Lipid Oxidation Pathways

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Preface

Autoxidation of unsaturated lipids received much attention in the 20th century because it has many applications in the rancidity of foods and stability of lipids in biological tissues and compartments. The foundations of the chemistry of the reactions were established after excellent studies by workers in the British Rubber Producers' Research Association at the beginning of that century and subsequent research elsewhere in the world. As a result, the oxidation reaction is recognized as a chain reaction that can be inhibited by antioxidants. Despite this common nature of the reaction, the addition of small amounts of different chemicals and alterations in the microenvironment of the reaction can cause significant changes in the reaction rate and the relative levels of different reaction products. Thus, the original lipid oxidation models must be "refined" or "tuned" to be able to accommodate existing knowledge and to unravel a number of paradoxes associated with the kinetics of oxidation of different lipids.

The aim of this book is to review state-of-the-art developments in the understanding of the oxidation of lipids and its connection with the oxidation of other biological molecules such as proteins and starch. The various chapters illustrate the special features associated with different lipids, antioxidants, reaction conditions, and lipid environments. The first chapter provides an overview of the importance of hydroperoxides as products and catalysts in the chain reactions involved. The second chapter discusses the challenge resulting from the high vulnerability of the n-3 fatty acids of fish oils to oxidation and how tocopherols can be used synergistically with naturally occurring antioxidants to inhibit this oxidation. Chapter 3 show how tocopherols and fatty acids compete for oxidizing radicals under thermoxidation conditions. Chapter 4 presents a kinetic model that was widely used by the authors to evaluate the activity of antioxidants as inhibitors of lipid oxidation. In Chapter 5, a kinetic analysis of the oxidation of β-carotene in lipids in the absence and presence of an antioxidant inhibitor is given. The occurrence, mechanism of formation, analysis and biological significance of core aldehydes, one of the persistent secondary oxidation products, is discussed in Chapter 6. Chapters 7 and 8 discuss lipid oxidation in two defined systems, i.e., emulsions and dried microencapsulated oils. During the last decade, much interest has been paid to other biomolecules that co-exist with lipids. Chapter 9 is dedicated to protein alterations induced by lipid oxidation and Chapter 10 considers the question of radical formation due to radiolysis of starch and lipid-containing starch. A better understanding of lipid oxidation pathways will result from the synthesis of knowledge on that topic that is provided here.

With sincere gratitude, the editor thanks all of the authors for their contributions to this book and the efforts that made this publication possible. The editor also gratefully acknowledges the professional help and support of Mary Lane and her colleagues at AOCS Press who brought this book to its present state.

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Contents

Chapter 1

The Challenging Contribution of Hydroperoxides to the Lipid Oxidation Mechanism

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Introduction

Lipid hydroperoxides were identified as autoxidation products of polyunsaturated fatty acids (PUFA) in the early work of Farmer (1945) and Bolland (1946 and 1949). Farmer and his group developed the free radical theory of autoxidation, which involves an attack of oxygen at the allylic position with the formation of unsaturated hydroperoxides. Although hydroperoxides are more stable than radical species, they are still weak oxidizing agents that decompose to peroxyl and alkoxyl radicals, leading to secondary oxidation products including aldehydes, ketones, alcohols, acids, and lactones (Benzie 1996). The secondary oxidation products are responsible for impaired taste, flavor and texture in foods and for a number of disasterous reactions in biological tissues and the human body.

However formed, hydroperoxides do take part in autoxidation and initiate new chain reactions (Chan and Coxon 1987, Gardner 1987). It is thus of extreme importance to find ways to stabilize lipid hydroperoxides in biological material and to inhibit their reactions with PUFA and their cascade into other hazardous oxidation products. To be able to achieve this goal, a proper understanding of the kinetics and mechanism of these reactions and how they are affected by surrounding chemical and physical conditions is of vital importance.

The classical kinetic scheme for the autooxidation of PUFA was illustrated by Labuza (1971) according to contemporary knowledge. On the basis of the original theory of Bolland, Bateman and co-workers at the British Rubber Producers' Research Association, it is accepted that autoxidation of PUFA occurs as a chain reaction that proceeds through three phases (Fig. 1.1), namely, (i) initiation, (ii) propagation, and (iii) termination (Bateman and Gee 1951, Bateman *et al.* 1951 and 1953, Bateman 1954, Bolland 1946 and 1949, Bolland and Gee 1946a, 1946b). The classical lipid oxidation scheme proposed by these workers (Scheme 1.1) has been used since then to explain most of the observations and research findings, but it remains unable to explain complicated secondary phenomena and some of the details encountered in lipid oxidation studies (Chan 1987). A number

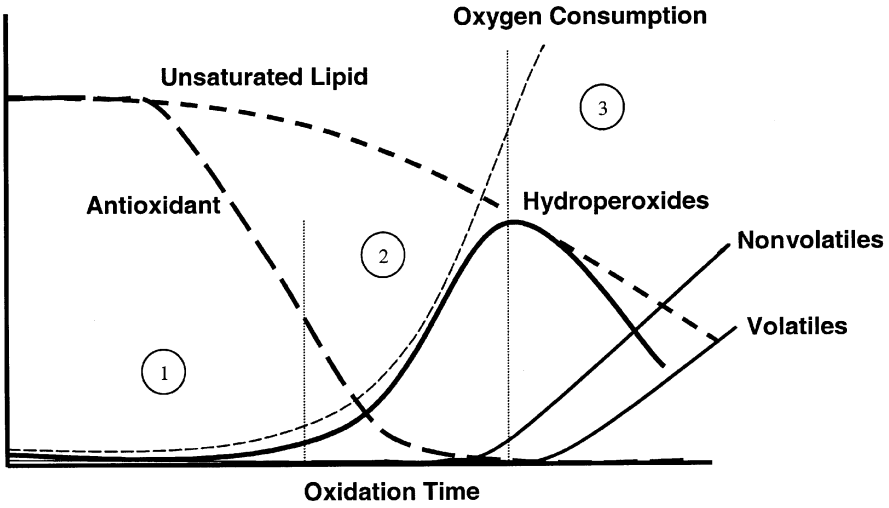


Fig. 1.1. The kinetic curve of autooxidation of polyunsaturated fatty acids as modified from Labuza (1971).

of empirical formulas were derived to describe the lipid oxidation reactions (Table 1.1), but a proper understanding of the kinetic and thermodynamic determinants of these reactions has not yet been achieved.

Throughout the years, additional reactions (Scheme 1.2) were added to the classical scheme to explain some of the mechanistic aspects awaiting resolution, e.g., the nature of the initiating radicals and how they are generated, the criticality in the oxidation phenomena, the paradoxical behavior of antioxidants, and the

Initiation

$$LH \longrightarrow L^{\bullet} + H^{\bullet} \qquad R_i \qquad (1)$$

Propagation

$$L^{\bullet} + O_2 \rightleftharpoons LOO^{\bullet} \qquad (2)$$

$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet} \qquad (3)$$

Termination

$$2LOO^{\bullet} \xrightarrow{k_t} \text{Inactive products} \qquad (4)$$

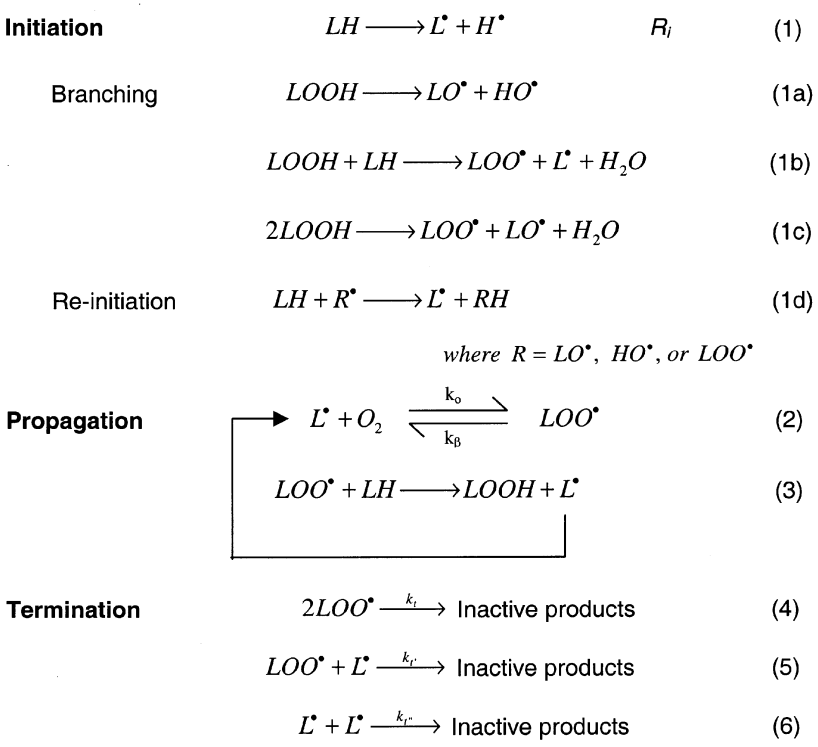
$$LOO^{\bullet} + L^{\bullet} \xrightarrow{k_{t'}} \text{Inactive products} \qquad (5)$$

$$L^{\bullet} + L^{\bullet} \xrightarrow{k_{t''}} \text{Inactive products} \qquad (6)$$

Scheme 1.1.

TABLE 1.1
Examples of the Logistic Equations Used to Describe the Rate of Lipid Oxidation

Equation	Explanation of terms	Comments	Reference
(1) Rate = $k[LH][LOOH][O_2]/[K + O_2]$	[LH], [LOOH], and $[O_2]$ are the concentrations of lipid substrate, hydroperoxides and oxygen; k and K are constants.	Applied to the oxidation of ethyl linoleate	Bolland 1949
(2) Rate = $KC[1 - C/C_{max}]$	C is the concentration of total oxidation products; C_{max} is the maximum attaintable concentration of parameter C due to consumption of substrate; K is a constant.	Applied to lipid oxidation in foods and the oxidation of cholesterol	Özilgen and Özilgen 1990 Chien *et al.* 1998
(3) Rate = $k[O_2]S(1 - x/n)\, f'(t)$	x is the number of moles of O_2 consumed per initial mole of substrate at time t; $[O_2]$ is the oxygen concentration in the substrate; $S(1 - x/n)$ is the amount of unreacted substrate at time t, n is the number of O_2 molecules that react with 1 mol of the substrate (for linoleate, $n = 2$); $f'(t)$ is a time function that is dependent on the state of the catalyst.	Applied to the oxidation of unsaturated fatty acids	Brimberg 1993a Brimberg 1993b
(4) Rate = $[LOOH]\,(1 - [LOOH])k[O_2]/(K + [O_2])$	[LOOH] and $[O_2]$ are the concentrations of hydroperoxides and oxygen; k and K are constants.	Modified from Bolland's equation and applied to the oxidation of fatty acids and their esters	Adachi *et al.* 1995 Borquez *et al.* 1997



Scheme 1.2.

mechanisms governing the distribution of hydroperoxide decomposition products. This knowledge is important with respect to free radicals and their harmful roles in health and disease but also from an analytical point of view as it relates to toxicological and/or biomarker aspects. This review aims to explore current knowledge about the lipid oxidation mechanism. Effects of temperature and antioxidants are included to explain mechanistic aspects. The concluding remarks cover controversies, unexplained phenomena, and mechanistic aspects awaiting further investigation.

The Oxidation Pathway and the Role of Hydroperoxides

Chain Initiation

When followed experimentally, autoxidation of unsaturated fatty acids usually begins with an initial period known as the lag phase or induction period (Cadenas and Sies 1998). When no initial hydroperoxides or other prooxidant species are present at this early stage of oxidation, the rates of oxygen uptake, the disappearance of the substrate, and the formation of hydroperoxides are very slow and do

generally agree with the stoichiometric ratio of one mole of oxygen for each mole of a PUFA (Chan and Levett 1977a, Porter *et al.* 1980, Yamamoto *et al.* 1982a, 1982b), i.e., with the overall reaction

$$LH + O_2 \rightarrow LOOH \quad [1]$$

Kinetic studies on peroxide-free tetralin showed that the initial rate of initiation can be expressed by the following formula where *AH* represents an antioxidant inhibitor (George and Robertson 1946, George *et al.* 1946)

$$\text{Rate} = k[LH]^2[O_2]^0/(1 + k_{AH}\,[AH]) \quad [2]$$

Despite the developments in our understanding of different parts of the lipid autoxidation reactions, the exact mechanism responsible for chain initiation, i.e., the formation of the first hydroperoxides in pure substrates is still not known (Labuza 1971, Privett and Blank 1962). It was suggested by Uri (1956) and Heaton and Uri (1961) that trace metal ions are responsible for the primary abstraction of a hydrogen from a PUFA. The glass walls of reaction vessels seem to act as reaction catalysts in the same way as transition metals (Davies *et al.* 1956). Ultraviolet (UV) light and temperature were found to increase the rates of direct attack of PUFA by oxygen (Bolland and ten Have 1947a and 1947b, Chahine and de Man 1971, Russel 1956, Shelton and Vincent 1963). Berg (1994) and Brimberg (1991, 1993a, and 1993b) postulated that unsaturated fatty acids are oxidized by hydrogen peroxide, produced from the decomposition of water by heterogeneous catalysis on metal surfaces. Rawls and van Santen (1968 and 1970) suggested that the first hydroperoxides are formed by direct combination of unsaturated substrates with trace amounts of singlet oxygen. The rate of initiation, R_i, is often considered to be constant, although it has been shown that this might not always be true (Abuja *et al.* 1997, Privett and Blank 1962). A nonconstant rate of initiation will certainly result from a multitude of possible initiating events, which are difficult to analyze due to the small degree of alterations at these early stages.

Once the first hydroperoxides are formed in a medium containing PUFA and/or other oxidizable substrates, they increase the rate of initiation tremendously due to generation of reinitiating radicals by monomolecular and bimolecular hydroperoxide decomposition (Labuza 1971). Hydroperoxide decomposition by these mechanisms is thought to be responsible for the linear and exponential parts of the kinetic curve during the induction period [Scheme 1.2, Eqs. (2) and (3)]. The lowest hydroperoxide concentration limit that causes a significant catalytic initaition was set by Knorre *et al.* (1957) to be as low as 10^{-6} M, corresponding to a peroxide value (PV) of 1–2 mEq/kg, whereas Crapiste *et al.* (1999) found the critical concentration to be 18.8 mEq/kg. Russian scientists attributed this effect of *branching* to bimolecular dissociation of hydroperoxides (Emanuel and Lyaskovskaya 1967), which is believed to follow hydrogen bond formation between the hydroperoxide

hydrogen of one molecule and an oxygen atom of the hydroperoxide function of the other molecule (Bateman *et al.* 1953, Hiatt 1975, Walling and Heaton 1965). Alkoxyl and peroxyl radicals generated by these decomposition reactions initiate new reaction chains by reacting with PUFA (LH) to produce the very reactive carbon-centered alkyl radicals [L•, reaction (2) in Schemes 1.1 and 1.2]. Once formed, L• reacts very rapidly with oxygen to form a peroxyl radical [LOO•, Reaction (3) in Scheme 1.2].

Propagation and Isomerization During Hydroperoxide Formation

The abstraction of a hydrogen atom from an allylic or bisallylic position of an unsaturated fatty acid (LH) by peroxyl radical (ROO•) to generate hydroperoxide (LOOH) and another radical (L•) is the slowest step in chain propagation (Reaction 3, Table 1.2). The susceptibility of different fatty acids to this hydrogen abstraction is dependent on the dissociation energies of C-H bonds present in the fatty acid. The presence of a double bond in the fatty acid weakens the C-H bonds on the carbon atom adjacent to the double bond and makes the hydrogen removal easier. The bond dissociation energy for the bisallylic C-H bond is ~85 ± 3 kcal/mol, whereas that of the monoallylic C-H bond is ~10 kcal/mol higher (Gardner 1989, Porter 1986, Reich and Stivala 1969, Wu *et al.* 1978). Thus, as shown by Cosgrove *et al.* (1987) and Wagner *et al.* (1994), the measured oxidizabilities of the PUFA are dependent on the number of bis-allylic methylenes present in the fatty acid. The oxidizability is much less for monounsaturated substrates such as oleic acid and choles-

TABLE 1.2
The Approximate Rate Constants of the Different Reactions Involved in the Autooxidation of Methyl Linoleate in the Absence or Presence of α-Tocopherol

No.	Reaction	$K/M^{-1}s^{-1}$	Reference
1	$LH + O_2 \rightarrow LOOH$	5.8×10^{-11}	Kasaikina *et al.* 1999
2	$LOOH + TOH \rightarrow LOO^{\bullet} + TO^{\bullet} + H_2O$	unknown	Yanishlieva *et al.* 2002
3	$LOOH + LH \rightarrow LOO^{\bullet} + L^{\bullet} + H_2O$	2.3×10^{-7}	Kasaikina *et al.* 1999
4	$LOOH + LOOH \rightarrow LOO^{\bullet} + LO^{\bullet} + H_2O$	2.4×10^{-6}	Kasaikina *et al.* 1999
5	$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$	3×10^{8}	Hasegawa and Patterson 1987
		5×10^{6}	Kasaikina *et al.* 1999
6	$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet}$	1×10^{6}	Niki *et al.* 1984
7	$LOO^{\bullet} + TO^{\bullet} \rightarrow$ TO-OOL	2.5×10^{6}	Kaouadji *et al.* 1987
8	$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$	31	Babbs and Steiner 1990
		90	Kasaikina *et al.* 1999
9	$2\ LOO^{\bullet} \rightarrow 2\ (LO) + {}^1O_2$	1×10^{5}	Barclay *et al.* 1989
		4.4×10^{6}	Kasaikina *et al.* 1999
10	$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet}$	1×10^{7}	Small *et al.* 1979
11	$TO^{\bullet} + LH \rightarrow TOH + L^{\bullet}$	0.02	Mukai and Okauchi 1989
		0.07	Remorova and Roginsky 1991
12	$TO^{\bullet} + LOOH \rightarrow TOH + LOO^{\bullet}$	0.1–0.5	Mukai *et al.* 1993
13	$2\ TO^{\bullet} \rightarrow (TO)_2$	3×10^{3}–5×10^{5}	Burton *et al.* 1985

terol than for polyunsaturated substrates because monoallylic methylene hydrogens are more resistant to abstraction. In addition to the bond dissociation energy, the supramolecular orientation of the hydroperoxides also seems to play a significant role. Paradoxically, tuna lipids were found to be more stable than soybean oil triacylglycerols (Miyashita *et al.* 1995), and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were more stable than linoleic acid in oil-in-water emulsions (Bruna *et al.* 1989, Endo *et al.* 1997, Yazu *et al.* 1996 and 1998). This phenomenon can be explained by the fact that the structurally "more-bent" hydroperoxides of the highly unsaturated fish lipids are more polar and have a higher tendency to migrate and assemble themselves in micelles. This behavior increases the oxidizability of these lipids vs. that of linoleic acid in bulk but decreases it in oil-in-water emulsions.

Reactions occuring during the chain propagation phase are also not fully understood. The major radical species present during this period are the peroxyl radicals produced by the autocatalytic cycle shown in Eqs. [2] and [3], in which the ratio of $[LOO^{\bullet}]$:$[L^{\bullet}]$ was $\sim 1.5 \times P[O_2]$. It was claimed that peroxyl radicals were detected directly by electron spin resonance at distinguished *g*-values in the range of 2.014–2.019 (Bascetta *et al.* 1982 and 1983, Chamulitrat and Mason 1989, Ingold 1969). However, by using the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), these radicals were reassigned to alkoxyl radicals (Dikalov and Mason 1999 and 2001) emphasizing their unrecognized role(s) in lipid oxidation as will also be discussed later.

As mentioned, peroxyl radicals react mainly with PUFA during this phase by removing easily abstractable hydrogen atoms. The relative rates of oxidation for oleic, linoleic, linolenic, and arachidonic acids were in the order 1:40:80:160 (Holman and Elmer 1947, Holman 1954). The rate of oxygen uptake in reaction [2] increases with increased oxygen concentration as shown by the rate equations extracted by the steady-state approximation given in the Appendix. For methyl linoleate, relevant values were obtained for k_3 by different researchers, e.g. 62 $M^{-1}s^{-1}$ at 30°C (Howard and Ingold 1967), 100 $M^{-1}s^{-1}$ at 37°C (Niki *et al.* 1984), and 230 $M^{-1}s^{-1}$ at 50°C (Yamamoto *et al.* 1982b). These values agree with the general rule that the rate of reaction doubles with every 10°C increase in temperature. For the series $-CH_2-(CH{=}CH-CH_2)_n-CH_2-$ with n = 1–6, the relative values of k_3 increased in the order 0.025:1:2:4:6:8, respectively (Witting 1965 and 1970).

Four hydroperoxide positional isomers (8-, 9-, 10-, and 11-) are produced by the abstraction of an allylic hydrogen with or without double bond migration during the autoxidation of oleate (Frankel *et al.* 1977a). These four hydroperoxides were later shown to be present as eight *cis* and *trans* isomers (Frankel *et al.* 1984, Haslbeck and Grosch 1983, Neff *et al.* 1978, Porter *et al.* 1994). A mechanism involving competition between hydrogen atom abstraction (chain propagation) and [2,3]-allylperoxyl radical rearrangement was proposed by Porter *et al.* (1994) to explain the isomerization and the relative dominance of the different isomers during the formation of oleate hydroperoxides. The mechanism involves hydrogen atom abstraction at the 8- and 11-positions of oleic acid to give two allylic radicals. The

addition of oxygen to these allylic radicals gives rise to four peroxyl radicals 11-*cis*, 9-*trans*, 8-*cis* and 10-*trans*. These peroxyl radicals may interconvert *via* an allylperoxyl rearrangement to 11-*trans* and 8-*trans* peroxyl radicals. Hydroperoxides result from peroxyl radicals undergoing hydrogen atom abstraction from oleic acid. The relative distribution of different hydroperoxide isomers is dependent on the hydrogen-donating ability of the medium because the formation of "kinetic" 11-*cis*, 9-*trans*, 8-*cis* and 10-*trans* hydroperoxides is favored in the presence of good hydrogen atom donors (Porter *et al.* 1995).

It is worth mentioning here that peroxyl radicals do not react with unsaturated fatty acid substrates only by the abstraction mechanism. For example, substrates with conjugated double bonds prefer oxidation by the addition mechanism rather than by the hydrogen abstraction mechanism (Mayo 1968). In addition to hydrogen abstraction in the oxidation of oleate and cholesterol, peroxyl radicals are also able to add directly to the double bond to form epoxides (Koelewijn 1972, Ozawa *et al.* 1986, Smith *et al.* 1982, Sugiyama *et al.* 1987, Walther and Spiteller 1993). Epoxides are known to represent the major proportion of the oxidation products of other monounsaturated substrates such as cholesterol and other Δ^5-sterols (Dutta 1997, Kim and Nawar 1993).

By 1945, Bolland and Koch had described the primary products of autoxidation of methyl linoleate at 37°C as monohydroperoxides whose major part contained a conjugated diene (CD) structure. Bergstöm and co-workers (1945 and 1950) found the oxidation of linoleate to give 9- and 13-hydroperoxides with CD structures resulting from positional isomerization of one double bond in the peroxyl radical. Primary oxidation of linoleate is largely (~95%) due to abstraction of the bisallylic hydrogen and isomerization of one of the double bonds. The presence of minor amounts of nonconjugated 8-, 10-, 12-, and 14-hydroperoxides due to abstraction of allylic hydrogens was also reported (Haslbeck and Grosch 1983, Haslbeck *et al.* 1983, Schieberle and Grosch 1981a,b). The formation of even smaller amounts of the bisallylic 11-hydroperoxide in the presence of α-tocopherol was shown only recently (Brash 2000).

Using high-performance liquid chromatography (HPLC) with UV detection at 234 nm, Chan and Levett (1977a) isolated four CD hydroperoxides from the autoxidation of methyl linoleate, *viz.*, 9-hydroperoxy-10*trans*,12*cis*-, 9-hydroperoxy-10*trans*,12*trans*-, 13-hydroperoxy-9*cis*,11*trans*-, and 13-hydroperoxy-9*trans*, 11*trans*-octadecadienoate. This was the first evidence that hydroperoxide isomers are formed not only from positional isomerization but also from geometrical isomerization of double bonds, which requires the opening of these double bonds and rotation of the resultant single bonds. Because peroxyl radicals have ample lifetimes (~7 s) (Pryor 1986), they have enough time to rearrange before abstracting hydrogen atoms from PUFA to form hydroperoxides. Using ^{18}O-enriched hydroperoxides, Chan *et al.* (1978 and 1979) showed that the geometrical isomerization of hydroperoxides is accompanied by an exchange with atmospheric oxygen and formation of a pentadienyl radical. Subsequent oxygen addition generates 9- and 13-*cis,trans* peroxyl

radicals, both of which have three possible options. Hydrogen atom abstraction from donors such as linoleic acid residues generates first the unstable 11-hydroperoxide, which rearranges to give 9- and 13-*cis,trans* hydroperoxides that are kinetic products (Tallman *et al.* 2001). The formation of *trans,trans* hydroperoxide isomers result from β-fragmentation of oxygen from the *cis,trans* hydroperoxyl radicals followed by bond rotation. Oxygen addition then leads to peroxyl radicals that give 9- and 13-*trans,trans* hydroperoxides, which are the thermodynamic products. As with oleic acid, the *cis,trans/trans,trans* hydroperoxide product ratio is determined by the competition between hydrogen atom abstraction to give the *cis,trans* kinetic hydroperoxides and β-fragmentation leading to *trans,trans* thermodynamic hydroperoxides (Porter 1986, Porter *et al.* 1980, 1981, and 1995, Tallman *et al.* 2001). This isomerization of lipid hydroperoxides occurs as part of the lipid autoxidation mechanism, mainly during chain propagation. It can be induced by initiators but can also operate autocatalytically (Scheme 1.2) and is inhibited by hydrogen donors in a concentration-dependent manner (*vide infra*).

Porter *et al.* (1980 and 1981) showed that the ratio of *cis,trans*-to-*trans,trans* hydroperoxides increases with increased concentration of PUFA and inhibitors, i.e., total hydrogen-donation ability of the reaction mixture, and proposed a schematic representation for the rearrangement of the pentadienyl radical during this reaction. Yamamoto *et al.* (1982a and 1982b) confirmed these findings and showed that the ratio of *cis,trans/trans,trans* hydroperoxides increased with increased dielectric constant of the solvent but was independent of oxygen partial pressure. The basic equation illustrating the *cis,trans*-to-*trans,trans* hydroperoxide ratio is

$$\frac{cis,trans\ \mathrm{LOOH}}{trans,trans\ \mathrm{LOOH}} = \frac{\Sigma K[\mathrm{RH}] + \alpha k_{\beta}^{III}}{k_{\beta}^{II}(1-\alpha)} \qquad [3]$$

where K is a constant for the ability of a hydrogen donor(s) to donate a hydrogen atom to the hydroperoxyl radical and inhibits its isomerization, k_{β}^{II} and k_{β}^{III} are the rate constants for β-scission of the cis,trans and *trans,trans* peroxyl radicals (Fig. 1.2), and $(1 - \alpha)$ represents the partitioning of the carbon radical between *cis,trans* and *trans,trans* peroxyl radicals. RH can be an antioxidant, e.g., α-tocopherol, or a PUFA. The degree of inhibition depends on the hydrogen-donation ability of the different hydrogen donors (K) and their concentrations [RH] in the reaction mixture (Barclay *et al.* 1997, Porter *et al.* 1980, 1981, 1995). The same rules governing *cis,trans*-to-*trans,trans* hydroperoxide isomerization were obtained in phospholipid membranes (Barclay *et al.* 1997) and in aqueous emulsions (Wang *et al.* 1999).

Allen *et al.* (1949) studied the oxidation of methyl linoleate at 30°C using oxygen absorption, PV, and CD as parameters describing the oxidative changes. The comparison of the results of these parameters in mol/mol methyl linoleate suggests that the 9- and 13-hydroperoxides with CD structures are in fact only part of the peroxides formed (Fig. 1.3). The other, not yet characterized, hydroperoxides

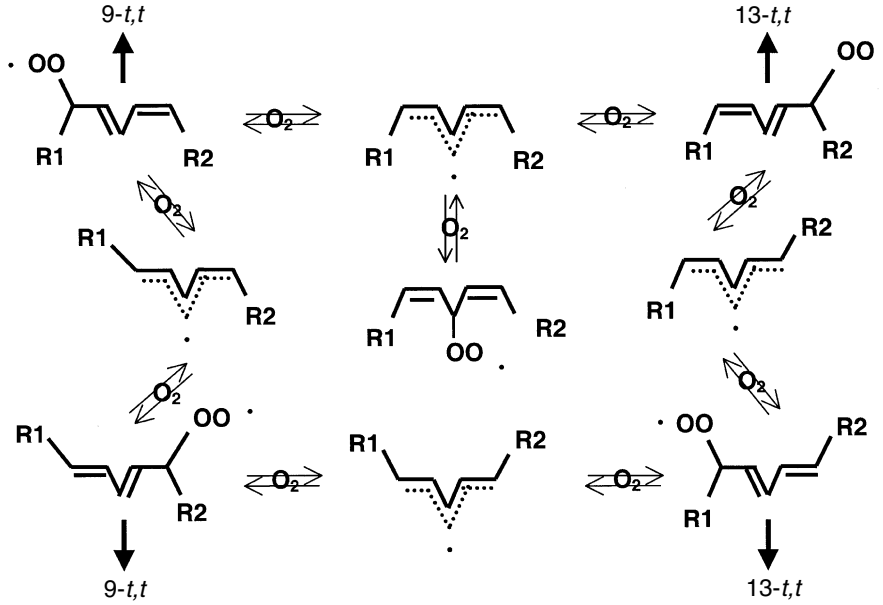


Fig. 1.2. The isomerization of methyl linoleate hydroperoxides as modified from Porter *et al.* (1995), and Tallman *et al.* (2001).

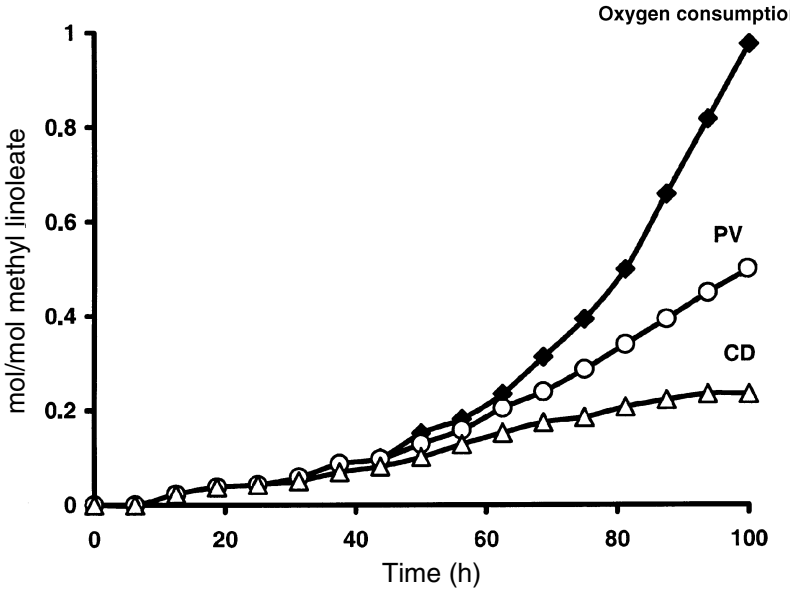


Fig. 1.3. The course of oxidation of methyl linoleate followed by measuring oxygen consumption, peroxide value (PV), and conjugated dienes (CD) in mol/mol substrate. Data taken from Allen *et al.* (1949).

should then be epoxy-hydroperoxides resulting from the cyclization of alkoxyl radicals formed by the decomposition of the conjugated hydroperoxides. The remaining oxygen is present in the epoxy functions, in water formed by decomposition of hydroperoxides, and the minor amounts of secondary oxidation products formed during this period. In an outstanding review, Gardner (1987) explained that epoxy hydroperoxide derivatives are formed by intramolecular rearrangement of alkoxyl radicals produced from primary hydroperoxides and that they account for the major part of oxygen consumption during the decomposition of conjugated linoleate hydroperoxides (Fig. 1.4). This figure also illustrates the analytical limitations associated with the commonly used methods of CD and PV as descriptive parameters for the kinetic evaluation of lipid oxidation. The use of these insensitive methods is likely the main reason that the exact oxidation mechanism remains obscure. Oxygen absorption is the only method that can provide accuracy in studies on the kinetics of lipid oxidation. Because PV (and less so CD) measurements do correlate with oxygen absorption, they can provide approximate data.

The presence of more than two isolated double bonds, as in linolenate, arachidonate, eicosapentaenoic acid, and docosahexaenoic acid, makes these fatty acid moeities to give different oxidation products than linoleate with two isolated double bonds. On autoxidation, linolenate, arachidonate, eicosapentaenoate, and

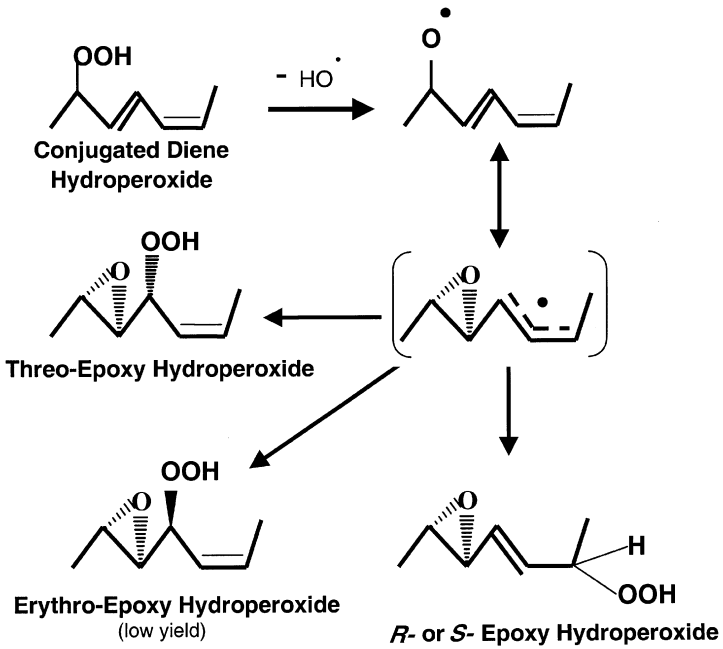


Fig. 1.4. Formation of epoxyhydroperoxides from linoleate moities as modified from Gardner (1987).

docosahexaenoate yield a wide variety of isomeric monohydroperoxides, cyclic epidioxides, and dihydroperoxide products (Chan *et al.* 1954, Coxon *et al.* 1981, Frankel *et al.* 1977c, Peers *et al.* 1981).

The autoxidation of methyl linolenate produced the following monohydroperoxides: 9-*cis,trans* (27%), 9-*trans,trans* (11%), 12-*cis,trans* (6%), 12-*trans,trans* (2%), 13-*cis,trans* (7%), 13-*trans,trans* (3%), 16-*cis,trans* (31%), and 16-*trans, trans* (13%) (Peers *et al.* 1981). Linolenic acid contains two separate 1,4-diene systems, a C-9 to C-13 system identical with the linoleic acid plus the C-12 to C-16 system. A mixture of 9-, 13-, 12-, and 16-hydroperoxides each as *cis,trans* and *trans,trans* isomers is formed during autoxidation (Chan and Levett 1977a and 1977b, Frankel *et al.* 1977b). However, the analysis of these isomers revealed that the "outer" 9- and 16-hydroperoxides are formed approximately four times more than "inner" 12- and 13-hydroperoxides (Frankel 1991). This uneven distribution of hydroperoxides is due to the tendency of 12- and 13-peroxyl radicals to undergo rapid 1,3-cyclization. Figure 1.5 shows that the *cis*-double bonds homoallylic to

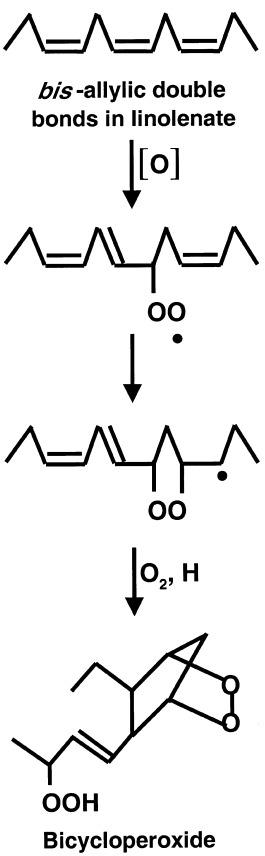


Fig. 1.5. Formation of bicycloperoxides from linolenate moities.

the peroxyl groups in the peroxyl radical of the 12- and 13-hydroperoxides permit a facile intramolecular 1,3-cyclization and the formation of new radicals (Chan *et al.* 1980, Coxon *et al.* 1981, Gardner 1989). In contrast to linoleic acid, the *cis,trans* hydroperoxides of linolenic acid are not readily isomerized to the *trans,trans* configuration, apparently because cyclization is favored much more than geometrical isomerization. The rate constants for cyclization are 4.5- to 6.5-fold higher than β-scission and thus outer 9- and 16-hydroperoxides and cyclic peroxides accumulate in media of low hydrogen-donating capacity (Gardner 1989). More than with linoleate, the measurements of PV or CD in the case of linolenate give only a limited measure of the degree of oxidation and might present a serious limitation to our understanding of the degree and mechanism of the oxidation reaction.

In addition to the degree of the unsaturation of the fatty acid, the autoxidation rate is dependent on the lipid structures. As an example, Miyashita and Takagi (1986) showed that oleic, linoleic, and linolenic acid as free fatty acids were autoxidized more rapidly than their corresponding methyl esters. They suggested that the higher oxidative rate of free fatty acids was due to the catalytic action of carboxyl groups on the decomposition of a small amount of hydroperoxides formed in the initial stage of autoxidation.

Decomposition and Further Oxidation of Hydroperoxides

Hydroperoxides are relatively stable compounds under favorable conditions, such as low temperature, dilute solution, the presence of antioxidants and the absence of catalyst. Normally such conditions are not encountered, and the hydroperoxides become susceptible to decomposition (Benzie 1996, Gardner 1987, Gruger and Tappel 1970). Hydroperoxide decomposition provides one of the most important catalysts for lipid oxidation; hence the name autoxidation (Schieberle *et al.* 1979). Once the hydroperoxide content in a system containing PUFA reaches a certain critical value, their decomposition becomes significant and the rate of lipid oxidation increases. During the propagation phase, the rate of hydroperoxide formation is greater than the rate of their decomposition and this situation is reversed in the decomposition stage. This was shown elegently in a recent study by Crapiste *et al.* (1999) who represented the rate of peroxide formation in sunflower triacylglycerols by a kinetic model composed of a first-order formation reaction and a second-order decomposition reaction

$$\frac{d[\mathrm{LOOH}]}{dt} = k_1[\mathrm{LOOH}] - k_2[\mathrm{LOOH}]^2 \qquad [4]$$

the reaction rate constants (k_1 and k_2) were found to be temperature-dependent in accordance with Arrhenius equation

$$k_i = k_{oi} \cdot \mathrm{e}^{-\Delta E_i/RT} \qquad [5]$$

where k_{oi} and ΔE_i represent the frequency factor and the activation energy for the rate constant [k_i (i = 1,2), respectively], R is the gas constant and T is the absolute temperature. The values of k_{01} and k_{02} for sunflower triacylglycerols were 1.04×10^5/d and 0.139×10^5 [mEqu/(kg · d)]$^{-1}$ and the ΔE_1 and ΔE_2 values were 38.35 and 48.47 kJ/mol K. The model applied from a critical PV of 18.8 mEq/kg to maximal values that are temperature dependent (PV = 414 mEq/kg at 30°C, 334 mEq/kg at 47°C and 267 mEq/kg at 67°C). After these maximal values, the reaction enters the active decomposition phase in which autoxidation reactions involve mainly hydroperoxides rather than PUFA. Trace metal ions, when present, catalyze the unimolecular decomposition of hydroperoxides (Hiatt *et al.* 1968c).

As mentioned *vide* supra, one feature of linoleate peroxidation that is not adequately discussed is the formation of monoenic epoxyhydroperoxides in an approximate 1:1 ratio to CD hydroperoxides during the exponential oxidation stage of methyl linoleate (Allen *et al.* 1949). Cyclization of alkoxyl radicals (LO$^\bullet$) yields epoxyallylic carbon-centered radicals (OL$^\bullet$), which capture oxygen to yield epoxyallylic peroxyl radicals (Gardner 1991, Wilcox and Marnett 1993). It has been shown that oxygen is absorbed during the degradation of linoleate hydroperoxides (Johnston *et al.* 1961, Kanazawa *et al.* 1973). Thus, measuring CD during lipid oxidation provides a less sensitive measure of the degree of peroxidation compared with PV.

At the last stage of autooxidation, the concentration of peroxyl radicals might be high enough that they start to form adducts. Reaction (4) (Scheme 1.1) is not a termination reaction leading to stable nonradical products as is generally believed; rather, it is a reinitiation/decomposition reaction. The conception that a combination of peroxyl radicals gives inactive products was supported by the Russel mechanism, which emphasized the formation of an alcohol and a ketone *via* a tetroxide, resulting from the combination of a pair of peroxyl radicals in a solvent cage (Hiatt *et al.* 1968a and 1968b, Russel 1957). Consideration of products formed from the decomposition of hydroperoxides, at low temperatures, reveals most of these products to be C18-epoxy derivatives, indicating that the dismutation of peroxyl radicals by the Russel mechanisms is not all benign and that the major products from the combination of peroxyl radicals are alkoxyl radicals. As just mentioned, alkoxyl radicals are known to undergo very fast rearrangement to form epoxy allyl radicals, which would capture oxygen and form epoxy peroxyl radicals that would participate in chain propagation (Gardner 1991, Wilcox and Marnett 1993). The work of Terao and Matsushita (1975) and Terao *et al.* (1975) showed that although 89% of the CD structure was lost by incubation of methyl linoleate hydroperoxides at 37°C for 4 d, only 36% of the PV was lost. This finding can be explained by the conversion of hydroperoxides to alkoxyl radicals, which act as precursors for a wide variety of secondary products. Alkoxyl radicals often cyclize to carbon-centered epoxy derivatives, which are fast to capture another molecule of oxygen to form monoenic epoxy hydroperoxides, epoxyoxoenes, and epoxy hydroxyenes (Gardner 1975, Hamberg and Gotthammar 1973, Hamberg 1975, Johnston *et al.* 1961, Kanazawa *et al.* 1973). Alkoxyl radicals are also the precursors for oxodienes,

hydroxydienes, oxohydroxyenes, dihydroxyenes, and peroxide dimers (Hamburg 1983, Miyashita *et al.* 1982a, 1982b, 1985a, and 1985b, Terao and Matsushita 1975, Terao *et al.* 1975, Yamamoto *et al.* 1984). Dimers of alkoxyl radicals have also been found to form at the initial stages of oxidation (Miyashita *et al.* 1982a, 1982b, 1985a, and 1985b). As the reaction temperature increases, the formation of C18-epoxy derivatives from alkoxyl radicals compete with fast β-scission and breakdown to shorter-chain volatile and nonvolatile products (Fig. 1.6).

A complex mixture of volatile, nonvolatile, and polymeric secondary oxidation products is formed through the decomposition reactions of hydroperoxides (Chan 1987). The structures of some of these decomposition products are known relatively well on the basis of the studies done in various model systems (see the reviews of Frankel 1998, Gardner 1987 and 1989). However, the exact mechanisms for their formation and the kinetic and thermodynamic factors governing their quantitative and qualitative distribution are not yet completely understood. Variables that may have an effect on the relative reaction rates and product distribution include temperature, reaction media, and antioxidative and prooxidative compounds (Gardner 1987). Most likely the relative distribution of the decomposition products is determined by several competitive reaction pathways whose relative importance depends on the reaction conditions. Most of these secondary oxidation products are formed from alkoxyl radicals as shown in Figure 1.7. A number of aldehydic products (including malondialdehyde, C3–C10 straight chain aldehydes, and α,β-unsaturated aldehydes, such as 4-hydroxynonenal and acrolein) are known to form as secondary oxidation

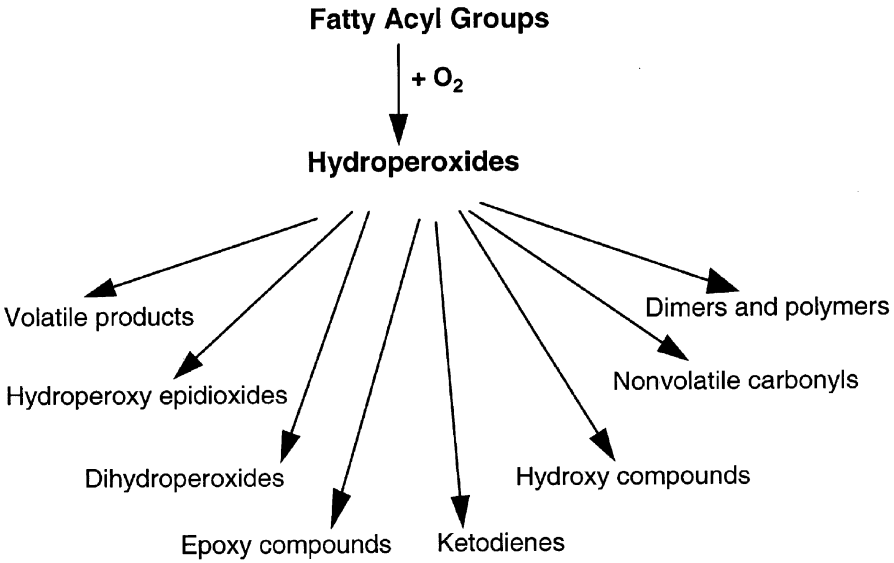


Fig. 1.6. The secondary decomposition products of hydroperoxides.

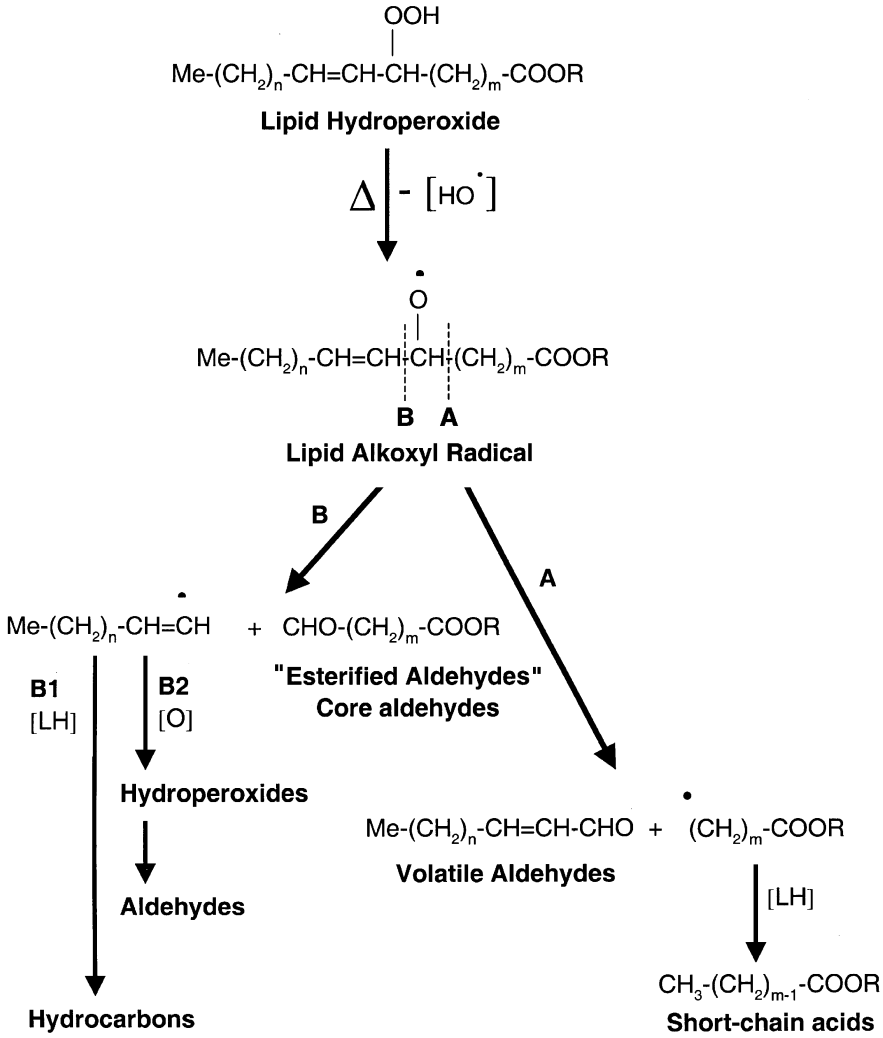


Fig. 1.7. The formation of some secondary oxidation products from alkoxyl radicals of hydroperoxides.

products of lipids (Esterbauer *et al.* 1991). Examples of toxic aldehydic products of lipid oxidation are shown in Figure 1.8.

Effect of Temperature on Hydroperoxide Reactions

As with all chemical reactions, temperature has pronounced effects on the rates and products of lipid oxidation. For example, the rates of oxidation were mentioned to

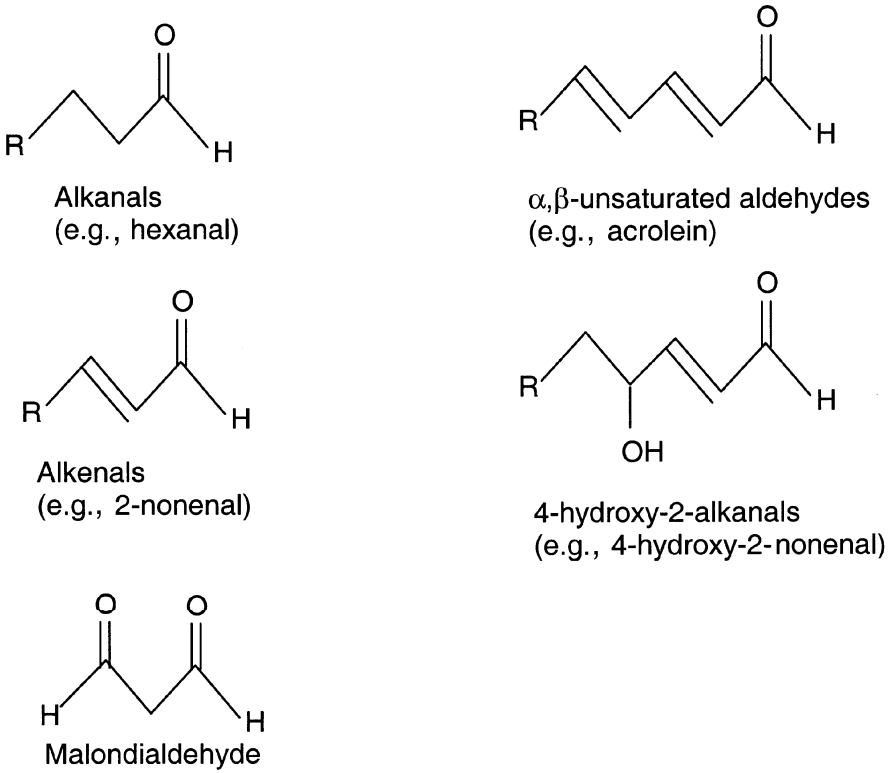


Fig. 1.8. Examples of toxic aldehydes formed by the decomposition of hydroperoxides.

increase up to twofold with every 20°C rise in temperature between 40 and 100°C (Lundberg and Chipault 1947). In agreement with the general empirical chemical rule, it was shown *vide supra* that k_p doubles with every 10°C increase in temperature. Although the propagation reaction is the rate-limiting step at ambient temperature, it is not necessarily so at high temperatures where the decomposition of hydroperoxyl radicals is enhanced and the availability of oxygen might be critical (Ragnarsson and Labuza 1977).

When oxidation experiments were performed at moderate temperatures (≤60°C) and without added catalysts, there were large variations in the length of induction period (Lea and Ward 1959, Lundberg and Chipault 1947). Frankel (1993) proposed that under mild conditions, many unknown variables control the beginning of oxidation, leading to poor reproducibility. At higher temperatures (≥80°C), the lengths of the induction periods are more stable and have been used as the criteria for stability in accelerated stability tests. However, oxidation at ≥100°C is too drastic to be relevant in studies on flavor stability and oxidative deterioration of edible oils and might mislead in mechanistic studies (Frankel 1993 and 1995). The optimum temperature of

noncatalyzed studies may be ~60°C, where the decomposition of hydroperoxides and other side reactions are minimized.

Temperature also has an effect on the further reactions of peroxyl radicals and isomerization of hydroperoxides. Allyl peroxyl radicals rearrange by a concerted 3,2 free radical pathway involving an intermediate allyl radical-dioxygen pair that collapses stereoselectively at a diffusion-controlled rate, forming a product with the opposite configuration (Porter *et al.* 1990 and 1994). Stereoselectivity of oleate peroxyl rearrangement was slightly higher at 20°C than at 50°C (Porter *et al.* 1990); in general, it improved at low temperatures and was especially high with *trans*-geometry (Porter *et al.* 1994). Dienyl peroxyl radicals rearrange by decomposing into pentadienyl radicals *via* β-fragmentation (Chan *et al.* 1979, Porter *et al.* 1986) after which the resonance-stabilized pentadienyl radicals react slowly with molecular oxygen. The rearranged hydroperoxides have *trans,trans*-geometry (Hasegawa and Patterson 1978, Simic *et al.* 1992). Thus, less β-fragmentation of dienyl peroxyl radicals occurred at 10°C than at 50°C because the ratio of *trans, cis-/trans,trans*-hydroperoxides of linoleic acid was higher at the lower temperature (Porter *et al.* 1980).

The rate of hydroperoxide decomposition increases greatly at 50–60°C (Holman 1954, Timms and Roupas 1982), indicating that there is a change in the mechanism at about this temperature. It has been shown that the ratio of all lipid molecules involved in oxidation to those radicals initiating the oxidation, which is expressed as the kinetic chain length, is increased as the temperature rises (al-Sheikhly and Simic 1989, Simic *et al.* 1992). Due to differences in activation energies of different reactions, the distribution of peroxide products is different at 50–70°C than at room temperature (Simic *et al.* 1992). In an oxidation study of methyl linoleate, most of the absorbed oxygen was always found in peroxides, but the fraction of nonperoxide oxygen increased as the oxidation temperature increased from 40 to 100°C (Lundberg and Chipault 1947).

Temperature also changes the decomposition routes of hydroperoxides. Monomolecular breakdown [reaction (1a), Scheme 1.2] is highly affected by temperature and is more relevant at high temperatures, whereas bimolecular breakdown [reactions (1b) and (1c), Scheme 1.2] are less sensitive to temperature (Chan *et al.* 1976, Hiatt and Irwin 1968, Labuza 1971). Grosch (1987) divided studies of volatile oxidation products into two categories: those performed at moderate temperatures (<80°C) and those performed at elevated temperatures (>130°C). The same oxidized flavor compounds were formed at from –10 to +50°C in milk fat (Hamm *et al.* 1968). However, more hexanal and less 2,4-heptadienal was formed at <75°C than at 85–210°C (Grosch 1987). Experiments conducted at moderate temperatures are relevant in the evaluation of storage stability of foods and those conducted at elevated temperatures in studying cooking, baking, and frying (Grosch 1987, Ragnarsson and Labuza 1977).

The temperature also has an effect on termination reactions by affecting the availability of oxygen (Badings 1970, Karel 1992, Labuza 1971). At 30°C and PV = 18,

dimers of methyl linolenate with peroxide linkages were the major products of oxidation (Miyashita *et al.* 1982a,b), suggesting that termination reactions involving peroxyl and/or alkoxyl radicals are the most important ones under mild conditions of oxidation. At 150°C, all dimers contained either ether or carbon linkages, suggesting that the termination reactions involving alkyl radicals become more important at elevated temperatures (Neff *et al.* 1988).

The Effects of Antioxidants, Examplified in Tocopherols, on Hydroperoxide Reactions

Antioxidants are compounds that are able to scavenge chain-propagating peroxyl radicals and inhibit the rate of PUFA oxidation. Although the term antioxidant is poorly defined, the broad definition of Halliwell and Gutteridge (1989), which recognizes an antioxidant as "*any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate*," is widely accepted. Antioxidants (AH) inhibit autoxidation of PUFA by competing significantly for propagating peroxyl radicals (Denisov and Khudyakov 1987). Phenolic antioxidants inhibit the propagation of PUFA oxidation because their radicals ($A^{\bullet}$) are orders of magnitude more stable than $PUFA^{\bullet}$, which react with oxygen and propagate oxidation. To be able to compete with PUFA in the above reaction, a good antioxidant should be able to easily donate a hydrogen atom to scavenge the propagating $PUFA\text{-}OO^{\bullet}$. The ease of hydrogen donation is inversely related to the bond dissociation energy (BDE) of the phenolic hydrogen. The PUFA-OO-H bond formed is characterized by a BDE of 87–88 kcal/mol (Golden *et al.* 1990), suggesting that effective antioxidants should have a BDE lower than that. Studies on the structure-activity relationship of antioxidants have shown that the presence of electron-releasing substituents (e.g. alkyl, hydroxyl, alkoxyl, amino) at the *ortho* or *para* positions enhances the antioxidant properties of phenols, demonstrating that unsubstituted and *meta*-substituted phenols are almost void of antioxidant activity (Rognisky *et al.* 1996, Zhang 1999). In this chapter, we discuss the mechanistic aspects of antioxidants exemplified in tocopherols. The discussion is complemented by Chapter 2, dedicated to the antioxidant efficacy of tocopherols in stabilizing fish oils. A more detailed examination of various antioxidants and antioxidant mechanisms is provided in Chapters 3 and 7.

Tocopherols are the best known fat-soluble antioxidants designed naturally to protect the PUFA of biological systems against oxidative degradation (Burton and Ingold 1981, Burton *et al.* 1985, Kamal-Eldin and Appelqvist 1996, Kamal-Eldin and Andersson 1997). In free radical reactions, tocopherols are known to scavenge propagating peroxyl radicals by at least two mechanisms, i.e., (i) hydrogen donation to peroxyl radicals to produce hydroperoxides Eq. [6] and (ii) formation of adduct compounds between the resulting tocopheroxyl radical and a second peroxyl radical Eq. [7].

$$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet} \quad [6]$$

$$LOO^{\bullet} + TO^{\bullet} \rightarrow TO\text{-}OOL \quad [7]$$

Evidence for the interception of peroxyl radicals ($LOO^{\bullet}$) by antioxidants was first provided by the work of Tappel (1962) and confirmed by all subsequent research. Tocopherols inhibit the propagation chains because their radicals ($TO^{\bullet}$) are several orders of magnitude more stable than those formed from the unsaturated fatty acids ($L^{\bullet}$), which react with oxygen at diffusion-controlled rates (Table 1.2). Very small levels of tocopherols are enough to provide significant inhibition of lipid oxidation (Lampi *et al.* 1997). The rate of inhibited oxidation is generally given by the following equation (Burton and Ingold 1981):

$$R_{inh} = \frac{k_p[LH]R_i}{nk_{inh}[TOH]} \quad [8]$$

The value for k_{inh} for the inhibition of oxidation of methyl linoleate by α-tocopherol was reported as 5.1×10^5 $M^{-1}s^{-1}$ at 37°C (Niki *et al.* 1984). It can be seen from Figure 1.1 that the autoxidation reaction does not enter the propagation phase until the antioxidant is nearly consumed, i.e., when the ratio $k_p[LH]/k_{inh}[TOH]$ reaches a certain critical value. As will be discussed later, this equation is not valid for high concentrations of many antioxidants (e.g., tocopherols) because these antioxidants and/or their radicals do participate in side reactions, which decrease their efficacy and cause a concentration-dependent increase in the rate of oxidation during the induction period (Fuster *et al.* 1998 and 2001, Kamal-Eldin *et al.* 2002, Lampi *et al.* 1999, Mäkinen *et al.* 2000, Yanishlieva and Marinova 1992). The inhibitory effects of tocopherols are also known to be dependent on the degree of unsaturation in the PUFA. For example, the yield of hydroperoxides per free radical initiation (the kinetic chain length) at optimum concentration of α-tocopherol was ~1–2 for oleate, ~70 for linoleate, ~140 for linolenate, and ~280 for arachidonate (Witting 1969).

In biological compartments with low oxygen concentrations, Dmitriev *et al.* (1994) suggested that the antioxidant effect of α-tocopherol is due to regeneration of the peroxyl radical back to an intact lipid molecule.

$$LOO^{\bullet} \rightarrow L^{\bullet} + O_2 \quad [9]$$

$$L^{\bullet} + TOH \rightarrow LH + TO^{\bullet} \quad [10]$$

Tocopherols also inhibit radical-catalyzed isomerization of hydroperoxides shown in Figure 1.2 by acting as hydrogen donors and inhibiting the abstraction of the hydroperoxyl hydrogen and the rearrangement of peroxyl radicals.

$$LOO_{ct}^{\bullet} \rightarrow LOO_{tt}^{\bullet} \quad [11]$$

$$LOO_{tt}^{\bullet} + LOOH_{ct} \rightarrow LOOH_{tt} + LOO_{ct}^{\bullet} \quad [12]$$

The ability of α-, γ-, and δ-tocopherols to inhibit this isomerization of methyl linoleate hydroperoxides was consistent with their hydrogen-donating ability (Mäkinen *et al.* 2001). α-Tocopherol stabilized hydroperoxides into the *cis,trans* configuration, whereas γ- and δ-tocopherols allowed partial conversion of *cis, trans*-hydroperoxides to *trans,trans* isomers. It is possible that the biological importance of α-tocopherol compared with other tocopherols is due in part to its greater efficacy in protecting the *cis,trans* configuration of hydroperoxides formed, for example, in the enzymatic oxidation of PUFA (Gardner 1996, Smith *et al.* 1991). All tocopherols inhibited hydroperoxide decomposition, but the effect was greater when tocopherols were present at intermediate than at high concentrations. At high concentrations, α-tocopherol was less efficient than γ- and δ-tocopherols as an inhibitor of methyl linoletate hydroperoxide decomposition. Thus as their concentration increased, all tocopherols experienced a loss of efficiency that was highest for α-tocopherol but was also evident for γ- and δ-tocopherols (Mäkinen *et al.* 2001).

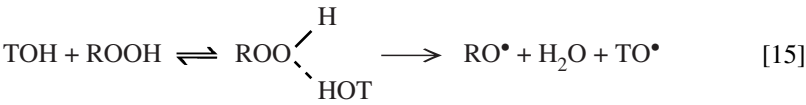
Tocopherols start to lose their antioxidant efficacy as their concentrations in lipid media increase beyond certain optima (Fuster *et al.* 1998, Gottstein and Grosch 1990, Huang *et al.* 1994, 1995, and 1996, Lampi *et al.* 1999, Mäkinen *et al.* 2000). The loss of efficacy in tocopherol antioxidant action was previously attributed to the tocopheroxyl radical ($TO^{\bullet}$) which was thought to react reversibly with unperoxidized lipids as well as with hydroperoxides by chain transfer, generating alkyl and peroxyl radicals, respectively,

$$TO^{\bullet} + LH \rightarrow TOH + L^{\bullet} \quad [13]$$

$$TO^{\bullet} + LOOH \rightarrow TOH + LOO^{\bullet} \quad [14]$$

The rate constants of the reverse reactions of the α-$TO^{\bullet}$ with the linoleate ethyl ester [13] was reported to be 1.82×10^{-2} (Mukai and Okauchi 1989, Mukai *et al.* 1993a) and the rate constant for its reaction with linoleate hydroperoxides [14] was in the range of $1–5 \times 10^{-1}$ $M^{-1}s^{-1}$ (Mukai *et al.* 1988 and 1993b). Thus, these two reactions cannot totally explain the loss of efficiency; it is sometimes called the "prooxidant effect" or "tocopherol-mediated peroxidation" that is encountered in many oxidation studies including those with low density lipoproteins (LDL) (Bowry and Stocker 1993). The reaction apparently involves abstraction of a hydrogen atom from the tocopherol and scission of the O-O bond of the hydroperoxide because this effect of tocopherols is related to their hydrogen atom-donating ability and is enhanced by the presence of high initial levels of hydroperoxides that slowly oxidize tocopherols (Hicks and Gebicki 1981). The reaction is proceeded

by H-bonding between the phenol and the peroxide (Denisov 1964, Denisov and Khudyakov 1987):

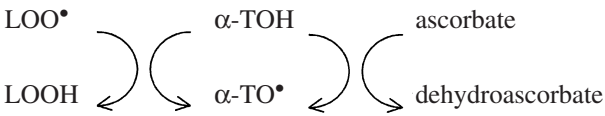


[15]

As a result of this reaction, free radicals that will initiate new reaction chains are formed. α-Tocopherol is expected to decompose the hydroperoxides at a faster rate compared with the other tocopherols because the kinetics of this reaction are determined by the hydrogen-donating power, which is greatest for this tocopherol. The decomposing effects of α-tocopherol on lipid hydroperoxides during the oxidation of methyl linoleate were studied in a model system using the response surface methodology statistical technique. Interactions among LOOH molecules and between LOOH and α-TOH molecules were found to have negative effects on the stability of α-TOH and the rate of LOOH formation and the rates of formation of hydroxy and ketodiene compounds during the induction period (Kamal-Eldin *et al.* 2002). This reaction was recently supported by kinetic evidence (Yanishlieva *et al.* 2002).

Tocopherols have characteristic effects on hydroperoxide decomposition products. As good hydrogen atom donors, all tocopherols scavenge alkoxyl radicals and inhibit their cyclization to epoxyallylic radicals as well as their decomposition to low-molecular-weight products. Frankel and Gardner (1989) showed that tocopherols inhibit the β-scission of the alkoxyl radical formed during hydroperoxide decomposition, and Hopia *et al.* (1996) suggested that tocopherols may inhibit hydroperoxide decomposition by scavenging the alkoxyl radical, thus increasing formation of hydroxy compounds.

In biological systems, α-tocopherol works as part of an antioxidant network that includes ascorbic acid and other antioxidants. The synergistic interactions between α-tocopherol and ascorbic acid are explained mainly by the regeneration and recycling of the tocopheroxyl radical intermediate to the parent tocopherol (Niki 1987, Packer *et al.* 1979, Packer 1997, Tappel 1962).



[16]

The interactions between tocopherols and ascorbic acid were first demonstrated in homogeneous systems (Packer *et al.* 1979). Later evidence was obtained of the synergism between α-tocopherol and ascorbic acid in methyl linoleate and linoleic acid micelles (Barclay *et al.* 1983) and in phosphatidylcholine liposomes (Niki *et al.* 1985). Because the decomposition of α-tocopherol occurred only after all ascorbic acid had been consumed, it was suggested that α-tocopheroxyl radicals near the aqueous phase

were regenerated by water-soluble ascorbate (Niki 1987). If the α-tocopheroxyl radicals reside in the same position as α-tocopherol, it is easy to understand the regeneration of α-tocopherol by water-soluble reducing agents such as ascorbic acid (Burton and Ingold 1986). In addition, the synergistic interactions between tocopherols and ascorbic acid may be due to the ability of ascorbic acid to act as a metal chelator and thus inhibit the initiation of oxidation chain reactions.

Interaction of α-tocopherol and ascorbic acid also accounts for the prevention of oxidation of LDL oxidation, which is of major importance in biology. Moreover, the α-tocopheroxyl radical may continue lipid oxidation instead of inhibiting it in the absence of ascorbate and ubiquinol because the radical cannot escape the LDL particle (Bowry and Stocker 1993, Ingold *et al.* 1993).

Concluding Remarks

Peroxyl radicals, alkoxyl radicals, and hydroperoxides are the major determinants of the lipid oxidation pathway, and their effects are very much related to their structures. Knowledge of the kinetics and mechanisms of hydroperoxide formation and decomposition is important for developing strategies for its control in food and biological systems including the human body and its compartments. To achieve a proper understanding, the basic lipid oxidation schemes developed in early 20th century after the innovative work at the British Rubber Research Institute must be updated and many details associated with the elementary steps of the reaction have to be specified. To date, we understand very little about the reactions that initiate lipid oxidation in the dark and in the absence of enzymes. Other aspects that require immediate research attention include the transformation of peroxide adducts into epoxy carbon-centered radicals and their role in the lipid oxidation pathway. The classical lipid oxidation mechanism also fails to explain the critical phenomena in the peroxidation reactions, e.g., the criticality in the change from the initiation to the exponential phase of oxidation, the criticality associated with minimum antioxidant concentration required for protection, and the loss of antioxidant activity beyond certain optimum concentrations. Detailed and more updated studies on the kinetics and thermodynamics of lipid oxidation that take into consideration these anomalies are clearly warranted in future research.

Appendix

Derivation of the Rate Law by the Steady-State Approximation

The steady-state approximation recognizes the oxidation as a chain reaction when the propagation reactions (1) and (2) are faster than the termination reactions (4)–(6) (Scheme 1.1). The overall oxidation rate can be given by the following expression (Labuza 1971):

$$-\mathrm{d}[O_2]/dt = d[\mathrm{LOOH}]/dt = -d[\mathrm{LH}]/dt \qquad [17]$$

From reactions [(1)–(6) Scheme 1.1],

$$d[\mathrm{LOOH}]/dt = k_3\,[\mathrm{LOO}^{\bullet}][\mathrm{LH}] \qquad [18]$$

For the solution of [18], the following three assumptions are necessary:

(i) The three rate termination steps are related by $k_5 = (k_4k_6)^{0.5}$. Thus

$$d[\mathrm{L}^{\bullet}]/dt = r_{i1} + k_3\,[\mathrm{LOO}^{\bullet}][\mathrm{LH}] - k_2\,[\mathrm{L}^{\bullet}][\mathrm{O}_2] - (k_4k_6)^{0.5}\,[\mathrm{L}^{\bullet}][\mathrm{LOO}^{\bullet}] - k_4[\mathrm{L}^{\bullet}]^2 \qquad [19]$$

and,

$$d[\mathrm{LOO}^{\bullet}]/dt = r_{i2} + k_2\,[\mathrm{L}^{\bullet}][\mathrm{O}_2] - k_3[\mathrm{LOO}^{\bullet}][\mathrm{LH}] - (k_4k_6)^{0.5}\,[\mathrm{L}^{\bullet}][\mathrm{LOO}^{\bullet}] - k_6[\mathrm{LOO}^{\bullet}]^2 \qquad [20]$$

where r_{i1}, and r_{i2} are the initial rates of production of alkyl ($L^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals, respectively.

(ii) That the Bodenstein or "steady-state" assumption holds, i.e., d[radical]$/dt \approx 0$. By assuming that the concentrations of alkyl ($L^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals do not change much with time (pseudo-steady state), then

$$R_i = r_{i1} + r_{i2} = (k_4)^{0.5}\,[\mathrm{L}^{\bullet}] + (k_6)^{0.5}\,[\mathrm{LOO}^{\bullet}]) \qquad [21]$$

(iii) When the reaction chains are long enough, the rate of reactions (2) and (3) are equal; thus

$$[\mathrm{L}^{\bullet}] = k_3\,[\mathrm{LOO}^{\bullet}][\mathrm{LH}]/k_2[\mathrm{O}_2] \qquad [22]$$

By simultaneously solving the last two equations, one obtains

$$[\mathrm{LOO}^{\bullet}] = k_2R_i^{0.5}[\mathrm{O}_2]/\{k_3(k_4)^{0.5}\,[\mathrm{LH}] + k_2(k_6)^{0.5}\,[\mathrm{O}_2]\} \qquad [23]$$

from which the rate of the overall reaction can be obtained as

$$-d[\mathrm{LH}]/dt = \frac{k_3R_i^{0.5}[\mathrm{LH}][\mathrm{O}_2]}{(k_6)^{0.5}([\mathrm{O}_2]) + \{k_3(k_3)^{0.5}[\mathrm{LH}]\}/[k_2(k_6)^{0.5}]} \qquad [24]$$

This expression is especially interesting for intermediate oxygen concentrations. For low oxygen concentrations, in which $[\mathrm{O}_2] <<< \{k_3(k_4)^{0.5}[\mathrm{LH}]\}/[k_2(k_6)^{0.5}]$, the expression can be simplified to the following form where the rate is dependent on the oxygen concentration but not the substrate concentration

$$-d[\mathrm{LH}]/dt = k_2(R_i/k_4)^{0.5}[\mathrm{O_2}] \quad [25]$$

At high oxygen concentrations, where $[\mathrm{O_2}] >>> \{k_3(k_4)^{0.5}[\mathrm{LH}]\}/[k_2(k_6)^{0.5})]$, the expression becomes dependent on the substrate concentration and independent of the oxygen concentration

$$-d[\mathrm{LH}]/dt = k_3(R_i/k_6)^{0.5}[\mathrm{O_2}] \quad [26]$$

Whenever this relationship applies, one can quantify the susceptibility of a substrate to peroxidation in terms of oxidizability {the slope of the curve of d[LOOH]/dt in mol/(L · s) (y-axis) vs. $R_i^{0.5}$[LH] (x-axis)}.

$$\text{Oxidizability} = \frac{-d[\mathrm{LH}]/dt}{R_i^{0.5}[\mathrm{LH}]} \quad [27]$$

The kinetic chain length (KCL), or the number of substrate molecules oxidized per initiating radical, can be obtained from the following equation:

$$\mathrm{KCL} = \frac{-d[\mathrm{LH}]/dt}{R_i} \quad [28]$$

The rate law [24] is changed by the addition of an inhibitor as is discussed in Chapter 3 of this book.

References

Abuja, P.M., Albertini, R., and Esterbauer, H. (1997) Simulation of the Induction of Oxidation of Low-Density Lipoprotein by High Copper Concentrations: Evidence for a Nonconstant Rate of Initiation, *Chem. Res. Toxicol. 10*, 644–651.

Adachi, S., Ishiguro, T., and Matsuno, R. (1995) Autoxidation Kinetics for Fatty Acids and Their Esters, *J. Am. Oil Chem. Soc. 72*, 547–551.

Allen, R.R., Jackson, A., and Kummerow, F.A. (1949) Factors Which Affect the Stability of Highly Unsaturated Fatty Acids. 1. Differences in the Oxidation of Conjugated and Non-Conjugated Linoleic Acid, *J. Am. Oil Chem. Soc. 26*, 395–399.

al-Sheikhly, M., and Simic, M.G. (1989) Chain-Propagation Length of Linoleic-Acid Peroxidation in Aqueous Monomeric and Micellar Systems, *J. Phys. Chem. 93*, 3103–3106.

Babbs, C.F., and Steiner, M.G. (1990) Simulation of Free Radical Reactions in Biology and Medicine, *Free Radic. Biol. Med. 8*, 471–485.

Badings, H.T. (1970) Principles of Autoxidation Processes in Lipids with Special Regard to the Development of Autoxidation Off-Flavours, *Neth. Milk Dairy J.14*, 215–242.

Barclay, L.R.C., Baskin, K.A., Locke, S.J., and Vinquist, M.R. (1989) Absolute Rate Constants for Lipid Peroxidation and Inhibition in Model Biomembranes, *Can. J. Chem. 68*, 2258–2269.

Barclay, L.R.C., Locke, S.J., and MacNeil, J. (1983) The Autoxidation of Unsaturated Lipids in Micelles. Synergism of Inhibitor Vitamins C and E, *Can. J. Chem. 61*, 1288–1290.

Barclay L.R.C., Vinqvist M.R., Antunes F., and Pinto R.E. (1997) Antioxidant Activity of Vitamin E Determined in a Phospholipid Membrane by Product Studies: Avoiding Chain Transfer Reactions by Vitamin E Radicals, *J. Am. Chem. Soc. 119*, 5764–5765.

Bascetta, E., Gunstone, F.D., Scrimgeour, C.M., and Walton J.C. (1982) E.S.R. Observation of Pentadienyl and Allyl Radicals on Hydrogen Abstraction from Unsaturated Lipids, *J. Chem. Soc. Chem. Commun. 2*, 110–112.

Bascetta, E., Gunstone, F.D., and Walton J.C. (1983) An Electron Spin Resonance Study of Fatty Acids and Esters. Part 1. Hydrogen Abstraction from Olefinic and Acetylenic Long-Chain Esters, *J. Chem. Soc. Perkin Trans. II*, 603–614.

Bateman, L. (1954) Olefin Oxidation, *Q. Rev. (London) 8*, 147–167.

Bateman, L., and Gee, G. (1951) Determination of Absolute Rate Constants for Olefinic Oxidations by Measurement of Photochemical Pre- and After-Effects. I. At High Oxygen Pressures, *Trans. Faraday Soc. 47*, 155–164.

Bateman, L., Gee, G., Morris, A.L., and Watson, W.F. (1951) The Velocity Coefficients of the Chain Propagation and Termination Reactions in Olefin Oxidation in Liquid Systems, *Disc. Faraday Soc. 10*, 250–259.

Bateman, L., Hughes, H., and Morris, A.L. (1953) Hydroperoxide Decomposition in Relation to the Initiation of Radical Chain Reactions, *Disc. Faraday Soc. 14*, 190–199.

Benzie, I.F.F. (1996) Lipid Peroxidation: A Review of Causes, Consequences, Measurement and Dietary Influences, *Int. J. Food Sci. Nutr. 47*, 233–261.

Berg, O.T.G. (1994) Heterogeneous Oxidation, *Tidskr. Teknisk Fysik 1*, 13–23.

Bergström, S. (1945) Autoxidation of Linoleic Acid, *Nature 156*, 717–718.

Bergström, S., Blomstrand, R., and Laurell, S. (1950) On the Autoxidation of Linoleic Acid in Aqueous Colloidal Solution, *Acta Chem. Scand. 4*, 245–250.

Bolland, J.L. (1946) Kinetic studies in the Chemistry of Rubber and Related Materials. I. The Thermal Oxidation of Ethyl Linoleate, *Proc. Royal Soc. (London) 186A*, 218–236.

Bolland, J.L. (1949) Kinetics of Olefin Oxidation, *Quarterly Reviews 3*, 1–21.

Bolland, J.L., and Gee, G. (1946a) Kinetic Studies in the Chemistry of Rubber and Related Materials. II. The Kinetics of Oxidation of Unconjugated Olefins, *Trans. Faraday Soc. 42*, 236–243.

Bolland, J.L., and Gee, G. (1946b) Kinetic Studies in the Chemistry of Rubber and Related Materials. III. Thermochemistry and Mechanisms of Olefin Oxidation, *Trans. Faraday Soc. 42*, 244–252.

Bolland, J.L., and Koch, H.P. (1945) Course of Autoxidation Reactions in Polyisoprenes and Allied Compounds. IX. The Primary Thermal Oxidation Product of Ethyl Linoleate, *J. Chem. Soc.*, 445–447.

Bolland, J.L., and Ten Have, P. (1947a) Kinetic Studies in the Chemistry of Rubber and Related Materials. IV. The Inhibitory Effect of Hydroquinone on the Thermal Oxidation Of Ethyl Linoleate, *Trans Faraday Soc. 43*, 201–210.

Bolland, J.L., and Ten Have, P. (1947b) Kinetic Studies in the Chemistry of Rubber and Related Materials. V. The Inhibitory Effect of Phenolic Compounds on the Thermal Oxidation of Ethyl Linoleate, *Disc. Faraday Soc. 2*, 252–263.

Bórquez, R., Koller, W.-D., Wolf, W., and Spieß, W.E.L. (1997) A Rapid Method to Determine the Oxidation Kinetics of n-3 Fatty Acids in Fish Oil, *Lebensm.-Wiss. Technol. 30*, 502–507.

Bowry, V.W., and Stocker, R. (1993) Tocopherol-Mediated Peroxidation. The Prooxidant Effect of Vitamin E on the Radical-Initiated Oxidation of Human Low-Density Lipoprotein, *J. Am. Chem. Soc. 115*, 6029–6044.

Brash, A.R. (2000) Autoxidation of Methyl Linoleate: Identification of the bis-Allylic 11-Hydroperoxide, *Lipids 35*, 947–952.

Brimberg, U.I. (1991) The Kinetics of the Autoxidation of Fats, *Fat Sci. Technol. 8*, 298–303.

Brimberg, U.I. (1993a) On the Kinetics of the Autoxidation of Fats, *J. Am. Oil Chem. Soc. 70*, 249–254.

Brimberg, U.I. (1993b) On the Kinetics of the Autoxidation of Fats. II. Monounsaturated Substrates, *J. Am. Oil Chem. Soc. 70*, 1063–1067.

Bruna, E., Petit, E., Beljean-Leymarie, M., Huynh, S., and Nouvelot, A. (1989) Specific Susceptibility of Docosahexaenoic Acid and Eicosapentaenoic Acid to Peroxidation in Aqueous Solution, *Lipids 24*, 970–975.

Burton, G.W., Doba, T., Gabe, E.J., Hughes, L., Lee, F.L., Prasad, L., and Ingold, K.U. (1985) Autoxidation of Biological Molecules. 4. Maximizing the Antioxidant Activity of Phenols, *J. Am. Chem. Soc. 107*, 7053–7065.

Burton, G.W., and Ingold, K.U. (1981) Autooxidation of Biological Molecules. I. The Antioxidant Activity of Vitamin E and Related Chain-Breaking Phenolic Antioxidants *In Vitro*, *J. Am. Chem. Soc. 103*, 6472–6477.

Burton, G.W. and Ingold, K.U. (1986) Vitamin E: Application of the Principles of Physical Organic Chemistry to the Exploration of Its Structure and Function, *Acc. Chem. Res. 19*, 194–201.

Cadenas, E., and Sies, H. (1998) The Lag Phase, *Free Radic. Res. 28*, 601–609.

Chahine, M.H., and de Man, J.M. (1971) Autoxidation of Corn Oil under the Influence of Fluorescent Light, *Can. Inst. Food Technol. J.4*, 24–28.

Chamulitrat, W., and Mason, R.P. (1989) Lipid Peroxyl Radical Intermediates in the Peroxidation of Polyunsaturated Fatty Acids by Lipoxygenase, *J. Biol. Chem. 264*, 20968–20973.

Chan, H.W.-S. (1987) *Autoxidation of Unsaturated Lipids*, Academic Press, London.

Chan, H.W.-S., and Coxon, D.T. (1987) Lipid Hydroperoxides, in *Autoxidation of Unsaturated Lipids* (Chan, H.W.-S., ed.) pp. 17–50. Academic Press, London.

Chan, H.W.-S., and Levett, G. (1977a) Autoxidation of Methyl Linoleate. Separation and Analysis of Isomeric Mixtures of Methyl Linoleate Hydroperoxides and Methyl Hydroxy Linoleates, *Lipids 12*, 99–104.

Chan, H.W.-S., and Levett, G. (1977b) Autoxidation of Methyl Linolenate: Analysis of Methyl Hydroxylinolenate Isomers by High-Performance Liquid Chromatography, *Lipids 12*, 837–840.

Chan, H.W.-S., Levett, G., and Matthew, A. (1978) Thermal Isomerization of Methyl Linoleate Hydroperoxides. Evidence of Molecular Oxygen as a Leaving Group in a Radical Rearrangement, *J. Chem. Soc. Chem. Commun.*, 756–757.

Chan, H.W.-S., Levett, G., and Matthew, A. (1979) The Mechanism of the Rearrangement of Linoleate Hydroperoxides, *Chem. Phys. Lipids 24*, 245–256.

Chan, H.W.-S., Matthew, J.A. and Coxon, D.T. (1980) A Hydroperoxy-Epidioxide from the Autoxidation of a Hydroperoxide of Methyl Linolenate, *J. Chem. Soc. Chem. Commun.*, 235–236.

Chan, H.W.-S., Prescott, F.A.A., and Swoboda, P.A.T. (1976) Thermal Decomposition of Individual Positional Isomers of Methyl Linoleate Hydroperoxide: Evidence of Carbon-Oxygen Bond Scission, *J. Am. Oil. Chem. Soc. 53*, 572–576.

Chan, N.A., Lundberg, W.O., and Khan, J.A. (1954) Displacement Analysis of Lipids. IX. Products of the Oxidation of Methyl Linoleate, *J. Am. Chem. Soc. 76*, 1179–1784.

Chien, J.T., Wang, H.C., and Chen, B.H. (1998) Kinetic Model of the Cholesterol Oxidation During Heating, *J. Agric. Food Chem. 46*, 2572–2577.

Cosgrove, J.P., Church, D.F., and Pryor, W.A. (1987) The Kinetics of the Autoxidation of Polyunsaturated Fatty Acids, *Lipids 22*, 299–304.

Coxon, D.T., Price, K.R., and Chan, H.W.-S. (1981) Formation, Isolation and Structure Determination of Methyl Linolenate Diperoxides, *Chem. Phys. Lipids 28*, 365–378.

Crapiste, G.H., Brevedan, M.I.V., and Carelli, A.A. (1999) Oxidation of Sunflower Oil During Storage, *J. Am. Oil Chem. Soc. 76*, 1437–1443.

Davies, D.S., Goldsmith, H.L., Gupta, A.K., and Lester, G.R. (1956) Radical Capture Agents in Tetralin: Measurement of Relative Efficiencies and Correlation with Structure, *J. Chem. Soc. (London)*, 4926–4933.

Denisov, E.T. (1964) The Role of Hydrogen Bonding in Generation of Free Radicals from Hydroperoxide, *Zh. Fiz. Khim. 38*, 2085.

Denisov, E.T., and Khudyakov, I.V. (1987) Mechanism of Action and Reactivities of the Free Radical Inhibitors, *Chem. Rev. 87*, 1313–1357.

Dikalov, S.I., and Mason, R.P. (1999) Reassignment of Organic Peroxyl Radical Adducts, *Free Radic. Biol. Med. 27*, 864–872.

Dikalov, S.I., and Mason, R.P. (2001) Spin Trapping of Polyunsaturated Fatty Acid-Derived Peroxyl Radicals: Reassignment to Alkoxyl Radical Adducts, *Free Radic. Biol. Med. 30*, 187–197.

Dmitriev, L.F., Ivanova, M.V. and Lankin, V.Z. (1994) Interaction of Tocopherol with Peroxyl Radicals Does Not Lead to the Formation of Lipid Hydroperoxides in Liposomes, *Chem. Phys. Lipids 69*, 35–39.

Dutta, P.C. (1997) Studies on Phytosterol Oxides. 2. Content in Some Vegetable Oils and in French Fries Prepared in These Oils, *J. Am. Oil Chem. Soc. 74*, 659–666.

Emanuel, N.M., and Yu. N. Lyaskovskaya (1967) *The Inhibition of Fat Oxidation Processes* (translated by K.A. Allen) Pergamon Press, Oxford.

Endo, Y., Hoshizaki, S., and Fujimoto, K. (1997) Kinetics for the Oxidation of Triacylglycerols Containing Eicosapentaenoic Acid, *Biosci. Biotechnol. Biochem. 61*, 1036–1037.

Esterbauer, H., Schaur, R.J., and Zollner, H. (1991) Chemistry and Biochemistry of 4-Hydroxynonenal, Malonaldehyde and Related Aldehydes, *Free Radic. Biol. Med. 11*, 81–128.

Farmer, E.H. (1945) Peroxidation in Relation to Olefinic Structure, *Trans. Faraday Soc. 42*, 228–236.

Frankel, E.N. (1991) Recent Advances in Lipid Oxidation, *J. Sci. Food Agric. 54*, 495–511.
Frankel. E.N. (1993) In Search of Better Methods to Evaluate Natural Antioxidants and Oxidative Stability in Food Lipids, *Trends Food Sci. Technol. 4*, 220–225.
Frankel, E.N. (1995) Natural and Biological Antioxidants in Foods and Biological Systems. Their Mechanism of Action, Applications and Implications, *Lipid Technol. 7*, 77–80.
Frankel, E.N. (1998) *Lipid Oxidation*, The Oily Press, Dundee, Scotland.
Frankel, E.N., and Gardner, H.W. (1989) Effect of α-Tocopherol on the Volatile Thermal Decomposition Products of Methyl Linoleate Hydroperoxides, *Lipids 24*, 603–608.
Frankel, E.N., Garwood, R.F., Khambay, B.P., Moss, G.P., and Weedon, B.C. (1984) Stereochemistry of Olefin and Fatty Acid Oxidation. Part 3. The Allylic Hydroperoxides from the Autoxidation of Methyl Oleate, *J. Chem. Soc. Perkin Trans. 1*, 2233–2240.
Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weedon, B.C.L. (1977a) Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: I. Methyl Oleate, *Lipids 12*, 901–907.
Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weedon, B.C.L. (1977b) Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: II. Methyl Linoleate, *Lipids 12*, 908–913.
Frankel, E.N., Neff, W.E., Rohwedder, W.K., Khambay, B.P.S., Garwood, R.F., and Weedon, B.C.L. (1977c) Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: III. Methyl Linolenate, *Lipids 12*, 1055–1061.
Fuster, M.D., Lampi, A.-M., Hopia, A., and Kamal-Eldin, A. (1998) Effects of α- and γ-Tocopherols on the Autoxidation of Purified Sunflower Triacylglycerols, *Lipids 33*, 715–722.
Gardner, H.W. (1975) Decomposition of Linoleic Acid Hydroperoxides: Enzymic Reactions Compared with Nonenzymic, *J. Agric. Food Chem. 23*, 129–136.
Gardner, H.W. (1987) Reactions of Hydroperoxides: Products of High Molecular Weight, in *Autoxidation of Unsaturated Lipids* (Chan, H.W.-S., ed.) pp. 51–93, Academic Press, London.
Gardner, H.W. (1989) Oxygen Radical Chemistry of Polyunsaturated Fatty Acids, *Free Radic. Biol. Med. 7*, 65–86.
Gardner, H.W. (1991) Recent Investigations into the Lipoxygenase Pathway of Plants, Biochim. Biophys. Acta 1084, 221–239.
Gardner, H.W. (1996) Lipoxygenase as a Versatile Biocatalyst, *J. Am. Oil Chem. Soc. 73*, 1347–1357.
George, P., Rideal, E.K., and Robertson, A. (1946) The Oxidation of Liquid Hydrocarbons. I. The Chain Formation of Hydroperoxides and Their Decomposition, *Proc. R. Soc. (London) A185*, 288–309.
George, P., and Robertson, A. (1946) The Oxidation of Liquid Hydrocarbons. II. The Energy-Chain Mechanism for the Thermal Oxidation of 1,2,3,4-Tetrahydronaphthalene, *Proc. R. Soc. (London) A185*, 309–336.
Golden, D.M., Bierbaum, V.M., and Howard, C.J. (1990) Comments on "Reevaluation of the Bond Dissociation Energies (DHBDE) for H-OH, H-OOH, H-O$^-$, H-O$^\bullet$, H-OO$^-$, H-OO$^\bullet$," *J. Phys. Chem. 94*, 5413–5415.
Gottstein, T., and Grosch, W. (1990) Model Study of Different Antioxidant Properties of α- and γ-Tocopherol in Fats, *Fat Sci. Technol. 92*, 139–144.

Grosch, W. (1987) Reactions of Hydroperoxides—Products of Low Molecular Weight, in *Autoxidation of Unsaturated Lipids* (Chan, H.W.-S., ed.), pp. 95–139, Academic Press, London.

Gruger, E.H., and Tappel, A.L. (1970) Reactions of Biological Antioxidants: I. Fe(III)-Catalyzed Reactions of Lipid Hydroperoxides with α-Tocopherol, *Lipids 5*, 326–331.

Halliwell, B., and Gutteridge, J.M.C. (1989) *Free Radicals in Biology and Medicine*, 2nd edn., p. 236, Clarendon Press, Oxford.

Hamberg, M. (1975) Decomposition of Unsaturated Fatty Acid Hydroperoxides by Hemoglobin: Structures of Major Products of 13L-Hydroperoxy-9,11-Octadecadienoic Acid, *Lipids 10*, 87–92.

Hamberg, M. (1983) Autoxidation of Linoleic Acid. Isolation and Structure of Four Dihydroxyoctadecadienoic Acids, *Biochim. Biophys. Acta 752*, 353–356.

Hamberg, M., and Gotthammar, B. (1973) New Reaction of Unsaturated Fatty Acid Hydroperoxides. Formation of 11-Hydroxy-12,13-epoxy-9-octadecenoic Acid from 13-Hydroperoxy-9,11-octadecadienoic Acid, *Lipids 8*, 737–744.

Hamm, D.L., Hammond, E.G., and Hotchkiss, D.K. (1968) Effect of Temperature on Rate of Autoxidation of Milk Fat, *J. Dairy Sci. 51*, 483–491.

Hasegawa, K., and Patterson, L.K. (1978) Pulse Radiolysis in Model Lipid Systems: Formation and Behaviour of Peroxyl Radicals in Fatty Acids, *Photochem. Photobiol. 28*, 817–823.

Haslbeck, F., and Grosch, W. (1983) Autoxidation of Phenyl Linoleate and Phenyl Oleate: HPLC Analysis of the Major and Minor Monohydroperoxides as Phenyl Hydroxystearates, *Lipids 18*, 706–713.

Haslbeck, F., Grosch, W., and Firl, J. (1983) Formation of Hydroperoxides with Uniconjugated Diene Systems During Autoxidation and Enzymatic Oxygenation of Linoleic Acid, *Biochem. Biophys. Acta 750*, 185–193.

Heaton, F.W., and Uri, N. (1961) The Aerobic Oxidation of Unsaturated Fatty Acids and Their Esters: Cobalt Stearate-Catalyzed Oxidation of Linoleic Acid, *J. Lipid Res. 2*, 152–160.

Hiatt, R.R. (1975) Hydroperoxide Destroyers and How They Work? *CRC Crit. Rev. Food Sci. Nutr. 7*, 1–12.

Hiatt, R.R., and Irwin, K.C. (1968) Homolytic Decomposition of Hydroperoxides. V. Thermal Decomposition, *J. Org. Chem. 33*, 1436–1441.

Hiatt, R.R., Mill, T., Irwin, K.C., and Castleman, J.K. (1968b) Homolytic Decomposition of Hydroperoxides. III. Radical-Induced Decompositions of Primary and Secondary Hydroperoxides, *J. Org. Chem. 33*, 1428–1430.

Hiatt, R.R., Mill, T., Irwin, K.C., and Gould, C.W. (1968c) Homolytic Decomposition of Hydroperoxides. IV. Metal-Catalyzed Decompositions, *J. Org. Chem. 33*, 1430–1436.

Hiatt, R.R., Mill, T., and Mayo, F.R. (1968a) Homolytic Decomposition of Hydroperoxides. I. Summary and Implications for Autoxidation, *J. Org. Chem. 33*, 1416–1420.

Hicks, M., and Gebicki, J.M. (1981) Inhibition of Peroxidation in Linoleic Acid Membranes by Nitroxide Radicals, Butylated Hydroxy Toluene and α-Tocopherol, *Arch. Biochem. Biophys. 210*, 56–63.

Holman, R.T. (1954) Autoxidation of Fats and Related Substances, *Prog. Chem. Fats Other Lipids 2*, 51–98.

Holman, R.T., and Elmer, O.C. (1947) The Rates of Oxidation of Unsaturated Fatty Acids and Esters, *J. Am. Oil Chem. Soc. 24*, 127–129.

Hopia, A., Huang, S.-W., and Frankel, E.N. (1996) Effect of α-Tocopherol and Trolox on the Decomposition of Methyl Linoleate Hydroperoxides, *Lipids 31*, 357–365.

Howard, J.A., and Ingold, K.U. (1967) Absolute Rate Constants for Hydrocarbon Autoxidation. VI. Alkyl Aromatic and Olefinic Hydrocarbons, *Can. J. Chem. 45*, 793–802.

Huang, S.W., Frankel, E.N., and German, J.B. (1994) Antioxidant Activity of Alpha-Tocopherols and Gamma-Tocopherols in Bulk Oils and in Oil-in-Water Emulsions, *J. Agric. Food Chem. 42*, 2108–2114.

Huang, S.W., Frankel, E.N., and German, J.B. (1995) Effects of Individual Tocopherols and Tocopherol Mixtures on The Oxidative Stability of Corn-Oil Triglycerides, *J. Agric. Food Chem. 43*, 2345–2350.

Huang, S.W., Hopia, A., Schwarz, K., Frankel, E.N., and German, J.B. (1996) Antioxidant Activity of Alpha-Tocopherol and Trolox in Different Lipid Substrates: Bulk Oils vs Oil-In-Water Emulsions, *J. Agric. Food Chem. 44*, 444–452.

Ingold, K.U. (1969) Peroxy Radicals, *Acc. Chem. Res. 2*, 1–9.

Ingold, K.U., Bowry, V.W., Stocker, R., and Walling, C. (1993) Autoxidation of Lipids and Antioxidation by α-Tocopherol and Ubiquinol in Homogeneous Solution and in Aqueous Dispersions of Lipids: Unrecognized Consequences of Lipid Particle Size as Exemplified by Oxidation of Human Low-Density Lipoprotein, *Proc. Natl. Acad. Sci. USA 90*, 45–49.

Johnston, A.E., Ziltch, K.T., Selke, E., and Dutton, H.J. (1961) Analysis of Fatty Acid Oxidation Products by Countercurrent Distribution Methods. V. Low-Temperature Decomposition of Methyl Linoleate Hydroperoxide, *J. Am. Oil Chem. Soc. 38*, 367–371.

Kamal-Eldin, A., and Andersson, R. (1997) A Multivariate Study of the Correlation Between Tocopherol Content and Fatty Acid Composition in Different Vegetable Oils, *J. Am. Oil Chem. Soc. 74*, 375–380.

Kamal-Eldin, A., and Appelqvist, L.-Å. (1996) The Chemistry and Antioxidant Properties of Tocopherols and Tocotrienols, *Lipids 31*, 671–701.

Kamal-Eldin, A., Mäkinen, M., Lampi, A-M., and Hopia, A. (2002) A Multivariate Study of α-Tocopherol and Hydroperoxide Interaction During the Oxidation of Methyl Linoleate, *Eur. Food Res. Technol. 214*, 52–57.

Kanazawa, K., Mori, T., and Matsushita, S. (1973) Oxygen Absorption at the Process of the Degradation of Linoleic Acid Hydroperoxides, *J. Nutr. Vitaminol. 19*, 263–275.

Kaouadji, M.N., Jore, D., Ferradini, C., and Patterson, L.K. (1987) Radiolytic Scanning of Vitamin E/Vitamin C Oxidation Reduction Mechanisms, *Bioelectrochem. Bioenerg. 18*, 59–70.

Karel, M. (1992) Kinetics of Lipid Oxidation, in *Physical Chemistry of Foods* (Schwartzberg, H.G., and Hartel, R.W., eds.) pp. 651–668, Marcel Dekker, New York.

Kasaikina, O.T., Kortenska, V.D., and Yanishlieva, N.V. (1999) Effect of Chain Transfer and Recombination/Disproportionation of Inhibitor Radicals on Inhibited Oxidation of Lipids, *Russ. Chem. Bull. 48*, 1891–1896.

Kim, S.K., and Nawar, W.W. (1993) Parameters Influencing Cholesterol Oxidation, *Lipids 28*, 917–922.

Knorre, D.G., Maizus, Z.K., Obukhova, L.K., and Emanuel, N.M. (1957) Modern Concepts of the Mechanism of Oxidation of Hydrocarbons in Liquid Phase, *Usp. Khim. 26*, 416–458.

Koelewijn, P. (1972) Epoxidation of Olefins by Alkylperoxy Radicals, *Recl. Trav. Chim. Pays-Bas 91*, 759.

Labuza, T.P. (1971) Kinetics of Lipid Oxidation in Foods, *CRC Crit. Rev. Food Technol. 2*, 355–405.

Lampi, A.-M., Hopia, A., and Piironen, V. (1997) Antioxidant Activity of Minor Amounts of γ-Tocopherol in Natural Triacylglycerols, *J. Am. Oil Chem. Soc. 74*, 549–555.

Lampi, A.-M., Kataja, L., Kamal-Eldin, A., and Piironen, V. (1999) Antioxidant Activities of α- and γ-Tocopherols in the Oxidation of Rapeseed Oil Triacylglycerols, *J. Am. Oil Chem. Soc. 76*, 749–755.

Lea, C.H., and Ward, R.J. (1959) Relative Antioxidant Activities of the Seven Tocopherols, *J. Sci. Food Agric. 10*, 537–548.

Lundberg, W.O., and Chipault, J.R. (1947) The Oxidation of Methyl Linoleate at Various Temperatures, *J. Am. Oil Chem. Soc. 69*, 833–836.

Mäkinen, M., Kamal-Eldin, A., Lampi, A-M., and Hopia, A. (2000) Effects of α- and γ-Tocopherols on Formation of Hydroperoxides and Two Decomposition Products from Methyl Linoleate, *J. Am. Oil Chem. Soc. 77*, 801–806.

Mäkinen, M., Kamal-Eldin, A., Lampi, A-M., and Hopia, A. (2001) α-, γ- and δ-Tocopherols as Inhibitors of Isomerization and Decomposition of *cis,trans* Methyl Linoleate Hydroperoxides, *Eur. J. Lipid Sci. Technol. 103*, 286–291.

Mayo, F.R. (1968) Free Radical Autoxidation of Hydrocarbons, *Acc. Chem. Res. 1*, 193–201.

Miyashita, K., Fujimoto, K., and Kaneda, T. (1982a) Formation of Dimers During the Initial Stage of Autoxidation in Methyl Linoleate, *Agric. Biol. Chem. 46*, 751–755.

Miyashita, K., Fujimoto, K., and Kaneda, T. (1982b) Structures of Dimers Produced from Methyl Linoleate During Initial Stage of Autoxidation, *Agric. Biol. Chem. 46*, 2293–2297.

Miyashita, K., Hara, N., Fujimoto, K., and Kaneda, T. (1985a) Dimers Formed in Oxygenated Methyl Linoleate Hydroperoxides, *Lipids 20*, 578–587.

Miyashita, K., Hara, N., Fujimoto, K., and Kaneda, T. (1985b) Decomposition Products of Dimers Arising From Secondary Oxidation of Methyl Linoleate Hydroperoxides, *Agric. Biol. Chem. 49*, 2633–2640.

Miyashita, K., Hirao, M., Nara, E., Ota, T. (1995) Oxidative Stability of Triglycerides from Orbital Fat of Tuna and Soybean Oil in An Emulsion, *Fish. Sci. 61*, 273–275.

Miyashita, K., and Takagaki, T. (1986) Study on the Oxidation Rate and Prooxidant Activity of Free Fatty Acids, *J. Am. Oil Chem. Soc. 63*, 1380–1384.

Mukai, K., Kohno, Y., and Ishizu, K. (1988) Kinetic Study of the Reaction Between Vitamin E Radical and Alkyl Hydroperoxides in Solution, *Biochem. Biophys. Res. Commun. 155*, 1046–1050.

Mukai, K., and Okauchi, Y. (1989) Kinetic Study of the Reaction between Tocopheroxyl Radical and Unsaturated Fatty Acid Esters in Benzene, *Lipids 24*, 936–939.

Mukai, K., Morimoto, H., Okauchi, Y., and Nagaoka, S. (1993a) Kinetic Study of Reactions Between Tocopheroxyl Radicals and Fatty Acids, *Lipids 28*, 753–756.

Mukai, K., Sawada, K., Kohno, Y., and Terao, J. (1993b) Kineitic Study of the Prooxidant Effect of Tocopherol. Hydrogen Abstraction from Lipid Hydroperoxides by Tocopheroxyls in Solution, *Lipids 28*, 747–752.

Neff, W.E., Frankel, E.N., and Fujimoto, K. (1988) Autoxidative Dimerization of Methyl Linolenate and Its Monohydroperoxides, Hydroperoxy Epidioxides, and Dihydroperoxides, *J. Am. Oil Chem. Soc. 65*, 616–623.

Neff, W.E., Frankel, E.N., Scholfield, C.R., and Weisleder, D. (1978) High-Pressure Liquid Chromatography of Autoxidized Lipids: I. Methyl Oleate and Linoleate, *Lipids 13*, 415–421.

Niki, E. (1987) Interaction of Ascorbate and α-Tocopherol, Ann. N.Y. Acad. Sci. 498, 186–199.

Niki, E., Kawakami, A., Yamamoto, Y., and Kamiya, Y. (1985) Oxidation of Lipids. VIII. Synergistic Inhibition of Oxidation of Phosphatidylcholine Liposome in Aqueous Dispersion by Vitamin E and Vitamin C, B*ull. Chem. Soc. Jpn. 58*, 1971–1975.

Niki, E., Saito, T., Kawakami, A., and Kamiya, Y. (1984) Inhibition of Oxidation of Methyl Linoleate in Solution by Vitamin E and Vitamin C, *J. Biol. Chem. 259*, 4177–4182.

Ozawa, T., Hayakawa, M., Takamura, T., Sugiyama, S., Suzuki, K., Iwata, M., Taki, F., and Tomita, T. (1986) Biosyntehsis of Leukotozin, 9,10-Epoxy-12-octadecenoate by Leukocytes in Lung Lavages of Rat After Exposure to Hyperoxia, *Biochem. Biophys. Res. Commun. 134*, 1071–1078.

Özilgen, S., and Özilgen, M. (1990) Kinetic Model of Lipid Oxidation in Foods, *J. Food Sci. 55*, 498–501.

Packer, J.E., Slater, T.F., and Willson, R.L. (1979) Direct Observation of a Free Radical Interaction Between Vitamin E and Vitamin C, *Nature 278*, 737–738.

Packer, L. (1997) Vitamin C and Redox Cycling Antioxidants, in *Vitamin C in Health and Disease* (Packer, L., and Fuchs, J., eds.) pp. 95–107, Marcel Dekker, New York.

Peers, K.E., Coxon, D.T., and Chan, H.W.-S. (1981) Autoxidation of Methyl Linolenate and Methyl Linoleate: The Effect of α-Tocopherol, *J. Sci. Food Agric. 32*, 898–904.

Porter, N.A. (1986) Mechanisms for the Autoxidation of Polyunsaturated Lipids, *Acc. Chem. Res. 19*, 262–268

Porter, N.A., Caldwell, S.E., and Mills, K.A. (1995) Mechanisms of Free Radical Oxidation of Unsaturated Lipids, *Lipids 30*, 277–290.

Porter, N.A., Kaplan, J.K., and Dussault, P.H. (1990) Stereoselective Acyclic 3,2 Peroxyl Radical Rearrangements, *J. Am. Chem. Soc. 112*, 1266–1267.

Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) Unified Mechanism for Polyunsaturated Fatty Acid Autooxidation of Peroxy Radical Hydrogen Atom Abstraction, β-Scission and Cyclization, *J. Am. Chem. Soc. 103*, 6447–6455.

Porter, N.A., Mills, K.A., and Carter, R.L. (1994) A Mechanistic Study of Oleate Autooxidation: Competing Peroxyl H-Atom Abstraction and Rearrangement, *J. Am. Chem. Soc. 116*, 6690–6696.

Porter, N.A., Weber, B.A., Weenen, H., and Khan, J.A. (1980) Autoxidation of Polyunsaturated Lipids. Factors Controlling the Stereochemistry of Product Hydroperoxides, *J. Am. Chem. Soc. 102*, 5597–5601.

Privett, O.S., and Blank, M.L. (1962) The Initial Stages of Autooxidation, *J. Am. Oil Chem. Soc. 39*, 465–469.

Pryor, W.A. (1986) Oxy Radicals and Related Species: Their Formation, Lifetimes and Reactions, *Annu. Rev. Physiol. 48*, 656–667.

Ragnarsson, J.O., and Labuza, T.P. (1977) Accelerated Shelf-Life Testing for Oxidative Rancidity In Foods—A Review, *Food Chem. 2*, 291–308.

Rawls, H.R., and van Santen, P.J. (1968) Singlet Oxygen and the Initiation of Fatty Acid Autoxidation, *Tetrahedron Lett. 14*, 1675–1678.

Rawls, H.R., and van Santen, P.J. (1970) A Possible Role for Singlet Oxygen in The Initiation of Fatty Acid Autoxidation, *J. Am. Oil Chem. Soc. 47*, 121–125.

Reich, L., And Stivala, S.S. (1969) *Autoxidation of Hydrocarbons and Polyolefins*, Marcel Dekker, New York.

Remorova, A.A., and Roginsky, V.A. (1991) Rate Constants for the Reaction of α-Tocopherol Phenoxy Radicals with Unsaturated Fatty Acid Esters and the Contribution of this Reaction to the Kinetics of Inhibition of Lipid Peroxidation, *Kinet. Catal. 32*, 726–731.

Roginsky, V.A., Barsukova, T.K., Remorova, A.A., and Bors, W. (1996) Moderate Antioxidative Efficiencies of Flavonoids During Peroxidation of Methyl Linoleate in Homogeneous and Micellar Solutions, *J. Am. Oil Chem. Soc. 73*, 777–786.

Russel, G.A. (1956) The Rates of Oxidation of Aralkyl Hydrocarbons. Polar Effects in Free Radical Reactions, *J. Am. Chem. Soc. 78*, 1047–1054.

Russell, G.A. (1957) Deuterium-Isotope Effects in The Autoxidation of Aralkyl Hydrocarbons. Mechanism of the Interaction of Peroxyl Radicals, *J. Am. Chem. Soc. 79*, 3871–3877.

Schieberle, P., and Grosch, W. (1981a) Detection of Monohydroperoxides with Unconjugated Diene Systems as Minor Products of the Autoxidation of Methyl Linoleate, *Z. Lebensm.-Unters.-Forsch 173*, 199–203.

Schieberle, P., and Grosch, W. (1981b) Decomposition of Linoleic Acid Hydroperoxides by Radicals. II. Breakdown of Methyl 13-Hydroperoxy-*cis,trans*-9,11-octadecadienoate by Radicals of Copper II Ions, *Z.-Lebensm.-Unters.-Forsch 173*, 192–198.

Schieberle, P., Tsoukalas, B., and Grosch, W. (1979) Decomposition of Linoleic Acid Hydroperoxides by Radicals. I. Structures of Products of Methyl 13-Hydroperoxy-*cis,trans*-9,11-octadecadienoate, *Z.-Lebensm.-Unters.-Forsch 168*, 448–456.

Shelton, J.R., and Vincent, D.N. (1963) Retarded Autoxidation and the Chain-Stopping Action of Inhibitors, *J. Am. Chem. Soc. 85*, 2433–2439.

Simic, M.G., Jovanovic, S.V., and Niki, E. (1992) Mechanisms of Lipid Oxidative Processes and Their Inhibition, in *Lipid Oxidation in Food*, ACS Symposium Series 500 (St. Angelo, A.J., ed.) pp. 14–32, American Chemical Society, Washington.

Small, R.D. Jr., Scaiano, J.C., and Patterson, L.K. (1979) Radical Processes in Lipids. A Laser Photolysis Study of t-Butoxy Radical Reactivity Toward Fatty Acids, *Photochem. Photobiol. 29*, 49–51.

Smith, L.L., Teng, J.I., Lin, Y.Y., Seitz, P.K. (1982) Lipid Peroxidation of Cholesterol, in *Proc. Int. Conf. on Lipid Peroxides in Biology and Medicine 1980* (Yagi, K., ed.) pp. 89–105, Academic Press, New York.

Smith, W.L., Borgeat, P., and Fitzpatrick, F.A. (1991) The Eicosanoids: Cyclooxygenase, Lipoxygenase, and Epoxygenase Pathways in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D.E., and Vance, J., eds.) pp. 297–325, Elsevier Science Publishers, B.V., Amsterdam.

Sugiyama, S., Hayakawa, M., Nagai, S., Ajioka, M., and Qzawa, T. (1987) Leukotoxin (9,10-Epoxy-12-octadecenoate) Causes Cardiac Failure in Dogs, *Life Sci. 40*, 225–231.

Tallman, K.A., Pratt, D.A., and Porter, N.A. (2001) Kinetic Products of Linoleate Peroxidation: Rapid β-Fragmentation of Nonconjugated Peroxyls, *J. Am. Chem. Soc. 123*, 11827–11828.

Tappel, A.L. (1962) Vitamin E as the Biological Antioxidant, *Vitam. Horm. 20*, 493–510.
Terao, J., and Matsushita, S. (1975) Further Oxygenated Compounds Produced from Methyl Linoleate Monohydroperoxides at the Process of Autoxidation, *Agric. Biol. Chem. 39*, 2027–2033.
Terao, J., Ogata, T., and Matsushita, S. (1975) Degradation Process of Autoxidized Methyl Linoleate, *Agric. Biol. Chem. 39*, 397–402.
Timms, R.E., and Roupas, P. (1982) The Application of Chemiluminescence to Study of the Oxidation of Oils and Fats, *Lebensm.-Wiss. Technol. 15*, 372–377.
Uri, N. (1956) Metal-Ion Catalysis and Polarity of Environment in the Aerobic Oxidation of Unsaturated Fatty Acids, *Nature 177*, 1177–1178.
Wagner, B.A., Buettner, G.R., and Burns, C.P. (1994) Free Radical-Mediated Lipid Peroxidation in Cells: Oxidizability Is a Function of Cell Lipid *bis*-Allylic Hydrogen Content, *Biochemistry 33*, 4449–4453.
Walling, C., and Heaton, L. (1965) Hydrogen Bonding and Complex Formation in Solutions of t-Butyl Hydroperoxides, *J. Am. Chem. Soc. 87*, 48–51.
Walther, U., and Spiteller, G. (1993) Formation of Oleic Acid Epoxide During the Storage of Technical Quality Oleic Acid, *Fat Sci. Technol. 95*, 472–474.
Wang, X.-H., Ohshima, T., Ushio, H., and Koizumi, C. (1999) Proportion of Geometrical Hydroperoxide Isomers Generated by Radical Oxidation of Methyl Linoleate in Homogeneous Solution and in Aqueous Emulsion, *Lipids 34*, 675–679.
Wilcox, A.L., and Marnett, L.J. (1993) Polyunsaturated Fatty Acid Alkoxyl Radicals Exist as Carbon-Centered Epoxyallylic Radicals: A Key Step in Hydroperoxide-Amplified Lipid Peroxidation, *Chem. Res. Toxicol. 6*, 413–416.
Witting, L.A. (1965) Lipid Peroxidation *In Vivo*, *J. Am. Oil Chem. Soc. 42*, 908–913.
Witting, L.A. (1969) Oxidation of α-Tocopherol During the Autoxidation of Ethyl Oleate, Linoleate, Linolenate, and Arachidonate, *Arch. Biochem. Biophys. 129*, 142–151.
Witting, L.A. (1970) The Interrelationship of Polyunsaturated Fatty Acids and Antioxidants *In Vivo*, *Prog. Chem. Fats Other Lipids 9*, 519–553.
Wu, G.S., Stein, R.A., and Mead, J.F. (1978) Autoxidation of Fatty Acid Monolayers Absorbed on Silica Gel. II. Rates and Products, *Lipids 12*, 971–978.
Yamamoto, Y., Niki, E., and Kamiya, Y. (1982a) Oxidation of Lipids. I. Quantitative Determination of the Oxidation of Methyl Linoleate and Methyl Linolenate, *Bull. Chem. Soc. Jpn. 55*, 1548–1550.
Yamamoto, Y., Niki, E., Kamiya, Y. (1982b) Oxidation of Lipids. III. Oxidation of Methyl Linoleate in Solution, *Lipids 17*, 870–877.
Yamamoto, Y., Saeki, N., Haga, S., Niki, E., and Kamiya, Y. (1984) Oxidation of Lipids. IX. Decomposition of Methyl Linoleate and Methyl Linolenate Hydroperxides in Solution, *Bull. Chem. Soc. Jpn. 57*, 3177–3181.
Yanishlieva, N.V., Kamal-Eldin, A., Marinova, E.M., and Toneva, A.G. (2002) Kinetic Study of the Antioxidant Activity of α- and γ-Tocopherols in Sunflower and Soybean Triacylglycerols, *Eur. J. Lipid Sci. Technol. 104*, 262–270.
Yanishlieva, N.V. and Marinova, E.M. (1992) Inhibited Oxidation of Lipids. I. Complex Estimation and Comparison of the Antioxidative Properties of Some Natural and Synthetic Antioxidants, *Fat Sci. Technol. 94*, 374–379.

Yazu, K., Yamamoto, Y., Niki, E., Miki, K., and Ukegawa, K. (1998) Mechanism of Lower Oxidizability of Eicosapentaenoate than Linoleate in Aqueous Micelles. II. Effect of Antioxidants, *Lipids 33*, 597–600.

Yazu, K., Yamamoto, Y., Ukegawa, K., and Niki, E. (1996) Mechanism of Lower Oxidizability of Eicosapentaenoate than Linoleate in Aqueous Micelles, *Lipids 31*, 337–340.

Zhang, H.-Y. (1999) Theoretical Methods Used in Elucidating Activity Differences of Phenolic Antioxidants, *J. Am. Oil Chem. Soc. 76*, 745–748.

Chapter 2

Oxidation of Fish Lipids and Its Inhibition with Tocopherols

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Introduction

The consensus of evidence over the last two decades is that increasing the dietary intake of longer-chain ω-3 (n-3) polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), is beneficial in various human disorders including cardiovascular and inflammatory conditions. A related issue is the requirement of DHA for normal neural development in the human fetus and premature infants. Fatty fish and fish oils are rich sources of EPA and DHA.

Unfortunately, the high degree of unsaturation also renders the PUFA in fish oil highly susceptible to oxidation. Thus, lipid oxidation is the most critical parameter affecting the shelf-life of fish oils and of food products in which marine lipids have been incorporated. As discussed in Chapter 1, lipid autoxidation leads to the formation of fatty acid hydroperoxides, which then decompose to a wide range of secondary oxidation products. Many of these are low-molecular-weight compounds with strong olfactory attributes, imparting the characteristic and disagreeable flavor of rancid fish oil. As discussed in Chapter 8, encapsulation in gelatin is frequently used to decrease the oxidation of fish oils and make them palatable. This may give a false sense of security, however, because even encapsulated oils have been found to contain high levels of fatty acid oxidation products (Sagredos 1991 and 1992, Shukla and Perkins 1998).

The general trend toward the use of all-natural ingredients in foods has caused renewed interest in naturally occurring antioxidants such as the tocopherols and ascorbic acid (vitamin C). Of the tocopherols, α-tocopherol (α-TOH) is now defined as the only one that contributes to meeting the human requirement for vitamin E (Institute of Medicine 2000). Another aspect of lipid oxidation is *in vivo* peroxidation, which has been implicated in DNA and protein modification and in the deposition of arterial plaque associated with low density lipoprotein modification (Porter *et al.* 1995). An increased intake of PUFA increases the physiologic requirements for antioxidants that are also effective *in vivo* (Muggli 1994).

Fish oil is an important ingredient in aquaculture feeds. Due to the rapid growth in the aquaculture industry, this now constitutes a major area of application of fish oil. Recent years have seen the introduction of so-called "high-energy" diets in salmon aquaculture. Such diets contain 30–40% of the feed as oil and result in enhanced growth rates of the fish. This enhances the susceptibility of the components of the feed, including the red pigment astaxanthin, to oxidative degradation and increases the oxidative stress to the fish. To overcome these problems, better antioxidant protection is desirable.

Unlike in most vegetable oils, α-TOH is generally the only tocopherol naturally present in oils from wild fish (Ackman and Cormier 1967). Commercial refining and deodorization substantially reduces the level of α-TOH in fish oils, generally to <100 μg/g. The effect of added antioxidants on the oxidation of fats and oils is dependent on the concentration and type of antioxidants naturally present. The addition of tocopherols to refined vegetable oils often does not improve their oxidative stability because these oils seem to contain adequate levels of tocopherols (Hudson and Ghavami 1984, Pokorný 1987). Fish oils, on the other hand, require additional stabilization by antioxidants if intended for human consumption.

Oxidation of Fish Lipids

Marine oils originate from the bodies of fatty fish, the livers of lean fish, and the blubber layers of marine mammals such as seal and whale. Commercial fish oils are produced mainly from sardine/pilchard, anchovy, menhaden, horse mackerel, sand launce, capelin, herring, and cod livers. The principal fatty acids of some commercial fish oils are shown in Table 2.1 (Bimbo 1990). Refined and deodorized fish oils consist mainly of triacylglycerols with a very complex fatty acid

TABLE 2.1
Principal Fatty Acids of Some Commercial Fish Oils[a]

Fatty acid[b]	Menhaden	Sardine	Anchovy	Cod liver	Capelin	Herring
	% (w/w)					
14:0	9	8	9	3	7	7
16:0	20	18	19	13	10	16
16:1n-7	12	10	9	10	10	6
18:1[c]	11	13	13	23	14	13
20:1n-9	1	4	5	0	17	13
22:1n-11[d]	Trace	3	2	6	14	20
20:5n-3	14	18	17	11	8	5
22:6n-3	8	9	9	12	6	6

[a]*Source:* Adapted from Bimbo (1990).
[b]The shorthand notation refers to the carbon chain length, the number of double bonds and the position of the first double bond from the hydrocarbon end of the molecule.
[c]25% 18:1n-7, 75% 18:1n-9.
[d]Also accompanied by other isomers of 22:1.

composition, but eight fatty acids usually dominate. The concentrations of EPA and DHA (molecular structures shown in Fig. 2.1) in fish oils differ significantly among species. Within-species variations also occur, reflecting geographical factors, nutritional status, season of catch, and reproductive status. Minor refined fish oil components may include cholesterol/cholesterol esters, α-TOH, free fatty acids, mono- and diacylglycerols, phospholipids, hydrocarbons, wax esters, and alcohols, as well as polymers and other oxidation products.

The oxidation of PUFA occurs readily at room temperature. The rate of autoxidation of PUFA increases approximately twofold for each active bis-allylic methylene group (Frankel 1998), and the reactivity of DHA is thus ~5 times greater than that of linoleic acid. In view of the particularly low flavor threshold values found for volatile secondary oxidation products with an n-3 double bond (Frankel 1982, Table 2.2), this high rate of oxidation makes fish oil quality difficult to maintain.

EPA has four and DHA has five active *bis*-allylic methylene groups, and four and five different pentadienyl radicals, respectively, may form by hydrogen abstraction from these methylene groups. Oxygen attachment on either side of these pentadienyl radicals produces a mixture of 8 positional hydroperoxide isomers for EPA and 10 for DHA. These hydroperoxides have either a *cis,trans* or a *trans,trans* configuration, and a complex system of 16 and 20 monohydroperoxide isomers is therefore expected from the autoxidation of EPA and DHA, respectively. Dihydroperoxides and epoxy hydroperoxides may also be formed.

Hydroperoxides of unsaturated lipids are unstable, particularly at high temperatures and in the presence of transition metal ions (Frankel 1998). They decompose to a wide variety of shorter-chain volatile flavor compounds, to other oxygenated compounds of the same chain length (including hydroxy compounds, epoxides, epoxy hydroperoxides, and ketodienes), as well as to oxidation products of higher molecular weight. The complexity of the degradation pathways of EPA and DHA makes it difficult to identify and quantify the large number of possible reaction products. Data obtained from studies of model systems of the less unsaturated n-3 fatty acid, 18:3n-3, have provided valuable information about the reaction pathways and products expect-

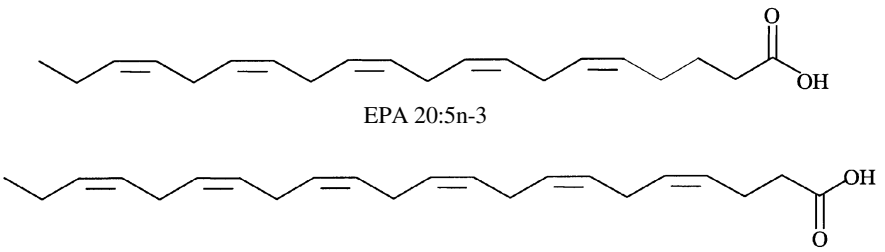

Fig. 2.1. Structures of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids.

TABLE 2.2
Some Volatile Secondary Oxidation Products Expected from the Autoxidation of n-3 Fatty Acids and Their Flavor Threshold Values in Oil

Oxidation product	Flavor/odor description	Flavor threshold value in oil (μg/g)
Propanal	Sharp, irritating[a]	1.6[b]
c-3-Hexenal	Fresh green leaves[a]	0.09[a]
c-4-Heptenal	Creamy, putty[a], stale, burnt[c], fishy[d]	0.0005[a]
t,t-2,4-Heptadienal	Fatty, oily[a]	0.10[a]
t,c-2,4-Heptadienal	Frying odor[a], fishy[d]	0.04[a]
t,t-2,6-Nonadienal	Cucumber, tallowy[a]	0.02[a]
t,c-2,6-Nonadienal	Fresh cucumbers[a]	0.0015[a]
t,t,c-2,4,7-Decatrienal	Burnt/fishy[c]	—
t,c,c-2,4,7-Decatrienal	Burnt/fishy[c]	—
1-Penten-3-one	Sharp, fishy[a]	0.003[a]
1,*c*-5-Octadien-3-one	Metallic, geranium-like[e]	0.00003[e]

[a]*Source:* Badings (1970).
[b]*Source:* Meijboom (1964).
[c]*Source:* Karahadian and Lindsay (1989a).
[d]*Source:* Hartvigsen *et al.* (2000).
[e]*Source:* Rørbæk (1994).

ed from the longer-chain PUFA. Peroxyl radical cyclization is an important process in the autoxidation of fatty acids or esters having three or more methylene-interrupted double bonds, e.g., linolenic acid, arachidonic acid, EPA, and DHA (Chan *et al.* 1982, Neff *et al.* 1981, Porter *et al.* 1981). Intramolecular peroxyl radical addition to a *cis* homoallylic double bond and subsequent reaction with oxygen yield 5-membered hydroperoxy epidioxides as oxidation products, and this pathway explains the uneven distribution of positional isomers of hydroperoxides of linolenate. Hydroperoxy epidioxides, in fact, represented as much as 25% of the total hydroperoxides found in autoxidized methyl linolenate (Neff *et al.* 1981), and their formation is expected to be a major reaction pathway for EPA and DHA also. Hydroperoxy bicycloendoperoxides, also formed through intramolecular radical addition, comprise another group of oxidation products characteristic of PUFA with three or more methylene-interrupted double bonds.

Fatty acid monohydroperoxides decompose through the formation of peroxyl and alkoxyl radicals, and cleavage of the alkoxyl radicals by homolytic β-scission, generating shorter-chain secondary oxidation products. Less is known about the decomposition mechanisms for the more complex cyclic and oligomeric oxidation products. The many different fatty acid hydroperoxide positional and geometrical isomers formed during autoxidation of the n-3 fatty acid PUFA in fish oils give rise to a complex mixture of volatile secondary oxidation products. The hydroperoxide precursors of some of the volatile aldehydes formed from EPA are shown in Figure 2.2. Volatile oxidation products formed during the storage of fish oil have been identified and their individual flavors characterized in a number of studies

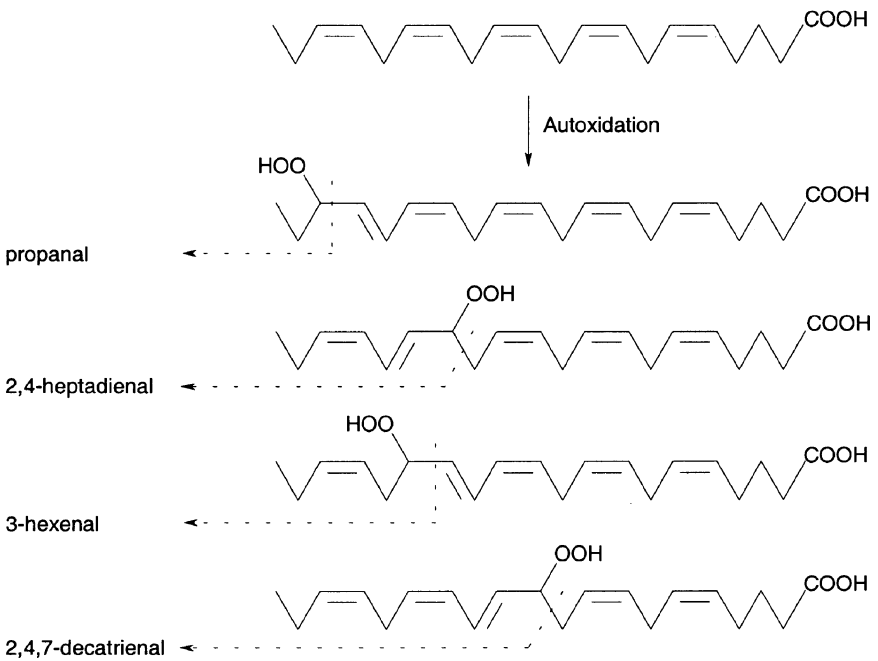

Fig. 2.2. Autoxidation sites associated with major aldehydes expected to form from eicosapentaenoic acid (EPA).

(Hartvigsen *et al.* 2000, Hsieh *et al.* 1989, Karahadian and Lindsay 1989a, Olsen 2000, Rørbæk 1994). Together with information about the threshold values for the different flavor compounds, this enabled researchers to pinpoint some of the principal volatile products contributing to fishy off-flavors in fish oil (Hartvigsen *et al.* 2000, Hu and Pan 2000, Karahadian and Lindsay 1989a, Meijboom and Stroink 1972, Nakamura *et al.* 1980, Rørbæk 1994).

The formation of unpleasant flavor notes in fish oil during storage has also been satisfactorily modeled on the basis of the concentrations of only a few potent flavor compounds (Macfarlane *et al.* 2001, Olsen 2000).

Some important flavor compounds formed from the autoxidation of n-3 fatty acids together with their flavor description and threshold values in oil are listed in Table 2.2. *cis*-4-Heptenal, an extremely potent flavor compound, and *trans, cis,cis*- and *trans,trans,cis*-2,4,7-decatrienal impart the characteristic fishy flavor of oxidized fish oil, whereas the 2,4-heptadienal geometrical isomers as well as 1-penten-3-one contribute generally undesirable oxidized flavors. Another potent flavor compound, 1,*cis*-5-octadien-3-one, imparts a heavy geranium-like flavor note. A more desirable fresh fish-like character is contributed by *trans,cis*-2,6-nonadienal.

The conclusions from any particular study undertaken to characterize important contributors to off-flavors in fish oils depend on which compounds were actually detected by the analytical methodology used and at what concentration. Even though some of the compounds identified as important for the formation of fishy off-flavors most likely have a major effect on the sensory quality, the different volatiles may interact unpredictably, modifying the flavor attributes of an oil in a way that complicates efforts to control the formation of off-flavors.

Freshly deodorized fish oil has a bland fresh fish-like flavor. Deodorization removes flavor-potent volatile oxidation products, but fish oils previously damaged from inadequate or prolonged storage of the fish itself or of the product oil will again develop unpleasant rancid flavors shortly after refining. This situation is true even though the results from methods commonly used to evaluate fish oil oxidative quality, such as the peroxide value (POV) and the *p*-anisidine value (*p*-AV), indicate a high quality. For fish oil applications in which the sensory quality is of major importance, such as incorporation into food products or consumption as a health supplement in liquid forms, every effort must be taken in the processing of the fish itself and of the fish oil to ensure speedy handling without exposure to oxygen or elevated temperatures. A relatively recent and valuable resource for high-quality fish oil is farmed salmon. The live fish is brought directly to the onshore slaughtering and processing facility. By-products of the filleting line are very fresh, and further processing using mild processing conditions yields salmon oil, protein hydrolyzates, and other products. The planned salmon processing allows by-products to become an integrated part of the production line to ensure a high quality and relatively stable product oil.

Oxidation of lipids in emulsions, such as mayonnaises and salad dressings, is a much more complex process than lipid oxidation in bulk oils and is influenced by a number of factors such as pH, type of emulsifier used, droplet size distribution, and properties of the other ingredients, as well as the partitioning of antioxidants, prooxidants, and oxidation products between the water phase, the oil phase, and the oil-water interface. Lipid oxidation in complex food systems is covered in detail in other parts of this book. Some important and intriguing findings regarding the relative stability of PUFA in multiphase systems are worth mentioning here, however. As expected, triacylglycerols from the orbital fat of tuna were more susceptible to oxidation than soybean oil triacylglycerols (Miyashita *et al.* 1995). In an oil-in-water emulsion, on the other hand, the oxidative stability of the tuna triacylglycerols was substantially prolonged and was higher than that of emulsified soybean triacylglycerols. A similar discrepancy with regard to relative order of oxidative stability of different PUFA has been observed when comparing homogeneous systems and aqueous micelles (Bruna *et al.* 1989, Yazu *et al.* 1996 and 1998). The surprisingly high oxidative stability of EPA and DHA in aqueous micelles was ascribed to the high polarity of their peroxyl radicals and the migration of these radicals to the micelle surface with a subsequent reduction in the rate of chain propagation and an increase in the rate of chain termination (Yazu *et al.* 1996 and 1998).

Properties of Tocopherols in Fish Oil

Tocopherols

The tocopherols are the most important natural antioxidants in fats and oils. They occur as homologs (α-, β-, γ-, and δ-) with varying extent and patterns of methylation of a chroman ring having a saturated phytyl side chain (Fig. 2.3). The phytyl tail has three chiral centers (position 2, 4′ and 8′), making a total of eight stereoisomeric

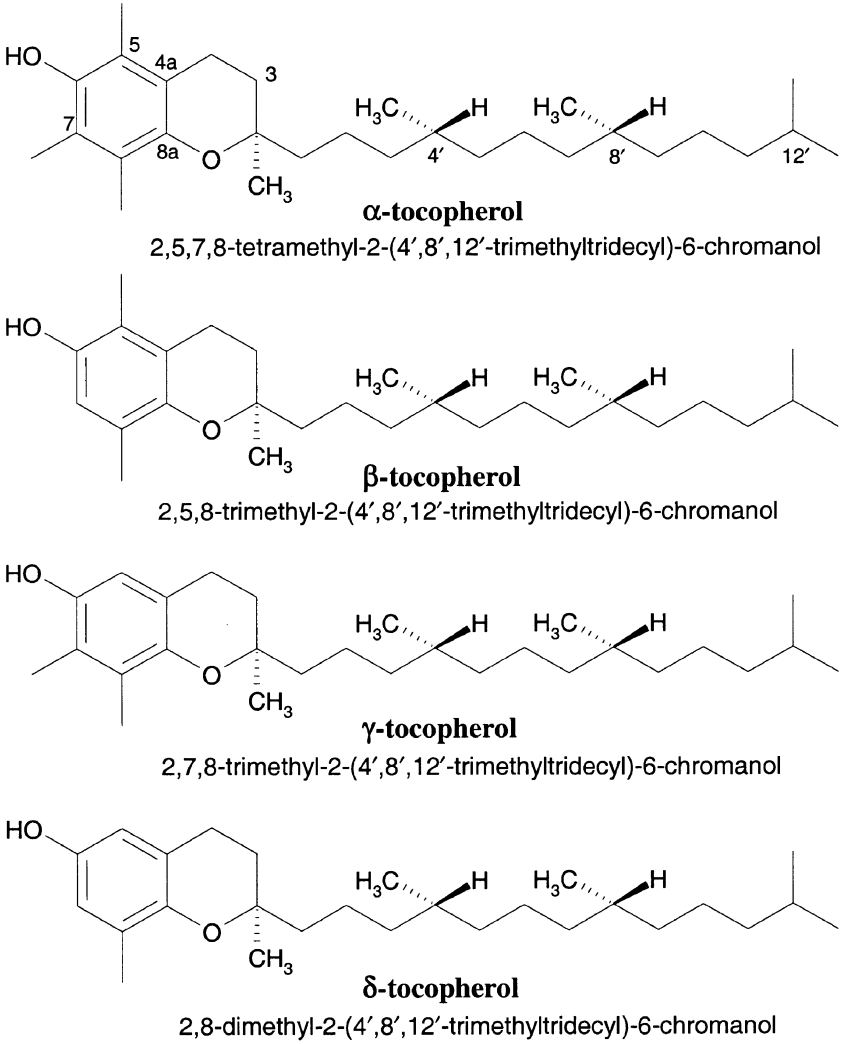

Fig. 2.3. Structures of α-, β-, γ-, and δ-tocopherol (TOH).

forms possible. All naturally occurring tocopherols have the same molecular configuration, i.e., *RRR* or *d*-tocopherols. The tocotrienols have the same chroman heads as the corresponding tocopherols but contain three isolated double bonds in their phytyl tails.

Tocopherol concentrations in animal fats are generally much lower than in vegetable oils. Because fish do not synthesize tocopherol, the levels in fish oil are directly related to the fish's diet (Ackman and Cormier 1967). α-Tocopherol is generally the only tocopherol naturally present in marine fish (Ackman and Cormier 1967, Aminullah Bhuiyan *et al.* 1993, Hemre *et al.* 1997), and thus also in commercial fish oils. The other tocopherols are likely filtered out by the many invertebrates intermediate between phytoplanktons and fish (Sigurgisladottir *et al.* 1993). The concentration of α-TOH determined in samples of five commercial fish oils is given in Table 2.3 (Kinsella, 1987). Commercial refining and deodorization, however, are known to substantially reduce the concentration of α-TOH. Because oilseed meals are now commonly added to commercial aquaculture feeds, farmed fish may have somewhat higher levels of tocopherols other than α-TOH.

The tocopherols react with lipid radicals and intercept lipid oxidation mainly by reactions [1] and [2]:

$$ROO^{\bullet} + TOH \rightarrow ROOH + TO^{\bullet} \quad [1]$$

$$ROO^{\bullet} + TO^{\bullet} \rightarrow \text{nonradical products} \quad [2]$$

The resonance-stabilized tocopheroxyl radical formed upon transfer of the phenolic hydrogen to a peroxyl radical [1] combines with a second peroxyl radical [2], thus yielding an antioxidant stoichiometric factor of two (Burton and Ingold 1981, Liebler and Burr 1995).

The tocopherols are all excellent hydrogen donors. Their reactivity with peroxyl radicals is controlled by the fused 6-membered heterocyclic ring, which confers additional stabilization to the radical formed, compared with that of a simpler phenoxyl radical through an interaction between the *p*-orbitals on the two *para*-oxygen atoms (Burton *et al.* 1983 and 1985). Differences in the relative activities

TABLE 2.3
α-Tocopherol (α-TOH) Found in Some Commercial Fish Oils[a]

Fish oil	α-TOH (mg/kg)
Anchovy	291
Capelin	140
Cod liver	220
Menhaden	75
Herring	92

[a]*Source:* Adapted from Kinsella (1987).

of the tocopherols are related to the number and position of the methyl groups on the phenolic ring. The presence of electron-releasing substituents in positions *ortho* and/or *para* to the hydroxyl group of a phenol facilitates rapid hydrogen donation to peroxyl radicals and increases the stability of the phenoxyl radical (Pokorný 1987). The fully methylated α-TOH is, therefore, structurally expected to be the most potent tocopherol homolog. Accordingly, the reactivity of the tocopherols with peroxyl radicals was found to decrease in the order α-TOH > β-TOH ≈ γ-TOH > δ-TOH (Table 2.4, Burton and Ingold 1981, Niki *et al.* 1986).

α-Tocopherol is one of the most reactive phenolic antioxidants known (Burton and Ingold 1981, Burton *et al.* 1983), and the rate constant for its reaction with peroxyl radicals is ~250 times that of the well-known synthetic antioxidant butylated hydroxytoluene (BHT) (Burton and Ingold 1989). Not surprisingly, considering its peroxyl radical scavenging ability, α-TOH is the major lipid-soluble chain-breaking antioxidant in mammalian membranes (Burton and Ingold 1989, Kamal-Eldin and Appelqvist 1996). The vitamin E activity of the tocopherols (Table 2.4) is governed not only by their chemical reactivity but also by their bioavailability, and there is a preference for the incorporation of the natural *RRR*-α-TOH in tissues and membranes. This transfer mechanism has been found to be mediated by an α-TOH transfer protein (α-TTP) expressed in the liver and other tissues (Azzi and Stocker 2000). Although not important for the rate of reaction with peroxyl radicals *in vitro*, the phytyl tail is essential for retention of the tocopherols in cell membranes (Burton and Ingold 1989). The tocopherols also inhibit photosensitized oxidation, predominantly by physical quenching of singlet oxygen (Kamal-Eldin and Appelqvist 1996). Their relative quenching efficiency is α-TOH > β-TOH > γ-TOH > δ-TOH.

Effect of Individual Tocopherols on Fish Oil Fatty Acid Autoxidation

From their established rates of reaction with lipid peroxyl radicals (α-TOH > γ-TOH > δ-TOH), α-TOH was expected to be the better inhibitor of hydroperoxide formation in fish oil. This order of antioxidant activity was in fact observed on the basis of the formation of hydroperoxides in purified fish oil at a low level of toco-

TABLE 2.4
Relative Tocopherol Activity

Tocopherol homolog	Relative reactivity with peroxyl radicals[a] (%)	Relative biological activity[b] (%)
α-TOH	100	100
β-TOH	71	50
γ-TOH	68	10
δ-TOH	28	3

[a]Determined by Burton and Ingold (1981) for the oxidation of styrene in chlorobenzene solution.
[b]*Source:* Kamal-Eldin and Appelqvist (1996).

pherol addition of 100 μg/g (Kulås and Ackman 2001b). However, the reverse order of antioxidant activity was found when the initial tocopherol concentration was ≥500 μg/g (Kulås and Ackman 2001b and 2001c). This change in relative antioxidant activity is caused by the existence of concentrations for maximum antioxidant activity of α-TOH and of γ-TOH. At levels of addition above these tocopherol concentrations, there is an increase in the rate of hydroperoxide formation.

The formation of hydroperoxides, as conjugated dienes (CD) at 30°C in purified fish oil without any antioxidant and with different concentrations of α-TOH, is shown in Figure 2.4A (Kulås and Ackman 2001b). The substrate oil was a freshly processed South American Pacific fish oil (refined, deodorized, and winterized) that had been further purified by column chromatography to remove minor components such as free fatty acids, preformed oxidation products, and antioxidants originally present (Kulås and Ackman 2001a). This allows better control over factors known to influence the autoxidation process. Of particular importance is the removal of the α-TOH originally present to below the limit of detection by high-performance liquid chromatography. It is likely that the effect of tocopherols originally present in fats and oils is an important contributing factor to previous conflicting results in the literature with regard to the relative activities of added antioxidants. In the presence of >100 μg/g α-TOH, there was a significant increase in the initial rate of formation of hydroperoxides (Fig. 2.4A). Hence, at the concentrations tested, α-TOH displayed an inversion of activity at 100 μg/g. C3-aldehydes (propanal + 2-propenal) were determined by static headspace/GC analysis as markers of the formation volatile secondary oxidation products. Propanal and 2-propenal are both typical of the oxidation of n-3 PUFA. Contrary to the effect on the formation of hydroperoxides, the amount of C3-aldehydes formed decreased with increasing α-TOH concentration (Fig. 2.4B).

On the basis of the formation of hydroperoxides, maximum antioxidant activity of γ-TOH was observed at 500 μg/g (Fig. 2.5A), whereas the antioxidant activity of δ-TOH increased with the level of addition up to 1500 μg/g, when no further improvement of oil stability was obtained (Fig. 2.6A). Maximum antioxidant activity of α-TOH at 100 μg/g was also observed in several vegetable oils oxidized in bulk (Blekas *et al.* 1995, Huang *et al.* 1994, Jung and Min 1990, Lampi *et al.* 1999). In addition to their degree of unsaturation, these oils are likely to have differed with regard to the concentration of minor constituents expected to influence the autoxidation process, as well as storage conditions. Taking this into account, the influence of substrate composition on concentrations for maximum tocopherol activity appears to be small and of little practical importance. As shown in Figure 2.4A, the addition of α-TOH at concentrations >100 μg/g to fish oil leads to a substantial increase in the accumulation of hydroperoxides, and fish oils may be particularly susceptible to the adverse effects of high levels of α-TOH. This α-TOH–mediated accumulation of hydroperoxides has been explained previously by the participation of α-TOH and/or α-TO$^{\bullet}$ in reactions other than with lipid peroxyl

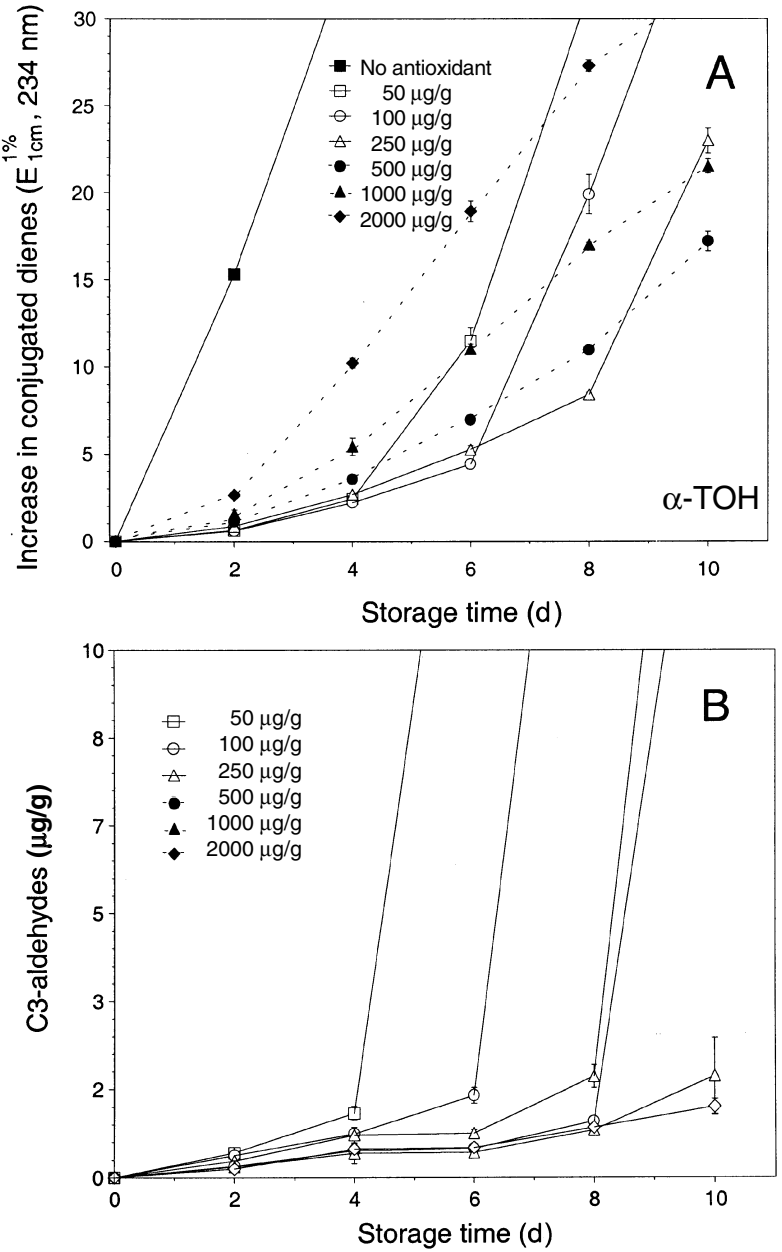

Fig. 2.4. Formation of hydroperoxides, as conjugated dienes (A) and C3-aldehydes (B) during storage of purified fish oil with and without α-tocopherol (TOH) at 30°C in the dark. Data points are means ± SD (n = 3). *Source:* Reprinted with permission from Kulås and Ackman (2001b).

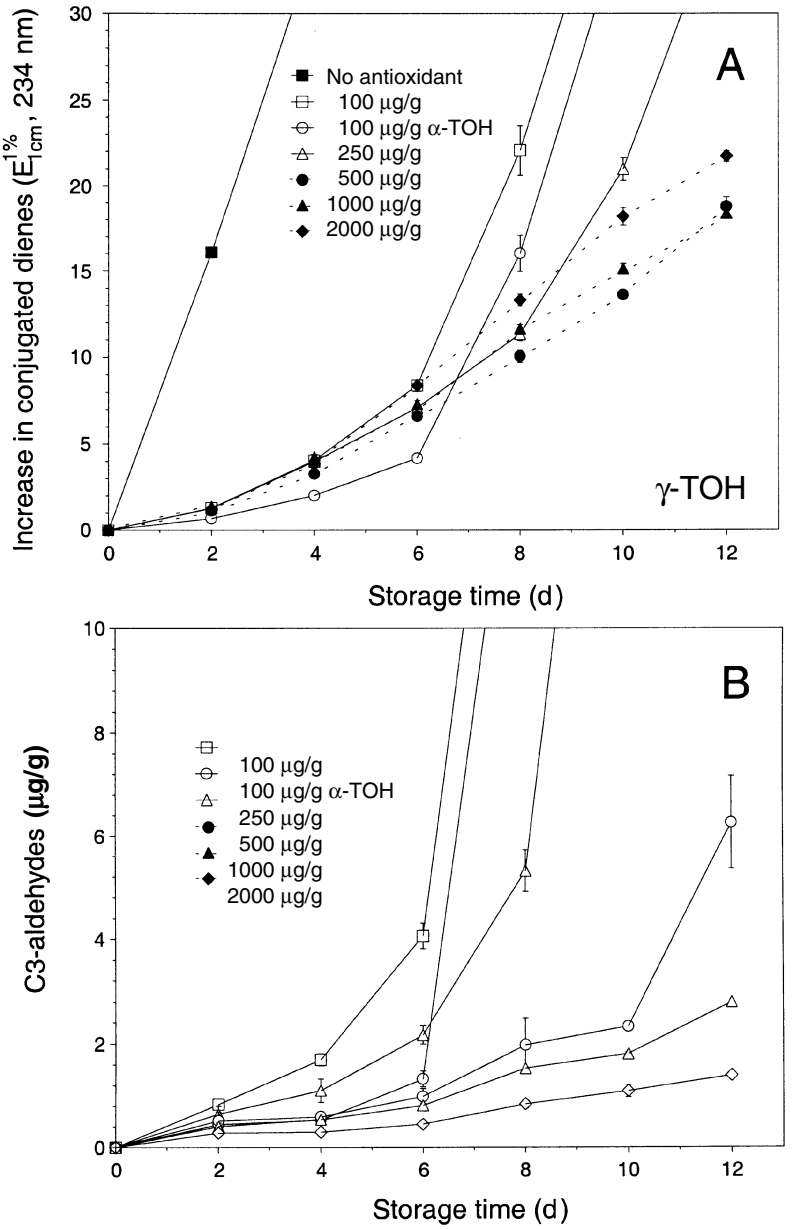

Fig. 2.5. Formation of hydroperoxides, as conjugated dienes (A) and C3-aldehydes (B) during storage of purified fish oil with and without γ-tocopherol (TOH) at 30°C in the dark. An oil sample with α-TOH (100 µg/g) was included for comparison of TOH activity. Data points are means ± SD (n = 3). *Source:* Reprinted with permission from Kulås and Ackman (2001b).

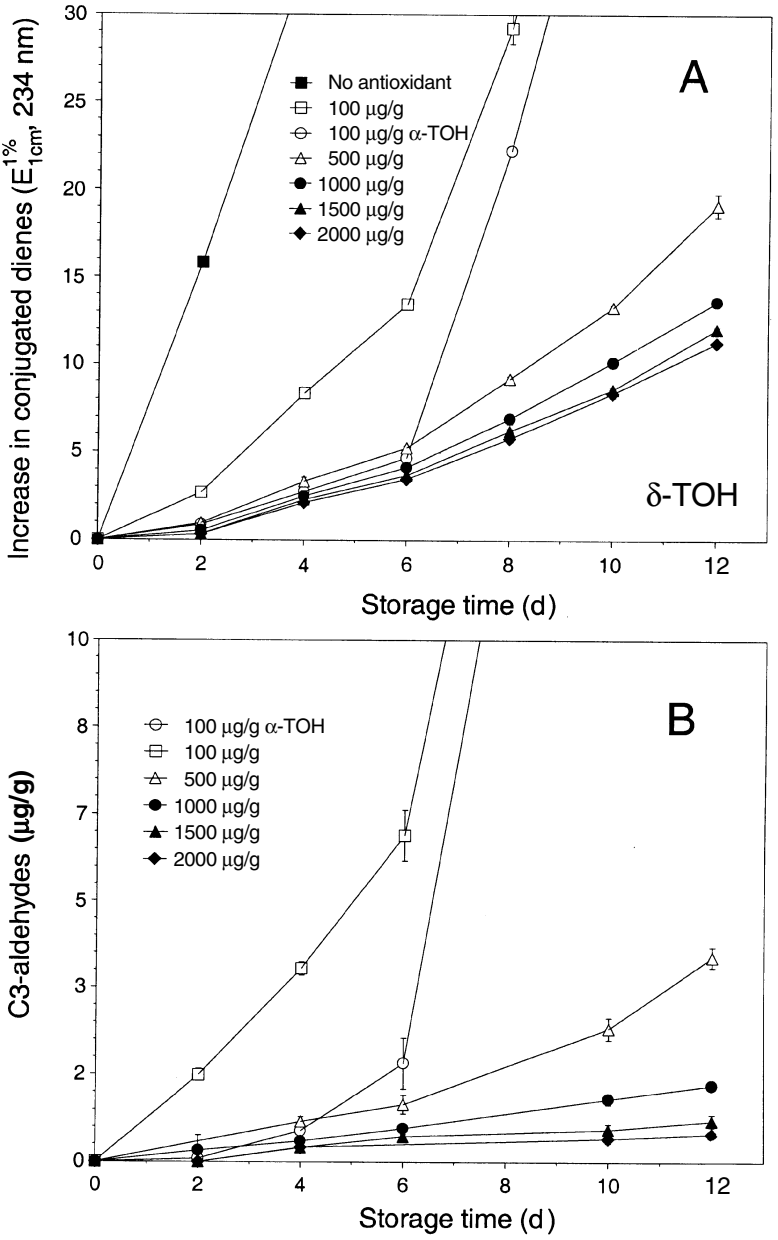

Fig. 2.6. Formation of hydroperoxides, as conjugated dienes (A) and C3-aldehydes (B) during storage of purified fish oil with and without δ-tocopherol (TOH) at 30°C in the dark. An oil sample with α-TOH (100 µg/g) was included for comparison of TOH activity. Data points are means ± SD (n = 3). *Source:* Reprinted with permission from Kulås and Ackman (2001b).

and alkoxyl radicals (Fuster *et al.* 1998, Kamal-Eldin and Appelqvist 1996, Marinova and Yanishlieva 1992, Yanishlieva *et al.* 2002), and appears to be related to the extent of hydrogen-donation by antioxidant.

As was found for α-TOH, γ-TOH and δ-TOH inhibited the formation of C3-aldehydes from hydroperoxide decomposition in a concentration-dependent manner (Figs. 2.5B and 2.6B, respectively). It was previously demonstrated that the decomposition of methyl linoleate hydroperoxides is inhibited by α-TOH (Hopia *et al.* 1996, Mäkinen and Hopia 2000), and by γ-TOH and δ-TOH (Mäkinen *et al.* 2001a), most likely through hydrogen donation to both peroxyl and alkoxyl radicals. Hydrogen donation inhibits β-scission of alkoxyl radicals, and the addition of α-TOH to methyl linoleate hydroperoxides was found to reduce the formation of hexanal in a concentration-dependent manner (Hopia *et al.* 1996). Accordingly, it is likely that the formation of C3-aldehydes in fish oil, also products of β-scission of alkoxyl radicals, is inhibited by increased stabilization of the hydroperoxides and by scavenging of alkoxyl radicals. We have demonstrated that both the tocopherol type and concentration influence the composition of volatile secondary oxidation products in fish oil (Kulås *et al.* 2002). The sensory quality of the oil may, therefore, not be related to the concentration of C3-aldehydes.

Fish oil without any antioxidant oxidizes very rapidly, and the tocopherols retard the formation of both hydroperoxides and volatile secondary oxidation products. Although an inversion of activity does take place above certain concentrations of α-TOH and γ-TOH, neither of the tocopherols displays prooxidant activity, up to concentrations $\leq$2000 μg/g, compared with a control oil devoid of antioxidants.

Contrary to what may be expected, considering that antioxidants retard oxidation by being oxidized preferentially, high tocopherol antioxidant activity in fish oil was related to a low rate of consumption of the antioxidant. The average rates of consumption of α-TOH and γ-TOH [μg/(g · d)] were strongly correlated with the amount of tocopherol added (Fig. 2.7, Kulås and Ackman 2001b). Above a certain initial concentration, ~250 μg/g for α-TOH and 500 μg/g for γ-TOH, the relationships appeared linear and the value of the slope was highest for α-TOH. The rate of consumption of δ-TOH reached a constant value of ~50 μg/(g · d) (δ-TOH $\geq$ 1000 μg/g) and became independent of the initial tocopherol concentration. The assumption that α-TOH and/or α-TO$^{\bullet}$ participate in side reactions is in accordance with the high rate of tocopherol consumption observed at high initial α-TOH concentrations. The γ-TOH, which is a less active hydrogen donor than α-TOH, is consumed to a lesser extent in side reactions. Because the rate of tocopherol consumption was independent of the level of addition, the more stable δ-TOH seems not to participate in side reactions. It is considered likely that, in addition to the participation of TOH/TO$^{\bullet}$ in side reactions, stabilization by α-TOH and γ-TOH of hydroperoxides already formed also contributed to the observed accumulation of hydroperoxides at high concentrations of these antioxidants (Mäkinen *et al.* 2001a).

The influence of the method used for the evaluation of antioxidant activity on the results that may be obtained is illustrated in Figure 2.8, where the induction

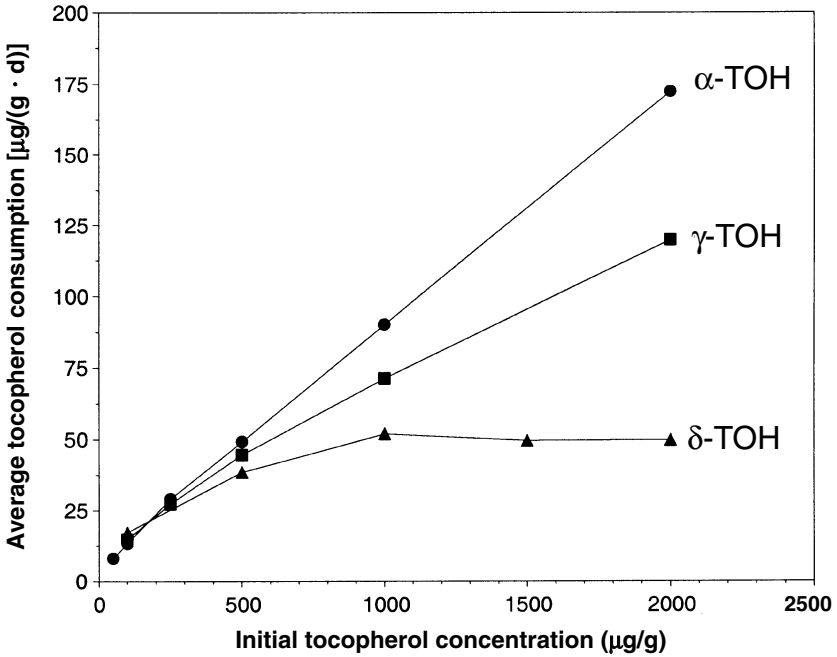

Fig. 2.7. Relationships between the average rate of tocopherol consumption and the initial tocopherol concentration in purified fish oil with α-tocopherol (TOH), γ-TOH, or δ-TOH held at 30°C in the dark. *Source:* Reprinted with permission from Kulås and Ackman (2001b).

periods, as well as the initial rates of formation of CD and C3-aldehydes, determined in fish oil held at 30°C, are plotted vs. initial α-TOH concentration (Kulås and Ackman 2001b). Above ~250 µg/g α-TOH, there appears to be a linear relationship between the initial rate of hydroperoxide formation and the initial α-TOH concentration. The induction period, on the other hand, increases in a curvilinear fashion with α-TOH concentration, and the extension at high antioxidant concentrations (α-TOH > 500 µg/g) is small. This can be explained by the high rate of α-TOH consumption in this concentration range (Fig. 2.7). The stabilization of hydroperoxides by α-TOH and the retardation of C3-aldehyde formation are also apparent from Figure 2.8. At relatively high rates of hydroperoxide formation (α-TOH > 500 µg/g), the inhibition of hydroperoxide decomposition by α-TOH is somewhat reduced.

As expected, the antioxidant activity of tocopherol blends is strongly affected by their composition. We found that at 1500 µg/g total tocopherol, the rate of formation of hydroperoxides in purified fish oil at 30°C decreased markedly with an increasing ratio of γ-TOH + δ-TOH to that of α-TOH. However, the addition of more tocopherol, in the form of 500 µg/g γ-TOH and 500 µg/g δ-TOH, to fish oil already containing 500 µg/g α-TOH, had a negative effect on the oil stability eval-

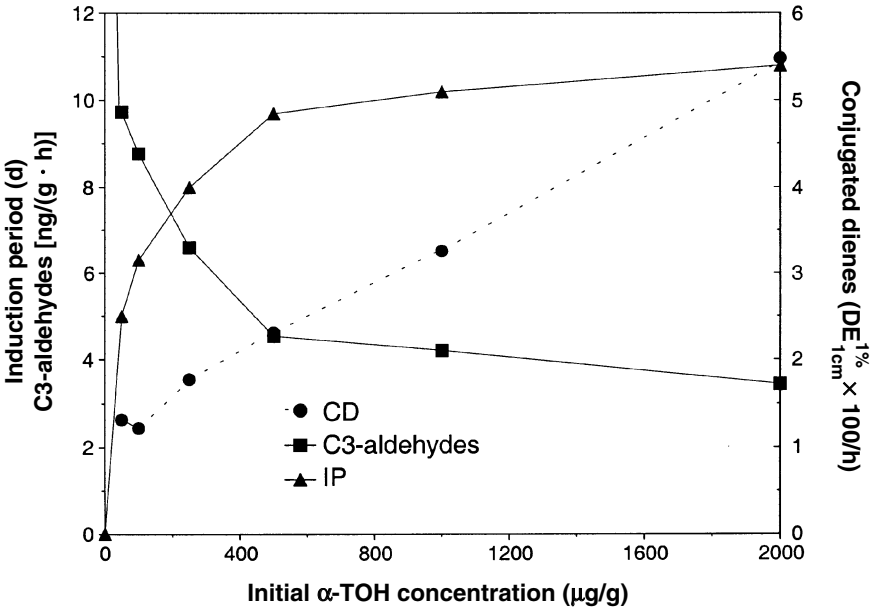

Fig. 2.8. Comparison of the induction period (IP) and initial rates of formation of conjugated dienes (CD) and C3-aldehydes in purified fish oil with different concentrations of α-tocopherol (TOH). Initial rates of formation of oxidation products were estimated from the measurements obtained after 2 d of storage at 30°C. *Source:* Reprinted with permission from Kulås and Ackman (2001b).

uated solely in terms of hydroperoxide formation. At low initial tocopherol concentrations (≤100 μg/g), α-TOH effectively retards the formation of primary oxidation products in purified fish oil; substituting some of this α-TOH with the less active hydrogen donors γ-TOH and δ-TOH is expected to reduce the oxidative stability of fish oil. On the other hand, the accumulation of hydroperoxides observed at high levels of addition of α-TOH makes the substitution of some α-TOH with γ-TOH and/or δ-TOH increasingly effective in reducing the formation of primary oxidation products.

POV and CD measurements are both well-established methods for the determination of primary oxidation products in fats and oils. The widely used iodometric titration method (AOCS methods Cd 8-53 and Cd 8b-90) for POV determination is a measure of the fatty acid peroxides formed during oxidation. Peroxidation of unsaturated fatty acids is accompanied by a shift in the position of double bonds to form conjugated hydroperoxides, and the conjugated structure absorbs strongly at 232–234 nm. Although POV is a direct measure of peroxides, it appears likely that oxidation products with CD structures of different origin, e.g., fatty acid hydroxy compounds, contribute to the CD value. The tocopherols were found to affect the relationship between the data obtained by these two methods to different

extents, by affecting the formation of the different oxidation products detected in fish oils (Kulås and Ackman 2001c). The order of antioxidant activity of the tocopherols (2.32 mmol/kg oil; ~1000 μg/g) in purified menhaden oil was δ-TOH > γ-TOH > α-TOH on the basis of both POV and CD measurements. α-TOH–mediated accumulation of hydroperoxides was also observed by both methods when testing different concentrations of this tocopherol in anchovy oil at 30°C. Linear relationships between the POV and CD values were found for all three tocopherols tested (Fig. 2.9A) as well as for the levels of α-TOH (Fig. 2.9B). The values of the regression line slopes, however, differed substantially and decreased with increasing hydrogen-donating ability of the tocopherols and with increasing α-TOH concentration. It appears, therefore, that an increased hydrogen-donating capacity results in the formation of a higher proportion of oxidation products with a CD structure, other than fatty acid hydroperoxides, and/or a lower proportion of oxidation products with more than one peroxide group per CD unit.

Although the product mixtures from the autoxidation of all PUFA, and EPA and DHA in particular, are extremely complex, it may be speculated that at least two groups of oxidation products contribute to the observed dependence on tocopherol homolog and concentration, namely, fatty acid hydroxy compounds and hydroperoxy epidioxides (Fig. 2.10). Fatty acid hydroxy compounds, contributing to the CD value only, are formed upon hydrogen donation to alkoxyl radicals, which are intermediates in the decomposition of lipid hydroperoxides (Pokorný 1987). On the basis of previous observations from the study of the decomposition of methyl linoleate hydroperoxides (Hopia *et al.* 1996), the concentration of fatty acid hydroxy compounds in fish oil is expected to increase with increased tocopherol hydrogen-donating power. It is well established that the formation of hydroperoxy epidioxides is a major pathway of the autoxidation of polyunsaturated fatty acids with three or more methylene-interrupted double bonds (Frankel 1991, Porter *et al.* 1981). Both peroxide groups of the hydroperoxy epidioxides are expected to contribute to the POV, the cyclic peroxide unit to a lesser extent than the hydroperoxide group (Sanchez and Myers 1996). The hydrogen-donating power of α-TOH (5%) was found to completely inhibit the 1,3-cyclization of methyl linolenate peroxyl radicals and the formation of hydroperoxy epidioxides by trapping the peroxyl radicals as monohydroperoxides (Peers *et al.* 1981). On the basis of these results, an increase in the tocopherol hydrogen-donating capacity may be expected to reduce the formation of hydroperoxy epidioxides relative to that of hydroperoxides, and thereby reduce the POV relative to the CD value, which is in accordance with the observations in fish oil.

Effect of Individual Tocopherols on the Composition of Volatile Secondary Oxidation Products

A complex mixture of volatile secondary oxidation products most likely causes the disagreeable flavor of oxidized fish oil. However, certain carbonyl compounds,

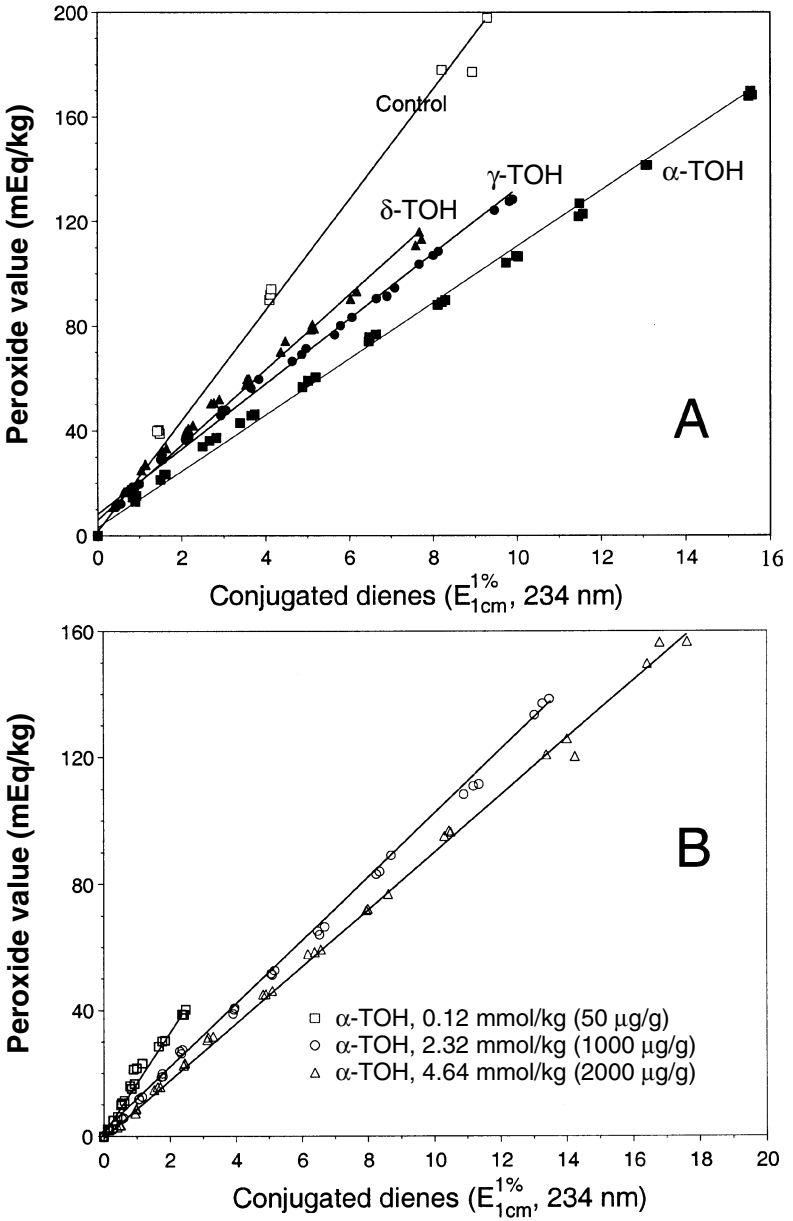

Fig. 2.9. Relationships between peroxide value and conjugated diene measurements in (A) purified menhaden oil with and without 2.32 mmol α-, γ-, or δ-tocopherol (TOH)/kg oil, and (B) anchovy oil with different levels of α-TOH. *Source:* Reprinted with permission from Kulås and Ackman (2001c). Copyright 2001 American Chemical Society.

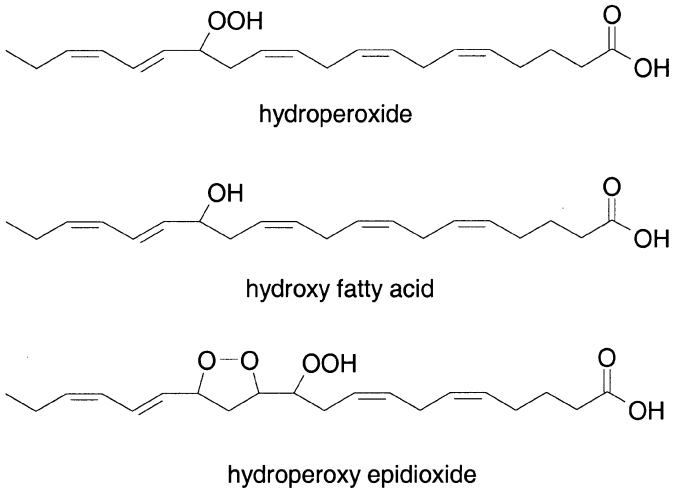

Fig. 2.10. Structures of a typical hydroperoxide, hydroxy fatty acid, and hydroperoxy epidioxide expected to be formed from the autoxidation of eicosapentaenoic acid (EPA).

including the 2,4-heptadienal isomers, the 2,4,7-decatrienal isomers, and *c*-4-heptenal, have emerged as principal contributors (Hsieh *et al.* 1989, Hu and Pan 2000, Karahadian and Lindsay 1989a, Meijboom and Stroink 1972).

The tocopherol type and concentration affect not only the overall formation of volatile secondary oxidation products in fish oil, but also the composition of this group of oxidation products. This again is likely to influence the formation of off-flavors during storage. The type of volatile secondary oxidation products formed in an oil is dependent on the structure of their hydroperoxide precursors. During fatty acid autoxidation, the *cis,trans* peroxyl radicals initially formed rearrange to the thermodynamically more stable *trans,trans* isomers (Porter *et al.* 1980). The addition of tocopherols affects the composition of hydroperoxides of methyl linoleate and methyl linolenate by rapid hydrogen donation to the *cis,trans* peroxyl radicals, thereby increasing the proportions of hydroperoxide geometrical isomers with a *cis,trans* configuration (Mäkinen and Hopia 2000, Mäkinen *et al.* 2001a, Peers *et al.* 1981). Accordingly, high concentrations of α-TOH were found to increase the formation of *trans,cis,cis*-2,4,7-decatrienal relative to the *trans,trans,cis* isomer in oxidizing fish oil (Karahadian and Lindsay 1989b). For inhibiting the isomerization of *cis,trans* hydroperoxides of methyl linoleate (Mäkinen *et al.* 2001a), the order of tocopherol activity was in accordance with their reactivity with fatty acid peroxyl radicals (α-TOH > γ-TOH > δ-TOH). The presence of α-TOH was demonstrated to decrease the formation of hydroperoxy epidioxides of methyl linolenate relative to monohydroperoxides compared with an oil without any antioxidant (Peers *et al.* 1981). The relative formation of hydroperoxy epidioxides is also relevant for the composition of volatile secondary oxidation products.

The tocopherols, on the basis of their different hydrogen-donating abilities, are expected to some extent to affect the relative formation of the different groups of precursors of the volatile secondary oxidation products in fish oil, and thereby also influence the composition of the volatiles formed. This was confirmed using dynamic headspace/GC-MS analysis to determine the composition of volatile secondary oxidation products formed during storage at 30°C for up to 8 d of purified anchovy oil samples (6.0 g) with α-, γ-, or δ-TOH added as antioxidant (Kulås *et al.* 2002). The major volatile compounds identified in the oxidizing fish oil were propanal, 2-propenal, 1-penten-3-ol, and the two 2,4-heptadienal geometrical isomers. The 2,4,7-decatrienals were not detected. The composition of volatile secondary oxidation products in the oil is likely to be influenced by the sample oxidative state, and because antioxidants reduce the rate of oxidation to different extents, samples should ideally be compared after having reached the same overall level of oxidation. This is difficult to achieve, particularly because there is no simple definition of oxidative state. It was possible to separate the effect of the tocopherols on the overall level of oxidation from their effect on the composition of volatile oxidation products by using principal component analysis (PCA), a procedure for observing sources of variation in complex data sets, to evaluate the dynamic headspace/GC-MS data.

The results from a PCA of the data from a dynamic headspace analysis of fish oil samples with 1000 μg/g initial tocopherol levels are shown in Figure 2.11. The volatile secondary oxidation products were grouped according to their chemical structure. The principal component 1 (PC1) explained the majority of the variability in the data set and is a latent variable describing the overall volatile formation, which increased with storage time. In the score plot, the samples were distributed as three bands along PC2 in the same order as the hydrogen-donating power of the added tocopherol. The α-TOH samples were characterized by relatively high hydrocarbon concentrations and diunsaturated and long-chain aldehydes levels. α-Tocopherol was also found to direct the formation of the *trans,cis* geometrical isomer of 2,4-heptadienal. The δ-TOH samples, on the other hand, were characterized by preferable formation of the shorter and more saturated aldehydes, particularly propanal. The concentration of α-TOH used (100 or 1000 μg/g) also had an effect on the distribution of volatile oxidation products (Fig. 2.12). Again, PC1 was an indicator of general product size and described the overall oxidative state based on volatile oxidation product formation. PC2, on the other hand, described differences in sample volatile composition. The loading plot was similar to that from the PCA of the 1000 μg/g tocopherol samples (Fig. 2.11). Decreasing the antioxidant hydrogen-donating capacity by decreasing the α-TOH concentration appeared to affect the volatile distribution in the same direction as decreasing the tocopherol hydrogen-donating power by using a different tocopherol homolog. A high initial α-TOH level was characterized by a higher *trans,cis*-/*trans,trans*-2,4-heptadienal ratio, hydrocarbon concentration, and higher levels of long and diunsaturated aldehydes compared with shorter and saturated aldehydes, respectively.

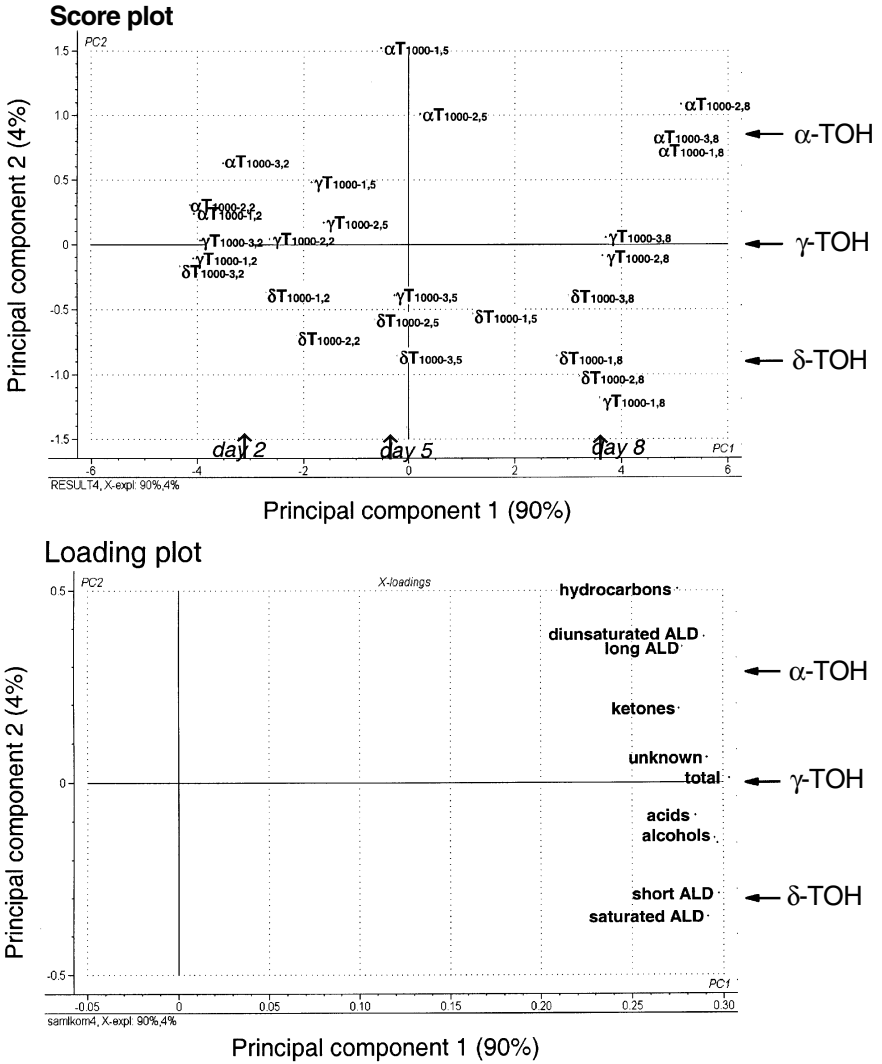

Fig. 2.11. Score plot and loading plot from the principal component analysis of the data obtained from dynamic headspace analysis of fish oil samples with nominal initial concentration 1000 μg/g of α-tocopherol (TOH), γ-TOH or δ-TOH after 2, 5, or 8 d of storage at 30°C. ALD: aldehydes. *Source:* Reprinted with permission from Kulås *et al.* (2002).

On the basis of these results, some general conclusions about the different properties of the tocopherols can be made. The hydrocarbons formed during lipid autoxidation are quite neutral with regard to their flavor impact. More relevant for fish oil flavor is the influence of tocopherol type and concentration on the forma-

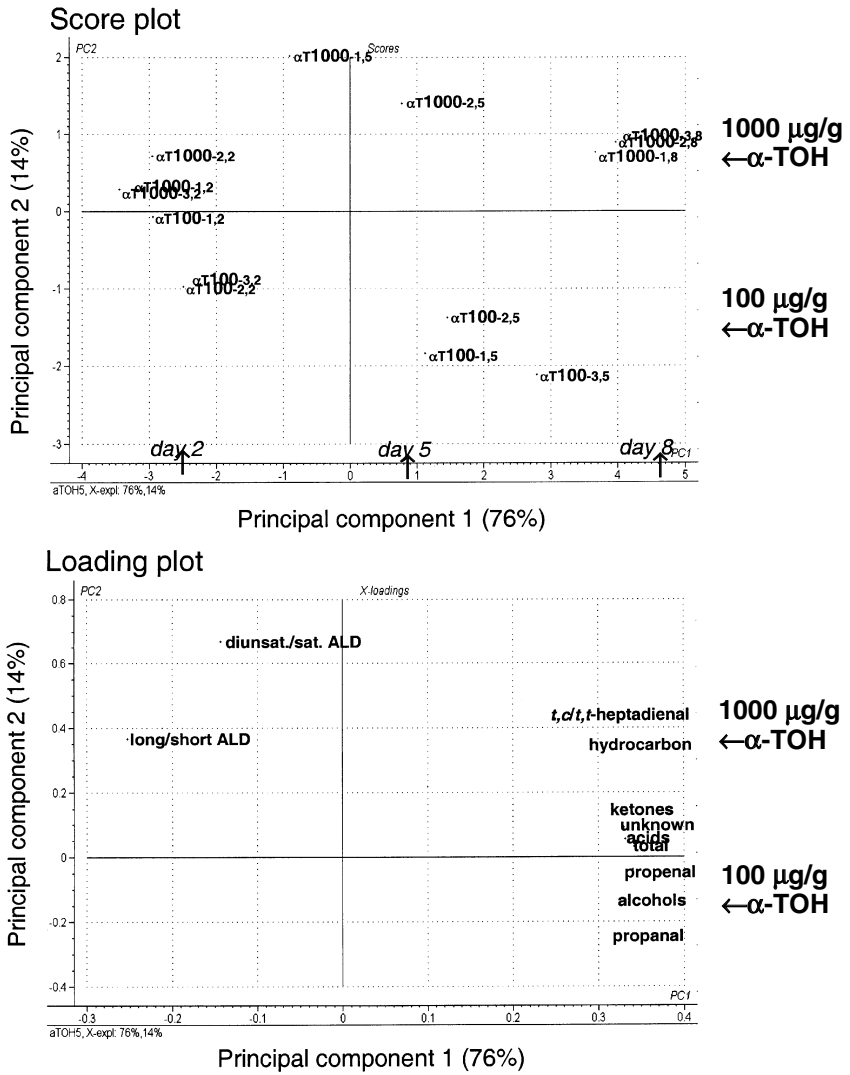

Fig. 2.12. Score plot and loading plot from the principal component analysis of the data obtained from dynamic headspace analysis of fish oil samples with a nominal initial α-tocopherol (TOH)-concentration of 100 or 1000 µg/g after 2, 5, or 8 d of storage at 30°C. ALD, aldehydes; diunsat./sat. ALD, diunsaturated/saturated aldehydes. *Source:* Reprinted with permission from Kulås *et al.* (2002).

tion of the flavor-potent long-chain and unsaturated aldehydes relative to that of short-chain and saturated aldehydes, as well as the relative formation of different geometrical isomers of unsaturated aldehydes. The mechanisms for the formation of lower saturated aldehydes were reviewed by Frankel (1982). The relative forma-

tion of lower saturated aldehydes was dependent on both the type of tocopherol added as antioxidant and the α-TOH concentration, and increased with decreasing antioxidant hydrogen-donating capacity (Figs. 2.11 and 2.12). This suggests that the tocopherol hydrogen-donating capacity influences the further oxidative degradation of already formed unsaturated aldehydes, and/or the decomposition of primary hydroperoxides. An expected preferable formation of hydroperoxy epidioxides in the presence of δ-TOH may also have contributed to the directed formation of the shorter and more saturated aldehydes in these samples. The observed increased ratio of *trans,cis/trans,trans*-2,4-heptadienal with antioxidant hydrogen-donating capacity is in accordance with the expected relative rate of scavenging of *cis,trans* peroxyl radicals by the tocopherols before isomerization to the *trans,trans* isomers (Mäkinen *et al.* 2001a). The flavor threshold in oil is lower for *t,c*-2,4-heptadienal than for the *trans,trans* isomer (Table 2.2). It is considered likely that the tocopherols influence the relative formation of the geometrical isomers of other unsaturated aldehydes expected to be formed in fish oils, including the 2,4,7-decatrienals, in the same direction as the 2,4-heptadienal isomers. The *trans,cis,cis*-2,4,7-decatrienal was assessed as having a fishy or cod liver–like flavor, whereas the *trans,trans,cis* isomer, in addition to a less pronounced fishy flavor, had sweet, green, cucumber- and melon-like flavor notes (Meijboom and Stroink 1972). We speculate that α-TOH, especially at high levels of addition, directs the formation of the more flavor-potent aldehyde geometrical isomers and of the more flavor-potent unsaturated aldehydes compared with γ-TOH and δ-TOH.

At low tocopherol levels, both the rate of formation of hydroperoxides and the overall rate of formation of volatile secondary oxidation products are highly dependent on the type of tocopherol added as antioxidant, and the antioxidant activity decreases in the order α-TOH > γ-TOH > δ-TOH (Figs. 2.4–2.6). Any influence of tocopherol type on the distribution of the volatile secondary oxidation products is thus considered to be of minor importance compared with the effect of the particular tocopherol on the overall oxidative state of the oil. At high initial tocopherol levels, the tocopherols also inhibit the formation of hydroperoxides to different extents, but the order of activity is reversed. The overall rates of formation of volatile secondary oxidation products, on the other hand, were similar for the three tocopherols (Fig. 2.11). The influence of the particular tocopherol used (or the composition of tocopherol blends) on the distribution of the volatile compounds formed may therefore be of greater importance for flavor formation at high tocopherol concentrations.

Sensory Impact of Tocopherol-Directed Reactions in the Autoxidation of Fish Oil

The work presented in the previous section demonstrated that the tocopherols do influence the composition of volatile secondary oxidation products in fish oil when small oil samples were kept under conditions of free oxygen access. More relevant

from the point of view of fish oil consumers is the relationship between chemical findings and sensory data of fish oils and the storage time for their suitability for human consumption.

A fish oil storage experiment carried out under conditions resembling real-life storage was undertaken by Olsen (2000). Freshly refined and deodorized cod liver oil was stored in capped retail glass bottles with a small headspace of air. The control oil without any added antioxidant was compared with cod liver oil to which was added 800 μg/g of a commercially available mixed tocopherol concentrate (Coviox T-70 from Henkel), or the same amount of the tocopherol concentrate plus 200 μg/g ascorbyl palmitate. The amount of α-TOH present in the control oil after refining and deodorization was not determined, but was considered to be very low. At the end of the storage period (14 wk), the results from the chemical evaluation of the oxidative quality of the oils (POV < 3.5 mEq/kg and *p*-AV < 14) were indicative of a fish oil of acceptable quality; the oils were also considered to be of acceptable sensory quality by the trained sensory panel. There was only a small increase in the POV and the *p*-AV during storage of the oils, and the changes in these parameters were similar for the three antioxidant treatments. This was thought to reflect the limited amount of oxygen present for reaction with the PUFA (and hence a reaction rate for hydroperoxide formation controlled by the availability of oxygen). The development of unpleasant herring oil–like and painty flavor notes in the oil was successfully modeled on the basis of the concentrations of all or a selection of the volatile compounds. A dynamic headspace/GC-MS analysis of volatile secondary oxidation products may thus replace sensory analysis in the evaluation of cod liver oil quality. The 2,4,7-decatrienal isomers, also regarded as important contributors to fishy off-flavors, were not detected in this work.

Despite the small changes in the parameters commonly used to evaluate oxidative quality in fish oils, and the small difference between the antioxidant treatments, a marked effect of the different antioxidant treatments on the composition of the volatile secondary oxidation products detected by dynamic headspace/GC-MS analysis was observed. The addition of a tocopherol mixture to the refined cod liver oil reduced the formation of a number of volatile compounds during storage (including acetaldehyde, 2-propenal, and 1-penten-3-ol), but increased the formation of several hydrocarbons and of certain flavor-potent volatile compounds, such as the 2,4-heptadienal isomers. This is in agreement with the results obtained by Kulås *et al.* (2002) presented in the previous section. These demonstrated that tocopherol addition (i.e., increased tocopherol hydrogen-donating capacity) affects volatile composition by protecting unsaturated carbonyl compounds from further oxidation and/or influences the composition of the precursors of the volatile compounds, at storage times well within the desirable shelf life of a fish oil.

The addition of the tocopherol mixture to the refined cod liver oil did not, however, cause a detectable difference in the flavor properties of the oil. The degree of development of unpleasant herring oil–like and painty off-flavors during the storage

period was similar for both treatments, and the effect of the tocopherols on the concentration and composition of volatile compounds was not extensive enough in this case to influence the sensory attributes of the oil. The presence of ascorbyl palmitate in addition to the tocopherols, on the other hand, directing the formation of hexanal, *trans*-2-hexenal, and 2,6-nonadienal, resulted in the development of a more pronounced grass/cucumber flavor. The mechanism responsible for this effect of ascorbyl palmitate is not known.

The chemical and sensory effect of tocopherol-directed reactions in menhaden and cod liver oils was also evaluated by Karahadian and Lindsay (1989b). The effect of the addition of α-TOH (670 μg/g) or Trolox C (1000 μg/g) to refined and deodorized fish oils held at 65°C was studied. The refined and deodorized fish oils most likely contained a small amount of α-TOH from the presence of this antioxidant in the fish itself. Trolox C is a synthetic antioxidant that is structurally related to α-TOH, but lacking the long alkyl side chain. An α-TOH–mediated accumulation of hydroperoxides was observed in both oils, whereas Trolox C suppressed hydroperoxide formation. Trolox C directed the formation of the potent flavor compound 1,5-octadien-3-one, which imparts a metallic, geranium-like, character to fish oil, and a mechanism for this action of the antioxidant that involves opening of the phenoxyl ring of the antioxidant to form the quinone was proposed. Both tocopherol-type compounds markedly influenced the proportion of geometrical isomers of unsaturated aldehydes in favor of the *trans,cis* isomer of 2,4-heptadienal and the *trans,cis,cis* isomer of 2,4,7-decatrienal compared with the refined control oils, in agreement with the results obtained by Kulås *et al.* (2002). As outlined in the previous section, this effect is due to an increase in the ratio of *cis,trans* to *trans,trans* hydroperoxides caused by rapid antioxidant hydrogen donation to fatty acid peroxyl radicals. The presence of the tocopherol-type compounds also enhanced the overall formation of the 2,4-heptadienals and of the 2,4,7-decatrienals, in agreement with the positive correlation between the accumulation of long-chain unsaturated aldehydes and the tocopherol concentration found by Kulås *et al.* (2002) (see previous section). The relatively high levels of these diunsaturated and triunsaturated aldehydes, and *trans,cis,cis*-2,4,7-decatrienal in particular, suggests that they contribute strongly to the unpleasant burnt/fishy flavor that developed in the samples with α-TOH. Trolox C also directed the formation of these aldehydes, but a less objectionable green, metallic flavor was dominant in the fish oils containing this antioxidant. The high levels of 1,5-octadien-3-one found in the samples with Trolox C suppressed the burnt/fishy flavor notes imparted by the decatrienals.

In conclusion, the findings presented in this section and the one previous demonstrate that antioxidants can direct oxidative processes in fish oils and thereby modify flavor development during storage. It appears that the addition of α-TOH alone at high concentrations should be avoided in order to minimize the formation of fishy off-flavors. It may be speculated that the less active hydrogen donors γ-TOH and δ-TOH reduce the accumulation of the more "fishy" volatiles. The use of ascorbyl palmitate in addition to tocopherol(s), on the other hand, appears to direct the forma-

tion of volatile secondary oxidation products to produce less objectionable green-type flavor notes, as also may be the case for Trolox C.

Tocopherols in Combination with Other Antioxidants

The effect of added antioxidants to any fat or oil is dependent on the type and concentration of antioxidants naturally present. Unless purified or stripped, fish oil will contain some α-TOH, and it has been demonstrated that even minor amounts of tocopherol have a pronounced effect on the rate of oxidation of PUFA (Kulås and Ackman 2001a, Lampi *et al.* 1997). When reviewing the literature, the effect of an added antioxidant to fish oil may therefore be difficult to distinguish from a combined, and sometimes synergistic, effect of the antioxidant in question and the α-TOH and other natural antioxidants originally present in the oil.

Ascorbyl palmitate, the oil-soluble derivative of ascorbic acid (vitamin C), is a commonly used food-grade antioxidant. It is well established that ascorbyl palmitate, as well as ascorbic acid, can act as antioxidant synergists to α-TOH (Frankel 1998, Schuler 1990). They readily reduce α-TO$^{\bullet}$, thereby regenerating α-TOH. The multiple effects of ascorbic acid may also include metal inactivation, scavenging of oxygen in aqueous systems, and reduction of hydroperoxides to stable alcohols (Frankel 1998). However, Mäkinen and Hopia (2000) demonstrated that a reduction of methyl linoleate hydroperoxides to more stable hydroxy compounds by ascorbyl palmitate did not occur to an extent significant enough to inhibit the radical chain reactions of hydroperoxide decomposition. On the basis of the results from a related study (Mäkinen *et al.* 2001b), they assumed that the antioxidant activity of ascorbic acid and ascorbyl palmitate is due mainly to their synergistic interaction with other antioxidants, such as tocopherols. At 100 μg/g α-TOH in purified menhaden oil, the concentration for inversion of activity, ascorbyl palmitate (at 250 μg/g) had no effect on the initial rate of formation of hydroperoxides (Fig. 2.13, Kulås and Ackman 2001a). At higher α-TOH concentrations, on the other hand, the presence of ascorbyl palmitate reduced the initial rate of hydroperoxide formation to approximately that in the oil with 100 μg/g α-TOH. Ascorbyl palmitate protected α-TOH from being consumed at all α-TOH concentrations tested, including the concentration for inversion of activity, probably due to the regeneration of α-TOH from α-TO$^{\bullet}$, a mechanism for which chemical evidence was presented by Lambelet *et al.* (1985).

The α-TOH–mediated promotion of oxidation frequently observed in fats and oils, as well as in fish oils, appears to be related to the excellent hydrogen-donating power of this tocopherol compared with that of other phenolic antioxidants, but the mechanism(s) responsible for the inversion of tocopherol activity has not been clarified. It is assumed that it is caused by the participation of α-TOH and/or α-TO$^{\bullet}$ in reactions other than with peroxyl radicals (Fuster *et al.* 1998, Kamal-Eldin and Appelqvist 1996, Marinova and Yanishlieva 1992, Yanishlieva *et al.* 2002). These so-called side reactions may include the interaction with minor oil con-

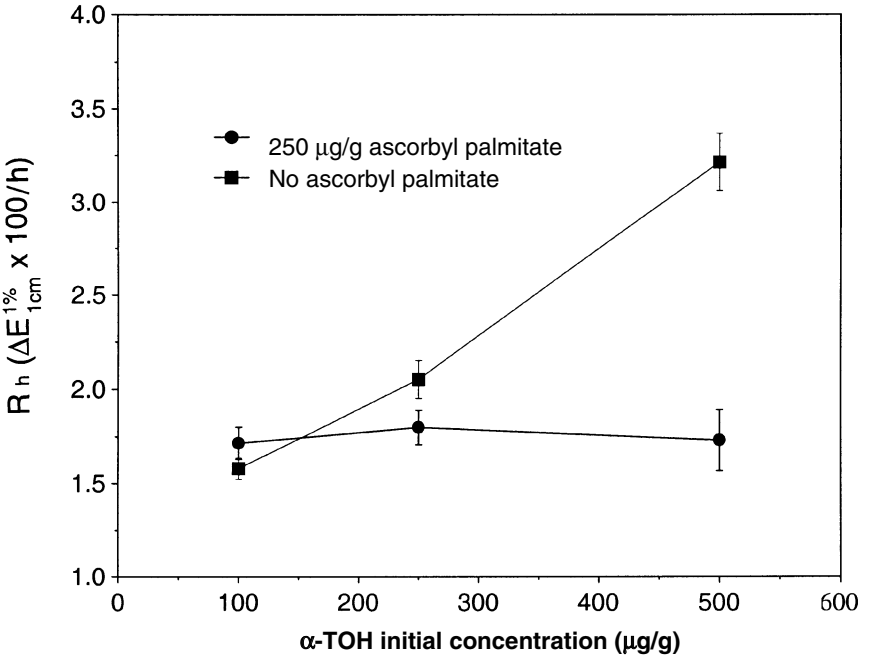

Fig. 2.13. Relationship between initial rate of hydroperoxide formation, R_i, and initial α-tocopherol (TOH) concentration in purified menhaden oil with and without ascorbyl palmitate. Data points are slopes ± SEM. *Source:* Reprinted with permission from Kulås and Ackman (2001a).

stituents such as metal ions and preformed hydroperoxides, the generation of new radicals during tocopherol oxidation to quinone/epoxyquinones, direct reaction with oxygen, as well as chain-transfer with intact fatty acids or hydroperoxides (reviewed by Kamal-Eldin and Appelqvist 1996). The addition of ascorbyl palmitate to fish oil also containing α-TOH eliminated this α-TOH–mediated accumulation of hydroperoxides (Fig. 2.13). By maintaining a high α-TOH concentration for a longer time, the initial rate of hydroperoxide formation was reduced in the oils with an α-TOH concentration >100 µg/g. This emphasizes the importance of the α-TO•, as opposed to α-TOH, in reactions inducing hydroperoxide formation, possibly by chain-transfer with hydroperoxides or intact fatty acids. The question remaining concerns why the initial rate of formation of α-TO• apparently increases with α-TOH concentration (Lambelet and Löliger 1984), when a low α-TOH level (e.g., 100 µg/g) appears to be sufficient for the scavenging of the peroxyl radicals generated by chain initiation and propagation.

The efficiency of rosemary extracts as antioxidants for fish oil was studied by Wada and Fang (1992), who found that an α-TOH/rosemary mixture delayed the onset of oxidation in sardine oil longer than either of the antioxidants alone. An effective inhibition of hydroperoxide formation in fish oil by two rosemary extracts and

their major active components, carnosol and carnosic acid, was also observed by Frankel *et al.* (1996).

The addition of nitrogen-containing phospholipids alone to fish oil had little antioxidant activity compared with a tocopherol mixture (Totani 1997). Phosphatidylethanolamine, however, shows a remarkable synergistic effect with tocopherols. The synergistic effect of tocopherols and phospholipids, and that of phosphatidylethanolamine in particular, was observed in a number of studies in fish oils, including the work of Ohshima *et al.* (1993), Hamilton *et al.* (1998), and Bandarra *et al.* (1999). Several mechanisms for this action of phosphatidylethanolamine have been proposed, including the regeneration of tocopherols from tocopheroxyl radicals, the involvement of Maillard reaction products, and metal chelation.

Ternary antioxidant systems containing tocopherol, ascorbic acid/ascorbyl palmitate, and phospholipid concentrates (lecithin) are promising as stabilizers of fish oils, due to the synergistic action of these antioxidants. However, many studies on the effect of such antioxidant systems in fish oils have relied on accelerated tests at elevated temperatures, and the results may therefore not be directly transferable to storage temperatures at or below the ambient temperature. The ternary mixtures were intermediate in extending the induction period of cod liver oil compared with a range of other natural antioxidants/antioxidant blends using the Rancimat at 80°C, but were superior when the relative change in chemiluminescence at 35°C was used as a measure of oxidative stability (Burkow *et al.* 1995). Yi *et al.* (1991) and Han *et al.* (1991) evaluated the antioxidant effects of δ-TOH and ascorbic acid in a sardine oil/lecithin/water system using the Rancimat at 80°C and storage as thin films at 30°C, respectively, and reported a pronounced synergistic effect of the three antioxidants. The addition of lecithin, ascorbyl palmitate, and tocopherol (preferably γ-TOH and preferably used in a ratio of 3:2:4 in a total amount of 3000 μg/g) to silica-treated fish oil substantially increased the Rancimat induction time (Koschinski and Macfarlane 1993).

In a study by Hamilton *et al.* (1998), the effects of tocopherols alone and in combination with ascorbyl palmitate and lecithin on the oxidative stability of refined and deodorized fish oil held at 20°C were investigated. The combination of δ-TOH (at 2%), ascorbyl palmitate (at 0.1%), and lecithin (at 0.5%) provided the greatest protection of the antioxidants tested, and the fish oil showed no significant oxidation as determined by the POV, over a period of 6 mo when stored in air. Off-flavors, on the other hand, developed in the oil within 3 wk despite the low POV, and no antioxidant treatment substantially improved the flavor stability. The lecithin itself also imparted certain initial off-flavors to the oil. The ratio of ascorbyl palmitate to lecithin, rather than the absolute level of lecithin, appears to be important for an optimal effect of such antioxidant blends, and the ability of phosphatidylethanolamine to interact with ascorbyl radicals was suggested to contribute to the observed effect (Hamilton *et al.* 1998). Several ternary antioxidant systems containing tocopherol, ascorbic acid/ascorbyl palmitate, and lecithin are available commercially.

Concluding Remarks

The tocopherols affect many stages of the autoxidation process in fish oils, including the formation of hydroperoxides, the decomposition of hydroperoxides, the formation of nonvolatile secondary oxidation products, as well as the composition of the volatile secondary oxidation products formed. The number of methyl groups on the chroman ring of the tocopherols has a remarkable effect on their antioxidant properties in fish oil. The results obtained in oxidation tests addressing these different aspects of lipid autoxidation can be related to the relative hydrogen-donating ability of the tocopherols, which decreases in the order of α-TOH > γ-TOH > δ-TOH.

Antioxidants may be evaluated by their ability to retard both the formation of hydroperoxides and of secondary oxidation products, as well as the extent to which they increase the induction period of oil. An antioxidant for oils high in PUFA should also be active *in vivo*. Ideally, an antioxidant should perform well according to all of these criteria. α-Tocopherol is active in retarding the overall formation of secondary oxidation products, but induces hydroperoxide formation at concentrations relevant for addition, and should not be used alone as an antioxidant additive to fish oils, particularly not at high concentrations. In addition, this tocopherol appears to direct the formation of the more flavor-potent volatile secondary oxidation products. In contrast, δ-TOH at high levels of addition is an efficient antioxidant on the basis of both primary and secondary oxidation, as well as the duration of the induction period, but its biological activity is low. Due to its high activity *in vivo*, α-TOH should continue to be used as an antioxidant additive to fish oils. Methods to protect α-TOH, as well as γ-TOH, from rapid consumption and corresponding induction of hydroperoxide formation, and, moreover, to make the most of its excellent peroxyl radical–scavenging activity, therefore, become important. Ascorbyl palmitate substantially increases the antioxidant strength of α-TOH, as well as that of tocopherol blends, in fish oil. Ternary antioxidant systems (containing tocopherol, ascorbyl palmitate, and lecithin) are also very promising as stabilizers of fish oils. Alternatively, α-TOH may be added to fish oil, in addition to other antioxidants, as the commercially available ester of acetic acid. This compound is not an antioxidant *in vitro* but possesses high activity as vitamin E *in vivo*.

Antioxidant addition is an important factor in any attempt to increase the stability of fish oils. However, for antioxidants to substantially reduce flavor deterioration, the oil to which they are added must be of high initial quality with regard to oxidative deterioration. This implies the use of freshly produced oil that has been subjected to a gentle refining process and kept in the absence of light and oxygen. The use of high temperature and/or oxygen access will induce the formation of artifacts such as dimers and oligomers, as well as the conjugation and isomerization of double bonds. Although not detected by the most common methods for determining fatty acid oxidation, these products are likely to contribute to a more rapid formation of fishy off-flavors.

References

Ackman, R.G., and Cormier, M.G. (1967) α-Tocopherol in Some Atlantic Fish and Shellfish with Particular Reference to Live-Holding Without Food, *J. Fish. Res. Board Canada 24*, 357–373.

Aminullah Bhuiyan, A.K.M., Ratnayake, W.M.N., and Ackman, R.G. (1993) Nutritional Composition of Raw and Smoked Atlantic Mackerel (*Scomber scombrus*): Oil- and Water-Soluble Vitamins, *J. Food Comp. Anal. 6*, 172–184.

Azzi, A., and Stocker, A. (2000) Vitamin E: Non-Antioxidant Roles, *Prog. Lipid Res. 39*, 231–255.

Badings, H.T. (1970) Cold-Storage Defects in Butter and Their Relation to the Autoxidation of Unsaturated Fatty Acids, *Neth. Milk Dairy J. 23–24*, 146–256.

Bandarra, N.M., Campos, R.M., Batista, I., Nunes, M.L., and Empis, J.M. (1999) Antioxidant Synergy of α-Tocopherol and Phospholipids, *J. Am. Oil Chem. Soc. 76*, 905–913.

Bimbo, A.P. (1990) Processing of Fish Oils, in *Fish Oil in Nutrition* (Stansby, M.E, ed.) p. 181, Van Nostrand Reinhold, New York.

Blekas, G., Tsimidou, M., and Boskou, D. (1995) Contribution of α-Tocopherol to Olive Oil Stability, *Food Chem. 52*, 289–294.

Bruna, E., Petit, E., Beljean-Leymarie, M., Huynh, S., and Nouvelot, A. (1989) Specific Susceptibility of Docosahexaenoic Acid and Eicosapentaenoic Acid to Peroxidation in Aqueous Solution, *Lipids 24*, 970–975.

Burkow, I.C., Vikersveen, L., and Saarem, K. (1995) Evaluation of Antioxidants for Cod Liver Oil by Chemiluminescence and the Rancimat Method, *J. Am. Oil Chem. Soc. 72*, 553–557.

Burton, G.W., Doba, T., Gabe, E.J., Hughes, L., Lee, F.L., Prasad, L., and Ingold, K.U. (1985) Autoxidation of Biological Molecules. 4. Maximizing the Antioxidant Activity of Phenols, *J. Am. Chem. Soc. 107*, 7053–7065.

Burton, G.W., Hughes, L., and Ingold, K.U. (1983) Antioxidant Activity of Phenols Related to Vitamin E. Are There Chain-Breaking Antioxidants Better than α-Tocopherol? *J. Am. Chem. Soc. 105*, 5950–5951.

Burton, G.W., and Ingold, K.U. (1981) Autoxidation of Biological Molecules. 1. The Antioxidant Activity of Vitamin E and Related Chain-Breaking Phenolic Antioxidants *In Vitro*, *J. Am. Chem. Soc. 103*, 6472–6477.

Burton, G.W., and Ingold, K.U. (1989) Vitamin E as *In Vitro* and *In Vivo* Antioxidant, *Ann. N.Y. Acad. Sci. 570*, 7–22.

Chan, H.W.-S., Coxon, D.T., Peers, K.E., and Price, K.R. (1982) Oxidative Reactions of Unsaturated Lipids, *Food Chem. 9*, 21–34.

Frankel, E.N. (1982) Volatile Lipid Oxidation Products, *Prog. Lipid Res. 22*, 1–33.

Frankel, E.N. (1991) Recent Advances in Lipid Oxidation, *J. Sci. Food Agric. 54*, 495–511.

Frankel, E.N. (1998) *Lipid Oxidation*, The Oily Press, Dundee, Scotland.

Frankel, E.N., Huang, S.-W., Prior, E., and Aeschbach, R. (1996) Evaluation of Antioxidant Activity of Rosemary Extracts, Carnosol and Carnosic Acid in Bulk Vegetable Oils and Fish Oil and Their Emulsions, *J. Sci. Food Agric. 72*, 201–208.

Fuster, M.D., Lampi, A.-M., Hopia, A., and Kamal-Eldin, A. (1998) Effects of α- and γ-Tocopherols on the Autoxidation of Purified Sunflower Triacylglycerols, *Lipids 33*, 715–722.

Hamilton, R.J., Kalu, C., McNeill, G.P., Padley, F.B., and Pierce, J.H. (1998) Effects of Tocopherols, Ascorbyl Palmitate, and Lecithin on Autoxidation of Fish Oil, *J. Am. Oil Chem. Soc. 75*, 813–822.

Han, D.H., Yi, O.-S., and Shin, H.-K. (1991) Solubilization of Vitamin C in Fish Oil and Synergistic Effect with Vitamin E in Retarding Oxidation, *J. Am. Oil Chem. Soc. 68*, 740–743.

Hartvigsen, K., Lund, P., Hansen, L.F., and Hølmer, G. (2000) Dynamic Headspace Gas Chromatography/Mass Spectrometry Characterization of Volatiles Produced in Fish Oil Enriched Mayonnaise During Storage, *J. Agric. Food Chem. 48*, 4858–4867.

Hemre, G.-I., Juell, J.E., Hamre, K., Lie, Ø., Strand, B., Arnesen, P., and Holm, J.C. (1997) Cage Feeding of Atlantic Mackerel (*Scomber scombus*): Fatty Acid Composition, Oxidation Status and Vitamin E Concentration, *Aquat. Living Resour. 10*, 365–370.

Hopia, A., Huang, S.-W., and Frankel, E.N. (1996) Effect of α-Tocopherol and Trolox on the Decomposition of Methyl Linoleate Hydroperoxides, *Lipids 31*, 357–365.

Hsieh, T.C.Y., Williams, S.S., Vejaphan, W., and Meyers, S.P. (1989) Characterization of Volatile Components of Menhaden Fish (*Brevoortia tyrannus*) Oil*, J. Am. Oil Chem. Soc. 66*, 114–117.

Hu, S.-P., and Pan, B.S. (2000) Modification of Fish Oil Aroma Using Macroalgal Lipoxygenase, *J. Am. Oil Chem. Soc. 77*, 343–348.

Huang, S.-W., Frankel, E.N., and German, J.B. (1994) Antioxidant Activity of α- and γ-Tocopherols in Bulk Oils and in Oil-in-Water Emulsions, *J. Agric. Food Chem. 42*, 2108–2114.

Hudson, B.J.F., and Ghavami, M. (1984) Stabilising Factors in Soyabean Oil—Natural Components with Antioxidant Activity, *Lebensm. Wiss. Technol. 17*, 82–85.

Institute of Medicine, Panel on Dietary Antioxidants and Related Compounds (2000) *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids*, National Academy Press, Washington.

Jung, M.Y., and Min, D.B. (1990) Effects of α-, γ- and δ-Tocopherols on Oxidative Stability of Soybean Oil, *J. Food Sci. 55*, 1464–1465.

Kamal-Eldin, A., and Appelqvist, L.-Å. (1996) The Chemistry and Antioxidant Properties of Tocopherols and Tocotrienols, *Lipids 31*, 671–701.

Karahadian, C., and Lindsay, R.C. (1989a) Evaluation of Compounds Contributing Characterizing Fishy Flavors in Fish Oils, *J. Am. Oil Chem. Soc. 66*, 953–960.

Karahadian, C., and Lindsay, R.C. (1989b) Action of Tocopherol-Type Compounds in Directing Reactions Forming Flavor Compounds in Autoxidizing Fish Oils, *J. Am. Oil Chem. Soc. 66*, 1302–1308.

Kinsella, J.E. (1987) *Seafoods and Fish Oils in Human Health and Disease*, Marcel Dekker, New York.

Koschinski, I., and Macfarlane, N. (1993) Stability of Marine Oils by Treatment with Silica and Addition of Lecithin, Ascorbyl Palmitate and Tocopherol, International Patent WO 93/10207.

Kulås, E. (2000) Antioxidant Properties of Tocopherols in Fish Oils, Ph.D. Thesis, Dalhousie University, Halifax, Nova Scotia.

Kulås, E., and Ackman, R.G. (2001a) Protection of α-Tocopherol in Nonpurified and Purified Fish Oil, *J. Am. Oil Chem. Soc. 78*, 197–203.

Kulås, E., and Ackman, R.G. (2001b) Properties of α-, γ- and δ-Tocopherol in Purified Fish Oil Triacylglycerols, *J. Am. Oil Chem. Soc. 78*, 361–367.

Kulås, E., and Ackman, R.G. (2001c) Different Tocopherols and the Relationship Between Two Methods for Determination of Primary Oxidation Products in Fish Oil, *J. Agric. Food Chem. 49*, 1724–1729.

Kulås, E., Olsen, E., and Ackman, R.G. (2002) Effect of α-, γ- and δ-Tocopherol on the Distribution of Volatile Secondary Oxidation Products in Fish Oil, *Eur. J. Lipid Sci. Technol. 104*, 520–529.

Lambelet, P., and Löliger, J. (1984) The Fate of Antioxidant Radicals During Lipid Autoxidation. I. The Tocopheroxyl Radicals, *Chem. Phys. Lipids 35*, 185–198.

Lambelet, P., Saucy, F., and Löliger, J. (1985) Chemical Evidence for Interactions Between Vitamins E and C, *Experientia 41*, 1384–1388.

Lampi, A.-M., Hopia, A., and Piironen, V. (1997) Antioxidant Activity of Minor Amounts of γ-Tocopherol in Natural Triacylglycerols, *J. Am. Oil Chem. Soc. 74*, 549–555.

Lampi, A.-M., Kataja, L., Kamal-Eldin, A., and Piironen, V. (1999) Antioxidant Activities of α- and γ-Tocopherols in the Oxidation of Rapeseed Oil Triacylglycerols, *J. Am. Oil Chem. Soc. 76*, 749–755.

Liebler, D.C., and Burr, J.A. (1995) Antioxidant Stoichiometry and the Oxidative Fate of Vitamin E in Peroxyl Radical Scavenging Reactions, *Lipids 30*, 789–793.

Macfarlane, N., Salt, J., Birkin, R., and Kendrick, A. (2001) The FAST Index—A Fishy Scale. A Search for a Test to Quantify Fish Flavor, *Inform 12*, 244–249.

Mäkinen, M., and Hopia, A. (2000) Effects of α-Tocopherol and Ascorbyl Palmitate on the Isomerization and Decomposition of Methyl Linoleate Hydroperoxides, *Lipids 35*, 1215–1223.

Mäkinen, M., Kähkönen, M., and Hopia, A. (2001b) Ascorbic Acid and Ascorbyl Palmitate Have Only Minor Effect on the Formation and Decomposition of Methyl Linoleate Hydroperoxides, *Eur. J. Lipid Sci. Technol. 103*, 683–687.

Mäkinen, M., Kamal-Eldin, A., Lampi, A.-M., and Hopia, A. (2001a) α-, γ- and δ-Tocopherols as Inhibitors of Isomerization and Decomposition of *cis,trans* Methyl Linoleate Hydroperoxides, *Eur. J. Lipid Sci. Technol. 103*, 286–291.

Marinova, E.M., and Yanishlieva, N.V. (1992) Effect of Temperature on the Antioxidative Action of Inhibitors in Lipid Autoxidation, *J. Sci. Food Agric. 60*, 313–318.

Meijboom, P.W. (1964) Relationship Between Molecular Structure and Flavor Perceptibility of Aliphatic Aldehydes, *J. Am. Oil Chem. Soc. 41*, 326–328.

Meijboom, P.W., and Stroink, J.B.A. (1972) 2-*trans*,4-*cis*,7-*cis*-Decatrienal, the Fishy Off-Flavor Occurring in Strongly Autoxidized Oil Containing Linolenic Acid or ω3,6,9, etc., Fatty Acids, *J. Am. Oil Chem. Soc. 49*, 555–558.

Miyashita, K., Hirao, M., Nara, E., and Ota, T. (1995) Oxidative Stability of Triglycerides from Orbital Fat of Tuna and Soybean Oil in an Emulsion, *Fish. Sci. 61*, 273–275.

Muggli, R. (1994) Polyunsaturated Fatty Acids and Requirements of Vitamin E, *Fat Sci. Technol. 96,* 17–19.

Nakamura, K., Iida, H., and Tokunaga, T. (1980) Separation and Identification of Odor in Oxidized Sardine Oil, *Bull. Japan Soc. Sci. Fish. 46*, 355–360.

Neff, W.E., Frankel, E.N., and Weisleder, D. (1981) High-Pressure Liquid Chromatography of Autoxidized Lipids: II. Hydroperoxy-Cyclic Peroxides and Other Secondary Products from Methyl Linolenate, *Lipids 16*, 439–448.

Niki, E., Tsuchiya, J., Yoshikawa, Y., Yamamoto, Y., and Kamiya, Y. (1986) Oxidation of Lipids. XIII. Antioxidant Activities of α-, β-, γ-, and δ-Tocopherols, *Bull. Chem. Soc. Jpn. 59*, 497–501.

Ohshima, T., Fujita, Y., and Koizumi, C. (1993) Oxidative Stability of Sardine and Mackerel Lipids with Reference to Synergism Between Phospholipids and α-Tocopherol, *J. Am. Oil Chem. Soc. 70*, 269–276.

Olsen, E. (2000) Cod Liver Oil Oxidation Products (Oksidasjonsprodukter i tran), Master of Science Thesis, University of Oslo, Oslo, Norway.

Peers, K.E., Coxon, D.T., and Chan, H.W.-S. (1981) Autoxidation of Methyl Linolenate: The Effect of α-Tocopherol, *J. Sci. Food Agric. 32*, 898–904.

Pokorný, J. (1987) Major Factors Affecting the Autoxidation of Lipids, in *Autoxidation of Unsaturated Lipids* (Chan, H.W.-S., Ed.), pp. 141–206, Academic Press, London.

Porter, N.A., Caldwell, S.E., and Mills, K.E. (1995) Mechanisms of Free Radical Oxidation of Unsaturated Lipids, *Lipids 30*, 277–289.

Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) Unified Mechanism for Polyunsaturated Fatty Acid Autoxidation. Competition of Peroxy Radical Hydrogen Atom Abstraction, β-Scisssion, and Cyclization, *J. Am. Chem. Soc. 103*, 6447–6455.

Porter, N.A., Weber, B.A., Weenen, H., and Khan, J.A. (1980) Autoxidation of Polyunsaturated Lipids. Factors Controlling the Stereochemistry of the Product Hydroperoxides, *J. Am. Chem. Soc. 102*, 5597–5601.

Rørbæk, K. (1994) Oxidation and Flavours in Fish Oil, Ph.D. Thesis, Technical University of Denmark, Lyngby, Denmark.

Sagredos, A.N. (1991) Fatty Acid Composition of Fish Oil Capsules, *Fat Sci. Technol. 93*, 184–191.

Sagredos, A.N. (1992) On the Quality and Contamination of Fish Oil Capsules, *Fat Sci. Technol. 94*, 101–111.

Sanchez, J., and Myers, T.N. (1996) Peroxides and Compounds (Organic), in *Kirk-Othmer Encyclopedia of Chemical Technology* (Kroschwitz, J.I., and Howe-Grant, M., eds.) 4th ed., Vol. 18, pp. 230–310, John Wiley and Sons, New York.

Schuler, P. (1990) Natural Antioxidants Exploited Commercially, in *Food Antioxidants* (Hudson, B.J.F., ed.) pp. 99–170, Elsevier Applied Science, Essex, UK.

Shukla, V.K.S., and Perkins, E.G. (1998) Rancidity in Encapsulated Health-Food Oils, *Inform 9*, 955–961.

Sigurgisladottir, S., Ackman, R.G., and O'Keefe, S.F. (1993) Selective Deposition of α-Tocopherol in Lipids of Farmed Blue Mussels (*Mytilus edulis*), *J. Food Lipids 1*, 97–109.

Totani, Y. (1997) Antioxidative Effect of Nitrogen-Containing Phospholipids on Autoxidation of Polyunsaturated Oil, *J. Jpn. Oil Chem. Soc. 46*, 3–15.

Wada, S., and Fang, X. (1992) The Synergistic Antioxidant Effect of Rosemary and α-Tocopherol in Sardine Oil Model System and Frozen-Crushed Fish Meat, *J. Food Process. Preserv. 16*, 263–274.

Yanishlieva, N.V., Kamal-Eldin, A., Marinova, E.M., and Toneva, A.G. (2002) Kinetic Study of the Antioxidant Activity of α- and γ-Tocopherols in Sunflower and Soybean Triacylglycerols, *Eur. J. Lipid Sci. Technol. 104*, 262–270.

Yazu, K., Yamamoto, Y., Niki, E., Miki, K., and Ukegawa, K. (1998) Mechanism of Lower Oxidizability of Eicosapentaenoate Than Linoleate in Aqueous Micelles. II. Effect of Antioxidants, *Lipids 33*, 597–600.

Yazu, K., Yamamoto, Y., Ukegawa, K., and Niki, E. (1996) Mechanism of Lower Oxidizability of Eicosapentaenoate Than Linoleate in Aqueous Micelles, *Lipids 31*, 337–340.

Yi, O.-S., Han, D., and Shin, H.-K. (1991) Synergistic Antioxidant Effects of Tocopherol and Ascorbic Acid in Fish Oil/Lecithin/Water System, *J. Am. Oil Chem. Soc. 68*, 881–883.

Chapter 3

Competitive Oxidation between α-Tocopherol and Unsaturated Fatty Acids under Thermoxidation Conditions

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Introduction

Lipid oxidation is accepted as a free radical–mediated reaction. At low temperatures, hydroperoxides are the main products and catalysts of the reaction. Their catalytic effect results from their unimolecular and bimolecular decomposition to peroxyl and alkoxyl radicals. Although thermoxidation proceeds by the same basic mechanism as autoxidation, its rate is much higher and the quantitative nature of the reaction products is significantly different. This is because of the extreme instability of hydroperoxides and the lower solubility of oxygen in lipids at high temperatures. Consequently, there is an increased participation of alkyl and alkoxyl radicals and diminished participation of peroxyl radicals. Products of oxidation at high temperatures are mainly oligomers and polymers with carbon-carbon, epoxy, and ether bonds as opposed to the hydroperoxides formed at low temperatures.

Purified triacylglycerols of sunflower, rapeseed and high-oleic sunflower oils were found to be less stable than their nonpurified forms containing endogenous tocopherols. The α- and γ-tocopherols exerted an antipolymerization effect in model systems composed of purified high-oleic sunflower triacylglycerols at 180°C (Lampi and Kamal-Eldin 1998). Vegetable oils have different susceptibilities toward thermoxidative degradation depending on their fatty acid composition, endogenous antioxidants, and conditions of the physical environment. At high temperatures, such as those of frying, the fatty acid composition is more important for the stability than the tocopherol content and composition. It was suggested that high-oleic/high-α-tocopherol oils (such as high-oleic sunflower oil) have lower thermoxidative stability than high-oleic/high-α-tocopherol oils (such as high-oleic canola and high-oleic soybean oils). Because of its instability, it is of primary interest to know the evolution of α-tocopherol degradation in relation to the fatty acid oxidation status.

A marked loss of α-tocopherol upon heating of the model system, even under low oxygen concentrations, was observed. The α-tocopherol loss was strongly related to the heating temperature and heating time. Additional experiments, using a synthetic antioxidant *tert*-butyl hydroquinone (TBHQ) and flushing with nitrogen, were per-

formed to determine whether the tocopherol loss is inevitable due to a thermal breakdown or whether it can be explained by oxidative degradation. Experiments under nitrogen indicated that under specific conditions in which the atmosphere is constantly renewed, α-tocopherol is thermally stable in a triacylglycerol matrix up to temperatures of 240°C. Tocopherols could be protected against degradation only when nitrogen, as an inert gas, was constantly blown through the triolein (Verleyen *et al.* 2001b).

Tocopherol Degradation in Oils with Different Degrees of Unsaturation

Scattered research on the influence of triacylglycerol unsaturation on the α-tocopherol loss and triacylglycerol polymerization during frying is available. In some studies, it was found that tocopherols degraded faster in less unsaturated oils and a tocopherol exhaustion was observed at lower oil degradation (Jorge *et al.* 1996a and 1996b), whereas in other studies tocopherol losses were independent of the fatty acid matrices of triolein and trilinolein (Barrera-Arellano *et al.* 1999).

α-Tocopherol degradation in relation to the formation of polar and nonpolar triacylglycerol oxidation products was studied in four vegetable oils, namely, palm, high-oleic sunflower, sunflower, and flaxseed oils (Fig. 3.1). The difference in the rate of tocopherol degradation, as affected by triacylglycerol unsaturation, was evident at 240°C but not at 180°C (Verleyen *et al.* 2002). At 240°C, α-tocopherol was more stable in the more unsaturated flaxseed and sunflower oils compared with the more saturated high-oleic sunflower and palm oils, which is in agreement with the conclusions of Barrera-Arellano *et al.* (1999) who did not observe any differences in tocopherol losses during simulated deep-frying experiments at 180°C in different

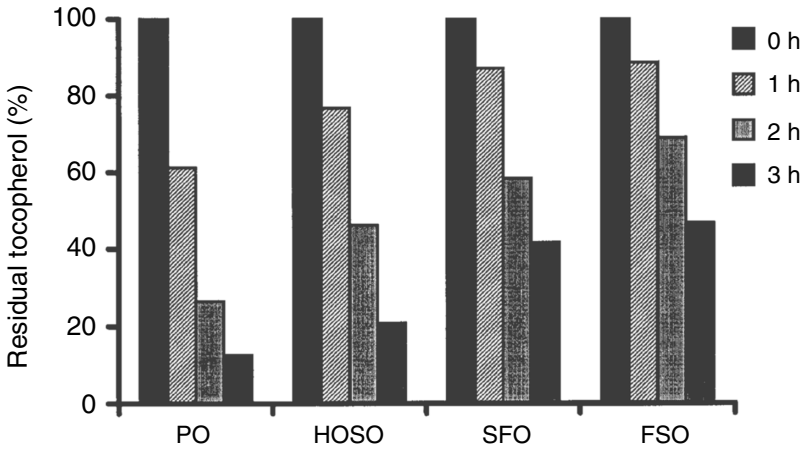


Fig. 3.1. Degradation of α-tocopherol in four vegetable oils during 3 h of heating at 240°C. Initial tocopherol concentration was 1000 μg/g. PO = palm oil, HOSO = high-oleic sunflower oil, SFO = sunflower oil, and FSO = flaxseed oil.

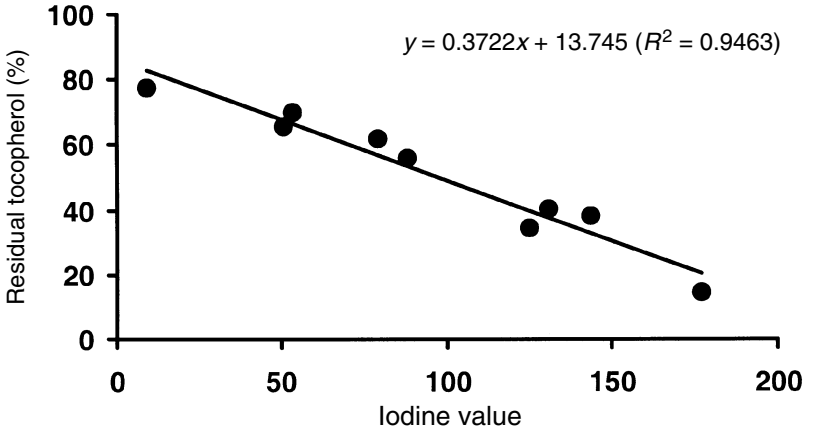


Fig. 3.2. Relationship between iodine value and α-tocopherol degradation (%) in purified triacylglycerols from nine vegetable oils after 2 h of heating at 240°C. The oils were coconut oil, palm oil, tallow, olive oil, high-oleic sunflower oil, sunflower oil, corn oil, soybean oil, and flaxseed oil.

levels of triolein and trilinolein. At these high temperatures, the rate of tocopherol degradation was not significantly affected by the tocopherol concentration, which is in agreement with Lampi and Kamal-Eldin (1998).

The influence of triacylglycerol unsaturation on the tocopherol loss was also investigated at 240°C using nine purified oils with different triacylglycerol unsaturation (coconut oil, palm oil, tallow, olive oil, high-oleic sunflower oil, sunflower oil, corn oil, soybean oil, and flaxseed oil). After heating for 2 h at 240°C, a considerable difference in the stability of tocopherol was observed between the different oils and, again, the tocopherol was more stable in the more unsaturated oils. A linear correlation ($y = 86.25 - 0.37x$, $R^2 = 0.9463$) was obtained between the iodine value (x) and percent tocopherol degradation (y) (Fig. 3.2).

Synthesis of α-Tocopherol Oxidation Products

On the basis of the results obtained above, it was interesting to study the degradation products of α-tocopherol. Because standards of tocopherol oxidation products are not commercially available, several oxidation products of α-tocopherol have been synthesized according to existing methods and used to characterize and authenticate the oxidation products formed in triacylglycerols upon heating.

Preparation of α-Tocopherol Spirotrimer by Oxidation of α-Tocopherol with Potassium Ferricyanide

A schematic overview of the oxidation of α-tocopherol toward α-tocopherol-spirotrimer upon oxidation with potassium ferricyanide is presented in Figure 3.3.

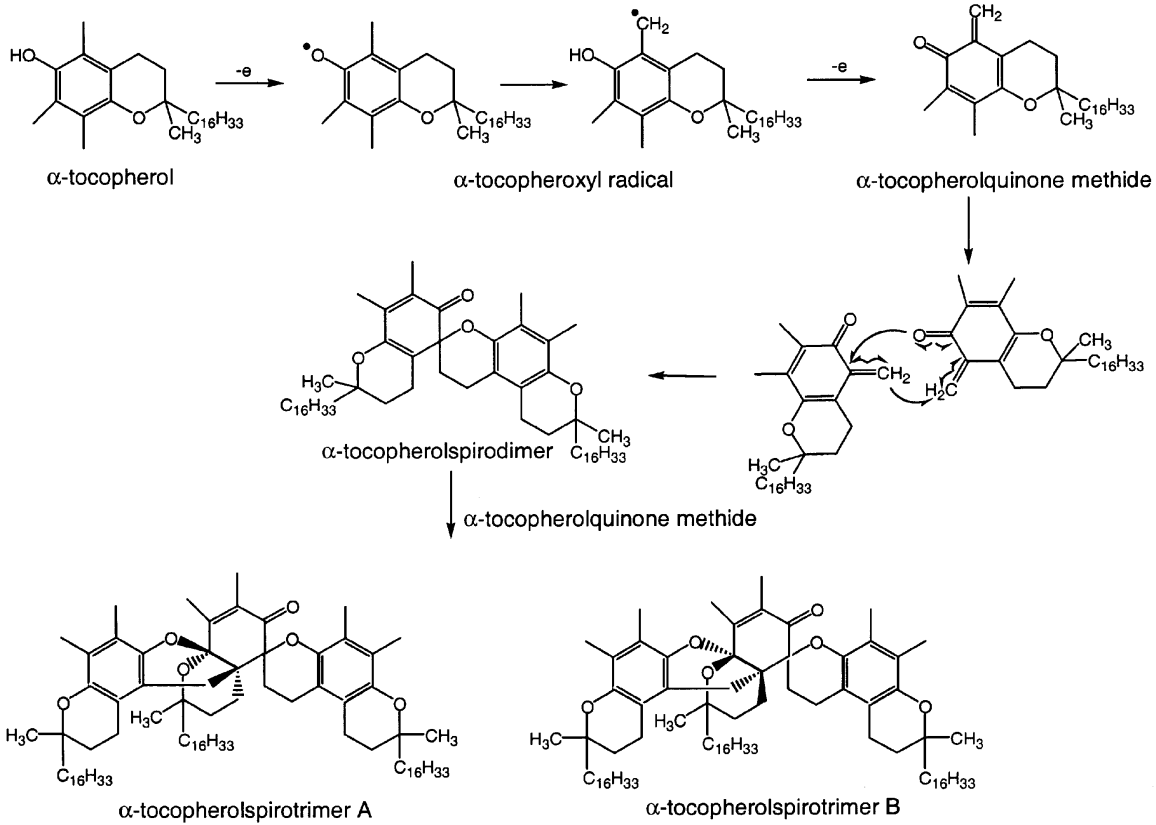


Fig. 3.3. Autoxidation of α-tocopherol to α-tocopherolspirotrimers A and B.

The quinone methide, which has a very short lifetime, is supposed to be a key intermediate in the trimerization (Bolon 1970, Skinner and Alaupovic 1963, Skinner and Parkhurst 1964). Because the third *ortho*-quinone methide can add to the dimer in two ways, it leads to the formation of two tocopherol spirotrimer isomers.

Oxidation of α-tocopherol with potassium ferricyanide was carried out according to a modified procedure of Nelan *et al.* (1962). Briefly, α-tocopherol (1 g), dissolved in 30 mL petroleum ether, was shaken in a separatory funnel with a potassium ferricyanide solution [3 g $K_3Fe(CN)_6$ dissolved in 150 mL of 0.5 N NaOH]. The solution was shaken vigorously for 3 min and instantly colored dark green followed by a bright yellow color. The petroleum ether layer was subsequently washed with a ferrous sulfate solution (5%, 2 × 20 mL) and water (3 × 20 mL). After being dried over anhydrous sodium sulfate, the solvent was evaporated leaving a bright yellow residue.

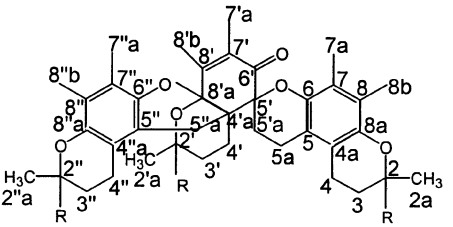
Analytical high-performance liquid chromatography (HPLC) analysis of the crude oxidation mixture with *n*-hexane/isopropyl alcohol (99.5:0.5, vol/vol) as mobile phase and ultraviolet (UV) detector set at 292 nm indicated almost a complete disappearance of α-tocopherol (retention time, 7.6 min); a major peak at 3.7 min and several minor peaks at 5.3, 5.6, 8.2 and 9.3 min appeared in the chromatogram. Nonpolar α-tocopherol oxidation products were isolated by silica gel column chromatography (40 g silica, column 40 × 1 cm, i.d.) developed with 150 mL petroleum ether/ethyl acetate (97:3, vol/vol). Purity of the different fractions was monitored by analytical HPLC. The component with a retention time of 3.7 min in the HPLC chromatogram eluted in the first fractions and spectral data indicated this component to be α-tocopherolspirotrimer (*vide infra*). The concentration of the other oxidation products was too low for identification.

α-Tocopherolspirotrimer was further separated by preparative normal-phase (NP) HPLC with *n*-hexane/isopropyl alcohol (98:2, vol/vol) as isocratic eluent at a flow of 15 mL/min, yielding two trimers A (54%) and B (46%) at a retention time of 5.4 min and 6.9 min, respectively. α-Tocopherolspirotrimer had a UV λ_{max} (*n*-hexane) 290 nm, infrared (IR; NaCl) 1680, 1654 cm^{-1} and ^1H NMR and ^{13}C NMR spectral data (Table 3.1) in agreement with Yamauchi *et al.* (1988). The geometric relationship between trimer A and trimer B was established by interpretation of the spectra. If the third tocopherol quinone methide adds *trans* to the 2′a-methyl group as in trimer A, the 2′a-methyl group is localized in a shielding environment above the plane of the enone system and is found at 1.22 μg/g in the ^1H NMR. In trimer B, the tocopherol quinone methide residue is present in the *cis* configuration to the 2′a-methyl group, resulting in a deshielding of the 2′a-methyl group at 1.44 μg/g (Yamauchi *et al.* 1988).

Preparation of α-Tocopherolquinone Oxidation of α-Tocopherol with Ferric Chloride

α-Tocopherolquinone was obtained by oxidation of α-tocopherol with ferric chloride (Frampton *et al.* 1954 and 1960). α-Tocopherol (2 g) was added to 30 mL of a

TABLE 3.1
Nuclear Magnetic Resonance (NMR) Spectral Data of α-Tocopherol Spirotrimers A and B



^{1}H NMR chemical shifts	α-Tocopherolspirotrimer A	α-Tocopherolspirotrimer B
4, 4″	2.42 (t, 2 × 3H, J = 7.26)	2.42 (t, 2 × 3H, J = 7.26)
	2.23 (s, 3H)	2.23 (s, 3H)
7a, 8b, 7″a, 8″b	2.19 (s, 3H)	2.19 (s, 3H)
	2.13 (s, 3H)	2.13 (s, 3H)
	2.09 (s, 3H)	2.09 (s, 3H)
7′a	1.99 (s, 3H)	1.99 (s, 3H)
8′b	1.67 (s, 3H)	1.67 (s, 3H)
3, 3′, 3″	1.79 (t, 2H, J = 7,26)	1.79 (t, 2H, J = 7,26)
2′a	1.22 (s, 3H)	1.44 (s, 3H)
2a	1.25 (s, 3H)	1.25 (s, 3H)
2″a	1.20 (s, 3H)	1.20 (s, 3H)
R, R′, R″	0.81–1.4	0.81–1.4
^{13}C NMR chemical shifts		
6′	202.5	202.4
8′	149.8	150.2
6, 8a, 6″, 8″a	145.8, 145.5,144.7, 142.9	145.9, 145.5,144.7, 142.9
7′	127.1	127.0
5, 7, 5″, 7″	123.5, 123.4, 122.1	123.5, 123.4, 122.1
4a, 8, 4″a, 8″	116.1, 115.8, 115.7, 115.0	118.1, 115.7, 115.2, 115.0
8′a	99.5	99.2
5′	81.0	80.9
2′	76.8	76.2
2, 2″	74.4, 74.3	74.4, 74.3
R, R′, R″	40.7–19.5	39.8–19.5

6% solution of ferric chloride in 50% ethanol. The solution was stirred (3 h at 60°C) under nitrogen, diluted with 50 mL water, and extracted with petroleum ether (2 × 40 mL).

The black residue, obtained after evaporation of the petroleum ether, was separated on a silica gel column (40 cm, i.d.) developed first with 150 mL petroleum ether/ethyl acetate (90:10, vol/vol) followed by 200 mL petroleum ether/ethyl acetate (70:30, vol/vol). Purity of the different fractions was determined by HPLC, using *n*-hexane/isopropyl alcohol (99:1, vol/vol) as a mobile phase and UV detection at 275 nm, which indicated an almost complete removal of α-tocopherol

(retention time, 5.2 min) and formation of one major product (retention time, 8.9 min). The compound was identified as α-tocopherolquinone (Fig. 3.4) and had a UV λ_{max} (*n*-hexane) 275 nm, IR (NaCl) cm^{-1}: 3495 (OH), 2925, 2845, 1642 (C=O), 1460, 1374, 1309, and ^{1}H NMR/^{13}C NMR spectral data listed in Table 3.2.

Preparation of α-Tocopherolquinone Oxidation of α-Tocopherol with Azo-iso-butyronitrile

4a,5-Epoxy-α-tocopherolquinone and 7,8-epoxy-α-tocopherolquinone were prepared according to a method modified from Csallany and Ha (1992). α-Tocopherol (0.3 g) was dissolved in 100 mL of a mixture acetonitrile/water (3:1, vol/vol), cooled to 0–3°C and oxygen was slowly blown into the solution through a capillary for 15 min until saturated. The radical initiator azo-iso-butyronitrile (AIBN, 1.8 g) was added, and the solution was slowly heated to 50°C and stirred overnight. Then the oxidation mixture was poured in water (50 mL), extracted with petroleum ether (2 × 100 mL), and dried over anhydrous sodium sulfate. After evaporation of the petroleum ether, the crude residue was dissolved in *n*-hexane (5 mL) and filtered over a cotton cloth to remove residual traces of AIBN crystals.

The crude α-tocopherol oxidation mixture thus obtained was cleaned up by preparative HPLC analysis into different classes of α-tocopherol oxidation products. The preparative HPLC column eluted at a flow rate of 15 mL/min with a gradient of *n*-hexane/isopropyl alcohol ranging from 99.5:0.5 (vol/vol) to 99:1 (vol/vol) over a period of 8 min. The eluent was monitored at different wavelengths between 240 and 290 nm, and a wavelength of 275 nm was found optimal to determine the different tocopherol oxidation products. The gradient separated the crude oxidation mixture into four fractions eluting at 5, 11.5, 17.3, and 18.7 min, respectively.

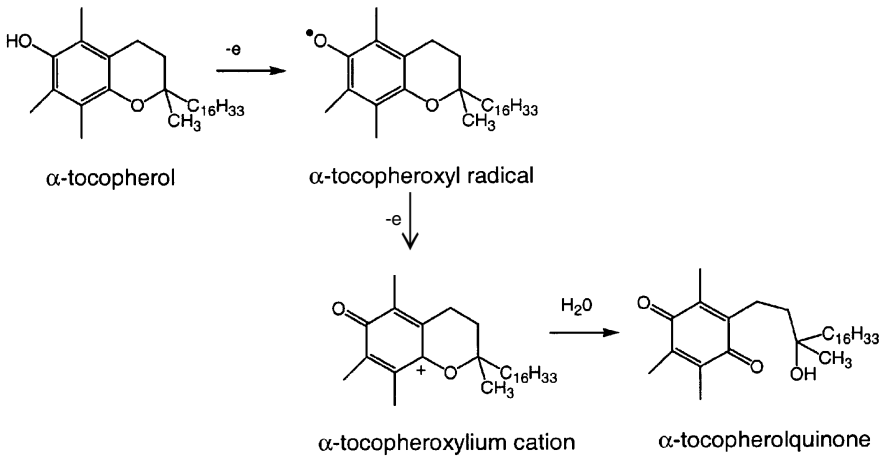


Fig. 3.4. Oxidation of α-tocopherol toward α-tocopherolquinone.

TABLE 3.2
Nuclear Magnetic Resonance (NMR) Spectral Data of Tocopherolquinone (TQ), 4a,5-Epoxy-tocopherolquinone (4a,5-ETQ) and 7,8-Epoxy-tocopherolquinone (7,8-ETQ)

	TQ	4a,5-ETQ	7,8-ETQ
^{1}H NMR chemical shifts			
5a	2.0 (s, 3H)	1.15 (s, 3H)	1.9 (s, 3 H)
7a	2.04 (s, 3H)	1.52 (s, 3 H)	1.16 (s, 3H)
8b	2.0 (s, 3H)	1.54 (s, 3H)	1.19 (s, 3H)
4	2.54 (t, 2H, J = 4.95)		2.32 (t, 2H, 4.1)
R	1.00–1.42	1.00–1.42	1.00–1.42
	0.81–0.88	0.81–0.88	0.81–0.88
^{13}C NMR chemical shifts			
2	72.6	72.3	72.5
2a	26.54	26.63	26.53
3	32.74	32.74	32.74
4	21.39	21.35	21.35
4a	144.5	63.4	144.8
5	140.2	65.8	140.6
5a	11.93	11.52	11.36
6	187.3	193.9	194.2
7	140.4	140.9	65.4
7a	12.28	13.26	12.28
8	140.50	141.20	65.50
8a	187.7	194.3	194.7
8b	12.33	14.09	13.17
R	19.50–42.23	20.67–42.23	20.02–42.23

The first fraction, which eluted at 5 min, represented 7% of all oxidation products and could not be identified. The second fraction, eluting at 11.5 min, corresponded to 17% of the oxidation products formed. Analytical HPLC analysis showed that the second fraction consisted of the co-elution of four components. Preparative HPLC with a linear gradient of *n*-hexane/isopropyl alcohol ranging from 99.75:0.25 (vol/vol) to 99:1 (vol/vol) over a period of 12 min gave a complete separation of these isomers. All isomers showed the presence of two tertiary ethers (± 77 μg/g), a carbon bearing two oxy substituents (± 98 μg/g), a cyano function (± 120 μg/g), and a single carbonyl function (± 185 μg/g). The spectral data obtained allowed identification of these components as isomers of 8a-cyano-isopropylperoxy-α-tocopherone (data not shown). Spectral data were in agreement with those of Liebler *et al.* (1990), which established a relationship between the structures and the corresponding ^{1}H NMR spectral data. The oxidation pathway is presented in Figure 3.5. The fractions eluting at 17.3 and 18.7 min represented 76%. According to the spectral data, these fractions were identified as 4a,5-epoxy-α-tocopherolquinone and 7,8-epoxy-α-tocopherolquinone and were present in a ratio of 9:1. The 4a,5- and 7,8-epoxy-α-tocopherolquinones both had UV λ_{max} (*n*-hexane): 273 nm, IR (NaCl) cm^{-1}: 1682, 1461, 1377, and the ^{1}H NMR and ^{13}C NMR spectral data listed in Table 3.2.

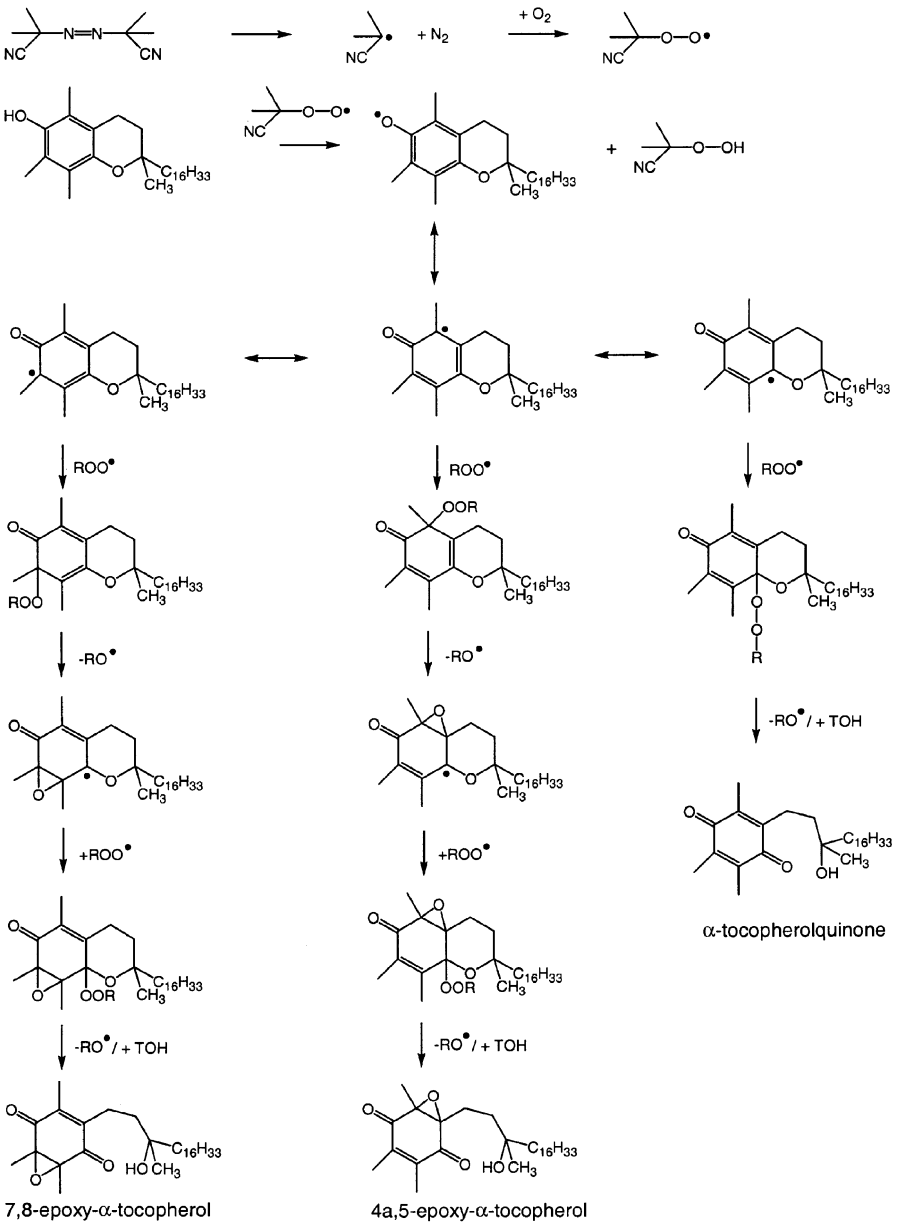


Fig. 3.5. Autoxidation of α-tocopherol toward 7,8-epoxy-α-tocopherolquinone, 4a,5-epoxy-α-tocopherolquinone and 8a-cyano-isopropylperoxy-α-tocopherone. (ROO• = Cyanoisopropylperoxyl radical.)

Preparation of α-Tocopherol-α-Tocopheroxyl Dimer and α-Tocopherol-α-Tocopherolquinone Dimer by Oxidation of α-Tocopherol with tert*-Butyl Hydroperoxide*

α-Tocopherol (1 g) was dissolved in 30 mL chloroform saturated with water and a solution of *t*-butyl-hydroperoxide (340 mg dissolved in 5 mL $CHCl_3$) was slowly added. After the solution was refluxed for 3 h at 60°C, the mixture was washed with 5% ferrous sulfate (2 × 20 mL) and subsequently with water (3 × 20 mL). After being dried over anhydrous sodium sulfate, the solvent was evaporated, yielding a light brown viscous residue. This crude oxidation mixture was separated by preparative NP HPLC. The mobile phase, *n*-hexane/isopropyl alcohol (99.5:0.5, vol/vol) was pumped at a flow rate of 15 mL/min and UV detection was done at 290 nm. This allowed the isolation of six fractions with retention times at 3.7, 5.6, 7.6, 9, 11.2, and 13.2 min. The fraction eluting at 3.7 min, corresponding to 17% of all oxidation products, was identified as α-tocopherol spirotrimer. Natural α-tocopherol (40%) eluted at 7.6 min. The fraction eluting at 13.2 min (6%) was identified as α-tocopherolquinone with spectral data listed in Table 3.2. Several other α-tocopherol oxidation products (9%) were present in low concentrations and could not be identified. The unknown fraction eluting at 5.6 min, corresponding to 11% of all oxidation products, was identified as α-tocopherol-α-tocopheroxyl dimer and is formed by radical addition of two tocopheroxyl radicals (Fig. 3.6). It had an UV λ_{max} 290 nm; IR (NaCl) 3380 (OH), 2978, 2930, 1465, 1379, 1258, 1213, 1170, 1086 (C-O-C), 925 cm^{-1} and the spectral data (^1H NMR/^{13}C NMR) are listed in Table 3.3.

The spectral data of the product eluting at 11.2 min (9%) indicated that this component had an aromatic and quinonoid structure. It was identified as α-tocopherol-α-tocopherol quinone dimer by examination of the NMR spectra and the data published by Suarna *et al.* (1988). The α-tocopherol-α-tocopherol quinone dimer was formed by Diels Alder dimerization of α-tocopherolquinone methide with α-tocopherolquinone (Fig. 3.7). α-Tocopherol-α-tocopherol quinone dimer had UV λ_{max} 297 nm and IR (NaCl) 3450 (OH) cm^{-1}, 2925, 2845, 1670 (CH=CH-C=O), 1625,

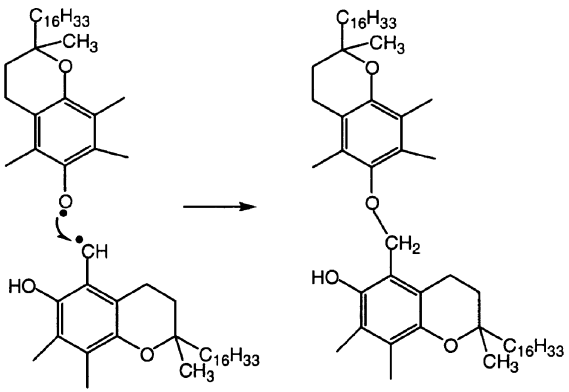


Fig. 3.6. Autoxidation of α-tocopherol to α-tocopherol-α-tocopheroxyl dimer.

TABLE 4.3
Nuclear Magnetic Resonance (NMR) Spectral Data of α-Tocopherol-α-Tocopheroxyl Dimer and α-Tocopherol-α-Tocopherol Quinone Dimer

	α-Tocopherol-α-tocopheroxyl dimer	α-Tocopherol-α-tocopherol quinone dimer	
^{1}H NMR chemical shifts			
5a	4.6 (s, 2H)	4	2.7 (t, 2H, J = 6.8 Hz)
4, 4′	2.6 (t, 2H, J = 6.9 Hz) 2.51 (t, 2H, J = 6.9 Hz)	4′	2.4 (t, 2H, J = 4.1 Hz)
8b, 8b′, 7a, 7a′, 5a′	2.13 (s, 2 × 3H)		
	2.1 (s, 3 × 3 h)	7a, 8b, 7a′, 8b′	2.0 (s, 3h), 2.04 (s, 3 h), 2.11 (s, 3h), 2.21 (s, 3h)
3, 3′	1.76 (t, 2H, J = 6.6 Hz) 1.70 (t, 2H, J = 6.9 Hz)		
R,R′	0.81–1.4 (2 × 33 H)	R	0.81–1.4 (33 H)
^{13}C NMR chemical shifts			
8a, 6, 6′, 8a′	147.4, 145.6, 144.7, 144.6	8a′, 6′	201.1, 198.3
8, 7, 5, 4a, 5′, 7′, 8′, 4a′	125.6, 123.1, 122.6, 121.11, 118.5, 117.3, 116.1, 115.1	8b, 6, 7′, 8′, 8, 4a, 5	145.7, 142,8, 142.1, 142.0, 124.2, 123.1, 114.8, 112.7
2, 2′	74.5, 74.4	4a′	85.0
5a	68.0	2, 2′	72.1, 70.3
4, 4′	21.39	5′	51.5
5a′	11.72	7a, 8a, 7′a, 8′a	11.9, 11.8, 12.3, 12.2
7a, 7a′	11.91, 11.79	5a	29.5
8b, 8b′	12.31, 12.22	5a′	25.9
R, R′	42.23, 18.59	R, R′	42.23, 18.59

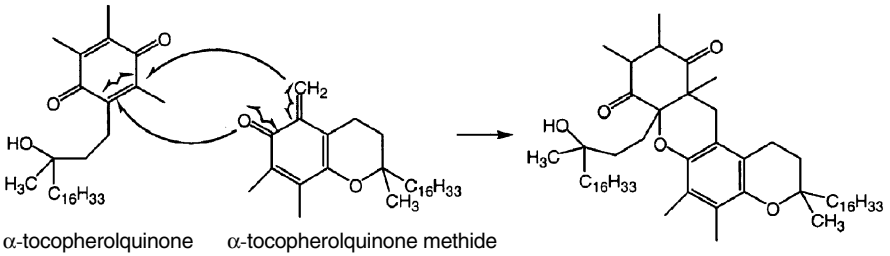


Fig. 3.7. Autoxidation of α-tocopherol to α-tocopherol-α-tocopherol quinone dimer.

1450, 1370, 1255, 1215, 1165, 1090 (C-O-C). ^{1}H NMR and ^{13}C NMR spectral data are listed in Table 4.3.

Tocopherol Degradation in a Model System of Triolein-Tripalmitin

The effect of fatty acid unsaturation on the degradation of α-tocopherol was studied in mixtures of triolein and tripalmitin using a full factorial central composite design. Five levels were used for each of three factors, i.e., percentage of triolein in tripalmitin (0, 25, 50, 75, 100%), temperature (150, 175, 200, 225, 250°C), and heating time (0, 1, 2, 3, 4 h). The initial tocopherol concentration was 1000 μg/g in all mixtures and heating was performed in OSI tubes.

Heating of the triacylglycerol mixtures caused a marked reduction in residual α-tocopherol level (66–98%) and increased the formation of total polar materials, mainly dimers and oxidized monomers, from triacylglycerols (Verleyen *et al.* 2001a). Polymerization of triacylglycerols increased with increasing temperature, heating time, and triacylglycerol unsaturation. Oxidation causes α-tocopherol to degrade to a wide range of oxidation products, many of which are not easy to analyze due to lack of the strong fluorescence of the chromanol structure. Enrichment was performed by extraction of residual α-tocopherol and its polar oxidation products with hot methanol in an attempt to quantify tocopherol oxidation products (Murkovic *et al.* 1997). Several peaks were present in the reversed-phase HPLC chromatogram of the methanol extract (Fig. 3.8). The components eluting at 10.4, 10.7, 12.5, 15.5 min were identified as 7,8-epoxy-α-tocopherolquinone, 4a,5-epoxy-α-tocopherolquinone, α-tocopherolquinone, and α-tocopherol. These results are in agreement with the report of Murkovic *et al.* (1997) who noted a marked formation of epoxy-α-tocopherolquinones during thermoxidation. Identification of the other peaks was not possible but these may include oxidation products from triolein. No tocopherol dimers, tocopherol trimers, or tocopherol-tocopherol quinone dimer were found by NP HPLC of the oxidized lipids. Nevertheless, the sum of residual tocopherol, 7,8-epoxy-α-tocopherolquinone, 4a,5-epoxy-α-tocopherolquinone, and α-tocopherolquinone is far less than 100% in the heated samples. This may be

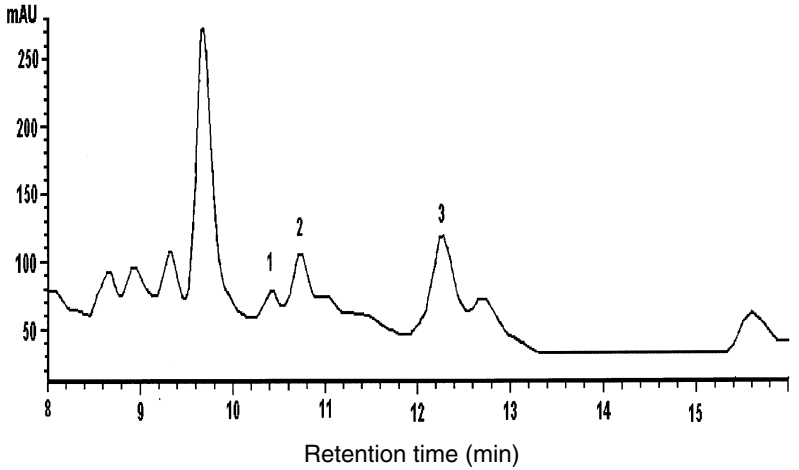


Fig. 3.8. Reversed-phase high-performance liquid chromatography chromatogram of extracted oil sample (275 nm): (1) 7,8-epoxy-α-tocopherolquinone, (2) 4a,5-epoxy-α-tocopherolquinone, and (3) α-tocopherolquinone.

due to analytical limitations in the analysis of the tocopherol dimers and polymers or to the presence of other unknown tocopherol degradation products.

Mechanism of Oxidation

The formation of polar monomers and polar dimers from unsaturated fatty acid moieties in the triacylglycerols and from α-tocopherol indicates that peroxyl radicals are involved as active species in the oxidation under the conditions of the studies. Thus, there was enough oxygen available for formation of peroxyl radicals despite the lowering effect of temperature on oxygen solubility.

Peroxyl radicals react selectively with α-tocopherol by hydrogen donation and formation of adducts with the resulting tocopheroxyl radical (Kamal-Eldin and Appelqvist 1996). The formation of 4a,5- and 7,8-epoxy-α-tocopherolquinones and α-tocopherolquinone suggests that adducts are formed between α-tocopheroxyl radicals and peroxyl radicals at the 5, 7, and 8a positions, respectively. The origin of these peroxyl radicals is not known because both peroxyl radicals and hydroperoxides are very unstable at these high temperatures. Moreover, the fast degradation of α-tocopherol in reaction mixtures containing only tripalmitin casts doubt on the participation of peroxyl radicals from fatty acyl moieties. The predominance of 4a,5-epoxy over 7,8-epoxy α-tocopherolquinone is in agreement with the work of Nilsson *et al.* (1969), showing increased reactivity at the 5-position compared with the 7-position.

The hydroperoxide adducts, formed as a result of the above-mentioned reactions, are not stable and degrade at high temperatures to form alkoxyl radicals. The alkoxyl radicals generated in the case of 5 and 7 adducts will cyclize to epoxy radical species

with carbon-centered radicals at the 8a position (Liebler *et al.* 1990, Yamauchi *et al.* 1995). Carbon-centered radicals capture oxygen and form 8a-hydroperoxyl derivatives. Further degradation of 8a-hydroperoxy-4a,5-epoxy-α-tocopherol, 8a-hydroperoxy-7,8-epoxy-α-tocopherol, and 8a-hydroperoxy-α-tocopherol will generate 4a,5-epoxy-α-tocopherolquinone, 7,8-epoxy-α-tocopherolquinone, and α-tocopherolquinone, respectively. The scheme for these reactions with peroxyl radicals is similar to that used for the synthesis of 4a,5-epoxy α-tocopherolquinone, 7,8-epoxy α-tocopherolquinone, and α-tocopherolquinone using butyronitrile peroxyl radical (Fig. 3.5). A modification of the mechanism proposed by Yamauchi *et al.* (1995) can explain the possible reactions behind the competitive oxidation between α-tocopherol and unsaturated fatty acids observed. Because the alkoxyl and the epoxyalkoxyl radicals are less selective in their reactions than peroxyl radicals, some of these radicals may be able to "leak out" from the protection provided by α-tocopherol and react with unsaturated acyl groups, resulting in a sparing effect on the tocopherol.

Concluding Remarks

α-Tocopherol is highly susceptible to degradation under thermoxidation conditions. α-Tocopherolquinone and 4a,5- and 7,8-epoxy-α-tocopherolquinones, but no dimeric or trimeric derivatives, were identified as oxidation products. The mechanism behind the degradation of α-tocopherol and perhaps the identity of some of its oxidation/degradation products are not fully elucidated. Both α-tocopherolquinone and epoxy-α-tocopherolquinones were not stable, and upon prolonged heating at high temperatures, they degraded to other unknown oxidation products. The rate of α-tocopherol oxidation decreased in less unsaturated fatty acyl substrates due to competitive oxidation with unsaturated triacylglycerols. At high temperatures, the protection provided to unsaturated fatty acids by α-tocopherol is reduced due to the increased instability of the antioxidant.

References

Barrera-Arellano, D., Ruiz-Mendez, V., Marquez-Ruiz, G., and Dobarganes, C. (1999) Loss of Tocopherols and Formation of Degradation Compounds in Triacylglycerol Model Systems Heated at High Temperature, *J. Sci. Food Agric. 79*, 1923–1928.

Bolon, D. (1970) Generation of *o*-Quinone Methides in Solution: Trimerization, *J. Org. Chem. 35*, 715–719.

Csallany, A., and Ha, Y. (1992) α-Tocopherol Oxidation Mediated by Superoxide Anion (O_2^-). I. Reactions in Aprotic and Protic Conditions, *Lipids 27*, 195–200.

Frampton, V., Skinner, W., and Bailey, P. (1954) The Production of Tocored upon the Oxidation of α-Tocopherol with Ferric Chloride, *J. Am. Chem. Soc. 76*, 282–284.

Frampton, V., Skinner, W., Cambour, P., and Bailey, P. (1960) α-Tocopurple, an Oxidation Product of α-Tocopherol, *J. Am. Chem. Soc. 82*, 4632–4634.

Jorge, N., Marquez-Ruiz, G., Martin-Polvillo, M., Ruiz-Mendez, M.W., and Dobarganes, M.C. (1996a) Influence of Diemethylpolysiloxane Addition to Edible Oils: Dependence of The Main Variables of The Frying Process, *Grasas Aceites 47*, 14–19.

Jorge, N., Marquez-Ruiz, G., Martin-Polvillo, M., Ruiz-Mendez, M.W., and Dobarganes,

M.C. (1996b) Influence of Diemethylpolysiloxane Addition to Frying Oils: Performance of Sunflower Oil in Discontinuous and Continuous Laboratory Frying, *Grasas Aceites 47*, 20–25.

Kamal-Eldin, A., and Appelqvist, L. (1996) The Chemistry and Antioxidant Properties of Tocopherols and Tocotrienols, *Lipids 31*, 671–701.

Lampi, A.-M., and Kamal-Eldin, A. (1998) Effects of α- and γ-Tocopherols on Thermal Polymerization of Purified High Oleic Sunflower Triacylglycerols, *J. Am. Oil Chem. Soc. 75*, 1699–1703.

Liebler, D., Baker, P., and Kaysen, K. (1990) Oxidation of Vitamin E: Evidence for Competing Autoxidation and Peroxyl Radical Trapping Reactions of the Tocopheroxyl Radical, *J. Am. Chem. Soc. 112*, 6995–7000.

Murkovic, M., Wiltschko, D., and Pfannhauser, W. (1997) Formation of α-Tocopherolquinone and α-Tocopherolquinone Epoxides in Plant Oil, *Fett/Lipid 99*, 165–169.

Nelan, D., and Robeson, C. (1962) The Oxidation Product from α-Tocopherol and Potassium Ferricyanide and Its Reaction with Ascorbic and Hydrochloric Acids, *J. Am. Chem. Soc. 84*, 2963–2965.

Nilsson, J.L.G., Sievertsson, H., and Selander, H. (1969) On the Preferential Oxidative Coupling at the 5- Versus the 7-Position in Tocopherols and Related 6-Chromanols, *Acta Chem. Scand. 23*, 859–870.

Skinner, W., and Alaupovic, P. (1963) Oxidation Products of Vitamin E and Its Model, 6-Hydroxy-2,2,5,7,8-pentamethylchroman. V. Studies of the Products of Alkaline Ferricyanide Oxidation, *J. Org. Chem. 28*, 2854–2858.

Skinner, W., and Parkhurst, R. (1964) Oxidation Products of Vitamin E and Its Model Compound 6-Hydroxy-2,2,5,7,8-pentamethylchroman. VII. Trimer Formed by Alkaline Ferricyanide Oxidation, *J. Org. Chem. 29*, 3601–3603.

Suarna, C., Craig, D., Cross, K., and Southwell-Keely, P. (1988) Oxidation of Vitamin E (α-Tocopherol) and Its Model Compound 2,2,5,7,8-Pentamethyl-6-hydroxychroman: A New Dimer, *J. Org. Chem. 53*, 1281–1284.

Verleyen, T., Kamal-Eldin, A., Dobarganes, C., Verhe, R., Dewettinck, K., and Huyghebaert, A. (2001a) Modeling of Loss and Products of α-Tocopherol During Thermoxidation in Mixtures of Triolein and Tripalmitin, *Lipids 36*, 719–726.

Verleyen, T., Kamal-Eldin, A., Mozuraityte, R., Verhe, R., Dewettinck, K., Huyghebaert, A., and De Greyt, W. (2002) Oxidation at Elevated Temperatures: Competition Between α-Tocopherol and Unsaturated Triacylglycerols, *Eur. J. Lipid Sci. Technol. 104*, 228–233.

Verleyen, T., Verhe, R., Huyghebaert, A., Dewettinck, K., and De Greyt, W. (2001b) Identification of Alpha-tocopherol Oxidation Products in Triolein at Elevated Temperatures, *J. Agric. Food Chem. 49*, 1508–1511.

Yamauchi, R., Kato, K., and Ueno, Y. (1988) Formation of Trimers of α-Tocopherol and Its Model Compound, 2,2,5,7,8-Pentamethylchroman-6-ol in Autoxidizing Methyl Linoleate, *Lipids 23*, 779–783.

Yamauchi, R., Kato, K., and Ueno, Y. (1995) Free Radical Scavenging Reactions of α-Tocopherol During the Autoxidation of Methyl Linoleate in Bulk Phase, *J. Agric. Food Chem. 43*, 1455–1461.

Chapter 4

Kinetic Evaluation of the Antioxidant Activity in Lipid Oxidation

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Introduction

One of the major concerns in food technology is the autoxidation of lipids that occurs autocatalytically through free radical intermediates and is generally initiated by trace metals and peroxides present as ubiquitous impurities in food systems (Yanishlieva-Maslarova 2001). Several factors such as ultraviolet or ionizing radiation are also known to bring about initiation of free radical reactions.

Lipid oxidation products in foods are not only the reason for the unpleasant rancid odor, but they might also initiate oxidative chain processes in the human body, which are implicated in the progress of carcinogenesis, atherosclerosis, myocardial infarction, allergies, inflammatory bowel and other diseases (Gordon 1996, Rice-Evans and Burdon 1993). The problem of oxidative instability of lipids and its inhibition by antioxidant addition is important for food stability as well as for health protection.

Lipids occur in almost all foodstuffs and most of them are in the form of triacylglycerols. The two major components involved in lipid oxidation are unsaturated fatty acids and oxygen. Molecular oxygen is a biradical; it has two unpaired electrons in the ground state and is said to be in the triplet state. Its direct reaction with unsaturated fatty acids is a spin-forbidden transition and has to be carried by free radical intermediates. The free radical chain mechanism of autoxidation can be described by the reactions of noninhibited oxidation presented in Scheme 4.1 in which LH is an oxidizing lipid substrate, and $LOO^{\bullet}$ is a peroxyl radical.

The lipid oxidation reaction is said to be in a kinetic regime when the oxygen concentration and appropriate diffusion are high enough to ensure that oxygen limitation does not influence the process rate. The only products formed during the initial stage of lipid oxidation in a kinetic regime are the hydroperoxides (Popov and Yanishlieva 1976), and the kinetics of their accumulation is indicative of autoxidation kinetics. In a kinetic regime of oxidation, reproducible results are achieved through interpretation of the kinetic behavior of the unsaturated lipids during autoxidation. The kinetic regime can be ensured by blowing oxygen or air through the samples, or by performing the process in thin layers, e.g., 1 mm (Yanishlieva *et al.* 1999).

(1)	$2LH + O_2$	$\rightarrow$	$2L^{\bullet} + H_2O_2$
(1a)	LOOH	$\rightarrow$	$LO^{\bullet} + {}^{\bullet}OH$
(1b)	LOOH + LH	$\rightarrow$	$LO^{\bullet} + H_2O + L^{\bullet}$
(2)	$L^{\bullet} + O_2$	$\rightarrow$	$LOO^{\bullet}$
(3)	$LOO^{\bullet}$ + LH	$\rightarrow$	$LOOH + L^{\bullet}$
(4)	$LOO^{\bullet} + LOO^{\bullet}$	$\rightarrow$	products
(5)	$L^{\bullet} + LOO^{\bullet}$	$\rightarrow$	L-O-O-L
(6)	$L^{\bullet} + L^{\bullet}$	$\rightarrow$	L-L

Scheme 4.1. Noninhibited autoxidation.

The primary oxidation products, the hydroperoxides LOOH, are odorless and tasteless. However, they are initiators of the oxidative chains through decomposition to free radicals [Scheme 4.1, reactions (1a) and (1b)]. As a result of further oxidation and cleavage of the hydroperoxide molecules, low-molecular-weight products, e.g., aldehydes, ketones, acids, alcohols, esters, furans, and lactones are formed, causing rancidity. These products may react further in the organism with functional groups of proteins or DNA, impairing their function (Stahl 2000).

Antioxidants and Lipid Protection

In nature, oxidatively sensitive unsaturated fatty acids are accompanied by a wide range of compounds and defensive mechanisms capable of protecting them against free radical attack. The free radical chain process of autoxidation can be retarded by two categories of inhibitors, i.e., chain-breaking inhibitors and preventive inhibitors (Yanishlieva-Maslarova 2001). Of particular interest are phenolic chain-breaking inhibitors, which have electron-releasing substituents in the *ortho*- and/or *para*-positions on the phenolic rings and are generally known as antioxidants. In addition to their structure, many other factors are of utmost importance in determining the efficacy of antioxidants in inhibiting the lipid oxidation process.

The introduction of an antioxidant AH into the oxidizing system leads to a change in mechanism and kinetics of the process (Denisov and Khudyakov 1987). This can be illustrated by comparing Scheme 4.1 for noninhibited oxidation with Scheme 4.2 for inhibited oxidation. Under the conditions of a kinetic regime of oxidation, the system being oxidized contains no short-lived radicals ($L^{\bullet}$), and the termination proceeds according to reaction (4) (Scheme 4.1) and/or reactions (7) and (8) (Scheme 4.2). It was found that the antioxidant efficacy depends on the participation of its molecules and its radicals in a series of reactions presented in Scheme 4.2 (Denisov and Khudyakov 1987, Roginskii 1990).

Although the reactions in which the inhibitor moieties participate can be many in number (Scheme 4.2), the mechanism of the process is determined by only some of them. Depending on the structure of the antioxidant, on the oxidizing substrate, and on the oxidation conditions, different side reactions may play a major role in

(7)	$LOO^{\bullet} + AH$	$\rightarrow$	$LOOH + A^{\bullet}$
(*7)	$A^{\bullet} + LOOH$	$\rightarrow$	$AH + LOO^{\bullet}$
(8)	$A^{\bullet} + LOO^{\bullet}$	$\rightarrow$	A-OOL
(9)	$A^{\bullet} + A^{\bullet}$	$\rightarrow$	products
(10)	$A^{\bullet} + LH$	$\rightarrow$	$AH + L^{\bullet}$
(11)	AH + LOOH	$\rightarrow$	products
(12)	$AH + O_2$	$\rightarrow$	$A^{\bullet} + HO_2^{\bullet}$
(13)	AOOL	$\rightarrow$	$AO^{\bullet} + LO^{\bullet}$
(14)	$A^{\bullet} + O_2$	$\rightarrow$	$AOO^{\bullet}$

Scheme 4.2. Inhibited autoxidation.

the process (Yanishlieva and Marinova 1998). The most widely used antioxidants in foods are able to compete with the substrate for the chain-carrying species normally present in highest concentration in the system, the peroxyl radical $LOO^{\bullet}$, reaction (7) in Scheme 4.2. The efficient inhibitors terminate free radical chain oxidation by trapping two peroxyl radicals according to reactions (7) and (8). The stoichiometric inhibition factor *f* (the number of kinetic chains broken per molecule of antioxidant) is normally ≥2 (Scott 1985). The probability that reactions (7)–(14) take place depends not only on the inhibitor structure but also on the type, degree of lipid unsaturation, and binding the fatty acids to triacylglycerols, on antioxidant concentration, on temperature, and on the participation of different microcomponents (present or added to the lipid systems) in the oxidation process (Popov and Yanishlieva 1976).

The main type of unsaturated lipids in food are monoenic fatty acyl moieties (primarily oleate), and fatty acids with two or more methylene interrupted double bonds (mainly linoleate with two double bonds). By interpretation of the kinetic results, one must consider that the oxidation of linoleate is ~10 times faster than that of oleate (Gunstone and Hildich 1945, Stirton *et al.* 1945), and that linoleate peroxyl radicals react several times faster than oleate peroxyl radicals (Yanishlieva *et al.* 1970). Moreover, oleate hydroperoxides are much more stable than linoleate hydroperoxides (Yanishlieva 1973). It has also been established that both the oleate and linoleate moieties are oxidized during the initial stage of autoxidation of triacylglycerols and methyl esters of lard and olive oil, whereas linoleate units are oxidized with high selectivity in the case of triacylglycerols and methyl esters of sunflower oil (Yanishlieva and Popov 1973). That is why LH, $LOO^{\bullet}$, and LOOH, by having different compositions and reactivities in the different lipid systems, may strongly influence the kinetic behavior of the antioxidants in the various lipid substrates.

To obtain objective information about the activity and mechanism of action of the antioxidants and the effect of test conditions, we have carried out the experiments in a kinetic regime of oxidation and with pure triacylglycerols or methyl esters of fatty acids. To obtain pure triacylglycerols, the lipid substrates were freed by adsorption chromatography from pro- and antioxidative microcomponents known to participate in and contribute to the autoxidation process (Popov *et al.* 1968,

Yanishlieva and Marinova 1995b). The following pure lipid systems were used: triacylglycerols and methyl esters of lard (TGL and MEL), triacylglycerols and methyl esters of olive oil (TGOO and MEOO), triacylglycerols and methyl esters of sunflower oil (TGSO and MESO), and triacylglycerols of soybean oil (TGSBO). Some of the investigations were performed with fats and oils without purification (Marinova and Yanishlieva 1997, Yanishlieva and Marinova 1996b, Yanishlieva *et al.* 1997, 2001a, and 2001b) so as to obtain practical information concerning the possibility for further stabilization of real lipid systems.

Our investigations on the effects of antioxidants on the oxidative stability of lipid substrates started 30 years ago. Several compounds (Fig. 4.1) and some plant extracts were studied. These include butylated hydroxyanisole (BHA) **1**, butylated hydroxytoluene (BHT) **2**, hydroquinone **3**, propyl gallate **4**, and quercetin **5** in various lard samples (Yanishlieva and Popov 1974), and α-tocopherol **6** and α-tocotrienol **7** in MESO (Yanishlieva-Maslarova *et al.* 1977). The following antioxidants were studied at different concentration levels: α-tocopherol (Marinova and Yanishlieva 1992a and 1998, Yanishlieva and Marinova 1992 and 1996a, Yanishlieva *et al.* 1994 and 2002), ascorbyl palmitate **8** (Marinova and Yanishlieva 1992c), *p*-coumaric **9**, ferulic **10**, caffeic **11**, and sinapic **12** acids (Marinova and Yanishlieva 1992a, 1992b, 1994b, and 1996, Yanishlieva and Marinova 1995b and 1996b, Yanishlieva *et al.* 1994), 3,4-dihydroxybenzoic **13**, vanillic **14** and syringic **15** acids (Marinova and Yanishlieva 1992b, 1994b, Yanishlieva and Marinova 1995b and 1996b), carnosol **16** (Marinova *et al.* 1991), esculetin **17** (Marinova *et al.* 1994, Yanishlieva and Marinova 1996b), esculin **18** (Marinova *et al.* 1994), fraxetin **19** and fraxin **20** (Marinova *et al.* 1994, Yanishlieva and Marinova 1996b), quercetin and morin **21** (Yanishlieva and Marinova 1996a, Marinova and Yanishlieva 1998), 3,4-dihydroxyphenylacetic acid **22** (Yanishlieva *et al.* 1998), thymol **23** and carvacrol **24** (Yanishlieva *et al.* 1999), β-carotene **25** (Yanishlieva *et al.* 2001b), β-apo-8′-carotenoic acid **26** and its esters (Yanishlieva *et al.* 2001a), and *trans*-resveratrol **27** (Marinova *et al.* 2002).

Recently our research has been directed toward the elucidation of the dependency of antioxidant activity of different natural antioxidants on their structure (Marinova and Yanishlieva 1992a, 1992b, 1994a, 1994b, and 1998, Marinova *et al.* 1994 and 2002, Yanishlieva and Marinova 1992 and 1996a, Yanishlieva *et al.* 1998 and 1999), on temperature (Marinova and Yanishlieva 1992a, 1992c, 1998, Yanishlieva and Marinova 1996a), on the type of lipid system being oxidized (Marinova and Yanishlieva 1992c, 1994a and 1996, Marinova *et al.* 1994 and 2002, Yanishlieva and Marinova 1995b, Yanishlieva *et al.* 1994 and 1999), and on the binding of fatty acids to natural triacylglycerols (Yanishlieva and Marinova 1995b, Marinova and Yanishlieva 1996).

Different extracts and individual compounds from natural sources were also investigated, e.g., broad beans (Yanishlieva *et al.* 1983), propolis (Yanishlieva *et al.* 1984, Marinova *et al.* 1989), algae *Scenedesmus acutus* (Yanishlieva and Marinova 1985), *Silibum marianum* seed oil (Yanishlieva *et al.* 1985), *Capsicum annum* L. (red pepper) (Yanishlieva and Marinova 1986), *Rosemary officinalis* L.

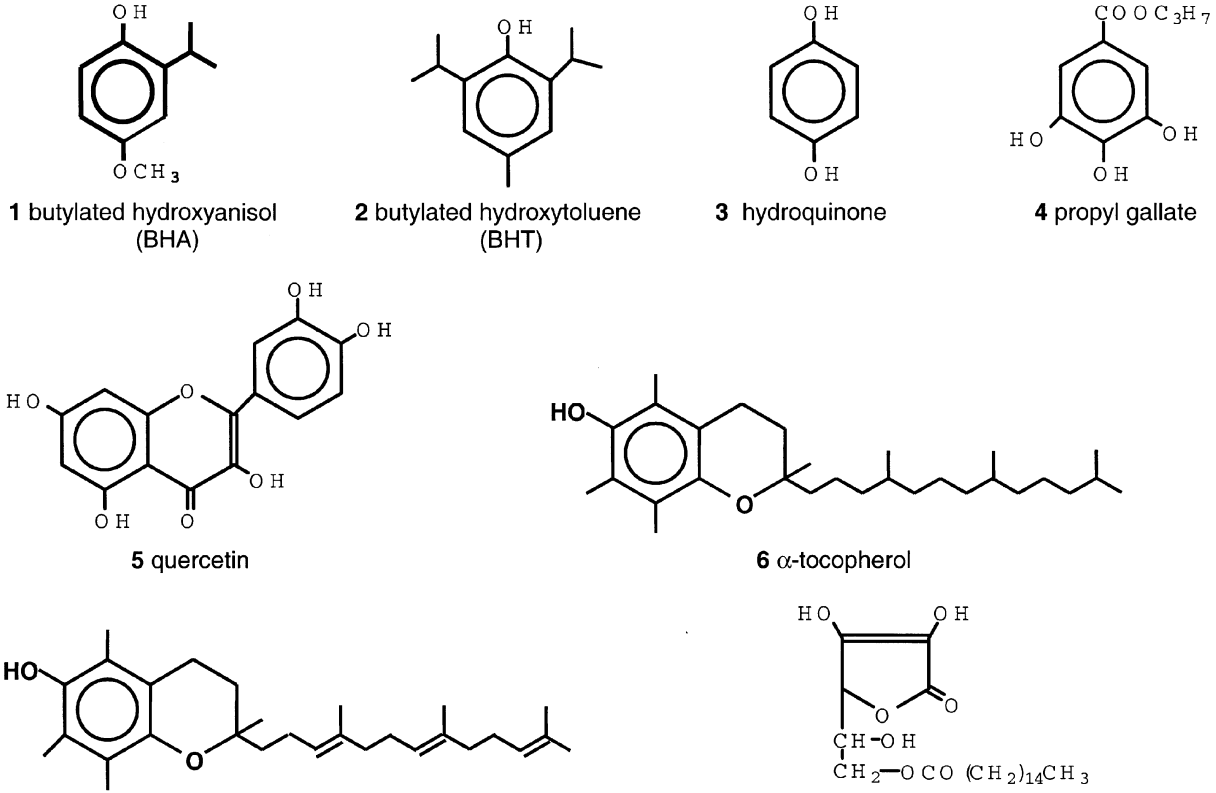
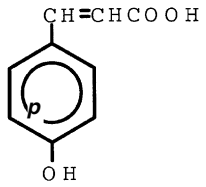
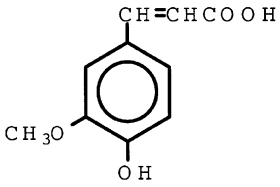


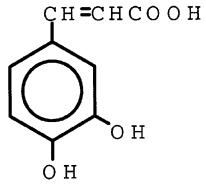
Fig. 4.1. Chemical structures of the oxidation inhibitors discussed in the text. (*Continued pp. 90–91*).



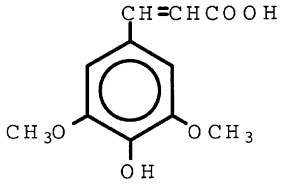
9 p-coumaric acid



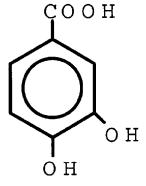
10 ferulic acid



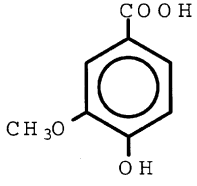
11 caffeic acid



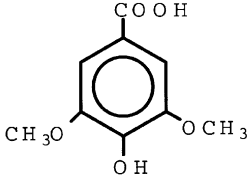
12 sinapic acid



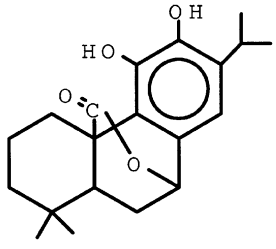
13 3,4-dihydroxybenzoic acid



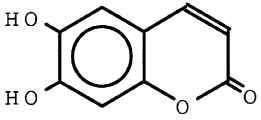
14 vanillic acid



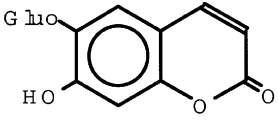
15 syringic acid



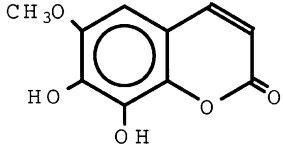
16 carnosol



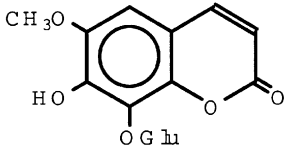
17 esculetin



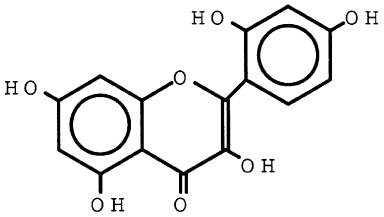
18 esculin



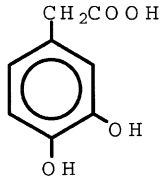
19 fraxetin



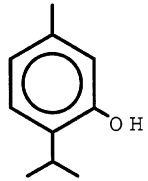
20 fraxin



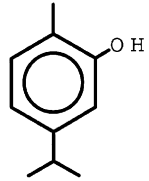
21 morin



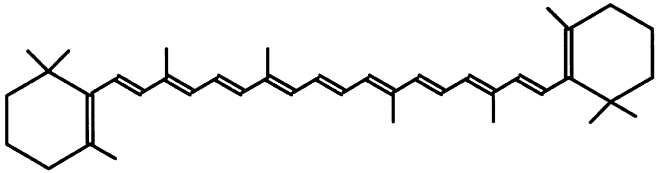
22 3,4-dihydroxyphenylacetic acid



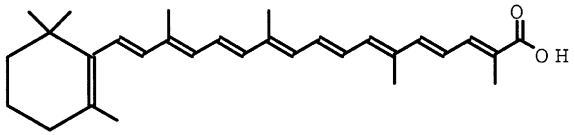
23 thymol



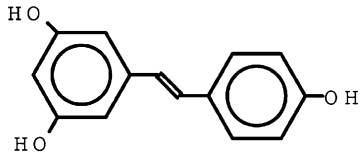
24 carvacrol



25 β-carotene



26 β-apo-8'-carotenoic acid



27 *trans*-resveratrol

Fig. 4.1. (*Cont.*).

(Marinova *et al.* 1991), bark ash from *Fraxinus ornus* L. (Marinova *et al.* 1994), and selected species of the family *Lamiaceae* grown in Bulgaria and used as spices, e.g., *Melissa officinalis* L. (common balm), *Mentha piperita* L. (peppermint), *Mentha spicata* L. (spearmint), *Ocimum basilicum* L. (common basil), *Origanum vulgare* L. (oregano), and *Saturejeae hortensis* L. (summer savory) (Yanishlieva and Marinova 1995a, Marinova and Yanishlieva 1997, Yanishlieva *et al.* 1997).

Main Kinetic Parameters Characterizing the Antioxidative Action of the Inhibitors

A characteristic feature of antioxidants is that they produce a lag period, the so-called induction period (IP), when the extent of oxidation is plotted vs. time. The IP continues until the antioxidant has been destroyed and its duration is proportional to the antioxidant concentration. During this lag period, lipid peroxidation proceeds at a very low rate, but at its end, the oxidation continues at a rate equal to that of the unprotected lipid. The end of the IP is characterized by a transition from a steady-state to an autocatalytic oxidation regime. During the IP, the inhibitor exhibits its effectiveness.

The effectiveness of an antioxidant is estimated on the basis of the IP, usually determined in time units by the method of tangents to the two parts of the kinetic curve (Le Tutour and Guedon 1992, Yanishlieva and Popov 1971). The effectiveness represents the possibility of blocking the chain radical process by interaction with the peroxyl radicals which are responsible for the duration of the IP [reactions (7) and (8), Scheme 4.2].

From a kinetic point of view, the antioxidant has an inhibiting effect in the lipid system when the following inequality is observed during the IP (Emanuel *et al.* 1965):

$$fk_7[\mathrm{AH}] >> (k_6 W_i)^{0.5} \qquad [1]$$

where W_i is the mean rate of initiation during the induction period of inhibited oxidation (M/s), and f is the stoichiometric coefficient of inhibition which determines how many radicals perish per inhibitor molecule.

In addition to the length of the IP, the initial oxidation rate during the IP (the rate of inhibited oxidation, W_{inh}), is the other kinetic parameter characterizing the antioxidant action. In a kinetic regime of oxidation W_{inh} can be given by the expression:

$$W_{\mathrm{inh}} = k_2[\mathrm{LH}]W_i/k_7 f[\mathrm{AH}] \qquad [2]$$

where k_2 is the rate constant of chain propagation and k_7 is the rate constant of inhibition. W_{inh} depends on the possibility of the inhibitor moieties participating in the reactions of chain initiation (11)–(13), and of chain propagation (*7), (10), (14) (Scheme 4.2). W_{inh} characterizes the strength of the antioxidant.

The main types of oxidation kinetics during the initial stage of the process depend on, e.g., the antioxidant structure and concentration, the character of the lipid system or the temperature, and can be presented as follows: (i) With increasing concentration, antioxidant addition results in lengthening of the IP, as well as in decreasing of the initial W_{inh}. Such behavior is demonstrated by ferulic acid during oxidation of TGL at 100°C (Fig. 4.2). (ii) With increasing concentration, antioxidant addition results in lengthening of the IP, whereas no change in the initial W_{inh} is observed. An example is presented with caffeic acid in TGL oxidation at 100°C (Fig. 4.3). (iii) With increasing antioxidant concentration, the duration of the IP increases, and the rate of inhibited oxidation W_{inh} increases as well. Such behavior is demonstrated by α-tocopherol during oxidation of TGL at 25°C (Fig. 4.4).

To compare the action of various antioxidants in different lipid systems and under different oxidation conditions, the relative parameters stabilization factor F and oxidation rate ration ORR are used:

$$F = \mathrm{IP}_{inh}/\mathrm{IP}_o \qquad [3]$$

$$\mathrm{ORR} = W_{inh}/W_o \qquad [4]$$

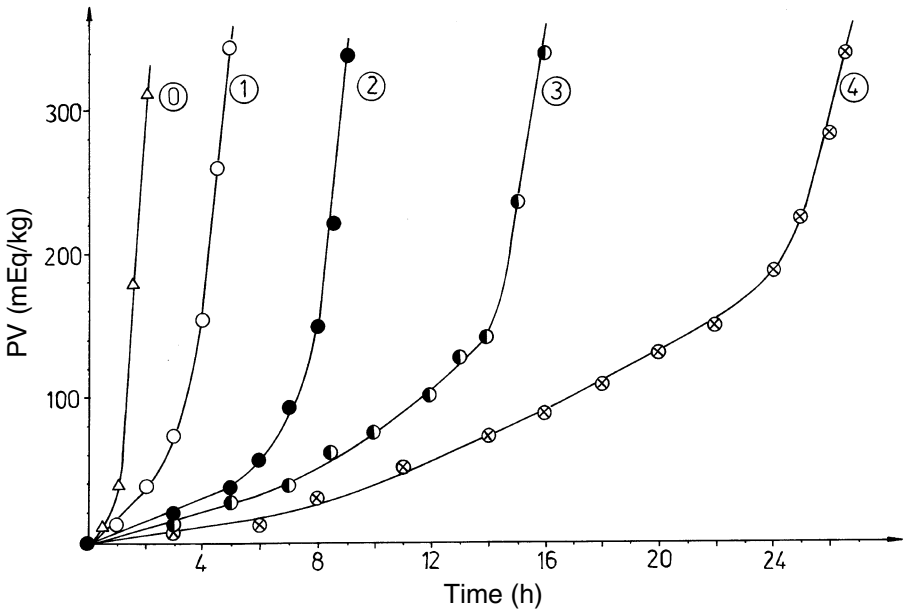


Fig. 4.2. Kinetic curves of peroxide accumulation during oxidation of triacylglycerols of lard (TGL) at 100°C in the presence of ferulic acid: 0–0%; 1–0.02%; 2–0.05%; 3–0.1%; 4–0.2%.

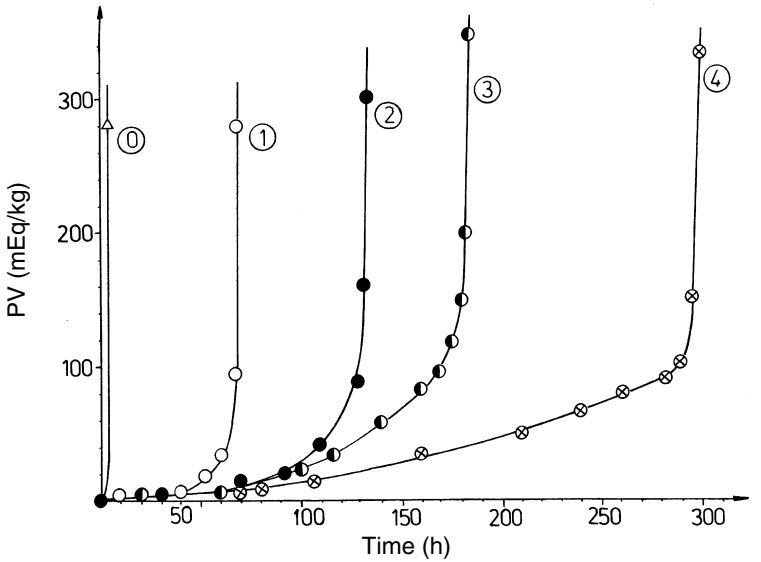


Fig. 4.3. Kinetic curves of peroxide accumulation during oxidation of triacylglycerols of lard (TGL) at 100°C in the presence of caffeic acid: 0–0%; 1–0.02%; 2–0.05%; 3–0.1%; 4–0.2%.

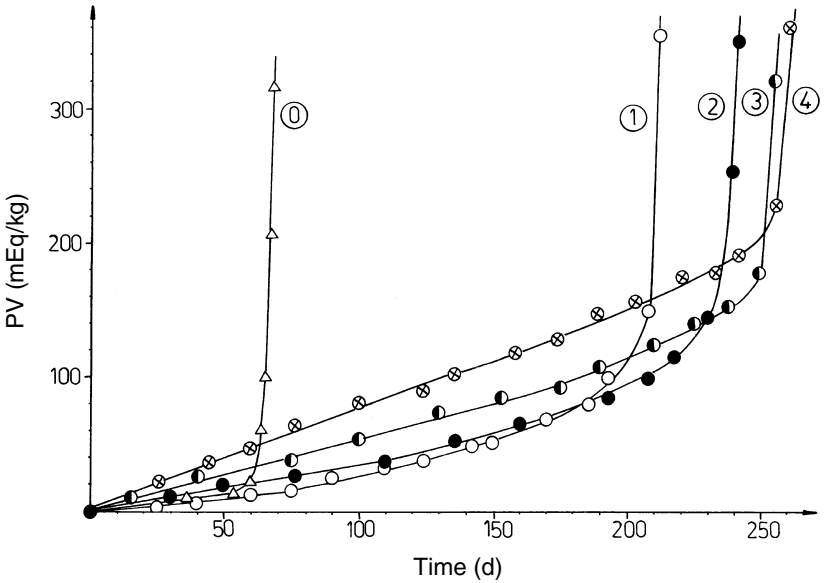


Fig. 4.4. Kinetic curves of peroxide accumulation during oxidation of triacylglycerols of lard (TGL) at 25°C in the presence of α-tocopherol: 0–0%; 1–0.02%; 2–0.05%; 3–0.1%; 4–0.2%.

TABLE 4.1
Kinetic Parameters Characterizing the Inhibited Oxidation of the Triacylglycerols of Sunflower Oil (TGSO) at 100°C[a,b]

	Antioxidant concentration				
Antioxidant	[AH] (%)	[AH] × 10^3 (M)	*F*	ORR	*A*
Esculetin	0.01	0.56	14.8	0.15	99
	0.02	1.12	20.8	0.09	231
	0.05	2.81	37.6	0.06	626
	0.10	5.56	41.2	0.05	842
Fraxetin	0.01	0.48	13.2	0.15	88
	0.02	0.95	27.2	0.09	302
	0.05	2.38	72.0	0.04	1800
	0.10	4.76	125.0	0.02	6250

[a]*Source:* Marinova *et al.* (1994).
[b]The induction period of the noninhibited system (IP_o) = 0.25 h; the oxidation rate of noninhibited oxidation (W_o) = 1.8×10^{-5} M/s.

where IP_{inh} is the induction period in the presence of an inhibitor, IP_o is the induction period of the noninhibited system, W_{inh} is the initial oxidation rate in the presence of an inhibitor, and W_o is the oxidation rate of the noninhibited oxidation.

F is a measure of the effectiveness, and ORR is an inverse measure of the strength of the antioxidant (the lower the ORR, the stronger the inhibitor). When ORR > 1, the oxidation proceeds more quickly in the presence of an inhibitor than in its absence. For example, this is observed at high α-tocopherol concentrations during TGL oxidation at room temperature (Marinova and Yanishlieva 1992a).

Taking into account the complicated changes in the kinetic parameters of inhibited oxidation and the fact that the estimation of the antioxidative effect on the basis of IP or on the process rate may lead in many cases to different results, we proposed a general kinetic parameter antioxidant activity *A* (Yanishlieva and Marinova 1992). This parameter, *A*, unifies the effectiveness of an inhibitor in termination of the autoxidation chain, on the one hand, and its ability to decrease the oxidation rate during the IP on the other:

$$A = F/\text{ORR} \qquad [5]$$

By way of example, *F*, ORR, and *A* are presented in Tables 4.1 and 4.2 for esculetin and fraxetin during oxidation of TGSO at 100°C, and for thymol and carvacrol during oxidation of TGSO at 22°C, respectively. The influence of the lipid substrate on the antioxidant activity is illustrated in Table 4.3, in which some of the data obtained for the activity of various antioxidants at concentration levels 0.02, 0.05 and 0.10% during oxidation of different lipid systems at 100°C are presented.

TABLE 4.2
Kinetic Parameters Characterizing the Inhibited Oxidation of the Triacylglycerols of Sunflower Oil (TGSO) at 22°C[a,b]

Antioxidant	Antioxidant concentration		*F*	ORR	*A*
	[AH] (%)	[AH] × 10^3 (M)			
Thymol	0.02	1.33	6.0	0.08	75
	0.05	3.33	7.9	0.08	99
	0.10	6.67	9.2	0.08	115
	0.20	13.33	7.4	0.08	92
Carvacrol	0.02	1.33	2.0	0.71	2.8
	0.05	3.33	3.4	0.42	8.1
	0.10	6.67	5.9	0.29	20.3
	0.20	13.33	10.5	0.17	62

[a]*Source:* Yanishlieva *et al.* (1999).
[b]The induction period of the noninhibited system (IP_o) = 5 d; the oxidation rate of noninhibited oxidation (W_o) = 4.63×10^{-8} M/s.

TABLE 4.3
Activity of Various Antioxidants at Concentration Levels 0.02, 0.05 and 0.10% During Oxidation of Different Lipid Systems at 100°C[a,b]

Antioxidant	Lipid system	*A*		
		0.02%	0.05%	0.10%
α-Tocopherol	TGL	50	43.5	21.6
α-Tocopherol	TGSO	211	215	220
3,4-Dihydroxybenzoic acid	TGL	191	705	1477
3,4-Dihydroxybenzoic acid	TGSO	6.0	11.6	17.6
p-Coumaric acid	TGL	3.9	8.8	17.9
p-Coumaric acid	TGOO	11.0	23.2	43.1
Ferulic acid	TGL	5.2	17.6	52
Ferulic acid	TGOO	20.0	57.5	148
Ferulic acid	TGSO	4.3	5.3	7.8
Sinapic acid	TGL	95	333	1015
Sinapic acid	TGSO	28.1	34.8	48.1
Caffeic acid	TGL	10350	20350	28917
Caffeic acid	TGOO	4867	10182	29167
Caffeic acid	TGSO	448	900	1364
Esculetin	TGL	712	1290	1462
Esculetin	TGSO	231	627	824
Fraxetin	TGL	2877	10400	34000
Fraxetin	TGSO	302	1800	6250
Resveratrol	TGL	3750	6675	10675
Resveratrol	TGSO	79	119	178

[a]*Sources:* Marinova and Yanishlieva (1992b, 1996), Yanishlieva and Marinova (1992, 1995b), Marinova *et al.* (1994, 2002), and Yanishlieva *et al.* (2002).
[b]TG = triacylglycerol; SO = sunflower oil; L = lard; OO = olive oil.

Participation in Side Reactions

If the antioxidant participates in chain termination only, the stabilization factor F increases linearly with concentration (Fig. 4.5A), and the mean rate of inhibitor consumption W_{InH} is given by the formula $W_{InH} = W_i/f$ (Emanuel *et al.* 1965). With some of the antioxidants studied we have observed a nonlinear dependence of F on the antioxidant concentration (Fig. 4.5B). The absence of linearity of the dependences is due to the participation of the inhibitor molecules in reactions other than the main reaction (7) of chain termination, namely reaction (11) or/and (12). In this case there is a relationship between the mean rate of inhibitor consumption W_{InH} and the inhibitor concentration [AH]:

$$W_{InH} = W_i/f + k_{eff}[AH]^n \qquad [6]$$

where W_i is the mean rate of initiation during the IP (M/s), f is the stoichiometric coefficient of inhibition, and n is the number of side reactions in which the antioxidant participates.

After processing of the kinetic curves, the mean rates of inhibitor consumption W_{InH} are determined according to the formula [7]:

$$W_{InH} = [AH]_o/IP, \text{ M/s} \qquad [7]$$

where $[AH]_o$ is the initial molar concentration of the antioxidant, and IP is the duration of the induction period in seconds.

The W_{InH} obtained for different initial concentrations of the antioxidants are presented as dependence [6] for different n. As an illustration, Figure 4.6 shows the

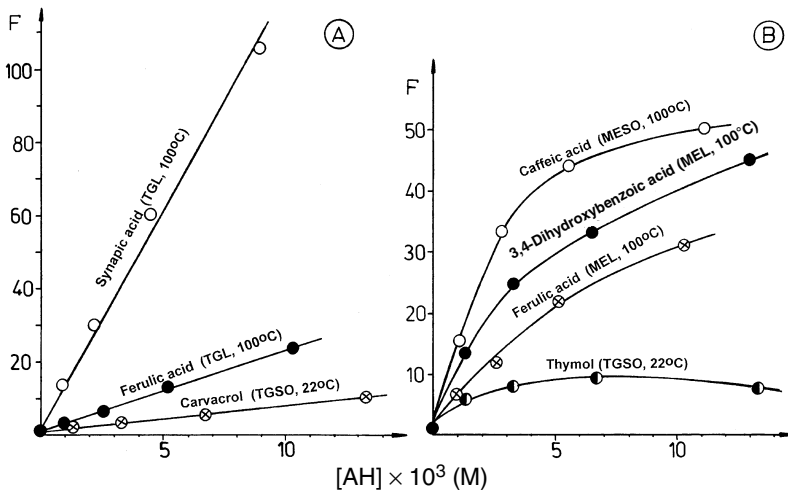


Fig. 4.5. Dependence of the stabilization factor F on the concentration of various antioxidants at different oxidation conditions. Adapted from Marinova and Yanishlieva (1992b, 1994b), Yanishlieva *et al.* (1999).

dependence of W_{InH} on the concentration of vanillic, *p*-coumaric, 3,4-dihydroxybenzoic, and caffeic acids ($n = 1$).

The kinetic results showed that for most of the investigated antioxidants $n = 1$ or $n = 0$, for example, their molecules participate in one side reaction, (11) or (12), or do not participate in such reactions. From dependence [6], the kinetic parameters W_i/f and k_{eff} were also found and discussed. W_i/f was determined by extrapolation to zero concentration of the antioxidant, and k_{eff} was obtained from the slope of the dependence [6]. In Table 4.4, some of the data obtained for W_i/f and k_{eff} are presented.

The consumption of the inhibitors according to the reaction of chain initiation (12) presupposes that k_{eff} should not depend on the character of the lipid medium, which is not the case (Table 4.4). Therefore, the antioxidant molecules take part in side reactions with the hydroperoxides, reaction (11). This statement is confirmed by the different composition, and hence, different stability of the hydroperoxides formed during oxidation of various types of lipid substrates (Yanishlieva 1973, Yanishlieva and Popov 1973), previously discussed. This means that the rate constants of consumption of the inhibitors should be greater (higher) in TGSO than in TGL, which is demonstrated by the k_{eff} values obtained (Table 4.4).

From Table 4.4, it can be seen that ferulic and sinapic acids, and fraxetin in TGL oxidation at 100°C, as well as fraxetin in TGSO oxidation at 100°C, do not change the rate of their consumption with rising concentration ($n = 0$), and $k_{eff} = 0$, respectively. The molecules of these antioxidants do not participate in side reactions under these oxidation conditions.

Previous research (Denisov and Khudyakov 1987) showed that if the antioxidant radical ($A^{\bullet}$) participates in one reaction of chain propagation [reaction (*7), or (10), or (14)], the dependence [8] is valid:

$$W_{inh} \approx [AH]^{-0.5} \qquad [8]$$

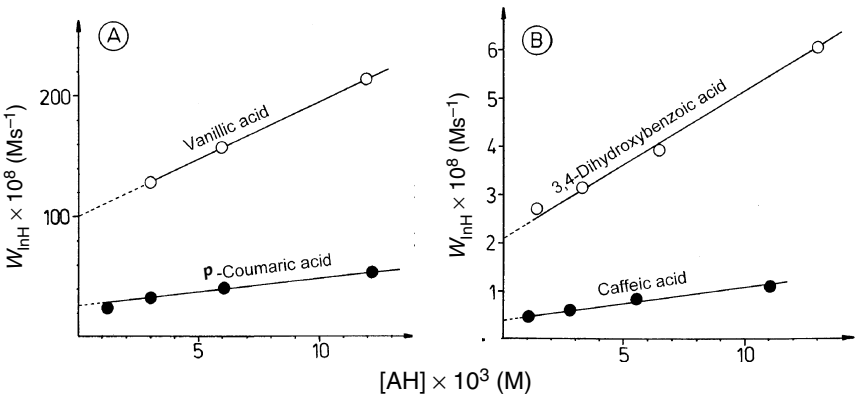


Fig. 4.6. Dependence of the rate of consumption, W_{InH}, of different phenolic acids on their concentration [AH]. Oxidation of triacylglycerols of lard (TGL) at 100°C (Marinova and Yanishlieva 1992b).

TABLE 4.4
Kinetic Parameters k_{eff} and W_i/f Determined for Various Antioxidants During Oxidation of Different Lipid Substrates at 100°C

	k_{eff} (s^{-1})			W_i/f (M/s)		
Antioxidant	TGL	TGOO	TGSO	TGL	TGOO	TGSO
Caffeic acid	7.0×10^{-7}	7.0×10^{-7}	8.2×10^{-6}	0.04×10^{-7}	0.27×10^{-8}	0.1×10^{-7}
Ferulic acid	0	3.8×10^{-6}	10.6×10^{-5}	1.2×10^{-7}	0.40×10^{-7}	3.0×10^{-7}
p-Coumaric acid	2.6×10^{-5}	2.1×10^{-5}	—	2.5×10^{-7}	0.98×10^{-7}	—
Sinapic acid	0	—	3.2×10^{-5}	0.2×10^{-7}	—	0.6×10^{-7}
Esculetin	2.5×10^{-6}	—	2.0×10^{-5}	0.05×10^{-7}	—	0.35×10^{-7}
3,4-Dihydroxybenzoic acid	2.8×10^{-6}	—	7.3×10^{-5}	0.20×10^{-7}	—	1.5×10^{-7}
Fraxetin	0	—	0	0.03×10^{-7}	—	0.41×10^{-7}
α-Tocopherol	0.98×10^{-5}	—	1.5×10^{-5}	0.35×10^{-8}	—	0.15×10^{-7}
Resveratrol	2.3×10^{-6}	—	3.4×10^{-5}	0.14×10^{-7}	—	0.9×10^{-7}

[a]*Sources:* Marinova and Yanishlieva (1992b, 1996), Yanishlieva and Marinova (1995b), Marinova *et al.* (1994, 2002), Yanishlieva *et al.* (2002).
[b]TG = triacylglycerol; L = lard; OO = olive oil; SO = sunflower oil.

When A$^{\bullet}$ does not participate in chain propagation, dependence [9] is valid:

$$W_{inh} \approx [AH]^{-1} \quad [9]$$

The W_{inh} values for vanillic acid during oxidation (100°C) of MEL and for ferulic acid during oxidation (100°C) of MESO did not show linear dependence on either $[AH]^{-0.5}$ or $[AH]^{-1}$ (Marinova and Yanishlieva 1994b), which indicated that the radicals of these phenolic acids were involved in more than one reaction of chain propagation. The same was true for α-tocopherol in TGL oxidation (Marinova and Yanishlieva 1992a), as well as for α- and γ-tocopherols at higher concentrations in TGSO and TGSBO oxidation (Yanishlieva *et al.* 2002).

It has been established that the radical of esculetin does not participate in chain propagation during oxidation of TGL and TGSO, and the radical of fraxetin does not participate in chain propagation during TGL oxidation (Marinova *et al.* 1994). On the other hand, the radical of fraxetin took part in one reaction of chain propagation in TGSO oxidation (Marinova *et al.* 1994). The same was true for 3,4-dihydroxybenzoic and caffeic acids in TGSO (Yanishlieva and Marinova 1995b), for *p*-coumaric, ferulic and caffeic acids in TGOO and MEOO (Marinova and Yanishlieva 1996), and for vanillic, *p*-coumaric, ferulic, syringic, and 3,4-dihydroxybenzoic acid in TGL (Marinova and Yanishlieva 1992b). The interpretation of the kinetic results obtained for the oxidation of different lipid substrates in the presence of the antioxidants studied led to the assumption that this reaction should be reaction (10) (Marinova and Yanishlieva 1994b, Marinova *et al.* 1994, Yanishlieva and Marinova 1995b).

Influence of the Lipid System on Antioxidative Action

As can be seen from Table 4.3, all of the antioxidants studied, with the exception of α-tocopherol, show lower activity in TGSO than in the more saturated lipid system TGL. Moreover, *p*-coumaric and ferulic acids are more active antioxidants in TGOO than in TGL.

The antioxidative action of some phenolic acids in triacylglycerols and methyl esters of sunflower and olive oils and lard at 100°C is compared in Table 4.5. These results illustrate that the activity of the antioxidants is in most cases higher in TGL and TGSO than in MEL and MESO, respectively, indicating that the binding of the fatty acids to the triacylglycerol structure offers a greater stabilizing effect by the antioxidants. The opposite is true for TGOO and MEOO. This result leads to the assumption that the oleate moiety plays a specific role with respect to the antioxidative stability of lipids, which should be examined in connection with the triacylglycerol structure of the olive oil.

The influence of the lipid substrate on the kinetic parameters W_i/f and k_{eff} is presented in Table 4.4. It can be seen that in TGL W_i/f and k_{eff} have lower values than in TGSO. Table 4.6 summarizes the data for the antioxidant activity and mechanism of action of some of the investigated antioxidants in different lipid substrates at 100°C.

TABLE 4.5
Antioxidative Activity (*A*) of Some Phenolic Acids During Oxidation of Different Lipid Substrates at 100°C[a,b]

Antioxidant	Concentration	*A*					
		TGL	MEL	TGSO	MESO	TGOO	MEOO
3,4-Dihydroxy-benzoic acid	1.3×10^{-3} M (0.02%)	191	55	6.0	2.0	—	—
	3.25×10^{-3} M (0.05%)	705	210	11.6	3.7	—	—
	6.49×10^{-3} M (0.10%)	1477	412	17.6	6.8	—	—
Ferulic acid	1.03×10^{-3} M (0.02%)	5.2	11.1	4.3	1.0	20.0	42.0
	2.53×10^{-3} M (0.05%)	17.6	35.7	5.3	1.7	58	150
	5.15×10^{-3} M (0.10%)	52	100	7.8	2.2	149	322
Sinapic acid	0.89×10^{-3} M (0.02%)	95	103	28.1	3.4	—	—
	2.23×10^{-3} M (0.05%)	333	315	34.8	7.3	—	—
	4.46×10^{-3} M (0.10%)	1015	588	48.1	14.2	—	—
Caffeic acid	11.1×10^{-3} M (0.02%)	10350	2652	448	74	4867	7786
	2.78×10^{-3} M (0.05%)	20350	6444	900	237	10182	17000
	5.56×10^{-3} M (0.10%)	28917	9500	1364	400	29167	25600

[a]*Sources:* Marinova and Yanishlieva (1992b, 1994a, 1994b, 1996), Yanishlieva and Marinova (1995b).
[b]TG = triacylglycerol; L = lard; ME = methyl ester; SO = sunflower oil; OO = olive oil.

TABLE 4.6
Antioxidant Activity and Mechanism of Action of Some of the Investigated Antioxidants at 100°C[a,b]

Antioxidant	Concentration interval (M)	Conditions	A (range)	Participation in side reactions of autoxidation
Caffeic acid	$1.1–11.1 \times 10^{-3}$ (0.02–0.20%)	TGL	10350–45900	Molecules are consumed in one side reaction (11) $K_{eff} = 7.0 \times 10^{-7}$ s^{-1} $W_i/f = 0.04 \times 10^{-7}$ M/s
Fraxetin	$0.5–4.8 \times 10^{-3}$ (0.01–0.10%)	TGL	764–34000	Molecules are not consumed in side reactions $W_i/f = 0.03 \times 10^{-7}$ M/s Radicals do not participate in chain propagation
3,4-Dihydroxy-benzoic acid	$1.3–13.0 \times 10^{-3}$ (0.02–0.20%)	TGL	191–2890	Molecules are consumed in one side reaction (11) $K_{eff} = 2.8 \times 10^{-6}$ s^{-1} $W_i/f = 0.2 \times 10^{-7}$ M/s Radicals participate in one side reaction of chain propagation (10)
Carnosol	$0.3–6.1 \times 10^{-3}$ (0.01–0.20%)	TGL	590–1643	Molecules are consumed in one side reaction (11) $K_{eff} = 3.6 \times 10^{-6}$ s^{-1} $W_i/f = 0.03 \times 10^{-7}$ M/s
Sinapic acid	$0.9–8.9 \times 10^{-3}$ (0.02–0.20%)	TGL	95–2617	Molecules are not consumed in side reactions $W_i/f = 0.2 \times 10^{-7}$ M/s Radicals participate in one side reaction of chain propagation (10)
Esculetin	$0.6–5.6 \times 10^{-3}$ (0.01–0.10%)	TGL	324–1462	Molecules are consumed in one side reaction (11) $K_{eff} = 2.5 \times 10^{-6}$ s^{-1} $W_i/f = 0.05 \times 10^{-7}$ M/s Radicals do not participate in chain propagation
p-Coumaric acid	$1.2–12.2 \times 10^{-3}$ (0.02–0.20%)	TGOO	11.0–62	Molecules are consumed in one side reaction (11) $K_{eff} = 2.14 \times 10^{-5}$ s^{-1} $W_i/f = 0.98 \times 10^{-7}$ M/s Radicals participate in one side reaction of chain propagation (10)

Ferulic acid	$1.0–10.3 \times 10^{-3}$ (0.02–0.20%)	TGOO	20.0–296	Molecules are consumed in one side reaction (11) $K_{eff} = 0.38 \times 10^{-5}$ s^{-1} $W_i/f = 0.40 \times 10^{-7}$ M/s Radicals participate in one side reaction of chain propagation (10)
Caffeic acid	$1.1–11.1 \times 10^{-3}$ (0.02–0.20%)	TGOO	4867–37833	Molecules are consumed in one side reaction (11) $K_{eff} = 0.07 \times 10^{-5}$ s^{-1} $W_i/f = 0.027 \times 10^{-7}$ M/s Radicals participate in one side reaction of chain propagation (10)
3,4-Dihydroxy-benzoic acid	$1.3–13.0 \times 10^{-3}$ (0.02–0.20%)	TGSO	6.0–17.6	Molecules are consumed in one side reaction (11) $K_{eff} = 7.30 \times 10^{-5}$ s^{-1} $W_i/f = 1.5 \times 10^{-7}$ M/s Radicals participate in one side reaction of chain propagation (10)
Ferulic acid	$1.0–10.3 \times 10^{-3}$ (0.02–0.20%)	TGSO	4.3–9.0	Molecules are consumed in one side reaction (11) $K_{eff} = 10.6 \times 10^{-5}$ s^{-1} $W_i/f = 3.0 \times 10^{-7}$ M/s The radicals are involved in more than one reaction of chain propagation
Sinapic acid	$0.89–8.93 \times 10^{-3}$ (0.02–0.20%)	TGSO	28.1–448	Molecules are consumed in one side reaction (11) $K_{eff} = 3.2 \times 10^{-5}$ s^{-1} $W_i/f = 0.6 \times 10^{-7}$ M/s The radicals are involved in more than one reaction of chain propagation
Caffeic acid	$1.1–11.1 \times 10^{-3}$ (0.02–0.20%)	TGSO	448–1463	Molecules are consumed in one side reaction (11) $K_{eff} = 0.82 \times 10^{-5}$ s^{-1} $W_i/f = 0.1 \times 10^{-7}$ M/s Radicals participate in one side reaction of chain propagation (10)

[a] *Sources:* Marinova and Yanishlieva (1992b, 1996), Marinova *et al.* (1991, 1994), Yanishlieva and Marinova (1995b).
[b] TG = triacylglycerol; L = lard; OO = olive oil; SO = sunflower oil.

It was established that the effectiveness of the antioxidants differed depending on whether the process took part in a bulk phase or in a liposome bilayer (Yanishlieva *et al.* 1994). It was also found that the effectiveness of α-tocopherol and caffeic acid was considerably lower in the case of liposome oxidation. In addition, the sequence of effectiveness of α-tocopherol and caffeic acid was reversed when passing from bulk phase oxidation to liposome oxidation. In the first case, caffeic acid was twice as effective as α-tocopherol, whereas in the second case, α-tocopherol was 2.5 times more effective than caffeic acid.

Comparison of the data for *F* of different extracts from some species of the family Lamiaceae during oxidation of sunflower oil at 100°C (Marinova and Yanishlieva 1997) with *F* of the extracts in TGSO oxidation (Yanishlieva and Marinova 1995a) shows that the natural sunflower oil is much more difficult to stabilize than are its pure triacylglycerols. The tocopherol concentration in sunflower oil is close to the optimal concentration required for its stabilization, which explains the effect observed.

Influence of Temperature on Antioxidative Action

The influence of temperature on the antioxidative action was examined with α-tocopherol and ferulic acid. Figure 4.7 illustrates the kinetic curves of peroxide accumulation during inhibited oxidation of TGL in the presence of equal molar concentrations (2.4×10^{-3} M) of α-tocopherol and ferulic acid at 100 and 25°C. The unnumbered curves present noninhibited oxidation at the same temperatures. It can be seen that the

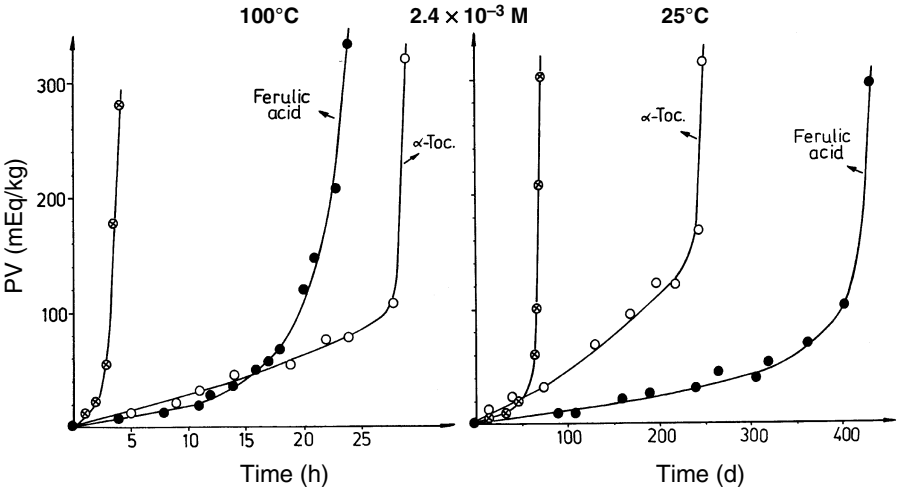


Fig. 4.7. Kinetic curves of peroxide accumulation during oxidation of TGL in the presence of equal molar concentration of α-tocopherol and ferulic acid at 100 and 25°C. The curves without number present noninhibited oxidation of triacylglycerols of lard (TGL) at the same temperatures (Marinova and Yanishlieva 1992a).

variation of temperature changes the order of antioxidant effectiveness; ferulic acid was more effective at 25°C, whereas α-tocopherol exhibited higher effectiveness at 100°C. Moreover, in the presence of α-tocopherol at 25°C, the oxidation rate during the IP was higher than in the noninhibited system, which was not observed at 100°C.

After processing the kinetic results for TGL oxidation at 25, 50, 75, and 100°C in the presence of different concentrations of ferulic acid and α-tocopherol (Marinova and Yanishlieva 1992a), the data for *F* and ORR were determined (Figs. 4.8 and 4.9). It is clear that *F* and ORR for ferulic acid do not depend on temperature (Fig. 4.8), whereas *F* increased, and ORR decreased with increasing temperature for α-tocopherol (Fig. 4.9). These results show that the change in temperature does not affect the activity of ferulic acid, and with rising temperature, the activity of α-tocopherol increases (Fig. 4.10).

The results obtained allowed the following conclusion to be made (Marinova and Yanishlieva 1992a): A change in temperature does not affect the mechanism of action of ferulic acid; therefore, its effectiveness and strength, i.e., activity, remain stable at different temperatures. With rising temperature both the effectiveness and the strength, i.e., activity, of α-tocopherol increase, which is due to the change in mechanism of its participation in the different reactions of inhibited oxidation. Thus, the results for the oxidative stability of lipids obtained at high temperature can be used for quantitative estimation of the stability at room temperature only when no change occurs in the mechanism of participation of the antioxidant and its radical in the reactions of inhibited oxidation.

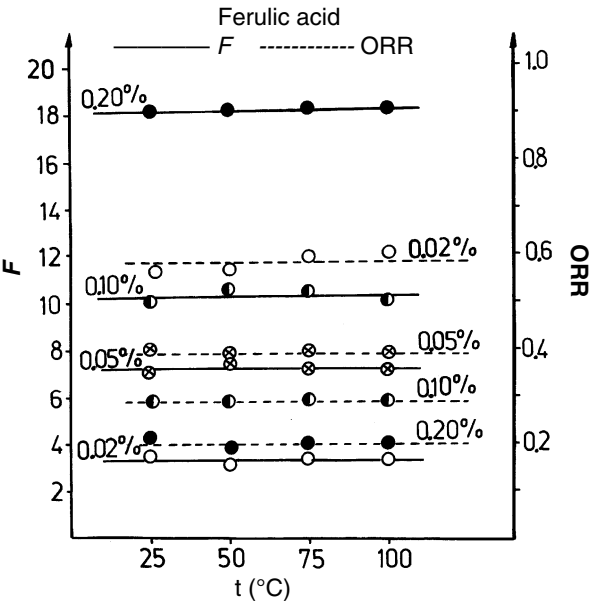


Fig. 4.8. *F* and ORR for various concentrations of ferulic acid during oxidation of triacylglycerols of lard (TGL) at different temperatures. Adapted from Marinova and Yanishlieva (1992a).

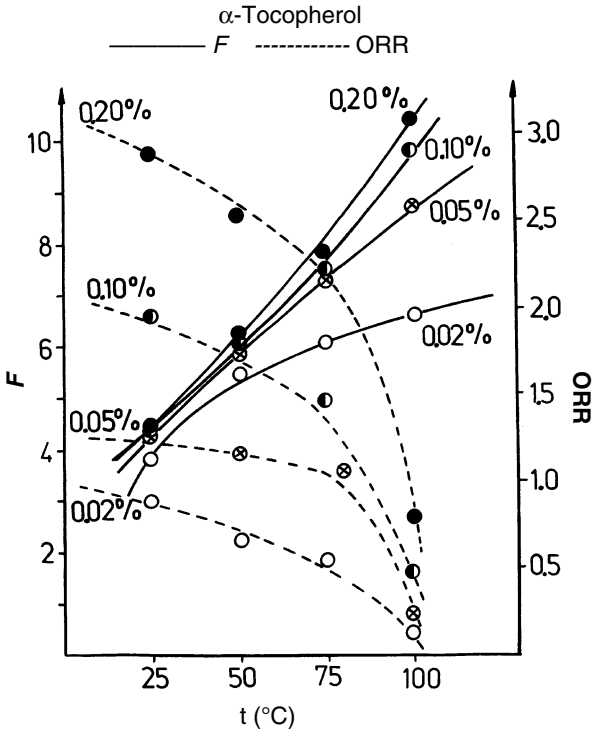


Fig. 4.9. *F* and ORR for various concentrations of α-tocopherol during oxidation of triacylglycerols of lard (TGL) at different temperatures. Adapted from Marinova and Yanishlieva (1992a).

We investigated the oxidation kinetics of TGL and TGSO, containing 0.05% α-tocopherol, in the presence of different concentrations of ascorbyl palmitate (AP) at 25 and 100°C (Marinova and Yanishieva 1992c). It has been established that the rise of temperature was associated with an increase in *A* of AP, which was more pronounced with the lipid system of lower oxidizability, e.g., TGL.

The influence of temperature on the antioxidative action of quercetin and morin in TGL (Yanishlieva and Marinova 1996a) and in TGSO (Marinova and Yanishlieva 1998) was also studied. At 22°C and in the concentration interval $2.2–8.9 \times 10^{-4}$ M (0.0075–0.03%), the values of *A* for morin and quercetin in TGL did not differ significantly, whereas they differed at 90°C for both inhibitors by one order of magnitude (in morin's favor) (Yanishlieva and Marinova 1996a). Quercetin was a more active antioxidant than morin in TGSO at both temperatures. In addition, with rising temperature, the activity of both antioxidants increased significantly (Marinova and Yanishlieva 1998). The investigation of the antioxidative effect of the ethanol extract from *Saturejae hortensis* L. (summer savory) in lipids showed that the effect of the additive was stronger at room temperature than at 100°C (Yanishlieva and Marinova 1998).

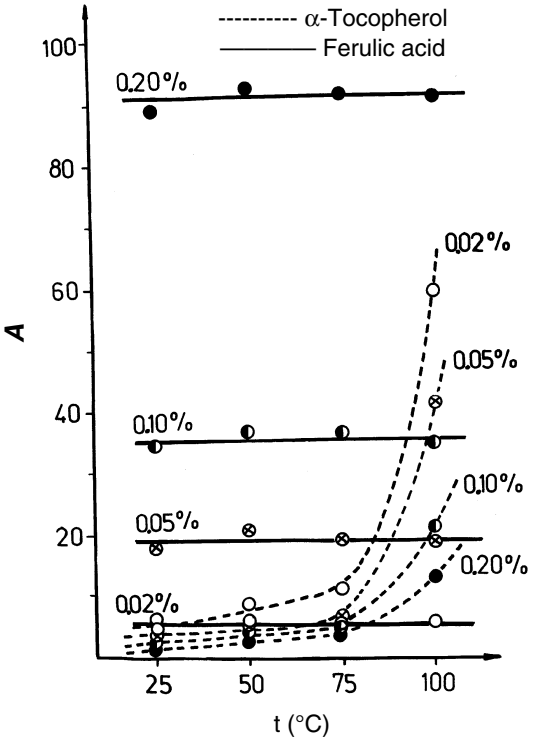


Fig. 4.10. Activity A for various concentrations of α-tocopherol and ferulic acid during oxidation of triacylglycerols of lard (TGL) at different temperatures. Adapted from Marinova and Yanishlieva (1992a).

Concluding Remarks

The general parameter activity, *A*, allows a complex estimation of the effect of antioxidants in lipid oxidation. It unifies the effectiveness of an inhibitor in the termination of the autoxidation chain (*F*) and its ability to change the oxidation rate during the induction period (ORR). Together with the other parameters (*F* and ORR), *A* enables the identification of some of the major events associated with the inhibitory action of antioxidants as well as the participation of the antioxidant molecules and/or radicals in side reactions that decrease the antioxidant potential. The three parameters (*F*, ORR, and *A*) also enable the evaluation of the effect of other factors, e.g., type of substrate and temperature, on the efficacy of antioxidants.

References

Denisov, E., and Khudyakov, I. (1987) Mechanism of Action and Reactivities of the Free Radicals of Inhibitors, *Chem. Rev. 87*, 1313–1357.

Emanuel, N.M., Denisov, E.T., and Maizuss, Z.K. (1965) *Chain Radical Oxidation of Hydrocarbons in Liquid Phase*, Nauka, Moscow.

Gordon, M.H. (1996) Dietary Antioxidants in Disease Prevention, *Bioorg. Chem. 14*, 265–273.

Gunstone, F.G., and Hilditch, T.P. (1945) The Union of Gaseous Oxygen with Methyl Oleate, Linoleate, and Linolenate, *J. Chem. Soc*. 836–841.

Le Tutour, B., and Guedon, D. (1992) Antioxidative Activities of *Olea europaea* Leaves and Related Phenolic Compounds, *Phytochemistry 31*, 1173–1178.

Marinova, E., Yanishlieva, N., Bankova, V., and Popov, S. (1989) On the Antioxidative Activity of Phenolic Acids and Their Esters of Propolis During the Autoxidation of Lard, *Proceedings of the Fifth International Conference on Chemistry and Biotechnology of Biologically Active Natural Products, Vol. 2*, pp. 244–251, Varna, Bulgaria.

Marinova, E., Yanishlieva, N., and Ganeva, I. (1991) Antioxidative Effect of Bulgarian Rosemary and Inhibiting Activity of Its Carnosol, *Oxid. Commun. 14*, 125–131.

Marinova, E.M., and Yanishlieva, N. (1992a) Effect of Temperature on the Antioxidative Action of Inhibitors in Lipid Oxidation, *J. Sci. Food Agric. 60*, 313–318.

Marinova, E.M., and Yanishlieva, N.V. (1992b) Inhibited Oxidation of Lipids. II: Comparison of the Antioxidative Properties of Some Hydroxy Derivatives of Benzoic and Cinnamic Acids, *Fat Sci. Technol. 94*, 428–432.

Marinova, E.M., and Yanishlieva, N.V. (1992c) Inhibited Oxidation of Lipids. III: On the Activity of Ascorbyl Palmitate During the Autoxidation of Two Types of Lipid Systems in the Presence of α-Tocopherol, *Fat Sci. Technol. 94*, 448–452.

Marinova, E.M., and Yanishlieva, N.V. (1994a) Antioxidative Action of Some Phenolic Acids in Three Different Type Lipid Systems, in *Bioactive Substances in Food of Plant Origin*, Vol. 1 (Kozlowska, H., Fornal, J., and Zdunczyk, Z., eds.), pp. 157–160, Polish Academy of Sciences, Olsztyn, Poland.

Marinova, E.M., and Yanishlieva, N.V. (1994b) Effect of Lipid Unsaturation on the Antioxidative Activity of Some Phenolic Acids, *J. Am. Oil Chem. Soc. 71*, 427–434.

Marinova, E.M., and Yanishlieva, N.V. (1996) Antioxidative Activity of Phenolic Acids on Triacylglycerols and Fatty Acid Methyl Esters from Olive Oil, *Food Chem. 56*, 139–145.

Marinova, E.M., and Yanishlieva, N.V. (1997) Antioxidative Activity of Extracts from Selected Species of the Family Lamiaceae in Sunflower Oil, *Food Chem. 58*, 245–248.

Marinova, E.M., and Yanishlieva, N.V. (1998) Antioxidative Action of Quercetin and Morin in Triacylglycerols of Sunflower Oil at Ambient and High Temperatures, *Seifen Öle Fette Wachse 124*, 10–16.

Marinova, E.M., Yanishlieva, N.V., and Kostova, I.N. (1994) Antioxidative Action of the Ethanolic Extract and Some Hydroxycoumarins of *Fraxinus ornus* Bark, *Food Chem. 51*, 125–135.

Marinova, E.M., Yanishlieva, N.V., and Totzeva, I.R. (2002) Activity and Mechanism of Action of *trans*-Resveratrol in Different Lipid Systems, *Int. J. Food Sci. Technol. 37*, 145–152.

Popov, A., and Yanishlieva, N. (1976) *Autoxidation and Stabilization of Lipids*, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Popov, A., Yanishlieva, N., and Slavceva, J. (1968) Methode zum Nachweis von Antioxidantien in Methyloleat für kinetische Untersuchungen, *Compt. Rend. Acad. Bulg. Sci. 21*, 443–446.

Rice-Evans, C., and Burdon, R. (1993) Free Radical-Lipid Interactions and Their Pathological Consequences, *Prog. Lipid Res. 32*, 71–110.

Roginskii, V.A. (1990) Kinetics of Polyunsaturated Fatty Acid Esters Oxidation Inhibited by Substituted Phenols, *Kinet. Catal. 31*, 546–552.

Scott, G. (1985) Antioxidants *In Vitro* and *In Vivo*, *Chem. Brit. 21*, 648–653.

Stahl, W. (2000) Lipid Oxidation and Antioxidants, *Curr. Opin. Clin. Nutr. Metab. Care 3*, 121–126.

Stirton, A.J., Turer, H., and Riemenschneider, R.W. (1945) Oxygen Absorption of Methyl Esters of Fatty Acids and the Effect of Antioxidants, *Oil Soap 22*, 81–83.

Yanishlieva, N. (1973) Über einige Eigentümlichkeiten in der Kinetik zu Beginn der Autoxydation von Estern ungesättigter Fettsäuren. 5. Mitt. Über den Mechanismus der Hydroperoxidabbaus, *Nahrung 17*, 313–322.

Yanishlieva, N., and Marinova, E. (1985) Einfluss verschiedener Produkte von *Scenedesmus Acutus* auf die Autoxidation der Lipide, *Seifen Öle Fette Wachse 111*, 637–639.

Yanishlieva, N., and Marinova, E. (1986) Effect of Ground Red Pepper on the Autoxidation of Lipid Systems of Low Oxidation Stability, *Food Ind. Sci. 2*, 44–48.

Yanishlieva, N., and Marinova, E. (1992) Inhibited Oxidation of Lipids. I: Complex Estimation of the Antioxidative Properties of Natural and Synthetic Antioxidants, *Fat Sci. Technol. 94*, 374–379.

Yanishlieva, N., and Marinova, E. (1995a) Antioxidant Activity of Selected Species of the Family *Lamiaceae* Grown in Bulgaria, *Nahrung 39*, 458–463.

Yanishlieva, N., and Marinova, E. (1995b) Effect of Antioxidants on the Stability of Triacylglycerols and Methyl Esters of Fatty Acids of Sunflower Oil, *Food Chem. 54*, 377–382.

Yanishlieva, N., and Marinova, E. (1996a) Antioxidant Activity of Some Flavonoids at Ambient and High Temperatures, *Riv. Ital. Sostanze Grasse 73*, 445–449.

Yanishlieva, N., and Marinova, E. (1996b) Antioxidative Effectiveness of Some Natural Antioxidants in Sunflower Oil, *Z. Lebens.-Unters.-Forsch. 203*, 220–223.

Yanishlieva, N., and Marinova, E. (1998) Activity and Mechanism of Action of Natural Antioxidants in Lipids, *Recent Res. Dev. Oil Chem. 2*, 1–14.

Yanishlieva, N., and Popov, A. (1974) Sur la Stabilite l'Oxydation du Saindoux, *Rev. Fr. Corps Gras 21*, 553–557.

Yanishlieva, N.V., Kamal-Eldin, A., Marinova, E.M, and Toneva, A.G. (2002) Kinetic Study of the Antioxidative Activity of α- and γ-Tocopherols in Sunflower and Soybean Triacylglycerols, *Eur. J. Lipid Sci. Technol. 104*, 262–270.

Yanishlieva, N., Marinova, E., Antonova, V., Gardev, M., and Petrov, G. (1983) Method for Obtaining of Antioxidative Preparation from Broad Beans, *Oil Soap Indust. 19*, 37–44.

Yanishlieva, N., Marinova, E., Bankova, V., Popov, S., and Marekov, N. (1984) Does the Antioxidative Activity of Propolis Depend on the Flavonoids Present, Groupe Polyphenols, *J. Int. D'Etudes et Assemblees Generales—Bulletin de Liason 12*, 481–486.

Yanishlieva, N.V., Marekov, I.N., and Christie, W.W. (1994) Comparison of the Antioxidative Effectiveness of α-Tocopherol and Caffeic Acid During Bulk Phase and Liposome Oxidation of Acylglycerols with High Linoleic Acid Content, *Seifen Öle Fette Wachse 120*, 662–665.

Yanishlieva, N.V., Marinova, E.M., Gordon, M.H., and Raneva, V.G. (1999) Antioxidant Activity and Mechanism of Action of Thymol and Carvacrol in Two Lipid Systems, *Food Chem. 64*, 59–66.

Yanishlieva, N.V., Marinova, E.M., Marekov, I.N., and Gordon, M.H. (1997) Effect of an Ethanol Extract from *Saturejae hortensis* L. on the Stability of Sunflower Oil at Frying Temperature, *J. Sci. Food Agric. 74*, 524–530.

Yanishlieva, N.V., Marinova, E.M, Raneva, V.G., Partali, V., and Sliwka, H.-R. (2001a) β-Apo-8′-carotenoic Acid and Its Esters in Sunflower Oil Oxidation, *J. Am. Oil Chem. Soc. 78*, 641–644.

Yanishlieva, N., Marinova, E., and Rankov, D. (1985) On the Antioxidative Action of *Silibum marianum* Seed Oil Unsaponifiables, *Communications of the Third International Conference on Chemistry and Biotechnology of Biologically Active Natural Products*, Vol. 5, pp. 12–15, Sofia, Bulgaria.

Yanishlieva, N., and Popov, A. (1971) Über einige Eigentümlichkeiten in der Kinetik zu Beginn der Autoxydation von Estern ungesättigter Fettsäuren. 3. Mitt. Inhibierte Autoxydation, *Nahrung 15*, 671–681.

Yanishlieva, N., and Popov, A. (1973) La Spectrophotometrie Ultraviolette en Tant que Methode d'Estimation de l'Etat d'Oxydation des Lipides Insaturee, *Rev. Fr. Corps Gras 20*, 11–26.

Yanishlieva, N., Rafikova,V., and Skibida, I. (1970) Etude de la Cinetique de l'Oxydation Competitive de l'Oleate et du Linoleate de Methyl, *Rev. Fr. Corps Gras 17*, 741–746.

Yanishlieva, N., Raneva, V., Blekas, G., and Boskou, D. (1998) Antioxidative Properties and Mechanism of Action of 3,4-Dihydroxyphenylacetic Acid in Lipids, *Riv. Ital. Sost. Grasse 75*, 453–456.

Yanishlieva, N.V., Raneva, V.G., and Marinova, E.M. (2001b) β-Carotene in Sunflower Oil Oxidation, *Grasas Aceites 52*, 10–16.

Yanishlieva-Maslarova, N. (2001) Inhibiting Oxidation, in *Antioxidants in Food: Practical Applications* (Pokorný, J., Yanishlieva, N., and Gordon, M., eds.), pp. 22–70, CRC Press, Boca Raton.

Yanishlieva-Maslarova, N., Popov, A., Seher, A., and Ivanov, S. (1977) Natürliche Antioxidantien III. Über einige Kinetische Besonderheiten der Antioxydativen Wirkung der Tocotrienole, *Fette Seifen Anstrichm. 79*, 357–360.

Chapter 5

Kinetic Analysis of β-Carotene Oxidation in a Lipid Solvent With or Without an Antioxidant

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Introduction

β-Carotene, having biological activities as a provitamin A and an active oxygen quencher (Foote and Denny 1968), oxidizes easily and loses activity in the air. Prevention of the oxidation during processing and long-term storage is very important to utilize β-carotene as a food additive. When used as a food additive, β-carotene is commonly dissolved in a lipid because of its high solubility therein. As lipids oxidize, β-carotene oxidation proceeds *via* a complicated co-oxidation mechanism, accompanying the lipid oxidation. To prevent the β-carotene oxidation in a food system, an antioxidant such as α-tocopherol is usually added. When β-carotene exists together with lipids and antioxidants in a practical food system, not only does the co-oxidation of β-carotene and lipid take place but also a protection of β-carotene by the antioxidant occurs. For the determination of the appropriate amount of antioxidant required to protect β-carotene in a mixture, it is important to understand the following: (i) the oxidation mechanism of β-carotene itself; (ii) the co-oxidation mechanism of β-carotene in the lipid solvent; and (iii) the antioxidation mechanism for the protection of β-carotene by the antioxidant.

Although a large number of studies have been made on the oxidation kinetics of β-carotene, many researchers have used simple first-order kinetic models to describe the oxidation behavior (Arya *et al.* 1979, Chou and Breene 1972, Henry *et al.* 1998, Ramakrishnan and Francis 1979a). This is because most of the oxidation experiments have been performed under special conditions such as a high reaction temperature or in the presence of initiators. The oxidation of β-carotene in general proceeds *via* an autocatalytic free radical chain reaction that involves induction, propagation, and termination stages (Papadopolou and Ames 1994). Thus, the consumption curve of β-carotene is mainly sigmoidal. Kasaikina *et al.* (1975 and 1981) constructed a simplified free radical reaction model. Their model described the oxidation behavior of β-carotene in organic solutions except for the induction stage. Ozhogina and Kasaikina (1995) modified the model to describe the induction stage. However, their model could not describe the effect of the oxygen concentration on the oxidation rate, which is an important factor in the oxidation process.

Several researchers have investigated β-carotene oxidation in solutions containing lipids and many have discussed an antioxidant effect of β-carotene on the lipid oxidation (Kennedy and Liebler 1992, Palozza *et al.* 1995, Terao 1989, Tsuchihashi *et al.* 1995). When Budowski and Bondi (1960) and Ramakrishnan and Francis (1979b) investigated β-carotene oxidation in an organic solution containing a lipid such as cottonseed oil or methyl linoleate, the oxidation rate of β-carotene increased with the lipid content in solution. They suggested that the product generated by the lipid oxidation affected the oxidation rate of β-carotene. However, the co-oxidation mechanism of β-carotene with a lipid remains unclear, and a kinetic model based on the reaction mechanism has never been proposed.

Many research groups have also investigated the effect of antioxidant addition on lipid oxidation. An induction period, during which the lipid oxidation was suppressed by adding antioxidant, was generally observed. The length of the induction period and the oxidation rate during this period were determined to evaluate the activities of various antioxidants as shown in Chapter 3 of this book as well as other publications (Iwatsuki *et al.* 1994, Kortenska and Yanishlieva 1995, Marinova and Yanishlieva 1992). On the other hand, lipid oxidation was reported to be suppressed or accelerated by adding an antioxidant according to reaction conditions such as temperature and antioxidant concentration (Nagaoka *et al.* 1992, Terao and Matsushita 1986). The oxidation mechanism of lipids in the presence of antioxidant has not been elucidated and no kinetic model based on the reaction mechanism has been proposed.

This chapter presents a compilation of previous research on the oxidation kinetics of β-carotene in an inert organic solvent, *n*-decane, without initiators (Takahashi *et al.* 1999), in oleic acid as a lipid solvent (Takahashi *et al.* 2001), and in *n*-decane with the addition of α-tocopherol as an antioxidant (Takahashi *et al.* 2003). Rigorous kinetic models describing the oxidation behavior including co-oxidation of β-carotene in a lipid solvent and antioxidation for the protection of β-carotene by antioxidant were proposed. The kinetic and equilibrium constants in the models were estimated by fitting the models with the experimental results obtained under various conditions of temperature and oxygen composition. Validity of the proposed kinetic models was verified by comparison of the calculated and experimental results.

Construction of the Kinetic Model

Oxidation Mechanism of β-Carotene

The oxidation of β-carotene (Takahashi *et al.* 1999) is generally considered to follow an autocatalytic free radical chain reaction that involves chain initiation ($I1_A$), propagation ($P1_A$, $P2_A$), and termination ($T1_A$), similar to the oxidation of hydrocarbons (Alekseev *et al.* 1968). Kasaikina *et al.* (1975 and 1981) constructed a simplified model on the basis of the free radical reaction as

$$AH + O_2 \xrightarrow{k_{I1,A}} A^{\bullet} + HO_2^{\bullet} \quad [1]$$

$$A^{\bullet} + O_2 \xrightarrow{k_{P1,A}} AO_2^{\bullet} \quad [2]$$

$$AO_2^{\bullet} + AH \xrightarrow{k_{P2,A}} AOOH + A^{\bullet} \quad [3]$$

$$AO_2^{\bullet} + AO_2^{\bullet} \xrightarrow{k_{T1,A}} \text{nonradical stable product} \quad [4]$$

where AH is β-carotene, $A^{\bullet}$ and $AO_2^{\bullet}$ are β-carotene–derived carbon-centered and peroxyl radicals, and AOOH is β-carotene hydroperoxide.

The induction stage, in which the reaction rate is extremely small, is generally observed at the beginning of the oxidation. The period of the induction stage is known to be closely associated with the storage life of β-carotene (Toro-Vazquez *et al.* 1993). However, the kinetic model based on this reaction mechanism (Appendix I) cannot represent the induction stage because the consumption rate of β-carotene, Equation [A-1], is maximal at the beginning of the oxidation as will be described later. Therefore, it is necessary to take other reactions into account.

A hydroperoxide generated by the oxidation is considered to decompose to free radicals (Toro-Vazquez *et al.* 1993). These radicals are supposed to take part in reinitiation reactions (Encinar *et al.* 1993 and 1994) so that the initiation rate increases proportionally to the amount of hydroperoxide generated by the oxidation. These phenomena are considered to contribute to the appearance of the induction stage in the oxidation. In our study, the secondary initiation reaction by the decomposition of the hydroperoxide ($I2_A$) was as follows:

$$AOOH + O_2 \xrightarrow{k_{I2,A}} AO_2^{\bullet} + HO_2^{\bullet} \quad [5]$$

It has been reported that the secondary oxidation products such as epoxide, alcohol, or ketone are generated by the further reaction of the hydroperoxide (Handelman *et al.* 1991, Ito 1987). When β-carotene remains in the system, however, only small amounts of these secondary products are present (Britton 1995). In our study, about 10% of β-carotene remained at the end of the experiments; therefore, reactions concerned with the secondary products were not incorporated into the kinetic model.

The β-carotene–derived carbon-centered radical, $A^{\bullet}$, is more stable than the one that is hydrocarbon-derived because of resonance-stabilization. The propagation reaction concerned with the radical should be reversible (Burton and Ingold 1984) rather than irreversible as proposed by Kasaikina *et al.* (1975 and 1981). The reversible reaction of Equation [2] is given as

$$AO_2^{\bullet} \xrightarrow{k_{-P1,A}} A^{\bullet} + O_2 \quad [6]$$

Here, the minus symbol of $k_{-P1,A}$ represents a kinetic constant of the reversible reaction. The termination reaction with the resonance-stabilized radical, $A^{\bullet}$, ($T2_A$) should also be incorporated (Burton and Ingold 1984) as

$$A^{\bullet} + AO_2^{\bullet} \xrightarrow{k_{T2,A}} \text{nonradical stable product} \quad [7]$$

The oxidation mechanism of β-carotene is considered to consist of Equations [1]– [7]. Assuming steady state for the concentrations of the respective radicals, $A^{\bullet}$ and $AO_2^{\bullet}$, in the system, the rates of change in the concentrations of β-carotene and its hydroperoxide are

$$\frac{dC_{AH}}{dt} = -k_{I1,A}C_{AH}C_{O_2} - k_{PT,A}C_{AH}\sqrt{r_I / (1 + k_{R1,A} / C_{O_2})} \quad [8]$$

$$\frac{dC_{AOOH}}{dt} = -k_{PT,A}C_{AH}\sqrt{r_I / (1 + k_{R1,A} / C_{O_2})} - k_{I2,A}C_{AOOH}C_{O_2} \quad [9]$$

The initial conditions are

$$t = 0;\ C_{AH} = C_{AH}(0);\ C_{AOOH} = C_{AOOH}(0) \quad [10]$$

The initiation rate, r_I, and the kinetic constants, $k_{PT,A}$ and $k_{R1,A}$, in Equations [8] and [9] are given as

$$r_1 = k_{I1,A}C_{AH}C_{O_2} - k_{I2,A}C_{AOOH}C_{O_2} \quad [11]$$

$$k_{PT,A} = \frac{k_{P2,A}}{\sqrt{2k_{T1,A}}} \quad [12]$$

$$k_{R1,A} = \frac{k_{T2,A}\ k_{-P1,A}}{k_{T1,A}\ k_{P1,A}} \quad [13]$$

Co-Oxidation Mechanism of β-Carotene in Oleic Acid Solvent

The lipid solvent, oleic acid (Takahashi *et al.* 2001), was oxidized by an autocatalytic free radical chain reaction mechanism similar to β-carotene (Bateman 1954, Takahashi *et al.* 2000). The peroxyl radical, having a high reactivity, is considered to react preferentially with β-carotene rather than oleic acid due to the polyene structure of β-carotene. This reaction may contribute to an increase in the oxidation rate of β-carotene in the presence of oleic acid. On the basis of this concept, a kinetic model was constructed for the co-oxidation of β-carotene in this lipid solvent.

The co-oxidation of β-carotene in the lipid solvent is considered to consist of the oxidation of β-carotene itself, the oxidation of oleic acid itself and the cross reaction of

β-carotene and oleic acid. As described in the previous section, the oxidation of β-carotene itself proceeds by a multistep autocatalytic free radical chain reaction mechanism, Equations [1]–[7], in which a secondary initiation, a reverse reaction of propagation, and a termination concerned with the carbon-centered radical were involved.

The oxidation of oleic acid itself, LH, also proceeds by a multistep autocatalytic free radical chain reaction mechanism similar to β-carotene, in which the chain initiation ($I1_L$) is given as

$$LH + O_2 \xrightarrow{k_{I1,L}} L^{\bullet} + HO_2^{\bullet} \qquad [14]$$

The carbon-centered radical, $L^{\bullet}$, generated by Equation [14] reacts rapidly with oxygen to form the peroxyl radical $LO_2^{\bullet}$ as

$$L^{\bullet} + O_2 \xrightarrow{k_{P1,L}} LO_2^{\bullet} \qquad [15]$$

Although $A^{\bullet}$ is a resonance-stabilized radical, $L^{\bullet}$ is not. Thus, the propagation reaction concerned with $L^{\bullet}$ ($P1_L$), Equation [15], is considered not to be reversible. The oleic acid concentration decreased slightly throughout the experiment, suggesting that the oxidation of oleic acid remained in the initial stage of the chain reaction. Therefore, it is sufficient to take into account only Equations [14] and [15].

$LO_2^{\bullet}$, generated by Equation [15], reacts preferentially with β-carotene over oleic acid as described above. The cross-reaction of β-carotene and oleic acid (CR_{LA}) is as follows:

$$LO_2^{\bullet} + AH \xrightarrow{k_{CR,LA}} LOOH + A^{\bullet} \qquad [16]$$

where LOOH is oleic acid hydroperoxide.

The co-oxidation of β-carotene with oleic acid proceeds by a series of mechanisms as shown in Equations [1]–[7] and [14]–[16]. Assuming steady state for the concentrations of the respective radicals, $A^{\bullet}$, $AO_2^{\bullet}$, $L^{\bullet}$, $LO_2^{\bullet}$, in the system, the rate of change in the β-carotene concentration is

$$\frac{dC_{AH}}{dt} = -k_{I1,A}C_{AH}C_{O_2} - k_{PT,A}C_{AH}\sqrt{(r_I + r_{CR,LA})/(1 + k_{R1,A}/C_{O_2})} - r_{CR,LA} \qquad [17]$$

and the initial conditions are

$$t = 0;\ C_{AH} = C_{AH}(0) \qquad [18]$$

The initiation rate, r_I and the kinetic constants, $k_{PT,A}$ and $k_{R1,A}$, are given as Equations [11]–[13] in the previous section. The cross-reaction rate, $r_{CR,LA}$, in Equation [17] is expressed as

$$r_{CR,LA} = k_{I1,L}C_{LH}C_{O_2} \qquad [19]$$

The oleic acid concentration decreased slightly throughout the experiment as indicated by an oxidative conversion of less than 1% (data not shown), so that C_{LH} is assumed to be constant at the initial concentration, $C_{LH}(0)$. Thus, Equation [19] is substituted by the following equation:

$$r_{CR,LA} = k_{I1,L} C_{LH}(0) C_{O_2} \quad [20]$$

Antioxidation Mechanism for Protection of β-Carotene by α-Tocopherol

For the oxidation of β-carotene in the presence of α-tocopherol (Takahashi *et al.* 2003), not only the oxidation of β-carotene itself but also the antioxidation reaction by α-tocopherol are considered to occur. As described above, the oxidation of β-carotene proceeds by a multistep autocatalytic free radical reaction mechanism as in Equations [1]–[7].

α-Tocopherol, EH, is known to react with a peroxyl radical, having a high reactivity, and to change itself to the stable tocopheroxyl radical, $E^{\bullet}$ (Lampi *et al.* 1999). The peroxyl radical concerned with the chain propagation, $AO_2^{\bullet}$, decreases by this reaction, so that the chain oxidation of β-carotene is suppressed. Thus, the antioxidation reaction by α-tocopherol (Inh_{EA}) should be incorporated as

$$EH + AO_2^{\bullet} \xrightarrow{k_{Inh,EA}} E^{\bullet} + AOOH \quad [21]$$

However, the simple kinetic model based on Equations [1]–[7] and [21] (Appendix II) cannot describe not only the linear decrease in the α-tocopherol concentration but also the slight decrease in the β-carotene concentration during the induction period, as will be described later. Therefore, it is necessary to include other reactions involving β-carotene and α-tocopherol in the kinetic model.

α-Tocopherol might react with oxygen by itself and, therefore, it is reasonable to consider that the α-tocopherol oxidation proceeds by a multistep autocatalytic free radical chain reaction mechanism similar to β-carotene as

$$EH + O_2 \xrightarrow{k_{I1,E}} E^{\bullet} + HO_2^{\bullet} \quad [22]$$

$$E^{\bullet} + O_2 \underset{k_{-P1,E}}{\overset{k_{P1,E}}{\rightleftharpoons}} EO_2^{\bullet} \quad [23]$$

$$EO_2^{\bullet} + EH \xrightarrow{k_{P2,E}} EOOH + E^{\bullet} \quad [24]$$

$$EO_2^{\bullet} + EO_2^{\bullet} \xrightarrow{k_{T1,E}} \text{nonradical stable product} \quad [25]$$

$$E^{\bullet} + EO_2^{\bullet} \xrightarrow{k_{T2,E}} \text{nonradical stable product} \quad [26]$$

Here, $EO_2^{\bullet}$ is an α-tocopherol peroxyl radical and EOOH is α-tocopherol hydroperoxide. Equation [22] accounts for the chain initiation ($I1_E$), Equations [23] and [24] for the propagation ($P1_E$, $P2_E$), and Equations [25] and [26] for the termination ($T1_E$, $T2_E$) reactions.

In our system, the concentration of β-carotene was much higher than that of α-tocopherol, and hence the α-tocopherol peroxyl radical generated by Equation [23] reacts preferentially with β-carotene rather than with α-tocopherol itself. Thus, instead of Equation [24], the propagation reaction ($P2_{EA}$) of the α-tocopherol peroxyl radical with β-carotene is as follows:

$$EO_2^{\bullet} + AH \xrightarrow{k_{P2,EA}} EOOH + A^{\bullet} \quad [27]$$

Similarly, instead of Equation [26], the termination reaction ($T2_{EA}$) of the α-tocopherol peroxyl radical with the β-carotene peroxyl radical is incorporated as

$$EO_2^{\bullet} + AO_2^{\bullet} \xrightarrow{k_{T2,EA}} \text{nonradical stable product} \quad [28]$$

The α-tocopherol hydroperoxide formed by Equation [27] is considered to decompose as is the case for the β-carotene. Furthermore, by taking into consideration the slight decrease of β-carotene concentration during the induction period, it is reasonable to consider that the α-tocopherol hydroperoxide reacts with β-carotene and β-carotene hydroperoxide to generate free radicals taking part in the initiation reaction of the β-carotene oxidation. Thus, the secondary initiation reactions ($I2_{EA}$, $I3_{EA}$) are as follows:

$$EOOH + AH \xrightarrow{k_{I2,EA}} EO^{\bullet} + A^{\bullet} + H_2O \quad [29]$$

$$EOOH + AOOH \xrightarrow{k_{I3,EA}} EO_2^{\bullet} + AO^{\bullet} + H_2O \quad [30]$$

The alkoxyl radicals, $EO^{\bullet}$ and $AO^{\bullet}$, are newly generated by Equations [29] and [30]. The termination reactions concerned with these radicals ($T3_{EA}$, $T4_{EA}$) are also included in the model.

$$EO^{\bullet} + A^{\bullet} \xrightarrow{k_{T3,EA}} \text{nonradical stable product} \quad [31]$$

$$AO^{\bullet} + A^{\bullet} \xrightarrow{k_{T4,EA}} \text{nonradical stable product} \quad [32]$$

When two resonance-stabilized radicals are present, an exchange reaction between the radicals is known to occur (Denisov and Khudyakov 1987). Because two resonance-stabilized radicals, $E^{\bullet}$ and $A^{\bullet}$, exist in the system, the reversible exchange reaction (EX_{EA}) is as follows:

$$E^{\bullet} + AH \underset{k_{-EX,EA}}{\overset{k_{EX,EA}}{\rightleftharpoons}} EH + A^{\bullet} \quad [33]$$

The oxidation of β-carotene in the presence of α-tocopherol proceeds by a series of mechanisms as shown in Equations [1]–[7], [21]–[23], [25] and [27]–[33]. This is our rigorous model; it considers not only the antioxidation but also the co-oxidation and radical-exchange reaction of β-carotene and α-tocopherol. Assuming steady state for the concentrations of the respective radicals, $A^{\bullet}$, $AO_2^{\bullet}$, $AO^{\bullet}$, $E^{\bullet}$, $EO_2^{\bullet}$, and $EO^{\bullet}$, in the system, the rates of change in the concentrations of β-carotene and α-tocopherol are

$$\frac{dC_{AH}}{dt} = -k_{I1,A}C_{AH}C_{O_2} - k_{PT,A}C_{AH}\sqrt{r_{II}} - k_{I2,EA}C_{EOOH}C_{AH} - r_{III} \quad [34]$$

$$\frac{dC_{EH}}{dt} = -k_{I1,E}C_{EH}C_{O_2} - k_{IT,EA}C_{EH}\sqrt{r_{II}} \quad [35]$$

and the initial conditions are

$$t = 0;\ C_{AH} = C_{AH}(0);\ C_{EH} = C_{EH}(0) \quad [36]$$

The kinetic constant, $k_{PT,A}$, is given in Equation [12]. The initiation rates, r_{II} and r_{III}, and the kinetic constant, $k_{IT,EA}$, in Equations [34] and [35] are expressed as

$$r_{II} = \frac{k_{I1,A}C_{AH}C_{O_2} + k_{I2,A}C_{AOOH}C_{O_2} + k_{I1,E}C_{EH}C_{O_2}}{1 + k_{R1,A}/C_{O_2} + C_{EH}/(k_{R2,EA}C_{AH}) + C_{EH}^2/(k_{R3,EA}C_{AH}^2)} \quad [37]$$

$$r_{III} = k_{I1,E}C_{EH}C_{O_2} + k_{I3,EA}C_{EOOH}C_{AOOH} + k_{IT,EA}C_{EH}\sqrt{r_{II}} - \left(\frac{C_{EH}}{2k_{R2,EA}C_{AH}} + \frac{C_{EH}^2}{k_{R3,EA}C_{AH}^2}\right)r_{II} \quad [38]$$

$$k_{IT,EA} = \frac{k_{Inh,EA}}{\sqrt{2k_{T1,A}}} \quad [39]$$

The kinetic constant, $k_{R1,A}$, is also given in Equation [13]. Other constants, $k_{R2,EA}$ and $k_{R3,EA}$, in Equations [37] and [38] are

$$k_{R2,EA} = \frac{k_{T1,A}k_{P1,A}k_{-P1,E}k_{EX,EA}}{k_{T2,E}k_{-P1,A}k_{P1,E}k_{-EX,EA}} \quad [40]$$

$$k_{R3,EA} = \frac{k_{T1,A} k_{P1,A}^2 k_{-P1,E}^2 k_{EX,EA}^2}{k_{T1,E} k_{-P1,A}^2 k_{P1,E}^2 k_{-EX,EA}^2} \quad [41]$$

Experimental Procedures

Oleic acid, provided by the NOF Company, Japan, has purity of 92.3%. Other chemicals including β-carotene, α-tocopherol, and *n*-decane were of reagent grade and were used without further purification. A schematic diagram of the experimental apparatus is shown in Figure 5.1. The reaction vessel was made of stainless steel, and its volume was 5.0×10^{-4} m^3. The working volume was 3.0×10^{-4} m^3 and the initial β-carotene concentration was 0.75 mol/m^3. Aeration gas, N_2 or a mixture of O_2 and N_2, was supplied by bubbling through a porous filter at the rate of 1.7×10^{-6} m^3/s at atmospheric pressure. A condenser was installed at the gas outlet to reduce the loss of solvent due to evaporation. Before the beginning of the reaction, the reaction solution in the vessel was sufficiently aerated by inert N_2 gas to purge dissolved oxygen, and the vessel was immersed in an oil bath. After the solution temperature reached a given constant value, the reaction was started by supplying the reaction gas mixture in place of N_2 gas. The reaction was stopped when the β-carotene conversion reached 90%. *n*-Decane or oleic acid was used as a solvent. The reaction temperature, the oxygen composition in the supplied gas, and the initial α-toco-

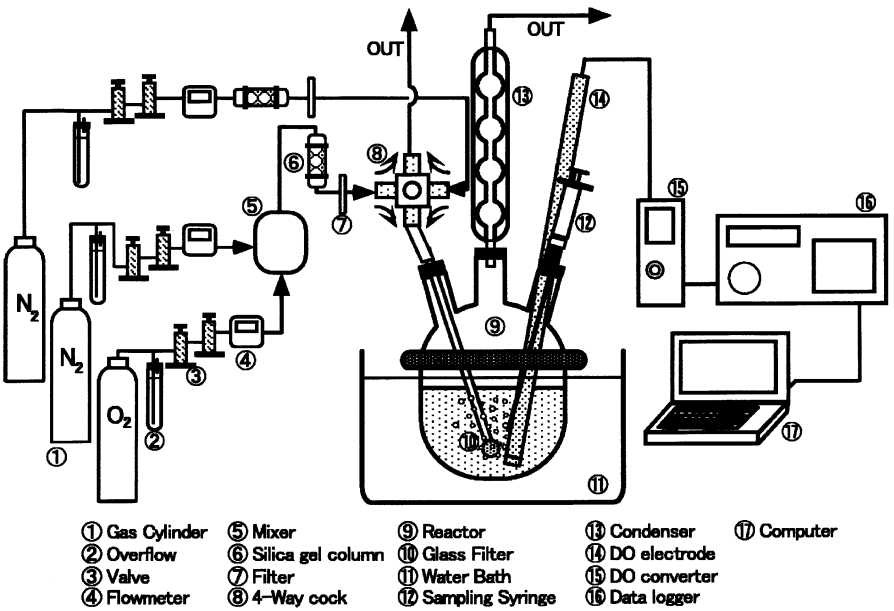

Fig. 5.1. Schematic diagram of experimental apparatus.

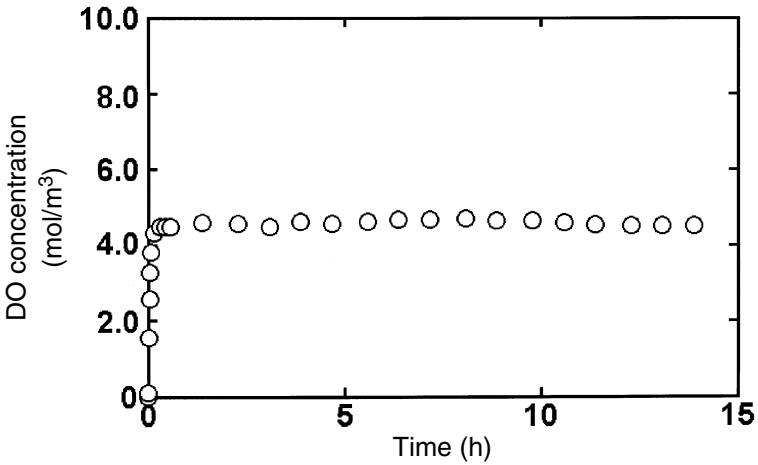

Fig. 5.2. Time course of the dissolved oxygen (DO) concentration measured at a temperature of 333 K and an oxygen composition in the supplied gas of 40 mol%.

$$k_{I2,A} = B_{I2,A}\exp(-E_{I2,A}/RT) \quad [44]$$

$$k_{R1,A} = B_{R1,A}\exp(-E_{R1,A}/RT) \quad [45]$$

These constants, B_i and E_i, were estimated by fitting Equation [8] with eight sets of the results for the oxidation experiments in *n*-decane according to the following fitting procedure. Using an arbitrary set of the constants, the differential equation, Equation [8], is numerically solved by means of the Runge-Kutta method. The time step for the numerical calculation was set at 60 s. The best-fitted values of the constants were determined using the Simplex method (Nelder and Mead 1964) by

TABLE 5.1
Asymptotical Constant Values of Dissolved Oxygen (DO) Concentration Measured Under Various Conditions

Temperature (K)	Oxygen composition in supplied gas (mol%)	DO concentration (mol/m^3)
323	40	4.81
333		4.58
343		4.89
333	10	1.02
	20	2.14
	30	3.19
	40	4.58
	70	8.12
	100	11.10

pherol concentration were regulated between 323 and 343 K, 10 and 100 mol%, and 0 and 7.5×10^{-3} mol/m^3, respectively. The oxidation experiments were also performed using a mixed solvent of 10 or 50 mol% oleic acid (the residual component was *n*-decane).

In each experiment, the dissolved oxygen (DO) concentration was measured *in situ* using a monitoring system with a DO electrode. Sample solutions were withdrawn at specific time intervals. The concentration of residual β-carotene was measured spectroscopically at 450 nm and that of residual oleic acid was measured using a high-performance liquid chromatography (HPLC) system equipped with an Inertsil ODS column and an ultraviolet (UV) detector at 210 nm. The mobile phase was acetonitrile/water (90:10, vol/vol) and the flow rate was 0.5×10^{-8} m^3/s. Whenever applicable, α-tocopherol concentration was determined with an HPLC system equipped with an Inertsil ODS column and a UV detector at 295 nm. The mobile phase was methanol and the flow rate was 0.5×10^{-8} m^3/s. In the oxidation experiments in *n*-decane without α-tocopherol, the β-carotene hydroperoxide concentration was also determined by standard titration (Lea 1931). This method is generally used for the analysis of the lipid hydroperoxide.

Results and Discussion

Oxidation Kinetics of β-Carotene

Measurement of Dissolved Oxygen Concentration. An example of the time course of the DO concentration obtained in the oxidation experiments of β-carotene in *n*-decane is shown in Figure 5.2. The concentration increased rapidly during the first 15 min and then approached a constant value asymptotically. This tendency was observed under all of the reaction conditions tested. The period in which the concentration increased (15 min) was negligibly short compared with the entire reaction time (15 h). Therefore, the DO concentration was assumed to be constant throughout the reaction period. The asymptotical constant values of the DO concentration measured under various conditions are listed in Table 5.1. The values increased with the oxygen composition in the supplied gas and depended little on the reaction temperature.

Estimation of Kinetic Constants. In the kinetic model for the β-carotene oxidation in *n*-decane, there are four unknown constants, $k_{I1,A}$, $k_{PT,A}$, $k_{I2,A}$, and $k_{R1,A}$. To describe the effect of the reaction temperature on the oxidation behavior, a temperature dependence of the Arrhenius type was introduced. Each constant is expressed using the preexponential factor, B_i, and the activation energy, E_i, as

$$k_{I1,A} = B_{I1,A}\exp(-E_{I1,A}/RT) \quad [42]$$

$$k_{PT,A} = B_{PT,A}\exp(-E_{PT,A}/RT) \quad [43]$$

minimizing the squared sum of the relative error between the calculated and experimental results for the β-carotene concentration. The experimental values obtained at the beginning of the reaction were used for the initial value of β-carotene concentration, $C_{AH}(0)$, and the measured values listed in Table 5.1 were used for the dissolved oxygen concentration, C_{O_2}, under each reaction condition.

The experimental and fitted results are shown in Figures 5.3 and 5.4. The former describes the effect of the oxygen composition in the supplied gas on the oxidation rate, and the latter describes the effect of the reaction temperature. The symbols and the solid lines represent the experimental data and the fitted results by our model, respectively. For reference, the fitted results by the model proposed by Kasaikina *et al.* (1975 and 1981) (Appendix I) are also shown in these figures by dotted lines. Under any conditions, the solid lines were in much better agreement with the experimental data than the dotted lines. Our model described not only the sigmoidal oxidation curve of β-carotene but also the effect of oxygen composition in the supplied gas on the oxidation rate. Thus, not only the secondary initiation reaction by hydroperoxide decomposition but also the propagation and termination reactions involving the β-carotene–derived carbon-centered radical should be incorporated.

The estimated values of each constant in the model are listed in Table 5.2. The kinetic constant, $k_{R1,A}$, defined by Equation [13], was combined with the constants for various reactions, and hence the value cannot be compared with the literature

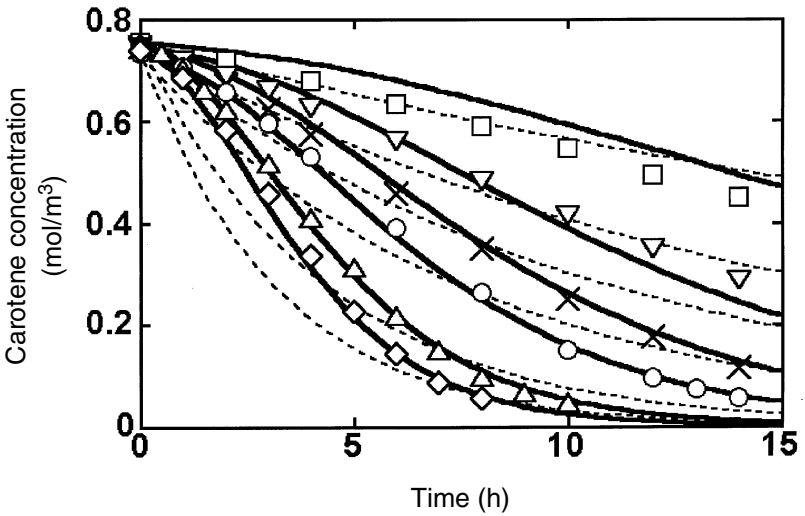

Fig. 5.3. Experimental data for β-carotene concentration obtained at a temperature of 333 K and fitted results by the models: □, 10; ▽, 20; ×, 30; ○, 40; △, 70; ◇, 100 mol% of oxygen composition in the supplied gas; solid lines, our model; dotted lines, model proposed by Kasaikina *et al.* (1975 and 1981).

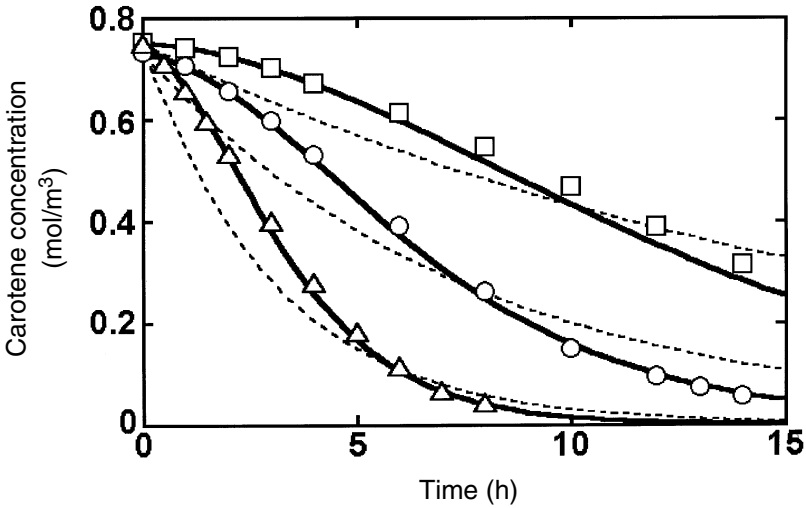

Fig. 5.4. Experimental data for β-carotene concentration obtained at an oxygen composition in the supplied gas of 40 mol% and fitted results by the models: □, 323; ○, 333; △, 343 K temperature; solid lines, our model; dotted lines, model proposed by Kasaikina *et al.* (1975 and 1981).

values. On the other hand, the activation energy of the kinetic constant, $k_{PT,A}$, defined by Equation [12], was reported to be in the range of 24 to 75 kJ/mol (Mill and Hendry 1980). In our study, the value was estimated to be 27.8 kJ/mol and was within range of the literature values. There were no literature values for the activation energy of the kinetic constants for the initiation, $k_{I1,A}$, and for the hydroperoxide decomposition, $k_{I2,A}$. However, the estimated values of 138 and 66.0 kJ/mol were within the range of the activation energy of the kinetic constant concerned with the oxidation, 20 to 150 kJ/mol (Mill and Hendry 1980). Therefore, these estimated values were considered to be reasonable.

Simulation of Hydroperoxide Concentration. The changing rate of the β-carotene hydroperoxide concentration is given as Equation [9]. In the model proposed by Kasaikina *et al.* (1975 and 1981), it is also given by Equation [A-2] in

TABLE 5.2
Estimated Values of Constants in Our Model for β-Carotene Oxidation in *n*-Decane

Constant	Preexponential factor B_i	Activation energy E_i (J/mol)
$k_{I1,A}$	5.82×10^{10} ($mol^{-1} \cdot m^3 \cdot s^{-1}$)	1.38×10^5
$k_{PT,A}$	2.30×10^4 ($mol^{-1/2} \cdot m^{3/2} \cdot s^{-1/2}$)	2.78×10^4
$k_{I2,A}$	5.30×10 ($mol^{-1} \cdot m^3 \cdot s^{-1}$)	6.60×10^4
$k_{R1,A}$	2.02×10^3 ($mol \cdot m^{-3}$)	1.87×10^4

Appendix I. These equations were solved numerically using the estimated values of the constants shown in Table 5.2 and Table 5-A.1, respectively. The experimental and simulated results obtained at a temperature of 333 K and an oxygen composition in the supplied gas of 40 mol% are shown in Figure 5.5. The dotted line, representing the model proposed by Kasaikina *et al.* (1975 and 1981), remained at almost zero throughout the reaction, suggesting that β-carotene hydroperoxide was scarcely formed. On the other hand, the solid line representing our model was in much better agreement with the experimental data.

Application of Kinetic Model to Other Experiments. Tanaka (1995) studied the β-carotene oxidation in *n*-hexane without an initiator at low temperature. The structure of *n*-hexane is a straight-chain hydrocarbon similar to that of *n*-decane used in our experiments; hence the difference of the kinetic constants between the two solvents can be ignored. The kinetic models were applied to their experimental result using the estimated values of the kinetic constants in *n*-decane. There was a difference in the dissolved oxygen concentration between the solvents; therefore, the literature values for the dissolved oxygen concentration in *n*-hexane (Battino 1981) were used in the numerical calculation. The results are shown in Figure 5.6. The reaction temperature and the oxygen composition in the supplied gas are 293 K and 20 mol%, respectively. There was a significant difference between the experimental data reported by Tanaka (1995) and the broken line calculated according to the model proposed by Kasaikina *et al.* (1975 and 1981). On the other hand, the solid line representing our model was

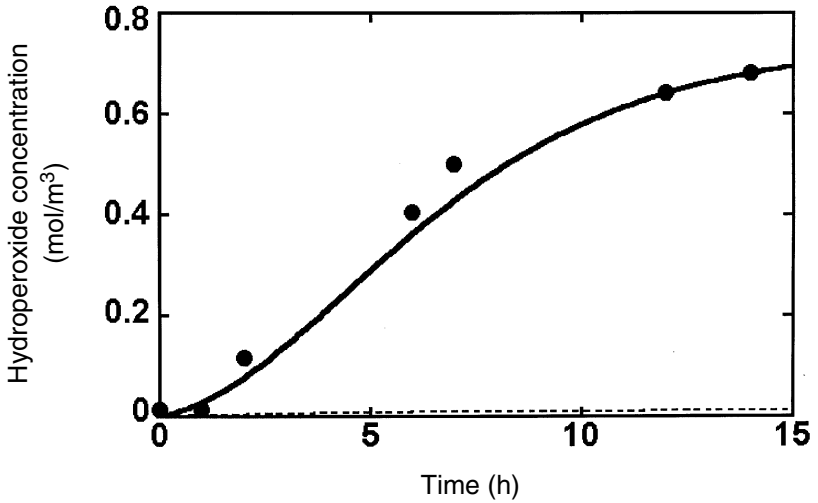

Fig. 5.5. Experimental data for hydroperoxide concentration obtained at a temperature of 333 K and an oxygen composition in the supplied gas of 40 mol% and simulated results by the models: solid line, our model; dotted line, model proposed by Kasaikina *et al.* (1975 and 1981).

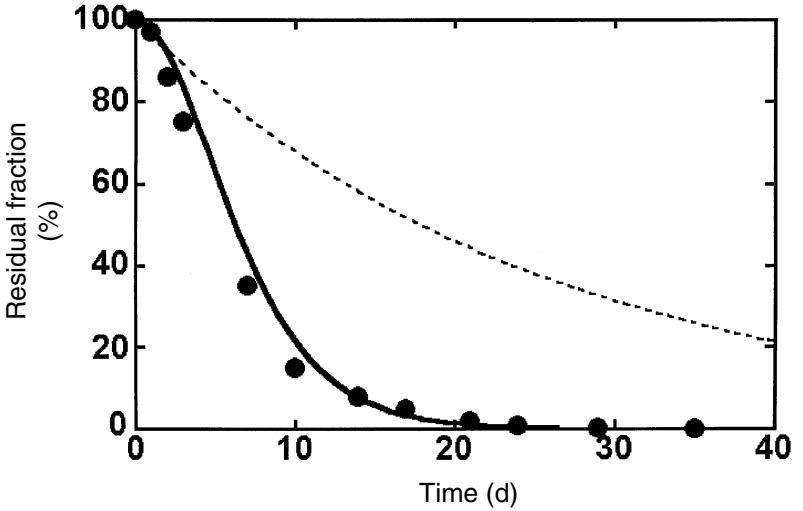

Fig. 5.6. Experimental data reported by Tanaka (1995) and simulated results by the models: solid line, our model; dotted line, model proposed by Kasaikina *et al.* (1975 and 1981).

in much better agreement with the experimental data. Therefore, when a straight-chain hydrocarbon compound is used as a solvent, the model can predict the behavior of β-carotene oxidation by incorporating only the difference in the dissolved oxygen concentration between the solvents.

Co-Oxidation Kinetics of β-Carotene in Oleic Acid Solvent

Estimation of Constants in Mass Balance Equation for Oxygen. In the oxidation experiments using oleic acid solvent, the period in which the DO concentration increased was about 1 h, compared with the total reaction time of 4–6 h. Thus, the variation in the DO concentration at the beginning of the reaction should be included in the model. The mass balance equation for oxygen in the solution is expressed as

$$\frac{dC_{O_2}}{dt} = k_L a(C^*_{O_2} - C_{O_2}) - (r_I + r_{CR,LA}) \qquad [46]$$

where $k_L a$ is the volumetric mass transfer coefficient and $C^*_{O_2}$ is the saturated DO concentration.

The second term on the right-hand side of Equation [46] is the rate of oxygen consumption by the reaction. Therefore, in the co-oxidation model, there are seven unknown constants including the kinetic constants for the oxidation of β-carotene itself, $k_{I1,A}$, $k_{I2,A}$, $k_{PT,A}$ and $k_{R1,A}$, the kinetic constant for the cross reaction, $k_{I1,L}$, and the constants in the mass balance equation for oxygen, $k_L a$ and $C^*_{O_2}$.

Generally, $k_L a$ is scarcely affected by the temperature or oxygen composition, and hence is assumed to be constant under all conditions. $C^*_{O_2}$ is assumed to be proportional only to the mole fraction of oxygen. The oxygen consumption rate by the reaction is considered to be negligible compared with that of the gas-liquid mass transfer during the period in which the DO concentration increases at the beginning of the reaction. Thus, the mass balance equation for oxygen in the solution, Equation [46], is simplified to

$$\frac{dC_{O_2}}{dt} = k_L a(y_{O_2} C^*_{pureO_2} - C_{O_2}) \qquad [47]$$

Here, y_{O_2} is the mole fraction of oxygen in the supplied gas and $C^*_{pureO_2}$ is the saturated DO concentration when 100 mol% O_2 gas is supplied to the solution. The constants, $k_L a$ and $C^*_{pureO_2}$, were estimated by fitting Equation [47] with six sets of the experimental results for the DO concentration in oleic acid solvent. In the calculation, the experimental results for reaction times up to 2 h were used. The fitting procedure was similar to that described above. The fitted and experimental results are shown in Figure 5.7. Under all conditions, the calculated lines were in agreement with the experimental results. The estimated values of $k_L a$ and $C^*_{pureO_2}$ are 1.31×10^{-3}/s and 10.2 mol/m^3, respectively.

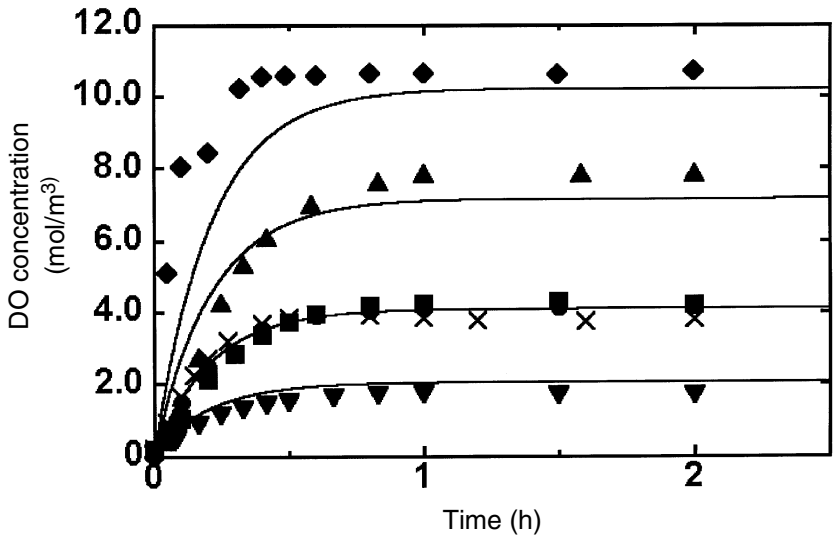

Fig. 5.7. Experimental data for DO concentration and fitted results by Equation [47]: ■, 323; ●, 333; ×, 343 K temperature at 40 mol% of oxygen composition in the supplied gas; ▼, 20; ▲, 70; ◆, 100 mol% of oxygen compositions at 333 K; solid lines, fitted results.

Estimation of Other Kinetic Constants. The differences in physical properties of solvents have little effect on the reaction rates in free-radical chain reactions such as in the oxidation of organic compounds (Stirling 1965). Thus, the kinetic constants for the oxidation of β-carotene itself in oleic acid, $k_{I1,A}$, $k_{I2,A}$, $k_{PT,A}$ and $k_{R1,A}$, were assumed to be the same as those in *n*-decane previously listed in Table 5.2.

To estimate the kinetic constant for the cross-reaction, $k_{I1,L}$, a temperature dependence of the Arrhenius type was introduced. The constant, $k_{I1,L}$, is expressed using the preexponential factor, B_i, and the activation energy, E_i, as

$$k_{I1,L} = B_{I1,L} \exp(-E_{I1,L}/RT) \quad [48]$$

These constants were estimated by fitting Equation [17] with six sets of the results for the oxidation experiment using pure oleic acid as a solvent.

Figures 5.8 and 5.9 show the effects of temperature and oxygen composition in the supplied gas on the oxidation of β-carotene in oleic acid. Under all conditions, the calculated lines were in good agreement with the experimental results. The model quantitatively described the oxidation behavior of β-carotene in oleic acid over a wide range of temperatures and oxygen compositions. The estimated value of $k_{I1,L}$ is given as

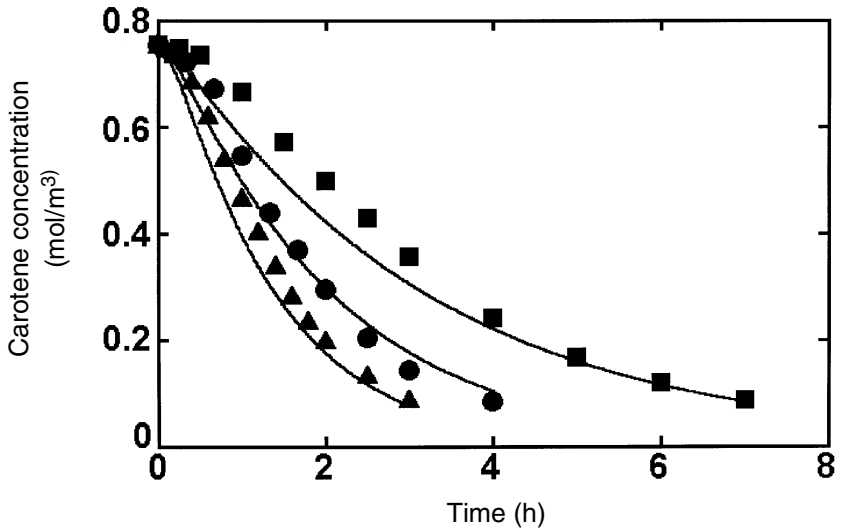

Fig. 5.8. Experimental data for β-carotene concentration obtained at an oxygen composition in the supplied gas of 40 mol% and fitted results by the model: ■, 323; ●, 333; ▲, 343 K temperature; solid lines, fitted results.

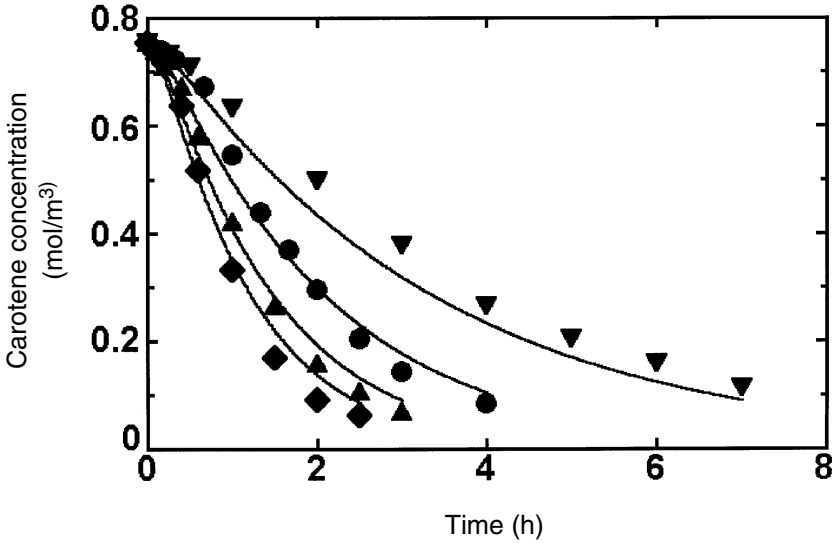

Fig. 5.9. Experimental data for β-carotene concentration obtained at a temperature of 333 K and fitted results by model: ▼, 20; ●, 40; ▲, 70; ◆, 100 mol% of oxygen compositions; solid lines, fitted results.

$$k_{I1,L}\ (\text{mol}^{-1} \cdot \text{m}^3 \cdot \text{s}^{-1}) = 2.03 \times 10^{-7} \exp(-3.16 \times 10^4/RT) \qquad [49]$$

The activation energy of the kinetic constant concerned with the oxidation is in the range of 20–150 kJ/mol (Mill and Hendry 1980). Therefore, the estimated value, 31.6 kJ/mol, is considered to be reasonable.

Simulation of Oxidation Behavior for Various Oleic Acid Contents. The cross-reaction rate of β-carotene with oleic acid, $r_{CR,LA}$, expressed by Equation [20], increases with the initial oleic acid concentration, $C_{LH}(0)$. Thus, the oxidation rate of β-carotene is considered to increase with $C_{LH}(0)$. The oxidation behavior was simulated using the model under various oleic acid contents in the solvent. The calculated results are shown in Figure 5.10, compared with the experimental results. For reference, the experimental and fitted results for the oleic acid contents of 0 and 100 mol% are also shown in Figure 5.10. The results for 0 mol% correspond to those in *n*-decane mentioned in the previous section. The oxidation rate of β-carotene was found to increase with the oleic acid content in the solvent, and the model simulated the experimental results well.

Antioxidation Kinetics for Protection of β-Carotene by α-Tocopherol

Measurement of Dissolved Oxygen Concentration. In the oxidation experiments with the addition of α-tocopherol, the DO concentration increased rapidly

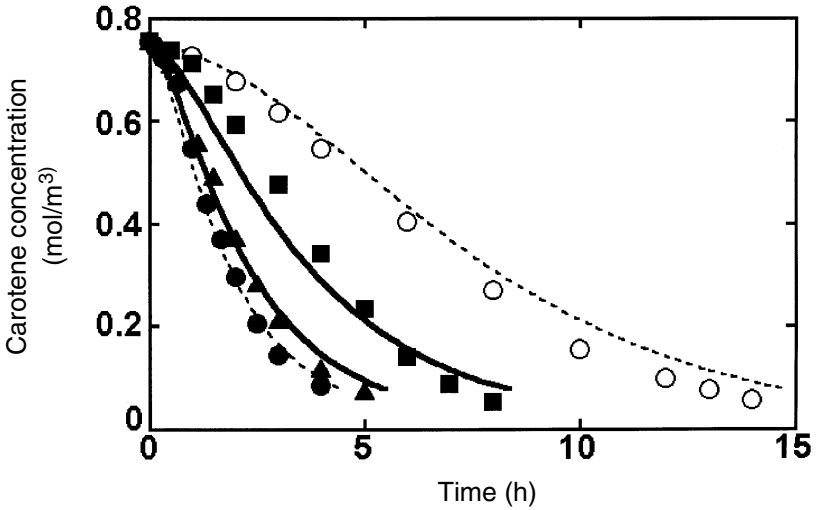

Fig. 5.10. Time course of β-carotene concentration obtained at an oxygen composition of 40 mol% and a temperature of 333 K: ○, 0 (in *n*-decane); ■, 10; ▲, 50; ●, 100 mol% of oleic acid content in solvent; solid lines, simulated results; broken lines, fitted results.

during the first 15 min and then asymptotically approached a constant value under all conditions (data not shown). The period in which the concentration increased was negligibly short compared with the entire reaction time. This kind of behavior and the asymptotical constant values of DO concentration were identical with those in the β-carotene oxidation in *n*-decane without α-tocopherol described above. Therefore, the DO concentration was assumed to be constant through all the reaction period and the constant values previously listed in Table 5.1 were used in the calculation.

There are ten unknown constants including the kinetic constant for the β-carotene oxidation, $k_{I1,A}$, $k_{PT,A}$, $k_{I2,A}$, and $k_{R1,A}$, the kinetic constant for the α-tocopherol oxidation, $k_{I1,E}$, and the kinetic constants for the reactions of β-carotene and α-tocopherol, $k_{I2,EA}$, $k_{I3,EA}$, $k_{IT,EA}$, $k_{R2,EA}$, and $k_{R3,EA}$ in the model. The kinetic constants for the β-carotene oxidation were scarcely affected by the existence of α-tocopherol; hence the estimated values without α-tocopherol previously listed in Table 5.2 were used.

Estimation of Kinetic Constant for α-Tocopherol Oxidation. The time courses of the α-tocopherol concentration obtained from the independent oxidation experiment for a long period (150 h) are shown in Figure 5.11. The concentration decreased more quickly with increasing temperature but the amount of α-tocopherol consumed throughout the experiment was not particularly large (<10%). This suggests that the independent oxidation of α-tocopherol remained in the ini-

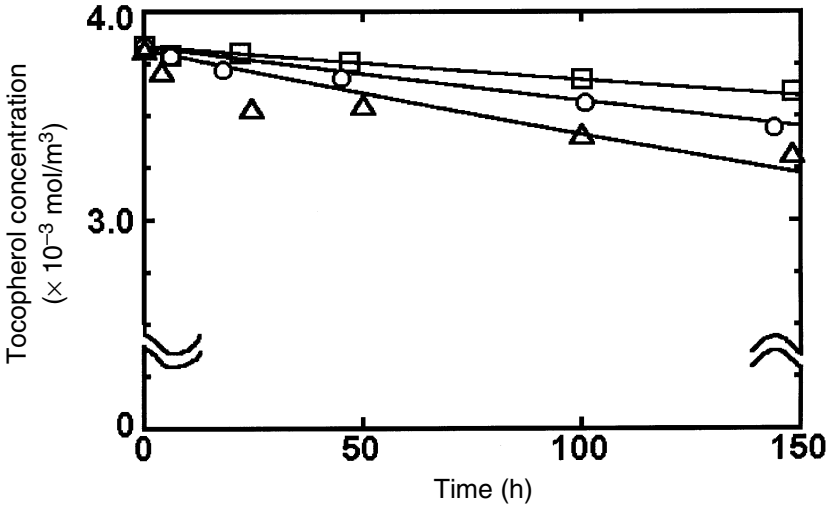

Fig. 5.11. Experimental and fitted results for α-tocopherol oxidation at 40 mol% of oxygen composition in the supplied gas and 3.8×10^{-3} mol/m^3 of initial α-tocopherol concentration: □, 323; ○, 333; △, 343 K; solid lines, fitted results.

tial stage of the chain reaction. Therefore, it is sufficient to take into account only Equations [22] and [23]. The rate of change in the α-tocopherol concentration is

$$\frac{dC_{EH}}{dt} = -k_{I1,E} C_{EH} C_{O_2} \qquad [50]$$

As in the above section, the kinetic constant, $k_{I1,E}$, is expressed using the preexponential factor, B_i, and the activation energy, E_i, as

$$k_{I1,E} = B_{I1,E} \exp(-E_{I1,E}/RT) \qquad [51]$$

These constants were estimated by fitting Equation [50] with three sets of the experimental results for the α-tocopherol oxidation.

The results are also shown in Figure 5.11 by the solid lines. Under all conditions, the calculated lines were in agreement with the experimental results. The estimated value of $k_{I1,E}$ is given as

$$k_{I1,E}\ (\text{mol}^{-1} \cdot \text{m}^3 \cdot \text{s}^{-1}) = 4.96 \times 10^{-1} \exp(-4.48 \times 10^4/RT) \qquad [52]$$

The activation energy of the kinetic constant concerned with the oxidation is in the range of 20 to 150 kJ/mol (Mill and Hendry 1980). Thus, the estimated value, 44.8 kJ/mol, was considered to be reasonable.

Estimation of Other Kinetic Constants. Similar to the previous sections, the kinetic constants for the reactions of β-carotene and α-tocopherol are expressed as

$$k_{I2,EA} = B_{I2,EA} \exp(-E_{I2,EA}/RT) \quad [53]$$

$$k_{I3,EA} = B_{I3,EA} \exp(-E_{I3,EA}/RT) \quad [54]$$

$$k_{IT,EA} = B_{IT,EA} \exp(-E_{IT,EA}/RT) \quad [55]$$

$$k_{R2,EA} = B_{R2,EA} \exp(-E_{R2,EA}/RT) \quad [56]$$

$$k_{R3,EA} = B_{R3,EA} \exp(-E_{R3,EA}/RT) \quad [57]$$

Equations [34] and [35] with five sets of the experimental results for the β-carotene oxidation in the presence of α-tocopherol were used for estimation.

The fitted and experimental results are shown in Figures 5.12 and 5.13. For reference, the fitted results according to the simple kinetic model based on Equations [1]–[7] and [21] (Appendix II) are also shown by the dotted lines. As noted above,

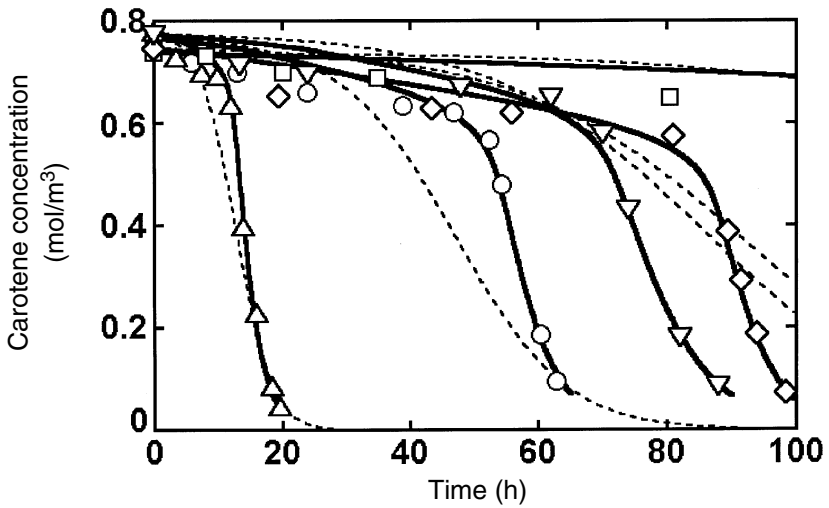

Fig. 5.12. Experimental data for β-carotene concentration and fitted results by the models: ○, 3.8×10^{-3}; ◇, 7.5×10^{-3} mol/m^3 of initial α-tocopherol concentration at 333 K and 40 mol% of oxygen composition; □, 323; △, 343 K at 40 mol% of oxygen composition and 3.8×10^{-3} mol/m^3 of initial α-tocopherol concentration; ▽, 20 mol% of oxygen composition at 333 K and 3.8×10^{-3} mol/m^3 of initial α-tocopherol concentration; solid lines, fitted results by our model based on Equations [1]–[7], [21]–[23], [25] and [27]–[33]; dotted lines, fitted results by simple model based on Equations [1]–[7] and [21].

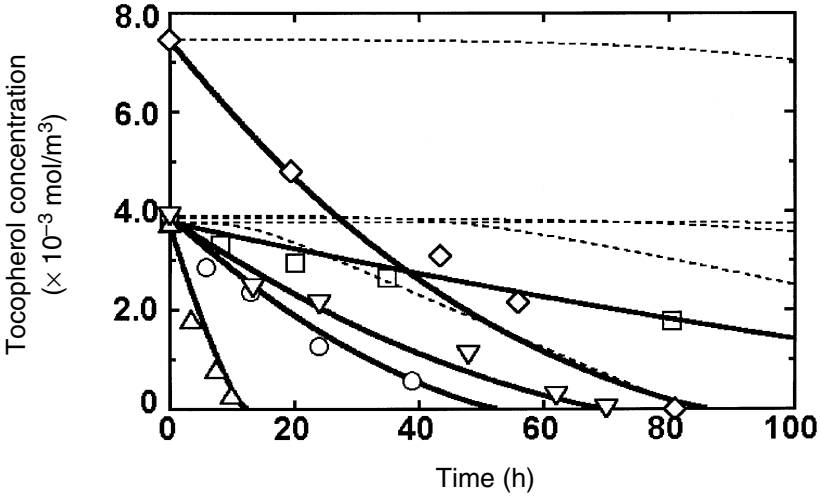

Fig. 5.13. Experimental data for α-tocopherol concentration and fitted results by the models: ○, 3.8×10^{-3}; ◇, 7.5×10^{-3} mol/m^3 of initial α-tocopherol concentration at 333 K and 40 mol% of oxygen composition; □, 323; △, 343 K at 40 mol% of oxygen composition and 3.8×10^{-3} mol/m^3 of initial α-tocopherol concentration; ▽, 20 mol% of oxygen composition at 333 K and 3.8×10^{-3} mol/m^3 of initial α-tocopherol concentration; solid lines, fitted results by our model based on Equations [1]–[7], [21]–[23], [25] and [27]–[33]; dotted lines, fitted results by simple model based on Equations [1]–[7] and [21].

the dotted lines cannot describe the slight decrease in the β-carotene concentration during the induction period and later the sharp decrease. The lines cannot describe the linear decrease in the α-tocopherol concentration. On the other hand, the solid lines representing our model were in much better agreement with the experimental results for the concentrations of both β-carotene and α-tocopherol.

The estimated values of each constant are listed in Table 5.3. The kinetic constants, $k_{R2,EA}$ and $k_{R3,EA}$, defined by Equations [40] and [41], were combined with

TABLE 5.3
Estimated Values of Constants in Our Model Based on Equations [1]–[7], [21]–[23], [25], and [27]–[33]

Constant	Preexponential factor B_i	Activation energy E_i (J/mol)
$k_{I2,EA}$	7.83×10^2 (mol^{-1} · m^3 · s^{-1})	2.61×10^4
$k_{I3,EA}$	7.77×10^{21} (mol^{-1} · m^3 · s^{-1})	1.29×10^5
$k_{IT,EA}$	8.91×10^6 (mol$^{-1/2}$ · m$^{3/2}$ · s$^{-1/2}$)	3.57×10^4
$k_{R2,EA}$	4.27 (–)	3.63×10^4
$k_{R3,EA}$	7.04×10^{12} (–)	1.35×10^5

the constants for various reactions, and hence the values cannot be compared with the literature values. The activation energy of the kinetic constant, $k_{IT,EA}$, defined by Equation [39] was reported to be in the range of 24–75 kJ/mol (Mill and Hendry (1980). In our study, this value was estimated to be 35.7 kJ/mol and was within each literature value range. There were no literature values for the activation energy of the kinetic constants, $k_{I2,EA}$, and $k_{I3,EA}$. However, the estimated values of 26.8 and 131 kJ/mol were within the range of the activation energy of the kinetic constant concerned with the oxidation, 20 to 150 kJ/mol (Mill and Hendry 1980). Therefore, these estimated values seemed to be reasonable.

Conclusion

The oxidation mechanism including co-oxidation of β-carotene in lipid solvent and antioxidation for the protection of β-carotene by antioxidant was proposed on the basis of the multistep autocatalytic free radical reaction. Not only the oxidation of β-carotene but also the oxidation of lipid and antioxidant was taken into consideration. Reactions between β-carotene and lipid or antioxidant involving radicals, having a high reactivity, were incorporated. In each oxidation mechanism, it was important to consider the secondary initiation reactions by decomposition of hydroperoxide and the reactions concerned with the resonance-stabilized radicals. Our models were constructed on the basis of the proposed reaction mechanism and quantitatively described the oxidation behavior of β-carotene over a wide range of temperatures, oxygen composition, lipid content, and initial antioxidant concentration. The estimated values of each model constant were verified to be reasonable by comparison with the literature values. The models can predict oxidation behavior in the practical system in which β-carotene, lipid, and antioxidant existed.

Appendix I

A simplified free radical reaction model proposed by Kasaikina *et al.* (1975 and 1981) consists of Equations [A-1]–[A-4]. Assuming steady state for the concentrations of the respective radicals in the system, the rates of change in the concentrations of β-carotene and its hydroperoxide are

$$\frac{dC_{AH}}{dt} = -k_{I1,A}C_{AH}C_{O_2} - k_{PT,A}C_{AH}\sqrt{r_{I,S}} \qquad [A\text{-}1]$$

$$\frac{dC_{AOOH}}{dt} = k_{PT,A}C_{AH}\sqrt{r_{I,S}} \qquad [A\text{-}2]$$

and the initial conditions are

$$t = 0;\ C_{AH} = C_{AH}(0);\ C_{AOOH} = C_{AOOH}(0) \qquad [A\text{-}3]$$

The initiation rate, $r_{I,S}$, and the kinetic constant, $k_{PT,A}$, in Equations [A-1] and [A-2] are

$$r_{I,S} = k_{I1,A} C_{AH} C_{O_2} \quad [A\text{-}4]$$

$$k_{PT,A} = \frac{k_{P2,A}}{\sqrt{2k_{T1,A}}} \quad [A\text{-}5]$$

The kinetic constants, $k_{I1,A}$ and $k_{PT,A}$, were estimated by fitting Equation [A-1] with eight sets of the experimental results for the β-carotene oxidation in *n*-decane as is the case for our model. The estimated values of each constant in the model proposed by Kasaikina *et al.* (1975 and 1981) are listed in Table 5-A.1.

Appendix II

In a simple kinetic model, the oxidation of β-carotene in the presence of α-tocopherol is considered to proceed by a series of mechanisms as shown in Equations [1]–[7] and [21]. Assuming steady state for the concentrations of the respective radicals in the system, the rates of change in the concentrations of β-carotene and α-tocopherol are

$$\frac{dC_{AH}}{dt} = -k_{I1,A} C_{AH} C_{O_2} - \frac{k_{PI,EA} C_{AH} r_{II,S}}{C_{EH}} \quad [A\text{-}6]$$

$$\frac{dC_{EH}}{dt} = -r_{II,S} \quad [A\text{-}7]$$

and the initial conditions are

$$t = 0;\ C_{AH} = C_{AH}(0),\ C_{EH} = C_{EH}(0) \quad [A\text{-}8]$$

The initiation rates, $r_{II,S}$, and the kinetic constant, $k_{PI,EA}$, in Equation [A-6] are given as

$$r_{II,S} = k_{I1,A} C_{AH} C_{O_2} + k_{I2,A} C_{AOOH} C_{O_2} \quad [A\text{-}9]$$

TABLE 5-A.1
Estimated Values of Constants in Model Proposed by Kasaikina *et al.* (1975 and 1981)

Constant	Preexponential factor B_i	Activation energy E_i (J/mol)
$k_{I1,EA}$	3.04×10^7 ($mol^{-1} \cdot m^3 \cdot s^{-1}$)	8.03×10^4
$k_{PT,A}$	1.59×10^4 ($mol^{-1/2} \cdot m^{3/2} \cdot s^{-1/2}$)	5.40×10^4

$$k_{PI,EA} = \frac{k_{P2,A}}{k_{Inh,EA}} \quad [A\text{-}10]$$

For the kinetic constants, $k_{I1,A}$ and $k_{I2,A}$, for the β-carotene oxidation, the estimated values listed in Table 5.2 were used. The kinetic constant, $k_{PI,EA}$, was estimated by fitting Equations [A-6] and [A-7] with five sets of the experimental results for the β-carotene oxidation in the presence of α-tocopherol as is the case for our model based on Equations [1]–[7], [21]–[23], [25] and [27]–[33]. The estimated value is given as

$$k_{P1,EA}\,(-) = 1.06 \times 10^7 \exp(-3.71 \times 10^4/RT) \quad [A\text{-}11]$$

References

Alekseev, E.V., Gagarina, A.B., Etveeva, N.M., Vakulova, L.A., Samokhvalov, G.I., and Emanuel, N.M. (1968) Kinetic Principles of the Oxidation of Polyenic Hydrocarbons. Communication 1. Decomposition of β-Carotene in the Presence of Free Radical Initiators, *Izv. Akad. Nauk USSR, Ser. Khim. 11*, 2474–2480.

Arya, S.S., Natesan, V., Psrihar, D.B., and Vijayaraghavan, P.K. (1979) Stability of Beta-Carotene in Isolated Systems, *J. Food Technol. 14*, 571–578.

Bateman, L. (1954) Olefin Oxidation, *Q. Rev. 8*, 147–167.

Battino, R. (1981) Oxygen and Ozone. Hydrocarbons, *Solubility Data Series 7*, 219.

Britton, G. (1995) Structure and Properties of Carotenoids in Relation to Function, *FASEB J. 9*, 1551–1558.

Budowski, P., and Bondi, A (1960) Autooxidation of Carotene and Vitamin A. Influence of Fat and Antioxidant, *Arch. Biochem. Biophys. 89*, 66–73.

Burton, G.W., and Ingold, K.U. (1984) β-Carotene: An Unusual Type of Lipid Antioxidant, *Science 224*, 569–573.

Chou, H., and Breene, W. (1972) Oxidative Decoloration of Beta-Carotene in Low-Moisture Model System, *J. Food Sci. 37*, 66–68.

Denisov, E.T., and Khudyakov, I.V. (1987) Mechanisms of Action and Reactivities of the Free Radicals of Inhibitors, *Chem. Rev. 87*, 1313–1357.

Encinar, J.M., Beltran, F.J., and Frades, J.M. (1993) Liquid Phase Oxidation of α-Pinene Initiated by Ozone. 1. Conversion of α-Pinene and Formation of Its Hydroperoxide, *Chem. Eng. Technol. 16*, 68–74.

Encinar, J.M., Beltran, F.J., and Frades, J.M. (1994) Liquid Phase Oxidation of α-Pinene Initiated by Ozone. 2. Formation of Verbenol, Verbenone and Acid Products, *Chem. Eng. Technol. 17*, 187–194.

Foote, C.S., and Denny, R.W. (1968) Chemistry of Singlet Oxygen. VIII. Quenching by β-Carotene, *J. Am. Chem. Soc. 90*, 6233–6235.

Handelman, G.J., van Kuuk, F.J.G.M., Chatterjee, A., and Krinsky, N.I. (1991) Characterization of Products Formed During the Autoxidation of β-Carotene, *Free Radic. Biol. Med. 10*, 427–437.

Henry, L.K., Catignani, G.L., and Schwartz, S.J. (1998) Oxidative Degradation Kinetics of Lycopene, Lutein, and 9-*cis* and all-*trans* β-Carotene, *J. Am. Oil Chem. Soc. 75*, 823–829.

Ito, Y. (1987) Knowledge for β-Carotene, *New Food Ind. 29*, 22–29.

Iwatsuki, M., Tsuchiya, J., Komuro, E., Yamamoto, Y., and Niki, E. (1994) Effects of Solvents and Media on the Antioxidant Activity of α-Tocopherol, *Biochem. Biophys. Acta 1200*, 19–26.

Kasaikina, O.T., Gagarina, A.B., and Emanuel, N.M. (1975) Reactivity of β-Carotene in the Interaction with Free Radicals, *Izv. Akad. Nauk USSR, Ser. Khim. 10*, 2243–2246.

Kasaikina, O.T., Kartasheva, Z.S., and Gagarina, A.B. (1981) Polyene Compounds as Free-Radical Acceptors, *Izv. Akad. Nauk USSR, Ser. Khim. 3*, 536–540.

Kennedy, T.A., and Liebler, D.C. (1992) Peroxyl Radical Scavenging by β-Carotene in Lipid Bilayers. Effect of Oxygen Partial Pressure, *J. Biol. Chem. 267*, 4658–4663.

Kortenska, D.K., and Yanishlieva, N.V. (1995) Effect of the Phenol Antioxidant Type on the Kinetics and Mechanism of Inhibited Lipid Oxidation in the Presence of Fatty Alcohols, *J. Sci. Food Agric. 68*, 117–126.

Lampi, A.-M., Kataja, L., Kamal-Eldin, A., and Vieno, P. (1999) Antioxidant Activities of α- and γ-Tocopherols in the Oxidation of Rapeseed Oil Triacylglycerols, *J. Am. Oil Chem. Soc. 76*, 749–755.

Lea, C.H. (1931) The Effect of Light on the Oxidation of Fats, *Proc. Soc. London Ser. B. 108,* 175–189.

Marinova, E.M., and Yanishlieva, N.V. (1992) Effect of Temperature on the Antioxidative Action of Inhibitors in Lipid Autoxidation, *J. Sci. Food Agric. 60*, 313–318.

Mill, T., and Hendry, D.G. (1980) Kinetics and Mechanisms of Free Radical Oxidation of Alkanes and Olefins in the Liquid Phase, in *Comprehensive Chemical Kinetics* (Bamfold, C.H., and Tipper, C.F.H., eds.), Vol. 16, pp. 34–81, Elsevier, Amsterdam.

Nagaoka, S., Kuranaka, A., Tsuboi, H., Nagashima, U., and Mukai, K. (1992) Mechanism of Antioxidant Reaction of Vitamin E. Charge Transfer and Tunneling Effect in Proton-Transfer Reaction, *J. Phys. Chem. 96*, 2754–2761.

Nelder, J.A., and Mead, R. (1964) A Simplex Method for Function Minimization, *Comput. J. 7*, 308–313.

Ozhogina, O.A., and Kasaikina, O.T. (1995) β-Carotene as an Interceptor of Free Radicals, *Free Radic. Biol. Med. 19*, 575–581.

Palozza, P., Luberto, C., and Bartoli, G.M. (1995) The Effect of Fatty Acid Unsaturation on the Antioxidant Activity of β-Carotene and α-Tocopherol in Hexane Solutions, *Free Radic. Biol. Med. 18*, 943–948.

Papadopoulou, K., and Ames, J.M. (1994) Kinetics of all-*trans*-β-Carotene Degradation on Heating With and Without Phenylalanine, *J. Am. Oil Chem. Soc. 71*, 893–896.

Ramakrishnan, T.V., and Francis, F.J. (1979a) Stability of Carotenoids in Model Aqueous Systems, *J. Food Qual. 2*, 177–189.

Ramakrishnan, T.V., and Francis, F.J. (1979b) Coupled Oxidation of Carotenoids in Fatty Acid Esters of Varying Unsaturation, *J. Food Qual. 2*, 277–287.

Stirling, C.J.M. (1965) *Radicals in Organic Chemistry*, p. 14, Oldbourne Press, London.

Takahashi, A., Shibasaki-Kitakawa, N., and Yonemoto, T. (1999) Kinetic Model for Autoxidation of β-Carotene in Organic Solutions, *J. Am. Oil Chem. Soc. 76*, 897–903.

Takahashi, A., Shibasaki-Kitakawa, N., and Yonemoto, T. (2000) Kinetic Analysis for Oxidation of Oleic Acid, *J. Chem. Eng. Jpn. 33*, 481–488.

Takahashi, A., Suzuki, J., Shibasaki-Kitakawa, N., and Yonemoto, T. (2001) A Kinetic Model for Co-Oxidation of β-Carotene with Oleic Acid, *J. Am. Oil Chem. Soc. 78*, 1203–1207.

Takahashi, A., Shibasaki-Kitakawa, N., and Yonemoto, T. (2003) A Rigorous Kinetic Model for β-Carotene Oxidation in the Presence of an Antioxidant, α-Tocopherol, *J. Am. Oil Chem. Soc.* in press.

Tanaka, Y. (1995) Physical Property and Function of Palm Carotene, *New Food Ind. 37*, 59–70.

Terao, J. (1989) Antioxidant Activity of β-Carotene-Related Carotenoids in Solution, *Lipids 24*, 659–661.

Terao, J., and Matsushita, S. (1986) The Peroxidizing Effect of α-Tocopherol on Autoxidation of Methyl Linoleate in Bulk Phase, *Lipids 21*, 255–260.

Toro-Vazquez, J.F., Castillo-M, A.A., and Hernandez-C, R. (1993) A Multiple-Variable Approach to Study Corn Oil Oxidation, *J. Am. Oil Chem. Soc. 70*, 261–267.

Tsuchihashi, H., Kigoshi, M., Iwasaki, M., and Niki, E. (1995) Action of β-Carotene as an Antioxidant against Lipid Peroxidation, *Arch. Biochem. Biophys. 323*, 137–147.

Chapter 6

Glycerophospholipid Core Aldehydes: Mechanism of Formation, Methods of Detection, Natural Occurrence, and Biological Significance

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Introduction

The investigation of glycerophospholipid (GPL) peroxidation is a rapidly growing field in medicine and biology, encouraged by increasing evidence that lipid oxidation is involved in the pathogenesis of many chronic diseases including atherosclerosis, Alzheimer's disease, Parkinson's disease, stroke, and aging (Spiteller 1998). GPL are major components of biological membranes and lipoproteins. A vast array of aldehydes is produced by oxidative cleavage of polyunsaturated GPL (Esterbauer *et al.* 1991). The short-chain alkanals and alkenals and their hydroxy or epoxy derivatives have been isolated and fully characterized, including malonaldehyde (MDA), hydroxynonenal, and hydroxyhexenal (Frankel 1982, 1985 and 1999). Much less is known about the nonvolatile products of lipid peroxidation that remain esterified in GPL (Kuksis 1990, Pokorny 1984). Recent studies have shown that oxidative cleavage of the arachidonic acid ester of glycerophosphocholine (GroPCho) generates 5-oxovaleric acid ester of GroPCho, which mimics the platelet-activating factor (PAF) (Stremler *et al.* 1989 and 1991) that activates endothelial cells to bind monocytes (Watson *et al.* 1997). Despite the high molecular weight, core aldehydes generated by cleavage of the phospholipids avidly bind covalently with proteins, leading to protein modifications that may interfere with their biological function (Kaur *et al.* 1997, Kuksis 2000, Ravandi *et al.* 1997). Covalent binding of the core aldehydes to the apoproteins of both low density (LDL) and high density lipoproteins (HDL) apparently facilitates the entry of monocytes into vascular vessels, an event believed to be important in the development of atherosclerosis (Ahmed *et al.* 2003b).

By 1990, Myher *et al.* (1989a and 1989b) had published an essentially complete account of the composition of the molecular species of the GPL of plasma lipoproteins and red blood cells and identified the oxygenation products of intact lipid esters as a remaining analytical challenge. Grzelinska *et al.* (1979) had already called attention to the core aldehydes of GroPCho as potential modifiers of the structure and function of lipoproteins and cell membranes, but no specific

oxophospholipid species were isolated or identified. In fact, until recently, there was no methodology available for the isolation of oxoesters from natural sources, and there was a general lack of commercial standards for their identification. Before 1990, lipid ester core aldehydes were mentioned only in passing and largely as theoretically possible secondary products of lipid peroxidation. Unlike the volatile aldehydes, which have been studied extensively, the core aldehydes were ignored and regarded as being of too low reactivity to be bothered with in biological assays.

The situation has changed during the last 10 years, accompanying development of superior methods for the preparation of standards, for the isolation and quantification of both neutral and polar lipid ester core aldehydes and other oxo-derivatives from natural sources, and for the subfemtomolar identification and quantification by immunochemical techniques of the core aldehydes of GroPCho. Moreover, several physiologic activities were demonstrated, including similarity to the PAF. Other studies have demonstrated that both C_5 and C_9 core aldehydes possess sufficient chemical reactivity to form Schiff bases with amino phospholipids, amino acids, and polypeptides. As a result, the core aldehydes along with other high-molecular-weight oxidation products of lipid esters, such as isoprostanes, are now considered to be chemical modifiers of various enzyme and receptor systems, as well as gene activators, although only a few specific instances have thus far been identified.

Preparation of Reference Core Aldehydes

To identify the lipid ester core aldehydes among the oxidation products of natural GPL, it was essential to synthesize reference standards for their characterization by chromatographic and mass spectrometric (MS) methods. The core aldehydes of diacylglycerols (DAG), triacylglycerols (TAG), and of phosphatidylcholine (PtdCho) have been prepared with high efficiency by ozonolysis and reductive cleavage during structural investigations of natural glycerolipids by Privett and Blank (1963), who also introduced the term "core aldehydes." This method has been employed extensively in more recent investigations.

Stremler *et al.* (1989) ozonized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (50 mg, 64 μmol) or the 2-[1–^{14}C]-labeled compound (10 μCi, 0.182 μmol) at –78°C in dichloromethane. After a reductive workup, solvent and excess dimethylsulfide were removed under nitrogen flow. The resulting residue was purified by reversed-phase high-performance liquid chromatography (HPLC) using a 5-μm Ultrasphere column (25 cm × 0.46 cm i.d.) and methanol/water/acetonitrile (930:70:50, by vol) containing 20 mM choline chloride as the developing solvent (Brash *et al.* 1987). The product was shown to be 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine by the following properties: thin-layer chromatography (TLC; R_f = 0.28, twofold development, chloroform/methanol/acetic acid/water, 90:10:0.5:0.5, by vol); appropriate spectra upon examination by infrared (IR), ultraviolet (UV) (λ max = 243); ^{1}H nuclear magnetic resonance (NMR), ^{31}P NMR, and ^{13}C NMR spectroscopy; and characteristic ions by MS.

Boechzelt *et al.* (1998 and 1999) reported the use of dimethylsulfide as the reducing and dimethoxylating reagent for the production of short-chain dimethoxy fatty acids from ozonides. The dimethylacetals can be condensed with acylglycerols or lysophospholipids to yield the dimethoxy derivatives of the corresponding core aldehydes. The aldehydes are regenerated by passage through an ion exchange column (Amberlist-15). This procedure is suitable for the preparation of core aldehydes of glycerolipids containing unsaturation sites that would otherwise be destroyed upon direct ozonization.

Ravandi *et al.* (1995a) prepared core aldehydes by reductive ozonization from synthetic and natural GPL of known structure. The ozonization was performed as originally described by Privett and Blank (1963), except that a Tesla coil described by Beroza and Bieri (1969) was used. The lipid ester (20–30 mg) was dissolved in 4 mL of chloroform/methanol (2:1, vol/vol) and cooled in a dry ice/acetone bath for 10 min. O_2 gas containing 3–4% ozone was then bubbled through the solution at a rate of 150 mL/min for 5 min. The appearance of a faint blue color indicated the saturation of the dichloromethane solution with ozone, at which time the reaction was stopped. The hexane was evaporated under nitrogen and the residue redissolved in chloroform. The ozonides of PtdCho and phosphatidylethanolamine (PtdEtn) were isolated by TLC on Silica Gel H using a polar solvent system made up of chloroform/methanol/acetic acid/water (75:45:12:6, by vol) (Skipski and Barclay 1969). Triphenylphosphine (20 mg) was added to the ozonides (10–20 mg in 4 mL chloroform) and the reaction mixture was kept at room temperature for 1 h (Beroza and Bierl 1969, Privett and Blank 1963). At the end of this time, the aldehyde and any unreacted ozonides were recovered from the chloroform solution and resolved by TLC on silica gel H using a polar solvent system (see above). The aldehyde bands were located by spraying the plate with Schiff reagent, which gave a purple color. The phospholipid aldehydes were recovered by extraction of the gel with chloroform/methanol/water (65:25:4, by vol) (Kuksis and Marai 1967). Both palmitoyl and stearoyl homologs are obtained for each of the aldehydes. The aldehyde peaks eluted over a period of 14–16 min under the HPLC conditions and were retained longer than the parent PtdCho, which emerged over a period of 11–13 min, and were well resolved from the core acids, which emerged later (15–16 min). The core acids were made up of the ω-carboxy homologs corresponding to the core aldehydes and had resulted from further oxidation of the core aldehydes.

Part of the aldehyde preparation was converted into the dinitrophenylhydrazone (DNPH) derivatives by reaction with dinitrophenylhydrazine in the dark (0.5 mg in 1 mL of 1 N HCl) for 2 h at room temperature and 1 h at 4°C (Esterbauer *et al.* 1990). The DNPH derivatives were extracted with neutral chloroform/methanol (2:1, vol/vol) (Kamido *et al.* 1992b). The PtdEtn aldehydes should be reacted without delay to protect the aldehyde groups from forming N=C bonds by interacting with the ethanolamine head group. The DNPH derivatives of the phospholipid core aldehydes were purified by rechromatography in the phospholipid solvent system. For

the preparation of DAG containing the aldehyde cores and their hydrazones, which can also be used for identification of the molecular species, the core aldehydes of PtdCho and PtdEtn were hydrolyzed with phospholipase (PL)C (*Bacillus cereus*) to release the polar head groups as described by Kamido *et al.* (1992b). The hydrolysis products were recovered by extraction with chloroform and were immediately trimethylsilylated for gas–liquid chromatography (GLC) (Myher and Kuksis 1984). The hydrazones were analyzed without further derivatization by HPLC with UV detection (Kamido *et al.* 1992b) or by reversed-phase LC/MS with thermospray ionization (Kuksis *et al.* 1993). All of the analyzed derivatives provided consistent quantitative proportions of the core aldehydes of egg yolk PtdCho and PtdEtn identified as the mono-C_5 and mono-C_9 aldehydes of palmitoyl- and stearoyl-GPL. Friedman *et al.* (2002) concluded that the core aldehydes of PtdEtn and PtdSer undergo rapid internal cyclization or polymerization because they do not bind the EO6 antibody.

Originally Kamido *et al.* (1992b) prepared glycerolipid-bound aldehydes by oxidation with osmium tetroxide followed by periodic acid cleavage. The oxoalkanoates were prepared by adding osmium tetroxide in dioxane (10 mg/mL, 0.2 mL) to a solution of the unsaturated phosphatide (1.5 mg in 0.2 mL pyridine/dioxane, 1:8, vol/vol). After 2 h at room temperature, 6 mL of a suspension of sodium sulfite (made by adding 8.5 mL of 16% sodium sulfite to 2.5 mL of methanol) was added and mixed. After another 2 h at room temperature, the mixture was centrifuged and lipids were extracted from the residue with chloroform/ methanol (2:1, vol/vol). The lipid extracts containing the alcohols were evaporated under nitrogen and dissolved in 0.5 mL of 95% ethanol. To this solution, 0.5 mL of 1% periodic acid in 95% ethanol was added, mixed and left at room temperature for 1 h. The mixtures were diluted with 2 mL of chloroform and washed three times with water. The lower chloroform layer containing the phospholipid core aldehydes was evaporated under nitrogen, and the aldehydes were purified by TLC on silica gel H using chloroform/methanol/water (65:35:6, by vol) as the developing solvent. The lipid areas were located by spraying the plate with 2,7-dichlorofluorescein or with Schiff reagent (Skipski and Barclay 1969). The fluorescent silica gel zones were scraped off the plate and the lipids were eluted with chloroform/methanol (2:1, vol/vol). The phospholipid extract was reduced to small volume and hydrolyzed with PLC for 1 h at 37°C (Kuksis *et al.* 1969). The DAG core aldehydes were recovered by solvent extraction and characterized by various chromatographic and mass spectrometric methods. The 1-palmitoyl-2-(9-oxononanoyl)GroPEtn was prepared as described for the corresponding PtdCho-derived core aldehyde, except that the PtdEtn was first converted to the dinitrophenyl (DNP) derivative by treatment with 1-fluoro-2,4-dinitrobenzene to avoid interaction between the amino group and the aldehyde function to be introduced. The core aldehyde resulting from the osmium oxidation and periodate cleavage of PtdEtn DNP was purified by TLC using chloroform/acetone/methanol/ acetic acid/water (50:20:10:10:5, by vol).

Osmium tetroxide/periodic acid oxidation of the DNP derivative of synthetic 16:0/18:1 GroPEtn gave two bands on TLC, i.e., one corresponding mainly to the core aldehyde (R_f 0.53) and another one (R_f 0.60) corresponding to the diethyl acetal of the core aldehyde (Kamido *et al.* 1992b). The ion at *m/z* 796 represented $[M+Na]^+$ of the free aldehyde molecule, whereas those at *m/z* 870 and 865 represented the $[M+Na]^+$ and $[M+NH_4]^+$ ions, respectively. The *m/z* 824 ion is due to loss of ethanol from the diethyl acetal group of $[M+Na]^+$, whereas the *m/z* 802 ion is due to $[M+H–C_2H_5OH]^+$. The presence of the DNP group on the ethanolamine moiety of PtdEtn allowed UV monitoring of the presence of nonaldehyde peaks formed as minor by-products in the oxidation reaction, which were identified as the acids and their ethyl esters of the core aldehydes.

Wang and Tai (1990) used a multistep synthesis of an aldehydic analog of PAF with a reactive aldehyde group at the ω-end of the *sn*-1-position. A critical step involved ozonization of a monounsaturated fatty acid in the *sn*-1-position to yield the 9-oxo-nonanoyl core aldehyde. Kern and Spiteller (1996) reported the synthesis and properties of naturally occurring α-hydroxy-aldehydes. Deng and Salomon (2000) reported the synthesis of the (11*E*)-9-hydroxy-13-oxotrice-11-enoate ester of 2-lysoPtdCho. A lactone, 8-(3-oxo-1H,6H-2-oxinyl)octanoic acid, believed to be generated through an intermediate (11*E*)-9-hydroxy-13-oxotridec-11-enoic acid, was produced upon oxidation of linoleic acid. The synthesis of the methyl ester of the lactone was accomplished from the intermediate by a novel *trans-cis* isomerization that was driven to completion by cyclization to a hemiacetal.

Mechanism of Formation of Primary and Secondary Products

Biological oxidation of lipoprotein and membrane phospholipids is believed to be primarily a result of a chemical reaction. Porter *et al.* (1980) reported that the peroxidation products, 1–4 (Fig. 6.1), that form when a neat film of 1-palmitoyl-2-linoleoyl GroPCho is incubated in an atmosphere of air at 37°C, originate from linoleoyl side-chain oxidation. The mechanism by which the four peroxidation products of linoleate form was described in detail by Porter *et al.* (1981 and 1995) and was summarized by Milne and Porter (2001). Initially, hydrogen atom extraction at the C_{11} position on the linoleoyl side chain yields a pentadienyl radical. A molecule of oxygen then adds to this stable radical at either the C_9 or the C_{13} positions to give *cis,trans* peroxyl radicals; in the presence of a good hydrogen atom donor (such as α-tocopherol), these will be trapped as the *cis,trans* hydroperoxides 2 and 1, respectively. In the absence of hydrogen atom donors, the *trans,trans* hydroperoxides 3 and 4 form (Fig. 6.1). The ratio of *cis,trans* to *trans,trans* products formed in a reaction mixture is a good measure of the competition between hydrogen atom abstraction from hydrogen donor molecules and β-fragmentation.

The oxidation of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine yields six hydroperoxides in the presence of a good hydrogen donor (Milne and Porter 2001). The main mechanism for the formation of aldehydes from lipid per-

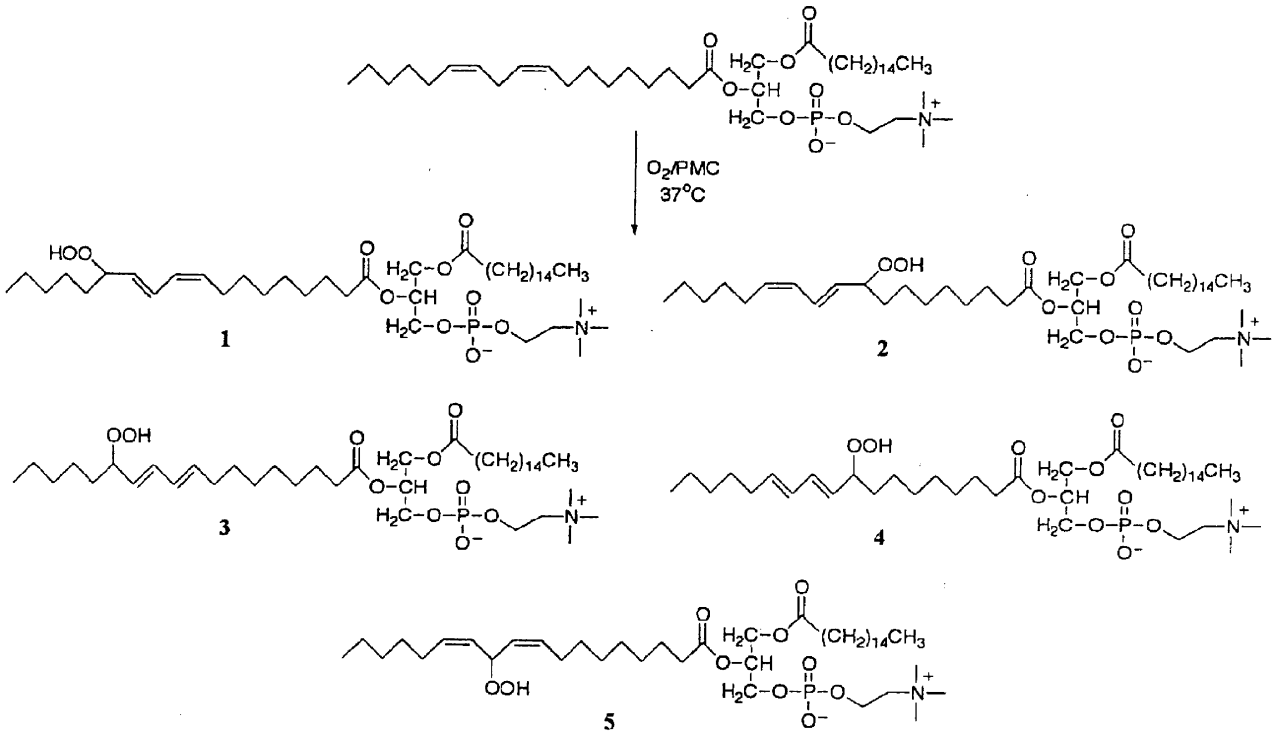


Fig. 6.1. Oxidation products of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine formed in the presence of 0.1 equivalents of pentamethylchromanol (hydrogen donor). In the presence of a good hydrogen donor, the *cis,trans* peroxyradicals are trapped as *cis,trans*-hydroperoxides (1 and 2); in the presence of large amounts of hydrogen donor, the 11-hydroperoxide (5) is obtained. In the absence of a hydrogen donor, the *trans,trans* hydroperoxides (3,4) are obtained. Reproduced from Milne and Porter (2001) with permission of the publisher.

oxides follows hemolytic scission (β-cleavage) of the two C-C bonds on either side of the hydroperoxy group (Gardner 1989). Applying this mechanism to the linoleate ester of GroPCho, it would be anticipated that the cleavage of the carbon-carbon bond would result in aldehydes derived from the methyl terminus of the fatty chain and of aldehydes still bound to the parent lipid molecule. The major core aldehydes derived from 9- and 13-hydroperoxides of linoleates would, therefore, be expected to be 8-nonanoic acid and 9-oxononanoyl-, (12-oxo)-9,10-doedecenoyl, and (13-oxo)-9,11-tridecadienoyl GroPCho. Figure 6.2 shows the proposed formation of the major core aldehydes from linoleoyl GroPCho attributable to peroxidation. More complex schemes for the generation of core aldehydes from linoleoyl esters have been discussed by Sjovall *et al.* (2002).

Peroxidation of the arachidonoyl GroPCho would lead to formation of the monohydroperoxides at positions 5, 7, 9, 11, 13, and 15 (Milne and Porter 2001), which would yield the saturated (5-oxo)valeroyl GroPCho as the major core aldehyde product, with the unsaturated core aldehydes undergoing further oxidation to produce complex mixtures of minor core aldehydes as demonstrated by Kamido *et al.* (1992b). The oleoyl GroPCho would be anticipated to yield (8-oxo)octanoyl and (11-oxo)-9,10-undecenoyl GroPCho, as well as (9-oxo)nonanoyl and (10-oxo)-8,9-decenoyl GroPCho. This is because it has been shown that 9- and 10-hydroperoxides are formed in amounts approximately similar to those of the 8- and 11-hydroperoxides in the oxidation of methyl oleate (Frankel 1984 and 1985, Porter *et al.* 1995). The formation of the core aldehydes is accompanied by the formation of small amounts of the corresponding core acids, which result from further oxidation of the aldehydes.

Girotti (1998) reviewed the important routes of lipid hydroperoxide (LOOH) formation and turnover in oxidatively challenged cells. Reactive oxygen species such as $^1O_2$ generated by photodynamic action (Sens/hv) or $HO^\bullet$ generated by Fenton chemistry (H_2O_2/iron) give rise to primary stage peroxyl radicals ($LOO^\bullet$) (Fig. 6.3). These $LOO^\bullet$ may undergo iron-mediated one-electron reduction and oxygenation to give epoxyallylic peroxyl radicals ($OLOO^\bullet$), which trigger exacerbating rounds of free radical–mediated lipid peroxidation. For $^1O_2$ systems, radical chemistry would start here, whereas for $HO^\bullet$ systems, radical chemistry would continue to be propagated. Alternatively, lipid hydroperoxides (LOOH) may undergo two-electron reduction to redox-inert alcohols (LOH); these reactions are typically catalyzed by glutathione (GSH)-dependent selenoperoxidase(s) (SePX), most prominently phospholipid hydroperoxide glutathione peroxidase (PHGPX). As shown in Figure 6.3, involvement of reduced oxygen species in lipid peroxidation may commence with the reduction or dismutation of metabolically or photochemically generated O_2^- to H_2O_2· Fenton-type reduction of H_2O_2 by suitably chelated iron (e.g., membrane-associated Fe^{2+}) produces $HO^\bullet$, which can trigger chain peroxidation by abstracting allylic hydrogens from proximal unsaturated lipids (LH). For phospholipids, these hydrogens would typically come from the *sn*-2-fatty acyl groups. Rapid addition of $^3O_2$ to the resulting lipid alkyl radicals ($L^\bullet$)

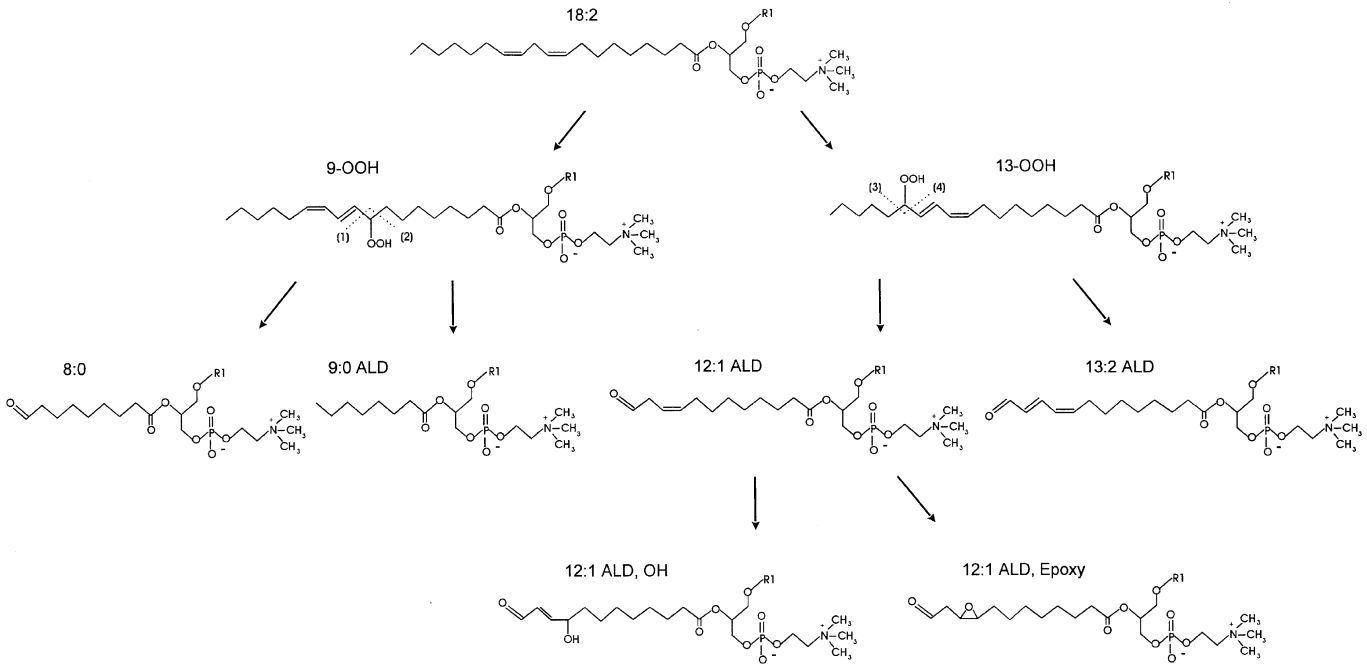


Fig. 6.2. Postulated formation of core aldehydes from 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine. Positions of cleavage of the fatty acid chain of hydroperoxides are numbered 1 to 3. Modified from Sjovall *et al.* (2001) with permission of the publisher.

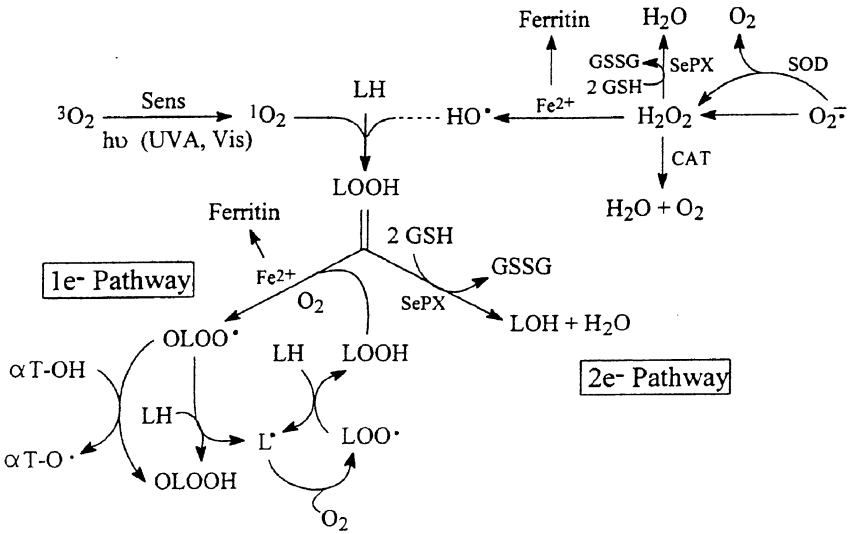


Fig. 6.3. Postulated routes of lipid hydroperoxide (LOOH) formation and turnover in oxidatively challenged cells. ROS such as $^1O_2$ generated by photodynamic action (Sens/hv) or HO• generated by Fenton chemistry (H_2O_2/iron) give rise to primary stage LOOH. These LOOH may undergo iron-mediated one-electron reduction and oxygenation to give epoxyallylic peroxy radicals (OLOO•), which trigger exacerbating rounds of free radical–mediated lipid peroxidation. Alternatively, LOOH may undergo two-electron reduction to redox-inert alcohols (LOH). GSH, glutathione; SePX, GSH-dependent selenoperoxidase; CAT, catalase; SOD, superoxide dismutase; α-TOH, α-tocopherol; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide. Reproduced from Girotti (1998) with permission of the publisher.

propagates the reaction *via* LOO• intermediates, with concomitant formation of LOOH species.

Girotti (1998) points out that a similar overall mechanism would apply for ONOOH• or O_3-induced lipid peroxidation. In the case of ONOOH, homolytic decomposition gives HO• and O_2N•, either of which could act as an H-abstracting initiator, although an activated isomer of ONOOH with properties similar to HO• has been suggested as a more probable effector. Ozone can add across an unsaturated fatty acid double bond to give a 1,2,3-trioxolane, which on O-O homolysis gives an oxygen-centered diradical that may undergo β-scission to split out a carbon-centered radical or rearrange to a carbonyl oxide leading to radicals *via* β-scission. In contrast to HO• or ONOOH, $^1O_2$ can react directly with unsaturated fatty acyl groups to give LOOH with double bonds shifted to the allylic position. This is an example of the "ene" reaction of $^1O_2$ with olefins in which all atoms of the hydroperoxyl group derive from $^1O_2$ and the target lipid. This contrasts with a free radical–generated hydroperoxyl group, which derives from $^3O_2$ and another H-donating

lipid. Girotti (1998) points out that many articles and reviews continue to give the impression that all lipid peroxidation reactions are free radical in nature, not withstanding the fact that $^1O_2$-mediated peroxidation does not involve free radicals *per se*. Interestingly, Mashima *et al.* (1998) reported the reduction of PtdCho hydroperoxide by apolipoprotein (apo)AI and purification of the hydroperoxide-reducing protein from human plasma.

Spiteller (1998) proposed a cascade of reactions to accommodate the early formation of 13-oxotrideca-9,11-dienoic acid and ultimate generation of 9-oxononanoic acid through Michael addition of water to the conjugated dienal. The resulting hydroxylated nonenal gives nonanal through retro-aldol fragmentation, and lactone through further oxidation and lactonization. With completion of the synthesis of (11*E*)-9-hydroxy-13-oxotridec-11-enoate (HOT), HOT 2-lysoPtdCho (HOT-PtdCho), and lactone 1 by Deng and Salomon (1998 and 2000) and the dienal and the derived phospholipid to be reported elsewhere (Salomon *et al.*, unpublished), all of the compounds in the putative cascade as well as the corresponding phospholipids have now been synthesized. However, the natural formation of the various oxidized phospholipids upon oxidation of linoleoyl GroPCho and determination of their natural occurrence remain to be demonstrated.

Free radical oxidation of arachidonoyl GroPCho *in vitro* also yields prostaglandin (PG)-like structures (Morrow *et al.* 1990a) as was first observed for free arachidonic acid (Porter and Funk 1975). *In vivo* isoprostanes, which are initially formed in cell membranes at the site of free radical attack and then cleaved by phospholipases, circulate until they are excreted in urine as isomers of PGF_2 (Morrow *et al.* 1990b). These compounds, termed F_2-isoprostanes, possess a 1,3-dihydroxycyclopentane ring (PGF ring) with hydroxyls mainly in the *syn* configuration. Lawson *et al.* (1999) briefly reviewed the two routes of peroxidation of arachidonates (Fig. 6.4), which are believed to lead to isoprostane formation. In the endoperoxide mechanism, the first oxygen molecule is incorporated into the endoperoxide ring to form the two hydroxyl groups on the PGF ring. In the dioxetane/endoperoxide mechanism, by contrast, it is the second oxygen molecule that is incorporated into the PGF ring. Also 5- and 15-hydroperoxy radicals can form Groups VI and III only by the dioxetane/endoperoxide mechanism. The radical at position 10 of arachidonic acid, by contrast, can yield isoprostanes by both mechanisms. Thus, hydroperoxy radicals formed at 8 and 12 have the option to proceed to form a dioxetane ring or a dioxypentane ring on a competitive basis, although it is not yet clear which is favored. Other compounds may be derived from a 9-hydroperoxy radical by the endoperoxide mechanism or from a 5-hydroperoxyl radical by the dioxetane/endoperoxide mechanism, both of which are derived from an initial hydrogen atom abstraction at position 7 of arachidonic acid. Abstraction at carbon 13 can give rise to 11- and 15-hydroperoxy radicals, yielding only Group III isoprostanes. A radical at position 10 of arachidonic acid rearranges to a radical at 8 or 12, which yield groups V and IV, respectively. Compounds analogous to the F_2-isoprostanes are formed from other fatty acid substrates, e.g., eicosapentaenoic acid (EPA) and

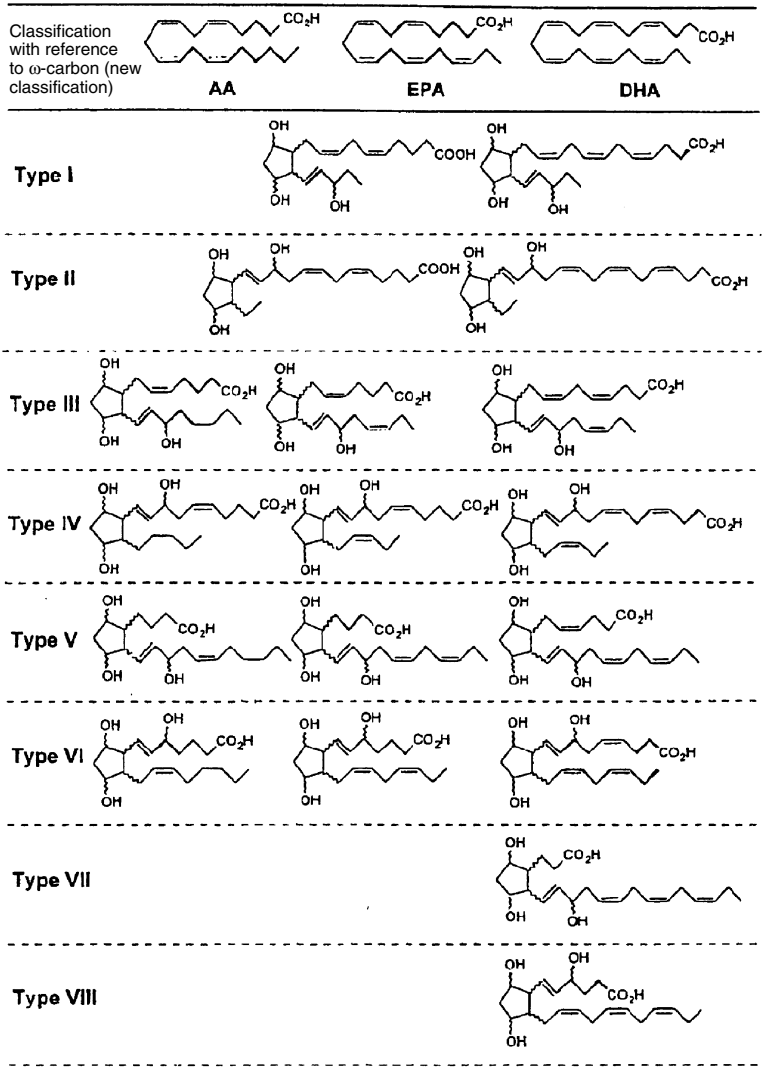


Fig. 6.4. Structural comparison of isoprostanes from arachidonic, eicosapentaenoic, and docosahexaenoic acids. Reproduced from Lawson *et al.* (1999) with permission of the publisher.

docosahexaenoic acid (DHA) (Roberts *et al.* 1998). The isoprostane-containing GroPCho are readily recognized among the peroxidation products of polyunsaturated GroPCho (see below).

It may be noted that linolenic acid–containing GroPCho would be anticipated to yield phytodienoic acid esters, which are precursors of jasmonic acid, a substi-

tuted pentenylcyclopentane derivative (Stelmach *et al.* 2001). Burke *et al.* (2000) indicated that 2,3-*dinor*-5,6-dihydro-isoprostane $F_{2\alpha}$-III was a metabolite of isoprostane $F_{2\alpha}$-III and an oxidation product of γ-linolenic acid (GLA). Nourooz-Zadeh *et al.* (1997) presented evidence for the formation of F_3-isoprostanes during peroxidation of EPA. Two classes of F_1 isoprostanes, each composed of eight isomers, are theoretically possible (Rokach *et al.* 1997). Reich *et al.* (2000) reported the formation of the novel D-ring and E-ring and Fam *et al.* (2002) the highly reactive A-ring and J-ring isoprostane-like compounds *in vitro* and *in vivo* from DHA.

Methods of Detection and Quantification

Hydroperoxides

The extent of lipid peroxidation has been quantified by measuring various analytes, including LOOH, in terms of peroxide value (Helbock *et al.* 1993), conjugated dienes (Ahotupa *et al.* 1998, Holley *et al.* 1993), expired hydrocarbons (Cailleux and Allain 1993), and/or aldehydes (Yeo *et al.* 1994). The most direct methods for assessing lipid peroxidation are the measurement of lipid hydroperoxides by iodometric titration and spectrophotometric analysis of conjugated dienes, but these are comparatively insensitive techniques. A sensitive method involving HPLC with isoluminol chemiluminescence detection has been developed (Yamamoto 1994) but it is not easy to perform. Other commonly used methods for detecting lipid peroxidation products are based upon detection of lipid hydroperoxide breakdown products, such as the aldehydes (MDA, nonenal, and hexanal) or the alkanes (ethane and pentane) and are consequently indirect methods of analysis. In biological samples, the presence of these compounds must be interpreted with caution because it is possible for them to result from processes other than lipid peroxidation and because their recoveries tend to be variable. For example, the thiobarbituric acid (TBA) assay frequently used for the quantification of MDA has been criticized due to the cross-reactivity of thiobarbituric acid with other compounds and the generation of MDA from the oxidation of biological compounds other than lipids (Halliwell and Gutteridge 1989).

The oxidation of the arachidonates also leads to the formation of MDA, which is frequently assayed as a marker of lipid peroxidation (Yeo *et al.* 1999). The aldehydes, in addition to being markers of lipid oxidation, have generated biochemical interest because of their intrinsic potential for toxicity (Woutersen *et al.* 1986). In foods, most of the attention has been directed towards measurement of the volatile carbonyls but also MDA, a well-known peroxidation product in rancid foods. In biological systems, it is a product of pathological lipid oxidation and normal PG biosynthesis. MDA is commonly assayed by the unspecific TBA assay as an indirect measure of lipid oxidation (Draper *et al.* 1993, Janero 1990). Although the HPLC method addresses the issue about specificity, it nevertheless permits temper-

ature-dependent oxidation artifacts that severely limit the validity of the method (Yeo *et al.* 1998). Assays involving HPLC for the detection of a variety of aldehydes are considerably more sensitive and specific (Kinter 1995, Lang *et al.* 1985); nevertheless, they serve only as indirect indicators of lipid peroxidation.

Very sensitive assays for the detection of low-molecular-weight aldehydes by gas chromatography (GC), MS or a combination of the two methods (GC/MS) have been described despite some concern about the generation of artifacts, the required manipulation of the sample, and variable recoveries from biological samples. Yeo *et al.* (1999) described an improved method for GC/MS measurement of MDA. Briefly, hepatocytes are lysed with phosphate buffered saline (PBS) containing 2.8 mM butylated hydroxytoluene (BHT) and 1% (wt/vol) SDS, pH 7.4. The protein-bound MDA is hydrolyzed with H_2SO_4. MDA is converted to a stable derivative by reacting with pentafluorophenylhydrazine at room temperature. The derivative is detected with a DBWAX capillary column (15 m × 0.25 mm i.d., 0.15-μm film thickness) in the negative ion chemical ionization (NICI) mode. The results were related to protein, which was measured by using the biocinchoninic acid (BCA) protein assay kit. Nevertheless, the development of a direct and sensitive method for the analysis of lipid peroxides would still be of considerable benefit.

Liu *et al.* (1997) compared the TBA assay measuring TBA-reactive substances (TBARS), and a new GC/MS assay measuring MDA. Both GC/MS and TBA assays gave parallel results for oxidation of unsaturated fatty acids and biological samples. The GC/MS assay was approximately two- to sixfold more sensitive than the TBA assay for oxidation of unsaturated fatty acids. In contrast, the TBA assay gave two- to sixfold higher TBARS than MDA by GC/MS assay in biological samples, possibly due to the unspecificity and artifact formation during the acid hydrolysis step of the TBA assay. GC analysis with electron impact (EI) ionization MS requires hydrolysis or transesterification of the lipid, reduction of peroxides to hydroxides, and sample derivatization to produce volatile species, thus introducing additional manipulation steps in which artificial oxidation can occur. Moreover, the high energy EI/MS commonly used causes fragmentation of the molecules, thus complicating the analysis of samples containing multiple components. However, the fragmentation can be useful for the determination of the position of the peroxidation. By contrast, electrospray ionization (ESI) MS is a soft ionization technique that does not normally cause fragmentation of analytes and is therefore more suitable for analysis of the complex mixtures that comprise biological membranes, especially because prior derivatization is not required. In addition, collisionally induced decomposition techniques can be used to cause fragmentation if required. Previously, ESI/MS was used for analysis of the ozonides formed from GroPCho lipids (Harrison and Murphy 1996), detection of breakdown products of oxidized palmitoylarachidonoyl GroPCho (Watson *et al.* 1997), and detection of peroxidized fatty acids on the cyclooxygenase/lipoxygenase pathway, such as hydroperoxyeicosatetraenoic acid (MacMillan and Murphy 1995). The first direct

observation of lipid hydroperoxides in phospholipid vesicles by ESI/MS was reported by Spickett *et al.* (1998).

Spickett *et al.* (1998) used positive ion ES/MS to obtain a lipid profile of vesicles prepared from egg yolk lecithin and enriched with arachidonoylstearoyl GroPCho and dipalmitoyl GroPCho. The vesicles were oxidized by treatment with *tert*-butyl hydroperoxide (*t*-ButOOH) and iron (II) sulfate, and the formation of hydroperoxides of the polyunsaturated lipid arachidonoylstearoyl GroPCho was observed. The native lipid signal at 832 amu decreased and new signals appeared at 864, 896, and 928 amu, corresponding to the addition of one (+32), two (+64), and three (+96) molecules of oxygen, respectively. The dihydroperoxide was found to be the most favorable peroxide product; however, it appeared that a degradation of the hydroperoxides was occurring concomitant with their formation, but only their net formation was observed. The rate of depletion of the polyunsaturated lipid and the rate of accumulation of the hydroperoxides were found to increase with the Fe^{2+} concentration between 10 μM and 2 mM, and was also dependent on the *t*-ButOOH concentration.

Hall and Murphy (1998) used on-line normal phase LC/ESI/MS to separate phospholipid classes and analyze the distribution of the major polyunsaturated fatty acyl groups and corresponding oxidation products. Arachidonic acid was observed primarily in plasmalogen GroPEtn, whereas linoleic acid was equally distributed in 1,2-diacyl-GroPEtn and GroPCho lipids. The additions of one and two oxygen atoms to polyunsaturated phospholipid molecular species were observed as the major, stable products after incubation with *t*-ButOOH. Tandem mass spectrometry (MS/MS) was utilized for further structural characterization of the oxidized fatty acyl groups, identified as 5-, 8-, 9-, 11-, 12-, and 15-hydroperoxyeicosatetraenoate (HETE) and 5-, 12-, and 15-hydroperoxyeicosatetraenoate (HpETE) in addition to 9- and 13-hydroxyoctadecadienoate (HODE) and 9- and 13-hydroperoxyoctadecadienoate (HpODE).

Schneider *et al.* (1997) developed an LC/ESI/MS strategy for the detection of fatty acid hydroperoxides in complex samples followed by identification of the corresponding regioisomers. The fatty acid hydroperoxides generated by soybean lipoxygenase were detected by LC/ESI/MS without derivatization before analysis. Localization of fatty acid hydroperoxides in complex mixtures was achieved by monitoring the loss of hydrogen peroxide using constant neutral loss scanning. In the presence of 5 mM NH_4OAc in methanol/water, the ion adducts $[M + NH_4]^+$ were formed almost exclusively, directly revealing the molecular mass of the thermolabile hydroperoxides. Low-energy collision-induced dissociation of precursor ions $[M - NH_4]^+$ led to characteristic product ions from both of the 9- and 13-regioisomers.

Yamauchi *et al.* (2000) developed a chemiluminescence-based HPLC method for the analysis of the addition products of α-tocopherol with PtdCho-peroxyl radicals (TOO-PC). The TOO-PC eluted from a reversed-phase column was reacted with a chemiluminescent reagent consisting of a *Cypridina* luciferin analog and lipid-soluble iron chelate in acidic methanol at 50°C, and the chemiluminescence

generated was monitored. The detection limit for TOO-PC by this method was ~1 pmol. This method was applied to the detection of TOO-PC in the peroxidized membranes prepared from rabbit erythrocyte ghosts.

Isoprostanes

F_2-isoprostanes are complex metabolites of arachidonic acid generated *via* nonenzymatic free radical oxidation and are isomeric to $PGF_{2\alpha}$, which is enzymatically produced by PGH_2 synthase. Theoretically, four distinct regioisomeric families are possible. These regioisomeric families have a common 1,3-diol cyclopentane structural feature, but differ by the comparative length of two attached alkyl chains and the position of a third hydroxyl group. Eight synthetic $PGF_{2\alpha}$ isomers were found separable by capillary GLC and reversed-phase HPLC.

Kayganich-Harrison *et al.* (1993) showed that intact phospholipids containing fatty acyl groups of the isoprostane structure can be readily detected with MS/MS even when present as minor components in a biological extract. Although specific isomer identification could be made from the complex mixture, these techniques establish the existence of these novel metabolites of arachidonic acid esterified to GPL. Waugh and Murphy (1996) used electrospray MS/MS to detect the elution of these isomers from the HPLC column by monitoring the characteristic loss of 44 u (C_2H_4O) from the 1,3-diol cyclopentane ring. Catalytic reduction, derivatization, and ESI/MS techniques were used to obtain definitive information concerning the location of the side chain hydroxyl position in these isomers through abundant α-cleavage ions. The isoprostanes were generated by free radical oxidation of arachidonic acid and were separated by HPLC and capillary GLC techniques. Members of each of the four specific regioisomeric isoprostane families could be identified in this mixture from the predicted α-cleavage ions. Although many epimers within a single family type could be separated, the four regioisomeric families were substantially superimposed in the HPLC and GLC elution. Type I and Type IV regioisomers were the major F_2-isoprostane products, but the complexity of the isomers requires more than a simple GC/MS assay to identify precisely a particular stereoisomer within a regioisomeric family (e.g., 8-epi $PGF_{2\alpha}$). Type I F_2-isoprostanes are unique noncyclooxygenase products and may be more specific targets with which to measure lipid peroxidation *in vivo*. Figure 6.5 shows a direct LC/ES/MS/MS analysis for the loss of 44 u from the carboxylate anion of F_2-isoprostanes (*m/z* 353) (Waugh and Murphy 1996).

Nourooz-Zadeh *et al.* (1997) presented evidence for the formation of F_3-isoprostanes during peroxidation of eicosapentaenoic acid. F_3-isoprostanes were analyzed by GC/NICI/MS using tetradeuterated $PGF_{2\alpha}$ as the internal standard, following conversion to pentafluorobenzyl (PFB) ester/trimethylsilyl (TMS) derivatives. Morrow and Roberts (1999) reported an MS method of quantification of F_2-isoprostanes in biological fluids and tissues as a measure of oxidant stress, and Murphy *et al.* (2001) discussed the analysis of nonvolatile lipids by MS. Finally,

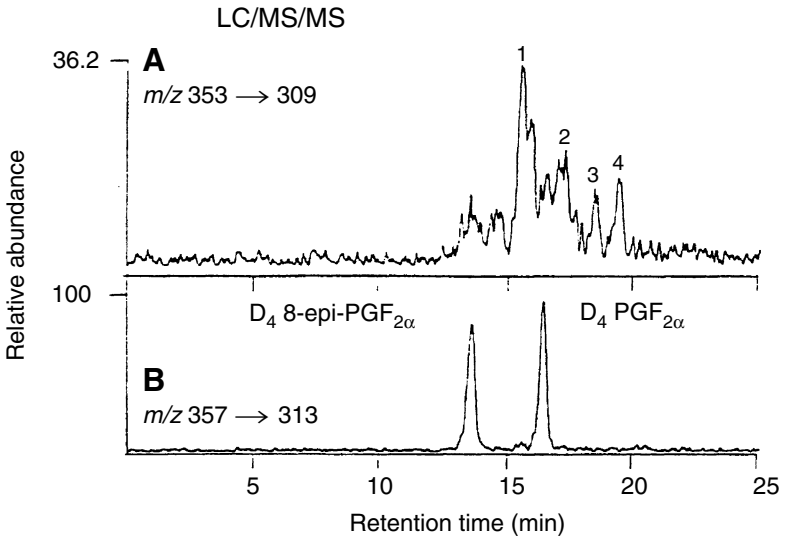


Fig. 6.5. Direct liquid chromatography (LC) mass spectrometry (MS)/MS analysis *via* electrospray ionization (ESI) of a mixture of synthetic Type IV regioisomers of prostaglandin (PG)$F_{2\alpha}$ separated by reversed-phase high-performance liquid chromatography (HPLC; upper panel). The loss of 44 u from the carboxylate anion (*m/z* 353) was used to detect the elution of each synthetic isomer (A); the loss of 44 u from *m/z* 357 was used to detect the elution of the deuterium-labeled internal standards (B); F_2-isoprostanes obtained by free radical oxidation (Fenton) or arachidonic acid. Lower panel: Fractions collected during elution of the isoprostanes indicated by the numbers 1–4 were collected (A) and analyzed by ES after catalytic reduction and derivatization; the loss of 44 u from *m/z* 357 that corresponds to the deuterium-labeled internal standards. Reproduced from Waugh and Murphy (1996) with permission of the publisher.

Reich *et al.* (2000) reported the formation of novel D-ring and E-ring isoprostane-like compounds (D_4/E_4-neuroprostanes) *in vitro* and *in vivo* from DHA. Free radical oxidation of DHA increased putative D_4/E_4-neuroprostane levels produced 380-fold. The levels of D_4/E_4-neuroprostanes increased 54-fold after oxidation of fresh brain tissue. The D_4/E_4-neuroprostanes were also detected in rat and human brain tissue at 9–12 ng/g of tissue. The identity of the D_4/E_4-neuroprostanes was confirmed by chemical and LC/MS approaches.

Core Aldehydes

The detection of GPL core aldehydes in natural lipid extracts depends upon one or more of the factors involved in the formation, destruction, and stabilization of lipid peroxides. Theoretically, there should be a mole of core aldehyde produced for each mole of short-chain aldehyde measured (Halliwell and Gutteridge 1989).

Most of the quantitative measurements of aldehyde content of plasma lipoproteins have been confined to the short-chain aldehydes. However, the improved methods developed for their measurement are of interest to the quantification of the high-molecular-weight ester-bound aldehydes. Thus, Bailey *et al.* (1997) described a modification of the earlier procedure of Holley *et al.* (1993) for improved determination of nanomolar concentrations of *n*-alkanals, hydroxyalkenals, MDA, and furfural in biological fluids. Aldehydes are reacted with 1,3-cyclohexanedione to produce fluorescent derivatives, which are separated by gradient reversed-phase HPLC. Analysis time was reduced by shortening the sample preparation, and sensitivity was increased by miniaturization of the derivatization procedure, reducing required sample size. This method allowed the separation and determination of the short-chain aldehydes in a human LDL sample (see below). Seppanen and Csallany (2001) reported a very sensitive HPLC method for the determination of the nonpolar and polar lipophilic secondary lipid peroxidation products in vegetable oil. Seventeen nonpolar and 13 polar lipophilic aldehydes and related carbonyl compounds, derived from thermally oxidized soybean oil as 2,4-dinitrophenylhydrazones, were separated simultaneously and resolved by reversed-phase HPLC. The above methods do not appear to have been utilized for the determination of core aldehydes in natural or artificial samples.

The presence of the lipid ester core aldehydes may be demonstrated by the isolation and identification of the intact aldehyde molecule, its derivatives, or degradation products. Usually, several methods may be necessary to establish unequivocally the presence of lipid ester core aldehydes *in vivo*. Thus, Stremler *et al.* (1989) identified 5-oxo-valeroyl GroPCho among the *in vitro* peroxidation products of PtdCho with arachidonic acid at the *sn*-2-position, and Kuksis (1990) identified the 9-oxo-nonanoyl GroPChos among the *in vitro* peroxidation products of plasma PtdCho with linoleic acid at the *sn*-2-position. Furthermore, Itabe *et al.* (1988) had earlier isolated 2-azelaoyl GrPCho as one of the cytotoxic products generated during hemoglobin-induced peroxidation of PtdCho, whereas Tokumura *et al.* (1988) determined the structure of a group of phospholipids with vasopressor activity and found that these are PtdChos with 4–9 carbon dicarboxylic acids at the *sn*-2-position. It is obvious that these products originated from the oxidation of PtdChos containing polyunsaturated fatty acids (PUFA) in the *sn*-2-position. Furthermore, Stremler *et al.* (1989) found that *sn*-2-(5-oxo)valeroyl GroPCho was a substrate for the purified PAF acetylhydrolase.

Stremler *et al.* (1989) extracted the phospholipid core aldehydes using chloroform/methanol 1:1 (vol/vol) as described by Bligh and Dyer (1959). After removal of the solvent under nitrogen flow, the resulting residue was examined by reversed-phase HPLC using a method described by Brash *et al.* (1987) and synthetic 1-palmitoyl-2(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine was used as reference standard (R_t = 9.75 min). The reversed-phase HPLC was carried out isocratically in methanol/water/acetonitrile (930:70:50, by vol) with 20 mM choline chloride at 1 mL/min. The effluent was monitored for UV absorption at 235 nm with a flow-through detector, and for

estimation of radioactivity by liquid scintillation spectroscopy using collected fractions.

Kuksis (1990) identified the plasma PtdCho core aldehydes after extraction with chloroform/methanol (2:1, vol/vol) as described by Folch *et al.* (1957). After solvent evaporation, the lipid residue was resolved by TLC (Silica gel H) using chloroform/methanol/water (65:35:6, by vol) as solvent and synthetic PtdCho core aldehydes as standards. The PtdCho core aldehyde (R_f 0.26) was clearly resolved from native PtdCho (R_f 0.33). The identity of the core aldehydes was established by dephosphorylation of the PtdCho core aldehydes with PLC followed by GC/MS or LC/MS analysis of the DAG derived. For this purpose, the PtdCho core aldehydes were recovered from the TLC plate by elution with chloroform/methanol (2:1, vol/vol); the extract was reduced to a small volume and hydrolyzed with PLC for 12 h at 37°C as described by Kuksis *et al.* (1969). For GC and GC/MS, the DAG core aldehydes were converted into the methoxime (MOX) derivatives by heating the PLC digests with 100 µL of the methoxylamine · HCl reagent at 60°C for 3 h (Horning *et al.* 1968). The reaction mixture was evaporated under nitrogen and the residue was extracted with 5 mL hexane and washed with water. The solvent layer was dried over sodium sulfate and evaporated to dryness before preparation of TMS derivatives for GLC or GC/MS analysis. The TMS ethers were prepared by treating the lipid fractions or their MOX derivatives with a silylating reagent made up of one part BSTFA + 1% TMSC and one part pyridine for 30 min at room temperature. Nonpolar capillary GC/EI/MS or GC/NICI/MS was performed as previously reported (Kuksis and Myher 1980). Figure 6.6A shows the separation of the DAG containing the core aldehydes as the TMS and MOX-TMS derivatives by nonpolar capillary GLC (Kamido *et al.* 1992b). Two major peaks are seen, which were shown by GC/MS to be due to the 9-oxononanoyl esters of palmitoyl and stearoylglycerols. The presence of the aldehyde function was indicated by the shift of both peaks to longer retention times after conversion to the MOX derivatives. Figure 6.6B gives the GC/EI/MS spectra of the TMS ethers of 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycerol and its MOX derivative (Kamido *et al.* 1992b).

Kamido *et al.* (1995) used acidified chloroform/methanol containing 2,4-dinitrophenylhydrazine to extract PtdCho core aldehydes generated by copper-oxidation of human LDL and HDL. The DNPH derivatives formed were resolved by reversed-phase HPLC and identified by on-line quadrupole LC/MS. The LDL and HDL with minimum peroxidation were isolated as described by Jurgens *et al.* (1990) and were subjected to copper oxidation according to Lenz *et al.* (1990). The ester-bound lipid aldehydes were isolated using a method originally described by Esterbauer *et al.* (1987) for the isolation of low-molecular-weight aldehydes. To the oxidized lipoprotein solution (1 mL containing 1.5 and 4.5 mg protein/mL of LDL and HDL, respectively), EDTA (0.1 mL of 1%), BHT (10 µL of 2%), and freshly prepared DNPH in 1 N HCl (0.5 mg/mL, 1 mL) were added, mixed vigorously, and allowed to stand in the dark for 2 h at room temperature and then

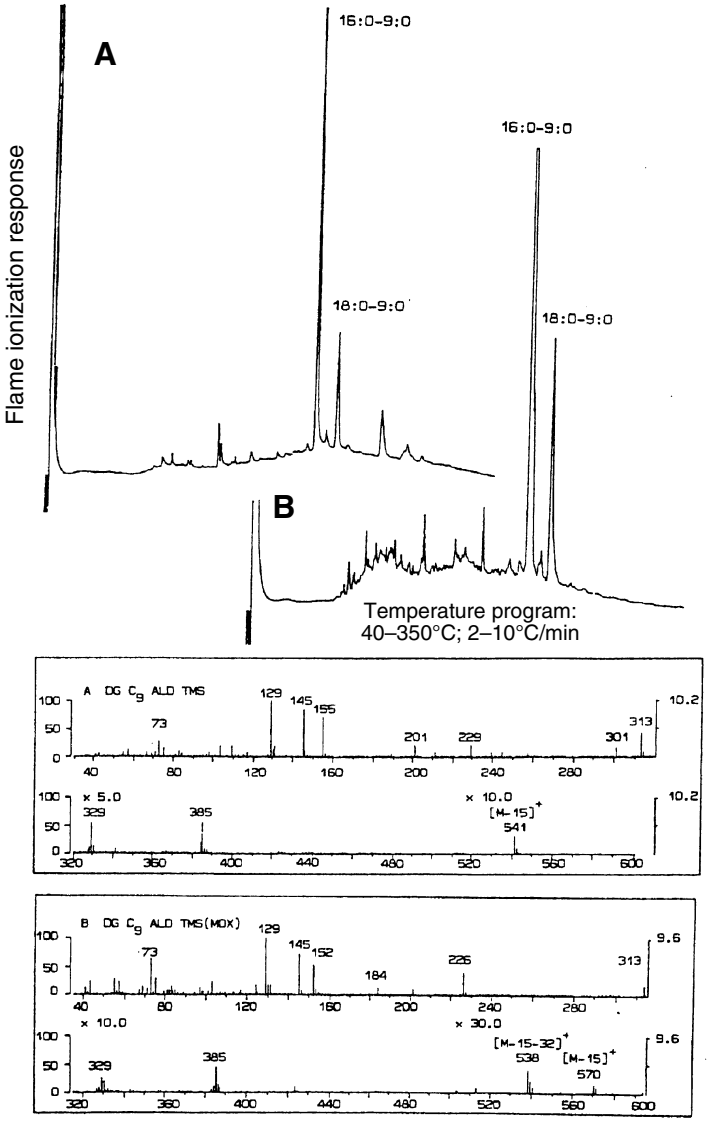


Fig. 6.6. Upper panel: Nonpolar capillary gas–liquid chromatography (GLC) profiles of diacylglycerol moieties of oxidized egg yolk phosphatidylcholine (PtdCho) containing core aldehydes (ald). (A) trimethylsilyl (TMS) ethers; (B) methoxime (MOX)-TMS ethers. GLC conditions: nonpolar methyl silicone column (8 m × 0.30 mm); temperature program, 40–350°C; flame ionization detection (FID). Lower panel: Full mass spectra of 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycerol as obtained by gas chromatography (GC)/mass spectrometry (MS) for the TMS ether (A) and MOX-TMS ether (B). Reproduced from Kamido *et al.* (1992b) with permission of the publisher.

overnight at 4°C. The reaction mixture was extracted with chloroform/methanol 2:1 (vol/vol). The DNPH derivatives were separated by TLC on Silica gel H plates (20 × 20 cm) using a double development with dichloromethane (to a height of 10 cm) and, after solvent evaporation, with toluene (to a height of 17 cm). The DNPH derivatives of the GroPCho-bound aldehydes were recovered from the origin of the plate (R_f 0.0–0.05). Aliquots of the extracts of the origin of the TLC plate and of the total peroxidation mixture were digested with PLC (*Bacillus cereus*) for 2 h at 37°C, and the released DNPH derivatives of the DAG core aldehydes were purified by TLC using chloroform/methanol (95:5, vol/vol) as the developing solvent (Kamido *et al.* 1995). The DNPH derivatives (R_f 0.20) were resolved from residual DAG (R_f 0.30) and ceramides (R_f 0.25) and were recovered separately by extraction with chloroform/methanol (2:1, vol/vol) after location by UV absorption and fluorescein spraying. Reversed-phase HPLC of the TLC fractions of the DNPH derivatives gave two major peaks for both LDL and HDL, corresponding to the C_{16} and C_{18} homologs of the oxoalkylglycerols. Figure 6.7 gives the total NICI/MS profiles (Total) and single ion plots (*m/z*) for the DNPH derivatives of the major DAG core aldehydes derived from the GPL of oxidized human HDL. The ion chromatogram shows that the aldehydes are resolved on the basis of molecular weight, with the palmitoyl derivatives eluting ahead of the stearoyl derivatives in each core aldehyde class. Overlaps occur among isobaric species (e.g., 16:0/C_{9ALD} and 18:0/C_{7ALD}) and species with similar partition properties (e.g., 16:0/C_{9ALD} and 18:1C_{9ALD}).

Alternatively, oxidized LDL and HDL lipids were extracted with chloroform/methanol in the absence of DNPH, and aliquots of the extract were digested with PLC, separated by TLC on the basis of polarity, recovered from the silica gel, converted to the TMS ethers, and resolved by high temperature GLC (Kuksis 1990). Meyer *et al.* (1992) demonstrated in model experiments that plasmalogens, which make up a minor proportion of lipoprotein phospholipids but are major components of the membranes of red blood cells, platelets, and heart and kidney tissue, are attacked by lipid peroxides (lipid peroxy radicals), which transform them to their epoxides. These epoxides decompose to α-hydroxyaldehydes. Felde and Spiteller (1995) determined the plasmalogen aldehydes using the dithiolane method. The crude lipid extracts were first separated by preparative TLC on silica gel plates (1.5 mm thick layer) using cyclohexane/diethyl ether (90:10, vol/vol) as developing solvent. The phospholipids were recovered from the origin (R_f 0.0) by elution with methanol. The solvent was evaporated and the residue redissolved in diethyl ether; the resulting solution was stirred with ethane dithiol/ BF_3 etherate to prepare 2-alkyl-1,3-dithiolanes. The 2-alkyl-1,3-dithiolanes of the corresponding plasmalogen aldehydes were obtained by extracting the neutralized reaction mixture with diethyl ether and purifying by TLC on silica gel plates using cyclohexane/ethyl acetate (15:1, vol/vol) as developing solvent. The TLC bands were located by UV absorption and spraying with 10% ethanolic ammonium molybdate orthophosphorus acid (R_f 0.7–0.9). The dithiolanes were recovered with diethyl

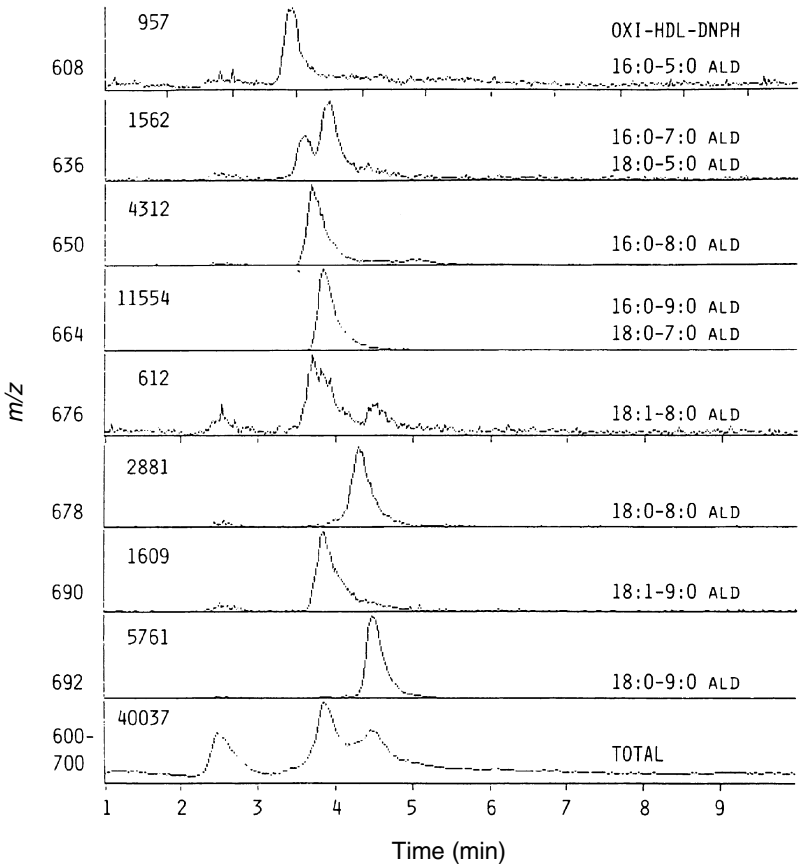


Fig. 6.7. Total negative ion current profiles and single ion plots (*m/z*) for the dinitrophenylhydrazone (DNPH) derivatives of the major diacylglycerol core aldehydes (ald) derived from the glycerophospholipids of oxidized human high density lipoprotein (HDL). Peak identification is given in figure. Liquid chromatography (LC)/mass spectrometry (MS) conditions: Supelco Lc-18 column (250 m × 4.6 mm i.d.); eluant, linear gradient of 30–90% propionitrile in acetonitrile in 30 min; MS conditions: Hewlett-Packard Model 1086 B liquid chromatograph interfaced with Hewlett-Packard Model 5985 B quadrupole mass spectrometer *via* a direct liquid inlet interface. Negative chemical ionization (electron capture) mass spectra were taken every 5 s over the entire chromatogram in the mass range 200–900. Single-ion plots were extracted from total ion current. Reproduced from Kamido *et al.* (1995) with permission of the publisher.

ether and were identified by GLC and GC/MS, using single ion monitoring of mass 105 corresponding to the α-cleavage product.

In parallel, Felde and Spiteller (1995) determined the α-hydroxyaldehydes as the pentafluorobenzyloxime (PFBO) derivatives as reported by Van Kuijk *et al.*

(1986) and Loidl-Stahlhofen and Spiteller (1994). The pentafluorobenzylamine derivatives must be prepared immediately to avoid loss of the hydroxyaldehyde due to dimerization (Effenberger *et al.* 1992). The PFBO derivatives of the hydroxyaldehydes were quantified by adding 300 μg of 1-hydroxy-2-dodecanone/g lipoprotein and 20 μg of the hydroxyundecanal-PFBO derivative/g lipoprotein to natural or oxidized lipoproteins as internal standards. After TLC purification (cyclohexane/ethylacetate, 3:1, vol/vol), the PFBO derivatives were dissolved in dimethylformamide (DMF) (100 μg/mL and incubated with the same volume of MTBSFA for 12 h at 60°C to convert the hydroxy groups into *t*-butyldimethylsilyloxy (TBDMS) derivatives. The resulting products were purified and enriched by TLC (cyclohexane/ethylacetate, 98:2, vol/vol) and analyzed by GC/MS. The detection limit was ~5 ng aldehyde equivalent/g lipid.

Weisser *et al.* (1997) reported a dramatic increase in α-hydroxyaldehydes derived from plasmalogens in the aged human brain. Plasmalogens that occur in brain tissue in substantial amounts suffer degradation either by hydrolysis under production of aldehydes or by oxidation with lipid peroxyradicals by generation of plasmalogen epoxides. The latter react by the addition of pentafluorobenzylhydroxylamine (PFBHA) · HCl under hydrolysis to α-hydroxyaldehydes, which are immediately transformed to pentafluorobenzyloximes. Similarly, free aldehydes are transformed to PFBO-derivatives. PBFO-derivatives of free aldehydes and PFBO-derivatives of α-hydroxyaldehydes were extracted and quantified by GC/flame ionization detection (FID) and by GC/MS after trimethylsilylation. The plasmalogens, along with other lipids remaining in the aqueous phase, were hydrolyzed by treatment with acid. The resulting long-chain aldehydes react with PFBHA · HCl to produce PFBO-derivatives.

Natural Occurrence

Isolation of Core Aldehydes from In Vitro *Peroxidation Products*

The methods of detection and mechanism of formation of core aldehydes were first explored by analyzing the products of *in vitro* peroxidation of unsaturated GPL and GPL-containing lipoproteins and cell membranes. Several reports have appeared concerning the *in vitro* peroxidation products of chemically pure phospholipids and of lipoproteins. Itabe (1998) reviewed these studies including the identification of PtdCho containing a *sn*-2-azelaoyl residue as a peroxidation product of the linoleoyl species with oxyhemoglobin. Kamido *et al.* (1993) subjected purified egg yolk phospholipids to oxidation with *t*-ButOOH. A homologous series of core aldehydes was produced; their generation required the intermediate formation of desaturated derivatives that would undergo further oxidation, as well as the conversion of the aldehydes into acids by overoxidation. Tanaka *et al.* (1994) demonstrated that various kinds of derivatives of PtdCho having an *sn*-2-butyrate, oxovalerate, *O*-methylglutarate, or 4-hydroxybutyrate residue were detectable by GC/MS after peroxidation of 1-hexadecyl-2-arachidonoyl or docosahexaenoyl GroPCho

with $FeSO_4$/ascorbate/EDTA. Copper oxidation is another alternative and commonly used procedure for the preparation of peroxidized phospholipids.

Originally, peroxidation of lipids was assessed on the basis of identification and quantification of short-chain compounds derived from oxidized phospholipids, such as water-soluble TBARS. Many laboratories continue to use these procedures as quantitative indicators of the overall peroxidation. Kawai *et al.* (1999) recently employed TBARS to measure the decomposition of phospholipids and the formation of large amounts of TBARS after peroxidation of red cell membranes with XO/XOD/Fe^{3+}. In contrast, hydroperoxy and aldehydic PtdCho were formed in human red blood cell membranes peroxidized with an azo initiator, 2,2-azobis(2-amidinopropane)-dihydrochloride (AAPH). Aldehydic PtdCho was preferentially generated from arachidonoyl GroPCho rather than from linoleoyl GroPCho in AAPH-peroxidized membranes. The core aldehydes were analyzed as the fluorescent 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) derivatives. A typical chromatogram of the DBD-H derivatives of aldehydic PtdCho showed clear peaks of 1-acyl-2-(5-oxopentanoyl) and 1-acyl-2-(9-oxononanoyl)-*sn*-GroPCho. Other aldehydic peaks appeared to be present in the chromatograms as well, but they were not identified in the absence of reference standards and MS. Other methods of identifying and quantifying core aldehydes are based on GLC resolution of the aldehyde acids released from oxidized phospholipids by saponification and methylation. This method has proven adequate for the identification of the core aldehydes in oxidized cholesteryl esters and in used and unused frying oils (Kamal-Eldin *et al.* 1997). These procedures do not permit determination of the exact origin of the core aldehyde, unless the appropriate molecular species is isolated first.

Recently, *in vitro* peroxidation studies have become increasingly more sophisticated, and in many instances, the oxidation conditions and the products obtained have been difficult to distinguish from the *in vivo* conditions and products. Thus, Guy *et al.* (2001) made extensive use of a peroxynitrite generating system (SIN) to mimic the peroxidation of LDL GPL, whereas Ahmed *et al.* (2001 and 2002b) used this system to mimic the *in vivo* peroxidation of HDL. Borowitz and Montgomery (1989) showed that exposure of microsomes to *t*-ButOOH was associated with a rapid accumulation of TBARS, which peaked at 10 min and then reached a plateau. Accumulation of dienes was somewhat slower and continued to increase throughout the 30-min incubation, suggesting continual initiation or reinitiation of peroxidation. Exposure of microsomes to *t*-ButOOH was associated with activation of endogenous PLA_2. When PLA_2 was inhibited with chlorpromazine, mepacrine, or *p*-bromophenacyl bromide, the accumulation of TBARS was reduced in a dose-dependent manner. In contrast, the accumulation of conjugated dienes was not affected by chlorpromazine. Pretreatment with exogenous PLA_2 reduced both dienes and TBARS. Thus, the process of peroxidation activated PLA_2, which facilitated the propagation of the peroxidative process. In contrast, peroxidation was inhibited when PLA_2 was activated before initiation of peroxidation.

Felde and Spiteller (1995) compared the plasmalogen aldehyde contents in the different plasma lipoprotein fractions of very low density lipoproteins (VLDL) and LDL and HDL before and after oxidation. Incubation with Fe^{2+}/ascorbate resulted in a dramatic decrease in plasmalogens that correlated with an appropriate increase in α-hydroxyaldehydes. The greatest decrease in plasmalogens was observed for VLDL and LDL, whereas HDL was rather resistant. Huber *et al.* (2002) showed that oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. Specifically, it was shown that oxidized phospholipids, such as 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC) generated in *t*-ButOOH–treated endothelial cells and apoptotic blebs, are responsible for biological activity in membrane vesicles and apoptotic blebs. Natural antibodies from apoE-null mice that recognize POVPC also recognized oxidized membrane vesicles (MV), and pretreatment of MV with these antibodies inhibited their ability to activate endothelial cells. Quantitative analysis of the polar lipid fraction by ES/MS showed increased levels of the oxidized phospholipids, palmitoyloxovaleroyl, palmitoylepoxyisoprostane, and palmitoylglutaroyl GroPCho in oxidized MV compared with native MV. Among these oxidized phospholipids, only palmitoyloxovaleroyl GroPCho was shown to mimic the overall action of minimally modified (MM)-LDL in terms of induction of leukocyte-endothelial interactions.

Ahmed *et al.* (2003a and 2002b) peroxidized apoA1-containing proteoliposomes by means of a peroxynitrite generating system and demonstrated the formation of both C_5 and C_9 aldehydes of PtdCho present in total lipid extracts of HDL or added as a purified palmitoyl arachidonoyl and palmitoyl linoleoyl GroPCho. Ahmed *et al.* (2001) observed that apoA1 increases the formation of PtdCho core aldehydes during peroxynitrite oxidation of proteoliposomes and HDL. PtdCho core aldehydes, which were present only in small amounts, increased 10-fold during oxidation of native HDL, compared with trypsinized HDL, and fourfold compared with HDL lipid suspensions. Similarly, incubation of apoA1 with 10-palmitoyl-2-linoleoyl GroPCho proteoliposomes in the presence of the peroxynitrite donor resulted in a significant increase in PtdCho core aldehyde. Earlier, Rubbo *et al.* (1995) showed that oxidation of 1-octadecanoyl-2-arachidonoyl GroPCho by soybean lipoxygenase in the presence of oxygen and nitric oxide [•NO] resulted in the formation of monohydroxy (*m/z* 798), monohydroperoxy (*m/z* 846), dihydroperoxy (*m/z* 846), nitroso (*m/z* 827), and nitrosoperoxy (*m/z* 843) derivatives of the parent ion molecule ($[M + H]^+$, *m/z* 782). The detection of adducts of nitrogen dioxide and nitric oxide with free radical intermediates of GroPCho was somewhat simplified by the fact that the major ions observed at $[M + H]^+$ or $[M - 15]^-$ were at an odd mass number because of the presence of an additional nitrogen atom in these species.

Isolation of Core Aldehydes from Natural Sources

Studies on the natural occurrence of GPL core aldehydes have centered mainly on plasma lipoproteins, although isolated cell membranes, atheromas, and tissue cul-

tures have also been examined. Among the methods proposed for measurement of lipid hydroperoxides in biological samples, a combination of chemiluminescence detection and HPLC is one of the most promising because of high sensitivity and selectivity (Miyazawa *et al.* 1987, Yamamoto *et al.* 1987). Akasaka *et al.* (1995) developed an automatic method for the determination of hydroperoxides by injection of a deproteinized plasma sample onto a reversed-phase column and subsequent introduction into two analytical columns and postcolumn reaction with diphenyl-1-pyrenylphosphine. They reported the presence of 6–7 pmol of PtdCho hydroperoxides in human plasma. The drawbacks of this technique include the complicated chromatography apparatus and the lack of detailed information about the molecular species of the phospholipid. Yasuda and Narita (1997) reported the simultaneous determination of phospholipid hydroperoxides and cholesteryl ester hydroperoxides in human plasma by HPLC with chemiluminescence. LOOH were extracted quantitatively from human plasma with a mixture of *n*-hexane and ethyl acetate, and separated by column-switching HPLC using one aminopropyl column and two octyl columns followed by chemiluminescence detection. LOOH could be completely separated from each other and detected at picomole levels. Application of the method to normal human plasma gave the following levels of PtdCho OOH and cholesteryl ester (CE)-OOH: 36.0 ± 4.0 nM (n = 6) and 12.3 ± 3.1 nM (n = 6), respectively.

To demonstrate the presence of aldehydic PtdCho in lipoproteins and in biomembranes prepared from natural tissues, specific methods are necessary for the isolation and identification of phospholipids that contain short-chain carbonyl moieties, such as aldehydes. Esterbauer *et al.* (1989) used the UV-absorbing DNPH derivatives to resolve and quantify the volatile and water-soluble aldehydes by TLC and HPLC. Kamido *et al.* (1992a and 1992b) were the first to isolate lipid ester core aldehydes from minimally peroxidized LDL. Both C_5 and C_9 core aldehyde esters of GroPCho were recovered as the DNPH by extraction with chloroform/methanol (2:1, vol/vol) containing DNPH. Kamido *et al.* (1992a and 1992b) established conditions for HPLC fractionation of the DNPH derivatives of the oxo-DAG released by PLC from the phospholipids of oxidized LDL and HDL. The resolved peaks were identified by reversed-phase LC/MS with thermospray ionization (TSI) MS. Subsequently, core aldehydes attached to the *sn*-2-position of the GroPCho were reported in LDL (Frey *et al.* 2000, Heery *et al.* 1995, McIntyre *et al.* 1999, Schlame *et al.* 1996, Stremler *et al.* 1991, Subbanagounder *et al.* 2000, Watson *et al.* 1997), in HDL (Ahmed *et al.* 2001 and 2002b, Kamido *et al.* 1993 and 1995), and in plasma and red cells of diabetics (Kuksis *et al.* 1995).

Hahn and Subbiah (1994) demonstrated a significant association of lipid peroxidation products with HDL isolated by a rapid precipitation procedure; losses of oxidation products were encountered during ultracentrifugation. Under basal conditions, HDL isolated from human plasma or from the total lipoprotein fraction (d >1.21) using the dextran sulfate/Mg^{2+} precipitation technique (Warnick *et al.* 1982) carried nearly 35–40% of the total plasma fatty acid peroxidation product

(measured as MDA). The samples were subjected to the measurement of TBARS as described by Arshad *et al.* (1991). In this method, reaction products are extracted into butyl alcohol after reaction of MDA with TBA and absorbance is read at 530 nm. As noted above, TBARS determination is not a sensitive method for core aldehyde determination.

Hörkkö *et al.* (1996) reported that antiphospholipid antibodies are directed against epitopes of oxidized phospholipids. The monoclonal antibodies to epitopes of oxidized LDL allowed the recognition even of cardiolipin. Itabe (1998) reviewed the preparation and utilization of mono- and polyclonal antibodies that specifically recognize aldehydic PtdCho in plasma lipoproteins and in human atherosclerotic lesions. Haginaka *et al.* (2001) developed an anion-exchange HPLC method for assaying plasma lipoproteins and copper-oxidized LDL using a Prot-Ex-DEAE column. The method was applied to assay mildly oxidized LDL from Watanabe heritable hyperlipidemic rabbits, and promising separations were obtained. The nature of the oxidation products in the mildly oxidized rabbit LDL was not established.

Felde and Spitteller (1995) estimated the α-hydroxyaldehyde content of natural lipoproteins. In all fractions, α-hydroxyaldehydes were detected in trace amounts (up to 800 ng/g lipid). Because plasmalogens are major compounds in heart and brain tissue, in contrast to serum lipids, it was felt that in the case of oxidative stress, their high susceptibility to oxidation might cause a much higher production of α-hydroxyaldehydes than in lipoproteins, with the consequence of possible deleterious effects. The α-hydroxyaldehydes and unsubstituted C_{16} and C_{18} aldehydes are readily isolated and identified by HPLC of the DNPH derivatives and identified by LC/MS (Kamido *et al.* 1994). Pryor *et al.* (1996) reported the detection of small amounts of low-molecular-weight aldehydes (heptanal, nonanal, and hexanal) in bronchoalveolar lavage of rats exposed to ozone. However, the method was not sensitive or reproducible enough to be used as a measure of oxidative stress.

It has been claimed that oxidative reactions *in vivo* are associated with pathologic events. To this end, much attention has been focused on the products of free radical damage *in vivo*. Because phospholipids are an abundant source of PUFA, the products of phospholipid oxidation can be markers of peroxidative damage. Ravandi *et al.* (1995a, 1999a and b) established an LC/ES/MS system for the identification of hydroperoxides of the GPL, their aldehyde esters, as well as their isoprostane esters. Figure 6.8 shows the separation of oxidized phospholipids of human LDL on a normal phase silica column, which allows the identification and quantification of PtdCho oxidation products. It is seen that the various oxidation products are well resolved from the residual PtdChos. Interestingly, the oxidation of PtdCho is accompanied by an extensive release of lysoPtdCho. The isoprostanes are produced only from the arachidonate-containing species. The oxidation products of other long-chain polyunsaturated esters were not identified. The relatively saturated SM does not appear to be affected by mild oxidation with copper (Ravandi *et al.* 1995a, 1999b). Ahmed *et al.* (2001) successfully extended this method to the identification and quantification of the lipid peroxidation products of

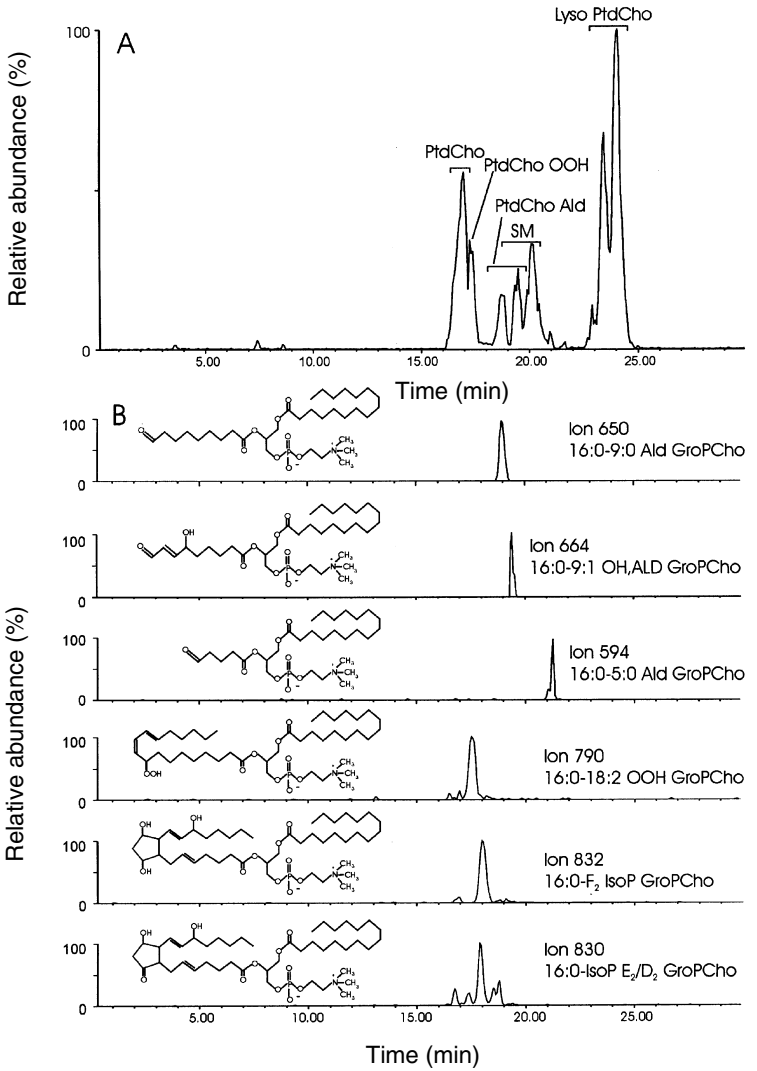


Fig. 6.8. Liquid chromatography (LC)/electrospray ionization (ESI)/mass spectrometry (MS) analysis of oxidized phosphatidylcholine (PtdCho) in oxidized low density lipoproteins (LDL). (A) Total positive ion current profile of oxidized LDL. (B) Single-ion plots of representative PtdCho oxidation products. Peak identification is given in the figure. LDL was oxidized by incubation with 5 μM $CuSO_4$ in 0.1 M phosphate buffered saline for 12 h at 37°C. Total lipid extract of the oxidized LDL was dissolved in chloroform/methanol (2:1, vol/vol) and 20 μL of the same sample containing 10 μg lipid was analyzed. Structural assignments for aldehydes and hydroperoxides are according to reference standards. Ions at *m/z* 832 and *m/z* 830 were identified on the basis of retention time and molecular weight. Reproduced from Ravandi *et al.* (1999b) with permission of the publisher.

human HDL resulting from treatment with peroxinitrite. Lynch *et al.* (1994) previously demonstrated the formation of the hydroperoxides and isoprostanes in plasma and LDL exposed to oxidative stress *in vitro*, but they did not analyze intact lipid esters. Ravandi *et al.* (1999b) demonstrated the presence of hydroperoxides, core aldehydes, as well as isoprostanes of GPL in samples of atheroma. Figure 6.9 shows a representative single-ion chromatogram. Single-ion monitoring allowed the detection limit to be lowered to 10–50 fmol for the different oxidized phospholipid classes. A more detailed account of the aldehydic PtdCho content of atheroma is provided elsewhere (Ravandi and Kuksis, 2003, unpublished). Analysis of lipid extracts obtained from advanced human atherosclerotic lesions revealed the presence of 9-oxononanoyl cholesterol (28 ± 14 μmol/mol cholesterol) in all tissue samples analyzed despite the presence of α-tocopherol (4 ± 1.2 mmol/mol cholesterol).

Ou *et al.* (1995) and Kawai *et al.* (1999) established an HPLC method for the fractionation of oxidized PtdCho that contain aldehyde residues, after derivatization with the fluorescent reagent, DBD-H, which reacts efficiently with the aldehyde residues of phospholipids at room temperature. Fluorescent derivatives of aldehydic PtdCho were well separated into species that contained aldehyde groups at different sites. The method could determine as little as several picomoles of aldehydic PtdCho and was used to demonstrate for the first time the presence of core aldehydes in peroxidized red blood cell membranes. Karten *et al.* (1998) described the preparation of fluorescent decahydroacridine derivatives of cholesteryl ester core aldehydes by reaction with 1,3-cyclohexanedione. These derivatives were stable for at least 160 h. The limit of quantification of the method was at the low (<50) femtomole level, with an absolute limit of detection of 15 fmol. Weisser *et al.* (1997) utilized the reactivity of the aldehydes and hydroxyaldehydes with pentafluorobenzylhydroxylamine for their sensitive measurement in postmortem brain tissue. In fresh brain tissue samples, α-hydroxyaldehydes were detected at all age periods, varying from 15 ng to 18 μg/g of lipid in brain tissue samples stored for 2–4 y at –68°C because they had already reacted with other biomolecules.

F_2-Isoprostanes in plasma and urine are generally determined by labor-intensive methods requiring sample purification by solid phase extraction and TLC. Walter *et al.* (2000) streamlined a more sensitive method for the measurement of esterified plasma F_2-isoprostanes by replacing the TLC steps with HPLC using an amino column with a hexane/2-propanol gradient. Pentafluorobenzyl (PFB) esters of F_2-isoprostanes were prepared and purified by HPLC, silylated, and then analyzed by GLC with NICI/MS. With this procedure, esterified plasma F_2-isoprostanes were found to be 8.3-fold higher in an end-stage renal failure patient on hemodialysis and urinary 8-iso-$PGF_{2\alpha}$ was 7.1-fold higher in a cigarette smoker than respective control subjects.

Chemical and Biochemical Reactivity

It has been well established that during peroxidation, PUFA are converted to LOOH, which decompose to form highly reactive breakdown products, such as

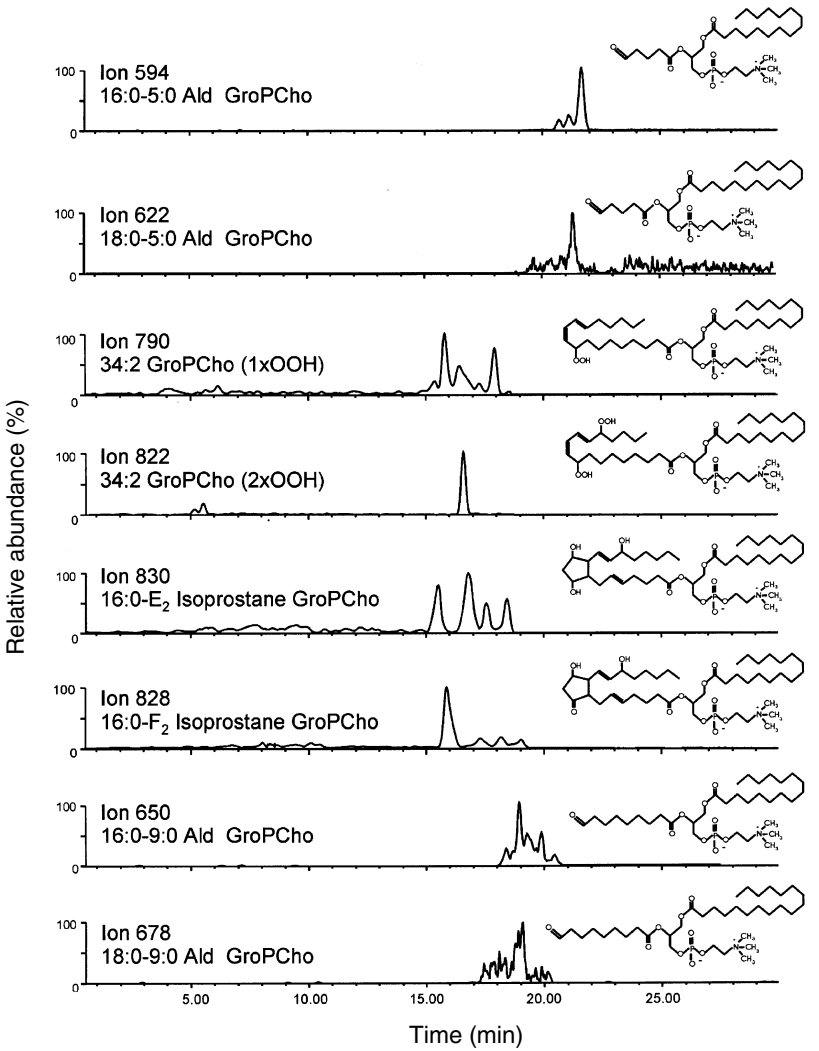


Fig. 6.9. Normal phase liquid chromatography (LC)/electrospray ionization (ESI)/mass spectrometry (MS) single-ion monitoring of oxidized phosphatidylcholines in human atheroma. Peak identification is as given in the figure. LC/ESI/MS conditions are as given in Figure 6.10. Ald, core aldehyde; GroPCho, glycerophosphocholine. Reproduced from Ravandi and Kuksis (2000) with permission of the publisher.

MDA and 4-hydroxynonenal (4-HNE) (Esterbauer *et al.* 1987). Such reactive aldehydes can form covalent Schiff base and Michael-type adducts with lysine residues of apoB, the protein moiety of LDL (Esterbauer *et al.* 1991). The core aldehydes of cholesteryl esters (CE), TAG and GPL also readily react with aldehyde group

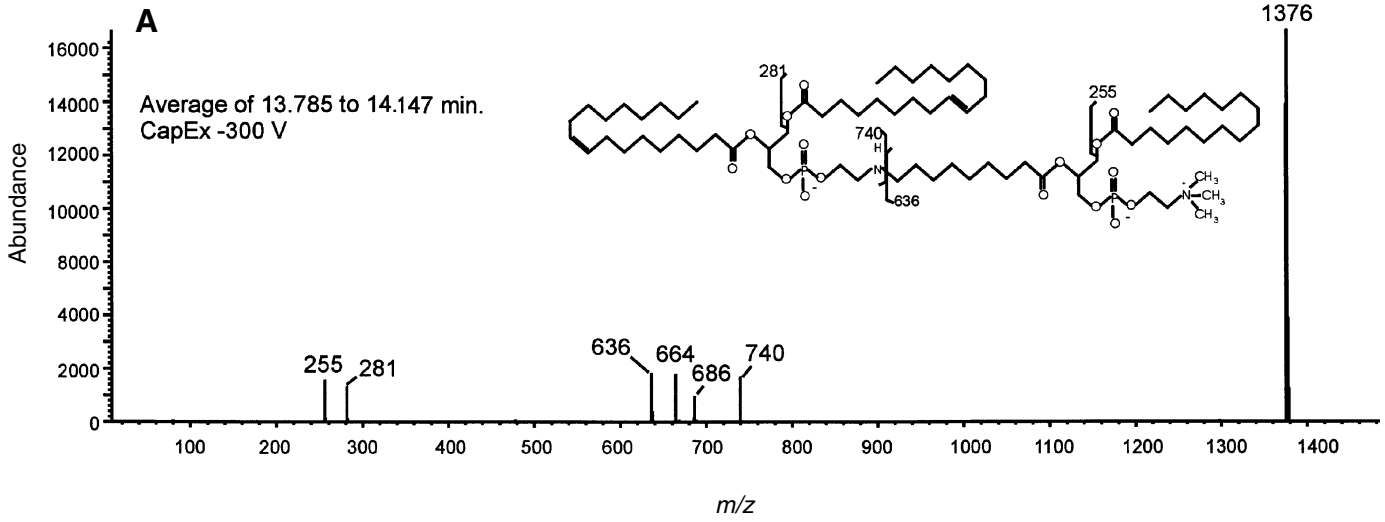
reagents despite their relatively high molecular weight, as already noted for the formation of UV-absorbing and fluorescent derivatives (see above). In addition, the core aldehydes participate in aldol condensation–type reactions and are subject to further oxidation to acids and reduction to alcohols.

Schiff Base Formation

The reactive phospholipid core aldehydes form Schiff base adducts with amino acids, polypeptides, and with amino group–containing phospholipids, such as PtdEtn and phosphatidyl serine (PtdSer) at room temperature (Ravandi *et al.* 1997). Figure 6.10 shows the normal phase LC/ESI/MS fragmentation spectra of (A) the reduced Schiff base of dioleoyl GroPEtn and 16:0/9:0Ald GroPCho at negative Cap Ex voltage of –300 V, and (B) of positive Cap Ex voltage of +300 V. The generated ions correspond to the anticipated cleavages depicted in the sketches accompanying the figures. Figure 6.11 shows the total positive ion current profile, the single-ion mass chromatograms, and the full ESI mass spectrum of the reduced Schiff base adduct between free lysine and the 16:0–9:0Ald GroPCho (reduced, *m/z* 778; nonreduced, *m/z* 780). Similarly, the Schiff bases of other α-amino and ε-amino acids are readily formed and can be isolated and characterized after *in situ* hydrogenation. Hazen *et al.* (1999) reported the synthesis, isolation, and characterization of the adduct formed in the reaction of *p*-hydroxyphenylacetaldehyde with the amino headgroup of PtdEtn and PtdSer. Ravandi *et al.* (1997) also demonstrated that the PtdCho core aldehydes yield covalently bonded complexes with myoglobin. Earlier, Wang and Tai (1990) successfully conjugated a *sn*-1-(9-oxo)nonanyl-*sn*-2-acetyl GroPCho with thyroglobulin *via* reductive amination and then used it to immunize rabbits for the production of specific antibodies.

Earlier, Beppu *et al.* (1987) had detected oxidized lipid-modified erythrocyte membrane proteins by radiolabeling with tritiated borohydride. The tritium incorporation closely correlated with membrane lipid oxidation as assessed by formation of TBARS and fluorescent substances. Possible candidates for producing borohydride-reducible functions in the proteins are various mono- and bi-functional aldehydes, as well as those for producing fluorescence and cross-links. The most probable candidates for them are Schiff's bases formed by the reaction of aldehydes with amino groups of proteins. They are susceptible to borohydride reduction, which stabilizes the bases. Hörkkö *et al.* (1997) showed that incubation of human β_2 glycoprotein 1 with oxidized 1-palmitoyl-2-linoleoyl [1-^{14}C]GroPCho, but not dipalmitoyl-[1-^{14}C]GroPCho, led to the formation of covalent adducts recognized by antiphospholipid sera. It was concluded that the reactive groups of oxophospholipids, such as core aldehydes generated during decomposition of oxidized PUFA, form covalent adducts with β_2-glycoprotein1 and other proteins.

High reactivity has since been demonstrated for the C_9 core aldehyde of 2-monoacylglycerol (Kurvinen *et al.* 1999) and cholesteryl esters (Kamido *et al.* 1995). These *in vitro* findings are consistent with the demonstration that oxidized LDL and HDL, as well as atheroma tissue, retain a certain amount of core aldehydes



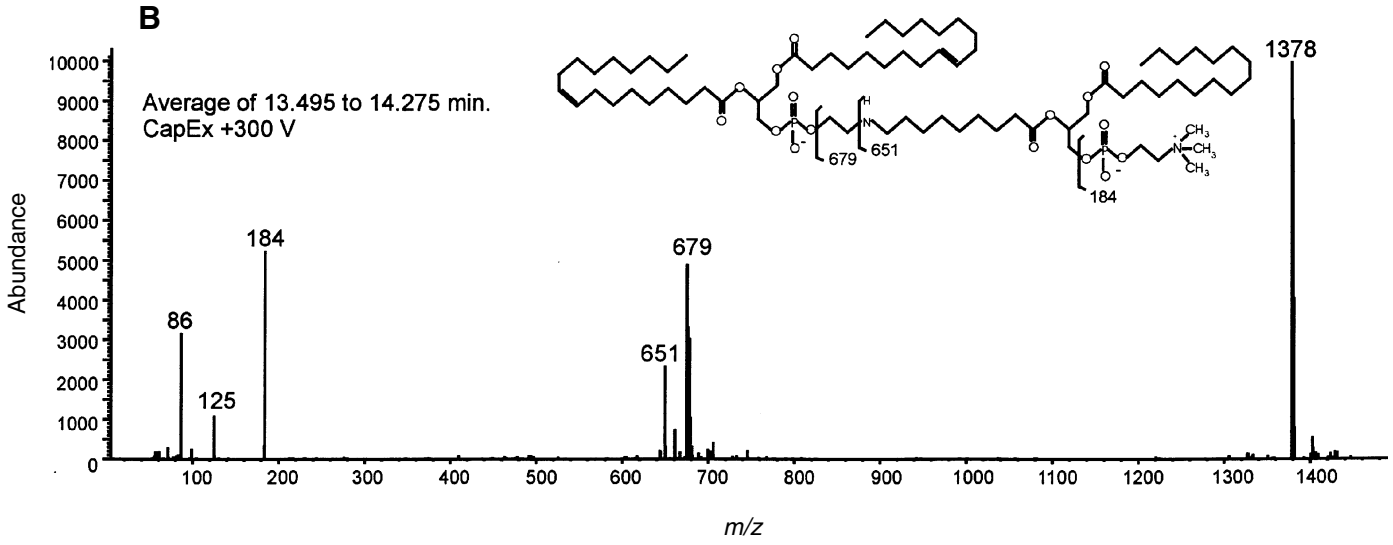


Fig. 6.10. Normal phase liquid chromatography (LC)/electrospray ionization (ESI)/mass spectrometry (MS) fragmentation spectra of the reduced Schiff base of dioleoyl glycerophosphoethanolamine (GroPEtn) and 16:0/9:0Ald glycerophosphocholine (GroPCho) at (A) a negative Cap Ex voltage of –300 V and (B) a positive Cap Ex voltage of +300 V. LC/ESI/MS conditions are as given in the figure. Other chromatographic and mass spectrometric conditions are as given in Figure 6.8. The double bonds in the *sn*-1-fatty chains are *cis* and not *trans* as would appear from the artwork. Reproduced from Ravandi *et al.* (1997) with permission of the publisher.

in the form of Schiff's bases, from which they can be displaced by brief incubation with dinitrophenylhydrazine (Kamido *et al.* 1996). Buldt and Karst (1997) showed that 1-methyl-(2,4-dinitrophenyl)hydrazine (MDNPH) reacts with aldehydes to give the corresponding hydrazones in the presence of an acid as catalyst. In contrast to other hydrazine reagents, MDNPH is oxidized by both ozone and nitrogen dioxide quantitatively to *N*-methyl-2,4-dinitroanaline, which can be separated from the hydrazones of the lower aldehydes by means of HPLC. This reaction has not been evaluated for lipid ester core aldehydes.

Ahmed *et al.* (2003a and 2003b) reported that the formation of AI-PtdCho core aldehyde Schiff's base adducts promotes uptake by an acute monocytic leukemia cell line (THP-1) macrophages. Oxidation of HDL increased formation of PtdCho core aldehyde covalent binding to HDL protein from 0.96 to 8.5 phosphorus/HDL protein (mole/mole). The binding and uptake of PtdCho core aldehyde-apoAI proteoliposomes by THP-1 macrophages was similar to that observed for oxidized HDL and oxidized LDL. Greilberger and Jurgens (1998) reported a similar covalent binding of MDA and 4-HNE to apoAI and apoAII, which influences binding of oxidized LDL to type I and type III collagen *in vitro*. Silva *et al.* (2001) reported that peroxynitrite-modified ^{99M}TC-β-VLDL is rapidly cleared from plasma and accumulates in several tissues, mainly in the liver and kidney. The exact nature of the free radical modification of the VLDL was not established.

Aldol Condensation

Friedman *et al.* (2002) demonstrated that the core aldehydes may follow a postoxidation chemical pathway involving aldol condensation. An immunoglobulin M monoclonal auto-antibody (EO6) to oxidized phospholipids blocks the uptake of oxidized LDL by macrophages. Friedman *et al.* (2002) relied on this property to define the structural motifs of oxidized phospholipids responsible for antigenicity for EO6 using synthetic substrates. Oxidized phospholipids containing *sn*-1-long-chain fatty acids were not antigenic unless the *sn*-2-position contained an aldehyde that first reacted with a peptide yielding a Schiff base or the *sn*-2-oxidized fatty acid underwent an aldol-type self-condensation. Friedman *et al.* (2002) suggested that upon oxidation, similar reactions occur in phospholipids on the surface of LDL, generating ligands for macrophage recognition. Synthetic immune adducts of oxidized phospholipids of this type were capable of blocking the uptake of oxidized LDL.

Other Chemical Reactions

Uppu *et al.* (1997) examined the reactions of peroxynitrite with short-chain aliphatic aldehydes to model the reaction of the peroxynitrite anion ($ONOO^-$) with CO_2. Aliphatic aldehydes, such as CO_2, react rapidly with peroxynitrite and catalyze its decomposition. The pH dependence of the reaction is consistent with the addition of $ONOO^-$ to the carbonyl carbon atom of the free aldehyde forming a

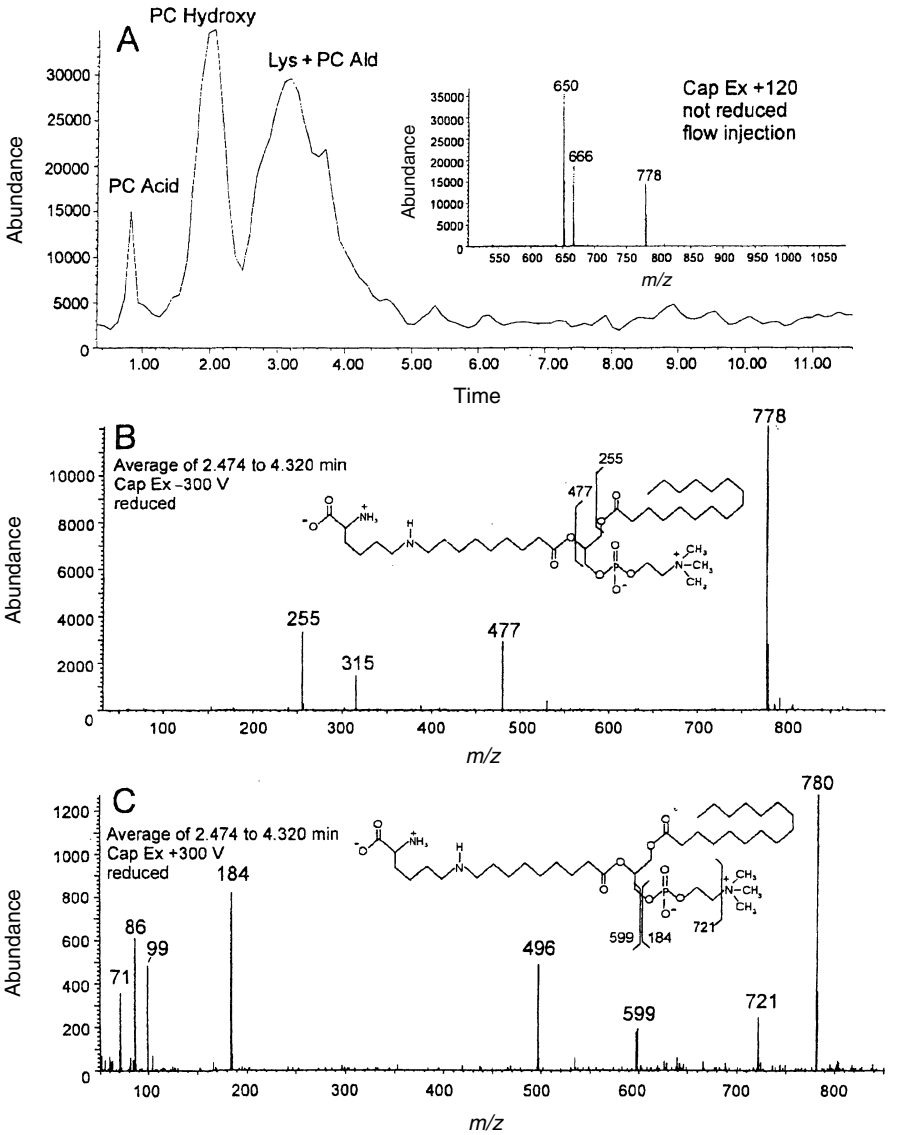


Fig. 6.11. Reverse phase liquid chromatography (LC)/electrospray ionization (ESI)/mass spectrometry (MS) of (A) the reduced reaction products of free lysine and 16:0/9:0Ald glycerophosphocholine (GroPCho) and (B) fragmentation spectra of the reduced Schiff base free lysine and 16:0/9:0AldGroPCho at negative Cap Ex voltage of –300 V and (C) at positive Cap Ex voltage of +300 V. Chromatographic and mass spectrometric conditions are as given in Figure 6.8. The generated ions correspond to the plausible bond cleavage products depicted in the sketches accompanying the figures. Reproduced from Ravandi *et al.* (1997) with permission of the publisher.

1-hydroxyalkylperoxynitrite anion adduct, which structurally resembles the nitrosoperoxycarbonate adduct formed from the reaction of $ONOO^-$ with CO_2. In analogy with the peroxynitrite/CO_2 system, Uppu *et al.* (1997) suggested that the anion adduct undergoes hemolytic or heterolytic cleavages at the O-O bond, giving a caged radical pair [$RCH(OH)ONO_2$] that can dissociate to give nitrate and regenerate the aldehyde. Zurek and Karst (2000) described the quantification of aldehydes and ketones by LC/MS using an internal calibration approach with stable isotope–labeled standards and HPLC-APCI/MS. 2,4-Dinitro-3,5,6-trideuterophenylhydrazine and many of its hydrazone derivatives were synthesized for this purpose. The results exhibited good agreement with quantification data obtained with UV detection.

Biological Significance

Oxophospholipids containing carbonyl moieties, derived from oxidatively fragmented PUFA in phospholipids, initially attracted attention mainly as mimics of PAF. At present, oxophospholipids are known to be involved in such effects as the activation of neutrophils, aggregation of platelets, lysis of red blood cells, gene activation, and development of atherosclerosis.

Enzyme Substrates

The core aldehydes of PtdCho are effective substrates for PLA_2 and PAF acetyl hydrolase (Tokumura 1995) and lecithin-cholesterol acyltransferase (LCAT) (Smiley *et al.* 1991, Subramanian *et al.* 1999, Itabe *et al.* 1999), while the core aldehydes of TAG are subject to hydrolysis by pancreatic lipase (Kurvinen *et al.* 1999). The core aldehyde esters containing GroPCho are also subject to hydrolysis by PLC and PLD. It is not known whether the core aldehyde esters of glycerol are also subject to hydrolysis by lipoprotein lipase and hepatic lipase. The aldehydes are also subject to hydrolysis by paraoxonase (Ahmed *et al.* 2003a and 2002b). In addition, the core aldehydes are subject to endogenous oxidases and reductases because both the corresponding acids and alcohol are found among the autoxidation products of polyunsaturated GPL.

Paraoxonase-1 is a HDL-bound enzyme, which acts on a variety of aromatic and aliphatic substrates. Ahmed *et al.* (2003a and 2002b) recently showed that paraoxonase 1 hydrolyzed 1-palmitoyl-2-(9-oxo)nonanoyl GroPCho and 1-palmitoyl-2-(5-oxo) valeroyl GroPCho to lysoPtdCho. The hydrolysis was not affected by Pefabloc, a serine esterase inhibitor. There was no detectable release of linoleate, arachidonate, or their hydroperoxyl or hydroxyl derivatives in the presence of paraoxonase-1; hence the enzyme activity could not be attributed to a simple PLA_2. Reddy *et al.* (2001) reported the identification of human paraoxonase-3 as an HDL-associated enzyme with biological activity similar to the paraoxonase-1 protein, whose expression is not regulated by oxidized lipids in HepG2 cells. Grune *et al.* (1997) identified GSH conjugation as a pathway for the metabolism of 4-hydroxynonenal and demonstrated its selective excretion by the kidney as the secondary product hydroxynonenal-mercapturic acid conjugate. The formation of the GSH-HNE adduct is a GSH-transferase–catalyzed reaction.

HNE-mercapturic acid is a stable product of HNE metabolism (Alary *et al.* 1995). These HNE-metabolism pathways are in accordance with those found by Siems *et al.* (1995). A possible metabolism of the phospholipid-bound aldehydes by pathways parallel to those just described for 4-HNE has not been investigated, and core aldehyde conjugation to glutathione has not been demonstrated. Subramanian *et al.* (1999) oxidized 16:0-[^{14}C]-18:2 GroPCho, equilibrated with plasma PtdCho, to obtain labeled short- and long-chain OxPtdCho. The truncated OxPtdCho were shown to be hydrolyzed by PAF acetylhydrolase. Furthermore, a labeled *sn*-2-acyl group from the long-chain OxPtdCho was not only hydrolyzed to free fatty acid, but was preferentially transferred to DAG in both the normal and PAF-AH–deficient plasmas.

Enzyme Inhibition

Halliwell and Gutteridge (1989) pointed out that the aldehydes produced during lipid peroxidation can attack the primary amino groups of proteins. Bifunctional aldehydes such as MDA can cross-link protein, whereas hydroxyalkenals can destroy sulfhydryl groups in proteins. Lipid peroxidation inactivates many enzymes, both cytosolic and membrane bound. Thomas and Poznansky (1990) prepared an early listing of the affected enzymes. In addition, they showed that lipid peroxidation inactivates rat liver microsomal glycerol-3-phosphate acyl transferase. The mechanism of this effect, however, was not established. Apparently, unaware of the previous work on the lipid peroxide inactivation of glyceraldehyde dehydrogenase by Thomas and Poznansky (1990), Morgan *et al.* (2002) reported the inhibition of glyceraldehyde-3-phosphate dehydrogenase by peptide and protein peroxides generated by singlet oxygen attack. Although the formation of lipid ester aldehydes as secondary products of lipid peroxidation, which must have also taken place, was not specifically mentioned, it was not excluded. It is likely that both lipid and peptide peroxidation lead to the same mechanism of inactivation of the glyceraldehydes-3-phosphate dehydrogenase.

The report of Morgan *et al.* (2002) is important in view of the demonstration of Gieseg *et al.* (2000) that peroxidation of proteins occurs before lipids in U937 cells exposed to peroxyl radicals. The peroxyradicals were generated by the thermal decomposition of a water-soluble azo compound. This led to the gradual accumulation of hydroperoxide groups. There was no lag period before the onset of peroxidation, indicating that cell antioxidants could not protect the proteins. The half-life of protein hydroperoxides in cell suspensions was ~4 h at 37°C. Hoppe *et al.* (1997) reported that the C_9 cholesteryl ester core aldehyde resisted hydrolysis by cholesteryl ester hydrolase and inhibited its activity. Millar and Leaver (2000) found that the cytotoxic peroxidation product, 4-HNE, specifically inhibited decarboxylating dehydrogenases in the matrix of plant mitochondria.

Cell Lysis and Cell Adhesion

Cell lysis was one of the effects anticipated by the early workers (Grzelinska *et al.* 1979) who first predicted adverse effects of core aldehydes on membrane structure

and function. Clemens and Waller (1987) reviewed the early evidence for lipid peroxidation of erythrocytes and concluded that it was minimal in view of the extensive protective mechanisms found there. Aldehydic GroPCho containing an oxovaleroyl moiety at the *sn*-2-position have the ability to activate the adhesive response of neutrophils, as does PAF (Tanaka *et al.* 1994, Tokumura 1995). Hexadecyl 2-succinyl glycerophosphocholine, like PAF, induces the aggregation of platelets very effectively at the extremely low concentration of 10^{-7} mol (Tanaka *et al.* 1994). There is evidence for accumulation of PtdCho core aldehydes in plasma of burn patients (Gasser *et al.* 1995) and for an increased core aldehyde production in the stomach by decomposition of dietary hydroperoxides (Kanazawa and Ashida 1998).

Watson *et al.* (1999) recognized three compounds present in minimally oxidized LDL, in oxidized 1-palmitoyl-2-arachidonoyl GroPCho, and in rabbit atherosclerotic lesions, that stimulate endothelial cells to bind monocytes *in vitro*. All were derived from the oxidation of arachidonic aid–containing GroPCho in LDL. Specifically, Watson *et al.* (1999) found that antibodies to these lipids were produced spontaneously *in vivo* by the apoE knockout mice that were genetically predisposed to develop atherosclerosis. Two of the biologically active compounds were produced by oxidative fragmentation of the arachidonic acid moiety in the *sn*-2-position of palmitoylarachidonoyl GroPCho and were identified as 1-palmitoyl-2-(5-oxo)valeroyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine. Chang *et al.* (1999) found that the binding of oxidized LDL to mouse CD36 is mediated in part by oxidized phospholipids that are associated with both the lipid and protein moieties of the lipoprotein. Gilotte *et al.* (2000) followed up this report by claiming that oxidized phospholipids, linked to apoB of oxidized LDL, are ligands for macrophage scavenger receptors.

Balasubramanian *et al.* (2001) demonstrated Ca^{2+}-dependent binding of annexin V to MDA-lipid adducts, which is comparable to its binding to phosphatidylinositol (PtdIns). Similar to oxidized LDL and arachidonate-containing PtdCho, MDA-lipid adducts also promoted the "PtdSer-dependent" prothrombinase assay. The authors suggested the possibility that aldehyde-modified lipids might participate in various "PS-dependent" processes. These results indicate that annexin V binding cannot be used as an exclusive indicator of cell surface PtdSer and raise the possibility that some phenomena attributed to PtdSer may, in fact, also involve aldehyde-lipid products. Camandola *et al.* (2000) reported that exposure of cultured rat cortical neurons to an apoptotic concentration of HNE resulted in a large increase in activating protein-1 (AP-1) DNA-binding activity.

Antigen Formation and Inflammatory Response

Phospholipids isolated from oxidized LDL that induced monocyte-endothelial cell interaction were found to be 1-hexadecanoyl-2-(5-oxo)pentanoyl)-GroPCho and I-hexadecanoyl-2-(5-carboxy)pentanoyl-GroPCho (Watson *et al.* 1997). Biologically

active oxidized phospholipids in LDL with short alkyl chains in the *sn*-2-position (acetate and butanoate) and an alkyl ether substituent at the *sn*-1-position were also identified (Marathe *et al.* 2000). Leitinger *et al.* (1999) and Subbanagounder *et al.* (2000) reported regulation of endothelial cell inflammatory functions by oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (Ox-PAPC) and three component oxidized phospholipids, containing oxovaleroyl (POVPC), glutaroyl (PGPC), and epoxyisoprostane (PEIPC) groups at the *sn*-2-position of oxidized phospholipids. Subbanagounder *et al.* (2002) subsequently demonstrated the presence of γ-hydroxy-α,β-unsaturated aldehydic phospholipid, 1-palmitoyl-2-(5-hydroxy-8-oxooxt-6-enoyl)-*sn*-glycero-3-phosphocholine (HOOA-PC; *m/z* 650.4), in Ox-PAPC by LC/MS, LC/MS/MS, derivatization, and MS/MS analyses. HOOA-PC dose dependently activated human aortic endothelial cells (HAEC) to bind monocytes (twofold at 10 μg/mL) and caused a dose-dependent increase (two- to threefold) in levels of monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8), chemokines that are important in monocyte entry into chronic lesions. It was concluded that the HOOA-PC exerts its effects on endothelial cells as a free lipid.

Friedman *et al.* (2002) correlated the antiphospholipid antibody recognition with the structure of synthetic oxidized phospholipids and recognized the importance of Schiff base formation and aldol condensation in this process. Oxidized phospholipids containing *sn*-1 long-chain fatty acids were not antigenic unless the *sn*-2-oxidized fatty acid contained an aldehyde that first reacted with a peptide yielding a Schiff base or the *sn*-2-oxidized fatty acid underwent an aldol-type self-condensation. In contrast to previous reports (Hörkkö *et al.* 1999), Friedman *et al.*(2002) observed that freshly prepared POVPC failed to bind EO6 antibody.

C-reactive protein (CRP) is an acute phase protein that binds specifically to PtdCho as a component of microbial capsular polysaccharide and precipitates the innate immune response against microorganisms. Chang *et al.* (2002) reported that CRP binds to oxidized LDL (OxLDL) and oxidized PtdCho, but does not bind to native, nonoxidized LDL or to nonoxidized PtdCho, yet its binding is mediated through the recognition of a PC moiety. Chang *et al.* (2002) suggested that CRP binds OxLDL and apoptotic cells by recognition of a PC moiety that becomes accessible as a result of oxidation of a PtdCho molecule, and that analogous to EO6 and scavenger receptors, CRP is a part of the innate immune response to oxidized PC-bearing phospholipids within OxLDL and on the plasma membranes of apoptotic cells. For further discussion, see Hazen and Chisholm (2002).

Gene Expression

Bochkov *et al.* (2002a and 2002b) reported that oxidized phospholipids stimulate tissue factor (TF) expression in human endothelial cells *via* activation of ERK/EGR-1 and Ca^{2+}/nuclear factor of activated T cells (NFAT). Specifically, it was shown that expression of TF was elevated by oxidized palmitoylarachidonoyl GroPCho, and that this induction was mediated mainly by early growth response factor-1 and NFAT-dependent transcription, but was independent of NF-κB activation. Upstream

mechanisms activated by oxidized palmitoyl-arachidonoyl GroPCho were elevation of cytosolic Ca^{2+}, activation of protein kinase C, and the mitogen-activated protein kinase/extracellular signal-regulated kinase cascade. Thus, oxidized phospholipids may contribute to inflammation by activating pathways alternative to the classical NF-κB pathway.

Kadl *et al.* (2002) recently analyzed the expression of inflammatory genes induced by oxidized L-α-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (OXPAPC) *in vitro* and *in vivo* using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Cultured human umbilical vein endothelial cells (HUVEC) and monocyte-like U937 cells were treated with OxPAPXC or lipopolysaccharide (LPS) for 3 h. For *in vivo* studies, OxPAPC or LPS was injected intravenously into female C57B1/6J mice and different tissues were isolated after 3 h. It was found that both OxPAPC and LPS induced expression of early growth response factor 1 (EGR-1) and monocyte chemoattractant protein 1 (MCP-1) in HUVEC, and of JE, the mouse homolog of MCP-1, in liver and heart. OxPAPC, but not LPS, increased expression of heme oxygenase 1 (HO-1) in U937 cells, HUVEC, aorta, heart, liver, and isolated blood cells. Furthermore, OxPAPC-induced expression of HO-1 was blocked by a PAF receptor antagonist.

Ehara *et al.* (2001) estimated that oxidized LDL in patients was only 0.25% of the total LDL, as estimated using a sandwich enzyme-linked immunosorbent assay (ELISA) with the murine monoclonal antibody DLH3. This antibody recognizes oxidized phosphatidylcholine, including adducts with proteins that are not apoB (Itabe *et al.* 1996). Tsimikas and Witztum (2000) also observed up to a 60% increase in autoantibody titers to several model-epitopes of oxidized LDL, such as MDA-LDL, copper-oxidized LDL, and 1-palmitoyl-2-(5-oxoglutaroyl)-3-GroPCho, whereas Hörkkö *et al.* (1999) showed up to a 35% increase of circulating levels of an oxidation-specific epitope of oxidized LDL, defined by EO6, a natural murine monoclonal auto-antibody that likely binds an oxidized phospholipid epitope similar to DLH3. Finally, Fuhrman *et al.* (2002) demonstrated that oxidative stress increases the expression of the CD36 scavenger receptor and the cellular uptake of oxidized LDL in macrophages from atherosclerotic mice, and that antioxidants and paraoxonase play a protective role.

Atherosclerosis

There is increasing evidence that lipid oxidation products may play an important role in the pathogenesis of atherosclerosis as well as other chronic diseases (Berliner 2002, Mackness *et al.* 2002, Navab *et al.* 2002, Uchida 2000). Subbanagounder *et al.* (1999) presented evidence that phospholipid oxidation products play an important role in early atherogenesis. These observations suggest that extensive oxidation of biomembranes produces proinflammatory phospholipids similar to PAF *via* the oxidative fragmentation of PUFA in phospholipids to short-chain carbonyl residues. Furthermore, there is evidence that lipid ester core aldehyde formation is increased under certain dietary and disease conditions.

Bartfay *et al.* (1999) correlated cardiac function and cytotoxic aldehyde production in a murine model of chronic iron overload. Although no single mechanism was likely to account for the complex pathophysiology of iron-induced heat failure, the findings showed that chronic iron overloading in a murine model results in dose-dependent alterations to cardiac function, and results in free radical–mediated damage to the heart, as measured by excess concentrations of cytotoxic aldehyde-derived peroxidation products. Kamido *et al.* (2002) correlated platelet aggregation with physiologically relevant concentrations of alkylGroPCho core aldehydes. Aggregations induced by C_5 alkyl GroPCho core aldehydes were completely inhibited by two different PAF receptor antagonists. 1-Palmitoyl-2-(5-oxovaleroyl)-*sn*-GroPCho induced platelet shape change but not aggregation. In contrast, C_5 alkyl and C_5 acyl GroPCho core aldehydes both inhibited endothelium-dependent relaxation of rabbit artery by 50%, whereas endothelium independent relaxation was not affected.

Nishi *et al.* (2002) made a comparable correlation between lipid peroxidation and carotid plaque formation, except that in this case, TBARS was used as a measure of lipid peroxidation and the plaque formation was examined morphometrically. The data showed that lipid peroxidation in carotid plaques was significantly associated with carotid atherosclerosis, especially plaque instability. Furthermore, glucosylation of PtdEtn promotes lipid peroxidation and core aldehyde formation in LDL and possibly in atheroma in diabetes (Ravandi *et al.* 1996).

Concluding Remarks

The previous 10 years have seen much progress made in the determination of the core aldehydes and other oxidation products of intact GPL. The work with the oxygenated PtdChos demonstrated their biological activity and association with plasma lipoproteins and atherosclerotic lesions. There is now evidence that PAF and/or PAF-like phospholipid oxidation products are important mediators of atherosclerotic lesion development *in vivo* and that specific receptor antagonists for these molecules may represent a novel therapeutic approach. Specifically, the core aldehydes or their Schiff bases may serve as model epitopes for antibodies that block the uptake of oxidized LDL by macrophages and smooth muscle cells. Similarly, the role of core aldehydes in the activation of transcription factors and the induction of specific gene expression may be of interest in studies of inflammatory response. The current opinion now held is that HDL- and LDL-derived oxidized phospholipids may be part of a system of nonspecific innate immunity. The most important outcome of the studies of oxophospholipid formation and function is evidence that both can be manipulated to prevent or minimize disease.

Acknowledgments

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References

Ahmed, Z., Babaei, S., Maguire, G.F., Dragonov, D., Kuksis, A., La Du, B.N., and Connelly, P.W. (2003a) Paraoxonase-1 Reduces Monocyte Chemotaxis and Adhesion to Endothelial Cells Due to Oxidation of Palmitoyl, Linoleoyl Glycerophosphocholine, *Cardiovasc. Res. 57*, 225–231.

Ahmed, Z., Ravandi, A., MaGuire, G.F., Emili, A., Draganov, D., La Du, B.N., Kuksis, A., and Connolly, P.W. (2001) Apolipoprotein A-I Promotes the Formation of Phosphatidylcholine Core Aldehydes That Are Hydrolyzed by Paraoxonase (PON-1) During High Density Lipoprotein Oxidation with a Peroxynitrite Donor, *J. Biol. Chem. 276*, 24473–24481.

Ahmed, Z., Ravandi, A., Maguire, G.F., Emili, A., Draganov, D., La Du, B.N., Kuksis, A., and Connolly, P.W. (2002b) Multiple Substrates for Paraoxonase-1 During Oxidation of Phosphatidylcholine by Peroxynitrite, *Biochem. Biophys. Res. Commun. 290*, 391–396.

Ahmed, Z., Ravandi, A., Maguire, G.F., Kuksis, A., and Connelly, P.W. (2003b) Formation of Apolipoprotein A1—Phosphatidylcholine Core Aldehyde Schiff Base Adducts Promotes Uptake by THP-1 Macrophages, *Cardiovasc. Res.*, in press.

Ahotupa, M., Marniemi, J., Lehtimaki, T., Talvinen, K., Raitakari, O.T., Vasankari, T., Viikari, J., Luoma, J., and Yla-Herttula, S. (1998) Baseline Diene Conjugation in LDL Lipids as Direct Measure of *In Vivo* LDL Oxidation, *Clin. Biochem. 31*, 257–261.

Akasaka, K., Ohata, A., Ohrui, H., and Meguro, H. (1995) Automatic Determination of Hydroperoxides of Phosphatidylcholine and Phosphatidylethanolamine in Human Plasma, *J. Chromatogr. B 665*, 37–43.

Alary, J., Bravais, F., Cravedi, J.P., Debrauwer, L., Rao, D., and Bories, G. (1995) Mercapturic Acid Conjugates as Urinary End Metabolites of the Lipid Peroxidation Product 4-Hydroxynonenal in the Rat, *Chem. Res. Toxicol. 8*, 34–39.

Arshad, M.A.Q., Bhadra, S., Cohen, R.M., and Subbiah, M.T.R. (1991) Plasma Lipoprotein Peroxidation Potential: A Test to Evaluate Individual Susceptibility to Peroxidation, *Clin. Chem. 37*, 1756–1758.

Bailey, A.L., Wortley, G., Southon, S. (1997) Measurement of Aldehydes in Low Density Lipoproteins by High Performance Liquid Chromatography, *Free Radic. Biol. Med. 23*, 1078–1085.

Balasubramian, K., Bevers, E.M., Willems, G.M., and Schroit, A.J. (2001) Binding of Annexin V to Membrane Products of Lipid Peroxidation, *Biochemistry 40*, 8672–8676.

Bartfay, W.J., Dawwood, F., Wen, W.H., Lehotay, D.C., Hou, D., Bartfay, E., Luo, X., Backx, P.H., and Liu, P.P. (1999) Cardiac Function and Cytotoxic Aldehyde Production in a Murine Model of Chronic Iron-Overload, *Cardiovasc. Res. 43*, 892–900.

Beppu, M., Murakami, K., and Kikugawa, K. (1987) Detection of Oxidized Lipid-Modified Erythrocyte Membrane Proteins by Radiolabeling with Tritiated Borohydride, *Biochim. Biophys. Acta 897*, 169–179.

Berliner, J. (2002) Lipid Oxidation Products and Atherosclerosis, *Vasc. Pharmacol. 38*, 187–191.

Beroza, M., and Bieri, B.A. (1969) Ozone Generator for Microanalysis, *Microchem. Acta (Wien) 4*, 720–723.

Bligh, E.G., and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37*, 911–917.

Bochkov, V.N., Kadl, A., Huber, J., Gruber, F., Binder, B.R., and Leitinger, N. (2002a) Protective Role of Phospholipid Oxidation Products in Endotoxin-Induced Tissue Damage, *Nature 419*, 77–81.

Bochkov, V.N., Mechtcheriakova, D., Lucerna, M., Huber, J., Malli, R., Graier, W.F., Hofer, E., Binder, B.R., and Leitinger, N. (2002b) Oxidized Phospholipids Stimulate Tissue Factor Expression in Human Endothelial Cells *via* Activation of ERK/EGR-1 and Ca^{++}/NFAT, *Hem. Thromb. Vasc. Biol. 99*, 199–206.

Boechzelt, H., Karten, B., Abuja, P.M., Sattler, W., and Mittelbach, M. (1998) Synthesis of 9-Oxononanoyl Cholesterol by Ozonization, *J. Lipid Res. 39,* 1503–1507.

Boechzelt, H.G., Bauman, F., Karten, B., Abuja, P.M., Sattler, W., and Mittelbach, M. (1999) Synthesis of Core Aldehydes in: *Abstracts, 90th AOCS Annual Meeting and Expo*, Orlando, Florida, 9–12 May 1999, AOCS Press, Champaign, IL.

Borowitz, S.M., and Montgomery, C. (1989) The Role of Phospholipase A_2 in Microsomal Lipid Peroxidation Induced with *t*-Butylhydroperoxide, *Biochem. Biophys. Res. Commun. 1258*, 1021–1028.

Brash, A.R., Ingram, C.D., and Harris, T.M. (1987) Analysis of a Specific Oxygenation Reaction of Soybean Lipoxygenase-1 with Fatty Acids Esterified in Phospholipids, *Biochemistry 26*, 5465–5471.

Buldt, A., and Karst, U. (1997) 1-Methyl-1-(2,4-dinitropenyl)hydrazine as a New Reagent for the HPLC Determination of Aldehydes, *Anal. Chem. 69*, 3617–3622.

Burke, A., Lawson, J.A., Meagher, E.A., Rokach, J., and FitzGerald, G.A. (2000) Specific Analysis in Plasma and Urine of 2,3-Dinor-5,6-dihydro-isoprostane $F_{2\alpha}$-III, a Metabolite of Isoprostane $F_{2\alpha}$-III and an Oxidation Product of γ-Linolenic Acid, *J. Biol. Chem. 275*, 2499–2504.

Cailleux, A., and Allain, P. (1993) Is Pentane a Normal Constituent of Human Breath? *Free Radic. Res. Commun. 18*, 323–327.

Camandola, S., Poli, G., and Mattson, M.P. (2000) The Lipid Peroxidation Product 4-Hydroxy-2,3-nonenal Increases AP-1-Binding Activity Through Caspase Activation in Neurons, *J. Neurochem. 74*, 159–168.

Chang, M,-K., Binder, C.J., Torzewski, M., and Witztum, J.L. (2002) C-Reactive Protein Binds Both Oxidized LDL and Apoptotic Cells Through Recognition of a Common Ligand: Phosphorylcholine of Oxidized Phospholipids, *Proc. Natl. Acad. Sci. USA 99*, 13043–13048.

Clemens, M.R., and Waller, H.D. (1987) Lipid Peroxidation in Erythrocyte, *Chem. Phys. Lipids 45*, 251–268.

Deng, Y., and Salomon, R.G. (1998) Total Synthesis of γ-Hydroxy-α,β-Unsaturated Aldehydic Esters of Cholesterol and 2-Lysophosphatidylcholine, *J. Org. Chem. 63*, 7789–7794.

Deng, Y., and Salomon, R.G. (2000) Total Synthesis of Oxidized Phospholipids. 3. The (11*E*)-9-Hydroxy-13-Oxotridec-11-Enoate Ester of 2-Lysophosphatidylcholine, *J. Org. Chem. 65*, 6660–6665.

Draper, H.H., Squires, E.J., Mahmoodi, H., Wu, J., Agarwal, S., and Hadley, M. (1993) A Comparative Evaluation of Thiobarbituric Acid Methods for the Determination of Malonaldehyde in Biological Materials, *Free Radic. Biol. Med. 15*, 353–363.

Effenburger, F., Null, V., and Ziegler, T. (1992) Preparation of Optically Pure L-2-Hydroxyaldehydes with Transketolase, *Tetrahedron Letters 33*, 5157–5160.

Ehara, S., Ueda, M., Naruko, T., Haze, K., Itoh, A., Otsuka, M., Komatsu, R., Matsuo, T., Itabe, H., Takano, T., Tsukamoto, Y., Yoshiyama, M., Takeuchi, K., Yoshikawa, J., and Becker, A.E. (2001) Elevated Levels of Oxidized Low Density Lipoprotein Show a Positive Correlation with the Severity of Acute Coronary Syndromes, *Circulation 103*, 1955–1960.

Esterbauer, H., Dieber-Rotheneder, M., Waeg, G., Striegl, G., and Jurgens, G. (1990) Biochemical Structural and Functional Properties of Oxidized Low-Density Lipoprotein, *Chem. Res. Toxicol. 3*, 77–92.

Esterbauer, H., Jurgens, G., Quechenberger, O., and Koller, E. (1987) Autoxidation of Human Low Density Lipoprotein: Loss of Polyunsaturated Fatty Acids and Vitamin E and Generation of Aldehydes, *J. Lipid Res. 28*, 495–509.

Esterbauer, H., Schaur, R.J., and Zollner, H. (1991) Chemistry and Biochemistry of 4-Hydroxynonenal, Malonaldehyde and Related Aldehydes, *Free Radic. Biol. Med. 11*, 81–128.

Esterbauer, H., H. Zollner, and R.J. Schaur (1989) Aldehydes Formed by Lipid Peroxidation, in *Membrane Lipid Oxidation* (Vigo-Pelfrey, C., ed.) Vol. 1, pp. 239–268, CRC Press, Boca Raton, FL.

Fam, S.S., Murphey, L.J., Terry, E.S., Zackert, W.E., Chen, Y., Gao, L., Pandalai, S., Milne, G.L., Roberts, L.J., Porter, N.A., Montine, T.J., and Morrow, J.D. (2002) Formation of Highly Reactive A-Ring and J-Ring Isoprostane-Like Compounds (A_4/J_4-Neuroprostanes) *In Vivo* from Docosahexaenoic Acid, *J. Biol. Chem. 277*, 36076–36084.

Felde, R., and Spiteller, G. (1995) Plasmalogen Oxidation in Human Serum Lipoproteins, *Chem. Phys. Lipids 76*, 259–267.

Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226*, 497–509.

Frankel, E.N. (1982) Volatile Lipid Oxidation Products, *Prog. Lipid Res. 22*, 1–33.

Frankel, E.N. (1984) Lipid Oxidation: Mechanisms, Products and Biological Significance, *J. Am. Oil Chem. Soc. 61*, 1908–1917.

Frankel, E.N. (1985) Chemistry of Free Radical and Singlet Oxidation of Lipids, *Prog. Lipid Res. 23*, 197–221.

Frankel, E.N. (1999) *Lipid Oxidation*, pp. 13–22, The Oily Press, Dundee, Scotland.

Frey, B., Haupt, R., Alms, S., Holzmann, G., Konig, T., Kern, H., Kox, W., Rustow, B., and Schlame, M. (2000) Increase in Fragmented Phosphatidylcholine in Blood Plasma by Oxidative Stress, *J. Lipid Res. 41*, 1145–1153.

Friedman, P., Hörkkö, S., Steinberg, D., Witzum, J.L., and Dennis, E.A. (2002) Correlation of Antiphospholipid Antibody Recognition with the Structure of Synthetic Oxidized Phospholipids. Importance of Schiff Base Formation and Aldol Condensation, *J. Biol. Chem. 277*, 7010–7020.

Fuhrman, B., Volkova, N., and Aviram, M. (2002) Oxidative Stress Increases the Expression of the CD36 Scavenger Receptor and the Cellular Uptake of Oxidized Low-Density Lipoprotein in Macrophages from Atherosclerotic Mice: Protective Role of Antioxidants and Paraoxonase, *Atherosclerosis 161*, 307–316.

Gardner, H.W. (1989) Oxygen Radical Chemistry in Polyunsaturated Fatty Acids, *Free Radic. Biol. Med. 7*, 65–86.

Gasser, H., Hallstrom, S., Red, H., and Schlag, G. (1995) Oxidatively Modified Plasma Phospholipids Containing Relative Carbonyl Functions Measured by HPLC: Evidence for Phosphatidylcholine-Bound Aldehydes in Plasma of Burn Patients, *Free Radic. Res. 22*, 327–336.

Gieseg, S., Duggan, S., and Gebicki, J.M. (2000) Peroxidation of Proteins Before Lipids in U937 Cells Exposed to Peroxyl Radicals, *Biochem. J. 350*, 215–218.

Gillotte, K.L., Hörkkö, S., Witzum, J.L., and Steinberg, D. (2000) Oxidized Phospholipids, Linked to Apopoprotein B of Oxidized LDL, Are Ligands for Macrophage Scavenger Receptors, *J. Lipid Res. 41*, 824–833.

Girotti, A.W. (1998) Lipid Hydroperoxide Generation, Turnover, and Effector Action in Biological Systems, *J. Lipid Res. 39*, 1529–1542.

Greilberger, J., and Jurgens, G. (1998) Oxidation of High-Density Lipoprotein HDL_3 Leads to Exposure of Apo-AI And Apo-AII Epitopes and to Formation of Aldehyde Protein Adducts, and Influences Binding of Oxidized Low-Density Lipoprotein to Type I and Type III Collagen *In Vitro*, *Biochem. J. 331*, 185–191.

Grune, T., Siems, W.G., and Petras, T. (1997) Identification of Metabolic Pathways of the Lipid Peroxidation Product of 4-Hydroxynonenal in *In Situ* Perfused Rat Kidney, *J. Lipid Res. 38*, 1660–1665.

Grzelinska, E., Bartosz, G., Gwozdzinski, K. and Leyko, W. (1979) A Spin-Label Study of the Effect of Gamma Radiation on Erythrocyte Membrane. Influence of Lipid Peroxidation on Membrane Structure, *Int. J. Radiat. Biol. 36*, 325–334.

Guy, R.A., Maguire, G.F., Crandall, I., Connelly, P.W., and Kain, K.C. (2001) Characterization of Peroxynitrite-Oxidized Low Density Lipoprotein Binding to Human CD36, *Atherosclerosis 155*, 19–28.

Haginaka, J., Yamaguchi, Y., and Kunitomo, M. (2001) Anion-Exchange High-Performance Liquid Chromatography Assays of Plasma Lipoproteins and Modified Low-Density Lipoproteins Using a ProtEx-DEAE Column, *J. Chromatrogr. B 751*, 161–167.

Hahn, M., and Subbiah, M.T.R. (1994) Significant Association of Lipid Peroxidation Products with High Density Lipoproteins, *Biochem. Mol. Biol. Int. 33*, 699–704.

Hall, L.M., and Murphy, R.C. (1998) Analysis of Stable Oxidized Molecular Species of Glycerophospholipids Following Treatment of Red Blood Ghosts with *t*-Butylhydroperoxide, *Anal. Biochem. 258*, 184–194.

Halliwell, B., and Gutteridge, J.M.C. (1989) *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford.

Harrison, K.A., and Murphy, R.C. (1996) Direct Mass Spectrometric Analysis of Ozonides: Application to Unsaturated Glycerophospholipids, *Anal. Chem. 68*, 3224–3230.

Hazen, S.L., and Chisolm, G.M. (2002) Oxidized Phosphatidylcholines: Pattern Recognition Ligands for Multiple Pathways of the Innate Immune Response, *Proc. Natl. Acad. Sci. USA 99*, 12515–12517.

Hazen, S.L., Heller, J., Hsu, F.-F., d'Avignon, A., and Heinecke, J.W. (1999) Synthesis, Isolation and Characterization of the Adduct Formed in the Reaction of *p*-Hydroxyphenylacetaldehyde with the Amino Headgroup of Phosphatidylethanolamine and Phosphatidylserine, *Chem. Res. Toxicol. 12*, 19–27.

Heery, J.M., Kozak, M., Stafforini, D.M., Jones, D.A,., Zimmerman, G.A. McIntyre, T.M., and Prescott, S.M. (1995) Oxidatively Modified LDL Contains Phospholipids with Platelet-Activating Factor-Like Activity and Stimulates the Growth of Smooth Muscle Cells, *J. Clin. Investig. 96*, 2322–2330.

Helbock, H.J., Motchnik, P.A., and Ames, B.N. (1993) Toxic Hydroperoxides in Intravenous Lipid Emulsions Used in Preterm Infants, *Pediatrics 91*, 83–87.

Holley, A.E., Walker, M.K., Cheesemena, K.H., and Slater, T.F. (1993) Measurement of *n*-Alkanals and Hydroxyalkenals in Biological Samples, *Free Radic. Biol. Med. 15*, 281–289.

Hoppe, G., Ravandi, A., Herrera, D., Kuksis, A., and Hoff, H.F. (1997) Oxidation Products of Cholesteryl Linoleate Are Resistant to Hydrolysis in Macrophages, Form Complexes with Proteins and Are Present in Human Atherosclerotic Lesions, *J. Lipid Res. 38*, 1347–1360.

Hörkkö, S., Bird, D.A., Miller, E., Itabe, H., Leitinger, N., Subbanagounder, G., Berliner, J.A., Friedman, P., Dennis, E.A., Curtis, L.K., Palinski, W., and Witzum, J.L. (1999)

Monoclonal Autoantibodies Specific for Oxidized Phospholipids or Oxidized Phospholipid Protein Adducts Inhibit Macrophage Uptake of Oxidized Low-Density Lipoproteins, *J. Clin. Invest. 103*, 117–128.

Hörkkö, S., Miller, E., Branch, D.W., Palinski, W., and Witzum, J.L. (1997) The Epitopes for Some Antiphospholipid Antibodies Are Adducts of Oxidized Phospholipid and β2 Glycoprotein 1 (and Other Proteins), *Proc. Natl. Acad. Sci. USA 94*, 10356–10361.

Hörkkö, S., Miller, E., Dudl, E., Reaven, P., Curtiss, L.K., Zvaifler, N.J., Terkeltaub, R., Pierangeli, S.S., Branch, D.W., Palinski, W., and Witzum, J.L. (1996) Antiphospholipid Antibodies Are Directed Against Epitopes of Oxidized Phospholipids. Recognition of Cardiolipin by Monoclonal Antibodies to Epitopes of Oxidized Low Density Lipoprotein, *J. Clin. Invest. 98*, 815–825.

Horning, M.G., Moss, A.M., and Horning, E.C. (1968) Formation and Gas–Liquid Chromatographic Behavior of Isomeric Steroid Ketone Methoxime Derivatives, *Anal. Biochem. 22*, 284–294.

Huber, J., Vales, A., Mitulovic, G., Blumer, M., Schmid, R., Witzum, J.L., Binder, B.R., and Leitinger, N. (2002) Oxidized Membrane Vesicles and Blebs from Apoptotic Cells Contain Biologically Active Oxidized Phospholipids That Induce Monocyte-Endothelial Interactions, *Arterioscler. Thromb. Vasc. Biol. 22*, 101–107.

Itabe, H. (1998) Oxidized Phospholipids As a New Landmark in Atherosclerosis, *Prog. Lipid Res. 37*, 181–207.

Itabe, H., Hosoya, R., Karasawa, K., Jimi, S., Saku, K., Takebayasi, S., Imanaka, T., and Takano, T. (1999) Metabolism of Oxidized Phosphatidylcholines Formed in Oxidized Low Density Lipoprotein by Lecithin-Cholesterol Acyltransferase, *J. Biochem. 126*, 153–161.

Itabe, H., Kudo, I., and Inoue, K. (l988) Preferential Hydrolysis of Oxidized Phospholipids by Peritoneal Fluid of Rats Treated with Casein, *Biochim. Biophys. Acta 963*, 192–200.

Itabe, H., Yamamoto, H., Suzuki, M., Kawai, Y., Nakagawa, Y., Suzuki, A., Imanaka, T., and Takano, T. (1996) Oxidized Phosphatidylcholines That Modify Proteins: Analysis by Monoclonal Antibody Against Oxidized Low Density Lipoprotein, *J. Biol. Chem. 271*, 33208–33217.

Janero, D.R. (1990) Malonaldehyde and Thiobarbituric Acid-Reactivity as Diagnostic Indices of Lipid Peroxidation and Peroxidative Injury, *Free Radic. Biol. Med. 9*, 515–540.

Jurgens, G., Ashy, A., Esterbauer, H. (1990) Detection of New Epitopes Formed Upon Oxidation of Low-Density Lipoprotein, Lipoprotein (A) and Very Low Density Lipoprotein, *Biochem. J. 265*, 605–608.

Kadl, A., Huber, J., Gruber, F., Bochkov, V.N., Binder, B.R., and Leitinger, N. (2002) Analysis of Inflammatory Gene Induction by Oxidized Phospholipids *In Vivo* by Quantitative Real-Time RT-PCR in Comparison with Effects of LPS, *Vasc. Pharmacol. 38*, 219–227.

Kamal-Eldin, A., Marquez-Ruiz, G., Dobarganes, C., and Appelqvist, L.-A. (1997) Characterization of Aldehydic Acids in Used and Unused Frying Oils, *J. Chromatogr. A 776*, 245–254.

Kamido, H., Eguchi, H., Ikeda, H., Imaizumi, T., Yamana, K. Hartvigsen, K., Ravandi, A., and Kuksis, A. (2002a) Core Aldehydes of Alkyl Glycerophosphocholines in Atheroma Induce Platelet Aggregation and Inhibit Endothelium-Dependent Arterial Relaxation, *J. Lipid Res. 43*, 158–166.

Kamido, H., Koyama, K., Nonaka, K., Yamana, K., Kosuga, K., Kuksis, A,. and Marai, L. (1994) Cholesteryl Ester Core Aldehydes (Components of Oxidized Low Density

Lipoproteins) Exist in Human Atherosclerotic Lesions and Are Cytotoxic to Cultured Human Endothelial Cells, in *Abstracts, Xth International Symposium on Atherosclerosis*, Montreal, Quebec, 9–14 October 1994.

Kamido, H., Kuksis, A., Marai, L. and Myher, J.J. (1992) Identification of Cholesterol-Bound Aldehydes in Copper-Oxidized Low Density Lipoprotein, *FEBS Lett. 304*, 269–272.

Kamido, H., Kuksis, A., Marai, L., and Myher, J.J. (1993) Identification of Core Aldehydes Among *In Vitro* Peroxidation Products of Cholesteryl Esters, *Lipids 28*, 331–336.

Kamido, H., Kuksis, A., Marai, L., and Myher, J.J. (1995) Lipid Ester-Bound Aldehydes Among Copper-Catalyzed Peroxidation Products of Human Plasma Lipoproteins, *J. Lipid Res. 36*, 1876–1886.

Kamido, H., Kuksis, A., Marai, L., Myher, J.J., and Pang, H. (1992b) Preparation, Chromatography and Mass Spectrometry of Cholesteryl Ester and Glycerolipid-Bound Aldehydes, *Lipids 27*, 645–650.

Kamido, H., Nonaka, K., Kuksis, A., Marai, L., and Ravandi, A. (1996) Identification of Lipid Ester Core Aldehydes and Hydroperoxides in Human Atherosclerotic Lesions, *Circulation 94 (Suppl.)*, I-707.

Kanazawa, K., and Ashida, H. (1998) Dietary Hydroperoxides of Linoleic Acid Decompose to Aldehydes in Stomach Before Being Absorbed into the Body, *Biochim. Biophys. Acta 1393*, 349–361.

Karten, B., Boechzelt, H., Abuja, P.M., Mittelbach, M., Oettl, K., and Sattler, W. (1998) Femtomole Analysis of 9-Oxononanoyl Cholesterol by High Performance Liquid Chromatography, *J. Lipid Res. 39*, 1508–1519.

Kaur, K., Salomon, R.G., O'Neil, J., and Hoff, H.F. (1997) Carboxyalkylpyrroles in Human Plasma and Oxidized Low-Density Lipoproteins, *Chem. Res. Toxicol. 10*, 1387–1396.

Kawai, Y., Ogamo, A., and Nakagawa, Y. (1999) Formation of the Aldehydic Choline Glycerophospholipids in Human Red Blood Cell Membrane Peroxidized with an Azo Initiator, *J. Biochem. 126*, 115–120 (1999).

Kayganich-Harrison, K.A., Rose, D.M., Murphy, R.C., Morrow, J.D., and Roberts, L.J., II (1993) Collision-Induced Dissociation of F_2-Isoprostane-Containing Phospholipids, *J. Lipid Res. 34,* 1229–1235.

Kern, W., and Spiteller, G. (1996) Synthesis and Properties of Naturally Occurring α-Hydroxyaldehydes, *Tetrahedron 52*, 4347–4362.

Kinter, M. (1995) Analytical Technologies for Lipid Oxidation Products Analysis, *J. Chromatogr. B 671*, 223–236.

Kuksis, A. (1990) Core Aldehydes—Neglected Products of Lipid Peroxidation, *INFORM 1*, 1055–1060.

Kuksis, A. (2000) Core Aldehydes—No Longer Neglected, *INFORM 11*, 746–752.

Kuksis, A., Breckenridge, W.C., Marai, L., and Stachnyk, O. (1969) Molecular Species of Lecithins of Rat Heart, Kidney and Plasma, *J. Lipid Res. 10*, 25–32.

Kuksis, A., and Marai, L. (1967) Determination of Complete Structure of Natural Lecithins, *Lipids 2*, 217–224.

Kuksis, A., and Myher, J.J. (1980) New Approaches to Lipid Analysis of Lipoproteins and Cell Membranes, in *Control of Membrane Fluidity* (Kates, M., and Kuksis, A., eds.) pp. 3–32, Humana Press, Clifton, NJ.

Kuksis, A., Myher, J.J, Marai, L., and Geher, K. (1993) Analyses of Hydroperoxides and Core Aldehydes of Triacylglycerols, in *Proceedings, 17th Nordic Symposium* (Malkki, Y., ed.) pp. 230–238, Lipidforum Publication, Imatra, Finland.

Kuksis, A., Ravandi, A., Kamido, H., Marai, L., and Myher, J.J. (1995) Increased Phospholipid-Bound (Core) Aldehydes in Plasma and Red Cells of Diabetic Subjects, in *Oils Fats Lipids Proceedings* (Castenmiller, A.M., ed.) Vol. 3, pp. 279–284, 21st World Congress and Exhibition of the International Society for Fat Research, The Hague.

Kurvinen, J.-P., Kuksis, A., Ravandi, A., Sjovall, O., and Kallio, H. (1999) Rapid Complexing of Oxoacylglycerols with Amino Acids, Peptides and Aminophospholipids, *Lipids 34*, 299–305.

Lang, J., Celotto, C., and Esterbauer, H. (1985) Quantitative Determination of the Lipid Peroxidation Product 4-Hydroxynonenal by High Performance Liquid Chromatography, *Anal. Biochem. 150*, 369–378.

Lawson, J.A., Rokach, J., and FitzGerald, G.A. (1999) Isoprostanes: Formation, Analysis and Use as Indices of Lipid Peroxidation *In Vivo*, *J. Biol. Chem. 274*, 2441–2444.

Leitinger, N., Tyner, T.R., Oslund, L., Rizza, C., Subbanagounder, G., Lee, H., Shih, P.T., Mackman, N., Tigyi, G., Territo, M.C., Berliner, J.A., and Vora, D.K. (1999) Structurally Similar Oxidized Phospholipids Differentially Regulate Endothelial Binding of Monocytes and Neutrophils, *Proc. Natl. Acad. Sci. USA 96*, 12010–12015.

Lenz, M.L., Hughes, H., Mitchell, J.R., Via, D.P., Guyton, J.R., Taylor, A.A., Gotto, A.M., Jr., and Smith, C.V. (1990) Lipid Hydroperoxy and Hydroxy Derivatives in Copper-Catalyzed Oxidation of Low Density Lipoprotein, *J. Lipid Res. 31*, 1043–1050.

Liu, J., Yeo, H.C., Diniger, S.J., and Ames, B.N. (1997) Assay of Aldehydes from Lipid Peroxidation: Gas Chromatography-Mass Spectrometry Compared to Thiobarbituric Acid, *Anal. Biochem. 245*, 161–166.

Loidl-Stahlhofen, A., and Spiteller, G. (1994) Generation of α-Hydroxyaldehydic Compounds in the Course of Lipid Peroxidation, *Biochim. Biophys. Acta 1213*, 140–148.

Lynch, S.M., Morrow, J.D., Robertson, L.J., II and Frei, B. (1994) Formation of Non-Cyclooxygenase-Derived Prostanoids (F_2-Isoprostanes) in Plasma and Low Density Lipoprotein Exposed to Oxidative Stress *In Vitro*, *J. Clin. Investig. 93*, 998–1004.

Mackness, B., Durrington, P.N., and Mackness, M.I. (2002) The Paraoxonase Gene Family and Coronary Heart Disease, *Current Opin. Lipidol. 13*, 357–362.

MacMillan, D.K., and Murphy, R.C. (1995) Analysis of Lipid Hydroperoxides and Long-Chain Conjugated Keto Acids by Negative Ion Electrospray Mass Spectrometry, *J. Am. Soc. Mass Spectrom. 6*, 1190–1201.

Marathe, G.K., Harrison, K.A., Murphy, R.C., Prescott, S.M., Zimmerman, G.A., and McIntyre, T.M. (2000) Bioactive Phospholipid Oxidation Products, *Free Radic. Biol. Med. 28*, 1762–1770.

Mashima, R., Yamamoto, Y., and Yoshimura, S. (1998) Reduction of Phosphatidylcholine Hydroperoxide by Apolipoprotein A-I: Purification of the Hydroperoxide-Reducing Proteins from Human Blood Plasma, *J. Lipid Res. 39*, 1133–1140.

McIntyre, T.M., Zimmerman, G.A., and Prescott, S.M. (1999) Biologically Active Oxidized Phospholipids, *J. Biol. Chem. 274*, 25189–25192.

Meyer, C., Lutz, A., and Spiteller, G. (1992) α-Hydroxyaldehyde Derivatives Arising from Oxidation of Plasmalogens, *Angew. Chem. Int. Ed., Engl. 31*, 468–470.

Millar, A.H., and Leaver, C.J. (2000) The Cytotoxic Lipid Peroxidation Product, 4-Hydroxy-2-Nonenal, Specifically Inhibits Decarboxylating Dehydrogenases in the Matrix of Plant Mitochondria, *FEBS Lett. 481*, 117–121.

Milne, G.L., and Porter, N.A. (2001) Separation and Identification of Phospholipid Peroxidation Products, *Lipids 36*, 1265–1275.

Miyazawa, T., Yasuda, K., and Fujimoto, K. (1987) Chemiluminescence-High Performance Liquid Chromatography of Phosphatidylcholine Hydroperoxide, *Anal. Lett. 20*, 915–918.

Morgan, P.E., Dean, R.T., and Davies, M.J. (2002) Inhibition of Glyceraldehydes-3-Phosphate Dehydrogenase by Peptide and Protein Peroxides Generated by Singlet Oxygen Attack, *Eur. J. Biochem. 269*, 1916–1925.

Morrow, J.D., Harris, T.M., and Roberts, L.J., II (1990a) Noncyclooxygenase Oxidative Formation of a Series of Novel Prostaglandins: Analytical Ramifications for Measurement of Eicosanoids, *Anal. Biochem. 184*, 1–10.

Morrow, J.D., Hill, K.E., Burk, R.F., Nammour, T.M., Badr, K.F., and Roberts, L.J., II (1990b) A Series of Prostaglandin F_2-Like Compounds Are Produced *In Vivo* in Humans by a Non-Cyclooxygenase, Free-Radical Catalyzed Mechanism, *Proc. Natl. Acad. Sci. USA 87*, 9383–9387.

Morrow, J.D., and Roberts, L.J., II (1999) Mass Spectrometric Quantification of F_2-Isoprostanes in Biological Fluids and Tissues as Measure of Oxidant Stress, *Methods Enzymol. 300*, 3–12.

Murphy, R.C., Fiedler, J., and Hevko, J. (2001) Analysis of Nonvolatile Lipids by Mass Spectrometry, *Chem. Rev. 101*, 479–526.

Myher, J.J., and Kuksis, A. (1984) Resolution of Alkenylacylglycerol Moieties of Natural Glycerophospholipids by Gas–Liquid Chromatography on Polar Capillary Columns, *Can. J. Biochem. Cell Biol. 62*, 352–362.

Myher, J.J., Kuksis, A., and Pind, S. (1989a) Molecular Species of Glycerophospholipids and Sphingomyelins of Human Erythrocytes: Improved Method of Analysis, *Lipids 24*, 396–407.

Myher, J.J., Kuksis, A., and Pind, S. (1989b) Molecular Species of Glycerophospholipids and Sphingomyelins of Human Plasma: Comparison to Red Blood Cells, *Lipids 24*, 408–418.

Navab, M., Hama, S.Y., Ready, S.T., Ng, C.J., Van Lenten, B.J., Laks, H., and Fogelman, A.M. (2002) Oxidized Lipids as Mediators of Coronary Heart Disease, *Curr. Opin. Lipidol. 13*, 363–372.

Nishi, K., Uno, M., Fukuzawa, K., Horiguchi, H., Shinno, K., and Nagahiro, S. (2002) Clinocopathological Significance of Lipid Peroxidation in Carotid Plaques, *Atherosclerosis 160*, 289–296.

Nourooz-Zadeh, J., Halliwell, B., and Anggard, E.E. (1997) Evidence for the Formation of F_3-Isoprostanes During Peroxidation of Eicosapentaenoic Acid, *Biochem. Biophys. Res. Commun. 236*, 467–472.

Ou, Z., A. Ogamo, L. Guo, Y. Konda, Y. Harigaya, and Y. Nakagawa (1995) Identification and Quantification of Choline Glycerophospholipids That Contain Aldehyde Residues by Fluorometric High Performance Liquid Chromatography, *Anal. Biochem. 227*, 289–294.

Pokorny, J. (1984) Non-Volatile Lipid Oxidation Products, in *CRC Handbook of Chromatography, Vol. 2, Lipids* (Mangold, H.K., ed.) pp. 205–217, Boca Raton, FL.

Porter, N.A., Caldwell, S.E., and Mills, K.A. (1995) Mechanisms of Free Radical Oxidation of Unsaturated Lipids, *Lipids 30*, 277–290.

Porter, N.A., and Funk, M.O. (1975) Peroxy Radical Cyclization as a Model for Prostaglandin Biosynthesis, *J. Org. Chem. 40*, 1614–1615.

Porter, N.A., Lehman, L.S., Weber, B.A., and Smith, K.J. (1981) Unified Mechanism for Polyunsaturated Fatty Acid Autoxidation. Competition of Peroxy Radical Hydrogen Atom Abstraction, β-Scission, and Cyclization, *J. Am. Oil Chem. Soc. 103*, 6445–6447.

Porter, N.A., Wolf, R.A., and Weenen, H. (1980) The Free Radical Oxidation of Polyunsaturated Lecithins, *Lipids 15*, 163–167.

Privett, O.S., and Blank, M.L. (1963) A Method for the Structural Analysis of Triglycerides and Lecithins, *J. Am. Oil Chem. Soc. 40*, 70–75.

Pryor, W.A., Bermudez, E., Cueto, R., and Squadrito, G.L. (1996) Detection of Aldehydes in Bronchoalveolar Lavage of Rats Exposed to Ozone, *Fund. Appl. Toxicol. 34*, 148–156.

Ravandi, A., and Kuksis, A. (2000) Phospholipids of Plasma Lipoproteins, Red Cells, Platelets and Atheroma, in *Encyclopedia of Analytical Chemistry: Instrumentation and Applications* (Myers, R.A., ed.), Vol. 2, pp. 1531–1570, John Wiley and Sons, Chichester, West Sussex, UK.

Ravandi, A., Kuksis, A., Myher, J.J., and Marai, L. (1995a) Determination of Lipid Ester Ozonides and Core Aldehydes by High-Performance Liquid Chromatography with On-Line Mass Spectrometry, *J. Biochem. Biophys. Methods 30*, 271–285.

Ravandi, A., Kuksis, A., Myher, J.J., and Marai, L. (1995b) Preparation and Characterization of Glucosylated Aminoglycerophospholipids, *Lipids 30*, 885–891.

Ravandi, A., Kuksis, A., Myher, J.J., Marai, L., Steiner, G., Lewis, G., and Kamido, H. (1996) Isolation and Identification of Glycated Aminophospholipids from Red Cells and Plasma of Diabetic Blood, *FEBS Lett. 381*, 77–81.

Ravandi, A., Kuksis, A., and Shaikh, N.A. (1999a) Analysis of Core Aldehydes and Isoprostane Esters of Phospholipids in Plasma Lipoproteins and Human Atheroma by Electrospray Mass Spectrometry with Single Ion Monitoring, *Clin. Chem. 45*, A16.

Ravandi, A., Kuksis, A., and Shaikh, N.A. (1999b) Glycosylated Aminophospholipids of Low-Density Lipoprotein Promote Macrophage Uptake and Accumulation of Cholesteryl Esters and Triacylglycerols, *J. Biol. Chem. 274*, 16494–16500.

Ravandi, A., Kuksis, A., and Shaikh, N.A. (2000) Glucosylated Aminophospholipids Are the Major LDL Glycation Products and Increase LDL Susceptibility to Oxidation: Evidence for Their Presence in Atherosclerotic Lesions, *Arterioscler. Thromb. Vasc. Dis. 20*, 467–477.

Ravandi, A., Kuksis, A., Shaikh, N.A., and Jackowski, G. (1997) Preparation of Schiff Base Adducts of Phosphatidylcholine Core Aldehydes and Aminophospholipids, Amino Acids and Myoglobin, *Lipids 32*, 989–1001.

Reddy, S.T., Wadleigh, D.J., Grijalva, V., Ng, C., Hama, S., Gangopadhyay, A., Shih, D.M., Lusis, A.J., Navab, M., and Fogelman, A.M. (2001) Human Paraoxonase-3 Is an HDL-Associated Enzyme with Biological Activity Similar to Paraoxonase-1 Protein but Is Not Regulated by Oxidized Lipids, *Arterioscler. Thromb. Vasc. Biol. 21*, 542–547.

Reich, E.E., Zackert, W.E., Brame, C.J., Chen, Y., Roberts, L.J., Hachey, D.L., Montine, T.J., and Morrow, J.D. (2000) Formation of Novel D-Ring and E-Ring Isoprostane-Like Compounds (D4/E4-Neuroprostanes) *In Vivo* from Docosahexaenoic Acid, *Biochemistry 39*, 2376–2383.

Roberts, L.J., II, Montine, T.J., Markesbery, W.R., Tapper, A.R., Hardy, P., Chemtob, S., Dettbarn, W.D., and Morrow, J.D. (1998) Formation of Isoprostane-Like Compounds (Neuroprostanes) *In Vivo* from Docosahexaenoic Acid, *J. Biol. Chem.* 13605–13612.

Rokach, J., Khanapure, S.P., Hwang, S.-W., Adiyama, M., Lawson, J.A., and FitzGerald, G.A. (1997) The Isoprostanes: A Perspective, *Prostaglandins 54*, 823–851.

Rubbo, H., Parthasarathy, S., Barnes, S., Kirk, M., Kalynaraman, B., and Freeman, B.A. (1995) Nitric Oxide Inhibition of Lipoxygenase-Dependent Liposome and Low Density Lipoprotein Oxidation: Termination of Radical Chain Propagation Reactions and Formation of Nitrogen-Containing Oxidized Lipid Derivatives, *Arch. Biochem. Biophys. 324*, 15–25.

Rubbo, H., Raddi, R., Trujillo, M., Telleri, R., Kalynaraman, B., Barnes, S., Kirk, M., and Freeman, B.A. (1994) Nitric Oxide Regulation of Superoxide and Peroxynitrite-Dependent Lipid Peroxidation, *J. Biol. Chem. 269*, 26066–26075.

Schlame, M., Haupt, R., Wiswedel, I., Kox, W.J., and Rustow, B. (1996) Identification of Short Chain Oxidized Phosphatidylcholine in Human Plasma, *J. Lipid Res. 37*, 2608–2615.

Schneider, C., Schreier, P., and Herderich, M. (1997) Analysis of Lipoxygenase-Derived Fatty Acid Hydroperoxides by Electrospray Ionization Tandem Mass Spectrometry, *Lipids 32*, 331–336.

Seppanen, C.M., and Csallany, A.S. (2001) Simultaneous Determination of Lipophilic Aldehydes by High Performance Liquid Chromatography in Vegetable Oil, *J. Am. Oil Chem. Soc. 78*, 1253–1260.

Siems, W.G., Grune, T., and Esterbauer, H. (1995) 4-Hydroxynonenal Formation During Ischemia and Reperfusion of Rat Small Intestine, *Life Sci. 57*, 785–789.

Silva, E.L., Meneghetti, J.C., Celho, I.J.C., and Abdalla, D.S.P. (2001) Peroxynitrite-Modified ^{99M}TC-beta-VLDL: Tissue Distribution and Plasma Clearance Rate, *Free Radic. Biol. Med. 31*, 440–449.

Sjovall, O., Kuksis, A., and Kallio, H. (2002) Formation of Triacylglycerol Core Aldehydes During Rapid Oxidation of Corn and Sunflower Oils with *tert*-Butyl Hydroperoxide/Fe^{2+}, *Lipids 37*, 81–94.

Skipski, V.P., and Barclay, M. (1969) Thin-Layer Chromatography, *Methods Enzymol. 14*, 542–548.

Smiley, P.L., Stremler, K.E., Prescott, S.M., Zimmerman, G.A., and McIntyre, T.M. (1991) Oxidatively Fragmented Phosphatidylcholines Activate Human Neutrophils Through the Receptor for Platelet-Activating Factor, *J. Biol. Chem. 266*, 11104–11110.

Spickett, C.M., Pitt, A.R., and Brown, A.J. (1998) Direct Observation of Lipid Hydroperoxides in Phospholipid Vesicles by Electrospray Mass Spectrometry, *Free Radic. Biol. Med. 25*, 613–620.

Spiteller, G. (1998) Linoleic Acid Peroxidation—the Dominant Lipid Peroxidation Process in Low Density Lipoprotein—and Its Relationship to Chronic Diseases, *Chem. Phys. Lipids 95*, 105–162.

Stelmach, B.A., Muller, A., Hennig, P., Gebhardt, S., Schubert-Zsilavecz and Weiler, E.W. (2001) A Novel Class of Oxylipids, *sn*-1-*O*-(12-Oxophytodienyl)-*sn*-2-*O*-(hexadeca-trienyl)-monogalactosyl Diglyceride, from *Arabidopsis thaliana*, *J. Biol. Chem. 276*, 12832–12838.

Stremler, K.E., Stafforini, D.M., Prescott, S.M., and McIntyre, T.M. (1991) Human Plasma Platelet-Activating Factor Acetylhydrolase: Oxidatively Fragmented Phospholipids As Substrates, *J. Biol. Chem. 266*, 11095–11103.

Stremler, K.E., Stafforini, D.M., Prescott, S.M., Zimmerman, G.A., and McIntyre, T.M. (1989) An Oxidized Derivative of Phosphatidylcholine Is a Substrate for the Platelet-Activating Factor Acetylhydrolase from Human Plasma, *J. Biol. Chem. 264*, 5331–5334.

Subbanagounder, G., Deng, Y., Borromeo, C., Dooley, A.N., Berliner, J.A., and Solomon, R.G. (2002) Hydroxy Alkenal Phospholipids Regulate Inflammatory Functions of Endothelial Cells, *Vasc. Pharmacol. 38*, 201–209.

Subbanagounder, G., Leitinger, N., Schwenke, D.C., Wong, J.W., Lee, H., Rizza, C., Watson, A.D., Faull, K.F., Fogelman, A.M., and Berliner, J.A. (2000) Determinants of Bioactivity of Oxidized Phospholipids: Specific Oxidized Fatty Acyl Groups at the *sn*-2-Position, *Arterioscler. Thromb. Vasc. Biol. 20*, 2248–2254.

Subbanagounder, G., Leitinger, N., Shih, O.T., Faull, K.F., and Berliner, J.A. (1999) Evidence That Phospholipid Oxidation Products and/or Platelet-Activating Factors Play an Important Role in Early Atherogenesis, *Circ. Res. 85*, 311–318.

Subramanian, V., Goyal, J., Miwa, M., Sugatami, J., Akiyama, M., Liu, M., and Subbaiah, P.V. (1999) Role of Lecithin-Cholesterol Acyltransferase in the Metabolism of Oxidized Phospholipids in Plasma: Studies with Platelet-Activating Factor-Acetyl Hydrolase-Deficient Plasma, *Biochim. Biophys. Acta 1439*, 95–109.

Tanaka, T., Iimori, M., Tsukatani, H., and Tokumura, A. (1994) Platelet Aggregating Effects of Platelet-Activating Factor-Like Phospholipids Formed by Oxidation of Phosphatidylcholines Containing an *sn*-2-Polyunsaturated Fatty Acyl Group, *Biochim. Biophys. Acta 1210*, 202–208.

Thomas, P.D., and Poznansky, M.J. (1990) Lipid Peroxidation Inactivates Rat Liver Microsomal Glycerol-3-Phosphate Acyl Transferase, *J. Biol. Chem. 265*, 2684–2691.

Tokumura, A. (1995) A Family of Phospholipid Autacoids: Occurrence, Metabolism and Bioactions, P*rog. Lipid Res. 34*, 151–184.

Tokumura, A., Asai, T., Takauchi, K., Kamiyasu, K., Ogawa, T., and Tsukatani, H. (1988) Novel Phospholipids with Aliphatic Dicarboxylic Acid Residues in a Lipid Extract from Bovine Brain, *Biochem. Biophys. Res. Commun. 155*, 863–869.

Tsimikas, S., and Witztum, J.L. (2000) The Oxidative Modification Hypothesis of Atherosclerosis, in *Oxidative Stress and Vascular Disease* (Keany, J.F., ed.) pp. 1955–1960, Kluwer Academic Publishers, Boston.

Uchida, K. (2000) Role of Reactive Aldehyde in Cardiovascular Diseases, *Free Radic. Biol. Med. 28*, 1685–1696.

Uppu, R.M., Winston, G.W., and Pryor, W.A. (1997) Reactions of Peroxynitrite with Aldehydes as Probes for the Reactive Intermediates Responsible for Biological Nitration, *Chem. Res. Toxicol. 10*, 1331–1337.

Van Kuijk, F.J., Thomas, D.W., Stephens, R.J., and Dratz, E.A. (1986) Occurrence of 4-Hydroxyalkenals in Rat Tissues Determined as Pentafluorobenzyl Oxime Derivatives by Gas Chromatography-Mass Spectrometry, *Biochem. Biophys. Res. Commun. 139*, 144–149.

Walter, M.F., Blumberg, J.B., Dolnikowski, G.D., and Handelman, G.J. (2000) Streamlined F_2-Isoprostane Analysis in Plasma and Urine with High-Performance Liquid Chromatography and Gas Chromatography/Mass Spectroscopy, *Anal. Biochem. 280*, 73–79.

Wang, C.-J., and Tai, H.-H. (1990) A Facile Synthesis of an Aldehydic Analog of Platelet Activating Factor and Its Use in the Production of Specific Antibodies, *Chem. Phys. Lipids 55*, 265–273.

Warnick, G.R., Benderson, J., Albers, J.J. (1982) Dextran Sulfate-Mg^{2+} Precipitation Procedure for Quantitation of High Density Lipoprotein Cholesterol, *Clin. Chem. 28*, 1379–1388.

Watson, A.D., Leitinger, N., Navab, M., Faull, K.F., Hörkkö, S., Witzum, J.L., Palinski, W., Schwenke, D., Salomon, R.G., Sha, W., Subbanagounder, G., Fogelman, A., and Berliner, J.A. (1997) Structural Identification by Mass Spectrometry of Oxidized Phospholipids in Minimally Oxidized Low Density Lipoprotein That Induce Monocyte/Endothelial Interactions and Evidence for Their Presence *In Vivo*, *J. Biol. Chem. 272*, 13597–13607.

Watson, A.D., Subbanagounder, G., Welsbie, D.S., Faull, K.F., Navab, M., Jung, M.J., Fogelman, A.M., and Berliner, J.A. (1999) Structural Identification of a Novel Pro-

Inflammatory Phospholipid in Mildly Oxidized Low Density Lipoprotein, *J. Biol. Chem. 274*, 24787–24798.

Waugh, R.J., and Murphy, R.C. (1996) Mass Spectrometric Analysis of Four Regioisomers of F_2-Isoprostanes Formed by Free Radical Oxidation of Arachidonic Acid, *J. Am. Soc. Mass Spectrom. 7*, 490–499.

Weisser, M., Vieth, M., Stolte, M., Riederer, P., Pfeuffer, R., Leblhuber, F., and Spiteller, G. (1997) Dramatic Increase of α-Hydroxyaldehydes Derived from Plasmalogens in the Aged Human Brain, *Chem. Phys. Lipids 90*, 135–142.

Woutersen, R.A., Appelman, L.M., Van Garderen-Hoetmer, A., and Feron, V.J. (1986) Inhalation Toxicity of Acetaldehyde in Rats. III. Carcinogenicity Study, *Toxicology 41*, 213–231.

Yamamoto, Y. (1994) Chemiluminescence-Based High Performance Liquid Chromatography Assay of Lipid Hydroperoxides, *Methods Enzymol. 233*, 319–324.

Yamamoto, Y., Brodsky, M.H., Baker, J.C., and Ames, B.N. (1987) Detection and Characterization of Lipid Hydroperoxides at Picomole Levels by High Performance Liquid Chromatography, *Anal. Biochem. 160*, 7–13.

Yamauchi, R., Hara, Y., Murase, H., and Kato, K. (2000) Analysis of the Addition Products of α-Tocopherol with Phosphatidylcholine-Peroxyl Radicals by High-Performance Liquid Chromatography with Chemiluminescent Detection, *Lipids 35*, 1405–1410.

Yasuda, M., and Narita, S. (1997) Simultaneous Determination of Phospholipid Hydroperoxides and Cholesteryl Ester Hydroperoxides in Human Plasma by High Performance Liquid Chromatography with Chemiluminescence Detection, *J. Chromatogr. B 693*, 211–217.

Yeo, H.C., Helbock, H.J., and Ames, B.N. (1998) Toxic Carbonyl Compounds, in *Chromatography Science Series 77: Chromatographic Analysis of Toxicants in the Environment and Food* (Shibamoto, T., ed.) pp. 289–322, Marcel Dekker, New York.

Yeo, H.C., Helbock, H.J., Chyu, D.W., and Ames, B.N. (1994) Assay of Malonaldehyde in Biological Fluids by Gas Chromatography-Mass Spectrometry, *Anal. Biochem. 220*, 391–396.

Yeo, H.C., Liu, J., Helbock, H.J., and Ames, B.N. (1999) Assay of Malonaldehyde and Other Alkanals in Biological Fluids by Gas Chromatography-Mass Spectrometry, *Methods Enzymol. 300*, 70–78.

Zurek, G., and Karst, U. (2000) 2,4-Dinitro-3,5,6-trideuterophenylhydrazones for the Quantitation of Aldehydes and Ketones in Air Samples by Liquid Chromatography-Mass Spectrometry, *J. Chromatogr. A. 869*, 251–259.

Chapter 7

Lipid Oxidation in Emulsions

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Introduction

For several years, there has been an increasing market for formulated foods and an expected development of functional foods. This increase corresponds to a huge demand for easy-to-use tasty products with a long shelf life that also fit nutritional recommendations such as decreased total fat content and increased amounts of iron and n-3 fatty acids. These contradictory requirements exacerbate the problem of lipid oxidation and emphasize the need for effective solutions ensuring adequacy of both shelf life and sensory quality of the new products.

A large number of these formulated foods consist of a lipid phase dispersed in an aqueous medium and can therefore be described as oil-in-water emulsions. These emulsions are stabilized by the use of emulsifiers and surfactants adsorbed at the oil-water interface. Proteins as well as smaller molecules, such as monoacylglycerols and phospholipids, play a major role in the physical stabilization of food emulsions. Before they reach the consumer, food emulsions undergo various thermal or mechanical treatments and are stored under various conditions. These treatments induce modifications of the components and structure of the emulsion, including physical destabilization (Friberg and Larsson 1997), denaturation, aggregation and/or polymerization of protein (Genot *et al.* 1990, Monahan *et al.* 1993), interface aging (Leaver *et al.* 1999b and 1999c), and other chemical reactions.

Among the reactions that can take place in emulsion systems, lipid oxidation has an appreciable influence on the technological, sensory, and nutritional qualities of the products. It first targets unsaturated lipids and can take place under a range of environmental conditions, provided that oxygen is present. It induces many side effects generally considered to be detrimental to product quality. It gives rise to undesirable off-flavors (Jacobsen *et al.* 1999a, van Ruth *et al.* 1999b) and potentially toxic reaction compounds (Aw 1998, Kubow 1992) directly derived from fatty acid degradation. First, the off-flavor compounds make the food no longer acceptable for human consumption when they are present in amounts higher than the acceptance threshold. Second, in complex systems such as emulsions, the reaction also affects the neighboring nonlipid molecules, especially proteins, and may alter their functional and nutritional properties.

The mechanisms of the lipid oxidation reaction in homogeneous systems have been studied and documented extensively since Nicolas Théodore de Saussure, in 1804, related the first observations on the chemical behavior of plant lipids in the presence of oxygen (Recherches chimiques sur la végétation, cited in http://www.cyberlipid.org/perox/oxid0002.htm#1). Even if some of the reaction pathways are not yet completely elucidated, the general schemes and reactions of lipid oxidation in bulk oils and fats can be found in other chapters in this book and elsewhere (Chan 1987, Frankel 1980, 1991, and 1998, Gardner 1989, Hsieh and Kinsella 1989, Porter *et al.* 1995). Other recent reviews and articles have focused on lipid oxidation in emulsions or dispersed systems compared with bulk lipid (Coupland and McClements 1996, Frankel 1998, Fritsch 1994, McClements and Decker 2000). These reviews highlight the fact that, compared with bulk oils, lipid oxidation in emulsions is influenced by a number of additional factors and that these factors are far from being completely elucidated. These observations prompt renewed interest in a better understanding of lipid oxidation in emulsified systems to optimize our ability to prolong their shelf life as much as possible and enhance their quality.

This chapter reviews current knowledge concerning the development of oxidation in food emulsions, especially oil-in-water emulsions. Both the mechanisms of lipid oxidation and the factors that affect it in such heterogeneous systems are described with special attention to their expected consequences on the sensory qualities of the products. For a better understanding of the specificity of oxidation in emulsions, the main physical and structural characteristics of these systems are summarized initially.

Emulsions

Definition

Food products are commonly composed of different phases, i.e., water, air, fat, and solid particles. Emulsions are a specific type of multiphase system and are formed from two immiscible liquids, with one dispersed as droplets in the second one. Because the two phases are generally water and oil, emulsions can be classified according to the composition of the continuous phase. Two types of emulsions are recognized: (i) when oil droplets are dispersed in an aqueous phase, the system is classified as an oil-in-water (O/W) emulsion (Fig. 7.1), and (ii) when water droplets are dispersed in an oil phase, the system is known as a water-in-oil (W/O) emulsion. In food products, milk, mayonnaise, and salad dressing represent the first type of emulsion, whereas butter and margarine are examples of the second type. In the following discussion, only O/W emulsions will be considered.

Formation

With the exception of milk, which is a natural emulsion, manufacturers or consumers generally produce emulsions by a combination of an aqueous phase and an oil phase. When water and oil are poured into a container, they do not mix or dissolve, but

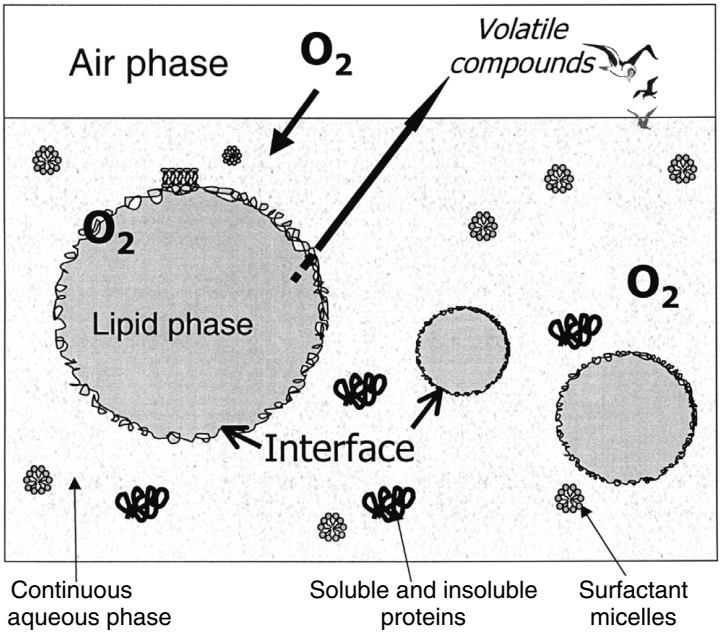

Fig. 7.1. Schematic representation of an oil-in-water emulsion.

spontaneously adopt the thermodynamically most stable form. Because the oil molecules cannot establish hydrogen bonds with the neighboring water molecules, the contact surface between lipid and aqueous phases tends to be minimized to reduce the total free energy (G) of the system. In an emulsion, many small droplets are dispersed in a continuous phase, so that the interface as well as the free energy is very large. G depends on the interfacial tension between the two phases and can be reduced by the presence of tensioactive molecules. As a consequence, the formation of a stable emulsion requires both an important input of energy and the presence of tensioactive molecules. The energy can be supplied by various technologies. Their main effect is droplet elongation and rupture in laminar or turbulent shear flow. Coarse emulsions characterized by large-sized droplets can be produced by mechanical agitation of the two phases as in home-made salad dressing or mayonnaise, but finer emulsions with smaller droplet size and increased stability generally require a second step of emulsification to reduce the droplet size.

Several types of homogenizers have been developed for the production of emulsions in a large range of volumes for different purposes. Some of these homogenizers are explained below: (i) *High-speed blenders*, producing 2- to 10-μm oil droplets, are the most commonly used in the food industry for directly homogenizing oil and water phases. They are available in large volumes for industrial applications and in small volumes for laboratory applications. (ii) *Colloid mills*, producing 1- to 5-μm oil droplets, are based on the formation of a high-shear

laminar flow between rotor and stator disks, which makes it possible to reduce droplet size of coarse or high-viscosity emulsions. (iii) *High-pressure valve homogenizers* are used by the food industry to produce fine emulsions (0.1- to 2-μm oil droplets). A coarse emulsion is forced through a valve by a high-pressure pump, and undergoes a combination of intense shear, cavitational, and turbulent flow conditions, which break the large droplets into smaller ones (Phipps 1985, cited by McClements 1999). (iv) *Ultrasonic homogenizers* are well suited to produce small volumes of emulsions and are used in research laboratories when materials used to prepare emulsions are expensive. The main droplet disruption mechanism of these homogenizers is cavitation and the droplet sizes obtained can be very small (0.1 μm). (v) *Membrane homogenizers* are used to form emulsions by forcing one phase into the other through a solid membrane of controlled pore size. The major advantage of this technique is a very high-energy yield because less energy is lost *via* viscous dissipation compared with the other techniques.

The size of droplets produced by these homogenizers varies between 0.3 and 10 μm. Whatever the technique chosen, the size of the droplets produced during homogenization depends on the viscosity ratio between the two phases, the volume fractions of dispersed phases, and the emulsifier type and concentration.

Characterization

First, emulsions should be characterized by the composition of the water and oil phases and of emulsifiers, the dispersed phase volume fraction, and the droplet size distribution. Knowledge of the *dispersed-phase volume fraction* (ϕ) is important because this factor greatly influences appearance, flavor, texture, and stability of emulsions. The overall ϕ of an emulsion is determined by formulation, but physical destabilization of emulsions during conservation (creaming) may induce a nonhomogeneous dispersed phase volume fraction (ϕ) in the entire volume of the emulsion. Because droplets of emulsions are often formed in turbulent flows, statistical theories imply that the *droplet size distribution* should follow a log normal distribution, which is roughly observed in practice (Gopal 1968, cited by McClements 1999). To describe this distribution, droplets are gathered in size classes according to droplet diameter (d_i) and the result is plotted as a histogram giving the number of droplets (n_i) in each class (i) (number frequency distribution). Droplet distribution is often expressed as the volume frequency distribution, which gives a better view of the quantity (volume) of dispersed phase product in each class. Droplet size distribution is often measured by laser diffraction using Mie theory, which allows the calculation of the volume distribution from measurements of the intensity of scattered light vs. scattering angle. It is often convenient to use a mean size and dispersion of droplets to characterize emulsion size distribution. The most often used mean droplet sizes are as follows:

$$D_n = \frac{\Sigma n_i d_i}{\Sigma n_i} \qquad D_{43} = \frac{\Sigma n_i d_i^4}{\Sigma n_i d_i^3} \qquad D_{32} = \frac{\Sigma n_i d_i^3}{\Sigma n_i d_i^2}$$

D_n is the real mean diameter in number; it is useful for collision calculation. D_{43} is the mean diameter weighed in volume; it amplifies the importance of large globules and is often used in creaming studies. D_{32} is the volume-surface mean diameter; it is used in studies concerned with the influence of the interface on emulsion properties. The interfacial surface area (A_s; m^2/cm^3 oil) is related to D_{32} by the following relationship:

$$A_s = \frac{6}{D_{32}}$$

Generally, laboratories optimize the conditions for preparing emulsions so that the products have homogeneous droplet size distributions with a single narrow peak (monomodal distribution). However, food emulsions are often heterogeneous and have either broad or multimodal size distributions so that it may be important to characterize the products with an entire distribution and not only with averaged values.

Other parameters of great significance in determining the properties of emulsions include partitioning of the emulsifiers in the different phases of the emulsions, and the composition and characteristics of the interface layer (e.g., charge, thickness, structure, viscoelasticity, diffusivity). Viscoelastic properties of the product may also be of great significance.

Physical Stability and Destabilization

Emulsions are subject to physical and chemical changes during processing and storage. Physical instability involves modification of emulsion structure, whereas chemical instability concerns the constituents at the molecular level that may in turn modify physical stability. The principal chemical modifications are hydrolysis and oxidation; the latter is the primary topic of this chapter.

Physical instability of emulsions results from the high level of energy they store as interfacial free energy. According to thermodynamics, high-energy systems tend to relax to reach their minimal energy level. In emulsions, that corresponds to the complete separation of oil and water phases. However, this return to a low-energy state can be delayed by the presence of an energy barrier as shown schematically in Fig. 7.2. Accordingly, food emulsions are thermodynamically unstable but kinetically stable because the activation energy (ΔG^*) is appreciably larger than the thermal energy (20 kT). This explains why emulsions can be kept for several days to months without dephasing (Friberg 1997).

Three main processes play a role in emulsion destabilization, i.e., flocculation, creaming, and coalescence. Flocculation is often the first stage of emulsion destabilization because it furthers creaming and coalescence. It corresponds to the aggregation of two or more droplets that keep their integrity in the aggregate. Flocculation modifies the apparent droplet size distribution, dispersed-phase volume fraction, and

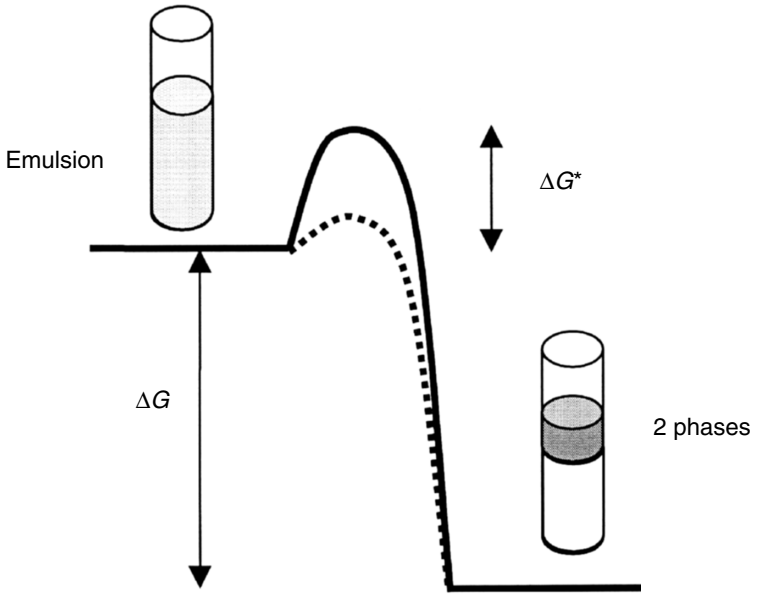

Fig. 7.2. Comparison of activation energy for a kinetically stable emulsion (straight line) and an unstable emulsion (dotted line).

rheological properties of emulsions. It depends on the relative magnitude of repulsive and attractive interactions between droplets. These colloidal interactions are of five major types: electrostatic, steric, Van der Waals, hydrophobic, and depletion. The first two forces are repulsive, whereas the other three are attractive.

Van der Waals forces occur when molecules have a temporary or permanent dipole moment. These interactions are attractive and relatively long-range forces (Israelachvili 1992). Hydrophobic interactions are caused by the tendency of hydrophobic domains located at the surface of different droplets to reduce contacts with water molecules. They manifest themselves as strong attractive forces between hydrophobic domains. Depletion flocculation occurs when the continuous phase contains nonadsorbed colloidal particles. Particles, such as micelles or hydrocolloids, are excluded from a volume surrounding the droplets whose thickness is approximately equal to the radius of the particles. The particle concentration is lower in the exclusion volume than in the bulk of the continuous phase. The induced concentration gradient provokes the migration of water molecules from less to more particle-concentrated zones, forcing adjacent droplets to come into contact. Electrostatic repulsion between droplets occurs when the interfaces of droplets are electrically charged; in the presence of salt as in many foods, however, this effect is hindered. The best way to reduce the occurrence of flocculation phenomena is to make use of polymer steric interactions. When proteins or other high-molecular-weight emulsifiers stabilize emulsions, they adopt conformations maximiz-

ing contacts between their hydrophobic domains and oil on one hand and between their hydrophilic parts and water on the other hand (Damodaran 1996). Thus, expanded thick layers are formed at the interface. When two droplets come into contact, the two interface layers are compressed and they interpenetrate, generating osmotic repulsion. When attractive forces prevail over repulsive ones, droplets remain in contact after collision and aggregates, or flocs, are formed.

Creaming results from the difference in densities between the oil and water phases, which induces gravitational forces on droplets. Because oils normally have lower densities than the water phase, droplets move upward and accumulate at the top in a creamed layer. The creaming velocity depends on the density difference between the phases, the droplet diameter, and the viscosity of the continuous phase (McClements 1999). In most food products, the density difference cannot be modified; thus, food manufacturers must either reduce the droplet diameter or add thickening agents to counteract creaming.

Coalescence occurs when droplets merge into larger droplets. In the absence of an emulsifier, droplets may coalesce at each collision but in surfactant stabilized emulsions, the emulsifiers adsorbed at the interface generate short-range repulsion, preventing rapid coalescence. In practice, coalescence takes place only within droplet aggregates and after a relatively long delay because formation of a hole across the film separating the two droplets is required. The coalescence rate is governed by the probability of opening such a hole. This probability is reduced by high interfacial viscosity of the film surrounding the droplets.

Structure and Composition of the Interface

Emulsions are systems composed of at least three distinct domains: two isotropic phases, water and oil, and a thin extended one, the interface (Fig. 7.1). In a first approach, the interface can be likened to a surface separating the two main phases, but to understand many droplet properties, it is important to describe and characterize the interface composition and structure more precisely. Amphiphilic molecules contain both hydrophobic and polar domains. These molecules adopt their lowest energetic state when adsorbed at an interface to minimize contact between their hydrophobic part and water. Such molecules accumulate at the interface, thereby separating oil and water molecules and reducing the interfacial tension.

Two main types of emulsifiers are used for emulsion stabilization. The first type comprises small molecules with a polar head and one or more aliphatic chains, whereas the second type includes high-molecular-weight polymers with alternation of hydrophobic and polar segments. In food emulsions, the first group is represented by phospholipids, free fatty acids, and monoacylglycerols, as well as synthetic surfactants often used in model emulsions. The second group consists mainly of proteins, although hydrocolloids may sometimes adsorb and participate in interface structure. Small emulsifiers form a very compact film at the interface and efficiently reduce the interfacial tension by having their polar heads in direct

contact with the water phase and their aliphatic chain immersed in or located close to the oil phase. The thickness of the interface is directly related to the length of the chain and the diameter of the polar head. When proteins are adsorbed, the interface is thicker but more heterogeneous, depending on protein conformation. Indeed, proteins present different structures and positioning at the interface depending on interfacial concentration, water phase properties (ionic strength, pH), presence of small emulsifiers, and temperature. In the presence of small amounts of proteins, the proteins spread at the interface to cover maximal area, and layer thickness is reduced (Dalgleish and Leaver 1993). When proteins and small emulsifiers are present simultaneously at the interface, several structures can be obtained as a function of the protein/surfactant ratio (Mackie 2001, Murray and Dickinson 1996). In the presence of small quantities of emulsifiers, the small surfactants fill the empty domains between proteins, and the film looks homogeneous. When the surfactant concentration increases, surfactants progressively occupy distinct domains, compressing protein domains until they collapse and form multilayers (Mackie 2001, Rodiguez Niño and Rodriguez Patino 1998). A schematic representation of the structure on the interface composed of both proteins and small emulsifiers is shown in Figure 7.3. It illustrates the hydrophobicity gradient, which takes place within the entire thickness of the interface, allowing water molecules to interact or participate directly with the hydrophilic side, whereas triacylglycerols may be dissolved to a small extent in the hydrophobic domain.

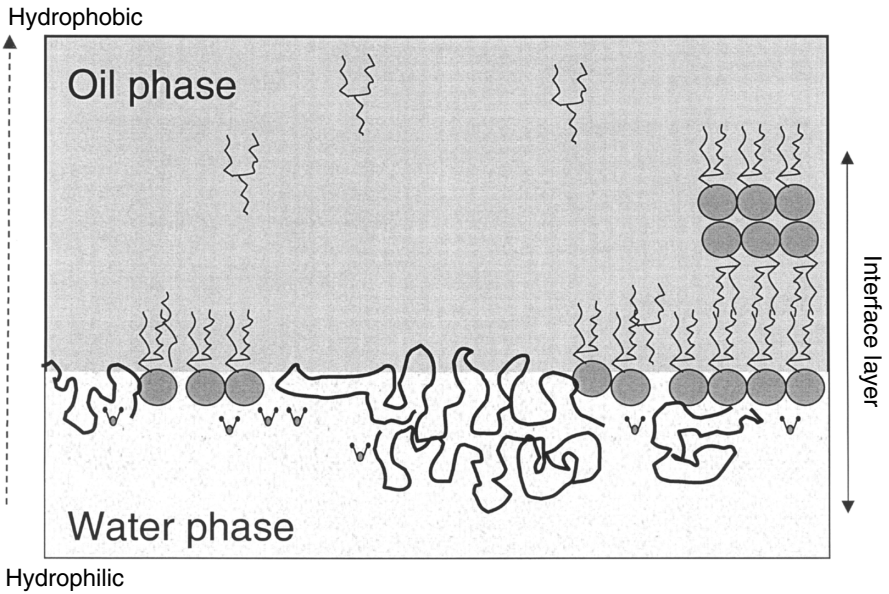

Fig. 7.3. Schematic representation of the interface of an oil-in-water emulsion.

Lipid Oxidation in Oil-in-Water Emulsions

In oil-in-water emulsions, the lipids are not directly in contact with air, but they are surrounded by an interfacial film and dispersed in the aqueous phase. Thus, oxygen, water-soluble antioxidants and prooxidants must diffuse through the aqueous phase and go through the interface before reaching the lipid substrate. As a consequence, even if the mechanism of autoxidation in emulsions is not fundamentally different from that identified in bulk oils and fats (see Chapter 1), a number of additional factors intervene in the process of lipid oxidation (Table 7.1). These factors include the structure of the emulsion (i.e., size distribution of the oil droplets), the physicochemical properties of the aqueous phase, the structure and organization of the oil phase, and the properties of the droplet's membrane. The partitioning of antioxidant and prooxidant molecules among the oil, the aqueous phase, the interfacial region, and anti- and prooxidant interactions with the emulsifiers and other constituents at the interface or in the aqueous phase may also play an important role in the development of oxidation (Jacobsen *et al.* 1999a, Mancuso *et al.* 1999a, Schwarz *et al.* 2000). The polar paradox described by Porter (1980 and 1993) and Frankel *et al.* (1994) is a good illustration of the effect of antioxidant partitioning on antioxidant activities. It corresponds to the observation that the efficiency of a lipophilic antioxidant is generally greater in oil-in-water emulsions than in bulk oils, whereas the opposite is often observed for hydrophilic antioxidants. Finally, lipid reaction products and free radical intermediates can react with other components, such as proteins, present either at the interface or in the aqueous

TABLE 7.1
Additional Factors That Influence the Rate and Course of Oxidation in Oil-in-Water Emulsions

Aqueous phase	Interface	Lipid phase
pH →	Charge	Chemical structure
Metal ions →	Colloidal interactions	Liquid or solid state
Salts/ionic strength		Accessibility of unsaturated FA
Viscosity	Interfacial area ←	Droplet size
Thickeners/chelators	Composition (surfactants)	Oil volume fraction
Soluble proteins	Organization, structure	
Micellar phases	Thickness, permeability	
	Microviscosity, interfacial pressure, polarity	

Partition, interactions or reaction, diffusion of:
Oxygen
Metal ions
Antioxidants
Radicals
Primary products of oxidation
Secondary products (aldehydes)

phase of the emulsion. This leads to deviations in lipid oxidation pathways and kinetics, changing the proportions of the reaction products or even giving rise to other products and apparent or true antioxidant activities (see Chapter 9).

Real food emulsions are, indeed, scarcely described by such a relatively simple three-phase system comprising the oil phase, the water phase, and the interface. Other colloidal phases such as surfactant micelles, and protein micelles, or aggregates are also present. Because the oxidation reaction is a very local phenomenon, the influence of these objects, through their interactions with metal ions or antioxidants, should also be taken into account to describe precisely and predict further the development of oxidation.

In the following discussion, we pay particular attention to the results obtained with systems in which the lipid phase is made of natural triacylglycerols. However, a number of studies focused on simplified model systems consisting of either free fatty acids dispersed as a micellar phase in a buffer solution, fatty acid methyl esters emulsified by synthetic surfactants, or hydrocarbon-in-water emulsions containing nonesterified unsaturated fatty acids (Table 7.2). These studies often give a poor idea of the effective consequences of lipid oxidation in real products because kinetics and products of oxidation are different. But they provide relevant information on the mechanisms that may influence the progress of the reaction in emulsions. For similar reasons, studies related to the development of oxidation in liposomes, membrane extracts, lipoproteins, or even lipid monolayers have been included when appropriate.

Influence of Emulsification, Structure and Treatment of Emulsions

Emulsified vs. Bulk Oils. When lipids are dispersed in the aqueous phase of an oil-in-water emulsion, oxidation rates are generally markedly higher than those found in bulk oils (Frankel *et al.* 2002, Hopia *et al.* 1996, Lethuaut *et al.* 2002, van

TABLE 7.2
Foods or Model Systems Used to Study Lipid Oxidation

Mayonnaises
Homogenates (fish or meat)
Natural oil-in-water emulsions stabilized by various emulsifiers
Synthetic triglyceride-in-water emulsions
Fatty acid methyl ester emulsion stabilized by surfactant (e.g., SDS, Tween 20, triton, CTAB) (mainly linoleic acid methyl esters)
Lipoproteins from various origins
Membrane extracts
Liposomes
Micellar solutions (i.e. linoleic acid micelles)
Monomolecular films
Mixture (solution or dispersion) of hydroperoxides or secondary products of oxidation (e.g., aldehydes) + proteins and/or peptides

Ruth *et al.* 1999b). This observation may be attributed to several causes, including the emulsification process itself and the creation of a large interfacial area between the oil and aqueous phases, which is a main characteristic of the structure of emulsions. In some cases, emulsified oils do not present higher oxidizability compared with bulk oils, probably due to the presence of hydrophobic antioxidants that are more efficient in a multiphase system (Khan and Shahidi 2000).

Effect of Emulsification. The intense mechanical agitation during homogenization of the oil and aqueous phases with high-pressure valve homogenizes or colloid mills may promote lipid oxidation due to the high-shear stress, local temperature elevation, and incorporation of oxygen. For instance, after emulsification of soybean oil with a microfluidizer or when the same procedure was applied to the oil, volatile compounds typical of lipid oxidation were identified and quantified, evidencing the prooxidant effect of the treatment (Leaver *et al.* 1999a). In addition, the blending process tends to introduce air bubbles, which are trapped within the emulsion. Therefore, the time and condition of emulsification should be optimized with a special effort to limit temperature increase and air incorporation. Under such well-controlled conditions, the emulsification of stripped sunflower oil with aqueous protein solution using either valve or rotor-stator homogenizers induced the formation of only low levels of conjugated dienes (<10 μmol conjugated dienes/g oil). Conjugated diene formation was not significantly influenced ($P > 0.05$) by the emulsification process (rotor-stator vs. 15 bar vs. 200 bar valve-homogenizer; n = 3) (Genot and Lethuaut, and unpublished results).

Ultrasound homogenization can also induce lipid oxidation. In aqueous media, the chemical effects of ultrasound are due to acoustic cavitation, which leads to thermal dissociation of water vapor into $^{\bullet}$OH radicals and $^{\bullet}$H atoms (Riesz and Kondo 1992). According to the very high rate constants of the reaction between lipids and hydroxyl radicals, ultrasound treatment promotes lipid oxidation and hydrolysis as was demonstrated during liposome preparation (Jana *et al.* 1986 and 1990, Klein 1970). The reaction can be minimized by using radical scavengers, antioxidants, nitrogen atmosphere, and controlled temperature (Genot *et al.* 1999, Jana *et al.* 1986 and 1990).

Influence of Emulsion Structure (Size Distribution of Oil Droplets). The structure of the emulsion is ascertained primarily by the size distribution of the oil droplets, which determines the surface area of the interface membrane according to an inverse relationship. For instance, emulsions with monomodal droplet size ranging between 50 and 0.1 μm would develop interfaces varying from 0.065 to 33 m^2/g oil, directly in contact with 0.02–10% of the lipid molecules (McClements 1999, McClements and Decker 2000). In practice, emulsions are often multimodal, and a small fraction of the lipids dispersed as small size droplets may account for a significant part of the interfacial area.

Small droplet size signifies a large surface area and a high potential of contact between diffusing oxygen, water-soluble free radicals, antioxidants, and the inter-

face. It also means that the ratio of oxidizable fatty acids located near the interface to those embedded in the hydrophobic core of the droplet increases, thereby increasing the oxidation rate (Coupland *et al.* 1996, Schuster *et al.* 1995). Accordingly, early results related to autoxidation of unsaturated fatty acids adsorbed to silica gel demonstrated that the rate of oxidation was higher when the lipid arrangement was close to a monolayer, exposing a maximum surface area to oxygen (Porter *et al.* 1967 and 1972). Nevertheless, when the droplet size decreases, the number of lipid molecules per droplet also diminishes and that could limit propagation chains. The adsorption of surface-active compounds at the interface is also favored, resulting in membranes that might protect lipids by acting as barriers to the penetration and diffusion of the molecular species that initiate lipid oxidation. For instance, proteins or polysaccharides adsorbed on the droplet membrane may shield the core lipids from oxygen and catalysts (Matsumara *et al.* 2000). Proteins can also act as antioxidants due to various reactions with oxidizing lipids leading to chain polymerization, amino acid damage, and formation of protein-lipid cross-links (Gardner 1979 and Chapter 9 in this book). Accordingly, homogenization is reported to protect milk fat from oxidation catalyzed by metal complexes (Hegenauer *et al.* 1979) because casein, which adsorbs to the surface of droplets, is an efficient antioxidant in milk (Allen and Wrieden 1982) due probably to chelation of prooxidant metal ions by phosphoseryl residues.

According to these contrasting views, there is some confusion surrounding the few studies concerned with the influence of the oil droplet size distribution and interfacial surface area on lipid oxidation in emulsions. Jacobsen *et al.* (1999b) found that the addition of propyl gallate to fish oil–enriched mayonnaise both increased the mean droplet diameter and promoted a slight increase in lipid hydroperoxides and certain volatile oxidation products as well as producing flavor deterioration. Shimada *et al.* (1996) observed no effect of droplet size (mean size, 7–21 μm) of soybean oil emulsions stabilized by polysaccharides on the time required for 50% oxygen uptake. Ponginebbi *et al.* (1999) found no change in oxidation rates when the emulsifier concentration was increased in linoleic acid/Tween 20 emulsions, which induced a decrease in oil droplet size (from 0.3 to 0.15 μm). This lack of effect was interpreted as resulting from a balance between the prooxidant influence of decreased droplet size and the antioxidant effect of the emulsifier in the water phase. Roozen *et al.* (1994) also noticed no influence of oil droplet size on the protection afforded by emulsified chemically stable oils on linoleic acid-surfactant micelles. In contrast, Gohtani *et al.* (1999) measured slower development of peroxides in large droplet size (6.4 μm) docosohexaenoic acid (DHA)-in-water emulsions stabilized by xanthan than in 3.4-μm emulsions. Similarly, Jacobsen *et al.* (2000a and 2000b) observed, in the early stage of the storage of fish oil–enriched mayonnaises, a positive correlation between the increase of the size of the oil droplets induced by an antioxidant mixture and that of oxidative stability, evaluated from concentrations of free radicals and several volatile compounds and the development of rancid off-flavors and odors. After 3–4

wk of storage, the oxidation of the products had progressed almost equally, whatever the mayonnaise composition. The authors concluded that once oxidation has been initiated, propagation becomes independent of droplet size (Jacobsen *et al.* 2000b).

Very recently, we studied the influence of oil droplet size (volume-surface mean diameter: 0.5, 1.9, and 2.7 μm) on emulsions made of stripped sunflower oil, stabilized by aqueous bovine serum albumin (BSA; pH 4.4) and stored in sealed vessels (Genot *et al.* 2002, Lethuaut *et al.* 2002, Rampon *et al.* 2001 and 2002). In the first stage of the reaction, when oxygen concentration was not the limiting factor, the rates of oxygen consumption and conjugated diene formation increased with decreasing droplet size (Lethuaut *et al.* 2002). However, the multiplying factor for oxidation rates was far from that calculated for interfacial area, probably due to the protective influence of the protein adsorbed at the interface. Accordingly, protein degradation, as evaluated from the decrease in tryptophan fluorescence, was favored in small droplet size emulsions due to the higher proportion of protein at the interface (Rampon *et al.* 2002). Jacobsen *et al.* (2001b) showed that a decrease in the oil droplet size in mayonnaise accelerates oxidation only when the iron is located at the interface and not inactivated by EDTA. In a later stage in the reaction, when most of the oxygen had been consumed, conjugated diene levels became very similar and were kept steady, regardless of the size of the oil droplets (Lethuaut *et al.* 2002). The formation of fluorescent oxidized lipid-protein adducts, and, to a lesser extent, decreased protein fluorescence, were favored when the average droplet diameter decreased (Rampon *et al.* 2001). The formation of volatile compounds was not influenced by the droplet size when measured in the headspace of the emulsions using the manual static headspace method (Lethuaut *et al.* 2002). The more sensitive solid-phase microextraction (SPME) technique suggested that droplet size influences volatile formation in ways that depend on the type of measured volatile (Genot *et al.* 2002).

Influence of Storage Conditions and Physical Destabilization of Emulsions on Oxidation. The first parameters to be considered when studying oxidation in emulsified systems are light and temperature. In the presence of a photosensitizer and light illumination, photooxidation may become the prominent phenomenon (Bradley and Min 1992). For every 10°C increase in temperature, the rate of reaction of oxygen with lipids roughly doubles in bulk oils and fat (Allen 1994). The same tendency is found in emulsions, even if the multiplying factor depends greatly on the method used to evaluate oxidative degradations (Rampon *et al.* 2001).

Agitation of the emulsions modifies the kinetics of lipid oxidation when diffusion phenomena is a limiting factor (McClement and Decker 2000). This is illustrated in Figure 7.4, which shows the formation of conjugated dienes in emulsions stored under various agitation conditions in closed or open vessels (Genot and Longuet, unpublished results). According to the expected influence of diffusion of the reactants, oxidation development was the slowest under nonagitated conditions (Fig. 7.4A). Agitation influences not only the diffusion of oxygen and other com-

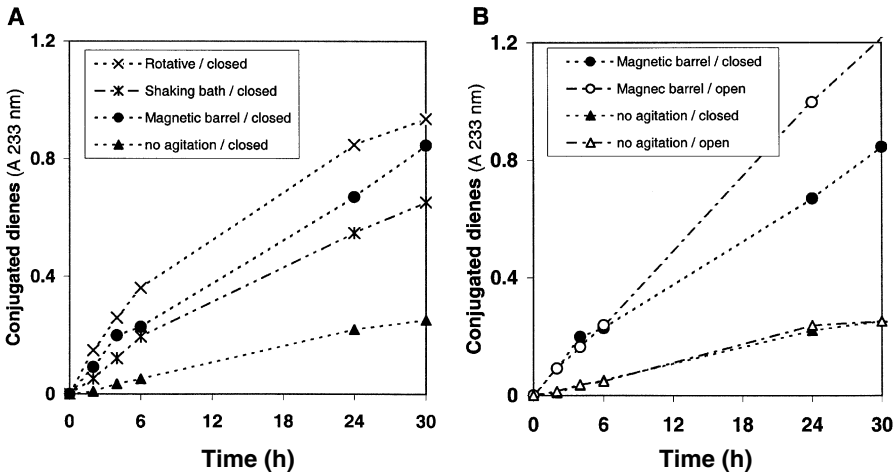

Fig. 7.4. Formation of conjugated dienes in stripped sunflower oil emulsions stabilized by bovine serum albumin during aging at 47°C under various conditions: (A) influence of agitation; (B) open or closed vials.

ponents, but also the eventual physical destabilization of the emulsions with consequent influences on oxidation development. When emulsions are not agitated, they tend to form a cream layer with an increased viscosity, which may hinder the diffusion of reactants. Accordingly, Sims *et al.* (1979) observed an increasing resistance of emulsions to oxidation measured by oxygen uptake, when the oil volume fraction increased in the range of 6–44%. However, as a result of creaming, the smallest droplets remain in the bottom phase, not directly in contact with the atmosphere. Thus, one may expect that levels of lipid oxidation are different in the creamed layer than in the bottom phase. When emulsions are agitated with a shaking bath or by magnetic stirring, droplets will likely come into contact, favoring flocculation and then coalescence of the oil droplets. To our knowledge, the influence of flocculation and droplet-droplet interactions on lipid oxidation has not been documented.

Effect of Composition and Organization of the Lipid Phase

As mentioned above, the oxidative stability of unsaturated lipids generally decreases with an increasing degree of unsaturation. Initiation occurs most likely at a carbon atom adjacent to a double-bonded carbon atom because the energy required to split a hydrogen atom is higher for molecules containing only methylene groups than for molecules with one allylic group, which in turn is higher than that for molecules with two or more allylic groups. This results in the well-accepted principle that unsaturated lipids are far more susceptible to autoxidation than those that are

saturated. However, results obtained by several authors appear to contradict this view. In multiphase systems, the rate of lipid oxidation is determined not only by the unsaturation of the fatty acid chains, but also by their conformation, their local environment, and their location in the hydrophobic core of the droplets or at the interface.

When dispersed as micellar structures in aqueous systems, the very long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA) and DHA, were more stable than linoleic and linolenic acids (Miyashita *et al.* 1993). This phenomenon could result from the conformation of the fatty chains in the micelles, i.e., the unsaturated part of the fatty acid is buried in the hydrophobic interior of the micelles (Miyashita *et al.* 1993). Another hypothesis is that the peroxidized LC-PUFA diffuse to the micelle surface, favoring chain termination reactions (Terao 2001). This low oxidizability of nonesterified LC-PUFA in an aqueous environment led Terao (2001) to propose the lipase hydrolysis of fish oil emulsions or the addition of free PUFA to prevent the development of oxidation in these n-3 fatty acid–enriched systems. The position of the unsaturated fatty acids on the glycerol, the chain length of neighboring fatty acids in the lipid molecule, or the position of the double bond on the fatty chain also can affect the oxidative stability of unsaturated fatty acids (Endo *et al.* 1997). The fact that n-9 fatty acids in micelles were more susceptible to oxidation than n-6 and n-3 fatty acids can be explained by the closeness of their double bond to the carboxylic end. This position places them nearer the aqueous solution, making them more vulnerable to oxidation (Miyashita *et al.* 1995, cited by McClements and Decker 2000). Other experiments showed linoleate residues to be less susceptible to oxidation when in the hydrophobic core of the oil droplets than when located on their surface or present as fatty acid micelles (Coupland *et al.* 1996, Roozen *et al.* 1994). The parallel or perpendicular orientation of the oxidizable molecules at the surface of the oil droplet may also be important. More generally, in multiphase systems such as emulsions, one would expect a higher oxidizability of polar or amphiphilic lipids compared with lipids that are neutral or hydrophobic (McClements and Decker 2000).

The physical state of the lipid may also influence oxidation rates. For example, the oxidation rate of arachidonyl-phosphatidylcholine or arachidonic acid included in dipalmitoyl phosphatidylcholine (DPPC) or dipalmitoyl phosphatidylethanolamine (DPPE) liposomes was greater at temperatures below rather than above the solid-liquid phase transition temperature (Cervato *et al.* 1988, McLean and Hagaman 1992). Segregation of the unsaturated fatty acid chains in the solid state, thereby increasing the local concentration of oxidation substrate, could explain this effect. On the other hand, Ramos *et al.* (1995) observed no breaks in the Arrhenius plots of oxidation parameters at the melting point of low density lipoproteins (LDL), whereas the fluid physical state of LDL core lipids was shown to enhance the susceptibility of the lipoproteins to oxidation on the basis of differential scanning calorimetry and oxidation measurements as a function of temperature (Schuster *et al.* 1995).

Lag time and conjugated diene production rates showed break points at the core transition temperature, and resistance to oxidation was greater below the phase transition temperature. The decrease in oxygen transfer rate below the transition temperature of the lipids, the decreased diffusion of PUFA toward the interface, and changes in the effective volume and cluster organization of the molecules upon freezing, associated with the formation of defect regions in which radicals and antioxidants may concentrate, were offered as explanations.

As a consequence of these observations, one may predict that factors such as solid fat content of the oil droplets, crystal morphology, and the presence of fat crystals at the interface should influence the development of oxidation of emulsified systems. Finally, the key role played by the lipid-soluble constituents in the development of oxidation in emulsions should be acknowledged. For example, lipid-soluble antioxidants, such as tocopherols and hydroperoxides, still present in oils and fats, have opposite effects on lipid oxidation; these are discussed below and in other chapters of this book.

The case with emulsions is similar to that of microencapsulated oils as discussed in Chapter 8. Even if oxidation in emulsions is recognized as being a local phenomenon, all studies consider the lipid phase to be a homogenous phase even if dispersed among a large number of droplets. Yet, it would be relevant to compare the kinetics of oxidation in the small and large oil droplets in the same emulsion or to be able to compare the development of lipid oxidation at the surface layer of the oil droplets and in the hydrophobic core.

Partition and Diffusion of Oxygen and Initiators of Oxidation (Metal Ions and Free Radicals)

Oxygen. The oxygen concentration at the site of reaction and the total amount of oxygen available, both in the emulsion and its headspace, are factors of great significance for oxidation. In emulsions stored in sealed vials, the total amount of oxygen is critical for the development of lipid oxidation. Oxygen dissolved in air-saturated emulsions at 25°C was calculated as a function of oil volume fraction using a reference value for oxygen solubility in air-saturated water (Hitchman 1978) and a solubility of oxygen in oil 4.4 times higher than that in water (Battino *et al.* 1968, Gros *et al.* 1992) (Fig. 7.5). The calculation did not take into account the decrease in oxygen solubility in the presence of salts or other constituents dissolved in the aqueous phase (Hitchman 1978). Dissolved oxygen, reported in the lipid phase, varies from ~900 µg O_2/g lipid to 41 µg/g when the oil volume fraction increased from 0.01 to 0.8 (Fig. 7.5). It reached ~40 µg/g for the pure lipids. Thus, even when the emulsion was saturated with air, the total oxygen dissolved in the emulsion remains relatively low and the oxygen content in the headspace becomes prominent as soon as the headspace volume is ~5% of the emulsion volume (Fig. 7.6). In emulsions stored in closed vessels, the total amount of peroxides

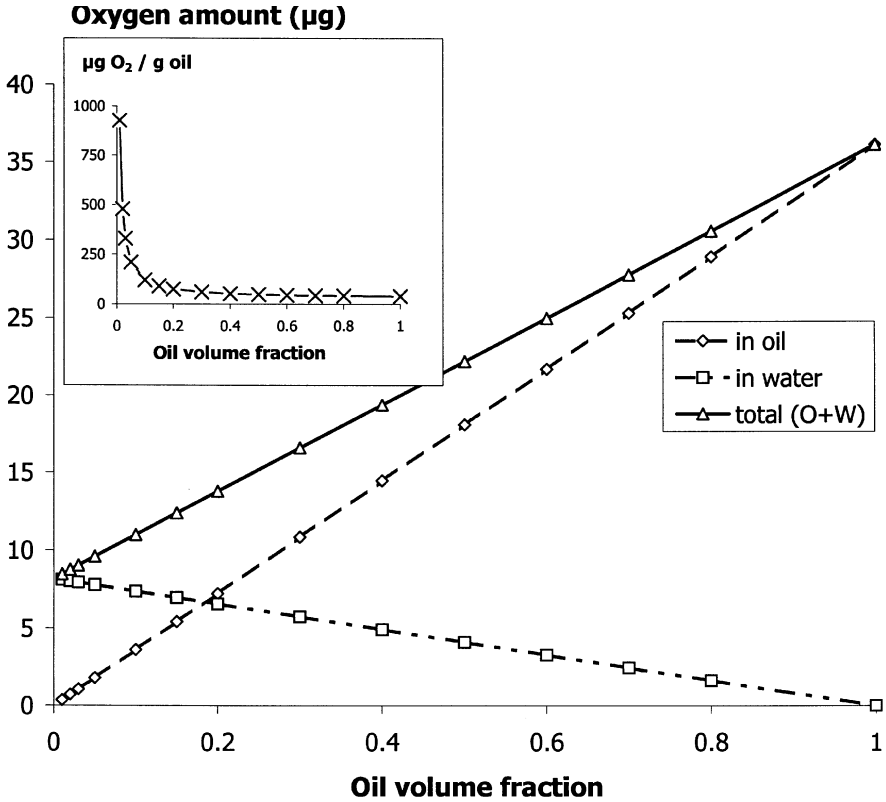

Fig. 7.5. Oxygen dissolved in 1 mL oil-in-water emulsion saturated with air at 25°C as a function of the oil volume fraction.

that can be produced is determined by the molar ratio of oxidizable fatty acids to oxygen. At the beginning of oxidation, the oxygen partial pressure is high, and the oxidation rate is relatively independent of the oxygen partial pressure. However, the oxygen initially dissolved in the lipid phase and in the aqueous phase may be rapidly consumed. Then, the reaction would require the gas to be transported across the aqueous phase and the interface from the exterior environment. Under these conditions, the viscosity of the aqueous phase and that of the emulsion, the formation of a highly viscous creamed layer, the surface area of the emulsion in contact with air, as well as possible agitation can determine the oxidation rate. Accordingly, when total available oxygen decreases or when the emulsion is stored at a low oxygen concentration, the rate-limiting effect of oxygen diffusion becomes prominent (Karel 1992, Labuza 1971). At low oxygen partial pressure, there is a direct correlation between the oxidation rate and the oxygen partial pressure (Andersson and Lingnert 1997, Marcuse and Fredrikson 1968). For instance, the degradation rate of linoleic acid was almost halved after 18 h of incubation at

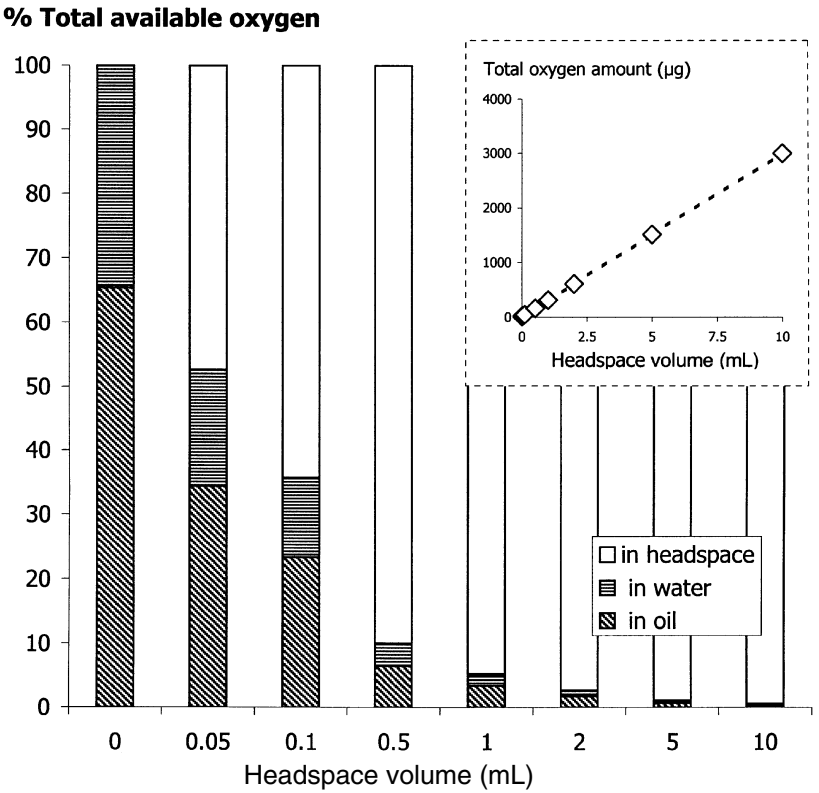

Fig. 7.6. Influence of headspace volume on total available oxygen. (Data were tabulated for 1-mL oil-in-water emulsions with an oil volume fraction of 0.3 and equilibrated with air at 25°C).

25°C, when the oxygen pressure was decreased from atmospheric pressure (21%, vol/vol) to 1% (Marcuse and Fredriksson 1968). The oxygen dependence of the oxidation rate increases with increasing temperature. At elevated temperature, hydrogen abstraction from unsaturated fatty acids is favored, whereas the solubility of oxygen decreases. Hence, the rate-limiting influence of oxygen diffusion and oxygen pressure increases. Also the secondary products of oxidation differ according to oxygen pressure (Andersson and Lingnert 1999). For instance, 2,4-decadienal is a favored decomposition product of linoleic hydroperoxides over hexanal in the absence of oxygen, whereas the reverse situation is true in the presence of oxygen (Grosch 1987, Schieberle and Grosch 1981).

Metal Ions and Oxygen Free Radical Species. Transition metals (iron, copper) intervene in the initiation step through the formation of reactive oxygen free radicals by the Fenton reaction, activation of perferryl ions, or hydrogen abstraction.

They also act during the propagation step by inducing the homolytic scission of hydroperoxides. The mechanisms involved, although they remain controversial, have been discussed in several reviews and articles (Ahn and Kim 1998a, Halliwell and Gutteridge 1990, Samokyszyn *et al.* 1990, Schaich 1992, Tadolini *et al.* 1997). Metal ions are present in the ingredients (i.e., oil, water, or emulsifiers), gained during emulsification treatment, or even added as a supplement for nutritional reasons. For instance, vegetable oils for human consumption may contain from 0.2 to 15 mg/kg iron (Pokorny 1987).

Ferrous iron and heme-bound iron of hemoglobin have strong prooxidant activity in vegetable oil emulsions (Tween 20, pH 6.5), whereas ferric iron or ferritin has no catalytic effect as long as reducing agents were not present (Ahn and Kim 1998a and 1988b). In addition to the redox state (Fe^{2+} *vs.* Fe^{3+}) and overall concentration, the location of the metal ions in the different phases of the emulsions, as well as their ligands and/or neighboring molecules should be considered (Decker and McClements 2001). The addition of metal chelators to the aqueous phase of emulsions showed that the metal ions present in the aqueous phase or bound to the interface are major prooxidants of the lipid oxidation reaction in emulsions (Decker and McClements 2001, Frankel 1998, Jacobsen *et al.* 2001b). Indeed, metal ions are not freely dissolved in the aqueous phase, but coordinate with various ligands, maintaining the metal in solution. Iron complexes differ in their metal affinity, the valence state of the metal they stabilize, and their redox potential (Buettner 1993). Factors such as pH or metal/ligand molar ratio either enhance or inhibit the prooxidant activity of the metal. For instance, complexes of iron with ligands such as EDTA or histidine may have pro- or antioxidant activity or even no effect on lipid oxidation, depending on several chemical or physical factors and on the method used to evaluate oxidation (Hsieh and Kinsella 1989, Schaich 1992). An important point is the distance between the sites of generation of the activated oxygen species and the site of their reaction with the substrate. This distance depends on the ligand that maintains the metal ion in solution and the reactivity of the free radicals. In fact, the deleterious effects of the most reactive free radicals produced during lipid oxidation are restricted to their immediate environment due to their rapid reaction with neighboring molecules. For example, the radius of diffusion of $^{\bullet}OH$ is a few nanometers from their site of formation (Li *et al.* 1999, Slater and Cheeseman 1988). This distance is on the order of the thickness of a small surfactant monolayer at the interface of oil droplets. In contrast, less reactive radicals such as $O_2^{\bullet-}$ ions may diffuse at distances >100 μm (Slater and Cheeseman 1988). In addition, $O_2^{\bullet-}$ and H_2O_2 are reported to be capable of crossing biological and model membrane, whereas oxyradicals, including $^{\bullet}OH$, were suggested not to penetrate into the lipid phase of liposome membranes (Strul *et al.* 1993). Thus, only the free radicals of low reactivity are able to diffuse further, but they are not reactive enough to produce important deterioration of their environment. Because initiation of lipid oxidation most likely involves unsaturated fatty chains located at the interface, the interface properties and reaction conditions increasing the binding of metals to the interface should increase their catalytic activity. Accordingly, when

the charge of the interface is negative due to pH and/or the nature of the emulsifier, rates of iron-induced lipid oxidation are higher than when it is positive (Donnelly *et al.* 1998, Mei *et al.* 1998 and 1999a). Location of less soluble iron at the interface, in interaction with emulsifiers, was proposed to explain the higher oxidizability of neutral pH emulsions compared with acid emulsions in which the metal ion is dissolved in the water phase (Mancuso *et al.* 1999a).

In addition, agents allowing solubilization of the metals in the lipid phase such as fatty acids could be very effective in either promoting or inhibiting lipid oxidation in the hydrophobic core (Schaich 1992). When lipid oxidation is promoted directly in the hydrophobic phase, oxidation pathways and products would be different from those arising when oxidation is initiated in the aqueous phase or at the interface. To gain a better understanding of the effect of lipid oxidation on the variety of constituents normally found within food emulsions, the mobility and solubility of the free radicals, and the primary and secondary products of oxidation in the different phases of the emulsions should be considered as well as the localization of initiators such as metal ions.

Peroxyl Radicals and Hydroperoxides. In contrast to the hydroxyl radical, the lifetime of peroxyl radicals may be as long as several seconds, which allows them to diffuse an appreciable distance from their site of formation. Methyl to ethenyl peroxyl radicals have relatively high dipole moments (in the range 2.3–2.6 D) and are more polar than water (1.85 D) (Boyd *et al.* 1990). As a consequence, lipid peroxyl radicals are highly amphiphilic and are expected to diffuse rapidly from the hydrophobic core of droplets or the hydrophobic layers of the interface in which they are produced, to the more polar regions of the interface or even to the aqueous phase (Boyd *et al.* 1990, Buettner 1993). When they reach these hydrophilic environments, hydroperoxides can either form hydrogen bonds with water molecules, moderating their recombination rate, or abstract a hydrogen atom from a neighboring hydrophilic antioxidant. Simultaneously, they become closer to aqueous metal ions that favor their homolytic cleavage. The overall consequence of this partitioning of hydroperoxides is that it favors oxidative deterioration as shown by the increased susceptibility to oxidation of emulsified lipids compared with bulk oils. Moreover, hydroperoxides may also be present as impurities in surfactants such as Tweens or lecithins (Mancuso *et al.* 2000, Nuchi *et al.* 2001) and in the raw lipid material (Decker and McClements 2001). These peroxides are brought directly to the interface of the oil droplets during emulsification and consequently exert an efficient prooxidant activity in the presence or absence of added metal ions according to the pH conditions.

The Role of the Aqueous Phase

In oil-in-water emulsions, the aqueous continuous phase in which the oil droplets are dispersed is far from being a passive medium when the development of lipid

oxidation is considered. Through its physicochemical properties and the molecules it solubilizes, the water phase makes active or inactive hydrophilic initiators of oxidation, allows the diffusion of atmospheric oxygen, and eventually participates in the protection against oxidation.

pH. Among the physicochemical characteristics of the aqueous phase that influence the oxidative stability of emulsions, pH plays an extremely important role. It affects the chemical stability of emulsions in a variety of ways, including reactivity, solubility, partitioning, and interactions of a number of reactive species. Therefore, according to the operating conditions, i.e., the composition of the system (nature of the surfactants, oxidation catalysts, antioxidants), apparently contradictory results have been obtained. First, pH may evolve during the development of oxidation. Lipid oxidation generally tends to decrease the pH in nonbuffered systems due to the formation of volatile and nonvolatile acids. For example, van Ruth *et al.* (1999a) observed that during storage of nonbuffered sunflower oil/Tween 60 emulsions for 6 d at 60°C, the pH decreased by 3.0 or 0.1 pH units when the initial pH was 6.0 or 3.0, respectively. Small pH decreases were noticed by Huang *et al.* (1996c) and Medina *et al.* (2002) in emulsions buffered by phosphate buffer and emulsified by Tween 20 or lecithin. In contrast, in pH 4.4 sunflower emulsions emulsified by nonbuffered BSA solution, we noticed a small increase of pH (~0.2 pH units) during oxidation (Lethuaut *et al.*, unpublished results). The control protein stored under similar conditions showed a similar pH increase. The buffering capacity of the protein and changes of its conformation and/or aggregation state during storage could impair the pH decrease linked to the formation of acidic lipid oxidation products.

Previous studies showed a differential influence of pH on oxidation of linoleic acid aqueous dispersions according to their composition and the concentration of oxidation catalyst (Wills 1965 and 1966), i.e., the rate of oxidation of linoleate micelles was unaffected by pH changes over the range 5.0–8.0 when no catalyst and no hemoglobin was added, but a marked effect was observed on oxidation catalyzed by metal or by liver homogenate. The rate of oxidation was maximum at ~pH 5.5 when the oxidation was catalyzed by Fe^{3+} and ascorbic acid, but when the oxidation was catalyzed by Fe^{3+} and cysteine, the rate decreased continuously from pH 4 to 8. A pH over the range 5.5–8.0 had little influence on the oxidation rate when the concentration of liver homogenate was small, whereas at high homogenate concentration, the rate of oxidation was very rapid at pH 5.8 but decreased sharply when pH increased. At low oxygen pressure, oxygen consumption in fatty acids/Tween 20 micellar systems showed a maximum at ~pH 6 (Marcuse and Fredriksson 1968). The oxidation of phospholipid liposomes induced by ferrous iron increased with lowering pH, whereas the opposite was observed when no iron was added to phosphatidylethanolamine liposomes (Kawakatsu *et al.* 1984, Lu and Baker 1987). Similar contradictory results were obtained with true oil-in-water emulsions. Some authors reported increased rates of oxidation when pH decreased (Frankel *et al.* 1996, Mei *et al.* 1998, Sims *et al.* 1979,

Yamauchi *et al.* 1988), whereas others observed the opposite effect (Huang *et al.* 1996c, Mabrouk and Dugan 1960, cited by van Ruth *et al.* 1999a; Mancuso *et al.* 1999a; Saunders *et al.* 1962). From the general rules of chemistry, it could be expected that oxidation is favored as the pH decreases. The numerous examples that contradict this rule in multiphase systems demonstrate that in these systems, pH intervenes in one direction or another through a wide range of underlying mechanisms.

One main aspect of pH intervention on emulsion oxidative stability is its influence on the solubility, chemical stability, and redox-state of metal ions and antioxidants. Iron is more soluble at acidic pH than at neutral pH; therefore, at neutral or basic pH, unsolubilized iron ions would bind to anionic or nonionic surfactants at the interface and would be more active in initiating oxidation (Mancuso *et al.* 1999a). In the presence of reducing agents, a low pH promotes activation of metal ions due to their displacement from chelators that made them inactive at higher pH even if they are located near the interface. Such a mechanism was illustrated by Jacobsen *et al.* (1999a) while studying the influence of ascorbic acid on the stability of fish oil–enriched mayonnaise in the presence of vinegar or lemon juice. Ascorbic acid was shown to enhance lipid oxidation at low pH (Jacobsen *et al.* 1999a and 2001c). This effect paralleled the reduction by ascorbic acid of the ferric ion bound to phosvitin, release of the ferrous metal from the interface likely due to the disruption of the egg yolk lipoprotein structure, and the production of free radicals (Jacobsen *et al.* 1999a and 2001c, Thomsen *et al.* 2000). This resulted in hydroperoxide decomposition and immediate formation of rancid and fishy off-flavors.

At the same time, antioxidants such as tocopherols, phenolic compounds, and ascorbic acid are more stable and have better reducing capacities at low than at neutral or basic pH (Frankel et al. 1996, Mei *et al.* 1999a), which may sometimes induce prooxidative activities of these compounds. Indeed, the redox potential of antioxidants is pH-dependent according to Nernst's Law. Moreover, partitioning of antioxidants such as phenolic compounds and Trolox in the aqueous and lipid phases is also affected by pH due to the higher affinity of nondissociated compounds for the lipid phase in contrast to the affinity of dissociated forms for water. Therefore, pH greatly modulates the efficiency of these antioxidants (Huang *et al.* 1996c). Accordingly, inhibition of hydroperoxides and hexanal formation in stripped corn oil emulsified by Tween 20 by carnosic acid, carnosol, α-tocopherol and Trolox depended on the presence of phosphate buffer and on its pH (Frankel *et al.* 1996, Huang *et al.* 1996a and 1996c). In nonbuffered emulsions, α-tocopherol had the highest antioxidant activity followed by carnosol and carnosic acid, whereas in buffered emulsions of pH 4 or 5, carnosic acid and carnosol had the best performance. In emulsions of pH 7, the antioxidants exhibited very little, if any, activity. At the lower pH, partitioning of carnosic acid and carnosol would be in favor of the oil phase or of the oil-water interface, enhancing their antioxidant efficiencies.

A number of recent studies have emphasized the role of pH in the charge of the interface and its ability to bind metal ions or charged antioxidants. The effect

of pH depends on the surfactant: oxidation decreased with decreasing pH in whey protein isolate (WPI)–stabilized emulsions, but the contrary was observed with a Tween 20–stabilized emulsion (Donnelly *et al.* 1998, Frankel *et al.* 1996). Proteins, at pHs below their pIs, cover the interface with positively charged membranes that repel the cationic metal ions but attract negatively charged ascorbic acid. Nevertheless, oxidation of oil-in-water emulsions stabilized with Tween 20 increased with increasing pH (Huang *et al.* 1996c, Mancuso *et al.* 1999a). The effect has been attributed to an increased reactivity, in the absence of added iron, of the peroxides originating from the surfactant at a neutral pH compared with pH 3 (Mancuso *et al.* 1999b). Also, hydroperoxides and volatiles increased more rapidly in nonbuffered pH 6.0 than in nonbuffered pH 3.0 sunflower oil emulsions stabilized by Tween 60 (van Ruth *et al.* 1999a). Indeed, pH also affects the partition of volatile compounds and their release. For instance, the release of hexanal, 3-pentanol, 1-octen-3-ol was enhanced at pH 3 compared with pH 6; however, opposite effects were found for other compounds (van Ruth *et al.* 1999a).

In some cases, the influence of pH has been attributed to its effect on the physical stability of the emulsions. For instance, the decrease in oxygen absorption of emulsions stabilized by anionic surfactants (sodium stearoyl-2-lactylate), when pH increased from 6.2 to 8.2, was attributed to the decrease in the rate of creaming caused by the greater negative charge of the oil droplets (Sims *et al.* 1979). Finally, in multiphase systems (such as biological models, meat and dairy products, and related model systems) in which prooxidant activities of enzymatic proteins or heme proteins may become prominent, the influence of pH is directly related to its effect on the prooxidant proteins (Ahn and Kim 1998a, Allen 1994, Chan *et al.* 1997). For instance, low pHs induce release of iron from the heme proteins and increase prooxidant efficiency of the metal ions.

Salts and Ionic Strength. Salt (NaCl) may adversely affect the formation and physical stabilization of protein-stabilized emulsions because it screens electrostatic repulsion between droplets and may promote aggregation. Too much salt may also cause proteins to aggregate in the aqueous phase of the emulsions. Salt can also influence the rate of autoxidation. It decreased the rate of lipid oxidation in the absence of added iron probably by hindering the binding of the metal ions to the interface (Mei *et al.* 1998). In contrast, high NaCl concentrations favored the reaction in the presence of added iron probably because it increased the catalytic activity of the metal ion (Mei *et al.* 1999b). Buffers used to prepare the aqueous phase may also intervene in the progress of oxidation. Some buffers, such as phosphate and citrate buffers, may exhibit iron-chelating properties and also influence the efficiency of antioxidants acting as metal chelators. For instance, the protective effect of the iron-binding protein lactoferrin was enhanced in the presence of Tris buffer compared with phosphate buffer when evaluated on the basis of conjugated diene formation (Huang *et al.* 1999). Phosphate buffer would interfere in the antioxidant properties of the protein through competitive binding and activation of

the iron. However, the inverse effect was noted with hexanal production. Other buffers (e.g., Tris or Hepes buffers) might act as free radical scavengers (Dawra *et al.* 1989, Fiorentini *et al.* 1989, Yoshimura *et al.* 1992).

Metal Chelators and Emulsifiers. Metal chelating activities may be found in a number of small molecules or macromolecules that are dissolved in the aqueous phase of the emulsions. For instance, EDTA, citric acid, phosphate ions, proteins (particularly phosphorylated casein and phosvitin), and several hydrocolloids have the potential to protect emulsified systems against oxidation. Many chain-breaking antioxidants also exert a part of their activity through metal chelation. Emulsifiers present in the aqueous phase as individual molecules (concentration lower than critical micelle concentration) or in micellar states may also interfere with oxidation, through interactions with metal ions or formation of mixed micelles with oxidizable lipid molecules such as fatty acids, protecting them from oxidation (Ponginebbi *et al.* 1999).

Amino Acids, Sugars and Polysaccharides (Viscosity vs. Metal Chelation). Amino acids and reducing sugars possess both antioxidant and prooxidant activities depending mainly on their concentrations, pH, and the presence of metal ions. In the presence of trace metal ions, both initiation of oxidation and decomposition of hydroperoxides are favored in aqueous emulsion systems by reducing sugars because of their capacity to reduce transition metals. The co-presence of chelators, such as EDTA, efficiently inhibits this prooxidant activity (Yamauchi *et al.* 1984). Similarly, amino acids, especially histidine and cysteine, exhibit prooxidant activities in the presence of metal ions depending on concentration and pH (Farag *et al.* 1978, Simpson and Dean 1990, Yamashoji *et al.* 1979).

On the other hand, several studies have shown that emulsion stabilizers, sugars, and sugar alcohols (e.g., glycerol) can have the capacity to improve the chemical stability of the emulsions. This effect was initially interpreted to result from the increase in the viscosity of the aqueous phase, which hinders the diffusion of reactants such as oxygen (Sims *et al.* 1979). In fact, experimental evidence for the influence of viscosity on oxidation is lacking, and further experiments tend to contradict this assumption. Metal chelation, free radical scavenging, synergism with antioxidant activities, and physical protection by the interface are thought to play a large part in this protection (Matsumara *et al.* 2000, Ponginebbi *et al.* 1999, Shimada *et al.* 1992, Sirendi *et al.* 1998, Xue *et al.* 1998). These conclusions were drawn on the basis of studies performed on various multiphase systems. For example, 10–60% dextrin modulated the formation of hydroperoxides in methyl linoleate emulsions (pH 6.8) in the presence of proteins (1%) (Fujii *et al.* 1995). The protection was effective with 60% dextrin with low dextrose equivalent, whereas dextrin with high dextrose equivalent and mono- and di-saccharides (60%) increased the peroxide formation. A viscoelastic film of gum arabic adsorbed on the surface of the droplet of methyl linoleate emulsified by β-casein

(pH 7) caused decreased oxygen uptake during oxidation initiated by lipoxygenase or by water-soluble 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) (Matsumura *et al.* 2000). No or slight inhibition was observed under similar conditions with maltodextrin and pullulan, which did not form such a protective film at the interface. The ability of the peptide moieties of gum arabic to scavenge the radical species should also explain in part its antioxidative activity on AAPH-induced oxidation. On these bases, Xue *et al.* (1998) demonstrated that several water-soluble marine polysaccharides (alginate and alginate derivatives, water-soluble chitosan derivatives, and lipid-soluble chitin derivatives) displayed antioxidant activity during AAPH-induced oxidation of liposomes. The protection by alginate derivatives increased with decreasing molecular weight and with increasing sulfate content. Free amino groups of water-soluble chitosan derivatives as well as hexanoyl and hydroxyl groups of lipid-soluble chitin derivatives were thought to participate in the protective effects. Chitin derivatives also showed peroxide radical trapping activity in organic solvents. Xanthan, glucomannan, and locust bean gum greatly inhibited the formation of peroxides in pH 4.0 Tween 20/methyl linoleate emulsions, whereas no effect or only a small effect was found with carrageenan and chitosan (Sirendi *et al.* 1998). The authors found the polysaccharides to have no radical-scavenging activity but iron-binding capacities, which increased in the order glucomannan < carrageenan < locust bean gum < xanthan. Accordingly, Shimada *et al.* (1992) showed that oxidation of soybean oil emulsified by cyclodextrin (pH 4, pH 7, or nonbuffered) was synergistically inhibited by xanthan in the presence of tocopherols. The stabilizer was assumed to act with tocopherols by inactivation of the metal ions due to the capacity of the pyruvate groups of xanthan to chelate iron.

Most amino acids have a significant antioxidant potential due to their free amino groups or to their side chains (Riison *et al.* 1980, Taylor and Richardson 1980, Yamashoji *et al.* 1979). These activities result mainly from the hydrogen-donating capacity of these functional groups and their capacity to quench free radicals, but metal chelation could also intervene, especially when synergistic activities with primary antioxidant occur (Rousseau *et al.* 1988).

Interface and Oxidation

The interface consists of a narrow region surrounding each oil droplet (Fig. 7.3). It comprises emulsifier molecules but also other molecules whose location within the interface depends on their hydrophobicity. For instance, a certain percentage of water may be found in the hydrophilic domain of the interface and up to 2–3% triglycerides can be dissolved in the phospholipid layers. Other surface-active molecules include antioxidants, amphiphilic products of lipid oxidation such as hydroperoxides, core-aldehydes, other secondary products, and various ligands that may be bound to the emulsifiers (e.g., metal ions). For relatively small droplets, this interfacial region represents a significant volume fraction of the droplets

(McClements and Decker 2000). For example, when the droplet diameter is 0.4 μm and the interfacial thickness varies from 2 to 10 nm, the interfacial region occupies 3–14% of the total volume of the droplets, and ~10% of the oil molecules are in direct contact with the interface.

The interface acts as a barrier against the diffusion of prooxidants from the aqueous to the lipid phase. Hence, a thick and tight membrane effectively retards lipid oxidation (Donnelly *et al.* 1998, Matsumura *et al.* 2000, Silvestre *et al.* 2000). Accordingly, the thickness of the interface, provided by surfactants differing in the size of their polar head groups, was shown to correlate with oxidation development in emulsions (Silvestre *et al.* 2000). Lower emulsifier levels of small ionic emulsifiers, such as soybean lecithin, gave higher peroxide levels in fish oil emulsions (pH 7), whereas the effect was not found for whey protein concentrate (Fomuso *et al.* 2002). A commercial emulsifier added at moderate concentration (200 mg/kg) to fish oil–enriched mayonnaise also led to lower lipid peroxides and modified volatile profiles, although it did not significantly affect flavor deterioration or free radical formation (Jacobsen *et al.* 2001b). At high concentrations, the tighter packing of the surfactant molecules or the presence of multilayers of the phospholipids at the interface could act as a barrier to the diffusion of the oxidation catalysts into the oil droplets.

The electrical charge of the interface, which is determined by the composition of the droplet membrane and the pH and ionic strength of the aqueous phase, is another significant factor in controlling oxidation. For example, if the interfacial membrane is positively charged, positively charged trace metal ions are repulsed, and their ability to promote lipid oxidation in the oil phase or at the interphase is limited. The inverse effect is observed when repulsive electrostatic interactions between an antioxidant and a droplet prevent the antioxidant from being active at the interface (Mei *et al.* 1999a). As a consequence, differences in the oxidative stability of emulsions vary with the surface charge of the emulsifier (with decreasing rates in the order anionic, nonionic, and cationic surfactants) and with the pH (Mancuso *et al.* 1999a, Mei *et al.* 1998).

The interface may also act as a promoter of oxidation if it comprises unsaturated fatty acids and prooxidant proteins. Milk fat globule membrane was shown to favor the development of oxidation in dairy emulsions (e.g., cream). Indeed, it contains membrane lipids with higher unsaturation than neutral lipids, as well as membrane metalloproteins such as xanthine oxidase that exert prooxidant activity in the presence of copper ions (Allen 1994, Chen and Nawar 1991). Surfactants, such as Tweens, may contain substantial amounts of peroxides that could be involved in the development of oxidation of emulsions in the presence of metal ions or free radicals at the interface (Mancuso *et al.* 1999b, Nuchi *et al.* 2001). Natural lecithins from egg yolk or soybean vary greatly in their fatty acid and phospholipid composition. They often contain appreciable levels of hydroperoxides, which could promote emulsion oxidative instability. Also, both the high unsaturation of the fatty chain and the nature of the polar head groups may contribute to emulsion

oxidizability, as was shown in models and in muscle foods for muscle polar lipids (Chan *et al.* 1997, Gandemer *et al.* 1997).

Phospholipids and nonionic surfactants also exert antioxidant activity in oils, liposome systems, and emulsions (Saito and Ishihara 1997, Terao 2001). Dipalmitoyl phosphatidic acid reduced the oxidation of fish oil emulsions (pH 7.4) induced by methemoglobin, metmyoglobin, or free iron, whereas bovine brain phosphatidyl serine was active only against iron-catalyzed oxidation (Dacaranhe and Terao 2001). The inhibition of the prooxidant activity of nonheme iron could result from interactions of the phospholipid polar heads with the metal ions. Also, synergistic activity of phospholipids containing primary amine group(s) with primary antioxidants has been evoked (Lambelet *et al.* 1994). Choline and ethanolamine residues were also assumed to enhance hydroperoxide decomposition, leading to decreasing amounts of peroxides and conjugated dienes (Miyazawa *et al.* 1984). Nonionic surfactants such as sucrose monoesters, sorbitans, Spans, Tweens, and monoglycerides could stabilize hydroperoxides due to hydrogen bond formation between the hydroperoxides and the surfactants (Pokorny 1987).

Antioxidant Activity in Emulsions

Antioxidants have been defined as "substances that when present in small quantities are able to prevent or greatly retard the oxidation of oxidizable material such as lipids" (Chipault 1962). The main types of antioxidants are chain-breaking antioxidants, metal chelators, hydroperoxide destroyers, ultraviolet light deactivators, singlet oxygen quenchers, and synergists (Frankel 1998). Chain-breaking antioxidants are often considered as true antioxidants and classified as primary antioxidants. Their antioxidant activity is based on their ability to break radical chains by donating a hydrogen atom to the peroxyl (chain-breaking electron donors) or to the lipid radicals (chain-breaking reaction acceptors). In addition, reactions with alkoxyl radicals either by hydrogen donation or by the combination termination reaction can be involved (Frankel and Meyer 2000). Most chain-breaking antioxidants are natural or synthetic substituted phenolic compounds such as tocopherols, butylated hydroxyanisole, butylated hydroxytoluene (BHT), carnosol, carnosic acid, flavonoids, and phenolic compounds. Other antioxidants are classified as preventive antioxidants. Metal chelators complex with transition metal ions, preventing them from promoting the metal-catalyzed initiation reactions and the decomposition of hydroperoxides. This group of antioxidants inactivates the prooxidative metal ions and thus retards the formation of hydroperoxides (Frankel 1998).

Increasing concentrations of antioxidants normally yield better stability toward oxidation. However, high concentrations of α-tocopherol were claimed to be prooxidant because α-tocopherol can abstract the hydrogen atom from the hydroperoxides (Cillard *et al.* 1980, Debal 1992, Porter *et al.* 1995). In Chapters 1 and 2 of this book, this effect was qualified as a loss of efficacy and not a prooxi-

dant effect. As discussed in Chapter 4, the same kind of phenomenon was observed with phenolic antioxidants. In addition to the oxidation rate, antioxidants also affect the nature of the reaction products. For example, the presence of α-tocopherol changed the relative ratio of *cis*, *trans* to *trans*, *trans* hydroperoxide isomers formed from linoleic acid oxidation at the expense of the *trans*, *trans* configuration (Porter *et al.* 1995).

The antioxidant mechanism depends first on the chemical structure of the antioxidants. Structure-activities relationships of antioxidants have given rise to a number of studies that have been reviewed elsewhere (Hall and Cuppett 1997, Rice-Evans *et al.* 1996, Zhang 1999). Antioxidant activity also depends on their interactions, location, and orientation in the emulsion (Coupland and McClements 1996). According to the "polar paradox," nonpolar antioxidants are more effective in general in an emulsion system than polar antioxidants due to their affinity toward the oil-water interfaces where they form a protective layer around the droplets. In contrast, polar antioxidants are more active in bulk oils than in emulsions because of their higher proportions in the water phase where they are not directly in contact with oxidizing lipids (Frankel *et al.* 1994, Huang *et al.* 1996a and 1996b, Porter *et al.* 1980 and 1993). The great importance of the affinity of the antioxidants for the interface and/or the substrate, and its effect(s) on the antioxidant efficiency, has been demonstrated in several studies performed on various models (Frankel and Meyer 2000). In an ethanol-buffer homogeneous system and in liposomes, the activity of ferulates or gallates was observed to depend on their affinity for the substrate (Kikuzaki *et al.* 2002, Nakayama *et al.* 1998). Hydroxycinnamic acids exerted efficient antioxidant activity in liposomes because their capacity to scavenge free radicals was more effective due to their affinity for the phospholipids (Kikuzaki *et al.* 2002). In contrast, they were less efficient than BHT in delaying hydroperoxide formation in corn oil/Triton X100 emulsions (Chen and Ho 1997). Flavonoids showed an efficient antioxidant activity in liposomes (Terao *et al.* 1994, cited by Terao 2001) and in fish oil/bile salt emulsions (Hoshino *et al.* 1997) due to their affinity for the phospholipid bilayers and the interface. On the other hand, despite its higher rate constant for scavenging peroxyl radicals, α-tocopherol displayed a lower antioxidant activity than the tested flavonoids because the molecule is more hydrophobic and remained in the hydrophobic core of the emulsions (Hoshino *et al.* 1997, Terao 2001). Carnosic acid and carnosol, which are more polar than α-tocopherol, were less effective antioxidants in emulsions made of corn oil, methyl linoleate, or linoleic acid and stabilized by Tween 20 (Hopia *et al.* 1996). Carnosic acid was more effective than carnosol in hydrophobic substrates (e.g., corn oil and methyl linoleate), but less effective in linoleic acid, possibly due to interactions of the free acid group of the fatty acid with the carboxyl group of carnosic acid.

The partitioning of antioxidants has been studied in model and food emulsions (Jacobsen *et al.* 1998 and 1999c, Pekkarinen *et al.* 1999, Richards *et al.* 2002, Rodis *et al.* 2002, Stöckmann and Schwarz 1999, Stöckmann *et al.* 2000). The par-

titioning of the antioxidants at the interface depends on the emulsifier type; entrapment of antioxidants is made possible due to specific interactions with other constituents of the interface. For instance, caffeic acid, with low lipid solubility in water-oil systems, partitioned to the lipid phase in Tween 20–stabilized emulsions (Pekkarinen *et al.* 1999). It greatly inhibited hydroperoxide formation in bulk methyl linoleate, whereas it slightly promoted their formation when the lipid was emulsified. Because these interactions often involve hydrogen bonds, the efficiency of the antioxidant is consequently influenced (Schwarz *et al.* 2000, Stöckmann *et al.* 2000). This may give unexpected results in view of the polar paradox (Stöckmann *et al.* 2000). Micelles of excess surfactant in the continuous phase of oil-in-water emulsions can displace polar antioxidant molecules from the interface (Richards *et al.* 2002). In addition, electrostatic repulsion between charged antioxidants and emulsifiers may hinder the protecting effect of antioxidant compounds (Pryor *et al.* 1993). Proteins, both in the continuous phase and adsorbed at the interface may also markedly modify the activity of antioxidant molecules through specific or unspecific interactions (Bartolomé *et al.* 2000, Heinonen *et al.* 1998). In fact, the use of antioxidants in real food emulsions often gives rise to unexpected and fluctuating results due to the complexity of the systems (Jacobsen *et al.* 2000a, 2001a and 2001b).

Actually, it is too simplistic to represent food or cosmetic emulsions by two- or even three-phase models. Other dispersed organized molecular structures that may be present are lamellar, hexagonal, or cubic liquid-crystalline phases of polar lipids, surfactants, or protein micelles. These structures can develop specific interactions with metal ions and antioxidant molecules, affecting their prooxidant or antioxidant activities. To our knowledge, only a few studies have attempted to take into account this aspect of the structure of multiphase systems. The antioxidant activity of α-tocopherol in emulsions was greater when the antioxidant was added to a lecithin emulsifier dispersed as liposomes than when it was dissolved in the oil before emulsification (Ruben and Larsson 1985). The improved antioxidant activity was attributed to the probable location of α-tocopherol in the multilayer lamellar liquid-crystalline phase surrounding the oil droplets. According to Terao (2001), the preventive effect of nonesterified EPA during the oxidation of fish oil emulsion may result in part from the interception of the lipid peroxyl radicals responsible for the chain propagation in the hydrophobic core of the oil droplets by the PUFA micelles.

Effects of Oxidation on Odor and Flavor of Emulsions

Lipid oxidation in emulsified systems leads to an overall decrease in the nutritional, sensory, and functional qualities of the products. However, in food science, negative consequences of lipid oxidation on the odor and the flavor of the products are generally considered as having priority, given the very low detection threshold of certain volatile products of oxidation. This is why we decided to focus on the

effects of lipid oxidation on odor and flavor and not to discuss the other consequences on other quality parameters of the products, e.g., functional properties and nutritional value.

Flavor Release in Foods and Emulsions

The term flavor refers to the sensations experienced by consumers when volatile (aroma compounds) and nonvolatile (tasty molecules) components of the foods they are eating reach their nose and mouth receptors. As in other lipid-containing foods, lipid oxidation in emulsion leads to the development of rancid and off-flavor notes that reduce their acceptability by the consumer. One of the key parameters influencing the flavor perception of an emulsion is the partitioning and diffusion of volatile compounds through the different phases of the system. Aroma compounds must be present in the continuous phase to be released to the gaseous phase, where they can be perceived.

Flavor release can be defined as the process whereby flavor molecules move out of a food and into the surrounding saliva or vapor phase during mastication. This release occurs under extremely complex and dynamic conditions. The food usually spends a relatively short period, typically 1 to 30 s, in the mouth before being swallowed. During this period, it is diluted with saliva, undergoes temperature changes, and is subjected to a variety of mechanical forces. Mastication may therefore cause dramatic changes in the structural characteristics of a food. During mastication or consumption, the flavor molecules must move from the food, through the saliva, and into the gas phase, where they are carried to the aroma receptors in the nasal cavity. For comparison, the time scale for the diffusion of molecules out of oil droplets has been calculated to be in the range from 10 μs to 10 ms when the droplet diameter varies from 1 to 20 μm (McClements and Decker 2000).

The two major factors that determine the rate at which these processes occur are the equilibrium partition coefficient and the mass transfer coefficient. The partition coefficient determines the magnitude of the flavor concentration gradients at the various boundaries. The mass transfer coefficient determines the speed at which the molecules move from one environment to another (de Ross and Wolswinkel 1994, McClements 1999, van Ruth and Roozen 2000a). The nature and amounts of odor-active compounds present determine the perceived aroma intensity of a food. Furthermore, the intensity is influenced by the availability of the compounds to the sensory system, which depends on factors influencing aroma release (e.g., temperature, mastication, salivation) and on factors influencing the transport of released volatiles *via* the respiratory cycle to the olfactory epithelium. Most studies in this area have dealt with partition phenomena, in particular with the effect of medium composition on the equilibrium concentration, e.g., in relatively simple systems containing proteins, lipids, and carbohydrates. The effect of mass transport on aroma release has received less attention.

Food lipids have been reported to influence the perception of flavor in food products in terms of both flavor release and textural change (Tuorila *et al.* 1995). In bulk lipids, the volatile compounds are present in, and released from, only one phase. In emulsions, they may be distributed and released from several phases, i.e., the aqueous phase, the oil phase, and the interface. Many volatile flavor compounds possess a lower vapor pressure in oil and, therefore, a higher odor threshold than they do in aqueous systems (Buttery *et al.* 1973). Release of hydrophobic flavor compounds from the oil phase proceeds at a lower rate than from the aqueous phase. This may be attributed to the higher resistance to mass transfer in oil than in water. Furthermore, flavor compounds in oil-in-water emulsions have to diffuse from the oil to the aqueous phase before they can be released from the aqueous phase of the emulsion to the vapor phase (de Ross 1997).

Volatile Products and Aroma Compounds Derived from Oxidation

The primary products of autoxidation, the hydroperoxides, are odorless and tasteless and do not greatly impair the sensory properties of the emulsion until they are decomposed into secondary oxidation products, particularly aldehydes (Henick *et al.* 1954). The widely accepted pathways for the decomposition of monohydroperoxides are presented in Figure 7.7. Decomposition of hydroperoxides involves free radical mechanisms and results in the formation of nonradical products including both volatile and nonvolatile compounds. It begins with homolytic cleavage to give an alkoxy radical ($RO^{\bullet}$) and a hydroxy radical ($^{\bullet}OH$); this is followed by cleavage of the fatty acid chain adjacent to the alkoxy radical (β-scission). According to Grosch (1982), the nature of volatile compounds for a particular hydroperoxide depends on the composition of the alkyl chain and the position at which β-scission takes place (A or B in Fig. 7.7). The volatile decomposition products from hydroperoxides have a noticeable influence on the odor and flavor of the products. Thus, this part of the chapter will focus on the formation of volatile compounds arising from lipid oxidation and on their sensory impact. Other products such as core-aldehydes are the subjects of other chapters in this book. Physicochemical as well as flavor properties of volatile compounds arising from lipid autoxidation are presented below according to the nature of the fatty acid precursors.

In fats and oils, oleic acid (18:1n-9) is the major n-9 fatty acid, and it predominates in olive oil, high-oleic sunflower oil, canola oil, lard, and butter oil. Oleyl hydroperoxides of oxidizing oils decompose at about the same rate (if not more quickly) than more unsaturated hydroperoxides and contribute appreciably to the volatile profile of the oxidized oils (Lampi and Piironen 1999, Lee *et al.* 1995). Identified compounds are alkanes, alcohols, alkanals, and 2-alkenals, with chain length varying from 7 to 11. Physicochemical characteristics of volatile compounds arising from autoxidation of n-9 fatty acids are summarized in Table 7.3. All of these compounds are very hydrophobic as revealed by log P values >3, where P refers to the partition coefficients. This factor is important to keep in mind

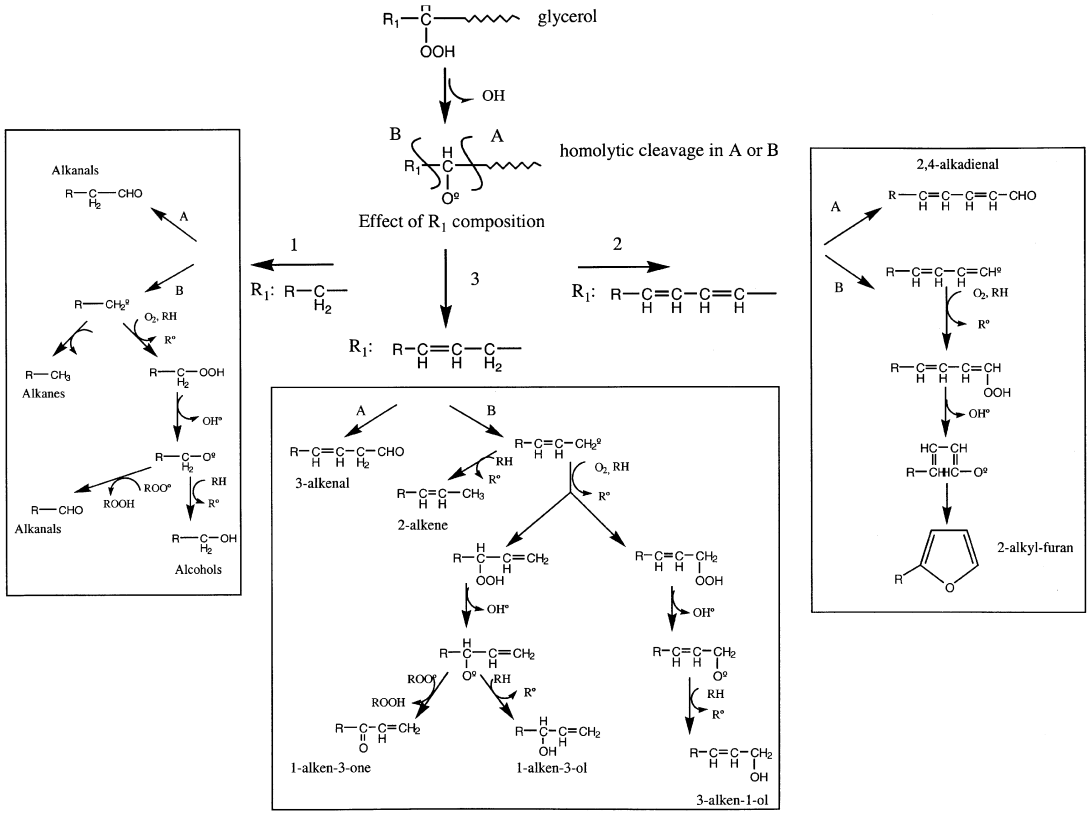

Fig. 7.7. Pathways of decomposition of monohydroperoxides of triacylglycerols. Effect of alkyl substituent. (Adapted from Grosch 1987 and Mottram 1991). Note: Remaining acyl chains represented nonvolatile oxidation products such as core aldehydes.

TABLE 7.3
Physicochemical Characteristics of Volatile Compounds Arising from Oxidation of n-9 Fatty Acids[a,b,c]

Volatile compound	CAS[a]	Physicochemical data[b]					Parent HP	Relative proportions of volatiles in different oxidizing lipids (%)		
		MW	S (g/L)	Psat mm Hg	log P	h atm·m^3/mol		Oleate HP	Triolein[d]	Oil (Olive)[e]
Heptane	142-82-5	100.21	3.40E-03	46	4.66	2.00E+00	11-OOH	9.6	9.5	4.5
Heptanal	111-71-7	114.19	1.2	3.52	2.29	2.70E-04	11-OOH	1.1	5.6	3.6
Heptanol	111-70-6	116.2	1.67	0.22	2.69	1.88E-05	11-OOH	0.9	1.8	—
Octane	111-65-9	114.23	6.60E-04	14.05	5.18	3.21	10-OOH	5.9	10.8	8.3
Octanal	124-13-0	128.21	0.56	1.18	2.78	5.14E-04	10,11-OOH	23.9	9.4	2.2
Octanol	111-87-5	130.23	0.54	0.079	3.0	2.45E-05	10-OOH	0.9	2.4	—
1-Nonen	124-11-8	126.24	1.12E-03	5.4	5.15	0.8	9-OOH	—	—	—
Nonanal	124-1-6	142.24	9.60E-02	0.37	3.27	7.34E-04	9,10-OOH	32.6	24.3	5.7
1-Decen	872-05-9	140.27	1.15E-04	1.67	5.7	2.68	8-OOH	—	—	—
Decanal	112-31-2	156.27	6.08E-02	0.103	3.76	1.80E-05	8-OOH	8.5	3.1	—
2-Decenal	3913-71-1	154.25	—	—	3.55	—	9-OOH	11.8	18.8	1.4
2-Unedecenal	2463-77-6	168.28	—	—	4.04	—	8-OOH	3.7	12.1	0.4

[a]CAS: Chemical Abstract Service registry number.
[b]HP, hydroperoxide; S, water solubility at 25°C; log P, log of partition coefficient between octanol and water; Psat, vapor pressure at 25°C; h, Henry's law constant at 25°C.
[c]—, data not found.
[d]*Source:* Selke *et al.* 1978.
[e]*Source:* Snyder *et al.* 1985.

when looking at the partition and release of volatile compounds arising from lipid oxidation in emulsions. Relative proportions of the volatile compounds produced during oxidation of different lipids containing oleic acid as the major fatty acid are also reported. Table 7.3 shows that results obtained with purified compounds such as oleate or triolein cannot be applied directly to complex lipids such as olive oil. The nature of the volatile compounds is similar, but large differences in the relative proportion and quantities exist. Flavor properties of volatile compounds arising from n-9 fatty acid oxidation are tabulated in Table 7.4. C_9-C_{10} alkanals possess fruity notes such as orange and citrus, whereas C_7 possesses green notes. Their odor thresholds in water range from 0.3 to 80 ng/g. In oil, the odor thresholds were much higher and ranged from 40 ng/g to 4 μg/g.

The major n-6 fatty acid of edible fats and oils is linoleic acid, the main fatty acid in the vegetable oils of corn (60%), cottonseed (57%), safflower (82%), soybean (54%), and sunflower (73%) (Snyder *et al.* 1985). Physicochemical characteristics of volatile compounds arising from autoxidation of n-6 fatty acids are summarized in Table 7.5. The compounds identified include alkanes, unsaturated alcohols, saturated and unsaturated aldehydes, and ketones, with chain lengths ranging from 5 to 10, and hydrophobicities ranging from 1.31 to 4. The relative proportion of volatile compounds differs greatly depending on the esterification of the fatty acid and on the nature of the alcohol moiety. Thus, the proportion of pentane formed in the oxidation products varied from 10% from the degradation of linoleate hydroperoxide to 55% from the degradation of oxidizing safflower oil. Surprisingly, the proportion of hexanal was similar regardless of the oxidizing system (~15%). This observation is of importance in regard to the flavor properties of oxidizing lipids (Table 7.6). The data indicated that pentane, the major volatile compound arising from safflower oil, possessed a very high odor threshold (up to 100 μg/g) compared with 0.07 ng/g for 2,4-decadienal (accounting for 1.8% of oxidation products of safflower oil). Flavor notes associated with n-6 oxidation ranged from green, to pungent, mushroom, or deep-fried. The odor thresholds in water ranged from 0.008 to 0.09 μg/g, and in oil, they ranged from 0.077 to 14 μg/g.

Most vegetable oils, except the rarely used flaxseed and perilla seed oils, contain a relatively low proportion of n-3 fatty acids. Conversely, fish oils contain relatively high proportions of these fatty acids, especially long-chain fatty acids such as EPA (20:5n-3) and DHA (22:6n-3), which account for at least one third of total fatty acids. Linolenic acid autoxidation is known to some extent, whereas the mechanism of autoxidation and the structure of the hydroperoxides produced from long-chain n-3 fatty acids are still poorly understood. Nevertheless, the major volatile compounds arising from linolenate oxidation have been identified in various fish oils (Horiuchi *et al.* 1998, Hsieh *et al.* 1989, Karahadian and Lindsay 1989) or in emulsions prepared with fish oil (Hartvigsen *et al.* 2000). Table 7.7 summarizes the physicochemical data and occurrence of volatile compounds from n-3 origin in various oxidized lipids. Oxidation of fish oil–containing systems led to the formation of numerous volatile compounds including unsaturated aldehydes,

TABLE 7.4
Flavor Properties of Volatile Compounds Arising from Oxidation of n-9 Fatty Acids[a,b,c]

Volatile compound	Odor description[a,b]	Odor threshold (μg/g)[b]		Flavor threshold (μg/g)[c]			
		Oil	Water	Paraffin oil	Vegetable oil/butter	Water	Milk
Heptanal	Fresh, green, herbal, sweet, oily, putty, soapy, fruity	3.2	0.003–0.008	0.042–0.055	—	0.031	0.12
Heptanol	Violet, herbal, green, sweet, fresh, woody	—	—	—	—	0.52	—
Octanal	Sharp, citrus, fruity, sweet, fatty, soapy, fruity	0.32	0.0003–0.0007	0.04–0.6	0.9	0.005–0.047	0.46
Octanol	Strong, orange, waxy, fatty, rose, sweet	—	0.11	—	—	0.047	—
Nonanal	Fresh, fatty, rose, iris, lily, orange, verbena, tallowy, soapy, fruity	13.5	0.001–0.008	0.20–0.32	—	0.045	0.22
Decanal	Sweet, waxy, orange, peel, citrus, orange peels	6.7	0.001–0.08	0.7–1	0.6	0.007	0.24
2-Decenal	Fatty, orange, rose, top note floral, green, tallowy, orange	33.8	—	—	—	—	—
2-Unedecenal	Fresh, citrus, fruity, orange peel	150	—	4.2	—	—	—

Sources: [a]The Good Scents Company (http://www.thegoodscentscompany.com); [b]Fazzaralli 1978, Grosch 1987; [c]Forss 1972.
[d]—, data not found.

TABLE 7.5
Physicochemical Characteristics of Volatile Compounds Arising from Oxidation of n-6 Fatty Acids[a,b,c]

Volatile compound	CAS[a]	Physicochemical data[b]					Parent HP	Relative proportions of volatiles in different oxidizing lipids (%)			
		MW	S (g/L)	Psat mm Hg	log P	h atm-m^3/mol		Linoleate HP[f]	Triolein[d]	Oil[e]	
										Corn	Safflower
Pentane	109-66-0	72.15	3.80E-02	514	3.39	1.25E+00	13-LOOH	9.9	12	38.5	54.3
Pentanal	110-62-3	86.13	11.7	26	1.31	1.47E-04	13-LOOH	0.8	3.6	1.6	1.9
Hexanal	66-25-1	100.16	5.6	11.26	1.78	2.13E-04	9,12,13-LOOH	15	17.2	15.5	11.1
t-2-Heptenal	18829-55-5	112.3	—	—	2.07	—	12-LOOH	tr	15.3	3	5.6
t-2-Octenal	2363-89-5	126.2	0.61	0.86	2.57	7.34E-05	9-LOOH	2,7	4.4	0.8	0.6
1-Octen-3-ol	3391-86-4	128.21	—	—	2.6	—	10-LOOH	tr	1.7	0.5	0.1
1-Octen-3-one	4312-99-6	126.2	—	—	2.37	—	10-LOOH	—	—	—	—
t,t-2,4-Decadienal	—	152.23	—	—	—	—	9-LOOH	14	19	2	1.8
2-Pentyl-furan	3777-69-3	138.21	—	—	3.87	—	9-LOOH	2.4	0.8	0.8	0.7

[a]CAS: Chemical Abstract Service registry number.
[b]HP, hydroperoxide; S, water solubility at 25°C; log P, log of partition coefficient between octanol and water; Psat, vapor pressure at 25°C; h, Henry's law constant at 25°C; tr, trace.
[c]—, data not found.
[d]*Source:* Selke *et al.* 1978.
[e]*Source:* Snyder *et al.* 1985.
[f]*Source:* Frankel 1985.

TABLE 7.6
Flavor Properties of Volatile Compounds Arising from Oxidation of n-6 Fatty Acids[a,b,c]

Volatile compound	Odor description	Odor threshold (μg/g)		Flavor threshold (μg/g)			
		Oil	Water[c]	Paraffin oil	Vegetable oil/butter	Water	Milk
Pentane	—	—	22–110	—	—	—	—
Pentanal	Glue, green, sharp, *bitter-almond*	0.24	0.024	0.07–0.15	—	0.07	0.13
Hexanal	Pungent, green, fatty, *green-fruity-bitter-almond*	0.32	0.008	0.08–0.6	0.3	0.03	0.049
t-2-Heptenal	*Putty-fatty, bitter-almond*	14	0.053	0.2–0.63	—	—	0.077
t-2-Octenal	*Woodbugs-putty, nutty*	7	0.004	0.15–1	—	—	—
1-Octen-3-ol	Pungent, soil, fruity	—	—	0.0075	0.1	0.001	0.01
1-Octen-3-one	Mushroom, *metallic, moldy-mushroom-metallic*	0.077	0.089	0.0001	0.001	0.0001	0.01
2,3-Octanedione	Pungent, sour	—	—	—	—	—	—
t,t-2,4-Decadienal	Deep-fried	2.15	—	0.1	—	0.0005	—
2-Pentyl-furan	Green, *buttery, beany, rancid*	2	—	—	—	—	—

Sources: [a]Hartvigsen *et al.* 2000; *italics* denote Grosch 1987; [c]Devos *et al.* 1990; see also http://www.leffingwell.com and Fazzaralli 1978.
[b]—, data not found.

alcohols and ketones. These compounds are less hydrophobic than compounds of n-9 or n-6 origin because their log P values range from 0 to 2.84. The flavor properties of volatile compounds of n-3 origin are tabulated in Table 7.8. Odor thresholds ranged from 0.01 ng/g to 1.5 µg/g and the dominant notes were green, tomato, cucumber, fishy, and fatty.

Partitioning of Volatile Products of Lipid Oxidation in Emulsions

In emulsions, it can be assumed that lipid oxidation compounds are produced in the dispersed lipid phase. Thereafter, according to their physicochemical and thermodynamic properties, the compounds disperse among the different phases of the emulsion until they reach equilibrium. As a consequence, during the time course of oxidation, hydrophilic lipid oxidation products diffuse from the core of the oil droplets to the interface, where they then diffuse to the water phase to finally be released, if sufficiently volatile, into the exterior gas phase.

The partition coefficient of a volatile compound between air and a solvent is defined as the ratio of concentrations of the volatile in the air phase and in the solvent. Partition coefficients in pure solvents of some aldehydes formed during lipid oxidation are listed in Table 7.9. Buttery *et al.* (1973) proposed the following equations to evaluate the partition coefficient above the emulsion from the partition above the constitutive phases:

$$K_{aem} = \frac{1}{\frac{\Phi_w}{K_{aw}} + \frac{\Phi_o}{K_a}} \quad \text{or} \quad K_{aem} = \frac{K_{aw}}{(\Phi_w + \Phi_o \cdot K_{ow})}$$

where K_{aem} is the partition coefficient between air and emulsion, K_{aw} is the partition coefficient between air and water, K_{ao} is the partition coefficient between air and oil, K_{ow} is the partition coefficient between oil and water ($K_{ow} = K_{aw}/K_{ao}$), Φ_w is the volume fraction of water, and Φ_o is the volume fraction of oil.

The variation of the partition coefficient of some aldehydes between air and emulsion vs. oil volume fraction is presented in Figure 7.8. In most cases, for highly hydrophobic compounds, an increase in the oil volume fraction induces a decrease in the volatility of the aroma over the emulsion. However, the effect of volume fraction on the partition between air and emulsion depends on the nature and especially on the log P value of the aroma compounds. Hydrophilic compounds (log P < 1, Fig. 7.8A) were characterized by a progressive change of partition coefficient over the entire range of volume fraction. The variation was either negative (e.g., for butanal) or positive (e.g., for 2-butenal). Partition coefficients of compounds with intermediate hydrophobicity were dramatically reduced when the oil volume fraction varied from 0 to 0.4 and then remained almost stable (Fig. 7.8B). Partition coefficients of hydrophobic compounds (log P > 2, Fig. 7.8C) were by far the most sensitive to the addition of an oil phase to the aqueous solution and

TABLE 7.7
Physicochemical Characteristics of Volatile Compounds Arising from Oxidation of n-3 Fatty Acids

Volatile compound[a]	CAS[c]	Physicochemical data[b,c]					Parent HP	Relative proportions of volatiles in different oxidizing lipids (%)				
		MW	S (g/L)	Psat (mm Hg)	log P	*h* (atm·m^3/mol)		LNA HP[d]	Lino-lenate HP[e,f]	Trilino-lenin[g]	Men-haden oil[h,i]	Cod liver oil[j]
Propanal	123-38-6	58.08	3.06E+02	316.8	0.59	7.34E-05	15-LnOOH	NQ	2.3–7.7	36.5	0.2	—
2-Propenal	107-02-8	56	212	274.1	–0.01	1.22E-04	—	—	—	5	21.7	—
t-2-Butenal	4170-30-3	70.09	181	30	0.6	9.68E-06	15-LnOOH	1.3	0.8–0.5	2	8.6–0.4	0.1
1-Penten-3-ol	616-25-1	86.13	90.05	9.68	1.12	9.88E-06	13-LnOOH	—	—	3	10.2	—
1-Penten-3-one	1629-58-9	84.12	—	—	0.9		13-LnOOH	3.8	—	—	1.6–0.7	5.7
t-2-Pentenal	764-39-6	84.12	—	—	1.09		13-LnOOH	4.5	–1.6	4	10.4–2.8	1.4
c-2-Pentenal	—		—	—	—			5.8				
t-2-Penten-1-ol	—		—	—	—		13LnOOH	—	—	—	3.8	0.3
c-2-Penten-1-ol	1576-95-0	86.13	—	—	1.12	—						
c-3-Hexenal	4440-65-7	98.14		1.58			13-LnOOH	13.4	–1.4	—	0.25	—
t-2-Hexenal	505-57-7	98.14	5.26	6.6	1.58	4.89E-05	11,13-LnOOH	1.9		0.5	1.9–1.5	1.4
2,4-Hexadienal	142-83-6	96.13	8.13	4.81	1.37	9.78E-06	—	—	—	—	0.14	0.1
2,4-Heptadienal	413-03-5	110.15	—	—	1.86		11-LnOOH	50	60.8–9.3	21	7.2–11.4	25.6

1,5-Octadien-3-ol	—	—	—	—	—	—	—	—	—	—	—	0.7
1,5-Octadien-3-one	65767-22-8	124.18	—	—	—	—	—	—	—	—	—	0.01
t,c-2,6-Nonadienal	557-48-2	138.21	—	—	2.84	—	10-LnOOH	1.3	–0.5	—	—	1.1
2,4,7-Decatrienal	—	—	—	—	—	—	9-LnOOH	11	14	22	0.2	2.3
2-Ethyl-furan	3208-16-0	96.13	1.0	25.9	2.4	7.88E-05	11-LnOOH	—	—	1	1.9	—

[a]*Source:* Hartvigsen *et al.* 2000, Horiuchi *et al.* 1998.
[b]*Source:* The Physical Properties Database (PhysProp) of the Syracuse Research Corporation, http://esc-plaza.syrres.com/ interkow/physdemo.htm, http://esc.syrres.com/efdb/Datalog.htm.
[c]CAS: Chemical Abstract Service registry number; S, water solubility at 25°C; log P, log of partition coefficient between octanol and water; Psat, vapor pressure at 25°C; *h*, Henry's law constant at 25°C; HP, hydroperoxide; LNA, linolenic acid; —, data not found.
[d]*Source:* Grosch 1982 and 1987.
[e]*Source:* Frankel *et al.* 1987 (oxidized with Fe ascorbate).
[f]*Source:* Frankel *et al.* 1981.
[g]*Source:* Frankel *et al.* 1992.
[h]*Source:* Horiuchi *et al.* 1998.
[j]*Source:* Karahadian and Lindsay 1989.
[j]*Source:* Hsieh *et al.* 1989.

TABLE 7.8
Flavor Properties of Volatile Compounds Arising from Oxidation of n-3 Fatty Acids[a,b]

Volatile compound	Odor description	Odor threshold (ng/g)	Odor threshold (µg/g)		Flavor threshold (µg/g)			
			Oil	Water	Paraffin oil	Vegetable oil/butter	Water	Milk
Propanal	*Sharp-irritating*	9.5–37	3.6	0.16–0.009	1.0	0.2	0.17	0.43
2-Propenal	—	174–407	—	—	—	—	—	—
t-2-Butenal	Old cheese	135–389	—	—	0.7	1.4	1.6	—
1-Penten-3-ol	Sweet	400	—	—	4.2	10	3	3
1-Penten-3-one	Pungent, rancid green, glue, *sharp fishy*	1–1.3	—	—	0.003	0.005	0.01	0.003
t-2-Pentenal	Pungent, glue, green, grassy, *sharp-paint-green, apple*	1500	2.3	—	0.32	—	—	—
c-2-Pentenal	Fruity	—	—	—	0.8	—	—	—
t-2-Penten-1-ol	Green	—	—	—	—	—	—	—
c-2-Penten-1-ol	Musty, compost-like	—	—	—	—	—	—	—
c-3-Hexenal	Sour, old cheese, *fresh green leaves, green-beans-tomato-green*	0.25	—	—	0.09	—	—	—
t-2-Hexenal	Sour, green, *green*	17	10	0.316	2.5	—	—	0.067
2,4-Hexadienal	—	10–60	—	—	0.04	—	—	—
2,4-Heptadienal	*t,c*: Fishy, fatty, burnt, *frying odor, rotten apples*	4.2–19	4	—	0.04	—	—	—
	t,t: Nasty, green, fatty, *fatty-oily, rancid hazelnuts*	—	10	—	0.10–0.46	—	—	0.049
1,5-Octadien-3-ol	Citrus, green	—	—	—	—	—	—	—
1,5-Octadien-3-one	Geranium, metallic	—	—	—	—	—		
t,c-2,6-Nonadienal	Cucumber, *fresh cucumbers; cucumber-like*	0.01	0.01	—	0.0015	—	0.0001	—
2-Ethyl-furan	Flower	—	—	—	0.018–0.020	—	0.01	—

[a]*Sources:* Hartvigsen *et al.* 2000, *italics* denote Grosch 1987; http://www.leffingwell.com; Devos *et al.* 1990; Grosch 1987; Forss 1972.
[b]—, data not found.

to the small variations in oil volume of low-fat products. For example, the addition of 0.1% oil to an aqueous solution of octanal would induce a sixfold decrease in the aroma volatility, whereas the addition of 1% oil would reduce it by 53-fold. Most of the aroma compounds issued from lipid oxidation have intermediate or low hydrophilic character (Tables 7.3, 7.5, and 7.7). Thus, when the oil volume fraction is >0.5, the concentration of the aroma in the air phase above the emulsions depends mainly on the total aroma concentration in the emulsion and only slightly on the oil volume fraction.

The equations proposed by Buttery *et al.* (1973) do not take into account the other factors that might influence the partition and release of the aroma compounds, for example, the droplet size of the dispersed phase or the interface characteristics. For instance, in the case of emulsions stabilized by protein, the presence of proteins absorbed at the interface or solubilized in the aqueous phase makes possible the irreversible binding of aroma compounds such as α,β-unsaturated aldehydes (see Chapter 9). Thus, differences between the measured and calculated partition coefficients can be expected, whereas reliable quantitative data related to binding of α,β-unsaturated aldehydes to proteins are lacking. No clear conclusion can be drawn concerning the effect of droplet size on the partition behavior of aroma compounds over emulsions despite some recent publications on the subject (Charles *et al.* 2000, McClements 1999, van Ruth *et al.* 2002).

Oxidation and Flavor of Mayonnaise and Other Emulsions

The effects of lipid oxidation on the flavor of emulsions are indeed very complex because both the kinetics of volatile compound formation and their partitioning and release must be considered. Examples of oxidation in mayonnaise prepared with fish oil (Jacobsen and co-workers 1998–2001) and in emulsions prepared with vegetable oil (van Ruth and co-workers, 1999–2000) are presented and analyzed below.

Evidence from several investigations suggests that n-3 PUFA and especially EPA and DHA are beneficial to the human body. The physiologic benefits have been associated mainly with a reduced risk of cardiovascular diseases and with the neural and visual development of children. Thus efforts have been made to increase the consumption of n-3 PUFA by incorporating fish oil into different food products such as bread, yogurt, salad dressing, and mayonnaise (Jacobsen *et al.* 1999b). Traditional mayonnaise is a mixture of egg, vinegar, vegetable oil and spices (especially mustard). Typically, mayonnaise contains 70–80% fat rich in n-6 FA and despite high oil content is an oil-in-water emulsion. The studies of Jacobsen and co-workers (1998–2001) demonstrated that mayonnaise prepared with fish oil did not oxidize more quickly than mayonnaise without fish oil according to the chemical parameters tested (peroxide values). However, the fish oil–enriched mayonnaise developed unpleasant off-odors and off-flavors much more quickly than mayonnaise without fish oil (Jacobsen *et al.* 1999b). The fishy off-flavor in mayonnaise may be caused by small amounts of specific volatile off-flavor compounds, with low threshold values,

TABLE 7.9
Partition Coefficient of Some Volatile Aldehydes Arising from Lipid Oxidation[a]

	K_{aw}	K_{ao}	K_{ow}	$K_{octanol\text{-}water}$	log P
Butanal	4.7E-03	2.3E-03	2.0	7.6	0.88
Pentanal	6.0E-03	1.0E-03	6.0	20.4	1.31
Hexanal	8.7E-03	3.5E-04	24.9	60.3	1.78
Heptanal	1.1E-02	1.0E-04	110.0	195	2.29
Octanal	2.1E-02	4.0E-05	525.0	603	2.78
2-Butenal	8.0E-04	1.4E-03	0.6	4	0.6
2-Bexenal	2.0E-03	1.6E-04	12.5	38	1.58

[a]K_{aw}, partition coefficient between air and water (Buttery *et al.* 1969 and 1971); K_{ao}, partition coefficient between air and vegetable (safflower) oil (Buttery *et al.* 1973).

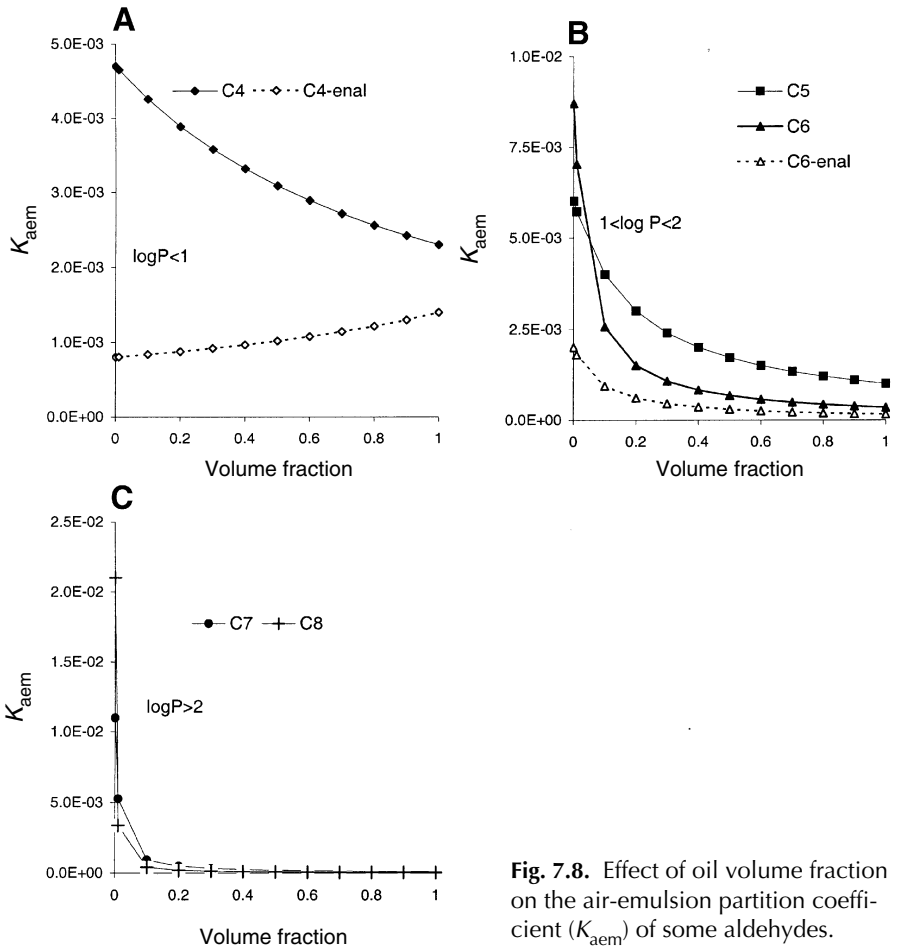

Fig. 7.8. Effect of oil volume fraction on the air-emulsion partition coefficient (K_{aem}) of some aldehydes.

present in the water phase of the emulsions. These off-flavor compounds apparently stem from the oxidation of EPA and DHA (Jacobsen *et al.* 1999a and 1999b). As previously mentioned, volatile compounds arising from n-3 fatty acids generally possess lower log P values than compounds arising from n-6 fatty acid oxidation. Consequently, their concentrations in the continuous aqueous phase of the mayonnaise are higher than the concentration of compounds from n-6 fatty acid oxidation.

Studies by van Ruth and co-workers (van Ruth *et al.* 1999b, van Ruth and Roozen, 2000a) emphasized differences in the formation rate of volatile compounds in oils and in emulsions. The researchers also observed differences in the release of volatile compounds, including differences in partitioning. The effect of the dilution of an emulsion with water or artificial saliva was considered to mimic phenomena occurring during the consumption of emulsions. Volatile lipid oxidation products are important for the aroma of oils and emulsions. The perception of aroma of the latter depends on the formation rate of aroma compounds throughout lipid oxidation, as well as on the release of these compounds (van Ruth and Roozen 2000b). Decreasing the lipid fraction in foods may aggravate the sensory consequences of lipid oxidation due to favored release in the air phase. Low-fat foods may consequently be perceived as being more oxidized than high-fat foods even though the overall concentration of volatile reaction products is similar (Roozen 1994).

To conclude, emulsification, fatty acid composition of the oil, and emulsion structure influence the formation of volatile secondary oxidation products, which contribute to the aroma. Emulsification, structure, and the texture of emulsions also influence the release of aroma compounds; these include volatility and mass transfer factors. Further studies are required to evaluate the importance of each parameter. The final objective of this research area should be the development of a mathematical model to predict the flavor properties of formulated emulsions.

Concluding Remarks

From the user's or manufacturer's point of view, a number of practical measures can be applied to minimize the development of oxidation in emulsions during their processing and storage. The main measures necessitate the possible combination of the following actions:

- To minimize the contact between the emulsion and air, which is often difficult to achieve during processing.
- To reduce the risks of metal contamination during processing. However, in most cases, trace metals cannot be avoided in formulations due to their presence in the ingredients or even their voluntary addition for nutritional reasons.
- To keep the temperature as low as possible.
- To avoid light exposure, especially at shorter wavelengths.

These very general measures should be combined with optimized use of ingredients and antioxidants, taking into account the specificity of the oxidation in heterogeneous systems. In this way, the partitioning and activities of prooxidant and antioxidant substances in the emulsified systems, the influence of the interactions of the constituents at various levels of organization, and the alterations of other constituents during lipid oxidation should be better known, especially from a quantitative point of view. The effects of these phenomena on the sensorial and nutritional qualities of the products also should be better understood. An improved appreciation of the numerous factors that intervene in oxidation development in emulsions and other multiphase systems will enable the building of operational predictive models that can be used to improve emulsion quality.

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References

Ahn, D.U., and Kim, S.M. (1998a) Prooxidant Effects of Ferrous Iron, Hemoglobin, and Ferritin in Oil Emulsion and Cooked-Meat Homogenates Are Different from Those in Raw-Meat Homogenates, *Poult. Sci. 77*, 348–355.

Ahn, D.U., and Kim, S.M. (1998b) Effect of Superoxide and Superoxide-Generating Systems on the Prooxidant Effect of Iron Emulsion and Raw Turkey Homogenates, *Poult. Sci. 77*, 1428–1435.

Allen, J.C. (1994) Rancidity in Dairy Products, in *Rancidity in Foods* (Allen, J.C., and Hamilton, R.J., eds.) pp. 179–190, Blackie Academic and Professional, London.

Allen, J.C., and Wrieden, W.L. (1982) Influence of Milk Proteins on Lipid Oxidation in Aqueous Emulsion. I. Casein, Whey Protein and α-Lactalbumin, *J. Dairy Res. 79*, 239–248.

Andersson, K., and Lingnert, H. (1997) Influence of Oxygen Concentration on the Storage Stability of Cream Powder, *Lebensm.-Wiss. Technol. 30*, 147–154.

Andersson, K., and Lingnert, H. (1999) Kinetic Studies of Oxygen Dependence During Initial Lipid Oxidation in Rapeseed Oil, *J. Food Sci. 64*, 232–266.

Aw, T.Y. (1998) Determinants of Intestinal Detoxication of Lipid Hydroperoxides, *Free Radic. Res. 28*, 637–646.

Bartolomé, B., Estrella, I., and Hernandez, M.T. (2000) Interaction of Low Molecular Weight Phenolics with Proteins (BSA), *J. Food Sci. 65*, 617–621.

Battino, R., Evans, F.D., and Danforthe, W.F. (1968) The Solubilities of Seven Gases in Olive Oil with Reference to Theories of Transport Through the Cell Membrane, *J. Am. Oil Chem. Soc. 45*, 830–833.

Boyd, S.L., Boyd, R.J., and Barclay, R.C. (1990) A Theoretical Investigation of the Structures and Properties of Peroxyl Radicals, *J. Am. Chem. Soc. 112*, 5724–5730.

Bradley, D.G., and Min, D.B. (1992) Singlet Oxygen Oxidation of Foods, *Crit. Rev. Food Sci. Nutr. 31*, 211–236.

Buettner, G.R. (1993) The Pecking Order of Free Radicals and Antioxidants: Lipid Peroxidation, α-Tocopherol, and Ascorbate, *Archiv. Biochem. Biophys. 300*, 535–543.

Buttery, R.G., Ling, L.C., and Guadagni, D.G. (1969) Volatilities of Aldehydes, Ketones, and Esters in Dilute Water Solution, *J. Agric. Food Chem. 17*, 385–389.

Buttery, R.G., Bomben, J.L., Guadagni, D.G., and Ling, L.C. (1971) Some Considerations of the Volatilities of Organic Flavor Compounds in Foods, *J. Agric. Food Chem. 19*, 1045–1048.

Buttery, R.G., Guadagni, D.G., and Ling, L.C. (1973) Flavor Compounds: Volatilities in Vegetable Oil and Oil-Water Mixtures. Estimation of Odor Threshold, *J. Agric. Food Chem. 21*, 198–201.

Cervato, G., Viani, P., Masserini, M., Di Iorio, C., and Cestaro, B. (1988) Studies on Peroxidation of Arachidonic Acid in Different Liposomes Below and Above Phase Transition Temperature, *Chem. Phys. Lipids 49*, 135–139.

Chan, H.W.S. (1987) The Mechanism of Autoxidation, in *Autoxidation of Unsaturated Lipids* (Chan, H.W.S., ed.) pp. 1–15, Academic Press, London.

Chan, W.K.M., Faustman, C., and Renerre, M. (1997) Model Systems for Studying Pigment and Lipid Oxidation Relevant to Muscle-Based Foods, in *Natural Antioxidants, Chemistry, Health Effects, and Applications* (Shahidi, F., ed.) pp. 319–330, AOCS Press, Champaign, IL.

Charles, M., Rosselin, V., Beck, L., Sauvageot, F., and Guichard, E. (2000) Flavor Release from Salad Dressing: Sensory and Physicochemical Approaches in Relation with the Structure, *J. Agric. Food Chem. 48*, 1810–1816.

Chen, J.H., and Ho, C.-T. (1997) Antioxidant Activities of Caffeic Acid and Its Related Hydroxycinnamic Acid Compounds, *J. Agric. Food Chem. 45*, 2374–2378.

Chen, Z.Y., and Nawar, W.W. (1991) Role of Milk Fat Globule Membrane in Autoxidation of Milk Fat, *J. Food Sci. 5*2, 398–426.

Chipault, J.R. (1962) Antioxidants for Use in Foods, in *Autoxidation and Antioxidants Vol. II* (Lundberg, W.O., ed.) pp. 477–542, Wiley Interscience, New York.

Cillard, J., Cillard, P., and Cormier, M. (1980) Effect of Experimental Factors on the Prooxidant Behavior of α-Tocopherol, *J. Am. Oil Chem. Soc. 57*, 255–261.

Coupland, J.N., and McClements, D.J. (1996) Lipid Oxidation in Food Emulsions, *Trends Food Sci. Technol. 7*, 83–91.

Coupland, J.N., Zhu, Z., Wan, H., McClements, D.J., Nawar, W.W., and Chinachoti, P. (1996) Droplet Composition Affects the Rate of Oxidation of Emulsified Ethyl Linoleate, *J. Am. Oil Chem. Soc. 73*, 795–801.

Dacaranhe, C.D., and Terao, J. (2001) Effect of Phosphatidic Acid and Phosphatidylserine on Lipid Oxidation in Beef Homogenate During Storage and in Emulsified Sardine Oil, *J. Food Sci. 66*, 422–427.

Dalgleish, D.G., and Leaver, J. (1991) Dimensions and Possible Structures of Milk Proteins at Oil-Water Interfaces, in *Food Polymers, Gels and Colloids* (Dickinson, E., ed.) pp. 113–122, The Royal Society of Chemistry, Cambridge.

Damodaran, S. (1996) Amino Acids, Peptides, and Proteins, in *Food Chemistry* (Fennema, O.R., ed.) pp. 321–429, Marcel Dekker, New York.

Dawra, R.K., Sharma, O.P., and Makkar, H.P.S. (1989) Protein-Phosphate Complexes as an Inhibitor of Iron-Induced Lipid Peroxidation, *Biochem. Int. 18*, 537–544.

Debal, A. (1992) Chimie et Physicochimie des Tocophérols, in *Vitamine E—Tocophérols et Composés Apparentés* (Léger, C.L., ed.) pp. 1–20, Polytechnica, Paris.

Decker, E.A., and McClements, D.J. (2001) Transition Metal and Hydroperoxide Interactions, *INFORM 12*, 251–256.

de Ross, K.B. (1997) How Lipids Influence Food Flavor, *Food Technol. 51*, 60–62.
de Ross, K.B., and Wolswinkel, K. (1994) Non-Equilibrium Partition Model for Predicting Flavour Release in Mouth, in *Trends in Flavour Research* (van der Heijd, H., and Maarse, G., eds.) pp. 15–32, Elsevier, Amsterdam.
Devos, M., Patte, F., Rouault, J., Laffort, P., and Van Gemert, L.J. (1990) *Standardized Human Olfactory Thresholds*, pp. 1–165, IRL Press, Oxford.
Donnelly, J.L., Decker, E.A., and McClements, D.J. (1998) Iron-Catalysed Oxidation of Menhaden Oil as Affected by Emulsifiers, *J. Food Sci. 63*, 997–1000.
Endo, Y., Hoshizaki, S., and Fujimoto, K. (1997) Oxidation of Synthetic Triacylglycerols Containing Eicosapentaenoic and Docosahexaenoic Acids: Effect of Oxidation System and Triacylglycerol Structure, *J. Am. Oil Chem. Soc. 74*, 1041–1045.
Farag, R.S., Osman, S.A., Hallabo, S.A.S., and Nasr, N.A.A. (1978) Cottonseed Oil Oxidation Catalysed by Amino Acids and Albumin in Aqueous and Non-Aqueous Media, *J. Am. Oil Chem. Soc. 55*, 613–615.
Fazzalari, F.A. (1978) *Compilation of Odor and Taste Threshold Values Data*, pp. 1–499, American Society for Testing and Materials, Philadelphia.
Fiorentini, D., Landi, L., Barzanti, V., and Cabrini, L. (1989) Buffers Can Modulate the Effect of Sonication on Egg Lecithin Liposomes, *Free Radic. Res. Commun. 6*, 243–250.
Fomuso, L.B., Corredig, M., and Akoh, C. (2002) Effect of Emulsifier on Oxidation Properties of Fish Oil-Based Structured Lipid Emulsions, *J. Agric. Food Chem. 50*, 2957– 2961.
Forss, D.A. (1972) Odor and Flavor Compounds from Lipids, *Prog. Chem. Fats Other Lipids 13*, 177–258.
Frankel, E.N. (1980) Lipid Oxidation, *Prog. Lipid Res. 19*, 1–22.
Frankel, E.N. (1985) Chemistry of Autoxidation: Mechanism, Products and Flavor Significance, in *Flavor Chemistry of Fats and Oils* (Min, D.B., and Smouse, T., eds.) pp. 1–37, American Oil Chemists' Society, Champaign, IL.
Frankel, E.N. (1991) Recent Advances in Lipid Oxidation, *J. Sci. Food Agric. 54*, 495–511.
Frankel, E.N. (1998) *Lipid Oxidation*, pp. 1–303, The Oily Press, Dundee, Scotland.
Frankel, E.N., Huang, S.W., Aeschbach, R., and Prior, E. (1996) Antioxidant Activity of a Rosemary Extract and Its Constituents, Carnosic Acid, Carnosol, and Rosmarinic Acid, in Bulk Oil and Oil-in-Water Emulsion, *J. Agric. Food Chem. 44*, 131–135.
Frankel, E.N., Huang, S.W., Kanner, J., and German, J.B. (1994) Interfacial Phenomena in the Evaluation of Antioxidants: Bulk Oils *vs.* Emulsions, *J. Agric. Food Chem. 42*, 1054–1059.
Frankel, E.N., and Meyer, A.S. (2000) The Problems of Using One-Dimensional Methods to Evaluate Multifunctional Food and Biological Antioxidants, *J. Sci. Food Agric. 80*, 1925–1941.
Frankel, E.N., Neff, W.E., and Selke, E. (1981) Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: VII. Volatile Thermal Decomposition Products of Pure Hydroperoxides from Autoxidized and Photosensitized Oxidized Methyl Oleate, Linoleate and Linolenate, *Lipids 16*, 279–285.
Frankel, E.N., Neff, W.E., Selke, E., and Brooks, D.D. (1987) Thermal and Metal-Catalyzed Decomposition of Methyl Linolenate Hydroperoxides, *Lipids 22*, 323–327.
Frankel, E.N., Satué-Gracia, T., Meyer, A.S., and German, J.B. (2002) Oxidative Stability of Fish and Algae Oils Containing Long-Chain Polyunsaturated Fatty Acids in Bulk and in Oil-in-Water Emulsions, *J. Agric. Food Chem. 50*, 2094–2099.

Frankel, E.N., Selke, E., Neff, W.E., and Miyashita, K. (1992) Autoxidation of Polyunsaturated Triacylglycerols. IV. Volatile Decomposition Products from Triacylglycerols Containing Linoleate and Linolenate, *Lipids 27*, 442–446.

Friberg, S. (1997) Emulsion Stability, in *Food Emulsions* (Friberg, S., and Larsson, K., eds.) pp. 1–55, Marcel Dekker, New York.

Fritsch, C.W. (1994) Lipid Oxidation—the Other Dimensions, *INFORM 5*, 423–436.

Fujii, N., Hamano, M., and Yuasa, K. (1995) Inhibition of the Autoxidation of Methyl Linoleate by Protein and Highly Concentrated Dextrin in an Emulsion System, *Biosci. Biotechnol. Biochem. 59*, 1761–1763.

Gandemer, G., Genot, C., and Meynier, A. (1997) Phospholipid, Lipolysis, Oxidation and Flavour of Meat, *Proceedings of SCI Oil and Fat Group Symposium: Animal Fats—BSE and After,* pp. 116–136, P.J. Barnes and Associates, Bridgewater, UK.

Gardner, H.W. (1979) Lipid Hydroperoxide Reactivity with Proteins and Amino Acids: A Review, *J. Agric. Food Chem. 27*, 220–229.

Gardner, H.W. (1989) Oxygen Radical Chemistry of Polyunsaturated Fatty Acids, *Free Radic. Biol. Med. 7*, 65–86.

Genot, C., Gandemer, G., Paternoster, D., and Marion, D. (1990) Etude en Système Modèle des Modifications Biochimiques de la Sérum Albumine Bovine en Présence de Lipides Faiblement Oxydés, *Sci. Aliment. 10*, 403–415.

Genot, C., Métro, B., Viau, M., and Bouchet, B. (1999) Characterisation and Stability During Storage of Liposomes Made of Muscle Phospholipids, *Lebensm.-Wiss. Technol. 32,* 167–174.

Genot, C., Viau, M., Villère, A., Bronnec, I., Lethuaut, L., and Brossard, C. (2002) Effect of Droplet Size on Oxidation and Release of Volatiles in Protein-Stabilised Food Emulsions, in *Proceedings of the Third World Congress on Emulsion*, Lyon, France.

Gohtani, S., Sirendi, M., Yamamoto, N., Kajikawa, K., and Yamamo, Y. (1999) Effect of Droplet Size on Oxidation of Docosahexaenoic Acid in Emulsion System, *J. Dispers. Sci. Technol. 20*, 1319–1325.

Gros, J.B., Achard, C., and Dussap, C.G. (1992) Solubilité de l'Oxygène dans les Milieux Alimentaires Liquides. Mesure et Prédiction, *Sci. Aliment. 12*, 47–61.

Grosch, W. (1982) Lipid Degradation Products and Flavour, in *Developments in Food Science. Food Flavours. Part A. Introduction* (Morton, I.D., and MacLeod, A.J., eds.) pp. 325–398, Elsevier, Amsterdam.

Grosch, W. (1987) Reactions of Hydroperoxides-Products of Low Molecular Weight, in *Autoxidation of Unsaturated Lipids* (Chan, H.W.S., ed.) pp. 95–139, Academic Press, London.

Hall, C.H., III, and Cuppett, S.L. (1997) Structure-Activities of Natural Antioxidants, in *Antioxidant Methodology: In Vivo and In Vitro Concepts* (Aruoma, O.I., and Cuppett, S.L., eds.) pp. 141–172, AOCS Press, Champaign, IL.

Halliwell, B., and Gutteridge, J.M.C. (1990) Role of Free Radicals and Catalytic Metal Ions in Human Diseases: An Overview, *Methods Enzymol. 186*, 1–85.

Hartvigsen, K., Lund, P., Hansen, L.F., and Holmer, G. (2000) Dynamic Headspace Gas Chromatography/Mass Spectrometry Characterization of Volatiles Produced in Fish Oil-Enriched Mayonnaise During Storage, *J. Agric. Food Chem. 48*, 4858–4867.

Hegenauer, J., Saltman, P., Ludwig, D., Ripley, L., and Bajo, P. (1979) Effects of Supplemental Iron and Copper on Lipid Oxidation in Milk. 1. Comparison of Metal Complexes in Emulsified Homogenized Milk, *J. Agric. Food Chem. 27*, 860–867.

Heinonen, M., Rein, D., Statué-Gracia, M.T., Huang, S.-W., German, J.B., and Frankel, E.N. (1998) Effect of Protein on the Antioxidant Activity of Phenolic Compounds in a Lecithin-Liposome Oxidation System, *J. Agric. Food Chem. 46*, 917–922.

Henick, A.S., Benca, M.F., and Mitchell, J.H. (1954) Estimating Carbonyl Compounds in Rancid Fats and Foods, *J. Am. Oil Chem. Soc. 31*, 88–91.

Hitchman, M.L. (1978) *Measurement of Dissolved Oxygen*, pp. 1–255, John Wiley and Sons, New York.

Hopia, A.I., Huang, S.-W., Schwarz, K., German, J.B., and Frankel, E.N. (1996) Effect of Different Lipid Systems on Antioxidant Activity of Rosemary Constituents Carnosol and Carnosic Acid with and Without α-Tocopherol, *J. Agric. Food Chem. 44*, 2030–2036.

Horiuchi, M., Umano, K., and Shibamoto, T. (1998) Analysis of Volatile Compounds Formed from Fish Oil Heated with Cysteine and Trimethylamine Oxide, *J. Agric. Food Chem. 48*, 5232–5237.

Hoshino, C., Tagawa, Y., Wada, S., Oh, J.-H., Park, D.-K., Nagao, A., and Terao, J. (1997) Antioxidant Activity of Quercitin Against Metmyoglobin-Induced Oxidation of Fish Oil-Bile Salt Emulsion, *Biosci. Biotechnol. Biochem. 61*, 1634–1640.

Hsieh, R.J., and Kinsella, J.E. (1989) Oxidation of Polyunsaturated Fatty Acids: Mechanisms, Products, and Inhibition with Emphasis on Fish, *Adv. Food Nutr. Res. 33*, 233–341.

Hsieh, T.C.Y., Williams, S.S., Vejaphan, W., and Meyers, S.P. (1989) Characterization of Volatile Components of Menhaden Fish (*Brevoortia tyrannus*) Oil, *J. Am. Oil Chem. Soc. 66*, 114–117.

Huang, S.W., Frankel, E.N., Schwarz, K., Aeschbach, R., and German, J.B. (1996a) Antioxidant Activity of Carnosic Acid and Methyl Carnosate in Bulk Oils and Oil-in-Water Emulsions, *J. Agric. Food Chem. 44*, 2951–2956.

Huang, S.W., Frankel, E.N., Schwarz, K., and German, J.B. (1996b) Effect of pH on Antioxidant Activity of α-Tocopherol and Trolox in Oil-in-Water Emulsions, *J. Agric. Food Chem. 44*, 2496–2502.

Huang, S.W., Hopia, A., Schwarz, K., Frankel, E.N., and German, J.B. (1996c) Antioxidant Activity of α-Tocopherol and Trolox in Different Lipid Substrates: Bulk Oils *vs.* Oil-in-Water Emulsions, *J. Agric. Food Chem. 44*, 444–452.

Huang, S.W., Satué-Gracia, M.T., Frankel, E.N., and German, J.B. (1999) Effect of Lactoferrin on Oxidative Stability of Corn-Oil Emulsions and Liposomes, *J. Agric. Food Chem. 47*, 1356–1361.

Israelachvili, J.N. (1992) *Intermolecular and Surface Forces*, 2nd edn., pp. 1–450, Academic Press, London.

Jacobsen, C., Adler-Nissen, J., and Meyer, A.S. (1999a) Effect of Ascorbic Acid on Iron Release from the Emulsifier Interface and on the Oxidative Flavor Deterioration in Fish-Oil-Enriched Mayonnaise, *J. Agric. Food Chem. 47*, 4917–4926.

Jacobsen, C., Hartvigsen, K., Lund, P., Adler-Nissen, J., Holmer, G., and Meyer, A.S. (2000a) Oxidation in Fish-Oil-Enriched Mayonnaise 2. Assessment of the Efficacy of Different Tocopherol Antioxidant Systems by Discriminant Partial Least Squares Regression Analysis, *Eur. Food Res. Technol. 210*, 242–257.

Jacobsen, C., Hartvigsen, K., Lund, P., Meyer, A.S., Adler-Nissen, J., Holstborg, J., and Holmer, G. (1999b) Oxidation in Fish-Oil-Enriched Mayonnaise: 1. Assessment of Propyl Gallate as an Antioxidant by Discriminant Partial Least Squares Regression Analysis, *Eur. Food Res. Technol. 210*, 13–30.

Jacobsen, C., Hartvigsen, K., Lund, P., Thomsen, M.K., Skibsted, L.H., Adler-Nissen, J., Holmer, G., and Meyer, A.S. (2000b) Oxidation in Fish-Oil-Enriched Mayonnaise: 3. Assessment of the Influence of the Emulsion Structure on Oxidation by Discriminant Partial Least Squares Regression Analysis, *Eur. Food Res. Technol. 211*, 86–98.

Jacobsen, C., Hartvigsen, K., Lund, P., Thomsen, M.K., Skibsted, L.H., Holmer, G., Adler-Nissen, J., and Meyer, A.S. (2001a) Oxidation in Fish-Oil-Enriched Mayonnaise: 4. Effect of Tocopherol Concentration on Oxidative Deterioration, *Eur. Food Res. Technol. 212,* 308–318.

Jacobsen, C., Hartvigsen, K., Thomsen, M.K., Hansen, L.F., Lund, P., Skibsted, L.H., Holmer, G., Adler-Nissen, J., and Meyer, A.S. (2001b) Lipid Oxidation in Fish-Oil-Enriched Mayonnaise: Calcium Disodium Ethylenediaminetetracetate, but Not Gallic Acid, Strongly Inhibited Oxidative Deterioration, *J. Agric. Food Chem. 49*, 1009–1019.

Jacobsen, C., Meyer, A.S., and Adler-Nissen, J. (1998) Oxidation Mechanisms in Real Food Emulsions: Method for Separation of Mayonnaise by Ultracentrifugation, *J. Food Lipids 5*, 87–101.

Jacobsen, C., Schwarz, K., Stöckmann, H., Meyer, A.S., and Adler-Nissen, J. (1999c) Partitioning of Selected Antioxidants in Mayonnaise, *J. Agric. Food Chem. 47*, 3601–3610.

Jacobsen, C., Timm, M., and Meyer, A.S. (2001c) Oxidation in Fish-Oil-Enriched Mayonnaise: Ascorbic Acid and Low pH Increase Oxidative Deterioration, *J. Agric. Food Chem. 49,* 3947–3956.

Jana, A.K., Agarwal, S., and Chatterjee, S.N. (1986) Ultrasonic Radiation Induced Lipid Peroxidation in Liposomal Membrane, *Radiat. Environ. Biophys. 25*, 309–314.

Jana, A.K., Agarwal, S., and Chatterjee, S.N. (1990) The Induction of Lipid Peroxidation in Liposomal Membrane by Ultrasound and the Role of Hydroxyl Radicals, *Radiat. Res. 124*, 7–14.

Karahadian, C., and Lindsay, R.C. (1989) Evaluation of Compounds Contributing Characterizing Fishy Flavors in Fish Oils, *J. Am. Oil Chem. Soc. 66*, 953–960.

Karel, M. (1992) Kinetics of Lipid Oxidation, in *Physical Chemistry of Foods* (Schwartzberg, H.G., and Hartel, R.W., eds.) pp. 651–668, Marcel Dekker, New York.

Kawakatsu, M., Terao, J., and Matsushita, S. (1984) Phospholipid Oxidation Catalysed by Ferrous Iron and Ascorbic Acid, *Agric. Biol. Chem. 48*, 1275–1279.

Khan, M.A., and Shahidi, F. (2000) Oxidative Stability of Stripped and Nonstripped Borage and Evening Primrose Oils and their Emulsions in Water, *J. Am. Oil Chem. Soc. 77*, 963–968.

Kikuzaki, H., Hisamoto, M., Hirose, K., Akiyama, K., and Taniguchi, H. (2002) Antioxidant Properties of Ferulic Acid and Its Related Compounds, *J. Agric. Food Chem. 50*, 2161–2168.

Klein, R.A. (1970) The Detection of Oxidation in Liposome Preparations, *Biochim. Biophys. Acta 210*, 486–489.

Kubow, S. (1992) Routes of Formation and Toxic Consequences of Lipid Oxidation Products in Foods, *Free Radic. Biol. Med. 12*, 63–81.

Labuza, T.P. (1971) Kinetics of Lipid Oxidation in Foods, *CRC Crit. Rev. Food Technol. 2*, 355–405.

Lambelet, P., Saucy, F., and Löliger, J. (1994) Radical Exchange Reactions Between Vitamin E, Vitamin C and Phospholipids in Autoxidizing Polyunsaturated Lipids, *Free Radic. Res. 20*, 1–18.

Lampi, A.M., and Piironen, V. (1999) Dissimilarity of the Oxidations of Rapeseed and Butter Oil Triacylglycerols and Their Mixtures in the Absence of Tocopherols, *J. Sci. Food Agric. 79*, 300–306.

Leaver, J., Law, A.J.R., and Brechany, E.Y. (1999a) Covalent Modification of Emulsified β-Casein Resulting from Lipid Peroxidation, *J. Colloids Interface Sci. 210*, 207–214.

Leaver, J., Law, A.J.R., Brechany, E.Y., and McCrae, C. (1999b) Chemical changes in β-Lactoglobulin Structure During Ageing of Protein-Stabilized Emulsions, *Int. J. Food Sci. Technol. 34*, 503–508.

Leaver, J., Law, A.J.R., and Horne, D.S. (1999c) Caseinate-Stabilized Emulsions: Influence of Ageing, pH and Oil Phase on the Behaviour of Individual Protein Components, in *Food Emulsions and Foams: Interfaces, Interactions and Stability* (Dickinson, E., and Rodriguez Patino, J.M., eds.) pp. 258–268, Royal Society of Chemistry, Cambridge.

Lee, I., Fatemi, S.H., Hammond, E.G., and White, P.J. (1995) Quantitation of Flavor Volatiles in Oxidized Soybean Oil by Dynamic Headspace Analysis, *J. Am. Oil Chem. Soc. 72*, 539–546.

Lethuaut, L., Métro, F., and Genot, C. (2002) Effect of Droplet Size on Lipid Oxidation Rates of Oil-in-Water Emulsions Stabilized by Protein, *J. Am. Oil Chem. Soc. 79*, 425–430.

Li, H., Jacques, A., Wang, F., and Byrnes, R.W. (1999) Diffusion Distances of Known Iron Complexes in Model Systems, *Free Radic. Biol. Med. 26*, 61–72.

Lu, C.-L., and Baker, R.C. (1987) Effect of pH and Food Ingredients on the Stability of Egg Yolk Phospholipids and the Metal-Chelator Antioxidant Activity of Phosvitin, *J. Food Sci. 52*, 313–316.

Mackie, A.R., Gunning, A.P., Wilde, P.J., and Morris, V.J. (2001) Scratching the Surface: Imaging Interfacial Structure Using Atomic Force Microscopy, in *Food Colloids: Fundamentals of Formulation* (Dickinson, E., and Miller, R., eds.) pp. 13–21, Royal Chemical Society, Cambridge.

Mancuso, J.R., McClements, D.J., and Decker, E.A. (2000) Iron-Accelerated Cumene Hydroperoxide Decomposition in Hexadecane and Trilaurin Emulsions, *J. Agric. Food Chem. 48*, 213–219.

Mancuso, J.R., McClements, D.J., and Decker, E.A. (1999a) The Effect of Surfactant Type, pH, and Chelators on the Oxidation of Salmon Oil-in-Water Emulsions, *J. Agric. Food Chem. 47*, 4112–4116.

Mancuso, J.R., McClements, D.J., and Decker, E.A. (1999b) Ability of Iron to Promote Surfactant Peroxide Decomposition and Oxidize α-Tocopherol, *J. Agric. Food Chem. 47*, 4146–4149.

Marcuse, R., and Fredriksson, P.-O. (1968) Fat Oxidation at Low Oxygen Pressure: I. Kinetic Studies on the Rate of Fat Oxidation in Emulsions, *J. Am. Oil Chem. Soc. 45*, 400–407.

Matsumura, Y., Satake, C., Egami, M., and Mori, T. (2000) Interaction of Gum Arabic, Maltodextrin and Pullulan with Lipids in Emulsions, *Biosci. Biotechnol. Biochem. 64*, 1827–1835.

McClements, D.J. (1999) *Food Emulsions: Principles, Practice, and Techniques*, pp. 1–378, CRC Press, Boca Raton, FL.

McClements, D.J., and Decker, E.A. (2000) Lipid Oxidation in Oil-in-Water Emulsions: Impact of Molecular Environment on Chemical Reactions in Heterogenous Food Systems, *J. Food Sci. 65*, 1270–1282.

McLean, L.R., and Hagaman, K.A. (1992) Effect of Lipid Physical State on the Rate of Peroxidation of Liposomes, *Free Radic. Biol. Med. 12*, 113–119.

Medina, I., Tombo, I., Statué-Gracia, M.T., German, J.B., and Frankel, E.N. (2002) Effects of Natural Phenolic Compounds on the Antioxidant Activity of Lactoferrin in Liposomes and Oil-in-Water Emulsions, *J. Agric. Food Chem. 50*, 2392–2399.

Mei, L., McClements, D.J., and Decker, E.A. (1999a) Lipid Oxidation in Emulsions as Affected by Charge Status of Antioxidants and Emulsion Droplets, *J. Agric. Food Chem. 47*, 2267–2273.

Mei, L., McClements, D.J., and Decker, E.A. (1999b) Evidence of Iron Association with Emulsion Droplets and its Impact on Lipid Oxidation, *J. Agric. Food Chem. 47*, 2267–2273.

Mei, L., McClements, D.J., and Wu, J. (1998) Iron-Catalyzed Lipid Oxidation in Emulsion as Affected by Surfactant, pH and NaCl, *Food Chem. 61*, 307–312.

Miyashita, K., Nara, E., and Ota, T. (1993) Oxidation Stability of Polyunsaturated Fatty Acids in Aqueous Solution, *Biosci. Biotechnol. Biochem. 57*, 1638–1640.

Miyazawa, T., Yamaguchi, M., Lee, J.-H., Fujimoto, K., and Kaneda, T. (1984) Decomposition of Lipid Hydroperoxide by Choline and Ethanolamine, *Agric. Biol. Chem. 48*, 1375–1377.

Monahan, F.J., McClements, D.J., and Kinsella, J.E. (1993) Polymerization of Whey Proteins in Whey Protein-Stabilized Emulsions, *J. Agric. Food Chem. 41*, 1826–1829.

Mottram, D.S. (1991) Meat, in *Volatile Compounds in Foods and Beverages* (Maarse, H., ed.) pp. 107–177, Marcel Dekker, New York.

Murray, B.S., and Dickinson, E. (1996) Interfacial Rheology and the Dynamic Properties of Adsorbed Films of Food Proteins and Surfactants, *Food Sci. Technol. Int. 2*, 131–145.

Nakayama, T., Ono, K., and Hashimoto, K. (1998) Affinity of Antioxidative Polyphenols for Lipid Bilayers Evaluated with a Liposome System, *Biosci. Biotechnol. Biochem. 62*, 1005–1007.

Nuchi, C.D., McClements, D.J., and Decker, E.A. (2001) Impact of Tween 20 Hydroperoxides and Iron on the Oxidation of Methyl Linoleate and Salmon Oil Dispersions, *J. Agric. Food Chem. 49*, 4912–4916.

Pekkarinen, S.S., Stöckmann, H., Schwarz, K., Heinonen, M., and Hopia, A. (1999) Antioxidant Activity and Partitioning of Phenolic Acids in Bulk and Emulsified Methyl Linoleate, *J. Agric. Food Chem. 47*, 3036–3043.

Pokorny, J. (1987) Major Factors Affecting the Autoxidation of Lipids, in *Autoxidation of Unsaturated Lipids* (Chan, H.W.S., ed.) pp. 141–205, Academic Press, London.

Ponginebbi, L., Nawar, W.W., and Chinachoti, P. (1999) Oxidation of Linoleic Acid in Emulsions: Effect of Substrate, Emulsifier and Sugar Concentration, *J. Am. Oil Chem. Soc. 76*, 131–138.

Porter, N.A., Caldwell, S.E., and Mills, K.A. (1995) Mechanisms of Free Radical Oxidation of Unsaturated Lipids, *Lipids 30*, 277–290.

Porter, W.L. (1980) Recent Trends in Food Application of Antioxidants, in *Autoxidation in Food and Biological Systems* (Simic, M.G., and Karel, M., eds.) pp. 295–365, Plenum Press, New York.

Porter, W.L. (1993) Paradoxical Behavior of Antioxidants in Food and Biological Systems, in *Antioxidants: Chemical, Physiological, Nutritional and Toxicological Aspects* (William, G.M., ed.) pp. 93–122, Princeton Scientific, Princeton.

Porter, W.L., Henick, A.S., and Clifford, M. (1967) The Autoxidative Contraction of Monolayers of Linoleic Acid at Gas-Water Interface, *J. Am. Oil Chem. Soc. 44*, 185–190.

Porter, W.L., Levasseur, L.A., and Henick, A.S. (1972) Effects of Surface Concentration, Metals and Acid Synergists on Autoxidation of Linoleic Acid Monolayers on Silica, *Lipids 7*, 699–709.

Pryor, W.A., Cornicelli, J.A., Devall, L.J., Tait, B., Trivedi, B.K., Witiak, D.T., and Wu, M. (1993) A Rapid Screening Test to Determine the Antioxidant Potencies of Natural and Synthetic Antioxidants, *J. Org. Chem. 58*, 3521–3532.

Ramos, P., Gieseg, S.P., Schuster, B., and Esterbauer, H. (1995) Effect of Temperature and Phase Transition on Oxidation Resistance of Low Density Lipoprotein, *J. Lipid Res. 36*, 2113–2128.

Rampon, V., Lethuaut, L., Mouhous-Riou, N., and Genot, C. (2001) Interface Characterization and Aging of Bovine Serum Albumin Stabilized Oil-in-Water Emulsions as Revealed by Front-Surface Fluorescence, *J. Agric. Food Chem. 49*, 4046–4051.

Rampon, V., Mouhous-Riou, N., and Genot, C. (2002) Modification of Bovine Serum Albumin and Formation of Fluorescent Pigments in Oxidizing Emulsions as Revealed by Front-Surface Fluorescence, in *Recent Research Developments in Agricultural and Food Chemistry: Food Emulsions and Dispersions* (Anton, M., and Pandalaï, S.G., eds.) pp. 93–102, Res. Signpost, Kerala, India.

Riaublanc, A., Llamas, G., Lopez, C., and Brossard, C. (2002) Influence of Temperature on Rheological Properties of Sodium Caseinate Stabilised Emulsions, in *Recent Research Developments in Agricultural and Food Chemistry: Food Emulsions and Dispersions* (Anton, M., and Pandalaï, S.G., eds.) pp. 41–48, Res. Signpost, Kerala, India.

Rice-Evans, C.A., Miller, N.J., and Paganga, G. (1996) Structure-Antioxidant Activity Relationships of Flavonoids and Phenolic Acids, *Free Radic. Biol. Med. 20*, 933–956.

Richards, M.P., Chaiyasit, W., McClements, D.J., and Decker, E.A. (2002) Ability of Surfactant Micelles to Alter the Partitioning of Phenolic Antioxidants in Oil-in-Water Emulsions, *J. Agric. Food Chem. 50*, 1254–1259.

Riesz, P., and Kondo, T. (1992) Free Radical Formation Induced by Ultrasound and Its Biological Implications, *Free Radic. Biol. Med. 13*, 247–270.

Riisom, T., Sims, R.J., and Fioriti, J.A. (1980) Effect of Amino Acids on the Autoxidation of Safflower Oil in Emulsions, *J. Am. Oil Chem. Soc. 57*, 354–359.

Roddis, P.S., Karathanos, V.T., and Mantzavinou, A. (2002) Partitioning of Olive Oil Antioxidants Between Oil and Water Phases, *J. Agric. Food Chem. 50*, 596–601.

Rodriguez Niño, M.R., and Rodriguez Patino, J.M. (1998) Surface Tension of Protein and Insoluble Lipids at the Air-Aqueous Phase Interface, *J. Am. Oil Chem. Soc. 75*, 1233–1239.

Roozen, J.P., Frankel, E.N., and Kinsella, J.E. (1994) Enzymic and Autoxidation of Lipids in Low Fat Foods: Model of Linoleic Acid in Emulsified Hexadecane, *Food Chem. 50*, 33–38.

Rousseau, C., Richard, C., and Martin, R. (1988) Synergy Effects of Glutathione and Amino Acids on the Antioxidant Efficiency of Vitamins C and E in the Induced Oxidation of Methyl Linolenate, *J. Chim. Phys. 85*, 145–146.

Ruben, C., and Larsson, K. (1985) Relation Between Antioxidant Effect of α-Tocopherol and Emulsion Structure, *J. Dispersion Sci. Technol. 6*, 213–221.

Saito, H., and Ishihara, K. (1997) Antioxidant Activity and Active Sites of Phospholipids as Antioxidants, *J. Am. Oil Chem. Soc. 74*, 1531–1536.

Samokyszyn, V.M., Miller, D.M., Reilf, D.W., and Aust, S.D. (1990) Iron-Catalysed Lipid Peroxidation, in *Membrane Lipid Oxidation, Volume I* (Vigo-Pelfrey, C., ed.) pp. 101–127, CRC Press, Boca Raton, FL.

Saunders, D.H., Coleman, J.E. Hampson, J.W., Wells, P.A., and Riemenschneider, R.W. (1962) Autoxidation of Fatty Materials in Emulsions. I. Pro-oxidant Effect of Histidine and Trace Metals on the Oxidation of Linoleate Esters, *J. Am. Oil Chem. Soc. 39*, 434–439.

Schaich, K.M. (1992) Metals and Lipid Oxidation: Contemporary Issues, *Lipids 27*, 209–218.

Schieberle, P., and Grosch, W. (1981) Model Experiments About the Formation of Volatile Carbonyl Compounds, *J. Am. Oil Chem. Soc. 58*, 602–607.

Schuster, B., Prassl, R., Nigon, F., Chapman, M.J., and Lagoner, P. (1995) Lipid Structure Is a Major Determinant of the Oxidative Resistance of Low Density Lipoprotein, *Proc. Natl. Acad. Sci. USA 92*, 2509–2513.

Schwarz, K., Huang, S.-W., German, J.B., Tiersch, B., Hartmann, J., and Frankel, E.N. (2000) Activities of Antioxidants Are Affected by Colloidal Properties of Oil-in-Water and Water-in-Oil Emulsions and Bulk Oils, *J. Agric. Food Chem. 48*, 4874–4882.

Selke, E., Frankel, E.N., and Neff, W.E. (1978) Thermal Decomposition of Methyl Oleate Hydroperoxides and Identification of Volatile Components by Gas Chromatography-Mass Spectrometry, *Lipids 13,* 511–513.

Selke, E., Rohwedder, W.K., and Dutton, H.J. (1980) Volatile Components from Trilinolein Heated in Air, *J. Am. Oil Chem. Soc. 57*, 25–30.

Shimada, K., Fujikawa, K., Yahara, K., and Nakamura, T. (1992) Antioxidative Properties of Xanthan on the Autoxidation of Soybean Oil in Cyclodextrin Emulsion, *J. Agric. Food Chem. 40*, 945–946.

Shimada, K., Okada, H., Matsuo, K., and Yoshioka, S. (1996) Involvement of Chelating Action and Viscosity in the Antioxidative Effect of Xanthan in an Oil/Water Emulsion, *Biosci. Biotech. Biochem. 60*, 125–127.

Silvestre, M.P.C., Chaiyasit, W., Brannan, R.G., McClements, D.J., and Decker, E.A. (2000) Ability of Surfactant Headgroup Size to Alter Lipid and Antioxidant Oxidation in Oil-in-Water Emulsions, *J. Agric. Food Chem. 48*, 2057–2061.

Simpson, J.A., and Dean, R.T. (1990) Stimulatory and Inhibitory Actions of Proteins and Amino Acids on Copper-Catalysed Free Radical Generation in the Bulk Phase, *Free Radic. Res. Commun. 10*, 303–312.

Sims, R.J., Fioriti, J.A., and Trumbetas, J. (1979) Effect of Sugars and Sugar Alcohols on Autoxidation of Safflower Oil in Emulsions, *J. Am. Oil Chem. Soc. 56*, 742–745.

Sirendi, M., Gohtani, S., and Yamamo, Y. (1998) Effect of Some Polysaccharides on Oxidative Stability of Methyl Linoleate in Emulsion, *J. Dispers. Sci. Technol. 19*, 679–694.

Slater, T.F., and Cheeseman, K.H. (1988) Free Radical Mechanisms of Tissue Injury and Mechanisms of Protection, in *Reactive Oxygen Species in Chemistry, Biology, and Medicine* (Quintanilha, A., ed.) pp. 1–14, Plenum Press, New York.

Snyder, J.M., Frankel, E.N., and Selke, E. (1985) Capillary Gas Chromatographic Analyses of Headspace Volatiles from Vegetable Oils, *J. Am. Oil Chem. Soc. 62*, 1675–1679.

Stöckmann, H., and Schwarz, K. (1999) Partitioning of Low Molecular Weight Compounds in Oil-in-Water Emulsions, *Langmuir 15*, 6142–6149.

Stöckmann, H., Schwarz, K., and Huynh-Ba, T. (2000) The Influence of Various Emulsifiers on the Partitioning and Antioxidant Activity of Hydrobenzoic Acids and Their Derivatives in Oil-in-Water Emulsions, *J. Am. Oil Chem. Soc. 77*, 535–542.

Strul, G., Frimer, A.A., and Weiner, L. (1993) Spin-Trapping Study of Free Radical Penetration into Liposomal Membranes, *J. Chem. Soc. Perkin Trans. 2*, 2057–2059.

Tadolini, B., Cabrini, L., Menna, C., Pinna, G.G., and Hakim, G. (1997) Iron(III) Stimulation of Lipid Hydroperoxide-Dependent Lipid Peroxidation, *Free Radic. Res. 27*, 563–576.

Taylor, M.J., and Richardson, T. (1980) Antioxidant Activity of Skim Milk: Effect of Heat and Resultant Sulfhydryl Groups, *J. Dairy Sci. 63*, 1783–1795.

Terao, J. (2001) Factors Affecting the Oxidative Stability of Emulsified Oil and Membranous Phospholipids, *J. Oleo Sci. 50*, 393–397.

Thomsen, M.K., Jacobsen, C., and Skibsted, L.H. (2000) Mechanism of Initiation of Oxidation in Mayonnaise Enriched with Fish Oil as Studied by Electron Spin Resonance Spectroscopy, *Eur. Food Res. Technol. 211*, 381–386.

Tuorila, H., Sommardahl, C., Hyvönen, L., Leporanta, K., and Merimaa, P. (1995) Does Fat Affect the Timing of Flavour Perception? A Case Study with Yoghurt, *Food Qual. Pref. 6*, 55–58.

van Ruth, S.M., King, C., and Giannouli, P. (2002) Influence of Lipid Fraction, Emulsifier Fraction, and Mean Particle Diameter of Oil-in-Water Emulsions on the Release of 20 Aroma Compounds, *J. Agric. Food Chem. 50*, 2365–2371.

van Ruth, S.M., and Roozen, J.P. (2000a) Aroma Compounds of Oxidised Sunflower Oil and its Oil-in-Water Emulsion: Volatility and Release Under Mouth Conditions, *Eur. Food Res. Technol. 210*, 258–262.

van Ruth, S.M., and Roozen, J.P. (2000b) Release of Volatile Oxidation Products from Sunflower Oil and Its Oil-in-Water Emulsion in a Model Mouth System, in *Flavor Release* (Roberts, D.D., and Taylor, A.J., eds.) pp. 309–320, American Chemical Society, Washington DC.

van Ruth, S.M., Roozen, J.P., Posthumus, M.A., and Jansen, F.J.H.M. (1999a) Volatile Composition of Sunflower Oil-in-Water Emulsions During Initial Lipid Oxidation: Influence of pH, *J. Agric. Food Chem. 47*, 4365–4369.

van Ruth, S.M., Roozen, J.P., Posthumus, M.A., and Jansen, F.J.H.M. (1999b) Influence of Ascorbic Acid and Ascorbyl Palmitate on the Aroma Composition of an Oxidized Vegetable Oil and Its Emulsion, *J. Am. Oil Chem. Soc. 76*, 1375–1381.

Wills, E.D. (1965) Mechanisms of Lipid Peroxide Formation in Tissues. Role of Metals and Haematin Proteins in the Catalysis of the Oxidation of Unsaturated Fatty Acids, *Biochim. Biophys. Acta 98*, 238–251.

Wills, E.D. (1966) Mechanisms of Lipid Peroxide Formation in Animal Tissues, *Biochem. J. 99*, 667–676.

Xue, C., Yu, G., Hirata, T., Terao, J., and Lin, H. (1998) Antioxidative Activities of Several Marine Polysaccharides Evaluated in a Phosphatidylcholine-Liposomal Suspension and Organic Solvents, *Biosci. Biotechnol. Biochem. 62*, 206–209.

Yamashoji, S., Yoshida, H., and Kajimoto, G. (1979) Effects of Amino Acids on the Photosensitized Oxidation of Linoleic Acid in an Aqueous Ethanol Solution, *Agric. Biol. Chem. 43*, 1505–1511.

Yamauchi, R., Goto, Y., Kato, K., and Ueno, Y. (1984) Prooxidant Effect of Dihydroxyacetone and Reducing Sugars on the Autoxidation of Methyl Linoleate in Emulsions, *Agric. Biol. Chem. 48*, 843–848.

Yamauchi, R., Tatsumi, Y., Asano, M., Kato, K., and Ueno, Y. (1988) Effects of Metal Salts and Fructose on the Autoxidation of Methyl Linoleate in Emulsions, *Agric. Biol. Chem. 52*, 849–850.

Yoshimura, Y., Matsuzaki, Y., Watanabe, T., Uchiyama, K., Ohsawa, K., and Imaeda, K. (1992) Effects of Buffer Solutions and Chelators on the Generation of Hydroxyl Radical and the Lipid Peroxidation in the Fenton Reaction System, *J. Clin. Biochem. Nutr. 13*, 147–154.

Zhang, H.-Y. (1999) Theoretical Methods Used in Elucidating Activity Differences of Phenolic Antioxidants, *J. Am. Oil Chem. Soc. 76*, 745–748.

Chapter 8

Oxidation in Dried Microencapsulated Oils

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Introduction

Microencapsulation of oils is a technological approach used to protect sensitive oils, mask or preserve flavors and aromas, and transfer liquids into easily handled solids (Balassa and Fanger 1971, Dziezak 1988, Gibbs *et al.* 1999, Jackson and Lee 1991, Matsuno and Adachi 1993, Shahidi and Han 1993). The process of oil microencapsulation is based on the preparation and drying of an oil-in-water emulsion to obtain a powdery ingredient in which oil droplets are surrounded by a dry matrix of proteins and/or carbohydrates.

The most relevant formulated microencapsulated oils are infant formulas (prepared with vegetable oils), flavoring additives, and pigments (prepared with essential oils from fruits and spices) and microencapsulated fish oils, used as functional ingredients in a growing number of milk and bakery products because of their beneficial physiologic effects. There is also another type of microencapsulated oil obtained from drying natural foods such as milk powders, dried eggs, or dehydrated soups and sauces.

Lipid oxidation in microencapsulated oils is of paramount importance because it results in the loss of nutritional value and the development of flavors that are unacceptable to consumers. However, few studies have been published on the variables involved in oxidation and the action of antioxidants in these lipid systems. The process of lipid oxidation leads to formation of a multitude of compounds of different molecular weight and polarity, thus making it difficult to evaluate the degree of oxidation (Frankel 1993, 1998a, and 1998b, Frankel and Meyer 2000, Rossell 1994). This situation becomes even more complicated in the case of microencapsulated oils due to the involvement of additional factors of great relevance, essentially derived from the presence of the other matrix components and the heterogeneous lipid distribution. Most of the studies published on microencapsulated oils have focused on the influence of the type and concentration of encapsulating agents, amount of lipids, and drying conditions on the encapsulation efficiency, oil globule size, and microstructural characteristics of microencapsulated oils. However, few studies have examined oxidation of microencapsulated oils;

furthermore, it is difficult to deduce general conclusions due to the great variety of matrixes, the different drying procedures used and, above all, the diversity of oxidation conditions and analytical methods applied to evaluate oxidation.

This chapter includes a general overview of the variables involved in the oxidation of microencapsulated oils and the analytical methods normally used to evaluate oxidation. As the main objective of the chapter, differences in the oxidation profile of bulk oils and microencapsulated oils, and the heterogeneity of lipid oxidation in microencapsulated systems are discussed in light of results obtained recently through a new analytical approach.

Variables Affecting Oxidation in Microencapsulated Oils

In addition to the numerous variables that influence lipid oxidation in bulk oils (unsaturation, surface area, prooxidants, antioxidants, oxygen, light, and temperature), specific variables may exert an important effect in microencapsulated oils. The most relevant of these are presented below with references to the supporting literature.

Type and Concentration of the Matrix Components

The influence of matrix characteristics on microencapsulation efficiency is undoubtedly the main factor of interest with respect to microencapsulated oils because the fraction of oil that remains free or unencapsulated after preparation of dried microencapsulated oils is theoretically more susceptible to oxidation than are the oil globules surrounded, and hence protected, by the matrix. Microencapsulation efficiency can be evaluated indirectly by measuring the oil fraction accessible to simple extraction, e.g., by washing with an organic solvent, usually hexane, under well-established conditions (Buma 1971a, Sankarikutty *et al.* 1988). A number of studies comparing the effectiveness of microencapsulating agents, such as proteins, carbohydrates or gums (Bangs and Reineccius 1990, Bhandari *et al.* 1998, Dian *et al.* 1996, Faldt and Bergenstahl 1995, Flink and Karel 1970a, Imagi *et al.* 1990, Keogh and O'Kennedy 1999, Keogh *et al.* 2001, Kim and Morr 1996, Kim *et al.* 2000, Kopleman *et al.* 1977 and 1992, Rosenberg and Young 1993, Sheu and Rosenberg 1998, Young *et al.* 1993a and 1993b), the influence of the solid content in the previous emulsion (Chang and Ha 2000, McNamee *et al.* 1998, Pauletti and Amestoy 1999, Rosenberg *et al.* 1990, Sheu and Rosenberg 1995), and the effect of particle size and porosity (Buma 1971a, 1971b, 1971c, and 1971d) on microencapsulation efficiency have been reported.

Concerning specific studies on the influence of matrix characteristics on oxidative deterioration, the main objective has been to compare effectiveness of different carbohydrates using linoleic acid as a model lipid (Imagi *et al.* 1990 and 1992, Iwami *et al.* 1987b, Kim *et al.* 2000, Minemoto *et al.* 1999 and 2001, Reichenbach and Min 1997) or oils (Anandaraman and Reineccius 1986, Lin *et al.* 1995a and 1995b, Moreau and Rosenberg 1996, Strange *et al.* 1997, Wanasundara

and Shahidi 1995). Among the carbohydrates tested, certain cyclodextrin isomers (Kim*et al.* 2000, Reichenbach and Min 1997, Wanasundara and Shahidi 1995) and maltodextrin of high dextrose equivalents (Anandaraman and Reineccius 1986) were found to provide acceptable protection against oxidation. Also, some authors have investigated the effect of different proteins, such as whey (Keogh and O'Kennedy 1999, Moreau and Rosenberg 1996), gelatin, albumin, and sodium caseinate (Imagi *et al.* 1990), and sodium vs. calcium caseinate (Keogh *et al.* 2001).

Drying Procedure

In general, the influence of the drying procedures more commonly used, i.e., spray-drying and freeze-drying, on the oxidative stability of microencapsulated oils, has received little attention under conditions that allow comparisons, i.e., using the same process parameters and starting emulsions. Some authors have reported that oxidation proceeded more rapidly in freeze-dried than in spray-dried samples, attributing such results to the greater surface area of the former (Fioriti *et al.* 1975, Sims 1994, Taguchi *et al.* 1992a), whereas others found the opposite (Desobry *et al.* 1997, Minemoto *et al.* 1997, Tang and Chen 2000), starting either from samples with similar microencapsulation efficiency (Desobry *et al.* 1997) or even from freeze-dried samples with a higher content of surface oils than their spray-dried analogs (Minemoto *et al.* 1997); the lower oxidative stability of spray-dried samples was attributed to the high temperatures used during the process.

A recent study on freeze-drying, potentially most suitable for sensitive oils because of the low temperature used, showed that the increase in microencapsulation efficiency was inversely related to freezing rate (Heinzelmann *et al.* 2000b). The study agrees with results obtained in previous studies (Flink and Karel 1970b, Menting *et al.* 1970, Rulkens and Thijssen 1972) although, interestingly, higher microencapsulation efficiencies did not necessarily render higher oxidative stability (Heinzelmann *et al.* 2000b).

Addition of Antioxidants

Although variables of general influence on lipid oxidation are not the subject of this chapter, specific comments on antioxidants have been included here because their action in microencapsulated oils is poorly understood and is not predictable from the results obtained in bulk oils. This is due to the particular characteristics of these complex lipid systems, e.g., the heterogeneous lipid distribution. Moreover, polarity and partitioning of antioxidants may play an important role as in other disperse systems such as emulsions (Frankel 1998b, Frankel and Meyer 2000).

The addition of antioxidants is a powerful means of enhancing oxidative stability of microencapsulated oils, which are highly susceptible to oxidation; it is of great importance, therefore, in dried eggs (Guardiola *et al.* 1995 and 1997, Huber

et al. 1995), infant formulas enriched with polyunsaturated fatty acids (Bendich and Brock 1997), and microencapsulated fish oils (Heinzelmann *et al.* 2000a and 2000b, Keogh *et al.* 2001, Velasco *et al.* 2000a and 2000b). It is important to note that special care must be taken when adding antioxidants to some formulated microencapsulated oils. For example, ascorbic acid is added to iron-fortified infant formulas because of its antioxidant effect *in vivo*, although it may act as a prooxidant in the presence of nonprotein-bound iron (Almaas *et al.* 1997, Galdi *et al.* 1987, Satué-Gracia *et al.* 2000).

Tocopherols, ascorbyl palmitate, and gallates are probably the most widely used antioxidants in these products, although much research work is required to clarify the type and amounts of antioxidants needed for successful protection of these lipid systems.

Water Activity

According to Labuza and co-workers (Karel *et al.* 1967, Labuza 1968), lipid oxidation is lowest at water activity values close to the water monolayer (0.2–0.3 for most foods), due to a decrease in the catalytic effect of transition metals, quenching of free radicals and singlet oxygen, and/or retardation of hydroperoxide decomposition. During manufacturing and storage, the quality of powdery foods may be affected by changes in water activity; hence, the oxidative stability of such products at water activity values between 0.11 and 0.34 has been investigated in milk powders (Burvall *et al.* 1978, Stapelfeldt *et al.* 1997). Recently, a growing number of studies have focused on the effect of relative humidity on oxidation of microencapsulated lipids (Desobry *et al.* 1997, 1999, Minemoto *et al.* 1997 and 2001, Ponginebbi *et al.* 2000, Velasco 2001).

An aspect of considerable interest is the effect of moisture content on physical changes of the solid matrix of microencapsulated oils that may affect the oil distribution and, consequently, the accessibility of oxygen to the oil. After drying, a high-viscosity solid matrix in the glassy amorphous state is obtained. The protection provided by matrices in the glassy state was reported recently (Orlien *et al.* 2000, Selim *et al.* 2000). However, when either moisture content or temperature increases, the solid changes from the glassy state to the rubbery amorphous state with a high molecular mobility. Temperature at the state change, called the glass transition temperature, depends on the solid matrix nature and decreases as water content increases (Roos *et al.* 1996). Because molecular mobility is increased by the plasticizing effect of water or by temperature, crystallization of sugars or the so-called "collapse" may occur (Chuy and Labuza 1994, Levine and Slade 1990, Orford *et al.* 1989). These physical changes are associated with the partial release of encapsulated lipids (Chirife and Karel 1974, Gejl-Hansen and Flink 1977, Kopelman *et al.* 1977, Menting *et al.* 1970, Rosenberg *et al.* 1990, Shimada *et al.* 1991); the released oil may then be more exposed and undergo rapid oxidation (Karel 1980, Labrousse *et al.* 1992, Shimada *et al.* 1991).

Oil Globule Size

Even though measurement of oil globule size is a well-controlled characteristic of microencapsulated oils and usually reported for starting samples, its influence on lipid oxidation has not been studied extensively. In general, increases in homogenization pressures during preparation of the emulsion lead to smaller oil globule sizes and higher microencapsulation efficiencies; hence, the results obtained may be confounded. Imagi and coworkers found that those microencapsulating agents that led to smaller oil globule size and higher microencapsulation efficiency (Imagi *et al.* 1990) did not always retard oxidation (Imagi *et al.* 1992). On the other hand, some authors have attributed the less extensive oxidation found at high relative humidities in part to coalescence of oil droplets (Ponginebbi *et al.* 2000).

Interactions Between Matrix Components

In microencapsulated oils, the main reactions between matrix components that may have relevant influence on lipid oxidation are nonenzymatic browning or Maillard reactions, whose resulting products act as antioxidants (Eriksson 1987, Karel 1984). On the other hand, reactions between oxidized lipids and proteins lead to the loss of essential amino acids and hence impairment of nutritional value (Eriksson 1987, Frankel 1998b, Gardner 1979, Hidalgo *et al.* 1992, Karel 1984).

The only published works found regarding Maillard reactions refer to milk powders, but these studies have limited relevance to this subject because they were carried out at water activity values above that of the monolayer (0.3–0.7) (Iwami *et al.* 1987a, Karel 1984, Riisom *et al.* 1980, Wang *et al.* 1991). With respect to reactions between oxidized lipids and amino acids or proteins, a great number of studies have been reported on model systems subjected to high relative humidity, i.e., ≥80% (Gardner 1979). The results obtained, however, are difficult to extrapolate to foods (Frankel 1998b) especially due to the poor understanding of how these reactions proceed in foods at low water activity values. In this context, the only study found on microencapsulated oils showed considerable losses of methionine, followed by tryptophan, histidine, and lysine, exclusively at 50°C and high relative humidity (80%) (Matoba *et al.* 1984).

Others

One important variable affecting oxidation in microencapsulated oils is lipid distribution, but this subject will be extensively discussed below, in connection with the results found on oxidation in different lipid phases of microencapsulated oils. Although pH is considered one important factor affecting oxidation in foods (Fritsch 1994), no references were found on its effect on the oxidation of microencapsulated oils. In addition, the fact that an increase in particle size and hence a decrease in surface area delays oxidation (Desobry *et al.* 1997) should not rule out the potential effect of changes in other parameters concurrent with modifications in particle size, e.g., in the content of surface oil (Fritsch 1994).

Analytical Methods Used to Evaluate Oxidation in Microencapsulated Oils

Although there are a large number of analytical methods available to evaluate lipid oxidation in fats and oils, selection of suitable methods for complex lipid systems such as microencapsulated oils is a difficult task because many factors must be considered.

In general, the measurement of hydroperoxides as primary oxidation products is meaningful only under conditions that favor peroxide formation over decomposition and may thus provide insufficient information when applied alone (Frankel 1993). Therefore, some authors have found it difficult to follow the progress of oxidation in microencapsulated oils on the basis of peroxide values when only low (Orlien *et al.* 2000) or very high (Iwami *et al.* 1988) peroxide values are obtained. Determination of the peroxide value yields results that are difficult to interpret in microencapsulated oils compared with bulk oils (Sims 1994) when it is correlated with oxygen uptake, and, in general, it is not recommended for low-fat foods or foods containing a noncontinuous lipid phase (Fritsch 1994).

Loss of substrate is generally used as an oxidation parameter in most of the studies involving microencapsulated lipid model systems, such as microencapsulated linoleic acid or methyl linoleate (Imagi *et al.* 1992, Iwami *et al.* 1987b, Minemoto *et al.* 1997, 1999 and 2001, Ponginebbi *et al.* 2000). However, it is not sensitive enough because a significant decrease of polyunsaturated fatty acids is not detected until well within the advanced oxidation stage (Angulo *et al.* 1998, Lin *et al.* 1995a, Taguchi *et al.* 1992a, Thomkinson and Mathur 1990).

Among the analytical methods that evaluate formation of secondary oxidation products in microencapsulated oils, measurement of volatile compounds has been commonly applied to milk powders (Hall and Andersson 1985, Min *et al.* 1989, Park and Goins 1992, Shiratsuchi *et al.* 1994, Ulberth and Roubicek 1995). Also, cholesterol oxides are usually evaluated in products of high surface area such as milk powders (Chan *et al.* 1993, McCluskey *et al.* 1997, Przygonski *et al.* 2000, Rose-Sallin *et al.* 1995) and dried eggs (Huber *et al.* 1995, Li et al. 1996, Wahle *et al.* 1993). A variety of methods, normally applied to nonquantitative extracts, have been used for infant formulas. These methods include determination of thiobarbituric acid reactive substances (TBARS), loss of unsaturated fatty acids (Angulo *et al.* 1998, Giammaroli *et al.* 1997, Thomkinson and Mathur 1986, 1989 and 1990), measurement of cholesterol oxides (Przygonski *et al.* 2000, Rose-Sallin *et al.* 1995), loss of tocopherols (Angulo *et al.* 1998), hexanal (Giammaroli *et al.* 1995 and 1997), and oxidative stability of the extracted oils by Rancimat (Presa-Owens *et al.* 1995).

One of the main problems in the evaluation of lipid oxidation is that each of the methods commonly used is applicable only to particular stages of the process, and the information provided depends on the type of method chosen (Frankel 1993). In recent years, we have developed and widely applied a methodology based on the combination of adsorption and exclusion chromatographies to enable concomitant quantitation of primary and secondary oxidation products (Dobarganes *et al.* 1988, 2000, Márquez-

Ruiz *et al.* 1996a), thus providing a good measurement for early and advanced stages of oxidation (Dobarganes and Márquez-Ruiz 1993, 1995 and 1998). Application of this procedure has proved to be of great utility for quality evaluation of refined oils (Dobarganes *et al.* 1989, Ruiz-Mández *et al.* 1997) and for the study of the evolution of oxidation in model systems (Márquez-Ruiz *et al.* 1996b), oils (Martín-Polvillo *et al.* 1996, Martín-Polvillo 2000), fried foods (Márquez-Ruiz *et al.* 1999, Pérez-Camino *et al.* 1991), and microencapsulated oils (Márquez-Ruiz *et al.* 2000, Velasco *et al.* 2000a, Velasco 2001). The advantages of this analytical procedure to evaluate oxidation in microencapsulated oils will be discussed below.

Oxidation in Microencapsulated Oils vs. Oxidation in Bulk Oils

One important factor influencing oxidation in foods in which there is a noncontinuous lipid phase as in microencapsulated oils, is the coexistence of two portions of lipid phases, i.e., (i) a portion that can be easily extracted with hexane (usually called free, surface, or nonencapsulated oil), and (ii) a portion of noncontinuous lipid phase (known as encapsulated oil) wherein extraction of lipids in droplets requires previous disruption of the matrix structure (Fritsch 1994). Therefore, the main problem in the evaluation of oxidation in microencapsulated oils is the difficulty in interpreting the real oxidation status of the sample from analytical data obtained. For example, external oxidation (of the surface oil) might induce rancidity even if the encapsulated oil has a low oxidation level; otherwise, however, rancidity might not be detected until the oxidized encapsulated oil is released. Hence it is not strange to find that analysis of the total lipids often yields poor and confusing results.

Moreover, evolution of oxidation in the noncontinuous or dispersed lipid phase may become very complex due to the heterogeneity in the composition of lipid droplets isolated from one another in the matrix. Consequently, different oxidation rates can occur in different droplets depending on the many variables outlined above. However, after extraction of the encapsulated fraction, a continuous oily phase is analyzed and substantial information on the oxidation in the different droplets is lost. Some of the analytical methods of general application might not be useful in detecting the oxidation taking place in the lipids embedded in the matrix, and careful selection of methods that provide complementary information is essential to gain insight into the oxidation status of this lipid phase, in comparison with the results obtained in monophasic systems. Such limitations in the evaluation of oxidation of microencapsulated oils could explain in part why some authors using more than one analytical method have found results difficult to interpret (Fioriti *et al.* 1975, Moreau and Rosenberg 1996, Ponginebbi *et al.* 2000).

Profile of Oxidation in Bulk Oils

As already discussed, we have approached the evaluation of oxidation in bulk oils through the application of an analytical methodology that permits quantitation of

primary and secondary oxidation products. First, unoxidized triglycerides are separated by adsorption chromatography; then the more polar fraction is analyzed by high-performance size-exclusion chromatography, thus allowing quantitation of oxidized triglyceride monomers, dimers, and higher oligomers. Figure 8.1 shows a representative profile of the oxidation of sunflower oil at 25°C in the dark (Martín-Polvillo 2000). Values for oxidized triacylglycerol monomers and polymers (sum of dimers and higher oligomers) as well as tocopherol content were included. As can be observed, oxidized triglyceride monomers showed a progressive increase during the earlier stages of oxidation, attributable to the increment in hydroperoxides. Thus, an excellent correlation was found between the amount of oxidized triglyceride monomers and peroxide value until oxidation accelerated, at ~400 d. This point, defined as the end of the induction period, was clearly marked by the initiation of polymerization and the exhaustion of tocopherol. During the advanced oxidation stage, a multitude of secondary oxidation products (containing epoxy-, hydroxy-, keto- and other oxygenated functions) contributed greatly to increase the amount of oxidized triacylglycerol monomers.

This oxidation pattern has been found repeatedly in numerous experiments conducted to study evolution of oxidation of bulk oils (Martín-Polvillo *et al.* 1996, Martín-Polvillo 2000) or foods in which the oil is in a continuous phase, such as fried foods (Márquez-Ruiz *et al.* 1999, Pérez-Camino *et al.* 1991). Although the amount of oxidized monomers found at the end of the induction period depends on the degree of unsaturation and the temperature of the oil, it is commonly observed that formation of polymeric compounds denotes the end of the induction period, which is practically concurrent with the exhaustion of antioxidants.

Profile of Oxidation in Dried Microencapsulated Oils

As already mentioned, differences in oxidation rates and profiles can be analyzed in two lipid fractions obtained from these products, i.e., the surface oil accessible to organic solvents, usually constituting a small portion of the total lipids, and the

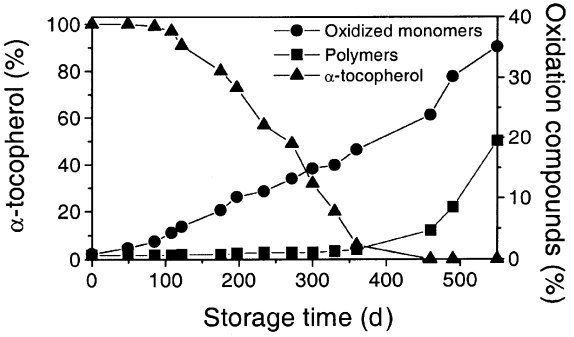

Fig. 8.1. Evolution of oxidized monomers (wt% on oil), polymers (wt% on oil) and α-tocopherol contents (mg/kg oil) in sunflower oil stored at 25°C.

encapsulated portion, which is nonaccessible to organic solvents. Once oxidation can be differentiated, two questions are of interest:

Are There Different Oxidation Profiles in the Accessible and the Nonaccessible Oil? The answer to this question is not difficult considering that two independent extracted fractions can be analyzed. Nevertheless, it is surprising that only a few researchers have approached the study of oxidation in microencapsulated oils by separate extraction of these two fractions.

Geijl-Hansen and Flink (1977) carried out separate extraction only in initial samples; after storage of intact samples and those devoid of surface oil, results clearly showed that the initial elimination of the surface oil led to more stable samples. Other authors found that the surface oil increased under certain storage conditions and that this fraction oxidized more rapidly than did the encapsulated fraction (Shimada *et al.* 1991). Similarly, it was reported recently that oxidation was more rapid in surface than in encapsulated oil in samples exposed to ultraviolet light (Hardas *et al.* 2000). In another study, oxidation also seemed to be higher in the surface oil fraction compared with the encapsulated oil, but there was a discrepancy between the results obtained through determination of conjugated dienes and residual amount of unoxidized substrate (Ponginebbi *et al.* 2000). In other studies, oxidation of microencapsulated oils was compared with that in mixtures constituted by the same components and used as models of lipids in a continuous phase. The results showed higher stability for the total oil (Taguchi *et al.* 1992a and 1992b) or the surface oil (Iwami *et al.* 1988) extracted from microencapsulates compared with the corresponding oil in the homogenous mixtures, conflicting with results reported by Yoshii *et al.* (1997).

Unfortunately, in other studies, it is not possible to establish clear differences in oxidation because separation of phases was conducted only in initial samples (Desobry *et al.* 1999, Lin *et al.* 1995a) or because the determination of oxidation was applied exclusively to the surface oil fraction (Labrousse *et al.* 1992) or to the total lipids extracted (Minemoto *et al.* 1997). For example, in the last-mentioned study, the relative oxidation of both fractions was deduced from the ratio of total oxidized methyl linoleate-to-surface oil fraction, leading to the suggestion that not only the surface oil fraction was oxidized. Other problems encountered have been due to the application of inappropriate analytical methods and/or rapid oxidation of the substrate used (Lin *et al.* 1995a).

Are There Different Oxidation Rates and/or Oxidation Profiles in the Oil Droplets Immersed in the Solid Matrix? No published information was found on this aspect. According to Fritsch (1994), there is no question that in foods containing a noncontinuous lipid phase, a portion of the lipids will oxidize rapidly and other portions either slowly or not at all. For example, under similar conditions of air accessibility, the expected oxidation rate would increase with surface-to-volume ratio and, consequently, as the oil globule size decreases. However, the main

problem encountered is how differentiation of distinct oxidation status can be made once the encapsulated lipids have been extracted from the matrix as a continuous lipid phase. In recent years, we have directed our efforts toward improving the evaluation of oxidation in microencapsulated oils during storage (Heinzelmann *et al.* 2000b, Márquez-Ruiz *et al.* 2000, Velasco *et al.* 2000a, Velasco 2001) and toward the application of an accelerated oxidative test to predict shelf-life and efficiency of antioxidants (Velasco *et al.* 2000b).

To illustrate the complexity of oil oxidation in microencapsulated systems, we have selected from our studies three samples among hundreds that showed distinct oxidation patterns. Their main characteristics are presented in Table 8.1. The samples were stored at 25°C in the dark until the powders were highly rancid.

Sample 1 is microencapsulated sunflower oil devoid of antioxidants; sample 2 is a spray-dried infant formula prepared using a mixture of polyunsaturated oils; and sample 3 is microencapsulated sunflower oil containing its naturally occurring tocopherols. First, Figure 8.2 shows the profiles of oxidation for the total oils extracted from the three samples. Only polymers and tocopherol data have been represented because, as noted before, they both clearly show the end of the induction period. As expected, sample 1 was rapidly oxidized, with rancidity appearing at 8 d when the oxidation accelerated and polymers showed a marked increase. In contrast, the other two samples did not follow the oxidation profile found for bulk oils. Sample 2 maintained nearly the initial levels because only a slight increase of polymers (~1%) and a small loss of tocopherols (~5%) occurred after 100 d of storage. However, samples were highly rancid earlier than that, at ~2 mo of storage. The reverse pattern was observed in sample 3 because after 100 d of storage, polymers had increased significantly and ~40% of the tocopherols was lost, but rancidity was detected much later, after almost 1 y of storage. Unlike bulk oils, in this sample, rather high levels of tocopherols remained despite the significant polymerization. These results reflect the enormous complexity of oxidation in multiphasic lipid systems and the difficulties in deducing the status of oxidation starting from total oil samples. After separation of the surface and encapsulated fractions, some specific facts became evident and were of utility to explain the results in Figure 8.2.

Results obtained for the surface and encapsulated fractions extracted quantitatively are shown in Figure 8.3. To simplify this figure, results for total oils were omitted

TABLE 8.1
Characteristics of Microencapsulated Oils

Sample	Drying process	Encapsulation matrix	Microencapsulation efficiency (%)[a]	α-Tocopherol (mg/kg oil)
1	Freeze-drying	Lactose/casein	75.1 ± 0.75	0
2	Spray-drying	Skim milk powder	95.1 ± 0.81	410
3	Freeze-drying	Lactose/casein	70.3 ± 1.29	625

[a]Percentage of encapsulated oil fraction in total oil.

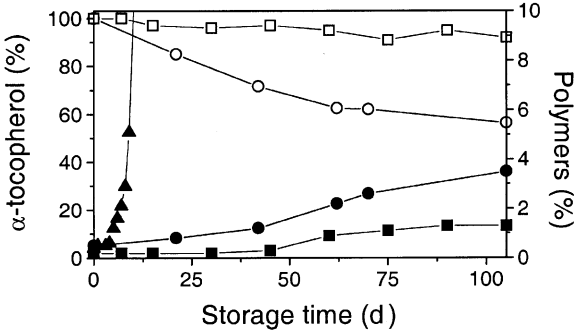

Fig. 8.2. Evolution of oxidation in total oils extracted from microencapsulated sunflower oil devoid of antioxidants (polymer content: ▲), infant formula (polymer content: ■, and loss of α-tocopherol: □) and microencapsulated sunflower oil (polymer content: ●, and loss of α-tocopherol: ○). Polymer contents are expressed as wt % on oil, and loss of α-tocopherol as percentages of remaining α-tocopherol. Samples were stored at 25°C in the dark.

(data up to 105 d are included in Fig. 8.2). In sample 1, oxidation was very rapid and apparently both fractions oxidized at approximately the same rate; in sample 2, the surface oil oxidized much more quickly, whereas the reverse was observed in sample 3. These results indicate that although theoretically the more accessible (external or surface) oil is not protected by the matrix and is more exposed to oxidation, the great number of variables influencing oxidation in these systems exert a crucial role in the relative oxidation rate of the surface and encapsulated fractions. This notion was already pointed out by Fritsch (1994) in a paper that stressed the fact that although lipid distribution is of paramount importance in food oxidation, it is still too often ignored.

As can be observed, the rapid surface oil oxidation in sample 2 was masked when the total oil was analyzed because of the low contribution of surface oil (~5%) to the total oil. In fact, no analytical method applied to the total oil would be valid to explain the obvious rancidity in the sample. It is usual to forget that analytical data obtained from multiphasic systems are based on the evaluation of the monophasic oil extracted, whereas an increase in volatiles may proceed from advanced oxidation occurring in a very small fraction of the sample. Interestingly, the oxidation profile of this surface fraction was very similar to that obtained for bulk oils, thus typical of lipids in continuous phase, showing a clear end of the induction period as marked by initiation of polymerization and exhaustion of tocopherols, at about 50–60 d. Unfortunately, it was not possible to obtain the oxidation profile in the encapsulated oil because the rapid development of rancidity in the surface oil denoted the end of the storage period.

For sample 3, the oxidation profile of surface oil was again similar to that found for samples 1 and 2, although the rate of oxidation was much slower than

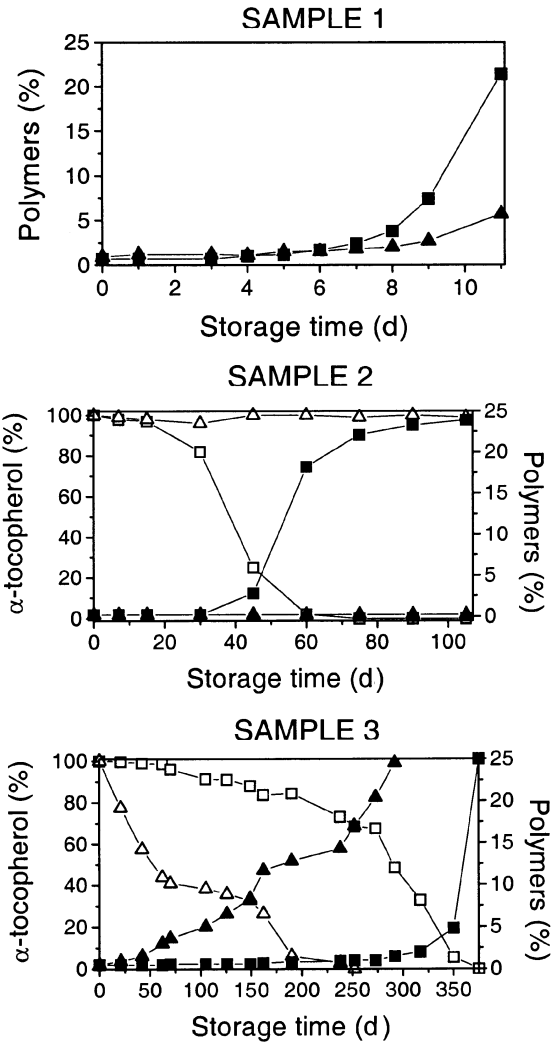

Fig. 8.3. Evolution of oxidation in microencapsulated sunflower oil devoid of antioxidants (sample 1), infant formula (sample 2), and microencapsulated sunflower oil (sample 3): Polymer contents (wt % on oil) in surface (■) and encapsulated (▲) oil fractions and losses of α-tocopherol (% of remaining α-tocopherol) in surface (□) and encapsulated (△) oil fractions. Samples were stored at 25°C in the dark.

that found for the encapsulated oil. For this reason, contrary to the situation in sample 2, this pattern was reflected in the analysis of total oil due to the high proportion of encapsulated oil in the total oil (~70%). However, the oxidation profile of encapsulated oil was rather unusual, i.e., considerably high polymer values were found in samples that continued to contain high levels of residual tocopherol. For example, after 150 d, the encapsulated oil fractions contained an amount of polymers as high as 8% and continued to have ~30% residual tocopherol. It is now evident that such samples did not have objectionable odor when tested as intact samples despite the high level of oxidation because rancidity was detected only when the encapsulated oil was released. It

was also appreciable that both polymer increase and tocopherol loss showed a "shifting" or "uneven" profile. Overall results reflected the coexistence of oil globules in a wide range of oxidation states, likely including some at low oxidation stages that were still protected by the presence of tocopherol and others devoid of antioxidants and well within the advanced oxidation stage. Therefore, analysis of the noncontinuous phase of microencapsulated oils provided a profile typical of a mixture of oil samples showing different oxidation rates.

This situation is also applicable to other disperse systems in which oil droplets may have different susceptibility to oxidation. Unfortunately, despite the high number of papers published on lipid oxidation in emulsions, no comments were found on this simple and predictable fact. Another field of importance for this finding could be oxidation in biological systems; thus, it has been found that α-tocopherol and highly oxidized lipids coexist in lipoproteins of advanced human atherosclerotic plaques (Niu *et al.* 1999), possibly supporting the notion of oxidation in noncontinuous lipid phases.

Concluding Remarks

Oxidation in microencapsulated oils is affected by numerous variables; of these, the heterogeneous lipid distribution is of outstanding influence, making its analytical evaluation require a careful selection of methods suitable for these complex lipid systems. In this chapter, the analytical methods described enable the detection of differences in oxidation in the continuous lipid phase (monophasic lipid systems) and noncontinuous lipid phase. Oxidation in the continuous lipid phase is characterized by the following pattern: an increase in hydroperoxides (oxidized triglyceride monomers) and the initiation of polymerization in detectable amounts at the end of the induction period, when oxidation accelerates and a rapid loss of antioxidants also occurs. In contrast, the coexistence of antioxidants with polymerization compounds in the noncontinuous oil phase would indicate the presence of a mixture of oils with different oxidation status. This situation could also apply to other disperse systems in which oil droplets may have different susceptibility to oxidation, such as oil-in-water emulsions and certain biological systems.

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References

Almaas, R., Rootwell, T., Oeyasaeter, S., and Saugstad, O.D. (1997) Ascorbic Acid Enhances Hydroxyl Radical Formation in Iron-Fortified Infant Cereals and Infant Formulas, *Eur. J. Pediatr. 156*, 488–492.

Anandaraman S., and Reineccius G.A. (1986) Stability of Encapsulated Orange Peel Oil, *Food Technol. 40*, 88–93.

Angulo, A.J., Romera, J.M., RamÌrez, M., and Gil, A. (1998) Effects of Storage Conditions on Lipid Oxidation in Infant Formulas Based on Several Protein Sources, *J. Am. Oil Chem. Soc. 75*, 1603–1607.

Balassa, L.L., and Fanger, G.O. (1971) Microencapsulation in the Food Industry, *CRC Crit. Rev. Food Technol*. 245–265.

Bangs, W.E., and Reineccius, G.A. (1990) Characterization of Selected Materials for Lemon Oil Encapsulation by Spray Drying, *J. Food Sci. 55*, 1356–1358.

Bendich, A., and Brock, P.E. (1997) Rationale for the Introduction of Long Chain Polyunsaturated Fatty Acids and for the Concomitant Increase in the Level of Vitamin E in Infant Formulas, *Int. J. Vitam. Nutr. Res. 67*, 213–231.

Bhandari, B.R., D'Arcy, B.R., and Bich, L.L.T. (1998) Lemon Oil to β-Cyclodextrin Ratio Effect on the Inclusion Efficiency of β-Cyclodextrin and the Retention of Oil Volatiles in the Complex, *J. Agric. Food Chem. 46*, 1494–1499.

Buma, T.J. (1971a) Free Fat in Spray-Dried Whole Milk 2: An Evaluation of Methods for the Determination of Free-Fat Content, *Neth. Milk Dairy J. 25*, 42–52.

Buma, T.J. (1971b) Free Fat in Spray-Dried Whole Milk 8: The Relation Between Free-Fat Content and Particle Porosity of Spray-Dried Whole Milk, *Neth. Milk Dairy J. 25*, 123–140.

Buma, T.J. (1971c) Particle Structure of Spray-Dried Milk Products as Observed by a Scanning Electron Microscope, *Neth. Milk Dairy J. 25*, 75–80.

Buma, T.J. (1971d) Free Fat in Spray-Dried Whole Milk. 3. Particle Size: Its Estimation, Influence of Processing Parameteres and Its Relation to Free-Fat Content, *Neth. Milk Dairy J. 25*, 53–72.

Burvall, A., Asp, N.G., Bosson, A., San José, C., and Dahlqvist, A. (1978) Storage of Lactose-Hydrolysed Dried Milk: Effect of Water Activity on the Protein Nutritional Value, *J. Dairy Res. 45*, 381–389.

Chan, S.H., Gray, J.I., Gomaa, E.A., Harte, B.R., Kelly, P.M., and Buckley, D.J. (1993) Cholesterol Oxidation in Whole Milk Powders as Influenced by Processing and Packaging, *Food Chem. 47*, 321–328.

Chang, P.S., and Ha, J.S. (2000) Optimization of Fish Oil Microencapsulation by Response Surface Methodology and Its Storage Stability, *Korean J. Food Sci. Technol. 32*, 646–653.

Chirife, J., and Karel, M. (1974) Effect of Structure Disrupting Treatments on Volatile Release from Freeze-Dried Maltose, *J. Food Technol. 9*, 13–20.

Chuy, L.E. and Labuza, T.P. (1994) Caking and Stickiness of Dairy-Based Food Powders as Related to Glass Transition, *J. Food Sci. 59*, 43–46.

Desobry, S.A., Netto, F.M., and Labuza, T.P. (1997) Comparison of Spray-Drying, Drum-Drying and Freeze-Drying for β-Carotene Encapsulation and Preservation, *J. Food Sci. 62*, 1158–1162.

Desobry, S.A, Netto, F.M., and Labuza, T.P. (1999) Influence of Maltodextrin Systems at an Equivalent 25DE on Encapsulated β-Carotene Loss During Storage, *J. Food Process. Preserv. 23*, 39–55.

Dian, N.L.H.M., Sudin, N., and Yusof, M.S.A. (1996) Characteristics of Microencapsulated Palm-Based Oil as Affected by Type of Wall Material, *J. Sci. Food Agric. 70*, 422–426.

Dobarganes, M.C., and Márquez-Ruiz, G. (1993) Size Exclusion Chromatography in the Analysis of Lipids, in *Advances in Lipid Methodology*, vol. 2 (Christie, W.W., ed.) pp. 113–137, The Oily Press, Dundee, Scotland.

Dobarganes, M.C., and Márquez-Ruiz, G. (1995) High Performance Size Exclusion Chromatography Applied to the Analysis of Edible Fats, in *New Trends in Lipid and Lipoprotein Analysis* (Perkins, E.G. and Erickson. M.D., eds.) pp. 89–111, AOCS Press, Champaign, IL.

Dobarganes, M.C., and Márquez-Ruiz, G. (1998) Analytical Evaluation of Fats and Oils by Size-Exclusion Chromatography, *Analusis 26*, M61–M65.
Dobarganes, M.C., Pérez-Camino, M.C., and Márquez-Ruiz, G. (1988) High Performance Size Exclusion Chromatography of Polar Compounds in Heated and Non-Heated Fats, *Fat Sci. Technol. 90*, 308–311.
Dobarganes, M.C., Pérez-Camino, M.C., Márquez-Ruiz, G., and Ruiz-Méndez, M.V. (1989) New Analytical Possibilities in Quality Evaluation of Refined Oils, in *Edible Fats and Oils Processing: Basic Principles and Modern Practices* (Erikson, D.R., ed.) pp. 427–429, AOCS Press, Champaign, IL.
Dobarganes, M.C., Velasco, J., and Dieffenbacher (2000) The Determination of Polar Compounds, Polymerized Triacylglycerols, Oxidized Triacylglycerols and Diacylglycerol in Fats and Oils, *Pure Appl. Chem. 72*, 1563–1575.
Dziezak J.D. (1988). Microencapsulation and Encapsulated Ingredients, *Food Technol. 42*, 136–151.
Eriksson, C.E. (1987) Oxidation of Lipids in Food Systems, in *Autoxidation of Unsaturated Lipids* (Chan, H.W.-S., ed.) pp. 207–231, Academic Press, London.
Faldt, P., and Bergenstahl, B. (1995) Fat Encapsulation in Spray-Dried Food Powders, *J. Am. Oil Chem. Soc. 72*, 171–176.
Fioriti, J.A., Stahl, H.D., Cseri, J., and Sims, R.J. (1975) Chemical and Organoleptic Properties of Dried Emulsions, *J. Am. Oil Chem. Soc. 52*, 395–399.
Flink, J., and Karel, M. (1970a) Retention of Organic Volatiles in Freeze-Dried Solutions of Carbohydrates, *J. Agric. Food Chem. 18*, 295–297.
Flink, J., and Karel, M. (1970b) Effects of Process Variables on Retention of Volatiles in Freeze-Drying, *J. Food Sci. 35*, 444–447.
Frankel, E.N. (1993) In Search of Better Methods to Evaluate Natural Antioxidants and Oxidative Stability in Food Lipids, *Trends in Food Sci. Technol. 4*, 220–225.
Frankel, E.N. (1998a) Methods to Determine Extent of Oxidation, in *Lipid Oxidation*, pp. 79–98, The Oily Press, Dundee, Scotland.
Frankel, E.N. (1998b) Foods, in *Lipid Oxidation*, pp. 187–225, The Oily Press, Dundee, Scotland.
Frankel, E.N., and Meyer, A.S. (2000) The Problems of Using One-Dimensional Methods to Evaluate Multifunctional Food and Biological Antioxidants, *J. Sci. Food Agric. 80*, 1925–1941.
Fritsch, C.W. (1994) Lipid Oxidation—The Other Dimensions, *INFORM 5,* 423–431.
Galdi, M., Valencia, M.E., and Sambucetti, M.E. (1987) Changes in the Physicochemical State of Iron and Nutritional Deterioration in Powdered Infant Formulas During Storage, *Nutr. Rep. Int. 35*, 1279–1284.
Gardner, H.W. (1979) Lipid Hydroperoxide Reactivity with Proteins and Amino Acids: A Review, *J. Agric. Food Chem. 27*, 220–229.
Gejl-Hansen, F., and Flink J.M. (1977) Freeze-Dried Carbohydrate Containing Oil-in-Water Emulsions: Microstructure and Fat Distribution, *J. Food Sci. 42*, 1049–1055.
Giammarioli, S., Bellomonte, G., Denaro, M., and Milana, M.R. (1995) Determination of Hexanal in Infant Formulas by Headspace-Gas Chromatography, *Ital. J. Food Sci. 7*, 69–76.
Giammarioli, S., Lammardo, A.M., Sanzini, E., and Bellomonte, G. (1997) Preliminary Study on Lipid Oxidation in Infant Formulas During Storage, *Riv. Sci. Aliment. 26*, 80–88.
Gibbs, B.F., Kermasha, S., Alli, I., and Mulligan, C.N. (1999) Encapsulation in the Food Industry: A Review, *Int. J. Food Sci. Nutr. 50*, 213–224.

Guardiola, F., Codony, R., Manich, A., Rafecas, M., and Boatella, J. (1995) Stability of Polyunsaturated Fatty Acids in Egg Powder Processed and Stored Under Various Conditions, *J. Agric. Food Chem. 43*, 2254–2259.

Guardiola, F., Codony, R., Rafecas, M., Grau, A., Jordan, A., and Boatella, J. (1997) Oxysterol Formation in Spray-Dried Egg Processed and Stored Under Various Conditions: Prevention and Relationship with Other Quality Parameters, *J. Agric. Food Chem. 45*, 2229–2243.

Hall, G., and Andersson, J. (1985) Flavor Changes in Whole Milk Powder During Storage. III. Relationships Between Flavor Properties and Volatile Compounds, *J. Food Qual. 7*, 237–253.

Hardas, N., Danviriyakul, S., Foley, J.L., Nawar, W.W., and Chinachoti, P. (2000) Accelerated Stability Studies of Microencapsulated Anhydrous Milk Fat, *Lebensm.-Wiss. Technol. 33*, 506–513.

Heinzelmann, K., Franke, K., Jensen, B., and Haahr, A.-M. (2000a) Protection of Fish Oil from Oxidation by Microencapsulation Using Freeze-Drying Techniques, *Eur. J. Lipid Sci. Technol. 102*, 114–121.

Heinzelmann, K., Franke, K., Velasco, J., and M·rquez-Ruiz, G. (2000b) Microencapsulation of Fish Oil by Freeze-Drying Techniques and Influence of Process Parameters on Oxidative Stability During Storage, *Eur. Food Res. Technol. 211*, 234–239.

Hidalgo, F.J., Zamora, R., and Alaiz, M. (1992) Modificaciones Producidas en las ProteÌnas Alimentarias por su Interacción con Lípidos Peroxidados. III. Consecuencias Nutricionales y Toxicológicas, *Grasas Aceites 43*, 97–100.

Huber, K.C., Pike, O.A., and Huber, C.S. (1995) Antioxidant Inhibition of Cholesterol Oxidation in a Spray-Dried Food System During Accelerated Storage, *J. Food Sci. 60*, 909–916.

Imagi, J., Kako, N., Nakanishi, K., and Matsuno, R. (1990) Entrapment of Liquid Lipids in Matrixes of Saccharides, *J. Food Eng. 12*, 207–222.

Imagi, J., Muraya, K., Yamashita, D., Adachi, S., and Matsuno, R. (1992) Retarded Oxidation of Liquid Lipids Entrapped in Matrixes of Saccharides or Proteins, *Biosci. Biotechnol. Biochem. 56*, 1236–1240.

Iwami, K., Hattori, M., and Ibuki, F. (1987a) Prominent Antioxidant Effect of Wheat Gliadin on Linoleate Peroxidation in Powder Model Systems at High Water Activity, *J. Agric. Food. Chem. 35*, 628–631.

Iwami, K., Hattori, M., Nakatani, S., and Ibuki, F. (1987b) Spray-Dried Gliadin Powders Inclusive of Linoleic Acid (Microcapsules): Their Preservability, Digestibility and Application to Bread Making, *Agric. Biol. Chem. 51*, 3301–3307.

Iwami, K., Hattori, M., Yasumi, T., and Ibuki, F. (1988). Stability of Gliadin-Encapsulated Fatty Acids Against Autoxidation, *J. Agric. Food Chem. 36*, 160–164.

Jackson, L.S., and Lee, K. (1991) Microencapsulation and the Food Industry, *Lebensm.-Wiss. Technol. 24*, 289–297.

Karel, M. (1980) Lipid Oxidation, Secondary Reactions and Water Activity of Foods, in *Autoxidation in Foods and Biological Systems* (Simic, H.G. and Karel, M., eds.) pp. 191–206, Plenum Press, New York.

Karel, M. (1984) Chemical Effects in Food Stored at Room Temperature, *J. Chem. Educ. 61*, 335–339.

Karel, M., Labuza, T.P., and Maloney, J.F. (1967) Chemical Changes in Freeze-Dried Foods and Model Systems, *Cryobiology 3*, 288–296.

Keogh, M.K., and O'Kennedy, B.T. (1999) Milk Fat Microencapsulation Using Whey Proteins, *Int. Dairy J. 9*, 657–663.

Keogh, M.K., O'Kennedy, B.T., Kelly, J., Auty, M.A., Kelly, P.M., Fureby, A., and Haahr, A.-M. (2001) Stability to Oxidation of Spray-Dried Fish Oil Powder Microencapsulated Using Milk Ingredients, *Food Chem. Toxicol. 66*, 217–224.

Kim, S.J., Park, G.B., Kang, C.B., Park, S.D., Yung, M.Y., Kim, J.O., and Ha, Y.L. (2000) Improvement of Oxidative Stability of Conjugated Linoleic Acid (CLA) by Microencapsulation in Cyclodextrins, *J. Agric. Food Chem. 48*, 3922–3929.

Kim, Y.D., and Morr, C.V. (1996) Microencapsulation Properties of Gum Arabic and Several Food Proteins: Spray-Dried Orange Oil Emulsion Particles, *J. Agric. Food Chem. 44*, 1314–1320.

Kopelman, I.J., Meydav, S., and Wilmersdorf, P. (1977) Freeze-Drying Encapsulation of Water Soluble Citrus Aroma, *J. Food Technol. 12*, 65–72.

Labrousse, S., Roos, Y., and Karel, M. (1992) Collapse and Crystallization in Amorphous Matrices with Encapsulated Compounds, *Sci. Aliments 12*, 757–769.

Labuza T.P. (1968) Sorption Phenomena in Foods, *Food Technol. 22*, 15–24.

Levine, H., and Slade, L. (1990) Influences of the Glassy and Rubbery States on the Thermal, Mechanical, and Structural Properties of Doughs and Baked Products, in *Dough Rheology and Baked Product Texture* (Faridi, H. and Faubión, J.M., eds.) pp. 157–330, AVI, New York.

Li, S.X., Cherian, G., and Sim, J.S. (1996) Cholesterol Oxidation in Egg Yolk Powder During Storage and Heating as Affected by Dietary Oils and Tocopherol, *J. Food Sci. 61*, 721–725.

Lin, C. Ch., Lin, S.Y., and Hwang, L.S. (1995a) Microencapsulation of Squid Oil with Hydrophilic Macromolecules for Oxidative and Thermal Stabilization, *J. Food Sci. 60*, 36–39.

Lin, S.Y., Hwang, L.S., and Lin, C.C. (1995b) Thermal Analyzer and Micro FTIR-DSC System Used to Determine the Protective Ability of Microencapsulated Squid Oil, *J. Microencapsul. 12*, 165–172.

Márquez-Ruiz, G., Jorge, N., Martín-Polvillo, M., and Dobarganes, M.C. (1996a) Rapid, Quantitative Determination of Polar Compounds in Fats and Oils by Solid-Phase Extraction and Exclusion Chromatography Using Monostearin as Internal Standard, *Grasas Aceites 749*, 55–60.

Márquez-Ruiz, G., Martín-Polvillo, M., and Dobarganes, M.C. (1996b) Quantitation of Oxidized Triglyceride Monomers and Dimers as a Useful Measurement for Early and Advanced Stages of Oxidation, *Grasas y Aceites 47*, 48–53.

Márquez-Ruiz, G., Martín-Polvillo, M., Jorge, N., Ruiz-Méndez, M.V., and Dobarganes, M.C. (1999) Influence of Used Frying Oil Quality and Natural Tocopherol Content on Oxidative Stability of Fried Potatoes, *J. Am. Oil Chem. Soc. 76*, 421–425.

Márquez-Ruiz, G., Velasco, J., and Dobarganes, M.C. (2000) Evaluation of Oxidation in Dried Microencapsulated Fish Oils by Combination of Adsorption and Size Exclusion Chromatography, *Eur. Food Res. Technol. 211*, 13–18.

Martín-Polvillo, M. (2000) Evolución de la Alteración Oxidativa y Formación de Nuevos Compuestos en Sistemas Modelo, Aceites y Alimentos Grasos, Ph.D. Thesis, Universidad de Sevilla, Seville.

Martín-Polvillo, M., Márquez-Ruiz, G., Jorge, N., Ruiz-Méndez, M.V., and Dobarganes, M.C. (1996) Evolution of Oxidation During Storage of Crisps and French Fries Prepared with Sunflower Oil and High Oleic Sunflower Oil, *Grasas Aceites 47*, 54–58.

Matoba, T., Yonezawa, D., Nair, B.M., and Kito, M. (1984) Damage to Amino Acid Residues of Proteins After Reaction with Oxidizing Lipids: Estimation by Proteolytic Enzymes, *J. Food Sci. 49*, 1082–1084.

Matsuno, R., and Adachi, S. (1993) Lipid Encapsulation Technology—Techniques and Applications to Food, *Trends Food Sci. Technol. 41*, 256–261.

McCluskey, S., Connolly, J.F., Devery, R., O'Brien, B., Kelly, J., Harrington, D., and Stanton, C. (1997) Lipid and Cholesterol Oxidation in Whole Milk Powder During Processing and Storage, *J. Food Sci. 62*, 331–337.

McNamee, B.F., O'Riordan, E.D., and O'Sullivan, M. (1998) Emulsification and Microencapsulation Properties of Gum Arabic, *J. Agric. Food Chem. 46*, 4551–4555.

Menting, L.C., Hoogstad, B., and Thijssen, H.A.C. (1970) Aroma Retention During the Drying of Liquid Foods, *J. Food Technol. 5*, 127–139.

Min, D.B., Lee, S.H., Lindamood, J.B., Chang, K.S., and Reineccius, G.A. (1989) Effects of Packaging Conditions on the Flavor Stability of Dry Whole Milk, *J. Food Sci. 54*, 1222–1224, 1245.

Minemoto Y., Adachi, S., and Matsuno, R. (1997) Comparison of Oxidation of Methyl Linoleate Encapsulated with Gum Arabic by Hot-Air-Drying and Freeze-Drying, *J. Agric. Food Chem. 45*, 4530–4534.

Minemoto, Y., Adachi, S., and Matsuno, R. (1999) Autoxidation of Linoleic Acid Encapsulated with Polysaccharides of Differing Weight Ratio, *Biosci. Biotechnol. Biochem. 63*, 866–869.

Minemoto, Y., Adachi, S., and Matsuno, R. (2001) Effect of Relative Humidity During Storage on the Autoxidation of Linoleic Acid Encapsulated with a Polysaccharide by Hot-Air-Drying and Freeze-Drying, *Food Sci. Technol. Res. 7*, 91–93.

Moreau D.L., and Rosenberg M. (1996) Oxidative Stability of Anhydrous Milkfat Microencapsulated in Whey Proteins, *J. Food Sci. 61*, 39–43.

Niu, X., Zammit, V., Upston, J.M., Dean, R.T., and Stocker, R. (1999) Coexistence of Oxidized Lipids and α-Tocopherol in All Lipoprotein Density Fractions Isolated from Advanced Human Atherosclerotic Plaques, *Arterioscler. Thromb. Vasc. Biol. 19*, 1708–1718.

Orford, P.O., Parker, R., Ring, S.G., and Smith, A.C. (1989) Effects of Water as a Diluent on the Glass Transition Behaviour of Malto-Oligosaccharides, Amylose and Amylopectin, *Int. J. Biol. Macromol. 11*, 91–96.

Orlien, V., Andersen, A.B., Sinkko, T., and Skibsted, L.H. (2000) Hydroperoxide Formation in Rapeseed Oil Encapsulated in a Glassy Food Model as Influenced by Hydrophilic and Lipophilic Radicals, *Food Chem. 68*, 191–199.

Park, P.S.W., and Goins, R.E. (1992) Determination of Volatile Lipid Oxidation Products by Dynamic Headspace-Capillary Gas Chromatographic Analysis with Application to Milk-Based Nutritional Products, *J. Agric. Food Chem. 40*, 1581–1586.

Pauletti, M.S., and Amestoy, P. (1999) Butter Microencapsulation as Affected By Composition of Wall Material and Fat, *J. Food Sci. 64*, 279–282.

Pérez-Camino, M.C., M·rquez-Ruiz, G., Ruiz-Méndez, M.V., and Dobarganes, M.C. (1991) Lipid Oxidation in Fats and Fatty Foods: Quantitative Determination of Oxidized Triglycerides, in *Proceedings of Euro Food Chem VI*, Vol. 2 (Baltes, W., Ekland, T., Fenwick, R.W., eds.) pp. 569–574, Lebensmittel-chemische Gesellschaft, Frankfurt.

Ponginebbi, L., Nawar, W.W., and Chinachoti, P. (2000) Effect of Relative Humidity on Lipid Oxidation in Freeze-Dried Emulsions, *Grasas Aceites 51*, 348–354.

Presa-Owens, S., López-Sabater, M.C., and Rivero-Urgell, M. (1995) Shelf-Life Prediction of an Infant Formula Using an Accelerated Stability Test (Rancimat), *J. Agric. Food Chem. 43*, 2879–2882.

Przygonski, K., Jelen, H., and Wasowicz, E. (2000) Determination of Cholesterol Oxidation Products in Milk Powder and Infant Formulas by Gas Chromatography and Mass Spectrometry, *Nahrung 44*, 122–125.

Reichenbach, W.A., and Min, D.B. (1997) Oxidative Stability and Nuclear Magnetic Resonance Analyses of Linoleic Acid Encapsulated in Cyclodextrins, *J. Am. Oil Chem. Soc. 74*, 1329–1333.

Riisom, T., Sims, R.J., and Fioriti, J.A. (1980) Effects of Amino Acids on the Autoxidation of Safflower Oil in Emulsions, *J. Am. Oil Chem. Soc. 57*, 354–359.

Roos, Y.H., Karel, M., and Kokini, J.L. (1996) Glass Transitions in Low Moisture and Frozen Foods: Effects on Shelf Life and Quality, *Food Technol. 50*, 95–108.

Rose-Sallin, C., Hugget, A.C., Bosset, J.O., Tabacchi, R., and Fay, L.B. (1995) Quantification of Cholesterol Oxidation Products in Milk Powders Using [^{2}H-7]-Cholesterol to Monitor Cholesterol Autoxidation Artifacts, *J. Agric. Food Chem. 43*, 935–941.

Rosenberg, M., and Young, S.L. (1993) Whey Proteins as Microencapsulating Agents: Microencapsulation of Anhydrous Milkfat-Structure Evaluation, *Food Structure 12*, 31–41.

Rosenberg, M., Kopelman, I.J., and Talmon, Y. (1990) Factors Affecting Retention in Spray-Drying Microencapsulation of Volatile Materials, *J. Agric. Food Chem. 38*, 1288–1294.

Rossell, J.B. (1994) Measurements of Rancidity, in *Rancidity of Foods* (Allen, J.C., and Hamilton, R.J., eds.), pp. 22–53, Chapman and Hall, Glasgow.

Ruiz-Méndez, M.V., Márquez-Ruiz, G., and Dobarganes, M.C. (1997) Relationships Between Quality of Crude and Refined Edible Oils Based on Quantitation of Minor Glyceridic Compounds, *Food Chem. 60*, 549–554.

Rulkens, W.H., and Thijssen, H.A.C. (1972) Retention of Volatile Compounds in Freeze-Drying Slabs of Maltodextrin, *J. Food Technol. 7*, 79–93.

Sankarikutty, B., Sreekumar, M.M., Narayanan, C.S., and Mathew, A.G. (1988) Studies on Microencapsulation of Cardamon Oil by Spray-Drying Technique, *J. Food Sci. Technol. 25*, 352–356.

Satué-Gracia, M.T., Frankel, E.N., Rangavajhyala, N., and German, J.B. (2000) Lactoferrin in Infant Formulas: Effect on Oxidation, *J. Agric. Food Chem. 48*, 4984–4990.

Selim, K., Tsimidou, M., and Biliaderis, C.G. (2000) Kinetic Studies of Degradation of Saffron Carotenoids Encapsulated in Amorphous Polymer Matrices, *Food Chem. 71*, 199–206.

Shahidi, F., and Han, X.-Q. (1993) Encapsulation of Food Ingredients, *Crit. Rev. Food Sci. Nutr. 33*, 501–547.

Sheu, T.-Y., and Rosenberg, M. (1995) Microencapsulation by Spray-Drying Ethyl Caprylate in Whey Protein and Carbohydrate Wall Systems, *J. Food Sci. 60*, 98–103.

Sheu, T.-Y., and Rosenberg, M. (1998) Microstructure of Microcapsules Consisting of Whey Proteins and Carbohydrates, *J. Food Sci. 63*, 491–494.

Shimada, Y., Roos, Y., and Karel, M. (1991) Oxidation of Methyl Linoleate Encapsulated in Amorphous Lactose-Based Food Model, *J. Agric. Food Chem. 39*, 637–641.

Shiratsuchi, H., Shimoda, M., Imayoshi, K., Noda, K., and Osajima, Y. (1994) Off-Flavor Compounds in Spray-Dried Skim Milk Powder, *J. Agric. Food Chem. 42*, 1323–1327.

Sims, R.J. (1994) Oxidation of Fats in Food Products, *INFORM 5*, 1020–1028.

Stapelfeldt, H., Nielsen, B.R., and Skibsted, L.H. (1997) Effect of Heat Treatment, Water Activity and Storage Temperature on the Oxidative Stability of Whole Milk Powder, *Int. Dairy J. 7*, 331–339.

Strange, E.D., Konstance, R.P., Lu, D., Smith, P.W., Onwulata, C.I., and Holsinger, V.H. (1997) Oxidative and Functional Stability During Storage of Butter Oil Encapsulated with Sucrose or Flour, *J. Food Lipids 4*, 245–260.

Taguchi, K., Iwami, K., Ibuk, F., and Kawabata, M. (1992a) Oxidative Stability of Sardine Oil Embedded in Spray-Dried Egg White Powder and Its Use for n-3 Unsaturated Fatty Acid Fortification of Cookies, *Biosci. Biotechnol. Biochem. 56*, 560–563.

Taguchi, K., Iwami, K., Ibuki, F., and Kawabata, M. (1992b) Preservability and Utilization of Powdered α-Linoleic Acid with Egg White, *Biosci. Biotechnol. Biochem. 56*, 672–673.

Tang, Y.C., and Chen, B.H. (2000) Pigment Change of Freeze-Dried Carotenoid Powder During Storage, *Food Chem. 69*, 11–17.

Thompkinson, D.K., and Mathur, B.N. (1986) Oxidative Stability of Vegetable Oils in Combination with Milk Solids, *Indian J. Dairy Sci. 39*, 431–433.

Thompkinson, D.K., and Mathur, B.N. (1989) Co-Relation of Chemical Parameters for Measurement of Oxidation in PUFA Rich Dried Food System, *Indian J. Dairy Sci. 42*, 659–660.

Thompkinson, D.K., and Mathur, B.N. (1990) Stability of Fatty Acids and Nutritional Adequacy of PUFA Rich Infant Formula During Storage, *Indian J. Dairy Sci. 43*, 203–206.

Ulberth, F., and Roubicek, D. (1995) Monitoring of Oxidative Deterioration of Milk Powder by Headspace Gas Chromatography, *Int. Dairy J.5*, 523–531.

Velasco, J. (2001) Ingredientes Funcionales Constituidos por Aceites Microencapsulados: Evaluación de la Oxidación Lipídica y Eficacia de Antioxidantes en Estudios de Conservación y Oxidación Acelerada, Ph.D. Thesis, Universidad de Sevilla, Seville.

Velasco, J., Dobarganes, M.C., and Márquez-Ruiz, G. (2000a) Oxidation of Free and Encapsulated Oil Fractions in Dried Microencapsulated Oils, *Grasas Aceites 51*, 441–448.

Velasco, J., Dobarganes, M.C., and Márquez-Ruiz, G. (2000b) Application of the Accelerated Test Rancimat to Evaluate Oxidative Stability of Dried Microencapsulated Oils, *Grasas Aceites 51*, 261–267.

Wahle, K.W.J., Hoppe, P.P., and McIntosh, G. (1993) Effects of Storage and Various Intrinsic Vitamin E Concentrations on Lipid Oxidation in Dried Egg Powders, *J. Sci. Food Agric. 61*, 463–469.

Wanasundara, U.N., and Shahidi, F. (1995) Storage Stability of Microencapsulated Seal Blubber Oil, *J. Food Lipids 2*, 73–86.

Wang, J.Y., Fujimoto, K., Miyazawa, T., and Endo, Y. (1991) Autoxidative Mechanism of Maize in Powder Model Systems Against Methyl Linoleate: Effect of Water Activity and Coexistence of Antioxidants, *J. Agric. Food Chem. 39*, 351–355.

Yoshii, H., Furuta, T., Kawasaki, K., Hirano, H., Funatsu, Y., Toyomi, A., and Nakayama, S. (1997) Oxidative Stability of Powdery Tridocosahexanoin Included in Cyclodextrin and Its Application to Fish Meal Paste, *Biosci. Biotechnol. Biochem. 61*, 1376–1378.

Young, S.L., Sarda, X., and Rosenberg, M. (1993a) Microencapsulating Properties of Whey Proteins. 1. Microencapsulation of Anhydrous Milk Fat, *J. Dairy Sci. 76*, 2868–2877.

Young, S.L., Sarda, X., and Rosenberg, M. (1993b) Microencapsulating Properties of Whey Proteins. 2. Combination of Whey Proteins with Carbohydrates, *J. Dairy Sci. 76*, 2878–2885.

Chapter 9

Protein Alterations Due to Lipid Oxidation in Multiphase Systems

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Introduction

Proteins are abundant in all organisms and are indeed fundamental to life because of their involvement in transport, storage, regulation, and enzymatic catalysis and their behavior as messengers or antibodies. Proteins are also key components in foods because they have a high nutritional value and are involved in food structure, texture, and sensory quality through their functional properties and a wide range of interactions with other constituents.

Many biological compartments and food products are complex media containing both protein and lipid as major components, solubilized or dispersed in a more or less aqueous medium, and organized into complex structures such as biological membranes, lipoproteins, polar lipid mesophases, oil-in-water, or water-in-oil emulsions. These structures are physically stabilized *via* the interfacial forces and lipid-protein interactions that contribute to the functioning of the biological machinery or to the functionality of the food. However, the closeness of lipid and protein molecules also favors their interreactivity. An example is the related oxidation of proteins, lipids, and cholesterol that proceeds in lipoproteins *in vivo* and contributes to atherosclerosis. Another example is the complex relationship between lipid oxidation and myoglobin oxidation in meat and meat products that makes the two phenomena almost impossible to dissociate. In food products, reactions of proteins with oxidizing lipids may lead to losses of selected free amino groups of nutritional interest, decrease of protein solubility, changes in hydrophobicity of soluble or insoluble proteins, increase of protein molecular mass, and loss of enzyme activities (Karel 1980, Liang 1999, Neukom 1980).

The objective of this chapter is to provide an overview of the actual knowledge concerning the degradations of proteins induced by lipid oxidation and their possible antioxidant and prooxidant activities in multiphase food systems such as emulsions. The lack of data concerning the influence of protein partitioning on their degradation is also emphasized.

Basic Aspects of Protein Chemistry and Structure

Protein Structure

Proteins are linear heteropolymers of fixed chain length. A single type of protein always has the same number and composition of monomers, but different proteins have a range of monomer (amino acid) units varying from a few tens to ~1000. Twenty kinds of amino acids varying in size, shape, charge, hydrogen-bonding capacity, and chemical reactivity are commonly found in proteins. All proteins in all species are constructed from this set of 20 amino acids. They are generally self-folding, i.e., the linear chains fold into specific three-dimensional conformations that are determined by the sequence of amino acids. The three-dimensional structures of proteins are also extremely diverse, ranging from completely fibrous to globular. Interested readers may consult a number of more detailed references and books (Birkbeck College homepage: http://pps01–1.cryst.bbk.ac.uk, Chothia 1984, Doolittle 1985, Goldberg 1985, Harrison and Durbin 1985, Pauling and Corey 1951, Stryer 1988).

Amino Acids

All amino acids have an amino group, a carboxyl group, a hydrogen atom, and a distinctive R group bonded to the α-carbon atom. Amino acids in solution at neutral pH are predominantly dipolar ions, zwitterions, rather than unionized molecules. Amino acids in the protein are linked by peptide bonds; the three-dimensional structure and function of a protein are dependent on the sequence of amino acid side chains in the polypeptide. The types of amino acids and their distribution in the proteins is also very important for the oxidation kinetics and pathways.

Amino acids can be divided into several different classes based on their physicochemical properties.

(i) Hydrophobic-Aliphatic Amino Acids. The side chains of these amino acids consist of nonpolar methyl or methylene groups. These amino acids are usually located on the interior of the protein because they are hydrophobic in nature. This group of amino acids includes glycine, alanine, valine, leucine, isoleucine, proline, and methionine. Methionine contains a sulfur atom in a thioether linkage ($-S-CH_3$). It could reasonably be classed as a hydrophobic residue because it is nearly always associated with the hydrophobic cores of proteins.

Glycine and proline are unique amino acids in that they appear to influence the conformation of the polypeptide. Glycine essentially lacks a side chain and can therefore adopt conformations that are sterically forbidden for other amino acids. This confers a high degree of local flexibility on the polypeptide. In contrast, proline is the most rigid of the 20 naturally occurring amino acids because its side chain is covalently linked with the main chain nitrogen.

(ii) Hydrophobic-Aromatic Amino Acids. This group includes three amino acids, i.e., phenylalanine, tryptophan, and tyrosine. Of these, only phenylalanine is entirely nonpolar. Tyrosine's phenolic side chain has a hydroxyl substituent and tryptophan has a nitrogen atom in its indole ring system. These residues are often found largely buried in the hydrophobic interior of a protein because they are predominantly nonpolar in nature. However, the polar atoms of tyrosine and tryptophan allow hydrogen-bonding interactions with other residues or even solvent molecules. These two amino acids are among those that are targeted by the oxidative reactions in biological and food systems because their phenol and indol rings allow electron delocalization and are chemically reactive. Tyrosine also has some hydrogen-donating activity.

(iii) Neutral-Polar Side Chains. A number of small aliphatic side chains contain polar groups that cannot ionize readily. Serine and threonine possess hydroxyl groups that can form hydrogen bonds with the main chain, which influences the local conformation of the polypeptide. Asparagine and glutamine possess amide groups, which are usually hydrogen-bonded whenever they occur in the interior of a protein. Cysteine contains a sulfhydryl group (-SH). It has the unique property of being able to form a covalent cross-link with another cysteyl residue elsewhere in the protein. These disulfide bridges involve the formation of -S-S- bonds between spatially adjacent cysteyl residues. Disulfide bridges are sensitive to reducing agents, which convert the two sulfur atoms back to their original -S-H form. Cysteine frequently takes an active part in metal binding sites because its sulfur atoms can form covalent bonds with certain metal ions. The sulfhydryl groups are also a target for oxidative reactions through hydrogen atom departure and formation of thiyl radicals.

(iv) Acidic Amino Acids. Aspartic acid and glutamic acid have carboxyl side chains and are therefore negatively charged at physiologic pH (~neutral). The strongly polar nature of these residues means that they are most often found on the surface of globular proteins where they can interact favorably with water molecules. These residues can also take part in electrostatic interactions with positively charged basic amino acids. Aspartic and glutamic acids can also take on catalytic roles in the active sites of enzymes and are able to bind metal ions.

(v) Basic Amino Acids. Of the basic amino acid side chains, histidine has the lowest pK_a (~6) and is therefore neutral at around physiologic pH. This amino acid acts as a metal ion ligand in numerous protein families. It also has hydrogen-donating capacity, which was shown to give it some antioxidant activity in natural or synthetic peptides. Lysine and arginine are more strongly basic and are positively charged at physiologic pH. They are generally solvated, but do occasionally occur in the interior of a protein where they are usually involved in electrostatic interactions with negatively charged groups such as Asp or Glu. Lys and Arg have important roles in anion-binding proteins because they can interact electrostatically with the ligand. The free NH_2 group of lysine is also very reactive with aldehydes, for example.

The Conformation of Proteins

In its native state, each type of protein molecule has a characteristic three-dimensional shape, referred to as its conformation. Depending on their conformation, proteins can be placed into two major classes, fibrous and globular. The fibrous proteins consist of polypeptide chains arranged in parallel along a single axis, to yield fibers or sheets. Fibrous proteins are physically tough and are insoluble in water or dilute salt solutions. They are the basic elements in the connective tissue of higher animals. In globular proteins, the polypeptide chains are tightly folded into compact spherical or globular shapes. Most globular proteins are soluble in aqueous systems.

Primary structure of protein refers to the covalent backbone of the polypeptide chain and the sequence of its amino acid residues. Secondary structure refers to a regular, recurring arrangement in space of the polypeptide chain along one dimension. Secondary structure is particularly evident in the fibrous proteins in which the polypeptide chains have an extended or longitudinally coiled conformation; it also occurs in segments of the polypeptide chains in globular proteins. Tertiary structure refers to how the polypeptide chain is bent or folded in three dimensions to form the compact, tightly folded structure of globular proteins. Quaternary structure refers to how individual polypeptide chains of a protein having two or more chains are arranged in relation to each other.

Pauling and Corey (1951) evaluated a variety of potential polypeptide conformations by building precise molecular models. They proposed two periodic polypeptide structures, called the α-helix and the β-pleated sheet. The α-helix is a rod-like structure. The tightly coiled polypeptide main chain forms the inner part of the rod, and the side chains extend outward in a helical array. The α-helix is stabilized by hydrogen bonds between the NH and CO groups of the main chain. The α-helix content of proteins of known three-dimensional structure is highly variable. In some, such as myoglobin and hemoglobin, the α-helix is the major structural motif. Other proteins, such as chymotrypsin, are virtually devoid of α-helix. In most proteins, the single-stranded α-helix is usually a short rod, less than 40 Å in length.

The β-pleated sheet differs markedly from the α-helix in that it is a sheet rather than a rod. A polypeptide chain in the β-pleated sheet is almost fully extended rather than being tightly coiled as in the α-helix. The axial distance between adjacent amino acids is 3.5 Å, in contrast with 1.5 Å for the α-helix. Another difference is that the β-pleated sheet is stabilized by hydrogen bonds between the NH and CO groups in different polypeptide chains, whereas in the α-helix, the hydrogen bonds are between the NH and CO groups in the same polypeptide chain. Adjacent chains in a β-pleated sheet can run in the same direction (parallel β-sheet) or in opposite directions (antiparallel β-sheet).

Most proteins have compact, globular shapes due to numerous reversals of direction of their polypeptide chains. Many of these chain reversals are accomplished by a common structural element called the β-turn. For more information, the reader is

referred to the original literature (Birbeck College: http://pps01–1.cryst.bbk.ac.uk, Chothia 1984, Doolittle 1985, Goldberg 1985, Harrison and Durbin 1985, Stryer 1988).

The Behavior of Proteins in Solution

Proteins in solution show profound changes in solubility as a function of pH, ionic strength, the dielectric properties of the solvent, and temperature. Compared with other food macrocomponents, proteins have several characteristics, e.g., the isoelectric effect, salting-in and salting-out effects, and denaturation by temperature.

Isoelectric Precipitation. The solubility of most globular proteins is profoundly influenced by the pH of the system. For example, the solubility of β-lactoglobulin is at a minimum at pH 5.2–5.3, regardless of the concentration of sodium chloride present. On either side of this critical pH, the solubility rises very sharply. The pH at which a protein is least soluble is its isoelectric pH, defined as that pH at which the molecule has no net electric charge. At pH values below the isoelectric point, the net charge of the protein is positive, and at pH values above the isoelectric point, the net charge of the protein is negative. When a protein solution is thoroughly dialyzed against distilled water to remove all small ions that proteins can bind, the pH of the resulting solution is known as the isoionic pH. The isoionic pH is a constant for any given protein.

Salting-In and Salting-Out. At a low concentration, salts increase the solubility of many proteins, a phenomenon called salting-in. The salts of divalent ions are far more effective at salting-in than salts of monovalent ions. The ability of neutral salts to influence the solubility of proteins is a function of their ionic strength. Increasing further the ionic strength decreases the solubility of a protein, an effect called salting-out. The high concentration of salt may remove water of hydration from the protein molecules, thus reducing their solubility, but other factors are also involved. Proteins precipitated by salting-out retain their native conformation and can be dissolved again, usually without denaturation.

Effect of Temperature. Within a limited range from ~0 to 40°C, most globular proteins increase in solubility with increasing temperature. Above 40–50°C, most proteins become increasingly unstable and undergo a physical change known as denaturation, in which the most visible effect is a decrease in solubility. Denaturation is the unfolding of the characteristic native folded structure of the polypeptide chain of globular protein molecules.

Adsorption and Partition of Proteins in Emulsions

Proteins present in the ingredients used to prepare emulsions are distributed between the continuous water phase (nonadsorbed proteins) and the interface where they pro-

vide emulsion stability (adsorbed proteins). This partitioning depends on many parameters, such as the concentration and the interfacial properties of the protein, the oil phase volume fraction, and the emulsification process. The adsorption process leads to changes in protein structure that influence the properties of the interface and also further chemical changes of the protein during emulsion aging.

Adsorption of proteins to interfaces can be described as a three-phase process. In the first stage, proteins diffuse from the bulk water phase to the interface. In the second stage, they rotate to expose their accessible hydrophobic domains to the oil surface. Finally, they adsorb on the interface. During this last stage, the protein changes its conformation to increase the number of hydrophobic amino acid residues in contact with the oil phase and reduces the free energy (Damodaran 1996). Unlike small emulsifiers that present only one or two highly hydrophobic binding sites made of the aliphatic chains, proteins have a large number of weak binding domains, i.e., the hydrophobic amino acids. As a consequence, small emulsifiers can be exchanged rapidly between the interface and the water phase, whereas proteins are more firmly attached to the interface and are more difficult to exchange with those that are nonadsorbed (McClements 1999). Interchange also depends on the age of the interface. The aptitude of proteins to interact and adsorb onto the interface depends on their amino acid composition and sequence. On the one hand, when a high proportion of hydrophobic amino acids is distributed along the polypeptide chain, proteins generally adopt a globular conformation with a compact structure. Further rearrangements require exposition of hydrophobic residues to water before direct contact with the oil phase can occur, making protein unfolding thermodynamically unfavorable. When adsorbed to an interface, globular proteins undergo some rearrangements but keep roughly their globular and compact form. On the other hand, when hydrophobic and hydrophilic residues are clustered in large domains as in caseins, proteins adsorb to the oil-water interface through their hydrophobic domains, whereas hydrophilic domains form tails or loops in the water phase (Damodaran 1996). Rearrangements of adsorbed proteins at the interface make various residues more accessible for chemical reactions. For instance, tryptophanyl residues can be displaced to a more hydrophobic environment upon their adsorption (Castelain and Genot 1994). Disulfide bonds and free thiol residues may also be more exposed. As the proteins become highly concentrated in the interface layer, intermolecular cross-linking through disulfide bond exchanges or formation of new intra- or intermolecular disulfide bonds is favored, leading to a progressive formation of a protein network at the interface (Monahan *et al.* 1993). This slow reaction takes place during emulsion aging and induces an increase of interfacial viscosity and of emulsion stability (Dickinson and Tanai 1992).

Proteins may be present either in solution or in a colloidal state. Food emulsions are generally produced with industrial protein powders, which have various degrees of heat denaturation and may be highly aggregated. Major milk proteins, such as caseins, are also in a micellar state. A part of these protein aggregates is adsorbed at the interface. As protein aggregates become relatively large in relation to the interface thick-

ness, they can be adsorbed simultaneously onto the interfaces of two or more oil droplets, leading to the formation of bridges between droplets and large droplet aggregates or flocs (Mulder and Walstra 1974). To minimize this effect, dairy manufacturers use double-stage homogenizers that break the flocs. Other protein aggregates remain in a colloid state in the water phase. These aggregates are large enough to induce depletion forces between globules and emulsion flocculation (Riaublanc *et al.* 2002). When the same proteins are both in the water phase and adsorbed at the interface, which is often observed in food products, a change in physicochemical conditions (e.g., pH, temperature) can lead to gel formation, with the droplets acting as active fillers (Chen and Dickinson 1998).

Possible Degradations of Proteins Induced by Oxidized Lipids

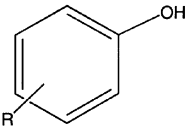
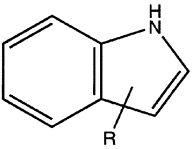
Considering the complexity of the problem, most of the studies related to protein modifications induced by oxidized lipids have been performed on very simple model systems. Because of the importance of a better understanding of the role of free radicals and oxidation *in vivo*, many studies have been performed on biological systems. In fact, only a few studies were dedicated to these phenomena in food or model emulsions. Nevertheless, the results obtained with model and biological systems are indicative of what can happen in emulsions.

Reaction of Proteins with Primary Products of Oxidation or with Oxidizing Lipids

According to Gardner (1979 and 1983), radical reactions of proteins promoted by lipid hydroperoxides can be divided into three main categories: (i) protein-protein or protein-lipid cross-linking; (ii) protein scission; and (iii) protein oxidation. The molecular bases of these damages were established on model systems involving single amino acids and peroxidized lipids. They involved free radical processes comprising H-abstraction followed by β-scission of amino acid oxy radical and possibly free radical addition. These reactions target the reactive groups listed in Table 9.1. Gardner (1979) stated that the most labile amino acids are histidine, cysteine, methionine, lysine, tyrosine, and tryptophan. The degradation of cysteine likely proceeds through the thiyl radical by H-abstraction from the thiol group (Gardner 1983). Further reaction of the $RS^{\bullet}$ can lead to the formation of a disulfide bond and thus, protein cross-links. The degradation of tryptophan involves the indole ring (Yong *et al.* 1980). Histidine reacts mainly through its imidazole side chain. For detailed reactions and mechanisms involving radical damages to amino acids, see the review of Gardner (1979).

Quantitative data concerning losses of amino acid residues in proteins exposed to peroxidized fatty acid or lipid oxidation products are reported in Table 9.2, which highlights the most sensitive amino acids, i.e. cysteine, histidine, lysine, and methionine. The extent of damage depends highly on protein type, oxidizing system, and

TABLE 9.1
Reactive Groups Involved in Chemical Reactions Between Oxidized Lipids and Proteins[a]

Oxidized lipid	Protein
Hydroperoxides: R-OOH	Primary amines: R-NH_2
Saturated aldehydes: R-CH_2-CHO	Secondary amines: R-NH-R′
Unsaturated aldehydes: R-CH=CH-CHO	Thiols: R-SH
	Sulfides: R-S-R′
	Disulfides: R-S-S-R′
	Phenols: 
	Indoles: 

[a]*Source:* Adapted from Pokorny 1977.

experimental conditions, making generalization of results difficult. Furthermore, the susceptibility of tryptophan to radical damage could not be determined in these papers because amino acid composition was generally determined after acid hydrolysis. Nevertheless, it is known that tryptophan is sensitive to oxidative degradation as demonstrated from its decrease in fluorescence upon exposure to oxidized lipids (Hidalgo and Kinsella 1989, Stapelfeldt and Skibsted 1994, Rampon *et al.* 2001 and 2002).

Reaction of Proteins with Secondary Products of Oxidation

Secondary products of oxidation is a generic term often used to describe a mixture of compounds obtained from the decomposition of lipid hydroperoxides. Some of these compounds react with proteins, but in peroxidized mixtures, it is difficult to assess the relative importance of secondary products compared with the primary lipid hydroperoxides (Gardner 1979). Among secondary products, aldehydes are the most reactive. The main targets of aldehydes are the sulfhydryl and amino groups (Alaiz and Giron 1994, Esterbauer *et al.* 1976). Reactions with amino groups, which form Schiff's base, concern both ε-NH_2 of lysyl residues and N-terminus residues.

Zhou and Decker (1999a and 1999b) studied the ability of various amino acids, dipeptides, polyamines, and sulfhydryls to bind hexanal and *trans*-2-hexenal. Among the amino acids tested, only histidine was able to reduce concentrations of hexanal in

TABLE 9.2
Damage to Amino Acids (AA) Exposed to Oxidized Lipids or Oxidation Products[a]

Protein or AA	Benzyl oxy amino acid	γ-Globulin	BSA	Ovalbumin	Ovalbumin	Casein	Lysozyme
Lipid substrate	LOOH	Peroxidizing ethyl arachidonate			Peroxidized ethyl linoleate		Hexanal
Reaction conditions	Met Hemoglobin 24 h, 37°C, pH 7.5	Aqueous solution 37°C			Aqueous solution 55°C, 24 h		Vaporized 50°C, 10 d
Reference	Kikugawa *et al.* 1991a	Roubal and Tapel 1966, cited by Gardner 1979			Horigone *et al.* 1974 cited by Gardner 1979		Kato *et al.* 1983
Ala	—[b]	—	50	—	8	9	—
Arg	2.9	—	—	—	—	8	—
Asp	—	—	—	—	—	8	—
Cys	—	33	64	—	—	—	—
Gly	—	—	83	28	—	—	—
His	13	52	54	38	—	—	—
Leu	—	—	—	—	8	—	—
Lys	12.9	59	59	—	9	10	27
Met	—	38	48	80	17	—	—
Phe	—	—	—	—	—	8	—
Pro	—	—	—	28	—	—	—
Ser	—	—	—	—	10	8	—
Thr	—	—	—	28	10	—	—
Trp	17.6	ND	ND	ND	ND	ND	ND
Tyr	5.7	51	—	—	—	—	—
Val	—	—	48	—	—	—	26

[a]BSA, bovine serum albumin; ND, not determined.
[b]Differences, if any, were considered to nonsignificant by the authors and therefore not reported in the referenced papers.

the headspace (Table 9.3). Histidine-containing dipeptides decreased headspace hexanal 3.0- to 8.5-fold more than histidine alone. This effect increased with the size of the aliphatic side group of the amino acid adjacent to histidine, and Leu-His had the greatest activity. The decrease of aldehyde headspace concentration by His-peptides was enhanced when *trans*-2-hexenal was tested, with β-Ala-His (carnosine) and Ile-His having the highest activities. The quenching of aldehydes was even more intense with sulfhydryls. The result with His-peptides can be attributed to the high reactivity of the *trans*-2-hexenal double bond with the amino-group of the imidazole ring of histidine. It also can explain a part of the antioxidant activity of the natural dipeptide, carnosine, under different conditions (Kansci *et al.* 1997).

Formation of Fluorescent Compounds

In addition to the protein damage mentioned above, formation of brown pigments, blue-fluorescence, and protein cross-linking due to reactions between oxidizing lipids and proteins have been demonstrated since the late 1960s (Chio and Tappel 1969a and 1969b, Fletcher and Tappel 1971, Fletcher *et al.* 1973, Kikugawa and Beppu 1987). Under conditions in which oxygen was not a limiting factor for oxidation, the development of fluorescence was linearly related to oxygen absorption (Biddlack and Tappel 1973, Dillard and Tappel 1973) and correlated with the decrease in diene conjugation (Shimasaki *et al.* 1977) and the increase in thiobarbituric acid reactive substances (Liang 1999). However, when available oxygen was

TABLE 9.3
Decrease in Headspace Concentration of Aldehydes After 1 Hour Incubation of Amino Compounds (5 mM) with 0.5 mM Aldehyde at pH 7.4 and 40°C[a]

		Aldehydes remaining in the headspace (%)	
Amino compound		Hexanal	t-2-hexenal
DL-Lysine		99.9	92.7
L-Histidine		97.7	91.7
β-Alanyl-histidine		96.8	56.0
γ-Aminobutyryl-histidine		100.0	77.5
Glycyl-histidine		93.0	75.5
Alanyl-histidine		91.6	77.0
Valyl-histidine		90.4	59.0
Leucyl-histidine		82.2	62.1
Isoleucyl-histidine		89.5	54.4
Spermine		95.2	80.4
Glutathione	1 mM	98.2	52.7
	5 mM	92.6	5.6
Thioctic acid	1 mM	99.6	24.5
	5 mM	96.2	2.8

[a]*Source:* Adapted from Zhou and Decker 1999a and 1999b.

the limiting factor for hydroperoxide formation, the amounts of conjugated dienes and hydroperoxides remained almost stable, whereas the fluorescence continued to increase steadily (Rampon *et al.* 2001 and 2002, Fig. 9.1).

Two major pathways of reaction of alkenals with proteins have been recognized (Amarnath *et al.* 1998). The first starts with the Michael addition of a protein nucleophile to the enals and with the imine formation (Schiff's base) between the aldehyde and a lysine (Stadtman and Berlett 1997). The significant aspect of the second pathway is the formation of 2-pentyl-pyrrole on the lysyl residues of protein (Sayre *et al.* 1993).

Fluorescent and Cross-Linked Proteins Derived from Lipid Hydroperoxides. The reaction between oxidizing lipids and proteins gives rise to lipid-soluble and water-soluble fluorescent chromophores (Bidlack and Tappel 1973, Dillard and Tappel 1973). The wavelengths of excitation and emission maxima of these pigments increased with increasing unsaturation of fatty acids. Kikugawa and Beppu (1987) reported that linoleic, linolenic, and arachidonic acids produced fluorescent substances with excitation maxima at 355–370 nm and emission maxima at 420–440 nm by reaction with methylamine. Table 9.4 summarizes the characteristics of the fluorophores formed by the reaction of amino compounds and oxidizing lipids, mainly peroxides. Excitation maxima ranged from 319 to 362 nm and emission maxima from 383 to 433 nm. Two groups can be distinguished. The first group corresponds to fluorophores having excitation maxima ranging from 340 to 350 nm and emission maxima ranging from 410

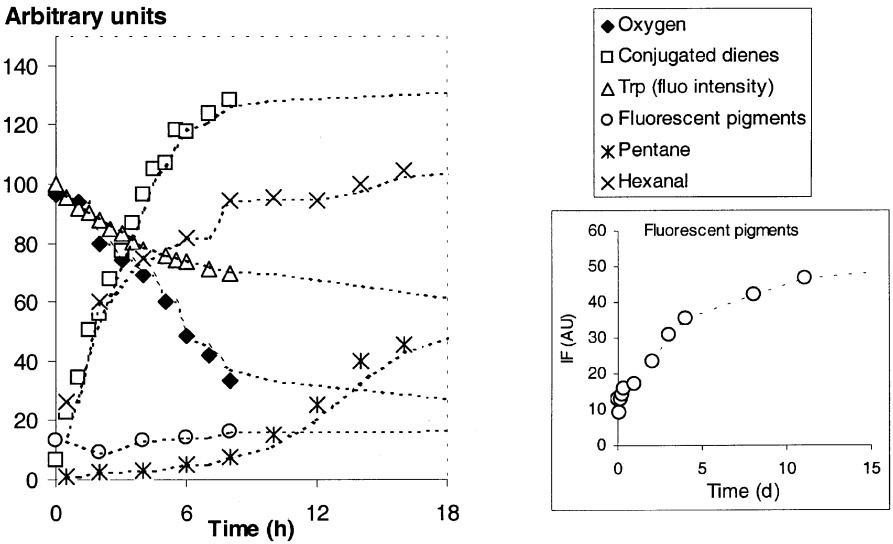


Fig. 9.1. Kinetics of oxidation in a sunflower oil-in-water emulsion followed by different methods and the formation of fluorescent compounds.

TABLE 9.4
Characteristics of Fluorophores Formed from the Reaction of Amino Compounds with Hydroperoxides and Oxidizing Lipids[a]

Excitation max (nm)	Emission max (nm)	Nature of amino compound	Nature of oxidizing lipids	Reference
348	420	1-Amino-pentane	Methyl linoleate hydroperoxides	Iio and Yoden 1988b
350	420	1-Amino-pentane	PLPC	Iio and Yoden 1988a
340–350	410–420	1-Amino-pentane	LLL	
319	383	Arginine	12-OOH	Fukuzawa *et al.* 1985
353	430	Arginine	LOOH/MetHemoglobin	Kikugawa *et al.* 1991b
342	414	Glycine	12-OOH	Fukuzawa *et al.* 1985
338	410	Histidine	Linoleic acid hydroperoxides	Shimasaki *et al.* 1982
360	432	Histidine	LOOH/MetHemoglobin	Kikugawa *et al.* 1991b
339	413	Histidine	12-OOH	Fukuzawa *et al.* 1985
360	430	Lysine	Linoleic acid hydroperoxides	Shimasaki *et al.* 1982
358	432	Lysine	LOOH/MetHemoglobin	Kikugawa *et al.* 1991b
349	419	Lysine	12-OOH	Fukuzawa *et al.* 1985
362	433	Nα-Z-Lysine	LOOH	Yamaki *et al.* 1992
361	433	Nα-Z-Lysine	Methyl LOOH	Yamaki *et al.* 1992
362	432	Nα-Z-Lysine	PCOOH	Yamaki *et al.* 1992
334	408	Polylysine	12-OOH	Fukuzawa *et al.* 1985
347	425	Polylysine	13-LOOH	Kikugawa *et al.* 1985
350–360	420–430	β-Lactoglobulin	13-LOOH	Hidalgo and Kinsella 1989
350	435	BSA	Oxidizing methyl linoleate	Fletcher and Tappel 1971
360	430	BSA	Linoleic acid hydroperoxides	Shimasaki *et al.* 1982
350	420	BSA	12-OOH	Fukuzawa *et al.* 1985
355	416	BSA	Oxidizing sunflower oil	Rampon *et al.* 2001
350	440	Milk	Milk fat globule membrane	Bouzas *et al.* 1985

[a]Abbreviations: PLPC, 1-palmitoyl-2-linoleoyl-phosphatidylcholine; LLL, trilinolein; BSA, bovine serum albumin.

to 420 nm; this group includes mainly simple amino compounds such as 1-amino-pentane and free amino acids. The second group corresponds to compounds with excitation maxima located in the 350–360 nm range and emission maxima between 420 and 430 nm. It includes mainly proteins and polypeptides.

Fluorescent and Cross-Linked Proteins Derived from Aldehydes. Malondialdehyde (MDA), which is a typical secondary oxidation product of unsaturated fatty acids with more than two double bonds, is also capable of producing fluorescence by cross-linking to proteins. The spectral characteristics of the fluorescent pigments differ significantly from those measured with hydroperoxides and oxidizing lipids, with higher excitation and emission maximum wavelengths.

Aldehydes other than MDA can also contribute to the formation of blue fluorescence due to their reaction with proteins (Kikugawa and Beppu 1987, Kikugawa *et al.* 1991a and 1991b). Characteristics of fluorophores formed by reaction of proteins with aldehydes are summarized in Table 9.5. Excitation maxima ranged from 372 to 387 nm for 2-alkenals, whereas they varied from 340 to 355 nm for alkanals. The emission wavelength depends on the nature of aldehyde and amino compounds. The maxima are generally shifted to higher wavelengths with 2-alkenals compared with alkanals. These differences in excitation and emission spectra suggest that the structure of the fluorophore depends on the reacting aldehyde. For example, 2-alkenals can react with the indole ring of histidine, whereas this reaction cannot take place with alkanals.

Mechanisms and Pathways of Reactions

Several pathways of reaction of aldehydes with protein side chains have been suggested (Figs. 9.2–9.6). For a long time, many food scientists thought that only

TABLE 9.5
Characteristics of Fluorophores Formed from the Reaction of Amino Compounds with Secondary Lipid Oxidation Products[a]

Excitation max (nm)	Emission max (nm)	Nature of amino compound	Nature of oxidizing lipids	Reference
340	416	1-Amino-pentane	2,4-Decadienal	Iio and Yoden 1988b
344	408	1-Amino-pentane	2-Hexenal	Iio and Yoden 1988b
366	442	1-Amino-pentane	Hexanal	Iio and Yoden 1988b
340	411	Glycine	Hexanal	Fukuzawa *et al.* 1985
326	411	Glycine	2-Hexenal	Fukuzawa *et al.* 1985
348	416	Nα-Z-Lysine	Hexanal	Yamaki *et al.* 1992
375	453	Nα-Z-Lysine	2-Hexenal	Yamaki e*et al.* 1992
380	434	Nα-Z-Lysine	2,4-Hexadienal	Yamaki *et al.* 1992
372	453	Nα-Z-Lysine	2-Octenal	Yamaki *et al.* 1992
370	450	Polylysine	MDA	Kikugawa *et al.* 1985
357	430	Polylysine	Acetaldehyde	Kikugawa *et al.* 1985
340	417	Polylysine	Hexanal	Kikugawa *et al.* 1985
350	410	β-Lactoglobulin	Pentanal, hexanal, heptanal	Stapelfeldt and Skibsted 1994
374	442	BSA	2-Hexenal	Yamaki *et al.* 1992
387	445	BSA	2-Hexenal	Inoue and Kikugawa 1998
375	434	BSA	2-Octenal	Yamaki *et al.* 1992
350	440	BSA	2-Octenal	Alaiz and Barragan 1995
382	401	BSA	Hydroxyhexenal	Inoue and Kikugawa 1998
385	443	BSA	2-Nonenal	Inoue and Kikugawa 1998
360	425	BSA	Hydroxynonenal	Inoue and Kikugawa 1998
360	435	BSA	2,4-Hexadienal	Fletcher and Tappel 1971
355	425	Lysozyme	Hexanal	Tashiro *et al.* 1985

[a]Abbreviations: MDA, malondialdehyde; BSA, bovine serum albumin.

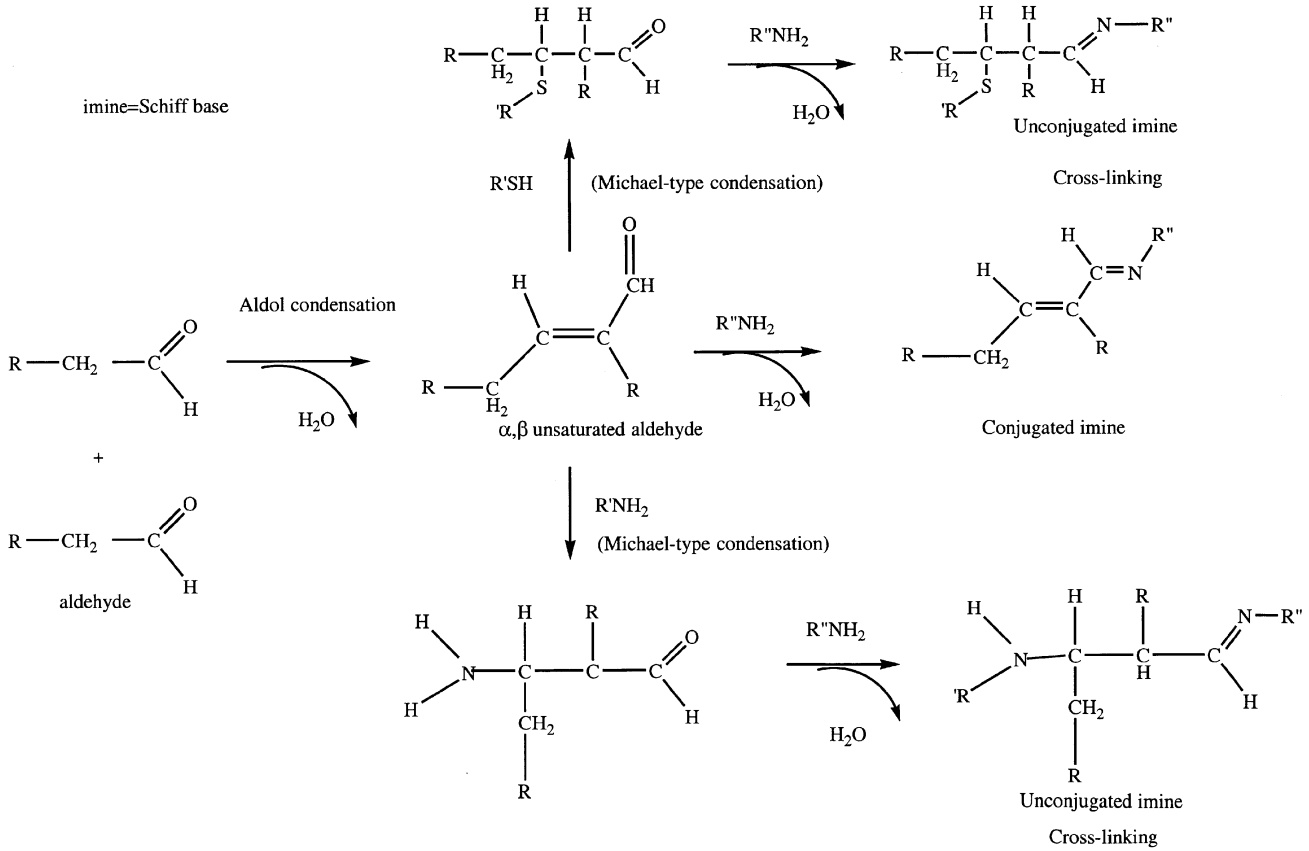


Fig. 9.2. Possible reactions involved in cross-linking of proteins with aldehydes (adapted from Stapelfeldt 2001).

monofunctional aldehydes were able to modify the side chain of amino acids and especially of lysine.

However, α,β-unsaturated aldehydes, which are produced from the aldol condensation of monofunctional aldehydes, react through several pathways. These compounds may react with the nucleophilic side chains of numerous amino acids, giving rise to intramolecular or intermolecular cross-linking as shown in Figure 9.2 (Stapelfeldt 2001). The latter gives rise to the formation of protein polymers.

Another type of reaction concerns the reaction of unsaturated aldehydes with the imidazole ring of histidine through their α-double bond (Alaiz and Giron 1994; Fig. 9.3). Baker *et al.* (1998) studied the reaction of 2-alkenals with *N*-acetyl-glycyl-methyl-lysine. Apart from Schiff's base and Michael addition compounds, an unexpected complex mixture of reaction products was formed. On the basis of nuclear magnetic resonance and mass spectrometry, up to seven compounds were identified (Fig. 9.4). The stoichiometry of the reaction varied from one product to another. For example, compounds I to IV were formed by reaction of one peptide with two aldehydes, compounds V and VI by reaction of two peptides and three aldehydes, and finally compound VII was the result of the reaction of one peptide with three aldehydes (Baker *et al.* 1998). Alkenals are also able to react with cysteyl, histidyl, and lysyl residues of proteins. The different products of reaction of 4-hydroxy-2-nonenal with apomyoglobin at pH 4.4 are shown in Figure 9.5 (Bolgar and Gaskell 1996).

Malondialdehyde is also able to react with various primary amines. Two models have been proposed for the reaction scheme and structure of the fluorescent pigments resulting from the reaction of MDA with proteins: conjugated Schiff's bases and 1,4-dihydropyridine 3,5-dicarbaldehyde derivatives (Fig. 9.6). The last pigment possesses excitation and emission wavelengths at 398 and 470 nm, respectively (Kikugawa and Beppu 1987). This pigment was formed during reaction with proteins under physiologic conditions, after treatment of polylysine with excess MDA at pH 7

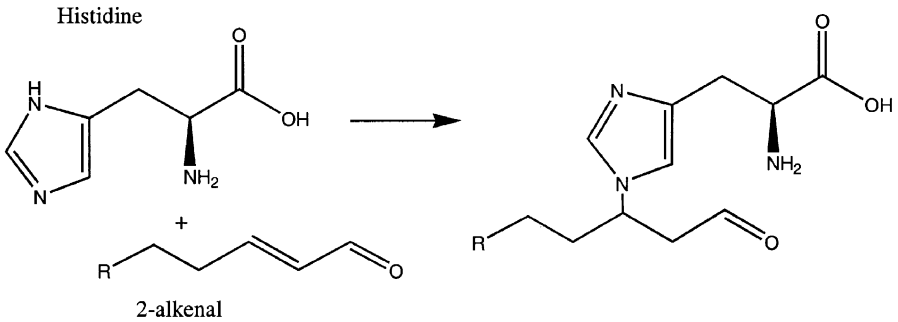


Fig. 9.3. Reaction of 2-alkenals with histidine (adapted from Alaiz and Giron 1994).

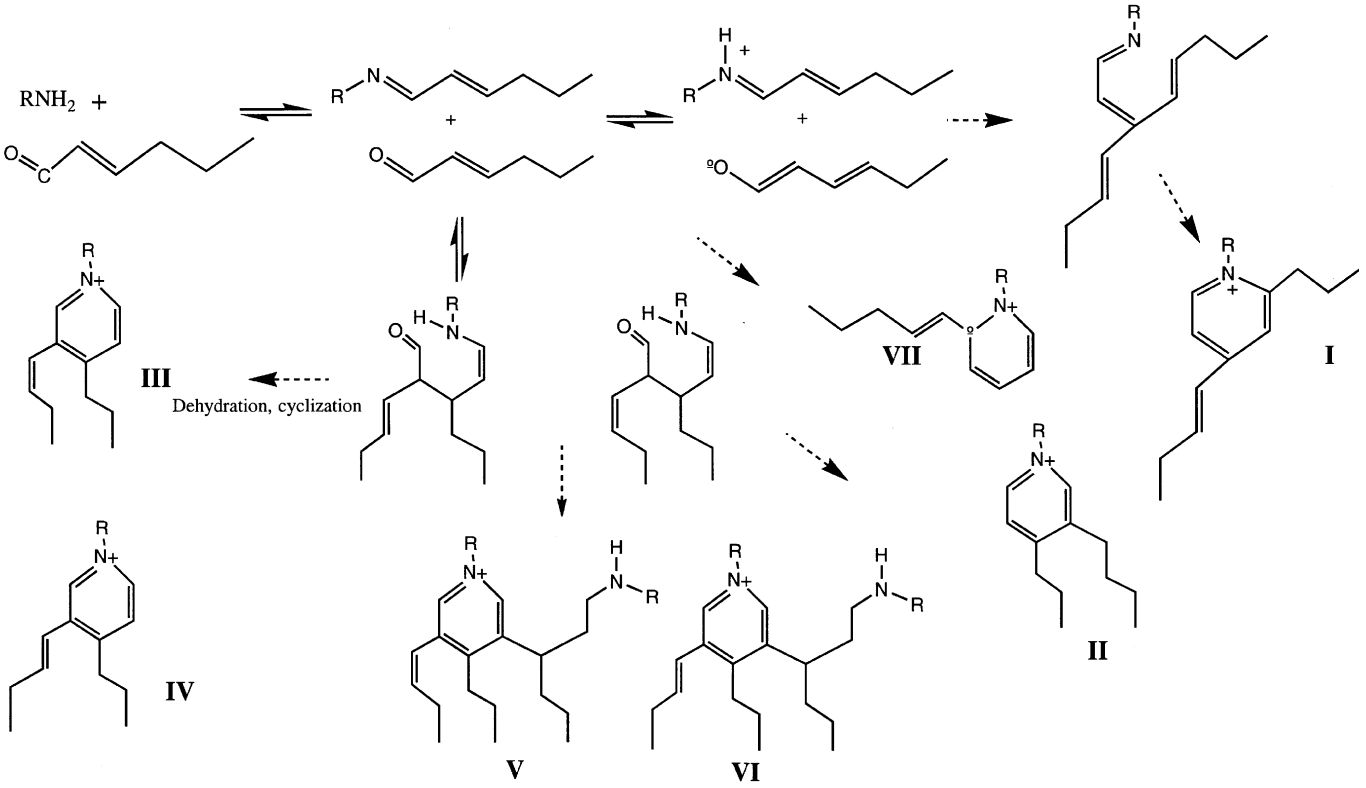


Fig. 9.4. Proposed reaction mechanism leading to the formation of pyridimium derivatives from lysine-containing peptides with 2-alkenals (from Baker *et al.* 1998).

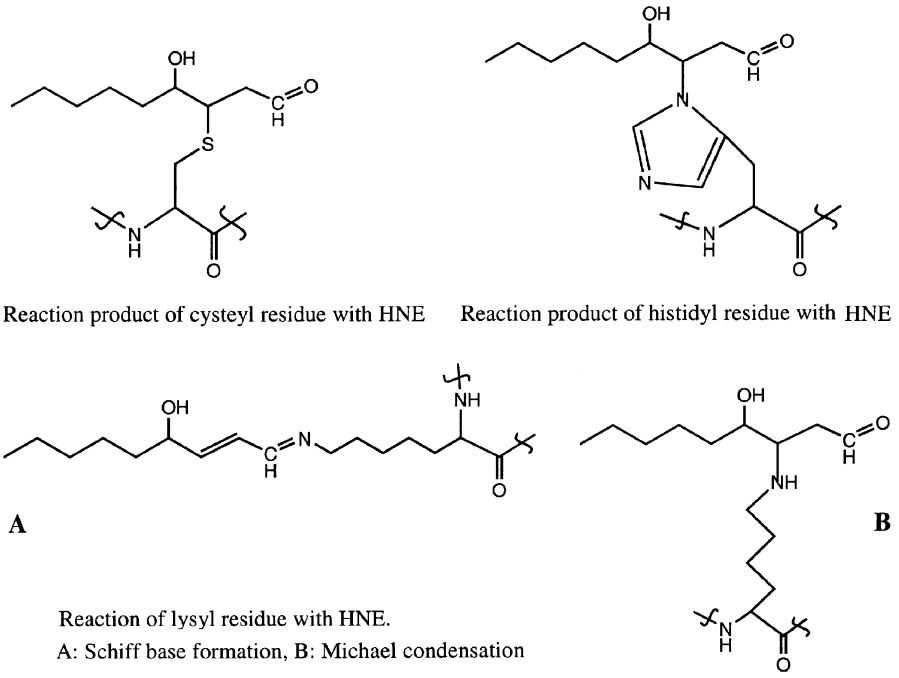


Fig. 9.5. Literature proposed structures of amino acid residues modified by reaction with 4-hydroxy-2-nonenal (HNE) (adapted from Bolgar and Gaskell 1996).

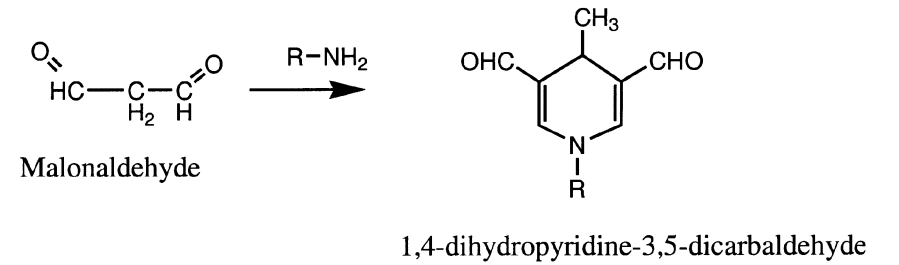


Fig. 9.6. Reaction of primary amine with malonaldehyde (adapted from Kikugawa 1991).

(Kikugawa and Beppu 1987) and in a model system containing simple amines such as methylamine (Kikugawa 1991). Baker *et al.* (1998) confirmed the formation of this derivative.

Protein Alteration Due to Lipid Oxidation in Emulsions

Protein alteration can take place even during the early stages of lipid oxidation. Modifications in the thermal stability of protein components of low density

lipoproteins (LDL) occurred from the end of the lag phase, when antioxidants were consumed but only minor amounts of lipid peroxidation products could be detected (Prassl *et al.* 1998). The alteration was similar whether the initiating agent was located in the lipid core of the lipoprotein or in the aqueous phase. It could involve unfolding of some regions of the apoprotein and lipid-protein interactions. Exposure of an initially buried hydrophobic region of the polypeptide chain to the aqueous environment was assumed to render it more accessible to oxidative attack, favoring modifications of its primary structure through reactions with peroxyl and alkoxyl radicals. Other modifications may also target the soluble proteins when water-soluble initiators are involved (Dean *et al.* 1991). Therefore, the use of global techniques to quantify total amino acid losses in the oxidizing emulsions may be extremely limited in evaluating these early and selective modifications.

To our knowledge, very few studies have been performed to evaluate protein modifications during lipid oxidation in real food emulsions and their consequences on emulsion physicochemical properties. Early modifications of proteins in oxidizing emulsions have not yet been demonstrated as in lipoproteins. Indeed, the protein modifications during the early stages of oxidation may be limited and involve specific targets, such as the loops of adsorbed proteins directly in contact with the oxidizing lipids or the sites of free radical formation.

The fluorescence intensity of the tryptophanyl residues of bovine serum albumin was shown to decrease progressively during oxidation of sunflower oil emulsions stabilized by the protein. The shape of the curves and rates of decrease depended on both the temperature and the size of the oil droplets (Rampon *et al.* 2001 and 2002). It is noteworthy that the fastest decrease in fluorescence was observed with small size droplet emulsions during the initial step of lipid oxidation, parallel to a rapid formation of primary products of lipid oxidation (Fig. 9.1). During the later steps of oxidation, when amounts of primary products of oxidation remained steady, the decrease in fluorescence slowed but remained greater in emulsions with small droplet size than in larger ones. Deconvolution of fluorescence spectra using fourth derivative calculations showed that the tryptophanyl residues of the protein, adsorbed at the interface but placed in a hydrophilic environment, were preferentially altered during both the initial and later stages of oxidation (Fig. 9.7). Covalent modifications of the tryptophanyl residues were probably involved, but other mechanisms leading to fluorescence quenching cannot be excluded.

The formation of fluorescent products due to reaction between protein and oxidizing lipids is also directly related to the droplet size of the emulsions. The smaller the droplets, the larger the interface and the amount of adsorbed protein, and the intensity resulting from covalent products of reaction (Rampon *et al.* 2001 and 2002). Contrary to the formation of primary products and the decrease of tryptophan fluorescence, a lag phase was observed for the appearance of these fluorescent products. Their formation continued during the later stages of long-term storage, when oxygen was no longer available in the system (Fig. 9.1). This allows the conclusion that the fluorescent compounds formed during reactions between pro-

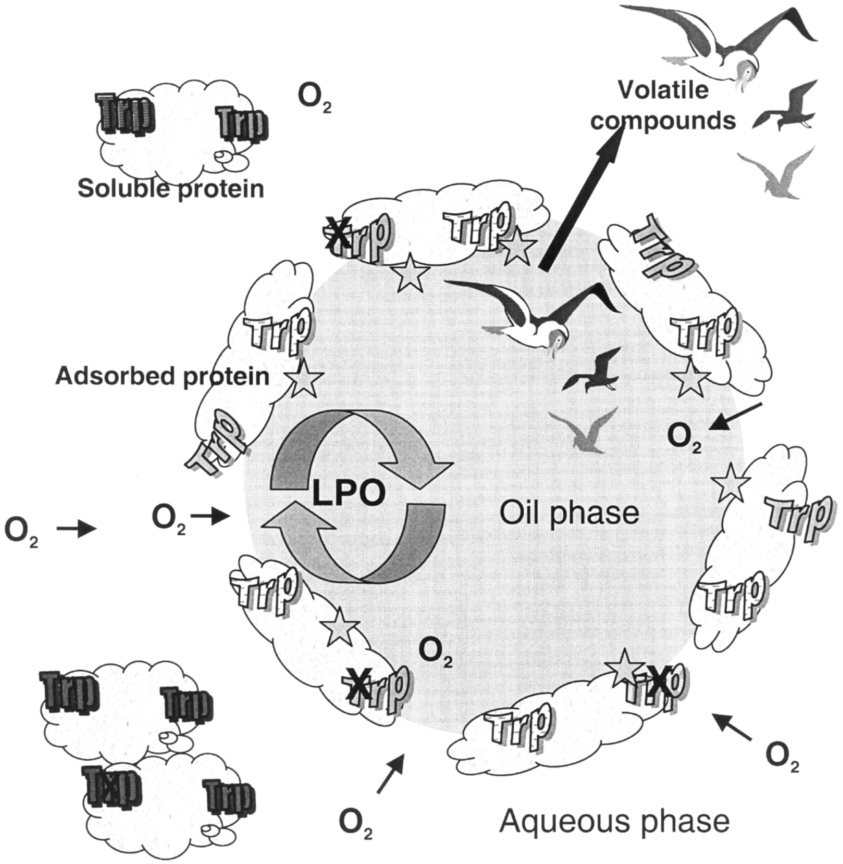


Fig. 9.7. Schematic representation of protein alteration during lipid oxidation in an oil-in-water emulsion stabilized by proteins.

teins and oxidizing lipids are produced during the later stages of oxidation and not during the initiation and propagation phases. These changes are likely accompanied by protein cross-linking involving either intermolecular bonds or covalent reactions with oxidized lipids. Accordingly, when sunflower oil emulsions stabilized by lecithin and bovine serum albumin were stored in the dark for 7 d at room temperature (18–22°C), significant losses of thiol groups of the protein accompanied by formation of protein polymers were observed (Genot *et al.* 1990). These polymers are made of dimers and trimers, but also of higher-degree polymers due to both aggregation phenomena and formation of intermolecular disulfide bonds. Such intermolecular disulfide bonds probably resulted from changes in the protein conformation at the interface (Monahan *et al.* 1993) favored by the presence of phospholipids. Similar results were also observed with whey proteins (Genot *et al.*,

unpublished results). However, the structure of the emulsions (droplet size distribution) and their physical stability were not controlled in these studies, and the difference in the behavior of the adsorbed and nonadsorbed proteins was not evaluated. Some losses of basic amino acids (lysine + histidine + arginine) were also measured, but they were similar in control protein and emulsion (Genot *et al.* 1990). Protein changes may arise during aging of pure protein solutions even in the absence of lipid and lipid oxidation phenomena. These changes, which comprise losses in labile groups, aggregation, and polymerization of proteins are not within the scope of this review.

Changes affecting proteins adsorbed at the interface of emulsions due to lipid oxidation during ageing were also studied by reversed-phase high-performance liquid chromatographic analysis of β-lactoglobulin, β-casein and caseinate and their tryptic hydrolysates after their competitive displacement from the interface by Tween 20 (Leaver *et al.* 1999a, 1999b, and 1999c, Stevenson *et al.* 1997). Emulsions made of oxidizable and unoxidizable apolar phases were also compared. Displacement of the proteins in the emulsions by the nonionic surfactant was dependent on the age of the emulsion, with the protein being displaced more easily from freshly made samples (Stevenson *et al.* 1997). Although lipid oxidation was not measured in these studies, it clearly appeared to be the major cause of the observed phenomena from the composition of the volatiles extracted from the aged emulsions (Leaver *et al.* 1999b). In addition to the overall decrease in the likelihood of protein being displaced by a surfactant, its covalent modification was demonstrated by a 300-Da increase in molecular mass. This modification probably involved attachment to an α,β-unsaturated aldehyde. The great importance of the amount of protein adsorbed at the interface and of the droplet size of the emulsions was underlined in these studies.

Antioxidant and Prooxidant Activities of Proteins in Emulsions

In emulsions, proteins are either adsorbed at the interface and participate in the physical stabilization of the emulsions or they are present in the aqueous phase as individual molecules, micelles, or aggregates (Fig. 9.7). They participate in the chemical stability of emulsions in a variety of ways. Some proteins have prooxidant activities. In milk and dairy products, the metalloenzymes, xanthine oxidase and lactoperoxidase, may induce oxidation in the presence of small amounts of divalent copper ion (Allen 1994, Bradley and Min 1992). Lactoferrin can be prooxidant in the presence of excess iron (Huang *et al.* 1999). In meat emulsions and related models, the catalytic activity of heme proteins (myoglobin, hemoglobin) may become prominent compared with metal-catalyzed oxidation (Ahn and Kim 1998a, Allen 1994, Chan *et al.* 1997, Verma *et al.* 1985). Lactoferrin is also prooxidant in the presence of excess iron and reductants (Ahn and Kim 1998b).

Meyer and Isaksen (1995) reviewed the potential application of enzymes as antioxidants in foods. The review highlighted that in addition to difficulties linked

to legislative rules, the main problem for using antioxidant enzymes in emulsions is their loss of activity upon denaturation when adsorbed at the oil-water interfaces. Apart from antioxidant activities due to enzymatic mechanisms, proteins exhibit antioxidant activities through various other mechanisms (Kansci 1996, Shahidi 1997). These activities have been demonstrated in various biological and model systems. Proteins have the following capabilities: (i) scavenging free radicals due to the ability of amino acid side chains to provide hydrogen; (ii) chelating metal ions, making them unable to initiate oxidation or to decompose hydroperoxides; (iii) reacting with primary and secondary products of oxidation, making them inactive; (iv) binding antioxidants, such as phenolic compounds, and possibly affect their antioxidant or prooxidant activities; (v) altering the interfacial characteristics, such as charge, thickness, density, thus giving a physical protection against the access of aqueous prooxidants and oxygen to the oxidizable substrates or facilitating their activities; and (vi) interacting specifically with various ligands (i.e., free fatty acids, volatile compounds), making some substrates inaccessible to initiators of oxidation and/or modifying partition and release of oxidized flavors.

It should be underscored that while reacting with lipid intermediates, proteins may be modified to function as "suicide" sinks. This may have a number of consequences for the nutritional and functional properties of the products. The mechanisms involved in covalent modification of proteins during lipid oxidation was discussed above. Another mechanism for antioxidant activity of protein was proposed recently to explain the protective effect of pulmonary surfactant proteins. The proteins could interfere directly with lipid oxidation by inhibiting the formation of lipid radicals or by acting as free radical chain terminators through a catalytic mechanism (Bridges *et al.* 2000). These antioxidant activities of proteins depend on many factors, such as pH and presence of metal ions (see Chapter 7 of this book). The presence of low-molecular-mass surfactants may also have an effect because they can displace adsorbed proteins or modify their structures and interactions (Courthaudon *et al.* 1991, Dalgleish *et al.* 1995).

Only a few studies have been performed on true oil-in-water emulsions, generally on dairy and egg proteins. These studies were performed either with purified native proteins, industrial protein concentrates of more complex composition, or chemically modified proteins. The relationship between proteins and other constituents (surfactants or phenolic antioxidants) in modifying the time course of lipid oxidation has been investigated in only a few cases. Egg yolk high density lipoproteins (EY-HDL) were found more effective than EY-LDL at inhibiting iron-induced oxidation of emulsified linoleic acid (Yamamoto and Omori 1994). Egg yolk phosvitin inhibited metal-induced oxidation of phospholipid aqueous dispersions (Lu and Baker 1987). Chelation of the iron ions by the phosphoseryl residues of EY-HDL and phosvitin could be involved in this activity. Changes in the structure of egg yolk lipoprotein and phosvitin at the interface of the oil droplets were assumed to be involved in the release of iron and enhanced formation of free radicals and volatiles in fish oil–enriched mayonnaise (Jacobsen *et al.* 1999 and 2001).

Within milk proteins, caseins and to a lesser extent α-lactalbumin were found to inhibit copper-induced oxidation of trilinolein or sunflower oil emulsions stabilized by lysophosphatidyl choline (Allen and Wrieden 1982). Sodium caseinate was also found to be the emulsifier that offered the best protection against methyl linoleate oxidation compared with egg albumin and Tween 20 (Fujii *et al.* 1995). The high-molecular-mass fraction of whey proteins exhibited antioxidant properties in Tween-stabilized fish oil emulsions by the intervention of sulfhydryl and/or iron chelation (Tong *et al.* 2000). This confirms previous studies showing that sulfhydryls of proteins were involved in protecting linoleate emulsions against oxidation induced by hemoglobin (Taylor and Richardson 1980a and 1980b). When whey protein isolates, stabilizing the oil droplets of fish oil emulsions, were displaced from the interface by Tween 20, the oxidation rate increased significantly, suggesting that the adsorbed protein probably afforded some physical protection (Donnelly *et al.* 1998). This protection was the highest when the pH of the emulsion was below the isoelectric point of the proteins probably because under these conditions, the interfaces are positively charged and metal ions are repulsed, depressing their efficiency to initiate oxidation and decompose hydroperoxides. The protein could be either prooxidative or antioxidative when added to stabilized emulsions, depending on the concentrations of surfactant (Tween 20) and protein added to the aqueous phase. The protective effect against oxidation of proteins and protein hydrolysates stabilizing docosahexaenoic acid (DHA)-containing triacylglyceride aqueous dispersions was assumed to involve a barrier effect at the interface (Hirose and Miyashita 1999). Soybean protein hydrolysates afforded the highest protection compared with soybean protein, albumin, and casein; these last two proteins were inefficient with the most unsaturated triacylglycerides, which contained 40% DHA. Lactoferrin, the milk iron-binding glycoprotein, binds ferric iron very tightly. At physiological concentrations, it protected emulsified lipids from iron-induced peroxidation. However, it exerted prooxidant activity in the presence of high concentrations of metal ions, at pH <5.6 or in the presence of reductants such as ascorbate (Huang *et al.* 1999).

The simultaneous presence of proteins and antioxidants, such as phenolic compounds or tocopherol, in multiphase systems can modify their respective influence toward lipid oxidation. In corn oil/lecithin emulsions (pH 6.6), phenolic compounds and α-tocopherol reinforced the antioxidant activity of lactoferrin (Huang *et al.* 1999, Medina *et al.* 2002). Bicarbonate also increased the antioxidant activity of the protein. The synergistic effect of phenolic compounds, lower in emulsions than in liposomes, was attributed to a protection of phenols by the protein. Bovine serum albumin enhanced the antioxidant activities of some phenolic compounds in liposomes, but tended to promote prooxidant effects of others (Heinonen *et al.* 1998). This enhancement could result from the capacity of the protein to selectively bind certain low-molecular-mass phenols (Bartolomé *et al.* 2000). β-Lactoglobulin markedly improved the antioxidant activity of α-tocopherol and Trolox in linoleic acid emulsions stabilized by Tween 20 (Yamamoto *et al.*, 1998). This was attributed to the

formation of a linoleic acid/β-lactoglobulin complex at the interface that would be efficiently protected by the antioxidants.

The presence of other constituents, such as polysaccharides, can also influence the antioxidant activities of proteins. When dextrin was present in the aqueous phase, the inhibition of oxidation by casein and ovalbumin was strongly enhanced (Fujii *et al.* 1995). The antioxidant potential of ovalbumin was also improved when the protein was modified by covalent binding with dextran or galactomannan (Nakamura *et al.* 1992); however, the modified protein was not tested in "true" emulsions.

Conclusion

A number of pathways for chemical reactions involving oxidized or oxidizing lipid and proteins have been proposed and evidenced in model systems. However, their actual occurrence in biological compartments and formulated foods remains unclear. Data are lacking to really appreciate the consequences of protein modifications resulting from lipid oxidation in emulsions and more complex systems. For instance, the consequences on droplet aggregation, emulsion physical stability, partition and release of volatiles, and on the flavor of the products have not yet been elucidated. The influence of proteins in the development of lipid oxidation is also poorly understood. Their efficiency to protect, or, in some cases, to enhance lipid oxidation, as well as their influence on the activity of antioxidants in different systems warrants further research.

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References

Ahn, D.U., and Kim, S.M. (1998a) Prooxidant Effects of Ferrous Iron, Hemoglobin, and Ferritin in Oil Emulsion and Cooked-Meat Homogenates Are Different from Those in Raw-Meat Homogenates, *Poult. Sci. 77*, 348–355.

Ahn, D.U., and Kim, S.M. (1998b) Effect of Superoxide and Superoxide-Generating Systems on the Prooxidant Effect of Iron Emulsion and Raw Turkey Homogenates, *Poult. Sci. 77*, 1428–1435.

Alaiz, M., and Barragan, S. (1995) Changes Induced in Bovine Serum Albumin Following Interactions with the Lipid Peroxidation Product *E*-2-Octenal, *Chem. Phys. Lipids 77*, 217–223.

Alaiz, M., and Giron, J. (1994) Modification of Histidine Residues in Bovine Serum Albumin by Reaction with (*E*)-2-Octenal, *J. Agric. Food Chem. 42*, 2094–2098.

Allen, J.C. (1994) Rancidity in Dairy Products, in *Rancidity in Foods* (Allen, J.C. and Hamilton, R.J., eds.) pp. 179–190, Blackie Academic and Professional, London.

Allen, J.C., and Wrieden, W.L. (1982) Influence of Milk Proteins on Lipid Oxidation in Aqueous Emulsion. I. Casein, Whey Protein and α-Lactalbumin, *J. Dairy Res. 79*, 239–248.

Amarnath, V., Valentine, W.M., Montine, T.J., Patterson, W.H., Amarnath, K., Basset, C.N., and Graham, D.G. (1998) Reactions of 4-Hydroxy-2(*E*)-Nonenal and Related Aldehydes with Proteins Studied by Carbon-13 Nuclear Magnetic Resonance Spectroscopy, *Chem. Res. Toxicol. 11*, 317–328.

Baker, A., Zidek, L., Wiessler, D., Chmelik, J., Pagel, M., and Novotny, M.V. (1998) Reaction of *N*-Acetylglycyllysine Methyl Ester with 2-Alkenals: An Alternative Model for Covalent Modification of Proteins, *Chem. Res. Toxicol. 11*, 730–740.

Bartolomé, B., Estrella, I., and Hernandez, M.T. (2000) Interaction of Low Molecular Weight Phenolics with Proteins (BSA), *J. Food Sci. 65*, 617–621.

Biddlack, W.R., and Tappel, A.L. (1973) Fluorescent Products of Phospholipids During Lipid Peroxidation, *Lipids 8*, 203–207.

Birkbeck College (1996) http://pps01–1.cryst.bbk.ac.uk

Bolgar, M.S., and Gaskell, S.J. (1996) Determination of the Sites of 4-Hydroxy-2-Nonenal Adduction to Protein by Electrospray Tandem Mass Spectrometry, *Anal. Chem. 68*, 2325–2330.

Bouzas, J., Kamarei, A.R., and Karel, M. (1985) Effect of Extraction Procedures on Fluorescent Chromophores in Milk, *J. Food Sci. 50*, 1515–1516.

Bradley, D.G., and Min, D.B. (1992) Singlet Oxygen Oxidation of Foods, *Crit. Rev. Food Sci. Nutr. 31*, 211–236.

Bridges, J.P., Davis, H.W., Damodarasamy, M., Kuroki, Y., Howles, G., Hui, D.Y., and McCormack, F.X. (2000) Pulmonary Surfactant Proteins A and D Are Potent Endogenous Inhibitors of Lipid Peroxidation and Oxidative Cellular Injury, *J. Biol. Chem. 275*, 38848–38855.

Castelain, C., and Genot, C. (1994) Conformational Changes of Bovine Serum Albumin upon Its Adsorption in Dodecane-in-Water Emulsions as Revealed by Front-Face Steady-State Fluorescence, *Biochim. Biophys. Acta 1199*, 59–64.

Chan, W.K.M., Faustman, C., and Renerre, M. (1997) Model Systems for Studying Pigment and Lipid Oxidation Relevant to Muscle-Based Foods, in *Natural Antioxidants, Chemistry, Health Effects, and Applications* (Shahidi, F., ed.) pp. 319–330, AOCS Press, Champaign, IL.

Chen, J., and Dickinson, E. (1998) Heat-Set Whey Protein Emulsion Gels, *J. Texture Stud. 29*, 285–304.

Chio, K.S., and Tappel, A.L. (1969a) Synthesis and Characterization of the Fluorescent Products Derived from Malonaldehyde and Amino Acids, *Lipids 8*, 2821–2827.

Chio, K.S., and Tappel, A.L. (1969b) Inactivation of Ribonuclease and Other Enzymes by Peroxidizing Lipids and by Malonaldehyde, *Lipids 8*, 2827–2832.

Chothia, C. (1984) Principles That Determine the Structure of Proteins, *Annu. Rev. Biochem. 53*, 537–572.

Courthaudon, J.-L., Dickinson, E., and Dalgleish, D.G. (1991) Competitive Adsorption of β-Casein and Nonionic Surfactants in Oil-in-Water Emulsions, *J. Colloids Interface Sci. 145*, 390–395.

Dalgleish, D.G., Srinivasan, M., and Singh, H. (1995) Surface Properties of Oil-in-Water Emulsion Droplets Containing Casein and Tween 60, *J. Agric. Food Chem. 43*, 2351–2355.

Damodaran, S. (1996) Amino Acids, Peptides, and Proteins, in *Food Chemistry* (Fennema, O.R., ed.) pp. 321–429, Marcel Dekker, New York.

Dean, R.T., Hunt, J.V., Grant, A.J., Yamamoto, Y., and Niki, E. (1991) Free Radical Damage to Proteins: The Influence of The Relative Localization of Radical Generation, Antioxidants, and Target Proteins, *Free Radic. Biol. Med. 11*, 161–168.

Dickinson, E., and Tanai, S. (1992) Temperature Dependence of the Competitive Displacement of Protein from the Emulsion Droplet Surface by Surfactants, *Food Hydrocolloids 6*, 163–171.

Dillard, C.J., and Tappel, A.L. (1973) Fluorescent Products from Reaction of Peroxidizing Polyunsaturated Fatty Acids with Phosphatidylethanolamine and Phenylalanine, *Lipids 8*, 183–189.

Donnelly, J.L., Decker, E.A., and McClements, D.J. (1998) Iron-Catalysed Oxidation of Menhaden Oil as Affected by Emulsifiers, *J. Food Sci. 63*, 997–1000.

Doolittle, R.F. (1985) Proteins, *Sci. Am. 253*, 88–99.

Esterbauer, H., Ertl, A., and Scholz, N. (1976) The Reaction of Cysteine with α,β-Unsaturated Carbonyls, *Tetrahedron 32*, 285–289.

Fletcher, B.L., Dillard, C.J., and Tappel, A.L. (1973) Measurement of Fluorescence Lipid Peroxidation Products in Biological Systems and Tissues, *Anal. Biochem. 52*, 1–9.

Fletcher, B.L., and Tappel, A.L. (1971) Fluorescent Modification of Serum Albumin by Lipid Peroxidation, *Lipids 6*, 172–175.

Fujii, N., Hamano, M., and Yuasa, K. (1995) Inhibition of the Autoxidation of Methyl Linoleate by Protein and Highly Concentrated Dextrin in an Emulsion System, *Biosci. Biotechnol. Biochem. 59*, 1761–1763.

Fukuzawa, K., Kishikawa, K., Tokumura, A., Tsukatani, H., and Shibuya, M. (1985) Fluorescent Pigments by Covalent Binding of Lipid Peroxidation By-Products to Protein and Amino Acids, *Lipids 20*, 854–861.

Gardner, H.W. (1979) Lipid Hydroperoxide Reactivity with Proteins and Amino Acids: A Review, *J. Agric. Food Chem. 27*, 220–229.

Gardner, H.W. (1983) Effects of Lipid Hydroperoxides on Food Components, in *Xenobiotics in Foods and Feeds* (Finley, J.W., and Schwess, D.E., eds.) pp. 63–84, A.C.S. Symposium Series, New York.

Genot, C., Gandemer, G., Paternoster, D., and Marion, D. (1990) Etude en Système Modèle des Modifications Biochimiques de la Sérum Albumine Bovine en Présence de Lipides Faiblement Oxydés, *Sci. Aliment. 10*, 403–415.

Goldberg, M.E. (1985) The Second Translation of the Genetic Message: Protein Folding and Assembly, *Trends Biochem. Sci. 10*, 388–391.

Harrison, S.C., and Durbin, R. (1985) Is There a Single Pathway for the Folding of a Polypeptide Chain? *Proc. Nat. Acad. Sci. USA 82*, 4028–4030.

Heinonen, M., Rein, D., Statué-Gracia, M.T., Huang, S.-W., German, J.B., and Frankel, E.N. (1998) Effect of Protein on the Antioxidant Activity of Phenolic Compounds in a Lecithin-Liposome Oxidation System, *J. Agric. Food Chem. 46*, 917–922.

Hidalgo, F.J., and Kinsella, J.E. (1989) Changes Induced in β-Lactoglobulin B Following Interactions with Linoleic Acid 13-Hydroperoxide, *J. Agric. Food Chem. 37*, 860–866.

Hirose, A., and Miyashita, K. (1999) Inhibitory Effect of Proteins and Their Hydrolysates on the Oxidation of Triacylglycerols Containing Docosahexaenoic Acids in Emulsion, *J. Jpn. Soc. Food Sci. Technol. 46*, 799–805.

Huang, S.W., Satué-Gracia, M.T., Frankel, E.N., and German, J.B. (1999) Effect of Lactoferrin on Oxidative Stability of Corn-Oil Emulsions and Liposomes, *J. Agric. Food Chem. 47*, 1356–1361.

Iio, T. and Yoden, K. (1988a) Formation of Fluorescent Substances from Degradation Products of Methyl Linoleate Hydroperoxides with Amino Compound, *Lipids 23*, 1069–1072.

Iio, T., and Yoden, K. (1988b) Fluorescence Formation from Hydroperoxide of Phosphatidyl-choline with Amino Compounds, *Lipids 23*, 65–67.

Inoue, T., and Kikugawa, K. (1998) Blue Fluorescence Generated During Lipid Oxidation of Rat Liver Microsomes Cannot Be Derived from Malonaldehyde but Can Be from Other Aldehyde Species, *Biol. Pharm. Bull. 21*, 319–325.

Jacobsen, C., Adler-Nissen, J., and Meyer, A.S. (1999) Effect of Ascorbic Acid on Iron Release from the Emulsifier Interface and on the Oxidative Flavor Deterioration in Fish Oil Enriched Mayonnaise, *J. Agric. Food Chem. 47*, 4917–4926.

Jacobsen, C., Timm, M., and Meyer, A.S. (2001) Oxidation in Fish Oil Enriched Mayonnaise: Ascorbic Acid and Low pH Increase Oxidative Deterioration, *J. Agric. Food Chem. 49*, 3947–3956.

Kansci, G. (1996) Effets Antioxydants de Peptides et d'Hydrolysats de Protéines sur l'Oxydation des Phospholipides. Thèse de Docteur de l'Ecole Nationale Supérieure Agronomique de Rennes, France.

Kansci, G., Genot, C., Meynier, A., and Gandemer, G. (1997) The Antioxidant Activity of Carnosine and Its Consequences on the Volatile Profiles of Liposomes During Iron-Induced Phospholipid Oxidation, *Food Chem. 60*, 165–170.

Karel, M. (1980) Lipid Oxidation, Secondary Reactions, and Water Activity of Foods, in: *Autoxidation in Food and Biological Systems* (Simic, M.G., and Karel, M., eds.) pp. 191–206, Plenum Press, New York.

Kato, H., Tashiro, Y., Okitani, A., and Utsunomyia, N. (1983) Polmerization of Lysosyme by Reacting with Vaporized Hexanal, *Agric. Biol. Chem. 47*, 2137–2139.

Kikugawa, K. (1991) Involvement of Lipid Oxidation Products in the Formation of Fluorescent and Cross-Linked Membrane Proteins, in *Membrane Lipid Oxidation*, Vigo-Pelfry, C., ed.) pp. 171–189, CRC Press, Boca Raton, FL.

Kikugawa, K., and Beppu, M. (1987) Involvement of Lipid Oxidation Products in the Formation of Fluorescent and Cross-Linked Proteins, *Chem. Phys. Lipids 44*, 277–296.

Kikugawa, K., Kato, T., Beppu, M., and Hayasaka, A. (1991a) Development of Fluorescence and Cross-Links in Eye Lens Crystallin by Interaction with Lipid Peroxyl Radicals, *Biochim. Biophys. Acta 1096*, 108–114.

Kikugawa, K., Kato, H., and Hayasaka, A. (1991b) Formation of Dityrosine and Other Fluorescent Amino Acids by Reaction of Amino Acids with Lipid Hydroperoxides, *Lipids 26*, 922–929.

Kikugawa, K., Takayanagi, K., and Watanabe, S. (1985) Polylysines Modified with Malonalde-hyde, Hydroperoxylinoleic Acid and Monofunctional Aldehydes, *Chem. Pharm. Bull. 33*, 5437–5444.

Leaver, J., Law, A.J.R., and Brechany, E.Y. (1999a) Covalent Modification of Emulsified β-Casein Resulting from Lipid Peroxidation, *J. Colloids Interface Sci. 210*, 207–214.

Leaver, J., Law, A.J.R., Brechany, E.Y., and McCrae, C. (1999b) Chemical Changes in β-Lactoglobulin Structure During Ageing of Protein-Stabilized Emulsions, *Int. J. Food Sci. Technol. 34*, 503–508.

Leaver, J., Law, A.J.R., and Horne, D.S. (1999c) Caseinate-Stabilized Emulsions: Influence of Ageing, pH and Oil Phase on the Behaviour of Individual Protein Components, in *Food Emulsions and Foams: Interfaces, Interactions and Stability* (Dickinson, E., and Rodriguez Patino, J.M., eds.) pp. 258–268, Royal Society of Chemistry, Cambridge.

Liang, J.H. (1999) Fluorescence Due to Interactions of Oxidizing Soybean Oil and Soy Proteins, *Food Chem. 66*, 103–108.

Lu, C.-L. And Baker, R.C. (1987) Effect of pH and Food Ingredients on the Stability of Egg Yolk Phospholipids and the Metal-Chelator Antioxidant Activity of Phosvitin, *J. Food Sci. 52*, 313–616.

McClements, D.J. (1999) *Food Emulsions: Principles, Practice, and Techniques*, pp. 1–378, CRC Press, Boca Raton.

Medina, I., Tombo, I., Statué-Gracia, M.T., German, J.B., and Frankel, E.N. (2002) Effects of Natural Phenolic Compounds on the Antioxidant Activity of Lactoferrin in Liposomes and Oil-in-Water Emulsions, *J. Agric. Food Chem. 50*, 2392–2399.

Meyer, A.S., and Isaksen, A. (1995) Application of Enzymes as Food Antioxidants, *Trends Food Sci. Technol. 6*, 300–304.

Monahan, F.J., McClements, D.J., and Kinsella, J.E. (1993) Polymerization of Whey Proteins in Whey Protein-Stabilized Emulsions, *J. Agric. Food Chem. 41*, 1826–1829.

Mulder, H., and Walstra, P. (1974) *The Milk Fat Globule: Emulsion Science as Applied to Milk Products and Comparable Foods*, pp. 1–296 Commonwealth Bureau of Dairy Science and Technology, Belfast.

Nakamura, S., Kato, A., and Kobayashi, K. (1992) Enhanced Antioxidative Effects of Ovalbumin Due to Covalent Binding of Polysaccharides, *J. Agric. Food Chem. 40*, 2033–2037.

Neukom, H. (1980) Oxidative Crosslinking of Proteins and Other Biopolymers, in *Autoxidation in Food and Biological Systems* (Simic, M.G., and Karel, M., eds.) pp. 249–259, Plenum Press, New York.

Pauling, L., and Corey, R.B. (1951) Configurations of Polypeptide Chains with Favored Orientations Around Single Bonds: Two New Pleated Sheets, *Proc. Natl. Acad. Sci. USA 37*, 729–740.

Pokorny, J. (1977) Interactions of Oxidized Lipids with Proteins, *Riv. Ital. Sostanze Grasse 54*, 389–393.

Prassl, R., Schuster, B., Laggner, P., Flamant, C., Nigon, F., and Chapman, M.J. (1998) Thermal Stability of Apoprotein B100 in Low-Density Lipoprotein Is Disrupted at Early Stages of Oxidation While Neutral Lipid Core Organization Is Conserved, *Biochemistry 37*, 938–944.

Rampon, V., Lethuaut, L., Mouhous-Riou, N., and Genot, C. (2001) Interface Characterization and Aging of Bovine Serum Albumin Stabilized Oil-in-Water Emulsions as Revealed by Front-Surface Fluorescence, *J. Agric. Food Chem. 49*, 4046–4051.

Rampon, V., Mouhous-Riou, N., and Genot, C. (2002) Modification of Bovine Serum Albumin and Formation of Fluorescent Pigments in Oxidizing Emulsions as Revealed by Front-Surface Fluorescence, in *Recent Research Developments in Agricultural and Food Chemistry: Food Emulsions and Dispersions* (Anton, M., and Pandalaï, S.G., eds.) pp. 93–102, Res. Signpost, Kerala, India.

Riaublanc, A., Llamas, G., Lopez, C., and Brossard, C. (2002) Influence of Temperature on Rheological Properties of Sodium Caseinate Stabilised Emulsions, in *Recent Research Developments in Agricultural and Food Chemistry: Food Emulsions and Dispersions* (Anton, M., and Pandalaï, S.G., eds.) pp. 41–48, Res. Signpost, Kerala, India.

Sayre, L.M., Arora, P.K., Iyer, R.S., and Salomon, R.G. (1993) Pyrrole Formation from 4-Hydroxynonenal and Primary Amines, *Chem. Res. Toxicol 6*, 19–22.

Shahidi, F. (1997) Natural Antioxidants: An Overview, in *Natural Antioxidants, Chemistry, Health Effects, and Applications* (Shahidi, F., ed.) pp. 1–11, AOCS Press, Champaign, IL.

Shimasaki, H., Privett, O.S., and Hara, I. (1977) Studies of the Fluorescent Products of Lipid Oxidation in Aqueous Emulsion with Glycine and on the Surface of Silica Gel, *J. Am. Oil Chem. Soc 54*, 119–123.

Shimasaki, H., Ueta, N., and Privett, O.S. (1982) Covalent Binding of Peroxidized Linoleic Acid to Protein and Amino Acids as Models for Lipofuscin Formation, *Lipids 17*, 878–883.
Stadtman, E.R. and Berlett, B.S. (1997) Reactive Oxygen-Mediated Protein in Aging and Disease, *Chem. Res. Toxicol 10*, 485–494.
Stapelfeldt, H. (2001) Searching for Proper Balance Between Maillard Reaction and Lipid Oxidation in Milk Powder, in *Melanoidins in Food And Health, Cost Action 919*, (Ames, J.A., ed.) pp. 43–52, European Commission, Brussels.
Stapelfeldt, H., and Skibsted, L.H. (1994) Modification of β-Lactoglobulin by Aliphatic Aldehydes in Aqueous Solutions, *J. Dairy Res. 61*, 209–219.
Stevenson, E., Horne, D.S., and Leaver, J. (1997) Displacement of Native and Thiolated β-Casein from Oil-Water Interfaces: Effect of Heating, Ageing and Oil Phase, *Food Hydrocolloids 11*, 3–6.
Stryer, L. (1988) *Biochemistry*, 3rd edn., W.F. Freeman and Company, New York.
Tashiro, Y., Okitani, A., Utsunomyia, N., Kaneko, S., and Kato, H. (1985) Changes in Lysozyme Due to Interaction with Vaporized Hexanal, A*gric. Biol. Chem. 49*, 1739–1747.
Taylor, M.J., and Richardson, T. (1980a) Antioxidant Activity of Cysteine and Protein Sulfhydryls in a Linoleate Emulsion Oxidized by Hemoglobin, *J. Food Sci. 45*, 1223–1230.
Taylor, M.J., and Richardson, T. (1980b) Antioxidant Activity of Skim Milk: Effect of Heat and Resultant Sulfhydryl Groups, *J. Dairy Sci. 63*, 1783–1795.
Tong, L.M., Sasaki, S., McClements, D.J., and Decker, E.A. (2000) Mechanisms of the Antioxidant Activity of a High Molecular Weight Fraction of Whey, *J. Agric. Food Chem. 48*, 1473–1478.
Verma, M.M., Paranjape, V., and Ledward, D.A. (1985) Lipid and Haemoprotein Oxidation in Meat Emulsions, *Meat Sci. 14*, 91–104.
Yamaki, S., Kato, T., and Kikugawa, K. (1992) Characteristics of Fluorescence Formed by the Reaction of Proteins with Unsaturated Aldehydes Possible Degradation Products of Lipid Radicals, *Chem. Pharm. Bull. 40*, 2138–2142.
Yamamoto, Y., Kataoka, A., and Kitora, M. (1998) Enhancing Effect of β-Lactoglobulin on the Antioxidative Activity of α-Tocopherol in an Emulsion of Linoleic Acid, *Biosci. Biotechnol. Biochem. 62*, 1912–1916.
Yamamoto, Y., and Omori, M. (1994) Antioxidative Activity of Egg Yolk Lipoprotein, *Biosci. Biotechnol. Biochem. 58*, 1711–1713.
Yong, S.H., Lau, S., Hsieh, Y., and Karel, M. (1980) Degradation Products of L-Tryptophan Reacted with Peroxidizing Methyl Linoleate, in *Autoxidation in Food and Biological Systems* (Simic, M.G., and Karel, M., eds.) pp. 237–246, Plenum Press, New York.
Zhou, S., and Decker, E.A. (1999a) Ability of Amino Acids, Dipeptides, Polyamines, and Sulfhydryls to Quench Hexanal, a Saturated Aldehydic Lipid Oxidation Product, *J. Agric. Food Chem. 47*, 1932–1936.
Zhou, S. and Decker, E.A. (1999b) Ability of Carnosine and Other Skeletal Muscle Components to Quench Unsaturated Aldehydic Lipid Oxidation Products, *J. Agric. Food Chem. 47*, 51–55.

Chapter 10

Radical Formation in the Radiolysis of Starch and Lipid-Containing Starch

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Introduction

Carbohydrates are the major components of many foods, and their diversity of structure is matched by their diversity of function. They have been traditionally categorized into simple sugars and polysaccharides: The former are used for their sweetening power, preservative action, and crystallinity, whereas the latter confer body, texture, and colloid properties. The polysaccharides, consisting of monoglycerides bound to each other by glycosidic linkages, include cellulose, collagen, glucans, alginates, pectin, and starch.

Of the different carbohydrates, the functional properties of starch in food are very much related to its interactions with other components, particularly lipids. In cereals, two types of lipids are generally recognized: surface and internal lipids (i.e., lipids extractable only by polar solvents after gelatinization of starch). Analysis of the surface lipids of wheat and maize starches shows significant qualitative differences from the internal lipids (Galliard and Bowler 1987). Triglycerides are, presumably, derived from the oil storage bodies (spherosomes) that occur in the different tissues of cereal grain. A clear distinction among potato, maize, and wheat starches can be made on the basis of internal lipids. First, of all starch granules, those of maize and wheat contain significant amounts of internal lipids, whereas starch granules from potato and other tubers (e.g., cassava) as well as from legume seeds (e.g., peas, beans) do not contain this type of internal lipids.

On the other hand, the significance of the monoacyl character of starch internal lipids (Galliard and Bowler 1987) lies in the fact that these can form helical inclusion complexes with amylose, whereas di- or triacyl lipids do not form such complexes. But the presence of lipids also can give rise to anomalous values for the amylose content of starch if this is determined by the conventional I_2-binding method because the lipid occupies the same site within amylose helices as those available to iodine in lipid-free amylose, leading to apparent values of 21.4 instead of 27.5 in the case of maize, and 20.4 instead of 27.2 in the case of wheat (Galliard and Bowler 1987).

Consequently, lipids often alter rheological properties of cereal starches (Colonna *et al.* 1987). If present on the surface of starch granules, lipids may affect

the diffusion of water into the granule or, when present in inclusion complexes with amylose, may induce more pronounced changes in structural organization. This may lead to an increase (maize and wheat) or a decrease (rice) in the viscosity of defatted starches.

Carbohydrates may also be used in a number of drugs and cosmetic products as excipients (Crowley and Martini 2001), or in the form of starch-lipid complexes to modify some properties of a product (Dongowski *et al.* 1998, Sintzel *et al.* 1997). Any factor that contributes to an increase in the viscosity of the continuous phase of an emulsion causes a significant delay in flocculation and coalescence. Gelatin and many gums, some of which are not surface active, are extremely useful in stabilizing oil-in-water (O/W) emulsions because of their effect on the aqueous phase (Nawar 1985).

In many cases, the carbohydrates or their lipid complexes may be irradiated when the product must be debacterized or sterilized. As an example, the chemical studies undertaken from 1965 to 1990 to ascertain the wholesomeness of irradiated foods (Anonymous 1981 and 1999a) were first conducted using models. Among carbohydrates, starch was held as a model for the following reasons (Raffi *et al.* 1981a): (i) It is one constituent that is utilized in the production of a great number of food products and ready-cooked dishes, or (ii) it is the main component of various agricultural products such as corn, bread wheat, rice, green beans, manioc (cassava) or potatoes.

In this chapter, we shall first discuss the principles of the irradiation treatment, pointing out its main applications, and then introducing the irradiation of starch-lipid complexes and the formation of radicals during these processes. Next, we will present the main results of research conducted on the radiochemistry of starches and lipid-containing starches. Consequently we shall demonstrate the commonality in the radiolysis of starch components and make a comparison with some other molecules before we conclude.

Basic Concepts of Radiation Processing

The potential practical interest in irradiation was recognized soon after the discovery of X-rays by Röentgen and of radioactivity by Becquerel. Although known in principle since the 1900s, the industrial development of the process was very slow due to the lag in the development of research and industrial radiation sources.

Today, two main irradiation techniques are used, i.e., high-energy electrons, generated by a particle accelerator (energy up to 10 MeV), and γ-rays produced by a cobalt-60 or cesium-137 radioactive source. X-Rays (energy up to 5 MeV) may also be utilized but commercial X-ray facilities have not been used to date even though such an irradiator was recently built in the United States. The main effect of γ- and X-rays is Compton scattering, i.e., ionization of atoms giving rise to "secondary electrons." These electrons, like those generated in an accelerator, lead to a number of other secondary electrons and ions. This cascade of secondary electrons loses energy in ionizing the foodstuff molecules, with consequent production of

radicals and thereby of radiolytic products. The physical, chemical, and biological effects are linked to the irradiation dose, which is the quantity of energy absorbed by the material, measured in gray: 1 Gy = 1 J/kg.

The irradiation process may be used for a number of purposes such as radiochemistry (polymerization, cross-linking and grafting, scission), treatment of effluents, and many biochemical applications, e.g., radiation sterilization of cultures and disposable medical supplies and, now, in the preservation of food (Anonymous 1981and 1999a, Raffi and Siadous 1998), and radiosterilization of drugs (Gibella *et al.* 2000), (Jacobs 1985, Piccerelle *et al.* 2000), medicinal preparations (Fang and Wu 1998), and related products.

For example, in the case of foodstuffs, the doses normally applied for different technological reasons are as follows (Raffi and Siadous 1998, WHO 1980 and 1997): (i) 0.0–0.15 kGy for sprout inhibition (of potatoes, for example); (ii) 0.2–1 kGy for disinfestation; (iii) 1–10 kGy for radurization and radicidation, terms invented to cover the process of pasteurization by irradiation, and (iv) up to 50 kGy for sterilization.

Assuming that all of these applications are theoretically possible, irradiation would be used mainly in developed countries to increase the hygienic quality of foods (i.e., doses generally between 5 and 10 kGy) or instead of fumigation treatments (e.g., by ethylene oxide, methyl bromide). Doses >10 kGy, i.e., for sterilization, would not be used for the general public but in hospitals for immunosuppressive patients. Many investigations have been devoted to possible health hazards and, in 1980, a Joint Food and Agriculture Organization (FAO)/International Atomic Energy Agency (IAEA)/World Health Organization (WHO) Expert Committee meeting concluded that "the irradiation of any food commodity presents no toxicological hazard; hence, toxicological testing of foods so treated is no longer required" (WHO 1980, 1997 and 1999).

The different mechanisms of radiolysis have been widely studied (Elias and Cohen 1977 and 1983) and the relative results can be used to propose potential identification tests. However, the most difficult problem has been that the changes that occur in irradiated foodstuffs are quite insignificant and generally similar to those produced by classic food treatment processes (heating, freezing) or natural spoilage (autoxidation).

Some facts about food irradiation have to be understood, i.e., (i) the effects of the irradiation treatment are dependent, at constant temperature, only on the energy delivered to the food, i.e., the irradiation dose, and (ii) the lower the temperature, the smaller the quantities of radiolytic products produced.

Irradiation of Starch-Lipid Complexes

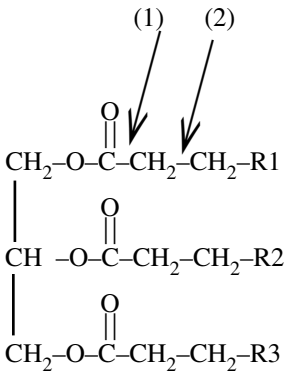
Although considered pharmacologically inert, carbohydrates (like other excipients) can initiate, propagate, or participate in chemical reactions (Crowley and Martini 2001, Hombrercher and Schell 1998, Singh *et al.* 2000), particularly during steril-

ization treatments such as heating or radiation. The stronger the interaction, the more the different molecules are complexed together (Agheli *et al.* 1998, Illum *et al.* 2001, Mangala *et al.* 1999, Tuvesson and Eliasson 2000].

Starch is isolated from plant sources as water-insoluble discrete granules. Starch solutions are easily obtained by passing starch-water slurries through a continuous-stream jet cooker. The co-jet cooking of starch with lipids allows the uniform dispersion of the lipid component within the starch-water matrix as small droplets; these compositions can be used as fat replacers and stabilizers in foods and as components in cosmetics, drug delivery systems, and adhesives (Fanta and Eskins 1995). Amylose also creates inclusion complexes with monoglycerides and fatty acids (Liu *et al.* 1997, Mangala *et al.* 1999, Tufvesson and Eliasson 2000). In the case of foods, starch-lipid inclusion complexes may vary under irradiation and/or alter the functional properties of flour (Singh *et al.* 2000). On the other hand, in pharmacology, oligosaccharides or starch complexes may regulate fatty acid synthase activity (Agheli *et al.* 1999), enhance the nasal absorption of polypeptides (Illum *et al.* 2001), or delay propanolol transport (Dongowski *et al.* 1998).

Before we discuss the case of irradiation of lipids, let us remember that γ-rays, X-rays, or electron beams lead to the same radiolytic reactions, with only quantitative changes among the ratios of the different chain reactions. Ions and excited molecules are the first species formed when ionizing radiation is absorbed by matter. Because of this similarity, we will show results of experiments conducted mainly with γ-ray facilities. The following reactions are not the result of a statistical distribution of random cleavages of chemical bonds; rather, they follow preferred pathways largely influenced by molecular structure. Even if the lipid molecules are very different from the starch molecules, there is a commonality in their respective radiolysis because they both contain similar –C–O– and –C–C– bonds.

For the applied treatments, although the mechanisms initially involved are different, many of the compounds that are produced from fats by irradiation are similar to those formed by heating. Far more decomposition products, however, have been identified from heated and thermally oxidized fats than from irradiated fats (Nawar 1985). For example, if we look at a triglyceride such as the following,



the two radio-induced linkage breaks at positions 1 and 2 lead to the formation of two main hydrocarbons with one or two carbon atoms less than the parent lipid. This property is used in the detection of irradiated foodstuffs containing lipids (Raffi and Siadous 1998). Breaks in other positions lead to the formation of aldehydes, cyclobutanones, or esters, for example. But these radiolytic products are not characteristic of an irradiation treatment because they are also inducible by other reactions such as oxidation, with the possible exception of cyclobutanones (Raffi and Siadous 1998).

However, in fat-containing foods, the amount of lipids, the state and environment of the lipids, and its fatty acid composition influence the radiation chemistry. However, the radiolytic products that appear to arise from the effects of irradiation on the lipid fraction in complex foods are on a quality level similar to those formed by the irradiation of simple lipids in model systems (Delincée 1983). Thus, we shall first discuss the study of radiolysis mechanisms in starch and starch components, before we return to the case of lipids and lipid-starch complexes.

Research Results on the Radiochemistry of Starch

Radiolytic products have been determined in γ-irradiated starches derived from different foods [maize, bread wheat, manioc (cassava), rice] (Raffi *et al.* 1978 and 1981a). The nature and concentration of the main radio-induced products (malonaldehyde, formaldehyde, acetaldehyde and total carbonyl, formic acid, free and total acidity, hydrogen peroxide) show no important differences among the various starches. The origin of the starch is in fact less important than the irradiation parameters (dose, oxygen, water content, irradiation and storage temperatures, storage time before and after treatment), which exercise similar roles in the formation of a given radio-induced product (Figs. 10.1 and 10.2). The ratio of maximum to minimum values of the radio-induced products is generally <8 (Table 10.1).

Recent studies are oriented more toward the technological effects of irradiation such as disintegration properties (Ciesla *et al.* 1999, De Kerf *et al.* 2001, Pietranera and Narvaiz 2001) and the influence of the irradiation and packaging atmosphere (Stecchini *et al.* 1995). The radio-depolymerization of starch has been studied at the experimental and theoretical levels (Kang *et al.* 1999, Raffi *et al.* 1981b, Roushdi *et al.* 1983, Sokhey *et al.* 1993). All of the starch varieties behave similarly under irradiation and, the previously described theory of maize starch depolymerization, in particular, (Raffi *et al.* 1981b) can be extrapolated to other varieties. For instance, the quantities of radio-induced dextrins are always on the same order of magnitude (Table 10.2). The effects of the radio-depolymerization on technological properties have also been studied (Langhout *et al.* 2000, Pietranera and Narvaiz 2001).

Electron spin resonance (ESR) studies have been conducted for a long time (Raffi and Agnel 1983) on the "polysaccharide radicals" induced by γ-radiation and/or other treatments. The ESR spectra of these products, which are in solid

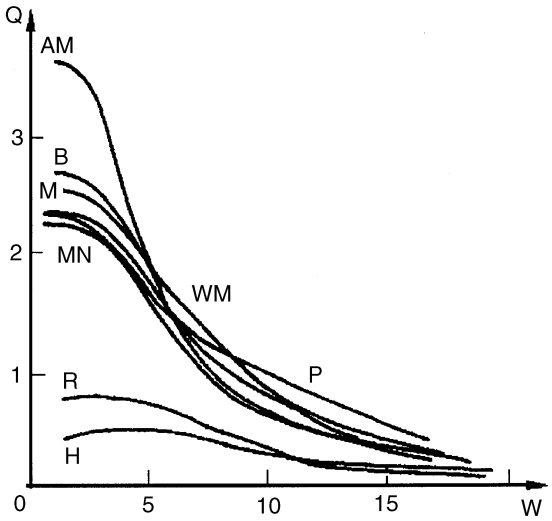


Fig. 10.1. Quantities Q (arbitrary units) of radio-induced malonaldehyde with regard to the water content (%) of the starch. See the starch notation in Table 10.1.

phase, are very easy to record but difficult to interpret, i.e., to be linked to chemical structures. The powder spectra are poorly resolved due to the overlapping of many spectra induced by the random directions of radicals with regard to the magnetic field. However, it is possible to draw some conclusions from these studies, even if spin-trapping experiments on sugars of low molecular weight are necessary to determine the nature of the radio-induced radicals.

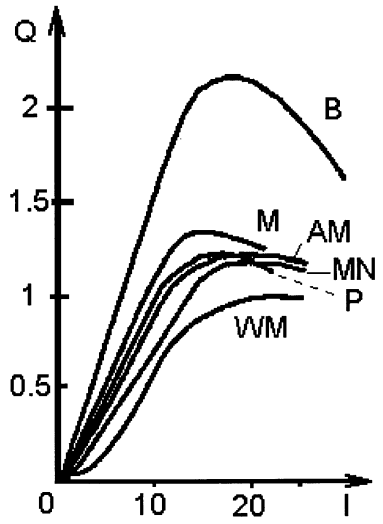


Fig. 10.2. Quantities (µeq/mL) of radio-induced formic acid with regard to the irradiation dose I (kGy). See the starch notation in Table 10.1.

TABLE 10.1
Radio-Induced Quantities (μg/g) at 1 kGy of Some Products in Starches Derived from Different Foodstuffs

Product starch	Water content	MDA[a]	Formaldehyde	Acetaldehyde	Formic acid	H_2O_2
Maize (MN)	12.2	0.125	0.486	0.729	0.135	0.246
Waxymaize (WM)	12.5	0.125	0.569	1.328	0.179	0.154
Amylomaize (AM)	14.4	0.083	0.410	0.625	0.115	0.171
Manioc (M)	3.9	0.104	0.444	0.469	0.185	0.242
Bread wheat (B)	11.4	0.018	0.465	0.781	0.344	0.071
Potato (P)	18.4	0.063	0.319	0.469	0.129	0.313
Rice (R)	12.1	0.042	0.153	0.885	0.156	0.113
Green beans (H)	11.8	0.042	0.222	0.313	0.110	0.038
Average		0.104	0.382	0.703	0.160	0.167
±		50%	37%	45%	35%	56%

[a]A, malondialdehyde.

Two main types of radicals are radio-induced in starches, linked to the so-called AA′ and BB′ ESR shapes (Fig. 10.3) (Raffi *et al.* 1983). Their ESR characteristics (g-factor, peak-to-peak widths) (Table 10.2) and the kinetic laws of their disappearance are the same regardless of the source of starch. Moreover, the ESR shape, i.e., predominance of AA′ or BB′ shapes (Fig. 10.4), is more linked to the presence of pseudo-crystalline or amorphous parts than to the origin of the starch (Gol'din *et al.* 1971). For example, short crystalline glucose oligomers provide acceptable models for the AA′ and BB′ shapes (maltotriose G3 and above) and even for the kinetic laws of disappearance (G5 and above) (Table 10.3). Notably, this is not the case for glucose and maltose (G2), thus emphasizing the importance of the glycosidic linkage. Some other radicals induced in weak concentrations were

TABLE 10.2
Radio-Depolymerization and Radio-Induced Radicals in Starches Derived from Different Foodstuffs

Product starch	Dextrins (μg/g at 1kGy)	AA′ peak-to-peak width (mT)	BB′ peak-to-peak width (mT)
Maize (MN)	232	2.61	0.851
Waxymaize (WM)	432	2.59	0.928
Amylomaize (AM)	175	2.53	0.844
Manioc (M)	391	2.61	0.854
Bread wheat (B)	208	2.61	0.864
Potato (P)	138	2.60	0.845
Rice (R)	297	2.49	0.910
Green beans (H)	326	2.55	0.856
Average	276	2.57	0.867
±	38%	1.9 %	5.2%

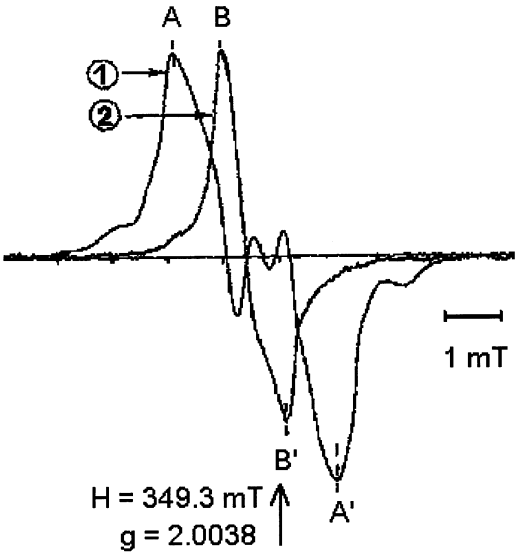


Fig. 10.3. Electron spin resonance (ESR) spectra of 20 kGy irradiated green bean starch: "initial" AA′ shape (curve 1) recorded just after treatment and "final" BB′ shape (curve 2) recorded 95 d later (gain × 100).

also studied (Korkmaz and Polat 2000), showing that the main radiolytic mechanism is accompanied by a number of side reactions. This is not surprising because the energy deposit during irradiation is not performed on a single molecule but on a number of molecules, and the subsequent reactions happen statistically in relation to the breaking energy of the different links in the molecule.

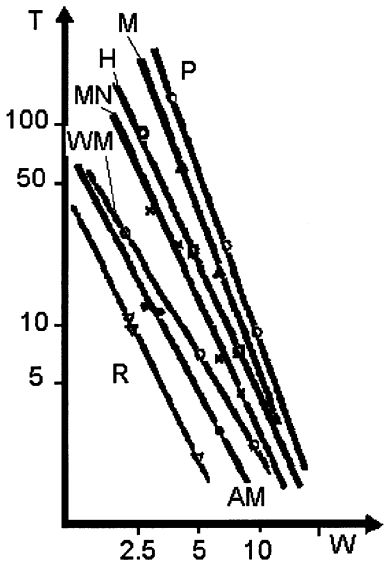


Fig. 10.4. Relation (log scales) between the water content W (%) and the time t (d) when AA′ and BB′ signal areas are equivalent. See the starch notation in Table 10.1.

TABLE 10.3
Kinetic Constants of Radicals Induced in Starch and Glucose Oligomers[a]

ESR signal	AA′		BB′	
Sugar	Peak-to-peak width (mT)	K_{III} (10^{-3}/d)	Peak-to-peak width (mT)	K_{III} (10^{-3}/d)
Starch	2.57	68	0.87	34
G7	2.63	61.6	0.96	ND
G6	2.66	72.6	0.85	ND
G5	2.60	72.7	0.85	21.2
G4	2.65	77.4	0.92	ND
G3	2.65	37	0.90	ND

[a]G7, maltoheptaose, to G3, maltotriose; ND, not determined.

Because the irradiated starches and glucose oligomers recorded by powder ESR spectra are poorly resolved, spin-trapping experiments were conducted on glucose oligomers from G3 to G7, using the reaction

$$^{\bullet}G_n + Bu^t\text{–}N{=}O \rightarrow Bu^t\text{–}N(\text{–}G_n)\text{–}O^{\bullet}$$

A water-ethanol solution of the trap, 2-methyl-2-nitrosopropane (Bu^t–N=O), is added to the irradiated sugar powder (with $^{\bullet}G_n$ radicals), leading to an adduct Bu^t–N(–G_n)–O$^{\bullet}$, which is quite stable in the liquid medium, thus allowing the recording of well-resolved ESR spectra. These studies have allowed a chemical interpretation of the main radicals induced from the glucose oligomers (Thiéry *et al.* 1983, 1990), which are comparable to those from irradiated starches. Among these, the most important radical is the following:

$$HO\text{–}C^{\bullet}_{(1)}(\text{–}H)\text{–}C_{(2)}(\text{–}H)(\text{–}OH)^{-}$$

which is consecutive to the glycosidic linkage break.

Note that a similar technique, spin-probe, may also be used to study the consequences of irradiation (Wang and You 2000). In this case, a nitroxide molecule was introduced into the medium as a spin-probe before the irradiation treatment, and the relative ESR spectrum was recorded. The difference between the spectra recorded after and before irradiation shows the differences of the surroundings of these probe molecules, e.g., the changes in the starch macromolecules and/or starch complexes, depending on the chemical structure of the probe.

Commonality in the Radiolysis of Starch and Other Carbohydrates

Among the ~40 products that have been analyzed in irradiated starches, none is characteristic of irradiation because all of these products are more or less present in

nonirradiated samples of starch induced by natural oxidation or by heating treatment. This is the reason why no unique chemical determination can provide proof of an irradiation treatment. Consequently, no detection test for irradiated foodstuffs is based on the determination of a unique radiolytic product from starch.

Moreover, if we now consider the radio-induced radicals, there is very often a signal in the nonirradiated sample that is very close to the BB′ signal. Without the reference sample, BB′ cannot be used as proof of irradiation under commercial conditions. If AA′ is characteristic, when present in relatively high concentrations, its life time (up to 10–12 mo in highly crystallized starches) is not sufficient for products such as cereals to be stored for several years (Raffi *et al.* 1987). The same changes in the physicochemical characteristics of starches may occur with natural fermentation, drying (Sokhey *et al.* 1993, Raffi *et al.* 1987, Ciesielski and Tomasik 1996, Mestres and Rouaux 1997, Eerlingen *et al.* 1997, Ciesielski *et al.* 1998, Sriburi *et al.* 1999), and the action of some chemical agents (Srirubi and Hill 1997). The grinding of cereals also leads to the same radicals (Raffi and Rouaux, unpublished results).

The radicals themselves are not characteristic of γ-irradiation because cosmic rays or ultraviolet (UV) radiation also induced the same signals. For instance, the NASA Long Duration Exposure Facility (LDEF) satellite, which spent nearly 6 y in space (1984–1990), provided an important opportunity for testing ionizing radiation models. A comparison with the equivalent γ-induced radicals was used to calculate the "γ-equivalent-dose" absorbed by rice starches (Bayonove *et al.* 1994).

UV radiation also induced the AA′ and BB′ signals, even if the radicals were concentrated in only a small external layer of the starch. They were studied for purposes of grafting (Merlin and Fouassier 1981) or for UV-depolymerization of cereal or legume starches (Bertolini *et al.* 2001a and 2001b, Cakir *et al.* 1991). Microwave radiation may also induce the same radicals because the effect on the physicochemical properties of starches seems to be equivalent, or even smaller (Lewandowicz and Jankowski 2000). On the other hand, grinding also induces the same AA′ radicals in starches (Raffi *et al.* 2002). The different treatment consequences upon starch properties were also studied and compared (Erlandsson *et al.* 1997, Fanta and Eskins 1995, Inouye *et al.* 1999, Jauho *et al.* 2000).

The very initial radicals may not be the same because the mechanism of energy deposit is not identical. But the successive reactions always lead to the breakage of the weakest linkages and provide the same radicals. When radicals are formed in a medium, regardless of the inducing treatment, they always lead to the more stable species common to the different treatments.

As for starch, the main effect of γ-radiation on cellulose is depolymerization (Cakir *et al.* 1991, Han *et al.* 1981, Ross and Engeljohn 2000). The decrease in the degree of polymerization is not the same for cellulose I and II; this is related to the different microstructures of the polysaccharides, which are very similar to the differences between amylose and amylopectin parts of starch. Moreover, here too, relatively high doses effectively accelerate enzymatic hydrolysis of cellulose material such as rice

straw, chaff, and sawdust. In the case of long-chain dextrans or Arabic gums, for example, the results are also comparable to those found for starch (Aliste *et al.* 2000).

To improve the knowledge about radiolysis mechanisms, several approaches have been taken, including the following:

- Electron spin resonance/electron nuclear double resonance (ESR/ENDOR) studies of single crystals to detect the very primary radicals or ion-radicals (Madden *et al.* 1982)
- Flow-systems (Gilbert *et al.* 1982, Gilbert 1995) and spin-trapping studies (Raffi and Agnel 1983, Thiéry *et al.* 1983, Triolet *et al.* 1992), for the detection of intermediate radicals
- The study of radiolytic products to make hypotheses (Baugh *et al.* 1976, Von Sonntag *et al.* 1976, Von Sonntag 1980) concerning the structures of the "final" radicals

Monosaccharides with no glycosidic bond such as glucose react differently, with breakage of the glycosidic ring as the major pathway. But the chain reactions in dimers, such as maltose, and in longer oligomers more closely resemble the chain reactions found in polysaccharides (Aliste *et al.* 2000, Grant and d'Appolonia 1991, Han *et al.* 1981). This is also true for other monomers with regard to the relative polysaccharide, for instance, cellulose. As for starches, grinding and UV radiation induce equivalent radicals even if the quantities are very low compared with those induced by ionizing radiation (Raffi *et al.* 2002). In fact, for low-molecular-weight sugars, the main pathway leads to polymerization, whereas for poly-saccharides, the main pathway involves radio-depolymerization.

In most foods, the two mechanisms occur simultaneously. The pure carbohydrate-induced radicals are also induced in complex foods. For example, the ESR spectrum of achenes from irradiated strawberries presents an apparent doublet (Fig. 10.5) which has been shown (Raffi and Agnel 1989) to be due to a relatively long-lived pure cellulose-induced radical, showing the commonality of these reactions. This is very important in considering the generic clearance of irradiated foods (chemiclearance) but also to be used as proof of an irradiation treatment in foods containing cellulose (European protocol NF EN 1787).

When collagen (Kato *et al.* 1995) and more generally proteins and long chains of amino acid components (Köksel 1998) are γ- or UV-irradiated, the same phenomenon occurs (Bonner *et al.* 1979, Von Sonntag *et al.* 1976). It involves degradation of the chain, depolymerization by breakage of the most labile linkage(s), and simultaneous polymerization leading to aggregations (Kakehashi 1993) that can be detected by electrophoresis (Tzaphlidou *et al.* 1997).

For example, radio-induced structural changes can be studied by performing capillary electrophoresis on fragments of collagen, which are obtained by CNBr attack of the polypeptide chain. The electropherogram shows the disappearance of the original shape (Fig. 10.6), which is a consequence of the degradation of the chain. Moreover,

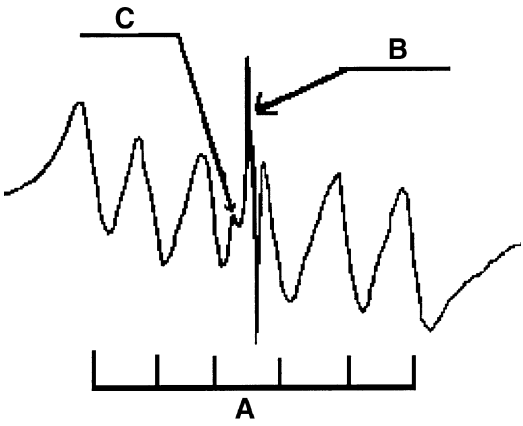


Fig. 10.5. Electron spin resonance (ESR) spectrum of strawberries achenes. A and B, respectively, due to Mn^{2+}, and to hydroxy quinone radicals, are not characteristic from irradiation; only C, due to a cellulose radical, is characteristic of an irradiation treatment.

it is possible to observe the presence of low-molecular weight molecules (small polypeptides) on the electropherogram and the parallel gelatinization of the medium. In the case of smaller molecules such as amino acids, radicals that are induced are too close together under these conditions to allow a good separation of the different irradiated compounds; however, the amount of radicals and their stability are tightly bound to the chemical structure of the parent amino acid (Raffi *et al.* 2002).

Research Results on the Radiochemistry of Starch-Lipid Complexes

If we now reconsider lipids and lipid-complexes, we must first note that the radiolytic products that are obtained from an isolated lipid are usually also observed when the complex food containing that fat is irradiated. However, the concentration of these substances in the irradiated food will be considerably reduced by the

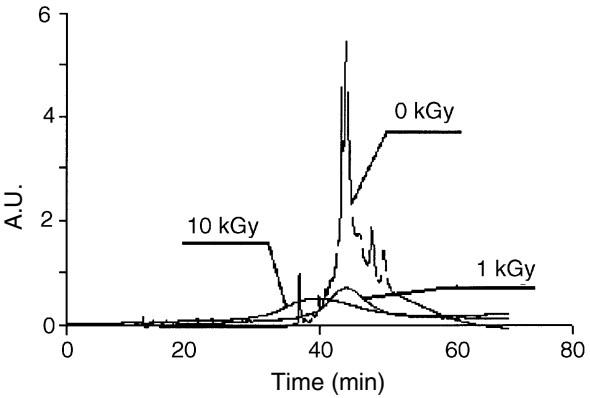


Fig. 10.6. Electropherogram of CNBr fragments from collagen.

diluting effect of the other substances present. Of course, additional changes can be anticipated from radiolysis of the nonlipid constituents and from the interactions between these and the lipids (Nawar 1977, 1983 and 1985). For instance, a review of irradiated cereals claimed that few changes, if any, would occur in the lipids of cereal grains. No appreciable changes were found in the total lipids in wheat (Delincée 1983) irradiated up to 10 kGy, even though this was far in excess of the practical dose for wheat disinfestation.

Because the changes are quite insignificant, they are very difficult to study, and few experiments exist that are trying to study the influence of lipid-starch complexes on the radiolysis of each component or of the whole mixture. Due to the nutritional importance of flour, some differential scanning calorimetry experiments have been performed (Ciesla *et al.* 1999); these showed only quite insignificant differences between gelatinization thermal effects before and after γ-irradiation.

The most recent studies have used the most modern analytical techniques (and apparatus) such as high-performance liquid chromatography and reversed-phase liquid chromatography (Hauville *et al.* 2001). The interactions between starch and lipid have been studied not only in food products (Ciesla *et al.* 1999, Nam *et al.* 2001) but also in products of biological interest, such as membranes (Inouye *et al.* 1999, Jauho *et al.* 2000, Yin *et al.* 1999), blood (Cicha *et al.* 2000), and model products (Manno *et al.* 2001). It is possible that the future will provide opportunities to use ESR probe techniques in these important studies (Wang and You 2000, Wasserman and Le Meste 2000).

Even if the lipid molecules are very different from those of starch, there is a commonality in their respective radiolysis because they contain similar –C–O– and –C–C– chains; the radio-induced breaks are also on or close to these links, leading to starch depolymerization and, in the case of lipids, to aldehydes and hydrocarbons (Nawar *et al.* 1986, see also other chapters in this book). In fact, these radiolytic mechanisms are very similar as can be seen in Figure 10.7. Any differences in the reactions of lipids and starch may be related to the degree of unsaturation in the lipids, which affects the propagation reactions and/or the supramolecular orientation of the two types of molecules.

Concluding Remarks

From a general point of view, the effect of irradiation on starches and starch-lipid complexes is not basically different from that of other food components including lipids. Radio-induced reactions in similar foods are similar, leading to similar radiolytic products *via* similar radicals. Moreover, these radicals and radiolytic products are not characteristic of irradiation treatment. The pathway induced by the energy deposit, ~1 MeV compared with tens of keV for the weakest link breakage energies, leads to the more likely radicals and to final products that are also inducible by other treatments, such as heating or simple autoxidation. The reason why the methods used to prove whether a food has been irradiated took so long to establish (the first European protocols were not published until 1997) was because there is no characteristic radi-

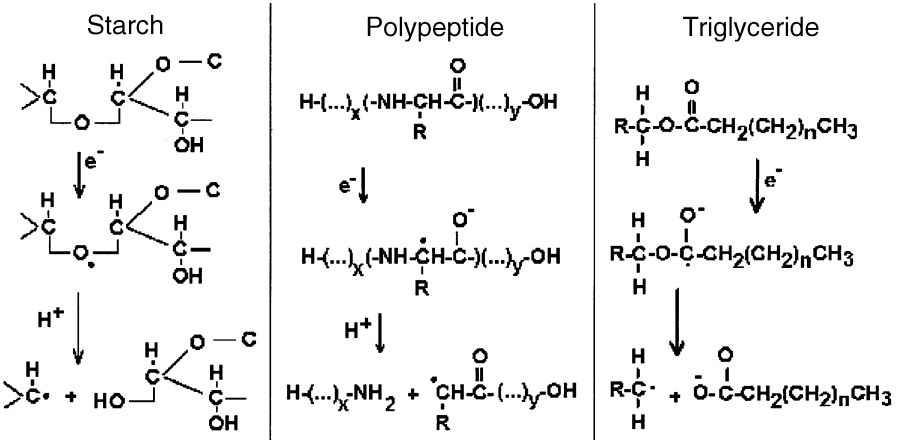


Fig. 10.7. Mechanism of radiolysis.

olytic product, i.e., no irradiation marker. For instance, the cellulose radicals induced in the NF EN 1787 protocol are not characteristic of γ-irradiation (they are also induced by UV rays) but their great number in the whole sample (not only on the surface) is characteristic of an irradiation treatment; the hydrocarbons used in the NF EN 1784 protocol are also not characteristic of γ-irradiation (they are also induced by heating) but their composition is more strictly bound to the lipid composition than in the case of the heating treatment.

All of these facts support the conclusions drawn by the experts of the last Joint FAO/IAEA/WHO study group on food irradiation, justifying the "use of the chemiclearance approach for granting broadly-based, generic approvals of high-dose irradiated foods" (Anonymous 1999a). In the same way, it becomes easier for the European Commission to reach an agreement for the commercial irradiation of foods (Anonymous 1999b). Moreover, the WHO is greatly in favor of irradiation treatment because it is concerned about the increase in contamination accidents in the food industry (WHO 1997 and 2000).

More recently, the position of the European Commission has changed with the publication of a European Directive (Anonymous 1999b and 2001); in addition, the number of allowances has increased in the United States (Neyssen 2000). At the same time, the Australia New Zealand Food Authority (ANZFA) announced that the use of ethylene oxide for debacterization of herbs, spices, and herbal teas will be phased out by October 2003. The combination of these facts and conclusions should lead to the development of commercial applications of food irradiation during the next few years.

However, it is likely that our knowledge concerning irradiated starch and starch-lipid complexes will be improved by future studies on biological molecules as well as the development of commercial irradiation of drugs, cosmetics, and related products. These applications will be more and more important and will probably occur more quickly than those concerning food irradiation.

References

Agheli, N., Kabir, M., Berni-Canani, S., Petitjean, E., Boussairi, A., Luo, J., Bornet, F., Slama, G., and Rizkalla, S.W. (1999) Plasma Lipids and Fatty Acid Synthesase Activity Are Regulated by Short-Chain Fructo-Oligosaccharides in Sucrose-Fed Insulin-Resistant Rats, *J. Nutr. 128*, 1283–1288.

Aliste, A., Vieira, F., and Del Mastro, N. (2000) Radiation Effects on Agar, Alginates and Carrageenan to Be Used as Food Additives, *Radiat. Phys. Chem. 57*, 305–308.

Anonymous (1981) Wholesomeness of Irradiated Foods, *WHO Technical Report Series 659*, WHO, Geneva.

Anonymous (1999a) High-Dose Irradiation: Wholesomeness of Food Irradiated with Doses Above 10 kGy, *WHO Technical Report Series 890*, WHO, Geneva.

Anonymous (1999b) Directive 1999/2/CE of the European Commission, *J. Off. Comm. Eur.* 13 March.

Anonymous (2001) Liste des Autorisations des Etats Membres Relatives aux Denrées et Ingrédients Alimentaires Pouvant Être Soumis à un Traitement par Ionisation, *J. Off. Comm. Eur.* 28 Avril.

Baugh, P., Goodall, J., Phillips, G., Von Sonntag, C., and Dizdaroglu, M. (1976) Gamma Irradiation-Induced Ring-Opening of Polycrystalline Cycloamylose Hydrates, *Carbohydr. Res. 49*, 315–323.

Bayonove, J.F., Raffi, J., and Agnel, J.-P. (1994) Investigation on Rice Embryos and Seeds after the LDEF Flight: ESR Identification, *Adv. Space Res. 14*, 53–57.

Bertolini, A.C., Mestres, C., Colonna, P., and Raffi, J. (2001a) Free Radicals Formation in the UV and Gamma Irradiated Cassava Starch, *Carbohydr. Polymer. 44*, 269–271.

Bertolini, A., Mestres, C., Raffi, J., Buléon, A., Lerner, D., and Colonna, P. (2001b) Photodegradation of Cassava and Corn Starches, *J. Agric. Food Chem. 49*, 675–682.

Bonner, W., Blair, N., and Lemmon, R. (1979) The Radioracemization of Amino Acids by Ionizing Radiation: Geochemical and Cosmochemical Implications, *Orig. Life 9*, 279–290.

Cakir, S., Köksal, F., Tapramaz, R., and Cakir, O. (1991) ESR of Free Radicals in Some Legumes, Cereals and Their Aqueous Solutions Under Photolysis, *Radiat. Phys. Chem. 38*, 17–21.

Cicha, L., Suzuki, Y., Tateischi, N., Shiba, M., Muraoka, M., Tadokoro, K., and Maeda, N. (2000) Gamma-Ray Irradiated Red Blood Cells Stored in Mannitol-Adenine-Phosphate Medium: Rheological Evaluation and Susceptibility to Oxidative Stress, *Vox sanguinis 79*, 75–82.

Ciesielski, W., Achremowicz, B., Tomasik, P., Baczkowicz, M., and Korus, J. (1998) Starch Radicals. Part II: Cereals Native Starch Complexes, *Carbohydr. Polym. 34*, 303–308.

Ciesielski, W., and Tomasik, P. (1996) Starch Radicals. Part I. Thermolysis of Plain Starch, *Carbohydr. Res. 31*, 205–210.

Ciesla, K., Svensson, E., and Eliasson, A.-C. (1999) Preliminary Studies Using Differential Scanning Calorimetry of Radiation-Induced Transformations in Starch and Flour, *J. Therm. Anal. Calor. 56*, 1197–1202.

Colonna, P., Buléon, A., and Mercier, M. (1987) Physically Modified Starches, in *Starch: Properties and Potential* (Galliard, T., ed.) pp. 79–114, John Wiley and Sons, Chichester, UK.

Crowley, P., and Martini, L. (2001) Drug-Excipient Interactions, *Pharm. Technol. Eur. 13*, 26–28.

De Kerf, M., Mondelaers, W., Lahorte, P., Vervaet, C., and Remon, J.P. (2001) Characterization and Disintegration Properties of Irradiated Starch, *Int. J. Pharmaceut. 221*, 69–76.

Delincée, H. (1983) Recent Advances in Radiation Chemistry of Lipids, in *Recent Advances in Food Irradiation* (Elias, P.S., and Cohen, A.J., eds.) pp. 89–114, Elsevier Biomedicals, Amsterdam.

Dongowski, G., Neubert, R., Plaetzer, M., Schwarz, M., Schnorrenberger, B., and Anger, H. (1998) Interaction Between Food Components and Drugs. Part 6: Influence of Starch Degradation Products on Propanol Transport, *Pharmaziz 53*, 817–875.

Eerlingen, R., Jacobs, H., Block, K., and Delcour, J. (1997) Effects of Hydrothermal Treatments on the Rheological Properties of Potato Starch, *Carbohydr. Res. 297*, 347–356.

Elias, P.S., and Cohen, A.J. (1977) *Radiation Chemistry of Major Food Components*, Elsevier, Amsterdam, 220 pp.

Elias, P.S., and Cohen, A.J (1983) *Recent Advances in Food Irradiation*, Elsevier, Amsterdam, 361 pp.

Erlandsson, B., Karsson, S., and Albertsson, A.-C. (1997) Mode of Action of Corn Starch and a Pro-Oxidant System in LDPE: Influence of Thermo-Oxidation and UV-Irradiation on the Molecular Weight Changes, *Polym. Degrad. Stab. 55*, 237–245.

Fang, X., and Wu, J. (1998) Feasibility of Sterilizing Traditional Chinese Medicines by Gamma-Irradiation, *Radiat. Phys. Chem. 52*, 53–58.

Fanta, G., and Eskins, K. (1995) Stable-Starch-Lipid Compositions Prepared by Steam Jet Cooking, *Carbohydr. Polym. 28*, 171–175.

Galliard, T., and Bowler, P. (1987) Morphology and Composition of Starch, in *Starch: Properties and Potential* (Galliard, T., ed.) pp. 55–78, John Wiley and Sons, Chichester, U.K.

Gibella, M., Crucq, A.-S., Tilquin, B., Stocker, P., Lesgards, G., and Raffi, J. (2000) Electron Spin Resonance Studies of Some Irradiated Pharmaceuticals, *Radiat. Phys. Chem. 58*, 69–76.

Gilbert, B. (1995) ESR Studies of the Generation and Reactions of Free Radicals in Chemical and Biochemical Systems, *Radiat. Phys. Chem. 45*, 889–890.

Gilbert, B., King, D., and Thomas, C. (1982) Radical Reactions of Carbohydrates. Part 3. An ESR Investigation of Base-Catalysed Rearrangements of Radicals Derived from D-Glucose and Related Compounds, *J. Chem. Soc. Perkin Trans. II*, 169–179.

Gol'din, S., Shampryi, V., and Markecich, S. (1971) Formation and Conversion of Radicals in Glucose Polymers on Gamma Radiolysis, *Dok. Akad. Nauk. SSSR 201*, 133–136.

Grant, L., and d'Appolonia, B. (1991) Effects of Low-Level Gamma Radiation on Water-Soluble Nonstarchy Polysaccharides Isolated from Hard Red Spring Wheat Flour and Bran, *Cereal Chem. 68*, 651–652.

Han, Y., Ciegler, A., Courtney, J., Curry, W., and Lambremont, E. (1981) Gamma-Ray-Induced Degradation of Lignocellulosic Materials, *Biotechnol. Bioeng. 23*, 2525–2535.

Hauville, C., Rémita, S., Théroud, P., Jore, D., and Gardès-Albert, M. (2001) Radiation Induced Peroxidation of Polyunsaturated Fatty Acid: Recent Results on Formation of Hydroperoxides, *Can. J. Physiol. Pharmacol. 79*, 176–179.

Hombrercher, H.K., and Schell, C. (1998) Carbohydrate Substituted Porphyrins. Synthesis, Characterization and Lipoprotein Binding, *Carbohydr. Polym. 34*, 422–442.

Illum, L., Fisher, A.N., Jabbal-Gill, I., and Davis, S. (2001) Bioadhesive Starch Microspheres and Absorption Enhancing Agents Act Synergistically to Enhance the Nasal Absorption of Polypeptides, *Int. J. Pharmaceut. 222*, 109–119.

Inouye, M., Mio, T., and Sumino, K. (1999) Formation of 9-Hydroxy Linoleic Acid as a Product of Phospholipid Peroxidation in Diabetic Erythrocyte Membranes, *Biochem. Biophys. Acta 1438*, 204–212.

Jacobs, G.P., (1985) Current Trends in the Radiosterilization of Pharmaceuticals, *Isr. Pharm. J. 25*, 25–30.

Jauho, E.S., Boas, U., Wiuff, C., Wredstrom, K., Pedersen, B., Andresen, L.O., Heegaard, P.M., and Jakobsen, M.H. (2000) New Technology for Regiospecific Covalent Coupling of Polysaccharide Antigens in ELISA for Serological Detection, *J. Immunol. Methods 242*, 133–143.

Kakehashi, A., Akiba, J., Ueno, N., and Chakrabarti, B. (1993) Evidence for Singlet Oxygen-Induced Cross-Links and Aggregation of Collagen, *Biochem. Biophys. Res. Commun. 196*, 1440–1446.

Kang, I., Byun, M., Yook, H., Bae, C., Lee, H., Kwon, J., and Chung, C. (1999) Production of Modified Starches by Gamma Irradiation, *Radiat. Phys. Chem. 54*, 425–430.

Kato, Y., Nishikawa, T., and Kawakishi, S. (1995) Formation of Protein-Bound 3,4 Dihydroxyphenylalanine in Collagen Types I and IV Exposed to UV Light, *Photochem. Photobiol. 61*, 367–372.

Köksel, H., Sapirstein, H., Celik, S., and Bushuk, W. (1998) Effects of Gamma-Irradiation of Wheat on Gluten Proteins, *J. Cereal Sci. 28*, 243–250.

Korkmaz, M., and Polat, M. (2000) Free Radical Kinetics of Irradiated Durum Wheat, *Radiat. Phys. Chem. 58*, 169–179.

Langhout, D.J., Schutte, J.B., de Jong, J., Sloetjes, H., Verstegen, M.W., and Tamminga, S. (2000) Effect of Viscosity on Digestion of Nutrients in Conventional and Germ-Free Chicks, *Br. J. Nutr. 83*, 533–540.

Lewandowicz, G., Jankowski, T., and Fornal, J. (2000) Effect of Microwave Radiation on Physico-Chemical Properties and Structure of Cereal Starches, *Carbohydr. Polym. 42*, 193–199.

Liu, H., Arntfield, S., Holley, R., and Aime, D. (1997) Amylose-Lipid Complex Formation in Acetylated Pea Starch-Lipid Systems, *Cereal Chem. 74*, 159–162.

Madden, K., and Berhard, W. (1982) Thermally Induced Free-Radical Reactions in α-D-Glucopyranose Single Crystals. An ESR-ENDOR Study, *J. Phys. Chem. 86*, 4033–4036.

Mangala, S., Udayasankar, K., and Tharanathan, R. (1999) Resistant Starch from Processed Cereals: The Influence of Amylopectin and Non-Carbohydrate Constituents in Its Formation, *Food Chem. 64*, 391–396.

Manno, M., Emanuele, A., Martorana, V., San Biagio, P.L., Bulone, D., Palma-Vittorelli, M.B., McPherson, D.T., Xu, J., Parker, T.M., Urry, D.W. (2001) Interaction Processes on Different Length Scales in a Bioelastomer Capable of Performing Energy Conversion, *Biopolym. 59*, 51–64.

Merlin, A., and Fouassier, J.-F. (1981) Etude de Radicaux Libres Formés par Irradiation UV de l'Amidon: Application aux Réactions de Photo-Dégradation et de Photo-Greffage, *Makromol. Chem.; Macromol. Symp. 182*, 3053–3068.

Mestres, C., and Rouaux, X. (1997) Influence of Natural Fermentation and Drying Conditions on the Physicochemical Characteristics of Cassava Starch, *J. Sci. Food Agric. 74*, 147–155.

Nam, K.C., Du, M., Jo, C., and Ahn, D.U. (2001) Cholesterol Oxidation Products in Irradiated Raw Meat with Different Packaging and Storage Time, *Meat Sci. 28*, 431–435.

Nawar, W. (1977) Radiation Chemistry of Lipids, in *Radiation Chemistry of Major Food Components* (Elias, P.S., and Cohen, A.J., eds.) pp. 21–61, Elsevier, Amsterdam.

Nawar, W. (1983) Comparison of Chemical Consequences of Heat and Irradiation Treatment of Lipids, in *Recent Advances in Food Irradiation* (Elias, P.S., and Cohen, A.J., eds.) pp. 115–127, Elsevier, Amsterdam.

Nawar, W. (1985) Lipids, in *Food Chemistry* (Fennema, O.W., ed.) pp. 139–244, Marcel Dekker, New York.

Nawar, W., Zhu, Z., Wan, H., DeGroote, E., Chen, Y., and Aciukewicz, T. (1986) Progress in the Detection of Irradiated Foods by Measurement of Lipid-Derived Volatiles, in *Detection Methods for Irradiated Foods: Current Status* (McMurray, C.H., Stewart, E.M., Gray, R., and Pearce, J., eds.) pp. 241–248, Royal Soc. Chem., Cambridge, UK.

Neyssen, P. (2000) Practical Implications of Developments in Legislation on Food Irradiation in the European Union, *Radiat. Phys. Chem. 57*, 215–217.

Piccerelle, P., Reynier, J.-P., Joachim, J., Tilquin, B., and Raffi, J. (2000) Radio-Stérilisation de Médicaments: Intérêt, Législation et Travaux à Entreprendre, *J. Pharm. Belg. 55*, 131–136.

Pietranera, A., and Narvaiz, P. (2001) Examination of Some Prospective Conditions on Technological Properties of Irradiated Food Grade Polysaccharides, *Radiat. Phys. Chem. 60*, 195–201.

Raffi, J., and Agnel, J.-P. (1983) Influence of the Physical Structure of Irradiated Starches on their ESR Spectra Kinetics, *J. Phys. Chem. 87*, 2369–2373.

Raffi, J., and Agnel, J.-P. (1989) Electron Spin Resonance Identification of Irradiated Fruits, *Radiat. Phys. Chem. 34*, 891–894.

Raffi, J., Agnel, J.-P., and Kassis, S.-R. (1987) Identification par RPE de Céréales Irradiées, *Sci. Aliment. 7*, 657–663.

Raffi, J., Agnel, J.-P., Thiéry, C., Fréjaville, C., and Saint-Lèbe, L. (1981a) Study of Gamma Irradiated Starches Derived from Different Foodstuffs: A Way for Extrapolating Wholesomeness Data, *J. Agric. Food Chem. 29*, 1227–1232.

Raffi, J., Dauberte, B., d'Urbal, M., Pollin, C., and Saint-Lèbe, L. (1981b) Gamma Radiolysis of Starches Derived from Different Foodstuffs. Part IV. Study of Radio-Depolymerization, *Stärke 33*, 301–306.

Raffi, J., Gelly, S., Barral, L., Burger, F., Piccerelle, P., Prindere, P., Baron, M., and Chamayou, A. (2002) ESR Study of Radicals induced in Drugs and Relative Products by Different Treatments, *Spectrochimica Acta Part A 58*, 1313–1320.

Raffi, J., Saint-Lèbe, L., and Berger, G. (1978) Radiolyse de l'Amidon, in *Food Preservation by Irradiation,* Vol. 1, pp. 517–527, IAEA, Vienna.

Raffi, J., and Siadous, R. (1998) 1997–1998: A Turn in Food Irradiation, *Agro. Food Industry Hi-Tech 9*, 31–35.

Ross, R., and Engeljohn, D. (2000) Food Irradiation in the United States: Irradiation as a Phytosanitary Treatment for Fresh Fruits and Vegetables and for the Control of Microorganisms in Meat and Poultry, *Radiat. Phys. Chem. 57*, 211–214.

Roushdi, M., Harras, A., El-Meligi, A., and Bassim, M. (1983) Effect of High Doses of Gamma Rays on Corn Grains. Part II. Influence on Some Physical and Chemical Properties of Starch and Its Fractions, *Stärke 35*, 15–18.

Singh, N., Kaur, K., Singh, H., and Singh, H. (2000) Effect of Starch-Lipids Inclusion Complex Formation on Functional Properties of Flour in Tandoori Roti, *Food Chem. 69*, 129–133.

Sintzel, M.B., Merkli, A., Nabatabay, C., and Gurny, R. (1997) Influence of Irradiation Sterilization on Polymers Used as Drug Carriers: A Review, *Drug Dev. Ind. Pharm. 23*, 857–878.

Sokhey, A., and Chinnaswamy, R. (1993) Chemical and Molecular Properties of Irradiated Starch Extrudates, *Cereal Chem. 70*, 260–268.

Sriburi, P., Hill, S., and Barclay, F. (1999) Depolymerisation of Cassava Starch, *Carbohydr. Polym. 38*, 211–218.

Stecchini, M.L., Sarais, I., Del Torre, M., and Fuochi, P.G. (2000) Effect of Electron Irradiation and Packaging Atmosphere on the Survival of *Aeromonas hydrophila* in Minced Poultry Meat, *Radiat. Phys. Chem. 46*, 779–784.

Thiéry, C., Agnel, J.-P., Fréjaville, C., and Raffi, J. (1983) ESR Spin-Trapping Analysis of Gamma Induced Radicals in Polycrystalline Alpha-D-Glucose, *J. Phys. Chem. 87*, 4485–4488.

Thiéry, J.-M., Thiéry, C., Agnel, J.-P., Vincent, P., Battesti, C., Raffi, J., and Evans, J.C. (1990) Gamma Radiolysis of Glucose Oligomers: A Spin-Trapping Study, *Magn. Res. Chem. 28*, 594–600.

Triolet, J., Thiéry, C., Agnel, J.-P., Battesti, C., Raffi, J., and Vincent, P. (1992) ESR Spin Trapping Analysis of Gamma Induced Radicals in Sucrose: II, *Free Radic. Res. Commun. 16*, 183–196.

Tufvesson, F., and Eliasson, A.-C. (2000) Formation and Crystallization of Amylose-Monoglyceride Complex in a Starch Matrix, *Carbohydr. Polym. 43*, 359–365.

Tzaphlidou, M., Kounadi, E., Leontiou, I., Matthopoulos, D., and Glaros, D. (1997) Influence of Low Doses of Gamma-Irradiation on Mouse Skin Collagen Fibrils, *Int. J. Radiat. Biol. 71*, 109–115.

Von Sonntag, C. (1980) Free-Radical Reactions of Carbohydrates as Studied by Radiation Techniques, *Adv. Carbohydr. Chem. Biochem. 37*, 7–77.

Von Sonntag, C., Dizdaroglu, M., and Schulte-Frohlinde, D. (1976) Radiation Chemistry of Carbohydrates. VIII. Gamma Radiolysis of Cellobiose in N_2O Saturated Aqueous Solution. Part II. Quantitative Measurements: Mechanisms of the Radical-Induced Scission of the Glycosidic Linkage, *Z. Naturforsch. 31b*, 857–864.

Wang, Z.-N., and You, R.-L. (2000) Changes in Wheat Germination Following Gamma-Ray Irradiation: An *In Vivo* EPR Spin-Probe Study, *Environ. Exp. Bot. 43*, 219–225.

Wasserman, L.A., and Le Meste, M. (2000) Influence of Water on Potato Starch-Lipid Interactions. An ESR Probe Study, *J. Sci. Food Agric. 80*, 1608–1616.

World Health Organization (1980) Wholesomeness of Irradiated Food, *WHO Technical Report Series No. 659*, WHO, Geneva.

World Health Organization (1997) Foodborne Diseases—Possibly 350 Times More Frequent Than Reported, *Press Release 58*, 13 August.

World Health Organization (1999) High-Dose Irradiation: Wholesomeness of Food Irradiated with Doses Above 10 kGy, *WHO Technical Report Series No. 890*, WHO, Geneva.

World Health Organization (2000) Salubrité des Aliments et Maladies d'Origine Alimentaire, *Ann. Fals. Exp. Chim. 952*, 361–366.

Yin, J., Mossoba, M., Kramer, J.K., Yurawecz, M., Eulitz, K., Morehouse, K., and Ku, Y. (1999) Effects of Conjugated Linoleic Acid on Oxygen Diffusion-Concentra-tion Product and Depletion in Membranes by using ESR Spin-Label Oximetry, *Lipids 34*, 1017–1023.