data were examined on the basis of individual long-chain PUFA, participants with the highest EPA had a 17% lower total mortality risk, those with the highest DHA had a 20% lower risk, and those with the highest DPA had a 23% lower risk. When the data were examined relative to CVD, the combined PUFA was associated with a 35% lower risk of CVD. For the different subtypes of CVD, high DHA levels appeared most important. DHA was associated with a 40% lower CHD risk and a 45% lower risk of cardiac arrhythmia. Interestingly, neither EPA nor DPA was associated with a decreased risk of CHD, indicating potential unique effects of the different *n*-3 PUFA. Further evidence of unique effects was shown when, relative to nonfatal myocardial infarction, only EPA levels were significantly associated with a reduced risk. Likewise, relative to death from stroke, DPA was most strongly related (47% lower risk) to reduced death rate. The Cardiovascular Health Study [34] also found a significant inverse association only between DPA and cancer mortality, and between total *n*-3 PUFA and death from infections.

Other studies have also found that EPA and DHA can have dissimilar effects. A meta-analysis of 10 trials examining effects of *n*-3 supplementation on attention-deficit/hyperactivity disorder (ADHD) found a significant association between the amount of EPA and improved ADHD symptoms. DHA, however, did not have a significant effect on treating ADHD symptoms [57]. Similarly, EPA but not DHA showed significant benefits in treating depression [58]. One possible mechanism to explain these findings is that both EPA (20:5*n*-3) and arachidonic acid (20:4*n*-6) compete for the same elongase (see Figure 23.1) as well as several enzymes in the prostaglandin synthesis pathway (e.g., prostaglandin endoperoxide H synthase, PGHS-1 [59]). Therefore, EPA can inhibit production of inflammatory prostaglandins derived from arachidonic acid. In contrast, DHA (22:6*n*-3) is not recognized by these enzymes.

IV. INFLAMMATORY PATHWAYS AND CHRONIC DISEASE

The inflammatory process involves several steps, starting with increased blood flow to the damaged area. Inflammatory mediators such as bradykinin, thrombin, histamine, and vascular endothelial growth factor cause the junctions between endothelial cells to widen [60]. These wider intercellular gaps allow the passage of plasma proteins such as albumin, an osmotic protein, to enter the surrounding tissue [61]. Inflammatory cytokines also stimulate endothelial cells to synthesize adhesion molecules and lipid chemoattractants that recruit circulating leucocytes to the region [62]. In turn, leucocytes release chemical mediators to assist in host defense. While leukocytes produce many chemical mediators (e.g., histamine, cytokines, reactive oxygen species), some mediators such as prostaglandins and leukotrienes (types of eicosanoids) are derived from *n*-6 PUFA [63].

Eicosanoids are a group of bioactive signaling molecules derived from membrane-bound PUFA. This group includes more than 100 different compounds, and although the name eicosanoid indicates they are fatty acids containing 20 carbons, eicosanoids also include similar metabolites derived from other PUFAs [64]. To initialize eicosanoid pathways, PUFAs such as *n*-6 arachidonic acid are cleaved from a membrane-bound phospholipid by phospholipase A_2 (PLA_2); with regard to eicosanoid production, cytosolic PLA_2 is most likely [65]. Metabolically, eicosanoids are produced when PUFAs are converted by a variety of enzymatic pathways. These enzymes include cyclooxygenases (COX-1 and COX-2, creating prostaglandins and thromboxanes), lipoxygenases (LOX, creating leukotrienes, lipoxins, and others), cytochrome P450s (CYP), and nonenzymatic

pathways [64]. Notably, COX-2 can also recognize EPA in addition to arachidonic acid. With regard to prostaglandins, the PUFA precursor defines the final products; dihomo-γ-linolenic acid (20:3*n*-6) is the precursor to the 1-series of prostaglandins, arachidonic acid is the precursor for the 2-series, and EPA is the precursor for the 3-series (see Figure 23.1, [66]). The 2 series is generally considered inflammatory [67], although a single prostaglandin molecule may have many roles, including both inflammatory and anti-inflammatory functions, due partially to several unique receptors and differential effects among various cell and tissue types [68]. As an example of these complex effects, prostaglandin E2 interacting with receptor EP4 is associated with several inflammatory mechanisms such as inhibiting interleukin-12 production by macrophages, as well as anti-inflammatory mechanisms such as suppressing macrophage cytokine production [69].

When arachidonic acid is released from cell membranes and transformed into eicosanoids such as prostaglandins, leukotrienes, and thromboxanes, these compounds signal immune cells and trigger immune responses [70]. One hypothesis is that an abundance of *n*-6 PUFAs in the diet may cause more of these inflammatory compounds to be produced, while consuming more *n*-3 PUFAs may reduce production of these mediators. While the inflammatory response to infection or injury is essential for proper immune function, high *n*-6 PUFA intake is associated with bronchial constriction, pain, increased blood clotting, blood vessel constriction, and increased blood viscosity [55]. Due to this inflammatory association, *n*-6 PUFAs have been implicated in chronic diseases such as arthritis, CVD, and cancer [71].

However, it is important to note the beneficial aspects of *n*-6 PUFA and instances where they have no apparent negative health impacts. For example, epoxyeicosatrienoic acids (EETs) are other eicosanoids derived from arachidonic acid (see Figure 23.1). EETs are synthesized by epoxygenase enzymes and appear to have unique vascular effects depending on the organ in question. Some of these vascular effects include vasodilation, stopping platelet aggregation, or acting as an anti-inflammatory agent [72].

Johnson and Fritsche [73] conducted a qualitative review of fifteen randomized, placebo-controlled intervention studies examining how healthy subjects older than 1 year responded to alterations in dietary *n*-6 linoleic acid in terms of inflammatory biomarkers. The most frequently examined biomarkers in these studies included C-reactive protein (CRP), fibrinogen, and plasminogen activator inhibitor-1. There were no differences between the control and linoleic acid groups for any of these biomarkers or for several other markers tested less frequently, such as TNF-α, intercellular adhesion molecule-1, or several series 2 prostaglandins. Although these studies were shorter than 6 weeks in duration and had, on average, fewer than 35 participants, there was little evidence suggesting that dietary linoleic acid increased inflammatory biomarkers in healthy individuals. This can possibly be explained by a lack of statistical association between dietary linoleic acid and changes in plasma arachidonic acid levels, even when dietary levels were decreased by 90% or increased sixfold [74].

Interestingly, even in 269 individuals who exhibited clinical signs of coronary artery disease, there was no significant relationship between the inflammatory biomarker CRP and granulocyte arachidonic acid content [75]. A lack of significant *n*-6 PUFA (including arachidonic acid) associations with inflammation is also seen in other disease-related studies. A multiethnic cohort study of more than 2800 U.S. adults found that while circulating EPA and DHA were inversely associated with CVD events, there were no significant associations for either circulating linoleic acid or circulating arachidonic acid with CVD [76]. The SELECT Trial examined plasma phospholipid fatty acids in relation to prostate cancer risk in a group of 834 men diagnosed with prostate cancer, and almost 1400 men matched to those cases. While the study found higher *n*-3 levels were associated with an increased risk of prostate cancer, *n*-6 linoleic acid was actually associated with a reduced cancer risk in the upper three quartiles, although there was no linear trend. This study also found arachidonic acid was not associated with increased prostate cancer risk [77].

It is possible these two schools of thought, one suggesting *n*-6 fatty acids are inflammatory as compared to the idea that *n*-6 fatty acids do not influence inflammation, may both be valid. An analysis of a combined subset from the Health Professionals Follow-up Study and the Nurse's

Health Study II examined 859 subjects including both healthy subjects and those with chronic inflammatory diseases such as rheumatoid arthritis and Crohn's Disease. Men and women with the highest EPA + DHA intakes had the lowest levels of soluble tumor necrosis factor receptors (sTNF-R1 and sTNF-R2); neither ALA (*n*-3) nor linoleic acid (*n*-6) was significantly related to any measured inflammatory markers [78]. However, when the data were further separated based on two factors, namely, intake levels of EPA + DHA relative to linoleic acid intake, higher amounts of *n*-6 fatty acids were associated with the highest levels of soluble tissue necrosis factor receptors at the lowest levels of *n*-3 PUFA intake. Conversely, at the highest levels of *n*-3 PUFA intake, high *n*-6 intake was related to the lowest levels of both sTNF-R1 and sTNF-R2 inflammatory markers [78]. It is possible that *n*-6 intakes are associated with inflammation when *n*-3 intakes are low but may have anti-inflammatory roles when *n*-3 intakes are higher. Despite the developing pattern seen when examining sTNF relative to both *n*-3 and *n*-6, there were no correlations between *n*-3 or *n*-6 fatty acid intake and other inflammatory biomarkers such as CRP or interleukin-6.

The Rotterdam study [79] also supports these findings that high total (*n*-3 and *n*-6) PUFA intake is associated with lower levels of inflammatory markers. More than 2900 participants over the age of 55 were monitored for serum CRP level at the start of the study and at a third visit approximately 7 years later. Their total dietary PUFA was assessed by a validated food frequency questionnaire. Findings indicate that both higher total PUFA intake and higher *n*-6 PUFA intake were significantly associated with lower CRP levels. This study, however, found no consistent trends relating either *n*-3 PUFA or the ratio of *n*-3 to *n*-6 to CRP [79].

A. How *n*-3 PUFA May Elicit Anti-Inflammatory Benefits

Regardless of the cause of inflammation, *n*-3 PUFAs are well supported in their role as anti-inflammatory agents. Membrane-bound PUFAs are a source of biologically active molecules. Replacing some membrane-bound *n*-6 fatty acids with *n*-3 fatty acids increases competition between released PUFA for enzymes in the eicosanoid pathway [80]. For example, treating endothelial cells with EPA resulted in COX enzymes producing prostaglandin D3. This compound antagonized the prostaglandin D2 receptor on neutrophils, stopped their migration, and thus suppressed inflammation [8].

Released membrane-bound DHA and EPA appear to influence more than eicosanoid biosynthesis. DHA and EPA can also reduce inflammation when they bind and activate various compounds and receptors. G protein–coupled receptors (GPCR and GPR) are signaling molecules that can activate G proteins (guanine nucleotide–binding proteins). In turn, G proteins transmit signals from a cell's exterior to its interior. Ligands binding to GPCRs induce various cellular responses *via* secondary messengers such as cAMP [3]. While several G proteins can be activated by various free fatty acids, GPR120 is specifically activated by long-chain fatty acids such as DHA and EPA; this receptor is highly expressed in both adipose tissue and pro-inflammatory macrophages. When bound by DHA or EPA, activated GPR120 inhibits inflammatory responses mediated by both TLR4 (toll-like receptor 4, associated with the production of pro-inflammatory cytokines) and TNF-α (tumor necrosis factor, a cytokine associated with the acute phase of inflammation [3]). However, it has been noted that GPR120 is also activated by *n*-6 PUFA such as γ-linolenic acid, saturated fatty acids such as stearic acid, as well as ALA [81]. These findings suggest that additional mechanisms other than activating GPR120 may account for the anti-inflammatory effects of DHA and EPA.

Another possible molecular target of *n*-3 PUFA is PPARγ (peroxisome proliferator–activated receptor). PPARγ is a type II nuclear receptor that, when bound to *n*-3 PUFA, inhibits inflammatory pathways by blocking the activation of NF-kB (nuclear factor [63]). The NF-kB pathway is important in the expression of cytokines, chemokines, and adhesion molecules that are typical of inflammatory pathways [82]. However, PPARγ also binds with ALA and the *n*-6 arachidonic acid, so *n*-3 PUFA effects on PPARγ may not completely explain *n*-3's anti-inflammatory effects [81].

Other interactions with NF-kB and its inhibitor protein, IkB may contribute to *n*-3 PUFA's anti-inflammatory effects. Under normal conditions, NF-kB is found in the cytoplasm and is associated

with an inhibitor protein (IkB). IkB keeps NF-kB inactive by masking a nuclear localization sequence. When macrophages are induced, IkB kinase-α phosphorylates IkB and causes it to dissociate from NF-kB. NF-kB is then able to relocate to the nucleus and initiate transcription of several genes, including TNF-α. However, pretreatment with *n*-3 PUFA inhibits phosphorylation of IkB, significantly reducing NF-kB activity and causing a 46% drop in TNF-α protein expression and thus a decrease in cytokine production [83]. Decreased phosphorylation of IkB (and thus decreased NF-kB activation) is also thought to be involved in the decreased expression of adhesion molecules after exposure to marine-derived *n*-3 PUFA [80].

DHA and EPA released from cell membranes can also be modified to form *specialized pro-resolving mediators*. These pro-resolution compounds control both the duration and magnitude of acute inflammation and return the inflamed area to normal by influencing macrophage-based clearance of the damaged area [84] and stopping neutrophil infiltration of the area [81]. While inflammation in response to injury or invasion is beneficial, this process must be controlled and resolved to avoid problems associated with chronic inflammation (e.g., CVD, asthma, diabetes, rheumatoid arthritis, Alzheimer's [85]). Several DHA-derived pro-resolving mediators have been identified, including the protectins, neuroprotectins, and macrophage-produced maresins. Both EPA and DHA can be modified to form compounds called resolvins. It is important to note that while these *n*-3-derived resolvins are anti-inflammatory in nature, the *n*-6 PUFA arachidonic acid is also a precursor for a group of pro-resolving mediators called lipoxins [84].

Impressive progress has been made in our understanding of the anti-inflammatory effects of long-chain *n*-3 fatty acids. Additional research is needed to determine the amount of each *n*-3 fatty acid that elicits the optimal anti-inflammatory response. Based on the present information, it seems prudent that all of these fatty acids including *n*-6 PUFA be consumed in the context of a healthy dietary pattern.

V. *n*-3 PUFA AND THE EVOLUTION OF THE HUMAN BRAIN

There are many theories on why the human brain increased to almost three times the volume of early Australopithecines, and twice the volume of *Homo habilis*, the earliest known *Homo* genus [86]. One theory suggests exposure to colder temperatures allowed for better brain thermoregulation, and thus bigger brains [87]. Other selection-based theories suggest those with bigger and better brains increased their chances of survival in situations of competition, helped in problem solving, or allowed for increased cooperation due to better social and language skills [88].

From a nutritional viewpoint, however, increased human brain volume can only result from ingesting sufficient raw materials obtained from high-quality diets. Brain construction appears closely tied to DHA availability. When the brain chemistry of 42 mammalian species was examined, species were similar in their consistent use of DHA [89]. Crawford et al. [89] hypothesize that if adequate DHA was unavailable in a species' diet, the species sacrificed brain size instead of altering brain chemistry. It should be noted that if an individual is deficient in DHA, it does not cause microcephaly [90]. Instead, a study on rats indicates DHA deficiency during fetal and postnatal development strongly compromises adult brain plasticity [91].

The "expensive tissue hypothesis" suggests that brain size increased as gut size decreased; this smaller gut size is thought to be related to a diet higher in animal products [92]. Although this hypothesis is being debated [93], there is still evidence suggesting that dietary changes that increased animal product consumption were required in order for humans to afford bigger, and thus more costly, brains. Plant-based diets are associated with lower basal metabolic rates (BMRs) [94]. While a lower BMR results from the energy costs of digesting cellulose-based materials, it also reflects the higher energy costs associated with detoxification of plant secondary metabolites (i.e., toxins) [95]. Therefore, diverting high amounts of energy toward digestive processes potentially reduces available energy for brain development. Cooking foods would also allow for easier digestion, and thus more energy could be available for brain development after humans adopted cooking [96].

Even more intriguing is the hypothesis that consuming fish and seafood played an integral role in the evolution of the human brain and intelligence [97]. Stable isotope analysis of bone collagen indicates the proportion of dietary protein derived from terrestrial, freshwater, or marine sources that a person consumed in the decade prior to their death [98]. Analysis of Mid–Upper Paleolithic human skeletons indicates freshwater or marine sources made up 10%–50% of their diet, and this most likely resulted in greater DHA consumption. While this DHA could have been utilized in other tissues or burned for energy, higher DHA consumption could also have been directed toward brain development. Conversely, Neanderthals consumed mostly red meat; their skeletal remains show no evidence of consuming aquatic species even though they lived in similar environments to humans [98].

For our early ancestors, fish were thought to be abundant, consistently available, and easier to catch than land animals [99], thus providing not only ample calories but ample DHA. When adequate DHA was consistently present in the diets of early humans, it is thought their babies were born with not only sufficient subcutaneous fat to provide energy for postnatal brain development, but also sufficient levels of DHA in their subcutaneous fat to provide building blocks for improved neuronal development and potentially larger brains [99].

A. PUFA Distribution within the Brain and Patterns in Fetal and Postnatal Development

Sixty percent of the brain's dry weight consists of phospholipids [97]. The main fatty acid constituents of these phospholipids include arachidonic acid and DHA; DHA alone makes up 30% of the brain's total fatty acid content [90]. These fatty acids are not equally distributed within the brain. There is relatively more DHA than arachidonic acid in gray matter (cell bodies of neurons), while in white matter (axons covered with myelin sheath) there is relatively more arachidonic acid than DHA [100].

DHA, when coupled to lysophosphatidylcholine, appears to be taken up from the blood and moved across the blood–brain barrier via MFSD2A, a transporter that is highly expressed in brain endothelial cells. Humans with a mutation in this transporter suffer from microcephaly and high plasma levels of lysophosphatidylcholine, a symptom associated with PUFAs that were apparently unable to cross the blood–brain barrier [90]. Monitoring DHA uptake using positron emission tomography indicated that healthy adult humans took up 3.8 (±1.7) mg DHA/day [101], although this did not account for DHA production by astrocytes or cerebral endothelial cells [97]. Umhau et al. [101] also estimated that the half-life of DHA within the brain was 2.5 years, suggesting that a diet lacking DHA for 7 weeks might result in a 5% loss of DHA in the brain.

DHA is not only associated with specific regions of a cell, but also with specific regions within the brain. In a study examining the DHA content of 26 brain regions in 4-week-old breast-fed baboons, researchers found the highest DHA levels were (in descending order) found in the gray matter of the globus pallidus (regulates voluntary movement), superior colliculus (involved in gaze shifting), putamen (controls motor skills), pre-centralis (motor cortex), inferior colliculus (convergence of auditory pathways), and caudate (voluntary movement) [102]. It is important to recognize that these allocation patterns may not be permanent and may actually reflect the parts of the brain undergoing the greatest development at that time. During the first month of their lives, baboon infants spend most of their time clinging to their mother with very brief periods spent on the ground [103]. In other words, this first month in a baboon's life is important in gaining motor skills and learning maternal communication, commitments that may temporarily require greater amounts of DHA for periods of active neuronal development. By adulthood, DHA-rich regions may shift to reflect the brain regions used most. Within the adult rat brain, most DHA is found in brain regions associated with memory and attention (e.g., cerebral cortex and hippocampus) [104,105]. During human fetal development, major DHA deposition in the fetal brain begins in the sixth month of pregnancy, a period marked by major fetal neurogenesis. DHA deposition continues for at least 2 years postnatally, a period marked by intense synapse formation [106,107].

DHA and EPA are not only important to early brain development but also to brain aging. For example, reduced hippocampal volume is an indicator of Alzheimer's disease before symptoms appear. A cross-sectional study of more than 1100 postmenopausal women determined that a greater combined DHA + EPA content of red blood cells was associated with a larger hippocampal volume 8 years later [108].

B. How *n*-3 PUFA Might Benefit Neurons and the Brain

In addition to previously mentioned protective mechanisms common to most cells, DHA imparts some unique characteristics that improve neuronal function. While *n*-3 PUFA incorporation into membranes occurs in all tissues, some tissues such as the retina, brain, and myocardium are especially high in *n*-3 fatty acids such as DHA, suggesting not only cellular mechanisms for incorporating these specific fatty acids, but their critical role in cell function [109,110]. With regard to the brain and the retina, maintaining normal neuron function depends greatly on membrane fluidity; membranes become less fluid with added cholesterol, but more fluid with the incorporation of long-chain *n*-3 PUFA [111].

Besides fluidity, DHA also alters gene expression in the rat brain [112]. A short-term supplementation experiment examined both mouse spatial-recognition memory and gene expression within the hippocampus, a brain region associated with spatial navigation and transmitting information from short-term memory to long-term memory. After 3 weeks of supplementing their diet with fish oil, mice showed improved cognitive function in a Y-maze. These fish oil–supplemented mice also showed an upregulation of several genes [113]. Upregulated genes included α-synuclein, a widely recognized but poorly understood brain protein [114]; calmodulin, a gene with many roles including short- and long-term memory; and transthyretin, a gene coding for a protein that carries the hormone thyroxine within serum and cerebrospinal fluid. This study also found that an *n*-3 deficiency induced by feeding mice butter in place of fish oil not only reduced their cognitive performance but also increased expression of ZnT3. Upregulation of this gene in *n*-3-deficient mice indicates abnormal zinc metabolism, possibly leading to increased zinc transport into synaptic vesicles and neuronal injuries [113].

In a mouse model, not only was dietary DHA supplementation associated with increased DHA in the hippocampus, but DHA also enhanced synaptic transmission [115]. Likewise, in a rat model, a profound *n*-3 PUFA deficiency decreased the number of vesicles in dopaminergic presynaptic terminals of the frontal cortex [116]; this, in turn, could explain behavioral abnormalities exhibited by deficient rats.

Ion exchange, necessary for sending messages along nerves, is also directly affected by *n*-3 PUFAs. For example, in rat neurons, *n*-3 fatty acids directly activate the ion channel TRPV1 [117]. TRPV1 is a nonselective cation channel involved in transmitting pain messages when activated by compounds such as capsaicin. Interestingly, *n*-3 PUFAs differed in their ability to stimulate or suppress the TRPV1 ion channel; DHA was most effective as an agonist, while EPA and linolenic acid were effective inhibitors. These cellular actions were supported by studies injecting capsaicin, capsaicin + DHA, or capsaicin + EPA into the footpad of mice. Only EPA was effective in reducing behavior associated with capsaicin-induced pain [117].

Other possible functions of DHA within the brain include protecting CNS function during trauma. In a study of induced spinal cord injury in mice, Paterniti et al. [118] found treating mice with DHA post injury significantly decreased several key indicators of spinal cord trauma, including inflammation, expression of pro-inflammatory tissue necrosis factor α, nitrotyrosine (a marker of cell damage) production, expression of glial fibrillary acidic protein (produced by astrocytes and thought to be important in CNS repair), and apoptosis. In an *in vitro* model, treating neurons with DHA also reduced the effects of oxidative stress on dorsal root ganglion cells [118]. A likely cause of DHA's neuroprotection is its conversion to specialized pro-resolving mediators such as neuroprotectin D-1 [119].

C. *n*-3, Alzheimer's and Dementia

The relationship between *n*-3 fatty acid intake and dementia and/or Alzheimer's disease (AD) has been of interest for the past two decades. Numerous prospective cohort studies have reported an inverse association between fish intake and risk of dementia, AD, and/or cognitive decline [120–126]. Although fatty fish are a rich source of long-chain *n*-3 fatty acids, it is unclear whether they are responsible for the observed associations [126,127]. A meta-analysis of six prospective cohort studies (n = 22,402) reported higher fish intakes were associated with a 36% lower risk of AD (RR: 0.64; 95% CI 0.44-0.92), and a dose–response analysis showed that fish intakes of an increment of 100 g/week were associated with an 11% lower risk of AD (RR: 0.89; 95% CI 0.79–0.99) [128]. However, dietary *n*-3 fatty acid intake was not associated with a reduced incidence of AD or dementia.

The suggested protective benefits of fish and the role of *n*-3 fatty acids have been evaluated recently in randomized clinical trials. Jiao et al. [129] conducted a meta-analysis of 34 randomized controlled trials, 12 of which examined elderly volunteers (median age 71.4 years; IQR 68.9–74.2 years), to evaluate the effects of *n*-3 fatty acids on cognitive performance throughout the life span. A subgroup analysis of six elderly studies reported no effect of *n*-3 fatty acid supplementation over a 6–40 month period on cognitive decline (standard mean difference: 0.04; 95% CI −0.02 to 0.10). The range of doses in this analysis included EPA (0.24–1.08 g/day) plus DHA (0.16–1.72 g/day), DHA (0.9–1.1 g/day), ALA (2 g/day), or 0.24 g/day EPA plus 0.16 g/day DHA plus 2 g/day ALA. Moreover, a meta-analysis of ten randomized, double-blind, placebo-controlled trials reported no benefit of *n*-3 fatty acid supplementation in healthy or AD subjects over 12.8–108 weeks, with doses including EPA (19.75–1670 mg/day) plus DHA (59–1550 mg/day), or DHA only (40–800 mg/day) [130]. A 2012 Cochrane review of the effects of *n*-3 supplementation for the prevention of dementia and cognitive decline assessed three randomized controlled trials of *n*-3 interventions for 6–40 months in volunteers 60 years and older (n = 3536) who were cognitively healthy at baseline [131]. The authors concluded there was no benefit of the *n*-3 fatty acid interventions that included margarine containing 400 mg/day EPA + DHA, 2 g/day ALA, or both EPA + ALA, or capsules of EPA (200–800 mg) + DHA (500–800 mg) and suggested the need for longer duration studies to detect effects. The largest (n = 4000) and longest randomized controlled trial to date reported *n*-3 fatty acid supplementation of 350 mg DHA + 650 mg EPA did not delay cognitive decline in older persons with age-related macular degeneration over 5 years [132].

Interest remains about the effects of longer-term long-chain *n*-3 fatty acid supplementation on dementia and AD. A meta-analysis of 15 *n*-3 fatty acid supplementation studies of EPA plus DHA or DHA in animal models found that long-term (minimum of 10% of the life span) *n*-3 fatty acid supplementation decreased the *n*-6/*n*-3 ratio, decreased brain plaques characteristic of AD pathology, improved cognitive function, and deceased neural loss [133]. Further studies are needed to determine the relationship between *n*-3 fatty acid intake and AD and dementia, optimizing disease stage for intervention and supplementation type and duration, especially in those with low dietary *n*-3 fatty acid intakes [131].

VI. *n*-3 PUFAs AND CHD, EPIDEMIOLOGICAL AND RCT EVIDENCE

There is convincing epidemiological evidence of a cardioprotective benefit of fish consumption. A meta-analysis of 11 prospective cohort studies evaluating fish consumption and CHD mortality in more than 222,000 individuals found an inverse association with fish consumption and fatal CHD [134]. The authors reported a dose–response relationship; each 20 g/day increase in fish consumption was associated with a 7% lower risk of CHD mortality. Moreover, a meta-analysis of 14 cohort and 5 case–control studies reported fish consumption versus little-to-no consumption was associated with a lower risk of fatal CHD (RR: 0.83; 95% CI 0.76–0.90; $p < 0.005$) and total CHD (RR: 0.86; 95% CI 0.81–0.92; $p < 0.005$) [135]. A lower risk of heart failure is also associated with fish intake. A meta-analysis of seven prospective studies (n = 176,441) found the pooled relative risk

for incident heart failure comparing the highest to lowest category of fish intake was 0.85 (95% CI 0.73–0.99; p = 0.04), while the pooled relative risk for heart failure comparing the highest to lowest category of omega-3 fatty acids intake was 0.86 (95% CI 0.74–1.00; p = 0.05) [136].

There is some epidemiological evidence of a benefit of ALA, the plant-derived precursor for EPA and DHA, on CHD. Pan et al. [137] conducted a meta-analysis of ALA and CVD risk in 27 prospective and retrospective dietary and biomarker studies totaling over 250,000 individuals and over 15,000 CVD events. The authors reported that higher ALA intake was associated with a moderately lower risk of CVD (RR: 0.86; 95% CI 0.77–0.97); however, there was a high unexplained between-study heterogeneity [137]. Similarly, a meta-analysis of five prospective cohort studies evaluating the association between ALA and heart disease mortality reported that high ALA intake was associated with a 21% reduced risk of heart disease mortality (95% CI 0.60–1.04) [138].

Despite promising results from both prospective cohort studies and mechanistic studies, the results from recent randomized controlled trials (RCTs) and meta-analyses often fail to support cardiovascular benefits of *n*-3 PUFAs. RCTs and meta-analyses provide stronger evidence than observational prospective cohort studies since observational studies are limited by confounding factors such as a lack of randomized exposures, effects related to dietary displacement, and collinearity with other dietary intakes [139].

Rizos et al. [140] conducted a meta-analysis of 20 RCTs that included more than 68,000 patients and found no statistically significant associations between *n*-3 supplementation and all-cause mortality, cardiac death, sudden death, myocardial infarction, or stroke. Similarly, a meta-analysis of 10 RCTs evaluating sudden cardiac death in more than 33,000 CVD patients indicated that *n*-3 fatty acids did not reduce sudden cardiac death risk in patients with guidelines-adjusted therapy [141]. Guidelines-adjusted therapy includes routine use of drugs such as β-blockers, statins, and angiotensin-converting enzyme inhibitors, as well as lifestyle changes [142].

While a lack of significant *n*-3 effects may be due to limitations in the experimental design of RCTs (e.g., dose, timing of the exposure, length of follow-up), other factors may also influence outcomes. For example, in CVD patients with non-guidelines-adjusted therapy, *n*-3 fatty acids significantly reduced the risk of sudden cardiac death [141]. This may explain why early studies conducted prior to current treatment guidelines (e.g., Burr et al. [143]) found that male survivors of a myocardial infarction who consumed fatty fish twice a week had a 29% reduction in all-cause mortality over a 2-year period. There is also some evidence suggesting that statin drugs may attenuate the biological effects of *n*-3 PUFAs [144]. Other reasons for lack of significance in recent RCTs may reflect differences in *n*-3 PUFA amounts consumed as a result of both experimental doses administered as well as background dietary intake. A recent RCT conducted in Japan showed a significant 19% reduction in CVD mortality in those consuming *n*-3 PUFAs [145]. Sekikawa et al. [145] indicate Western studies typically administer 300–900 mg/day with an average background dietary intake of less than 300 mg/day, while the Japanese study administered 1800 mg/day with an average background dietary intake of more than 1000 mg/day. Indeed, dose may play a significant role in helping to modulate other cardiac dysfunctions. A small RCT of 43 patients found higher doses (4 g/day) of *n*-3 PUFAs increased the left ventricular ejection fraction, improved endothelial function, and decreased interleukin-6 [146]. Although recent RCT findings suggest *n*-3 are not effective in treating CHD, there are still unanswered questions regarding dosage and concomitant drug use to warrant further investigations.

Long-chain *n*-3 PUFAs also interact with ion channels, thus moderating blood pressure [147]. A meta-analysis of 70 randomized controlled trials examining the effects of DHA and EPA on blood pressure found these *n*-3 PUFAs significantly decreased both systolic and diastolic blood pressures in both untreated hypertensive and non-hypertensive subjects, although the effects were greater in those with hypertension [148]. In a mouse model, blood pressure lowering in vascular smooth muscle cells was associated with cellular uptake of DHA. Within smooth muscle cells, DHA reversibly activated large-conductance Ca^{2+} and voltage-activated K^+ channels. Activating these channels keeps the cell membrane hyperpolarized, creating negative feedback on the cell's ability to contract and thus lowering blood pressure [147].

However, it is important to recognize that while *n*-3 PUFAs can influence ion channels, there are a variety of ion channels triggering widely differing cellular responses. For example, RCTs examining atrial fibrillation indicate *n*-3 PUFAs do not alter the risk of atrial fibrillation [149,150]. These results most likely reflect the opposing effects of *n*-3 PUFA on different ion channels. For example, *n*-3 effects on sodium and calcium channels shorten cardiac action potential, while *n*-3 effects on potassium channels lengthen cardiac action potentials [151].

VII. ADDITIONAL SYSTEMS AFFECTED BY *n*-3 PUFA

As the beneficial effects of *n*-3 PUFA were recognized, other studies began examining if and how *n*-3 PUFA influenced an array of health issues. While a complete coverage of these is beyond the scope of this chapter, a brief comparison of some affected systems allows us an appreciation of the comprehensive nature of *n*-3 PUFA's effects. For example, increased levels of dietary *n*-3 PUFA are associated with increased muscle protein synthesis in older adults [152], and increased bone formation in animal models due to *n*-3 PUFA's ability to alter osteoblast function [153].

In addition to their influence on gene expression [4,112], *n*-3 PUFAs also influence telomeres. Telomeres are uniform stretches of DNA capping the end of chromosomes. These caps become shorter with each cell division. Longer telomeres are associated with better health in humans aged 97–108 years [154], while shorter telomeres are associated with more degenerative diseases. Telomere length is not only heritable [155], but it is also influenced by diet. Over a 5-year period, individuals with the lowest blood DHA + EPA levels exhibited the most rapid telomere shortening, while those in the highest DHA + EPA quartile experienced the slowest telomere shortening rate [156].

VIII. CONCLUSION

The health benefits of consuming recommended amounts of *n*-3 PUFA are extensive due to their unique impact on a variety of biochemical pathways, physiological processes, and molecular mechanisms [157]. Biochemically, *n*-3 PUFAs are precursors to a variety of chemicals that are anti-inflammatory or resolve inflammation (Figure 23.1). The importance of balanced intake must be stressed, since there is also evidence suggesting that *n*-6 PUFA can have anti-inflammatory effects, as long as sufficient *n*-3 PUFAs are available. Physiologically, *n*-3 PUFAs can impact the fluidity of cell membranes. Although DHA and EPA are distributed throughout the body, higher concentrations of DHA are found in the brain and myocardium. *n*-3 PUFAs in the brain impact the function of neurons and the nervous system, with DHA playing a vital role in fetal and infant brain development. Epidemiological evidence suggests fish intake, the primary source of dietary *n*-3 fatty acids, may be cardioprotective. However, RCTs of *n*-3 supplementation are conflicting and warrant further investigation. At the molecular level, *n*-3 PUFAs have been shown to decrease expression of proteins such as the inflammatory marker tissue necrosis factor. *n*-3 PUFAs can impact DNA not only by altering gene expression, but also by altering telomeres, our personal biological clock. The combined biological actions of these *n*-3 fatty acids in the body converge to promote health and reduce the risk of chronic disease and, as such, support the importance of achieving dietary recommendations for all *n*-3 fatty acids.

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24 Dietary Fatty Acids, Lipid Mediators, Immunity, and Inflammation

Philip C. Calder

CONTENTS

I. THE IMMUNE SYSTEM

A. Overview

Immune response is the main means by which an individual is protected from the harmful effects of infectious organisms (such as bacteria, viruses, fungi, and parasites) and other noxious insults. It is a complex system, involving many cell types dispersed across many locations in the body and moving between those locations in the lymph and the bloodstream. In some locations, immune cells are organized into distinct organs, termed primary lymphoid organs, where immune cells arise and mature (bone marrow and thymus), and secondary lymphoid organs (lymph nodes, spleen, gut-associated lymphoid tissue), where mature immune cells interact and respond to antigens. Functionally, the immune system has two divisions, called the innate (also called the natural) and the acquired (also called the specific or adaptive) immune systems.

B. Innate Immunity

Innate immunity includes physical barriers (e.g., skin), soluble factors present in the bloodstream and in secretions like saliva and tears (e.g., complement), and phagocytic cells; the latter include granulocytes (subclassified into neutrophils, basophils, eosinophils), monocytes, and macrophages. Innate immunity occurs quickly but has no memory and is therefore not influenced by previous exposure to a pathogen. Phagocytic cells recognize common structures on bacteria via surface receptors called pattern recognition receptors; the bacterial structures they recognize are called microbe-associated molecular patterns. Binding of bacteria to one of these receptors triggers phagocytosis (engulfing) and subsequent destruction of the organism by toxic chemicals, such as superoxide radicals and hydrogen peroxide. Natural killer cells also possess surface receptors and destroy their target cells by the release of cytotoxic proteins. In this way, innate immunity provides a first line of defense against invading pathogens. However, an immune response often requires the coordinated actions of both innate and acquired immunity.

C. Acquired Immunity

In contrast to innate immunity that is not very specific, acquired immunity involves the specific recognition of molecules (termed antigens) derived from an invading pathogen and which distinguish it as being foreign to the individual. Acquired immunity is also involved in ensuring that there is no active immune response to sources of nonthreatening antigens such as nonpathogenic bacteria, food, and the individual's own tissues. The main cells involved in acquired immunity are lymphocytes, which are classified into T- and B-lymphocytes (also called T cells and B cells). B-lymphocytes undergo development and maturation in the bone marrow before being released into the circulation, while T-lymphocytes mature in the thymus. From the bloodstream, lymphocytes can enter secondary lymphoid organs. Immune responses occur largely in these lymphoid organs, which are highly organized to favor the interactions between immune cells and antigens that are required for an effective immune response.

The acquired immune response is highly specific and becomes effective over several days after its initial activation. It also persists for some time after the removal of the initiating antigen. This persistence gives rise to immunological memory, which is a characteristic feature of acquired immunity. Memory is the basis for the stronger, more effective immune response to re-exposure to an antigen (i.e., reinfection with the same organism) and is the basis for vaccination.

D. B- and T-Lymphocytes

B-lymphocytes produce antibodies that are soluble antigen-specific immunoglobulins. This form of protection is termed humoral immunity. Antibodies bind to the surface of microorganisms bearing the antigen the antibody was raised against, and this promotes the recognition and phagocytosis of the organism by phagocytic cells. Thus, the organisms being dealt with by humoral immunity are extracellular until they are phagocytosed. However, some pathogens, particularly viruses, but also certain bacteria, infect individuals by entering cells. These pathogens will escape humoral immunity and are instead dealt with by cell-mediated immunity, which is the role of T-lymphocytes. T-lymphocytes express antigen-specific T-cell receptors on their surface. They are only able to recognize antigens that are presented to them on another cell surface (the cell presenting the antigen to the T-lymphocyte is called an antigen-presenting cell). Activation of the T-cell receptor results in proliferation of the T cell and secretion of the cytokine interleukin-2 (IL)-2, which promotes proliferation and differentiation. This process greatly increases the number of antigen-specific T-lymphocytes. There are three main types of T-lymphocytes: cytotoxic, helper, and regulatory. Cytotoxic T-lymphocytes carry the surface protein marker CD8 and kill infected cells and tumor cells by secretion of cytotoxic enzymes, which cause lysis of the target cell. Helper T-lymphocytes

carry the surface protein marker CD4 and eliminate pathogens by stimulating the phagocytic activity of macrophages and the proliferation of, and antibody secretion by, B-lymphocytes. Helper T-lymphocytes have traditionally been subdivided into two broad categories according to the pattern of cytokines they produce. Th1 cells produce IL-2 and interferon (IFN)-γ, which activate macrophages, natural killer cells, and cytotoxic T-lymphocytes. Antigens derived from bacteria, viruses, and fungi tend to induce Th1 activity. Th2 cells produce IL-4, which stimulates immunoglobulin (Ig)E production, and IL-5, an eosinophil-activating factor. Th2 cells are responsible for defense against helminthic parasites, which is due to IgE-mediated activation of mast cells and basophils. Other categories of helper T cells, including Th17 cells, have been described recently. Regulatory T cells produce IL-10 and transforming growth factor-β and suppress the activities of B cells and other T cells preventing inappropriate activation.

E. The Immune System Changes over the Lifecourse

Newborn babies have an immature immune system. After birth, immunologic competence is gained partly as a result of maturation factors present in breast milk and partly as a result of exposure to antigens (from the mother's skin, from food, and from environmental microorganisms) [1]. Some of the early encounters with antigens play an important role in assuring tolerance and a breakdown in this system of "immune education" can lead to disease such as allergies [1]. At the other end of the life course, older people experience a progressive dysregulation of the immune system, leading to decreased acquired immunity and a greater susceptibility to infection [2]. This age-related decline in acquired immunity is termed immunosenescence [2]. An additional consequence of immunosenescence is an impaired response to vaccination. Innate immunity appears to be less affected by aging than acquired immunity.

F. The Immune System in Health and Disease

Clearly, a well-functioning immune system is essential to health and serves to protect the individual from the effects of ever-present pathogenic organisms. Cells of the immune system also have a role in identifying and eliminating cancer cells. There are, however, some undesirable features of immune responses. First, in developing the ability to recognize and eliminate foreign antigens effectively, the immune system is responsible for the rejection of transplanted tissues. Second, the ability to discriminate between "self" and "non-self" is an essential requirement of the immune system and is normally achieved by the destruction of self-recognizing T- and B-lymphocytes before their maturation. However, since lymphocytes are unlikely to be exposed to all possible self-antigens in this way, a second mechanism termed clonal anergy exists, which ensures that an encounter with a self-antigen induces tolerance. In some individuals, there is a breakdown of the mechanisms that normally preserve tolerance; a number of factors contribute to this, including a range of immunological abnormalities and a genetic predisposition. As a result, an inappropriate immune response to host tissues or to normally benign environmental antigens is generated and this can lead to autoimmune and inflammatory diseases, which are typified by ongoing chronic inflammation and a dysregulated T-cell response [3].

G. Inflammation

Inflammation is a physiological response to infection, injury, or irritants. It is part of the host's innate defense mechanism, acting to initiate pathogen killing. Inflammation also plays an important role in tissue repair processes, so helping to restore homeostasis at infected or damaged sites. Thus, in its physiological context, inflammation is protective. Inflammation involves interactions among many cell types and the production of, and responses to, a number of chemical mediators. Without inflammation, pathogens would not be efficiently eliminated and wounds would not heal.

Chronic inflammation can, however, damage host tissues and is a central element in a number of diseases, such as rheumatoid arthritis (RA), Crohn's disease, ulcerative colitis, asthma, dermatitis, and even atherosclerosis and cancer [3]. Because of its potential to cause damage, it is important that inflammation is closely regulated. The five classical signs of inflammation are pain (dolor), heat (calor), redness (rubor), swelling (tumor), and loss of function (functio laesa). Redness and heat are due to increased blood flow to the inflamed site, swelling is caused by accumulation of fluid at that site, and pain is due to release of chemicals that stimulate nerve endings.

Inflammation may be classified as acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli. It is a short-term process, usually appearing within a few minutes or hours and ceasing upon the removal of the injurious stimulus. The exact processes by which inflammation is terminated (called resolution) are rather poorly understood. Resolution is, however, important to prevent unnecessary damage to host tissues. This self-regulation of inflammation involves the activation of negative-feedback mechanisms including the production of pro-resolving mediators, inhibition of pro-inflammatory signaling cascades, shedding of receptors for inflammatory mediators, and activation of regulatory cells. Pathological chronic inflammation involves a loss of these regulatory processes, and, where this becomes excessive, irreparable damage to host tissues and disease can occur [3].

II. OXIDIZED FATTY ACID DERIVATIVES ARE IMPORTANT MEDIATORS AND REGULATORS OF IMMUNE AND INFLAMMATORY RESPONSES

A. Eicosanoids

Eicosanoids are oxidized derivatives of 20-carbon polyunsaturated fatty acids (PUFAs). They are produced by enzyme-catalyzed reactions and the most common substrate for their biosynthesis is the omega-6 (n-6) PUFA arachidonic acid (20:4n-6), but other substrates include dihomo-γ-linolenic acid (20:3n-6) and eicosapentaenoic acid (EPA; 20:5n-3). Eicosanoids include prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs). Because of differences in substrate fatty acid structure (i.e., different number of double bonds), the eicosanoids produced from each fatty acid have a unique structure. Eicosanoids are produced in a cell-type and stimulus-dependent manner, have short half-lives, and act on target cells via cell surface receptors, often members of the G-protein-coupled receptor family. The initial substrate for eicosanoid synthesis is a 20-carbon PUFA esterified at the *sn*-2 position of a membrane phospholipid. The substrate PUFA is released from the phospholipid by the action of phospholipase A_2 enzymes, which are often activated by inflammatory stimuli. The free PUFA then acts as a substrate for cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome P450 enzymes (Figure 24.1). COX enzymes initiate the pathway that produces PGs and TXs while 5-LOX initiates the pathway that produces LTs. Other eicosanoid derivatives are produced by pathways initiated by 12- and 15-LOX and by cytochrome P450 enzymes (Figure 24.1). There are two main COX enzymes, COX-1 and COX-2. COX-1 is responsible for maintaining housekeeping functions of PGs and TXs while COX-2 is responsible for the increased production of PGs seen in infection or inflammation. COX metabolism of arachidonic acid yields 2-series PGs and TXs (e.g., PGE_2) while 5-LOX metabolism yields 4-series LTs (e.g., LTB_4). Table 24.1 lists some of the eicosanoids produced from arachidonic acid, along with their receptors. The analogous eicosanoids produced from dihomo-γ-linolenic acid are the 1-series PGs (e.g., PGE_1) and the 3-series LTs (e.g., LTA_3), while EPA gives rise to the 3-series PGs (e.g., PGE_3) and the 5-series LTs (e.g., LTB_5).

Effects and mechanisms of action of the eicosanoids produced from arachidonic acid have been much more widely studied than those of the eicosanoids produced from other PUFA substrates. Because there are many different eicosanoids acting on many target cell types through many different receptors, eicosanoids have a huge variety of effects on immune cell responses and on components of the inflammatory process [4,5]. Sometimes the action of one eicosanoid directly opposes that of another. PGE_2 has wide ranging effects. It acts to suppress the activity of T cells, especially

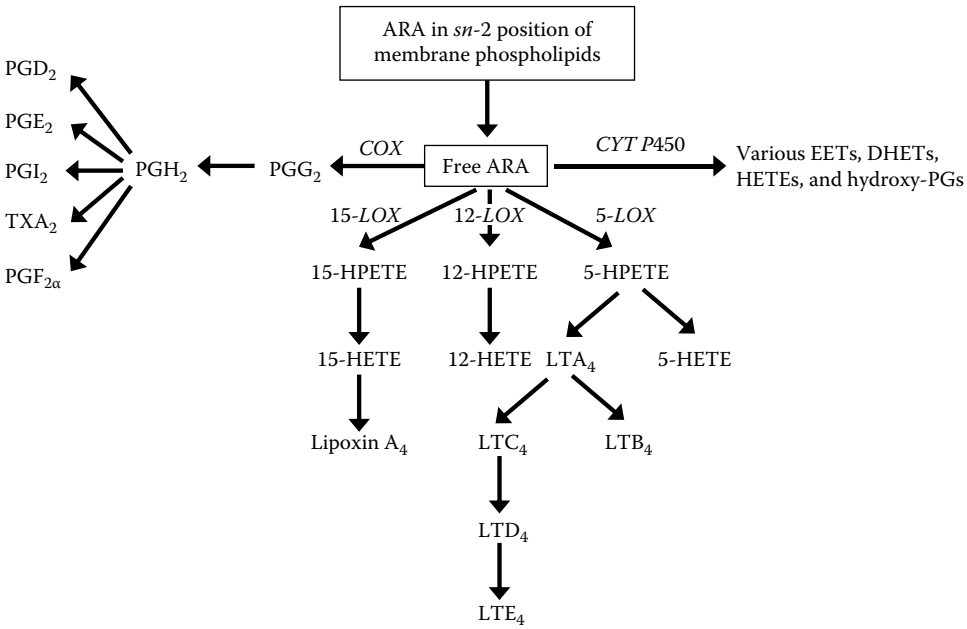

FIGURE 24.1 Overview of the pathway of eicosanoid synthesis from arachidonic acid (ARA). *Abbreviations:* EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid.

TABLE 24.1
Examples of Eicosanoids Produced from Arachidonic Acid and Their Receptors

Eicosanoid	Pathway	Receptor
PGD_2	COX	DP1, DP2
PGE_2	COX	EP1, EP2, EP3, EP4
$PGF_{2\alpha}$	COX	FP
PGI_2	COX	IP
TXA_2	COX	TP
5-HETE	5-LOX	BLT2
LTB_4	5-LOX	BLT1, BLT2
LTC_4, LTD_4, LTE_4	5-LOX	CysLT1, CysLT2
15-HETE	15-LOX	BLT2
15-HPETE	15-LOX	BLT2
12-HETE	12-LOX	BLT2
LXA_4	15-LOX/5-LOX or 5-LOX/12-LOX	FPR2/ALX

Abbreviations: COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; PG, prostaglandin; TX, thromboxane.

Th1-type cells involved in defense against bacteria and viruses, and is an active mediator of inflammation, causing pain and fever. Thus, PGE_2 can contribute to states of immunosuppression and hyperinflammation and is believed to favor allergic responses. Excessive production of PGE_2 has been linked to immunosenescence and to immune paralysis in some clinical settings such as following major burns. Several common anti-inflammatory medications, including aspirin and other nonsteroidal anti-inflammatory drugs, target the COX enzymes responsible for PGE_2 production, showing the clear role of such metabolites in inflammation. It is important to note, however, that this role is now known to be more complex than previously thought. As inflammation proceeds PGE_2 suppresses 5-LOX, so decreasing production of pro-inflammatory 5-series LTs, and induces 15-LOX, so favoring synthesis of the pro-resolving mediator lipoxin A_4 [6]. In this regard, PGE_2 is acting in an anti-inflammatory manner. While we usually think of eicosanoids as being produced by the host's own cells, it is interesting to note that several eukaryotic organisms including helminths can produce eicosanoids, including PGE_2, and this seems to form part of their mechanism of evading the host immune response [7].

Eicosanoids produced from dihomo-γ-linolenic acid or EPA usually have less potent effects than those produced from arachidonic acid, and they sometimes may have opposite effects. However, not all possible effects of all eicosanoids produced from dihomo-γ-linolenic acid or EPA have been studied to date. Consumption of γ-linolenic acid or dihomo-γ-linolenic acid leads to enrichment of dihomo-γ-linolenic acid in cell membrane phospholipids while consumption of EPA from foods like fatty fish or from fish oil supplements leads to enrichment of EPA in cell membrane phospholipids (see [8] for a review). This favors the production of the eicosanoids from these alternative sources [8] and can even decrease production of the eicosanoids from arachidonic acid [8]. This is considered to be a core mechanism of the anti-inflammatory actions of γ-linolenic acid, dihomo-γ-linolenic acid, and EPA [8].

B. Resolvins, Protectins, and Maresins

One of the important steps forward in the field of n-3 fatty acid biology has been the elucidation of the structures, actions, and mechanisms of so-called "pro-resolving lipid mediators" (sometimes called "specialized pro-resolving mediators") produced from EPA and docosahexaenoic acid (DHA; 22:6n-3). These include the resolvins produced from EPA (E-series) and DHA (D-series) and protectins and maresins produced from DHA (Table 24.2); protectins are also referred to as neuroprotectins when generated within the neural tissue. The synthesis of resolvins, protectins, and maresins involves the COX and LOX pathways, with different epimers being produced in the presence and absence of aspirin [9,10] (Figure 24.2). These pathways operate in a transcellular manner with the early steps occurring in one cell type and the latter in another [9,10]. More recently, analogous compounds have been shown to be produced from a third n-3 fatty acid, docosapentaenoic acid (DPA; 22:5n-3) [11]. Pro-resolving lipid mediators have been demonstrated to occur at detectable levels in human blood [12], adipose tissue [13], and breast milk [14].

TABLE 24.2
Examples of Pro-Resolving Mediators Produced from EPA and DHA

	Substrate	
Pro-resolving mediator family	EPA	DHA
Resolvin	RvE1, RvE2	RvD1, RvD2, RvD3, RvD4, RvD5, RvD6
Protectin	—	PD1 (also called NPD1), PDX
Maresin	—	MaR1, MaR2

Abbreviations: MaR, maresin; NP, neuroprotectin; P, protectin; Rv, resolvin.

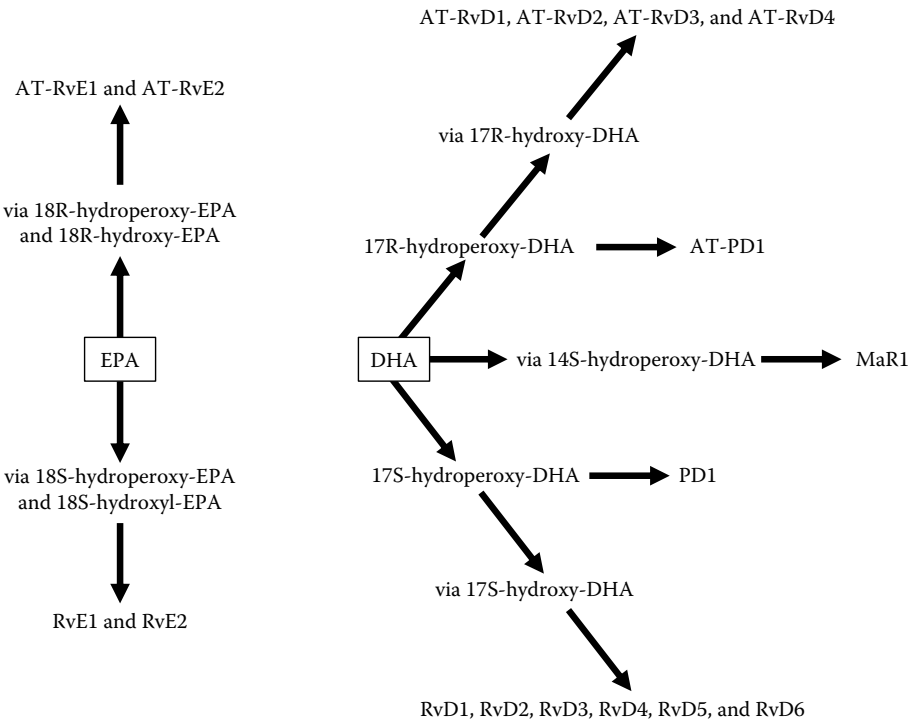

FIGURE 24.2 Overview of the pathways of synthesis of pro-resolving lipid mediators from EPA and DHA. *Abbreviations:* AT, aspirin-triggered; MaR, maresin; P, protectin; Rv, resolvin.

The biological effects of resolvins, protectins, and maresins have been examined extensively in cell culture and animal models of inflammation [9,10]. These models have shown them to be anti-inflammatory and inflammation resolving with high potency. For example, resolvin E1, resolvin D1, and protectin D1 all inhibited the transendothelial migration of neutrophils, so preventing the infiltration of neutrophils into sites of inflammation; resolvin D1 inhibited IL-1β production; and protectin D1 inhibited TNF-α and IL-1β production [9,10]. The biological activities of pro-resolving mediators occur via specific G-protein-coupled receptors. The production of resolvins, protectins, and maresins is enhanced by increased consumption of EPA and DHA [12] and so these novel mediators represent a significant mechanism for the anti-inflammatory action of n-3 PUFAs.

III. FATTY ACIDS, IMMUNITY, AND INFLAMMATION

A. Effect of Dietary Fat Quantity

A small number of fairly old studies in humans reported that putting volunteers on a diet containing a lower amount of total fat than normally consumed resulted in enhanced T-cell responses and natural killer cell activity [15], suggesting that high-fat diets suppress immune responses. However, this area is not well explored in controlled trials in humans.

B. Effect of Essential Fatty Acid Deficiency

The membranes of cells involved in immune responses contain significant amounts of the essential fatty acid (EFA) linoleic acid (18:2n-6) and of the PUFA derivatives of linoleic and α-linolenic acids (arachidonic acid, EPA, DPA, DHA). These derivatives play important roles in immune cell

membrane structure and function (see the following) and are precursors of bioactive mediators (see Section II). Thus, it is reasonable to assume that EFA deficiency will compromise immune cell membrane structure and function and result in dysregulation of immune and inflammatory responses. Therefore, in common with other essential nutrient deficiencies, EFA deficiency is likely to result in modified lymphoid organ structure, altered numbers of immune cells in the bloodstream and in lymphoid organs, impaired immune cell function, and increased susceptibility to infection.

C. Effects of Dietary Fatty Acids

1. Introduction

Numerous cell culture, animal feeding, and human studies have been performed over the last 40 years exploring the role of individual fatty acids in many aspects of the immune response, including its inflammatory component. Reviews of these studies can be found elsewhere [8,15–17]. In surveying this literature, it is clear that findings are not always consistent. This reflects differences in study design, models employed, technical aspects of the methodologies used, and the precise nature of the comparisons being made. The inconsistencies make it difficult to draw firm conclusions about the influence of specific fatty acids on specific aspects of the immune response, with a few exceptions.

2. Phagocytosis

During phagocytosis, the membrane of a host's phagocytic cell moves to engulf a microorganism or other particle. The rate and efficiency of phagocytosis is determined in part by the physical nature of the membrane (its "fluidity"), which in turn is determined in part by the fatty acid composition of the membrane phospholipids. Enrichment of phagocyte membranes with saturated fatty acids impairs phagocytosis, while enrichment with PUFAs (either n-6 or n-3) enhances phagocytosis (see [17] for discussion). Among PUFAs, n-3 PUFAs have a greater enhancing effect than n-6 PUFAs. However, it seems that making the phagocyte membrane too unsaturated also impairs phagocytosis, suggesting a "bell-shaped" relation between membrane unsaturation/fluidity and phagocytosis (see [17]).

3. T-Cell Responses

There has been a long history of the study of fatty acids and T-cell responses. This is for a variety of reasons. First, T cells are of fundamental importance to the host's immune responses. Second, T cells are recognized as being central to many inflammatory conditions. Third, T cells are simple to isolate from the blood of humans or from lymphoid organs of laboratory animals. Fourth, T cells are highly proliferative in culture and this can be used as a simple functional readout. Furthermore, T-cell proliferation can be triggered by exposure to several easily obtained chemical stimulants. Much interest in the effects of fatty acids on T-cell proliferation, and the related production of cytokines like IL-2, IFN-γ, and IL-4, has focused on n-6 and n-3 PUFAs. Many studies have shown that high exposure to either n-6 or n-3 PUFAs can suppress T-cell responses, but careful studies with low concentrations of n-3 PUFAs like EPA and DHA have identified enhanced T-cell responses (see [17,18] for discussion). These effects might be linked to decreased production of PGE_2, which suppresses T-cell proliferation. However, non-eicosanoid-dependent effects on early signal transduction processes in the membranes of activated T cells also seem to be involved in the effects of n-3 PUFAs [17,18]. Membrane rafts are platforms that assemble in response to cell stimulation. They are important because they form structures that enable signaling proteins to interact with one another. In vitro and laboratory animal studies have identified that the effects of EPA and DHA on early signaling events in T cells and on T-cell responses seem to involve the disruption of the formation of lipid rafts, although the exact effects of EPA and DHA on rafts may be different [17,18].

4. Inflammation

In vitro studies have suggested that inflammatory cells like monocytes, macrophages, and dendritic cells can be activated directly by saturated fatty acids, including lauric acid (12:0) and palmitic acid (16:0), in a process involving the endotoxin receptor, toll-like receptor (TLR)-4, and subsequent activation of the pro-inflammatory transcription factor nuclear factor kappa B (NFκB) (see [8] for discussion). Studies on overweight or obese humans report a positive association between circulating saturated fatty acids and IL-6 concentration, but lean individuals did not show this (see [19] for references). An intervention study feeding diets rich in stearic acid (18:0) or in the combination of lauric, myristic (14:0), and palmitic acids to men for 5 weeks showed higher concentrations of C-reactive protein (CRP), fibrinogen, IL-6, and soluble E-selectin (sE-selectin) compared with a diet enriched in oleic acid [20]. There are few other intervention studies chronically increasing saturated fatty acid intake in humans and reporting inflammatory markers.

There were positive relationships between dietary *trans* fatty acid intake and concentrations of six inflammatory markers, including CRP, IL-6, and three soluble adhesion molecules, in the Nurses' Health Study (see [19]). In a 5-week intervention study in healthy men, a *trans* fatty acid–enriched diet resulted in higher CRP and IL-6 concentrations than diets rich in oleic acid, stearic acid, or the combination of lauric, myristic, and palmitic acids [20]. Furthermore, the concentration of sE-selectin was higher than in all other dietary groups including the stearic acid and lauric + myristic + palmitic groups. Thus, it appears that dietary *trans* fatty acids elevate the concentrations of a range of inflammatory markers, including CRP, IL-6, and adhesion molecules, and that *trans* fatty acids may be more "pro-inflammatory" than saturated fatty acids.

Association studies suggest a limited anti-inflammatory effect of α-linolenic acid over the normal range of intakes from the diet. However, the significantly increased consumption of α-linolenic acid results in an increased content of EPA in the membranes of inflammatory cells (see [21] for discussion) in parallel with a decreased content of arachidonic acid. This might be expected to affect inflammation. Several intervention studies have involved high α-linolenic acid intakes usually by providing flaxseed oil in capsules or in liquid form or foodstuffs made using flaxseed oil. Frequently, these studies have used a control group with a high intake of linoleic acid, with the comparison essentially being replacement of linoleic acid with α-linolenic acid. These studies have produced inconsistent findings with some identifying effects of α-linolenic acid on some markers of inflammation and not others, and some studies finding no effects (see [21]). Caughey et al. [22] reported that 13.7 g per day α-linolenic acid for 4 weeks resulted in decreased production of TNF and IL-1β by endotoxin-stimulated mononuclear cells by 27% and 30%, respectively. By comparison, fish oil providing 2.7 g per day EPA + DHA decreased the production of these inflammatory cytokines by 70% and 78%, respectively [22]. Thus, on a gram per day basis, marine n-3 fatty acids are about 10 times more potent than α-linolenic acid. These data suggest that a marked increase in α-linolenic acid intake is required in order for anti-inflammatory effects to be seen. Even then, the effects will be much more modest than those exerted by EPA and DHA. The observed effects of high intakes of α-linolenic acid on inflammation seem to be due to its derivative EPA rather than α-linolenic acid itself [23]. Thus, the likely explanation for the lack of anti-inflammatory effect of α-linolenic acid at modest, and even at rather high, intakes is that there has been insufficient conversion to the more active EPA.

Incorporation of EPA and DHA into inflammatory cells is partly at the expense of arachidonic acid, resulting in less substrate available for synthesis of the classic inflammatory eicosanoids (see earlier). Through altered eicosanoid production, EPA and DHA could affect immune responses, inflammation, and inflammatory processes, although they also exert non-eicosanoid-mediated actions on cell signaling and gene expression (Figure 24.3). These actions include modification of formation of signaling platforms (lipid rafts) in the inflammatory cell membrane, activation of the anti-inflammatory transcription factor peroxisome proliferator activated receptor (PPAR)-γ, and inhibition of activation of the pro-inflammatory transcription factor NFκB [8]. Recent studies

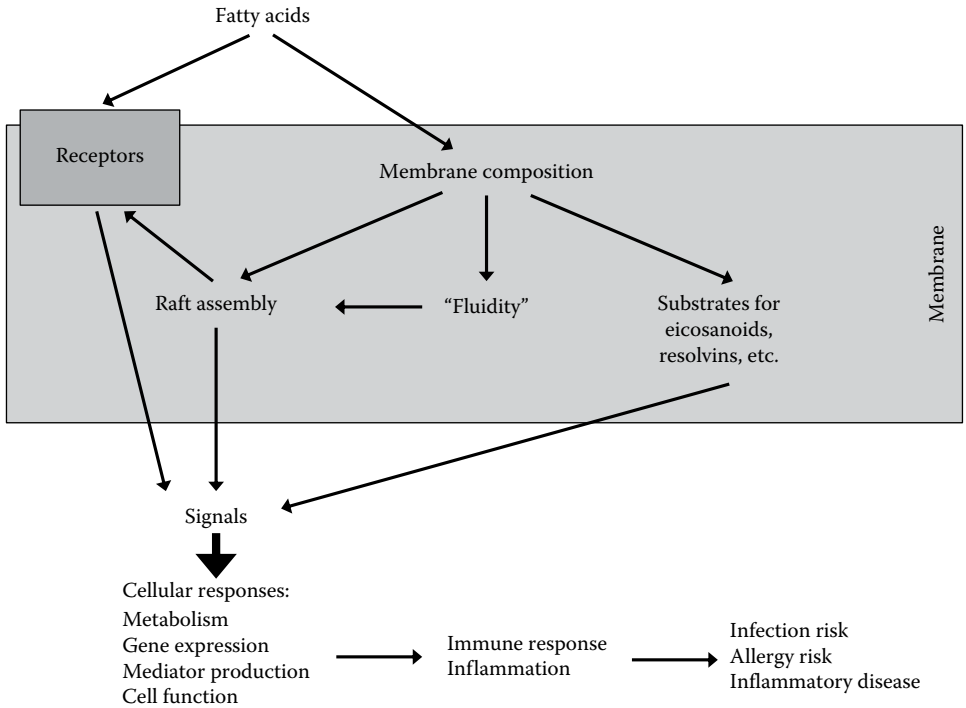

FIGURE 24.3 Depiction of mechanisms by which fatty acids can influence immune cell function and inflammation.

indicate a membrane-bound G-protein-coupled receptor called GPR120 that is important to the anti-inflammatory effects of DHA in macrophages [24]. The effects of EPA and DHA have been examined in many model systems, and findings from cell culture systems and from animal models are generally consistent in identifying anti-inflammatory actions (see [8,15–17] for discussion). Cross-sectional studies consistently show an inverse association between intake or status of EPA and DHA and markers of inflammation [19]. The ready availability of fish oil capsules has facilitated numerous supplementation studies of EPA and DHA in various subject and patient groups. They have been shown to decrease leukocyte chemotaxis and inflammatory cytokine production [8]. Clinical trials have demonstrated anti-inflammatory effects and some clinical benefit from fish oil administration in diseases with a frank inflammatory basis, including RA, inflammatory bowel diseases (IBDs), and childhood asthma [8]. Through their effects on T cells, EPA and DHA might also affect susceptibility to and severity of infection. Indeed, providing EPA + DHA to school children in Thailand or South Africa decreased the risk of respiratory illness [25,26]. There is a developing evidence base suggesting that early exposure to EPA + DHA reduces the risk of developing allergic disease in children [27].

IV. CONCLUSIONS

Changes in fatty acids in immune cell membranes can affect eicosanoid levels, immune responses, and inflammatory processes. However, non-eicosanoid mechanisms of action are now recognized to also play a role in the effects of fatty acids seen. For example, altered transcription factor activities as a result of impacts on membrane signaling processes are now seen to be very important mechanisms of fatty acid bioactivity, while recent discoveries of fatty acid receptors suggest exciting novel actions yet to be discovered. Effects of fatty acids may be seen as being generic to families, but it is

important to note that fatty acids have individual effects and individual potencies. The most well-explored family of fatty acids is the n-3 family, particularly EPA and DHA. They have effects on eicosanoid profiles, phagocytosis, T-cell responses, and inflammatory processes; they oppose the actions of both saturated and n-6 PUFAs; and they are substrates for the synthesis of highly potent pro-resolving lipid mediators. The effects of EPA and DHA are well recognized and systematic reviews of studies on clinical endpoints in diseases such as RA have concluded that they are effective if the dose is sufficient [28,29]. Further exploration of the effects of a range of dietary fatty acids on immune responses and inflammatory processes in humans is warranted.

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25 Dietary Fats and Coronary Heart Disease

Ronald P. Mensink and Jogchum Plat

CONTENTS

I. INTRODUCTION

Many factors are associated with increased risk for coronary heart disease (CHD), a major cause of morbidity and mortality in Western countries. Some of these factors, such as aging or a family history of premature CHD, are not amenable to preventive intervention, but other factors are. Three of these preventable factors—the distribution of plasma cholesterol over low-density and high-density lipoproteins (LDLs and HDLs), the oxidizability of LDLs, and hemostasis—can be modified by changing the sources and amount of fats and oils in the diet. The purpose of this chapter is to review some of the most recent and important findings on the effects of dietary fatty acids on these three cardiovascular risk markers.

II. DIETARY FATS

Although fat and oils are complex mixtures of fatty acids, each fat or oil has its characteristic fatty acid composition. Dairy fat, for example, is relatively rich in fatty acids with 14 or less carbon atoms, whereas olive oil has a high oleic acid content (Table 25.1). Sunflower oil, on the other hand, is rich in linoleic acid, although varieties exist that contain large amounts of oleic acid. In normal, regular diets, palmitic and stearic acids are the most prevailing saturated fatty acids, whereas oleic and linoleic acids are, respectively, the most widespread dietary monounsaturated and polyunsaturated fatty acids. About 30%–40% of total energy intake is provided by fat. For a person consuming 10 MJ (2400 kcal)/day, this corresponds to a fat intake of 80–107 g.

III. LIPOPROTEINS

The solubility of cholesterol in water is very low, approximately 5.2×10^{-3} mmol/L. The actual cholesterol concentration in the watery plasma of healthy subjects, however, is about 3.9–5.2 mmol/L and increases to more than 10 mmol/L in severely hypercholesterolemic people. This high degree of solubilization is achieved by the formation of lipoproteins.

Lipoproteins are globular, high-molecular-weight particles that are complex aggregates of lipid and protein molecules. A lipoprotein consists of a hydrophobic core, which mainly contains

TABLE 25.1
Major Fatty Acids in Some Edible Fats and Oils

Formula	Fatty Acid	Source
Saturated fatty acids		
	Medium-chain fatty acids	Dairy fat, coconut oil, palm kernel oil
C12:0	Lauric acid	Dairy fat, coconut oil, palm kernel oil
C14:0	Myristic acid	Dairy fat, coconut oil, palm kernel oil
C16:0	Stearic acid	Meat, cocoa butter
Monounsaturated fatty acids		
C18:1, *n*-9	Oleic acid	Olive oil, rapeseed oil, high oleic acid sunflower oil
Polyunsaturated fatty acids		
C18:2, *n*-6	Linoleic acid	Sunflower oil, corn oil, soybean oil, corn oil
C18:3, *n*-3	α-Linolenic acid	Rapeseed oil, soybean oil
C20:5, *n*-3[a]	Timnodonic acid	Fatty fish, fish oil capsules
C22:5, *n*-3[b]	Cervonic acid	Fatty fish, fish oil capsules

[a] Trivial names, eicosapentaenoicacid (EPA).
[b] Trivial name, docosahexaenoicacid (DHA).

TABLE 25.2
Some Physical Characteristics and Mean Composition of Lipoprotein Fractions from Normotriglyceridemic Subjects

	Chylomicrons	VLDL	IDL	LDL	HDL
Density (g/mL)					
Lower limit	—	0.96	1.006	1.019	1.063
Upper limit	0.96	1.006	1.019	1.063	1.21
Size (nm)	75–1200	30–80	25–35	19–25	5–12
		% of Total Lipoprotein Mass			
Core components					
Triacylglycerols	87	52	29	6	6
Cholesterylester	3	9	28	40	21
Surface components					
Phospholipids	6	23	22	22	24
Free cholesterol	2	6	7	9	3
Apolipoprotein	2	10	14	23	46

Abbreviations: HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low density lipoprotein.

TABLE 25.3
Some Major Apolipoprotein and Their Functions

Apolipoprotein	Lipoprotein	Function
ApoC-II	Chylomicrons, VLDL	Activator of LPL
ApoE	Chylomicron remnants, VLDL, IDL	Ligand for remnant and LDL receptors
ApoB-100	IDL, LDL	Ligand for LDL receptor
ApoA-I	Chylomicron, HDL	Cofactor for LCAT

Abbreviations: LCAT, lecithin cholesteryl acyltransferase; LPL, lipoprotein lipase.

triacylglycerols and cholesterylesters, and a polar, hydrophilic coat composed of phospholipids, unesterified cholesterol, and specific apolipoproteins. In this way, the hydrophobic core is protected from the watery surrounding, and transport of large amounts of cholesterol and triacylglycerols through the blood vessels is possible.

Lipoproteins are a heterogeneous group, which can be divided into five major classes: chylomicrons, very-low-density lipoproteins (VLDLs), intermediate-density lipoproteins (IDLs), LDLs, and HDLs. Each class has its own characteristic lipid and apolipoprotein composition, size, and density, whereas each apolipoprotein has its own specific metabolic functions (Tables 25.2 and 25.3).

IV. METABOLISM OF DIETARY FATTY ACIDS AND LIPOPROTEINS

A. Exogenous Pathway

The metabolism of dietary fatty acids, dietary and biliary cholesterol, and lipoproteins is depicted in Figure 25.1. In the duodenum, dietary triacylglycerols are dissolved with the help of bile salts, as well as small quantities of fatty acids and monoglycerides. The enzyme pancreatic lipase then hydrolyzes the dietary triacylglycerols into mono- and diglycerides, free fatty acids, and glycerol.

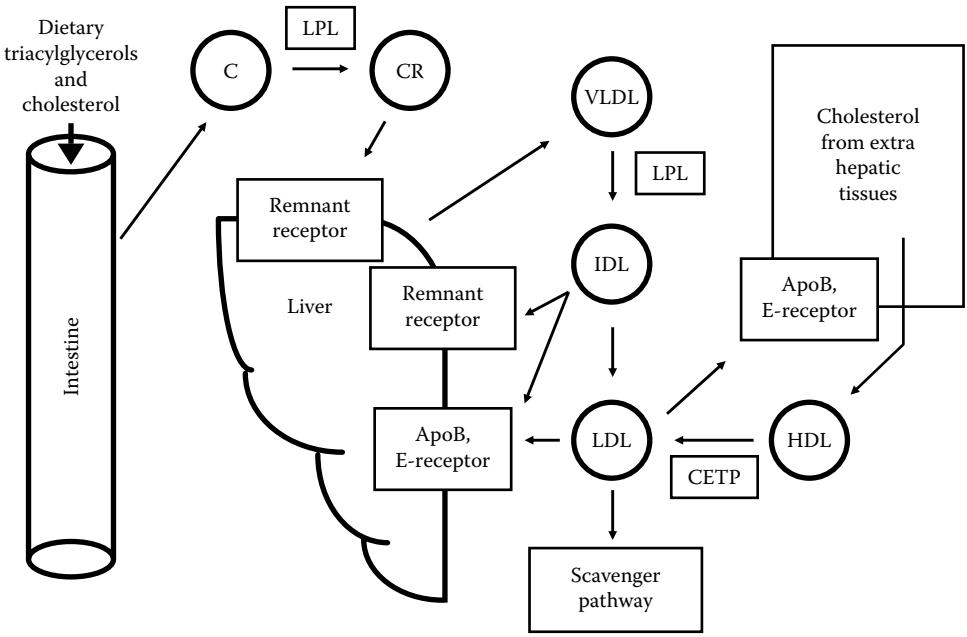

FIGURE 25.1 Overview of the metabolism of dietary fatty acids, dietary cholesterol, and plasma lipoproteins. Dietary triacylglycerols and cholesterol enter the blood circulation in chylomicrons (C). The triacylglycerols from the chylomicron core are hydrolyzed by lipoprotein lipase (LPL) and the so-formed chylomicron remnants (CRs) are removed from the circulation by the hepatic remnant receptor. The liver excretes cholesterol and triacylglycerols into the circulation in very-low-density lipoproteins (VLDLs). These lipoproteins also interact with LPL, and VLDL remnants—also called intermediate-density lipoprotein (IDLs)—are formed, which are taken up by the liver or converted into low-density lipoprotein (LDL). Most of the LDL is removed from the circulation by the (hepatic) LDL receptor pathway, whereas a smaller part is removed via the scavenger pathway. High-density lipoprotein (HDL) binds cholesterol from tissues, which can be transferred with the assistance of cholesterol ester transfer protein (CETP) to LDL. HDL particles may also be taken up in the liver by a putative HDL receptor or lose a part of its content by the action of hepatic lipase and then reenter the circulation again (not drawn).

The so-formed emulsion of lipids, which also contain the dietary cholesterol, passes the mucous membrane of the intestinal cells. Within the cell, further hydrolysis of lipids takes place and new triacylglycerols are formed by reesterification of the free fatty acids with glycerol. The newly synthesized triacylglycerols and cholesterylesters, derived from the intestinal cholesterol pool, are incorporated into chylomicrons, which enter the lymph and subsequently the blood circulation in the subclavian vein. In the blood, the triacylglycerols from the chylomicron core are hydrolyzed by lipoprotein lipase (LPL), an enzyme adhered to the endothelial cells of the blood vessels, which is activated by apolipoprotein C-II. The free fatty acids pass the endothelial cells and enter adipocytes or muscle cells. In these cells, the fatty acids are respectively stored as triacylglycerols or oxidized. The core of an emptied chylomicron mainly consists of cholesterylesters and is called a chylomicron remnant. These remnant particles are removed from the circulation by the hepatic remnant receptor, which has a high affinity for apolipoprotein E (apoE) from the chylomicron surface.

B. Endogenous Pathway

The liver secretes cholesterol and triacylglycerols into the circulation by the formation of VLDL particles. These lipoproteins also interact with LPL, triacylglycerols become hydrolyzed, and VLDL remnants (also called IDLs) are formed. A part of the IDL is taken up by the liver, whereas the remaining part is converted in the circulation into LDL. LDLs are nearly devoid of triacylglycerols and carry

about 60%–70% of the total amount of cholesterol in the plasma. Most of the LDL is now removed from the circulation through the hepatic LDL receptor pathway, which recognizes apolipoprotein B-100 (apoB-100). LDL uptake from the blood by the LDL receptor–mediated pathway is highly controlled, and this pathway will be downregulated if the amount of cholesterol in the cell becomes too high. A smaller part, however, is removed via the scavenger pathway. Uptake via this pathway is not saturable and is positively related to the LDL cholesterol concentration. Thus, the higher the plasma LDL cholesterol concentration, the more LDL will be taken up via the scavenger pathway. When too much LDL is taken up via the scavenger pathway from macrophages, cells loaded with cholesterol are formed—so-called foam cells—which are frequently found in atherosclerotic lesions.

Cholesterol can also be transported out of tissues by reverse cholesterol transport. This system is mediated by HDL, lecithin cholesteryl acyltransferase (LCAT), and cholesterol ester transfer protein (CETP). HDL binds free cholesterol from tissues, which is esterified by LCAT, a protein associated with apolipoprotein A-I (apoA-I). The formed cholesterylesters move to the core of the HDL particle, and the HDL is converted to a larger particle. The acquired cholesterylesters can now be transferred with the assistance of CETP to apoB-100-containing lipoproteins in exchange for triacylglycerols. The apoB-100-containing lipoproteins are further metabolized, as has already been described. The large HDL particles may lose a part of its cholesterol content through the interaction with the scavenger receptor BI (SR-BI).

V. PLASMA LIPOPROTEINS AND CHD

LDL and HDL are differently related to the risk for CHD. High concentrations of LDL cholesterol are atherogenic, whereas high levels of HDL cholesterol are negatively associated with the risk for CHD. As LDL carries most of the plasma cholesterol, the total plasma cholesterol is also a good index for the risk of CHD. It should be realized, however, that some people have high total cholesterol concentrations due to high HDL cholesterol levels. Therefore, the total cholesterol to HDL cholesterol might be the most efficient predictor for the risk for CHD [1]. In addition, high levels of triacylglycerols, which are in the fasting condition mainly found in the VLDLs, are positively related to the risk for CHD [2].

VI. DIETARY FATS AND PLASMA LIPOPROTEINS

A. Earlier Studies

In the 1950s, Keys and coworkers started a series of well-controlled experiments to examine the effects of dietary fatty acids on plasma total cholesterol concentrations [3,4]. Groups of physically healthy men were fed diets that differed widely in the amount of fat and in dietary fatty acid composition. During the studies, individual allowances were adjusted weekly to keep body weight stable so that changes in plasma total cholesterol concentrations could be attributed solely to dietary changes. At the end of the studies, an empirical formula to predict for a group of subjects' changes in plasma cholesterol concentrations from changes in dietary fatty acid composition was derived:

$$\Delta\text{Plasma total cholesterol (mmol/L)} = 0.03 \times (2 \times \Delta\text{Sat}' - \Delta\text{Poly})$$

or

$$\Delta\text{Plasma total cholesterol (mg/dL)} = 1.2 \times (2 \times \Delta\text{Sat}' - \Delta\text{Poly})$$

where

Sat′ is the percentage of energy provided by saturated fatty acids with 12, 14, or 16 carbon atoms (lauric, myristic, and palmitic acid, respectively)

Poly refers to the amount of polyunsaturated fatty acids in the diet

How should this formula be interpreted? First, it should be realized that effects are expressed relative to those of carbohydrates. A hypercholesterolemic fatty acid is therefore defined as a fatty acid that causes an increase in the plasma cholesterol level when substituted in the diet for an isocaloric amount of carbohydrates. Thus, when 10% of energy from Sat′ is replaced by carbohydrates, ΔSat′ equals −10, and the expected decrease in plasma total cholesterol concentrations is $0.03 \times 2 \times -10 = -0.60$ mmol/L (−24 mg/dL). If this amount of carbohydrates is then replaced by linoleic acid, a further decrease of $-0.03 \times 10 = -0.30$ mmol/L (−12 mg/dL) is expected. Direct replacement of Sat′ by Poly yields the sum of these two effects, a fall of 0.90 mmol/L (36 mg/dL). Furthermore, this formula suggests that—because they are not part of the equation—the effects on plasma total cholesterol concentrations of saturated fatty acids with fewer than 12 carbon atoms, of stearic acid, and of monounsaturated fatty acids are similar to those of carbohydrates. Finally, it can be seen that the cholesterol-raising effect of Sat′ is about twice as compared with the cholesterol-lowering effect of Poly.

Similar types of studies were carried out in the 1960s by Hegsted and colleagues [5]. The results were essentially similar, but it was also concluded that myristic acid was more cholesterolemic than palmitic and lauric acids.

These and other studies have led to recommendations that the most effective diet for lowering plasma total cholesterol concentration should contain a low proportion of the cholesterol-raising saturated fatty acids and a high proportion of linoleic acid. In addition, a reduction in cholesterol intake was advocated as dietary cholesterol increases plasma total cholesterol concentrations [5,6].

However, these earlier well-controlled studies were not specifically designed to examine the effects of specific dietary fatty acids on plasma cholesterol concentrations and over the various lipoproteins. Therefore, new studies were initiated that compared side-by-side effects of specific fatty acids on the plasma lipoprotein profile.

B. Later Studies

1. Saturated Fatty Acids

To discuss the effects of saturated fatty acids on plasma lipid and lipoproteins, the saturated fatty acids are, in agreement with the earlier studies, categorized into three classes: medium-chain fatty acids (MCFAs), fatty acids with 12, 14, or 16 carbon atoms, and stearic acid.

a. Medium-Chain Fatty Acids

Saturated fatty acids with fewer than 12 carbon atoms are called short- and medium-chain saturated fatty acids and are found in relatively large amounts in coconut fat, palm kernel oil, and butterfat, but also in certain structured lipids, parenteral nutrition preparations, and sport drinks.

McGandy and coworkers [7] have carefully compared the effects of MCFAs, mainly capric acid, on plasma total and LDL cholesterol levels. Eighteen physically healthy men were fed several diets, each for 4 weeks. Diets contained several low-fat food items to which the experimental fats were added. It was shown that modest amounts of MCFAs in the diet have comparable effects on the plasma total and LDL cholesterol and on triacylglycerol concentrations as have carbohydrates. However, large amounts of MCFAs increased triacylglycerol concentrations. The results of two other studies [8,9], however, suggested that a mixture of MCFAs slightly increases LDL cholesterol concentrations relative to oleic acid (Figure 25.2). No effects on HDL cholesterol were found, whereas serum triacylglycerol concentrations were slightly increased.

b. Lauric, Myristic, and Palmitic Acids

Palm kernel oil, coconut oil, and dairy fat are rich in lauric acid, but also contain relatively high amounts of myristic acid. Therefore, it is hardly possible to study the specific effects of these two saturated fatty acids on plasma lipoproteins with natural fats. A diet enriched in palm kernel oil,

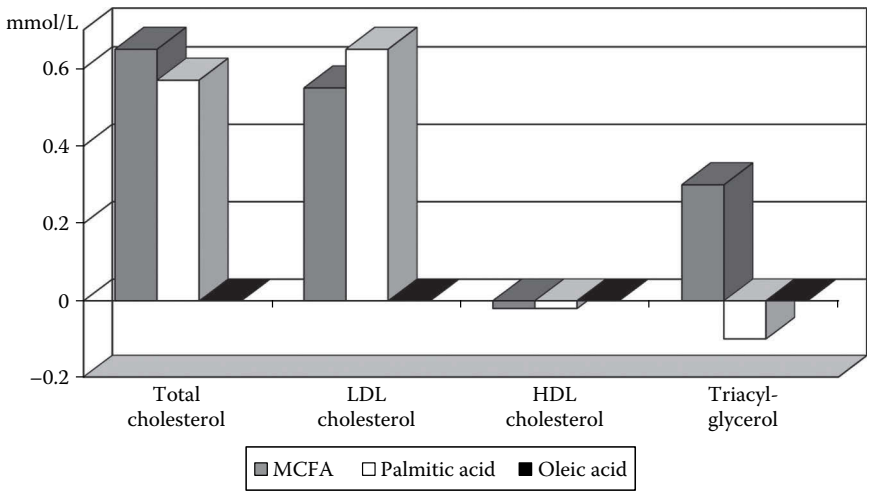

FIGURE 25.2 Effects of medium-chain fatty acids (MCFAs) and palmitic acid on plasma total, LDL cholesterol, HDL cholesterol, and triacylglycerol concentrations relative to those of *cis* monounsaturated fatty acids (oleic acid). Nine men received three mixed natural diets, each for 3 weeks, in random order. The composition of the diets was identical, except for 43% of daily energy intake, which was provided as MCFAs (C8:0 and C10:0), or palmitic acid (C16:0), or oleic acid (*cis*-C18:1). (From Cater, N.B. et al., *Am. J. Clin. Nutr.*, 65, 41, 1997.)

for example, contains high amounts of both lauric and myristic acids, and it will subsequently be impossible to ascribe the effects on the plasma lipoprotein profile to either lauric or myristic acid. To circumvent this problem, several studies have used synthetic fats to examine the cholesterolemic effects of these two saturated fatty acids. In this way, a fat with any desired fatty acid composition can be made. For example, when one interesterifies trilaureate with high oleic acid sunflower oil, the resultant will be a high lauric acid fat without any myristic acid.

In a recent meta-analysis, the effects of the individual saturated fatty acids on the serum lipoprotein profile have been estimated [10]. It was found that lauric, myristic, and palmitic acids all increased serum total and LDL cholesterol concentrations (Figure 25.3). These effects decreased with increasing chain length. Thus, lauric acid was more hypercholesterolemic than myristic acid, which on its turn was more hypercholesterolemic acid than palmitic acid. For HDL cholesterol, the effects did also depend on chain length. All three saturated fatty acids increased HDL cholesterol and effects of lauric acid were the strongest. Because the effects of lauric acid were proportionally higher on HDL than on LDL cholesterol, replacement of carbohydrates by lauric acid resulted in a significantly lower total to HDL cholesterol ratio. Myristic and palmitic acids did not affect the ratio of total to HDL cholesterol. Compared with carbohydrates, these three saturated fatty acids lowered triacylglycerol concentrations to the same extent.

c. Stearic Acid

Keys and coworkers already demonstrated that stearic acid did not increase plasma total cholesterol concentrations [4,11]. However, Bonanome and Grundy were the first to study the effects of stearic acid on the distribution of cholesterol over the various lipoproteins [12]. From that study, it was concluded that stearic acid exerted comparable effects on the plasma lipoprotein profile as oleic acid, a monounsaturated fatty acid. In fact, stearic acid significantly lowered total, LDL, and HDL cholesterol concentrations compared with the other saturated fatty acids (Figure 25.3). Furthermore, it has been reported that stearic acid did not change the total to HDL cholesterol ratio when compared with carbohydrates [10].

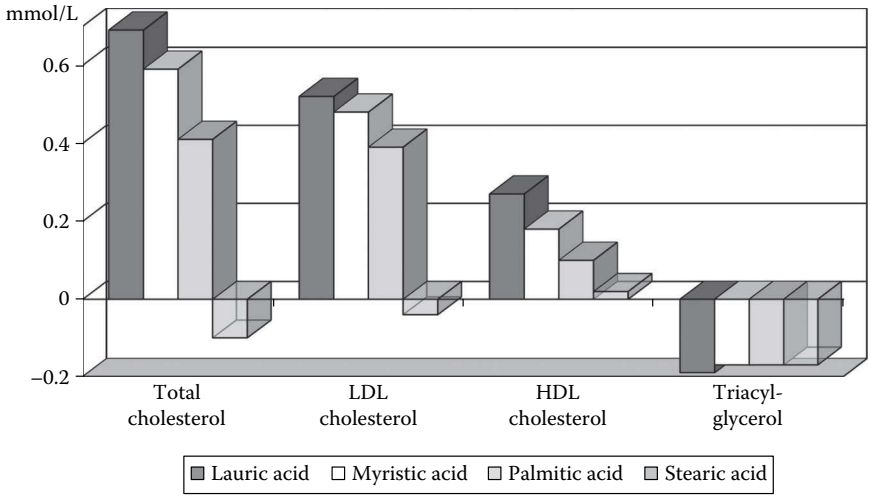

FIGURE 25.3 Effects of exchanging 10% of energy from carbohydrates by lauric (C12:0), myristic (C14:0), palmitic (C16:0), or stearic (C18:0) acid on plasma total, LDL cholesterol, HDL cholesterol, and triacylglycerol concentrations. (From Mensink, R.P. et al., *Am. J. Clin. Nutr.*, 77, 1146, 2003.)

2. Monounsaturated Fatty Acids

The most abundant monounsaturated fatty acid in the human diet, oleic acid, has 18 carbon atoms and 1 double bond. Although olive oil probably is the most well-known source of oleic acid, animal fats are in many countries a major contributor to total oleic acid, but also to palmitic and stearic acids, intakes.

Effects of monounsaturated fatty acids on plasma total cholesterol levels are often described as neutral. This term is often misinterpreted. It does not mean that the plasma total cholesterol level does not change when monounsaturated fatty acids are added to the diet. Neutral indicates that monounsaturated fatty acids have the same effect on plasma total cholesterol as compared with an isocaloric amount of carbohydrates. Although this may be correct for plasma total cholesterol levels, oleic acid and carbohydrates do not have similar effects on the distribution of cholesterol of the various lipoproteins [10,13] (Figure 25.4). This was also shown in a study with young healthy volunteers [14]. Increasing the intake of oleic acid at the expense of carbohydrates increased plasma HDL cholesterol concentrations and decreased those of triacylglycerols. The increase in HDL cholesterol was compensated for by a decrease in VLDL cholesterol. Effects of carbohydrates and oleic acid on plasma total and LDL cholesterol concentrations were comparable.

3. Polyunsaturated Fatty Acids

Polyunsaturated fatty acids in the diet belong to either the (*n*-6) or (*n*-3) family. About 90% of all polyunsaturated fatty acids in the diet is linoleic acid, which is found in vegetable oils like sunflower oil, corn oil, and soybean oil. The mean daily intake of fatty acids from the (*n*-3) family is only 1–3 g. These polyunsaturated fatty acids are either from vegetable or animal origin. α-Linolenic acid is found in rapeseed and soybean oils, whereas the very-long-chain fatty acids timnodonic or eicosapentaenoic acid (EPA) and cervonic or docosahexaenoic acid (DHA) are only present in fish oils.

a. n-6 Polyunsaturated Fatty Acids

Earlier studies found that linoleic acid was hypocholesterolemic as compared with carbohydrates and monounsaturated fatty acids [3,5]. The study of Mattson and Grundy [15], however, suggested that, as compared with monounsaturates, part of the cholesterol-lowering effect of linoleic acid was

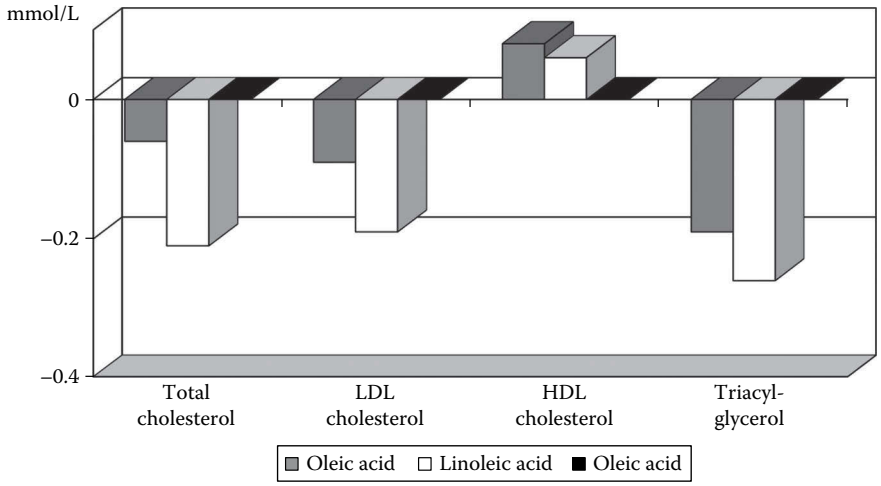

FIGURE 25.4 Effects of exchanging 10% of energy from carbohydrates by oleic (*cis*-C18:1) or linoleic (*cis*-C18:2) acid on plasma total, LDL cholesterol, HDL cholesterol, and triacylglycerol concentrations. (From Mensink, R.P. et al., *Am. J. Clin. Nutr.*, 77, 1146, 2003.)

due to a decrease in HDL cholesterol. However, linoleic acid intake in that study was unrealistically high (28% of energy intake), which may have influenced the results. Studies at lower intakes found similar effects of linoleic and oleic acids on HDL cholesterol, but also on LDL cholesterol [16–18]. Thus, these more recent studies suggested that replacement of saturated fatty acids in the diet by monounsaturated fatty acids causes the same favorable change in plasma lipoprotein cholesterol levels as replacement by polyunsaturated fatty acids. However, it should be noted that in a meta-analysis, effects of linoleic acid on plasma LDL cholesterol were slightly more favorable than those of oleic acid (Figure 25.4) [10].

b. n-3 Polyunsaturated Fatty Acids

The effects of α-linoleic acid on the plasma lipoprotein profile are similar to those of linoleic acid [19]. The highly unsaturated fatty acids from fish oils, however, have different effects. In normocholesterolemic subjects, these fatty acids do not change plasma LDL or HDL cholesterol concentrations but do lower plasma triacylglycerol concentrations and those of cholesterol in VLDL. In hyperlipidemic subjects, and in particular in patients with elevated triacylglycerol concentrations, fish oils also lower plasma triacylglycerols but raise LDL and HDL cholesterol concentrations [20].

4. *Trans* Fatty Acids

Most unsaturated fatty acids found in nature have the *cis* configuration. This means that the two carbon side chains attached to the double bond point to the opposite (*cis*) direction. However, in some fatty acids, the carbon side chain points to the same (*trans*) direction. In this way, two compounds are formed that have exactly the same number and type of atoms but have different chemical, physical, and physiologic characteristics.

Trans fatty acids are formed when vegetable oils are hardened by hydrogenation. These hydrogenated fats are used for the production of certain types of margarines, frying fats, and foods prepared with these fats. Most *trans* fatty acids in the diet have 18 carbon atoms and 1 double bond (*trans*-C18:1). However, *trans* fatty acids are not only found in hydrogenated oils but also in milk fat and body fat from ruminants, formed from dietary polyunsaturated fatty acids by the action of bacteria in the rumen of these animals. The *trans* polyunsaturated fatty acids in the diet mainly originate from hydrogenated fish oils.

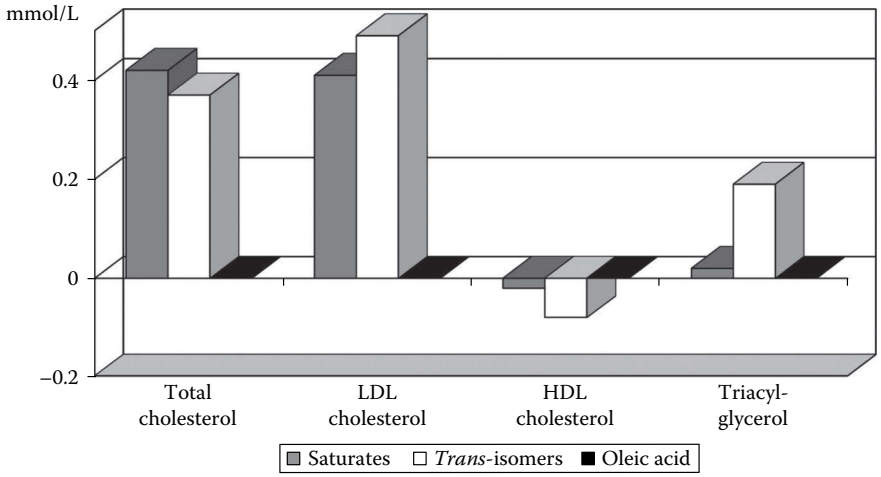

FIGURE 25.5 Effects of a mixture of saturated fatty acids and *trans* isomers of oleic acid on plasma LDL cholesterol, HDL cholesterol, and triacylglycerol concentrations relative to those of *cis* monounsaturated fatty acids (oleic acid). (From Mensink, R.P. et al., *Am. J. Clin. Nutr.*, 77, 1146, 2003.)

LDL cholesterol concentrations increase when *cis*-monounsaturated fatty acids in the diet are replaced by *trans* monounsaturated fatty acids [10,21]. In most studies, a decrease in HDL cholesterol was also observed. Although the LDL cholesterol–raising effect of *trans* monounsaturated fatty acids is less than the effect of a mixture of saturated fatty acids, *trans* monounsaturated fatty acids also lowered HDL cholesterol relative to a mixture of saturated fatty acids (Figure 25.5). Therefore, it was concluded that both types of fatty acids have an unfavorable effect on the plasma lipoprotein profile.

C. Conclusion

Dietary fatty acid composition affects the distribution of cholesterol over LDL and HDL. As compared with an isoenergetic amount of carbohydrates, lauric (C12:0), myristic (C14:0), and palmitic acids (C16:0) have a hypercholesterolemic effect, whereas stearic acid (C18:0) seems to be neutral. Linoleic acid (*cis*,*cis*-C18:2) and probably also oleic acid (*cis*-C18:1) have an LDL cholesterol–lowering effect. *Trans* fatty acids have a strong plasma total and LDL cholesterol–increasing effect. Effects on LDL cholesterol levels are positively related to those on HDL cholesterol concentrations. An exception is *trans* fatty acids that do not have an effect on HDL cholesterol as compared with carbohydrates. Thus, a reduction in the intake of the cholesterol-raising saturated fatty acids and *trans* fatty acids is more important for optimizing the plasma lipoprotein profile than a reduction in total fat intake per se.

VII. OXIDIZABILITY OF LDLs

As has already been mentioned, elevated plasma LDL cholesterol concentrations are associated with increased risk for CHD. However, the atherogenicity of the LDL particle increases after oxidative modification of its polyunsaturated fatty acids.

A. LDL Oxidation

Oxidation of LDL is a free-radical-driven process that may initiate a cascade of reactions (Figure 25.6). For the in vivo situation, it is not clear where the initiating radical species is derived from, but several suggestions, based on in vitro experiments, have been made.

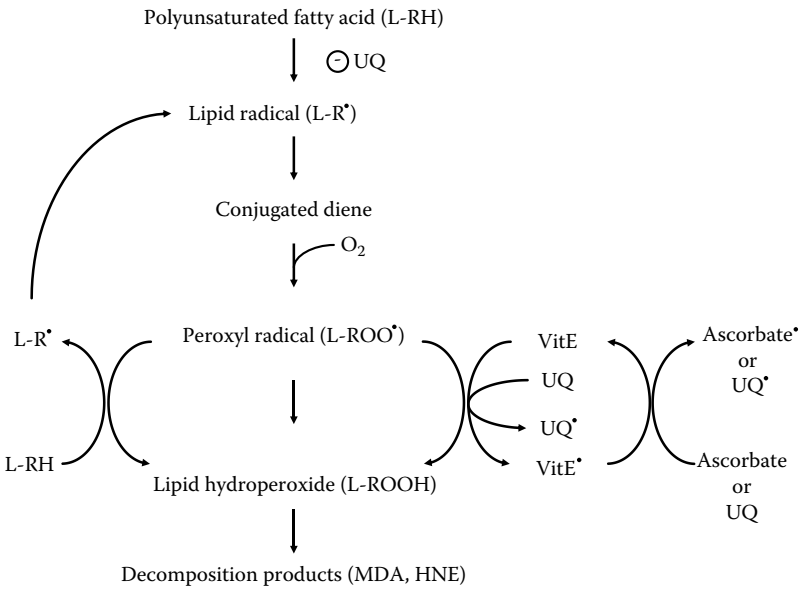

FIGURE 25.6 Schematic representation of lipid peroxidation. Polyunsaturated fatty acids are converted into lipid radicals, a process that can be inhibited by ubiquinol-10 (UQ). After molecular rearrangement, the lipid radical becomes a conjugated diene and then a peroxyl radical. This highly reactive species can attack other polyunsaturated fatty acids, thereby initiating a chain reaction. Vitamin E and ubiquinol, however, scavenge lipid peroxyl radicals, thereby breaking the chain reaction. Vitamin E can be regenerated by ascorbic acid (vitamin C) or ubiquinol-10.

Some experiments have suggested that cellular production of superoxide anions ($O_2^{-\bullet}$) or hydroxyl radicals ($OH^\bullet$), which are intermediates in several metabolic processes from the mitochondrial respiratory chain or the cytochrome P450 system, initiates the lipid peroxidation reaction. Other experiments have proposed that lipid peroxidation is initiated by lipoxygenase activity, as this enzyme forms radicals as intermediate products in the formation of eicosanoids. These hypotheses are not necessarily contradictory because lipoxygenase activity might be particularly important in endothelial cells and peroxide initiation by superoxide anions and hydroxyl radicals in smooth muscle cells [22].

In vitro oxidation of LDL results in alterations in both the lipid and the protein components of LDL. The amount of unsaturated cholesterylester content decreases, especially cholesteryl arachidonate and cholesteryl linoleate. In addition, phosphatidylcholine—the main phospholipid in LDL—is converted to lysophosphatidylcholine after cleavage of a fatty acid from the *sn*-2 position by phospholipase A_2. It has been postulated that the released fatty acid is readily oxidized and might then become responsible for the propagation of lipid peroxidation chain reaction, as inhibitors of phospholipase A2 block the generation of not only lysophospholipids but also lipid peroxides [23].

After peroxidation, lipid peroxides decompose and break down products, such as malondialdehyde (MDA) and several aldehydes, such as 4-hydroxynonenal (4-HNE), are formed. These products can react with the ε-amino groups of apoB-100, which causes an irreversible modification of the apolipoprotein, as the number of free cysteine and charged lysine residues of apoB-100 decreases. This results in reduced recognition and uptake of LDL by the LDL receptor, since the affinity of the LDL receptor is based on binding of positively charged apoB-100 to the negatively charged binding domain of the LDL receptor. This reduced uptake is compensated for by an increased affinity of these modified LDLs to the acetyl or scavenger receptors on the cell surface of macrophages. This uptake is not downregulated and may lead to extensive lipid loading and the transformation of macrophages into foam cells (see also Section IV.B).

Peroxidation products are cytotoxic, and chronic irritation of endothelial cells results in lesions of the endothelial cell layer. In addition, lysophosphatidylcholine from oxidized LDLs and the expression of chemoattractant proteins like monocyte chemoattractant protein-1 (MCP-1), other chemoattractants, and inflammatory cytokines by damaged endothelial cells and leukocytes attract leukocytes from the circulation and initiate a local inflammation. Animal studies have shown that inflammatory cell recruitment and activation is critical for the development of atherosclerosis. For example, MCP-1 knockout mice and macrophage colony-stimulating factor (M-CSF) knockout mice are less susceptible for the development of atherosclerosis.

One of the earliest inflammatory steps in the atherogenesis is a slower rolling of leukocytes along the vascular endothelium, which proceeds by a subsequent attachment of rolling leukocytes to the vascular endothelium. In this process, several adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and P-selectin play an important role. At least in vitro, these adhesion molecules are rapidly synthesized in response to several proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1). Moreover, HDL particles can inhibit the cytokine-induced expression of adhesion molecules (VCAM-1 and E-selectin) on endothelial cells in vitro [24]. This finding may be a possible link with the antiatherogenic effect of high serum HDL cholesterol concentrations in vivo.

The presence of adhesion molecules on endothelial cells alone, however, is not enough for attachment of leukocytes to the endothelium. For this interaction, leukocytes need to express ligands for these adhesion molecules. These ligands, expressed on lymphocytes and monocytes, are known as integrins. For example, very late antigen-4 (VLA-4), a β1-integrin, is a ligand for VCAM-1 and lymphocyte function associated-1 (LFA-1), a β2-integrin, for ICAM-1. After attachment, the proinflammatory leukocytes infiltrate into the activated endothelium, followed by further progressing of the inflammatory response. Interestingly, blocking VLA-4 by antibodies indeed decreased leukocyte entry and fatty streak formation in mice fed an atherogenic diet [25], which shows that this integrin plays a causal role during atherosclerosis. In conclusion, this process results in a continuous recruitment of new monocytes and T lymphocytes to the place of oxidation in the endothelium and in accumulation of macrophages filled with (oxidized) LDL in the arterial intima. In addition, several other processes are activated, which results in platelet aggregation, disturbance of eicosanoid homeostasis, and release of growth factors. These factors cause smooth muscle cell to proliferate and ultimately to migrate from the media to the intima. All these mechanisms together result in the formation of fatty streaks and atherosclerotic plaques (Figure 25.7).

1. Measurement of LDL Oxidation

LDL oxidation is thought to be initiated and to proceed primarily in the endothelial layer, whereas oxidized LDL particles are rapidly removed from the circulation. Thus, it is very difficult to quantify the LDL oxidation process in vivo. However, several assays have been developed to measure LDL oxidation.

a. In Vitro Copper-Mediated LDL Oxidation

In vitro oxidation of LDL can be induced by exposure of LDL particles to redox-active metal ions, such as copper (Cu^{2+}), or to reactive oxygen species, such as superoxide anion.

Esterbauer et al. [26] have developed a method—frequently used in earlier studies—to determine in vitro the susceptibility of LDL to oxidation by continuous monitoring of the formation of conjugated dienes, the products formed after oxidation of the polyunsaturated fatty acids from the LDL particle. After LDL isolation, copper is added to the test tube to initiate the oxidation process, and the formation of conjugated dienes is then quantified by measuring the change in absorbance at 234 nm (Figure 25.8). This curve can be divided into three consecutive phases: the lag phase, the propagation phase, and the decomposition phase.

During the lag phase, LDL-bound lipophilic antioxidants protect the polyunsaturated fatty acids from oxidation. When the LDL particle becomes depleted of antioxidants, the peroxidation reaction

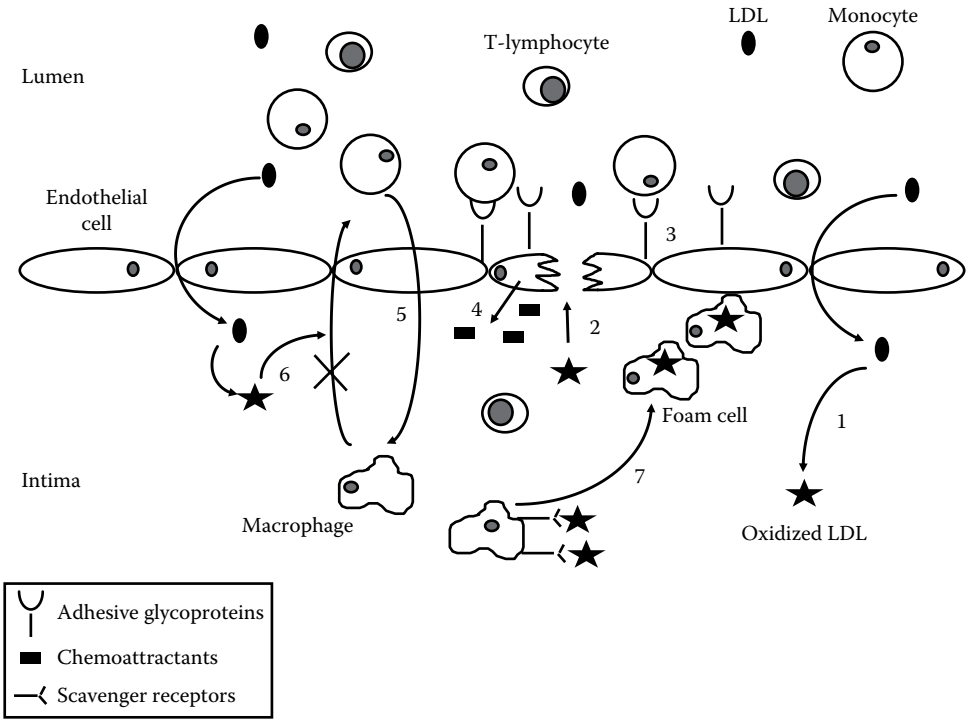

FIGURE 25.7 Schematic representation of the formation of an atherosclerotic plaque. (1) LDL enters the intimal layer and can be oxidized by several factors, such as lipoxygenase or reactive oxygen species. (2) Oxidized LDL is cytotoxic and causes endothelial damage, (3) which results in the expression of adhesive glycoproteins to which monocytes and T-lymphocytes attach. (4) The damaged endothelial cells excrete chemoattractants, which cause a continuous recruitment of monocytes and T-lymphocytes. (5) These cells pass the endothelial cell layer, and monocytes may become macrophages. (6) Oxidized LDL prevents return of macrophages back to the lumen, and (7) the arrested macrophages absorb large amounts of oxidized LDL via the scavenger receptors and become foam cells, which may eventually lead to the formation atherosclerotic plaques.

shifts to an autocatalytic process. Now the propagation phase starts during which the polyunsaturated fatty acids are oxidized and converted into conjugated dienes. The absorbance at 234 nm then reaches a maximum and may eventually decrease, since the produced conjugated dienes are labile and decompose to several products, such as MDA and 4-HNE. The oxidation profile of LDL is described by the duration of the lag time, the maximal amount of dienes formed, and the maximal rate of oxidation. A short lag time and a large amount of dienes are considered to reflect a high oxidative susceptibility of the LDL particle. However, interpretation of the oxidation rate is not clear.

b. Thiobarbituric Acid–Reactive Substances

LDL oxidation is also estimated by measuring in plasma the amount of thiobarbituric acid–reactive substances (TBArs), such as MDA and MDA-like substances, which can be formed during the in vivo peroxidation process. The detection of TBArs in plasma and LDL might therefore be an indication of the possible occurrence of peroxidative injury. However, these assays are considered to be nonspecific and subject to interferences with other compounds [27].

c. Antibodies

Oxidation of LDL in vivo can be measured by the detection of autoantibodies against MDA-modified LDL in human plasma [28]. Another approach for the determination of in vivo LDL oxidation is to use monoclonal antibodies that selectively bind to specific epitopes of oxidized LDL [29].

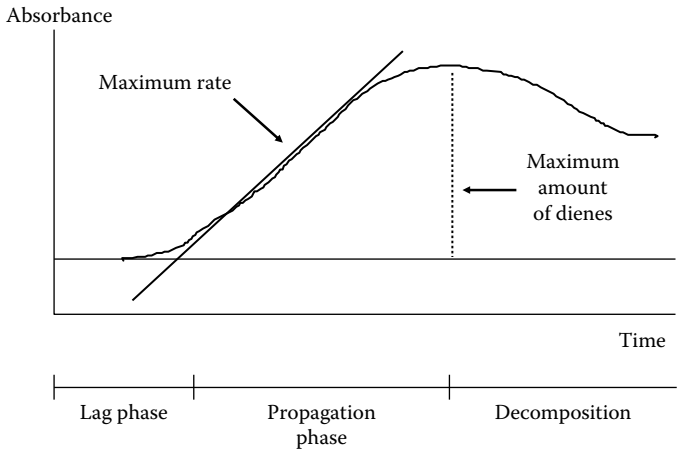

FIGURE 25.8 In vitro LDL oxidation. Formation of conjugated dienes during copper-catalyzed oxidation of LDL in vitro is monitored spectrophotometrically at 234 nm. The lag time before onset of rapid oxidation, the maximum rate of oxidation during the propagation phase, and the maximum amount of dienes formed are used to describe LDL oxidation characteristics.

d. Isoprostanes

Isoprostanes are isomers of prostaglandins (PGs), which are produced in vivo primarily—if not exclusively—by free radical–mediated peroxidation of polyunsaturated fatty acids. Especially the F2-isoprostanes, which are isomers of the PGF2a, derived from peroxidation of arachidonic acid, are considered suitable markers for oxidative modifications in vivo [30]. F2-isoprostanes can be analyzed in the circulation as well as in the urine by gas chromatography/mass spectrometry (GC-MS) or an immunoassay.

2. LDL Oxidation and CHD

Although causality has not been proved, it would be unwise not to consider a possible role of LDL oxidation in the genesis of CHD. In fact, Lobbes et al. [31] concluded from a systematic review that oxidized LDL is significantly associated with an increased relative risk of developing cardiovascular disease. However, it should be noted that especially the results from later epidemiological studies are not consistent. Tsimikas et al. [32] observed in a cross-sectional study a strong positive association between circulating concentrations of oxidized LDL with angiographically documented coronary artery disease. Furthermore, Meisinger et al. [33] found in a prospective, nested, case–control study that increased concentrations of oxidized LDL predicted future CHD events in apparently healthy men at the start of the study. Holvoet et al. [34], however, measured cross-sectionally the association between oxidized LDL and cardiovascular disease in the Multi-Ethnic Study of Atherosclerosis cohort. It was concluded that the relationship between oxidized LDL and subclinical CVD was largely explained by the relationship between oxidized LDL and other cardiovascular risk factors such as dyslipidemia, inflammation, and smoking. A comparable conclusion was drawn by Koenig et al. [35], based on findings in their prospective population-based case-cohort study. After adjustment for conventional cardiovascular risk markers, the associations between increased oxidized LDL concentrations and incident CHD became strongly attenuated. Also in the Framingham Offspring Study, no relationship was reported between autoantibodies to oxidized LDL and incident CHD [36]. These discrepant findings raise the question whether oxidized LDL is an independent risk predictor for CHD.

Not only oxidation of polyunsaturated fatty acids but also certain oxidized forms of cholesterol (oxysterols) are atherogenic and may play a role in plaque development. Especially, 7β-hydroxycholesterol may be a good marker for free radical–related lipid peroxidation.

Several studies have now shown that in humans 7β-OH-cholesterol is associated with the risk for atherosclerosis [37]. However, so far no causal relation has been established between oxysteroids and lesion formation.

3. Dietary Effects

The fatty acid composition of the diet, and in particular the amount of polyunsaturated fatty acids, is reflected by the fatty acid composition of the LDL particle (Tables 25.4 and 25.5). As polyunsaturated fatty acids are more easily oxidized than monounsaturated or saturated fatty acids, it can be envisaged that LDL oxidizability is influenced by changing the dietary fatty acid composition. However, when interpreting the results, it should be realized that the diet always induces multiple changes in the fatty acid composition of the LDL particle. For example, the proportion of linoleic acid in the LDL particle increases after enrichment of the diet with this fatty acid, whereas the proportion of oleic acid, arachidonic acid, palmitoleic acid, and palmitic acid decreases [38].

TABLE 25.4
Fatty Acid Composition (g/100 g Fatty Acid) of an LDL Particle after Consumption of a Diet Rich in Oleic Acid or Linoleic Acids

	Diet	
Fatty Acid	**Oleic Acid**	**Linoleic Acid**
Saturated	24.7	24.8
Monounsaturated	25.0	20.4
Polyunsaturated	50.3	54.8
(*n*-6)	45.7	51.2
(*n*-3)	4.1	3.3

Source: Abbey, M. et al., *Am. J. Clin. Nutr*., 57, 391, 1993.

TABLE 25.5
Fatty Acid Composition (g/100 g Fatty Acid) of an LDL Particle after Consumption of a Diet Rich in (*n*-6) or (*n*-3) Polyunsaturated Fatty Acids from Respectively Corn or Fish Oils

	Diet	
Fatty Acid	**Corn Oil**	**Fish Oil**
Saturated	25.5	26.1
Monounsaturated	23.5	22.3
Polyunsaturated	48.9	49.3
(*n*-6)	46.4	40.7
(*n*-3)	2.5	806

Source: Adapted from Suzukawa, M. et al., *J. Lipid Res*., 36, 473, 1995.

a. *Effects of Linoleate-Rich versus Oleate-Rich Diets*

Linoleic acid, an *n*-6 polyunsaturated fatty acid, contains more unsaturated bonds than oleic acid and is preferentially incorporated into tissue lipids. Accordingly, increasing the amount of linoleic acid in the diet at the expense of oleic acid leads to a higher proportion of linoleic acid in the LDL particle (Table 25.4) [38]. Therefore, it can be expected that, at least in vitro, LDL is more easily oxidized after consumption of linoleic acid–enriched diets. Indeed, several studies have demonstrated that replacement of oleic acid in the diet for linoleic acid may result in a decreased lag time, a higher production of dienes, and a reduced oxidation rate. In addition, LDL uptake by macrophages was increased after consumption of linoleic acid–enriched diets, suggesting that in vivo the LDL was modified to a greater extent [39].

b. *Effects of* n-*3 Polyunsaturated Fatty Acids*

Consumption of fish oils or fish oil capsules, which are rich in EPA and DHA, also affects the fatty acid composition of the LDL particle [40]. The amount of these two *n*-3 polyunsaturated fatty acids increases mainly at the cost of *n*-6 polyunsaturated fatty acids (Table 25.5). Effects of fish oil relative to other fatty acids on LDL oxidation are contradictory. In some studies, no effects on susceptibility of LDL to oxidation were observed after fish oil supplementation, whereas in other studies decreases or increases in susceptibility of LDL to oxidation were reported [39]. Currently, there is no explanation for these contradictory results.

c. *Antioxidants*

Fat-soluble antioxidants, especially α-tocopherol (vitamin E), are a main protecting factor against in vitro LDL oxidation. Supplementation of vitamin E increases the tocopherol content of LDL, resulting in a higher oxidation resistance of LDL, as evidenced from an increased lag time. However, oxidized LDL concentrations were not changed after α-tocopherol supplementation [39]. Other antioxidants, such as carotenoids, ascorbic acid, and ubiquinol-10, also have an important impact on the oxidative resistance of LDL.

Ascorbic acid, a hydrophilic antioxidant, and ubiquinol-10, a lipophilic antioxidant, are capable of regenerating tocopherols, and addition of these antioxidants to the test tube results in a longer lag time [41,42].

Oils rich in polyunsaturated fatty acids by nature contain relatively high concentrations of antioxidants. An exception are fish oils, which have relatively low levels of fat-soluble antioxidants, but their potentially harmful effects on LDL oxidation can be counteracted by addition of vitamin E to these oils.

B. Conclusion

Several studies strongly suggest that oxidation of LDL in vivo takes place, which results in even more harmful LDL particles. Therefore, LDL oxidation may play an important role in the formation of atherosclerotic lesions. However, a causal role of oxidized LDL in the genesis of CHD has not been established.

Replacing saturated fatty acids in the diet with linoleic acid reduces plasma LDL cholesterol concentrations. However, this dietary intervention also increases the proportion of linoleic acid in the LDL particle, thereby its in vitro oxidizability. Increased in vitro oxidizability of the LDL particle might also be observed when *n*-3 polyunsaturated fatty acids from fish oils are added to the diet, which can be overcome by simultaneously increasing the intake of vitamin E. The results for fish oils, however, are not consistent. Although these studies clearly demonstrate that the diet affects in vitro LDL oxidizability, the importance of these findings for the in vivo situation is less evident. In fact, there is no good evidence that increased intakes of antioxidants protect against cardiovascular disease [43].

VIII. HEMOSTASIS

Hemostasis, derived from the Greek words for blood and standing, is a complex, delicately balanced system of interactions to keep the blood circulating as a fluid through the blood vessels. In case of imbalance, such as when a vessel is damaged, the blood stands, starts to clot, and a stable thrombus forms. This, of course, is necessary to stop a wound from bleeding. However, if a stable thrombus is formed in a small coronary artery, the artery becomes occluded, blood and oxygen supply are hampered, and heart attack results.

The hemostatic system involves interacting processes for the formation of a stable thrombus—platelet aggregation and blood clotting—but also a mechanism to dissolve the thrombus, that is, fibrinolysis (Figure 25.9). In vivo these processes are associated and the interplay defines the prethrombotic state of the blood.

Hemostatic factors are difficult to measure. Due to the venipuncture and subsequent blood sampling and plasma preparation, platelets might become activated, which makes it very difficult to obtain a true reflection of the in vivo situation. In addition, measurements are usually made in venous fasting blood, while one is interested in thrombotic tendency in the arteries. In addition, many different methods are used, which makes a comparison between studies difficult.

A. Platelet Aggregation

The activity of blood platelets is an important factor for thrombus formation. Aggregated platelets adhere to the injured blood vessel to form a hemostatic plug, excrete substances such as thrombin and calcium, and provide a phospholipid surface—all of which are important for blood coagulation.

Several mechanisms are being proposed to explain the effects of fatty acids on platelet aggregation. Differences in fatty acid composition can change the arachidonic acid content of platelet and endothelial phospholipids. Arachidonic acid acts as a substrate for thromboxane A_2 (TxA_2) in platelets and prostacyclin (PGI_2) in endothelial cells, and the balance between these two eicosanoids affects platelet aggregation (Figure 25.10). Fatty acids have also been reported to directly affect TxA_2 receptors on platelet membranes. A third mechanism is that differences in fatty acid composition can affect the cholesterol content of membranes and consequently affect the fluidity of platelet membranes and platelet activation.

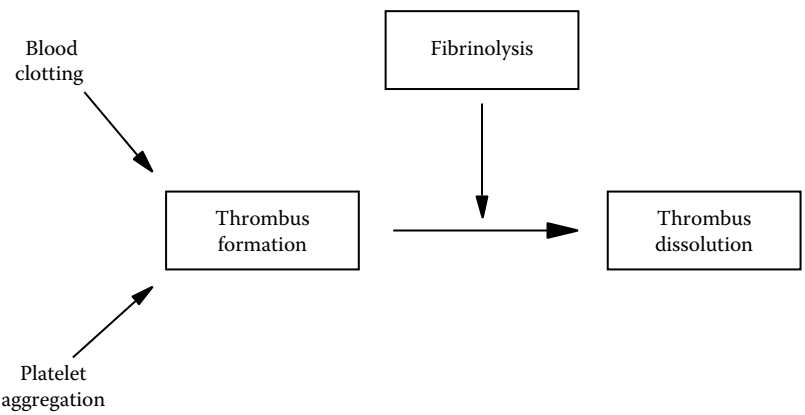

FIGURE 25.9 Processes involved in thrombus formation. The hemostatic system involves interacting processes for the formation of a stable thrombus—platelet aggregation and blood clotting—but also a mechanism to dissolve the thrombus, fibrinolysis.

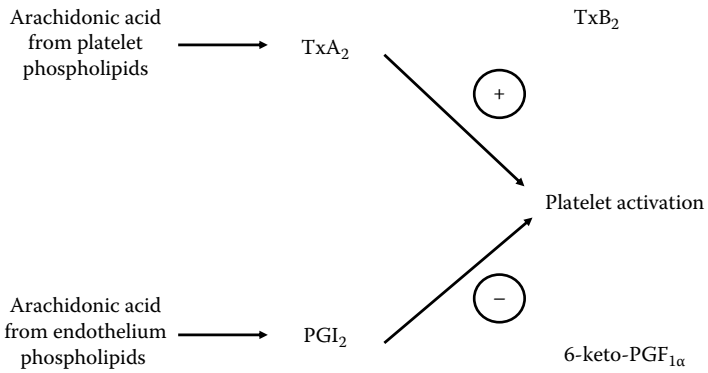

FIGURE 25.10 Schematic representation of platelet activation. Arachidonic acid from platelet acts as a substrate for thromboxane A_2 (TxA_2) and arachidonic acid from the endothelial cells for prostacyclin (PGI_2). The balance between these two eicosanoids affects platelet activation. TxB_2 is the stable metabolite of TxA_2, and 6-keto-$PGF_{1\alpha}$ of PGI_2.

1. Measurement of Platelet Aggregation

A broad scale of methods is available to measure platelet aggregation in vitro. First, the blood sample needs to be anticoagulated to avoid clotting of the blood in the test tube or in the aggregometer. Different anticoagulants are used, such as citrate, which depletes the sample from calcium, and heparin or hirudin, which causes an inhibition of the conversion of prothrombin to thrombin. In vitro platelet aggregation can then be measured in whole blood, in platelet-rich plasma, or—to remove the influence of possible interfering constituents from the plasma—in a washed platelet sample. Finally, the aggregation reaction in the test tube can be triggered with many different compounds, such as collagen, adenosine diphosphate (ADP), arachidonic acid, and thrombin. The use of all these different methods complicates comparisons of findings between studies.

2. Platelet Aggregation and CHD

In vitro platelet aggregation has been reported to be an important marker for the prediction of reoccurrence of coronary events [44]. In addition, platelet aggregation measured in whole blood was strongly associated with the prevalence of ischemic heart disease [45]. Furthermore, aspirin, which is known to inhibit platelet aggregation, has been reported to reduce nonfatal myocardial infarction, but not CHD [46]. However, studies have also been reported that did not find an association between platelet aggregability and CHD [47].

3. Dietary Fats and Platelet Aggregation

a. Total Fat Content of Diets

Renaud and coworkers [48] studied nine groups of farmers from different areas in France and Britain, who differed with respect to dietary intakes of total and saturated fatty acids. In the groups with a high consumption of total and saturated fatty acids, an increased thrombin-induced aggregation in platelet-rich plasma was observed as compared with the groups of farmers with lower intakes. In a later intervention study [49], the diets of French farmers were reduced in saturated fat content by replacing the habitually consumed dairy fat by high linoleic acid margarine. A control group of farmers was advised not to change their diets. In the intervention group, total fat intake decreased along with the intake from saturated fatty acids, whereas the intake of dietary linoleic acid and a-linolenic acid was increased compared with initial values. In agreement with the previous study [48], a decreased thrombin-induced aggregation was observed in the intervention group, whereas platelet aggregation did not change in the control group. However, platelet aggregation induced by ADP was significantly increased in the intervention group. From this dietary intervention, however,

it was not clear whether the changes of total fat content or the changed fatty acid composition of the diets were responsible for the changes observed in platelet aggregation.

b. Dietary Fatty Acid Composition

The effects of dietary fatty acid composition were further evaluated in well-controlled dietary intervention studies, in which total fat content of the diets was kept constant and only the dietary fatty acid composition changed. The results however are inconsistent.

When saturated fatty acids were replaced by oleic acid or linoleic acid, platelet aggregation was increased, decreased, or not changed. Comparable conflicting results have been found when oleic and linoleic acids were compared side by side. Other studies have examined the effects of different saturated fatty acids. Compared with oleic acid, MCFAs, lauric, myristic, or palmitic acids did not change collagen-or ADP-induced whole blood aggregation [50]. In another study, similar effects of stearic, oleic, and linoleic acids were found [51]. Thus, dietary fatty acids can modulate platelet aggregation, but the use of many different in vitro methods makes comparison between studies and extrapolation to the in vivo situation difficult. However, Bachmair et al. [52] concluded that effects of oleic and linoleic acids on agonist-induced platelet aggregation were more favorable than those of saturated fatty acids and *trans* fatty acids. More consistent are the effects of fish oils, which decrease platelet aggregation tendency [53].

Effects of specific saturated fatty acids on stable metabolites of TxA_2 and PGI_2—TxB_2 and 6-keto-$PGF_{1\alpha}$, respectively—in urine showed that lauric, myristic, palmitic, and stearic acids had similar effects on urinary thromboxane and PG excretion [54,55]. In addition, effects of *trans* fatty acids were comparable with those of stearic acid [56]. Furthermore, *n*-6 polyunsaturated fatty acids increased urinary 11-dehydro-TxB_2 excretion compared with saturated and monounsaturated fatty acids, which may be related to ADP-induced platelet aggregation [57].

B. Coagulation

Several pathways for blood coagulation exist. The tissue factor pathway of blood coagulation, previously known as the extrinsic pathway of blood coagulation, appears to be the most important one. The factors involved in this pathway are depicted in a simplified scheme in Figure 25.11. Most coagulation factors are mainly present in inactivated form, except for factor VII, of which 1% circulates as activated factor VII (factor VIIa). However, Figure 25.11 only shows the activated coagulation factors, except for factor VII.

Thrombus formation in vivo is initiated when factor VII or factor VIIa contacts thromboplastin tissue factor, expressed, for example, after vessel injury or inflammation. A tissue factor, such as thromboplastin, is a procoagulant that is expressed only on activated endothelium. Once bound, factor VII is rapidly activated into factor VIIa. The complex of factor VIIa with tissue factor initiates a cascade of reactions, which ultimately results in the conversion of factor X into factor Xa and the generation of thrombin from prothrombin. Thrombin finally cleaves fibrinogen into fibrin, which stabilizes a thrombus. However, thrombin also inhibits some coagulation factors, and the coagulation cascade is thus inhibited by one of its end products so as to prevent uncontrolled formation of fibrin.

The coagulation cascade is further regulated by the action of coagulation inhibitors. An important inhibitor of coagulation is the tissue factor pathway inhibitor (TFPI), which inhibits the activity of the tissue factor–factor VIIa complex, and TFPI therefore prevents further activation of the coagulation cascade. The tissue factor–factor VIIa complex is also inhibited by antithrombin III, which also suppresses the activation of thrombin and other activated coagulation factors.

1. Measurement of Coagulation

Most assays measure the total amount (e.g., factor VII-antigen or fibrinogen concentrations) or the activity of circulating coagulation factors. However, these factors are normally present in large

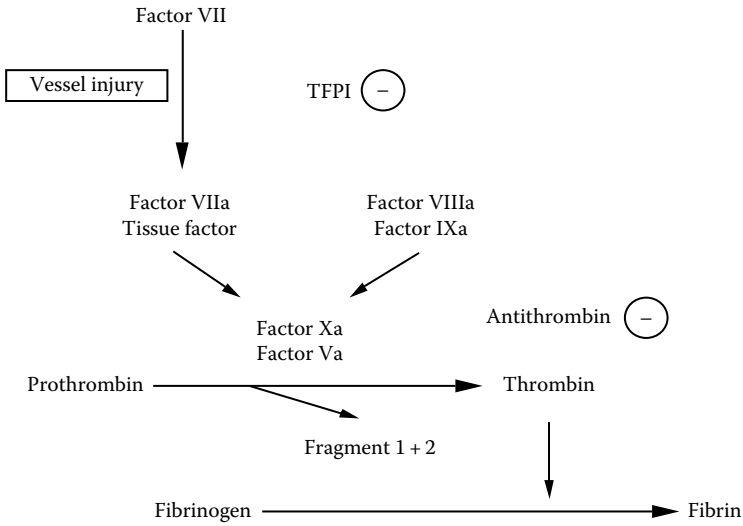

FIGURE 25.11 Schematic representation of the coagulation cascade. When factor VII contacts a tissue factor, expressed, for example, after vessel injury or inflammation, factor VII is rapidly activated to factor VIIa. The complex of factor VIIa with tissue factor initiates a cascade of reactions, which ultimately results—after cleavage of fragment 1 + 2—in the conversion of prothrombin into thrombin. Thrombin cleaves fibrinogen into fibrin, which stabilizes a thrombus. Tissue factor pathway inhibitor (TFPI) and antithrombin-III inhibit the coagulation cascade.

excess in the blood, and only a small percentage is converted to active enzymes under in vivo situations. Nowadays, other assays are also available, which reflect actual in vivo coagulation. The plasma fragment 1 + 2 concentration reflects the amount of prothrombin actually converted to thrombin, whereas fibrinopeptides A and B concentrations reflect the conversion of fibrinogen to fibrin. However, in healthy subjects, concentrations of most of these markers are very low and just above detection limits, which sometimes make it difficult to implement them in dietary studies.

Many methods are available to measure factor VII. In many studies, factor VII is measured with a coagulant assay (factor VII coagulant activity). Factor VII activity, however, is measured with a two-step chromogenic assay, which depends on the rate of generation of factor Xa from factor X by factor VIIa. Both methods may give different results and do not differentiate between factor VIIa and factor VII antigen concentrations.

2. Coagulation and CHD

Long-term prospective epidemiological studies have consistently reported that in healthy males factor VII coagulant activity and fibrinogen concentrations were higher in subjects who developed cardiovascular diseases at a later stage of the study. Factor VII coagulant activity was particularly associated with an increased risk of dying from cardiovascular disease [58,59]. In addition, from the Northwick Park Heart Study (NPHS), it has been reported that low and, unexpectedly, also high concentrations of antithrombin III were associated with increased deaths from CHD [60]. Furthermore, other coagulation factors such as factor II, factor V, and the von Willebrand factor have shown significant association with an increased risk for CHD [47,61].

3. Dietary Fats and Coagulation

a. Total Fat Content of Diets

Marckmann and colleagues [62] have investigated both shorter- and longer-term effects of low-fat/high-fiber diets on human blood coagulation. In an 8-month study, it was found a low-fat/high-fiber

diet significantly decreased plasma factor VII coagulant activity by 5%–10%, but only in the first 2 months and in the last months of dietary intervention. The absence of effects in the middle study period was explained by the fact that the subjects in this period did not follow the dietary guidelines strictly because of allowed study holidays. Plasma fibrinogen concentrations were not changed. The results of this study were confirmed in a study of shorter duration [63].

In another study, Marckmann et al. [64] investigated whether the low-fat or the high-fiber component of diets was responsible for decreased factor VII coagulant activity found in earlier studies. The experimental diets of this trial only differed in their fat content (39% vs. 31% of energy) and carbohydrate content (47% vs. 54% of energy). Factor VII coagulant activity was similar on the low-fat and high-fat diets. In addition, no changes in fibrinogen concentrations were found. This limited number of studies indicates reducing effects on factor VII coagulation activity of low-fat/high-fiber diets. However, more controlled studies are needed to definitely address whether the reduced fat content of low-fat diets, the increased fiber content, or a combination of these two dietary factors is responsible for the decreased factor VII coagulant activity of such diets.

b. Dietary Fatty Acid Composition

The effects of saturated compared with unsaturated fatty acids have been studied in several experiments. Irrespective of the marker used, effects were small, whereas results varied between studies. Diets enriched in *n*-3 fatty acids from fish did also not change factor VII coagulant activity [65,66] or fibrinogen concentrations [65–67].

A study of Almendingen et al. [68] investigated the effects of *trans* fatty acids in diets enriched with hydrogenated fish oil or hydrogenated soybean oil compared with a diet enriched in butterfat. The diet enriched in butterfat showed slightly increased fibrinogen concentrations as compared with the hydrogenated fish oil diet. No significant differences in the levels of factor VII or fibrin degradation products were observed.

Well-controlled studies investigating effects of specific saturated fatty acids have also been published. Tholstrup et al. [69] reported that diets rich in lauric plus myristic acids or palmitic acid increased factor VII coagulant activity as compared with a diet rich in stearic acid. It was also suggested that the turnover of prothrombin was increased on the lauric plus myristic acids diet as fragment 1 + 2 concentrations were higher on such a diet than on a stearic acid diet [70]. In a second study, Tholstrup et al. [71] investigated diets rich in myristic or palmitic acid. On the myristic acid diet, subjects showed an increase in factor VII coagulant activity. Thus, these studies suggest that saturated fatty acids, except for stearic acid and probably MCFA, increase fasting factor VII coagulant activity (Figure 25.12). However, effects during the postprandial phase may be different, as a lesser increase in FVIIa was reported after the consumption of saturated fats than after unsaturated test fats [72]. The biological significance of these findings should be investigated further.

C. Fibrinolysis

The process involved in thrombus dissolution, and thus the conversion of fibrin into fibrin degradation products, is called fibrinolysis. A simplified scheme of the fibrinolytic pathway is given in Figure 25.13. The central reaction in the fibrinolytic process is the conversion of plasminogen into plasmin, which is regulated by the action of tissue plasminogen activator (t-PA). The fibrinolytic capacity of blood is regulated by inhibiting t-PA activity by the action of plasminogen activator inhibitor type-1 (PAI-1), whereas plasmin is inhibited mainly by α_2-antiplasmin.

1. Measurement of Fibrinolytic Capacity of Plasma

The fibrinolytic capacity of plasma can be measured by global tests or more specific assays. The global tests include the dilute clot lysis time, the euglobulin clot lysis time, and the fibrin plate assay. The dilute and euglobulin clot lysis time measure total fibrinolytic capacity. The total blood sample or the insoluble protein sample (euglobulin fraction) is diluted with a buffer, clotted, and the lysis

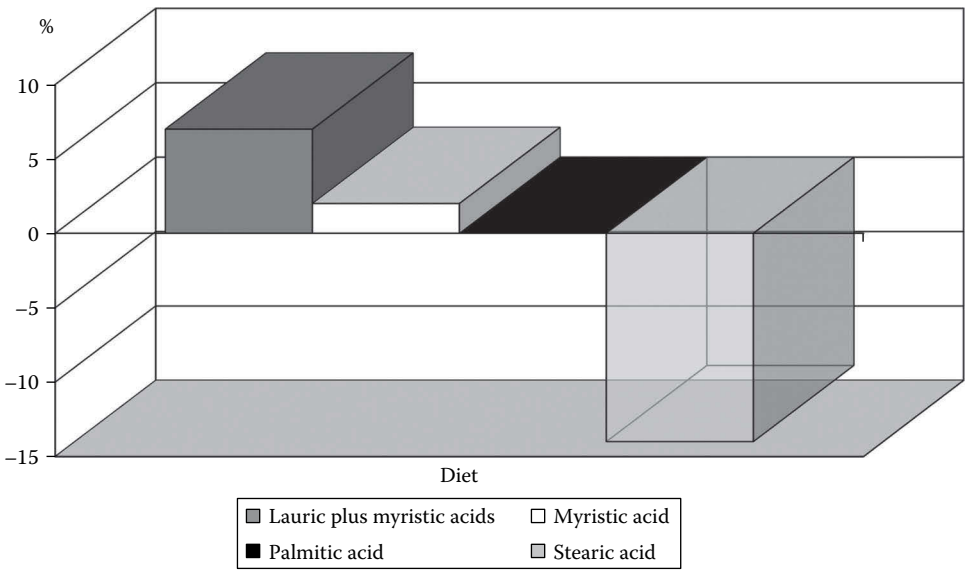

FIGURE 25.12 Effects of lauric plus myristic acid, myristic acid, and stearic acid on factor VII coagulant activity relative to those of palmitic acid. Healthy young men consumed diets enriched in lauric plus myristic acids (C12:0 and C14:0; 14% of energy), myristic acid (C14:0; 14% of energy), palmitic acid (C16; 15%–16% of energy), or stearic acid (C18:0; 14% of energy) for 3 weeks. (From Tholstrup, T. et al., *Am. J. Clin. Nutr.*, 59, 371, 1994; Tholstrup, T. et al., *Am. J. Clin. Nutr.,* 60, 919, 1994.)

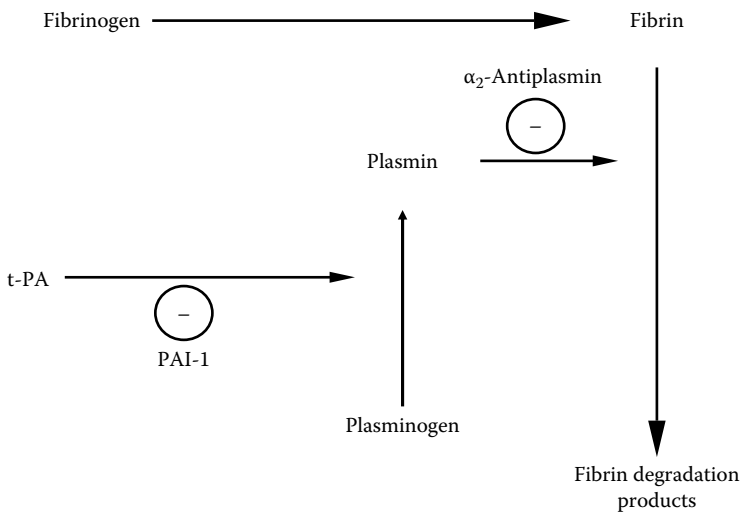

FIGURE 25.13 Schematic representation of the fibrinolytic pathway. Fibrin is degraded by plasmin. The conversion of plasmin from plasminogen is regulated by the action of tissue plasminogen activator (t-PA). t-PA activity is suppressed by plasminogen activator inhibitor type-1 (PAI-1), while plasmin is inhibited by α_2-antiplasmin.

time of the clot recorded. In the fibrin plate assay, a standard volume of the euglobulin fraction of plasma is added to standardized plasminogen-rich fibrin plates and the amount of lysis is recorded. In more specific assays, total plasma concentrations of t-PA and PAI-1 can be determined with enzyme-linked immunosorbent assays. Plasma t-PA and PAI-1 activities can be estimated with a chromogenic assay.

2. Fibrinolysis and CHD

From the NPHS, it has been reported that low fibrinolytic capacity of plasma, measured as clot lysis time, was significantly associated with increased CHD risk in men aged 40–54 [73]. In addition, Hamsten et al. [74] have reported that higher concentrations of PAI-1 were associated with increased risk of reoccurrence of coronary events. Furthermore, t-PA antigen levels were related to an increased risk for CHD [61]. High t-PA antigen levels indicate a low fibrinolytic capacity.

3. Dietary Fats and Fibrinolysis

a. Total Fat Content of Diets

Marckmann et al. [62,63] found in both a shorter-term (2 week) and a longer-term trial (8 months) that plasma euglobulin fibrinolytic capacity and plasma t-PA activity were increased on low-fat/high-fiber diets as compared with high-fat/low-fiber diets (Figure 25.14). No changes were observed in t-PA and PAI-1 antigen concentrations. In agreement with these results, Mehrabian et al. [75] reported decreased plasminogen and PAI-1 activities, after consumption of a low-fat/high-fiber/low-cholesterol diet with less than 10% of energy from fat for 21 days. This was associated with decreased t-PA antigen concentrations. However, from these studies, it is not possible to conclude whether the changes observed were due to the lower fat or cholesterol contents, or the higher fiber content of the diets.

In another study, Marckmann et al. [64] have also compared two diets, which differed in total fat content though fiber intake and the relative fatty acid composition were comparable. Their results indicated that plasma euglobulin fibrinolytic capacity on a high-fat diet (39% of energy from fat) did not change compared with a low-fat diet (31% of energy). It was concluded that an isolated reduction of total fat content of the diet does not affect the fibrinolytic capacity of the blood but that concomitant changes in the diet, as, for example, changes in fatty acid composition or fiber content, are necessary to provoke changes of fibrinolytic factors.

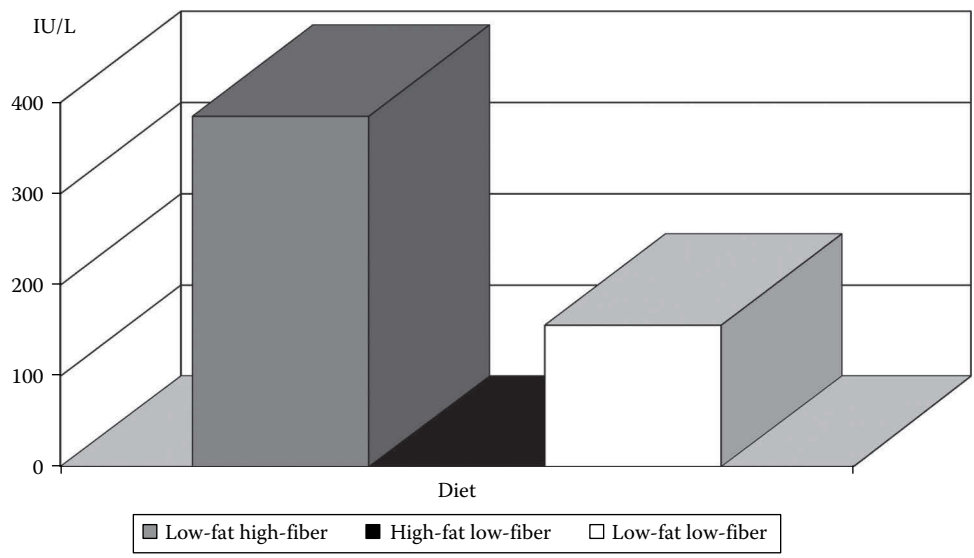

FIGURE 25.14 Effects of fat and fiber on the plasma euglobulin fibrinolytic activity relative to those of a high-fat/low-fiber diet. Healthy individuals consumed a low-fat/high-fiber (28% of energy from fat and 3.3 g/MJ fiber), a high-fat/low-fiber (39% of energy from fat and 2.1–2.3 g/MJ fiber), or a low-fat/low-fiber (31% of energy from fat and 2.2 g/MJ fiber) diet for 2 weeks. (From Marckmann, P. et al., *Am. J. Clin. Nutr.*, 59, 935, 1994; Marckmann, P. et al., *Arterioscler. Thromb.*, 12, 201, 1992.)

b. Dietary Fatty Acid Composition

Effects of different fatty acid composition on fibrinolytic capacity have been investigated extensively for fatty acids from fish, although recently additional data have become available for the effects of other fatty acids.

High intakes of fish may affect fibrinolytic capacity. Brown and Roberts [66] studied the effects of a daily consumption of 200 g of lean fish, with or without fish oil supplement. They observed an apparent enhancement of plasma euglobulin fibrinolytic capacity, which tended to be accentuated with fish oil supplementation. However, compared with meat diets, diets enriched with fatty fish have also been associated with an unfavorable increase in PAI-1 activity [65,67]. Increased PAI-1 activity has also been reported on diets enriched in partially hydrogenated soybean oil compared with a butterfat diet [68].

In other studies, no unfavorable effects of lauric plus myristic acids, palmitic acid, or stearic acid diets relative to other unsaturated fatty acids were reported [51,69,71].

D. Conclusion

Results of platelet aggregation studies are difficult to interpret because of large differences in the methods used. Lowering dietary fat intake decreases in vitro platelet aggregation tendency induced by some antagonists but increases in vitro platelet aggregation by other antagonists. In addition, the effects of dietary fatty acid composition on in vitro platelet aggregation are contradictory. Effects of fish oils are however more consistent and these oils may decrease platelet aggregation tendency.

To affect coagulation and fibrinolytic factors, some studies suggest that lowering fat intake must be accompanied by changes in dietary fatty acid composition and increased fiber intakes. Though results between studies are less consistent, favorable effects on blood coagulation may be obtained by lowering the content of the cholesterol-raising saturated fatty acids and replacing them with unsaturated fatty acids.

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26 Conjugated Linoleic Acid

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CONTENTS

SUMMARY

With great interest in conjugated linoleic acid (CLA) as a dietary supplement and food application in the past few decades, it is important to understand not only its biological functions but also the potential adverse health implications. This chapter summarizes CLA's effects on preventing cancer, reducing cardiovascular disease risk, preventing obesity, improving muscle metabolism, improving bone health, and modulating immune and inflammatory responses. In addition, currently identified potential adverse health implications with CLA, impaired glucose homeostasis, increased oxidative stress markers, liver dysfunction, and reduced milk fat are reviewed.

I. INTRODUCTION

Conjugated linoleic acid (CLA) refers to conjugated isomers of linoleic acid, octadecadienoic acid. Reports on CLA can be traced back to the 1930s, while CLA's structure was first identified in 1966 as an intermediate of the biohydrogenation of linoleic acid to stearic acid during rumenic fermentation [1,2]. However, it is not until 1987 that the first bioactivity of CLA as an anticancer component of ground beef extract was reported [3]. Since then, various activities of CLA have been reported and its health impact still attracts attention [4]. This chapter reviews the origins, isomers, intake, and health benefits of CLA as well as its potential health concerns.

II. CLA ISOMERS

CLA is not a single fatty acid; rather the name refers to a number of geometric and positional isomers, with two double bonds at position 7, 8, 9, 10, 11, 12, or 13 [5,6]. Among them, the first reported CLA is the *cis*-9,*trans*-11 isomer [1]. This isomer is one of two stable intermediates produced during the biohydrogenation of linoleic acid (*cis*-9,*cis*-12) to stearic acid by rumen bacteria [1]. In addition, it is also known that the *trans*-11 vaccenic acid, the other stable intermediate of biohydrogenation, can be converted to the *cis*-9,*trans*-11 CLA by Δ^9-desaturase in mammalian tissue [7]. Thus, this isomer is naturally present and represents more than 80% of total CLA in food of rumen origin such as beef, milk, and other dairy products [8–10].

Among other CLA isomers, the *trans*-10,*cis*-12 isomer has gotten much attention due to its activity on body fat reduction [11]. This isomer is present at very low levels in food; however, when CLA is prepared by chemical processes, the *cis*-9,*trans*-11 and this isomer make up about 90% of total CLA in approximately equal quantities [12,13]. Thus, chemically prepared CLA is often referred to as "CLA-mixture," "CLA 1:1," or "50:50." Beyond its effect on body fat reduction, the *trans*-10,*cis*-12 isomer has been linked to the reduction of apolipoprotein B secretion from human hepatoma cells, inhibition of Δ^9-desaturase, and prevention of cancer development [14–16].

Among other isomers, *trans*,*trans* CLA isomers (*trans*-9,*trans*-11 or mix of all *trans*,*trans*) are linked to anticancer effects, antiinflammatory responses, antiplatelet aggregation, hypocholesterolemia, and prevention of fatty liver [17–25]. There are only few reports on the bioactivities of other CLA isomers, and most studies focused on the two major CLA isomers: *cis*-9,*trans*-11 and *trans*-10,*cis*-12.

III. REGULATORY ASPECTS OF CLA

The U.S. Food and Drug Administration specifically defines *trans* fat as "all unsaturated fatty acids that contain one or more isolated double bonds (i.e., nonconjugated) in a *trans* configuration" [26]. As CLA has *trans* configuration as part of its "conjugated" form, current *trans* fat definition excludes CLA. Moreover, CLA has had approval as a generally recognized as safe (GRAS) additive in the United States since 2008. CLA GRAS claim is "use of CLA-rich oil in certain specified foods within the general categories of soymilk, meal replacement beverages and bars, milk products, and fruit juices at levels not to exceed 1.5 g per serving" [27]. Based on previous reports, consumption of CLA as triglyceride (TG) form of mixture of two main isomers, up to 6 g CLA/day for 1 year or 3.4 g/CLA for 2 years, is currently considered safe [28–31]. However, with CLA's usage as a dietary supplement or a food additive, intake of CLA will likely increase significantly; therefore, it is important to review the current knowledge on CLA health benefits and potential adverse effects.

IV. CLA INTAKE AND SERUM/TISSUE LEVELS

Table 26.1 summarizes estimated CLA intake reported in publications, which is mainly the *cis*-9,*trans*-11 CLA isomer as this is the major isomer found naturally in food [8–10]. CLA intake corresponds to dietary patterns; higher CLA serum levels are reported with a high CLA diet such as dairy [32]. In addition, due to the conversion of *trans*-11 vaccenic acid to the *cis*-9,*trans*-11 CLA by Δ^9-desaturase, it has been reported that Δ^9-desaturase polymorphism contributes to serum CLA levels [7,33,34].

CLA concentrations reported in human serum range from 2 to 20 μM with no supplementation and 50–180 μM after supplementation with 0.8–3.2 g CLA per day for 2 months [34–37]. Both major CLA isomers are detected in human serum without supplementation [34,37]. Abdelmagid et al. [34] reported that serum CLA levels were 16–19 μM *cis*-9,*trans*-11 and 3.9–4.2 μM *trans*-10,*cis*-12, as well as 1.8–2.1 μM *cis*-11,*trans*-13 isomer. Alternatively, others reported that CLA made up

TABLE 26.1
Estimated Dietary CLA Intake

		CLA Intake (mg/Day)	
Reference	**Subjects**	**Women**	**Men**
Fritsche and Steinhart [10]	German	350	430
Herbel et al. [35]	United States	127–151	
Salminen et al. [38]	Finland	310	
Park et al. [32]	United States	15–291	n.d.
Aro et al. [188]	Finland	127–142	n.d.
Ritzenthaler et al. [189]	United States	104–151	176–212
Voorrips et al. [190]	Netherlands	70–290	n.d.
McCann et al. [191]	United States	134–161	n.d.
Larsson et al. [52]	Sweden	70–150	n.d.
Mushtaq et al. [9]	United Kingdom	68	127
Smit et al. [43]	Costa Rica	230–330	

n.d.: Not determined.

0.1%–0.57% of the total fatty acids in serum [38–40]. Without further supplementation, serum CLA levels returned to control level within 2 months in both human and animal studies [36,41].

As an indicator of long-term CLA exposure, 0.35%–0.78% of the *cis*-9,*trans*-11 CLA from total fatty acids in adipose tissue were reported with no detectable *trans*-10,*cis*-12 [42,43]. Meanwhile, Chajes et al. [44] reported 0.44% CLA (ranging 0.19%–0.75%) of total fatty acids in breast adipose tissues.

V. HEALTH IMPLICATIONS OF CLA

A. Anticancer Effects of CLA

The first reported biological effect of CLA was its preventive effects on 7,12-dimethylbenz[*a*] anthracene (DMBA)-induced mouse epidermal carcinogenesis [3]. Since then numerous animal studies have proven that CLA prevents the development of various types of cancer [45–48]. Among them, the effect of CLA on DMBA-induced mammary cancer has been extensively studied. As low as 0.1% CLA in the diet significantly reduced mammary tumor incidence, and CLA's effects are independent of the content or type of dietary fat [49,50]. CLA's preventive effects on carcinogenesis are suggested to be through its influence on one or more stages of cancer development: initiation, promotion, progression, and/or metastasis [45,46,48,51].

Compared to strong evidence of the link between CLA and cancer prevention in animal studies, there are currently only limited reports on CLA and cancer in humans as summarized in Table 26.2 [13,46,47]. Among 11 human studies on this topic, 8 were on breast cancer. With the exception of the study from McGowan et al. [37], all reported breast cancer were linked to dietary, serum, or tissue levels of CLA. Overall, there was a weak association between CLA and a reduction of breast cancer incidence.

A study by Larsson et al. [52] reported a negative link between CLA-rich food consumption and incidence of colorectal cancer. Three reports discussed supplementing CLA to cancer patients along with cancer treatment(s), although the supplementation period was relatively short (up to 8 weeks) with limited sample size [37,53,54]. These studies suggest potential benefits of CLA along with cancer treatment; however, these data are too preliminary to conclude that CLA presents benefits on certain types of cancer in humans.

TABLE 26.2
Summary of Human CLA Studies in Cancer

Reference	Study Design and Subject Information	n (Sex)	CLA	Study Duration	Results
Breast cancer					
Knekt et al. [192]	Cohort	4,697 (F)	Milk	25 years	Milk consumption is inversely associated with breast cancer incidence.
Aro et al. [188]	Case–control; pre- and postmenopausal	403 (F)	CLA from diet	4 years	CLA-rich foods may protect against breast cancer in postmenopausal women.
Chajes et al. [44]	Case–control; invasive breast carcinoma or benign breast pathologies	297 (F)	Tissue CLA levels	5 years	CLA levels in breast adipose tissue do not associate with breast cancer risk.
Chajes et al. [193]	Follow-up of Chajes et al. [44]; metastasis	209 (F)	Tissue CLA levels	7.5-year follow-up	Breast adipose tissue CLA level is not linked to prognostic factor or risk of metastasis or death.
Voorrips et al. [190]	Cohort; postmenopausal	941 (F)	CLA from diet	6.3 years	CLA intake is positively correlated with breast cancer incidence; total *trans* fatty acids and saturated fatty acids are positively associated with breast cancer incidence.
Rissanen et al. [40]	Case–control; pre- and postmenopausal	369 (F)	Serum CLA levels	10-year follow-up	Serum CLA levels are not associated with breast cancer incidence; serum PUFA is inversely associated with breast cancer incidence.
McCann et al. [191]	Case–control; pre- and postmenopausal	3,158 (F)	CLA from diet	6 years	Dietary CLA intake is not associated with breast cancer risk; CLA may reduce estrogen receptor–negative tumor in premenopausal women.
McGowan et al. [37]	Open; invasive nonmetastatic breast cancer	23 (F)	7.5 g/day	~12 days (10–28 days)	CLA supplementation is linked with decreased S14 and Ki-67 in tumor tissue (decreased fatty acid synthesis and tumor cell proliferation).
Colorectal cancer					
Larsson et al. [52]	Cohort study	60,708 (F)	CLA from diet	15 years	CLA-rich food may decrease colorectal cancer incidence.
Mohammadzadeh et al. [53]	Randomized; double-blind; inflammatory rectal cancer patients (Stage II–III)	32 (F/M)	3 g/day	6 weeks	CLA supplementation is linked with decreased inflammatory and angiogenesis biomarkers (TNF-α, IL-1β, CRP, and MMP-9).
HPV-induced laryngeal papillomatosis					
Louw [54]	Randomized; double-blind; cross-over (6 weeks washout); children	8 (F/M)	2.5 g/day	8 weeks	CLA supplementation is linked with decreased anatomical scores for mild/moderate aggressive papillomatosis.

B. Reduced Cardiovascular Disease Risk by CLA

The first report of CLA reducing the development of rabbit aorta atherosclerotic lesions led to a series of reports determining the role of CLA in atherosclerosis and cardiovascular diseases risks [13,46,47,55,56]. Human studies mainly focused on observing blood markers of cardiovascular diseases such as total, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) cholesterols, and/or TG, as well as blood pressure [13,46,47,56–59]. Most human studies reported none to minimal effects of CLA on the earlier mentioned markers. However, there are reports of increased C-reactive protein (CRP) by CLA supplementation. Since increased CRP is one of the adverse cardiovascular disease markers, there has been considerable attention paid to determining the potential role of CLA in cardiovascular disease risk [13,47,56,60–62]. However, as mentioned earlier, there is no concurrent increase of other markers for cardiovascular diseases with CLA, suggesting that increased CRP with CLA supplementation may not indicate altered risk for cardiovascular diseases [56]. Consistently, a recent randomized double-blind study with 0.9% of energy from the *cis*-9,*trans*-11 CLA reported no effect of CLA on cholesterol levels but CLA reduced TG levels, although the study concluded that CLA presented overall neutral effects on cardiovascular disease risk [63]. In addition, the inverse association between adipose tissue *cis*-9,*trans*-11 CLA and risk of myocardial infarction among subjects from Costa Rica further supports that CLA may not increase cardiovascular disease risks in humans [43].

Compared to minimal effects of CLA on blood lipid markers, there are reports that CLA reduced blood pressure [13,46,47,56,64]. In particular, two publications by the same group reported that CLA supplementation significantly reduced blood pressure for subjects with pregnancy-induced hypertension [65,66]. In addition, supplementation of CLA to subjects taking a hypertensive drug, ramipril, further improved blood pressure reduction [67]. However, a recent meta-analysis failed to support the effectiveness of CLA on blood pressure regulation in humans [68]. Overall, it is not conclusive whether CLA modulates blood pressure. However, based on currently limited reports, CLA may have the potential to be used to improve blood pressure for patients at risk along with current treatment options.

C. Antiobesity Effects of CLA

One unexpected bioactivity of CLA is its ability to reduce body fat, 60%–70% body fat reduction after CLA supplementation compared to control in mice [4]. This effect of CLA has gained tremendous attention and has been consistently reported in various animal species [46,69]. Body fat reduction by CLA is independent of the animal age, type of fat, or fat contents in the diets [46,69–71]. However, it is apparent that the effects of CLA are not observed when the animals were in negative energy balance [71]. In addition, this effect of CLA is linked to the *trans*-10,*cis*-12 isomer, independently of the presence of the *cis*-9,*trans*-11 isomer [11].

A number of publications suggest that CLA effectively reduces body fat by multiple mechanisms, including increased basal metabolic rate, enhanced overall energy expenditure (in part by increasing activity), modulated energy metabolism, and/or targeted adipocyte and skeletal muscle metabolisms [46,69]. Although there are reports of CLA reducing food intake in animals, particularly during the first week of CLA supplementation, it has been confirmed that CLA's effect on food intake is not linked to its effect on body fat reduction [71]. Along with suggested mechanisms of increased energy expenditure, the current focus of CLA research is on both adipocyte and skeletal muscle metabolisms [13,46,69]. CLA effectively reduces lipogenesis, increases lipolysis, and enhances fat utilization as a fuel [69]. Moreover, it was recently suggested that the *trans*-10,*cis*-12 CLA potentiates adipocyte browning in mice, which can further extend the contribution of CLA to decreased adiposity [72,73].

In contrast to dramatic body fat reduction in mice, human studies with CLA show none to minimal effects on markers of body fatness such as body mass index (BMI), body weight, body fat mass (BFM), abdominal adiposity, and/or lean body mass (LBM) [13,28,31,46,47,56,74].

Three meta-analyses of clinical trials all concluded that CLA supplementation resulted in mild, although significant, changes in body weight or body fat in humans [28,31,74]. The major differences among energy metabolism, doses and durations used, and experimental design, including dietary regimens, may have contributed to reduced effectiveness of CLA in humans compared to rodents [13,46,69]. However, several human studies reported the combination of CLA and other supplements, exercise, or different delivery methods improved the efficacy of CLA in body fat regulation [75–78]. Overall, CLA may not be an effective weight loss tool by itself in humans; however, there is potential for CLA to support weight management in conjunction with current efforts to control obesity.

D. Improved Muscle Metabolism and Exercise Outcome by CLA

Animal studies reporting reduction of body fat show significant improvement of overall body protein mass (as representative of muscle mass) [4,46,69]. Although earlier data were presented as percentage of total body composition, total protein contents from a number of animal studies were reanalyzed and confirmed significant improvement of total body protein in animals [4]. CLA also prevented age-associated skeletal muscle loss [46,69,79,80] and the changes in body protein by CLA followed by changes in body fat [41]. Moreover, CLA-fed animals had increased voluntary activity and improved endurance capacity [70,81–84]. As the skeletal muscle is about 40% of the total body weight and is a significant contributor to overall energy metabolism, the previously listed effects of CLA suggest that CLA modulates skeletal muscle metabolism [85].

During energy demand, AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1, a conserved nicotinamide adenine dinucleotide $[NAD]^+$-dependent deacetylase) mediate the cellular responses to provide proper energy in the skeletal muscle [86–88]. Few reports indicate that CLA modulates AMPK and SIRT1 in the skeletal muscle [89–92]. In addition, effects of CLA on peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), one of the major downstream markers of AMPK and SIRT2 and a primary regulator of mitochondrial biogenesis, have been inconsistent [89–95]. In addition to these cellular changes described earlier, the muscle fiber type changes as an adaptive muscle response to physiological and metabolic demands, which is potentially mediated through peroxisome proliferator-activated receptor-δ (PPARδ) [96–98]. CLA supplementation has been previously linked to increased oxidative slow-twitch type I fibers, but not glycolytic fast-twitch type IIb and IIx fibers in pig skeletal muscle [99,100], although effects of CLA on PPARδ are still inconclusive [83,89,95]. In addition, there have been reports of increased voluntary activity and/or endurance capacity in animals following CLA supplementation, particularly associated with the *trans*-10,*cis*-12 isomer [81,83,84,101].

Table 26.3 summarizes clinical CLA trials with exercise outcome. Overall, there is a trend indicating that CLA supplementation improves resistance exercise outcome better than endurance exercise outcome, although studies are relatively limited and inconsistent. Most of the studies in Table 26.3 used a relatively short time, lesser than 12 weeks, of supplementation, except one (6 months). Thus, it is not clear whether the minimal effect of CLA on exercise outcome is due to short-term study design as previously suggested [28,31,74]. It is important to note that CLA may be used in conjunction with other supplements to improve the efficacy of muscle metabolism and exercise outcome [76,77,102,103]. It is also interesting that CLA increased testosterone levels, which can be linked to the effect of CLA on the earlier mentioned muscle metabolism or exercise outcome [104,105]. Overall, there are limited reports on CLA and muscle metabolism; however, these effects of CLA may be important to understand its effect on overall changes in body composition.

E. Improved Bone Health by CLA

CLA has been reported to improve bone health in various animals, including old and ovariectomized animal models, although not consistently [13,56,106–110]. This inconsistency has been suggested to be, in part, associated with the interaction of CLA with calcium [111–115]. In addition,

TABLE 26.3
Summary of Human CLA Studies with Exercise Outcome

Reference	Subjects and Exercise Type	CLA Dose (g/Day) and Duration	Co-supplementation	Results[a]	Note[a]
Blankson et al. [194]	Overweight/obese and standardized training	1.7/3.5/5.1/6.8 for 6 and 12 weeks	N/A	↑ Training time (5.1 g for light and 6.8 g for intensive training)	↓ BFM ↑ LBM (6.8 g) No change BMI and BW
Kreider et al. [119]	Normal/overweight and resistance	6 for 4 weeks	N/A	No change in strength No change in creatine kinase (muscle catabolism marker)	No change BW, BFM, and LBM
Colakoglu et al. [195]	Normal and endurance	3.6 for 6 weeks	N/A	No change in running velocities corresponding to 65% and 80% of heart rate reserve (V-HRR65, V-HRR80) No change in 30 min running performance (V-30 min)	↓ BMI and BW w/exercise ↓ BFM and waist ↑ LBM
Pinkoski et al. [78]	Normal/overweight and resistant	5 for 7 weeks	N/A	↑ Bench press in men No change in leg press and knee extension torque	↓ BFM ↑ LBM No change in RMR
Tarnopolsky et al. [76,102]	Normal/overweight and resistance older subjects (>65 years)	5.4 for 6 months	5 g creatine monohydrate	↑ Capacity and strength ↑ Various measures of endurance	No change in BMI and BW ↓ BFM ↑ LBM
Diaz et al. [103]	Overweight/obese and endurance	1.8 for 12 weeks	0.4 mg chromium picolinate	No change in peak oxygen consumption (VO_2)	No change in BW, BFM, and LBM
Cornish et al. [77]	Obese and resistance	4.3 for 5 weeks	9 g creatine monohydrate and 36 g whey protein	↑ Bench press and leg press strength	No change in BMI, BW, and BFM ↑ LBM
Macaluso et al. [104]	Normal/overweight and resistance	4.8 for 3 weeks	N/A	No change in training volume (leg press, curl, extension, lat pull-down, bench and shoulder press, barbell bicep curl, supine triceps extension, and total volume)	No change in BFM and LBM
Jenkins et al. [59,196]	Normal/overweight and endurance	5.63 for 6 weeks	N/A	No change in peak oxygen consumption (VO_2), gas exchange threshold, respiratory compensation point, and fatigue threshold	

Abbreviations: BFM, body fat mass; BMI, body mass index; BW, body weight; LBM, lean body mass; RMR, resting metabolic rate; V-HRR65 and V-HRR80, running velocities corresponding to 65% and 80%, respectively, of heart rate reserve.

[a] ↑, increase; ↓, decrease.

CLA has been reported to improve osteoblastogenesis (bone formation) and to decrease osteoclastogenesis (bone resorption), which are linked to its ability to inhibit adipogenesis of the bone marrow mesenchymal stem cells [106–108,116–118].

Reports of the effects of CLA and human bone health are limited and have been summarized previously [13]. Most of them reported no effects of CLA on markers of bone health, except two [119,120]. Kreider et al. [119] observed a nonsignificant increase in overall bone mass due to CLA, but this study included resistance training, which may have contributed to the outcome of CLA as seen in a mouse study [121]. Another study by Brownbill et al. [120] is a cross-sectional study of dietary CLA in postmenopausal women, and they reported the positive role of dietary CLA intake on bone mineral density. The subjects in this study were likely taking dietary calcium supplements, which may be one of the important factors for the beneficial effects of CLA on bone health [114,115,122]. It is also important to note that the study by Brownbill et al. [120] primarily targeted the *cis*-9,*trans*-11 isomer from natural food sources. It was previously reported that the *trans*-10,*cis*-12 CLA is responsible for improving bone health by modulating osteoblastogenesis and/or osteoclastogenesis, while the *cis*-9,*trans*-11 isomer may contribute to improve certain types of calcium transport along with the *trans*-10,*cis*-12 isomer [108,114,116]. Thus, further studies analyzing the effect of CLA on bone health are needed, but inclusion of calcium intake (including supplementation) and degree of resistance exercise should be carefully evaluated to determine the role of CLA in bone health.

F. Modulating Immune and Inflammatory Responses by CLA

One of the earlier reports of CLA's bioactivity was to determine if CLA reduced adverse effects of immune challenge in animals [123,124]. Since then, a number of publications reported various effects of CLA on immune as well as inflammatory responses [13,46,125–127]. In human studies, effects of CLA on symptoms of allergy, atopic dermatitis, rhinovirus exposure, antibody production, or serum inflammatory markers including subjects with rheumatoid arthritis have been reported [13,47,128–133]. Overall, there are minimal benefits of CLA on immune and inflammatory responses in humans, while others reported that CLA is associated with reduced symptoms of allergy or cold, improved antibody production after hepatitis B vaccination, and reduced inflammatory responses associated with *Helicobacter pylori* infection in gastric epithelial cells [134–137].

Along with increased interest in gut health and reports of CLA and inflammatory bowel disease in animal models, potential applications of CLA in the treatment of inflammatory bowel diseases have been explored [133,138,139]. Currently, approximately 1.4 million Americans are presented with inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, but treatment options for these diseases are rather limited [140]. CLA supplementation is linked to decreased Crohn's disease activity index and improved quality of life, which corresponded with decreased serum inflammatory cytokines [133]. Similar effects were reported after ingestion of CLA-enriched butter (thus, primarily the *cis*-9,*trans*-11 isomer) [131]. In addition, based on the facts that intestinal gut microbiota play a critical role in inflammatory bowel disease progression and CLA ingestion alters the microbiota composition [141–143], CLA may have a positive effect on gut health via microbiota-mediated mechanisms. Though there are only two human reports of CLA and colorectal cancer (Table 26.2), based on the positive association between inflammatory bowel disease and colorectal cancer [144], understanding the role of CLA in inflammatory bowel disease may further extend its potential benefit to prevent the development of colorectal cancer.

VI. POTENTIAL ADVERSE EFFECTS OF CLA

Even though CLA presents a number of health benefits as described in the previous section, CLA supplementation also presents concerning adverse effects such as impaired glucose homeostasis, increased oxidative stress markers, liver dysfunction, and milk fat depression.

A. Impaired Glucose Homeostasis

Effects of CLA supplementation on glucose homeostasis have been controversial in both animal and human studies [13,46,47,56,69]. Most clinical studies reported no change in glucose or insulin levels after CLA supplementation, including long-term studies (longer than 6 months up to 2 years) [13,29,30,56,145].

The role of the two major isomers has been studied for their potential contribution to glucose homeostasis. The *trans*-10,*cis*-12 CLA isomer is linked to insulin resistance [146,147], while the *cis*-9,*trans*-11 isomer is not [13,148–151]. Pronounced effects of *trans*-10,*cis*-12 CLA on lipid metabolism are suggested to be associated with its negative effects on glucose homeostasis [152–154]. It is important to point out that when CLA-mixture (1:1 ratio of these two isomers) was used, no significant effects of CLA on glucose homeostasis were observed suggesting that the *cis*-9,*trans*-11 isomer may antagonize the adverse effects of the *trans*-10,*cis*-12 CLA isomer on glucose homeostasis [13,146]. Consistently, Castro-Webb et al. [42] reported decreased risk of diabetes and the adipose tissue levels of the *cis*-9,*trans*-11 isomer. Overall, application of CLA as a CLA-mixture is considered safe for glucose homeostasis.

B. Increased Oxidative Stress Markers

CLA supplementation has been consistently linked to increased isoprostanes, markers of oxidative stress, in human studies [13,56]. Isoprostanes, such as 8-iso-prostaglandin $F_{2\alpha}$ and 15-ketodihydro-$PGF_{2\alpha}$ from human serum or urine samples, are used as nonenzymatic and enzymatic oxidative stress markers, respectively. In contrast to these effects of CLA, other markers of oxidative stress such as oxidized LDL, serum malondialdehyde (MDA, a lipid peroxidation marker), or serum antioxidant concentrations were not altered with CLA supplementation in other human studies [61,132,155–160]. In addition, CLA itself can be metabolized to analogs of isoprostanes due to its structural similarity to ω-6 fatty acids; thus, increased isoprostanes may be a metabolic consequence of CLA supplementation rather than a representative of oxidative stress [161,162].

C. Liver Dysfunction

CLA supplementation, particularly the *trans*-10,*cis*-12 isomer, has been linked to enlarged liver, primarily as the form of nonalcoholic fatty liver, in mice [163–168]. It is reported to be an adaptive response to the effects of CLA on the tremendous mobilization of fat from adipose tissue along with increased hepatic lipogenesis, increased long-chain fatty acid uptake, and increased TG synthesis [165,166,168]. In animal studies, CLA's effect on the liver was reversible and, interestingly, CLA reduced high-fat diet-induced steatosis [169,170].

Human studies reported none-to-minimal changes in markers of the liver functions after CLA supplementation, which included a long-term study (6 months to 2 years) or a high dose of CLA (19.3 g CLA/day; 14.6 g *cis*-9,*trans*-11 and 4.7 g *trans*-10,*cis*-12) [13,29–31,53,60,64,171]. A recent meta-analysis of serum levels of liver enzymes and CLA supplementation concluded that CLA as a mixture, but not the *cis*-9,*trans*-11, is linked to increased serum alanine aminotransferase (AST) levels (approximately 0.224 U/L), while no association was found between CLA and aspartate aminotransferase (ALT) and alkaline phosphatase (ALP) [172]. Currently, three isolated human cases of CLA-associated hepatitis have been reported, which are summarized in Table 26.4. Based on these reports, it is likely that rodents may be more sensitive to CLA's effects in the liver; however, it is important to monitor the potential adverse effects of CLA on liver health by regularly monitoring serum markers of liver functions.

TABLE 26.4
Reports of CLA and Human Cases of Hepatitis

Reference	Subject	CLA Information	Symptoms: General	Symptoms: Examination[a]	Diagnosis	Treatment and Recovery
Ramos et al. [197]	46-year-old female	None, 14 days' supplementation	Asthenia, nausea, anorexia, jaundice, pruritus	• ↑ AST, ALT, GGT, total bilirubin, and conjugated bilirubin • Normal ALP; ultrasound and magnetic resonance cholangiopancreatography were normal • Biopsy showed hyperplasia of Kupffer cells and hydropic degeneration of hepatocytes	Toxic hepatitis	Stopped CLA supplement; 14 days post-CLA discontinuation, enzyme levels started to decline and returned to normal after 2 months
Nortadas and Barata [198]	63-year-old female	None, 1 month supplementation	Nausea, anorexia, jaundice, choluria for 3 weeks	• ↑ AST, ALT, ALP, GGT, total bilirubin, and conjugated bilirubin • Ultrasound and CT scan were normal; after 1 week of hospitalization, the patient developed hepatic encephalopathy and worsening cholestasis	Toxic hepatitis	Liver transplant; tissue showed hepatic parenchymal with hemorrhage and necrosis, septal mononuclear cell infiltration, massive hepatic necrosis
Bilal et al. [199]	26-year-old female, lost 50 lb in 6 months	None, 7 days' supplementation	Right upper quadrant abdominal pain, nausea, vomiting for 1 day	• ↑ AST, ALT, ALP (mild), GGT, and total bilirubin (mild); mild right upper quadrant abdominal tenderness, no jaundice, or hepatomegaly; ultrasound was normal; biopsy showed increase in sinusoidal macrophages and lymphocytes in the lobules, no fibrosis	Acute hepatitis	Discontinued CLA and general symptoms resolved the next day

Abbreviations: ALP, alkaline phosphatase; ALT, aspartate aminotransferase; AST, alanine aminotransferase; CLA, conjugated linoleic acid; CT, computed tomography; GGT, γ-glutamyl transferase.

[a] ↑, increase.

D. Milk Fat Depression

Similar to its effect on body fat reduction, CLA reduced milk fat contents, particularly in cow's milk [173,174]. This effect of CLA may have significant implications for infant health; thus, careful evaluation is needed. However, it is known that human milk fat is primarily derived from the diet, while cow's milk fat is mainly derived from *de novo* fatty acid synthesis, which is significantly influenced by CLA [173–175]. Thus, it is likely that CLA's effect on milk fat depression is greater in cows compared to that in humans.

Current reports on the effects of CLA on human milk fat content are limited. Only four studies have been published with a relatively short supplementation period of 5 days [176–179]. One publication reported significant reduction of milk fat with CLA supplementation, although within the normal milk fat range (about 3% in placebo vs. about 2.3% in CLA group) [176]. The other three publications reported no reduction of milk fat after CLA supplementation [177–179], including one study using the *cis*-9,*trans*-11 CLA isomer, which is known to not influence fat levels as discussed in the earlier section [11]. Thus, it is difficult to draw any conclusions regarding the potential effects of CLA on human milk fat content eventually influencing the health of infants based on these limited reports.

VII. POTENTIAL APPLICATIONS OF CLA TO IMPROVE AGE-ASSOCIATED HEALTH ISSUES

Based on the described bioactivities of CLA, there is great potential that CLA can be used for age-associated health issues, particularly for menopause, sarcopenia, and sarcopenic obesity. At menopause, the significant decline of estrogen production is linked to a number of major health issues such as weight gain, osteoporosis, and metabolic disorders [180]. CLA reduced body fat in ovariectomized mice, suggesting that CLA may be used to prevent menopause-associated weight gain in animals [80,109,181]. Similarly, previous animal studies indicated that CLA reduced bone loss associated with aging or ovariectomy [106,109,121]. In fact, mild, but significant, beneficial effects of CLA supplementation on body fat and bone mineral density were reported in postmenopausal subjects [120,182,183]. Moreover, CLA may influence the estrogen receptor signaling pathway, thereby negatively influencing estrogen receptor-mediated breast cancer development, which further strengthens the benefits of CLA with regard to health of women [184].

In addition, sarcopenia and sarcopenic obesity are significant health concerns for the aged population [185]. Sarcopenia is a condition with significant loss of skeletal muscle and, often, sarcopenia is accompanied with obesity, defined as sarcopenic obesity [185]. It is estimated that approximately 50% of the population over 80 years of age have sarcopenia or sarcopenic obesity [186]. Based on animal studies reporting that CLA prevented age-associated muscle loss and body fat gain, CLA may have great potential to prevent sarcopenia and sarcopenic obesity [70,79,80]. Among published human studies, Tarnopolsky et al. [76] reported that CLA with creatine monohydrate reduced BFM and increased lean mass in older subjects. However, others reported no benefits from CLA intake with regard to body fat in older men (average age of 56 years) [187]. Taken together, these results suggest CLA may be helpful for managing age-associated health issues along with current efforts to control these conditions.

VIII. CONCLUSIONS

In almost 30 years since the first report of CLA's anticancer properties, many of CLA's various roles in biological systems have been explored. As a relatively simple-structured fatty acid, it is still not clear how CLA exerts its various biological effects. With the existence of various isomers, it is possible that CLA isomers may have various cellular and molecular targets. Thus, more studies are warranted to uncover the exact mechanism(s) of CLA for efficient applications to improve human health.

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27 Dietary Fats and Obesity

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CONTENTS

I. INTRODUCTION

Overweight and obesity are defined by the World Health Organization as having abnormal or excessive body fat accumulation that may impair health, with obesity further classified based on having a body mass index (weight [kg]/height [m]^2) of 30 or more [1]. Obesity is associated with many chronic diseases and alterations in physiological function, including cardiovascular disease, hypertension, diabetes mellitus, gallbladder disease, osteoarthritis, and certain types of cancer [2]. Obesity is characterized as a major public health problem in the United States and Europe and is designated as a disease by the American Medical Association, although the designation is still a matter of debate [3]. According to recent estimates (2011–2014), the prevalence of obesity among U.S. adults was 36.5% overall, with higher prevalence reported for Hispanic (42.5%) and non-Hispanic Black (48.1%) adults [4]. Obesity is also an emerging worldwide health concern, with the prevalence of obesity doubling since 1980 to reach 600 million people (13% of the world's adult population) in 2014 [1].

The etiology of human obesity is quite complex, involving genetic, metabolic, behavioral, and environmental factors. Although obesity is believed to have a strong genetic component [5], it is

recognized that genetic, epigenetic, and evolutionary influences together provide a template against which lifestyle and environmental insults operate in the development of the disease [6]. Among dietary factors, both total energy intake and fat intake have been correlated with increases in BMI during obesity development in some population groups [7]. However, increased intake of fat energy is associated with a greater per unit increase in body mass than is increased intake of energy from nonfat sources. Therefore, much attention regarding dietary influences on obesity development or prevention has focused dietary fat, both quantity and quality [8].

II. DIETARY FAT AND BODY WEIGHT

The effect of dietary fat on body weight has been intensively studied, and in experimental animals, it generally has been shown to be obesogenic. In humans, however, despite thorough investigation, significant debate still exists regarding dietary fat and energy balance.

The literature in this area has been thoroughly reviewed [9–11], and for the most part, dietary fat at sufficient levels results in increased body weight. A genetic component to this effect is illustrated by studies with animals that are either resistant or susceptible to developing obesity when eating a high-fat diet [12]. The caloric density of the diet plays a major role in promoting weight gain. If fat density is about 25% or more, excess weight gain is more likely to occur [13]. In addition, younger rats may exhibit greater responses than mature animals [14]. Multiple mechanisms have been proposed to explain the obesogenic effect of high-fat feeding in experimental animals, including impaired regulation of glucose and insulin homeostasis [15,16], increased parasympathetic activity [14,17,18], and decreased sensitivity to central insulin and leptin [19]. The endocannabinoid system has also been implicated in the effect of dietary fat on body weight [20,21].

Human studies, because they are more difficult to control, have reported conflicting results. Lissner et al. [22] demonstrated that subjects consuming a high-fat diet gained weight, whereas those eating a low-fat diet lost weight. Others [23,24] have reported that switching from a high- to a low-fat diet promotes relative weight loss. Indirect evidence provided from epidemiologic studies shows positive correlations between body weight and fat intake [25]. The Leeds Fat Study [26] reported that, of individuals consuming high-fat versus low-fat foods, there were 19 times more individuals classified as obese in the high-fat group. When Japanese men living in Honolulu were compared with those living in Hiroshima and Nagasaki, the prevalence of obesity was greater among the men in Honolulu than those in Japan, presumably due to the higher level of fat in the Honolulu diet [27]. More recently, a meta-analysis of 32 randomized controlled trials and 25 cohort studies showed a small but stable effect of lower fat intake on decreased body weight in children and adults [28].

Conversely, in some randomized controlled studies, short-term weight loss has been greater in obese subjects who were advised to restrict carbohydrate rather than fat intake [29–32]. For example, Shai et al. [32] followed participants for 2 years who were on a low-carbohydrate, low-fat, or Mediterranean diet and reported the greatest weight loss for the low-carbohydrate group. A recent meta-analysis of randomized controlled trials supports the benefit of a low-carbohydrate over a low-fat diet [33]. In a comprehensive review, Shikany et al. [34] reported that, under isocaloric but caloric-deficient conditions, increasing dietary fat leads to decreased body weight. When diets are isocaloric or hypercaloric, altering dietary fat has no effect on body weight. However, when subjects have unlimited access to high- or low-fat diets, body weight is decreased when subjects are told to consume a low-fat diet. The effects of type of fat on energy balance have also been examined with varying results [35–38].

These conflicting results are due in part to the fact that obesity is a complex disease, with many contributing factors. In addition, even randomized controlled trials are not fully controlled and cannot account for subjects' lack of compliance or inaccuracies in self-report data. Comparisons across countries, where dietary fat intake is correlated to obesity rates, cannot account for the multitude of other contributing factors such as food availability, activity, genetics, and culture.

In an attempt to unequivocally determine if dietary fat enhances body weight to a greater degree than other macronutrients, investigators have utilized metabolic wards where subjects are confined and diet composition and adherence can be well controlled. Previous studies of in-patient adults who are given isocaloric diets varying in macronutrient composition have been reported (for review, see Reference 39) and many show a decrease in body weight with a decrease in dietary fat. In a very recent report, Hall et al. [39] studied adults who were confined to a metabolic ward for two 2-week periods and placed subjects, in a cross-over design, on either a low-carbohydrate or low-fat diet. Subjects in the low-carbohydrate group lost more body weight but less body fat than those in the low-fat group, as measured by metabolic balance. Other studies in metabolic wards show that low-carbohydrate diets lead to greater weight losses than low-fat diets do, but this loss has been attributed to decreases in water loss or protein loss [39].

Whether people living freely in society will lose body weight or body fat on a low-fat diet is difficult to determine, as weight or fat loss is a product of many factors, which vary between individuals. Thus, studies that compare populations or even randomized controlled trials will likely have conflicting results regarding dietary macronutrient content and body weight or body composition.

III. DIETARY FAT AND FOOD INTAKE

A. Does Dietary Fat Affect Food Intake?

Excessive calorie intake without compensatory energy expenditure will result in positive energy balance and weight gain. Whether or not dietary fat leads to overconsumption and thus weight gain is still debated, but certain circumstances have been shown to couple dietary fat with increases in body weight. Studies designed to investigate the effect of dietary fat on food intake generally employ one of two approaches: short-term studies examining the influence of dietary fat on meal size or frequency and long-term investigations of the effects of dietary fat on energy intake over days or weeks. Short-term effects are typically examined in one of two ways: (1) fat is given as a preload, and subsequent feelings of hunger or food intake are reported or (2) fat is given as part of a mixed diet and concurrent food intake is measured. In the first instance, satiety, that is, the ability of a substance to suppress further eating, is measured by the time elapsed or amount of food eaten at the next meal. In the second case, satiation is assessed and is defined as the size of the current meal [40].

Many studies have been carried out examining the effect of a preload, often in the form of a liquid on subsequent intake, typically over a short time period. Others have investigated the effect of high- or low-fat meals or snacks, or meals supplemented with fat or carbohydrate, on subsequent feeling of fullness and food intake. The results of these studies have been quite variable (for review, see Blundell et al. [41]). Many reports indicate that with a preload, individuals do not fully compensate for the calories ingested, that is, they do not reduce their intake in accurate proportion to the calories previously consumed [41–45]. This seems to be truer for fat versus carbohydrate preloads [44–50], though some studies have indicated that fat has satiety value equal to carbohydrate [51,52] or even greater than carbohydrate [53,54]. In addition, compensation seems to occur initially but then decreases over time [51–53].

It is important that the volume, sensory characteristics, and protein content of the preloads be similar when investigating the satiety effect of fat. A number of studies have not controlled for all of these factors [43,50,55,56]. However, when preloads are similar, investigators have found that individuals vary substantially in their response to preloads and that body weight may play a role in these responses. For example, some have reported that males who are of normal weight and not concerned about their body weight or food intake (unrestrained eaters) appear to compensate adequately for the caloric content of a preload [50]. Porrini et al. [57] report that a high-protein food given as a snack 2 h before a meal exerts a higher effect on both intrameal satiation and postingestive satiety than a high-fat snack. When a first course is consumed as part of a meal, the

sensory characteristics of the food play an important role in controlling subsequent food intake. Lawton et al. [46] have shown that in obese individuals, fat exerts only a weak action on satiety. Data such as these have led to the speculation that obesity may be the result of insensitivity to satiety signals generated by ingestion of fat.

In studies of satiation, dietary fat is an integral variable of the test diets. Studies show that caloric intake is greater with dietary fat than with carbohydrate. When subjects are fed diets in which fat, fiber, and simple sugars are manipulated to obtain low-energy versus high-energy diets, energy intake is greater on the high-energy diet than the low-energy diet [58]. Caputo and Mattes [59] reported that individuals consuming high-fat meals consume more calories than those consuming high- or low-carbohydrate or low-fat meals. Brennan et al. [60] reported that after a high-protein, high-fat, or a high-carbohydrate lunch, both hunger and calorie intake were higher after the high-carbohydrate lunch compared to the high-fat or high-protein lunch. Thomas et al. [61] have shown that individuals consuming high-fat or high-carbohydrate diets for 1 week consume more calories on the high-fat versus high-carbohydrate diets. This hyperphagic effect of dietary fat has been observed in many studies [62–67].

The excessive intake of dietary fat primarily occurs during a meal to increase meal size rather than between meals to increase meal frequency [64]. Overconsumption is particularly high when fat is combined with alcohol [68]. Studies indicate that palatability and energy density of high-fat diets play an important role in overfeeding; however, other factors may also influence this behavior [69]. Over the long term, many investigators have shown that this passive overconsumption of dietary fat can lead to obesity (for review, see References 12, 64, 70, and 71).

Relatively few long-term studies on humans have been conducted because of difficulties such as inability of the investigator to control for the subjects' current or past food intake, activity level, or genetic background. However, Kendall et al. [23] reported that women consuming either a low- or high-fat diet for two separate 11-week periods consume more calories on the high-fat diet. Lissner et al. [22] also showed that subjects eating a low-, medium-, or a high-fat diet for 2 weeks consume the most calories on the high-fat diet.

In addition, animal studies have shown that the type of fat may influence the satiation effects of dietary fat. In both chickens [72] and rats [73], medium-chain triglycerides have been shown to have a greater satiating effect than long-chain triglycerides. However, when medium- or long-chain fatty acids were infused into the hepatic portal vein, the medium-chain fatty acids had no effect on feeding, whereas the long-chain fatty acids robustly inhibited feeding [74]. Nonetheless, many studies with rodent models have shown that a preference for dietary fat occurs when texture, olfaction, and postingestive cues are controlled (for review, see Reference 75). Pittman et al. [75] have suggested that orosensory cues play a major role in dietary fat preference in rats. In humans, Rolls et al. [76] and Stubbs and Harbron [77] report that substitution of long-chain triglycerides with medium-chain triglycerides depresses food intake. In addition, the physical form as well as the type of fat contributes to its satiation effects [53,78]. Overeating occurs with diets containing saturated fats [79–82] as well as mixed fats [83–86]. Lawton et al. [87] showed that in human subjects, polyunsaturated fatty acids (PUFAs) may exert a stronger control over appetite than monounsaturated fatty acids (MUFAs) or saturated fatty acids (SFAs). Kozimor et al. [88] reported that liquid meals rich in SFAs produce a stronger feeling of satiety compared to PUFA- or MUFA-rich meals in normal weight women. However, others have reported no differences in an SFA or MUFA fat load on postprandial satiety scores or the time to next meal [89]. Others have also failed to find an effect of fatty acid saturation on postprandial satiety [90] or food intake [91–94].

Some have suggested that the hyperphagic effect of high-fat diets is not due solely to the fat but is also influenced by the presence of carbohydrate and overall caloric density [95]. Ramirez and Friedman [95] fed rats diets varying in carbohydrate, fat, cellulose, or caloric density and found that energy intake varies directly as a function of caloric density regardless of the fat or cellulose content of the diets. They concluded that high levels of fat, carbohydrate, and energy interact to produce overeating in animals fed high-fat diets. In support of this hypothesis, Emmet and Heaton [96]

examined food records from 160 subjects who had weighed their food for 4 days. They reported that an increase in refined sugar intake is associated with a linear increase in the intake of fat combined with carbohydrate. Others [97] demonstrated that allowing free access to sugar as a separate choice, along with the high fat, is important for the hyperphagic effect. This suggests that refined sugar may act as a vehicle for fat intake by increasing fat palatability.

Some have reported that there is an inverse relationship between consumption of sugar and fat, terming it the "see-saw" effect. This suggests that carbohydrates, per se, are protective against obesity, as sugars may displace fat energy from the diet [98–100]. When consumption of fat and sugar is expressed as a percentage of total intake, this inverse relationship is present. However, when expressed in absolute terms, there is a positive relationship between dietary fat and carbohydrate intake [101]. When high-fat and high-carbohydrate diets of equal caloric density are compared, both contribute to increased, uncompensated caloric intake [71].

B. Mechanisms for Fat-Induced Food Intake

1. Caloric Density

The higher caloric density of many high-fat diets may play a role in inducing a hyperphagic response [63,64,102]. In humans, Duncan et al. [58] reported that adult subjects eat almost twice as many calories on a high-density diet compared with a low-density diet. In studies using experimental animals, hyperphagia is typically observed only when the caloric density of the diet is high, greater than approximately 5.8 kcal/g (for review, see Warwick and Schiffman [9] and Geary [103]). In addition, when caloric density is constant, rats fed a diet high in corn oil have a caloric intake similar to that of animals fed low-fat diets [99,104]. When the energy density of high-fat diets is reduced by adding nonnutritive fillers, energy intake and body weight typically do not increase (for review, see Geary [103]). However, a more recent review [105] reports that rats given a nonnutritive fat, Olestra, will compensate with an increase consumption to account for the loss in calories, while humans do not.

Several investigators have reported that a preference develops for a flavor that is paired with a high number of calories versus one paired with a low number of calories. A study by Johnson et al. [106] indicated that children report increased flavor pleasantness because of association with a high density of fat calories. In rats, a flavor associated with corn oil consumption is preferred over a flavor that is not paired with oil [107,108]. In studies in which oils or high-fat foods are given, animals often initially do not consume greater quantities of the fat. However, over time the rats do consume more of the fat as they learn about the associated postingestive consequences (i.e., greater caloric value) [109–113]. In contrast, others have suggested that dietary fat is overconsumed even when compared with an isoenergetic carbohydrate diet of similar palatability [69,114,115]. Furthermore, Lucas et al. [116] report that, relative to an isocaloric high-carbohydrate diet, the postingestive effect of high-fat diets stimulates overeating and conditions a stronger flavor preference in rats, suggesting that some quality in fat per se may be inducing intake.

2. Stomach Distention

Dietary fat's effect on stomach distention may also account for its hyperphagic effect [9]. Warwick and Schiffman [9] suggested that due to the greater caloric density of dietary fat, a high-fat meal has a smaller volume than an isocaloric high-carbohydrate meal, resulting in less stomach distention. This lesser distention would lead to an attenuation of satiety signals. In addition, Cunningham et al. [117] reported that the rate of stomach emptying increases when a high-fat diet is habitually consumed. When the energy density of high-fat diets is reduced by adding noncaloric fillers to rats, the hyperphagia and weight gain do not occur [82, 118–121]. Similar effects have been reported in humans [122,123]. It has been suggested that distention of the stomach by fat is simply a volumetric signal, which sends a neural signal to the central nervous system via the nucleus of the solitary tract [124].

Dietary fat has also been found to influence relative consumption of carbohydrate and protein. Crane and Greenwood [125] allowed rats to select from either high-carbohydrate or high-protein diets. Half of the diets contained 20% soybean oil as the fat component and the other half contained 20% lard as the fat component. Animals that selected from the soybean oil diets consumed more carbohydrate and less protein than those that chose from the lard-based diets. Grossman et al. [126] further showed that rats gavaged with either beef tallow or corn oil 2 h before selecting from either high-carbohydrate or high-protein diets consume less carbohydrate and more protein if given tallow versus corn oil. They [126] also demonstrated that the hepatic vagus must be intact for this selection to occur and that mercaptoacetate (a fatty acid oxidation inhibitor) can blunt the effect.

3. Metabolic Signals

In an attempt to understand the mechanisms underlying the influence of dietary fat on appetite, investigators have examined its absorptive and postabsorptive effects. If fat absorption is inhibited, then fat's effects on promoting body weight and oxygen consumption are attenuated [127]. Hydrolysis of lipids in the intestine appears to be essential in order for fat to induce satiety. For example, studies using Orlistat, a lipase inhibitor, abolished the satiating effects of fat infusion into the small intestine of humans [128,129]. Gut epithelial cells exhibit a wide variety of fatty acid–sensing receptors, mainly of the G protein–coupled receptor type, which have been found in the gut as well as orally [130,131]. In studies in rodents in which the receptor is missing, oral signaling to the CNS is decreased, implying a role for fatty acid signaling in mediating fat's influence on food intake [132,133]. The receptor CD36 has been shown to play a role in fat absorption in the small intestine and is essential for the production of oleoylethanolamine (OEA). OEA is a lipid messenger and is produced from oleic acid as a result of intestinal exposure to fat. The mobilization of OEA from the mucosa suppresses food intake via the activation of the nuclear receptor, PPAR-α [134,135]. Romano et al. [136] have shown that rats fed a high-fat diet are more sensitive to the hypophagic action of OEA, and this may be due to decreased sensitivity to vagal afferent fibers. Multiple other receptors have been reported and their signaling peptides identified [137,138].

Studies in rodents and humans have shown that a diet rich in diacylglycerols increases fatty acid oxidation and decreases food intake (for review, see Rudkowska et al. [139]). Scharrer and Langhans [140] demonstrated that, in rats, consumption of a high-fat diet can be stimulated by inhibiting fatty acid oxidation with 2-mercaptoacetate. Friedman et al. [141] also reported a stimulation of food intake when fatty acid oxidation is inhibited with methyl palmoxirate. This feeding effect, labeled lipoprivic feeding, has been shown to be impaired by hepatic vagotomy [142] and subdiaphragmatic vagotomy [143]. In addition, Ritter and Taylor [144] reported that capsaicin can block this effect, implying that vagal sensory neurons appear to be involved in lipoprivic feeding. Studies utilizing brain lesions indicate that lipoprivic feeding involves the lateral parabrachial nucleus and possibly the area postrema/nucleus of the solitary tract [145]. Type of fat may influence this response as Wang et al. [146] reported that food intake is stimulated by mercaptoacetate in rats given corn oil but not tallow diets. Finally, Suzuki et al. [147] have shown that rats given mercaptoacetate exhibit reinforcing and palatability effects to sucrose, but not to corn oil, suggesting postingestive energy signals play a role in these behaviors.

Other investigators have examined aspects of fat metabolism as potential satiety signals by administering fat or fat metabolites centrally or peripherally. Central administration of oleic acid inhibits food intake, though shorter-chain fatty acids do not have this effect [148]. Some evidence suggests that malonyl-CoA and long-chain fatty acid CoA are fuel sensors in the hypothalamus, which help regulate feeding behavior [149]. However, in both animal and human studies, medium-chain triglycerides typically decrease food intake more than long-chain triglycerides (for review, see Jambor de Sousa et al. [74]). Arase et al. [150] reported that intracerebroventricular infusions of the four-carbon β-hydroxybutyrate (β-OHB) reduce food intake in Sprague–Dawley or Osborne–Mendel rats consuming either a high- or a low-fat diet. However, such infusions do not reduce food intake in S5B/PI rats that are resistant to weight gain when consuming high-fat diets. Peripheral injections

of β-OHB decrease food intake in S5B/PI rats but not Osborne–Mendel animals, and glycerol has no effect in either strain [151]. Peripheral injections of glycerol in Wistar rats [152] and β-OHB in Sprague–Dawley rats [153] also decrease food intake. In addition, central administration of inhibitors of fat synthesis results in decreased food intake in rodents [154,155].

Fat, when infused into the intestine, can suppress hunger, induce satiety, or delay gastric emptying [42,156–160]. Fat's effect on satiety is especially pronounced when infused into the ileum and has been termed the "ileal brake" [160]. However, as stated previously, dietary fat appears to be overconsumed and often can lead to obesity. The disparate effects of intraintestinal infusions of fat versus dietary fat have been termed the fat paradox [161]. It has been suggested that high-fat foods have high palatability and orosensory stimulation [162–164], leading to overconsumption before the nutrients can enter the intestine to generate satiety signals. Oral sensing of fat is influenced by texture and olfactory signals [165]. Pittman et al. [75] have shown that free fatty acids depolarize taste receptor cells and may increase concomitant tastants. In support of this hypothesis, satiety signals potentially arising from fat metabolism appear to be blunted in comparison with other nutrients. For example, it has been reported that carbohydrate and protein consumption is followed by an increase in their oxidation [121,166,167], whereas oxidation of fat is not generally stimulated until 3–7 days following consumption of a high-fat diet [168,169]. Furthermore, it has been reported that fat oxidation is especially limited in obese as compared with lean individuals [61,170].

4. Effects of Hormones and Pharmacological Agents

Use of hormones and pharmacological agents has further illuminated factors that may play a role in the effect of dietary fat on food intake. Pancreatic procolipase is a cofactor for lipase, an enzyme necessary for proper fat digestion. A pentapeptide produced by the cleavage of procolipase, Val–Pro–Asp–Pro–Arg, or enterostatin, has been shown to reduce food intake in rats [171], especially when consuming a high-fat diet [172]. Peripheral or intracarotid injection of enterostatin [173] or injection into the lateral ventricle [174] suppresses fat intake in fat-adapted rats, suggesting both a gastrointestinal (GI) site and a central site of action. In addition, high-fat feeding and cholecystokinin-8 (CCK-8) increase intestinal enterostatin levels [175]. Lin et al. [176] report that β-casomorphin 1–7 stimulates intake of a high-fat diet in rats, and this effect is inhibited by enterostatin or naloxone.

Much research has been focused on CCK and its effects on satiety (for review, see Smith and Gibbs [177] and Geary [178]). It has been well documented that consumption of fat stimulates the release of CCK from endocrine cells located in the proximal small intestine, activating receptors in the stomach. This signal is transmitted along the vagus to the nucleus of the solitary tract, where it is forwarded to the hypothalamus. Vagotomy can block these effects of systematically administered CCK [179], as can CCK-A receptor antagonists [180]. CCK is also produced in the central nervous system (reviewed in Beinfeld [181]) and is released from the hypothalamus during feeding [182,183]. CCK administered into the cerebral ventricles inhibits food intake in primates [184].

Morphine has also been shown to have specific effects on intake of dietary fat. Rats given morphine injections subsequently increase fat intake while suppressing carbohydrate intake when given separate sources of macronutrients [185–187] or mixed diets [188]. Continuous infusion of morphine also stimulates fat intake [189]. An opioid agonist, butorphanol, also increases consumption of a high-fat diet [86]. Administration of opioid antagonists suppresses fat intake with little effect on protein or carbohydrate intake [190,191]. In humans, opioids also appear to play a role in regulating fat intake. Opiate antagonists cause a decrease in intake of fat calories with less effect on carbohydrate consumption [192–194]. A diet high in fat and sucrose increases gene expression of the opioid, dynorphin in the arcuate nucleus [195]. It appears that intake of highly palatable foods affects opioid activity.

Corticosterone has also been implicated in the regulation of fat intake. Castonguay et al. [196,197] reported that adrenalectomy reduces total caloric intake in rats, particularly fat intake, and that corticosterone can restore the fat consumption. Dallman et al. [198] have shown that insulin must be present for corticosterone to stimulate fat intake. Devenport et al. [199] have reported that the

type 1 adrenocorticoid receptor mediates corticosterone's effect on fat appetite. Kumar et al. [200] have suggested that corticosterone acts to enhance carbohydrate rather than fat intake. It has been suggested [201] that the differing levels of micronutrients added to the diets in these studies may account for these disparate findings.

Investigators have also reported that the peptide galanin influences appetite for fat. Leibowitz [202] first demonstrated that galanin stimulates fat intake, especially at the end of the nocturnal cycle. Galanin is thought to work in concert with norepinephrine, which is colocalized with galanin in paraventricular neurons [203]. Smith et al. [204] report that centrally injected galanin induces fat intake only in fat-preferring rats, that is, baseline feeding preferences are important in determining the feeding response to galanin. A high-fat meal or increasing circulating lipids increases galanin in the paraventricular nucleus [205].

Leptin, the gene product of *ob* gene, is shown to regulate body fat in mice and is produced in human adipose tissue as well (for review, see Harris [206]). Reports in humans indicate that leptin is negatively associated with fat intake. Havel et al. [207] report that in women, high-fat/low-carbohydrate meals result in a lowering of 24-h circulating leptin concentration. Other research shows that there is a negative correlation between leptin levels and dietary fat (7-day records), when controlling for body weight [208]. Finally, Niskanen et al. [209] indicate that serum leptin concentrations in obese humans are inversely related to dietary fat intake. In mice, however, high-fat feeding or a high-fat diet increases serum leptin levels [210,211]. It has long been shown that high-fat feeding results in secretion of a protein, apo A-IV, by enterocytes, which in turn, can regulate secretion of triglycerides and inhibit food intake (for review, see Tso and Lui [212]). Doi et al. [213] have reported that increased levels of apo A-IV are attenuated by intravenous leptin infusions. Thus, it has been suggested that leptin may regulate fat-induced apo A-IV levels and food intake in animals.

Neuropeptide Y (NPY) increases intake of a preferred diet when given in the paraventricular nucleus or cerebral ventricles. When given in the amygdala to satiated rats, NPY causes a decrease in fat intake [214]. A meal rich in fat has been shown to induce the release of peptide YY (PYY) from the GI tract, which inhibits NPY/agouti-related peptide neurons and, at the same time, stimulates the proopiomelanocortin/cocaine and amphetamine regulated transcript neurons, all of which induces satiety [215–217]. Artmann et al. [218] have shown that, in rats, short-term feeding of diets resembling human diets can affect tissue levels of endocannabinoids. Neuromedin U in the PVN may also play a role in regulating preference of high-fat foods [219]. A variety of hypothalamic peptides and their dietary effects have been reviewed in the encyclopedic work of Leibowitz and Wortley [220].

IV. DIETARY FAT AND METABOLISM

Obesity is the final result of increased deposition of fat through increased de novo lipogenesis and increased fatty acid esterification relative to lipolysis and oxidation. In the following sections, the critical literature that characterizes the role of dietary lipid in altering these metabolic events will be reviewed.

A. Influence of Dietary Fat on Lipogenesis

Dietary lipid level influences the rate of lipogenesis. Early studies showed that de novo synthesis of fatty acids is decreased by high dietary lipid level [221,222]. Two key enzymes in the lipogenic pathway, fatty acid synthetase and acetyl-CoA carboxylase, are reduced in animals receiving a high-fat diet. In addition, the pentose phosphate pathway and malic enzyme, both of which provide reducing equivalents for de novo lipogenesis, are also influenced by dietary lipid level. Malic enzyme, the pentose phosphate pathway, and the rate-limiting enzyme in this pathway, glucose-6-phosphate dehydrogenase, are decreased in rats fed a diet containing high levels of dietary fat and increased in diets high in carbohydrate [223–225].

In experimental animals, the lipid content and composition of the diet can cause a shift in the source of stored lipid. On a diet rich in lipids, rat adipose tissue fatty acids come mainly from dietary fat, whereas on a high-carbohydrate diet the fatty acids come from hepatic lipogenesis [226]. In genetically obese rats, both hepatic and adipose tissue lipogenic rates are decreased by a high-fat diet. Nonetheless, the animals still deposit more fat because of the increased uptake of fatty acids from the diet. High-fat dietinduced inhibition of lipogenesis is also influenced by dietary fatty acid type. For example, unsaturated fatty acids inhibit de novo lipogenesis to a greater extent than do SFAs [227–229], with inhibitory effects further influenced by fatty acid chain length, degree of unsaturation, and double-bond location [230]. In addition, greater inhibition of lipogenesis is observed with *n*-3 as compared with *n*-6 PUFAs [225,231–233]. PUFA-mediated inhibition of lipogenesis may also be influenced by metabolic status as obese mice appear to be somewhat resistant to PUFA-induced feedback control of gene expression [234].

Mechanistic studies in experimental animals indicate that ingestion of *n*-6 or *n*-3 PUFA causes a rapid inhibition of the expression/activation of many enzymes involved in lipogenesis (see reviews by Jump and Clarke [224] and Clarke [225]) and a coordinate induction of genes encoding proteins involved in lipid oxidation and thermogenesis (discussed in Section IV.C). PUFA regulation of lipogenic genes is mediated at both the transcriptional level, as for pyruvate kinase, pyruvate dehydrogenase, acetyl-CoA carboxylase, and fatty acid synthase, as well as the posttranscriptional level, as for glucose-6-phosphate dehydrogenase (see reviews by Jump and Clarke [224] and Clarke [225]). Dietary PUFAs regulate hepatic lipogenesis by mediating changes in the expression and nuclear abundance of the transcription factor, sterol regulatory element-binding protein 1 (SREBP-1) [233–237], and by targeting other transcriptional regulatory networks (see review by Jump [237]). Emerging evidence indicates that PUFAs also influence hepatic lipogenesis upstream from cytosolic lipogenic processes by modulating the expression of the mitochondrial citrate carrier (CIC) through transcriptional and posttranscriptional mechanisms [238]. In experimental animal studies, both *n*-6 and *n*-3 PUFAs have been shown to be potent inhibitors of mitochondrial CIC with the degree of inhibition depending on the amount of added PUFA and length of dietary treatment [238]. In contrast, SFA and MUFA have no influence on mitochondrial CIC.

The effect of dietary fat on lipogenic processes in humans is not as well studied as de novo lipogenesis was believed to be an insignificant metabolic pathway particularly when consuming a Western, high-fat diet (for review, see Murphy [239]). Some studies in human subjects suggested a minor role of hepatic lipogenesis in energy balance but did not address the issue of extrahepatic lipogenesis (for review, see Hellerstein et al. [240]). In contrast, Chascione et al. [241], comparing subjects fed a high-carbohydrate diet with those fed a high-fat diet after a period of energy restriction, suggested that adipose tissue may account for up to 40% of whole-body lipogenesis.

Consistent with animal studies, lipogenesis in humans appears to be influenced differentially by the health status of the subjects as well as the quantity and quality of dietary fat [242–244]. Four to five times higher rates of de novo lipogenesis and higher fasting triacylglycerol concentrations have been reported for hyperinsulinemic obese subjects consuming a high-fat Western diet as compared with normoinsulinemic lean or obese subjects consuming the same diet [242]. Change to a low-fat, high-carbohydrate diet resulted in an increase in triacylglycerol concentrations that was correlated with increased fractional rates of de novo lipogenesis in both normoinsulinemic lean and hyperinsulinemic obese subjects. A recent randomized controlled trial demonstrated that 12-week consumption of a low-fat high-carbohydrate diet (LFHCC) enhanced fasting expression of the lipogenic genes SREBP-1 and stearoyl-CoA desaturase D9-desaturase (SCD1) in adipose tissue of patients with metabolic syndrome, whereas supplementation of the diet with 1.24 g/day PUFA prevented this effect [243]. In skeletal muscle, both the expression of several lipogenic genes (SREBP-1 and ACC2) and tissue lipid content were reduced in insulin-resistant subjects after 12 weeks on an LFHCC diet supplemented with *n*-3 PUFA [244]. These positive changes in lipid metabolism did not, however, translate to changes in insulin sensitivity, which suggests that higher levels of PUFA supplementation or longer period of dietary

manipulation may be required to demonstrate the full potential of these fatty acids to modulate insulin resistance (see Section IV.E).

B. Influence of Dietary Fat on Lipid Uptake

Lipoprotein lipase (LPL) has been called the gate keeper enzyme because it controls the rate of uptake of lipid by adipose cells [245]. This enzyme acts primarily during the postprandial period to hydrolyze chylomicron and VLDL triglycerides, facilitating fatty acid uptake and decreasing circulating lipid concentrations. An interaction between dietary fat intake and LPL genotype on modifying HDL-cholesterol (HDL-C) concentrations has been reported, with greatest HDL-C observed in LPL GG homozygotes consuming lower total, saturated and monounsaturated fat intakes [246]. The action of LPL is also thought, at least by some, to account for the ability of *n*-3 PUFA to lower serum triglycerides. Increases in LPL gene expression [247] or activity are observed with *n*-3 fatty acid supplementation in both healthy and hypertriglyceridemic patients [247–249] and experimental animals [250,251]. In contrast, others observed either no effect of *n*-3 fatty acids on LPL activity in humans [252,253] or rats [254], or effects that were adipose depot specific [255]. In addition, LPL activity may not necessarily correspond to mRNA levels, as posttranscriptional events, such as glycosylation and binding of LPL to cell surface heparan sulfate proteoglycans, modulate expression and activity of the enzyme [256]. Furthermore, although alterations in LPL activity or expression may function in the lowering of serum lipid levels associated with fish oil (*n*-3 fatty acid) consumption, it is at odds with the finding of reduced adipose tissue mass in animals fed the same diet. Conversely, the reduced obesity associated with diets high in *n*-3 PUFA may be due to the influence of these fatty acids on reducing hepatic fatty acid synthetase activity [250,251,257] and stimulating fatty acid oxidation (see Section IV.C).

As recently reviewed [258], there is a net uptake of dietary fat into adipose tissue for about 5 h following a single test meal or throughout the day in a typical three-meal intake pattern. While the action of LPL increases steadily after meal intake, the uptake of fatty acids increases to a greater extent, suggesting that upregulation of fatty acid esterification pathways is also involved in postprandial lipid uptake [258,259]. Studies investigating uptake of different fatty acid species in a single test meal have found that adipose tissue did not discriminate between fatty acid type or positioning on the triacylglycerol molecule [258]. However, net uptake was proportional to the molar proportion of fatty acids in the test meal, and the rank order of uptake was MUFA > *n*-6 PUFA > SAT > *n*-3 PUFA [258]. A greater uptake and storage of oleate (a monounsaturated fat) from a test meal, which was suggested to be due to a greater availability of this fatty acid fraction, was also observed in stable isotope tracer studies [258].

Obesity is associated with a delayed plasma clearance and reduced adipose tissue uptake of fat from the diet or test meals [reviewed in References 258 and 260]. Reduced adipose tissue uptake of dietary fat is accounted for by both a lower and less responsive rate of adipose tissue blood flow and a reduced rate of action of LPL in obese as compared to lean individuals [258]. The reduction in LPL action may be further explained by both a lower expression of the enzyme [258] and increased inhibition from apolipoprotein C-I [260] in obese individuals. Decreased efficiency of adipose tissue fat uptake and storage in obesity and/or with excessive fat intake has important implications as resultant increases in postprandial lipids can lead to the uptake and storage of fat not only in adipose tissue but also in nonfat tissue such as the liver and skeletal muscle.

Many investigators have reported increases in intramyocellular lipid (IMCL) content in skeletal muscle with both short- and long-term high-fat diet feeding regimes (for review, see Schrauwen-Hinderling et al. [261]). In some subjects, an increase in IMCL oxidation may occur with high-fat feeding in response to the increase in IMCL content [261]. In contrast, many obese subjects are characterized by increased adipose tissue and plasma lipid concentrations, high IMCL, and low muscle oxidative capacity [262, reviewed in Reference 263]. The obesity-associated increase in IMCL is believed to be due to an imbalance between fat delivery to and oxidative capacity of

skeletal muscle [261,263]. Excess accumulation of fat in skeletal muscle can be pathophysiological as high IMCL has been indicated as an early marker of the development of insulin resistance and type II diabetes [261]. Indeed, overexpression of muscle LPL has been associated, in some studies, with increased intramyocellular triacylglycerol concentrations and insulin resistance (for review, see Corcoran et al. [263]). Conversely, there is emerging evidence that it is the increased dependence on and utilization of fatty acids for energy within the muscle mitochondria, and not the accumulation of IMCL per se, that serves as a stimulus for the development of insulin resistance under conditions of high dietary fat intake (for review, see Rindler et al. [264]).

C. Influence of Dietary Fat on Fatty Acid Oxidation and Energy Expenditure

It has been known since the early days of calorimetry that diets high in fat lower the respiratory quotient, an indicator of increased fatty acid oxidation [265]. Fatty acid oxidative rates are dependent in part on chain length and degree of unsaturation. For example, the results of stable isotope studies in normal weight men indicate that oxidation rates of individual fatty acids are highest for lauric acid, followed by PUFA and MUFA, and least for longer-chain SFA for which oxidation decreases with increasing chain length [266]. Results of indirect calorimetry studies in healthy nonobese to moderately obese men or postmenopausal women indicate higher postprandial fat oxidation rates in response to a high-MUFA test meal as compared with high-SFA [267,268] or high-PUFA [269] test meals. In a recent review of short- and long-term dietary and single-meal challenge studies, Krishnan and Cooper [270] report that while not all studies agree, the majority indicate that unsaturated fats induce greater fat oxidation, diet-induced thermogenesis, and energy expenditure than saturated fats, with the preferred order of oxidation being MUFA $\geq$ PUFA > SFA. These authors further conclude that based on preferential effects on increasing postprandial energy expenditure and fat oxidation, medium-chain FA and MUFA may have beneficial influences on body weight maintenance and fat balance.

Dietary lipid stimulation of fatty acid oxidation is thought to act through the sympathetic nervous system [271] and the carnitine palmitoyltransferase (CPT) system involved in the transport of cytosolic long-chain fatty acids into the mitochondria for β-oxidation [272,273]. Both systems are influenced by the source of dietary fat. For example, safflower oil-fed rats have the highest sympathetic activity when compared with coconut oil- or medium-chain triglyceride-fed rats [271]. Similarly, feeding fish oil as compared with corn oil causes a marked increase in carnitine acyltransferase activity (CACT, a component of the CPT system) in hepatic mitochondria [272]. Recently, Priore et al. [273] reported an increase in hepatic CACT activity in mice-fed fish oil-enriched diets and decrease in CACT activity in those fed safflower-enriched diets with the effects associated with translational and posttranslational modifications, respectively. Peroxisomal oxidation is also increased by diets containing fish oils when compared with vegetable oils [231]. Studies in rodents have demonstrated that the *n*-3 fatty acids EPA and DHA both stimulate peroxisomal β oxidation in the liver, whereas EPA also increases mitochondrial β-oxidation [274]. Substitution of *n*-3 PUFA for a portion (~35%) of SFA in a high-fat diet (28% w/w) stimulates fatty acid oxidation in the liver and to a lesser extent in skeletal muscle [275], presumably due to the upregulation of genes encoding proteins for mitochondrial and peroxisomal enzymes (see reviews by Clarke [225] and Baillie et al. [276]). Adipose depot-specific effects of dietary EPA and DHA on upregulating the expression of genes associated with mitochondrial biogenesis and β-oxidation have also been reported [277].

There is mounting evidence that a reduced capacity for fat oxidation may be a contributing factor in the development of diet-induced obesity [278–284]. Ji and Friedman [278] observed that the whole-body fat oxidation rates predicted the propensity of susceptible rats to develop obesity in response to high-fat feeding. They [279] also observed that treatment of obesity-prone rats with fenofibrate (a peroxisome proliferator-activated receptor alpha [PPAR-α] agonist that promotes fatty acid oxidation) increased whole-body fatty acid oxidation and reduced food intake, weight gain, and adiposity to levels seen in control obesity-resistant rats. Studies by Jackman et al. [280] demonstrated

that while obesity-resistant rats increase fat oxidation in response to high-fat feeding, obesity-prone rats tend to preferentially partition dietary fat for storage in adipose tissue. Furthermore, Iossa et al. [281] observed that adult rats have a compromised ability to resist high-fat diet-induced obesity due, in part, to a reduced capacity to increase fat oxidation, an adaptive mechanism that counteracts obesity development in younger animals.

In humans, whole-body fat oxidation, as measured over a 12-h period after a breakfast containing stable isotope labeled palmitic acid, is negatively related to percentage body fat [282]. Other studies report a reduced capacity to oxidize fat after a high-fat meal in both obese insulin-resistant subjects [283] and in individuals who are not, and have never been obese, but who have a strong familial predisposition to overweight [284]. In contrast, Bergouignan et al. [285] observed a similar increase in both whole-body fat oxidation and skeletal muscle oxidative capacity in healthy lean and obese adults in response to an acute increase in dietary fat under isocaloric conditions. They suggested that the ability to adapt to an increase in dietary fat with changes in whole-body and skeletal muscle oxidative capacity is not impaired in obesity. Likewise, Draznin et al. [286] found an increase in phosphorylation of AMPK and deacylation of PGC1α in skeletal muscle of both healthy normal weight and obese adults in response to a short-term increase in dietary fat under hypercaloric and hypocaloric conditions, respectively, supporting the notion that the energy sensing network within muscle is not impaired in obesity. In contrast, Boyle et al. [287] reported that short-term high-fat feeding induced a differential response in the expression of several genes involved in lipid oxidation in skeletal muscle from healthy lean and insulin-resistant obese humans. Differences between lean and obese individuals with regard to hepatic fatty acid partitioning and oxidation have also been reported [288]. Interestingly, these investigators [288] found greater rates of oxidation, elongation, and desaturation of palmitic acid in abdominally obese as compared with lean individuals and suggested that this may be an adaptive mechanism to reduce liver accumulation by redirecting fatty acids for oxidation and/or export.

In addition to fat oxidation per se, energy expenditure can also be influenced by dietary lipids. In general, high-fat diets have a lower heat increment than diets high in carbohydrate or protein [265]. This may lead to a decrease in dietary energy utilization and an increase in body weight gain when consuming a high-fat diet. Although evidence exists for high-fat diets causing obesity, not all high-fat diets affect energy metabolism and body weight in the same manner. For example, diets high in essential fatty acids result in a lowering of body weight and increase in thermogenin content in rat brown adipose tissue [289]. Diets containing safflower oil are associated with an increase in thermogenesis [290] and uncoupling protein (UCP) content [291] of brown adipose tissue in comparison with diets containing beef tallow. As their name implies, uncoupling proteins dissociate mitochondrial oxidative phosphorylation from energy production, leading to energy loss as heat. *n*-3 PUFAs have been demonstrated to increase expression of UCPs beyond that observed with other types of dietary fats in several studies [254,276,292,293]. Thus, Takahashi and Ide [254] reported an increase in UCP-1 mRNA in brown adipose tissue of rats fed high-fat diets containing fish oil as compared with safflower oil. Likewise, Baillie et al. [276] reported an increase in skeletal muscle UCP-3 mRNA in fish oil-fed rats, which was inversely correlated with a decrease in body fat mass. It has been postulated that one mechanism by which *n*-3 fatty acids decrease adipose tissue mass in rats and mice is by increasing thermogenesis. However, dissipation of consumed energy as heat does not explain the lack of variance in body weights in some studies.

D. Influence of Dietary Fat on Adipose Tissue Lipolysis

Adipose tissue lipolysis, a catabolic process resulting in the hydrolysis of stored triglycerides and release of fatty acids and glycerol from fat cells, has been described as a complex cyclic AMP-dependent signal transduction cascade catalyzed by hormone sensitive lipase and regulated primarily by stimulation from catecholamines and inhibition by insulin (see review by Vernon [294]). More recent work has identified additional endocrine and paracrine factors, such as natriuretic peptides,

and molecular mechanisms involved in lipolytic regulation (for review, see Langin [295]). Two major lipases are involved in the process: hormone-sensitive lipase (HSL) that catalyzes catecholamine and natriuretic peptide-stimulated lipolysis and adipose tissue triglyceride lipase (ATGL) that mediates triglyceride hydrolysis under basal conditions [295]. The influence of the level and type of dietary fat on hormone-stimulated lipolysis and other lipolytic processes is detailed in the following text.

1. Level of Dietary Fat

Both hormone-stimulated lipolysis and the antilipolytic effects of insulin are influenced by the quantity and type of dietary fat with these effects varying somewhat according to species and adipose tissue depot. For example, feeding rats high levels of dietary fat leads to a decrease in both catecholamine- [296–304] and glucagon- [296] stimulated lipolysis. The effect of high-fat feeding on adipose tissue lipolysis in the rat is believed to be due to changes in β-adrenoceptor number or to an uncoupling between the hormone receptor and adenylate cyclase, rather than to differences in hormone binding [297,298,304]. In contrast, several studies have indicated an increase in cyclic AMP-dependent signal transduction and lipolytic response with an increase in dietary fat [301,305–309]. More specifically, adenylate cyclase activity [305] and isoproterenol-stimulated lipolysis [306] are increased and phosphodiesterase activity is decreased in a depot-dependent manner [307] in pigs fed added fat diets. There are few studies regarding the effect of high-fat feeding on adipose tissue lipolysis in humans. In one short-term study, Kather et al. [310] observed no differences in either sensitivity or response to catecholamines in adipose tissue of subjects eating fat-rich diets as compared with carbohydrate-rich diets. In a second short-term study, Suljkovicova et al. [311] reported no effect of dietary macronutrient composition on β-adrenergic responsiveness of adipose tissue to catecholamine action at rest, but a higher rate of lipolysis in adipose tissue of high-fat-fed subjects as compared with high-carbohydrate-fed subjects during exercise. Recently, van Hees et al. [312] reported reduced ATGL and HSL protein expression in adipose tissue from men and women with metabolic syndrome fed low-fat high-carbohydrate diets as compared to several types of high-fat diets for 12 weeks. They suggested that under isoenergetic conditions dietary fat quantity, rather than composition, may be most important for modulating expression of these lipolytic enzymes.

Fat feeding has been shown to influence the antilipolytic effects of insulin in adipose tissue and skeletal muscle from several species; however, study results are somewhat inconsistent. Smith et al. [299] observed a decreased response to the antilipolytic effects of insulin in adipose tissue from high-fat as compared with high-carbohydrate-fed rats, whereas subsequent studies by Susini et al. [300] and Tepperman et al. [298] failed to observe an effect of dietary fat on the antilipolytic effects of insulin. In pigs, a decrease in the antilipolytic action of insulin in response to added fat feeding is observed in the subcutaneous fat depot but not for the perirenal site [313]. In humans, a greater sensitivity to the antilipolytic action of insulin is observed in adipocytes of subjects consuming fat-rich diets as opposed to energy-restricted diets [310]. Contradictory effects on basal lipolysis in skeletal muscle have been reported in response to high-fat feeding in rats, that is, an increase in one study [314] and a decrease in the other [315]. Nonetheless, both studies observed a resistance to the antilipolytic action of insulin in skeletal muscle of the high-fat-fed animals [314,315].

2. Type of Dietary Fat

Lipolytic response may be influenced by the type as well as the level of fat included in the diet. The composition of dietary fat selectively influences fatty acid deposition in adipose tissue [316]. In turn, the composition of the fat tissue influences lipid mobilization and release of fatty acids into the circulation. Lipid mobilization from adipose tissue is not a random event, but instead is influenced by chain length, degree of saturation, and positional isomerization of the fatty acids [317–321]. The relative mobilization of fatty acids from adipose tissue is correlated positively with unsaturation and negatively with chain length [319]. The most easily mobilized fatty acids are those with 16–20 carbon atoms and 4 or 5 double bonds, whereas very long-chain MUFAs and PUFAs are less readily

mobilized [320]. Higher rates of lipolysis are also observed with *trans* as opposed to *cis* isomers of octadecenoic acid [318]. It has been suggested that the decreased fat accumulation in animals fed *trans* fatty acids may be associated with direct effects of the *trans* isomer on fat cell metabolism [318]. Likewise, decreased visceral fat accumulation in fish oil-supplemented sucrose-fed rats is believed to be due, at least in part, to an effect of *n*-3 PUFA on increasing lipolytic responsiveness [251]. Adipose tissue from animals fed diets high in PUFA generally exhibit a greater lipolytic response to catecholamines and synthetic β-adrenoceptor agonists as compared with tissue from animals fed diets high in SFA [299,322–327]. The decreased responsiveness of fat cells from rats fed saturated fat diets is associated with reductions in adenylate cyclase, cyclic AMP phosphodiesterase, and HSL activity [322]. β-Adrenergic receptor binding is also lower in fat cell membranes from rats fed high-SFA as compared with high-PUFA diets [323,328], due to decreased binding affinity, correlated with a reduction in membrane fluidity [323], rather than changes in receptor number. In contrast to the aforementioned studies, several investigators failed to observe an effect of dietary fat type on lipolytic response [302,306,329,330]. Lipinski and Mathias [302] observed that norepinephrine-stimulated lipolysis in rat adipocytes is depressed by an increase in fat calories but is unaffected by the degree of saturation of the fat. Likewise, Mersmann et al. [306,329] reported an increase in the number of β-adrenergic receptors, with no change in receptor affinity or receptor-mediated function (i.e., lipolysis) in adipose tissue from pigs fed high levels of SFA. Portillo et al. [331] observed that under energy-controlled feeding conditions, various dietary fat regimes caused major changes in rat adipose tissue phospholipid composition, but no important changes in lipolysis. Similarly, van Hees et al. [312] reported that only dietary fat quantity, but not dietary fat composition, influenced adipose tissue mRNA and protein expression of the lipolytic enzymes ATGL and HSL in metabolic syndrome patients fed under isoenergetic conditions for 12 weeks. Interestingly, Camargo et al. [243], using the same diets and experimental protocol in metabolic syndrome patients, observed that long-term consumption of high-SFA diets increased adipose tissue ATGL in both the fasted and postprandial states relative to high-MUFA or low-fat/high-carbohydrate diets. Reason for the discrepant results between these studies is unknown, and requires further investigation.

In a recent study, Diaz-Villasenor et al. [332] observed that the type of protein as well as the type and amount of dietary fat may selectively modify adipose tissue lipolytic activity. Specifically, soy protein increased HSL phosphorylation independent of the amount or type of dietary fat in genetically obese Zucker (fa/fa) rats fed soy protein or casein-based experimental diets for 2 months. It remains to be determined whether similar dietary protein/fat interactions influence lipolytic processes in other animal models of obesity and in human subjects. Nonetheless, these results demonstrate the extreme complexity of the potential regulatory influence of diet composition on lipolysis and other metabolic pathways.

E. Influence of Dietary Fat on Insulin Action

1. Level of Dietary Fat

An association between high-fat diets and impaired insulin action has been observed in numerous in vivo and in vitro studies. Early studies in human subjects indicate that diets high in fat lead to a reduction in glucose tolerance [333,334]. More recent epidemiologic data suggest that individuals with higher fat intakes are more likely to develop disturbances of glucose metabolism, type 2 diabetes, and impaired glucose tolerance than individuals consuming lower amounts of fat, although obesity and physical inactivity may be confounding factors [335]. Studies in experimental animals indicate that high-fat feeding induces both a decline in insulin sensitivity [336–338] and the development of insulin resistance in a variety of tissues [339–345].

Cellular mechanisms responsible for the decline in insulin responsiveness in association with high-fat feeding have not been fully defined. Euglycemic, hyperinsulinemic clamp studies in human subjects and experimental animals indicate that high-fat feeding significantly impairs insulin action

by a variety of mechanisms including skeletal IMCL (for review, see Corcoran et al. [263]), a reduction in skeletal muscle insulin signaling [346,347] and glucose metabolism [346], and a decreased ability of insulin to suppress hepatic glucose production [346,348]. Such studies also indicate that diet-associated development of peripheral insulin resistance may be modulated by age [349] and genetics (see reviews by Lopez-Miranda et al. [350] and Roche et al. [351] and more recent reports [352–357]).

Reductions in insulin binding in tissues from rats fed high-fat diets as compared with high-carbohydrate diets have been observed by several groups of investigators [339,358–365]. However, other investigators failed to observe significant alterations in insulin binding in response to high-fat feeding [344,366,367]. Several postreceptor defects in insulin action [339,340,342,362–365] are observed in tissues from animals fed high-fat diets. Specifically, reductions in insulin receptor kinase activity [363], in the intracellular glucose transport system [342,362,364], and in the intracellular capacity to utilize glucose for lipogenesis [340] have all been reported in association with high-fat feeding.

Tissue expression and serum levels of resistin and tumor necrosis factor-alpha (TNF-α), cytokines that oppose insulin action (see reviews by Steppan and Lazar [366] and Borst [367]), are frequently increased with high-fat feeding [368–370], whereas expression or circulating concentrations of adiponectin, an insulin-sensitizing hormone, are decreased [370–372]. Interestingly, Pitombo et al. [370] observed that removal of visceral fat reversed the impairment in glucose homeostasis and insulin action in high-fat diet-induced obese rats and returned circulating levels of TNF-α, adiponectin, and other cytokines to near-control levels. More recently, van Dijk et al. [373] reported that proinflammatory gene expression in adipose tissue of abdominally obese men at risk for metabolic system was influenced by dietary fat consumption. Taken together, this suggests that proinflammatory and other adipose-derived factors may contribute to peripheral insulin resistance observed with high-fat diet-induced and other forms of obesity.

2. Type of Dietary Fat

The effect of dietary fat on insulin action is greatly influenced by the type of fatty acid consumed (for review, see [263,374–377]). High-SFA intakes are consistently associated with insulin resistance, whereas MUFAs and PUFAs are less deleterious in this regard [263,335,376–379]. van Amelsvoort et al. [380] observed that insulin response is greater in epididymal fat cells from rats fed diets high in PUFA as compared with SFA. Likewise, diets with increasing ratios of PUFAs to SFAs induce alterations in the composition of adipocyte plasma membranes that are associated in a dose-dependent manner with increases in insulin binding, insulin receptor signaling, and glucose transporter activity [381–384]. More recently, van den Berg et al. [385] demonstrated differential effects between stearic acid (C18:0) and palmitic acid (C16:0) on inducing adiposity and whole-body insulin resistance in high-fat-fed mice, characterized by hepatic insulin resistance in those fed the stearic acid containing diets. Evidence from both animal and human studies suggest a link between dietary fat intake and alterations in skeletal muscle plasma membrane and IMCL composition that interfere with cellular signaling leading to the development of insulin resistance (see reviews by Corcoran et al. [263], Haag and Dippenaar [375] and Ritter et al. [386]). Interestingly, recent evidence suggests that such alterations may be muscle fiber type dependent [387] and mediated by different metabolic pathways dependent on specific lipid moieties (see review by Ritter et al. [386]).

Studies in both animal models [345,380,388,389] and humans [374,378,379,390] indicate that substitution of more saturated with less saturated dietary fat sources can improve or ameliorate high-fat diet-induced impairment in insulin function. In rats, substitution of safflower oil (high PUFA) for beef tallow (high SFA) in a moderate-fat diet leads to an increase in glucose uptake in response to insulin [345] and to alterations in gene expression of several insulin signal transduction pathway intermediates [391], but not to changes in insulin receptor mRNA or relative expression of insulin receptor mRNA isoforms [391]. In healthy men and women, isocaloric substitution of MUFAs for SFAs improves insulin sensitivity [378,379,390], with the effect dependent on both

total dietary fat intake [378] and metabolic status of study subjects [390]. Thus, Vessby et al. [378] observed that increasing dietary MUFA content improved insulin sensitivity in individuals with a moderate-fat intake while having no beneficial effect in individuals with a high-fat intake (>37% energy). Lovejoy et al. [390] observed no impact of dietary fat type on insulin sensitivity in lean subjects, but susceptibility to develop insulin resistance on the high-SFA diets in overweight individuals. In contrast, Tierney et al. [392] observed no effect of 12-week substitution of dietary SFA intake with MUFA on insulin sensitivity or other metabolic risk factors in obese subjects with metabolic syndrome participating in a large pan-European controlled intervention study. They postulate that obese individuals with preexisting metabolic syndrome may be resistant to this dietary intervention and require a greater or more prolonged modification to benefit from a reduction in SFA.

There is mounting evidence that *n*-3 PUFAs may afford a protective effect against diet-induced insulin resistance [263,335,377,384,393–397]. In animal studies, replacement of a small portion (6%–11%) of the fatty acids in a high-safflower oil diet with long-chain *n*-3 fatty acids from fish oil prevents the accumulation of intramuscular triglyceride and development of insulin resistance typically observed in association with consumption of a very high-fat (59% calories) diet [389,390, reviewed in Reference 263]. Likewise, substitution of a portion of lard and safflower oil with fish oil in diets fed at energy restricted levels over 12 weeks to diet-induced obese mice resulted in a greater decrease in body weight and greater improvement in glucose tolerance as compared with energy restriction alone [398]. Many studies have demonstrated that substitution of a portion of dietary fat with *n*-3 PUFA (fish oil) prevents or corrects the inhibitory effects of high-sucrose/high-fat feeding on insulin action [384,393,395,399], as evidenced by higher rates of insulin-stimulated glucose transport and other metabolic indices that were positively correlated with the fatty acid unsaturation index of adipocyte membrane phospholipids [398]. In contrast, Fickova et al. [400] observed a reduction in insulin-stimulated glucose transport and lower number of insulin binding sites on adipocytes from rats fed high levels of *n*-3 as compared with *n*-6 PUFAs. Likewise, Ezaki et al. [401] reported that although substitution of a portion of the safflower oil in a high-fat diet with fish oil led to a transient (at week 1) increase in both insulin-stimulated glucose uptake and glucose transporter distribution in rat adipocytes, further fish oil feeding (4 weeks) resulted in a reappearance of insulin resistance and adipose cell enlargement comparable with that observed with the high-safflower diet.

Human studies have demonstrated promising effects of *n*-3 PUFA on improving lipid profiles, but less consistent effects on insulin resistance (reviewed by Riccardi et al. [376], Lombardo and Chicco [395], and Nettleton and Katz [396]). Epidemiologic evidence suggests that moderate fish oil consumption may have a protective effect against the development of impaired glucose tolerance and diabetes mellitus (see review by Lombardo and Chicco [395]). However, *n*-3 PUFA supplementation in patients with type 2 diabetes and obesity has produced inconsistent results [263,376,394,395,397,402,403]. Some studies [394,397, reviewed in Reference 395] report an effect of *n*-3 PUFAs on improving insulin sensitivity. Thus, in a recent hypocaloric low-fat dietary intervention that emphasized increased lean fish consumption in obese subjects, Haugaard et al. [394] observed that skeletal muscle membrane phospholipid *n*-3 PUFA content was an independent predictor of improved insulin sensitivity. Likewise, Jimenez-Gomez et al. [397] recently reported that addition of *n*-3 PUFA to a high complex carbohydrate diet fed to individuals with metabolic syndrome for 12 weeks led to an improvement in insulin sensitivity and to changes in adipose tissue expression of several genes in the insulin signaling pathway. Other studies in type 2 diabetics (reviewed by Corcoran et al. [263], Riccardi et al. [376], and Lombardo and Chicco [395]), however, report no effect of *n*-3 PUFA on improving insulin sensitivity, whereas the authors of a recent systematic review [404] found that evidence for an association between *n*-3 PUFA and type 2 diabetes remains inconclusive. Still others [reviewed in References 395,402,403] report a deterioration of glycemic control as evidenced by moderate increases in blood glucose concentrations and decreased insulin sensitivity in type 2 diabetics receiving large daily doses of fish oil. Although there is no definitive proof that *n*-3 fatty acids can reverse insulin resistance, it has been suggested [396] that

regular consumption of modest amounts of *n*-3 PUFAs by persons with type 2 diabetes may result in beneficial lipid-lowering effects without adversely affecting glycemic control.

Epidemiological evidence reveals an association between a high intake of *trans* fatty acids and an increased risk of developing type 2 diabetes [405]. Likewise, a 12-week diet study in rats [406] demonstrated a greater impairment in insulin sensitivity in adipocytes from *trans* fat-fed animals as compared with control-fed or SFA-fed animals. Several controlled intervention studies in humans (for review, see Riserus [407]) suggest that *trans* fatty acids may impair insulin sensitivity in insulin-resistant overweight subjects, while having had no effect on insulin sensitivity in lean healthy subjects. Clearly, additional studies are needed to confirm the effect of *trans* fatty acids on insulin sensitivity and to determine mechanisms of action.

3. Reversibility of Diet-Induced Alterations in Insulin Action

Prolonged consumption of a high-fat diet impairs insulin action and leads to the development of obesity. Conversely, a reduction in dietary fat content may improve insulin sensitivity and reduce obesity development. Harris and Kor [337] observed an impaired insulin response to a glucose challenge in rats fed high-fat (40% energy) diets for 8 weeks. This effect is reversed within 3 days following a modest reduction in dietary fat content (30% energy). As alterations in body weight or fat content are not observed until 14 days following the switch to the lower-fat diet, the improvement in insulin sensitivity is not believed to be secondary to a reduction in obesity [337]. Though these results are encouraging, an improvement in insulin response on reduction in dietary fat content has not been consistently observed. In rats previously fed high-fat (60% energy) diets for 6 months, Yakubu et al. [338] failed to detect either an improvement in insulin response or a reduction in body weight following subsequent consumption of a low-fat (20% energy) diet for 3 or 6 months. Thus, alterations in insulin response induced by a high-fat diet and the reversibility of these effects appear to be influenced by both level and type of dietary fat and the duration of the high-fat feeding.

V. DIETARY FAT AND ADIPOSE TISSUE CELLULARITY

The expansion of adipose tissue during growth or the development of obesity is achieved through an increase in adipose tissue size (cellular hypertrophy), an increase in adipose tissue number (cellular hyperplasia), or through a combination of both processes. Whether adipose tissue expands through hypertrophy (enlargement) of existing adipocytes or through hyperplasia (proliferation resulting in smaller adipocytes) has important metabolic consequences. An emerging body of evidence suggests an association between increasing adipose size and adipose tissue dysfunction, inflammation, changes in adipose secretory profile, and pathogenesis of metabolic disease (reviewed in References [408–410]), whereas adipocyte hyperplasia, characterized by more numerous smaller adipocytes, or a reduction in adipocyte size is associated with improvements in insulin sensitivity and metabolic profiles [407–416]. Numerous studies provide evidence that variations in the level and type of fat included in the diet can lead to alterations in adipose cell size and number, and to subsequent changes in metabolic health. As quantification of total adipose tissue cell number in human subjects is not readily obtainable, these studies have been primarily conducted in experimental animals.

A. Level of Dietary Fat

Many animal studies confirm that high-fat feeding leads to an expansion of adipose tissue mass through an increase in fat cell size and number and to the subsequent development of obesity. Studies of adipose tissue development in rodents indicate that increases in fat pad weight are typically associated with increases in both fat cell size and number until approximately 10–18 weeks of age [417–419]. Body and fat pad weights and fat cell size and number then plateau and remain fairly constant in the adult animal. High-fat feeding influences both the dynamic stage of adipose tissue development early in life [417,420–422] and also the more static phase associated with

adulthood [417,423,424]. In mice fed high-fat diets from birth, increased fat pad weights are associated with a greater fat cell size through 18 weeks of age, followed by an increase in fat cell number through 52 weeks of age [417]. At that time, fat pad weight is sixfold greater in the high-fat than the control-fed mice, whereas fat cell size and number are increased 2.3- and 2.5-fold, respectively. An even greater effect on adipose tissue cellularity is observed in young Osborne–Mendel rats, a strain susceptible to high-fat feeding [82], with a 4- to 16-fold increase in adipocyte number (dependent on the specific fat pad studied) observed between 24 and 105 days of age when the animals are fed a high-fat diet [417]. A more recent study [425] also reported a strain-dependent response to high-fat feeding in young animals, further noting that whereas changes in cell size were largely dependent on diet, changes in cell number were strain dependent, suggesting an interaction of diet and genetics.

Dramatic alterations in adipose tissue cellularity are also observed in adult rats subjected to high-fat feeding [417,423,424]. In adult rats, high-fat diet-induced obesity is associated with increases in both fat cell size and fat cell number, with increases in cell size preceding changes in cell number. In 5-month-old rats, an increase in fat cell size is detected as early as 1 week after the introduction of a high-fat diet, followed by increases in cell number in the perirenal and epididymal fat pads after 2 and 8 weeks, respectively [423]. Likewise, Faust et al. [424] report increases in cell size in several fat pad depots after 3 weeks of high-fat feeding in adult rats but an increase in cell number only after 9 weeks of dietary treatment.

Depot-dependent differences in the expansion of adipose tissue by hypertrophy and/or hyperplasia in response to high-fat feeding have been reported [426,427], which may be explained by intrinsic depot-dependent differences in the abundance and proliferative activity of adipogenic progenitor (AP) cells [427]. Thus, Joe et al. [427] report that APs are eightfold more abundant in subcutaneous as compared with visceral fat and that the expansion of subcutaneous, but not visceral fat, in response to high-fat feeding is highly correlated with AP proliferation. This relative lack of progenitor cells may underlie the hypertrophic growth of visceral adipose tissue and associated metabolic consequences during obesity development [427].

B. Type of Dietary Fat

It is generally accepted that a high level of fat in the diet may induce adipose cell hypertrophy and hyperplasia. However, the influence of dietary fat type on adipose tissue cellularity and the development of obesity are less definitive, particularly with respect to diet-induced alterations in fat cell hyperplasia. Several studies reported a greater effect of unsaturated as compared with saturated fat diets on increasing fat cell number [428–430], whereas other investigations report a greater degree of fat cell hyperplasia with saturated as compared with unsaturated fat diets [421,431,432]. Other studies [380,433–435] in various rodent models suggest that the alterations in fat cell size and number associated with high-fat feeding are not influenced by type of dietary fat. Interestingly, Kim et al. [415] found a similar degree of cellular hypertrophy and impairment in insulin-dependent glucose uptake in adipocytes treated with saturated or MUFAs, but an induced inflammatory response only with the SFA treatment.

In contrast to the conflicting effects on adipose cellularity observed with saturated or unsaturated fat diets, more consistent effects are observed with dietary *n*-3 PUFA, which reportedly limit hypertrophy and/or hyperplasia [255,400,416,436–442], in a depot-dependent manner [255,437]. Whether differences in fat cell size and number are observed appears to be dependent on the duration of the study. Cell size is decreased during short-term *n*-3 PUFA-feeding studies in rats [255,400,438,439], whereas fat cell number is also reduced during longer-term studies [437]. Reductions in fat cell size in association with adipose tissue and dietary *n*-3 PUFA content [443] and with short-term *n*-3 PUFA supplementation [444] have been reported for human studies.

The mechanism by which *n*-3 PUFAs affect adipose tissue cellularity has not been totally defined. Alterations in both β-oxidation and lipolytic response (detailed in Sections IV.C

and IV.D) are associated with the reduction in adipose tissue mass observed with *n*-3 PUFA diets. In addition, *n*-3 PUFA may influence adipose tissue cellularity indirectly by preventing adipose tissue matrix remodeling and adipocyte enlargement [445] or directly by inhibiting adipogenesis [422,441] (reviewed by Ailhaud et al. [446]). Thus, Hensler et al. [441] recently suggested that the decrease in adiposity observed in obese mice in response to *n*-3 PUFA diets may be due to an influence on both differentiation and proliferation of adipose cell, mediated by changes in fatty acid composition of cellular membranes and altered formation of PUFA-derived metabolites such as eicosanoids. In vitro studies support this contention, demonstrating potent effects of arachidonic acid (*n*-6 PUFA) on stimulating adipogenesis, which are mediated through conversion to prostacyclin, increased cAMP production, and activation of the protein kinase A pathway [422] (reviewed by Ailhaud et al. [446]). Conversely, *n*-3 fatty acids, (EPA, and to a lesser extent DHA) inhibit the stimulatory effect of arachidonic acid on cAMP production and thereby attenuate arachidonic acid-stimulated adipogenesis [422]. Accordingly, mice born to dams consuming a high-fat linoleic (arachidonic acid precursor) acid-rich diet and reared to the same diet were 50% heavier at weaning and had an increased adipose tissue mass at 8 weeks, characterized by an increased adipose cell size as compared with pups from mothers fed a high-fat diet containing a mixture of linoleic and α-linolenic (*n*-3 fatty acid, DHA, and EPA precursor) acids [422]. Likewise, Ruzickova et al. [447] observed a reduction in adipose tissue cellularity and obesity development with the addition of *n*-3 PUFA of marine origin (EPA and DHA) to a high-fat linoleic acid-rich diet. Interestingly, studies in obese diabetic db/db mice [445] demonstrate that inclusion of *n*-3 PUFA in diets rich in SFA and MUFA or *n*-6 PUFA could completely prevent the upregulation of adipose tissue expression of genes involved in matrix remodeling and the increase in adipocyte size typically observed with these high-fat diets. More recent studies [416,440] provide evidence that supplementation of high-fat diets with *n*-3 PUFA is effective not only in suppressing adipose hypertrophy and hyperplasia but also in ameliorating adipose tissue inflammation.

C. Cellularity Changes: Reversibility of Obesity and Insulin Resistance

The reversibility of obesity induced by high-fat feeding [424,448,449, reviewed in Reference 450] may be dependent on the duration and severity of the dietary treatment. For example, diet-induced obesity is reversed when the animals are returned to a low-fat diet following 16 [448] but not 30 weeks [449] of high-fat feeding. Diet-induced alterations in adipose tissue cellularity may be a major factor dictating the reversibility of the obese state [424,449]. Thus, obesity associated with changes in fat cell size only is readily reversed on return to a low-fat regime [424,429]. Interestingly, diet-induced changes in fat cell number may have short-term beneficial effects, but long-term adverse consequences. Accordingly, an increased number of small insulin-sensitive fat cells may afford protection against insulin resistance and other obesity-associated comorbidities by expanding lipid storage and oxidation capacity and preventing further expansion of larger insulin-resistant adipocytes and ectopic accumulation of lipid [445,451]. Nonetheless, several animal studies indicate that diet-induced changes in fat cell number appear to be permanent, as switching from a high-fat to a low-fat diet leads to a reduction in body weight and fat cell size but not fat cell number [424,448–450]. Thus, an increased capacity for energy storage, although perhaps beneficial in the short-term, would increase the capability or capacity of adipose tissue mass to expand with subsequent episodes of increased caloric intake. This would suggest that high-fat diet-induced obesity, particularly of extended duration, may be resistant to intervention. It remains to be determined if these observations are indeed applicable to humans, as results of several studies in humans demonstrated a reduction in fat cell number following reversal of obesity [450]. It is clear that additional investigation is required to determine whether and under what conditions significant weight loss accompanied by a reduction in fat cell number can be consistently achieved in human subjects.

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28 Influence of Dietary Fat on the Development of Cancer

Howard Perry Glauert

CONTENTS

LIST OF ABBREVIATIONS

AAF	2-Acetylaminofluorene
ACF	Aberrant crypt foci
AFB	Aflatoxin B_1
AOM	Azoxymethane
APC	Adenomatous polyposis coli
BOP	*N*-nitrosobis(2-oxopropyl)amine
BP	Benzo(*a*)pyrene
DAB	*p*-Dimethylaminoazobenzene
DEN	Diethylnitrosamine
DMBA	7,12-Dimethylbenz(*a*)anthracene
DMH	1,2-Dimethylhydrazine
GGT	γ-Glutamyl transpeptidase
LXR	Liver X receptor
MAM	Methylazoxymethanol
MNU	Methylnitrosourea
PAH	Polycyclic aromatic hydrocarbon(s)
PanIN	Pancreatic intraepithelial neoplasia
PGST	Placental glutathione *S*-transferase

PKC Protein kinase C
PPAR Peroxisome proliferator-activated receptor
TPA 12-*O*-tetradecanoylphorbol-13-acetate

I. INTRODUCTION

Cancer is currently the second leading cause of death in the United States. It is estimated that more than 1,658,000 people will be diagnosed with cancer in the United States in 2015 and that more than 589,000 will die from it [1]. One of the primary mechanisms for reducing cancer deaths may be by altering the diet, and one proposed way is by decreasing the consumption of dietary fat. In this chapter, the role of dietary fat on the development of human and experimental cancer is discussed. Because of the large number of studies published, reviews are cited where possible.

II. EPIDEMIOLOGICAL STUDIES

Numerous epidemiological studies have examined the effect of dietary fat on human cancer. Several correlational studies have noted an increase in the rates of colon, breast, and other cancers in areas where dietary fat consumption is high [2]. In addition, studies with immigrant populations have identified dietary fat intake as a causative factor in the development of these cancers [2].

A. Colon Cancer

For colon cancer, epidemiological studies have not reached a clear consensus about the influence of dietary fat. Case–control studies overall have not found a positive association with dietary fat, although many have observed a positive association with meat intake [3]. Prospective epidemiological studies have conflicting results: some studies found a positive association [4–6], others saw no effect [7–24], and others saw an actual protective effect of high-fat intake [25–27]. In several of these studies, the consumption of red meat was found to be significantly correlated with colon cancer risk, but independently of fat intake. The International Agency for Research on Cancer recently classified red meat as probably carcinogenic to humans and processed meat as carcinogenic to humans, with the colon being the main target organ [28]. In the Women's Health Initiative intervention study published in 2006 [29], 19,500 women lowered their fat intake by about 10% compared to 29,000 women who did not alter their diet for a follow-up period that averaged 8 years. The intervention group had a relative risk of 1.08, which was not statistically significant, indicating that a diet lower in fat did not inhibit the development of colon cancer in this study.

B. Breast Cancer

Numerous epidemiological studies have attempted to identify factors that influence breast cancer risk in humans. Established breast cancer risk factors include age of first pregnancy, body mass index, age at menarche or menopause, and family history of breast cancer [30]. The effect of dietary fat has been studied in correlational, case–control, and prospective epidemiological studies. Studies examining international correlations between dietary fat intake and breast cancer risk and migrant studies have reported a positive association between dietary fat intake and breast cancer risk [31]. A meta-analysis of 27 case–control studies found no significant association between breast cancer risk and saturated fat intake [32]. Most prospective studies did not find any link between total dietary fat intake and the development of breast cancer [33–60]. Furthermore, a combined analysis of many of these prospective studies did not find any evidence of a link between total dietary fat intake and breast cancer risk, although an elevated risk was observed with higher consumption of polyunsaturated fat [32]. The Women's Health Initiative intervention study (described in the

preceding section) examined the effect of low-fat diets on the development of breast cancer [61]. Although dietary fat did not significantly affect the development of breast cancer, there was a relative risk of 0.91 in the low-fat intervention group.

C. Pancreatic Cancer

For the pancreas, international comparisons do not show as strong of a trend as with colon or breast cancer [62]. Overall, neither case–control nor prospective studies that examined total fat intake observed an effect [63]. However, diets high in polyunsaturated fat were found to have an inverse association in a meta-analysis, whereas diets high in saturated fatty acids or monounsaturated fatty acids had no effect [64]. However, studies examining the consumption of cholesterol tend to show a positive correlation [65,66]. Several prospective studies have examined the relationship of meat consumption to pancreatic cancer; a meta-analysis of these studies observed a positive association [67].

D. Prostate Cancer

A number of case–control and prospective studies have examined the role of dietary fat in prostate cancer. Most case–control studies have observed a positive correlation between the intake of total and saturated fat and the development of prostate cancer, although others did not see an effect [68,69]. Increased total and saturated fat intake were associated with a higher risk of advanced prostate cancer in case–control studies [70]. Several prospective studies have been performed, with most observing no effect; a meta-analysis also did not find an effect of total, saturated, monounsaturated, or polyunsaturated intake [71,72].

E. Other Cancers

Fewer studies have been conducted for other major forms of human cancer. For endometrial cancer, several but not all case–control studies have noted an association with dietary fat; a meta-analysis did not observe an association [73]. Similarly, no association was observed in prospective studies. No significant effects were observed with either plant fat or animal fat intake [73]. Case–control studies examining dietary fat and bladder cancer showed an association in some but not all studies; a prospective study did not observe a correlation between dietary fat intake and the development of bladder cancer [74]. Dietary fat has been found to be a risk factor for ovarian cancer in some epidemiological studies but not in others [75–84]. Lung cancer risk was not found to be significantly affected by total dietary fat in prospective studies, but several case–control studies have observed an association [85–89], although several investigators indicated that their results may have been affected by confounding from smoking. In pooled analyses, neither total nor polyunsaturated fat was found to affect lung cancer risk [90,91]. Using case–control and cohort study designs, Granger et al. [92] found that increased dietary fat consumption protected against the development of skin cancer. Davies et al. [93] and Gamba et al. [94], however, found that dietary fat did not influence nonmelanoma skin carcinoma development. The development of esophageal cancer was found to be increased by dietary fat in two case–control studies [95,96].

III. EXPERIMENTAL CARCINOGENESIS STUDIES

A. Skin Carcinogenesis

Mouse skin is one of the oldest and most widely used systems for studying chemical carcinogenesis, including multistage carcinogenesis. Two-stage carcinogenesis (initiation–promotion) was first observed in mouse skin and involves initiation by a subcarcinogenic dose of radiation or of a chemical such as a polycyclic aromatic hydrocarbon (PAH) followed by the long-term administration of

croton oil or its active ingredient 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [97]. More recently, transgenic skin carcinogenesis models have been developed [98,99].

Most studies examining dietary fat have studied complete carcinogenesis by PAH or ultraviolet light. Early studies demonstrated that high-fat diets enhanced skin carcinogenesis induced by tar [100] or PAH [101–107]. In studies where skin tumors were induced by ultraviolet (UV) light, Mathews-Roth and Krinsky [108] and Vaid et al. [109] found that high-fat diets increased skin carcinogenesis, whereas Black et al. [110] found that high-fat diets did not increase skin carcinogenesis, but that feeding a saturated fat inhibited tumorigenesis. Lou et al. [111] found that mice fed a diet high in ω-3 fatty acids developed fewer UVB-induced skin tumors than mice fed a diet high in ω-6 fatty acids. In a transgenic model, skin tumors in mice overexpressing the oncogenic human papillomavirus type 16 were increased when the mice were fed a diet high in *n*-6-polyunsaturated fatty acids (corn oil) [112].

The effect of fatty acids on the initiation and promotion of skin carcinogenesis has also been studied. Certain fatty acids—oleic acid and lauric acid—were found to have promoting activity when applied daily to mouse skin after a single application of 7,12-dimethylbenz(*a*)anthracene (DMBA); stearic acid and palmitic acid did not have any effect [113]. When diets varying in their fat content were fed during the promotion stages of DMBA-initiated, TPA-promoted mouse skin carcinogenesis, high-fat diets were found to enhance the promotion of skin carcinogenesis in some studies [114,115] but not in others [116,117]. High-fat diets also partially offset the tumor inhibitory effects of caloric restriction [118]. Locniskar et al. [119] found that substituting menhaden oil for corn oil or coconut oil did not affect skin tumor promotion by TPA. When benzoyl peroxide was used as the promoting agent, mice fed mainly coconut oil had the highest tumor incidence and mice fed corn oil had the lowest tumor incidence, with those fed mainly menhaden oil having intermediate tumor incidence [120]. In a study using mezerein as the promoting agent, high-fat diets did not increase the skin carcinogenesis [121]. High-fat diets were found to not affect or slightly inhibit initiation [114,122], and substituting coconut oil for corn oil did not influence UV-induced skin carcinogenesis [123].

B. Hepatocarcinogenesis

Many early studies of dietary fat and cancer used the liver as the target organ. In these studies, aromatic amines and azo dyes were frequently used to induce hepatocellular carcinomas. In later studies, effects of dietary fat on initiation and promotion in the liver or in transgenic models were examined. In initiation–promotion protocols, the administration of a single subcarcinogenic dose of a carcinogen such as diethylnitrosamine [DEN] or DMBA along with a proliferative stimulus (such as partial hepatectomy) followed by the long-term feeding of chemicals such as phenobarbital, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, or polyhalogenated biphenyls leads to a high incidence of hepatocellular adenomas and carcinomas [124,125]. In addition, foci of putative preneoplastic hepatocytes appear before the development of gross tumors. These foci, known as "altered hepatic foci" or "enzyme-altered foci," contain cells that exhibit qualitatively altered enzyme activities or alterations in one or more cell functions [124]. The enzymes most frequently studied include γ-glutamyl transpeptidase (GGT) and placental glutathione-*S*-transferase (PGST), which are normally not present in adult liver but are often present in foci, and ATPase and glucose-6-phosphatase, which are normally present but are frequently missing from foci [126,127]. Altered hepatic foci can also be identified on hematoxylin and eosin–stained tissue [128,129]. The appearance of foci has been correlated with the later development of malignant neoplasms [130,131]. Transgenic mouse models of liver carcinogenesis have also been developed [132,133].

The first studies examined the effect of dietary fat on the induction of hepatocellular carcinomas by complete hepatocarcinogens. In the liver, increasing the fat content of the diet enhances the development of 2-acetylaminofluorene (AAF)-, *p*-dimethylaminoazobenzene (DAB)-, and aflatoxin B_1 (AFB)-induced tumors and GGT-positive foci in rats [134–138]. Furthermore, hepatocarcinogenesis

by DAB is enhanced by feeding a diet that contains a greater proportion of polyunsaturated fatty acids [139,140]. In these studies, however, the diets were administered at the same time as the carcinogen injections, so that the stage of carcinogenesis, which was affected, could not be determined.

Other studies have examined whether this enhancement of hepatocarcinogenesis is caused by an effect on the initiation of carcinogenesis, the promotion of carcinogenesis, or both. Misslbeck et al. [141] found that increasing the corn oil content of the diet after the administration of 10 doses of aflatoxin increased the number and size of GGT-positive foci, but Baldwin and Parker [142], using a similar protocol, found no effect of dietary corn oil. Glauert and Pitot [143] similarly found that increasing the safflower oil or palm oil content of the diet did not promote DEN-induced GGT-positive foci or greatly affect phenobarbital promotion of GGT-positive foci. The promotion of GGT-positive foci by dietary tryptophan also is not affected by dietary fat [144]. Newberne et al. [145] found that increasing dietary corn oil (but not beef fat) during and after the administration of AFB increased the incidence of hepatic tumors, but not when the diets were fed only after AFB administration. Baldwin and Parker [142] also found that increasing the corn oil content of the diet before and during AFB administration increased the number and volume of GGT-positive foci. When rats are fed diets high in polyunsaturated fatty acids (but not in saturated fatty acids) before receiving the hepatocarcinogen DEN, they develop more GGT-positive and ATPase-negative foci than rats fed low-fat diets [146]. The feeding of diets high in corn oil but not lard enhanced the initiation of PGST-positive foci induced by azoxymethane (AOM) [147]. Finally, the feeding of a high-fat diet inhibited the initiation of hepatic tumors induced by DEN [148]. The results of these studies suggest that the enhancement of chemically induced hepatocarcinogenesis by dietary fat is primarily due to an effect on initiation and that polyunsaturated fats have a greater effect than do saturated fats.

Other studies have fed diets sufficiently high in fat to induce nonalcoholic steatohepatitis [149,150]. Liquid high-fat diets increased the numbers of DEN-initiated PGST-positive foci in rats compared to rats fed a liquid control diet [151,152]. In male C57Bl/6J mice, feeding high-fat diets induced liver tumors, as compared to mice fed a standard chow diet [150]. Therefore, the induction of steatohepatitis appears to alter the effect of high-fat diets.

C. Colon Carcinogenesis

Studies in experimental animals have produced differing results. A variety of chemicals have been used to induce colon tumors, usually in rats or mice. These include 1,2-dimethylhydrazine (DMH) and its metabolites AOM and methylazoxymethanol (MAM); 3,2′-dimethyl-4-aminobiphenyl (DMAB); methylnitrosourea (MNU); and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) [153–156]. DMH and AOM have been used most frequently to study nutritional effects. Both can induce colon tumors by single [157–161] or multiple [162–166] injections. The Min mouse, which has a mutation in the mouse homolog of the adenomatous polyposis coli (APC) gene, develops small intestinal and colon tumors spontaneously and has been used as a model of colon carcinogenesis [167]. Mice with mutations at other locations of the APC gene have also been developed [168]. In addition to tumors, putative preneoplastic lesions, aberrant crypt foci (ACF), are induced by colon carcinogens [169]. ACF, which are identified by fixing the colon in formalin and then staining with methylene blue, are stained darker and are larger than normal crypts [169]. Some but not all studies have shown that ACF correlates well with the later appearance of adenocarcinomas [170–173].

Animal studies examining the effect of dietary fat have used a variety of protocols, and the results obtained often have been dependent on the investigator's protocol. In these studies, rats or mice were subjected to multiple doses of a colon carcinogen, with the dietary fat content being varied (isocalorically) during, and frequently before or after, the carcinogen injections. Some of these studies found an enhancement when the dietary fat content of the diet was increased, but others saw no effect or even an inhibition of tumor development [164,174–183]. High-fat diets were found to influence the early stages of carcinogenesis more than the later stages [184]. In several studies

where fat was found to enhance colon carcinogenesis, fat either was added to an unrefined (chow) diet or was substituted for carbohydrate on a weight basis, so that the ratio of calories to essential nutrients was altered; therefore, the effect could have been due to a lower consumption of essential nutrients rather than to an effect of fat [162,163,185–191]. In the Min model, high-fat diets were found to increase colon carcinogenesis in two studies but not another [192–194]. Increasing the fat content of the diet has been found to increase the number of ACF induced by colon carcinogens in several but not all studies [182,195–205]. The type of fat (unsaturated vs. saturated) in the diet also produced conflicting results [180,201,206]. ω-3 Fatty acids can also influence colon carcinogenesis: feeding fish oil, microalgal oil, or flaxseed oil in place of corn oil, or eicosapentaenoic acid in place of linoleic acid, decreases the development of DMH- or AOM-induced colon tumors, but adding menhaden oil to a low-fat diet does not affect colon carcinogenesis [182,207–216]. A transgenic model (fat-1 mouse) that has high endogenous levels of ω-3 fatty acids was found to have lower induction of colon tumors induced by AOM and dextran sodium sulfate [217]. Olive oil, high in ω-9 fatty acids, was also found to inhibit colon carcinogenesis when substituted for polyunsaturated fatty acids [212].

D. Pancreatic Carcinogenesis

Dietary fat has been studied extensively in animal models of pancreatic cancer. A common model is induction of pancreatic tumors by azaserine; however, azaserine produces tumors in acinar cells [218], whereas the primary site in humans is the ductal cell. Tumors can be produced in pancreatic ductal cells in rats, by DMBA [219], or in hamsters, by the chemicals *N*-nitroso-bis-(2-oxypropyl) amine (BOP) and *N*-nitroso-bis-(2-hydroxypropyl)amine (BHP) [218]. A number of transgenic models have been developed [218,220]. A number of models used regulatory elements from the rat elastase gene, which targets acinar cells. These constructs produced acinar tumors or mixed acinar/ductal tumors [218,220]. Another model uses an oncogenic K-ras ($KRAS^{G12D}$) inserted into the endogenous K-ras locus [221]. The gene has a Lox-STOP-Lox (LSL) construct inserted upstream. These mice are interbred with mice containing the Cre recombinase downstream from a pancreatic-specific promoter, either PDX-1 or P48. The PDX-1-Cre;LSL-$KRAS^{G12D}$ mice develop pancreatic intraepithelial neoplasia (PanINs), which progress over time [221]. In addition, when these mice are crossed to mice containing p53 mutations or Ink4a/Arf deficiency, a rapid development of pancreatic adenocarcinomas is observed [222,223].

Dietary fat has been found to influence tumorigenesis in mice, rats, and hamsters. In rats, feeding high-fat diets after, or during and after, the injection of azaserine enhances the development of pancreatic tumors and putative preneoplastic lesions [224–232]. Pancreatic carcinogenesis induced in rats by *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine [233] or by DMBA [234] is also enhanced by feeding high-fat diets. In hamsters, BOP-induced pancreatic carcinogenesis is also increased by feeding high-fat diets [230–232,235–239]. Roebuck and colleagues [224,226,229] found that polyunsaturated fat, but not saturated fat, enhanced pancreatic carcinogenesis and that a certain level of essential fatty acids is required for the enhancement of pancreatic carcinogenesis. Increased linoleic acid was also found to increase metastases to the liver in hamsters [240]. Appel et al. [241], however, found that increasing the linoleic acid content of the diet did not increase pancreatic carcinogenesis in either rats or hamsters. Birt et al. [238] found that feeding a saturated fat (beef tallow) enhanced pancreatic carcinogenesis in hamsters greater than a polyunsaturated fat (corn oil). In transgenic models, increasing dietary corn oil led to a higher incidence and size of pancreatic ductal neoplasia in elastase-Kras mice [242] and in PDX-1-Cre;LSL-$KRAS^{G12D}$ mice [243].

Studies using fish oil or *n*-3 fatty acids have produced differing results, depending on the experimental protocol. Substituting fish oil for oils high in polyunsaturated fats decreases [244,245] or does not affect [246] the development of azaserine-induced preneoplastic lesions in rats. Adding fish oil to a diet containing adequate polyunsaturated fatty acids enhances azaserine-induced carcinogenesis in rats and BOP-induced carcinogenesis in hamsters [247–249]. However, Heukamp et al. [250]

found that increasing dietary *n*-3 fatty acids inhibited the incidence but not the number of liver metastases in BOP-treated hamsters compared to hamsters fed with a low-fat diet or a diet enriched in *n*-3, *n*-6, and *n*-9 fatty acids; the incidence of pancreatic adenocarcinomas did not differ among the diets. In addition, Strouch et al. [251] found that increasing dietary *n*-3 fatty acids inhibited precancerous lesions similar to PanINs in elastase-mutant Kras transgenic mice.

Finally, it has been observed in 2-year carcinogenesis studies in which corn oil gavage has been used as the vehicle for the carcinogen that a higher incidence of pancreatic acinar cell adenomas is present in corn oil gavage-treated male Fischer-344 control rats than in untreated controls [252,253]. This association was not observed in female rats or in male or female $B6C3F_1$ mice.

E. Mammary Carcinogenesis

The effect of dietary fat on mammary carcinogenesis in experimental animals has been examined extensively: over 100 experiments have been conducted [254–256]. The primary model used is a rat model (usually the Sprague–Dawley strain) in which mammary tumors are induced by DMBA or MNU. Genetically engineered models have also been developed, including models in which the *Erbb2* gene or simian virus 40 (SV40) T/t-antigens are overexpressed in mammary epithelial cells [257]. The use of these models is advantageous because tumor latency, tumor size, and tumor progression can easily be quantified by palpation of mammary tumors as they appear. Increasing the fat content of the diet clearly enhances the development of mammary tumors [254–256]. In the rat model, a high-fat diet increases tumorigenesis both when it is fed during and after carcinogen administration and when it is fed only after carcinogen injection. More recent studies have examined the role of high-fat diets fed before and/or during gestation, and/or during lactation, or during puberty on mammary carcinogenesis in the offspring. Increasing the level of unsaturated fat (e.g., corn oil) during gestation and lactation increased the number of mammary tumors developing in the offspring, whereas monounsaturated fat (olive oil) had less of an effect, and saturated fat (lard) produced conflicting results [258–261]. Feeding high-fat diets during puberty was sufficient to enhance mammary carcinogenesis [262,263]. Feeding a diet high *n*-3 fatty acids decreases experimental mammary carcinogenesis in experimental animals [254–256,264–266]. A meta-analysis of experimental animal studies found that *n*-6 fatty acids strongly enhanced carcinogenesis, saturated fatty acids were weaker at enhancing carcinogenesis, monounsaturated fatty acids had no effect, and *n*-3 fatty acids weakly (but nonsignificantly) inhibited carcinogenesis [256].

F. Other Sites

Dietary fat has also been studied for its effect on experimental carcinogenesis in other organs. In the lung, dietary fat enhanced BP- or BOP-induced carcinogenesis in hamsters [236,267], whereas in mice a high-fat diet did not affect spontaneous carcinogenesis in one study [106] and produced different results in 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx)-induced tumors [268,269]. Tumor metastasis of Lewis lung carcinoma cells was increased by high-fat diets [270–272]. In the prostate, several studies have found that high-fat diets enhance the growth of transplantable prostate tumors but that inconsistent effects are seen in chemically induced prostate carcinogenesis models [273–279].

IV. SUMMARY AND CONCLUSIONS

Clearly, there is much variability in studies of dietary fat and cancer, both in epidemiological and experimental studies. In epidemiological studies, a relationship between dietary fat and breast cancer has been found in correlational studies, but prospective studies do not support a role for dietary fat. Prospective epidemiological studies examining the role of dietary fat in the development of colon, pancreatic, and prostate cancers have produced conflicting results. The Women's Health Initiative

intervention studies did not show any significant effects for dietary fat in the development of either colon or breast cancer in women. In experimental studies, dietary fat generally enhances chemically induced skin, liver, pancreatic, and mammary carcinogenesis, whereas conflicting results have been seen in colon carcinogenesis. Dietary fat appears to act primarily during the promotional stage of carcinogenesis in all of these models except the liver, where the effect of dietary fat is primarily on initiation.

Because of the variability seen in studies of dietary fat and cancer (particularly prospective epidemiological studies), recommendations for preventing human cancer should not include decreasing the fat content of the diet. This is reflected in recent recommendations for reducing cancer risk by dietary means. For example, in 1997 the American Institute for Cancer Research stated to "limit consumption of fatty foods, particularly those of animal origin" [280]. In their updated 2007 report [281], however, there is no specific recommendation for dietary fat. In addition, the American Cancer Society no longer specifically recommends lowering fat intake, and instead advises individuals to "consume a healthy diet, with an emphasis on plant sources" [282].

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29 Brain Lipids in Health and Disease

Jessica H. Hall and John L. Harwood

CONTENTS

ABBREVIATIONS

Aβ	β-Amyloid protein
AD	Alzheimer's disease
ALA	α-Linolenic acid (n-3 18:3)
APP	Amyloid precursor protein
ARA	Arachidonic acid (n-6 20:4)
DHA	Docosahexaenoic acid (n-3 22:6)
DPA	Docosapentaenoic acid (n-3 or n-6 22:5)
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid (n-3 20:5)
FA	Fatty acid (abbreviated with the number before the colon giving carbon atoms and the figure after giving the number of double bonds. Thus, 16:0 is palmitate, 18:0 is stearate, and 18:1 is octadecenoic acid, usually oleate)
LA	Linoleic acid (n-6 18:2)
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
PtdIns	Phosphatidylinositol
PtdGro	Phosphatidylglycerol
PtOH	Phosphatidic acid
PtdSer	Phosphatidylserine
PUFA	Polyunsaturated fatty acid

I. INTRODUCTION

Next to adipose tissue, the brain is the most lipid-rich organ. Much of this is due to typical membrane lipids—phosphoglycerides, sphingolipids, and cholesterol (Table 29.1). Most of these molecules are commonly found in other tissues [1–3]. However, certain features in the brain are characteristic. For example, this tissue has a high concentration of ether lipids (e.g., ethanolamine plasmalogens) and polyunsaturated fatty acids (PUFAs) (especially arachidonate [ARA] and the n-3 PUFA docosahexaenoic acid [DHA]). In contrast, brain sphingolipids are enriched in saturated or monounsaturated fatty acids. There are also a number of unusual or "atypical" lipids in the brain; *N*-acylphospholipids [4] and certain glycosphingolipids [5] are examples. One aspect of the brain that bears emphasizing is its complexity, whereas, for example, the liver is predominantly one cell type—hepatocytes—the brain has many different cells of varying shapes and sizes. Even when particular brain areas are analyzed and studied, the results are often complicated by the various cells present. This should be borne in mind when attempting to interpret analytical data.

II. LIPID COMPOSITION OF THE BRAIN

Early studies of mammalian brain (Table 29.1) showed that it contained relatively high concentrations of cholesterol and, within the phosphoglycerides, while PtdCho and PtdEtn were major, the concentrations of PtdSer were double those of PtdIns (Table 29.2). Sphingomyelin was a major sphingolipid, while others were, in quantitative order, cerebrosides > gangliosides > sulfatides [6], with cerebrosides being relatively abundant in the white matter [7]. The higher concentration of all lipid classes in the "white" rather than the "gray" matter on a fresh weight basis was also noted [8] (see Table 29.1). If myelin is analyzed specifically, it has a rather similar phosphoglyceride composition to other brain fractions, except PtdSer, which tends to be enriched there (Table 29.3). Cholesterol, sphingomyelin, and cerebrosides are also major lipids (Table 29.1).

TABLE 29.1
Major Lipids in the Human Brain

	Brain Fraction Analyzed		
	Gray Matter	White Matter	Myelin
	(% Dry Weight)		
Total lipids	39.6	64.6	78.0
Phosphoglycerides	21.1	21.5	24.8
Etn	9.2	9.1	11.2
Cho	9.0	8.2	8.3
Ser	2.9	4.2	5.3
Sphingolipids	5.5	21.5	24.5
Sphingomyelin	1.9	5.2	4.4
Cerebroside	2.3	12.3	16.0
Cerebroside sulfate	0.8	3.0	3.4
Ceramides	0.5	0.8	0.7
Cholesterol	7.2	15.1	19.7

Source: Data from O'Brien, J.S. and Sampson, E.L., *J. Lipid Res.*, 6, 537, 1965.
Etn, Cho, and Ser represent ethanolamine, choline, and serine phosphoglycerides, respectively, with no division between diacyl- and ether-phosphoglycerides.

TABLE 29.2
Comparison of the Percentage Phospholipid Composition of Brain from Different Mammals

	Human	Sheep	Rat	Bovine	Rabbit	Guinea Pig
PtdCho	30.3	41.0	36.8	29.8	31.2	35.6
Cho Plasmalogen	n.m	1.0	n.m	n.m	1.0	tr
PtdEtn	36.2	8.3	36.4	12.3	12.7	14.1
Etn Plasmalogen	n.m	18.1	n.m	21.5	22.6	19.7
PtdIns	2.6	2.3	3.1	3.2	3.0	3.2
PtdSer	17.7	10.0	11.8	16.9	15.8	13.5
Cardiolipin	n.d	2.1	2.2	0.7	2.0	1.5
PtdOH	n.d	2.8	1.2	0.5	n.d	1.0
Sphingomyelin	13.2	14.0	5.7	12.7	12.4	9.1
Others	tr	0.4	2.8	2.4	tr	2.3

Source: Taken from White, D.A., The phospholipid composition of mammalian tissues, in Ansell, G.B., Dawson, R.M.C., and Hawthorne, J.N., eds., *Form and Function of Phospholipids*, Elsevier, Amsterdam, the Netherlands, 1973, pp. 441–482, where primary sources will be found.

n.m, not measured; n.d, not detected; tr, trace. In some cases minor lipids such as lysoPtdCho or phosphorylated PtdIns were reported. Where plasmalogens were not measured, the corresponding diacyl-phosphoglyceride will contain this phospholipid.

TABLE 29.3
Percentage of Phospholipid Distributions in Myelin from Brain White Matter

	Human	Bovine	Rat	Guinea Pig	Rabbit
PtdCho	18.6	23.7	25.4	32.4	18.7
Cho Plasmalogen		0.9			
PtdEtn	45.9	10.0	43.6	14.2	36.3
Etn Plasmalogen		33.3		24.6	
PtdSer	16.6	14.3	15.1	14.3	15.4
PtdIns	1.4	2.4	5.0	3.0	2.6
Sphingomyelin	17.2	15.2	8.3	7.5	16.8
Cardiolipin	0.4	n.d	2.5	n.d	7.8
Others	tr	0.2	0.1	4.0	2.4

Source: Recalculated from White, D.A., The phospholipid composition of mammalian tissues, in Ansell, G.B., Dawson, R.M.C., and Hawthorne, J.N., eds., *Form and Function of Phospholipids*, Elsevier, Amsterdam, the Netherlands, 1973, pp. 441–482, where primary sources will be found.

In several cases, the plasmalogen derivatives were not separated from their diacyl analogs.

n.d, not detected; tr, trace.

TABLE 29.4
Changes in the Percentage Distribution of Lipids (% Dry Weight) with Age in Human Brain White Matter

Age	10 Months	6 Years	9 Years	55 Years
Total lipids	49.0	58.4	66.3	64.6
Total phosphoglycerides	20.3	20.4	25.9	21.5
Etn	9.4	8.6	12.0	9.1
Cho	8.6	8.3	8.8	8.2
Ser	2.4	2.5	5.1	4.2
Total sphingolipids	14.3	19.2	19.9	21.5
Sphingomyelin	2.1	2.7	4.9	5.2
Cerebroside	8.5	12.8	10.5	12.5
Cerebroside sulfate	2.5	2.7	3.9	3.0
Ceramide	1.1	0.9	0.5	0.8
Cholesterol	11.5	13.4	13.2	15.1

Source: Taken from O'Brien, J.S. and Sampson, E.L., *J. Lipid Res.*, 6, 537, 1965.
Data expressed at % of dry weight.

As one might anticipate, with age (and, hence, brain development), there are subtle changes in the pattern of lipid classes (Table 29.4). Undoubtedly, this reflects (at least in part) the changing cell content and the cellular distribution of lipids within brain tissue. In the period from 20 to 100 years of age, all major lipid classes (phosphoglycerides, cholesterol, gangliosides) decreased on a wet weight basis in the frontal and temporal cortices [9]. Further analysis revealed subtle changes in the relative proportions of different gangliosides. The effect of the alteration in lipid composition in age-dependent cognitive decline has been reviewed [10]. Of course, because most brain lipids are acyl lipids, the changes in their fatty acid composition are also relevant. The PUFAs, ARA, and DHA are prominent in brain and are considered conditionally essential in the diet of the newborn (see later in this chapter). Observations in both humans and animals suggest that there is proportional increase in fatty acid length and degree of unsaturation from maternal liver to fetal liver, and then, brain. Moreover, the mother's milk composition influenced strongly the fatty acid composition of fetal brain [11]. For cerebral cortex, the fatty acid composition has been studied for subjects aged 2–88 years [12]. Up to 18 years, PUFAs generally decreased with age, with the exception of DHA, which increased. For subjects above 18 years, linoleic acid percentages increased steadily (by 50%) up to 90 years of age, while ARA decreased (about 30%). Other PUFAs, such as DHA, decreased with age but not to a great degree [12].

In agreement with numerous studies on acyl lipid classes in different tissues [3], brain phosphoglycerides have distinct fatty acid contents. For example, phosphatidylethanolamine (PtdEtn) is enriched in 18:0 and 22:6, phosphatidylserine (PtdSer) likewise, while phosphatidylcholine (PtdCho) contains high concentrations of 16:0, 18:0, and 18:1 (Table 29.5, [13]). Notably, white matter has a different fatty acyl distribution by lipid class compared to gray matter (Table 29.5). This is yet another example of the subtle regulation of lipid metabolism within the brain. In addition, age-related changes were found for some phosphoglyceride classes and, moreover, whereas PtdEtn and PtdSer contained an abundance of 18:0 and 22:6 in human cerebral gray matter, in white matter 18:1 was also a major constituent. PtdCho had significant 16:0 in both brain tissues and much less PUFA [14].

Two recent advances in technology have impacted on lipid analysis. First, the development of mass spectrometry has led to the so-called lipidomics revolution. Second, application of mass

TABLE 29.5
Fatty Acid Compositions of Different Phosphoglycerides from Human Brain

	Gray Matter			White Matter		
	Etn	Cho	Ser	Etn	Cho	Ser
14:0	0.2	2.9	0.3	0.5	1.3	0.3
16:0	6.7	45.0	2.3	6.7	34.3	1.7
16:1	0.4	5.1	0.3	1.4	1.0	0.4
18:0	26.0	9.3	25.4	9.0	13.4	35.8
18:1	11.9	31.4	21.5	42.4	45.2	39.7
20:4 n-6	13.8	4.1	1.6	6.4	1.3	2.0
22:5 n-6	14.3	—	5.0	13.7	—	4.8
22:5 n-3	tr	—	3.3	0.5	0.3	0.9
22:6 n-3	24.3	3.1	36.6	3.4	0.1	5.6

Data as % of total fatty acids. Lipids were extracted from the brain of a 55-year-old human and data represent gray and white matter. See O'Brien and Sampson [13] for details. Only the main fatty acids are listed.

Abbreviations: tr, trace. Fatty acids are listed with the carbon number and the number of double bonds separated by a colon. Where the double bond position was reported, it is noted.

spectrometry (and analogous) techniques has allowed sophisticated analysis of individual areas of the brain—thus revealing differences in metabolism (and function) within different regions.

When detailed analysis of phosphoglyceride molecular species was carried out for cerebral cortex and hippocampus (both regions of high relevance to Alzheimer's disease (AD) and other complaints affecting cognition), there were few significant differences in the distribution of arachidonate (ARA) or DHA-containing phosphoglyceride fractions. Indeed, any differences between the cerebral cortex and hippocampus were almost entirely due to the quantity (per gram fresh weight) rather than to the percentage distribution of individual molecular species. The overall content of ARA and DHA within phosphoglycerides is shown in Table 29.6. Thus, it will be seen readily that DHA tends to be esterified in

TABLE 29.6
Distribution of Arachidonate and Docosahexaenoate within Different Phosphoglycerides of Mouse Brain

	μmol/g Tissue	
	Arachidonate	Docosahexaenoate
PtdCho	2.49	0.76
PtdSer	0.32	1.06
PtdEtn	3.30	5.02
PtdIns	2.19	1.02
Nonpolars	1.90	0.83

Source: Calculated from Axelsen, P.H. and Murphy, R.C., *J. Lipid Res.*, 51, 660, 2010.

PtdEtn and PtdSer preferentially (as noted earlier) while ARA is enriched in PtdCho and, particularly, in phosphatidylinositol (PtdIns). A detailed analysis of molecular species was also reported [15]. The latter examination has revealed that, in many cases, the predominant molecular species distributions in cerebral cortex or hippocampus are rather similar [15]. Thus, for example, PtdSer really consisted of almost entirely (>90%) of single molecular species (Table 29.7) in both regions [15].

These distinct fatty acid distributions within different phosphoglyceride classes reflect well earlier analysis of human gray or white matter (and comparisons with other mammals). Thus, the major phosphoglyceride, PtdCho, contained palmitate, stearate, and oleate as major fatty acids and rather small levels of PUFAs (Table 29.8). In contrast, the other major phosphoglyceride, PtdEtn, was enriched in DHA but still with significant (8%–13%) ARA (Table 29.9). The phosphoglyceride with the highest content of ARA was PtdIns (Table 29.10). The enrichment of stearate in this phosphoglyceride also accounted for the high level of the 18:0/20:4 molecular species in PtdIns (Table 29.7). PtdSer in human brain was reported to contain high levels of 18:0 and 22:6 in gray matter (Table 29.11). Again, this is reflected well in the very high levels of 18:0/22:6 in the PtdSer fraction of mouse cerebral cortex (Table 29.7, [15]).

Further analyses of molecular species of phosphoglycerides and sphingomyelin have been documented for bovine tissue [16] and humans [17]. The enrichment of DHA in PtdSer may be partly explained by the substrate preference of phosphatidylserine synthase 2 [18]. Moreover, the characteristic molecular species profiles of phosphoglycerides from different tissues have been

TABLE 29.7
Major Molecular Species of Phosphoglycerides Containing Arachidonate or Docosahexaenoate in Mouse Cerebral Cortex

Lipid	ARA Species	DHA Species
PtdGro	16:0 = 18:0	16:0 > 18:0 = 18:1
PtdOH	18:0 > 16:0	18:0[a]
PtdIns	18:0 > 16:0	20:0 = 22:0 > 16:0 = 18:0 = 20:0p
PtdSer	18:0[a]	18:0[a]
PtdCho	18:0 = 16:0 > 18:1	16:0 > 18:0 > 18:1
PtdEtn	18:0 > 16:0 = 16:0p = 18:0p = 18:1p	18:0 = 18:0p > 16:0 = 16:0p

Data recalculated from Reference 15. The "p" denotes ether derivatives (e.g., plasmalogens).
[a] Essentially a single molecular species.

TABLE 29.8
Major Fatty Acids of Brain Phosphatidylcholine (% Total by Weight)

	14:0	16:0	16:1	18:0	18:1	18:2	20:4	22:5	22:6
Human (white matter)	1.3	34.3	1.0	13.4	45.2	0.4	1.3	0.3	0.1
Human (gray matter)	2.9	45.0	3.1	9.3	31.4	0.4	4.1	—	3.1
Rat	0.3	45.0	1.4	13.8	32.3	—	5.1	—	—
Chicken	0.7	51.0	tr	16.5	26.3	0.7	3.9	0.4	0.6

Source: Taken from White, D.A., The phospholipid composition of mammalian tissues, in Ansell, G.B., Dawson, R.M.C., and Hawthorne, J.N., eds., *Form and Function of Phospholipids*, Elsevier, Amsterdam, the Netherlands, 1973, pp. 441–482, where primary sources will be found.
Abbreviation: tr, trace.

TABLE 29.9
Major Fatty Acids of Brain Phosphatidylethanolamine (% Total by Weight)

	14:0	16:0	16:1	18:0	18:1	18:2	20:4	22.4	22:5	22:6
Human (white matter)	—	5.9	0.4	30.4	8.7	0.5	13.2	8.3	2.0	28.6
Human (gray matter)	—	6.2	1.1	13.8	43.2	0.5	7.9	13.4	0.8	3.0
Bovine (gray matter)	0.3	8.4	0.8	28.5	13.2	0.2	13.2	—	6.7	27.3
Chicken	1.6	17.9	1.1	28.5	12.4	0.4	12.6	4.4	2.0	17.5

Source: Taken from White, D.A., The phospholipid composition of mammalian tissues, in Ansell, G.B., Dawson, R.M.C., and Hawthorne, J.N., eds., *Form and Function of Phospholipids*, Elsevier, Amsterdam, the Netherlands, 1973, pp. 441–482, where primary sources will be found.

TABLE 29.10
Major Fatty Acids of Brain Phosphatidylinositol (% Total by Weight)

	16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:4	22:6
Human (white matter)	15.9	2.1	32.4	19.5	0.5	5.1	17.3	2.4	1.1
Human (gray matter)	14.0	1.8	34.9	12.6	0.6	1.8	28.2	1.5	4.0
Bovine	7.9	0.2	37.7	10.0	1.2	4.8	33.8	tr	1.0
Cat	10.5	2.9	31.4	10.5	3.7	—	30.8	—	—

Source: Taken from White, D.A., The phospholipid composition of mammalian tissues, in Ansell, G.B., Dawson, R.M.C., and Hawthorne, J.N., eds., *Form and Function of Phospholipids*, Elsevier, Amsterdam, the Netherlands, 1973, pp. 441–482, where primary sources will be found.
Abbreviation: tr, trace.

TABLE 29.11
Major Fatty Acids of Brain Phosphatidylserine (% Total by Weight)

	16:0	16:1	18:0	18:1	20:1	20:3	20:4	22:4	22:6
Human (white matter)	1.8	0.6	45.7	38.1	3.5	0.9	1.4	2.0	0.9
Human (gray matter)	2.9	0.6	43.6	10.7	0.7	0.9	2.0	5.0	30.7

Source: Taken from White, D.A., The phospholipid composition of mammalian tissues, in Ansell, G.B., Dawson, R.M.C., and Hawthorne, J.N., eds., *Form and Function of Phospholipids*, Elsevier, Amsterdam, the Netherlands, 1973, pp. 441–482, where primary sources will be found.

suggested to be useful for their identification [19]. In further developments of mass spectrometry, adaptations can be used for the *in situ* analysis of lipids in brain. Early studies used the positive ion mode for PtdCho, PtdEtn, sphingomyelin, and cholesterol, while the negative ion mode was utilized for PtdIns, sulfatide, and gangliosides [20,21]. Further refinements allowed the structural definition of molecular species and suggested regional differences in concentrations of particular molecular species [22]. Quantitative aspects of the measurements still remain a problem [22], although step-wise advances are being made [23]. Of note is the widespread use of formalin fixation before MALDI-MS (matrix-assisted laser desorption/ionization mass spectroscopy).

The knowledge gained from detailed studies of this procedure should permit the future analysis of archival tissue [24].

In a further analytical advance concerning direct imaging of the lipid distribution in brain, positron emission tomography (PET) has been used. Positron-emitting [^{11}C]-derivatives of ARA and DHA were synthesized and used in human volunteers. The data not only did show differences in various brain regions but also permitted calculation of rates of uptake and of half-lives of the fatty acids in brain. The latter was estimated to be around 2.5 years for DHA in human brain [25].

III. METABOLIC CONSIDERATIONS

Pathways for the synthesis and catabolism of brain lipids are the standard ones in mammals. Details can be found in [1,2,26]. However, there are particular aspects that have attracted attention. Notably, because of the high and functionally important content of long-chain PUFAs (ARA, DHA) in the brain, there have been questions about whether the brain is capable of synthesizing all of its own ARA and DHA from the usual dietary 18C precursors, linoleate and α-linolenate (ALA), respectively, or whether it is reliant on plasma-derived and, hence, liver-produced material. Moreover, because ARA and DHA are functionally important and the desaturases involved in their formation are low in activity, there is also the question about whether dietary ARA and DHA are needed or whether the more abundant dietary levels of linoleate (and ALA) are sufficient. This aspect is addressed below.

Nutrient deprivation of n-3 PUFAs in rats has been found to have several effects. First, the half-life of DHA in brain phosphoglycerides was increased two-fold or greater, thus conserving the existing brain levels [27]. In contrast, docosapentaenoate (n-6 DPA) showed an increased turnover as well as elevated levels [28]. At the same time, dietary n-3 PUFA deprivation did not affect the rate of conversion of linolenate to DHA within the brain. Under these conditions though, the liver converts more linolenate to DHA and is the main source when DHA is absent from the diet [29]. In parallel, experiments on the production of ARA indicate that linoleate is a negligible source in adult rats where this acid is largely β-oxidized [30]. It will also be interesting to discover the quantitative function of the G protein–coupled receptor 120 (GPR 120), which acts as an n-3 PUFA receptor/sensor, in brain tissue [31].

A detailed review has addressed specifically the evidence as to whether dietary ALA can act as an adequate metabolic source for the very long-chain n-3 PUFAs, eicosapentaenoic acid (EPA), and DHA of brain [32]. The conclusion was that ALA is an important dietary PUFA that, under most circumstances, can generate DHA in sufficient quantities despite its high rate of β-oxidation. The importance of the diet in providing suitable amounts of PUFAs for brain function is addressed in the next section.

IV. DIETARY EFFECTS AND RECOMMENDATIONS

Cunnane, about 10 years ago, raised the following question: "What is an essential fatty acid (EFA) ?" [33]. Following on from the pioneering work of Burr and Burr around 1930, one could say that two acids fit the definition: linoleic acid and α-linolenic acid [34,35]. However, there are conditions where other PUFAs may be conditionally essential. A notable example would be for infants and, indeed, infant milk formulae routinely include ARA and DHA to make sure they are not in deficit [33]. The main problem here is that infants cannot make sufficient ARA and DHA to guarantee normal brain development [36–38]. There is also the question of getting the correct dietary ratio of n-6 to n-3 PUFAs [39,40]. Modern thinking suggests that a ratio of around 4 is desirable. This is due to the fact that conversion of ARA to biologically active metabolites (eicosanoids, etc.) produces mainly proinflammatory molecules while EPA (and DHA) forms non- or anti-inflammatory substances [39–41].

Over 40 years ago, it was noted that there was a reciprocal relationship between n-6 and n-3 PUFAs when feeding different diets [42]. This could be expected since production of ARA and EPA (as well as DHA) uses the same enzymes [1,39], so as ARA goes up EPA will go down, and vice versa. It should also be noted that diet also often affects expression of (lipid-metabolizing) genes [1]. In addition, n-3 PUFA-enriched diets could have different effects dependent on the precise nature of the n-3 PUFA. Thus, perilla oil (enriched in ALA) had little effect on PtdEtn composition in contrast to fish oil (enriched in EPA and DHA) [43]. Nevertheless, both diets affected expression (increased or decreased) of around 100 genes [43]. In addition, increased dietary n-3 PUFAs resulted in less enhancement in the striatum compared with the cortex and hippocampus. There were also marked differences in the response of different lipid classes with PtdCho and PtdEtn showing significant changes in rat brain. A lipidomics approach added further details to these dietary-induced alterations [44].

Fish oil–enriched diets increased DHA (and reduced ARA) in brain phosphoglycerides such as PtdEtn. Simultaneously, there was better cognitive behavior [45]. Conversely, n-3 PUFA deficiency led to differential effects on brain hippocampus phosphoglycerides. Thus, while DHA was usually replaced by n-6 DPA, molecular species alterations could be quite distinct. Although the overall phosphoglyceride content was unchanged, PtdSer was decreased and PtdCho increased. It was suggested that the overall loss of DHA molecular species along with PtdSer reductions could be key biochemical changes underlying losses in hippocampal function [46]. Whereas many animal dietary-deficiency experiments have used two generations of feeding, use of a first generation model has been suggested to resemble human conditions better [47]. In such regimes, with n-3 deficient diets, brain DHA was replaced with n-6 DPA as in previous observations.

The aforementioned reciprocal changes in DHA and n-6 DPA are of interest because one source of DHA for infant formulae and other nutraceuticals is from the microalgae *Schizochytrium* species that contains both fatty acids. When n-6 DPA was fed, it *only* replaced DHA in the latter's dietary absence. The implication of this observation is, of course, that nutritional supplements made from *Schizochytrium* oil would be a suitable dietary source of DHA [48]. In other experiments, it was shown that not only n-6 DPA but also other n-6 PUFAs such as ARA would replace DHA in the brain of rats on n-3 PUFA-deficient diets [49]. Of interest also is the demonstration that changes in brain DHA brought about by diet occur significantly quicker in young animals compared to adults [50].

Two of the problems with using "plant PUFAs" as a source of EFA under all conditions is that they are poorly converted to ARA and DHA and, in addition, are oxidized at high rates (Table 29.12). Indeed, human males convert lesser than 5% of available dietary ALA to DHA. Thus, for good health, an intake of "fish oil type" n-3 PUFAs (EPA, DHA) is recommended. Not only are such

TABLE 29.12
β-Oxidation Rates of Common Dietary Fatty Acids

	PA	SA	OA	LA	ALA
In vivo					
Human	87	30–70	106–160	100	146–169
Rat	53–63	21–49	116	100	135
In vitro					
Rat liver mito	7–75	4–29	15–54	100	140–157

Source: Data taken from Cunnane, S.C., *Prog. Lipid Res.*, 42, 544, 2003, where more details can be found.

Data were compared to results for linoleic acid. PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid.

TABLE 29.13
Cunnane Classification of PUFAs

Pregnancy, Lactation, Childhood		Adults (>20 Years Old)	
Conditionally Dispensable	**Conditionally Indispensable**	**Conditionally Dispensable**	**Conditionally Indispensable**
EPA	LA	LA	ALA
	ALA	ARA	
	ARA	EPA	
	DHA	DHA	

Source: Data taken from Cunnane, S.C., *Prog. Lipid Res.*, 42, 544, 2003, where further discussion will be found.
For fatty acid abbreviations, see Abbreviations section.

PUFA needed for adequate brain function but also they are thought to be beneficial for cardiovascular and other diseases. The varying needs for dietary PUFAs led Cunnane to propose the additional criteria of "conditionally" indispensable to reflect the extra needs of a young mammal where brain development is active [33] (Table 29.13).

The widespread evidence from epidemiology and dietary intervention studies for the health-beneficial properties of very long-chain n-3 PUFAs (EPA, DHA) [51–54] has led to an increased demand for fish oils. The latter come either from cold water marine fish (which are under threat and considered unsustainable) or from aquaculture, which, ironically, needs fish-derived oils in the feed. Although the primary source of n-3 very long-chain PUFAs are marine microbes such as algae, their culture to supply such acids is currently too expensive for the supply of bulk nutraceutical markets. Accordingly, various groups in Australia, Europe, and the United States have developed methods to modify land crops to produce EPA and DHA [55–58]. This is not a trivial matter because it involves the production and subsequent introduction of multiple gene cassettes [59] and overcoming certain constraints in the plant metabolism of PUFAs. Nevertheless, manipulations of crops such as camelina, oilseed rape, and soybean have shown good success in field trials [60].

Roughly 1% of energy uptake as linoleate is thought to satisfy the n-6 PUFA requirements of healthy adults. Conditions such as pregnancy or early development raise this figure up to 3%. However, present consumption in Western countries is around 8% for adults, that is, much more than needed [33]. As mentioned previously, a ratio of n-6/n-3 PUFA of about 4 is also considered optimal.

V. EFFECTS OF PUFAs ON COGNITIVE FUNCTION

Due to an aging population, dementia is increasingly becoming a major health problem with an estimated 24 million sufferers worldwide, and there is a growing body of evidence that indicates a link between dementia and lipids [62]. AD is the most frequent cause of dementia, accounting for approximately 60% of all cases [63]. The two major pathological hallmarks of AD are neurofibrillary tangles and amyloid plaques, with gradual changes in levels of β-amyloid (Aβ) being thought to initiate the amyloid cascade [64]. A buildup of soluble forms of Aβ causes plaque formation, which, upon deposition, causes inflammatory responses resulting in an observed synaptic loss and neuritic dystrophy, ultimately leading in turn to oxidative stress [65]. Neurofibrillary tangles are then thought to be induced secondary to plaques, due to altered kinase and phosphatase activity, which causes hyperphosphorylation of tau, thus culminating in widespread neuronal dysfunction [66].

Short-term memory loss is the most prevalent clinical feature in the early stages of AD, with cognitive impairments extending to other domains as the illness progresses, such as affecting movement, recognition, social behavior, and even manifesting as mood and behavioral changes [67]. There is evidence for an important role of lipids, specifically PUFAs and their metabolites in brain function and cognition, with PUFAs playing a key part in the regulation of neuronal structure and function [68]. The ability of lipids to be manipulated by dietary intervention provides an opportunity to explore a preventative approach, an important prospect based on the current lack of ability to improve an AD prognosis post-diagnosis.

A. Cholesterol and Sphingolipids

The brain is highly enriched in lipids [69]; in order to understand the potential therapeutic benefits of dietary lipid alterations, it is important to consider how lipids influence the neurological molecular mechanisms, leading to Aβ accumulation. One important brain lipid is cholesterol, which is an essential component of cell membranes. It has, however, also been observed that cholesterol stimulates β and γ secretase and, therefore, can have a strong impact on APP processing [70]. Thus, high levels of cholesterol have been shown to promote amyloid aggregation, whereas reduced cholesterol leads to a decline in β-amyloid aggregation in AD [71].

In addition, the sphingolipids are a group of lipids (synthesized from palmitoyl-CoA), which are essential for normal brain function. Moreover, evidence suggests altered sphingolipid metabolism during AD progression [70]. The sphingolipids play a major role in cellular signaling pathways; for example, sphingomyelin is a major source of ceramides, which are generated upon the cleavage of sphingomyelin by sphingomyelinases [72]. Sphingomyelinases are activated upon oxidative stress and, thus, ceramides are increased, which can stimulate apoptosis [73]. Data from studies in which neurons were exposed to Aβ also suggest that this neurotoxic peptide may be responsible for membrane lipid abnormalities in AD [74]. Thus, normal aging (together with genetic and environmental factors) results in increased oxidative stress and increased production of Aβ, which in turn perturbs cholesterol metabolism, activating sphingomyelinases and, thus, increasing ceramide production, which ultimately further increases the accumulation of cholesterol in cells [74]. The increased levels of ceramide and cholesterol then further exacerbate Aβ production and, thus, oxidative stress and ultimately trigger synaptic dysfunction and apoptosis [74]. Therefore, the role of altered metabolism of cholesterol and sphingomyelins in AD may suggest a therapeutic target downstream of Aβ.

B. Docosahexaenoic Acid in the Brain

It is also important to consider lipid changes that may be of benefit prior to the accumulation of Aβ aggregates. Notably, PUFAs are implicated in several processes within the brain, including neurotransmission, cell survival, and neuroinflammation, which, thereby, affect cognition and mood [68]. Thus, a role for PUFAs in cognition has been explored. DHA has been extensively implicated in AD [75]. DHA cannot be synthesized *de novo*, but must be provided by the diet either directly or by synthesis from dietary α-linolenic acid [76], thus providing an opportunity to alter DHA levels easily. The main dietary source of DHA currently is from fatty fish and fished-based supplements such as cod liver oil [77]. DHA and its metabolites are known to have potent antiinflammatory neuroprotective properties. It has been shown to reduce the production and activity of proinflammatory cytokines [78] and, in addition, gives rise to lipid signaling molecules including the resolvins RvD1–RvD4 and neuroprotectin D1, which are potent antiinflammatory mediators [79].

The role of DHA has been studied both by epidemiologic studies focusing on DHA levels and AD incidence and also by investigating the impact of DHA supplementation on AD symptoms. Three major epidemiological studies point toward an increased risk of AD when DHA in the diet is low. The Rotterdam study [80] focused on different components of fat intake (including total fats, saturated fat, cholesterol, and PUFAs) by assessing food intake in nondemented participants and

following up with a dementia screening protocol. They concluded an increased risk of dementia with a high saturated fat and cholesterol diet, and high fish consumption (19 g fish/day) was inversely related to incident dementia. More specifically, Barberger-Gateau and colleagues [81] studied data in older fish consumers from Bordeaux and found that regular fish consumers were, in general, more educated and had better cognitive performance. The Chicago study [82] supported the conclusion that high dietary intake of n-3 PUFAs can reduce the risk of AD incidence when they found that participants who consumed fish once or more per week had a 60% lesser risk of AD compared to participants who rarely ate fish. This conclusion was similar to the Rotterdam and Bordeaux studies. A further study also examined the association between DHA levels on cognitive function in a cohort of institutionalized elderly people [83]. Fatty acid intake was measured and cognitive ability was subsequently assessed and revealed that DHA consumption was predictive of the level of cognitive impairment. Ultimately, an appreciable fish consumption in the diet acts as a good source of dietary PUFAs, resulting in a significantly reduced risk of developing AD.

Considering that the risk of AD is increased when dietary DHA is low, then it follows that large reductions in brain DHA have been shown to be associated with impaired cognitive function [84]. A number of studies have also focused on phospholipid profiles in the postmortem brains of AD patients, and there is evidence for a significant reduction in phosphatidylethanolamine (PtdEtn), one of the main phospholipids containing DHA, in the frontal cortex and hippocampus of AD patients [85]. A further postmortem study by Han [86] utilized multidimensional mass spectrometry in various brain regions to establish that PtdEtn was significantly lower in the cortex of AD patients than in control individuals. Consequently, alterations of DHA in the brain appear to occur in AD.

The evidence that high dietary DHA is consistent with a decreased risk of AD, together with studies demonstrating low DHA in the brains of AD patients means that it is sensible to examine the effects of DHA supplementation on cognitive parameters. A study conducted by Fontani et al. [87] tested healthy subjects with a battery of cognitive tasks prior to supplementation and 35 days following supplementation with n-3 PUFAs. Following supplementation, there was an improvement of attention, with participants showing significant improvements on go-no-go and sustained attention tests. In further animal experiments, when neurons were treated with DHA, there was promotion of hippocampal neurite growth and synaptogenesis [88], which emphasizes the importance of DHA in supporting hippocampus-related cognitive function. However, it is important to note that dietary DHA only appears to be of benefit to healthy individuals [89]; once AD symptoms are exhibited, there seems to be no beneficial effect [90]. Consequently, dietary PUFAs should be considered a preventative measure as opposed to a tool by which AD symptoms could be alleviated. Not only were cognitive abilities improved with DHA, but Fontani et al. [87] also examined mood profiles both prior to and following the dietary addition of n-3 fatty acids and found that the mood profile was also improved, with increased vigor and reduced anger, anxiety, and depression states. Whether improved mood was a direct result of n-3 fatty acids in the diet or a result of reduced frustration due to an improvement in cognitive abilities is unclear, but it is important to note that the benefits of dietary lipid changes are not restricted to executive function.

C. Plasmalogens

Plasmalogens are essential membrane phospholipids in the human nervous system, and decreases in brain plasmalogens are one of the earliest biochemical changes that are found in AD [91]. Amyloid precursor protein (APP) processing has been found to increase the expression of the enzymes necessary for regulating the synthesis of plasmalogens [70] and, thus, plasmalogen changes are likely a consequence of AD. Studies have examined the relationship between plasmalogen levels and scores on AD-cognitive assessment scales and discovered that low plasmalogen levels were associated with poor scores on cognitive tasks [91]. The severity of plasmalogen decreases has also been linked to the severity of the dementia symptoms displayed by AD patients [92]. Thus, serum plasmalogen levels could essentially be used as a diagnostic predictor of cognitive decline in AD patients.

One mechanism by which plasmalogen reduction is thought to affect cognition is abnormal ion channel function; it is thought that decreases in plasmalogen levels may underlie subsequent cholinergic dysfunction, which underlies cognitive deficits [92]. The decreased neurotransmitter release associated with reduced plasmalogens can, however, be corrected with a DHA-rich diet [93].

VI. USE OF ANIMAL MODELS FOR THE STUDY OF LIPIDS IN ALZHEIMER'S DISEASE

In order to study and understand the etiology and pathology of AD, animal models of the disease have been developed. Many models have been generated using a mutated human APP gene in order to cause APP overexpression in mouse models. For example, the Tg2576 mouse model [94] expresses the human APP with the Swedish mutation, while the PDAPP mouse [95] expresses human APP with the Indiana mutation; both present with Aβ pathology. Other APP models also exist (e.g., APP23 mouse, TgCRND8 mouse) [96], alongside models that express APP together with other mutant transgenes, such as presenilin 1, and mouse models that express mutant tau (for a review, see [97]). Animal models provide the opportunity for elegant experimental designs, which control for variables that can seldom be achieved in human studies. Rodent studies allow for the establishment of a cognitive deficit in the animals and, as the models show many features of the human AD pathology, they provide an opportunity to study the effects of lipid profiles on Aβ aggregation. Animal models also allow an examination of the effects of dietary intervention on AD pathology and cognition.

Rodent models have frequently been used to study the effect of DHA on learning and memory and on Aβ pathology. As in humans, rats deficient in DHA have been shown to display cognitive deficits [98]. In a study carried out by Lim and colleagues [99], newborn rat pups, which were reared to be deficient in DHA, were found to have reduced brain DHA and showed poor performance in the Morris water maze compared to rats that were reared with adequate levels of DHA. This demonstrates the importance of DHA for an optimal performance on spatial learning tasks. Emphasizing this requirement of DHA for optimal cognitive function, supplementation with DHA has also been shown to improve cognition in transgenic mouse models of AD. When Tg2576 mice were supplemented with DHA, their ability to remember the location of a hidden platform in the Morris water maze significantly improved in comparison to Tg2576 mice that were not supplemented with DHA [100], indicating a beneficial effect of dietary DHA in AD mouse models.

Animal models have also allowed for the study of the mechanism(s) by which changes in cognition occur with varying dietary DHA levels. A study carried out on aged Tg2576 mice explored how different levels of DHA supplementation (none, low, or high) affected aggregation of Aβ in the brains of the animals [99]. It was evident that a high DHA diet was effective in significantly reducing Aβ burden, decreasing total Aβ by as much as 70% when compared to low DHA and control diets. In addition, imaging studies revealed a 40% reduction in plaques. The greatest changes were seen in the hippocampus, again supporting the role of DHA in aiding in hippocampal-dependent forms of learning and memory. Further studies have also supported this claim. Nine-month-old APPswe/PS1dE6 mice were given different experimental diets for 3–4 months, varied in n-3 PUFAs, cholesterol, and saturated fats [101]. Hippocampal Aβ levels were then assessed using an enzyme-linked immunosorbent assay, and it was shown that a diet rich in DHA decreased Aβ levels, whereas diets rich in cholesterol and saturated fat increased Aβ levels. This emphasizes the role of DHA as a potential tool for reducing Aβ production in AD. Furthermore, DHA has also been shown to improve the rate of tau hyperphosphorylation in addition to Aβ reductions in 3xTg-AD mice [102]. 3xTG-AD mice were fed with different DHA-containing diets: DHA, DHA-DPA, DHA-ARA, or a control diet. DHA-containing diets reduced Aβ accumulation up until 6 months of age, after which DHA diets, which were combined with n-6 fatty acids, started to show reduced efficacy, whereas the DHA diet alone continued to show Aβ reduction up until 12 months of age. It appeared that this decrease in Aβ as a result of DHA supplementation occurred as a result of a reduction in PS1

expression as opposed to an alleviation of APP expression. In addition, it was also shown that there was a reduction in the accumulation of phosphorylated tau in the brains of the animals when DHA was combined with DPA.

In summary, lipid alterations in the brain, namely, alterations in cholesterol, sphingolipids, plasmalogens, and PUFAs, are linked to an increased risk of AD. In particular, there is considerable evidence for a role for DHA in cognitive processing. Postmortem and animal studies have linked low levels of DHA with poor cognition. Moreover, it appears that dietary supplementation with DHA can have beneficial effects on cognition and mood, and these cognitive changes are paralleled with biochemical changes in the form of lower Aβ levels, reduced plaque formation, and decreased hyperphosphorylation of tau. Thus, understanding lipid changes, which occur in animal models of AD, allows the opportunity to manipulate lipid consumption in order to utilize dietary supplementation as a preventative tool for cognitive deficits. Such data provide additional support for the translation of results from animal experiments to humans.

VII. CONCLUSIONS

It will be clear from the foregoing text that lipids not only are major constituents of mammalian brain but also have vital functions there. PUFAs are an important example, particularly as there is uncertainty as to whether sufficient amounts of ARA and DHA can be synthesized *in vivo*. Therefore, dietary requirements have occupied much attention. As a supplement to the discussion in Section V, Tables 29.14 and 29.15 summarize recommendations for dietary advice. Both n-6 and n-3 EFA are needed, but the balance is very important. Furthermore, requirements differ with age as well as in relation to diseases.

Although our knowledge of brain lipid metabolism and function has increased considerably in the past decade, there is much more to learn. For a research scientist, the future is exciting in that many really fundamental questions need to be solved. We hope that the information in this chapter will help to facilitate experiments to gain the necessary understanding of brain lipid biochemistry.

TABLE 29.14
Consensus of Recommendations on n-3 PUFA Intakes (2015)

	EPA and DHA	n-3 PUFA	Fish Portions
General population	200–2000 mg/day	1%–2% energy as n-3 PUFA	2 fish portions/week (at least 1 oily fish)
Individuals with CVD risk	0.5–1 g/day		
Pregnant and lactating females	100–300 mg/day	1.8 g/day	
Infants	70–150 mg/day		

Expert bodies from world (e.g., WHO, NATO), Australia, Europe, Brazil, United States, China, Malaysia, Japan surveyed.

TABLE 29.15
ISSFAL Recommendations

Adequate Linoleic Acid Intake	*Healthy* α-Linolenic Acid Intake	*Minimum* EPA and DHA Combined Intake
2% energy	0.7% energy	500 mg/day

Recommendations for intake of polyunsaturated fatty acids in healthy adults—July 2004.

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30 Biotechnologically Enriched Cereals with PUFAs in Ruminant and Chicken Nutrition

Milan Čertík, Tatiana Klempová, Dušan Jalč, Zora Váradyová, and Slavomír Marcinčák

CONTENTS

I. INTRODUCTION

Cereals are staple foods used directly for human consumption and indirectly via livestock feed. Cereals as one of the most important energy sources and the main human source of nutrition provide various macronutrients (proteins, lipids, carbohydrates, and dietary fiber) and biologically active micronutrients (minerals, vitamins, phenolic compounds, and phytosterols) [1]. Nevertheless, cereals are deficient in certain health-promoting components such as essential polyunsaturated fatty acids (PUFAs) and contain only limited amounts of other lipophilic constituents (e.g., carotenoids, coenzyme Q10). Therefore, a diet based primarily on cereal grains may not only encourage an improper dietary equilibrium of these metabolites but also lead to increased incidence of various diseases resulting from insufficient intake of PUFAs [2]. From this point of view, could cereals be

considered as useful sources of functional foods/feed with biologically active PUFAs? Surely yes, providing that metabolic composition of cereals will be modified appropriately.

Natural construction or modification of cereals for preparation of functional cereal-derived food/feed products containing biologically active PUFAs might be promising and challenging technology in this field. Biotechnological methods are convenient techniques to reach this goal. The chapter focusses on preparation of functional cereal-based materials by fungal solid-state fermentations (SSFs) and their application in ruminant and chicken nutrition.

II. IMPORTANCE AND SOURCES OF PUFAs

A. Importance of PUFAs

PUFAs are indispensable for human well-being due to their healthy, dietary, and functional properties [3]. Attention is mainly paid to γ-linolenic acid (18:3 n-6, GLA), dihomo-γ-linolenic acid (20:3 n-6, DGLA), arachidonic acid (20:4 n-6, AA), eicosapentaenoic acid (20:5 n-3, EPA), docosapentaenoic acid (22:5 n-3 or n-6, DPA), and docosahexaenoic acid (22:6 n-3, DHA). Because mammals do not possess the enzymes Δ12-desaturase (catalyzes conversion of oleic acid to linoleic acid) and Δ15-desaturase (transforms linoleic acid to α-linolenic acid), other PUFAs belonging to both n-6 (GLA, DGLA, AA) and n-3 (EPA, DHA) pathways cannot be synthesized by mammals when linoleic and α-linolenic acids are not supplemented in the diet. Significances of PUFAs (n-6 and n-3) due to their unique structural and functional properties are clear from their two main roles in cells: (1) regulation of membranes architecture and permeability and (2) precursors of metabolites controlling critical biological functions [3].

It is apparent that PUFAs are required in every organ in the mammalian body. Therefore, insufficient dietary consumption of PUFAs leads to abnormalities in the skin, diabetes, hypertension, coronary heart disease, abnormalities in the endocrine, nervous, inflammatory, immune, respiratory, and reproductive systems, rheumatoid arthritis, and premenstrual syndrome [4]. Besides the overall intake of PUFAs, the ratio of n-6/n-3 PUFAs families is important for balanced synthesis of eicosanoids and for proper maintenance of body health. Optimal n-6/n-3 ratio is always a matter of debate and may depend on the age, physiological, and clinical status of the individual. Nutritional scientists suggest the ideal n-6/n-3 ratio as from 2:1 to 4:1, although the ratio is strongly influenced by a local PUFA sources and consumer habitats [5].

B. Plant and Animal Sources of PUFAs

Some plant seeds are the main oil sources of C_{18} PUFAs with dominant synthesis of linoleic acid (18:2 n-6, LA) and α-linolenic acid (18:3 n-3, ALA) [5]. GLA is naturally occurring only in a few plants such as oils from seeds of evening primrose (8%–10%), borage seeds (24%–25%), black currant seeds (16%–17%), and echium (10%–13%) and these are the most commercially important sources of GLA. PUFAs above C_{18} cannot be synthesized by higher plants in any significant amounts owing to a lack of necessary enzymes [6].

The marine fish oils (cod, tuna, and mackerel) have been described for their content of C_{20} and C_{22} n-3 PUFAs (notably, EPA, DPA n-3, and DHA). Amount of n-3 fatty acids depends on the fish species, ability to use microorganisms as their primary food source, season, and geographic location [6]. Freshwater fish accumulates mainly n-6 PUFAs [7].

Common animal meat (pork, beef, lamb, and poultry) does not contain considerable amounts of PUFAs, and fatty acid profile is strongly affected mainly by the diet composition and the digestive system of the animals. Also, cow milk and dairy products are poor sources of PUFAs. Only human milk contains small but significant levels of PUFAs, particularly GLA, AA, and DHA [5] necessary for optimal development of the brain, the retina, and other infant tissues. On the other hand, egg

yolks could be considered as good source of PUFAs, mainly EPA (0.1%) and DHA (0.7%), especially when chicken are fed with either fish oil or plant oils rich in ALA [6].

C. Microbial Producers of PUFAs

Oleaginous fungi, marine bacteria, heterotrophic and phototrophic microalgae, and mosses synthesize a variety of PUFAs [8,9]. Fungi belonging to Zygomycetes (*Thamnidium* sp., *Cunninghamella* sp., *Mucor* sp., *Mortierella* sp.) could be economically valuable because they are able to utilize various cheap-cost substrates and convert them into GLA, DGLA, AA, and EPA. Microalgae *Spirulina platensis* as source of GLA is sold as a specialty food in several countries [10]. Marine bacterium *Shewanella marinintestina* is known for its significant synthesis of EPA [5]. On the other hand, stramenopile protists *Thraustochytrium* sp., unicellular protist *Shyzochytrium* sp. and *Entomophthora* sp., and bacterium *Moritella marina* have been described as microorganisms accumulating high amounts of DHA.

III. BIOTECHNOLOGICAL STRATEGY FOR CEREALS ENRICHED WITH PUFAs

Biotechnological modification of cereals for natural preparation of functional cereal-derived food/feed products containing biologically active PUFAs is challenging. Basically, there are two biotechnological possibilities to enhance the content of PUFAs in cereal diet. The first way is based on the genetic engineering approach with the aim to construct new cereal varieties with tailor-made profile of desired fatty acids. This technique requires more sophisticated strategies for overcoming difficulties with gene transformation in various cereals. In any case, application of such genetically modified cereals must be approved in many countries in order to use them for food and feed purposes. However, construction of transgenic barley and wheat synthesizing both GLA and stearidonic acid (SDA, 18:4 n-3) by introduction of the fungal Δ6-desaturase gene was reported recently [11,12]. The second possibility to prepare cereals enriched with PUFAs rely on the ability of suitable microorganisms to utilize these materials and convert them to new bioproducts with a high content of valuable metabolites during the process known as "solid-state fermentation" (SSF) [13,14]. This technology has been successfully developed using lower filamentous fungi and prefermented cereals containing PUFAs have been tested as food and feed additives.

A. Solid-State Fermentations

Fermentation is one of the oldest methods of food processing. The extensive uprise of biotechnology over the past years has opened several strategies that could be applied to the formation of functional food/feed. Biotechnological approach based on SSFs is one of the most promising techniques to enrich cereals with desired metabolites. During the process, useful microorganisms grow on various agro-materials by utilization of carbon, nitrogen, minerals, and other nutrients from these substrates. As a result of microbial growth and metabolism, various types of value-added substances of microbial origin with the desired properties are formed in the fermented food/feed, including PUFAs, pigments, sterols, organic acids, alcohols, esters, and enzymes [13]. An advantage of the SSF process is that fermented materials can be directly used for food/feed applications without any downstream process. Such fermentation is also attractive because of its low cost.

B. Microorganisms for Cereal Utilization

One of the key factors to attain commercial feasibility of SSF is selection of appropriate microorganisms that are able to grow on various cereal substrates and simultaneously synthesize a range of compounds in large enough quantities. Therefore, suitable microorganisms should meet the following criteria [15]: (1) sufficient cover of the cereal substrates surface and penetration into the cereal

particles for nutrient utilization, (2) production of necessary enzymes (e.g., amylases, proteases, and lipases) for the hydrolysis of polymeric compounds of the substrates, (3) adequate growth at reduced water activity, (4) active biosynthetic machinery for formation of desired compounds, (5) cannot produce any toxins, and (6) ability to decrease amounts of antinutrient compounds in the substrates.

To fulfill these criteria, screening of microorganisms has led to the selection of lower filamentous fungi especially those belonging to *Mucorales* as the best candidates for SSF processes. Among them, *Thamnidium elegans*, *Cunninghamella echinulata*, *Cunninghamella elegans*, and *Mortierella isabellina* have been used as producers of GLA and *Mortierella alpina* as a producer of DGLA, AA, and EPA [13]. Moreover, several *Mucor* strains were found to form both GLA and β-carotene [14].

C. Cereal Substrates

Cereals contain various sources of assimilable carbon with adequate levels of organic nitrogen and other nutrients necessary for fungal proliferation. However, cereals differ in chemical composition that might affect microbial growth and the final product properties. Therefore, cultivation of selected microorganisms on various substrates usually leads to different microbial effectiveness for the formation of prefermented materials with desired attributes. A variety of cereal materials (e.g., rice bran, wheat bran, oat flakes, and peeled barley) have been tested as useful substrates to achieve prefermented bioproducts with a high PUFA content [16]. In all cases, amount of PUFAs in prefermented mass were constantly lower when cereals were used as sole substrates only and/or without any pretreatment. Therefore, microbial transformation of cereal substrates into desired prefermented mass requires optimization of the cultivation process.

D. Effect of Cultivation Conditions on Production of PUFA-Enriched Cereals

Accumulation and amount of microbial PUFAs in prefermented cereals depends on the substrates, microorganisms, and cultivation conditions. SSFs are often carried out with the help of an internal solid support or a matrix (e.g., spent malt grains) that is required for improving the efficiency of respiration and aeration, the elimination of heat formed during fermentation, and the reduction of substrate particle agglomeration [16]. Adequate oxygen availability is also necessary for high-activity enzymes that transform carbon to PUFA. The appropriate moistening of a substrate is a significant factor for optimal fungal growth and the evaporative cooling of fermentation mass. Water serves as a solvent for nutrients and is also necessary for the intracellular transport of mass and physical protection against turgor forces. In addition, a proper water activity of the cereal substrate prevents the growth of undesired microorganisms and also considerably alters both the formation of air mycelium and the yield of PUFAs [13,16].

Heterogeneity of the cereal substrates and requirement for well-balanced utilization of nutrients from cereals is a basic problem of fungal SSFs. An increase in carbon source availability for microorganisms might be solved by either partial hydrolysis (chemical and enzymatic) of cereal substrates or gradual elevation of the carbon/nitrogen ratio using appropriate carbon source supplementation to the cereal substrates. When either glucose or whey was added to cereal substrates, the lipid accumulation and GLA content were improved by 80% and 60%, respectively [13]. Addition of PUFA precursors in the form of extracellular plant oils is another strategy that was applied for enrichment of cereals with GLA by *T. elegans* or EPA by *M. alpina* [17]. Nutritional regulation of SSF also includes the supplementation of cereals with various ions (e.g., Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+}) and with activators or inhibitors (e.g., isolated from plants) that modify activities of enzymes involved in the carbon flow to the target PUFAs [15]. Depending on the substrate and cultivation conditions, several prefermented cereal products enriched with PUFAs or with simultaneous production of GLA/β-carotene have been prepared. The maximal accumulations of individual PUFAs in prefermented cereals are summarized in Table 30.1.

TABLE 30.1
Accumulation of γ-Linolenic Acid (GLA), Dihomo-γ-Linolenic Acid (DGLA), Arachidonic Acid (AA), and Eicosapentaenoic Acid (EPA) in Cereal-Based Prefermented Bioproduct (BP) Prepared by Fungal Solid-State Fermentations of Selected Fungi Utilizing Various Cereal Substrates

Strain	Cereal Substrate	PUFA	Yield (g/kg BP)	References
Thamnidium elegans	Spelt flakes/SMG	GLA	7.2	[16]
	Wheat bran/SMG/sunflower oil	GLA	10.0	[13]
	Wheat bran/SMG/sunflower oil/plant extract	GLA	20.0	[17]
	Crushed corn	GLA	10.0	[15]
Mortierella isabellina	Barley	GLA	18.0	[17]
Cunninghamella elegans	Barley	GLA	7.0	[15]
	Barley/SMG/peanut oil	GLA	14.2	[18]
Mucor circinelloides	Rye bran/SMG/sunflower oil	GLA	24.2	[14]
Mortierella alpina	Wheat bran/SMG	AA	42.3	[15]
	Dehulled millet	AA	44.7	[19]
	Oat brans	AA	87.0	[20]
	Crushed sesame seeds	DGLA	21.3	[15]
	Peeled barley/SMG/linseed oil	EPA/AA	23.4/36.3	[15]

SMG, spent malt grains.

IV. APPLICATION OF PUFA SOURCES IN RUMINANT NUTRITION

A. Supplementation of Ruminant Diet with PUFA Oils

A current world trend in human nutrition is the increased demand for human diets containing health beneficial essential PUFAs that are not produced by the body and must be obtained through animal feeding. However, ruminant diets contain relatively small amount of lipids. It is clear that supplementation of ruminant diets with dietary lipid sources influences the concentration of fatty acids (FA), the *trans* FA, and conjugated linoleic acid (CLA) contents of ruminant products [21]. The main form of CLA present in ruminant foods is the CLA isomer (*cis*9, *trans*11-C18:2), which is formed as an intermediate product during rumen biohydrogenation of linoleic acid (LA, C18:2) to *trans*-vaccenic acid (*trans*11-C18:1) and stearic acid (C18:0) by rumen microorganisms [22]. Diurnal FA profiles of rumen fluid and microbial fractions from sheep fed diets supplemented with PUFA oils (sunflower, rapeseed, and linseed) were reported previously [23,24]. The PUFA oils were used as they are rich in LA (sunflower oil, 533 g/kg of FA), oleic acid (rapeseed oil, 605 g/kg of FA), and ALA (linseed oil, 504 g/kg of FA). Diet of sheep consisted of meadow hay (960 g dry matter (DM)/day) and of barley grain (240 g DM/day) supplemented (60 g/day) with PUFA oils and overall effect of the oil supplements on increased concentrations of *trans*11-C18:1 and CLA isomers in rumen fluid and microbial fractions were evident [23,24]. Cieslak et al. [25] reported higher concentrations of PUFAs in rumen fluid *in vitro*, when fresh lucerne (300 g/kg DM) or meadow hay (900 g/kg DM) and wheat meal (600:400, w/w) were used as the components of the diet supplemented with black currant oil or grape seed oil up to 5% of DM of diet. These oils were selected due to high content of LA (grape oil 696 g/kg of FA, black currant oil 586 g/kg of FA).

Ruminant diets are usually composed of plants that are rich in LA and ALA; however, the conversion efficiency of LA and ALA to other essential PUFAs is low and therefore direct uptake appears to be significantly more effective.

Recent reviews described the importance of microorganisms (fungi, marine bacteria, and heterophytic and photophytic microalgae and mosses) as a source of microbial oils rich in essential PUFAs including GLA [5,26]. GLA is involved in maintaining proper cell functions in mammals and its insufficient supply from agricultural and animal sources has resulted in a search for appropriate microorganisms suitable for producing GLA in high yields. Compared to plant oils rich in GLA (evening primrose oil, 90 g/kg of FA; borage oil, 230 g/kg of FA), the content of GLA in microbial oil is 84 g/kg of FA. Limited research is available on direct effect of microbial oil on rumen fermentation and lipid metabolism; however, according to previous studies [27,28], ruminant diets supplemented directly with microbial oils from fungus *T. elegans* increased the production of *trans*11-C18:1 and CLA isomers resulting in incomplete FA biohydrogenation. Jalč and Čertík [29] also showed that the direct supplementation of the purified microbial oil (30 g/kg DM) did not affect the digestibility of the diet in artificial rumen (RUSITEC). This is in contrast to lipid supplementation of the diet with fish oil (60 g/kg DM) and linseed or coconut oil (up to 40 g/kg DM), respectively, which can reduce sheep ruminal organic matter and neutral detergent fiber digestibility [30,31]. The direct supplementation of purified microbial oil (30 g/kg DM) into the ruminant diets also decreased the ciliated protozoal population [32], reduced the molar proportion of acetate, and increased molar proportion of propionate *in vitro* [28,29,33]. These finding are consistent with the effect of various PUFA oils as diet supplements on rumen fermentation parameters examined *in vitro* [25,33], as well as *in vivo* [23].

B. Supplementation of Ruminant Diet with Prefermented Cereals Enriched with PUFAs

Concentrates used in ruminant diets are rich in proteins and carbohydrates, but many of them are deficient in essential nutrients such as PUFAs. One of the possible solutions is using cereal substrates enriched through SSF with oleaginous fungi as a source of GLA [17] in ruminant nutrition. In the recent *in vitro* studies [34–36], prefermented cereal substrates (PCS) enriched with two oleaginous fungi (*T. elegans* and *C. echinulata*) in ruminant diets were used. PCS as wheat bran, wheat bran with brewer's spent grains (BSG); corn meal, corn meal with BSG; barley flakes, barley flakes with BSG; or rye bran were used as components of a ratio together with meadow hay or lucerne hay, respectively. The effect of PCS on ruminal digestibility, fermentation, and lipid metabolism was examined using a RUSITEC [37] or batch culture system of incubation [38]. Results clearly indicated that PCS added as a dietary component to ruminal fermentation significantly improved the output of GLA from ruminal effluent. Further results obtained using these two *in vitro* methods are discussed in the following text.

Recent studies [34–36] showed that the effectiveness of different PCS added to feed ration depends on several factors such as the type of PCS, the method and duration of the experiment, the forage-to-concentrate ratio, and source of GLA. These studies also showed that PCS are less digestible than untreated cereal substrates for higher contents of dry matter and detergent fiber in the fungal cereal substrates that are covered by fungal mycelium. The digestibility of PCS is lower compared to no fungal–treated substrates, however, without detectable differences in the major rumen fermentation parameters. Ruminal diets with PCS have specific antiprotozoal fungal effects, which do not interfere with hydrogenesis, since methane production in all experiments was not reduced [34–36], although the reduction in the protozoal population usually decreases methanogens. However, based on 16S-PCR-DGGE method, the *C. echinulata* is unable to produce sufficient concentrations of bioactive compounds to affect the eubacterial community [36]. It is clear that in the rumen fluid no relationship exists between ruminal protozoal population depression and suppression of bacterial activity by PUFA sources [39] since rumen ciliates and bacteria have different metabolic responses to different forms and concentrations of PUFA sources [40,41]. Based on the available literature, supplemented fats was one of the main factors reducing ammonia N concentrations [42]. However, PCS and especially PCS with BSG produces higher concentrations of ammonia N in rumen fluid compared to control diet probably because of the presence

TABLE 30.2
Daily Outputs of γ-Linolenic Acid of the PCS Enriched with Oleaginous Fungi in RUSITEC Effluents (Means ± SEM)

	Prefermented Cereal Substrates (PCS)						
	WB	**WB + BSG**	**BF**	**BF + BSG**	**CM**	**CM + BSG**	**RB**
	Thamnidium elegans						
GLA (mg/day per vessel)	ND	0.34 ± 0.030	0.02 ± 0.006	ND	0.18 ± 0.008	ND	0.04 ± 0.003
	Cunninghamella echinulata						
GLA (mg/day per vessel)	0.21 ± 0.067	0.26 ± 0.050	0.11 ± 0.015	1.38 ± 0.175	0.21 ± 0.021	0.23 ± 0.033	ND

WB, wheat bran; WB + BSG, wheat bran with brewer's spent grains; BF, barley flakes; BF + BSG, barley flakeswith brewer's spent grains; CM, corn meal; CM + BSG, corn meal with brewer's spent grains; RB, rye bran; GLA, γ-linolenic acid (C18:3n-6); ND, not determined.

of hyperammonia-producing bacteria [36]. Cozzi and Polan [43] and Miyazawa et al. [44] also reported increase in the concentration of ammonia N at 2 h after feeding by partial replacement of concentrates with BSG in the diet of cows.

To date, only limited research is available on the effect of PCS with oleaginous fungi on lipid metabolism in ruminants [34–36]. These data clearly indicate that PCS might positively enhance daily outputs of GLA from RUSITEC effluent. However, the effectiveness of GLA sources in increasing ruminal GLA outputs varied with the filamentous fungi used (*C. echinulata* > *T. elegans*) and efficiency depends on the cereal substrate type used (Table 30.2). The values for concentrations of the GLA ranked by prefermented substrates were barley flakes + BSG > wheat bran + BSG > corn meal + BSG > wheat bran > corn meal > rye bran > barley flakes. The majority of PCS have no effect on the concentration of CLA isomers. Dietary lipids are subjects of hydrolysis and biohydrogenation by rumen microbial population, and the majority of CLA isomers in ruminant-derived food products originate from the isomerization of GLA and ALA in the rumen. The experiments associated with dietary addition of microbial oil (GLA-enriched oil) from oleaginous fungi in rumen fluid *in vitro* resulted in higher production of PUFAs and incomplete FA biohydrogenation [27,28]. However, this has not been fully confirmed in diet with PCS, because the effect of PCS on ruminal biohydrogenation varied according to the type of fermented substrates added to the ruminal diet. Furthermore, the fatty acid composition of various PCS is not constant and can, in many cases, be enhanced by the ratio in diets.

It can be concluded that the ability of the fungal strains *T. elegans* and *C. echinulata* to grow and utilize various agroindustrial substrates might be useful in developing new animal diets enriched with fungal GLA in the area of ruminant nutrition. However, *in vivo* studies are needed to determine the impact of prefermented cereal-derived substrates as components of ruminant diets and to support the *in vitro* results. Research is required to fully characterize the benefits associated with using these substrates in ruminant diets and to understand how the levels in diets can be enhanced.

V. APPLICATION OF PUFA SOURCES IN CHICKEN NUTRITION

Today's trend of healthy nutrition is to consume less fat, especially fats with higher proportion of PUFA. This theory is based on scientific studies that confirm the positive effect of PUFAs on human health. Diet and nutrition are important determinants of health, so inadequate and bad eating habits are risk factors for lifestyle diseases. The results of recent research show that diet and lifestyle

contribute to the development of many noninfectious diseases, including obesity, cardiovascular disease, and degenerative diseases [45]. Inadequate intake of essential and PUFA, as well as unsuitable ratio of n-6/n-3 in foods also contribute to these diseases.

The enrichment of broiler chicken meat with PUFA is a viable means of increasing PUFA consumption in European diets because population intake of broiler chicken meat are high, and broiler meat is amenable to manipulation of its fatty acid composition by altering the fatty acid composition of broiler diet [46]. The lipid digestion in animals has significant effects on the transfer of fatty acids from the food to the animal products. In period, when the animals do not starve and they have sufficient amount of fatty acid in the feed, fatty acids are not synthesized in the body, but they are directly incorporated from the feed into the fatty tissue of animals. Feeding of fat, therefore, has a direct and predictable effect on the general composition of fatty acids in poultry production [47] and influence on unsaturated fatty acids in the tissues can be achieved simply by increasing their share in the feed. The ability to increase the share of major PUFA in the fatty tissue of animals by changing the fat proportion of compound feed is another challenge to the scientific community and is directing research projects.

A. Effect of PUFA Feeding on the Quality and Composition of Chicken Meat

In chicken, the composition of fatty acids stored in adipose tissues reflects that of ingested lipids [45]. Grain feed, a conventional fed to poultry, supplies mainly n-6 PUFA and a small amount of n-3 PUFAs [48]. This is reflected in the fatty acid composition of meat produced with a high percentage of linoleic acid. To change the fatty acid composition of poultry meat requires adding the PUFA source to feed. Previously published papers on the fattening of poultry used fish oil [49,50], linseed oil [47,51], camelina (*Camelina sativa*) oil [52], and marine algae [46]. All the sources of n-3 PUFA added to the fodder increased the proportion of n-3 PUFA in the fatty tissue of animals and meat and also improved the ratio of n-6/n-3 PUFA. However, the feeding of soybean [53] and sunflower oil [52], containing a higher proportion of linoleic acid, increased the proportion of n-6 PUFA, especially linoleic acid in abdominal fat and muscle tissues of broilers. The addition of rapeseed [54] and olive oils [55] mainly increased proportion of monounsaturated fatty acid (MUFA) and oleic acid in the abdominal fat and meat of chicken.

Linseed oil feeding in broiler chickens has a positive impact on increasing the content of n-3 PUFA, especially ALA in fatty tissues of animals. Zelenka et al. [51] stated that feeding of linseed oil in quantities of 1%, 3%, 5%, and 7% had a favorable effect on the increase of n-3 PUFA in intramuscular fat in breast and thigh muscles. As the amount of linseed oil in feed increased, also the proportion of n-3 PUFA in meat chickens was higher. Another important source of ALA is camelina (*C. sativa*). Jaskiewicz et al. [52] supplemented diet for broiler chickens with camelina oil, and it was compared with diets supplemented with soybean oil and rapeseed oil. The addition of camelina oil to the diet increased the ALA content of the muscle tissues and abdominal fat. The addition of soybean oil increased the linoleic acid and rapeseed the oleic acid content in the muscle tissues and abdominal fat.

Adding camelina and linseed oils with a high proportion of ALA into poultry feed, it is possible to increase the proportion of ALA and other n-3 PUFAs in the produced meat and fat of animals. Conversely, the proportion of n-6 PUFA in the produced meat is decreased, thus the ratio of n-6/n-3 PUFA is greatly improved [45]. As the ALA is a precursor to other n-3 PUFA, it can be assumed that increased amounts of the fatty acid in feed will also increase the content of other n-3 PUFA in intramuscular fat and the fatty tissue of animals. It is possible that content of EPA and DPA, through the feeding of linseed oil, will be increased in the meat and adipose tissue. However, the content of DHA is not modified by feeding of linseed oil [50]. Significant increases in DHA and EPA were reported by few authors [46,49], who used fattening fish oil or algae [46]. In this case, there is no synthesis of DHA in the body of animals, but DHA was directly incorporated from the feed into the fatty tissue of animals. To increase levels of DHA in the intramuscular fat and in the adipose

tissue, it is necessary to include the fatty acid in the feed. Formation of DHA in the body appears to be tightly regulated metabolically and not influenced by the intake of the precursors in the feed. Elongation and desaturation of n-3 fatty acids are probably blocked at the level of the formation of DHA [56]. The addition of fish oil and algae to chickens' feed has the effect of increasing the content of DHA and EPA in the meat, but the content of the other n-3 PUFAs by feeding has not been affected.

It is well known that the oxidation of fats is one of the main problems of spoilage of food rich in fats. Increased amount of PUFA in intramuscular and abdominal fat may have a negative effect on the oxidative stability and shelf life of produced meat and fat. Emerging oxidation products directly affect the characteristics of the meat such as unpleasant change in flavor and taste, discoloration, and textural change. The addition of antioxidants in the feed or water is the best way to protect intramuscular fat of animals during storage. The most effective and the most commonly used antioxidant in animal feed is vitamin E [49]. Effective antioxidant ingredients added to feed or water are also plant extracts [57] and essential oils from plants [58].

B. Effect of Prefermented Cereals Enriched with PUFAs on Feeding Parameters and the Quality and Composition of Chicken Meat

The increased interest in feed and food containing high amount of PUFA has been observed, because PUFA are considered as functional ingredients to prevent coronary heart disease and other chronic diseases [59]. A current world trend in the production of diets with supplemented components of PUFA has increased the demand for feeds containing GLA for animal nutrition [34]. GLA, via conversion to prostaglandin E1, shows antiinflammatory, antithrombotic, antiproliferative, and lipid-lowering potential [60]. However, GLA is rarely found in common foods. Therefore, interest has been focused on oleaginous microorganisms that are able to accumulate GLA-rich oils in their biomass [8]. Alternatively, various agricultural products and by-products can be enriched in PUFAs through SSF or semisolid state fermentation with PUFA-producing microorganisms (*C. echinulata*, *M. isabellina*, and *T. elegans*) and directly used as feed supplements [26] to modify the fatty acids profile in poultry [61]. The cheap cereal materials such as rice bran, wheat bran, oat flakes, malted draff, and peeled or pearled barley provide a suitable source of nutrients for fungal growth and lipid production. During SSF, the fungus produce enzymes necessary for hydrolysis of sources bound in biopolymers. Moreover, fungi also simultaneously decrease antinutrient compounds and partially hydrolyze substrate biopolymers, which can positively influence the animal production parameters [13].

The aim of our experiments was to raise content of significant PUFA, especially GLA, in the meat of broiler chickens after feeding of prefermented cereal feed and/or extract from agrimony (*Agrimonia eupatoria* L.) as an antioxidant. Another aim was to investigate the effect of adding prefermented feed on poultry production parameters.

Prefermented cereal feed was prepared by SSF using *T. elegans* CCF 1456, while the basic substrate was wheat bran. Organic product formed by SSF contained 15% GLA and showed a lower proportion of LA as compared to the standard feed mixture (Table 30.3). After the cereal substrate (3%) addition in compound feed, an increase in content of GLA, and a reduction in LA were found. Control chickens were fed with standard feed mixtures (starter and grower) for 42 days. Experimental chickens were fed with standard feed mixtures for 20 days, after that the mixture of standard feed enriched with 3% of prefermented cereals was used for feeding up to 42 days. The results of the fatty acid composition showed a positive effect of the addition of the prefermented cereal substrate on the fatty acid composition in fat of breast and thigh muscle [61]. GLA content, which has been supplied in large quantities in the feed, was significantly increased in experimental groups compared to control. The positive effects include increasing the amount of DGLA, EPA, and DPA and reducing in LA in meat of experimental groups. The amount of MUFAs was decreased, mostly due to reduction of oleic acid (C18:1). The ratio of n-6/n-3 in the breast muscle of experimental groups was significantly better than the control. In the thigh meat, the ratio was only marginally improved.

TABLE 30.3
Fatty Acids Profile (%) of Feed Mixtures Used in Experiments for Feeding of Broilers

Fatty Acid (%)	PCF	SF	SF + 3% of PCF	SF +5% of PCF
C14:0	0.406 ± 0.1	0.106 ± 0.02	0.107 ± 0.01	0.022 ± 0.06
C16:0	15.101 ± 0.5	12.544 ± 0.1	12.609 ± 0.1	12.422 ± 0.2
C16:1	0.566 ± 0.2	0.072 ± 0.02	0.078 ± 0.01	0.079 ± 0.03
C16:1 n-7	0.532 ± 0.1	0.127 ± 0.05	0.131 ± 0.04	0.151 ± 0.08
C18:0	2.764 ± 0.5	2.841 ± 0.1	2.845 ± 0.1	2.454 ± 0.1
C18:1 n-9, OA	18.687 ± 1.8	22.827 ± 0.1	22.855 ± 0.5	22.839 ± 0.4
C18:1 n-7	0.592 ± 0.1	0.833 ± 0.1	0.822 ± 0.1	0.926 ± 0.1
C18:2 n-6, LA	43.039 ± 0.4	55.858 ± 0.3	54.693 ± 0.3	55.155 ± 0.3
C18:3 n-6, GLA	15.296 ± 4.5	ND	0.135 ± 0.01	0.571 ± 0.5
C18:3 n-3 ALA	2.297 ± 0.7	3.786 ± 0.1	4.745 ± 0.01	3.404 ± 0.2
C20:0	T	0.395 ± 0.01	0.391 ± 0.00	0.356 ± 0.01
C20:1 n-9	0.711 ± 0.1	0.325 ± 0.04	0.306 ± 0.00	0.303 ± 0.08
C22:0	T	0.288 ± 0.03	0.247 ± 0.04	0.241 ± 0.02
∑SFA	18.280 ± 1.42	16.173 ± 0.25	16.237 ± 0.23	15.676 ± 0.28
∑MUFA	21.089 ± 2.00	24.183 ± 0.02	24.191 ± 0.03	24.217 ± 0.07
∑PUFA	60.633 ± 1.25	59.644 ± 0.27	59.573 ± 0.46	60.107 ± 0.45

PCF, prefermented cereal feed; SF, standard feed used for feeding of broilers; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; ∑SFA, sum of saturated fatty acids; ∑MUFA, sum of monounsaturated fatty acids; ∑PUFA, sum of polyunsaturated fatty acids; T, traces; ND, not determined.

Meat of experimental group after feeding cereal substrate with increased PUFA underwent oxidative changes faster. The administration of the extract of agrimony (0.1%) in water of chickens in one of the experimental group during the fattening period significantly reduced the oxidation processes in the meat during storage in the refrigerator. Application of agrimony extract appears to be suitable as an antioxidant against peroxidation of GLA [62]. Sensory evaluation of meat was not affected by the feeding of prefermented feed and in combination with an extract of agrimony achieved better sensory properties than control. Our study demonstrated that prefermented cereal product exerted a positive effect on production parameters of chicken. In the experimental group, its administration resulted in increased performance and feed conversion [61].

In another study, prefermented substrate was prepared by SSF using *T. elegans* CCF 1456, while the basic substrate was spelt bran. Organic product formed by SSF contained 8.5% γ-linolenic acid and showed a lower proportion of linoleic acid and a greater percentage of oleic acid compared to the commercial feed. Using the base cereal ingredient and also fermentation conditions had an impact on lower proportion of GLA in the produced substrate, and modification of FAs profile in the substrate [13]. Created cereal substrate was added in chicken in the amount of 5%, and simultaneously about this amount was reduced in commercial feed. Mixing of the prefermented feed with commercial feed caused an increase in the content of GLA and LA declined in this mixture (Table 30.3). Commercial feed mixtures fed in industrial conditions of farming contain high levels of LA due to the use of feed ingredients such as wheat and corn.

Feeding with prefermented feed at 5% concentration resulted in an increase in GLA and stearidonic acid (C18:4 n-3) in meat and abdominal fat of chickens from treatment groups. A higher proportion of GLA was in the feed but do not affect significantly the content of other important fatty acids (DGLA, AA). The breast and thigh meat recorded slightly higher values compared to

the control. Feeding fermented feed in the experimental group increased MUFA and PUFA content and vice versa reduced saturated fatty acids (SFA) in meat and also in abdominal fat of chickens. Increased proportion of PUFA in chickens' meat had an effect on fat oxidation during storage of meat in the refrigerator, which was reflected particularly after storage of thigh muscle.

Feeding prefermented feed at 5% concentration affected the production parameters. Chickens on the 35th day of fattening had the highest body weight, gains, and the best feed conversion, which was caused by lower food consumption. Chickens fed with prefermented feed also achieved better slaughter yield. This shows that the fermented feed is full-value feed, which is not only the source of GLA but also a source of easily digestible fiber, protein, trace elements, vitamins, and at 5% concentration is fully usable for poultry and can replace commercial feed mixture. Comparing our results with the results of other works is difficult. These experiments were the first studies using prefermented cereal feed for fattening of broiler chickens. However, while using other sources (linseed oil, camelina, fish oil, and algae biomass), PUFA authors [46,51,52] did not record the effect of added oils to the improvement of the production parameters. On the contrary, the deterioration of basic indicators of fattening (final weight, conversion, and gains) was found. In this situation, prefermented feed can be, for manufacturers of feed and poultry farmers, an appropriate supplement for enrichment of meat with major PUFAs and an improvement in the production parameters of poultry. However, more research is required to provide a better understanding of the contribution of prefermented feeds in poultry nutrition.

VI. CONCLUSIONS AND PERSPECTIVES

Development of functional cereals enriched with PUFAs and their applications in ruminant and chicken nutrition is one of the strategic targets in food and feed industry. Growing interest in employing of natural technologies for preparation of healthy and dietary cereal-based materials containing PUFAs has focused on biotechnological methods. SSFs combining the power of oleaginous fungal strains to form PUFAs and effectively utilize various cereal substrates have demonstrated considerable potential for their applications in numerous food/feed fields. Current success with establishment of this biotechnological process for tailor-made PUFA-cereals with their subsequent testing in ruminant and chicken nutrition represents a challenging and potentially rewarding subject for preparation of new types of food/feed with required nutritional design and thus may fill marketing claims in food, feed, and veterinary fields. However, further development of biotechnologically prepared cereals functionally enriched with PUFAs will undoubtedly depend on their acceptability in the market, regulatory approval, and the size of the capital investment required for prosperous commercialization and effective delivering of the bio-based cereals to market.

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Section V

Biotechnology and Biochemistry

31 Lipid Biotechnology and Biochemistry

Sampson Anankanbil, Fan Suo, Peter Ruhdal Jensen, and Zheng Guo

CONTENTS

I. INTRODUCTION

In a broad sense, in addition to the production and biotransformation of lipids by microorganisms and isolated enzymes, lipid biotechnology covers modern techniques in genetic engineering for the modification of conventional oilseed crops for altering fatty acid, improving oil productivity, or enriching specific fatty acids with the aim of better agricultural economy and improved application in food, biofuels, or biochemical industry. Genetic engineering of oil crops, cereals, and other plants is addressed in Chapters 36 and 37.

The foci of this chapter are (1) the production of lipids in microorganisms and engineering of microbial cell factory for improved production or production of functional lipids. Microorganisms include yeast, fungi, microalgae, and prokaryotic microorganism bacteria, while the lipid products extend from fatty acid and triacylglycerols (TAGs) to steryl lipids, waxes, ketones and lactones, and biosurfactants. (2) Modification and biotransformation of natural lipids with isolated enzymes as biocatalysts, particularly the reactions and applications of lipases/esterases in the production of structured lipids, design and production of value-added functional lipids such as bioesters, flavor esters, wax and steryl esters, lactones and estolides, sugar esters and alkylglycoside, and lipophilic phenolics. Other enzymes for the production of fatty acid derivatives such as lipoxygenase (LOX), oxygenase, and epoxide hydrolase and phospholipase A1/A2/D for the modification of phospholipids or generation of new phosphatidyl-based molecular species are also discussed. New technical advances in the application of green media such as supercritical fluids (SCFs), ionic liquids (ILs), and deep eutectic solvents (DES) for biocatalytic biotransformation of lipids are also reviewed.

II. MICROBIAL PRODUCTION OF FATS AND OTHER LIPIDS

Microorganisms are often considered as the ideal cell factory for renewable energy sources due to their properties of fast metabolism, little environmental pollution, and good accessibility to generic engineering. Lipids as an essential ingredient of cell structure are distributed widely in eukaryotic and prokaryotic microorganisms and also have huge commercial value in many fields. In this section, production, distribution, and application of various types of lipids including TAG, fatty acid, sterols, ketones, lactone, wax, and surfactants would be introduced. Microbial lipid production is also addressed in Chapter 14.

A. Production of Fats and Fatty Acids in Microbial Cell Factory

1. Triacylglycerol

TAG is a water-insoluble fatty acid ester of glycerol, and its type, property, and diversity depend on fatty acid composition [1]. TAG is a more efficient storage pool for energy and carbon compared to carbohydrate and protein for the reason that cells can yield more caloric value at the expense of oxidizing less TAG. TAG mainly as reserve compound is distributed widely in eukaryotes, such as plants, animals, yeast, and fungus; they accumulate TAG mostly under the condition of metabolic stress, while TAG of bacteria origin has been rarely reported [1–3]. Nowadays, alternative renewable biodiesel production as a viable substitute for fossil fuel, especially using microbial lipids, which are known as single cell oil (SCO), is receiving considerable attention [4]. Several microorganisms with the potential for microbial oil production are listed in Table 31.1.

a. Yeast

Some yeast strains such as *Rhodosporidium* sp., *Rhodotorula* sp., and *Lipomyces* sp. are capable of accumulating intracellular lipids as much as 70% of their dry cell weight [4]. *Cryptococcus curvatus* as the most efficient oleaginous yeast species could accumulate up to 60% storage lipid of biomass dry weight under limited supply of nitrogen, consisting of similar components to plant oil seeds [5]. Many studies have been carried on using metabolic engineering techniques such as

TABLE 31.1
Oil Content of Some Microorganisms

Microorganisms	Oil Content (% Dry Weight)
Yeast	
Yarrowia lipolytica	33[a]
Candida curvata	58
Cryptococcus albidus	65
Lipomyces starkeyi	64
Rhodotorula glutinis	72
Microalgae	
Botryococcus braunii	25–75
Chlorella sp.	28–32
Cr. cohnii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	>20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis sueica	15–23
Fungi	
As. oryzae	57
Mo. isabellina	86
Humicola lanuginose	75
Mortierella vinacea	66
Bacteria	
Arthrobacter sp.	>40
Acinetobacter calcoaceticus	27–38
Rhodococcus opacus	24–25
Bacillus alcalophilus	18–24

Sources: Meng, X. et al., *Renew. Energy*, 34(1), 1, 2009; Chisti, Y., *Biotechnol. Adv.*, 25(3), 294, 2007; Aggelis, G. and Komaitis, M., *Biotechnol. Lett.*, 21(9), 747, 1999.

[a] Growing in the presence of *Teucrium polium* L. aqueous extract.

overexpressing enzymes relevant to fatty acid and TAG biosynthesis pathways, regulating of TAG biosynthesis, bypass and blocking competing pathways, and transgenic approach to enhance TAG production in yeast [6].

A previous research showed that *Escherichia coli* acetyl-CoA carboxylase (ACC) protein could catalyze the rate-limiting step for fatty acid biosynthesis [7]. The gene encoding ACC originated from the oleaginous fungus *Mucor rouxii* that was overexpressed successfully in the nonoleaginous yeast *Hansenula polymorpha*, resulting in a 40% increase in the total fatty acid yield [8]. Tai and Stephanopoulos constructed a high expression platform in the oleaginous yeast *Yarrowia lipolytica* using a translation elongation factor-1α (TEF) promoter and overexpressed diacylglycerol acyltransferase (DGA1), the final step of the TAG synthesis pathway, resulting in significant increase in fatty

acid and lipid production [9]. Similarly, the fatty acid and TAG yields increased more than twofold in *Aspergillus oryzae* by replacing the promoter regulating fatty acid synthesis for highly expressed gene *tef1* promoter [10]. In addition, the deletions of several genes in oleaginous yeast *Y. lipolytica*, such as GUT2 gene coding the glycerol-3-phosphate dehydrogenase isomer and POX1 and POX6 genes encoding six acyl-coenzyme A oxidases (Aox), promote lipid production [11].

Formation of TAG in the yeast *Saccharomyces cerevisiae* requires the two key intermediates of lipid metabolism, its precursor phosphatidic acid (PA) and diacylglycerol (DAG). Two major de novo synthesis pathways for PA could occur either through glycerol-3-phosphate-(G-3-P) or dihydroxyacetone-phosphate (DHAP)-biosynthetic pathway [12]. TAG could be synthesized through acylation of DAG, converted by PA biosynthetic pathway [13].

An engineered *S. cerevisiae* strain by overexpression of glycerol kinase increased glycerol utilization and G-3-P accumulation, to further promote TAG synthesis [6]. In the same paper, the overall content of TAG in the other engineered *S. cerevisiae* YPH499 strain by overexpressing diacylglycerol acyltransferase (DGA1) and phospholipid diacylglycerol acyltransferase (LRO1) represented a 2.3-fold improvement, compared with the wild-type strain [14].

b. Microalgae

Many microalgae have the capability of producing substantial amount of neutral lipids, mainly in the form of TAG (approximately 20%–50% dry biomass weight in response to adverse environmental stress like photooxidative stress [15,16]). As many microalgae have been found to grow at a high rate and produced large quantities of TAG or oils, thus oleaginous microalgae were employed as ideal cell factories for biofuels derived from microalgae oil and other biomaterials [16–19].

In view of slow phototrophic growth rate of microalgae due to light limitation, heterotrophic cultivation in conventional fermenters offered several advantages including good control of cultivation process, low cost for harvesting biomass, and little light requirement and has been favorably considered [20–22]. The yield of crude bio-oil produced from fast pyrolysis by metabolically controlling heterotrophic *Chlorella protothecoides* (through the addition of the organic carbon source [glucose] to the medium and the decrease of the inorganic nitrogen source in the medium) was 3.4 times higher than autotrophic cells (57.9% dry biomass weight) [20]. Some cheap raw materials, nitrogen source regulation, initial carbon to nitrogen (C/N) ratio, and other environmental factors for microalgal production were also considered to lower the cost and increase bio-oil yield [16]. Corn powder hydrolysate instead of glucose has been used as organic carbon source in heterotrophic *C. protothecoides* medium; as a result, cell biomass increased significantly under the heterotrophic growth and the crude lipid content in fermenters was up to 55.2% [23]. As complex nitrogen source had amino acids, vitamins, and growth factors, it should be superior to simple nitrogen source in cultivation of heterotrophic microalgae [23]. The diluted monosodium glutamate waste water as a cheap fermentation broth was used for *Rhodotorula glutinis* for biosynthesis of biodiesels [24]. It was reported in many literatures that nitrogen starvation played a helpful role in promotion of total lipid content and triggered lipid accumulation [25–28]. Cell lipid content would be at a minimum C/N ratio of approximately 20 and would enhance at both higher and lower C/N ratios than at the value of 20 in the heterotrophic cultivation conditions of *Chlorella sorokiniana* [21]. Other environmental factors such as light intensity, pH, salinity, dissolved O_2, temperature, and cultivation modes would also have a great effect on lipid composition and accumulation in microalgae [25,29–32].

Historically, most molecular and genetic physiological researches for modifying the lipid content mainly focused on the green microalga *Chlamydomonas reinhardtii* as the model [33]. However, other microalgae and diatoms also attracted greater interests in industrial application nowadays. A variety of transformation methods have been successfully applied into more than 30 kinds of microalgae to date, and in most cases, transformation of transgenes resulted in stable expression [34–38]. Understanding microalgae lipid biosynthesis and catabolism, as well as pathways, was necessary for the ultimate lipid production, so some genetic engineering techniques such as gene knockout and overexpression have been employed to clarify gene functions in lipid accumulation [33].

c. Fungi

Fungus, especially mold, was considered as a favorable oleaginous microorganism. Although many types of fungi have the ability to accumulate lipids, most fungi were applied to produce specific lipids, such as DHA (docosahexaenoic acid, *all-cis*-4,7,10,13,16,19-docosahexaenoic acid), GLA (γ-linoleic acid, *all-cis*-6,9,12-octadecatrienoic acid), EPA (eicosapentaenoic acid, *all-cis*-5,8,11,14,17-eicosapentaenoic acid), and ARA (arachidonic acid, *all-cis*-5,8,11,14-eicosatetraenoic acid) [6]. In ancient times, people started to use filamentous fungi as lipid producer from their biomass [39]. The composition and content of lipids in filamentous fungi were very heterogeneous depending on the taxonomic position of fungi, development stage, and growth conditions, sometimes reaching up to 86% dry biomass weight [4,40]. TAG was still a main component of lipid fraction in fungi, and neutral lipids in fungi were presented by monoacylglycerols, diacylglycerols, and free fatty acids [41]. The mucoralean fungus *Cunninghamella japonica* was reported as a promising lipid producer, and it could produce up to 16 g/L biomass and over 7 g/L lipids in the inexpensive medium with ammonium nitrate as nitrogen source [42]. The biomass and SCO yields of *Mortierella isabellina* cultivated in nitrogen starvation media with initial sugar concentration resulted in increased values [43]. Many other mold species such as *Aspergillus terreus*, *Cunninghamella echinulata*, *Claviceps purpurea*, *Tolyposporium*, and *Mortierella alpina* could also accumulate lipids [44].

Some metabolic engineering approaches in lipid production were also reported in fungus. Malic enzyme (ME) played a major role in NADPH production, an essential substrate for storage lipid by *Aspergillus nidulans* [45]. The mutant *As. nidulans* strain without ME activity (acuK248) could only accumulate half as many lipids as parent strain [45]. After the genes encoding the isoform of ME in *Mucor circinelloides* and *Mo. alpina*, two commercially useful oil-producing fungi, were cloned and transformed into *M. circinelloides* separately, the lipid contents in both recombinants were enhanced from 12% of the biomass to 30% [46].

d. Prokaryotic Microorganisms

The eukaryotic yeast, mold, and microalgae could produce TAG similar in composition to vegetable oils, but generally, most bacteria were able to accumulate storage lipids and mainly synthesized specific lipids such as poly(3-hydroxybutyric acid) and other polyhydroxyalkanoic acids (PHAs) [47,48]. Only a few certain prokaryotic species like the actinomycetes group, such as *Streptomyces*, *Nocardia*, *Rhodococcus*, *Mycobacterium*, *Dietzia*, or *Gordonia*, have been described to accumulate TAG recently, which has been considered to be uncommon storage lipid in bacteria, and TAG was also detected in aerobic heterotrophic bacteria and *Cyanobacteria* [1]. Up to now, yeast and fungi were considered to be the preferred producer of fats and oils for industrial applications, but most types of lipids found in their cells are still unknown. However, some oleaginous bacteria such as TAG-accumulating bacterium *Rhodococcus opacus* strain PD630 could have 52% TAG content in the cells and approximately 37.5 g dry matter per liter, so prokaryotic microorganisms should also be considered as a lipid source in the future [49].

The abundance of fatty acids and the easier accessibility of genetic engineering in bacteria offer a lot of advantages in commercial applications [1]. The recombinant PHA and PHB biosynthesis vectors based on *the E. coli-Rhodococcus* shuttle vectors were transferred and expressed successfully into *Rhodococcus opacus* PD630 [50].

2. Fatty Acids

Saturated fatty acids have no double bonds, while unsaturated fatty acids have one or more. The biosynthesis of saturated fatty acids (SFAs) in bacteria is performed by multiple conserved enzymes in a multistep process [51]. The mechanisms of SFA synthesis in both eukaryotes and bacteria are quite conservative although the catalytic entities reside in markedly various protein arrangements and meanwhile the bacterial fatty acid synthesis is very much similar to that in most abundant species of eukaryote except slightly shorter chain, more saturated fatty acids, and different double-bond positions in C18 acids [52].

Among unsaturated fatty acids, the polyunsaturated fatty acids (PUFAs) have attracted increasing interest in nutrient especially in the cognitive development of infants [53]. Among PUFAs, docosahexaenoic acid (22:6n-3; DHA) and arachidonic acid (20:4n-6; ARA) are of particular importance in the development of neural function and retinal acuity to infants [54,55]. Eicosapentaenoic acid (20:5n-3; EPA) always occurs with DHA as it is effective in improving DHA status and growth [56].

A few species of marine microorganisms have been investigated to produce a high proportion of DHA and EPA in their total lipids and low content of saturated fatty acids, such as *Schizochytrium* sp. [57–61], *Aurantiochytrium limacinum* [62,63], and *Crypthecodinium cohnii* [64]. *Schizochytrium* sp. s31 could accumulate up to 40% lipid of biomass and 13% DHA at initial pH 7.0 [58]. Reinforced acetyl-CoA and NADPH supply at a specific fermentation stage would promote the DHA content accounting for 60% of total fatty acid [59]. A DHA productivity of 3.7 g/L/day was reached by optimization of DHA production by the strain *Au. limacinum* SR21 [62]. A *Cr. cohnii* fed-batch fermentation could produce 1.9 g/L DHA after 100 h using diluted carob pulp syrup (1:10.5, v/v) supplemented with yeast extract and sea salt [64]. Compared to traditional photosynthetic mass culture, heterotrophic cultivation of microalgae had more advantages especially in commercial application [65]. Vazhappilly and Chen screened 20 microalgal strains for EPA and DHA production under heterotrophic growth condition. As a result, *Cr. cohnii* UTEX L1649 had the highest production of 19.9% DHA (of total fatty acids) and followed by *Amphidinium carterae* UTEX LB 1002 (17%) and *Thraustochytrium aureum* (16.1%) [66]. Unlike the aforementioned results, the highest EPA proportion was obtained from *Monodus subterraneus* UTEX 2341 (34.2% of total fatty acids), followed by *Chlorella minutissima* UTEX 2341 (31.3%) and *Phaeodactylum tricornutum* UTEX 642 (21.4%) [66]. *Nitzschia laevis* was identified by Tan and Johns [67] as the ideal EPA producer among nine diatom strains examined using heterotrophic growth conditions, yielding 0.017 g/g dry cell weight.

Some fungi have also been reported to produce DHA and EPA in their biomass. The filamentous fungi *Mortierella* species could produce large amount of EPA when growing at low temperature [68]. The EPA and DHA in *Mo. alpina* 1S-4 contents could attain up to 29.2% and 20%, respectively, of total fatty acids when growing on salmon oils [69]. The level of DHA increased to 40% of total lipid by growth optimization of a marine fungus, *Thraustochytrium aureum* (ATCC 34304) [70]. With Vogel's medium, the fungus *Pythium ultimum* as EPA and ARA producer could yield 133 ± 27 ARA and 138 ± 25 mg/L EPA, respectively [71].

Many studies using microorganisms such as *E. coli* and *S. cerevisiae* as a cell factory for fatty acids production have been done recently. Compared with other microorganisms, *E. coli* has the highest titer in fatty acid production (Table 31.2). However, yeast as a robust industrial organism has many advantages so it has a good potential in fatty acid production. Recently, there has been an increasing interest in developing yeast as a cell factory for fatty acid production (Table 31.3). The fatty acids biosynthesis pathway in *E. coli* and *S. cerevisiae* are shown in Scheme 31.1.

TABLE 31.2
Metabolic Engineering of *E. coli* for Fatty Acid Production

Strategies	Titer (g/L)	Ref.
Overexpression of thioesterases	0.7161	[72]
Overexpression of thioesterases and Δ*fadD*	0.7	[73]
Overexpression of thioesterases and *accABCD,* Δ*fadD*	0.81	[74]
Overexpression of thioesterases and Δ*fadE*	1.1	[73]
Overexpression of fabZ and thioesterases, Δ*fadD*	1.7	[75]
Overexpression of *fadR* and thioesterases, Δ*fadE*	**5.2**	[**76**]

TABLE 31.3
Metabolic Engineering of Yeast for Fatty Acid Production

Strain	Strategies	Titer (g/L)	Ref.
BY4741	Overexpression of *Mus musculus ACOT5*	0.493	[77]
BY4727	Overexpression of *TesA*, *ACC1*, *FAS1*	0.4	[78]
BJ5464	Overexpression of FAS2	0.33	[79]
YPH499	Overexpression of $ACC1^{S1157A}$	0.14	[80]
CEN.PK2	Δ*FAA1* and Δ*ADH1*	0.011	[81]
	Overexpression of the reversed β-oxidation pathway and SeAcsL641P, Δ*ADH1*, Δ*ADH4*, Δ*GPD1*, and Δ*GPD2*		

B. Other Lipids Produced by Microbiological Cell Factory

1. Sterols

Sterols are vital membrane components of eukaryotes and belong to the vast family of isoprenoids. Sterols can regulate membrane fluidity and permeability and also participate in the control of membrane-associated metabolic processes. Moreover, sterols play a specific role in signal transduction, synthesis of second-metabolites, and cell prolongation in animals, fungi, and higher plants [82].

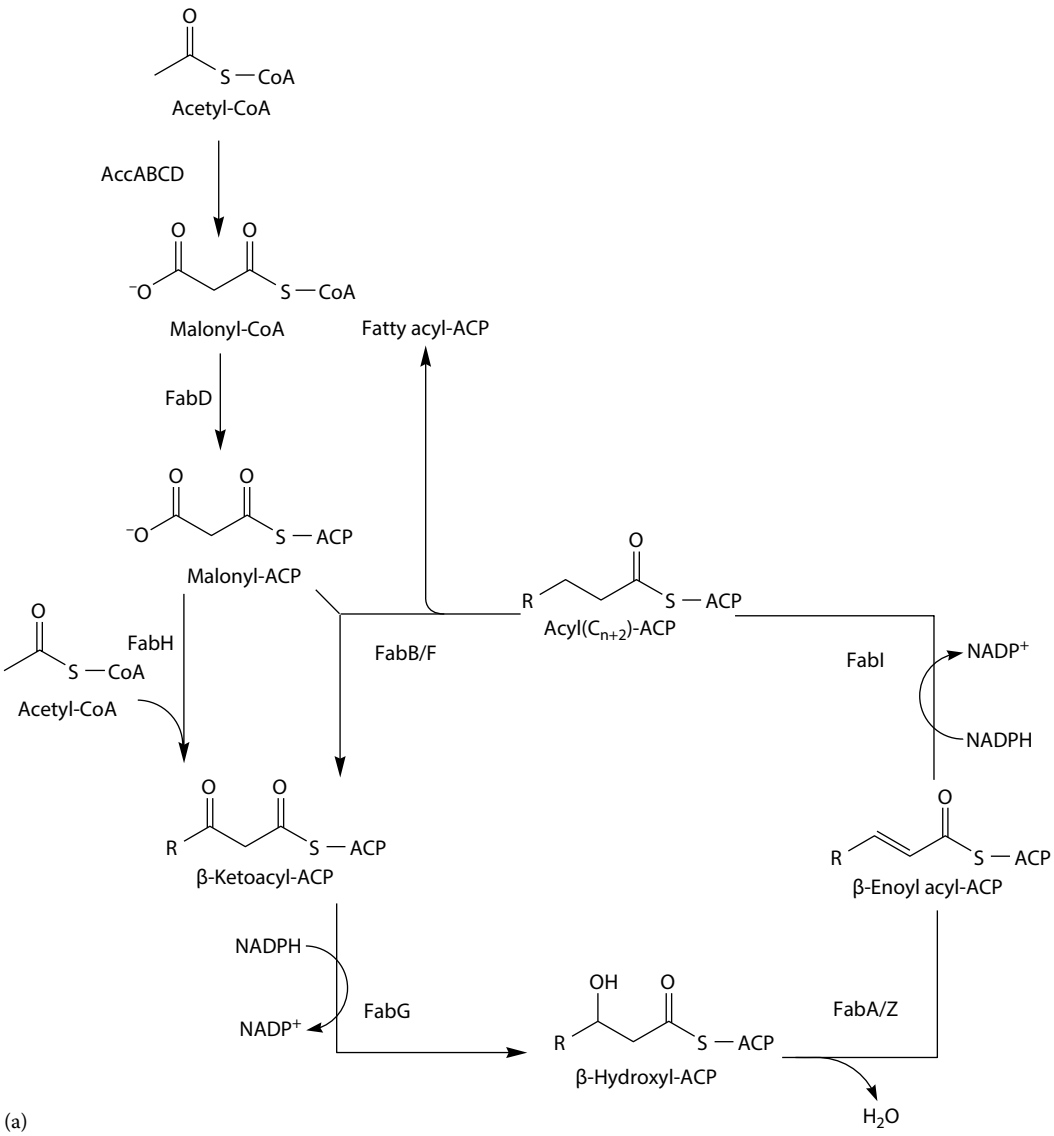
Microalgae can produce various sterols with different compositions, which are influenced by internal factors such as species and other external conditions such as light, intensity, and growth phase. For a more comprehensive review on the diverse sterol compositions of marine microalgae, the reader is referred to References [83–86]. Species of heterotrophic microalgae, which could grow in conventional fermentation systems, have more important role in commercial applications due to much lower cost than microalgae produced in most aquaculture nurseries [87,88]. The *Thraustochytrium* [89,90], *Crypthecodinium* [91], and *diatums* [92,93] have been used for commercial production of sterols. In addition, the spray-dried products, including cholesterol, 24-ethylcholesta-5,22E-dien-3b-ol, and 24-methylcholesta-5,22Edien-3b-ol, were available commercially in microalga heterotrophic *Schizochytrium* sp. by generous fermentation [88]. The composition of lipids produced by *Thraustochytrium* sp. ATCC 26185, as reported by Weete et al. [89], consists of the following: 63% of the nonsaponifiable matter was squalene and 41% of the total sterols were cholesterol. Moreover, 20 sterols were found in a docosahexaenoic acid–producing *Thraustochytrid* strain ACEM 6063, 13 of which were identified, and the predominant sterols were cholesterol, 24-ethylcholesta-5,22E-d ien-3b-ol, 24-methylcholesta-5,22E-dien-3b-ol, and 24-ethylcholesta-5,7,22-trien-3b-ol, comprising 50%–90% of the total sterols [90].

Among heterotrophic dinoflagellates, *Cr. cohnii* have never had any indication of pathogenicity or toxigenicity in all studies and could grow in the conventional fermenter at a low cost [91]. So it could be used in the commercial production of lipids such as DHA and/or stearidonic acid (18:4n-3) SDA-enriched algal oils [91,93]. Moreover, *Cr. cohnii* could produce some typical dinoflagellate sterols such as 4α-monomethyl compound dinosterol (4α-23, 24-trimethylcholest-22-en-3P-ol) and steroid ketones [94].

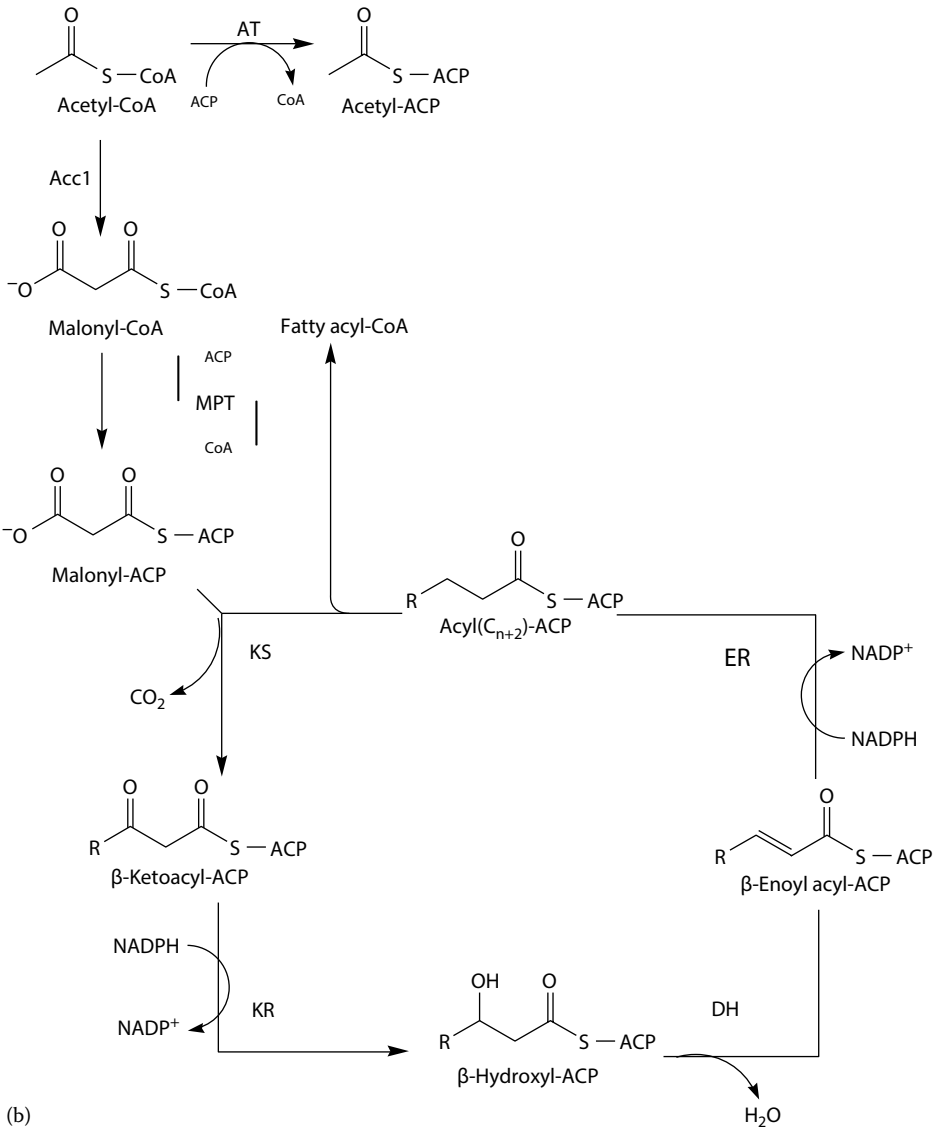
Orcutt et al. [95] compared the sterol percentage in the genera diatoms *Nitzschia*, *Phaeodactylum*, *Amphora*, *Navicula*, *Thalassiosira*, *Biddulphia*, and *Fragilaria* and identified a total of 11 kinds of sterols in the 11 organisms. 24S-Ergosta-5,22-dienol was the major sterol in five species, while stigmasterol was the major sterol in two species and cholesterol was the major sterol in one species [95].

Varied sterol profiles could be found in different fungi, protozoans, and yeasts, whereas ergosterol is the dominating component. The yield of ergosterol depends on the concentration as well as the type of sugars used as carbon source [96]. Sterols were essential cell components during the growth of *trypanosomatid protozoans* and could be applied for the therapy of the diseases resulting from this organism [97]. The sterols of fungi had several distinct

patterns [98]. The ergosterol (24b-methylcholesta-5,7,22E-trien-3b-ol) is distributed abundantly in the most advanced ascomycetes and basidiomycetes and some unicellular green algae [99,100]. Meanwhile, ergosterol was also the main lipid component in isolated melanized fungi such as *Hortaea werneckii*, *Alternaria alternata*, *Cladosporium cladosporioides*, *Cladosporium* sp., and *Aureobasidium pullulans*. These melanized fungi also contain different subordinate sterols such as 24-methyl-5α-cholest-7-en-3β-ol, 24-methyl-5α-cholesta-7,24,28-dien-3β-ol, and 4α,24-dimethyl-5α-cholest-7-en-3β-ol inside cells [101].



SCHEME 31.1 Schematic overview of *E. coli* type II (a) and *S. cerevisiae* type I (b) fatty acid biosynthesis pathway. (a) In type II fatty acid synthase (FAS) systems, the enzymes exist as distinct, individual proteins, where each protein catalyzes a single step in the reaction pathway. AccABCD initiates the formation of malonyl-ACP. Malonyl-CoA:ACP transacylase (FabD) substitutes the CoA for an acyl carrier protein (ACP). Then malonyl-ACP is iteratively fed into the elongation cycle. In order to continue the elongation reaction, acyl-ACPs are dehydrated by FabB/F. The end product is released as a fatty acyl-ACP after several rounds of elongation. (*Continued*)



SCHEME 31.1 (*Continued*) Schematic overview of *E. coli* type II (a) and *S. cerevisiae* type I (b) fatty acid biosynthesis pathway. (b) Type I FAS systems are multienzyme complexes that contain all the catalytic units as distinct domains. In *S. cerevisiae*, acetyl-CoA is activated by ACP acyltransferase (AT) and then malonyl-CoA is iteratively fed into the reaction cycle by malonyl/palmitoyl transferase (MPT). The elongation process is consecutively catalyzed by ketoacyl reductase (KR), dehydratase (DH), and enoyl reductase (ER). Different from *E. coli* fatty acid biosynthesis, the end product is released from the enzyme as a fatty acyl-CoA after back transfer to CoA from ACP by the double-functional MPT.

2. Wax

Many commercial products (i.e., cosmetics, candles, printing inks, lubricants, and coating stuffs) are made from biological waxes [102]. Specifically, some *Acinetobacter* strains accumulated wax in enormous quantity when they were grown on nitrogen-limited media and used *n*-alkanes or long-chain alkanols as carbon sources [102,103]. *Acinetobacter* sp. strain M-1 could reach 0.17 g hexadecyl hexadecanoate per gram in the cell (dry weight) with *n*-hexadecane as the substrate under optimized conditions [102]. With *n*-alkanes (C_{16}–C_{20}) as substrates, *Acinetobacter* sp. HO1-N could

form a series of mixed saturated and unsaturated wax esters containing several carbon–carbon double bonds, and they had a good chemical similarity to those of sperm whale and jojoba oils [103]. In general, the chain length of wax esters had a positive correlation with carbon sources. For example, when *n*-alkanes with longer carbon chains as substrates were used, the chain length of produced wax esters would be longer [103,104]. Besides, the ratio of unsaturated to saturated wax ester could increase with increasing chain length of *n*-alkane substrate [102].

The filamentous fungal cells *Rhizopus arrhizus* could employ TAG hydrolysates as sources to produce insolubilized lipase on the continuous reactor [105]. Légier and Comeau [106] designed a loop fixed-bed reactor to fulfill a continuous ester synthesis. A yield of 90% of dodecanoic dodecyl ester from long-chain alcohol and oleic acid could be produced and catalyzed by immobilized *Penicillium cyclopium* and *Rhizopus arrhizus*. *Rhizopus niveus* fungal cells immobilized on cellulose biomass support particles have also been used for wax ester synthesis [107].

With glucose, malate, and glutamate as the main carbon sources and gained ATP from the conversion of paramylon by fermentation, anaerobic *Euglena gracilis* SM-ZK cells could accumulate saturated wax ester ranging from C_{24} to C_{32}, among which the main component was C_{28} esters [108].

Grown in yeast malt extract medium, microalga *Eu. gracilis* ATCC 12716 could synthesize wax esters with saturated even carbon numbered fatty acids and alcohols ranging from C_{12} to C_{18} as carbon source resulting in predominant wax product tetradecanoyl tetradecanoate [109].

The four bacteria isolated from the marine environment (*Acinetobacter* sp. strain PHY9, *Pseudomonas nautica* IP85/617, *Marinobacter* sp. DSMZ 11874, and *Marinobacter hydrocarbonoclasticus* ATCC 49840) could generate isoprenoid wax esters with some acidic and alcoholic metabolites as sources during an aerobic degradation of 6,10,14-trimethylpentadecan-2-one and phytol [110]. Other microorganisms such as *Corynebacterium* sp. could also catalyze the esterification reaction among fatty acids with long-chain alcohols, yielding wax esters [111].

3. Ketones and Lactones

Used as flavor compounds and solvents, methylalkylketones (MAKs) have attracted much attention. It could be produced from bacterial and yeast secondary alcohols [112,113] and fatty acids by filamentous fungi [114–120]. But there were only a few reports about the commercial applications of this chemical. In 1989, Takashi Yagi et al. [121] observed that a fungus strain *Trichoderma* sp. SM-30 could accumulate remarkable amount of methylheptylketone with a maximal yield of approximately 36% with tricaprin substrate.

Cell suspensions of thermophilic obligate methane-oxidizing *bacterium* H-2, *Candida utilis* ATCC 26387, *H. polymorpha* ATCC 26012, *Pichia* sp. NRRL-Y-11328, *Torulopsis* sp. strain A1, and *Kloeckera* sp. strain A2 were able to oxidize secondary alcohols (C_3–C_{10}) into their corresponding methyl ketones [111,112].

The spores of fungus strain, *Penicillium roqueforti* ATCC 64380316, having excellent biocatalyst activity, could transform C_6–C_{10} fatty acids and their esters (TAGs) into various kinds of methylalkylketones (21 g 2-pentanone, 73 g 2-heptanone, and 57 g 2-nonanone per liter) by solid-state fermentation on buckwheat seeds [122]. The filamentous fungi, *Aureobasidium* SM-25, could generate *n*-alkane-2-one from both synthetic and natural TAGs [123]. The *Fusarium avenaceum f.* sp. fabae IFO 7158 could metabolize tricaprin for accumulation of *n*-nonane-2-one and monohydroxy-*n*-nonane-2-ones, a mixture of 8-hydroxy-2-one, 7-hydroxy-2-one, and 6-hydroxy-nnonane-2-one in an approximate ratio of 10:7:0.5 [124].

High-grade macrocyclic lactones C_{14}–C_{16} are expensive aromatic substances responsible for musky fragrance. Some attempts have been made to lactonize appropriate hydroxyacids esters with lipases as biocatalysts and further forming macrocyclic lactones [125–127]. Gatfield first synthesized pentadecanolide in 1984, by using *Mucor miehei* lipase to catalyze intramolecular esterification of 15-hydroxypentadecanoic acid [125]. The fungi *Mucor javanicus* and *M. miehei* catalyzed the lactonization of ω-hydroxy fatty acids, 15-hydroxypentadecanoic acids, and 16-hydroxyhexadecanoic acids to the corresponding macrocyclic monolactones and oligolactones that also been reported [126].

Pseudomonas sp. K-10 lipase was found to be able to catalyze the reaction of ciscyclohexane-1,2-dimethanol with 1,12-dodecanedicarboxylic acid to give 41% monolactone and 25% dilactone, while *Candida cylindracea* lipase could catalyze the condensation of *trans*-cyclohexane-1,4-dicarboxylic acid and 1,16-hexadecanediol to produce 42% monolactones and 8% dilactones [127].

4. Biosurfactants

Biosurfactants are amphiphilic compounds of microbial origin with considerable potential in commercial applications, including oil recovery, food processing, environmental bioremediation, and cosmetic and pharmaceutical industries [128]. The microorganisms make high-molecular-weight bioemulsifiers that bind tightly to surfaces, which are composed of amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins, or complex mixtures of these biopolymers, and low-molecular-weight bioemulsifiers, which are generally glycolipids that could lower surface and internal tensions [129]. To make biosurfactants economically attractive, many strategies have been used, such as using cheaper raw materials, optimizing bioprocess, and improving biosurfactant productivity of microorganisms via genetic engineering [130]. A few recombination and mutant hyperproducers are shown in Table 31.4.

The largest market for biosurfactants application is the petroleum industry, typically including oil production and oil bioremediation [139]. Petroleum bioremediation and other environmental bioremediation such as heavy metals mainly relied on the capability of microorganisms to degrade hydrocarbons [140–142]. Yeast, fungus, and bacteria have been reported capable of degrading hydrocarbons. A *Mycobacterium* sp. strain was first reported to be able to mineralize pyrene and other polycyclic aromatic hydrocarbons (PAHs) [143]. Other *Mycobacterium* sp. strains, such as *Mycobacterium* sp. CH1, is also capable of utilizing PAH including phenanthrene, pyrene, and fluoranthene [144]. Moreover, *Alcaligenes denitrificans* [145], *Paenibacillus* sp. [146,147] *Neptunomonas naphthovorans* [148], *Stenotrophomonas maltophilia* species [149], *Sphingomonas* genus [150], *Rhodococcus* sp. [151], and several *Pseudomonas* species [152,153] have been isolated and examined for metabolization of PAHs.

Different from bacteria, fungi could transform PAHs into detoxified metabolites rather than utilizing PAHs as their sole carbon source [154]. The fungus PHA-degrading studies mainly focused on white-rot fungi such as *Phanerochaete chrysosporium* [155–157], *Pleurotus ostreatus* [158], and *Trametes versicolor* [159]. The ability of white-rot fungi *Lentinus subnudus* found in Nigeria to mineralize soil contaminated with various concentrations of crude oil was also examined [160].

TABLE 31.4
Biosurfactants Production from Modified Microorganisms by Genetic Engineering

Microorganisms	Production Enhancement	Strategy	Ref.
Pseudomonas aeruginosa PG201	*Two times more rhamnolipids*	Transposon Tn5-GM-induced mutants	[131]
Bacillus subtilis	Lichenysin production	Whole module swapping	[132]
Bacillus subtilis ATCC 21332	Ipohexapeptide production with reduced toxicity	Genetic recombination	[133]
Pseudomonas aeruginosa PTCC1637	Ten times more rhamnolipids	Random mutagenesis	[134]
Gordonia amarae NRRL B-8176	Four times more trehalose lipid	Recombinant plasmids	[135]
P. aeruginosa PEER02 and *E. coli* TnERAB	Rhamnolipid production	Transposome-mediated chromosome integration	[136]
Bacillus licheniformis	Twelve times more lipopeptide	Random mutagenesis	[137]
Pseudomonas aeruginosa EBN-8	Three to four times more hydrocarbon emulsification	Gamma ray–induced mutant	[138]

A fission yeast isolated from palm wine was demonstrated to be able to utilize diesel, kerosene, and methanol as growth substrates [161].

III. MODIFICATION OF LIPIDS USING ISOLATED ENZYMES

A. Lipases/Esterases and Catalytic Specificities for Multipurpose Applications

Lipases (EC 3.1.1.3) are a class of enzymes that have the capability to catalyze both hydrolysis of oils and fats and synthesis of esters from fatty acids and alcohols [163]. Furthermore, they catalyze a myriad of transesterification and interesterification reactions including acidolysis, alcoholysis, aminolysis, or glycerolysis [163–166]. Lipases are characterized by their high regio- and/or enantioselectivity. The substrate specificities and regioselectivities in the hydrolysis of TAGs by some commercially available lipases are summarized in Table 31.5 [167–172]. Scheme 31.2 illustrates some representative lipase-catalyzed reactions [171]. Lipases have found enormous applications in biotechnology due to the fact that they (1) possess a wide range of substrate specificities, (2) are stable in organic solvents, (3) do not require cofactors, and (4) exhibit a high enantioselectivity. The following section outlines some commercial applications of lipase-catalyzed reactions.

B. Structured Triacylglycerols

1. Cocoa Butter Substitutes

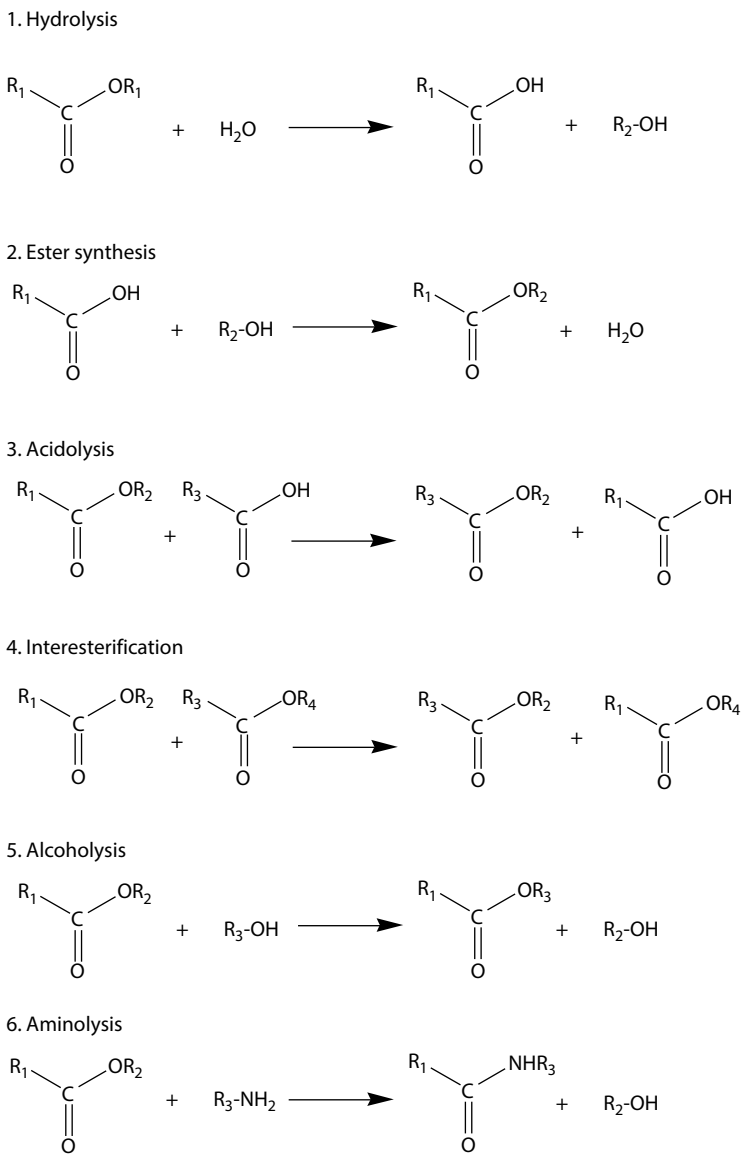
Cocoa butter is very important for food industry, particularly for chocolate manufacture. The TAG composition profile of cocoa butter is characterized by the fact that saturated fatty acids (mainly palmitic and stearic acids) are located at the *sn*-1 and *sn*-3 positions of glycerol backbone, whereas monounsaturated fatty acids such as oleic acid are at the *sn*-2 position. The rheological and sensory attributes of cocoa butter are dependent on this unique structure of TAGs. The interesterification

TABLE 31.5
Specificity of Triacylglycerol Lipases from Different Sources toward Various Fatty Acids/ Acyl Moieties

Source of Lipase	Lipase	Fatty Acid Selectivity
Microorganisms	*Candida rugosa* (syn. *C. cylindracea*)	Nonpreferable for all *cis*-PUFAs, such as DHA, GLA, SDA, and EPA
	Penicillium cyclopium	Preferable for Δ9 *cis*-unsaturated fatty acids
	Penicillium sp. (*lipase G*)	Preferable for Δ9 *cis*-unsaturated fatty acids
	Rhizomucor miehei	No fatty acids preference
	Rhizopus arrhizus	No fatty acids preference
Plants	Rape (*Brassica napus*) seedlings	Nonpreferable for DHA, petroselinic, GLA, SDA, and dihomo-γ-linolenic acids
	Papaya (*Carica papaya*) latex	Nonpreferable for DHA, petroselinic, GLA, SDA, and dihomo-γ-linolenic acids
Animal tissues	Porcine pancreas	Nonpreferable for DHA, petroselinic acid, GLA, and SDA

Sources: McNeill, G.P., Enzymic processes, in: Gunstone, F.D., ed., *Lipid Synthesis and Manufacture*, Academic Press, Cambridge, MA, 1998, p. 288; Godfrey, T., Lipases for industrial use, *Lipid Technol.*, 7, 58, 1995; Villeneuve, P. et al., *Chem. Phys. Lipids*, 76, 109, 1995; Hills, M.J. and Mukherjee, K.D., *Appl. Biochem. Biotechnol.*, 26(1), 1, 1990; Villeneuve, P. et al., *J. Am. Oil Soc.*, 72(6), 753, 1995; Villeneuve, P. and Foglia, T., *INFORM*, 8, 640, 1997.

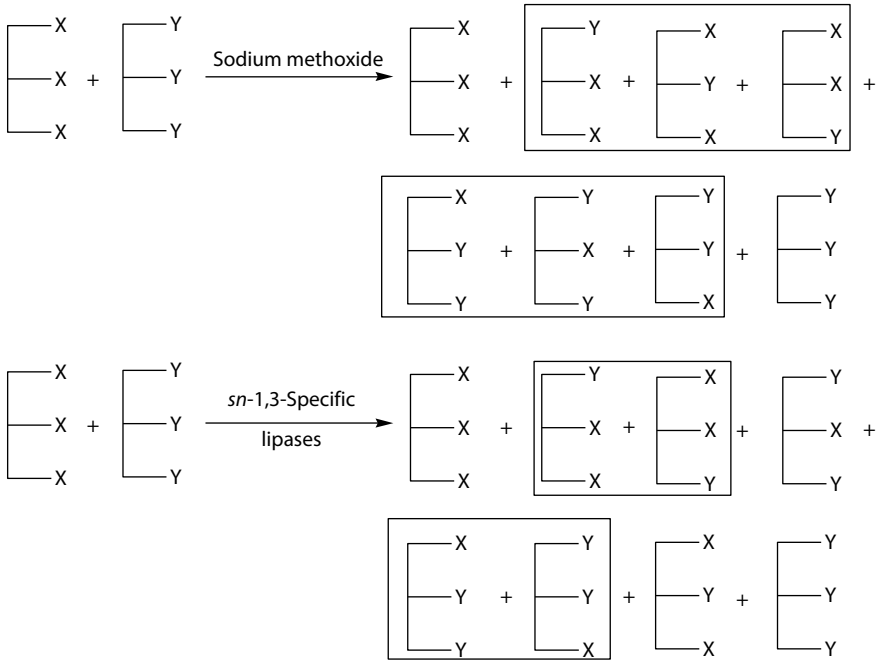
DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; SDA, stearidonic acid.



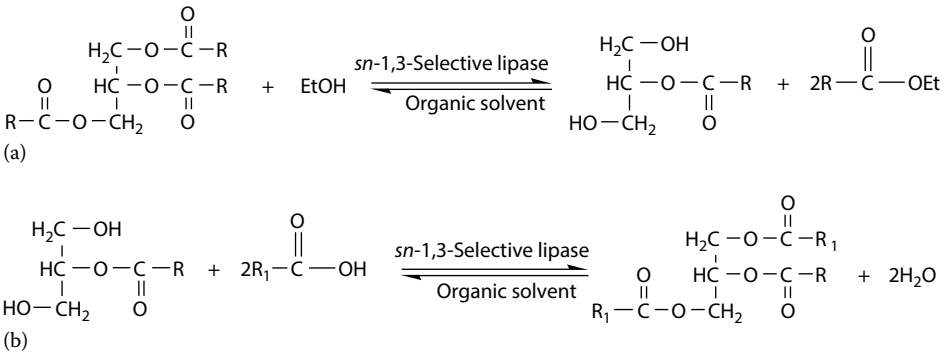
SCHEME 31.2 Diverse reactions catalyzed by lipases.

reaction of lipases has been explored for the production of shortenings, margarines, and other specialty fats. A new trend that has emerged is the use of enzymatic interesterification to produce fats, which resemble cocoa butter in terms of their physical properties and TAG structures [173]. The use of lipases has gained prominence in the area over the use of chemical catalysts due to their regioselectivity. Furthermore, the product obtained by enzymatic interesterification better resembles cocoa butter, and it is possible to carry the reactions at lower temperatures (see Scheme 31.3 for a comparison of the interesterification of TAGs by chemical catalysis and *sn*-1,3-specific lipase).

Many industrial productions of cocoa butter substitutes involve the use of palm midfraction and tristearate or stearic acid and the vegetable oils rich in oleic acid in *sn*-2 position using *sn*-1,3 lipases. Two products with a similar TAG composition as cocoa butter have been obtained through (1) interesterification of tea seed oil with methyl stearate and methyl palmitate [174] and (2) acidolysis reaction involving olive pomace oil and mixtures of palmitic and stearic acids in *n*-hexane [175].



SCHEME 31.3 Schematic representation of chemical and *sn*-1,3-specific lipase-catalyzed interesterification of two triacylglycerols (TAGs). Chemical catalysis yields randomized products, while lipase-catalyzed reaction gives similar products. TAG species in the rectangles have the same amount of randomized products and also the same amount of *sn*-1,3-specific lipase products provided that the specificities of the lipase for *sn*-1,3 positions and the different fatty acids are similar. X and Y are different fatty acids. (From Chimni, S.S. et al., *J. Mol. Catal. B: Enzym.*, 63, 128, 2010.)



SCHEME 31.4 Lipase-catalyzed synthesis of structured TAG by (a) alcoholysis and (b) esterification. (From Yu, C.Y. et al., *Ind. Eng. Chem. Res.*, 53, 7923, 2014.)

Scheme 31.4 illustrates the preparation of structured TAG via a two-step process of acidolysis followed by esterification.

2. Human Milk Fat Replacers

Approximately 50%–60% of the energy needs of infants are provided by human milk fat (HMF) [176,177]. Palmitic acid (C16:0) is the predominant fatty acid in HMF making up about 20%–25% of total milk fatty acid composition. In HMF, palmitic acid predominantly occupies the *sn*-2 position

of the glycerol backbone (>60%) with *sn*-1,3 positions occupied by unsaturated fatty acids [178]. The regioselectivity of lipases (*sn*-1,3-specific lipases) has made it possible to synthesize HMF replacers. Some typical HMF replacers can be produced in the following ways: (1) interesterification of tripalmitin from palm oil or lard with plant oils [179]; (2) acidolysis of butterfat, tripalmitin, lard, palm stearin, or palm oil with unsaturated fatty acids [180–185]; or (3) use of multistep reactions to synthesize 1,3-dioleoyl-2-palmitoylglycerol, a structured TAG in infant formulas [186]. *Betapol*™ and InFat™ are two commercial HMF replacers from *Loders Croklaan* (*Unilever*) and advanced lipids, respectively. They are both synthesized through an acidolysis reaction catalyzed by *sn*-1,3-specific lipases from *Rhizomucor miehei* and *Rhizopus oryzae* [187].

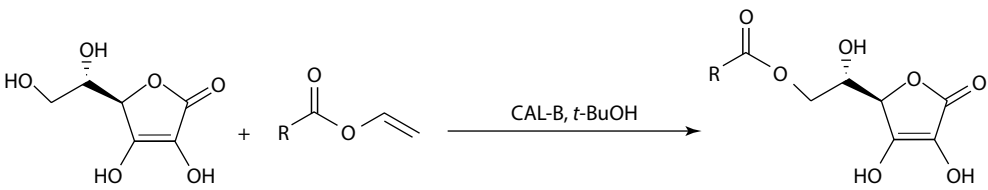
3. Nutraceuticals

Structured lipids for specific dietary purposes can be synthesized through transesterification reactions catalyzed by *sn*-1,3-specific lipases. The enrichment of TAGs with bioactive fatty acids such as conjugated linoleic acid, γ-linolenic acid, and ω-3 PUFAs has been reported in several studies [188–190]. The property of regioselectivity of lipases has further been used to synthesize low-calorie and dietetic TAGs that contain short-/medium-chain fatty acids at *sn*-1 and *sn*-3 positions of the glycerol backbone and a long-chain unsaturated fatty acid at the *sn*-2 position or TAGs containing three medium-chain fatty acids [191]. They have caloric values between 21 and 29 kJ/g (5.0–6.9 kcal/g) compared to conventional TAG, which have caloric values around 38 kJ/g (9.1 kcal/g) [191]. The difference is due to the lower caloric content of short- and medium-chain fatty acids compared to long-chain fatty acids [191]. One of the most common ways to produce dietetic TAG is through acidolysis reactions of vegetable or fish oils, where medium-chain fatty acids such as caprylic or caproic acids serve as acyl donors.

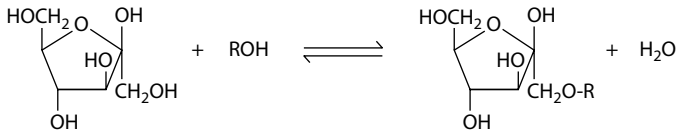
C. Bioesters, Long-Chain Esters, and Flavor Esters

Short-chain fatty acid alkyl esters form important components of natural fragrances and flavors. They are widely used in cosmetics, pharmaceuticals, foods, and beverages. Traditional methods such as chemical synthesis and extraction from various sources have provided these esters for a long time [192]. However, the increasing interest in natural foods has prompted the use of biotechnology to provide flavor esters. Fermentation procedures are a source of esters but the yields are often low [192,193].

A different approach is enzymatic synthesis, which is more productive and efficient. At the same time, flavor esters prepared by enzymatic synthesis are labeled as "natural" [194]. Lipase-catalyzed transesterification and esterification for the preparation of flavor esters has received considerable attention. Particularly, the nonspecific lipases from *Candida antarctica* and *Rhizopus* spp. have been applied commercially to produce a wide variety of esters including *n*-butyl acetate, cetyl esters, octyl stearate, isopropyl myristate, isopropyl palmitate, octyl palmitate, and citronellyl flavor esters [195–199]. Examples of the preparation of bioesters are shown in Schemes 31.5 and 31.6 for esters of vitamin C and fructose, respectively.



SCHEME 31.5 Lipase-catalyzed synthesis of vitamin C esters by reaction of vitamin C with vinyl esters. (From Rickert, W.K. and Klinman, P.J., *Biochemistry*, 38, 12218, 1999.)

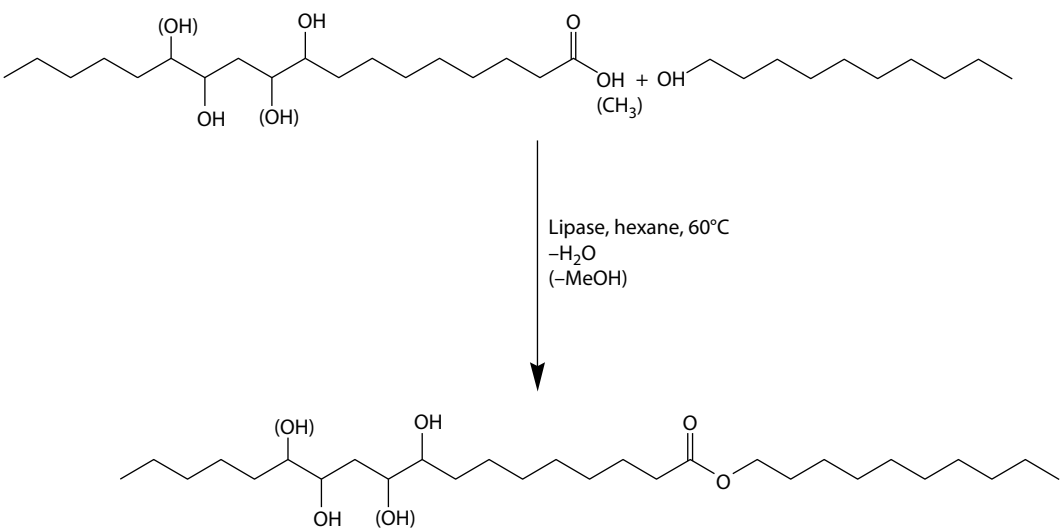


SCHEME 31.6 Lipase-catalyzed synthesis of fructose esters. (From Radzi, M.S. et al., *J. Oleo Sci.*, 54(4), 203, 2005.)

D. Wax Esters and Steryl Esters

Waxes are esters of long-chain alcohols and long-chain fatty acids with chain lengths ≥12 [110]. Natural sources of waxes include plant and animal materials such as beeswax, jojoba oil, and spermaceti oil. Waxes have found wide applications in pharmaceutical products and cosmetics or as lubricants and plasticizers mainly due to their softness [200]. The division of wax esters into saturated and unsaturated is dependent on their chemical structures. Waxes from jojoba oil and spermaceti are unsaturated. Generally, natural unsaturated waxes such as jojoba oil are scarcely available and expensive to be exploited commercially. Alternative means are therefore being sought [201,202]. Chemical synthesis of wax replacements has been investigated for many years. However, chemical catalyzed synthesis has many drawbacks. Among them are the corrosive nature of the chemicals, hazards associated with chemicals, high energy consumption, and sometimes degradation of synthesized wax esters. The use of lipases for the synthesis of waxes has attracted much attention due to their mild reaction conditions and environmental benignity. Various studies have reported the synthesis of waxes using lipases. Two main routes have been intensively applied for lipase-catalyzed reactions. The first is alcoholysis of TAGs with fatty alcohols, and the second is the direct esterification of long-chain fatty acids with long-chain alcohols. Wax ester synthesis through direct esterification provides better yields than that through alcoholysis especially when the reactions take place in a low water medium [203].

Multzsch et al. [204] obtained yields of wax esters up to 90% through acylation of long-chain fatty alcohols with long-chain fatty acids or methyl ester (Scheme 31.7). Lipases from *Rhizomucor*



SCHEME 31.7 Lipase-catalyzed acylation of decanol with 9,10,12,13-dihydroxy octadecanoic methyl fatty acid esters in *n*-hexane. (From Ron, E.Z., Microbial life on petroleum, in: Seckbach, J., ed., *Journey to Diverse Microbial Worlds: Adaptation to Exotic Environments*, Springer, New York, 2000, pp. 303–315.)

miehei and *Candida antarctica* were used. Similarly, Gunawan et al. [200] synthesized palm-based wax esters from palm oil and oleyl alcohol by Lipozyme IM. Radzi et al. [205] obtained yields in excess of 90% when they esterified oleic acid and oleyl alcohol to form a liquid ester. Using immobilized *Candida antarctica* lipase B (Novozym 435), the optimized reaction conditions for such yields were as follows: reaction time 30 min, temperature 50°C, agitation 400 rpm, and a mole ratio of oleic acid to oleyl alcohol of 1:2. Wax esters resembling jojoba oil were also obtained through lipase-catalyzed alcoholysis of seed oils from *Sinapis alba*, *Lunaria annua* [206], or *Crambe abyssinica* [207]. In addition, immobilized lipases from *Candida antarctica* or *papaya* were used to synthesize wax esters through transesterification of oleyl alcohols with seed oils from crambe (*Crambe abyssinica*) and camelina (*Camelina sativa*) [208]. Lipase-catalyzed alcoholysis of triolein to produce wax esters has been intensively investigated [209–211].

Alkyl esters of fatty acids have been obtained in high yields through esterification reactions catalyzed by lipases from *R. miehei* [212,213] and *Candida rugosa* [214]. Moreover, high yields of alkyl esters were obtained through lipase-catalyzed transesterification of *n*-butanol, ethanol, or isopropanol with TAGs [215,216]. It is possible to enhance the yields of alkyl esters by removing glycerol using silica gel [216]. Alcoholysis of low-erucic-acid rapeseed oil with 2-ethyl-1-hexanol produced 2-ethyl-1-hexyl esters that could be used in printing ink [217]. Similarly, butyl esters (e.g., butyl oleate), which have found applications as lubricants, biodiesel additives, hydraulic fluids, and plasticizers for polyvinylchloride, can be synthesized by lipase-catalyzed esterification of oleic acid with *n*-butanol [217]. Esterification of long-chain alcohols with castor oil fatty acids or transesterification of castor oil with long-chain alcohols produced long-chain alkyl ricinoleic acid esters in high yields [218].

Medium- and long-chain dialkyl thia-alkanedioate antioxidants (e.g., dialkyl 3,3′-thiodipropionates such as dioctyl-, didodecyl-, dihexadecyl-, and dioleyl-3,3′-thiodipropionate) can be prepared either by a direct esterification of 3,3′-thiodipropionic acid with long-chain alkanols or by transesterification of dimethyl esters of 3,3′-thiodipropionic acid with long-chain alkanols [219]. Immobilized *Candida antarctica* lipase B (Novozym 435) is most effective for preparation of medium- and long-chain dialkyl thia-alkanedioate antioxidants. Lipases from *Thermomyces lanuginosus* (Lipozyme TL IM) and *R. miehei* (Lipozyme RM IM) are less active in this regard.

Steryl esters, which are used as blood cholesterol lowering supplement, can be obtained through lipase-catalyzed esterification reactions [220–224]. Lipase from *Pseudomonas* sp. catalyzed the synthesis of steryl esters of PUFAs in good yields but at low reaction rates [225]. Using *Candida rugosa* lipase as catalyst, fatty acyl esters of phytosterols and phytostanols have been obtained in near quantitative yields through esterification of sterols with fatty acids or by transesterification with fatty acid alkyl esters, both under vacuum [226–229].

E. Monoacylglycerols and Diacylglycerols

Monoacylglycerols and their derivatives are important emulsifiers for the food industry. They comprise approximately 75% of the world production of food emulsifiers [230]. Glycerolysis is the common method for production of monoacylglycerols. This reaction involves transesterification of TAGs with glycerol [231]. High temperatures (200°C–250°C) and inorganic alkaline catalysts under nitrogen atmospheres are often employed in conventional batchwise glycerolysis. The composition of the finished product is usually made up of 35%–60% monoacylglycerols, 35%–50% diacylglycerols, 1%–20% unreacted TAGs, 3%–4% residual glycerol, and 1%–10% free fatty acids [232].

In addition to side products, the major drawbacks of the conventional method for monoacylglycerol production are low yields, high energy consumption, and poor product quality [233]. However, the synthesis of monoacylglycerols by enzymatic process is rather environmentally friendly. Various approaches have been developed by means of lipase catalysis. For instance, monoacylglycerols could be synthesized by selective glycerolysis or hydrolysis using *sn*-1,3 regiospecific lipases [234],

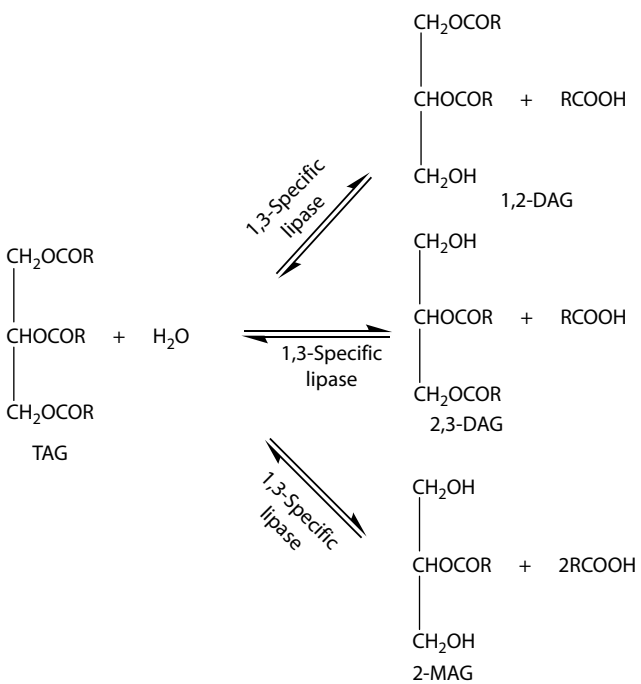
esterification of fatty acids with glycerol [235], and glycerolysis of fats and oils [236]. Higher yields, superior product quality, and lower energy consumptions could be achieved with the use of enzymes.

Kapoor and Gupta [237] compared the performances of different preparations of lipase from *Candida antarctica* (CALB) for the synthesis of monopalmitin with glycerol and palmitic acid in a low water medium (1% v/v) for 24 h (Scheme 31.8 illustrates one of general pathways for the preparation of mono- and diacylglycerols by *sn*-1,3-specific lipases). They reported yields of 81%, 82%, and 87% by means of cross-linked enzyme aggregates, protein-coated microcrystals, and cross-linked protein-coated microcrystals, respectively. In addition, the amounts of diacylglycerols obtained were, respectively, 4.5%, 4%, and 3.3%. Similarly, Valéro et al. [238] obtained a yield of ~70% (wt.%) monoacylglycerols for Novozym 435-catalyzed glycerolysis of olive oil in compressed *n*-butane within a short reaction time of 2 h.

The enrichment of beneficial fatty acids such as eicosapentaenoic acid and docosahexaenoic acid in monoacylglycerols has been studied. The glycerolysis of tuna oil with lipase AK, obtained from *Pseudomonas fluorescence*, produced a low yield of monoacylglycerols (24.6%) but contained 56.0% PUFAs [239]. The esterification of highly unsaturated fatty acids with glycerol has been reported to produce diacylglycerols in high yields [239–241]. Immobilized lipase from *R. miehei* (Lipozyme RM IM)-mediated reaction could produce improved diacylglycerol yields (60%–75%) by the interesterification of rapeseed oil with commercial monoacylglycerols such as Nutrisoft 55, Monomuls 90-O18, and Mulgaprime 90 [242]. Short-path distillation was used thereafter to obtain pure diacylglycerols with the yield of 66%–70% [242].

F. Lipophilic Phenolics

There has been a growing interest in the extraction of antioxidants from natural sources such as vegetable oils, rice hulls, spices, and tea leaves [243]. The reason for this is the alarming health concerns regarding the use of synthetic antioxidants such as butylated hydroxyl anisole and butylated hydroxyl

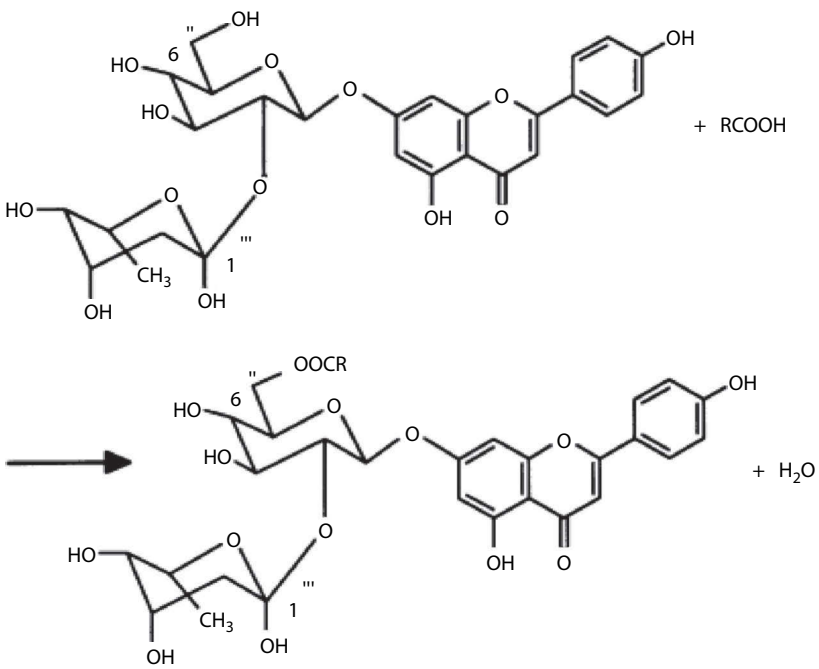


SCHEME 31.8 General scheme for the preparation of mono- and diacylglcyerols by *sn*-1,3-specific lipases. (From Dias-Ferreira, S. et al., *Electron. J. Biotechnol.*, 16, 3, 2013.)

toluene [244]. Attractively, some studies have shown that phenolic compounds and their derivatives are very efficient antioxidants at least in biological systems. They occur widely in plant cell walls as esters of fatty acids, carbohydrates, and proteins [245,246]. The ability of phenolics to serve as antioxidants in biological systems implies that they are somewhat soluble in aqueous media.

The practical applications of phenolic antioxidants in hydrophobic media such as in lubricants are limited by their lipophilic solubility. This limitation can be overcome through esterification of phenolics with aliphatic acyl chains. Properties such as solubility, miscibility, and their activity in hydrophobic formulations and emulsions are altered as a consequence [247]. Enzymatic esterification of phenolic compounds with fatty alcohols was first reported by Guyot et al. [248]. Cinnamic acid and benzoic acid derivatives were esterified with fatty alcohols with chain lengths between C4 and C12 using CALB (*Candida antarctica* lipase B) [249]. Their results indicated that CALB has a preference for the phenolics with primary hydroxyl groups compared to the polyphenolics with secondary or tertiary hydroxyl groups. Furthermore, they reported improved solubilities and stabilities of lipophilized derivatives of cinnamic acid and benzoic acid. In addition, lipase B from *Candida antarctica* was used to acylate several phenolics (rutin, esculin, quercetin, and hesperidin) in 2-methly-2-butanol at 60°C. Conversion yields of 38%, 70%, and 80% were reported for hesperidin, rutin, and esculin, respectively [250]. When carbon numbers of acyl donors were increased from 6 to 12, conversion of rutin rose from 42% to 76% but dropped when chain lengths were longer than 12. See Scheme 31.9 for lipase-catalyzed lipophilization of naringin.

Moreover, it has been possible to synthesize medium- or long-chain alkyl cinnamates, as well as hydroxycinnamates such as oleyl *p*-coumarate, oleyl ferulate, and palmityl ferulate in high yields via lipase-catalyzed transesterification of equimolar mixtures of fatty alcohols (e.g., lauryl, palmityl, and oleyl alcohols) with short-chain or long-chain alkyl cinnamates [251]. Novozym 435 (immobilized enzyme from *Candida antarctica*) is the most efficient enzyme for this kind of esterification. The relative transesterification activities found for *C. antarctica* lipase are of the following order: hydrocinnamate > cinnamate > 4-hydroxyhydrocinnamate > 3-methoxycinnamate >



SCHEME 31.9 Lipase-catalyzed lipophilization of naringin. (From Lu, D.Q. et al., *Afr. J. Biotechnol*., 10, 3436, 2011.)

2-methoxycinnamate ≈ 4-methoxycinnamate ≈ 3-hydroxycinnamate > hydrocaffeate ≈ 4-hydroxycinnamate > ferulate > 2-hydroxycinnamate > caffeate ≈ sinapate. Additionally, it prefers hydroxyl substituents in the following order: *meta* > *para* > *ortho*. Several other studies have also reported the synthesis of lipophilic phenolics using isolated enzymes as catalysts [252–256].

G. Lactones and Estolides

Lactones are intramolecular esters of hydroxy fatty acids. They are naturally available as γ- and δ-lactones. Lactones form important aroma substances in musky fragrances. Besides, lactones are used as scaffolds for the synthesis of antibiotics such as roxithromycin or erythromycin as well as pesticides [257]. Enzymatic synthesis of lactones was first reported by Gatfield using a lipase from *M. miehei* with 15-hydroxy pentadecanoic acid as substrate [257]. Lactone synthesis has been carried out using lipases as biocatalysts mostly based on ω-hydroxy fatty acids as substrates.

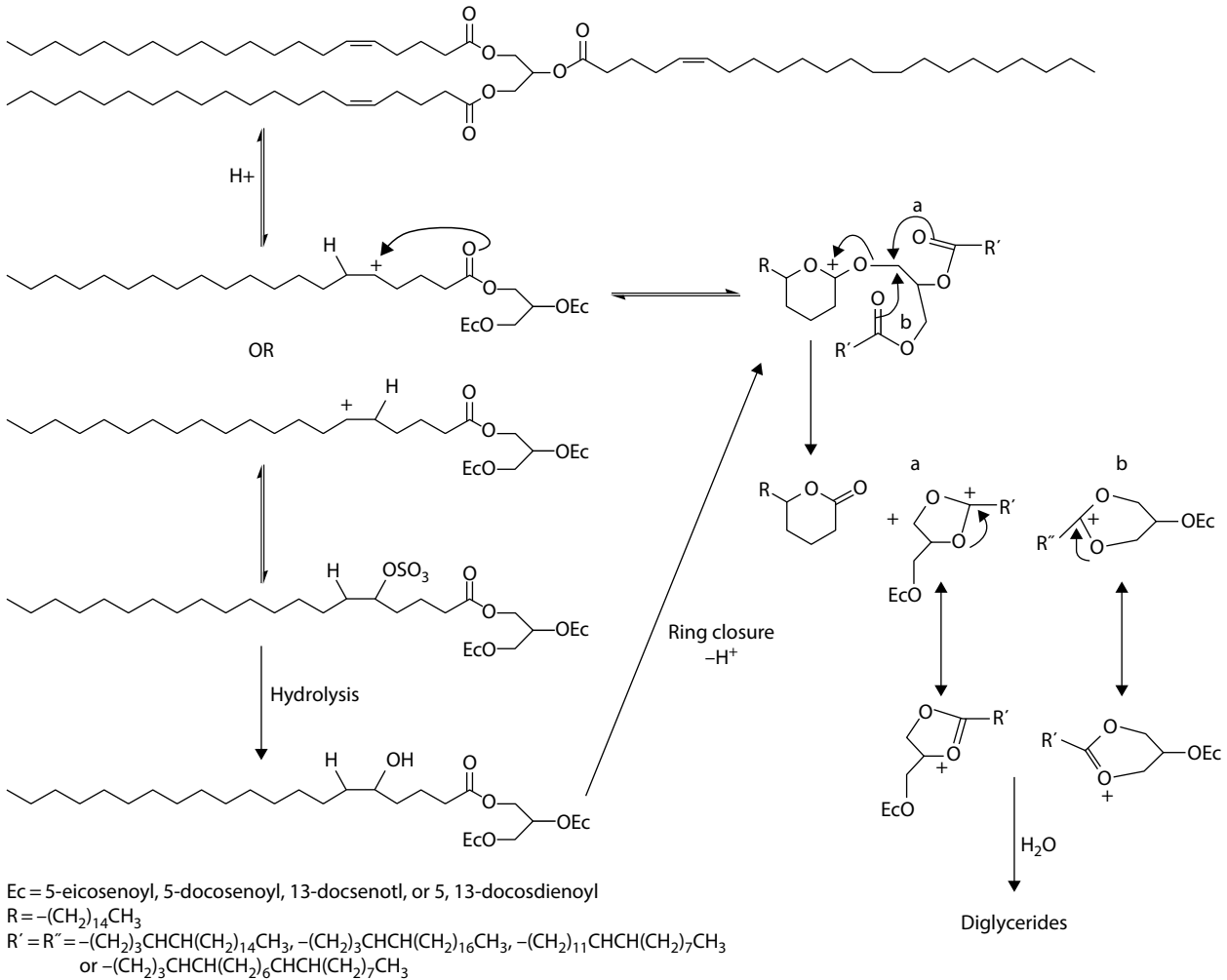
A novel macrolactone (13*S*-octadeca-(9*Z*,11*E*)-dienolide) was obtained through the intraesterification of the hydroxyl fatty acid (*R*)-13-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid catalyzed by immobilized lipase B from *Candida antarctica* (Scheme 31.10). Higher yields were obtained at 35°C using diisopropyl-ether as a solvent [258]. Similarly, a hexadecanolide lactone was synthesized from the corresponding 16-hydroxyhexadecanoic acid using Novozym 435. An optimization of the synthesis parameters (use of nonpolar solvent and under low water activity) gave monoester lactone a yield of around 41%. Attempts were made by the same authors to improve the yield by means of microemulsion technique but were not successful [259,260].

Preparation of chiral lactones using molecular distillation has been investigated. It was suggested that it is possible to separate enantiometric γ- and δ-lactones mixture, produced by *Pseudomonas cepacia* lipase-catalyzed reaction, by molecular distillation [261]. However, the yields from such processes are often low besides the poor enantioselectivities and the use of expensive chiral catalysts. A successful one-pot reduction of γ-ketoesters, δ-ketoesters, and lactones to the corresponding 1,4- and 1,5-diols followed by a lipase-catalyzed kinetic resolution coupled with hydrolysis to afford optically active diols was reported [262].

Estolides are polymeric molecules that are composed of hydroxyl fatty acids. Through esterification, the hydroxyl group of one fatty acid moiety is covalently linked with a carboxylic group from another fatty acid. Estolides have important applications in the manufacture of lubricants, plasticizers, printing ink, and cosmetic products mainly due to their low melting points and high viscosities. Lipases from *Rhizomucor miehei*, *Aspergillus niger*, *Pseudomonas* spp., and *Candida rugosa* have been used as biocatalysts for estolide synthesis [263,264].

H. Sugar Fatty Acid Esters, Alkylglycosides, and Other Hydroxy Compounds

Sugar fatty acid esters are synthesized from renewable sources, and their nontoxicity, odorless nature, biodegradability, and broad hydrophilic–lipophilic balance value spectrum have made them preferable emulsifiers for a number of food applications [265,266]. In addition, they are used as low-calorie sweeteners and as antimicrobial agents in pharmaceutical and food industries [265–269]. Carbohydrates are known multihydroxyl compounds; consequently, a regioselective acylation to produce pure sugar esters is very difficult [270]. Therefore, chemical synthesis at high temperatures is less selective and leads to colored by-products due to caramelization of sugars [271]. Enzymatic synthesis of sugar esters is one of the best options due to high catalytic selectivity of enzymes, lipases in this case. Lipases from *Candida antarctica* B and *Thermomyces lanuginosus* have been intensively used for this purpose [265]. Sugar esters (sucrose esters, maltose esters, and glucose esters) were synthesized in good yields by transesterification of the corresponding sugars with fatty acid vinyl esters using immobilized *Candida antarctica* lipase B (Novozyme 435) and *Thermomyces lanuginosus* lipase in 2-methyl-2-butanol:dimethylsulfoxide solvent mixtures. Novozyme 435 was particularly selective toward the synthesis of sucrose esters (6,6′-diacylsucrose),



Ec = 5-eicosenoyl, 5-docosenoyl, 13-docsenotl, or 5, 13-docosdienoyl
R = $-(CH_2)_{14}CH_3$
R′ = R″ = $-(CH_2)_3CHCH(CH_2)_{14}CH_3$, $-(CH_2)_3CHCH(CH_2)_{16}CH_3$, $-(CH_2)_{11}CHCH(CH_2)_7CH_3$
or $-(CH_2)_3CHCH(CH_2)_6CHCH(CH_2)_7CH_3$

SCHEME 31.10 Lactonization of Δ5 unsaturated triacylglycerols by a lipase in the production of δ-lactones. (From Chen, W.J. et al., *J. Biotechnol.*, 162, 183, 2012.)

while lipase from *T. lanuginosus* selectively catalyzed synthesis of 6-*O*-acylglucose. However, both lipases regioselectively catalyzed the synthesis of 6-*O*-palmitoylmaltose and 6-*O*-lauroylglucose. The synthesized sugar esters were effective antimicrobial agents against gram-positive and gram-negative bacteria [265].

Candida antarctica lipase B was also identified as the best lipase for the synthesis of glucose esters of palmitic acid and lauric acid in binary mixture of *tert*-butanol and IL ([BMIM][BF4] or [BMIM][PF6]). Synthesis of sugar esters in this case was possible with both free fatty acids and their vinyl esters. After optimization of reaction conditions (acyl donor chain length and type, temperature, reaction time, and solvent ratios), conversion rates of up to 60% were achievable using vinyl esters of fatty acids as acyl donors at a temperature of 60°C [266].

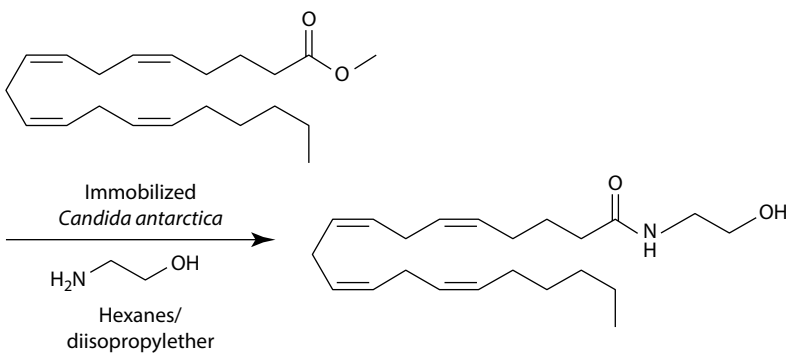
Synthesis of sugar esters in supercritical fluids has also been explored. A series of sugar esters (fructose palmitate, fructose laurate, sucrose laurate, and sucrose palmitate) were synthesized in high yields in supercritical carbon dioxide at 10 MPa and 2-methyl-2-butanol at atmospheric pressure. Water formed during the esterification process was removed by the use of molecular sieves. Sucrose laurate had growth inhibitory effects on *Bacillus cereus* at a concentration of 9.375 mg/mL [269].

Candida antarctica lipase B (CALB) immobilized on chitosan and acrylic resin was applied in the synthesis of lactose, fructose, and sucrose esters [272]. CALB immobilized on chitosan gave the highest conversion yield of lactose ester (84.1%) and a lower yield of fructose ester (70%). However, CALB immobilized to the acrylic resin gave a higher yield for both sucrose and fructose esters. The applications of the sugar esters as emulsion stabilizers for coconut milk emulsions were further investigated and lactose ester was revealed as the best biosurfactant [272]. Synthesis of various sugar esters either through transesterification or esterification reactions has also been investigated by other authors [273–284].

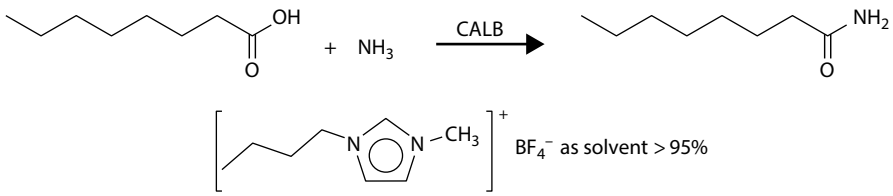
I. Amides

Fatty acid amides have gained increasing attention due to their potential applications in surfactant, lubricant, and cosmetic and shampoo sectors [285–287]. Direct amidation of fatty acids are often achieved by reacting fatty acids with anhydrous ammonia under high pressures (340–700 kPa) and high temperatures around 200°C. Additional purification steps are usually needed in order to obtain pure amides, free of numerous side products. The use of enzymes counteracts these drawbacks. Lipases have been used in the biosynthesis of both primary and secondary amides either through direct amidation of fatty acids or ammoniolysis of TAGs (Schemes 31.11 and 31.12).

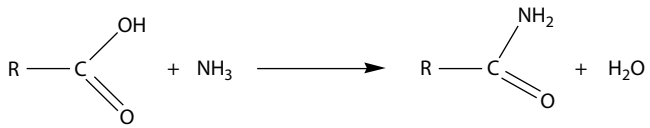
The lipase (CALB)-catalyzed ammoniolysis of olive oil TAGs, trilaurin, tributyrin, and jojoba wax gave the primary amides such as oleamide, lauranamide, butanamide, and *cis*-11-eicosanamide in yields of 90%, 97%, 55%, and 90%, respectively [288]. CALB was used in the direct amidation



SCHEME 31.11 CALB-catalyzed amidation of esters. (From Yildirim, D. et al., *Enzyme Microb. Technol.*, 49, 555, 2011.)



SCHEME 31.12 A scheme for the CALB-catalyzed ammoniolysis of octanoic acid in an ionic liquid. (From Yildirim, D. et al., *Enzyme Microb. Technol.*, 49, 555, 2011.)



SCHEME 31.13 Lipase-catalyzed amidation (direct) of a carboxylic acid with ammonia in the preparation of an amide. (From Yamada, Y. et al., *J. Am. Oil Chem. Soc.*, 76, 713, 1999.)

of various carboxylic acids (including acetic acid, butyric acid, and oleic acid) with ammonia (Scheme 31.13). Yields of 98%, 91%, and 94% were obtained for amidation involving acetic acid, butyric acid, and oleic acid, respectively [289]. Oleamide and erucamide have been obtained in good yields by lipase-catalyzed amidation of oleic acid and ammoniolysis of erucic acid with urea, respectively [290,291]. Primary amides from novel multihydroxy fatty acids such as 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) and 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) have been obtained through direct amidation catalyzed by *Pseudozyma* (*Candida*) *antarctica* lipase B [292]. The use of lipases in the synthesis of secondary amides has been reported as well [293]. The CALB-mediated direct amidation of a hydroxy oleic acid derivative (7,10-dihydroxy-8(*E*)-octadecenoic acid [DOD]) with *N*-methylethanol amine at 50°C and after 72 h reaction time gave a secondary amide in high yields (95%) [293]. More interestingly, the new amide had potent antimicrobial and antioxidant activities [293].

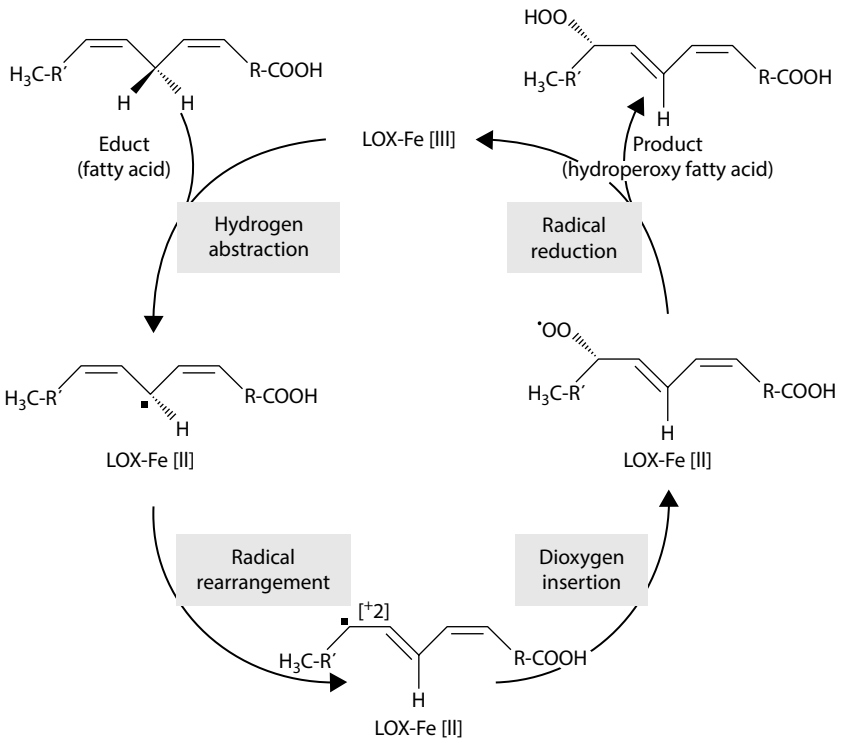
J. Other Enzymes Related to Lipid Processes

1. Lipoxygenases

LOXs are a group of nonheme iron-containing enzymes [294–296] that catalyze the stereo-specific peroxidation of PUFAs with at least one 1-*cis*,4-*cis*-pentadiene structure. The oxygenation of fatty acids by LOX consists of four reactions that include hydrogen abstraction (rate-limiting step), radical rearrangement, oxygen insertion, and peroxy radical reduction (Scheme 31.14) [297]. The hydroxyperoxides from LOX reactions are substrates for further reactions such as isomerization, reduction to hydroxydienoic acids, and hydroperoxide cleavage.

LOXs offer a cheap route to the preparation of moderate- to high-value products. For instance, they are applied in the synthesis of prostaglandins owing to an analogy in the hydrogen abstraction step of LOX reactions to the initial step of the enzyme involved in prostaglandin synthesis [298]. Soybean LOX has been used to prepare prostaglandin $F_{2\alpha}$ from arachidonic acid through its action on arachidonic acid followed by a chemical reduction [299].

Recently, soybean LOX has been applied to generate 9-hydroperoxy-γ-linolenic acid from GLA [300], dimers of linoleic acid [301], and hydroperoxides of acylglycerols and phospholipids [302,303]. In addition, a batch reaction consisting of a mixture of linoleic acid and soybean LOX was used to generate hydroperoxy-octadecadienoic acid, which can be reduced in situ into 13(*S*)-hydroxy-9-*cis*,12-*cis*-octadecadienoic acid in high yield [304]. A review of reactions catalyzed by LOX is available [305].



SCHEME 31.14 LOX-catalyzed oxidation of unsaturated fatty acids. (From Yu, Y.C. et al., *J. Biotechnol.*, 166, 12, 2013.)

2. Oxygenases

Oxygenases are enzymes that add molecular oxygen to organic molecules. They have become quite attractive for chemical synthesis as well as bioremediation because of their activity on a wide range of organic substrates. Moreover, oxygenases may be regio-, enantio-, or chemoselective, which has seen them being used to prepare pure compounds. Some reviews are available on the applications of oxygenases [306–308]. Monooxygenases (P-450) have been used to prepare epoxidized oils, which have attracted substantial commercial interest. The best known nonheme monoxygenase is cyclohexanone monooxygenase, which is obtained from *Acinetobacter* spp. NCIB 9871 [309]. Nonheme monooxygenases (Baeyer–Villiger monooxygenases) are used for the commercial production of bicyclic lactones, sulfoxides, thiosulfinates, and cyclic sulfates [310–313]. Furthermore, a peroxygenase from oat (*Avena sativa*) has been used for the epoxidation of oleic acid with hydrogen peroxide as oxidant [314]. Reactions catalyzed by oxygenases have been described in a review [305].

3. Epoxide Hydrolases

Epoxide hydrolases are cofactor-independent enzymes that catalyze the enantioselective hydrolysis of epoxides to vicinal diols. The asymmetric hydrolysis of epoxides yields enantiomerically pure and chiral vicinal diols, which have a wide range of applications in pharmaceuticals, liquid crystals, pesticides, and flavors [315]. For instance, (*R*)-1-phenyl-1,2-ethanediol ((*R*)-PED), a chiral vicinal diol is an important synthon for the manufacture of arylalkylamine calcimimetic (*R*)-(+)-NPS-R-568, NK-1 receptor antagonists (+) CP-99,994, as well as the nucleoside analogs with antiviral activity [316]. Moreover, S-PED, the opposite enantiomer, is used in the preparation of chiral biphosphines and as a chiral initiator of stereoselective polymerization [317].

A premier application of epoxide hydrolases was in the asymmetric hydrolysis of styrene oxide to R-PED using epoxide hydrolases from *As. niger.* However, the product enantiomeric excess (e.e.%)

was low (about 51%) [318]. That notwithstanding, epoxide hydrolases from *Bacillus alcalophilus*, plants (e.g., potato and crest), and animal tissues (e.g., duck and mouse liver) have been used for kinetic resolution of epoxides with higher e.e.% being achieved [319–321].

In addition, it has been possible to improve e.e.% to around 95% for epoxide hydrolase (isolated from mung bean)-catalyzed hydrolysis of styrene oxide to R-PED in the presence of various ILs [322]. Similarly, cross-linked epoxide hydrolase from mung bean catalyzed the hydrolysis of styrene oxide to R-PED with the product yield of 46% and an e.e.% of 93.5% and the product yield of 49% and an e.e.% of 95.8% in the absence and presence of ILs, respectively [323,324]. Furthermore, some studies have been conducted on the use of epoxide hydrolases for kinetic resolution of epoxides [325–327].

4. Phospholipases A1/A2/C/D

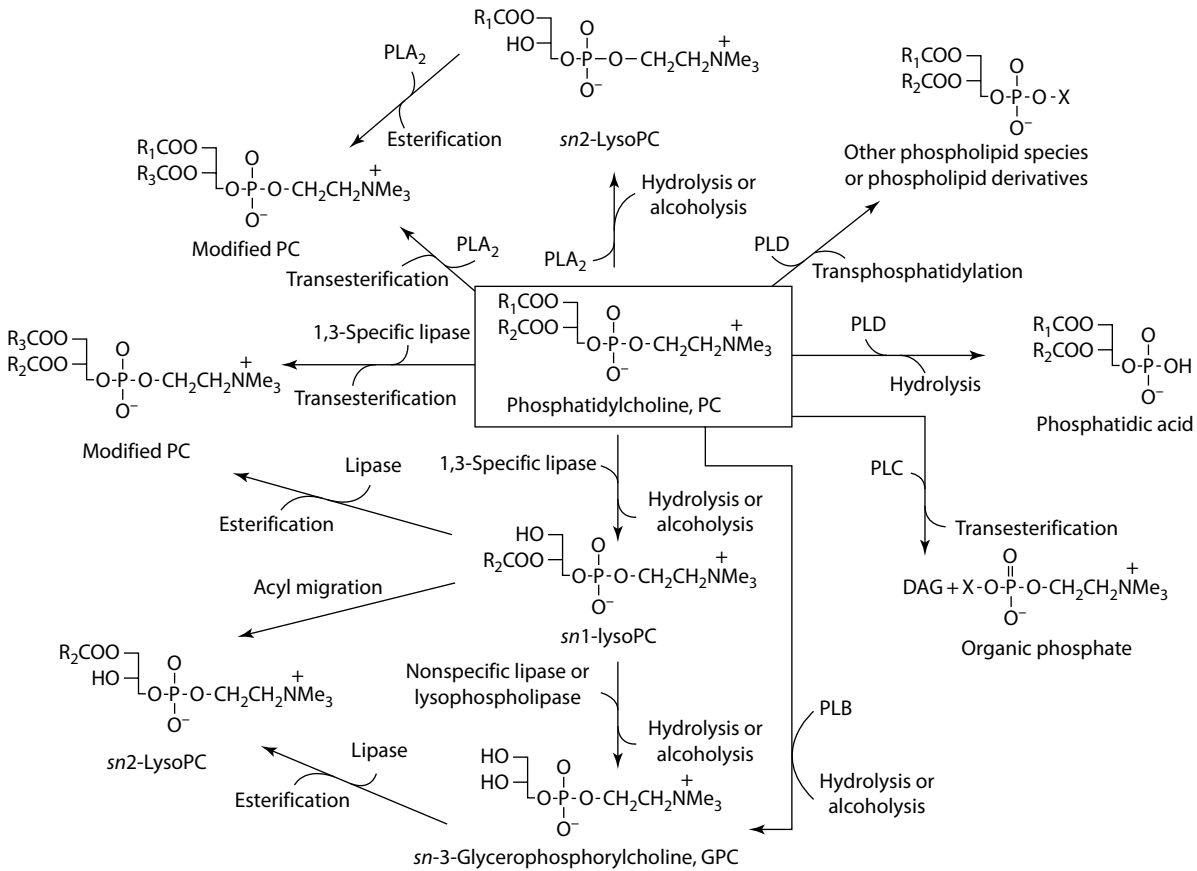
Phospholipases are classified into four types depending on the site of their action on phospholipids. Phospholipase A1 (PLA1) was initially identified in livers and rat brains but is currently obtained from microorganisms [328]. PLA1 hydrolyzes phospholipids at the *sn*-1 position of the glycerol backbone. Phospholipase A2 (PLA2) cleaves phospholipids at the *sn*-2 position, which can be found in porcine pancreas, snake venom, bee venom, and sheep red blood cell membrane. Hydrolysis of phospholipids by PLA2 yields lysophospholipids, which have excellent emulsification properties [329]. Phospholipase C (PLC) can be obtained from bacteria such as *Clostridium perfringens* and *Bacillus cereus*. PLC can catalyze the cleavage of the glyceryl-phosphate bonds in phospholipids producing diacylglycerols and phosphoryl hydroxyl base compounds [329]. The phospholipase D (PLD) can be found in plants such as cabbage leaves and microorganism [330–332]. However, PLD from *Actinomyces* and *Streptomyces* spp. showed high thermostability and broad spectrum of substrates [330–332]. PLD hydrolyzes phosphodiester bonds in phospholipids producing PA and choline or ethanolamine [328] (Scheme 31.15 illustrates the various modifications of phospholipids by phospholipases).

PLA1 and PLA2 have been used for the industrial degumming of oils and fats. They both release lysophospholipids, which easily become hydrated, thereby reducing the phospholipid contents in oils and fats to <10 ppm. Furthermore, PLC has been used for degumming of oils and fats. PLC attacks C—O—P bonds in phospholipids producing *sn*-1,2(2,3)diacylglycerols and a phosphate residue retaining the corresponding head group. The use of PLC is as efficient as using either PLA1 or PLA2. However, there is less oil loss accompanying this process [333].

Furthermore, PLA1 has been used to synthesize phospholipids enriched with *n*-3 PUFAs such as EPA, docosapentaenoic acid, and DHA. This was achieved by the modification of phosphatidylcholine in a solvent-free system using PLA1 from *Thermomyces lanuginosus*/*Fusarium oxysporum* as biocatalyst [334]. Similarly, an immobilized PLA1 from *Thermomyces lanuginosus*/*Fusarium oxysporum* was used to enrich phosphatidylcholine with *n*-3 PUFAs. After 24 h of incubation, the content of *n*-3 PUFA incorporated into phosphatidylcholine (PC) was 57% (mol.%) and the yield of PUFA-enriched PC product was 16.7% (mol.%) [335]. Furthermore, esterification of *sn*-1-acyllysoglycerophosphocholine with DHA and EPA in an emulsion system with a low water activity catalyzed by porcine pancreatic PLA2 produced diacylglycerophosphocholine product containing over 30% *n*-3 PUFAs [336].

In addition, PLA2 has been used to incorporate nervonic acid, a major fatty acid in brain sphingolipids, into phosphatidylcholine by transesterification in various organic solvent systems including toluene, butyl acetate, *n*-hexane, ethyl acetate, and diethyl ether. PLA2 was found to be very stable in these solvents [337]. Furthermore, an immobilized PLA2-catalyzed acidolysis was used for the acyl modification of the *sn*-2 position of soybean phosphatidylcholine [338]. Acyl donors such as conjugated linoleic acid, DHA, and caprylic acid were incorporated into PC [338]. A number of similar studies have been carried out on PLA1- or PLA2-catalyzed acyl modification of phospholipids [339–345].

PLD has been applied to the enzymatic production of special phospholipids due to its ability to catalyze transphosphatidylation of phospholipids. PLD was used to synthesize phosphatidylglycerols



SCHEME 31.15 Lipase-catalyzed modification of phospholipids. (From Yildirim, D. et al., *J. Mol. Catal. B: Enzym.*, 88, 84, 2013.)

by the transphosphatidylation of egg lecithin with glycerol [346,347]. A platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) analog has been synthesized by PLD-catalyzed exchange of choline in phospholipids with primary cyclic alcohols [347]. Furthermore, transphosphatidylation of pure glycerophospholipids with glucose in *tert*-butanol or diethylether using PLD from *Actinomadura* sp. as a catalyst produced phosphatidylglucose in high yields [348]. Also, many drugs and biological compounds containing reactive hydroxyl group have been successfully phosphatidylated via PLD-catalyzed polar head exchange of phospholipids [349–354].

IV. NEW MEDIA IN LIPID PROCESSING

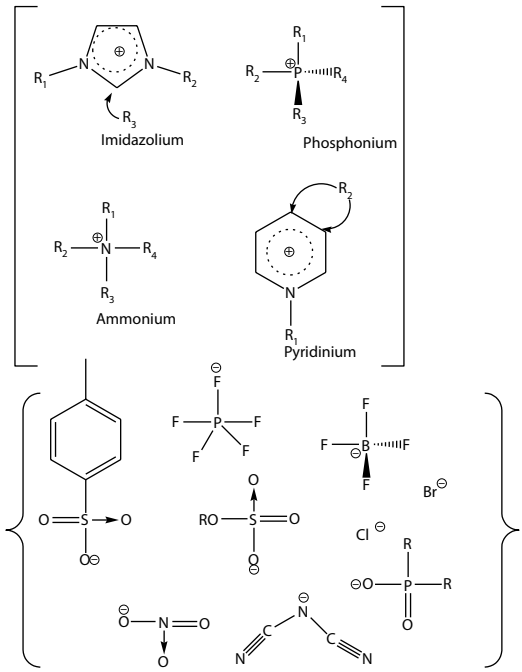
A. Supercritical Fluids (SCf's)

The focus on supercritical fluids as new reaction media has increased [355]. Enzymes, particularly hydrolytic enzymes such as lipases, have become useful biocatalysts under SCf's with several advantages over conventional media being reported. For instance, they increase the rate of mass transfer in reactions, are environmentally friendly, and require only simple separation of products. Furthermore, there are reports of higher enzyme activity than in conventional media [356].

Crude hog pancreatic lipase was used to study the esterification of myristic acid with ethanol in a solvent-free system compared to SCf's and acetonitrile. Under optimal conditions, conversions rates were 76%, 37%, and 4% for solvent-free, SCf's, and acetonitrile, respectively. Interestingly, at the same enzyme loading, conversion rates were higher in SCf'c than in the solvent-free system [357]. In addition, there was a report of improved enantio- and stereoselectivity of lipases under SCf's. A lipase-catalyzed asymmetrization of 1,3-propanediacetate was observed to be more enantioselective in SCf (e.e. of 50%) but nonselective in conventional media. This was attributed to a possible conformational change at the active site where lysine could be transformed into carbamic acid [358]. On the other hand, lipase-catalyzed (*Candida antarctica* [CALA and CALB] and *M. miehei* lipases) synthesis of glycidyl esters in biphasic systems of supercritical carbon dioxide and ILs slightly reduced enzyme activity but enantioselectivity remained unchanged compared to conventional media [359]. Recently, three immobilized lipases from *Candida antarctica*, *Thermomyces lanuginosus*, and *Rhizomucor miehei* were tested for the intramolecular synthesis of the macrolactone oxacyclohexadecan-2-one in supercritical fluids with good yields [360,361].

B. Ionic Liquids

ILs have gained increased attention in biocatalysis area due to their capability to support a variety of enzyme-catalyzed reactions with good results [362–364]. ILs are salts that are liquid at room temperature due to their low melting temperatures (<100°C) [301]. Typical cations of anionic liquids include imidazolium, phosphonium ammonium, and pyridinium ions, while common anions for ILs include hexafluorophosphate, tetrafluoroborate, ethyl sulfate, and methosulfate. Examples of ILs combinations are 1-butyl-3-methyl hexafluorophosphate and 1-methyl-3-methylimidazolium methyl sulfate [365]. See Scheme 31.16 for examples of ILs combinations. ILs differ from conventional solvents, apart from the obvious fact that they are solely made up of ions, in that they do not evaporate during use due to their negligible vapor pressures. Consequently, the use of ILs does not release volatile organic compounds into the atmosphere and is therefore regarded as "green solvents" [366]. An additional advantage of the use of ILs over conventional solvents is their wide range of liquid (from −96°C to 300°C). This unique property makes them flexible and lends them to a myriad of applications. Furthermore, they have high solvation powers and therefore dissolve a wide range of organic, inorganic, and polymeric materials [366]. In addition, ILs can be tailor made for specific applications due to variety of cation and anion combinations that are available. Through a judicious selection of cations and anions, the properties of ILs such as melting point, viscosity, polarity, hydrophobicity, water miscibility, and density can be fine-tuned [366].



SCHEME 31.16 Representation of some cations and anions of ionic liquids. (From Yu, Y.C. et al., *J. Biotechnol.*, 166, 12, 2013.)

Current research has demonstrated that some functional ILs have found applications in several areas such as lubricant, pharmaceutical, plasticizers, electrochemical, and medical industries. In the ensuing section, their applications in lipid processing will be highlighted.

A major challenge in the manufacture of sugar esters is the solubility gap between the sugars and fatty acids. One area of application of ILs is in the production of sugar esters from the esterification of carbohydrates and fatty acids because of their solvation power. Generally, regioselectivities of lipase-catalyzed esterification of sugars and fatty acids are higher in ILs compared to esterification in conventional solvents. For instance, the lipase-catalyzed esterification of glucose with vinylacetate [EMIM][BF4] yielded 6-*O*-acetylglucose as the main product demonstrating the increased selectivity of esterification reactions in ILs [365]. Furthermore, ILs have been used for the synthesis of monoacylglycerols, which represent an important group of emulsifiers in the food industry. Over recent years, enzymatic procedures for the production of monoacylglycerols have replaced the traditional high-temperature glycerolysis method, due to the attendant problems of the high-temperature process such as off-flavor generation, high energy consumption, low yields, and unwanted side reactions. The enzymatic procedure has its own drawbacks such as low reaction rates and the use of organic solvents. The use of ILs in the production of monoacylglycerols provides a "greener technology" besides the higher yields than enzymatic glycerolysis in organic solvents [366]. Enzymatic glycerolysis in a tetraammonium-based IL resulted in monoacylglycerol yields in excess of 90%, much higher than that in *tert*-butanol (72%) and solvent-free systems (36%) [368]. Several other studies have investigated the use of ILs in lipid processing with encouraging results, advocating ILs as the new designer solvents for the future [360,368–371].

C. Deep Eutectic Solvents

DESs are systems formed from a eutectic mixture of Lewis or Brønsted acids and bases that can contain a variety of anionic and/or cationic species. DESs have lower melting points than their

individual constituents, thus causing their liquid state [372]. The first reported DES was made by mixing choline chloride with urea [373]. DESs have some merits over ILs such as their higher biodegradability, less toxicity, and cheap price. Consequently, DESs have gained prominence as alternative over ILs as media for lipase-catalyzed reactions since their definition in 2003 [374]. DESs were first shown to be benign for lipase-catalyzed reaction by Gorke et al. [375] through studies on a variety of transesterification and ammoniolysis reactions using lyophilized and immobilized lipases. Detailed studies on immobilized CALB-catalyzed polymerization, transesterification, and perhydrolysis reactions in DESs then followed [376–378]. Using soybean oil as substrate, a biodiesel yield of 88% was obtained within 24 h with Novozym 435 as biocatalyst in a DES [379]. Recently, lipase-catalyzed transesterification reactions between phenolic acid and fatty acid methyl esters have been demonstrated to be optimum in DES [380]. Furthermore, the feasibility of lipase-catalyzed esterification reactions in DES has been highlighted recently in a few studies [374,381–383]. DES is expected to be more attractive as the new solvents for the near future due to their added ability to serve as substrate and solvent at the same time [335].

V. PERSPECTIVES

In the past decades, lipid biotechnology has witnessed a tremendous progress both in technique progress and in commercialization. The momentum to promote the commercialization of industrial lipid biotechnology comes from both a technology push and industry and market pulls. Rapid and unprecedented progress in the key technologies of modern biological sciences, such as metabolic engineering and synthetic biology, is driving lipid bioproducts and processes to be more efficient and cost competitive and is fueling innovations in food lipid industry. The progress in genetic engineering of oil crops has significantly improved the quality and quantity of plant oils and fats for specialty application. The industrial potential of microalgae oils is moving forward to industrialization and commercialization for both food and biofuels applications. Through engineering of lipid metabolic pathways and optimization of fermentation conditions, genetically engineered microorganisms are able to produce functional lipids, fatty acids, or lipidic ingredients in industrial relevant quantity. In combination with essential chemical processes, isolated enzymes, mainly lipases, are applied in enzymatic production of cocoa butter substitutes, HMF replacers, and *trans*-free plastic fats, as well as enzymatic degumming of vegetable oils. However, compared to chemical process, the biotechnological process is still higher cost-wise, which mainly comes from the downstream process and expensive enzymes. To reduce the cost of enzyme, the old concept to improve the performance and reusability of immobilized enzyme still works as an important strategy. However, with the significant progress in enzyme genetics and fermentation, scientists start to use free enzyme instead of immobilized enzyme to obtain a better cost efficiency. Indeed, lipid biotechnology offers a clear value proposition; a number of hurdles need to be addressed to fully realize the commercial potential of functional lipids, bio-based lipid products, and oleochemicals over the coming decades. Cost-effective solutions will depend on continued research and development, government and private sector investment, and market acceptance tied to compatibility with existing infrastructure. Fortunately, we seem to be on the path to commercialize these solutions and to promote a bio-based economy.

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32 Enzyme-Catalyzed Production of Lipid-Based Esters for the Food Industry

Emerging Processes and Technologies

Ran Ye

CONTENTS

I. ESTERS

Esters are chemical compounds obtained from the condensation reaction between acyl donor and acyl acceptor, forming a carbonyl group adjacent to an ether linkage to the alkyl group. Enzyme-catalyzed esterification receives considerable attention in dozens of food-related applications, since its enzymatic synthesis offers numerous advantages including chiral (stereo-selectivity), positional (region-selectivity), and functional group specificity (chemo-selectivity), compared to the conventional chemical synthesis [1]. In addition, the approach enables the elimination of environmental concerns, minimizes the generation of byproducts, lower the cost of downstream separation,

and improves the operation safety [1,2]. This chapter emphasizes immobilized enzyme-catalyzed esterification reaction in nonaqueous media for the synthesis of food-related products, highlighting emerging processes and technologies as well as methodological advancements.

II. LIPASES

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that synthesize or hydrolyze esters for the modification of the structures of fats and oils [3,4]. Lipases do not require any cofactor for its catalytic ability [5]. In general, lipases display inactive state in the absence of substrate since the active site of lipase is covered and protected by a "lid," a conservative sequence of hydrophobic amino acid residues. When contacting with the substrates at the lipid–water interface, the "lid" is activated, undergoes a conformational change, moves away, and the active site becomes accessible for the substrate molecules, this conformation is called "open" or active [6–8]. Due to their catalytic capabilities in fats and oils, lipases have received a great deal of attention acting as biocatalysts in enormous food-related applications such as oils and fats, baking, cheese making, and in synthesizing food emulsifiers. In addition, as the most used enzymes in synthesis of esters for food application, lipase-catalyzed esterification displays chemo-, regio-, and/or stereo-selectivity [4]. Regardless of the type of reaction (i.e., hydrolysis, esterification, or interesterification), it is well-recognized that the most accurate mechanism for lipase-catalyzed reaction is a Ping-Pong Bi-Bi mechanism, containing two main steps: (1) nucleophilic contact on the substrate ester bond through the oxygen atom of the hydroxyl group of Ser at the active site when the lid is open, leading to the formation of an acylated enzyme complex, and release of the alcohol moiety of the original substrate and (2) hydrolysis of the acylated enzyme complex, causing the release of the product and regeneration of the enzyme. The tentative mechanism is represented in Figure 32.1 using Cleland's schematic representation [9–12].

A. *Candida rugosa* Lipase

As one of the hydrolase-based enzymes, lipases from *Candida rugosa* (CRL) were firstly documented in the 1960s, by isolating the yeast from natural soils [13,14]. Until now, at least five genes (Lip1–Lip5) in the CRL have been biochemically characterized [15–17]. In fact, all the commercial CRL samples contain a mixture of several isoenzymes in different ratios. The active site of the enzyme included a catalytic triad composed of serine, aspartic acid, and histidine residues [6,7,18].

B. *Candida antarctica* Lipase

As the most widely used lipase in esterification reaction, lipases could be produced by different strains of genus *Candida* sp., especially *CAL*B (CAL-B), which is one of the 154 species of the genus *Candida* [19,20]. It was initially isolated from the lake Vanda, in Antarctica [19]. Two isoenzymes that were cloned and overexpressed in *Aspergillus oryzae* as host organism (lipases A and B) were commercially purified and characterized [21]. Due to the high stereoselectivity, thermal stability and activity, *Candida antarctica* lipase (CAL) is commonly used in nonaqueous media for different industrial applications as a biocatalyst. *Candida antarctica* lipase A, a calcium-dependent enzyme, with a molecular weight of 45 kDa, has a pH optimum of approximately 7 and an isoelectric point

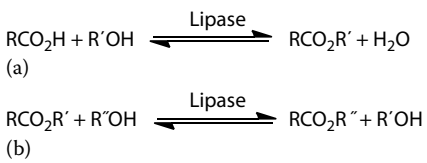


FIGURE 32.1 Reversible lipase-catalyzed (a) esterification and (b) transesterification reaction.

(pI) of 7.5 [21,22]. *Candida antarctica* Lipase B (CALB) has been extensively employed to catalyze biotransformation reactions. By immobilizing on solid supports such as macroporous resins for achieving large surface area, the enzyme activity, selectivity, stability, and reusability of CALB in nonaqueous media could be improved, compared to the native enzyme [23]. CALB contains 317 amino acid residues with a molecular mass of 33 K [24]. In addition, a serine–histidine–asparate catalytic triad contributes to the catalytic activity of CALB [24]. In detail, the active site of CALB has the acyl side and the alcohol side, corresponding to the binding location of the substrate. Four hydrophobic residues (two isoleucine residues, leucine and alanine) bordering the alcohol side, generate a narrow tunnel where the substrates have to travel through before the formation of tetrahedral intermediates [25,26]. Furthermore, the hydrogen bond between Ser^{105} and His^{224} and the hydrogen bond between Asp^{187} and His^{224} at the active site of CALB are mainly responsible for the stabilization of the transition state of the enzymatic reaction. The conformation change in the active site is greatly associated with the breakdown of the hydrogen bond between Ser^{105} and His^{224}, resulting in the low enzymatic activity of CALB in polar solvents [27]. Novozym-435 (N-435) is a commercial heterogeneous lipase from CALB immobilized onto macroporous resin poly(methyl methacrylate-co-divinylbenzene) with an average particle size of 315–1000 μm, a surface area of 130 m^2/g, and a pore diameter of ~150 Å, respectively [23]. N-435 is the most common biocatalyst utilized in the esterification and transesterification reaction with high activity, solvent tolerance, thermal stability, and regioselectivity reaction [27–30]. The active site of CALB is composed of serine, histidine, and aspartic acid or glutamic acid. A helical oligopeptide chain serves as a lid, blocking the contact between the reaction medium and the active site of enzyme. When interacting with a hydrophobic surface, lipase attaches on the surface and impel the equilibrium to shift toward the open state of enzyme. In addition, CALB display the α/β hydrolase fold within the catalytic domain, where a parallel β sheet is bordered by several α helices on each side [25,31].

C. *Rhizomucor miehei* Lipase

Lipase from *Rhizomucor miehei* (RML), an extracellular lipase, was originally reported in 1973 [32], commercially available from Novozymes as Lipozyme RM IM lipase immobilized onto a weak anion-exchange resin (Duolite ES 562) based on phenol–formaldehyde copolymers [33,34], or in free state (Palatase 2000 L) [35]. RML has a molecular weight of 31,600 Da with an isoelectric point of 3.8 with a single polypeptide chain of 269 residues [36]. RML owns a Ser-His-Asp trypsin-like catalytic triad with an active serine remaining under the cover of a short helical fragment of the lid, which is long surface loop, forming a singly wound β-sheet domain with predominantly parallel strands, linked with a myriad of hairpins, loops, and helical segments [35]. All right-handed loops generate the asymmetric central sheet where all the connecting fragments are positioned on one side with a single N-terminal α-helix. Three disulfide bonds (residues 29–268, 40–43, 235–244, *cis*-peptide bonds containing proline residues) are considered to stabilize the enzyme molecule [11,37–39].

D. Carboxylesterases

Carboxylesterases (EC 3.1.1.1, carboxyl ester hydrolases) are enzymes widely distributed in nature with different physiological functions for instance, pathogenicity, and detoxification [40]. Carboxylesterases display substrate specificity, regio-specificity, and chiral selectivity. Except for the hydrolysis of esters, they also can facilitate ester, and transesterification reactions [41–43].

E. Acyltransferases

Wax synthase, long-chain-alcohol O-fatty-acyltransferase (EC 2.3.1.75) is an enzyme that catalyzes the esterification of acyl-CoA and long-chain alcohol, generating CoA and long-chain ester [44]. It has been utilized in the production of wax esters [44] and biofuels [45,46].

III. WATER REMOVAL METHODS

Water concentration is considered as exerting a dual effect on the enzyme-catalyzed synthesis of esters. A certain amount of water is needed to maintain the conformational stability of enzyme [47,48] for both immobilized and free cell enzymes. A thin water layer attaching on the surface of enzyme serves as a protective enclosure for the structural integrity, protein stability, and active site polarity [49]. The presence of water can prevent the concrescence of polar residues on active sites of enzyme [50]. In contrast, excessive amount of water during ester reaction has an adverse impact on the thermodynamic equilibrium of the reaction [51] and the distribution of the desirable products in the media [48,52]. In addition, the water concentration is highly associated with thermo-stability of enzymes. The low water content in the reaction media could benefit the stability of enzyme at elevated temperatures [53]. Thus, the water concentration in the reaction media needs to be rationally controlled through the time courses of enzyme-catalyzed reactions. A myriad of means have been applied for controlling water partitions between the biocatalyst and reaction media during lipase-catalyzed esterification: molecular sieves (MS) [54–58], silica gel [52], azeotropic distillation [59], salt hydrate pairs [60], dry air or N_2 [54], free evaporation [61], and pervaporation using membranes [62]. In a recent publication, Ye and Hayes compared the different approaches listed in the order for the water removal: vacuum + N_2 bubbling > molecular sieves > N_2 bubbling > vacuum [63].

IV. DIFFERENT INNOVATIVE AND EMERGING TECHNOLOGIES FOR ENZYMATIC SYNTHESIS OF ESTERS

A. Supercritical Carbon Dioxide

Supercritical fluid technology is developed as an environmentally friendly replaceable technology for the enzyme-catalyzed synthesis of ester. As the most widely used supercritical fluid, CO_2 is selected since it has several advantages over the conventional method, such as availability in large quantities, replacement of organic solvent, moderate critical temperature and pressure (31°C and 7.4 MPa), nontoxicity, low cost, tunable solving properties, and nonflammability [64]. Supercritical CO_2 (SC—CO_2) as a reaction medium for the enzyme-catalyzed synthesis of ester could enhance the miscibility of lipids and other substrates, and improve interphase mass transfer properties of the reactants due to higher diffusivity and lower viscosity, and thus increase the reaction kinetics [64,65]. In addition, due to the insolubility of enzymes in supercritical CO_2, the catalyst particles are readily collected and separated from the system for scale up of the process [66] in Figure 32.2. Moreover, due to the high volatility of CO_2, it is readily removed from the final product [67].

Pressure and temperature are highly related with the reaction rate since they can affect the partitioning of substrates between the two phases [68]. Visual evidence of the phase behavior of the reaction mixture containing methanol and corn oil at 55°C demonstrated that the immiscible biphasic liquid phase transformed into a more homogeneous liquid phase with the increase of pressure in SC—CO_2. Habulin et al. successfully synthesized sucrose palmitate, sucrose laurate, fructose palmitate, and fructose laurate in supercritical CO_2 at 10 MPa in the presence of immobilized lipase Novozym 435 CALB [66]. Rodrigues et al. developed a packed bed reactor containing CALB for the continuous production of fatty acid methyl ester (FAME) using corn oil in SC—CO_2 at 19.4 MPa and 62.9°C with a yield of 93.3% ± 1.1% [69] in Figure 32.3. With increasing pressure, the miscibility of substrates was greatly enhanced, as depicted in Figure 32.4. Another packed bed reactor consisting of lipase from *Rhizomucor miehei* (Lipozyme RM IM) in continuous mode was employed to produce n-octyl oleate with a 93% conversion in supercritical CO_2 at 10 MPa and 323.15 K [70].

Lauryl oleate was synthesized by oleic acid esterification with 1-dodecanol in the presence of RML in a sapphire window batch stirred tank reactor (BSTR) using dense CO_2 as reaction medium [67].

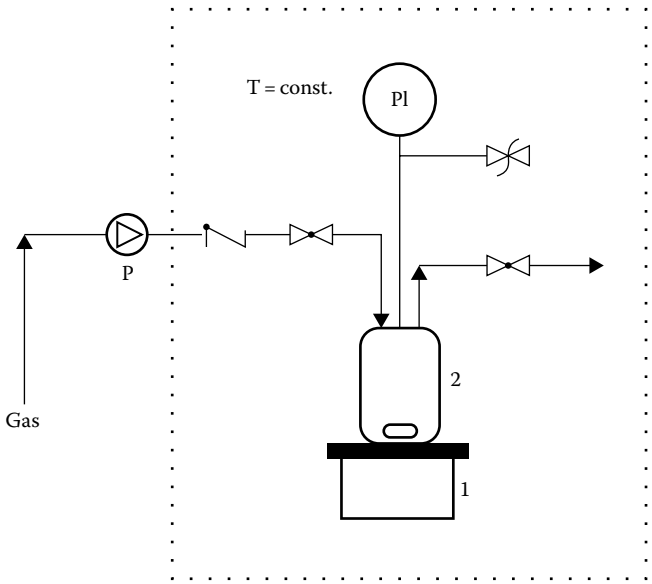


FIGURE 32.2 Schematic diagram of the SC—CO_2 device. 1, reactor; P, high-pressure pump; 2, magnetic stirrer and heater; PI, pressure indicator. (From Habulin, M. et al., *J. Supercrit. Fluids*, 45(3), 338, 2008.)

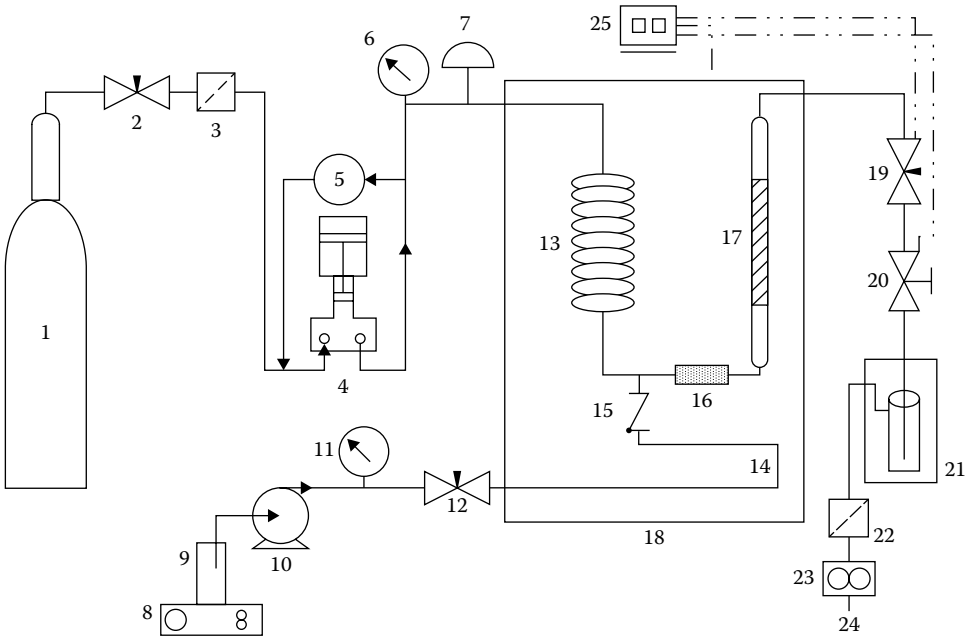


FIGURE 32.3 Schematic diagram of the SC–CO_2 bioreactor. 1, CO_2 cylinder; 2, 12 and 19, needle valve; 3, filter; 4, gas booster pump; 5, back pressure regulator; 6 and 11, pressure gauge; 7, rupture disc; 8, magnetic stirrer; 9, substrate mixture; 10, high pressure pump; 13, CO_2 preconditioning line; 14, substrate mixture preheating line; 15, check valve; 16, static mixer; 17, packed bed enzyme reactor; 18, oven; 20, micrometering valve; 21, cold trap; 22, oil and moisture trap; 23, digital mass flowmeter equipped with totalizer; 24, vent; 25, temperature controller. (From Ciftci, O.N. and Temelli, F., *J. Supercrit. Fluids*, 58(1), 79, 2011.)

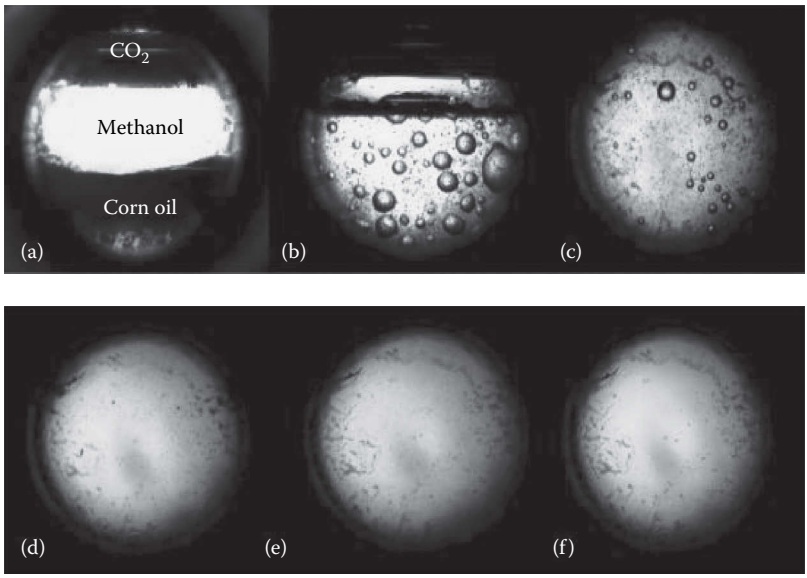


FIGURE 32.4 Visual observation of corn oil, methanol, and CO_2 mixture at different pressures at 55°C. (a) 0.102 MPa, (b) 3 MPa, (c) 7 MPa, (d) 11 MPa, (e) 20 MPa, (f) 35 MPa. (From Ciftci, O.N. and Temelli, F., *J. Supercrit. Fluids*, 58(1), 79, 2011.)

The enzyme retained high stability after 70 days in dense CO_2 for the production of the wax ester [67]. Furthermore, dense CO_2 in reaction mixture under subcritical conditions resulted in higher performance compared to the diluted gas in a single supercritical phase [67]. *Rhizopus oryzae* lipase (1.5%, w/v) immobilized on the support prepared from the mixture of hydroxylpropyl methyl cellulose (HPMC) and polyvinyl alcohol (PVA) was used for the synthesis of the flavor ester (citronellol esters) in supercritical carbon dioxide (SC–CO_2) at 45°C and a pressure of 8 MPa [71]. Terpinyl acetate, which is a short-chain flavor ester, was synthesized from α-terpineol and acetic anhydride in SC–CO_2 using five different lipases (*Candida rugosa* type VII, Amano PS, Amano AP-6, Amano G and Lipozyme RM IM) [72]. Diaz et al. studied the effect of the fatty acid chain length on different hexyl esters prepared from Novozym 435 in SC–CO_2 at 14 MPa. They concluded that the long fatty acid chain length favors the ester reaction and in contrast, the fatty acid with the short chain length may inhibit the enzyme activity [73].

Although supercritical fluid technology is recognized as an effective and promising process for replacing the employment of organic solvents, the capital cost for the instrument system is very high, impeding the utilization of supercritical fluid technology. In addition, another main drawback of SC—CO_2-based reaction is the inadequate solubility of numerous organic compounds in CO_2, requiring extremely high process pressures in order to benefit the solubility of substrates, which raise the safety and overwhelming energy input concerns [67].

B. Ionic Liquid

Ionic liquid (ILs) are chemicals, consisting of ions or salts in the liquid state below 100°C, offering environmentally friendly media for enzymatic esterification as alternatives to organic solvents because assorted ILS with different structures could enhance the solubility and miscibility of acyl donors and acceptors [74]. The analogous structural patterns in both the solid and liquid phases were detected in imidazolium ILs, caused by an ionic network composed of monomeric units of a cation surrounded by three anions and vice versa [75]. When molecules are incorporated into the IL, the structures of network would be altered and generate polar and non-polar regions. Hence, ILs are

also defined as nano-structured materials, allowing for the storage of neutral molecules in less polar regions, while fast diffusion of ionic or polar compounds occurs in the polar-oriented regions [56,75]. Recent spectroscopy evidence demonstrated the free volume increases with temperature since the molecules need more space to move in amorphous and crystalline phases [76]. The employment of ILs possesses several advantages, for instance, low melting points, low vapor pressures, high chemical and thermal stability, and non-toxic properties [74].

Hydrophobic ILs based on cations with large alkyl side-chains (e.g., 1-methyl-3-octadecylimidazolium bis(trifluoromethylsulfonyl)imide, [C_{18}mim][NTf_2]) have been used to form two-phase reaction systems, enabling easy product recovery [77] since hydrophobic ILs with large alkyl side-chains can transform from the solid to the liquid phase at certain temperature in the presence of enzymes [56]. Therefore, this knowledge could be employed to develop novel protocols for extraction and separation of ester products from the enzymatic synthesis reaction medium. In literature, flavor esters were catalyzed by Novozym 435 via esterification of citronellol, geraniol, nerol, and isoamyl alcohol, respectively, with acetic acid in 50% (w/w) [C_{16}tma][NTf_2] at 50°C [56]. For the citronellyl acetate, the yield could reach up to 89%. In particular, the desired flavor esters could be quickly recovered from the reaction medium, as described in Figure 32.5. Recently, the lipase-catalyzed synthesis of isoamylacetate in the 1-butyl-3-methylpyridinium dicyanamide/n-heptane two-phase system in a continuous microreactor was reported, yielding a threefold increase in the reaction rate when compared to the mixing bench mode [78]. Cinnamic acid derivatives, phenolic acid esters, were achieved from lipase-catalyzed esterification of aliphatic alcohols of various chain lengths (from 1 to 10 carbon atoms) with cinnamic acid in different ionic liquids, containing imidazolium-based cations, with various acyl chain lengths (C_2—C_8), and two different anions (either BF_4^- or PF_6^-) [79]. They reported that for the tested lipases, only Novozym 435® and RML could launch the esterification of phenolic acids with octanol [79].

In addition to the production of flavor esters, IL-based suspensions for lipase-catalyzed synthesis of sugar esters were successfully formed in literature since the occurrence of ILs improved

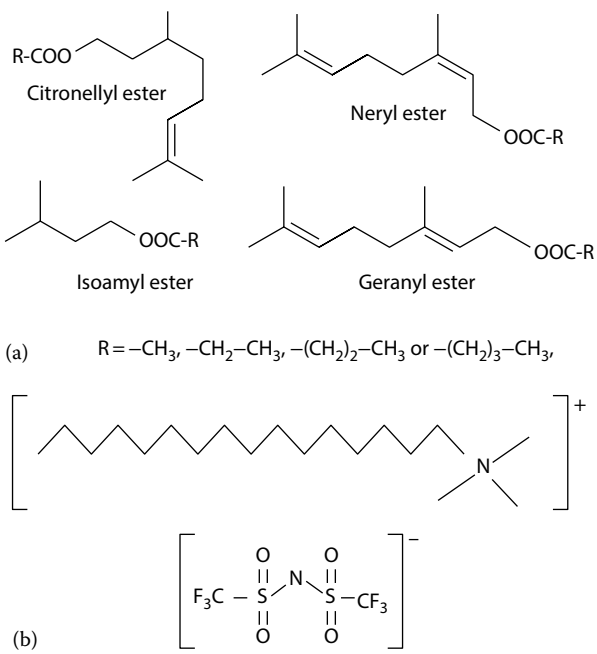


FIGURE 32.5 (a) Flavor esters synthesized by lipase-catalyzed esterification. (b) The IL [C_{16}tma][[NTf_2], as an example of switchable ionic liquid/solid phase. (From Lozano, P. et al., *Green Chem.*, 14(11), 3026, 2012.)

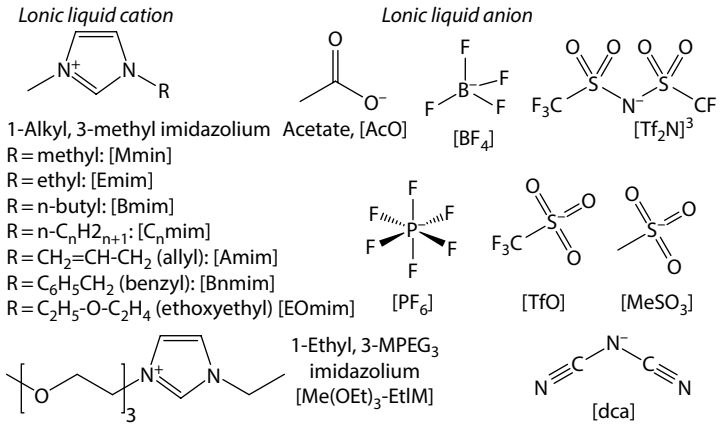


FIGURE 32.6 Molecular structure of ionic liquids used for conducting lipase-catalyzed synthesis of sugar esters. (From Ye, R. and Hayes, D.G., *J. Oil Palm Res.*, 26(4), 355, 2014.)

the solubility of polysaccharides. Several kinds of ILs (Figure 32.5) could potentially facilitate the lipase-catalyzed esterification of saccharides with fatty acids, because of the high solubility of saccharides in the reaction medium [80]. In detail, the IL's anion is highly linked with the solubility of saccharides, with effectiveness of anions toward glucose solubilization, described in the sequence (Figure 32.6): [dca] > [TfO] > [BF_4] > [PF_6] > [TF_2N] [80]. Lee et al. reported that the solubilization of glucose in [Bmim][TfO] was improved approximately 10-fold higher than that in 2-methyl 2-butanol and 400% higher than its saturated solution, leading to an increase in the conversion of glucose laurate from 8% to 96% in one day, using CALB as the biocatalyst [81]. Fisher et al. used the mixture of ILs and solvent (acetone/DMF and [Emim][$MeSO_3$]/tetrahydrofuran) to host the lipase catalyzed synthesis of maltose oleate mono- and di-esters and achieved the highest yields (65%–75%) [82]. Esterification of starch with long-chain fatty acids was also catalyzed by CRL in ionic liquids 1-butyl-3-methylimidazolium chloride ([BMIm]Cl) and 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIm][BF_4]) [83]. It was indicated that the maximum solubility of amylose starch was 11.0/100 g of [BMIm]Cl [83]. For further purification, the pregelatinized starch and starch esters could be rinsed and precipitated in the presence of absolute ethanol. As mentioned previously, ILs can be recycled by filtration and evaporation since they display high thermal stability and negligible vapor pressure. This conclusion was verified by the esterification of starch laurates in the recycled ILs [83]. Another starch fatty acid ester, starch palmitate was prepared in ionic liquid mixtures consisting of 1-butyl-3-methyl-imidazolium acetic ([BMIm]Ac) and 1-butyl-3-methyl-imidazolium tetraflouroborate ([BMIm][BF_4]) using lipase from *Candida rugosa*, type VI [83].

The employment of enzyme in ILs has numerous advantages over the organic solvents. However, they still suffer several drawbacks, limiting its application in industrial scale, for example, high cost, enzyme inactivation, and biocompatibility.

C. Solvent-Free Method

The most ideal strategy for the enzymatic esterification is to employ solvent-free systems because of their advantages including safe operation condition, high volumetric production, minimizing the deactivation of enzymes, environmental benefits, and high substrate concentration [85]. Moreover, the absence of organic solvent favors the purification of products from the reaction mixture in downstream processing and the manufacturing cost would be substantially reduced [49]. Furthermore, the safety and biocompatibility of the product obtained from the solvent-free system are superior to those prepared from the reaction mixture in the presence of organic solvent.

Lipid-based ester synthesis in solvent-free systems is presumably achieved through transesterification reaction. Two different mechanisms pertaining to transesterification in the literature, consisting of (1) one-step reaction: transesterification is considered as the alcoholysis of triacylglycerols; (2) two-step reaction: transesterification includes hydrolysis of triacylglycerols and subsequent esterification of liberated fatty acids [86]. In a solvent-free system, polarity of substrates such as short-chain alcohols and fatty acids could inhibit the enzymatic activity of lipase [87–89].

However, the major hurdle impeding the application of solvent-free system for enzymatic esterification reaction is the poor miscibility of polar and non-polar substrates, leading to slow reaction rates [52]. To overcome this hurdle, a series of solvent-free bioreactor systems were developed by making use of the desired product, monoester, to improve the miscibility of acyl acceptor (saccharide) and donor (fatty acid) in the presence of trace amount of organic solvent with low toxicity, for instance tert-butanol (t-BuOH) at fed-batch mode [61], or complete solvent-free reaction medium [57,58,63,90,91]. Recently, a closed-loop bioreactor system was developed and operated under continuous recirculation for the production of sugar-fatty acid esters using a stoichiometrical feed of substrates, consisting of a reservoir open to atmosphere at 65°C on a hot plate, a peristaltic pump and a packed-bed bioreactor (PBBR) filled with RML particles maintained at 53°C in an oven [57]. The original reaction medium contained solvent-free suspensions (1.5–2.0 wt% saccharide in mixtures of oleic acid/sugar ester). At specified 4–10 h intervals, the pump in the bioreactor system was stopped, and the reaction medium was removed. Meanwhile, additional grinded-saccharide was introduced into the medium at 800 rpm and 60°C for 6 h, for supplying consumed acyl acceptor in the reaction and re-form suspensions, followed by centrifugation to remove larger-sized particles. The medium was then returned to the PBBR system [57]. Upon reaching approximately 60 wt% ester, molecular sieves were added into the reservoir for the water removal. After 6 days of esterification reaction, 88% fructose oleate was synthesized, with 92% of monoester [57]. In addition, the combination of N_2 bubbling and vacuum pressure or incorporation of a packed column of MS into the bioreactor system were used to reduce the water concentration and maintained the water concentration from ~0.8 to ~0.4 wt in the liquid phase, leading to the yields ranging from 69 to 92 wt% saccharide fatty acid esters (typically 90 wt% monoester and 10 wt% diester) within 132 h, with a productivity of 0.15–0.30 mmol Ester/h/g lipase [63]. Moreover, the different acyl donors (oleic, caprylic, lauric, and myristic acids) and acceptors (fructose, sucrose, glucose, and xylose) were used for lipase-catalyzed synthesis of saccharide-fatty acid esters using solvent-free (50–200 μm) suspensions [58]. It was demonstrated that a positive linear relationship between initial saccharide concentration and initial rate of reaction and final ester conversion were obtained, regardless of acyl donor or acceptor type [52]. Subsequently, the closed-loop PBBR bioreactor system was redesigned for in situ addition of acyl acceptor into the reservoir. After 8.4 days, the reaction mixture was composed of 84 wt% ester with a productivity of 0.195 mmol/h/g [90]. High-speed homogenization, high-intensity ultrasound, and their combination were applied for the reduction of the particle size of sucrose crystals to enhance solvent-free lipase-catalyzed synthesis of sucrose oleate at 65°C [91]. The combination of homogenization and ultrasound could highly reduce the particle size of suspended sucrose crystals ranging from 88 to 18 μm in mixtures of oleic acid/sucrose oleate [91]. Of note, the pre-incubation of RML and CBL in substrate (oleic acid) in a stirred tank bioreactor prior to use was able to facilitate the lipase-catalyzed synthesis of sucrose oleate [91].

Octanoic acid esters, desirable fruity flavor obtained from the esterification of coconut oil and ethanol catalyzed by Lipozyme TL IM, a *sn*-1,3-specific lipase (450 interesterification units (IUN)/g) from *Thermomyces lanuginosa* granulated onto silica, was supplied by Novozymes A/S) in solvent-free system [92,93]. Another flavor ester, cinnamyl acetate was synthesized using Novozym 435 through transesterification between ethyl acetate and cinnamyl alcohol in the solvent-free system [94]. The reaction followed the Ping-Pong Bi-Bi mechanism. Excellent stability and reusability of lipases were also reported in the solvent-free system [94]. A medium-chain ester, hexyl laurate, with fruity flavor was obtained from the esterification of hexanol and lauric acid in the presence of RML in a solvent-free system [95]. Solvent-free enzymatic transesterification of ethyl ferulate and monostearin for the production of feruloyl fatty acyl esters at 98.3% ± 1.1% with the highest conversion

was conducted when Novozym 435 was applied [96]. Feruloylated structured lipids were catalyzed by Novozym 435 using ethyl ferulate (EF) and castor oil as substrates, in solvent-free system [97]. Monoacylglycerols were produced from olive oil in a solvent-free system containing Novozym 435 and sodium (bis-2-ethylhexyl) sulfosuccinate (Aerosol-OT or AOT) as surfactant [98]. Glyceryl ferulate (FG) and oleic acid were successfully synthesized by Novozym 435 for phenolic feruloylated monoacylglycerols in solvent-free system [99].

D. Microwave

Microwave radiation is used as an efficient heating source for different chemical reactions for obtaining high yields and reaction selectivity in a short period of reaction time [100]. For the enzyme-catalyzed reaction, microwave irradiation might facilitate the enzymatic activities to some extent because the conformational modification in the enzyme structure caused by microwave irradiation assists the substrates in approaching the active site of the enzyme readily [101]. Enzymatic transesterification of methyl benzoate with different alcohols (n-butanol, n-pentanol, n-hexanol, n-octanol, benzyl alcohol, isoamyl alcohol, and 2-ethyl-1-hexanol) was successfully performed for the synthesis of alkyl benzoate esters, using Novozym 435 under microwave irradiation. The highest conversion was 97% at 60°C in 6 h when the final product was n-hexyl benzoate. This product has gained great importance due to its wide application in industries including flavor, cosmetics, and pharmaceuticals [101]. Transesterification of ethyl cinnamate and geraniol in the presence of Novozym 435 was carried out for cinnamate esters under microwave irradiation. Compared to the control, a 4.2-fold increase in initial rate was observed using microwave irradiation [102]. Microwave-assisted lipase-catalyzed esterification of starch with different acyl donors: acetic, lauric, and stearic acid was conducted in DMF and DMSO [103]. Disruptions of the granular structure of starch particles were observed caused by solvent diffusion into the granule pores (at μν) in Figure 32.7. The small amount of water molecules in the starch granules interacted with μν, resulting in a fast increase of temperature and the quick evaporating water from particles. This phenomenon led to the disruptions [103].

E. Ultrasound

Ultrasound is generally defined as sound with a frequency pitched above human hearing. Based on the frequency level, it could be classified into two groups: (1) high frequency and low power (2–10 MHz; Hertz = cycle per second) ultrasound, developed for medically diagnostic imaging and chemical analyses and (2) low frequency and high power (20–100 kHz) ultrasound, used for cleaning and welding purposes, along with also for sonochemistry [104–106]. Ultrasound-assisted synthesis or sonochemistry is currently considered as a novel process technology widely used in food-related applications because ultrasound wave with compression and rarefaction cycles impact the molecules of the liquid. When the negative pressure of the rarefaction cycle surpasses the attractive interaction between the molecules of the liquid, a void or cavity is generated, facilitating a tiny amount of vapor from the liquid medium and the formation of an acoustic cavitation bubble [105,106]. Given that massive bubbles implode simultaneously, considerable amount of heat and pressure occur. The micro jet is generated, benefiting turbulence in the reaction medium [105]. Thus, ultrasound wave could substantially improve the interaction between phases through cavitation caused by the collapse of bubbles in liquid phase, disrupt the boundary phase, and eliminate the mass transfer resistance [105–108]. In particular, the low frequency ultrasound would not inactivate enzymes, but ultrasound irradiation at a mild level accelerated the activity and selectivity of enzymes [109] as well as the conversion of the final product, depending on the sonication parameters, the method of enzyme preparation and the type of enzyme [110]. When a high ultrasound power of 500 W was applied for the reaction (typical power used is 25–150 W), ultrasound could deactivate some enzymes, including alkaline protease, glucose-6-phosphate dehydrogenase, and trypsin [111]. This was probably attributed to the violent reaction of hydroxyl or hydrogen radicals

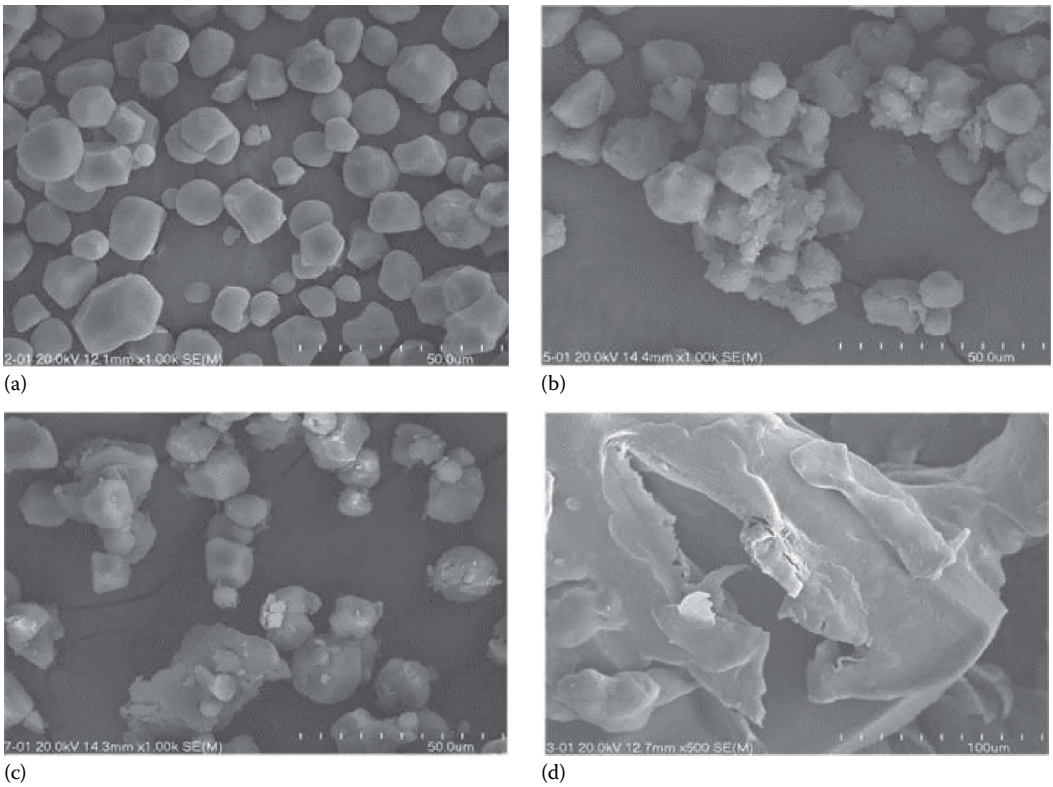


FIGURE 32.7 SEM microphotographs of starch esters: (a) native starch; (b) μν modification (lauric acid, DMF, 80 mW/g, 120 min); (c) Δ modification (lauric acid, DMF, 80°C, 120 min); and (d) μν modification (lauric acid, DMSO, 80 mW/g, 120 min). μν, microwave-assisted process; Δ, conventional heating process. (From Lukasiewicz, M. and Kowalski, S.: Low power microwave-assisted enzymatic esterification of starch. *Starch Stärke*. 2012. 64(3). 188–197. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.)

in the protein backbone [111], resulting in enzyme aggregation, thereby blocking the active sites and reducing protein stability [112]. Moreover, excessive shear forces caused by ultrasound irradiation also negatively affect enzyme stability [113]. Of note, for the enzymatic esterification reaction, mild agitation is required for the high yield of reaction product because gentle agitation could maintain the suspension of the particles in reaction mixture, preventing the coagulation of enzyme particles [114,115]. For instance, the ultrasound of 25 kHz frequency with a power of 70 W assisted in the synthesis of isoamyl butyrate, a fruity banana flavor, from isoamyl alcohol and butyric acid in the presence of immobilized Novozym 435, at 60°C with the stirring speed of 80 rpm, resulting in a 96% conversion in 3 h in Figure 32.8 [114]. Another flavor ester synthesis of acetic acid and butanol was carried out in low-frequency ultrasound (40 kHz) with Novozym 435 at 46°C, obtaining approximately 94% conversion in 2.5 h [116]. In addition, the productivity of the reaction was significantly increased around 7.5-fold, compared to the control (standard mechanical agitation) [116].

Caffeic acid phenethyl ester with antioxidant activities was successfully produced in a continuous packed-bed reactor containing Novozym 435 at ultrasonic power (1.20–1.98 W/cm^2). Lipase displayed a high stability over a 6-day period, leading to a 93%–97% conversion [117]. MAG and DAG from olive oil were generated at mild irradiation power supply (~130 W) in the presence of Novozym 435 [118]. In addition to the productions of MAG and DAG, ultrasound-mediated synthesis of sugar fatty acid esters was achieved using alkaline protease from *Bacillus subtilis* in

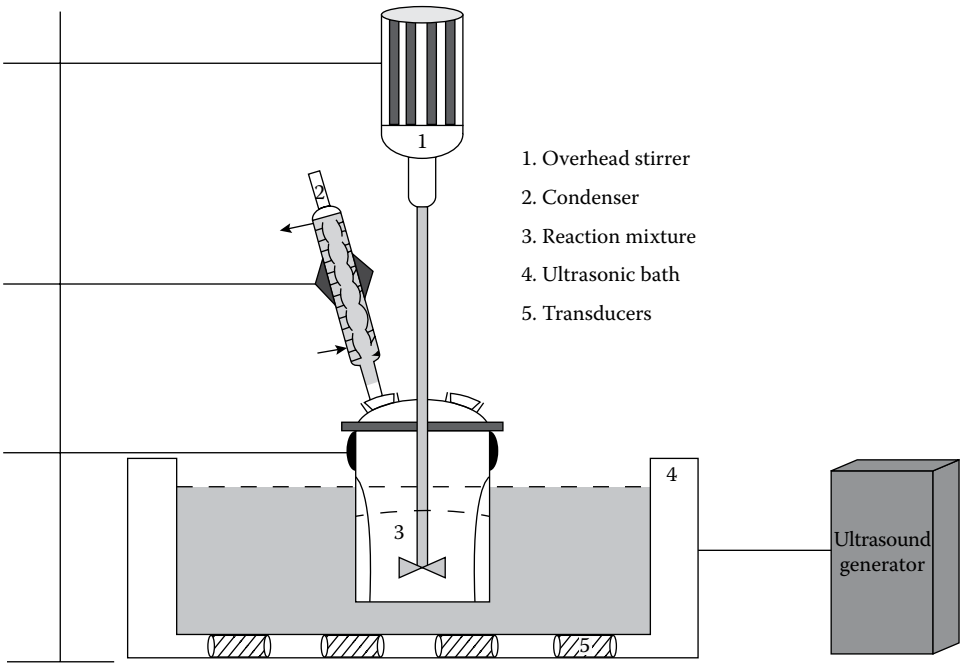


FIGURE 32.8 Schematic of experimental set up of esterification of isoamyl alcohol and butyric acid catalyzed with enzyme (Novozym 435) assisted with ultrasonic system. (From Bansode, S.R. and Rathod, V.K., *Process Biochem.*, 49(8), 1297, 2014.)

pyridine and Novozym 435 in tert-butanol [119]. Of interest, the positive effect from ultrasound is inversely proportional to the chain length of acyl donors from C_{10} to C_4 [119]. Furthermore, the ultrasound combined with molecular sieves [120] or ionic liquids [109] were successfully utilized in the enzyme-catalyzed synthesis of esters.

V. DIFFERENT TYPES OF LIPID-BASED ESTERS FOR THE FOOD INDUSTRY

A. Starch Fatty Acid Ester/Polysaccharide Fatty Acid Ester

Starch belongs to abundant, inexpensive, nontoxic, and renewable polysaccharides readily available from natural resources, for instance, corn, rice, and potato. The hydroxyl groups of starch allow for the esterification reaction with fatty acids [121]. Starch and polysaccharide fatty acid esters have been widely utilized in food, cosmetic, and pharmaceutical industries since they are nontoxic, biodegradable, and biocompatible biobased surfactants and emulsifiers. In general, dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), and pyridine are employed to increase the dissolubility of the starch, enabling the esterification readily [122].

Udomrati and Gohtanic [214] three fatty acids: decanoic acid (C-10), lauric acid (C-12), and palmitic acid (C-16) to react with maltodextrin using *Thermomyces lanuginosus* lipase at 60°C for 4 h with a degree of substitution (DS) 0.015–0.084. The DS reached the highest value at the ratio 1:0.5 of maltodextrin and fatty acids. Esterified maltodextrin displayed a higher viscosity than that of native maltodextrin. Moreover, the emulsification evaluation of esterified maltodextrin demonstrates they could act as emulsifiers to form stable n-hexadecane O/W emulsions.

The esterification reaction of vernonia oil methyl ester (epoxy ester) with cassava starch in a mixture of 1-butyl-3-methylimidazolium hexafluorophosphate, $[C_{(4)}C_{(1)}Im][PF_6]$ ionic liquid (IL) and DMSO, using Novozym 435, at 40°C for 72 h, with a degree of substitution (DS) of 0.9. The SEM micrographs indicate the morphology of the cassava starch granulate particles (Figure 32.9) is

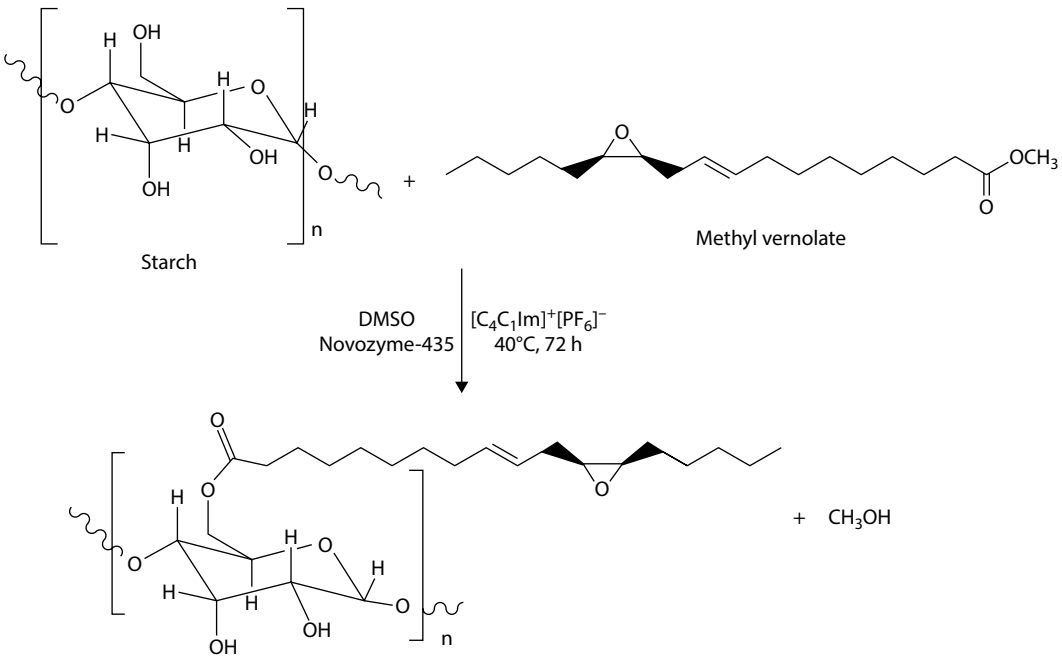
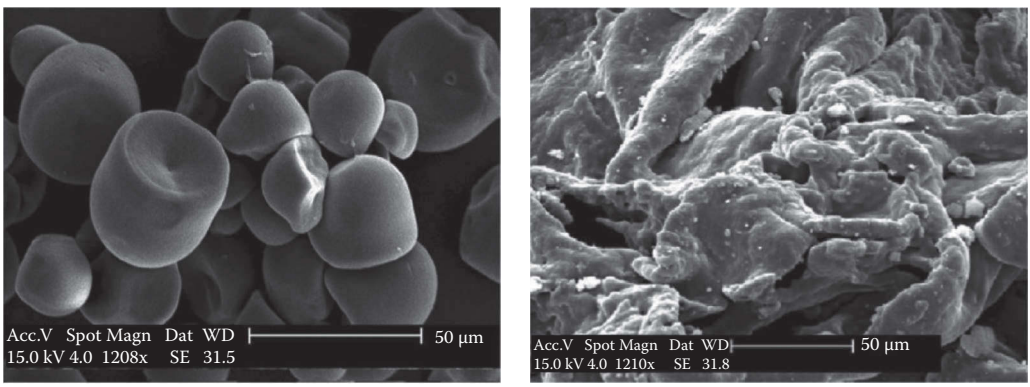


FIGURE 32.9 Reaction scheme for the synthesis of starch vernolate. (From Lukasiewicz, M. and Kowalski, S.: Low power microwave-assisted enzymatic esterification of starch. *Starch Stärke*. 2012. 64(3). 188–197. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.)



(a) (b)

FIGURE 32.10 SEM micrographs of (a) native starch and (b) starch vernolate. (From Desalegn, T., Garcia, IJV., Titman, J., Licence, P., Diaz, I., and Chebude, Y.: Enzymatic synthesis of epoxy fatty acid starch ester in ionic liquid–organic solvent mixture from vernonia oil. *Starch Stärke*. 2014. 66(3–4). 385–392. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.)

substantially modified after esterification, leading to the formation of a more irregular and shapeless morphology (Figure 32.10) in the starch vernolate at ambient temperature.

In another study, corn starch pretreated with NaOH/Urea was esterified with palmitic acid using 5% (w/w) lipase, Novozym 435, in solvent-free system at 70°C for 24 h, with a maximum DS of 1.04. The modified starch obtained more ideal emulsion capacity (67.6%) and emulsion stability (79.6%) than NS that is only 5.3% and 3.9%, respectively. The emulsion capacity and emulsion stability of starch palmitate did not change significantly during storage [124]. Authors also proposed

a reaction kinetics mechanism for the esterification of starch palmitate. The initial rate studies illustrated that the kinetics follow the Ping-Pong Bi-Bi mechanism [122]. Low power microwave reactor was developed to assist in the *hog pancreas* lipase-catalyzed esterification of glacial acetic acid, lauric acid, and stearic acid with starch, respectively, at 80 and 160 mW/g of the sample for 1 and 2 h, respectively, in DMSO and DMF [103]. Corn starch and oleic acid were mixed with immobilized *Staphylococcus aureus* lipase in microwave oven (Haier, HR-6702D; frequency, 2450 MHz; power consumption, 1150 W; maximum out power, 700 W), heating for 10S, followed by reacting at 200 rpm at 45°C for 4 h, to produce starch oleate with a DS of 2.86 [125].

Surfactants are applied to reduce the surface tension for enhancing the contact between fatty acid and starch. The gelatinized cassava starch (40%, w/v) and hydrolyzed coconut oil rich in lauric acid (2:1 ratio) in presence of Triton X-100 and lipase from *T. lanuginosa* was mixed for 20 min at 80 rpm at 60°C, followed by treating in a domestic microwave oven of frequency 2450 MHz, 80 W for 1 min. Subsequently, the esterification reaction was performed for 72 h to 6 days at 40°C in DMSO. DSC and TG results illustrated a dramatic increase in thermal stability after microwave esterification [126,127].

B. Structured Triacylglycerols

Structured lipids (SL) could be defined as the restructured triacylglycerols (TAG) with reorganized fatty acid compositions and different positional distributions in the glycerol backbone by chemical or enzymatic modification. SL could possess useful approaches of incorporating desired fatty acids into TAG for nutritive or therapeutic purposes [128,129]. SL can also be rationally designed to improve the physical and chemical properties of TAG for instance, melting point, iodine, and saponification number and thus, modifying the nutritional and textural attributes. In general, SL includes medium- and long-chain TAGs (MLCTs) [130], human milk fat substitute (HMFS) [131–133], cocoa butter [134], structurally modified phospholipids, health-beneficial fatty acid-rich fats/oils [135], low calorie fats/oils [136], low-*trans* or *trans*-free fats [129]. In this book (Chapter 35), Akoh and Kim will give a comprehensive overview on SL.

C. Wax Esters

Wax esters could be defined as high-molecular weight oxo esters of biodegradable, biocompatible, and nontoxic long-chain fatty acids esterified with long-chain alcohols. They are of considerable commercial importance due to their multiple applications in industry, for instance, alternatives for lubricating oils and co-plasticizer, along with emulsifiers in the food (sauces, beverage, and creams), cosmetics (lotions, creams, and ointments), leather, pharmaceuticals, agrochemicals (phytosanitary products), and printing (odor-free paint) industries [67]. Moreover, wax esters display dozens of biological functions, consisting of preventing from desiccation and ultraviolet (UV) light, inhibiting pathogens, as well as regulation of buoyancy and/or sound transmission [137,138]. They are naturally obtained from jojoba oil, beeswax, sperm whale oil, and seafowl feathers, using organic solvent extraction [139–141], followed by manifold expensive purification procedures [142]. However, the use of organic solvent raises environment concern and lead to high cost of production.

Wax esters with nearly 100% yields after 10 h at 50°C were prepared from long-chain fatty acids (myristic, palmitic, and stearic) and long-chain alcohols (1-dodecanol, 1-tetradecanol, 1-hexadecanol, and 1-octadecanol) in the presence of PEG-modified CRL. In general, CRL gives less preference to the esterification reaction of longer-chain fatty acids (C16 and C18) [143]. However, PEG-modified CRL has no preference toward the chain length of the acyl acceptor or acyl donor, resulting in broader industrial applications. Moreover, the addition of surfactant into the reaction medium favor the enzymatic performance of CRL [143], probably because of the improved mass transfer efficiency between substrates and the active site of enzyme. Palm stearin-based wax esters were formed via esterification with cetyl alcohol using an immobilized *Rhizopus oryzae* in

n-hexane, yielding a highest conversion of 98.52% for 2 h at 30°C [144]. Oleyloleate was successfully synthesized by Lipozyme TL IM® in a solvent-free system, using oleic acid and oleyl alcohol. Furthermore, the antimicrobial and emulsifying properties of this wax ester were assessed, indicating that emulsifying ability of the synthesized oleyloleate was lower than Tween 80 and sodium dodecyl sulfate, and no antimicrobial activity was detected [145]. Another study compared the physical features of oleyloleate prepared from immobilized lipase from *Candida* sp. with those of jojoba oil. Due to its high dynamic viscosity and flash point, oleyloleate could serve as a significant composition of lubricating oils [146]. As the major extract from seafowl feather oil used in cosmetics as a base oil due to its lubricity, moisture retention and non-toxic characteristics, cetyl-2-ethylhexanoate was prepared by the esterification of cetyl alcohol with 2-ethylhexanoic acid and Novozym 435 as a catalyst in n-hexane [140]. Jojoba oil was esterified with ethyl acetate for lipase (Novozym 435)-catalyzed synthesis of long-chain monounsaturated fatty acid ethyl esters and fatty alcohol acetates, which are essential functional ingredients of cosmetics, lubricants, fragrances, paints, and pharmaceutical formulations [147,148].

Palm esters were considered as another important ingredient in cosmetic industry. In the literature, palm esters were obtained from enzymatic transesterification of different palm oil fractions with oleyl alcohol in the presence of RML [139]. In particular, the density, slip melting point, and saponification value of the synthesized wax esters were inferior to those of palm oil because of the lower molecular weight in palm-based esters, leading to weak Van der Waals interaction per unit weight and less amount of ester bond in an ester molecule [139]. The overall refractive index values of palm esters were lower than palm oil. Since refractive index was improved with alkyl carbon chain length, unsaturation and conjugation [149], the overall refractive index values of palm esters were lower, comparing to palm oil groups with longer total carbon chain length. In addition, the iodine values of the palm esters were higher than that of palm oil because of the presence of additional double bond from oleyl alcohol. Of note, when used in cosmetic products, higher iodine value of wax esters will contribute to the higher permeation rate of the ingredient into stratum corneum of skin. All palm esters with a Human Irritancy Equivalent (HIE) score <0.9 demonstrate good skin compatibility. Furthermore, the moisturizing evaluation experiment of palm esters show they can maintain at 11.50 corneometric unit or 40.4% hydration after 2 h, indicating the excellent moisture retention ability. Therefore, it is a promising component for use in cosmetics and personal care products [139].

D. Monoacylglycerols and Diacylglycerols

Mono- (MAG) and diacylglycerols (DAG) are amphiphilic molecules, composed of lipophilic and hydrophilic portions. They are widely used as nonionic biobased surfactants or emulsifiers in the pharmaceutical, food, and cosmetic industries [150]. In addition, MAG and DAG have positive physiological functions for human health. MAG is able to impede an increase in triacylglycerol and insulin concentrations of postprandial blood [151]. In particular, monoesters containing oleic acid attached to the *sn*-2 position could exhibit significant bioactivities, such as *in vitro* and *in vivo* antioxidant, antiatherogenic, and antidiabetic activities [152]. DAG is considered as a minor constituent in vegetable oils and it is naturally generated by the cleavage of triacylglycerols (TAG) by lipases from plants [153]. DAG could inhibit excessive body fat accumulation and decrease serum TAG concentrations, therefore reducing and preventing obesity which trigger many health issues including heart attack and stroke, especially the 1,3-isomer DAG [154], because the intake of DAG-enriched oils enhances the rate of β-oxidation of fatty acids and interferes with the synthesis of fatty acids by inactivating the activity of enzymes [155–157].

In edible oil refinery industries, crude oils generally consist of different undesired compounds, depending on the resources and processing methods of oilseeds [158]. Among them, free fatty acids (FFA) in edible oils are usually released by hydrolysis of ester bonds in the presence of enzyme or/and heat and moisture during refining, processing, and storage. FFA reduces the oxidative stability

of the oils, resulting in oxidative rancidity in edible oils and fat-rich foods. In addition to oxidation susceptibility, the occurrence of short-chain fatty acids is associated with an undesirable rancid flavor (hydrolytic rancidity) in oil. Hence, the removal of the FFA is critical for the quality of the oil in most instances. The chemical and physical de-acidification is well-documented in oil industry. The chemical methods cause a high consumption of water and chemicals, excessive loss of neutral oil, and the undesirable byproducts (soapstock). Meanwhile, the physical approaches suffer several disadvantages, including the high requirements for pretreatments, the damage of heat-sensitive oil, and thermal polymerization [158]. In contrast, the enzyme-assisted de-acidification contributes to the synthesis of DAG and the decrease of FFA in the presence of glycerol.

von der Haar successfully reduced the concentration of FFA (oleic acid) in the pre-degummed rapeseed oil from 2% to 0.3%, using RML with a maximum DAG content of 23%. Furthermore, the immobilized lipase could be reused in 14 consecutive batches after washing with isopropanol and hexane [153]. Subsequently, authors screened Novozyme 435, RML and Lipozyme TL IM for the de-acidification of a refined rapeseed oil containing 6% of oleic acid (w/w) in the presence of the double stoichiometric amount of MAG [159]. In a recent study, distearin was used as a feruloyl acceptor for the preparation of lipophilic feruloylated lipid esters by the enzymatic transesterification of ethyl ferulate (EF) with distearin in a solvent-free system [160]. The final conversion was 97.6% ± 2.2%, consisting of 43.2% ± 0.9% MAG and 33.3% ± 1.3% DAG, respectively [160]. Another study indicated that Novozym 435 can effectively catalyze the transesterification of vinyl palmitate and glycerol [161]. To strengthen the health values of MAG and DAG, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids from menhaden oil were incorporated into the glycerol in a solvent-free system catalyzed by Novozym 435 [150].

E. Steryl Esters and Phytosteryl Esters

Steryl esters are fundamental components in the manufacture of functional foods. They are considered as cholesterol-lowering ingredients with high bioactivities based on the suggestion from the European Food Safety Authority (EFSA) [162]. The pressure-driven membrane processes enable the separation of steryl esters (650 < MW < 800 g/mol), free sterols (400−415 g/mol) and contaminant pesticides (150 < MW < 400 g/mol) based on size exclusion.

As the residual stream produced by the industry of vegetable oil refining, bioactive compounds such as sterols, tocopherols, and squalene in deodorizer distillates may vary from 2% to 20% [163]. Conventionally, classical approaches including organic solvent extraction, crystallization [164], chemical treatment [165], supercritical fluid [166,167] and molecular distillation [168,169] are used for the recovery or synthesis of value-added chemicals from deodorizer distillates with different compositions. Recently, enzymatic catalysis have been developed for lipase-catalyzed methyl or ethyl esterification of deodorizer distillate [170], to catalyze abundant and inexpensive fatty acids and sterols into their corresponding fatty acid methyl or ethyl esters, combining with molecular distillation and/or supercritical fluid extraction [8,170,171], for instance, soybean oil [170], vegetable oil [8], and sunflower oil [172]. In addition, the esterification of the sterols with free fatty acids in the deodorizer distillates allows for better separation of tocopherols and sterols using supercritical fluid extraction, short-path distillation [170] and the membrane-based separation technology, for instance, nanofiltration [162]. Moreover, compared to free sterols, steryl esters have more bioactive properties [162] and superior solubility in oil phases [173].

Phytosterols comprise plant sterols and stanols including β-sistosterol, campesterol, and stigmasterol. In general, they have ring structures with carbon side chains [164]. Due to the high similarities of chemical structures between cholesterol and phytosterols, phytosterols can lower cholesterol by preventing cholesterol absorption through competing with cholesterol for solubilization in micelles, competitive reactions during hydrolysis with cholesterol esterases and crystallizing with cholesterol to produce insoluble crystals [174–177]. However, since free phytosterols from vegetable oils

have high melting point and low solubility both in water and in oil phases, they are underutilized in food industry [162]. Phytosterols esters with free fatty acids have higher physical reactivity and solubility in water and oils [178]. Different lipases have been utilized to catalyze the phytosterols and free fatty acids from pine nut oil using CRL [178]; oleic acid in the presence of *CAL* [179] or *CRL* in hexane [180,181]. A two-step chemoenzymatic synthesis of phytosteryl caffeates was developed, including (1) the production of an intermediate vinyl caffeate; (2) subsequently esterified with phytosterols through lipase-catalyzed reaction [182]. In addition, phytosteryl caffeates showed higher antioxidant activity than caffeic acid [182]. Similarly, phytosteryl sinapates, vanillates, and ferulates synthesized by a chemoenzymatical method rendered superior antioxidant activity than their corresponding controls and intermediates [183,184]. Of particular importance, the phytosteryl docosahexaneates exerted anti-atherogenic impact on apo-E deficient mice [185].

F. Flavor/Aroma Esters

Esters with low boiling points and low molecular weights can emanate pleasant flavor/aroma. They have been extensively used in the flavor and fragrance industry. The unique aroma properties of esters are highly associated with their structures since flavor sensory recognition by humans is caused by the interaction between the olfactory receptors in the nose and aroma compounds [10]. For the food application, esters facilitate the presence of desirable fruity and sweet aromas in enormous food products including cheese, cakes, beer, wine, soft-drinks, and candies [186–188]. The customer demand for flavor and fragrances, such as aroma chemicals, essential oils, and other natural extracts, was increased 4.3% per year, and reached up to $23.5 billion in 2014 [189].

In general, the flavor esters are separated from natural resources using organic solvents in low concentrations [188]. However, extraction procedures using organic solvents are expensive due to trace amount of flavor/aroma esters in natural materials, and the employment of organic solvent causes the environment concerns and the formation of certain undesired side products and color impurities. Of note, flavor/aroma esters have been successfully synthesized via esterification of short chain acids with short chain alcohols. In addition, flavor esters catalyzed by enzymes are categorized as "natural flavors" [74,190]. Table 32.1 summarized lipase-catalyzed synthesis of flavor esters using pure acids, vinyl acetate, and alcohols from the recent publications.

In addition to the pure substrates, the mixtures of natural oils and lipids have been successfully used to synthesize flavor esters via esterification reaction using enzymes. Among different substrates, coconut oil or coconut cream, the inexpensive and sustainable chemicals, is composed of considerable amount of octanoic acid (C8 fatty acid), allowing for the substitution of expensive dairy fats for the production of octanoic acid esters. Coconut cream and fusel oil were employed to generate flavoroctanoic acid esters (ethyl-, butyl-, isobutyl-, and (iso)amyl octanoate) using Palatase®, a *sn*-1,3-specific lipase (22,500 Lipase Units/g) derived from a fungus, *Rhizomucor miehei* [204]. Additionally, coconut cream and 2-phenylethanol were converted into 2-phenylethyl hexanoate and 2-phenylethyl octanoate in the presence of Palatase [205]. Except for Palatase, lipase (Lipozyme TL IM) was involved in the synthesis of flavor-active octanoic acid esters in a solvent-free system during the transesterification reactions, using coconut oil and fusel oil [93] or fusel alcohols as the starting materials [93].

G. Phenolic Esters

The antioxidant capacity of phenolic acids is substantially beneficial for human health [206,207]. The antioxidant capacity and other bioactivities of these phenolic compounds could be enhanced by esterification with fatty alcohols, causing an increase in lipophilicity [208,209]. Cinnamic acid and its two derivatives, including hydroxylated, p-coumaric, and methoxylated, p-methoxycinnamic acid were esterified with ethanol and/or butanolin isooctane, catalyzed by Novozym 435 for the

TABLE 32.1
The Lipase-Catalyzed Synthesis of Flavor Esters Using Pure Acids, Vinyl Acetate, and Alcohols

Name	Flavor/Aroma	Acyl Donor and Acceptor	Type of Enzyme	Reference
Isoamyl acetate	Banana	Acetic acid and isoamyl alcohol	*R. miehei* (Lipozyme RM IM)	[191]
Isoamyl acetate	Banana	Acetic acid and isoamyl alcohol	*C. antarctica* (Novozym 435)	[192]
Isoamyl acetate	Banana	Acetic acid and isoamyl alcohol	*Thermomyces lanuginosus* (Lipozyme TL IM)	[193]
Isoamyl acetate	Banana	Acetic acid and isoamyl alcohol	*C. rugosa* (CRL)	[194]
Pentyl acetate	Banana and apple	Acetic acid and pentanol	Novozym 435	[195]
Pentyl butyrate	Pear and apricot flavors	Butyric acid and pentanol	Lipozyme RM IM	[196]
Ethyl butyrate	Pineapple and mango	Butyric acid and ethanol	Lipozyme TL IM	[196]
Ethyl butyrate	Pineapple and mango	Butyric acid and ethanol	*Candida rugosa* lipase	[197]
Ethyl valerate	Green apple	Valeric acid and ethanol	*Candida rugosa* lipase	[197]
2-Phenylethyl acetate	Rose aroma	Vinyl acetate and 2-phenethyl alcohol	Novozym 435	[198]
Acetoin, Decanoate	Fruity and buttery aroma	Dodecanoic acid and acetoin	Novozym 435	[199]
N-butyl acetate	Fruity aroma	Vinyl acetate and n-butanol	Lipase from *Rhizopus oligosporus* NRRL 5905	[200]
N-propyl acetate	Fruity aroma	Vinyl acetate and n-propanol	Lipase from *Rhizopus* oligosporus NRRL 5905	[200]
Butyl butyrate	Pineapple	Butyric acid and n-butanol	*R. miehei* (Lipozyme RM IM)	[201]
2-Phenylethyl acetate	Rose aroma	Vinyl acetate and 2-phenethyl alcohol	Lipase from *Candida rugosa*	[202]
Ethyl cinnamate	Fruity and balsamic aroma	Cinnamic acid and ethanol	Novozym 435	[203]
Butyl acetate	Pineapple	Acetic acid and butanol	Lipase from *Rhizopus oryzae*	[10]

production of different phenolic acid esters (ethyl and n-butyl cinnamate, ethyl p-coumarate, and n-butyl p-methoxycinnamate) [210]. All enzymatically synthesized esters showed superior antioxidant potential compared to cinnamic acid. The alkyl chain length is highly associated with the antioxidant activity of esters. In detail, their radical-scavenging abilities are inversely proportional to the alkyl chain length [210]. In addition, the structured phenolic lipids was synthesized by lipase-catalyzed transesterification of selected phenolic acids, including p-hydroxyphenyl acetic, p-coumaric, sinapic, ferulic and 3,4-dihydroxybenzoic acids, with triolein. The enzymatic production of phenolic lipid esters possessed a higher scavenging potency [211]. Weitkamp et al. employed Novozym 435 to prepare the analogous (hydroxy)phenylacetates using esterification and transesterification reactions between short-chain alkyl hydroxyphenylacetates and fatty alcohols in equimolar ratios [212]. Another study investigated the synthesis of lard-based ascorbyl esters in a tert-amyl alcohol solvent, using a packed bed reactor filled with Novozym 435 [213]. Moreover, the introduction of one or more hydrocarbon chains into the ascorbic acid ring, preferably in the 6-position, could improve the bioactive properties of L-ascorbic acid. Lard-based ascorbyl esters exhibited good scavenging activities on hydroxyl radical and superoxide anion radical. They can effectively prevent lipid oxidation in the oil system [213].

VI. CONCLUSIONS

The conventional chemical synthesis of esters for foods leads to health issues, environmental contamination, high energy consumption, dangerous operation condition, undesirable side reaction, byproducts, and high cost of downstream purification. With the goal of addressing these concerns, biocatalytic approach has proven successful in a large portion of esters used in foods with high selectivity and sustainability, mild and safe operation condition, and minor purification process required. Much work remains to be performed in future from an economical and environmental, sustainability standpoint. For instance, genetic modification for significant improvement of enzymatic activities, immobilization strategies for the enhancement of recycling the enzymes and their tolerance toward organic solvent, harsh environment (temperature and pH) and inhibitors, and the development of novel, cost-effective technologies for the increase of the immiscibility of substrates and/or enzymatic activity are desired.

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33 Microbial Lipases

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CONTENTS

I. INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes that catalyze the reversible hydrolysis of triacylglycerols (TAGs) under natural conditions. Widely distributed in animals, plants, and microbes, lipases differ from other esterases and are unique in that their activity is greatest against water-insoluble substrates and is enhanced at the substrate (oil)–water interface; that is, they exhibit "interfacial activation." Optimum activities are obtained in systems such as emulsions, where high surface areas of the substrate can be obtained. Lipases are active not only in normal-phase emulsions where the substrate is emulsified into an aqueous system (oil-in-water) but also, often more active, in invert (water-in-oil) emulsions and in reverse micelle systems containing an organic solvent solution of the substrate. Furthermore, lipases are exceedingly versatile in that they can also catalyze transesterification reactions and the stereospecific synthesis of esters, and they can act on a broad range of substrates.

Major advances have been made in our understanding of lipolytic enzymes over the past few years through solving the crystal structures of lipases from several sources. Knowledge of their structures has given insight into the mechanism of action, interfacial activation, specificity, and the nature of the active site. The high current interest in lipases, shown by the large volume of recent scientific literature on the topic, is driven by the great potential in a diversity of commercial applications for these enzymes. This chapter gives an overview of lipases and their properties, with a focus on the topics of current research interest such as lipase structure and its implications in interfacial activation and selectivity. The emphasis is on microbial lipases, particularly from fungi, with references to human pancreatic lipase for comparison.

II. LIPASES FROM DIFFERENT SOURCES

A. Animals

Lipases from various organs and tissues of several mammalian species have been investigated, but human and other pancreatic lipases are the most thoroughly studied. Pancreatic lipases are secreted into the duodenum and active on dietary TAGs. They are a class of structurally similar 50 kDa glycoproteins that are characterized by their specificity toward TAGs with little or no activity toward phospholipids, activation at the oil (substrate)–water interface and by colipase, and inhibition at micellar concentrations of bile salts. Pancreatic lipase can catalyze the complete breakdown of TAGs to free fatty acids (FFA) and glycerol. Nonhuman pancreatic lipases may differ from corresponding human lipases; for example, guinea pig pancreatic lipase differs in that it exhibits phospholipase A activity [1].

Other mammalian lipases have been studied and, while similar, they exhibit some characteristics that differ from those of pancreatic lipases. In addition to pancreatic lipase, fat digestion is aided by a series of lingual, pharyngeal, and gastric lipases that may be responsible for up to 50% of dietary fat breakdown [2]. Hence, in newborns, especially the premature and patients with cystic fibrosis where pancreatic lipase and bile acid levels are low, gastric lipolysis remains normal and a major portion of the fat digestion occurs without the presence of pancreatic lipase. Highly stable at low pH, these enzymes are activated by bile salts and show a preference for the *sn*-3 position of the substrate. Human lipoprotein lipase, which functions in hydrolyzing TAGs in chylomicrons and very low-density lipoproteins, shows many similarities to pancreatic lipase (e.g., high sequence homology and presence of the lid). However, this lipase is not responsive to colipase but instead requires apolipoprotein C-II (apoC-II) for activity, functions as a dimer, and is activated by heparin [3–5]. Hepatic lipase is confined to the liver, where it is also involved in the metabolism of lipoproteins, but not activated by apoC-II [6]. Human milk lipase, which functions in the digestion of milk fat ingested by infants, is activated by bile salt [7]. It is a glycoprotein, containing 10 wt% of carbohydrate [8], has an isoelectric point of 4 [9] and contains 12 and 10 mol% of proline and glycine, respectively, in its amino acid composition [8,9]. The amount of this lipase secreted in milk is ~0.2 mg/mL or 1% of the milk protein [9].

B. Plants

Plant lipases have not received the same attention as those from other sources, but they have been recently reviewed by Seth et al. [10]. Oilseed lipases have been of greatest interest among the plant lipases, and those from a variety of plant species show differences in their substrate specificity, pH optima, reactivity toward sulfhydryl reagents, hydrophobicity, and subcellular location [11]. In wheat, rice, cereals, and barley, for example, most of the lipolytic activities are found in the embryo (germ) and aleurone layer (the bran) of the grains. These lipases are relatively specific for the native TAGs of the species from which they were isolated. They are absent from the ungerminated seed and formed during germination to hydrolyze the reserve TAGs stored in lipid bodies. This provides energy and carbon skeletons during embryonic growth.

C. Microbes

There is substantial current interest in developing microbial lipases for use in biomedical and industrial applications because of their versatility and availability; in addition, they can be produced less expensively than corresponding mammalian enzymes. Animal and plant lipases are generally less thermostable than microbial extracellular lipases [12]. Hou and Johnston [13] screened 1229 bacteria, yeasts, actinomycetes, and fungi and found that about 25% were lipase-positive. Microbial lipases that have received the greatest attention are inducible, extracellular enzymes having properties that are generally similar to those of human pancreatic lipase, despite differences in detail. At least some of the microbes produce a mixture of extracellular lipases formed from multiple genes, [14–18] and some lipases vary by degrees of glycosylation. Fungal lipases typically exist as monomers with molecular masses ranging from about 30 to 60 kDa. They vary in specificity, specific activity, temperature stability, and other properties; however, dimeric lipases have been reported (Table 33.1) [19,20]. The characteristics of fungal lipases have been reviewed by Antonian [21], whereas the versatility of fungal lipases (from genera such as *Candida*, *Geotrichum*, *Rhizopus*, and *Thermomyces*) in biotechnology was illustrated extensively by Gandhi [22], Benjamin and Pandey [23], and Pandey et al. [24]. Lipases from *Rhizomucor*, *Rhizopus*, and *Candida* are currently used commercially. Examples of such commercially available fungal lipases include Lipozyme RMIM from *Rhizomucor miehei* (Novozymes A/S, Bagsvaerd, Denmark), Lipase A "Amano" (Amano Enzyme Inc., Nagoya, Japan) and Lipolase 100T (Novozymes A/S, Bagsvaerd, Denmark) from *Aspergillus niger*, Lipomod and Lipase F-AP 15 from *Rhizopus oryzae* (Novozymes A/S, Bagsvaerd, Denmark), Lipase AYS "Amano" (Amano Enzyme Inc., Nagoya, Japan), and Lipase MY (Meito Sangyo, Tokyo, Japan) from *Candida rugosa*, Novozym 435 from *Candida antarctica* (Novozymes A/S, Bagsvaerd, Denmark), and so on.

III. CLASSIFICATION OF MICROBIAL LIPASES

Microbial lipases can be classified based on a comparison of the amino acid sequences as well as some fundamental biological properties [25]. Such classification allows one to predict (1) important structural features such as catalytic site residues or the presence of disulfide bonds, (2) types of secretion mechanisms and requirement for lipase-specific foldase, and (3) the potential relationship to other enzyme families. The classification will contribute to a faster identification and easier characterization of novel bacterial lipolytic enzymes. Microbial lipases have been classified into eight different families (Table 33.2).

Family I is the largest and consists of six subfamilies. Subfamilies I.1 and I.2 consist of lipases from the *Pseudomonas* family. These lipases usually show pronounced differences in region and enantioselectivities despite a high degree (>40%) of amino acid sequence homology [26]. Lipases from subfamily I.1, also known as true lipases, have molecular masses in the range of 30–32 kDa and display higher sequence similarity to *Pseudomonas aeruginosa* lipase. Lipases from subfamily

TABLE 33.1
Properties of Some Recently Characterized Extracellular Microbial Lipases

Organism	Molecular Weight (kDa)	pH Optima	Optimum Temperature (°C)	Specific Activity (U = mg)	Specificity	Reference
Neurospora sp. TT-241	55	6.5	45	8,203	No specificity but preferred 1- and 3-positions	[74]
Rhizopus homothallicus	29.5	7.5	40	11,720	Preferred triacylglycerols with medium-chain fatty acid (C8)	[64]
Pseudomonas fluorescens AK102	33	8–10	55	6,200	Nonspecific for fatty acids	[65]
Rhizopus niveus lf04759						[241]
Lipase I	34	6.0–6.5	35	4,966	—	
Lipase II	30	6.0	40	6,198	—	
Candida parapsilosis	160	6.5	45	—	High specificity for long-chain fatty acids, particularly polyunsaturated fatty acids	[242]
Propionibacterium acidipropionici	6–8	7.0	30	—	Preferred substrates with high saturated fatty acids	[243]
Neurospora crassa	54	7.0	30	44	Preferred triglycerides with C16 and C18 acyl chains	[244]
Pythium ultimum #144	270	8.0	30	63	1,3-Specific, preferring substrates with higher unsaturation	[19]
Rhizopus delemar ATCC 34612	30.3	8.0–8.5	30	7,638	—	[66]
Fusarium heterosporium	31	5.5–6.0	45–50	2,010	1,3-Specific; preferred triacylglycerols with C6–C12 fatty acids	[68]
Penicillium roquefortii	25	6.0–7.0	30	4063	Preferred triacylglycerols with C4–C6 fatty acids	[67]
Penicillium sp. uzim-4	27	7.0	25	1,001	1,3-Specific, discriminates against diglycerides and active at low surface pressures	[245]

I.2 have slightly larger size (33 kDa). All lipases in subfamilies I.1 and I.2 require lipase-specific chaperon or lipase-specific foldase (Lif) for expression. Lipases from subfamily I.3 secrete lipases with higher molecular mass than the previous two, lack in the N-terminal signal peptide and Cys residue. Most of the Gram-positive bacteria such as those from *Bacillus* and *Staphylococcus* families are classified in Groups I.4–I.6. For example, *Bacillus subtilis*, *Bacillus pumilus*, *Geobacillus stearothermophilus*, *Geobacillus thermocatenulatus*, *Staphylococcus aureus*, and *Staphylococcus hyicus* were classified in these subfamilies. The subfamily I.7 consisted of lipolytic enzymes from *Propionibacterium acnes* and *Streptomyces cinnamoneus*, which show significant similarity to

TABLE 33.2
Classification of Microbial Lipolytic Enzymes

Family	Subfamily	Enzyme-Producing Strain	Accession Number	Similarity (%)		Properties
				Family	Subfamily	
I	1	*Pseudomonas aeruginosa*[a]	D50587	100		True lipases
		P. fluorescens C9	AF031226	95		
		Vibrio cholera	X16945	57		
		Acinetobacter calcoaceticus	X80800	43		
		Pseudomonas fragi	X14033	40		
		Pseudomonas wisconsinensis	U88907	39		
		Proteus vulgaris	U33845	38		
	2	*Bulkholderia glumae*[a]	X70354	35	100	
		Chromobacterium viscosum[a]	Q05489	35	100	
		Bulkholderia cepaciaa	M58494	33	78	
		Pseudomonas luteola	AF050153	33	77	
	3	*P. fluorescens* SIK W1	D11455	14	100	
		Serratia marcescens	D13253	15	51	
	4	*Bacillus subtilis*	M74010	16	100	
		Bacillus pumilus	A34992	13	80	
		Bacillus licheniformis	U35855	13	80	
	5	*Geobacillus stearothermophilus*	U78785	15	100	
		Geobacillus thermocatenulatus	X95309	14	94	
		Geobacillus thermoleovorans	AF134840	14	92	
	6	*Staphylococcus aureus*	M12715	14	100	Phospholipases
		Staphylococcus haemolyticus	AF096928	15	45	
		Staphylococcus epidermidis	AF090142	13	44	
		Staphylococcus hyicus	X02844	15	36	
		Staphylococcus xylosus	AF208229	14	36	
		Staphylococcus warneri	AF208033	12	36	
	7	*Propionibacterium acnes*	X99255	14	100	
		Streptomyces cinnamoneus	U80063	14	50	
II (GDSL)		*Aeromonas hydrophila*	P10480	100		Secreted acyltransferase
		Streptomyces scabies[a]	M57579	36		Secreted esterase
		P. aeruginosa	AF005091	35		Outer membrane-bound esterase
		Salmonella typhimurium	AF047014	28		Outer membrane-bound esterase
		Photorhabdus luminescens	X66379	28		Secreted esterase
III		*Streptomyces exfoliates*[a]	M86351	100		Extracellular lipase
		Streptomyces albus	U03114	82		Extracellular lipase
		Moraxella sp.	X53053	33		Extracellular esterase 1

(*Continued*)

TABLE 33.2 (*Continued*)
Classification of Microbial Lipolytic Enzymes

Family	Subfamily	Enzyme-Producing Strain	Accession Number	Similarity (%)		Properties
				Family	Subfamily	
IV (*HSL*)		*Alicyclobacillus acidocaldarius*	X62835	100		Esterase
		Pseudomonas sp. B11-1	AF034088	54		Lipase
		Archaeoglobus fulgidus	AE000985	48		Carboxylesterase
		Alcaligenes eutrophus	L36817	40		Putative lipase
		Escherichia coli	AE000153	36		Carboxylesterase
		Moraxella sp.	X53868	25		Extracellular esterase 2
V		*Pseudomonas oleovorans*	M58445	100		PHA-depolymerase
		Haemophilus influenzae	U32704	41		Putative esterase
		Psychrobacter immobilis	X67712	34		Extracellular esterase
		Moraxella sp.	X53869	34		Extracellular esterase 3
		Sulfolobus acidocaldarius	AF071233	32		Esterase
		Acetobacter pasteurianus	AB013096	20		Esterase
VI		*Synechocystis* sp.	D90904	100		Carboxylesterase
		Spirulina platensis	S70419	50		
		P. fluorescens[a]	S79600	24		
		Rickettsia prowazekii	Y11778	20		
		Chlamydia trachomatis	AE001287	16		
VII		*Arthrobacter oxydans*	Q01470	100		Carbamate hydrolase
		B. subtilis	P37967	48		*p*-Nitrobenzyl esterase
		Streptomyces coelicolor	CAA22794	45		Putative carboxylesterase
VIII		*Arthrobacter globiformis*	AAA99492	100		Stereoselective esterase
		Streptomyces chrysomallus	CAA78842	43		Cell-bound esterase
		P. fluorescens SIK WI	AAC60471	40		Esterase III

Source: Arpigny, J.L. and Jaeger, K.-E. *Biochem. J.*, 343, 1999, 177. With permission.
GDSL, Gly–Asp–Ser–(Leu); HSL, hormone-sensitive lipase.
[a] Lipolytic enzyme with known three-dimensional structure.

each other. The central region of these proteins (residues 50–150) is ~50% similar to lipases from *B. subtilis* and from subfamily I.2. No similarity was observed between the *S. cinnamoneus* lipases and other *Streptomyces* lipases known so far [25].

Family II exhibits a Gly–Asp–Ser–(Leu) [GDSL] motif containing the active site serine residues and not the conventional pentapeptide Gly–Xaa–Ser–Xaa–Gly, whereas Family III enzymes display the canonical fold of α/β-hydrolases and contain a typical catalytic triad usually formed by Ser, His, and Asp residues. This triad is functionally (but not structurally) identical with that of trypsin and subtilisin.

Several bacterial enzymes in Family IV show similar amino acid sequence to the mammalian hormone-sensitive lipase (HSL) [27]. It was once thought that the relatively high activity of enzymes in the HSL family at temperatures below 15°C was because of the sequence blocks that are highly conserved in these enzymes. However, the distinct sequence similarity between esterases from psychrophilic (*Moraxella* sp., *Psychrobacter immobilis*), mesophilic (*Escherichia coli*, *Alcaligenes*

eutrophus), and thermophilic (*Alicyclobacillus acidocaldarius*, *Archaeoglobus fulgidus*) indicates that the microorganisms' temperature adaptation is not responsible for the extensive conserved sequence motifs of these enzymes.

Enzymes grouped in Family V originate from mesophilic bacteria (*Pseudomonas oleororans*, *Haemophilus influenzae*, *Acetobacter pasteuriannus*) as well as from cold-adapted (*Sulfolobus acidocaldarius*) organisms. They show similarity to bacterial nonlipolytic enzymes such as epoxide hydrolases, dehalogenases, and haloperoxides, which also possess the α/β-hydrolase fold and a catalytic triad [28].

The enzymes classified in Family VI are among the smallest esterases known with molecular masses in the range of 23–26 kDa, active in dimer form, has the α/β-hydrolase fold and the typical Ser–Asp–His catalytic triad. Very little information is reported on the other enzymes in this family.

Bacterial esterases in Family VII have large molecular masses (55 kDa) and share similar amino acid sequence homology with eukaryotic acetylcholine esterases and intestine/liver carboxylesterases [25]. The esterase from *Arthrobacter oxydans* is active against the phenylcarbamate herbicides by hydrolyzing the central carbamate bond [29]. It is plasmid encoded and has the potential to be transmitted to other strains or species.

The enzymes in the last family, Family VIII, show striking similarity to several class C β-lactamases and has about 150 residues that are similar to *Enterobacter cloacae ampC* gene product, suggesting that the esterases in this family have an active site similar to that found in class C β-lactamases, which involves a Ser–Xaa–Xaa–Lys motif in the N terminus of both enzymes. However, Kim et al. [30] reported that the consensus sequence Gly–Xaa–Ser–Xaa–Gly in *Pseudomonas fluorescens* esterase is involved in the active site of the enzyme. This motif, which is not conserved in *Arthrobacter globiformis*, is also found in the *Streptomyces chrysomallus* esterase but lies near the C terminus and no histidine residue follows it in the sequence, implying that the order of the catalytic sequence (Ser–Asp–His) found in the entire superfamily of lipases and esterases is not conserved in this case. Obviously, more structural information is required to describe the catalytic mechanism of Family VIII esterases [25].

The attempt by Arpigny and Jaeger [25] in classifying bacterial esterases and lipases based on information available from protein and nucleotide databases showed that lipolytic enzymes display a wide diversity of properties and relatedness to other protein families, despite a highly conserved tertiary fold and sequence similarities. As more structural and kinetic information is made available from the continuing genome-sequencing projects, it is hoped that such classification will serve as a basis for a more complete and evolving bacterial lipolytic enzymes' classification.

IV. PRODUCTION, ISOLATION, AND PURIFICATION

A. Lipase Production

Microbial extracellular lipase production for laboratory study is typically carried out under liquid shake culture conditions or in small fermentors. Conditions for optimum lipase production seem to be variable depending on the species. Typically, TAGs (olive or soybean oil are commonly used) are placed in the culture medium to induce lipase production [19,31,32], but fatty acids (FA) may also induce lipase production [33–35]. However, lipase production has been studied in the absence of lipids (e.g., with sugars as the carbon source) [36,37]. Chang et al. [38] reported that Tween 80 and Tween 20 in the culture medium promoted lipase production and a change in the multiple forms of lipase produced by *C. rugosa*. The following is a specific example of conditions for the maximal production of lipases by *Geotrichum candidum*: growth for 24 h in liquid medium containing 1% soybean oil, 5% peptone, 0.1% $NaNO_3$, and 0.1% $MgSO_4$ at pH 7.0, 30°C, and shaking at 300 rpm [14]. In short, lipase production is influenced by both nutritional as well as physical factors and these factors are described as follows.

1. Effect of Nutritional Factors

a. Nitrogen Sources

Nitrogen source is required in large quantities because it amounts to ~10% of the dry weight of bacteria. It can be supplied from organic or inorganic sources in the form of ammonia, nitrate, nitrogen-containing compounds, and molecular nitrogen. Lin et al. [39] reported an extracellular alkaline lipase produced by *Pseudomonas alcaligenes* F-111 in a medium that contained peptone (1.5%, v/v) and yeast extract (0.5%, v/v), whereas Chander et al. [40] and Hiol et al. [41] showed enhanced *Aspergillus wentii* and *R. oryzae* lipase production using peptone as the nitrogen source. Organic nitrogen source such as corn-steep liquor has been found to increase the lipase production in *A. niger* [42], *Syncephalastrum racemosum* [36], and *R. oryzae* [36]. Researchers have also used inorganic nitrogen sources such as ammonium nitrate and sodium nitrate in their effort to maximize lipase production in *Candida* sp. and *Yarrowia* sp. [43] and ammonium sulfate in the production of *Burkholderia* sp. C20 lipase [44].

b. Carbon Sources

Carbon sources are important substrates for the energy production in microorganisms. According to Chopra and Chander [36], fructose 1% (w/v) is a good promoter to *S. racemosum* lipase production but not raffinose, galactose, maltose, lactose, mannitol, and glucose. However, in certain cases, it was observed that media supplemented with carbohydrates promoted good growth of the microorganism but not lipase activity [45–47]. Nahas [46] reported that the presence of monosaccharides or disaccharides or glycerol suppressed *Rhizopus oligosporus* lipase production in the growth medium. Similar observations were made by Macrae [47] on *G. candidum* lipase using glucose as the carbon source. Lipase production occurred only after glucose has been exhausted from the medium and growth almost ceased.

c. Lipid Sources

As mentioned in Section IV.A earlier, TAGs and FAs both can be used to induce lipase production. Olive oil, soybean oil, sunflower oil, and other oil related substrates significantly enhanced the activities of *Rhizopus chinensis* lipase [48], whereas rapeseed and corn oil at 3% were found to be the best in promoting growth of *R. oryzae* and 2% was the optimal for lipase production [49]. Another interesting observation was made on *Humicola lanuginosa* lipase by Omar et al. [50]. Oleic and eliadic acid (a stereoisomer of oleic acid) were found to have different effect on lipase production in *H. lanuginosa* with oleic acid exhibiting a higher stimulating effect than eliadic acid.

d. Surfactants

Lipase–substrate–detergent interaction can be complex due to many parameters that affect the interaction, such as micelle formation, concentration of free and micellar substrate, their availability to enzyme, enzyme denaturation/inactivation by detergent, degree and mode of enzyme activation by the hydrophobic interactions, and the structure of the enzyme at the water–oil interface. Cationic surface active agent such as hexadecyltrimethylammonium bromide (Cetrimide) stimulated production of *Flavobacterium odoratum* lipase 175% higher relative to the control (absence of detergent) compared with Tween 80, which increased the lipase activity to 170% of the control [51]. In contrast, Tweens and sulfonates were found to inactivate *Pseudomonas* sp. lipase completely in 90 min [52] while *Aspergillus carneus* lipase was markedly inhibited by ionic surfactants such as sodium dodecylsulfate (SDS), *N*-lauroyl sarcosine, and cholic acid [53].

e. Metal Ions

Metal ions play an important role in about one-third of enzymes. They modify electron flow in a substrate or enzyme, thus effectively controlling an enzyme-catalyzed reaction. They also serve to bind and orient substrate with respect to functional groups in the active site and provide a site for redox activity if the metal has several valence states [54]. When magnesium ions were added into

the medium, lipase production in *Pseudomonas pseudoalcaligenes* F-111 [39] and *P. aeruginosa* [55] was enhanced, and in *Bacillus* sp., the production of lipase was considerably enhanced when magnesium, manganese, and calcium ions were added into the production media [56].

2. Effect of Physical Factors

The growth and lipase production in microorganisms are significantly affected by certain physical parameters such as incubation temperature, initial pH of medium, inoculum size, and agitation speed.

a. Incubation Temperature

Temperature is a critical parameter that has to be controlled if maximum growth and lipase production have to be achieved, and it varies according to the microorganisms. *Pseudomonas* sp. lipases exhibit maximum production at 45°C, with substantial activity between 20°C and 60°C and a little activity at 80°C [57]. On the other hand, *Pseudomonas aurantiogriseum* exhibited highest lipase production at 29°C, and at 26°C and 32°C, the lipase production decreased by 1–2 orders of magnitude. This result showed the importance of temperature control during fermentation, since relatively small variations can greatly influence the productivity [58]. For some microorganisms, lipase production is growth-associated, as in the case of *Acinetobacter radioresisten*, which showed the highest yield between 20°C and 30°C, which coincided with the exponential growth of the microorganism [58].

b. Initial pH of Medium

The initial pH of the medium contributes to efficient lipase production by microorganisms as reported in many published works. Optimum pH of *Pseudomonas* sp. lipase with olive oil as substrate was pH 9, a characteristic shared by many lipases from the *Pseudomonas* genus [52]. Such lipases have great potential in the detergent industry, since it has the capability to function at alkaline pH.

c. Inoculum Size

The effect of inoculum size on lipase production can be demonstrated experimentally using different inoculum sizes. The lipase production rate by *Rhizopus delemar* is highest at low inoculum sizes, since the rate of oxygen transfer in the flask can be rate limiting at high inoculum size due to high oxygen consumption uptake by the microorganisms [59]. In addition, greater inoculum size can lead to an impaired nutrient supply and thereby decreasing production capacity [60].

d. Shaking Conditions

Shaking of growth culture during cultivation also gave contradictory effect on different microorganisms. Lipase production increases with the shaking condition literally. Shaking enhanced the production of extracellular lipases by *R. oryzae* [41]. For extracellular lipase production, 100 rpm was the optimal shaking rate, whereas for intracellular lipases, 150 rpm was the best. In shaken cultures, lipase production was enhanced by 70% for *Penicillium chrysogenum* [61], 50% for *A. wentii* [40], while the stationary culture experienced decreased growth and poor lipase production [62]. In contrast, when *S. racemosum* was grown in the nutrient medium in static condition, the enzyme production per unit growth was comparatively high [36].

B. Isolation and Purification

Extracellular lipases have been isolated, purified, and characterized from numerous microbial species. Some examples of recently characterized lipases are illustrated in Table 33.1. The specific isolation and purification methodology differs in detail from study to study, but it generally involves the ammonium sulfate precipitation of proteins from the culture medium after removal of cells or mycelium, and then fractionating the proteins through a series of ion exchange, affinity, and gel filtration columns. About 80% of purification schemes attempted and reported so far in the literature have used a precipitation step, with 60% of these using ammonium sulfate and 35% using ethanol,

acetone, or an acid (usually hydrochloric) [63]. The precipitation is usually used as an initial step to purification followed by chromatography separation. Increase in lipase activity depends on the concentration of ammonium sulfate solution used. This step is less affected by interfering nonprotein materials than chromatographic methods. Chromatographic separation involves passing a solution of enzymes through a medium that shows selective absorption for different solutes. Most of the time, a combination of chromatographic steps has proved to be efficient in purifying enzymes to homogeneity. The most common column used in the first chromatography step was the ion exchange chromatography. Gel filtration chromatography, which separates protein based on molecular size, is usually used after ion exchange chromatography. Affinity chromatography is an efficient technique used to purify enzymes based on ligand binding, but only 27% of studies reported had used affinity chromatography as a purification step. This is due to the high cost of the resins used. Most researchers preferred the less-expensive resins such as those used in ion-exchange and gel filtration chromatography. Several recent publications can be consulted for specific procedures for isolating lipase [19,64–73]. The following is given as an example of a lipase from *Neurospora* sp. TT-241 that was isolated and purified 371-fold [74].

1. *Ammonium sulfate fractionation*. Add solid ammonium sulfate to the clarified aqueous extract (or culture medium) up to 60% saturation. The resulting precipitate is collected by centrifugation at 12,000 × *g* for 30 min and dissolved in a minimal volume of buffer A (50 mM phosphate buffer, pH 7.0). The enzyme solution is dialyzed overnight against a 50-fold excess of the same buffer.
2. *Sephadex G-100 gel filtration chromatography*. The enzyme solution (70 mL) is applied to a Sephadex G-100 column (4 cm × 120 cm), eluted with buffer A at a flow rate of 40 mL/h, and 8 mL fractions are collected.
3. *Toyopearl phenyl-650M column chromatography*. Add ammonium sulfate to the pooled active fractions from gel filtration to a final concentration of 1 M. The enzyme solution is then applied to a Toyopearl phenyl-650M column (2.0 cm × 18 cm) that has been preequilibrated with buffer A containing 1 M ammonium sulfate. The column is washed with 300 mL of buffer A containing 1 M ammonium sulfate and then eluted with 1 L of a linear gradient from 1 to 0 M ammonium sulfate in buffer A at a flow rate of 60 mL/h; 4 mL fractions are collected.
4. *Ultrogel-HA hydroxyapatite column chromatography*. The pooled active fractions from the Toyopearl column are further purified by passage through an Ultrogel-HA column (3.0 cm × 9 cm), preequilibrated with 10 mM phosphate buffer (pH 7.0). After being washed with 10 mM phosphate buffer, the column is eluted with a 1 L linear gradient from 10 to 500 mM phosphate buffer at a flow rate of 50 mL/h, and 5 mL fractions are collected. The active fractions are pooled and stored at −20°C.

These procedures are carried out at 4°C. The purity of lipase active fractions is determined by SDS polyacrylamide gel electrophoresis.

V. ASSAY OF LIPASES

A. Review of Emulsion Systems

Lipases are assayed in emulsions where high substrate surface areas can be achieved. Since, in addition to high interfacial area, the chemical and physical environments at the substrate–water interface are important to the adsorption and activity of lipases, it is useful to understand the nature of the different types of emulsions used for lipase assay or in various applications. Emulsions are mixtures of two immiscible liquids (e.g., oil and water); one of the components is dispersed as very small droplets, or particles, and the mixture is stabilized by a surface-active agent, or surfactant. Emulsions are classified as macro- or microemulsions where the dispersed particles are either

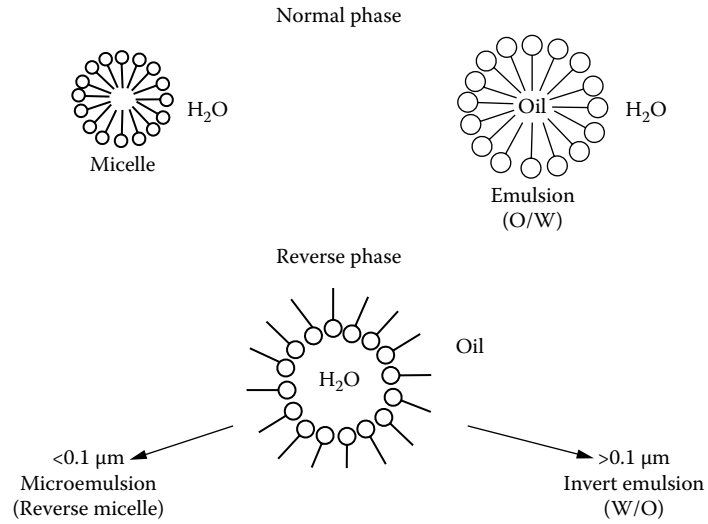

FIGURE 33.1 Diagrammatic representation of globules (particles) in various types of emulsions. In the ball-and-stick model of amphiphilic (surfactant) molecules, the ball represents the polar head group and the stick the hydrophobic region of the molecule.

greater or smaller than 1 mm in diameter, respectively. Macroemulsions are turbid, milky in color, and thermodynamically unstable (i.e., they will ultimately separate into the two liquid phases). On the other hand, microemulsions are homogeneous and stable.

Depending on the conditions of formation, and particularly the nature of the surfactant, macroemulsions may be normal phase (oil-in-water) or reversed phase (water-in-oil, invert). In the former, the oil is emulsified into the aqueous phase, and the surfactant forms a monolayer film around the dispersed oil droplets, whereby the hydrophobic moiety of the surfactant extends into the oil and the polar moiety is at the droplet surface (Figure 33.1). In reversed-phase emulsions, the aqueous phase is dispersed in the oil with the orientation of the surfactant molecules reversed (Figure 33.1).

Amphiphilic (surfactant) molecules undergo self-organization into spheroidal particles when dissolved in certain organic solvents such as isooctane with the polar head groups oriented inward and hydrophobic tails outward. These particles are referred to as reverse micelles and are <1 μm in diameter (Figure 33.1). Water can be "solubilized" in the organic solvent by becoming entrapped in the particles at up to several dozens of molecules per molecule of surfactant. Typically, reverse micelles are formed when the molar ratio of water to surfactant is <15, which is expressed as water activity, wo (i.e., molarity of water to molarity of surfactant) or R. Enzymes such as lipases can be entrapped within the aqueous phase particles of the invert emulsions or reverse micelles of microemulsions, where they retain their activity. In the latter case, the enzymes are isolated from the organic solvent. Luisi et al. [71] and Sánchez-Ferrer and Garcia-Carmona [75] can be consulted for more information on microemulsions.

B. Normal-Phase Emulsions

Most lipase assays for hydrolytic reactions are carried out in normal-phase emulsions whereby the water-insoluble oil substrate is emulsified by sonication into a buffered aqueous enzyme preparation. The emulsion is stabilized with an emulsifier and the aqueous phase often contains Ca^{2+}. The specific ingredients, buffer types, pH, and relative amounts of components vary widely with the author and specific lipase being assayed. An example of such an assay is as follows: 2 mL 0.2 M Tris maleate–NaOH buffer (pH 8.2), 1 mL 0.03 M Ca_2Cl, 5 mL distilled water, 1 mL olive oil, and 1 mL

enzyme solution. The emulsion is incubated at 30°C for 60 min or an appropriate time depending on lipase activity, and the reaction is stopped with 20 mL of acetone ethanol (1:1). Common emulsifiers used in lipase assays include polyvinyl alcohol and gum Arabic at 1%–2% by volume of assay mixture. Hydrolytic activity is most often determined by titration of the FA products of the reaction with NaOH. Other methods and assay conditions have been reviewed by Jensen [76].

C. Invert Emulsions

Using lipases from several fungi, Mozaffar and Weete [77] reported a reaction mixture containing 5 mL olive oil, 0.1 mL 520 mM taurocholic acid in 50 mM sodium phosphate buffer (pH 7.5), and 0.1 mL enzyme preparation. Final concentrations of taurocholic acid and water in the 5.2 mL of reaction mixture were 10 mM and 4%. The mixture was emulsified by vortexing for about 30 s and incubated at 45°C without shaking for 30 min, whereon the reaction was stopped as described earlier. Up to 90% of the substrate was hydrolyzed under these conditions by *C. rugosa* lipase when the incubation time was extended to 48 h with periodic additions of buffer (0.2 mL) during the incubation.

D. Microemulsion: Reverse Micelles

1. Hydrolysis

The desired amount of concentrated buffered lipase solution is poured into 5 mL of 50 mM aerosol optical thickness (AOT) [aerosol-OT, bis(2-ethylhexyl) sodium sulfosuccinate]–isooctane solution containing 10% v/v of the substrate. The amount of enzyme solution depends on the R value (e.g., 10.5). The reaction is initiated by vortexing until clear, and the mixture is incubated at 30°C for 15 min; then 0.4 mL is added to 4.6 mL benzene and 1.0 mL cupric acetate–pyridine solution, and the reaction is stopped by vortexing [78]. FAs liberated by the hydrolytic reaction are determined according to Lowry and Tinsley [79]. Other examples of studies involving hydrolysis by lipases in organic solvent–reverse micelle systems are cited in Table 33.3.

2. Transesterification

Using a lipase from *R. delemar*, Holmberg and Osterberg [66] used the following system for transesterifying TAGs with stearic acid: isooctane (91.65 wt%) was mixed with AOT at 100–200 mM, aqueous 0.066 M phosphate buffer (pH 6) (1.0%), and substrate (5.0%). The enzyme in the buffer was used at 1.5 U/mg substrate. The reaction was carried out at 35°C under nitrogen with magnetic stirring and was stopped by raising the temperature to 100°C and holding at that temperature for 10 min. Other examples of studies involving transesterification and synthetic reactions in organic solvent–reverse micelle systems are cited in Table 33.3.

VI. PROPERTIES AND REACTIONS

A. Structure

Over the past several years, the crystal structures of several mammalian and microbial lipases have been determined. Generally, lipases are α/β-proteins with a central core of a mixed β-sheet containing the catalytic triad composed of Ser …His…Asp, and a surface loop restricting access of the substrate to the active site.

1. Animal Lipases

Human pancreatic lipase is folded into two domains, a larger N-terminal domain (comprising residues 1–335) and a smaller C-terminal domain (residues 336–449). The core of the N domain is formed by a nine-stranded, β-pleated sheet in which most of the strands run parallel to one another. Seven α-helical

TABLE 33.3
Representative Applications of Microbial Lipases Using Organic Solvent Reverse Micelle Systems

Reaction Type	Topic	References[a]
Synthesis	Wax ester synthesis	[246]
	Synthesis of starch palmitate ester (polyol–fatty acid esters)	[247]
	Esterification reactions catalyzed by lipases in microemulsions: role of enzyme localization in relation to selectivity	[248]
	Esterification of oleic acid with glycerol in monolayer and microemulsion systems	[249]
	Synthesis of sugar esters	[250]
	Polyunsaturated fatty acid glyceride synthesis	[251]
	Monoacylation of fructose	[252]
	Enantioselective esterification of racemic acids and alcohols	[253]
	Esterification of oleic acid and methanol in hexane	[254]
	Esterification of glycerol: synthesis of regioisomerically pure 1,3-*sn*-diacylglycerols and monoacylglycerols	[255]
	Synthesis of DHA-rich triglycerides	[57]
	Synthesis of acylated glucose	[256]
	Synthesis of mono- and diglycerides	[232]
	Concentration of EPA by selective esterification	[257]
	Synthesis of water soluble plant stanol sorbitol succinate	[258]
	Synthesis of sialic acid (monosaccharide)	[259]
	Synthesis of food flavor	[260]
	Esterification of lauric acid	[261]
Interesterification	Interesterification of butterfat	[262]
	Interesterification of triglyceride between flaxseed oil and tricaprylin	[263]
	Enzymatic interesterification of fish oil in soybean lecithin	[264]
	Alteration of melting point of lard–rapeseed oil	[265]
	Interesterification of phosphatidylcholine	[266]
	Incorporation of medium-chain capric fatty acid into long-chain tripalmitin glyceride	[267]
	Kilogram-scale ester synthesis	[268]
	Incorporation of *n*-3 polyunsaturated fatty acids into vegetable oils	[269]
	Incorporation of EPA into tricaprin	[270]
	Interesterification of milk fat with oleic acid	[271]
	Incorporation of exogenous DHA into bacterial phospholipids	[272]
	Interesterification of phospholipids and fatty acids by surfactant-modified lipase	[273]
	Transesterification of rapeseed oil and 2-ethylhexanol	[274]
	Transesterification of cocoa butter	[212]
	Modification of phospholipids	[275]
	Diacylglycerol formation	[232]
	Transesterification of palm oil	[197]
	Transesterification of high oleic sunflower oil	[276]
	Transesterification of soybean oil	[277]
	Transesterification of trilinolein and trilinolenin with selected phenolic acids	[278]
Hydrolysis	Hydrolysis of phosphatidylcholine by an immobilized lipase	[279]
	Hydrolysis of vegetable oil	[280]
	Olive oil hydrolysis	[281]
	Production of polyunsaturated fatty acid-enriched fish oil	[282]
	Enrichment of γ-linolenic acid from evening primrose oil and borage	[283,284]
	Hydrolysis of vegetable oil	[280]

[a] The references cited are representative; they are not intended to be inclusive.

segments of varying length occur in the strand connections, and six of them pack against the two faces of the core sheet. The C domain is formed by two layers of antiparallel sheets, the strands of which are connected by loops of varying length. The N domain contains the active site, a glycosylation site, a Ca^{2+}-binding site, and possibly a heparin-binding site. The active site is buried beneath a short amphipathic α-helical surface loop, termed the "flap" or "lid" [80]. Colipase (see later) binds exclusively to the C domain of the protein through hydrophobic interactions and ion pairing [81], and the binding does not induce a conformational change [82–84]. Calcium ions activate and reduce the lag phase of human pancreatic lipase, particularly for mixed bile acid–lipase complexes [85].

The structures of pancreatic lipase from other systems have been recently reported, including guinea pig [1], horse [86], and ostrich [87]. Overall, the nonhuman pancreatic lipases are structurally similar to that from humans but may differ in detail. For example, the guinea pig pancreatic lipase does not possess the lid that is typical of other lipases [1]. In addition, unlike the pancreatic lipases from humans and pigs, the horse enzyme is not glycosylated [86].

2. Fungal Lipases

The crystal structures of several fungal lipases have also been determined: for example, *R. miehei* [88,89], *G. candidum* [90,91], *C. rugosa* [92], *C. antarctica* [93], *H. lanuginosa* [94], *Yarrowia lipolytica* [95], and *Malassezia globosa* [96]. Although there are no obvious sequence similarities between pancreatic and fungal lipases, except for the Gly–X–Ser–X–Gly consensus sequence in the active site region [87], there are structural similarities. Fungal lipases are α/β-proteins and have similar topologies based on a large central mixed β-pleated sheet pattern, mainly parallel, but the connectivity between strands vary. The two lipases (see later) of *G. candidum* have 554 amino acids and share about 85% sequence homology [16,97]. In addition, many fungal lipases possess the serine protease catalytic triad Ser…His…Asp in their active sites; however, glutamic acid is substituted for aspartic acid in the lipases from *G. candidum* [90] and *C. rugosa* [92]. Lipase II gene from *G. candidum* has been cloned [16,98], and the results of probing the active site by site-directed mutagenesis are consistent with x-ray crystallography data in that the Ser…His…Glu is the active site [99].

Fungal lipases also possess a lid that prevents access of the substrate to the active site, form a functional oxyanion hole, and have an interfacial-binding site (Gly–X–Ser–X–Gly), but the motion of the lid differs between fungal and pancreatic lipases. Lid rearrangements by human pancreatic lipase [83,84], *C. rugosa* [100,101], and *G. candidum* [90] and *M. globosa* [96] involve more than one loop. The lid is closed and covers the active site in *G. candidum* and is open in *C. rugosa*; otherwise, lipases from these two sources are very similar in sequence and structure. The three-dimensional structure of the extracellular lipase from *R. delemar* based on x-ray crystallographic coordinates showed that the lid partially open exposing the active site residues Ser^{145}, Asp^{204}, and His^{257} and residues 86–92 in the partially closed conformation [99].

3. Bacterial Lipases

Lipases from several *Pseudomonas* species have been crystallized, and preliminary x-ray crystallographic analyses of such species as *Pseudomonas cepacia* [102], *Pseudomonas glumae* [103], *P. fluorescens* [104], and *Psychrobacter* sp. [105] have been conducted. Their molecular masses, which are in 30–35 kDa range, show extensive sequence homology to one another but little to those of other lipases; the common G–X–S–X–G sequence in the active site region is conserved, and activity appears at the substrate–water interface. On the other hand, lipases from *Bacillus* species have molecular weights at about 19 kDa, have the sequence A–X–S–X–G instead of the characteristic sequence, and do not exhibit interfacial activation [106].

B. Interfacial Activation and the Hydrolytic Reaction

In 1958, Sarda and Desnuelle [107] showed that pancreatic lipase does not exhibit normal Michaelis–Menten kinetics with respect to substrate concentration. Lipases are inactive in aqueous media

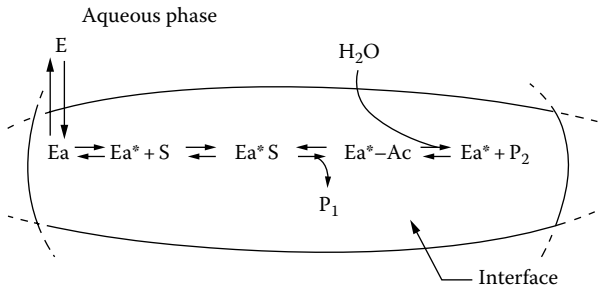

FIGURE 33.2 Schematic representation of the adsorption and hydrolytic activities of lipases at the oil (below the plane) and aqueous (above the plane) interface: E, enzyme in the aqueous phase; Ea, enzyme adsorbed at the interface; Ea*, activated enzyme; S, Substrate; Ea*S, enzyme–substrate complex; Ea*–Ac, acylenzyme; P_1, product (diacylglycerol); P_2, product (fatty acid).

with the substrate present in its monomeric form, but there is a sharp increase in activity when the substrate exceeds the critical micelle concentration. The inactive enzyme must first adsorb to the surface of the bulk substrate, which initiates interfacial activation (Figure 33.2). Interfacial enzyme kinetics in lipolysis was reviewed by Verger and de Haas [108].

Solving the crystal structures of lipases has given insight into the mechanism of interfacial activation [109]. It is believed that the preferred conformation in aqueous solution is with the lid covering the active site, thus denying access to the substrate. Adsorption of the lipase to the interface involves a conformational change in the enzyme, whereas the lid, which covers a cavity containing the active site and is held in place by mostly hydrophobic and some hydrogen bonds, undergoes reorientation. This is accompanied by additional conformational changes that expose the active site and a larger hydrophobic site, and allow access of the substrate to the active site. The working hypothesis for the mechanism of interfacial activation is based on the three-dimensional structural analyses of lipases from *R. miehei* [88,110], human pancreatic lipase [111], *G. candidum* [90,91], and *C. rugosa* [92]. The two latter lipases, for example, are globular, single-domain proteins built around an 11-stranded-mixed β-sheet with domains of 45 Å × 60 Å × 65 Å [112].

Activation of *R. miehei* lipase involves the movement of a 15 amino acid long lid in a hingetype, rigid-body motion that transports some of the atoms of a short α-helix by more than 12 Å. This, combined with another hinge movement, results in the exposure of a hydrophobic area representing 8% of the total molecular surface. In *C. rugosa*, comparison of open [92] and closed [113] conformation indicates that activation of the lipase requires the movement and refolding, including a *cis*-to-*trans* isomerization of a proline residue, of a single surface loop to expose a large hydrophobic surface where the substrate likely interacts. Lid reorganization contributes to the formation of a catalytically competent oxyanion hole and creation of a fully functional active site [112]. The scissile fatty acyl chain is bound in a narrow, hydrophobic tunnel, where modeling studies suggest that the substrate must adopt a tuning fork conformation [112,114]. There is a tryptophan residue at the tip of the lid (Trp89) in the lipase from *H. lanuginosa* that plays an important role in hydrolytic activity [115]. When Trp89 is substituted with other amino acids, activity drops substantially and variably depending on the substituent amino acid.

Visualizing how the hydrophobic substrate molecule, which is buried in the oil surface, gains access to the active site, which is buried within the water-soluble lipase molecule, is difficult. Blow [116] has suggested that on activation, a "hydrophobic seal" forms at the interface that allows the substrate to enter the active site without interacting with the bulk water; that is, the enzyme partially withdraws the substrate molecule from the bulk oil with at least some of the acyl chains projecting into the lipid.

Cygler et al. [112] have postulated that there are two tetrahedral intermediates in the lipase-catalyzed hydrolysis of esters. Formation of a noncovalent Michaelis complex between the lipase and TAG is followed by formation of a tetrahedral, hemiacetal intermediate resulting from

a nucleophilic attack by the serine Oγ. The oxyanion resulting from formation of the enzyme–substrate complex is stabilized by the amide groups of the oxyanion hole (e.g., Gly123 in *C. rugosa*) and α-helix following the active site serine [112]. Intermediate formation is followed by cleavage of the substrate ester bond, breakdown of the tetrahedral intermediate to the acyl enzyme, and protonation and dissociation of the diacylglycerol (DAG). The serine ester of the acylated enzyme is attacked by an activated water molecule to form a second tetrahedral intermediate, which is cleaved to give rise to the protonated enzyme (serine residue) and FA.

C. Activation/Inhibition

Calcium may stimulate lipase-catalyzed hydrolytic activity by (1) binding to the enzyme resulting in a change in conformation, (2) facilitating adsorption of the lipase to the substrate–water interface, and/or (3) removing from the interface FA products of hydrolysis that may reduce end-product inhibition of the reaction. Activation of human pancreatic lipase by calcium is complex and variable depending on the substrate and presence or absence of bile acids [85,117]. Calcium effects on microbial lipase activity may be variable depending on the enzyme source and assay conditions. For example, stimulation of *C. rugosa* lipase activity was attributed to the formation of calcium salts of FA products in a normal-phase emulsion, with olive oil as the substrate but not tributyrin; however, calcium had no effect in an invert emulsion [118]. In a nonemulsion system (i.e., without emulsifier), calcium had no effect on *C. rugosa* lipase activity with olive oil as the substrate but tended to offset the inhibitory effects of bile acid (Mozaffar and Weete, unpublished).

Human pancreatic lipase is inhibited by the bile salts, and the inhibition can be overcome by 10 kDa protein colipase. Bile salt coating of the substrate micelles creates a negatively charged surface that is believed to inhibit adsorption of the bile salt–lipase complex to the interface [119]. Colipase overcomes the inhibitory effect of the bile salts through formation of a 1:1 complex with lipase that facilitates adsorption at bile salt–coated interfaces [82,119]. Naka and Nakamura [120] found that although the bile salt sodium taurodeoxycholate inhibited pancreatic lipase activity when tributyrin was the substrate, a result that has been widely reported and cited by others, and colipase could reverse the inhibition, the bile salt actually stimulated hydrolytic activity when triolein was the substrate. This was attributed to the fact that triolein is a more natural substrate for the lipase than tributyrin. On the other hand, when sodium taurocholate was added to an emulsion assay of the lipase from *C. rugosa* with olive oil as the substrate, activity was progressively inhibited from 0.1 to 0.8 mM concentration of the bile salt [121]. Relatively high activity at the lowest concentration was attributed to the role of the bile salt in the stabilization of oil particles in the emulsion and providing high interfacial area for adsorption of the lipase, and inhibition was due to interaction with the enzyme such that adsorption was reduced.

A variety of substances have been shown to inhibit lipase activity; examples include anionic surfactants, certain proteins, metal ions, boronic acids, phosphorus-containing compounds such as diethyl *p*-nitrophenyl phosphate, phenylmethyl sulfonylfluoride, certain carbamates, β-lactones, and diisopropylfluorophosphate [122].

D. Selectivity

Lipases can be separated into three groups according to specificity [47,123]. The first group shows no marked specificity with respect to the position of the acyl group on the glycerol molecule, or to the specific nature of the FA component of the substrate. Complete breakdown of the substrate to glycerol and FAs occurs with nonspecific lipases. Examples of such lipases are those from *C.* (*cylindracae*) *rugosa*, *Corynebacterium acnes*, and *S. aureus*. The second group attacks the ester bonds specifically at the 1- and 3-positions of the substrate, with mixtures of DAG and monoacylglycerol (MAG) as products. Because of the instability of intermediate 1,2-DAG, 2,3-DAG, and 2-MAG (i.e., migration of the FA from the 2-position to the 1- or 3-position), these lipases may

catalyze the complete breakdown of the substrates. The positional specificity of lipases on *sn*-1 and *sn*-3 positions of the TAGs is due to the steric hindrance conflict that prevents the FA located at the *sn*-2 from binding to the active sites [124,125]. Most microbial lipases fall into this group; examples include those from *A. niger*, *R. delemar* (*oryzae*), *R. miehei*, and *Mucor javanicus*. Members of the third group of lipases show preference for a specific FA or chain length range, and are less common. The most widely studied lipase in this regard is that from *G. candidum*, which shows specificity for long-chain FAs with a *cis* double bond in the C9 position (see later) [125]. Other lipases that show some preference for specific FAs are those from *C. rugosa* (C18:2 *cis*-9, *trans*-11) [126], strains of *A. niger* (C10 and C12 or C18:1 *cis*-9), *Mucor miehei* (C12), *Rhizopus arrhizus* (C8, C10) [123], *H. lanuginosa* #3 [127], *Y. lipolytica* (C8, C18:1) [128], and human gastric lipase (C8 and C10) [129]. A lipase from *M. globosa* hydrolyzes only MAG and DAG [130]. Recombinant *C. antarctica* type lipase from strain *Ustilago maydis* is selective for *trans* FA [131]. The positional and FA selectivity of some fungal lipases are given in Table 33.4.

Many new bacterial lipolytic enzymes have been studied and published, but there has been very few attempts made to organize all the information published. Usually, lipolytic enzymes are characterized by their ability to catalyze a broad range of reactions and the different assay methods used such as pH-stat, monolayer technique, and hydrolysis of *p*-nitrophenyl esters prevented direct

TABLE 33.4
Positional and Fatty Acid Selectivity of Some Fungal Lipases

Organism	Positional Selectivity	Fatty Acid Selectivity	References
Aspergillus niger	1,2 and 2,3	SCFA, LCFA	[285]
Candida rugosa	Nonspecific	18 (*n*:3)	[286]
Candida antarctica A	—	C6-C12	[287]
Geotrichum candidum	Nonspecific	18:1 (*cis*-9)	[129]
		10:0 and 18:0	[288]
		cis-9 Unsaturated acids	[14]
		Double bond *cis*-9	[18]
	Double bond *cis*-9		
	2-Specific and nonspecific	Triolein and tricaprylin	[288]
		cis-Δ^9 Fatty acid	[146]
		Triolein, tricaprylin, and methyl oleate	[289]
		Triolein and methyl oleate inside ester bond of triolein	[290]
	—	C12, C14, C18	[148]
Humicola lanuginosa	1,3-Specific	12:0	[50]
Mucor javanicus	1,3 > 2	MCFA, LCFA > SCFA	[291]
Mucor miehei	1,3-Specific	12:0	[136]
		PUFA	[137]
N. crassa	1,3-Specific	16:0, 18:0	[244]
Rhizomucor miehei	1 > 3 ≫ 2	SCFA > MCFA, LCFA	[292]
Rhizopus arrhizus	1,3-Specific	16:0, 18:0	[293]
		18:1 (*cis*-6)	[294]
		SCFA, MCFA > LCFA	[292]
Rhizopus oryzae	1,3-Specific	C8:0 and C10:0	[295]
R. delemar	1,3	LCFA, SCFA	[296]
Penicillium camembertii	1,3-Specific	MAG, DAG > TAG	[292]

Abbreviations: PUFA, polyunsaturated fatty acids; SCFA, short-chain fatty acid; MCFA, medium-chain fatty acids; LCFA, long-chain fatty acid; MAG, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols.

comparison of the results obtained. To standardize the methods used, comparative studies have been conducted [129], but these studies only focused on a small number of bacterial lipases [132]. However, through the elucidation of many gene sequences and the resolution of numerous crystal structures, efforts have been made to classify the lipolytic enzymes. The work of Arpigny and Jaeger [25] as described in Section III is an example of such efforts. Arpigny and Jaeger [25] attempted to classify the lipolytic enzymes based on conserved sequence motifs and to relate them to the three-dimensional structural elements involved in substrate recognition and catalysis. Shimada and Akoh [133] categorized industrial lipases into five groups based on the homologies of their primary structures deduced from the nucleotide sequences (Table 33.5). According to him, homology of the primary structure shows similarity of tertiary structure, and lipases with similar tertiary structure are assumed to have similar properties. It is interesting to note that although Shimada had taken a simpler approach to categorizing the lipolytic enzymes, there were some similarities in his classification with that of Arpigny and Jaeger [25].

Negative selectivity has also been observed where the lipase from *Candida cylindracea* (*rugosa*) selected against TAG molecules containing docosahexaenoic acid (DHA) [134]; *G. candidum* lipase preparations have shown similar discrimination for γ-linolenate (GLA) in borage oil [135], and erucic acid from rapeseed oil [136]. Lipases from *Brassica napus* and *M. miehei* were also found to discriminate against polyunsaturated acids, such as GLA and DHA [137].

Lipases that are specific to FA for their chain lengths and unsaturation in catalyzing hydrolysis reaction of TAG have been used to develop concentrated forms of FFA including polyunsaturated fatty acid (PUFA) and GLA. Shimada et al. [138] used lipase of *G. candidum* to produce PUFA-rich oil from tuna oil. The principle involved is that if there is a lipase that does not digest PUFA esters, then the PUFA will be concentrated in glycerides by hydrolyzing PUFA-containing oil with

TABLE 33.5
Classification of Industrial Lipases Based on Their Primary Structure

Group	Microorganism	Property
Bacteria		
Group 1	*Bulkholderia cepacia*	Positional specificity: nonspecific or 1,3-position preferential
	B. glumae	
	P. aeruginosa	Acts somewhat on PUFA
Group 2	*P. fluorescens*	Positional specificity: nonspecific or 1,3-position preferential
	S. marcescens	Acts somewhat on PUFA
Yeast		
Group 3	*C. rugosa*	Positional specificity: nonspecific
	G. candidum	Act very weakly on C20 FA and PUFA
		FA specificity: relatively strict
		Acts on sterol and L-menthol
		Hydrolysis activity: strong
Group 4	*C. antarctica*	Positional specificity: 1,3-position preferential, 1,3-position specific in a reaction
		Acts strongly on PUFA and short-chained alcohols
Fungi		
Group 5	*R. miehei*, *R. oryzae*	Positional specificity: 1,3-position specific
	Thermomyces lanuginose	Acts weakly on PUFA
	Fusarium heterosporum	Acts strongly on C8–C24 saturated and monoenoic FA
	P. camembertii	Positional specificity: 1,3-position specific Does not act on TAG

Source: Shimada, Y., in *Handbook of Functional Lipids*, C.C. Akoh, ed., CRC Press, Boca Raton, FL, 2006, pp. 437–455. With permission.

that lipase. Thus, using the selective specificity of a lipase, certain FA can be enriched in glycerides. The work done by Shimada et al. [138] proved that *G. candidum* lipase hydrolyzed palmitic and oleic acids more effectively than *Fusarium heterosporum* and *C. cylindracea* lipases, and less effectively for eicosapentaenoic acid (EPA) and DHA esters. Therefore, *G. candidum* lipase enriched EPA and DHA, as its reactivity on DHA and EPA esters were very low. The substrate selectivity and regioselectivity of lipase can be exploited advantageously for use in structural determination of TAG, synthesis of a specific and defined set of MAG and/or DAG [96], and for the preparation of FA.

In addition, there have been numerous studies on the enantioselectivity catalyzed by lipases and esterases for the formation of optically pure chiral compounds. The fungal lipase from *C. rugosa* has been shown to hydrolyze octyl-2-chloropropionate with high stereoselectivity on a large scale, yielding 46% (R) acid and 45% of (S) ester [139]. The formation of optically active synthons and the ready availability of such materials for synthesis are important because optically pure end products are often prerequisite for incorporation in ethical drug formulations and important agricultural aids in the future [140].

In some instances, it is possible to couple FA selectivity to positional selectivity, for example, when a natural TAG has a high proportion of a particular FA in the primary positions. In one case, *R. delemar* lipase was employed in a biomembrane reactor to remove erucic acid from the primary positions of crambe oil [368]. Similarly, lesquerolic acid (14-hydroxy-*Z*-11-eicosanoic acid) has been removed from lesquerella oil by means of the positionally selective lipase of *R. arrhizus* [141]. Contrastingly, a lipase of *C. rugosa* was reported as positionally nonselective but has sufficient FA selectivity that it could be used to prepare 1,3-dierucin acid from rapeseed oil, that is, the lipase is positionally nonselective but favors hydrolysis or acid residues shorter than that of erucic acid, most of which occur at the 2-position [193]. Likewise, the lipases of *G. candidum*, *C. rugosa*, porcine lipase, and *Y. lipolytica* are useful in the production of oil containing high concentrations of PUFA. *G. candidum* lipase can enrich DHA and EPA [142], and *C. rugosa* lipase can enrich DHA, but not EPA [138,143], porcine lipase enrich DPA rapidly than EPA and DHA [144], and *Y. lipolytica* can enrich DHA.

Lipase selectivity/specificity may be due to structural features of the substrate (e.g., FA chain length, unsaturation, and stereochemistry), physicochemical factors at the interface, and/or differences in the binding sites of the enzyme. Stereoselectivity of enzymes can be influenced by temperature and hydrophobicity of the solvent. Recently, Rogalska et al. [145] showed that the enantioselectivity of lipases from *R. miehei*, *C. antarctica* B, lipoprotein lipase, and human gastric lipase toward monolayers of racemic dicaprin was enhanced at low surface pressures, while catalytic activity decreased.

Variations in specificity of lipase preparations from different fungi, different strains of the same species, and the same strain cultured under different conditions may be due to the production of multiple isoforms with differing specificities [146]. The lipase(s) of *G. candidum* have been of particular interest because those from some strains exhibit a relatively high preference for ester bonds involving FAs with a *cis*-9 double bond (e.g., oleic acid). Purification of *G. candidum* lipases and isolation of isozymes have been carried out [147]. *Geotrichum* species and strains produce at least two glycosylated lipases, most often designated lipases I and II, or less often A and B, which are coded for by two genes [148]. Additional isoforms may be produced, which differ by degrees of glycosylation. There are conflicting reports on the specificities of the two lipases. For example, of the two lipases produced by *G. candidum* CMICC 335426, lipase B showed high specificity for the *cis*-9 unsaturates than lipase A of this strain and according to Sidebottom et al. [18] lipases I and II of ATCC 34614 showed no preference. Bertolini et al. [149] cloned lipases I and II from *G. candidum* ATCC 34614 into *Saccharomyces cerevisiae*, then isolated and purified the two lipases from this yeast and determined their substrate specificities. Lipase I showed higher specificity than lipase II for long-chain unsaturated fatty acyl chains with *cis*-9 double bond, and lipase II showed a preference for substrates having short acyl chains (C8–C14). These investigators also showed that sequence variation in the N-terminal amino acids of these lipases, or the lid, does not contribute to variation

TABLE 33.6
Selectivities of Multiple Extracellular Lipases from *Geotrichum* Species/Strains and *M. miehei*

Fungus/Strain	Molecular Mass (kDa)	Selectivity	References
Hydrolysis			
G. candidum ATCC 34614			
Lipase I	50.1	No preference for C18:1	[18]
Lipase II	55.5	No preference for C18:1	
G. candidum CMICC 335426			
Lipase A	53.7	Preference for C16:0 relative to C18:1Δ^9	
Lipase B	48.9	Selectivity for fatty acids with *cis*-9 double bond	
G. candidum ATCC 66592			
Lipase I	61	Hydrolyzed C16:0 methyl ester at 60% of initial velocity to that of C18:1 methyl ester	[150]
Lipase II	57	Hydrolyzed C16:0 methyl ester at initial velocity that was only 7% of that of C18:1 methyl ester	
G. candidum ATCC 34614			
Lipase I	64	Showed high preference for triolein and TAG with C8	[151]
Lipase II	66	Same as lipase I	
Geotrichum sp. F0401B			
Lipase A	62	Incompletely 1,3-specific toward triolein but able to hydrolyze at 2-position at slower rate	[152]
Lipase B	—	Nonspecific positional specificity (presumed mixture of A and C)	
Lipase C	58	Incompletely 2-specific	
Synthesis			
G. candidum NRRL Y-553		Reactivities slow for γ-linolenic and ricinoleic acids relative to oleic acid; oleic acid esterifies 2.5 times faster than C16:0 to 1-butanol, but 50 times faster with 2-methyl-1-propanol or cyclopentanol	[153]

in substrate preference. Not all strains of *G. candidum* exhibit the preference for unsaturated FAs [11]. The specificities of isoforms of lipases from *Geotrichum* are shown in Table 33.6 [18,150–153].

Cygler et al. [112] suggested that the basis for the selectivity differences between lipases I and II from *G. candidum* involves key amino acid residues along the internal cavity presumed to be the binding site of the scissile acyl chain and that selectivity does not involve the lid. Studies with a cloned lipase from *R. delemar* [154] appear to support this suggestion. For example, substitution of certain amino acids (e.g., Phe95 → Asp) through site-directed mutagenesis in the substrate-binding region resulted in an almost twofold increase in the preference for tricaprylin relative to tributyrin and triolein [155]. In addition, substitution of Thr83 is believed to be involved in oxyanion binding with Ala-eliminated activity.

At least five genes encoding lipases have been found in *C. cylindracea* (*rugosa*), but the individual isoforms have not been purified and had their specificities determined [156]. Based on gene sequences, the lipase genes of *C. cylindracea* and *G. candidum* appear to belong to the cholinesterase family.

E. Immobilization

One of the limitations to the industrial uses of lipases is cost-effectiveness. This can be improved by reuse of the lipase, which can be accomplished by immobilizing the enzyme on an inert support. Immobilized lipases have been studied for hydrolytic [15,157–163], synthetic [164–168], and inter-/transesterification [169–172] reactions in both aqueous and organic media. Materials tested as solid supports for lipases include ion-exchange resins (DEAE-Sephadex A50 or Amberlite IRA94) [161] and others [173], adsorbents such as silica gel [174,175], microporous polypropylene [157], silk fiber [176], and nanofiber [177]. In other cases, lipases have been immobilized by entrapping them in gels of photo-cross-linkable resins (ENT and ENTP) [178]. Immobilization using methods described in the references cited earlier does not involve chemical modification of the enzyme. Recently, Braun et al. [166] achieved immobilization of *C. rugosa* lipase on nylon by covalent attachment to the support after conversion of carbohydrate groups on the enzyme to dialdehydes. More recently, Goto et al. [179] proposed a novel method of immobilizing enzymes onto porous hollow-fiber membrane modified using radiation-induced graft polymerization. An ion-exchange group containing the grafted hollow-fiber membrane extends from the pore surface toward the pore interior and through mutual repulsion, captures the enzymes via electrostatic interaction.

Besides the use of immobilized purified enzymes, there are studies reported on the use of whole cells, or the so-called naturally immobilized enzymes. Cells of microorganisms such as bacteria, yeast, and filamentous fungi produce their own enzymes within the confines of the peripheral plasma membrane, the so-called intracellular or naturally bound enzymes. Such naturally immobilized enzymes can be used directly without the laborious procedures of extraction, purification, and immobilization and thus are inexpensive and time saving. It can be used directly after physical separation from the growth medium and after drying if the condition requires low moisture content. Since extraction from the source cells is not required, loss of catalytic activity can be prevented. Such enzymes also do not require the addition of cofactors. Studies on the use of mycelium-bound lipase (MBL) have been reported [180–182].

Whole cell-immobilized enzymes can be obtained in alternative forms of growing cultures, resting cultures, spore cultures, or immobilized cultures [140]. Growing cultures are based on conventional fermentation practice where substrate to be biotransformed is added to the batch-grown cultures, and the incubation is continued until all of the added substrate disappears and/or the bioconversion cease. In this technique, it is crucial to avoid overmetabolism, which serves to erode the yield of desired products. The use of resting cells minimizes these problems. Resting cells are nongrowing live cells obtained by removing growing cells from liquid growing medium at a time in the growth phase when the potential of the cells to undertake the desired biotransformation is optimal. Spore cultures, even though metabolically inert, like resting cells can undertake desired reactions when suspended in dilute buffer.

Immobilized culture of microorganism has been much studied and reviewed [183–186]. Immobilized microbial cells are attractive in that they are likely to remain operationally active for very much longer than an actively growing culture, are easily removed from the reaction mixture, and can be reused repeatedly [187]. Long et al. [188] demonstrated that the MBL of *Aspergillus flavus* can be stabilized by cross-linking with glutaraldehyde, methylglyoxal, and ethylenediamine. However, the activity and stability of the MBL was affected by the concentration of the bifunctional reagent used and exposure times. The potential for enhancement of enzyme stability by immobilization and its limitations has been discussed in length by Klibanov [189].

In naturally bound enzymes, fungal mycelia are used as a direct source of the enzyme and thereby eliminate the need for isolation and external immobilization procedures in cases where the lipase is already bound to the cell wall. Batches of mycelia are grown in shake-flask culture, harvested, freeze-dried, defatted, ground into fine particles, and stored in vacuo at room temperature until required for use [190]. There have been reports of usage of microbial cells having lipase in microaqueous systems in bioreactor. The microbial cells used can be wet or in semidried forms

and suspended in water-immiscible organic solvent [191], packed in a column [192], or immobilize [193,194]. It has also been reported that an extracellular thermostable lipase produced by *Pseudomonas mephitica* var. *lipolytica* retains its activity in the supernatant fluid separated by centrifugation from a mixture obtained when fully grown cells are homogenized with small glass beads (under cooling provided by solid carbon dioxide) and disrupted with a sonic disintegrator [195]. Binding of lipases to whole dried cells or the so-called MBL has been successfully employed for exogenous lipases produced by fungal species [196], yeast [197], *R. oryzae* [193], *A. niger* [198], or bacteria [150,195]. For example of application in industry scale, the sugar beet industry uses immobilized α-galactosidase to hydrolyze the indigestible sugar raffinose into sucrose and galactose. This enzyme is fixed within pellets of the fungus *Mortierella vinaceae*, which are formed under particular growth conditions, and the untreated cells can be used directly as biocatalyst [199].

Szczesna-Antczak et al. [200] compared the stability and performance of *Mucor circinelloides* lipase either immobilized in situ in the mycelium pellets or isolated from mycelium and immobilized on solid carriers (cellulose palmitate and octate, diatomaceous earth, and modified porous glass) and found that lipase isolated and immobilized on solid supports gave lower activity in the synthesis of oleic and caprylic esters of propanol and sucrose, and weaker hydrolytic activity compared with the MBL preparation. They explained that the higher activity of MBL was due to the more favorable spatial arrangement of the lipase molecules anchored in the cell membranes, and this facilitated the binding of substrates in the catalytic site of the enzyme. Another reason for the lower synthetic and hydrolytic activity of the purified immobilized lipase was due to the physicochemical properties of the carriers, which were different from the natural matrix, thus changing the enzyme's microenvironment with a negative impact on its catalytic activity. The activity and operational stability of *M. circinelloides* lipase derived by defatting and dehydrating the mycelium pellets was superior and enhanced after entrapment of the MBL in polyvinyl pyrrolidone containing chitosan beads solidified with hexametapolyphosphate [200].

VII. INDUSTRIAL APPLICATIONS

A. General Uses

The current industrial enzyme market is estimated over $600 million, with lipases representing about 4% of the worldwide market. The three major industrial enzyme companies worldwide are Novozymes (>50%), Genencor (35%), and Solvay. Lipases are currently used, or have the potential for use, in a wide range of applications: in the dairy industry for cheese flavor enhancement, acceleration of cheese ripening, and lipolysis of butterfat and cream; in the oleochemical industry for hydrolysis, glycerolysis, and alcoholysis of fats and oils; and for the synthesis of structured triglycerides, surfactants, ingredients of personal care products, pharmaceuticals, agro-chemicals, and polymers [201,202]. Review of lipase applications has been enormous. Industrial applications of microbial lipases have been discussed by Andualema and Gessesse [203]; hydrolysis of glycerides by lipases has been reported by Nielsen [204]; the present and future applications of lipases have been reviewed by Macrae and Hammond [205]; applications of lipase-catalyzed hydrolysis have been listed by Iwai and Tsujisaka [206]; the importance of biotechnology in relation to the fats and oils industry has been reviewed by Macrae [369]; various aspects of enzymes that are useful in the lipid industry including sources, properties, reaction catalysis, some applications, and engineering aspects have been discussed by Yamane [12]; and some applications of lipase in industry have been discussed by Ferreira-Dias [207]. Many of these processes have been patented extensively since the early 1900s. Lai et al. [208] reviewed the patent literature on lipid technology.

The Colgate–Emery process, currently used in the steam fat-splitting of TAGs, requires 240°C–260°C and 700 psi, has energy costs, and results in an impure product requiring redistillation to remove impurities and degradation products. In addition, this process is not suitable for highly unsaturated TAGs [209]. Lipase-catalyzed reactions offer several benefits over chemical

reactions, including stereospecificity, milder reaction conditions (room temperature and atmospheric pressure), cleaner products, and reduced waste materials [210–212].

The largest current use of industrial enzymes is in laundry detergents, where they combine environmental friendliness and biodegradability with a low energy requirement and efficiency at low concentrations. The current U.S. market share of enzymatic laundry detergents is approaching 80%, and the U.S. detergent enzyme market is about $140 million. Essentially four types of enzymes are used in detergents: proteases, amylases, lipases, and cellulases. These enzymes perform multiple functions (e.g., stain removal, antiredeposition, whiteness/brightness retention, and fabric softening). Proteases were the first and are the most widely used enzymes in detergent formulations. Lipases are relatively new introductions to detergents, where they attack oily and greasy soils and contribute to making the detergents particularly effective at lower wash temperatures. However, a current limitation is that most lipases are unstable in alkaline conditions in the presence of anionic surfactants used in laundry detergents [65]. However, some lipases may be relatively resistant to certain surfactants [213].

B. New Lipases/Modification of Known Lipases

Early studies with fungal lipases focused on the isolation and characterization of extracellular lipases from various species. Some of the thoroughly studied fungal lipases include those from *C.* (*cylindracea*) *rugosa*, *R. miehei*, *Penicillium camembertii*, *H. lanuginosa*, *C. antarctica* B, *R. delemar*, and *G. candidum*, all of which are commercially available. Many of these lipases have relatively high specific activities: 3485 U/mg for *M. miehei* lipase A [15] and 7638 U/mg for *R. delemar* [27] (see Table 33.1 for other examples). Nevertheless, the research and development concerning lipase application in the lipid industry is not as dynamic as those in the area of carbohydrate, protein, and amino acids. Currently, lipase technology is mainly restricted to those operations where the cost of the product is high, making the enzyme cost low in relation, that is, this technology is applicable to high-value fine chemicals. Apart from this, lipase employment may be attractive in processes that involve thermolabile substrates or products, such as phospholipids, or that may entail a number of side reactions, such as oxidation, racemization, and dehydration, and in processes, where high enantio-and/or regioselectivity is required. The latter is a problem frequently faced with the high temperatures and/or mineral acid catalysts, which are highly nonspecific with respect to the types of reactions catalyzed. Lipases that have received the most attention are mainly those having relatively high activities or certain properties that make them commercially attractive. Other than additional strains of known lipase producers, there seems to be no pattern among the fungi or yeasts from a taxonomic point of view that would direct future studies on where to find prolific lipase producers or lipases with specific properties.

Lipases have been modified using either chemical or molecular approaches to alter their properties and to identify structure–activity relationships. For example, lipases have been chemically modified with polyethylene glycol to render them more soluble in organic media. Recently, Kodera et al. [214] produced amphipathic chain-shaped and copolymer derivatives of lipases from *Pseudomonas fragi* or *P. cepacia* that were soluble in aqueous and hydrophobic media and exhibited catalytic activities for esterification and transesterification reactions, as well as for hydrolysis. The modified lipase showed preference for the R isomer of secondary alcohols in esterification reactions.

Molecular approaches have been used to increase the production of a lipase from the fungus, *R. delemar* [154]. The gene for this lipase codes for a preproenzyme that is posttranslationally modified to the mature enzyme. A cloned cDNA for the precursor polypeptide of the lipase [215] was altered by site-directed mutagenesis to produce fragments that code for the proenzyme and mature enzyme [154]. When inserted into *E. coli* BL21 (DE3), the quantities of lipase from a 1 L culture exceeded those obtained from the fungal culture by 100-fold. Other examples of gene modification of lipases are given in Section VI.D, while Table 33.7 [53,216–221] lists some of the reported properties of cloned lipase genes.

TABLE 33.7
Properties of Cloned Lipase Genes

Source	Host	Vectors	Recombinant Plasmid	Size of Insert (bp)	Reference
Pseudomonas sp. Strain KB700A	*E. coli* TG1	pUC18	KB-lip	1422	[216]
Pseudomonas sp. Strain B11-1	*E. coli* C600	pUC118	pPL2–1	924	[218]
P. fluorescens No. 33	*E. coli* JM109	pUC19	pSHL2	1434	[218]
P. fluorescens SIK W1	*E. coli* JM83	pUC19	pJH92	1600	[219]
Pseudomonas KWI-56	*E. coli* HB101	pUC19	pLP64	1092	[53]
S. haemolyticus L62	*E. coli* XL1 Blue	pBluescript II SK (+)	pSHL	2136	[220]
Bacillus stearothermophilus L1	*E. coli* RR1	pUC19	pLIP1	1254	[221]

C. Production Synthesis/Modification

There are many examples of uses for lipases in product synthesis/modification. One of the major areas of interest is in the use of lipase-catalyzed interesterification to improve the nutritional value, or alter the physical properties, of vegetable or fish oils. This is achieved, for example, by increasing the content of DHA or EPA of these oils. These long-chain ω-3 (n-3) FAs have been incorporated into several vegetable oils using a lipase from *M. miehei* [109,268], medium-chain triglycerides [222], cod liver oil [223], borage, and evening primrose oil [224]. Kahveci and Xu [225] have also used repeated lipase-catalyzed hydrolysis to separate and enrich DHA and EPA in salmon fish oil. The n-3 FA content of tuna oil [226,227], cod liver oil [228], and sardine oil [229] has also been increased by lipase-catalyzed interesterification and esterification. Another FA of interest is GLA and ALA, which is applicable in a wide range of clinical disorders. GLA has been enriched in evening primrose and borage oil by several fungal lipases [230]. Table 33.8 summarizes some of the work done on the production of PUFA-enriched acylglycerols by lipase-catalyzed esterification.

Other research involving synthesis/modification includes the synthesis of mono- and diglycerides [231–234] including regioisomerically pure products [235], synthesis of acetylated glucose [236], modification of phospholipids into biosurfactants [237], hydrolysis of phosphatidylcholine [238], and production of high-value specialty fats such as cocoa butter substitutes or hardened vegetable oils with butterfat properties [239,240]. The production of high-value fats takes advantage of the 1,3-specificity of lipases that could not be achieved by chemical synthesis [212]. Some recent examples of research involving synthesis/modification by lipases were given in Tables 33.3 and 33.9.

VIII. PATENTS ON PRODUCTION OF MICROBIAL LIPASES

High-yield production of microbial lipases to satisfy the rising global demand for lipase is a challenging task. Lipases account for 21% of the world industrial enzyme market [323]. Initial exploitation of enzyme lipase with different catalytic characteristics always involves screening techniques in which new strains of productive microorganisms were identified and isolated for lipase production with desired properties [324]. Rahman et al. [325] reported on the isolation of *Geobacillus* Strain T1 bacteria T1-DSM 17139, a novel microorganism from palm oil mill effluent (POME), which is capable of producing thermostable enzyme lipase. Screening and isolation technique is normally followed by the optimization of culture conditions in which the composition of the growth medium, cultivation conditions, pH, temperature, dissolved O_2 concentration, and the kind of carbon and nitrogen sources in order to develop a cost-effective process for the production of enzyme lipase [324,326]. Optimization is an important stage as optimum process conditions for microbial lipase production can be predicted rapidly and accurately in order to reduce the overall operating cost and processing time. Consequently, most of the patent literatures focused on the optimization of the culture

TABLE 33.8
Production of Polyunsaturated Fatty Acid-Enriched Acylglycerols Using Lipase-Catalyzed Esterification

Lipase	Fatty Acid Incorporated	Product	References
C. antarctica lipase B, *M. miehei*, *Pseudomonas cepacia*, *P. camembertii*	EPA, DHA	Production of EPA and DHA TAG	[297]
R. oryzae, *C. antarctica*	DHA from cod liver oil	Production of PUFA-enriched MAG, TAG	[298]
C. antarctica lipase B and *M. miehei*	EPA, DHA	Production of EPA and DHA TAG	[299]
C. antarctica lipase B	EPA, DHA	Production of PUFA-enriched LML TAG	[300]
C. viscosum, *M. miehei*, *Pseudomonas* sp., *C. rugosa*, *R. niveus*, *A. niger*, and *R. oryzae*	ω-3 Fatty acids	Production of acylglycerols containing ω-3 fatty acids	[301]
Novozym 435	*n*-3 PUFA from menhaden oil	Enrichment of *n*-3 PUFA in hazelnut oil	[302]
Pseudomonas sp.	EPA	EPA was found to be incorporated mainly in the *sn*-1,3 positions of the TAG molecules and suitable for applications where quick energy release and EPA supplementation are required	[303]
Novozym 435, Lipozyme RMIM, *Pseudomonas* sp., *A. niger*, *C. rugosa*	EPA and DHA	Highest DHA and EPA incorporation into high-laurate canola oil was 37.3% and 61.6%, respectively	[304]
M. miehei (Lipozyme RMIM)	DHA	Production of high DHA acylglycerols	[305]
Lipozyme TLIM	ARA and DHA	Production of human milkfat substitute with ARA and DHA attached in *sn*-1 and *sn*-3 position	[306]
R. oryzae	DHA from tuna oil	Production of DHA-enriched acylglycerol	[307]

conditions and have been summarized as in Table 33.10. Besides that, the bioreactor operations and process design are often investigated prior to scaling-up of the lipase production. Both submerged fermentation (SF) and solid-state fermentations (SSF) are employed for the production of microbial lipase in which the former is suitable for the cultivation of bacteria and yeast, whereas the latter is used to cultivate fungal species [324,327–329]. Different bioreactor configurations are also investigated and employed in the last decade for the production of enzyme lipase and have been reviewed by Treichel et al. [370]. For instance, Voit and Mersmann [330] patented the application of centrifugal field bioreactor to produce microbial lipases from *Staphylococcus carnosus* strain (pLipPS1). According to this invention, higher lipase yield and substrate yield can be achieved with reduced residence time and reactor volume, thereby reducing the overall investment and operating costs.

Nevertheless, original microbial lipases produced normally have limited applications due to low yields, low specific activity, and stability, as well as limited range of activities. Several technical methods have therefore been proposed to improve the strain in order to enhance yield and stability. The simplest strategy of improving the microbial strain to enhance the lipase production yield is by conventional physical and chemical mutagenesis methods such as UV-irradiation, γ-ray, fast neutron irradiation, neodymium-doped yttrium aluminium garnet (Nd:YAG) laser, nitrosoguanidine (NTG), diethyl sulfate, and nitrous acid and these techniques have been reviewed by Turki [327] and Shu et al. [331]. Wu et al. [332] disclosed the use of ultraviolet (UV) irradiation with lithium chloride as mutant conditions to obtain mutant *A. niger* strain S-7749, which is capable of producing enzyme

TABLE 33.9
Lipase-Catalyzed Production of Structured Lipids

Lipase	Substrate	Product	References
Lipozyme IM	Canola oil and caprylic acid	Products containing ≈40% caprylic acid with 7.9% caprylic acid in *sn*-2 position	[308]
Lipozyme IM	Menhaden oil	Products containing 40% caprylic acid and 30% EPA and DHA	[309]
Lipozyme IM	Canola oil and caprylic acid (acidolysis); Lipozyme IM-hydrolyzed canola oil and caprylic acid (esterification)	59.9% New TAG with acidolysis and 82.8% new TAG with esterification	[310]
Lipozyme IM	MCT (cinnamomum camphora seed) and oleic acid	59.68% oleic acid-incorporated in MCT	[311]
Lipozyme IM	Sunflower, safflower, borage, linseed oils, and capric and caprylic acids	35%–47% incorporation, 40% dimedium-chain fatty acid incorporated TAG	[312]
R. arrhizus immobilized on Celite	Palm oil mid fraction, palmitic and stearic acid	Production of POP 30.7%, POS 40.1%, POO 9%, SOS 14.5%, SOO 5.7%	[239]
Carica papaya lipase	Tripalmitin + alkyl esters of caprylic acid	Transesterification with *n*-butyl and *n*-propyl was faster than when ethyl and methyl caprylates were used	[313]
R. miehei	Palm stearin and palm kernel olein	Margarine prepared from the transesterified blends had acceptable PV levels although slight posthardening was observed after 3 months storage	[314]
Lipozyme IM 60	Palm stearin and palm kernel olein	Production of low melting TAG caused a sharp drop in SFC which shifted from the range of 15°C–20°C to 10°C–15°C	[315]
R. miehei, *A. niger*, *Rhizopus javanicus*, *R. niveus*, *Alcaligenes* sp., *C. rugosa*, and *Pseudomonas* sp.	Palm stearin + anhydrous milk fat	Highest degree and rate of transesterification was obtained when *Pseudomonas* sp. lipase was used followed by *R. miehei* lipase	[316]
Lipozyme IM 20	Glycerol and triolein	Monoolein synthesis enhanced from 10.6 mol% in *n*-hexane to 64 mol% in 2-methyl-2-butanol	[317]
Mixtures of *R. miehei* and *Alcaligenes* sp.; mixtures of *Pseudomonas camembertii* and *Alcaligenes* sp.	Glycerol and conjugated linoleic acid (CLA)	TAG containing CLA reached 82%–83% after 47 h using 1 wt% lipases	[318]
Lipozyme TLIM, Lipozyme RMIM, Novozym 435	Menhaden oil and pinolenic acid	Incorporation of pinolenic acid was 19.4 mol% for Novozym 435, 16.1, and 13.6 mol% for Lipozyme TLIM and Lipozyme RMIM, respectively	[319]
Lipozyme RMIM	Stearic acid and blends of palm olein and palm kernel oil	An incorporation of 42% stearic acid into blends of palm olein and palm kernel oil can be achieved	[320]
Malassezia globosa/*P. camembertii*	CLA and glycerol	A high degree of esterification (54.3%) was achieved for DAG production	[321]
Lipozyme TLIM and Novozym 435	Phosphatidylcholine (PC), palmitic and stearic acids	58.6% and 57.1% palmitic acid was incorporated using Lipozyme TLIM and 56% and 61% using Novozym 435 in egg and soybean PC from an initial content of 37.4% and 16.8%, respectively. Stearic acid incorporation was 44.7% and 46.3% with Lipozyme TLIM and 37.2% and 55.8% using Novozym 435	[322]

TABLE 33.10
Production of Microbial Lipases Patents

Patent No.	Microorganism Species	Optimum Fermentation Conditions					Optimum Lipase Activity		Description	References
		Time (h)	Temp (°C)	pH	Agitation	Aeration	Temp	pH		
CN104726427A	*Fusarium oxysporum*	96	30	6.5	180	—	—	—	Optimization of culture medium for cultivation of *F. oxysporum*	[341]
CN101210235B	*Aspergillus carneus*	96	28	8	—	—	30	10	Lipase having thermal stability and pH stability. Low temperature lipase for cold washing detergent	[342]
CN103571808A	Wild bacteria strain	35	30	7	350	1.5 L/min	30	—	Low temperature lipase	[343]
CN102994472A	*Metarrhizium anisopliae*	72	25	6	150	—	—	—	Culture media for *M. anisopliae*	[344]
US2888385	*Aspergillus oryzae*	60–70	28–30	—	—	Yes	—	—	An improved process to produce ferment mixture rich in lipases and oxidases for used in food products	[345]
US3262863A	*R. delemar*	72	28	5–6	Yes				Stable lipase with reduced contamination of protease	[346]
US3513073A	*R. arrhizus* var. *delemar*	20–35		5–7	Yes	Yes	37	3.57	Lipase has two optima pH which is 3.5 similar to stomach pH and 7 similar to intestinal pH. Used to treat gastrointestinal disturbances	[347]

(*Continued*)

TABLE 33.10 (*Continued*)
Production of Microbial Lipases Patents

Patent No.	Microorganism Species	Optimum Fermentation Conditions					Optimum Lipase Activity		Description	References
		Time (h)	Temp (°C)	pH	Agitation	Aeration	Temp	pH		
US3649455A	*C. viscosum* var. *paralipolyticum*	96	26 (24–26)	6.5	300	Yes	50	6–7	Process for production of lipase from *C. viscosum paralipolyticum*	[348]
US4019959A	*Pseudomonas* sp. NRRL B-8110	68	30	6.5	600	0.2vvm	50	9	Production of lipase using hydrocarbon substrate *n*-paraffins	[349]
US4665029A	*Rhizopus chinensis* FER BP-936	96	27	6	—	0.5–1 L/min	40–60	6.5–7	Thermostable 1,3-specific lipase	[350]
US5827718	*Pseudomonas* strain FERM BP-4772	24	35	7–10	1000	Yes	55–65	10–11	Detergent stable lipase for used in detergent	[351]
US5063160A	*Pseudomonas alcaligens* SD2, ATCC 53877	24	30	—	150	Yes	50	10	Surfactant and pH stable lipase	[352]
US4999289A	*Penicillium cyclopium* (ATCC 34613)	40	25	—	—	—	35–40	5–7	Lipase for hydrolyzing triglyceride in serum	[353]
US5240851A	*P. fluorescens* IAM 1057	7 h (batch) 72 h (continuous)	27	6–7	500	1% saturation	60 (titrimetric) 47 (colorimetric	6–8	Thermostable and pH stable lipase for used in detergent	[354]
US5079158A	*B. stearothermophilus* H-165	25	50	—	Yes		75	5	Thermostable and pH stable lipase hydrolyzing specifically on monoglyceride	[355]
US3634195A	*Absidia butleri*	144	32–37	4.8–8.2	228 strokes/min (2 in. stroke)	Yes	—	—	Lipase with preferences to LCFA than SCFA	[356]

(*Continued*)

TABLE 33.10 (*Continued*)
Production of Microbial Lipases Patents

Patent No.	Microorganism Species	Optimum Fermentation Conditions					Optimum Lipase Activity		Description	References
		Time (h)	Temp (°C)	pH	Agitation	Aeration	Temp	pH		
US3619372A	*Torulopris ernobii* ATCC No. 20000	24	33	5.2	500	15 L/min	45	6.5	Acid-resistant lipase	[357]
WO2011081513 A2	*Burkholderia* sp.	24	30	9–11	200–240	—	—	—	Culture medium comprise of fermentation media and a composition for aqueous twophase system (ATPS) for simultaneous cultivation and purification purposes	[358]
WO2013109136A1	*Candida cylindracea*	50	30	6	500	1vvm	—	—	Method of producing lipase with palm oil as substrate	[359]
EP0204284A2	*P. fragi* 22.39B (FERM BP-1051)	—	—	—	—		65–70	9	Lipase with high activity at alkaline condition of pH 9 and active for decomposition of low molecular weight monoester	[360]
EP0442558A1	*G. candidum*	24–32	30	6.4	400	0.32vvm (0.16vvm air flow)	—	—	Two types of Lipase A (nonspecific) and Lipase B (specific for 9-*cis* fatty acid)	[361]

(*Continued*)

TABLE 33.10 (*Continued*)
Production of Microbial Lipases Patents

Patent No.	Microorganism Species	Optimum Fermentation Conditions					Optimum Lipase Activity		Description	References
		Time (h)	Temp (°C)	pH	Agitation	Aeration	Temp	pH		
EP0872548A1	*Pseudomonas solanacearum*	24	30	—	1000	—	85–100, 80–90 (absence of Ca ion)	6.5–9.6	Thermostable lipase	[362]
EP2450458A2	*Geobacillus* sp. strain ARM	24	52.3	5.8	Static	—	65	8	Thermostable lipase	[363]
	Aneurinibacillus thermoaerophilus strain AFNA	48	52.7	6.9	103					
EP0384717A1	*Bacillus* sp. A30-1 ATCC No. 53841		60–65	7–9	600	0.6 mL/min	60	9.6	Thermostable and pH stable lipase	[364]
EP0385401A1	*Pseudomonas* sp. ATCC 53848, 53849, 53850, 53851, 53853, 53854	—	—	—	—	—	40–60	9–10.5	Lipase surviving in conditions of 3,4-dichlorobenzoate	[365]

lipase with wider optimal pH range and higher pH stability from original microbial strain (*A. niger* s-8341). Another similar invention was reported by Oester et al. [333] wherein a mutant species of *G. candidum* NRRL Y-552 was obtained under UV irradiation for 90 s and the lipase secreted has high selectivity toward oleic acid.

The recent rapid development in genetic engineering has given a new impetus to microbial strain improvement for the high-yield production of enzyme lipase [327]. Aoyama and Yoshida [334] reported on the production of microbial lipase originated from *P. cepacia* IFO 14595 strain using recombinant DNA using *E. coli* as host genus bacterium. The advantage of this invention lies in its production of enzyme lipase with high utilization value and possibility of recovering the lipase from the cultured filtrate at the time of commercial production. Due to low production yield and the need for high temperature fermentation equipment for thermophiles to produce thermostable lipase, Rahman et al. [325] also described the use of recombinant DNA technology by subcloning the thermostable T1 lipase gene from *Geobacillus* bacterium into the *E. coli* as host strain to produce the thermostable T1 lipase. A similar approach for the production of enzyme lipase with improved thermostability and stability in the presence of alkaline proteases involving cultivation of *Aspergillus* sp. host with DNA sequence encoding the *Humicola* sp. lipase has been invented by Boel and Huge-Jensen [335].

Previous literature research also revealed that combined chemical and genetic mutagenesis approach is a promising strategy in obtaining relative high production yield as in the strain derived from LIP2 gene amplification in *Y. lipolytica* mutant named LgX64.81 mutant [336]. Overproduction of microbial lipases via heterologous expression in bacterial or yeast host especially in *Aspergillus* system is well explored in industry [337]. As an example, Novozymes A/s Co. describes the commercial production of lipase from *Thermomyces lanuginosus* and *C. antarctica* using *Aspergillus oryzae* expression system [338]. Despite dozens of commercial lipases available in the market, the search for novel microbial lipases with tailored properties continues, and it has led to the development of molecular biology techniques in which classical error-prone polymerase chain reaction (ep-PCR) was the conventional technique employed to improve the properties of lipases [307]. A lipase mutant with enhanced catalytic activity originated from *R. chinensis* CCTCC No. M201021 by changing the amino acid sequences of the enzyme lipase through ep-PCR method was invented by Yu et al. [339]. In the following year, another similar technique was employed by Yu et al. [340] to obtain lipase mutant with greater thermal resistance from *R. chinensis* CCTCC No. M201021.

IX. SUMMARY

Lipases are exceedingly interesting enzymes because the relationship between their structure and activity presents an intellectual challenge and because their versatility offers a broad range of possible industrial applications. However, interest in the lipases has begun to move from academic curiosity to full commercialization in terms of the availability of lipases and their industrial use. For example, 50 t/year of the chiral intermediate methyl methoxyphenyl glycidate is produced based on a lipase-catalyzed process [166].

Although lipases have high potential for a variety of industrial applications, their use at the present time is limited by several factors, such as lack of cost-effective systems or processes for producing sufficient enzyme, heterogeneity of available preparations, and absence of lipases with properties required for certain applications [154]. As with proteases, protein engineering can be applied to lipases to target numerous specific characteristics. Alteration of amino acid sequences will result in variants with modified specific activity, increased k_{cat}, altered pH and thermal activity profiles, increased stability (with respect to temperature, pH, and chemical agents such as oxidants and proteases), and show altered pI, surface hydrophobicity, and substrate specificity [366,367]. Currently, lipase genes from fungal sources (e.g., *G. candidum* and *C. rugosa*) are cloned and subjected to site-directed mutation to gain insight into structure–activity relationships, mainly with respect to selectivity, on which to base protein engineering strategies. Despite the enormous progress that has been made in this regard, the molecular basis for selectivity is still not well understood.

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34 Enzymatic Interesterification

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CONTENTS

I. INTRODUCTION

The development of methods to improve the nutritional and functional properties of fats and oils is of great interest to food processors. The molecular weight, unsaturation, and positional distribution of fatty acid residues on the glycerol backbone of triacylglycerols (TAGs) are the principal factors determining the physical properties of fats and oils [1,2]. Chemical interesterification produces a complete positional randomization of acyl groups in TAGs. It is used in the manufacture of shortenings, margarines, and spreads to improve their textural properties, modify melting behavior, and enhance stability [3,4]. Interest in interesterification from a nutritional and functional standpoint is increasing since it can be used to produce margarines with no *trans* unsaturated fatty acids, synthesize cocoa butter substitutes, and improve the nutritional quality of some fats and oils [5]. Recently, research efforts have been directed to substituting some chemical interesterification applications with enzymatic interesterification because of the inherent advantages associated with its enzymatic process. Enzymatic reactions are more specific, require less severe reaction conditions, and produce less waste. In addition, when immobilized, enzymes can be reused, thereby making them economically attractive [6]. Interesterification, whether chemical or enzymatic, is the exchange of acyl groups between an ester and an acid (acidolysis), an ester and an alcohol (alcoholysis), and an ester and an ester (transesterification) [7].

The major components of fats and oils are TAGs, the composition of which is specific to the origin of each fat or oil. The physical properties of various fats and oils are different because of the structure and distribution of fatty acids in the TAGs [8]. In natural fats, acyl groups are distributed in a nonrandom fashion. During chemical or enzymatic interesterification, acyl groups are redistributed first intramolecularly and then intermolecularly until a random distribution is achieved. With enzymatic interesterification, more control of final product composition is possible, and glyceride mixtures that cannot be obtained using chemical interesterification can be produced [9,10]. At present, randomization of acyl group distribution using chemical interesterification is used to produce changes in crystal structure, solid fat content (SFC), and melting point of fats. Interesterification using lipase with particular specificities is used to produce high-value specialty fats, such as cocoa butter substitutes and confectionary fats [5].

Enzymatic interesterification is accomplished using lipases, which are enzymes obtained predominantly from bacterial, yeast, and fungal sources. Extracellular microbial lipases are produced by microorganisms and released into their growth environment to digest lipid materials [9]. Lipases are defined as glycerol ester hydrolases (EC 3.1.1.3) because they catalyze the hydrolysis of carboxyl ester bonds in acylglycerols. Depending on the degree of hydrolysis, free fatty acids, monoacylglycerols, diacylglycerols (DAGs), and glycerol are produced. Lipases are differentiated from esterases in that they act only on insoluble substrates. Long-chain TAGs, the natural substrates of lipases, are insoluble in water, forming aggregates or dispersions in aqueous media. Lipases have a high affinity for hydrophobic surfaces and can be completely adsorbed from aqueous solution by emulsified long-chain TAGs [11]. In the presence of excess water, lipases catalyze the hydrolysis of long-chain TAGs, but under water-limiting conditions, the reverse reaction, ester synthesis, can be achieved [8,12]. Enzymatic interesterification systems are composed of a continuous water-immiscible phase, containing the lipid substrate, and an aqueous phase containing the lipase. Lipase-catalyzed interesterifications have been extensively studied in systems using organic solvents. However, if such a process is to be used in the food industry, solvent-free systems must be developed. Hence, the emphasis of this chapter will be on enzymatic interesterification performed in solvent-free systems.

A. Transesterification

As previously defined, transesterification is the exchange of acyl groups between two esters, namely, two triacylglycerols (Figure 34.1). Transesterification is used predominantly to alter the physical properties of individual fats and oils or fat–oil blends by altering the positional distribution of fatty acids in the TAGs. Transesterification of butter using a nonspecific lipase has been reported to improve the plasticity of the fat [13]. Kalo et al. [14] found that transesterification of butterfat with a positionally nonspecific lipase at 40°C increased the level of saturated C48–C54 TAGs, monoene C38 and C46–C52 TAGs, and diene C40–C54 TAGs. These authors also found that the DAG content increased by 45% whereas the free fatty acid content doubled. Overall, lipase-catalyzed transesterification of butterfat at 40°C produced an increase in the SFC below 15°C and a decrease in the SFC above 15°C (Figure 34.2).

In another study, lipase-catalyzed transesterification of butter increased the relative proportion of C36 and C40–C48 saturated TAGs, as well as triunsaturated TAGs [15]. The resulting product had a 114% greater SFC at 20°C than the starting butter, with the SFC increasing from 22% to 46%. In general, lipase-catalyzed transesterification produces fat with a slightly lower SFC compared with chemical interesterification. This is attributed to contamination by monoacylglycerols, DAGs, and free fatty acids, which are produced in the early stages of transesterification [8,13]. Kalo et al. [13] compared lipase-catalyzed transesterification with chemical interesterification of butter. They found that the SFC of butter increased from 41.2% to 42.2% at 20°C using lipase-catalyzed transesterification, whereas chemical interesterification produced butter with a SFC of 57.8% at 20°C. Transesterification has also been used to improve the textural properties of tallow and rapeseed oil mixtures as well as in the development of cocoa butter equivalents [16,17]. Forssell et al. [18] found that transesterification of tallow and rapeseed oil blend decreased the SFC and melting point. The extent of melting point reduction was dependent on the mass fraction of the two lipid components.

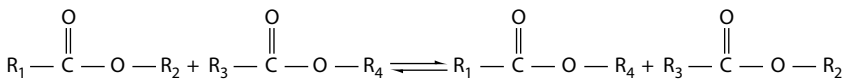

FIGURE 34.1 Lipase-catalyzed transesterification between two different triacylglycerols.

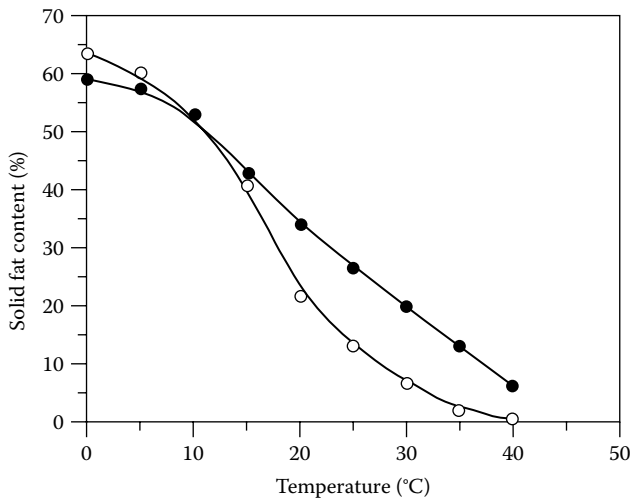

FIGURE 34.2 SFC versus temperature profiles for native and enzymatically interesterified butterfat in the absence of solvent using lipase from *P. fluorescens*. Nontransesterified butterfat (o); transesterified butterfat (●). (From Kalo, P. et al., *Milchwissenschaft*, 41(2), 82, 1986. With permission.)

With a mass fraction of tallow to rapeseed oil of 0.8, the melting point was reduced by 6°C, whereas a mass fraction of 0.5 produced a 12°C decrease in melting point. A decrease in the SFC has also been observed on transesterification between palm oil and canola oil, due to a decrease in the level of triunsaturated TAGs [19].

The attractiveness of cocoa butter to the chocolate and confectionary industry is based on the limited diversity of TAGs in this fat, which gives it a unique, narrow melting range of 29°C–34°C. Chocolate can contain 30% cocoa butter, meaning that this fat determines the crystallization and melting properties of the chocolate. At 26°C, cocoa butter is hard and brittle, but when eaten it melts completely in the mouth with a smooth, cool sensation. The major TAGs in cocoa butter are 1-palmitoyl-2-oleoyl-3-stearoylglycerol (POS), 1,3-dipalmitoyl-2-oleoylglycerol (POP), and 1,3-distearoyl-2-oleoylglycerol (SOS) with levels of 41%–52%, 16%, and 18%–27%, respectively [8,17]. The main disadvantage of using cocoa butter in chocolate and confections is its high cost. A cocoa butter equivalent can be made from inexpensive fats and oils by interesterification. By transesterifying fully hydrogenated cottonseed and olive oil, Chang et al. [17] were able to produce a cocoa butter substitute with similar POS levels and slightly higher SOS levels than those found in cocoa butter. The melting range of the transesterified product was 29°C–49°C, compared with 29°C–34°C for cocoa butter. In order to remove the desired TAG product from the other TAGs, trisaturated TAGs were removed by crystallization in acetone. High-oleic sunflower oil (HOSFO) and palm oil fraction have also been transesterified to obtain cocoa butter equivalents [5].

B. Acidolysis

Acidolysis, the transfer of an acyl group between an acid and an ester, is an effective means of incorporating novel-free fatty acids into TAGs (Figure 34.3). Acidolysis has been used to incorporate free fatty acid or ethyl ester forms of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into vegetable and fish oils to improve their nutritional properties. The nutritional benefits of consuming polyunsaturated fatty acids (PUFAs), such as EPA and DHA, derived from fish oils have been proven. When consumed, EPA reduces the risk of cardiovascular disease by reducing the tendency to form blood clots, whereas DHA consumption is required for proper nervous system and visual functions, due to its accumulation in the brain and retina [20,21]. Concentrations of EPA and DHA in fish oils to levels approaching 30% can be achieved using molecular distillation, winterization, and solvent crystallization. However, performing an acidolysis reaction between cod liver oil and free EPA and DHA, Yamane et al. [22] were able to increase the EPA content in the oil from 8.6% to 25% and the DHA content from 12.7% to 40% using immobilized lipase from *Mucor miehei*. Using ethyl esters of EPA, fish oil has been enriched by interesterification to contain 40% EPA and 25% DHA (wt%) [23]. During acidolysis in a fixed bed reactor, Yamane et al. [24] increased the PUFA content of cod liver oil by reducing the temperature to between −10°C and −20°C in the product reservoir. This led to crystallization and removal of more saturated fatty acids present in the fish oil. Lipases with strong specificities against EPA or DHA have also been used to enrich their content in fish oils [25]. Future development in lipase-catalyzed interesterification using EPA and DHA is directed to improving the nutritional quality of vegetable oils by enrichment with these fish oil-derived fatty acids. Acidolysis has also been used by Oba and Witholt [26] to incorporate oleic acid into milk fat. This process led to an increase in the level of unsaturated fatty acids in butter without losses in the characteristic flavor of butter. Acidolysis of milk fat with oleic acid was also found to decrease the crystallization temperature and lower the melting range of the milk lipids.

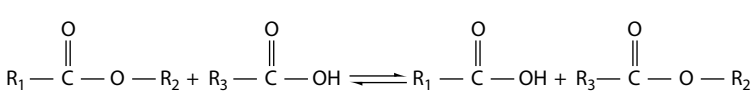

FIGURE 34.3 Lipase-catalyzed acidolysis reaction between an acylglycerol and a fatty acid molecule.

Along with the enrichment of oils, acidolysis using EPA and DHA has also been useful in the synthesis of structured lipids. Structured lipids are composed of medium- and long-chain fatty acids, which meet the nutritional needs of hospital patients and those with special dietary needs. When consumed, medium-chain fatty acids (MCFAs), such as capric and caproic acids, are not incorporated into chylomicrons and are therefore not likely to be stored but will be used for energy. They are readily oxidized in the liver and constitute a highly concentrated source of energy for premature babies and patients with fat malabsorption disease. MCFAs also possess a nutritional advantage compared with other fatty acids in that they are nontumor-producing forms of fat [27]. Long-chain fatty acids are also required by the body, especially in the form of PUFAs in the form of ω-3 and ω-6 fatty acids, which have been associated with reduced risk of platelet aggregation and cardiovascular disease, and the lowering of cholesterol [27,28]. When PUFAs are present in the *sn*-2 position and MCFAs are present in the *sn*-1,3 positions, they are rapidly hydrolyzed by pancreatic lipase, absorbed, and oxidized for energy, whereas essential PUFAs are absorbed as 2-monoacyglycerols. Therefore, structuring TAGs with MCFAs and PUFAs can dramatically improve the nutritional properties of TAGs [29]. Producing a TAG rich in EPA or DHA at the *sn*-2 position, with MCFAs in the *sn*-1 or *sn*-3 positions, would provide maximal benefit, especially for intravenous use in hospitals [30]. Structured lipids that are reduced in caloric content have also been developed by esterifying long-chain monoacylglycerols containing behenic acid with capric acid. The produced TAGs contain half the calories relative to natural TAGs due to the incomplete absorption of behenic acid during digestion [31].

Acidolysis is also a common method for production of cocoa butter substitutes. The most common method is acidolysis of palm oil mid fraction, which contains predominantly POP with stearic acid to increase the level of POS [32]. Chong et al. [33] also incorporated stearic acid into palmolein to produce 25% cocoa butter-like TAGs.

C. Alcoholysis

As previously mentioned, alcoholysis is the esterification reaction between an alcohol and an ester (Figure 34.4). Alcoholysis has been used in the production of methyl esters from esterification of TAGs and methanol with yields of up to 53% [34]. During alcoholysis, hydrolysis of TAGs to produce DAGs and monoacylglycerols can occur, in some cases reaching levels as high as 11%, although the presence of small amounts of alcohol can inhibit hydrolysis. The main use of alcoholysis is in the performance of glycerolysis reactions.

Glycerolysis is the exchange of acyl groups between glycerol and a TAG to produce monoacylglycerols, DAGs, and TAGs. There are several ways to produce monoacylglycerols, which are of great importance in the food industry as surface-active agents and emulsifiers. Monoacylglycerols can be produced by ester exchange between TAGs and glycerols, or by free fatty acids and glycerol, although only the former reaction is termed glycerolysis (Figure 34.5). Glycerolysis is usually performed using nonspecific lipases, giving a wide range of reaction products (Figure 34.6).

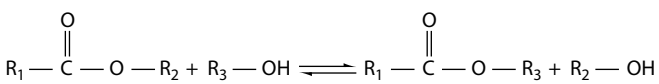

FIGURE 34.4 Lipase-catalyzed alcoholysis reaction between an acylglycerol and an alcohol.

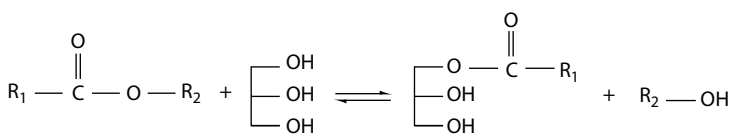

FIGURE 34.5 Lipase-catalyzed glycerolysis reaction between glycerol and a triacylglycerol to produce monoacylglycerols.

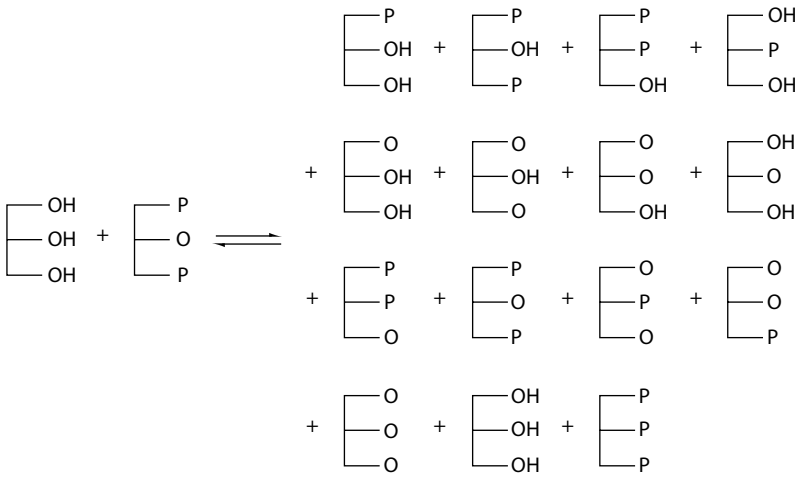

FIGURE 34.6 Products of a nonspecific lipase-catalyzed glycerolysis reaction between glycerol and 1,3-dipalmitoyl-2-oleoylglycerol.

High yields in lipase-catalyzed monoacylglycerol synthesis are achieved by temperature-induced crystallization of newly formed monoacylglycerols from the reaction mixture. This pushes the equilibrium of the reaction toward increased monoacylglycerol production. During glycerolysis, lipids containing saturated monoacylglycerols in the reaction product mixture crystallize at lower temperatures than unsaturated monoacylglycerols [35]. *Pseudomonas fluorescens* and *Chromobacterium viscosum* have been shown to have high glycerolysis activity [36]. In glycerolysis reactions, T_c is defined as the critical temperature below which monoacylglycerols formed by glycerolysis crystallize out of the reaction mixture. Removal of monoacylglycerols from the reaction mixture pushes the equilibrium of the reaction toward increased monoacylglycerol production. Vegetable oils with low melting points due to the presence of long-chain unsaturated fatty acids have a much lower T_c than animal fats. The T_c for vegetable oils ranges from 5°C to 10°C, whereas it is between 30°C and 46°C for animal fats. By reducing the temperature below T_c, yields of monoacylglycerols can be increased from 30% up to yields as high as 90% [35,36]. Water content can have an effect on glycerolysis since the reaction is an esterification. McNeill et al. [36] found that increasing the water content from 0.5% to 5.7% increased the production of monoacylglycerols, whereas higher levels of water did not increase the rate of reaction further. The main problem with lipase-catalyzed glycerolysis is the long reaction time in the order of 4–5 days required to produce high yields [36].

II. LIPASES

A. Three-Dimensional Structure

While lipases can be derived from animal, bacterial, and fungal sources, they all tend to have similar three-dimensional structures. In the period from 1990 to 1995, crystallographers solved the high-resolution structures of 11 different lipases and esterases including 4 fungal lipases, 1 bacterial lipase, and human pancreatic lipase [12]. Comparison of the amino acid sequences has shown large differences between most lipases, yet all have been found to fold in similar ways and have similar catalytic sites. The characteristic patterns found in all lipases studied so far have included α/β-structures with a mixed central β-sheet containing the catalytic residues. In general, a lipase is a polypeptide chain folded into two domains: the C-terminal domain and the N-terminal domain. The N-terminal domain contains the active site with a hydrophobic tunnel from the catalytic serine to the surface that can accommodate a long fatty acid chain.

In solution, a helical segment covers the active site of lipase, but in the presence of lipids or organic solvent, there is a conformational change in which the lid opens, exposing the hydrophobic core containing the active site. The structure of the lid differs for lipases in the number and position of the surface loops. For example, human pancreatic lipase has one α-helix (residues 237–261) in the loop covering the active site pocket [37,38]. The fact that the α-helix in the lid is amphipathic is very important in terms of the ability of the lipase to bind to lipid at the interface. If the amphiphilic properties of the loop are reduced, the activity of the enzyme is decreased [39]. The outside of the loop is relatively hydrophilic, whereas the side facing the catalytic site is hydrophobic. On association with the interface, the lid folds back, revealing its hydrophobic side that leads to increased interactions with the lipid at the interface [40]. The substrate can then enter the hydrophobic tunnel containing the active site.

B. Active Site

Koshland's modern induced fit hypothesis states that the active site does not have to be a preexisting rigid cavity, but instead can be a precise spatial arrangement of several amino acid residues that are held in the correct orientation by the other amino acids in the enzyme molecule [41]. The main component of the catalytic site is an α/β-hydrolase fold that contains a core of predominantly parallel β-sheets surrounded by α-helices. The folding determines the positioning of the catalytic triad composed of serine, histidine, and either glutamic acid or aspartic acid along with several oxyanion-stabilizing residues. The nucleophilic serine rests between a β-strand and an α-helix, whereas histidine and aspartic acid or glutamic acid rest on one side of the serine [12].

The importance of the serine residue for the catalytic activity of lipase has been demonstrated using site-directed mutagenesis. Substitution of Ser153 in human pancreatic lipase produces a drastic decrease in the catalytic activity of the enzyme but has no effect on the ability of the enzyme to bind to micelles. As well, the presence of a highly hydrophobic sequence of amino acid residues has been verified in the vicinity of the active site, which is important in the interaction of the enzyme with the interface [42]. The chemical properties of the groups within the catalytic triad are consistent with a hydrophobic environment [11]. The process of opening the lid covering the active site causes the oxyanion hole to move into proper positioning for interaction with the substrate. For example, lipase from *Rhizomucor miehei* has a serine side chain at position 82 that assumes a favorable conformation for oxyanion interactions only after the lid has moved away from the active site [43]. During binding of the substrate with the enzyme, an ester binds in the active site, so that the alcohol portion of the substrate rests on a floor formed by the end of the β-strand, while the acyl chain arranges itself in the hydrophobic pocket and tunnel region [42] (Figure 34.7).

In the lipase from *M. miehei*, the substrate-binding region is seven-carbon long. When longer chains are encountered, the rest of the carbons in the chain hang outside the hydrophobic tunnel [44]. When the lipase approaches the interface and the lid is folded back, an oxyanion-stabilizing residue is placed in proper orientation [12]. During hydrolysis, the tetrahedral intermediate is stabilized by hydrogen bonds with backbone amide groups of oxyanion-stabilizing residues. One stabilizing residue in the oxyanion hole is the amino acid following the catalytic serine, whereas the other one comes from a separate loop [37].

C. Activation by Interfaces

As previously stated, an advantage of enzyme-catalyzed interesterification in comparison with chemical methods is that it can operate effectively under relatively mild conditions. Enzyme-catalyzed reactions can increase the rate of a reaction by 10^6–10^{15} times even at 25°C [41]. The kinetics of lipase-catalyzed interesterification can get complicated due to many factors that can affect the reaction. Activation by interfaces as well as participation of multiple substrates in the interesterification reaction must all be considered when describing the action of lipases at interfaces.

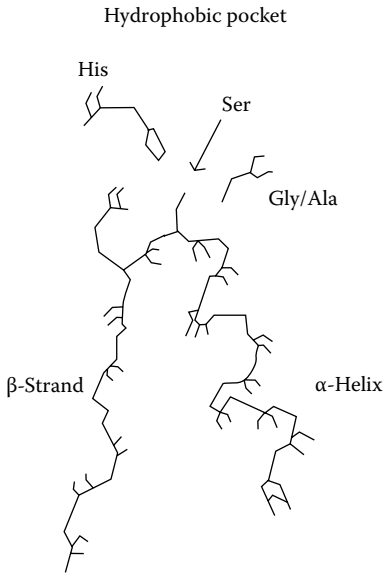

FIGURE 34.7 Crystal structure and location of catalytic residues of the active site of *C. rugosa* lipase. (Adapted from Kaslauskas, A.J., *Trends Biotechnol*., 12, 464, 1994.)

The natural substrates of lipases, long-chain TAGs, are uncharged and insoluble in water and as such form two phases in aqueous solutions. The property of being active at lipid–water interfaces is unique to lipases. At low concentrations of lipids, termed *monomeric solutions*, the lipids are dissolved in aqueous phase. The maximal concentration of monomers in aqueous solution is the solubility limit or critical micelle concentration, after which TAGs form emulsions. For example, the critical micelle concentration for triacetin in aqueous solution is 0.33 M, whereas for long-chain TAGs, it can be as low as 1.0 mM [12,45]. It has been shown that lipases display almost no activity toward monomeric solutions of lipids, whereas the lipids are dissolved and do not form interfaces. Once the level of lipids exceeds the critical micellar concentration, the reaction rate increases dramatically by a factor of 10^3–10^4 in some cases, depending on the quality of the interface (Figure 34.8).

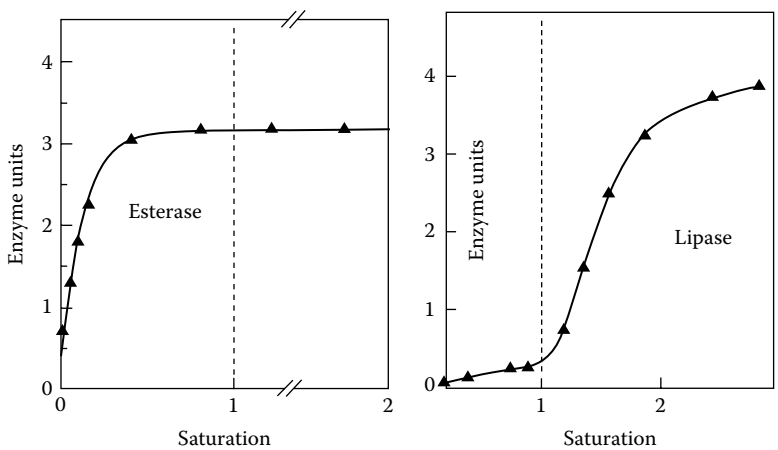

FIGURE 34.8 Comparison of the effect of substrate concentration on lipase and esterase activity at monomeric and saturation levels (beyond vertical dashed lines). (From Sarda, L. and Desnuelle, P., *Biochim. Biophys. Acta*, 30, 513, 1958. With permission.)

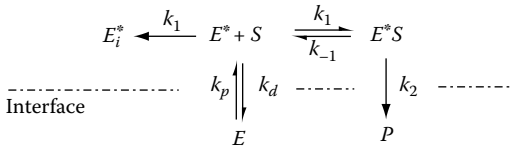

FIGURE 34.9 Model for activation and action of lipases at interfaces.

Lipases have been found to act at several interfaces, including emulsions, bilayers, and micelles [46]. Action of lipases at the lipid–water interface is believed to follow two successive equilibria involving penetration of lipase into the interface, followed by the formation of the enzyme–substrate complex (Figure 34.9).

Initially, the enzyme penetrates the interface and undergoes a conformational change, folding back the lid and thereby increasing the hydrophobic surface area of the lipase making contact with the interface. The enzyme adsorbs to the interface following a Langmuir adsorption isotherm. Once adsorption has taken place, the enzyme is in its catalytically active form, meaning that interfacial activation has taken place. The lipid substrate can then fit into the active site and be transformed into product. The product is believed to be water soluble and leave the interface rapidly by diffusion into the surrounding solution. Several mechanisms have been proposed to explain interfacial activation of lipases. The first theory relates interfacial activation to a conformational change of the enzyme, where the lid moves to make the active site available to substrate molecules at the interface. The second theory points to changes in the concentration and organization of substrate molecules at the interface to cause activation of the lipase. In the presence of a nonsubstrate lipid interface, a lipase will not be active, but once the concentration of substrate in the interface exceeds that of nonsubstrate lipids to become the continuous phase, lipase activity increases. There are several other theories as to why lipase activity is increased at an interface. One theory states that the higher substrate concentration at the interface produces more frequent collisions between the lipase and substrate than in monomeric solutions. Other theories involve decreased energy of activation induced by substrate aggregation, reduced hydration of the substrate, and progressive lipid-induced lipase aggregation at the interface [46].

In considering the action of lipases at interfaces, several factors have to be considered, including the reversibility of adsorption, the possibility of inactivation, and the quality of the interface. In general, lipases are considered to be reversibly adsorbed at interfaces, since by increasing surface pressure, lipases have been found to desorb from the interface [46]. The quality of the interface can affect the activity of lipases. Any factor that affects the affinity of the enzyme for the interface as well as packing and orientation of the molecules at the interface can affect activity [11].

D. Problem of Substrate Concentration

Since long-chain TAGs are insoluble in water and form aggregates, lipase-catalyzed interesterification cannot be strictly governed by the Henri–Michaelis rule relating the rate of the reaction to the molar concentration of substrate in solution. In interesterification reactions, the insoluble substrate is in large excess as the continuous solvent phase, making it difficult to define its concentration in the reaction mixture. Since the substrate is insoluble, only the concentration of the substrate present at the interface, which is available to the lipase, is considered. Lipase activity is controlled by the concentration of micellar substrates at the interface and is independent of the molar concentration of the substrate [47]. In contrast, esterases, acting only on water-soluble substrates, have a Michaelis–Menten dependence on substrate concentration [42,48]. The dependence of lipase activity on the surface area of the interface as a measure of substrate concentration was proven by Benzonana and Desnuelle [49], who measured the rates of hydrolysis in coarse and fine emulsions (Figure 34.10). When the rate of the reaction for the two emulsions was plotted as a function of substrate mass, the

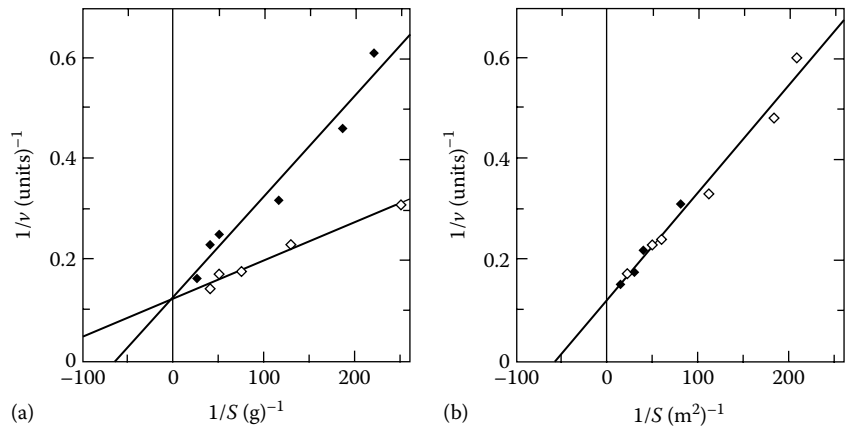

FIGURE 34.10 Lineweaver–Burk plot of lipase activity as a function of (a) mass of substrate at the interface and (b) area occupied by substrate at the interface. The comparison was made with assays containing a coarse emulsion (⬥) and a fine emulsion (◇). (Adapted from Benzonana, G. and Desnuelle, P., *Biochimie*, 105, 121, 1965.)

same maximal rates of hydrolysis were obtained; however, there was a difference in the values for K_m. In contrast, when initial velocities were plotted as a function of interfacial area, the values of K_m and V_{max} were constant for both fine and coarse emulsions, indicating that the concentration of substrate at the interface (mol/m^3) directly determines the rate of the reaction [11,12,46,50]. Reaction rates have also been shown to be a function of the emulsion concentration. If a stock emulsion is diluted to different concentrations, a progress curve of rate versus concentration will be obtained, which on plotting gives a straight line in the Lineweaver–Burk plot. A relative K_m can be obtained from this plot, but to obtain the absolute value for K_m, the area of the interface must be known [11]. It is very difficult to obtain an accurate assessment of the interfacial area due to several factors. In free enzyme solutions, the size distribution of emulsion droplets and the degree of adsorption of enzyme to the interface must be known. It is difficult to estimate the surface area of the interface due to size heterogeneity and the possibility of coalescence of emulsion droplets. With immobilized enzyme, the size distribution and surface area of support particles and pores must be determined, as well as the degree of loading of the lipase [47]. Due to the difficulty in measuring these factors accurately, only relative K_m values are determined.

E. Kinetics and Mechanism of Action

Interesterification is a multisubstrate reaction, with the main substrates being glycerides, fatty acids, and water. This reaction can be considered a special case of chemical group transfer, involving sequential hydrolysis and esterification reactions [51]. Lipase-catalyzed interesterification follows a Ping-Pong Bi–Bi reaction for multisubstrate reactions [50,51]. The actual mechanism of acylation and deacylation of the glyceride in the active site is shown in Figure 34.11. During acylation, a covalent acyl–enzyme complex is formed by nucleophilic attack of the active site serine on the carbonyl carbon of the substrate. The serine is made a stronger nucleophile by the presence of histidine and aspartic acid residues. The histidine imidazole ring becomes protonated and positively charged, stabilized by the negative charge of the active site aspartic acid or glutamic acid residues. A tetrahedral intermediate is subsequently formed, stabilized by two hydrogen bonds formed with oxyanion-stabilizing residues [12]. A break in the carbon–oxygen bond of the ester causes release of the alcohol. During the reaction, the acylglycerol is associated with the catalytic triad through covalent bonds. Histidine hydrogen bonds with both serine and oxygen of the leaving alcohol. Nucleophilic attack by water or an alcohol causes the addition of a hydroxyl group to the carbonyl carbon, producing a tetrahedral intermediate, which will rearrange, releasing the altered acylglycerol and regenerating the active site serine [42,52].

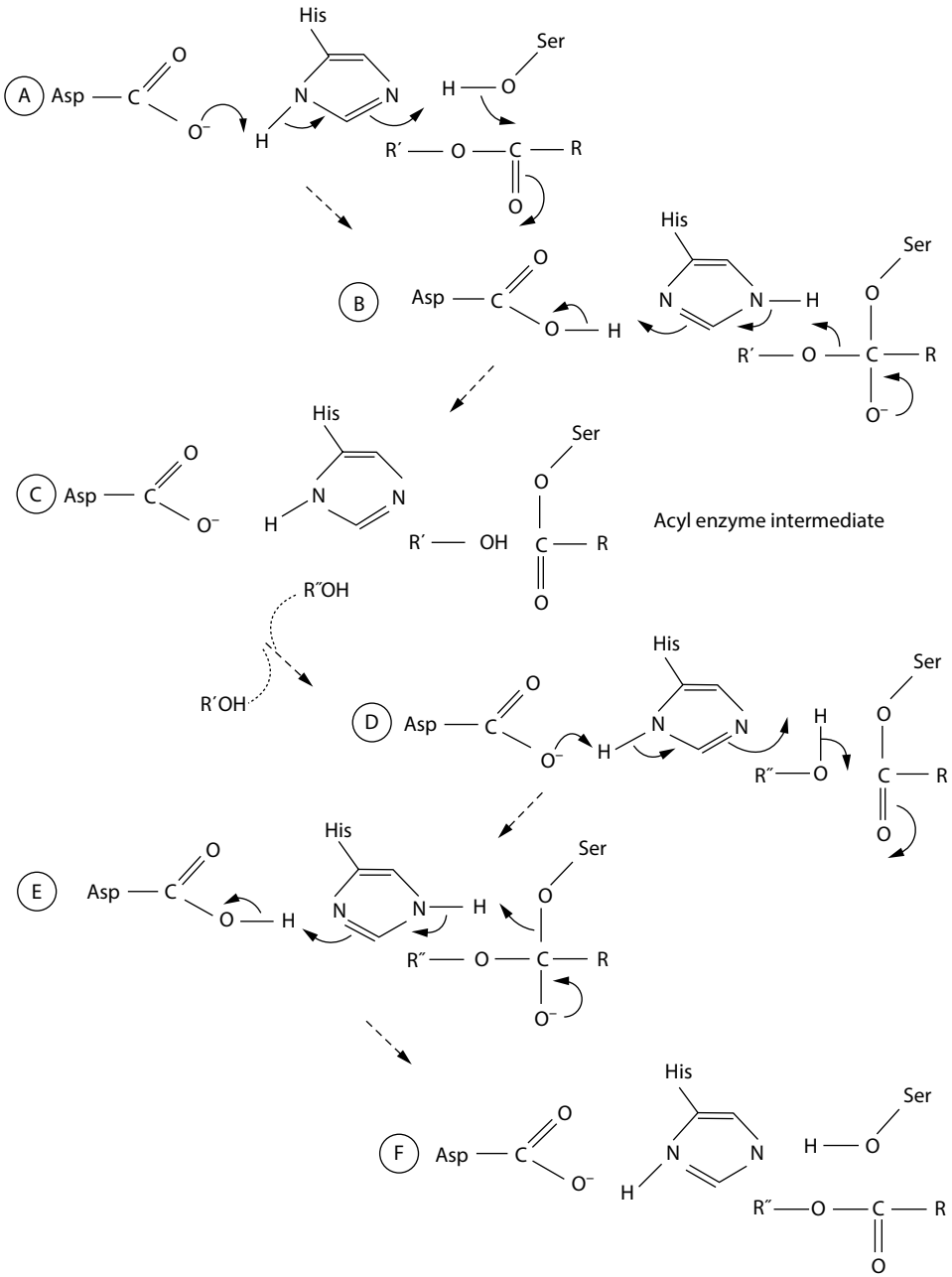

FIGURE 34.11 Catalytic mechanism for lipase-catalyzed interesterification, showing the catalytic site containing Asp/Glu, His, and Ser residues. (Adapted from Marangoni, A.G. and Rousseau, D., *Trends Food Sci. Technol*., 6, 329, 1995.)

The first stage of interesterification involves hydrolysis of TAGs with consumption of water to produce DAGs, monoacylglycerols, and free fatty acids. Accumulation of hydrolysis products will continue during interesterification until an equilibrium is established [51]. Since lipases are involved in multisubstrate, multiproduct reactions, more complex kinetic mechanisms are required. Interesterification involves acylation and deacylation reactions, either of which can be

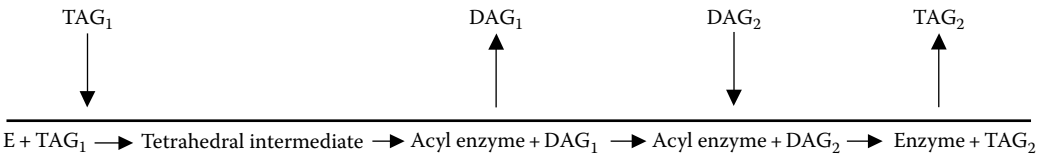

FIGURE 34.12 The Ping-Pong Bi–Bi mechanism for lipase-catalyzed transesterification, with the transfer of an acyl group from one triacylglycerol (TAG_1) to a diacylglycerol (DAG_2) to form a new triacylglycerol (TAG_4).

the rate-limiting step [50,53]. The basic mechanism for a Ping-Pong Bi–Bi reaction using multiple substrates is shown in Figure 34.12.

Under steady-state conditions,

$$\frac{\upsilon}{V_{\max}} = \frac{[AX][BX]}{K_{mBX}[AX] + K_{mBX}[B] + [A][B]}$$

where

AX is the first substrate

BX is the second substrate [41]

It is difficult to study the kinetics of Ping-Pong Bi–Bi mechanisms due to the presence of two substrates. In order to study the kinetics, one substrate concentration is usually held constant, whereas the other one is altered. In the case of lipase-catalyzed interesterification under aqueous conditions, there is the additional difficulty that the lipid substrate is also the reaction medium, which is in excess compared with other components. Even with measurable amounts of lipid substrate, it is difficult to develop rate equations since all species involved have to be considered [50].

F. Specificity

The main advantage of lipases that differentiates enzymatic interesterification from chemical interesterification is their specificity. The fatty acid specificity of lipases has been exploited to produce structured lipids for medical foods and to enrich lipids with specific fatty acids to improve the nutritional properties of fats and oils. There are three main types of lipase specificities: positional, substrate, and stereo. Positional and fatty acid specificities are usually determined by partial hydrolysis of synthetic TAGs and separation by thin-layer chromatography with subsequent extraction and analysis of the products. Other methods include conversion of the fatty acids produced during hydrolysis to methyl esters for gas chromatographic analysis [54].

1. Nonspecific Lipases

Certain lipases show no positional or fatty acid specificity during interesterification. Interesterification with these lipases after extended reaction times gives complete randomization of all fatty acids in all positions and gives the same products as chemical interesterification (Figure 34.13). Examples of nonspecific lipases include lipases derived from *Candida cylindracea*, *Corynebacterium acnes*, and *Staphylococcus aureus* [9,10].

2. Positional Specificity

Positional specificity, that is, specificity toward ester bonds in positions *sn*-1,3 of the TAG, results from an inability of lipases to act on position *sn*-2 on the TAG, due to steric hindrance (Figure 34.14). Steric hindrance prevents the fatty acid in position *sn*-2 from entering the active site [9,55].

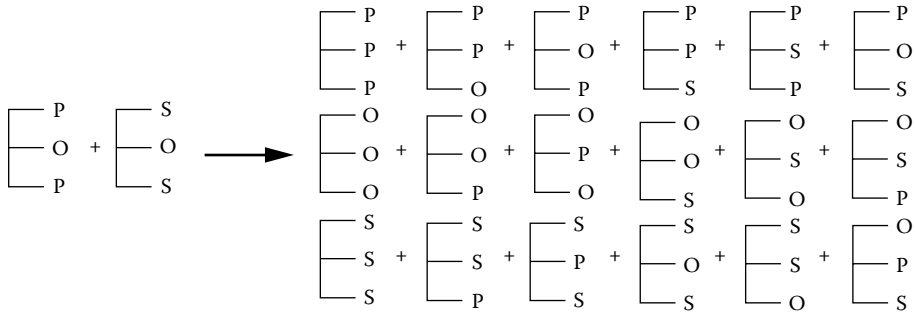

FIGURE 34.13 Triacylglycerol products from the transesterification of two TAGs, 1,3-dipalmitoyl-2-oleoylglycerol and l,3-distearoyl-2-oleoylglycerol, using either a nonspecific lipase or chemical esterification.

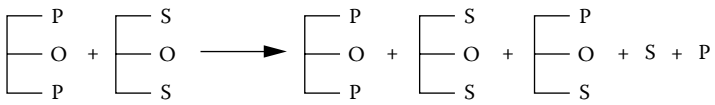

FIGURE 34.14 Transesterification products of 1,3-dipalmitoyl-2-oleoylglycerol and 1,3-distearoyl-2-oleoylglycerol using a 1,3-specific lipase.

An interesterification reaction using a 1,3-specific lipase will initially produce a mixture of TAGs, 1,2- and 2,3-diacylglycerols, and free fatty acids [55]. After prolonged reaction periods, acyl migration can occur with the formation of 1,3-diacylglycerols, which allows some randomization of the fatty acids existing at the middle position of the TAGs. In comparison with chemical interesterification, 1,3-specific lipase-catalyzed interesterification of oils with a high degree of unsaturation in the *sn*-2 position of the TAGs will decrease the saturated to unsaturated fatty acid level [56]. Lipases that are 1,3-specific include those from *Aspergillus niger*, *M. miehei*, *Rhizopus arrhizus*, and *Rhizopus delemar* [9]. The specificity of individual lipases can change due to microenvironmental effects on the reactivity of functional groups or substrate molecules [57]. For example, lipase from *Pseudomonas fragi* is known to be 1,3-specific but has also produced random interesterification, possibly due to a microemulsion environment. As of yet, lipases that are specific toward fatty acids in the *sn*-2 position have been difficult to identify. Under aqueous conditions, one such lipase from *Candida parapsilosis* hydrolyzes the *sn*-2 position more rapidly than either of the *sn*-1 and *sn*-3 positions, and is also specific toward long-chain PUFAs [58].

The differences in the nutrition of chemically interesterified fats and oils compared with enzymatically interesterified samples can be linked to the positional specificity exhibited by some lipases. In fish oils and some vegetables oils that contain high degrees of essential PUFAs, these fatty acids are usually found in greater quantities in the *sn*-2 position. In the intestines, 2-monoacylglycerols are more easily absorbed than *sn*-1 or *sn*-3 monoacylglycerols. Using a 1,3-specific lipase, the fatty acid composition of positions 1 and 3 can be changed to meet the targeted structural requirements while retaining the nutritionally beneficial essential fatty acids in position 2. Using random chemical interesterification, retention and improvement in beneficial fatty acid content cannot be accomplished due to the complete randomization of the fatty acids in the TAGs [59].

3. Stereospecificity

In TAGs, the *sn*-1 and *sn*-3 positions are sterically distinct. Very few lipases differentiate between the two primary esters at the *sn*-1 and *sn*-3 positions, but when they do, the lipases possess stereospecificity. In reactions where the lipase is stereospecific, positions 1 and 3 are hydrolyzed at different rates. Stereospecificity is determined by the source of the lipase and the acyl groups, and can

also depend on the lipid density at the interface, where an increase in substrate concentration can decrease specificity due to steric hindrance. Differences in chain length can also affect the specificity of the lipase [12]. Lipase from *Pseudomonas* species and porcine pancreatic lipase have shown stereoselectivity when certain acyl groups are hydrolyzed [60].

4. Fatty Acid Specificity

Many lipases are specific toward particular fatty acid substrates. Most lipases from microbial sources show little fatty acid specificity, with the exception of lipase from *Geotrichum candidum*, which is specific toward long-chain fatty acids containing *cis*-9 double bonds [9]. Lipases can also demonstrate fatty acid chain length specificity, with some being specific toward long-chain fatty acids and others being specific toward medium- and short-chain fatty acids. For example, porcine pancreatic lipase is specific toward short-chain fatty acids, whereas lipase from *Penicillium cyclopium* is specific toward long-chain fatty acids. As well, lipases from *A. niger* and *Aspergillus delemar* are specific toward both medium- and short-chain fatty acids [11,61]. Other lipases have been found to be specific toward fatty acids of varying lengths. Marangoni [62] found that in the hydrolysis of butter oil, lipase from *Candida rugosa* showed specificity toward butyric acid compared with *P. fluorescens* lipase. With interesterification reactions in organic media, lipases can also be specific toward certain alcohol species. A large group of lipases from sources such as *C. cylindracea*, *M. miehei*, and *R. arrhizus* have been found to be strongly specific against fatty acids containing the first double bond from the carboxyl end at an even-numbered carbon, such as *cis*-4, *cis*-6, and *cis*-8, resulting in slower esterification of these fatty acids in comparison with other unsaturated and saturated fatty acids. Fatty acid specificity by certain lipases can be used in the production of short-chain fatty acids for use as dairy flavors and in the concentration of EPA and DHA in fish oils by lipases with lower activity toward these fatty acids.

III. REACTION SYSTEMS

A. Enzymatic Interesterification in Microaqueous Organic Solvent Systems

Since the main substrates of lipases are long-chain TAGs, which are insoluble in water, many experiments have been conducted in the presence of organic solvents. Organic solvents allow the fat or oil to be solubilized and convert two-phase systems to one-phase systems [63]. Stability can be improved by covalent attachment of polyethylene glycol (PEG) to free amino groups of the lipase, giving lipases amphiphilic properties and allowing their dissolution in organic solvents [64]. It has been reported that the thermal stability of lipases can be improved in microaqueous organic solvent systems since the lack of water prevents unfolding of the lipase at high temperatures [65]. Elliott and Parkin [65] found that porcine pancreatic lipase had optimal activity at 50°C in an emulsion, whereas the optimum increased to 70°C in a microaqueous organic solvent system using hexane. Lipase activity in organic solvents depends on the nature and concentration of the substrate and source of enzyme [63]. The specific organic solvent used can dramatically affect the activity of the lipase [66]. Lipases are more active in *n*-hexane and isooctane than other solvents, such as toluene, ethyl acetate, and acetylnitrile [28,44]. The polarity of solvents can be described by *P*, the partition coefficient of a solvent between water and octanol. This is an indication of the hydrophobicity of the solvent. No lipase activity is observed in solvents with a value for log $P < 2$ [67,68]. The hydrophobicity of the solvent can also affect the degree of acyl migration during interesterification using a 1,3-specific lipase. Hexane tends to promote acyl migration due to the low solubility of free fatty acids and partial glycerides in hexane, which forces them into the microaqueous region around the lipase, providing optimum conditions for acyl migration. In contrast, the use of diethyl ether, in which free fatty acids and partial glycerides are more soluble, removes the products from the microaqueous environment and reduces the risk of acyl migration [6]. Since the choice of organic

solvents based on minimization of acyl migration may conflict with maximization of interesterification, acyl migration is usually minimized simply by reducing reaction times. Lipases can be made more active and soluble in organic solvent systems by attachment of an amphiphilic group such as PEG. PEG reacts with the N-terminal or lysine amino groups, rendering the lipase more soluble in organic solvents [69]. The activity of lipases in organic solvent depends on the solubility of the solvent in water. Lipases are only active in water-immiscible solvents, since water-miscible organic solvents extract the water of hydration layer from the vicinity of the enzyme, thereby inactivating them [44]. Since the success of an interesterification reaction depends on the concentration of water in the system, the hydration state of the lipase plays a key role because a minimal amount of water is needed to maintain the enzyme in its active form. The use of hydrophobic solvents limits the flexibility of the enzyme, preventing it from assuming its most active conformation. Therefore, if organic solvents are used, the enzyme must be in its active conformation before the addition of the organic solvent. This can be accomplished by exposing the enzyme to an inhibitor or substrate, then drying it in its active conformation [12,70]. The advantage of using organic solvents in lipase-catalyzed interesterification reactions is that the water content can be carefully controlled. A water content higher than 1% can produce high degrees of hydrolysis, whereas water levels lower than 0.01% can prevent full hydration of the lipase and reduce the initial rate of hydrolysis [1]. Therefore, water levels between these two extremes are necessary to maximize the effectiveness of enzymatic interesterification in organic solvents. In microaqueous organic solvent systems, the effect of pH on lipase activity is complex because water levels are so low. It has been proven that enzymes in organic solvent systems have a memory of the pH of the last aqueous environment in which they were. Elliott and Parkin [65] found that porcine pancreatic lipase has an optimum activity in hexane after being exposed to pH values between 6.5 and 7.0. At pH 8.5, the decrease in activity was attributed to a change in the ionization state of the histidine in the active site.

A common form of organic solvent system used in lipase-catalyzed interesterification is that of reverse micelles. Reverse micelles, or microemulsions, are defined as nanometer-sized water droplets dispersed in organic media with surfactants stabilizing the interface [29,71]. A common surfactant used is an anionic double-tailed surfactant called sodium-bis(2-ethylhexyl)sulfosuccinate (AOT). Reverse micelles are used in interesterification reactions because they increase the interfacial area and improve the interaction between lipase substrates [29]. As well, the use of microemulsions makes it possible to use polar and nonpolar reagents in the same reaction mixture [72]. Reverse micelles can be formed by gently agitating a mixture of AOT, lipid substrate, organic solvent, and lipase dissolved in buffer until the solution becomes clear. The lipase is trapped in an aqueous medium in the core of the micelle, avoiding direct contact with the organic medium [61]. Lecithin has been used to promote the formation of reverse micelles and to protect the lipase from nonpolar solvents [73,74]. At ionic strengths higher than 1 M, activity is decreased due to decreased solubility and activity of the lipase. The water content required for microemulsion systems is dependent on the desired reaction, although some level of water is necessary to hydrate the enzyme. For example, Holmberg et al. [75] found that 0.5% water was the optimum for the production of monoacylglycerols from palm oil in a microemulsion. The composition of the substrate can also affect the rate of interesterification in reverse micelles. Substrates with more amphiphilic properties are better because they can partition to the interface. More polar substrates tend to stay in the water phase and interact less with the interface [76]. The disadvantages of reverse micelle systems are that lipase activity is decreased rapidly, and the system can alter lipase specificity [73,76,77]. Reverse micelles can also be used with immobilized lipases, where the reverse micelle is formed around the support and immobilized lipase. This method has been used with hexane to produce cocoa butter equivalents [73]. Although they have been used in experimental form to produce TAGs from DAGs and oleic acid [78], as well as TAGs suitable for use as cocoa butter substitutes [74], reverse micelles are not used in industrial enzymatic interesterification applications.

IV. IMMOBILIZATION

Immobilization of lipases has become increasingly popular for both hydrolysis and synthesis reactions. The advantages of immobilized enzyme systems compared with free enzyme systems include reusability, rapid termination of reactions, lowered cost, controlled product formation, and ease of separation of the enzyme from the reactants and products. In addition, immobilization of different lipases can affect their selectivity and chemical and physical properties. Immobilization also provides the possibility of achieving both purification of the lipase from an impure extract and immobilization simultaneously, with minimal inactivation of the lipase [79]. Methods for immobilization of enzymes include chemical forms, such as covalent bonding, and physical forms, such as adsorption and entrapment in a gel matrix or microcapsules [7,80].

The easiest and most common type of immobilization used in interesterification reactions is adsorption, which involves contacting an aqueous solution of the lipase with an organic or inorganic surface-active adsorbent. The objective of immobilization is to maximize the level of enzyme loading per unit volume of support. The process of adsorption can be accomplished through ion exchange or through hydrophobic or hydrophilic interactions and van der Waals interactions [81]. After a short period of mixing of the free enzyme and support, the immobilized enzyme is washed to remove any free enzyme that is left, after which the product is dried [79]. The same adsorption process can be accomplished by precipitating an aqueous lipase solution onto the support using acetone, ethanol, or methanol, then drying as previously described [9,81]. Although desorption can occur, most immobilized lipase preparations are stable in aqueous solutions for several weeks. The preparations are stable because as the lipase adsorbs to the support, it unfolds slightly, allowing several points of interaction between the lipase and support. In order for desorption to occur, simultaneous loss of interactions at all contact sites must occur, which is unlikely [82].

The degree of immobilization depends on several conditions, including pH, temperature, solvent type, ionic strength, and protein and adsorbent concentrations. The choice of carrier is dependent on its mechanical strength, loading capacity, cost, chemical durability, functionality, and hydrophobic or hydrophilic character [83]. In general, lipases retain the highest degree of activity when immobilized on hydrophobic supports, where desorption of lipase from the support after immobilization is negligible, and improved activity has been attributed to increased concentrations of hydrophobic substrate at the interface [7,50]. The disadvantages of using hydrophilic supports include high losses of activity due to changes in conformation of the lipase, steric hindrance, and prevention of access of hydrophobic substrates [7]. Common hydrophobic supports include polyethylene, polypropylene, styrene, and acrylic polymers, whereas hydrophilic supports include Duolite, Celite, silica gel, activated carbon, clay, and Sepharose [7]. The effectiveness of the immobilization process is influenced by the internal structure of the support. If a support with narrow pores is used, most of the enzyme will be immobilized on the surface of the support, which prevents the occurrence of internal mass transfer limitations. If a support containing larger pore sizes is used, such as Spherosil DEA, with an average diameter of 1480 A, some lipase will be immobilized inside the pores, which can prevent access of the substrate to some of the lipase. This is due to preferential filling of pores and crevices by the lipase during immobilization [84,85]. The activity of lipases tends to decrease on immobilization, with activity being reduced by 20%–100% [79,81]. The activity of an immobilized enzyme relative to the free form can be compared by an effectiveness value, which is defined as the activity of immobilized enzyme divided by the activity of an equal amount of free enzyme determined under the same operating conditions. The effectiveness value can be used as a guide to the degree of inactivation of the enzyme caused by immobilization. For values close to 1.0, very little enzyme activity has been lost on immobilization, whereas values much lower than 1 indicate high degrees of enzyme inactivation [80].

The performance of an immobilized lipase can also be affected by handling and reaction conditions. Freeze drying of the immobilized enzyme before interesterification to substantially reduce

the moisture content has been reported to dramatically improve activity. Molecular sieves can also be added to reaction systems to reduce the amount of water that accumulates during the reaction, which would in turn reduce the degree of hydrolysis [4]. The main disadvantage associated with adsorption as an immobilization method is that changes in pH, ionic strength, or temperature can cause desorption of lipase that has been adsorbed by ion exchange. Lipases adsorbed through hydrophobic or hydrophilic interactions can be desorbed by changes in temperature or substrate concentration [79].

A. Factors Affecting Immobilized Lipase Activity

Immobilization can have an impact on the activity of lipases through steric, mass transfer, and electrostatic effects. During immobilization, the enzyme conformation can be affected and parts of the enzyme can be made inaccessible to the substrate due to steric hindrance.

1. Mass Transfer Effects

The kinetics of lipase-catalyzed interesterification can be affected by mass transfer limitations. The substrate must diffuse through the fluid boundary layer at the surface of the support into the pore structure of the support and react with the lipase. Once products have been released by the lipase, they must diffuse back out of the pore structure and away from the surface of the support. Mass transfer limitations fall into two categories: internal and external mass transfers. Internal mass transfer is the transport of substrate and product within the porous matrix of the support; it is affected by the size, depth, and smoothness of the pores. Internal mass transfer is diffusion-limited only. When the rate of diffusion inward is slower than the rate of conversion of substrate to product, the reaction is diffusion-limited, as there is not enough substrate available for the amount of enzyme present [86]. A diffusion coefficient for internal mass transfer in immobilized enzyme systems compared with free enzyme systems is defined as

$$D_e = \frac{D\psi}{\tau}$$

where

D_e is the effective diffusion coefficient inside the support particles
D is the diffusion coefficient in free solution
ψ is the porosity of the particles
τ is the tortuosity factor, defined as the distance of the path length traveled by molecules between two points in a particle

The effective diffusion coefficient varies inversely with the molecular weight of the substrate [80]. Internal diffusional limitations can be recognized if the activity increases when the support particles are crushed, since crushing would decrease the length of the pathway that the substrate would have to travel to reach the enzyme. The Thiele modulus, w, can be used to evaluate the extent of internal mass transfer limitations:

$$\phi = L\lambda = L\left(\frac{V_{\max}}{K_m D_e}\right)^{1/2}$$

where L is the half-thickness of the support particles. Internal mass transfer limitations can also be identified by measuring the initial velocity of the reaction at increasing enzyme concentrations. If the rate of the reaction remains constant at increasing enzyme concentrations (amount of enzyme per gram of support), the reaction is mass transfer-limited. If the rate of reaction increases linearly with increasing enzyme concentration, the reaction is kinetically limited.

Internal diffusion limitations can be reduced by decreasing the support particle size, increasing pore size and smoothness, using low-molecular-weight substrates, and using high substrate concentrations [80]. The difficulty with using smaller support particles in fixed bed reactors where internal mass transfer limitations are high is that it tends to increase the back pressure of the system [84].

External mass transfer limitations are the resistance to transport between the bulk solution and a poorly mixed fluid layer surrounding each support particle. External mass transfer can occur in packed bed and membrane reactors and is affected by both convection and diffusion [84]. If the reaction is faster than the rate of diffusion of substrate to the surface or product from the surface, this can affect the availability of substrate for lipase catalysis. If inadequate substrate quantities reach the enzyme, the rate of reaction will be lower than that of free enzyme. An increasing external mass transfer coefficient can be identified during kinetic analysis by an increasing slope of a Lineweaver–Burk plot [87]. In stirred reaction systems, external mass transfer limitations have been eliminated when there is no increase in the reaction rate with increasing rates of stirring. External mass transfer limitations can be reduced in packed bed reactors by increasing the flow rate, reducing the viscosity of the substrate, and increasing substrate concentration [80]. Changing the height-to-diameter ratio of a fixed bed reactor can also reduce external mass transfer limitation as it increases the linear velocity of the substrates.

2. Nernst Layer and Diffusion Layer

Immobilized lipases are surrounded by two different layers, which can create differences in substrate concentration between them and the bulk phase. The Nernst layer is a thin layer located directly next to the surface of the support. In the case of hydrophobic supports and hydrophobic substrates, such as TAGs, the concentration of substrates in the Nernst layer is more than in the bulk solution since the hydrophobic substrate tends to partition toward the hydrophobic support material. Another layer surrounding the support particles is a diffusion or boundary layer. A concentration gradient is established between the diffusion layer and the bulk phase as substrate is converted to product by the lipase. The product concentration in the diffusion layer is higher than in the bulk phase as it must diffuse from the surface of the support into the bulk phase. Consequently, due to the higher product concentration in the diffusion layer, the substrate concentration is lower than in the bulk phase, producing concentration gradient with more substrate diffusing toward the support and immobilized lipase. Differences in substrate concentration between the Nernst layer and/or the boundary layer and the bulk phase can affect the determination of K_m since substrate concentration will be measured in the bulk layer, which may not be the concentration of substrate closer to the lipase. With a lower substrate concentration at the support in comparison with the bulk phase, the apparent K_m will appear higher and the activity will appear lower than its actual values. The opposite will occur with a higher substrate concentration at the interface.

A third factor that can affect the activity of immobilized lipase is electrostatic effects. If the support and substrate possess the same charge then they will experience repulsion, whereas if they have opposite charge they will be attracted. This factor can have an effect on the apparent K_m. In addition, electrostatic effects can have an impact on other components in the reaction. For example, if the support was anionic, the local concentration of hydrogen ions would be higher in the vicinity of the immobilized lipase, which would cause a decrease in the pH around the enzyme.

Combining the electrostatic effects and the effect of the Nernst layer, the value of the apparent K_m can be modified as follows [88]:

$$K'_m = \left(K_m + \frac{x}{D} V_{\max} \right) \frac{RT}{RT - xzFV}$$

where
K'_m is the apparent K_m of the lipase
x is the thickness of Nernst layer
R is the universal gas constant
T is the absolute temperature
z is the valence of the substrate
F is Faraday's constant
V is the magnitude of the electric field around the enzyme support
D is the diffusion coefficient of the substrate

If the thickness of the Nernst layer decreases, then the ratio x/D would decrease and K'_m would decrease, approaching K_m.

B. Stability of Immobilized Enzymes

The stability of immobilized enzymes depends on the method of immobilization and the susceptibility of the enzyme to inactivation. Inactivation can be caused by contaminants and changes in temperature, pH, and ionic strength. High shear, microbial contamination, fouling, and breakage of support particles have also been found to inactivate immobilized enzymes. Depending on the strength of the immobilization method, the enzyme can also be desorbed from the support. The stability of immobilized enzymes is evaluated by determining the half-life of the enzyme under the reaction conditions. In diffusion-limited systems, there is a linear decay in enzyme activity in time, as enzymes on the surface of the support are inactivated and the substrate diffuses further into the pores to reach enzyme molecules that have not been inactivated. In systems free of diffusional limitations, enzyme inactivation follows a first-order decay:

$$\ln \frac{N}{N_0} = -\lambda t$$

where
N_0 is the initial enzyme activity
N is the activity at time t
λ is the decay constant

Using λ, the half-life of the immobilized lipase can be determined as follows:

$$\text{Half-life} = \frac{0.693}{\lambda}$$

The half-lives of lipases in interesterification systems have been reported to range from 7 min to 7 months, with the large variability attributed to the source of lipases and different reaction conditions [50]. As previously stated, the half-life of the immobilized enzyme can be used to determine the productivity of the system. In order to avoid losses in productivity as the activity of the immobilized lipase decreases, the temperature can be raised to increase the reaction rate or, in fixed bed reactor systems, the flow rate can be reduced [80]. While these measures can improve the conversion rate, they can also increase the rate of enzyme inactivation in the case of temperature increases, or decrease the throughput in the case of reduced flow rate.

C. Immobilized Enzyme Kinetics

The previous discussion on the kinetics of lipase action was developed for soluble lipases acting on insoluble substrate, but assuming that diffusional and mass transfer effects are not rate-limiting, the

same theories can be applied to immobilized lipases. When using immobilized lipases, the level of substrate in comparison with the level of enzyme must be considered. In general, there is a low average concentration of substrate in direct contact with the immobilized lipase due to high conversion rates, producing first-order, mixed first- and zero-order, or zero-order kinetics as opposed to zero-order Michaelis–Menten kinetics [80]. The rate of the reaction, *y*, is proportional to the substrate concentration at the interface where

$$\upsilon = \frac{V_{\max}\left[S\right]}{K_m + \left[S\right]}$$

The kinetics of immobilized lipases is also affected by the type of reactor used, since reactors differ in the amount of immobilized lipase used and in the method of substrate delivery, product removal, and degree of mixing.

V. ENZYMATIC INTERESTERIFICATION REACTORS

Reactors designed for immobilized enzyme reactions differ from one another based on several criteria. Reactors can be batch or flow-through systems and can differ in the degree of mixing involved during the reaction. For all reactor systems, the productivity of the system is defined as the volumetric activity × the operational stability of the immobilized enzyme, with units of kilograms of product per liter of reactor volume per year. The volumetric activity is determined as the mass of product obtained per liter of reactor per hour, whereas the operational stability is the half-life of the immobilized enzyme [80]. The most common reactor systems used include fixed bed, batch, continuous stirred tank, and membrane reactors.

A. Fixed Bed Reactor

A fixed bed reactor is a form of continuous flow reactor, where the immobilized enzyme is packed in a column or as a flat bed, and the substrate and product streams are pumped in and out of the reactor at the same rate. The main advantages of fixed bed reactors are their easy application to large-scale production, high efficiency, low cost, and ease of operation. A fixed bed reactor also provides more surface area per unit volume than a membrane reactor system [7]. A model fixed bed reactor for interesterification would consist of two columns in series: one for the reaction and a precolumn for fat-conditioning steps such as incorporation of water. Reservoirs attached to the columns would contain the feed streams and product streams. A pump would be required to keep the flow rate through the system constant, and the system would have to be water-jacketed to keep the reaction temperature constant (Figure 34.15).

Since water is required in minimal amounts for hydration of the enzyme during the reaction, the oil is first passed through a precolumn containing water-saturated silica or molecular sieves, which would allow the oil to become saturated with sufficient water to allow progression of the interesterification reaction without increasing the rate of hydrolysis. Interesterification in a fixed bed reactor can lead to increases in product formation through increased residence time in the reactor. Complete conversion to products will never be achieved, and with an increase in product levels, a loss in productivity will occur [89]. Using a fixed bed reactor with a silica precolumn for water saturation of the oil phase, Posorske et al. [89] produced a cocoa butter substitute from palm stearin and coconut oil. These authors found that decreasing the flow rates to increase the total product concentration caused a decrease in productivity. Decreasing the flow rates to increase product levels from 20% to 29% leads to a significant decrease in productivity. Fixed bed reactors are more efficient than batch reactors but are prone to fouling and compression. Dissolution of the oil in an organic solvent to reduce viscosity for flow through the packed bed may be required [89]. In addition, the substrate has to be treated to

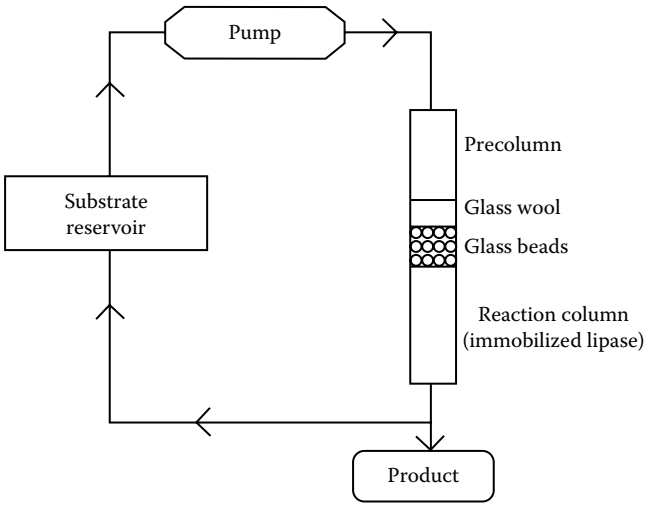

FIGURE 34.15 Fixed bed reactor for immobilized lipase-catalyzed interesterification.

remove any particulates, inhibitors, and poisons that can build up over time and inactivate the lipase [8]. Macrae [9] found that after treatment of palm oil mid fraction and stearic acid to remove particulates, inhibitors, and poisons, acidolysis reached completion after 400 h, and there was not appreciable loss in lipase activity even after 600 h of operation. Wisdom et al. [90] performed a pilot scale reaction using a 2.9 L fixed bed reactor to esterify shea oleine with stearic acid. It was found that with high-quality substrates, only a small loss of activity was exhibited after 3 days with the production of 50 kg of product. However, when a lower grade shea oil was used, there was rapid inactivation of the lipase.

The kinetics of a packed bed reactor are assumed to be the same as for a soluble lipase, where

$$\frac{dS}{dt} = \frac{V_{\max}\left[S\right]}{K'_m + [S]}$$

This can be rearranged and integrated to

$$\left[S_0\right]X = K'_m \ln(1-X) + \frac{k_{cat}E_\tau}{Q}$$

where

$[S_0]$ is the initial substrate concentration
X is the fraction of substrate that has been converted to product at any given time $(1 - [S]/[S_0])$
Q is the volumetric flow rate
E_τ is the total number of moles of enzyme present in the packed bed [80,88]

The residence time, τ, is based on the porosity of the packed bed and is defined as [91]

$$\tau = V_{tot}\frac{P}{Q}$$

where

V_{tot} is the volume of the reactor
P is the porosity of the bed
Q is the flow rate of the substrate

The porosity of the bed in a fixed bed reactor can produce internal transfer limitations. Ison et al. [84] studied the effects of pore size on lipase activity in a fixed bed reactor using Spherosil with a mean pore size of 1480 Å and Duolite with a mean pore size of 190 Å. The larger pore size of the Spherosil was found to produce a decrease in lipase activity. This loss in activity was due to the higher degree of enzyme loading during immobilization, making some of the lipase inaccessible to substrate. With the smaller pore size of Duolite, the lipase was immobilized only on the surface of the support, eliminating internal mass transfer limitations.

B. Stirred Batch Reactor

A stirred batch reactor is a common system used in laboratory experiments with lipase-catalyzed interesterification due to its simplicity and low cost. No addition and removal of reactants and products are performed except at the initial and final stages of the reaction (Figure 34.16). The equation to characterize the kinetics of a stirred batch reactor is

$$\left[S_0\right]X - K'_m \ln(1-X) = \frac{k_{cat}E_\tau t}{V}$$

where

$[S_0]$ is the initial substrate concentration
X is the fraction of substrate converted to product at any given time $(1 - [S]/[S_0])$
t is the reaction time
E_τ is the total number of moles of enzyme present in the reactor
V is the volume of the reactor

Kurashige [92] found that a batch reactor was useful in reducing the DAG content in palm oil by converting existing DAGs and free fatty acids to TAGs. Using lipase coadsorbed with lecithin on Celite under vacuum to keep the water content below 150 ppm, the author was able to increase the TAG content from 85% to 95% in 6 h. The rate of conversion in a stirred batch reactor decreases over time since there is a high initial level of substrate, which is reduced over time, with conversion to product. In order to maintain the same rate of conversion throughout the reaction, it would be necessary to add more immobilized enzyme to the reaction mixture [80]. A stirred batch reactor has the advantage of being relatively easy to build and free enzymes can be used, but it has the

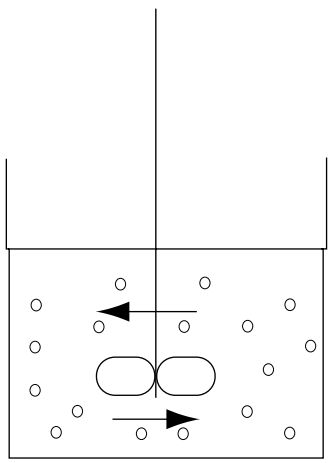

FIGURE 34.16 Stirred batch reactor for immobilized or free lipase-catalyzed interesterification.

disadvantage that, unless immobilized, the enzyme cannot be reused. As well, a larger system or longer reaction times are required to achieve equivalent degrees of conversion in comparison with other systems, and side reactions can be significant [63]. Macrae [9] used a batch reactor to produce cocoa butter equivalents from the interesterification of palm oil mid fraction and stearic acid. While product yields were high, by-products such as DAGs and free fatty acids were formed. Therefore, it was necessary to isolate the desired TAG products using fat fractionation techniques.

C. Continuous Stirred Tank Reactor

A continuous stirred tank reactor combines components of both fixed bed and batch reactors. It is an agitated tank in which reactants and products are added and removed at the same rate, while providing continuous stirring to eliminate mass transfer limitations encountered in fixed bed reactors (Figure 34.17). Stirring also prevents the formation of temperature and concentration gradients between substrates or products. A continuous stirred tank reactor can be in the form of a tank with stirring from the top or bottom, or a column with stirring accomplished by propellers attached to the sides of the column [68]. The kinetics for a continuous stirred tank reactor, developed by Lilly and Sharp [93], first encompass the substrate balance in the system as

$$Q[S_i] - Q[S_0] = \frac{dS}{dt} V$$

where
Q is the flow rate
$[S_i]$ is the initial substrate concentration entering the reactor
$[S_0]$ is the substrate concentration leaving the reactor
V is the steady-state liquid volume in the tank

Rearrangement gives modified equation here:

$$\left[S_0\right] X + K'_m \left(\frac{X}{1-X}\right) = \frac{k_{cat} E_\tau}{Q}$$

where
$[S_0]$ is the initial substrate concentration
X is the amount of substrate converted to product at any particular time $(1 - [S]/[S_0])$
Q is the flow rate
E_τ is the total number of moles of enzyme present in the reactor

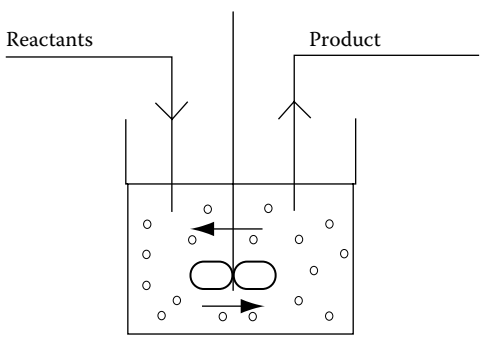

FIGURE 34.17 Continuous stirred tank reactor for immobilized lipase-catalyzed interesterification.

The main disadvantages of continuous stirred tank reactors are the higher power costs associated with continuous stirring, the possibility of breaking up support particles with agitation, and the requirement for a screen or filter at the outlet to prevent losses of the immobilized lipase [7,80].

D. Membrane Reactors

Immobilization of enzymes onto semipermeable membranes is an attractive alternative for lipase-catalyzed interesterification reactions. Membrane reactors involve two-phase systems, where the interface of two phases is at a membrane. The advantages of membrane systems are reduced pressure drops, reduced fluid channeling, high effective diffusivity, high chemical stability, and a high membrane surface-area-to-volume ratio [94]. Membranes are commonly produced in the form of a bundle of hollow fibers and can be hydrophilic of hydrophobic in nature. Materials used in membrane systems are polypropylene, polyethylene, nylon, acrylic resin, and polyvinyl chloride. In a membrane such as microporous polypropylene, the pores have dimensions of 0.075 by 0.15 mm, and the fibers have an internal diameter of 400 mm, providing 18 m^2 of surface area per gram of membrane [82]. With a hydrophilic membrane such as cellulose, the oil phase circulates through the inner fiber side, whereas the aqueous components circulate on the shell side [63]. Immobilization of lipase can be accomplished by submerging the fibers in ethanol, rinsing them in buffer, then submerging them in lipase solution [82]. Another method involves dispersing the enzyme in the oil phase and using ultrafiltration to deposit the lipase on the inner fiber side. One of the substrates can diffuse through the membrane toward the interface where the enzyme is immobilized. Van der Padt et al. [63] used hollow fibers made from cellulose to perform glycerolysis of decanoic acid. Using a hydrophilic membrane bioreactor, the lipase activity was similar to the activity in emulsion systems. The hydrophilic membrane was found to be more effective for glycerolysis since the lipase was immobilized on the oil phase side, with the membrane preventing it from diffusing into the glycerol phase and being lost. Hoq et al. [95] used a hydrophobic polypropylene membrane to esterify oleic acid and glycerol. The lipase was adsorbed on the glycerol side, resulting in the loss of some enzyme in this phase. Therefore, use of a hydrophobic membrane would require the addition of more lipase to prevent losses in activity [7,64]. Membrane reactors have been used in glycerolysis and acidolysis reactions and have an advantage over more conventional stirred tank reactors in that the reaction and separation of substrates and product can be accomplished in one system. Having the substrates and products separated during the reaction is especially useful during the esterification reaction where water is produced. Hoq et al. [95,96] found that during esterification of oleic acid and glycerol, the excess water produced could be removed by passing the oleic acid stream through molecular sieves, thereby preventing losses in productivity from hydrolysis.

E. Fluidized Bed Reactor

Fluidized bed reactors are reactors in which the immobilized enzyme and support are kept suspended by the upward flow of substrate or gas at high flow rates [80] (Figure 34.18). The advantages of fluidized bed reactors are that channeling problems are eliminated and that there is less change in pressure at high flow rates and less coalescence of emulsion droplets. In addition, particulates do not have to be removed from the oil and there are no concentration gradients [7]. The main disadvantage of fluidized bed reactors is that small concentrations of enzyme can be used since a large void volume is required to keep the enzyme and support suspended. Mojovic et al. [97] used a gas lift reactor to produce a cocoa butter equivalent by interesterifying palm oil mid fraction. These authors immobilized lipase encapsulated in lecithin reverse micelles in hexane; the reaction in the gas lift reactor was more efficient than in a stirred batch reactor. Equilibrium was reached 25% earlier and productivity was 2.8 times higher in the gas lift reactor.

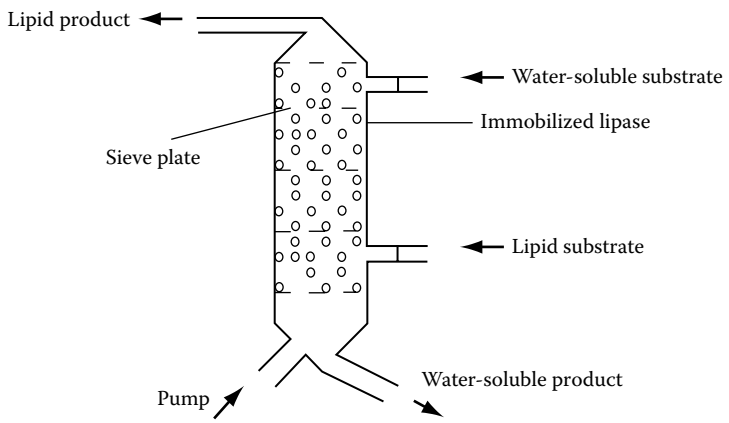

FIGURE 34.18 Fluidized bed reactor for immobilized lipase-catalyzed interesterification.

VI. FACTORS AFFECTING LIPASE ACTIVITY DURING INTERESTERIFICATION

In considering all of the factors involved in enzymatic interesterification, all components of the system must be examined, namely, pH, water content, temperature, substrate composition, product composition, and lipase content.

A. pH

Lipases are only catalytically active at certain pH, depending on their origin and the ionization state of residues in their active sites. While lipases contain basic, neutral, and acidic residues, the residues in the catalytic site are only active in one particular ionization state. The pH optima for most lipases lies between 7 and 9, although lipases can be active over a wide range of acid and alkaline pH, from about pH 4 to 10 [50,98]. For example, the optimum pH for lipase from *Pseudomonas* species is around 8.5, whereas fungal lipases from *A. niger* and *R. delemar* are acidic lipases [99]. The effect of immobilization on the pH optimum of lipases is dependent on the partitioning of protons between the bulk phase and the microenvironment around the support and the restriction of proton diffusion by the support. If the lipase is immobilized on a polyanionic, the concentration of protons in the immediate vicinity of the support will be higher than in the bulk phase, thereby reducing the pH around the enzyme in comparison with the pH of the bulk phase. Since there is a difference in the perceived pH of the solution as measured by the pH of the bulk phase, the lipase would exhibit a shift in pH optimum toward a more basic pH. For instance, for a free lipase that has a pH optimum of 8.0, when immobilized on a polyanionic matrix, with the bulk solution at pH 8.0, the pH in the immediate vicinity of the lipase might be only 7.0. Therefore, while the reaction pH is 8.0, the lipase is operating at pH 7.0, which is below its optimum. The pH of the bulk solution would have to be increased to pH 9.0 to get the pH around the lipase to its optimum of 8.0. This phenomenon is only seen in solutions with ionized support and low ionic strength systems [100]. If protons are produced in the course of interesterification, the hydrogen ion concentration in the Nernst layer can be higher than in the bulk phase, thereby decreasing the pH in the vicinity of the lipase. Running an interesterification reaction with lipases at a pH well removed from the optimum can lead to rapid inactivation of the enzyme.

B. Temperature

In general, increasing the temperature increases the rate of interesterification, but very high temperatures can reduce the reaction rates due to irreversible denaturation of the enzyme. Animal and plant lipases are usually less thermostable than extracellular microbial lipases [98]. In a solvent-free system,

the temperature must be high enough to keep the substrate in the liquid state [84,101]. Temperatures do not need to be as high in systems containing organic solvents since they easily solubilize hydrophobic substrates. However, for food industry applications, where organic solvents are avoided, the reaction temperatures are usually higher. Sometimes, the temperature has to be increased to as high as 60°C to liquify the substrate. Such high temperatures can seriously reduce the half-life of the lipase, although immobilization has been found to improve the stability of lipases under high temperature conditions. Immobilization fixes the enzyme in one conformation, which reduces the susceptibility of the enzyme to denaturation by heat. The optimal temperature for most immobilized lipases falls within the range of 30°C–62°C, whereas it tends to be slightly lower for free lipases [50]. Immobilized lipases are more stable to thermal deactivation because immobilization restricts movement and can reduce the degree of unfolding and denaturation. Hansen and Eigtved [102] found that even at a temperature of 60°C, immobilized lipase from *M. miehei* has a half-life of 1600 h.

C. Water Content and Water Activity

The activity of lipases at different water contents or water activity is dependent on the source of the enzyme. Lipases from molds seem to be more tolerant to low water activity than bacterial lipases. The optimal water content for interesterification by different lipases ranges from 0.04% to 11% (w/v), although most reactions require water contents of <1% for effective interesterification [15,50,103]. The water content in a reaction system is the determining factor as to whether the reaction equilibrium will be toward hydrolysis or ester synthesis. Ester synthesis depends on low water activity. Too low a water activity prevents all reactions from occurring because lipases need a certain amount of water to remain hydrated, which is essential for enzymatic activity [34,104]. As stated previously, lipases tend to retain the greatest degree of original activity when immobilized on hydrophobic supports. When the immobilized lipase is contacted with an oil-in-water emulsion, the oil phase tends to associate with and permeate the hydrophobic support, so that there is no aqueous shell surrounding the enzyme and support. It can be assumed that there is an ordered hydrophobic network of lipid molecules surrounding the support. Any water that reaches the enzyme for participation in hydrolysis and interesterification reactions must diffuse there from the bulk emulsion phase. Therefore, to avoid diffusional limitations, the oil phase must be well saturated with water [50]. Too much water can inhibit interesterification, probably due to decreased access of hydrophobic substrates to the immobilized enzyme. Abraham et al. [105] found that in a solvent-free system, interesterification dominated hydrolysis up to a water-to-lipase ratio of 0.9, after which hydrolysis became the predominant reaction. During interesterification, the reaction equilibrium can be forced away from ester synthesis due to accumulation of water, 1 mol of which is produced for every mole of ester synthesized during the reaction. The equilibrium can be pushed back toward ester synthesis by continuous removal of water produced during the reaction. Water activity can be kept constant by having a reaction vessel with a saturated salt solution in contact with the reaction mixture via the gas phase to continuously remove the water produced in the course of interesterification. Another method of water activity control that has proven useful with interesterification reactions is the use of silicone tubing containing the salt solution, immersed in the reaction vessel. Water vapor can be transferred out of the reaction system across the tubing wall and into the salt solution [105]. A very simple method for water removal involves adding molecular sieves near the end of the reaction, or running the reaction under a vacuum so that the water produced is continuously removed, while still allowing the lipase to retain its water of hydration [44,92,106]. Kurashige [92] ran an effective interesterification reaction with <150 ppm water maintained by running the reaction under vacuum.

D. Enzyme Purity and Presence of Other Proteins

During immobilization, adsorption of protein to surface-active supports is not limited solely to lipases. Other protein sources in the lipase solution can be adsorbed, and this can have an effect on

the loading and activity of the immobilized enzyme. Use of a pure lipase solution for immobilization has been found to reduce activity of the lipase, whereas the presence of other proteins on the support can increase the activity of the immobilized lipase [90]. Nonprotein sources of contamination during immobilization are usually not a problem because the lipase is preferentially adsorbed to the support.

E. Substrate Composition and Steric Hindrance

The composition of the substrate can have an effect on the rate of hydrolysis and interesterification by lipase. The presence of a hydroxyl group in the *sn*-2 position has a negative inductive effect, so that TAGs are hydrolyzed at a faster rate than DAGs, which are hydrolyzed at a faster rate than monoacylglycerols [11]. While the nucleophilicity of substrate is important to the rate of reaction, steric hindrance can have a much greater negative effect. If the composition of the substrate is such that it impedes access of the substrate to the active site, any improvements in the nucleophilicity will not improve the activity [107].

The conformation of the substrate can also have an effect on the rate of reaction. The hydrophobic tunnel in the lipase accepts aliphatic chains and aromatic rings more easily than branched structures [11,44]. For example, using carboxylic acids of differing chain lengths, Miller et al. [44] found that increasing the acyl group chain length up to seven carbons increased the esterification rate for lipase from *M. miehei*.

Oxidation of substrates, especially PUFAs, is possible and can cause inhibition and a decrease in activity of lipases, especially in reactions containing organic solvents. Inhibition is seen at hydroperoxide levels greater than 5 mequiv/kg oil and is attributed to the breakdown of hydroperoxides to free radicals [108]. Therefore, before running interesterification reactions, especially in flow-through systems such as fixed bed reactors that are more susceptible to poisoning and inactivation, oils containing high levels of PUFAs must be highly refined [89].

F. Surface-Active Agents

The presence of surface-active agents used during the immobilization process can improve lipase activity during interesterification. The addition of lecithin or sugar esters as surface-active agents during the immobilization process can increase activity 10-fold when the preparation is used under microaqueous conditions [19]. In contrast, using surface-active agents to form an emulsion can dramatically decrease the rate of interesterification because they prevent contact between the lipase and substrate [109]. Adsorption at the interface can be inhibited by the presence of other nonsubstrate molecules, such as proteins. The presence of proteins other than lipase at the interface reduces the ability of the lipase to bind to the interface. Addition of protein in the presence of lipase can cause desorption of lipase from the interface.

Phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, can be found as minor components in oil, in quantities of 0.1%–3.2%. The presence of phospholipids can have a negative effect on lipase activity. The initial rate of reaction can be decreased due to initial competition between phosphatidylcholine and the TAGs for the active site of the lipase. Phosphatidylethanolamine seems to have the most inhibitory effect on lipase action possibly due to the presence of the amine group. Due to their effects, the phospholipid content of oils must be <500 ppm to prolong the half-life of immobilized lipases during interesterification [110].

G. Product Accumulation

During interesterification of two TAGs, the production of monoacylglycerols and DAGs can lead to an increase in the rate of reaction, whereas the presence of high levels of free fatty acids can inhibit the initial hydrolysis of TAGs [51]. In lipase-catalyzed interesterification, where hydrolysis

is extensive, or in acidolysis reactions, the level of free fatty acids has an impact on the rate of the reaction. During acidolysis of butter oil with undecanoic acid, Elliott and Parkin [65] reported that concentrations of undecanoic acid greater than 250 mM decreased the activity of porcine pancreatic lipase. Inhibition of lipase activity by free fatty acids agrees with the Michaelis–Menten model for uncompetitive inhibition by a substrate [65]:

$$\nu = \frac{V_{\max}[S_0]}{[S_0]\left(1 + ([S_0]/K_i)\right) + K_m}$$

where

$[S_0]$ is the initial free fatty acid concentration
K_i is the inhibition constant
K_m is the Michaelis constant

The loss of activity by lipase in the presence of high concentrations of free fatty acids has been attributed to several factors. High levels of free fatty acids would produce high levels of free or ionized carboxylic acid groups, which would acidify the microaqueous phase surrounding the lipase or cause desorption of water from the interface. In addition, with short- and medium-chain fatty acids, there could be partitioning of fatty acids away from the interface into the surrounding water shell due to their increased solubility in water. This would limit access by the substrate to the interface [111]. Kuo and Parkin [111] found that there was less inhibition when longer chain fatty acids, such as C13:0 and C17:0, were used during acidolysis, compared with C5:0 and C9:0. The decrease in lipase activity was attributed to both increased solubility of the short-chain fatty acids and acidification of the aqueous phase.

VII. APPLICATIONS OF ENZYMATIC INTERESTERIFICATION

Vegetable oils and animal fats have numerous applications in the food industry. However, there is a limitation in their application based on their inherent physical and chemical properties. Since the composition of TAGs, the main constituent of oils and fats is diverse and is dependent of the kind of fatty acid attached to the glycerol backbone and their distribution within the molecule. Fatty acids can have a wide range of chain lengths and number of double bonds in their structure. Therefore, for fats, physical properties such as melting point, crystallization temperature, viscosity, SFC, crystal polymorphism, and functionality such as plasticity, spreadability, snap, and glossiness are strongly related to their TAG compositions.

Most of the time, modification of TAG composition in natural fats and oils is a necessity to obtain desirable properties and functionality. The main strategies for the modification of fatty acid and TAG composition in oils and fats include genetic engineering of oilseeds, physical separation of TAGs using fractionation and distillation techniques, and chemical or enzymatic interesterification. Engineering TAGs by placing selected fatty acids at specific locations within the TAG molecule has attracted much attention due to the many potential food and medicinal applications. For instance, TAGs molecules with two MCFAs in *sn*-1,3 position and one polyunsaturated fatty acids in the middle *sn*-2 position (M-PUFA-M) can be used for feeding patients who have difficulty digesting fat, such as victims of cystic fibrosis [112].

Enzymatic transesterification can be catalyzed by many different lipases, such as *R. miehei*, *C. rugosa*, *Rhizopus oryzae*, *Thermomyces lanuginosus*, and *Candida antarctica* lipase. These have been used to create oils and fats for edible applications such as *trans*-free margarines and shortenings, structured lipids for infant formulas, low-calorie fats, enteral and parenteral nutrition, and cocoa butter equivalents, emulsifiers (monoacylglycerols [MAGs]), anti-blooming agents [113].

A good example of enzymatic transesterification is the glycerolysis reaction used for producing MAGs. The annual world production of MAGs (mainly GMS and GMP) is about 180,000–250,000

metric tons. MAGs not only have many applications in the food industry as surface active components but also exhibit special properties such as antimicrobial activity (monolinolenin, monolinolein, monomyristin, and monolaurin), antioxidation and antiatherosclerotic properties (2-monoolein) [114]. Glycerolysis of oils leads to the formation of mixtures of MAGs and DAGs, and thus there is a need to separate MAGs from the final reaction mixture. The most common techniques used are crystallization, adsorption, and distillation.

Ethanolysis of oils using 1,3-specific lipase can also be used for making 2-MAGs. For example, Wang et al. synthesized 2-MAGs after optimizing enzymatic ethanolysis of HOSFO using Novozym 435 lipase from *C. antarctica* as the catalyst. At the end of the reaction, solvent fractionation in hexane at −20°C was used to separate 2-MAGs from ethyl esters, DAGs, and TAGs. The final product was 2-monoolein (97.5% purity) with 78.3% yield (w/w) [114].

A. Cocoa Butter Equivalents

The main fatty acids in cocoa butter include palmitic, stearic, and oleic acids, while the three major TAGs are POP, POS, and SOS. The typical molar fatty acid composition of cocoa butter is C16:0%–24.4%, C18:0%–33.6%, C18:1%–37.0%, C18:2%–3.4%, and the classic molar TAG profile is POP-16%, POS-35%, SOS-26%, POO-4%, and SOO-6%. This TAG composition imparts a special physical property and polymorphism to cocoa butter and chocolate. For example, cocoa butter melts sharply between 32°C and 35°C, and has typical SFCs at different temperatures: 82.1% (at 20°C), 78.7% (at 25°C), 58.3% (at 30°C), and 2.4% (at 37°C) [115]. The optimal polymorphic crystal form for cocoa butter is β-V, which greatly affects the physical properties and functionality of chocolate, specifically gloss, snap, sharp melting, and bloom-resistance.

A limited and uncertain supply of natural cocoa butter, high price, and quality variability has prompted the search for alternatives. Among the different cocoa butter mimetics (cocoa butter substitutes [CBS], cocoa butter equivalents [CBE], and cocoa butter replacements [CBR]), CBEs have physical and sensory properties similar to CB since their TAGs are similar to or compatible with CB TAGs. The most common CBEs include palm mid fraction (PMF), illipe (*Shorea stenoptera*) fat, shea (*Butyrospermum parkii*) butter, sal (*Shorea robusta*) fat, kokum (*Garcinia indica*) butter, and mango fat [116,117].

However, the use of CBEs in confectionary products, and especially their addition to chocolate, is highly regulated in many countries. For example, in the United States, it is not possible to add CBE to chocolate, while it is allowed to use it in chocolate coatings. CBEs can be added at levels higher than 5%, but then these products must then be labelled as compound chocolate. Based on Directive 2000/36/EC, the Member States of the European Union, only 5% CBE is allowed to be added to chocolate [116].

The key factor for synthesizing a CBE is retaining oleic acid at the *sn*-2 position of the TAGs. 1,3-Specific lipases such as those derived from *A. niger*, *Mucor javanicus*, *M. miehei*, *R. arrhizus*, *R. delemar*, and *Rhizopus niveus* are usually used for esterification of palmitic and stearic acids to the *sn*-1,3 positions of TAGs. It is also important to minimize acyl migration that usually occurs during interesterification, which would convert POP to PPO or SOS to SSO. This migration would have a great effect on the physical and functionality of final products. For example, PPO and SSO have higher melting points and display a faster crystallization rate than POP and SOS [116].

Industrial scale research into the application of lipase for modification of fats and oils began in the early 1980s at Unilever and Novozyme companies [118]. Back in 1981, Unilever published a patent on acidolysis of PMF, rich in POP with stearic acid using a 1,3-specifc lipase [119]:

$$\mathrm{POP} + \mathrm{S} \leftrightarrow \mathrm{POS} + \mathrm{SOS}$$

$$\mathrm{OOO} + \mathrm{S} \leftrightarrow \mathrm{SOO} + \mathrm{SOS}$$

High-oleic oils could be a suitable base stock for acidolysis or interesterification reactions. For example, Kim et al. [120] used enzymatic transesterification of HOSFO with a mixture of ethyl palmitate and ethyl stearate esters (EEs) to produce cocoa butter equivalents. In their study, response surface methodology (RSM) was used to optimize reaction conditions for maximizing total POS contents while minimizing DAG formation and acyl migration. At the end of the reaction, the amount of POS and DAG in the products were 25.1% and 9.4%, respectively. Since a higher DAG content in CBE leads to a reduction in the crystallization rate of cocoa butter TAGs and a faster bloom development in chocolate, DAG content needs to be minimized. Xua et al. [121] showed that the most important factors that affect formation of DAGs during enzymatic interesterification were water content and lipase load, meanwhile increasing reaction temperature and time lead to formation of higher 1,3-DAGs contents compared to 1,2-DAGs.

In other research, a CBE was synthesized by using HOSFO and a mixture of stearic acid (53.4%) and palmitic acid (44.8%) in a solvent-free system using Lipozyme RM IM as the catalyst. The effects of substrate ratio, enzyme load, water content, reaction temperature, and time on the resulting SOS and SSO contents were studied. They found the highest SOS (59%) and lowest SSO (3%) contents were obtained at a 10% lipase load, 1% water content, 1:7 substrate mole ratio, 65°C reaction temperature, and 6 h reaction time [116]. A palm oil/fully hydrogenated soybean oil (FHSBO) blend was enzymatically interesterified to make cocoa butter equivalents by Abigor et al. [122]. The optimal mass ratio of palm oil/FHSBO in the blend (1.6/1 w/w) was determined based on the maximum production of POS at the end of reaction. Fractional distillation in acetone was used to remove high melting TAGs and silica chromatography was used to eliminate MAGs and DAGs. Under optimal conditions, the amount of POP, POS, and SOS produced was 11%, 39%, and 23%, respectively. The melting point of this CBE was 33.9°C [122].

Another product that is used in chocolate industry as anti-bloom agent is Bohenin (1,3-behenate 2-oleate, BOB). It is manufactured by the Fuji Oil Company in Japan by enzymatic acidolysis of triolein and behenic (22:0) acid or interesterification of triolein with behenic acid or behenic ethyl ester in the presence of a 1,3 stereospecific lipase [119]. Behenic acid can be produced from fully hydrogenated high-erucic-acid rapeseed oil after fat splitting and fractionation [115].

B. Medium-Chain Triacylglycerols

Structured lipids have important applications in health and nutrition. For example, they have been used in food products especially formulated for patients with HIV or impaired gastrointestinal function, liver disease, or congestive heart failure. These tailor-made structured lipids can also be used for people recovering from surgery, preterm infants, or infants with food allergies [123].

MCFAs defined as caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0) and, possibly, lauric acid (C12:0) can be naturally found in coconut and palm kernel oils. MCFAs are metabolized differently than long-chain fatty acids due to their smaller size and higher water solubility. MCFAs are transported directly via the portal vein in the stomach to the liver and metabolized immediately for energy, in a similar fashion as glucose. Studies have shown that consuming MSFAs can lead to control of obesity because as MCFAs are not re-esterified into TAGs and have little tendency to deposit as adipose fat in the body. Moreover, they are not carnitine-dependent for transport across the mitochondria, are not incorporated into chylomicrons, and are not readily stored in adipose tissue or accumulate in the reticuloendothelial (RES) system [124]. Although medium-chain triacylglycerols (MCTs) have these advantages, a minimum amount of long-chain fatty acids (preferably PUFA) need to be esterified along with MCFAs to provide a more balanced nutritional profile in enteral and parenteral food products and also to fulfill essential fatty acid requirements [117,123]. Consuming pure MCTs can cause gastrointestinal problems, such as abdominal pain and diarrhea. At high doses, they may even be toxic, leading to a condition known as ketosis or ketonemia [124,125].

During the past few years, different types of interesterification reactions (acidolysis, alcoholysis, interesterification) have been applied to the synthesis MCTs. Munio et al. studied the synthesis of

structured TAGs in two steps. The first step was ethanolysis of fish oil using 1,3-specific lipases (Novozym 435 from *C. antarctica* and *Lipase D* from *R. oryzae*) to obtain 2-MAGs with 90% recovery after purification by solvent precipitation and extraction (ethanol and hexane). The second step was esterification of 2-MAGs with caprylic acid using lipase D as catalyst to produce Ca-PUFA-Ca with 90% yield [126].

Acidolysis of fish oil with capric acid in hexane has been studied by Jennings and Akoh. They showed an average of 43 mol% incorporation of capric acid into fish oil, while EPA and DHA contents decreased to 27.8 and 23.5 mol%, respectively. But in a solvent-free reaction, about 32 mol% of capric acid was esterified, while EPA and DHA decreased to 33.2 and 28.3 mol%, respectively. The highest yield attained in this study was 65.4 mol%, at a molar ratio of 1:8 (fish oil/capric acid). For the solvent-free reaction, the optimal mol% capric acid incorporation (56.1 mol%) occurred at a molar ratio of 1:6 [127].

In another study, low-calorie MCT oil was produced by interesterification of tristearin and tricaprin in hexane using *R. miehei* lipase (IM 60) as the catalyst. After optimizing reaction parameters (1:1 mole ratio of tristearin to tricaprin, 10% w/w of lipase, and zero percent adding water), the amount of C10-C18-C10 TAGs was 44.3%, and only 2.3% tristearin was left, showing an excellent conversion and yield [21].

Enzymatic acidolysis of lard with capric acid was studied by Zhao et al. They ran the reaction in different nonpolar solvents using 5%–10% w/w lipase TL IM from *T. lanuginosus*. After a 24 h reaction at 50°C–55°C, results showed 37.4 mol% of capric acid incorporated into lard TAGs [125].

The enzymatic interesterification of tricaprylin (CCC) and trilinolenin (LnLnLn) was carried out to synthesize structured TAGs containing linolenic acid using a lipase from *R. miehei* (Lipozyme RM IM) in hexane. After optimization of the reaction, CLnC and CLnLn were the main TAGs in the final product [128]. The same type of products can be produced via an acidolysis reaction. For example, MLM type TAGs were synthesized by enzymatic acidolysis of virgin olive oil with caprylic and capric acids, catalyzed by the 1,3-selective *R. oryzae* lipase. The reaction was performed at 25°C and 40°C in both solvent-free media and in hexane. The highest incorporation of C8 (21.6%) and C10 (34.8%) was attained in solvent-free media at 40°C [129].

Many studies have shown that CLA has anti-carcinogenic, growth-promoting, anti-atherogenic, anti-diabetic, and lean body mass-enhancing properties [130]. CLA decreased benzo(a)pyrene-induced forestomach tumors by 50%. Consumption of 3.5 g CLA/day (for a 70 kg person) could protect the body from certain forms of cancer. Garcia et al. enriched milk fat by acidolysis of butter oil with CLA using *C. cylindracea*, *Pseudomonas* sp., *M. miehei*, and *C. antarctica* lipases. They increased the CLA content of milk fat from 0.6 to 15 g/100 milk fat [131]. γ-Linolenic acid (GLA) is an intermediate precursor of hormones such as prostaglandins, thromboxanes, and leukotrienes [119]. Evening primrose oil and Borage oil contain high amount of GLA. Research showed that GLA can modulate immune and inflammatory responses [132]. Consequently, there has been significant interest in synthesizing structured lipids containing GLA and MCFAs. In one study, acidolysis was carried out on a mixture of borage oil/caprylic acid (1:2, w/w) using immobilized *R. oryzae* lipase at 30°C. After ending the reaction, free fatty acids, MCTs, and partial acylglycerols (around 16% MAGs and DAGs) were removed by molecular distillation. The maximum CLnC content in the purified sample was 52.6 mol% [132].

Another interesting property of MCT oils is that they have less caloric per unit mass than regular fats and oils [133]. MCT oils can be used to formulate low-calorie foods. For example, a low-calorie structured lipid was produced by interesterification of tributyrin and methyl stearate, catalyzed by Lipozyme RM IM. The optimum reaction conditions obtained using RSM were a reaction time 6.5 h, substrate molar ratio (ME:TB) of 1.77:1, and a lipase amount of 10.3% at 65°C. At the end of the interesterification, MAGs, DAGs, and methyl esters were removed by molecular distillation. The maximum conversion of methyl stearate was 78.4% and only 5% tributyrin remained at the end of reaction. The SFC of the final product was similar to that of cocoa butter equivalents that displayed a sharp melting slope between 20°C and 32.5°C, decreasing from an SFC of 84.5% to 3.5%, respectively [133].

C. Human Milk Fat Substitute

Human milk contains 3%–5% fat, 0.8%–0.9% protein, 6.9%–7.2% carbohydrates, and 0.2% minerals, vitamin, and other minor substances. The main component in human milk fat is TAGs (>98%) that provide 50% of the total energy used by infants followed by phospholipids (0.4%–1.0%). Since the composition of human milk fat changes during different lactation periods, a typical fatty acid composition of human milk fat is capric acid (1%–2%), lauric acid (3%–7%), myristic acid (4%–9%), palmitic acid (20%–30%), palmitoleic acid (1%–3%), stearic acid (5%–9%), oleic acid (25%–35%), and linoleic acid (10%–20%). Also, long-chain polyunsaturated fatty acids (DHA, arachidonic acid [AA], docosapentaenoic acid, and EPA) can be found in human milk fat [134]. Studies have shown that MCFAs in human milk fat can protect infants from microorganisms, since they exhibit antiviral and antimicrobial properties [135]. Human milk fat TAG composition is distinctive from vegetable oil sources, since palmitic acid is mainly esterified to the *sn*-2 position (about 70%), while unsaturated fatty acids (mainly oleic and linoleic acids) are located at the *sn*-1,3 positions of the glycerol backbone (OPO and OPL). In the infants' digestive tract, TAGs are hydrolyzed to 2-MAGs and FFAs in the presence of gastric lipases. The 2-MAGs can be absorbed directly by the intestine [119].

A human milk fat substitute (HMFS) named Betapol® was developed by *Loders-Croklaan* in the Netherlands by enzymatic acidolysis of tripalmitin with oleic acid. This product contained 53.5% palmitic acid at the *sn*-2 position [119,135]. Although human milk fat has not been fully matched at the molecular level, many current research efforts are directed toward the synthesis of human milk fat analogues by different enzymatic reaction methods.

In order to mimic the fatty acid and TAG composition of human milk fat, a blend consisting of lard sunflower oil, canola oil, palm kernel oil, palm oil, algal oil, and a microbial oil at a 1:0.1:0.5:0.13:0.12:0.02:0.02 w/w ratio was prepared. The blended oil was then enzymatically interesterified by the addition of 11 (wt%) Lipozyme RM IM (1,3-regiospecific) at 60°C for 3 h. In the final TAG composition, 63% palmitic acid was esterified to the *sn*-2 position [136].

In another study, after screening the activity of two immobilized lipases from *T. lanuginosus* (TL IM) and *R. miehei* (RM IM) in three reactions (acidolysis, glycerolysis, and interesterification), lipase TL IM was selected as the catalyst for interesterification of tripalmitin with EPA ethyl esters to produce a HMFS. In the optimized reaction, a substrate molar ratio of 5 (EE/PPP), a lipase load of 20 wt%, and a reaction time of 20 h were used, and the maximum acyl incorporation obtained was 42% [137].

HMFSs were made using lard as a base stock via ethanolysis and interesterification with Novozyme 435 and Lipozyme RM IM in a two-step reaction. First, after ethanolysis of lard, 2-palmitoyl monoacylglycerol (90% purity) was obtained. Second, 2-palmitoyl monoacylglycerol, oleic acid, linoleic acid, and lard were dissolved in hexane and reacted in the presence of Lipozyme RM IM to produce a HMFS. Optimum reaction conditions were 7 mmol of lard, 3 mmol of 2-P-MAG, 5.2 mmol of oleic acid, 3.5 mmol of linoleic acid, 10 mL of hexane, and 10 wt% of Lipozyme RM IMR at 37°C for 6 h. Oxidative stability of the HMFS was compared to a commercial powdered formula for infants by an auto-oxidation test. The test showed HMFS had a greater oxidative stability than the powdered milk [138].

A mathematical model for the evaluation of HMFSs was developed using FA and TAG composition data of different human milk fats by RP-HPLC-APCI-MS at different lactation periods. After validating the model with selected fats and oils with specific fatty acid and TAG compositions and finding the degree of similarity, this model could be used for finding compatibility and similarity of commercially formulated HMFS based on their FA and TAG composition with human milk fat [134].

Although lard is considered as a good choice for formulation of HMFS, vegetable oil–based stocks have a greater appeal for diverse reasons. A good choice to replace lard could be palm stearin and its fractions, since they have a high amount of palmitic acid (45%–75%). In one study, palmitic-acid-enriched fat (produced by acidolysis of palm stearin with palmitic acid) was used as base

stock for enzymatic acidolysis with oleic acid (molar ratio of oleic acid to palmitic acid enriched fat was 6:1) to make a high OPO TAG [139]. Another example is a study by Pina-Rodriguez and Akoh. They developed a two-step enzymatic interesterification procedure to make HMFS. In the first step, amaranth oil containing 18.6%–23.4% palmitic acid and ethyl palmitate at a 1:4 molar ratio were interesterified using 10% Novozym 435 at 60°C for 3 h to increase the amount of palmitic acid at the *sn*-2 position of TAGs. In the second step, DHA was incorporated into the *sn*-1,3 positions of TAGs using Lipozyme RM IM. At the end of the reaction, the structured lipid contained 20% palmitic acid in the *sn*-2 position and 2.4% DHA in the *sn*-1,3 positions of TAGs [140].

Another potential strategy to synthesis HMFS is by saponifying oils of interest and interesterifying them with a fat rich in palmitic acid at the *sn*-2 position. A good example of this method is research by Pande et al. In this study, extra-virgin olive oil free fatty acids and DHA single-cell oil free fatty acids were produced by saponification of the oils followed by acidification of the soaps using hydrochloric acid. Enzymatic acidolysis between the FFA mixture and tripalmitin was then carried out using Lipozyme TL IM for 24 h at 65°C. The final structured lipids had about 60 mol% palmitic acid at the *sn*-2 position and oleic acid positioned mainly at the *sn*-1,3 positions (OPO). The amount of incorporated DHA in the HMFS was from 5.9 to 7.5 mol% [141].

In another study, a structured lipid that could be used as a HMFS was prepared in a two-step reaction. HOSFO and palm stearin were interesterified using Lipozyme TL IM as catalyst to increase the amount of palmitic acid in the *sn*-2 position (higher OPO content). In the second step, tricaprin was interesterified with the TAGs produced in the first step with the aim of increasing the amount of capric acid in the *sn*-1,3 positions. The final structured lipid contained 20% palmitic acid, of which nearly 40% was located at the *sn*-2 position and total capric acid content was 21%. The main TAGs in the final product were OPO and CLC [135].

D. Other Applications of Structured Lipids

Research has shown that symmetrical TAGs with LC-PUFAs at the *sn*-2 position have many advantages in clinical nutrition and health [4]. Tang et al. synthesized symmetrical TAGs in a two-step reaction. A fungal oil rich in AA (48.8%) was used to make 2-MAGs with AA at the *sn*-2 position via an ethanolysis reaction using Novozym 435 as the catalyst. In the second step, a symmetrical TAGs was synthesized by enzymatic transesterification of 2-MAGs with vinyl palmitate (as acyl donor instead of palmitic acid) to synthesize pure PAP. The highest obtained was 89% [142]. Increasing the oxidative stability of vegetable oils by incorporation of oleic acid, or removal of linoleic and linolenic acids to make stable frying oils is another target for the synthesis of structured lipids. Lin and Huey used different lipases to run acidolysis reactions between oleic acid and palm olein in order to increase the oxidative stability and also enhance the nutritional properties of palm olein.

The molar ratio of palm olein/oleic acid used in this study was 3:1 and 5% w/w lipase was used as the catalyst. Results showed that the OLO/OOL content was increased at least four times and OOO content was increased at least threefold. The amount of oleic acid in palm olein increased from 49% before acidolysis to 56% at the end of the reaction [143].

Ghazali et al. [4] studied the effects of interesterification of neat palm olein with 1,3-specific and nonspecific lipases on TAG profiles before and after interesterification. They found that using both types of enzymes lead to the formation of PPP, which was not detected in palm olein prior to interesterification, and also lead to an increase in OOO (1.3–2.1-fold), OOL (1.7–4.5-fold), and OLL (1.7–4.3-fold) contents. SOS was another TAG that was not detected in palm olein before interesterification. Upon interesterification catalyzed by *R. miehei* and *Pseudomonas* lipases, SOS content increased to 1.3% and 1.6%, respectively. Since the new high melting TAG fraction formed can be removed by solvent fractionation, the remaining TAGs will have higher amounts of oleic and linoleic acid compared to the original palm olein.

E. Zero-*trans* Margarine and Shortening

For formulation of table margarine or shortening, the presence of a high melting fraction is necessary. Partially hydrogenated oils have been used as hardstocks for many years, but this is not acceptable, or allowed, nowadays, and zero *trans* alternatives are necessary. Using naturally highly saturated oils such as palm oil and animal fat, or fully hydrogenated oils would be a good choice, if it was not for their lack of functionality in their native state.

For example, different ratios of palm stearin/coconut oil, soybean oil/fully hydrogenated soybean oil, palm stearin/palm kernel olein, palm stearin/soybean oil, palm stearin/palm kernel oil/sunflower oil, palm stearin/sunflower oil, low-erucic-acid rapeseed oil/tallow, butterfat/canola oil, and lard/HOSFO have been enzymatically interesterified to create different base stock fats for margarines and shortening [113,144–147].

Table margarines should be solid in the refrigerator, but spread easily upon removal from refrigeration and melt quickly in the mouth. The SFC at specific temperatures is a good predictor of spreadability of margarine. The hardness of margarine at refrigeration temperatures can be predicted by measuring the SFC content at 2°C and 10°C, while the SFC at 25°C provides an indication of plasticity at room temperature. Enzymatic interesterification is an appropriate tool for modification of oils to make plastic fats suitable for margarine and shortening manufacture [119].

Many studies have been carried out using enzymatic interesterification for the manufacture of zero-*trans* margarine. *T. lanuginosus* lipase was used to interesterify corn oil and tristearin at a 90:10 (w/w) ratio at 45°C. After 30 min of reaction, the amount of tristearin decreased from 6% to 0.5%, while the plastic range of the product was increased [148]. In another research, a blend of 70:30% w/w palm stearin/soybean oil was used as the base stock. After enzymatic interesterification, the amount of solids at temperatures below 15°C decreased, while it increased at temperatures above 15°C [144]. In margarine formulations, the incorporation of long-chain polyunsaturated fatty acids such as EPA and DHA into base stocks by enzymatic interesterification has great potential to provide human health benefits, such as prevention of heart disease and the reduction of inflammatory conditions [146].

Chemical interesterification is also an alternative method for the modification of oils used in margarine production. Since the reaction temperature in this method is higher than that of enzymatic interesterification, and a strong alkali is used, chemically interesterified oils usually have lower oxidative stability. In this regard, a study showed that margarines produced from chemically interesterified fat had a higher PV after 4, 8, and 10 weeks compared to the margarines made using enzymatic interesterification. These differences were not caused by different contents of tocopherols in the hardstocks [149].

To compare the effects of chemical versus enzymatic interesterification on oxidative stability, a canola oil/tallow blend (6:4, w/w) was used for chemical and enzymatic interesterification. TAGs produced at the end of the reaction were separated by column chromatography into a pure TAG fraction, and a non-TAG fraction that contained free fatty acids and monoacylglycerol and DAGs. Then, the non-interesterified blend and the purified TAGs were oxidized and their oxidative stability was examined by DSC. Purified TAGs prepared after EIE had a higher oxidative stability compared to TAGs prepared by CIE. The authors concluded that while the starting blend showed higher oxidative stability, the presence of the non-TAG fraction in the interesterified products was the reason for the decreased oxidative stability [150]. Moreover, the tocopherols content in palm stearin/sunflower oil blend (20/80, w/w) was affected differently by CIE versus EIE. The tocopherol content decreased from 639 ppm in blended oil to 205 ppm in the CIE samples, while it only decreased to 603 ppm in EIE samples [151].

For formulation of bakery shortening, three blends made with fully hydrogenated soybean oil, canola oil, and palm stearin in the ratio of 15:20:65, 15:40:45, and 15:50:35 w/w/w were enzymatically interesterified. Melting points of the interesterified fats after completion of interesterification were 45.5°C, 42.3°C, and 39.3°C, respectively. SFCs of these products at specific temperatures were similar to those of four commercial shortenings. Moreover, fat crystals in all interesterified products were in the β′ polymorphic form [152].

VIII. CONCLUSIONS

Despite the benefits of using lipase-catalyzed interesterification, it is unlikely that it will replace chemical interesterification in the future. This is due to the higher cost associated with enzymatic interesterification and the low cost of products, such as margarine and shortening, that are currently produced using chemical interesterification. The main attraction of lipase-catalyzed interesterification reactions is in the specificity of individual lipases and their use for the development of novel fats and oils that cannot be produced by chemical means. Future applications will involve continued development of reduced-calorie products, enriched lipids, and structured lipids to target specific functionalities. In addition, research will continue in the area of the characterization of fatty acid specificities of new lipases particularly in the identification of a *sn*-2-specific lipase. In order for any of these new applications to be useful in the food industry, scale-up studies simulating industrial processes are necessary.

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35 Structured Lipids

Casimir C. Akoh and Byung Hee Kim

CONTENTS

I. INTRODUCTION

A. What Are Structured Lipids?

In a broad sense, structured lipids (SLs) are lipids that have been chemically or enzymatically modified from their natural biosynthetic form. In this definition of SLs, the scope of lipids includes triacylglycerols (TAGs) (the most common types of food lipids) as well as other types of acylglycerols, such as diacylglycerols (DAGs), monoacylglycerols (MAGs), and glycerophospholipids (phospholipids). The term "modified" means any alteration in the structure of the naturally occurring lipids. This definition includes the topics covered in Chapters 31, 32, and 34. In a narrower sense, and in many cases, SLs are specifically defined as TAGs that have been modified by incorporation of new fatty acids, restructured to change the positions of fatty acids, or the fatty acid profile,

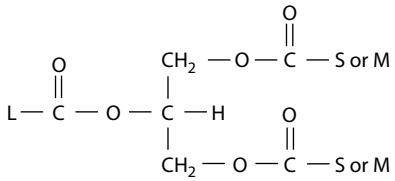

FIGURE 35.1 General structure of structured lipids: S, L, and M: short-, medium-, and long-chain fatty acid, respectively; the positions of S, L, and M are interchangeable.

from the natural state, or synthesized to yield novel TAGs. The fatty acid profiles of conventional TAGs are genetically defined and unique to each plant or animal species. In this chapter, SLs preferentially refer to TAGs containing mixtures of fatty acids (short-chain and/or medium-chain, plus long-chain) esterified to the glycerol moiety, preferably in the same glycerol molecule. Figure 35.1 shows the general structure of SLs; their potency increases if each glycerol moiety contains both short- (SCFAs) or medium-chain fatty acids (MCFAs) and long-chain fatty acids (LCFAs). SLs combine the unique characteristics of component fatty acids such as melting behavior, digestion, absorption, and metabolism to enhance their use in foods, nutrition, and therapeutics. Individuals unable to metabolize certain dietary fats or with pancreatic insufficiency may benefit from the consumption of SLs.

SLs are often referred to as a new generation of fats that can be considered as nutraceuticals: food or parts of food that provide medical or health benefits, including the potential for the prevention and treatment of diseases [1]. Sometimes, they are referred to as functional foods or in the present context, as functional lipids. Functional foods is a term used to broadly describe foods that provide specific health benefits beyond basic nutrition. Medical foods (medical lipids) are foods (lipids) developed to use under medical supervision to treat or manage particular disease or nutritional deficiency states. Other terms used to describe functional foods are physiologic functional foods, pharmafoods, and nutritional foods. The nomenclature is still confusing and needs to be worked out by scientists in this field. SLs can be designed for use as medical or functional lipids, and as nutraceuticals.

B. Rationale for Structured Lipid Development

Over the past several decades, long-chain triacylglycerols (LCTs), predominantly soybean and safflower oils, have been the standard lipids used in making fat emulsions for total parenteral nutrition (TPN) and enteral administration. The emulsion provides energy and serves as a source of essential fatty acids (EFAs). However, LCFAs are metabolized slowly in the body. It was then proposed that medium-chain triacylglycerols (MCTs) may be better than LCTs because the former are readily metabolized for quick energy. MCTs are not dependent on carnitine for transport into the mitochondria. They have higher plasma clearance, higher oxidation rate, improved nitrogen-sparing action, and less tendency to be deposited in the adipose tissue or to accumulate in the reticuloendothelial system (RES). One major disadvantage of using MCT emulsions is the lack of EFAs (18:2*n*-6). In addition, large doses of MCTs can lead to the accumulation of ketone bodies, a condition known as metabolic acidosis or ketonemia. It was suggested that combining MCTs and LCTs in the preparation of fat emulsions enables utilization of the benefits of both TAGs and may be theoretically better than pure LCT emulsions. An emulsion of MCTs and LCTs is called a physical mixture; however, a physical mixture is not equivalent to an SL. When MCTs and LCTs are chemically interesterified, the randomized product is called an SL. SLs are expected to be rapidly cleared and metabolized compared with LCTs.

For an SL to be beneficial, a minimum amount of LCFA is needed to meet EFA requirements. With the SL, LCFAs, MCFAs, and SCFAs can be delivered without the associated adverse effects

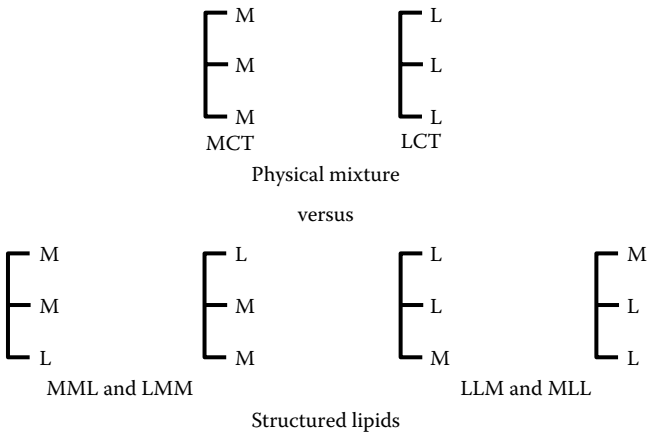

FIGURE 35.2 Structure of a physical mixture of medium-chain triacylglycerol and long-chain triacylglycerol, and structured lipid molecular species: M, medium-chain fatty acid; L, long-chain fatty acid. Note that physical mixture is not equivalent to structured lipid.

of pure MCT emulsions. This is especially important when intravenous administration is considered [2,3]. TAGs containing specific balances of medium-chain, *n*-3, *n*-6, *n*-9, and saturated fatty acids can be synthesized to reduce serum low-density lipoprotein (LDL) cholesterol and TAG levels, prevent thrombosis, improve immune function, lessen the incidence of cancer, and improve nitrogen balance [1,4]. Although physical mixtures of TAGs have been administered to patients, an SL emulsion is more attractive due to the modified absorption rates of the SL molecule. Figure 35.2 shows the difference between a physical mixture of two TAGs and SL pairs of molecular species.

SLs can be manipulated to improve their physical characteristics such as melting points. SLs are texturally important in the manufacture of plastic fats such as margarines, modified butters, and shortenings. Caprenin, an SL produced by Procter & Gamble Company (Cincinnati, OH), consists of 8:0–10:0–22:0; it has the physical properties of cocoa butter but only about half the calories. Benefat, originally produced as Salatrim (see Section II.B.2), consists of SCFAs (2:0–4:0) and LCFAs (18:0). Both products can be used as cocoa butter substitutes. Currently, they are manufactured through a chemical transesterification process. Due to the low caloric value of the SCFAs and the partial absorption of stearic acid on Salatrim, this product has strong potential for use as a low-calorie fat substitute in the future. The caloric content of Caprenin and Benefat is about 5 kcal/g (versus 9 kcal/g for a regular TAG). These SLs can also be manipulated for nutritive and therapeutic purposes, targeting specific diseases and metabolic conditions [4]. In the construction of SLs for nutritive and therapeutic use, it is important that the function and metabolism of various fatty acids be considered. This chapter focuses mainly on SLs and MCTs, emphasizing the use of enzymes for SL synthesis as an alternative to chemical processing.

II. PRODUCTION OF STRUCTURED LIPIDS

A. Sources of Fatty Acids for Structured Lipid Synthesis

SLs have been developed to optimize the benefit of fat substrate mixtures [5]. A variety of fatty acids are used in the synthesis of SLs, taking advantage of the functions and properties of each to obtain maximum benefits from a given SL. These fatty acids include SCFAs, MCFAs, polyunsaturated fatty acids (PUFAs), saturated LCFAs, and monounsaturated fatty acids (MUFAs). Table 35.1 gives the suggested levels of some of these fatty acids in SLs intended for clinical applications. The component fatty acids and their position in the TAG molecule determine the functional and physical

TABLE 35.1
Suggested Optimum Levels of Fatty Acids for Structured Lipids in Clinical Nutrition

Fatty Acid	Levels and Function
n-3	2%–5% to enhance immune function, reduce blood clotting, lower serum triacylglycerols, and reduce risk of coronary heart disease
n-6	3%–4% to satisfy essential fatty acid requirement in the diet
n-9	Monounsaturated fatty acid (18:1*n*-9) for the balance of long-chain fatty acid
SCFAs and MCFAs[a]	30%–65% for quick energy and rapid absorption, especially for immature neonates, hospitalized patients, and individuals with lipid absorption disorders

Source: Modified from Kennedy, J.P., *Food Technol*., 45, 76, 1991.

[a] Structured lipid containing short-chain fatty acids (SCFAs) and medium-chain fatty acids (MCFAs) as the main component.

properties, the metabolic fate, and the health benefits of the SL. It is therefore appropriate to review the function and metabolism of the component fatty acids.

1. Short-Chain Fatty Acids

The SCFAs range from 2:0 to 6:0. They occur ubiquitously in the gastrointestinal tract of mammals, where they are the end products of microbial digestion of carbohydrates [6]. In the human diet, SCFAs are usually taken in during consumption of bovine milk, which has a TAG mixture containing approximately 5%–10% butyric acid and 3%–5% caproic acid [7,8]. Butyric acid is found in butterfat, where it is present at about 30% of the TAG [9]. SCFAs, also known as volatile fatty acids, are more rapidly absorbed in the stomach than MCFAs because of their higher water solubility, smaller molecular size, and shorter chain length. Being hydrophilic, SCFAs have rates and mechanisms of absorption that are clearly distinguishable from those of lipophilic LCFAs [10]. SCFAs are mainly esterified to the *sn*-3 position in the milk of cows, sheep, and goats [7]. Under normal conditions, the end products of all carbohydrate digestion are the three major straight-chain SCFAs: acetate, propionate, and butyrate [11,12]; the longer SCFAs are generally found in smaller proportions except with diets containing high levels of sugar [13]. Microbial proteolysis followed by deamination also produces SCFA.

Using synthetic TAGs, Jensen et al. [14] have shown that human pancreatic gastric lipase can preferentially hydrolyze *sn*-3 esters over *sn*-1 esters in the ratio of 2:1. This enzyme has also shown some hydrolytic specificity for SCTs and MCTs, although later studies [15] reported in vitro optimal conditions for the hydrolysis of LCFAs by gastric lipase. Pancreatic lipase has been reported to attack only the primary ester group of TAG, independent of the nature of fatty acid attached [16]. Therefore, due to the positional and chain length specificity of the lipase, SCFAs attached to the *sn*-3 position of TAGs are likely to be completely hydrolyzed in the lumen of the stomach and small intestine. SCFAs are useful ingredients in the synthesis of low-calorie SLs such as Benefat because from heats of combustion, SCFAs are lower in caloric value than MCFAs and LCFAs. Examples of caloric values of SCFAs are as follows: acetic acid, 3.5 kcal/g; propionic acid, 5.0 kcal/g; butyric acid, 6.0 kcal/g; and caproic acid, 7.5 kcal/g.

2. Medium-Chain Fatty Acids and Triacylglycerols

MCTs contain 6:0–12:0 fatty acids esterified to glycerol backbone. MCTs serve as an excellent source of MCFAs for SL synthesis. MCTs are used for making lipid emulsions either alone or by blending with LCTs for parenteral and enteral nutrition. The MCT structure is given in Figure 35.3. MCTs are liquid or solid at room temperature, and their melting points depend on the fatty acid composition. MCTs are used as carriers for colors, flavors, vitamins, and pharmaceuticals [17]. MCFAs are

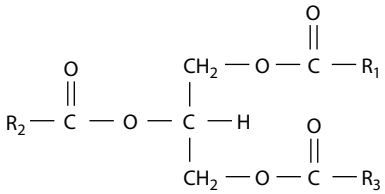

FIGURE 35.3 General structure of medium-chain triacylglycerols: R, alkyl group of medium-chain fatty acids (MCFAs) 6:0–12:0.

commonly found in kernel oils or lauric fats; for example, coconut oil contains 10%–15% 8:0–10:0 acid and is a raw material for MCT preparation [3]. MCT is synthesized chemically by direct esterification of MCFA and glycerol at high temperature and pressure, followed by alkali washing, steam refining, molecular distillation, and further purification. Enzymatically, MCTs have been synthesized with immobilized *Mucor miehei* lipase in a solvent-free system [18]. MCFAs have a viscosity of about 25–31 cP at 20°C and a bland odor and taste; as a result of the saturation of the fatty acids, they are extremely stable to oxidation [3]. MCTs have a caloric value of 8.3 kcal/g compared with 9 kcal/g for LCTs. This characteristic has made MCTs attractive for use in low-calorie desserts. MCTs may be used in reduced-calorie foods such as salad dressings, baked goods, and frozen dinners [17].

MCTs have several health benefits when consumed in mixtures containing LCTs. Toxicological studies on dogs have shown that consuming 100% MCT emulsions leads to the development of adverse effects in dogs, which include shaking of the head and vomiting and defecation, progressing to a coma [19]. It was theorized that these symptoms arose from elevated plasma concentration of MCFA or octanoate [19]. Some advantages of MCFA/MCT consumption include the following: (1) MCFAs are more readily oxidized than LCFAs; (2) carnitine is not required for MCT transport into the mitochondria, thus, making MCT an ideal substrate for infants and stressed adults [20]; (3) MCFAs do not require chylomicron formation; and (4) MCFAs are transported back to the liver directly by the portal system. Absorption of SLs is discussed later in this chapter.

MCTs are not readily reesterified into TAGs and have more than twice the caloric density of proteins and carbohydrates, yet can be absorbed and metabolized as rapidly as glucose, whereas LCTs are metabolized more slowly [3]. Feeding diets containing 20% and 30% lipid concentrations in weight maintenance studies indicate that MCTs may be useful in the control of obesity [21]. MCTs appear to give satiety and satisfaction to some patients. Thermogenesis of MCT may be a factor in its very low tendency to deposit as depot fat [3].

Some reports suggest that MCTs can lower both serum cholesterol and tissue cholesterol in animals and man, even more significantly than conventional polyunsaturated oils [22]. However, a study by Cater et al. [23] showed that MCTs indeed raised plasma total cholesterol and TAG levels in mildly hypercholesterolemic men fed MCT, palm oil, or high oleic acid sunflower oil diets. A suggested mechanism for the cholesterol-raising ability of MCTs is as follows: acetyl CoA, which is the end product of MCT oxidation, is resynthesized into LCFAs; the LCFAs then mix with the hepatic LCFA pool; and the newly synthesized LCFA may then behave like dietary LCFA. In addition, the 8:0 may serve as precursor for de novo synthesis of LCFAs such as 14:0 and 16:0, which were detected in the plasma TAG [23]. There were no differences in the high-density lipoprotein (HDL) cholesterol concentrations among the subjects.

Evidence is pointing against the advisability of using MCTs in weight control because the level of MCTs (50%) required to achieve positive reduction is unlikely in human diet [24]. An SL containing MCFA and linoleic acid bound in the TAG is more effective for cystic fibrosis patients than safflower oil, which has about twice as much linoleic acid as the SL [25]. It appears that mobility, solubility, and ease of metabolism of MCFAs were responsible for the health benefits of the SL in these cases. In the SL, MCFAs provide not only a source of dense calories but also potentially fulfill a therapeutic purpose.

3. Omega-6 Fatty Acids

A common *n*-6 fatty acid is linoleic acid (18:2*n*-6). Linoleic acid is mainly found in most vegetable oils and in the seeds of most plants except coconut, cocoa, and palm nuts. Linoleic acids have a reducing effect on plasma cholesterol and an inhibitory effect on arterial thrombus formation [26]. The *n*-6 fatty acids cannot be synthesized by humans and mammals and are therefore considered EFAs. The inability of some animals to produce 18:2*n*-6 is attributed to the lack of a D12 desaturase, required to introduce a second double bond in oleic acid. Linoleic acid can be desaturated further and elongated to arachidonic acid (20:4*n*-6), which is a precursor for eicosanoid formation, as shown in Figure 35.4.

Essentiality of fatty acids was reported by Burr and Burr in 1973 [27]. It is suggested that 1%–2% intake of linoleic acid in the diet is sufficient to prevent biochemical and clinical deficiency in infants. Adults consume enough 18:2*n*-6 in the diet, and deficiency is not a problem. The absence of linoleic acid in the diet is characterized by scaly dermatitis, excessive water loss via the skin, impaired growth and reproduction, and poor wound healing [28]. Nutritionists have suggested a 3%–4% content of *n*-6 fatty acids in SLs to fulfill the EFA requirements of SLs [1].

4. Omega-3 Fatty Acids

Omega-3 fatty acids are also known as EFAs because humans, like all mammals, cannot synthesize them and therefore must obtain them from their diets. The *n*-3 fatty acids are represented by linolenic acid (18:3*n*-3), which is commonly found in soybean and linseed oils and in the chloroplast of green leafy plants. Other polyunsaturated *n*-3 fatty acids (*n*-3 PUFAs) of interest in SL synthesis are eicosapentaenoic acid, 20:5*n*-3 (EPA), and docosahexaenoic acid, 22:6*n*-3 (DHA), which are commonly found in fish oils, particularly fatty fish. Children without enough *n*-3 PUFAs in their diet may suffer from neurological and visual disturbances, dermatitis, and growth retardation [29]. Therefore, *n*-3 PUFAs such as DHA must be included in their diet and in SL design.

SLs containing *n*-3 PUFAs and MCFAs have been synthesized chemically by hydrolysis and random esterification of fish oil and MCTs. They have been shown to inhibit tumor growth and to improve nitrogen balance in Yoshida sarcoma–bearing rats [30]. Lipases have been successfully used as biocatalysts to synthesize position-specific SLs containing *n*-3 PUFAs with an ability to improve immune function and reduce serum cholesterol concentrations [31,32]. EPA is important in

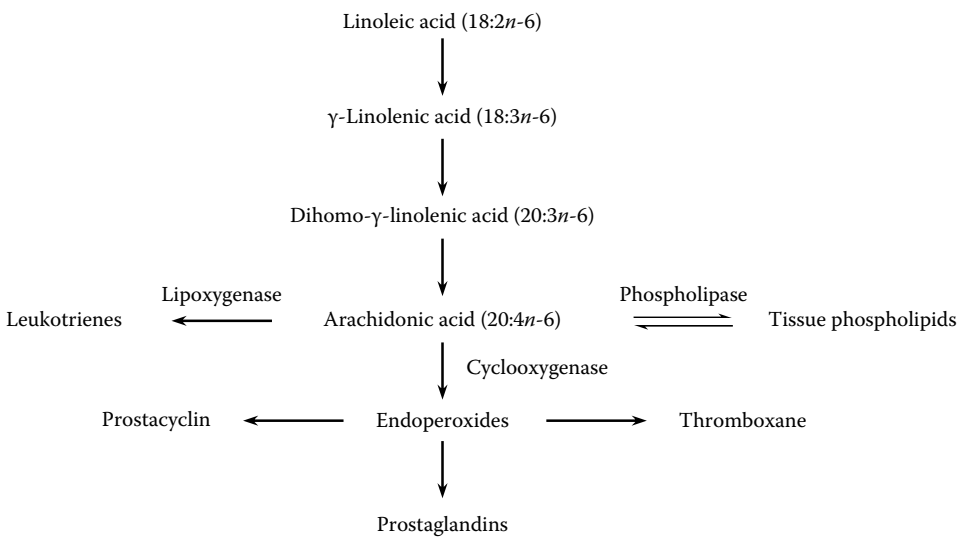

FIGURE 35.4 Pathway for eicosanoid biosynthesis.

preventing heart attacks primarily due to its antithrombotic effect [33]. It was also shown to increase bleeding time and to lower serum cholesterol concentrations [33]. Studies with nonhuman primates and human newborns suggest that DHA is essential for the normal functioning of the retina and brain, particularly in premature infants [34]. Other studies have shown that *n*-3 fatty acids can decrease the number and size of tumors and increase the time elapsed before the appearance of tumors [35].

The *n*-3 fatty acids are essential in growth and development throughout the life cycle of humans and therefore should be included in the diet. Nutritional experts consider a level of 2%–5% of *n*-3 fatty acids optimum in enhancing immune function in SL, as shown in Table 35.1. PUFAs of the *n*-3 series are antagonists of the arachidonic acid (20:4*n*-6) cascade (Figure 35.5). The mode of action of fish oil *n*-3 PUFAs on functions mediated by *n*-6 PUFAs is summarized in Table 35.2 [36]. The *n*-3 PUFAs inhibit tissue eicosanoid biosynthesis by preventing the action of δ-6 desaturase and cyclooxygenase/lipoxygenase enzymes responsible for the conversion of 18:2*n*-6 to 20:4*n*-6 and 20:4*n*-6 to eicosanoids, respectively. The amount of 18:2*n*-6 determines the 20:4*n*-6 content of tissue phospholipid pools and affects eicosanoid production. Eicosanoids are divided into prostanoids (prostaglandins, prostacyclins, and thromboxanes), which are synthesized via cyclooxygenase, and leukotrienes (hydroxy fatty acids and lipoxins), which are synthesized via lipoxygenase, as illustrated in Figure 35.4.

A proper balance of *n*-3 and *n*-6 fatty acids should be maintained in the diet and SL products. High concentrations of dietary 18:2*n*-6 may lead to increased production of immunosuppressive eicosanoids of the 2- and 4-series (prostaglandin E_2 [PGE_2], thromboxane A_2 [TXA_2],

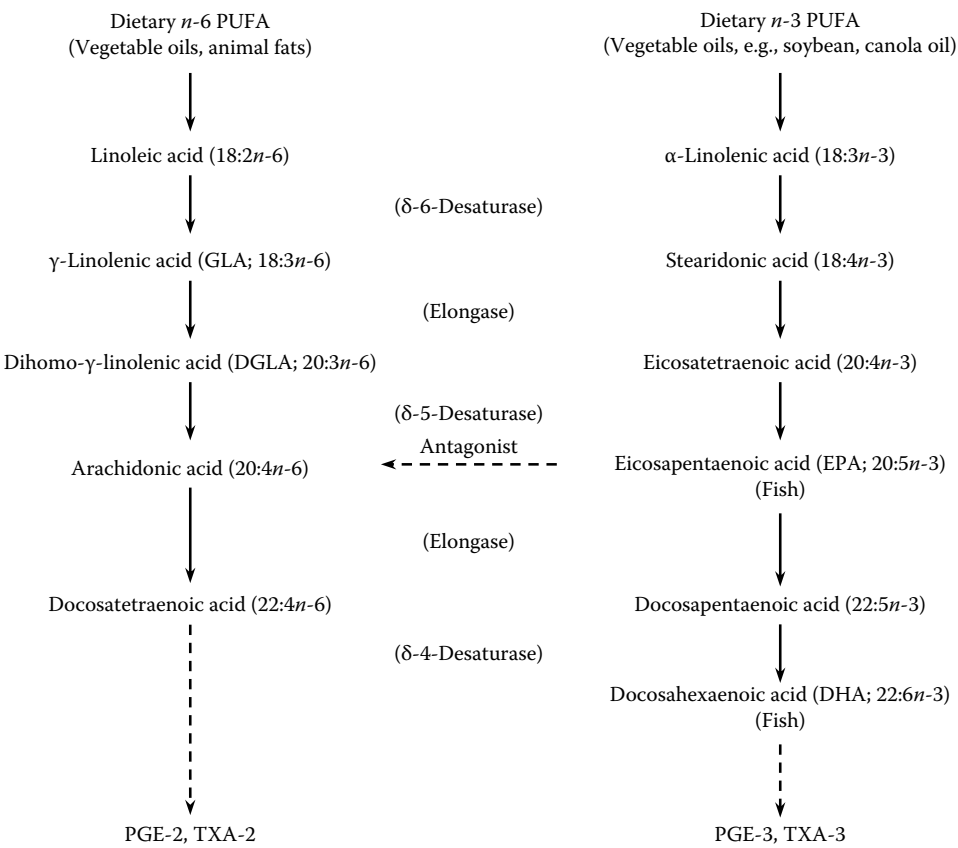

FIGURE 35.5 Pathways leading to the metabolism of dietary *n*-6 and *n*-3 polyunsaturated fatty acids.

TABLE 35.2
Mode of Action of *n*-3 PUFAs on Functions Mediated by *n*-6 PUFAs

Impair uptake of *n*-6 polyunsaturated fatty acid (PUFA).
Inhibit desaturases, especially δ-6-desaturase.
Compete with *n*-6 PUFAs for acyltransferases.
Displace arachidonic acid (20:4*n*-6) from specific phospholipid pools.
Dilute pools of free 20:4*n*-6.
Competitively inhibit cyclooxygenase and lipoxygenase.
Form eicosanoid analogs with less activity or competitively bind to eicosanoid sites.
Alter membrane properties and associated enzyme and receptor functions.

Source: Adapted from Kinsella, J.E., Sources of omega-3 fatty acids in human diets, in: Lees, R.S. and Karel, M. (eds.), *Omega-3 Fatty Acids in Health and Disease*, Dekker, New York, 1990.

leukotriene B_4 [LTB_4]). However, diets high in 20:5*n*-3 inhibit eicosanoid production and reduce inflammation by increasing production of TXA_3, prostacyclin (PGI_3), and LTB_5. Diets including *n*-3 PUFAs also increase HDL-cholesterol and interleukin-2 (IL-2) levels. On the other hand, they inhibit or decrease the levels of IL-1, LDL-cholesterol, and very low-density lipoprotein cholesterol (VLDL-cholesterol).

5. Omega-9 Fatty Acids

The *n*-9 fatty acids or MUFAs are found in vegetable oils such as canola, olive, peanut, and high-oleic sunflower as oleic acid (18:1*n*-9). Oleic acid can be synthesized by the human body and is not considered an EFA. However, it plays a moderate role in reducing plasma cholesterol in the body [26]. Oleic acid is useful in SLs for fulfilling the LCT requirements of SLs, as given in Table 35.1.

6. Long-Chain Saturated Fatty Acids

Generally, saturated fatty acids are believed to increase plasma cholesterol levels, but it has been claimed that fatty acids with chains 4–10 carbon atoms do not raise cholesterol levels [37,38]. Stearic acid (18:0) has also been reported not to raise plasma cholesterol levels [39]. TAGs containing high amounts of LCFAs, particularly stearic acid, are poorly absorbed in man, partly because stearic acid has a melting point higher than body temperature; they exhibit poor emulsion formation and poor micellar solubilization [40]. The poor absorption of saturated LCTs [40] makes them potential substrates for low-calorie SL synthesis. Indeed, Nabisco Foods Group used this property of stearic acid to make the group of low-calorie SLs called Salatrim (now Benefat) (see Section II.B.2), which consists of short-chain aliphatic fatty acids and LCFAs, predominantly 18:0 [41]. Caprenin, an SL produced by Procter & Gamble, contains 22:0, which is also poorly absorbed. An SL containing two behenic acids and one oleic acid has been used in the food industry to prevent chocolate bloom and to enhance fine crystal formation of palm oil and lard products [42].

B. Synthesis of Structured Lipids

1. Chemical Synthesis

Chemical synthesis of SLs usually involves hydrolysis of a mixture of MCTs and LCTs and then reesterification after random mixing of the MCFAs and LCFAs has occurred, by a process called transesterification (ester interchange). The reaction is catalyzed by alkali metals or alkali metal alkylates. This process requires high temperature and anhydrous conditions. Chemical transesterification results in desired randomized TAG molecular species, known as SLs, and in a number of unwanted products, which are difficult to remove. The SL product

consists of one (MLL, LML) or two (LMM, MLM) MCFAs, in random order (Figure 35.2), and small quantities of pure unreacted MCTs and LCTs [19].

The starting molar ratios of the MCTs and LCTs, and the source or type of TAG, can be varied to produce new desired SL molecules. Coconut oil is a good source of MCTs, and soybean and safflower oils are excellent sources of (*n*-6)-containing fatty acids for SL synthesis. Isolation and purification of the products is tedious because of unwanted coproducts. SLs are also produced by physical blending of specific amounts of MCTs and LCTs, except there is no exchange or rearrangement of fatty acids within the same glycerol backbone. When consumed, the blend will retain the original absorption rates of the individual TAG. Positional specificity of fatty acids on the glycerol molecule is not achieved by chemical transesterification, and this is a key factor in the metabolism of SLs. A possible alternative is the use of enzymes—specifically lipases—as described later in this chapter (Section II.B.3).

2. Examples of Commercial Products Prepared by Chemical Synthesis

a. Caprenin

Caprenin is a common name for caprocaprylobehenin, an SL containing 8:0, 10:0, and 22:0 esterified to glycerol moiety. It is manufactured from coconut, palm kernel, and rapeseed oils by a chemical transesterification process. The MCFAs are obtained from the coconut oil and the LCFAs from rapeseed oil. Caprenin's caloric density is 5 kcal/g compared with 9 kcal/g for a conventional TAG. Behenic acid is partially absorbed by the body and thus contributes few calories to the product. The MCFAs are metabolized quickly, like carbohydrates.

Procter & Gamble filed a Generally Recognized as Safe (GRAS) affirmation petition to the U.S. Food and Drug Administration (FDA) for use of Caprenin in soft candies such as candy bars, and in confectionery coatings for nuts, fruits, cookies, etc. Caprenin is made up of 95% TAGs, 2% DAGs, and 1% MAGs with 8:0 + 10:0 contributing 43%–45% and 22:0 40%–54% of the fatty acids. Caprenin has a bland taste, is liquid or semisolid at room temperature, and is fairly stable to heat. It can be used as a cocoa butter substitute. The structure of Caprenin is shown in Figure 35.6. Swift et al. [43] showed that Caprenin fed as an SL diet to male subjects for 6 days did not alter plasma cholesterol concentration but decreased HDL-cholesterol by 14%. However, the MCT diet raised plasma TAGs by 42% and reduced HDL-cholesterol by 15%.

b. Benefat/Salatrim

Benefat contains 2:0–4:0 and 18:0 esterified to glycerol moiety. Benefat is a brand name for Salatrim (short and long acyl triglyceride molecule), developed by Nabisco Foods Group (Parsippany, NJ), but now marketed as Benefat by Cultor Food Science (New York, NY). Benefat is produced by base-catalyzed interesterification of highly hydrogenated vegetable oils with TAGs of acetic, propionic, and butyric acids [44]. The product contains randomly distributed fatty acids attached to the glycerol molecule. Due to the random distribution of fatty acids, each preparation contains many molecular species. The ratio of SCFAs such as acetic, propionic, and butyric acids to LCFAs such as stearic acid can be varied to obtain SLs with physical and functional properties resembling those of conventional fats such as cocoa butter. The FDA accepted for filing in 1994 a GRAS affirmation petition by Nabisco.

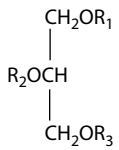

FIGURE 35.6 Structure of Caprenin (caprocaprylobehenin) with three randomized acyl groups: R_1, R_2, R_3, acyl part of capric acid, 10:0, caprylic acid, 8:0, and behenic acid, 22:0 in no particular order.

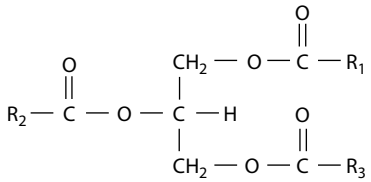

FIGURE 35.7 Structure of Benefat (brand name for Salatrim): R, alkyl part of 2:0–4:0 and C18:0; must contain at least one short-chain 2:0 or 3:0 or 4:0 and one long-chain (predominantly 18:0).

Benefat is a low-calorie fat like Caprenin, with a caloric availability of 5 kcal/g. The caloric availability of 2:0, 3:0, 4:0, glycerol, and LCFA in the Benefat molecule is 3.5, 5.0, 6.0, 4.3, and 9.5 kcal/g, respectively. Stearic acid is poorly or only 50% absorbed [45], especially if it is esterified to the *sn*-1 and *sn*-3 positions of the glycerol. Acetyl and propionyl groups in Benefat are easily hydrolyzed by lipases in the stomach and upper intestine and readily converted to carbon dioxide [46]. Benefat is intended for use in baking chips, chocolate-flavored coatings, baked and dairy products, dressings, dips, and sauces, or as a cocoa butter substitute in foods. The consistency of Benefat varies from liquid to semisolid, depending on the fatty acid composition and the number of SCFAs attached to the glycerol molecule. The structure of Benefat is given in Figure 35.7.

c. Others

Other commercially available chemically synthesized SLs and lipid emulsions are listed in Table 35.3. These include Captex, Neobee, and Intralipid 20%. Typical fatty acid profiles of selected SL products and MCTs are given in Table 35.4. Applications of these products vary depending on the need of the patient or the function of the intended food product. Enzymes can be used to custom-produce SLs for specific applications. Unfortunately, many enzymatically synthesized SLs are not commercially available, although the potential is there. This technology needs to be commercialized.

3. Enzymatic Synthesis

a. Lipases in Fats and Oils Industry

TAG lipases, also known as TAG acylhydrolases (EC 3.1.1.3), are enzymes that hydrolyze TAGs to DAGs, MAGs, free fatty acids (FFAs), and glycerol. They can catalyze the hydrolysis of TAGs and the transesterification of TAGs with fatty acids (acidolysis) or direct esterification of FFAs with glycerol [47–49]. Annual sales of lipases account for more than $20 million, which corresponds to less than 4% of the worldwide enzyme market estimated at $600 million [50]. Two main reasons for the apparent misconception of the economic significance of lipases are as follows: (1) lipases have

TABLE 35.3
Commercial Sources of Structured Lipids and Lipid Emulsions

Product	Composition	Source
Caprenin	8:0, 10:0, 22:0	Procter & Gamble Co., Cincinnati, Ohio
Benefat	2:0–4:0, 18:0	Cultor Food Science, New York, New York
Captex	8:0, 10:0, 18:2	ABITEC Corp., Columbus, Ohio
Neobee	8:0, 10:0, LCFA	Stepan Company, Maywood, New Jersey
Intralipid	20% soybean oil emulsion	KabiVitrum, Berkeley, California Pharmacia AB, Stockholm, Sweden
FE 73403	Fat emulsion of 8:0, 10:0, LCFA	Pharmacia AB, Stockholm, Sweden

Abbreviations: LCFA, long-chain fatty acid (may vary from 16:0 to18:3*n*-3); FE, fat emulsion.

TABLE 35.4
Fatty Acid Composition of Typical Lipid Emulsions and Medium-Chain Triacylglycerol

	Composition (%)		
Fatty Acid	**FE Emulsion 73403**	**Intralipid 20%**	**MCT**
8:0	27		65–75
10:0	10		25–35
12:0			1–2
16:0	7	13	
18:0	3	4	
18:1*n*-9	13	22	
18:2*n*-6	33	52	
18:3*n*-3	5	8	
Other	2	1	1–2

Abbreviation: MCT, medium-chain triacylglycerol.

been investigated extensively as a route to novel biotransformation and (2) diversity of the current and proposed industrial applications of lipases by far exceeds that of other enzymes such as proteases or carbohydrases [51].

Although enzymes have been used for several years to modify the structure and composition of foods, they have only recently become available for large-scale use in industry, mainly because of the high cost of enzymes. However, according to enzyme manufacturers, progress in genetics and in process technology may now enable the enzyme industry to offer products with improved properties and at reduced costs [51]. For lipases to be economically useful in industry, enzyme immobilization is necessary to enable enzyme reuse and to facilitate continuous processes. Immobilization of enzymes can simply be accomplished by mixing an aqueous solution of the enzyme with a suitable support material and removing the water at reduced pressure, after which small amounts of water are added to activate the enzyme. Suitable support materials for enzyme immobilization include glass beads, Duolite, acrylic resin, and Celite.

In spite of the obvious advantages of biological catalysis, the current level of commercial exploitation in the oleochemical industry is disappointing, probably because of the huge capital investments involved, and until recently, the high cost of lipase [51]. The introduction of cheap and thermostable enzymes should tip the economic balance in favor of lipase use for the commercial production of SL and lipid modifications.

b. Mode of Action of Lipases

TAG lipases are probably among the most frequently used enzymes in organic synthesis. This is in part because they do not require coenzymes and because they are stable enough in organic solvents at relatively high temperatures [52]. Lipases act at the oil–water interface of heterogeneous reaction systems. This property makes them well suited for reactions in hydrophobic media. Lipases differ from esterases in their involvement of a lipid–water interface in the catalytic process [53]. Some regions of the molecular structure responsible for the catalytic action of lipase are presumed to be different from those of ordinary enzymes that act on water-soluble substrates in a homogeneous medium [54]. Because lipases work at substrate–water interfaces, a large area of interface between the water-immiscible reaction phase and the aqueous phase that contains the catalyst is necessary to obtain reasonable rates of interesterification [55]. This is exemplified by the greater tendency for lipase to form off-flavors in homogenized milk than in unhomogenized milk.

Theoretical interpretations of the activation of lipase by interfaces can be divided into two groups: those assuming that the substrates can be activated by the presence of an oil–water interface and those assuming that the lipase undergoes a change to an activated form on contact with an oil–water interface. The first interpretation assumes higher concentrations of the substrate near the interface rather than in the bulk of the oil, and the second involves the existence of separate adsorption and catalytic sites for the lipase such that the lipase becomes catalytically active only after binding to the interface. More information on the action of microbial lipases is available in Chapter 34.

c. *Enzymes in Organic Solvents*

It is now commonly accepted that enzymes can function efficiently in anhydrous organic solvents. When enzymes are placed in an organic environment, they exhibit novel characteristics, such as altered chemo- and stereoselectivity, enhanced stability, and increased rigidity [56]. Lipases have also been shown to catalyze peptide synthesis, since they can catalyze the formation of amide links while lacking the ability to hydrolyze them [57]. Lipase can be used in several ways in the modification of TAGs [48]. In an aqueous medium, hydrolysis is the dominant reaction, but in organic media esterification and interesterification reactions are predominant. Lipases from different sources display hydrolytic positional specificity and some fatty acid specificity. The positional specificity is retained when lipases are used in organic media.

One application of lipases in organic solvents is their use as catalysts in the regiospecific interesterification of fats and oils for the production of TAGs with desired physical properties [58]. Lipases can also be used in the resolution of racemic alcohols and carboxylic acids by the asymmetric hydrolysis of the corresponding esters. An example of stereoselectivity of lipases is the esterification of menthol by *Candida cylindracea*. This enzyme was shown to esterify L-menthol while being catalytically inactive with the D-isomer [59,60]. Table 35.5 lists advantages of employing lipases in organic solvents for the modification of lipids as opposed to aqueous media [61].

d. *Strategies for the Enzymatic Production of Structured Lipids*

Various methods can be used for lipase-catalyzed production of SLs [4]. The method of choice depends to a large extent on the type of substrates available and the products desired.

Direct esterification: Direct esterification can be used for the preparation of SLs by reacting FFAs with glycerol. The major problem is that the water molecules formed as a result of the esterification reaction must be removed as they are formed to prevent them from hydrolyzing back the

TABLE 35.5
Advantages of Lipase Modification of Lipids in Organic Solvents

Increased solubility of nonpolar lipid substrates in organic solvents such as hexane and isooctane.
Shift of thermodynamic equilibria to the right in favor of synthesis over hydrolysis.
Reduction in water-dependent side reactions, since very little water is required by lipases in synthetic reactions.
Enzyme recovery is made possible by simple filtration of the powdered or immobilized lipase.
If immobilization is desired, adsorption onto nonporous surfaces (e.g., glass beads) is satisfactory; enzymes are unable to desorb from these surfaces in nonaqueous media.
Ease of recovery of products from low-boiling-point solvents.
Enhanced thermal stability of enzymes in organic solvents.
Elimination of microbial contamination.
Potential of enzymes to be used directly within a chemical process.
Immobilized enzyme can be reused several times.

Source: Modified from Dordick, J.S., *Enzyme Microb. Technol.*, 11, 194, 1989.

product, leading to low product yield. Direct esterification, rarely used in SL synthesis (except in the synthesis of DAG oils; see Section VI.B), is presented in equation form as follows:

Structured lipids

$$\text{Glycerol} + \text{MCFA} + \text{LCFA} \xrightarrow{\text{Lipase}} \text{SL} + \text{Water},$$

where
MCFA is the medium-chain fatty acid
LCFA is the long-chain fatty acid
SL is the structured lipid moieties

Transesterification–acidolysis: Acidolysis is a type of transesterification reaction involving the exchange of acyl groups or radicals between an ester and a free acid:

$$\text{MCT} + \text{LCFA} \xrightarrow{\text{Lipase}} \text{SL} + \text{Water},$$

$$\text{LCT} + \text{MCFA} \xrightarrow{\text{Lipase}} \text{SL} + \text{LCFA},$$

where
MCT is the medium-chain triacylglycerol
LCT is the long-chain triacylglycerol

Figure 35.8 shows an example of acidolysis reaction [62], in this case between caprylic acid and triolein. Shimada et al. [63] used acidolysis reaction catalyzed by immobilized *Rhizopus delemar* lipase to synthesize an SL containing 22:6*n*-3 (DHA) and caprylic acids. Product isolation is easy after acidolysis. FFAs are removed by distillation or by other appropriate techniques.

Transesterification–ester interchange: This reaction involves the exchange of acyl groups between one ester and another ester:

$$\text{MCT} + \text{LCT} \xrightarrow{\text{Lipase}} \text{SL},$$

$$\text{LCT} + \text{MCFAEE} \xrightarrow{\text{Lipase}} \text{SL} + \text{LCFAEE},$$

$$\text{MCT} + \text{LCFAEE} \xrightarrow{\text{Lipase}} \text{SL} + \text{MCFAEE},$$

where
MCFAEE is the medium-chain fatty acid ethyl ester
LCFAEE is long-chain fatty acid ethyl ester

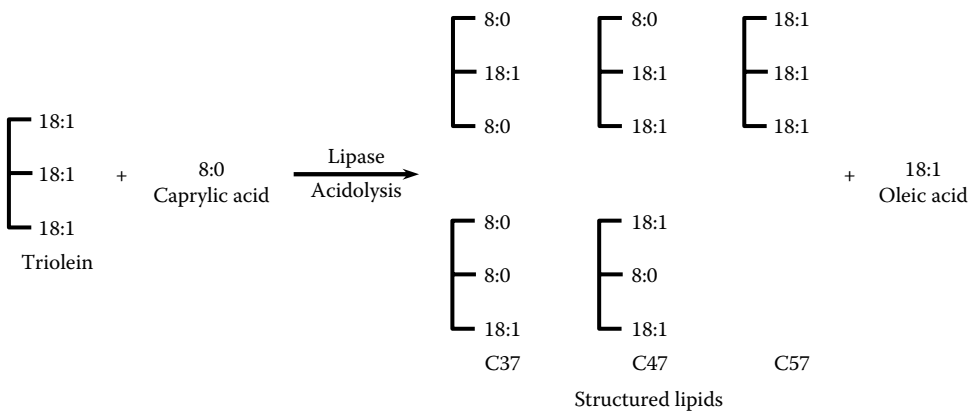

FIGURE 35.8 Reaction scheme showing acidolysis reaction in the synthesis of structured lipids from triolein and caprylic acid. (From Akoh, C.C. and Huang, K.H., *J. Food Lipids*, 2, 219, 1995.)

This method is widely used in lipid modifications and in the synthesis of SLs [4,47,64,65].

In a transesterification reaction, generally, hydrolysis precedes esterification. In all the preceding examples, SCTs and SCFAs can replace MCTs and MCFAs, respectively, or can be used in combination. Figures 35.9 and 35.10 give examples of the suggested strategies involving interchange reactions between a TAG (trilinolein) and a TAG (tricaproin) ester and between EPA ethyl ester and tricaprin, respectively. We have successfully used enzymes to synthesize position-specific SLs containing *n*-3 PUFAs with ability to improve immune function and reduce serum cholesterol [31,32].

e. *Factors That Affect Enzymatic Process and Product Yield*

Water: It is generally accepted that water is essential for enzymatic catalysis. This status is attributed to the role water plays in all noncovalent interactions. Water is responsible for maintaining the

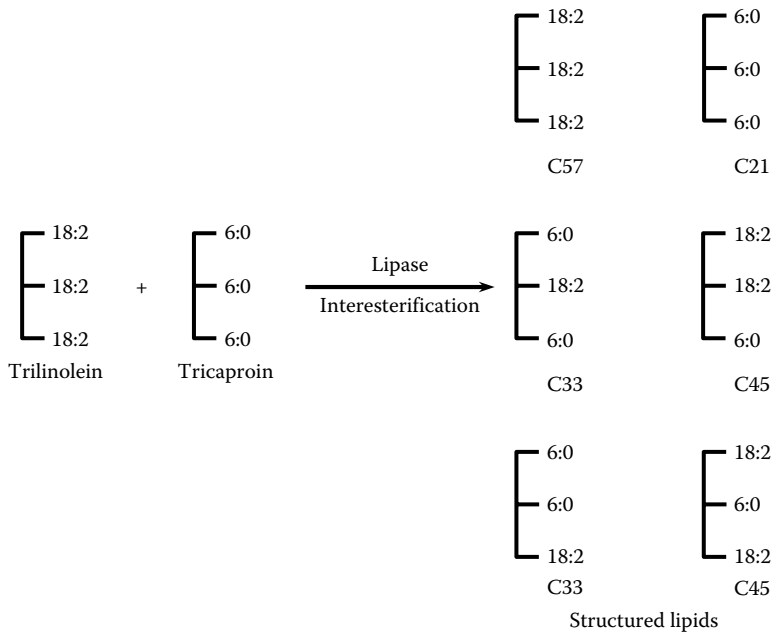

FIGURE 35.9 Ester interchange reaction between two triacylglycerols, trilinolein, and tricaproin in the enzymatic production of structured lipids.

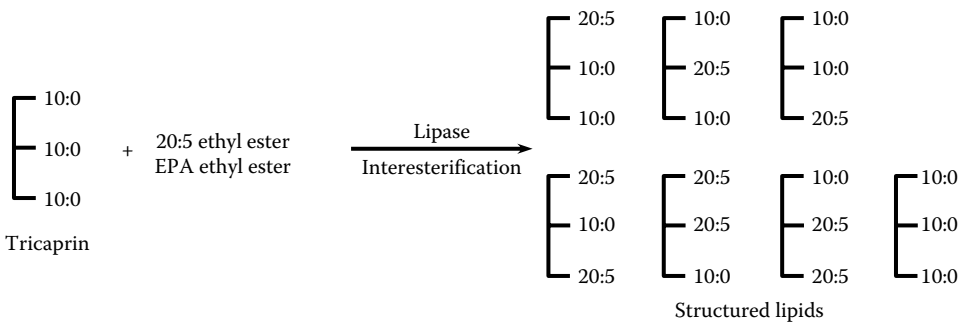

FIGURE 35.10 Ester interchange reaction in the production of structured lipids containing eicosapentaenoic acid (EPA) with tricaprin and EPA ethyl ester as substrates. An immobilized *Candida antarctica* lipase, SP 435, was the biocatalyst. Note EPA esterified to the *sn*-2 position.

active conformation of proteins, facilitating reagent diffusion, and maintaining enzyme dynamics [66]. Zaks and Klibanov [67] reported that for enzymes and solvents, tested enzymatic activity greatly increased with an increase in the water content of the solvent. The absolute amount of water required for catalysis for different enzymes varies significantly from one solvent to another [56]. Hydration levels corresponding to one monolayer of water can yield active enzymes [68]. Although many enzymes are active in a variety of organic solvents, the best nonaqueous reaction media for enzymatic reactions are hydrophobic, water-immiscible solvents [67,69,70]. Enzymes in these solvents tend to keep the layer of essential water, which allows them to maintain their native configuration, and therefore catalytic activity.

Solvent type: The type of organic solvent employed can dramatically affect the reaction kinetics and catalytic efficiency of an enzyme. Therefore, the choice of solvent to be used in biocatalysis is critical. Two factors affecting this choice are the extent to which the solvent affects the activity or stability of the enzyme and the effect of the solvent on the equilibrium position of the desired reaction [71]. The equilibrium position in an organic phase is usually different from that in water due to differential solution of the reactants. For example, hydrolytic equilibrium is usually shifted in favor of the synthetic product because the product is less polar than the starting materials [71]. The nature of the solvent can also cause inhibition or inactivation of enzymes by directly interacting with the enzymes. Here, the solvent alters the native conformation of the protein by disrupting hydrogen bonding and hydrophobic interactions, thereby, leading to reduced activity and stability [72].

Lipases differ in their sensitivity to solvent type. An important solvent characteristic that determines the effect of solvent in enzymatic catalysis is the polarity of the solvent. Solvent polarity is measured by means of the partition coefficient (P) of a solvent between octanol and water [73], and this is taken as a quantitative measure of polarity, otherwise known as log P value [74]. The catalytic activity of enzymes in solvents with $\log P < 2$ is usually lower than that of enzymes in solvents with $\log P > 2$. This is because hydrophilic or polar solvents can penetrate into the hydrophilic core of the protein and alter the functional structure [75]. They also strip off the essential water of the enzyme [67]. Hydrophobic solvents are less able to remove or distort the enzyme-associated water and are less likely to cause inactivation of enzymes [61].

In choosing a solvent for a particular reaction, two important factors must be taken into consideration: the solubility of the reactants in the chosen solvent and the need for the chosen solvent to be inert to the reaction [61]. Other factors that must be taken into account in determining the most appropriate solvent for a given reaction include solvent density, viscosity, surface tension, toxicity, flammability, waste disposal, and cost [61]. A report by Akoh and Huang [62] on the effect of solvent polarity on the synthesis of SLs using Lipozyme RM IM (immobilized lipase from *Rhizomucor miehei*) showed that nonpolar solvents such as isooctane and hexane produced 40 mol% of disubstituted SL, whereas a more polar solvent such as acetone produced 1.4% of the same SL. Claon and Akoh [76] found that with SP 435 lipase from *Candida antarctica*, a higher log P value does not necessarily sustain a higher enzyme activity. Some experimentation is therefore necessary in selecting solvents for enzymatic reactions.

pH: Enzymatic reactions are strongly pH dependent in aqueous solutions. Studies on the effect of pH on enzyme activity in organic solvents show that enzymes remember the pH of the last aqueous solution to which they were exposed [65,70]. That is, the optimum pH of the enzyme in an organic solvent coincides with the pH optimum of the last aqueous solution to which it was exposed. This phenomenon is called pH memory. A favorable pH range depends on the nature of the enzyme, the substrate concentration, the stability of the enzyme, the temperature, and the length of the reaction [77].

Thermostability: Temperature changes can affect parameters such as enzyme stability, affinity of enzyme for substrate, and preponderance of competing reactions [78]. Thermostability of enzymes is a major factor the industry considers before commercialization of any enzymatic process, mostly due to the potential for saving energy and minimizing thermal degradation. Thermostability of lipases varies considerably with enzyme origin: animal and plant lipases are usually less thermostable than microbial extracellular lipases [49].

Several processes that lead to the irreversible inactivation of enzymes involve water as a reactant [79]. This characteristic of enzymes makes them more thermostable in water-restricted environments such as organic solvents. Enzymes are usually inactivated in aqueous media at high temperatures. Several studies have been reported on the effect of temperature on lipase activity [64,76,80]. Zaks and Klibanov [80], who studied the effect of temperature on the activity of porcine pancreatic lipase, showed that in aqueous solution at 100°C, the lipase is completely inactivated within seconds, whereas in dry tributyrin containing heptanol, the lipase had a shelf life at 100°C of 12 h. These investigators concluded that in organic solvents, porcine pancreatic lipase remains rigid and cannot undergo partial unfolding, which causes inactivation. The heat stability of a lipase also depends on whether a substrate is present. This is because substrates remove excess water from the immediate vicinity of the enzyme, thus restricting its overall conformational mobility [81].

Most lipases in nonimmobilized form are optimally active between 30°C and 40°C [82]. Immobilization confers additional stability to the lipase compared with nonimmobilized lipase. Excellent reviews on the immobilization procedures and bioreactors for lipase catalysis were published recently [48,83,84]. The immobilization support must possess the following properties: high surface area to allow maximum contact with enzyme, high porosity to allow good flow properties, high physical strength, solvent resistance, high flow properties, and chemical and microbiological inertness [85,86].

Other factors: Other factors that affect yield of products are substrate molar ratio; enzyme source, activity, and load; incubation time; specificity of the enzyme to substrate type and chain length; and regiospecificity.

4. Examples of Commercial Products Prepared by Enzymatic Synthesis

a. Human Milk Fat Substitutes (HMFSs)

A typical human milk fat primarily comprises of oleic (18:1*n*-9, ~40% of the total fatty acids) and palmitic acids (16:0, ~25% of the total fatty acids) [87]. The *sn*-2 position of the human milk fat consists mainly of 16:0 (55%, w/w), whereas the *sn*-1,3 position is predominantly esterified with 18:1*n*-9 (45%, w/w) [87]. Because the human milk fat has such a unique distribution pattern of fatty acid, it gives infants several health benefits, such as improved fat (especially 16:0) absorption, improved mineral (especially calcium) absorption, softer stools, and less constipation [87,88].

HMFSs are SLs with a similar fatty acid composition and distribution as human milk fat. They are mad for use in infant formulas. Betapol, which was developed by Loders Croklaan (Channahon, IL, the Netherlands), is a representative example of commercial HMFSs. This product has been produced through the *sn*-1,3-specific lipase (e.g., Lipozyme RM IM)-catalyzed acidolysis of tripalmitin-rich fats (e.g., palm stearin) with 18:1*n*-9 obtained from high oleic sunflower acid oil, as illustrated in Figure 35.11 [89].

b. Cocoa Butter Equivalents (CBEs)

Cocoa butter, which is the main component of chocolate, is composed predominantly of symmetric monounsaturated TAGs including 1,3-dipalmitoyl-2-oleoyl-glycerol (POP; 15%–19%, w/w),

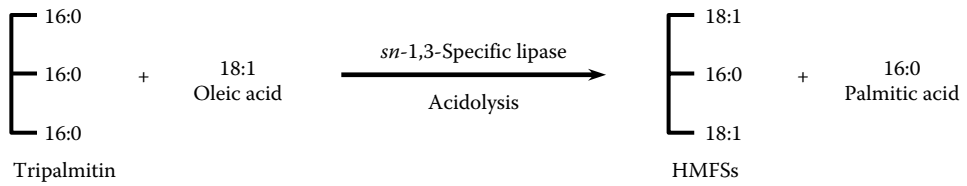

FIGURE 35.11 Acidolysis reaction in the production of human milk fat substitutes (HMFSs) from tripalmitin and oleic acid. Note that an *sn*-1,3-specific lipase was used as the biocatalyst for the reaction.

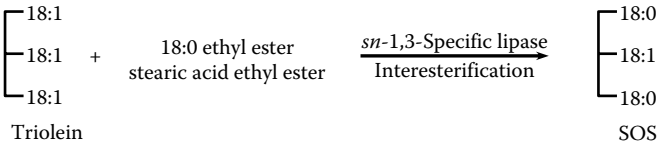

FIGURE 35.12 Ester interchange reaction in the production of 1,3-distearoyl-2-oleoyl-glycerol (SOS) with triolein and stearic acid ethyl ester as substrates. SOS-rich fats are used in the manufacture of cocoa butter equivalents. Note that an *sn*-1,3-specific lipase was used as the biocatalyst for the reaction.

1-palmitoyl-2-oleoyl-3-stearoyl-*rac*-glycerol (POS; 36%–41%, w/w), and 1,3-distearoyl-2-oleoyl-glycerol (SOS; 25%–31%, w/w) [90]. Large amounts (~80%, w/w) of symmetric monounsaturated TAGs in cocoa butter are responsible for its unique physical attributes, such as the dominating existence of stable β (or form V) crystals and rapid melting at body temperature, thereby, providing chocolate with gloss, snap, and a smooth mouthfeel [91].

CBEs are SLs with a similar TAG composition to cocoa butter but are produced from low-cost plant oils. They are used as an alternative to natural cocoa butter in the manufacturing of chocolate products because of high cost and fluctuations in the supply and demand for cocoa butter. Commercial CBEs (such as Melano from Fuji Oil, Tsukubamirai-City, Japan) are generally produced by blending fats rich in POP with SOS-rich fats. Enzymatically produced SLs are sometimes used as the SOS-rich fats in the manufacturing of CBEs. As shown in Figure 35.12, the SLs are prepared through the *sn*-1,3-specific lipase-catalyzed interesterification of triolein-rich plant oils (e.g., high oleic acid sunflower oil) with stearic acid ethyl esters [89].

5. Chemical versus Enzymatic Synthesis

The most useful property of lipases is their regio- and stereospecificity, which results in products with better defined and more predictable chemical composition and structure than those obtained by chemical catalysis. Potential advantages of using enzymes over chemical procedures may be found in the specificity of enzymes and the mild reaction conditions under which enzymes operate [92]. Enzymes form products that are more easily purified and produce less waste, and thus make it easier to meet environmental requirements [92]. Chemical catalysts randomize fatty acids in TAG mixtures and do not lead to the formation of specialty products with desired physicochemical characteristics [51]. The specificities of lipase have classically been divided into five major types: lipid class, positional, fatty acid, stereochemical, and combinations thereof [81]. Enzymes have high turnover numbers and are well suited for the production of chiral compounds important to the pharmaceutical industry.

Transesterification using *sn*-1,3-specific lipase results in SL products with fatty acids at the *sn*-2 position remaining almost intact. This is significant from a nutritional point of view because the 2-MAGs produced by pancreatic lipase digestion are the main carriers of fatty acids through the intestinal wall [93]. Fatty acids esterified at the *sn*-2 position are therefore more efficiently absorbed than those at the *sn*-1 and *sn*-3 positions. A TAG containing an EFA at the *sn*-2 position and SCFA or MCFA in the *sn*-1 and *sn*-3 positions has the advantage of efficiently providing an EFA and a quick energy source [94].

Some studies have shown that the rate of autoxidation and melting properties of TAGs can be affected by the position of unsaturated fatty acids on the glycerol molecule [95,96]. TAGs having unsaturated fatty acids at the *sn*-2 position of glycerol are more stable toward oxidation than those linked at the *sn*-1 and *sn*-3 positions.

The energy saved and minimizations of thermal degradation are probably among the greatest attractions in replacing the current chemical technology with enzyme biotechnology [51]. Table 35.6 shows some of the potential advantages of the enzymatic approach to SL design. Potential food applications of SL are listed in Table 35.7.

TABLE 35.6
Advantages of Enzymatic Approach to Structured Lipid Design

Position-specific SL (i.e., desirable fatty acids can be incorporated at specific positions of triacylglycerol).
Enzymes exhibit regioselectivity (discriminate based on bond to be cleaved), enantioselectivity (optical activity), chemoselectivity (based on functional group), and fatty acid chain length specificity.
Can design SL on case-by-case basis to target specific food or therapeutic use—custom synthesis.
Products with defined structure can be produced.
Novel products not possible by conventional plant breeding and genetic engineering can be obtained (e.g., by inserting specific fatty acid at the *sn*-2 position of glycerol molecule).
Mild reaction conditions.
Few or no unwanted side reactions or products.
Can control the overall process.
Ease of product recovery.
Add value to fats and oils.
Improve functionality and properties of fats.

TABLE 35.7
Potential Food Uses of Structured Lipids

Margarine, butter, spreads, shortening, dressings, dips, and sauces.
Improve melting properties of fats.
Cocoa butter substitute.
Confectioneries.
Soft candies.
As reduced- or low-calorie fats (e.g., Caprenin, Benefat).
Baking chips, baked goods.
Snack foods.
Dairy products.

6. Analysis of Structured Lipids

Figure 35.13 presents a purification and analysis scheme for enzymatically produced SLs. Method of analysis depends on whether the SL is synthesized by acidolysis or by interesterification reaction. The crude SL product can be analyzed with silica gel G or argentation $AgNO_3$ (based on unsaturation), thin-layer chromatography (TLC), gas–liquid chromatography (GLC) of the fatty acid methyl or ethyl esters for fatty acid profile, and by reversed-phase high-performance liquid chromatography (RP-HPLC) of molecular species based on equivalent carbon number (ECN) or total carbon number (TCN). A typical HPLC chromatogram of SL products is shown in Figure 35.14.

Other methods of typical lipid analysis described in this book can be applied to studies of SLs. The choice of fractionation or purification technique depends on substrate or reactant types, products formed, overall cost, and whether a small-scale or large-scale synthesis was employed. The need for improved methodologies for the analysis of SCFA and MCFA components of SLs is emphasized here because of their volatility during extraction and GLC analysis.

a. Stereospecific Analysis

Figure 35.15 shows the stereochemical configuration of a TAG molecule, with *sn* notation indicating the stereochemical numbering system. The positional distribution of SFCA, MCFA, and LCFA on the glycerol moiety of SL is important in relation to the physical and functional properties of the SL and its metabolism. As indicated in Section III, the absorption and transport pathway of the

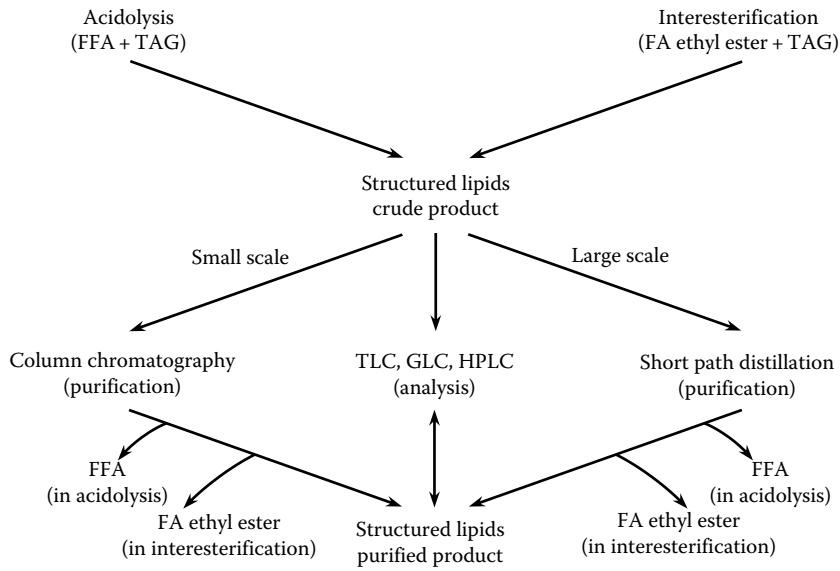

FIGURE 35.13 Purification and analysis scheme for enzymatically produced structured lipids.

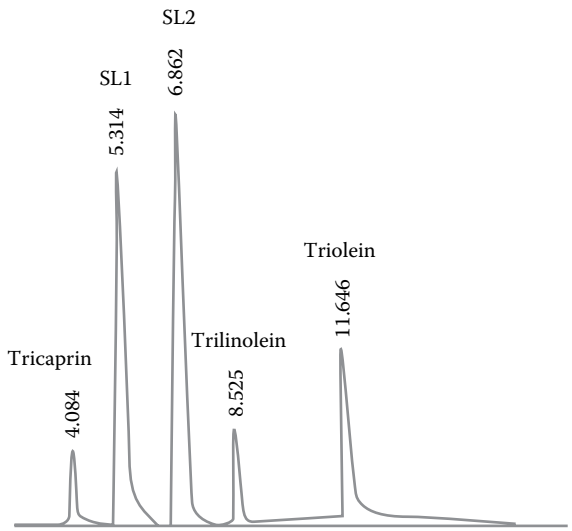

FIGURE 35.14 High-performance liquid chromatographic separation of structured lipid products from the reactants using a reversed-phase column: SL1, structured lipid containing two medium-chain fatty acids; SL2, structured lipid containing one medium-chain fatty acid. Trilinolein and tricaprin were the reactants, and triolein was the internal standard.

SL depend somewhat on the fatty acid at the *sn*-2 position. In most vegetable oils, unsaturated fatty acids occupy the *sn*-2 position, and saturated fatty acids are located in the *sn*-1 and *sn*-3 positions [97–100]. The *sn*-2 position of TAG is determined by pancreatic lipase hydrolysis of the fatty acids at the *sn*-1 and *sn*-3 positions, followed by GLC analysis of the 2-MAG fatty acid methyl or ethyl ester. Detailed stereospecific analysis of the fatty acids at all three positions of the glycerol molecule was excellently reviewed by Small [98] and is not discussed in detail here. ^{13}C-NMR was used to determine acyl position of fatty acids on glycerol molecule [101].

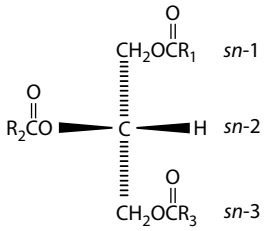

FIGURE 35.15 Stereochemical configuration of triacylglycerols or structured lipids with *sn* notation indicating stereochemical numbering of the carbon atoms of glycerol moiety. When the carbon in the 2-position is in the plane of the page and the 1- and 3-carbons behind the plane of the page, if the OH on the 2-position of glycerol is drawn to the left, the top carbon becomes 1 and the bottom becomes 3. Thus, a structured lipid with octanoic acid on the 1-position, oleic acid on the 2-position, and decanoic acid on the 3-position is named *sn*-glycerol-1-octanoate-2-oleate-3-decanoate.

Grignard reagent or Grignard degradation [102,103] is useful in obtaining the complete stereochemical structure of any TAG following pancreatic hydrolysis. In general, phospholipid derivatives (phosphatidylcholine, PC) of 1,2-DAG and 2,3-DAG are made by reacting with phospholipase A_2 (PLA_2). Since the *sn*-2 fatty acid is known, chemical analysis of the 2,3-diacyl-PC PLA_2 hydrolysis product gives the fatty acid at the *sn*-3 position. Similarly, chemical analysis of the 1,2-DAG hydrolysis product of PLA_2 gives the fatty acid at the *sn*-1 position. Alternatively, pancreatic hydrolysis of the 1,2-DAG followed by chemical analysis can give the fatty acid at the *sn*-1 position, since this enzyme is *sn*-1,3 specific.

III. ABSORPTION, TRANSPORT, AND METABOLISM OF STRUCTURED LIPIDS

The influence of TAG structure on lipid metabolism has been the subject of recent reviews and research efforts [97,98,104–106]. SLs may be targeted for either portal or lymphatic transport. In one widely accepted pathway, 2:0–12:0 fatty acids are transported via the portal system and 12:0–24:0 via the lymphatic system [2]. There is growing evidence that MCFAs may indeed be absorbed as 2-MAG, especially if they are esterified to the *sn*-2 position of the SL. The rate of hydrolysis at the *sn*-2 position of TAG is very slow, and as a result the fatty acid at this position remains intact as 2-MAG during digestion and absorption. Indeed, close to 75% of *sn*-2 fatty acids are conserved throughout the process of digestion and absorption [107].

LCTs are partially hydrolyzed by pancreatic lipase and absorbed slowly as partial acylglycerols in mixed micelles [98]. The resulting LCFAs are reesterified and incorporated into chylomicrons in the enterocyte, whereupon they enter the lymphatics to reach the general circulation through the thoracic duct. However, MCTs are nearly completely hydrolyzed and absorbed faster, mainly as FFAs and rarely as 2-MAGs. These MCFAs are then transported as FFAs bound to serum albumin in portal venous blood.

Figure 35.16 shows a proposed modified pathway for MCT, LCT, and SL metabolism. The metabolism of an SL is determined by the nature and position of the constituent fatty acids on the glycerol moiety. This may account for the differences in the pathway of absorption: lymphatics versus portal.

Evidence for lymphatic absorption of MCFAs and storage in adipose tissue is accumulating [108–112]. Jensen et al. [112] observed the presence of more 10:0 than 8:0 in the lymph of canine model fed an SL containing MCTs and fish oil versus its physical mixture, despite an overall ratio of 10:0–8:0 of 0.3 in their diets. Analysis of the SL molecular species revealed that MCFAs in lymph were present as mixed TAGs, suggesting that the MCFAs at the *sn*-2 position may account for the improved absorption. The 2-MAGs apparently were reesterified with endogenous or circulating LCFAs and subsequently absorbed through the lymphatic system. In addition, feeding high levels of MCTs can lead to lymphatic absorption and presence of MCFAs in the chylomicrons.

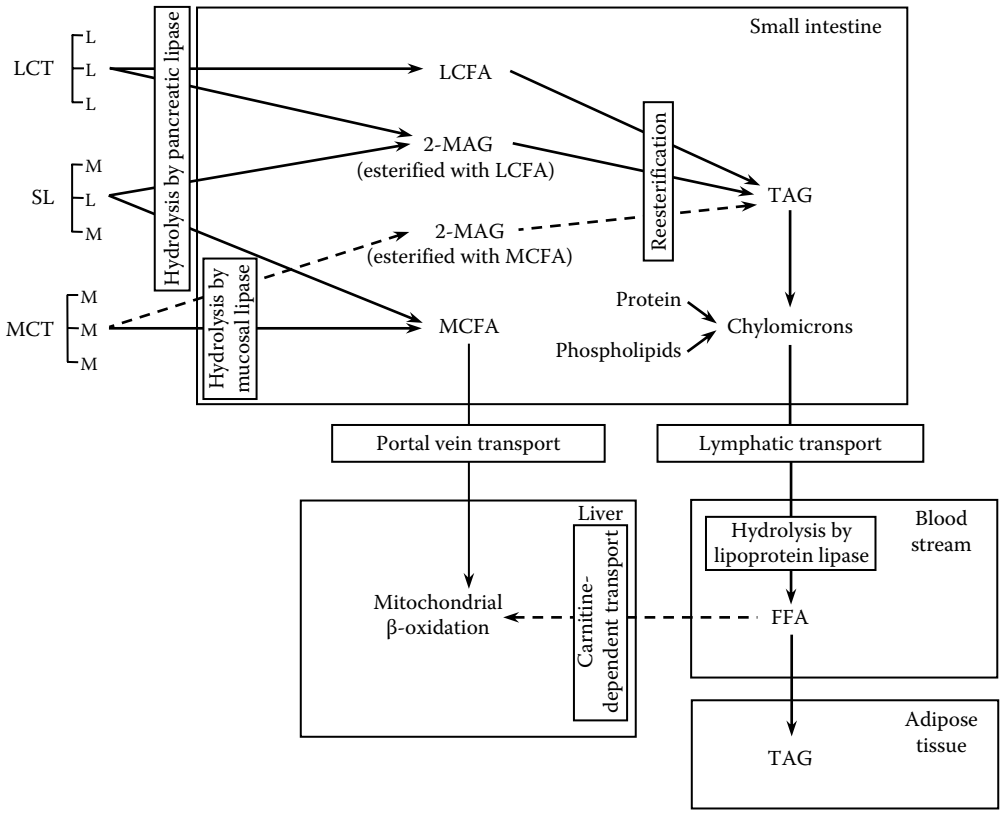

FIGURE 35.16 Proposed modified metabolic pathways for medium-chain and long-chain triacylglycerols and structured lipids.

Enhanced absorption of 18:2*n*-6 was observed in cystic fibrosis patients fed SL containing LCFAs and MCFAs [94,113]. Rapid hydrolysis and absorption of an SL containing MCFAs at the *sn*-1 and *sn*-3 positions and an LCFA at the *sn*-2 position has been reported [94,114,115]. To improve the absorption of any fatty acid, its esterification to the *sn*-2 position of the glycerol moiety is suggested. Mok et al. [116] reported that the metabolism of an SL differs greatly from that of a similar physical mixture. The purported benefit of fish oil *n*-3 PUFAs can be attributed to their absorption as 2-MAGs. This factor is important in the construction of novel or designer SL molecules for food, therapeutic, and nutritional use.

IV. NUTRITIONAL AND MEDICAL APPLICATIONS

SLs can be synthesized to target specific metabolic effects or to improve physical characteristics of fats. An SL made from fish oil and MCTs was compared with conventional LCTs and found to decrease tumor protein synthesis, reduce tumor growth in Yoshida sarcoma–bearing rats, decrease body weight, and improve nitrogen maintenance [30]. In addition, the effects of fish/MCT on tumor growth were synergistic with tumor necrosis factor (TNF). A similar study by Mendez et al. [117] compared the effects of an SL (made from fish oil and MCFAs) with a physical mixture of fish oil and MCTs and found that the SL resulted in improved nitrogen balance in animals, probably because of the modified absorption rates of SL. Gollaher et al. [118] reported that the protein-sparing action associated with SL administration is not seen when the SLs provide 50% of protein calories and suggested that the protein-sparing action of SLs may be dependent on the ratio of MCTs to LCTs used to synthesize the SL.

TABLE 35.8
Potential and Reported Benefits of Structured Lipids

Benefit	References
Superior nitrogen retention	[116]
Preservation of reticuloendothelial system (RES) function	[125]
Attenuation of protein catabolism and the hypermetabolic stress response to thermal injury	[128–130]
Enhanced absorption of the fatty acid at the *sn*-2 position (e.g., 18:2*n*-6 cystic fibrosis patients)	[25,131]
Reduction in serum TAG, LDL-cholesterol, and cholesterol	[32,111]
Improved immune function	[1,132]
Prevention of thrombosis	[1]
Lipid emulsion for enteral and parenteral feeding	[132,133]
Calorie reduction	[134]
Improved absorption of other fats	[94,111,112]

Source: Modified from Akoh, C.C., *Inform*, 6, 1055, 1995.

Jandacek et al. [94] demonstrated that an SL containing caprylic acid at the *sn*-1 and *sn*-3 positions and an LCFA in the *sn*-2 position is more rapidly hydrolyzed and efficiently absorbed than a typical LCT. They proposed that the SL may be synthesized to provide the most desirable features of LCFAs and MCFAs for use as nutrients in cases of pancreatic insufficiency [94]. Metabolic infusion of an SL emulsion in healthy humans showed that the capacity of these subjects to hydrolyze SL is at least as high as that to hydrolyze LCT [119]. This finding is significant due to evidence of interaction and interference in the metabolism of LCT and MCT when both are present in a physical mixture [120,121]. An investigation into the in vivo fate of fat emulsions based on SL showed potential for use of SL as core material in fat emulsion–based drug delivery systems [122].

An SL made from safflower oil and MCFAs was fed to injured rats, and the animals receiving the SL were found to have greater gain in body weight, greater positive nitrogen balance, and higher serum albumin concentration than controls receiving a physical mixture [116]. Enhanced absorption of 18:2*n*-6 was observed in cystic fibrosis patients fed SLs containing LCFAs and MCFAs [25]. A mixed acid type of TAG composed of linoleic acid and MCFAs has been reported to improve immune functions [123], and evaluations in clinical nutrition are ongoing. However, a 3:1 admixture of MCT-LCT emulsions was reported to elevate plasma cholesterol concentrations compared with LCT emulsions in rats fed by intravenous infusion [124].

SL appears to preserve reticuloendothelial function while improving nitrogen balance as measured by the organ uptake of radiolabeled *Pseudomonas* in comparison with LCT [125]. Long-term feeding studies with an SL containing MCFAs and fish oil fatty acids showed that SL modified plasma fatty acid composition, reflecting dietary intake and induced systemic metabolic changes that persisted after the diet was discontinued [126]. An SL made by reacting tripalmitin with unsaturated fatty acids using an *sn*-1,3-specific lipase that closely mimicked the fatty acid distribution of human milk was commercially developed for application in infant formulas under the trade name Betapol [89,127]. HDL cholesterol decreased by 14% when a diet containing Caprenin as 40% of total calories was fed to healthy men, compared with no change in levels when an LCT diet was fed [43]. Table 35.8 lists the potential and other reported benefits of SL [1,25,32,94,111,112,116,125,128–134].

V. SAFETY AND REGULATORY ISSUES

The problem with consuming large doses of pure MCTs or their emulsions is the tendency to form ketone bodies (i.e., to induce metabolic acidosis). This outcome can be circumvented by using SLs or their emulsions. SL is safe and well tolerated in the body. Physiological and biochemical data

suggest that SL emulsions, intralipid 20%, and fat emulsion 73403 (Kabi Pharmacia AB, Stockholm, Sweden), when fed to postoperative patients, were rapidly cleared and metabolized [135]. The safety of Benefat was assessed, and no significant clinical effects were reported in subjects consuming up to 30 g/day [136]. Other studies also indicate that SLs are safe [137].

SLs that provide fewer calories (<9 kcal/g) than conventional TAGs (9 kcal/g) pose a great challenge to the FDA and other regulatory agencies around the world. These SLs include Caprenin, Benefat, and Captex. The issue is complicated by the labeling requirements for reduced fats. The big problem is how to establish uniform digestibility and absorbability coefficients for all available and soon-to-be-available SL molecules and other fat substitutes [138]. The current dietary guidelines recognize total fat and saturated fat, but not digestibility coefficients. The FDA needs to develop new guidelines for SLs and genetically engineered vegetable oils or to modify existing guidelines for TAGs to reflect the new generation of fats. FDA accepted for filing the GRAS petition for Benefat/Salatrim in 1994.

VI. DIACYLGLYCEROL OILS

A. What Are DAG Oils?

DAGs have been used as the principal emulsifiers in the mixture form with MAGs in the food industry, as covered in Chapters 3 and 32. In addition to such traditional applications of DAGs based on their chemical properties, recently, the specialty SL products called DAG oils were developed for the purpose of obtaining positive effects on blood lipid profiles and obesity [139,140]. The physiological benefits of DAG oil will be described later (see Section VI.C).

DAG oils generally refer to the edible oils containing high concentrations of DAGs (>80%, w/w) and smaller amounts of TAGs (<20%, w/w) and MAGs (<3%, w/w) [140,141]. The predominant isoform of DAG present in the DAG oils is 1,3-DAGs (~70%, w/w) as compared with 1,2-DAGs (~30%, w/w) due to its enzymatic synthesis process using *sn*-1,3-specific lipase [140]. DAG oils are similar in taste, appearance, and fatty acid composition to conventional vegetable oils (TAG oils) [141].

DAG oils were first introduced to the Japanese market by Kao Corporation in February 1999 [141,142]. DAG oils produced by an enzymatic process are approved as food for specified health use (FOSHU) in Japan [143,144]. In the United States, DAGs have the status of GRAS as food ingredients according to the notice by FDA in 2000 [143,144]. DAG oil is also marketed as Enova oil by ADM Kao LLC, a joint venture of Archer Daniels Midland (ADM) Company and Kao Corporation [145].

B. Enzymatic Synthesis of DAG Oils

For the synthesis of DAG oils, enzymatic procedures using lipases are preferred to chemical procedures since the positional specificity of lipases results in oils rich in 1,3-DAGs, which cannot be obtained by chemical catalysts. DAGs can be synthesized by several enzymatic procedures via partial hydrolysis of TAGs in the presence of water [146], reaction of TAGs with glycerols (glycerolysis) [147], and direct esterification of glycerol with FFAs [148–151].

Plou et al. [146] prepared mixtures of DAGs and MAGs in high yields (~79%) through partial hydrolysis of TAGs using *sn*-1,3-specific lipases from porcine pancreas, *Rhizopus* sp., and *M. miehei* and nonspecific lipases from *Candida rugosa*. However, partial hydrolysis of TAGs is usually not a proper way to specifically synthesize 1,3-DAGs. Yamane et al. [147] obtained high yield (~85%) of 1,3-DAGs from hydrogenated beef tallow and glycerol via enzymatic glycerolysis using a *Psesudomonas* sp. lipase. However, their procedure based on the selective crystallization of 1,3-DAGs is limited to the synthesis of DAGs of saturated fatty acids having relatively high melting points.

Direct esterification of glycerol with FFAs has been the most successful enzymatic procedure for the production of oils high in 1,3-DAGs [148–151]. Rosu et al. [148] performed the direct

esterificaion of glycerol with several kinds of FFAs having relatively low melting points (<45°C), such as MCFAs and unsaturated fatty acids, in a solvent-free system. They obtained high yields (up to 85%) of 1,3-DAGs using Lipozyme RM IM (*sn*-1,3-specific lipase) as the biocatalyst. Watanabe et al. [149] synthesized 1,3-DAGs (maximal yield, ~75%) through Lipozyme RM IM-catalyzed direct esterification of glycerol with oleic and linoleic acids in a stirred batch reactor system. They removed water in the reaction mixture using a vacuum pump that helped shift the reaction equilibrium to the direction of esterification and thereby attained high yield (~75%) of 1,3-DAGs. Lo et al. [150] reported on the direct esterification of glycerol with fatty acids from soybean oil deodorizer distillates in a solvent-free system using several kinds of *sn*-1,3-specific lipases. They obtained maximal yield (47%) of 1,3-DAGs with Lipozyme RM IM. Yamada et al. [151] patented an enzymatic procedure for the industrial manufacture of DAG oils. Their procedure involves the partial hydrolysis of natural oils to obtain a partial hydrolysate with concentrated FFAs, followed by an *sn*-1,3-specific lipase-catalyzed direct esterification of glycerol with the FFAs.

However, in spite of using *sn*-1,3-specific lipase, a side reaction called acyl migration is known to occur during the esterification reaction. Acyl migration in the synthesis of 1,3-DAGs includes conversions of 1,3-DAGs to 1,2-DAGs or conversion of 1-MAGs to 2-MAGs. Therefore, acyl migration plays an important role in decreasing the yield of 1,3-DAGs in the enzymatic production of DAG oils. Recently, several studies have attempted to elucidate the reaction factors that influence acyl migration and to reduce it during laboratory-scale or pilot-scale lipase-catalyzed esterification reaction [152–154].

C. Metabolic Characteristics and Physiological Benefits of DAG Oils

Over the last few years, DAG oils have received much attention as one important class of SLs used as functional foods and nutraceuticals because it has unique physiological benefits compared with conventional TAG oils as follows: (1) suppressive effects on postprandial elevated blood TAG levels (known as hypertriglyceridemia) and (2) suppressive effects on body fat accumulation and obesity [139,140].

Taguchi et al. [155] compared the energy values of DAG oil containing 87% (w/w) DAGs and TAG oil with a similar fatty acid composition by measuring the combustion energies with a bomb calorimeter. According to their study, the energy values of DAG oil and TAG oil were 9.29 and 9.46 kcal/g, respectively, and the energy difference (<2%) was shown to be negligible in the total energy value of the practical diet. They also found that there was no difference between the absorption coefficients (weight percentage of ingested fat which was not excreted in the feces) of DAG oil and TAG oil in male Sprague–Dawley rats fed the diets containing DAG or TAG oils (20% of diet weight) [155]. Because the digestibility of fats is very similar for humans and rats, their findings might also be compatible in the case of humans [156,157]. Furthermore, the digestibilities of dietary DAG and TAG oils were proven to be nearly same to each other in another animal model, such as mice [158]. These results suggest that the physiological differences between DAG and TAG oils are due to their different metabolic characteristics after their digestion and absorption.

1. Hypotriglyceridemic Effect

Both dietary DAGs and TAGs are hydrolyzed to MAGs and fatty acids by pancreatic lipase in the small intestine. However, the 1-MAGs are produced during digestion of 1,3-DAGs, whereas, the main products of TAGs digestion are 2-MAGs [140]. The produced MAGs and fatty acids are absorbed by small intestinal epithelial cells (enterocytes) and are transported into the endoplasmic reticulum, where they are used to resynthesize TAGs for chylomicron formation and secretion [159]. The resynthesis of TAGs is predominantly catalyzed by two types of enzymes: MAG acyltransferase (MGAT) and DAG acyltransferase (DGAT). Sequentially, MGAT catalyzes the formation of DAGs from MAGs and fatty acyl-CoAs, and DGAT catalyzes the production of TAGs from DAGs and fatty acyl-CoAs [159]. MGAT has a preference for 2-MAGs produced from dietary TAGs as

the substrates, whereas 1-MAGs derived from 1,3-DAGs are not readily used for the resynthesis of DAGs [136]. In addition, 1,3-DAGs are little utilized by DGAT as substrates in the resynthesis of TAGs compared with 1,2-DAGs, which are the common substrates for DGAT [160]. Therefore, the lower postprandial blood TAG levels by dietary DAG oils rich in 1,3-DAGs may be due to their suppressive resynthesis of TAGs and retarded lymphatic transport of TAGs as chylomicrons compared with those of TAG oils [139].

2. Antiobesity Effect

Some experimental studies in humans and animals demonstrated that dietary DAG oils have antiobesity effects, such as reductions of body weight and body fat accumulation [158,161–165]. However, despite such observed beneficial effects of DAG oils, the evident antiobesity mechanisms are still not known in detail. As compared with TAG oils, the antiobesity effects of dietary DAG oils are shown to be related to the activated energy metabolism via the enhanced β-oxidation and energy expenditure phenomena found in some animal studies. That is, some researchers reported that dietary DAG increased hepatic fat oxidation [166], increased activities of enzymes involved in β-oxidation in the liver [158,163,167], and increased messenger RNA expression of some enzymes (e.g., acyl-CoA oxidase, medium-chain acyl-CoA dehydrogenase, acyl-CoA synthase) involved in β-oxidation and lipid metabolism in the small intestine and liver [158,163]. Furthermore, Kamphuis et al. [168] demonstrated that dietary DAG oil increases (hepatic) fat oxidation and decreases respiratory quotient (RQ) (i.e., carbon dioxide production divided by oxygen consumption) in humans. Their results suggest that the utilization of DAG oil as an energy source is greater than TAG oil even though a change in energy expenditure was not found. However, further studies are still required to determine the precise mechanism behind the antiobesity effects of DAG oils.

VII. PERSPECTIVES

This chapter discussed the currently available SLs, methods of synthesis, raw materials considerations, metabolism, and SL applications. An understanding of the functional properties and metabolic fate of the component fatty acids will aid in the synthesis of new SL molecules with beneficial end-use properties. The key to efficient absorption rests on the stereochemical structure of the SL. With this in mind, the outlook and potential for commercialization (Table 35.9) of the enzymatic process is bright.

Enzymes allow scientists to design SLs intended for various applications, which may include treatment of cystic fibrosis patients; individuals with pancreatic insufficiency; acquired immune deficiency syndrome (AIDS) patients, who need to boost their immune system by consuming SLs

TABLE 35.9
Factors That Affect Outlook for Commercialization of the Enzymatic Process

Specialty needs or niche market as food ingredient, fine chemical use, nutritional supplement, enteral and parenteral feeding.
Cost of enzymatic versus chemical process and product yield.
Ease with which the enzymatic process can be scaled up.
Position specific with enzymes versus randomized products with chemical synthesis.
Consumer preference: natural versus synthetic products.
Cost–benefit assessment: investment and potential returns.
Catalyst reuse: immobilized enzyme can be reused several times without significant loss of activity.
Side or unwanted products of the reaction.
Processing costs to obtain products of high purity.
Regulation by the FDA (time-consuming process).
Competition with genetically engineered crops that produce structured lipids (e.g., high lauric acid canola oil).

containing 20:5*n*-3 at the *sn*-2 position; stressed and septic and hospital patients; obese patients; and preterm infants. Potential nonmedical applications include foods and nutritional supports. SLs will continue to play a role in enteral and parenteral nutrition.

More research is needed on the effect of all lipid emulsions, especially SL emulsions, on the immune system. The notion that MCTs do not go via lymphatic transport is becoming less acceptable in the scientific community. The use of enzymes in constructing SLs destined for either portal or lymphatic transport will greatly enhance our knowledge on how SLs are metabolized. Chemical synthesis will lead to randomized SLs. Since the position and type of fatty acids in the TAG are key to their metabolism, the best alternative to chemical synthesis is the use of lipases. More applications of SLs in our regular diets is encouraged, meaning that food technologists need to explore this further. Genetic engineering of vegetable oil producing plants as covered in Chapter 37 will play a role in future commercial availability of SL.

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36 Production of Edible Oils through Metabolic Engineering

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CONTENTS

I. INTRODUCTION

Plant oil is predominantly composed of triacylglycerol (TAG) that consists of a glycerol backbone with fatty acids (FAs) esterified at the *sn*-1, *sn*-2, and *sn*-3 positions. The nature of these FAs and their position on the glycerol backbone can impact the physical properties, functionality, nutritional value, and physiological effects of the oil [1,2]. Recent estimates indicate that global vegetable oil production is about 177 million metric tons (mt) [3], which is closely matched by demand at about 173 million mt [4]. Although most of this oil is used for food and feed, some is also used for the production of biofuel and other industrial bio-products [5]. Thus, in the future, it will be a challenge to provide sufficient quantities of edible high quality vegetable oil for our growing global population. All of this oil is derived from seed or mesocarp tissue. In 2013, four major oil crops producing multipurpose oils accounted for 85% of the market share (Table 36.1) [6]. Palm (*Elaeis guineensis*) (mesocarp and kernel) accounts for most of the oil produced followed by the seeds of soybean (*Glycine max*), oilseed rape (*Brassica napus*), and sunflower (*Helianthus annuus*). Most vegetable oils currently consumed by humans and livestock are from cultivars that have been produced through plant breeding. Within the last three decades, however, plant biotechnology and genomics

TABLE 36.1
Vegetable Oil Production in 2013

Oil Crop			
Common Name	**Major Cultivated Species**	**Production (mt)**	**Percentage Contribution**
Palm	*Elaeis guineensis* L.	56.2	35
Soybean	*Glycine max* L.	42.6	26
Oilseed rape[a]	*Brassica napus* L.	25.0	15
Sunflower	*Helianthus annuus* L.	13.9	9
Other oil crops		25.1	15
	Total	162.8	

Source: 2013 global vegetable oil production—Palm oil, soya, sunflower, rapeseed http://www.slideshare.net/GreenPalmOil/2013-global-vegtable-oil-production.

[a] Mainly low-erucic/low-glucosinolate cultivars. mt, million (metric) tons. Palm values include both palm oil and palm kernel oil.

have opened up new possibilities for changing the proportions of existing FAs in oils or introducing FAs that are not normally present in the oil.

This chapter mainly deals with the use of metabolic engineering to produce edible seed TAG with desired FA composition. Our coverage of the area is not comprehensive but rather is based on discussion of numerous examples of genetic intervention. Examples of metabolic engineering interventions leading to seed oils with desired FA compositions in oil crops are presented in Table 36.2. Brief comments on the potential nutritional value or health benefits of introducing certain FAs into seed TAG will be included in our discussion. Although the major focus of our discussion will involve established oil crops, in some cases we will present proof of concept work based on studies using the model oilseed plant, *Arabidopsis thaliana* (*Arabidopsis*). We will also comment on recent strategies to increase seed TAG content or to produce TAG in vegetative tissue using metabolic engineering. Prior to discussing these molecular strategies, we present a brief overview of FA and TAG biosynthesis.

II. OIL FORMATION IN OLEAGINOUS PLANTS

High levels of oil can accumulate in the zygotic embryos of developing seeds of oleaginous plants. Our brief discussion of this process is based on more detailed information from a few recent reviews [20–23]. A generalized scheme, which emphasizes TAG biosynthesis in relation to aspects of membrane metabolism, is shown in Figure 36.1. During embryo development, FAs are formed in a subcellular organelle known as the plastid. Sucrose derived from photosynthesis is delivered to the embryos to provide precursors for FA formation. In some cases (e.g., olive [*Olea europaea*]), *in situ* photosynthesis is important in addition to leaf-derived sucrose. Malonyl-CoA is produced through the catalytic action of acetyl-CoA carboxylase (ACCase) and provides two carbon fragments for the growth of the fatty acyl chain, catalyzed by the enzymes of the fatty acid synthase complex, using acyl-acyl carrier protein (acyl-ACP) intermediates. A soluble (Δ9) desaturase can catalyze the introduction of a single double bond into fatty acyl-ACP. Unesterified (free) FAs are liberated from fatty acyl-ACP through the catalytic action of acyl-ACP thioesterases and can move across the inner membrane of the plastidial envelope with the help of FA export1 (FAX1). In soybean and oilseed rape, the main FA produced in the plastid is oleic acid (18:1Δ^{9cis}; hereafter 18:1), with lower levels of palmitic acid (16:0) and stearic acid (18:0) produced. On the outside of the plastid, long-chain acyl-CoA synthetase (ACS) catalyzes the ATP-dependent esterification of free FA with coenzyme A (CoA) to form acyl-CoA, which can be regarded as an "activated" FA. In some seeds, 18:1-CoA can

TABLE 36.2
Examples of Metabolic Engineering Interventions Resulting in Modified Fatty Acid Compositions of Seed Oils from Oil Crops

Engineered Crop	Molecular Strategy	Fatty Acid Modifications in the Seed Oil	References
Canola-type *Brassica napus*	Seed-specific antisense downregulation of the gene encoding stearoyl-ACP-Δ9-desaturase	Up to ~40% stearic acid (18:0)	[7]
Canola-type *B. napus* (DH12075)	Seed-specific overexpression of native acyl-ACP thioesterase B [BnaFatB(2)] combined with artificial microRNA-mediated downregulation of 8 native genes encoding putative stearoyl-ACP- Δ9-desaturases	37.3%–45.6% total saturated fatty acid content (up from 7.4% in the control)	[8]
Canola-type *B. napus*	Seed-specific expression of cDNA encoding *Cuphea hookeriana* FatB2 acyl-ACP thioesterase	Up to 11 mol% caprylic acid (8:0) and 27 mol% capric acid (10:0), respectively	[9]
Canola-type *B. napus*	Seed-specific co-expression of cDNAs encoding California bay laurel (*Umbellularia californica*) 12:0-acyl-ACP thioesterase and coconut (*Cocos nucifera*) 12:0-CoA-preferring LPAAT	Up to 67 mol% lauric acid (12:0)	[10]
Canola-type *B. napus* L. cv Westar	Seed-specific expression of *Anacystis nidulans DES9* gene encoding glycerolipid desaturase with KKSS endoplasmic reticulum retrieval and retention signal	Saturated fatty acid content of 4.3 mol% (down from 7.2 mol% in the wild type)	[11]
Solin flax (*Linum usitatissimum*) variety "Linola™ 1084" with ~78% linoleic acid ($18{:}2\Delta^{9cis,12cis}$) in the seed oil	RNA interference-mediated silencing of *FATTY ACID DESATURASE 2* genes	Up to 80% oleic acid ($18{:}1\Delta^{9cis}$)	[12]
Camelina (*Camelina sativa* L. cv Sunesson)	Seed-specific co-expression of cDNAs encoding a mutant Δ9-acyl-ACP and acyl-CoA desaturase with increased preference for 16:0-containing substrates combined with seed-specific suppression of genes encoding 3-keto-acyl-ACP synthase II and FatB 16:0-ACP thioesterase	60%–65% ω-7 monounsaturated fatty acids (up from ~2% in the wild type)	[13]
B. carinata with about 40% erucic acid ($22{:}1\Delta^{13cis}$) in the seed oil	Seed-specific expression of cDNA encoding bittercress (*Cardamine graeca*) 3-ketoacyl-CoA synthase	Up to 44% nervonic acid ($24{:}1\Delta^{15cis}$)	[14]
Camelina (*C. sativa*)	Seed-specific co-expression of several genes encoding various desaturases and elongases	>12% docosahexaenoic acid ($22{:}6\Delta^{4cis,7cis,10cis,13cis,16cis,19cis}$)	[15,16]
High α-linolenic acid ($18{:}3\Delta^{9cis,12cis,15cis}$) (~75%) flax (*L. usitatissimum*) line F06396B	Seed-specific expression of the gene encoding Δ6-desaturase from *Pythium irregular*	~26% stearidonic acid ($18{:}4\Delta^{6cis,9cis,12cis,15cis}$)	[17]
High linoleic acid (~80%) safflower (*Carthamus tinctorius* L. cv Centennial)	Seed-specific expression of the gene encoding Δ6-desatuase from *Saprolegnia diclina*	Up to >70% (v/v) γ-linolenic acid ($18{:}3\Delta^{6cis,9cis,12cis}$)	[18]
Soybean (*Glycine max* L. cv Jack)	Seed-specific expression of cDNA encoding fatty acid conjugase from *Calendula officinalis*	~20% calendic acid ($18{:}3\Delta^{8trans,10trans,12cis}$)	[19]

Abbreviations: ACP, acyl-carrier protein; *DES*, *DESATURASE*; LPAAT, acyl-CoA *lyso*-phosphatidic acid acyltransferase.

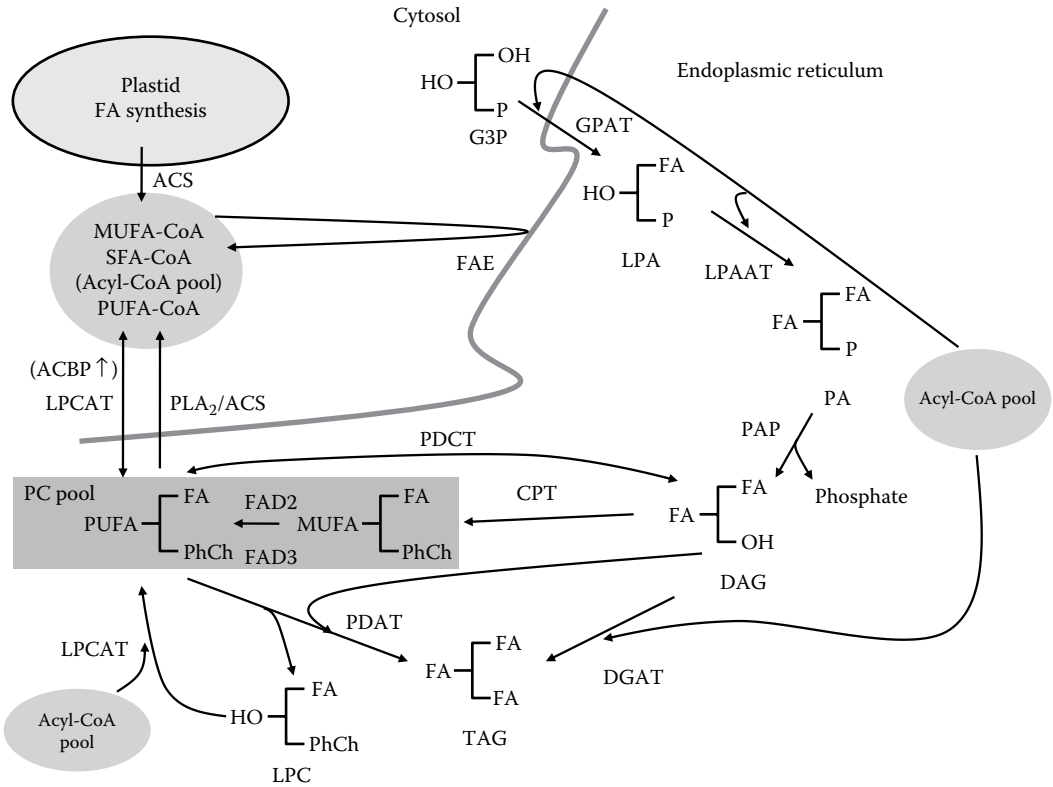

FIGURE 36.1 Generalized scheme for triacylglycerol(TAG) biosynthesis in developing seeds of oleaginous plants. Monounsaturated FAs (MUFAs) and saturated FAs (SFAs) are synthesized in the plastid and, following export from this organelle, are converted to acyl-coenzyme As (CoAs) FA elongase (FAE) FA elongase on the ER. TAG can be synthesized via the acyl-CoA-dependent acylation of the glycerol backbone derived from *sn*-glycerol-3-phosphate (G3P). Phosphatidic acid phosphatase catalyzes the dephosphorylation of phosphatidic acid (PA) to produce *sn*-1,2-diacylglycerol (DAG) prior to the final acylation catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT). DAG can also be converted to phosphatidylcholine (PC) via the action of *sn*-1,2-diacylglycerol:cholinephospho-transferase (CPT) and/or phosphatidylcholine:diacylglyce rol cholinephosphotransferase (PDCT). MUFA at the *sn*-2 position of PC can undergo desaturation catalyzed by FA desaturases 1 and 2 (FAD1 and FAD2), respectively. PC enriched in PUFA can be returned as DAG into the linear part of the G3P pathway leading to TAG via the action of PDCT and DGAT. Phospholipase A_2 (PLA_2) may also catalyze the removal of polyunsaturated FA (PUFA) from the *sn*-2 position of PC that in turn is converted to acyl-CoA via the action of acyl-CoA synthetase (ACS). In addition, acyl-CoA:*lyso*-phosphatidylcholine acyltransferase (LPCAT) may catalyze acyl-exchange between the *sn*-2 position of PUFA-enriched PC and the acyl-CoA pool. Low molecular mass-soluble acyl-CoA-binding protein (ACBP) may interact with acyl-CoA and encourage the reverse reaction of LPCAT leading to acyl-CoA. Phospholipid:diacylglycerol acyltransferase (PDAT) catalyzes the transfer of FA at the *sn*-2 position of PC to DAG to also generate TAG. PLA_2 and PDAT have been implicated in catalyzing the removal of unusual FAs from the PC. LPCAT may catalyze the reacylation of *lyso*-phosphatidylcholine produced through the action of PLA_2 or PDAT. Other abbreviations: GPAT, acyl-CoA:*sn*-glycerol-3-phosphate acyltransferase; LPA, *lyso*-phosphatidic acid; LPAAT, acyl-CoA:*lyso*-phosphatidic acid acyltransferase; PhCh, phosphocholine head group. (Reproduced from Taylor, D.C. et al., Plant triacylglycerol synthesis, AOCS Lipid Library, http://lipidlibrary.aocs.org/content.cfm?ItemNumber=40314. With permission from the American Oil Chemists' Society.)

be further elongated on the ER via the catalytic action of elongases. Membrane-bound acyltransferases in the endoplasmic reticulum (ER) can use fatty acyl groups from acyl-CoA as a source of acyl donor to acylate the glycerol backbone. The pathway leading from *sn*-glycerol 3-phosphate (G3P) to TAG is known as the Kennedy or G3P pathway. In oilseed crops producing TAG containing polyunsaturated FAs (PUFAs), there is a complex interplay between the Kennedy pathway and membrane metabolism that involves acyl-editing. *sn*-1,2-Diacylglycerol (DAG) enriched in 18:1 can be converted to phosphatidylcholine (PC) that in turn becomes the site for PUFA formation through the sequential catalytic action of FA desaturase (FAD)2 and FAD3 resulting in the formation of linoleic acid (18:2$\Delta^{9cis,12cis}$; hereafter 18:2) and α-linolenic acid (18:3$\Delta^{9cis,12cis,15cis}$; hereafter α-18:3), respectively. Various mechanisms have been identified for channeling of PC-formed PUFA into TAG. For example, phospholipid:diacylglycerol acyltransferase (PDAT) can catalyze the transfer of PUFA from the *sn*-2 position of PC to DAG to form TAG enriched in PUFA. Other examples of channeling PUFA into TAG are described in the legend to Figure 36.1. TAG produced in the Kennedy pathway and through PDAT action accumulates between the outer leaflets of the ER eventually pinching off to form cytosolic oil bodies, coated with a monolayer of phospholipid with specialized proteins, ranging in size from about 0.2 to 2 μm in diameter.

III. INTRODUCTION OF NEW OIL TRAITS AND TRAIT STABILITY

In order to modify FA composition in a desired model plant or oil crop, it is often necessary to introduce cDNAs or genes encoding enzymes from other sources, preferably under direction of a seed-specific promoter. *Agrobacterium*-mediated transformation and direct-DNA transfer using microprojectile bombardment with DNA-coated particles represent conventional approaches for random insertion of foreign DNA into plants [25]. With more recent technologies, foreign genes can be targeted to precise locations in the genome through homologous recombination [26]. A newly introduced foreign enzyme may have to compete with an endogenous enzyme that catalyzes the same general reaction. Therefore, it may be useful to suppress the expression of the gene encoding the endogenous form of the enzyme in order to obtain maximum benefit from introduction of the foreign gene [27].

Random chemical mutagenesis has been used as a tool in conventional breeding to produce desirable mutations although extensive selection is needed to remove unwanted mutations [28]. For example, flax (*Linum usitatissimum*) oil is typically enriched in α-18:3, but high 18:2 lines have been generated by inactivation of *FAD3* through mutagenesis [29]. The technique of Targeted Induced Local Lesions IN Genomes was developed to specifically detect such mutations [30]. The mutations can be exploited in breeding programs by the use of back-crossing to eliminate most undesirable mutations [31]. Moreover, genome editing techniques are now available to modify a specific base in a gene thereby specifically affecting the function of the gene [32]. Genome editing is particularly useful for loss-of-function approaches where it is desirable to inactivate an encoded enzyme.

Once a desired FA composition trait is established under growth chamber or greenhouse conditions, it is important to monitor the stability of the trait through subsequent generations. In addition, field conditions represent the "real environment" under which a genetically engineered oil crop will be commercially propagated, and, therefore, it is critical to include field studies at later stages in the development of such a crop. Abiotic factors that are known to influence TAG accumulation and FA composition of TAG can further complicate the situation [33].

IV. MODIFYING SATURATED FATTY ACID CONTENT

A. Health and Physiological Effects of Saturated Fatty Acids

Saturated fatty acids (SFAs) are often considered less healthy to consume than unsaturated fatty acids (UFAs) with individual SFAs having different reported effects on promoting cardiovascular disease. For example, myristic acid (14:0) and palmitic acid generally increase the cholesterol

content of low-density lipoprotein and high-density lipoprotein to a similar extent, whereas stearic acid has a neutral effect on the cholesterol content of low-density lipoprotein and has the least effect in increasing the cholesterol content of high-density lipoprotein [34]. However, recent data have raised doubts about the long-held opinion that dietary long-chain SFAs are potentially harmful from a cardiovascular disease standpoint. Unlike long-chain FAs (14 carbons or greater in length), medium-chain SFAs (6–12 carbons), such as those found in coconut oil, are readily absorbed and metabolized by the liver without being incorporated directly into body fat and can be converted to ketone bodies [1,35]. Although glucose is an important source of energy for the brain, ketones also provide an important source of energy and appear to be beneficial in the prevention of Alzheimer's disease where glucose uptake by the brain is compromised [35]. Increased production of ketone bodies due to metabolism of medium-chain SFAs have also been shown to be useful in treating convulsive disorders [36]. Indeed, ketogenic diets were shown to be effective in the treatment of epilepsy. In addition, medium-chain FA and SFAs have potential applications in clinical medicine [37,38]. Thus, the effect of dietary SFAs on health depends on the FA chain length.

B. Increasing Saturated Fatty Acid Content

Temperate oilseed plants such as *Arabidopsis*, oilseed rape, soybean, and sunflower contain mainly UFAs in their seed oils, while tropical oils such as palm, coconut (*Cocos nucifera*), and cocoa (*Theobroma cacao*) have relatively high proportions of SFAs [39–41]. Coconut oil and palm kernel oil differ from other commodity oils and are known collectively as lauric oils due to their high content of lauric acid. Over 3 mt of coconut oil are produced annually mainly in the Philippines, Indonesia, and India [42]. It provides income for over 10 million small farmers [43]. Coconut and palm kernel oils have identical contents of lauric (48%) and myristic (17%) acids, but coconut oil has a higher content of 6–10C SFA than palm kernel oil [42]. Because of its economic importance for smallholders, there has been much interest in biotechnology to improve the crop. This has focused in five main areas: embryo culture, clonal propagation, anther-culture, cryopreservation of germplasm, and genetic transformation. Both biolistics [44] and *Agrobacterium*-mediated transformation [45] have been used with oil productivity being a particular target [43]. Coconut, as well as palm kernel oil, has important nonfood uses such as for soaps and other surface-active products.

Palm oil itself (from the fruit mesocarp) is high in SFAs, especially palmitic (typically 45% 16:0 with 4% 18:0). Because of this, palm oil is used extensively in the food industry for products such as spreads. It is an exceptionally useful crop since it yields about ten times the average oil production per hectare as a typical seed [46]. At present, two countries (Indonesia and Malaysia) account for around 90% of production, which lends a certain level of vulnerability to supplies. As with coconut, palm can be transformed by both biolistics and *Agrobacterium*-mediated events [47,48], with attention being focused presently on high oleic or better yielding lines (see Section VIII).

Temperate oilseed species have been successfully engineered or bred to produce high proportions of SFAs in their seed oils. These investigations were driven mainly by the interest in developing a temperate source of SFA for both industrial and edible applications. Various metabolic engineering approaches for increasing the SFA content of *B. napus* have been reviewed by Stoll et al. [49].

There have been concerns regarding the process of partial hydrogenation of oils from temperate crops to produce spreads such as margarine because of the simultaneous production of deleterious *trans* FAs [50]. Increasing the palmitic acid and stearic acid content of the seed oil of a temperate oilseed crop could potentially provide a source of saturates for blending or interesterification with TAG enriched in UFAs to provide a better alternative to spreads and oils for deep-frying to those based on partially hydrogenated oils [8,51].

Early metabolic engineering studies focused on boosting the stearic acid content of *B. napus* seed oil. Downregulation of the gene encoding stearoyl-ACP desaturase [7] (see Table 36.2) and the introduction of a modified acyl-ACP thioesterase from *Garcinia mangostana* [52] were two strategies that resulted in substantially increased proportions of stearic acid in the seed oil. The Mexican

shrub, *Cuphea hookeriana*, which accumulates 75% caprylic acid (8:0) and 10% capric acid (10:0), was found to contain two acyl-ACP thioesterases (ChFatB1 and ChFatB2) [9,53]. ChFatB1 exhibited enhanced specificity for 16:0-ACP, and when the encoding cDNA was expressed in canola-type *B. napus*, the seed oil of 25 independent transgenic lines contained 7–34 mol% palmitic acid [53].

Over a decade later, downregulation of β-ketoacyl-ACP synthase II in the model oilseed plant, *Arabidopsis*, resulted in the production of seed oil containing up to 53% palmitic acid, which was similar to that of palmlike tropical oil [41]. β-Ketoacyl-ACP synthase II catalyzes the conversion of 16:0-ACP to 18:0-ACP in the plastid [20]. Reducing this conversion would make more 16:0 available for eventual incorporation into TAG. Recently, *B. napus* DH12075 was engineered to overexpress a cDNA encoding native fatty acyl-ACP thioesterase in combination with artificial microRNA-mediated downregulation of eight endogenous genes encoding putative 18:0-ACP desaturases [8] (see Table 36.2). (MicroRNAs are small noncoding RNAs that control gene expression by directing their target mRNAs for degradation or translational repression.) The SFA content of the oil shifted from 7.4% in the control to 37.3%–45.6% in the transgenic lines. Seed oil from the transgenic line with the highest SFA content contained about 30% palmitic acid and exhibited a melting point 25°C higher than the control oil.

Mutagenesis breeding has also been used to increase the SFA content in soybean and sunflower. Soybean lines ranging from 19% to 30% stearic acid were developed, whereas standard soybean oil contained 2%–4% stearic acid [51]. Mutagenesis breeding was also used to generate sunflower lines with 30% stearic acid and 26% palmitic acid, respectively, in their seed oils [54].

Engineering *B. napus* to produce caprylic and capric acid is an early example of medium-chain SFA production in a temperate oilseed crop. Transformation (seed specific) of canola-type *B. napus* with the cDNA *CpFatB2* resulted in T_2 seeds producing both caprylic and capric acid [9] (see Table 36.2). The highest expressing line accumulated 11 mol% caprylic acid and 27 mol% capric acid.

Production of *B. napus* seed oil enriched in lauric acid (12:0) is another notable example of engineering medium-chain SFA accumulation in a temperate oilseed crop. Over 50% lauric acid in the seed oil was attained through the introduction of a California bay laurel (*Umbellularia californica*) 12:0-ACP thioesterase [55]. Lauric acid incorporation into TAG, however, was limited by the induction of both β-oxidation and glyoxylate cycle enzymes indicating that medium-chain SFA production can override developmental programs that typically suppress the expression of genes encoding these enzymes during seed development [56]. FA biosynthesis, however, also increased, apparently to compensate for 12:0 lost through β-oxidation. Somewhat later, the 12:0 content of the oil was further boosted in *B. napus* by seed-specific co-expression of cDNAs encoding both the 12:0-ACP thioesterase and a 12:0-CoA-preferring *lyso*-phosphatidic acid acyltransferase (LPAAT) from coconut [10] (see Table 36.2). LPAAT operates in the Kennedy pathway to catalyze the acyl-CoA-dependent acylation of the *sn*-2 position of lysophosphatidic acid to generate phosphatidic acid (PA) [57] (see Figure 36.1). The introduced LPAAT with increased preference for 12:0-CoA resulted in enhanced production of TAG with 12:0 at the *sn*-2 position of the glycerol backbone since endogenous LPAAT activity in *B. napus* was not effective in utilizing 12:0-CoA. Typical modified lines contained 60% laurate but, even so, such crops could not compete commercially with traditional laurate supplies from palm kernel oil.

C. Decreasing Saturated Fatty Acid Content

In the United States, vegetable oils with a SFA content of <3.5% can be labeled as "zero sat" [11]. This has already been achieved by Dow AgroSciences LLC for ω-9 sunflower oil [58]. Lowering the SFA content of canola-type *B. napus* seed oil from 6% to 7% to below 3.5% could potentially enhance the marketing of canola oil based on the general public's perception that a "zero sat" oil would provide additional health benefits due to further reduction in "harmful" SFAs [25] (see also Section IV.A). Stearoyl-ACP desaturase catalyzes the generation of monounsaturated FAs (MUFAs) in the plastid during seed development [20]. Palmitoyl-ACP desaturase from the seed of the forest vine, cat's claw (*Doxantha unguis-cati*), was introduced during seed development in *Arabidopsis* and canola in attempts to lower the SFA content of the seed oil by converting 16:0 to

the ω-7 FA, palmitoleic acid ($16:1\Delta^{9cis}$; hereafter 16:1) [59]. Palmitoleic acid was considered to be healthier than palmitic acid [60,61]. Although 16:1 and its apparent elongation product, *cis*-vaccenic acid ($18:1\Delta^{11cis}$), were formed, the overall SFA content of the seed oils remained similar to the wild-type controls. Somewhat later, the same research group introduced a cyanobacterial glycerolipid desaturase (encoded by the *DES9* gene) [62] into canola and generated seed oil with 4.3 mol% SFA content [11] (see Table 36.2). *FAT-5* from *Caenorhabditis elegans* encodes a desaturase that is highly specific to 16:0 as substrate [63]. Expression of *FAT-5* in *Arabidopsis* under the control of a strong seed-specific promoter resulted in seed oil with one-third of the palmitic acid content of the wild-type control [63]. Overall, the SFA content of the *Arabidopsis* seed oil was reduced by 50% compared to the wild-type control, but seed oil content and seed size were also somewhat reduced.

V. INCREASING MONOUNSATURATED FATTY ACID CONTENT

A. Oleic Acid

Oil enriched in oleic acid from the mesocarp of olive is one of the constituents of the "Mediterranean diet," which is known to have a protective effect against metabolic syndrome [64,65]. Metabolic syndrome is characterized by cluster of risk factors for cardiovascular disease and type 2 diabetes mellitus including abdominal obesity, high blood pressure, high fasting glucose, low fasting high-density lipoprotein cholesterol, and high fasting serum TAG [64].

Vegetable oils enriched in oleic acid with reduced α-linolenic acid content also display enhanced stability during frying and can be blended or interesterified with saturated fats to generate shortenings and margarine [66]. These oils offer the consumer an alternative source of oleic acid over olive oil. Recently, seed mutagenesis has been used to develop low α-linolenic acid *Brassica oleracea* lines with mutated *FAD3* genes [67].

Canola-type *B. napus* DH12075 contains about 60% oleic acid in its seed oil [8,68], which reflects what is typical of the oleic acid content of canola oil. Canola oil highly enriched in oleic acid (>85%) was generated through reduced expression of *FAD2* that encodes a desaturase catalyzing the conversion of 18:1–18:2 in PC [69,70]. Recently, RNA interference was used to silence *FAD2* genes in high-18:2-Linola-type flax, resulting in seed oil with up to 80% oleic acid [12] (see Table 36.2). (In RNA interference, RNA molecules can inhibit gene expression through degradation of specific mRNA molecules.) Seed oil from the high-18:2 control contained 12% oleic acid. It was suggested that the high oleic acid flax oil had the potential to replace less stable oils in deep-frying applications. "Zero sat" ω-9 Sunflower commercially available from Dow AgroSciences LLC exhibits an ultrahigh oleic acid content of 93% [58].

Other approaches for increasing the oleic acid content of seed oil might be possible through the use of acyltransferases with enhanced selectivity for substrates containing oleic acid. A phenylalanine insertion at position 469 in a type-1 diacylglycerol acyltransferase (DGAT) from maize (*Zea mays*) was associated with embryo oil with increased oleic acid content [71]. DGAT catalyzes the acyl-CoA-dependent acylation of *sn*-1,2-diacylglycerol to produce TAG and free CoA [72] (see Figure 36.1). The acyl-CoA-dependent acylation of the *sn*-1 position of G3P in both the plastid and ER is catalyzed by *sn*-glycerol-3-phosphate acyltransferase (GPAT) [20] (see Figure 36.1 for GPAT action in the ER). Recombinant plastidial GPAT from *Erysimum asperum* can utilize acyl-CoA as an acyl donor even though the natural substrate of the enzyme is acyl-ACP [73]. The biochemical properties *E. asperum* GPAT suggest that cytosolic introduction of the plastidial enzyme in developing seed may be useful for increasing 18:1 content at the *sn*-1 position of TAG.

Section IV.B discussed how efforts to manipulate the oil quality of palm oil have focused on raising oleic acid content. In order to give mesocarp oil a FA composition enriched in oleic acid, it will be necessary to manipulate at least three genes (*palmitoyl thioesterase,* β*-ketoacyl-ACP synthase II*, and *stearoyl-ACP desaturase*), and possibly more [74]. At present, the highest palm oleate fractions obtained during processing are only about 52% oleic acid (39% in regular palm oil) [42].

B. ω-7 Monounsaturated Fatty Acids

Palmitoleic acid has been shown to decrease inflammation, increase muscle response to insulin, increase membrane fluidity, and inhibit cancer development [61]. Higher plasma levels of *cis*-vaccenic acid, which can be derived from apparent elongation of 16:1 [59], were associated with a lower risk of heart failure in male physicians [75].

Although palmitoleic acid does not occur widely in terrestrial plants, edible oils enriched in palmitoleic acid include oil of macadamia (*Macadamia integrifolia*) nuts (30% 16:1) [61] and oil from the pulp (32–43 mol% 16:1) of the sea buckthorn (*Hippophae rhamnoides* L.) berry [76]. *cis*-Vaccenic acid accounts for 5–7 mol% of the FAs in oil from sea buckthorn pulp.

Recently, Wu et al. reviewed the metabolic engineering of higher plants to produce increased 16:1 and other ω-7 MUFAs in their seed oils [61]. *Arabidopsis* seed oil containing up to 56% ω-7 MUFA content was engineered through the introduction of a modified 16:0-ACP desaturase combined with downregulation of β-ketoacyl-ACP synthase II [77]. This strategy involved boosting the level of plastidial 16:0-ACP, the substrate of the modified desaturase with enhanced specificity for catalyzing the desaturation of 16:0-ACP. The resulting seed oil contained high levels of palmitoleic acid and *cis*-vaccenic acid, the elongation product of 16:1. Further increases in ω-7 MUFA content were achieved though the cytosolic introduction of fungal desaturases from *Stagonospora nodorum* and *Aspergillus nidulans*. More recently, a similar strategy was used to engineer camelina (*Camelina sativa*) with 60%–65% ω-7 MUFA content in the seed oil [13] (see Table 36.2). In addition, total SFA content was reduced from about 12% to about 5% in the high ω-7 transgenic lines. The same study reported the metabolic engineering of soybean to produce about 17% ω-7 MUFA in the seed oil.

C. Very-Long-Chain Monounsaturated Fatty Acids

In early studies, high-erucic-acid (22:1Δ^{13cis}; hereafter 22:1) oil was shown to lead to myocardial lesions in feeding experiments with rats [78–80]. These unfavorable dietary effects associated with rapeseed oil led to the development of modern day canola that has <2% erucic acid in the oil coupled with low glucosinolate content in the meal [25]. Available evidence, however, does not indicate such an association in humans ingesting rapeseed oil containing relatively high levels of erucic acid [80].

The movie *Lorenzo's Oil* (released in 1992) suggested that an oil composed of the TAGs of oleic acid and erucic acid (four parts to one) was useful in decreasing the progression of adrenoleukodystrophy, which is X-linked peroxisomal disease characterized by brain demyelination [81–83]. Adrenomyeloneuropathy is a related disorder involving the spinal cord and peripheral nervous system [81,83]. Both disorders are associated with impaired degradation of very-long-chain SFAs, which results in high *in vivo* concentrations of these FAs leading to a number of unfavorable physiological effects [83]. Although dietary erucic acid therapy was shown to reduce very-long-chain FA concentrations to normal levels in patients with adrenoleukodystrophy [84,85] or adrenomyeloneuropathy [81], trials have shown that treatment with Lorenzo's oil had limited effectiveness in preventing the progression of these neurological disorders [81,86].

The elongation product of erucic acid is also considered to offer potential therapeutic value. Brain myelin and nerve tissue contain nervonic acid (24:1Δ^{15cis}; hereafter 24:1), which is formed by elongation of erucic acid [87]. Ingestion of nervonic acid can contribute to myelin synthesis and function [87,88]. Thus, nervonic acid could have nutraceutic value in treating neurological disorders such as adrenoleukodystrophy [88]. cDNAs encoding 3-ketoacyl-CoA synthase have been isolated from the "money plant" (*Lunaria annua*) and bittercress (*Cardamine graeca*), and expressed in *Brassica carinata* to produce 24:1-enriched oil [14,89]. The introduced 3-ketoacyl-CoA synthase catalyzed the elongation of 22:1-CoA to 24:1-CoA during seed development. The best transgenic lines generated using the bittercress cDNA reached 44% nervonic acid with 6% residual erucic acid (see Table 36.2), which was near the acceptable level of 5% [14].

VI. INCREASING POLYUNSATURATED FATTY ACID CONTENT

A. ω-3 Polyunsaturated Fatty Acids

The very-long-chain ω-3 PUFAs, eicosapentaenoic acid (EPA; 20:5$\Delta^{5cis,8cis,11cis,14cis,17cis}$) and docosahexaenoic acid (DHA; 22:6$\Delta^{4cis,7cis,10cis,13cis,16cis,19cis}$), are well known for numerous health benefits including reduction of cardiovascular disease, anticancer effects, prevention of obesity and metabolic syndrome, and improved cognitive function in patients with very mild Alzheimer's disease [34,90–93]. DHA, in particular, is involved in the proper function of the nervous system and the retina [92,94]. Recently, EPA, DHA, and the ω-3 intermediates SDA and eicosatetraenoic acid (20:4$\Delta^{8cis,11cis,14cis,17cis}$; ETA) were all shown to reduce the survival of two human breast cancer cell lines [95]. In addition, DHA may be useful in promoting the action of chemotherapy drugs [96,97]. In humans, EPA and DHA are produced from dietary α-linolenic acid, but conversion is limited due to the inefficient Δ6-desaturation step leading to stearidonic acid (SDA; 18:4$\Delta^{6cis,9cis,12cis,15cis}$) [98]. Although dietary α-linolenic acid is insufficient under most circumstances [99], there are certain conditions where EPA and DHA become "conditionally essential" [100].

Metabolic engineering of oil crops to produce EPA and DHA in seeds has been driven by the need to develop a land-based source of oil to replace EPA-/DHA-enriched fish oils that are dwindling [94,101,102]. The most popular approach involves installing a metabolic pathway of alternating desaturation and elongation that is referred to as the aerobic pathway because molecular oxygen is required for the desaturation reactions. The alternative approach, which is primarily for DHA production, is through the anaerobic polyketide synthase pathway that does not require oxygen-dependent desaturation. A polyketide synthase pathway was previously identified in the protist *Schizochytrium* spp. [103]. The process involves several catalytic sites, in a large complex, that drive chain extension and insertion of double bonds. Metabolic engineering to introduce an aerobic pathway, however, has garnered most interest because of the ease of cloning genes encoding relatively small desaturases and elongases from various microbial sources rather than having to deal with a gene encoding a large polyketide synthase [101]. A major challenge of engineering the aerobic pathway in plants is the issue of "substrate dichotomy" [94,101,104]. Desaturation usually occurs on PC, whereas elongation utilizes acyl-CoA as a substrate. Thus, there is a need for engineering additional pathways for effective trafficking of modified FAs between PC and acyl-CoA. For example, flax appears to have several routes for channeling α-linolenic acid from PC into TAG [105–107]. Flax contains PDATs that are highly selective for *sn*-2-α-18:3-PC and di-α-18:3 DAG so as to produce a TAG highly enriched in α-linolenic acid [105]. The flax PDATs also effectively utilize substrates containing SDA or EPA suggesting that the encoding genes could be useful potentially in metabolic engineering strategies to further boost ω-3 PUFA levels. Some researchers have attempted to install an exclusive acyl-CoA desaturation/elongation pathway leading to very-long-chain ω-3 PUFA in plants [108], but yields were low. Recently, two research groups were able to produce >12% DHA in the seed oil of *C. sativa* through heterologous expression of several genes [15,16] (see Table 36.2). More recently, field studies under varied environmental conditions were conducted at Rothamsted Research (Harpenden, Hertfordshire, UK) with *C. sativa* engineered to produce EPA and DHA in the seed oil in order to gain insight into the effect of environment on the stable production of these ω-3 FAs [109]. Despite the substantial variation in environmental conditions with respect to temperature and rainfall, there was no significant difference in the proportions of EPA and DHA in seeds grown under different field conditions versus those grown in the greenhouse. Thus, although there are known effects of the environment on FA composition [33], it is possible to produce stable levels of EPA and DHA in field-produced *C. sativa* oil. In addition, there was no difference in the seed oil content of transgenic lines and wild-type *C. sativa* grown under field conditions. Several companies are also working toward the development of canola-type *B. napus* producing EPA and DHA in the seed oil [110]. Soybean has also been targeted successfully for EPA and DHA accumulation [111].

Intake of oils containing SDA provide a direct source of the ω-3 intermediate that bypasses the metabolic limitations on accumulation of this FA imposed by mammalian Δ-6 desaturase [95]. Dietary SDA has been shown to increase the EPA content of red blood cells and exhibit similar effects to dietary EPA when compared to α-linolenic acid [98,112,113]. Recently, treatment of two human breast cancer cell lines with SDA resulted in about a 40% reduction in cell viability that was similar to the effects of EPA or DHA *in vitro* [95] and *in vivo* [17].

Soybean [114] and flax [17,104,115,116] oils enriched in SDA have been produced using metabolic engineering. In 2009, SDA-enriched soybean oil produced by the Monsanto Company was awarded "generally regarded as safe" status by the United States Food and Drug Administration [117]. Recently, flax oil enriched in about 26% SDA was generated through heterologous expression of a *Pythium irregular* Δ*-6 DESATURASE* during seed development in a high-α-18:3 line of flax [17] (see Table 36.2). Immunodeficient 6-week-old female nu/nu mice (*Mus musculus*) were implanted with MDA-MB-231 human breast cancer cells and fed a diet containing the SDA-enriched oil to assess the dietary effects of this oil on tumor growth [17]. The SDA-enriched diet resulted in a substantial and significant reduction in tumor growth after 4 weeks of feeding in comparison to the control diet. Immunochemical analysis of tumor cells revealed that Fas ligand receptor (CD95)-induced apoptosis contributed to reduced cell viability.

B. ω-6 Polyunsaturated Fatty Acids

Both ω-3 and ω-6 PUFA provide health benefits, although excessive dietary ω-6 PUFA can also lead to some deleterious health effects [34,118,119]. γ-Linolenic acid (GLA; 18:3$\Delta^{6cis,9cis,12cis}$) has nutraceutical value as an anti-inflammatory [118]. GLA is metabolized to di-homo-γ-linolenic acid (DGLA; 20:3^{8cis,11cis,14cis}) and arachidonic acid (ARA; 20:4$\Delta^{5cis,8cis,12cis,14cis}$) [118,119]. The lipid-peroxidizing enzyme, COX, catalyzes free radical reactions that convert DGLA and ARA into prostaglandins 1 and 2, respectively. Prostaglandins 1 exhibit anti-inflammatory and anticancer activities, while prostaglandins 2 are associated with pro-inflammatory activity [119]. Recently, treatment of two human breast cancer cell lines with GLA or DGLA resulted in significantly reduced cell viability although the effects were not as pronounced as in cancer cells treated with ω-3 PUFA [95].

Seeds of borage (*Borago officinalis*), evening primrose (*Oenothera biennis*), and blackcurrant (*Ribes nigrum*) represent higher plant sources of GLA, but these plants lack the agronomic characteristics associated with major oil crops such as soybean and canola [18]. If oilseed species containing α-18:3 and 18:2 are transformed by heterologous expression with Δ*-6 DESATURASE*, usually both SDA and GLA are formed in the seed oil [17,104,115]. Although various research groups have engineered oleaginous plants to produce GLA (see [18]), the highest levels were produced through metabolic engineering of high 18:1 canola-type *B. napus* [120] and high-18:2 safflower (*Carthamus tinctorius*) [18]. Transformation of high-18:2 safflower with a Δ*-6 DESATURASE* from *Saprolegnia diclina* resulted in seed oil with >70% (v/v) GLA [18] (see Table 36.2). This GLA-enriched (to a lesser extent than 70%) safflower oil was commercialized as SONOVA™400 by Arcadia Biosciences after receiving Food and Drug Administration approval in the United States [121]. The Company has also developed a transgenic safflower that produces ARA-enriched seed oil.

C. Conjugated Fatty Acids

Some plant species contain seed oils enriched in PUFA with conjugated non-methylene-interrupted double bonds [122]. Conjugated fatty acids (CFAs) have been shown to possess nutraceutic properties. Calendic acid (18:3$\Delta^{8trans,10trans,12cis}$) was shown to reduce the viability of human colon cancer cells [123], while catalpic acid (18:3$\Delta^{9trans,11trans,13cis}$), from the seed oil of species in the Bignoniaceae family, was shown to reduce abdominal fat deposition and improve glucose homeostasis [124]. In addition, punicic acid (18:3$\Delta^{9cis,11trans,13cis}$), which is highly enriched in pomegranate (*Punica granatum*)

seed oil, inhibited the growth of both human prostate cancer cells [125] and breast cancer cells [126]. Furthermore, *B. napus* that was metabolically engineered to produce a low level of punicic acid in its seed oil was shown to reduce body fat when fed to mice [127].

In developing seeds of plants producing CFAs, FA conjugase (FADX) catalyzes the conversion of 18:2 into CFA at the *sn*-2 position of PC [122], similar to the formation of PUFA at this position (see Figure 36.1). Calendic acid or α-eleostearic acid (18:3$\Delta^{9cis,11trans,13trans}$) was produced in soybean and in an *Arabidopsis fad3/fae1* mutant through expression of *FADXs* from developing seeds of *Calendula officinalis*, *Momordica charantia*, and *Vernicia fordii* [19]. A calendic acid level of about 20% was produced in the total seed lipid of soybean transformed with the *C. officinalis FADX* [19] (see Table 36.2). *Arabidopsis* [128,129] and *B. napus* [127] were metabolically engineered to produce punicic acid in the seed oil. About 20% punicic acid was produced in T_3 transgenic seed of the *Arabidopsis fad3/fae1* mutant co-expressing pomegranate *FADX* and *FAD2* [129]. Only about 10% punicic acid was produced with *FADX* expression alone due to apparent suppression of *Arabidopsis FAD2* expression by the pomegranate *FADX*. Analyses of CFAs in the PC and TAG of seeds of plants engineered to produce CFAs in their seed oils has shown that CFAs are not effectively removed from their site of synthesis in PC [19,129]. Further increases in the proportions of CFAs in *Arabidopsis* or oil crops engineered to produce CFAs could come from improving the channeling of CFA from the *sn*-2 position of PC into TAG.

VII. ENGINEERING PRODUCTION OF ANTIOXIDANTS IN OILSEEDS

Hydrophobic antioxidants that partition into vegetable oils during the extraction process can protect PUFAs from oxidation and serve as nutraceuticals in their own right [25]. However, it is well accepted that polyphenolics and tocopherols, which are the main antioxidants in freshly pressed olives, play an important role in stabilizing virgin olive oil during processing [130]. Furthermore, the introduction of hydrophobic antioxidants could be an important consideration in the metabolic engineering of oil crops to produce seed oils with enhanced PUFA content.

Ingestion of phytosterols and phytostanols (hydrogenated phytosterols) has been shown to result in reduced cholesterol content in serum and low-density lipoprotein [131]. Phytosterols are present at low concentrations in vegetable oils with phytostanols being absent or present at only trace levels [132]. A cDNA encoding a bacterial 3-hydroxy steroid oxidase was plastidially expressed during seed development in *B. napus* to produce seed oil with significant phytostanol content [133].

There has also been interest in increasing the carotenoid content of crops to increase resistance to high light intensity and increase the nutraceutical value of vegetable oils [134–136]. Carotenoids have strong antioxidant properties and pro-vitamin A activity and are used in preventing cancer and macular degeneration [137]. In one study, carotenoids were enriched in flax seed by about 19-fold (over the control) through the introduction of a bacterial phytoene synthase [138]. Recently, soybean was engineered to produce the pink ketocarotenoid, canthaxanthin, at levels up to 52 μg/g (dry weight) seed [136]. The transformation involved the heterologous expression of genes encoding phytoene synthase in combination with genes encoding ketolases. Ketocarotenoid-enhanced soybean could prove to be a value-added animal feed. Inclusion of carotenoids in feed can result in increased pigmentation and nutritive value of fish, eggs, and lobster [139].

Naturally occurring vitamin E is composed of tocopherols and tocotrienols that serve as antioxidants in photosynthetic organisms [140,141]. Tocopherol content was increased in *B. napus* through metabolic engineering of the tocopherol biosynthetic pathway by heterologous introduction genes from various organisms [142,143]. *BnaX.TE4* was the first gene cloned from *B. napus* that encoded an enzyme involved in tocopherol biosynthesis [144]. The functionality of this gene was assessed in transformation experiments with *Arabidopsis*. *BnaX.TE4*, which encoded a γ-tocopherol methyltransferase, was mapped to a position in the genome where two quantitative trait loci (QTL) for α-tocopherol were previously mapped. This is an example of how a functionality investigation with transgenic plants can complement marker-assisted selection in an oilseed breeding program.

Although no efforts have been made to increase the carotenoid or antioxidant content of palm oil, it is noteworthy that virgin palm oil is already enriched in carotenoids (mainly β- and α-carotene), lycopene, tocopherols, and tocotrienols. These are often partly removed as valuable by-products during processing [42].

The presence of hydrophilic phenolics can also stabilize plant oils even more effectively than hydrophobic tocopherol [145] by a phenomenon described as the "polar paradox" [146]. Phenolic compounds were increased in transgenic high-18:2-flax by metabolic engineering of the phenylpropanoid pathway [147]. Phenolic compounds were identified in the oil with increased oxidative stability; caffeic acid was the most effective of the phenolics. Moreover, mention has already been made of the importance of polyphenolics in preventing oxidation of virgin olive oils [130].

VIII. ENGINEERING INCREASED SEED OIL CONTENT

Increasing the oil content of seed and mesocarp tissue is an important consideration in meeting the demand for edible oil in the future. In Canada, if canola seed oil content could be increased by 1% on an absolute basis, this could potentially add up to over $90 million annually to the oilseed extraction and processing industry (Canola Council of Canada). The enrichment of oil crops to produce bioactive FAs with nutraceutic properties could also potentially compromise the total level of oil accumulated. Therefore, it will be critical to develop oil-boosting molecular strategies to maintain the oil content of the wild type in these particular situations.

For the world's most important oil crop (Table 36.1), oil palm, the possibility of increasing yields to match continued global demand is vital. Currently yields are about 4.5 t/ha but better management could double this; in addition, new palm varieties are being developed that can produce 10–20 t/ha oil. There are realistic prospects of achieving significant increases in oil yield via genetic selection and, maybe, transformation [46]. Alteration is currently focused on disease resistance and tree architecture, but the recent discovery of rapeseed varieties with much higher oil accumulation [148] has led to further interest in the ability of drupes to accumulate oil. Thus, while a freshly picked oil palm bunch is generally 21%–23% (w/w) oil, some olive varieties can contain up to 28%. In addition, palm kernel (about 45% oil), compared to some nut varieties, can have as much as 75% oil [46]. Increasing knowledge of the metabolic control characteristics of oil palm [149,150] should aid this work.

In *B. napus*, seed oil content is controlled by multiple gene loci (QTL) that mainly exhibit an additive effect [151,152]. QTL × environment interactions are also involved in controlling seed oil content [151,152]. Interestingly, there are, however, numerous examples of single gene manipulations in oleaginous plants that resulted in significant increases in seed oil content [21,151,153,154]. Multiple gene manipulations were shown to result in greater increases in seed oil content in *Arabidopsis* [155] and *B. napus* [154] when compared to overexpression of individual genes governing oil formation. In addition, analysis of global gene expression in *B. napus* accessions differing in seed oil content has been used as a basis for identifying genes influencing seed oil content [156–158]. The combination of transcriptome and metabolome analysis is particularly useful in characterizing regulators that influence seed oil content since transcript abundance alone does not always correlate with metabolic outcome [159]. Combined analysis of the transcriptome and metabolome from different developmental stages of oil palm mesocarp versus date palm (*Phoenix dactylifera*) revealed insights into carbon partitioning between lipid and carbohydrate [160].

Additional approaches for increasing TAG accumulation in developing seeds could come from deciphering regulatory mechanisms influencing primary metabolism leading to TAG. In some cases, it may be possible to reduce metabolite-induced feedback inhibition. Indeed, 18:1-ACP was shown to directly inhibit plastidial ACCase in an investigation using microspore-derived cell suspension cultures and developing seeds of *B. napus* [161]. If plastidial ACCase could be desensitized to the allosteric effect of 18:1-ACP, this might result in increased production of FAs to fuel enhanced TAG accumulation.

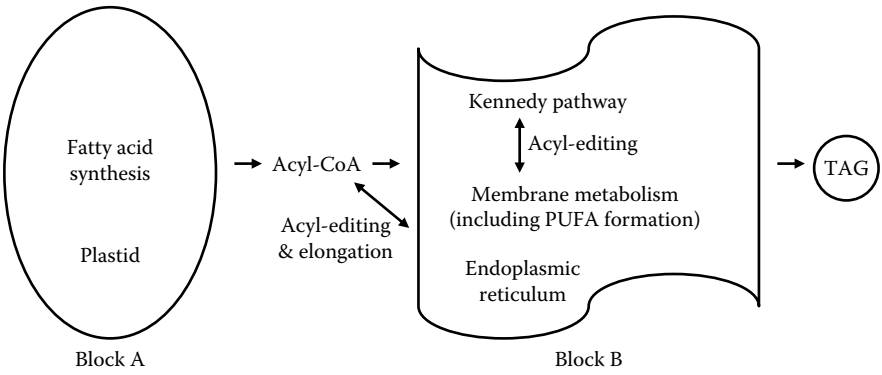

FIGURE 36.2 Simplification of FA and triacylglycerol formation reactions for top-down control analysis. *Abbreviations:* CoA, coenzyme A; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol.

Metabolic control analysis has been useful in studying the relative contributions of different enzyme-catalyzed reactions to TAG accumulation. In top-down control analysis, TAG biosynthesis can be divided into the two main blocks, with Block A representing FA biosynthesis and Block B representing glycerolipid biosynthesis leading to TAG [162,163] (Figure 36.2). Interestingly, in olive, oil palm, and soybean, Block A exerted more control over TAG production than Block B, but in *B. napus* Block B exerted the greatest control [163]. Overexpression of either *B. napus DGAT1* or *Arabidopsis DGAT1* in *B. napus* L. cv Westar and DH12075 resulted in increased seed oil content under both greenhouse and field conditions [164,165]. In addition, in a field study, the overexpression of *DGAT1* reduced the penalty on seed oil content caused by drought [164]. Top-down control analysis of *B. napus* L. cv Westar overexpressing *B. napus* DGAT1 revealed that control of TAG production by Block B decreased to about 50% in transgenic lines from 70% control in the wild type. Several variants of *B. napus* DGAT1 exhibiting enhanced activity over the nontransgenic control have been generated through directed evolution [166]. It is anticipated that some of these variants may be useful in further boosting seed oil content in *B. napus* and possibly other oil crops. Recently, one of four forms of *B. napus* DGAT1 [167,168] (BnaC.DGAT1.a) was highly purified in an active form [169] setting the stage for structure/function studies, which could potentially lead to rationale engineering of the enzyme for enhanced activity and/or altered substrate specificity.

IX. PRODUCTION OF EDIBLE OILS IN VEGETATIVE TISSUE

Typically vegetative tissues contain low levels of TAG of about 0.5% (dry weight) [170], although there are a few exceptions including the stems of *Tetraena mongolica* [171] and *Cyperus esculentus* tubers [172] that accumulate about 9 and 25% TAG, respectively. Within the last 10 years however, substantial advances have been made in the metabolic engineering of vegetative tissue to accumulate substantial levels of TAG [173–175]. Recently, tobacco (*Nicotiana tabacum*) was engineered to produce 15% TAG content in the leaves, without compromising plant development, through combined introduction of *Arabidopsis* WRINKLED1 transcription factor, *Arabidopsis* DGAT1, and sesame (*Sesamum indicum*) oleosin, which is an oil body protein [176]. WRINKLED1 upregulated genes encoding enzymes catalyzing the production of precursors for FA synthesis and enzymes involved in FA synthesis. Increased DGAT1 activity encouraged the utilization of FAs for TAG production, whereas the introduction of oleosin presumably encouraged the production of oil bodies with increased resistance to degradation. In addition to increasing the global supply of plant oil, production of increased lipid in forage vegetative tissue has the potential to increase the energy value of this livestock feed [177]. Furthermore, the incorporation of 3% lipid into high-forage

diets has been shown to result in decreased methane emissions in cattle [178]. Thus, there are both potential dietary and environmental benefits associated with producing increased TAG levels in vegetative tissue.

X. CLOSING COMMENTS

Given the health benefits of many bioactive FAs and the growing consumer demand for dietary sources of these FAs, metabolic engineering of oleaginous plants to produce these FAs has the potential to result in an enormous contribution to preventative medicine (as well as the sustainable production of industrial feedstocks). Although many plant species accumulate FAs with desirable properties, these species typically lack the favorable agronomic characteristics of major oil crops. Most metabolic engineering innovations have focused on alterations of FA synthesis and/or modification (e.g., PUFA formation). In cases where the target FA is derived from an endogenous precursor FA, it is clearly advantageous to have the wild-type seed oil enriched in the precursor FA (see Table 36.2). It is anticipated that the genetic manipulation of acyltransferase action involved in TAG assembly and acyl-editing will gain increasing prevalence in engineering increased levels of bioactive FAs. Acyltransferases may prove to be particularly useful in overcoming "bottlenecks" in PUFA incorporation into TAG and in the generation of intentionally structured TAGs. For example, the positional distribution of very-long-chain ω-3 PUFA within the TAG molecule can influence the functionality of the oil [102]. The melting point of oil is increased when it has DHA present at the *sn*-2 position of TAG and DHA in this position is more resistant to oxidation than if it were at the *sn*-1/3 positions. The melting point effect has implications in the production of margarines and *trans*-free fat substitutes for baking. The increased absorption of FAs at the *sn*-2 position of TAG [1,2] could have implications in the overall bioactivity of modified oil. Would TAG, enriched in DHA or punicic acid at the *sn*-2 position, be a more effective anticancer agent than TAGs with bioactive FAs at the *sn*-1/3 positions? Co-expression of genes encoding enzymes involved in bioactive PUFA formation and antioxidant production could help to produce more stable oils while at the same time providing for a source of valuable antioxidants to benefit human health.

Most oil crops engineered to produce modified oils for food and feed applications are at the experimental stage. Eventually, these crops will need to obtain regulatory approval for large-sale propagation under field conditions. In addition, the resulting oil has to be deemed safe for consumption, especially if the oils contain FAs that are not normally found in the seed oils of the normal crop. As indicated in Sections VI.A and VI.B, inroads here have already been made for the regulatory approval of soybean oil enriched in SDA and safflower oil enriched in GLA.

Finally, substantially greater amounts of vegetable oil will be required as the population of the world increases. Thus, it will be of paramount importance to complement breeding for higher seed oil content with biotechnological approaches, which could be particularly useful in instances where seed oil content has been reduced because of the genetic interventions used to produce vegetable oil with bioactive FAs. Producing oil in vegetative tissue can be considered a disruptive technology that has the potential to substantially increase the global supply of oil for food, feed, medical, and industrial applications.

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37 Genetically Engineered Cereals for the Production of Polyunsaturated Fatty Acids

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CONTENTS

I. INTRODUCTION

Cereal grains are the most important dietary energy source globally where wheat, rice, and maize provide about half of the dietary energy source for mankind. With a global increase in sedentary lifestyles and metabolic disorders (e.g., obesity, cardiovascular diseases, diabetes), the relationship between the energy and nutrient composition of food products has become a major theme in the development of cereal-derived diet with a proper nutritional balance and useful functional attributes. Cereals as a major food supply for humanity contribute to the intake of several macronutrients (proteins, carbohydrates, dietary fiber) and micronutrients (minerals, vitamins). However, they are quite low in fats (average 3%–5% in grains) lacking some essential polyunsaturated fatty acids (PUFAs) and other lipophilic compounds (e.g., carotenoids). A diet based primarily on cereal grains might not only encourage an improper dietary equilibrium of these metabolites, but it may also finally lead to an increased incidence of various diseases resulting from an insufficient intake of PUFAs. Therefore, the availability of cereal types with proper compositional balance, the consumption of such cereal foods, and their contribution to a healthy diet should be the bottleneck to good nutrition. In all cases, a well-balanced compositional quality of cereals plays a "gate-keeping" role in providing cereal foods for consumers.

Polyunsaturated fatty acids are indispensable for human well-being due to their healthy dietary and functional properties [1]. Attention is mainly paid to γ-linolenic acid (18:3 n-6, GLA), dihomo-γ-linolenic acid (20:3 n-6, DGLA), arachidonic acid (20:4 n-6, AA), eicosapentaenoic

acid (20:5 n-3, EPA), docosapentaenoic acid (22:5 n-3 or n-6, DPA), and docosahexaenoic acid (22:6 n-3, DHA). Insufficient content of PUFAs in cereal grains in comparison with the high amount of consumed cereals has led to the search for an appropriate technology that would be able to enrich common cereals with these metabolites. Natural construction or modification of cereals for the preparation of functional cereal-derived food/feed products containing biologically active PUFAs might be a promising and challenging technology.

II. SIGNIFICANCE AND SOURCE OF POLYUNSATURATED FATTY ACIDS

Polyunsaturated fatty acids (PUFAs) cannot be synthesized by mammals because mammals do not possess the enzymes Δ12-desaturase (catalyzes conversion of oleic acid to linoleic acid) and Δ15-desaturase (transforms linoleic acid to α-linolenic acid). Apparently, other PUFAs belonging to both n-6 (GLA, DGLA, AA) and n-3 (EPA, DHA) pathways cannot be produced when linoleic and α-linolenic acids (C18:3 n-3, ALA) are lacking in the diet. PUFAs, due to their distinctive structural and functional properties, are characterized by several functions [1]:

- Regulate the architecture, dynamics, phase transition, and permeability of membranes.
- Modulate the behavior of membrane-bound proteins such as receptors, ATPases, transport proteins, and ion channels.
- Control the expression of certain genes and thus affect some processes including fatty acid biosynthesis and sterol metabolism and transport in the body.
- Inhibit synthesis of vasoaggressive low-density lipoprotein (LDL) without any influence on the vasoprotective high-density lipoprotein (HDL).
- Are precursors of a wide variety of metabolites (such as prostaglandins, leukotrienes, and hydroxy-fatty acids) that regulate critical biological functions.
- n-3 PUFAs have beneficial effects on cardiovascular diseases and are essential for normal visual and brain function.

The various roles played by PUFAs make it apparent that they are required in every organ in the body for the organs to function normally. Therefore, insufficient dietary consumption of PUFAs leads to abnormalities in the skin (atopic eczema), diabetes, hypertension, coronary heart disease, endocrine, nervous, inflammatory, immune, respiratory and reproductive systems, rheumatoid arthritis, and premenstrual syndrome [2]. Because mammals lack the ability to synthesize PUFAs, these must be supplied in the diet. Besides the overall intake of PUFAs and due to the distinct functions of the two PUFA families, the ratio of n-6/n-3 PUFAs is known to be of nutritional importance as it is the key index for balanced synthesis of eicosanoids and for proper maintenance of body health. Optimal n-6/n-3 ratio is always a matter of debate and obviously depends on age and physiological and clinical body status. Nutritional scientists suggest the ideal n-6/n-3 ratio from 2:1 to 4:1, although the ratio is strongly influenced by local PUFA sources' availability and consumer habitats (e.g., the ratio for Indian consumers is equal to 1:30–70, for Japanese 1:2–4, for Western communities 10–30:1) [3]. The most common sources of main PUFAs are summarized in Table 37.1.

III. BIOCHEMICAL PATHWAYS FOR PUFA FORMATION

A. Biosynthesis of Polyunsaturated Fatty Acids

Biosynthesis of PUFAs is associated with both membrane-bound desaturase and elongation enzymes. The desaturation system of the aerobic pathway is composed of three proteins: NAD(P)H-cytochrome b_5 reductase, cytochrome b_5, and the terminal cyanide-sensitive desaturase [11]. The exception could be found in organisms from the prokaryotic group since they contain ferredoxin

TABLE 37.1
Common (Non-GMO) Sources of PUFAs

Fatty Acid	Plant Source	Animal Source	Microbial Source
GLA	Echium, borage seeds, black currant seeds, evening primrose [4]	Human milk [3]	Zygomycetous fungi (*Thamnidium elegans*, *Cunninghamella echinulata*, *Mucor circinelloides*, etc.) [6]
AA		Human milk [3]	*Mortierella alpina* [7]
EPA		Cod, tuna, mackerel [5]	*Pythium* sp. [8]
DHA		Cod, tuna, mackerel, human milk [3]	*Thraustochytrium* sp. [9], *Thalassiosira pseudonana* [10]

Abbreviations: GLA, γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

instead of cytochrome b_5. Desaturation takes place in the endoplasmic reticulum where the fatty acid bound to phospholipids (especially phosphatidylcholine) is desaturated rather than thiol CoA ester [12]. Membrane-bound desaturases introduce double bond into fatty acids that are either esterified as acyl-CoA or bound to the glycerol moiety of glycerolipids [13].

Figure 37.1 represents the aerobic biosynthetic pathway of PUFAs. The first double bond is introduced into Δ9-position of saturated fatty acid, thus palmitoleic (C16:1n-7) and oleic (C 18:1 n-9) are the most common monoenes in organisms. Oleic acid is then, in general, transformed

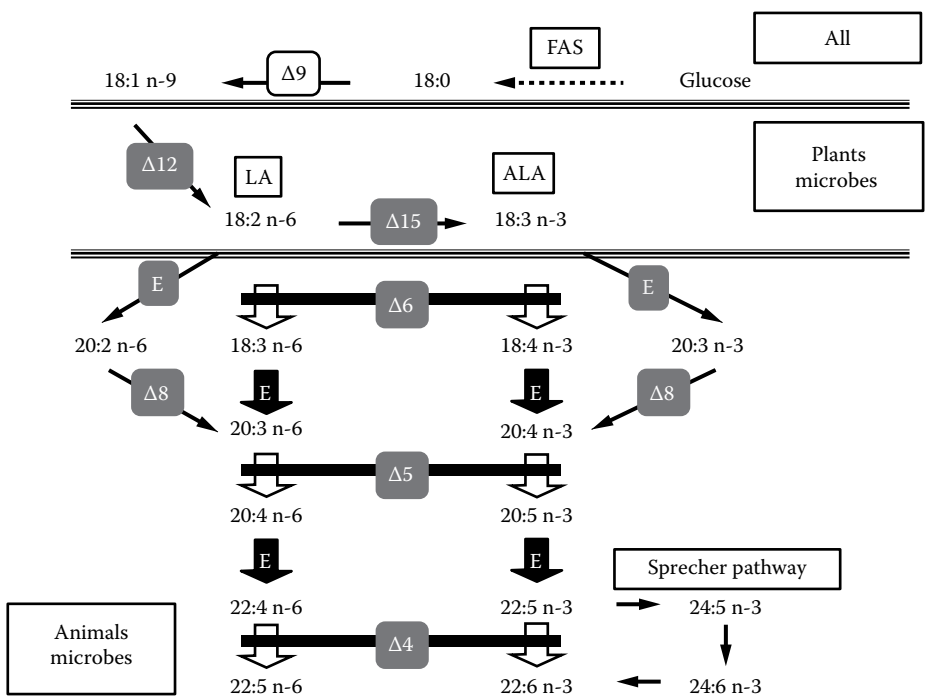

FIGURE 37.1 Biosynthetic pathway of polyunsaturated fatty acids. FAS, fatty acid synthase system; LA, linoleic acid; ALA, alpha linolenic acid; E, elongase; ΔX, desaturation enzyme (the number expresses the carbon position in acyl chain where desaturation occurs). (From Klempová, T. et al., *Gen. Physiol. Biophys.*, 32, 445, 2013.)

by Δ12-desaturase to yield linoleic (C18:2 n-6, LA), which may be further converted by Δ15 (ω-3)-desaturase to ALA. Thus, these fatty acids are the basic precursors of n-6 and n-3 fatty acid cascades. The next step is the desaturation of these fatty acids by Δ6-desaturase. LA in n-6 pathway is converted to GLA (C18:3 n-6), and ALA in n-3 pathway is converted into stearidonic acid (SDA, C18:4 n-3). GLA and SDA are subsequently elongated into C20 fatty acid. GLA is elongated into DGLA (C20:3 n-6), and SDA into eicosatetraenoic acid (ETA, C20:4 n-3). Then occurs another desaturation step using Δ5-desaturase. In n-6 pathway, ARA (C20:4 n-6) is synthesized and in n-3 pathway EPA (C20:5 n-3) is formed. These two fatty acids undergo the elongation step to create C22 fatty acids with a corresponding number of double bonds. The last step is catalyzed by Δ4-desaturase and docosapentaenoic acid (C22:5 n-6) and docosahexaenoic acid (DHA, C22:6 n-3) are produced [14]. Each fatty acid desaturase introduces a double bond at the specific position of the acyl chain [13]. The Δ9-desaturase is the only universally spread desaturase present in all living beings groups. The remaining desaturases are missing in some of the evolutionary lineages and occurrences of various types of desaturases depend on individual organisms. The Δ12- and Δ15-desaturase occurs only in marine bacteria and algae, cyanobacteria, zygomycetes fungi, and higher plants. This distribution makes linoleic and α-linolenic acid "essential fatty acids" for animals and Δ9-desaturase essential for life [15]. The Δ6- and Δ5-desaturases are present only in marine bacteria and algae, zygomycetes fungi, and animals. The Δ4-desaturase was identified only in marine protists, algae, and thraustochytrids such as *Thraustochytrium* sp. [9] and *Thalassiosira* [10]. For the last step, mammals use the "Sprecher pathway" instead of direct desaturation of C22:5 n-3 to DHA. Sprecher pathway has three distinct steps—first is elongation to 24:5 n-3 followed by desaturation with Δ6-desaturase and then one β-oxidation step to DHA [16]. In the late 1990s, the alternative Δ8-pathway was discovered in some protists and algae species, for example, *Euglena* [17] or *Isochrysis* [18]. This pathway involved the Δ9-elongation step of LA or ALA as the first one was followed by Δ8-desaturation to yield DGLA (in n-6 cascade) or eicosatetraenoic acid (ETA) (in n-3 cascade).

B. PUFA Storage in Triacylglycerols

Once desaturation has occurred, the acyl transfer reactions then facilitate distribution of newly synthesized PUFA to other cellular lipids. Fatty acids are selectively incorporated into complex lipids to overcome cell toxicity from the free acyl form. Membrane phospholipids as functional cell structures are one of the targets for PUFA incorporation. However, many oleaginous microorganisms accumulate large amounts of PUFAs as storage lipids in the form of triacylglycerols [19,20]. These fungi also have a unique cooperation of relevant metabolic processes to cope with their surrounding environment by adjusting pools of membrane and storage lipids. Triacylglycerols, as the main storage lipid pools, are synthesized from *sn*-glycerol-3-phosphate and acyl-CoA through a sequential process involving glycerol-3-phosphate acyltransferase, lysophosphatidate acyltransferase, phosphatidate phosphatase, and diacylglycerol acyltransferase [21,22]. Also, the acyl chains are bound to phospholipids where they become desaturated. Fatty acids from phospholipids are then available for triacylglycerol biosynthesis by one of the two mechanisms responsible for the regulation of triacylglycerol unsaturation [23]. In the first mechanism, acyl exchange occurs between acyl-CoA pool and acyl-phospholipid (mainly phosphatidylcholine) by the combined reverse and forward reactions of acyl-CoA:phospholipid acyltransferase. The resulting newly formed PUFA-CoAs enrich the acyl-CoA pool where they serve as acyl donors for triacylglycerol synthesis. The second mechanism involves donation of the newly formed PUFA-diacylglycerols from phosphatidylcholine catalyzed by reversible CDP-choline phosphotransferase that can be directly available for triacylglycerol synthesis. Thus, acyltransferases and desaturases may be tightly bound and their cooperation, including their specificities and selectivities to different acyl-CoA species, is responsible for PUFA distribution among individual lipid structures.

IV. GENE ENGINEERING APPROACH FOR CONSTRUCTION PLANTS CONTAINING PUFAs

Transgenic plants engineered to produce major PUFAs, such as GLA, DGLA, AA, EPA, and DHA, could be one way of overcoming the foreshadowed shortage of these nutritionally important fatty acids. During the last few years, many of the genes that are responsible for the biosynthesis of PUFAs have been cloned from various organisms, including fungi, algae, mosses, plants, nematodes, and mammals [14,24,25]. However, successful alteration of PUFA metabolic pathway would require the introduction of genes controlling each of the biosynthetic steps in the pathway for the synthesis of PUFAs from their adequate C18 precursors.

As a first step in the conventional PUFA pathway, effort was focused on the introduction of a double bond at the Δ6 position by the action of the Δ6-desaturase to produce both GLA from linoleic acid and stearidonic acid (18:4 n-3, SDA) from ALA [25,26]. Other genes of the conventional desaturation/elongation Δ6-desaturase, elongase, and Δ5-desaturase have been expressed in oilseeds for the biosynthesis of DGLA, AA, and EPA [27]. However, the elongation step was found as a "bottleneck" for more efficient production of very long-chain PUFAs. Fortunately, successful accumulation of AA and EPA in transgenic plants was carried out by "reverse-engineering" the PUFA pathway via the alternative nonconventional Δ9-elongase/Δ8-desaturase system [28,29]. This approach was initiated by utilization of endogenous linoleic acid and ALA in the acyl-CoA pool by Δ9-elongase to provide C20 substrates for the subsequent desaturases. The final transgenic lines accumulating C20 PUFAs were also the result of three sequential genetic transformations, in which the Δ9-elongase, Δ8-desaturase, and Δ5-desaturase were introduced as separate transgenes and integration events. In addition, some nonmethylene interrupted C20 fatty acids (sciadonic acid 20:3Δ5,11,12, n-6 and juniperonic acid 20:4Δ5,11,14,17, n-3) were also detected with parallel accumulation of AA and EPA [29]. These studies represent important biotechnological breakthroughs in a major step forward in the production of very long-PUFAs in transgenic plants. They also provide some new insights into the biochemical pathways under manipulation and provide useful new tools for the dissection of the underlying enzymatic reactions. However, these studies focus on manipulation within dicotyledonous plants. Gene transformation of monocotyledonous plants is still a challenge for scientists since such kinds of plants represent a major source of food for mankind.

V. GENETICALLY ENGINEERED CEREALS PRODUCING ESSENTIAL FATTY ACIDS

Cereals, as a major food supply, are a rich source of proteins, carbohydrates, minerals, and some vitamins. However, they are deficient in essential PUFAs. The diet predominantly based on consumption of cereal grains may therefore lead to increased incidence of diseases caused by insufficient intake of PUFAs. The profile of synthesized fatty acids in cereal grains is not possible to modify by classical breeding approaches used in plants. Therefore, it is a challenge for scientists to breakthrough this barrier in different cereals. An appropriate way to achieve this should be biotechnological approaches such as the solid state fermentations (SSF) or genetic engineering. The common element for both approaches could be the *Zygomycetes* fungi playing a crucial role as producers of PUFAs as well as suitable donors of genes-encoding relevant enzymes involved in biosynthesis of the desired PUFAs (e.g., fatty acid desaturases).

One of biotechnological approaches for the creation of cereal plants able to synthesize desired PUFAs using the cereal genome modification are genetic engineering techniques. Although the effective transfer of alien genes into the cereals is not as straightforward as in other agricultural crops, the delivery of fungal gene *D6D* encoding the enzyme Δ6-desaturase into barley and wheat genomes and production of PUFAs in their seeds have been successfully completed recently [30,31]. Successful modification of barley and wheat plants by transfer of the *D6D* gene were confirmed at all the genomic, transcriptomic, as well as metabolomic levels. Developed transgenic barleys and wheats were able to synthesize two PUFAs, γ-linolenic acid and stearidonic acid. Both biotechnological

strategies mentioned earlier offer real potential for the production of cereal-based products naturally enriched with PUFAs as well as tailor-made functional cereals, respectively. Both approaches may open new applications in the food and feed industry by the production of cereal-based products beneficial for consumers.

A. Gene Transfer in Cereals

Genetic transformation is a modern biotechnological approach enabling the creation of novel types of plants by the introduction of alien genes originating from microorganisms, animals, or other plant species not insertable into the plant genome by conventional plant breeding techniques. Plants adopting different foreign gene(s) acquire new traits and properties related to resistance against diseases and pests, tolerance to herbicides and abiotic stresses (drought, high temperature, low soil quality, soil contamination), higher yield, physiological changes, improved quality, nutritional value, technological properties, and biomass production [32,33]. Transgenic plants can be used also as producers of different biologically active compounds usable as nutraceuticals, enzymes, pharmaceuticals, vaccines, or raw materials with improved properties for industrial applications. Nowadays, the most advanced applications of the gene transfer technologies within cereals are in maize.

An effective plant transformation procedure has to meet general requirements. They include an effective *in vitro* regeneration system optimized for recipient plant species or specific genotypes, well-designed DNA vectors carrying the gene of interest as well as reporter and selection genes, and other specific DNA sequences needed for the improvement of transgene expression in plant. Another important requirement is an efficient technique for gene transfer into plant cells and tissues, reliable system for selection of transformed cells, and molecular methods for the analysis of gene transfer and expression at the levels of DNA, RNA, protein, and phenotype. The level of transgene expression in transformed plants strongly depends on the gene delivery vector and gene construct containing an appropriate promoter (constitutive, inducible, tissue-, organ-, or developmentally specific), attached signal peptide, and potentially modified codon usage with respect to target plant species. Expression of transgene and the final phenotype of the genetically modified plant are also affected by the number of copies and the position of inserted transgene within the plant host genome.

Most plant transformation protocols depend on the ability of plant species to regenerate from cells and tissues into the complete plants through the *in vitro* cultivation system and later to develop fertile plants. Cereals, as a member of monocotyledonous plants, are generally considered as recalcitrant species in *in vitro* tissue cultures as well as for genetic transformations. Some genotypes as the well-known barley cultivar Golden Promise [34,35] and the wheat cultivar Bobwhite [36] are considered as respondent, possessing relatively high *in vitro* regeneration ability, acceptable for genetic transformation experiments. However, both cultivars have none or very limited practical and economical value in agriculture. Even though significant progress has been made in the transfer of genes into the model cultivars of some cereals, commercially important cultivars still pose a serious problem. An effective *in vitro* regeneration within the elite cultivars was described only rarely [37–40]. Therefore, the selection of regenerable genotypes and the optimization of the *in vitro* regeneration and transformation protocols are obligatory prerequisites for successful genetic modification of genotypes in cereals, especially in barley and wheat. Different plant explants have been used for initiation of *in vitro* culture and subsequent transformation and regeneration within wheat and barley. Initial efforts to deliver foreign genes into their genomes were done by the transformation of protoplasts, but the recovery of fertile plants from protoplast has rarely been observed [41–43]. Most of the fertile transgenic wheat or barley plants were created using the immature embryos as targets for alien genes [39,44]. Nevertheless, plant biotechnologists are still looking for alternative tissues and cells to eliminate labor- and time-consuming cultivation of donor plants either in the field or in controlled conditions to obtain well-conditioned immature embryos.

Construction of the first recombinant plasmids [45] and discovery that the soil bacteria *Agrobacterium tumefaciens* acts as the natural plant vector able to transfer own genes located in

its Ti-plasmid into the genome of colonized plant cells [46] were the cornerstones of alien gene transfer and genetic modifications of plants. After this breakthrough, scientists attempted to use these bacteria for directed genetic transformation of plants and the first transgenic plant, the tobacco resistant to antibiotics, was created [47]. Unsuccessful genetic transformations of cereals by the *A. tumefaciens* in those years initiated the development of alternative methods for direct gene transfer into cereals. Many different direct gene transfer methods were developed such as electroporation, microinjection, polyethylene glycol–mediated transfer, imbibition, DNA uptake mediated by ultrasound, laser, and silicon-carbide fibers. Some of these approaches were successful in the generation of transgenic plants. Nevertheless, these methods are strongly dependent on specific types of cells possessing the ability to regenerate into complete plants [44]. The first transgenic plants of wheat [48] and barley [49] were created by the biolistic method (also called particle bombardment). This technique was first presented in 1987 [50,51] and has been based on the bombardment of plant cells with pressure or electrically accelerated particles coated with DNA-containing genes of interests. Commonly accepted equipment for cells and tissues bombardment is the PDS-1000/He™ System (Bio-Rad Laboratories, Hercules, CA) accelerating nucleic acid-coated gold or tungsten microparticles. Particles, as the microcarriers of plasmid DNA accelerated by the helium pressure, can penetrate into the targeted plant tissues and cells. The biolistic approach has been recognized as a more or less species- and tissue-independent method with the potential to transform a broad spectrum of plant species and genotypes including the elite ones and is routinely used for the creation of transgenic plants in cereals. Nevertheless, success in cereals was achieved usually only when immature embryos were used as targets for particle bombardment. The current status in cereals' genetic transformations, especially in barley, including the gene transfer techniques, targets, transgenic progeny stabilization, and field trials, has been reviewed recently [33,52].

The monocots were generally considered as being out of the host spectrum of *A. tumefaciens* and were transformed (rice plants) using this vector 10 years after the transformation of dicots [53]. Difficulties in transformation of monocots using the *A. tumefaciens* system are due to anatomical differences in the cell wall chemistry and wound response including the activation of virulence (*vir*) genes between monocots and dicots [54]. These barriers were later overcome using the supervirulent strains of *A. tumefaciens* and by the addition of chemicals such as acetosyringone, acting as the *vir*-activator [42]. The first transformation of barley by *A. tumefaciens*–mediated gene transfer was reported by Tingay et al. [55], and in the same year, wheat plants were successfully transformed also [56]. Since that time, an important improvement in *Agrobacterium*-mediated transformation techniques of monocots has been made. This method is now widely applicable due to its advantages such as higher transformation efficiency, lower transgene copy incorporation into the host genome, preference of the DNA integration into transcriptionally active regions, more stable expression, and inheritance of the transgene into the progeny [57].

B. Genetic Engineering of Fatty Acid Biosynthesis in Cereals

Higher plants contain higher variation in fatty acids composition in comparison with animals. Nevertheless, the number of different fatty acids common in plants is lower. The most common fatty acids in plants are saturated fatty acids such as palmitic and stearic acids and unsaturated fatty acids such as oleic acid, LA, and ALA [58]. Cereals can synthesize only linoleic and α-linolenic acids among PUFAs. The reason is the absence of relevant enzymes in their fatty acid biochemical pathway responsible for desaturation leading to the generation of other PUFAs and elongation leading to synthesis of very long-chain PUFAs.

Production and overproduction of PUFAs have been realized in the past 17 years by genetic transformation of model plants and also in several oil-seed plants. These results confirmed that genetically engineered plants are able to produce fatty acids, including PUFAs, nonnative to them. Several genes-encoding desaturases and elongases originating from different species were delivered into plants synthesizing and cumulating oils in seeds and after adoption of different transformation

events, they were able to modify the quantity and also quality of synthesized PUFAs. Oil-seed crops as the dicotyledonous plants are more accessible for genetic transformations in comparison with monocotyledonous plants. The first organism with modified fatty acids content created by genetic modification was the model plant *Arabidopsis thaliana* [59–61]. Their experiments focused on the study of fatty acid desaturases changing the composition of unsaturated fatty acids. These ground-breaking works showed that desaturation occurs in plants in two ways—prokaryotic-like and eukaryotic. Prokaryotic-like desaturation is located in plastids and eukaryotic at the endoplasmic reticulum [62–65].

Several plant species were transformed with genes-encoding desaturases and elongases later (1997–2015) to achieve either the production of uncommonly synthesized PUFAs or an increase production of natively produced PUFAs. The graphical review in Figure 37.2 reveals that dicotyledonous plant species were transformed with desaturases more frequently. Within cereals, the rice plants were modified by Δ12 and Δ15-desaturases to overproduce ALA, but only barley and wheat plants were transformed by Δ6-desaturase gene [30,31].

The most frequented object of genetic transformation within cereals is rice (*Oryza sativa* L.) and efforts were aimed at improvement of fatty acid content that are deficient in rice seeds. Wakita et al. [66] modified rice plants by gene construct containing the tobacco microsome n-3 fatty acid desaturase gene (*NtFAD3*) under the control of the CaMV 35S promoter. Content of the ALA in seeds of transgenic homozygous progenies in their experiment was 2.5-fold higher than in control [67].

The first cereal seeds enriched with PUFAs up to the level similar to flax (*Linum usitatissimum* L.) were also seeds of rice (*O. sativa* L., cv. Reiho) transformed by the *A. tumefaciens* system [68]. Plant transformations have been performed with the chimeric gene *GmFAD3* consisting of cDNA of soybean microsomal n-3 fatty acid desaturase (Δ15-desaturase) and the maize constitutive promoter *Ubi1-P-int*. The *GmFAD3* transgene was expressed effectively and transgenic plants accumulated ALA up to 37.5% of the total oils in seeds (compared to original ratio 2.1%–2.2%). This heterologous gene expression system was so effective that seeds of transgenic rice plants contained as much ALA as seeds of flax.

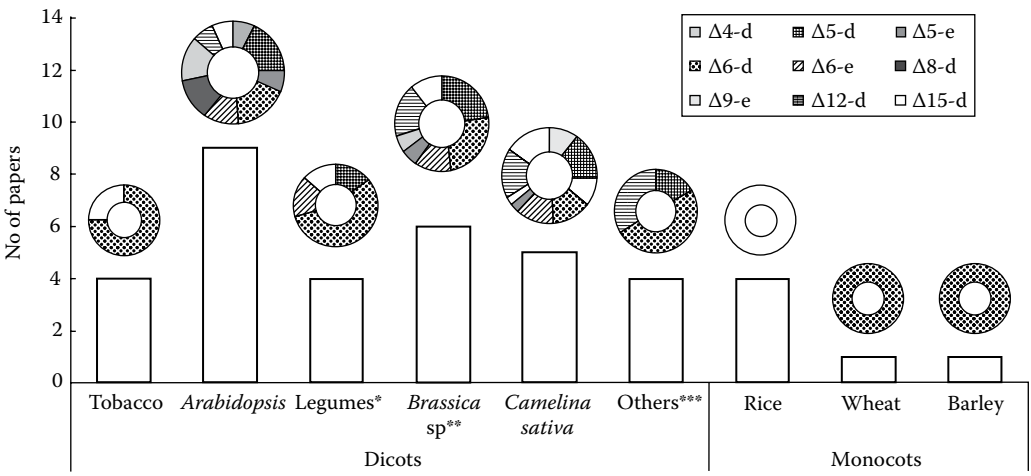

FIGURE 37.2 Number of scientific papers published from 1997 to November 2015 related to genetic modification of plants by desaturase ("d" in legend) and elongase ("e" in legend) genes to produce native and nonnative PUFAs. *Soybean (2 papers), *Lotus japonicus* (1), *Vigna angularis* (1), ***Brassica juncea* (2), *Brassica carinata* (1), *Brassica napus* (3), ***Safflower (1), flax (1), tomato (1), potato (1). (From Databases ScienceDirect, SpringerLink, Wiley Online Library, and PubMed, under search option: "transgenic" AND "plant" AND "PUFA.")

Liu et al. [69] achieved increased amounts of ALA in rice seeds by genetic transformations using six different n-3 fatty acid desaturase (*FAD*) genes cloned from rice and soybean under the control of endosperm-specific promoter *GluC* and constitutive promoter *Ubi-1*, respectively. Modified plants obtained by the *cis*-genesis contained 27.9 times more ALA, while the plants obtained by the *trans*-genesis had 23.8 times higher content of ALA in comparison to non-transgenic control plants. Authors confirmed the hypothesis that the endosperm-specific promoter increases destination of ALA production into the mature grains.

Yin et al. [70] transformed rice with fatty acid desaturase genes n-3/Δ15 originated from rice and soybean, under the control of embryo-specific promoter *REG*. The ALA expression in embryos and bran was up to 27.9-fold more than in nontransformed control. The ALA accounted for 46.7% and 44.3% of the total fatty acids in embryos and bran, respectively, which was also comparable with the flax seeds.

An opposite strategy to change fatty acid composition in rice seeds was presented by Zaplin et al. [71]. They modified rice plants to increase content of oleic acid in seeds at the expense of linoleic and palmitic acids, that is, to suppress the formation of fatty acids with double bonds. This was achieved by suppressing the microsomal Δ12-desaturase (*OsFAD2*) gene by RNA interference (RNAi). Expression of the *FAD2*-RNAi construct was driven by the wheat high molecular weight glutenin subunit (HMW-GS) promoter *Bx17*. Content of oleic acid in transgenic rice lines was raised to 51%–65% from the original 38% and contents of linoleic and palmitic acids were reduced.

The first genetic modification of barley (*Hordeum vulgarum* L., cv. Golden Promise) resulted in the expression of two nonnative PUFAs in seeds was presented recently [30]. Authors created transgenic barley plants producing γ-linoleic acid up to 0.14% and stearidonic acid up to 0.29% of the total amount of fatty acids in mature seeds. Barley plants were transformed with the artificial gene construct encoding the Δ6-desaturase (*D6D*) (GenBank accession HM640246.1) derived from the filamentous fungus *Thamnidium elegans*. Original gene sequence was modified in codon usage to be optimal for expression in cereals. The final destination of the *D6D* gene expression was supported by the endosperm-specific promoter of the HMW-GS *Dx5* as well as by the signal sequence for the same glutenin subunit. Protein sorting of HMW-GS is located at the endoplasmic reticulum and their signal sequences are relatively highly conserved [72–74]. This work was the breakthrough in the synthesis of non-native PUFAs in cereals. The PUFAs enrichment of cereals should be desired not only from nutritional, but also from growing point of view, as the factor contributing to cold tolerance. The same artificial gene construct encoding the Δ6-desaturase has been used also for genetic modification of fatty acid biosynthesis in wheat (*Triticum aestivum* L.) [31]. The artificial *D6D* gene was delivered into plants of the spring wheat line CY-45 and transcribed *D6D* mRNA was confirmed in T_0 and T_1 plants. Synthesized GLA was detected in mature grains up to 0.32% of the total fatty acids. Both newly synthesized GLA and SDA have been detected also in immature and mature grains of the T_2 generation at 0%–1.40% and 0%–1.53% of the total amount of fatty acids, respectively.

Increased demand for vegetable oils led to the interest in the lipid metabolism and storage in different plant species including cereals and redirecting of carbon from starch to oil in the endosperm of cereal seeds [75]. Improvement of cereal grains with PUFAs is an interesting idea to improve their nutritional value. However, another important reason for the interest in enrichment of cereal seeds with PUFAs is their possible role and relationships between the level of unsaturation of lipids and temperature during plant growth. Mano et al. [76] studied PUFAs as one of the main components of phospholipids in the *japonica* brown rice cultivars growing in colder regions. They contained phospholipids with higher amounts of PUFAs than the *indica* cultivars growing in locations with mild climatic conditions. These results are opening serious discussions about the level of unsaturation of fatty acids leading to higher biological membranes fluidity and related growth and survival of rice plants as well as other plant species at lower or higher temperatures.

VI. SOLID STATE FERMENTATION APPROACH FOR PREPARATION OF CEREAL-DERIVED MATERIALS WITH PUFAs

Fermentation approach based on solid state fermentations (SSF) is one of the prospective techniques to enrich cereals with desired PUFAs. During the process, useful microorganisms grow on various agro-materials (e.g., cereals) by the utilization of carbon, nitrogen, minerals, and other nutrients from these substrates and finally convert them to various types of value-added prefermented "bioproducts" with the desired properties [6,77,78]. An advantage of the SSF process is that it is not necessary to extract PUFAs from fermented materials, but these newly formed PUFA cereal materials can be directly used for applications, for example as food and feed additives. Therefore, the production of PUFA cereals by SSF could be a useful method and it might provide valuable opportunity to fill marketing claims in the food, feed, pharmaceutical, veterinary, and environmental fields.

Depending on the substrate and cultivation conditions, several prefermented cereal products enriched with PUFAs have been prepared. Under optimal conditions, the final prefermented cereals with *T. elegans* yielded up to 20 g GLA/kg bioproduct [79]. *Mortierella isabellina* sufficiently enriched barley with 18 g GLA/kg bioproduct [79], cultivation of *Cunninghamella elegans* on the mixture of barley/spent malt grain/peanut oil led to 14.2 g of GLA/kg product [80] and growth of *Mucor circinelloides* on the mixture of rye bran/SMG/sunflower oil yielded to 24.2 g of GLA/kg product [81]. In addition, the growth of *Mucor wosnessenskii* was optimized for the simultaneous production of GLA and β-carotene. Utilization of mixture of oat flakes/spent malt grains (3:1) resulted in 10.7 g GLA and 260 mg β-carotene/kg bioproduct, respectively. On the other hand, *Mortierella alpina* converted mixture of wheat bran/spent malt grains (3:1, w/w) to bioproduct with 4.2% AA, dehulled millet to prefermented mass containing 4.5% AA, and cracked barley to the final bioproduct consisting of 4.1% AA [7]. *M. alpina* was also used for enrichment of cereals with DGLA by addition of sesame seeds to peeled barley (17 g DGLA/kg bioproduct) [7]. SSF process has been developed to prepare EPA-rich cereals by *M. alpina* that rapidly consumed the mixture of peeled barley/linseed oil/spent malt grains (0.5:1:3, w/w) and simultaneously yielded up to 23.4 g EPA and 36.3 g AA/kg bioproduct, respectively [7]. *Mortierella antarctica* was also employed for enrichment of brewer's spent grain, however the final prefermented product contained only 0.28% EPA [82]. Thus, such strategy allows the preparation of oils with desirable n-6/n-3 PUFA ratio finally leading to more beneficial applications.

VII. CONCLUSIONS AND PERSPECTIVE

The development of biotechnological enrichments of cereals with PUFAs should reflect their commercial potential and demand. Flourishing accomplishments of gene engineering techniques carried out over recent years have enabled the construction of new tailor-made cereal varieties synthesizing desired PUFAs. These molecular methods represent a challenging and potentially rewarding subject for aimed modification of cereals with required nutritional design and claim. In addition, newly "bio-based" PUFA-enriched cereals naturally prepared by SSFs should, in general, be considered safe and may open novel prospects for such functional cereals in food, feed, pharmaceutical, and veterinary fields. Thus, commercial applications of biotechnologically prepared PUFA cereals will certainly depend on their acceptability in the market, regulatory approval, the size of the capital investment, and effective delivering of the product to the market.

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