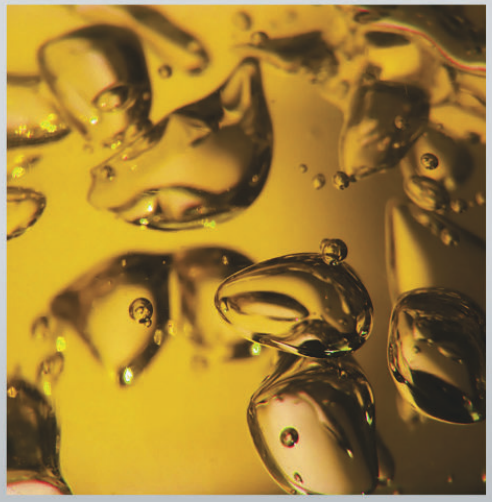
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Lipid oxidation

Second edition

Edwin N. Frankel

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Lipid oxidation

Second edition

EDWIN N. FRANKEL
University of California, Davis, California, USA





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Lipid Technology

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Preface

Preface to Second Edition

After more than six decades, the field of lipid oxidation continues to be very active and has expanded into broad areas of food science and technology, free radical chemistry, nutrition, biochemical and biomedical interests. Although there have been significant advances in this challenging field, many important problems remain unsolved. This second edition follows the example of the first edition in offering a summary of the many unsolved problems that need further research. New developments in lipid oxidation research and technology and new dietary guidelines have resulted in difficult and challenging problems of control with nutritional and biological implications. Various processed foods have been reformulated with oils rich in long-chain polyunsaturated fatty acids because of their recognized nutritional benefits, but lipid oxidation has seriously limited their utilization. Because partial hydrogenation produces *trans* fatty acids now considered nutritionally harmful, much effort has been made in developing alternate methods for the preparation of oxidatively stable oils. To avoid hydrogenation, special plant breeding has been used to develop several high-oleic and low-linolenic acid oils, and has improved their oxidative stability and nutritional value. In the last decade, enormous worldwide attention has been given to natural phenolic antioxidants, including flavonoids and various phytochemicals found in many fruits and vegetables and beverages. Although some new knowledge has been acquired about the absorption of these antioxidants in humans, their *in vivo* activity is still not well understood. The development of reliable biomarkers that can be better related to degenerative disease presents one of the most difficult challenges in this field.

The second edition includes important developments in the characterization by capillary gas chromatography-olfactometry of aroma and flavor impact of volatile decomposition products from polyunsaturated fatty acids and esters. Discussions are included on various mechanisms for the formation from linoleate of 4-hydroxy-2-nonenal, which has received much attention in the biochemical literature because of its cytotoxic properties, and its occurrence in oxidized LDL and in vegetable oils heated at frying temperatures. Some of the volatiles produced from fish oils are responsible for major problems in their utilization, because they produce very powerful ‘fishy’ odors and flavors perceptible at extremely low levels of oxidation.

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This second edition offers new material on methods of sensory detection (nasal: through the nose) or (retronasal: through the mouth and back of the oral cavity), different flavor release phenomena in the headspace versus the mouth, and matrix in flavor release from oils compared to emulsion systems. Advanced gas chromatographic methods are included, such as solid phase microextraction for the volatile analyses in foods and vegetable oils, gas chromatography-olfactometry, and aroma extraction dilution analyses.

A number of advanced instrumental methods have emerged for analysing mixtures of complex products of lipid oxidation. The second edition has a new chapter that examines these research methods, and how they provide new information on the stereochemistry of hydroperoxides from oleate and linoleate, polyunsaturated phospholipids and triacylglycerols, oils extracted from fried foods and their decomposition products (Chapter 6). The formation of the nonconjugated bis-allylic 11-hydroperoxide intermediate from linoleate was established unambiguously for the first time using α-tocopherol acting as hydrogen donor. Conjugated linoleic acid (CLA) has been claimed to be bioactive and to have beneficial nutritional effects. The mechanism of CLA autoxidation is discussed, together with the complex isomeric mixtures of endoperoxides and thromboxanes (prostaglandin-like compounds) produced from arachidonic acid, phospholipids and cholesterol. Many of these new research methods are useful for the direct mass spectral identification of intact labile polyunsaturated hydroperoxides and other oxidation products without the necessity of derivatization, but their quantitation has not been achieved without the availability of authentic standards. This new chapter includes important advances in the development of very sensitive methods for the analysis of volatile flavor compounds, linking their relative release to sensory perception in foods.

A new section in the chapter on Stability Methods (Chapter 7) covers electron spin resonance spectrometry and spin-trapping for measuring the tendency of foods to form radicals during early stages of oxidation, and a discussion on its merits and pitfalls. The chapter on Control Methods (Chapter 8) has new material on physical refining, and soft column deodorization, more structural details of oxidative deterioration compounds formed during refining of different vegetable oils obtained by high performance size exclusion chromatography, and *trans* isomers of polyunsaturated fatty acids, oxidized sterols and cyclic monomers. Novel processing technology includes special membranes for the separation of impurities in crude oil extracts and for refining oils, cold pressing and hot expeller-pressing oils that have attracted interest for environmental reasons as they avoid the use of chemical solvents in the extraction of seeds. There is also information on high-oleic and mid-oleic canola and sunflower oils now commercially available as substitutes for partially hydrogenated oils, and an expanded section on packaging.

Chapter 9 on Antioxidants presents more detailed information on metal

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inactivators including ethylenediamine tetraacetic acid (EDTA), new material on lactoferrin, an expanded discussion of synergism, problems with methodology to evaluate antioxidants in multi-phase systems, and more on the mechanism of rosemary antioxidants at elevated temperatures. A new section on the evaluation of natural antioxidants in plants was motivated by the explosion of published research on the antioxidant properties of phytochemicals, including new tables comparing free radical trapping methods and lipid oxidation methods for the evaluation of natural antioxidants.

Chapter 10 on Multiphase Systems includes the effects of food proteins that can inhibit oxidation of oil-in-water emulsions by several mechanisms and how pH affects their activity. The metal complexing activity of bovine lactoferrin in inhibiting oxidation in emulsions and liposome systems is compared with that of EDTA. Antioxidants are greatly influenced by the colloidal properties of lipid systems. Interactions of emulsifiers with antioxidants, hydrogen bondings, interphase transport and surface access are important parameters determining their activity in lipid-containing systems differing in their colloidal characteristics. Proteins have strong binding properties with flavonoid antioxidants in foods and biological systems, and generally increase the oxidative stability of liposomes. The partitioning behavior of different phenolic antioxidants in emulsions is greatly affected by interactions between oils and emulsifiers that determine their solubilization and their activity in inhibiting lipid oxidation.

There is an expanded discussion in Chapter 11 on Foods on the interactions between metmyoglobin, deoxyhemoglobin and deoxymyoglobin and their mechanism of initiating lipid oxidation in meat and fish. The antioxidant properties produced by the Browning products are discussed, together with the methodology problems involved with model systems generally used in the literature. New information is added on the structure of volatile compounds and their impact on the flavor of cooked meat. The effect of thermal processing of foods and beverages (tomato juice, wine, coffee and tea) are subject to complex interactions between lipid oxidation and reducing browning reaction products, proteins, phospholipids, and phenolic antioxidants that are influenced by multifactorial changes during processing and storage. The methodological problems of using simplistic model systems and unidimensional methods to evaluate antioxidants in foods are discussed here and throughout the second edition. New material addresses the problems of iron supplementation in infant formulas and the control of oxidation by lactoferrin and EDTA, and the relative oxidative stability of long chain polyunsaturated fish and algae oils and emulsions, including the antioxidant activity of roasted foods.

Chapter 12 on Frying contains new material on complex interaction compounds produced by non-enzymatic browning reactions between thermally oxidized lipids and amines, amino acids and proteins. This chapter

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also discusses the new varieties of vegetable oils containing higher levels of oleate and lower levels of linolenate being used for frying, because partial hydrogenation produces *trans* fatty acids, which are now considered nutritionally undesirable. New tables include aroma extraction dilution analysis of volatiles from French fries; analyses of total polar compounds and tocopherol in frying oils versus fried potatoes; fatty acid composition of new varieties of soybean, sunflower, and canola oils suitable for frying without partial hydrogenation. The controversial subject of whether or not natural antioxidants can account for the strong epidemiological evidence that fruits and vegetable consumption is associated with reduced coronary heart disease, cancer and other diseases is covered in Chapter 13 on Biological Systems. The absorption of antioxidants in human blood as metabolites, their molecular mechanisms of protection *in vivo*, and of disease intervention are under intensive investigation around the world. Important developments are included in discussions of new pathways of LDL oxidation, the metabolic interactions between LDL and HDL on cholesterol transport mechanisms, new evidence on the role of antioxidant enzymes in HDL, which can break down oxidized lipids and neutralize their proinflammatory effects, and the close link between the oxidation and inflammation hypotheses of cardiovascular disease. The new evidence of core aldehyde formation in oxidized LDL is examined, together with the bioactivity of oxidized phospholipids on the surface of LDL particles that are recognized by macrophages. More information is discussed on the cyclic oxidation products of arachidonate described as isoprostanes in biological samples as an index of oxidative status. Important nutritional topics have been added to the second edition, including the significant role of docosahexaenoic acid (DHA) in neural membrane phospholipid metabolism, immune responses and the aging process. The difference in antioxidant testing results between animal and human studies and between *in vitro* and *in vivo* effects is discussed in terms of different end-points of oxidation. This chapter concludes with seven important unsettled nutritional questions that need future research. With so many unanswered questions, the conclusion is drawn that it would be imprudent to make dietary recommendations to the public before the mechanisms of polyunsaturated lipid nutrition, *in vivo* activity of antioxidants, and *in vivo* lipid peroxidation are better understood.

The author gratefully acknowledges the invaluable editorial work of Bill Christie and Frances Daniel.

Edwin Frankel
Department of Food Science and Technology
University of California
Davis, California 95616 USA
enfrankel@ucdavis.edu
January 2005

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Preface to First Edition

The oxidation of unsaturated fatty acids is one of the most fundamental reactions in lipid chemistry. When unsaturated lipids are exposed to air, the complex, volatile oxidation compounds formed cause rancidity, decreasing the quality of foods containing lipids as well as foods in which oils are used as ingredients. Another important process in the oxidation of unsaturated fats proceeds through activated species of oxygen. Singlet oxygen produced by photooxidation, in the presence of a sensitizer such as chlorophyll, is an important reactant that has attracted much interest to organic and biological chemists. Products of lipid oxidation have been implicated in many vital biological reactions. The revival of the field of lipid oxidation in the last two decades may be attributed in large part to the accumulating evidence that free radicals and reactive oxygen species participate in tissue injuries and in degenerative diseases.

Since the early 1960s, our understanding of the autoxidation of unsaturated lipids has advanced considerably as a result of the application of powerful new analytical tools. The characterization of cyclic peroxides from autoxidized linolenate, structurally related to the physiologically active prostaglandins, has also generated widespread interest in the field. Much of the work reported on the oxidative decomposition products of unsaturated fats emphasizes the role they play in causing rancidity in foods and cellular damage in the body. Decomposition of fat hydroperoxides creates a wide range of carbonyl compounds, hydrocarbons, furans, and other materials that contribute to flavor deterioration of foods. However, materials from secondary oxidation are also implicated in biological oxidation. Although fat hydroperoxides are the recognized precursors of volatile secondary products, the mechanism of their decomposition is not clearly understood.

This book aims at integrating a large body of interdisciplinary information on the oxidation of unsaturated lipids in order to develop the basic principles involved in the methodology and mechanisms of free radical oxidation. Understanding these principles is necessary for gaining insights into how unsaturated lipids in foods are subject to oxidative deterioration, and for developing appropriate control measures. The question of how biological systems undergo oxidative damage is very complex and the literature accumulates at a geometric rate.

This is an advanced textbook for graduate students, academic and industrial scientists concerned with the many phases of the complex series of lipid oxidation reactions. Starting from the basics of free radical and hydroperoxide chemistry, and methodology, the book progresses into topics of increasing complexity including control methods, antioxidants, and oxidation in multiphase systems, foods, frying fats and biological systems. Investigators working with polyunsaturated fatty acids and lipids must be seriously concerned with their

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oxidation, since these products have caused numerous problems in analyses, particularly in stability tests. Unfortunately, in spite of the great strides made in the field of lipid oxidation, many contemporary studies employ nonspecific and unreliable methods to follow lipid oxidation. Lipid oxidation is often studied under drastic conditions and with simple model systems that do not simulate complex multi-phase foods and biological systems. Much of the classical concepts of lipid oxidation come as a result of elegant kinetic studies, in which a wholly theoretical picture is developed in support of mechanistic hypotheses. The conditions and systems often employed in these studies may be simplistic, however, and do not represent those of real complex food systems undergoing oxidation.

This book discusses these and other pitfalls inherent in basic studies that use artificial radical initiators with unrealistic models and homogeneous systems. The application of simple research models may lead to numerous problems in the ultimate interpretation of results, because lipid oxidation proceeds by a complex sequence of reactions influenced by many factors, all of which become extremely difficult to unravel in real food and biological systems. These systems are multi-phased and controlled by complex colloidal phenomena affecting different sites of oxidation and antioxidation. In interpreting the effects of prooxidant and antioxidant compounds their 'effective' concentrations in different phases must be considered. A dimension of lipid oxidation that is important to better understand control methods deals with the relative partition of oxidants and antioxidants in multiphase systems. This topic has not received sufficient attention.

One of the main objectives of this book is to develop the background necessary for a better understanding of what factors should be considered, and what methods and lipid systems should be employed, to achieve suitable evaluation and control of lipid oxidation in complex foods and biological systems. Now, and throughout its 50-year history, the field is still developing and more progress can be expected in improving our understanding of the complex phenomena of lipid oxidation.

Edwin N. Frankel,
California
1998

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Introduction

Lipids are important structural and functional components of foods. The specific definition used in this book for the term lipids refers to fatty acids or closely related derivatives or metabolites. In foods, lipids have a significant effect on quality even when they constitute a minor component. The main lipid components of foods are triacylglycerols and are commonly referred to as fats and oils. They not only contribute to flavor, odor, color and texture, but also confer a feeling of satiety and palatability to foods. Nutritionally, lipids provide a concentrated source of calories (9 kcal per gram) that is about two-fold higher than either carbohydrates or proteins. They also furnish essential nutrients, including linoleic acid, linolenic acid, and fat-soluble vitamins (A, D, E and K). The subject of lipid oxidation will be introduced briefly with the classification of lipid components in foods and biological systems, with minor non-glyceride components, and with a few basic concepts of free radical organic chemistry.

A. Classification of lipids

The principal classes of lipids with which we will be concerned include free fatty acids, triacylglycerols consisting of fatty acids esterified to glycerol, and phosphoglycerides consisting of fatty acids esterified to glycerol and containing phosphoric acid and organic bases.

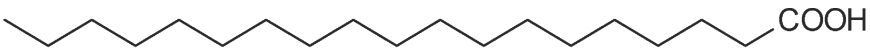
1. Fatty acids

These long chain aliphatic carboxylic acids are essential parts of most lipids in foods including triacylglycerols (also referred to as triglycerides) and phosphoglycerides (also referred to as phospholipids). Fatty acids can be either saturated or unsaturated (Figure 1). The fatty acids of major vegetable oils and animal fats include saturated, monounsaturated and polyunsaturated fatty acids (Table 1).

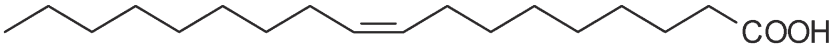
The common range of saturated fatty acids has a straight chain, even number of carbons and chain length of 12 to 22 carbons. Myristic acid with 14 carbons is designated as 14:0 (or 14 carbons with no double bond), palmitic acid with 16 carbons as 16:0, and stearic acid with 18 carbons, as 18:0.

The monounsaturated oleic acid with one double bond in the *cis* (or *Z*) configuration in the 9-carbon position (relative to the carboxyl group) is designated as 18:1 *n*–9. The "n" symbol indicates the number of carbons from the terminal end of the 18-carbon chain, a nomenclature favored by

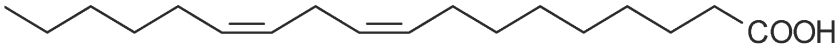
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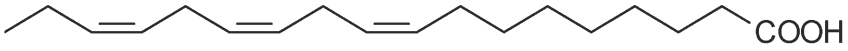
stearic acid: octadecanoic acid (18:0)



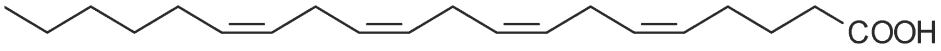
oleic acid: *cis*-9-octadecenoic acid (18:1 *n*–9)



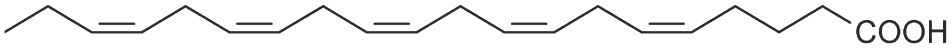
linoleic acid: *cis*-9,*cis*-12-octadecadienoic acid (18:2 *n*–6)



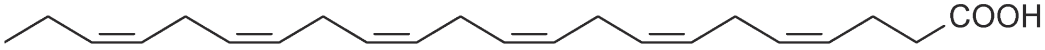
linolenic acid: *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid (18:3 *n*–3)



arachidonic acid: *cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid (20:4 *n*–6)



eicosapentaenoic acid: 20:5 *n*–3 (EPA)



docosahexaenoic acid: 22:6 *n*–3 (DHA)

Figure 1. Line drawing representations of the structures of saturated, monounsaturated and polyunsaturated fatty acids.

biochemists. Elaidic acid has the same structure as oleic acid except that the double bond is in the *trans* (or *E*) configuration. Although this acid is present in only small amounts in milk fat (~10% of bovine octadecenoic acids), vaccenic acid (11-*trans*-18:1) is the main *trans* fatty acid in milk fat (~36% of octadecenoic acids). Elaidic acid is also produced by partial hydrogenation of polyunsaturated fats for the manufacture of margarines and shortenings. These

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Table 1. Average fatty acid composition of vegetable oils, animal fats and fish oils (% by weight)[a]

Fats	14:0	16:0	18:0	18:1	18:2	18:3	Others
Corn	–	13	3	31	52	1	–
Cottonseed	–	27	2	18	51	trace	2
Peanut[b]	–	13	3	38	41	trace	$C_{20–24}$ 5
Olive	–	10	2	78	7	1	2
Palm	–	44	4	40	10	trace	2
Rapeseed[c]	–	4	2	56	26	10	$C_{20:1}$ 3
Safflower	–	7	3	14	75	–	1
Soybean	–	11	4	22	53	8	2
Sunflower	–	6	5	20	69	trace	–
Lard	2	27	11	44	11	–	5
Beef tallow	3	27	7	48	2	–	13
Salmon	6	15	3	19	3	2	52[d]
Tuna	4	20	6	18	2	2	48[e]

[a] Gunstone *et al.* (1994).
[b] Also termed groundnut oil in Europe.
[c] Low-erucic acid rapeseed oil is also known as canola oil in Canada and USA
[d] Frankel *et al.* (2002), includes: 7% 16:1, 2% 18:4, 2% 20:4 *n*–3, 2% 22:1 *n*–9, 8% 20:5 *n*–3 (EPA), 3% 22:5 *n*–3,10.5% 22:6 *n*–3 (DHA).
[e] Frankel *et al.* (2002), includes: 4% 16:1, 1% 18:4, 1% 20:4 *n*–3, 7% 20:5 *n*–3 (EPA), 2% 22:5 *n*–3, 26% 22:6 *n*–3 (DHA).

hydrogenated products contain other *cis* and *trans* isomers of monounsaturated fatty acids in which the double bond has migrated between the carbon-8 and carbon-12 positions.

The polyunsaturated fatty acids are characterized by having two or more *cis* double bonds separated by a single methylene group, or a 1,4-diene structure. Linoleic acid with two *cis* double bonds in the 9,12-positions is designated as 18:2 *n*–6 (9,12). Linolenic acid is 18:3 *n*–3 (9,12,15); arachidonic acid is 20:4 *n*–6 (5,8,11,14) and abbreviated as ARA; eicosapentaenoic acid is 20:5 *n*–3 (5,8,11,14,17) and abbreviated as EPA; and docosahexaenoic acid is 22:6 *n*–3 (4,7,10,13,16,19) and abbreviated as DHA. Linoleic acid and linolenic acid are essential fatty acids because they are not synthesised in the body. EPA and DHA are also considered important in modulating the biosynthesis of oxygenated derivatives of arachidonic acid called eicosanoids, which have hormone-like functions and play an important role in inflammation processes (see Chapter 13). More recently, infant foods have been supplemented with long-chain polyunsaturated oils, especially DHA from fish and algae, because of their recognized nutritional benefits in visual and brain functions.

Fatty acids occur mainly as esters of glycerol called glycerolipids, including triacylglycerols, or triglycerides, and phosphoglycerides or phospholipids. The alcohol can also be a long-chain compound or a sterol.

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2. *Triacylglycerols (*triglycerides*)*

Triacylglycerols are important storage lipids and the main constituents (~99%) of vegetable oils and food lipids. Natural triacylglycerols contain generally a mixture of different fatty acids and their composition reflects the relative fatty acid concentrations. However, when the proportion of one fatty acid is higher than 33%, the oil contains a large amount of triacylglycerols containing one fatty acid, such as trioleylglycerol (or triolein, designated as OOO) in olive oil, and tripalmitoylglycerol (or tripalmitin, designated as PPP) in palm oil (Table 1). The number of possible triacylglycerols increases markedly with the number of different fatty acids. With two different fatty acids, the number of possible triacylglycerols is eight. For example, with palmitate (P) and oleate (O), we can have the following triacylglycerols: PPP, POP, OPO, OOO, OPP, PPO, POO, and OOP. The pairs OPP/PPO and POO/OOP are unsymmetrical enantiomeric pairs (or mirror image forms). With three different fatty acids, the number of possible triacylglycerols is 27, or equal to n^3 for n = the number of different fatty acids. The physical, texture and rheological properties of triacylglycerols is greatly dependent not only on their fatty acid composition but also on their distribution among the 1-, 2- or 3- positions (also designated as *sn*-positions) in the glycerol molecule.

Triacylglycerols can be hydrolysed either chemically or enzymatically to produce a mixture of diacylglycerols (or diglycerides), monoacylglycerols (or monoglycerides), free fatty acids and glycerol. Chemical hydrolysis with aqueous alkali or acid occurs randomly to produce two fatty acids from the *sn*-1 and *sn*-3 positions (or α-positions) and one fatty acid from the *sn*-2 (or β-position). Enzymatic hydrolysis by pancreatic lipase occurs selectively at the α-positions to yield 1,2- and 2,3-diacylglycerols, followed by 2-monoacylglycerol and two fatty acids. Dietary lipids are assimilated in the body following the hydrolytic action of pancreatic lipase, which takes place in the small intestine (Figure 2).

3. *Phosphoglycerides (phospholipids)*

These are important structural lipids in foods and cell membranes, which are also known as phospholipids or phosphatides. They are based on phosphatidic acids, which are 1,2-diacyl esters of 3-glycerolphosphoric acid, linked to organic bases or other moieties (Figure 3).

Phospholipids are minor components of the crude vegetable oils obtained either by solvent extraction or by pressing oilseeds. A large portion of these materials is removed by a hydration process known as degumming (see Figure 8.1), where they are separated as a mixture with triacylglycerols known as lecithin. The *sn*-1 position in phosphatidylcholine (PC) of animal origin is largely esterified by saturated fatty acids, while the *sn*-2 position contains

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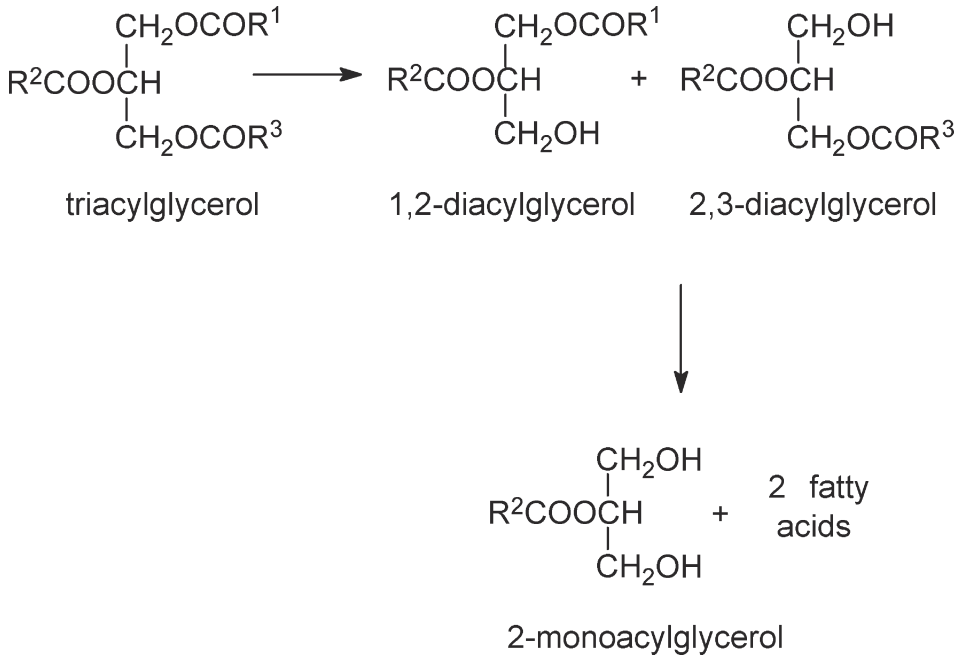


Figure 2. Hydrolysis of a triacylglycerol by the enzyme pancreatic lipase.

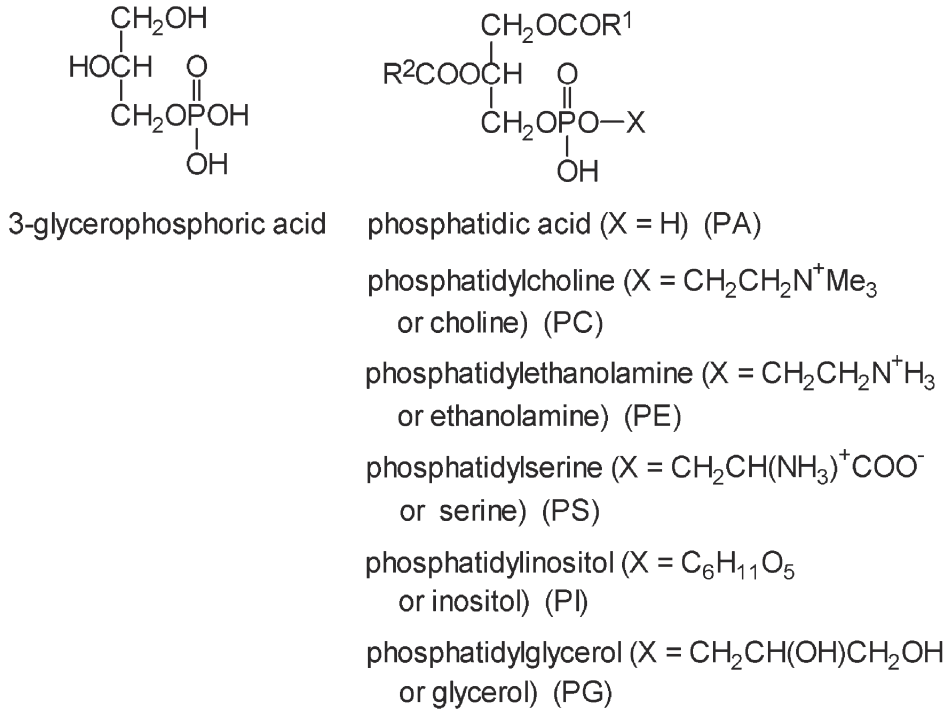
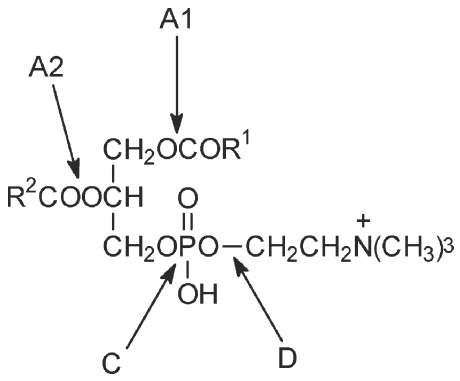


Figure 3. The structures of the common range of phospholipids.

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polyunsaturated fatty acids. The lecithin obtained from soybeans is a mixture of PC (20–23%), phosphatidylethanolamine (PE) (16–21%) and phosphatidylinositol (PI) (12–18%), and is used as an important emulsifier in foods. These phospholipids play an important role in stabilizing vegetable oils during processing (Chapter 8), and as synergists by reinforcing the activity of antioxidants (Chapter 9). When dispersed in water, phospholipids form liposomes consisting of multi-layers or bilayers, which are important components of cell membranes, meat and fish products (see Chapters 10 and 11).

Phosphatidylcholine can be completely hydrolysed with aqueous acid to produce fatty acids, glycerol, phosphoric acid and choline. With alkali, the fatty esters are preferentially hydrolysed, leaving the glycero-phosphorylcholines, which can in turn hydrolyse slowly into glycerophosphoric acid and choline. Enzymatic hydrolysis occurs selectively at different ester sites by several phospholipases to produce a range of products. Phospholipases A1 and A2 are obtained commercially from snake venom. They hydrolyse the fatty acid at the *sn*-1 and *sn*-2 positions respectively, to produce lysophosphatidylcholine. Phospholipase C catalyses the hydrolysis of the phosphate moiety to give 1,2-diacylglycerols and phosphorylcholine. Phospholipase D found in plant tissues mainly catalyses hydrolysis of the phosphate ester to produce choline and phosphatidic acids (1).



(1)

B. Minor components

Fats and oils contain varying amounts (1–2%) of non-glyceride components including hydrocarbons such as squalene and carotenoids, sterols, tocopherols and tocotrienols, chlorophyll, vitamin A (retinol), and vitamin D (cholecalciferol). Only the tocopherols, carotenoids and chlorophyll have a significant effect on the oxidative stability of fats and oils. The tocopherols (Figure 9.1 and Table 9.4) are the most important free radical chain-breaking antioxidants (Chapter 9). Because the tocopherols are thermally stable, a relatively large proportion (60–70%) remains in the oils after processing. The antioxidant

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activity of tocotrienols, present mainly in palm oil, is not fully established in food systems. The carotenoids are effective inhibitors of photosensitized oxidation by quenching singlet oxygen, a very active form of oxygen responsible for this type of photooxidation (Chapter 3). For most edible oils the carotenoids are largely removed and destroyed during conventional processing by deodorization at elevated temperatures. For some oils such as virgin olive oil and corn oil, the yellow color imparted by carotenoids is desirable and maintained by partial or full omission of the bleaching step, or by deodorizing at lower temperatures. The chlorophylls are very active promoters of photosensitized oxidation (Chapter 3). These greenish pigments must be removed as completely as possible by bleaching to prevent the damaging effects of light oxidation. Squalene and sterols have little effects on lipid oxidation, but some types of sterols are claimed to have protective effects by inhibiting polymerization of oils during frying (Chapter 12).

C. Chemical reactions of unsaturated fatty acids

The electronic representation of a double bond consists of two components (Figure 4):

i. a sigma (σ) bond that has an electronic symmetry that overlaps the two olefinic carbons endwise, or has σ-symmetry; this bond is as strong as an ordinary single bond.

ii. a pi (π) bond that consists of a pair of electrons which occupy a position in which one is above and one below the plane of the paper, and overlap sidewise, or has π-symmetry. The π-bond is weaker and of lower energy because the π electrons are less firmly held between the two nuclei and more exposed. The π electrons are consequently responsible for the greater reactivity of unsaturated compounds. Because the π bond is more readily polarizable than the σ bond, it imparts nucleophilic character to double bonds, which react with electrophilic reagents.

Organic reactions can be divided mechanistically by the formation of either heterolytic ionic or homolytic radical intermediates.

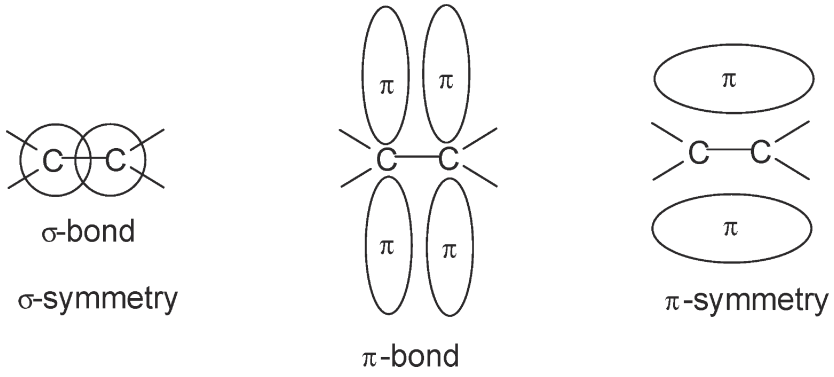


Figure 4. Electronic representation of an unsaturated bond.

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1. Heterolytic ionic reactions

In these reactions, a covalent bond is formed by one species donating and the other accepting an electron pair. Heterolytic cleavage of the resulting covalent bond produces two species, one negatively charged ion accompanied by the electron pair, and one positively charged ion.

$$X: + Y \longrightarrow X:Y \longrightarrow :Y^{-} + X^{+} \qquad (2)$$

The negatively charged ion Y^{-} is an electron-donating or nucleophilic species, and the positively charged ion X^{+} is electrophilic, or accepts an electron pair. These charged ions follow laws of electrostatic attraction and repulsion.

2. Homolytic radical reactions

In these reactions, a bond is formed by two free radical species with odd electrons, both donating a single electron. Homolytic cleavage or homolysis of the resulting covalent bond produces free radicals by splitting the pair of electrons.

$$X^{\cdot} + {}^{\cdot}Y \longrightarrow X:Y \longrightarrow X^{\cdot} + {}^{\cdot}Y \qquad (3)$$

These free radicals containing odd electrons are highly reactive but have no formal charge.

The free radicals and charged ionic intermediates have distinctly different characteristic behaviors that can be summarized as follows:

Free radical reactions:	Ionic reactions:
i catalysed by light, heat, by other free radicals and peroxides.	i unaffected by light, free radicals and peroxides; often catalysed by acids or bases.
ii inhibited by antioxidants (radical acceptors reacting with other free radicals).	ii unaffected by radical acceptors or antioxidants.
iii proceed in vapor phase or non-polar solvents.	iii rarely occur in the vapor phase; strongly affected by solvents.
iv autocatalytic and have an induction period.	iv follow first or second order kinetics.
Examples of free radical transformations include oxidation, most polymerization and photo-chemical reactions and reactions using peroxides as catalysts.	Examples of reactions involving ionic intermediates include alkali isomerization, dehydration, hydrolysis and saponification.

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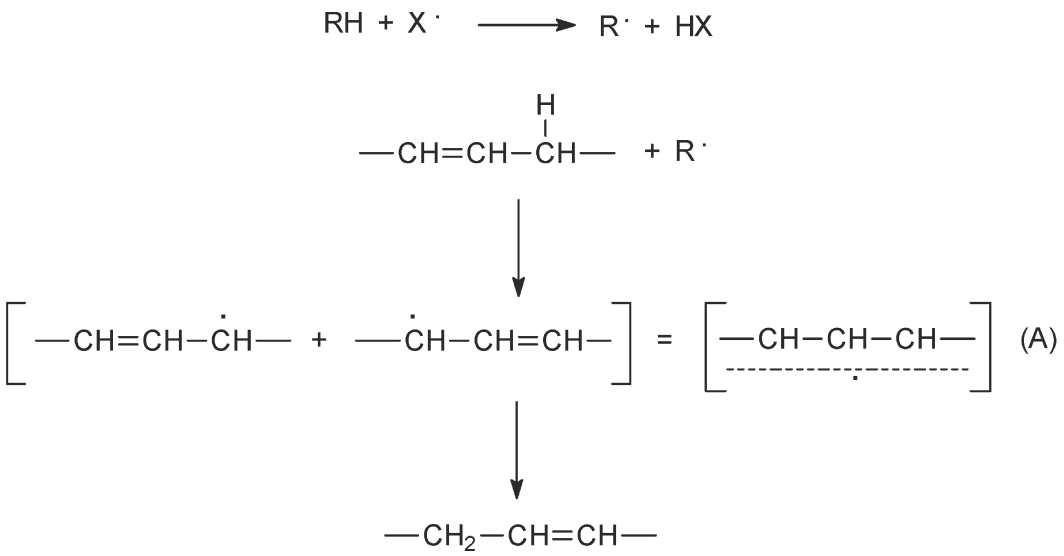


Figure 5. Double bond migration by hydrogen abstraction in a free radical mechanism.

The autoxidation of unsaturated fatty acids is a chain process occurring autocatalytically through free radical intermediates. Although autoxidation implies that this reaction occurs spontaneously under mild conditions, it is generally initiated by trace metals and peroxides or hydroperoxides present as ubiquitous impurities in food and biological lipid systems (see Chapter 1).

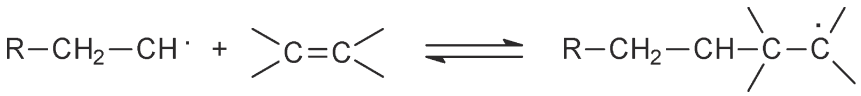
Several radical transformations can be considered in the context of lipid oxidation. These reactions are facilitated by the presence of an odd electron in the free radical intermediates that weakens the surrounding bonds. Two simple rules apply in free radical chemistry: (a) the combination of two radicals yields a new non-radical compound with paired electrons, and (b) the reaction of a radical with a non-radical produces a new radical compound.

*a. Hydrogen abstractions***.** Radicals can readily abstract a hydrogen from unsaturated fatty acids containing an allylic center, –CH=CH–CH_2– by a radical displacement reaction. The formation of allylic intermediates is an important characteristic of free radical reactions that determines the nature of lipid oxidation products (Figure 5).

The two allylic intermediates differing only in the distribution of electrons can be represented as one resonance hybrid structure A. The odd electron in this allylic conjugated carbon-centered radical (R˙) is delocalized and distributed through the three-carbon structure. This phenomenon contributes to its strong resonance stabilization. Radicals conjugated with polyunsaturated fatty acid systems are similarly stabilized by resonance.

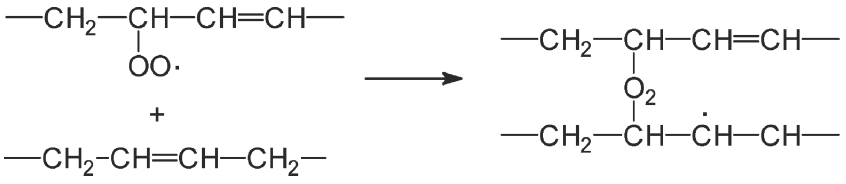
b. Addition and elimination reactions. One radical can attack the π electrons of a double bond to be transformed into another radical (4). The elimination reaction (–4) is the reverse of the addition reaction.

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(4)

The new radical thus formed can undergo further reactions, such as cyclization and polymerizations (Chapter 4). Allylic peroxyl radicals (ROO$^{\bullet}$) can thus add to an unsaturated fatty acid to produce a peroxide-linked dimer (5).



(5)

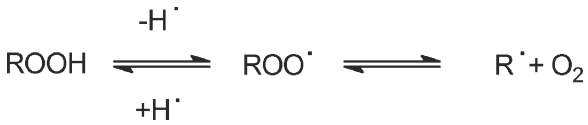
c. Fragmentation reactions. Secondary alkoxy radicals (RO$^{\bullet}$) may undergo decomposition by β-scission or elimination to form aldehydes and other volatile compounds together with simpler radicals.



(6)

These decomposition reactions become especially important with polyunsaturated fatty acids and at elevated temperatures or in the presence of metal catalysts (see Chapter 4).

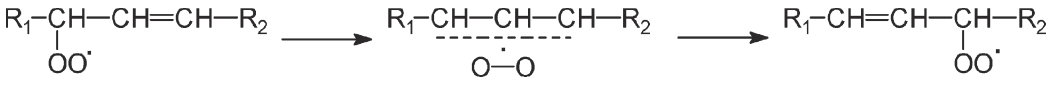
d. Rearrangement reactions. A hydroperoxide can rearrange by reversibly losing a hydrogen radical and eliminating oxygen from a peroxyl radical intermediate (7).



(7)

Lipid hydroperoxides undergo rearrangement of the OOH group readily to give mixtures of positional and geometric isomeric products (see Chapter 2).

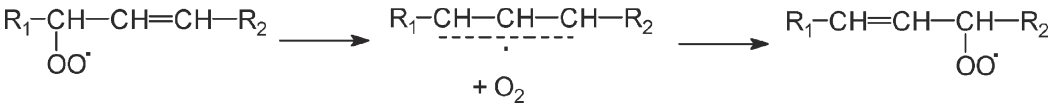
Allylic peroxides can also undergo 1,3-peroxyl transfer either by a concerted (one step) pathway (8):



(8)

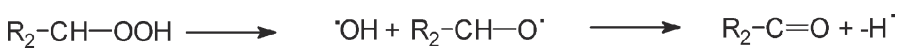
or by a dissociative pathway involving the removal and re-addition of oxygen (9):

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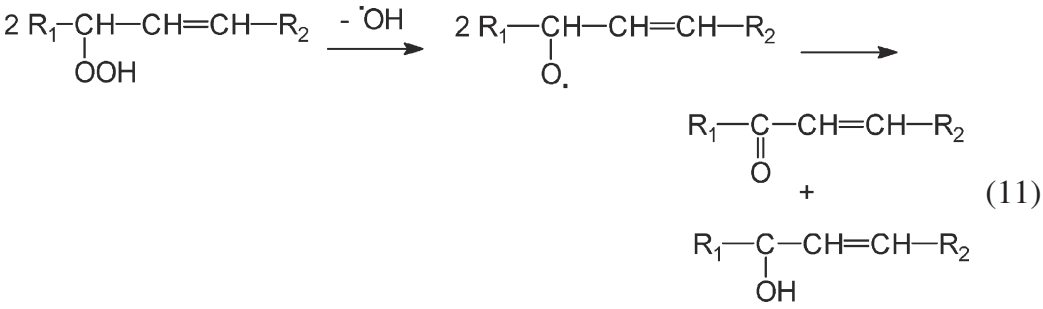
(9)

*e. Disproportionation reactions***.** A hydroperoxide can be converted to a ketone by disproportionation involving the transfer of hydrogen radical from an alkoxyl radical intermediate (10).



(10)

Similarly, two allylic hydroperoxides can undergo disproportionation, by transfer of hydrogen from two alkoxyl radical intermediates, into a mixture of ketone and hydroxy products (11).

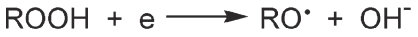


(11)

f. Oxidation–reduction. Transition metal catalysts can either initiate radicals by electron transfer reactions (12) and (13) or terminate radicals by reaction (14) by their ability to either oxidize or reduce compounds by one electron.



(12)



(13)



(14)

3. Radical stability

The stability of a radical depends on the rate of its disappearance or formation according to the reaction conditions. The thermodynamic stability of a radical is related to the ease of dissociation of the R–H bond to give $R^{\bullet}$ and $H^{\bullet}$ or the R–H dissociation energy. The thermodynamic stability of a radical depends on double bond conjugation and electron delocalization stabilized by resonance in allylic systems produced by hydrogen abstraction in polyunsaturated lipids (see Chapter 2).

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The kinetic stability of a radical is largely controlled by steric factors. When the radical center is crowded, the radical becomes less reactive and persists longer under normal conditions (it has a longer life-time). Aromatic compounds that can form allylic radicals show similar benzylic stabilization. If the radical center is sterically crowded by bulky tertiary butyl substituents, the allylic radical intermediates formed by hydrogen transfer have kinetic stability that imparts important antioxidant properties (see Chapter 9). Thus, when phenolic compounds contain three bulky tertiary butyl substituents, they form persistent radicals after hydrogen donation and inhibit lipid oxidation by interrupting the propagation of free radicals (see Chapter 9).

D. Lipid oxidation and health

Rancidity of edible oils is a serious problem in many sectors of the food industry, because of increasing emphasis on the use of polyunsaturated vegetable and fish oils, discontinuing the use of synthetic antioxidants, and fortification of cereals in adult and infant foods with iron. Oils rich in long-chain *n*–3 polyunsaturated fatty acids containing 20:5 *n*–3 and 22:6 *n*–3 derived from fish and algae have now been recognized for their nutritional importance, but lipid oxidation limits their utilization in processed foods, and as nutritional supplements. Oxidation of lipids not only produces undesirable rancid flavors in foods but can also decrease their nutritional quality and safety by the formation of secondary products after cooking and processing (Chapter 12). Effective control methods against lipid oxidation include the use of metal inactivators (or chelators), minimizing the loss of natural tocopherols and exposure to air and light during processing, hydrogenation and the use of antioxidants (Chapter 9). Hydrogenation of polyunsaturated oils is becoming less and less attractive because of recent evidence that *trans* isomers, which are formed during the process, may have adverse nutritional effects. Instead of hydrogenation, an alternate approach is to mix different proportions of high-oleic oils with polyunsaturated vegetable and fish oils to prepare more stable edible oils with a wide range of desired fatty acid compositions (Chapter 8). Several high-oleic oils have been developed recently by special plant breeding and genetic manipulations to improve their oxidative stability and nutritional value.

Much research has been conducted to better understand the basic processes of lipid oxidation and singlet oxygenation of polyunsaturated lipids, antioxidant action, and the effects of decomposition products of lipid oxidation. Enormous worldwide attention has been given recently to the use of natural phenolic antioxidants (Chapter 9). Flavonoids are phytochemicals with potent antioxidant activity that are found in many fruits and vegetables and beverages (red wine, grape juice and tea). When consumed in our diet these natural antioxidants may be associated with reduced risk of degenerative disease. Phytochemicals are now being widely investigated for their nutritional

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benefits. However, the occurrence of more than 4000 different flavonoids in plant foods presents a formidable challenge in attempting to better understand their potential nutritional properties on a molecular basis. Although much progress has been achieved in determining the relative absorption of flavonoids and metabolism in man, very little is known about their bioavailability and bioactivity.

Natural phenolic antioxidants inhibit biological oxidation by a complex multi-step mechanism that remains unclear. Unfortunately, many contemporary studies tend to use non-specific indicators of oxidation and antioxidation. A better understanding of the mechanism of action of flavonoids requires not only well-designed studies *in vitro*, but also demonstrations of their effectiveness *in vivo*. The development of reliable biomarkers that can be better related to degenerative disease presents one of the most difficult challenges in this field (Chapter 13).

In the developed countries, it appears that increased consumption of linoleate from vegetable oils in our diet may have reached too high a level, thus promoting a variety of chronic diseases, including coronary artery and inflammatory diseases. Excessive linoleate in our diet causes an undue release into blood of eicosanoids, oxygenated metabolites of arachidonic acid that have profound physiological effects. These eicosanoids produce abnormal cell functions in the body that result in predisposition to heart attacks (Chapter 13). Because *n*–3 polyunsaturated fatty acids are known to antagonize the metabolism of linoleic acid, many nutritionists have advocated that a portion of the vegetable oils in the diet be replaced with fish and fish oils rich in these *n*–3 fatty acids. However, this approach overlooks the possible risk factor from the high susceptibility to autoxidation of the polyunsaturated fatty acids in fish oils, especially 20:5 *n*–3 and 22:6 *n*–3. The oxidation of these highly unsaturated fatty acids causes undesirable rancid and fishy flavors and oxidative deterioration in fish oil products, which cannot be effectively controlled by supplementation with vitamin E and other antioxidants (Chapter 11).

Modifications of the polyunsaturated lipid intake in the diet require that we examine carefully the possible nutritional consequences of oxidative damage to biological systems. Oxidation of low-density lipoproteins (LDL) in circulating blood has been well recognized in the etiology of coronary heart diseases (Chapter 13). Some of the nutritional problems are due to lipid oxidation and oxidative stress resulting from reactive oxygen species (Chapter 13). These problems could be alleviated either by consuming more antioxidants, or by increasing the amounts of oxidatively stable oleic acid in our diet. There is evidence that diets rich in oleic acid and antioxidants increase the oxidative stability of LDL and may reduce coronary disease. Although in healthy subjects an important major antioxidant defense is to prevent metals from catalysing the generation of reactive oxygen species, excessive iron supplementation in the diet may overload this defense system. We need to improve

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our diet by reducing or minimizing the risk factors associated with oxidative deterioration of polyunsaturated dietary lipids that may also be aggravated by excessive iron supplementation. Flavonoid antioxidants represent a positive potential in our diet that requires further research to improve our understanding of their mechanism of action (Chapter 13).

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CHAPTER 1

Free radical oxidation

A. Mechanism

Autoxidation is the direct reaction of molecular oxygen with organic compounds under mild conditions. Oxygen has a special nature in behaving as a biradical by having two unpaired electrons ($^{\bullet}O–O^{\bullet}$) in the ground state and is said to be in a triplet state. The oxidation of lipids proceeds like that of many other organic compounds by a free radical chain mechanism, which can be described in terms of initiation, propagation, and termination processes. These processes often consist of a complex series of sequential and overlapping reactions.

1. Initiation

In the presence of initiators (I), unsaturated lipids (LH) lose a hydrogen radical ($H^{\bullet}$) to form lipid free radicals ($L^{\bullet}$).

$$LH \xrightarrow[R_I]{I} IH + L^{\bullet} \quad (1)$$

The direct oxidation of unsaturated lipids by triplet oxygen ($^3O_2$) is spin forbidden because the lipid ground state of singlet multiplicity has an opposite spin direction from that of oxygen of triplet multiplicity. This spin barrier between lipids and oxygen can be readily overcome in the presence of initiators that can produce radicals by different mechanisms.

(a) thermal dissociation of hydroperoxides (LOOH) present as impurities:

$$LOOH \longrightarrow LO^{\bullet} + {}^{\bullet}OH \quad (2)$$

(b) decomposition of hydroperoxides catalysed by ubiquitous redox metals (M) of variable valency:

$$LOOH + M^{2+} \longrightarrow LO^{\bullet} + OH^{-} + M^{3+} \quad (3)$$

$$LOOH + M^{3+} \longrightarrow LOO^{\bullet} + H^{+} + M^{2+} \quad (4)$$

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(c) exposure to light in the presence of a sensitizer such as a ketone (RCOR):

$$RCOR + h\nu \longrightarrow RCO^{\bullet} + {}^{\bullet}R \quad (5)$$

Because the reaction of unsaturated lipids with singlet oxygen ($^1O_2$) (Chapter 3) occurs at a significantly greater rate than their reaction with normal triplet oxygen ($^3O_2$), the hydroperoxides that are rapidly formed under photosensitized conditions can serve as important initiators of free radicals by reactions (2), (3) and (4).

The mechanism of initiation of lipid oxidation has been debated for many years. The most likely initiation process is the metal-catalysed decomposition of preformed hydroperoxides. The thermal oxidation of unsaturated lipids is usually autocatalytic and involves initiation by decomposition of hydroperoxides, which is generally considered metal-catalysed because it is very difficult or nearly impossible to eliminate trace metals that act as potent catalysts for reactions (3) and (4).

2. *Propagation*

The alkyl radical of unsaturated lipids ($L^{\bullet}$) containing a labile hydrogen reacts very rapidly with molecular oxygen (k_o) to form peroxyl radicals (6). This step is always much faster than the following hydrogen transfer reaction (k_p) with unsaturated lipids to form hydroperoxides (7).

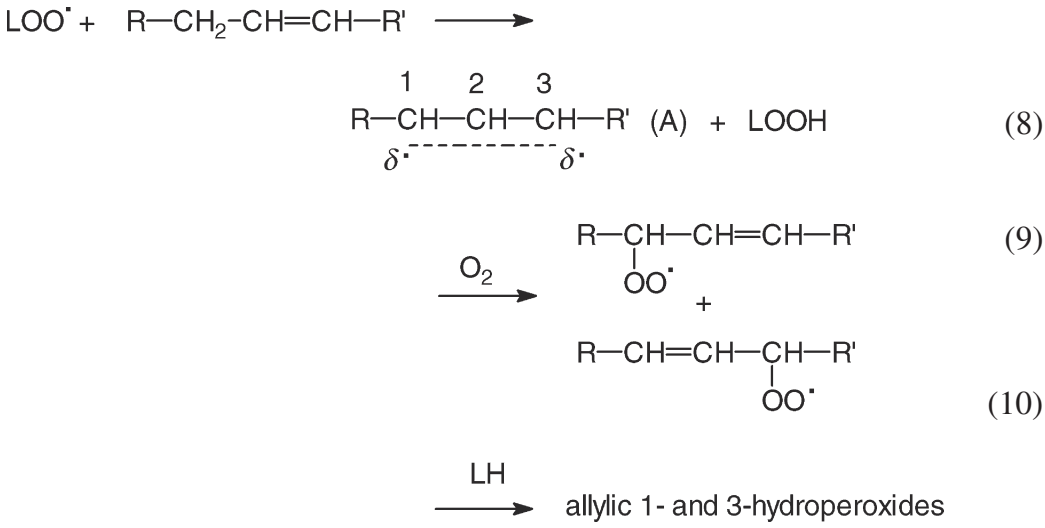
$$L^{\bullet} + O_2 \xrightarrow{k_o} LOO^{\bullet} \quad (6)$$

$$LOO^{\bullet} + LH \xrightarrow{k_p} LOOH + L^{\bullet} \quad (7)$$

This is the most widely occurring oxidation and describes the first stages of the oxidation of unsaturated lipids producing hydroperoxides as the fundamental primary products. Because this step is slow and rate-determining, hydrogen abstraction from unsaturated lipids becomes selective for the most weakly bound hydrogen.

The susceptibility of lipids to autoxidation thus depends on the availability of allylic hydrogens and their relative ease to react with peroxyl radicals to form hydroperoxides. A hybrid radical (A) is formed, with a partial free radical ($\delta^{\bullet}$) at each end of the allylic system (8). Oxygen attack at each end of the allylic system produces a mixture of allylic 1- and 3-hydroperoxides (10).

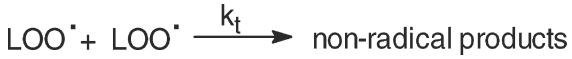
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We shall see in Chapter 2 that the hybrid radical (A•) formed by reaction (8) and the resulting peroxyl radicals by reaction (9) can assume different stereochemical conformations, which determine the geometric configurations (*cis* and *trans*) of the isomeric hydroperoxides from various unsaturated lipids oxidized under different conditions. The oxygen addition reactions (8) and (9) can be reversible for many radicals. The reverse reaction (–9) proceeds by *β*-scission or *β*-fragmentation of the carbon-oxygen bond (–C–|–OO•) of the peroxyl radicals and may also determine the stereochemical configuration of the isomeric hydroperoxides.

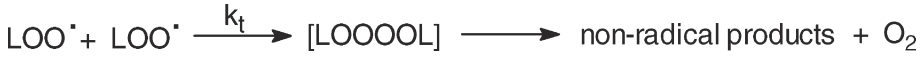
3. Termination

At the last stages of autoxidation the peroxyl radicals accumulate. At relatively high levels the peroxyl radicals interact with each other to form non-radical products by the termination reaction (11).



(11)

At atmospheric pressure, termination (k_t) occurs first by the combination of peroxyl radicals to an unstable tetroxide intermediate followed rapidly by its decomposition by the Russell mechanism, to yield non-radical products (12).



(12)

Many complexities can be considered when hydroperoxides undergo thermal or metal-catalysed homolysis generating peroxyl and alkoxyl radicals

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leading either to additional radicals that continue the chain or to non-radical end-products. Alkoxyl radicals can thus either react with the unsaturated lipid to form stable and innocuous alcohols (13), or undergo fragmentation into unsaturated aldehydes and other unstable products (14) causing rancidity in polyunsaturated lipids. Both of these *branching* reactions produce new radicals that continue the chain and further complicate the process of lipid autoxidation.

$$LO^{\bullet} + LH \longrightarrow LOH + L^{\bullet} \quad (13)$$

$$LO^{\bullet} \longrightarrow RCHO + L^{\bullet} \quad (14)$$

Other termination reactions can proceed by condensation of peroxyl, alkoxyl or alkyl radicals. At low temperatures, peroxyl radicals can combine to produce peroxyl-linked dimers (LOOL) with the formation of oxygen (15).

$$2\ LOO^{\bullet} \longrightarrow LOOL + O_2 \quad (15)$$

At low oxygen pressures and elevated temperatures, alkoxyl and alkyl radicals can combine to produce ether-containing dimers (16), and carbon–carbon linked dimers (17).

$$LO^{\bullet} + L^{\bullet} \longrightarrow LOL \quad (16)$$

$$2\ L^{\bullet} \longrightarrow L{-}L \quad (17)$$

The decomposition of hydroperoxides and dimers will be considered in detail in Chapter 4.

One of the most important termination reactions (18) involves an antioxidant (AH).

$$LOO^{\bullet} + AH \rightleftharpoons LOOH + A^{\bullet} \quad (18)$$

The application of antioxidants will be considered in Chapter 9 as one of the important practical measures for the control of lipid oxidation.

B. Kinetics

Many of the classical mechanistic concepts of lipid oxidation were formulated on the basis of kinetic studies. Later developments in support of the general free radical mechanism of oxidation were based on structural studies of the primary hydroperoxide products. To simplify the kinetics of linoleate oxidation, the reactions were studied at early stages of oxidation, at low levels of conversion, at lower temperatures and in the presence of an appropriate initiator. Under these conditions, the propagation reactions producing hydroperoxides in high yields are emphasized, and the decomposition of hydroperoxides is minimized

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or considered insignificant. However, the kinetics become much more complex when autoxidation is carried out to high conversions, or at elevated temperatures, or in the presence of metals. Hydroperoxides decomposition becomes significant at advanced levels of oxidation of linoleate, and at earlier stages with linolenate and other polyunsaturated fatty acids containing more than three double bonds. Hydroperoxides accumulate thus in significantly higher yields from methyl oleate and linoleate (>90%) than from methyl linolenate (about 30–40%) and from polyunsaturated fatty acids containing more than three double bonds. Very many low molecular weight and high molecular weight decomposition products are formed by complex pathways (13–17), which greatly complicate the kinetics of lipid oxidation (Chapter 4).

Hydroperoxide formation. At oxygen pressures greater than 100 mm Hg, the reaction of alkyl radicals $L^{\bullet}$ with oxygen is so rapid that the concentration of the resulting peroxyl radicals $LOO^{\bullet}$ is much greater than $L^{\bullet}$. Applying stationary state conditions, by assuming the concentrations of $L^{\bullet}$ and $LOO^{\bullet}$ do not change, the rate of change is zero.

$$d[L^{\bullet}]/dt = 0 \text{ and } d[LOO^{\bullet}]/dt = 0$$

Addition of these two equations leads to

$$R_i - 2k_t[LOO^{\bullet}]^2 = 0 \text{ and } [LOO^{\bullet}] = (R_i/2k_t)^{1/2}$$

Since $d[LOOH]/dt = k_p[LOO^{\bullet}][LH]$, the following expression can be derived for the rate of hydroperoxide formation:

$$d[LOOH]/dt = k_p(R_i/2k_t)^{1/2}[LH]$$

and the rate of oxidation is independent of oxygen pressure. The ratio of the rate constants $k_p/(2k_t)^{1/2}$ is referred to as "oxidizability" and used as a measure of the reactivity of unsaturated lipids to undergo autoxidation (Section C).

When oxygen is limited and the partial oxygen pressure (P_{O_2}) is below 100 mm Hg, assuming a constant rate of initiation and amount of lipid substrate, the rate equation can be simplified as follows:

$$d[LOOH]/dt = A[P_{O_2}/(P_{O_2} + B)]$$

where A and B are constant and the rate becomes oxygen dependent.

To study quantitatively the kinetics of lipid oxidation and antioxidation, a standard way of controlling and measuring the rate of free radical initiation is to use thermally labile azo compounds. These artificial initiators generate radicals at a reproducible, well-established and constant rate. In the presence of initiators such as α,α-azobisisobutyronitrile (AIBN) or benzoyl peroxide, the overriding initiation can be directly related to the rate of production of the initiator radical. Also, by using either water-soluble or lipid-soluble azo dyes, these compounds can initiate radicals at known specific micro-environments.

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For example, the water-soluble 2,2'-azobis-(2-amidino-propane) dihydrochloride (AAPH) generates free radicals in the aqueous phase, and the oil-soluble, 2,2'-azobis (2,4-dimethylvaleronitrile) (DMVN) generates free radicals within the lipid phase. These azo compounds decompose thermally at a known and constant rate (K_d), to produce two radicals ($A^{\bullet}$) and nitrogen.

$$A{-}N{=}N{-}A \xrightarrow{K_d} \underset{\text{cage}}{[A^{\bullet} N_2 A^{\bullet}]} \longrightarrow 2\ A^{\bullet} + N_2 \qquad (19)$$

The azo radicals ($A^{\bullet}$) react rapidly with oxygen to give peroxyl radicals ($AO_2^{\bullet}$), which attack lipids by abstraction of hydrogen atoms to form lipid radicals leading to lipid hydroperoxides.

$$A^{\bullet} + O_2 \longrightarrow AO_2^{\bullet} \xrightarrow{LH} AOOH + L^{\bullet} \qquad (20)$$

The initiator radicals initially formed in solution are held together briefly in a cage of solvent molecules. This cage effect causes radical molecules to recombine and slows down their diffusion through the solvent. Therefore, the rate of initiation (R_i) depends on the rate of decomposition of the initiator (k_d) and on the fraction *e* of the initiator radicals that escape the solvent cage affecting the efficiency of the chain initiation reaction (20).

$$e = R_i/2k_d\ [\text{Initiator}]$$

The rate of initiation is generally measured by an induction period method using an antioxidant (AH) that has a known stoichiometric factor n, defined as the number of radicals trapped by each molecule of antioxidant. Because α-tocopherol is known to have an n value of 2, a known concentration is used to determine the induction period, τ, during which the oxidation is inhibited.

$$R_i = n[AH]/\tau$$

This kinetic approach is generally used to study free radical oxidation in simple model systems. However, the azo initiators used in these studies are artificial systems that are not found in either foods or biological systems. The efficiency of these initiators is greatly affected by the lipid systems used for oxidation and by the solvent viscosity; the more viscous the solvent the lower their efficiency. The efficiency of initiators in escaping the solvent cage varies; DMVN is about 75% efficient whereas AIBN is 65% efficient. The diazo hydroperoxides (AOOH) formed by reaction (20) are known to interfere with the HPLC analyses of lipid hydroperoxides. Diazo initiators produce a big flux of peroxyl radicals that do not have time to branch and proceed to other reactions observed in real lipid systems. The quantitative kinetic data obtained with diazo initiators may thus be oversimplified and not relevant to either foods or biological systems.

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Table 1.1. Rates of autoxidation of unsaturated fatty esters [a,b]

Fatty esters	Number of $-CH_2-$	mole O_2 per 100 hr [a]	Relative rates [a]	Oxidizability $M^{-½}sec^{-½}$ [b]	Relative rates [b]
18:1	0	0.04	1		
18:2	1	1.63	41	0.020	1
18:3	2	3.90	98	0.041	2.1
20:4	3	7.78	195	0.058	2.9
22:6	5			0.102	5.1
Trilinolein	1 × 3	1.99	50	0.080	4.0

[a] Holman and Elmer (1947): neat methyl or ethyl esters were autoxidized at 37°C and rates were measured by oxygen absorption with a Warburg respirometer.
[b] Cosgrove *et al.* (1987): chlorobenzene solution were oxidized at 37°C in the presence of 2,2'-azobis(2-methylpropionitrile) and rates were measured by oxygen absorption with a pressure transducer in an automated recording gas apparatus.

C. Relative rates of autoxidation of unsaturated fatty acids

The relative rates of autoxidation of different unsaturated fatty acids and esters were compared on the basis of oxygen absorption measurements (Table 1.1). In neat systems without added initiator, 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. The oxidizability of polyunsaturated fatty acid (PUFA) esters was also compared on the basis of oxygen uptake measured kinetically by the induction period method described above, in solution in the presence of azo initiators. The oxidizability of 18:2, 18:3, 20:4, and 22:6 was linearly related to the number of bis-allylic positions present in the fatty esters. From this relationship, the oxidizability of each PUFA was increased approximately two fold for each active bis-allylic methylene group. Thus, the oxidizability of 22:6 was 5 times greater than that of 18:2.

The autoxidation of trilinolein was more complicated than that of the simple fatty esters and did not follow the same rate equation. The order of the reaction was about 0.84 compared to 1.0 for the simple fatty esters. The efficiency of the initiators used, DMVN, was increased to 100% in the trilinolein compared to about 75% in the simple fatty esters. The difference in kinetic behavior between trilinolein and methyl linoleate was attributed to the tendency of the triacylglycerol to form aggregates. The kinetic induction period approach used to measure oxidizability may be subject to errors because of the changes in efficiency of some of the artificial initiators used according to the system and the lipid substrate. Phenolic antioxidants such as α-tocopherol are also affected by the colloidal properties of the lipids used in the oxidation test system employed (Chapter 9).

Further complications arise in comparing the relative rates of oxidation of PUFA, which change with the medium and with the methods used to analyse

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oxidation products. The hydroperoxides from more highly unsaturated fatty acids accumulate in much lower yields because they are readily decomposed especially in the presence of contaminating metal catalysts. Thus, methods based on yields of hydroperoxides show decreasing rates with increasing number of double bonds. Although many elegant kinetic studies have contributed significantly to our current knowledge, it was only after the hydroperoxides were purified, characterized structurally and analysed quantitatively that the mechanism of free radical lipid oxidation obtained wide acceptance (Chapter 2). Further knowledge acquired on the structures of hydroperoxides led to important advances in clarifying the structures of their decomposition products that contribute to the problems of rancidity in foods, and to free radical damage in biological systems. Organic structural studies of oxidation products obtained with lipids relevant to foods and biological systems are thus more useful than kinetic studies relying on artificial initiators, and simplistic models carried out under improbable reaction conditions.

D. Metal catalysis

The above kinetic formulation is generally based on homogeneous systems in solution that require initiators generating radicals at a known and constant rate. However, the kinetics of lipid oxidation become complicated in the presence of metals. As the oxidation proceeds, the rate of lipid radical formation is not constant and increases by the accumulation of lipid hydroperoxides, which decompose in the presence of metals. Further complications arise in heterogeneous systems where the metal and hydroperoxide initiators are distributed in different phases (Chapter 10).

Trace amounts of heavy metals, such as iron and copper, have a marked accelerating effect on the rates of lipid oxidation. The homolytic decomposition of lipid hydroperoxides is generally considered to be metal-catalysed, because it is very difficult or nearly impossible to eliminate traces of metals that can act as potent catalysts. Metals may produce radicals by two pathways:

(a) by electron transfer:

$$M^{(n+1)+} + LH \longrightarrow M^{n+} + L^{\bullet} + H^{+} \quad (21)$$

(b) by formation of metal-oxygen transition complexes ($M–O_2$) or metal-hydroperoxide complexes (M–HOOR), which catalyse autoxidation and decomposition by one-electron redox reactions:

$$M^{n} + ROOH \longrightarrow M^{n+1} + RO^{\bullet} + OH^{-} \quad (22)$$

$$M^{n+1} + ROOH \longrightarrow M^{n} + ROO^{\bullet} + H^{+} \quad (23)$$

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Reactions (22) and (23) require metal ions which can exist in two oxidation states with a suitable redox potential. Reaction (22) is usually much more rapid than reaction (23), and the metal is converted mostly in its most oxidized state, so that the rate of chain initiation depends on reaction (22). In the presence of metals, these reactions thus initiate additional autoxidation chains, which accelerate the rates of lipid oxidation.

Kinetically, if the reaction of metal and hydroperoxides is assumed to be the overriding initiation reaction:

$$\mathrm{LOOH + M} \xrightarrow{k_i} \mathrm{X^{\cdot}} \xrightarrow{\mathrm{LH}} \mathrm{L^{\cdot}} \qquad (24)$$

The following rate expression can be derived for the rate of metal initiation:

$$R_i = k_i\,[\mathrm{LOOH}]\,[\mathrm{M}]$$

Substituting this equation into the rate expression formulated above for hydroperoxide formation, we obtain the following rate of the metal-catalysed oxidation:

$$d[\mathrm{LOOH}]/dt = k_p[\mathrm{LH}]\,k_i\,[\mathrm{LOOH}][\mathrm{M}]^{1/2}/2k_t$$

The rate of autoxidation is thus proportional to the square root of the metal ion concentration. However, this rate does not hold when the concentration of metal initiator is too small to measure. In many food and biological systems, metal catalysts may be coordinated with ligands as complexes or may exist as dimers or higher molecular weight compounds. Other chelating materials may form strong complexes with metals and inactivate their catalytic effects in promoting hydroperoxide decomposition (Chapter 4).

In actual lipid oxidation, one cannot overlook the critical role of trace metals, which complicate the kinetic sequences of initiation and decomposition of lipid hydroperoxides. These metals catalyse both initiation of free radicals and decomposition of hydroperoxides, which become particularly significant with polyunsaturated lipids containing more than two double bonds. With these polyunsaturated lipids, although the yields of hydroperoxides are reduced in the presence of metals, they produce volatile decomposition products that have a serious impact on flavor deterioration. In foods and biological systems, the mixture of trace metals and hydroperoxides is the most important initiator that plays a key part in the development of free radical oxidation and rancidity. The use of artificial azo compounds as initiators to study free radical oxidation is therefore not relevant.

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CHAPTER 2

Hydroperoxide formation

A. Oleate autoxidation

The classical mechanism for the free radical oxidation of methyl oleate involves hydrogen abstraction at the allylic carbon-8 and carbon-11 to produce two delocalized three-carbon allylic radicals (Figure 2.1). According to this mechanism, oxygen attack at the end-carbon positions of these intermediates produces a mixture of four allylic hydroperoxides containing OOH groups on carbons 8, 9, 10 and 11, in equal amounts:

9-hydroperoxy-*trans*-10-octadecenoate (*trans*-9-OOH)
11-hydroperoxy-*cis*-9-octadecenoate (*cis*-11-OOH)
10-hydroperoxy-*trans*-8-octadecenoate (*trans*-10-OOH)
8-hydroperoxy-*cis*-9-octadecenoate (*cis*-8-OOH)

However, studies based on gas-chromatography-mass spectrometry (GC-MS) and on high-performance liquid chromatography (HPLC) (Chapter 6) showed a small but consistently higher amount of the 8- and 11-OOH (25–29%) than the 9- and 10-OOH (22–25%) (Table 2.1). In the GC-MS

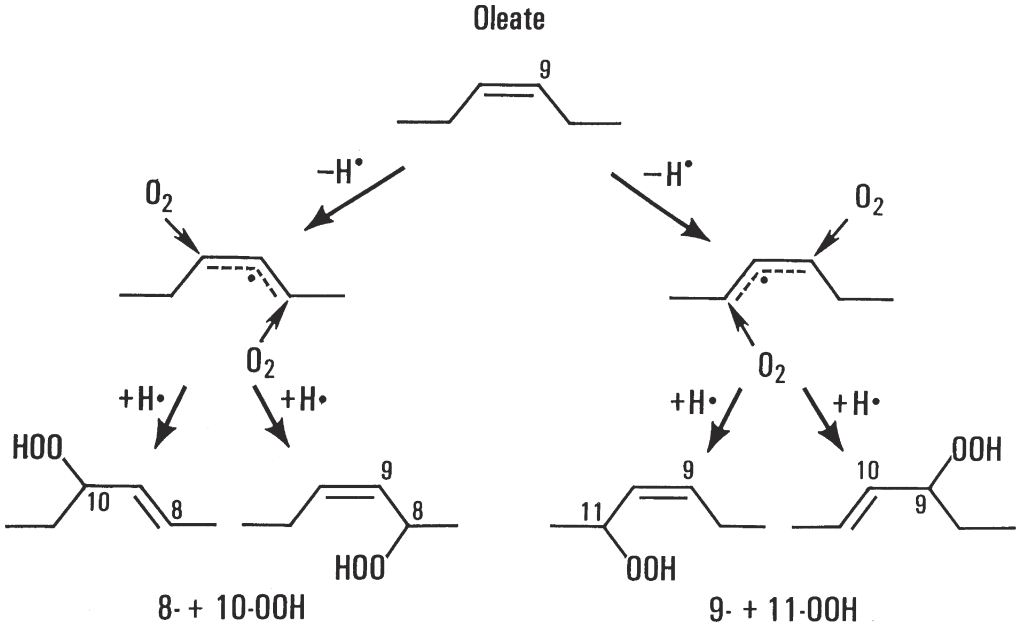


Figure 2.1. Mechanism of oleate autoxidation.

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Table 2.1. Hydroperoxides from autoxidation of methyl oleate (as % of total) [a]

Temp. °C	8-OOH			9-OOH			10-OOH			11-OOH		
	cis	*trans*	Total	*cis*	*trans*	Total	*cis*	*trans*	Total	*cis*	*trans*	Total
25	14.1	12.3	26.4	1.1	23.1	24.2	1.1	21.7	22.8	13.7	12.9	26.6
30[b]	13.0	16.0	29.0	1.0	21.0	22.0	1.0	21.0	22.0	13.0	16.0	29.0
40	10.6	16.0	26.6	1.6	22.0	23.6	1.7	21.7	23.4	10.1	16.0	26.4
50	8.3	17.8	26.1	2.2	22.5	24.7	2.2	21.3	23.5	8.3	17.4	25.7
75	6.1	19.0	25.1	2.7	22.5	25.1	2.9	22.0	24.9	5.4	19.5	24.9

[a] From Frankel *et al.* (1977, 1984): oxidation with neat methyl oleate; products analysed by ^{13}C-NMR as *cis*- and *trans*-hydroxyoctadecenoates separated by $AgNO_3$ thin layer chromatography followed by GC-MS of the hydroxyoctadecanoate derivatives.

[b] From Porter *et al.* (1994): oxidation of methyl oleate in hexane solution inititated with di-*tert*-butyl hyponitrite; products analysed as the hydroxyoctadecenoates by normal phase HPLC. Co-oxidation of oleate with *tert*-butyl hydroperoxide produced only the 11-*cis*, 9-*trans*,10-*trans* and 8-*cis* in a ratio of 1:1.2 for 11-*cis*:9-*trans* and 8-*cis*:10-*trans* products.

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method, a mass spectrometry-computer summation approach was employed for quantification using authentic synthetic samples of 8-, 9-, 10- and 11- hydroxy-octadecanoate, as the trimethylsilyl (TMS) ether derivatives. This computer summation approach was developed because the isomeric hydroxy TMS ethers were partially separated by GC, and all the mass spectra within the appropriate GC peak were added for proper quantification. The quantitative analyses by the HPLC method, carried out with a normal phase silica column on the hydroxyoctadecanoate derivatives, were in complete agreement with the GC-MS method.

Stereochemical studies based on ^{13}C-nuclear magnetic resonance spectroscopy (^{13}C-NMR) showed the presence of eight *cis* and *trans* allylic hydroperoxides (Table 2.1). To determine the isomeric distribution of allylic hydroxyooctadecenoate derivatives, *cis* and *trans* fractions were separated by silver nitrate-thin layer chromatography (TLC), a procedure that separates according to the number, position and geometry of double bonds, and they were hydrogenated prior to GC-MS analyses of the TMS ether derivatives. More recently, the six major hydroperoxide isomers of methyl oleate were partially separated by silica HPLC, and identified by chemical-ionization mass spectrometry and 1H NMR (Table 2.1). These hydroperoxide isomers were better separated as the hydroxy octadecenoate derivatives by the same silica HPLC method and re-analysed by GC-MS.

When pure neat methyl oleate was oxidized, with increasing temperature, the relative amounts of *cis*-8-OOH and *cis*-11-OOH isomers decreased, the *trans*-8-OOH and *trans*-11-OOH isomers as well as the *cis*-9- and cis-10-isomers increased, while the *trans*-9- and *trans*-10-OOH isomers did not change (Table 2.1). When a hexane solution of methyl oleate was oxidized at 30°C in the presence of the free radical initiator di-*tert*-butyl hyponitrite, the distribution of hydroperoxide isomers was similar to that obtained at 25°C, except for greater amounts of *trans*-11-OOH (plus 3%) and *trans*-8-OOH isomers (plus 4%) (Table 2.1). These changes in the configuration of hydroperoxides with temperature of oxidation, and with the concentration of the oleate substrate, can be attributed to both kinetic and thermodynamic factors that determine the stereochemistry of either the allylic carbon or the peroxyl radical intermediates, or both (Figure 2.2). The stereochemistry of the allylic hydroperoxides can depend on kinetic and thermodynamic factors, according to the conditions of oxidation. The temperature of oxidation has a significant effect on the *cis* and *trans* configurations of the initial hydroperoxide formed. Temperature effects reflect significant changes in the 8- and 11-hydroperoxides and very little in the 9- and 10-hydroperoxides isomers (Table 2.1).

A number of mechanisms have been advanced to explain the formation of all eight *cis* and *trans* isomers of 8-, 9-, 10- and 11-hydroperoxides in autoxidized methyl oleate. In one mechanism, the delocalized radicals formed by abstraction of the hydrogens allylic to the double bond of oleate lose their

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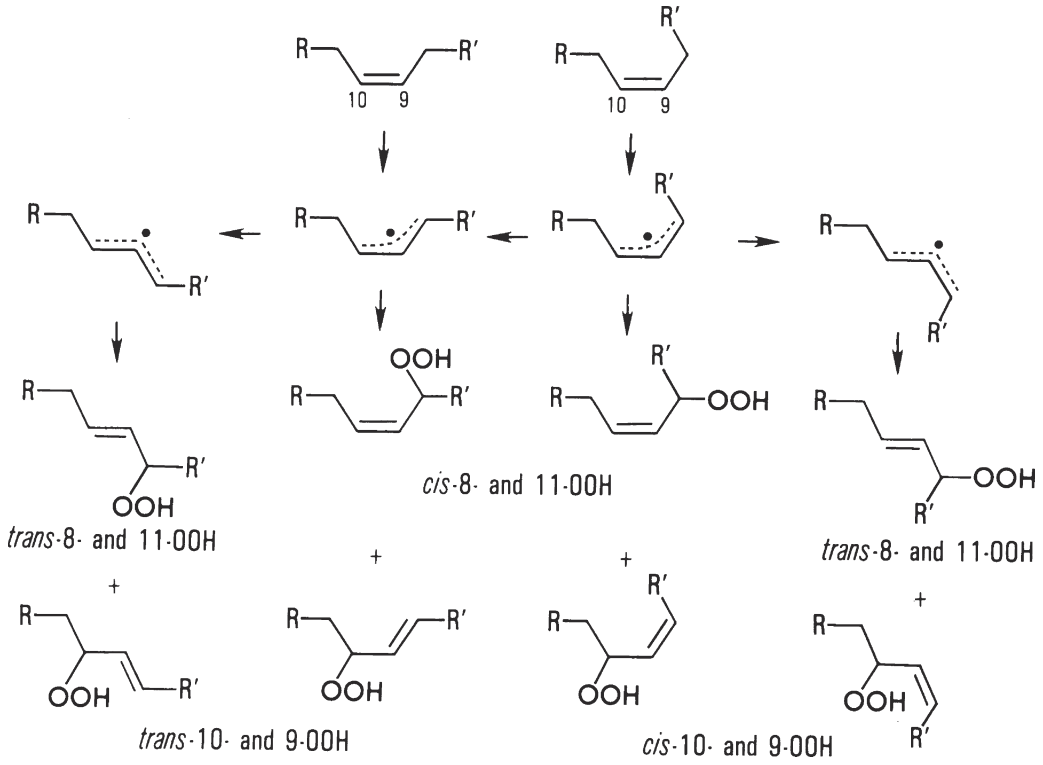


Figure 2.2. Mechanism II of oleate oxidation (Frankel *et al.*, 1984).

stereochemistry, resulting in a change of conformation, especially at elevated temperatures (Figure 2.2). The conformation of the allylic carbon radicals may thus be established initially by the conformation of the oleate according to the temperature of oxidation. The relative amounts of *cisoid* and *transoid* conformers of the hybrid allylic carbon radical intermediates formed from oleate will depend on the degree of oxidation. The small preference for the reaction of oxygen on carbon-8 and carbon-11 may indicate that some hydrogen abstraction occurs from conformers of non-planar radicals formed from methyl oleate at lower temperatures. Thus, the small preference for the 8- and 11-hydroperoxides may be explained by a reaction of oxygen with radicals before they rotate to a planar conformation.

According to another mechanism, the *cisoid* or *transoid* configurations of the peroxyl radicals rather than those of the allylic carbon radicals, which have a very short lifetime in the presence of oxygen, determine the stereochemistry of the hydroperoxides. Although the hydroperoxides of methyl oleate could be analysed directly by HPLC-chemical ionization mass spectrometry (CI-MS) producing fragmentation ions by scission alpha to the hydroperoxy group (Figure 6.17), better separation was obtained after reduction to the corresponding hydroxyoctadecenoate derivatives. A simpler mixture of 8-*cis*-OOH,

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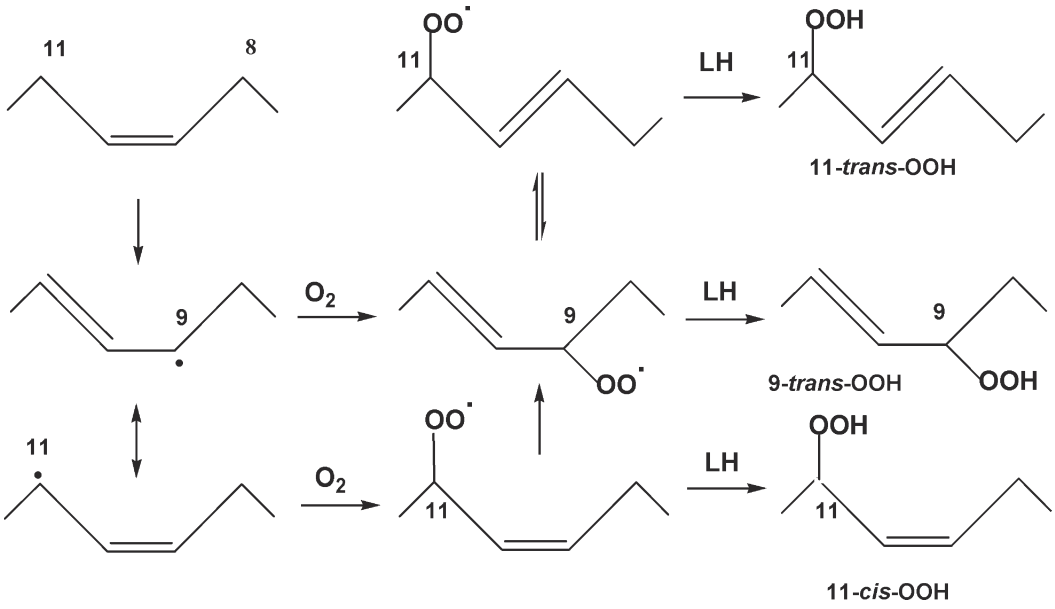


Figure 2.3. Mechanism III of oleate oxidation (Porter, 1994).

9-*trans*-OOH, 10-*trans*-OOH and 11-*cis*-OOH was produced by co-oxidation of methyl oleate with *tert*-butyl hydroperoxide to suppress rearrangement of the allyl peroxyl radicals. These results support a mechanism in which peroxyl H-atom abstraction competes with [2,3] allylperoxyl rearrangement (Figure 2.3). According to this mechanism, the allylic radicals in the *cisoid* configuration were considered kinetically more labile toward oxygen than in the *transoid* configuration. The most relevant mechanism however involves rearrangement of the allylic peroxyl radicals in the absence of the artificial co-oxidant *tert*-butyl hydroperoxide.

B. Linoleate autoxidation

Linoleate is 40 times more reactive than oleate (Chapter 1, Table 1.1), because it has an active bis-allylic methylene group on carbon-11 between two double bonds that can lose a hydrogen atom very readily. Hydrogen abstraction at the carbon-11 position of linoleate produces a hybrid pentadienyl radical, which reacts with oxygen at the end carbon-9 and carbon-13 positions to produce a mixture of two conjugated diene 9- and 13-hydroperoxides (Figure 2.4). The greater reactivity of linoleate to autoxidation is due to the formation of a pentadienyl radical intermediate, which is more effectively stabilized by resonance, and the resulting dienoic hydroperoxides produced that are stabilized by conjugation. These isomeric conjugated dienoic hydroperoxides are formed in equal concentrations at different levels of autoxidation and a wide range of temperatures.

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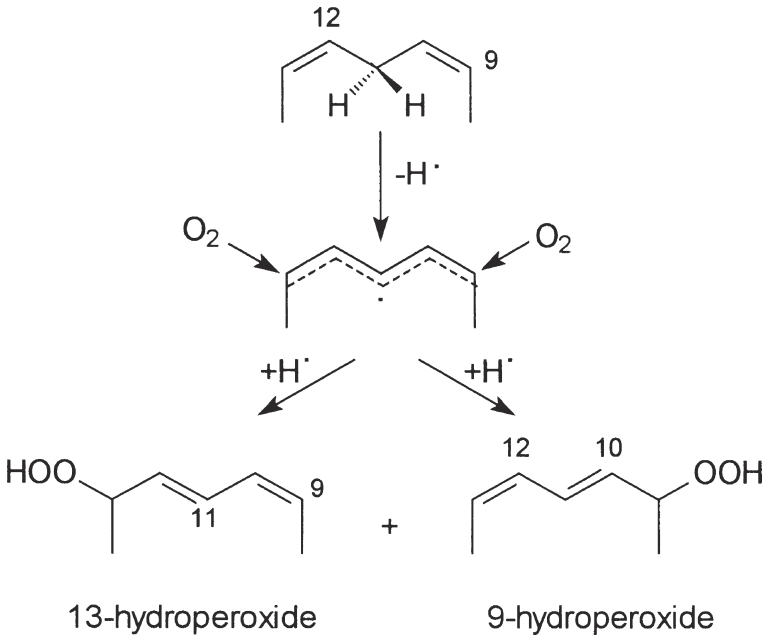


Figure 2.4. Mechanism I of linoleate autoxidation.

Stereochemical studies based on HPLC and ^{13}C-NMR (Chapter 6) showed the formation of a mixture of four *cis,trans* and *trans,trans* conjugated diene hydroperoxides:

9-hydroperoxy-*trans*-10, *cis*-12-octadecadienoate (*cis,trans*-9-OOH)
9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoate (*trans,trans*-9-OOH)
13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate (*cis,trans*-13-OOH)
13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoate (*trans,trans*-13-OOH)

Initially and at low temperatures, the *cis,trans*-OOH isomers are predominant. The proportion of *trans,trans*-OOH isomers increases with the level and

Table 2.2. Hydroperoxides from autoxidation of methyl linoleate (as % of total) [a]

Temp °C	13-OOH *trans,trans*	13-OOH *trans,trans*	9-OOH *cis,trans*	9-OOH *trans,trans*	Total 13-OOH	Total 9-OOH
25	31.0	20.1	29.7	19.2	51.2	48.9
50	19.5	28.0	22.9	29.6	47.5	52.5
65	18.6	33.5	17.5	30.4	52.1	47.9

[a] From Frankel *et al.* (1990); average analyses by preparative HPLC and by ^{13}C-NMR of the isomeric hydroxyoctadecadienoate derivatives obtained by sodium borohydride reduction of the purified hydroperoxides.

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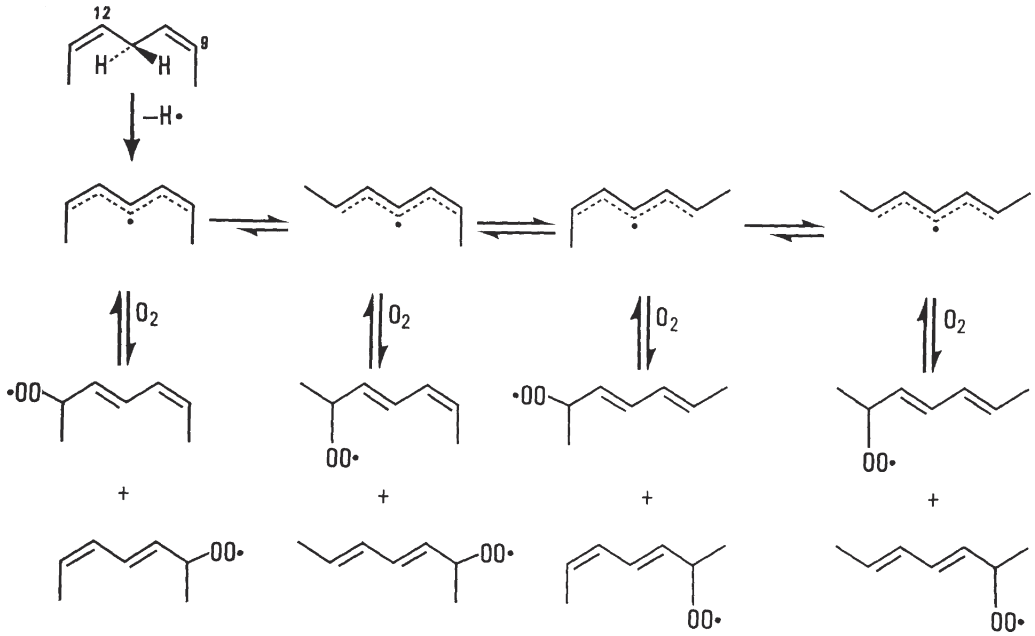


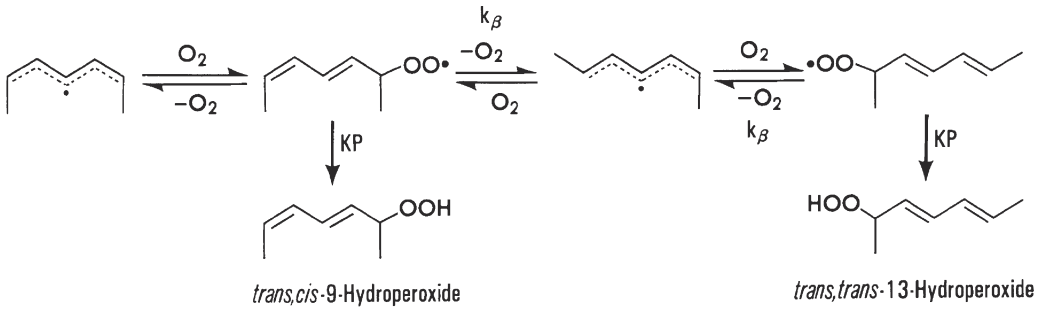
Figure 2.5. Mechanism II of linoleate autoxidation (Frankel, 1979).

temperature of oxidation (Table 2.2). Each of the *cis,trans*-9- and 13-OOH isomers are readily inter-converted into mixtures containing the corresponding *trans,trans*-OOH isomers.

As with oleate, several mechanisms have been advanced to explain the stereochemical formation of the four *cis,trans*- and *trans,trans* isomers of the 9- and 13-hydroperoxides in autoxidized methyl linoleate. In one mechanism, the initial pentadienyl radical assumes four conformations before reaction with oxygen (Figure 2.5). An initial *cis,cis*-pentadienyl radical is formed which is converted into *trans,cis/cis,trans*-pentadienyl radicals and into a *trans,trans* radical at higher levels and temperatures of oxidation. Under these conditions, the ratio of *cis,trans*- and *trans,trans*-hydroperoxides decreases but the proportion of the 9- and 13-hydroperoxides remains the same, because the pentadienyl radicals react equally on carbon-9 and carbon-13. These radicals react with oxygen reversibly to form the corresponding *cis,trans*- and *trans,trans*-peroxyl radicals. The loss of oxygen leads to a pool of pentadienyl radicals that undergo rearrangement.

In another proposed mechanism, the stereochemistry of linoleate hydroperoxides is determined by the configuration of the peroxyl radicals rather than that of the carbon pentadienyl radicals, which have a very short lifetime because they react very rapidly with oxygen (Figure 2.6). Accordingly, the interconversion of *cis,trans*- and *trans,trans*-hydroperoxides results from the loss of oxygen from the peroxyl radicals by reversible β-scission to produce carbon pentadienyl radicals of different conformations. The *trans,cis/trans,trans*

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k_β = β-scission of C—O bond
KP = H abstraction

Figure 2.6. Mechanism III of linoleate autoxidation (Porter *et al.*, 1980).

product ratio is determined by the relative rate of the reversible β-scission or β-fragmentation (k_β) of the carbon–oxygen bond of the peroxyl radicals and their rate of hydrogen abstraction (KP) to form the corresponding hydroperoxides. The interconversion of *cis,trans*- and *trans,trans*-hydroperoxides is inhibited in the presence of antioxidants, which act as hydrogen donors. In the presence of 5% α-tocopherol, the formation of *trans,trans*-hydroperoxides is completely inhibited during autoxidation of methyl linoleate.

In considering the merits of these mechanisms to explain the stereochemistry of linoleate autoxidation, we should appreciate the marked variation in conditions used by various workers to study autoxidation. In some studies conducted in neat viscous lipid systems where autoxidation was initiated in the presence of trace metals and hydroperoxide impurities, hydroperoxidation would be influenced by factors favoring the formation of thermodynamically stable *trans,trans* hydroperoxides. In other studies, thermodynamic factors were minimized or synthetically eliminated, by conducting the oxidation in solution in the presence of artificial non-lipid initiators that generate a flux of radicals and at very low levels of conversion. Under these conditions, the autoxidation was kinetically controlled and favored a higher *trans,cis/trans,trans* product ratio. In the oxidation of neat lipids or multi-phased emulsion systems, initiated by metal catalysts and oxidized lipid impurities, at elevated temperatures and at higher degrees of conversion, the reaction may become more controlled by thermodynamic than by kinetic factors.

The possible formation of the nonconjugated bis-allylic 11-hydroperoxide from linoleate after hydrogen abstraction from carbon-11 has been debated for a long time in the literature. Whether or not this proposed bis-allylic 11-hydroperoxide is unstable and immediately isomerized to the more thermally stable conjugated 9- and 13-hydroperoxides is not clear. Recently, the bis-allyl 11-hydroperoxide was produced in about 5–10% relative yield by *kinetically*

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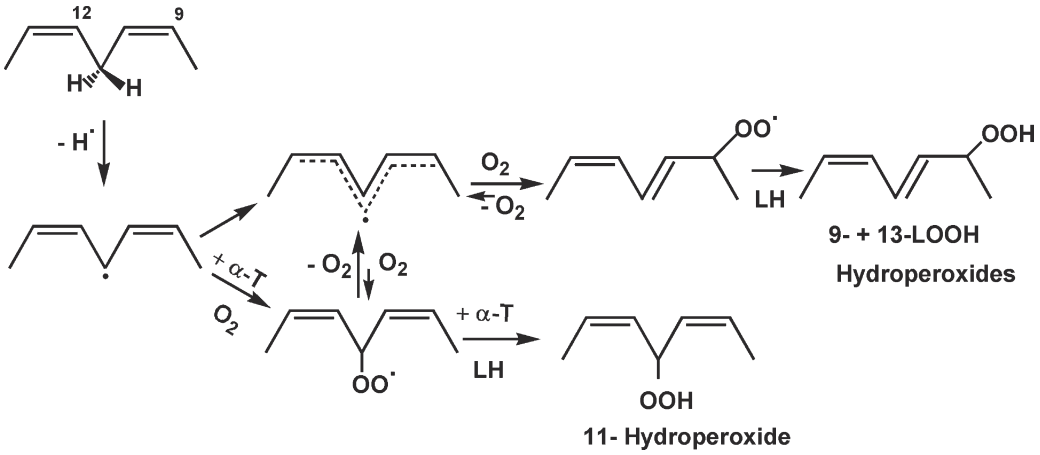


Figure 2.7. Mechanism IV of linoleate autoxidation. Based on evidence for bis-allylic 11-hydroperoxide in the presence of α-tocopherol (Brash, 2000). α-T = 5% α-tocopherol.

controlling the autoxidation of methyl linoleate in the presence of 5% α-tocopherol acting as hydrogen donor. The structure of this minor hydroperoxide was established after reduction and separation of the hydroxyoctadecadienoate by reversed phase HPLC, shown to be nonconjugated by UV, and characterized by GC-MS as TMS ether of the octadecanoate derivative, and by ^{1}H NMR of the bis-allylic hydroxy-octadecadienoate. In the absence of α-tocopherol, the bis-allyl pentadiene radical intermediate is either converted immediately into the *cis,cis*-pentadienyl radical according to mechanism II (Figure 2.5), or the 11-peroxyl radical rearranges by loss and re-addition of oxygen into the more thermodynamically stable conjugated 9- and 13-peroxyl radicals according to mechanism IV (Figure 2.7).

Conjugated linoleic acid (CLA), a mixture of 9-*cis*,11-*trans*- and 10-*trans*,12-*cis*-octadecadienoic acid, has been claimed to be bioactive and to have beneficial nutritional effects. However, the mechanism of CLA autoxidation is unclear and controversial. Recent studies showed that CLA produced a complex mixture of conjugated hydroperoxide isomers difficult to resolve and identify structurally. A simpler product mixture was identified by UV, GC-MS and NMR from pure methyl 9-*cis*-11-*trans*-octadecadienoate autoxidized in the presence of 20% (by wt) α-tocopherol as hydrogen donor. HPLC analysis of the purified hydroxyoctadecadienoate esters with UV detection at 234 nm showed a complex isomeric mixture composition (in relative peak area percentage) consisting of: 50% 13-OH-9-*cis*,11-*trans*-, <1% 13-OH-9-*trans*,11-*trans*-, 12% 12-OH-8-*trans*,10-*trans*-, 13% 9-OH-10-*trans*,12-*cis*-, 15% 9-OH-10-*trans*,12-*trans*-, 2% 8-OH-9-*cis*,11-*trans*-, and 8% 8-OH-9-*trans*,11-*trans*-octadecadienoate esters. Although this evidence cannot rule out the possible formation of nonconjugated hydroperoxides, the mechanism proposed involves allylic hydrogen abstraction producing *cis,trans*-pentadiene-

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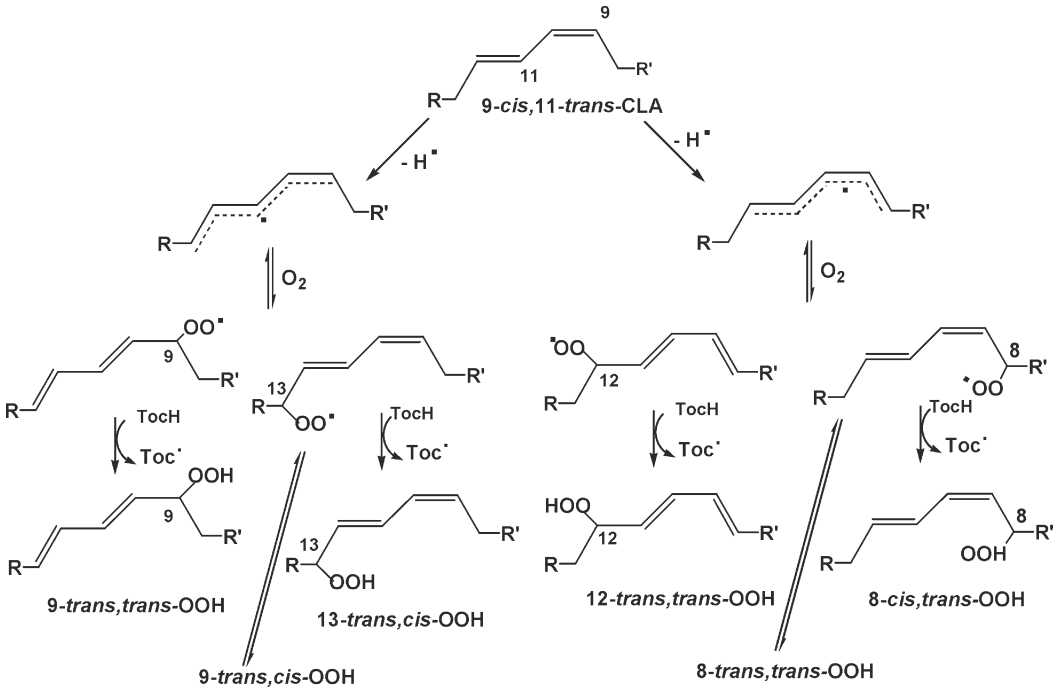


Figure 2.8. Mechanism proposed for the autoxidation of conjugated methyl 9-*cis*,11-*trans*-octadecadienoate in the presence of α-tocopherol (Hämäläinen *et al.*, 2002).

and *trans,trans*-pentadiene radical intermediates and geometric isomerization of peroxyl radicals (Figure 2.8). By the same mechanism, the hydroperoxides expected from methyl 10-*trans*,12-*cis*-octadecadienoate would include 10-*trans,trans*-OOH, 14-*cis,trans*-OOH, 9-*trans,cis*-OOH and 13-*trans,cis*-OOH. More quantitative analyses are needed however to clarify the mechanism of CLA oxidation and to rule out the possible formation of nonconjugated hydroperoxides in the absence of added α-tocopherol.

C. Linolenate autoxidation

Methyl linolenate has two bis-allylic methylene groups and reacts twice as fast with oxygen as linoleate (Chapter 1, Table 1.1). Linoleate was 40 times more reactive than oleate, and linolenate was 2.4 times more reactive than linoleate. When comparing the mono-allylic hydrogens of oleate with the bis-allylic hydrogens of linoleate, the rates increased 40 fold compared to two fold by introducing the second bis-allylic hydrogens of linolenate. Therefore, the two bis-allylic methylene groups in linolenate act independently and are not activated by each other. By the same mechanism as linoleate, two pentadienyl radicals are formed by hydrogen abstraction on carbon-11 and carbon-14 between the two 1,4-diene systems on carbon-9 and carbon-13, on one hand, and on carbon-12 and carbon-16, on the other (Figure 2.9). Reaction with

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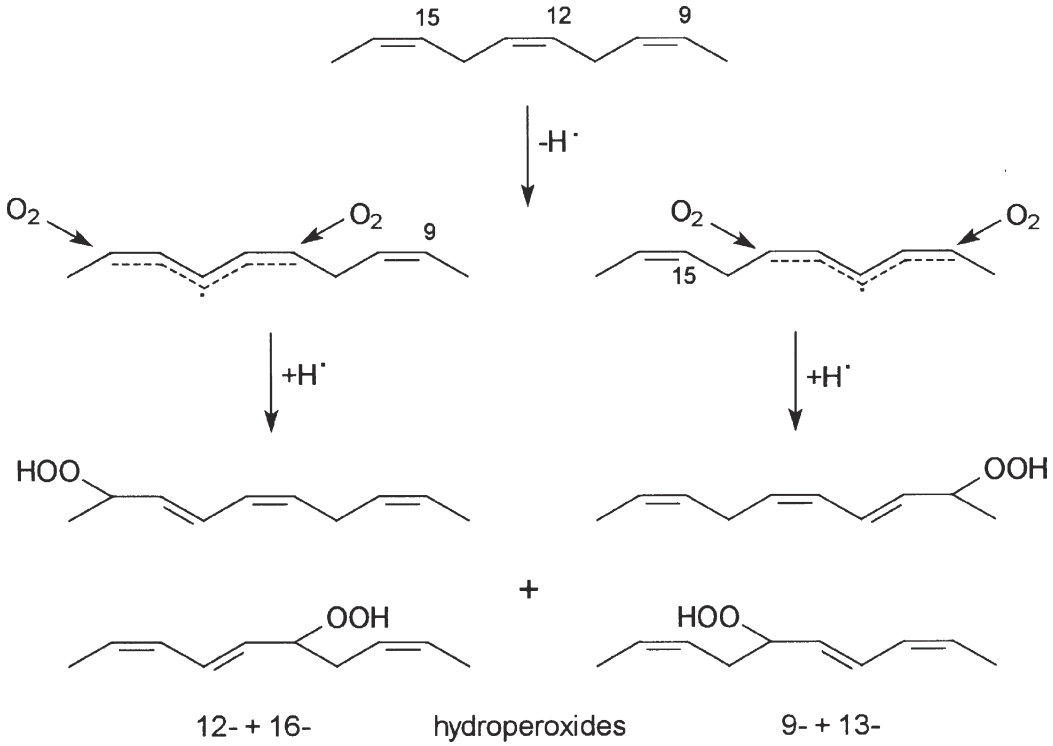


Figure 2.9. Mechanism of linolenate autoxidation (Frankel *et al.*, 1961).

oxygen at the end-carbon positions of each pentadienyl radical produces a mixture of four peroxyl radicals leading to the corresponding conjugated diene 9-, 12-, 13- and 16-hydroperoxides containing a third isolated *cis* double bond.

9-hydroperoxy-*trans*-10, *cis*-12, *cis*-15-octadecatrienoate (*trans,cis,cis*-9-OOH)
13-hydroperoxy-*cis*-9, *trans*-11, *cis*-15-octadecatrienoate (*cis,trans,cis*-13-OOH)
12-hydroperoxy-*cis*-9, *trans*-13, *cis*-15-octadecatrienoate (cis,*trans,cis*-12-OOH)
16-hydroperoxy-*cis*-9, *cis*-12, *trans*-14-octadecatrienoate (*cis,cis,trans*-16-OOH)

These hydroperoxides can be divided into two distinct groups: the "external" (or "outer") 9- and 16-hydroperoxide isomers, which have the OOH group outside the system of double bonds, and the "internal" (or "inner") 12- and 13-hydroperoxide isomers, which have the OOH group located between a *cis,trans*-conjugated diene system and a single *cis* double bond separated by a homoallylic methylene group. The analyses of linolenate autoxidized at a wide range of conversion (2 to 30%) and temperatures (25 to 80°C) showed that the amounts of external 9- and 16-hydroperoxides were about four times higher than the internal 12- and 13-hydroperoxides (Table 2.3). The GC-MS analyses

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Table 2.3. Hydroperoxides from autoxidation of methyl linolenate (as % of total) [a]

Temp. °C	9-OOH	12-OOH	13-OOH	16-OOH
25	30.6	10.1	10.8	48.6
37[b]	30	11	10	48
40	33.0	10.8	12.4	43.9
60	29.4	11.4	11.8	47.5
80	34.2	11.1	11.5	43.3

[a] From Frankel *et al.* (1977a); GC-MS analyses (as trimethylsilyl derivatives of hydroxyoctadecanoate esters) of samples autoxidized to different peroxides and temperatures were averaged.
[b] From Frankel *et al.* (1961); oxidative cleavage of monoenes derived by dehydration of hydroxyoctadecanoates derivatives.

of isomeric distribution of linolenate hydroperoxides are in complete agreement with earlier studies based on oxidative cleavage products, and later studies based on HPLC analyses of hydroxystearate derivatives (Figure 6.15).

This uneven distribution of isomeric hydroperoxides of linolenate is attributed to the tendency of the 12- and 13-peroxyl radicals to undergo rapid 1,3-cyclization and further oxidation to form 5-membered hydroperoxy epidioxides as major products (25%) (Figure 2.10). Because the peroxyl radicals of the 12- and 13-hydroperoxides have a homoallylic *cis* double bond, they undergo facile 1,3 cyclization by intramolecular radical addition to the double bond and formation of a new radical that reacts with oxygen. This rapid cyclization accounts for the lower concentrations of the internal 12- and 13-hydroperoxides observed under a wide range of conditions of oxidation (Table 2.3). In contrast to linoleate, during autoxidation, the *cis,trans*-hydroperoxides of linolenate are not readily isomerized to the *trans,trans* configuration, apparently because cyclization is much more favorable and competes with geometric isomerization. The mechanism for cyclization of the internal 12- and 13-hydroperoxyl radicals of linolenate is well demonstrated by the evidence that this cyclization is completely inhibited by the addition of 5% α-tocopherol to linolenate. The use of α-tocopherol as a good hydrogen donor that inhibits the cyclization results in an even distribution of the 9-, 12-, 13- and 16-hydroperoxides. The peroxyl radicals derived from the internal 12- and 13-hydroperoxides of linolenate can undergo 1,5-cyclization to form two isomeric 9- and 16-hydroperoxy bicycloendoperoxides (Figure 2.11). These hydroperoxy bicycloendoperoxides are characteristically formed from polyunsaturated fatty acids containing three or more double bonds, and are precursors of malonaldehyde and thiobarbituric acid (TBA)-reactive materials (Chapter 4, Table 4.1).

The significantly higher proportion of the 16-hydroperoxide relative to that of the 9-hydroperoxide of linolenate (Table 2.3) indicates that oxygen attack favors the terminal carbon-16 position of the 12,16-pentadienyl radical closest to the end of the fatty acid chain of linolenate. The same preference of oxygen

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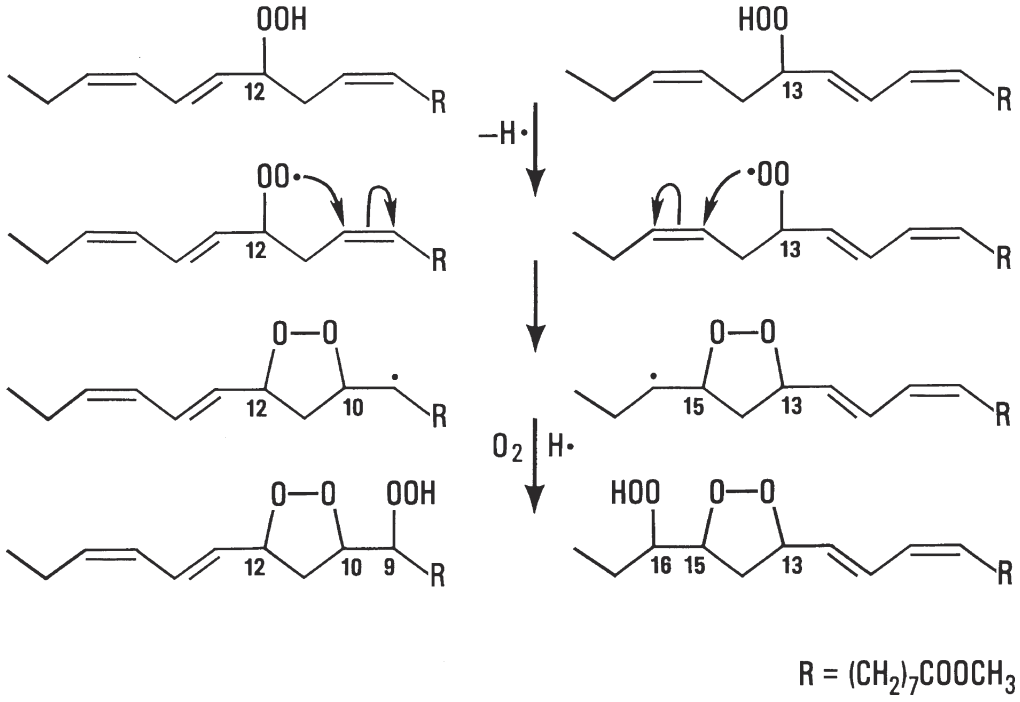


Figure 2.10. Cyclization of 12- and 13-hydroperoxides of linolenate (Neff *et al.*, 1981).

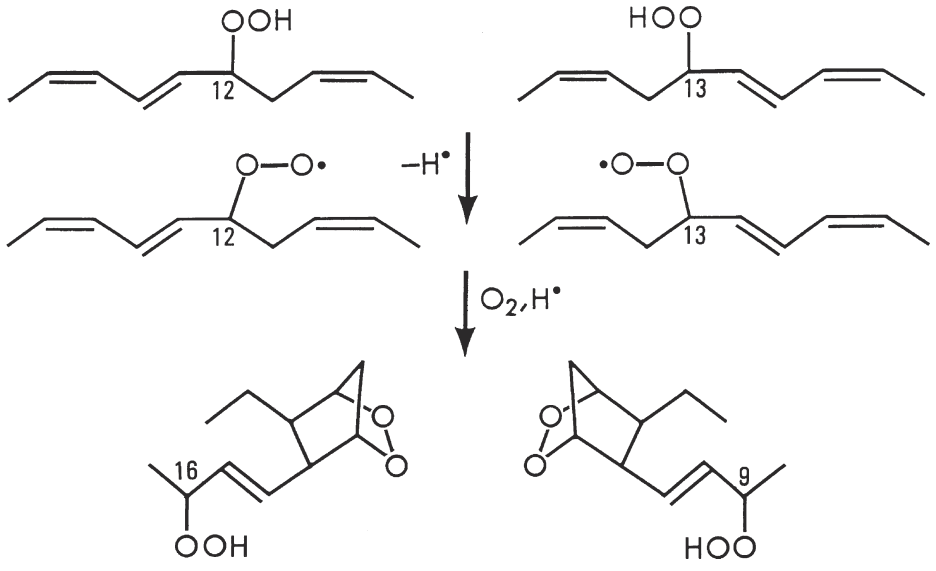


Figure 2.11. Formation of bicyclic endoperoxides from 12- and 13-hydroperoxides of linolenate.

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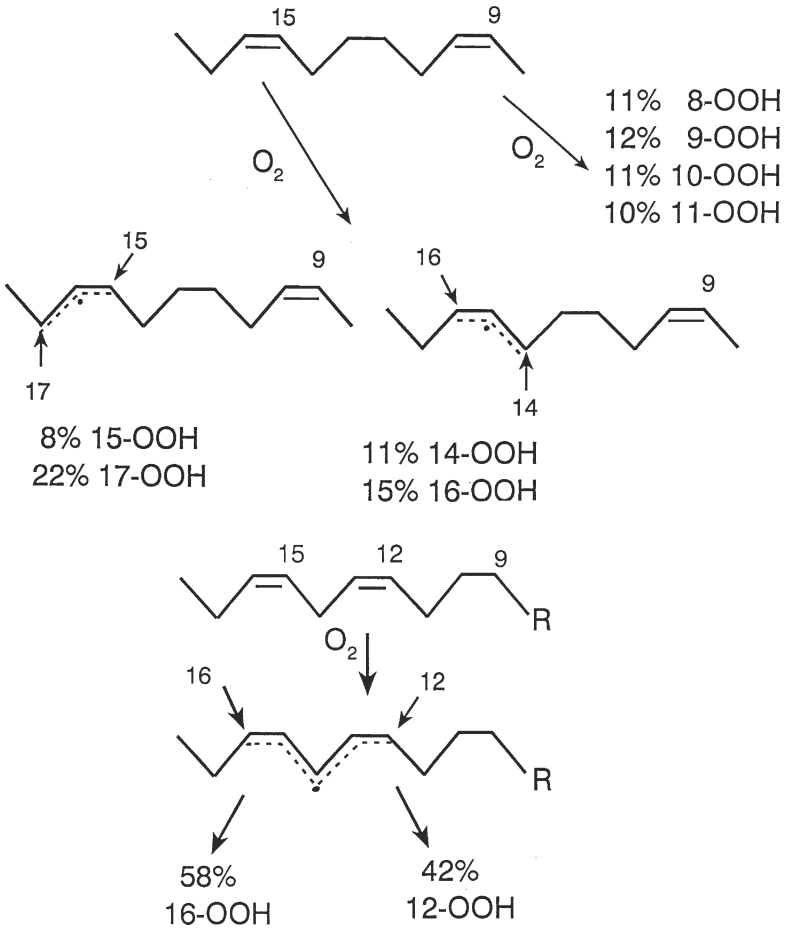


Figure 2.12. Hydroperoxidation of methyl *cis*-9, *cis*-15-octadedienoate and methyl *cis*-12, *cis*-15-octadedienoate (Frankel *et al.*, 1980).

attack at the end of the fatty acid molecule was confirmed in the autoxidation of *cis*-9, *cis*-15-octadecadienoate and *cis*-12, *cis*-15-octadecadienoate. These isomeric dienes are found in partially hydrogenated methyl linolenate. The 9,15-diene has two isolated *cis* double bonds, and oxidizes like oleate to form four isomeric 8-, 9-, 10- and 11- hydroperoxides by oxidation of the *cis*-9-double bond, and four isomeric 14-, 15-, 16- and 17-hydroperoxides by oxidation of the *cis*-15-double bond. The hydroperoxides derived from the 9-double bond are fairly evenly distributed (10–12%), as observed for oleate. However, the hydroperoxides derived from the *cis*-15 double bond are not evenly distributed; they show a significantly higher concentration of the 16-(15%) and 17-hydroperoxides (22%) than the 14- (11%) and 15-hydroperoxides (8%) (Figure 2.12). Oxidation of the terminal 16- and 17-allylic terminal carbons is thus greatly favored. Decomposition of the hydroperoxides derived from the 9,15-diene produces potent volatile compounds that are responsible

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for the characteristic so-called "hydrogenation" flavor of partially hydrogenated linolenate-containing oils (Chapter 4).

Because the 12,15-diene has a 1,4-diene system, it oxidizes like linoleate to form two conjugated dienoic 12- and 16-hydroperoxides. However, in contrast to linoleate, the external 16-hydroperoxide is formed at a higher concentration than the internal 12-hydroperoxide, (with normalized concentrations of 58% and 42%) (Figure 2.12). Therefore, both the 9,15- and 12,15-dienes produce allylic radicals in which the terminal carbons 16 and 17, closest to the end of the fatty acid chain, are the most reactive with oxygen. The same preference of oxygen attack at the terminal double bond position is also observed in other polyunsaturated fatty acids with *n*–3 double bonds (linolenate, eicosapentaenoic and docosahexaenoic acids), and *n*–6 double bonds (arachidonic acid). Volatile decomposition products derived from hydroperoxides containing an *n*–3 double bond are particularly significant for their impact on flavor (Chapter 4).

D. Arachidonate autoxidation

Methyl arachidonate reacts with oxygen about three times as fast as linoleate (Chapter 1, Table 1.1), because it has three active bis-allylic methylene groups and three 1,4-diene systems, i.e. between carbon-5 and carbon-9, between carbon-8 and carbon-12, and between carbon-11 and carbon-15. By the same mechanism for autoxidation as methyl linoleate and linolenate, hydrogen abstraction on the bis-allylic methylene groups on carbon-7, carbon-10, and carbon-13 of arachidonate produces three pentadienyl radicals between carbons 5 and 9, between carbons 8 and 12, and between carbons 11 and 15. Oxygen attack at either end of these pentadienyl radicals produces a mixture of six isomers with hydroperoxide substitution on carbons 5, 8, 9, 11, 12 and 15 (Figure 2.13). As with linolenate, the outer 5- and 15-hydroperoxides of arachidonate are formed at higher concentration (52%) than the inner 8-, 9, 11- and 12-hydroperoxides (10–16%). This distribution of hydroperoxides is similar to that of autoxidized methyl linolenate and can be attributed to the tendency of the internal 8-, 9-, 11- and 12-hydroperoxides to cyclize into four hydroperoxy epidioxides and bicycloendoperoxides (Figures 2.10 and 2.11). In addition to monocyclic and bicyclic peroxides, epoxy alcohols are formed as several stereoisomers. Also, as observed with linolenate, the 15-hydroperoxide in arachidonate is formed at a higher concentration (33.8%) than the 5-hydroperoxide (18.3%), indicating preferential oxygen attack on the carbon closest to the end of the fatty acid chain. As with methyl linolenate, the addition of 1 to 5% α-tocopherol to arachidonate inhibits the cyclization of the 8-. 9-, 11- and 12-hydroperoxyl radicals and results in an even distribution of the six hydroperoxide isomers of arachidonate.

The hydroperoxy bicycloendoperoxides of linolenic and arachidonic acids have structures that are related to the physiologically important prostaglandins

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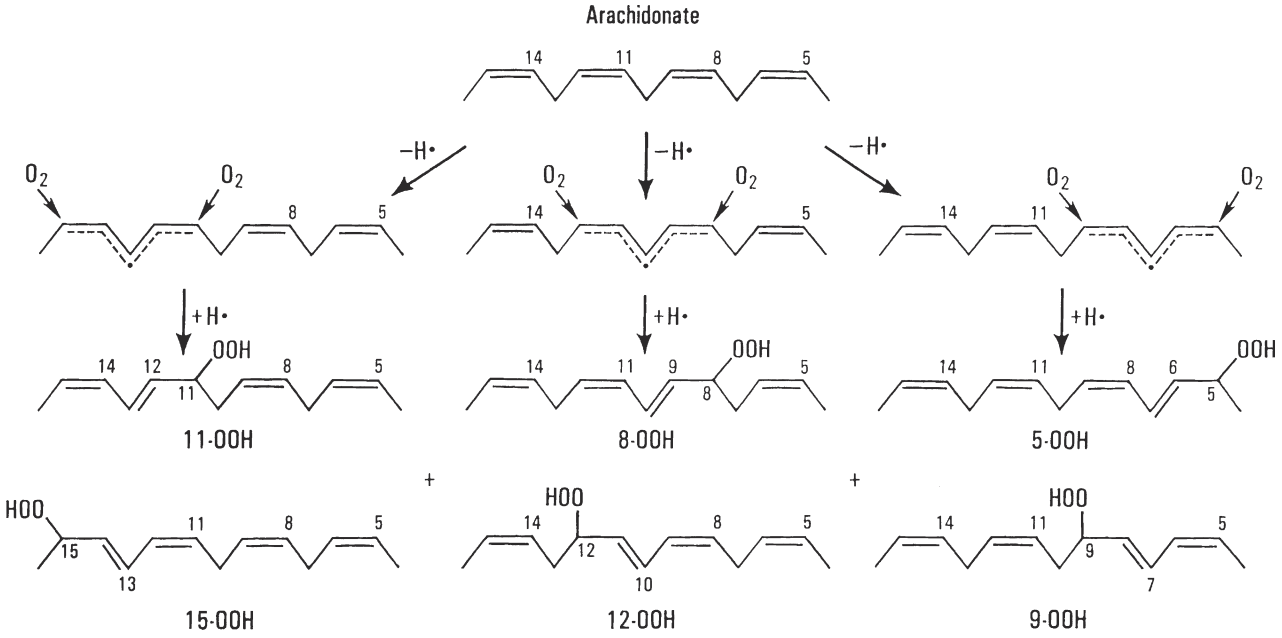


Figure 2.13. Mechanism of arachidonate autoxidation.

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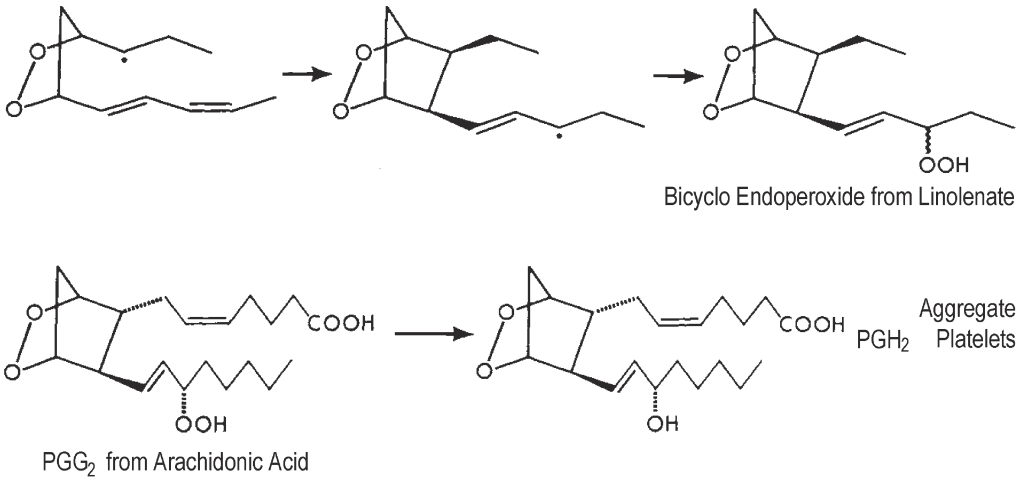


Figure 2.14. Bicyclic endoperoxides from oxidized linolenate and arachidonic acid.

PGG_2 and PGH_2, which promote platelet aggregation and can cause smooth muscle contraction and inflammation (Figure 2.14). Thromboxanes are important metabolites of PGG_2 and PGH_2 formed by ring opening (Figures 13.10, 13.11); they are also involved in platelet aggregation and implicated in thrombosis (see Chapter 13). The bicycloendoperoxides produced by autoxidation of pure 13-hydroperoxide of α-linolenate and 9-hydroperoxide of γ-linolenate were purified by HPLC and shown to have mainly *cis* substituents (also referred to as *syn* configuration), in contrast to the natural *trans* stereochemistry (or *anti* configuration) of prostaglandins derived exclusively by cyclooxygenase (COX) conversion from arachidonic acid. This difference in stereochemistry between the non-enzymatically and enzymatically produced bicycloendoperoxides may be important physiologically.

The complex mixture of regioisomers and stereoisomers structurally related to the endoperoxides and thromboxanes (prostaglandin-like compounds) produced from arachidonic acid and esterified to phospholipids and cholesterol are classified as *isoprostanes*. These compounds are used as markers of *in vivo* oxidative injury in biological systems and considered reliable because they are specific, stable and not affected by the lipid content of the diet (Chapter 13. E.4). However, these secondary oxidation products are produced in relatively small concentrations because they are derived from arachidonic acid, which is a minor polyunsaturated lipid compared to linoleic acid in biological samples. Very sensitive gas chromatography-mass spectrometric (GC-MS) and liquid chromatography-MS techniques are required to analyse isoprostanes in biological samples.

The bicyclic endoperoxides produced from the 5- and 9-OOH (Type I), the 8- and 12-OOH (Types II and III), and the 11- and 15-OOH (Type IV) of cholesteryl arachidonate have been identified recently by the use of electrospray mass spectrometry in the presence of silver ions (Figure 2.15). This new

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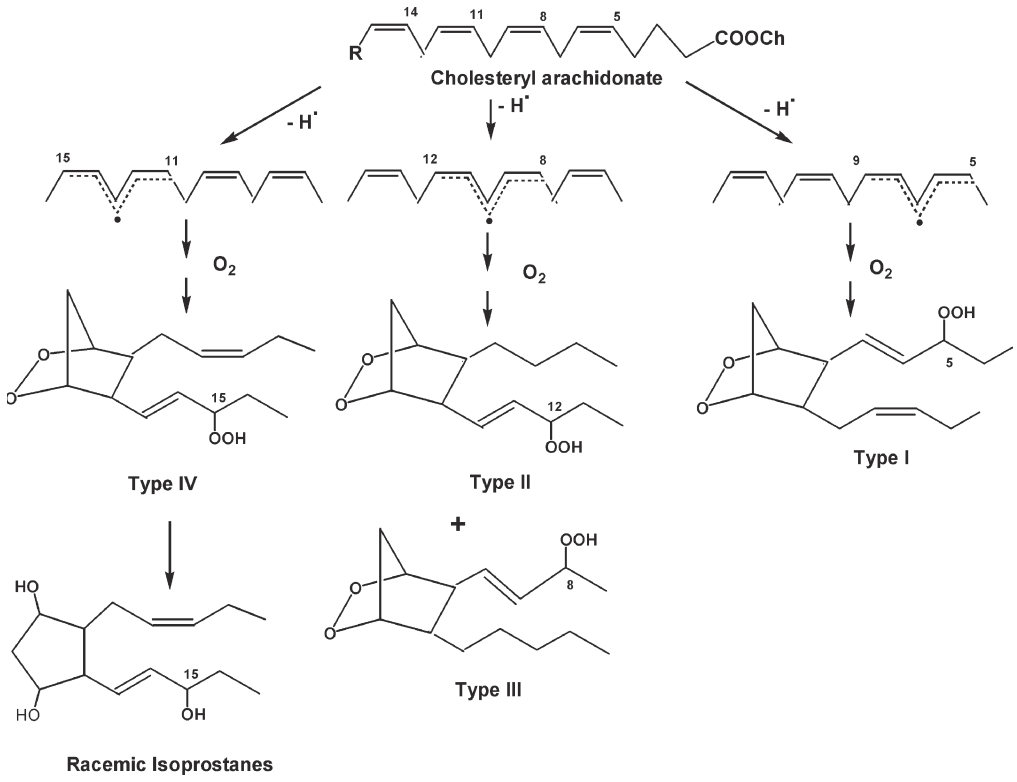


Figure 2.15. Types of bicyclic endoperoxides identified from the oxidation of cholesteryl arachidonate (Yin *et al.*, 2002).

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technique, known as Ag^+ coordination ion-spray mass spectrometry (CIS-MS) has also been used for the direct identification of mixtures of hydroperoxides (Chapter 6). The evidence supports the formation of isoprostanes from the 15-peroxyl radical by β-fragmentation followed by re-addition of oxygen (Figure 2.15).

E. Autoxidation of other long-chain polyunsaturated fatty acids

As the number of double bonds increases in polyunsaturated fatty acids (PUFA), they produce more complex mixtures of hydroperoxides, which are easily decomposed and become very difficult to analyse quantitatively. The most important long-chain *n*–3 PUFA found in fish, marine and algae oils include all *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA), and all *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA). The hydroperoxides produced from EPA and DHA have been identified but not quantified. By the same mechanism established for linolenate, EPA produced the eight 5-, 8-, 9-, 11-, 12-, 14-, 15- and 18-hydroperoxides. DHA produced the ten 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17- and 20-hydroperoxides.

We have seen in Chapter 1 that the oxidizability of 18:2, 18:3, 20:4 and 22:6 was linearly related to the number of bis-allylic positions in the fatty esters. From this relationship, the oxidizability of each PUFA is increased approximately two fold for each active bis-allylic methylene group. Thus, the oxidizability of 20:5 was four times greater, and that of 22:6 was five times greater than that of 18:2. There is current interest in supplementing infant formulas and clinical nutrition products with EPA and DHA-containing fish and algae oils for their nutritional benefits, but their susceptibility to oxidation may become an important problem, especially because commercial infant formulas are supplemented with iron (Chapter 11).

F. Autoxidation of triacylglycerols

Trilinolein formed monohydroperoxides located on the 1,3- and 2-positions of the glycerol moiety as the main initial products. The monohydroperoxides were further oxidized to form a mixture of 1,2- and 1,3-bis-hydroperoxides, which are also oxidized to tris-hydroperoxides by sequential oxygen addition (Figure 2.16). The hydroperoxides were composed of *cis,trans*-and *trans,trans*-9- and 13-isomers, which were located in the 1(3)- and 2-triacylglycerol positions in a ratio of two to one. Therefore, the oxidation of trilinolein showed no positional preference between the 1(3)- and 2-triacylglycerol positions.

Trilinolenin produced on autoxidation 1(3)- and 2-monohydroperoxides, 1,2- and 1,3-bis-hydroperoxides and tris-hydroperoxides by sequential oxidation (Figure 2.17). Trilinolenin produced also significant amounts of hydroperoxy epidioxides, formed by 1,3-cyclization (Section C). The

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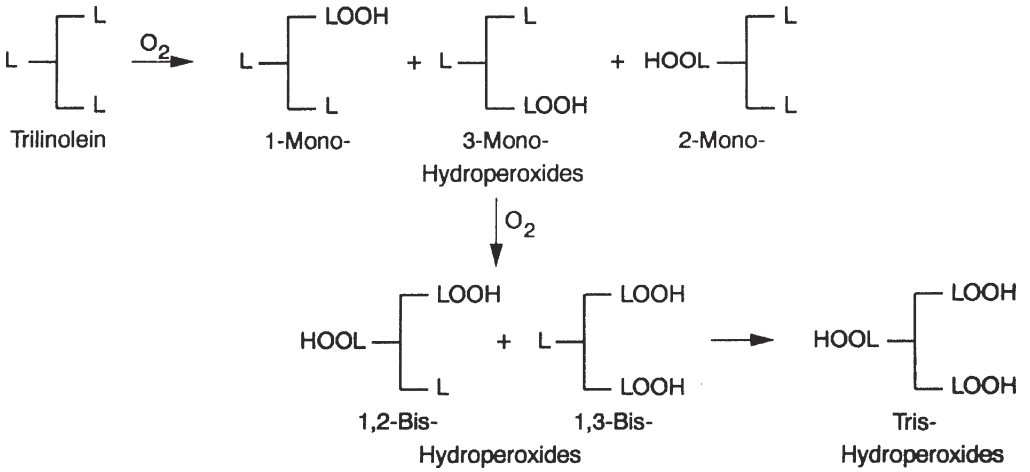


Figure 2.16. Mechanism of trilinolein autoxidation (L = linoleate) (Neff *et al.*, 1990).

hydroperoxides consisted of a mixture of 9-, 12-, 13- and 16-isomers, and the hydroperoxy epidioxides were mixtures of 9- and 16-hydroperoxy isomers. The *cis,trans*-16-linolenate hydroperoxide in the 1(3)-triacylglycerol positions was produced in a ratio of 2.3:1 relative to the 2-triacylglycerol position, compared to 1.8:1 for the corresponding *cis,trans* 9-linolenate hydroperoxide. These results support a small preferential attack of oxygen on the *n*–3 double bond of linolenate in the 1(3)-triacylglycerol positions. Triacylglycerols containing EPA and palmitic acid also produced monohydroperoxides as major products (70–80%) including hydroperoxy epidioxides and other unidentified secondary products. Tri-EPA were further oxidized to bis- and tris-hydroperoxides. These hydroperoxides were separated by HPLC and identified by liquid chromatography-mass spectrometry (LC-MS) with electrospray ionization.

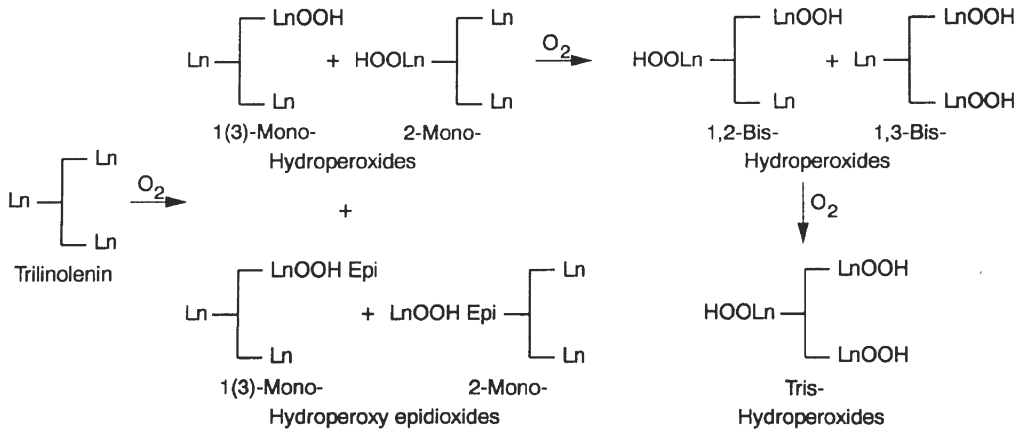


Figure 2.17. Mechanism of trilinolenin autoxidation (Ln = linolenate) (Frankel *et al.*, 1990).

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Four synthetic triacylglycerols containing linoleate (L) and linolenate (Ln) in specific 1,2 or 1,3-positions (LLLn, LLnL, LnLnL and LnLLn) were oxidized to determine the effect of fatty acid position on rates and products of autoxidation. The rates and yields of oxidation products decreased in the order: LnLnL > LnLLn > LLnL > LLLn (Figure 2.18). Therefore, triacylglycerols containing two Ln and one L were more easily oxidized when the Ln is in the 1,2- (2,3-) than the 1,3-triacylglycerol positions. On the other hand, triacylglycerols containing two L and one Ln were more easily oxidized when Ln is in the 2- than the 1,3-triacylglycerol position. Interactions in the 1,2-positions between either two Ln or one Ln and one L appear to increase the oxidative susceptibility of these triacylglycerols. This interaction in the 1,2-positions was supported by further studies with synthetic triacylglycerols containing linoleate and palmitate (P). Thus, the oxidation rates and yields of triacylglycerol hydroperoxides at 60°C were much greater for LLP than for LPL. In triacylglycerols containing two P and one L, PLP was more easily oxidized than PPL. Thus, when 1,2-interactions between L were eliminated, the triacylglycerols were more easily oxidized when L is in the 2- than in the 1,3-triacylglycerol position. This trend is the same as that observed above for the dilinoleoyl-monolinolenoyl glycerols (Figure 2.18).

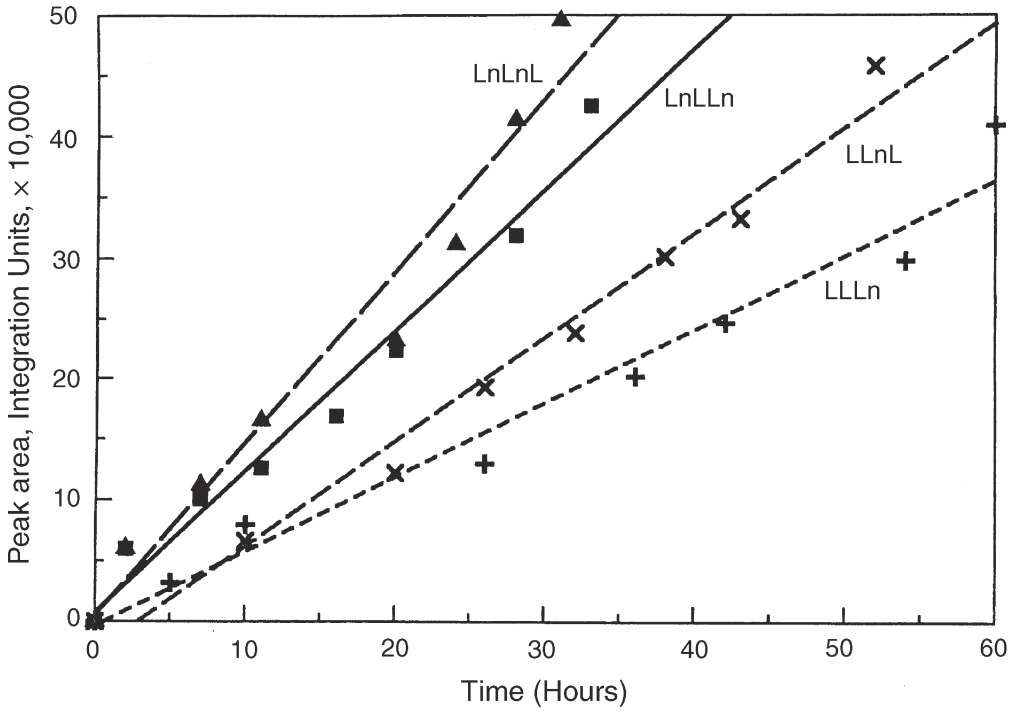


Figure 2.18. Oxidation of four synthetic triacylglycerols containing linoleate (L) and linolenate (Ln) in different specific positions at 40°C (Miyashita *et al.*, 1990). Formation of oxidation products was determined by HPLC as total peak area of hydroperoxides and hydroperoxy epidioxides. With permission of AOCS press.

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G. Autoxidation of phospholipids

The autoxidation of polyunsaturated phospholipids was shown, by reverse-phase HPLC of the hydroxylinoleate hydroperoxides, to follow a similar course as for the fatty esters. The liposome of oxidized dilinoleoyl-phosphatidylcholine produced two hydroxydienes derived from phosphatidylcholine containing one linoleate hydroperoxide, and a phosphatidylcholine containing two linoleate hydroperoxides. 1-Palmitoyl-2-linoleyl-phosphatidylcholine produced by air oxidation the four *cis,trans*- and *trans,trans*-9- and 13-linoleate hydroperoxides. 1-Stearoyl-2-arachidonyl-phosphatidylcholine forms conjugated diene hydroperoxides. In the presence of increasing proportions of saturated dipalmitoyl-lecithin, the oxidation of soybean lecithin produced increasing proportions of hydroxyepoxy fatty acids and their hydrolysis products, trihydroxy acids. Apparently, the saturated lecithin prevents intermolecular oxygen transfer. The yield of *trans,trans* hydroperoxides was much greater than that of the *cis,trans* isomers at all stages of oxidation of soybean phosphatidylcholine liposomes. Soybean phosphatidylcholine is a much poorer H donor than methyl linoleate in solution. The much slower oxidizability of egg lecithin initiated by di-*tert*-butyl hyponitrile in an aqueous liposome system (0.016 $M^{-½}s^{-½}$) than in chlorobenzene solution (0.61 $M^{-½}s^{-½}$) was attributed to the high microviscosity of the lecithin bilayer.

The direct separation of phospholipid hydroperoxides has now been achieved by further advances in HPLC and increased sensitivity by chemiluminescence detection. By reverse-phase HPLC, the hydroperoxides of phosphatidylcholine and phosphatidylethanolamine were separated and determined in stored spray-dried eggs (2.4–6.4%), fresh ground meat (0.2%), and raw fish (0.4%). Silica HPLC with post-column chemiluminescence detection has been used to determine phospholipid hydroperoxides in human blood plasma and in the low-density lipoprotein fraction.

H. Autoxidation of cholesterol

A voluminous literature has been published on a wide range of oxidation products of cholesterol because of their potential adverse effects on health and in compromising the safety of various food products (Chapters 12 and 13). This section is limited to the initial hydroperoxides serving as precursors to a complex mixture of oxygenated products, also referred to either as *oxysterols* or as *cholesterol oxides*. These products formed either enzymatically or non-enzymatically in biological and food systems will be discussed further in Chapters 11 to 13.

The hydroperoxides produced by oxygen attack at the allylic 7-position of cholesterol have been identified in low-density lipoprotein (LDL), oxidized *in vitro*, rat skin, marine animals and plant tissues. Cholesterol oxidation in solution or in aqueous dispersions is initiated by hydrogen abstraction to

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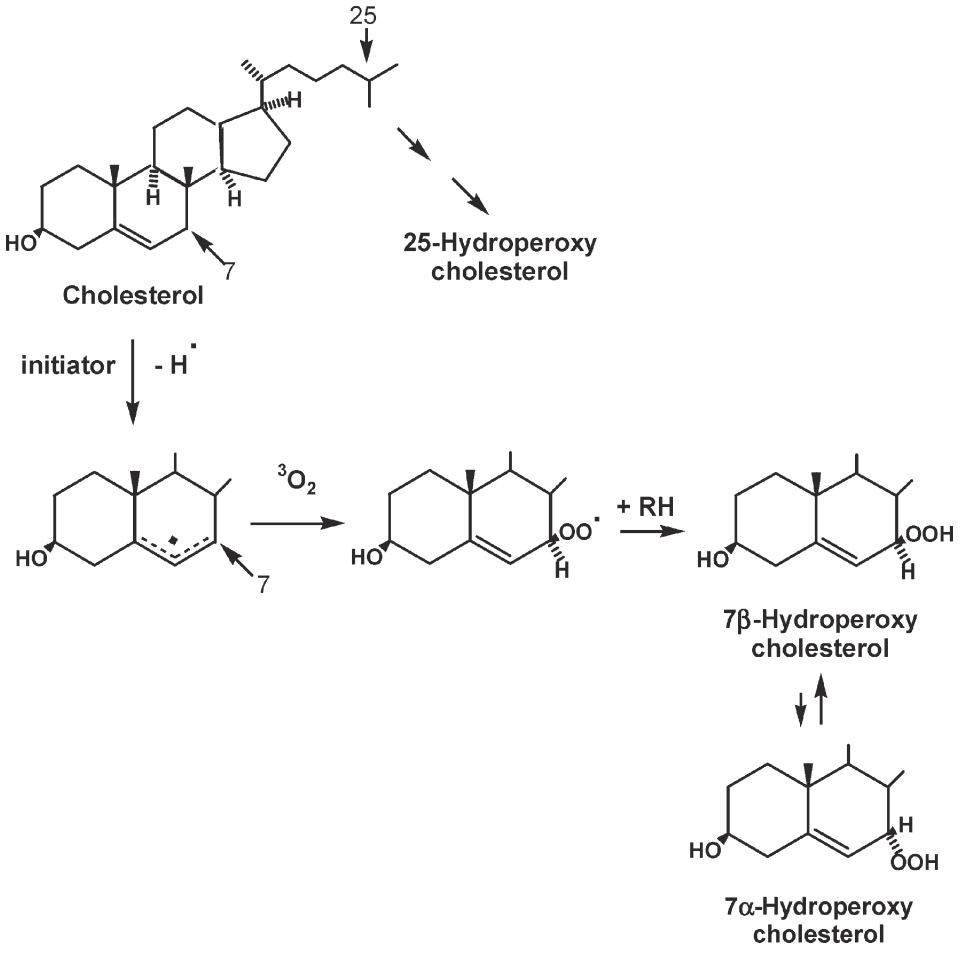


Figure 2.19. Products of cholesterol autoxidation ($^3O_2$) and singlet oxygenation ($^1O_2$).

produce a delocalized three carbon allylic radical followed by oxygen attack mainly at carbon-7 to produce the epimeric 7α- and 7β-hydroperoxycholesterol simultaneously, but after interconversion, the more thermodynamically stable 7β-hydroperoxide predominates (Figure 2.19). Oxygen attack at the allylic carbon-4 position is not favored, apparently because of steric hindrance by the trialkyl-substituted carbon-5 and perhaps the neighboring effect of the OH group on carbon-3. A unique hydroperoxide is formed on carbon 5 however by singlet oxygen-attack, and this mechanism will be discussed in the next chapter.

In the solid or crystalline phase, cholesterol side chain oxidation occurs at the tertiary carbon-25 position and yields the 25-hydroperoxycholesterol, which is readily degraded thermally into the corresponding 25-hydroxy-cholesterol. Two dihydroperoxides (3β-hydroxy-20*R*- and 20*S*-cholest-5-ene-20,25-dihydroperoxides) have also been isolated in autoxidized bulk cholesterol. The monohydroperoxides of cholesterol decompose into complex

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mixtures of hydroxy, keto, epoxyene and ketodiene secondary products that have received considerable attention because of their biological and potential adverse effects in certain foods (Chapters 11,13).

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CHAPTER 3

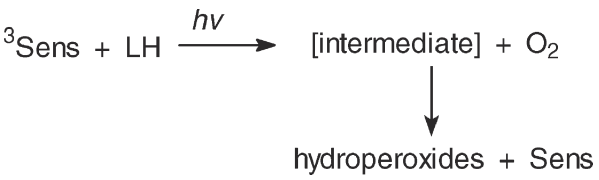
Photooxidation of unsaturated fats

The oxidation of unsaturated fats is accelerated by exposure to light. Direct photooxidation is due to free radicals produced by ultraviolet light irradiation, which catalyses the decomposition of hydroperoxides (ROOH) and other compounds such as peroxides (ROOR), carbonyl compounds (RCOR), or other oxygen complexes of unsaturated lipids. This type of oxidation proceeds by normal free radical chain reactions (Chapter 1) and can be inhibited by chain-breaking antioxidants, or by ultraviolet deactivators (e.g. *o*-hydroxybenzophenone) that absorb irradiation without formation of radicals. Direct photochemical oxidation of lipids is generally of little concern, because light absorption at less than 220 nm cannot reach lipids unless they are exposed to direct sunlight or fluorescent light without suitable protection by glass or opaque plastic containers. However, oxygen can become activated in the presence of metals or metal complexes and initiate oxidation either by formation of free radicals or singlet oxygen.

A. Photosensitized oxidation

Photooxidation provides an important way to produce hydroperoxides from unsaturated fatty acids and esters in the presence of oxygen, light energy and a photosensitizer. Pigments in foods can serve as photosensitizers by absorbing visible or near-UV light to become electronically excited. Sensitizers have two excited states; by absorption of light, the singlet (^{1}Sens) is converted to the triplet state (^{3}Sens), which has a longer life-time and initiates photosensitized oxidation. Pigments initiating photosensitized oxidation in foods include chlorophyll, hemeproteins, and riboflavin.

Two types of sensitizers are recognized for photosensitized oxidation. These sensitizers can proceed by two pathways. A Type I sensitizer serves as a photochemically activated free radical initiator. The sensitizer in the triplet state reacts with the lipid substrate by hydrogen atom or electron transfer to form radicals, which can react with oxygen (1). The hydroperoxides produced



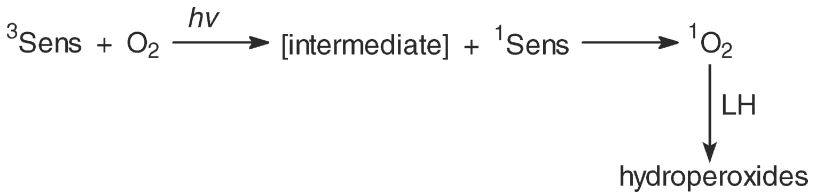
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from the conjugated lipid radical are the same as those from free radical autoxidation. Although free radicals in Type I reactions can initiate lipid oxidation chain reactions, in contrast to the free radical oxidation, this photosensitized reaction is not inhibited by chain-breaking antioxidants (Chapter 9).

Riboflavin reacts with unsaturated fatty acid esters by type I photosensitized oxidation to give the same isomeric hydroperoxides as free radical autoxidation.

The type II sensitizer in the triplet state interacts with oxygen by energy transfer to give nonradical singlet oxygen ($^1O_2$), a highly reactive species of molecular oxygen, which reacts further with unsaturated lipids (2). This type of photosensitized oxidation is also not inhibited by chain-breaking antioxidants.



(2)

Chlorophyll, methylene blue, protoporphyrins and erythrosine react with unsaturated fatty esters by type II photosensitized oxidation in which singlet oxygen produces hydroperoxides by an entirely different mechanism from free radical autoxidation. In marked contrast to autoxidation, the distribution of hydroperoxides produced from oleate, linoleate and linolenate in the presence of singlet oxygen is very different, and is discussed in Section B below.

Singlet oxygen can also be generated directly by non-photochemical processes, including the reaction of hydrogen peroxide and hypochlorous acid, by decomposition of triphenyl phosphite ozonides, and by radio-frequency gas discharge. Other sources of singlet oxygen may include the termination of peroxyl radicals via a tetroxide intermediate, known as the Russell mechanism (Chapter 1). The oxygen thus produced is activated in the singlet state. Another mechanism of singlet oxygen production is assumed in the decomposition of hydroperoxides by hemeproteins.

B. Hydroperoxide formation by singlet oxygen

Oxygen in the triplet state becomes activated by electronic excitation to the singlet state. Because singlet oxygen is highly electrophilic, it reacts very rapidly with unsaturated fatty acids by a different mechanism from free radical autoxidation. Singlet oxygen reacts with methyl linoleate at a rate 1500 times faster than normal triplet oxygen. Because of this high reactivity,the hydroperoxides formed by singlet oxygen may play an important part in the initiation of free radical oxidation.

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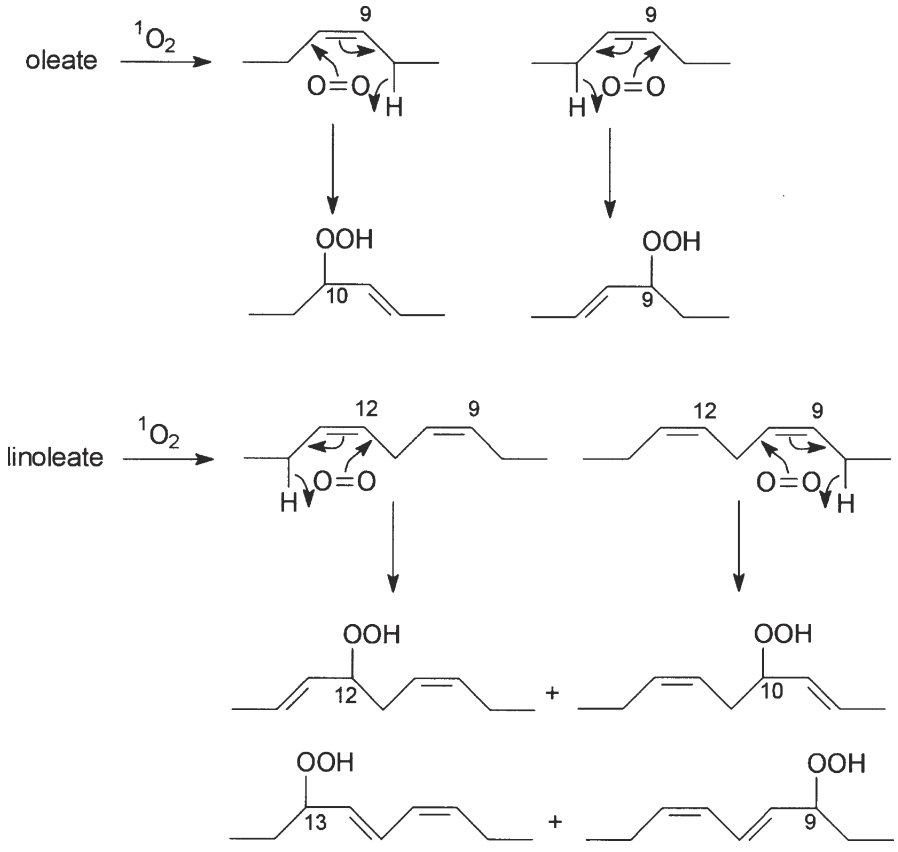


Figure 3.1. Oxidation of oleate and linoleate by singlet oxygen.

Singlet oxygen reacts directly with the double bonds of unsaturated fatty acids by a concerted "ene" addition mechanism. Accordingly, oxygen is inserted at either end carbon of a double bond, which is shifted to an allylic position in the *trans* configuration. The resulting hydroperoxides have an allylic *trans* double bond (Figure 3.1). In contrast to the free radical oxidation (Table 3.1), the relative rates of singlet oxidation increase in direct proportion to the number of double bonds, in ratios of 1: 1.7: 2.3 for 18:1, 18:2 and 18:3. According to this mechanism, the hydroperoxide distribution is different from that of free radical autoxidation (Chapter 2). Oleate produces a mixture of 9- and 10-hydroperoxides, linoleate a mixture of 9-, 10-, 12- and 13-hydroperoxides, and linolenate a mixture of 9-, 10-, 12-, 13-, 15- and 16-hydroperoxides. Oleate produces an equal mixture of 9- and 10-hydroperoxides as expected by the ene-addition mechanism (Table 3.1).

Linoleate treated with singlet oxygen produces a mixture of four hydroperoxide isomers:

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Table 3.1. Isomeric hydroperoxides in unsaturated fatty acid esters oxidized by singlet oxygen

Hydroperoxides	Me Oleate	Me Linoleate	Me Linolenate
trans-9-OOH	48–51		
trans-10-OOH	49–52		
trans,cis-9-OOH		28	
trans,cis-10-OOH		18	
cis,trans-12-OOH		21	
cis,trans-13-OOH		34	
trans,cis,cis-9-OOH			20–23
trans,cis,cis-10-OOH			13
cis,trans,cis-12-OOH			12–14
cis,trans,cis-13-OOH			14–15
cis,cis,trans-15-OOH			12–13
cis,cis,trans-16-OOH			25–26

Sources: Frankel *et al.* (1980,1982) and Neff *et al*. (1980).

9-hydroperoxy-*trans*-10-*cis*-12-octadecadienoate (*trans,cis*-9-OOH)
13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoate (*cis,trans*-13-OOH)
10-hydroperoxiy-*trans*-8-*cis*-12-octadecadienoate (*trans,cis*-10-OOH)
12-hydroperoxy-*cis*-9-*trans*-13-octadecadienoate (*cis,trans*-12-OOH)

The *trans,cis*-9-OOH and the *cis,trans*-13-OOH have conjugated double bonds and have the same structures as those formed by free radical autoxidation (Chapter 2). However, the *trans,cis*-10-OOH and *cis,trans*-12-OOH are not conjugated and are unique to singlet oxygenation. The external conjugated 9- and 13-hydroperoxides are formed in a ratio of 2 to 1 relative to the unconjugated internal 10- and 12-hydroperoxides (Table 3.1). The lower concentration of the internal 10- and 12-hydroperoxides is attributed to the tendency of their peroxyl radicals to cyclize readily into a mixture of hydroperoxy epidioxides (Figure 3.2). The internal 10- and 12-hydroperoxides have the same *cis* homoallylic structure (where the hydroperoxide and the double bond are separated by a methylene group) as the internal 12- and 13-hydroperoxides of linolenate, and undergo rapid 1,3-cyclization (Figure 2.10). Because cyclization of the pure hydroperoxides, prepared by photosensitized oxidation of linoleate, occurs rapidly in the dark at 0–5°C, this is evidently a free radical side reaction that is not photosensitized. The formation of hydroperoxy epidioxides is made possible as a consequence of the unique homoallylic structures of the 10- and 12-hydroperoxides of linoleate. In the presence of hydrogen donor antioxidants such as α-tocopherol, this free radical cyclization is inhibited and an even distribution is produced of the four 9-, 10-, 12- and 13-hydroperoxides.

Under conditions of photosensitized oxidation, the 9- and 13-hydroperoxides prepared by autoxidation of linoleate produced six-membered cyclic peroxides

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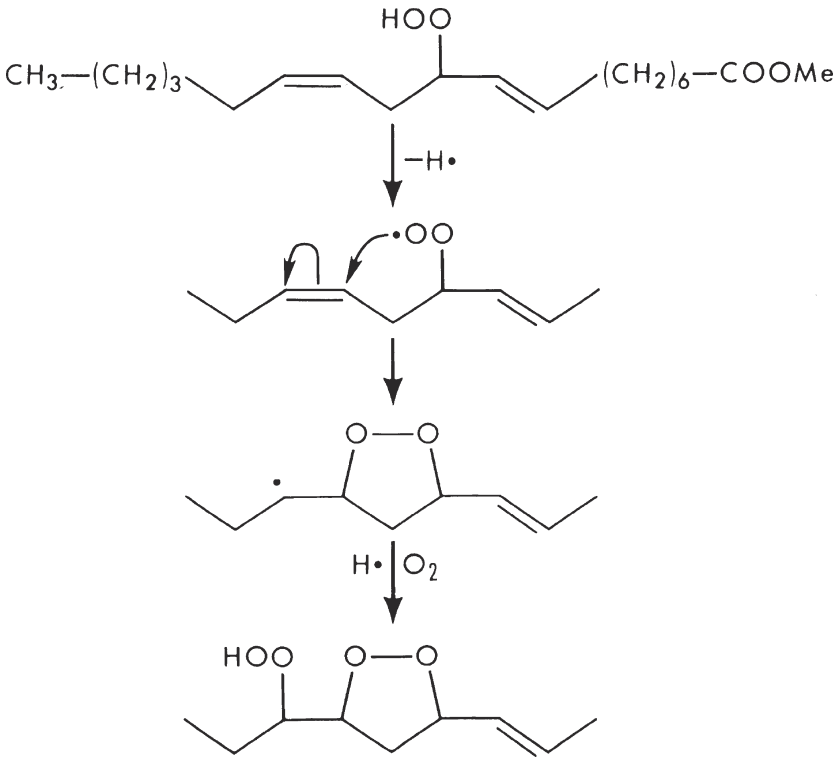


Figure 3.2. Cyclization of *trans*-8, *cis*-12-10-hydroperoxyoctadecadienoate produced by singlet oxidation of methyl linoleate (Frankel *et al.*, 1982).

by 1,4-addition of singlet oxygen to their conjugated diene systems (Figure 3.3). This reaction proceeds by photosensitized isomerization of the *cis,trans*-conjugated diene hydroperoxides to the *trans,trans* configuration, which can then assume a *cisoid conformation* that is required for 1,4-cycloaddition of singlet oxygen to the conjugated diene system of linoleate hydroperoxides.

Linolenate treated with singlet oxygen produces a mixture of six hydroperoxide isomers:

9-hydroperoxy-*trans*-10, *cis*-12, *cis*-15-octadecatrienoate (*trans,cis,cis*-9-OOH)
10-hydroperoxy-*trans*-8, *cis*-12, *cis*-15-octadecatrienoate (*trans,cis,cis*-10-OOH)
12-hydroperoxy-*cis*-9, *trans*-13, *cis*-15-octadecatrienoate (*cis,trans,cis*-12-OOH)
13-hydroperoxy-*cis*-9, *trans*-11,*cis*-15-octadecatrienoate (*cis,trans,cis*-13-OOH)
15-hydroperoxy-*cis*-9, *cis*-12, *trans*-16-octadecatrienoate (*cis,cis,trans*-15-OOH)
16-hydroperoxy-*cis*-9, *cis*-12, *trans*-14-octadecatrienoate (*cis,cis,trans*-16-OOH)

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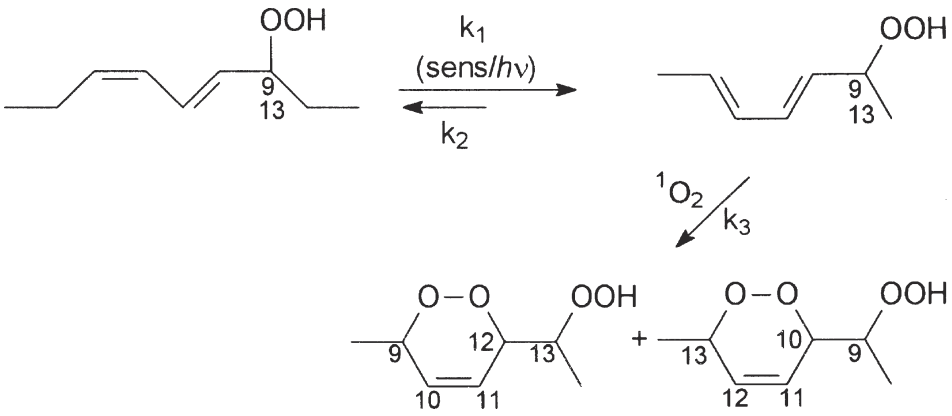


Figure 3.3. Formation of six-membered cyclic peroxides by photosensitized oxidation of 9- and 13-hydroperoxides of methyl linoleate (Neff *et al.*, 1983).

As with linolenate, the external 9- and 16-hydroperoxides are formed in significantly higher proportions than the internal 10-, 12-, 13- and 15-hydroperoxides (Table 3.1). The internal hydroperoxide isomers have *cis*-homoallylic structures that lead to 1,3-cyclization of their peroxyl radicals. The major hydroperoxy epidioxides formed are similar to those produced from the 12- and 13-hydroperoxyl radicals from autoxidized methyl linolenate, but have only one allylic double bond (Figure 2.10). The 10- and 15-hydroperoxyl radicals have homoallylic unsaturation and undergo serial cyclizations to produce a mixture of bis-epidioxides (Figure 3.4). The formation of hydroperoxy bis-epidioxides is a free radical cyclization that occurs as a rapid side reaction, only because of the unique homoallylic structures of the 10- and 15-hydroperoxides produced from linolenate treated with singlet oxygen. The photosensitized oxidation of methyl linolenate produces also small amounts of hydroperoxy bicyclo-endoperoxides (with the OOH group on either carbon-9 or carbon-16) (Figure 2.11), and isomeric mixtures of dihydroperoxides (9,12-, 10,12-, 13,15-, 13,16-, 10,16-, 9,15- and 9,16-) (Chapter 4, Figure 4.5).

Under conditions of photosensitized oxidation, the mixture of 9-, 12-, 13- and 16-hydroperoxides prepared by autoxidation of methyl linolenate produced cyclic peroxides and hydroperoxy bis-cyclic peroxides containing five- and six-membered rings. The six-membered rings are formed by 1,4-cycloaddition of singlet oxygen to the conjugated 1,3-diene systems of the terminal 9- and 16-hydroperoxides after *trans,trans* isomerization (Figure 3.5). The *trans,cis*-diene hydroperoxides undergo photosensitized isomerization to the *trans,trans* configuration which assumes a *cisoid* conformation as required

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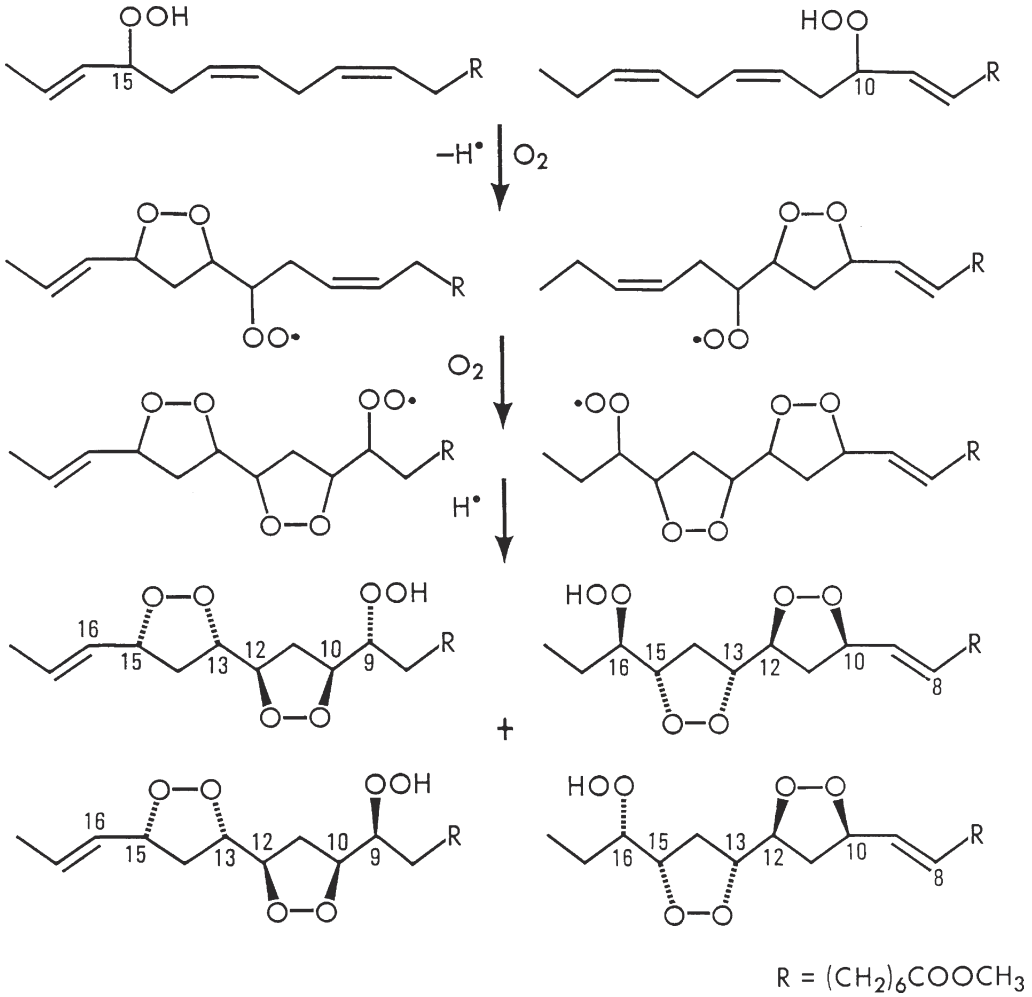


Figure 3.4. Serial cyclization of 10- and 15-hydroperoxides from photosensitized oxidized methyl linolenate (Neff *et al.*, 1982).

for cyclo-addition with singlet oxygen. The 1,4-cyclization of the 9- and 16-hydroperoxides is less favorable than the free radical cyclization of the 12- and 13-hydroperoxides of linolenate because the five-membered hydroperoxy mono-epidioxides are formed in higher proportion than the six-membered hydroperoxy mono-epidioxides. The hydroperoxy bis-epidioxides are formed by cyclo-addition of singlet oxygen to the conjugated diene systems of the hydroperoxy mono-epidioxides derived from the 12- and 13-hydroperoxides after *trans,trans* isomerization (Figure 3.6).

Photosensitized oxidation of long-chain PUFA produces the hydroperoxides expected by concerted addition of oxygen at each unsaturated carbon positions. Thus, methyl arachidonate produced a mixture of 8 isomers with hydroperoxide on carbons 5, 6, 8, 9, 11, 12, 14 and 15. Methyl 5,8,11,14,17-eicosapentaenoate (EPA) produced a mixture of 10 isomers with hydroperoxides on carbons 5, 6,

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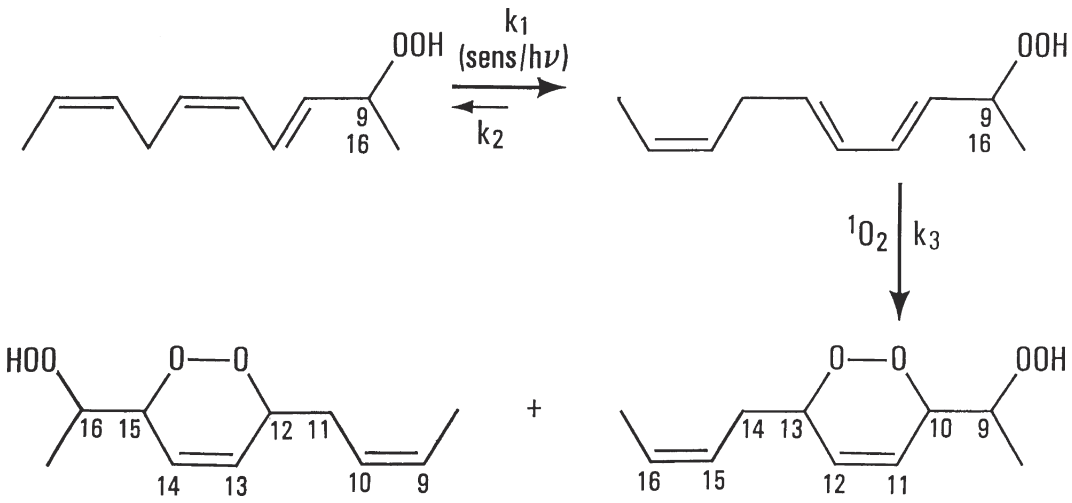


Figure 3.5. Formation of six-membered cyclic peroxides by photosensitized oxidation of 9- and 16-hydroperoxides of methyl linolenate (Neff and Frankel, 1984). With permission of AOCS Press.

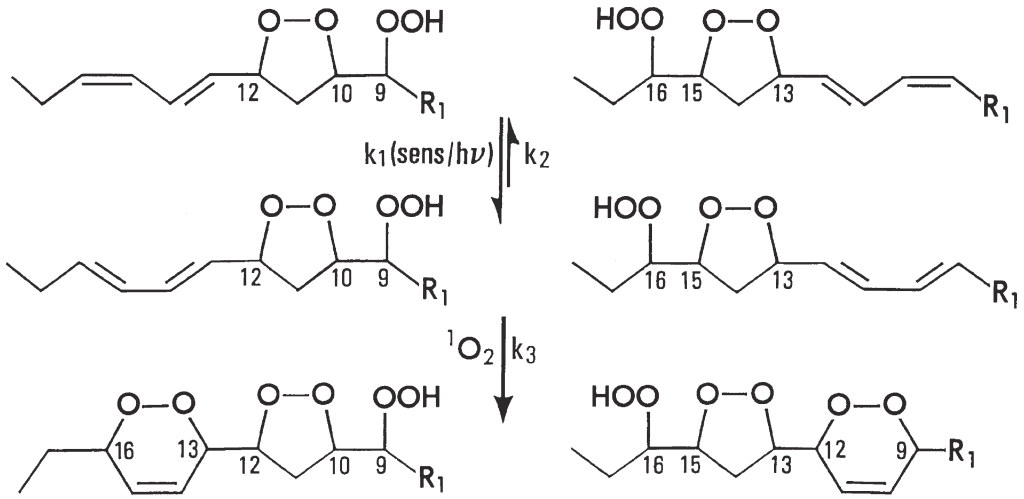


Figure 3.6. Formation of five- and six-membered cyclic peroxides by photosensitized oxidation of 9- and 16-hydroperoxy epidioxides of methyl linolenate (Neff and Frankel, 1984). With permission of AOCS Press.

8, 9, 11, 12, 14, 15, 17 and 18. However, no quantitative analyses of these isomers have been reported. Photosensitized oxidation of dilinoleoyl-phosphatidylcholine in methanol containing methylene blue produced the same isomeric mixture of monohydroperoxides as methyl linoleate. GC-MS analyses of the hydroxy octadecanoate derivatives gave a ratio of 9-OOH: 10-OOH: 12-OOH: 13-OOH of 34:16:16:34, indicating cyclization of the nonconjugated 10- and 12-OOH.

Photosensitized oxidation of cholesterol produces a unique hydroperoxide

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Table 3.2. Reactivity of cholesterol and fatty acid methyl esters towards singlet oxygen ($^1O_2$) [a]

Lipid	$^1O_2$ Reactivity	β values (m) [b]
Cholesterol	1.0	0.89
18:1	1.1	0.82
18:2	1.9	0.48
18:3	2.9	0.31
20:4	3.5	0.26

[a] From Doleiden *et al.* (1974). Photooxidation was carried out in pyridine with protophorphyrin IX as sensitizer. Reactivity was based on amount of lipids consumed measured by gas chromatography at nearly equal extent of reaction.
[b] β = rate of decay of $^1O_2$/bimolecular rate constant for reaction of lipid with $^1O_2$.

by singlet oxygen attack mainly on carbon-5 to form 3β-hydroxy-5α-cholest-6-ene 5-hydroperoxide. This hydroperoxide is accompanied by small amounts of the epimeric 3β-hydroxy-4-ene 6-hydroperoxide (1–2%) but with no detectable cholesterol 7α-hydroperoxide or 7β-hydroperoxide. This oxidation is in marked contrast to free radical oxidation, producing mainly the 7α- and 7β-hydroperoxides (Chapter 2, H, Figure 2.19). In cholesterol, the concerted "ene" addition of singlet oxygen becomes specific for the more highly congested side of the double bond and sterically hindered carbon-5. This difference in hydroperoxide formation in cholesterol oxidation has been suggested as a probe to distinguish between the free radical and singlet oxidation mechanisms. The relative reactivity of cholesterol and fatty acid methyl esters toward singlet oxygen showed a linear relationship with the number of doubly allylic hydrogen atoms (Table 3.2). The rate constant for the reaction of singlet oxygen as a function of the number of doubly allylic hydrogen atoms was 1 for cholesterol and 18:1, 2 for 18:2, 3 for 18:3 and 3.5 for 20:4.

C. Inhibition of photosensitized oxidation

Plants containing chlorophyll are well protected by carotenoids against the atmospheric damage of photosensitized oxidation. The same mechanism of protection is generally invoked for the inhibition of photosensitized lipid oxidation by carotenoids. Carotenoids are the most important inhibitors of type II photosensitized oxidation. They protect unsaturated lipids against photosensitized oxidation by interfering with the activation of triplet oxygen into singlet oxygen. This quenching effect takes place by an energy transfer mechanism from singlet oxygen to carotene. By a similar energy transfer mechanism, carotenoids also react with the triplet state of the excited sensitizers (Figure 3.7). α-Tocopherol is highly reactive toward singlet oxygen and inhibits photosensitized oxidation by quenching singlet oxygen and by forming stable addition products.

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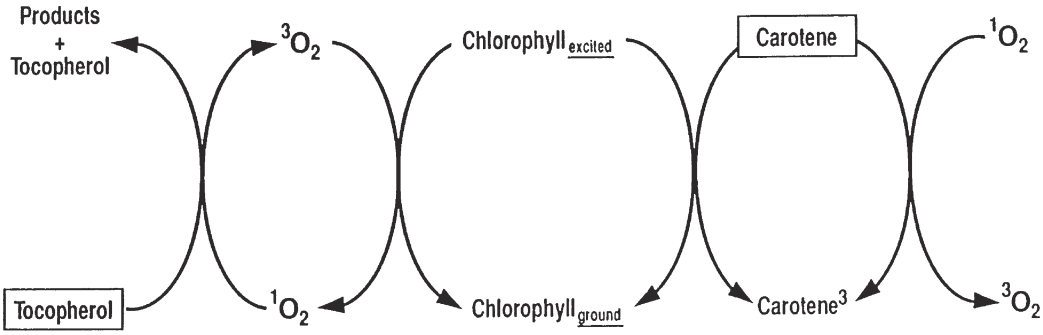


Figure 3.7. Inhibition of photosensitized oxidation by carotene and tocopherol.

The *chemical quenching* by the reaction with singlet oxygen to produce stable products can be distinguished from *physical quenching* without undergoing chemical changes. The rate of physical quenching by β-carotene approaches diffusion rate (1.5×10^{10} $M^{-1}sec^{-1}$) and is much greater than that for α-tocopherol (Table 3.3). On the other hand, the rate of chemical quenching is much greater for α-tocopherol than that for β-carotene. γ-Tocopherol and δ-tocopherol, present in soybean oil at much greater concentrations than α-tocopherol (Chapter 9), are less efficient singlet oxygen quenchers than α-tocopherol. Other carotenoids such as lycopene and lutein (Figure 3.8) also inhibit photosensitized oxidation of lipids with lycopene being the most potent inhibitor. However, their relative inhibitory activity in the oxidation of methyl linoleate photosensitized with methylene blue varied with the concentration used (Table 3.4). Although no difference was observed between the inhibitory effects of the three carotenoids at 10 ppm, β-carotene and lycopene were more active than lutein at 20 ppm, while lycopene was more active at 40 ppm. Lycopene was also more oxidatively stable at 40 ppm. The three carotenoids had no effect on the distribution of isomeric hydroperoxides. Their inhibition mechanism therefore does not involve hydrogen donation like α-tocopherol.

The inhibition of photosensitized oxidation by β-carotene is complicated because it is highly susceptible to autoxidation and is quickly destroyed in the presence of free radicals or hydroperoxides. To be effective in unsaturated

Table 3.3. Rates of quenching of singlet oxygen by tocopherols and β-carotene (in M^{-1} sec^{-1})

Type of quenching	α-Tocopherol	γ-Tocopherol	δ-Tocopherol	β-Carotene
Physical	2.6×10^8	1.8×10^8	1.0×10^8	1.5×10^{10}
Chemical	6.6×10^6	2.6×10^6	0.7×10^6	0.8×10^2

Source: Matsushita and Terao (1980).

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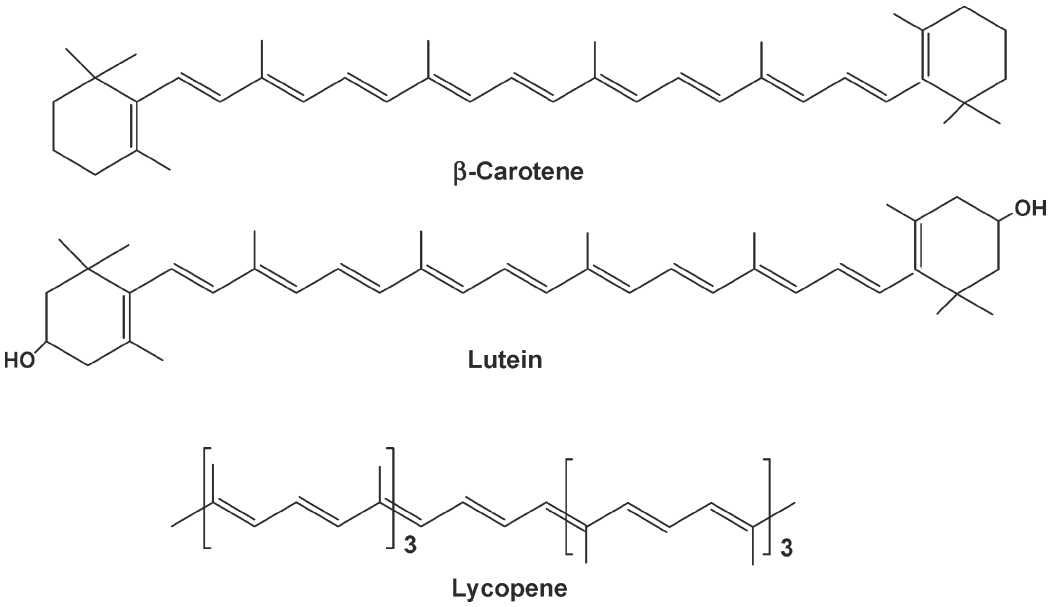


Figure 3.8. Structures of carotenoids.

lipids exposed to light irradiation, β-carotene must be protected by an antioxidant. The effectiveness of β-carotene as an inhibitor of photosensitized oxidation in unsaturated lipids (and most likely in biological systems) depends largely on its protection against oxidation by natural tocopherols, which also can reinforce the activity of β-carotene. The antioxidant activity of β-carotene is greatly influenced by the type of initiators and interactions with other antioxidants. Apparently without proper protection from vitamin E, vitamin C or other chain-breaking antioxidants, β-carotene and other carotenoids are rapidly oxidized and at high concentrations can promote rather than retard lipid oxidation. This prooxidant activity could be due to the oxidation products of β-carotene acting as free radical promoters of lipid oxidation.

Table 3.4. Inhibition of photosensitized oxidation of methyl linoleate by different carotenoids [a]

Carotenoids [b]	Inhibition (%)		
	10 ppm	20 ppm	40 ppm
β-Carotene	23.9	38.4	59.6
Lutein	22.6	32.6	55.7
Lycopene	25.7	38.6	68.0

[a] From Viljanen *et al.* (2002). Methyl linoleate in methanol solution was photooxidized in the presence of methylene blue at 3°C for 30 min. Hydroperoxide formation was measured by normal-phase HPLC and post-column detection with diphenyl-1-pyrenyl phosphine (Chapter 6).
[b] See structures in Figure 3.8.

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Table 3.5. Effect of β-carotene on flavor and oxidative stability of light-exposed soybean oil

β-carotene (ppm)	Flavor score 8 hr	Flavor score 16 hr	Peroxide value 8 hr	Peroxide value 16 hr
No citric acid				
0	5.0	4.3	1.3	3.0
1	5.2	4.2	1.0	2.0
5	5.5	4.5	0.6	1.8
10	5.7	4.3	1.0	2.2
15	5.5	4.7	1.1	1.4
20	6.5	6.0	1.1	2.1
+100 ppm citric acid				
0	5.4	5.4	2.2	3.5
1	6.2	5.9	0.9	3.3
5	6.8	6.3	0.4	2.9
10	6.7	6.3	1.1	0.7
15	6.2	6.4	0.9	1.3
20	6.2	6.4	1.0	2.3

Source: Warner and Frankel (1987); samples exposed to fluorescent lighting of 7535 lux; flavor scores are based on a 1–10 intensity scale, with 10 = bland and 1 = strong; peroxide values in meq kg.

In soybean oil containing natural tocopherols, β-carotene was effective in protecting against light oxidation (Table 3.5). With no citric acid, 20 ppm of β-carotene improved the flavor and oxidative stability of soybean oil; with citric acid, 5–10 ppm of β-carotene was sufficient to show an improvement. On the other hand, in distilled soybean methyl esters containing no natural tocopherols, a larger amount of β-carotene (1000 ppm) was necessary to show a protective effect against light oxidation (Figure 3.9). α-Tocopherol and β-carotene were effective singlet oxygen quenchers, but α-tocopherol was more effective in protecting soybean esters against light oxidation. Because β-carotene was rapidly bleached, its effect was confounded by its depletion during oxidation. When soybean oil was stored in the dark, β-carotene at high concentrations promoted autoxidation, apparently due to its oxidation products.

In soybean oil and rapeseed oils chromatographed to remove the natural tocopherols, β-carotene by itself acted as a prooxidant by promoting oxidation. However, β-carotene effectively inhibited light oxidation in the presence of added δ- or γ-tocopherols. The mixture of β-carotene and γ-tocopherol was more effective than γ-tocopherol alone in inhibiting light oxidation of chromatographed rapeseed oil. Therefore, β-carotene is an effective inhibitor of photosensitized oxidation in vegetable oils only when it is protected against oxidation or reinforced by natural tocopherols.

In addition to the singlet oxygen quenching property of carotenoids, other

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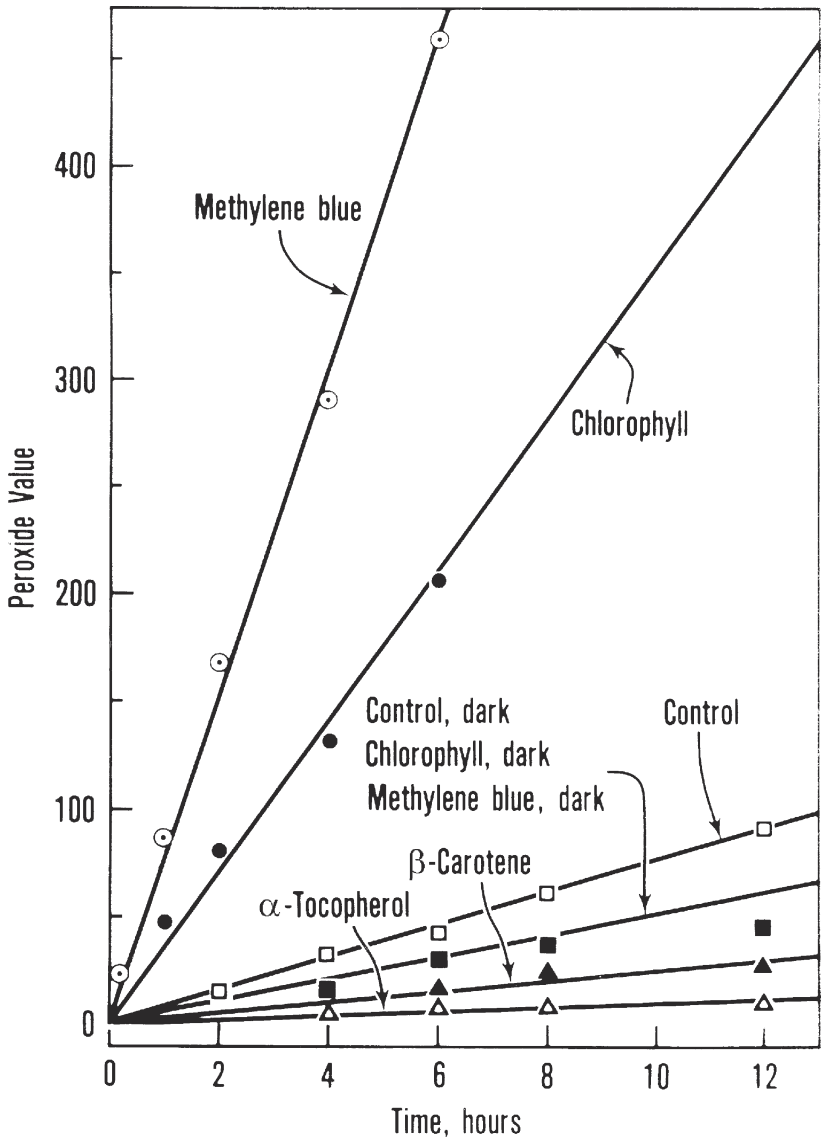


Figure 3.9. Effect of methylene blue and chlorophyll as photosensitizers, and β-carotene and α-tocopherol as singlet oxygen quenchers, on the photooxidation of distilled soybean esters at 35ºC (Frankel *et al*., 1979).

mechanisms have been invoked for their inhibition of photosensitized oxidation. Carotenoids may act either as built-in filters for light of short wavelengths, or by being preferentially oxidized, they may exert a sparing effect until they are completely oxidized. There is good evidence, however, that the main effect of carotenoids is their singlet oxygen quenching, because the rate of this quenching is a function of the number of conjugated double bonds in their chain. Carotenoids with nine or more double bonds are much more efficient than those with seven or less double bonds. The carotenoids are most effective in inhibiting photosensitized oxidation in this range of polyunsaturation.

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Table 3.6. Inhibition of isomeric hydroperoxides by β-carotene and α-tocopherol in dilinoleoyl phosphatidylcholine photooxidized in the presence of methylene blue [a]

Inhibitors	Concentration (mol%)	Inhibition (%) [b]	
		9-OOH	10-OOH
β-Carotene	0.45	89.5	91.0
α-Tocopherol	0.45	11.2	15.7
α-Tocopherol	4.5	94.4	97.2
β-Carotene + α-tocopherol	0.45	64.5	64.0

[a] From Stratton and Liebler (1997). Initial inhibition calculated from reported initial rate data for 0–75 min.
[b] The relative concentrations of 9-OOH and 10-OOH were assumed to be equal and to correspond to the relative concentrations of 13-OOH and 12-OOH.

Cold pressed virgin olive oil and unprocessed vegetable oils obtain their color from natural carotenoids and chlorophylls. However, carotenoids are completely removed by bleaching and deodorization during processing of vegetable oils. Therefore, for protection against light oxidation β-carotene must be added after deodorization in oils containing natural tocopherols, at concentrations below 20 ppm provided the resulting color is not objectionable to the consumer.

The oxidation of a liposome (see Chapter 10) prepared with 1,2-dilinoleoyl-glycero-3-phosphocholine sensitized with methylene blue was studied by measuring the ratio of 9- and 10-hydroxy linoleate to distinguish between the radical-mediated Type I and singlet oxygen-mediated Type II pathways (Table 3.6). At equal molar concentrations (0.45 mol%), β-carotene was more efficient in inhibiting both 9-OOH and 10-OOH than α-tocopherol, because the latter was more readily decomposed. At higher concentrations (4.5 mol%) however, α-tocopherol was more effective in inhibiting the 10-OOH than the 9-OOH. The presence of both β-carotene and α-tocopherol (0.45 mol%) inhibited 64–65% both 9- and 10-OOH. Although the formation of equal amounts of conjugated 9-OOH and nonconjugated 10-OOH was assumed, β-carotene and α-tocopherol were considered to inhibit both the nonspecific Type I and the singlet oxygen-mediated Type II photooxidation. However, this assumption may be invalidated if the nonconjugated 10-OOH and 12-OOH undergo cyclization in the phospholipid liposome as well established in solution (Figure 3.2). If cyclization of the 10-OOH (and 12-OOH) occurred in this liposome system, it would be inhibited by α-tocopherol acting as a good hydrogen donor but not by β-carotene (Section B).

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CHAPTER 4

Hydroperoxide decomposition

A complex mixture of monomeric, polymeric and small molecular weight volatile materials is produced when lipid oxidation is carried out to higher levels than 5–10%, especially with polyunsaturated fats containing more than two double bonds. Complicated pathways are recognized for hydroperoxide decomposition. However, the extensive literature on the nature of secondary reactions must be interpreted with care, because the conditions used in many studies are either too drastic or artificial and not relevant to real food lipid applications. In complex foods, the interaction of lipid hydroperoxides and secondary oxidation products with proteins and other components has a significant impact on oxidative and flavor stability and on texture during processing, cooking and storage. The volatile decomposition products contribute to the flavor and may affect the safety of lipid-containing foods by causing damage to proteins and enzymes.

A. Monomeric products

On prolonged oxidation oleate, linoleate and linolenate produce a multitude of secondary oxidation products.

1. Oleate

Methyl oleate produces small amounts of allylic keto-oleates (with CO on carbons 8-, 9-, 10- and 11), epoxy-stearate or epoxy-oleates (8,9-, 9,10- and 10,11-epoxy), dihydroxy-oleates (8,9-, 9,10-, and 10,11-diOH) and dihydroxy-stearates (between carbon-9 and carbon-11). The allylic keto-oleates may be derived by dehydration of the corresponding hydroperoxides. 9,10-Epoxy-stearate may be produced by the reaction of oleate and the hydroperoxides. The other epoxy products can be formed by cyclization of an alkoxy radical formed from the corresponding hydroperoxides of oleate (Figure 4.1). Accordingly, the 11-hydroperoxide forms the 10,11-epoxy ester, the 8-hydroperoxide forms the 8,9-epoxy ester, and the 9- and 10-hydroperoxides form the 9,10-epoxy ester. The 1,2- and 1,4-dihydroxy esters may be formed from a similar alkoxyl radical that undergoes hydroxyl and hydrogen radical substitution via an allylic hydroxy ester radical (Figure 4.1).

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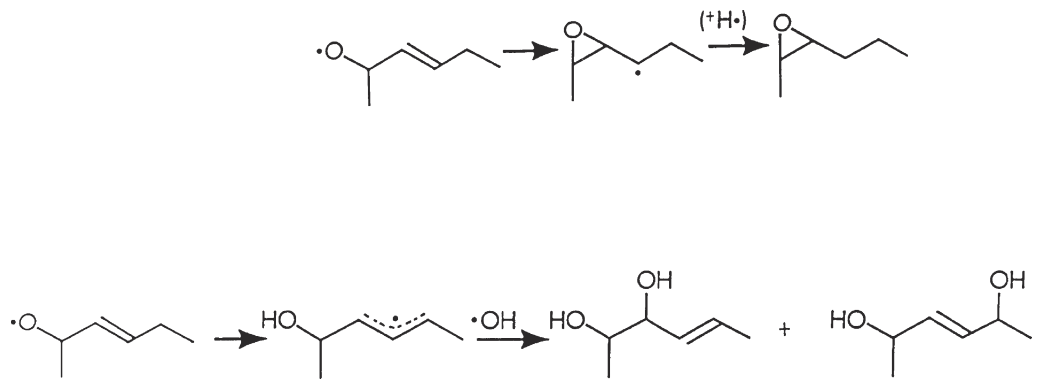


Figure 4.1. Formation of epoxystearate and dihydroxy oleate from oleate hydroperoxides.

2. *Linoleate*

A large number of compounds have been identified from autoxidized methyl linoleate and further oxidation of linoleate hydroperoxides, including keto-linoleate (with CO on carbon-9 and carbon-13), epoxyhydroxy-oleate (9,10-12,13-epoxy-11-hydroxy and 9,13-hydroxy-12,13-/9,10-epoxy), 9,13-dihydroxy, and trihydroxy (9,10,13- and 9,12,13-) stearate. The epoxy allylic hydroxy, hydroperoxy and keto products can be formed by cyclization of an alkoxy radical formed from the corresponding hydroperoxides of linoleate (Figure 4.2). The availability of the almost pure 13-hydroperoxide isomer produced from linoleic acid by soybean lipoxygenase (Chapter 11) has made it possible to clarify the mechanism of decomposition of linoleate hydroperoxides under different conditions. Decomposition of the 13-hydroperoxide isomer of linoleic acid in the presence of various free radical initiators or decomposers (e.g. hemoglobin, iron compounds, di-alkyl peroxy oxalate) produces 13-keto and 13-hydroxy dienes and mixtures of 11-hydroxy-12,13-epoxy-9-oleate and 9-hydroxy-12,13-epoxy-10-oleate isomers. The same hydroxy epoxy compounds are produced from autoxidized linoleic acid containing a mixture of 9- and 13-hydroperoxide isomers. Other polyoxygenated compounds formed by thermal decomposition of linoleate hydroperoxides include di- and trihydroxy esters, which are apparently formed from the corresponding dihydroperoxides, keto-hydroperoxides and hydroxy-hydroperoxides.

3. *Linolenate*

The formation of hydroperoxy epidioxides in significant yields during autoxidation of methyl linolenate was discussed previously (Chapter 2, Figure 2.10). Autoxidation studies with pure *cis,trans*-9-linolenate hydroperoxide (prepared enzymatically) led to the formation of a mixture of 16% 16-hydroperoxy

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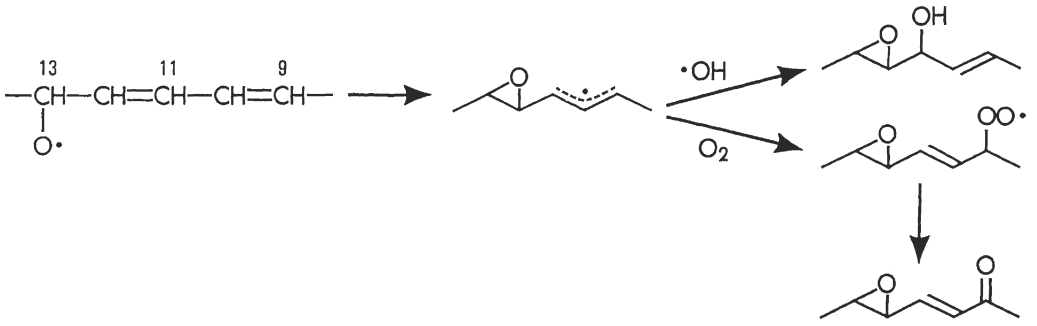


Figure 4.2. Formation of epoxy allylic hydroxy, epoxy allylic hydroperoxy and epoxy allylic keto oleate from linoleate hydroperoxides.

epidioxide and 7% 9-hydroperoxy epidioxide, expected from the scheme in Figure 2.10, while the pure *cis,trans*-13-linolenate hydroperoxide produced 7.5% 16-hydroperoxy epidioxide, accompanied by 1% of the 9-hydroperoxy epidioxide. These results were explained (Figure 4.3) by rearrangement of the pentadienyl radicals of linolenate (Figure 2.9), leading to the 12- and 13-hydroperoxy radicals that undergo cyclization (Figure 2.10). It is however important to distinguish between oxidation studies in which the substrate linolenate is present in large excess and studies with pure hydroperoxides. On the one hand, during autoxidation of linolenate the rapid cyclization of the 12- and 13-hydroperoxyl radicals is greatly favored, and the presence of a large amount of unreacted linolenate prevents the isomerization and rearrangement reactions. On the other hand, when pure linolenate hydroperoxides are oxidized, cyclization can only occur by isomerization of the 9- and 16-hydroperoxides to the 12- and 13-hydroperoxides. Under these conditions, dihydroperoxides are also produced in competition with cyclization (Figure 4.3). In the presence of α-tocopherol as hydrogen donor, 9,16-dihydroperoxides are formed selectively.

The peroxyl radicals leading to hydroperoxy epidioxides can undergo a second cyclization to produce bicyclo-endoperoxides (Figure 2.11). These cyclic peroxides are precursors of malonaldehyde, a volatile decomposition product characteristic of oxidation of polyunsaturated lipids containing three or more double bonds (Chapter 2). Many precursors of malonaldehyde have been suggested to explain what is measured by the thiobarbituric acid (TBA) test, a popular method to measure rancidity in polyunsaturated lipids (Chapter 5). The cleavage of monocyclic peroxides of linolenate and arachidonate on each side of the endoperoxide ring to form malonaldehyde was proposed as the main mechanism for the formation of the red-colored TBA-malonaldehyde adduct (Figure 4.4). The formation of pure malonaldehyde was tested by a GC procedure measuring the tetramethyl acetal derivative, which is stable under the GC conditions. Several purified oxidation products of methyl linoleate and linolenate subjected to either free radical autoxidation or photosensitized

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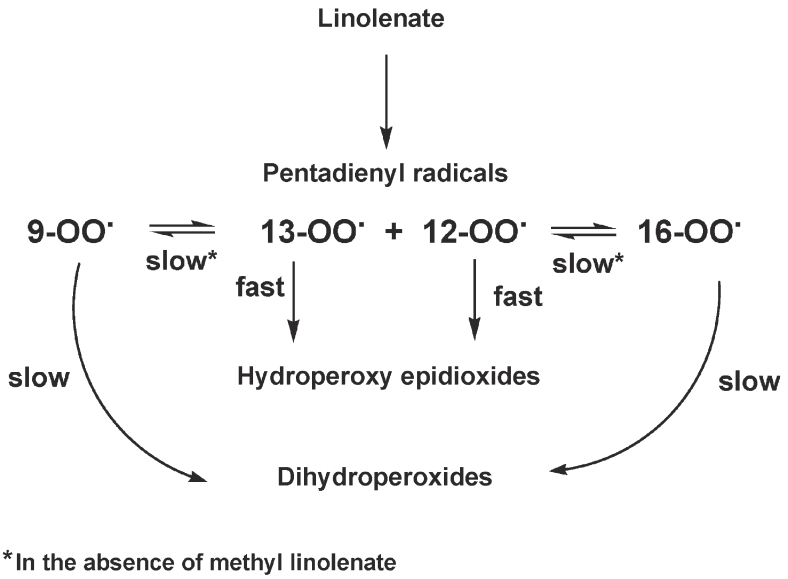


Figure 4.3. Secondary oxidation of methyl linolenate. *In the absence of methyl linolenate, oxidation of pure 9-OOH and 13-OOH produced mixtures of hydroperoxy epidioxides by rearrangement of the linolenate hydroperoxyl radicals (Coxon *et al.*, 1981). See text.

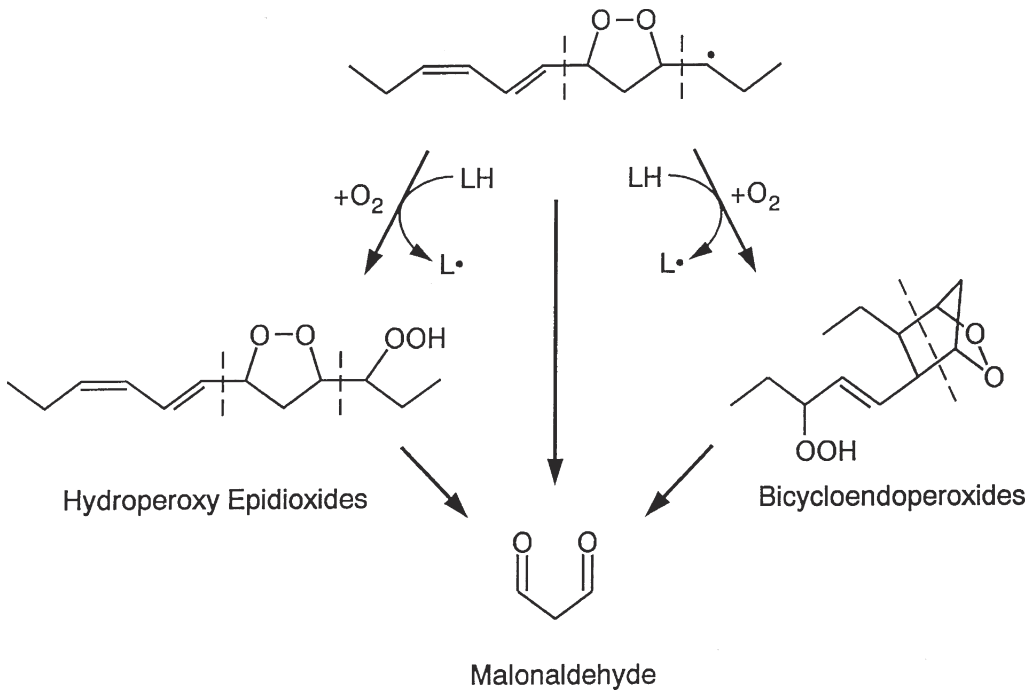


Figure 4.4. Formation of malonaldehyde from hydroperoxy epidioxides and bicyclo-endoperoxides of methyl linolenate (Pryor *et al.*, 1976).

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Table 4.1. Lipid oxidation precursors of malonaldehyde

Oxidation products	Malonaldehyde (MDA), mol %	TBARS as MDA, mol %
Monohydroperoxides		
Linoleate, $^3O_2$	0.18	0.53
Linoleate, $^1O_2$	0.26	0.18
Linolenate, $^3O_2$	0.22	1.85
Linolenate, $^1O_2$	0.44	1.41
Hydroperoxy epidioxides		
Linoleate, $^1O_2$	24.2	1.64
Linolenate, $^3O_2$	2.34	4.02
Linolenate, $^1O_2$	2.20	2.49
Hydroperoxy bis-epidioxides[a]	5.65	10.5
Hydroperoxy bicycloendoperoxides[a]	4.14	3.41
10,12-Dihydroperoxides[a]	4.76	3.77
9,12- & 13,16-Dihydroperoxides[a]	0.81	6.29
9,16-Dihydroperoxides[a]	0.0	7.63
10,16-Dihydroperoxides[a]	0.0	2.74

From Frankel and Neff (1983). $^3O_2$: produced by autoxidation; $^1O_2$: produced by oxidation with singlet oxygen.
[a] Products of oxidized methyl linolenate

oxidation were tested as precursors of malonaldehyde under the acidic methanol conditions used for acetalation (Table 4.1). Under these acidic decomposition conditions, the hydroperoxy epidioxides of linoleate were the most significant precursors of malonaldehyde, followed by the hydroperoxy bis-epidioxides of linolenate, 1,3-dihydroperoxides of linolenate (see below), hydroperoxy bicycloendoperoxides of linolenate, and the hydroperoxy epidioxides of linolenate. The monohydroperoxides of linoleate and linolenate were not significant sources of malonaldehyde. Completely different trends of malonaldehyde formation were observed by the TBA test.

A mixture of 9,12-, 9,16- and 13,16-dihydroperoxides is formed in smaller yields than the cyclic peroxides by secondary oxidation of the pentadienyl systems of the 9- and 16-hydroperoxides of methyl linolenate. The 12,15-pentadienyl radical produced by hydrogen abstraction on carbon-14 of the 9-hydroperoxide reacts with oxygen at the end carbon positions to form the 9,12- and 9,16-dihydroperoxides (Figure 4.5). The corresponding pentadienyl radical produced by hydrogen abstraction on carbon-11 of the 16-hydroperoxide of linolenate reacts with oxygen to form the 9,16- and 13,16-dihydroperoxides. The 9,12- and 13,16-dihydroperoxides have a *cis,trans* conjugated diene system, while the 9,16-dihydroperoxide has a *trans,cis,trans* conjugated triene system. In the presence of 10% α-tocopherol, the 9,16-dihydroperoxide is produced selectively from methyl linolenate. As discussed previously (Chapter 2), the hydrogen donating ability of α-tocopherol inhibits the cyclization

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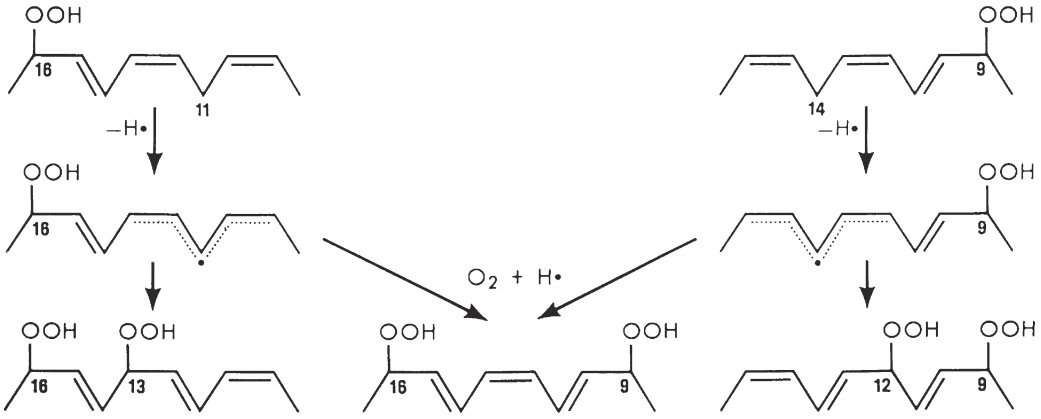


Figure 4.5. Formation of dihydroperoxides from autoxidized methyl linolenate (Neff *et al.*, 1981).

reaction, thereby promoting the formation of dihydroperoxides. Minor secondary oxidation products of methyl linolenate include epoxy-hydroxydienes and epoxy-hydroperoxydienes.

B. Oligomeric products of methyl linoleate and methyl linolenate

Dimeric compounds are formed during the autoxidation of methyl linoleate and linoleate hydroperoxides under ambient conditions. Dimers formed from methyl linoleate hydroperoxides are composed of unsaturated fatty esters cross-linked through either peroxide or ether linkages and containing hydroperoxy, hydroxy or oxo substituents (Figure 4.6).

Peroxy-linked dimers are also formed from linoleate hydroperoxides in the presence of free radical initiators and copper palmitate, and carbon–carbon linked dimers in the presence of copper catalysts. Decomposition of methyl linoleate hydroperoxides at 210°C under nitrogen produces mainly carbon–carbon linked dimers (82%), monomers with loss of diene conjugation, volatile compounds (4–5%) and water. The resulting dimers contain carbonyl and hydroxyl groups and double bonds scattered between carbon 8 and carbon 10. Linoleate hydroperoxides can dimerize by one of the termination reactions discussed in Chapter 1. The termination reactions involving combination of alkyl, alkoxyl, or peroxyl radical intermediates produce dimers with carbon–carbon, carbon ether, or peroxy links. The carbon–carbon and carbon–oxygen linked dimers are favored at elevated temperatures and the peroxy-linked dimers at ambient temperatures. The peroxy-linked dimers may also decompose to the ether-linked and carbon–carbon linked dimers via the corresponding alkyl and alkoxyl radical intermediates.

Dimers and oligomers are significant secondary products occurring in methyl linolenate highly oxidized under mild conditions (25–40ºC). These secondary products include conjugated diene-triene, dihydroperoxides or

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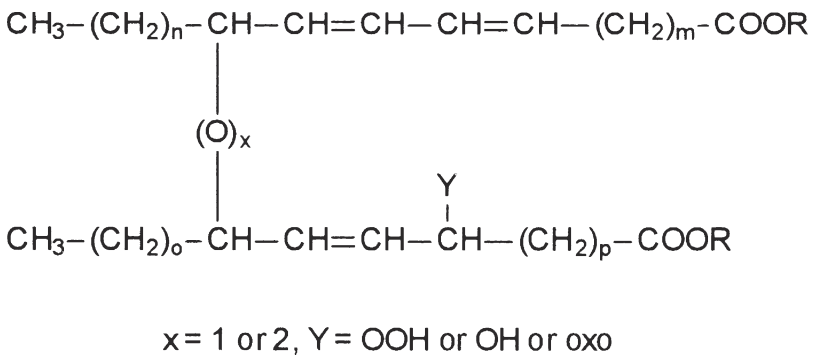


Figure 4.6. Dimers from methyl linoleate hydroperoxides.

hydroperoxy epidioxide units cross-linked with peroxide groups (Figure 4.7). Dimers are produced in yields of 6–8% from methyl linolenate autoxidized at 40ºC. These dimers are derived from hydroperoxides (80%) and from hydroperoxy epidioxides (20%), and are 90% peroxide-linked and 10% ether- and/or carbon-linked. Oligomers are the principal products formed by decomposition of pure hydroperoxides of methyl linolenate in the presence of ferric chloride and ascorbic acid (73%, molecular weight of 625), and thermally at 150°C (62%, molecular weight of 834). These oligomers contain monomeric units linked by either ether or carbon-carbon bonds (57%) or by peroxide bonds (43%). The peroxide-linked dimers can be easily cleaved thermally and produce volatile compounds contributing to flavor deterioration of linolenate-containing oils (Section D).

C. Oligomeric products of triacylglycerols

In contrast to methyl linoleate and its hydroperoxides, which tend to dimerize readily under ambient conditions, highly oxidized trilinoleoylglycerol or the monohydroperoxides from trilinoleoylglycerol (Chapter 2) do not dimerize under the same conditions. Dimerization at ambient conditions is evidently significant only in the methyl esters of unsaturated fatty acids, because intermolecular condensations of peroxyl radicals are favored. On the other hand, further oxidation of the monohydroperoxides of trilinoleoylglycerol to bis- and tris-hydroperoxides appears to be the preferred reaction (Chapter 2, Section F). Intramolecular hydrogen abstraction from the linoleoyl residues can evidently occur more favorably than intermolecular condensation of the peroxyl radicals to form dimers. There is also no evidence for dimerization of tris-hydroperoxides of trilinoleoylglycerol. Alternate pathways of decomposition of oxidized triacylglycerols may be more favorable. Therefore, the simple esters of unsaturated fatty acids do not necessarily provide valid models for the oxidative dimerization of unsaturated triacylglycerols. The extensive use of methyl

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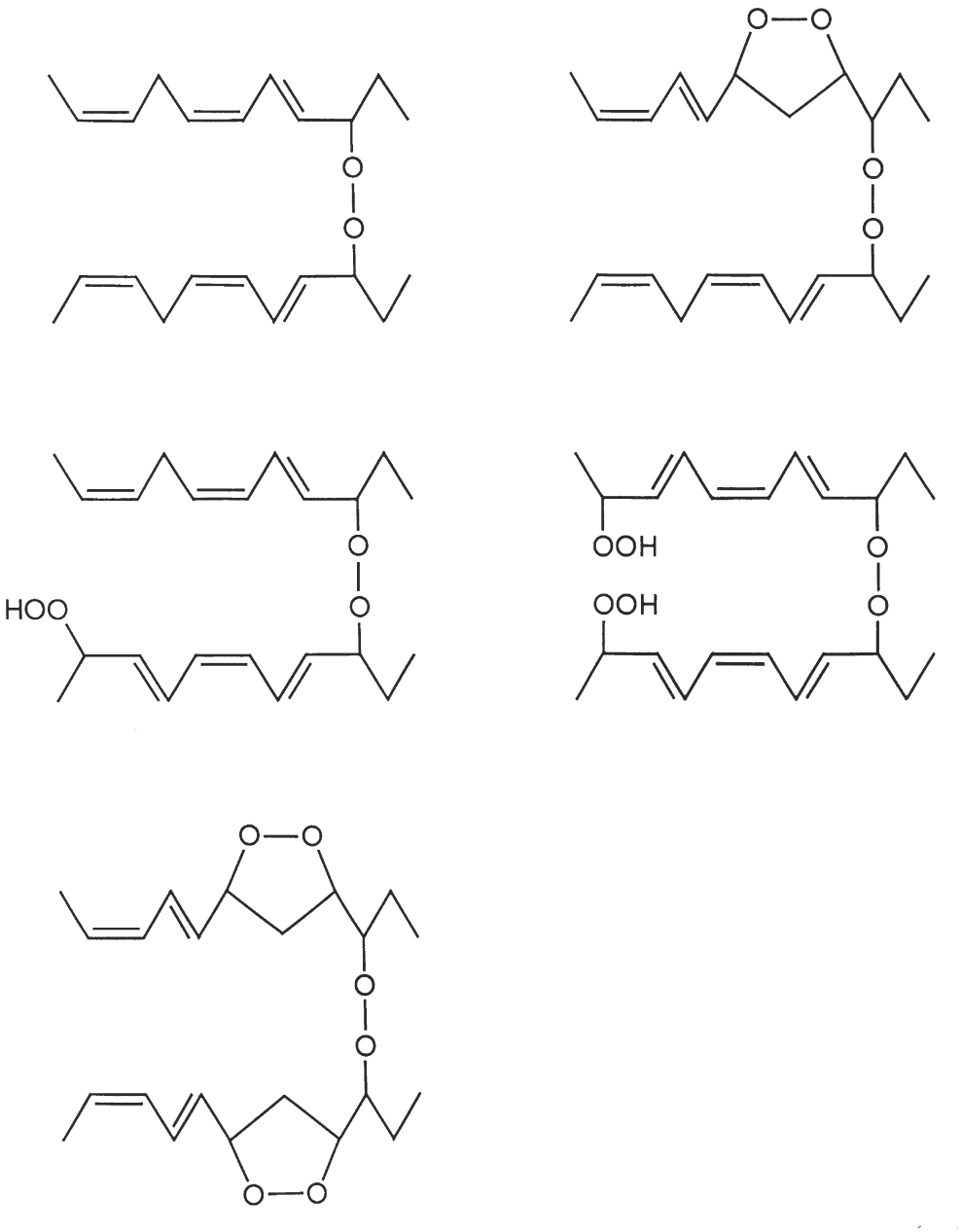


Figure 4.7. Dimers from autoxidized methyl linolenate (Neff *et al.*, 1988).

linoleate as a model lipid in decomposition studies may not apply to triacylglycerols and may not be relevant to fats and oils in general.

Although highly oxidized polyunsaturated triacylglycerols do not undergo dimerization and oligomerization under mild temperatures, these reactions

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become significant in oxidized soybean oil and other vegetable oils at the elevated temperatures of frying (Chapter 12), and of deodorization at 210°C and above. Column chromatographic analyses of refined-bleached and deodorized soybean oils show the presence of 1 to 3% high molecular weight compounds, consisting of both unpolar non-oxygenated and polar oxygenated fractions with average molecular weights of dimers. The non-oxygenated dimers, referred to as "thermal dimers", and the oxygenated dimers, referred to as "oxidative dimers", are complex high molecular weight decomposition mixtures that are not well characterized. Partial separation and analyses by size-exclusion chromatography show triacylglycerol dimeric compounds ranging from 0.5 to 2.8% in vegetable oils oxidized to high peroxide values (100–400). These high molecular weight compounds found in oxidized-deodorized vegetable and fish oils contain significant amounts of carbonyl function that can also be estimated by the anisidine value, which provides another measure of non-volatile carbonyl compounds (Chapter 5).

The presence of increasing levels of high molecular weight compounds in vegetable oils is related to the degree of oxidation prior to deodorization. This type of oxidation occurring during processing has been referred to as "hidden oxidation", because the hydroperoxides are completely decomposed at the elevated temperatures of deodorization into dimers and the resulting deodorized oils have a peroxide value of zero. The high molecular weight materials having an average molecular weight of "dimers" decrease the oxidative stability of the oils upon further storage. Dimers and oligomers can be measured in these oils by either silicic acid column chromatography or by size exclusion chromatography to obtain information on how much oxidative damage occurs during processing. These measurements can be used to estimate the future oxidative stability of fats and oils (Chapters 5 and 6).

D. Volatile products

Flavor deterioration of food lipids is caused mainly by the presence of volatile lipid oxidation products that have an impact on flavor at extremely low concentrations, often at the parts per billion (ppb) levels. An understanding of the sources of volatile oxidation products provides the basis for improved methods to control and evaluate flavor deterioration. The decomposition of lipid hydroperoxides produces carbonyl compounds, alcohols and hydrocarbons under various conditions of elevated temperatures and in the presence of metal catalysts.

Thermal decomposition of monohydroperoxides produces alkoxyl radicals undergoing homolytic β-scission to form aldehydes, alkyl and olefinic radicals (Figure 4.8). The alkyl radicals form either a hydrocarbon by hydrogen abstraction, or an alcohol by reacting with a hydroxyl radical. Similarly, the olefinic radical reacts either with a hydrogen radical to form an olefin, or reacts

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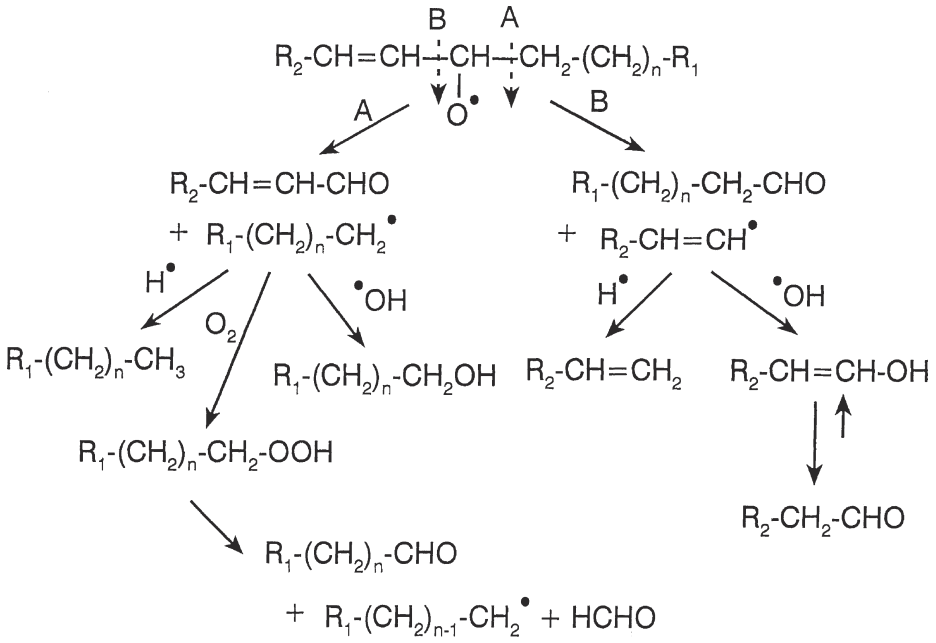


Figure 4.8. Homolytic β-scission of fatty ester monohydroperoxides.

with a hydroxyl radical to produce 1-enols which tautomerize into saturated aldehydes. The formation of lower aldehydes proceeds by reaction of the alkyl radicals with oxygen to produce primary hydroperoxides, which decompose further to produce aldehydes via an alkoxyl radical, or cleave to produce formaldehyde and a lower alkyl radical containing one less carbon.

Other mechanisms for the thermal decomposition of allylic hydroperoxides are recognized in free radical chemistry and photochemistry. The so-called Hock cleavage involves fragmentation of allyl hydroperoxides into two carbonyl products by oxygen insertion (Figure 4.9). Although generally catalysed by acids, this type of fragmentation is also known to occur thermally. This scheme has the advantage of not invoking the unstable vinyl radical intermediates (Figure 4.8) and is related to the acid heterolytic decomposition mechanism discussed below (Figure 4.19). However, the formation of alcohols, saturated hydrocarbons and lower fatty esters cannot be explained by the Hock cleavage reaction. Therefore, reactions of alkyl radicals and vinyl radicals with hydrogen obtained by abstraction or with hydroxyl radicals (Figure 4.8) must also be considered in lipid oxidation.

1. Oleate

The main volatile decomposition products formed from oleate, linoleate and linolenate are those expected from the cleavage of the alkoxyl radicals formed from the hydroperoxides of autoxidized and photosensitized oxidized fatty

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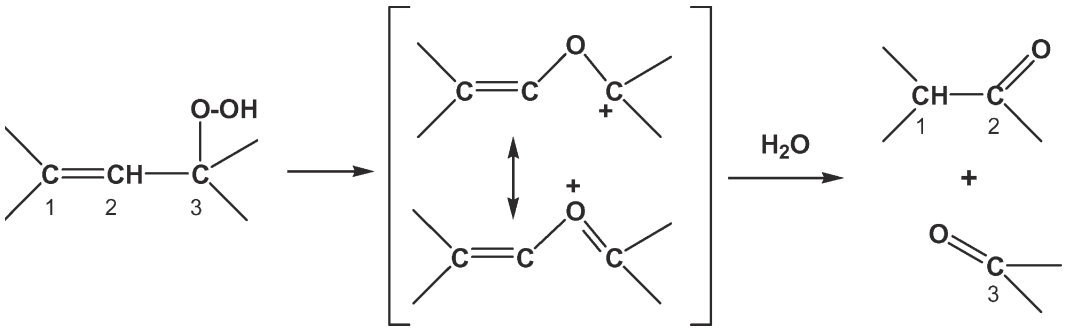


Figure 4.9. Hock cleavage mechanism for the fragmentation of allylic hydroperoxides into two carbonyl products.

esters (Figure 4.8). Gas chromatography (GC) by direct injection (see Chapter 5) is a useful technique used to study the volatile decomposition products of hydroperoxides that can be identified by GC-MS. This technique of anaerobic pyrolysis eliminates or minimizes secondary reactions between hydroperoxides and unsaturated fatty esters that would confound the interpretation of results. However, the volatile compounds analysed are those best amenable to the GC-MS technique used for analysis. The quantitative analyses are also affected by the thermal stability of the volatile aldehydes produced. A further complication arises from the ready interconversion of isomeric hydroperoxides that occurs as a side-reaction during thermal decomposition under the GC conditions.

With autoxidized oleate, decanal and 2-undecenal are produced by homolytic cleavages A and B on either side of the alkoxyl intermediate group from the 8-hydroperoxide, nonanal from either the 9- or 10-hydroperoxide, octane from the 10-hydroperoxide, and octanal and heptane from the 11-hydroperoxide (Figure 4.10). A mixture of 8-, 9-, 10- and 11-oxo esters is produced by cleavage A on the ester side of the hydroperoxides. With the monohydroperoxides of triglycerides containing oleate, the corresponding oxo glycerides or aldehydo-glycerides are formed; these high molecular weight nonvolatile compounds, referred also as "core" aldehydes, are useful predictors of quality of edible oils (Chapter 5).

The hydroperoxides of oleate subjected to photosensitized oxidation not only produced the volatiles expected from the 9- and 10-isomers, but also those derived from the 8- and 11-isomers. These results are explained by the interconversion between the 9- and 10-hydroperoxides of oleate formed by photosensitized oxidation into a mixture of 8-, 9-, 10-, and 11-hydroperoxides (Figure 4.11). Although both types of hydroperoxide produce the same major volatiles, the photosensitized oxidation-derived hydroperoxides produced more of the volatiles derived from the 9- and 10-hydroperoxide isomers (octane, 1-octanol, 2-decenal, and methyl octanoate). Small amounts of volatile

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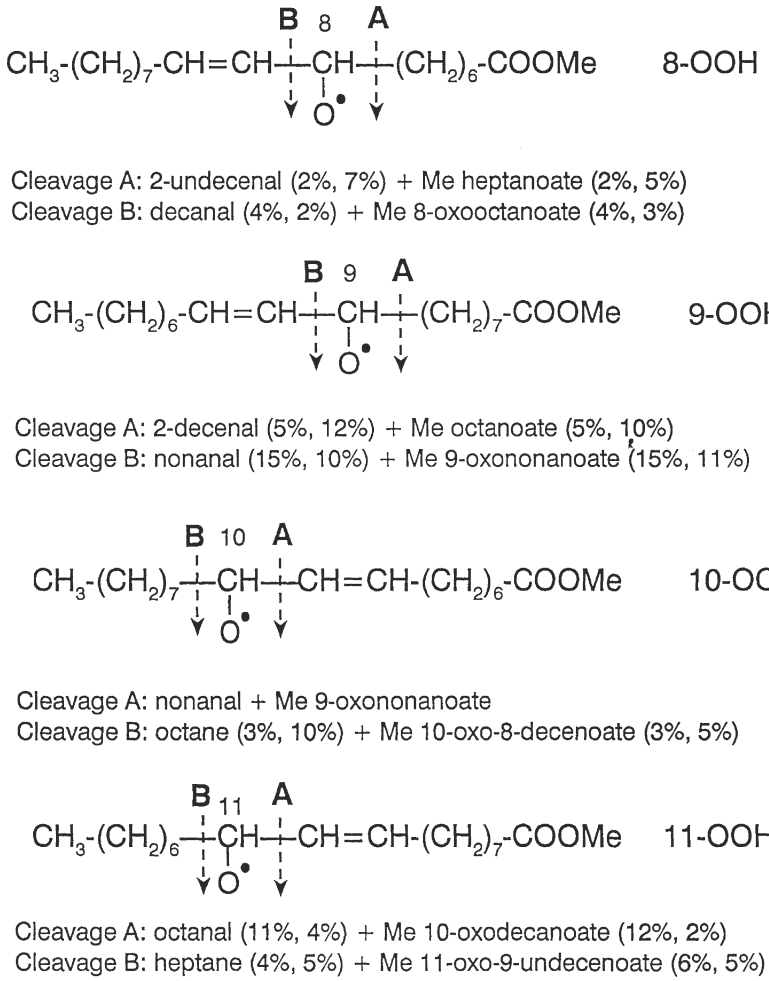


Figure 4.10. Cleavage products from methyl oleate hydroperoxides (Frankel *et al.,* 1981). Relative percent values shown in parentheses are for autoxidation and photosensitized oxidation respectively.

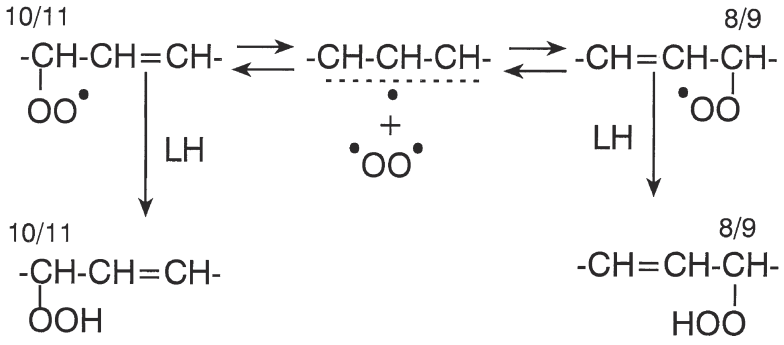
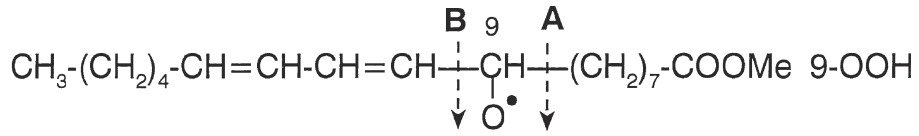
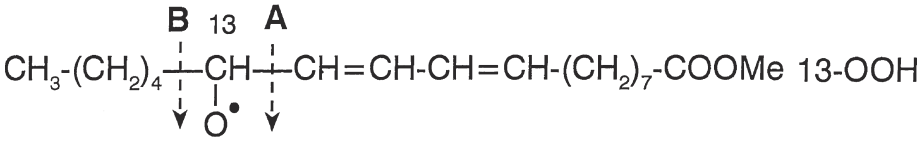


Figure 4.11. Thermal rearrangement of oleate hydroperoxides via allylic 3-carbon intermediates.

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Cleavage A: 2,4-decadienal (14%, 4%) + Me octanoate (15%, 8%)
Cleavage B: 3-nonenal (1%, 2%) + Me 9-oxononanoate (19%, 22%)



Cleavage A: hexanal (15%, 17%) + Me 12-oxo-9-dodecenoate (?)
Cleavage B: pentane (10%, 4%) + 1-pentanol (1%, 0.3%) +
pentanal (1%, 0.3%) + Me 13-oxo-9,11-tridecadienoate (?)

Figure 4.12. Cleavage products from 9- and 13-hydroperoxides formed by autoxidation of methyl linoleate (Frankel *et al.*, 1981). Relative percent values shown in parentheses are for autoxidation and photosensitized oxidation respectively; (?) = not detected.

decomposition compounds formed from oleate hydroperoxides cannot be explained by the classical A and B cleavage mechanism; these compounds include heptanal, 2-nonenal, and methyl nonanoate.

2. *Linoleate*

With autoxidized linoleate, 2,4-decadienal and methyl octanoate are produced by homolytic cleavage A on the 9-hydroperoxide, and 3-nonenal and 9-oxononanoate by cleavage B; hexanal, pentane, 1-pentanol, and pentanal are produced from the 13-hydroperoxide (Figure 4.12). With linoleate subjected to photosensitized oxidation, the 9- and 13-hydroperoxides produced less 2,4-decadienal, methyl octanoate and pentane, and similar amounts of hexanal. The unique unconjugated 10-hydroperoxide produced 9-oxononanoate and methyl 10-oxo-8-decenoate, and the 12-hydroperoxide produced a significant amount of 2-heptenal (Figure 4.13).

As with oleate, some volatile decomposition compounds are formed from linoleate hydroperoxides that cannot be explained by the classical A and B cleavage mechanism, including acetaldehyde, 2-pentylfuran, methyl heptanoate, 2-octenal, 2,4-nonadienal, methyl 8-oxooctanoate, and methyl 10-oxodecanoate. Although hydroperoxides derived from both autoxidation and from photosensitized oxidation of linoleate form the same volatile decomposition products, significant quantitative differences are noted. The autoxidized linoleate hydroperoxides produced more pentane, 2-pentyl furan, 2,4-decadienal and

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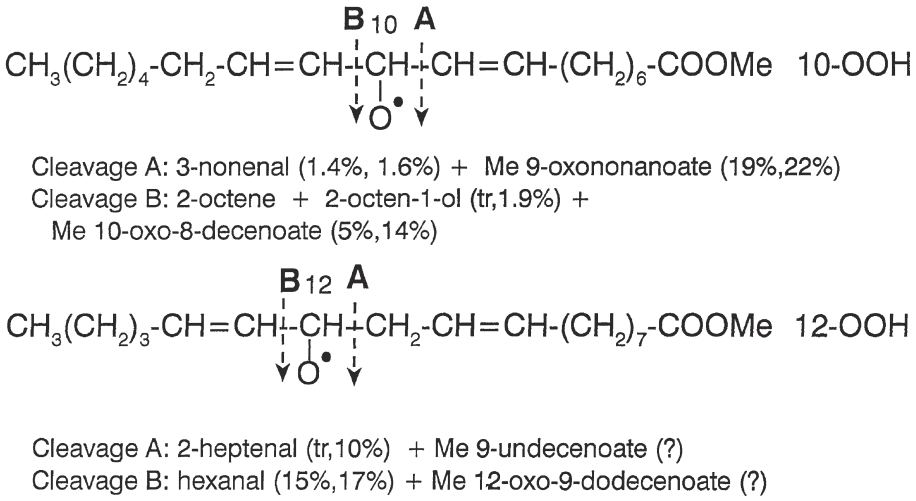


Figure 4.13 Cleavage products from 10- and 12-hydroperoxides formed by photosensitized oxidation of methyl linoleate (Frankel *et al.*, 1981). Relative percent values shown in parentheses are for autoxidation and photosensitized oxidation respectively.

methyl octanoate, and less 10-oxo-8-decenoate and 2-heptenal than the photooxidized linoleate hydroperoxides.

Volatile decomposition products from autoxidized linoleic acid and methyl linoleate were characterized for their intense aroma and flavor impact by capillary gas chromatography-olfactometry. This technique involves sniffing the gas chromatograph effluent after stepwise dilution of the volatile extract. The most intense volatiles included hexanal, *cis*-2-octenal, *trans*-2-nonenal, 1-octene-3-one, 3-octene-2-one and *trans*-2-octenal (Table 4.2). This analytical approach does not, however, consider the effects of complex interactions of volatiles occurring in mixtures produced in oxidized food lipids.

4-Hydroxy-2-nonenal produced by decomposition of linoleate hydroperoxides has received much attention in the biochemical literature because of its cytotoxicity and its occurrence in oxidized LDL (Chapter 13, C, E.2, Figure 13.7). 4-Hydroxynonenal has also been identified in vegetable oils heated at frying temperatures. Many mechanisms have been suggested for the formation of this hydroxy aldehyde. The autoxidation of the 9- and 13-hydroperoxides of linoleic acid produced a mixture of fragmentation products including 4-hydroperoxynonenal, 4-hydroxynonenal, together with C-11 and C-12 aldehyde acids and dihydroperoxides (Table 4.3). Fe(II)-mediated autoxidation of 13-linoleate hydroperoxide produced a mixture of 4-hydroperoxynonenal, 4-ketononenal and 4-hydroxynonenal. One suggested scheme involves allylic hydrogen abstraction at C-8 of the 13-hydroperoxide of linoleic acid to produce a 10,13-dihydroperoxide, which cleaves between C-9 and C-10 to produce 4-hydroperoxy-2-nonenal, which in turn is reduced to 4-hydroxy-2-nonenal (Figure 4.14). In another proposed scheme, 4-hydroxynonenal can be

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Table 4.2. Intense volatile flavor compounds from autoxidized linoleic acid and methyl linoleate [a]

Compound	Linoleic acid	Me linoleate
	D value [b]	
Hexanal	20	40
cis-2-Octenal	20	80
trans-2-Nonenal	20	80
1-Octene-3-ol	6	
1-Octene-3-one		18
2,4-Nonadienal	4	2
2,4-Decadienal	4	2
cis-2-Octenal		8
3-Octene-2-one	2	
trans-2-Octenal	2	2

[a] From Ullrich and Grosch (1987). Linoleic acid and methyl linoleate were oxidized at 22–24°C; the volatiles were isolated by column chromatography on alumina oxide, concentrated by vacuum distillation and analysed by capillary gas chromatography.
[b] D value = highest dilution in which a substance can be smelled from the gas chromatograph eluate after stepwise dilution of the volatile compounds. Linoleic acid was oxidized for 72 hr and methyl linoleate for 150 hr.

Table 4.3. Decomposition products from the autoxidation of linoleic acid hydroperoxides [a,b]

Decomposition products	Linoleic acid hydroperoxides
9-Hydroperoxy-12-oxo-10-dodecenoic acid[a]	13-OOH
11-Oxo-9-undecenoic acid[a]	13-OOH
4-Hydroperoxy-2-nonenal[a, b]	9-OOH and 13-OOH
4-Hydroxy-2-nonenal[a, b, c]	9-OOH and 13-OOH
8,13-Dihydroperoxy-octadecadienoic acid[a]	13-OOH
9,14-Dihydroperoxy-octadecadienoic acid[a]	9-OOH
4-Oxo-2-nonenal[b]	13-OOH

[a] See Figures 4.14, 4.15.
From Schneider *et al.* (2001). Autoxidation carried out at 37ºC; products separated by reverse phase HPLC and identified by UV, ^{1}H NMR, GC-MS, LC-ESI-MS [liquid chromatography-electrospray ionization-mass spectrometry (see Chapter 6)] of suitable derivatives.
[b] From Lee and Blair (2000). Autoxidation carried at 37°C in the absence and presence of $FeSO_4(NH_4)_2SO_4$; products separated by normal phase HPLC and identified by LC-MS of the oxime derivative.
[c] 4-hydroxy-2-nonenal was detected only as a minor product in autoxidation products but was readily produced by reduction with triphenylphosphine.

formed from 4-hydroperoxy-2-nonenal by oxidation of 3-nonenal produced from the 9-hydroperoxide of linoleate by either Hock or β-cleavage B (Figure 4.15). By similar pathways, the formation of 4-hydroxy-2-nonenal would be expected from other *n*–6 PUFA hydroperoxides, especially the 11- and 15-hydroperoxides of arachidonate (Figure 2.13).

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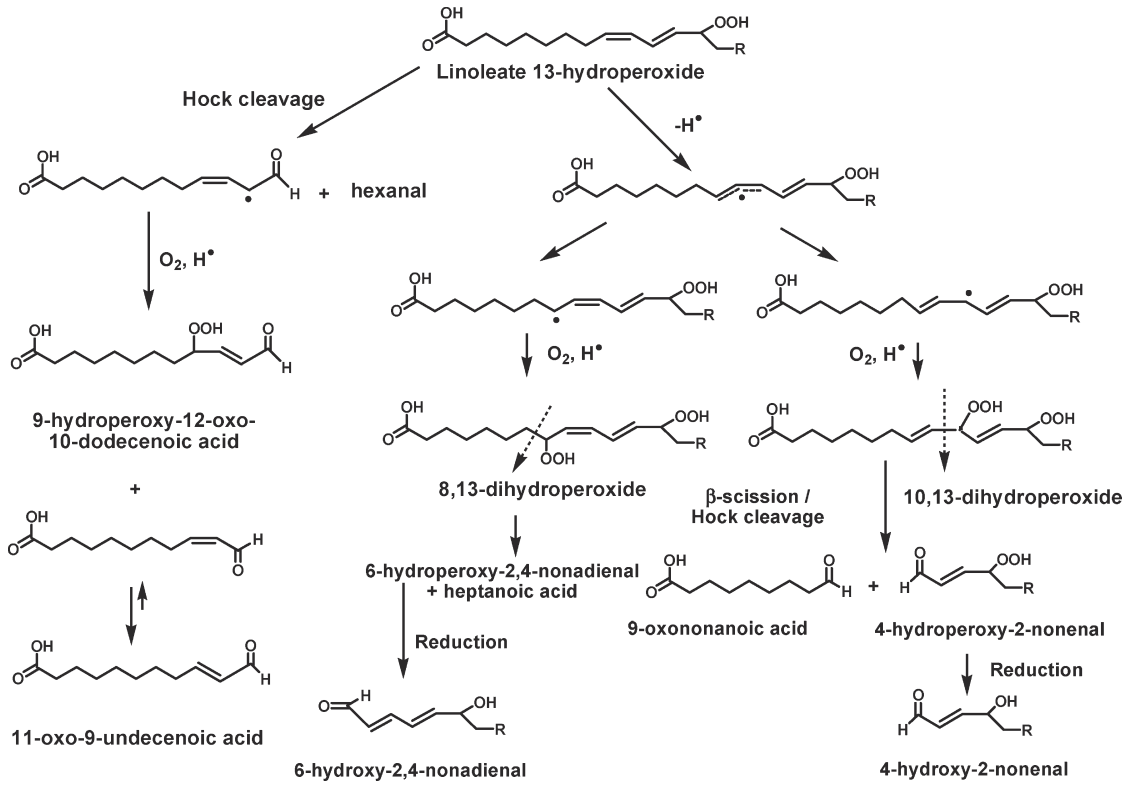


Figure 4.14. Scheme for the formation of 4-hydroxy-2-nonenal from linoleic acid 13-hydroperoxide. Adapted from Schneider *et al.* (2001).

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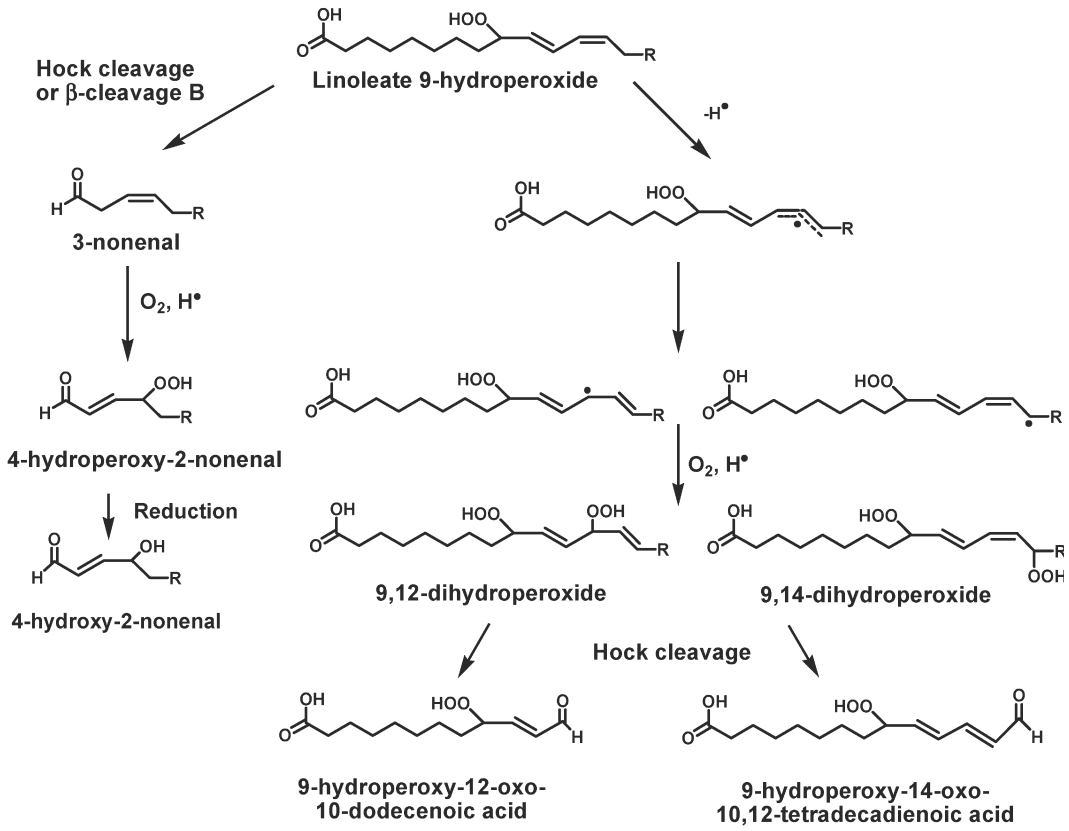


Figure 4.15. Scheme for the formation of 4-hydroxy-2-nonenal from linoleic acid 9-hydroperoxide and 3-nonenal. Adapted from Schneider *et al.* (2001).

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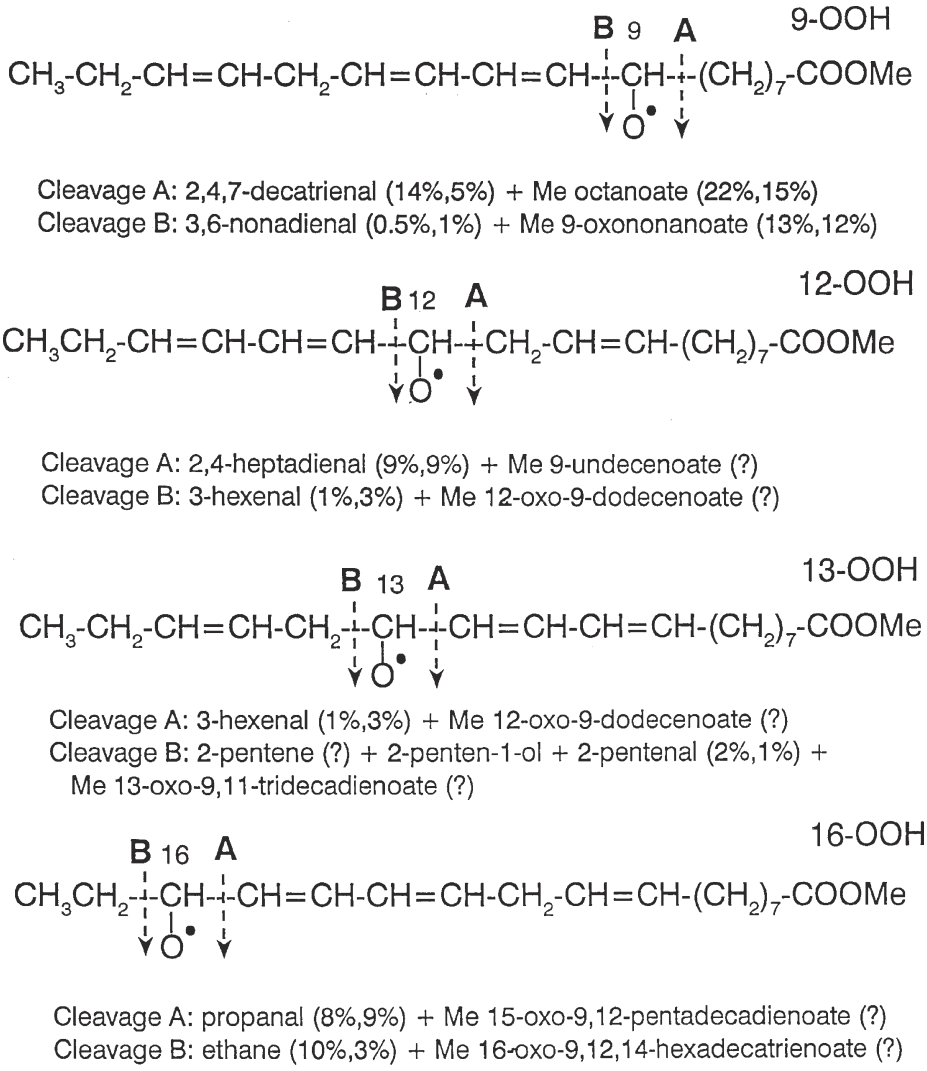


Figure 4.16. Cleavage products from 9-, 12-, 13- and 16-hydroperoxides formed by autoxidation of methyl linolenate (Frankel *et al.*, 1981). Relative percent values shown in parentheses are for autoxidation and photosensitized oxidation respectively.

Enzymatic biosyntheses have also been reported in plant extracts involving lipoxygenase producing the 9-hydroperoxide of linoleic acid, and lyase (an enzyme catalysing the decomposition of hydroperoxides) producing 3-nonenal and 3-alkenal oxygenase to give 4-hydroperoxy-2-nonenal, which is reduced to 4-hydroxy-2-nonenal.

3. Linolenate

With autoxidized methyl linolenate, decatrienal and methyl octanoate are derived from the 9-hydroperoxide; 2,4-heptadienal from the 12-hydroperoxide;

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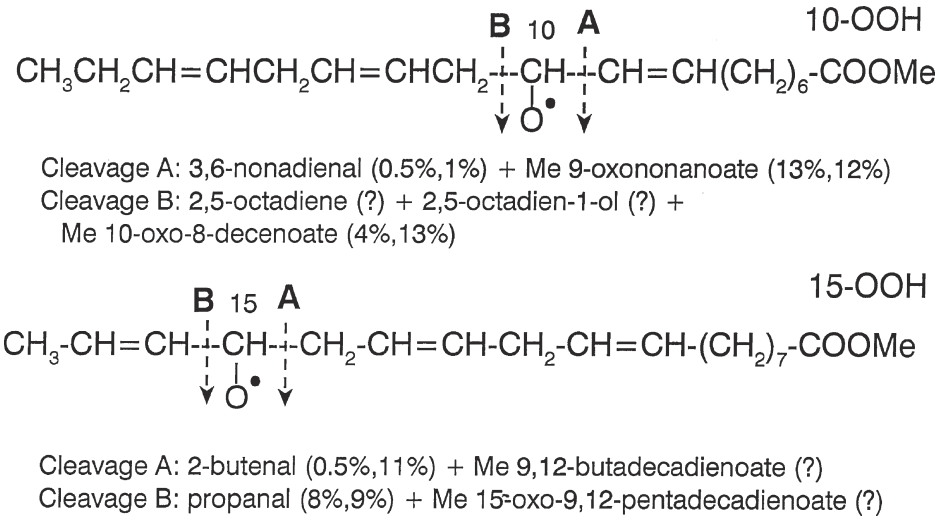


Figure 4.17. Cleavage products from 10- and 15-hydroperoxides formed by photosensitized oxidation of methyl linolenate (Frankel *et al.*, 1981). Relative percent values shown in parentheses are for autoxidation and photosensitized oxidation respectively.

3-hexenal and 2-pentenal from the 13-hydroperoxide; propanal and ethane from the 16-hydroperoxide (Figure 4.16). As with oleate and linoleate hydroperoxides, common volatiles are produced from both types of hydroperoxides derived by autoxidation and photosensitized oxidation. The autoxidation hydroperoxides of linolenate produced more decatrienal, methyl octanoate and ethane. With linolenate subjected to photosensitized oxidation, 9-oxononanoate and methyl 10-oxo-8-decenoate are produced from the 10-hydroperoxide, and a significant amount of 2-butenal is produced from the 15-hydroperoxide (Figure 4.17).

As with oleate and linoleate, some volatile decomposition compounds are formed from linolenate hydroperoxides that cannot be explained by the classical A and B cleavage mechanisms, including acetaldehyde, butanal, 2-butyl furan, methyl heptanoate, 4,5-epoxyhepta-2-enal, methyl nonanoate, methyl 8-oxooctanoate, and methyl 10-oxo-8-decenoate. Some of these minor volatile oxidation products can be attributed to further oxidation of unsaturated aldehydes. Other factors contribute to the complexity of volatile products formed from hydroperoxides, including temperature of oxidation, metal catalysts, stability of volatile products and competing secondary reactions including dimerization, cyclization, epoxidation and dihydroperoxidation (Section E).

Volatile decomposition products from autoxidized methyl linolenate were characterized for their intense aroma impact by capillary gas chromatography-olfactometry. The most intense volatiles included *trans,cis*-2,6-nonadienal, 1-*cis*-5-octadien-3-one, *trans,cis*-3,5-octadien-2-one and *cis*-3-hexenal (Table 4.4). The mechanism suggested for the formation of 1-*cis*-5-octadien-3-one assumes as precursor the 10-hydroperoxide formed by singlet oxidation that

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Table 4.4. Intense volatile odor compounds from autoxidized methyl linolenate [a]

Compound	FD-Factor [b]	
	48 hr	102 hr
1-Penten-3-one	1	4
cis-3-Hexenal	4	12
1-*cis*-5-Octadien-3-one	16	340
2,4-Heptadienal	<1	4
trans,cis-3,5-Octadien-2-one	2	2
trans,cis-2,6-Nonadienal	16	4

[a] From Ullrich and Grosch (1988). Methyl linoleate was oxidized at 22–24°C; the volatiles were isolated by column chromatography on silca gel and analysed by capillary gas chromatography-olfactometry. FD factor is determined by gas chromatographic analysis and effluent sniffing of a diluted series of the original extract

[b] FD-Factor = the number of dilution required before a compound can no longer be detected by sniffing the gas chromatograph effluent.

undergoes β-scission, followed by hydroxylation of the left fragment and oxidation to the keto compound.

4. Long-chain polyunsaturated fatty acids

Lipid oxidation of oils derived from fish and algae containing EPA (20:5) and DHA (22:6) produce complex mixtures of volatile decomposition compounds (Chapter 11, Section E.3) causing fishy off-flavors. Some of these volatiles cause very powerful "fishy" odors and flavors perceptible at concentrations of parts per billion (ppb). These off-flavors from volatiles produced at extremely low levels of oxidation are generally considered a major problem in the utilization of fish oils (Chapter 11). A short list of volatiles associated with fishy flavor in cod liver oil includes *cis*-heptenal, *trans,cis,cis*- and *trans,trans,cis*-decatrienal, *trans*-2-hexenal, *trans,cis*-decadienal, *trans,cis*-nonadienal and *cis*-3-hexen-1-ol (Table 4.5). Analyses of fish oils by dynamic headspace GC showed over 150 volatile compounds, but only 20 correlated with taste panel response. A simpler and manageable model restricting the "fishy scale" index to three compounds (2,6-nonadienal, 4-heptenal and 3,6-nonadienal), as key volatiles, was proposed by using gas chromatography and mass spectral detection with ammonia negative chemical ionization (Chapter 6) in the ppb ranges. A mechanism for the formation of *cis*-3,*cis*-6-nonadienal involves β-cleavage of a hydroperoxide produced from either EPA or DHA (Chapter 2), isomerization to *trans*-2,*cis*-6-nonadienal, followed by hydration and retro-aldol condensation to give *cis*-4-heptenal (Figure 4.18).

5. Heterolytic mechanisms

In contrast to thermal and metal-catalysed decomposition of hydroperoxides, which proceed by homolytic cleavage, decomposition under acid conditions

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Table 4.5. Volatile compounds associated with fishy flavor in oxidizing cod liver oil [a]

Compound	Concentration (ppb)
cis-4-Heptenal	2000
trans,cis,cis-2,4,7-Decatrienal	930
trans,trans,cis-2,4,7-Decatrienal	170
trans-Hexenal	720
trans,cis-2,4-Decadienal	30
trans,cis-2,6-Nonadienal	570
cis-3-Hexen-1-ol	80

[a] From Karahadian and Lindsay (1989). Deodorized cod liver oil samples were stored under fluorescent light at 21°C, and aliquots were analysed for volatiles by dynamic headspace gas chromatography (see Chapter 5).

proceeds by a different mechanism involving heterolytic cleavage producing ionic ether intermediates, also referred to as carbocations. The resulting products are simpler and consist mainly of aldehydes. The acid decomposition products of linoleate hydroperoxides are those expected by selective heterolytic cleavage between the hydroperoxide group and the allylic double bond (Figure 4.19). Acid decomposition with hydrochloric acid–methanol produced the dimethyl acetal derivatives that are stable under conditions of GC-MS. Thus, methyl 9-oxononanoate (51%) and 3-nonenal (2%) are formed from the 9-hydroperoxide of linoleate; and hexanal (21%) and methyl 12-oxo-9-dodecenoate (26%) are formed from the 13-hydroperoxide of linoleate (Table 4.6). Methyl 9-oxononanoate (22%) and 3,6-nonadienal (5%) are formed from

Table 4.6. Aldehydes produced by heterolytic acid decomposition of hydroperoxides of methyl linoleate and linolenate [a]

Aldehydes as (dimethyl acetals)	Methyl linoleate hydroperoxides		Methyl linolenate hydroperoxides	
	Relative %	Precursors	Relative %	Precursors
Hexanal	21	13-OOH		
3-Nonenal	2	9-OOH		
Me 9-oxononanoate	51	9-OOH		
Me 12-oxo-9-dodecenoate	26	13-OOH		
Propanal			22	16-OOH
3-Hexenal			1	12- + 13-OOH
3,6-Nonadienal			5	9-OOH
Me 9-oxononanoate			22	9-OOH
Me 12-oxo-9-dodecenoate			26	12- + 13-OOH
Me 15-oxo-9,12-pentadecadienoate			24	16-OOH

[a] From Frankel *et al.* (1984); relative % values were normalized to 100%;
-OOH = hydroperoxide.

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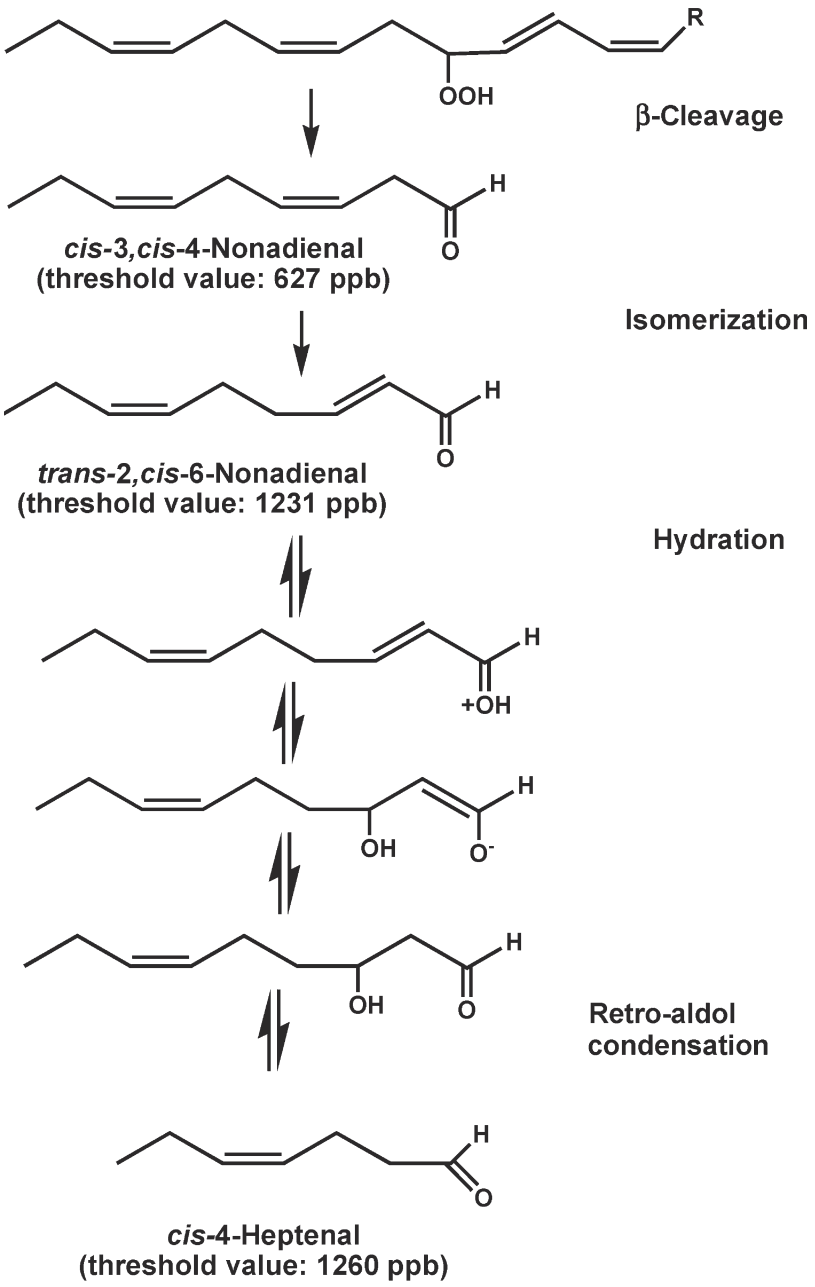


Figure 4.18. Mechanism for the formation of *cis*-3,*cis*-6-nonadienal, *trans*-2,*cis*-6-nonadienal, and *cis*-4-heptenal from a hydroperoxide produced from either EPA or DHA.

the 9-hydroperoxide of linolenate; methyl 12-oxo-9-dodecenoate (26%) and 3-hexenal (1%) are formed from the 12-hydroperoxide and 13-hydroperoxide of linolenate; and propanal (22%) and methyl 15-oxo-9,12-pentadecadienoate (24%) are formed from the 16-hydroperoxide of linolenate (Table 4.3).

The classical homolytic β-scission mechanism (Figure 4.8) has been

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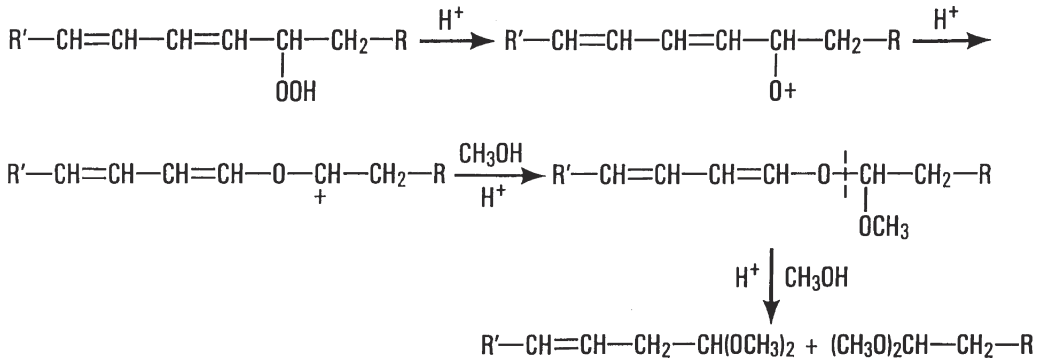


Figure 4.19. Acid heterolytic decomposition of linoleate hydroperoxdes (Frankel *et al.*, 1984). Stable dimethyl acetal derivatives are produced in the presence of HCl-methanol.

criticized, because β-scission on one side of the hydroperoxide group leads to stable alkyl radicals ($R^{\bullet}$), and on the other side of the hydroperoxide group it leads to vinyl radicals (R'-CH=$CH^{\bullet}$), which are unstable and unlikely. For this reason, a homolytic–heterolytic mechanism was postulated to avoid the formation of unfavorable vinylic radical intermediates (Figure 4.20). By this "mixed" mechanism, cleavage occurs homolytically on the alkyl side of the hydroperoxide to produce 2-alkenals, and heterolytically on the unsaturated side of the hydroperoxide to produce alkanals. This heterolysis may be favored because hydroperoxides are known to be weakly acidic.

E. Volatiles from secondary oxidation products

In addition to the monohydroperoxides, unsaturated aldehydes and ketones undergo autoxidation and provide additional sources of volatile compounds. Nonvolatile secondary products that undergo further decomposition into volatile products include dimers and oligomers, hydroperoxy epoxides, hydroperoxy epidioxides and dihydroperoxides. These secondary products contain one or more hydroperoxide groups and decompose the same way as monohydroperoxides to produce similar volatile materials. As with the monohydroperoxides, the multitude of volatile compounds formed from secondary products are important contributors to the flavor quality of lipid-containing foods.

1. Aldehydes

The oxidation of unsaturated aldehydes provides an important source of additional aldehydes from the decomposition of hydroperoxides. The oxidation products of 2-nonenal include alkanals, glyoxal, and mixtures of α-keto aldehydes. The same products are formed from the oxidation of 2,4-heptadienal,

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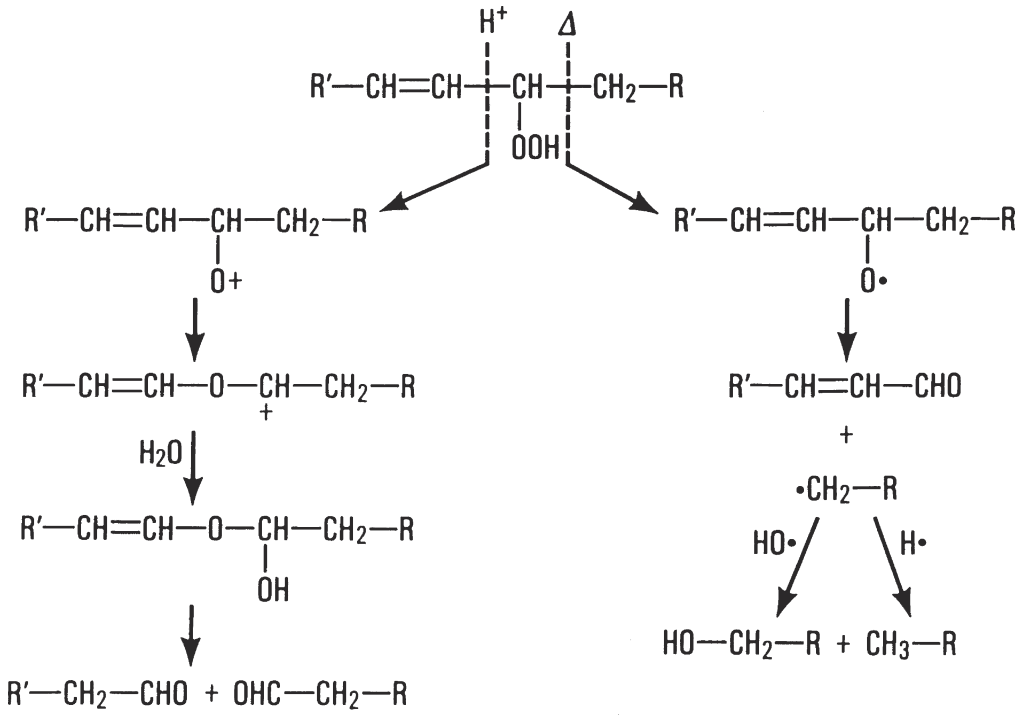


Figure 4.20. Homolytic–heterolytic mechanism for the decomposition of methyl linoleate hydroperoxides (Frankel *et al.,* 1984). With permission of AOCS Press.

which also produces dialdehydes (*cis*-2-butene-1,4-dial and glyoxal). The production of these volatile products is explained by the formation of α-hydroperoxide aldehydes or α-keto aldehydes undergoing cleavage into smaller chain aldehydes and malonaldehyde (Figure 4.21). By this mechanism, enals produce the next lower alkanal, and 2,4-dienals give the next lower enal and alkanal with four carbons fewer than the parent aldehyde, together with dialdehydes.

In mixtures, 2-nonenal and 2,4-heptadienal are more rapidly oxidized than methyl linoleate and linolenate, and these fatty esters are more readily oxidized than nonanal. Therefore, during the more advanced stages of oxidation the saturated aldehydes accumulate and the unsaturated aldehydes are further oxidized to lower aldehydes and dialdehydes. At elevated temperatures, saturated aldehydes and dialdehydes are further oxidized into mono- and dibasic acids. The formation of low molecular weight volatile acids is used as a measure of thermal oxidation of fats, and is the basis for detection in some high temperature stability tests (Chapter 7).

2. *Dimers and oligomers*

Mixtures of unsaturated dimers and oligomers isolated from thermally decom-

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posed linoleate hydroperoxides and oxidized linoleate produce hydroperoxides. Cleavage of these hydroperoxides produces the same volatile products as the monohydroperoxides. The formation of pentane seems to be favored. High molecular weight aldehydo-glycerides (or oxo-glycerides) found in oxidized fats are formed by the same cleavage mechanism producing aldehyde esters from the hydroperoxide esters. Aldehydo-glycerides are also referred to as "core" aldehydes.

Dimers isolated from autoxidized linolenate, monohydroperoxides, hydroperoxy epidioxides and dihydroperoxides produce volatile decomposition products that are similar to those formed from the corresponding monohydroperoxides, but their relative concentrations are different. Compared to the corresponding monomers, dimers from linolenate hydroperoxides produce more propanal and methyl 9-oxononanoate, but less methyl octanoate, and much less 2,4-heptadienal and 2,4,7-decatrienal. The major volatile decomposition products expected from different peroxide-linked dimers are shown in Figure 4.22. The formation of methyl 9-oxononanoate as the major volatile product can be explained by cleavage between the peroxide link and the olefinic side of the 9- and 16-hydroperoxide of linolenate dimers. The formation of propanal and methyl octanoate can be explained by cleavage on the opposite side of the peroxide link. Although early studies showed that the formation of dimeric materials after processing and deodorization of vegetable oils contribute to poor oxidative stability, little is known regarding the relative contribution of dimers and oligomers to the flavor deterioration of unsaturated fats.

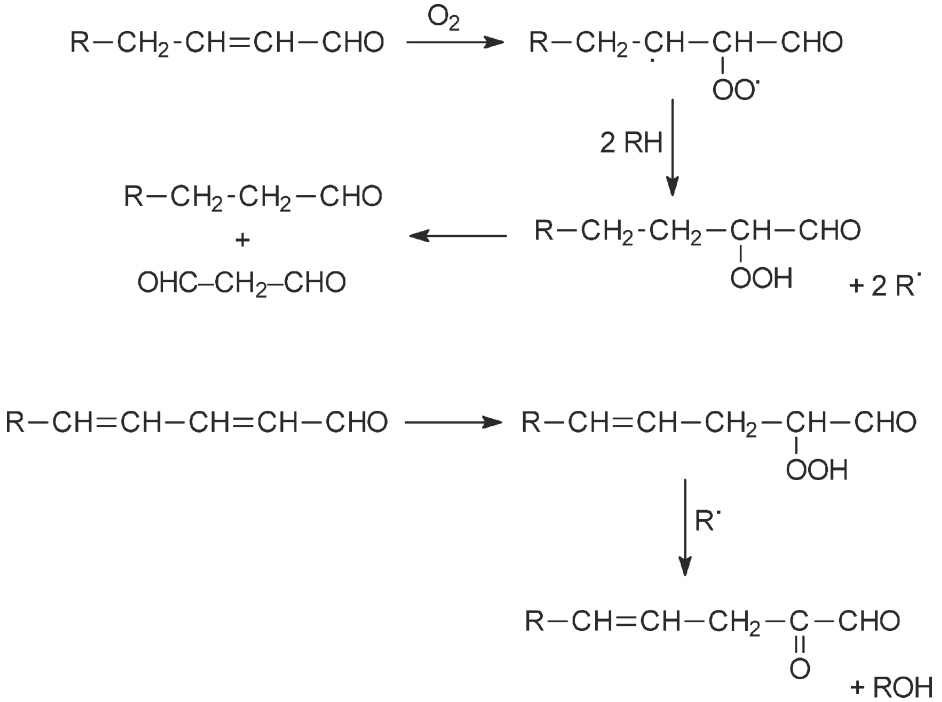


Figure 4.21. Oxidation of 2-alkenals.

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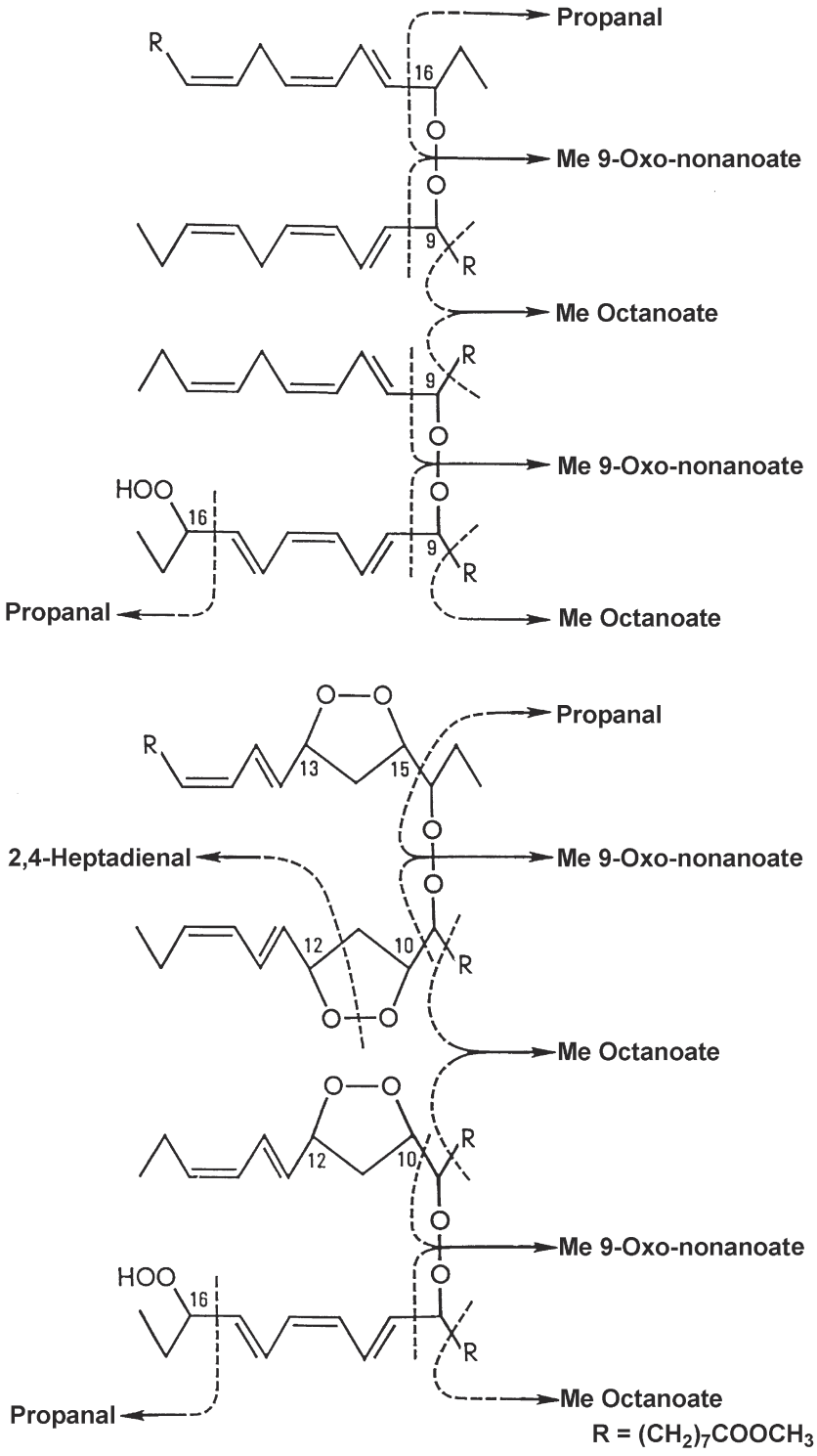


Figure 4.22. Volatile decomposition products expected from peroxide-linked dimers of methyl linolenate (Frankel *et al.*, 1988).

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3. *Hydroperoxy epoxides, epidioxides and bicyclo-endoperoxides*

A number of volatile epoxy acids and aldehydes are found in autoxidized linoleate and unsaturated fats. *Trans*-2,3-epoxyoctanoic acid is an important volatile compound produced by double bond cleavage of the epoxy-hydroperoxide found in autoxidized methyl linoleate. Cleavage at the allylic position of the hydroperoxide forms the corresponding unsaturated epoxy aldehyde (Figure 4.23).

Trans-4,5-epoxy-2-heptenal ($R_1 = C_2H_5$) found in oxidized butterfat is presumed to come from *trans*-15,16-epoxy-12-hydroperoxy-9,13-dienoic acid derived from the 16-hydroperoxide of linolenic acid, because 4,5-epoxy-2-heptenal is also found in thermally decomposed methyl linolenate hydroperoxides. 4,5-Epoxy-2-decenal identified in thermally oxidized trilinoleoyl glycerol comes from the corresponding 12,13-epoxy-9-hydroperoxy-10-enoic acid ($R_1 = C_5H_{11}$) from the 13-hydroperoxide of linoleate. Epoxyaldehydes are apparently important flavor precursors, because they have a significant sensory impact when tested by the sniffing technique at the exit port of a gas chromatograph (Chapter 5).

The hydroperoxy epidioxides formed from photosensitized oxidized methyl linoleate are important precursors of volatile compounds, which are similar to those formed from the corresponding monohydroperoxides. Thus, 13-hydroperoxy-10,12-epidioxy-*trans*-8-enoic acid produces hexanal and methyl 10-oxo-8-decenoate as major volatiles (Figure 4.24). The 9-hydroperoxy-10,12-epidioxy-*trans*-13-enoic acid produces 2-heptenal and methyl 9-oxononanoate. Other minor volatile products include two volatiles common to those formed from the monohydroperoxides, pentane and methyl octanoate, and two that are unique, 2-heptanone and 3-octene-2-one. The hydroperoxy epidioxides formed from autoxidized methyl linolenate produce the volatiles expected from the cleavage reactions of linolenate hydroperoxides, and significant amounts of the unique compound 3,5-octadiene-2-one. This vinyl ketone has a low threshold value or minimum detectable level, and may contribute to the flavor impact of fats containing oxidized linolenate (Chapter 5).

As observed with the monohydroperoxides (Section D), thermal decomposition of the hydroperoxy bicyclo-endoperoxides from methyl linolenate produced more complex mixtures of volatile compounds than acid decomposition with acidic methanol. The thermal decomposition products included methyl 9-oxononanoate, propanal, 2,4-heptadienal, methyl octanoate, methyl 13-oxo-9,11-tridecadienoate and ethane (Figure 4.25). The acid decomposition products, analysed as the di- and tetramethyl acetals, comprised only propanal, methyl 9-oxononaoate, and malonaldehyde. As with the monohydroperoxides, by thermal decomposition the bicyclo-endoperoxides are cleaved on either side of the hydroperoxide group, whereas by acid decomposition they are cleaved only between the hydroperoxide group and the

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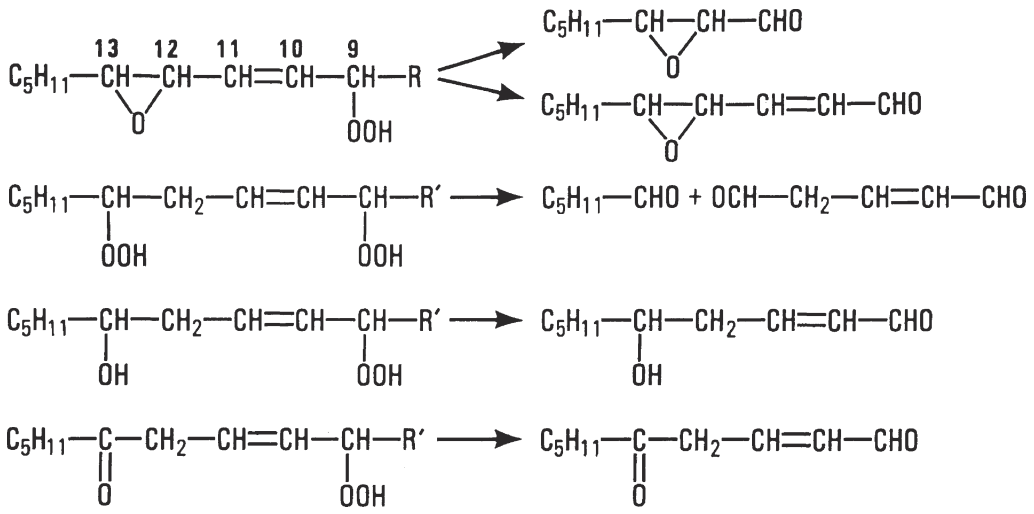


Figure 4.23. Formation of unsaturated epoxy aldehydes from the epoxyhydroperoxide of methyl linoleate.

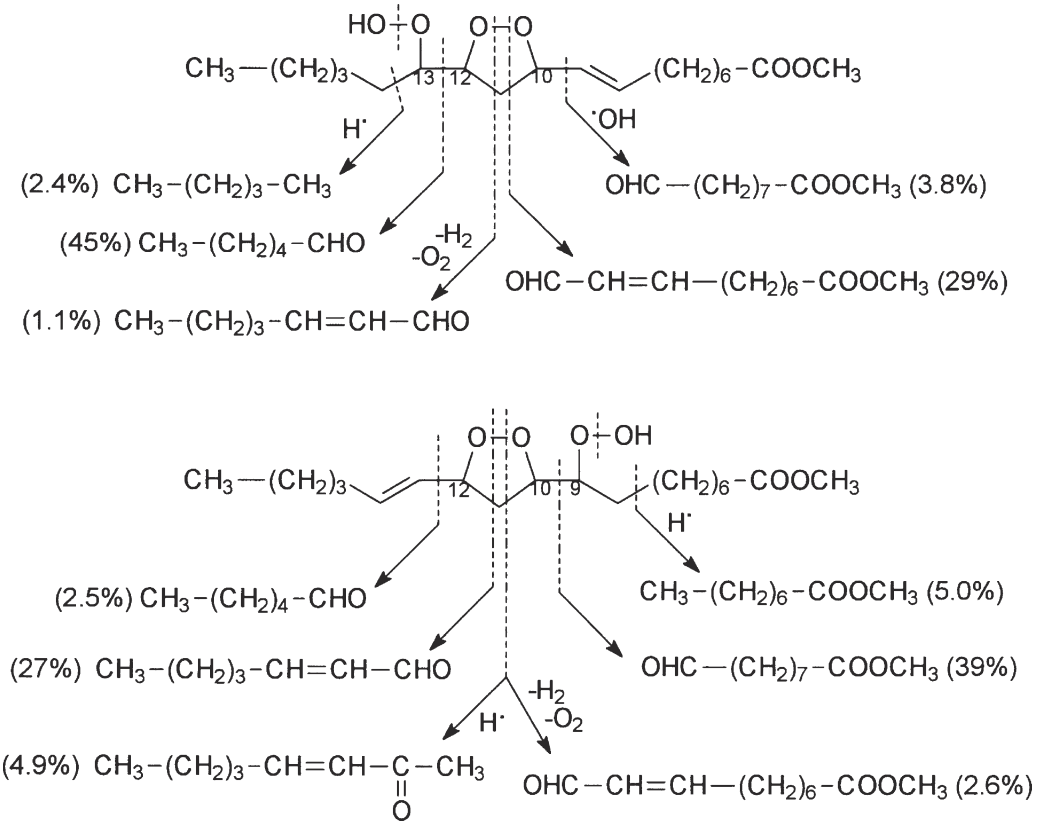


Figure 4.24. Volatile decomposition products from hydroperoxy epidioxides of methyl linoleate oxidized with singlet oxygen (Frankel *et al.*, 1982).

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allylic double bond. The bicyclo-endoperoxides undergo cleavage across the ring also to produce malonaldehyde (cf. Figure 4.4), which was only detected under the conditions of acid decomposition (as the tetramethyl acetal derivative). This dialdehyde was too unstable to be detected under the conditions of thermal decomposition.

4. Triacylglycerols

The main volatile compounds detected from autoxidized trilinolein monohydroperoxides include pentane, hexanal, 2-heptenal and 2,4-decadienal. The volatiles from trilinolenin and monohydroperoxides include propanal, 2,4-heptadienal and 2,4,7-decatrienal. Mixtures of 1:1 trilinolein and trilinolenin autoxidized at low peroxide values (PV 34) show equal contribution of volatiles derived from linoleate and linolenate hydroperoxides.

Soybean oil containing a mixture of oleate, linoleate and linolenate triacylglycerols can produce 14 positional hydroperoxides by autoxidation and photosensitized oxidation (Chapters 2 and 3). Soybean oil and other vegetable oils, such as safflower oil and corn oil, that do not contain linolenate, produce an unexpectedly high concentration of the12-hydroperoxide isomer at peroxide values below 50. The 12-hydroperoxide isomer appears to be derived from photosensitized oxidation because its concentration is decreased in the presence of singlet oxygen quenchers such as β-carotene and α-tocopherol (Chapter 3).

A large proportion of the volatiles identified in vegetable oils are derived from the cleavage reactions of the hydroperoxides of oleate, linoleate, and linolenate (Section D). A wide range of hydrocarbons (ethane, propane, pentane and hexane) appears to be formed in soybean oil oxidized to low peroxide values. A number of volatiles identified in vegetable oils that are not expected as primary cleavage products of monohydroperoxides include dialdehydes, ketones, ethyl esters, nonane, decane, undecane, 2-pentylfuran, lactone, benzene, benzaldehyde and acetophenone. Some of these volatiles may be derived from secondary oxidation products, but the origin of many volatiles still remains obscure. However, studies of volatile decomposition products should be interpreted with caution, because the conditions used for isolation and identification may cause artifacts, especially when fats are subjected to elevated temperatures.

Soybean oil and other linolenate-containing oils such as canola oil are notorious for their flavor deterioration noted at unusually low levels of oxidation, sometimes at peroxide values below 1. This flavor defect is known as “reversion”, an obsolete term derived from the flavor characteristic of crude soybean oil described as “beany” or “grassy”. Oils susceptible to this flavor defect contain oxidatively derived dimers and oligomers produced during deodorization by thermal decomposition of hydroperoxides present initially

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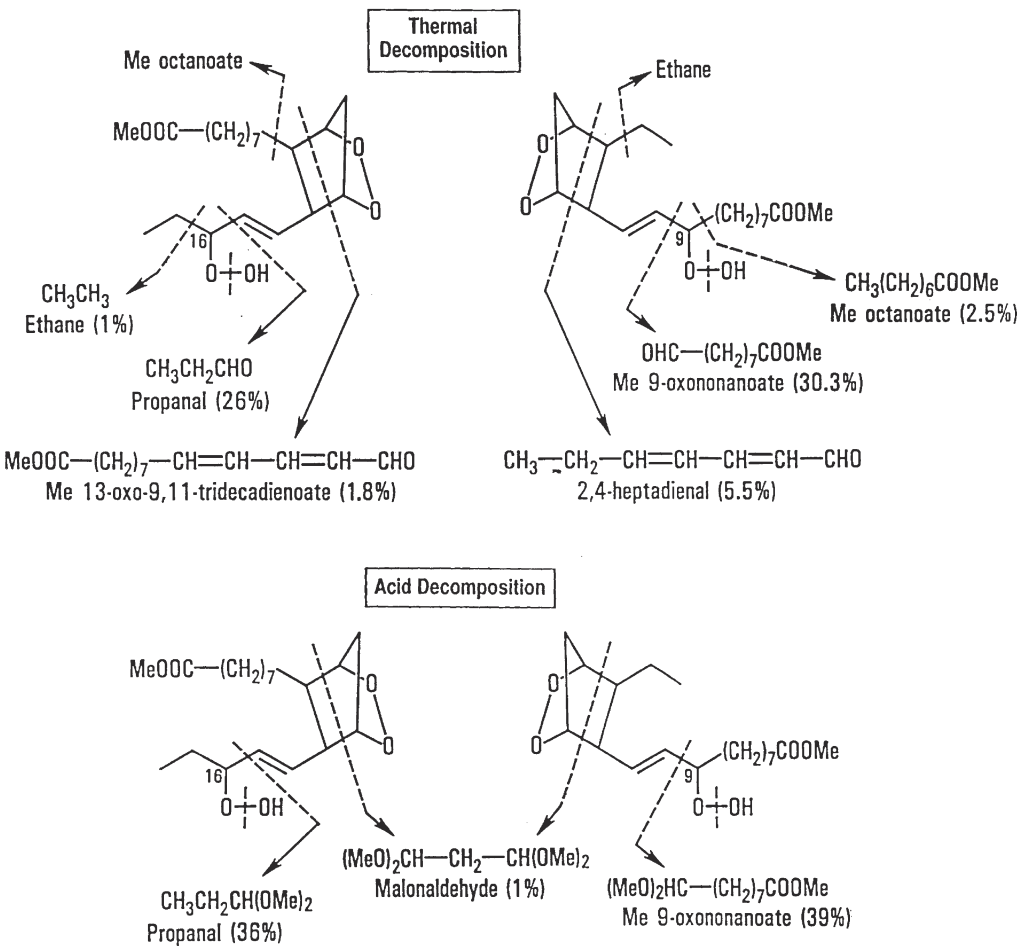


Figure 4.25. Volatile products formed by acid versus thermal decomposition of hydroperoxy bicyclo-endoperoxides of methyl linolenate (Frankel *et al.*, 1984). Aldehydes produced under acid conditions were analysed as the di- or tetramethyl acetals.

and formed during processing (Section C). These high molecular weight compounds, also known as oxidative dimers, are easily decomposed into volatile compounds with high impact on flavor. The volatile oxidation products containing an *n*–3 double bond derived from linolenate have unusually low threshold values, thus contributing to the flavor deterioration detected at low peroxide values (Chapter 5).

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CHAPTER 5

Methods to determine extent of oxidation

Previous chapters showed that lipids oxidized into complex mixtures of primary and secondary products that decompose into a multitude of low and high molecular weight compounds. These mixtures vary in nature and composition according to the conditions and extent of oxidation. The quality of food lipids is greatly influenced by the nature of these complex oxidation products and the levels and significance of their concentrations as they relate to the acceptability of the food. A large repertoire of methods is available to determine oxidative deterioration of food lipids. The usefulness of the results can be evaluated according to the chemical and physical properties measured, the specificity and precision of the analyses, and how they relate to real life storage of food products. For routine analyses, many methods have been developed to appraise the extent and nature of oxidative deterioration. The most commonly used methods are evaluated in this chapter according to the usefulness of the results in determining how closely they relate to flavor deterioration under ambient storage and processing conditions. Other advanced methods based on gas chromatography (GC), solid phase microextraction (SPME)-GC, GC-olfactometry (GC-O) and GC-mass spectrometry (MS) are also discussed in this chapter. Research methods to evaluate lipid oxidation and methods to determine the oxidative stability of unsaturated lipids are covered in Chapters 6 and 7.

A. Sensory methods

Sensory evaluation is a specialized discipline, using trained panels to measure and analyse the characteristics of food lipids evoked by the senses of taste, smell, sight and mouth feel. Sensory analyses are those most closely associated with the quality of food lipids, but their usefulness is limited because they are costly and require a well-trained taste and odor panel and the proper facilities. However, sensory analyses provide sometimes a useful approach to identifying flavor or odor defects in the processing of food lipids that cannot be detected by other more objective chemical or instrumental analyses. For example, certain flavor defects characterized as “grassy” or “fishy” in linolenate-containing oils such as soybean and low-erucic rapeseed (canola) oils (Chapter 1) occur at such low levels of oxidation that they can only be detected by sensory analyses. The old term “flavor reversion” for soybean oil is based on the characteristic of this oil undergoing flavor deterioration at unusually low levels of oxidation that cannot be measured by peroxide value determination. Oils derived from fish

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and algae containing long-chain polyunsaturated fatty acids such as EPA (20:5) and DHA (22:6) produce very powerful volatile compounds (also abbreviated as "volatiles"), causing "fishy" odors and flavors perceptible at concentrations of parts per billion (ppb) and extremely low levels of oxidation. Although these powerful volatiles can be detected and assessed by sensory odor evaluation with a trained panel, they require specialized gas chromatographic techniques with very sensitive detector systems (see Section F).

The two important types of taste panels include:

a) the analytical panel, using 5 to 20 experienced subjects who are trained to differentiate and rank samples according to a numerical scale, using reference standards; they should not be used to judge acceptability; and
b) the consumer panel, using 50 or more untrained subjects who are asked to express preference or acceptability of finished products; they should not be used to obtain descriptive information.

Liquid vegetable oils can be evaluated for odor and flavor according to a 10-point scale, to rate either intensity (bland to strong), or quality (excellent to bad). Freshly deodorized soybean oil is described as "bland"; weakly oxidized soybean oil is described as "buttery". As oxidation progresses, this oil is described as "beany" and "grassy". In the more advanced stages of oxidation, the oil becomes "rancid" and "painty". Because of the subjective nature of taste panel testing, different investigators vary widely in their descriptions of flavor defects in various oils. Further differences exist in the descriptive vocabulary or lexicon used by panels from different laboratories, different regions and different countries.

The types of flavors imparted by lipid oxidation in foods are more difficult to assess because there is wide variation in the sensory impact of different volatile products, and in the vocabulary used by taste or odor panels to describe their effect on quality. The diversity of sensory vocabulary used by different investigators to describe the same flavor defect in an edible oil has led to controversy on the origin of different flavor defects in soybean and other vegetable and fish oils.

The sensitivity of taste or odor panels can be measured by the ability of individuals to detect sensory characteristics. "Threshold values" are measures of the least concentrations of volatile compounds detected in a food matrix (oil or water) or minimum detectable level by at least 50% of the panelists. This definition is, however, now commonly used to refer to detection by 100% of the panelists. There is a considerable difference in the flavor significance of volatile decomposition products formed in oxidized or "rancid" lipids. Hydrocarbons have the highest threshold values ranging from 90 to 2150 ppm, and the least impact on flavor. Substituted furans with threshold values of 2–27 ppm, vinyl alcohols with threshold values of 0.5–0.3 ppm, and 1-alkenes

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Table 5.1. Comparison of threshold values between linoleate (*n*–6)-derived volatiles and linolenate (*n*–3)-derived volatiles

Linoleate volatiles	Threshold values (ppm)	Linolenate volatiles	Threshold values (ppm)
Pentanal	0.15	Propanal	1.6
Hexanal	0.15	2-Pentenal [a]	0.046
2-Heptenal	0.63	3-Hexenal [a]	0.09
2-Octenal	1.0	2,4-Heptadienal [a]	0.055
2-Nonenal/3-nonenal	0.1/0.03	2,6-Nonadienal [a]	0.002
2,4-Decadienal	0.28	3,6-Nonadienal [a]	0.0015
2-Pentyl furan	2.0	2,4,7-Decatrienal [a]	0.15

[a] Aldehydes with *n*–3 unsaturation

with threshold values of 0.02–9, are also relatively insignificant in flavor impact. Volatile compounds of increasing flavor intensity include 2-alkenals with threshold values of 0.04–2.5 ppm, alkanals with threshold values of 0.04–1.0 ppm, *trans,trans*-2,4-alkadienals with threshold values of 0.04–0.3 ppm, isolated alkadienals with threshold values of 0.0003–0.1 ppm, isolated *cis*-alkenals with threshold values of 0.0003–0.1 ppm, *trans*-2, *cis*-4-alkadienals with threshold values of 0.002–0.006 ppm and vinyl ketones with threshold values of 0.00002–0.007 ppm.

The volatiles derived from oils containing linolenic acid (soybean and canola oils) have significant sensory impact and lower threshold values than the volatiles derived from oils containing linoleic acid (cottonseed, corn and sunflower oils) (Table 5.1). The most sensory-significant linolenate-derived aldehydes (with lower threshold values) were characteristic in having *n*–3 unsaturation. These trends explain why linolenic acid oils develop undesirable odors and flavors at much lower levels of oxidation (peroxide value of less than 1) than linoleic acid oils (peroxide value of 10). Similarly, potent volatile aldehydes have been identified in fish oil oxidized at very low levels of oxidation by static and dynamic headspace GC (see F.2) and detected by GC-MS at parts per billion levels, including *cis*-4-heptenal (1250 ppb), *trans,cis*-2,6-nonadienal (1231 ppb) and *cis,cis*-3,6-nonadienal (627 ppb). *Cis*-4-heptenal is produced by decomposition of *trans,cis*-2,6-nonadienal, which can be produced in turn by the decomposition of *n*–7 and *n*–9 hydroperoxides derived from the oxidation of 20:4, 20:5 and 22:6 *n*–3 PUFA (Chapter 4, D4).

According to their relative threshold values, volatile compounds are also referred to as "aroma active". The aroma value $\mathbf{A}_x$ (also referred to as "odor unit") of a compound x is defined as:

$$\mathbf{A}_x = C_x/a_x$$

where C_x is the concentration of compound x in the food, and a_x is the odor threshold or the minimum odor detectable level of compound x in the food.

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This value is used to estimate the contribution of a volatile compound to the odor (or flavor) of the food sample.

Odor threshold values vary widely according to the structures of the carbonyl compounds, the matrix (oil or water), and according to the method of sensory detection (nasal: through the nose) or (retronasal: through the mouth and back of the oral cavity) (Table 5.2). In oil aldehydes have significantly higher odor thresholds and could be perceived at lower concentrations by retronasal rather than simple nasal sensations because of the different flavor release phenomena in the headspace *vs* the mouth and the volatiles are not lost between the headspace of the sample and the nose. Generally, the nasal response is simply referred to as "odor" and the retronasal response as "flavor". Flavor is due to the combination of the chemical senses from the oral, nasal cavities, including taste, smell, and chemical irritation perceived when the sample is placed in the mouth. All aldehydes have significantly lower threshold values in water than in oil matrices, because of their lower partition in water, resulting in greater concentrations detectable in the air cavities of the nose. Differences in responses between emulsions and oils are also explained by differences in flavor release from these systems. The descriptive vocabulary used for flavor quality in Table 5.2 is unfortunately too vague and not sufficiently specific for the use of panels in different sensory laboratories around the world.

The sensory data obtained from a panel are calculated by determining the overall mean scores for intensity or quality: total score points divided by the number of panelists for each sensory session. In some panel procedures, the scores are discarded if the means differ by more than two units from the average score. This procedure requires at least 10 or 12 final judgments for statistical analyses. The significance of the overall mean scores is calculated statistically by two-way analysis of variance (ANOVA). Sensory scores can also be correlated with the results of other tests of lipid oxidation by regression analyses. If an objective test correlates well with sensory analyses, it is usually interpreted as giving similar information regarding the level of oxidation. However, correlation data must be interpreted with care because they can only be used to show trends between two sets of analyses, and cannot be used to obtain cause and effect relationships.

More sophisticated statistical treatments of sensory data have been more commonly applied in studies of multiple factors of lipid oxidation on quality of foods, including "Multivariate" and "Principal Component" analyses. These procedures attempt to simplify complex relationships of several factors and sets of data into more understandable levels. Multivariate analysis is based on the fact that one measured property generally depends on more than one factor and the classical statistical "univariate" methods dealing with just one variable at a time are inadequate to analyse complex data**.**

The main limitations of sensory techniques include poor reproducibility of the data generated by a panel of human subjects who can occasionally and

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Table 5.2. Odor threshold values of volatile carbonyl compounds produced by lipid oxidation

Aldehydes	Odor threshold in oil (ppm)		Odor threshold in water (ppm)	Descriptions
	Nasal	Retronasal	Nasal	
Hexanal	320	75	12	Tallowy, green leafy
Heptanal	3200	50	5	Oily, fatty
Octanal	320	50	0.8	Oily, fatty, soapy
Nonanal	13500	260	5	Tallowy, soapy-fruity
Decanal	6700	850	5	Orange peel like
2-*t*-Pentenal	2300	600	–	Pungent, apple
2-*t*-Hexenal	10000	400	50	Apple
3-*c*-Hexenal	14	3	0.25	Green, leafy
2-*t*-Heptenal	14000	400	51	Fatty, bitter almond
2-*t*-Nonenal	900	65	0.8	Tallowy, cucumber
3-*c*-Nonenal	250	35	–	Cucumber
2-*t*-Decenal	33800	150	–	Tallowy, orange
2-*t*,4-*c*-Heptadienal	4000	50	–	Frying odor, tallowy
2-*t*,4-*t*-Heptadienal	10000	30	–	Fatty, oily
2-*t*,4-*t*-Nonadienal	2500	460	–	Fatty, oily
2-*t*,6-*c*-Nonadienal	4	1.5	–	Cucumber-like
2-*t*,4-*c*-Decadienal	10	–	–	Frying odor
2-*t*,4-*t*-Decadienal	180	40	0.2	Frying odor
2-*t*,4-*c*,7-*c*-Decatrienal	–	24	–	Cut beans
4-*t*,5-Epoxy-2-*t*-decenal	1.3	3	0.12	Metallic

From Belitz and Grosch (1999).
Abbreviations: *t* = *trans*, *c* = *cis*.

unexpectedly react erratically in spite of extensive training and expensive facilities. This method also requires relatively large amounts of samples. The approach currently recommended is to use precise chemical and instrumental methods to complement and support the sensory analyses (see Section F).

B. Peroxide value

This method is one of the oldest and most commonly used measurements of the extent of oxidation in oils. The standard iodometric procedures measure by titration, or colorimetric or electrometric methods, the iodine produced by potassium iodide added as a reducing agent to the oxidized sample dissolved in a chloroform–acetic acid mixture. The liberated iodine is titrated with standard sodium thiosulfate to a starch endpoint. The peroxide value (PV) is expressed as milliequivalents of iodine per kg of lipid (meq/kg), or as millimole of hydroperoxide per kg of lipid (referred to as peroxide). PV expressed as meq/Kg = 2 × PV mmol/kg.

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The standard iodometric method for peroxide value requires a sample of 5 g for peroxide values below 10, and about 1 g for higher peroxide values. The sensitivity of this method is about 0.5 meq/kg, and can be improved by determining the iodine starch-end point colorimetrically, or the liberated iodine electrometrically by reduction at a platinum electrode maintained at constant potential.

The results of peroxide value determinations vary according to the procedures used as a result of interference from oxygen in the air, exposure to light, and absorption of iodine by the unsaturated fatty acid in the oils. Although by carrying out the procedure in the absence of air and in the dark can reduce this interference, these precautions are not generally taken for routine analyses. The method is therefore highly empirical and dependent on the technique of the operator.

The ferric thiocyanate method for peroxide value is based on the oxidation of ferrous to ferric ions, which are determined colorimetrically as ferric thiocyanate. This method is more sensitive and requires a smaller sample (about 0.1 g) than does the iodometric method (Table 5.3). However, the values obtained by the ferric thiocyanate method are higher by a factor of 1.5 to 2 relative to those of the iodometric method. The peroxide values obtained by both methods are of only relative significance. The ferric thiocyanate method is commonly applied to dairy products, which undergo oxidative deterioration at relatively low peroxide values. Other colorimetric methods for peroxide values include the determination of the blue starch-iodine complex in the iodometric method, the red color developed with 1,5-diphenyl-carbohydrazide, the color developed with ferric ions and xylenol orange, ferrous chloride and 2,6-dichlorophenolindophenol, titanium dichloride, and xylenol orange.

The determination of peroxide value is useful for bulk oils that can be analysed directly. For foods, emulsions or muscle tissues, the lipid is extracted with mixtures of solvents that must be carefully removed by evaporation

Table 5.3. Comparison of different methods to determine extent of oxidation [a]

Method	Lo-OOH [b] Absorbance	Sens [c]	Me linoleate PV 475	Sens [c]	Me linolenate PV 450	Sens [c]
Conj. diene	7.42	1	0.55	1	0.91	1
Conj. triene	0.07	<0.1	0.06	0.1	0.29	0.3
Fe thiocyanate	26.4	3.6	5.2	9.4	5.7	6.3
TBA	0.07	<0.1	0.03	<0.1	0.42	0.46
Anisidine value	0.12	<0.1	0.16	0.3	0.75	0.8

[a] Adapted from Tsoukalas and Grosch (1977).
[b] Linoleic acid hydroperoxides in cyclohexane-diethyl ether solution.
[c] Sensitivity = calculated absorbance values divided by normalized diene absorption for a solution diluted 1:10.

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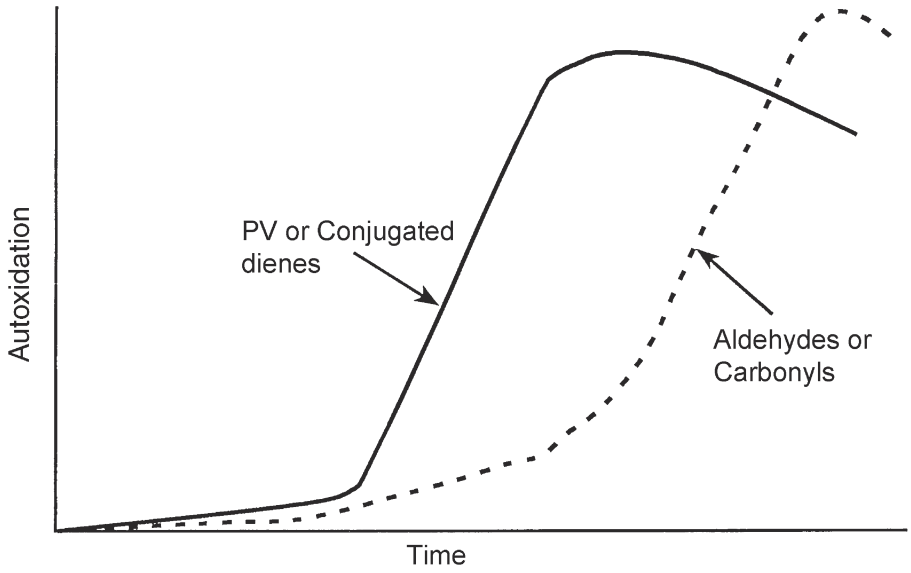


Figure 5.1. Development of hydroperoxides, as measured by peroxide value (PV) or conjugated dienes, and decomposition of hydroperoxides, as measured by aldehydes and carbonyls, during the autoxidation of an unsaturated fat.

without decomposition of hydroperoxides. This solvent extraction is not quantitative for samples that are highly oxidized or subjected to drastic conditions of storage or processing. Solvents should not contain antioxidants as stabilizers, which would act as reducing agents and interfere with the peroxide value determination.

The peroxide value of oil is an empirical measure of oxidation that is useful for samples that are oxidized to relatively low levels (peroxide values of less than 50), and under conditions sufficiently mild so that hydroperoxides are not markedly decomposed. During autoxidation, the peroxide value of oil reaches a maximum followed by a decrease at more advanced stages varying according to the fatty acid composition of the oil and the conditions of oxidation (Figure 5.1). The peroxide value maximum occurs at earlier stages of oxidation in the more polyunsaturated oils, such as soybean and rapeseed oils containing linolenate, and fish oils containing *n*–3 polyunsaturated fatty acids, because their hydroperoxides decompose more readily. Hydroperoxides are rapidly decomposed during oxidation at temperatures above 100°C, or in the presence of metals, or when exposed to light. The peroxide values of oils oxidized under these accelerated conditions are misleading and erroneously low especially for polyunsaturated oils because of significant decomposition of hydroperoxides. Therefore, knowledge of the history of the oil samples is important to better interpret the significance of peroxide value measurements (see Chapter 7).

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C. Conjugated dienes

The conjugated diene hydroperoxides produced from polyunsaturated lipids can be determined quantitatively by their strong absorption maximum at 234 nm (molecular extinction of 25,000–29000 for a solution of 1 mole hydroperoxides/l methanol). A weighed sample is simply diluted in methanol (for esters) or in isooctane (for triacylglycerols) and the absorbance at 234 nm measured spectrophotometrically. Aqueous emulsions can also be measured directly by dissolving in methanol even if the solutions becomes slightly cloudy, provided a suitable blank is used with the same diluted unoxidized sample. The conjugated diene value of lipids can be determined by their UV absorbance at 234 nm and expressed in μmol hydroperoxides/g sample.

The measurement of conjugated dienes is a sensitive method to follow the early stages of lipid oxidation under conditions in which hydroperoxides undergo little or no decomposition. The method requires 50 to 100 mg of sample, is simple and does not depend on chemical or color reactions. As with peroxide value determinations, the conjugated diene determination will reach a maximum during the progress of oxidation and decrease when the rate of decomposition of hydroperoxides exceeds the rate of their formation (Figure 5.1). At more advanced stages of oxidation, the hydroperoxides decompose into secondary and polymeric products that also absorb at 234 nm, but with lower extinction than the monohydroperoxides, and at 270 nm due to conjugated trienes. Although the hydroperoxides of oxidized methyl linolenate have conjugated diene structures that absorb mainly at 234 nm, they produce secondary products that absorb at 270 nm for conjugated trienes and carbonyl compounds that contribute to other tests (carbonyl, TBA and anisidine values) discussed below (Table 5.3).

As discussed above for the peroxide value, the conjugated diene method is not suitable for oils that have been heated under conditions that decompose hydroperoxides. Interference will occur from small amounts of conjugated diene and triene products found in hydrogenated fats, and small amounts of conjugated trienes in linolenate-containing oils (soybean and rapeseed oils) produced by deodorization at elevated temperatures (above 250°C). The conjugated diene method is also not suitable for oils containing low levels of polyunsaturated fatty acids such as olive oil, high-oleic sunflower oil and high-oleic safflower oil.

D. Carbonyl compounds

Total carbonyl content can be determined in oxidized lipids by the reaction with 2,4-dinitrophenylhydrazine, and the colored hydrazone (2,4-DNPH) derivatives are measured spectrophotometrically at 430–460 nm, and expressed as nmol hexanal/kg sample. The classical method for total carbonyl

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value using trichloroacetic acid catalyst causes further decomposition of hydroperoxides. This interference can be eliminated by reducing the hydroperoxides with stannous chloride, or by separating the hydrazone derivatives by vacuum distillation. During autoxidation of unsaturated lipids, the carbonyl value increases slowly followed by a rapid rate after the hydroperoxides reach a maximum corresponding to their decomposition into aldehydes and carbonyl products (Figure 5.1). Because the carbonyl value is related to decomposition carbonyl compounds, which contribute to flavor deterioration, it provides information relevant to sensory evaluation.

The anisidine test measures high molecular weight saturated and unsaturated carbonyl compounds in triacylglycerols. The anisidine value is defined as the absorbance of a solution resulting from the reaction of 1 g fat in 100 ml of isooctane solvent and reagent (0.25% anisidine in glacial acetic acid). The anisidine test is often attributed to 2-alkenals but no direct evidence is published for it. Oxidized methyl linolenate and other *n*–3 polyunsaturated fats produce significantly higher anisidine values than oxidized methyl linoleate because of the greater tendency of *n*–3 polyunsaturated hydroperoxides to decompose into carbonyl compounds (Table 5.3). Fish oils containing long-chain *n*–3 PUFA are particularly susceptible to oxidative deterioration during processing, and tend to have relatively high initial anisidine values. The test provides useful information on non-volatile carbonyl compounds formed in oils during processing. This type of deterioration can be serious in poorly processed oils containing linolenate (soybean and rapeseed) and long-chain *n*–3 PUFA (fish). Several high molecular weight decomposition products discussed previously, including oxidative dimers of triacylglycerols, aldehydoglycerides or core aldehydes (Chapter 4.C and 4.D), contribute to the anisidine test in poorly processed and thermally abused fats. Like the column chromatographic determination of dimers (see Chapter 6.B), the anisidine test on freshly processed oils can be used as a rough predictor of their future storage stability (Figure 5.2). This test is also useful for estimating "hidden oxidation", on the basis of high molecular weight decomposition compounds produced during vegetable oil processing and prior to deodorization (Figure 5.2) (see Chapter 4.C). These high molecular weight decomposition products can also be analysed by size exclusion chromatography (or gel permeation chromatography) (Section G and Table 8.3). The so-called Totox value (anisidine value + 2 PV) is used as an empirical measure of the precursor non-volatile carbonyls present in processed oils plus any further oxidation products developed after storage. Good quality oil should have a Totox value of less than four.

Other methods for determining carbonyl compounds involve derivatization followed by column chromatography or by HPLC or by GC. Total carbonyls in lipid extracts are converted to their 2,4-DNPHs by using a column of diatomaceous earth impregnated with 2,4-DNPH, followed by extraction and spectrophotometric measurement at 340 nm. By reaction with methylhydrazine,

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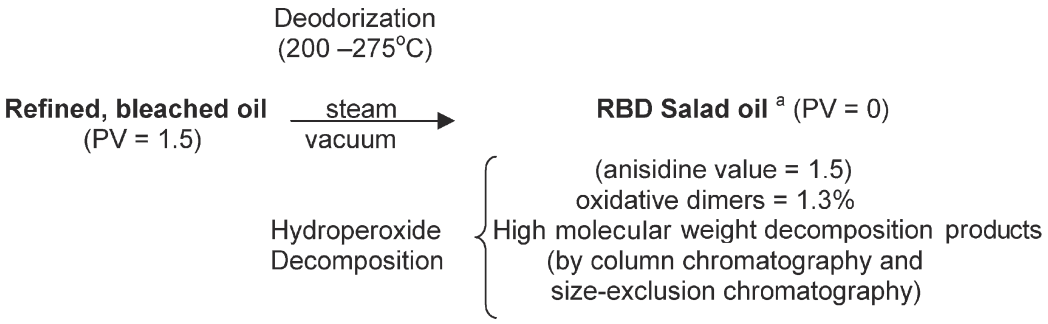


Figure 5.2. "Hidden oxidation" as a measure of future stability, based on anisidine value, oxidative dimers and high molecular weight decomposition products (relatively high in *n*–3 polyunsaturated oils).

saturated carbonyls (formaldehyde, acetaldehyde and hexanal) are converted to hydrazones, α,β-unsaturated carbonyls (acrolein and 4-hydroxy-nonenal) to pyrazolines, and β-dicarbonyls (malonaldehyde) to methyl pyrazole derivatives, and determined by GC.

E. 2-Thiobarbituric acid (TBA) value

The TBA test is an old and popular colorimetric method used to measure rancidity in some foods and oxidation products in biological systems. This test is based on the pink color absorbance at 532–535 nm formed between TBA and oxidation products of polyunsaturated lipids. Because this reaction is not specific and is produced by a large number of secondary oxidation products, these products are referred to as TBA-reactive substances or TBARS. The colored complex was originally characterized as being due to the condensation of two moles of TBA and one mole of malonaldehyde, a decomposition product of lipid hydroperoxides under the thermal acidic conditions of the test (Figure 5.3). On this basis, the test is standardized by using malonaldehyde generated by acid hydrolysis of 1,1,3,3-tetraethoxypropane, the tetraethoxyacetal of malonaldehyde. The TBA value is defined as the mg of malonaldehyde per kg of sample. However, malonaldehyde is in fact not detected in many oxidized lipids and is often a minor secondary oxidation product.

Many factors affect the production of the pink color in the TBA test, including temperature and time of heating, pH, metal ions, and antioxidants. Numerous variations of this test are aimed either at increasing the sensitivity by heating in acids and adding ferric ions, or at reducing the artifactual production of decomposition materials under the conditions of the test, by adding an antioxidant such as BHT or a chelator such as EDTA. Heating in acids with iron causes further oxidation and decomposition of oxidized lipids. Although BHT can reduce this decomposition, such addition confounds the application of the

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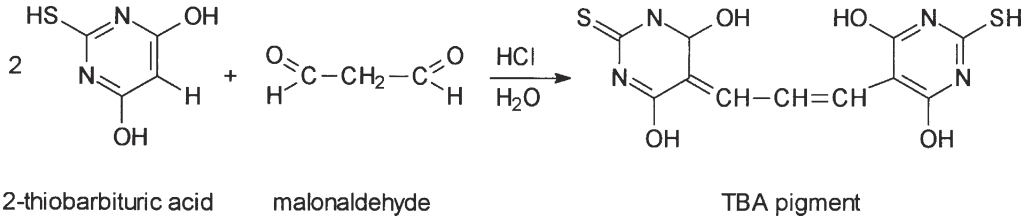


Figure 5.3. Proposed structure of TBA pigment as a colored adduct between TBA and malonaldehyde. From Sinnhuber *et al.* (1958).

TBA test in studies of the effect of natural or added antioxidants. Other variations are aimed at increasing the selectivity of the test by separating TBARS by steam distillation of the volatile components, or by HPLC of the peak corresponding to the TBA-malonaldehyde adduct.

Many precursors of malonaldehyde have been suggested including monocyclic endoperoxides and bicyclic endoperoxides, which are produced as secondary products in polyunsaturated lipids containing three or more double bonds (Chapter 4). The TBA test is consequently more sensitive when used with these polyunsaturated lipids and underestimates the oxidation products from lipids containing mainly oleic and linoleic acids.

Pure malonaldehyde has been determined by acid decomposition of various pure fatty acid oxidation products by GC as the tetramethylacetal derivative produced by acetalation under acid methanolic conditions (Table 4.1). The yields of malonaldehyde varied from 0.18–0.26 mol% for the monohydroperoxides of methyl linoleate to 0.22–0.44 mol% for the monohydroperoxides of methyl linolenate, from 2.20–2.34 mol% for the hydroperoxy epidioxide of methyl linolenate to 24.2 mol% for the hydroperoxy epidioxide of methyl linoleate oxidized with singlet oxygen, 4.14 mol% for the hydroperoxy bicyclo-endoperoxides of linolenate, and from 0.81 for the 1,3-dihydroperoxides of linolenate to 4.76 for the 1,3-dihydroperoxides of linolenate. This determination of pure malonaldehyde as the tetramethylacetal derivative by GC showed no correlation with the malonaldehyde determined by the TBA method, which was not specific for most of the pure fatty acid oxidation products examined (Table 4.1, Table 5.4). As expected from the acid cleavage mechanism discussed in Chapter 4, the 1,7- and 1,8-dihydroperoxides of linolenate produced no malonaldehyde by the GC analysis of the tetramethyl acetal derivative. In contrast, the TBA method gave values corresponding to 2.74 and 7.63 mol% malonaldehyde. Several other studies compared the TBA test and methods measuring pure malonaldehyde as stable derivatives by GC and HPLC. Determinations of malonaldehyde in oxidized vegetable oils were 2 to 17 times higher by the TBA test than by HPLC of the dansyl hydrazine (Table 5.4). For autoxidized fatty esters, the malonaldehyde values by the TBA test were higher than by HPLC by a factor of 6 for methyl oleate, 7 for methyl

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Table 5.4. Determination of malonaldehyde (MDA) by the TBA test and by HPLC of the dansyl hydrazine derivative [a]

Samples [b]	Peroxide value (meq/Kg)		MDA by TBA (ppm)		MDA by HPLC (ppm)	
	20 hr	50 hr	20 hr	50 hr	20 hr	50 hr
Soybean	11.2	31.4	0.86	4.31	0.28	0.79
Corn	48.0	92.2	0.70	6.09	0.23	0.35
Rapeseed	17.1	28.8	5.00	6.39	0.82	1.93
Olive	8.8	21.6	0.70	1.05	0.27	0.33
Peanut	20.4	37.9	0.80	1.10	0.33	0.35
Methyl oleate	18.5	–	2.58	–	0.44	–
Methyl linoleate	62.5	–	5.36	–	0.75	–
Methyl linolenate	799.3	–	147.0	–	2.63	–

[a] From Hirayama *et al.* (1983).
[b] Vegetable oils were oxidized at 100°C and fatty esters at 37°C in the dark.

linoleate and 56 for methyl linolenate. Malonaldehyde levels determined in muscle tissues by the TBA test were 4 to 5 times higher than HPLC analyses of malonaldehyde. The TBA test evidently measures many other decomposition products than malonaldehyde. Other derivatives have been used to analyse free malonaldehyde by HPLC and GC (pyrazole and thiazolidine) (See Section D).

In addition to malonaldehyde, yellow and orange pigments are formed by other decomposition products under the TBA conditions, including alkanals, 2-alkenals, and 2,4-alkadienals absorbing at 450 nm, and by alkenals and 2,4-alkadienals absorbing at 532 nm. Other components present in foods and biological systems, such as browning reaction products, protein and sugar degradation products, amino acids, nucleic acids and nitrite, interfere with the formation of the TBA color complex. Amino acids and carbohydrates decompose in the presence of iron to produce TBARS. Significant interference is observed with the TBA test by the unavoidable presence of metals in food lipids, which also contain natural antioxidants. The literature reports conflicting studies correlating TBA values with sensory evaluations of food lipids. The TBA test is thus notoriously unspecific and unsuitable for complex food materials and biological systems containing non-lipid materials contributing to the color reaction.

F. Gas chromatographic methods

GC methods are capable of determining volatile oxidation products that are either directly responsible for or serve as markers of flavor development in oxidized lipids. GC analyses for volatile compounds not only correlate with flavor scores by sensory analyses, but also provide sensitive methods to detect low levels of oxidation in various oils and food lipids. Volatile compounds

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suitable for GC determinations are mainly aldehydes, ketones and hydrocarbons. Three commonly used GC methods for volatiles are discussed below.

1. Static headspace method

This method includes four steps: i) measuring and sealing a sample into a vial or suitable container; ii) heating to a given temperature until the components of interest diffuse and vaporize into the gas phase and reach or approach equilibrium; iii) injecting an aliquot of the gas phase headspace directly into the gas chromatographic column, either manually with a heated gas-tight syringe, or by using automated systems after pressurizing the heated sample container; and iv) separating the volatile compounds on either a packed or capillary column heated isothermally or by temperature programming.

After equilibration, the concentration determined in the headspace is proportional to the concentration of the volatiles in the sample. Proportionality is based on the equilibrium established when the sample is heated. The method depends on the mass balance of solutes in the sealed system by taking account of their distribution or partition constants, K,

$$K = C_l/C_g$$

where C_l = concentration of solute in liquid phase, and C_g = concentration of solute in gas phase.

Quantification is thus based on the distribution of the volatile compounds between the static gas phase and the sample matrix under defined discontinuous gas extraction conditions, keeping the volumes of sample phase and of the gas phase constant. Because this method is very dependent on the nature of the sample matrix, the identity and quantity of each component are determined by comparison with known pure standards added in the same matrix as the sample analysed.

This simple technique permits the quantitative analysis of volatile compounds in various liquid, semi-liquid or solid foods, in biological fluids and tissues, and environmental contaminants in water, air and soils. The method is very sensitive to the equilibrium solute distribution between phases at the temperature selected for the analysis. Equilibration is greatly dependent on the solubility and viscosity of the samples. This method is particularly suited to highly volatile compounds because they have a favorable equilibrium between liquid (or solid) phase and its headspace, producing a higher concentration of volatile compounds in the headspace.

The static headspace method is rapid and suitable for routine consecutive analyses of many samples. This method has the important advantage in not requiring any cleaning between sample injections, because only the volatile components are injected into the gas chromatograph and the nonvolatile portion of the sample is retained in the vial. Since this method is applicable to

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a wide range of liquid, solid and semi-solid samples without manipulation, complex food systems can be analysed directly without manipulations or extractions. This is an important advantage over other methods designed for pure oil samples, or that require extraction of the lipids from complex food or biological systems. Such extractions can create many artifacts and poor recoveries that may confound the analyses of rancid foods. The main disadvantage of the static headspace method is the difficulty of reaching complete equilibrium with viscous and semi-solid samples and with oxidized polyunsaturated lipid samples that can be easily decomposed during the equilibration-heating step. Although heating can accelerate equilibration of volatiles, the volatile precursors decompose rapidly at temperatures above 100°C and additional volatiles are then produced. For rapid routine analyses, the samples are heated between 40 and 60°C for 15 to 20 min prior to injection without achieving complete equilibration. Under these non-equilibrium conditions, rigorous standardized control of temperature and time of heating is required to obtain reproducible results.

2. Dynamic headspace method

This method, also known as "purge-and-trap", is more elaborate than the static headspace method. It includes four steps: i) purging or sweeping a liquid sample with nitrogen or helium in a heated tube or vessel; ii) trapping the vaporized volatiles into a short column containing a porous polymer (Tenax, Chromosorbs or Porapaq) or charcoal with or without cooling; iii) desorbing the volatiles from the trap at elevated temperatures and transferring, by backflushing with carrier gas, into the capillary inlet of the gas chromatograph; and iv) separating the volatile compounds by GC. Commercial instruments are available that allow collection of volatiles from many samples into a series of traps, which are subsequently desorbed sequentially in an automatic temperature-controlled system, prior to injection into a cryoscopically cooled capillary GC column for separation of volatiles. Quantification of the volatile compounds is based on the recovery of a suitable internal standard subjected to the same conditions as the sample. The recovery of volatile compounds by this method is based on their relative adsorption and desorption from the trap used under defined continuous gas extraction conditions. The "breakthrough volume" of the porous sorbent used in the trap is defined as the volume of gas required to move a particular compound through the trap column. This property of the sorbent determines the type and amount of volatile compound retained by the trap.

The sampling temperature used in the dynamic headspace method had a significant effect on the volatile profiles of soybean oil (Figure 5.4). At 60°C, the volatiles derived from linoleate (pentane, hexanal, 2-heptenal and 2,4-decadienal) contributed 50 to 54%, and the volatiles derived from linolenate

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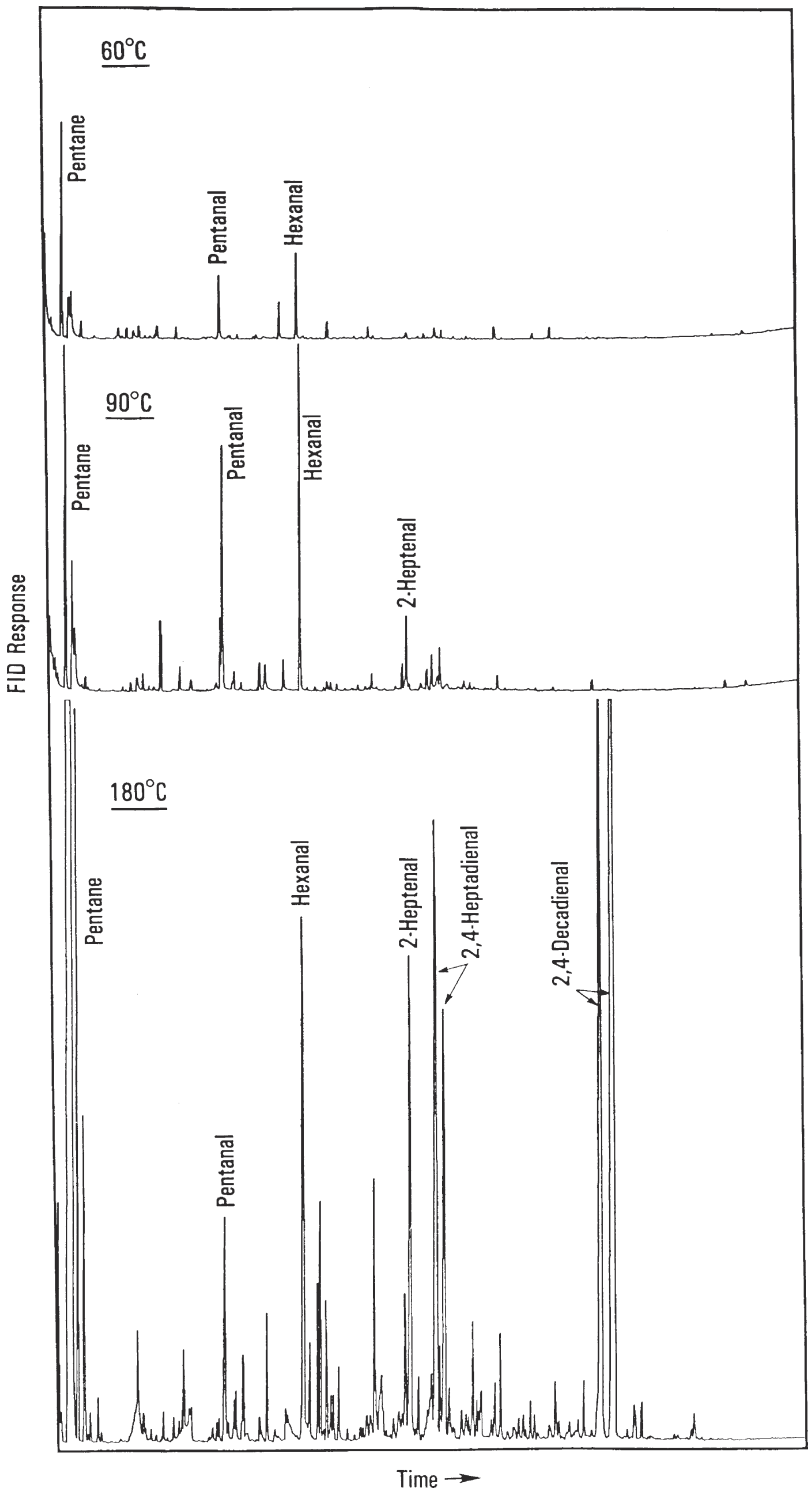


Figure 5.4. Effect of temperature on the dynamic headspace analyses of volatile compounds in oxidized soybean oil (PV 9.5) (Selke *et al.*, 1987). Courtesy of the *Journal of the American Oil Chemists' Society*.

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(2-/3-hexenal and 2,4-heptadienal) contributed 16% of the total peak area. At 180°C, the proportion of linoleate volatiles increased to 74–76% and the linolenate volatiles to 19%.

The dynamic headspace method has the advantage of using lower temperatures than the static headspace method, and permitting enhancement of trace components in complex mixtures of a wide range of volatile compounds. However, this method is slow and not suitable for routine analyses of many samples. Direct purging of liquid oil samples must be avoided to prevent fogging of the volatile compounds, which results in serious contamination of the transfer lines. The dynamic headspace method requires heated transfer lines and valves and multi-step manipulation of volatiles that must be controlled rigorously. The transfer lines and valves must be cleaned between analyses. Several blank runs are usually necessary to avoid sample contamination causing memory effects in the gas chromatograms. This method thus requires appreciable instrument downtime and maintenance. Newer models of automated dynamic headspace instruments overcome some of the problems of sample contamination, by collecting volatiles from different samples into a series of individual trap columns stacked in a rotating magazine. The trap columns are then successively heated in an automatic thermal desorber attached to a GC system.

A specialized dynamic headspace technique known as "solid-phase microextraction" (SPME) has been developed to accelerate the analyses of volatiles and reduce problems of sample contamination in the transfer lines and valves used in some instruments. This technique consists of extracting the volatiles from a sample by adsorption on a special unit with fused silica fiber coated with either a polymer or solid adsorbent. The fiber is attached to a stainless steel plunger surrounded by a protective needle. The needle is injected into a sample vial through a septum. The plunger is depressed into either the liquid sample or the headspace above the sample. Volatiles are allowed to adsorb on the fiber for 2–30 min. The fiber is retracted into the needle that is removed from the sample vial, and inserted into the GC port. The plunger is then depressed to expose the fiber to the heated zone of the injector to desorb and transfer the volatiles onto the GC column. The fiber is retracted and needle is removed from the GC port. Commercial automated versions of this technique have been developed. The GC chromatograms obtained by this technique are similar to those obtained by conventional dynamic headspace GC showing higher proportions of high molecular weight volatiles and lower proportions of low molecular weight volatiles (Figure 5.5). The technique of SPME permits high throughput and does not require extended sample preparations. However, analyses of complex mixtures of volatiles may be skewed if some compounds are present at relatively high concentrations, or have high affinity for the adsorbing fiber. Better estimates of true headspace concentrations can be obtained by short-time sampling (1 minute). This technique has been

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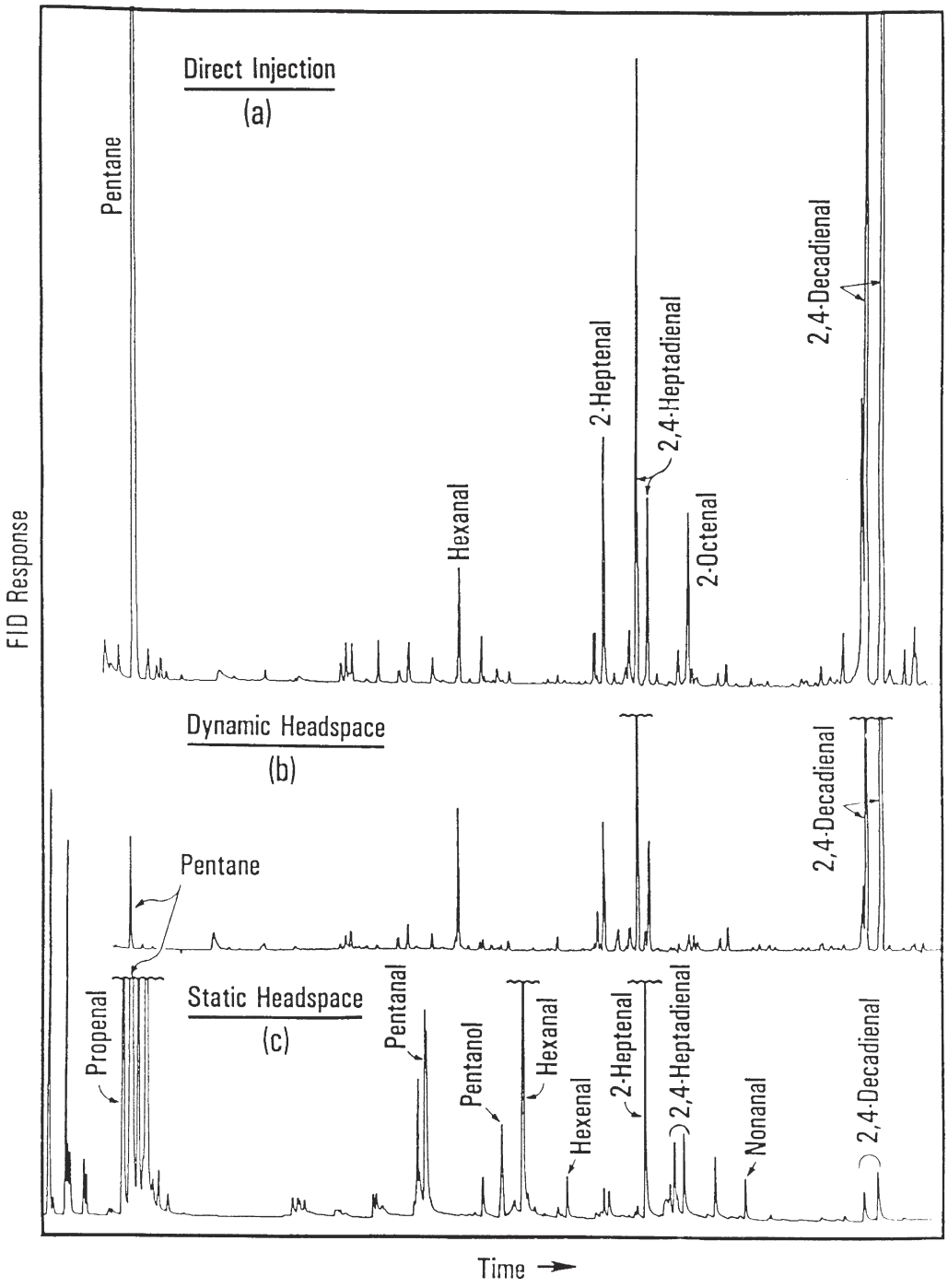


Figure 5.5. Comparison of three gas chromatographic methods for the analyses of volatile compounds in oxidized soybean oil (PV 9.5, heated to 180°C) (Snyder *et al.*, 1988). Courtesy of the *Journal of the American Oil Chemists' Society.*

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automated to avoid the problems from contamination between sample injections, and in heated transfer lines and valves requiring instrument downtime.

3. Direct injection method

The sample is transferred into the GC instrument, either through an injector insert packed with glass wool or column packing, or through a temperature-controlled external unit. The volatiles generated at the elevated temperature of the injector port are trapped at the head of a packed or capillary GC column either maintained at ambient temperature or cooled cryoscopically. The column is then subjected to temperature programming as required for separation. The direct injection method was first developed to analyse only hydrocarbons by using alumina columns, which completely adsorb the carbonyl and other volatile oxygenated polar compounds in oxidized oils. By this approach, ethane and pentane can be exclusively separated and analysed in oils and foods. The determination of pentane has correlated well with sensory evaluations of oxidized vegetable oils. The direct injection method has been applied successfully for the determination of pentane and ethane in the breath of animals as a non-invasive method to follow *in vivo* oxidation (Chapter 12). This method provided a useful approach to the study of hydroperoxide precursors of volatile decomposition products (Chapter 4).

The direct injection method operates by the same steps as the dynamic headspace method, including purging at the injector port, trapping at the head of the GC column and desorbing by heating the column. However, desorption of the volatiles is slower depending on the temperature program used for separation of the volatiles. For capillary GC columns, better separation of volatile compounds can be achieved by cryoscopic cooling of the head of the column or the entire oven of the gas chromatograph with liquid nitrogen or dry ice before temperature programming.

The direct injection method has similar advantages to the dynamic headspace method in detecting both low molecular weight and high molecular weight volatile compounds. Although the direct injection method permits some enrichment of the volatiles, it is not as efficient as the dynamic headspace method. The direct injection method is much simpler than the dynamic headspace method, more rapid and inexpensive because it does not require modification of the gas chromatograph. The direct injection method is therefore suitable for routine analyses of many samples. However, this method requires elevated temperatures, which cause further thermal decomposition of hydroperoxides and other lipid oxidation precursors. The sample inserts must be thoroughly cleaned or replaced between injections. It is necessary to run blanks between analyses to avoid or minimize contamination between injected samples.

The three GC methods described above were compared in the analysis of volatile compounds in a sample of oxidized soybean oil (Table 5.5). Each

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Table 5.5. Flavor significance of volatile compounds in oxidized soybean oil (peroxide value: 9.5) [a]

Major volatiles	Threshold values	Relative %			Weighted % [b]			Relative order		
		SHS	DHS	DI	SHS	DHS	DI	SHS	DHS	DI
t,*t*-2,4-Decadienal	0.10	0.3	40.5	46.9	0.03	4.1	4.7	7	2	2
t,*c*-2,4-Decadienal	0.02	1.0	21.5	23.8	0.5	10.8	11.9	3	1	1
t,*t*-2,4-Heptadienal	0.04	2.0	13.3	6.5	0.5	3.3	1.6	3	3	3
t-2-Heptenal	0.20	8.3	6.7	3.1	0.4	0.33	0.16	5	7	7
t,*c*-2,4-Heptadienal	0.10	2.5	5.4	3.1	0.25	0.54	0.31	6	6	6
Hexanal	0.08	24.7	5.4	6.9	3.1	0.68	0.86	2	5	5
Pentane	340	38.6	3.7	4.8	1.1[c]	0.11[c]	0.14[c]	10	10	11
t-2-Pentenal	1.00	1.2	1.4	1.9	0.01	0.01	0.02	8	8	9
1-Octen-3-ol	0.01	0.3	1.1	1.4	0.3	1.1	1.4	4	4	4
2-Pentylfuran	2.00	0.5	1.0	1.2	2.5[c]	6.0[c]	6.0[c]	9	9	10
Propanal	0.06	20.6	0.0	0.5	3.4	0.0	0.08	1	0	8

From Frankel (1985).

[a] Abbreviations: SHS = static headspace, DHS = dynamic headspace, DI = direct injection, *t* = *trans*, *t*,*c* = *trans*,*cis*, *t*,*t* = *trans,trans*

[b] Calculated on the basis of 1-octen-3-ol which has the lowest threshold value

[c] $\times 10^{-3}$

method produced significantly different GC profiles (Figure 5.5). The direct injection and dynamic headspace techniques produced much higher proportions of 2,4-decadienal and 2,4-heptadienal than the static headspace method. The static headspace method showed more low molecular weight and larger proportions of 2-heptenal, hexanal, pentane and propanal.

As discussed in Section A, the volatile lipid oxidation products vary widely in their sensory impact. It is therefore important to estimate the relative flavor significance of different volatile compounds in addition to their relative concentration in oil. The flavor significance of the volatiles listed in Table 5.5 were estimated by calculating weighted percentages on the basis of 1-octen-3-ol, which has the lowest threshold value of 0.01. The volatile compositions showed wide differences in flavor significance according to the GC method employed. The direct injection method and the dynamic headspace methods showed the same seven most flavor significant compounds decreasing in the order: *trans,cis*-2,4-decadienal, *trans,trans*-2,4-decadienal, *trans,trans*-2,4-heptadienal, 1-octen-3-ol, hexanal, *trans,cis*-2,4-heptadienal, and 2-heptenal. This similarity in trend indicates that the direct injection and dynamic headspace methods operate on the same principle of purging, trapping and desorbing steps. However, by the static headspace method, which operates by equilibration, the seven most flavor significant and volatile compounds are propanal, followed by hexanal, *trans,trans*-2,4-heptadienal, 1-octen-3-ol, 2-heptenal, *trans*,*cis*-2,4-heptadienal and *trans*,*trans*-2,4-decadienal. Results of volatile GC analyses by the three methods described above correlated very well with sensory analysis of vegetable oils oxidized at different levels.

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Table 5.6. Potent volatile compounds identified in fish oil enriched milk stored at 2°C by dynamic headspace gas chromatography-mass spectrometry/gas chromatography-olfactometry [a]

Compound	Odor description	Detection frequency [b]
1-Penten-3-one	Plastic, leather	8
cis-4-Heptenal	Sweet	6
1-Octen-3-one	Mushroom	7
1-*cis*-5-Octadien-3-one	Green, leafy, geranium	6
trans,trans-2,4-Heptadienal	Rancid, fatty	6
trans,*cis*-Nonadienal	Cucumber, green	8

[a] Selected data from Venkateshwarlu *et al.* (2004). A panel of 12 assessors evaluated the original fish oil enriched milk to have a fishy odor (scores 1.5–1.4) and fishy taste (scores 2.1–2.4) after storage for 4 and 8 days.
[b] Number of panelists out of 10 detecting the odor compound after storage for 1 to 14 days.

4. Advanced GC methods

Gas chromatography-olfactometry (GC-O) techniques, also referred to as GC-sniffing port (GC-SP), have been developed to estimate potent odorants in foods. These techniques are based on the odor detection and description of volatile compounds by one or more trained panelists, as they emerge from each individual peak in GC effluents. Although these techniques eliminate the effect of interactions between mixtures of volatiles, the information may not be relevant to the flavor and odor psychophysical perception of complex mixtures of flavor compounds in a food or emulsion. The odor intensity of various compounds does not follow the same linear relationship. Volatile odor and flavor compounds in mixtures can interact by either intensifying or masking their effects on human perception. Complex flavor and odor defects such as "fishy", "fried flavor" and "hydrogenation", can be derived from a mixture of volatiles in which individual components do not impart the same impression or perception.

A case in point is illustrated by a recent study of the volatiles produced by cold storage of milk emulsions enriched with fish oil. Analyses for volatiles by dynamic headspace followed by GC-MS and GC-O after storage at 2°C showed that, although a panel of 10 assessors found 1-penten-3-one, *cis*-4-heptenal, 1-octen-3-one, 1-*cis*-5-octadiene-3-one, *trans*,*trans*-2,4-heptadienal, and *trans*-2, *cis*-6-nonadienal to be potent, they reported that none of these compounds have a fishy or metallic odor (Table 5.6). The original fish oil enriched milk was however evaluated by the sensory panel to have a fishy and metallic odor and taste after storage for four and eight days at 2°C. *Trans*,*cis*,*cis*- and *trans*,*trans*,*cis*-decatrienal were also identified in the fish oil enriched milk emulsions at peroxide values of 0.8 and 3.4, but were not described as fishy. Yet, these isomers of decatrienal have been previously recognized for their fishy odors and flavors in linolenate-containing vegetable oils, and in fish oils.

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Another powerful technique known as aroma extract dilution analysis is used to determine the most significant odor and flavor compounds in a complex mixture in a food product. This method determines the odor activity of volatile compounds in an extract eluted from a high-resolution capillary GC-SP column (see Table 11.9). The odor activity or impact of a compound is expressed as the flavor dilution factor (FD), which is the ratio of its concentration in the initial extract to its concentration in the most dilute extract in which the odor can be detected by GC-SP. However, the information from this technique may be of limited practical value, because it ignores the significant effect of food matrices on flavor and odor perception of mixtures of flavor and odor compounds. Advanced instrumental techniques have been developed for flavor analysis during food consumption. These techniques permitting direct mass spectrometry at atmospheric pressure are discussed in Chapter 6.

G. Fluorescence methods

Schiff base compounds formed by the interaction of oxidation products with proteins, phospholipids and nucleic acids produce chromophores showing characteristic fluorescence spectra. The Schiff base formed between malonaldehyde and amino acids is attributed to the conjugated structure: –NH=CH–CH=CH–NH–. Lipid-soluble fluorescence chromophores are produced from oxidized phospholipids and from oxidized fatty acid esters in the presence of phospholipids. These chromophores have fluorescence emission maxima at 435–440 nm and excitation maxima at 365 nm. The Schiff base of malonaldehyde and phospholipids has a higher wavelength maximum for emission (475 nm) and excitation (400 nm). The interaction between oxidized arachidonic acid and dipalmityl phosphatidylethanolamine produce similar fluorescence spectra (maximum excitation at 360–90 nm and maximum emission at 430–460 nm). The products from oxidized arachidonic acid and DNA have characteristic fluorescence spectra, with excitation maximum at 315 nm and emission maximum at 325 nm. Similar fluorescence spectra, with excitation maximum at 320 nm and emission maximum at 420 nm, are obtained from the interactions of either lipid hydroperoxides or secondary oxidation products with DNA in the presence of metals and reducing agents, or different aldehydes, ketones and dimeric compounds from oxidized linolenate. Therefore, the Schiff base produced from various oxidized lipids and phospholipids and DNA may be considered to be due to a mixture of closely related chromophores.

Several fluorescence techniques have been used to investigate oxidation products in animal tissues, in frozen fish muscle during storage, in freeze-dried meats, and in oxidatively damaged soybeans. Mixtures of tissues are extracted with chloroform–methanol (2:1, by volume), homogenized, mixed with water and centrifuged. The chloroform layer is analysed for fluorescence using

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quinine sulfate to calibrate fluorescence intensity and wavelength. Fluorescence has also been measured directly in the chloroform–methanol extract of ground samples of oxidatively damaged soybeans (Table 5.7). Fluorescence spectra for oxidized meats show maximum excitation at 350 nm and emission at 440 nm, and for damaged beans maximum excitation at 364 nm and emission at 437 nm. Similar fluorescent chromophores are observed in oxidized phosphatidylethanolamine and phosphatidylserine with excitation maxima at 365–370 nm and emission maxima at 435–440.

Interactions of lipid oxidation products with DNA related to aging or tissue injuries can also be determined by fluorescence. Fluorescence measurements are useful for estimating how DNA interacts with lipid hydroperoxides, secondary oxidation products and carbonyl decomposition products in the presence of metals and reducing agents (Table 5.8). Monohydroperoxides of linoleate and linolenate produced the highest levels of fluorescence, and hydroperoxyepidioxides of linolenate and dihydroperoxides of linoleate and linolenate were among the most active secondary products in forming fluorescence with DNA. Under the same test conditions, malonaldehyde produced very little fluorescence, yet malonaldehyde has been generally considered responsible for the cross-linking properties with DNA causing fluorescence. The importance of malonaldehyde in its cross-linking properties with DNA may thus have been exaggerated in the literature.

Various carbonyl compounds and decomposition products of methyl linolenate hydroperoxides were tested for their interaction with DNA by measuring fluorescence in the presence of ferric chloride and ascorbic acid. 2,4-Alkadienals and 2,4,7-decatrienals were among the most active decomposition products of linolenate hydroperoxides (Table 5.8). To determine the type of reactive species involved in fluorescence formation with DNA, the effect of free radical antioxidants and a singlet oxygen quencher were examined. β-Carotene, α-tocopherol and phenolic antioxidants strongly inhibited DNA fluorescence formed by decomposition of linolenate hydroperoxides in the presence of ferric chloride and ascorbic acid (Table 5.9). These results indicate that singlet oxygen and free radical species are important intermediates in the interaction of linolenate hydroperoxides with DNA in the presence of iron and ascorbic acid.

Fluorescence techniques are very sensitive. On a molar basis, the amount of malonaldehyde detected by fluorescence is 10 to 100 times more sensitive than the colorimetric TBA assay. However, this method is not specific, as it measures complex mixtures resulting from the interactions of oxidized lipids, unsaturated aldehydes and malonaldehyde with proteins, peptides, amino acids, phospholipids, DNA and nucleic acids. These interactions involve oxidized or polymerized species of proteins or amino acids. The fluorescence method provides a non-specific but sensitive measure of oxidative deterioration in complex foods such as meats and fish, which are often difficult to analyse for

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Table 5.7. Quality of soybeans stored at different moisture levels [a]

Storage time (days)	Fluorescence (relative units) [b]	Peroxide values (meq/kg)	Hexanal (peak areas) [c]
13% moisture			
22	603	0.0	1.3
49	621	1.0	1.5
20% moisture			
19	632	4.2	4.6
24	1213	4.3	0.6
26	1451	5.7	1.6

[a] From Frankel *et al.* (1987).
[b] Excitation 364 nm, emission 437 nm relative to quinine sulfate.
[c] Integrated areas × 10^3.

Table 5.8. Formation of fluorescence by DNA in the presence of lipid oxidation products, ferric chloride and ascorbic acid

Oxidation products	DNA fluorescence intensity [a]
Study I [b]	
Methyl linoleate monohydroperoxides	472
Methyl linolenate monohydroperoxides	725
Methyl linolenate hydroperoxyepidioxides	365
Methyl linolenate bicycloendoperoxides	246
Methyl linolenate dihydroperoxides	245
Malonaldehyde	8
Study II [c]	
Methyl linolenate monohydroperoxides	362
Autoxidized methyl linolenate volatiles [d]	115
Propanal	24
Hexanal	20
Methyl 9-oxo-nonanoate	23
2-Hexenal	49
2,4-Heptadienal	37
2,4-Decadienal	77
2,4,7-Decatrienal	115

[a] DNA fluorescence after 72 hours (excitation 315 nm, emission 420 nm)
[b] From Fujimoto *et al.* (1984).
[c] From Frankel *et al.* (1987).
[d] Low molecular weight fraction separated by gel permeation chromatography (or size exclusion chromatography) after decomposition of autoxidized methyl linolenate with iron-ascorbate at room temperature (mean molecular weight: 150, mixture of methyl heptanoate and octanoate, methyl 9-oxo-nonanoate, 2,4-heptadienal, and 2,4,7-decatrianal).

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Table 5.9. Effect of β-carotene, α-tocopherol and phenolic antioxidants on fluorescence formation by DNA in the presence of methyl linolenate hydroperoxides, ferric chloride and ascorbic acid [a]

Samples	Concentration (μM × 10^{-6})	DNA fluorescence intensity [b]
Methyl linolenate hydroperoxides	Control	356
+ β-Carotene	2–1900	40–65
+ α-Tocopherol	2–1800	25–49
+ β-Carotene + α-tocopherol	190 + 190	28
+ BHA	280–560	39–29
+ BHT	230–460	44–36
+ TBHQ	300–600	43–34

[a] From Frankel *et al.* (1987). Abbreviations: BHA, butylated hydroxyanisole, BHT, butylated hydroxytoluene, TBHQ, *tert*-butyl hydroquinone.
[b] Fluorescence intensity of DNA after 72 hours (excitation 315 nm, emission 420 nm).

carbonyl compounds, because they are complexed and covalently bonded with proteins.

H. Octanoate method

This method determines the residual ester product remaining attached to triacylglycerol after thermal cleavage of oxidized triacylglycerols. We have seen in Chapter 4 that decomposition of hydroperoxides produce aldehydes and esters by cleavage on either side of the hydroperoxide group (Figure 4.8). Methyl octanoate was an important cleavage product of hydroperoxides of fatty acid esters. The corresponding hydroperoxides of triacylglycerol produce methyl octanoate after alkali transmethylation**.** In order to reflect the total oxidation of an oil, the oxidized sample is heated under vacuum at 180°C to decompose all oxidation compounds and remove interfering volatile decomposition products. After thermal decomposition, methyl octanoate can be determined quantitatively by GC, as a measure of oxidation of triacylglycerols after the initial hydroperoxides are decomposed during processing. The octanoate assay thus provides a measure of the oxidative state of crude oils and oils at different stages of processing prior to deodorization. The results provide the same information as the anisidine method for aldehyde esters and aldehydo glycerides. Although the octanoate method is more specific than the colorimetric anisidine test, it may not be as sensitive**.**

I. Conjugatable oxidation products

More conjugated chromophores are produced by converting PUFA hydroperoxides and oxodienes in oxidized lipids into the corresponding allylic

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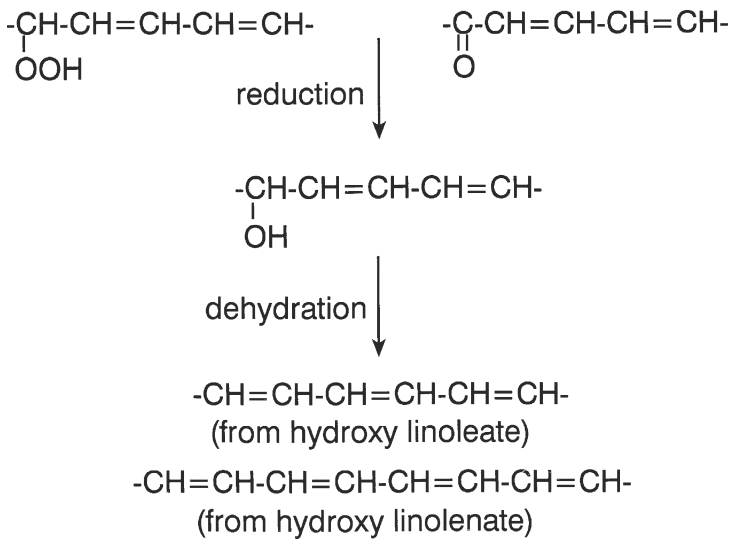


Figure 5.6. Production of conjugatable oxidation products by reduction and dehydration sequence.

alcohols by chemical reduction with sodium borohydride, followed by acid dehydration with ethanolic sulfuric acid (Figure 5.6). This reaction sequence has been used to characterize hydroperoxides of oleate, linoleate, and linolenate.

Accordingly, conjugated trienes absorbing at 268 nm are produced by dehydration of hydroxy linoleate, and conjugated tetraenes absorbing at 301 nm are produced by dehydration of hydroxy linolenate and hydroxy esters from PUFA containing more than three double bonds. The chromophores produced are measured by absorbance in the ultraviolet. The sum of the absorbance

Table 5.10. Analyses of conjugable oxidation products (COP) in different food lipids [a]

Food lipids [b]	Storage at 25°C, time	PV	Oxodiene	COP
Margarine	0 time	nil	nil	0.5
Margarine	120 days	31.4	0.3	11.9
Pastry mix	0 time	nil	0.3	0.5
Pastry mix	147 days	2.0	0.5	1.9
Salad cream	0 time	1.2	0.2	2.3
Salad cream	110 days	51.0	0.8	23.0
Roasted peanuts	0 time	nil	0.2	4.5
Roasted peanuts	112 days	17.3	0.3	10.3
Soup powder	0 time	2.4	0.2	1.0
Soup powder	113 days	2.3	0.3	1.0

[a] Adapted from Parr and Swoboda (1976)
[b] Lipid extracted with a 1:2:0.8 (v/v) mixture of chloroform, methanol and water.

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increases at 268 and 301 for a 1% (w/v) lipid solution after the dehydration step, is defined as "conjugatable oxidation product value". The concentration of oxodienes present in the original mixture is estimated by determining the decrease in absorbance at 275 nm observed after reduction. This procedure is useful as a quality control measure to distinguish between types of unsaturated fatty acids and unsaturated carbonyls in oxidized food lipids (Table 5.10).

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CHAPTER 6

Research methods for lipid oxidation

Before advanced instrumental methods became available, conventional liquid adsorption and partition column chromatographic methods, as well as countercurrent distribution techniques, were generally used for the concentration and purification of fatty ester hydroperoxides to study the mechanism of lipid oxidation. Thin-layer chromatography was also developed as a useful tool in the separation and purification of mixtures of oxidation products, but its application was mainly qualitatitve. A number of powerful instrumental methods emerged later to analyse mixtures of complex products of lipid oxidation. These methods combined various improved chromatographic techniques that require sensitive detection systems with mass spectrometry (MS). They include high performance liquid chromatography (HPLC), and size-exclusion chromatography (HPSEC), combinations of GC and mass spectrometry (GC-MS), HPLC and ^{13}C nuclear magnetic resonance (HPLC-^{13}C NMR), HPLC-MS, chemical ionization MS (CI-MS), tandem-mass spectrometry or MS-MS or collision-induced dissociation (CID), electrospray MS (ESI-MS), and electro and coordination-ion spray MS (CIS-MS). The last section of this chapter deals with recently developed advanced techniques of direct MS, including atmospheric pressure chemical ionization-MS (APCI-MS) for volatile flavor analysis during food consumption.

Significant improvements have been achieved in the separation of isomeric hydroperoxides and other labile oxidation products from complex lipids, and their direct characterization by various advanced MS techniques avoiding sample manipulation, problems of variable recoveries from biological samples and tedious derivatization. However, the direct analyses of lipid oxidation products achieved with many of these advanced MS techniques have not yet produced reliable quantitative information. Although a few synthetic and enzymatically-derived polyunsaturated lipid hydroperoxides have been described in the literature, they have not been generally used as standards for quantitative MS analyses. This chapter is organized in chronological order of method development.

A. Countercurrent distribution

In early studies, although the hydroperoxides could be purified from autoxidized methyl oleate and linoleate by adsorption column chromatography or countercurrent distribution with a 29-tube apparatus, the same techniques failed with

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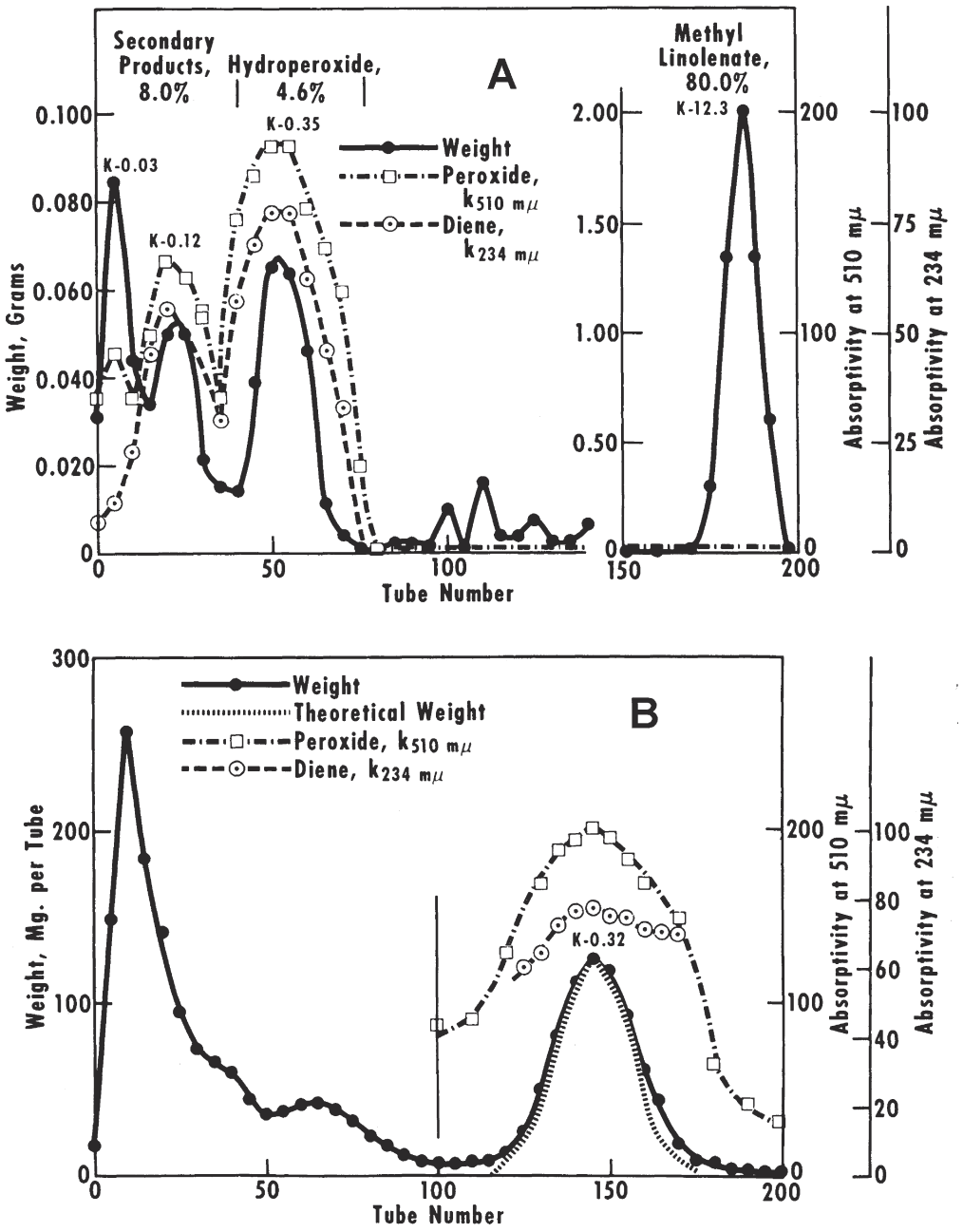


Figure 6.1. A. Separation of hydroperoxides by countercurrent distribution of autoxidized methyl linolenate (200 transfers between 8:2 water:ethanol and petroleum ether). B. Countercurrent distribution of hydroperoxide fraction from autoxidized methyl linolenate (600 transfers). From Frankel *et al.* (1961b). Courtesy of the American Chemical Society.

autoxidized linolenate. These results led to a controversy as to whether or not the oxidation of methyl linolenate produces hydroperoxides by the same mechanism as methyl linoleate. Later studies were more successful in isolating

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a hydroperoxide fraction partially separated from secondary oxidation products with a 200-tube countercurrent distribution apparatus (Figure 6.1A). By determining the partition coefficient of the linolenate hydroperoxide fraction (K = 0.35) and that of the secondary oxidation products (K = 0.12) it was calculated that 600 transfers were required to achieve complete separation of pure linolenate hydroperoxides (Figure 6.1B). Under the same conditions of oxidation the yield of pure linolenate hydroperoxides was much lower (36%) than that of linoleate hydroperoxides (90%) because linolenate produced much higher concentrations of secondary oxidation products. A major portion of these secondary products was later identified as a mixture of hydroperoxy epidioxides (Chapter 2.C).The yield of 2 g of pure hydroperoxides obtained by the countercurrent distribution technique permitted a detailed structural and quantitative study of the isomeric composition of linolenate hydroperoxides (Chapter 2, Figure 6.15).

B. Conventional column liquid chromatography

Adsorption (solid–liquid) and normal-phase liquid partition (liquid–liquid) column chromatography run at low pressures were widely used to separate and analyse quantitatively hydroperoxides and secondary products of lipid oxidation. Adsorption column chromatography with silica and alumina used in early studies gave low yields of hydroperoxides due to various degrees of decomposition. Better results were obtained by combining adsorption and partition chromatography using a silicic acid column with methanol as a partial "stationary" phase to purify and analyse the hydroperoxides of fatty acids and esters, and dimeric and polymeric products in vegetable oils. By this procedure, silicic acid was treated with methanol as the stationary phase and benzene containing 2 to 4% by volume methanol as the mobile phase. The use of an insufficient amount of methanol to saturate the silicic acid column support resulted in a sharp gradient of methanol concentration in the eluate with a single mobile phase of methanolic benzene. Adsorption and partition effects were both indicated because the methanol concentration of the effluent was always lower than that of the eluent. This "semi-partition" chromatography was effective in yielding pure monomeric hydroperoxides efficiently separated from the unoxidized esters and secondary decomposition products (Figure 6.2). The chromatographic yields of hydroperoxides were significantly higher for methyl linoleate (89 to 95%) than for methyl linolenate (30 to 36%), and the relative concentration of secondary products was correspondingly higher for linolenate than for linoleate. This difference was in agreement with previous countercurrent distribution analyses (Section A) and was later accounted for by the significant formation of hydroperoxy epidioxides in linolenate, proportionately about 25% relative to the total monomeric hydroperoxides (Chapter 2. C).

The same column chromatographic procedure was used to determine

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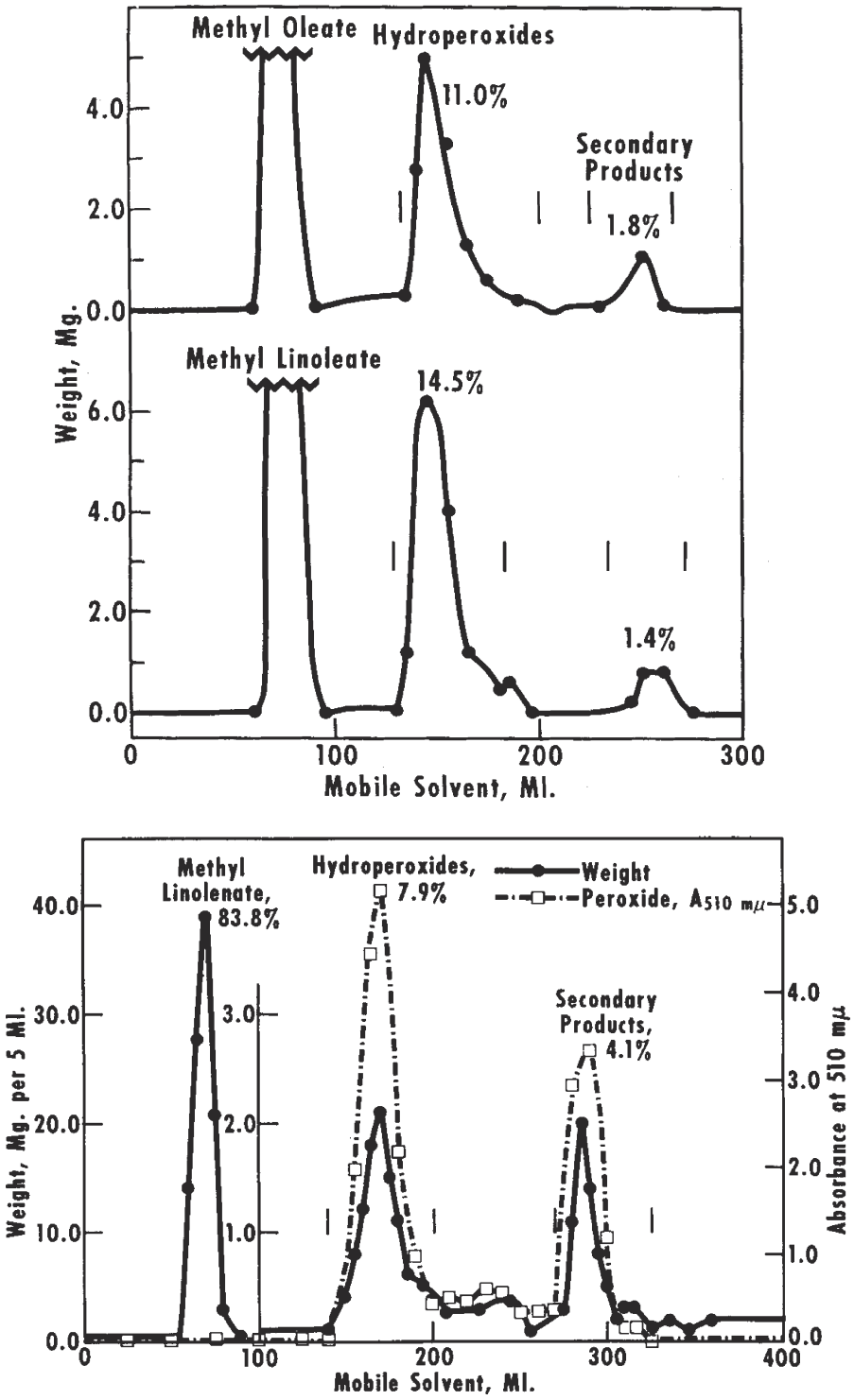


Figure 6.2. Chromatographic separation of autoxidized methyl oleate, linoleate and linolenate on a silicic acid column prepared with 2:8 methanol:benzene as immobile solvent and 2% methanol in benzene (v/v) as mobile solvent. Top graph: from Frankel *et al.* (1961a), courtesy of the American Oil Chemists' Society, and bottom graph: Frankel *et al*. (1961b), courtesy of the American Chemical Society.

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compounds of average molecular weight corresponding to dimers in the free fatty acids obtained from deodorized vegetable oils (Figure 5.2), and thermally polymerized fatty acids and esters. The dimer contents of different vegetable oils varied from 1 to 3%. In oxidized–deodorized oils, a dimer concentration of 1% corresponded to a peroxide value of 40. The significant amounts of dimeric compounds produced after deodorization from hydroperoxides formed during processing was shown to decrease the flavor and oxidative stability of soybean oil (see Chapter 8). This determination is thus designated as "hidden oxidation", and reflects the extent of oxidative abuse during processing (see Figure 5.2, Chapter 5.D).

The use of small efficient chromatographic columns was later developed to separate and concentrate lipid oxidation products. Reproducible columns known as solid-phase extraction (SPE) became popular among lipid analysts when they became available commercially with a variety of polar and non-polar adsorbents having different functionality by bonding to the silanol groups on the surface of silica gel. Pure hydroperoxides from autoxidized methyl linoleate, methyl linolenate, and hydroperoxy epidioxides from methyl linolenate were prepared in 20–40 mg quantities by a rapid and convenient chromatographic method using a silica cartridge (Table 6.1). These fractions of purified hydroperoxides were used to induce oxidation of *n*–6 polyunsaturated fatty acids in biological samples and compared with *tert*-butyl hydroperoxide. This synthetic hydroperoxide is commonly used as a convenient "oxidant" in biological oxidation studies because it is readily available commercially. The fatty acid hydroperoxides are, however, more appropriate and relevant sources of biological oxidants than the more stable and artificial *tert*-butyl hydroperoxide.

Table 6.1. Chromatographic separation of hydroperoxides and hydroperoxy epidioxides on solid-phase extraction (SPE) column [a]

Autoxidized methyl esters (200 mg)[b]	Eluting solvents, ml (ether:hexane, v/v) [c]	Hydroperoxide absorptivity (A_{nm}) [d]	Hydroperoxy epidioxides (A_{nm}) [d]
Methyl linoleate	4, 4 (5:95)	A_{231}: 28,640	
Methyl linolenate	4 (6:94), 3.5 (12:88)	A_{235}: 23,772	
Methyl linolenate	4 (12:88), 4 (25:75)		A_{235}: 24,975

[a] From Frankel *et al.* (1989), using a Waters Sep-Pak silica cartridge.
[b] Methyl esters were shaken with oxygen at 40°C until 20–25% oxidation was obtained on the basis of conjugated diene absorptivity.
[c] Isolated pure fractions are underlined.
[d] Literature values: 26,000–28,600 for pure *cis,trans* and *trans,trans* isomers of methyl linoleate 9- and 13-hydroperoxides (Chan and Levett, 1977); 24,600 for isomeric mixture of *cis,trans* and *trans,trans* methyl linolenate hydroperoxides (Frankel *et al.*, 1961a), and 24,200–28700 for pure *cis,trans* and *trans,trans* isomers of methyl linolenate hydroperoxy epidioxides (Neff *et al.*, 1981).

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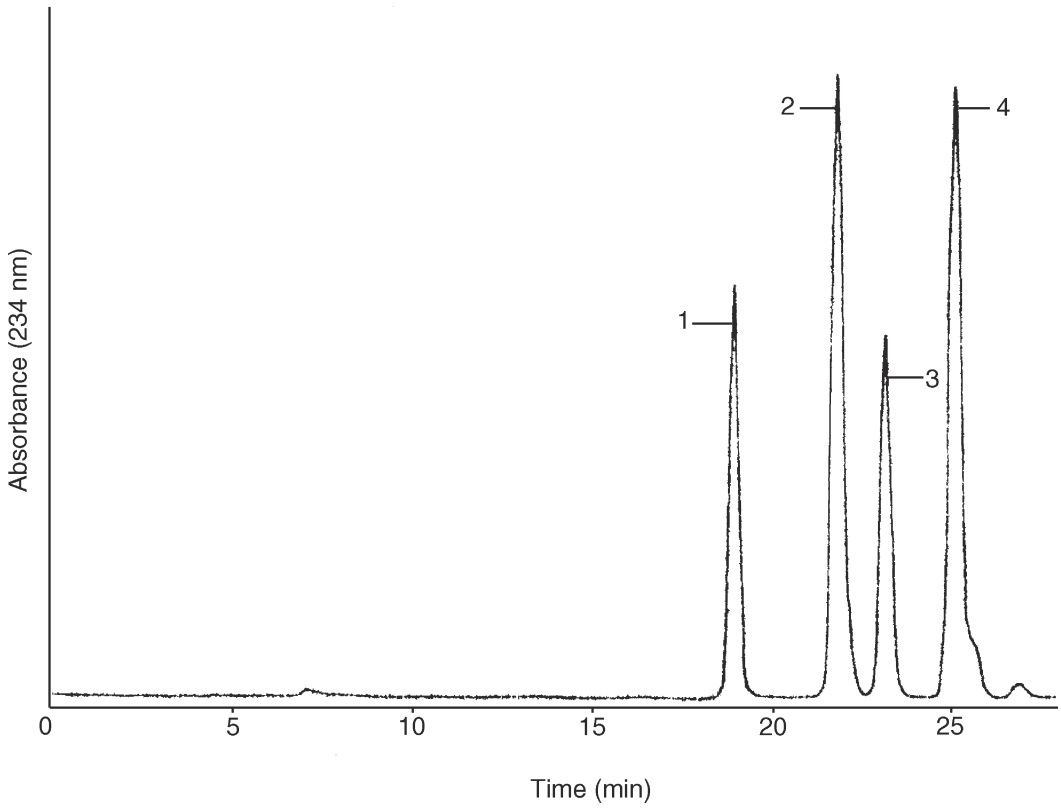


Figure 6.3. Normal phase HPLC separation of the four *cis,trans* and *trans,trans* isomeric 9- and 13-hydroperoxides as the hydroxyoctadecadienoate derivatives: 1, methyl 13-hydroxy-*cis*-9, *trans*-11-; 2, methyl 13-hydroxy-*trans*-9, *trans*-11-; 3, methyl 9-hydroxy-*trans*-10, *cis*-12-; 4, methyl 9-hydroxy-*trans*-10, *trans*-12-octadecadienoate, from autoxidized methyl linoleate with a microporous 5 μm silica (Partisil-5) column and 0.75% ethanol in hexane (v/v) as eluting solvent, UV detector at 234 nm. From Chan and Levett (1977a). Courtesy of the American Oil Chemists' Society.

C. High performance liquid chromatography (HPLC)

When more advanced HPLC systems became available, the four *cis,trans* and *trans,trans* isomeric 9- and 13-hydroperoxides were partially separated from autoxidized methyl linoleate by adsorption chromatography with a microporous 5 μm silica acid column. The corresponding hydroxyoctadecadienoate isomers obtained by sodium borohydride reduction were better and completely separated with the same silica column (Figure 6.3). Similarly, the isomeric hydroperoxides from autoxidized methyl linolenate were not resolved with the same adsorption column used with autoxidized methyl linoleate. However, after reduction, the resulting hydroxyoctadecatrienoate derivatives were completely separated into the expected eight *cis,trans* and *trans,trans* isomers (Figure 6.4). The corresponding hydroxyoctadecanoate derivatives obtained by catalytic hydrogenation of different mixtures of isomeric hydroperoxides were sufficiently separated with a 10 μm silica column to permit quantitative analyses of the isomeric composition, in samples of autoxidized and photo-

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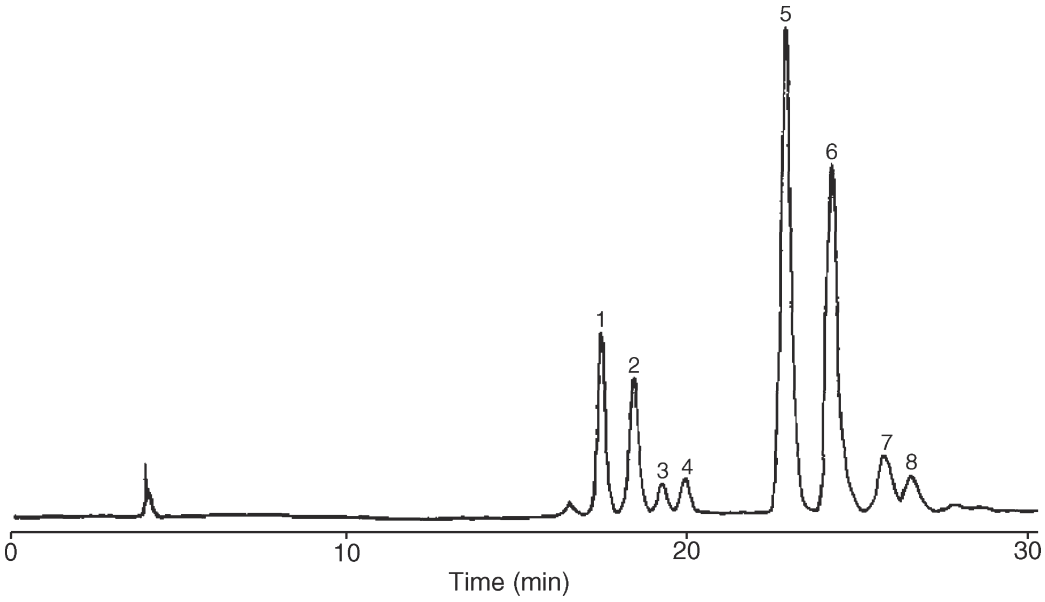


Figure 6.4. Normal phase HPLC of hydroxyoctadecatrienoate isomers from autoxidized methyl linolenate as described in Figure 6.3: 1, 13-*cis*, *trans*-; 2, 12-*cis*, *trans*-; 3, 12-*trans*,*trans*-; 4, 13-*trans*,*trans*-; 5, 16-*cis*,*trans*-; 6, 9-*cis*,*trans*-; 7, 16-*trans*,*trans*-; 8, 9-*trans*,*trans*. From Chan and Levett (1977b). Courtesy of the American Oil Chemists' Society.

sensitized-oxidized methyl linoleate and linolenate (Figure 6.5). The isomers of hydroperoxy epidioxides from oxidized linolenate (Figure 6.6) and from photosensitized-oxidized linoleate (Figure 6.7) were effectively separated by HPLC on microporous 5 or 10 μm silica columns. Partial resolution of stereoisomers of linolenate hydroperoxyepidioxides is indicated in peaks III and IV (Figure 6.6). The same HPLC systems were used to demonstrate the effect of α-tocopherol in inhibiting the formation of *trans,trans*-hydroperoxides and hydroperoxy epidioxides in autoxidized methyl linolenate (Figure 6.8) (Chapter 2, B,C).

Autoxidized trilinolein was separated into mono-, bis- and tris-hydroperoxides by preparative reversed phase HPLC using a 5 μm C-18 column with UV at 235 nm and refractive index detectors (Chapter 2, F; Figure 6.9). The mono-hydroperoxides of trilinolein were further resolved into the positional isomers components by normal phase HPLC on a 5 μm silica column with UV detection (Figure 6.10). Complete and partial separation of various *cis,trans*- and *trans,trans*-2- and 1(3)-mono-13- and 9-hydroperoxides were identified by lipolysis and capillary GC. The ratio of 1(3)- to 2-monohydroperoxides estimated by normal phase HPLC averaging 2.0 indicated that oxidation of trilinolein was not selective toward either the 1(3)- or 2-position.

Analyses of oxidized trilinolenin by reversed phase HPLC with a 5 μm C-18 column showed that monohydroperoxides and hydroperoxy epidioxides are the only products formed initially. Bis- and tris-hydroperoxides were detected

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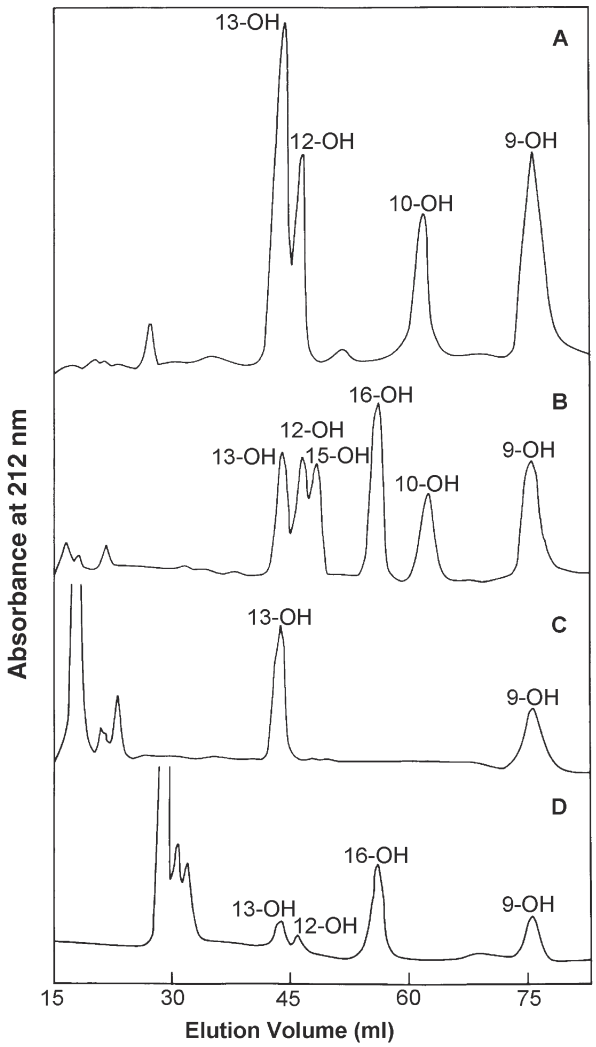


Figure 6.5. Normal phase HPLC of hydroxyoctadecanoate derivatives of isomeric hydroperoxides of autoxidized and photosensitized-oxidized methyl linoleate and linolenate with a 10 μm silica Porasil column and 0.25% isopropanol in hexane (v/v), variable wavelength detector at 212 nm. A. photosensitized-oxidized methyl linoleate; B. photosensitized-oxidized methyl linolenate; C. autoxidized methyl linoleate; D. autoxidized methyl linolenate. From Neff and Frankel (1980). Courtesy of the American Oil Chemists' Society.

at peroxide values above 30, and hydroperoxy bicycloendoperoxides and mono-dihydroperoxides at peroxide values above 75 (Chapter 2, Figure 6.11). The monohydroperoxide mixture of oxidized trilinolenin was resolved by analytical normal phase HPLC into six peaks identified as 1,3- and 2,3-dilinolenoyl glycerol with 2-and 1(3)-substituted [12(13)]-, 2-(16)-, and 9-hydroperoxides (Figure 6.12). These HPLC analyses enabled to estimate the relative triacylglycerol positions of the hydroperoxides isomers. The *cis,trans*-16-hydroperoxides favored the 1(3)- over the 2-triacylglycerol positions by a ratio of 2.3, compared to 1.8 for the *cis,trans*-9-hydroperoxides (Chapter 2, F).

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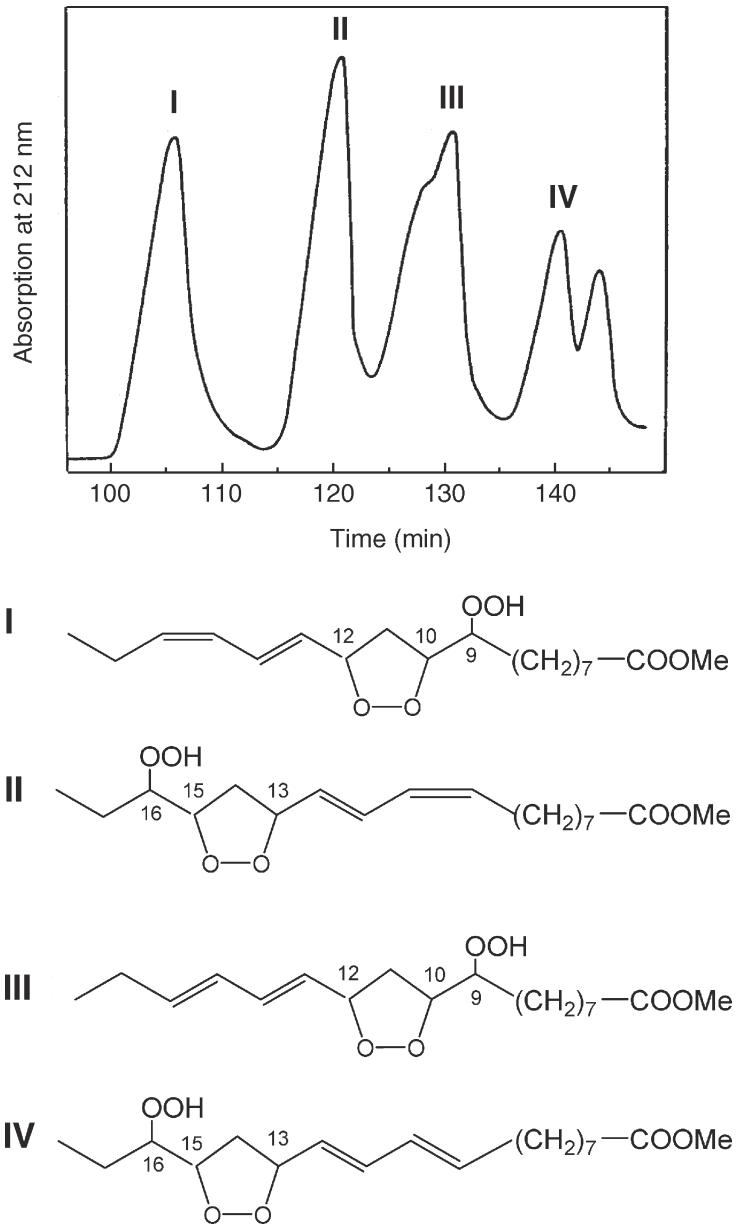
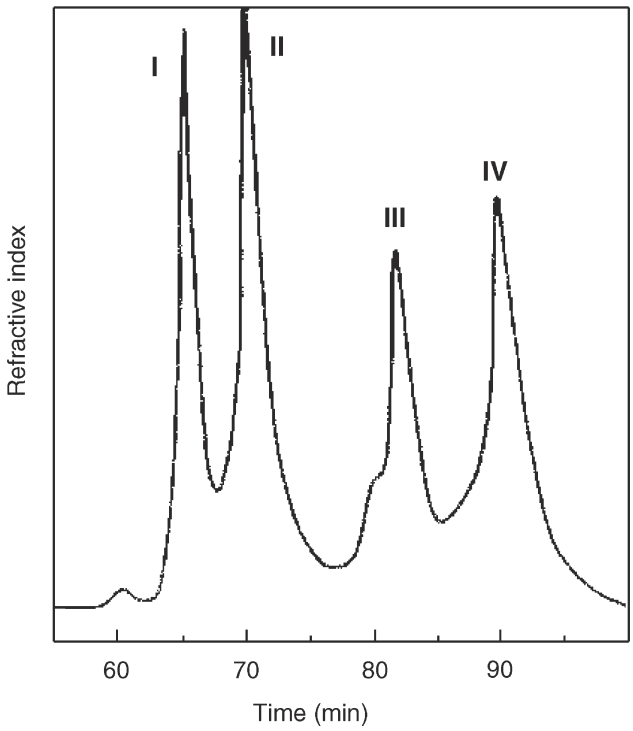


Figure 6.6. Normal phase HPLC isomers of hydroperoxy epidioxide isomers from autoxidized linolenate on microporous 10 μm silica Partisil 10 column, 0.3% ethanol in hexane (v/v) as eluting solvent, variable wavelength detector at 212 nm. From Frankel *et al.* (1981). Courtesy of the American Oil Chemists' Society.

The use of HPLC with post-column chemiluminescence (CL) detection permits more sensitive and direct analyses of hydroperoxides from oxidized complex lipids in biological samples. By this technique, the HPLC effluent is mixed with different CL cocktails at a post column tee and is monitored by a CL detector measuring light emitted by the reaction of hydroperoxides with a heme

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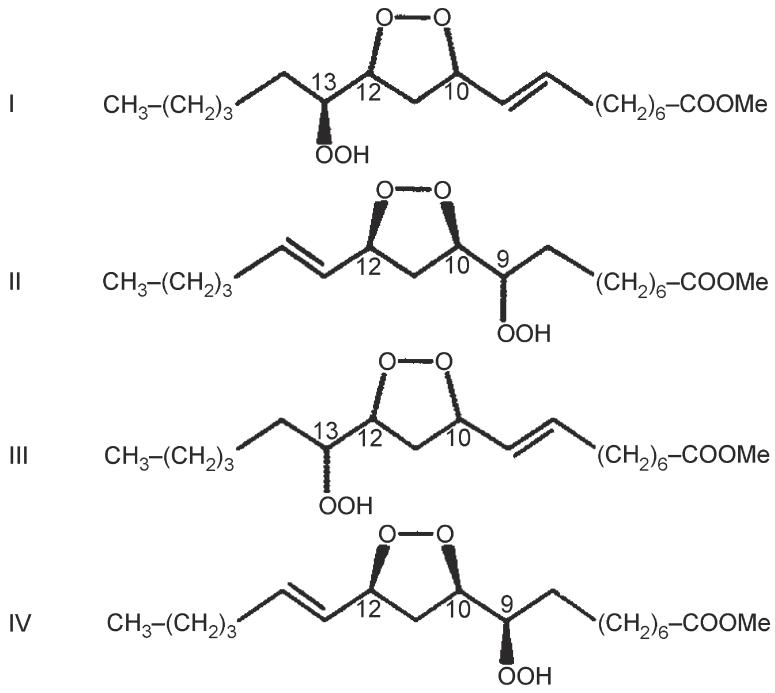


Figure 6.7. HPLC of hydroperoxy epidioxide isomers from photosensitized-oxidized linoleate with a microporous 10 μm silica Partisil 10 column and 6:4:1 hexane:methylene chloride:ethyl acetate (v/v) as mobile solvent, refractive index detector. From Neff *et al.* (1982). Courtesy of the American Oil Chemists' Society.

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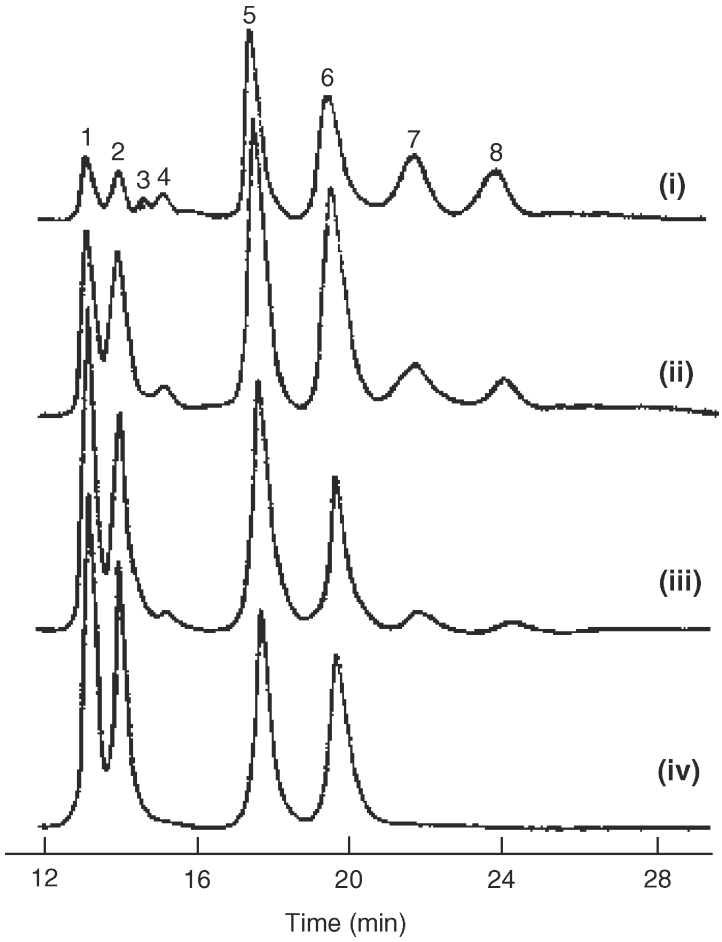


Figure 6.8. Normal phase HPLC of hydroxyoctadecatrienoate isomers to study the effect of α-tocopherol in inhibiting the formation of *trans,trans*-hydroperoxides and hydroperoxy epidioxides in autoxidized methyl linolenate with a 5 μm Partisil-5 column, and 0.4% ethanol in hexane (v/v) as eluting solvent, UV detector at 234 nm. (i) no α-tocopherol added, (ii) + 0.05% α-tocopherol, (iii) + 0.5% α-tocopherol, (iv) + 5% α-tocopherol. From Peers *et al.* (1981). Courtesy of Society of Chemical Industry.

protein (cytochrome c or peroxidase) and an oxidized dye (luminol or isoluminol). A cytochrome c and luminol CL cocktail was used to analyse phospholipid hydroperoxides in human plasma, human blood LDL, and animal liver and brain tissues. With this mixture, spectrophotometric evidence supports the formation of singlet oxygen in the decomposition of peroxyl radicals by the Russell mechanism (Chapter 1, A.3).

Although the HPLC-CL approach allows the detection of hydroperoxides at the picomole levels in oxidized lipid extracts from complex biological samples, many problems arise in recovery of different hydroperoxides, in differences in substrate specificities between CL cocktails, mistaken structural assignment for different HPLC peaks, and spurious results resulting from some artificial

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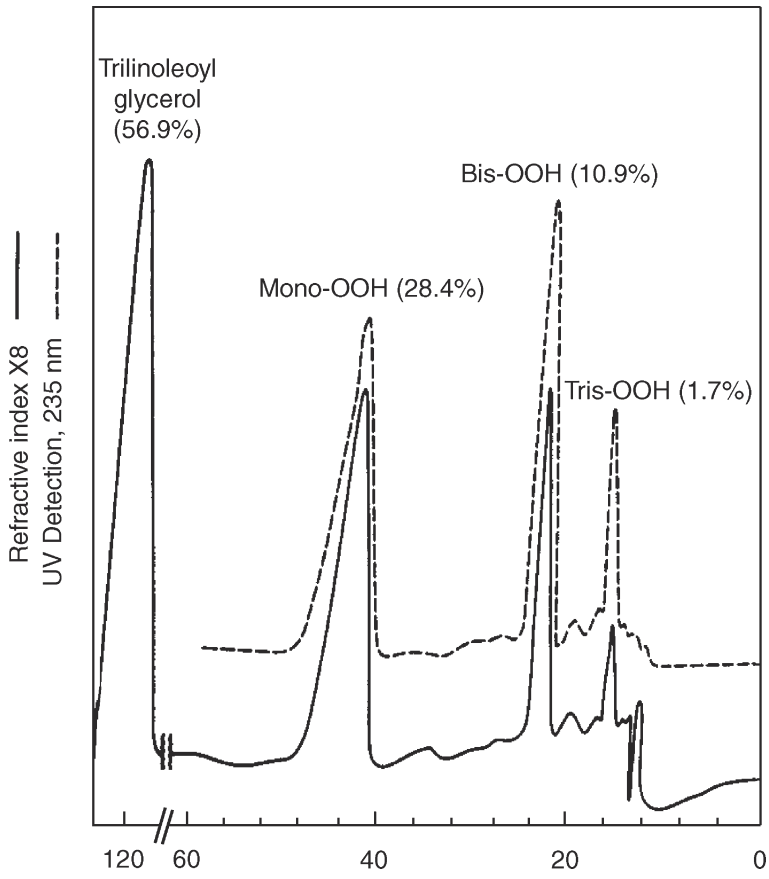


Figure 6.9. Preparative reversed-phase HPLC of autoxidized trilinolein with a 5 μm C-18 column, 30:70 methylene chloride:acetonitrile (v/v) as eluting solvent; broken line, UV detection at 235 nm; solid line, refractive index detection. From Neff *et al.* (1990). Courtesy of the American Oil Chemists' Society.

oxidizing agents. Less polar hydroperoxides derived from triacylglycerols and cholesterol esters can be effectively extracted with hexane and methanol, but more polar phosphatidylcholine (PC) hydroperoxides require Folch extraction with chloroform and methanol. The cytochrome c–luminol cocktail is 100 times more responsive in the CL detection of PC hydroperoxides than the peroxidase-isolumionol CL cocktail. A peak assigned erroneously to cholesteryl ester hydroperoxides in blood plasmas by using the microperoxidase-isoluminol CL reagent was shown to be actually due to the hydroquinone of ubiquinol-10. Further analyses by LC-thermospray MS revealed that PC samples oxidized with the free radical initiator azobis-dimethylvaleronitrile (AMVN) produced AMVN-derived hydroperoxides that were not formed when the same samples were photooxidized with the sensitizer methylene blue (Table 6.2, Section F). Although this study demonstrated that the use of AMVN produced misleading results, this and other artificial free radical azo initiators are unfortunately commonly used to promote oxidation in biological systems (see Chapter 13).

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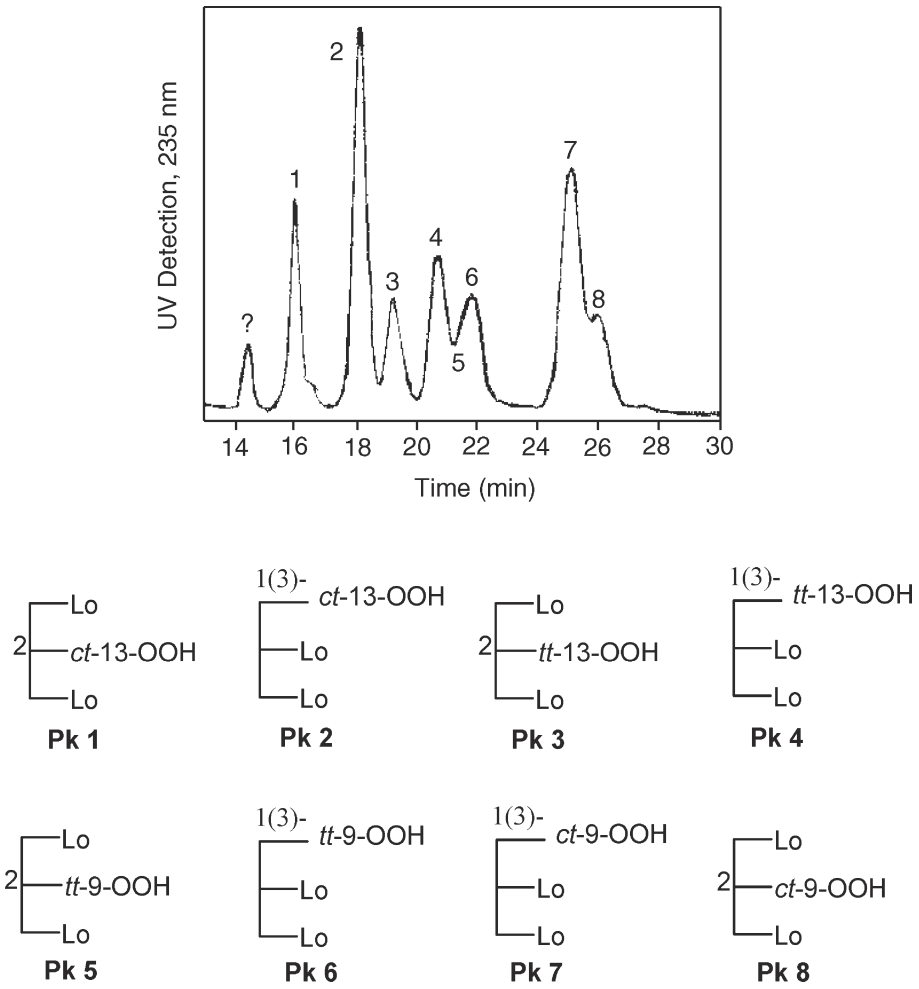


Figure 6.10. Analytical normal phase HPLC separation of monohydroperoxide isomers of autoxidized trilinolein with a 5 μm silica column, 0.5:99.5 2-propanol:hexane (v/v) as eluting solvent, UV detection at 235 nm. Lo, linoleoyl residue; *ct* 13-OOH, *cis,trans* linoleic 13-hydroperoxides; *tt* 13-OOH, *trans,trans* linoleic 13-hydroperoxides; *ct* 9-OOH, *cis,trans* linoleic 9-hydroperoxides; *tt* 9-OOH, *trans,trans* linoleic 9-hydroperoxides. From Neff *et al.* (1990). Courtesy of the American Oil Chemists' Society.

An HPLC-fluorescence method using post-column detection was effectively applied in the analyses of hydroperoxide mixtures containing conjugated and non-conjugated diene structures. The fluorescent reagent diphenyl-1-pyrenylphosphine (DPPP) reacts with lipid hydroperoxides to form DPPP oxide with exciting (352 nm) and emission (380 nm) absorptions. This reagent allowed the detection of both conjugated 9- and 13-hydroperoxides and the non-conjugated 10- and 12-hydroperoxides produced by photosensitized oxidation of methyl linoleate (Figure 6.13). This method was also applied to the detection of conjugated and non-conjugated hydroperoxides produced by photosensitized oxidation of methyl linolenate and the non-conjugated hydroperoxides from autoxidized methyl oleate. Other post-column detection systems

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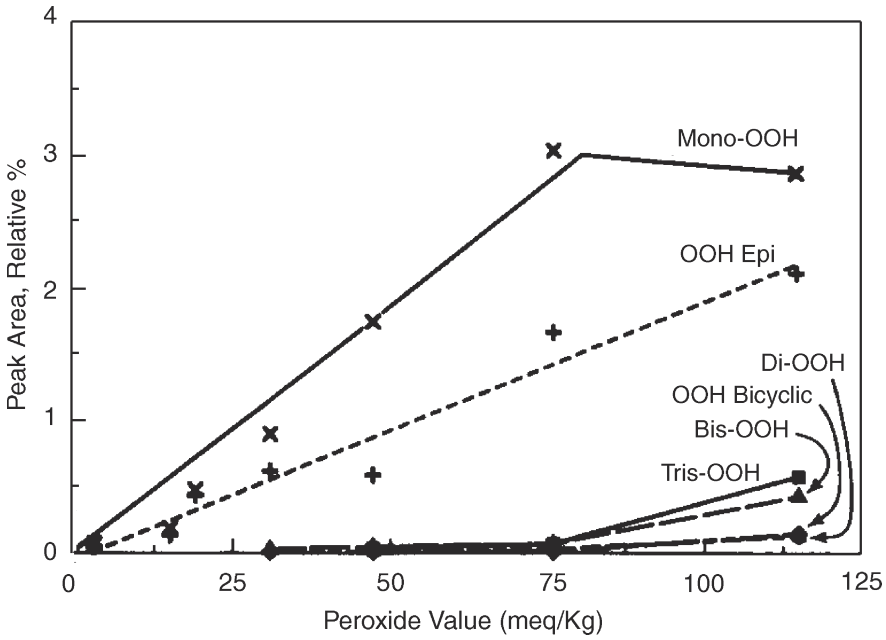


Figure 6.11. Autoxidation of trilinolenin at 40°C. Analyses by reversed-phase HPLC as described in Figure 6.10. Mono-OOH, monohydroperoxides; OOH Epi, hydroperoxy epidioxides; di-OOH, dihydroperoxides; Bis-OOH, bis-hydroperoxides; Tris-OOH, tris-hydroperoxides; OOH Bicyclic, hydroperoxy bicycloendoperoxides. From Frankel *et al.* (1990). Courtesy of the American Oil Chemists' Society.

Table 6.2. Analyses of hydroperoxide in phosphatidyl choline (PC) oxidized with azobis-dimethylvaleronitrile (AMVN) and photooxidized with methylene blue [a]

Type of oxidation	Substrate	PCOOH (pmole)	AMVN-OOH [b] (pmole)	Other X-OOH	% of PC-OOH
Photosensitized	di-18:2 PC	18.0	–	2.0	90.0
Photosensitized	18:0, 18:2-PC	18.6	–	1.4	93.0
AMVN	di-18:2 PC	5.1	61	34.9	5.1
AMVN	18:0, 18:2-PC	4.8	54	41.2	4.8
AMVN	di-18:2 PC	29.4	36	34.6	29.4
AMVN	18:0, 18:2-PC	27.5	51	21.5	27.5

[a] From Zhang *et al.* (1995). PCOOH were determined by HPLC-CL comparing PC-OOH peak with standard purified from PC photosensitized oxidized with methylene blue. AMVN-OOH were determined by HPLC-CL comparing the peak with standard AMVN-OOH. Identification of peak due to AMVN-OOH was based on thermospray LC-MS showing ions at m/z 238, 270, 302 and 334 absent in the MS of PC-OOH showing m/z 814,818 or 846.
X-OOH = other hydroperoxides, remaining from total hydroperoxide subtracted by PCOOH and AMVN-OOH.
[b] AMVN-OOH are very responsive to the microperoxidase–isoluminol but not to the cytochrom c–luminol CL cocktail, which in turn is more sensitive to PC-OOH.

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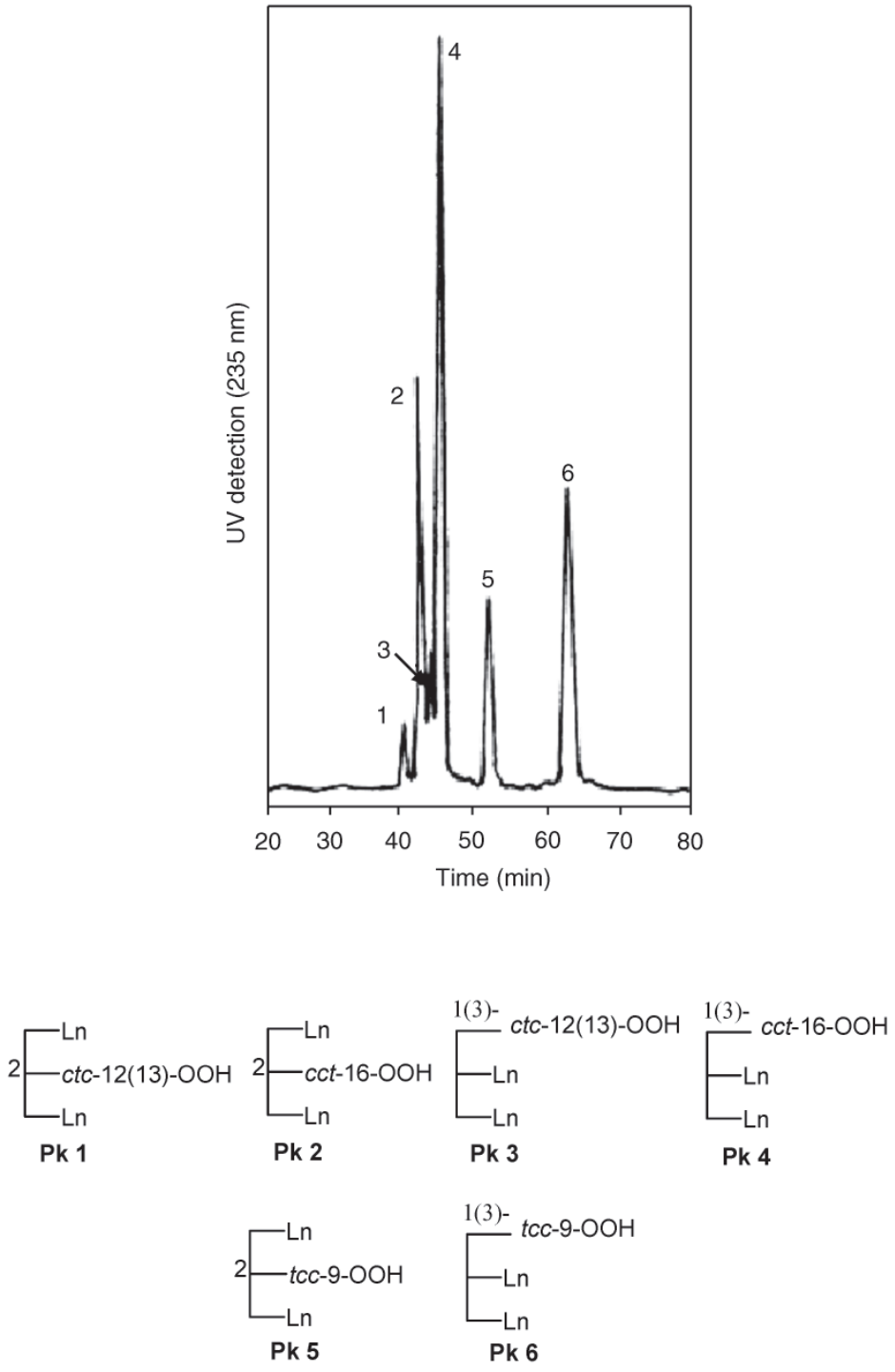


Figure 6.12. Analytical normal phase HPLC separation of monohydroperoxide isomers of autoxidized trilinolenin, as described in Figure 6.10. Ln, linolenic acid residue; *ctc* 12(13)OOH, *cis,trans,cis* linolenic 12+13-hydroperoxides; *cct* 16-OOH, *cis,cis,trans* linolenic 16-hydroperoxide; *tcc* 9-OOH, *trans,cis,cis* linolenic 9-hydroperoxide. From Frankel *et al*. (1990). Courtesy of the American Oil Chemists' Society.

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Table 6.3. Quantitative analyses of monohydroperoxides (mg/g) isolated by reversed-phase HPLC from trilinolein oxidized at different levels [a]

PV (meq/kg)	CL detector	UV detector	ELS detector
2.6	0.10	0.55	0.55
8.3	0.32	0.74	1.00
22.0	0.93	1.82	1.75
47.4	2.04	4.38	3.50
63.2	2.42	6.39	4.46

[a] From Mäkinen *et al.* (1996).
Abbreviations: PV, peroxide value; CL, chemiluminescence, UV, ultraviolet; ELS, evaporative light-scattering.
Analytical conditions: The polar hydroperoxides were first separated from non-polar material by solid-phase extraction on ammonium columns, followed by reversed phase HPLC on C-18 columns using either methanol:2-propanaol (90:10, v/v) or methanol-2-propanol: dichloromethane (80:10:10, v/v). The post-column CL reagent contained isoluminol and microperoxidase in borate buffer (pH 10)–methanol (30:70, v/v). Methyl linoleate hydroperoxides were used as external standards for quantification by CL and UV detectors, and 1,3-diolein by ELS detector.

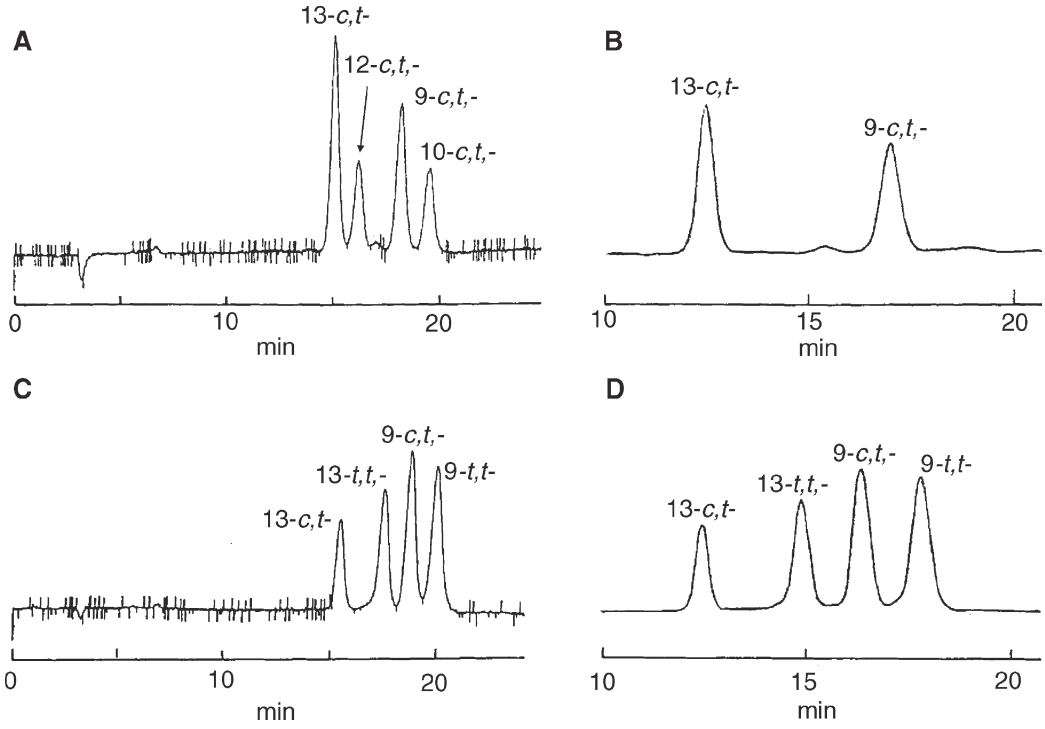


Figure 6.13. Normal phase HPLC analyses of hydroperoxide with a 5 μm silica column, 34:500 diethyl ether:hexane (v/v) as eluting solvent. A, photosensitized oxidized methyl linoleate detected by post-column fluorescent reagent diphenyl-1-pyrenylphosphine (DPPP), exciting (352 nm) and emission at (380 nm) absorptions and B, by UV detection at 234 nm, C, Autoxidized methyl linoleate detected DPPP fluorescence, and D, UV detection at 234 nm. See Figure 6.10 caption for abbreviations. From Ohshima *et al.* (1996). Courtesy of the American Oil Chemists' Society.

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Table 6.4. ^{13}C NMR and preparative HPLC analyses (rel %) of dienol isomers from hydroperoxides of methyl linoleate oxidized at different temperatures [a]

Temperature and method	13-OH-*c*-9,*t*-11	13-OH-*t*-9,*t*-11	9-OH-*t*-10,*c*-12	9-OH-*t*-10,*t*12-	Ratio *c,t*/*t,t*
25°C					
NMR	31.6	18.9	31.7	17.8	1.72
HPLC	30.5	21.4	27.6	20.5	1.39
Average	31.0	20.2	29.7	18.9	1.56
50°C					
NMR	18.2	28.1	23.5	30.2	0.72
HPLC	20.8	27.8	22.3	29.1	0.76
Average	19.5	28.8	22.9	29.7	0.74
65°C					
NMR	17.7	34.0	18.1	30.2	0.56
HPLC	19.5	33.0	16.9	30.6	0.57
Average	18.4	33.5	17.5	30.4	0.57

[a] From Frankel *et al.* (1990)
Abbreviations: *c*, *cis*; *t*, *trans*
Analytical conditions: Hydroperoxide isomeric mixtures from autoxidized methyl linoleate are reduced to hydroxydiene derivatives by sodium borohydride, and separated by preparative TLC on plates coated with silica gel treated with UV marker and developed with diethyl ether–hexane (6:4, v/v) and UV active hydroperoxides are eluted with diethyl ether. The dienol isomers are separated by HPLC on a preparative 6 μm porous silica column with ethanol–hexane (0.5:99.5, v/v) as mobile phase with a UV detector set at 234 nm. The weight percent composition is based on the weight of each fraction collected.

varied significantly for the quantitative analyses of triacylglycerol hydroperoxides separated by reverse phase HPLC. UV and CE detectors were more sensitive and selective than the evaporative light-scattering (ELS) detector for measuring the hydroperoxides from trilinolein at peroxide values ranging between 3 and 8 (Table 6.3). At higher peroxide values (22–63), detection by CE and ELS was more sensitive than by UV.

The separation of conjugated diene hydroperoxides or the hydroxydiene derivatives by HPLC and their quantification by UV detection requires calculations based on the different molar absorptivities of each isomer, which are lower for the *cis,trans* (27,200–28,300) than the *trans,trans* (30,500–31,600) isomers. In contrast, separation of the hydroxydiene derivatives by preparative HPLC allows direct quantification based on the weight of fractions. ^{13}C NMR studies of the four geometric hydroxydiene isomers isolated by preparative HPLC showed characteristic differences in resonance for their olefinic carbons and for the methylene carbons allylic to the diene systems. ^{13}C NMR analyses based on the integrals of the olefinic carbons were in good agreement with the gravimetric HPLC analyses on samples of methyl linoleate oxidized at different temperatures (Table 6.4). The ratios of *cis,trans*- to *trans,trans*-hydroxydiene isomers decreased from 1.7 at 25°C to 0.56 at 65°C.

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D. High performance size exclusion chromatography (HPSEC)

Separation by HPSEC (also known as gel permeation chromatography) is based on the differences in effective diameter of molecules and is not affected by their functionality. Different solutes are separated by their physical restriction in column packing consisting of crosslinked copolymers of styrene divinyl benzene. This stationary phase discriminates molecules by eluting the larger ones first and separating the smaller ones by mechanical diffusion into pores of different sizes. Ideally, the separation is only affected by the size of the molecules without the interactions that occur with adsorption, partition and ion exchange chromatography. Several detection systems are used for lipid oxidation products based on UV absorption for conjugated dienes, infrared, refractive index, flame ionization, or evaporative light scattering. Detection and quantitative analyses of hydroperoxides by post-column reactions include the use of luminol with a catalyst that generates chemiluminescence, reduction with potassium iodide into I^{3-} detected spectrophotometrically, by the ferric thiocyanate complex formation and reduction with triphenyphosphine.

An important application of HPSEC includes analyses of dimers, trimers, oligomers, partial glycerides and cyclic fatty acids in heated, thermally oxidized and frying fats and fish oils. This technique has become an accepted method for evaluating the quality of frying fats (Chapter 12), crude and refined oils and the effectiveness of processing. Analyses by HPSEC showed significant increases in oxidized triglyceride monomers, triglyceride dimers and triglyceride polymers in extracts from potato chips fried in sunflower oil after storage at room temperature, but none in the corresponding potato chips fried in high-oleic sunflower oil and palmolein (Table 6.5).

Table 6.5. High performance size exclusion chromatography (HPSEC) analyses of oils extracted from potato chips after storage at room temperature (wt%) [a]

Oils	Storage (weeks)	Total polar compounds	Ox TG monomers	TG dimers	TG polymers	DG	FA
Sunflower	0	5.2	15.3	15.0	1.7	12.7	6.9
	15	7.5	37.3	16.9	1.9	12.9	6.2
HiOlSun	0	4.9	12.4	10.3	1.0	18.9	5.9
	15	4.8	12.4	10.0	1.3	18.1	5.8
PalmOl	0	8.9	11.4	12.5	1.0	60.3	3.4
	15	8.6	11.6	12.2	1.2	58.2	3.1

[a] From Marquez-Ruiz *et al.* (1996)
Abbreviations: OxTG, oxidized triglycerides; DG, diglycerides; FA, fatty acids; HiOlSun, high-oleic sunflower; PalmOl, palmolein.
Analytical conditions: A solid-phase extraction silica cartridge is used to separate the nonpolar components by elution with petroleum ether:diethyl ether (90:10) and the polar components with diethyl ether. The polar components are resolved by HPSEC on 2 columns in series of highly cross-linked styrenedivinylbenzene copolymers, using tetrahydrofuran as mobile phase and a refractive index detector.

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Table 6.6. Molecular weight and concentration distribution of decomposition products in vegetable oils used for frying [a]

Frying oil	Frying time (h)	Polar compounds (%)	Mol.Wt./ Conc. (g/100g oil)	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6
SBO	0	2.45	MW	nd	3465	2450	1740	1190	nd
			Conc.	nd	0.17	0.90	4.20	7.07	1.69
	20	29.5	MW	2475	2220	1900	990	925	nd
			Conc.	1.79	4.02	15.3	5.44	0.84	0.30
	40	46.1	MW	6315	4960	2390	2310	1615	nd
			Conc.	4.51	6.65	15.9	4.39	0.57	nd
HOSBO	0	1.03	MW	nd	1860	1285	1110	1010	660
			Conc.	nd	0.13	0.52	1.59	0.30	0.27
	20	9.60	MW	3230	3030	2235	1325	915	nd
			Conc.	0.17	0.46	1.50	3.80	0.69	nd
	40	15.9	MW	2980	2820	2140	1285	910	nd
			Conc.	0.21	0.49	1.32	2.87	0.38	nd

[a] From Abidi and Warner (2001)
Oils were used to fry French fried potatoes and tortilla chips at 190°C. Polar compounds were determined by column chromatography.
Abbreviations: MW, molecular weight; Conc, concentrations; nd, not determined.
Compositions: soybean oil (SBO): contained 13.2% 16:0+18:0, 26.2% 18:1, 53.1% 18:2, 5.90% 18:3; high-oleic soybean oil (HOSBO) contained 10.0% 16:0+18:0, 84.0% 18:1, 1.60% 18:2, 2.40% 18:3.
Analytical conditions: The polar fraction from frying oils was separated by silica gel chromatography by removing the nonpolar components with petroleum ether:diethyl ether (87:13 v/v) followed by chloroform:methanol (1:1). The polar fraction was fractionated by HPSEC with 3 columns in series packed with high-efficiency mixed-bed PLGEL columns, eluting with THF as mobile phase. The chromatograph was interfaced with a differential viscometer and a refractometer connected in parallel.

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The molecular weight distribution of macromolecular triglyceride polymers formed in frying oils was recently analysed by HPSEC with viscometric and refractometric detection. The molecular weight (MW) distribution is determined by plotting the log of (intrinsic viscosity × MW) vs. retention volume using fatty acids, di- and triglycerides and several polystyrene standards. By this novel technique, the nonvolatile polar components of heated and frying fats may be better represented by their molecular weight distribution rather than by the oversimplified and ambiguous assignments as monomer, dimer, trimer, tetramer and highly polymeric triglycerides. As expected, the molecular weight distribution was a function of frying time and vegetable oil type (Table 6.6). There was no evidence of formation of distinct dimer, trimer and tetramer species in the oils used in these frying experiments. As expected, soybean oil showed a higher degree of polymerization than high-oleic soybean oil and other oils containing no linolenate. This novel method may be useful to clarify the chemical nature of high molecular weight carbonyl compounds produced during oxidation followed by polymerization during the processing of polyunsaturated vegetable and fish oils (Chapter 8).

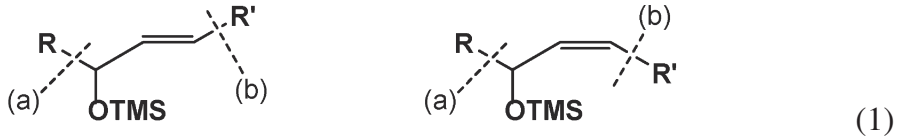
E. Gas chromatography-mass spectrometry (GC-MS)

The combination of GC and MS has been used to analyse qualitatively and quantitatively various derivatives of oxidation products that can be separated in the vapor phase and emerge from fused silica capillary columns, either directly into the ion source of the MS or through membranes or molecular jet separators to concentrate the components of each GC peak. In the mass spectrometer, the organic molecules in the vapor state are bombarded by electron-impact ionization (EI) to form positively charged ions, which fragment into smaller ions. The fragmentation patterns obtained by the "hard" EI method are most useful to characterize the volatile compounds of low-molecular weight separated by capillary GC. Much work has been published to characterize qualitatively by GC-MS hydroperoxides and secondary oxidation products from various unsaturated lipids. Quantitative analyses by GC-MS require however careful standardization with stable derivative compounds of well-defined structures such as hydroxy fatty acid derivatives that can be readily synthesized unambiguously. The EI method is not suitable for molecules of larger molecular weight because they produce excessive fragmentation of molecular ions. Chemical ionization (CI) and other "soft" ionization MS methods cause less fragmentation with lipid hydroperoxides and other labile oxidation products. More advanced MS techniques described in Section F permit the direct qualitative analysis of hydroperoxides with less fragmentation, but their quantification is still elusive.

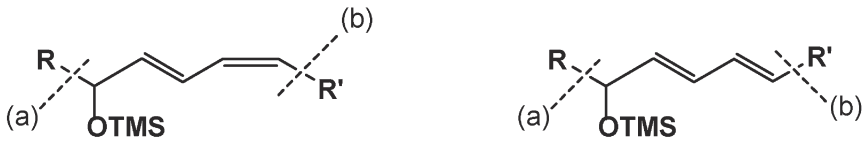
Allylic unsaturated hydroperoxides and the corresponding hydroxy esters cannot be analysed directly by GC and GC-MS. The thermally labile

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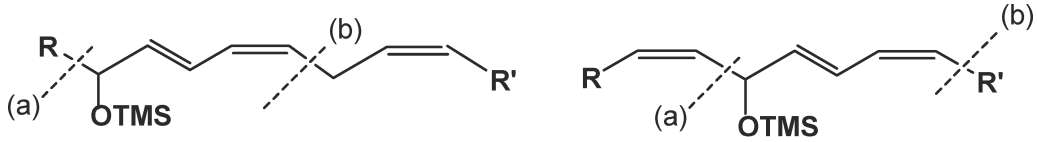
hydroperoxides are readily decomposed, and EI-MS provides no useful molecular weight information. The allylic hydroxy derivatives are also labile and thermally dehydrated into conjugated polyenes under the GC conditions. The trimethylsilyl (TMS) derivatives of hydroxyoctadecenoates, hydroxyoctadecadienoates and hydroxyoctadecatrienoates from the corresponding hydroperoxides are most suitable for GC and GC-MS. These derivatives produce intense ions due to α-scission (a) and a much less intense fragment (b) allylic to the single double bond of oleate (1), or the conjugated diene systems of linoleate (2) and linolenate (3):



(1)



(2)



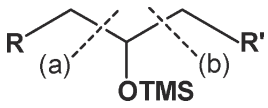
(3)

The relative intensity of the two fragments varies because of 1,3-rearrangement of the allylic TMS ethers (4) under the conditions of GC-MS:



(4)

For quantitative MS analyses, the confounding effect of 1,3-rearrangement of allylic TMS ethers can be completely avoided by catalytic hydrogenation of the allylic alcohols. The resulting saturated hydroxy compounds are suitable derivatives for direct quantitative GC-MS analysis of the positional isomers of the hydroperoxides because they produce two intense fragments by α-scissions at both sides (a) and (b) of the TMS ether position:



(5)

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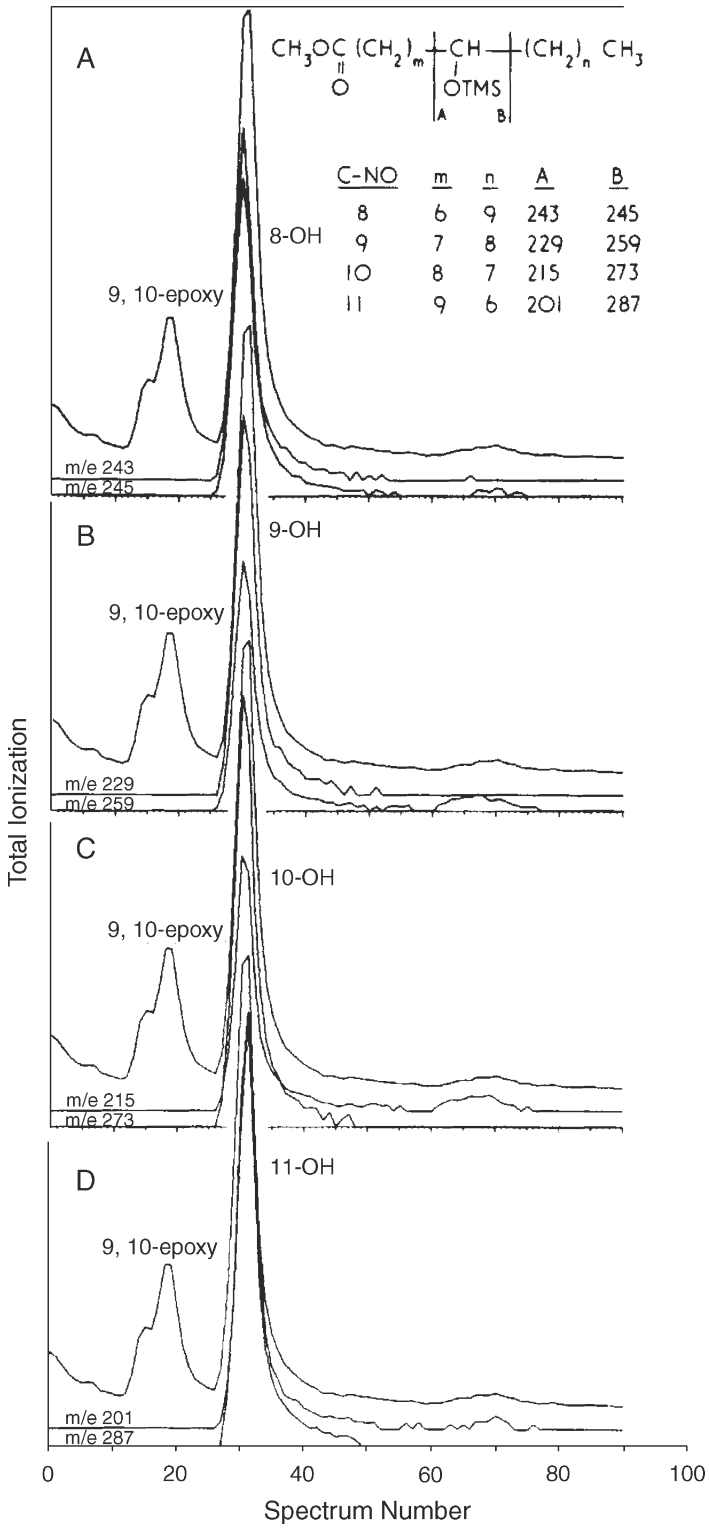


Figure 6.14. Mass chromatography of TMS ethers of methyl hydroxy octadecanoate isomers from autoxidized methyl oleate. From Frankel *et al.* (1977a). Courtesy of the American Oil Chemists' Society.

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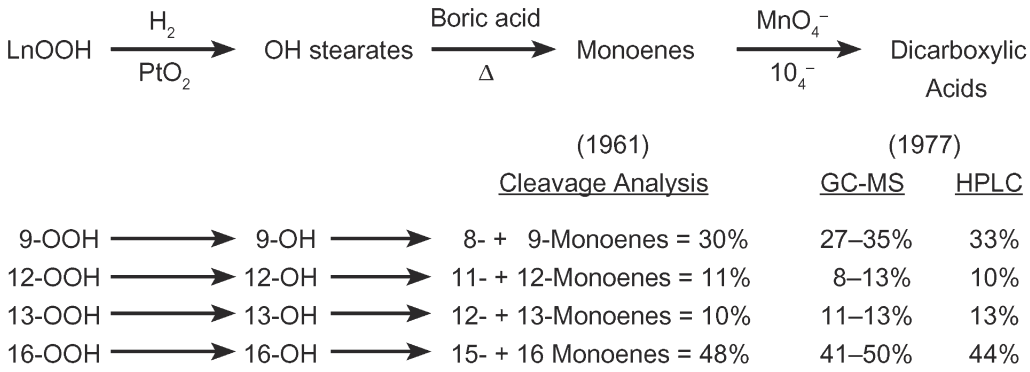


Figure 6.15. Reaction schemes and different methods used to determine the isomeric composition of hydroperoxides from autoxidized methyl linolenate. From Frankel (1999).

However, quantitative studies required standardization with authentic synthetic standards of the positional hydroxyoctadecanoate isomers. The TMS ethers of the four positional hydroxy isomers from autoxidized methyl oleate were analysed quantitatively by standardizing the intensity of both α-scission fragments (A and B) obtained with synthetic methyl 8-, 9-, 10-, and 11-hydroxyoctadecanoates after computer summation of all the spectra within the GC peak due to hydroxyocadecanoate (Figure 6.14). A similar GC-MS computer summation method was standardized with synthetic 9- and 13-hydroxyoctadecanoate. Equal amounts of 9- and 13-hydroperoxides were found in all samples of methyl linoleate oxidized at different temperatures and peroxide levels (Table 2.2, Chapter 2, A, B). With methyl linolenate oxidized at different temperatures and peroxide values, the standardized GC-MS computer summation method applied on the hydroxyoctadecanoate isomers showed that the proportion of 9- and 16-hydroxy esters was significantly higher (75–82%) than that of the 12- and 13-hydroxy esters (18–25%) (Table 2.3). These GC-MS results were in complete agreement with earlier studies based on analyses of cleavage products of monoenes derived by dehydration of hydroxyoctadecanoates derivatives with boric acid, and HPLC of the hydroxyoctadecatrienoate derivatives (Figure 6.15). The uneven distribution of isomeric hydroperoxides of methyl linolenate was later accounted for by the significant formation of hydroperoxy epidioxides derived by cyclization of the 12- and 13-hydroperoxides (Chapter 2, C).

Solid phase microextraction coupled to gas chromatography-mass spectrometry (SPME-GC/MS) has been used as a sensitive method for measuring volatiles in the headspace of milk powders. Headspace measurements on two samples of infant milk powders gave values of hexanal initially of 466 and 1181 parts per billion (ppb), and 617 to 3430 after accelerated storage at 37°C for 1 to 4 weeks (Table 6.7). These levels are far above the hexanal perception thresholds of 4.5 ppb in water and 50 ppb in homogenized whole milk.

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Table 6.7. Analyses of hexanal by solid-phase microextraction (SPME) /MS and by isotope dilution in milk powders after accelerated storage [a]

Storage (weeks)	Hexanal (ppb)
Sample A	
0	1181
1	1848
3	3430
Sample B	
0	466
2	617
4	1022

[a] From Fenaille *et al.* (2003).
Infant formula milk powder samples were stored ungassed in the dark at 37°C. Analytical conditions: Milk powder was reconstituted in bidistilled water and an aliquot was sealed in a glass vial and equilibrated 1 h at 25°C, before analysis by SPME inserting a PDMS/DVB fiber and volatiles sampled during 10 min at 25°C, followed by desorption in the GC inlet interfaced with a mass selective detector. Quantification was carried out by isotope dilution using deuterated d_2-hexanal (m/z 58). Sample A at 0-time gave a value of 1316 ppb by standard addition using non-deuterated hexanal (m/z 56).

F. High performance liquid chromatography and mass spectrometry (HPLC-MS)

MS coupled with normal phase and reversed-phase HPLC is a powerful tool that permits the direct characterization of thermally labile hydroperoxides and nonvolatile high molecular weight secondary oxidation products of triacylglycerols, cholesterol and phospholipids without the necessity of prior derivatization. The problems of removing the solvent from these labile compounds emerging from an HPLC column prior to MS have been overcome by using thermospray, or electrospray ionization (ESI), or atmospheric-pressure chemical ionization (APCI) interfaces. The thermospray interface consists of a rapidly heated capillary tube connecting the HPLC column and the MS that produces a supersonic vapor jet carrying particles or droplets of solute. By traveling at high speed through a lens system, the droplets acquire an electronic charge and the high density ions are ejected into the MS. With the more recently developed ESI interface, a high electric field is applied to nebulize the sample solution and impart a negative or positive charge to the droplets that builds up as the solvent evaporates. This relatively mild and sensitive "soft" ionization technique provides useful structural information on molecular ions with little fragmentation based on both positive and negative ions spectra obtained with sodium or ammonium salts. With the APCI technique, the sample is ionized either similarly to the ESI technique, or by a charge-transfer method.

The ESI technique was applied to the analyses of synthetic isomers of triacylglycerol hydroperoxides of eicosapentaenoic acid, and other oxidation products, including hydroxides, epoxides and triglyceride core aldehydes

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(aldehydo glycerides, Chapters 4, 5, 7 and 11, also called "2½ glycerides"). The APCI technique was used to analyse qualitatively oxidation products from triacylglycerols separated by reversed-phase HPLC. Triacylglycerol hydroperoxides, epoxides, bishydroperoxides, epidioxides and diepoxides from oxidized triolein, trilinolein and trilinolenin could be identified from the protonated molecular ions, near-molecular ions and molecular ion adducts, and from characteristic diacylglycerol fragment ions. Analyses of oxidized sunflower oil by reverse-phase HPLC/ESI/MS showed the presence of a mixture of triglyceride core aldehydes, dialdehydes, hydroxy and epoxy aldehydes.

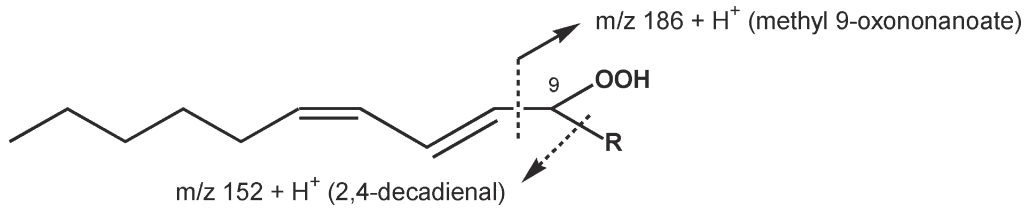
HPLC-CL (Section C) and HPLC-thermospray MS were used to analyse hydroperoxides generated from PC with either the free radical initiator azobisdimethylvaleronitrile (AMVN), or by photooxidation sensitized with methylene blue. Mono-and di-PC hydroperoxides were established by HPLC-CL in the sample produced by photosensitized oxidation. Over 90% of the hydroperoxide peak was shown to be PC hydroperoxides and reacted preferentially with the cytochrome c–luminol CL cocktail (Section C). In contrast, the sample prepared with AMVN showed that 70–95% of the hydroperoxides was not PC-OOH, but was largely due to spurious AMVN-derived hydroperoxides (Table 6.2).

Lipid hydroperoxides in phospholipid vesicles prepared from egg lecithin enriched with arachidonylstearoylphosphatidylcholine (ASPC) were analysed directly by ESI-MS. The positive ESI of phospholipid vesicles obtained in the presence of excess sodium chloride showed mass 832 corresponding to the sodiated form M+23 of ASPC. Oxidation with a mixture of *tert*-butyl hydroperoxide and Fe^{2+} produced new species at mass 864, 896, and 928, corresponding to the addition of one (+32), two (+64) and three (+96) dioxygen molecules to ASPC, consistent with the formation of mono-, di-, and trihydroperoxides.

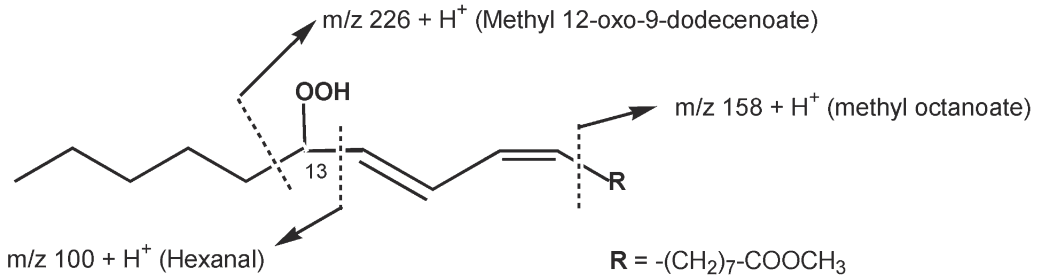
Various lipid oxidation products have been separated by HPLC and the purified compounds characterized by chemical ionization mass spectrometry (CI-MS) with a direct exposure probe. CI-MS yields the protonated molecular ion $[M+H]^+$ with isobutane and the adduct ion $[M+NH_4]^+$ with ammonia as the reagent gases. Because the ionic species formed by chemical ionization have much less energy than the molecular ion formed by electron impact ionization, fragmentation is greatly reduced and provides useful structural information and more intense mass fragments with intact hydroperoxides, mono- and di-hydroxy fatty acids without derivatization. The CI-MS technique produces simpler and more straightforward fragmentation patterns that may be easier to interpret than by GC-electron ionization MS.

With the isomeric methyl 9- and 13-hydroperoxides of linoleate purified by HPLC, the direct exposure CI technique with isobutane as reagent gas provided structural information on the location of the hydroperoxide group and fragmentation patterns that could be explained by the homolytic and heterolytic

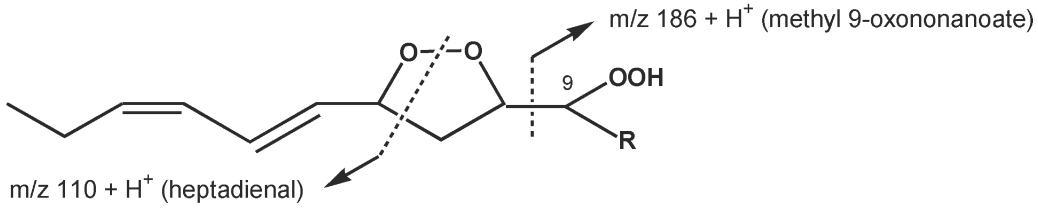
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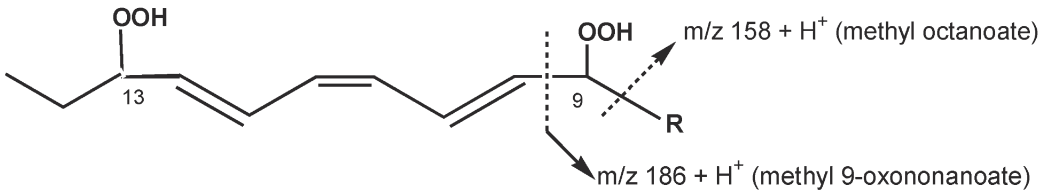
Methyl 9-hydroperoxy- *trans*-10,*cis*-12-octadecadienoate



Methyl 13-hydroperoxy- *cis*-9,*trans*-11-octadecadienoate



Methyl 9-hydroperoxy-10,12-epidioxy-13,15-octadecadienoate



Methyl 9,16-dihydroperoxy-10,12,14-octadecatrienoate

R = -$(CH_2)_7$-$COOCH_3$

Figure 6.16. Mass fragment ions obtained by chemical ionization-mass spectrometry (CI-MS) analysis of 9- and 13-hydroperoxides from oxidized methyl linoleate, from Plattner and Gardner (1985) (note: both hydroperoxide isomers produced intense ions at m/z 309 and 311 in the isobutane spectrum), and 9-hydroperoxy epidioxide and 9,16-dihydroperoxide from oxidized methyl linolenate, from Frankel *et al.* (1986).

decomposition pathways established by GC-MS (Chapter 4, D) (Figure 6.16). With ammonia as the CI gas, both 9- and 13-hydroperoxides showed a more stable molecular adduct ion $(M+NH_4)^+$ at m/z 344. The 9-hydroperoxide

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produced methyl 9-oxononanoate (m/z 204) and decadienal (m/z 170), and the 13-hydroperoxide produced methyl 13-tridecadienoate (m/z 256), expected by homolytic cleavage. The same analytical approach was applied to analyse hydroperoxy epidioxides and dihydroperoxides obtained from methyl linoleate and linolenate (Figure 6.16). The fragmentation patterns observed under CI-MS conditions were in complete agreement with those established by decompositions under GC-electron ionization MS conditions (Chapter 4, D). Similarly, the sites of hydroperoxy substitution in isomeric hydroperoxides of methyl oleate were identified directly by CI-MS (Figure 6.17). The fragmentation ions (m/z 213, 199, 155 and 169) were attributed to scission α to the hydroperoxy group with loss of water. However, previous CI-MS analyses of the hydroperoxides of linoleate and secondary oxidation products gave ions with isobutane for protonated fragments, produced by either homolytic β-scission or heterolytic (also referred to as Hock cleavage) decomposition pathways established under thermal and acid conditions (Chapter 4, D). Thus, the fragmentation ion m/z 213 from the 11-oleate hydroperoxide can be attributed to the protonated ion ($212+H^+$) due to methyl 11-oxo-9-undecenoate, the ion m/z 199 from the 10-oleate hydroperoxide to the protonated ion ($198+H^+$) due to methyl 10-oxo-8-decenoate, the ion m/z 155 from the 9-oleate hydroperoxide to the protonated ion ($154+H^+$) due to 2-decenal, and the ion m/z 169 from the 8-oleate hydroperoxide to the protonated ion ($168+H^+$) due to 2-undecenal (Chapter 4, D). The corresponding fragmentation patterns obtained from linoleate hydroperoxides were also supported unambiguously by tandem MS discussed below.

By tandem mass spectrometry or MS/MS a specific parent ion is ionized again into "daughter" ions, which are separated and monitored with higher sensitivity of detection. Fragments arising from CI of intact hydroperoxides were thus isolated and studied by tandem spectrometry. Although both the 9- and 13-hydroperoxides gave intense CI ions at m/z 309 and 311 with isobutane (Figure 6.16), the daughters of these ions obtained by MS/MS were clearly different. From the ion at m/z 309 relatively intense daughter fragments were obtained at m/z 99 (apparently due to hexanal–H^+) in the 13-hydroperoxide and at m/z 185 (apparently due to methyl 9-oxononanoate–H^+) in the 9-hydroperoxide. Similar information was obtained from the CI ion at m/z 328 with ammonia giving intense daughter fragments at m/z 185 and 99 from the 9- and 13-hydroperoxides, respectively. Tandem mass spectrometry and CI-MS can thus be used as powerful tools for establishing the position of hydroperoxides and other complex oxygenated functions in isomeric mixtures of oxidized unsaturated lipids without the tedious problems of derivatization.

Although valuable and useful, the structural information from the advanced MS techniques APCI, CI and other "soft" MS techniques has been mainly qualitative. For quantitative analyses, more careful work is needed to standardize spectra with authentic reference compounds and to better

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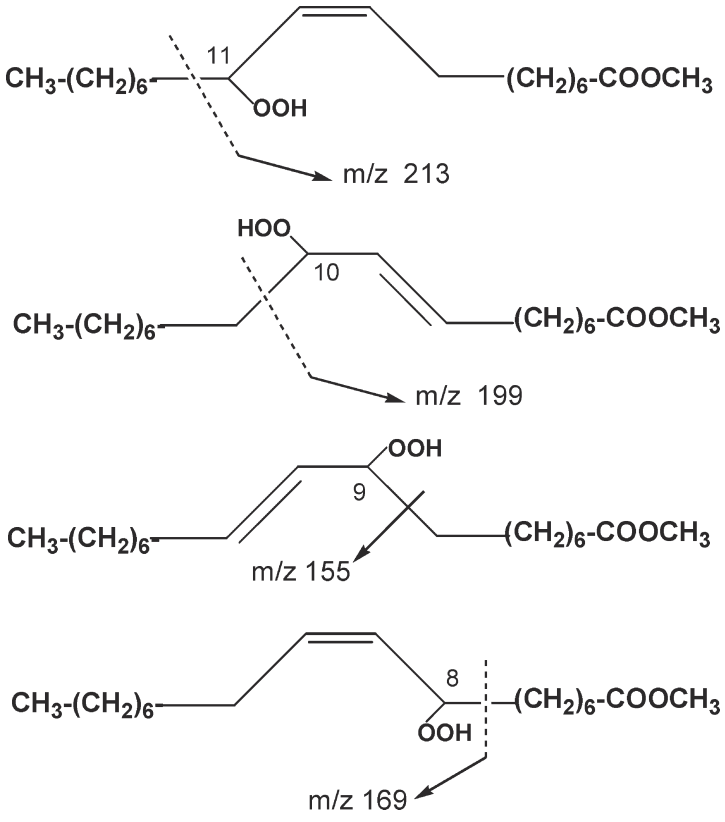


Figure 6.17. Mass fragment ions obtained by chemical ionization-mass spectrometry (CI-MS) analysis of 8-, 9-, 10- and 11-hydroperoxides from oxidized methyl oleate. From Porter *et al.* (1994). Fragmentation ions m/z 213, 199, 155 and 169 were attributed to scission α to the hydroperoxy group with loss of water (see discussion in text).

interpret the complicated fragmentation patterns obtained by these MS techniques. Although these methods are useful for the direct MS identification of intact labile polyunsaturated hydroperoxides and other oxidation products without the necessity of derivatization, authentic standards of these compounds required for quantification are very difficult to synthesize and therefore not readily available.

G. Coordination ion-spray-mass spectrometry (CIS-MS)

This new technique of chemical ionization is based on the formation of charged complexes with silver or other transition metal ions, producing stable coordination compounds with olefinic compounds that can be detected by MS. The use of silver ions with CIS-MS proved to be a useful technique for the analysis of complex lipid oxidation products, which readily form Ag^+ coordination

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compounds. Lipid hydroperoxides and cyclic peroxides form stable Ag^+ adducts producing dominating molecular ions $[Mg+Ag]^+$ and fragmentation ions by heterolytic decomposition pathways. These pathways were previously established by GC and GC-MS (Chapter 4) under either thermal or acid conditions and by CI-MS (Section F); they are also referred to as Hock fragmentation (see Chapter 4).

The CIS-MS technique was used to study the hydroperoxides produced from cholesterol linoleate, cholesterol arachidonate and the corresponding polyunsaturated fatty esters of phospholipids, by introducing samples either by direct liquid infusion or by normal phase HPLC. Analyses by direct liquid injection of solutions of 13- and 9-hydroperoxides containing $AgBF_4$ produced molecular ions $[M+Ag]^+$ adducts from the Ag isotopes 107 and 109. Collision-induced dissociation experiments (also known as tandem MS, or MS-MS, section F) on the Ag adduct ions gave fragment ions (m/z 687, 689) corresponding to 12-oxo-9-dodecenoate cholesteryl ester for the 13-hydroperoxides of linoleate, and fragment ions (m/z 647, 649) corresponding to 9-oxononanoate cholesteryl ester for the 9-hydroperoxides of linoleate (Figure 6.18). The same analytical approach was used to identify the corresponding aldehyde esters from the 9- and 13-hydroperoxides of linoleoyl phosphatidylcholine and from the 9- and 12-hydroperoxides of arachidonyl phosphatidylcholine (Figure 6.19). The same aldehyde esters (also known as core aldehydes) were previously identified among the thermal decomposition products of methyl linoleate hydroperoxides (Chapter 4). The CIS-MS technique was also used to characterize the fragment ions corresponding to aldehydes and epoxides from cholesterol arachidonate hydroperoxides as well as the cholesteryl 7α- and 7β-hydroperoxides (Chapter 2, Section D) produced by direct oxidation of the cholesterol backbone. How much competition occurs between oxidation of the side chain of arachidonate esters and the cholesterol backbone is not established. More quantitative analyses are needed to clarify the mechanism of lipid hydroperoxide decomposition under CIS-MS conditions.

H. Advanced instrumental techniques for volatile flavor analysis

Important advances have been achieved recently in developing very sensitive methods for analysing volatile flavor compounds to link their relative release to sensory perception in foods. Volatile compounds can now be measured "in-nose" by introducing the whole mixture into the mass spectrometer and resolving them entirely by mass followed by APCI-MS based on proton transfer. This powerful technique also referred to commercially as the "MS-Nose", allows *in vivo* sampling of moist air from the panelist to the source and characterizing the effect of lipds and food emulsions on flavor release and delivery from food matrixes during consumption.

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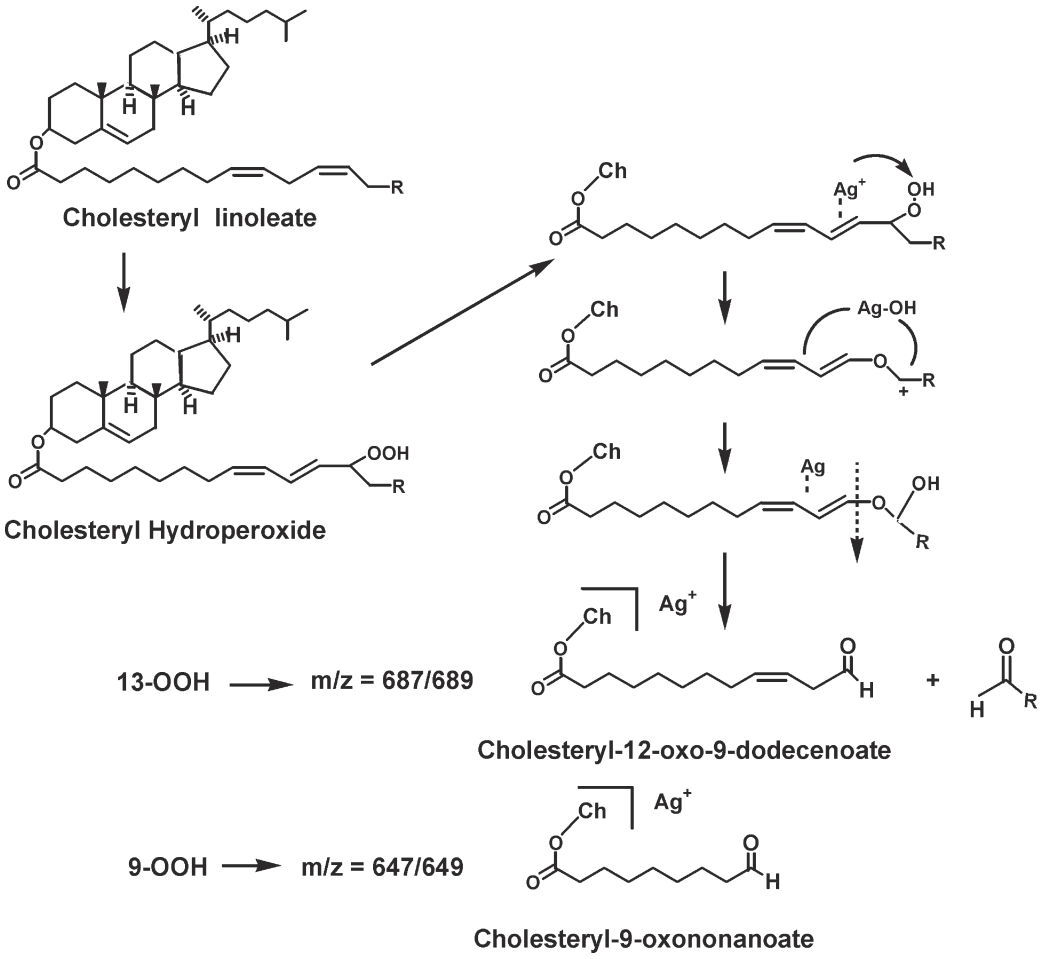


Figure 6.18. Coordination (Ag^+) ion spray-mass spectrometry (CIS-MS) of oxidized cholesteryl linoleate showing heterolytic cleavage of silver adducts of cholesteryl linoleate hydroperoxides. Adapted from Havrilla *et al.* (2000).

Monitoring flavor release from foods during eating requires a rapid and sensitive response in the range of ppb. Direct MS techniques have been developed for this purpose by sampling directly into the source and resolving the ions by mass alone. The current MS-nose method used for sensitive flavor analysis by APCI or proton transfer reaction (PTR) followed by MS, can tolerate water and air, produce single ions for most compounds and operate at pressures that allow interfacing between people and the ion source. This powerful technique, also referred to as "nosepace analysis", can measure the actual concentration of compounds close to the olfactory receptors, and allows the study of relationships between concentration of volatile compounds and perceived intensity and any synergy or masking between compounds during food consumption.

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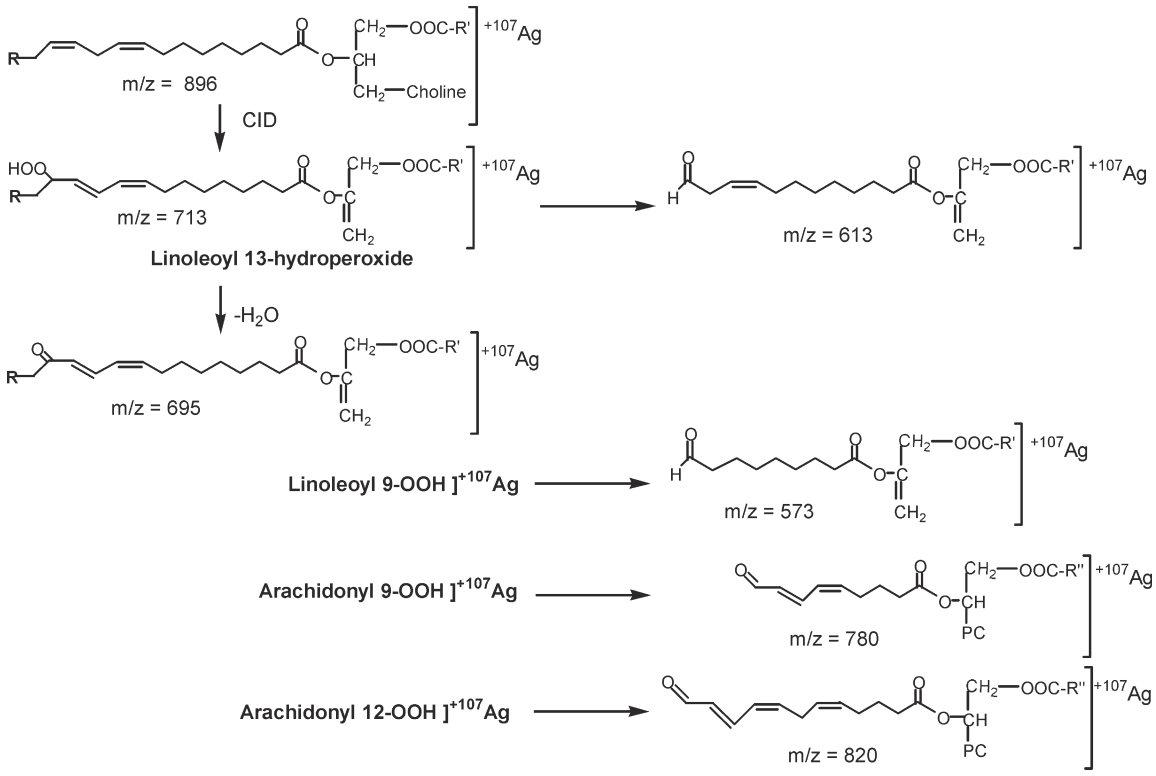


Figure 6.19. Coordination (Ag^+) ion spray mass spectrometry (CIS-MS) of oxidized 1-palmitoyl -2-linoleoyl-*sn*-glycero-3-phosphatidylcholine and 1-stearoyl -2-arachidonyl-*sn*-glycero-3-phosphatidylcholine showing heterolytic cleavage of silver adduct of linoleoyl and arachidonyl hydroperoxides (CID = collision induced dissociation). Adapted from Milne and Porter (2001). See Figure 6.18.

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Table 6.8. Nosespace release (NR) and perceived intensity (PI) of hexanal in four different matrices at three different concentrations [a]

Matrix	Low hexanal (1 mg/l)		Medium hexanal (4 mg/l)		High hexanal (16 mg/l)	
	NR	PI	NR	PI	NR	PI
Water	6.3	29	33.0	52	131	84
Skim milk	6.3	31	28.5	49	110	86
2.7% Fat milk	5.4	5	33.4	38	114	65
3.8% Fat milk	5.9	6	26.1	28	81	48

[a] From Roberts *et al.* (2003).
NR, nosespace release in nl/l; PI, perceived intensity in line scale value, 0–100.
Nosespace analysis: The air exhaled through the nose was drawn up into a special heated stainless steel tube and split into two fractions. One fraction was analysed by proton reaction mass spectrometry, and the remainder was released into the laboratory air.
Perceived intensity determination: Five experienced panelists rated the intensity of the added hexanal with the sample's background of milk flavor. They marked their PI on a line anchored at either end with a line scale of 0 (weak) to 100 (strong).

Nosespace analyses can measure the actual compound concentration that comes out of the nose after passing through the nose cavity. Nosespace release analysis of hexanal was compared with perceived intensity by trained panelists at three concentrations to determine the effect of fat concentration on flavor absorption (Table 6.8). This analysis showed only significant differences between water and whole milk at the high hexanal concentration. The perceived intensity by the sensory panel was significantly different over water with whole milk and low-fat milk but not with skim milk, decreasing in the order: 3.8% fat milk < 2.7% fat milk < skim milk = water. This reduction in nosespace release with increasing fat concentration was lower than that observed by static headspace-GC. This difference was attributed to several possible factors, including the physiology of swallowing liquid samples, dilution with saliva, transfer of the volatile compound from the sample to the air and through the retronasal route to the nasal cavity and final exhalation through the nostrils. These matrix effects on relative perceived intensity are consistent with the well-recognized lower threshold values of flavor volatile compounds in oil than in aqueous systems (Chapter 11, C.4). The same phenomenon, referred to as "flavor paradox", is reflected by the higher impact of flavor volatiles in low-fat foods than in higher fat foods, due to their higher headspace concentration in the former than in the latter foods (Chapter 11, C.4).

Comparison of the amounts of volatiles in the headspace above a solution and the breath volatile concentration after consumption of the solution showed that the volatiles exhaled from the mouthspace and nosespace had significantly lower intensities relative to those for headspace analysed by APCI-MS (Table 6.9). Breath exhaled from the mouth contained 8-fold more volatiles than

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Table 6.9. Breath analysis of mouthspace and nosespace signal intensities relative to headspace intensities by atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) [a]

Compound	Mouthspace (%)	Nosespace (%)
Acetaldehyde	19	3.0
Butanal	13	3.4
Hexanal	14	3.2
2-Hexenal	13	3.4
Octanal	5.4	0.80
Decanal	2.1	0.38

[a] From Linforth *et al.* (2002)
Headspace analysis: The headspace of volatile solutions (0.1 to 5 ppm) were analysed by APCI-MS by drawing an aliquot into the heated sampling line of the mass spectrometer and determining the maximum signal within 20 seconds.
Breath analysis: Aliquots (15 ml) of the solutions were placed in-mouth and swallowed immediately. Breath was exhaled through the nose or mouth through a tube connected to the end of the mass spectrometer sampling line and the maximum signal intensity was compared with the signal during headspace analysis.

breath exhaled from the nose. This difference was not due to dilution of the sample by saliva in the mouth. The most important factor appeared to be mass transfer equilibration affecting volatile delivery from the solution to the gas phase where it is diluted through the upper airway. The concentration of volatiles exhaled from the nose was affected by absorption to the nasal epithelia, which increased with lower air/water partition coefficients.

The studies published in this promising area of flavor chemistry and physiology have been limited to a few volatile compounds. They need to be extended to the multitude of other volatile compounds derived from lipid oxidation. Many of these volatiles are known to have a significant impact on quality and acceptability of lipid-containing foods. The complex volatiles produced by food lipids containing *n*–3 polyunsaturated fatty acids reported to impart nutritional and health benefits are especially important because they develop undesirable fishy odors and flavors at extremely low levels of oxidation (Chapter 4.D; Chapter 5.F; Chapter 11.E).

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CHAPTER 7

Stability methods

A. Stability as parameter of quality control

Stability can be defined as the resistance of a lipid to oxidation and to the resulting deterioration due to the generation of flavor and odor causing oxidative rancidity and decreasing food quality. Tests for *oxidative stability* attempt to accelerate the normal oxidation process to yield results that can be translated into quality parameters for different food lipids. Stability methods thus constitute an important quality control tool for predicting the shelf life of foods. Stability methods are also important to evaluate the effect of changes in food formulations, processing parameters and other factors on lipid oxidation. Stability methods are particularly valuable in research and evaluation of antioxidants and their effects on protection of foods against lipid oxidation (Chapter 9).

The literature on the evaluation of oxidative stability of food lipids is extensive, but the results of different investigators are difficult to interpret, because of variations in test conditions and end-point of lipid oxidation. Conditions used by many workers for accelerated oxidation have been too drastic. The use of stability tests requiring elevated temperatures of oxidation (100°C and higher) is particularly questionable, because samples develop excessive levels of rancidity, which are not relevant to normal storage conditions. Further complications arise in multi-component foods in which interactions between lipids, carbohydrates and proteins at elevated temperatures produce materials that can act as either prooxidants or inhibitors. As new oil phases are formed, the solubility and partition of different reactants between different phases change with temperature. Metals and exposure to light may cause decomposition of hydroperoxides and produce misleadingly low peroxide values even though rancidity increases and sensory quality decreases.

The oxidative stability of food lipids has been evaluated by a variety of methods using a wide range of conditions and techniques for measuring oxidation. The choice of appropriate methods and oxidation conditions is critical in the interpretation of stability data. Thus, interpretation of data pertaining to lipid oxidations should take into account the limitation of the methodology used. The oxidative stability of unsaturated lipids can be estimated by determining the extent of oxidation produced under various defined and standardized conditions. The susceptibility of the samples to oxidation can be measured reliably if the samples are initially fresh (with a peroxide value approaching 0) and the conditions of oxidation are sufficiently mild and

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relevant to normal storage conditions. Excessive deteriorations beyond normal levels are to be avoided for many reasons discussed below. The results of stability measurements are useful in comparing the quality of raw materials in various food applications, in attempting to predict future quality performance of fats used as food ingredients, and in comparing the performance of antioxidants and stabilizers (Chapters 9 and 11).

For the judicious choice of stability methods, we need to address the following questions:

a) What is happening to the unsaturated lipid or food under a range of realistic storage conditions?
b) What oxidative stress should be used? What are the effects of temperature, metals, and light?
c) What end-point is relevant to the sample in question? What is an acceptable level of quality deterioration?
d) Which lipid oxidation methods should be used? How significant are the results with respect to quality deterioration? Different products should be measured, including primary and secondary decomposition products.
e) How is stability influenced by the interfacial relationship between food components, oxidants and antioxidants?

Methods to measure lipid oxidation have generally been unspecific and not sufficiently sensitive to measure flavor and low levels of oxidative deterioration of food lipids. Several specific complementary methods are needed to determine the contribution of lipid oxidation products formed at different stages of the multi-step process of oxidative deterioration in foods. Since polyunsaturated hydroperoxides decompose more readily at higher temperatures to form aldehydes causing rancidity, it is important to measure both aldehydes and hydroperoxides to monitor lipid oxidation. For the reliable prediction of shelf life of foods containing polyunsaturated lipids, it is essential to use more than one specific method to determine oxidation at different levels and to store samples at several temperatures, preferably between 40 and 60°C.

B. Development of rancidity in foods during storage

Lipid oxidation in food products develops slowly initially, and then accelerates at later stages during storage (Figure 7.1). The induction period (IP), or shelf life at room temperature, is defined as the time to reach a constant percent oxidation of the fat or the end of shelf life. From an industrial point of view, shelf life is also defined as the storage time before the quality of a product becomes unacceptable to consumers.

The induction period is measured, as the time required reaching an end-point of oxidation corresponding to a level of detectable rancidity. Alternatively, the induction time is taken as the time required for a sudden change in rate of

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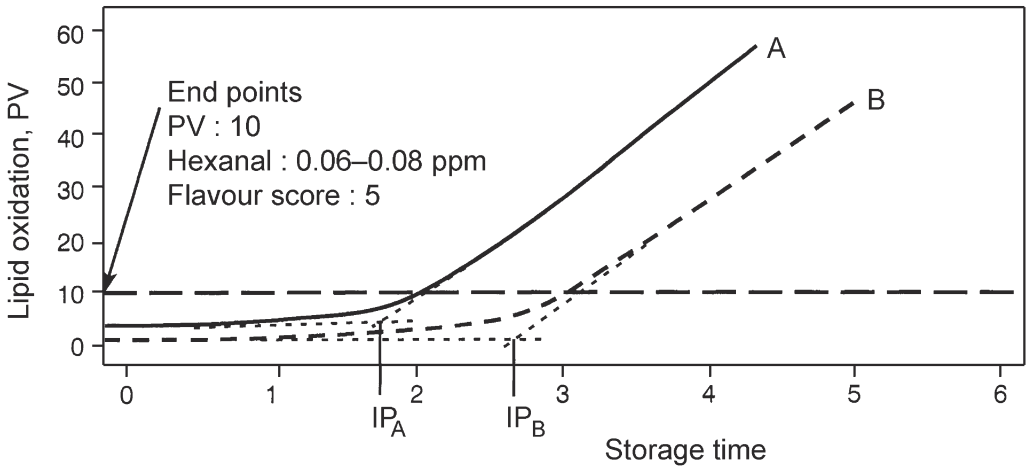


Figure 7.1. Development of rancidity in foods during storage. Storage times are given in arbitrary values. PV = peroxide value; IP = induction period.

oxidation, by estimating the intersect or cross-point between the initial and final rates of oxidation (IP_A and IP_B). The end-points for rancidity or acceptability limits for vegetable oils containing linoleic acid as the main polyunsaturated fatty acid are shown in Figure 7.1 as a broken line: peroxide value of 10, or hexanal content of 0.06–0.08 ppm, or flavor score of 5. These end-points are reached after 2 days/weeks/months (depending on conditions) in sample A, compared to 3 days/weeks/months in sample B. For vegetable oils containing linolenic acid (e.g. soybean and canola oils) and fish oils containing EPA and DHA, the end-points for acceptability will occur at significantly lower levels of oxidation. If we measure peroxide value or hexanal or propanal contents, we refer to this evaluation as *oxidative stability*, if we measure a flavor score, then we refer to it as *flavor stability*. The difference in stability between samples A and B could be due to their different initial levels of oxidation at the start of the storage period. Other factors that will be discussed in later chapters include formulation, processing and packaging. From the crude stage to different stages of processing, some oxidation can take place. This oxidation during processing is also known as "hidden oxidation" (see Chapter 4.C and Chapter 5.D), and will affect the subsequent stability of the food product during storage.

The distinction between oxidative stability and flavor stability is particularly important in the evaluation of vegetable oils containing linolenate, such as soybean and canola oils, and fish oils containing long-chain *n*–3 PUFA (see Table 1). These oils undergo flavor deterioration much sooner and at an earlier stage of oxidation than vegetable oils containing linoleate, such as cottonseed and corn oils. The difference between these oils can be attributed to the significantly lower threshold values of odor- and flavor-significant volatiles produced from oxidation of linolenate and EPA and DHA compared to volatiles produced from oxidation of linoleate (Chapter 5.A; Table 5.1).

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C. Stability of different systems

Lipids exist in most foods as multiphased colloidal systems bound by surface-active phospholipids, proteins and emulsifiers. The oxidative stability of food lipids is greatly affected by the partitioning of the lipid substrates, metal initiators and antioxidants, which is complex and depends on the physical properties of the food. We may consider three types of food systems (see Chapter 10):

Bulk oil systems: Oil is the continuous phase and air bubbles or air-surface is the dispersed phase. Salad vegetable oils are examples of bulk oil systems. Hydrophilic antioxidants and prooxidants may be oriented in the air-oil interface, and lipophilic antioxidants and prooxidants remain in solution in the oil phase.

Oil-in-water emulsions: Water is the continuous phase and oil is the dispersed phase. This dispersed phase is stabilized by an emulsifier. Milk and mayonnaise are examples of oil-in-water emulsions. Lipophilic antioxidants and prooxidants are generally sufficiently surface-active to be oriented in the oil-water interface, whereas hydrophilic antioxidants and prooxidants move to the water phase and become diluted.

Liposomes: Phospholipids spontaneously form bilayers called liposomes when dispersed in water. Meat and biological membranes are examples of liposomes. Hydrophilic antioxidants and prooxidants would be oriented at the polar end of the phospholipid bilayers, while the lipophilic antioxidants and prooxidants would be located between the long chain of the lecithin and the water phase. Multiphase food systems will be discussed further in Chapter 10.

D. Accelerated parameters

To estimate the oxidative stability or susceptibility of a fat to oxidation, the sample is subjected to an accelerated oxidation test under standardized conditions and a suitable end-point is chosen to determine appropriate levels of oxidative deterioration (Figure 7.1). Several parameters such as temperature (60–140°C), metal catalysts (5–100 ppm), oxygen pressure (3–165 psi), or variable shaking to increase reactant contact, are manipulated to accelerate oxidation and development of rancidity in oils and emulsions. The oxidation level used for an end-point varies widely according to the time desired to obtain stability data. For practical purposes, predictions of oxidative stability in foods and oils based on measurements of induction period should be related to actual product shelf life, and the conditions used should be as close as possible to those under which the food is stored. To translate the induction period obtained under accelerated conditions to the actual shelf life of a product, it is necessary to use an arbitrary factor based on prior experience with the desired product. Much effort has been devoted to more accurately estimate the shelf life of foods

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by optimizing formulation, processing, packaging and environmental storage conditions. Food acceptability by consumers is generally inferred from practical experience. More accurate testing is needed, however, and the cost can be justified by avoiding or minimizing wasteful discarding foods before they become unacceptable to consumers.

E. Effect of temperature

Temperature is the most important factor to be considered in evaluating the oxidative stability of unsaturated fats, because the mechanism of oxidation changes with temperature. Different hydroperoxides act as precursors of volatile flavors, and decompose to different extents at different temperatures. Because the rate of oxidation is exponentially related to temperature, the shelf life of a food lipid decreases logarithmically with increasing temperature (Section I). At ambient temperatures, the rate of lipid oxidation is independent of the oxygen pressure (Chapter 1). However, at elevated temperatures, the rate of lipid oxidation becomes dependent on oxygen pressures, because the solubility of oxygen decreases and becomes a significant limiting factor during the progress of the reaction. In stability tests, it is important therefore to use several storage temperatures and in a range as low as practical.

The activation energy (or the additional energy needed by reactants to form products) of lipid oxidation is higher in the presence of antioxidants (about 20–25 kcal/mole) than in their absence (about 18 kcal/mole), because antioxidants lower the rates of oxidation by increasing the overall energy of activation. Conversely, metal catalysts lower the activation energy (about 10–14 kcal/mole) by increasing the rate of lipid oxidation. An Arrhenius plot of log induction period versus the reciprocal of absolute temperature shows that the effectiveness of antioxidants increases as temperature decreases (Figure 7.2). At a given elevated temperature, the lines corresponding to inhibited and uninhibited lipid oxidation will cross and the effectiveness of antioxidants will vanish and may even act as prooxidants (see Chapter 9.1a). The overall rate constant for autoxidation is proportional to the log of the induction period. The temperature coefficients (or how much faster a sample is oxidized by raising the temperature) are different for a fat according to the relative concentration and effectiveness of natural or added antioxidants. Thus, the temperature coefficient is higher for an animal fat, which has very low levels of antioxidants, than a vegetable oil containing natural antioxidants. Consequently, any prediction for lower temperatures requires testing at several different temperatures. Stability tests run at three temperatures about 5 or 10°C apart are thus generally recommended.

The accelerated temperature used for stability tests is greatly dependent on the relative fatty acid composition of the oils tested: in general, the more polyunsaturated the oils, the lower the temperatures that should be used to test

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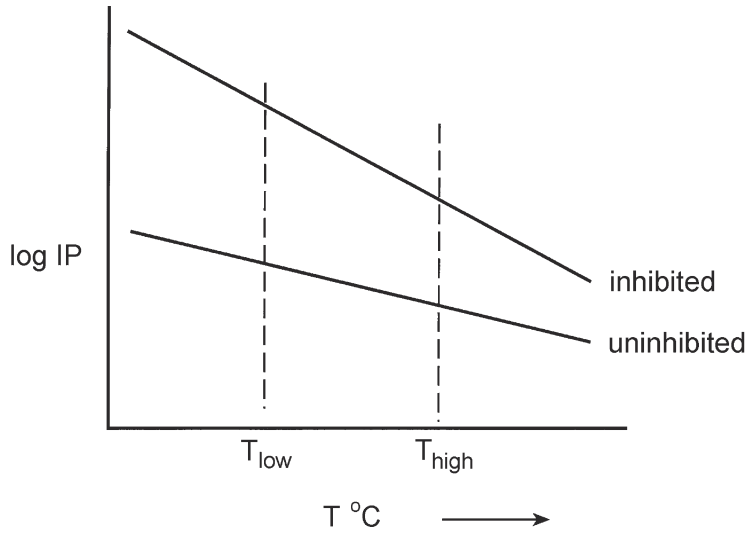


Figure 7.2. Arrhenius plots for inhibited and uninhibited lipid oxidation. IP = induction period; T = temperature. Adapted from Ragnarsson and Labuza (1977).

their oxidative stability. The significance of the induction period depends on the degree of unsaturation of the oils tested. With highly polyunsaturated oils, the level of oxidation detected at the end-point at high temperatures is too high and beyond the point at which rancid flavors are detected. Therefore, vegetable oils should be oxidized between 40 and 60°C, and fish oils at 40°C or below.

F. Stability of oxidation products

The large number of precursors of volatile decomposition products affecting the flavor of oils has been discussed in Chapter 4. Only qualitative information is available on the relative oxidative stability of hydroperoxides, aldehydes and secondary oxidation products. As observed with the unsaturated fatty ester precursors, the stability of hydroperoxides and unsaturated aldehydes decreases with higher unsaturation. Different hydroperoxides of unsaturated lipids, acting as precursors of volatile flavor compounds, decompose at different temperatures. Hydroperoxides of linolenate and long-chain *n*–3 PUFA decompose more readily and at lower temperatures than hydroperoxides of linoleate and oleate. Similarly, the alkadienals are less stable than alkenals, which in turn are less stable than alkanals. The short-chain fatty acids produced by oxidation of unsaturated aldehydes will further decrease the oxidative stability of polyunsaturated oils. For secondary products, dimers are less stable than dihydroperoxides, which are less stable than cyclic peroxides.

Common vegetable oils containing mixtures of oleate, linoleate and linolenate produce 14 positional isomers of hydroperoxides by autoxidation and photosensitized oxidation (Chapters 2 and 3). More secondary products, dimers and

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oligomers are produced from linolenate-containing oils than from linoleate-containing oils at lower levels and temperatures of oxidation. Fish oils containing long-chain *n*–3 polyunsaturated fatty acids produce volatile decomposition products at lower temperatures (below 40°C) than vegetable oils containing linoleate and linolenate (above 60°C). Fish oils have thus lower activation energies of volatile formation than vegetable oils and high-oleic oils.

G. Standard accelerated stability tests

To estimate oxidative stability or susceptibility of a fat to oxidation, the sample is subjected to an accelerated oxidation test under standardized conditions and a suitable end-point is chosen to determine signs of oxidative deterioration.

1. Storage at ambient conditions

Although ambient conditions are ideal, this test is too slow to be of practical value. Results at ambient conditions are very difficult to reproduce because environmental variables are difficult to control over prolonged periods of storage.

(a) *Light oxidation.* By exposure to light, the stability of vegetable oils can be screened rapidly. This test is especially useful for soybean oil and other vegetable oils that are very sensitive to light oxidation. However, the mechanism of photosensitized oxidation is different from that of autoxidation (Chapter 3), and different flavor precursors are formed with different volatile breakdown products, which may have different flavor significance.

(b) *Metal accelerated oxidation.* The addition of metal catalysts such as iron or copper salts provides a rapid screening test for stability, which emphasizes the breakdown products more than the flavor precursors. Because lipid oxidation is generally catalysed by trace metals, they become the dominating catalyst at moderately elevated temperatures such as 40°C, and adding metals may be considered as a meaningful way of accelerating oxidation. Hemin and metallic catalysts are useful for estimating the oxidative stability of food emulsions.

2. Storage at elevated temperatures

(a) *Weight-gain method.* This method consists of measuring the increase in weight of an oil sample due to oxygen absorption. The test is not sensitive and the end-point is questionable because it requires too high a level of oxidation that is beyond the point where flavor deterioration occurs, especially with vegetable oils containing linolenic acid and fish oils containing *n*–3 polyunsaturated fatty acids.

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(b) *Schaal oven.* This test, also referred to as *oven test*, involves heating the sample at 50 to 60°C until it becomes rancid by smell or taste, or reaches a suitable end-point based on peroxide value, conjugated dienes or carbonyl value. This test uses relatively mild temperatures and has the fewest problems. The results of this test correlate best with actual shelf life because the end-point represents a lower degree of oxidation. The determinations of peroxide values, conjugated dienes or carbonyl values of oils are more meaningful with oils heated at 60°C or below. At 100°C and higher temperatures, the results of these determinations become questionable and difficult to interpret because the hydroperoxides are thermally decomposed, and side-reactions forming polymers and breakdown products become significant.

(c) *Active Oxygen Method (AOM), Rancimat and Oxidation Stability Index (OSI).* Air is bubbled through a sample of oil in special test tubes heated at 98–100°C and the progress of oxidation is followed by peroxide value determination in the AOM test, and by conductivity measurements in the Rancimat and OSI tests. The automated Rancimat and OSI tests may be run at temperatures ranging from 100–140°C, and the effluent gases are led through a vessel containing deionized water and the increase in conductivity measured are due to the formation of volatile acids (mainly formic acid) by thermal oxidation. The OSI is defined as the time in hours required to reach the maximum change of conductivity.

(d) *Oxygen absorption method (Oxidograph).* A sample of oil is heated around 100°C in a sealed vessel containing a known volume of oxygen and the reduction in pressure, recorded automatically, is measured as oxygen absorbed. The induction period is taken as the time in hours to reach the maximum change in rate of oxygen uptake.

The AOM, Rancimat, OSI and Oxidograph tests are not reliable, because they are run at elevated temperatures at which the mechanism of lipid oxidation changes. At these high temperatures, oxidation becomes more dependent on oxygen concentration, the solubility of oxygen decreases, and the concentration of available oxygen changes drastically because of the rapid rates of oxidation. The dependence on oxygen increases with degree of oxidation of the oils. As the oxygen pressure is reduced, the concentration of peroxyl radicals ($ROO^{\bullet}$) decreases and dimeric and polymeric compounds are produced by condensation of the resulting alkyl radicals ($R^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$) (see Chapter 12). Lipid oxidation proceeds erratically at elevated temperatures by increasing the activity of contaminating metals and by promoting the prooxidant activity of tocopherols in vegetable oils (Chapters 9 and 12). At elevated temperatures, polymerization and cyclization become important, and these side-reactions are not significant at normal storage temperatures. Volatile acids measured by the automated Rancimat and OSI methods are only produced in

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significant amounts at elevated temperatures and, therefore, are not relevant to normal storage conditions. In spite of these serious pitfalls, the AOM, the Rancimat and OSI methods are commonly used to compare the relative stability of very similar samples. However, the results of these high-temperature stability tests can be misleading in the evaluation of the effectiveness of antioxidants (Chapter 9). Antioxidants are markedly influenced by the effect of oxidation temperature, and widely diverging results are frequently obtained when antioxidants are compared at temperatures below 60°C with temperatures above 60°C (see Table 9.3). Several attempts have been made in the literature to predict oil performance during frying based on measures of thermal oxidation products such as polymers formed at elevated temperatures (170–180°C) without bubbling air. Such methods are very misleading because frying is a complex process influenced by a large number of factors, including the foods, which incorporate significant amount of water converted into steam at frying temperatures (See Chapter 12).

H. Alternative stability methods

1. Gas analysis

This method measures oxygen in the headspace of oil stored in a suitable container under variable conditions by gas chromatography using a thermal conductivity detector. This is a more sensitive and sophisticated method than oxygen uptake by the classical manometric methods. The information provided is limited, however, because it measures a reactant rather than breakdown products that are directly related to and cause flavor deterioration.

2. Analyses of volatiles by gas chromatography

These tests are the most useful and objective for estimating oxidative and flavor stability, because the volatile products measured correlate well with rancidity if they are formed under relatively mild conditions of oxidation (60°C or below). These methods are discussed in detail in Chapter 5, Section F.

3. Hydroperoxides

These primary products of oxidation can be determined by gas chromatography, after preparing suitable derivatives, and by HPLC using either an ultraviolet or refractive index detector. Although hydroperoxides are the most important precursors of flavor compounds, their importance depends on their relative stability and the composition of the volatiles they produce under different conditions. The yields of hydroperoxides decrease as their rates of decomposi-

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tion increase, in the order: oleate, linoleate, linolenate, higher polyunsaturated fatty acids (containing more than three double bonds).

4. Carbonyl compounds

These secondary products of oxidation can also be determined by gas chromatography or HPLC, either directly or after preparing suitable derivatives. The HPLC method is less sensitive than the direct gas chromatography methods. HPLC can be carried out under mild conditions and is subject to less artifacts than gas chromatographic methods, which require higher temperatures. However, HPLC requires the use of organic solvents, which cause losses of volatile carbonyl compounds. HPLC is also unsuitable for the determination of volatile hydrocarbons formed by decomposition of hydroperoxides.

5. Thermal analyses

These techniques measure the effect of temperature on physical or chemical changes, such as specific heat and sample weight, in a lipid material. *Differential scanning calorimetry* (DSC) measures the heat absorbed or gained (ΔH) by the sample to obtain the temperatures and heat of the exothermic oxidation. This technique involves heating the sample and reference compounds isothermally or continuously to increasingly elevated temperatures (120–150°C), with either purged oxygen or oxygen pressure (500 psig), for the rapid estimation of oxidative stability. The end-point of DSC is measured as the time where a rapid exothermic reaction of oil and oxygen occurred.

Thermogravimetric analysis measures the effect of temperature on change in weight resulting from oxygen absorption during oxidation. The technique can also be carried out as an accelerated stability test, either isothermally or by linear temperature programming from ambient to elevated temperatures. When run isothermally, the end-point is the time where a rapid weight gain is observed. When run by temperature programming, the end-point is the temperature where the rapid weight gain is detected. Although these methods correlate well with the standard AOM test, they have the same problems in requiring elevated temperatures where oxidation is too high and beyond the level of detection of rancidity flavors.

6. Electron spin resonance (ESR) spectrometry and spin-trapping

This sensitive technique, also known as electron paramagnetic resonance (EPR), is a powerful method that can specifically detect free radicals formed by autoxidation. Because radicals have unpaired electrons, they display different paramagnetic properties or energy levels (measured as magnetic moment)

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under a varying magnetic field. Solid or liquid samples are irradiated and the absorption of electromagnetic radiation at different energy levels is measured as a function of the applied magnetic field. Although this analytical technique can be used to predict the stability of lipids by measuring their initial tendency to form radicals during early stages of oxidation, it has many limitations as discussed below.

Although radicals may be measured directly in certain solid foods of low water activity, such as milk powder, indirect methods are required in liquid foods, such as emulsions and beer, because the radicals are very short lived (several seconds for peroxyl radicals). In solid samples, the ESR signals are, however, difficult to identify because of line broadening. In liquid samples, the use of artificial traps is required to form stable adducts that can be readily detected by ESR by reacting covalently with unstable radicals. Spin traps are usually nitroso compounds (e.g. *tert*-nitrosobutane), or nitrones (e.g. α-phenyl-*tert*-butyl nitrone). The applications of spin traps are limited, however, because their efficiencies vary widely with different radicals.

Spin trapped radicals were previously misidentified by ESR as due to peroxyl radicals but were later shown to be due to alkoxyl radical adducts. Attempts to spin trap peroxyl radicals of PUFA led to the formation of PUFA alkoxyl radicals. Spin traps can also perturb the systems under investigation by introducing foreign substances that not only confuse the identity of the original radical adducts, but also can either accelerate or inhibit oxidation. Other special techniques to detect radicals by ESR using rapid freezing, lyophilization, and continuous flow are not often used because they suffer from problems of artefactual signals, and consuming very large samples.

ESR spectroscopy was used to evaluate the oxidative status of milk powders stored at different temperatures and water activities. Samples prepared at low heat and high water activity developed a significantly higher relative radical concentration than those prepared at medium and high heat and low water activity. As expected, high free radical content was observed after storage at 45°C, but not at ambient temperature. The relative concentration of free radicals measured directly in milk powder correlated with measures of TBARS and sensory scores in the reconstituted milk. With fish oil separated from mayonnaise, the induction period for generation of radicals was determined by spin trapping. ESR spectroscopy and spin trapping techniques were also used to determine induction times of food emulsions after storage at 50°C, and to demonstrate the stabilizing effect of EDTA.

The ESR techniques are limited because they involve large size and relatively expensive equipment beyond the capability of many laboratories. They also require conditions that perturb the food systems and may not reflect actual storage stability. The information provided is further limited because these methods measure initial reactants rather than breakdown products that are more directly related to and cause oxidative and flavor deterioration.

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Table 7.1. Ranking of lipid oxidation methods

Methods	Sensitivity	Precision	Information
Sensory	High	Low	High
Volatiles by GC	High	Low	High
Ultraviolet absorption	High	High	Low
Carbonyls	Low	High	Low
Anisidine value	Low	High	Low
Peroxide value	Low	High	Low
Oxygen uptake	Low	High	Low
TBA	Low	High	Low
Carotene bleaching	High	Low	Low
Volatile acids (Rancimat)	Low	Low	Low

I. Lipid oxidation methods

To evaluate oxidative stability, different methods are used to measure lipid oxidation after the sample is oxidized under standardized conditions to a suitable end-point. In Table 7.1, different lipid oxidation tests are ranked in decreasing order of usefulness in predicting the stability or shelf life of a food product. Methods for sensory evaluations, conjugated diene, gas chromatography of volatiles, peroxide values and thiobarbituric acid-reacting substances were discussed in Chapter 5.

1. Sensory methods

These methods provide the most useful information because it is related to consumer acceptance of the food product based on odor and taste. These techniques are very sensitive and provide information on flavor stability, but they are unfortunately expensive and require a relatively large number of samples. They are dependent on taste panels that must be properly trained and on scoring, which is not reproducible from laboratory to laboratory. Human tasters may also be sensitive to the potential allergic effects or toxicity of certain food ingredients and additives.

2. Analysis of volatiles by gas chromatography

This approach is most closely related to flavor evaluation and suitable for comparison with results of sensory panel tests. Although it is more precise than sensory methods, the results vary with different unsaturated oils, and with different additives such as antioxidants and metal inactivators. This method provides useful data on the origin of volatiles and flavor precursors. While total and individual volatiles can be correlated between oxidation time (Table 7.2)

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Table 7.2. Most suitable marker volatiles in virgin olive oil and their correlation with oxidation time at 60°C [a]

Volatile compounds	Slope [b]	Correlation (r)	Source [c]
Nonanal	9.00	0.975	9-18:1-OOH
Hexanal	8.27	0.970	13-18:2-OOH
2-Heptenal	5.01	0.962	12-18:2-OOH
Octane	4.14	0.970	10-18:1-OOH
2-Pentylfuran	2.99	0.966	18:2-OOH
2-Propenal	2.89	0.925	16-18:3-OOH
c,t-2,4-Heptadienal	1.99	0.888	12-18:3-OOH
t,t-2,4-Heptadienal	1.60	0.931	12-18:3-OOH
Heptanal	1.22	0.929	18:1-OOH

[a] From Vichi *et al.* (2003).
[b] Slope of linear regression × 100 between concentration (μg/g) and oxidation time in weeks.
[c] See Chapter 4.D.

and flavor scores, their flavor significance and impact on flavor stability are not clearly established and are difficult to evaluate. More advanced techniques of solid-phase microextraction (SPME) using GC capillary columns with mass spectrometry detection and multivariate analysis (see Chapter 5) are useful to predict the shelf life of milk products. SPME proved useful in determining suitable volatile compounds to use as markers that correlated significantly with peroxide value, UV absorbance at 232 and 270 nm, and loss of unsaturated fatty acids (Table 7.2). Volatile compounds that showed the most significant correlation ($r > 0.900$) between concentration and time of oxidation included, nonanal, hexanal, 2-heptenal, octane, 2-pentylfuran, 2-propenal, *cis,trans*-2,4-heptadienal and *trans,trans*-heptadienal and heptanal. Nonanal, derived from oleate hydroperoxides, and hexanal, derived from linoleate hydroperoxides, increased the most rapidly during oxidation at 60°C.

3. Ultraviolet absorption measurements

The determination of conjugated dienes by ultraviolet spectrophotometry is related to the contents of polyunsaturated hydroperoxides, which act as flavor precursors. These measurements are sensitive and reproducible, but the information is only useful to determine precursors of volatiles formed from polyunsaturated oils.

4. Carbonyls and anisidine values

These tests provide similar information as gas chromatographic analyses for volatiles, but they include also non-volatile precursors. The results are less

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sensitive but more precise than gas chromatographic analyses. Although the information may be related to flavor scores, the proportion of flavor significant carbonyls is difficult to assess.

5. Peroxide values

This measurement is not sensitive, but its precision is relatively high. The results are related to flavor precursors such as hydroperoxides, but not to the flavor compounds. This method is empirical, and the results are misleading in samples that have been thermally abused or subjected to light oxidation. The peroxide value of samples heated under the AOM or OSI conditions is not related to the actual level of oxidation in the same samples stored under either ambient or lower temperature (below 60ºC) conditions.

6. Oxygen absorption methods

These methods measure the amount of oxygen absorbed during lipid oxidation. Because they measure a reactant and not a product of oxidation, they are of limited sensitivity and require high levels of oxidation as end-point for induction periods and are unsuitable for detecting subtle effects of long-chain *n*–3 PUFA producing fishy odors. The information provided is therefore not closely related to flavor stability.

7. Thiobarbituric acid (TBA) method

Although less sensitive, this method is relatively precise. However, the test is not specific, and the results can be misleading because many primary and secondary oxidation products form TBA-reactive substances. This test is more sensitive with polyunsaturated fats containing three or more double bonds and not sensitive for the oxidation products of oleic and linoleic acids. The results overestimate the amount of lipid oxidation, because extraneous materials in foods such as browning reaction products, protein and sugar degradation products interfere with the TBA test (see Chapter 5).

8. Carotene bleaching

This spectrophotometric method measures the rate of carotene destruction by bleaching occurring by co-oxidation of linoleic acid at 450–470 nm. It is simple and sensitive, but not specific and subject to interference from oxidizing and reducing agents present in crude extracts. Linoleic acid is not an appropriate substrate for food lipids consisting mainly of triacylglycerols, because it forms micelles in aqueous systems. Micelles have markedly different colloidal properties and oxidative stabilities from emulsified triacylglycerols;

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antioxidants and oxidants behave differently in micelles compared to their behaviour in triacylglycerol systems (see Chapter 10). Certain natural phenolic antioxidants show prooxidant activity at low concentrations in contrast to synthetic antioxidants.

9. Volatile acids by Rancimat and OSI

These automated instrumental tests measure the conductivity of low molecular weight fatty acids (mainly formic acid) produced during autoxidation of fats at elevated temperatures (Section G.2c). The results obtained by these methods require relatively high levels of oxidation (peroxide values over 100); the end-points are unreliable because high temperatures are necessary to obtain detectable amounts of organic acids, which are produced beyond the normal level for rancidity.

J. Shelf life prediction

We have seen earlier that since the rate of oxidation is exponentially related to temperature, the shelf life of a food lipid decreases logarithmically with increasing temperature (Section E). Assuming the Arrhenius model, the true shelf life of a food product at ambient temperature can be thus approximated by extrapolating measurements of rancidity obtained at higher temperatures projected to a lower temperature (Figure 7.3).

A well recognized temperature acceleration factor, known as Q_{10}, is based on the increase in oxidation rate produced by a 10°C increase in temperature (T). The shelf life is defined as the reciprocal of the accelerator factor, Q_{10}, as follows:

Q_{10} = quality measure at T + 10/quality measure at T

Q_{10} = shelf life at T/shelf life at T + 10

For a constant Q_{10} of 2, the shelf life of a product is doubled for every 10°C decrease in storage temperature, e.g. if the shelf life is 2 weeks at 50°C, a shelf life of 4 weeks can be predicted at 40°C (Table 7.3). For storage studies carried out at one temperature, shelf life estimates can be estimated if Q_{10} values are known from the literature. The Q_{10} of oxidation is related to the activation energy, which can be determined from an Arrhenius plot (Section E) of ln k versus the reciprocal of absolute temperature. The activation energy of oxidation and Q_{10} can be calculated from the slope of the Arrhenius equation. Lipid oxidation in foods has activation energy between 15 and 25°kcal/mole, depending of course on the presence of prooxidant metal catalysts, which lower the activation energy, or the presence of antioxidants, which increase it. Unusual effects may occur from competitive reactions occurring at lower

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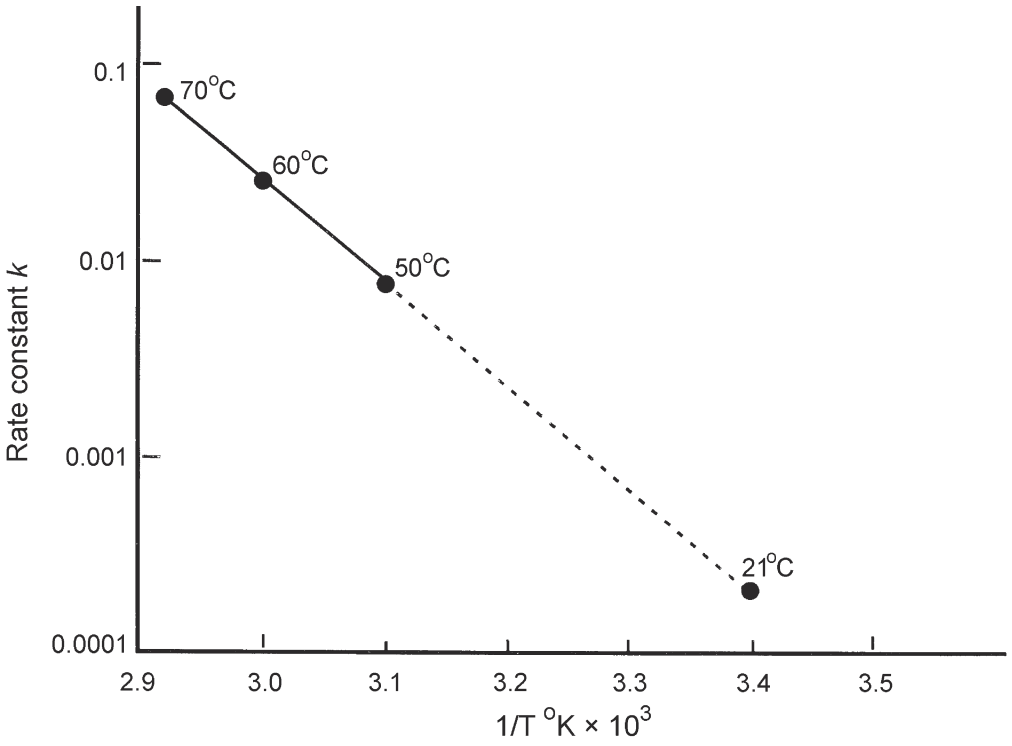


Figure 7.3. Application of Arrhenius plot to approximate shelf life of a food. Adapted from Labuza, 1984).

temperatures, such as during freezing; the concentration of catalysts in the unfrozen liquid may accelerate the oxidation. On the other hand, if oxygen availability is a limiting factor in a food, raising the temperature decreases the solubility of oxygen in fat or water by about 25% for every 10°C increase, and may result in a decrease in quality deterioration. Other factors include changes in water activity and viscosity with temperature and changes in moisture due to humidity differences in storage chambers.

Table 7.3. Effect of storage temperature on shelf life [a]

Temp. (°C)	Shelf life (weeks)		
	$Q_{10} = 2$	$Q_{10} = 2.5$	$Q_{10} = 3$
60	1	0.8	0.67
50	2	2	2
40	4	5	6
30	8	12.5	18
20	16	31.3	54

[a] Adapted from Labuza and Riboh (1982).

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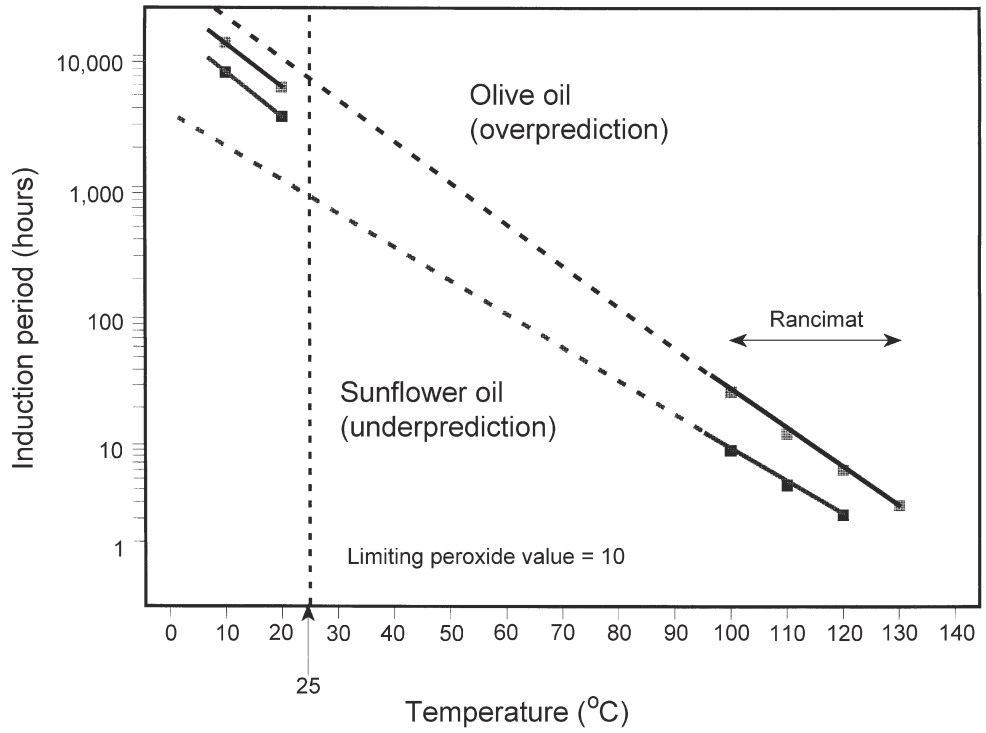


Figure 7.4. Shelf life plots based on induction periods of olive oil and sunflower oil extrapolated to long time storage at 25°C Adapted from Kaya *et al*. (1993).

The shelf life of a food at ambient conditions has been estimated by plotting the log of induction periods (or other suitable end-point of stability) versus accelerated temperature and extrapolating to room temperature. However, this approach can lead to errors, if the stability test is based on an end-point that is beyond the rancidity detected by sensory methods. Extrapolation of the stability results obtained by the Rancimat test to ambient storage leads to either overprediction or underprediction of the actual shelf life, depending on the fatty acid composition of the oils (Figure 7.4). This discrepancy in prediction can be attributed to the different end-point between ambient conditions (peroxide value of 10 for rancidity of oils), and the much higher oxidation levels (peroxide value of 50 or higher under conditions of the automated AOM test) occurring at the elevated temperatures of the Rancimat test. This test run between 100 and 130°C estimates the shelf life of a much more oxidized oil than other oven tests carried out between 40 and 60°C.

Shelf life testing is a relatively expensive undertaking that for most practical purposes must be applied to specific food products. Results of such tests are sparse in the literature. Many complex factors affect the prediction of shelf life of foods. The effect of heating foods containing fats, proteins, carbohydrates and phospholipids is confounded by many factors that may either underpredict or overpredict their shelf life, because accelerated conditions may not reflect

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Table 7.4. Effect of increasing temperature on prediction of shelf life of foods [a]

Type of food	Changes	Oxidation	Shelf life prediction
Fats	Solid fat to liquid oils	Increase	Overpredict
Carbohydrates	Amorphous to crystalline	Increase / decrease	Over- or underpredict
Proteins	Denature	Increase / decrease	Over- or underpredict
Frozen foods	Unfrozen liquid formed	Increase	Overpredict
Dry foods	Water activity increases	Increase	Overpredict

[a] Adapted from Labuza and Schmidt (1985)

what happens during storage at ambient temperatures (Table 7.4). If two reactions affect quality, the reaction with higher activation energy may predominate and may produce different volatile end-products affecting flavor stability. Phase changes due to melting of fats at elevated temperatures do not reflect normal changes at lower temperatures. The shelf life of liquid *vs* solid fats is thus underpredicted at low temperatures due to phase changes and mobility of reactants. On the other hand, the shelf life of frozen foods can be overpredicted because reactants in the unfrozen liquid portion oxidize faster. At increasing temperatures, phase changes may occur (e.g. from solid fat to liquid oil) that affect mobility and oxidizability of food components and carbohydrates may crystallize out. The effect of storage temperature on the moisture content of foods and their water activity (discussed in Chapter 11) may be larger than predicted by the Arrhenius model. The water activity in dry foods increases with temperature at constant moisture, with a resulting increase in rate of oxidation. Similarly, the heating of moist foods drives off moisture changing the water activity and affecting the oxidative stability (see Figure 11.2). Among other factors influencing the shelf life of foods we may include differential partitioning of catalysts and antioxidants between different phases, changes in moisture, pH and protein denaturation at high temperatures. Moisture in foods enhances the interactions of hydroperoxide radicals with proteins resulting in increased stability in some cases. The formation of browning reaction products that have reducing properties generally increase the oxidative stability of lipids (see Chapter 11).

K. Recommended stability testing protocol

The desired objective is to use sensitive methods to detect end-points at low levels of oxidation relevant to flavor deterioration. To evaluate the oxidative or flavor stability of food lipids, it is critical to establish how the experimental data are influenced by the methodology used and the nature of the sample materials. No short cut approach can be used to food stability tests because it is influenced by complex phenomena. The stability of each system has to be researched under various test conditions. The following factors must be considered:

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(a) Substrates can include triacylglycerols or phospholipids, in bulk, or emulsions or liposomes. To eliminate the confounding effects of natural antioxidants, evaluations should be made with polyunsaturated edible oils stripped of (treated to remove) natural tocopherols. Various methods are available to remove natural tocopherols, including carbon bleaching, bleaching earth, or silicic acid chromatographic methods and molecular distillation. These methods should remove the tocopherols efficiently without significantly affecting the original fatty acid composition of the oil. Stripped corn oil is commercially available for nutritional studies of antioxidants and vitamin E, but care must be taken that samples have low initial peroxide values and stored properly under inert gas at freezer temperatures. Although fatty acid methyl esters are commonly used as model substrates for stability studies, they are not recommended because they do not simulate very well the oxidation of triacylglycerols. Fatty acid methyl esters are more rapidly oxidized and the effects of antioxidants and metal catalysts on their oxidative susceptibility are significantly different from the corresponding triacylglycerols.

(b) Different storage temperatures are required for testing to obtain reliable comparison of oxidative stabilities of food lipids, preferably between 40 and 60°C, with efficient shaking depending on the rate of oxidation. Each test should be calibrated for each individual fat and accelerated conditions must be kept as close as practical to the actual storage conditions.

(c) More than one method should be used to determine the degree of oxidation at suitable time intervals by measuring different types of products, including initial products of lipid oxidation (e.g. hydroperoxides) and their decomposition products (e.g. hexanal). Initially formed hydroperoxides can be estimated either by peroxide values or by conjugated dienes. Decomposition products of oxidation can be measured by analyses of carbonyl compounds or volatiles by gas chromatographic methods. Initial products provide information on precursors of flavor compounds, and decomposition products are directly related to the flavor compounds affecting quality. Flavor stability is measured by sensory evaluations, which provide the most appropriate criterion of consumer quality.

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CHAPTER 8

Control of oxidation

The development of rancidity in edible oils is a serious problem in some sectors of the food industry, because of increasing use of polyunsaturated vegetable and fish oils, discontinuing of the use of synthetic antioxidants, decreasing or eliminating the use of partial hydrogenation, and fortification of some foods with iron. Important nutritional and economic benefits can be expected by improved strategies to control lipid oxidation in foods, including improvement in safety, by protecting unstable polyunsaturated fatty acid components and vitamins, and reduction of food product loss by deterioration during processing and storage.

Effective control methods against lipid oxidation include:

a) Inactivating prooxidant metals
b) Processing by reducing or eliminating metal contamination and light exposure, and minimizing the loss of natural tocopherols
c) Partially hydrogenating polyunsaturated fats
d) Blending polyunsaturated fats with more stable monounsaturated fats
e) Breeding and genetic modification of fatty acid composition and tocopherol content
f) Packaging to minimize exposure to air and moisture
g) Using antioxidants (see Chapter 9).

Significant progress has recently been achieved by breeding and the genetic modification of soybeans, rapeseeds and sunflowers to produce edible oils of improved oxidative stability, by changing their fatty acid compositions and the contents of tocopherols and other antioxidants. High-oleic and low linolenic acid vegetable oils have now been commercialized to a limited extent in the US and Europe. Further commercialization of genetically modified new crops by breeding will require further improvements in their agronomic and economic properties in various climatic environments and countries. Increased interest in using stable vegetable oils containing no *trans* unsaturation is expected, as a result of future mandatory Government requirements to label the content of *trans* fatty acids in foods containing partially hydrogenated oils (see Section C).

A. Metal inactivation

The use of citric acid was one of the earliest measures discovered for significantly stabilizing soybean oil against oxidation. The effectiveness of citric acid was attributed to its metal chelation properties. Many other metal chelators

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were developed to inactivate prooxidant metals in oils, including tartaric, phytic, phosphoric and carboxymethyl-mercaptosuccinic acids. By forming stable coordination complexes with prooxidant metals, these chelating compounds inhibit effectively both the metal-catalysed initiation of free radical oxidation, and the decomposition of hydroperoxides producing volatile compounds that lower the flavor and oxidative stability of oils (see Chapters 1 and 4).

Citric acid and phosphoric acid are the most commonly used commercially because they are economical. Although citric acid is less oil-soluble than phosphoric acid, it is very effective and most widely used during the processing of vegetable oils. Isopropyl citrate, stearyl citrate and monoglyceride citrate have been used to increase the solubility of citric acid in oils. However, in properly processed oils, free citric acid is generally effective at levels below 50 ppm. The effectiveness of citric acid in vegetable oils is also increased by a synergistic action with tocopherols that are naturally occurring in vegetable oils in varying concentrations. Because traces of copper can completely inactivate the effects of natural and added synthetic antioxidants, metal inactivators such as citric acid act as "preventive antioxidants", by reinforcing the effect of and stabilizing tocopherols and added synthetic antioxidants. This well-established phenomenon is referred to as "synergism" and will be discussed in Chapter 9.

Phosphatides and purified lecithin are also useful metal inactivators, but they must be used at low concentrations (a few ppm) to minimize brown color formation. The use of phosphatides and phosphoric acid is also limited to low concentrations because above 0.02–0.05% these materials impart a "melony" or "cucumber" off-flavor.

Crude extracted oils contain varying amounts of iron and copper, in either ionized or complex form (such as heme complexes), and these are very detrimental to the oxidative and flavor stability of the processed oils. Crude soybean oil may contain as much as 3 to 5 ppm of iron and 0.1 to 0.2 ppm of copper. Alkali refining removes 95 to 98% of the initial iron and copper found in crude oils. However, the flavor stability of soybean oil and canola oil can be significantly reduced with as little as 0.1 ppm of iron and 0.01 ppm of copper. Citric acid can often be shown to inhibit lipid oxidation, even when iron and copper are present at levels too low to be detected. The judicious use of metal inactivators is therefore essential to the production of reasonably stable vegetable oils. Although citric acid is active as a metal inactivator in oils subjected to normal storage conditions, it is decomposed and becomes ineffective at the elevated temperatures of cooking and frying.

Citric acid and other metal inactivators are more effective in vegetable oils after they are heated, apparently because metals form prooxidant complexes with preformed hydroperoxides, which must be destroyed thermally before the chelating agents become effective.

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$$\text{Me} \rightleftharpoons [\text{Me—OOL}] + \text{citric acid} \xrightarrow{210^\circ\text{C}} [\text{citric acid—Me}] + \text{LOOH}$$
$$\text{LOOH} \xrightarrow{210^\circ\text{C}} \text{decomposition products} \qquad (1)$$

By this mechanism the chelating agent replaces the hydroperoxides by ligand exchange after its thermal decomposition to form a more stable metal coordination complex that can no longer catalyse lipid oxidation. If added before deodorization, citric acid is thermally decomposed into a mixture of organic acids (aconitic, itaconic and citraconic acids) that are less effective as metal chelators. For this reason citric acid is generally added to vegetable oils on the cooling cycle of deodorization below 100°C. Because citric acid is added during processing, it is considered a processing aid in the US and is not required to be declared on food labels.

Water-soluble and hydrophilic metal chelators, such as ethylenediamine tetraacetic acid (EDTA) and its calcium disodium salt, phosphoric acid, polyphosphates, phytate and ascorbic acid, are effective in improving the oxidative stability of aqueous food emulsion systems (e.g. salad dressings, mayonnaise and margarine). The EDTA salts are also used to inactivate metal contaminants in salt, spices and other food ingredients. The salt purified in the presence of EDTA to remove heavy metals can be added to foods without a labeling requirement. Ascorbic acid has multiple functions in aqueous foods, and its effectiveness as a metal chelator may be compromised, because it is known to have prooxidant effects in the presence of significant amounts of metals (Chapter 9). Ascorbic acid can act as a prooxidant by reducing Fe^{3+} to Fe^{2+}, which is a more active catalyst of lipid oxidation (see Chapter 1.D). Similarly, in food emulsions care must be exercised that EDTA is used at sufficiently high concentrations to completely chelate iron catalysts, especially if they are supplemented or highly contaminated with iron. At low concentrations, EDTA can act as a prooxidant because it changes the ratio of Fe^{3+} to Fe^{2+} by chelating Fe^{3+} (stability constant for EDTA-Fe^{3+} = 14.6) more favorably than Fe^{2+} (stability constant for EDTA-Fe^{2+} = 9.5). At low EDTA concentrations, therefore, any free and uncomplexed Fe^{2+} will promote oxidation because it is a more active lipid oxidation catalyst than Fe^{3+} (Chapter 1. Section D).

B. Processing

Any oxidation during processing is injurious to the flavor and oxidative stability of processed oils, either directly by the formation and accumulation of oxidation products, or indirectly by the loss of protective naturally occurring tocopherol antioxidants. Optimum oxidative stability can be achieved therefore by proper handling of oilseeds and crude oils to minimize exposure to air,

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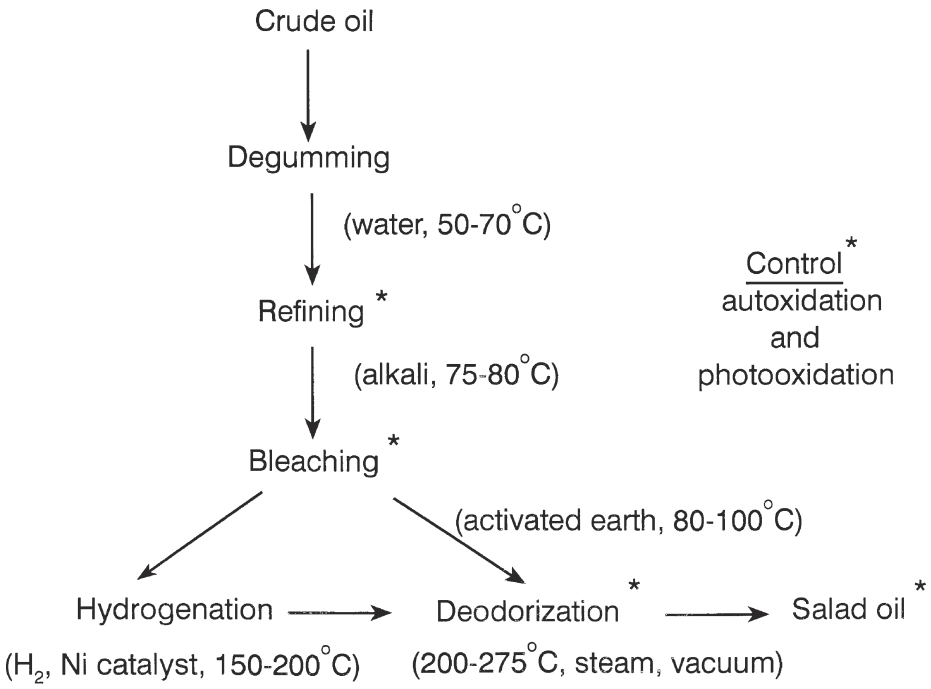


Figure 8.1. Key steps in processing of soybean oil that require control of autoxidation and photooxidation.

light, metal contaminants, during processing and to retain natural antioxidants. Processing of crude soybean and other vegetable oils requires the control of lipase and lipoxygenase activity without delay during the cracking, tempering, flaking, and solvent extracting of beans to minimize free fatty acids and peroxide formation (Chapter 11.A.3). Good processing measures for the resulting crude oil are required to minimize oxidation. These measures include careful control of refining temperature, vacuum bleaching, nitrogen blanketing between processing steps, and minimizing light exposure (Figure 8.1). During the initial water-degumming step to remove phospholipids and some metals, polyunsaturated vegetable oils become more susceptible to oxidation after removal of most of the phospholipids. It is important, therefore, to minimize oxidation during the subsequent alkali refining to eliminate fatty acids, bleaching and deodorization steps. Although bleaching removes several impurities, including oxidized components, this step lowers the oxidative stability of partially processed oil by complete removal of the phospholipids and by exposure to the large surface area of the adsorption clays at elevated temperatures. On one hand, bleaching removes chlorophylls that decrease oil stability to light oxidation. On the other hand, this step also removes carotenoids that inhibit photosensitized oxidation (Chapter 3). Deaeration of the bleaching earth and vacuum bleaching are thus desirable. After bleaching, soybean oil is particularly susceptible to oxidation and the oil must be carefully protected by

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Table 8.1. Effect of storing refined and bleached soybean oil in tanks with air or nitrogen atmospheres [a]

Characteristics		Nitrogen	Air
Oil temperature:	range	16–34°C	18–31°C
	average	23ºC	24ºC
Oxygen in headspace		1.4%	21%
Peroxide value:	initial	1.0	1.0
	final	1.5	5.0

[a] From Going (1968).

nitrogen blanketing or by minimizing storage in air before deodorization. The oxygen content and peroxide value of refined and bleached soybean oil are significantly decreased by storage in nitrogen compared to air (Table 8.1).

Deodorization at elevated temperatures (200–275°C) under vacuum is the last and most critical step in vegetable oils processing. The quality of vegetable oils can be appreciably improved by deaerating the oil at low temperature to reduce dissolved oxygen (to less than 0.1% vol.), avoiding any contact of hot oil with air, and cooling the deodorized oil to below 40°C before exposure to air or cooling under nitrogen atmosphere. Storage of the deodorized oils or packaging under nitrogen is of course most desirable to minimize oxidation.

The alternate process of physical refining is generally applied to crude palm oil and other tropical oils that contain relatively large levels of free fatty acids. This process involves a more efficient degumming (also referred to as super-degumming) in the presence of phosphoric acid or citric acid as metal chelators, and omission of the alkali-refining step to avoid excessive losses incurred by the formation of large amounts of soaps. The free fatty acids are removed and trapped by distillation with a deodorizer of higher capacity and at higher temperatures than alkali refining. Volatile compounds, off-flavors, pesticides and polycyclic hydrocarbon contaminants may also be removed by physical refining. The quality of physically refined vegetable oils is generally comparable to that of the corresponding alkali refined oils. During physical refining, however, special precautions are required to avoid oxidation by minimizing exposure to air and delays between steps because the partially processed oils are better degummed and not as well protected by lecithin.

Although processing of vegetable oils is aimed at improving their oxidative stability by removing undesirable impurities such as prooxidant metals and chlorophyll photosensitzers, significant losses may occur of desirable antioxidants such as tocopherols in vegetable oils and phenolic compounds in virgin olive oil. Tocopherol losses vary from 30 to 70%, depending on the severity of different processing steps and the initial contents of individual tocopherols in various vegetable oils. Crude soybean oil is an exception because it contains

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Table 8.2. Effect of different processing steps on loss of total tocopherols in sunflower oil (ppm) [a]

Processing steps [b]	Chemical refining	% Loss	Physical refining	% Loss	Soft column deodorization [c]	% Loss
Crude	842.6		755.2		854.9	
Neutralization	803.2	4.7			840.7	1.7
Bleaching	739.5	12.2	728.8	3.5	812.2	5.0
Winterization	705.6	16.3	722.7	4.3	792.9	7.3
Deodorization	621.3	26.3	569.3	24.6	746.4	12.7

[a] From Alpaslan *et al.* (2001).
[b] Neutralization = alkali refining.
[c] Soft column deodorization = reaction of oil with steam for a short time at 230–240°C.

relatively high levels of tocopherols, ranging between 1300 to 1500 ppm. The removal of 30 to 50% of the tocopherols in soybean oil by deodorization can actually improve its oxidative stability, because the tocopherol concentration needed for optimum stability ranges between 400 to 700 ppm (see Chapter 9). This finding has been commercially exploited by recovering tocopherols from soybean oil in the deodorizer distillate as a profitable source of tocopherol concentrates for food antioxidant applications. The removal of tocopherols from other vegetable oils that contain much less than soybean oil can be of concern, however, especially with oils such as sunflower oil rich in α-tocopherol, which is not as effective an antioxidant as the mixture of γ- and δ-tocopherols in soybean and canola/rapeseed oils (Table 9.4). New deodorization processes have been developed to decrease or minimize losses of tocopherols in sunflower. A new technique called soft column deodorization was developed to reduce losses of tocopherols by reacting the oil with steam for a short time at 230–240°C (Table 8.2). Soft column deodorization of sunflower oil resulted in 12.7% loss of total tocopherols, compared with 26.3% with conventional chemical refining with alkali, and 24.6% with physical refining without alkali.

Any hydroperoxides and other secondary oxidation products formed in oils during processing are volatilized and polymerized during and after deodorization. The non-volatile materials are found at levels of 1 to 2% and include 70% dimers, trimers and higher molecular weight compounds, aldehydo-glycerides, and esters (also referred to as "core" aldehydes), carbonyls and alcohols. These high molecular weight compounds are readily decomposed during storage to produce volatile compounds contributing to the flavor deterioration of oils (Chapters 4, 5, 7).

The formation and decomposition of oxidative polymers during soybean oil processing has been referred to as "hidden oxidation" because it cannot be measured by the conventional peroxide value determination. However, these

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Table 8.3. Effect of oxidation prior to deodorization on dimer and secondary products in soybean oil [a]

Peroxide value [b]	Dimer, %	Secondary products, %
8.1	1.8	0.0
8.8	1.6	0.0
43.6	2.4	0.6
94.6	3.8	1.4
859.0	11.9	12.5

[a] From Frankel *et al.* (1961)
[b] Oils oxidized at 60°C before deodorization, and analysed for dimers chromatographically after deodorization.

oxidative polymers can be determined by silicic acid chromatography, or size-exclusion chromatography or estimated by the anisidine test (Chapter 5, Figure 5.2). Such high molecular weight materials are detrimental to the oxidative and flavor stability of vegetable oils and particularly fish oils. The peroxide value of soybean oils before deodorization is directly related to the dimer content determined chromatographically after deodorization (Table 8.3).

More structural details of oxidative deterioration compounds formed during refining of different vegetable oils were obtained by separating the polar compounds by high performance size exclusion chromatography (Table 8.4). The anisidine and polar compounds varied extensively among different vegetable oils reflecting a wide range of oxidative status. Although the total polar compounds and oxidized triglycerides decreased after each processing step, the triglyceride oligopolymers increased significantly after bleaching and deodorization. This increase in triglyceride oligopolymers was apparently related to the increase of the anisidine value reaching a maximum after bleaching. After deodorization, in contrast to the decrease observed in the anisidine value, the oxidized triglycerides and polar compounds, the triglyceride oligopolymers increased. Polymeric triglycerides are mainly dimers and together with oxidized triglycerides constitute the major components of the total polar compounds. This increase in oligopolymers with deodorization is in agreement with the dimer and secondary products for soybean oil (Table 8.3) and, like the anisidine value, can be useful for indicating the amount of oxidation occurring during processing.

The production of oxidative dimers and triglyceride oligopolymers by oxidation prior to deodorization decreases the stability of oils. Flavor and oxidative stability of soybean oil correlates with the concentration of oxidative dimers determined chromatographically. The oxidative stability of vegetable oils can therefore be markedly improved, if any oxidation products present in partially processed oils can either be removed or chemically reduced to stable hydroxides before deodorization.

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Table 8.4. Effect of processing of vegetable oils on different analyses of oxidative deterioration [a]

Samples	PV (meq/kg)	Anisidine value	Polar compds (%)	TG oligo-polymers (%)	Oxidized TG (%)	DG (%)
Soybean 1						
Crude	5.8	4.47	5.33	0.15	2.72	1.24
Degummed-alkali refined	6.3	5.21	4.52	0.14	2.64	1.24
Bleached	0.1	9.60	4.29	0.31	2.26	1.25
Deodorized	0.0	5.46	3.78	0.74	1.46	1.23
Sunflower 1						
Crude	12.0	3.77	4.16	0.12	1.28	1.16
Degummed-alkali refined	15.0	2.90	2.73	0.08	1.33	1.00
Bleached	1.2	11.96	2.64	0.23	1.01	1.02
Deodorized	0.3	5.26	2.62	0.37	0.93	1.02
Corn						
Crude	2.6	7.75	6.76	0.15	1.77	2.48
Degummed-alkali refined	5.7	4.89	4.99	0.13	1.80	2.43
Bleached	0.7	11.62	4.78	0.21	1.57	2.41
Deodorized	0.0	3.48	4.18	0.65	0.89	2.32
Soybean, crude [b]	–	–	5.90	0.63	3.54	0.51
Sunflower, crude [b]	–	–	4.48	0.40	2.57	1.00
Corn, crude [b]	–	–	5.37	0.20	1.10	2.33
Rapeseed, crude[b]	–	–	3.94	0.35	2.21	0.83
Palm, crude [b]	–	–	10.75	0.53	2.20	4.95
Olive, crude [b]	–	–	6.71	0.32	2.73	2.58

[a] Selected data from Gomes *et al.* (2003). PV, peroxide value; TG, triglycerides; DG, diglycerides. Polar compounds determined by high performance liquid chromatography. Polar compounds were analysed for TG oligopolymers, oxidized TG, and diglycerides by high performance size exclusion chromatography (see Chapter 6).
[b] Selected data from De Greyt *et al.* (1999).

In addition to polar and oligomeric triglycerides, a number of undesirable compounds are formed during deodorization, including *trans* isomers of polyunsaturated fatty acids, oxidized sterols and cyclic monomers (Table 8.5). Deodorization produced significant amounts of *trans* dienes and trienes and cyclic monomers with increasing temperatures, but the concentration of oxyphytosterols did not change significantly. Fish oils, containing large amounts of oxidizable long-chain *n*–3 polyunsaturated fatty acids, are sometimes processed to reduce polymer formation by molecular distillation at lower temperatures than conventional deodorization (150–180°C or below) under high vacuum.

Several patented processes have been developed to improve the oxidative

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Table 8.5. Effect of deodorization temperatures on the formation of *trans* fatty acids, oxyphytosterols and cyclic monomers in low erucic rapeseed oil [a]

Analyses	Refined oil	6 h at 200°C	6 h at 225°C	6 h at 250°C
C18:2 *trans* (%)	0.08	0.03	0.17	1.03
C18:3 *trans* (%)	0.47	0.29	1.26	4.19
Oxyphytosterols (μg/g)	70.1	68.7	74	72.5
Cyclic monomers (μg/g)	48	68	224	669

[a] Selected data from Lambelet *et al.* (2003). Fatty acid composition of refined low-erucic rapeseed oil (also known as canola oil)(%): C16:0, 4.7; C18:0, 1.7; C18:1, 61.7; C18:2, 20.0; C18:3, 8.3; C20:1, 1.3; other, 2.3. Oxyphytosterols include oxides of brassicasterol, campesterol and sitosterol. Cyclic monomers include *cis* and *trans* methyl 9-(2-butylcyclopentyl)nonanoate and *cis* and *trans* methyl 10-(2-propylcyclopentyl) decanoate, *cis* methyl 10-(2-propylcyclopentenyl) decanoate, and *cis* methyl9-(2-propylcyclohexyl) nonanoate.

and flavor stability of vegetable oils by reducing lipid oxidation during processing. In some processes, oxidation products are removed chromatographically or by improved bleaching with better adsorbents such as silicic acid. In other processes, degummed oils are treated with alkaline sodium borohydride to reduce the peroxide content of oils and improve the oxidative stability of the processed oils. The use of special membranes has been developed to separate impurities in crude oil extracts and to refine oils. The main advantages of membrane technology and other so-called "minimal processed" foods is to save on energy consumption, minimize degradation and retain natural antioxidants. Before this new technology is adopted for vegetable oil refining, further developments are required to justify significant environmental and economic advantages in energy and other costs.

The physical processes of cold pressing and hot expeller-pressing oils have attracted some interest recently for environmental reasons, as ways of avoiding the use of chemical solvent in the extraction of seeds. Cold-pressed virgin olive oil contains significant amounts of phenolic compounds that impart antioxidant activity. Claims of nutritional benefits and enhanced stability for these olive oils have not been substantiated in the literature, however. Although fresh virgin olive oils are relatively stable, the quality of stored commercial olive oils can vary significantly because their shelf life is not well controlled. Commercial olive oils are assumed to be relatively stable because of their high level of oleic acid and phenolic contents, but their shelf life varies significantly (Table 8.6). A commercial refined bleached and deodorized (RBD) olive oil having the lowest initial peroxide value was the most stable. Although the commercial virgin olive oils contained higher levels of phenolic compounds (518 and 534 ppm total phenols) than commercial refined olive oils (63 and 81 ppm total phenols) and the RBD olive oil (8 ppm total phenols), their oxidative stability was significantly decreased by their relatively higher initial peroxide values.

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Table 8.6. Oxidative stability of commercial olive oils [a]

Oils [b]	Initial peroxide value (PV)	Peroxide value (meq/kg)		Hexanal (mmol/kg)	
		Day 3	Day 11	Day 3	Day 11
Control, RBD	0.4	4.8	24.7	0.2	0.9
Refined, extra light	11.0	13.8	53.4	0.2	0.9
Refined, pure	12.7	22.3	61.6	0.4	1.0
Extra virgin 1	15.6	17.6	36.2	0.4	0.6
Extra virgin 2	32.5	46.5	89.3	1.0	0.9

[a] From Satué *et al.* (1995). Oxidation at 60°C.
[b] RBD = refined, bleached, deodorized. RBD and extra virgin 1 olive oils came from California, refined extra light and refined pure olive oils from Italy, and extra virgin 2 olive oil from Spain.

C. Hydrogenation

The treatment of polyunsaturated vegetable oils with hydrogen gas in the presence of activated nickel catalysts reduces their linolenate and linoleate components into mixtures of isomeric diunsaturated and monounsaturated oils. According to the temperature, hydrogen pressure and activity of the catalyst, linolenate and linoleate triacylglycerols can be more or less selectively hydrogenated without the formation of saturated products. Partial hydrogenation of linolenic acid in soybean and rapeseed oils, and of *n*–3 polyunsaturated fatty acids in fish oils provides a practical means of increasing their oxidative and flavor stability. However, hydrogenation does not completely control the flavor deterioration of these oils because this process produces isomeric diunsaturated fatty acids that can oxidize and decompose into volatile compounds contributing to flavor defects designated as "hardening" or "hydrogenation" that are considered objectionable, especially during frying. Furthermore, hydrogenation of polyunsaturated oils forms varying amounts of products with *trans* double bonds according to the conditions used in this process.

On catalytic hydrogenation, linoleate produces conjugated dienes (diunsaturated fatty acids), and linolenate produces conjugated diene-trienes (with two conjugated double bonds and one isolated double bond) as intermediates. These conjugated products are more reactive with hydrogen and become rapidly reduced. The conjugated dienes of linoleate produce isomeric monoenes (monounsaturated fatty acids) with *cis* and *trans* double bonds distributed between carbon-9 and carbon-12 (Figure 8.2). The conjugated diene-trienes of linolenate produce two kinds of isomeric dienes (Figure 8.3). The "conjugatable dienes" are methylene interrupted and produce conjugated dienes that can hydrogenate into a mixture of monoenes with double bonds distributed between carbon-9 and carbon-15. The "non-conjugatable dienes" have two isolated

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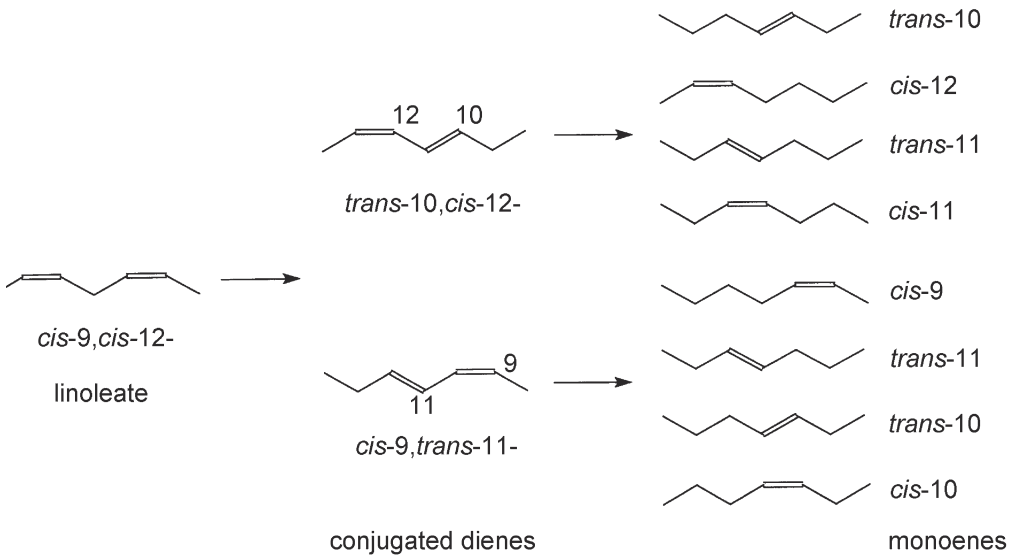


Figure 8.2. Formation of isomeric *cis* and *trans* monoenes by hydrogenation of linoleate.

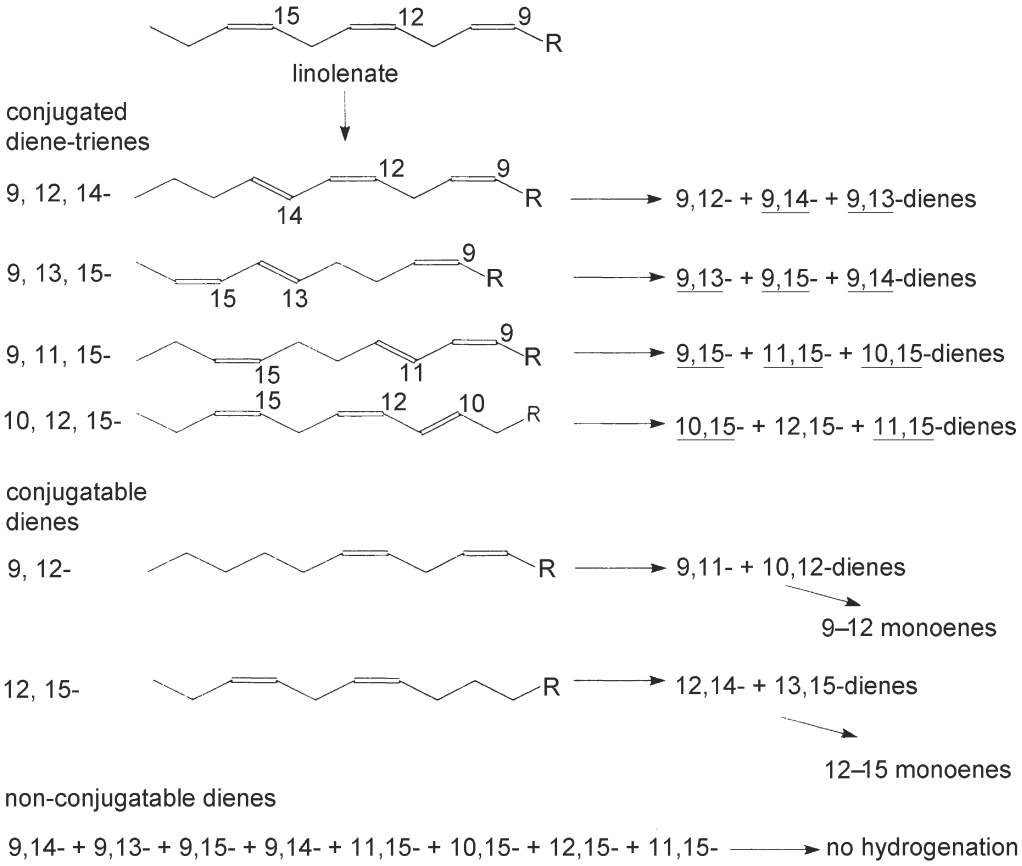


Figure 8.3. Formation of isomeric monoenes, and dienes by hydrogenation of linolenate. The "conjugatable dienes" are hydrogenated into isomeric monoenes. The "nonconjugatable dienes" or "isolinoleate" are not hydrogenated and accumulate in the product.

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double bonds separated by more than one methylene group. These dienes are referred to as "isolinoleate" and accumulate in the partially hydrogenated oils because they cannot be conjugated and do not react with hydrogen. These isomeric dienes behave like two oleate functions in the same molecule and are, therefore, difficult to oxidize. Soybean oil is thus stabilized by hydrogenating linolenate to produce isolinoleate. However, the oxidation of isolinoleate produces hydroperoxides that are readily decomposed into undesirable volatile flavor compounds.

The oxidation of methyl 9,15-octadecadienoate (Chapter 2. C), used as a model for isolinoleate, produced a mixture of 8-, 9-, 10- and 11-hydroperoxides by oxygen attack at the 9-double bond, and a mixture of 14-, 15-, 16- and 17-hydroperoxides resulting from oxidation of the 15-double bond (Figure 2.9). Thermal decomposition of the external 14-, 15-, 16- and 17-hydroperoxides would produce a mixture of acetaldehyde, propanal, 2-butenal and 2-pentenal, which is similar to that produced from the hydroperoxides of linolenate (Figure 8.4). On the other hand, decomposition of the internal mixture of 8-, 9-, 10- and 11-hydroperoxides produces a mixture of unsaturated aldehydes, including 2,8-undecadienal, 2,7-decadienal, 6-nonenal and 5-octenal (Figure 8.5). Because these unsaturated aldehydes have particularly low threshold values, ranging from 0.00032 to 0.32 ppm, they are very potent flavor compounds that contribute to the undesirable "hardening" or "hydrogenation" off-flavors of partially hydrogenated polyunsaturated vegetable and fish oils. Hydrogenation provides therefore a practical but incomplete measure to control oxidatively derived flavor problems of polyunsaturated oils.

For most consumer applications that do not require elevated temperatures, polyunsaturated vegetable oils, such as soybean, rapeseed and sunflower oils, can be used without hydrogenation when they are properly processed. These oils can also be used for either cooking or for single (without re-using) or pan-frying foods (see Chapter 12). However, the flavor of polyunsaturated oils will deteriorate, especially if the oils contain linolenic acid, when they are used repeatedly for cooking, deep-fat frying, and continuous frying. These oils produce undesirable fishy odors after prolonged and repeated heating that are derived from accumulation of oxidation polar compounds. Partially hydrogenated linolenate-containing oils are commonly used commercially for continuous or intermittent frying operations, even when mixed with fresh make up oils. In current industrial practices, these polyunsaturated oils are lightly hydrogenated to a linolenate content of 2 to 4%, and blended with more stable oils to minimize the *trans* isomers and the "hydrogenation" flavor that may be objectionable in some consumer markets.

More recently, the hydrogenation of vegetable oils has become less attractive, since *trans* isomers are considered nutritionally undesirable because they behave as saturated fatty acids by increasing the ratio of LDL to HDL in blood (see Chapter 13). For this reason, the commercial use of hydrogenated oils has

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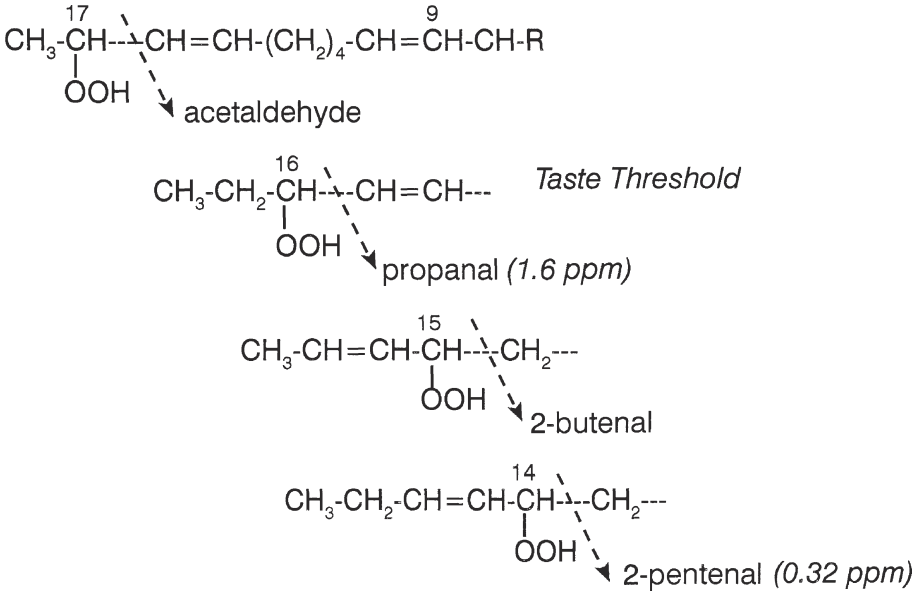


Figure 8.4. Volatile decomposition products from 14-, 15-, 16- and 17-hydroperoxides formed from the autoxidation of methyl *cis*-9, *cis*-15-octadecadienoate.

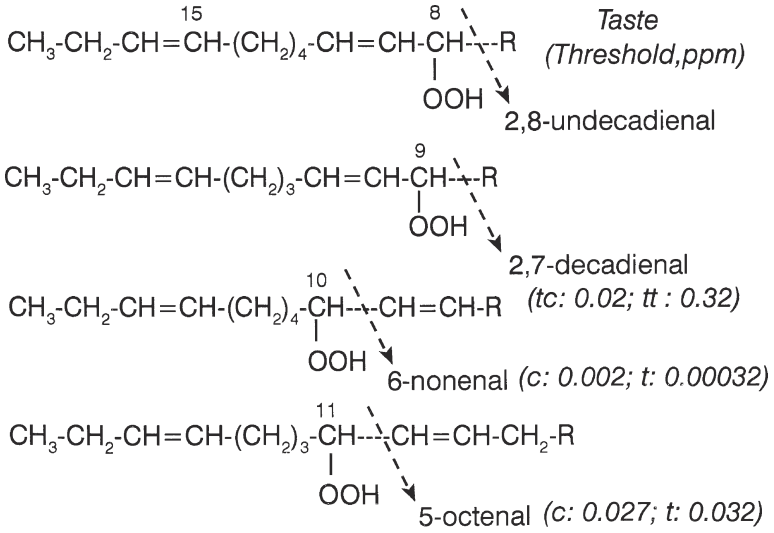


Figure 8.5. Volatile decomposition products from 8-, 9-, 10- and 11-hydroperoxides formed from the autoxidation of methyl *cis*-9, *cis*-15-octadecadienoate.

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Table 8.7. Fatty acid composition (%) and oxidizability of standard baking and interesterified shortenings [a]

Fatty acids	Standard shortening	Interesterified shortening
C-14:0	1.0	0.41
C-16:0	11.0	16.5
C-18:0	13.0	34.9
C-18:1	62.0	14.6
C-18:2	12.0	30.8
C-18:3	0.5	1.43
Total saturates	25.0	51.8
Total *trans* fatty acids	32.0	0.0
Oxidizability [b]	0.14	0.34

[a] From Gupta (2003). Standard shortening is made by partial hydrogenation; interesterified shortening is a blend of 45% fully hydrogenated cottonseed oil and 55%% low-linolenic soybean oil.
[b] Oxidizability = [(% C18:2 × 1) + (% C18:3 × 2)] ÷ 100
(Based on data from Table 1.1)

decreased significantly especially in Europe. The same trend is undergoing in the US because the Food and Drug Administration (FDA) has mandated that, by 2006, labels of package foods disclose the content of *trans* fats. This label requirement is expected to reduce and eventually discontinue hydrogenation in the future by producers of vegetable oils. However, food formulations containing small amounts of partially hydrogenated oils contributing less than 0.5% *trans* fatty acids per serving (e.g. peanut butter) do not have to declare it in the label.

Several commercial processes have been developed to prepare higher melting plastic shortenings and margarines containing no *trans* (or low *trans*, 2–3%) by either interesterifying or blending more saturated palm oil or saturated completely hydrogenated oils (also called "base" stocks) with various unsaturated vegetable oils (Table 8.7). These products may have similar oxidative stability as the partially hydrogenated products, but more care is required to completely remove any prooxidant alkali transesterification catalysts that will lower oxidative stability. The present trend to eliminate *trans* fatty acids from edible vegetable oils is becoming accepted as a useful practice to improve their nutritional value. This nutritional goal is defeated, however, if *trans*-containing oils are replaced in margarines and shortenings with saturated fats rich in palmitic acid that are equally undesirable nutritionally (see Chapter 13).

D. Blending with monounsaturated oils

The flavor and oxidative stability of linolenate-containing oils can be improved substantially by blending them with different levels of high-oleic oils to lower the linolenate content. High-oleic sunflower and safflower oils are relatively

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Table 8.8. Fatty acid composition and oxidizability of mixtures of soybean (SBO), canola (CA), corn (CO) and cottonseed (CSO) oils with high-oleic sunflower oil (HOSO) (Relative %) [a,b]

Oils and mixtures	Saturates	C18:1	C18:2	C18:3	Oxidizability [c]
HOSO	8.9	77.4	12.5	0.1	0.13
SBO	14.6	24.7	53.8	6.9	0.68
SBO/HOSO, 27:73	10.5	63.5	24.0	2.0	0.28
SBO/HOSO, 41:59	11.4	55.6	30.0	3.0	0.36
SBO/HOSO, 63:37	12.7	43.7	39.1	4.5	0.48
CA[d]	5.7	61.3	21.9	9.5	0.41
CA/HOSO, 7:93	8.3	78.7	12.0	1.0	0.14
CA/HOSO, 17:83	8	76.8	13.2	2.0	0.17
CA/HOSO, 27:73	7.8	75.0	14.2	3.0	0.20
CO	12.9	26.3	59.8	1.0	0.62
CO/HOSO, 18:82	9.2	70.4	20.0	0.4	0.21
CO/HOSO, 59:41	11.1	48.2	40.0	0.7	0.41
CSO[b]	26.5	16.4	54.2	0.1	0.54
CSO/HOSO, 67:33[b]	19.2	42.9	35.7	0.1	0.36
CSO/HOSO, 33:67[b]	11.7	67.5	18.7	0.1	0.19
HOSO[b]	8.0	78.1	12.1	0.1	0.12

[a] From Frankel and Huang (1994)
[b] From Warner *et al.* (1997)
[c] Oxidizability = [(% C18:2 × 1) + (% C18:3 × 2)] ÷ 100
[d] Includes erucic acid (22:0) for the balance to 100%

stable because they have a fatty acid composition similar to that of olive oil with an oleate content ranging from 65 to 80%. The mixing of different proportions of high-oleic sunflower oil with soybean, rapeseed (canola), corn and cottonseed oils provides a simple method for the preparation of more stable edible oils with a wide range of desired fatty acid compositions (Table 8.8). The relative oxidizability of oils and mixtures can be calculated on the basis of their fatty acid compositions, by assuming that oleate does not oxidize significantly (in the presence of polyunsaturated fatty acids) and linolenate oxidizes approximately twice as fast as linoleate (based on data in Table 1.1). As expected, blending polyunsaturated oils with high-oleic sunflower oil lowers their calculated oxidizability significantly. Mixtures of soybean and canola oils with high-oleic sunflower oil containing lower levels of linolenate can be prepared with the same or better oxidative stability than that of partially hydrogenated oils. Catalytic hydrogenation of linolenate-containing vegetable oils can be avoided by blending with high-oleic sunflower oil or other stable oils. Oxidatively stable all *cis* oils can, therefore, be produced by blending high-oleic sunflower oil with polyunsaturated oils as an alternative technology to partial hydrogenation. Mixing different proportions of high-oleic sunflower or safflower oils with soybean, canola, corn and cottonseed oils provides a simple

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method to prepare more stable edible oils with a wide range of desired fatty acid compositions (Table 8.8).

A number of vegetable oil blends are now available on the US markets that are produced from mixtures of soybean, canola, sunflower, corn, cottonseed and olive oils. These blended oils are designed for improved nutritional value by lowering their saturated fatty acid and raising their oleic acid contents. However, blends of soybean and canola oils containing significant amounts of linolenic acid (more than 3–4%) cause flavor deterioration and poor frying performance, when used for repeated and prolonged frying, and reduce oxidative stability. Although the detrimental effects of oxidation products of linolenic acid to the stability and ultimately to the safety of the products are well known, the amounts of linolenic acid needed in the diet for its nutritionally benefits are not adequately established (Chapter 13).

E. Breeding and genetic modification

For many years, numerous varieties of soybeans have been developed to improve their oxidative stability and frying performance by lowering the content of linolenic acid. Conventional breeding did not succeed in producing variety lines that maintained this characteristic in succeeding generations. Recent breeding programs were more successful and recurrent selection with mutation breeding produced mutant soybean varieties low in linolenic acid. New cultivars of soybean and canola oils containing linolenic acid ranging from 0.8 to 4.8 % showed higher oxidative and flavor stability than the control soybean oil containing 7.7 % linolenic acid (Table 8.9). High-oleic corn and sunflower oils have also been developed that would be comparable to olive oil in oxidative stability. These new low-linolenic soybean oils show even more significant improvements in frying performance (Chapter 12). Therefore, genetically modified vegetable oils provide improved fatty acid compositions and oxidative stability. However, these new varieties are reportedly not resistant to environmentally changes and produce lower yields than the conventional crops.

New high-oleic soybeans have been developed recently by introducing cloned mutant genes into soybeans to create transgenic lines. The oils from these transgenic soybeans contain more than 80 % oleic acid, less than 4 % stearic acid and less than 2% linolenic acid, and would be expected to be oxidatively stable. Such transgenic soybeans are not yet commercially available. Modified oils (Table 8.10) have become or are close to becoming commercially available. As expected, these new oils are more oxidatively stable and free of *trans* fatty acids. They are all designed to avoid hydrogenation and to satisfy future labeling requirements mandated by the FDA in the US. Although the high-oleic oils are more stable, the medium-oleic oils are considered to be more favorable for frying because they impart a desirable "fried

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Table 8.9. Fatty acid composition and stability of new varieties of vegetable oils [a]

Oils	16:0	18:0	18:1	18:2	18:3	Other	Flavor score (PV)[b]	Oxidizability
SBO	10.5	3.6	23.8	54.4	7.7	–	5.5 (5.8)	0.70
LoLnSBO(1983 A5)	10.8	4.5	45.0	36.4	3.3	–	6.6 (3.0)	0.43
LoLnSBO(1985 C1640)	10.6	3.6	24.6	56.4	4.2	–	6.6 (4.9)	0.65
LoLnSBO(1982 PI)	13.1	3.6	16.6	61.9	4.8	–	7.0 (5.4)	0.72
LoLnSBO[c]	11.3	4.7	22.4	59.7	2.0	–	–	0.64
ULoLnSBO[c]	11.5	4.6	24.8	58.2	0.8	–	–	0.60
LoSatSBO[d]	3–4	1–3	28–31	57–61	3–9	–	–	0.64–0.75
HiOlSBO[d]	6–9	3	79–86	2–3	2–6	–	–	0.04–0.09
HiStSBO[d]	9–11	21–26	18–63	1–39	3–8	–	–	0.04–0.47
LoLnCA[e] (IMC 01–4.5)	4.0	2.3	64.2	23.6	2.8	2.0	–	0.29
LoLnCA[e] (IMC 01–3)	4.0	2.0	66.8	21.3	2.9	1.8	–	0.27
LoLnCA[e] (IMC 129)	3.5	2.3	78.3	8.5	4.2	2.1	–	0.17
HiOl corn[f]	9.0	2.3	65.6	22.5	0.6	–	7.2 (2.0)	0.24
HiOl sun[f] (80%)	3.6	3.9	81.9	3.7	0.0	–	–	0.04
HiOl sun[f] (90%)	2.9	3.9	89.1	3.7	0.0	–	–	0.04

[a] From Mounts *et al.* (1988). Abbreviations: SBO = standard soybean oil, HWSBO = hydrogenated-winterized soybean oil, n.r. = not reported, PV = peroxide value
[b] Flavor evaluations and PV determinations made after storage at 60°C for 8 days.
[c] From Warner *et al.*. (2003). LoLnSBO = low-linolenic acid soybean oil, ULoLnSBO = ultra-low-linolenic acid soybean oil, evaluated for frying potato chips (Table 12.10).
[d] From Gunstone (2002). LoSatSBO = Low saturates soybean oil, HiOlSBO = high-oleic soybean oil, HiStSBO = high-stearate soybean oil.
[e] From Warner *et al.* (1994): canola oils (InterMountain Canola, Cinnaminson, NJ), also referred to as mid-oleic canola oil (see Table 8.10).
[f] From Warner and Knowlton (1997): HiOl corn = high-oleic corn oil, HiOl sun = high-oleic sunflower oil, evaluated for frying potato chips (Table 12.9).

flavor" to potato chips and French fries (see Chapter 12). This fried flavor is attributed to *cis, trans*-2,4-decadienal produced from linoleic acid at relatively low levels of oxidation. If oxidation becomes excessive, however, the flavor is described as "rancid" and becomes undesirable. Therefore, the frying process has to be carefully optimized by a relatively fast turnover consisting of replacing the fat used by food frying with fresh fat (Chapter 12.C.1). With the increasing use of unhydrogenated oils for frying, additional fresh fat turnover may be necessary to lower the total polar compounds derived from polyunsaturated fatty acid components to improve the shelf life of fried foods.

F. Packaging

Ideal containers for oils, shortenings and other lipid-rich foods should be impermeable to air and moisture, and should be opaque to light to prevent further oxidation during prolonged storage under ambient conditions. Liquid

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Table 8.10. Fatty acid composition (relative %) and oxidizability of commercial new generation high-oleic (HO), medium-oleic (MO) and low-linolenic (LL) vegetable oils

New oils	Sat [a]	C18:1	C18:2	C18:3	Oxidizability
LL-soybean [b]	14	27	55	4	0.63
LL-soybean [b]	15	41	41	2	0.45
HO-soybean [b]	11	81	4	4	0.25
LL-canola [b]	7	65	23	3	0.29
HO-canola [b]	7	75	11	7	0.36
HO/LL canola [b]	7	75	13	5	0.23
MO-sunflower [c]	8	60	29	0	0.29
MO-sunflower [c]	8	79	11	<0.5	0.12
HO-sunflower [d]	6–11	75–91	2–17	0.1	–

[a] Sat = C18: + C18:0
[b] From Gunstone (2001)
[c] From Warner *et al*.(2003). Averages of NuSun oils from 8 US States.
[d] From Warner *et al.* (2003). Ranges of high-oleic sunflower oils from Codex Alimentarius (2001).

oils were traditionally packaged in clear glass containers; brown bottles were sometimes used to protect unstable oils from light oxidation. Consumer perception indicated later that clear glass containers were preferred, however. Plastic containers have now generally replaced glass bottles to save on cost and to incorporate various organic barrier coatings into the packaging polymers to retard oxidation during storage. This practice is now referred to either as "active packaging", or the application of "barrier polymers". Active packaging techniques deal with substances that absorb oxygen, and moisture, and those that release antioxidants and desirable flavors.

The storage stability of vegetable oils was significantly better in glass bottles than in plastic bottles containing BHA and BHT (Table 8.11). Both BHA and BHT leached out however from the plastic films into the vegetable oils during storage. The ranking in stability of virgin olive oil and sunflower oil samples decreased in the order: polyvinyl chloride (PVC), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS). PVC (monomer free) and PET are preferred because they are less permeable to oxygen and superior to PP and PS, which are permeable to oxygen and to moisture (Table 8.12). Although PVC and high-density polyethylene (HDPE), are used extensively in the US, PET has now become a plastic material of choice because of economic reasons, and it is considered to be superior to PVC in protecting oils from oxidation. Because HDPE is more permeable to oxygen than PVC, an oxygen barrier has been added to HDPE to extend shelf life of oils. Barrier polymers have been used either as organic thin coatings, or in blends with standard polymers.

Although polymers excluding light or colored or opaque plastic material are preferable to protect the oils from light oxidation, clear plastic containers are generally used because of consumer preference. Ultraviolet absorbers have been used in PET bottles for oil to reduce rates of photooxidation. The additive

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Table 8.11. Effect of different kinds of plastic bottles on the stability of vegetable oils after 60 days of storage at 37°C [a]

OilsTests	Glass	PET	PVC	PP	PS
Olive oil					
PV (meq/kg)	8.64	12.57	11.69	23.78	25.02
BHA (ppm)	nd	4.61	45.24	23.71	17.03
BHT (ppm)	nd	5.13	47.4	25.72	19.65
Sunflower oil					
PV (meq/kg)	18.75	27.28	24.06	75.84	75.84
BHA (ppm)	nd	5.10	46.26	24.74	17.85
BHT (ppm)	nd	5.62	48.67	25.12	21.02

[a] From Tawfik and Huyghebaert (1999). PET = polyethylene terephthalate, PVC = polyvinylchloride, PP, polypropylene, PS = polystyrene. Oils = commercial virgin olive and refined sunflower oils. Levels (% w/w) of BHA = PET, 0.017; PVC, 0.180; PP, 0.070; PS, 0.060; levels (% w/w) of BHT = PET, 0.035; PVC, 0.180; PP, 0.105; PS, 0.087.

Table 8.12. Permeability of polymers used in packaging for vegetable oils [a]

Polymer	O_2 permeability [b] [$cm^3 mm/(m^2 day\ atm)$]	Moisture permeability [c] [$(gmm/(m^2 day)$]
Polyethylene terephthalate (PET)	1–5	0.5–2
Polyvinyl chloride (PVC)	2–8	1–2
High density PE (HDPE)	4	–
Polypropylene (PP)	50–100	0.2–0.4
Polystyrene (PS)	100–150	1–4

[a] From Lange and Wyser (2003).
[b] At 23°C 50% relative humidity.
[c] Water vapor permeability at 23°C 85% relative humidity.

2-(2'-hydroxy-3'-tert-butyl-5'-methylphenyl)-5-chlorobenzotriazole (Tinuvin 326), useful for this purpose, has been approved by the European Union. α-Tocopherol has also been used in low-density polyethylene film to protect both the polymer and the food against oxidative deterioration during storage.

Several protective polymer barriers have been developed to decrease oxygen and moisture permeability of plastic containers by incorporating either thin, discreet silica or organic coatings, or blends with standard polymers. These innovative barrier technologies have not been generally adopted in foods, however. Tin or aluminum cans and paper cartons are also used for shortenings and margarine, which may be wrapped in aluminum foil-laminated paper. Environmental factors affecting lipid oxidation may be controlled by novel

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packaging techniques, e.g. by incorporation of oxygen scavengers, or desiccants, or volatile antioxidants in packaging materials.

Highly oxidizable oils such as fish oils can be protected by a process known as microencapsulation, which coats the oil with a matrix of protein (gelatin, casein), carbohydrates (starch, cellulose, carboxymethylcellulose or cellulose derivatives) and lecithin. Microencapsulation provides protection against oxidation and imparts oxidative stability. The use of carboxymethylcellulose and cyclodextrins as coatings is claimed to provide better protection of oils by improved oxygen barrier properties. For special applications as nutritional supplements, fish oils enriched in *n*–3 PUFA are microencapsulated, in the presence of antioxidants, into a powder that is relatively stable at ambient temperatures. However, encapsulated fish oils can impart undesirable fishy taste when incorporated into food emulsions. More research and development is needed to evaluate potential applications and benefits of active packaging to increase the shelf life of fish oils and other highly oxidizable oils in foods.

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CHAPTER 9

Antioxidants

The inhibition of free radical autoxidation by antioxidants is of considerable practical importance in preserving polyunsaturated lipids from oxidative deterioration. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ) (Figure 9.1) have been commonly used in the US and several other countries to inhibit lipid oxidation and to retard the development of rancidity in foods. Although these synthetic antioxidants are efficient and relatively cheap, special attention has been given to the use of natural antioxidants, because of a worldwide trend to avoid or minimize the use of synthetic food additives. The presence of important natural antioxidants in plant foods has attracted considerable interest, because of their potential benefits as anticarcinogenic agents and as inhibitors of biologically harmful oxidation reactions in the body (Chapter 13). Epidemiological evidence has accumulated that diets rich in plant antioxidants derived from fruits and vegetables are associated with lower risks of coronary heart disease and cancer.

A. Mechanism of antioxidants

1. Chain-breaking antioxidants (AH)

These types of antioxidants inhibit or retard lipid oxidation by interfering with either chain propagation or initiation by hydrogen-atom transfer and readily donating hydrogen atoms to lipid alkyl, alkoxyl and peroxyl radicals [Chapter 1 and reactions (1)–(4) below].

$$LH \longrightarrow L^{\cdot} \quad (1)$$

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot} \quad (2)$$

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \quad (3)$$

$$LOO^{\cdot} + AH \rightleftharpoons LOOH + A^{\cdot} \quad (4/-4)$$

$$L^{\cdot} + AH \longrightarrow LH + A^{\cdot} \quad (5)$$

$$A^{\cdot} + LOO^{\cdot} \longrightarrow LOOA \quad (6)$$

$$A^{\cdot} + A^{\cdot} \longrightarrow A\text{-}A \quad (7)$$

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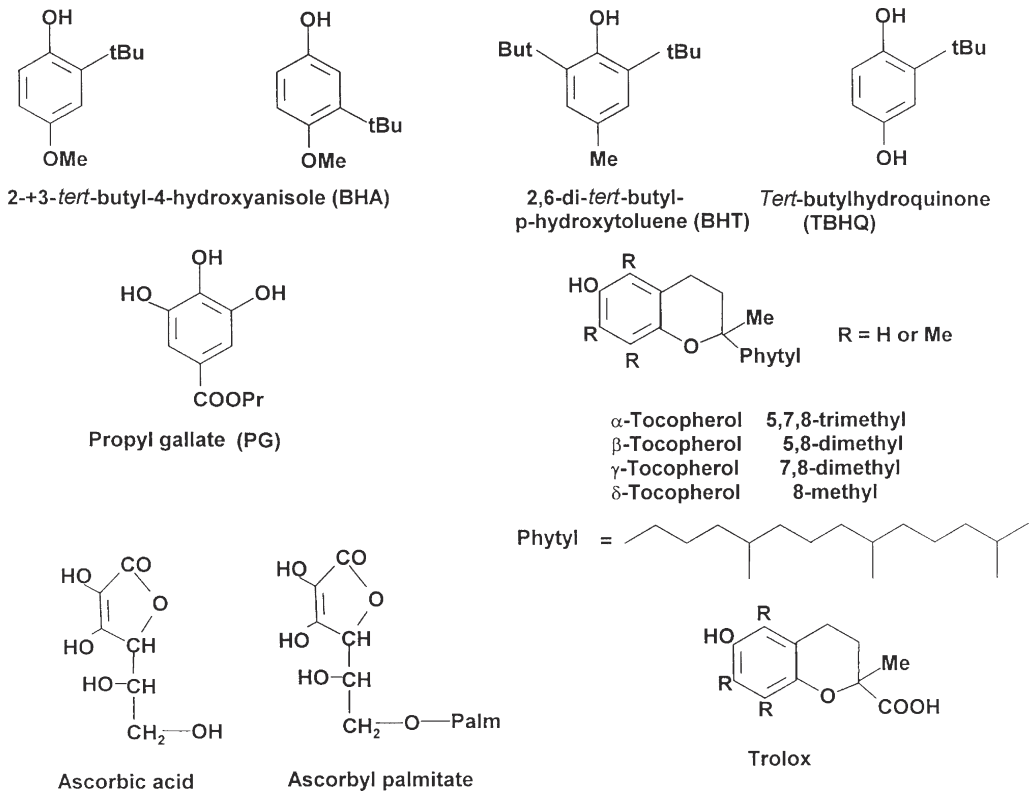


Figure 9.1. Structures of major synthetic and natural antioxidants. Trolux is a trade name of Hoffmann La Roche Inc. for 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

To be effective, chain-breaking antioxidants produce a relatively stable radical A· to react slowly with the lipid substrate LH by reaction (3) and rapidly with peroxyl radical LOO· by reaction (4). Reaction (5) is unimportant at atmospheric pressure, because in the presence of oxygen the alkyl radical R· has a very short life and is immediately converted to the peroxyl radical LOO· by reaction (2). Reaction (5) becomes significant, however, at low oxygen pressure and elevated temperatures. The antioxidant radical A· will either react again with peroxyl radicals to form stable peroxides LOOA by reaction (6) or dimerize with another antioxidant radical to produce A-A by reaction (7).

Phenolic compounds with bulky alkyl substituents near the hydroxyl group, such as BHA, BHT, TBHQ (Figure 9.1), are effective chain-breaking antioxidants because (a) they produce stable and relatively unreactive antioxidant radicals A·, by reactions (4) and (5), that are too unreactive to propagate the chain, and (b) they are able to compete with the lipid substrate (LH), present in much higher concentration in reaction (3), for the chain-carrying peroxyl radicals LOO·. Effective phenolic antioxidants with bulky *ortho* substituents have a weak OH bond to facilitate their reactions with free radicals.

In the presence of an antioxidant, the rate of hydroperoxide formation is

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related to the ratio of lipid (LH) concentration to that of the antioxidant (AH) concentration.

$$d[LOOH]/dt = k_3\ R_1[LH]/(k_4[AH])$$

where k_3 = rate constant for propagation reaction (3); R_1 = rate of initiation (1) and k_4 = rate of inhibition reaction (4).

At elevated temperatures, the oxygen concentration is limiting, inhibition reaction (5) becomes more important, and the rate of hydroperoxide formation is then related to the ratio of oxygen concentration to that of the antioxidant concentration.

$$d[LOOH]/dt = k_3\ R_1[O_2]/(k_5[AH])$$

where k_5 = rate of inhibition reaction (5).

To effectively break the free radical chain, the structure of an active antioxidant is designed to produce phenoxyl radicals in which the unpaired electron is delocalized around the aromatic structure and is stabilized by high resonance energy (Figure 9.2). The antioxidant radicals are thus stabilized by electron delocalization of their phenoxyl structures. The antioxidant effectiveness is directly related to the resonance stabilization (see Introduction C.3) of the phenoxy radicals. Phenolic compounds with electron-donating substituents at the *ortho* and *para* positions are more effective than those with substituents at the *meta* position. *Ortho*- and *para*-dihydric phenols have high antioxidant activity because of their ability to donate hydrogen atoms and to form semiquinone structures that are readily delocalized (Figure 9.2).

Products studies provided more direct evidence than the kinetic approach for

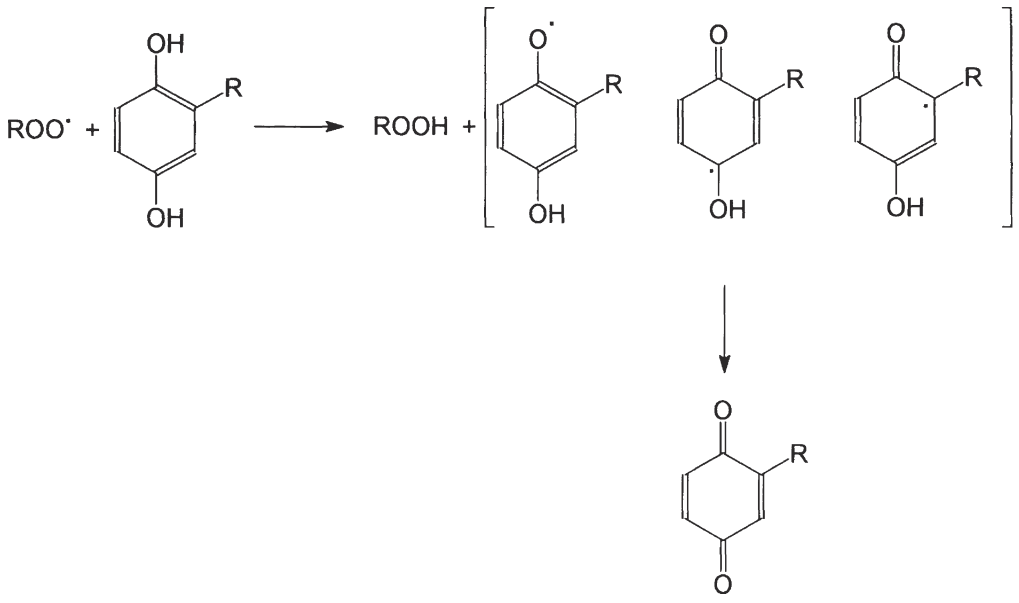


Figure 9.2 Stabilization of an alkylated hydroquinone by electron delocalization.

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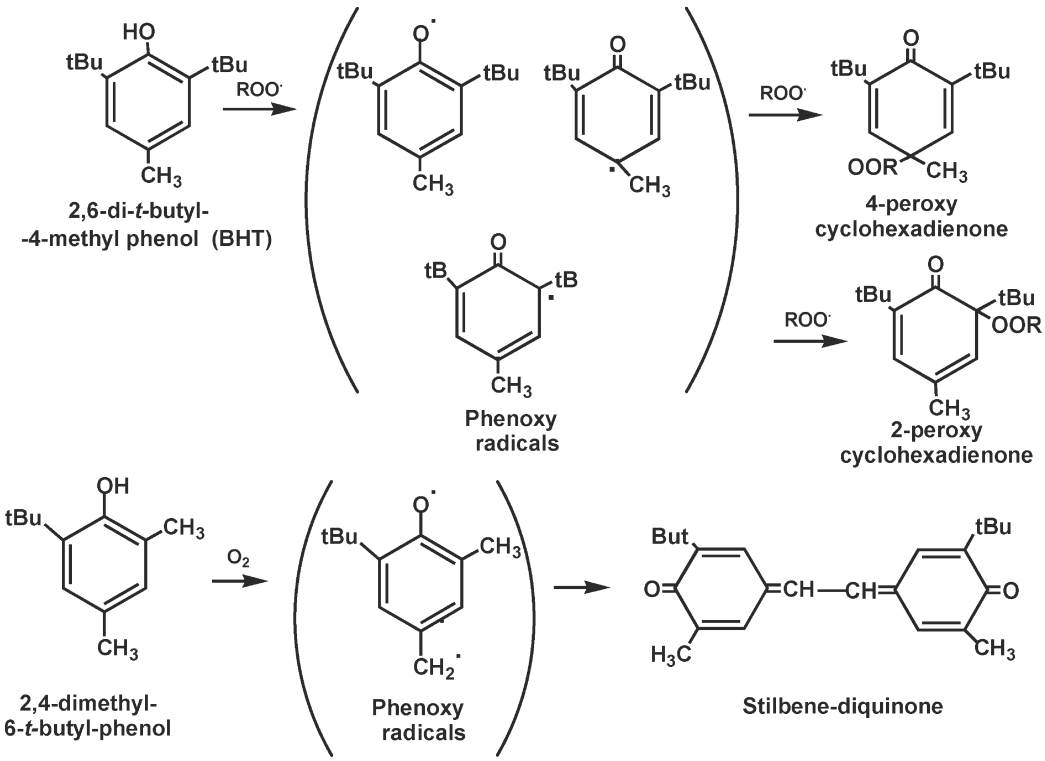


Figure 9.3. Formation of 4-peroxy cyclohexadiene by oxidation of BHT. From Bickel and Kooyman (1953).

the stoichiometry of lipid oxidation inhibited by phenolic antioxidants. Both the peroxide products (LOOA) of reaction (6) and the dimers (A-A) of reaction (7) were identified from tri-substituted phenols (BHT) (Figure 9.3) and from less hindered phenols containing more methyl than *tert*-butyl substituents and from α-tocopherol. Phenolic antioxidants are most effective when all the *ortho* and *para* positions are substituted and when one or two *ortho* substituent is a branched group such as *tert*-butyl. When the *para* substituent is methyl, ethyl or *n*-butyl the phenol is more effective than when it is *tert* butyl. Electron donor substituents increase and electron acceptor substituents decrease activity. With heavily hindered phenolic compounds (e.g. BHT), the extent of inhibition is directly related to the antioxidant concentration.

a) *Prooxidant activity*. Under certain conditions phenolic antioxidants are less effective, because they are less resonance stabilized and have a tendency to act as chain-carriers by generating new radicals and become prooxidants. An important factor affecting the effectiveness of an antioxidant is the tendency of the radical it produces to undergo transfer with the lipid substrate. The effectiveness of antioxidants can thus be decreased by the direct oxidation reaction (8), and by a hydrogen chain transfer reaction (9) of the antioxidant radical with the lipid substrate to reinitiate the chains.

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$$AH + O_2 \longrightarrow A^{\cdot} + HO_2^{\cdot} \quad (8)$$

$$A^{\cdot} + LH \longrightarrow AH + L^{\cdot} \quad (9)$$

Reactions (8) and (9) become important with less substituted phenols, at high concentrations of antioxidants and at elevated temperatures, such as frying, or in the presence of metal catalysts or other oxidation promoters such as hydroperoxides and free fatty acids (see Figure 7.2 and Chapter 12). With less hindered phenolic compounds such as α-tocopherol, inhibition increases with increasing concentration and goes through a maximum. At high concentrations, these phenolic antioxidants can become prooxidant and chain-carriers by regenerating peroxyl radicals by reaction (–4) and by participating in the chain transfer reaction (9). The effectiveness of antioxidants depends therefore on the balance between the rates of inhibition reactions (4) and (6) and the rates of chain transfer reactions (–4) and (9).

Antioxidants also lose their efficiency at elevated temperatures because of homolytic decomposition of hydroperoxides formed by reversing reaction (4) and by producing a chain-carrying peroxyl radical. However, the homolytic decomposition of hydroperoxides producing alkoxyl radicals by reaction (10) is more important than reaction (–4), because it requires less energy to cleave an LO-OH bond (~44 kcal/mol) than an LOO-H bond (90 kcal/mol). Cleavage by reaction (10) is also facilitated by metal catalysts and by heat (Chapter 4).

$$LOOH \longrightarrow LO^{\cdot} + OH^{\cdot} \quad (10)$$

The alkoxyl radicals produced by cleavage reaction (10) can undergo further reactions, including: (11) β-cleavage to produce aldehydes and other decomposition product (short-chain hydrocarbons, and shorter chain fatty acids) that contribute to flavor deterioration of unsaturated lipids (Chapter 4, Figure 4.7); (12) cyclization to produce epoxy derivatives, and formation of ketone and hydroxy derivatives that may or may not contribute to flavor deterioration of unsaturated lipids (Chapter 4); and (13) hydrogen abstraction from the lipid substrate to form innocuous alcohols and a chain-carrying lipid radical.

$$LO^{\cdot} \longrightarrow \text{Aldehydes \& other decomposition products} \quad (11)$$

$$LO^{\cdot} \longrightarrow \text{Secondary products (epoxy, keto, hydroxy)} \quad (12)$$

$$LO^{\cdot} + LH \longrightarrow LOH + L^{\cdot} \quad (13)$$

b) *Effect on hydroperoxide decomposition.* Antioxidants can inhibit the decomposition reaction (11) by different pathways, including: (14) hydrogen donation to form stable hydroxy compounds, or (15) by trapping alkoxyl radicals with antioxidant radicals.

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$$LO^{\cdot} + AH \longrightarrow LOH + A^{\cdot} \quad (14)$$

$$LO^{\cdot} + A^{\cdot} \longrightarrow LOA \quad (15)$$

The overall rate of aldehyde formation (11) is directly related to the concentration of hydroperoxides and inversely related to the rate of formation of stable products that do not form aldehydes.

$$d[\text{aldehydes}]/dt = k_{10}\, k_{11}\, [\text{LOOH}]/k_{12} + k_{14}\, [\text{AH}] + k_{15}\, [A^{\cdot}]$$

Reaction (14) producing stable hydroxy fatty acid derivatives is important, because it inhibits the decomposition of hydroperoxides into aldehydes and other volatile products that decrease the oxidative and flavor stability of polyunsaturated food lipids and contribute to biological damage (see Chapter 13).

We have seen earlier that in the presence of an antioxidant the rate of hydroperoxide formation is related to the ratio of lipid concentration to that of the antioxidant concentration, which is dependent on the rate of inhibition reaction (4). The effectiveness of an antioxidant is generally based on the balance between the inhibition rate (k_{AH}) of reaction (4) and the transfer reactions (–4), (9) and (13). Therefore, the effect of antioxidants on hydroperoxide decomposition reactions (10) and (11) is an important property that needs to be evaluated. However, most studies of antioxidant actions measure initial events of lipid oxidation based on oxygen absorption, hydroperoxide formation, and peroxide values (Chapters 5 and 7). Very few studies have measured the effect of antioxidants on decomposition products of hydroperoxides, such as aldehydes and carbonyl compounds. Yet these volatile decomposition aldehydes are most relevant to the development of rancidity and to the ultimate quality and stability of food lipids.

For example, α-tocopherol exhibited prooxidant activity with stripped corn oil (that has been treated to remove natural tocopherols) and with corn oil-in-water emulsions at concentrations of 250–1000 ppm, based on conjugated diene formation, but it had antioxidant activity between 100 and 1000 ppm, based on hexanal formation (Table 9.1). γ-Tocopherol was also more effective in inhibiting hexanal than hydroperoxide formation in bulk corn oil, but not in the corn oil emulsion. Contrasting results are thus obtained according to the methods used to measure products at different stages of lipid oxidation and depending on the lipid system used in the test. Assuming that hexanal determinations, measuring decomposition of hydroperoxides, are more closely related to flavor deterioration than conjugated diene measurements of hydroperoxide formation, the effects of antioxidants in inhibiting hydroperoxide decomposition may better reflect their activity in reducing flavor deterioration due to lipid oxidation.

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Table 9.1. Effect of concentration of α- and γ-tocopherols on inhibition (in percentage) of hydroperoxide and hexanal formation in bulk corn oil and oil-in-water emulsions oxidized at 60°C

Corn oil	Bulk oils			Oil-in-water emulsions			
	Hydroperoxides		Hexanal	Hydroperoxides		Hexanal	
	day 3	day 7	day 7	day 2	day 4	day 2	day 4
+ α-Tocopherol							
100 ppm	9	88	88	6	70	9	30
250 ppm	–26	85	99	–5	81	24	63
500 ppm	–39	82	99	–24	75	25	72
750 ppm	–85	79	100	–101	57	36	73
1000 ppm	–97	77	99	–100	69	37	85
+ γ-Tocopherol							
100 ppm	26	73	56	–5	66	–2	47
250 ppm	27	83	85	26	86	23	69
500 ppm	24	82	99	45	88	20	82
750 ppm	23	80	99	29	86	24	84
1000 ppm	10	79	99	7	82	31	86

From Huang *et al.* (1994). Negative values represent prooxidant activity.

c) *Effectiveness.* An effective antioxidant can stop the reaction of two chain-carrying peroxyl radicals by reactions (4) and (6), and thus break two kinetic chains (reactions producing new radicals) per molecule. The stoichiometry of the antioxidant reaction can be estimated by controlling the initiation reaction with a standard artificial azo initiator such as α,α-azobis-isobutyronitrile (Chapter 1). Under conditions in which free radicals are produced only by decomposition of the added initiator and not from the products of oxidation, any added antioxidant (AH) that can cause an induction period can be considered to be removing propagating free radicals. From the length of the induction period and the known rate of production of initiating free radicals, the number of free radicals reacting with each antioxidant molecule may be determined.

$$n\mathrm{LOO}^{\cdot} + \mathrm{AH} \longrightarrow \text{Stable products} \qquad (16)$$

where n = the number of radicals that react with a single molecule of antioxidant. An n value of 2 is found for a number of phenolic antioxidants, including α-tocopherol, that corresponds to the two peroxyl radicals trapped by the antioxidant in reactions (4) and (6).

The limitations and pitfalls of using artificial azo initiators were discussed in Chapter 1. Unfortunately, the use of such artificial initiators to evaluate antioxidants is common (see Section E). The so-called "radical trap" methods (also known as antioxidant status or capacity) are dependent on the use of either water-soluble or lipid-soluble radical trapping agents. These methods measure the relative reactivity of antioxidants toward artificial radicals, but they provide

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no quantitative information on what lipid oxidation products are inhibited by the antioxidants. The artificial azo initiators produce a big flux of peroxyl radicals, thereby promoting the propagation phase leading to hydroperoxide formation and minimizing their decomposition. In marked contrast to metals, the artificial azo compounds used as initiators in kinetic studies have no effect on the decomposition of hydroperoxides, whereas metals catalyse the initiation as well as the decomposition of hydroperoxides. Therefore, in the real world of lipid oxidation in foods and biological systems containing unstable polyunsaturated fatty acids in the presence of trace metals and hydroperoxides, artificial azo initiators are not relevant and their use to evaluate antioxidants kinetically is misleading. Antioxidants also show a marked variation in activity with different lipid systems and under different oxidizing conditions. The radical trap methods may not be useful without knowing what specific lipid substrate is protected and what lipid oxidation products are inhibited (see Section E).

Products studies provide more direct evidence than the kinetic approach for the stoichiometry of lipid oxidation inhibited by phenolic antioxidants. Thus, n equals 2 with various phenolic compounds, because both the peroxide products (LOOA) of reaction (6) and the dimers (A-A) of reaction (7) were identified from tri-substituted phenols and from less hindered phenols containing more methyl than *tert*-butyl substituents and from α-tocopherol (Figure 9.3). Reactions (6) and (7) are supported by product studies with various trialkylphenols varying in stability according to their substituents.

To learn about the real effects of antioxidants, it is therefore important to obtain specific chemical information about which products of lipid oxidation are inhibited. Several specific assays are needed to elucidate how lipid oxidation products act in the complex multi-step mechanism of lipid oxidative deterioration of foods. The results of several complementary methods are required to determine lipid oxidation products formed at different stages of the free radical chain. Since antioxidants show different activities toward hydroperoxide formation and decomposition, it is important that more than one method be used to monitor lipid oxidation.

2. *Other classes of antioxidants*

Several types of compounds can inhibit lipid oxidation by mechanisms that do not involve deactivation of free radical chains. They sometimes have multiple effects and their mechanisms of action are therefore often difficult to interpret.

a) *Initiator inhibitors or preventive antioxidants.* Metal inactivators are the most important compounds of this type; they deactivate metal ions, which promote the initiation and decomposition of hydroperoxides, and thus retard the formation of deleterious aldehydes. They function either by coordinating

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the metals and changing their potential by suppressing the redox reactions producing peroxyl and alkoxyl radicals [Reactions (3) and (4), Chapter 1], or by blocking complex formation with hydroperoxides and preventing their decomposition. Care must be taken, however, with some coordination compounds that have a prooxidant effect at low concentrations by changing the redox properties of metals and promoting the oxidative catalytic properties of metals.

Common inactivating chelating compounds include citric acid, phosphoric acid, ethylenediamine tetraacetic acid (EDTA), polyphosphates, phytate, and 8-hydroxy-quinoline (Chapter 8.A). Citric acid and other metal inactivators are more effective in soybean oil after heating, apparently to decompose metal-hydroperoxide complexes (Chapter 8); for this reason, citric acid is added to vegetable oils after heating during the cooling stage of deodorization. Hemin metal complexes behave generally as prooxidants in lipid oxidation. Ascorbic acid may behave either as an antioxidant or prooxidant, depending on its relative concentration, the amounts of metals present and the lipid system (Section D.2). By reducing iron or copper to a lower valence state, ascorbic acid becomes a potent prooxidant catalyst of lipid oxidation in foods and biological systems. Similarly, EDTA at low concentrations can act as a prooxidant because it will promote oxidation by chelating Fe^{3+} more favorably than Fe^{2+}, which is a more active lipid oxidation catalyst than Fe^{3+} (Chapter 8.A). In a fish oil emulsion supplemented with 100 and 200 μM of Fe^{2+}, EDTA promoted oxidation when used at equimolar ratios of EDTA and iron, and effectively inhibited oxidation at higher molar ratios of EDTA and iron (2:1 and 4:1) (Table 9.2). It is important, therefore, to use excess molar concentrations of EDTA and ascorbic acid to avoid prooxidant effects. In combination, ascorbic

Table 9.2. Effect of iron and EDTA on the oxidation of an oil-in-water lecithin emulsion prepared with mackerel oil containing long-chain polyunsaturated fatty acids at 40°C[a]

Emulsified oil	Increase in peroxide value (meq/kg)			
	18 hr	24 hr	48 hr	72 hr
Control	14.7	21.6	52.1	203.6
+ 100 μM Fe	32.1	32.5	62.8	234.3
+ 100 μM EDTA	0.0	0.2	1.0	2.9
+ 100 μM Fe + 100 μM EDTA	39.2	61.3	253.0	327.7
+ 50 μM Fe + 100 μM EDTA	1.9	2.9	6.2	13.2
+ 25 μM Fe + 100 μM EDTA	0.1	1.0	2.9	7.0

[a]From Frankel *et al.* (2002).
Emulsions were prepared with 5% mackerel oil, 1% lecithin and homogenized with phosphate-citrate buffer (25 mM, pH 6.6). The mackerel oil contained 40.2% polyunsaturated fatty acids consisting of 9.7% EPA and 14.5% DHA, and 11 ppm of α-tocopherol.

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and citric acids also behave synergistically at lower concentrations when applied in the form of dipping solutions to preserve seafoods.

Various proteins inhibit lipid oxidation in different lipid systems by their capacity to bind or chelate metal ions. The relative antioxidant potency of phenolic compounds in liposomes was mediated to different extents by the presence of protein. At high relative metal concentrations, however, these complexes can also promote lipid oxidation (see Chapter 10.B.2).

Lactoferrin is an iron transport non-heme glycoprotein present in human milk and many biological secretions that can effectively inhibit the oxidation of various lipid systems by its iron-binding capacity. However, this protein can also have prooxidant activity, depending on the lipid system, and its concentration relative to the concentration of metal ions. Lactoferrin effectively inhibited oxidation and increased the oxidative stability of infant formulas supplemented with iron (see Chapter 11.C). Other known proteins that chelate or inactivate transition metals are found in biological systems including transferrin, albumin, and ceruloplasmin (Chapter 13).

b) *Hydroperoxide destroyers*. These compounds inhibit oxidation by induced decomposition of hydroperoxides by forming either stable alcohols or inactive products by non-radical processes, e.g. by reduction or hydrogen donation. α-Tocopherol and its water-soluble carboxylic acid analog called Trolox (Figure 9.1), and other phenolic compounds are weak hydroperoxide destroyers by reducing linoleate hydroperoxides into stable hydroxylinoleate in low yields. We have seen above that α-tocopherol is more efficient in inhibiting hexanal formation than hydroperoxide formation during oxidation of polyunsaturated vegetable oils (Table 9.1). Sodium borohydride has been used to reduce hydroperoxides accumulated during vegetable oil processing but has not been adopted commercially (Chapter 8). Other examples of effective hydroperoxide destroyers include reducing agents, sulfur and selenium compounds, phosphites, and phosphines, which cannot be used in foods because of their toxicity and production of undesirable flavors, however.

c) *Ultraviolet light deactivators*. Certain compounds that can absorb light energy without formation of radicals can deactivate photo-initiators of lipid oxidation such as hydroperoxides and traces of carbonyl compounds. Examples of compounds useful in non-food applications include, pigments such as carbon black used in polymers, phenyl salicylate, and aromatic ketones such as *ortho*-hydroxybenzophenone.

d) *Synergists.* In multi-components systems, antioxidant compounds can reinforce each other by cooperative effects known as *synergism*. Many antioxidant combinations are known to impart more protection against lipid oxidation than the sum of the activities of the components when used separately. Effective synergistic inhibition can be achieved if both initiation and propagation

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are suppressed. Combinations of a preventive metal inactivator such as citric acid and a chain breaking antioxidant, such as BHA, BHT, TBHQ, or tocopherols (Figure 9.1), are commonly used in foods. Thus, vegetable oils containing varying concentrations of α-, β- and γ- tocopherol mixtures are effectively stabilized by added citric acid. In these combinations synergism may also arise by the protective effect of citric acid against the metal catalysed oxidation of the tocopherol mixtures. A strong synergistic or combined effect was observed between natural tocopherols in soybean oil in the presence of added citric acid (Table 9.3). The percent synergism was calculated on the basis of the induction periods (IP) observed, as follows:

$$\% \text{ synergism} = \frac{\left(\begin{matrix}\text{IP tocopherol + citric acid}\\ \text{combined}\end{matrix}\right) - \left(\begin{matrix}\text{IP tocopherol} + \text{IP citric acid}\\ \text{alone} \qquad\quad \text{alone}\end{matrix}\right)}{\left(\begin{matrix}\text{IP tocopherol} + \text{IP citric acid}\\ \text{alone} \qquad\quad \text{alone}\end{matrix}\right)}$$

The calculated synergism between tocopherols and citric acid in soybean oil was 285%, and increased at the lower concentrations of tocopherol (200–500 ppm) in the oil treated with carbon black (Table 9.3). This synergistic effect between a chain-breaking antioxidant and citric acid is attributed to metal chelation. Another well-recognized synergism between a free radical acceptor such as α-tocopherol and a reducing agent such as ascorbic acid is explained by the regeneration and recycling of the tocopheroxyl radical intermediate to the parent phenol, α-tocopherol (Section C.1).

Other types of synergism are observed with mixtures of phenolic compounds and lecithin, polyols and amino acids. These synergistic components may act as

Table 9.3. Synergistic effects of tocopherols and citric acid on the oxidative stability of carbon-treated soybean oil at 60°C[a]

Samples[b]	Tocopherol (ppm)	Induction period (hr)	Initial rate (PV/hr)	Synergism[c] (%)
Control	1500	20	0.14	–
Control + CA	1500	104	0.023	285
C-treated 1× + CA	750	235	0.018	226
C-treated 2× + CA	468	244	0.018	328
C-treated 3× + CA	214	160	0.032	566
C-treated 4× + CA	0	7	0.31	–

[a]From Frankel *et al.* (1959a).
[b]CA = citric acid, 0.01%
[b]C-treated 1× = carbon-treated once to remove tocopherols
[b]C-treated 2× = carbon-treated twice to remove tocopherols
[b]C-treated 3× = carbon-treated three times to remove tocopherols
[b]C-treated 4× = carbon-treated four times to remove tocopherols
[c]See text for calculation.

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metal scavengers. Lecithin may act as a synergist in oils by metal chelation, and in emulsions by its surface-active properties to improve the contact between the polar phenolic antioxidants and the less polar lipid substrate. Phenolic antioxidants also have synergistic properties in reinforcing the antioxidant properties of lactoferrin [Section 2 (a)] in various lipid systems supplemented with iron for nutritional purposes. The recognized multi-effects of flavonoids include also metal chelation (Section D.4).

B. Antioxidant evaluations

The effectiveness of antioxidants in protecting foods against oxidative deterioration is very dependent on complex phenomena determined by the relative physical states of the lipid substrates, the conditions of oxidation, the methods used to follow oxidation and the stages of oxidation. Assessment of the bioactivity of antioxidants in humans is a different complex issue that will be discussed in Chapter 13. In foods, antioxidant effectiveness is evaluated by measuring inhibition of oxidation of a suitable substrate under appropriate and standard conditions, and by choosing a proper method to determine an end point of oxidative spoilage. The methods used to evaluate antioxidants in foods are complicated by many factors (Chapter 7): (i) the use of different lipid substrates has a significant impact on the activity of various antioxidants according to their hydrophilic or lipophilic nature; (ii) solubility and partition properties affect the activity of antioxidants in heterogeneous systems where they are distributed differently between aqueous and lipid phases; (iii) different results can be obtained at different temperatures because the mechanism of oxidation and hydroperoxide decomposition change with temperature; and (iv) different methods used to follow oxidation can give varying results according to the different effects of antioxidants on formation of hydroperoxides and their decomposition (Table 9.1). Relative antioxidant efficiencies vary markedly from one oxidizing lipid substrate to another. In the same lipid substrate, the relative activities of antioxidants often depend on the antioxidant concentrations.

The activity of natural antioxidants is greatly affected by complex interfacial phenomena in emulsions and multi-component foods. The methodology to evaluate natural antioxidants must be carefully interpreted depending on whether oxidation is carried out in bulk oils or in emulsions, and which analytical method is used to determine extent and end-point of oxidation (Chapter 10). To understand and better predict how natural antioxidants may protect foods against oxidation, the following complex questions need to be carefully considered for the judicious choice of antioxidant evaluation protocols: a) What are the protective properties of antioxidants? b) What substrates are oxidized and what products of oxidation are inhibited? c) In a multiphase food system is the antioxidant located where oxidation takes place? d) Are there any

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other interacting components that may affect the results? e) What conditions are relevant to real-life applications?

Meaningful interpretation of antioxidant action thus requires: a) specifying the oxidizing substrate protected; b) measuring the correct extent of oxidation and inhibition; c) choosing an appropriate end-point of oxidation; and d) determining any possible adverse prooxidant effects from the antioxidants by using a range of concentrations.

Each antioxidant evaluation should thus be carried out under various conditions of oxidation, using several methods to measure different products of oxidation related to real food quality. There cannot be a short cut approach to determining the activity of antioxidants. Various testing protocols should consider the following parameters and recommendations:

a) *Substrates*. Use triacylglycerols or phospholipids, in bulk, emulsions, or liposomes systems because they are most important in foods and biological systems. Free fatty acids should be avoided because they influence the relative activities of different antioxidants, and because they form micelles that behave differently from triacylglycerols.

b) *Conditions*. Test under different temperatures (below 60°C), metal catalysts, or surface exposure (e.g. agitation) to simulate real food storage as close as possible.

c) *Analyses*. Measure initial products (hydroperoxides, peroxide value, conjugated dienes) and secondary decomposition products (carbonyls, volatile compounds) at several time periods during different stages of lipid oxidation, including the initiation and early stage of the propagation phases of oxidation. Because the effects of different antioxidants vary in their kinetic of oxidation, it is important for valid comparisons to analyse at different time points for the development of primary oxidation and decomposition products. For foods, chemical analyses should be confirmed by sensory evaluations. Sensory tests are particularly important and sensitive for vegetable oils containing linolenic acid and fish oils rich in long-chain polyunsaturated fatty acids that produce fishy responses at very low levels of oxidation.

d) *Concentrations*. Compare antioxidants at the same molar concentration of active components and at several levels adequately spaced using appropriate structurally related reference compounds. With crude extracts, compare their total phenol content and compositional data of different samples. Where possible characterize antioxidant oxidation and breakdown products.

e) *Calculations*. Quantify on the basis of induction period, or percent inhibition or rates of hydroperoxide formation or decomposition at several levels of oxidation, or I_{50} (antioxidant concentration required to obtain 50% inhibition).

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Because of the complexity of real foods, accelerated test systems are difficult to standardize for antioxidants. Each test should be calibrated for each lipid or food. Accelerated oxidation conditions should be as close as practical to the storage conditions under which the food is to be protected (Chapter 7). Ultimately, antioxidants should be evaluated on the food itself.

In the last ten years, the literature on antioxidant evaluations has expanded tremendously, because of the cumulating evidence that they may contribute to the recognized nutritional benefits of fruits, vegetable and beverages containing antioxidants. The large amount of effort expended in testing new natural antioxidants emphasizes the need for improved test methods and their standardization. Significant variations in test results have been reported that created much confusion in this field. To better understand and compare the results of various test methods each antioxidant types will be first described below (see Section F).

C. Synthetic antioxidants

For food applications, synthetic antioxidants must be sufficiently active to be used at low concentrations (below 0.02%) and cannot be toxic. To be effective in foods, synthetic antioxidants must partition favorably between the oil–air interfaces in bulk oil systems, and between oil–water interfaces in emulsion systems (Chapter 10). Food antioxidants must also be stable to processing and cooking conditions. The term "carry through effect" refers to the ability of antioxidants to withstand the thermal treatments of frying or baking to be absorbed by the food and have sufficient activity to stabilize the fried or baked food. Compared to natural antioxidants, synthetic antioxidants are generally more effective and can be used at lower concentrations; they are less expensive, can be prepared with consistent quality without effect on flavor, color and aroma of the final product.

The most important synthetic antioxidants for food applications worldwide include BHA, BHT, PG and ascorbyl palmitate (Figure 9.1). The dihydric TBHQ can be used in the US and its approval is now being considered in Europe. Although octyl and dodecyl gallate are permitted in Europe they are not in the US. BHA is more soluble in fats than BHT and both are considered to be more effective in less unsaturated animal fats than polyunsaturated vegetable oils. The trihydric PG is less oil-soluble than the monohydric BHA and BHT. The dihydric TBHQ is more effective in soybean oil than BHA, BHT or PG, and more stable to heat processing. Antioxidants of different activities, such as BHA and BHT, or BHA and PG show useful synergistic activity when used in mixtures. This synergism may be explained by an increase in oxidative stability of the antioxidants by the mutual protection of the antioxidant radicals in a mixture.

Commercial synthetic antioxidants are commonly formulated with propylene glycol, glyceryl monooleate, mono- and diglycerides, or vegetable oils as

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carriers to increase their solubility or dispersibility in foods. On the one hand, these carriers facilitate the uniform incorporation of antioxidants into oils and foods, but their effect on activity in different lipid systems is not well established. On the other hand, even if polar and hydrophilic antioxidants such as ascorbic acid (Section D.2) and carnosic acid (Section D.3) are only partially miscible in bulk oils, they are very effective without the use of carriers provided they are suspended uniformly in the oil matrix preferably by shaking during storage (see *polar paradox*, Chapter 10.C.2.c).

Several synergistic mixtures are used commercially including BHA and BHT, BHA and PG, BHA, BHT and TBHQ, with various levels of citric acid. PG reinforces the antioxidant activity of BHA but not that of BHT. Ascorbyl palmitate (Figure 9.1) is a weak antioxidant when used alone, but it shows synergistic activity in mixtures with natural tocopherols.

The reported and claimed activities of different antioxidants and formulations are difficult to evaluate because of the different testing conditions used. For example, when tested at 45°C, ascorbic acid was less effective in stabilizing soybean oil than ascorbyl palmitate, which was in turn more effective than BHA and BHT, but not as active as PG and TBHQ. TBHQ was the most active of the antioxidants tested (Table 9.4). However, when tested at 98°C under conditions of the active oxygen method (AOM), the relative antioxidant activities of ascorbic acid and ascorbyl palmitate were reversed and ascorbic acid became more active than PG and TBHQ. These results must be interpreted with caution. The peroxide value used to follow oxidation is not reliable when oils are heated at 98°C because a large fraction of the hydroperoxides is decomposed at this temperature (Chapter 7). The peroxide value of 70 used as an end-point is also questionable since flavor deterioration occurs in soybean oil at peroxide values below 10.

Table 9.4. Effect of temperature on antioxidant activity in soybean oil[a]

Antioxidants (0.02%)	Antioxidant activity days at 45°C [b]	Antioxidant activity hours at 98°C [b]
Control	7	5
Ascorbic acid	12	43
Ascorbyl palmitate	19	13
BHA	9	9
BHT	10	11
PG	15	14
TBHQ	26	26

[a]From Cort *et al.* (1982)
[b]To reach a peroxide value of 70. Oxidation in thin layer at 45°C; oxidation under conditions of the active oxygen method (AOM) at 98°C.
BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; PG, propyl gallate; TBHQ, *tert*-butyl hydroquinone (Figure 9.1).

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Phospholipids are generally useful as synergists in reinforcing the antioxidant activity of phenolic compounds. Various patented combinations between ascorbyl palmitate, tocopherols and lecithin are particularly effective for the stabilization of polyunsaturated fish oils at the elevated temperatures of the Rancimat test (Chapter 8) and based on peroxide value. However, these ternary mixtures are not effective in preventing the development of fishy odors and flavors at ambient storage conditions based on sensory measurements. Binary systems of ascorbyl palmitate/lecithin and γ/δ- or δ-tocopherol are very effective synergistic systems for fish oils. The antioxidant synergism of the ternary mixtures in oils is explained by their interactions between all three components. The lecithin not only functions as an emulsifier for improving the contact between ascorbic acid and tocopherol, but also participates in redox cycling with the tocopheroxyl radical (see Section D.1) and ascorbic acid by radical exchange reactions of a primary amine group in phosphatidylserine or phosphatidylethanolamine. A mixture of α-tocopherol with phosphatidylethanolamine exhibits higher antioxidant activity than with phosphatidylcholine. This difference is attributed to better regeneration of the tocopheroxyl radical to tocopherol by easier hydrogen transfer from the amino group to the radical. However, the effects of phospholipids are complicated by their multiple functions; they are effective metal chelators, and good emulsifying agents. As emulsifiers, the inhibiting effects of phospholipids may be explained by their ability to improve the affinity of the tocopherols and other phenolic antioxidants toward the lipid substrate. Another confounding effect of phospholipids is their tendency to produce browning materials when heated at the elevated temperatures used for the AOM and Rancimat tests (Chapter 7). Browning materials act as reducing agents, which are active food antioxidants (Chapter 11).

D. Natural antioxidants

Much interest has developed in naturally occurring antioxidants, because of the adverse attention received by the synthetic antioxidants, and because of the worldwide trend to avoid or minimize the use of artificial food additives. In the last 15 to 25 years, special attention has been given to the use of natural antioxidants because of the possible, but not well established, hazardous effects of synthetic antioxidants. The action of food processors to remove synthetic antioxidants from their formulations has also been motivated by economic concerns to save on testing costs. The advantages of natural antioxidants include unquestioned safety by their GRAS status ("generally recognized as safe"), higher concentrations allowed, worldwide acceptance, and lower volatility in heated foods. The disadvantages include untested toxicity, wide variation in concentration of active components with source and extraction method, poor oil solubility, and undesirable effects on flavors, colors and odors

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Table 9.5. Tocopherols in vegetable oils, fish and animal fats (ppm or mg/kg)[a]

Oils	α	β	γ	δ	Total
Corn	112	50	602	19	783
Cottonseed	389	–	387	–	776
Olive	119	–	7	–	126
Palm[b]	256	–	316	70	642
Peanut	130	–	214	21	365
Rapeseed/canola	210	1	42	–	252
Safflower	342	–	71	–	413
Soybean	75	15	797	266	1153
Sunflower	487	–	51	8	546
Menhaden	75	–	–	–	75
Salmon[c]	82	–	–	–	82
Mackerel[c]	11	–	–	–	11
Lard	12	–	–	–	12
Tallow	27	–	–	–	27

[a]From Gunstone *et al.* (1994).
[b]Includes 149 ppm of tocotrienols.
[c]From Frankel *et al.* (2002).

in foods. Although many epidemiological studies support the nutritional benefits of fruits and vegetables rich in phytochemicals showing strong *in vitro* antioxidant activity, their *in vivo* activity is not well understood and has not been well confirmed by human intervention studies.

1. Tocopherols

Tocopherols are the most important natural antioxidants found in vegetable oil-derived foods. They occur as different homologs varying in the extent of methylation of the chroman ring (α, β, γ and δ) containing a saturated phytyl (trimethyltridecyl) side chain (Figure 9.1). Vegetable oils are good sources of tocopherols and their concentrations and homologous compositions vary widely (Table 9.5). Soybean oil is rich in γ- and δ-tocopherol whereas sunflower oil is rich in α-tocopherol. Tocotrienols contain a tri-unsaturated side chain and occur in palm oil (about 150 to 500 ppm), rice bran oil (about 200 ppm), oats (about 30 to 200 ppm), barley (about 40 ppm as α) and corn oil (about 35 ppm), but their relative antioxidant activities in different oils are not well established. Animal fats and fish oils contain mainly α-tocopherol and at much lower levels than vegetable oils.

α-Tocopherol behaves like a chain-breaking electron donor antioxidant by competing with the substrate for the chain-carrying peroxyl radicals, normally present in the highest concentration in the lipid system. α-Tocopherol reacts rapidly with peroxyl radicals by reaction (4) with a rate of 10^6 $M^{-1}s^{-1}$, which is much faster than the reaction (3) of peroxyl radicals with the lipid, typically

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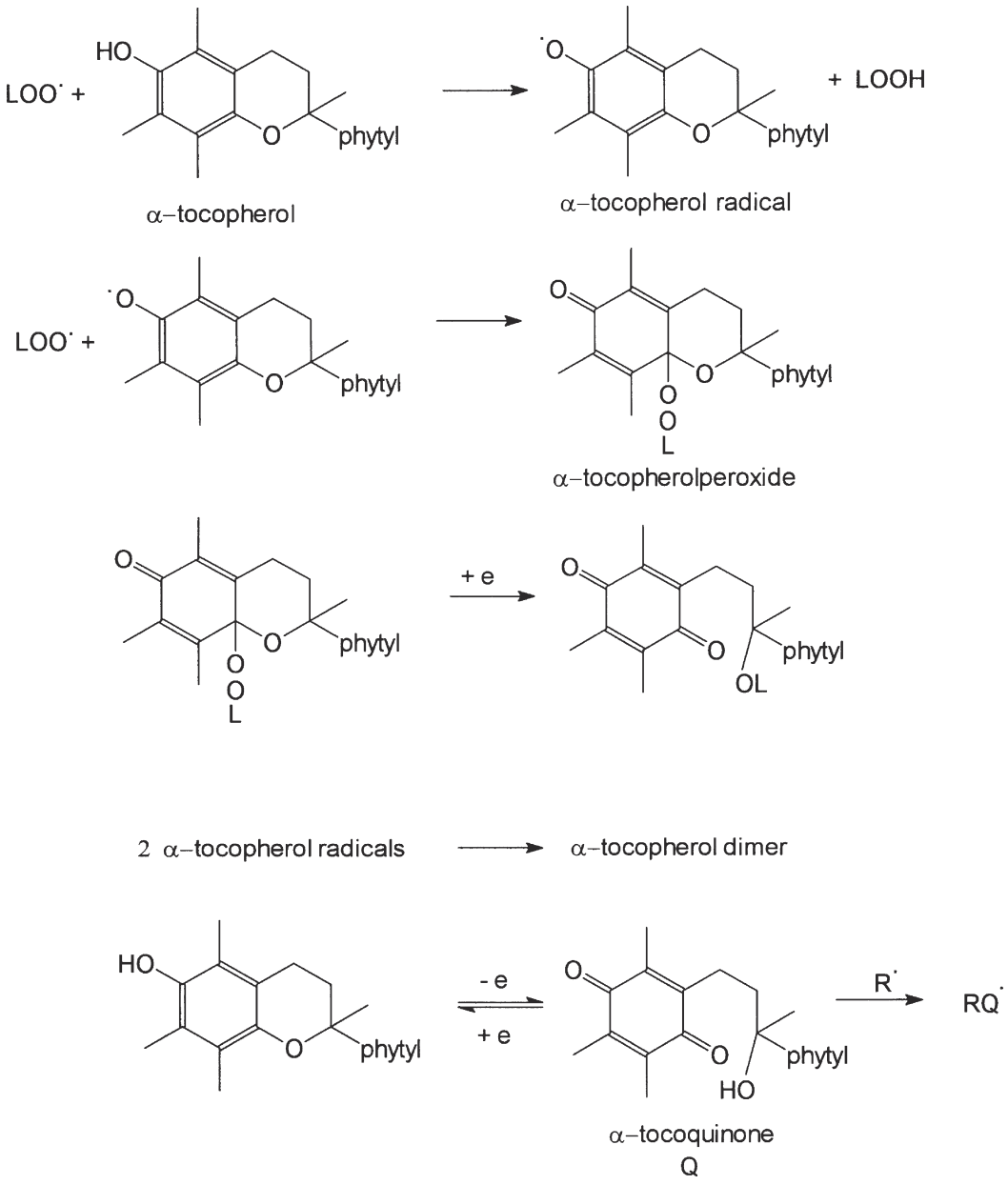


Figure 9.4. Mechanism of α-tocopherol action

10^1 $M^{-1}s^{-1}$. By reaction (4), α-tocopherol donates a hydrogen atom to produce lipid hydroperoxides and a tocopherol radical that is stabilized by resonance. This tocopherol radical does not propagate the chain but forms non-radical products, including stable peroxides, which can be reduced to a tocoquinone adduct and to tocopherol dimers (Figure 9.4). α-Tocopherol is also oxidized by reaction (8) to form α-tocopheroquinone, which acts as an electron acceptor antioxidant by competing with oxygen for alkyl radicals ($RQ^{\bullet}$). This competitive reaction would only become important at low oxygen pressure (e.g. elevated

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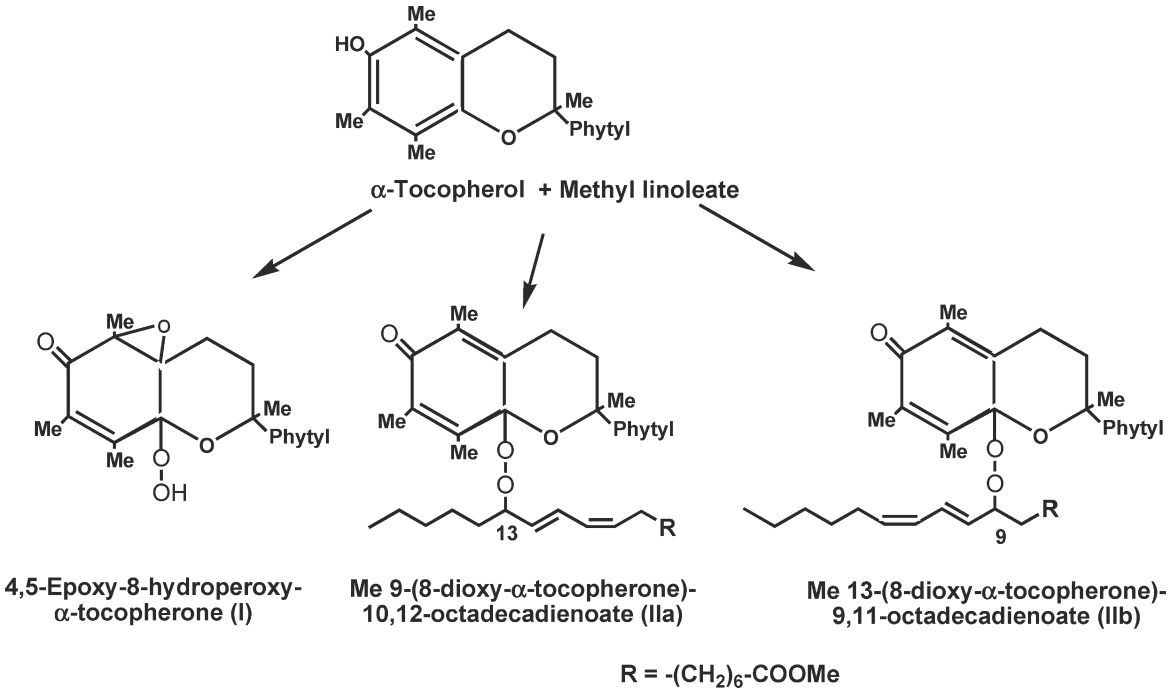


Figure 9.5. Oxidation products formed from methyl linoleate and α-tocopherol at 37 and 60°C. Adapted from Yamauchi *et al*. (1995).

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temperatures), because the alkyl radicals react extremely rapidly with oxygen under atmospheric conditions and much more readily than the tocopherol radicals. The oxidation of pure methyl linoleate in the presence of 0.1% α-tocopherol at 37 and 60°C yields mainly epoxy-hydroperoxy-α-tocopherones (I) and a mixture of 9- and 13-peroxyl octadecadienyl radicals (II) (Figure 9.5). During autoxidation, α-tocopherol radicals can thus react with both alkoxyl and peroxyl radicals of unsaturated lipids.

The well-recognized synergism between α-tocopherol and ascorbic acid is explained by the regeneration and recycling of the tocopherol radical intermediate to the parent α-tocopherol. During the oxidation of methyl linoleate in the presence of α-tocopherol and ascorbic acid, the induction period was prolonged and the propagation stage was delayed by the sparing effect of ascorbic acid, which is oxidized first followed by the oxidation of α-tocopherol (Figure 9.6). In a liposome system in which lecithin forms lamellar bilayers separated by water layers (see Chapter 10), ascorbic acid regenerates α-tocopherol only with a water-soluble initiator, but not with a lipid-soluble initiator. This observation suggests that ascorbic acid can probably only react with the tocopherol radicals at the surface of the liposome.

In natural food systems, the interaction of tocopherols and ascorbic acid occurs favorably because initiation of lipid oxidation occurs also by metal catalysts present in the water phase. Mixtures of tocopherols with rosemary extracts (Section 3) are also synergistic in bulk oil resulting probably by their mutual reinforcing activity. Although β-carotene when used alone in bulk and emulsion systems is either inactive or acts as a prooxidant, it becomes a good

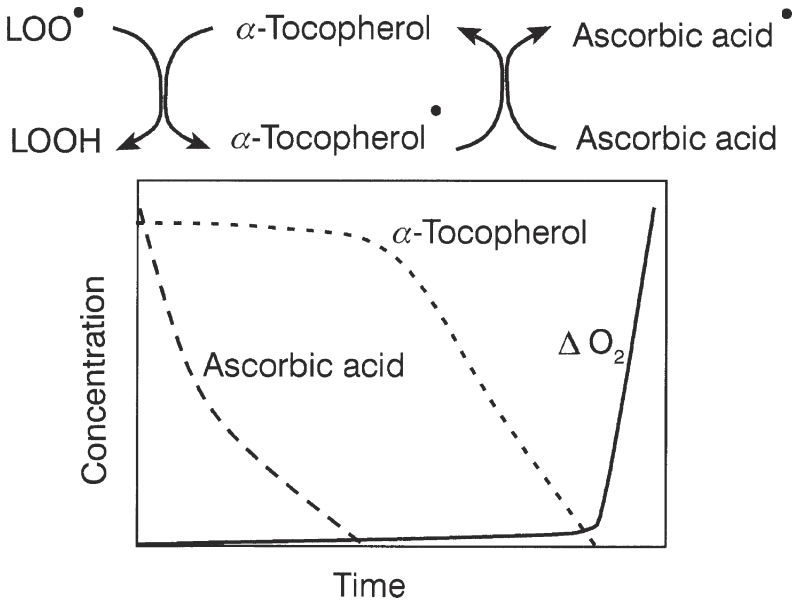


Figure 9.6. Disappearance of α-tocopherol and ascorbic acid during oxidation of methyl linoleate. From Niki *et al.* (1987).

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antioxidant and shows synergism in the presence of α- and γ-tocopherols. The effect of γ- and other tocopherols in protecting β- and other carotenoids during oxidation is recognized, but the reason why β-carotene increases the antioxidant activity of tocopherols is not clear. In biological systems, synergism takes place in a cascade of reactions where ascorbic acid is also regenerated at the expense of more oxidizable substrates (Chapter 13).

Tocopherols can interrupt lipid autoxidation by interfering with either the chain propagation or the decomposition processes. In addition to its free radical scavenging activity, α-tocopherol is highly reactive toward singlet oxygen and protects food lipids against photosensitized oxidation (Chapter 3). Although about 20 to 40 % of the tocopherols are lost during processing of vegetable oils, the levels of tocopherols left are usually adequate for protection against oxidation. In many studies in the literature, α-tocopherol has been shown to have a prooxidant effect at high concentrations, on the basis of methods measuring hydroperoxide formation during the early stages of lipid oxidation. Although the mechanism for the antioxidant effects of α-tocopherol in intercepting peroxyl radicals is well accepted, the prooxidant effects observed at high concentrations are not well understood.

In polyunsaturated vegetable oils, tocopherol mixtures are generally regarded as poor antioxidants. With crude soybean oil containing about 1300 to 1500 ppm mixed tocopherols, the removal of a portion of the natural tocopherols with carbon-black treatment markedly increased the oxidative stability (Table 9.6). The oxidative stability of soybean oil was optimum at tocopherol concentrations between 400 to 600 ppm. The antioxidant activity of the tocopherol mixture diminished when the tocopherol levels exceeded their optimal concentrations especially with increasing temperature. This observation is the basis of the commercial preparation of tocopherols, which are recovered as antioxidant concentrates from the deodorization distillates during soybean oil processing. In soybean oil treated with carbon black to remove the natural tocopherols almost completely, added α-tocopherol was a less effective antioxidant than either ascorbic acid or citric acid (Table 9.7). The mixture of α-tocopherol and citric acid gave the same induction period as citric acid alone. The effect of metal chelation by citric acid was, therefore, more important than the antioxidant effect of α-tocopherol in soybean oil. Although the activity of BHT alone was about the same as that of α-tocopherol, the mixture of BHT and citric acid was not as effective as the mixture of α-tocopherol and citric acid. The addition of 0.01% citric acid decreased the loss of tocopherol in soybean oil and significantly increased its oxidative stability (Table 9.8). The addition of 0.3 ppm of iron caused the rapid loss of tocopherols and a significant decrease in oxidative stability of soybean oil. The addition of 0.01% citric acid was sufficient to almost completely eliminate the prooxidant effect of iron.

The relative antioxidant activity of different tocopherol homologs has received much attention in the literature. On the basis of induction period

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Table 9.6. Effect of tocopherol concentration on oxidative stability of soybean oil at 60°C[a]

Samples[b]	Tocopherol (ppm)	Oxygen absorption Induction period, hr	Peroxide value Induction period, hr
Control	1500	39	30
C-treated 1×	1050	84	60
C-treated 2×	544	88	78
C-treated 3×	196	6	40
C-treated 4×	45	–	18

[a]From Frankel *et al.* (1959a).
[b]C-treated 1× = carbon-treated once to remove tocopherols
[b]C-treated 2× = carbon-treated twice to remove tocopherols
[b]C-treated 3× = carbon-treated three times to remove tocopherols
[b]C-treated 4× = carbon-treated four times to remove tocopherols

Table 9.7. Effect of antioxidants and metal inactivators on oxidative stability of soybean oil at 60°C[a]

Samples[c]	Peroxide value Induction period, hr
Control A (1500 ppm tocopherol)	26
C-treated (45 ppm tocopherol)[b]	17
+ α-Tocopherol	21
+ BHA	18
+ BHT	23
+ Propyl gallate	26
+ Citric acid (CA)	50
+ Ascorbic acid	43
+ α-Tocopherol + 0.01% CA	53
+ BHA + 0.01% CA	56
+ BHT + 0.01% CA	53

[a]From Frankel *et al.* (1959a).
[b]C-treated = carbon treated to remove tocopherol
Antioxidants concentrations = 0.57 mmol/kg

Table 9.8. Effect of citric acid (CA) and iron on oxidative stability and tocopherol oxidation in soybean oil at 60°C[a,b]

Samples	Induction period (hr)	Initial rate (PV/hr)	Tocopherol loss (ppm/hr)
Control	16	0.1	3.5
+ 0.01% CA	105	0.02	1.4
+ 0.3 ppm Fe^{3+}	4	0.41	43
+ 0.01% CA + 0.3 ppm Fe^{3+}	100	0.02	1.5

[a]From Frankel *et al.* (1959b).
[b]Tocopherol content of soybean oil: 1530 ppm; induction period based on peroxide value (PV).

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Table 9.9. Relative order of antioxidant activities of tocopherols

References	Conditions	α-	β-	γ-	δ-
Lea (1960)	Me linoleate, 50°C	4	3	1	2
	Lard esters, 90°C	4	3	2	1
Lea and Ward (1959)	Cottonseed esters, 37°C	4	3	1	2
	Cottonseed esters, 60°C	4	3	1	2
	Linseed esters, 37°C	1	3	2	4
	Linseed esters, 60°C	2	3	1	4
Olcott and Van der Veen (1968)	Menhaden oil 37°C	3	–	1	2
Burton and Ingold (1981)	Styrene, 30°C	1	2	3	4
Kulås and Ackman (2001)[a]	Purified anchovy oil, 30°C, 100 ppm	1	–	2	3
	Purified anchovy oil, 30°C, 1000 ppm	3	–	2	1
Evans *et al.* (2002)[b]	Stripped soybean oil, 40–60°C	1	–	2	3

[a]The anchovy oil was stripped of tocopherols by chromatography. The relative antioxidant activity was based on inhibition of hydroperoxides measured by conjugated diene formation.
[b]The soybean oil was stripped of tocopherols by short-path molecular distillation. The relative potency was based on inhibition of hydroperoxides measured by conjugated diene formation at optimal concentrations of about 100 ppm for α-tocopherol, about 300 ppm for γ-tocopherol. The optimal concentration for δ-tocopherol was not clearly defined and the antioxidant activity increased with concentration up to ~1900 ppm.

measurements, γ- and δ-tocopherols were relatively more effective than β- and α-tocopherols (Table 9.9). On the basis of initial rates of oxidation of styrene in chlorobenzene solution in the presence of azo-bis(isobutyronitrile) as initiator, the relative activities of tocopherol homologs increased in the same order as their vitamin E activities: $\alpha > \beta > \gamma > \delta$. The temperature of oxidation had a marked effect on the relative activities of tocopherol homologs in linseed esters high in linolenate, but not in cottonseed esters in which linoleate was the main unsaturated fatty acid. The discrepancies concerning the relative antioxidant activities of tocopherol homologs can be attributed to the wide differences in unsaturated substrates tested, the level of oxidation used in the tests, the method used to analyse oxidation, and their relative concentrations. Thus, in purified fish oil triacylglycerols the relative activity of tocopherols in inhibiting hydroperoxide formation decreased in the order α->γ->δ-tocopherol at 100 ppm, but the reverse order occurred at 1000 ppm (Table 9.9). Apparently, α- and γ-tocopherols have different optimal concentrations in the particular fish oil substrate tested. With soybean oil stripped of tocopherols by short-path molecular distillation, the relative potency based on conjugated diene at optimal concentrations (shown in parentheses) decreased from α- (~100 ppm) > γ- (~300 ppm) > δ-(~1900 ppm) tocopherol.

Tocopherols can behave as antioxidants or prooxidants depending on the test system, the concentrations, the stages of oxidation tested and the methods used to follow oxidation. Initially, α-tocopherol inhibited hydroperoxide formation at 100 ppm, but promoted hydroperoxide formation at 750 and 1000 ppm

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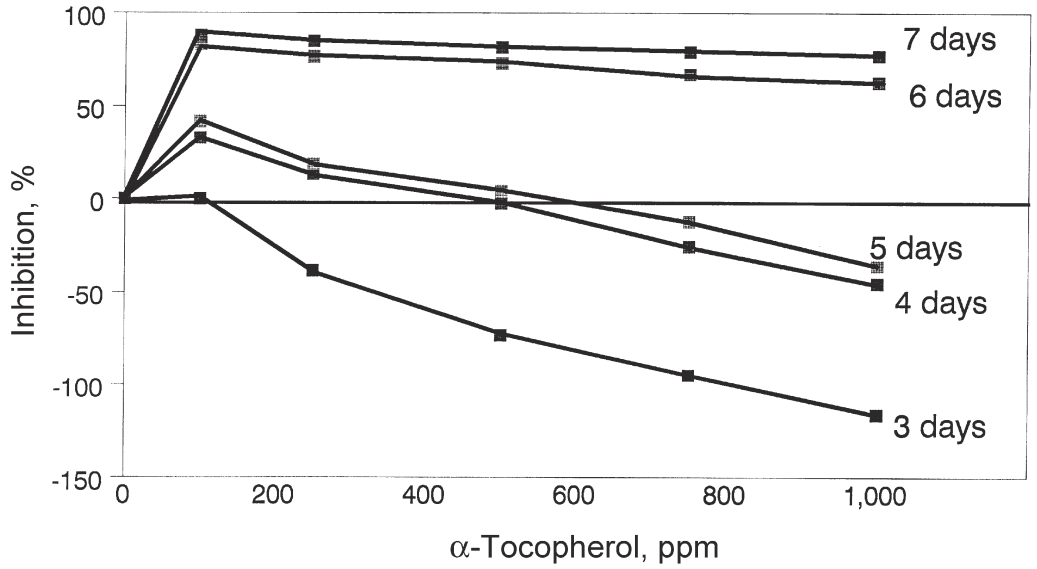


Figure 9.7. Effect of different concentrations of α-tocopherol on the inhibition of hydroperoxide formation at different stages of oxidation of bulk corn oil. From Frankel *et al.* (1994).

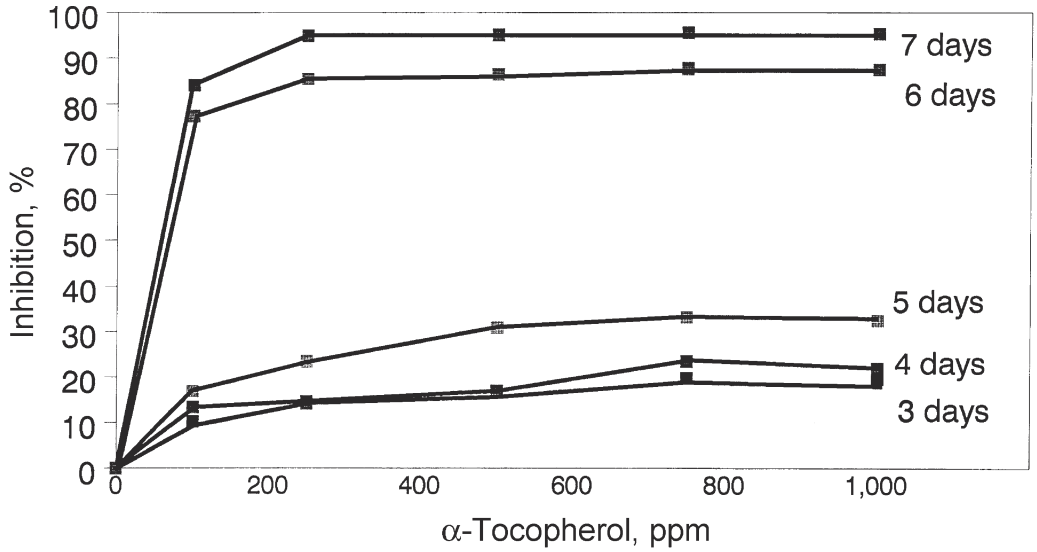


Figure 9.8. Effect of different concentrations of α-tocopherol on the inhibition of hexanal formation at different stages of oxidation of bulk corn oil. From Frankel *et al.* (1994).

(Figure 9.7, 3–5 days). At later stages of oxidation (6–7 days), α-tocopherol inhibited hydroperoxide formation at all concentrations tested. After 7 days, α-tocopherol showed its highest inhibition of hydroperoxide formation at 100 ppm. In mixtures, whether tocopherols acted as antioxidants or prooxidants seemed to depend on the relative concentration of α-tocopherol. In contrast to hydroperoxide formation, α-tocopherol inhibited hexanal formation at all levels tested (Figure 9.8). Inhibition of hexanal formation increased with

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concentrations of α-tocopherol and with oxidation time. After 6 and 7 days, hexanal formation was almost completely inhibited at all concentrations of α-tocopherol tested. The inhibition of hexanal formation by tocopherol mixtures was also more effective at higher concentrations. The same trends were observed in corn oil-in-water emulsions. Although α-tocopherol at high concentrations promoted hydroperoxide formation, its ability to inhibit hexanal formation improved at increasing concentrations. The ability of antioxidants to inhibit hexanal formation may thus be more relevant to flavor development.

In contrast to α-tocopherol, γ-tocopherol inhibited hydroperoxide and hexanal formation at all levels tested. The inhibition was significantly higher at 250–500 ppm and lower at 100 ppm in both bulk corn oil and in the corresponding oil-in-water emulsions. γ-Tocopherol had no prooxidant activity in either bulk oil or emulsion systems (Table 9.1). In bulk corn oil, δ-tocopherol, which is present in significant amounts in soybean oil (Table 9.5), did not show prooxidant activity at higher concentrations (1000–2000 ppm). At these concentrations, δ-tocopherol was also more effective than α- and γ-tocopherols in inhibiting the decomposition of hydroperoxides. With mixtures of soybean tocopherols, maximum antioxidant activity was observed between 250 and 3000 ppm. The prooxidant activity of tocopherols can be explained by the chain transfer reactions (–4) and (9), which become significant at high concentrations of α-tocopherol. Tocopherol oxidation by reaction (8) is also greater for α-tocopherol than for γ-tocopherol.

The effect of different lipid substrates has a significant impact on the activity of different antioxidants according to their hydrophilic or lipophilic character. For example, α-tocopherol (Figure 9.1) is a lipophilic antioxidant that behaved quite differently in various lipid substrates from its carboxylic acid analog Trolox, which is hydrophilic. In bulk corn oil triacylglycerols, Trolox was a better antioxidant than α-tocopherol, but the opposite trend was observed in

Table 9.10. Percent inhibition of hydroperoxides and hexanal formation by α-tocopherol and Trolox in bulk corn oil and corn oil-in-water emulsion at 60°C[a]

Antioxidants	Bulk oil		Emulsion	
	Hydroperoxides	Hexanal	Hydroperoxides	Hexanal
Corn oil				
+ α-Tocopherol				
150 μM	15	35	26	54
300 μM	9	40	88	75
+ Trolox				
150 μM	30	6	15	2
300 μM	84	88	13	–27

[a]From Huang *et al.* (1996). Hydroperoxides and hexanal measured after 6 days of oxidation in bulk corn oil, and after 4 days in corn oil emulsion. Negative values represent prooxidant activity.

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the corresponding oil-in-water emulsions (Table 9.10). In emulsified methyl linoleate, Trolox behaved the same way as in corn oil, and was less effective than α-tocopherol. However, in emulsified linoleic acid, Trolox was a better antioxidant than α-tocopherol. Linoleic acid constitutes a unique substrate because of its tendency to form mixed micelles (Figure 10.1).

The physical states of lipid systems affect the distribution of antioxidants and influence their activity. α-Tocopherol and Trolox exhibit complex interfacial properties between air–oil and oil–water interfaces that significantly affect their relative activities in different lipid systems (see Chapter 10). In the bulk oil system, the hydrophilic Trolox is apparently more protective by being oriented in the air–oil interface (Figure 10.8). In the emulsion system, the lipophilic α-tocopherol is more protective by being oriented in the oil–water interface. Because of its tendency to form micelles, linoleic acid is not an appropriate lipid for testing antioxidants since their behavior in this substrate would be significantly different from that in foods composed mainly of triacylglycerols.

2. *Ascorbic acid*

This organic acid is easily oxidized by one-electron transfer to produce the resonance-stabilized ascorbyl radical ($AH^{\bullet}$), which readily dismutates into a mixture of ascorbic (AH) and dehydroascorbic acid (DHA) (Figure 9.9). The ascorbyl radical is a strong acid (pK 0.45) and at pH 7 exists as a radical anion ($A^{\bullet -}$). In reducing unsaturated lipid radicals one electron is transferred to the free radical from AH^-, producing the anion radical $A^{\bullet -}$. Aqueous solutions of AH^- contain low concentrations of the anion radical producing a 1:1 mixture of AH and DHA.

Ascorbic acid is known for its complex multi-functional effects, as an antioxidant, a prooxidant, and a metal chelator, reducing agent or as an oxygen scavenger. A combination of these effects may predominate in many food applications. In aqueous media, ascorbic acid is active as an antioxidant at relatively high concentrations (approximately 1 mM), but it can act as a prooxidant at lower concentrations (approximately 0.01 mM), especially in the presence of metal ions. At a given concentration, there is a "crossover effect"

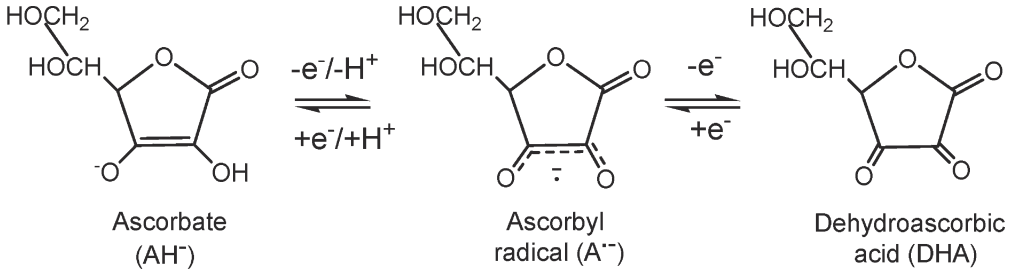


Figure 9.9. Oxidation and dismutation of ascorbic acid.

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where the effect of ascorbic acid switches from pro- to antioxidant. The concentration of ascorbic acid at which this crossover effect occurs varies widely according to the presence and relative concentration of catalytic metals. Ascorbic acid can both chelate Fe^{3+} and reduce it to the much more catalytically active Fe^{2+}, in the initiation and homolytic decomposition of hydroperoxides (Chapter 4). Ascorbic acid interacts with Cu^{2+} also to accelerate the decomposition of linoleate hydroperoxides.

In soybean oil which had been treated with carbon black to remove most of the natural tocopherols, ascorbic acid was more effective as an antioxidant than α-tocopherol, BHA, BHT and propyl gallate, but less effective than citric acid during oxidation at 60°C (Table 9.6). However, the temperature of oxidation markedly influenced the relative antioxidant activity of ascorbic acid (Table 9.3). Ascorbic acid was less effective at 45°C and more effective at 98°C than ascorbyl palmitate.

Ascorbic acid acts as an efficient reducing agent by regenerating antioxidants and increasing their effectiveness. The mixture of ascorbic acid and α-tocopherol thus shows strong synergism, an effect that has been known for a long time and has been explained by different mechanisms. During oxidation, α-tocopherol produces tocopheroxyl radical intermediates that can be readily reduced by ascorbic acid and regenerate α-tocopherol (Figure 9.4). This redox mechanism has been confirmed by electron spin resonance (ESR), a technique that provides direct evidence for the tocopheroxyl and ascorbyl radicals. Another mechanism involves the metal inactivation properties of ascorbic acid. Tocopherols are inactivated in the presence of metals and their antioxidant activities can be greatly improved in the presence of metal inactivators (citric acid in Table 9.8).

The synergism exhibited by the ternary mixture of α-tocopherol, ascorbic acid and phospholipids has been shown to be due to the stabilization of α-tocopherol, on the basis of ESR studies with methyl linolenate oxidized at 90°C to detect the free radicals of α-tocopherol and ascorbic acid. Evidence was obtained by this technique for the formation of nitroxide radicals ($R\text{-}N\text{-}O^{\bullet}$) in the presence of phosphatidylserine or phosphatidylethanolamine or soybean lecithin and oxidized methyl linolenate. However, as pointed out earlier (Section C), the synergistic activity of this ternary mixture may be derived from antioxidant products formed from the phospholipids at elevated temperatures by the Maillard browning reaction (Chapter 11).

The antioxidant activity of ascorbic acid requires the 2-and 3-hydroxyl groups to be unsubstituted. We observed previously that α-tocopherol behaved differently from its carboxylic acid analog, Trolox, in bulk corn oil triacylglycerols compared to the corresponding oil-in-water emulsions. The same behavior is observed when ascorbic acid is compared to ascorbyl palmitate. Although esterification of the 5- or 6-hydroxyl increases the lipid solubility of ascorbic acid, the resulting esters are less active as antioxidants in bulk oil

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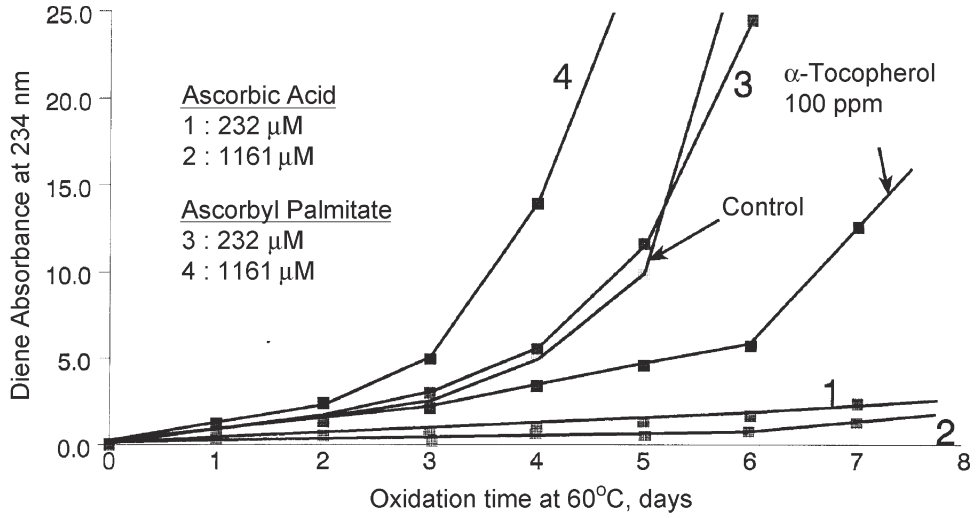


Figure 9.10. Effect of ascorbic acid, ascorbyl palmitate and α-tocopherol on the oxidative stability of bulk corn oil. Adapted from Frankel *et al.* (1994).

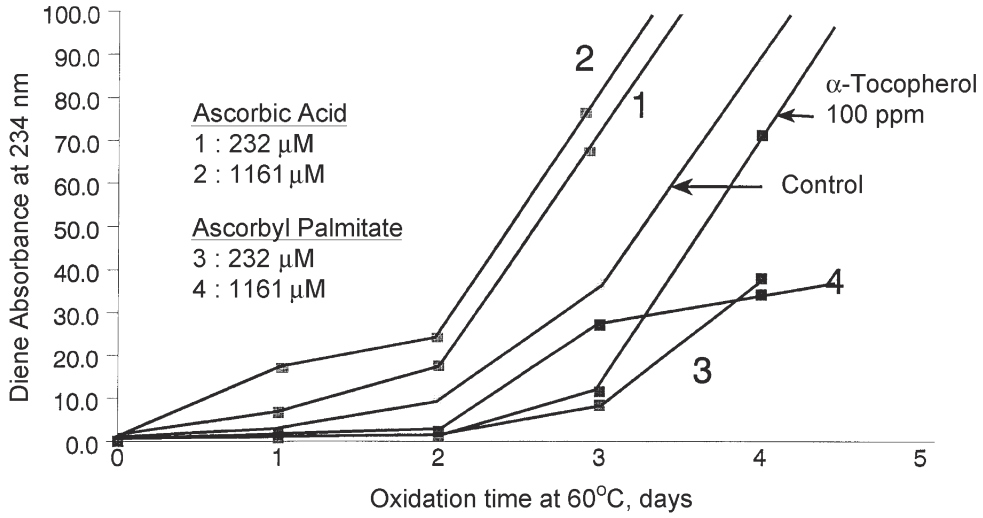


Figure 9.11. Effect of ascorbic acid, ascorbyl palmitate and α-tocopherol on the oxidative stability of corn oil-in-water emulsion. Adapted from Frankel *et al.* (1994).

than the free ascorbic acid. The hydrophilic ascorbic acid was a more effective antioxidant in bulk corn oil than in emulsified corn oil. In contrast, the lipophilic ascorbyl palmitate was a more effective antioxidant in the corn oil emulsion than in bulk corn oil (Figures 9.10, 9.11). In the bulk oil system, the hydrophilic ascorbic acid is apparently more protective by being oriented in the air–oil interface. In the emulsion system, however, the lipophilic ascorbyl palmitate is more protective by being oriented in the oil–water interface (Figure

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10.8). This interfacial phenomenon provides an explanation for the so-called "*polar paradox*", which is discussed in more detail in Chapter 10.

In aqueous solution, ascorbic acid is a good oxygen scavenger. After shaking for 24 hours, an ascorbic acid solution consumed oxygen at a ratio close to the theoretical value of 3.3 mg per cm^3 of headspace. By its oxygen scavenging activity, ascorbic acid is effective in protecting flavor compounds in wine, beer, fruits and vegetables against oxidation. However, anaerobic conditions are necessary for the effectiveness of ascorbic acid because it is readily decomposed in aqueous solution in the presence of air. Ascorbyl palmitate is too insoluble in water to function as an oxygen scavenger.

Ascorbic acid reduces other food components including nitrous acid (HO-NO) and oxidized phenolic compounds. The reduction of nitrous acid with high concentrations of ascorbic acid (>1000 ppm) to nitric oxide occurs during curing of meat and prevents the formation of N-nitrosamine, considered to be mutagenic. The reduction of quinones by ascorbic acid to regenerate phenolic compounds is important in preventing the enzymatic browning reactions and in restoring the antioxidant activity of polyphenolic compounds by a synergistic mechanism [Section 2 (d)].

In summary, the multiple effects of ascorbic acid include: (i) hydrogen donation to phenolic antioxidant radicals to regenerate the antioxidant; (ii) metal inactivation to reduce the rate of initiation and decomposition by metals; (iii) reduction of hydroperoxides to stable alcohols; and (iv) oxygen scavenging in aqueous systems.

3. Rosemary and other spice extracts

Rosemary extracts provide a major source of natural antioxidants used commercially at present in foods. The antioxidant activity of commercial rosemary extracts is mainly related to its content of two phenolic diterpenes, carnosic acid and carnosol, which are also found in sage. Carnosic acid has a structure consisting of three six-membered rings including a dihydric phenolic ring, and a free carboxylic acid (Figure 9.12). Carnosol is a derivative of carnosic acid containing a lactone ring. Rosemary and other herbs and spices are commercially extracted by using organic solvents (e.g. hexane and ethanol) and and deodorized or bleached. More recently, rosemary concentrates have been obtained by supercritical extraction with liquid carbon dioxide. They are available commercially in the form of powders, paste, or liquids. Some formulations contain various proprietary carriers such as propylene glycol, medium chain triacylglycerols or vegetable oils.

In bulk corn oil, the rosemary extract, carnosic acid, rosmarinic acid and α-tocopherol were significantly more active than carnosol (Table 9.11). In contrast, in corn oil-in-water emulsion, carnosol was significantly more active than in bulk oil, whereas the rosemary extract and carnosic acid were less active

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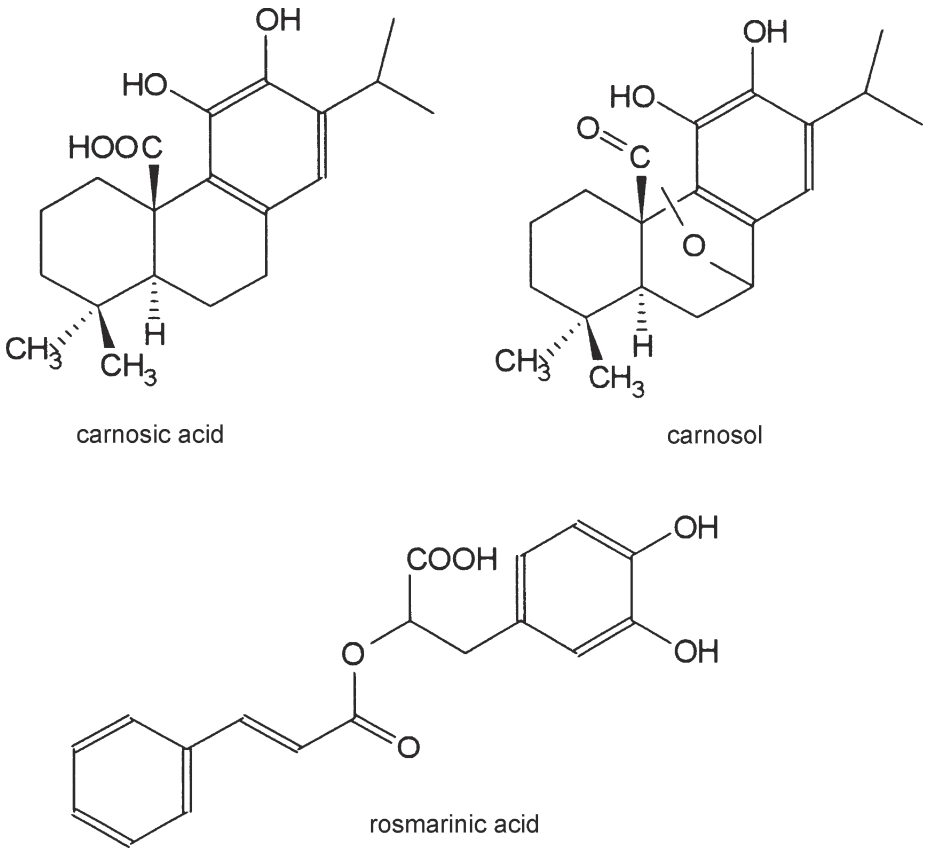


Figure 9.12. Structures of rosemary antioxidants: carnosic acid, carnosol, and rosmarinic acid.

and rosmarinic acid showed prooxidant activity. The polar hydrophilic rosemary compounds were less active in emulsion systems because they undergo interfacial partitioning into the water thus becoming less protective than in the bulk oil system (see Chapter 10).

Table 9.11. Antioxidant activity of a rosemary extract, carnosic acid, carnosol and rosmarinic acid in stripped corn oil and emulsified corn oil at 60°C (% inhibition)[a]

Samples	Hydroperoxides		Hexanal	
	Bulk oil (6 days)	Emulsion (4 days)	Bulk oil (6 days)	Emulsion (4 days)
Rosemary extract[b]				
250 ppm	64	11	–3	35
500 ppm	81	12	98	28
Carnosic acid, 50 ppm	70	37	29	83
Carnosol, 50 ppm	6	42	–20	84
Rosmarinic acid, 50 ppm	68	–11	78	–53

[a]From Frankel *et al.* (1996a). Negative values represent prooxidant activity.
[b]Contains 10.3% carnosic acid and 4.5% carnosol.

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The antioxidant activities of rosemary extracts, carnosol and carnosic acid were also significantly influenced by the oil substrates and the type of system tested, bulk oils versus oil-in-water emulsions, the methods used to measure oxidation and the concentration of test compounds. The rosemary extracts, carnosol and carnosic acid effectively inhibited hydroperoxide formation in corn oil, soybean oil, peanut oil and fish oil, when tested in bulk (Table 9.12). The rosemary extract and pure constituents were more active antioxidants in bulk corn, peanut and fish oils than in bulk soybean oil. This difference may be attributed to the relatively higher concentrations of tocopherols in soybean oil that are known to have a negative effect on the antioxidant activity of rosemary constituents. Test compounds also inhibited hexanal formation in bulk vegetable oils, and propanal and pentenal formation in bulk fish oils. In marked contrast, these test compounds were either inactive or promoted oxidation in the corresponding vegetable oil-in-water emulsions. In fish oil emulsions, however, the rosemary compounds inhibited conjugated diene and pentenal formation, but not propanal.

Interfacial phenomena may explain these differences in activities. Rosemary extracts, carnosol and carnosic acid behaved like other hydrophilic antioxidants such as ascorbic acid and Trolox in being more effective in bulk oil than in oil-in-water emulsion systems. In the bulk oil systems where oil is the main phase, the hydrophilic rosemary antioxidants may be more protective by being oriented in the air–oil interface. In contrast, in the oil-in-water emulsion systems, where water is the main phase, the hydrophilic rosemary antioxidants remain in the water and are less effective in the oil–water interface where oxidation takes place (Chapter 10). The higher antioxidant activities of rosemary antioxidants observed in fish oil emulsions than in vegetable oil emulsions may be explained by their greater affinity toward the more polar oil interface with the water of the fish oil systems.

In sunflower oil oxidized at 60°C a rosemary concentrate (36.3% carnosic acid and 4.8% carnosol) showed strong synergistic activity in inhibiting hydroperoxide formation when mixed with ascorbyl palmitate and weak synergistic activity with citric acid. α-Tocopherol had no effect or a slightly negative effect on the activity of the rosemary concentrate (Table 9.13). In bulk corn oil, pure carnosic acid and carnosol were more active antioxidants than α-tocopherol and in mixtures α-tocopherol had no effect or a slight negative effect on their antioxidant activity.

Although carnosic acid and carnosol are readily oxidized at 60°C and higher temperatures, their antioxidant activities are maintained. The oxidation products formed are apparently active antioxidants at high temperatures and protect oils during frying. They also have carry-over activity by protecting the fried foods. This carry-over activity can be attributed to the formation of various oxidation and isomerization products. Indeed, the radical [2,2'-azobis-(2,4-dimethylvalerate)] initiated or iron-catalysed oxidation of carnosic acid in the

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Table 9.12. Percent inhibition of hydroperoxide formation by rosemary extracts (R-E), carnosol and carnosic acid in bulk oils and oil-in water emulsions[a]

Samples	Corn oil		Soybean oil		Peanut oil		Fish oil	
	Bulk	Emulsion	Bulk	Emulsion	Bulk	Emulsion	Bulk	Emulsion
R-E:300 ppm	83.9	–81.2	55.0	–211	90.1	–17.7	78.9	25.3
R-E:500 ppm	89.3	–11.9	76.3	–59.6	94.8	–107	88.0	42.1
Carnosol, 30 ppm	38.4	–165	32.6	–102	37.3	–135	16.6	–2.5
Carnosol, 50 ppm	51.0	–27.5	34.0	–189	65.5	–136	10.3	20.3
Carnosic, 30 ppm	74.4	–29.6	5.5	–131	87.6	–107	28.0	36.1
Carnosic, 50 ppm	87.7	–14.9	70.5	–97.2	91.7	–104	45.7	32.7

[a]From Frankel *et al.* (1996b). Oxidation of vegetable oils were carried out at 60°C for 20 days in bulk and for 12 days in emulsions; oxidation of fish oil were carried out at 40°C for 10 days. Negative values represent prooxidant activity.

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Table 9.13. Effect of a rosemary concentrate and mixtures with ascorbyl palmitate, citric acid and α-tocopherol on the oxidation of sunflower oil at 60°C[a]

Inhibitors	Stabilisation Factor	Synergism (%)
Rosemary concentrate[b]	2.14	–
Ascorbyl palmitate (AP)	1.84	–
Citric acid (CA)	1.16	–
α-Tocopherol (Toc)	0.86	–
Rosemary + AP	5.50	56.0
Rosemary + CA	2.34	2.6
Rosemary + Toc	1.80	–0.2

[a]From Hraš *et al.* (2000). Stabilisation factor = IP with inhibitor/IP control, where IP = induction period; Synergism = (Ipm – Ipc) – (Ipi – Ipc)/(Ipm – Ipc)] × 100
where Ipm, Ipc and Ipi = induction periods with mixtures, with control, with inhibitor, respectively.
[b]Concentrate contained 36.33% carnosic acid and 4.79% carnosol.

presence of methyl linoleate produced an *ortho*-quinone and a hydroxy-para-quinone (Figure 9.13). Although these quinone products had weak antioxidant activity, their solution in acetonitrile or methanol showed strong activity upon standing for 4 hours at 37°C. This activity was shown to be due to the isomerization product carnosic 8-lactone, the redox product rosmanol and related diterpene quinones (Figure 9.13). Although these products were produced under artificial conditions, similar and related products may account for the carry-over antioxidant activity of rosemary extracts when added to oils under frying conditions (Chapter 12). Phenolic compounds are highly reactive and oxidize under different conditions into a wide range of quinones, dimers and aldehydes. The relative contribution of these complex oxidation derivatives produced under normal storage conditions to antioxidant activity is not yet well understood.

Plant extracts obtained mechanically with either medium-chain triacyl-

Table 9.14. Antioxidant activity of plant extracts (1%) obtained in different carriers in bulk and emulsified corn oil systems[a]

Plant extracts	MCT[b] carrier		PG[b] carrier	
	Bulk	Emulsion	Bulk	Emulsion
Rosemary	1.7	12	1.7	32
Sage	1.5	4	1.5	19
Thyme	1.1	1.3	1.2	8.4
Oregano	1.0	1.2	1.1	7.0
Cloves	1.0	8.9	1.0	13

[a]From Löliger *et al.* (1996). Activity values expressed as "antioxidant index" (AI) = induction period of oil + antioxidant/induction period of control oil.
[b]MCT = medium chain triglycerides, PG = propylene glycol.

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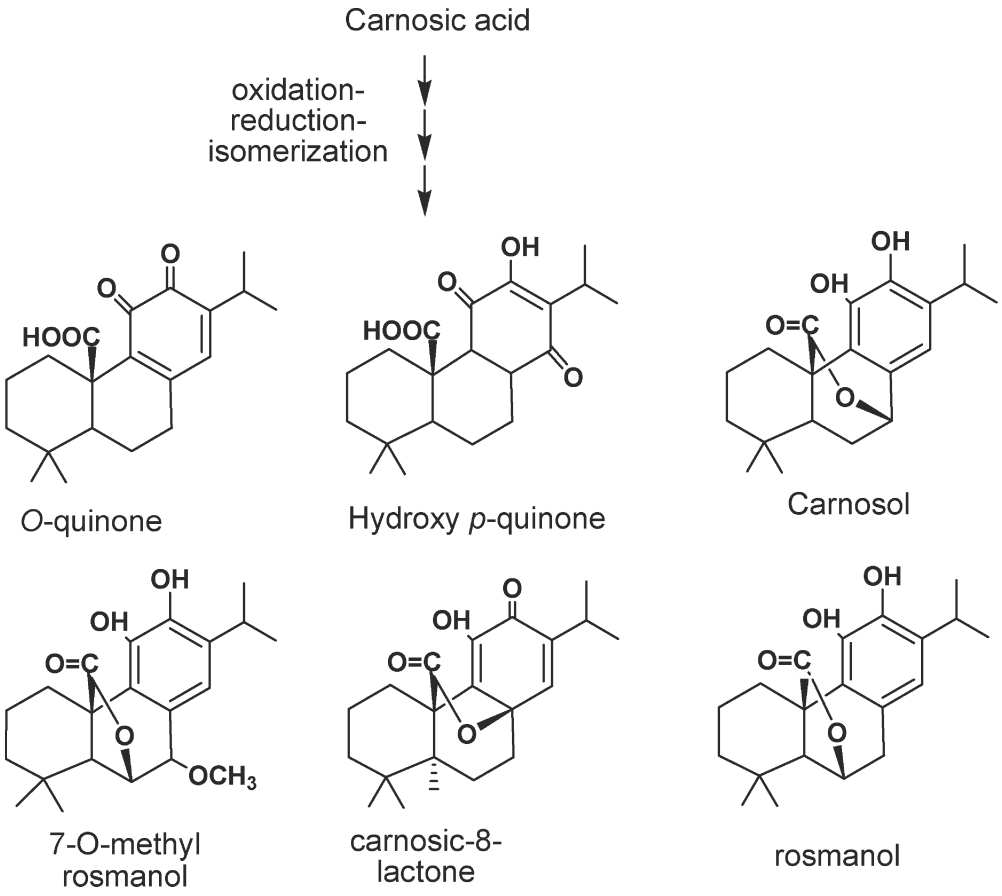


Figure 9.13. Products of oxidation, reduction and isomerization of carnosic acid. From Masuda *et al.* (2002).

glycerols or propylene glycols exhibited different antioxidant activities and were more active in corn oil emulsion than in bulk corn oil (Table 9.14). In the emulsion system, the rosemary extract in propylene glycol was more active than the extract in medium chain triacylglycerols. The higher activity of the rosemary extract with propylene glycol was attributed to the presence of more polar substances such as rosmarinic acid and flavonoids.

Rosemary extracts are also particularly effective in stabilizing dried oats, meat products and roasted nuts. The activities of natural antioxidants are thus very system-dependent and their effectiveness in different real food systems is difficult to predict.

4. Flavonoids and green tea catechins

Flavonoids are among the most active and extremely diverse natural antioxidants found in plant foods. Their basic structures have a flavan nucleus (2-phenyl-benzo-γ-pyrane), consisting of two benzene rings (A and B) combined

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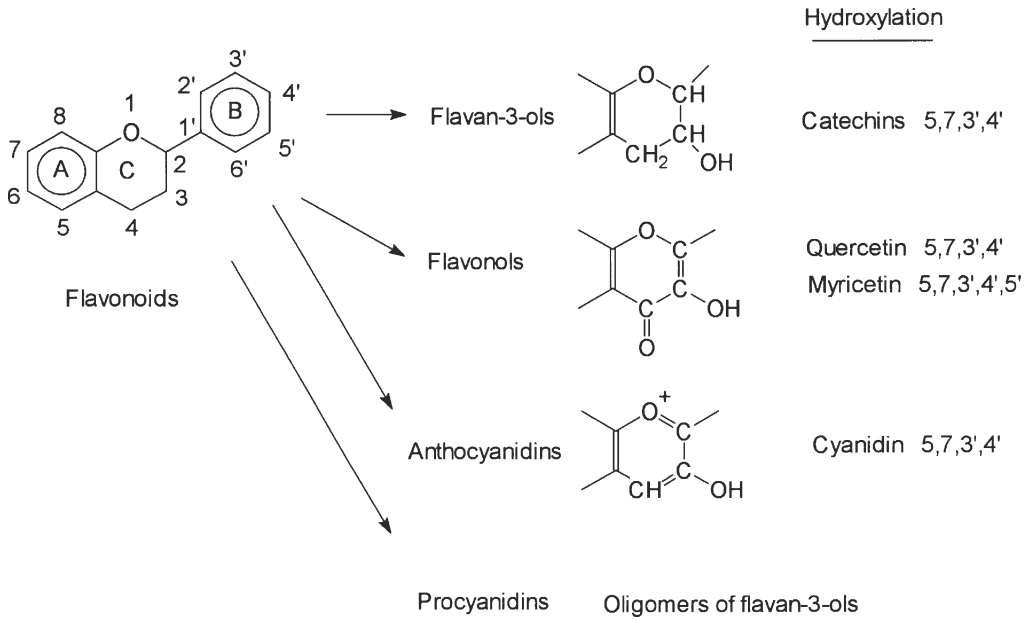


Figure 9.14. Structures of flavonoids.

by an oxygen-containing pyran ring C (Figure 9.14). Differences in substitution on ring C define the different classes of flavonoids, which include flavan-3-ols, containing a saturated C ring with a methylene at position 4 (e.g. catechins with OH on 5,7,3',4'-positions); flavonols, containing a keto group at position 4 in ring C (e.g. quercetin with OH on 5,7, 3',4'-positions, myricetin with OH on 5,7,3',4',5'-positions generally glycosylated); anthocyanidins, having lost the oxygen at carbon-4 of ring C to form fully aromatic flavylium compounds, which exist as cation-forming salts with organic acids (e.g. cyanidin with OH on 5,7,3',4' and glucoside on the 3-position); and procyanidins, which include oligomers of flavan-3-ols. Additional flavonoids found in fruits contain a very large and diverse number of glycosides, which arise from glycosylation on the 3 and 7 positions.

Flavonoids are recognized for both their antioxidant activity and their metal chelating properties. Antioxidant activity is closely related to hydroxylation of the 5,7 positions of the A-ring, 3,4'-dihydroxylation of the B-ring flavone, and 3- and 5-hydroxylation in ring C (Table 9.15). Additional structures that determine antioxidant activity include the 2,3 double bond conjugated with the 4-oxo function causing electron delocalization from the B ring. The metal chelation properties of flavonoids are attributed to two points of coordination between metals and the flavonoid molecule: the *ortho*-diphenol (3',4'-diOH-) structure in ring B and the ketol (3-OH-4-keto or 5-OH-4-keto) structures in ring C (Figure 9.14). Metal complexing is also possible with an *ortho*-quinol grouping in B-ring. The importance of metal chelation activity of flavonoids is supported by studies showing greater inhibition of metal ion-induced oxidation than peroxyl radical-induced oxidation.

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Table 9.15. Antioxidant activities of flavonols (0.01%) in lard oxidized at 60°C [a]

Flavones (Figure 9.14)	I.P.[b]
Control	90
3,3',4-Trihydroxy-	145
3,7,3',4'-Tetrahydroxy-	320
3,5,7,3',4'-Pentahydroxy-[c]	410
3,7,8,3',4'-Pentahydroxy-	555
3,5,7,3',4',5'-Hexahydroxy-[d]	685
3,5,6,7,3',4'-Hexahydroxy-	698

[a]From Mehta and Sheshadri (1959).
[b]I.P. = induction period in hours at 60°C to reach a peroxide value of 25.
[c]Quercetin.
[d]Myricetin.

Flavonoids produce phenoxy radicals that exhibit a much lower reduction potential (540 to 700 mV) and can thus inactivate various reactive oxygen species with higher potentials (950 to 2000 mV). Several antioxidant mechanisms have been considered for flavonoids in food and biological systems, including scavenging lipid radicals ($ROO^{\bullet}$ and $RO^{\bullet}$) and reactive oxygen species ($HO^{\bullet}$, $O_2^{-\bullet}$, $^1O_2$), inactivating catalytically active metal ions by chelation, and complexing with proteins (enzymes, apoprotein of low-density lipoproteins, metal binding sites of enzymes). Flavonoids also exhibit synergism by reducing and regenerating oxidized α-tocopherol and ascorbic acid, and partitioning according to their polarity to become distributed at different oxidation sites. By chelating one or two copper atoms, flavonoids inhibit copper-activating enzymes including ascorbic acid oxidase, polyphenol oxidase, hyaluronidase, lysine oxidase, lysosomal hydrolases and ATPases. Additional biological properties of flavonoids will be discussed in Chapter 13.

Much work has been published on evaluations of natural flavonoids in various lipid systems, but most evaluations were done either at elevated temperatures, under AOM or Rancimat conditions, or were based on the unspecific bleaching of β-carotene by co-oxidation with linoleic acid. Unfortunately, from the results of these assays, the usefulness of flavonoids as antioxidants is difficult to predict for foods containing mainly triacylglycerols and stored under ambient or near ambient conditions (Chapter 8).

Extracts of different grapes were tested for their antioxidant activities in a copper-catalysed lecithin liposome system (Table 9.16). Extracts of red wine grape varieties contained higher concentrations of total phenolic compounds and higher relative antioxidant activities (67–100%) than the white wine and table grapes (25–27%). The relative percent inhibition of conjugated dienes and hexanal correlated with total phenols. Anthocyanins were the most abundant

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Table 9.16. Antioxidant activities and phenolic composition (mg/l) of grape extracts in lecithin liposome oxidized at 37°C in the presence of 3 μM copper acetate[a]

Grape	Type	Total phenols	Inhibition, %		Hydroxy cinnamates (as caffeic acid)	Anthocyanins (as malvin)	Flavonols (as rutin)
			Hydro-peroxides	Hexanal			
Petite Sirah	RW	1115	100	100	10.4 (0.6)	1685 (98)	19.8 (1.1)
Cabernet Sauvignon	RW	565	71	47	0.8 (0.1)	640 (98)	9.3 (1.4)
Merlot	RW	569	67	46	5.3 (1.1)	437 (93)	26.8 (5.7)
Sauvignon Blanc	WW	264	43	25	6.5 (39)	0	10.0 (61)
Chardonnay	WW	230	27	18	1.4 (25)	0	4.2 (75)
Red Globe	BT	179	26	15	2.7 (11)	19.6 (79)	2.6 (10)
Thompson seedless	WT	198	25	15	3.8 (30)	0	8.9 (70)

[a] From Yi *et al.* (1997). Abbreviations: RW, red wine; WW, white wine; BT, blush table; WT, white table. Total phenols expressed as gallic acid equivalents/l. Values in parentheses are the relative percentage of each compound. The extract of Petite Sirah grapes was the only sample containing flavan-3-ols (10.6 mg/l as catechin).

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Table 9.17. Antioxidant activities and phenolic composition of berry extracts in lecithin liposome oxidized at 37°C in the presence of 3 μM copper acetate[a]

Berry extracts (10μM GAE)	Total phenols (mg/l)	Inhibition, %		Hydroxy cinnamates (as caffeic acid)	Anthocyanins (as malvin)	Flavonols (as rutin)	Flavan-3-ols (as catechin)
		Hydro-peroxides	Hexanal				
Sweet cherry	805	68.8	92.6	133	40	18	25
Red raspberry	3030	51.8	74.2	3	2200	19	480
Blackberry	4350	41.8	67.8	15	7650	83	108
Blueberry	3480	38.1	77.1	316	4840	139	70
Strawberry	2940	27.4	60.5	27	786	78	126
Catechin (pure)	–	57.4	41.1	–	–	–	–

[a]From Heinonen *et al.* (1998). Extracts obtained with 70% acetone. Total phenols expressed as gallic acid equivalents/l. The cherry and strawberry extracts also contained 2.5 and 2.1 mg/kg respectively of benzoates (as gallic acid).

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Table 9.18. Inhibition of hydroperoxides and hexanal by green tea extracts in bulk and emulsified corn oil at 50°C[a]

Samples[b]	Conc.[c] (ppm)	Bulk oil		Oil-in-water emulsion	
		Hydro-peroxides	Hexanal	Hydro-peroxides	Hexanal
G. tea extract I	98	–28	7	–42	–23
G. tea extract II	112	46	29	–45	–46
G. tea extract III	120	31	44	–66	–53
G. tea extract IV	155	52	3	–145	–93
Catechin	125	88	29	–	–

[a]From Frankel *et al.* (1997). Negative values represent prooxidant activity.
[b]Samples contained equivalent of approximately 125 ppm of total catechin.
[c]Total catechin concentration. Concentrations of tea extracts used in emulsions were about ten fold less than in the bulk systems.

phenolic compounds in extracts of red grapes and flavonols were most abundant in extracts of white grapes. Extracts of berries showed high antioxidant activity in a lecithin liposome system by inhibiting hydroperoxide and hexanal formation (Table 9.17). When the extracts were diluted and compared at the same molar concentration of total phenols, the relative antioxidant activity decreased in the order: sweet cherries > red raspberries > blackberries > blueberries > strawberries. In contrast to the grape extracts, the total phenolic content of berries did not correlate with their antioxidant activity, emphasizing the need of further characterization of the antioxidants present in berries.

Green tea extracts consist of a complex mixture of catechin gallates, including (+)-catechin, (+)-gallocatechin, (–)-epicatechin, (–)-epicatechin gallate, (–)-epigallocatechin, and epigallocatechin gallate (Figure 9.15). Tea catechins showed different trends in relative antioxidant activity in different lipid systems. In corn oil oxidized at 50°C, epigallocatechin, epigallocatechin gallate and epicatechin gallate were better antioxidants than epicatechin and catechin (Figure 9.16). However, in the corresponding corn oil-in-water emulsions, the tea catechins were prooxidant as were propyl gallate and gallic acid. In contrast, in lecithin liposomes, epigallocatechin gallate was the best antioxidant, followed by epicatechin, epigallocatechin, epicatechin gallate and catechin. Different commercial green teas were active antioxidants in bulk corn oil oxidized at 50°C, but were prooxidant in the corresponding oil-in-water emulsions (Table 9.18). Green teas were also active antioxidants in soybean lecithin liposomes oxidized at 37°C in the presence of a copper catalyst. More inhibition of hexanal than hydroperoxide formation was observed, suggesting that the green tea catechins may act by effectively inhibiting alkoxyl radicals formed as precursors of aldehydes [see Reaction (11)].

The marked variation in activity among green tea samples may be due in part to differences in their relative partition between phases in different lipid

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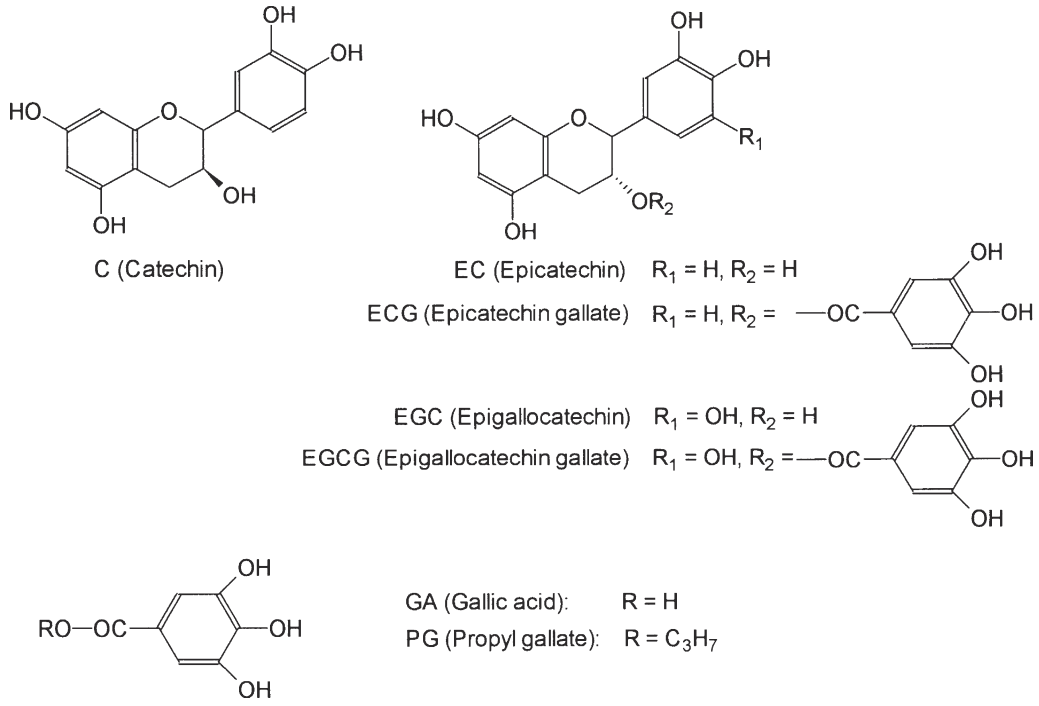


Figure 9.15. Structures of green tea catechins and reference compounds gallic acid and propyl gallate.

systems. The improved antioxidant activity observed for green teas in lecithin liposomes compared to corn oil emulsions was explained by the greater affinity of the polar tea catechin gallates with the polar surface of the lecithin lamellar bilayers (see Chapter 10), thus affording better protection against oxidation. Liposomes may thus be appropriate lipid models to evaluate hydrophilic antioxidants for foods containing phospholipids.

E. Evaluation of natural antioxidants in plants

An explosion of research has emerged on the antioxidant properties of phytochemicals based on the potential health benefits of fruits and vegetables that may be due to their antioxidant components. Although natural antioxidants from plant extracts are generally multifunctional, a wide range of one-dimensional methods has been developed to evaluate their activity. Several protocols have been developed for measuring the free radical-trapping activity of antioxidants. These methods use a wide variety of free radical generating systems and methods of inducing oxidation and measuring end-point of oxidation (Table 9.19). The validity of these test methods may be questionable because they do not take into account the complexity of antioxidant actions (see Section B). The results obtained confuse the interpretation of antioxidant activity. Although the DPPH and TEAC methods are commonly used to test the activity of antioxidants in plant materials, they are subject to significant interference

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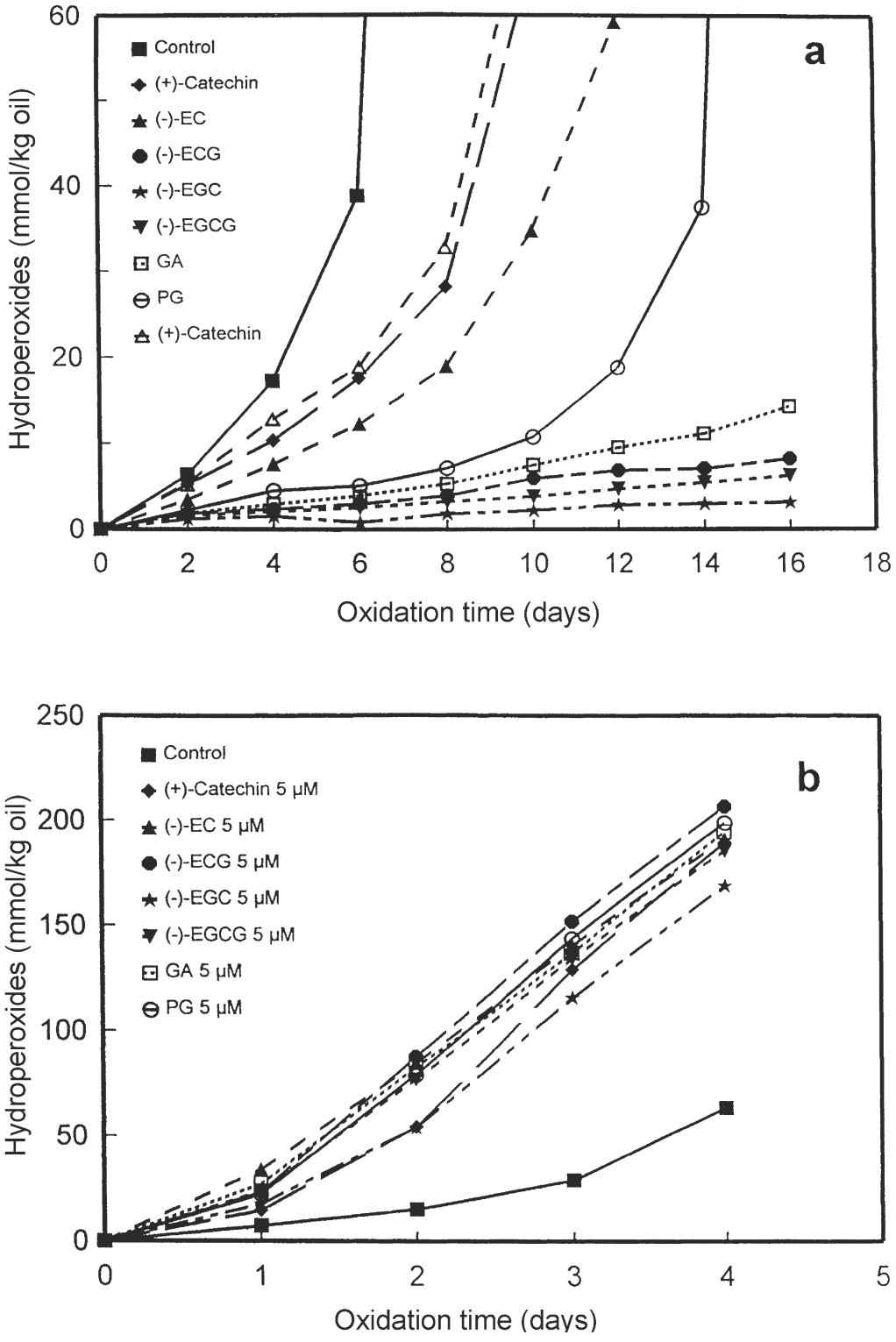


Figure 9.16. Antioxidant activity of tea catechins in (a) bulk corn oil and (b) corn oil-in-water emulsion. Huang and Frankel (1997), with permission from the American Oil Chemists' Society. See structures and abbreviations in Figure 9.15.

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Table 9.19. Free radical trapping methods to evaluate natural antioxidants[a]

Methods[b]	Principles	Limitations	References
DPPH	Decay of artificial radical chromogen at 515 nm	No substrate, not specific, interference from other radicals and plant pigments	Blois (1958), Arnao (2000)
TRAP	Decay of artificial radical in presence of linoleic acid, electrode end-point or luminol chemiluminescence	Not suitable to test lipophilic antioxidants and metal chelators, not sensitive	Wayner *et al.* (1985, 1986)
TEAC	Decay of artificial radical chromogen at 734 nm by ferrylmyoglobin, H_2O_2, peroxidase, or K persulfate	No substrate, interference from antioxidants, not specific	Miller *et al.* (1993, 1995), Rice-Evans and Miller (1994)
ORAC	AAPH induced oxidation of β-phyco-erythrin, fluorescence decay curve	Non specific substrate, Protection of photoreceptor not equal to protein protection	Delange and Glazer (1989), Glazer (1990) Cao *et al.* (1993)
FRAP	Ferric chromogenic complex reduction at 593 nm	No substrate, Not specific measure of reducing capacity	Benzie and Strain (1996)
Superoxide	Scavenging of $O_2^{\bullet -}$ generated by xanthine oxidase	Not specific, no equilibrium when $O_2^{\bullet -}$ is generated continuously	Costantino *et al.* (1992)

[a]From Frankel and Meyer (2000). DPPH: uses 2,2-diphenyl-1-picrylhydrazyl radical; TRAP: total radical-trapping parameter, uses peroxyl radicals generated by 2,2'-azobis(2-amidinopropane) hydrochloride (ABAP); TEAC: Trolox equivalent antioxidant capacity, uses radical cation 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonate) ($ABTS^{\bullet +}$) produced continuously by reacting either with ferrylmyoglobin, H_2O_2 and peroxidase or with K persulfate; ORAC: oxygen radical absorbance capacity, based on fluorescence produced by oxidation of β-phycoerythrin induced by 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH); FRAP: ferric reducing antioxidant power, by reducing ferric tripyridyltriazine complex to the ferrous complex.

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Table 9.20. Lipid oxidation methods to evaluate natural antioxidants[a]

Methods[b]	Principles	Limitations	References
β-Carotene-linoleic acid	Bleaching of β-carotene in linoleic acid emulsified with Tween-20, monitored by carotene destruction (460 nm)	Not specific, influenced by physical micelle properties of emulsion, high ratio of Tween-20/linoleic acid, inappropriate substrate	Kanner *et al.* (1977)
SDS emulsion of linoleic acid	Linoleic acid micelle oxidation induced by AAPH monitored by conjugated diene (234 nm)	Not specific, influenced by physical micelle properties of emulsion, not real oil-in-water emulsion, artificial inducer, inappropriate substrate	Pryor *et al.* (1988, 1993)
Methyl linoleate	Dodecane solution oxidized at 110°C with O_2 bubbling, monitor residual linoleate	Not sensitive, significant thermal decomposition of hydroperoxides, favors lipophilic antioxidants, inappropriate substrate, not sensitive or specific	Cuvelier *et al.* (1990, 1992)
Methyl linoleate	Oxidized at 40°C in bulk or oil-in-water emulsions, monitor conjugated diene (234 nm), 72 hr oxidation	Inappropriate substrate, one time point oxidation	Kähkönen *et al.* (1999, 2001, 2003)
Various lipids	Oxidized at 50 or 98°C, monitor peroxide value	Not specific measure of reducing capacity	Benzie and Strain (1996)
Corn oil (stripped of tocopherols)	Oxidized at 40–60°C, in bulk or oil-in-water emulsions, monitor conjugated diene (234 nm) and hexanal	Relatively slow oxidation, difficulty in removing tocopherols from triglycerides	Huang *et al.* (1994), Frankel *et al.* (1994, 1996, see Figures 9.7, 9.8, Table 9.11)
Lecithin liposome	Oxidized at 37°C, in bulk or oil-in-water emulsions, monitor conjugated diene (234 nm) and hexanal	Difficulty in preparing reproducible liposome	Yi *et al.* (1997, see Table 9.16), Heinonen *et al.* (1998, see Table 9.17)

[a]Adapted from Frankel and Meyer (2000).

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with plants containing colored compounds (anthocyannins, carotenoids and other pigments). Colored samples cause smaller absorbance decreases resulting in significantly lower antioxidant activity measured. The scavenging effect of the TEAC assay based on one reaction time changes according to the structure of flavonoids containing different number of hydroxyl groups. The ORAC and other methods using artificial radical inducers are also of limited value. As discussed previously [Section A.1(c)], trace metals in foods are the most important catalysts of both oxidation and decomposition, whereas artificial diazo compounds work by initiating only the formation of hydroperoxides but have no effect on their decomposition. Many important questions required for the evaluation of antioxidants cannot be answered by chromogenic radical methods. These methods do not reflect the true protective properties of antioxidants; they provide no information on what food substrates are oxidized and what products are inhibited, what is the location of the antioxidant in a food system, what is the effect of interacting components, and what conditions are relevant to real food applications (see Section B).

Several protocols are used to test antioxidants in foods by oxidizing different lipid substrates under standard conditions and assessing activity by various methods to determine how much oxidation is inhibited (Table 9.20). Many of these test systems overlook compositional and interfacial phenomena concerning charge and solubility of multiple components in real food systems that have significant effects on antioxidant performance. As we shall see in Chapter 10, the relative activities of various antioxidants vary widely according to the site of action in different systems, the charge and solubility of components in micelles compared to emulsions and liposome systems (Table 10.10). Valid *in vitro* protocols to test the activity of plant antioxidants require careful design to obtain mechanistic information by employing relevant substrates and oxidation inducers.

The activity and mechanism of multifunctional natural antioxidants are affected by many factors including the particular system used, the oxidation conditions, the kinetics and the composition of the system. Other factors in multiphase systems include the partitioning properties of the antioxidants between lipid and aqueous phases, the oxidation conditions, and the physical state of the oxidizable substrate (Chapter 10). The influence of all these parameters cannot be evaluated by using only one-dimensional protocols. The conditions used to accelerate oxidation are of particular importance, including the temperature, the use of metal catalysts or initiators, the surface of exposure and the use of light of varying intensity (Chapter 7). Finally, the results of evaluation tests are influenced by the specificity and methods employed to analyse the progress of oxidation, including the degree of oxidation chosen as end-point for testing. The antioxidant capacity of phytochemicals based only on their scavenging ability towards artificial or inappropriate radical chromogens provides no useful information on which lipid or other substrate is protected.

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The recent literature shows a wide divergence of results on natural antioxidants in foods based on simplistic and shortcut methodology. More valid and specific antioxidant protocols are urgently needed to define what products are formed and inhibited depending on conditions, systems and targets of protection. Methods to evaluate natural antioxidants recommended in Section B include using a relevant specific oxidizing substrate to be protected, measuring the correct extent of oxidation and inhibition, choosing an appropriate end-point of oxidation, and testing a range of concentrations to determine any optimum level and any possible adverse prooxidant effects from the antioxidants tested.

F. Antioxidant enzymes

Enzymes have been evaluated as new types of natural antioxidants in some food applications. Enzymes can be used beneficially to remove oxygen and reactive oxygen species and to reduce lipid hydroperoxides. Glucose oxidase coupled with catalase is the best-known commercially available system to remove oxygen from foods. Glucose oxidase utilizes oxygen by catalysing the oxidation of β-D-glucose to produce 2-δ-gluconolactone and hydrogen peroxide. The gluconolactone is spontaneously hydrolysed to D-gluconic acid, and the hydrogen peroxide can be removed by catalase. As legally permitted for food use in the US, this glucose-oxidase system has been applied in fruit juices, mayonnaise and salad dressings to increase shelf life and prevent off-flavor development. In the presence of superoxide, an active species of oxygen (see Chapter 12), superoxide dismutase produces oxygen and hydrogen peroxide. When used in conjunction with catalase, to remove hydrogen peroxide, superoxide dismutase had antioxidant activity in a milk fat model system oxidized with iron. However, this system was not effective in food emulsions containing edible oils. Glutathione transferase was shown to reduce linoleic acid hydroperoxides in the presence of glutathione, but the usefulness of this enzyme was not demonstrated in a food system.

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CHAPTER 10

Oxidation in multiphase systems

Lipid oxidation in multi-component foods is an interfacial phenomenon affecting prooxidant or antioxidant constituents that is greatly influenced by the interactions between the lipid and water components and the interface between them. Chapter 9 discussed how the relative activities of lipophilic and hydrophilic antioxidants vary between bulk oil systems and oil-in-water emulsions. The classical mechanism of inhibited lipid oxidation does not predict the changes in antioxidant effectiveness between solutions and emulsion systems. The same interfacial phenomena influence the effects of prooxidant metal catalysts, which can exist either in the free form or as complexes with proteins. Model systems have been used extensively to simulate foods or biological samples in research on lipid oxidation and its control. Although research on model systems is important for the development of chemical principles that would be applicable to many foods, working with a variety of model systems other than the "real" foods can be misleading by oversimplifying the interfacial interactions of multiple components.

The term "interfacial oxidation" refers to the complex interaction between constituents in multiphase lipid systems in either promoting or inhibiting lipid oxidation. Interfacial oxidation is a "surface" reaction dependent on the rate of oxygen diffusion and its interactions with unsaturated lipids, metal initiators, radical generators and terminators, all of which are distributed in different compartments of colloidal systems.

Lipid oxidation is not well understood in systems in which the fat is dispersed as emulsion droplets. This interfacial oxidation affects a large number of foods, which exist partially or entirely in the form of emulsions. Examples include milk, cream, cheese, mayonnaise, margarine, butter, ice cream, soups, sauces and baby foods. For a better understanding of the lipid oxidation in multiphase systems, the following basic questions should be considered:

a) How does the physicochemical environment of the lipids affect the mechanism of lipid oxidation?
b) What are the differences between lipid oxidation in bulk fats and in emulsified fats?
c) How are prooxidant and antioxidant constituents in multiphase systems related to their concentrations in different phases?
d) What are the effects of surface-active compounds on the concentrations and interactions of prooxidants and antioxidants at oil–water interfaces and their activities?

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Knowledge on the sites of prooxidant and antioxidant action in multi-component systems is essential to better predict the oxidative stability of complex foods and biological systems. Foods of improved quality may be developed if the association and driving forces of prooxidants and antioxidants can be controlled in multiphase systems.

A. Nature of colloidal systems

An emulsion consists of two liquids such as oil and water in which one is dispersed in the other in the form of small droplets. Emulsions are thermodynamically unstable, because energy is required to increase the surface area between the oil and water phases. The dispersion of one liquid into another is achieved by the energy obtained from vigorous or ultrasonic stirring, or from homogenization. A mixture of oil and water is homogenized by forcing the mixture through a small valve opening to create small oil droplets. However, on standing with time the dispersion will separate into layers of different densities. To prepare emulsions that are kinetically stable on standing for a reasonable time, it is necessary to use emulsifiers, also referred to as surfactants, which are surface-active substances that have an affinity for the surface of droplets by forming a protective film or membrane covering the droplets to prevent them from aggregating on standing. Emulsifiers are "amphiphilic" molecules (contain both hydrophilic and hydrophobic moieties) consisting of a polar group oriented in one phase, and a non-polar group oriented in the other phase. In milk, the native milk fat globules can be decreased in size by homogenization under pressure to eliminate flocculation and cream formation.

We may consider four classical types of emulsion consisting of a distinct continuous phase and a discontinuous or dispersed phase (Figure 10.1):

i. *Oil-in-water emulsions* in which the oil droplets are dispersed in the continuous water phase. This dispersed phase is stabilized by an emulsifier composed of a lipophilic or hydrophobic part and a hydrophilic part. The lipophilic part consists of a long chain alkyl residue with good solubility in the oil phase. The hydrophilic part consists of a dissociable group or a number of hydroxyl groups with good solubility in the water phase. In an immiscible oil-in-water system, the emulsifier is located at the interface, where it decreases the interfacial tension, and facilitates a fine dispersion of one phase in the other. Common food emulsifiers include surface-active proteins (casein, whey, soy and egg), phospholipids (soy or egg lecithin), or surfactants (polyoxyethylene sorbitan monoesters named 'Tweens', sorbitan fatty acid esters named 'Spans' or fatty acids) (Figure 10.2). Examples of oil-in-water food emulsions include milk, mayonnaise, salad dressing, cream and soups.

ii. *Water-in-oil emulsions* in which water droplets are dispersed in the continuous oil phase. To stabilize water-in-oil emulsions, we need an emulsifier

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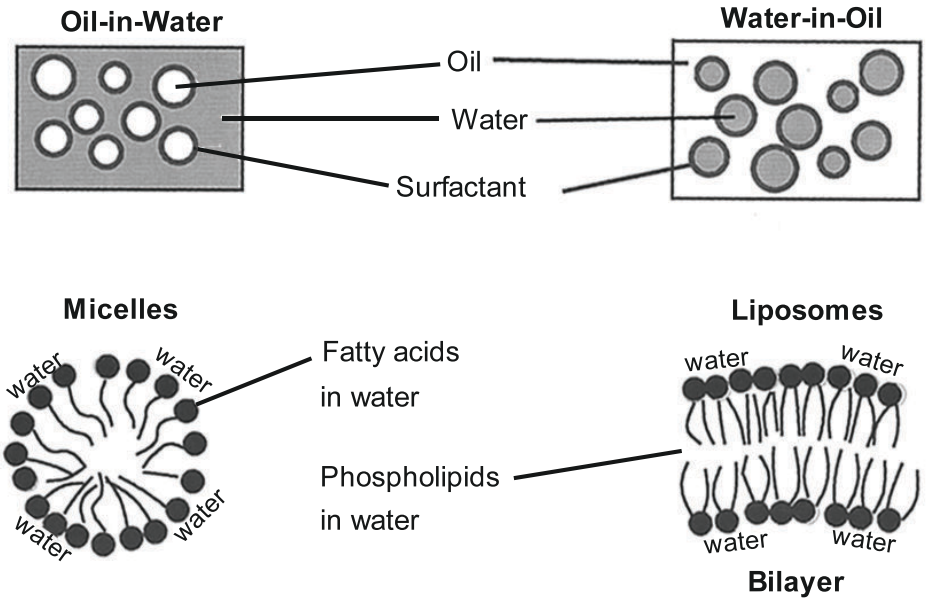


Figure 10.1. Four classical types of emulsion consisting of a distinct continuous phase and a discontinuous or dispersed phase.

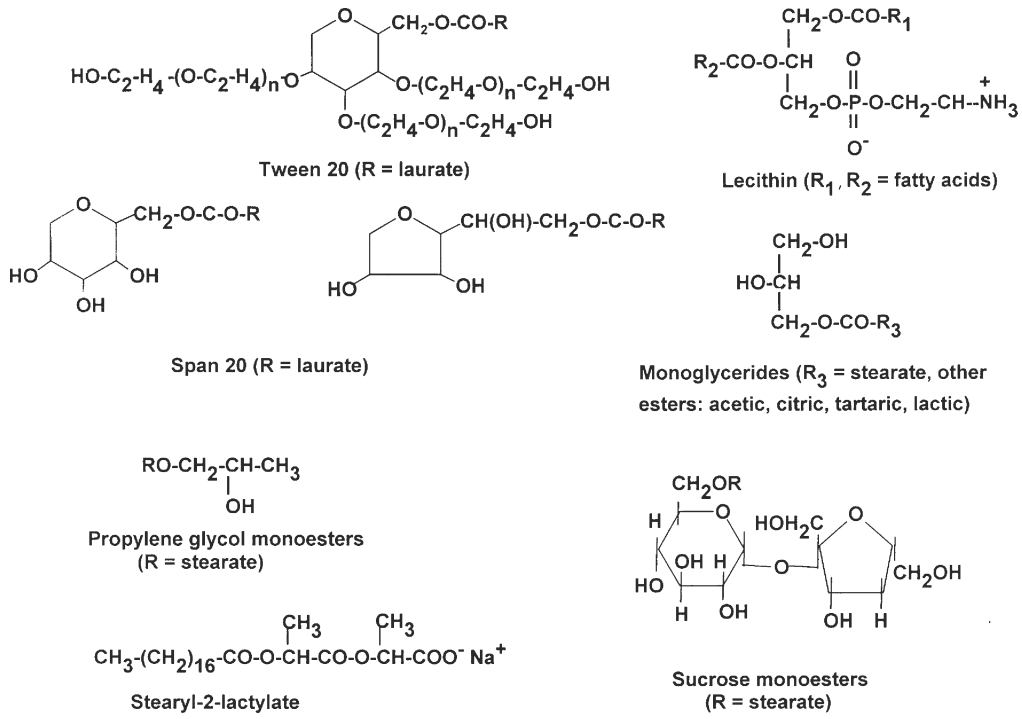


Figure 10.2. Common food emulsifiers: Tweens* (polyoxyethylene mono fatty acid esters of sorbitan or sorbitol anhydrides), Spans* (partial fatty acid esters and sorbitol anhydrides), lecithin (phosphatidylcholine), monoglycerides (monoacylglycerols), propylene glycol mono fatty acid esters (1,2-propanediol esters), sucrose mono fatty acid esters, stearyl-2-lactylate. *Tween/Span 20, monolaurate; Tween/Span 40, monopalmitate; Tween/Span 60, monostearate.

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with a relatively stronger hydrophilic group and weaker lipophilic group, and predominantly soluble in the water phase (examples: monoglycerides and Spans) (Figure 10.2). Examples of water-in-oil food emulsions include butter, margarine and spreads. Butter and margarines are unique water-in-oil emulsions because they are stabilized by the presence of crystals in the continuous phase.

iii. *Micelles*. When free fatty acids are dispersed in water they become arranged in the form of micelles, in which the polar head-groups lie mainly in the aqueous phase, and the non-polar tails lie mainly in the oil phase. This arrangement minimizes the free energy (or work content) of the system. Emulsified fatty acids can be either charged or ionic (example: sodium dodecyl sulfate, SDS) or uncharged (example: Tweens). Emulsified fatty acids can also be solubilized in surfactant micelles such as Tweens (Figure 10.3). Micelles can undergo rapid dynamic changes in size and shape involving the entering and leaving of emulsifier molecules within their structure.

Fatty acids ↔ Fatty acid micelles ↔ Emulsifier micelles ↔ Emulsifier

At high oil concentrations, the ternary mixture of oil, water and emulsifier can also exist as "inverted" micelles with the hydrocarbon chains of fatty acids or emulsifier (e.g. Tweens) located on the outside and the polar head-groups in the middle (Figure 10.3).

Micellar systems of free fatty acids are not common in foods, which are

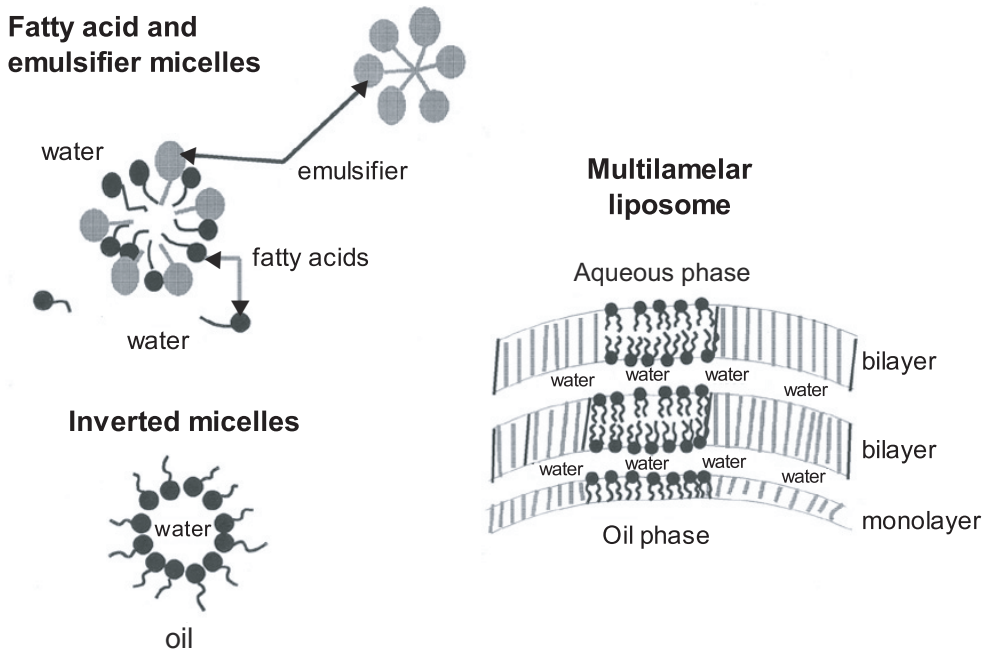


Figure 10.3. Structural pictures of emulsified fatty acids (with Tween 20), multilamellar liposome of aqueous lecithin and triglycerides, and inverted micelles.

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composed mainly of triacylglycerols or phospholipids. However, the presence of small amounts of micellar free fatty acids and moisture can greatly influence the oxidative stability of various foods, by solubilizing and facilitating the transfer of non-polar molecules and their reactions in the aqueous phase. Emulsification of triacylglycerols in the presence of small amounts of free fatty acids leads to the formation of mixed micelles and emulsions. These mixed colloidal systems may form during fat frying, and may either promote lipid oxidation by the interactions of micellar free fatty acids with metal catalysts, or retard it by the interactions with hydrophilic antioxidants (Chapter 12, Section C).

iv. *Liposomes*. When dispersed in water, phospholipids spontaneously form multi-layers consisting of bilayers between the water phase and the innermost layer next to the oil phase being in the form of a monolayer (Figures 10.1 and 10.3). The surface-active phospholipid molecules arrange themselves with the non-polar tails located away from the water, and the polar head groups located in contact with the water. "Hydrophobic interactions" between acyl tails of phospholipids are the major forces that lead to bilayer arrangements. These systems are commonly used to serve as models of meat and fish products, as well as cell membranes. The lipid bilayers of membranes containing phospholipids are well protected against oxidation by lipophilic antioxidants such as tocopherol. These lipophilic antioxidants can be regenerated by reaction with reducing agents such as ascorbic acid dissolved in the water phase. The interactions between mixtures of lipophilic and hydrophilic compounds provide efficient antioxidant systems in foods and biological materials (Section C.2).

B. Food lipid emulsions

Oil-in-water emulsions are stabilized by ionic emulsifiers, which are oriented on the interface with alkyl residues solubilized in the oil droplets, and charged end groups oriented in the aqueous phase (Figure 10.4). Counter ion groups form an electrostatic double layer, which prevents aggregation of the oil droplets. Nonionic emulsifiers are oriented on the surface of the oil droplets with the polar end projected into the aqueous phase. An anchored hydrated layer is formed around the polar groups thus stabilizing the droplets by preventing their coalescence. Nonionic emulsifiers are most commonly used because they do not lose their surface activity by interaction with salt in foods.

The various molecules in an emulsion system become distributed according to their polarity and surface activity between the different phases, which include the oil phase, the water phase and the interfacial region. The interface is a narrow region between the phases that consists of a mixture of oil, water and emulsifier (Figure 10.5). Non-polar molecules are distributed mainly in the oil

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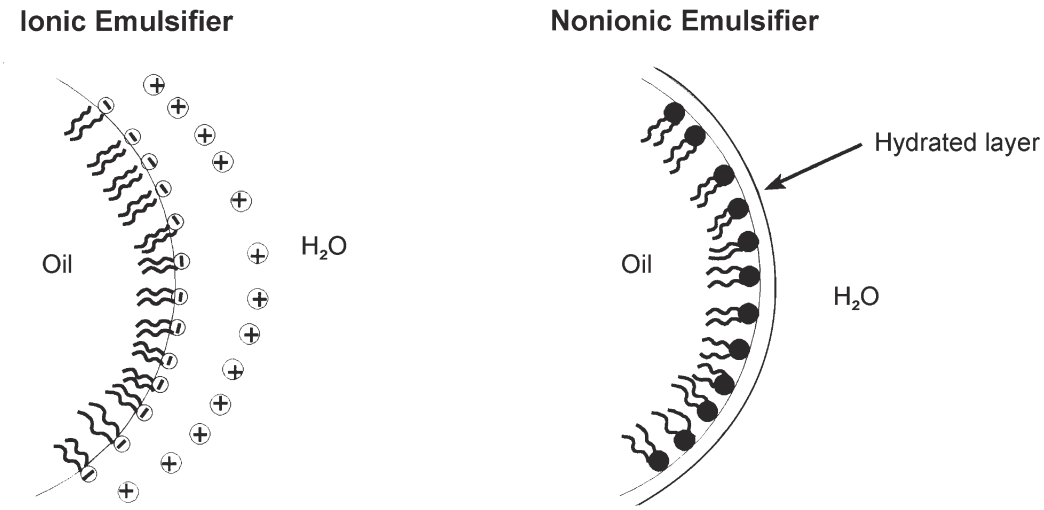


Figure 10.4. Stabilization of oil-in-water emulsion with an ionic emulsifier by electrostatic double layer, and with a nonionic emulsifier by formation of hydrated layer. Adapted from Belitz and Grosch (1987).

phase, polar molecule in the water phase, and amphiphilic molecules in the interface. Any surface-active solutes including proteins, phospholipids, surfactants, alcohols and solid particles tend to concentrate at the interface and may interact with the lipid components. The resulting mixture of oil, water and emulsifier becomes more chemically reactive at the interface than in the separate phases. The nature and interactions of the components of the interface can therefore significantly affect the rate of lipid oxidation and stability of multiphase systems. The properties of emulsions are also affected by their dynamic nature because the droplets can undergo collisions, and the emulsifier molecules can exchange at different rates between the interface and either the water or the oil phases.

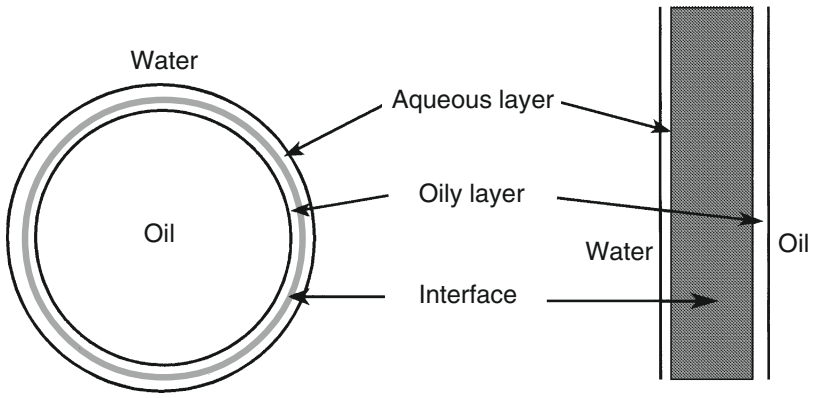


Figure 10.5. Diagrammatic representation of the interface components in an oil-in-water emulsion system. Interface = oil + water + emulsifier.

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Stable oil-in-water emulsions can also be obtained by dispersing polar lipids such as phospholipids into triglycerides and then emulsifying the oil in water. The presence of charged phosphatidylcholine components of phospholipids improves the stabilization of the emulsions. In most of these systems, the polar phospholipids form a separate phase at the interface where they form lamellar bilayers and a monolayer separated by triglyceride oil, between the outer water phase and the inner triglyceride oil phase (Figure 10.3).

1. Synthetic emulsifiers and surfactants

Numerous nonionic synthetic emulsifiers are used in the food industry. These materials are usually partial esters of medium or long-chain acids (C-12 to C-22) with polyhydric alcohols such as glycerol, propylene glycol, sucrose, or sorbitan (Figure 10.2). Monoglycerides are usually made by glycerolysis (transesterification) by heating triglyceride mixtures with glycerol (30% by wt) in the presence of an alkaline catalyst (sodium hydroxide or sodium methoxide), or by direct esterification of fatty acids and glycerol. Animal fats or palm oil or hydrogenated vegetable oils are used to increase the level of saturated fatty acids. The product is a mixture of mainly 1- and 3-monoglycerides (about 45–60%), diglycerides (about 36–43%) and triglycerides (about 6–12%). Commercial monoglycerides are available either as mixed glycerides (42–46%) or as concentrates following distillation (90% or higher plus 5% diglycerides). These commercial products are used to increase emulsion stability and volume and texture in breads, cake mixes, sauces, ice cream, frozen desserts, cereals, margarine and peanut butter. In doughs, monoglycerides interact with starch to increase dough strength and improve the texture of baked products.

The properties of monoglycerides can also be modified by esterification of one of the hydroxyl groups with acetic, lactic, fumaric, tartaric or citric acids. Similar emulsifiers are synthesised by reacting propylene glycol with saturated fatty acids to give a mixture of mono- and diacyl esters, which can be molecularly distilled to prepare concentrates containing about 90% propylene-glycol monostearate.

Other food emulsifiers are made by transesterification of fatty acid methyl esters with polyglycerols, sorbitan, sucrose and stearoyl lactate (Figure 10.2). Sugar, sorbitan fatty esters and polyoxyethylene sorbitan esters are used to stabilize oil-in-water food emulsions. Optimum emulsification is achieved with combinations of emulsifiers. Margarines are made with a combination of monoglycerides and lecithin, and cake mixes with a mixture of monoglycerides and propylene glycol monoesters.

2. Protein emulsifiers

Proteins are one of the most important natural food emulsifiers because they are

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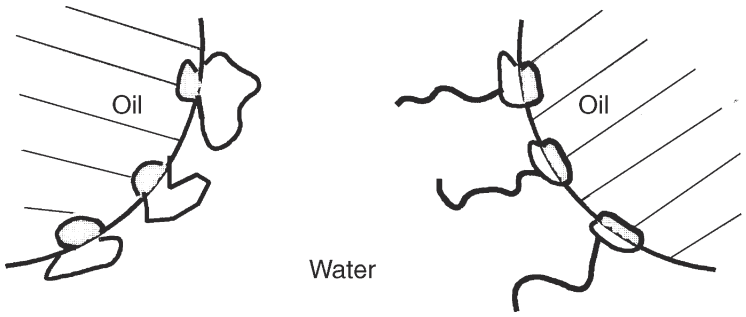


Figure 10.6. Schematic representation of adsorption of globular proteins such as β-globulin (left) and β-casein (right) at the interface of oil-in-water emulsions. Adapted from Larsson (1994).

surface-active and are effective in preventing coalescence of oil-in-water emulsions. Protein-stabilized oil-in-water emulsions are complicated because protein molecules have various conformations at the oil–water interface. Whey proteins such as β-lactoglobulin adsorb at the oil–water interface without changing their native conformation, whereas milk plasma proteins such as β-casein become unfolded and provide further stabilization by the presence of negative charges (Figure 10.6). These types of adsorption can either be reversible to different extents or be irreversible. The unfolding of certain proteins and exposing their peptide chain at the oil–water interface would be expected to greatly influence lipid oxidation in food emulsions (Chapter 11). Thermal denaturation causes the unfolding of proteins and exposing nonpolar amino acids and cysteine side groups forming disulfide bonds and changing the polarity of the interface.

Proteins can be displaced in food emulsions by other surface-active molecules or can exchange with other proteins. Homogenization of milk is an important process to control lipid oxidation by changing the interfacial composition of the fat globules. In the native state in raw milk, a complex mixture of lipoproteins, phospholipids, diacylglycerols, sterols and enzymes surrounds the fat globules. By homogenization, the milk fat membrane becomes resurfaced by adsorption of casein from the aqueous phase to form a protective layer that reduces oxidation. Lipid oxidation in emulsions can thus be inhibited to different degrees, according to the partitioning of proteins between the emulsion interface and the aqueous phase, the composition of the interfacial membrane and the conformation of the protein molecules at the surface (Figures 10.5 and 10.6). As discussed later (Section C. vi), when proteins are used as food emulsifiers, they have complex interfacial effects by either retarding or promoting lipid oxidation in oil-in-water emulsions.

3. Phospholipid emulsifiers

Phospholipids, also commonly referred to as lecithins (see Introduction), are

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important surface-active substances in the production of food emulsifiers, and are generally derived commercially either from soybeans or egg yolk. These substances and several derivatives are used generally in combination with other emulsifiers and stabilizing polymers, such as proteins, starches and gums, and with monoglycerides. For oil-in-water food emulsions, phospholipids are modified by acetylation or hydroxylation to increase their hydrophilic properties. The ethanol-soluble fraction of lecithin rich in phosphatidylcholine (about 65%) is suitable to stabilize oil-in-water emulsions, and the ethanol-insoluble fraction rich in phosphatidylethanolamine (about 33%) and phosphatidylinositol (about 63%) is suitable to stabilize water-in-oil emulsions.

Phospholipids are generally considered to have an antioxidant effect in foods, but this activity is confounded by the formation of reducing browning material at elevated temperatures (Chapter 9). Phosphatidylethanolamine appears to have synergistic activity in mixtures with natural tocopherols and synthetic antioxidants. This synergistic activity is often related to the metal scavenging ability of phospholipids.

4. Mixtures of emulsifiers

A mixture of emulsifiers can improve the stability of colloid food systems by strengthening the film membrane and by interaction between emulsifiers at the interface. Combinations of oil-soluble and water-soluble surfactants (Spans and Tweens) are often used in food formulations to produce more stable oil-in-water emulsions. Mixtures of phospholipids and proteins in foods undergo complex interactions during various thermal processing that can either promote or retard oxidation. At elevated temperatures phospholipids can produce antioxidant substances by the browning reaction, and proteins can denature to release reducing sulfhydryl groups that increase the oxidative stability of heated foods (e.g. evaporated milk).

C. Lipid oxidation in emulsions

Lipid oxidation in multiphase systems is greatly influenced by the nature of the interface, which in turn is affected by the composition of the oil, the water phases and surface-active solutes. In foods the membranes surrounding the emulsion droplets consist of surface-active substances or proteins or both. In addition to enhancing the physical stability of emulsion droplets, the film or membrane surrounding the droplets affect lipid oxidation processes in many ways.

i. *Protection of lipids*. The emulsifier provides a protective barrier to the penetration and diffusion of metals or radicals that initiate lipid oxidation. Food lipid radicals that can be generated by the action of metal catalysts in the

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aqueous phase markedly influence the oxidation of emulsions. Oxygenated decomposition products of lipid oxidation are more surface-active than the starting lipid and may become distributed in the interface and leach into the water phase. Hydroperoxides derived from partially oxidized phospholipids and emulsifiers become solubilized in the surfactant micelles and decrease the oxidative stability of emulsions. This "partitioning" of oxidation products promotes interactions with catalytic metals in the aqueous phase, and decreases the effectiveness of hydrophilic antioxidants (Chapter 9).

ii. *Emulsifier concentration*. The membrane increases the effectiveness of packing of emulsifier molecules at the interface. Higher concentrations of emulsifier can thus increase the oxidative stability of emulsified lipids against oxidation (Tables 10.1 and 10.2). Higher surfactant concentrations may cause tighter and thicker packing at the oil–water interface and the membrane becomes a more efficient interfacial barrier against diffusion of lipid oxidation initiators. At concentrations above the critical micellar concentration, a portion of polar emulsifiers, such as Tween-20, form micelles in the aqueous phase. These systems consist of an interface where the major part of the surfactant is located, and emulsifier micelles within the aqueous phase that are not located at the interface (Figure 10.3). Water-soluble and polar antioxidants can be solubilized effectively in bulk oil systems by formation of inverted micelles in the presence of low concentrations of moisture and emulsifiers such as lecithin. Ascorbic acid was thus solubilized effectively in oils in the presence of small amounts of water and emulsifiers such as lecithin. Synergistic effects were demonstrated with mixtures of ascorbic acid, lecithin and δ-tocopherol (Table 10.3). However, it is not clear whether the effects observed were due to solubilized ascorbic acid alone or due to the combination of ascorbic acid and lecithin necessary to prepare the inverted micelles. Phospholipids are generally useful as synergists in reinforcing the antioxidant activity of phenolic compounds (Chapter 9).

iii. *Sugars and amino acids*. Aqueous solutions of certain sugars and amino acids inhibit lipid oxidation in emulsions by scavenging free radicals in solution in the water phase. Some amino acids such as methionine, lysine and threonine retard lipid oxidation in emulsions, other amino acids such as histidine can promote lipid oxidation. However, these effects are very dependent on the type of emulsifier used, the presence of metals and the pH of the emulsion system. For example, the prooxidant activity of histidine was greatly accelerated in the presence of ferrous or ferric ions (Table 10.2). Phosphate buffers retarded lipid oxidation in these emulsions.

iv. *Effect of pH*. Lipid oxidation is affected by pH in oil-in-water emulsions and liposomes. In general, lipid oxidation is slowest at high pHs and the rate is accelerated as the pH decreases (Table 10.1). This acceleration may be due to

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Table 10.1. Effect of different factors on lipid oxidation of a safflower oil emulsion at room temperature[a]

Emulsifier %	Induction period[b]	pH	Induction period[b]	Sucrose %	Induction period[b]
0.5	22	6.2	14	0	3
1	28	7.2	25	17	3.5
2	38	8.2	49	33	5
4.0	47			50	10
				58	18
				67	40

From Sims *et al.* (1979).
[a]Emulsion contained 25% safflower oil homogenized with sodium stearoyl-2-lactylate.
[b]Induction period in days to absorb 20 meq oxygen per kg oil.

Table 10.2. Prooxidant effect of histidine on oxidation of methyl linoleate emulsions[a,b]

Histidine ± metal[a]	Induction period, hr[a]	Emulsifier[b]	Induction period, hr[b]	Concentration SDS[b]	Induction period, hr[b]
Control	38	Span 20	24	0.002 M	0.6
Cu^{2+}	18	Ethenoxylated tetradecanol	13	0.004 M	1.1
Fe^{2+}/Fe^{3+}	8	Tween 20	10	0.008 M	2.4
Histidine	3	K palmitate	1		
Histidine + Cu^{2+}	3	SDS	0.5		
Histidine + Fe^{3+}	1				
Histidine + Fe^{2+}	0				

From Saunders *et al.* (1962) [a] and Coleman *et al.* (1964).[b] Induction period in hr to absorb 20 mmoles oxygen per mole ester.
[a] Methyl linoleate (0.0024 mole) emulsified with 0.1% sodium dodecyl sulfate (SDS) oxidized at 30°C.
[b] Methyl linoleate (0.1 M) homogenized with different emulsifiers (0.002 M) in the presence of histidine (0.01 M) oxidized at 30°C.

Table 10.3. Effect of ascorbic acid solubilized in lecithin and water on antioxidant activity of δ-tocopherol in fish oil containing 0.1% water [a]

Sardine oil[b]	Induction period, days[c]
Control	2
+ L + 0.2% δ-tocopherol (LT)	6
+ LT + 0.01% AA	24
+ LT + 0.02 % AA	30
+ LT + 0.03 % AA	32

[a]From Han *et al.* (1991). Sardine oil oxidized at 30°C.
[b]Aqueous solutions of ascorbic acid (AA) were injected into the oil containing 0.3% lecithin (L).
[c]Estimated as days to reach a peroxide value of 10.

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solubilization of metal catalysts. Lipid oxidation is also affected to varying degrees by pH according to the charges of different emulsifiers, as discussed below.

v. *Electric charge of the membrane*. Electrostatic attraction or repulsion by the membrane environment influences significantly the effectiveness of prooxidants and antioxidants. The rate of oxidation is much higher in emulsions prepared with ionic emulsifiers (SDS, potassium palmitate) than with nonionic emulsifiers (Span 20, ethenoxylated tetradecanol, Tween 20) (Table 10.2). Oxidation is accelerated in the emulsions stabilized by anionic emulsifiers, such as SDS. In these emulsions, electrostatic attraction occurs between the negatively charged oil–water interface, and the positively charged metal ions present either as trace impurities or added. Metals in the water phase become hydrated and more reactive with polar hydroperoxides and water-soluble radicals (e.g. $^{\bullet}$OH, $^{\bullet}$OOH) at the oil–water interface.

vi. *Proteins*. As food emulsifiers, proteins have a complex interfacial effect on lipid oxidation. After saturating the oil–water interface, proteins at higher concentrations remain in the aqueous phase. Proteins can either retard or promote lipid oxidation in oil-in-water emulsions. By increasing the viscosity of the membrane surrounding the droplets, proteins may restrict the penetration and diffusion of radical initiators into the lipid phase. At certain concentrations when proteins are dispersed in the water phase, they may either scavenge free radicals or be preferentially oxidized and retard lipid oxidation. Proteins containing reducing sulfhydryl groups are particularly effective as antioxidants in milk, meat and fish. However, in the presence of metals some milk proteins may promote lipid oxidation (Chapter 11).

Various food proteins can inhibit oxidation of oil-in-water emulsions by several mechanisms, including metal neutralization by electrostatic repulsion between a positively charged interface and negatively charged metals or by metal binding, by physical separation to form a barrier, and by chemical reduction of hydroperoxides with sulfhydryl-containing amino acids. Thus, by lowering the pH of whey proteins below their isoelectronic point (pH 3.0) their protective activity could be increased, but the relative positive charge of the whey proteins did not correlate with their relative antioxidant activity (Table 10.4). Better correlation may be possible, however, by using purified rather than crude whey protein concentrates. Although the phosphoseryl residues of the phosphorus-enriched caseinophosphopeptides were expected to behave as metal cation chelators, they were less active in inhibiting lipid oxidation than the corresponding casein hydrolysate precursors. This trend may indicate that casein and casein hydrolysates may not only inactivate metals but also scavenge free radicals. The mechanisms to explain how these protein-stabilized emulsions inhibit lipid oxidation are still not well understood, however.

When oil-in-water emulsions of algae oil containing ω-3 PUFA stabilized

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Table 10.4. Relative activity of food proteins in inhibiting lipid hydroperoxide formation in oil-in-water emulsions

Oil-in-water emulsions	Food proteins
Salmon oil[a]	β-Lactoglobulin ≥ sweet whey > α-lactalbumin ≥ whey protein isolate
Corn oil[b]	Casein > whey protein isolate > soy protein isolate
Corn oil[c]	Casein hydrolysates > casein > P-enriched caseinophophopeptides

[a]From Hu *et al.* (2003a). Emulsion prepared with 5 wt% salmon oil, 0.2% protein, and 94.8% 5 mM acetate-imidazole buffer (pH 3.0). Sweet whey contained 12.1 wt% protein; whey protein isolate contained 97.6 wt% protein consisting of 55–61% β-lactoglobulin, 19–22% α-lactalbumin and 6–8% bovine serum albumin.
[b]From Hu *et al.* (2003b). Emulsion prepared with 5 wt% corn oil and 95% 5 mM acetate-imidazole buffer (pH 3.0) containing 0.2–1.5% proteins. Protein content and composition are the same as given by Hu *et al.* (2003a). The cationic charge of the whey protein isolate (measured as zeta potential) was higher (55.9 mV) than that of the casein (29.9 mV) and soy protein isolate (29.4 mV) and did not correspond to their relative antioxidant activity.
[c]From Díaz *et al.* (2003). Emulsion was prepared with 5% corn oil and 95% 10 mM imidazole-acetate buffer (pH 7.0), using 17 mM Brij 35 (polyoxyethylene lauryl ether) as emulsifier. Casein hydrolysates (91 P/N μg/mg) were obtained by trypsin hydrolysis of casein (52 P/N μg/mg), and 86% enriched caseinophophopeptides (237 P/N μg/mg) were prepared by precipitation of casein hydrolysates with $CaCl_2$ and ethanol.

with whey protein isolate were tested at pH 3 and pH 7, EDTA was the only effective metal chelator at concentrations higher than 1 μM (Table 10.5). At pH 3, less than the isoelectric point of the protein, tripolyphosphate and sodium citrate were prooxidant in accelerating both hydroperoxide and propanal formation, while hexametaphosphate inhibited hydroperoxide formation but promoted hexanal formation. At pH 7, similar results were obtained with EDTA and sodium citrate, and the phosphates had no effect on stability. The greater effectiveness of EDTA is due to its high ferric iron binding constant (1.3×10^{25} M), compared to that for citric acid (1.5×10^{11} M). EDTA can also inhibit lipid oxidation in emulsions by changing the redox potential, decreasing the

Table 10.5. Effect of different chelators on oxidative stability of algae oil-whey protein isolate-stabilized emulsions containing ω-3 polyunsaturated fatty acids[a]

Analyses (120 hours)	Control	TP	HP	SC	EDTA
Hydroperoxides (mmol/kg oil)	31	38	21	41	0
Propanal (μM)	75	100	110	82	25

[a]From Hu *et al.* (2004). Emulsions contained 5 wt% algal oil rich in ω-3 PUFA, 95% acetate-imidazole buffer (5 mM each, pH 3) and 0.2% whey protein isolate.
Experimental points were estimated from Figure 3 (Hu *et al.*, 2004).
Concentration of chelators, 10 μM, TP = tripolyphosphate,
HP = hexametaphosphate, SC = sodium citrate,
EDTA = ethylenediamine tetraacetic acid.

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solubility of iron and changing the partition of iron out of emulsion droplets into the aqueous phase.

1. Metal catalysis

The effects of metal catalysts as initiators of lipid oxidation in the presence of hydroperoxides were discussed in Chapter 1. Metal catalysis is not straightforward in various complex multiphase systems and the effects of a number of factors are difficult to unravel.

An important question to consider is where does metal catalysis takes place in multiphase systems? In bulk oils systems, the hydrophilic metals would be oriented in the air–oil interface to catalyse lipid oxidation (Figure 10.7). In emulsions and liposomes, the metals would be in solution in the aqueous phases and oriented in either the oil–water or phospholipid–water interfaces, where they may have an affinity for the hydrated layer around the droplets (Figure 10.4).

Metals become potent catalysts of lipid oxidation by forming complexes through the carboxylic acid groups of any free fatty acids present in oils. In liposome systems, metal catalysis may proceed by a different mechanism in the non-polar acyl chain moiety of the phospholipid molecule from that in the polar phosphate region. Different pathways for decomposition of lipid hydroperoxides

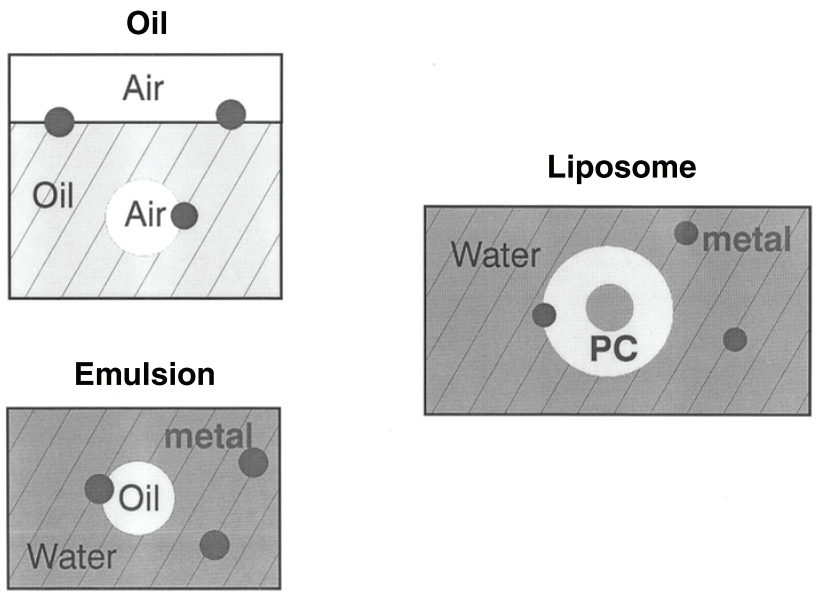


Figure 10.7. Metal catalysis in bulk oil and emulsion systems.

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Table 10.6. Effect of metals and heme compounds on the decomposition of linoleic acid hydroperoxides at 23°C and two pHs [a]

Metals ions	Relative rates		Heme compounds	Relative rates	
	pH 7	pH 5.5		pH 7	pH 5.5
Fe^{3+}	1	92	Hematin	4000	40000
Fe^{3+} + ascorbate	80	500	Hematin + ascorbate	64000	50000
Fe^{2+}	14	960	Methemoglobin	5000	76000
Cu^{2+}	0.2	1.6	Cytochrome c	2600	39000
Cu^{2+} + ascorbate	2200	6200	Oxyhemoglobin	1200	
Co^{2+}	600	0.2	Myoglobin	1120	

[a]From O'Brien (1969). Linoleic acid hydroperoxides (3.2×10^{-5} M) were emulsified in separate buffers. Relative rates were calculated with different concentrations of each catalyst and expressed as moles of metal ions or heme per min $\times 10^{-6}$; ascorbate concentration: 5.7×10^{-5} M.

occur also between bulk systems and emulsions. Water in emulsion systems may facilitate the breakdown of alkoxy radicals formed by decomposition of hydroperoxides. However, the effect of water in foods of varying moisture content shows a complex anomalous relationship that will be discussed in Chapter 11.

Metal ions decompose emulsified linoleic acid hydroperoxides at different rates according to the pH of the buffer (Table 10.6). Ferrous iron (Fe^{2+}) was 14 times more effective in decomposing linoleic hydroperoxides than ferric iron (Fe^{3+}). Copper was much less effective than iron in decomposing linoleic acid hydroperoxides. The activity of both iron and copper for this decomposition reaction was increased significantly by the addition of ascorbic acid and by lowering the pH from 7.0 to pH 5.5.

Heme proteins containing Fe^{2+} and hemin proteins containing Fe^{3+} are

Table 10.7. Prooxidant activity of free iron, hemin and metmyoglobin, before and after heating, in linoleic acid emulsions[a]

Concentration	$Fe(II)Cl_2$	Hemin	Metmyoglobin
Before heating			
2 μM	0.028	0.172	0.275
4 μM	0.049	0.079	0.29
8 μM	0.045		0.246
After heating[b]			
2 μM	0.036	0.227	0.114
4 μM	0.037	0.167	0.155
8 μM	0.02		0.182

[a]From Kristensen and Andersen (1997). Oxygen consumption expressed in μM/sec.
[b]Heated to 80°C at 5°C/min.

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widely distributed in animal tissue. Lipid oxidation is rapidly accelerated by hemoglobin, myoglobin and cytochrome C in fish, poultry and cooked meat (Chapter 11). Some heme compounds are much more active than iron ions in decomposing linoleic acid hydroperoxides (Table 10.7). These iron-protein complexes are thus much more efficient initiators of lipid oxidation. This higher activity can be attributed to a more favorable interaction of the iron-protein complexes at the micellar interface of the hydroperoxide acids.

Thermal denaturation of metmyoglobin (Mmb) containing Fe^{3+} causes structural changes to the heme protein that influences its prooxidant activity. Thus, heating hemin and metmyoglobin at 80°C produces considerable prooxidant activity, which decreases at high concentrations (Table 10.7). This thermal treatment is accompanied by the formation of free iron. By heating, the heme protein unfolds into several intermediate species, which eventually polymerize and precipitate.

$$\text{Mmb} \rightarrow \text{modified Mmb} + \text{Fe} \rightarrow \text{polymerized Mmb} \rightarrow \text{precipitate}$$

Thermal modifications of heme proteins and the resulting oxidative processes play an important part in the oxidative stability of heat-processed meat (Chapter 11).

The effects of metal chelators may be very complex in multiphase systems. Chelators vary in their metal affinities and charge and in their solubility in lipids; they partition to different degrees between the lipid and water phase. Thus, in aqueous systems EDTA can reduce lipid oxidation by changing the location and by reducing the oxidizing capacity of iron. However, in the presence of reducing agents, complex formation with EDTA may also promote lipid oxidation in certain systems by producing alkoxyl radicals from hydroperoxides and initiating the radical chain. The effects of antioxidants and reducing agents in the presence of different forms of metal complexes have important consequences for the lipid oxidation of multiphase systems, therefore.

Lactoferrin is a glycoprotein found in mammalian milk that tightly binds two ferric ions producing an iron complex more physically and chemically stable than the uncomplexed protein. Bovine lactoferrin inhibited oxidation in corn oil-in-water emulsions and lecithin liposome systems (Table 10.8). At the same molar concentration, lactoferrin was less effective than EDTA in inhibiting hydroperoxide formation in a corn oil emulsion. This lower antioxidant activity of lactoferrin may be explained by its partial iron saturation and lower affinity for ferric ions. The formation constant for ferric-EDTA is 1.3×10^{25} compared to $\sim 10^{20}$ for the ferric-lactoferrin complex. Lactoferrin was a better iron chelator in the liposome than in the emulsion systems. Inhibition in liposomes with iron-lactoferrin mixtures was in the order: 1:2 > 1:1 > 2:1. This order suggested that lactoferrin also chelated metal impurities as well as added iron to inhibit lipid oxidation. Lactoferrin did not inhibit the copper-catalysed

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Table 10.8. Inhibition (%) of hydroperoxide formation by lactoferrin in corn oil-in-water emulsions and in lecithin liposomes[a]

Additives	Emulsions	Liposomes
+ 1 μM Lactoferrin	40.7	–19.1
+ 5 μM Lactoferrin	51.5	79.9
+ 0.5 μM EDTA	27.9	–
+ 1 μM EDTA	51.4	–
+ 2 μM EDTA	85.9	–
+ 0.5 μM Fe^{2+}	–4.1	–47.4
+ 1 μM Fe^{2+}	–15.0	–105
+ 2 μM Fe^{2+}	–11.6	–242
+ 5 μM Fe^{2+}	–29.2	–241
+ 0.5 μM Fe^{2+} + 1 μM lactoferrin	20.8	84.4
+ 1 μM Fe^{2+} + 1 μM lactoferrin	2.3	54.7
+ 2 μM Fe^{2+} + 1 μM lactoferrin	–18.3	18.2
+ 5 μM Fe^{2+} + 1 μM lactoferrin	–13.6	–218

[a]From Huang *et al.* (1999). Oil-in water emulsions were prepared with 10% corn oil and 1% lecithin emulsifier. Liposomes were prepared with 1% lecithin. Both systems were made up in phosphate buffer (25 mM) (pH 6.6) and oxidized at 50°C. Inhibition values were taken after 2 days for runs with lactoferrin and lactoferrin + EDTA, and after 3 days for runs with Fe^{2+} and Fe^{2+} + lactoferrin, and for all liposome runs.

oxidation of corn oil-in-water emulsions. Whether lactoferrin did not chelate copper ions or its copper complexes catalysed oxidation is not clear.

2. *Antioxidants*

a. Initiators. Several dispersed systems have been used as models to study the interfacial behavior of various antioxidants on lipid oxidation induced with different artificial radical initiators. Quantitative kinetic studies of autoxidation are dependent on the use of an initiator (In) that decomposes into two radicals, which in solution are held together briefly in a cage of solvent molecules. The interactions of these radicals can retard or prevent a portion from diffusing into the bulk of the solution, and this phenomenon is known as the *cage effect.* Autoxidation chains are started only by the fraction of radicals that escape from the solvent cage. The radicals produced below are represented by a bar over the radical pairs for its caged nature.



In the decomposition of diazo initiators, the number of free radicals in the bulk solutions is estimated to be less than two for each molecule of initiator consumed. This cage effect is dependent on the solvent viscosity. The more

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Table 10.9. Effect of copper (II), azo initiators and BHT on oxidation of phosphatidylcholine (PC) liposomes at 37°C[a]

Oxidation promoters[b]	Rate of oxidation[c]		Induction period, min[d]
	PCOOH	Oxygen	
Control	7.8	7.4	
Cu (II)	24.3	21.5	
Cu (II) + BOOH	32.3	34.7	55
Cu (II) + BOOH + BHT			150
AAPH	23.5	30.5	65
AAPH + BHT			130
AMVN	31.2	35.9	40
AMVN + BHT			52

[a]From Yoshida and Niki (1992).
[b]Cu (II), 100 μM copper (II); BOOH, 3 mM *t*-butyl-hydroperoxide; BHT, 1 μM.
AAPH, 2mM 2,2'-azobis(2-amidinopropane) dihydrochloride;
AMVN, 2 mM 2,2'-azobis(2,4-dimethylvaleronitrile).
[c]Rates: d[PCOOH]/dt and d[O_2]/dt, in nM per sec.
[d]Time necessary for the formation of 100 μM phosphatidylcholine hydroperoxides.

viscous the solvent, the more difficult it will be for the radicals to escape through the walls of the solvent cage.

In contrast to solution, the higher viscosity of a lecithin liposome decreases the efficiency of free radical initiation, and retards their autoxidation. Artificial azo initiators have a very low efficiency (about 9%) when solubilized in the bilayer phase. For this reason, the oxidizability of lecithin dispersed in a liposome is much lower than in solution. Although the solvent cage effect may be unique to diazo initiators commonly used in kinetic studies, and is not necessarily relevant to food lipid systems, metal initiators which are relevant to foods and biological systems, may also be affected by solvent cage effects because of the hydrated layer in emulsions (Figure 10.4).

Antioxidants behave differently when oxidation is initiated with metals as compared to azo radical initiators. Thus, BHT was a more effective antioxidant in a phosphatidylcholine liposome oxidized with copper (II), in the presence of *t*-butyl-hydroperoxide, than in the same liposome oxidized with AAPH or with AMVN (Table 10.9). The inhibition by BHT was better with the water-soluble initiator AAPH than the oil-soluble initiator AMVN. This result suggests that radicals produced in the water phase by copper (II) were trapped by BHT in the same way as the radicals produced by AAPH. The rates of oxygen absorption in this liposome system were increased three fold with 100 μM copper (II) compared to four fold with 2 mM AAPH and about five fold with 2 mM AMVN. Such results must be interpreted with caution, because at sufficiently high concentrations metals react stoichiometrically and promote chain termination, a condition which is not observed at low and trace concentrations in

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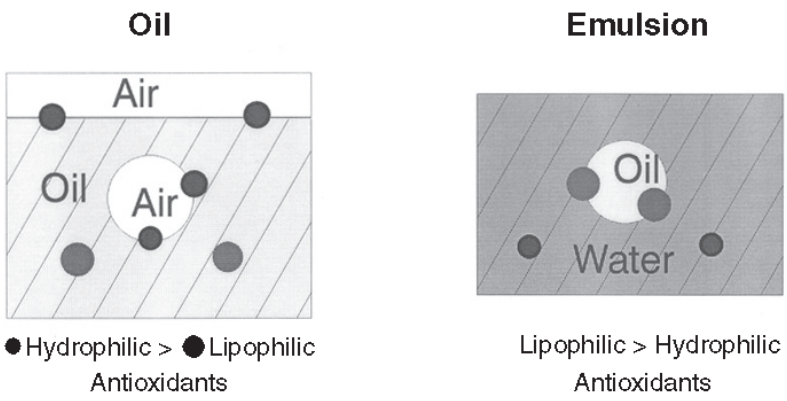


Figure 10.8. Interfacial distribution of hydrophilic and lipophilic antioxidants in bulk oil vs oil-in-water emulsion systems. From Frankel *et al.* (1994).

multiphase food systems. These model studies may, therefore, not be particularly applicable to food lipid systems where lipid oxidation is initiated predominantly by trace amounts of metals in the presence of small amounts of hydroperoxides.

b. Emulsions. The contrasting behavior of different antioxidants in bulk oil versus oil-in-water emulsions was discussed in Chapter 9. Antioxidant activity varies in different multiphase systems, which differ in the distribution of the lipid phase. In oil-in-water emulsions, hydrophilic antioxidants are generally less effective than lipophilic antioxidants, whereas in bulk oil systems hydrophilic antioxidants are more effective. Thus, the order of activity of α-tocopherol versus Trolox and ascorbic acid versus ascorbyl palmitate, is reversed when comparing bulk oil and emulsified systems (Table 9.9, Figures 9.10 and 9.11). This phenomenon was explained in relation to the affinities of the antioxidants toward the air–oil interfaces in bulk oil and the water–oil interfaces in emulsions. In the corn oil bulk system, the hydrophilic antioxidants Trolox and ascorbic acid are more protective against oxidation by being oriented in the air–oil interface; the lipophilic antioxidants α-tocopherol and ascorbyl palmitate are less protective by remaining in solution in the oil phase where they are present at low concentrations (Figure 10.8). In contrast, in the oil-in-water emulsions, the lipophilic antioxidants are oriented at the oil–water interface by virtue of their surface activity, and are more protective against oxidation than the hydrophilic antioxidants, which are dissolved and become diluted in the water phase.

The influence of the colloidal properties of lipid systems on the efficiency of antioxidants was demonstrated by comparing the activity of antioxidants in bulk oil, oil-in-water and water-in-oil emulsions, and by the addition of

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Table 10.10. Inhibition (%) of hydroperoxide formation by antioxidants in bulk oil, emulsions and bulk oils with and without emulsifier addition[a]

Antioxidants	Bulk oil (4 wks)[b]	O/W emulsion (5 wks)[b]	W/O emulsion (3 wks)[b]	Bulk oil (10 days)[c]	Bulk oil + PGMS (10 days)[c]	Bulk oil+ PGO 10 days)[c]
Tocopherol	38.9	31.5	7.5	10.8	32.4	–4.7
Trolox	98.2	43.2	22.2	58.0	59.5	7.2
Propyl gallate	86.7	89.9	–7.1	66.7	12.3	1.1
Gallic acid	87.2	–9.7	–45.8	63.7	44.4	–8.0
Carnosic acid	85.6	37.6	23.7	–.	–	–
Methyl carnosate	90.7	20.7	36.2	–	–	–

[a]From Schwarz *et al.* (2000). Bulk oil: tocopherol-stripped corn oil; emulsions contained 30% oil, 70% aqueous phase; oil phase: 72% stripped corn oil, 8% beeswax, 20% emulsifier; O/W: oil-in-water emulsion using polyglyceryl glucose methyl distearate (PGMS) emulsifier; W/O: water-in-oil emulsion using polyglycerol-3-oleate (PGO) emulsifier. Negative values represent prooxidant activity.
[b]Oxidation carried out at 37°C.
[c]Oxidation carried out at 60°C; emulsifier–oil ratio was equal to that of emulsions.

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emulsifiers in bulk oil (Table 10.10). α-Tocopherol, Trolox, carnosic acid and methyl carnosate showed higher activity in bulk oil than in emulsions. Propyl gallate had the same activity in the oil-in-water emulsion as in bulk oil. In both emulsions, the most polar antioxidants gallic acid had prooxidant activity. Methyl carnosate was the most active antioxidant in the water-in-oil emulsion but was less active than Trolox in the oil-in-water emulsion.

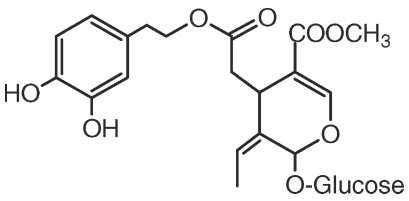
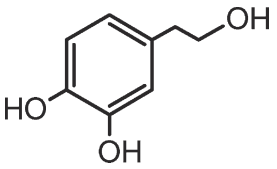
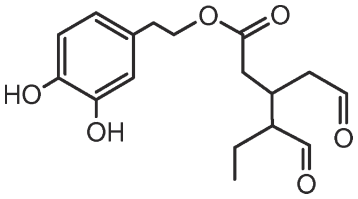
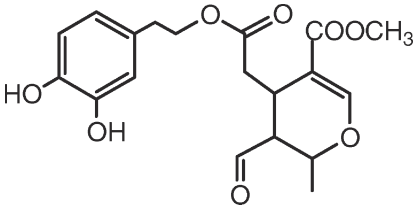
When added to bulk oil, the polar oil-in-water emulsifier polyglyceryl glucose methyl distearate (PGMS) increased the antioxidant activity of α-tocopherol, decreased the activity of propyl gallate and gallic acid but did not change the activity of Trolox. The less polar water-in-oil emulsifier polyglycerol-3-oleate (PGO) emulsifier strongly decreased the activity of all antioxidants tested. These results indicate that interactions of emulsifier with antioxidants, hydrogen bondings, interphase transport and surface access are important parameters determining their activity in lipid-containing systems differing in their colloidal characteristics. As shown previously when we compared polar vs non polar antioxidants (Figures 9.10 and 9.11), in bulk oil the polar antioxidants Trolox, propyl gallate and gallic acid were much more active than the non-polar α-tocopherol. In emulsions, the polar antioxidants became much less active, because they not only partition into the aqueous phase where they are diluted, but also can reduce metal catalysts into the more active lower valence state, especially gallic acid, and become prooxidants. The less oil-soluble polar antioxidants are more active in bulk oil because they accumulate at the air–oil surface (Figure 10.8).

On the one hand, this hypothesis is supported by the increased activity of α-tocopherol resulting from the added polar emulsifier PGMS and decreased activity from the non-polar emulsifier PGO. The polar emulsifier apparently enhanced the surface accessibility of α-tocopherol to become more active. Similarly, the very polar phospholipids are also known to enhance the antioxidant activity of tocopherols in bulk oil. On the other hand, the activity in bulk oil of the polar antioxidants gallic acid and propyl gallate decreased with both emulsifiers while the activity of Trolox decreased with PGO. In the presence of emulsifiers, these polar antioxidants are less active in bulk oil by increasing their solubility in the oil where they would be diluted and become less protective.

The effect of natural polyphenolic compounds from olives on the oxidative stability of oil-in-water emulsions prepared with stripped olive oil showed antioxidant activities decreasing in the order: 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA) ≈ 3,4-dihydroxyphenylethanol-elenolic acid (3,4-DHPEA-EA) > hydroxytyrosol ≈ oleuropein (Table 10.11). The order of polarity of the olive polyphenols based on the partition between octanol and water is hydroxytyrosol > oleuropein > DHPEA-EDA > 3,4-DHPEA-EA. Although the relative antioxidant trend agreed with the *polar paradox* (Section C) that more polar antioxidants are less effective in emulsions,

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Table 10.11. Effect of olive polyphenols and iron on the oxidative stability of stripped olive oil-in-water emulsions stored at 60°C[a]

Structures	Antioxidants	Iron (mg/kg)	Phenol (mM)	Induction period (days)[b]
–	Control	0	0	2.4–2.8
–	Control	1.2	0	1.5–2.3
	Oleuropein	0	1.2	28.0
		1.2	0.8	1.2
	Hydroxytyrosol	0	1.2	29.4
		1.2	0.8	1.5
	3,4-DHPEA-EDA	0	1.2	43.1
		1.2	0.8	2.2
	3,4-DHPEA-EA	0	1.2	41.2
		1.2	0.8	7.1

[a]From Paiva-Martins and Gordon (2002). Oil-in-water emulsions were prepared with 30% olive oil stripped of natural tocopherols and phenols in acetate buffer (0.05 M, pH 5.5) and 2% Tween 20. 3,4-DHPEA-EDA = 3,4-dihydroxyphenylethanol-elenolic acid dialdehyde, 3,4-DHPEA-EA = 3,4-dihydroxyphenylethanol-elenolic acid.
[b]Induction period = time for emulsions to achieve a conjugated diene content of 0.4%.

oleuropein was an exception because it may have "non-polar" effects due to its glucoside structure. The presence of ferric ions drastically reduced the stability for hydroxytyrosol, oleuropein and 3,4-DHPEA-EDA, but not so much for 3,4-DHPEA-EA. When tested at a pH of 3.5, hydroxytyrosol and oleuropein became prooxidants. The application of virgin olive oil in food emulsions

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Table 10.12. Relative effectiveness of antioxidants in lecithin liposomes versus polarity by thin-layer chromatography (TLC)[a]

Antioxidants	Relative efficiency[b]	R_f silica TLC
BHT	14.7	0.88
BHA	11.1	0.68
Propyl gallate	8.0	0.23
TBHQ	2.6	0.43
Quercetin	2.6	0.16
Methyl gallate	1.9	0.18
Caffeic acid	0.3	0.21
Gallic acid	0.3	0.06

[a]From Porter *et al.* (1989).
[b]Relative efficiency = $(IP_a / IP_c) - 1$, where IP_a is induction period with added antioxidant, and IP_c is induction period without added antioxidant.

(salad dressings and mayonnaise) that have an acidic pH requires therefore the judicious use of metal-chelating agents such as EDTA (Chapter 8).

c. Liposomes. The behavior of different types of antioxidants in emulsions, referred to as the *polar paradox*, is based on the phenomenological observation that non-polar antioxidants are more effective on polar lipids in emulsions, while polar antioxidants are more effective on non-polar lipids. In a lecithin liposome oxidized with hematin, the activities of different antioxidants showed a linear relation with polarity based on the R_f values in silica gel thin-layer chromatography (Table 10.12). The lipophilic BHA, BHT and propyl gallate were much more active antioxidants in protecting polar phospholipids in this liposome system than the hydrophilic TBHQ, caffeic and gallic acids.

A number of kinetic studies were carried out to elucidate the interaction between α-tocopherol and ascorbic acid in liposomes. By using either water-soluble or oil-soluble diazo radical initiators, the effect of radicals can be compared when they are produced either in the phospholipid bilayer or in the aqueous phase. Oxidation of a soybean phosphatidylcholine liposome in the presence of radicals produced in the water phase with a water-soluble radical initiator [2,2'-azobis(2-amidinopropane) dihydrochloride, (AAPH)], showed an induction period with both α-tocopherol (vitamin E) and ascorbic acid (vitamin C) (Figure 10.9). With a mixture of vitamin E and vitamin C, the length of the induction was close to the sum of the individual induction periods. This result indicates an additive effect in suppressing oxidation by both vitamin E and vitamin C. Vitamin C apparently traps radicals in the water phase. When oxidation was induced by radicals produced in the lipid phase with an oil-soluble radical initiator [2,2'-azobis(2,4-dimethylvaleronitrile), (AMVN)] incorporated into the membrane, ascorbic acid alone had no effect, while α-tocopherol had a greater effect because it is lipophilic. However, the mixture

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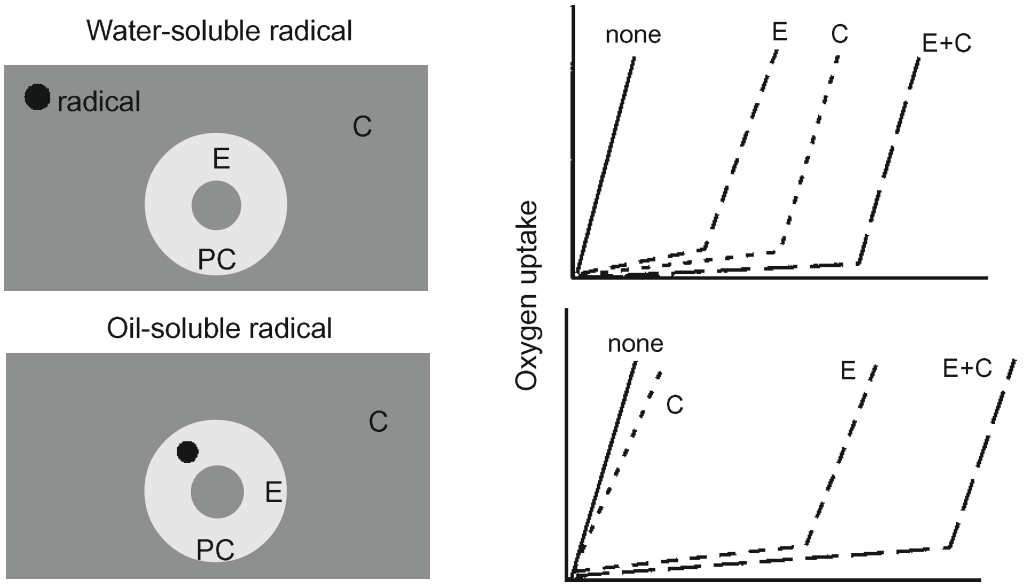


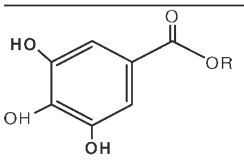
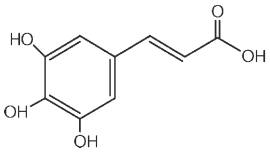
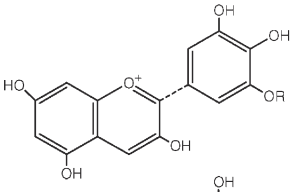
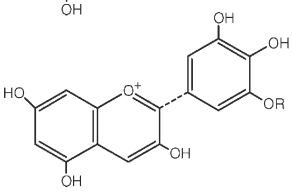
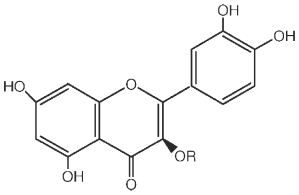
Figure 10.9. Effect of vitamin E (E) and vitamin C (C) and their mixtures on the oxidation of a soybean phosphatidylcholine (PC) liposome in the presence of a water-soluble radical initiator (AAPH) and oil-soluble radical initiator (AMVN). From Niki *et al.* (1985).

of α-tocopherol and ascorbic acid had a synergistic effect by increasing the induction period. Similar results were reported with liposomes of dilinoleoyl phosphatidylcholine. This effect can be explained by assuming that the radicals initiated in the lipid phase are first trapped by α-tocopherol within the liposome, and the resulting α-tocopherol radicals become oriented at the bilayer surface and are reduced by ascorbic acid in the aqueous phase to regenerate α-tocopherol.

The relative antioxidant activity of catechin gallates (Figure 9.15) in green tea changed significantly according to the test system and the conditions of oxidation. Tea catechins were shown previously to be active in bulk corn oil but were prooxidant in the corresponding corn oil-in-water emulsions, as were propyl gallate and gallic acid (Chapter 9, Section D.4, Table 9.18). Tea catechins were however active in liposomes under certain conditions. Thus, in soy lecithin liposomes oxidized at 50°C, epigallocatecin gallate (EGCG), epicatechin (EC) and propyl gallate (PG) were the best antioxidants, followed by catechin, epigallocatechin (EGC) and gallic acid (Table 10.16). In the oxidation of liposomes catalysed by copper, however, catechin and EC were better antioxidants than ECG, but EGCG, EGC, PG and gallic acid became prooxidants. These results suggest that the very polar antioxidants gallic acid, PG, EGC and EGCG may reduce copper ions into the more active lower valence state, causing them to become prooxidants. The improved antioxidant activity of tea catechins in liposomes compared to emulsions can be explained by the greater affinity of the polar catechins towards the polar surface of the lecithin bilayers to afford better protection.

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Table 10.13. Effect of bovine serum albumin (BSA) on inhibition (%) of lipid and protein oxidation on the antioxidant activity of phenolic compounds (5 μM) in lecithin liposome (PC)[a]

Structures	R	Antioxidants	Inhibition of hydroperoxides		Lysine loss	Protein carbonyls
			PC	PC+ BSA	PC+ BSA	PC+ BSA
	H	Gallic acid	–34.6	–23.8	–35.9	–15.7
	C_3H_7	Propyl gallate	5.6	–6.8	–25.2	–9.3
	H	Caffeic acid	19.2	4.7	–7.0	–4.2
	Me	Ferulic acid	54.6	57.8	97.5	40.9
	H	Delphinidin	–18.6	–28.3	–37.4	–31.9
	Me	Malvidin	23.7	40.7	57.1	21.5
	–	(+)-Catechin	44.0	18.5	21.5	5.9
	–	Epicatechin	47.5	16.0	11.7	–1.2
	H	Quercetin	19.1	16.5	6.8	2.0
	Rutinose	Rutin	23.3	31.7	83.3	10.8

[a] From Heinonen *et al.* (1998). Liposome contained 0.8% by wt PC, oxidized at 37°C, with 3 μM cupric acetate, in 25 mM succinate buffer at pH 4.7, without or with 0.16% BSA.

Proteins have strong binding properties with flavonoid antioxidants in foods and biological systems, and generally increase the oxidative stability of liposomes. The addition of bovine serum albumin (BSA) increased the oxidative stability of a lecithin-liposome and changed the relative antioxidant activity of various pairs of flavonoids and phenolic acids (Table 10.13). In the absence of BSA, the activity of phenolic antioxidants decreased in the order: ferulic acid > epicatechin > catechin > malvidin > caffeic acid ≈ quercetin > propyl gallate. In the presence of BSA, the activity of malvidin increased, followed by rutin and ferulic acid, but the activity of epicatechin and catechin decreased,

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followed by propyl gallate, caffeic acid, and quercetin. Although the highly polar gallic acid, and delphinidin were prooxidant with or without added BSA, BSA reduced the prooxidant activity of gallic acid. Ferulic acid was most effective in inhibiting protein oxidation (based on protein carbonyl gain and lysine loss), followed by rutin, malvidin and catechin. The less polar phenolic compounds (propyl gallate, ferulic acid and malvidin) appeared to be more effective antioxidants than their more polar counterparts. Ferulic acid and malvidin with methoxyl substituents showed the greatest antioxidant activity against both lipid and protein oxidation. The less polar member of structurally related pairs of phenolic compounds may be more active because their increased hydrogen donor ability and by forming more stable phenoxyl radical intermediates due to their methoxyl substituents. The lower polarity of the more active ferulic and malvidin may also impart better affinity for the liposome surface and better inhibit lipid oxidation. BSA may enhance the antioxidant activity of malvidin, rutin and ferulic acid by improving their association with the liposome interface undergoing oxidation.

In egg yolk phosphatidylcholine liposomes, using a water-soluble AAPH initiator, epicatechin gallate had the highest antioxidant activity, followed by epicatechin and quercetin, and α-tocopherol was the least active. In contrast, the reverse trend was obtained with methyl linoleate oxidized in hexane–isopropanol solution using a lipid-soluble AMVN initiator. In this solution, α-tocopherol had the highest activity, followed by quercetin, and epicatechin, which had the same activity as epicatechin gallate (Table 10.14; Figure 10.10). In liposomes, catechin gallates associate with the surface to protect the polar phospholipids, while α-tocopherol remains within the membrane and is less protective. Also, α-tocopherol was the least stable in the liposome system and disappeared first during oxidation, followed by quercetin, epicatechin and

Table 10.14. Effect of catechins, quercetin and α-tocopherol on the oxidation of phosphatidylcholine (PC) liposomes or solutions initiated by AAPH [a]

Antioxidants 10 μM	Liposomes[b]		Solutions[c]	
	Induction period, min	Decomposition time, min	Antioxidant efficiency (k_{inh}/k_p)	Inhibition time (t_{inh})
Epicatechin gallate	350	220	130	23
Epicatechin	290	110	135	27
Quercetin	250	100	280	13
α-Tocopherol	160	50	1700	5

[a]From Terao *et al.* (1994).
[b]PC oxidized with water-soluble AAPH; induction period for the formation of 0.25 mM phosphatidylcholine hydroperoxides; decomposition time for the complete loss of antioxidant.
[c]Hexane–isopropanol solution oxidized with oil-soluble AMVN.

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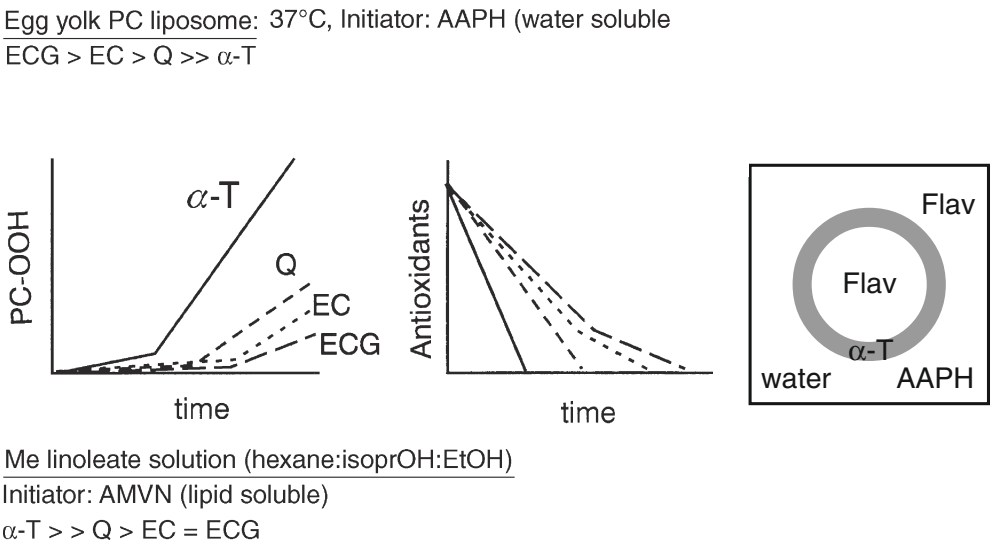


Figure 10.10. Relative antioxidant activities of epicatechin gallate (ECG), epicatechin (EC), quercetin (Q), and α-tocopherol (α-T) on the oxidation of egg yolk phosphatidylcholine liposome in the presence of a water-soluble initiator (AAPH) and in hexane–isopropanol solution in the presence of a lipid-soluble initiator (AMVN). From Terao *et al.* (1994). FLAV, flavonoids.

epicatechin gallate. During oxidation induced by a water-soluble azo initiator, EGC and EGCG were less stable than EC and ECG. Multiple factors are evidently involved to account for the wide range of activity of different tea catechin gallates, including their redox potentials, relative stability, metal chelation, and partition properties (see Section 2.e).

The activity of antioxidants is greatly enhanced by increasing the electrostatic attraction between the lipid surface and antioxidants. The charge of phospholipid liposomes can thus greatly affect the activity of charged antioxidants. Adjusting the pH of two types of phospholipids changed the charge of liposomes. The negatively charged Trolox had no antioxidant activity toward the zwitterionic (±) liposomes prepared with dilinoleoyl-phosphatidylcholine (DLPC) at pH 11 and with the negatively-charged dilinoleoyl-phosphatidylglycerol (DLPG) at pH 11 and 7, due to electrostatic repulsion between the surface charge and the antioxidant charge (Table 10.15). Conversely, Trolox became a much more active antioxidant toward the positively charged DLPC liposome at pH 4 due to electrostatic attraction. Similarly, Trolox was active by electrostatic attraction in the positively charged DPLC in the presence of stearyl amine at pH 7. Trolox was also active in dimyristoyl-phosphatidic acid in the presence of linoleic acid when it was positively charged at pH 2, but not when it was negatively charged at pH 7. Trolox was more active than the positively charged quaternary ammonium analog of α-tocopherol, hydroxychroman substituted with $-CH-(CH_2)_2-N^+-(CH_3)_3$, [6-hydroxy-2,5,7,8-

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Table 10.15. Effect of charge of liposome on the antioxidant activity of Trolox [a]

Liposome (charge)[b]	pH	Inhibition period, min	Inhibition rate constant, k_{inh} [c]
	Trolox (–)		
DLPC (±)	11	No	–
DLPC (±)	7	123	2.98
DLPC (+)	4	97	5.8
DLPC/SA (+)	7	83	4.16
DLPG (–)	7/11	No	–
DLPG (±)	4	109	1.22
DMPA/LA (+)	2	74	5.55
DMPA/LA (–)	7	No	–
	MDL (+) [d]		
DLPC (–)	7	170	0.95
DLPG (–)	7	96	1.62

[a]From Barclay and Vinqvist (1994). Liposome oxidized at 37°C in the presence of lipid-soluble initiator azo-bis-(2-amidinopropane hydrochloride) (ADVN).
[b]DLPC, dilinoleoyl-phosphatidylcholine; SA, stearyl amine; DLPG, dilinoleoyl-phosphatidylglycerol; DMPA, dimyristoyl-phosphatidic acid; LA, linoleic acid.
[c]Inhibition rate constants calculated from plots of oxygen uptake, in $M^{-1}\ s^{-1} \times 10^{-3}$.
[d]6-Hydroxy-2,5,7,8-tetramethyl-2-N,N,N-trimethylethanaminium methyl-benzenesulfonate.

tetramethyl-2-N,N,N-trimethyl-ethanaminium methyl-benzene sulfonate, (MDL)], in the DLPC liposome at pH 7 (Table 10.15). Conversely, MDL was more active than Trolox in the negatively charged DLPG liposome at pH 7.0.

d. Micelles. Although triacylglycerols are the major components in foods, polyunsaturated fatty acids and their esters are most often used as model lipid substrates in studies of antioxidants. However, free fatty acids are amphiphilic, and dissociate in aqueous systems according to the pH to partially water-soluble anions that form micelles. This micelle formation is in marked contrast to the water-insoluble fatty acid esters and triacylglycerols that are dispersed in water as emulsion particles. Although at neutral pH the oxidizability of linoleic acid anions remains constant within a wide range of concentration in SDS micelles, the oxidizability of methyl linoleate increases with increasing concentrations because it becomes solubilized within the micelles at higher concentrations. Above the critical micellar concentration, polar emulsifiers form mixed micelles with the free fatty acids in the aqueous phase (Figure 10.3). Polar antioxidants such as Trolox are more active in emulsified linoleic acid, consisting of mixed micelle systems of emulsifier and free fatty acids, than in oil-in-water emulsions of methyl linoleate or corn oil where they partition mainly in the water phase (Table 10.16, Section 2.e). For these reasons, linoleic acid cannot be used as a representative model system for

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Table 10.16. Effect of lipid systems on relative activities of hydrophilic versus lipophilic antioxidants

Lipid systems[a]	Antioxidant trends	Ref.[b]
Bulk corn oil	Trolox > α-tocopherol, ascorbic acid > ascorbyl palmitate	(1)
Oil/water emulsion	α-Tocopherol > Trolox, ascorbyl palmitate > ascorbic acid	(1)
Bulk corn oil	α-Tocopherol > carnosic acid > carnosol	(2)
Bulk linoleic acid	Trolox > α-tocopherol > carnosol > carnosic acid	(2)
Oil/water emulsion	α-Tocopherol > > carnosic acid and carnosol were inactive	(2)
Linoleic acid emulsion	Trolox > α-tocopherol = carnosol > carnosic acid	(2)
Bulk oil	Trolox > methyl carnosate > gallic acid ≈ propyl gallate ≈ carnosic acid > α-tocopherol	(3)
Oil/water emulsion	Propyl gallate > Trolox > carnosic acid > α-tocopherol > methyl carnosate > gallic acid (prooxidant)	(3)
Water/oil emulsion	Methyl carnosate > carnosic acid ≈ Trolox > gallic acid and propyl gallate (prooxidant)	(3)
Lecithin liposome	BHT > BHA > propyl gallate > TBHQ > gallic acid	(4)
DLPC liposome	Trolox = α-tocopherol	(5)
PC liposome	Epicatechin gallate > epicatechin > quercetin > α-tocopherol	(6)
Soy lecithin liposome	Epigallocatechin gallate > epicatechin = propyl gallate > catechin > epicatechin gallate > = gallic acid	(7)
LA/SDS micelles	Trolox >> α-tocopherol	(8)
SDS^- micelles	α-tocopherol = ascorbyl palmitate > Trolox > ascorbic acid	(8)
$HDTBr^+$ micelles	ascorbic acid > Trolox > ascorbyl palmitate > α-tocopherol	(8)
Solution[c]	α-tocopherol >> quercetin > epicatechin = epicatechin gallate.	(6)

[a] Abbreviations: DLPC, dilinoleoyl phosphatidylcholine; PC, phosphatidylcholine (egg); LA, linoleic acid; SDS, sodium dodecyl sulfate; HDTBr, hexadecyltrimethyl ammonium bromide.
[b]References: (1) Frankel *et al.* (1994) see Figure 9.7, 9.8, (2) Huang *et al.* (1996), Hopia *et al.* (1996), (3) Schwarz *et al.* (2000), see Table 10.10, (4) Porter *et al.* (1989), see Table 10.12, (5) Barclay *et al.* (1990), (6) Terao *et al.* (1994) see Figure 10.10, (7) Huang *et al.* (1997) see Figure 9.12, (8) Pryor *et al.* (1993) see Figure 10.11.
[c]hexane–isopropanol (1:1, v:v).

foods, since antioxidant behavior in linoleic acid micelles will be significantly different from that in foods composed mainly of triacylglycerols.

The charge of fatty acid micelles affects markedly the efficiency of charged and uncharged antioxidants. The negatively charged ascorbic acid and Trolox were significantly more effective antioxidants in a micelle system of linoleic acid stabilized with a positively charged emulsifier such as hexadecyltrimethyl-ammonium bromide (HDTBr), and much less effective when the system was stabilized with a negatively charged emulsifier, such as SDS (Figure 10.11). These charged antioxidants have an ionized carboxylate group which causes it to be repelled by the negatively charged SDS micelles. The stronger effect observed with ascorbic acid indicates greater attraction by the positively charged HDTBr micellar sphere where oxidation occurs. In this system the

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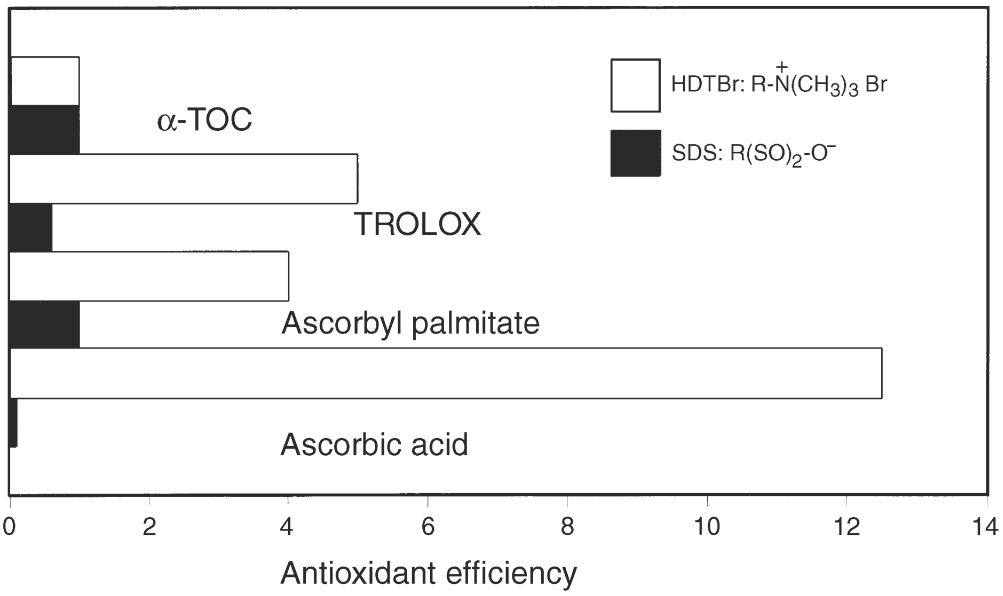


Figure 10.11. Relative antioxidant efficiencies of α-tocopherol (α-TOC), Trolox, ascorbic acid and ascorbyl palmitate on the oxidation of linoleic acid micelles stabilized with HDTBr and SDS. From Pryor *et al.* (1993).

activity of the uncharged and lipophilic antioxidant α-tocopherol was not affected by the charge of the emulsifiers. The same trend was observed, but to a smaller extent, with the hydrophilic ascorbyl palmitate as with ascorbic acid. With ascorbyl palmitate there may be a balancing effect between electrostatic and polar affinities for the linoleic acid–water interface.

The lipophilic α-tocopherol and its hydrophilic analog Trolox also behave quite differently in linoleic acid micelles compared to phosphatidylcholine liposomes. With micelles of linoleic acid in SDS initiated with a water-soluble initiator [2,2'-azobis-(2-amidino propane) dihydrochloride, ABAP], α-tocopherol was much less active than Trolox (Table 10.16). With phosphatidylcholine liposomes initiated with a lipid-soluble initiator AMVN, α-tocopherol and Trolox had about the same antioxidant activity. Linoleic acid and SDS form mixed micelles in the aqueous phase in which the polar antioxidant Trolox equilibrates more rapidly and becomes more effective than in liposomes. Also, Trolox can efficiently trap radicals from the water-soluble initiator ABAP.

In summary, the relative activities of various antioxidants vary widely according to the site of action in different systems, the charge and solubility of components in micelle compared to emulsions, liposome systems and solutions (Table 10.16). The interfacial behavior of antioxidants varies between bulk oil and different colloidal lipid systems. For example, polar antioxidants such as Trolox are more active in mixed micelle systems consisting of emulsifiers and free fatty acids than in corn oil-in-water emulsions, where they partition mainly in the water phase (see next Section). Although linoleic acid is commonly used

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in antioxidant evaluations, it is not a suitable lipid substrate because it gives misleadingly high activity results with polar antioxidants. Antioxidant activity is thus significantly affected by the physical state of different multiphase systems with varying lipid distributions and by the relative polarity of the antioxidants.

e. Partition studies. In multiphase systems, antioxidants partition between several environments, including the aqueous phase, lipid phase and surfactant-enriched environment, such as the interface and micelles. Antioxidants partition into the different phases according to their relative affinities toward these phases and their relative amounts. The partition behavior of different antioxidants in relation to their concentration and location in different phases is expected to change their effectiveness in food and biological systems. Although the literature on this subject has become more active recently, the relationship between the activity of antioxidants of different structures and their partition behavior in multiphase systems is still not well understood.

The concentration of Trolox in the water phase was lower in mixtures of linoleic acid and water and of methyl linoleate and water than in the mixture of corn oil triglycerides and water (Figure 10.12). The partition coefficient (ratio of concentrations between different phases) of Trolox between linoleic acid or methyl linoleate and water is twice as high (6.4) as between corn oil and water (3.8). Trolox has, therefore, a much lower affinity toward the triglyceride than toward either linoleic acid or methyl linoleate. In the corresponding oil-in-

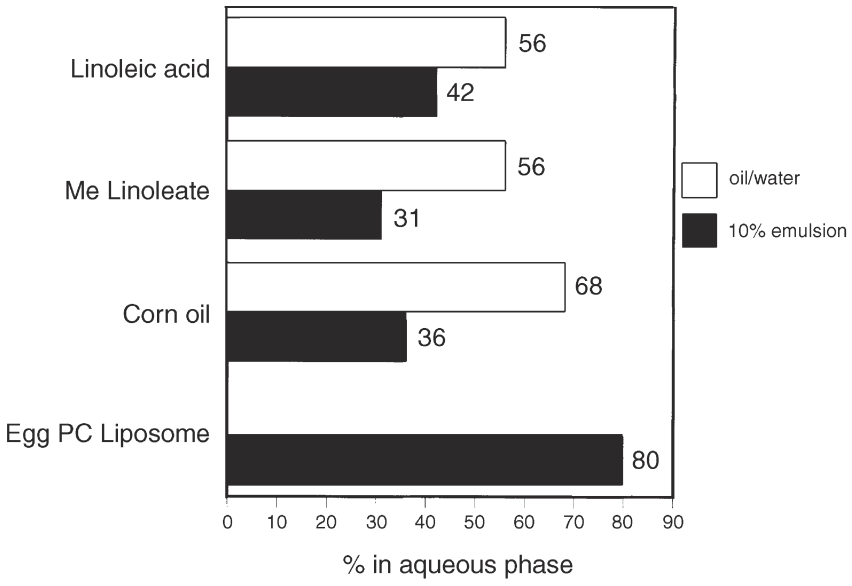


Figure 10.12. Partition of Trolox in different oil/water mixtures and in Tween 20 emulsions. From Huang *et al.* (1996) and Barclay *et al*. (1994).

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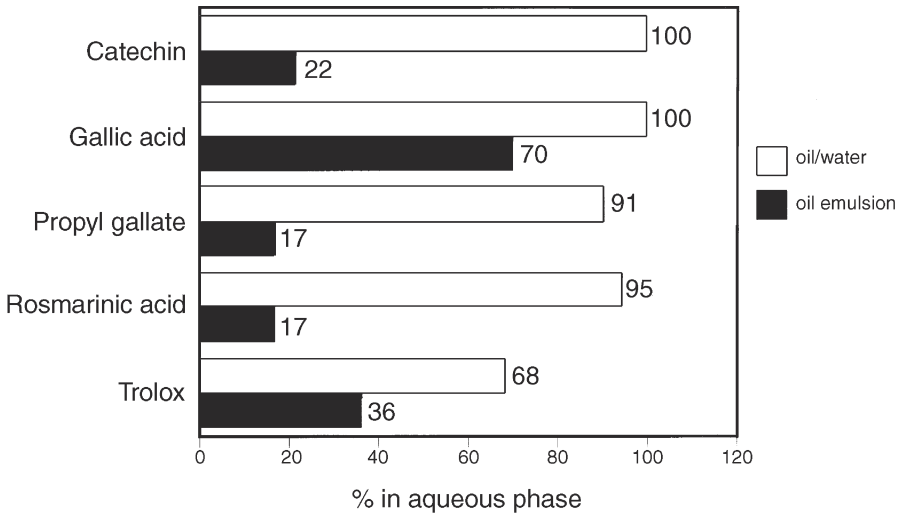


Figure 10.13. Partition of antioxidants in different oil/water mixtures and in Tween 20 emulsions. From Huang *et al.* (1997).

water emulsions, the concentration of Trolox in the water phase decreased because of its affinity toward the emulsifier Tween-20. The concentration of Trolox in the water phase was lower in the systems prepared with corn oil triacylglycerols and methyl linoleate than those with linoleic acid. In egg lecithin liposomes, the bulk of Trolox (80%) resided in the aqueous phase.

In corn oil–water mixtures, more than 90 % of the hydrophilic antioxidants catechin, gallate, propyl gallate and rosmarinic acid partitioned into the aqueous phase, compared to 68% for Trolox (Figure 10.13). The concentration of these antioxidants decreased markedly in the corresponding oil-in-water emulsions, in the order propyl gallate and rosmarinic acid, catechin, Trolox and gallic acid. The order of this decrease in partition in the water phase between oil-water mixtures and the corresponding emulsions can be attributed to the association or affinity of the antioxidants with the Tween 20 emulsifier.

In emulsified corn oil systems, the hydrophilic antioxidants favored the interface or surfactant enriched environment compared to the oil phase (Table 10.17). All these hydrophilic antioxidants showed lower affinity towards the SDS micelle systems, because of the lower bonding capacity of the negatively charged SDS compared to the other surfactants. The bulk of caffeic acid, gallic acid and catechin resided in the aqueous phase of SDS micelles. Caffeic acid, propyl gallate and gallic acid showed the highest proportion in the aqueous phase with the positively charged DTABr surfactant. These hydrophilic antioxidants thus favor the interface or surface-enriched environments compared to the oil phase. In the corn oil system emulsified with Tween 20, a significant proportion of this emulsifier was estimated to be not only in the oil–water interface (26–68 %) but also formed micelles (32–74 %). The low antioxidant activities of Trolox and other hydrophilic antioxidants (rosmarinic acid,

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Table 10.17. Partition of antioxidants between different surfactant environments in emulsion systems[a]

Antioxidants	Surfactant	% in water phase	% in oil phase	% in surfactant phase
Ferulic acid	Tween 20	48.6	8.9	42.5
Ferulic acid	SDS micelles	54.1	9.9	36
Ferulic acid	DTABr	51.4	9.4	39.2
Caffeic acid	Tween 20	54.6	1.5	43.9
Caffeic acid	SDS micelles	75.9	2.1	22
Caffeic acid	DTABr	42.2	1.2	56.6
Propyl gallate	Tween 20	42.9	9.6	47.5
Propyl gallate	SDS micelles	57.6	12.9	29.5
Propyl gallate	DTABr	19.4	21.1	59.5
Gallic acid	Tween 20	68.2	1.9	29.9
Gallic acid	SDS micelles	82.1	2.4	15.5
Gallic acid	DTABr	62.9	1.8	35.3
Trolox	Tween 20	26.7	23.6	49.7
Trolox	SDS micelles	38.6	34.4	27
Trolox	DTABr	26.4	23.6	50
Catechin	Tween 20	48.1	0.9	51
Catechin	SDS micelles	84	1.6	14.4
Catechin	DTABr	54.8	1.1	44.1

[a]From Schwarz *et al.* (1996). Proportions of antioxidants in the oil and surfactant phases were calculated on the assumption that the oil phase is identical to the bulk oil in biphasic systems.
Abbreviations: Tween 20, polyoxyethylene sorbitan monolaurate; SDS, sodium dodecylsulfate; DTABr, dodecyltrimethylammonium bromide.

catechin, propyl gallate, gallic acid and tea catechins) in oil-in-water emulsions were attributed to their partition into the Tween 20 micelles and into the aqueous phase.

The partitioning behavior of different phenolic antioxidants in oil-in-water emulsions is greatly affected by interactions between oils and emulsifiers that determine their solubilization equilibrium. The proportion of phenolic antioxidants solubilized in the lipid phase in oil-in-water emulsions varied according to the emulsifiers and polarity of the antioxidants (Table 10.18). The anionic polar emulsifier sodium dodecyl sulfate (SDS^-) solubilized antioxidants in the lipid phase of an emulsion in reverse order of their polarity according to the number of hydroxyl groups in their structures: *p*-hydroxybenzoic > 3,4-dihydroxy benzoic ethyl ester > ethyl gallate. The binding capacity of different emulsifiers for ethyl gallate with three hydroxyl groups decreased in the order: cationic cetyltrimethyl ammonium bromide ($CTAB^+$) > non-ionic Brij 58 > anionic SDS^- > zwitterionic partially hydrolysed lecithin ($PHLC^{\pm}$). The lipid phase solubilization capacity of different emulsifiers in oil-in-water emulsions is thus based on their relative charge and on the relative polarity of the antioxidants. However, the antioxidant activity of ethyl gallate did not

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Table 10.18. Lipid phase solubilization capacity of different emulsifiers for phenolic antioxidants in oil-in-water emulsions[a]

Antioxidants	Emulsifiers	% Solute in lipid phase	% Inhibition of hydroperoxide[a]
Ethyl gallate	SDS	49.9	95.6
3,4-Dihydroxy benzoic ethyl ester	SDS	76.0	–
p-Hydroxybenzoic ethyl ester	SDS	91.7	–
Ethyl gallate	CTAB	95.2	–
Ethyl gallate	Brij 58	72.3	69.8
Ethyl gallate	PHLC	45.1	73.9

[a]From Stöckmann and Schwarz (1999). Oil-in-water emulsions were prepared with 20% corn oil and 1% emulsifier in 0.2 M acetate buffer (pH 5). SDS = sodium dodecyl sulfate, CTAB = cetyltrimethyl ammonium bromide, Brij 58 = polyoxyethylene 20 cetyl ether, PHLC = partially hydrolysed soybean lecithin. Amount of solute solubilized in oil lipid phase of emulsions was calculated from the difference between the total amount in the system and the amount of oil solubilized in the aqueous phase determined by ultrafiltration and semiequilibrium dialysis.
[a]From Stöckmann *et al.* (2000). Emulsions were prepared with 20% corn oil stripped of tocopherols and 1% emulsifier, stored at 37°C for 24 days.

correspond to the binding capacity of these emulsifiers for ethyl gallate, and decreased in the order: SDS > PHLC> Brij 58 (no activity with CTAB). The relationship between the properties of emulsifiers and antioxidant activity may thus involve additional parameters, including hydrogen donating ability, hydrophobic interactions and structural properties, but their effect on antioxidant activity is complex and poorly understood.

Surface-active phenolic antioxidants can partition into different phases of emulsions according to their polarity. The oxidative stability of lipid emulsions can therefore be changed by the solubilizing effect of emulsifiers. When present in excess of the level needed to saturate the surface of emulsion droplets, emulsifiers form micelles that solubilize nonpolar compounds causing antioxidants to partition from the lipid to the aqueous phase. The phenolic content of the continuous phase in olive oil emulsions decreased in the reverse order of antioxidant polarity: propyl gallate > TBHQ > BHT (Table 10.19). In a salmon oil emulsion, although more TBHQ (13.5 ppm) was solubilized in the continuous phase by added emulsifier than in the olive oil emulsion (9.3 ppm), TBHQ was effective in inhibiting lipid oxidation. Since under the same conditions the emulsifier also inhibited lipid oxidation of the control salmon oil emulsion, the increased antioxidant activity obtained with TBHQ may be due to synergistic interactions with the polar emulsifier that may improve protection of the polyunsaturated salmon oil substrate. A similar synergistic antioxidant effect is known with mixtures of lecithin and phenolic antioxidants.

In foods, the partition of antioxidants between non-aqueous and aqueous phases depends on the solvent properties of the phases and their interaction

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Table 10.19. Partitioning of antioxidants by Brij 700 in continuous phase of olive oil and salmon oil-in-water emulsions[a]

Antioxidants in emulsion (20 ppm)	Added emulsifier (%)	Olive oil emulsion	Continous phase	Salmon oil emulsion	Continuous phase
		Emulsifier (%)	Antioxidant (ppm)	Emulsifier (%)	Antioxidant (ppm)
Control	0.0	0.2	0.8	0.3	0.1
	2.5	2.6	1.4	2.8	1.4
Propyl gallate	0.0	0.3	5.4	–	–
	2.5	2.8	12.5	–	–
TBHQ	0.0	0.2	2.6	0.3	5.4
	2.5	2.5	9.3	2.9	13.5
BHT	0.0	0.2	0.4	–	–
	2.5	2.8	2.0	–	–

[a]From Richards *et al.* (2002). Emulsions contained 5% lipid, 1% emulsifier (Brij 700 = polyoxyethylene fatty ether) and 10 mM acetate–imidazole buffer at pH 3.0. Partitioning by centrifugation was followed by analysing the continuous phase for emulsifier by density measurements and for antioxidants by total phenolic concentrations. Oxidations of emulsions were carried out at 32°C.

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with the emulsifier and other surface-active components in the system. Partition of antioxidants into the non-aqueous phase can exert an important effect on activity. Surfactants can significantly increase the solubility of hydrophilic antioxidants in the lipid phase and may exert a large positive effect on activity. A negative effect may occur, however, in the presence of catalytic metals that can be reduced and thus become more active in the presence of hydrophilic phenolic antioxidants. Partitioning of antioxidants according to polarity, surface accessibility and interactions with emulsifiers, are important parameters affecting their antioxidant activity in multiphase lipid-containing systems. However, the effects of antioxidant–emulsifier mixtures on their activity to inhibit lipid oxidation is still difficult to predict due to complex and multiple antioxidant or prooxidant interactions.

The oxidative stability of multiphase food lipids varies according to their colloidal location, because of their compartmentalized exposure to prooxidants and antioxidants. The partition behavior of antioxidants may provide, therefore, an important tool for the delivery of specific inhibitors in highly oxidizable lipid surfaces. The dynamic nature of the interface in multiphase systems may play an important part during storage and would affect lipid oxidation. More direct tools are required to measure antioxidant partition at the interfacial layer playing a critical role on lipid oxidation. Other factors that should be considered include the relative stability of antioxidants and their anomalous behavior in different colloidal phases. The critical role of discrete phases in lipid oxidation must now be recognized for a better understanding of the oxidative stability of food lipids and the effect of antioxidants, but the exact details still remain unclear. A great deal of research is needed to understand better the relationship between the multitude of factors that influence the partition of antioxidants, including the relative polarity of antioxidants and emulsifiers, their concentrations, type of lipid substrates, pH and conditions of oxidation. Recent studies on real food emulsions will be discussed in the next Chapter.

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CHAPTER 11

Foods

Lipid oxidation is the major form of deterioration in foods, even when the lipid content is very small. Quality problems due to lipid oxidation are in fact aggravated in low-fat foods, where oxidation decomposition products are more readily volatilized and perceived and have a greater impact on off-flavors. The rancidity developed in foods causes changes in quality that affect their flavor, odors, taste, color, texture and appearance. Thus, lipid oxidation is a decisive factor in the useful storage of food products. The changes due to oxidation occur at different stages of food preparation, ranging from the raw materials, through processing, packaging, storage, cooking, and various retail or large scale applications. An appreciation of various oxidation factors in food processing is necessary to control and minimize the impact of lipid oxidation on food quality.

A. Raw materials

1. Metals and metallo-proteins

Traces of catalytic metals are present in enzymes and bound with proteins. Heme (Fe^{2+}) and hemin (Fe^{3+}) proteins occur in many plant and animal foods. In animal tissues, lipid oxidation is catalysed by hemoglobin, myoglobin and cytochrome c. Heme catalysis is of widespread significance in meats, and poultry, frozen fish, milk and plant foods by causing rancidity and the destruction of natural tocopherols and carotenoids. Important heme and hemin proteins in plant foods include peroxidase and catalase. These heme compounds are much more active than the free metal ions in catalysing decomposition of lipid hydroperoxides (Table 10.5). The resulting alkoxyl radical intermediates are active in initiating lipid oxidation. However, when horseradish peroxidase is heated, the prooxidative activity of the denatured proteins is increased significantly (Table 11.1). Therefore, the mixture of free iron and heat-denatured proteins becomes an effective non-enzymatic catalyst of lipid oxidation. Thermal denaturation of heme proteins is a significant factor affecting adversely the stability and quality of processed meat and fish products.

2. Enzymatic oxidation in plant foods

Various hydrolytic and oxidative enzymes affect lipid oxidation in raw materials when tissues are disrupted or injured. Enzyme action occurs sequentially with

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Table 11.1. Effect of temperature on activity of horseradish peroxidase and on non-enzymatic lipid oxidation.[a]

Temperature (°C) [b]	Enzyme activity (%)	Non-enzyme activity (nmol O_2/min)
25	100	20
50	100	25
75	80	40
100	75	85
125	30	130

[a] From Eriksson (1987).
[b] Heating time 2 min.

the initial hydrolysis of triacylglycerols or lipolysis catalysed by lipases producing free fatty acids. The polyunsaturated free fatty acids are then oxidized by lipoxygenases, which represent important oxidizing enzymes found in plant raw materials, and in animal tissues. In plant foods, lipases and lipoxygenases are responsible for the formation of characteristic flavors. Lipolysis occurs readily after crushing or grinding oilseeds and vegetables. The resulting off-flavor production can be prevented by heat inactivation of lipases and lipoxygenases. For example, raw peanuts can be stored without lipid oxidation for over one year if they remain whole and intact. However, if the peanuts are ground, they undergo flavor deterioration readily due to enzyme activation. On the other hand, if the peanuts are dry-roasted to inactivate enzymes, they are oxidatively stable.

Lipoxygenases (Lox) are selective towards polyunsaturated fatty acids containing the *cis,cis*-1,4-pentadiene moiety to produce either the 9(*S*)-hydroperoxide, 13(*R*)-hydroperoxide, or a mixture of both from linoleic and linolenic acids. These enzymes contain an iron atom in their active center. They are activated by hydroperoxides, and the Fe^{2+} is oxidized to Fe^{3+}, according to a scheme in which the pentadiene radical of linoleic acid becomes bound to the enzyme, reacts with oxygen, and the peroxyl radical formed is reduced by the enzyme to produce a hydroperoxide after reaction with a proton (1).

$$\begin{aligned} &\text{Lox-Fe}^{2+} + \text{LOOH} \longrightarrow \text{Lox-Fe}^{3+} + \text{LH} \longrightarrow \text{Lox-Fe}^{2+}\text{-L}^{\cdot} \\ &\text{Lox-Fe}^{2+}\text{-L}^{\cdot} + \text{O}_2 \longrightarrow \text{Lox-Fe}^{2+}\text{-LOO}^{\cdot} + \text{H}^{+} \longrightarrow \text{LOOH} \end{aligned} \quad (1)$$

Several isoenzymes of lipoxygenase are known, including L-1 and L-2 from soybeans, with pH optima of 9.0 and 6.5, respectively. Isoenzymes L-1 with high pH optima prefer anionic free fatty acids, and L-2 with pH optima near neutral prefer neutral substrates such as triacylglycerols. L-1 produces the 13-hydroperoxide stereoselectively from linoleic acid, while L-2 produces a

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Table 11.2. Formation of volatile compounds produced by different lipoxygenases

Lipoxygenases	Substrates	Main volatiles [a]	Ref. [b]
Soy L-1	18:2 *n*–6	hexanal, *t*2,*t*4-decadienal	(1)
Soy L-2	18:2 *n*–6	hexanal, *t*2,*t*4-decadienal, *t*2,*c*4-decadienal, *t*-2-heptenal, *t*-2-octenal	(1)
Soy L-1	18:3 *n*–3	*t*-2-hexenal, propanal, pentenal	(2)
Soy L-2	18:3 *n*–3	propanal, *t*2,*c*4-heptadienal, *t*2-pentenal, *t*-2-hexenal, 2,4,6-nonatrienal, 3,5-octadien-2-one	(3)
Pea L	18:2 *n*–6	hexanal, 2,4-decadienal, 2-heptenal, 2-octenal, pentanal, 2,4-nonadienal	(2)
Pea L	18:3 *n*–3	*t*2,*c*4-heptadienal, propanal, 2-pentenal, acetaldehyde, crotonaldehyde, 2-hexenal	(2)
Potato L	18:3 *n*–3	2,6-nonadienal, 2-hexenal, propanal, 3,5-octadien-2-one, 2,4-heptadienal	(4)
Beans L	18:3 *n*–3	2-hexenal, propanal, 2,4-heptadienal	(4)
Peanut L	18:2 *n*–6	pentane, hexanal	(5)

[a] *t*, *trans*; *c*, *cis*
[b] (1) Fischer and Grosch, 1977); (2) Grosch (1968); (3) Grosch and Laskawy (1975); (4) Grosch *et al.* (1976); Singleton *et al.* (1976).

mixture of 9- and 13-hydroperoxides from linoleic acid and linoleic glycerides. Thus, isoenzyme L-2 does not require prior lipolysis of triacylglycerols to be active in foods. L-2 can also cooxidize carotenoids and chlorophyll into colorless products. This property of L-2 is used to bleach flour (Section F.2).

3. Enzymatic and non-enzymatic volatile decomposition products

The hydroperoxides produced by lipoxygenases undergo cleavage, either by hydroperoxide lyases or non-enzymatically, and other reactions including isomerization, and rearrangement to produce aldehydes, C-18 and short-chain alcohols, vinyl ethers, oxo acids (or aldehyde acids), epoxy-hydroxy esters, and dimers that contribute to rancid flavors. Hydroperoxides from both isoenzymes L-1 and L-2 produce a wide spectrum of volatile aldehydes from lipids similar to those formed by non-enzymatic autoxidation (Chapter 3). From linoleic acid, isoenzyme L-1 yields mainly hexanal and *trans*-2,*trans*-4-decadienal, while L-2 forms hexanal, *trans*-2-heptenal, *trans*-2-octenal, *trans*-2,*trans*-4-nonadienal and *trans*-2-*cis*-4-decadienal (Table 11.2). From linolenic acid, L-1 produces mainly *trans*-2-hexenal, propanal and pentenal, and L-2 produces the same aldehydes plus *trans*-2,*cis*-4-heptadienal, 2,4,6-nonatrienal, and 3,5-octadien-2-one.

Hydroperoxides from lipoxygenase isoenzymes in peas, potatoes and beans produce similar aldehyde mixtures from linoleic and linolenic acids. Peanut lipoxygenase forms pentane and hexanal. Under anaerobic conditions,

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Table 11.3. Flavor scores and gas chromatographic analyses of soybean oils [a]

Oils	Storage 60°C	Flavor score [b]		2,4-decadienal	
		Heated [c]	Unheated	Heated [c]	Unheated
Crude	0-time	6.4	6.6	20	20
	24 days	6.5	5.9	190	270
Degummed	0-time	6.5	6.7	20	20
	8 days	6.4	6.5	210	410
Refined	0-time	7.0	6.2	80	60
	4 days	6.0	6.4	620	430
Bleached	0-time	6.6	5.1	30	30
	2 days	6.3	5.6	140	150
Deodorized	0-time	8.1	8.5	6	8
	8 days	6.2	6.2	200	250
Defatted flour [d]	0-time	6.3	5.2	–	–

[a] From Frankel *et al.* (1988).
[b] Flavor score: 1–10; 10, bland; 1, strong. Crude and partially processed oil tasted after dilution 95:5 with good quality soybean oil; deodorized oils tasted undiluted.
[c] Dehulled, cracked soybeans heated with steam for 2 min.
[d] Tasted as 2% dispersion in water.

lipoxygenase reactions produce oxo acids, dimeric fatty acids and oxygenated dimers that may be derived by free radical intermediates. In many of these decomposition reactions, it is difficult to distinguish between enzymatic and non-enzymatic processes, which may occur either sequentially or simultaneously. After heat inactivation, lipoxygenases and other enzymes can act as non-enzymatic metallo-protein catalysts that may be activated under reducing conditions. During crushing and solvent extraction of oilseeds, metallo-proteins may dissociate, and the free metals may form metal salts with any free fatty acids and catalyse lipid oxidation during processing.

To prevent flavor deterioration due to the action of lipoxygenases and other enzymes, unprocessed plant foods such as beans and peas are blanched before canning or freezing to inactivate these enzymes. These heat treatments must be carried out under mild conditions to avoid undesirable changes in texture and loss of protein bioavailability.

Before solvent extraction, soybeans are cracked, heated (65–75°C) and the moisture content adjusted (10–11%) before flaking. This heat treatment is not sufficient to inactivate the lipases and lipoxygenase, which requires temperatures of 100–110°C. Treating soybean flakes with steam for 2 min inactivated L-1 and L-2 and produced higher initial flavor scores of refined and bleached oils and higher flavor scores after storage of the crude and bleached oils (Table 11.3). The crude, degummed, bleached and deodorized oils from the heated beans produced less 2,4-decadienal after storage at 60°C than the oils from unheated beans. The treatment of beans with steam was also important to

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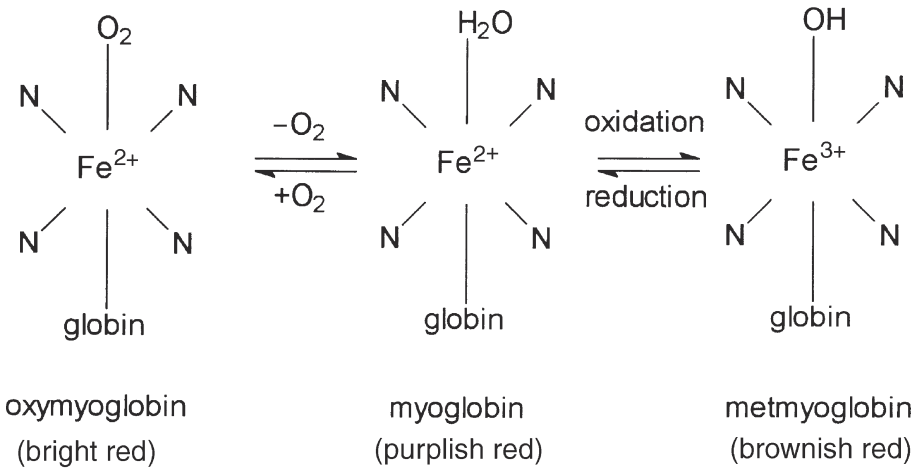


Figure 11.1. Schematic representation of the heme complex of myoglobin: iron bound to the nitrogen atoms of four pyrole rings in the porphyrin molecule and to the globin protein. From Cross *et al.* (1986).

produce defatted flour of higher flavor score and quality than the unheated beans.

Toasting of soybeans by heating at 100–110°C to inactivate lipoxygenase and antinutritional factors (e.g. trypsin inhibitor) is important to improve the quality of soybean protein products used for either animal feed or human consumption. However, this thermal enzyme inactivation is not carried out in the conventional processing of soybean oil. Therefore, the soybean flakes must be solvent extracted without delay to minimize free fatty acid and peroxide formation in the extracted crude oil and to produce a finished oil of improved oxidative and flavor stability.

4. Enzymatic and non-enzymatic oxidation in animal foods

Three major forms of metal-containing systems catalyse lipid oxidation in raw meat and seafood, namely heme iron, non-heme iron and microsomal enzymes. Myoglobin is the predominant hemoprotein responsible for the color of red meat. In myoglobin, the iron atom is bound covalently to the nitrogen atoms of the four pyrrole rings of the porphyrin structure, and one nitrogen of a histidine molecule of the globin protein (Figure 11.1)

The sixth bond orbital reacts with oxygen or water, involved in oxidation and reduction. The role of myoglobin in lipid oxidation in meat is still unclear and being debated. Microsomal enzymes in raw meat and fish catalyse lipid oxidation by maintaining iron in the reduced form. The relative activity of these metal-containing systems is strongly affected by heat treatments. The non-

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enzymatic catalytic system becomes more significant at higher temperatures and in cooked meat.

Many enzymes in milk are known to initiate lipid oxidation. *Trypsin* shows a protective effect that may be attributed to partial proteolysis increasing the copper-chelating capacity of milk. *Xanthine oxidase* is a flavin enzyme found as a significant component of the milkfat globule membranes that generates superoxide radicals ($O_2^{-\bullet}$). This reactive oxygen species is formed when oxygen takes up one electron, and occurs as an anion radical acting as a reducing agent. Under acidic conditions, $O_2^{-\bullet}$ has free radical activity producing the hydroperoxy radical ($HO_2^{\bullet}$), which undergoes dismutation to produce hydrogen peroxide (2).

$$O_2^{-\cdot} + H^+ \longrightarrow HO_2^{\cdot} + H^+ \longrightarrow H_2O_2 + O_2 \qquad (2)$$

Superoxide dismutase catalyses the dismutation of the superoxide radical $O_2^{-\bullet}$ to produce H_2O_2, a reaction that occurs in animal and plant tissues. The formation of metmyoglobin or ferric "methemoglobin" is also accompanied by the formation of $O_2^{-\bullet}$, which is readily converted to H_2O_2. The generation of H_2O_2 takes place in turkey muscle tissues, and increases during aging at low temperatures. By removing superoxide from tissues, superoxide dismutase can have antioxidant activity in the presence of catalase to remove H_2O_2 (Chapter 10. E).

Hydrogen peroxide produced by reduction of $O_2^{-\bullet}$ is relatively innocuous and has limited reactivity in the absence of metals. However, in the presence of Fe^{2+}, H_2O_2 produces the very reactive hydroxyl radical ($HO^{\bullet}$) by the so-called Fenton reaction (see Chapter 13). Iron compounds complexed with phosphate esters (adenosine di- and triphosphate, ADP and ATP) can decompose H_2O_2 to form free radicals. The presence of the ADP-Fe complex in plant and animal foods may trigger the reduction of H_2O_2 to form the very reactive and potentially damaging hydroxyl radicals (3).

$$\begin{aligned} &ADP\text{-}Fe^{3+} + O_2^{-\cdot} \longrightarrow ADP\text{-}Fe^{2+} + O_2 \\ &ADP\text{-}Fe^{2+} + H_2O_2 \longrightarrow HO^{\cdot} + OH^- \end{aligned} \qquad (3)$$

Hydroxyl radicals react non-selectively with all organic molecules. There is evidence that hydroxyl radicals are generated in muscle homogenates by heating, and in the presence of iron-ascorbate, but their importance with respect to lipid oxidation is unclear.

Myoglobin is an iron-heme protein that constitutes the most abundant pigment in muscle tissues. Autoxidation of myoglobin produces metheproteins, the ferric states of these proteins (Figure 11.1). The interaction of hydrogen

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peroxide with metmyoglobin (MetMb) or methemoglobin (MetHb) generates activated heme proteins, in which the iron is converted to an unstable hypervalent (higher oxidation state) by the formation of a porphyrin (P) cation radical, P^{+}-Fe^{+4}=O, a ferryl protein radical species considered to be more selective than the hydroxyl radical, as an initiator of lipid oxidation in muscle foods. The perferryl Fe^{+4} cation radical is rapidly reduced to the more stable ferryl-Fe^{4+} state. The mechanism of lipid oxidation proposed for hemoglobin and myoglobins involves activation of the iron catalyst by the formation of ferryl ion, as follows (4):

$$\begin{aligned} &P\text{-}Fe^{+3} + H_2O_2 \xrightarrow{H} P\text{-}Fe^{+3}\text{-}\overset{H}{O}\text{-}OH \\ &P\text{-}Fe^{+3}\text{-}\overset{H}{O}\text{-}OH \xrightarrow{H} P\text{-}Fe^{+4}\text{-}O^{\cdot} + H_2O \\ &P\text{-}Fe^{+4}\text{-}O^{\cdot} \longrightarrow P^{+}\text{-}Fe^{+4}\text{=}O \end{aligned} \qquad (4)$$

The ferryl-Fe^{4+} is slowly reduced to the met-form. If the globin structures of deoxy-, oxy-or met-forms of myoglobin are disturbed, they are reversibly or irreversibly converted into hemochrome-Fe^{2+} or hemichrome-Fe^{3+}. The role of these denatured species in lipid oxidation of meat is not clear. When ferric hemin is detached from the globin, the ferryl ion can initiate lipid oxidation by hydrogen abstraction to produce a lipid radical plus a proton. Iron can also be released from hemin in the presence of hydroperoxides to participate in lipid oxidation processes. Ascorbic acid and other reducing compounds in muscle cytosol effectively inhibit lipid oxidation promoted by ferryl ions in membranes.

Free iron and copper ions catalyse redox reactions in muscle lipids. Ferritin is the main iron storage protein in the cells that has a large cavity holding up to 4,500 ions of iron per mole. Iron can be released from ferritin by reducing agents. The formation of superoxide as a reductant may thus be a main pathway for the synthesis of myoglobin in muscle cells by mitochondria. A significant amount of iron is released from ferritin during storage of turkey muscle (Table 11.4).

Whether lipid oxidation in muscle foods is catalysed by the iron redox cycle or by formation of the ferryl ions is not clear. However, ferrous ions react with lipid hydroperoxides much faster than with hydrogen peroxide. As shown above, if the reaction of metmyoglobin with hydroperoxides produces ferryl radicals capable of initiating lipid oxidation, it is necessary to prevent the formation of metmyoglobin or methemoglobin. At acidic pH, ferric myoglobin can initiate lipid oxidation in the presence of lipid hydroperoxides.

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Table 11.4. Loss of iron from ferritin isolated from turkey muscle after storage at 4°C [a]

Storage time(days)	Dark muscle	Light muscle
0	1.50	0.55
2	1.14	0.42
9	0.60	0.08

[a] From Kanner (1992). Fe-ferritin in μg/g wet tissue.

Deoxyhemoglobin and deoxymyoglobin have a vacant iron-binding site and in the presence of air can catalyse lipid oxidation. Because fish and chicken have more deoxyhemoglobin than beef, they are generally more active catalysts of lipid oxidation (Table 11.5). In the presence of oxygen, ferrous deoxyhemoglobin undergoes rapid autoxidation to ferric methemoglobin with formation of superoxide. The rapid autoxidation rate observed in trout was attributed to its higher amounts of ferrous deoxyhemoglobin compared to chicken and beef hemoglobin. Fully oxygenated heme proteins should be thus more resistant to autoxidation than heme proteins containing higher amounts of ferrous deoxyhemoglobin.

The mechanisms of myoglobin-induced oxidation of polyunsaturated lipids are still being debated in the literature. Redox mechanisms were proposed that ferrous oxymyoglobin and deoxymyoglobin as well as ferrylmyoglobin may initiate lipid oxidation with formation of ferric metmyoglobin. Most of the empirical evidence for these species was based, however, on indirect experiments with hydrogen peroxide rather than lipid hydroperoxides. Decomposition products of polyunsaturated hydroperoxides such as 2-enals and 4-hydroxynonenal are known to oxidize oxymyoglobin to produce metmyoglobin. 4-Hydroxynonenal has been identified in stored meat. The mechanism proposed for the oxidation of oxymyoglobin involves protein modification by Michael addition with cysteine, histidine or lysine (Figure 13.7).

Table 11.5. Relative autoxidation rates of hemoglobin from trout, chicken and beef during storage at 4°C and formation of methemoglobin [a]

Hemoglobin type	Relative rate of autoxidation	Absorbance at 630 nm
Trout	1.00	0.027
Chicken	0.29	0.013
Beef	0.09	0.007

[a] From Richards and Dettmann (2003). Rates are derived from slopes of time plots vs % ferric methemoglobin.
Absorbance at 630 nm reflects the formation of ferric methemoglobin from ferrous hemoglobin.

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Most of the copper present in animal tissues is inactivated by chelation or binding to proteins in the form of ceruloplasmin, albumin, carnosine and other histidine-dipeptides. These chelating compounds are good inhibitors of lipid oxidation in meat and fish. Carnosine present in muscle tissues can also reduce ferryl to oxymyoglobin.

The 12-lipoxygenase and 15-lipoxygenase have been identified in fish gill tissue and in chicken muscle by their production of 12-hydroperoxide and 15-hydroperoxide from arachidonic acid and other polyunsaturated fatty acids in fish. The substrate dependency and product specificity toward other long-chain polyunsaturated fatty acids in fish (20:5 *n*–3, 22:4 *n*–6, 22:5 *n*–3) indicate that the 12-lipoxygenase is specific towards the methyl rather than the carboxyl end and can be described as an *n*–9 lipoxygenase. The hydroperoxides produced by these enzymes may be decomposed in the presence of metal catalysts to form volatile carbonyls that cause off-flavors in fish and frozen chicken meat.

B. Processed foods

A number of processing steps affect the oxidative stability of foods, including heat treatment, drying, fermentation, storage and packaging. The two most important factors due to processing include changes in water activity and the formation of non-enzymatic browning materials at elevated temperatures by the interactions of sugars or lipid oxidation products with proteins and amino acids. These compounds act as oxidation inhibitors, and their formation is influenced by the water activity, which affects the quality of foods and their nutritional value.

1. Water activity

Water is a key factor affecting lipid oxidation in foods during storage by a complex series of events. The water activity of a food is a more appropriate physicochemical property than water content, because in heterogeneous systems it determines the distribution of water between different components. Water activity (Aw) or the thermodynamic availability of water is defined as:

$$Aw = p/p_o$$

where, p = partial pressure of water in food, and p_o = vapor pressure of water.

Water activity is often used as a predictor of food quality, but problems arise from the inherent non-equilibrium nature of foods, and the kinetics of changes in moisture content must be followed as a function of temperature. The concept of water activity is rigorously applicable only in dilute aqueous solutions at equilibrium.

Lipid oxidation is high at low water activity (0.1–0.2) but decreases as water activity is increased to a minimum, below the monolayer value up to 0.2–0.4,

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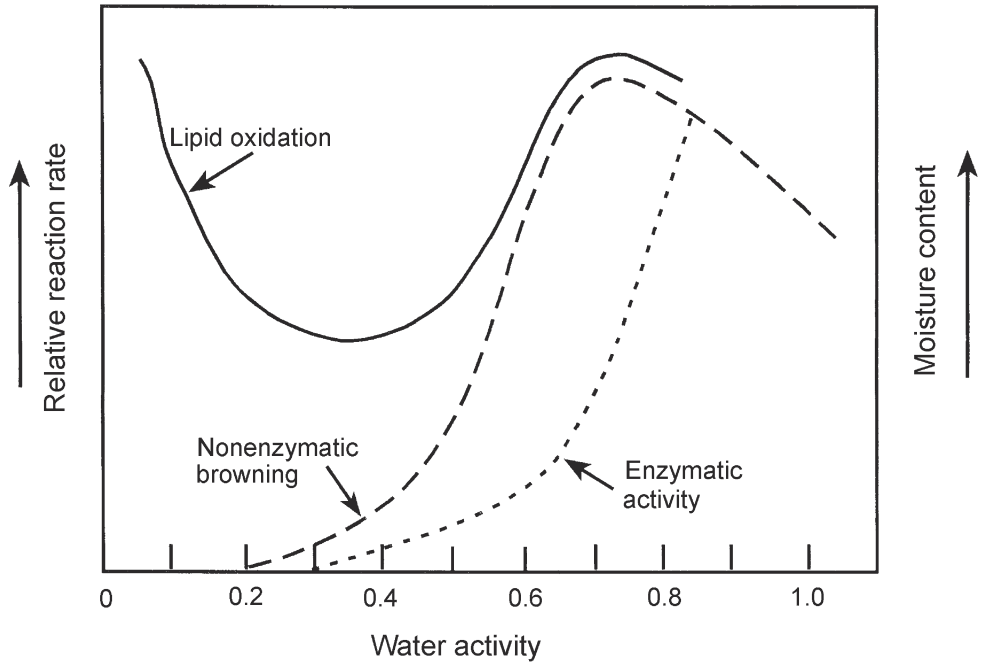


Figure 11.2. Food stability as a function of water activity. Adapted from Labuza (1971).

and then increases again above 0.4, reaching a maximum in the range 0.6–0.8 (Figure 11.2). This "food stability map" is problematic, because the relative rate of lipid oxidation, which is a kinetic property, cannot be plotted against water activity, which is an equilibrium thermodynamic function. The equilibrium nature of water activity should be clearly distinguished from the kinetics of lipid oxidation.

Several mechanisms have been suggested to explain the effect of water activity on lipid oxidation in foods. When dry, the metal catalysts are most active. At increasing moisture levels, lipid oxidation may decrease as a result of hydrogen bonding between water and hydroperoxides, thus retarding their decomposition into initiating radicals, and by decreasing the prooxidant activity of metal catalysts by hydration and changes in their coordination sphere. Metal salt hydrates are formed that are less lipid soluble and less active. At high water activity levels, lipid oxidation may be accelerated by increasing the diffusion and mobilization of the metals in the aqueous phase and by allowing more intimate association with the lipid–water interface where polar hydroperoxides concentrate. Above a water activity of 0.7, lipid oxidation may decrease again, apparently as a result of dilution of the metal catalysts. Accordingly, foods of intermediate moisture would be very susceptible to oxidation.

Water has a profound effect on interactions between lipid oxidation products

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and other food components, including heme compounds, amino acids and proteins. The oxidative stability of several foods is greatly affected by their water activity. For example, walnuts are oxidatively stable at a water activity of about 0.3, and become less stable at higher (0.45–0.54) and lower (0.26) water activities. In another example, potato chips oxidize much slower at a water activity of 0.4 compared to lower water activities, reaching a maximum rate at a water activity of 0.01. Foods that contain their natural moisture, such as refrigerated cooked meats, are readily oxidized. Meat proteins may also oxidize, and increasing the water content often accelerates this oxidation. The type of rancidity developed in foods in the dry state may be different compared to the normal moisture state. Carbonyls produced by lipid oxidation can also interact with proteins in the presence of moisture and lead to browning. Similarly, the effectiveness of antioxidants and metal chelators is affected by the moisture content of foods (Section C.3). Therefore, precautions are necessary in the preparation and storage of foods susceptible to lipid oxidation by using either packaging materials of low permeability to water vapor, or vacuum packaging, to reduce changes in the food water activity.

Although the concept of water activity and its effect on food stability is theoretically limited in its predictive capability, it continues to prove empirically useful. A better understanding of the relationship between moisture content and the rate of lipid oxidation may be obtained by a new approach known as the glass transition theory, which considers foods as polymers or a mixture of macromolecules. This theory has been used successfully to understand the physicochemical properties of synthetic polymers, and is now being exploited by food scientists to explain the complex relationship between water content and stability. Like polymers, foods can exist in either "glassy" or "rubbery" states according to their temperature and moisture content. The glass transition temperature (T_g) is the temperature at which amorphous regions of polymers change from a glassy state to a rubbery state. In the glassy state there is less free volume, or space in the food matrix that is not taken by the biopolymer chains of the macromolecules, than in the rubbery state, which has open or porous surfaces. The limited free volume present in the glassy state hinders the diffusion of a small molecule like oxygen through the food matrix (Figure 11.3). This temperature-composition state diagram shows zones of different stabilities, where the glass transition temperature is dependent on solute composition and water content.

According to the glass transition theory, an important property of water is to act as an excellent plasticizer, which swells and increases the free volume of the food matrix. An increase in moisture results in increased diffusion and molecular mobility in a multi-component food system, accelerating lipid oxidation by increasing the number of catalytic sites. Metals become more effective prooxidant catalysts by their increased mobility in the rubbery state. The molecular mobility decreases in solutes of increasing molecular weight. In

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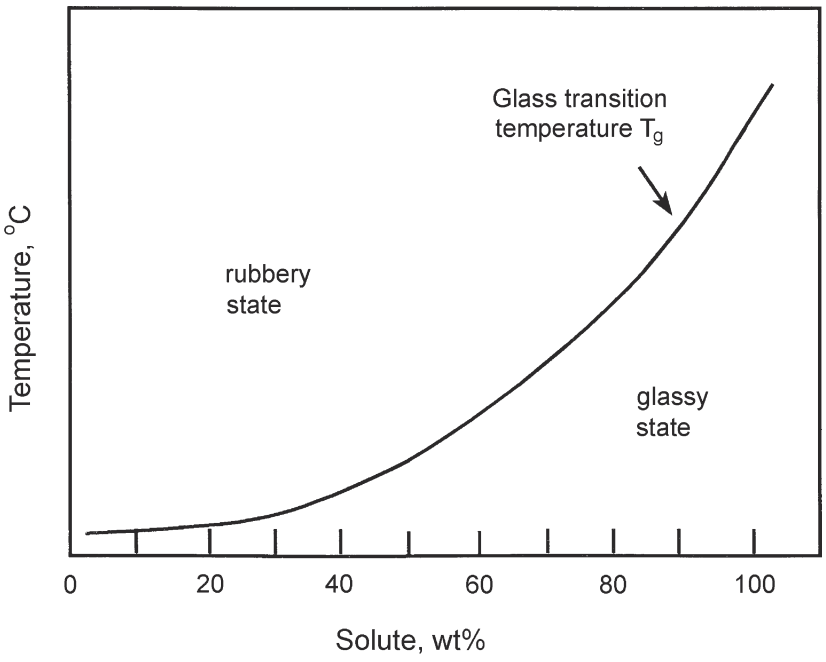


Figure 11.3. State diagram of a binary food system. Adapted from Nelson and Labuza (1992).

polymer science, the rate of chemical reactions increases at an exceedingly high value by raising the temperature above the glass transition temperature range. In the glassy state, diffusion in the food matrix is restricted and reaction rates are diminished. Accordingly, to increase food stability, formulations should be aimed at either bringing the matrix into the glassy state or increasing its glass transition temperature.

Freeze-dried or spray-dried sugar-containing foods remain in the glassy state at low moisture conditions. At high moisture conditions, the "glasses" can collapse, and mobility allows the glasses to be converted to an amorphous or rubbery state that facilitates the diffusion of oxygen and catalytic metals thus accelerating oxidation. Water thus acts as a plasticizer that facilitates diffusion of oxygen to lipids entrapped in the food matrix. The prediction of food stability is thus complicated by the interactive influences of moisture, water activity, the state of the water and other factors influencing the transition from glassy to rubbery states in moist foods.

According to the glass transition theory, lipids are dispersed in the free volume of the food matrix composed of carbohydrates and protein polymers. In the rubbery state, the lipids react readily with oxygen and become oxidized. In the glassy state, however, the lipids are stable to oxidation because they are encapsulated and there is no free volume. The glass transition temperature, which determines when the food matrix changes from one state to the other, increases with a decrease in moisture and water activity. In many foods the

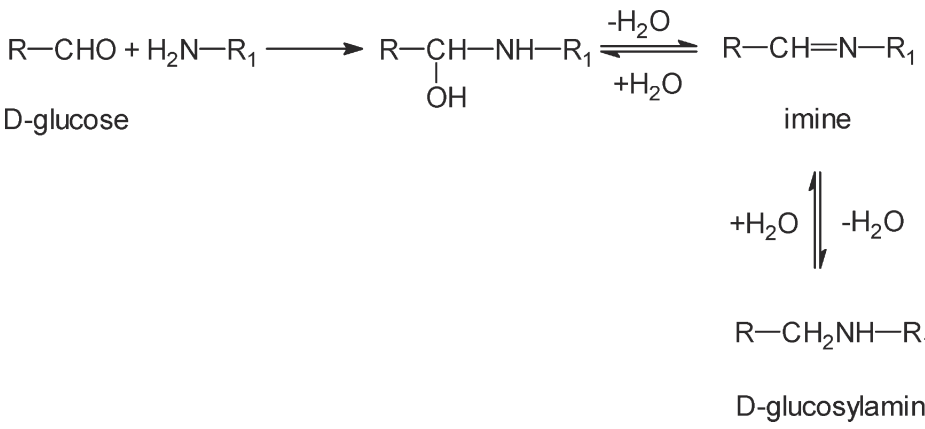
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difference in oxidizability of different portions of lipids may be due to their different transitions between rubbery and glassy states. Therefore, to control lipid oxidation in foods, it would be necessary to vary the formulation or processing conditions to minimize or eliminate the oxidizable lipid portions. However, this goal is difficult to achieve because the factors that determine how much of the lipids can be stabilized are not clear. To better control lipid oxidation, a fuller understanding is needed of the influence of moisture on the mechanism of transition between different states in various foods. The following section discusses how water activity also strongly affects food proteins and their interactions with oxidized lipids and sugars.

2. *Interactions of lipids, proteins and sugars*

Sugars and carbonyl compounds interact with amino acids or proteins in a sequence of complex reactions known as the Maillard reaction or as non-enzymatic browning. The browning products from this reaction have a marked influence on lipid oxidation. They generally retard lipid oxidation in foods, and contribute to meat flavors. Lipid oxidation products can also react with proteins and amino acids, leading to the loss of essential amino acids with impact on the oxidative stability and the nutritional quality of foods.

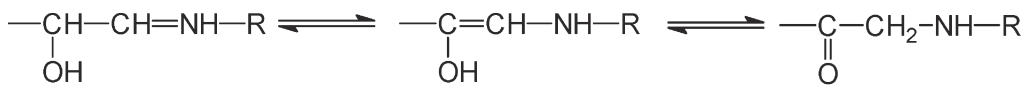
The interaction of amino compounds with reducing sugars proceeds by the addition of a carbonyl group to a primary amino group of an amino acid, peptide or protein, by water elimination through an intermediate imine, cyclizising to a glycosylamine (N-glycoside) (5).



(5)

N-glycosides are readily formed in foods containing a reducing sugar in the presence of proteins, amino acids and peptides, at elevated temperatures, or at low water activity. The glucosylamine undergoes rearrangement to a 1-amino-1-deoxyketose by a series of reactions known as the Amadori rearrangement, through an endiol-enaminol intermediate (6).

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1-amino-1-deoxy-ketose

(6)

Hundreds of complex brown reaction products are formed by a series of consecutive and parallel oxidation, reductions and aldol condensations. Complex Amadori products are found in dried fruits, dehydrated vegetables and milk products. In milk powder, the reaction between the σ-amino group of proteins and lactose leads to the formation of N-alkyl-1-amino-1-deoxy-lactulose. Color formation by the Maillard reaction is accompanied by aroma generation during cooking, frying, baking and roasting. These reactions lead also to the loss of essential amino acids, undesirable discoloration and development of objectionable flavor compounds in dehydrated foods during storage, or heat processing (milk sterilization, pasteurization and condensation, meat roasting). The reaction between water-soluble reducing sugars and amino acids generally occurs only in foods of water activity in the range above the monolayer (0.3–0.7). If foods of intermediate moisture contents are heated at sufficiently high temperatures, the rate of the browning reaction can be accelerated with the formation of Maillard reaction products that inhibit lipid oxidation effectively. This process is an effective way of stabilizing certain foods like evaporated milk, if the browning color does not detract from the acceptability of the product. When considered undesirable in certain foods, the Maillard reaction can be inhibited by lowering the pH, avoiding high temperatures and the critical range of water activity (0.7–0.8) during processing and storage (Figure 11.2), or by using formulations containing non-reducing sugars, and sulfite as an additive.

3. Antioxidant properties of browning reaction products

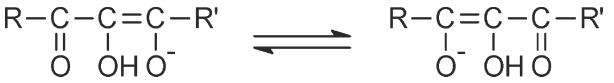
The antioxidant properties of Maillard reaction products are attributed to the formation of reductone (enaminone) structures that have both reducing and metal complexing properties. These compounds can also act as peroxide destroyers by reducing hydroperoxides into stable allylic alcohols. Reductones are intermediate compounds produced by the thermal reactions of reducing sugars that retain a carbonyl group in the vicinity of an endiol group. In the presence of amino compounds, reductones are produced under mild conditions. Reductones consist of vicinal diol compounds undergoing enolization and oxidation to the dehydro compounds (7).

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(7)

At pH below 6, reductones are resonance stabilized as anions (8).



(8)

Ascorbic acid is a reductone characterized by strong reducing properties under acidic conditions and at low temperatures. Basic amino acids (histidine, lysine and arginine) produce the most effective antioxidant products with sugars. Fractions from histidine–glucose reaction mixtures have good antioxidant properties. A range of these Maillard interaction products of amino acids and sugars are known to have antioxidant activity, and may be useful in food processing to retard oxidation in foods. Various heat treatments can improve the stability of bakery and cereal products and inhibit oxidation in milk products.

Various model systems were used to study the antioxidant activity of browning reaction products (Table 11.6). Unfortunately, the methodology employed to evaluate antioxidant activity of these complex browning reaction products is problematic. Linoleic acid used commonly as substrate is questionable because it forms micelles and oxidizes differently from triglycerides in foods and generally shows higher activity with polar hydrophilic antioxidants (Chapter 10). Peroxide values and TBARS used to measure oxidation would be subject to significant interference by the strong reducing power of Maillard reaction products. Other methods used, such as oxygen uptake, carotene and crocin bleaching, have analytical problems with poor sensitivity and selectivity (Chapter 7). Similarly, radical quenching provides no information on protective properties of antioxidants (Chapter 9). In most model systems the study of antioxidant activity of Maillard reaction products changed one variable at a time such as heating time, initial pH, sugar–amino acid combination and reactant ratio, whereas in foods the browning reaction products are formed by complex multivariable interactions.

On the one hand, various thermal processes can decrease the antioxidant activity of foods and beverages by the degradation of natural polyphenolic antioxidants. On the other hand, thermal treatments can increase their antioxidant activity by the formation of reducing browning reaction products. These effects can also be accompanied by interactions with lipid oxidation products, ascorbic acid and other phenolic antioxidants that are influenced by many factors and subject to complex changes during processing and storage. With tomato juice, although prolonged heat treatment (95°C) resulted in an overall increase in antioxidant potential due to formation of browning reaction products, short heat treatment caused initial reduction of the original antioxidant

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Table 11.6. Model systems used to evaluate antioxidant activity of browning reaction products

Model system	Conditions	Antioxidant activity	Oxidation method	Ref. [a]
Glucose–histidine	100°C – 5 hr	Linoleic acid oxidation	O_2 uptake, PV, MRP volatiles	(1)
Glucose–glycine	40°C – 40 hr a_w 0.23–0.82	Reducing power	K ferrycyanide test	(2)
Sucrose–lysine	100°C – 6 hr	Linoleic acid oxidation	O_2 uptake, PV, MRP volatiles	(3)
BSA–ribose–MeLo-OOH	25–120°C–pH 4,7,10	Soybean oil oxidation	TBARS	(4)
Glucose–lysine–starch	100°C – 90 min	Radical (ABAP) quenching	Crocin bleaching	(5)
Casein–glucose or fructose or ribose	55°C – 28 days	Radical (DPPH) and OH radicals quenching		(6)
Glucose–lysine or arginine or glycine	100°C – 60 min	LDL oxidation Cu induced	CD at 234 nm	(7)

[a] (1) Lingnert and Eriksson (1981), (2) Eicher (1981), (3) Kim and Harris (1989) (4) Alaiz *et al.* (1999), (5) Mastrocola and Munari (2000), (6) Jing and Kitts (2002), (7) Dittrich *et al.* (2003). Abbreviations: PV, peroxide value; MRP, Maillard reaction products; BSA, bovine serum albumin; MeLo-OOH, methyl linoleate hydroperoxides; TBARS, thiobarbituric reactive substances; ABAP, 2,2'-azo-bis(2-amidinopropane)dichloride; DPPH, 1,1-diphenyl-2-picrylhydrazyl; LDL, low-density lipoproteins; CD, conjugated dienes.

activity (Table 11.7). With wine, the progressive polymerization of polyphenols during aging resulted in a decrease in antioxidant activity, but the heating of must caused development of browning reaction products that enhanced the antioxidant activity. With green coffee, the antioxidant activity of phenolic compounds mainly associated with chlorogenic acid decreased with roasting. With roasted coffee, the formation of browning reaction products that have antioxidant activity is affected by the degree of roasting. Medium roasted coffee thus has higher antioxidant activity than light and dark roasted coffee. For a standard cup, the antioxidant activity in inhibiting LDL oxidation decreased in the order: coffee > cocoa > green tea > black tea. With peanuts, roasting enhanced antioxidant activity. Generally, in plant foods containing high levels of phenolic compounds such as tea, apple products and wine, the antioxidant activity resulting from complex interactions can increase initially and decrease with storage time.

Many studies of the effect of thermal processing used such a wide variety of non-specific methods to evaluate antioxidant activity that the results can be confusing and difficult to interpret (Tables 11.6 and 11.7). Because most natural antioxidants and those derived from browning reaction products are multifunctional, a reliable antioxidant protocol requires the measurement of

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Table 11.7. Antioxidant activity of browning reaction products in foods and beverages

Food systems	Conditions	Antioxidant activity	Oxidation method	Ref.[a]
Tomato juice	95°C 0–30 h	Radical (ABAP) quenching	Rancimat, O_2 uptake	(1)
White and red wines	Vintages: 1995, 1996, 1973	Radical (DPPH) quenching	O_2 uptake	(2)
Coffee (green, roasted)	Roasting 100–210°C	β-Carotene-Lo acid emulsion	Carotene absorbance	(3)
Peanuts (defatted)	180°C 0–60 min	Linoleic acid emulsion, DPPH	PV -(Fe-thiocyanate)	(4)
Coffee, cocoa, tea [b]	Standard beverage	LDL oxidation Cu or AAPH	Lag time CD at 234 nm	(5)
Coffee	Roasting 225–240°C 3 min	Radical ($ABTS^{\bullet+}$) quenching	Decolorization at 734 nm	(6)
Tomato pulp, puree, paste	30, 40, 50°C 3 months	Xanthine oxidase, Lo acid (Cu)	Ethane CD at 234 nm	(7)

[a] (1) Anese *et al.* (1999), (2) Manzocco *et al.*,1999, (3) Daglia *et al.* (2000), (4) Hwang *et al.* (2001) (5) Richelle *et al.* (2001), (6) Del Castillo *et al.* (2002), (7) Lavelli and Giovanelli (2003).
Abbreviations: ABAP, 2,2'-azo-bis(2-amidinopropane)dichloride; DPPH, 2,2'-diphenyl-1-picrylhydrazyl; LDL, low-density lipoproteins; Lo, linoleic; AAPH, azobis(2-amidinopropane)dihydrochloride; CD, conjugated dienes; ABTS, 2,2'=Azinobis(3-ethylbenzothiazoline-6-sulfonic acid).
[b] Antioxidant activity determined by the lag phase of LDL oxidation for a standard cup varied in the range: 292–948 min for coffee, 217–444 min for cocoa, 186–338 min for green tea and 67–277 min for black tea. Addition of milk did not alter the antioxidant activity.

more than one property relevant to either foods or biological systems (see Chapter 9). There is a great need, therefore, to standardize antioxidant testing to minimize the present confusion in the methodologies used to evaluate antioxidants. Several more specific methods are needed to obtain chemical information that can be related directly to oxidative deterioration of food and biological systems.

4. Interaction products in foods

Lipid oxidation products can interact with proteins and amino acids, and can affect the flavor deterioration and nutritive value of food proteins. Peroxyl radicals are very reactive with labile amino acids (tryptophane, histidine, cysteine, cystine, methionine, lysine and tyrosine), undergoing decarboxylation, decarbonylation and deamination. Methionine is oxidized to a sulfoxide; combined cysteine is converted to cystine to form combined thiosulfinate (Figure 11.4). Aldehydes, dialdehydes and epoxides derived from the decomposition of hydroperoxides react with amines to produce imino Schiff bases (R-CH=N-R'). Schiff bases polymerize by aldol condensation producing dimers

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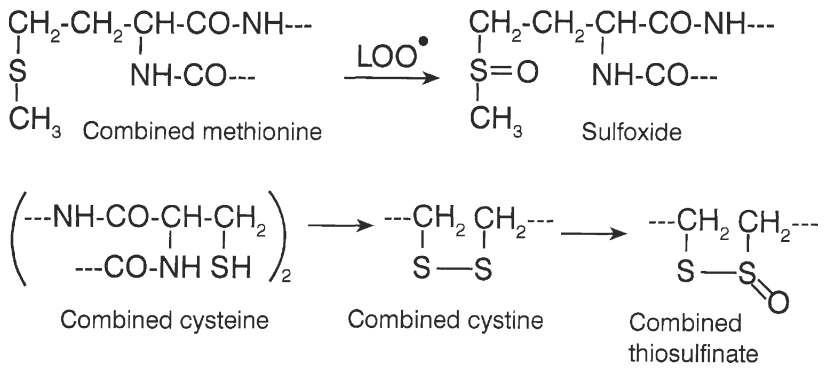


Figure 11.4. Interaction products between combined methionine and combined cysteine with lipid hydroperoxides.

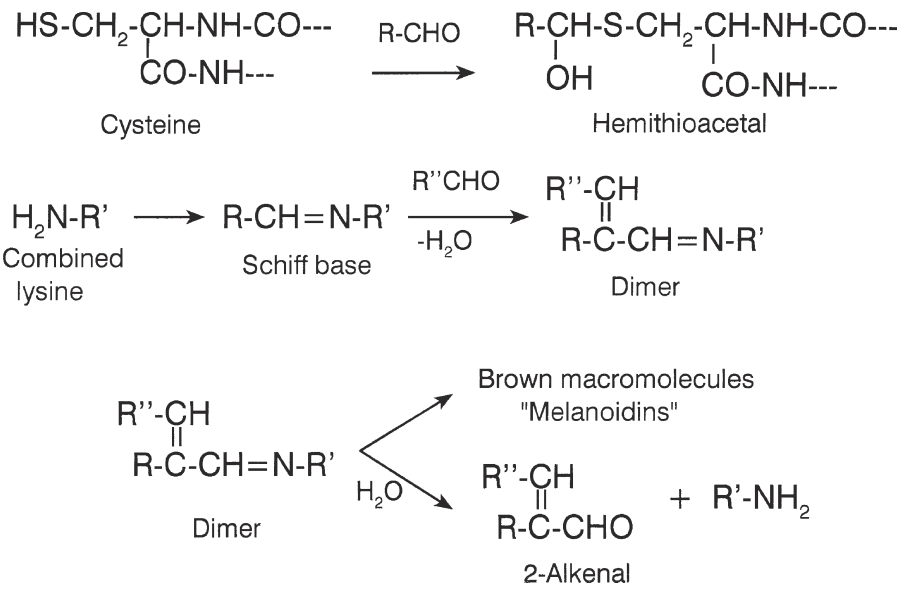


Figure 11.5. Interaction products between cysteine and aldehydes, and between a Schiff base and combined lysine.

and complex high molecular weight brown macromolecules known as melanoidins that are not well characterized (Figure 11.5). These polymeric brown materials are unstable and generate new volatiles, by scission of the macromolecule or dehydration, that affect the flavor characteristic of foods during cooking and processing. Hydroperoxides react with proteins to form protein free radicals, which cause the polymerization of the peptide chain to form protein–protein cross-links (Figure 11.6). The polymerization of lipids and peptide chains leads to browning of food proteins and changes in food texture and rheological properties.

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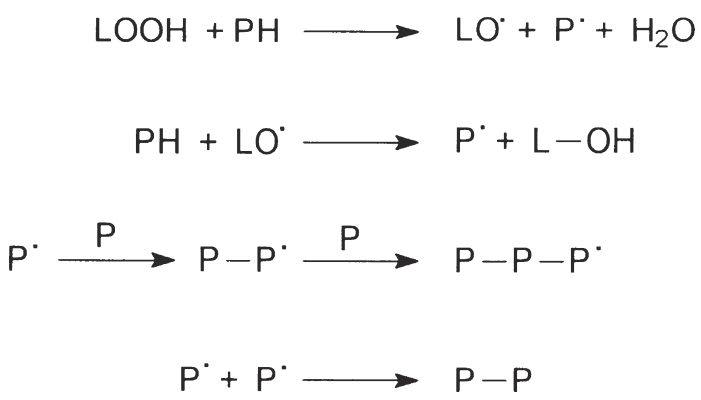


Figure 11.6. Protein–protein crosslink formation by interaction of protein radicals ($P^{\bullet}$) with lipid hydroperoxides.

Numerous studies have been published on the reactions of mixtures of lipid oxidation products and pure amino acids or proteins as model systems. The interactions involved in such complex mixtures are often difficult to understand and to relate to real food systems. Several factors influenced lipid oxidation of a model system consisting of a mixture of methyl linolenate and linoleate with whey proteins during storage in sealed tins under air (Table 11.8). Storage temperature had the most significant effect on hydroperoxide formation, with the highest amount formed after one week at 20°C, and no hydroperoxides left after the same period at 55°C. More hydroperoxides were produced as the water activity was increased, and surprisingly, when the oxygen level was reduced. Ethane formation (from decomposition of linolenate hydroperoxides) in the headspace decreased at lower storage temperature and water activity. Losses in amino acids (lysine and tryptophan) increased at higher water activity, and decreased at lower oxygen level. Tryptophan was more stable in this model system during storage.

The literature in this field is replete with a wide variety of model systems using for example cellulose, or silica, or amylopectin as solid supports, extruded mixtures of polyunsaturated fatty acids and esters with pure proteins, in the presence of humectants (to control moisture content) such as glycerol, or dextran as water-binding agent. Studies with such model systems are inherently problematic, because they do not simulate real foods where oxidation is naturally inhibited by the separation of lipids from catalysts in different cellular compartments. There is a serious lack of knowledge on the interactions of oxidized lipids and proteins in complex foods.

The structure of the food matrix is also known to affect the release of volatile compounds having an impact on flavors and aroma. Changes in flavor result from the interactions of lipid-derived carbonyl compounds by aldolization with the amino groups of proteins. Undesirable flavors are produced when beef or chicken are fried in oxidized fats by the interaction of secondary lipid oxidation

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Table 11.8. Lipid oxidation in a whey protein–linolenate/linoleate model system stored under different condition [a]

Temp. °C	Aw	Moisture g/kg	Oxygen mol/mol	Hydroperoxides [b]		Ethane [b]	
				7 days	14 days	7 days	14 days
20	0.90	170	4	5500	2900	3.6	6.6
37	0.90	170	1	2100	1000	5.9	6.6
37	0.90	170	4	1100	500	6.0	6.7
37	0.67	70	4	1600	750	8.2	9.0
37	0.30	20	4	1500	900	0.5	10.8
55	0.90	170	4	0	0	7.3	7.8

[a] Nielsen *et al.* (1985). Whey protein (from skimmed milk) was mixed with a mixture of methyl linolenate and linoleate in a hand mixer, the mixture was milled after spraying with water, and stored in enameled aluminum tins sealed under air.
[b] μmol/g lipid

products with meat proteins. Cooked meat undergoes flavor deterioration on storage through the oxidized fat adhering to the food surface. The complex interaction products formed during actual cooking and frying of meat products are not well characterized, and it is not clear how they degrade into volatile compounds that affect flavor. The chemistry of interactions between lipid oxidation products and proteins is very complex, and the impact on flavor deterioration is still not well understood.

Phospholipids contribute specific aroma to heated milk, meat and other cooked foods through lipid oxidation derived volatile compounds and interaction with Maillard reaction products. Most of the aroma significant volatiles from soybean lecithin are derived from lipid decomposition and Maillard reaction products including short-chain fatty acids, 2-heptanone, hexanal, and short-chain branched aldehydes formed by Strecker degradation (reactions of α-dicarbonyl compounds with amino acids). The most odor-active volatiles identified from aqueous dispersions of phosphatidylcholine and phosphatidylethanolamine include *trans*-4,5-epoxy-*cis*-2-decenal, *trans,trans*-2,4-decadienal, hexanal, *trans,cis,cis*-2,4,7-tridecatrienal (Table 11.9). Upon heating, these phospholipids produced *cis*- and *trans*-2-decenal and *trans*-2-undecenal. Besides fatty acid composition, other unknown factors apparently affect the formation of carbonyl compounds from heated phospholipids.

C. Milk products

Oxidation of milk lipids is an important cause of flavor deterioration of dairy products that is often referred to as oxidative rancidity, to distinguish it from hydrolytic rancidity resulting from lipolysis. Milk products have complex compositions, physico-chemical properties, and contain natural prooxidants

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Table 11.9. Odor active volatiles produced in heated aqueous dispersions of egg phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [a]

Volatiles	Conc. (mg/kg)		Odor threshold(ng/l)		Precursor fatty acids
	PC	PE	In air	In oil	
Hexanal	60.7	96.9	43	300	18:2, 20:4
1-Octen-3-one	7.4	1.8	0.07	10	18:2, 20:4
2-Octenal	20.2	8.1	47	7000	18:2
2-Nonenal	5.3	2.5	0.1	900	18:2, 20:4
2,4-Nonadienal	1.2	0.70	0.4	2500	18:3
2,4-Decadienal	108	4.8	0.1	180	18:2, 20:4
4,5-Epoxy-2-decenal	4.6	3,4	0.0015	1.3	18:2, 20:4
2,4,7-Tridecatrienal	2.8	1.7	0.07	180	20:4

[a] From Lin and Blank (2003). Odor threshold are orthonasal threshold taken from the literature.

and antioxidants. Milk fat consists mainly of triacylglycerols (95–96%) existing naturally in the form of fat globules, surrounded by a complex fat globule membrane composed of proteins (41%), phospholipids (30%), which are included in lipoproteins and glycoproteins, glycerides (14%), cerebrosides (3%), cholesterol (2%) and enzymes. The fatty acid composition of milk fat includes 63–69% saturated (24% palmitic, 13% stearic), 27–34% monounsaturated (25% oleic), and 2.5–3% polyunsaturated (2% linoleic) fatty acids. The milkfat globule membrane is a principal site of lipid oxidation, because the main phospholipid components contain about 6% linoleic acid and are associated with prooxidants in a lipoprotein matrix. This membrane contains traces of indigenous copper and iron, and its structure is dynamic and unstable to thermal treatments.

Flavor defects in milk and cream are collectively referred to as the "oxidized flavor", in butter as "metallic" or "tallowy". Oxidized flavors are noticeable in stored milk at very low levels of oxidation, usually at a peroxide value less than 1, because the low molecular weight off-flavor compounds are more readily volatilized in the predominantly aqueous matrix of milk. The development of oxidized flavor is most evident in fluid milk, cream and butter, because they have mild and more delicate flavors.

Oxidation occurs initially in the polyunsaturated phospholipid fraction associated with the fat globule membrane–water interface, followed by the main triacylglycerol fraction. Different milks vary widely in susceptibility to oxidation, and oxidative deteriorations in milk are classified empirically as: i. *spontaneous*, developing oxidized flavors within 48 hr after milking without added metal catalyst; ii. *susceptible*, developing oxidized flavors within 48 hr after contamination with copper, and iii. *resistant (*or *non-susceptible)*, not developing oxidized flavors after 48 hr even after addition of copper or iron.

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Many hypotheses have been advanced for these differences in oxidative susceptibility, including the oxidation potential of milks, and the action of xanthine oxidase and lactoperoxidase, which is controversial. However, there are no substrates for these enzymes in milk. The action of various metallo proteins in milk may be confused as enzymes. These metallo proteins act as powerful lipid oxidation catalysts in the presence of oxygen and redox systems involving ascorbic acid.

Although in fluid milk the phospholipid fraction is more susceptible to oxidation than the triacylglycerol fraction, in dry milk products, the triacylglycerol fraction is more susceptible to oxidation and the phospholipids act as antioxidants. Thus, solvent-extracted milkfat containing phospholipids is much more stable to oxidation than milkfat free of phospholipids, obtained by melting churned butter (also called butter oil). The susceptibility of milk phospholipids to oxidation appears to be dependent on whether they are suspended in water or fat. This difference of oxidative stability influences the development of different flavor defects in various dairy products. With butter, which is a water-in-oil emulsion system containing an aqueous phase of phospholipids dispersed in fat, the phospholipids oxidize more readily than the triacylglycerol components.

1. Metal catalysis

Metal catalysts are recognized as the most important factors in accelerating lipid oxidation in dairy products. Although copper occurs naturally in milk at a lower concentration (20–40 μg/l) than iron (100–250 μg/l), it is the most important catalyst for the development of oxidized flavors. These metals occur as complexes with proteins, and are associated with the fat globule membrane. Milks that develop oxidized flavors spontaneously contain more copper in the fat globule membrane. The most accepted mechanism of initiation of lipid oxidation in milk involves the catalytic action of lipoprotein–copper complexes at the fat globule membrane–aqueous interface. However, the integrity of this membrane as affected by processing must play an important part in determining the variation in oxidative susceptibility of milks. Metal chelators, such as ethylenediamine tetraacetic acid (EDTA), reduce the development of oxidized flavors in milk, but the use of these additives is not allowed in the US and other countries.

Ascorbic acid, found in milk at a concentration of 10–20 mg/l, is also apparently linked with the development of oxidized flavors in milk. Milk treated to remove ascorbic acid is more resistant to copper-catalysed lipid oxidation. The catalytic activity of copper is enhanced in the presence of ascorbic acid. However, when ascorbic acid is present in milk at sufficiently high concentrations (50–200 mg/l), it inhibits effectively the development of oxidized flavors. Combinations of ascorbate and copper have either prooxidant

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Table 11.10. Effect of mixtures of ascorbic acid and copper on the formation of conjugated dienes (CD) during oxidation of linoleate [a]

Ascorbic acid (M)	Copper (1.3 × M)	CD formation ($k_i \times 10^6$) [b]
1.8×10^{-6}	10^{-7}	1.0
1.8×10^{-6}	10^{-3}	6.6
1.8×10^{-5}	10^{-7}	5.3
1.8×10^{-5}	10^{-3}	9.8
1.8×10^{-4}	10^{-7}	11.4
1.8×10^{-4}	10^{-5}	0
1.8×10^{-3}	10^{-7}	13.8
1.8×10^{-3}	10^{-6}	0

[a] From Haase and Dunkley (1969).
[b] Initial rates in mol/l/min

effects, at relatively low levels of ascorbate, or antioxidant effects at relatively high concentrations of ascorbate (Table 11.10). Several mechanisms may explain these effects, including conversion of cupric ions to the more active cuprous state, formation of an active copper–ascorbic acid–oxygen complex catalyst, sparing lipid oxidation by preferential oxidation of ascorbic acid and depleting the available oxygen, and reduction of hydroperoxides by ascorbate into stable and innocuous allylic alcohols. Clearly, more work and especially data on volatile decomposition products are needed to clarify the paradoxical behavior of ascorbic acid.

Other reducing agents such as thiols may also have dual pro- and antioxidant effects in the presence of copper, similar to those of ascorbic acid. The ligands associated with copper and iron can have a profound effect on their catalytic activities. Significant portions of endogenous copper and iron are associated with proteins and the milk fat globule membrane. The formation of a copper-protein complex with the phospholipids of this membrane material has been suggested. The distribution of metals in various milk products and the effects of processing on their catalytic activities are influenced by a wide number of factors that are not clearly understood.

Iron supplementation to meet nutritional requirements can seriously limit the shelf life of milk products especially infant formulas. Lipid oxidation can be controlled in iron-supplemented milk by using lactoferrin, a non-heme iron-binding glycoprotein found in human (1.4 mg/ml) and bovine milk (0.1 mg/ml). Lactoferrin in bovine milk is 22% saturated with iron compared to 4% in mature human milk. Compared to human milk, infant formulas are more susceptible to lipid oxidation because they are supplemented with greater amounts of iron and do not contain lactoferrin. This antioxidant protein in milk has an important activity by binding prooxidant iron ions. Commercially available bovine lactoferrin, isolated from cheese whey, inhibited lipid

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Table 11.11. Effect of lactoferrin on the inhibition of hydroperoxide and hexanal formation in two infant milk formulas supplemented with different concentrations of iron [a]

Formulas	Iron (μM Fe)	Lactoferrin	Fe:lactoferrin (molar ratio)	% Inhibition	
				Hydroperoxides	Hexanal
Whey [b]	0	12.5	0:12.5	61.9	72.0
	0	25.0	0:25.0	92.1	95.2
Whey [b]	172	25.0	3.5:1.0	65.4	77.7
	172	44.0	2.0:1.0	94.7	95.8
Whey [b]	220	25.0	8.8:1.0	64.8	87.9
	220	110	2.0:1.0	101	97.8
Casein [c]	17	12.5	1.4:1.0	26.3	15.9
	17	25.0	0.7:1.0	63.9	63.3
Casein [c]	171	37.5	4.6:1.0	46.2	62.2
	171	85.5	2.0:1.0	92.7	89.7
Casein [c]	216	37.5	5.8:1.0	77.1	62.0
	216	108	2.0:1.0	95.8	89.5

[a] From Satué-Gracia *et al.* (2000). The whey-based and casein-predominant formulas were prepared on a pilot scale and supplemented with different concentrations of iron. The highest concentrations of iron are most used commercially. Oxidation was carried out at 50°C with shaking. Hydroperoxides were based on conjugated dienes and hexanal on headspace gas chromatography.
[b] Data calculated at 20, 12 and 11 days of oxidation for samples containing 0, 172 and 220 μM Fe, respectively.
[c] Data calculated at 7 days of oxidation for hydroperoxides and 8 days for hexanal data

oxidation when added to infant formulas in the absence and presence of different concentrations of supplemented iron (Table 11.11). Lactoferrin inhibited hydroperoxide and hexanal formation in a concentration-dependent manner. The inhibition of oxidation observed with control samples that contained no iron may be explained by chelation of copper present in all formulas. Since lactoferrin also has antimicrobial activity, it can be used as a dual-purpose antioxidant additive in infant formulas and other foods supplemented with iron.

2. *Light oxidation*

Exposure of milk and butterfat to light, especially sunlight, produces off-flavors, initially described as burnt, light-activated or sunlight flavor. On prolonged exposure to light, this flavor defect changes to the typical oxidized flavor of milk. The sunlight flavor originates in the serum proteins of milk and riboflavin is implicated as a potent water-soluble photosensitizer. On exposure to light in the presence of riboflavin, several amino acids (tryptophan, tyrosine, cysteine, and lysine) are destroyed in a low-density lipoprotein fraction associated with the milk fat globule membrane. Carbonyl compounds and methional,

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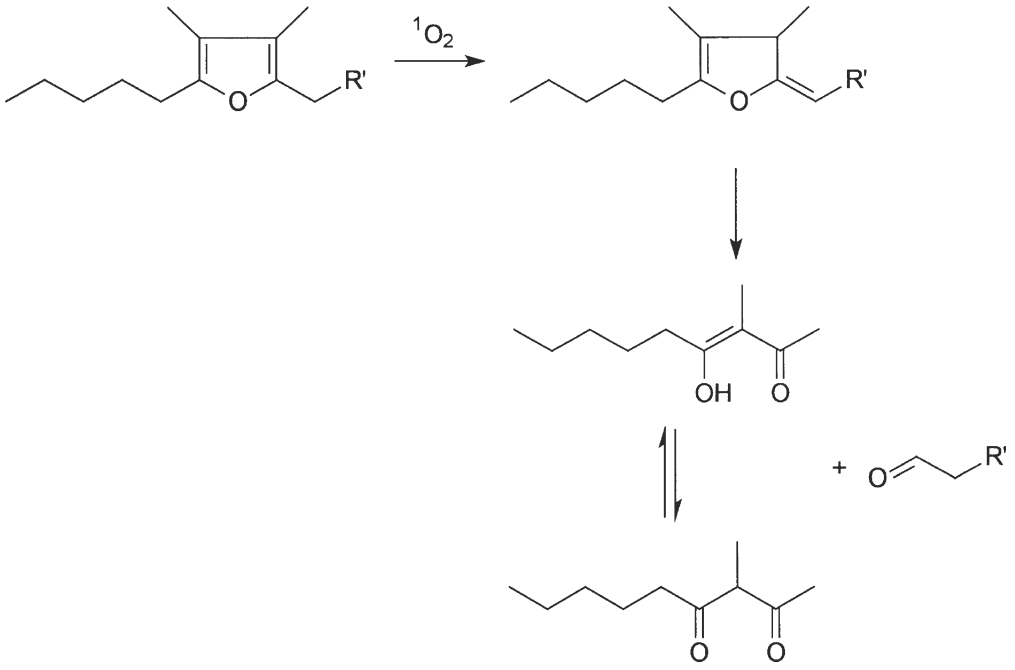


Figure 11.7. Proposed mechanism for the formation of 3-methylnonane-2,4-dione by photosensitized oxidation of furanoid fatty acids. From Grosch *et al.* (1992).

formed by degradation of methionine, are attributed to the development of sunlight flavor of milk, which is also related to thiols, sulfides, and disulfides in milk.

Recent studies of photooxidized butter and butter oil identified by aroma extract dilution analysis, 3-methylnonane-2,4-dione, a potent volatile compound derived from furanoid fatty acids (see Section C.4) (Figure 11.7). Six different furanoid fatty acids were established as dione precursors, and were found in various samples of butter made from either sweet cream (116–476 mg/kg), or from sour cream (153–173 mg/kg), or from butter oil (395 mg/kg). Similar precursors of the dione were identified in stored boiled beef and vegetable oils. This flavor defect arising by photooxidation of butter or butter oil is apparently different from the light-activated flavor in milk,that involves the interaction of sulfur-containing proteins and riboflavin. However, more sensory comparisons are needed to distinguish between these two flavor defects due to light oxidation.

3. Antioxidants

α-Tocopherol is the only important natural antioxidant in milk, which contains an average of 25 μg/g milkfat and 44 μg/g of fat globule membrane. The content of this antioxidant varies according to the feed of the animal and the season of the year. The oxidative stability of milk correlates well with the

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α-tocopherol level in milk, and especially in the lipids of the fat globule membrane. Supplementing the ration of animals with various forms of tocopherols provides an effective control measure against lipid oxidation in milk. However, direct addition of emulsified α-tocopherol to milk is a more efficient measure of controlling oxidized flavor in milk than ration supplementation. Several synthetic antioxidants (BHA, BHT, propyl gallate) and metal chelators (citric acid, phosphoric acid and salts of ethylenediamine tetraacetic acid) have proven effective in inhibiting lipid oxidation, but the use of these compounds is not legally permitted in dairy products in the US and other countries.

Ascorbic acid acts as an antioxidant in milk at normally low copper concentrations. However, during storage the concentration of ascorbic acid decreases continuously and is depleted by consuming dissolved oxygen. The concentrations of tocopherol and ascorbic acid in milk are thus affected by cow feeding and milk storage conditions.

Other components of milk may also inhibit lipid oxidation. Milk is thus protected from lipid oxidation by casein and other proteins capable of binding and inactivating copper and iron. One mechanism suggested for the improved oxidative stability of homogenized milk is the resurfacing of the fat globules with casein providing a protective membrane (see Section 5.d). Casein has an effective stabilizing action in preventing lipid oxidation in milk products. Its protective effect can be attributed to its binding capacity for copper and its association with the oil–water interface around the fat globule membrane. Heating milk products to activate sulfhydryl groups in proteins is another method of increasing the oxidative stability of dairy products.

4. Flavor compounds

The volatile carbonyl compounds developed from milk fat oxidation are detectable at extremely low levels (parts per billion). Flavor compounds are less volatile and mainly retained by the fat component. These compounds are thus much more easily perceived in milk, where they have much lower threshold values (0.004–0.1) than in oil systems (0.1–2.5). Because flavor compounds are very easily perceived in milk products, they can be detected at extremely low levels of oxidation. Fluid milk becomes rancid at peroxide values less than one.

Aldehydes found in various dairy products include those derived from the hydroperoxides of polyunsaturated fatty acid components, and further oxidation products of polyunsaturated aldehydes (Table 11.12). Flavors imparted by these carbonyl compounds are described as oxidized, fishy, metallic, cardboard, painty and tallowy. Higher alkanals and alkenals with more than six carbons and ketones are typical volatiles produced by lipid oxidation. A powerful technique, known as aroma extract dilution analysis, was used to

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Table 11.12. Aldehydes found in autoxidized dairy products [a, b]

Products	n-Alkanals	2-Alkenals	2,4-Alkadienals
Butter phospholipids	C_2 to C_4	C_5 to C_{11}	C_8, C_9
Butter oil	C_1 to C_{10}	C_3 to C_{11}	C_7, C_{11}
Skim milk	C_2, C_6	C_4 to C_{11}	C_6 to C_{11}
Nonfat dry milk	C_1, C_2, C_5–C_{10}, C_{12}, C_{14}		
Dry whole milk	C_1 to C_3, C_5–C_{10}, C_{12}	C_5 to C_{11}	
Fluid whole milk	C_5–C_{10}	C_6–C_{11}	C_8–C_{12}
Butter [b]	C_5–C_{12}	C_5 to C_{11}	C_7, C_9, C_{10}, C_{11}

[a] From Day (1966) and Parks (1974).
[b] Other carbonyls: *cis*-4-heptenal, *trans,cis*-2,6-nonadienal, 2,5-octadienal, 2,4,6-nonatrienal, 2,4,7-decatrienal, 1-penten-3-one, 1-octen-3-one, *trans,cis*-3,5-octadien-3-one, 3,5-undecadien-3-one, 1-octen-3-one, 1-octen-3-ol

determine potent odor and flavor compounds in various milk and food products. This method determines the odor activity of volatile compounds in an extract eluted from a high resolution capillary gas chromatograph (GC) by detection with a sniffing port (SP). The odor activity of a compound is expressed as the flavor dilution factor, which is the ratio of its concentration in the initial extract to its concentration in the most dilute extract in which the odor can be detected by GC-SP. By this technique, the sweet buttery flavor of fresh butter was attributed mainly to a mixture of diacetyl, acetic acid, butyric acid, guaiacol, vanillin, *cis*-6-dodecen-γ-lactone, δ-octalactone, δ-decalactone and various carbonyl compounds formed by lipid oxidation. The effect of storage of butter oil on the dilution factor of carbonyl compounds was determined by the technique of aroma extract dilution analysis (Table 11.13). On storage at room temperature, the dilution factor of carbonyl compounds increased, with 1-octen-3-one showing the most significant increase. Antioxidants (BHA, BHT, α- and γ-tocopherols) effectively inhibited the formation of *cis*- and *trans*-2-nonenal, *cis*-4-heptenal and 1-octen-3-one in butter oil stored at 35°C in the dark. Butter oil exposed to fluorescent light developed an off-flavor described as "green, strawy and fatty", which was attributed mainly to the production of 3-methylnonane-2,4-dione, *trans*-4,5-epoxy-*trans*-2-decenal and an increase in concentration of *trans*-2-nonenal and *trans,trans*-2,4-decadienal (Table 11.14). *Trans*-4,5-epoxy-*trans*-2-decenal was also identified as a significant volatile in butter and cheese. The formation of 3-methylnonane-2,4-dione was shown to be derived from a mixture of furanoid fatty acids occurring naturally in minor amounts (Figure 11.7).

Solid-phase microextraction (SPME) gas chromatography and mass spectrometry techniques (Chapters 5 and 6) were also used to study volatile compounds in reduced-fat milk exposed to light, heat and copper. Volatiles attributed to light exposure and copper-catalysed oxidation include pentanal, hexanal, heptanal, octanal, nonanal, 2-octenal and 2-nonenal. Mass spectral

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Table 11.13. Effect of storage of butter oil on increase in the dilution factor of carbonyl compounds [a]

Compounds	Fresh	Stored
Hexanal	16	64
cis-3-Hexenal	64	128
1-Octen-3-one	128	1024
cis-1,5-Octadiene-3-one	32	256
Nonanal	0	64
cis-2-Nonenal	64	128
trans-2-Nonenal	32	256
trans,trans-2,4-Nonadienal	8	128
trans,trans-2,4-Decadienal	32	64

[a] From Widder *et al.* (1991). Storage for 42 days at room temperature.
Dilution factor = dilution necessary to still detect compounds by GC olfactometry (using a sniffing port).

Table 11.14. Changes in dilution factors of flavor compounds formed in photooxidized butter oil [a]

Compounds	Fresh butter oil	Photooxidized butter oil
1-Hexen-3-one	128	64
Hexanal	<32	32
cis-3-Hexenal	64	64
cis-4-Heptenal	64	64
1-Octen-3-one	128	256
cis-2-Nonenal	64	64
trans-2-Nonenal	32	128
3-Methylnonane-2,4-dione	0	256
trans,trans-2,4-Decadienal	32	128
trans-4,5-Epoxy-*trans*-2-decenal	0	128

[a] From Grosch *et al.* (1992). Butter oil exposed to fluorescent light for 48 hours.

intensity lists obtained by averaging total masses of volatile compounds were used to predict the shelf life of commercial milk samples. SPME proved to be a sensitive method for the evaluation of various heat treatments of milk samples. The formation of methyl ketones correlated with heat treatments of pasteurized milks (Table 11.15). Conventional purge and trap GC gave higher values for 2-butanone than SPME and lower values for 2-heptanone. Using 2-heptanone as marker of severity of heat treatment, the relative concentration in μg/kg increased from 2.6 in fresh pasteurized milk, to 9.1 in "ultra-high temperature" (UHT) pasteurized milk, to 64.5 in sterilized milk in-bottle.

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Table 11.15. Volatile compounds analysed by dynamic headspace gas chromatography in milk products: purge and trap (PT) vs solid phase microextraction (SPME) [a]

Volatiles	Pasteurized milk		Ultra-high temperature	
	PT	SPME	PT	SPME
Dimethyl sulfide	0	1.2	1.5	0
Acetone	167	34	35.9	20.2
2-Butanone	81.9	14	8.2	5.7
2-Pentanone	3.1	0	3.9	9.4
Pentanal	1.8	–	2.3	–
Hexanal	3.8	–	2.6	–
2-Heptanone	2.6	10.4	9.1	67.5
Heptanal	2.4	–	2.1	–
2-Nonanone	–	2.5	–	16.7
2-Undecanone	–	0	–	4.0

[a] From Contarini and Povolo (2002). Units: in micrograms per kilogram. Pasteurized milk: at least 71.7°C for 15 sec; ultra high temperature milk: at least 135 °C for 1 sec.

Not all of the potent volatile compounds are derived from lipid oxidation, including a number of lactones that come from naturally occurring hydroxy fatty acids, diacetyl and vanillin in butter oil (from melted butter). The concentrations of the mixtures of carbonyl compounds exceed the flavor threshold values for individual aldehydes, and the "oxidized flavor" results from a combination of volatile compounds.

5. Factors affecting lipid oxidation

Lipid oxidation during processing and storage of milk products may influence prooxidants, antioxidants and the oxygen content of the final products. External factors include handling, agitation, storage, temperature, exposure to light and contamination by metals or microorganisms.

(a) Storage temperature. The effect of storage temperature on oxidative deterioration of dairy products is anomalous, because milk develops oxidized flavors more readily and shows higher TBA values at 0 to 4°C than at 4 to 20°C. Differences in activation energy between peroxide formation (10.3 kcal/mole) and increase in TBA values (13.9 kcal/mole) suggest that these methods measure two different events in the sequence of lipid oxidation. Initial hydroperoxide formation requires less energy than hydroperoxide decomposition into TBA-reactive products. Another explanation offered for the effect of storage temperature relates to the lowering of the oxygen content by bacterial activity at higher temperatures. However, other types of bacterial deteriorations would be expected at the higher storage temperatures.

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Table 11.16. Oxygen consumption by pasteurized milk during storage at 7°C in the dark for 6 days [a]

Consumed by:	Oxygen (mg/l)
Bacteria	1.48
Ascorbate	1.41
Peroxidation	0.003
Sulfhydryl groups	0.14
Total	4.10

[a] From Allen and Joseph (1983). 1.07 mg/l accounted for by loss through septum and other sources.

(b) Oxygen levels. The oxygen content of pasteurized milk varies from 9 mg/l at 20°C to 8 mg/l at 25°C, and storage in the dark consumes 4.1 mg/l after six days (Table 11.16). Bacteria and oxidation of ascorbic acid consume much more oxygen than oxidation of sulfhydryl groups or autoxidation.

The development of oxidized flavors in milk, butter oil and dry milk powder is effectively inhibited by removal of dissolved oxygen and by deaeration. The shelf life of dry whole milk is significantly extended by vacuum treatment, replacing air oxygen and packing with inert gas. Other effective techniques to remove oxygen from milk powder include the use of a mixture of glucose and catalase as an oxygen scavenger (see Chapter 9.E), in the presence of calcium as a desiccant, or the oxygen-absorbing mixture, sodium sulfite (Na_2SO_3) and copper sulfate ($CuSO_4$), enclosed in porous paper pouches, or by using a mixture of nitrogen and hydrogen in the presence of a palladium or a platinum catalyst. Dried milk stored in nitrogen shows slower development of off-flavors than unprotected samples.

(c) Heat treatments. Pasteurization of milk increases its susceptibility to oxidized flavors by increasing the copper content of the cream phase. However, heating milk to higher temperatures decreases this oxidative susceptibility because of the formation of sulfhydryl reducing compounds, derived from β-lactoglobulin, that impart a "cooked" flavor to milk. Heating milk also generates antioxidant-active browning reaction products that increase the oxidative stability of dairy products.

(d) Homogenization. The homogenization of milk disrupts the natural fat globule integrity and resurfaces the fat with a membrane consisting mainly of casein (93%) with some whey proteins. This process retards lipid oxidation and the development of oxidized flavors effectively. Several mechanisms have been advanced to explain this stabilization effect: (i) redistribution of catalytic metals, which may involve complex exchange between the fat globule membrane as a prooxidant catalyst, and casein in milk plasma as an inactive metal–protein complex; (ii) migration of the phospholipids into either the

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serum phase or the interior of the fat globules and replacement with casein; (iii) redistribution of the phospholipids in milk; (iv) denaturation of proteins with increase in available sulfhydryl groups; and (v) structural changes or decrease of the copper–protein complex that inactivates ascorbic acid in promoting lipid oxidation. Although considerable work has been published in this area, details of the structural changes resulting from homogenization of milk are still not clear.

(e) Packaging. In addition to conventional containers used for liquid milk including glass bottles, coated paper and plastic packaging, special ultra-high temperature (UHT) milk is packaged in flexible polyethylene pouches. These plastic containers may transmit light and may be more or less permeable to oxygen. New flexible packaging materials have been developed to effectively extend the shelf life of milk stored in pouches exposed to air and light, by using flexible monolayer low-density polyethylene (LDPE) clear or pigmented with titanium oxide, and multiple-layer coextruded LDPE/polyamide pigmented with carbon black and titanium oxide.

D. Meat and poultry products

The lipids in lean beef consist of about 2 to 4% triacylglycerols and 0.8 to 1% phospholipids containing 44% polyunsaturated fatty acids, which are mainly subject to oxidation. Oxidation occurs initially in the phospholipids of cellular and subcellular membranes, which are in close proximity to the heme catalysts of the mitochondria and microsomes. A typical fatty acid composition of beef membrane phospholipids includes 22% 18:2, 2% 18:3, 15% 20:4, less than 1% 20:5 and 2% 22:6. Phosphatidylethanolamine is the main phospholipid implicated in lipid oxidation of cooked meat. Chicken and turkey muscle are more susceptible to oxidation than beef, because of their higher polyunsaturated phospholipid fraction and relatively low levels of natural tocopherols. Red poultry muscles oxidize faster than white because of higher phospholipid and iron contents.

In addition to the phospholipid composition and contents, other factors affecting lipid oxidation in animal tissues include diet, processing and additives (salt, nitrite, spices and antioxidants). Desirable meat flavors appear to be positively correlated with the 18:3 content of meat obtained from grass fed cattle and sheep. Lipid oxidation and color development in ruminant meats are also correlated with the fatty acid composition and vitamin E content. Meat from grass-fed animals have higher levels of vitamin E that may improve the stability of both 18:3 PUFA and oxymyoglobin, and contribute to the desirable bright red color of fresh beef. By including linseed in the diet of pigs to increase the *n*–3 PUFA of pork, an adverse effect on lipid oxidation and meat quality was observed when the level of 18:3 PUFA approached 3% of neutral lipids or

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phospholipids. As expected, similar undesirable fishy flavor of steaks were obtained by feeding fish oil to steers.

Raw meat is resistant to oxidation when refrigerated. Lipid oxidation proceeds rapidly when the meat structure is disrupted by grinding, chopping or by heating, exposing the phospholipids to oxygen, enzymes, heme pigments and metal ions. When the cellular and biochemical integrity of muscle tissues is lost during food processing, there is a shift in the balance between prooxidants and antioxidants in favor of prooxidant activity. Pigment changes occurring by oxidation of the red oxymyoglobin and myoglobin to the brown metmyoglobin are important reactions affecting quality of meat (Figure 11.1). When meat tissue is minced in air, lipid oxidation is accelerated by any deoxyhemoglobin present. By heating meat, oxygen is released from oxymyoglobin, producing hydrogen peroxide that activates the porphyrin structure followed by the release of free iron. In the presence of reducing agents such as ascorbic acid, ferrous iron is produced that catalyses lipid oxidation.

The poor oxidative stability of meat may be due to some of the denatured species of oxymyoglobin and metmyoglobin, which become readily oxygenated in air and induce lipid oxidation, producing unsaturated and reactive aldehydes interacting with the amino groups of various myoglobin species. However, the significance of each myoglobin species in initiating lipid oxidation is not yet clear and much of the evidence based on simple model systems must be verified with real muscle foods.

Rancidity development in meat is distinguished between "normal oxidation", due to oxidation of the triacylglycerol fraction, and rapid oxidation referred to as the "warmed-over flavor", attributed mainly to the phospholipids. Normal oxidation occurs during long-term refrigeration storage, while the development of warmed-over flavor takes place in meat after cooking and varies at different stages of lipid oxidation. Rancidity due to lipid oxidation occurs also in uncooked meat when the muscle membranes are broken by mechanical separation and restructuring. Thermally induced lipid oxidation during initial cooking produces a complex mixture of carbonyl products contributing to the desirable flavor of meat due to interactions with amino groups and phosphatidyl ethanolamine. The interactions of carbonyl compounds from lipids in the browning Maillard reaction impart desirable flavor attributes to cooked meat. Lipid oxidation in meat is also associated with deterioration of the desirable beef flavor by formation of other flavor defects described as "cardboard" or "painty". Volatile compounds responsible for these flavors are summarized below (Section D.3).

1. Iron catalysts

Iron can be found either in the free form, non-heme iron, or bound to proteins. The free catalytically active iron represents 2 to 4% of the total muscle iron in

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beef, lamb, pork and chicken. Protein-bound iron exists in the form of soluble proteins, myoglobin, ferritin and other iron-complexed proteins (transferrin, ovotransferrin, lacto-transferrin). Cooking of meat extracts causes a release of iron from heme proteins. Ferritin releases ferrous iron in the presence of reducing agents such as ascorbate and thiols. Lipid oxidation is also catalysed in mixtures of ferritin isolated from meat extracts in the presence of ascorbate and cysteine. As in milk, depending on the level of catalytically active iron in meat, ascorbate can act either as a prooxidant at low concentrations by reducing iron to a low valence state, or as an antioxidant at high concentrations by scavenging lipid free radicals.

Although the free iron is catalytically active, the relative importance of protein-bound iron is being debated. Lipid oxidation in cooked meat is not only attributed to changes in iron distribution by protein denaturation and the release of catalytically active non-heme iron, but also to the disruption of cell membranes in meat that brings the polyunsaturated lipids in close contact to the catalysts. Unfortunately, in many studies using the TBA test to determine the effects of different forms of iron on lipid oxidation in meats, the results must be interpreted with caution, because the TBA reaction is significantly affected by iron and other metals, and is subject to serious interference by other factors. In addition to products of lipid oxidation, TBARS are also formed from proteins and nucleic acids and other non-lipid components in meat tissues that confound the results of the TBA test.

There is increasing evidence that degradation of proteins is associated with the development of the so-called "warmed-over flavor". Selective methods to measure protein oxidation may be useful as a complementary approach in evaluating oxidative deterioration of meat products. The determination of protein carbonyls (as dinitrophenyl hydrazine derivatives) is now used for this purpose. More reliable methods are needed, however, to determine specific products of lipid oxidation and their interaction products acting as precursors of flavor compounds to establish the relative contribution of heme and non-heme iron to the development of rancidity in various meat products.

2. *Enzymatic lipid oxidation*

Lipid oxidation in subcellular fractions can be mediated by enzyme systems in muscle microsomes that maintain iron in the ferrous form by reduced nicotinamide adenine dinucleotide (NADH). However, this redox system may not be enzymatic because, unlike lipoxygenase, no specific lipid oxidation products have been identified. Ascorbate and other reducing agents may have the same effects in the presence of heme-protein complexes. On the other hand, the presence of 15-lipoxygenase in chicken muscle may be responsible for oxidative deteriorations in uncooked chicken meat during frozen storage. Phospholipases

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inhibit lipid oxidation, apparently by forming iron complexes with the free fatty acids liberated by hydrolysis of phospholipids in the membrane.

Lipid oxidation in meats can also be accelerated by depletion of glutathione peroxidase and glutathione transferase, which decompose hydroperoxides by reduction into hydroxy compounds in the presence of glutathione. Lipoxygenases can participate in co-oxidation reactions at low oxygen levels that produce volatile products affecting the flavor quality of meat. The role of lipoxygenase in initiating lipid oxidation in meat systems is not clear yet and more detailed studies are needed to assess the significance of this enzyme.

3. Volatile flavor compounds

The interactions of lipid hydroperoxides and secondary products with proteins and amino acids have a considerable impact on the flavor of meat products during processing, cooking and storage. Hydroperoxide radicals react with sulfur and amine and amino acids, and secondary oxidation products with thiols from cysteine (Figures 11.4 and 11.5). Non-enzymatic browning reaction products and Schiff bases formed from the interactions of aldehydes with proteins are unstable and produce complex mixtures of volatile compounds that affect the flavor characteristics of meat products during cooking and processing.

A large number of volatile compounds have been identified in beef fat (Table 11.17). The flavor significance of individual volatile compounds can be calculated on the basis of their threshold values. Thus, two of the carbonyl compounds formed in the smallest concentrations, *cis*-4-heptenal and *trans,trans*-2,6-nonadienal, were the most flavor significant. More sensitive GC-MS analyses reported odor threshold values of 1260 ppb for *cis*-4-heptenal and 1231 for *trans,cis*-2,6-nonadienal found among fish oil volatiles (Section E.3 and Chapter 5). On the other hand, the most abundant carbonyl, 2-decenal, was the least flavor significant.

A direct and more advanced analytical approach to screening important volatile compounds in cooked beef is based on the aroma extract dilution analysis (Section C.4). By this technique 35 compounds were identified as having dilution factors greater than 4 (Table 11.18). This technique evaluates compounds on the basis of their relative aroma intensities. The most significant aroma compounds with dilution factors greater than 512 included, 2-methylfuran-3-thiol, methional, *trans*-2-nonenal, *trans,trans*-2,4-decadienal, β-ionone and bis-(2-methyl-3-furyl)-disulfide. 2-Methylfuran-3-thiol had an odor threshold of 0.0025–0.01 ng/l, and bis-(2-methyl-3-furyl)-disulfide, an odor threshold of 0.0007–0.0028 ng/l. In aged cured ham, methyl ketones representing 5% of the total volatiles are considered to have a positive impact on ham aroma.

The effects of lipid oxidation in cooked meat is to produce directly a number

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Table 11.17. Carbonyl compounds from beef fat [a]

Carbonyls (in order of concentration)	Flavor threshold	Flavor significance
2-Decenal	5.5	12
2-Undecenal	4.2	11
2-Nonenal	0.1	5
2-Octenal	1.0	10
Hexanal	0.15	6
2-Heptenal	0.63	9
Heptanal	0.042	3
Nonanal	0.32	8
Octanal	0.068	4
2,4-Decadienal	0.28	7
cis-4-Heptenal	0.001	1
trans-4-Heptenal	0.32	8
trans,trans-2,6-Nonadienal	0.001	1
trans,cis-2,6-Nonadienal	0.002	2

[a] From Frankel (1984). Flavor threshold in ppm.

of volatile compounds known to be derived from lipid hydroperoxides (e.g. alkanals, alkenals, alkadienals, and ketones), and indirectly decrease the levels of volatile compounds associated with desirable meaty odors (furans with sulfur substituents). The most important source of these furan compounds is the Amadori rearrangement and Maillard reactions between carbohydrates and amino acids, and further reactions with ammonia and hydrogen sulfide.

Volatiles that impart characteristic strong meat flavors and odor are formed in cooked meat by products from the reaction of cysteine and ribose and by the breakdown of thiamine. They include mixtures of 2-methyl-3-furanthiol (MFT), 2-furfurylthiol (FFT), 3-mercapto-2-pentanone (3M2P), and 2-mercapto-3-pentanone (2M3P). Cooked beef is distinguished from cooked pork, lamb and chicken by higher concentrations of MFT, FFT and 2M3P, but higher concentrations of 3M2P are found in chicken (Table 11.19). When the meat is refrigerated and reheated the concentrations of these four thiols are decreased, and off-flavors (described as cardboard and metallic) due to lipid oxidation appear. The four thiols MFT, FFT, 3M2P and 2M3P are also found in various heated foods including roasted coffee, pop-corn and wheat bread.

The volatiles identified in cooked chicken were the same as those of beef, but with higher levels of volatiles derived from unsaturated lipids (2,4-decadienal, and γ-dodecalactone), and lower beef aroma compound bis-(2-methyl-3-furyl)-disulfide. Chicken meat has a higher level of linoleic acid than beef and would be expected to contribute more volatile lipid decomposition products.

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Table 11.18. Volatile flavor compounds with high aroma values from cooked beef [a]

Flavor dilution factor	Volatile compound	Description
512	2-Methylfuran-3-thiol	Meaty, sweet
	Methional	Cooked potato
	trans-2-Nonenal	Tallowy
	trans,trans-2,4-Decadienal	Fried potato
	Bis(2-methyl-3-furyl)disulfide	Meaty
256	2-Acetyl-1-pyrroline	Roasted, sweet
	1-Octene-3-one	Mushroom
128	2-Acetylthiazole	Roasted
	trans,trans-2,4-Nonadienal	Fatty
64	2-Octanone	Fruity, musty
	trans-2-Octenal	Fruity, fatty, tallowy
	2-Decanone	Musty, fruity
	2-Dodecanone	Musty, fruity
32	*trans*-2-Heptenal	Fatty, tallowy
	1-*cis*-5-Octadien-3-one	Geranium, metallic
16	Hexanal	Green
	trans-2-Hexenal	Green
	2-Heptanone	Fruity, musty
	Heptanal	Green, fatty, oil
	trans-2-*cis*-6-Nonadienal	Cucumber
	2-Undecanone	Tallowy, fruity
	2-Tridecanone	Rancid, fruity, tallowy
8	1-Octen-3-ol	Mushroom
	2-Nonanone	Fruity, musty
	Nonanal	Tallowy, green
	2,4-Decadienal	Fatty
4	2-Methyl-3-(methylthio)furan	Sulfurous
	2-Acetythiophene	Sulfurous, sweet

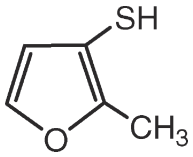
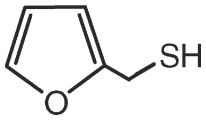
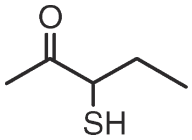
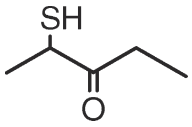
[a] From Gasser and Grosch (1988).

4. Antioxidants

Muscle tissues contain a multi-component antioxidant system consisting of lipid-soluble compounds (α-tocopherol, ubiquinone), water-soluble compounds (ascorbate, histidine-dipeptides) and enzymes (superoxide dismutase, catalase, glutathione peroxidase). Lipid oxidation in meats can be effectively controlled by the use of various phenolic compounds derived from spice extracts, by vitamin E supplementation of animal diets, and by processing of cured meat with sodium nitrite. Various natural antioxidant formulations containing mixtures of tocopherols, ascorbyl palmitate and citric acid show synergistic effects in stabilizing cooked and frozen meat. Synthetic antioxidants, BHA, TBHQ, propyl gallate (see Chapter 9) and combinations with citric acid, ascorbic acid or phosphates are also effective formulations used to retard lipid oxidation in

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Table 11.19. Concentrations (μg per kg) of 2-methyl-3-furanthiol (MFT), 2-furfurylthiol (FFT), 3-mercapto-2-pentanone (3M2P), and 2-mercapto-3-pentanone (2M3P) in cooked meat [a]

Structures	Volatiles	Beef	Pork	Lamb	Chicken
	MFT	7–28	6–9	5–11	4.5
	FFT	13–42	8–10	9–14	2.4
	3M2P	55–73	66–117	30	100
	2M3P	20–44	11–4	10	13

[a] From Kerscher and Grosch (1998). Samples of beef, pork and lamb were boiled for 45 min; chicken was boiled for 60 min.

meat. A number of metal chelators (EDTA, sodium pyrophosphate, tripolyphosphate, citric acid), and reducing agents (ascorbic acid, isoascorbic acid and their salts, sulfur dioxide) are effective protective agents in improving the sensory quality and color stability of stored meat products.

As in milk, ascorbic acid has either prooxidant activity at low concentrations (up to 250 ppm) by reducing iron, or antioxidant activity at higher levels (500 ppm) by acting as an oxygen scavenger. Higher concentrations of ascorbic acid (500–1000 ppm) may be detrimental by the formation of hydrogen peroxide in air that may not be removed by catalase in meat, leading to the destruction of myoglobin with bleaching of the meat color (Figure 11.1) and other quality deteriorations.

Supplementation of vitamin E in the diet has a significant effect in increasing the oxidative and color stability of poultry, beef and pork products. Dietary incorporation of α-tocopherol is more effective in stabilizing meat than exogenous addition of this antioxidant. Incorporation of α-tocopherol into the membrane lipids by the diet is considered to be an effective means of extending

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the shelf life of restructured and precooked meat products. The greater storage stability of chicken than turkey meat is attributed to the higher level of natural tocopherols in chicken meat. Vitamin E supplementation is also an effective means of reducing the formation of cholesterol oxidation products (cholesterol oxides) in raw and cooked veal muscle. Cholesterol oxides in foods are regarded as deleterious because of their association with health disorders.

Rosemary extracts containing potent antioxidants, including carnosic acid and carnosol (see Chapter 9), are effective in stabilizing cooked pork, beef, chicken and turkey meat. In cooked ground beef, rosemary extracts are effective in controlling the development of warmed-over flavor during storage. Many plant extracts and spices containing flavonoids and polyphenols reduce lipid oxidation and flavor deterioration, either when incorporated in the animal diets or when added directly to various ground meat products. However, the flavor associated with these plant extracts when added directly may limit their usefulness in muscle foods. Also, many current reports in the literature are difficult to interpret because no information is given on the amounts of active components in crude extracts. For example, pure Trolox and ascorbic acid are reported to be more effective than various commercial crude extracts of rosemary, but such comparisons made at different concentrations in weight percent are not valid without compositional information on the active ingredients, carnosic acid and carnosol. Another complication is the ready thermal oxidation of carnosic acid into antioxidant active products that have not been completely identified (see Figures 9.13 and 12.4).

Sodium nitrite ($NaNO_2$) used as a curing agent and as a preservative of red color in processing cured meat is an effective inhibitor of lipid oxidation and is an antimicrobial agent. The addition of nitrites to other meats (cooked ground beef, chicken and pork) retards oxidation, and inhibits the development of warmed-over flavor of stored cooked meat, and of rancidity in raw beef and chicken during storage. Nitrites produce a red color in cured meat due to the formation of nitrosylmyoglobin, which has antioxidant activity. The stability of cured meat decreases as the nitrosylmyoglobin becomes oxidized to produce metmyoglobin.

The chemistry of antioxidant activity of nitrite is complex and not completely understood. Although much of the literature evidence for this activity is based on the notoriously unreliable TBA test (see Chapter 5), there is more specific evidence based on GC volatile analyses indicating significant reduction of hexanal and other decomposition products of polyunsaturated fatty acids. A number of mechanisms have been suggested for the antioxidant activity of nitrite; it inhibits heme-catalysed lipid oxidation by forming a complex with the iron porphyrins, stabilizes the polyunsaturated lipids in membranes, chelates catalytic metals, forms inactive complexes with non-heme iron and copper, or low molecular weight iron fractions, produces

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nitrosated amines that possess antioxidant activity, stabilizes cell membranes, and scavenges free radicals by nitric oxide. The disadvantages of using nitrite in meat include the formation of undesirable N-nitrosamines, particularly in cooked bacon, altering the desirable color of myoglobin, and consumer objections.

Phytic acid and carnosine (histidine-containing dipeptide), obtained from cereal and meat by-products, are effective inhibitors of lipid oxidation by several mechanisms, including metal inactivation and free radical quenching. Uric acid obtained from the decomposition of adenosine triphosphate in muscle also inhibits lipid oxidation by the same mechanisms. However, the importance of uric acid as an endogenous antioxidant in muscle foods is not clear. Various protein concentrates from soybeans, cottonseed and peanuts inhibit lipid oxidation in muscle foods. In addition to their iron binding activity, these crude extracts contain complex polyphenolic flavonoids that have potent antioxidant activity.

5. Other control measures

Several factors can be manipulated to control and reduce flavor deterioration in meat due to lipid oxidation. Factors related to raw material include vitamin E content and age, while factors related to processing include addition of antioxidants, heat treatment and packaging. Oxidation in meat and other muscle foods is promoted by any processing that disrupts the natural cellular compartmental separation that controls oxidation. Heating and grinding raw meat thus accelerate lipid oxidation. It is therefore important to maintain the integrity of heated meat products to retard flavor deterioration from lipid oxidation.

Oxidative deteriorations of meat increase with increasing heating temperatures and heating time. However, above 100°C browning Maillard reaction products are formed at the surface of meat that inhibit lipid oxidation and the development of warmed-over flavor. This heat treatment changes the water-holding capacity and the appearance of the meat, which may not be desirable.

Packaging at reduced oxygen pressure and vacuum packaging are effective means of prolonging the oxidative stability of meat products such as precooked chill-stored, or freezer stored, sliced products. Modified-atmosphere (nitrogen or carbon dioxide) packaging is also an effective, but not commonly used, measure to increase the stability of meat products. Special modified atmosphere packaging has been developed to incorporate low levels of carbon monoxide (0.4–1%) together with nitrogen and carbon dioxide. Carbon monoxide binds strongly with ferrous myoglobin to produce carboxymyoglobin that has an attractive bright red color similar and more stable to that of oxymyoglobin. Low carbon monoxide concentrations can also inhibit lipid oxidation.

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Many controversial questions remain unanswered in evaluating the best measures to control flavor deterioration in processed meat. The effect of heating meat is explained by different mechanisms that accelerate the oxidation of phospholipids, by liberating iron from myoglobin and other metallo-proteins, by transforming myoglobin from an antioxidant to a prooxidant species, by disrupting in cell membranes the compartmental separation between the phospholipids and prooxidant metal catalysts, by thermal decomposition of hydroperoxides, and by denaturing antioxidant enzymes. Progress in this field has been hampered by the use of a number of over simplified model systems to study muscle tissue oxidation under unrealistic conditions of oxidation (e.g. heme catalysts activated with hydrogen peroxide and halides, combinations of iron and ascorbic acid). Transferring results obtained with such simplistic systems to the more complex meat systems may be questionable. The general and indiscriminate use of the TBA test to measure lipid oxidation is particularly problematic in complex foods such as meats, because they contain a multitude of prooxidant and antioxidant substances that seriously interfere with this test. Unfortunately, many studies do not run sufficient controls to correct for the interference from prooxidant metals and antioxidants and other factors, such as reducing browning reaction products on the colorimetric TBA method (see Chapter 5).

More recent studies determined hexanal and other volatile compounds by headspace gas chromatography (HS-GC) to measure lipid oxidation in meat. Although hexanal data may sometimes be in agreement with the results of the non-specific TBA method, the sensitive HS-GC method is more desirable because it determines specific decomposition products of lipid hydroperoxides. The same factors that influence lipid oxidation in meat such as pH, metal catalysts and antioxidants also affect and confound the interpretation of the results of the colorimetric TBA method. The TBA method is, therefore, not recommended to determine oxidation of meat and other complex foods because the degree to which non-lipid oxidation and degradation products, including browning reaction products, contribute to the TBA color remains unclear.

E. Fish products

Fish lipids are highly susceptible to oxidation because they contain high levels of polyunsaturated fatty acids (30–40%), including eicosapentaenoic acid (EPA) (5–12%) and docosahexaenoic acid (DHA) (10–26%). Concentrates containing 40 to 50% DHA are now obtained from special algae sources. These unique fatty acids distinguish fish lipids from other plant and animal lipids. Fish also contain prooxidants that generate lipid free radical initiators, and natural antioxidant systems. These pro-and antioxidant systems are affected by many factors, including storage temperature, oxygen concentration, pH, metal

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contamination, the use of salt, light, and processing causing cellular damage. Many of the same mechanisms of oxidative deterioration discussed for meat products (Section D) apply in varying degrees to fish products. Flavor deterioration in fish by lipid oxidation is the major problem in quality deterioration during processing and storage. Volatile decomposition products from long-chain *n*–3 polyunsaturated fatty acids in fish and fish oils have extremely low threshold values in the parts per billion range and are readily detectable at very low levels of oxidation (Section E.3).

The polyunsaturated fatty acids in phospholipids associated with the fish membranes also oxidize faster than the triacylglycerols found in fat deposits. Lipid oxidation in fish is influenced by several factors discussed below.

1. Metal catalysts

Iron is implicated in several stages of the lipid oxidation chain. Both heme (6–15 ppm) and non-heme iron (10 ppm) present in fish tissues catalyse lipid oxidation. Iron complexed to metabolites, referred to as low molecular weight iron, and iron complexed with myoglobin and hemoglobin, referred to as high molecular weight iron, participate in the initiation and propagation of lipid oxidation in fish. Increased rates of lipid oxidation in cooked fish muscles indicate the release of non-heme iron during cooking. Other prooxidant components present in fish include: mitochondria, nicotinamide adenine dinucleotide phosphate (NADP), lipoxygenase, ascorbate, ferritin and transferrin. Heme pigments are abundant in dark muscle of fish, which is also richer in unsaturated lipids and thus more susceptible to oxidation than white muscle.

As in meat, in cooked fish the myoglobin is activated into the catalytic metmyoglobin form, which accelerates the development of rancidity. On prolonged heating, the active heme iron is decomposed and the liberated free iron causes non-heme catalysis of lipid oxidation. Metmyoglobin can be activated in the presence of hydrogen peroxide to produce the hypervalent ferryl ion (Section A.4). However, there is no direct evidence showing the hydrogen peroxide-activated heme compounds in fish. By subjecting fish to freeze-thaw treatment, hemoproteins are oxidized and activated. The relative importance of hydrogen peroxide activation of metmyoglobin and the formation of free iron is not clear. EDTA inhibits the hemoprotein-catalysed oxidation in cooked fish due to exposure of the iron to the lipid substrate resulting from heating.

The inhibition of lipid oxidation from both heme and non-heme iron is difficult. In the early post mortem stages, fish tissues have sufficient reducing capacity to activate free iron. This reducing capacity of fish tissues is lost on storage and the ferric form of iron remains inactive as a catalyst. During

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Table 11.20. Oxidative stability of long-chain polyunsaturated fish and algae oils and oil-in-water emulsions at 40°C [a]

Samples	Fatty acids (%)			Peroxide value (meq/kg)	
	20:5 *n*–3	22:6 *n*–3	PUFA	48 hr	72 hr
Fish oil	11.8	16.13	34.6	3.0	11.3
Fish oil emulsion	–	–	–		
Algae oil [b]	–	42.4	42.4	1.0	1.3
Algae oil emulsion		–	–	40.5	–
Salmon oil	7.9	10.5	30.9	27.3	62.2
Salmon oil emulsion	–	–	–	37.4	74.5
Mackerel oil	9.7	14.5	40.2	34.9	148.1
Mackerel oil emulsion	–	–	–	52.1	203.6

[a] From Frankel *et al.* (2002). Oils were bleached and deodorized. Emulsions contained 5% oil, 1% lecithin and were homogenized with phosphate–citrate buffer (25 mM, pH 6.6).
[b] Contained 0.025% ascorbyl palmitate and 1937 ppm mixed tocopherols.

conditions of prolonged storage, myoglobin is oxidized to metmyoglobin (Figure 11.1), and hemoglobin to methemoglobin with iron in the ferric state. These oxidized hemes may be activated to the ferryl state by reacting with hydrogen peroxide and lipid hydroperoxides Metal catalysis in fish is further complicated by interfacial phenomena between multiphase and compartmentalized reactions in multiphase fish systems. New understanding of interfacial oxidation in complex fish tissues may be developed by partition studies with heme and non-heme iron by the same methodology used in partition studies with antioxidants (Chapter 9, 2.e).

Although oils rich in long-chain *n*–3 PUFA have attracted much interest because of their recognized nutritional importance, lipid oxidation limits their utilization in processed foods, especially in infant formula, which are also supplemented with iron (Section C.1). Fish and DHA-rich algae oils were initially much more stable to oxidation in bulk systems than in the corresponding oil-in-water emulsions (Table 11.20). With iron-supplemented fish and algae oil emulsions, addition of EDTA prevented oxidation in the control without added iron and at low iron:EDTA molar concentrations, but promoted oxidation at high iron:EDTA ratios. When the concentration of EDTA is insufficient to chelate all the iron, it becomes prooxidant by chelating Fe^{3+} preferentially over Fe^{2+}, which is a more active oxidation catalyst (see Table 9.2). Therefore, to be effective, EDTA must be added at molar concentrations higher than that of iron to inhibit oxidation of foods containing long-chain PUFA from either fish or algae and fortified with iron.

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2. *Enzyme oxidation*

Although lipoxygenases are recognized catalysts of lipid oxidation in plant tissues, as in meat tissues, in fish both enzymatic and non-enzymatic oxidation can take place. In general, lower temperatures favor enzymatic oxidation and higher temperatures promote non-enzymatic oxidation. This issue is confounded, however, because under conditions where enzymes are thermally inactivated, the denatured metallo-proteins provide active catalysts for the breakdown of hydroperoxides causing flavor deterioration. In stored fish muscle, enzyme-catalysed lipid oxidations are active for longer periods than non-enzymatic metal-catalysed oxidations. During frozen storage microsomal lipid oxidation is not significant, but after thawing, enzyme activity becomes an important factor for quality deterioration.

Lipoxygenases occurring in gill and skin tissues of fish produce conjugated diene hydroperoxides that may degrade, either by glutathione peroxidase to produce stable hydroxy fatty acids, or non-enzymatically by metal-heme catalysis to produce alkoxyl radicals which undergo cleavage into aldehydes and carbonyl compounds, causing rancidity. The 12-lipoxygenase and 15-lipoxygenase in fish gill tissue produce the 12- and 15-hydroperoxides from arachidonic acid and other polyunsaturated fatty acids in fish (Section A.4). The major volatile compounds produced from the oxidation of arachidonic acid and EPA by 12-lipoxygenase include: 1-octen-3-ol, 2-octenal, 2-nonenal, 2-nonadienal, 1,5-octadien-3-ol and 1,5-octadien-1-ol.

Gill lipoxygenase can be thermally inactivated above 60°C with a resulting improvement in shelf life stability of fish. However, heating increases non-enzymatic oxidation also, and this may exceed the oxidation due to lipoxygenase.

3. *Volatile compounds in stored fish, fish oils and fish oil enriched foods*

Oxidized fish oils, rich in *n*–3 polyunsaturated fatty acids, produced volatile compounds more readily than oxidized vegetable oils, rich in linoleic acid. Activation energy for the formation of propanal from fish oils was lower than for the formation of hexanal from vegetable oils. A mixture of aldehydes contributed to the characteristic odors and flavors of oxidized fish, described as rancid, painty, fishy and cod liver oil-like (Table 11.21). Oxidation of unsaturated fatty acids in fish was related to the formation of 2-pentenal, 2-hexenal, 4-heptenal, 2,4-heptadienal and 2,4,7-decatrienal. The fishy or trainy characteristic of fish oil was attributed to 2,4,7-decatrienal. Studies of volatiles from boiled trout after storage showed significant increases in potent volatiles by aroma extraction dilution analysis (Table 11.22). Volatiles with the highest odor impact included 1,5-octadien-3-one, 2,6-nonadienal, 3-hexenal, and 3,6-nonadienal. 3,6-Nonadienal and 3-hexenal were considered to contribute most to the fatty, fishy flavor in stored boiled fish.

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Table 11.21. Various carbonyl volatile compounds derived by lipid oxidation in fish tissues [a]

Compounds	Origin (PUFA) [b]	Conc. (ppb)	Threshold (ppb)	Descriptions
4-Heptenal	*n*–3	1–10	1	creamy
2,4-Heptadienal	*n*–3	1–10	10	rancid hazel nuts
2-Hexenal	*n*–3	1–10	17	green grass
2,4,7-Decatrienal	*n*–3	1–10	150	oxidized fish oil
1-Octen-3-ol	*n*–6	10–100	10	mushroom, melon
1,5-Octadien-3-ol	*n*–3	10–100	10	mushroom, seaweed
2,5-Octadien-1-ol	*n*–3	1–10	–	mushroom, seaweed
1,5-Octadien-3-one	*n*–3	0.1–5	0.001	mushroom
2-Nonenal	*n*–6	0–25	0.08	cucumber
2,6-Nonadienal	*n*–3	0–35	0.01	cucumber

[a] From Hsieh and Kinsella (1989); ppb, parts per billion.
[b] *n*–3 PUFA = 18:3 *n*–3, 20:5 *n*–3 and 22:6 *n*–3
n–6 PUFA = 18:2 *n*–6, 20:4 *n*–6.

Table 11.22. Effect of storage on potent odor compounds from boiled trouts analysed by aroma extraction dilution analysis [a]

Compounds	Concentration [b]		Odor activity value [c]	
	Fresh	Stored [d]	Fresh	Stored [d]
Butane-2,3-dione	268	363	54	73
Pentane-2,3-dione	140	167	28	33
cis-3-Hexenal	1.6	13	53	430
Hexanal	37	98	4	9
cis-4-Heptenal	2.8	6.0	46	100
Methional	5.8	7.5	145	188
1-Octen-3-one	0.6	0.9	60	90
1-*cis*-5-Octadien-3-one	0.36	0.64	900	1600
cis,cis-3,6-Nonadienal	<1.2	14.0	<24	280
trans-2-Nonenal	2.7	5.0	34	62
trans,cis-2,6-Nonadienal	8.0	10.5	400	525
trans,cis-2,4-Nonadienal	4.7	6.4	78	107

[a]From Milo and Grosch (1993).
[b]μg/kg boiled fish.
[c]odor activity value = concentration/odor threshold.
[d]stored 17 weeks at –13°C before boiling.

Milk emulsions enriched with 1.5% fish oil were evaluated after cold storage at 2°C. Although the cod liver oil contained less *n*–3 PUFA (26%) than the tuna oil (38%), the milk enriched with cod liver oil that had an initial peroxide value of 1.5 oxidized much faster than the same milk enriched with tuna oil that had

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Table 11.23. Oxidative stability of milk enriched with 1.5 wt% fish oils after storage at 2°C [a]

Fish oils	EDTA (ppm)	Peroxide values (meq/kg)		% Inhibition by EDTA	Fishy odor	Fishy flavor
		Day 7	Day 14	Day 14	Day 7	Day 7
Tuna	0	2.3	3.9	0	0.3	0.3
Tuna	50	2.7	3.7	6	0.4	0.9
Cod	0	4.7	5.3	0	3.2	3.2
Cod	50	4.3	4.3	18	1.7	2.4

[a] From Let *et al.* (2003).Tuna oil contained 38.1% *n*–3 PUFA, and cod liver oil 26% *n*–3 PUFA. EDTA = calcium disodium ethylenediamine acid.
% inhibition = (control-sample containing EDTA) / (control) × 100. Sensory scale: 0 (no intensity) to 9.

an initial peroxide value of 0.1 (Table 11.23). The milk enriched with tuna oil also did not develop a fishy oil odor during cold storage. Addition of EDTA significantly reduced the development of volatile oxidation products in the cod liver emulsions. The use of a fish oil with a sufficiently low initial peroxide value was therefore essential to prepare an oxidatively stable milk enriched with highly polyunsaturated fatty acids (*n*–3).

4. Fish processing

The fish processing industry is subject to many biological and environmental factors causing undue fluctuations in the quality of raw materials, which are extremely perishable. Many complex post mortem changes occur in fish muscles that disintegrate the membrane and affect lipid oxidation by mechanisms that are not well understood. A decrease in adenosine triphosphate (ATP) may initiate lipid oxidation by reducing nucleosides, conversion to hypoxanthine and change of xanthine dehydrogenase to xanthine oxidase. A decrease in reducing compounds such as ascorbate, NADH, and glutathione may affect lipid oxidation by changing the relative catalytic activity of heme iron and free iron, and the release of iron from ferritin.

(a) Effect of temperature. The storage life of fish is limited by the formation free fatty acids below 0°C due to hydrolysis of triacylglycerols by lipases and of phospholipids by phospholipases. Tissues containing free fatty acids are more readily oxidized and rancidity increases by long-term cold and frozen storage. Both free fatty acids and peroxide values increase in fish during frozen storage. By cooling just below the freezing point (–4°C), rancidity is accelerated in fish by a complicated process involving the removal of free

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Table 11.24. Effect of storing raw and baked minced carp at –18°C on free fatty acids (FFA), thiobarbituric acid (TBA) and carbonyl values [a]

Storage (weeks)	Raw			Baked		
	FFA	TBA	Carbonyl	FFA	TBA	Carbonyl
0	229	0.44	18.9	224	2.11	17.5
2	259	0.41	47.5	233	0.96	24.8
4	258	0.42	38.1	213	0.93	16.7
6	281	0.49	61.6	252	0.96	50.7
8	336	0.44	40.7	241	1.11	32.7

[a] From Flick *et al.* (1992). TBA and carbonyl values in μmoles/100 g wet sample.

water by crystallization, resulting in the concentration of catalytic metals. Other unfavorable changes that affect the quality of fish include protein denaturation, causing toughening of the fish muscle and yellow–brown discoloration, attributed to non-enzymatic browning due to the formation of lipid–protein complexes. The rate of oxidation in fish decreases rapidly at temperatures below –18°C. The development of rancidity in stored fish can be retarded by glazing with a thin layer of ice.

Cooking fish decreases the rate of rancid development on storage. This effect is attributed to the destruction of lipoxygenase, formation of water-soluble antioxidants, and destruction of heme compounds. Baked fish developed lower free fatty acids and carbonyl values but higher TBA values than raw fish after storage at –18°C for 8 weeks (Table 11.24). The TBA and carbonyl values reached a maximum and then decreased after 6 weeks storage, apparently because of further decomposition of carbonyl compounds. However, these determinations did not necessarily reflect the degrees of flavor deterioration, which needed to be determined by sensory analyses.

(b) Antioxidants. Lipid oxidation is retarded in fish by synthetic (BHA, BHT, TBHQ) and natural antioxidants (tocopherols, flavonoids) and by metal chelators (EDTA, ascorbate, phosphate, citrate, carnosine). Ascorbic acid retards the oxidation of herring during frozen storage, but may promote oxidation in cooked fish. Flavonoids are effective antioxidants in prolonging the shelf life of ground fish. EDTA and antioxidants inhibit enzyme-catalysed lipid oxidation (superoxide dismutase, catalase, and peroxidases) by removing iron and reducing hydrogen peroxide. Antioxidants are more effective in minced fish where they become more readily incorporated with the oxidizable lipids than in whole fish.

(c) Oxygen level and packaging. Other measures to retard development of rancidity in fish include minimizing exposure to air and using vacuum and opaque packaging. Vacuum and controlled atmosphere packaging (nitrogen

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and carbon dioxide) can improve the flavor score and shelf life of frozen stored fish. These measures are often more effective in fish products than the use of antioxidants.

To improve our understanding of the complex oxidation reactions in meat and fish products, evaluations should be carried out with each product stored under realistic conditions. Lipid oxidation should be followed by several and more reliable methods than the TBA test. These methods should measure specific lipid oxidation products (e.g. hydroperoxides or conjugated dienes) acting as precursors of rancid flavors, and specific volatile products (e.g. propanal, hexanal and other volatiles associated with fish flavors) that serve as reliable indicators of flavor deterioration. Instrumental analyses of volatile oxidation products should be supported and confirmed by sensory evaluations.

F. Cereal products

Cereals are more stable than other foods because they are low in total fat (2–5%) and contain relatively high levels of natural tocopherols (20–50 ppm α-, β- and γ-tocopherols). Cereals are also stabilized by antioxidant products formed during baking by the browning or Maillard reaction. Natural compartmentalization within plant cells and low water activity also contribute to the low susceptibility of cereal lipids to develop rancidity. As in other foods, since flavor deterioration in cereals is caused by minor amounts of lipid decomposition products, the amount of lipid in a product is much less important than its susceptibility to oxidation.

Cereal products from whole grain or those containing bran or germ components are more easily oxidized because they contain more lipoxygenases and more unsaturated lipids (40–60% linoleic acid and 1–10% linolenic acid). Recent popularity of whole grain products has increased the importance of rancidity and its control in cereal foods. Whole grains are stable during storage. However, after milling into flour the grain integrity is disrupted, lipids become subject to oxidation by exposure to hydrolytic and oxidative enzymes activated by air and moisture, and catalytic metal ions. Rancidity in cereal products results from hydrolytic and oxidative degradations, usually sequentially because hydrolytic products are more susceptible to oxidation.

1. Hydrolytic rancidity

The hydrolysis of polyunsaturated lipids in cereals produces free fatty acids that undergo further enzymatic or non-enzymatic oxidation to form volatile and non-volatile undesirable flavor compounds. Lipoxygenases act mainly on free fatty acids, which are also more easily oxidized than those esterified as triacylglycerols. In addition, free fatty acids are detrimental to functional properties of cereal products.

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Lipase activity is found mainly in the bran components, which readily accumulate free fatty acids during ambient storage. Bran lipase is most active at 17% moisture, in finely divided milled products; it is rapidly inactivated by heating at 100°C for 10 min. Lipase activity is high in other cereal grains including oats and rice.

2. Lipoxygenase action

This enzyme oxidizes linoleic and linolenic acids rapidly in whole flour or milling products containing wheat germ or bran mixed with water. The initial hydroperoxides formed by lipoxygenases in stored wheat bran are converted to secondary products, mono- and trihydroxy fatty acids. These oxidation products causing bitter and rancid flavors are formed in higher concentrations in hydrated products than in dry raw materials. Rancid flavors develop rapidly on hydration.

The addition of small amounts of unheated soy flour to wheat dough improves its rheological properties in the presence of air, but the mechanism for this improvement in bread quality is not clear. For the production of white bread, soybean flour containing lipoxygenase can be used, in small amounts (1%), to bleach the yellow pigment by co-oxidation of the carotenoids. However, heat inactivation of lipoxygenase is necessary in the preparation of pasta products to preserve the desirable yellow color of flour from durum wheat rich in carotenoids. Lipoxygenases, present in the germ component of most cereals, produce a mixture of secondary oxidation products in ground grain suspended in water. The formation of trihydroxy fatty acids cause bitter and rancid flavors in hydrated unheated milled oats.

3. Non-enzymatic oxidation

In contrast to enzymatic oxidation, free radical non-enzymatic oxidation of cereal lipids is readily inhibited by antioxidants. Undamaged grains and cereal germ oils are oxidatively stable because of their tocopherol contents. However, tocopherols are readily decomposed during storage of cereal products. Oats contain other flavonoid compounds. Certain processing steps can increase the susceptibility of grains to oxidation by decomposing tocopherols, disrupting the grain, redistributing the lipids and exposing them to catalytic metals. Natural metallo-proteins in cereals can also be activated during thermal processing in the presence of moisture to catalyse lipid oxidation.

4. Factors affecting lipid oxidation

(a) Raw materials. Cereal products from grains obtained under wet preharvest conditions are more susceptible to rancidity due to physical damage,

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resulting in cell disruption that promotes the activities of microbial and fungal lipases. Thermal inactivation of enzymes should be carried out at the lowest temperature to reduce susceptibility of the grains to non-enzymatic lipid oxidation and to avoid detrimental changes in functionality. Poor baking performance results from loss of gluten functionality by thermal denaturation of proteins.

(b) Processing. Enzymes are more easily inactivated by heat at higher water activity. Thus, at higher moisture content, oat lipoxygenase can be inactivated at a lower temperature (64°C at 12% moisture) than at low moisture (103°C at 8% moisture). Enzymatic lipolysis can also be prevented at storage temperatures sufficiently low to solidify the oil. Moisture contents below 5% are recommended to minimize non-enzymatic rancidity in cereal products.

(c) Antioxidants. Endogenous tocopherols are important to prevent rancidity during grain processing. Thermal processing designed to inactivate enzymes should be carried out at the lowest temperature necessary to minimize the oxidation of natural tocopherols. However, the tocopherol content of wheat flour decreases significantly during storage, and the lipids become more susceptible to oxidation. Added synthetic antioxidants such as BHA, TBHQ and natural antioxidants from rosemary extracts are effective in prolonging the shelf life of dry cereal products.

The presence of natural flavonoid antioxidants in oats and other cereals is known to minimize problems of rancidity. Phenolic compounds inhibit lipase activity, but they are readily decomposed by heating above 80°C. In roasted cereal products, the antioxidant activity varies according to the level of browning reaction products generated. The extracts of roasted wheat germ and roasted press cake from wheat germ processing were the most active when tested with stripped (tocopherol-free) maize oil oxidized at 50°C (Table 11.25). Ground and roasted hazelnut and sweet almond showed comparable antioxidant activity, while a coffee extract had the highest antioxidant activity. When the antioxidant activity of these extracts, based on inhibition of conjugated diene hydroperoxide formation, was compared with their anti-radical activity toward 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), the results were not strictly correlated (see Chapter 10).

Processing operations such as drying, blanching, roasting and extrusion destroy varying amounts of natural phenolic compounds in cereal products. Oat phenolic compounds are a mixture of free phenolic acids, esters, and phenolic glycosides as well as flavonols and polyphenols. Alcohol extracts of oat groat and flour have strong antioxidant activity, but 50% degradation of antioxidant phenolic compounds occurs during extrusion. Since heat-generated browning reaction products may be even more active as antioxidants than natural phenolic compounds, thermal processing of cereal products should be

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Table 11.25. Antioxidant activity of some roasted foods [a]

Antioxidant methods	Antioxidant trends (total phenol in μg/ml)
Inhibition of conjugated dienes	Wheat germ > AP > maize germ > barley germ
Inhibition of conjugated dienes	Roasted wheat germ cake (23.3) > roasted wheat germ (22.2) > defatted roasted wheat germ
Inhibition of conjugated dienes	Coffee (83.3) > roasted wheat germ > roasted hazelnut = roasted sweet almond (18.7)
Antiradical activity (DPPH)	Coffee = roasted hazelnut > roasted wheat germ cake > roasted wheat germ >> roasted sweet almond

[a] From Krings and Berger (2001). Ethanolic extracts tested at 20% for inhibition of conjugated diene formation on stripped maize oil free of tocopherols.
AP = ascorbyl palmitate; DPPH = 1,1-diphenyl-2-picrylhydrazyl

aimed at optimizing the time and temperature profile on the basis of valid antioxidant evaluations of the finished products (see Chapters 8 and 9).

(d) Iron fortification. Iron is added as a supplement to baby and to adult cereals, and to wheat flour as a nutritional supplement. These sources of iron, and any contamination from equipment during processing, provide the main catalyst for lipid oxidation in cereal products. Infant cereals are usually fortified with iron because it is considered the major source of iron during the weaning period. Adult cereals are also often supplemented with iron for presumed "energy" and "health" effects. Unfortunately, the most bioavailable forms of iron are soluble iron compounds, such as ferrous sulfate or ferrous gluconate that also promote lipid oxidation and cause flavor deterioration when added to stored cereals. Many less soluble and less bioavailable forms of iron (elemental iron, ferric pyrophosphate and ferric orthophosphate) are used commercially for economic reasons, and because they are relatively less active as oxidation catalysts. The prooxidant activity of soluble ferrous sulfate can be inhibited by encapsulation with either hydrogenated vegetable oils or mono- and diglycerides. Various iron complexes have proven to be suitable sources of iron fortification, including ferrous fumarate and ferrous succinate (Table 11.26). These iron chelates are bioavailable and do not promote lipid oxidation during storage at 37°C for 3 to 6 months. For either economic or legal reasons, these forms of iron supplement are not commonly used in baby cereals.

Because whole maize contains high levels of phytate, which inhibits the absorption of iron as ferrous sulfate, iron amino acid chelates that are more bioavailable have been investigated for infant nutrition. Although iron bisglycinate is more bioavailable than iron trisglycinate, when added to maize porridge, it produced more rancidity and hexanal after storage (Table 11.27). Ferric trisglycinate produced less rancidity and hexanal and was as stable as NaFeEDTA and the control, but it is insoluble and not well absorbed. The

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Table 11.26. Effect of iron fortification on pentane formation and odor evaluation of infant cereals stored for 3 months at 37°C [a]

Iron source (mg Fe/100g)	Headspace pentane (ppm)	Odor score[b]
Wheat cereal		
No iron	2.3	6.7
Dry ferrous sulfate (10)	2.3	6.7
Hydrated ferrous sulfate (10)	15.1	3.0
Encapsulated ferrous sulfate (10)[c]	2.0	6.7
Fe pyrophosphate (10)	21.5	3.3
Electrolytic Fe (10)	1.9	6.3
Rice cereal		
No iron	0.2	7.3
Ferrous fumarate (18.5)	0.2	7.0
Ferrous succinate (18.5)	0.2	7.7
Mixed cereal		
No iron	2.7	7.7
Ferrous fumarate (18.5)	4.7	5.8
Ferrous succinate (18.5)	5.4	5.3

[a] From Hurrell *et al.* (1989).
[b] Odor score: 9, excellent; 5, acceptable; 1, very strongly oxidized.
[c] Encapsulated in hydrogenated soybean oil.

Table 11.27. Effect of iron supplements on stability of maize porridge stored for 20 days [a]

Iron supplements (30 mg Fe/kg)	Temp.°C	Rancid flavor	Hexanal (peak area)
Control	30	0.7	111
$FeSO_4$	30	1.3	121
Fe Bisglycinate	30	2.5	5726
Fe Trisglycinate	30	1.2	164
EDTA	30	1.3	0
Control	50	0.8	682
$FeSO_4$	50	1.3	1907
Fe Bisglycinate	50	6.3	10081
Fe Trisglycinate	50	1.0	617
EDTA	50	1.1	232

[a] From Bovell-Benjamin *et al.* (1999).
Sensory scale: 0 = none to 10 = very intense.
Hexanal analysed by static headspace gas chromatography.

adverse effects of iron bisglycinate on rancidity and hexanal formation in the maize porridge were effectively minimized in the presence of 25 or 50 ppm BHA.

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Iron deficiency is apparently only a serious problem in developing countries that leads to a global prevalence of anemia in young children. Although the use of iron-fortified infant formulas and cereals contribute to the decline of iron deficiency in industrial countries, improved iron supplements have been sought that are more bioavailable than ferrous sulfate in baby cereals. In contrast to infant nutrition however, the frequent practice of iron supplementation of adult cereal products and flours may be questionable in view of the problems of rancidity development during storage and processing, and the possible nutritional and biological consequences of excessive iron in the body (Chapter 13).

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CHAPTER 12

Frying fats

Although the frying of foods is an important application of edible fats, it is one of the most complex and difficult processes to understand, because of the multitude of reactions taking place and the complexity of the products formed. During frying, the water released from the food into the heated oil produces steam that causes hydrolysis of the fat. On the one hand, the resulting free fatty acids are more rapidly oxidized and promote thermal oxidation by solubilizing metal catalysts. On the other hand, the steam blankets the surface of the frying oil and moderates the rate of lipid oxidation by reducing the availability of oxygen from the air. The steam stripping of volatile decomposition products also delays deterioration of the frying fat.

During frying, foods absorb oil in amounts varying from 5 to 40% by weight, and release some of their lipids into the frying fat. The composition and stability of the fat can thus be changed by the foods during frying. After frying chicken or fish, for example, the frying fat will contain chicken or fish lipids. An important step in commercial frying is the continuous addition of fresh oil to attain a steady state by replacing the oil absorbed by the food. The presence of food particles (e.g. breading and meat particles) will accelerate the thermal darkening process. The reactions of frying fats with food protein and carbohydrate components contribute to both desirable and undesirable flavors. The formation of desirable flavors, odors and texture is necessary for the fried foods to acquire their unique sensory characteristics. However, extensive thermal oxidative decomposition of fats during prolonged frying not only reduces the sensory quality of the fried foods, but also may diminish their nutritional value. Good frying practice is aimed, therefore, at carefully controlling the temperature, time and replacement of oil during frying to optimize desirable sensory qualities of the fried foods, and to minimize the accumulation of thermal deterioration products causing off-flavors and potential safety problems.

A. Chemistry of frying

The oxidation of unsaturated fats is not only greatly accelerated at high temperatures, but the free radical mechanism is changed by the decrease in oxygen concentration in heated fats. At elevated temperatures, the oxygen availability is lower and becomes limiting (see Chapter 7.E). The alkyl radicals, formed by initiation (1), become more important because the rate of the oxygenation reaction (2) is diminished at elevated temperatures. The termina-

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tion reactions (5–10) also become more important, because the alkyl radicals condense into stable products of higher molecular weights.

Initiation: $LH \longrightarrow L^{\cdot}$ (1)

Propagation: $L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$ (2)

$LH + LOO^{\cdot} \longrightarrow L^{\cdot} + LOOH$ (3)

$LOOH \longrightarrow LO^{\cdot}$ (4)

Termination: $2\ L^{\cdot} \longrightarrow L—L$ (5)

$LO^{\cdot} + L^{\cdot} \longrightarrow LOL$ (6)

$LOO^{\cdot} + L^{\cdot} \longrightarrow LOOL \longrightarrow LO^{\cdot} \longrightarrow L—L + LOL$ (7–10)

At temperatures above 100°C, the initial hydroperoxides formed by reaction (3) exist only transiently and decompose rapidly into a multitude of volatile and non-volatile products. Two mechanisms have been postulated for the thermal oxidation of unsaturated fats: (a) thermal decomposition by direct interactions of radicals, when unsaturated fats are continuously heated at elevated temperatures, and (b) induced decomposition through the intermediacy of hydroperoxides, when unsaturated fats are subjected to intermittent heating. Under these conditions, hydroperoxides accumulating at the lower temperatures contribute more radicals by decomposition when the fats are reheated. Intermittent heating of unsaturated fats is generally assumed to be more destructive than continuous heating. However, the evidence for these mechanisms are far from clear, due to the complexity of reactions products formed.

Three major reactions that occur during frying include: oxidation, polymerization and hydrolysis. Lipid oxidation at the elevated temperatures of frying (180–200°C), in the presence of moisture generated from foods, produces a multiplicity of compounds that contribute to desirable and undesirable changes affecting the flavor and quality of foods. During the frying of foods additional products may also be derived from interactions between lipid oxidation compounds and food components (proteins and carbohydrates). Thermal lipid oxidation and hydrolysis produce complex mixtures of volatile and non-volatile monomeric (cyclic and non-cyclic) and polymeric (polar and non-polar) substances.

Other changes in frying oils take place due to transfer of lipids from foods to the frying oil. The migration of minor constituents from the foods also occurs

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and can either inhibit or accelerate oxidation. Phospholipids and emulsifiers contribute to foaming; tocopherols and other phenolic spices inhibit oxidation; trace metals accelerate oxidation; volatile flavor compounds may contribute to off-flavors; breaded or battered foods produce particles that cause browning, burning and oil degradation.

1. Volatile compounds

During frying, a large mixture of volatile substances is produced by rapid decomposition of hydroperoxides and polyunsaturated aldehydes. These volatile decomposition products are found in relatively small amounts, because a large portion is removed from the oil by steam distillation and the sweeping action of steam generated during frying. The remaining volatile compounds are of concern, because they are partially absorbed by the fried foods and contribute to their flavor and to the odor of the room where frying is carried out, also referred to as room odor. Volatile compounds produced by thermal oxidation include aldehydes, ketones, alcohols, acids, esters, hydrocarbons, lactones, substituted furans, and aromatic compounds. Gas chromatographic analyses of fat samples after different frying treatments represent mainly the more stable volatile compounds remaining in the fats (Table 12.1). The major volatile constituents identified in frying oils are derived from the decomposition of lipid oxidation products and include 1-pentanol, hexanal, furfuryl alcohol, *trans*-2-heptenal, 5-methylfurfural, 1-octen-3-ol, octanal, 2-pentylfuran, *trans*-2-nonenal and hexadecanoic acid.

Additional minor volatile compounds are found in both frying oils and in fried foods, including *cis,trans*- and *trans,trans*-2,4-decadienal derived from oxidized linoleate and 2,4-heptadienal derived from linolenate. The isomers of 2,4-decadienal impart a desirable fried food flavor in fried potatoes when present in small amounts, but excessive amounts of this aldehyde would be expected to cause undesirable rancid flavors. Furfural compounds may be derived from interactions between food sugars and proteins. Minor amounts of sulfur compounds and nitrogen-containing heterocyclic compounds (methyl pyrazine and 2,5-dimethylpyrazine in potatoes) may originate from Maillard reactions (Chapter 11).

The potent volatiles from freshly prepared French fries were evaluated by aroma extraction dilution and headspace GC-olfactometry (Table 12.2). The most potent odorants included methional, 2-ethyl-3,6-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine, *trans,trans*-2,4-decadienal, dimethyltrisulfide, and 3-methylbutanal. Different pyrazines are formed as powerful aroma compounds by the Maillard reaction and as secondary products of the Strecker degradation between α-dicarbonyl compounds and amino acids, and by heating sugars with various amino acids and other N-containing components of foods (see Chapter 11.B.3) The deep-fried note attributed to *trans,trans*-2,4-

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Table 12.1. Volatile compounds identified in frying vegetable oils and in French fries [a,b]

Compounds found in frying oils	Relative amounts [c]	Compounds found in French fries [b]
1-Penten-3-ol	++	Hexanal
Pentanal	++	Methyl pyrazine
Heptane	++	2-Hexenal
1-Pentanol	+++	2-Heptanone
Hexanal	+++	Nonane
Octane	++	Heptanal
Furfural	++	2,5-Dimethylpyrazine
trans-2-Hexenal	++	2-Heptenal
Furfuryl alcohol	+++	2-Pentylfuran
Heptanal	++	Octanal
2-Acetylfuran	++	*trans,trans*-2,4-Heptadienal
trans-2-Heptenal	+++	2-Octenal
5-Methylfurfural	+++	Nonanal
1-Octen-3-ol	+++	2-Nonenal
Octanal	+++	Decanal
2-Pentylfuran	+++	2-Decenal
trans-2-Octenal	+++	*trans,cis*-2,4-Decadienal
Nonanal	+++	Undecanal
trans-2-Nonenal	+++	*trans,trans*-2,4-Decadienal
4-Oxo-nonanal	++	
trans,trans-2,4-Decadienal	++	
2-Undecenal	++	
Hexadecanoic acid	+++	

[a] From Takeoka *et al.* (1996). Soybean and cottonseed oils used to fry meat products. Analysed by capillary gas chromatography of fractions isolated by simultaneous steam distillation-extraction.
[b] From Perkins (1996).
[c] ++ = 0.5–2%, +++ = >2%.

decadienal predominated on nasal evaluation of the French fries, and the deep-fried and boiled potato-like odors caused by methional were perceived by the retronasal test (see Chapter 5).

2. *Monomeric compounds*

Thermal oxidation of unsaturated fats is accompanied by considerable isomerization of double bonds, leading to products containing *trans* double bonds and conjugated double bond systems. Non-volatile decomposition products are formed by thermal oxidation, hydrolysis and cyclization. A large number of hydroxy, alkoxy-substituted, epoxy and keto compounds are produced by oxidation, free fatty acids and mono- and diacylglycerols by hydrolysis, and cyclic monomers by cyclization of polyunsaturated fatty acids in heated fats. Aldehydo glycerides (also referred to as "core aldehydes") and keto glycerides

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Table 12.2. Aroma extaction dilution analysis of selected volatiles from French fries [a,b]

Compound	Conc.[a] (%)	Description [b]	Odor threshold value (μg/kg) [a]	Aroma value [a]	FD-factor [b]
3-Methylbutanal	5.0	Malty	13	3850	256
Hexanal	2.1	Green	120	175	16
Methional	2.0	Boiled potato	0.2	100,000	4096
Dimethyltrisulphide	–	Cabbage	–	–	512
Phenylacetaldehyde	18.0	Honey	22	8180	128
2-Octenal	–	Tallowy, nutty	–	–	32
3-Et-2,5-diMepyrazine	–	Roasty, earthy	–	–	512
cis-3-Nonenal	–	Cucumber	–	–	16
Nonanal	–	Fatty, green	–	–	64
trans-2-Nonenal	1.5	Tallowy, green	150	100	512
2,3-DiEt-5-Mepyrazine	–	Roasty, earthy	–	–	2048
2-Et-3,6-diMepyrazine [a]	7.4	Roasty, earthy	24	2720	2048
cis,trans-2,4-Nonadienal	–	Deep-fried	–	–	32
trans-2-Decenal	–	Fatty	–	–	64
trans,trans-2,4-Nonadienal	–	Tallowy	–	–	64
trans-4,5-Epoxy-2-nonenal	–	Metallic	–	–	32
cis,trans-2,4-Decadienal	–	Deep-fried	–	–	512
trans,trans-2,4-Decadienal	7.5	Deep-fried	135	560	2048
trans-4,5-Epoxy-2-decenal	–	Metallic	–	–	512

[a] From Belitz and Grosch (1986). Aroma value = concentration/odor threshold concentration; 2-Et-3,5-diMepyrazine was listed as 2-Et-3,6-diMepyrazine.
[b] From Wagner and Grosch (1997). FD factor = flavor dilution factor, determined on neutral/basic volatiles by gas chromatography-olfactometry after stepwise dilution with diethyl ether.

are also produced when triacylglycerol hydroperoxides undergo cleavage on the aldehyde (or oxo) group remains attached to the glyceride end of the chain. Free fatty acids are particularly damaging to the stability of the frying medium and the fried foods because they are rapidly oxidized; they catalyse further oxidation of polyunsaturated fats by solubilization and activation of catalytic metal salts, and by their surface activity, they may increase their contact with oxidizable components of foods and promote lipid–protein interactions. The increase in surface activity due to free fatty acids is also augmented by the formation of mono- and diacylglycerols arising from partial hydrolysis of triacylglycerols.

Cyclic monomers have received special attention because they are considered nutritionally harmful if they are present in sufficient quantities. However, the potential harmful effects of heated fats have been controversial, because many nutritional studies have been carried out under exaggerated conditions that do not represent actual home or commercial applications (Section F). A large number of cyclic fatty acids have been identified in heated vegetable oils containing linoleic and linolenic acids. Main structures include disubstituted five-membered (cyclopentyl) and six-membered (cyclohexyl) compounds with

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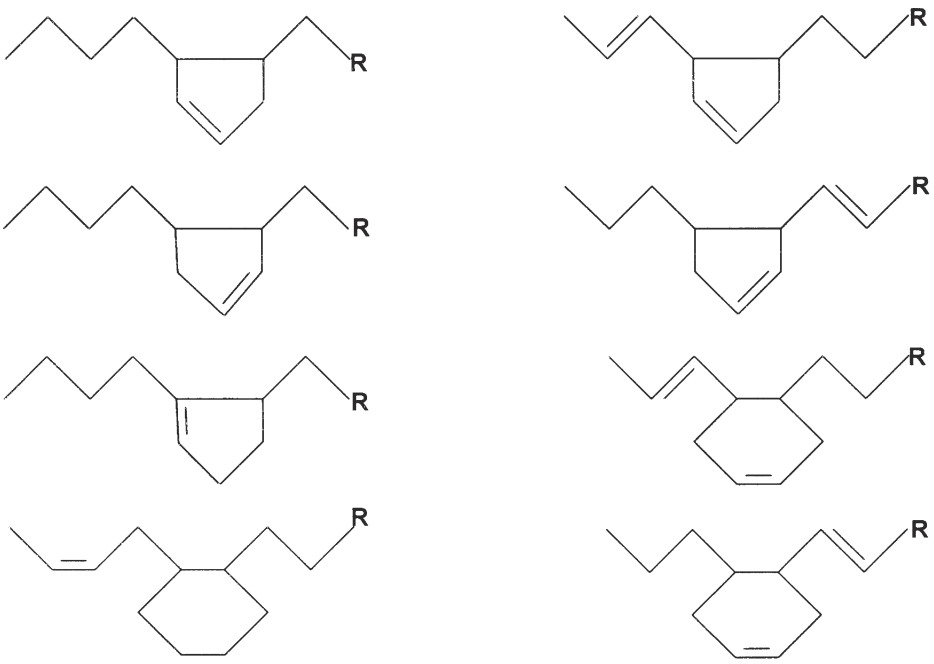


Figure 12.1. Structures of some cyclic monomers from linoleic and linolenic acids in heated oils. Adapted from Le Quéré and Sébédio (1996) and Christie and Dobson (2000).

unsaturation inside and outside the rings (Figure 12.1). About 0.3 to 0.6% cyclic monomers were found in partially hydrogenated soybean oil after prolonged frying of potato slices (Table 12.3). Polar materials varying from 0.4 to 0.9 % included oxygenated monomeric products. Non-eluted products ranging from 18 to 21% represented high molecular-weight polymeric compounds. Surveys of commercial frying oils in the US showed a range of 0.1 to 0.5% cyclic monomers and 1 to 8% polar and non-eluted materials.

Table 12.3. Gas chromatographic analysis of heated soybean oil [a]

Oils	Frying at 195°C [b]	Cyclic monomers	Polar compounds	Non-eluted material
Soybean oil	52 hr. intermittent	0.45	0.41	18.4
Hydrogenated soy. [c]	52 hr. intermittent	0.33	0.41	18.3
Hydrogenated soy. [c]	104 hr. continuous	0.57	0.91	20.7

[a] Meltzer *et al.* (1981). Gas chromatographic analyses carried out on completely hydrogenated samples after concentration by low-temperature crystallization.
[b] Oils were heated intermittently or continuously and potato slices were fried at different intervals.
[c] Hydrogenated soybean oil to an iodine value of 107.

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Basic studies with heated sunflower oil showed the formation of a wide variety of cyclopentenyl, cyclopentyl, cyclohexenyl, and cyclohexyl fatty acids (Figure 12.1). Cyclopentenyl fatty acids were apparently formed by cyclization from one double bond to the other, while cyclopentyl and cyclohexyl fatty acids were formed by cyclization in a direction away from the existing double bond. A range of saturated bicyclic fatty acids was also produced from the cyclic monoenes by formation of a bridge across a cyclopentyl or cyclohexyl ring. From heated linseed oil, two cyclohexene and two cyclopentene structures were identified, with one double bond in the ring and one in the aliphatic chain. The cyclohexenyl and cyclopentyl products were formed by cyclization from the first to the third double bond of linolenate or vice versa. Although a mechanism suggested for these cyclizations may involve intramolecular rearrangements catalysed by traces of hydroperoxides as initiators, another non-radical pathway may proceed by Diels–Alder cyclization of conjugated diene intermediates (Figure 12.2). Although the structures of cyclic monomeric compounds in frying oils have been well defined, there is evidence for a large number of oxygenated monomeric compounds that have not been completely characterized because of their complex composition. The oxidation of a synthetic diunsaturated C-18 cyclic fatty acid [9-(6-propyl-3-cyclohexenyl)-*Z*-8-noneoate] produced a mixture of 7 isomeric hydroperoxides under free radical conditions, and 5 isomeric hydroperoxides under photosensitized conditions (Table 12.4). Under free radical conditions, the ring double bond was preferentially oxidized over that of the side chain in a ratio of 8:1 because of greater steric hindrance at the side double bond. In contrast, under photosensitized oxidation, a ratio of 1.2:1 was found between side chain and ring oxidation. The possible toxic effects of these cyclic monomer hydroperoxides and other oxidation products have not yet been investigated.

3. Polymeric compounds

The predominant group of non-volatile compounds formed during frying of unsaturated fats includes dimers and oligomers. These high molecular weight compounds are mostly formed in the termination stages of free radical oxidation (Chapter 1). Non-polar dimers are formed by addition of alkyl radicals and linked by carbon–carbon bonds. According to the type of fatty acid precursors these carbon–carbon dimers include monoene, diene and tetraene structures (Figure 12.2). Polar dimers are oxygenated and form either by combining alkyl and alkoxyl radicals and linked by ether bonds (C–O–C), or by combining radicals containing oxygenated functions (hydroxy, keto, epoxy). Polar dimers linked by peroxy bonds (C–OO–C) are only formed at low temperatures and decompose at elevated temperatures (above 100°C). At these temperatures, peroxy-linked dimers may be formed as intermediates and produce either ether-linked or other oxygenated polar dimers.

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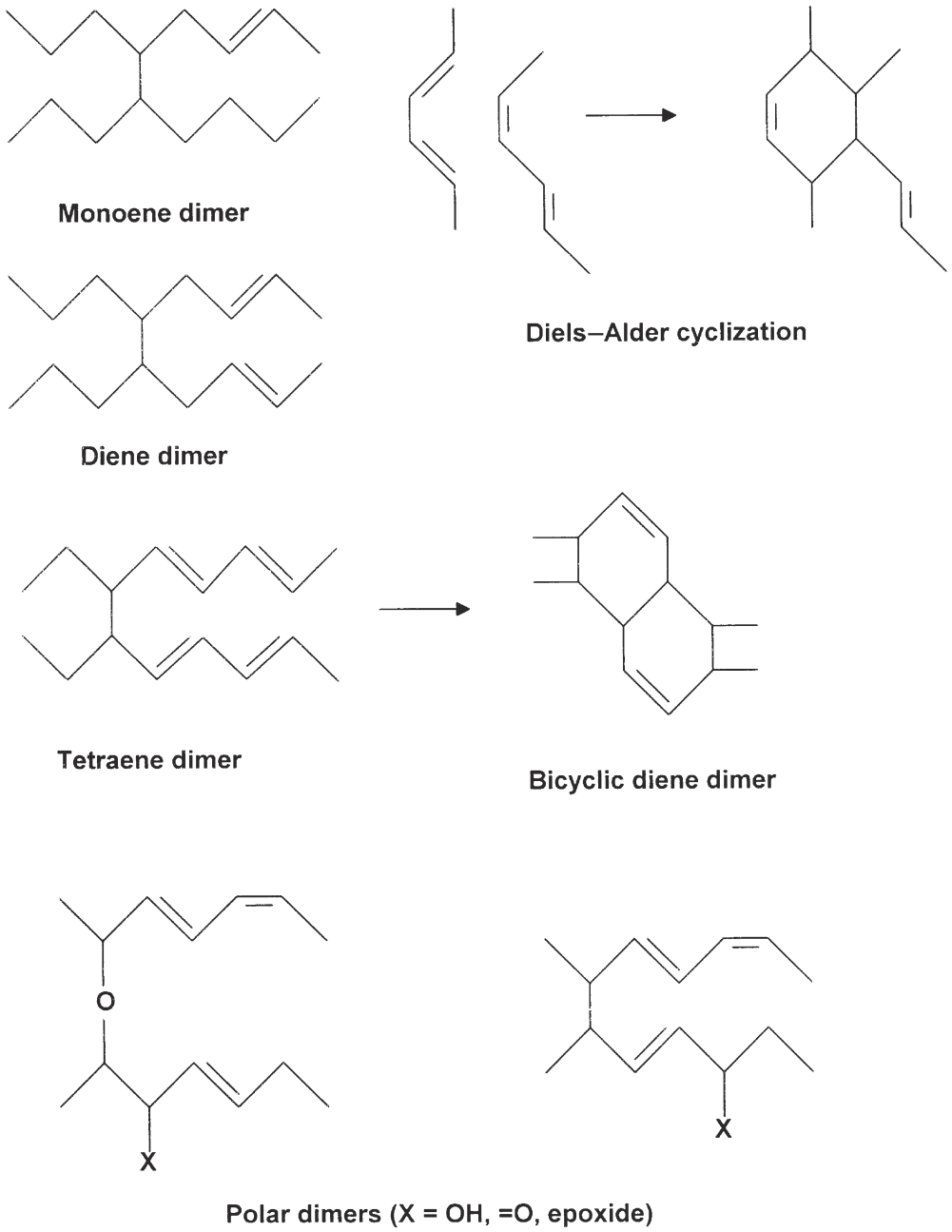
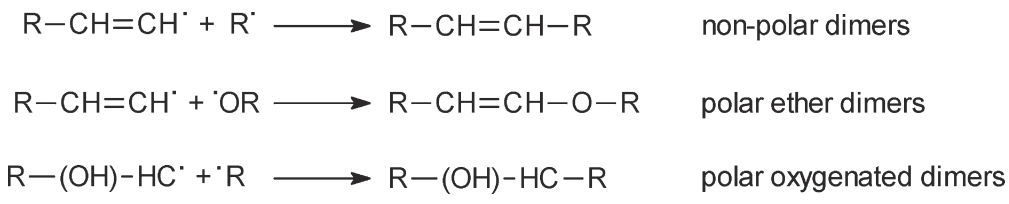


Figure 12.2. Structures of different dimers formed in heated oils.



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Cyclic dimers are also formed by intramolecular addition of an intermediate dimeric radical to the double bond in the same molecule.

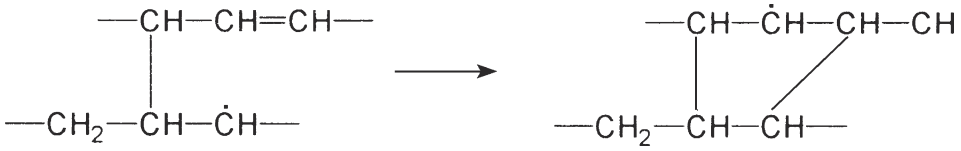
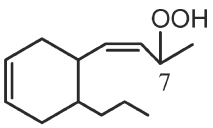
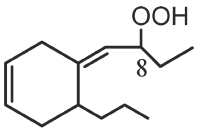
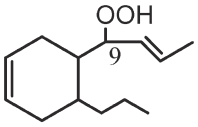
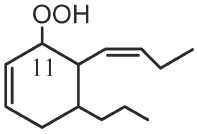
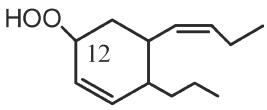
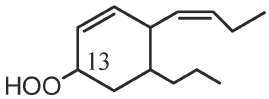
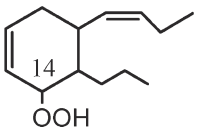


Table 12.4. Isomeric hydroperoxides produced from 9-(6-propyl-3-cyclohexenyl)-*Z*-8-noneoate by free radical and photosensitized oxidation [a]

Structures	Isomers	Relative %	
		Free radical	Photosensitized
	7-OOH	8.9	5.5
	8-OOH	1.9	3.5
	9-OOH	0.4	43.1
	11-OOH	4.2	
	12-OOH	37.8	19.0
	13-OOH	32.5	26.1
	14-OOH	14.3	

[a] From Awl *et al.* (1986, 1987).

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Non-radical polymerization can also take place at elevated temperatures by Diels–Alder reactions between either two conjugated diene fatty acids or between a conjugated and a non-conjugated diene fatty acid, to form non-polar substituted cyclohexene unsaturated dimers (Figure 12.2). These types of dimers are found in only small quantities because the reactive conjugated diene precursors may be formed in minor concentrations. More complex structures have been identified in oils used after frying, including unsaturated bicyclic compounds with conjugated and non-conjugated double bonds, trimers, acyclic polar dimers, and tetrahydrofuran tetrasubstituted dimers. These polar and non-polar dimeric and oligomeric compounds are not completely characterized because of their complex composition.

4. Interaction compounds

Complex mixtures are produced by non-enzymatic browning reactions between thermally oxidized lipids and amines, amino acids and proteins (see Chapter 11.B.4). Interactions between aldehydes, epoxides, hydroxy ketones, and dicarbonyls with proteins cause browning that has been related with losses of lysine, histidine, and methionine. Schiff base formation results in polymerization to form brown macromolecules. Interactions between epoxyalkenals formed at elevated temperatures and reactive groups of proteins produce protein pyrroles polymers and volatile heterocyclic compounds. Much of the published research in this complex chemical area was based on model systems. More structural information is needed however with real foods subjected to frying conditions.

B. Fats used for frying

A wide range of fats are used commercially, varying from unhydrogenated refined fats to blends of partially and "fully" hydrogenated fats. The selection is based on the fatty acid composition, the commercial application, and the ultimate performance in various fried foods. The main parameters for the selection of frying fats include the turnover rate of the fat (removal and replenishment during frying), the amount of fat absorbed by the food, and the shelf life of the fried foods. The fats used for foods consumed immediately or shortly after frying (e.g. restaurants) have different formulations ("light-duty") from fats used for foods consumed after storage (e.g. potato chips). Generally, the frying performance and stability of oils are greatly dependent on the amounts of total oxidizable polyunsaturated fatty acids. For repeated frying, to minimize the formation of undesirable rancid and fishy flavors as well as cyclic monomers, the level of linolenate must be below 2 to 3%. For this reason, soybean and canola oils are generally partially hydrogenated to reduce their linolenate content. If hydrogenation is used extensively to reduce the linolenate

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Table 12.5. Properties of commercial fats used for frying [a]

Characteristics	RBD soybean oil	All purpose shortening	Opaque shortening	Heavy duty shortening
Appearance	Clear, liquid	Plastic, solid	Plastic, pourable	Plastic, solid
M.p.(°C)	Liquid	43–48	33–37	40–43
AOM, hr	10–25	40+	35+	200+
Polyunsaturates	34–61	15–20	35–40	4–8
Saturates	13–27	20–30	15–20	22–25
Iodine value	130–135	80–85	102–108	65–75

[a] From Erickson (1996). RBD = refined, bleached and deodorized; AOM = active oxygen method.

content below 1%, the oils acquire undesirable "hydrogenation" off-flavors (see Chapter 8.C), and high *trans* unsaturation. Although the nutritional hazards of *trans* isomers in edible fats are controversial (see Chapter 13.F.3), current efforts in industry are aimed at either minimizing the amounts of these isomers or eliminating them in our food supply. The use of partially hydrogenated vegetable oils has in fact been practically eliminated in many European countries. The same trend is expected to occur in the future in the US because the US Food and Drug Administration will require industry to label the *trans* content of foods by 2006.

Frying fats can be formulated by blending partially hydrogenated vegetable oils (soybean, corn, cottonseed and canola) of varying iodine values (64 to 107) with almost completely hydrogenated fats (of iodine value of 1–8) referred to as *hardstock*. A fluid light-duty shortening may contain 98% partially hydrogenated vegetable oils (of iodine value 104–106), and 2% hardstock. A heavy-duty frying fat may be formulated to contain 97% partially hydrogenated vegetable oils (of iodine value 80–82), and 3% hardstock. Standard specifications for various commercial frying fats are based on melting points, AOM stability, polyunsaturated and saturated fat contents, and iodine values (Table 12.5). The fluid light-duty shortenings contain appreciably more polyunsaturated fatty acids (35–40%) than the heavy-duty shortenings (4–8%).

Plant breeders have developed new varieties of soybeans and canola seeds that produce oils with reduced linolenate contents. These new oils containing less than 3% linolenate have improved flavor and oxidative stability, and can be used successfully for frying without hydrogenation (Table 12.6). These so-called "zero trans" oils have been developed recently for frying and other uses in baking foods to avoid hydrogenation. High-oleic and mid-oleic vegetable oils have recently been developed that are particularly suitable for frying. These oils can be used either directly or after blending with the more readily available and cheaper polyunsaturated soybean and canola oils to reduce the linolenate content below 2 or 3% and obviate hydrogenation (Chapter 8.C, D, E). On the one hand, potato chips fried in mid-oleic vegetable oils have a more

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Table 12.6. Fatty acid composition of new varieties of soybean, sunflower and canola oils suitable for frying without partial hydrogenation

	Soybean [a]			Sunflower [b]		Canola [c]
Fatty acids %	**Low 18:3**	**Ultra low 18:3**	**High 18:1/ Low 18:3**	**Mid 18:1**	**High 18:1**	**High 18:1/ Low 18:3**
14:0	0.0	0.0	0.0	0.4–0.8	ND–0.1	0
16:0	10.8, 11.3	11.5	7.3	4.0–5.5	2.6–5.0	4
18:0	4.5, 4.7	4.6	3.4	2.1–5.0	2.9–6.2	2
18:1	22.4, 26.1	24.8	85.1	43.1–71.8	75–90.7	64–78
18:2	55.4, 59.7	58.2	1.3	18.7–45.3	2.1–17.0	9–24
18:3	2.0	0.8	2.0	ND–0.1	ND–0.3	3–4
Others	0.8	–	0.8	22:0, 24:0	22:0, 24:0	2

[a] From Warner and Gupta (2003, 2004).
[b] From www.sunflowernsa.com/research_statstics/research_workshop/documents/107.PDF (2003).
[c] From Warner *et al.* (1994).

desirable "fried food" flavor than those fried in high-oleic vegetable oils. Apparently, frying oils containing linoleic acid ranging from 25 to 30% are required to produce an optimum level of 2,4-decadienal that contributes to a desirable "fried food" flavor. On the other hand, the potato chips fried in high-oleic oils have a longer shelf life than those fried in mid-oleic oils.

C. Methods to assess frying deterioration

Several methods are used to determine compounds from thermal oxidation that cause significant changes in the physical, chemical and nutritional properties of frying fats. Gross changes resulting from frying include increase in viscosity and density, dark color development, tendency to foam and decrease in unsaturation. Fats used for frying show an increase in free fatty acids, a decrease in iodine value, small increases in peroxide value and conjugated diene absorption at 231 nm. The formation of polymers is one of the most important changes in fats during frying. Polymeric materials constitute more than 50% of the compounds formed by thermal deterioration due to frying.

A number of routine methods have been used to evaluate the extent of oxidative and thermal damage to frying fats. These methods can be divided into those that can be used later on oil samples collected some time after frying, those employed immediately after frying for quality control, and those applied to fried foods.

1. Post-frying methods

The degrees of alteration caused by frying foods are commonly estimated by determining total polar materials by simple column chromatography, polymers by gel-permeation chromatography, or size-exclusion chromatography, and

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petroleum–ether insoluble oxidized fatty acids. The amounts of free fatty acids produced during frying are generally too small to use as a basis to monitor the quality of food. More sensitive and quantitative techniques include column adsorption and partition chromatography and size exclusion chromatography to determine directly total polar material and non-polar monomeric and dimeric compounds. Gas-liquid chromatography is used to estimate indirectly the polymeric polar materials that do not elute ("non-eluted" materials) under standard conditions. These chromatographic techniques provide only semi-quantitative information owing to the complexity of the mixture of oxidized polar and non-polar fatty acid components formed under frying conditions.

The routine and well-recognized determination of total polar materials in heated fats is carried out by eluting the sample through a silica column (adsorption chromatography), and collecting first the non-polar or unchanged triacylglycerols with a mixture of 87% petroleum ether: 13% diethyl ether (by volume), followed by the polar materials with pure diethyl ether. The weight of the polar material fraction, isolated after evaporating the solvents, is a coarse measure of thermal deterioration. In practice, the first and larger fraction of unchanged triacylglycerols is isolated and weighed and the polar material fraction is calculated by difference. Several official methods approved in Europe and the US use column chromatography with silica inactivated with water. Because these methods are time consuming and require too much solvent, various rapid micromethods have been developed using disposable silica cartridges (Sep-Pak and SPE) to shorten the analysis time and reduce the cost.

On the basis of analyses of a large number of used frying fats, heated fats are acceptable until they reach a level of 25% polar materials. The formation of 25–30% polar materials in frying fats is recognized as an objectionable level of deterioration and should be rejected. The determination of total polar materials has been adopted as a standard reference method in several European countries to test for excessive use of fats for frying. However, the complex polar materials in frying fats have not been well characterized. Reverse-phase HPLC chromatograms have revealed the formation of "medium polarity" materials in various vegetable oils during frying. The nature of these materials and total polar materials has not been characterized. These determinations also do not measure non-polar materials that are formed in frying fats.

Different frying operations produced varying amounts of polar material (Figure 12.3). Pan frying with a shallow layer of fat forms the smallest amount of polar material (about 10%), because it proceeds quickly and usually without repeated use of the fat. Deep-fat frying in a household or restaurant produces an objectionable level of 30% polar materials after 40 hours, because the fat is used for intermittent frying and with slow turnover. Commercial deep-fat frying is carried out with a fast turnover by continuously replacing the fat used by food frying with fresh fat. By this operation, the fat approaches a steady and

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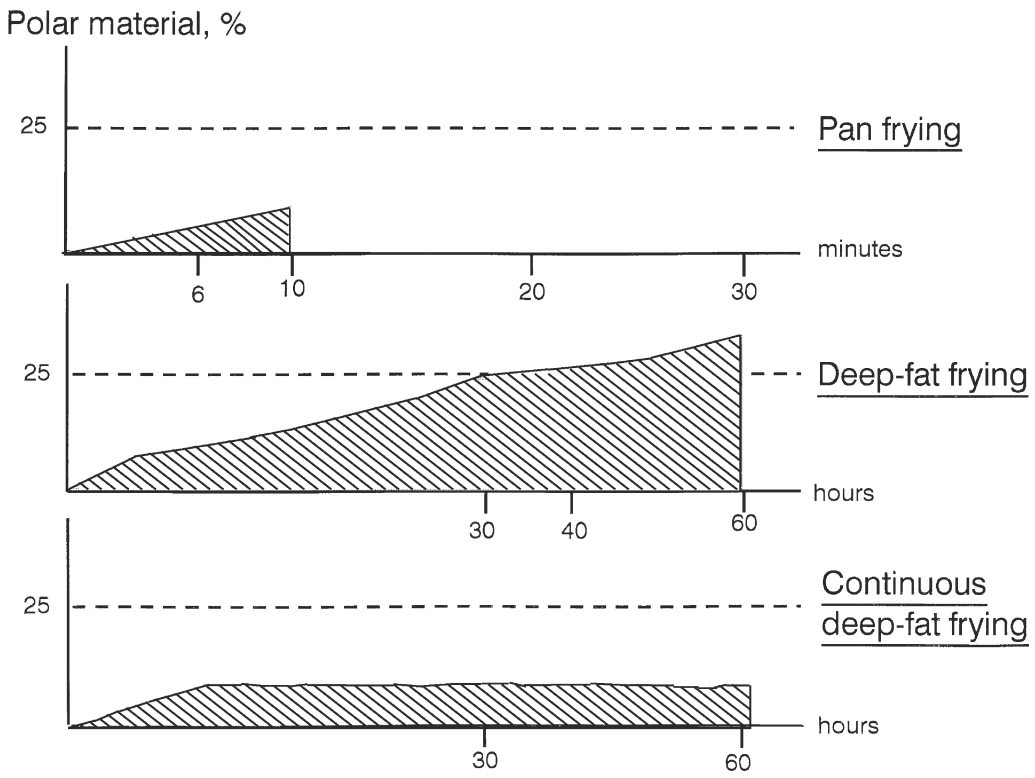


Figure 12.3. Effect of different treatments on development of polar materials during frying. Adapted from Billek (1983).

constant level of polar material that reached about 10% after a few hours of continuous frying.

2. Rapid control methods

These methods are employed during or immediately after frying, for either quality control or for an inspection purpose. Change in the dielectric constant has been used to monitor frying by a rapid instrument called the "food oil sensor". The dielectric constant increased with frying time much more in soybean oil than in hydrogenated vegetable oil, and a hydrogenated animal–vegetable oil blend (Table 12.7). The changes in dielectric constant correlated with other commonly used tests to assess frying fats, such as free fatty acids, peroxide values, conjugated dienes, polar material, foam heights and decrease in iodine value. The highest correlation with an increase in polar material indicated that oxidation products largely caused the changes in dielectric constant. Peroxide values and conjugated dienes are not reliable tests because the hydroperoxide precursors are largely decomposed during frying, and these measurements reflect mainly further oxidation taking place after sampling and cooling for analyses. Determinations of free fatty acids, foam heights and iodine value are the least sensitive and reliable measures of frying deterioration.

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Table 12.7. Analytical tests of various oils during frying of potatoes [a]

Frying at 190°C (hr)	Instrument reading [b]	FFA (%)	Peroxide Value	Conj. dienes	Polar material (%)	Foam height (mm)	Iodine value
Soybean oil [c]							
0	1.0	0.02	1.3	0.40	2.8	40	122.8
16	4.1	0.18	21.9	0.67	14.1	50	121.0
24	5.3	0.27	21.3	0.67	17.1	80	118.3
32	7.0	0.30	22.9	0.69	21.2	80	–
Change	6.0	0.28	21.6	0.29	18.4	40	–
Hydrogenated vegetable oil [d]							
0	1.0	0.02	0.9	0.11	3.7	30	70.2
16	1.6	0.17	2.4	0.18	5.0	35	69.3
24	1.8	0.28	3.4	0.19	5.5	40	69.7
32	2.0	0.38	3.9	0.22	7.6	25	–
Change	1.0	0.36	3.0	0.11	3.9	–5	–
Hydrogenated animal–vegetable oil blend [e]							
0	1.0	0.05	0.9	0.39	3.0	45	49.3
16	2.8	0.32	11.5	0.49	11.0	30	46.4
24	3.7	0.50	12.5	0.48	13.4	40	46.3
32	4.8	0.78	14.5	0.46	16.5	30	–
Change	3.8	0.73	13.6	0.07	13.5	–15	–

[a] From Fritsch *et al.* (1979). FFA = free fatty acids as C18:1; polar material was determined on a nylon chromatography column packed with neutral alumina, eluting neutral oil with toluene and hexane, cutting top section with razor and eluting polar material with chloroform/methanol mixture; foam height was read on the surface of a graduated cylinder cut off at the bottom and inserted into the hot oil until it almost touched the bottom of the fryer.
[b] Food oil sensor for detecting changes in dielectric constants.
[c] Refined, bleached, deodorized soybean oil: iodine value: 123, initial peroxide value: 1.3, AOM: 10 hr, saturates: 17.1%, 18:1: 23.1%, 18:2: 52.2%, 18:3: 6.9%.
[d] Commercial frying shortening containing dimethyl silicone: iodine value: 70.2, initial peroxide value: 0.9, AOM: over 200 hr, saturates: 26.2%, 18:1: 70.6%, 18:2: 2.5%, 18:3: 0.6%.
[e] Commercial animal–vegetable shortening containing 10 ppm BHA and 30 ppm BHT: iodine value: 49.3, initial peroxide value: 0.9, AOM: 65 hr, saturates: 50.7%, 18:1: 17.6%, 18:2: 4.0%.

The procedure for dielectric constant has several limitations. The initial dielectric constant varies among different fats, and changes with different food fats extracted into the frying fat. The fresh oil must be available for comparison with the heated sample. Caution is necessary in interpreting dielectric constant changes in heated fats because these measurements represent the net balance between polar and non-polar compounds, both of which are produced during frying. Although the polar compounds predominate, the relationship between both types of products and oil quality is confounded by several factors including moisture, mono- and diacylglycerols, and free fatty acids. Although good correlations have been reported between dielectric constant values obtained

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by the "food oil sensor" and total polar materials on large samples of frying oils, the dielectric constant values are influenced by too many factors including the type of fat, moisture, salt and minerals contributed by frying foods. Several other commercial colorimetric kits have been developed to rapidly test the heat abuse of frying fats. These tests are based on redox indicators, carbonyl compounds, free fatty acids, and alkaline materials formed by interaction of fried foods and deteriorated oils. These methods are, however, unspecific and confounded by the multitude of factors listed above and therefore not recommended.

3. Methods applied to fried foods

To establish more reliable quality criteria for consumer acceptability, the fried foods must be tested rather than the frying fats. More sensitive and reliable measures of frying performance of fats are based on sensory evaluations and gas chromatographic analyses of volatiles in fried foods carried out initially and after storage. Bread cubes proved to be a suitable model food because they can be prepared in uniform size and moisture content. After frying under standard conditions, bread cubes could be stored and evaluated by sensory techniques and by static headspace gas chromatography for volatiles. Although the flavor quality of bread cubes after initial frying did not differ between hydrogenated oils and unhydrogenated oils containing citric acid, the flavor quality of bread cubes used repeatedly for frying decreased significantly after accelerated storage at 60°C (Table 12.8). Initially, the bread cubes fried in hydrogenated oils had better flavor quality than those fried in unhydrogenated soybean oil. However, after storage, the flavor quality scores decreased significantly with bread cubes fried in all oils containing citric acid and *tert*-butyl hydroquinone (TBHQ). Those oils containing citric acid and methyl silicone or methyl silicone and TBHQ improved the stability of the fried bread cubes after storage. These results showed that if bread cubes are fried in repeatedly used oils, they could also be consumed immediately after frying. These bread cubes cannot be stored without marked deterioration of quality. The use of bread cubes as a food model to test frying fats was later confirmed with fried potato chips.

Gas chromatographic analyses of volatiles showed that bread cubes fried in all hydrogenated oils produced less volatile than non-hydrogenated control oil. Although TBHQ reduced volatile formation in all oils, methyl silicone had a much greater effect. The mixture of TBHQ and methyl silicone further decreased the total volatiles in bread cubes after storage. Therefore, gas chromatographic analysis of volatiles showed more significant improvement by hydrogenation and antioxidants than sensory evaluations. Volatile analyses need to be accompanied therefore by sensory evaluations.

When potato chips were fried in cottonseed oil, high-oleic sunflower oil, and

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Table 12.8. Effect of hydrogenation, methyl silicone and antioxidants on flavor and volatile formation in fried bread cubes after storage [a,b]

Oil samples + citric acid[c]	Flavor quality		Volatiles (peak area)[b]
	0-time	Stored	Stored
Soybean oil	5.5	2.7	246.6
Soybean oil + MS	4.8	3.8	6.2
Soybean oil + TBHQ	5.4	2.0	146.8
Soybean oil + TBHQ + MS	4.8	4.3	3.5
Hydrogenated (4.6)	6.5	3.8	127.0
Hydrogenated (4.6) + MS	6.6	4.8	4.8
Hydrogenated (4.6) + TBHQ	6.8	2.0	76.1
Hydrogenated (4.6) + TBHQ + MS	6.2	5.1	1.3
Hydrogenated (2.7)	6.6	3.0	130.5
Hydrogenated (2.7) + MS	7.0	6.4	1.9
Hydrogenated (2.7) + TBHQ	6.6	1.9	56.3
Hydrogenated (2.7) + TBHQ + MS	6.5	5.0	0.4
Hydrogenated (0.4)	5.9	2.8	31.7
Hydrogenated (0.4) + MS	5.9	4.5	1.7
Hydrogenated (0.4) + TBHQ	6.4	2.0	23.2
Hydrogenated (0.4)+ TBHQ + MS	5.5	5.2	0.2

[a] From Frankel *et al.* (1985).
Treatment: 2.5 hr frying bread cubes (1 inch) + 14 hr heating at 190°C.
Storage at 60°C for 4 days.
Flavor quality scale: 1–10, 10 = excellent,1 = extremely poor.
[b] From Snyder *et al.* (1986). By static headspace gas chromatography, peak area × 10^{-3}
[c] citric acid, 100 ppm, MS = methyl silicone, 5 ppm,
TBHQ = *tert*-butyl hydroquinone, 200 ppm; hydrogenated oils (%18:3).

their mixtures, the total polar compounds in the oils after frying decreased with increasing content of oleate (Table 12.9). However, the potato chips had better flavor quality and "fried flavor" intensity scores when evaluated fresh after frying in cottonseed oil, than chips fried in either high-oleic sunflower oil or in mixtures of these oils. After storage for 6 months at 25°C, potato chips fried in the oil blend containing 36% linoleate had the best flavor quality and fried flavor, but the potato chips fried in cottonseed oil had a higher intensity score for rancid flavor than in high-oleic sunflower oil and all the mixture with cottonseed oil. As expected, gas chromatographic analyses showed decreases in volatile formation with decreasing content of linoleate. Analyses of volatile compounds agreed with the total polar compounds in showing greater frying oil stability and oxidative stability of the fried chips. The higher flavor quality and fried flavor scores in the cottonseed oil and blends containing higher levels of linoleate were attributed to increased levels of 2,4-decadienal, a decomposition product of oxidized linoleate, but this aldehyde at high levels also contributes to rancidity. The relative concentrations of 2,4-decadienal necessary to optimize

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Table 12.9. Analyses of frying cottonseed oil (CSO) and high-oleic sunflower oils (HOSUN) and their mixtures, and sensory evaluations of potato chips after frying and storage [a]

Analyses	CSO	2:1 CSO/HOSUN	1:2 CSO/HOSUN	HOSUN
Fatty acids, %				
saturates	26.4	19.2	11.7	8.0
18:1	16.4	42.9	67.5	78.0
18:2	54.9	35.7	18.7	12.1
Polar compounds, %				
0-time	5.6	4.5	3.1	2.9
12 hours	8.9	7.1	6.5	4.8
Flavor quality				
0-time	7.7	7.6	7.0	6.6
6 months	5.0	5.7	5.4	5.1
Fried flavor intensity				
0-time	5.9	5.6	5.0	4.8
6 months	3.4	4.5	3.9	3.2
Rancid flavor intensity				
6 months	1.3	0.9	0.9	0.9
Volatiles (6 months)				
Hexanal	38.7	21.8	15.6	2.3
Pentanal	17.5	10.9	9.4	1.3
Octanal	1.2	0.6	0.5	0.0
2,4-Decadienal	62.5	36.5	20.2	6.7

[a] From Warner *et al.* (1997). Oils used to fry potato chips for 12 hours at 187–192°C.
Fatty acid composition on fresh oils; polar compounds on oils after frying
Flavor quality scores: 1 = bad, 10 = excellent; fried and rancid flavor intensity scores: 0 = none, 10 = strong.
Potato chips were tasted fresh at 0 time and after storage for 6 month at 25°C.
Potato chips were analysed directly for volatiles by static headspace gas chromatography after storage for 6 month at 25°C.

a desirable fried food flavor, without also producing rancidity are not known and are difficult to predict and control.

The frying performance of new varieties of low-linolenic acid soybean oils was compared to that of cottonseed oil (Table 12.10). The decrease in linolenic acid to 2 and 0.8% in the soybean oils improved the flavor quality and the oxidative stability of the potato chips. Although the total polar content of the cottonseed oil was higher, no differences were observed between the low linolenic acid soybean oils. Potato chips fried in ultra-low linolenic acid and stored for 7 weeks had significantly higher flavor quality, lower fishy flavor and lower hexanal than the low-linolenic acid soybean oil. In another study, low-linolenic acid and high-oleic soybean oils and their mixtures were compared with cottonseed oil (Table 12.11). The high-oleic acid soybean oil and its

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Table 12.10. Analyses of frying cottonseed oil (CSO), low- (LLSBO) and ultra-low linolenic acid (ULLSBO) soybean oils, and sensory evaluations of potato chips after frying and storage [a]

Analyses	CSO	LLSBO	ULLSBO
Fatty acids, %			
saturates	28.9	16.0	16.1
18:1	17.7	22.4	24.8
18:2	52.3	59.7	58.2
18:3	0.1	2.0	0.8
Polar compounds, %			
0-time	5.5	2.4	1.5
15 hours	15	6.8	6.8
Flavor quality score			
0-time	7.5	7.9	7.5
7 weeks	7.0	6.5	8.0
Fried food flavor			
0-time	1.0	2.0	1.3
7 weeks	2.1	1.8	1.3
Fishy flavor			
7 weeks	0.3	1.2	0.3
Hexanal, ppm (7 weeks)			
5 hours	6	9	6
15 hours	7	10	10

[a] From Warner and Gupta (2003). Oils used to fry potato chips for 5 hours at 190°C.
Fatty acid composition was determined on fresh oils; polar compounds on oils after frying 15 hours.
Flavor quality scores: 1 = bad, 10 = excellent; fried and rancid flavor intensity scores: 0 = none, 10 = strong.
Potato chips were tasted after frying 5 hours and storage for 7 weeks at 25°C.
Potato chips were analysed directly for volatiles by purge and trap gas chromatography after frying 5 and 15 hours and storage for 7 weeks at 25°C.

mixture with low-linolenic acid soybean oil developed less total polar compounds after 5 hour frying. After storage the potato chips fried in high-oleic soybean oil and its mixture with low-linolenic acid soybean oil had improved flavor quality score, less fishy flavor and hexanal than the low-linolenic acid soybean oil. Both low-linolenic acid soybean oils and canola oils as well as mid-oleic sunflower oils are expected to meet future goals of avoiding hydrogenation and *trans* isomers in frying oils (see Table 12.6).

D. Control measures

The degradation of fats during frying varies depending on the properties of fats, the conditions and wide variations possible in the handling and processing of fats. The extent of fat deterioration reflects complex interactions among many

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Table 12.11. Analyses of frying cottonseed oil (CSO), low-linolenic acid soybean oil (LLSBO), high oleic acid soybean oil (HOSBO), their blends, and sensory evaluations of potato chips after frying and storage [a]

Analyses	CSO	LLSBO	HOSBO	1:1 LLSBO:HOSBO
Fatty acids, %				
saturates	28.9	15.3	10.7	13.2
18:1	17.7	26.1	85.1	55.5
18:2	52.2	55.4	1.3	28.9
18:3	0.1	2.0	2.0	2.0
Polar compounds, %				
0-time	6.1	1.2	1.7	1.4
5 hours	11.6	8.5	5.6	6.9
25 hours	18.1	18.5	12.5	14.4
Flavor quality score				
1 week	7.6	8.0	5.5	8.4
7 weeks	6.7	5.9	4.8	6.8
Fishy flavor				
1 week	0.3	0.2	1.7	0.1
7 weeks	0.3	0.3	0.4	0.5
Hexanal, ppm (1 week)				
5 hours	2.6	1.8	1.2	1.0
25 hours	1.5	2.3	0.9	1.6
Hexanal, ppm (7 week)				
5 hours	1.9	2.4	1.2	1.5
25 hours	4.1	10.8	1.5	3.1

[a] From Warner and Gupta (2004). Oils used to fry potato chips for 5 and 25 hours at 190°C.
Fatty acid composition was determined on fresh oils; polar compounds on oils after frying 5 and 25 hours.
Flavor quality scores: 1 = bad, 10 = excellent; fried and rancid flavor intensity scores: 0 = none, 10 = strong.
Potato chips were tasted after frying 5 hours and storage for 1 and 7 weeks at 25°C.
Potato chips were analysed directly for volatiles by purge and trap gas chromatography after frying 5 and 25 hours and storage for 1 and 7 weeks at 25°C.

factors: degree of water volatilization and resulting air agitation, extent of protection by steam blanketing, level of hydrolysis depending on the type of food and temperature, the surface to volume ratio of the fat, the uniformity of heating in the frying equipment. The nature and oxidative stability of fats used for frying were discussed in Section B, as one of the critical factors controlling the quality of fried foods. Other important factors are discussed below.

1. Temperature

The temperature of the oil is controlled to allow for moisture bubbles to evolve after immersing the food until it is removed. Depending on the foods, for

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satisfactory results, frying temperatures range from 160 to 190°C. Continuous evolution of steam from the food is essential to minimize the penetration of oil into the surface of the food. During frying, fat deterioration is delayed by the removal of volatile decomposition products by the stripping action of the steam. Fats decompose rapidly if they are held at frying temperatures without foods. This deterioration can be delayed by reducing the temperature of the fat when it is not used for frying.

2. Turnover rate

This refers to the amount of fat added to the kettle to make up for the amount removed by the food during frying. The turnover rate is an important factor for the control of frying oil condition. Daily replacement of the oil absorbed by food is estimated as the proportion of the total oil capacity of the fryer. The turnover rate is a function of the absorption of fat by the frying food. The absorption of fat by the food during frying increases with the increase in viscosity. For any given rate of frying, the rate of fat turnover will increase as the viscosity increases, and the fat is thus protected by this effect. The fat reaches a steady state condition, through the removal and replenishment processes, and does not deteriorate further with continuous frying. Addition of fresh fat to used fat in good condition maintains the ability of the fat to contribute desirable sensory attributes to fried foods. A rapid turnover rate is important to minimize thermal deterioration of fats and to maintain the quality of fried foods. If a low turnover is necessary when an insufficient amount of food is fried daily, then a certain amount of fat must be discarded to increase the amount of make-up fat. The used oil must be replaced as needed to maintain good quality of fried foods. With the recent market demands for zero-*trans* polyunsaturated oils for frying, a higher turnover of non-hydrogenated oils is also required to obtain the same shelf life of fried food products as previously achieved with partially hydrogenated oils. Many reports in the scientific and patent literature have attempted to restore the quality of used frying oils with different bleaching adsorbents to remove polar oxidation products, but they have generally not been successful in achieving the same quality as the fresh oils.

3. Filtration

Daily filtration of frying fat to remove accumulated food particles, charred batter and breading is important to reduce deterioration, excessive color formation and development of undesirable bitter flavors and odors. Systems used for filtration include metal screens, paper filters and plastic cloths. The use of diatomaceous earth or filter aid is effective in reducing free fatty acids and color compounds.

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4. Antioxidants

Synthetic antioxidants such as BHA, BHT, propyl gallate and TBHQ (Chapter 9) are sometimes included in commercial shortenings, even though they are volatilized and decomposed at the temperatures of frying. The depletion rates in soybean oil heated under frying conditions was about the same for TBHQ as for BHA and BHT. Depending on the turnover rate with fresh oil during frying, these antioxidants are partially retained and stabilize the fried foods. There is evidence that the shelf life of potato chips is extended by the use of shortenings containing synthetic antioxidants such as TBHQ and BHT. BHA has the disadvantage of producing an objectionable phenolic odor.

The effect of natural tocopherols and of added tocopherols to frying fats is controversial. Natural occurring tocopherols in vegetable oils are generally not effective as antioxidants at high temperatures, and may even have prooxidant activity at high concentrations (Chapter 9.A.1). Tocopherols are lost during frying in varying degrees depending on the fats without apparently affecting the deterioration rate of the oils.

During oxidation at 100°C under AOM conditions, tocopherols were depleted more slowly in polyunsaturated than more saturated fats and after partial hydrogenation (Table 12.12). This trend was explained by the more rapid decomposition of polyunsaturated hydroperoxides before they react with the tocopherols. During simulated frying conditions by heating at 180°C with continuous water spraying, natural tocopherols were more slowly decomposed in polyunsaturated soybean, cottonseed and safflower oils than in the more saturated palm and coconut oils (Table 12.12). These results were confirmed in a recent study showing that α-tocopherol decomposed thermally more readily in saturated than in polyunsaturated purified vegetable oils (Table 12.12, footnote c). A linear relation was calculated between residual α-tocopherol (toc) and iodine value (IV) of different vegetable oils: toc = 3.72 IV + 137.5. The results were explained by the competition between α-tocopherol and unsaturated lipids in reactions with alkoxyl and hydroxyl radicals generated by decomposition of hydroperoxides.

Natural tocopherols, which are present at relatively high concentrations in soybean oil (Table 9.4), can produce undesirable red-colored tocoquinones during prolonged frying. Thus, the observation that the oxidative stability of heated olive oil and high-oleic safflower oil is significantly increased by tocopherol addition may not necessarily apply under conditions of frying foods. The frying life of soybean oil can actually be improved on the basis of reduced dark color formation after deep-frying of various foods, by removing a significant portion of the tocopherols, by vacuum steam distillation or deodorization at elevated temperatures for short times. The fat produced by this process is suitable for foods that are consumed shortly after frying such as in restaurants. However, the decreased level of tocopherols would be expected to

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Table 12.12. Loss of tocopherols in heated fats and fats treated under simulated frying [a,b,c]

Oils	Oxidation at 100°C (AOM)[a]	Simulated frying[b]	Heating at 240°C [c]
	Initial rate (ppm/hr)	Loss (%)	Loss (%)
Soybean oil	4.9	19.8	37.5
Soybean oil + CA[d]	3.6	–	–
Hydrogenated soybean oil + CA[d]	12.1	–	–
Safflower oil	3.9	8.8	–
Corn oil	21	46.5	39.8
Cottonseed oil	67	25.3	–
Lard + α-tocopherol[e]	180	–	–
Palm oil	–	67.4	66.5
Coconut oil	–	100	82.9

[a] From Frankel *et al.* (1959).
[b] From Yuki and Ishikawa (1976).
[c] From Verleyen *et al.* (2002). Vegetable oils were stripped of natural tocopherol by adsorption chromatography on alumina and 1000 ppm α-tocopherol added before heating. Loss of α-tocopherol (Toc) was calculated from the regression plot of residual tocopherol vs iodine value (IV) (Toc = 3.72IV + 137.5).
[d] From Evans *et al.* (1959); CA = citric acid, 0.01%; hydrogenated to iodine value of 101.7.
[e] 1500 ppm

reduce the shelf life of fried foods that are consumed after storage, such as packaged potato chips. Therefore, certain levels of tocopherols have a stabilizing effect on fried foods after storage, but would not be expected to increase the oxidative stability of fats during frying. For this reason, it is important to test the effectiveness of antioxidants on the oxidative stability of the fried foods and not that of the frying fats. The level of residual tocopherols in the fat absorbed by the fried foods would be expected to increase by more rapid rates of fat turnover during frying.

Rosemary extracts provide an important source of natural antioxidants used commercially in foods. These extracts are particularly active as antioxidants at the high temperatures in frying fats. They protect the oils (peanut and palm oils) during frying and their antioxidant activity is carried over into the fried foods (oriental noodles). Rosemary and sage extracts were equally effective in retarding deterioration of palm olein during frying of potato crisps on the basis of anisidine value, free fatty acids, polymer content, ratio of C18:2/C16:0 and sensory evaluations (Table 12.13). The active components of rosemary, carnosic acid and carnosol, are readily decomposed during thermal oxidation into products that remain active as antioxidants in heated fats. Electron spin resonance studies of carnosic acid and carnosol in oxidized methyl oleate heated at 130–160°C suggested the formation of *o*-semiquinone radicals (Figure 12.4), but the stable oxidation products of rosemary extracts that

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Table 12.13. Effect of rosemary and sage extracts on frying quality of palm olein [a]

Analyses	Day	Control	Rosemary	Sage
Anisidine value	0	0.96	0.95	0.96
	6	62.0	50.2	50.8
Free fatty acids (%)	0	0.05	0.05	0.05
	6	0.59	0.42	0.44
Polymer content (%)	0	0.01	0.01	0.01
	6	2.65	1.82	1.90
C18:2/C16:0 ratio	0	0.29	0.29	0.29
	6	0.17	0.20	0.21
Taste	1	6.2	6.2	6.2
	6	4.2	5.1	5.1
Odor	1	6.1	6.2	6.2
	6	4.9	5.1	5.2

[a] From Man and Jaswir (2000). Frying of potato slices carried out at 180°C. Sensory evaluations used a hedonic scale of 1 = dislike extremely, to 7 = like extremely.

remain active as antioxidants in frying fats have not been identified. More recently, an *ortho*-quinone and a hydroxy-*para*-quinone were identified from the azo-initiated and iron-catalysed oxidation of carnosic acid in the presence of methyl linoleate (Figure 9.13). Although these quinone products had weak antioxidant activity, their solution in acetonitrile or methanol showed strong antioxidant activity after standing for 4 hours at 37°C. This activity was shown to be due to the isomerization product carnosic 8-lactone, the redox product rosmanol and related diterpene quinones (Figure 9.13). Although similar and related products may account for the carry-over antioxidant activity of rosemary extracts when added to oils under frying conditions, they have not been identified in fried foods. Phenolic compounds are readily oxidized under different conditions into a wide range of quinones, dimers and aldehydes. The

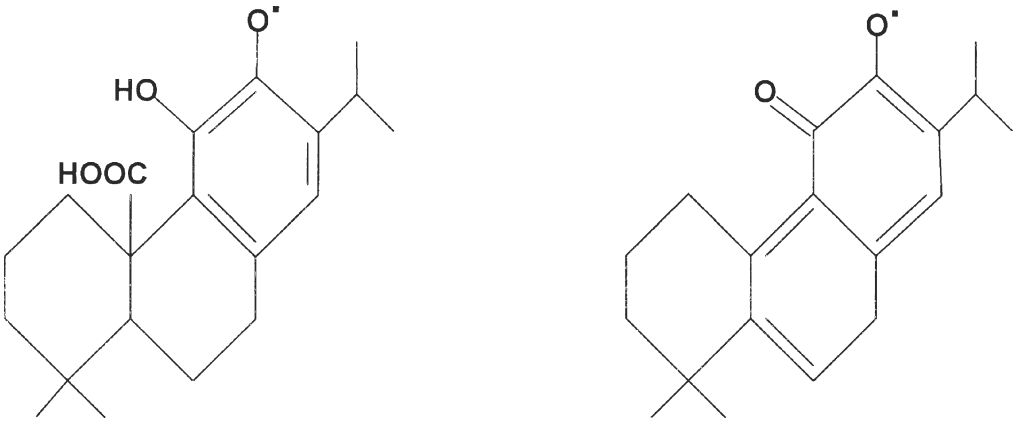


Figure 12.4. Structures of quinone radicals suggested by electron spin resonance studies of carnosic acid heated with oxidized methyl oleate. From Löliger *et al.* (1996). See also Figure 9.13.

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relative contribution of these complex oxidation derivatives produced under frying conditions to antioxidant activity is not yet understood.

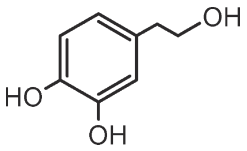
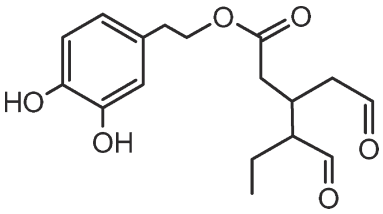
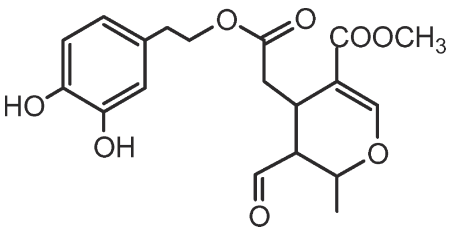
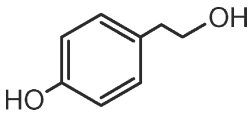
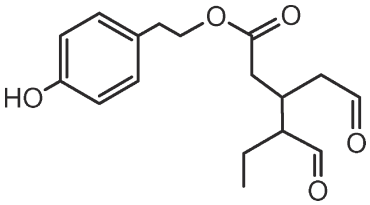
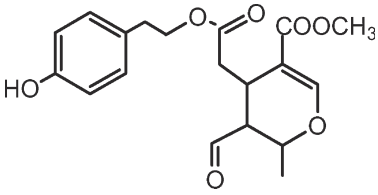
The natural phenolic antioxidant compounds of virgin olive oil undergo significant decomposition during multiple frying of potato slices (Table 12.14). On one hand, the concentration of hydroxytyrosol (3,4-DHPEA) and its derivatives 3,4-DHPEA-EDA and 3,4-DHPEA-EA diminished most rapidly with the number of frying operations, with less than 10% of the original content remaining after six fryings. On the other hand, tyrosol (*p*-HPEA) and its derivatives *p*-HPEA-EDA and *p*-HPEA-EA, were much more thermally stable. The much higher antioxidant activity of hydroxytyrosol and its derivatives compared to that of tyrosol and its derivatives in virgin olive oil may thus be related to their reactivity during frying. The effect of frying was also tested on the antioxidant activity of phenolic extracts from virgin olive oil. When the phenolic extract from fresh olive oil was tested in bulk oil oxidized at 60°C, the phenolic extract from the olive oil used for 12 frying operations not only lost all its antioxidant activity, but also showed a small prooxidant activity. This prooxidant effect can be attributed to oxidized triacylglycerols in the phenolic extract that increased 11 to 20 times after 12 frying operations. Quality evaluations of the fried potatoes are needed, however, to determine how much of the olive phenolic compounds are absorbed and to better predict their storage stability.

5. Polymerization inhibitors

Silicones, such as methyl silicone or poly(dimethylsiloxane), are very effective additives for retarding thermal oxidation and deterioration by polymerization in frying fats. Silicones are only effective at frying temperatures and at very low concentrations (0.5–5 ppm). Methyl silicone improved significantly the flavor quality and reduced the formation of volatile compounds in the bread cubes fried repeatedly in soybean oil and hydrogenated soybean oils (Table 12.8). The use of mixtures of antioxidants with silicones shows synergistic effects in improving the shelf life of fried foods. The frying performance of partially hydrogenated soybean oil was improved by adding TBHQ with methyl silicone (Table 12.8). This result suggests a synergistic effect. Other synergistic mixtures were used to improve the quality of oils used for frying, including mixtures of dimethyl silicone, BHA and ascorbyl palmitate, methyl silicone, citric acid and TBHQ, phenolic antioxidants, silicone and esters of EDTA. The effectiveness of these mixtures may be explained by the stabilizing effect of silicone in retarding the depletion of phenolic antioxidants during frying. The addition of 2 ppm dimethyl silicone to high-oleic sunflower oil (72% 18:1, 17% 18:2) had a significant effect in lowering the polar compounds and the loss of α-tocopherol of the oil and potatoes after intermittent fryings (Table 12.15).

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Table 12.14. Changes of phenolic components of virgin olive oil during frying [a]

Structures	Compounds	Concentrations after: (mg/kg)		
		0 Frying	6 Fryings	12 Fryings
	Hydroxytyrosol, 3,4-dihydroxyphenylethanol (3,4-DHPEA)	4.9	0.5	nd
	3,4-Dihydroxyphenylethanol-elenolic acid dialdehyde (3,4-DHPEA-EDA)	52.5	3.8	0.8
	3,4-Dihydroxyphenylethanol-elenolic acid(3,4-DHPEA-EA)	95.5	8.8	5.7
	Tyrosol, *p*-hydroxyphenylethanol (*p*-HPEA)	6.6	5.7	5.3
	*p*-HPEA-EDA	96.7	103	77.5
	*p*-HPEA-EA	53.1	29.9	12.9

[a] From Gómez-Alonso *et al.* (2003). Frying of potato slices carried out at 180°C for about 10 min, followed by cooling to 50°C. Twelve batches of potatoes were fried with the same oil during 6 days with two fryings per day.

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Table 12.15. Effect of dimethylsiloxane (DMPS) on total polar compounds and α-tocopherol content in frying oils and food lipids [a]

Frying oils	Polar compounds (%)		α-Tocopherol (mg/kg)	
	Frying oil	Food lipids	Frying oil	Food lipids
High-oleic sunflower oil	16.0	15.4	10	trace
+ DMPS (2 ppm)	5.5	4.9	535	569
Sunflower oil	19.0	18.7	185	155
+ DMPS (2 ppm)	7.0	6.4	543	584

[a] From Jorge *et al.* (1996). Discontinuous frying of potatoes starting at 175°C for 6 hours after 10 fryings.

After storage the potato chips prepared with high-oleic sunflower oil were as stable as those prepared with palm olein (43% 18:1, 11% 18:2).

Silicones increase the smoke point of frying oils apparently by providing a surface to air barrier. The mechanism for protection provided by silicones is not well understood. As antifoaming agents, silicones form a monomolecular protective film at the air–oil interface that acts as an oxygen barrier. The addition of silicones to oil inhibits foam formation by increasing the interfacial surface tension by forming a film at the surface of the deep-frying oil. There is also evidence that silicones slow down the degree of convection in fats during frying. Silicones cannot be used indiscriminately. They have disadvantages by causing failure in cake baking, poor performance in doughnut frying by defoaming the batter, and loss of desirable crispness in fried potato chips. The level of silicone is slowly depleted during frying by transfer to the surface of foods. The use of silicones is not recommended for potato chips because of their adverse effect on texture.

Plant sterols isolated from the unsaponifiable fraction of certain vegetable oils, and oat oil have been shown to retard oxidative polymerization of vegetable oils heated at frying temperature (180°C), on the basis of decrease in iodine value and polymer formation. The active sterols contain an ethylidene side chain (vernosterol, Δ^5-and Δ^7-avenasterol and fucosterol). However, no data are available on the effect of these sterols during actual frying of foods.

6. Fat uptake in fried foods

Recent research on the mechanism of fat uptake in fried foods has been stimulated by the growing demands for reduced fat foods. Among the physico-chemical changes occurring during frying, starch retrogradation (transition from soluble to insoluble microcrystalline state), Maillard reactions and glass transition are recognized to have desirable effects on the sensory properties of fried foods. The fatty acid composition of the fat taken up by the fried food is not significantly different from the fatty acid composition of the frying

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medium. A small increase in fat uptake occurs with oxidative deterioration of the frying fat. This fat uptake may be related to the decrease in oil–air surface tension and increase in viscosity caused by polar and polymeric products. The amount of fat uptake in fried foods can therefore be reduced by increasing the turnover rate with fresh oil and by delaying oxidative degradation with antioxidants and silicones.

E. Criteria for used frying fats

One of the most difficult quality control problems in frying operations is to decide when a fat is so thermally abused that it should be discarded and replaced. Many reasons are used for discarding used frying fats: culinary criteria based on appearance, color, odor, and quality of fried foods, excessive foam and smoke, and routine schedules for fat replacement. When fats are heated under practical frying conditions, the extent of deterioration is generally small and limited by changes that make the fat unsuitable for culinary reasons. Samples of used fats from commercial frying operations showed levels of free fatty acids of 1–3%, polar triglycerides ranging from 5 to 30%, and were judged to have rancid or "heated" or "burnt-type" flavors.

Different countries have established different guidelines for frying deterioration or rejection points (also referred to as maximum allowable or "cut-off" level), above which thermally abused fats should be discarded. One German standard is based on 1% oxidized fatty acids, measured as the petroleum ether-insoluble fatty acid fraction. This endpoint is equivalent to 15% polymeric triglycerides and 27–28% total polar compounds. The levels of polar and polymer compounds are now generally used as indications for rejection of used frying fats. Frying fats are recognized as objectionable and to be rejected if the level of polar materials exceeds 25–30% (most of Europe), or if the polymer level exceeds 16% (Netherlands). This range of polar material corresponds to 13–15% polymerized triglycerides and 0.7–1.0% oxidized petroleum–ether insoluble fatty acids in frying fats and oils (Table 12.16). Other rejection points include polymeric and dimeric triglyceride contents (not exceeding 10%) or dimeric triglycerides (not exceeding 6%), acid values (2.0–2.5) and smoke point. (170–180°C). Only a few countries have established formal regulations to control the quality of frying fats. However, the methods for approving or rejecting frying oils do not work in practice because they are almost impossible to enforce or exercise in practice.

F. Health effects of frying fats

The biological and nutritional effects of frying fats have been extensively investigated over the last five decades. Early studies with animals fed heat-abused fats, containing sometimes more than 50% polar materials, showed

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Table 12.16. Analytical criteria for abused frying fats [a]

Total polar materials	Polymerized triglycerides	Oxidized fatty acids	Number of samples
23–25	13–15	0.7–1.0	79
25	13–15	0.7–1.0	157
24.1	12.7	0.7	125

[a] From Gertz (2000). Various types of foods.

significant growth retardation and other severe consequences (irritation of gastrointestinal tract, diarrhea, high mortality) (Table 12.17). When fats that are used for normal frying of foods (containing 10–20% polar material) were fed to animals, no effects were observed even at high levels. Many studies were conducted with individual fractions separated from fats used for normal frying. The distilled non-urea adduct fraction, containing a high amount of cyclic monomers, was shown to be somewhat toxic in animal tests when used at concentrations much higher than the animal could consume in the whole fat. However, although heated linseed esters containing 1% cyclic fatty acid monomers fed to rats resulted in high mortality of the offspring, when the same quantity of purified cyclic fatty acid monomers were fed to the animals, the mortality of offspring was not different from the control animals (Table 12.17). Therefore, purified cyclic fatty acids did not display any ill effects in this study. The harmful effects of cyclic monomers to animals reported in early studies may have been due to contaminating hydroperoxides or other oxygenated compounds in the impure preparations of cyclic monomers.

The polar material separated chromatographically from fats used for normal frying caused small but significant reduction in growth and slight liver damage when the diets of animals contained 20% polar materials. Some of the adverse effects of the polar materials were later attributed to the presence of 0.2% polar cyclic monomers containing additional oxygen functions. Total polar materials are regarded as a good measure of deterioration of heated fats (see Section C.1). The dimeric fatty acids from heated vegetable oils were not toxic. The polymers present as major components in frying fats were regarded to be innocuous because they are not absorbed.

Most of the adverse effects observed in animal feeding experiments appear to be due to the use of fats abused by heating at high temperature without frying foods. Fats obtained under conditions of actual frying foods are subject to less heat damage, because of the moderating effect of steam generation from the food, and are considered not toxic. There is convincing evidence that frying fats prepared under these normal conditions are safe when consumed at reasonable levels, unless they are severely abused. However, some compounds produced from polyunsaturated fats (e.g. cyclic monomers) under frying conditions can

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Table 12.17. Selected animal studies testing the toxicity of heated fats

Heat treatment	Fractions	Observations	References
Heat abused, without frying foods	> 50% Polar material	Gastrointestinal irritations, diarrhea, growth retardation	Crampton *et al.* (1951) Chalmers *et al.* (1954)
Frying fats	10–20% Polar material	No effect	Billek *et al.* (1983)
Frying fats	DNUA[a] (cyclic monomers)	Growth retardation, diarrhea, toxic effects	Artman *et al.* (1972) Michael (1966) Nolen *et al.* (1967)
Methyl linolenate (195°C), N_2	Cyclic fatty acid monomers	Enlarged liver	Iwaoka and Perkin (1978)
Heated vegetable oils	Dimeric fatty acids	Not toxic	Perkin and Taubold (1978)
Frying fats	90% Polar fraction	Growth retardation, slight liver damage	Billek *et al.* (1983)
Frying fish	100% Polar fraction	Lower gain weight	Grandgirard and Juilard (1984)
Cyclic fatty acid monomers (CFAMs)	Purified CFAMS *vs* heated linseed esters	Purified CFAMS fraction was not different than control	Sebedio *et al.* (1996)

[a] DNUA = distilled non-urea adducts.

impair the nutritional value of frying fats, but the level and degree of toxicity are generally considered too low to be of any significance. Analyses of cyclic fatty acids in commercially frying oils from different countries ranged from 0.01 to 0.7% only (Table 12.18). The control of frying fats to prevent abusive conditions is, however, important to assure their safety. The level of 24–25% total polar materials is now recognized in Europe as an important rejection point in assessing frying oil quality (see Section E). Significant amounts of non-volatile aldehydes attached to the triacylglycerol molecule, also known as aldehydo-glycerides or core aldehydes (see Chapter 4), are expected in frying fats, and suggested by their high anisidine values. Although no information is published on their health effects, related core aldehydes produced by oxidation of phospholipids have been recently reported to be biologically active and may be atherogenic (see Chapter 13).

The presence of sterol oxidation products in foods fried in animal, and various mixed animal/vegetable fats and vegetable oils have received attention recently because of their possible effects on human health. Sterol oxidation products were reported to be mutagenic, cytotoxic and carcinogenic, to inhibit

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Table 12.18. Cyclic fatty acids in commercial frying oils from different countries

Countries	Amounts (%)	References
US	0.02–0.50	Frankel *et al.* (1984)
Egypt, Israel	0.17–0.66	Frankel *et al.* (1984)
Hungary	0.02–0.16	Gere *et al.* (1985)
France	0.01–0.25	Sébédio *et al.* (1987)

cholesterol biosynthesis and to cause arterial damage. Analyses of commercial French fries showed levels of sterol epoxides and diols ranging from 20 to 200 μg/g of lipids. However, no information is available on the level of toxicity of these compounds.

Hydrogenation of polyunsaturated vegetable oils has become less appealing because of recent evidence that *trans* isomers may have adverse nutritional effects. The linolenate content of vegetable oils such as soybean and canola oils can be easily reduced to less than 3% without hydrogenation, by blending with high-oleic sunflower or safflower oils (see Chapter 8.D). These blends of all *cis* vegetable oils are more thermally stable and have improved frying performance. Increasing the level of thermally stable monounsaturated fats used for frying is also recommended in view of accumulating evidence that these fats provide other nutritional benefits, a subject that will be discussed in the next chapter. A wide range of new varieties of high- and mid-oleic vegetable oils are thus becoming commercially available due to the potentially adverse health effects of *trans* isomers in partially hydrogenated vegetable oils (Table 12.6).

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CHAPTER 13

Biological systems

The role of polyunsaturated lipids in the diet and their effects on health has received much attention in the last three decades. Concerns about the problems of lipid oxidation stem from the deterioration of foods, and from the possible biological damage resulting from changes in diet and environment. Intense interest in this field has been generated by the compelling evidence that increased oxidative damage is implicated in many human diseases. In the early 1960s, vegetable oils became the main source of edible fats because of their higher contents of polyunsaturated fatty acids and lower contents of saturated fatty acids and lack cholesterol. When saturated fats and dietary cholesterol were shown to be associated with heart diseases in humans, diets high in polyunsaturated vegetable oils were recommended for a long time because they lower serum cholesterol. When low-erucic rapeseed oil, also known as canola oil, became readily available in the US, it was considered more desirable than soybean oil because it contained much less saturates (Table 1). However, canola oil contained more linolenate (averaging 10%) than soybean oil (averaging 7%), and both of these vegetable oils could not be used for frying without partial hydrogenation (Chapter 12). The present trend is to use either new, but more expensive and less available varieties of vegetable oils containing higher levels of oleate and lower levels of linolenate, or blends of vegetable oils, because partial hydrogenation produces *trans* fatty acids, which are now considered to be undesirable nutritionally (Section F.4).

More recently, too much linoleic acid in the diet was associated with a predisposition toward coronary and inflammatory diseases. Because *n*–3 polyunsaturated fatty acids moderated the adverse effects of excess *n*–6 polyunsaturated fatty acids, nutritionists have advocated an increase in consumption of fish and fish oils. The oxidation of polyunsaturated fatty acids components in lipoproteins in blood is now implicated in the development of atherosclerosis (see Sections D and E). Therefore, in recommending increased intakes of *n*–3 polyunsaturated fats in the diet, the potentially serious problems of their oxidation should not be overlooked by nutritionists and the general public.

This chapter considers the nutritional consequences of polyunsaturated lipids in the diet on free radical reactions in biological systems and diseases. The term lipid "peroxidation" is now used broadly to include both nonenzymatic and enzymatic oxidative reactions of free fatty acids, phospholipids, triacylglycerols, cholesterol and cholesteryl esters, lipoproteins, proteins,

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interaction products of proteins and reducing sugars, and DNA (see the Glossary at the end of this Chapter). An ultimate goal of nutrition is to improve our diet by reducing or minimizing the risk factors from oxidative deterioration of polyunsaturated lipids, proteins and DNA that are implicated in the developments of certain human chronic diseases, including atherosclerosis, cancer, cataract, diabetes, neurological and lung diseases, immune inflammatory disorders, and the universal process of aging.

A. Biological peroxidation

The biological significance of the free radical process known as lipid peroxidation has moved this field beyond our concerns with the problems of rancidity in foods. Lipid peroxidation is induced under certain conditions by the formation of more readily oxidizable substances, referred to collectively as reactive oxygen species. These oxidant species are responsible for the toxic effects of oxygen in the body, and together with the free radicals generated from unsaturated lipids (Chapter 1) may cause tissue damage accompanying disease states. Reactive oxygen species affecting lipid peroxidation include:

(a) Singlet oxygen ($^1O_2$). By photosensitization reactions, energy produced by exposure to light and an excited sensitizer, is transferred to oxygen, which becomes excited from the triplet state to the singlet state (Chapter 3). Many biological sensitizers are known to be effective, including chlorophylls, riboflavin and derivatives (flavin mononucleosides and flavin adenine dinucleotide), bilirubin, retinal, and porphyrins. The resulting $^1O_2$ reacts with linoleic acid at least 1500 times faster than normal triplet oxygen to form hydroperoxides causing damage to the membrane lipids in the retina. Visual cells can be damaged by exposure to light due to sensitization of retinal causing the formation of $^1O_2$. Exposure of cells to light causes damage to the mitochondria, which are rich in heme and flavin-containing proteins. In addition to polyunsaturated lipids, a large number of biologically important compounds are damaged by $^1O_2$, such as α-tocopherol, DNA, cholesterol, β-carotene, and proteins by reactions with several amino acids (tryptophan, methionine, cysteine and histidine).

(b) Superoxide radical ($O_2^{-\bullet}$). This reactive species is produced in aerobic cells by one-electron reduction of oxygen through the cellular electron transport chain of mitochondria, chloroplasts, and phagocyte cells. An important antibacterial mechanism proceeds by neutrophils, monocytes, and macrophages, which kill bacteria by producing $O_2^{-\bullet}$.

In aqueous solutions, superoxide may act as a weak base by accepting protons to form a hydroperoxyl radical ($HO_2^{-\bullet}$) that can dissociate to regenerate protons, by the equilibrium (1). However, not much superoxide is present at physiological pH.

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$$O_2^{-\cdot} + H^+ \rightleftharpoons HO_2^{-\cdot} \quad (1)$$

Superoxide can act either as a reducing agent or as a weak oxidizing agent. Superoxide reduces the iron in cytochrome c from Fe^{3+} to Fe^{2+}, and oxidizes ascorbic acid. In the oxidation of haemoglobin in erythrocytes to oxyhemoglobin, superoxide can be produced in its conversion to methemoglobin through an intermediate complex with oxygen (2).

$$\underset{\text{hemoglobin}}{} + O_2 \longrightarrow \underset{\text{oxyhemoglobin}}{[\text{hem-Fe}^{2+}\cdots O_2]} \longrightarrow \underset{\text{methemoglobin}}{\text{hem-Fe}^{3+}} + O_2^{-\cdot} \quad (2)$$

The cells are protected against the damage from excess superoxide by various enzymes in a sequence of reactions involving superoxide dismutase (SOD) producing hydrogen peroxide, which is removed by catalase, as discussed in Section B. More recently, superoxide has been suggested to regulate vascular tone as a vasoconstrictor, by interacting with nitric oxide ($NO^{\cdot}$), which acts on smooth muscle cells to produce relaxation.

(c) Hydrogen peroxide. The dismutation of superoxide (3) in the presence of SOD produces hydrogen peroxide, which occurs in whole bacteria, phagocytic cells, spermatozoa, mitochondria, microsomes, and chloroplasts.

$$2\,O_2^{-\cdot} + 2\,H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \quad (3)$$

Hydrogen peroxide crosses biological membranes such as red blood cell membranes, and is also found in human eye lens and human breath (from pulmonary macrophages). Although hydrogen peroxide *per se* is a weak oxidizing agent, in the presence of Fe^{2+} in the cell, it forms hydroxyl radicals, which are extremely reactive and responsible for the toxicity of hydrogen peroxide in killing bacteria. Catalase provides biological protection by removing hydrogen peroxide from the cell.

(d) Hydroxyl radicals ($^{\cdot}$OH). In the presence of transition metals, H_2O_2 produces hydroxyl radicals according to equation (4).

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + {}^{\cdot}OH + OH^- \quad (4)$$

Hydroxyl radicals are the most reactive and damaging oxygen species generated by a wide range of systems, including activated phagocytes, autoxidizing compounds, mixtures of hypoxanthine and xanthine oxidase (an important enzyme that synthesizes uric acid from xanthine). Hydroxyl radicals are so highly reactive that when produced *in vivo* they can react at or in close proximity to their site of formation.

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The decomposition of hydrogen peroxide by Fe^{2+} and some Fe^{3+} complexes, is known as the Fenton reaction and is represented by equations (5) and (6).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \quad (5)$$

$$Fe^{3+} + H_2O_2 \longrightarrow \text{ferryl} + H_2O_2 \longrightarrow \text{perferryl} + H_2O_2 \quad (6)$$

$$\downarrow$$

$${}^{\cdot}OH$$

The occurrence of the Fenton reaction *in vivo* has been long debated. If this reaction occurs, it is limited by the availability of iron ions. The hydroxyl radicals formed are so highly reactive that they combine at or near the location of the metal ions. If catalytic iron is bound to membrane lipids, hydroxyl radicals formed in the presence of hydrogen peroxide could initiate lipid peroxidation. However, the question of how much free metal ions are present *in vivo* is greatly debated in the current biochemical literature (see Section B.b).

(e) Hypochlorous acid (HOCl). This powerful oxidant is generated at sites of inflammation by the enzyme myeloperoxidase in activated phagocytic cells. The resulting process, known as phagocytosis, takes place by engulfing foreign particles such as bacteria with cells known as neutrophils. The myeloperoxidase can kill bacteria by oxidizing chloride ions in the presence of hydrogen peroxide to produce HOCl. Bacteria are killed by the strong oxidizing action of HOCl on proteins, especially the amino acids components containing sulfhydryl (–SH) groups, and other biological molecules such as ascorbic acid (vitamin C).

B. Biological antioxidants

Organisms have evolved antioxidant defense systems for protection against reactive oxygen species and repair systems to prevent the accumulation of products from oxidatively damaged lipids, proteins and DNA. A number of extracellular and intracellular antioxidant systems are known to effectively inactivate reactive oxygen species and oxidants, as discussed in Section A.

(a) Singlet oxygen quenchers. Carotenoids such as lycopene and β-carotene from fruits and vegetables protect lipids and other biological systems by interfering with the activation of oxygen. The mechanisms recognized for this effect include: quenching singlet oxygen and sensitizers, and scavenging peroxyl radicals at low oxygen pressures (Chapter 3). Relatively

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low concentrations of β-carotene are effective in preventing lipid peroxidation by singlet oxygen. Other compounds such as α-tocopherol, amino acids and glutathione behave as weak quenchers by oxidation with singlet oxygen. Although β-carotene is a poor inhibitor of free radical oxidation, it behaves synergistically in the presence of α-tocopherol in preventing lipid oxidation *in vitro*. Although the mechanism for this synergism is not clear, the possibility that this interaction between α-tocopherol and β-carotene applies *in vivo* should be investigated.

(b) Metal binders. Living organisms are well protected against the cytotoxic effects of iron by using transferrin as a transport protein and ferritin as a storage protein to minimize the intracellular pool of free iron. Transferrin is a glycoprotein carrier molecule, which under normal conditions is about 30% loaded with iron. No free iron salts are thus expected in blood plasma. The availability of iron and other transition metals necessary to generate reactive oxygen species *in vivo* has thus been much debated in the literature. Several *in vivo* mechanisms have been proposed for binding free transition metals and inactivating them as redox catalysts. However, under oxidative stress induced by superoxide or by hydrogen peroxide, small amounts of free iron may be released and mobilized from ferritin and other protein binders to catalyse free radical reactions. Cells may also contain small amounts of free iron that may catalyse the formation of hydroxyl radicals. Albumin can inhibit lipid peroxidation by binding copper ions. In addition to its metal binding capacity, albumin is a good scavenger of peroxyl radicals (by reacting with the sulfhydryl groups of its amino acids components) and of hypochlorous acid. Metal binding or chelation by plasma proteins is generally regarded as one of the most important antioxidant defense mechanisms.

(c) Superoxide scavengers. Superoxide dismutases (SOD) catalyse the dismutation of superoxide by reaction (3) to produce hydrogen peroxide, which is removed by catalase and glutathione peroxidase. Several SOD enzymes have been characterized, including the copper-zinc-SOD in mammalian tissues, fish and plant tissues, the manganese-SOD and the iron-SOD in higher organisms and bacteria.

(d) Enzymatic peroxide destroyers. Glutathione peroxidase removes hydrogen peroxide within cells by selectively using glutathione (GSH), a tripeptide of glutamic acid, cysteine and glycine, as a hydrogen donor. Two GSH molecules condense by reaction (7), through the –SH groups of cysteine to form a disulfide bridge, –S–S– .

$$2\,H_2O_2 + 2\,GSH \longrightarrow GSSG + H_2O \qquad (7)$$

Oxidized glutathione, GSSG, is reduced back to GSH by glutathione reductase to maintain a ratio of GSH/GSSG greater than 10 in normal cells.

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Glutathione peroxidase accepts other hydroperoxides and reduces lipid and steroid hydroperoxides, to form stable hydroxy lipids that do not decompose to form alkoxyl radicals and cell damaging aldehydes. There is ample evidence indicating that glutathione peroxidase provides an important protective mechanism against lipid peroxidation *in vivo*.

Two types of glutathione peroxidases are known, selenium and non-selenium containing, and these vary widely in different animals and organs. The former enzyme consists of four protein units, each containing one atom of selenium. The non-selenium glutathione peroxidase reacts with artificial hydroperoxides (e.g. cumene and *tert*-butylhydroperoxides) but not with hydrogen peroxide, and its activity may be due to glutathione-*S*-transferases. The selenium containing glutathione peroxidase is most important as indicated by the requirement of selenium in trace amounts as a cofactor in animal and human diets.

Catalases remove hydrogen peroxide by catalysing reaction (8).

$$2\,H_2O_2 \longrightarrow 2\,H_2O + O_2 \qquad (8)$$

Catalase activity is particularly high in the liver and erythrocytes of animals. These enzymes consist of proteins containing a heme group (Fe^{3+}-protoporphyrin), and can catalyse peroxidase-type reactions.

(e) Non-enzymatic peroxide destroyers. Ascorbic acid has multiple antioxidant effects in biological systems. It is an active reducing agent that can scavenge singlet oxygen, superoxide, hydrogen peroxide, hydroperoxyl radicals, hydroxyl radicals and hypochlorous acid, to give semidehydroascorbate. One of the multiple actions of ascorbic acid is to reduce lipid hydroperoxides to stable hydroxy lipids. It is regarded as the most effective water-soluble antioxidant in plasma. However, it can also reduce Fe^{3+} to Fe^{2+}, which can participate in the Fenton reaction by producing damaging hydroxy radicals in the presence of hydrogen peroxide, and stimulate iron-catalysed lipid peroxidation. This prooxidant effect is counterbalanced under certain conditions by the scavenging properties of ascorbic acid toward hydroxyl radicals. The main protective role of ascorbic acid may be the regeneration of vitamin E after its oxidation in plasma proteins (Section B.6).

Uric acid is a water-soluble antioxidant found in relatively high concentrations in plasma. It inhibits lipid peroxidation by tightly binding iron and copper ions into inactive forms, and by scavenging various oxidants such as hydroxyl radicals, peroxyl radicals, singlet oxygen and hypochlorous acid. By complexing with iron, uric acid stabilizes ascorbic acid in human serum.

Ubiquinol-10 (reduced form of ubiquinone-10, or coenzyme Q10) is a reactive lipid-soluble antioxidant that may act like ascorbic acid by regenerating vitamin E by reducing the vitamin E radicals produced during lipid peroxidation.

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(f) Radical chain breakers. α-Tocopherol (vitamin E) is considered the major lipid-soluble antioxidant in membranes, acting by several mechanisms, including: by scavenging free radicals (superoxide, hydroxyl radicals), which can initiate and propagate lipid peroxidation, by reacting with nitric oxide, and by deactivating singlet oxygen. Vitamin E reacts with lipid peroxyl and alkoxyl radicals by donating labile hydrogen and terminating the lipid peroxidation sequence (Chapter 9). The resulting α-tocopherol radical is stabilized by electron delocalization around the phenol ring structure. We shall see later that α-tocopherol is important in protecting lipoproteins against oxidation. To be effective *in vivo*, the α-tocopherol radicals are reduced back to α-tocopherol by ascorbic acid or other reducing agents, such as cysteine, or glutathione. This synergistic effect is assumed to take place at the membrane interface because the hydrophilic reducing agent, in solution in the aqueous phase, would have an affinity for the membrane phospholipids at the surface.

C. Oxidant–antioxidant balance

The field of lipid peroxidation has generated considerable attention because of compelling evidence that increased oxidative damage due to reactive oxygen species is implicated in many human diseases. Lipid peroxidation and degradation products may be involved in a number of biological effects, and may be relevant to certain types of cancer. Very many biological macromolecules, such as lipids, proteins and DNA, are susceptible to oxidative damage by free radicals and reactive oxygen species. A number of decomposition products of lipid hydroperoxides, such as malonaldehyde and hydroxynonenal, are known to be cytotoxic. The integrity of cell membranes provides an important protective mechanism by separating unsaturated lipids from biological oxidants and activated oxygen species, and by maintaining a low intracellular concentration of oxygen. Any factors that affect this structural separation will cause damaging lipid peroxidation. Lipid peroxidation occurs thus more rapidly in disrupted than in healthy tissues. However, it is often difficult to establish unequivocally if free radical lipid peroxidation is the cause of biological damage leading to diseases, or if it takes place as a consequence of this damage.

Protection against peroxidation comes from antioxidant nutrients and intracellular defenses from the enzymes, antioxidants, proteins acting as metal binders, reducing agents and cofactors discussed in Section B. *In vivo*, there is a constant competition between these oxidation and protective processes that depend on the polyunsaturated fatty acid composition of tissue lipids and their antioxidant levels (Figure 13.1). For optimum health, a balance is required between the oxidizing species and the antioxidants. An imbalance resulting from excess oxidants or oxygen species will lead to a disturbance, defined as oxidative stress, causing tissue damage and susceptibility to diseases.

Reactive oxygen species produced by several stimuli are now recognized as

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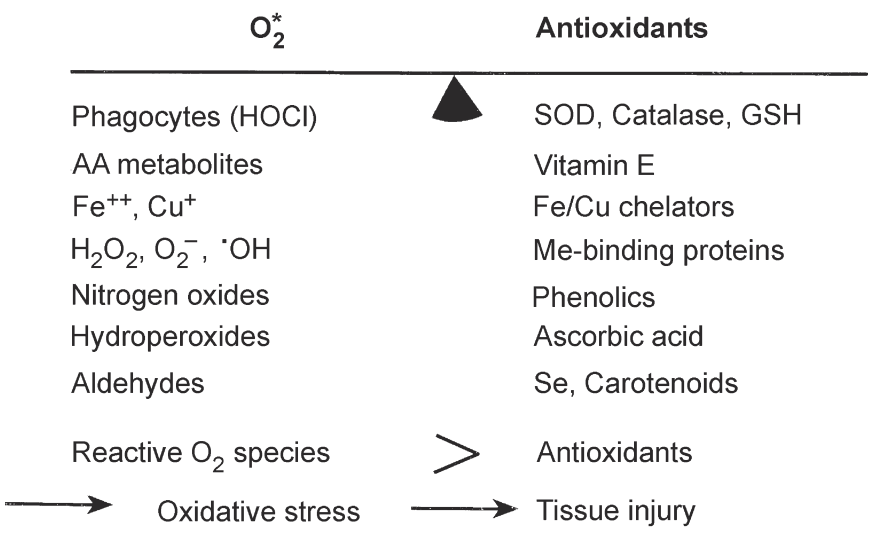


Figure 13.1. Oxidant – antioxidant balance. O_2^*, reactive oxygen species; AA, arachidonic acid; SOD, superoxide dismutase; Me, metals; Se, selenium.

important in the mechanism of toxicological responses of various compounds referred to as toxins. The reactivity of toxins could be neutralized by the concentrations of antioxidants in tissues to ensure the removal of excess oxidants. Many kinds of tissue injuries may initiate free radical reactions such as lipid peroxidation by destroying or inactivating cellular antioxidants, or by liberating metal catalysts from chelating proteins in cells. Oxygen radicals are recognized mediators of various degenerative disease and inflammatory diseases, including rheumatoid arthritis, diabetes, cancer, cataract formation, immune and brain dysfunctions, lung diseases, and the universal problem of aging.

Extensive studies are now carried out worldwide to test this oxidant–antioxidant balance hypothesis. The health of an individual may be influenced by the efficiency of various protection systems against repetitive and accumulated oxidant damage. The stress and tissue injuries resulting from oxidant damage may range in intensity from viral infections, trauma, inflammation, cigarette smoke or environmental pollution. Antioxidant requirements will vary in relation to the oxidative cellular damage or oxidant stress, and would be therefore very difficult to establish for a general population. The evidence is accumulating that antioxidants in fruits and vegetables may inhibit biologically harmful oxidation reactions in the body. Recently, the occurrence of relatively high levels of polyphenolic antioxidants in fruits, vegetables and certain beverages (especially red wine and tea) has received special attention, because of their potential protective effects against the damage from biological oxidants. The problems of oxidative stress could thus be alleviated by including in

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Table 13.1. Composition of lipoprotein particles in human blood plasma, expressed as % particle mass [a]

Components and characteristics	Chylomicrons	VLDL	LDL	HDL
Triacylglycerols	83	50	10	8
Total cholesterol	8	22	48	20
Phospholipids	7	20	22	22
Protein	2	7	20	50
Apoproteins	A, B-48, C	B-100, E	B-100	A
Mass (daltons)	$0.4\text{-}30 \times 10^6$	$10\text{–}100 \times 10^6$	2.35×10^6	$1.75\text{–}3.6 \times 10^5$
Density (g/ml)	>70	30–90	18–22	5–12

[a] From Gurr and Harwood (1991).

the diet more phenolic antioxidants found in fruit and vegetables. Although there is strong evidence that consumption of fruits and vegetables is associated with reduced coronary heart diseases, cancer and other diseases, very few studies have provided direct evidence that these benefits are actually due to antioxidants. The present knowledge is incomplete, however, on the great diversity of plant phenolic compounds or phytochemicals and their nutritional and biological effects. Although there is accumulating evidence that small amounts of these plant antioxidants are absorbed in human blood as metabolites (see Table 13.11), their molecular mechanisms of protection *in vivo* and of disease intervention are not well established. The evidence is inconsistent that supplements of antioxidant vitamins such as vitamins E and C can reduce the incidence and severity of of cancer and degenerative heart disease in humans. Studies of supplementations with individual antioxidants have thus far shown mainly negative results. There is a lack of reliable markers of *in vivo* oxidative damage to lipids (Section E.3). This subject is now under intensive investigation around the world.

D. Plasma lipoproteins

Since triacylglycerols and cholesterol are not soluble in blood, they are carried through it by lipoproteins serving as special transport systems. To understand the role of oxidized lipoproteins in the development of vascular heart diseases, it is necessary to review briefly some of the basic metabolic pathways of lipoproteins. Lipids are transported in the blood stream through a series of lipoprotein particles varying in size and the nature of the phospholipid and protein constituents (Table 13.1). These particles have a lipid core, containing mainly triacylglycerols and cholesteryl esters, and surrounded by a shell of phospholipids and proteins, called *apoproteins*. These lipoproteins are separated by ultracentrifugation into different classes, from the lowest to the highest

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density: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). These particles are described in terms of particle mass because they are structural aggregates of individual lipid and protein molecules. The apoprotein moieties of lipoproteins provide structural specificity to the particles by directing their metabolic pathways to various cell surface receptors throughout the body. The following functions are recognized for each lipoprotein: a) chylomicrons are the principal carriers of dietary lipids composed mainly of triacylglycerols in the blood stream; b) VLDL transport endogenous lipids synthesized in the liver and intestine; c) LDL are the main carriers of plasma cholesterol; and d) HDL carry excess cholesterol to the liver.

Lipoproteins are transported and metabolized through two pathways. The exogenous pathway concerns the fat from the diet; the endogenous pathway concerns the fats synthesized in the body from carbohydrates (Figure 13.2). After absorption, triacylglycerols, phospholipids and cholesteryl esters are packaged as chylomicrons. These large particles are removed from the plasma by the hydrolytic action of lipoprotein lipase forming fatty acids for either storage in adipose tissue or for fuel in the blood. The chylomicrons are thus partially degraded to smaller particles called chylomicron remnants, which are removed by the liver where they are taken up by remnant receptors and metabolized to LDL particles that are enriched in cholesterol. The concentration of cholesterol in the blood is controlled by reacting with specific LDL receptors on the cell surface, forming a lipoprotein-receptor complex in the cell, where the lipoprotein is broken down. Portions of chylomicrons are converted to HDL subfractions (HDL_1 and HDL_2), which remove excess cholesterol by converting it to cholesteryl esters by the action of another enzyme, lecithin-cholesterol acytransferase (LCAT) (Figure 13.3). By the endogenous pathway, the liver exports fat molecules as VLDL in the blood. These particles circulate in the blood and metabolize cholesterol by similar pathways as the chylomicrons, by forming smaller VLDL particles called intermediate density lipoproteins (IDL), which are metabolized into LDL and taken up by the LDL receptor.

The level of cholesterol in the body is delicately controlled by the number of LDL receptors, which varies according to the intracellular production of cholesterol. When cholesterol is present in blood in excessive amounts, it may lead to metabolic defects with undesirable nutritional consequences. The HDL particles play a vital part in removing excessive free cholesterol, converting it to cholesteryl esters by the action of LCAT and transporting it to the liver. The mechanism of this recognized process termed reverse cholesterol transport is not well established, however. In addition to this mechanism, HDL particles can transport antioxidant enzymes such as acetylhydrolase and paraoxonase, which can break down oxidized lipids and neutralize their proinflammatory effects (see Section E.3).

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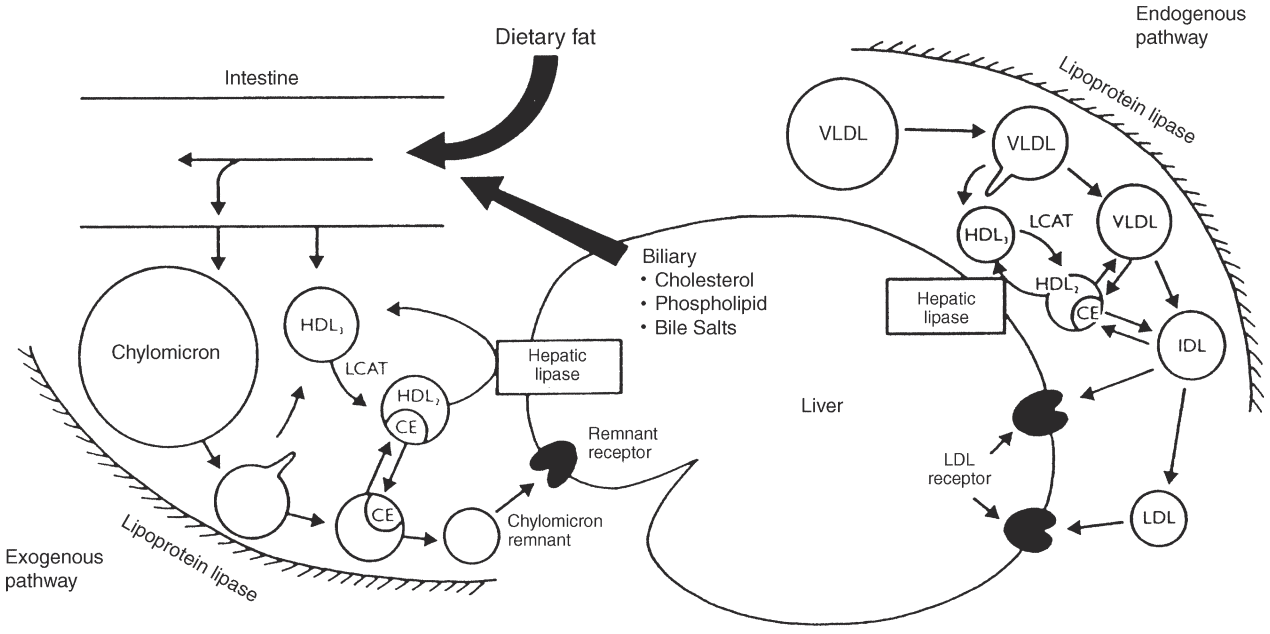


Figure 13.2. Metabolism of lipoproteins. From Gurr (1997) with permission. CE, cholesteryl esters; HDL, high density lipoprotein which is interconverted into two forms, HDL_2 and HDL_3; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein, LCA, lecithin cholesterol acyltransferase (see Figure 13.3).

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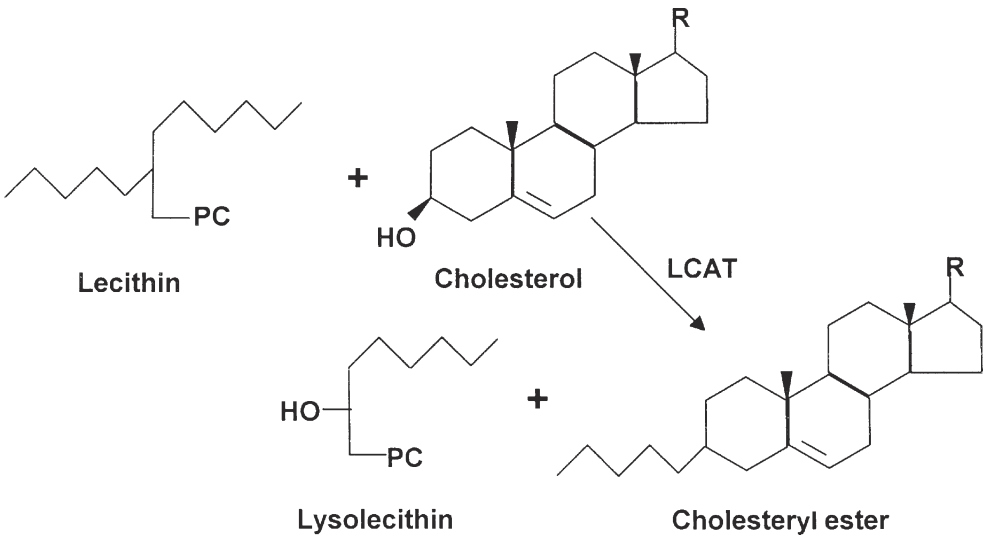


Figure 13.3. Lecithin cholesterol acyltransferase (LCAT). This enzyme esterifies cholesterol by transferring unsaturated fatty acids (mainly linoleic acid) from lecithin to form cholesteryl esters and lysolecithin.

E. Coronary heart disease

This disease is characterized by the thickening of the inner linings of arteries. Atherosclerotic lesions are initiated by an increase of LDL in plasma, which triggers an increase in circulating monocytes that adhere to endothelial cells in the artery (Figure 13.4). This process leads to more LDL particles entering into the intima (the next layer concentric to the endothelial layer and closest to the bloodstream), where they become oxidized by cells found in arterial lesions. The strong evidence that oxidation of LDL is a critically important event in the initiation and development of atherosclerosis has generated considerable interest in the biomedical significance of lipid peroxidation. Medical interventions against this disease are now based not only on lowering the serum level of LDL cholesterol, but also on reducing the susceptibility of LDL to oxidation and inflammation.

1. Development of the disease

Atherosclerosis is believed to develop by a response to injury mechanism, also referred to as the endothelial injury hypothesis, involving a chain of complex cellular interactions leading to the formation of fatty streaks.

(i) *Arterial injury*. An injury occurs initially to the endothelial lining of the artery in response to a range of factors, including aging, toxins, viral infection, immunological reactions, smoking, hypertension and lipid oxidation products

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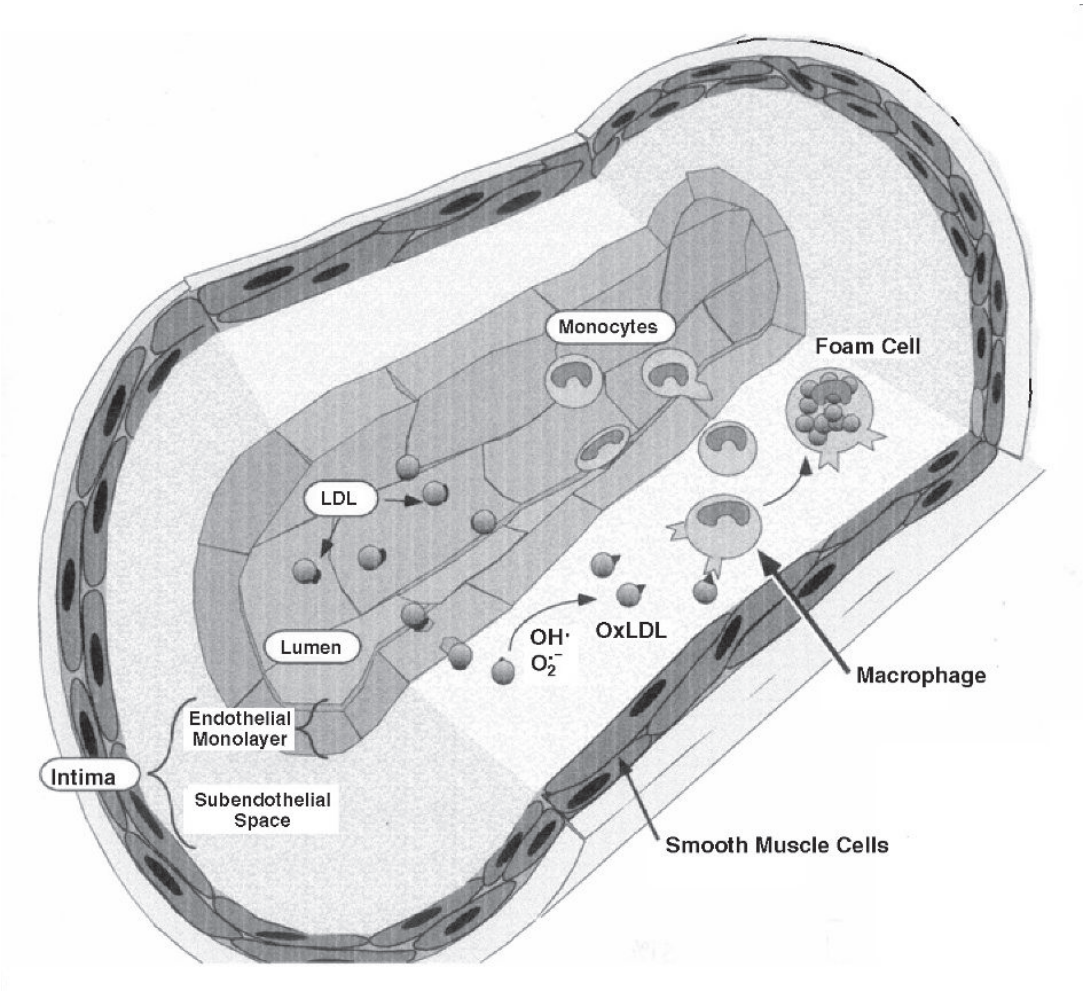


Figure 13.4. Oxidative modification of low lipoprotein. Formation of atherosclerotic lesions by oxidation of LDL in the intima, the inner layer concentric to the endothelial layer of the artery. From Pearson (1998).

in the diet and in oxidized LDL (oxidized cholesterol, phospholipids, triacylglycerols and free fatty acids). Although the origin of this arterial injury is unknown, it may be initiated by LDL particles, which when present at high concentrations in circulating plasma become more susceptible to oxidation.

(ii) *Monocyte adherence and macrophage production.* In response to injury, monocytes migrate from the blood into the intima and become macrophages that ingest particle cells, within the vessel wall. Monocytes attach themselves to the endothelium by the presence of adhesion molecules expressed in response to an injury. Activated monocytes and macrophages may cause further injury to endothelial and smooth muscle cells by secreting reactive oxygen species (superoxide, hydrogen peroxide).

(iii) *Oxidative modification of LDL.* When LDL particles accumulate in the

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intima of the arterial wall, their lipids undergo oxidation while their proteins undergo both oxidation and glycation (binding by sugars). The resulting oxidized LDL activates the endothelial and smooth muscle cells of the intima to secrete chemokines (chemicals that attract monocytes). When monocytes multiply and convert into active macrophages, they become recognized by the abnormal scavenger receptors. The lipoprotein fractions isolated from atherosclerotic lesions have increased electrophoretic mobility and reactivity with antibodies. Although the cause of oxidation of LDL is unclear, it may be the result of a local deficiency of antioxidants.

(iv) *Foam cell formation*. Oxidized LDL particles passing through into the lining of the blood vessel become engulfed and ingested by the macrophage and other cells, which are converted into cholesterol-engorged and distorted macrophages called foam cells.

(v) *Fatty streak formation*. Fatty streaks in the sub-endothelial area are formed by the prolific production of foam cells from macrophages. Platelets adhere to denuded areas in endothelium, recruit and proliferate smooth muscle cells.

(vi) *Intimal thickening*. Smooth muscle cells were assumed to produce foam cells. These cells derived from circulating macrophages adhere to the endothelium and get into the sub-endothelial space (Figure 13.4) where they assimilate lipoproteins laden with cholesteryl esters.

The injury cycle repeats with ultimate production of extensively raised plaques, which narrow and begin to obstruct the lumen of the artery. Advanced lesions can grow and block blood flow to the heart or brain and leading to acute occlusion by forming a blood clot termed a thrombus, resulting in myocardial infarction or stroke or heart attack. The process of atherosclerosis is very complex and takes several decades to develop. Nascent plaques have in fact been detected in humans in their early teens. Key steps that may promote this development include oxidative stress from excessive dietary cholesterol, elevated blood lipid concentrations, hypertension, cigarette smoking, or other genetic factors associated with this disease. Common forms of this disease may also result from polluted environments and the aging process. Other risk factors have been recently proposed including diabetes, smoking, obesity, high blood pressure and the resulting inflammation. Diabetes leading to increased glucose levels in blood can increase glycation and the inflammatory properties of oxidized LDL.

Oxidized LDL may have several other functions in the development of atherosclerosis, including the expression of chemotactic factors that attract macrophages in the endothelial space, to induce endothelial cells to secrete a chemotactic protein for monocytes activating inflammatory and immune responses, and altering the production of nitric oxide. The endothelial injury

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produces platelet aggregation, which is followed by the release of growth factors that initiate smooth muscle cell proliferation. Certain growth factors, eicosanoids (oxygenated products of arachidonic acid) and nitric oxide, can also affect the activities of the cells in atherosclerotic lesions. Macrophages release growth factors that affect the transport of cholesterol within the body. Endothelial cells release potent substances such as prostacyclin, through the prostaglandin cascade, and nitric oxide, that inhibit blood clot formation. The effect of dietary lipids through the conversion of polyunsaturated fatty acids to physiologically important molecules (eicosanoids, prostaglandins and leukotrienes) will be discussed further in Section F.2.b.

The oxidation hypothesis that oxidatively modified LDL particles are retained in the intima of arteries leading to atherosclerosis is supported by considerable evidence, but remains unproven. The inflammation hypothesis has recently been emphasized in the mediation of all stages of atherosclerosis including initiation, and acute thrombotic complications of atheroma. Since many lipid oxidation products are pro-inflammatory, the inflammation and oxidation hypotheses are closely linked. Oxidized LDL and the oxidized phosphatidylcholine components of LDL are in fact pro-inflammatory and have proatherogenic properties. In addition to LDL, other lipoprotein particles including VLDL and a subfraction called beta VLDL and IDL may undergo oxidation, activate inflammation and become atherogenic.

Growing evidence indicates that inflammation involves elevated blood circulatory C-reactive protein (CRP) that may be used as prognosis markers of cardiovascular inflammatory pathways. CRP is a normal plasma protein that increases significantly in concentration by tissue injuries resulting from infection, and inflammation. Oxidized LDL is known to bind to CRP. As a widely measured clinical index of disease activity, CRP values are considered an even more sensitive risk indicator of heart disease than blood cholesterol. Although CRP may have beneficial scavenging functions by binding specifically to phosphorylcholine (PC) of oxidized PC and to oxidized phosphatidylcholine, oxidized LDL, and lysophospholipids of damaged plasma membranes, it may also enhance tissue injury and have pro-inflammatory effects. Therefore, CRP may not only predict atherosclerosis, but also play a role in this disease.

Myeloperoxidase found within atherosclerotic lesions is also thought to provide a link between inflammation and cardiovascular disease. This enzyme is recognized as a major catalyst of lipid oxidation at sites of inflammation by the formation of nitric oxide derived oxidants, proatherogenic halogenated fatty aldehydes and unsaturated lysophospholipids. The oxidants produced by myeloperoxidase are considered as part of its normal function in host defense. Because the levels of this enzyme are significantly increased in persons afflicted with cardiovascular disease, it is emerging as an important marker of oxidant stress in assessing cardiovascular risk.

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2. *Oxidative modification of LDL*

The term oxidized LDL is poorly defined because the native plasma LDL particles are complex highly heterogeneous mixtures of lipid components, consisting of multiple subpopulations varying in degrees of oxidation and contents of antioxidants. LDL particles may be grouped into three subclasses of different density: light, large LDL (LDL1+2), intermediate LDL (LDL3), and small, dense LDL (LDL4+5). The average LDL particle consists of a very complex mixture consisting mainly of phospholipids, free cholesterol, cholesteryl esters, triacylglycerols, and apolipoprotein B (apoB) containing 4536 amino acid residues (Table 13.2). Minor antioxidant components include tocopherols, carotenoids and ubiquinol. Both lipid and apoB components become oxidized and the products of oxidation may interact to cause damage.

A voluminous literature has been recently published to show that constituents of oxidatively modified LDL are atherogenic and cytotoxic, leading to cell injury, cell death and arterial lesion formation. The type and sources of biological oxidants initiating LDL oxidation *in vivo* are unknown, however, and controversial.

Table 13.2. Chemical composition of human LDL [a]

Constituents	Mean mol/mol LDL
Phospholipids	700
Phosphatidylcholine	450
Lysophosphatidylcholine	80
Sphingomyelin	185
Free cholesterol	600
Cholesteryl esters	1600
Triacylglycerols	170
Free fatty acids	26
Apoprotein B	1
Fatty acid composition	
Saturated acids	880
Oleic acid	454
Linoleic acid	1100
Arachidonic acid	153
Docosahexaenoic acid	29
Antioxidants	
α-Tocopherol	6.4
γ-Tocopherol	0.5
β-Carotene	0.3
α-Carotene	0.1
Lycopene	0.2
Ubiquinol-10	0.1

[a] From Esterbauer *et al*. (1992).

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(a) Pathways of LDL oxidation. Several biological cellular pathways have been advanced for the oxidative modification of LDL, which can take place either in the arterial wall or at inflammatory sites. LDL particles can become oxidatively modified when they are retained in the artery wall or other cellular sites, and then return to the plasma. The oxidation of LDL is first initiated by reactive oxygen species, including linoleate and arachidonate hydroperoxides. After oxidative initiation, LDL particles become trapped in the subendothelial space of the artery wall where additional reactive species accumulate.

The resulting oxidized LDL particles trapped in the vessel wall can undergo a number of other reactions including lipolysis, proteolysis and aggregation, which contribute to inflammation and foam cell formation. A multitude of oxidation pathways have been proposed that involve 12/15-lipoxygenase, metals ions, myeloperoxidase producing hypochlorous acid, and NADP (nicotinamide adenine dinucleotide phosphate) oxidase.

In one pathway, the enzymes 12 and 15-lipoxygenases first oxidize cell lipids and the oxidized lipids transfer into the LDL in the medium and initiate chain reactions with extensive modification of the lipids in LDL (Figure 13.5). The resulting oxidized LDL becomes proinflammatory and is more rapidly taken up by macrophages. This hypothesis is supported on one hand by evidence that lipoxygenase inhibitors decrease the ability of macrophages to initiate LDL oxidation, and that stereospecific oxidation products are found in atherosclerotic lesions of rabbits and humans. This hypothesis has been questioned on the other hand, by experiments with animals having less atherosclerosis when genetically engineered to overexpress 15-lipoxygenase in their macrophages, and the negative effect when fed antioxidants. The latter evidence is disputed, however, by opposite reports on the effects of 12/15 lipoxygenase, and the inconsistent results of animal feeding studies with antioxidants that can become prooxidants under certain conditions (Section E.6).

A second pathway postulates the generation of active oxygen species such as superoxide within the cell, followed by release into the medium, where LDL lipids become oxidized. During oxidative modification, LDL phospholipids undergo extensive conversion to lysolecithin. Phospholipid hydroperoxides are selectively hydrolysed from the 2-position by phospholipase-A2, which is intrinsic to apoB of LDL. The resulting lysolecithin may be chemotactic to monocytes and produce lesions.

The third pathway involves metal catalysis. Metal catalysts appear to be necessary to initiate lipid peroxidation in cells since LDL oxidatively modified in the presence of cells has the same physico-chemical and biological properties as LDL oxidized by metals in the absence of cells. However, monocytes derived from macrophages can initiate both metal-induced and metal-independent oxidation processes. The polyunsaturated lipids (LH) in cholesteryl esters, phospholipids and triacylglycerols in LDL undergo oxidation by the same free radical chain mechanism as that established for food

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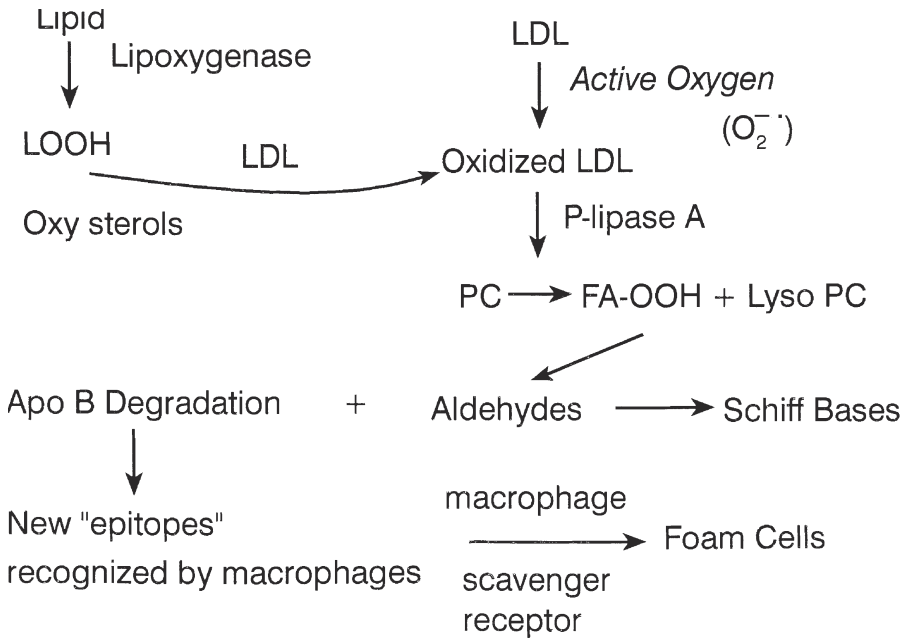


Figure 13.5. Pathways for the oxidative modification of low density lipoprotein. The lipoxygenase pathway produces hydroperoxides that catalyse the oxidation of LDL. The active oxygen pathway involves the direct oxidation of LDL with reactive oxygen species such as superoxide. The oxidized fatty acids (FA-OOH) of phosphatidylcholine (PC) in LDL are preferentially hydrolysed by phospholipase A and decomposed into aldehydes that interact with ApoB of LDL, leading to the formation of foam cells via macrophage and scavenger receptors.

lipids with formation and decomposition of hydroperoxides (Chapters 2 and 4) (Figure 13.6). Although many *in vitro* studies were carried out with media containing free metal ions, there is evidence that LDL oxidation does not always require media rich in free metals.

Several lines of evidence support the *in vivo* oxidation of LDL, but how and where LDL is oxidized *in vivo* is not yet clear. The LDL extracted from atherosclerotic lesions manifest the same physicochemical and biological properties as LDL oxidized under *in vitro* conditions. The susceptibility of plasma LDL to copper-induced oxidation *in vitro* correlates with the extent of coronary atherosoclerosis in patients with previous experience of myocardial infraction. However, the many cellular biological effects established for oxidized LDL *in vitro* could not be assumed to cause atherosclerosis *in vivo*.

Other cellular mechanisms postulated for the oxidation of human LDL involve the direct oxidation of apoB amino acid residues. The copper-mediated oxidation of tryptophan residues in apoB was suggested as an initiation step in

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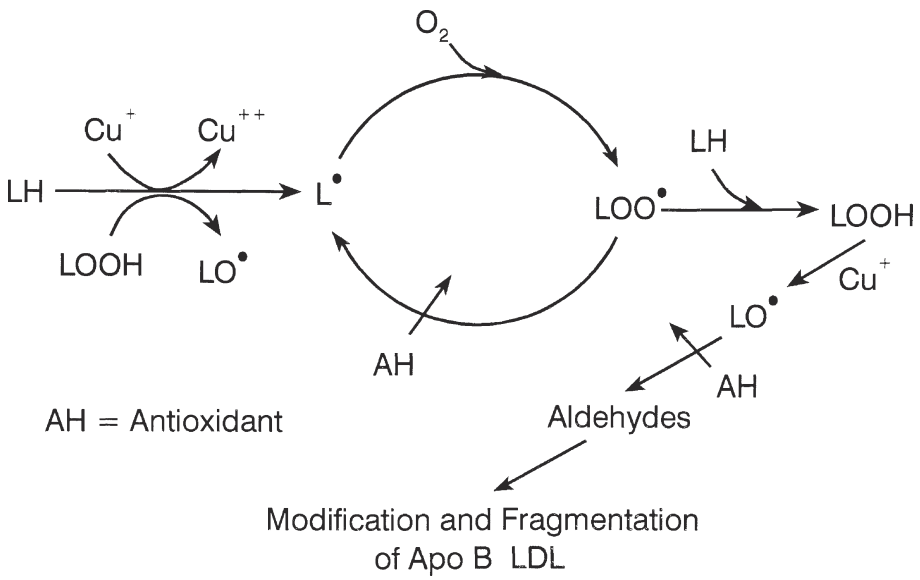


Figure 13.6. Free radical oxidation of polyunsaturated fatty acids (LH) components of low density lipoprotein (LDL). Formation of aldehydes that causes modification and fragmentation of amino acid residues in the ApoB of LDL. Antioxidants (AH) can inhibit LDL oxidation by reacting with both peroxyl radicals ($LOO^{\bullet}$) and alkoxyl radicals ($LO^{\bullet}$).

the oxidation of LDL particles. Hypochlorous/hypochlorite (HOCl) produced by myeloperoxidase in macrophages caused the preferential oxidation of lysine, tryptophan and cysteine residues of apoB. Although HOCl is found in atherosclerotic lesions, the direct intervention of myeloperoxidase in the *in vivo* oxidative modification of LDL is disputed.

Several mechanisms have been advanced for the atherogenic properties of oxidized LDL. In addition to macrophage uptake causing foam cell formation, oxidized LDL particles induce chemotactic proteins and growth factors in endothelial cells that cause cell adhesiveness. Oxidized LDL is chemotactic for monocytes and facilitates their migration into the arterial wall. As suggested previously, LDL can also become oxidatively modified at extra-cellular sites of inflammation and taken up into the artery to cause atherogenesis. In addition to the cytotoxicity of oxidized phospholipids in LDL, oxidized cholesterol and cholesterol esters, also named oxysterols, (see Section E.5) can cause cell destruction leading to lesion formation observed in atherosclerosis.

As discussed below, antioxidants can inhibit atherosclerosis in some experimental animal models (Section E.6). Much of the current research in this area is directed at accumulating evidence to support the potential antiatherogenic effects of antioxidants as preventive measures to slow atherosclerosis by inhibiting LDL oxidation *in vivo*.

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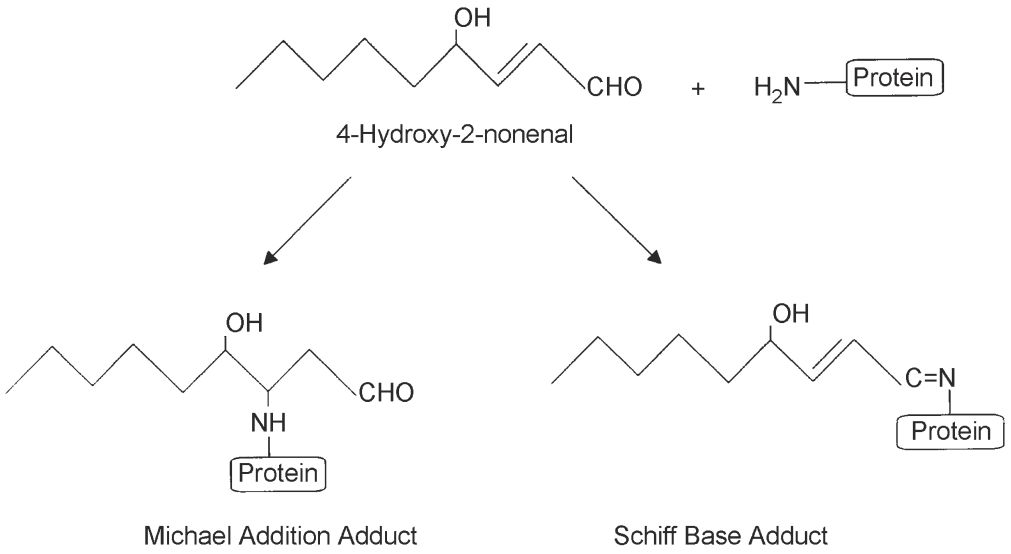


Figure 13.7. Formation of Michael addition and Schiff base adducts by interaction of 4-hydroxy-2-nonenal with amino residue of proteins.

(b) Products of LDL oxidation. In the presence of metals, the hydroperoxides undergo extensive fragmentation into aldehydes that react covalently with the apoB moiety of LDL by the formation of Schiff bases with the lysine ε-amino groups of apoB, and form cross-links between lipids and proteins. In addition to Schiff base formation, 4-hydroxy-2-nonenal and other 2,4-dienals can form protein adducts by Michael addition, leaving a free aldehyde moiety to undergo further condensation by intra- or intermolecular cross-linking changing the charge and configuration of the protein (Figure 13.7). The positive charge of lysine ε-amino residues is neutralized and the LDL particles acquire a net negative charge. When sufficient lysine amino acid residues are altered, a new epitope is formed on apoB that causes oxidized LDL to be recognized by the macrophage scavenger receptor and to be internalized and undergo endocytosis to become converted into lipid-engorged foam cells.

Hypercholesterolemia in animals and humans is characterized by elevated levels of cholesterol ester rich LDL subclasses of LDL1+2 and LDL3 that oxidize to provide rich sources of unstable hydroperoxides in the arterial wall. The oxidative susceptibility of LDL subfractions increased in the order LDL1+2 > LDL3 > LDL4 > LDL5, and the corresponding lag times were slightly shorter in the LDL subfractions of hyperlipidemic (HC) than normolipidemic (NC) patients (Table 13.3). The formation of hydroperoxides at different stages of oxidation from phosphatidylcholine (PCOOH) and cholesterol esters (CEOOH) in different LDL subclasses varied significantly between NC and HC subjects. Although the ratios of polyunsaturated PCOOH/polyunsaturated PC consumed were higher in LDL1+2, LDL3 and LDL4 subclasses from HC than NC

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Table 13.3. Lag time of LDL subfractions during copper initiated oxidation and ratios of phosphatidylcholine (PCOOH) and cholesterol ester (CEOOH) hydroperoxides to polyunsaturated fatty acids [a]

Hydroperoxides/ Polyunsaturated fatty acids consumed	LDL1+2		LDL3		LDL4		LDL5	
	NL	HC	NL	HC	NL	HC	NL	HC
Lag time (T_{lag}), min	54	47	43	41	41	35	36	32
Propagation half-time ($T_{½}$)								
PCOOH 18:2/PC 18:2	1.1	3.1	1.2	5.3	1.7	2.8	1.5	2.0
PCOOH 20:4+22.6/PC 20:4+22:6	0.8	3.3	1.0	4.4	1.5	2.2	1.1	2.1
CEOOH 18:2/CE:18:2	12.3	4.8	14.9	7.4	12.4	5.2	12.6	5.8
CEOOH 20:4/CE:20:4	7.7	3.5	8.4	5.4	9.0	3.3	5.3	4.2
Time at end of propagation (T_{max})								
PCOOH 18:2/PC 18:2	6.8	10.2	6.8	15.7	6.4	17.6	8.0	8.7
PCOOH 20:4:22.6/PC 20:4+22:6	8.0	23.8	9.2	27.1	8.5	25.7	10.0	15.0
CEOOH 18:2/CE:18:2	40.7	19.3	36.0	20.1	24.9	19.9	36.3	17.5
CEOOH 20:4/CE:20:4	73.3	21.3	47.1	27.1	31.1	22.5	36.0	17.5

[a] From Chancharme *et al.* (2002). Ratios of moles hydroperoxides per 100 moles of polyunsaturated fatty acids consumed calculated from Tables 2 and 4 in their paper. LDL subfractions had the following density ranges (g/ml): LDL1+2: 1.018–1.030, LDL3: 1.030–1.040, LDL4: 1.040–1.051, LDL5: 1.051–1.065.

Abbreviations: NL = normolipidemic subjects, HC = hypercholesterolemic, PCOOH = phosphatidylcholine hydroperoxides, CEOOH = cholesterol ester hydroperoxides. LDL subfractions were oxidized with copper at 37°C and monitored at 234 nm continuously, while time points were taken at the propagation half-time ($T_{½}$), and the end of the propagation time (T_{max}).

subjects, the opposite trend was observed in the corresponding ratios of polyunsaturated CEOOH/polyunsaturated CE consumed. Therefore, the CEOOH were much less stable than the corresponding PCOOH from HC subjects. The instability of CEOOH should be confirmed, however, by measuring the potentially bioactive aldehyde decomposition products.

A number of recent studies provided evidence for the formation of pro-inflammatory core aldehydes formed by β-cleavage of hydroperoxides of oxidized LDL phospholipids containing arachidonic acid in the *sn*-2 position. The three secondary products from oxidized 1-palmitoyl-2-arachidonyl-*sn*-glycero-3-phosphorycholine (PAPC), analysed by electrospray-mass spectrometry, included 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phophorylcholine (POVPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phophorylcholine (PGPC), and 1-palmitoyl-2-(5,6-epoxy-isoprostane E2)-*sn*-glycero-3-phophorylcholine (PEIPC) (Figure 13.8). These bioactive products increased significantly in minimally oxidized LDL (MM-LDL) (oxidized to a level before it is recognized by the scavenger receptor). The core aldehyde POVC and the corresponding glutaryl acid (PGPC) are pro-inflammatory and responsible for the immunoreactivity with certain antibodies in mildly oxidized LDL. The

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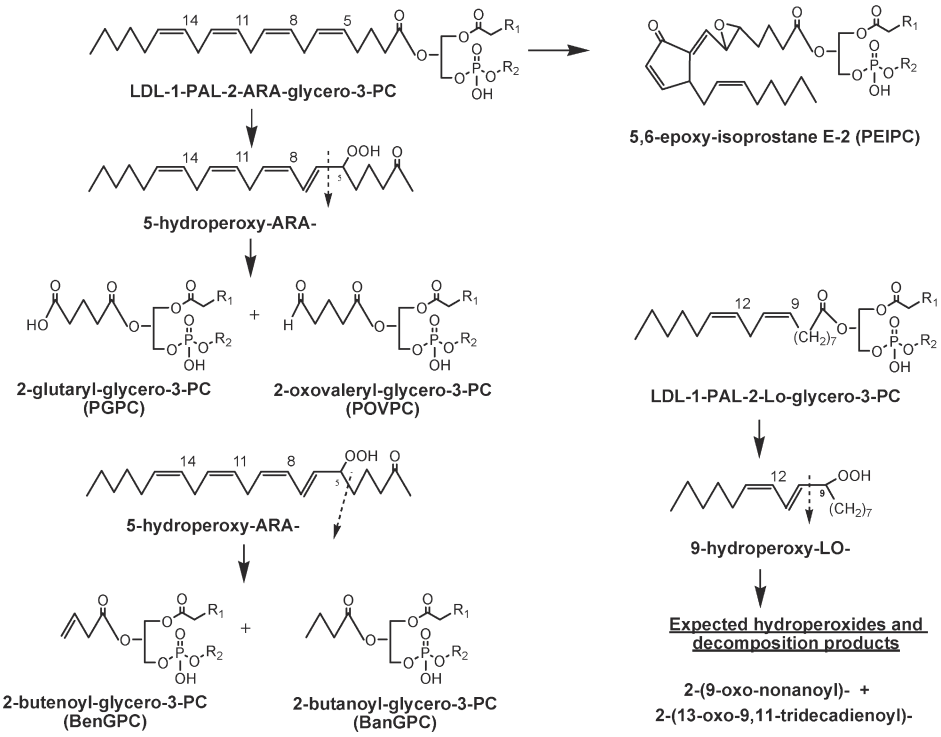


Figure 13.8. Formation of hydroperoxides and decomposition products from phospholipids in oxidized LDL. Adapted from Navab *et al.* (2004) and Marathe *et al.* (1999). Abbreviations: LDL, low-density lipoproteins; PAL, palmitoyl; ARA, arachidonyl; PC, phosphorylcholine; Lo, linoleoyl (see Chapters 2 and 3, and Table 13.4).

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Table 13.4. Oxidized phospholipids in human LDL, minimally oxidized LDL (MM-LDL) and in rabbit lesions [a]

Oxidized phospholipids	Concentrations (ng/mg lipoprotein or wet weight)			
	LDL	MM-LDL	Rabbit aorta (chow diet)	Rabbit aorta (atherogenic diet)
POVPC	172	986	27	116
PGPC	168	1966	21	62
PEIPC	351	742	16	85
SOVPC	84	650	16	85
SGPC	119	1004	15	45
SEIPC	198	364	14	58

[a] From Subbanagounder *et al.* (2000b). Quantitative analyses of phospholipids were carried out by electrospray ionization-MS in the positive mode.
POVPC, 1-palmitoyl-2-oxovaleroyl-*sn*-glycero-3-phophorylcholine, PGPC, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phophorylcholine, PEIPC, 1-palmitoyl-2-(5,6-epoxy-isoprostane E2)-*sn*-glycero-3-phophorylcholine, SOVPC , SGPC and SEIPC are the corresponding stearoyl derivatives.

LDL. The corresponding fragmented alkyl phosphatidylcholines, butanoyl GPC and butenyl GPC products are inflammatory platelet-activating factor (PAF)-like phospholipids and also named butanoyl PAF and butenyl PAF. These C-4 core aldehydes are probably the most stable and are readily analysed directly by LC-MS. Several additional and perhaps more bioactive unsaturated core aldehydes are also expected by non-enzymatic oxidation and fragmentation of PC and CE containing arachidonic, linoleic and *n*–3 PUFA acids (see Chapter 4) in oxidized LDL. These unsaturated core aldehydes may not have been detected because they may form covalent PC-apoprotein adducts that are not readily identified and analysed directly by LC-MS. However, these unsaturated core aldehydes may be potentially more bioactive and important than the saturated core aldehydes reported in oxidized LDL.

The bioactivity of oxidized phospholipids was tested in human LDL, MM-LDL and lipids from aorta of rabbits (Table 13.4). The levels of POVPC, PGPC, PEIPC (Figure 13.8) and the corresponding stearoyl derivatives (SOVPC, SGPC, SEIPC) were increased 3 to 6 fold in atherosclerotic lesions of animals fed an atherogenic diet. These oxidized phospholipids are strong activators of endothelium and play a significant role in atherogenesis.

Phospholipids on the surface of LDL particles generate ligands that are recognized by macrophages. There is now evidence that PC core ester aldehydes form covalent adducts with apolipoprotein B of oxidized LDL, that can be recognized by macrophage scavenger receptors and participate in antibody-antigen interactions. Because these oxidized PC–protein adducts are found in atherosclerotic lesions, the evidence suggests that oxidized phospholipid–apoB adducts are produced *in vivo*.

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Table 13.5. Relative proportions of core aldehydes recovered from oxidized LDL and HDL [a]

Molecular species	LDL	HDL
16:0-5:0 Aldehyde	ND	3.4
16:0-7:0 Aldehyde, 18:0-5:0 aldehyde	ND	7.1
16:0-8:0 Aldehyde	9.0	13.5
16:0-9:0 Aldehyde	32.6	36.1
18:1-8:0 Aldehyde, 18:0-8:1 aldehyde	ND	2.4
16:0-10:0 Aldehyde, 18:0-8:0 aldehyde	21.2	11.2
18:1-9:0 Aldehyde	ND	6.2
18:0-9:0 Aldehyde	36.5	19.0
18:0-10:0 Aldehyde	ND	0.5

[a] From Kamido *et al.* (1995). LDL and HDL were subjected to copper-catalysed oxidation, resulting in complete depletion of 20:4 in both LDL and HDL, 56% depletion of 18:2 in LDL and complete depletion in HDL. Values were estimated from peak areas in mass chromatograms obtained by LC-MS of DNPH derivatives of diacyglycerol core aldehydes.
ND, not detected

Previous reversed-phase HPLC analyses of highly oxidized LDL and HDL showed the formation of C-5, C-7, C-8, C-9 and C-10 core aldehydes of phosphatidylcholine (PC) (Table 13.5). The major C-9 core aldehyde produced from linoleyl PC is bioactive in providing a high affinity ligand to proliferating receptors of inflammation. Other less stable unsaturated core aldehydes may be expected by non-enzymatic oxidation and fragmentation of PC and CE containing arachidonate, linoleate as well as *n*–3 PUFAs and may also be bioactive. Several aldehydes and aldehyde–protein adducts and reactive carbonyls produced during hyperglycemia and hyperlipidemia by lipid oxidation may also induce oxidative stress in cells. The final step involves non-enzymatic oxidation producing the inflammatory POVPC, PGFPC and PEIPC that induce monocyte binding, chemotaxis and differentiation into macrophages.

3. The role of high-density lipoproteins (HDL)

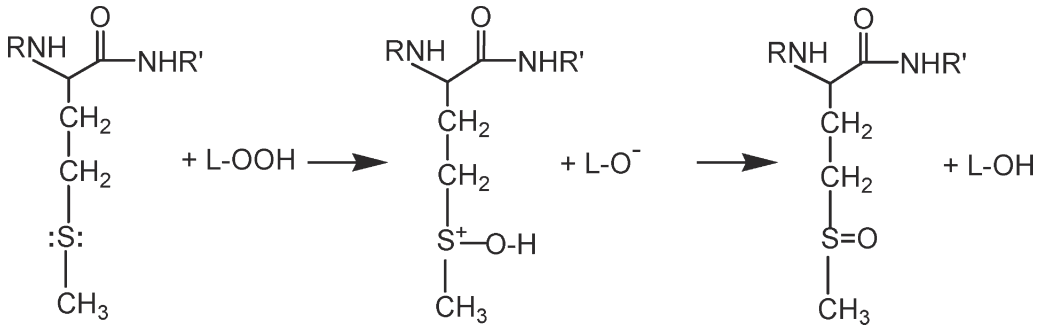
High-density lipoproteins consist of complex heterogeneous particles comprising about half protein and half lipid, a core containing cholesteryl esters and triglycerides surrounded by a monolayer of phospholipids and free cholesterol and several embedded apoproteins AI and AII. Several enzymes are found in HDL, including an esterase paraoxonase (PON1) that degrades bioactive oxidized phospholipids, lecithin-cholesterol acyltransferase (LCAT) and platelet-activating factor acetylhydrolase (PAF-AH).

HDL protects against atherosclerosis by counteracting the effects of oxidized LDL. The well-recognized reverse cholesterol transport mechanism

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of HDL involves removing excess cholesterol from peripheral tissues and transporting it back to the liver for excretion. Other enzymatic mechanisms include inhibition of LDL oxidation by PON1, antioxidant and anti-inflammatory properties of HDL. PON1 extends the lag phase of HDL oxidation, reduces HDL hydroperoxide and aldehyde formation. PAF-AH has anti-inflammatory activity by inactivating the phospholipid PAF causing inflammation. The mechanism of this enzyme action involves removing the acetyl group from the *sn*-2 position of PAF.

HDL and its main apoA-1 and peptides that mimic apoA-1 prevent atherogenesis in animal models and in humans by inhibiting the oxidation of LDL phospholipids responsible for the inflammatory response of atherosclerosis. LDL treated with apoA-1 inhibits the formation of hydroperoxides and the inducement of monocyte activity when added to human artery cell wall cultures. HDL is also reported to be a major carrier of lipid hydroperoxides in humans. ApoA-1 is able to "remove" linoleate and arachidonate hydroperoxides from human LDL, thus reducing significantly the inflammatory properties of LDL in animals and humans. This beneficial effect of HDL in decreasing the risk of atherosclerosis appears to be attributed to its ability to carry lipid hydroperoxides in the plasma and transport oxidized cholesterol esters to the liver for excretion. The anti-atherogenic activity of HDL can be explained by its ability to reduce cholesterol ester hydroperoxides transferred from oxidized LDL to the corresponding stable and inactive hydroxides. The proposed mechanism for hydroperoxide reduction involves the oxidation of methionine residues of apo AI and apo AII of HDL via the sulfide of thioethers to produce the corresponding methionine sulfoxide (reaction 9).



(9)

By oxidation with copper or hypochlorous acid, HDL loses its ability to remove cholesterol from cultured cells. This effect would be expected to be detrimental and promote cardiovascular disease. In contrast, however, HDL becomes more effective in protecting mice against atherosclerosis by exposing it to tyrosyl radicals generated by myeloperoxidase, a heme protein secreted by macrophages in human atherosclerotic lesions. The resulting tyrosylated HDL

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is also more active than native HDL at removing cholesterol from foam cells and macrophages *in vitro*. Tyrosylated HDL containing dityrosine is thus suggested to be more effective in promoting cholesterol efflux from macrophages and other tissues than HDL *in vivo*. The apoA-1 of HDL contains multiple amphipatic (containing both a hydrophilic and lipophilic moiety) α-helices that promote lipid association. Cross-linked heterodimers of apoA-1 in tyrosylated HDL have enhanced ability to remove cholesterol from cholesterol-rich cells. The protective activity of HDL may thus actually improve if oxidation enhances its ability to remove oxidized cholesterol from cells.

4. Methodology

If the LDL oxidation hypothesis is linked with the HDL reverse cholesterol transport hypothesis, measurements of oxidized lipids in lipoproteins, and the oxidation products of myeloperoxidase may predict the susceptibility to atherogenesis. A much larger body of literature is now available on the lipid oxidation products of LDL than on the oxidation products of apoproteins from both LDL and HDL. The results of *in vitro*, *ex vivo* and *in vivo* studies of LDL oxidation are extremely difficult to interpret in view of the great complexity of the LDL structure, which varies widely in particle heterogeneity. The degree of oxidation is greatly affected by the methods used to prepare the lipoprotein samples, and by the wide range of conditions used to induce LDL oxidation. The determinations of aldehydes, core aldehydes, aldehyde-protein adducts, reactive carbonyls, oxysterols, hydroxy-enals, linoleate and cholesteryl linoleate hydroperoxides, isoprostanes (derived from oxidized arachidonic acid), fluorescence, and oxidized proteins or protein carbonyls provide useful markers regarding oxidative damage most relevant to the modification of LDL. Oxysterols are found in oxidized LDL, in the blood of fasting humans, and in animals on a vitamin E-deficient diet (see Section E.4). Linoleate and arachidonate hydroperoxides are specific oxidation products formed initially from LDL (Figure 13.6), but they only act as precursors of aldehydes, which actually cause the damage to apoB.

Fluorescence formation is largely due to modification of apoB. Measurements of oxidized proteins, loss of amino acids (tryptophan and cysteine), or protein carbonyls are most relevant to the oxidative modification of apoB, but they are not sensitive and require significant oxidative changes for detection. Measures of protein carbonyls are also not specific and are influenced by factors other than peroxidation. Modification of LDL can be measured by macrophage uptake due to a process leading to aggregation resulting from the covalent cross-linking of apoB of LDL particles.

Other methods used to measure LDL oxidation include the loss of linoleic acid (18:2), arachidonic acid (20:4) and cholesteryl esters, formation of conjugated dienes and TBARS, and electrophoretic mobility. The loss of 18:2, 20:4

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and cholesteryl esters is an insensitive method, measuring a small change compared to a very large amount of starting material. Oxidative damage of lipids can in fact be observed before significant changes in 18:2 and 20:4 contents can be measured (above 5%). Determinations of conjugated dienes, derived from polyunsaturated fatty acids hydroperoxides, and several variations of the TBA test have been used most commonly to determine oxidation in LDL. Electrophoretic mobility measures the net negative charge of LDL resulting from the cross-linking of amino acid residues in apoB.

The rapid determination of conjugated dienes spectrophotometrically has been used extensively to make continuous measurements of LDL oxidation, and to determine duration of the lag phase as a sensitive method for oxidative susceptibility. Although diene conjugation is a useful method for the measurement of lipid peroxidation in pure lipids, it reflects hydroperoxide formation but not the decomposition aldehyde products that actually cause oxidative damage to apoB.

Because of its convenience, the TBA method has become a common assay to determine the degree of peroxidation and oxidative susceptibility of a wide range of biological materials, including LDL. However, the validity of the TBA determination as an index of lipid peroxidation in biological samples has been a matter of considerable debate in the literature. The determination of TBARS inherently lacks specificity, and is subject to interference by many compounds including materials that are not due to lipid peroxidation (see Chapter 5.E). This method is also flawed by analytical artifacts, and is affected by the same factors as lipid peroxidation.

The potential to be misled by inappropriate methodology is particularly critical when varying dietary unsaturated fats in animal studies. Measurements of TBA values and conjugated dienes are not appropriate if the effect of oleic acid is investigated since these methods do not measure oleic acid oxidation. Similarly, if the effect of *n*–3 PUFA is investigated, the degree of oxidation measured by TBARS may be exaggerated, because the results of this method are markedly affected by the oxidation of polyunsaturated fatty acids containing more than two double bonds (Chapter 5.E). Fish oil diets were reported to increases LDL oxidation on the basis of formation of TBARS. Because TBARS are formed by oxidative decomposition of polyunsaturated fatty acids containing more than two double bonds, the oxidation of any lipids containing *n*–3 polyunsaturated fatty acids would be expected to produce high levels of TBARS. Therefore, TBARS determinations cannot be used as the only determination of oxidative susceptibility to compare the effects of dietary *n*–6 versus *n*–3 polyunsaturated fatty acids on LDL oxidation (see Section F.2).

The technique of static headspace gas chromatography (Chapter 5.F.1) proved to be useful in our laboratory for the quantitative analysis of volatile aldehydes in oxidized human LDL. This method was used to distinguish volatile oxidation products of *n*–6 polyunsaturated fatty acids (pentane and

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hexanal) and *n*–3 polyunsaturated fatty acids (propanal) in LDL samples from hypertriglyceridemic human subjects fed fish oil supplements. This headspace assay of LDL oxidation thus permits the specific analysis of multiple products of lipid peroxidation (see Section F. 2.b).

More specific and sophisticated methodology has been developed recently to elucidate how lipid oxidation products and other bioactive compounds act in the complex mechanism of LDL oxidation. A large amount of work has been published recently on the GC-MS or LC-MS analyses of cyclic oxidation products of arachidonate described as isoprostanes in biological samples as an index of oxidative status. Because the level of arachidonate in LDL and blood is relatively small (Table 13.2), much effort has been made to use instrumental means to improve the sensitivity required to analyse isoprostanes. This instrumental approach is still beyond the capability of many laboratories, however. LC-MS has the advantage of direct measurements of isoprostanes in contrast to GC-MS (see Chapter 6), which requires the use of two or three thermally stable derivatives. On the one hand, LC-MS still requires purification by reversed-phase solid-phase extraction, followed by TLC to remove impurities, but this step can also be avoided if one can invest in a highly selective MS/MS system. On the other hand, the well-established methodology to measure oxidation products of linoleate, which is present in LDL at levels about seven fold higher than that of arachidonate (Table 13.2), is much more within the capability of many laboratories than the more expensive LC-MS and GC-MS and MS/MS approaches required for isoprostanes.

The bioactive core aldehydes derived from the oxidation of LDL phospholipids (see Section E.2) can be analysed either directly by LC-MS or after hydrolysis by phospholipases and sodium borohydride reduction. These aldehyde esters are purified by solid-phase extraction followed by preparative TLC. Unwanted ester containing phospholipids can be removed after treatment with phospholipase A1. The more polar oxidized phospholipids are eluted by HPLC after the class of phospholipids from which they are derived. The core aldehydes derived from oxidized phospholipids (Section E.2) are also known to be pro-inflammatory. Only a few C-4 and C-5 core aldehydes have been identified and analysed by LC-MS, either directly or after derivatization in oxidized LDL. Many more saturated and unsaturated core aldehydes can be expected in oxidized LDL, according to the degree and mode of oxidation. However, the ability of these core aldehydes to form covalent adducts with the apoB of LDL may prevent them from being detected directly by LC-MS. Chemical or enzymatic hydrolysis may therefore be required before they can be analysed by this technique.

Chemical analyses of specific oxidation products can be complemented by quantitative measures of biological changes that create an atherogenic LDL particle, such as increase macrophage intake, electrophoretic mobility, monocyte binding, or activation of platelets and human leucocytes. Also, many of the

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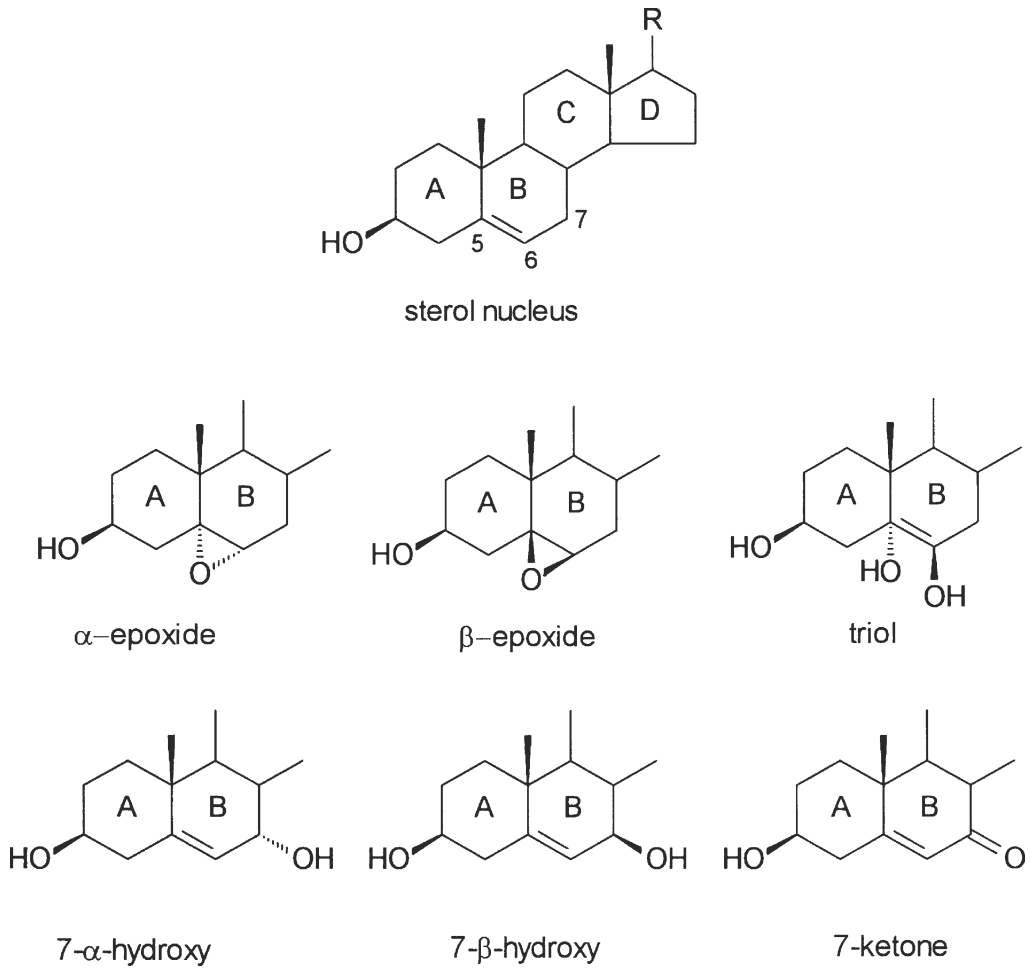


Figure 13.9. Common oxidation products of cholesterol (see also Chapter 2, Section H).

lipid oxidation products identified in blood plasma are not necessarily derived from oxidation of LDL, and could come from other sites and may not be linked to arterial diseases.

5. Autoxidation products of cholesterol

Oxidized cholesterol oxides have been suggested to initiate atherosclerotic lesions by stimulating LDL interactions with arterial smooth muscle cells and macrophages thus producing foam cells. Cholesterol is oxidized especially at the allylic 7-position, and a wide range of products have been identified and detected in LDL and other biological systems, including a mixture of epoxy, keto and hydroxy compounds (Figure 13.9 and Figure 2.19). These products also referred to either as oxysterols or as cholesterol oxides are formed

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enzymatically or by non-enzymatic processes involving active oxygen species. Side-chain oxidized oxysterols are also formed to a very small extent. In biological systems, evidence for oxysterols has been reported by oxidation induced with iron and carbon tetrachloride, and in tissues of animals on vitamin E-deficient diets. Although this type of evidence supports the involvement of oxysterols in human health, the physiological significance of cholesterol oxidation in normal tissues is not well understood. Cholesterol oxides have also been reported to be angiotoxic, mutagenic and to inhibit cholesterol biosynthesis.

Oxysterols are typically found in normal human blood plasma and tissues at levels below 1 ppm, and require analyses by isotopic dilution. Hepatic oxysterols and oxidized plant sterols in human plasma have been analysed by GC-MS. The main cytotoxic oxysterols identified in oxidized LDL include initially the 7β-hydroperoxycholesterol, and 7α- and 7β-hydroxycholesterol (see Figure 2.19). More oxidized LDL contains also 7-ketocholesterol, 5,6α- and 5,6β-epoxycholesterol. Various oxysterols have been shown to induce the process of programmed cell death called apoptosis in vascular, non-vascular and tumor cells. The most abundant cellular antioxidant glutathione (GSH) is significantly decreased during apoptosis. Therefore, as expected the addition of GSH or its precursor N-acetylcysterine as well as vitamin E protected the cells against exposure to oxysterols.

Variable amounts of 7α-hydroxycholesterol (10–20 ng/ml), and various oxidized sitosterol forms are found (in ratio of oxidized sitosterol to sitosterol of about 0.04) in soybean oil based lipid emulsions that are frequently used for intravenous feeding. Cholesterol oxides have also been detected at ppm levels in dehydrated foods, potatoes fried in tallow (Chapter 12) and cured animal products. However, there is no adequate evidence of any harmful biological effects on the amounts ingested in a normal diet and on the degree of absorption and metabolism of oxysterols found in foods. One of the most atherogenic oxysterols is 7β-hydroxycholesterol, but its possible harmful effects in humans are unknown.

6. Inhibition of LDL oxidation by antioxidants

Although LDL is well protected against oxidation in blood plasma by an ample supply of endogenous antioxidants and metal-binding proteins, this protection may not be adequate in the arterial wall where the oxidative modification of LDL is induced by endothelial cells. One hypothesis proposed is that natural antioxidants may be depleted within the arterial sub-endothelial space where oxidation takes place. The oxidative modification of LDL by endothelial cells can be completely inhibited in the presence of sufficient vitamin E or other antioxidants, such as butylated hydroxytoluene. These antioxidants can inhibit two steps in the free radical oxidation pathway, by reacting with the peroxyl

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radicals to stop the propagation chain, and with the alkoxyl radicals to inhibit the breakdown of the hydroperoxides, and the formation of aldehydes (Figure 13.6). Therefore, antioxidants can inhibit the formation of hydroperoxides and aldehydes, which in turn may prevent the oxidative modification of LDL.

(a) Endogenous antioxidants. In addition to the lipid-soluble antioxidants associated with LDL (Table 13.2), human plasma contains antioxidant proteins and enzymes and water-soluble enzymatic and non-enzymatic peroxide destroyers, including glutathione (see Section B). These antioxidants in plasma are also found in extracellular fluids and may inhibit *in vivo* oxidation of LDL in the intimal subendothelial space (Figure 13.4). The non-enzymatic antioxidants (metal-binding proteins, uric acid and ascorbic acid) are more important in extracellular fluids than the enzymatic antioxidants (superoxide dismutase and glutathione peroxidase). Plasma protein thiol groups are also active by trapping any aqueous peroxyl radicals found in plasma. Vitamin E is one of the most abundant lipid-soluble antioxidants in plasma and LDL that protects the lipids of LDL particles.

(b) Antioxidant supplementation. Several studies showed that antioxidants can reduce the oxidative modification of LDL *in vitro*, and can inhibit experimental atherosclerosis in animals. Various antioxidants react differently in LDL and their effectiveness varies greatly according to their concentration and the types of oxidant used (Table 13.6). When added to isolated LDL, vitamin C was an effective antioxidant against oxidation induced by endothelial cells, macrophages, copper and the aqueous azo initiator, AAPH (2'-azobis[2-amidinopropane] hydrochloride). Vitamin C may inhibit LDL oxidation by scavenging water-soluble peroxyl radicals and by regenerating α-tocopherol present in LDL. Vitamin E added *in vitro* inhibited LDL oxidation by endothelial cells and macrophages more effectively than oxidation induced by copper. In contrast, vitamin E showed a prooxidant effect when LDL oxidation was initiated by AAPH. With this artificial initiator, α-tocopherol appears to promote radical chain transfer.

Supplementation of vitamin C *in vivo* showed no effect on LDL oxidation tested *ex vivo* (Table 13.6). This result is expected with hydrophilic antioxidants such as Vitamin C, which cannot be tested with isolated LDL because they are removed from LDL when separated from plasma. Similar negative effects were observed in studies of the hydrophilic flavonoid antioxidants when tested in LDL after isolation from plasma (Section F.5). Supplementation with vitamin E resulted in a significant improvement of LDL resistance against oxidation by cells, macrophages and copper, but a prooxidant effect was found with AAPH. The relevance of using AAPH as an oxidant to test antioxidants for their biological effects is, therefore, questionable. This result provides another example showing that the use of AAPH can be very misleading, and adds to the

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Table 13.6. Inhibition of *in vitro* LDL oxidation following *in vitro* or *in vivo* supplementation of vitamin C and vitamin E[a]

In vitro LDL oxidant	*In vitro* supplementation		*In vivo* supplementation	
	Vitamin C	Vitamin E	Vitamin C	Vitamin E
Endothelial cells	++	++, partial	–	++
Macrophages	++	++	n.d.	++
Cu^{2+}	++	+, partial	–	++
AAPH [b]	++	Prooxidant	n.d.	Prooxidant

[a] From Frei (1995). ++ = yes; + = slight. N.d. = not determined.
[b] AAPH = 2,2'-azobis(2-amidinopropane) hydrochloride

many serious objections that artificial free radical initiators should never be used especially in biological systems.

LDL oxidation is effectively inhibited *in vitro* by a large number of natural antioxidants, including flavonoids (Chapter 9). Flavonoids may inhibit the oxidation of LDL by a multiplicity of mechanisms, including scavenging reactive oxygen species (superoxide, oxidized lipids, oxysterols), binding iron or copper catalysts, and protecting α-tocopherol present in LDL. Flavonoids are also known to inhibit lipoxygenase and cyclooxygenase enzymes that play key roles in eicosanoid synthesis (see Section F. 2). The evidence supports the possible use of antioxidants to ameliorate the stability of LDL oxidation, but the precise mechanism of how atherosclerosis is initiated *in vivo* needs to be clarified before antioxidants can be used as a mode of intervention to prevent this disease.

Although the administration of antioxidants in the diets of several experimental animals can inhibit LDL oxidation and retard the progression of atherosclerosis, a large number of human clinical trials have shown mixed results. Many confusing and confounding factors in these human trials can be attributed to the lack of reliable quantitative *in vivo* specific biomarkers of lipid oxidation and antioxidant activity, poor selection of human subjects, and invalid bases for antioxidant dose selection. These problems are discussed in further details in Section F.5.

F. Nutritional consequences

Several hypotheses have been advanced to explain the development of cardiovascular disease and the lipid hypothesis has been given the most attention. According to this hypothesis, the primary cause of atherosclerosis is the consumption of too much fat and particularly saturated fats in the diet. Hyperlipidemia and hypercholesterolemia in plasma are recognized as the most important symptoms of this disease. Modification of lipoproteins is

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another factor affecting the atherogenic potential, and high plasma LDL accelerates atherogenesis. Epidemiological and clinical studies showed that the effect of diet on the level of plasma LDL cholesterol has the strongest relationship to heart diseases. Plasma HDL is inversely related to heart disease risk. Thus, the main biochemical risk factors for cardiovascular diseases are high plasma LDL cholesterol and low plasma HDL cholesterol. On this basis, the generally accepted nutritional goals are a decrease of LDL cholesterol and an increase in HDL cholesterol. Reduction of LDL cholesterol would limit the uptake of cholesterol by cells in fatty streaks. An increase in HDL cholesterol would increase the rate of cholesterol removal from the arteries.

The nature and amount of lipids in the diet may not be the only causative factors, however. Heart disease is multifactorial and other risk factors of environment and life style as well as genetically inherited traits, may exert an even greater influence than diet, and these include stress, hypertension, obesity, and lack of exercise. Factors that interact to cause high blood pressure, hypertension, and blood toxins are all implicated in the development of heart diseases.

1. Effect of saturated fatty acids

Early work established a link between the amount and quality of dietary fats and plasma cholesterol concentration. The general consensus among nutritionists and epidemiologists is that saturated fats in the diet are the most important contributors to cardiovascular disease by influencing blood total cholesterol and LDL cholesterol. Although the mechanism is not clear, saturated fats may interfere with the ability of LDL receptors to clear LDL from the blood stream (Figure 13.2). Another hypothesis is based on evidence suggesting that abnormalities in lipoprotein metabolism predispose certain subjects to increased risk of cardiovascular disease. A poor ability to cope with postprandial (occurring after a meal) lipemia may increase this risk, because cholesteryl esters can be shunted from LDL and HDL into chylomicron remnants that become atherogenic as they are slow to clear from plasma. According to this hypothesis, the remnants of chylomicrons and VLDL (Figure 13.2) are more atherogenic than LDL. Subjects predisposed to this abnormal lipoprotein metabolism have higher blood triacylglycerol levels, low concentrations of HDL and a disturbance in lipoprotein lipase activity.

Chain length has a significant effect on the ability of saturated fatty acids to raise plasma total cholestrol and LDL cholestrol, and only lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids are effective, especially at low levels of linoleic acid. Stearic acid (C18:0) does not increase LDL cholesterol, apparently because it is rapidly converted to oleic acid by desaturation (Figure 13.10). Palmitic and stearic acids make up about 36–40% of the fatty acids of animal fats and 11–16% of the fatty acids of vegetable fats. Although myristic

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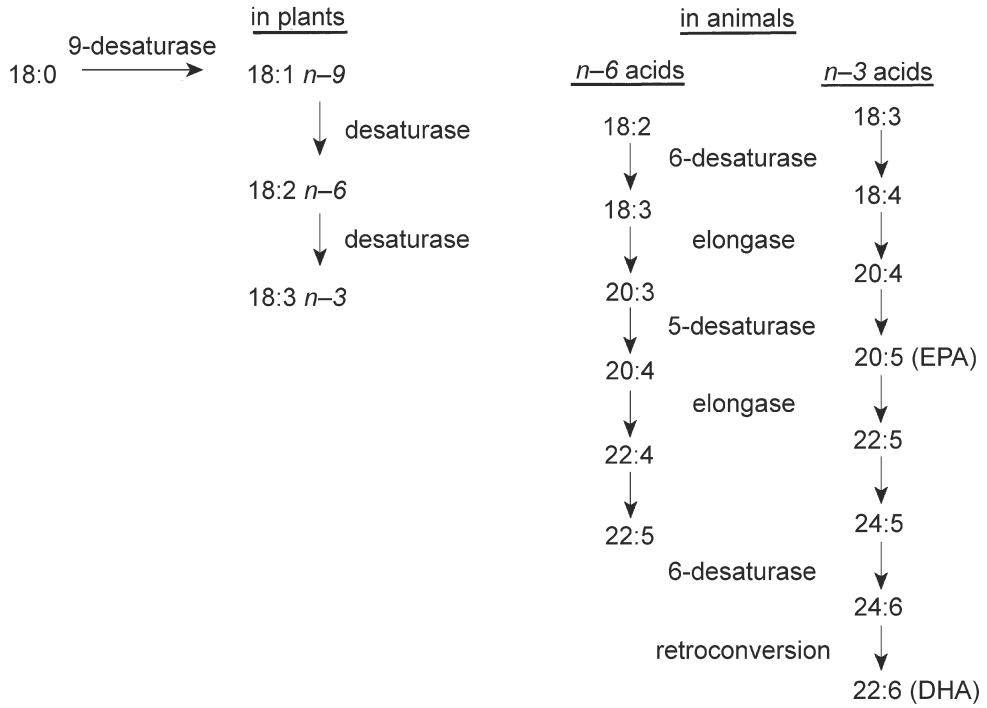


Figure 13.10. Biosynthesis of fatty acids. In plants, oleate (18:1) formed by desaturation of stearate (18:0) can undergo further desaturation to linoleate (18:2) and linolenate (18:3). The introduction of a new double bond occurs in the *n*-direction, between existing double bond and the terminal methyl group. In animals, the *n*–6 and *n*–3 polyunsaturated fatty acids undergo sequential desaturation and elongation to produce arachidonic acid (20:4 *n*–6), eicosapentaenoic acid (20:5 *n*–3) and docosahexaenoic acid (22:6 *n*–3). The introduction of a new double bond occurs in the Δ-direction, between the existing double bond and the carboxyl group.

acid is present at higher levels in butter fat (12%) and in palm kernel and coconut oils (45–48%), it is usually a rather minor component of the diet. Although stearic acid can now be considered neutral, it is generally and indiscriminately agreed that high levels of "all" saturated fatty acids in dietary fats constitute a risk factor for cardiovascular disease.

2. *Effect of polyunsaturated fatty acids*

The replacement of saturated fatty acids with polyunsaturated fatty acids is generally accompanied by a lowering of plasma total cholesterol and LDL cholesterol. Linoleic acid reverses the effects of saturated fatty acids in raising blood triacylglycerols and LDL. Although the results of many studies are inconsistent, most studies show that plasma HDL cholesterol levels tend to decrease when the saturated fatty acids in the diet are replaced with polyunsaturated fatty acids. However, the level of HDL cholesterol tends to be slightly raised by the saturated fatty acid content of the diet.

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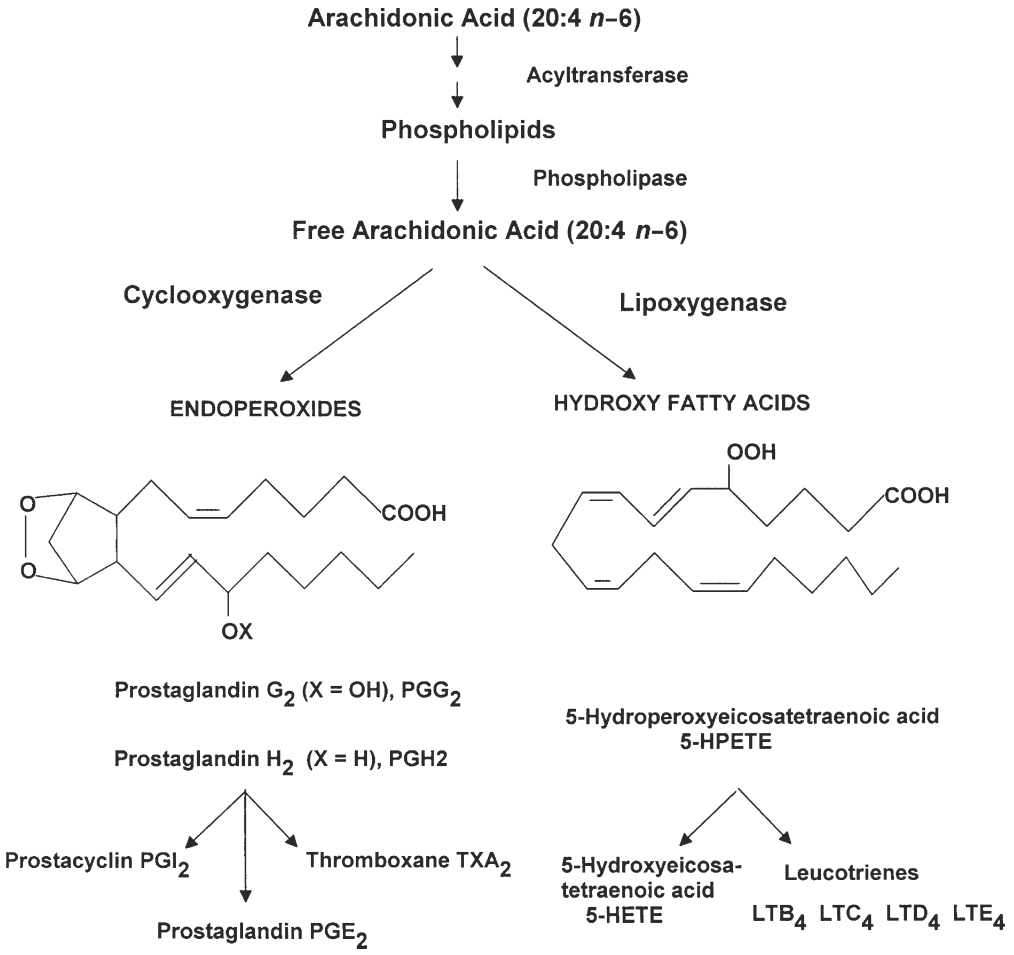


Figure 13.11. Eicosanoid synthesis by cyclooxygenase and lipoxygenase pathways. (See Table 13.7 for biological functions of eicosanoids).

Although polyunsaturated fats in the diet lower LDL cholesterol, they increase the susceptibility of LDL to oxidation. Higher levels of polyunsaturated fats in the diet also raise the requirements for vitamin E and other antioxidants. In view of the important role that oxidized LDL plays in the development of atherosclerotic lesions, the question remains unsettled: whether or not the effect of dietary polyunsaturated fatty acids in lowering plasma LDL is greater than the risk factor of increasing the oxidative susceptibility of LDL.

Linoleic acid (18:2 *n*–6) and linolenic acid (18:3 *n*–3) are essential fatty acids and are required for good health because animals lack the required desaturases to synthesise them (Figure 13.10). These polyunsaturated fatty acids must be supplied by plant foods because they are necessary for growth and reproduction and serve as precursors of various oxygenated fatty acids, called eicosanoids (see Figure 13.11). These compounds are physiologically

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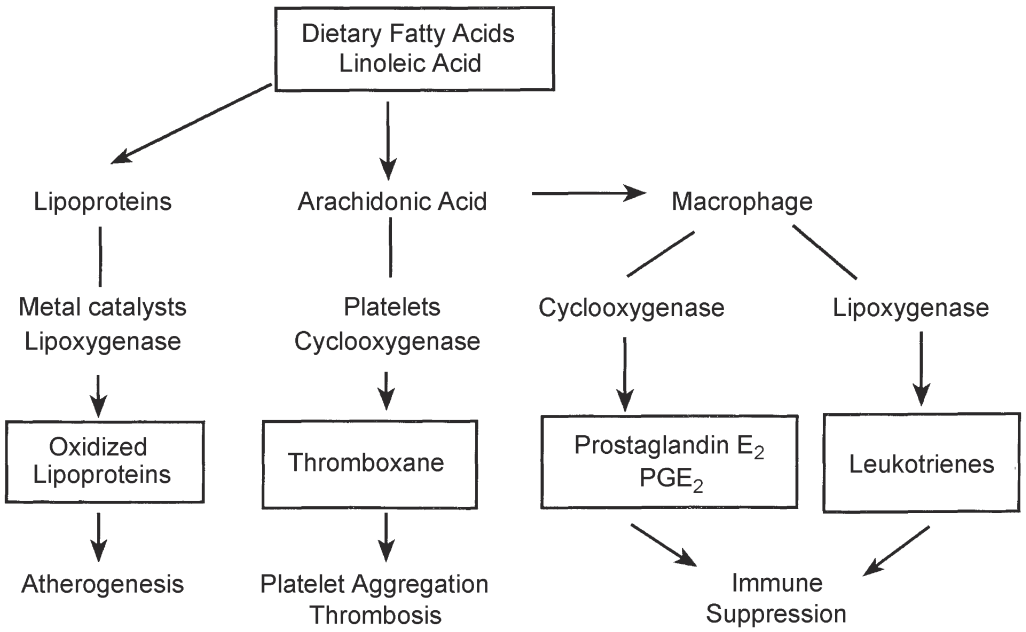


Figure 13.12. Metabolic pathways for the formation of oxidized lipoproteins and eicosanoids. These oxidation products contribute to atherogenesis, thrombosis and immune suppression.

active at extremely low concentrations. As an essential fatty acid, linoleic acid is required in the diet at a level of about 1% of dietary energy and linolenic acid at even lower levels of about 0.2%.

(a) ***n–6 Polyunsaturated fatty acids.*** In addition to its capacity to reduce the concentration of blood cholesterol and LDL cholesterol, linoleic acid in membrane phospholipids is the precursor of arachidonic acid, which is released by hydrolysis with phospholipase A_2 (Figure 13.11). The conversion of arachidonic acid (20:4 *n*–6) to biologically active eicosanoids occurs by a complex cascade of reactions catalysed by cyclooxygenase, producing prostaglandins, and by lipoxygenase, producing hydroperoxides and leukotrienes (Figures 13.11 and 13.12). The resulting mixtures of eicosanoids play a significant role in cellular signal communication. They are important mediators of leucocytes (monocyte, macrophage, neutrophil), which play a role in atherogenesis. They regulate immune function by altering activity of monocytes, macrophages and neutrophils, and are implicated in atherosclerosis, cancer, and autoimmune disorders (Table 13.7).

Lipid hydroperoxides play an important role in the biosynthesis of prostaglandins from arachidonic acid. The control of the concentration of hydroperoxides, also referred to as peroxide tone, is important for the maintenance of normal cell functions. Hydroperoxides can either stimulate cyclooxygenase activity at low concentrations (0.01 μM) or inhibit this enzyme at high concentrations (1 μM). The enzymes glutathione peroxidase and

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Table 13.7. Biological functions of eicosanoids [a]

System	Effects	Active species
Prostanoids and thromboxanes (cyclooxygenase products)		
Blood vessels	Vasodilation	$PGI_2 > PGI_3 > PGE_1$
	Vasoconstriction	TXA_2
Platelet	Adhesion, aggregation	TXA_2
	Antiaggregation	$PGI_2 > PGI_3 > PGE$
Tissue	Pain	PGI_2
	Cytoprotection	PGI_2, diMe PGE
Leukotrienes (lipoxygenase products)		
Vascular	Constriction	LTC_4, LTD_4
	Permeability	LTC_4, LTD_4
Neutrophils, monocytes	Adhesion	LTB_4
	Chemotaxis	LTB_4, HETE (5,9,11)
	Lysozyme secretion	LTB_4, HETE (5,12)

[a] From Kinsella (1987). See Figures 13.11, 13.12, 13.13.

glutathione transferase control prostaglandin synthesis in the cell by suppressing hydroperoxides and by protecting cells against the accumulations of undesirable levels of hydroperoxides.

To prevent coronary heart disease, increased dietary intakes of linoleic acid were recommended in the 1970s for lowering blood cholesterol. When the dietary level of linoleic acid is excessive, however, its conversion to arachidonic acid may cause serious pathophysiological actions by overproducing eicosanoids that generate abnormal signals to the cells (Figure 13.13). The enzyme prostacyclin synthetase is extremely sensitive to hydroperoxides, which control the activation and inhibition of cyclooxygenase. The basal rate of converting arachidonic acid to eicosanoids requires relatively low levels of linoleic acid (less than about 1% of energy). However, the Western diet, which includes about 8 to 10% of total calories linoleic acid, greatly favors the biosynthesis of arachidonic acid. In the US a significant dietary shift from animal to vegetable fats has resulted in a substantial and continuing increase in consumption of linoleic acid from about 8 g per person per day in 1910 to about 27 g per person per day in 1985 (National Research Council, 1989).

The blood level of arachidonic acid formed from dietary linoleic acid critically affects the response of vessels to stress. This response is delicately balanced by two prostaglandin systems (TXA_2 and PGI_2) (Figure 13.11, Table 13.7). The production of thromboxane TXA_2 causes vasoconstriction resulting from adhesion and excessive platelet aggregation that promote blood clotting, a critical event in preventing bleeding. This effect is counteracted by the production in the walls of blood vessels of prostacyclin PGI_2 causing

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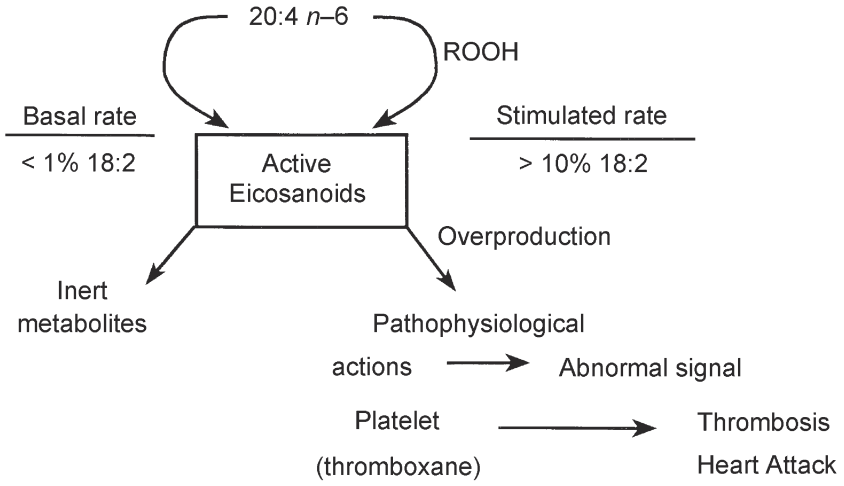


Figure 13.13. Effect of dietary linoleic acid on synthesis of active eicosanoids and metabolism. Pathophysiological actions result from faster eicosanoid formation by increased hydroperoxide levels. From Lands *et al*. (1986).

vasodilation and limiting the aggregation process away from an injury. The flow of blood is thus delicately controlled by the steady state formation of TXA_2 and PGI_2. Since they are both formed from arachidonic acid, the optimum level of dietary linoleic acid required to minimize the risk of thrombosis is uncertain.

(b) ***n*–3 Polyunsaturated fatty acids.** Eicosapentaenoic (20:5 *n*–3) (EPA) and docosahexaenoic (22:6 *n*–3) (DHA) acids from seafood and fish oils and linolenic acid (18:3 *n*–3) from soybean and canola oils are known to antagonize and compete with the metabolism of linoleic acid by lowering the biosynthesis of arachidonic acid. *n*–3 Polyunsaturated fatty acids compete with *n*–6 polyunsaturated fatty acids for the enzymes involved in the formation of arachidonic acid from linoleic acid. The eicosanoids derived from EPA and DHA in fish produce weakly inflammatory eicosanoids, in contrast to the strongly pro-inflammatory eicosanoids from the arachidonic acid produced by excessive linoleic acid in the diet (Figure 13.14). α-Linolenic acid (18:3 *n*–3) is also considered to have an important function in the nervous and vision systems. It is a significant component of the so-called Mediterranean diet where it may reduce the incidence of heart attacks, although this claim has not been proven.

n–3 Polyunsaturated fatty acids are known for their antithrombotic activity by inhibiting platelet aggregation and converting arachidonic acid to thromboxane. Because *n*–3 polyunsaturated fatty acids require a much higher level of hydroperoxides than *n*–6 polyunsaturated fatty acids to be converted to eicosanoids, the *n*–3 acids act as competitive inhibitors of prostaglandin biosynthesis from the *n*–6 acids. An increase in the *n*–3/*n*–6 PUFA ratio in cell membranes alters the composition of eicosanoids by the actions of the enzymes

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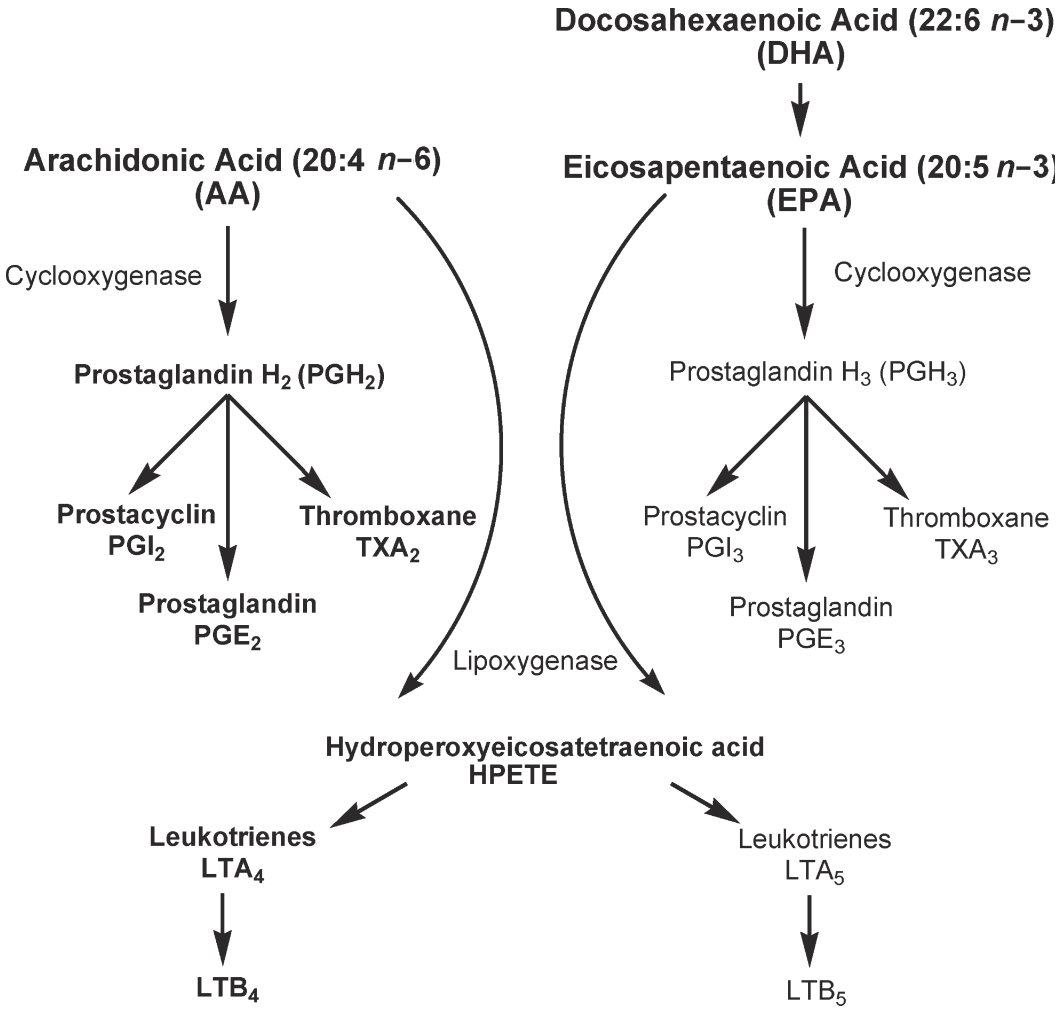


Figure 13.14. Competition between strongly inflammatory eicosanoids (shown in **bold** letters) derived from arachidonic acid and weakly inflammatory eicosanoids derived from eicosapentaenoic acid and docosahexaenoic acid. Adapted from Gurr (1999).

cyclooxygenase and lipoxygenase (Figures 13.11 and 13.12). These enzymes need more hydroperoxides in the presence of EPA than with arachidonic acid. The prostaglandins PGH_2 and PGE_2 derived from arachidonic acid are strongly inflammatory compared to the corresponding weakly inflammatory PGH_3 and PGE_3 derived from EPA (Figure 13.14). The corresponding leukotrienes LTA5 and LTB5 produced from EPA are less inflammatory and less effective as chemotactic agents than the corresponding leukotrienes LTA4 and LTB4 produced from arachidonic acid. Fatty acids of the *n*–3 PUFA series from fish also have a triacylglycerol-lowering effect in people with hyper-triglyceridemia, and play a vital role in vision and brain function.

As a major constituent of the visual system, DHA plays a significant role in

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neural membrane phospholipid metabolism, including visual membrane fluidity, receptor affinities, ion fluxes, and activities of membrane-bound enzymes. Feeding fish oil or DHA affects immune responses by decreasing lymphocyte (white blood cells) proliferation, and suppressing pro-inflammatory cytokines. The aging process attributed to free radical generation produces a detrimental decline in DHA levels in neural membranes, resulting in decline in memory and learning. DHA supplementation in aged rats improves their learning ability in maze performance. High doses of DHA without added vitamin E may, however, result in lipid peroxidation, forming aldehyde breakdown products with prooxidant effects.

The fatty acid composition of LDL is influenced by supplementation of fish oil, but the effect on LDL oxidation is not clear because when the level of EPA and DHA increases it is accompanied by a corresponding decrease in the level of *n*–6 polyunsaturated fatty acids. The assessment of susceptibility of LDL oxidation varies also according to the methods used to follow lipid peroxidation. When hyperglyceridemic subjects were supplemented with fish oil, LDL oxidation resulted in a significant increase in propanal formation. During *in vitro* oxidation of LDL with copper, propanal formation increased significantly in subjects supplemented with fish oil (Figure 13.15). This increase correlated directly with increases in *n*–3 polyunsaturated fatty acids in LDL, but the oxidative susceptibility of LDL did not change, based on total volatile oxidation products (Table 13.8). However, other studies reported significant increases in formation of TBARS resulting from fish oil diets. Because TBARS are formed by oxidative decomposition of polyunsaturated fatty acids containing

Table 13.8. Effect of fish oil supplementation on *in vitro* LDL oxidation determined by static headspace gas chromatography [a]

Analyses	Unsupplemented	Supplemented
Volatiles (nmol/ml LDL)		
Pentane	9.6	7.5
Propanal	3.7	13.4
Pentanal	14.7	11.4
Hexanal	138	108
Total volatiles [b]	189	189
Fatty acids (%)		
n–6 PUFA	43.7	35.0
n–3 PUFA	3.2	14.6
Total PUFA	46.8	49.6
Tocopherol (nmol/ml LDL)	14.0	18.0

[a] From Frankel *et al*. (1994). LDL obtained from blood of 9 subjects after consuming fish oil each day for 6 weeks (supplemented), or after consuming control diet without fish oil (unsupplemented). LDL samples oxidized with $CuSO_4$ were analysed by static headspace gas chromatography (Frankel *et al.*, 1992). See Figure 13.15.
[b] Expressed as hexanal.

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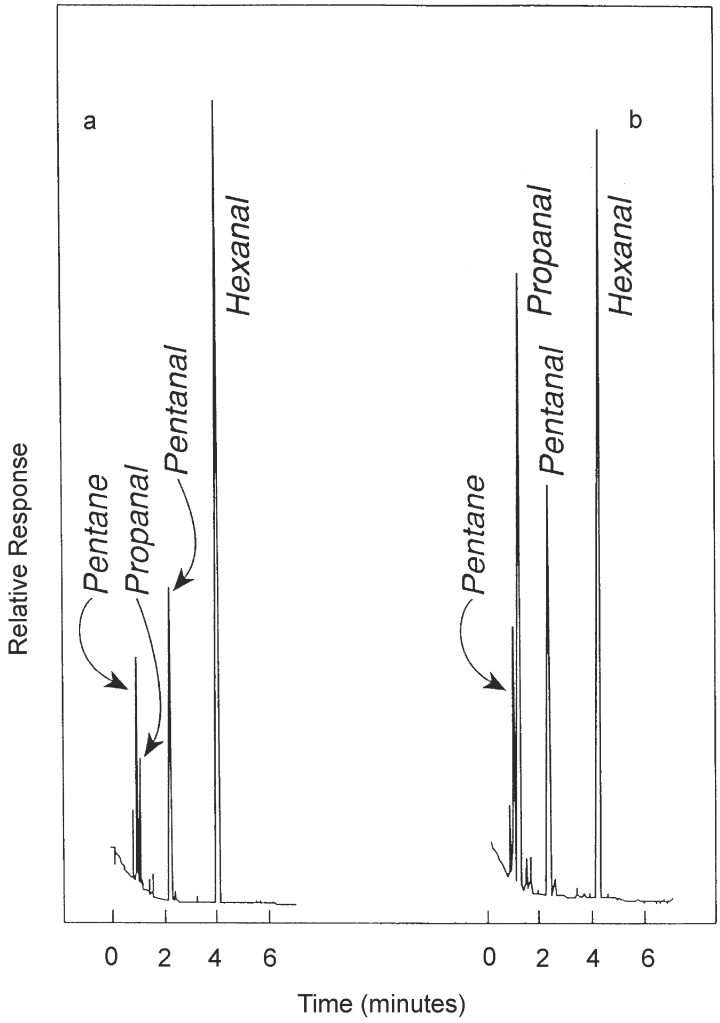


Figure 13.15. Headspace gas chromatograms of copper-oxidized low density lipoprotein isolated from a, unsupplemented subject, and b, fish oil supplemented subject. From Frankel *et al.* (1994), with permission from the AOCS Press.

more than two double bonds (Chapter 5), the oxidation of any lipids containing *n*–3 polyunsaturated fatty acids would be expected to produce high levels of TBARS. Therefore, better methodology is needed to obtain the basic chemical information necessary to understand the effects of dietary polyunsaturated fatty acids on the oxidation of LDL and other biological systems implicated in many diseases.

Dietary *n*–3 polyunsaturated fatty acids contribute additional effects on the cellular prooxidant activity of monocytes. Dietary supplementation of humans with *n*–3 polyunsaturated fatty acids increased the DHA and EPA of monocyte membrane and decreased arachidonic acid. These monocytes produce less superoxide and other reactive oxygen species. The mechanism for this effect of *n*–3 polyunsaturated fatty acids is related to the decrease in arachidonic acid and the resulting bioactive metabolites of arachidonic acid.

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Table 13.9. Reported effects of dietary *n*–3 polyunsaturated fatty acids on manifestations associated with cardiovascular disease [a]

Plasma lipids
Reduce hepatic fatty acid synthetase, triacylglycerol synthesis in liver, VLDL triacylglycerol production, ApoB synthesis
Increase tissue deposition of cholesterol, HDL cholesterol
Arachidonated metabolism
Inhibit 6-desaturase and arachidonic acid synthesis
Reduce peroxide tone
Compete with arachidonic acid for cyclooxygenase and lipoxygenase
Eicosanoids
Reduce TXA_2 synthesis (by platelets)
Convert EPA to TXA_3, a weak agonist
Form bioactive PGI_3 and LTB_5 from EPA
Decrease ratio TXA to PGI_2 and reduce platelet aggregation and vasoconstriction
Reduce LTB4 (chemotaxis and adhesion)
Platelets
Decrease arachidonic acid and increase EPA and DHA contents, TXA_2 synthesis
Decrease aggregation, adhesion and release reactions
Endothelium
Reduce PGI_2 synthesis (slightly)
Enhance PGI_3 synthesis
Monocytes and macrophages
Reduce adhesion, synthesis of LTB_4 (chemotaxis), inflammatory reactions
Other effects
Enhance bleeding tendency
Cause vasorelaxation

[a] From Kinsella *et al*. (1990). See Figures 13.10, 13.11, 13.12, 13.13.

Increased intakes of *n*–3 polyunsaturated fatty acids in the diet have been recommended because of their multitude of important biological functions and their known effects in competing with excessive linoleic acid (Table 13.9). Fish oils are presumed to reduce the risk of coronary heart disease by decreasing plasma lipids and by balancing eicosanoid metabolism. The cardiovascular system is closely regulated by a balance between the production of thromboxane (causing vasoconstriction) and prostacyclin (causing vasodilation). Although dietary α-linolenic acid is less effective than EPA and DHA in depressing eicosanoid synthesis, the more consistent consumption of linolenate-containing leafy vegetables and vegetable oils (soybean and canola oils) may be also desirable in low-fat diets. However, the great susceptibility of *n*–3

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polyunsaturated fatty acids to oxidation may constitute a potential risk factor in the development of cardiovascular disease. Studies with animals fed high amounts of menhaden or linseed oil for prolonged periods showed evidence of yellow discoloration of depot fat, referred to as yellow fat disease, which is attributed to the formation of lipofuscin by the polymerization of end-products of lipid peroxidation. This condition may also reflect a deficiency of vitamin E.

The higher requirement for vitamin E in diets containing higher levels of linoleic acid is generally satisfied by the consumption of vegetable oils rich in tocopherols. However, fish oils contain very little vitamin E (40–200 μg/g), and increased consumption of *n*–3 polyunsaturated fatty acids leads to an even higher requirement for vitamin E. Furthermore, vitamin E does not appear to provide sufficient protection to animals on diets high in *n*–3 polyunsaturated fatty acids. Practically, the production of oxidatively stable fish and fish oils is extremely difficult because of their high susceptibility to oxidation (see Chapter 11). This problem underscores the need to limit the amount of *n*–3 polyunsaturated fatty acids to the minimum required to antagonize the effects of linoleic acid, estimated at about 1% of calories. More effective methods are also needed to control the oxidation of fish oils and seafood products if their consumption is to be increased. Although the oxidation of foods enriched with fish oils is difficult to control with phenolic antioxidants, metal chelating agents such as EDTA are very effective (Chapter 9). Metal-binding proteins such as albumin and lactoferrin may also provide potentially important measures to control oxidation of fish oils.

3. Effect of monounsaturated fatty acids

Diets high in oleic acid are now recognized to be as effective as diets rich in linoleic acid in lowering serum cholesterol. However, in contrast to polyunsaturated fatty acids, monounsaturated fatty acids have the important advantages of not reducing plasma HDL concentration and of being much less susceptible to oxidation. These effects are both considered to be important risk factors. There are no apparent limits on how much monounsaturated fats can be safely used in our diet. The desirable effects of monounsaturated fats are maintained even when present in the diet at relatively high levels. Experiments with rabbits and humans fed diets rich in high-oleic sunflower oil compared to standard sunflower oil, showed that LDL was much less susceptible to copper-catalysed oxidation and underwent less degradation by macrophages after incubation with endothelial cells (Table 13.10). The increased resistance of LDL to oxidation was directly related to its increased oleate content. Several other studies showed that the linoleate content of LDL strongly correlated with the extent of oxidation and the reverse trend was observed with the oleate content of LDL. In studies with animal fed an atherogenic diet high in cholesterol, diets rich in oleic acid produced LDL more resistant to oxidation and significantly

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Table 13.10. Oxidation of LDL from animals and humans fed diets containing sunflower oil or high-oleic sunflower oil [a,b]

Feeding periods – oxidation tests	Sunflower oil	High-oleic sunflower oil
Rabbits on diets of 10% oil [a]		
6 weeks – Conj. dienes, 8 hr	0.41	0.5
10 weeks – Conj. dienes, 8 hr	0.48	0.7
6 weeks – TBARS, 8 hr	32	10
6 weeks – Macrophage degradation, 8 hr	4.5	0.8
Humans on diets containing 39.2 % fat [b]		
5 weeks – Conj. dienes, 8 hr	1.2–1.3	0.75–0.90
5 weeks – TBARS, 8 hr	43.6	37.6
5 weeks – Macrophage degradation, 8 hr	11.9	6.8

[a] From Parthasarathy *et al.* (1990).
[b] From Reaven *et al*. (1991).
Rabbits or humans fed either sunflower oil (67% 18:2, 20% 18:1) or high-oleic sunflower oil (83% 18:1, 8% 18:2). Conjugated dienes (absorbance at 234 nm) and TBARS (as nmol MDA/ml LDL protein) measured on LDL oxidized with 5 μM copper, and macrophage degradation of ^{125}I-LDL (μg/mg cell protein/5hr). MDA, malonaldehyde.

decreased fatty streak formation. Oleate-enriched diets may, therefore, reduce LDL oxidation and slow progress of atherosclerosis. Diets with lower linoleic acid and higher oleic acid contents have thus clear advantages, by lowering plasma cholesterol by the same degree as linoleic acid without lowering HDL, and at the same time decreasing the risk of LDL oxidation.

4. *Effect of* trans *unsaturated fatty acids*

Recent evidence showed that *trans* monounsaturated fatty acids are hypercholesterolemic in human studies (11% *trans* of energy intake) compared with oleic acid by raising the level of atherogenic LDL in serum and lowering the level of HDL. Further work confirmed that *trans* fatty acids from hydrogenation in margarines and shortening have similar effects to saturated fatty acids in the diet of humans (7.7% of energy), by lowering HDL cholesterol and increasing LDL cholesterol compared to oleic acid. The tendency of *trans* fatty acids to lower HDL cholesterol is in contrast to the saturated fatty acids, which tend to slightly raise HDL cholesterol. One explanation for the effect of *trans* fatty acids includes its competition for the desaturases converting linoleic acid into higher metabolites, such as arachidonic acid, which are precursors of important eicosanoids (Figure 13.11). Epidemiological studies showed no consistent association between *trans* fatty acid intake and coronary heart disease, however. In addition to the effect of *trans* fatty acids in elevating LDL cholesterol, its effect on the oxidative suscepitiblity of human LDL as another risk factor for cardiovascular disease remains to be determined. The nutritional effect of *trans* fatty acids remains controversial. In various experimental diets

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designed to maintain a constant level of total fat, the observed effect of *trans* fatty acids may be confounded by their exchange with monounsaturated or saturated fatty acids.

For a long time there was little concern that *trans* fatty acids may pose a problem for most of the population since the average consumption of *trans* fatty acids is estimated to be 3–4% of daily energy intake in the US and at the level of 2% of dietary energy common in many European countries. However, more recently under pressure from consumer advocates and nutritional authorities, the Food and Drug Administration in the US has mandated that by 2006 labels of all fat-containing foods must specify the content of *trans* fatty acids. The food industry has now reacted by marketing various "zero-*trans*"-containing fats prepared either directly by blending polyunsaturated vegetable oils with high-oleic vegetable oils, or by using new varieties of vegetable oils low in linolenic acid or high in oleic acid (see Table 12.6). For plastic fats used in margarine and shortenings, zero-*trans* products are prepared by interesterifying fully hydrogenated vegetable oils with different proportions of unhydrogenated oils to achieve the desired functionality. An important question that remains unanswered, however, is whether or not these interesterified products containing palmitate and more stearate are more nutritionally desirable than the partially hydrogenated fats containing *trans* fatty acids.

5. *Effect of antioxidants*

(a) *In vitro* versus *in vivo* studies. Many studies have tested directly the effects of antioxidants on the inhibition of atherosclerosis in animals and humans. With cholesterol-fed rabbits positive effects were reported with probucol (a dithio-butylated diphenol), butylated hydroxyanisole and diphenylenediamine. With humans, epidemiological evidence indicated that high intakes of vitamin E and quercetin (Chapter 9) correlate with reduced coronary disease. Several clinical trials with human subjects demonstrated that dietary supplementation with vitamin E produced significant enrichment of LDL vitamin E content and *ex vivo* protection of LDL against oxidation induced by copper ions, endothelial cells and smoking. The effect of vitamin C supplementation cannot be tested, because it is water-soluble and removed from LDL when isolated from plasma (Table 13.6). A large number of studies showed that natural phenolic antioxidants reduce the oxidative modification of LDL *in vitro*, and a few animal studies showed that they could inhibit experimental atherosclerosis. LDL particles from hypercholesterolemic individuals are believed to undergo more oxidant stress by prolonging their exposure to intravascular oxidation. Therefore, these individuals may require higher antioxidant intakes for protection against the damaging effects of higher circulating levels of oxidatively susceptible LDL.

Phenolic compounds may also have biological effects that are independent

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of their inhibition of LDL. Their "antioxidant" activity greatly depends on the test system, however. The effect of antioxidants on the *ex vivo* oxidizability of LDL by free metals cannot be assumed to be causally related to *in vivo* susceptibility to atherosclerosis and oxidative damage. Studies with animal models of atherosclerosis have demonstrated the antioxidant effect of vitamin E and phenolic compounds in delaying the progress of this disease. However, human clinical trials of antioxidants have given inconsistent and confusing results. Although the accumulation of circulating oxidized LDL by the artery, and the development of lesions within a few months, has been demonstrated in various animal models, human lesions develop much more slowly over periods of decades. Therefore, the results of animal testing of antioxidants do not agree with those of human studies, because they are based on significantly different end points. The same divergence may apply to studies comparing *in vitro* versus *in vivo* testing of antioxidants being based on different end points of oxidation.

Considerable epidemiological evidence has shown an association between diets rich in fruits and vegetables and decreased risk of cardiovascular disease and certain types of cancer. The protective effects of fruits and vegetables have been usually associated with the level of vitamins E and C and β-carotene. However, significant amounts of phenolic flavonoid compounds (Figure 9.10) are also present in fruits and vegetables, and their potential nutritional effect as antioxidants should not be overlooked and must also be considered for their cardioprotective actions. Similarly, flavonoid antioxidants from fruits and vegetables may be important in regulating eicosanoids. Because of the ability of flavonoids to inhibit cyclooxygenase and lipoxygenase activity, these antioxidants may exert anti-inflammatory effects (Figures 13.11, 13.12, 13.13), and may have broad metabolic and physiological effects. Our daily intake of plant flavonoids is estimated to range from 20 mg to 1 g. However, little is known about the *in vivo* antioxidant activity of flavonoid compounds in humans. The bioavailability of plant flavonoids may vary in different foods. How the food matrix and various flavonoid–nutrient interactions affect absorption and metabolism of flavonoid antioxidants, and the mechanisms by which they affect human health and disease is largely unknown.

The effects of phenolic compounds in wine on the oxidative susceptibility of human LDL were investigated *in vitro* by measuring the amounts of hexanal and conjugated dienes formed by Cu^{2+} catalysed-oxidation of human LDL. The addition of diluted de-alcoholized red wine containing 3.8 and 10 μM phenolic compounds to LDL inhibited 60 and 98% hexanal formation (Figure 13.16). The relative inhibition of LDL oxidation varied from 46 to 100% with red wines compared to 3 to 6% with white wines. The antioxidant activity of wines toward LDL oxidation was distributed widely among the principal phenolic compounds, including gallic acid, catechin, myricetin, quercetin, caffeic acid, rutin, epicatechin, cyanidin and malvidin-3-glucoside (see Chapter 9). The

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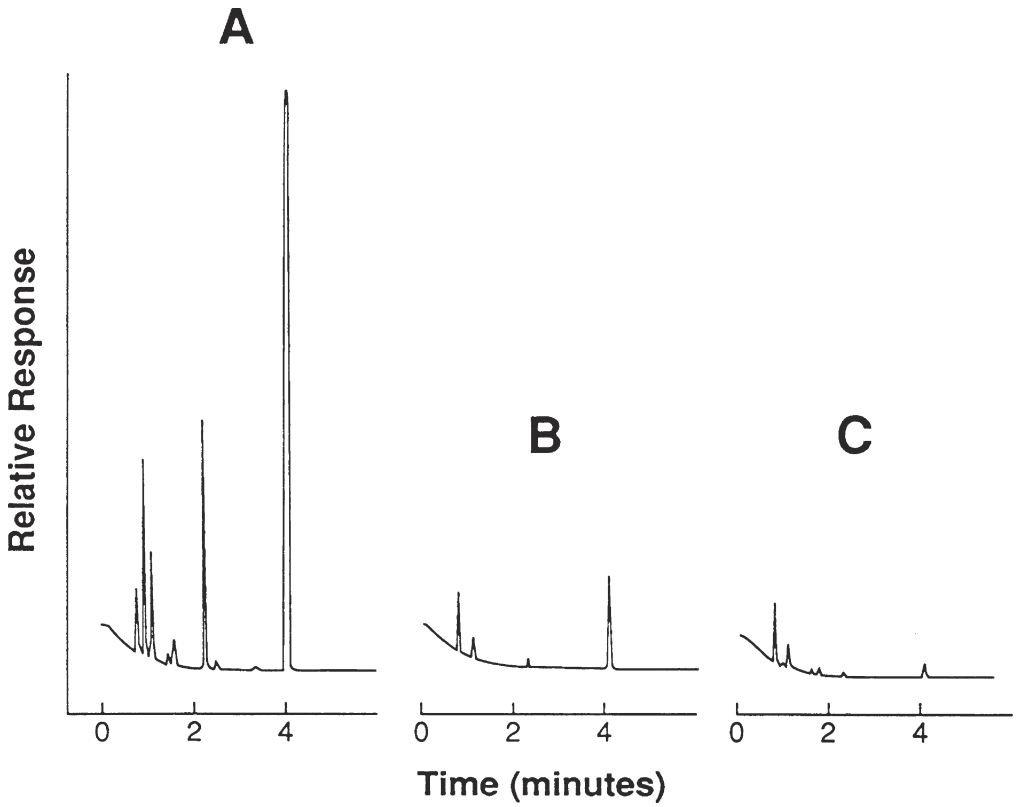


Figure 13.16. Headspace gas chromatograms of samples of oxidized human low density lipoprotein. A, control LDL, B, plus 3.8 μM wine phenolics, C, plus 10 μM wine phenolics. From Frankel *et al.* (1993), with permission from The Lancet Ltd.

antioxidant activities of extracts of different table and wine grapes in human LDL *in vitro* were comparable to those for wines. The extracts of various grape varieties showed different trends in antioxidant activity between the liposome and LDL systems. Rosemary extracts and components, tea catechins and commercial green teas had different antioxidant activities when compared in different lipid systems (bulk oil, lecithin liposomes, and human LDL). Therefore, the relative activity of natural phenolic antioxidants is greatly affected by the test system used and the biological target to be protected.

Although much evidence has been published to show that wine polyphenols have potent antioxidant activity by inhibiting LDL lipid oxidation *in vitro*, the important question of whether or not they can also inhibit LDL oxidation *in vivo* has been difficult to answer. Many attempts were based on measurements of oxidative susceptibility of human LDL isolated after consumption of red wine. Measurements of *ex vivo* LDL susceptibility to oxidation are confounded, however, because the phenolic acids and hydrophilic polyphenolic compounds of wine are water-soluble and lost by the standard methods used to isolate LDL, which remove soluble plasma constituents.

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In addition to preventing LDL oxidation, phenolic flavonoids are known to inhibit phospholipase, cyclooxygenase and lipoxygenase, which play a key role in the arachidonate cascade (Figure 13.12). By down-regulating cyclooxygenase and lipoxygenase, flavonoids and other phenolic compounds in fruits and vegetables may reduce thrombotic tendencies and inflammatory reactions in the body. By preventing or reducing monocytes from collecting in vessel walls, these phenolic compounds interfere with the immune response and with platelet aggregation involved in the blood clotting process and prevent thrombosis. However, the great difficulty of conducting *in vivo* experimental work to demonstrate the activity of plant antioxidants has added to the uncertainty of their nutritional benefits.

(b) Absorption studies. Catechin is a major flavonoid in grapes and wines and is considered responsible for part of the protective effect of red wine against atherosclerosis. The metabolism of catechin after consumption of red wine and the effect of alcohol was investigated by analysing metabolites in human plasma (Table 13.11). After consumption of one serving of red wine and de-alcoholized red wine, catechin was found to be present in plasma almost exclusively as methylated, glucuronide and sulfate conjugate metabolites. Less than 2% free catechin was detected one hour after consumption of both wine and de-alcoholized wine, and no free catechin after 3–4 hours. Maximum levels of total catechin (91 and 81 nmol/l for red wine and de-alcoholized red wine, respectively) were reached 1.5 hr after consumption, and varied widely among individuals. Ethanol did not significantly alter the distribution of the various catechin metabolites. After consumption of one serving of red wine, the small levels of flavonoid were rapidly absorbed (half-life of absorption of 0.7–0.8 hours) in plasma and excreted. Catechin in grapes and wine is therefore absorbed to varying degrees, but rapidly metabolized and conjugated. The results suggest that daily consumption of red wine would be required to sustain useful levels in plasma. In another study, after consumption of apples, the half-life of elimination ($E_{½}$) for quercetin was found to be significantly larger ($E_{½} = 23$) than for catechin after consumption of red wine ($E_{½} = 3.1$).

Human absorption studies also showed that glucuronide and sulfate metabolites were found in plasma after the consumption of green tea rich in flavan-3-ols and chocolate rich in epicatechin.

(c) Methodology. The results of various methods used to measure the effectiveness of dietary antioxidants *ex vivo* or *in vivo* do not always follow predictable patterns. Antioxidants have been commonly evaluated by determining their relative concentrations or the so-called "antioxidant status" in blood or other tissues after dietary supplementation. These analyses are confounded by variations in the relative stability of antioxidants and by the up-regulation of a wide array of antioxidant enzymes and scavenging systems in response to oxidative stress (Section B). The effectiveness of antioxidant

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Table 13.11. Pharmacokinetic values for catechin metabolites in red wine and quercetin in apples [a]

Parameters	Total catechin [b]		Quercetin [c]
	Red wine – ethanol	Red wine + ethanol	Apples
Dose (mg)	35	35	98
$E_{½}$ (h)	3.8	3.1	23
$A_{½}$ (h)	0.7	0.8	–
T_{max} (h)	1.5	1.5	2.5
C_{max} (nmol/l)	81	91	300

[a] From German and Walzem (2000). $E_{½}$ = half-life elimination, $A_{½}$ = half-life of absorption, T_{max} = time at maximum concentration, C_{max} = maximum concentration.
[b] From Donovan *et al.* (1999). Five male and four female volunteers consumed 120 ml wine containing 35 mg catechin. Analyses of plasma were carried out by GC-MS after silylation and selective ion monitoring. Total catechin included methylated, unmethylated (catechin and unmethylated metabolites), and sulfated metabolites.
[c] From Hollman *et al.* (1997).

intake may be more usefully evaluated by specific analyses of products formed at different stages of lipid oxidation than by the determination of their relative concentrations. Antioxidants have also been evaluated on the basis of their reactivity toward peroxyl radicals and their inhibition of hydroperoxide formation. However, their effects in scavenging alkoxyl radicals and in inhibiting aldehyde formation have been overlooked. Yet, the production of aldehydes is likely to be more relevant to oxidative damage due to their interaction with the apoB of LDL (Figures 13.5 and 13.6).

Several recent studies have produced mixed results in indicating *in vivo* antioxidant activity of phenolic compounds in red wine. However, the results of these studies are unconvincing because non-specific assays were used for antioxidant action that may be inadequate due to the confounding and indirect effects of many serum and plasma components. The effect of *in vivo* supplementation of polyphenols in red wine on the oxidizability of LDL cannot be tested *ex vivo* because, like vitamin C, these hydrophilic compounds are removed from LDL during isolation from plasma (Table 13.6). Not surprisingly red wine consumption showed no effect on LDL oxidation *ex vivo*.

Several non-specific assays known as "radical trap methods" have been used to test the "antioxidant capacity" of biological tissues and fluids by measuring the relative reactivity of antioxidants toward artificial nonphysiological radicals as chromogenic indicators. The relative antioxidant activity of many phenolic compounds varies widely according to different testing methods. There is no agreement between relative activity of various phenolic compounds on the inhibition of copper-induced human LDL oxidation *in vitro* compared to the total antioxidant capacity measured by the TEAC and ORAC assays (Table 13.12). The ranking of antioxidant activity is thus highly dependent on the test

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Table 13.12. Relative antioxidant activity of selected phenolic compounds by different methods [a]

Phenolic compounds (5 μM GA equivalent)	Inhibition (%) LDL oxidation [b]	TEAC (mM Trolox) [c]	ORAC (μM Trolox) [d]
Catechin	74.9	2.40	2.49
Myricetin	68.1	3.12	–
Epicatechin	67.6	2.50	2.36
Rutin	67.6	2.42	0.56
Gallic acid	63.3	3.01	1.74
Quercetin	61.4	4.72	3.29
α-Tocopherol	32.6	0.97	–

[a] From Frankel and Meyer (2000). TEAC, Trolox equivalent antioxidant capacity; ORAC, oxygen radical absorbance capacity; GA, gallic acid.
[b] Copper-catalysed oxidation of human LDL monitored by hexanal determination by headspace gas chromatography.
[c] TEAC assay uses $ABTS^{\bullet+}$ chromogen radical absorption decay at 734 nm, H_2O_2 and metmyoglobin as inducer, and Trolox as reference.
[d] ORAC assay uses β-phycoerythrin fluorescence decay, AAPH [2,2'-zobis(2-amidinopropane) dihydrochloride] as inducer and Trolox as reference.

system and on the substrate to be protected by the antioxidants. The discrepancy in ranking of antioxidants can be explained not only by the multiplicity of mechanisms effective for these phenolic compounds, but also by the effect of interfacial properties of multiphase LDL particles compared to the aqueous test systems used for the radical trap assays (see Chapter 10).

The total antioxidant activity of polyphenols of grapes, wines and green teas was thus evaluated by determining their scavenging ability toward various artificial radical systems. However, these approaches for testing natural antioxidants by artificial radical model systems provide no information on which lipid or protein or biological targets are protected by the antioxidants. Various flavonoids showed widely different antioxidant activities and even prooxidant activities according to their abilities to scavenge hydroxyl or superoxide radicals or lipid peroxidation. Because the properties of various phenolic compounds are very system-dependent, caution is required in interpreting the antioxidant activities determined with non-specific assays in the absence of suitable substrate targets. The validity of the antioxidant capacity or radical trap methods may be questionable without product analyses to indicate which specific biological substrate is protected.

To determine the real effects of natural antioxidants, it is important to obtain specific chemical information about which products of lipid peroxidation are inhibited. Several specific assays are needed to elucidate how lipid oxidation products act in the complex multi-step process of lipid peroxidation (Figure 13.6) and oxidative damage in biological tissues. The results of many complementary methods are required to determine oxidation products formed at

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different stages of the free radical chain. Since antioxidants show different activities toward hydroperoxide formation and decomposition, it is important that more than one method be used to monitor the oxidation process.

6. *Effect of lipid oxidation products*

Dietary lipid hydroperoxides are reported to be absorbed in humans and cause atherosclerosis in animals. They are known to be cytotoxic and cause various adverse effects to blood circulation and inflammation. Many studies supported that ingestion of a fat-containing meal contributes to the incidence of atherosclerosis, and that this link may be attributed to oxidized lipid in postprandial chylomicrons impairing arterial endothelial functions. Any significant dietary lipid oxidation would therefore be expected to greatly diminish the effectiveness of dietary antioxidants, especially if administered together with highly oxidizable polyunsaturated fats. Postprandial impairment of endothelial vasoactivity was prevented by the administration of vitamins C and E or the presence of antioxidant-rich foods in meals. The confounding effect of oxidized lipids may also account for the negative effects found in many epidemiological studies of dietary antioxidants, because giving antioxidant supplements will not overcome problems of lipid oxidation products. If the levels of hydroperoxides and aldehyde decomposition products were sufficiently elevated to cause foods to become rancid, consumers would reject them. However, many non-volatile polar secondary lipid oxidation products occurring in fried and thermally abused foods may not be detected readily by taste, and may cause adverse biological effects if absorbed by humans. The health implications for human nutrition of polar oxidation products in deep-fat fried foods (see Chapter 12) have concerned nutritionists for many decades. These potentially serious dietary problems due to lipid oxidation are not yet clear, however, and deserve much more research.

In a typical Western diet, the intake of lipid hydroperoxides has been estimated as 1.5 mmol per day. Diets that may be rich in lipid oxidation products may escape reduction and degradation when crossing the intestinal border and initiate further LDL oxidation in plasma. This hypothesis is supported by evidence that consumption of red wine antioxidants reduces the oxidative susceptibility of human plasma and LDL after consuming a fatty meal. A meal taken with red wine increased plasma concentration of vitamin E, sulfhydryl groups, uric acid and total plasma antioxidant capacity, but decreased total ascorbic acid (Table 13.13). Postprandial LDL after the wine-meal was more resistant to oxidation than that after the ethanol-meal. Red wine showed a sparing effect on vitamin E, but not on vitamin C. Wine polyphenolic compounds and other dietary antioxidants may therefore attenuate the risk of coronary heart disease by reducing the harmful pro-oxidant cytotoxic effects of hydroperoxides in fat-rich foods.

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Table 13.13. Effect of red wine consumed with a high-fat fried meal on levels of plasma antioxidants and antioxidant capacity [a]

Plasma components and antioxidant capacity	Red wine		Ethanol	
	Baseline	3 hrs	Baseline	3 hrs
Total ascorbic acid, μM	63	52	69	55
α-Tocopherol, μM	21	24	21	22
SH groups, μM	279	302	271	266
Uric acid, μM	291	357	292	321
Antioxidant capacity, mmoles/l plasma	1.1	1.3	1.2	1.1

[a] From Natella *et al.* (2001). Six men ate a test meal of meat and fried potatoes with either 400 ml red wine or with an isocaloric alcohol solution. Antioxidant levels and capacity were measured 3 h after the meal. Antioxidant capacity was based on the TRAP method (Chapter 9).

Although the resistance of postprandial LDL to copper oxidation appears to be unaffected by a meal rich in lipid oxidation products, several water-soluble antioxidants and low molecular weight proteins present in the subendothelial space cannot be properly evaluated by oxidation of isolated LDL *ex vivo* (Table 13.6). To overcome this problem, the copper-catalysed oxidation of dilute serum was studied to relate postprandial lipemia and atheroschlorosis (Table 13.14). On one hand, consuming meals rich in heated olive oils caused a more significant decrease in lag phase in conjugated diene formation (–12%) after four hours than the unheated oil. On the other hand, the same decrease (–11%) was observed with both heated and unheated safflower oil. Total conjugated diene formation increased significantly during postprandial lipemia after all meals, and the increase was significantly greater with safflower than with olive oil. Therefore, the oxidative susceptibility of lipoproteins may be increased in the postprandial period following meals rich in the polyunsaturated safflower oil whether they were heated or not, but not during meals rich in olive oil. These results support the use of olive oil in preference to polyunsaturated vegetable oils in diets aimed at reducing risk of cardiovascular disease. However, to better understand the nutritional effects of thermally oxidized fats, much more detailed information is required on the specific lipid oxidation products responsible for their effect during postprandial lipemia.

7. Effect of iron supplementation

Healthy subjects are well protected against oxidative damage from free iron by the strong metal binding properties of plasma proteins (Section B). However, metal ions may become available to promote oxidation as a result of tissue injury from bacterial infections, inflammations and human disease. Oxidative stress by tissue injury in unhealthy subjects can release enough free iron ions to promote the generation of active oxygen species causing tissue damage. Lowering the amount of iron storage *in vivo* may, therefore, decrease injury due

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Table 13.14. Baseline and postprandial changes in parameters during oxidation of dilute serum after consuming unheated and heat oil meals [a]

Parameters	Oil in meal	Olive oil		Safflower oil	
		Baseline	change (%)	Baseline	change (%)
Lag time (min)	Unheated	42	–5	46	–11
	Heated	59	–12	60	–12
Conjugated dienes (nmol)	Unheated	20	+9	20	+25
	Heated	20	+8	20	+21

[a] From Sutherland *et al.* (2002). Olive oil (67.3% 18:1, 12.4% 18:2) and safflower oil (12.7% 18:1, 77.6% 18:2) were heated in a deep-fryer for 8 hr at 210°C, with potato chips added after every hr. The heated and unheated oils were used in ice-cream milk shakes and fed to 16 healthy men. The serum separated from venous blood was diluted (0.67%) in phosphate–saline buffer, oxidized with copper (12.5 μmol/l) at 25°C and the formation of conjugated dienes was followed for 4 hr at 234 nm.

to oxygen radicals. In excess, dietary iron and iron supplementation may contribute to oxidative damage and may overload the defense system. The interaction of iron with superoxide, hydrogen peroxide and lipid hydroperoxides to produce more reactive oxygen species is now recognized as one of the most serious sources of free radical injury.

A major form of antioxidant defense in human plasma is the prevention of iron and other transition metal ions from promoting the generation of reactive oxygen species. Although many therapeutic measures to inactivate metals by chelation therapy have been advocated, the avoidance of iron supplementation in the diet may provide a simpler and more economical approach to this potential health problem. The current practice of iron supplementation of flour and cereal products may be imprudent, in view of the possible risk of excessive iron in generating oxidative stress in certain segments of the adult and older population. Iron deficiency was even advocated as a more effective and practical antioxidant treatment than supplementation with either synthetic or natural antioxidants.

G. Optimum dietary intakes

Nutritional advice on optimum fat intakes has been generally based on epidemiological, clinical and animal studies showing an association between dietary fat, serum cholesterol levels and coronary heart disease. These studies have concluded that an elevated level of serum cholesterol is a major risk factor for a heart attack. Keys *et al.* and Hegsted *et al.* (1965) have formulated equations based on a large number of human feeding trials indicating that saturated fatty acids raise serum cholesterol twice as much as polyunsaturated fatty acids decrease it, and that dietary cholesterol has a less important effect in raising serum cholesterol. These simplified relationships have been based on the assumption that the ratio of polyunsaturated to saturated fatty acids (p/s ratio)

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is fundamental to the control of coronary heart disease, by reducing elevatedserum cholesterol. This assumption has led to the unfortunate tendency in many Western countries to markedly increase the polyunsaturated fats in the diet.

We have seen in Section F that the dietary factors linked with the incidence of coronary heart disease reflect complex relations that cannot be simply based on ratios of polyunsaturated to saturated fats. It is now evident that not all saturated, polyunsaturated and monounsaturated fats play the same role in nutrition. *n*–3 Polyunsaturated fatty acids are not particularly effective in lowering serum cholesterol, but markedly reduce serum triacylglycerols in hypertriglyceridic subjects. The recent tendency has been to make nutritional recommendations on the basis of total fat intake and on the ratio of *n*–6 to *n*–3 polyunsaturated fatty acids. Reducing *n*–6 polyunsaturated fatty acids may be an effective way of utilizing relatively low and more practical amounts of fish oils.

Estimates of current fat intakes are considered to have high contents of saturated and PUFA and a high *n*–6 to *n*–3 PUFA ratio (Table 13.15). Recommendations have been made to reduce total fat, saturated fats, and decrease the *n*–6 to *n*–3 PUFA ratio from 9/1 to 3/1. More recently, dietary guidelines have been made from various international task forces on the basis of both epidemiological and intervention studies in humans (Table 13.16). They recommend consuming:

a) Less than 10% of total energy of saturated and PUFA by increasing the intake of monounsaturated fatty acids;
b) Small amounts of *n*–3 PUFA either as 18:3 from plant oils (soybean and canola oils) or as long-chain EPA and DHA (fish oils); and
c) Restricting as much as possible *trans* fatty acids derived primarily from partially hydrogenated fats.

These dietary recommendations reflect much guesswork, however. Dietary recommendations by committees of "experts" have been criticized for being biased towards literature citations that are supportive of the 'lipid hypothesis,' and overlooking those that are not supportive. Recommendations of fat modification to prevent cardiovascular disease are based on weak scientific evidence selected mainly by politicized public health committees.

Although diets rich in *n*–3 PUFA from fatty fish markedly reduce the blood triglyceride level of people with high blood triglycerides, they do not affect people with normal levels of blood triglycerides. However, unlike the *n*–6 PUFA, the effect of *n*–3 PUFA on blood cholesterol and HDL cholesterol is inconsistent. There is also a wide gap between these dietary recommendations and the typical diets in North American and European countries (Table 13.16). These recommendations are consistent with the so called Mediterranean diet that includes olive oil (containing up to 80% oleic acid) as the principal

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Table 13.15. Human consumption of dietary fats (percent calories)

Fats	Current intakes [a]	Recommended intakes				
		NATO[a]	Kinsella[b]	Dupin *et al.* [c]	COMA [d]	SCF [e]
Saturated	15	6–7	8	–	10	–
Monounsaturated	18	12–14	14	–	–	–
Polyunsaturated	7	6–7	8	–	–	max 15
n–6	7	4.8	6	3–6	no more than 10%	2 (min 0.5)
n–3	0.8	1.3 [f]	2	0.5–1	0.2 g/day	0.5 (0.1–5)
n–6/*n*–3	9	4	3	4–10	–	–
Total	40	24–28	30	–	35	–

[a] Galli and Simopoulos (1988).
[b] Kinsella (1991).
[c] Dupin *et al*. (1992). Centre national d'études et de recommandations sur la nutrition et l'alimentation (CNERNA) (France) (1992).
[d] Department of Health, UK (1994). Values expressed as % of energy. They also recommended no more than 2% *trans*.
[e] Scientific Committee for Food (SCF) (European Union) (1993).
[f] Includes 1.0 % 18:3, 0.27% EPA +DHA, and 18-3 *n*–3 / *n*–3 as EPA + DHA = 4/1.

Table 13.16. Optimal nutrient composition recommended by different guidelines [a]

Composition (% total energy intake)	ITF (1998)	GNS (2000)	USN (2001)
Carbohydrates	>50	>50	50–60
Total fat	≤30	30	25–35
Saturated FA	7–10	<10	<7
Monounsaturated FA	10–15	>13	≤20
Polyunsaturated FA	7–8	7	≤10
Trans FA	–	<1	As low as possible

[a] From Kratz *et al.* (2002). FA = fatty acids.
ITF: International Task Force for the Prevention of Coronary Heart Disease (1998);
GNS: German Nutritional Society (2000);
USN: US National Cholesterol Education Program Expert Panel (2001).

source of fat, small amount of meat and dairy products containing saturated fats, and high amounts of vegetables, legumes and fruits rich in natural antioxidants.

The traditional p/s ratio and now the *n*–6/*n*–3 PUFA ratio may be of little value because they fail to recognize the multiple effects of dietary PUFA and their metabolic interrelationships. In a multivariate system, attempts to describe a ratio of two variables have little meaning and are an inappropriate description of a mixture of fatty acids having a multiplicity of biosynthetic effects. These values should therefore be considered only as possible targets. It should be no longer valid to base dietary recommendations on the attainment of plasma

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polyunsaturated lipid concentrations, in view of the great multiplicity of functions mediated by eicosanoid metabolites.

The unduly increased level of dietary linoleate may be excessive in promoting a variety of chronic diseases including coronary artery disease by the excessive release of eicosanoids in blood and by increasing the oxidative susceptibility of *n*–6 polyunsaturated fatty acids in lipid components of LDL. *n*–3 PUFA have desirable anti-aggregatory and anti-inflammatory functions by inhibiting eicosanoid synthesis. On this basis, an increased intake of *n*–3 PUFA has been advocated. However, the levels necessary for these beneficial effects of *n*–3 PUFA are not well defined. Levels of total *n*–3 PUFA above 1% of calories in the diet may become a risk factor by increasing the oxidative susceptibility of LDL and other lipoproteins and oxidative stress. Any recommendations to increase the levels of *n*–3 PUFA in our diets should be moderated by the serious hazards of their greater susceptibility to oxidation affecting the stability of foods as well as the oxidative modification of lipoproteins in blood. One important question that concerns nutritionists today is whether we need additional or more effective natural antioxidants in our diet, to reduce oxidative stress from either excessive dietary polyunsaturated lipids or from other environmental factors. Although intervention studies have shown that the use of antioxidants do not reduce the risk of cardiovascular disease, there is a general consensus based mainly on epidemiological studies that we should increase the use of vegetable, fruit and whole grains (rich in antioxidants).

H. Conclusions and future perspectives

Knowledge of the effects of dietary fatty acids in the development of coronary artery disease is still incomplete. Atherosclerotic lesions develop slowly by a complex series of cellular reactions in which lipid peroxidation and various forms of oxidative stress may play an important part at each stage of lesion progression. It is now firmly established that coronary artery disease is reduced by diets that lower LDL cholesterol by replacing saturated fatty acids with polyunsaturated fatty acids. However, Western populations have tended to consume high amounts of polyunsaturated fats that are susceptible to oxidation. Excessive levels of polyunsaturated fatty acids in the diet, without providing sufficient protection from antioxidants and other inhibitors, can promote LDL oxidation and upset the oxidant–antioxidant balance (Section C) in favor of cellular prooxidants and promote coronary heart disease. On the other hand, diets rich in oleic acid including the Mediterranean diet have clear advantages in not only lowering serum cholesterol but also in decreasing the oxidative susceptibility of LDL.

The prevention of LDL oxidation by antioxidants has been viewed as an important potential means of decreasing the risk of heart disease. Plant phenolic antioxidants in fruits and beverages (wine, grapes, grape juice and tea)

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may have a protective effect against coronary heart disease and cancer, but the molecular mechanism of protection is not understood. There is now intensive interest in developing plant sources of polyphenolic antioxidants for foods and biological systems, and for their possible health benefits. There is, however, a significant gap in our knowledge on the relative absorption and in the activities of these natural antioxidants in the body and their bioavailability, and a lack of reliable biomarkers to test their activity *in vivo*. Although recent evidence indicates that small amounts of flavonoid antioxidants are absorbed as various metabolites in the blood of humans, their activities *in vivo* are unknown.

Polyunsaturated fatty acids from fish (EPA and DHA) may be useful to alleviate inflammation, reduce blood pressure and thrombosis. The recommended ratio of *n*–6 to *n*–3 PUFA in the diet is estimated to be around 3:1. Essential fatty acid requirements are less than 1% of calories. Therefore, current consumption in Western countries of more than 8 to 10% polyunsaturated fatty acids may be excessive and conducive to increased susceptibility to oxidative stress. Although the possible cardiovascular benefits of increased intake of vitamin E and other natural plants antioxidants are not yet well established, there is little doubt that increased consumption of monounsaturated fats may be highly recommended. Several population studies suggested that diets enriched in oleic acid and *n*–3 polyunsaturated fatty acids decrease coronary artery disease. The nutritional benefits from the Mediterranean diet are generally attributed to olive oil rich in oleic acid, α-linolenic acid and natural antioxidants from fruits and vegetables. However, the minimum requirements for the *n*–3 as well as the *n*–6 polyunsaturated fatty acids need to be more closely defined, because these fatty acids are highly susceptible to oxidation, especially during cooking and frying (see Chapter 12). Diets rich in EPA and DHA from fish should, therefore, be used prudently and in moderate amounts. An adequate supply of antioxidants from fruits and vegetables is also necessary to prevent peroxidation of polyunsaturated lipids.

There are many unsettled questions and in future research we need to:

(a) Define the role of lipid peroxidation in atherosclerosis and autoimmune disorders more precisely by using more useful and reliable *in vivo* markers;
(b) Define minimum or optimum requirements for *n*–3/*n*–6 PUFA without compromising their beneficial effects by the harmful effects of their oxidation;
(c) Reduce oxidation products in foods and establish how much oxidized fats ingested in our diet may be harmful;
(d) Optimize antioxidants from fruits and vegetables and determine if they play a significant role in preventing LDL and other biological oxidations *in vivo*, and their mechanism of protection and disease intervention (in

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the absence of possible confounding effects from oxidized dietary lipids);

(e) Re-define the requirements of iron supplementation, which may be excessive for some segments of the adult population especially among the older segment;

(f) Consider the oxidative susceptibility of polyunsaturated fats in the diet as an important risk factor;

(g) Establish if dietary antioxidants may play a role in preventing the damaging effects of oxidized fats in the diet;

(h) Determine what specific lipid oxidation products in polyunsaturated fats used for frying can affect postprandial lipema to injure endothelial cells and stimulate inflammation causing cardiovascular disease.

The dietary intake of polyunsaturated fats and minerals is clearly *not* a simple problem. Dietary recommendations for polyunsaturated fats and especially the highly oxidizable *n*–3 polyunsaturated lipids must consider their susceptibility to oxidation as a significant risk factor. When the interrelationships between the metabolic effects of different polyunsaturated fatty acids are better understood, an appropriate target may be to achieve a balanced eicosanoid metabolism. It would seem imprudent to make dietary recommendations to the public before the mechanisms of polyunsaturated lipid nutrition, *in vivo* activity of antioxidants, and *in vivo* lipid peroxidation are better understood.

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Glossary

Agonist	Compound which acts on a receptor to elicit a response
Aggregation	Process of forming adhesions between particles such as cells
Antigens	Foreign substance producing immune reaction or response
Atherogenesis	A long series of events that leads to development of atherosclerosis
Atherosclerosis	Condition of decreased blood flow in vessels in which the inner space has been diminished by thickening of the vessel wall by deposition of cholesterol and fat
Autoimmune	Immune to self antigens
Cardiovascular	Refers to the heart (cardio) and blood vessels (vascular) and the system for distributing blood, oxygen and nutrients to various tissues
Chemotactic	Chemical agents able to attract or induce cells
Choloroplasts	Plastid (photosynthetic cells) containing chlorophyll (site of photosynthesis)
Chylomicrons	Microscopic particles of fat occurring in blood
Chloroplast	Organelles of photosynthesis
Core aldehydes	(Also known as aldehydo glycerides, or 2½ glycerides): aldehydes attached to a glyceride or phospholipid
Cytochrome	Respirator enzyme that undergoes reduction and oxidation
Cyclooxygenase	Enzyme catalysing the formation of prostaglandins from arachidonic acid
Cytoplasm	Cell substance without the nucleus
Cytotoxic	Toxic to cells

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DNA	Deoxyribonucleic acid
Dysfunction	Impairment or abnormality in the function of an organ
Eicosanoids	Oxygenated derivatives of arachidonic acid
Endocytosis	Uptake of material into a cell by the formation of a membrane-bound vesicle
Endothelium	Layer of flat cells lining blood vessels
Epitope	Group recognized by an antibody formed in presence of foreign substance
Erythrocytes	Red blood cells
Essential fatty acids	Linoleic and linolenic acids cannot be biosynthesized by animals and must be obtained from plant sources
ex vivo	Outside the living body
Ferritin	Storage protein that bind iron in blood
Hemoglobin	Red respiratory protein of erythrocytes
Glutathione	Tripeptide of glutamic acid, cysteine, and glycine acting as a strong hydrogen donor
Hepatic	Acting on or in the liver
Hypercholesterolemia	Condition of elevated levels of serum cholesterol
Hyperlipidemia	Condition of elevated levels of serum low-density lipoprotein and triacylglcerols
Hypertriglyceridic	Condition of elevated levels of blood triglycerides
Hypoxanthine	A purine (precursor of uric acid) present in muscles and other tissues intermediate in the formation of uric acid
Immune	Defensive network of protective response against a pathogenic agent or antigen
Immune suppression	Reduction of immune response
Internalized	Taken into the cell or a specific organelle
Intima	Innermost layer of blood vessels or arteries
in vitro	In a test tube or artificial environment
in vivo	Within the living body
Ischemia	Localized tissue anemia due to obstruction of arterial blood
Leucocyte	White or colorless nucleated blood cell
Leucotriene	Eicosanoid formed by the action of lipoxygenase on arachidonic acid (Figure 13.11)
Lipemia	Presence of abnormal amount of lipid in circulating blood
Lipofuscin	Brown pigment granules lipid-containing residues of lysosomal digestion
Lipoprotein	Complex of lipid and protein separated by ultracentrifugation
Lipoxygenases	Enzymes catalysing the stereoselective formation of

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	hydroperoxides from polyunsaturated fatty acids
Lymphocyte	White blood cell, mediator of specific immunity
Lysosome	Cytoplasmic particle containing hydrolysing enzymes
Macrophages	Large mononuclear phagocytes (large scavenger cells that ingest dead tissue)
Microphages	Small leucocytes which ingest chiefly bacteria
Microsome	One of the granules in protoplasm with minute cellular structure
Mitochondria	Cellular organelles found outside nucleus
Monocyte	Large mononuclear leucocyte in circulating blood
Myeloperoxidase	A peroxidase occurring in leucocytes
Myocardial infarction	Condition of regional death of heart muscle due to impaired blood infarction flow
Neutrophil	Finely granular white cell (leucocyte) that is the chief phagocyte of blood
Organelle	A structurally discrete component of a cell
Oxyhemoglobin	Hemoglobin in combination with oxygen
Peroxide tone	Peroxide level required to activate or inhibit cyclooxygenase (Figure 13.14)
Patho-physiological	Causing deranged function seen in disease
Phagocyte	Cell that ingests bacteria, foreign particles, other cells and consumes debris
Phagocytosis	Ingestion and digestion of cells
Plasma	Fluid portion of blood in which blood cells are suspended
Platelets	Small disk particles irregular in shape that adhere to blood vessel walls
Postprandial	Occuring after a meal
Prostacyclin	Important antithrombotic prostaglandin produced by endothelial cells of blood vessels (Figure 13.11)
Prostaglandin	Oxygenated derivative of arachidonic acid that contains a five-membered ring (Figure 13.11)
Prostanoids	Metabolites of C_{20} acids, especially arachidonic acid, including prostaglandins, leukotrienes and thromboxanes
Rheumatoid	Inflammation on a joint
Serum	Fluid portion of blood remaining after coagulation
Singlet oxygen	Oxygen activated by electronic excitation to the singlet state (Chapter 3.B)
Spermatozoa	Male sexual cells
Superoxide	Reactive oxygen species produced through cellular transport chain of mitochondria
Superoxide dismutase	Enzyme catalysing the dismutation of superoxide into hydrogen peroxide

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Thrombi	Clot of blood formed within blood vessel remaining attached to place of origin
Thrombosis	Formation of blood clot of platelets and blood coagulation proteins within a blood vessel that restricts the flow of blood
Thromboxane	Prostaglandin intermediate causing platelet aggregation and contraction of muscles (Figure 13.11)
Transferrin	Transport protein that binds iron in blood
Vasoactive	Condition in which blood vessels respond to stimuli
Vasodilator	Dilates blood vessels causing decrease in blood pressure
Vasoconstrictor	Constricts blood vessels causing increase in blood pressure
Vesicle	A closed membrane shell, derived either physiologically (budding) or mechanically (by sonication)
Xanthine	2,6-dioxopurine, occurring in many organs and urine
Xanthine oxidase	Enzyme that synthesizes uric acid from xanthine

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Abbreviations

AAPH/ABAP	2,2' azobis-(2 amidino-propane) dihydrochloride
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AIBN	α,α-azobisisobutyronitrile
AMVN	2,2'-azobis(2,4-dimethylvaleronitrile)
ANOVA	analysis of variance
APCI-MS	atmospheric pressure chemical ionization-MS
AOM	active oxygen method
AV	anisidine value
ApoB	apolipoprotein B
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
CD	conjugated dienes
CI	chemical ionization
CL	chemiluminescence
CID	collision-induced dissociation
CI-MS	chemical ionization-MS
CIS-MS	coordination-ion spray MS
CLA	conjugated linoleic acid
COX	cyclooxygenase
DG	diglycerides
DHA	docosahexaenoic acid
DLPC	dilinoleoyl-phosphatidylcholine
DLPG	dilinoleoyl-phosphatidylglycerol
DMVN	2,2' azobis (2,4 dimethylvaleronitrile)
DNA	deoxyribonucleic acid
DNPH	2,4-dinitrophenylhydrazine
DNUA	distilled non-urea adducts
DSC	differential scanning calorimetry
DPPP	diphenyl-1-pyrenylphosphine
EDTA	ethylenediamine tetraacetic acid
EI	electron-impact ionization
ELS	evaporative light-scattering
EPA	eicosapentaenoic acid
ESI-MS	electrospray ionization-mass spectrometry
EPR	electron paramagnetic resonance

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ESR	electron spin resonance
FDA	Food and Drug Administration
FFA	free fatty acids
GC	gas chromatography
GC-EI-MS	gas chromatography-electron ionization-mass spectrometry
GC-MS	gas chromatography-mass spectrometry
GC-O	gas chromatography-olfactometry
GC-SP	gas chromatography-sniffing port
GSH	glutathione
HPLC	high performance liquid chromatography
HPSEC	high performance-size exclusion chromatography (also known as gel permeation chromatography)
HPLC-CL	HPLC-chemiluminescence
HPLC-^{13}C NMR	HPLC and ^{13}C nuclear magnetic resonance
HPLC-MS	high performance liquid chromatography-mass spectrometry
HDL	high density lipoproteins
IP	induction period
IDL	intermediate density lipoproteins
LCAT	lecithin-cholesterol acyltransferase
LC-MS	liquid chromatography-mass spectrometry
LC-ESI-MS	liquid chromatography-electrospray ionization-mass spectrometry
LDL	low-density lipoproteins
LOOH	hydroperoxides
LOX	lipoxygenase
MDA	malonaldehyde
MS-MS	tandem-mass spectrometry
MW	molecular weight
NADH	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
ND	not detected
NMR	nuclear magnetic resonance spectroscopy
^{1}H NMR	proton nuclear magnetic resonance
^{13}C-NMR	^{13}C -nuclear magnetic resonance spectroscopy
PC	phosphatidylcholine
PC-OOH	PC hydroperoxide
PE	phosphatidylethanolamine
PG	propyl gallate
PI	phosphatidylinositol
PV	peroxide value
PVC	polyvinylchloride
PET	polyethylene terephthalate
PP	polypropylene

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ppm	parts per million
ppb	parts per billion
PG	phosphatidylglycerol
PS	phosphatidylserine
psi	pounds per square inches
PUFA	polyunsaturated fatty acids
PV	peroxide value
RBD	refined, bleached and deodorized
$^1O_2$	singlet oxygen
SBO	soybean oil
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
SPE	solid-phase extraction
SPME	solid-phase micro extraction
$O_2^{-\bullet}$	superoxide radicals
$^3O_2$	triplet oxygen
TAG	triacylglycerols
TBA	thiobarbituric acid
TBARS	thiobarbituric reactive substances
TBHQ	*tert*-butylhydroquinone
TG	triglycerides
TLC	thin layer chromatography
TMS	trimethylsilyl
UV	ultraviolet
VLDL	very low density lipoproteins

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