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Volume 1: Understanding mechanisms of oxidation and antioxidant activity

Edited by
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D. Julian McClements

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Preface

Even though the involvement of peroxides in lipid oxidation has been known for over 100 years and the free radical mechanism of autooxidation was proposed over 60 years ago, many challenges still exist in controlling oxidative rancidity in foods. These challenges exist because the lipid oxidation process is influenced by a huge number of factors, many of which are specific for different food items. As was identified in the earliest studies on oxidative rancidity, these chemical pathways are dependent on temperature, oxygen concentrations and lipid type. In addition, the reactions often do not simply undergo autooxidation pathways, as they are also accelerated by various prooxidants which include enzymes, transition metals and reactive oxygen generators. Once the reaction starts, its rate is influenced by antioxidants that are endogenous to the food. These antioxidant systems can include free radical scavengers, metal chelators, singlet oxygen quenchers and antioxidant enzymes. Some of these antioxidant systems have been specifically designed by biological systems to inhibit lipid oxidation such as tocopherols, iron binding proteins (e.g. transferrin) and antioxidant enzymes. However, there are also numerous compounds in foods that were not specifically designed to inhibit oxidation but contain functional groups that can interact with free radicals and prooxidants. For example, proteins contain amino acids that can both scavenge free radicals and chelate transition metals. In addition, anthocyanidins and carotenoids, which are important in food color, can also scavenge free radicals, bind metals and inactivate singlet oxygen. These endogenous antioxidants are critical in the natural 'oxidative resistance' of foods.

The susceptibility of foods to lipid oxidation is increasing as food product developers attempt to make foods healthier by adding more polyunsaturated fatty acids, and by removing synthetic antioxidants that are causing consumer

concern for food safety. This means that to produce the new generation of healthier foods, new antioxidant technologies are needed to inhibit lipid oxidation. In order to create these new technologies, a better understanding of the basic chemical and physical attributes of foods that impact oxidative reactions is needed.

The first volume of this book provides a summary of the fundamental knowledge of lipid oxidation pathways. Part I provides an overview of the current state of understanding of lipid oxidation and then focuses on the major prooxidants in foods including transition metals, singlet oxygen, heme proteins and lipoxygenase. In addition, Part 1 contains chapters on how lipid oxidation impacts the sensory and health properties of foods and how lipid oxidation reactions can be monitored and predicted. How these oxidation pathways can be controlled is covered in Part 2. These chapters provide information on basic antioxidant mechanisms and discuss specific antioxidants such as proteins, phenolics and antioxidant additives. In addition, the impact of food structure and food processing operations on antioxidant activity are covered as are the methods that can be used to monitor the activity of antioxidants.

It is hoped that through a better understanding of the pathways that influence lipid oxidation in foods, better antioxidant technologies can be developed. Improved control of oxidation will be critical in meeting future challenges in the development of nutritious and healthy foods that will contain more oxidatively sensitive nutrients while being expected to meet the same – or better – shelf-life requirements without substantially increasing cost to the consumer.

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Understanding oxidation processes in foods

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Abstract: Oxidative deterioration in foods involves oxidation in both the aqueous phase (e.g., proteins) and the lipid phase (e.g., polyunsaturated lipids). Formation of free radicals is an early event that occurs prior to the progression of oxidation and is most often associated with the aqueous phase. Linear free energy relationships are found valuable for classification of such early events as electron transfer and hydrogen atom transfer. Inspiration for protection of processed foods against oxidative deterioration of their vulnerable constituents has been found in the antioxidant mechanisms appearing during evolution of aerobic life forms in an increasingly oxidizing atmosphere. A two-dimensional classification of antioxidants opens up for an understanding of the special role of carotenoids, and optimal protection seems to depend on a proper balance between antioxidants and antireductants.

Key words: food protection, protein oxidation, lipid oxidation, free radical kinetics, antioxidants, antireductants.

1.1 Introduction

Other forms of life than the aerobic forms dominating now were previously characteristic of our planet. When, almost three billion years ago, blue-green algae (*Cyanobacteria*) developed photosynthesis, oxygen accumulated in terrestrial atmosphere concomitant with oxygenation of the shallower surface ocean (Frei *et al.*, 2009). The oxygenation of the Earth's atmosphere seems to have fluctuated and lagged behind the development of oxygenic photosynthetic organisms in the upper ocean because bacteria became deprived of nitrogen

available for their growth and accordingly production of oxygen (Godfrey and Falkowski, 2009). Oxygen is a potent oxidant and changed the conditions for life dramatically initially in the oceans and in two spikes separated by a lower level period also in the atmosphere. The evolution of oxygenic photosynthesis caused the greatest selective pressure on primordial life (Benzie, 2003), since life dependent on one-electron Fe(III)/Fe(II) cycling and oxygen invariably will create reactive oxygen species (ROS), which need to be controlled by scavenging or through further reaction. During early evolution endogenous protection systems had accordingly to be developed for protection against oxidative stress together with development of chelators for tuning of Fe(III)/Fe(II) reduction potentials in electron transport systems and for prevention of precipitation of Fe(III) as hydroxide in the increasingly oxidizing atmosphere. It is the paradox of aerobic life, that oxidative damage occurs to key metabolic sites, and this continuing threat prompted the development of customized antioxidants concomitant with the appearance of various aerobic life forms.

1.2 Reactive oxygen and nitrogen species

Due to spin restrictions, atmospheric oxygen is rather unreactive, since ground state oxygen is a biradical and as such a triplet, whereas organic compounds are singlets. However, in a series of one-electron reductions as part of respiration, the reactive forms of oxygen such as superoxide, hydrogen peroxide, and the hydroxyl radical, have transient appearance during formation of water:

$$O_2 \xrightarrow{e^-} O_2^-/{}^\bullet O_2H \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} {}^\bullet OH \xrightarrow{e^-} H_2O \qquad 1.1$$

Especially the hydroxyl radical, $^\bullet$OH, is highly reactive with rates of reaction with most organic compounds like lipids approaching the diffusion limit. Such high reactivity is evident, for example, for wine and other alcoholic beverages in which any hydroxyl radicals formed during maturation or oxidative deterioration are converted to the l-hydroxyethyl radical by reaction with ethanol prior to any further reaction (Elias *et al.*, 2009). The spin restriction for the initial electron transfer to yield the superoxide radical as in the reaction sequence of eq. 1.1, is revoked by reaction of oxygen with transition metal ions either in enzymes like lipoxygenases (iron-based) or in simple hydrated ions under some conditions:

$$Fe^{2+}_{aq} + O_2 \rightarrow Fe^{3+}_{aq} + O_2^- \qquad 1.2$$

Likewise, the superoxide radical may leak from the mitochondria during respiration. The spin restriction, i.e. singlet/triplet reactions are forbidden, is also circumvented through reaction of organic material with singlet oxygen, $^1O_2$, which is electronically excited oxygen with spin pairing or with the allotropic form, ozone, O_3. Singlet oxygen is formed in some highly exergonic reactions but more importantν in photosensitized reactions like:

$$^1\text{Riboflavin} \xrightarrow{h\nu} {}^1\text{Riboflavin}^* \qquad 1.3$$

$$^{1}\text{Riboflavin}^{*} \xrightarrow{\text{ISC}} {}^{3}\text{Riboflavin}^{*} \quad 1.4$$

$$^{3}\text{Riboflavin}^{*} + {}^{3}O_2 \rightarrow {}^{1}\text{Riboflavin} + {}^{1}O_2 \quad 1.5$$

in which riboflavin, vitamin B_2, as present in tissue and many foods, absorbs light and through efficient intersystem crossing (ISC) yields the longer lived triplet-riboflavin subsequently reacting with ground state oxygen (or acting as an oxidant itself). Ozone is formed during electric discharge or by UV-light absorption by oxygen in the atmosphere:

$$3O_2 \xrightarrow{h\nu} 2O_3 \quad 1.6$$

Formation of reactive nitrogen species (RNS) is likewise linked to aerobic life manifestations in close interplay with formation and decay of ROS and with Fe(III)/Fe(II) redox cycling (Carlsen *et al.*, 2005). Although less studied than ROS, RNS such as peroxynitrite, $ONOO^-$, formed by reaction of the superoxide ion with nitric oxide, an important signalling molecule and also formed in nitrite-cured meat:

$$NO + O_2^- \rightarrow ONOO^- \quad 1.7$$

is involved in oxidative protein modifications like nitration of tyrosine residues (Ischiropoulus and Almehdi, 1995). Carbon dioxide present in the atmosphere throughout evolution may also be involved in oxidative stress through formation of free radicals (Goldstein and Czapski, 1998):

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow NO_2 + CO_3^- \quad 1.8$$

Transformations among ROS and RNS are often assisted by enzymes such as oxidoreductases depending on transition metal mediated electron transfer in the catalytic site. Myoglobin, traditionally considered solely as an oxygen storage protein, seems to have far wider functions as a chemical reactor for small molecules like O_2 and NO in relation to the balance between aerobic and anaerobic metabolism (Frauenfelder *et al.*, 2001). In Fig. 1.1 is shown as an example, the oxidation of nitrosylmyoglobin, which is also highly relevant for oxidative deterioration of cured meat. Both $O_2^{\bullet -}$ and $^{\bullet}NO$ are radicals (doublets), but in animal tissue (muscle/meat) they act differently, when they are released from iron(II) heme pigments like myoglobin, since $O_2^{\bullet -}$ is a prooxidant through conversion to H_2O_2 or $^{\bullet}OH$, while $^{\bullet}NO$ is an antioxidant through scavenging of other radicals (Kanner *et al.*, 1991):

$$\text{MbFe(II)}O_2 \rightarrow \text{MbFe(III)} + O_2^- \quad 1.9$$

$$\text{MbFe(II)NO} \rightarrow \text{MbFe(II)} + NO \quad 1.10$$

HNO, nitrosyl, also involved in regulation of oxidative stress, is hampered in proton dissociation, since NO^-, isoelectronic with O_2, is in a triplet state. NO^-, which otherwise would form the strong oxidant $ONOO^-$ by reaction with O_2, cf. eq. 1.7, is not produced under physiological conditions due to the spin restriction for the proton dissociation. HNO in contrast reacts as an oxidant rather

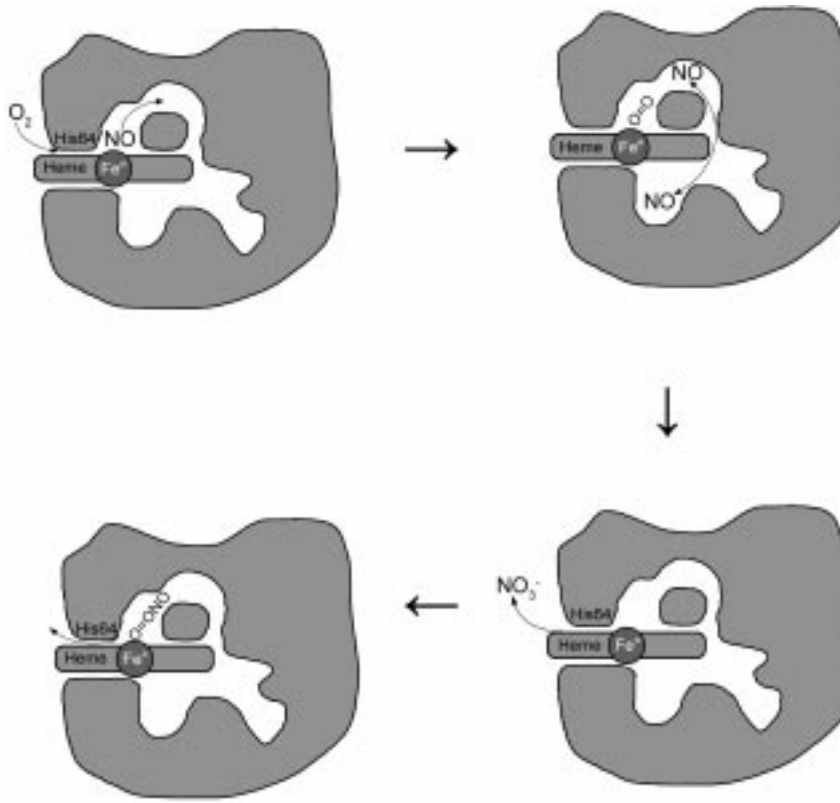

Fig. 1.1 Heme pigments are often involved in interaction between reactive oxygen species and reactive nitrogen species as shown for oxidation of NO by oxygen in nitrosylmyoglobin with the heme cavity acting as a chemical reactor as suggested by Frauenfelder *et al.* (2001) to yield peroxynitrite/nitrate and metmyoglobin. Absorption of visible light opens up for a parallel reaction pathway initiated by photodissociation of nitric oxide.

specifically with thiols and may be involved in thiol depletion and accordingly protein modification (Paolocci *et al.*, 2007). HNO is a unique molecule, as it may donate a hydrogen atom to a lipid peroxyl radical ($LOO^{\bullet}$):

$$HNO + LOO^{\bullet} \rightarrow NO + LOOH \qquad 1.11$$

in a process typical for chain-breaking antioxidants, as discussed in Section 1.4. $^{\bullet}NO$ formed, may further react as a radical scavenger and chain breaker in lipid autoxidation:

$$^{\bullet}NO + LOO^{\bullet} \rightarrow LOONO \rightarrow LONO_2 \qquad 1.12$$

The role of the unusual acid/base couple HNO/NO^{-} in oxidative processes should be further explored.

1.3 Evolution of antioxidants

Various foods are known to have strongly varying oxidative stability and since food resistance to oxidative deterioration is linked to the antioxidants present in the actual food raw-material, it is of relevance to try to follow the evolution of antioxidants as challenged by the changing conditions for aerobic life. Compounds now considered as valuable antioxidants may have appeared through evolution with other functions or with other functions in addition to be protectors against oxidative stress. In Table 1.1 an attempt is made to classify antioxidative protection into eight groups also in relation to contemporary food technology.

1.3.1 Physical barriers

Organisms may obtain protection against oxidation simply by exclusion of oxygen by physical barriers, as has been suggested as one of the first antioxidant mechanisms (Benzie, 2003). Notably, oxygen is only sparingly soluble in water but more freely soluble in organic phases like lipids and membranes; still oxygen penetration is not always controlled simply by diffusion, but may need thermal activation as has been shown for lipids encapsulated in glassy food matrices (Andersen *et al.*, 2000).

1.3.2 Photosynthesis

Photosynthesis, as the primary event in appearance of aerobic life and in all food production, converts the energy of sunlight through formation of electronically excited states to chemical energy by reduction of carbon dioxide to carbohydrates for energy storage with concomitant oxygen evolution. Plants and other photosynthetic organisms produce oxygen vital for life and at the same time damaging own tissue. Early photosynthetic organisms developed antioxidants such as carotenoids for their own protection. Carotenoids, which notably only are synthesized in the plant kingdom and in various forms, are associated with photosynthesis as developed in bacteria and plants. Carotenoids protect the phytosynthetic apparatus against oxidative damage caused by electronically excited states, but are in the marine feed chains transferred from phytoplankton to krill and further to shellfish and some fish, and similarly carotenoids are transferred from plants to land-based animals including humans. The role of carotenoids as a physical quencher of singlet oxygen converting the excitation energy to heat and consequently protecting tissue against light-induced oxidation is well-documented (Lee and Min, 1988):

$$^1O_2 + \text{car} \rightarrow {}^3O_2 + {}^3\text{car}^* \qquad 1.13$$

$$^3\text{car}^* \rightarrow \text{car} + \text{heat} \qquad 1.14$$

The role of carotenoids as radical scavengers in preventing oxidative changes is in contrast still controversial (Mortensen *et al.*, 2001). Carotenoids are

Table 1.1 Evolution of protection against oxidative stress for aerobic life as seen as inspiration for food technology

Type of protection/ protectants	Evolutionary step	Functions	Food protection
Physical barriers	Compartmentation in early life forms	Hampering access to oxygen	Encapsulation techniques Packaging systems
Carotenoids	Appearance of photosynthetic organisms and development of vision	Quenching of electronically excited states, scavenging of radicals	Light absorption (inner filter effects) Singlet oxygen quenching Synergistic radical scavenging
Iodide	Protection of (light exposed) marine life	Scavenging of ROS including ozone and singlet oxygen through iodide/ iodine cycling	To be explored
Siderophores, proteins	Acquisition and storage of iron and other minerals essential for aerobic life	Chelating iron(II)/iron(III) for tuning of redox-potentials for electron transport, iron homeostatis	Prevention of radical formation by chelation of transition metal ions
Enzymes (oxidoreductases)	Oxygen activation and deactivation in aerobic life forms, metabolic feed back systems	Controlling redox potentials in tissue, defence against infections, specific deactivation of ROS and NOS	Oxygen depletion, scavenging of superoxide and peroxides
Polyphenols	Development of allelopathy, surface protection of plants	Repairing plant tissue through oxidative polymerization (enzymatic), metal binding, radical scavenging, quenching of excited states	Addition of botanicals or their extracts to processed foods and beverages for multifunctional protection
Tocopherols/tocotrienols	Appearance of plant seeds with longevity	Radical scavenging in lipophilic compartments, chain-breaking in lipid autoxidation	Enhancement of oxidative stability of meat, milk and other animal based food items through change in animal feed Antioxidant supplementation to food
Uric acid, ascorbic acid, glutathione	Diversification in nitrogen excretion and herbivore/ omnivorous differentiation	Redox communication in hydrophilic compartments, protein function adjustment	Addition for oxygen depletion and tocopherol regeneration

unevenly distributed in animal tissue following intake as feed or food. The upconcentration of zeaxanthin and lutein in the yellow spot in the human eye is crucial for protection of the visual function against oxidative stress, while the importance of uptake and distribution of other carotenoids by animals is less evident except for the function of some carotenoids as provitamin A and for some birds of other carotenoids as plumage colorants.

1.3.3 Iodide

Iodide became important during early evolution as a protector against ROS, and iodide/iodine cycling is probably one of the most ancient mechanisms of defence against poisonous ROS. Coastal seawaters contain approximately 60 μg iodine per litre, and algae and kelp accumulate iodide, which together with peroxidases scavenges ROS (Venturi and Venturi, 2007). Iodide has recently been identified in *Laminariales* (kelp), the strongest accumulators of iodine among living organisms, as an important inorganic antioxidant, which upon oxidative stress submerged in seawater, scavenge oxygen radicals and peroxides, and in addition upon exposure to the atmosphere at low tide, reacts with ozone (Küpper *et al.*, 2008). Notably, iodide reacts with ozone, singlet oxygen and superoxide radicals at rates up to 500-fold higher than ascorbate and glutathione. Marine lipids as synthesized by algae and seaweed rank among the molecules most sensitive to especially ROS due to their high unsaturation linked to their low melting point necessitated by the often cold marine environment. Iodide seems throughout evolution to have had crucial roles for the protection of these energy-dense compounds of outmost importance for marine life. As may be seen from Table 1.2, the reaction between the ROS mentioned so far and iodide is all thermodynamically favourable, which is in contrast to the reactions with bromide (or chloride). The second-order rate constants for reaction with iodide indicate fast reactions even approaching the diffusion limit except for the reaction with hydrogen peroxide (Küpper *et al.*, 2008). For the reaction with hydrogen peroxide, enzymes like the iodoperoxidases found in kelp facilitate removal of hydrogen peroxide through formation of iodinated organic compounds like iodo-tyrosine:

$$2I^- + 2H_2O_2 + 2\text{tyrosine} + 2H^+ \rightarrow 2\ \textit{iodo} - \text{tyrosine} + 4H_2O \qquad 1.15$$

Table 1.2 Stoichiometry, reaction free energy and second-order rate constant for reaction of iodide with reactive oxygen species in aqueous solution at ambient conditions[a]

Reaction	ΔG^o (kJ mol^{-1})	k_2 (l mol^{-1} s^{-1})
$I^- + {}^\bullet OH \rightarrow I^\bullet + OH^-$	−37.7	$1.2 \cdot 10^{10}$
$I^- + {}^1O_2 + H^+ + H_2O \rightarrow HIO + H_2O_2$	−36.4	$8.7 \cdot 10^5$
$I^- + HO_2^\bullet + H^+ \rightarrow I^\bullet + H_2O_2$	−10.6	$1.0 \cdot 10^{8}$ [b]
$I^- + O_3 + H^+ \rightarrow HIO + O_2$	−210.8	$1.2 \cdot 10^9$
$2I^- + H_2O_2 + 2H^+ \rightarrow I_2 + 2H_2O$	−237.1	0.69

[a] Based on Küpper *et al.* (2008).
[b] Rate constant is for reaction between I_3^- med $O_2^{\bullet -}$.

Iodinated organic compounds are leached from the kelp into the sea and those compounds of low vapour-pressure are further transferred into the atmosphere as part of iodine circulation in nature.

1.3.4 Siderophores, proteins

Acquisition by aerobic life forms of the essential iron from the environment in order to maintain iron homeostatis depends on transport against concentration gradients. Bacterial and plant sideophores are strong chelators, in effect mobilizing iron from minerals. Iron redox catalysis including unintended oxygen activation is controlled likewise by chelation as in low-molecular-weight complexes with citrate and ATP as ligand and by *in situ* mineralization like ferrihydrit encapsulated in iron-storage proteins (Ghosh *et al.*, 2008). Chelation of transition metal ions like iron and copper together with mineralization accordingly also serve the purpose of antioxidative protection. Iron and copper may otherwise activate oxygen as through the reaction of eq. 1.2 or by cleavage of peroxides to yield the hydroxyl radical as shown in Fig. 1.2 for the ascorbate driven Fenton reaction.

1.3.5 Enzymes

Enzymatic oxygen activation as part of controlled metabolism depends mainly on two types of iron coordination exemplified by lipoxygenase with carboxamid/carboxylate/histidine binding of iron and by lactoperoxidase in

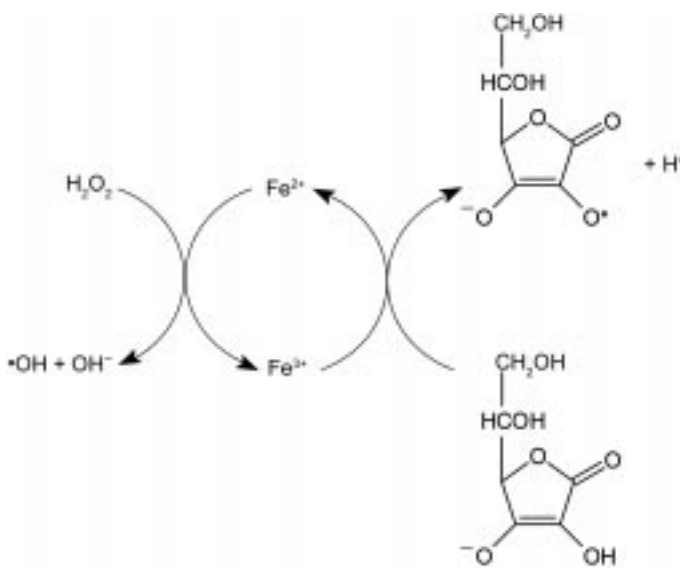

Fig. 1.2 Formation of the hydroxyl radical in the Fenton reaction by reductive cleavage of hydrogen peroxide showing prooxidative effects of ascorbate.

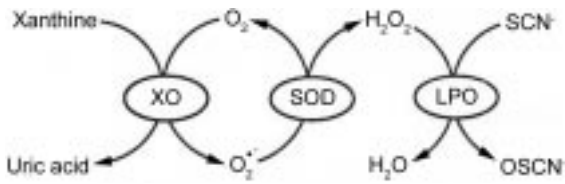

Fig. 1.3 Concerted action of enzymes in oxygen activation. Xanthine oxidase (XO) in fat globule membranes in milk reduces oxygen to superoxide anion through oxidation of xanthine to uric acid, while superoxide dismutate (SOD) converts the superoxide radical initially formed to hydrogen peroxide, the substrate of lactoperoxidase (adapted from Carlsen *et al.*, 2005). Lactoperoxidase (LPO) oxidizes thiocyanate to antimicrobial agents.

which the porphyrin ring system stabilizes even higher oxidation numbers than iron(II) and iron(III) creating strong prooxidants based on hypervalent iron(IV) and iron(V) (Carlsen *et al.*, 2005). An example of such controlled oxygen activation by concerted actions of enzymes is shown in Fig. 1.3. In milk, three enzymes fight in a concerted action growth of bacteria. Xanthine oxidase, a molybdenum containing enzyme associated with the fat globulus membrane, uses oxygen as an electron acceptor for oxidation of xanthine to yield the superoxide radical anion. Superoxide dismutase (SOD), a copper/zinc enzyme, accelerates the disproportionation of superoxide:

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \qquad 1.16$$

to yield oxygen and hydrogen peroxide. Hydrogen peroxide is, together with thiocyanate substrate for lactoperoxidase, an enzyme with porphyrin coordination of iron, which yields thiocyanogen and other antimicrobial agents protecting the milk.

1.3.6 Polyphenols

Polyphenols became ubiquitous in the plant kingdom for protection of plants against UV-irradiation. Polyphenols further provide a repair mechanism for plants through oxidative polymerization by enzymes following mechanical damage. Polyphenols also form the basis for important colorants for flowers and fruits like the anthocyanins and may in certain cases also be responsible for allelophathy. High concentrations of polyphenols in unripe fruits may further protect against insect attack. Polyphenols like the catechins from tea and quercetin from onions further chelate metal preventing Fenton reactions, which seem important for protection of sensitive tissue like root tips (Chabot *et al.*, 2009). Although polyphenols (φ-OH) may have appeared through evolution initially with other primary functions, they constitute in their rich structural diversity an important group of so-called chain-breaking antioxidants as the hydrogen-oxygen bond energy is rather small and smaller than the hydrogen oxygen bond energy in lipid hydroperoxides (LOOH):

$$\varphi - OH + LOO \rightarrow \varphi - O + LOOH \qquad 1.17$$

The phenoxyl radical formed is less reactive and acts as a dead-end for radicals of the chain reaction characteristic for lipid oxidation, as discussed in Section 1.4.

1.3.7 Tocopherols/tocotrienols

Tocopherols are more lipophilic than the polyphenols and especially than their glycosides and are as such associated with membranes including membranes in the photosynthetic apparatus. Tocopherols and tocotrienols protect unsaturated lipids such as phospholipids in membranes against oxidation according to the mechanism described for polyphenols (eq. 1.17). Tocopherols are abundant in seeds of various types and appear accordingly together with tocotrienols in oils made from such seeds. One of the four homologous tocopherols, α-tocopherol, is vitamin E. α-tocopherol is important in animal nutrition either from natural sources or as a supplement in order to obtain animal based food with good oxidative stability and better nutritive value (Sandström *et al.*, 2000). Notably, only one of the eight isomers is synthesized in nature (Fig. 1.4), and animals discriminate during uptake and excretion between the optical isomers in the synthetic mixtures most often used as feed supplements (Slots *et al.*, 2007).

1.3.8 Uric acid, ascorbic acid, glutathione

The last group of antioxidants included in Table 1.1, are a number of water-soluble compounds, belonging to different metabolic groups. Ascorbate, a reductone appearing in carbohydrate metabolism in plants and most animals, may act both as an antioxidant through depletion of oxygen or through regeneration of lipid-based antioxidants like α-tocopherol at lipid/water interfaces, and as a prooxidant being a reductor in the Fenton reaction (Fig. 1.2). For high concentrations of ascorbate, the antioxidant function normally dominates, while during depletion of ascorbate, the prooxidative effects become more important. Uric acid is less soluble than ascorbic acid/ascorbate in water,

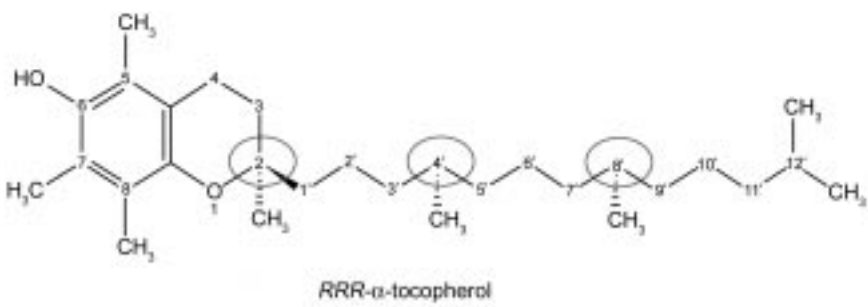

Fig. 1.4 α-tocopherol and homologe β, γ, and δ-tocopherols are synthesized in nature only as the R,R,R-isomer, while synthetic α-tocopherol used as feed supplement is a mixture of the eight optical isomers.

but is an effective radical scavenger. The concentration of urate, which is a breakdown product of nucleotides (cf. Fig. 1.3), may be increased in milk through feeding the dairy cows a low-fat feed with high rumen fermentability using wheat rather than maize, in effect increasing the oxidative stability of milk especially when exposed to light (Østdal *et al.*, 2008). Cystein containing peptides including glutathione became through evolution important as thiol/disulfide redox switches under conditions of oxidative stress in the cytosol or in relation to protein functionality adjustment (Wouters *et al.*, 2010).

1.4 Lipid phase oxidations

Lipids are more energy-dense than carbohydrates, which initially are formed as electron-rich compounds in photosynthesis, and especially the apolar lipids such as the triglycerides are important for energy storage in many plants and in animals. The more polar lipids including the phospholipids are building blocks for membranes. Besides classifications according to polarity, lipids may also be classified as saponifiable including triglycerides, phospholipids and waxes, and non-saponifiable including terpenes, carotenoids, tocopherols and steroids. Ease of oxidation for lipids depends on unsaturation, however, all lipids are subject to oxidation.

Oxidation of lipids is initiated by irradiation including exposure to visible light, by enzymes and metal catalysis. Heat and pressure accelerate lipid oxidation when initiated. From Fig. 1.5 the three major reaction paths involved in initiation of lipid oxidation may be identified:

(I) Oxygen activation by metal catalysis including activation by oxido-reductases to yield the hydroxyl radical subsequently abstracting an allylic or bis-allylic hydrogen from an unsaturated lipid (LH). Other radicals like the chlorine radical from disinfectants or generated by myeloperoxidases may also initiate the chain reaction through formation of a carbon-centered lipid radical ($L^{\bullet}$), which freely reacts with ground state oxygen.

(II) Lipoxygenases incorporating oxygen in the unsaturated lipid to yield lipid hydroperoxides like in chicken meat and vegetables.

(III) Photosensitized oxidation as described in eq. 1.3–1.5 for riboflavin, to yield singlet oxygen which as an electrophile may add to the double bond of unsaturated lipids to yield lipid hydroperoxides directly as for lipoxygenases. In addition to the singlet oxygen mediated (Type II, physical quenching) photosensitized lipid oxidation shown in Fig. 1.5, triplet excited photosensitzers may as strong oxidants form lipid radicals by hydrogen atom abstraction or by electron abstraction followed by deprotonation from the lipid substrate (Type I, chemical quenching) to form radicals as will initiate chain reaction as described for metal catalysis (I).

The lipid hydroperoxides formed in the lipoxygenase initiated lipid oxidation or in the Type II photosensitized lipid oxidation may be cleaved reductively by

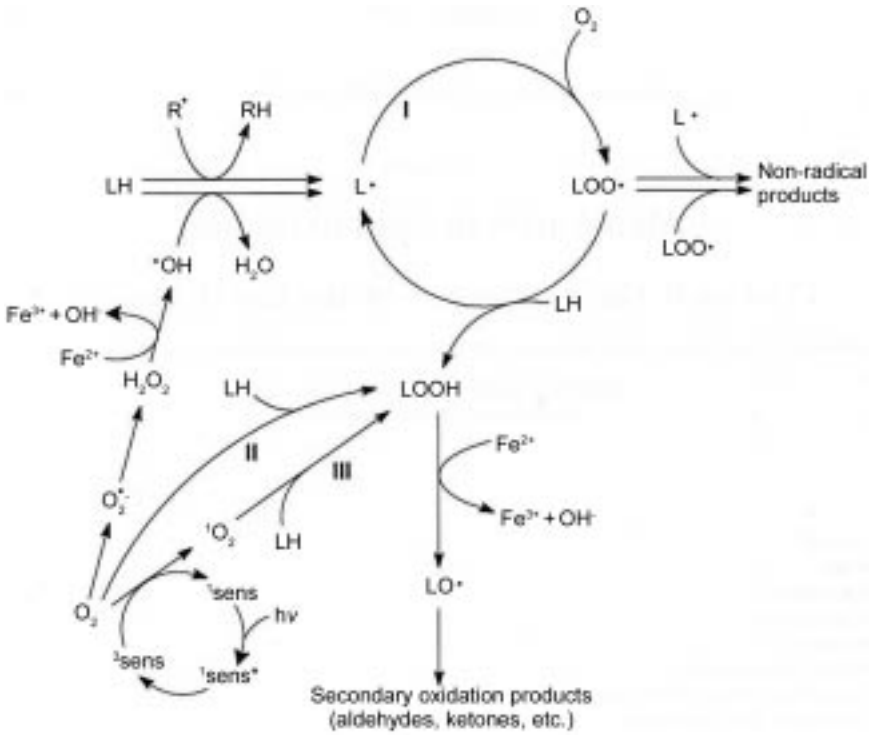

Fig. 1.5 Three major reaction paths are responsible for lipid oxidation in food: I) Free radical chain reaction initiated by oxygen activation to yield hydroxyl radicals or by radicals formed by irradiations or chemical oxidations. II) Enzymatic formation of lipid hydroperoxides through lipoxygenase activity. III) Photosensitized formation of lipid hydroperoxides (adapted from Carlsen *et al.*, 2005).

metal catalysis (Fe(II)/Fe(III) or Cu(I)/Cu(II)) as in the Fenton reaction of Fig. 1.2 to yield lipid alkoxyl radicals ($LO^{\bullet}$) initiating new chain reactions through

$$LO^{\bullet} + LOOH \rightarrow LOH + LOO^{\bullet} \qquad 1.18$$

or leading to secondary lipid oxidation products like aldehydes and ketones as are characteristic for rancid lipids. Lipid hydroperoxides may also be cleaved by oxidation by hypervalent heme pigments as in meat to initiate chain reactions from preformed lipid hydroperoxides:

$$MbFe(IV)=O + LOOH \rightarrow MbFe(III) + LOO^{\bullet} + OH^{-} \qquad 1.19$$

Reactions like the hydrogen atom transfer of eq. 1.18 depend on the relative strength of the oxygen/hydrogen bonds, and further the mechanisms of chain breaking antioxidants is a result of the ease by which the reaction of eq. 1.17 occurs. The relative bond strength for lipids and antioxidants are depicted in Fig. 1.6. Ascorbate will eventually transfer a hydrogen atom to phenoxyl radicals in effect regenerating the phenolic antioxidants. Notably, the oxygen/hydrogen

Table 1.3 Bond dissociation energies (BDE) for hydrogen-oxygen bonds in phenolic antioxidants in lipophilic environment[a] and standard reduction potentials in aqueous solution for their one-electron oxidized radicals[b]

Φ-OH	BDE (kJ mol^{-1})	E^{o} / V vs NHE
α-tocopherol	330.0	0.31
β-tocopherol	335.3	
γ-tocopherol	334.9	
δ-tocopherol	341.5	
Quercetin	343.0	0.29
(–) Epicatecin	343.2	0.33
Catecin	348.1	0.36
(–) Epigallocatecin	344.6	
(–) Epigallocatecholgallate	339.0	

[a] From Denisova and Denisov (2008). Solvent for tocopherols is styrene, for the plant polyphenols methyl linoleate micelles in water were used.
[b] From Jørgensen and Skibsted (1998). Solvent is aqueous solution with pH = 6.4 and 25 ºC. Value reported for α-tocopherol is based on analogy with the water-soluble Trolox.

bond strength shows large variation among individual plant phenols providing the rich diversity in antioxidant properties known for this group of secondary plant metabolites. The bond dissociation energy (BHE) may be based on quantum mechanical calculations and be valid for the gas phase only (Amié and Lucié, 2010), or be based on the rate for reaction with peroxyl radicals corresponding to the reaction of eq. 1.17 in lipophilic solvents or in a lipophilic environment (Denisova and Denisov, 2008). For a few phenolic antioxidants values for BHE in lipophilic solvents or in a lipophilic environment for discussion of antioxidant synergism in Section 1.6 are found in Table 1.3. α-tocopherol has the lowest BHE among the lipophilic antioxidants, which further is smaller than the BHE for plant phenols. For comparison, the BHE for the hydrogen-oxygen bond in lipid hydroperoxides is approximately 380 kJ mol^{-1}, establishing the sequence for reactivity of radicals shown in Fig. 1.6.

Oxidation of neat lipids is relatively well understood, and the rate of lipid oxidation depends mainly on the degree of unsaturation with fish oil oxidizing faster than plant oils, and with animal fat especially of ruminants being most stable. Lipid oxidation is characterized by a lag phase, which for comparable unsaturation depends mainly on the nature and concentration of antioxidants present in the oil or fat. In disperse systems initiation of oxidation is linked to the increased surface of the lipids and lipids become more vulnerable to oxidation especially in the presence of iron or copper ions which may bind to negatively charged surfactants in the lipid/water interface. For oil-in-water emulsions spatial location of antioxidants has been shown to be of outmost importance (Laguerre *et al.*, 2009). Traditionally the so-called polar paradox, i.e. the fact that hydrophilic antioxidants are efficient in bulk oil while lipophilic antioxidants are more efficient in emulsions, has been used for

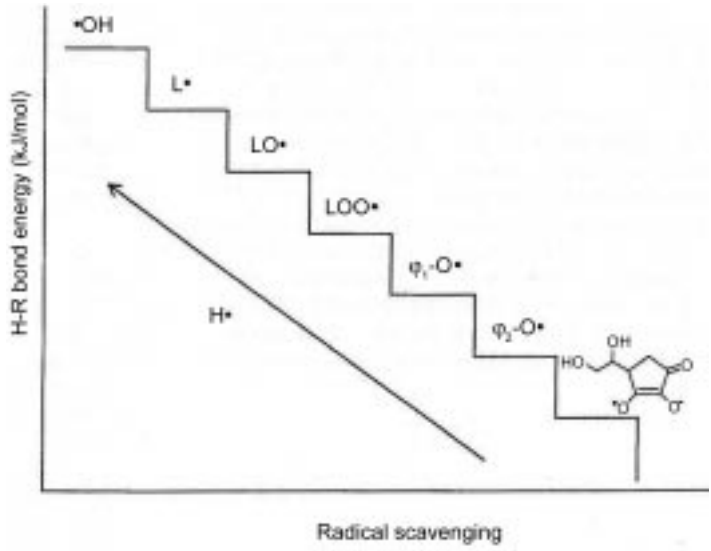

Fig. 1.6 A simplistic stairway for radical processes related to antioxidant scavenging of radicals, and antioxidant regeneration. Φ_1-O$^\bullet$ and Φ_2-O$^\bullet$ are phenoxyl radicals of antioxidants like flavonoids and α-tocopherol (adapted from Becker *et al.*, 2004). Ascorbate is the most reducing of the antioxidants and may regenerate any of the phenolic antioxidants depending on spatial location.

practical optimization of protection of lipids in food for long-term storage (Porter *et al.*, 1989).

The primary event in lipid oxidation is formation of free radicals followed by appearance of lipid hydroperoxides as primary lipid oxidation products further leading to secondary lipid oxidation products as shown in Fig. 1.5. The concentration of lipid hydroperoxides may go through a peak value during storage and again decrease when the rate of their breakdown to yield secondary lipid oxidation products becomes faster than their formation depending on temperature conditions (Aragao *et al.*, 2008). In food systems with low molecular mobility it is, however, possible to detect and quantify the otherwise highly reactive free radicals as precursors to lipid hydroperoxides providing further insight in the very early events of oxidation in food and beverages (Andersen and Skibsted, 2006). In fresh milk powder, the level of free radicals is thus very low; however, during storage even under the mildly accelerated conditions of 60 ºC, milk powder turns brown after approximately one week (Fig. 1.7). Once brown discoloration appears, the water activity increases suddenly in the closed containers indicating crystallization of the initially amorphous lactose in the milk powder resulting in decreased water binding and with the concomitant appearance of free radicals, as detected by electron spin resonance (ESR) spectroscopy (Thomsen *et al.*, 2005). Further changes in the milk powder include formation of secondary lipid oxidation products with characteristic off-flavours providing an example of a food for which early radical formation is

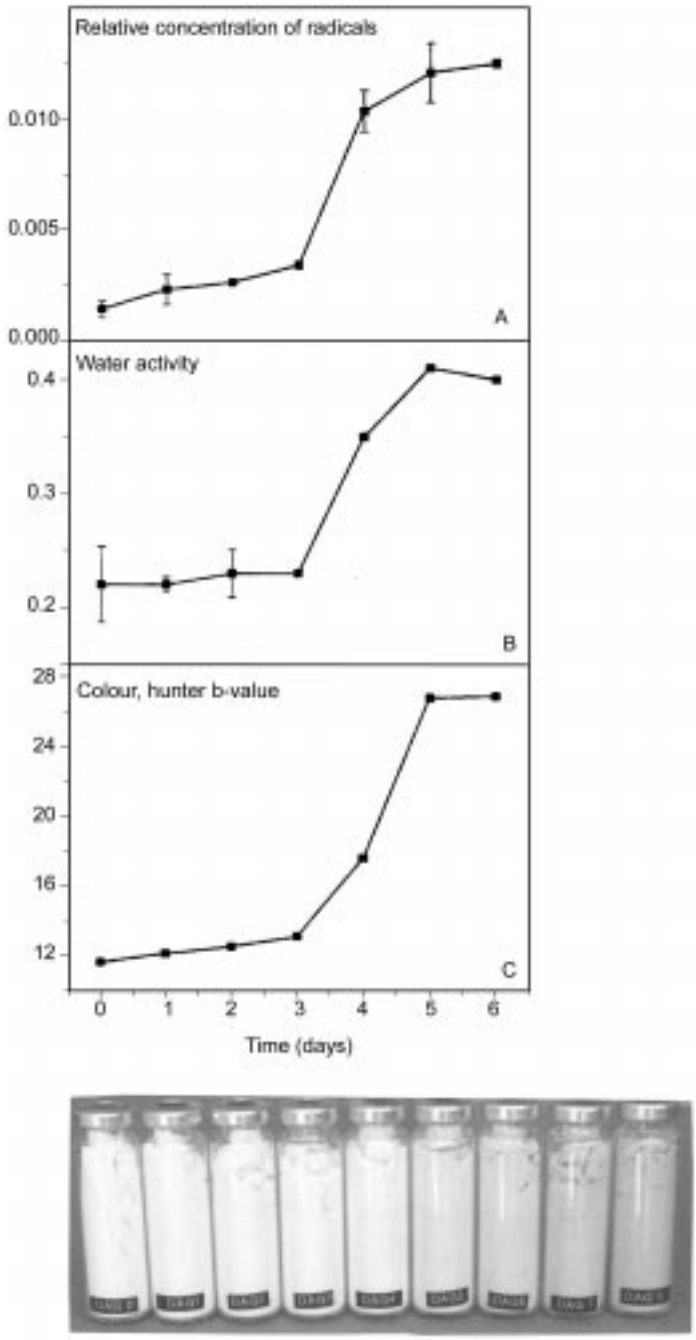

Fig. 1.7 Coupling between formation of free radicals (A), water activity (B), and browning (C) of milk powder during storage at 60 ºC for one week in closed containers as shown in photographs (partly from Thomsen *et al.*, 2005).

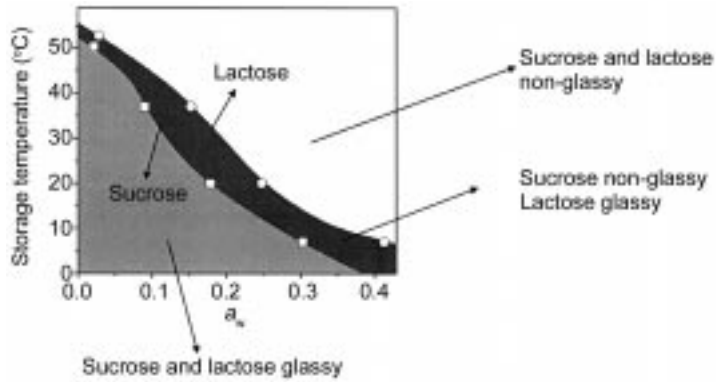

Fig. 1.8 A temperature/water-activity phase diagram for selection of storage conditions for a glassy food system based on calorimetric investigations (based on Kurtmann *et al.*, 2009). The milk powder shown in Fig. 1.7, for which lactose is mainly responsible for the glassy structure, is in the non-glassy domain for the actual storage conditions of 60 ºC and accordingly unstable with respect to browning and lipid oxidation.

clearly linked to oxidative deterioration of lipids. Deterioration of proteins in dry food products like milk powder is similar to lipid oxidation coupled to the increased water activity occurring as a result of possible glass transitions during storage (Thomsen *et al.*, 2005). Temperature/water-activity phase diagrams, such as those shown in Fig. 1.8, which combine the information normally presented in adsorption isotherms at varying temperatures with the information available from traditional so-called extended temperature/water-content phase diagrams, have recently been suggested to be used for selection of storage temperature for specified relative humidities for prolongation of shelf life for dried foods like milk powder and freeze-dried probiotic bacteria cultures (Kurtmann *et al.*, 2009). For foods with higher molecular mobility and for beverages, radicals need to be stabilized prior to ESR detection using the so-called spin-trapping technique as has been used for beer to predict beer staling and for wine to describe oxidative deterioration (Andersen and Skibsted, 2006; Elias *et al.*, 2009).

Among the non-saponifiable lipids, steroids like cholesterol and carotenoids are also subject to oxidation in food often coupled with oxidation of the phospholipids and other of the saponifiable lipids. Carotenoids with their many conjugated double-bonds are highly coloured and bleach upon oxidation, while lipid peroxyl radicals abstract one of the allylic hydrogens in cholesterol to yield 7-ketocholesterol as the stable oxidation end product (Smith *et al.*, 1973). Carotenoid bleaching may be used to monitor progression of oxidation in the lipid phase of processed dairy products, while 7-ketocholesterol is a valuable indicator of the total load of oxidative stress accumulated during production and subsequent storage (Sander *et al.*, 1989). Oxidized cholesterol in food causes some concerns due to their toxicity (Jacobsen, 1987). Carotenoids seem, however, to counteract the negative health effects of oxidized cholesterol (Palozza *et*

al., 2010). Carotenoids may further protect cholesterol in the product against singlet-oxygen, which otherwise adds to the single double bond of cholesterol to yield a specific 5-hydroperoxide often used as an indicator of involvement of singlet oxygen in oxidative deterioration of food lipids. Cholesterol is only present in food of animal origin. Dairy products with added plant oils are becoming increasingly popular and present a special challenge as cholesterol in such products are becoming exposed to lipids typically of lower oxidative stability than the milk fat with the risk of increased cholesterol oxidation through coupling with autoxidation of more highly unsaturated plant lipids.

1.5 Aqueous phase oxidations

Lipid oxidation is recognized as an important cause of quality deterioration of many foods like edible oils, muscle-based foods like fish and meat, and milk and dairy products. Most foods have besides the lipid phase also an aqueous phase with oxido-reductase activity, with redox-active metal ions present together with a steady formation of ROS and RNS. For buttermilk made by churning of milk from cows of two different feeding regimes to yield milk fat of varying unsaturation, it was found, not very surprisingly, that the buttermilk with the more saturated fat had a better resistance against oxidative rancidity during chill storage measured as formation of both lipid hydroperoxides and hexanal (Kristensen *et al.*, 2004). However, for both products, lipophilic antioxidants did not decrease during storage, while antioxidative capacity of the aqueous phase decreased significantly and with similar rates for the two products, as measured by ESR spectroscopy, indicating that radical formation as the early event of lipid oxidation occurs in the aqueous phase and independent of the degree of unsaturation of the lipids.

For meat systems, lipid oxidation seems also to be initiated in the aqueous phase. Myoglobins are moderate catalysts for lipid oxidation although their hypervalent forms may abstract allylic hydrogen from lipids (MbFe(V)=0) or cleave lipid hydroperoxides (MbFe(IV)=0, see eq. 1.19). Partly proteolyzed myoglobins show a dramatic increase in catalytic activity for lipid oxidation in heterogeneous systems (Fig. 1.9), and hydrolytic degradation of heme pigments during digestion may accordingly initiate oxidative damage in the digestive tract (Carlsen and Skibsted, 2004). During heat treatment of meat, iron seems to leak from metalloproteins opening up for simple Fe(II)/Fe(III) catalysis of lipid oxidation leading to warmed-over-flavour upon reheating (Tims and Watts, 1958).

For the aqueous phase, urate is important as an antioxidant in milk, in meat reducing co-factors like NADH determines the redox status of meat pigments, while in fruit products ascorbate and plant phenols together seems more important for the redox potential. Proteins may oxidize, and for the myofibrillar protein of meat, oxidation leads to cross-linking affecting water binding capacity and tenderness negatively (Decker *et al.*, 1993). Cystein side-chains of the

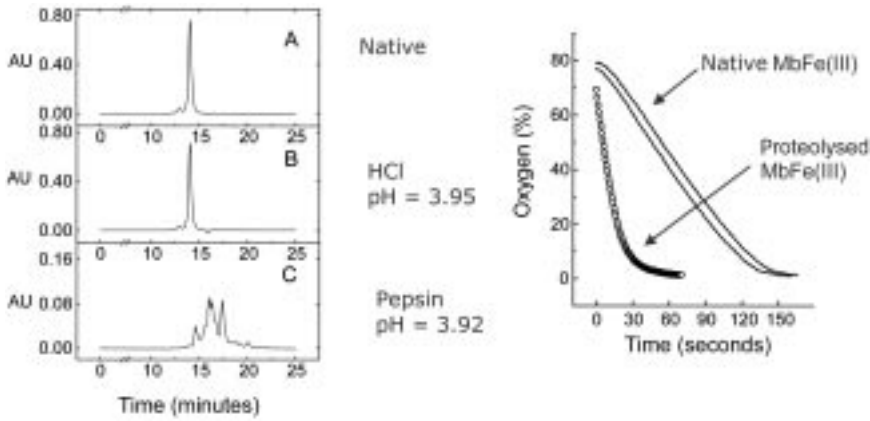

Fig. 1.9 Metmyoglobin is a moderate catalyst for lipid oxidation in an oil-in-water emulsion as followed electrochemically by oxygen depletion, the catalytic effect being strongly enhanced by proteolysis of metmyoglobin under stomach conditions (based on Carlsen and Skibsted, 2004). The three chromatograms (A, B and C) indicate hydrolytic cleavage of myoglobin and correspond to the oxygen depletion curves.

proteins are involved forming disulfide bridges and may also be involved in formation of dityrosine cross-links through a free radical mechanism (Baron and Lund, 2010). For the whey protein β-lactoglobulin, heat or pressure denaturation leads to polymerization through thiol/disulfide exchange reactions, while oxidation of denaturated β-lactoglobulin, leads to formation of inter-molecular ditryosine (Østdal *et al.*, 1996).

Besides becoming cross-linked through oxidation, protein side-chains may be modified oxidatively or undergo fragmentation as illustrated in Fig. 1.10 (Baron and Lund, 2010). The initial step in protein oxidation is the abstraction of an electron or a hydrogen atom by the actual oxidant. Using triplet-state riboflavin generated photochemically using a laser-flash technique, the second-order rate constants for electron transfer from a number of amino acids, peptides, and proteins to this strong aqueous oxidant have now become available (Cardoso *et al.*, 2004). As may be seen from Table 1.4, tryptophan, tyrosine, and the cystein anion are reacting with comparable rates approaching the diffusion limit for electron transfer. Cystein at physiological pH reacts much slower and by hydrogen atom transfer. Proteins are accordingly easily oxidized at the specific side chains with thiol groups or phenol groups leading to cross-linking, as is further supported by the insensitivity of the rate of oxidation to the incorporation of these amino acids in peptides (Table 1.4). The rate of electron transfer as the primary step in oxidative modification of proteins depends on the driving force for the electron transfer reaction (difference in redox potentials) as has been shown for histidine and closely related N-heterocycles as protein models (Huvaere and Skibsted, 2009). Such linear free energy relationships (Fig. 1.11) are together with demonstration of so-called isokinetic behaviour (activation entropy depends linearly on activation enthalpy) most valuable when assigning

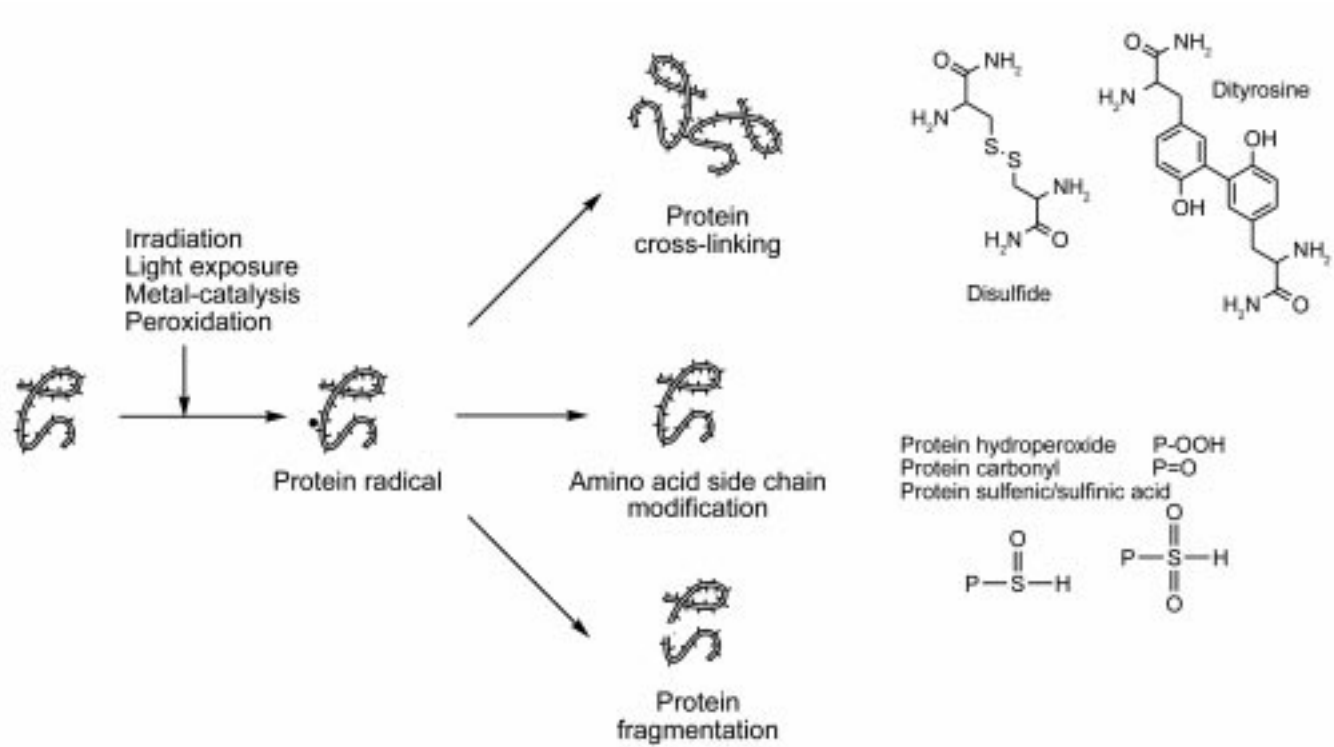

Fig. 1.10 Effects of protein oxidation (adapted from Baron and Lund, 2010).

Table 1.4 Second-order rate constant for initial step in oxidation of amino-acids, peptides, proteins and plant phenols by triplet-state riboflavin in pH = 6.4 aqueous solution at 25 °C[a]

Substrate	k_2 ($l\,mol^{-1}\,s^{-1}$)	Comment
Cystein (Cys)	$1.6 \cdot 10^6$	Low pH, hydrogen atom transfer
Cystein anion	$1.2 \cdot 10^9$	High pH, electron transfer
Cystin	–	No reaction
Methionine	$6.4 \cdot 10^7$	Electron transfer
Histidine	$5.2 \cdot 10^7$	Electron transfer – rate decreases for higher pH due to protonation
Phenylalanine	–	No reaction
Tyrosine (Tyr)	$1.4 \cdot 10^9$	Electron transfer
Tryptophan	$1.8 \cdot 10^9$	Electron transfer
H-Cys-Tyr-Cys-Tyr-OH	$1.9 \cdot 10^9$	Electron transfer
H-Cys-Gly-OH[b]	$6.7 \cdot 10^7$	Electron transfer
H-Tyr-Gly-OH	$1.3 \cdot 10^9$	Electron transfer
H-Gly-Tyr-OH	$1.6 \cdot 10^9$	Electron transfer
H-Gly-Tyr-Gly-OH	$2.1 \cdot 10^9$	Electron transfer
β-lactoglobulin	$3.6 \cdot 10^8$	Electron transfer
Bovine Serum Albumin	$2.3 \cdot 10^8$	Electron transfer
(−) Epigallocatechingallate	$1.7 \cdot 10^9$	Hydrogen atom transfer/electron transfer
(+) Catechin	$1.4 \cdot 10^9$	Hydrogen atom transfer/electron transfer
Rutin	$1.0 \cdot 10^9$	Hydrogen atom transfer/electron transfer

[a] From Cardoso *et al.* (2004), and Becker *et al.* (2005).
[b] Gly is glycine.

mechanisms for oxidative changes of aqueous phase components in foods and making distinction between electron transfer and hydrogen atom transfer as rate determining (Cardoso *et al.*, 2007). Proteins seem to be oxidized by initial electron abstraction, while some plant phenols rather transfer a hydrogen atom

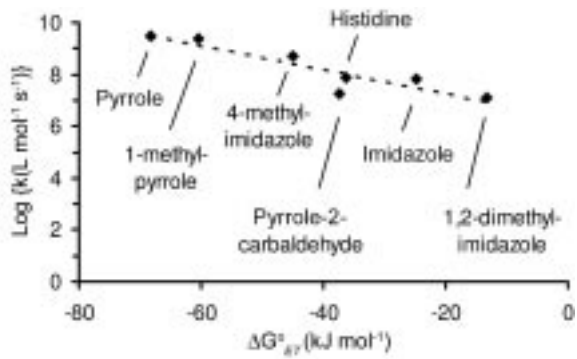

Fig. 1.11 Rate of electron transfer from histidine and other reducing nitrogen heterocycles to triplet riboflavin as photosensitizer shows a linear free energy relationship (LFER) indicative of a common reaction mechanism (from Huvaere and Skibsted, 2009).

to yield the phenoxyl radical in competition with electron transfer. Such information has not been available for oxidation of lipids, but a kinetic study of methyl linoleate quenching of triplet-riboflavin clearly indicates that lipid radicals are formed by hydrogen atom transfer (Huvaere *et al.*, 2010). Density functional calculations confirmed that electron transfer is endergonic, while hydrogen atom transfer is exergonic.

Iron(II)/iron(III) catalysis of protein oxidation by hydrogen peroxide becomes site specific through coordination of iron(II) to lysine side chain to yield protein carbonyls, which are often used as marker of oxidation of meat proteins (Stadtman, 1990). Protein oxidation is also important for protein functionality as in bread. The gluten network in wheat bread dough is damaged by reduction of the disulfide bridges by glutathione, and bromate and other oxidants have been used for flour improvement (cf. Fig. 1.12). Bromate is now being replaced by ascorbate. Notably, ascorbate is a reductant, but is oxidized enzymatically in the dough by oxygen to yield dehydroascorbate, which is the actual oxidant protecting the gluten disulfide bridges (Grosh and Weiser, 1999). Other oxidoreductases like laccase, a multicopper enzyme that catalyzes formation of phenolic radicals in lignin and from tyrosin in proteins may be used to oxidatively modify protein functionality (Steffensen *et al.*, 2008).

1.6 Antioxidants and antireductants

In relation to food, antioxidants were originally defined as 'substrates that in small quantities are able to prevent or greatly retard the oxidation of easily oxidizable nutrients such as fats' (Chipault, 1962). Antioxidants can prevent oxidative damage to food during processing, storage and preparation of meals. Antioxidants may accordingly provide more healthy food with low levels of lipid and protein oxidation products. Antioxidants may also have more direct health effects as part of the diet, but methodological shortcomings have been identified since both vitamin antioxidants (vitamin E and C) and non-vitamin antioxidants (polyphenols and carotenoids) are multifunctional in biological systems and cannot be evaluated by 'one-dimensional' methods (Frankel and Meyer, 2000). A four-step strategy for antioxidant evaluation has been proposed (Becker *et al.*, 2004). As seen from Fig. 1.13, the final evaluation depends on storage experiments for antioxidants for food protection, and on human intervention studies for health effects of antioxidants. Most standard assays for antioxidant evaluations in use deal with antioxidants as reductants or as scavengers of radicals (Wolfe and Liu, 2007). Screening of potential antioxidants for radical scavenging capacity or reducing activity using simple assays corresponding to step I (quantification), step II (radical scavenging) or step III (effects in model systems) of the scheme shown in Fig. 1.13 to predict protective effects on food stability or health effects in humans seems not scientific justified. Quantification of radical scavenging capacity or reducing activity alone only provides guidelines for the final evaluation in storage

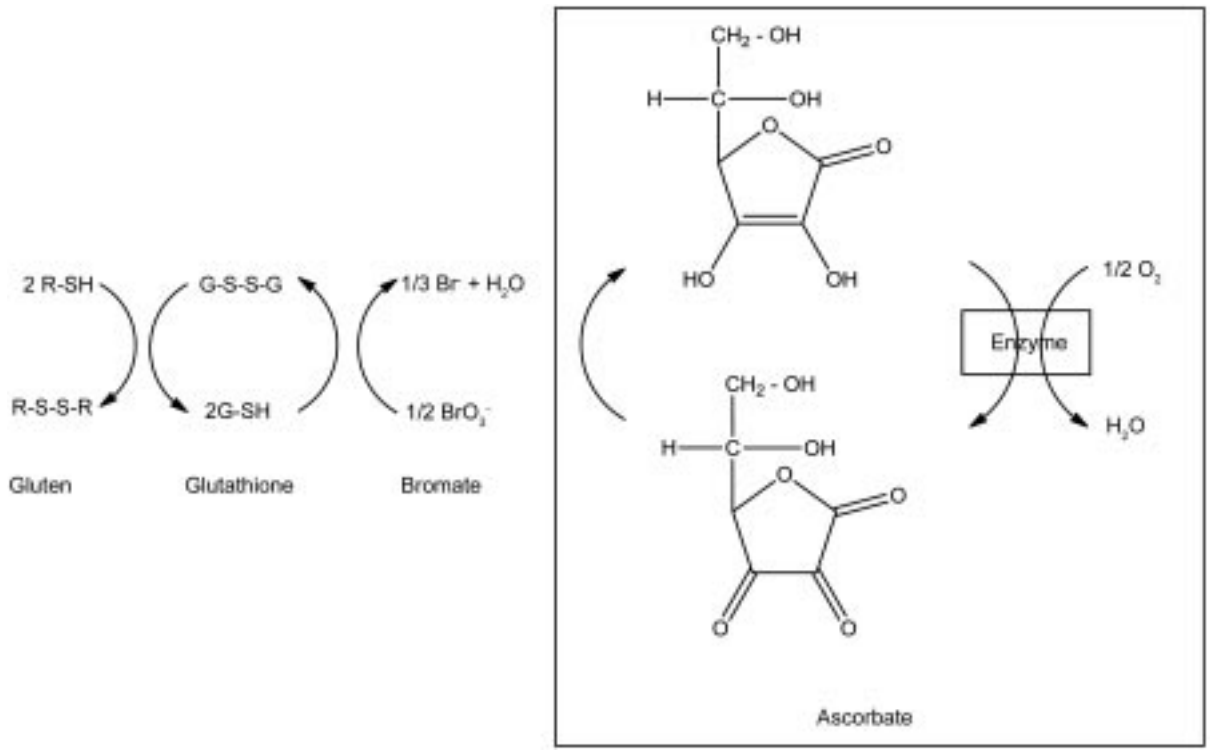

Fig. 1.12 Gluten network (R-S-S-R) in bread depends on oxidation of cystein residues to cross-linking cystin as mediated by glutathione. Reducing conditions in the dough have been avoided by using bromate as flour improvement. Bromate is now being replaced by ascorbate which, through enzymatic oxidation by oxygen, yields dehydroascorbate capable of acting as an oxidation in the dough.

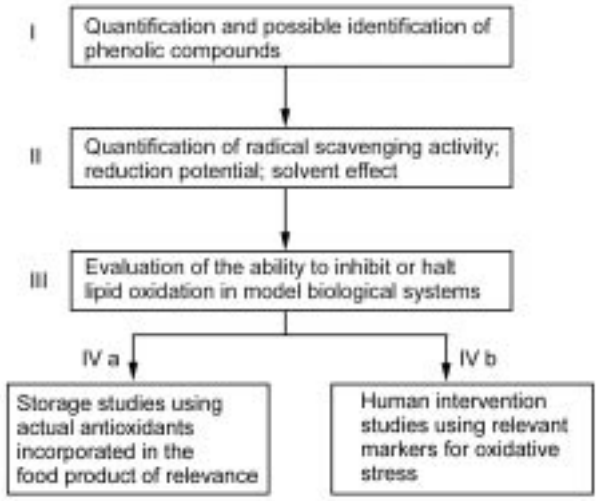

Fig. 1.13 Antioxidant evaluation strategy as proposed by Becker *et al.* (2004). The final evaluation of antioxidant protection of food and beverages depends on storage experiments, while the final evaluation of health effects of antioxidants depends on human intervention studies. Quantification of radical scavenging capacity or reducing efficiency alone only provides guidelines for the final evaluation.

experiments or in human intervention studies. For food systems this has become even clearer as it now appears that antioxidants protecting lipids do not necessarily protect proteins, at least in meat (Lund *et al.*, 2007).

Radical scavenging alone does not constitute a good antioxidant. Carotenoids are not chain-breaking antioxidants but are, besides being quenchers of singlet oxygen, efficient radical scavengers through three mechanisms (Galano, 2007):

$$R^{\bullet} + Car \rightarrow R^{-} + Car^{\bullet +} \quad 1.20$$

$$R^{\bullet} + Car \rightarrow [R - Car]^{\bullet} \quad 1.21$$

$$R^{\bullet} + Car \rightarrow RH + Car(-H)^{\bullet} \quad 1.22$$

Carotenoids may regenerate each other from their radical cations:

$$Car_1^{\bullet +} + Car_2 \rightarrow Car_1 + Car_2^{\bullet +} \quad 1.23$$

for which reaction the following hierarchy has been established (Mortensen *et al.*, 2001):

$$\text{lycopene} > \beta\text{-carotene} > \text{zeaxanthin} > \text{lutein} >> \text{canthaxanthin} > \text{astaxanthin} \quad 1.24$$

with lycopene being the most efficient radical scavenger. Notably astaxanthin, which is the least efficient radical scavenger among the carotenoids considered and with a moderate tendency of forming radical cations, is often found to have very positive effects on oxidative stability of food lipids (Jensen *et al.*, 1998). In

salmon muscles, astaxanthin was thus found to be of equal importance as α-tocopherol as antioxidant in protecting the highly unsaturated lipids. Astaxanthin is among the least reducing carotenoids and a very poor radical scavenger as shown in experiments establishing the ordering of carotenoids in eq. 1.24. Further theoretical calculations confirmed that astaxanthin is positioned low in the antioxidant hierarchy of carotenoids (Galano, 2007). Still astaxanthin has been found to be superior to the more reducing carotenoids as an antioxidant in protecting liposomes as models for cell membranes (Naguib, 2000).

Carotenoids should apparently not only be considered as an electron donor:

$$\mathrm{Car} + \mathrm{O_2}^{\bullet -} \rightarrow \mathrm{Car}^{\bullet +} + \mathrm{O_2}^{2-} \qquad 1.25$$

but also as an electron acceptor:

$$\mathrm{Car} + \mathrm{O_2}^{\bullet -} \rightarrow \mathrm{Car}^{\bullet -} + \mathrm{O_2} \qquad 1.26$$

Quantum mechanical calculations have provided a two-dimensional ranking of carotenoids and also of antioxidants like vitamin A, C and E according to their antiradical capacity (Martínez *et al.*, 2008). Based on a combination of ionization energy and electron affinity, an electron acceptance index, R_a, and an electron donating index, R_d, were defined relative to fluor and sodium, respectively (see Table 1.5). An electron acceptor/donor classification is shown in Table 1.6 with examples of compounds affecting oxidative processes in biological system. Compounds which are both good electron donors (R_d low) and good electron acceptors (R_a high) are the best antiradical compounds since they easily donate and accept an electron (corresponding to reactions of eq. 1.25 and eq. 1.26). β-carotene is such a good antiradical compound. Compounds with the opposite properties, i.e. R_d high and bad electron donors and R_a low and bad electron acceptors like ascorbic acid, are poor radical scavengers. The best antioxidants are compounds with low R_a as bad electron acceptors but which are good electron donors corresponding to a low R_d. α-tocopherol is such a compound. The last group is exemplified by astaxanthin: high R_a as a good electron acceptor and high R_d as a poor electron donor, and such compounds are now termed antireductants (Martínez *et al.*, 2008). Notably, R_a and R_d are not inversely proportional to each other and the novelty lies accordingly in the two-dimensional classification.

Among the carotenoids, the highly red-coloured like astaxanthin are the best antireductants, but the worst antioxidants. In contrast, the more colourless antioxidants are the best electron donors and the best antioxidants like α-tocopherol. This two dimensional ordering of antioxidants seems to explain the often unexpected positive effect of the highly coloured carotenoids. Even astaxanthin does not scavenge phenoxyl radicals (Table 1.5), astaxanthin as an antireductant may prevent the formation of the more reactive ROS from the less reactive. A proper combination of antioxidants like α-tocopherol and anti-reductants like astaxanthin as seen for salmon, may explain the positive effects seen for lipid stability (Jensen *et al.*, 1998). Ascorbic acid has in radical scavenging assays been found to be a rather poor antioxidant compared with

Table 1.5 Physico-chemical properties of carotenoids in comparison with α-tocopherol and ascorbic acid[a]

	R_d	R_a	$^1O_2$ quenching k_2 ($l\,mol^{-1}\,s^{-1}$)[b]	Phenoxyl scavenging Relative rate[c]	Relative tendency[d] to form $car^{\bullet+}$	E^o V vs. NHE[e]	$Car^{\bullet+}$/diadzein^{2-} k_2 ($l\,mol^{-1}\,s^{-1}$)[f]
β-carotene	1.40	0.46	$4.6 \cdot 10^9$	1	1	0.84	$5.8 \cdot 10^9$
zeaxanthin	1.44	0.49	$6.8 \cdot 10^9$	0.79	10	0.85	$8.3 \cdot 10^9$
canthaxanthin	1.93	0.82	$11.2 \cdot 10^9$	~0		0.95	$5.7 \cdot 10^{10}$
astaxanthin	2.10	0.94	$9.9 \cdot 10^9$	~0	3	0.97	$9.2 \cdot 10^{10}$
lycopene			$6.9 \cdot 10^9$	1.66	8	0.81	
α-tocopherol	0.31	0.15	$2.7 \cdot 10^7$			0.80	
ascorbate	1.29	0.11				0.22	

[a] $R_d = \omega^-/\omega^-_{Na}$ and $R_a = \omega^+/\omega^+_F$ relative to sodium and fluor, respectively. $\omega^- = (3I + A)^2/16$ (I-A) and $\omega^+ = (I + 3A)^2/16$ (I-A) is based on I, the (vertical) ionization energy, and A, the (vertical) electron affinity. From Martinez *et al.* (2008).
[b] Second-order rate constant at 25 ºC. From Min and Boff (2002).
[c] In di-*tert*-butyl peroxide/benzene (7/3, v/v) at 20 ºC, phenoxyl radical generated photochemically. From Mortensen and Skibsted (1997).
[d] Laser flash photolysis in $CHCl_3$ (490 nm, 120 fs pulses). From Han *et al.* (2002).
[e] Standard reduction potential of one-electron oxidized antioxidant, for $car^{\bullet+}$ determined in CH_2Cl_2, for oxidized α-tocopherol in DMF, and for the ascorbyl radical in water. From Han *et al.* (2002) and Jørgensen and Skibsted (1998).
[f] Bimolecular regeneration of carotenoid by the isoflavonoid dianion in methanol/chloroform (1/10) at 25 ºC. From Han *et al.* (2010).

Table 1.6 Electron donor/acceptor classification of potential radical scavengers[a]

R_d \ R_a	Low electron acceptor index (bad acceptors)	High electron acceptor index (good acceptors)
Low electron donation index (good donors)	Good radical scavengers Good antioxidants Example: Vitamin E	Best radical scavengers Example: β-carotene
High electron donation index (bad donors)	Poor radical scavengers Example: Vitamin C	Good radical scavengers Good antireductants Example: Astaxanthin

[a] R_a and R_d are relative to fluor and sodium, respectively, as defined in Table 1.5. Based on Martinez *et al.* (2008).

flavonoids like quercetin and the tea polyphenols (Wolfe and Liu, 2007) in agreement with the two-dimensional classification. Still ascorbic acid may prevent oxidation reaction but rather through oxygen depletion or through regeneration of phenolic antioxidants. The highly coloured anthocyanins have been classified as relatively good electron donors (R_d moderately high) and bad electron acceptors (R_a low) resulting in antioxidant properties between vitamin C and vitamin E (Table 1.6). However, solvents effect seems significant due to their positive charge (Martínez, 2009). It should further be noted (Table 1.5) that the most efficient singlet-quenchers are found among the good antireductants, while the best antioxidants are the best phenoxyl scavengers and best reductors.

Antioxidant synergism between α/β-carotene and tocopherols/tocotrienols has been observed in red palm oil (Schroeder *et al.*, 2006). Since α-tocopherol and α-tocotrienol were shown to regenerate the carotenes from their radical cations, rather than the opposite, and in agreement with their respective standard reduction potentials as seen in Table 1.5, it was concluded that the carotenoids were oxidized sacrificially, in effect protecting the tocopherols/tocotrienols as the better (chain-breaking) antioxidants. The two-dimensional classification of the actual compounds now available (Table 1.5) seems to confirm this previous conclusion with the carotenes being the best radical scavengers and the tocopherols/tocotrienols the best antioxidants. This important type of antioxidant synergism in model systems depends on differences in reaction rate and may be classified as a kinetic effect. For systems where the more efficient chain-breaking antioxidants are regenerated by the antioxidant less efficient as chain breakers but being more reducing, the synergism is rather the result of thermodynamic control. Such examples may be found in the interaction between α-tocopherol and plant phenols. Although the BDE predicts the tocopherols to be more reducing than the plant phenols, as seen for quercetin and the tea polyphenols in Table 1.3, solvent effects including pH are very important for the redox potential of plant phenols, which in solution may become the more reducing as seen for quercetin as compared to α-tocopherol (Pedrielli and Skibsted, 2002).

For lipid systems of increasing structural organization, antioxidant synergism may be caused by compartmentalization. For the less investigated interaction between carotenoids and plant phenols, clear antioxidant synergism has been observed and assigned to regeneration of carotenoids as active radical scavengers in the lipid phase by plant phenols at the lipid/water interface (Han *et al.*, 2007). For isoflavonoids in combination with carotenoid radical cations, regeneration rate at the diffusion limit was observed especially for astaxanthin as seen in Table 1.5. According to the two-dimensional classification of Table 1.5, astaxanthin has the highest electron accepting index among the carotenoids and accordingly should act as an antireductant in the lipid phase. Notably, the regeneration reaction for the astaxanthin radical cation by the moderately reducing isoflavonoid daidzein is faster for astaxanthin than for β-carotene in agreement with their reduction potentials, Table 1.5 (Han *et al.*, 2010).

1.7 Future trends

Lipid oxidation has often been investigated in food systems separately, and antioxidative protection directed solely towards the lipid phase of foods. Protein oxidation is now receiving attention and apparently antioxidants that protect lipids do not necessarily protect the proteins. However, future progress in protection of food against quality deterioration will benefit from a more holistic approach to the chemistry behind browning reactions, protein oxidation and lipid oxidation involving kinetic modelling. Degraded sugars as formed following reaction with proteins, produce Maillard products active as antioxidant but at the same time, proteins are degraded. The scheme presented as Fig. 1.14 for

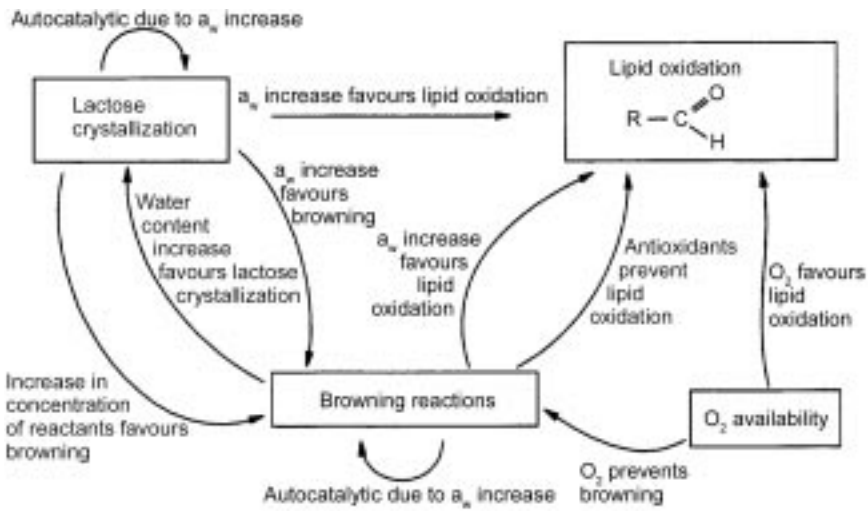

Fig. 1.14 Coupling between lactose crystallization, browning reaction and lipid (and protein) oxidation in milk powder (adapted from Thomsen *et al.*, 2005).

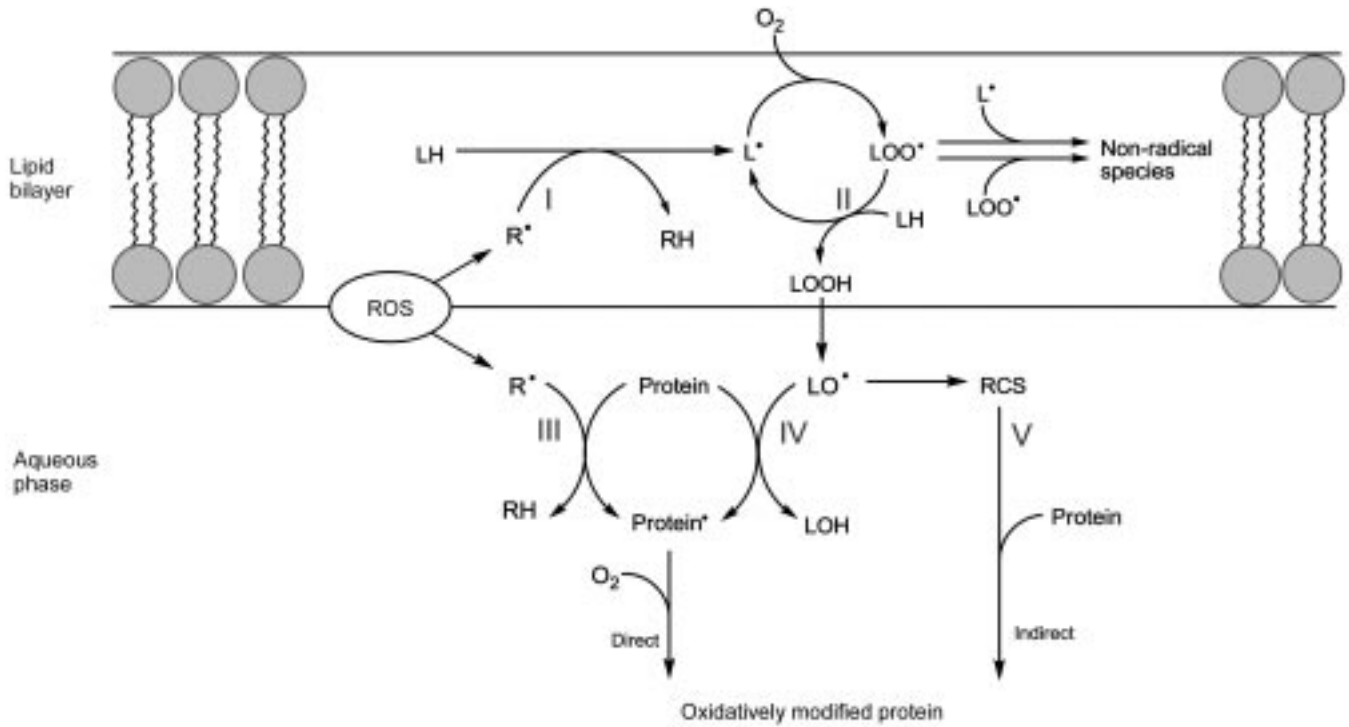

Fig. 1.15 Peptides as antioxidants in membranal systems with progressing lipid oxidation (I, II) initiated by reactive oxygen species (ROS) leading to direct protein modification (III, IV) or through formation of reactive carbonyl species (RCS) to indirect protein modification (V) (adapted from Jongberg *et al.*, 2009).

physical and chemical processes occurring in milk powder may serve as inspiration for such future studies (Thomsen *et al.*, 2005). For semi-dry systems, both Maillard reactions and lipid oxidation progress faster at increasing water activities, but at the same time, early stages of Maillard reactions produce water increasing the water activity. Water, as a plasticizer, further accelerates phase transitions resulting in less water binding and increasing water activity. Such foods provide examples of system with positive feed-back mechanism during their degradation.

Hydrolytic degradation of proteins also affects lipid oxidation, and certain peptides are considered to be effective antioxidants. The potential antioxidative mechanism of peptides in biological membrane systems involves interaction between lipid oxidation and protein oxidation as shown in Fig. 1.15 (Jongberg *et al.*, 2009). Peptides active as radical scavengers protect proteins from indirect oxidative modification, a protection which depends on a prevention of lipid oxidation derived carbonylation through formation of reactive carbonyl species (RCS) such as α,β-unsaturated aldehydes. Peptides are scavengers of aqueous phase radicals when present at high concentrations and may act as sacrificial rather than chain breaking antioxidants preventing direct protein oxidation.

Future food production will have more focus on raw material from plant and possibly algae due to the increasing world population. As for the plant lipids, the higher unsaturation calls for better oxidative protection with a special challenge for protection of food, where plant oils are used in combination with animal based lipids as in dairy spreads and new types of cheeses. Food science will also have to deal with the problems associated with radical formation from meat and meat products in the human digestive tract (Demeyer *et al.*, 2008). Optimizing use of plant-based antioxidants and milk proteins in processed meats as preventers of radical formation from meat pigments may lead to a new generation of meat products.

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2

Metals and food oxidation

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Abstract: Transition metals may initiate oxidation in foods by several mechanisms; they are able to interact directly with oxygen to generate reactive oxygen species (ROS), such as superoxide, perhydroxide and hydroxyl radicals. However, in fresh foods and especially in muscle foods, preformed hydrogen peroxide and hydroperoxides may act as the main species which are substrates for further oxidation, co-oxidation and propagation by transition metal catalyzers. Reducing agents are the most important cofactors turning transition metals such as Fe, Cu ions for acting as significant catalyzers of non-enzymatic oxidation in biological and food systems.

Hemeproteins at low concentration act as pro-oxidants, but at high concentration they work antioxidatively by auto-redaction. Reducing agents support the inversion of the activity of hemeproteins from pro-oxidation to antioxidation. In fresh foods many enzymatic and non-enzymatic molecules support the antioxidative tone of the material; however, heating and processing may increase the involvement of metal ions in food oxidation, changing the stability of the material to a pro-oxidative pathway. In order to prevent the pro-oxidative pathway, before heating and processing the food should be stabilized by a 'cocktail' of antioxidants.

Key words: food, oxidation, metal ions, hemeproteins, reducing compounds.

2.1 Introduction

Foods, and in general, biological oxidation, are almost exclusively metal-ion promoted reactions, and in this respect, iron, being the most abundant, is commonly involved. Iron is essential for aerobic life and is required for the biosynthesis of a variety of iron-containing proteins and for DNA synthesis. Iron

is utilized as a catalyst at the active site of numerous enzymes involved in oxygen metabolism and also within proteins involved in oxygen storage transport. The deleterious properties of iron are channeled and controlled within enzymes, whereas within iron binding and storage proteins, it is sequestrated in a poorly or non-reactive form, such as in transferrin, lactoferrin and ferritins. Ferritins are the main source of 'free' iron in cells of plant or animal origin (Kanner and Doll, 1991). When cells are injured, such as after harvesting, slaughtering, processing and storage, iron ions released from ferritins and the low molecular mass form of the 'free' iron increase significantly and attain micromolar concentration (Kanner *et al.*, 1987, 1988a; Kanner, 1994; Halliwell, 2007). Depending on the food, these 'free' iron ions have the potential to act as a pro-oxidant for the formation of reactive oxygen species (ROS) and oxidation of many biomolecules.

Foods also contain enzymes with transition metals at the active site and molecules such as hemeproteins which act very efficiently as metal catalyzers in food oxidation and antioxidation reactions. Many biomolecules bind transition metals, in particular, compounds containing non-bonding electrons around groups such as OH, NH and SH. The coordination of biomolecules by transition metals involves orbitals which also allow the simultaneous binding of oxygen, thereby providing a bridge between O_2 at triplet state and biomolecules at the singlet state. In such a way all the necessary ingredients to catalyze oxidation reactions at a particular site are present. The coordinative bonding of iron or other transition metals to different compounds and ligands affected the redox potential, spin state and ligand geometry of the metal, and by this, its efficiency as a catalyst of oxidation or reduction reactions. Iron ions can exist in a variety of redox states, but in foods and biological systems is mainly restricted to the ferrous (Fe^{2+}), ferric (Fe^{3+}) and ferryl (Fe^{4+}) states.

2.2 Sources of metal ions and products particularly affected

Iron is a transition metal, present at trace amounts in different foods and particularly in muscle foods, powder foods, cereals, eggs, nuts, oils, vegetables, fruits and beverages. The term transition metal refers to elements in the d-block of the periodic table which include groups 3 to 12 on the periodic table. There are a number of properties shared by the transition metals that are not found in other elements, which result from the partially filled d-shell. These include:

- Formation of compounds whose color is due to d-d electronic transitions.
- Formation of compounds in many oxidation states.
- Formation of many paramagnetic compounds due to the presence of unpaired electrons.

There are eight essential transition metals in foods required for our daily intake. These include: Fe (15 mg), Zn (~ 14 mg), Cu (3.2 mg), V (1–2 mg), Mn (2–9 mg), Mo (0.3 mg), Cr (0.05–0.1 mg) and Co (0.3 mg). A deficiency in the essential trace metals results in metabolic disorders that are primarily associated

with the absence or decreased activity of metabolic enzymes. Of greater practical significance is the amount of the element in a food that is available to the body. Iron requirement is about 1–3 mg/day. However, because only 10–30% of it is available, iron supplied in the diet must be in the range of 10–20 mg/day (sex/age) in order to meet this daily requirement. The most utilizable source of iron is the heme-iron because it is not affected by compounds such as polyphenols and tannins, phytic acid and other potential chelators found naturally in foods which bond non-heme iron and prevent its bioavailability.

In order to provide a sufficient supply of iron many foods, and especially cereals, are fortified with iron in the form of ferrous sulfate or ferrous gluconate. However, generally, iron is an undesirable element in food processing because it catalyzes oxidation of lipids and also others compounds such as antioxidant vitamins, polyphenols and many other reducing compounds found in food. Dehydrated foods (cereals snacks, biscuits, milk powder, eggs powder, etc.), fried products, oils, muscle foods, and many others such tea, coffee or orange juice are susceptible to oxidation by metal ions.

The amount of total iron (μg/g fresh weight) for different red muscle foods such as chicken, pork, turkey, lamb, beef, are: 5,10, 12, 16, 26, respectively from those heme-iron 3, 5, 8, 9, 16 and non-heme-iron 2, 5, 4, 7, 10, respectively. The amount of iron in fruits and vegetables, which is mostly non-heme iron is about 5–10 μg/g fresh weight. However, in cereals which are dehydrated the amount of iron mostly non-heme is about 20–30 μg/g dry weight.

2.3 Mechanism of metal oxidation in biological systems and foods

2.3.1 Free metal ions electron configuration

The 'free iron' ion pool seems to be chelated to small molecules. The exact chemical nature of this pool is not clear, but it may represent iron ions attached to phosphate esters (ATP, ADP, phospholipids) organic acids (citrate, fatty acids), membrane lipids, nucleic acids, amino acids, and reducing sugars (Spiro, 1969; Kakhlon and Cabantchik, 2002). Most recently it was suggested that saturated fatty acids are the pathologic mediators of iron translocation *in vivo* (Yao *et al.*, 2005).

Elementary iron has the following electronic configuration, $1s^2 2s^2 2p^6 3s^2 3p^6 3d^8$. The outermost valence shell is the 3d orbital. In ferric iron, there are five valence electrons and in ferrous there are six. Ligands binding to the iron, due to their electronegativity and special arrangement, alter the energies of the electrons in the 3d orbital. This is termed field splitting.

In most biological complexes, the orbitals occupy an octahedral orientation in which the d orbitals are split into two levels: a high energy pair of electron orbitals (eg) and a lower energy trio (+2g). The presence and magnitude of the energy differences between these orbitals are due exclusively to the presence of the ligand in the gas phase. The free 3d orbitals are all of equal energy. For the

most stable state of non-liganded iron, the valence electrons fill all possible orbitals with predominantly parallel spins, due to the orbital degeneracy and the Pauli exclusion principle. Ligand bonding, however, alters the energy of the orbitals and consequently, the lowest energy distribution of spin states is altered as well. This affect the high and low spin states of ferric and ferrous ions. The electrons in the 3d orbital of ferrous ion are in a high spin, however during the formation of a ligand, they adopt a low spin arrangement in the t2g level in such a way that the eg orbital remain free for occupation by the electrons of the ligand which form with iron coordinative bonding (Kanner *et al.*, 1987).

Transition metals, iron and copper, with their labile d-electron system, are well suited to catalyzed redox reactions. Stable paramagnetic states, resulting from the presence of impaired electrons, are common for transition metals and facilitate their reaction with free radicals or molecules at the triplet state such as oxygen.

2.3.2 Oxygen activation by metal ions

Oxygen is a vital component in oxidation of foods and biological matter. The electronic structure of oxygen has two unpaired electrons at energy level of π antibonding, in triplet state, $^3\Sigma g$. The reaction of oxygen, therefore, is spin forbidden with ground state molecules of singlet multiplicity, which are more than 99% of the molecules in biological matter. This barrier does not apply to reactions with single electrons, hydrogen atoms, molecules in triplet state or molecules containing unpaired electrons, such as free radicals or transition metals. Oxygen is a bi-radical and paramagnetic compound. Thus, transition metals are able to remove the spin restriction of oxygen and be a bridge between oxygen and other molecules such reducing compounds, poly-unsaturated fatty acids, proteins and sugars.

Transition metals may initiate oxidation in foods and other biological systems by several mechanisms:

1. They are able to interact directly with triplet oxygen to generate reactive oxygen species (ROS) such superoxide radical $(O_2)^-$, which by interaction with other O_2 to form $O_2^=$, and by addition of two protons to form hydrogen peroxide (H_2O_2).
2. Reduced metals reduce H_2O_2 to hydroxyl radical ($HO^\bullet$), the most reactive radical generated in biological systems. The redox potential of hydroxyl radical at pH7.0 ($HO^\bullet/H_2O$) is a +2.3V (Koppenol and Liebman, 1984), high enough to oxidize all bio-molecules. However, if the concentration of reduced metal is high enough, hydroxyl radical will oxidize the metal to form a hydroxyl anion by the following reactions 2.1–2.4:

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{\bullet -} \quad 2.1$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^\bullet + HO^- \quad 2.2$$

$$Fe^{2+} + HO^\bullet \longrightarrow Fe^{3+} + HO^- \quad 2.3$$

$$\text{net } 4Fe^{2+} + O_2 \longrightarrow 4Fe^{3+} + 2HO^- + 2H^+ \qquad 2.4$$

$$Fe^{2+} + O_2^{\bullet -} \xrightarrow{2H^+} Fe^{3+} + H_2O_2$$

Our work (Harel, 1994) showed that 100 μM of ferrous sulfate was oxidized during interaction of 50 μM of H_2O_2 with 200 μM of $FeSO_4$ at pH7.0 buffer acetate. This could be explained only if ferrous ions react by equations 2.3 and 2.4. The same stoichiometric ratio of Fe^{2+}/H_2O_2 of 2:1 was shown by Qian and Buettner (1999) to prevent oxidation of target molecules by $HO^{\bullet}$.

3. The most important reason to consider the oxidative chemistry initiated by $Fe^{2+} + O_2$ as a significant route to biological oxidation is that the overall steady state concentration of oxygen is much greater, about 10^3 higher than pre-existing H_2O_2 in living systems (O_2, 10 μM and H_2O_2, 10 nM). Data demonstrated by Qian and Buettner (1999) showed that when $[O_2]/[H_2O_2]$ >100, $Fe^{2+} + O_2$ chemistry is an important route to initiation of detrimental biological free radical oxidation, much more than Fe^{2+} with pre-existing H_2O_2 (Fenton reaction). This chemistry leads to the formation of ferryl ion from loosely bound iron by the following reactions:

   Reaction 2.1 will generate $Fe^{+3} + O_2^{\bullet -}$ which further generate a complex between Fe^{2+} and O_2.

$$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+}\text{-}O_2 \qquad 2.5$$

$$Fe^{2+}\text{-}O + Fe^{2+} \longrightarrow Fe^{2+}\text{-}O_2\text{–}Fe^{2+} \qquad 2.6$$

$$Fe^{2+}\text{-}O_2\text{–}Fe^{2+} \longrightarrow 2Fe^{4+}\text{=O oxo-ferryl ion} \qquad 2.7$$

   However, fresh muscle tissue after slaughtering and grounding at 37 ºC generates H_2O_2 at a very significant amount of about 0.9 nmole/g min and after 60 min produce a steady state concentration of abut 50 μM (Kanner and Harel, 1985b; Jorgenson and Skibsted, 1998; Harel and Kanner, 1985a). Aging muscle tissues at 4 ºC for a period of 5 days increases H_2O_2 production almost 2.3 fold. It seems that in muscle foods, endogenous generation of H_2O_2 plays an important role in the formation of the primary pool of biological catalysts.

4. Reducing agents are the most important co-factors turning transition metals such as Fe or Cu ions into significant catalysts of non-enzymic oxidation in biological and food systems. The most active reducing compounds involved in such reaction are ascorbic acid, cysteine, polyphenols, protein-SH, NADPH, NADH and dopa, dopamine and other minor reducing agents. We could assume that nearly all 'loosely bound' or catalytic iron is present as Fe^{2+} (Keyer and Imlay, 1996).

   The interaction between ascorbic acid and transition metals could be described by the following reaction:

$$Fe^{3+}/Cu^{2+} + 2AH_2 \longrightarrow Fe^{2+}/Cu^{1+} + 2AH^{\bullet} + 2H_2^{+} \qquad 2.8$$

$$AH^{\bullet} + AH^{\bullet} \longrightarrow AH_2 + A \qquad 2.9$$

The reduction of the transition metal generates ascorbyl radical which by disproportion form ascorbic acid and dehydroascorbic acid (DHAA). DHAA is not a toxic compound; however, in many foods it could increase non-enzymatic browning through Maillard reaction and Strecker degradation. Reduction of transition metal ions by some polyphenols generate free radicals with the potential to produce further oxidizing compounds by the following reactions:

$$Fe^{3+}/Cu^{2+} + PhOH \longrightarrow Fe^{2+}/Cu^{1+} + PhO^{\bullet} + Fe^{2+}/Cu^{+} \quad 2.10$$

$$PhO^{\bullet} + O_2 \longrightarrow Ph{=}O + O_2^{\bullet -} \quad 2.11$$

$$PhO^{\bullet} + PhO^{\bullet} \longrightarrow Ph{-}OH + Ph{=}O \quad 2.12$$

Equations 2.11 and 2.12 demonstrate that some polyphenols generate through reduction of transition metals, superoxide, H_2O_2 and oxidized polyphenols, which are well-known oxidizing and cytotoxic compounds (Galati *et al.*, 2006). Redox compounds are the ultimate driving force in obtaining high concentration of ferrous or cuprous ions, which are essential for the generation of oxygen reactive species such as superoxide, perhydroxyl radical, hydrogen peroxide and hydroxyl radicals, and the initiation of peroxidation of lipids, but also of proteins and carbohydrates. This reaction is also known as the 'metal-redox cycle' (Winterbourn, 1979; Kanner *et al.*, 1986; Harel, 1994).

5. Numerous iron (copper, zinc, manganese, molybdenum) containing enzymes and other iron-containing non-enzymatic compounds are important in ROS chemistry and biochemistry, in particular, lipoxygenase, cyclooxygenase, xanthine-oxidase, peroxidases, myoglobin and hemoglobin. They can directly or indirectly initiate ROS and lipid peroxidation. The enzymatic activity in foods is significant only in fresh products and is very important in the generation of 'preformed' H_2O_2 and hydroperoxides (ROOH). A great part of our food is processed by heating, which destroys the enzymatic activity and only catalysts such as free metal ions, myoglobin, hemoglobin or other hemeproteins still remain active for catalysis of ROS formation and lipid peroxidation (Kanner, 1994).

2.3.3 Hemeproteins

Activation of hemeproteins

Hemoglobin and myoglobin play an essential role in maintaining aerobic metabolism in animal tissues. Iron-hemeproteins, especially myoglobin, are very abundant in muscle tissues. After slaughtering, autooxidation of oxyhemoglobin and oxymyoglobin ($P{-}Fe^{2+}{-}O_2$) results in the formation of methemeproteins ($P{-}Fe^{3+}$) and superoxide and hydrogen peroxide. This process is accelerated by low pH, anions, low oxygen pressure, high temperature and is very much affected by the type of the myoglobin or hemoglobin (Kanner and Harel, 1985a; Kanner, 1994; Aranda *et al.*, 2009) by the following reactions: Auto-oxidation and oxygen activation:

$$P\text{–}Fe^{2+} - O_2 \xrightarrow[\text{Anions}]{H^+} P\text{–}Fe^{2+} + O_2 \qquad 2.13$$

$$P\text{–}Fe^{2+} + O_2 \xrightarrow[\text{Anions}]{H^+} P\text{–}Fe^{3+} + O_2^{\bullet -} \qquad 2.14$$

$$O_2^{\bullet -} + O_2^{\bullet -} \xrightarrow{2H^+} H_2O_2 + O_2 \qquad 2.15$$

where P = porphyrin–protein ligand.

Ferrous hemeproteins are oxidized by two-electron transfer when mixed by preformed H_2O_2 forming an oxo-ferryl specie by the following reaction:

$$P\text{–}Fe^{2+} + H_2O_2 \longrightarrow P\text{–}Fe^{4+}{=}O + H_2O \qquad 2.16$$

Ferrous hemeproteins should be regenerated from ferryl hemeproteins if a catalytic cycle reaction is expected. However, such regeneration is not expected as the ferryl state is preferentially reduced to ferric state in one electron reactions. The active form for the cycle reaction remains methemeproteins, $P\text{–}Fe^{3+}$.

Catalyst activation I

$$P\text{–}Fe^{3+} + H_2O_2 \longrightarrow P\text{–}Fe^{3+}\text{–}\overset{H}{\underset{|}{O}}\text{–}O\text{–}H \qquad 2.17$$

$$P\text{–}Fe^{3+}\text{–}\overset{H}{\underset{|}{O}}\text{–}O\text{–}H \longrightarrow P\text{–}Fe^{4+}{=}O^{\bullet} + H_2O \qquad 2.18$$

$$P\text{–}Fe^{4+}{=}O^{\bullet} \longrightarrow {}^{+\bullet}P\text{–}Fe^{4+}{=}O \qquad 2.19$$

Catalyst activation I is formed by the interaction of ferri-hemeproteins with H_2O_2 generating a porphyrin cation radical, also called perferryl or oxo-ferryl radical. The Fe^{3+} states of peroxidases, such as horseradish peroxidase, lactoperoxidase or myeloperoxidase form the oxo-ferryl state by a two-electron transfer. However, myoglobin and hemoglobin could form by two-electron transfer a pseudo-perferryl state generating a $^{\bullet}P\text{–}Fe^{4+}{=}O$, the radical on the tyrosine and not on the porphyrin (Egawa *et al.*, 2000). Myoglobin and hemoglobin interact with H_2O_2 ~10^5 slower than peroxidases, ~$10^2\,M^{-1}\,s^{-1}$ and ~$10^7\,M^{-1}\,s^{-1}$, respectively. Both myoglobin and hemoglobin could by one-electron transfer to form a ferryl and a $HO^{\bullet}$ by the following reaction:

$$P\text{–}Fe^{3+} + H_2O_2 \longrightarrow P\text{–}Fe^{4+} + HO^{\bullet} + HO^{-} \qquad 2.20$$

Hydroxyl radical forming near the porphyrin will most probably oxidize the heme ring or the protein formed a oxo-ferryl radical (Kanner and Harel, 1985a).

Hemeproteins, like myoglobin and hemoglobin, could be activated by hydroperoxides (Kanner and Harel, 1985a; Adachi *et al.*, 1993; Matsui *et al.*, 1999; Carlsen *et al.*, 2005). Reaction could activate $P\text{–}Fe^{3+}$ by two-electron or one-electron transfer (Fig. 2.1). The two-electron transfer will generate the following reactions:

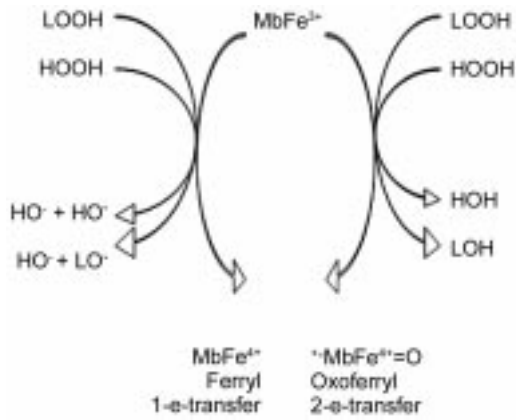

Fig. 2.1 Activation of metmyoglobin by one- or two-electron transfer.

$$P\text{–}Fe^{3+} + LOOH \longrightarrow P\text{–}Fe^{3+}\text{–}\overset{H}{\underset{|}{O}}\text{–}O\text{–}L \qquad 2.21$$

$$P\text{–}Fe^{3+}\text{–}\overset{H}{\underset{|}{O}}\text{–}O\text{–}L \longrightarrow P\text{–}Fe^{4+}\text{–}O^{\bullet} + LOH \qquad 2.22$$

$$P\text{–}Fe^{4+}\text{–}O^{\bullet} \longrightarrow {}^{\bullet}P\text{–}Fe^{4+}{=}O \qquad 2.23$$

where $^{\bullet}P$ is a protein radical and LOH is an alcohol such as cumyl alcohol.

However, the one-electron transfer of $P\text{–}Fe^{3+}$ to the hydroperoxide will produce two possible compounds by the following reactions:

$$P\text{–}Fe^{3+} + LOOH \longrightarrow P\text{–}Fe^{4+} + LO^{\bullet} + H_2O \qquad 2.24$$

$$P\text{–}Fe^{3+} + LOOH \longrightarrow P\text{–}Fe^{4+} + HO^{\bullet} + LOH \qquad 2.25$$

Both the alkoxyl radical or hydroxyl radical could oxidize the protein amino acids and be reduced to alcohol and H_2O.

Using a simple system activated met-myoglobin by cumene hydroperoxide, Adachi *et al.* (1993) and Matsui et al. (1999) found that ~70% of $MbFe^{3+}$ reacted in two-electron transfer producing cumyl alcohol and oxo-ferryl radical, $^{\bullet}P\text{–}Fe^{4+}{=}O$.

Both pathways generate active species which could initiate lipid peroxidation and co-oxidation of many other compounds such as carotenoids, cholesterol, other lipids, proteins and carbohydrates. The 1-e-transfer and 2-e-transfer pathways generate alkoxy, hydroxyl and oxoferryl radicals with redox potential high enough to initiate oxidation of unsaturated fatty acids (LH) to allyl ($L^{\bullet}$) radicals and further to propagate lipid peroxidation.

The redox cycle of oxo-ferryl radical and oxo-ferryl

The redox cycle of oxo-ferryl radical by two-electron transfer will regenerate $MbFe^{3+}$ (Fig. 2.2). If these donors are lipids or peroxides, the catalytic cycle will

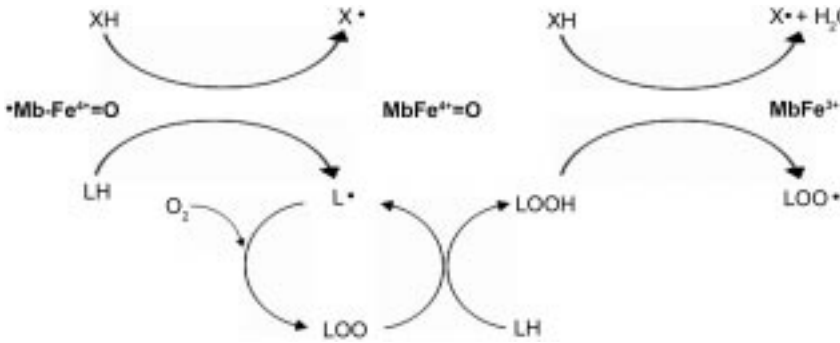

Fig. 2.2 The redox cycle of oxo-ferryl radical.

initiate and propagate oxidation. However, if the donors are antioxidants, such as ascorbic acid, tocopherols, polyphenols, or proteins containing amino acids such as tyrosine, tryptophan or SH group, relative unreactive radicals are formed, and the overall effect of the catalytic cycle will be antioxidative, removing the peroxides and hydroperoxides from the system (Lapidot *et al.*, 2005a).

A wide range of compounds including plant polyphenols and other antioxidants efficiently react with oxo-ferryl and regenerate metmyoglogin (Lapidot *et al.*, 2005a, 2005b; Carlsen *et al.*, 2000).

The interaction of $MbFe^{3+}$ with hydroperoxides leads to the generation by 1-e- or 2-e-transfer of alkoxyl, peroxyl and oxo-ferryl radicals. All of those species could be auto-reduced by the porphyrin ring and amino acids as electron donors of the hemeproteins. Such auto-reduction would produce free radical proteins and cause intra-molecular rearrangement and cross-linkage of the protein (Lewis and Wills, 1963; Kendrick and Watts, 1969; Harel and Kanner, 1989; Carlsen *et al.*, 2000; Mikkelsen and Skibsted, 1992). If the auto-reduction process is very efficient (in the presence of low concentration of hydroperoxides and relatively high concentration of hemeproteins) the antioxidant tone of hemeproteins will dominate in the system. The antioxidant effect has been found to be strongly supported by phenolic antioxidants such as catechin (Lapidot *et al.*, 2005a, 2005b).

The redox cycle of oxo-ferryl radical and oxo-ferryl was found to be dependent on pH (Mikkelsen and Skibsted, 1992; Reeder and Wilson, 1998; Kanner and Lapidot, 2001). At pH 3.0 metmyoglobin at low concentration (1:30), as compared to hydroperoxides in the lipid system, act pro-oxidatively almost 7×10^4 times as effective at pH 7.0. However, at a high concentration (~1:3), metmyoglobin acted antioxidatively, decomposing hydroperoxides whose concentration then remained at zero for a long period of time.

Polyphenols support the inversion of metmyoglobin catalysis, from pro-oxidation to antioxidation. During this reaction, polyphenols not only donate reducing equivalents to prevent lipid peroxidation, but also prevent the destruction and polymerization of metmyoglobin. The results of our research

highlighted the important and possible reaction of hemeproteins and polyphenols as couple antioxidants working as hydroperoxidases or as pseudo-peroxidases (Lapidot *et al.*, 2005b).

2.4 Prevention of reactions initiated by pro-oxidant metals in models and foods

The main protective mechanism used by plant and animal organisms *in vivo* against the toxic effects of 'free' iron ions are functional proteins which kept it highly chelated such as in ferritin (storage) and transferrin (transport). Turkey dark muscle contains ferritin almost three times more than the light muscle (Kanner and Doll, 1991).

Most of the copper in blood is bound by the enzyme caeruloplasmin which is a powerful inhibitor of iron redox-cycling-dependent lipid peroxidation (Gutteridge, 1983; Kanner *et al.*, 1988b). Its activity as a ferroxidase prevents the accumulation of ferrous iron by the following reaction:

$$4Fe^{2+} \xrightarrow[4H^{+} + O_2]{\text{caeruplasmin } Cu^{++}} 4Fe^{3+} + 2H_2O \qquad 2.26$$

In a model system of membranal lipid peroxidation containing iron ions, ascorbic acid, metmyoglobin and H_2O_2, caeruloplasmin inverts the pro-oxidative activity of the system to antioxidative. This was accepted because by removing ferrous ions, ascorbic acid turns metmyoglobin into an efficient pseudo-peroxidase couple which breaks down hydrogen-peroxide and hydroperoxides to water and alcohol products. The same inhibitory effect was obtained by introducing caeruloplasmin in a turkey meat homogenate (Kanner *et al.*, 1988b).

2.4.1 Chelating agents

Chelating agents can significantly affect the kinetics of lipid peroxidation induced by metal ions. Numerous studies with EDTA (a FDA approved chelator) have demonstrated the complexity which it imparts upon the reactivity of iron. Transition metals have a range of accessible oxidation states enabling them to transfer electrons. The redox potential for such a transfer can be varied by alteration of ligand-type geometry. Ethylenediaminetetraacetic acid and diethylenetriaminepentaacetic acid (DTPA) are widely used as iron chelators in biological research because they can drastically alter the efficiency of iron as a catalyst in oxidation reactions. Both chelators reduce the redox potential of Fe^{2+}. This increases the rate constant transfer of the electron from Fe^{2+} to oxygen or to H_2O_2 generating $O_2^{\bullet -}$, H_2O_2 and $HO^{\bullet}$ radical, and by this promote oxidation (Miller *et al.*, 1990; Welch *et al.*, 2002).

Another chelator known to work in a similar way is citrate anion used in oils to decrease lipid peroxidation (Belitz and Grosch, 1986). Paradoxally, EDTA,

DTPA and citrate, which enhance generation of $HO^{\bullet}$ (Harel, 1994) radicals in a system of iron 'redox cycle' (Girotti and Thomas, 1984; Kanner *et al.*, 1986; Ke *et al.*, 2009) inhibit membranal or triglyceride lipid peroxidation by the same system (Belitz and Grosch, 1986). It has become apparent that the chelate to iron ratio can markedly affect the mechanism of lipid peroxidation (Tien *et al.*, 1982), at EDTA to iron ratios greater than one, lipid peroxidation was inhibited. EDTA in excess de-activate the iron ion by surrounding it with tightly bound ligands that cannot be replaced by reagents such as fatty acid hydroperoxides. In addition to being affected by chelator to iron ratio, EDTA-iron is sensitive to lipid configuration. In liposomes, microsomes and triglycerides, EDTA prevent iron penetration through the membrane and the formation of a site-specific attachment of iron and further decomposition of hydroperoxides to alkoxyl and peroxyl radicals, preventing the initiation and propagation of lipid peroxidation (Tien *et al.*, 1982, Kanner *et al.*, 1988a, 1991b; Boon *et al.*, 2009). The inhibitory activity of EDTA at high concentration (5mM) against oxo-ferryl radicals stems from its characteristic to act as a weak electron donor (Frisell *et al.*, 1959; Harel and Kanner, 1985b).

Desferrioxamine (DFO) is currently the most effective iron chelator for use clinically in human; it binds Fe^{3+} ions with a high affinity constant (10^{31}) preventing effective reduction by reducing agents of iron to Fe^{2+} DFO. Desferrioxamine was found firstly by us to act as an electron donor for oxo-ferryl radicals, peroxidase compound I, lipid free radicals and cooxidation of β-carotene by lipoxygenase (Kanner and Harel, 1987). Desferrioxamine loses its electron donor capability after chelation of iron, forming the ferrioxamine complex (Kanner and Harel, 1987). This result indicates that the active redox electrons in DFO are affected by the non-bonding electrons around nitrogen and oxygen atoms, which are involved in iron coordinatively bonding. Later and most recently, many researchers have shown the electron donor capability of DFO (Morehouse *et al.*, 1987; Miller *et al.*, 1996; Steward *et al.*, 1996; Reeder and Wilson, 2005). Most recently Reeder *et al.* (2008) found other iron chelators as electron donors, including the clinically used dehydroxy-pyridinone.

There are several chelators, such as ferrozine, which stabilize the ferrous ions in a low spin state, preventing electron transfer to oxygen (Kanner and Doll, 1991; Meir *et al.*, 1995). Our group found that ligation of iron by Cl^{-} anions very much stabilizes the ferrous state oxidation in the presence of oxygen and the generation of H_2O_2 and $HO^{\bullet}$ radicals. However, Cl^{-} anion did not prevent the interaction of ferrous ion with preformed H_2O_2 or LOOH, generating $HO^{\bullet}$ and alkoxyl ($LO^{\bullet}$) radicals (Harel, 1994). As it is known, NaCl very much enhanced lipid peroxidation of meat during incubation or storage (Chang and Watts, 1950; Kanner *et al.*, 1991b). This effect could be explained by the fact that Cl^{-} anion turns Fe^{2+} ions from interaction with oxygen to interaction with preformed hydroperoxides, generating free radicals which enhance lipid peroxidation.

2.4.2 Nitric oxide

Another ligand which very much affects Fe^{2+} reactions with oxygen, H_2O_2 and LOOH is nitric oxide ($NO^{\bullet}$). Nitric oxide contains an unpaired electron, is paramagnetic, is a relatively stable radical and it is surrounded by three pairs of non-bonding electrons, with a high affinity to form coordinative bonds with transition metals and especially with iron. $NO^{\bullet}$ can undergo numerous reactions; it can act as either a weak oxidizing compound or as a reducing agent forming NO^- (nitroxyl anion) or NO^+ (nitrosonium cation), respectively. Since the discovery of $NO^{\bullet}$ biosynthesis, the strong affinity of $NO^{\bullet}$ toward hemeproteins and ferrous complexes has been used to trap and determine $NO^{\bullet}$ in biological systems (Stamler *et al.*, 1992). The rate constants for binding of NO with iron-heme at 20 ºC is $5 \times 10^7\ M^{-1}\ s^{-1}$. The results demonstrated that at 37 ºC and with more open iron complexes, $NO^{\bullet}$ is bonded at a rate constant that reaches the diffusion control limit. Once the $NO^{\bullet}$ adducts are generated, they are relatively stable. The dissociation rate constant of nitric oxide myoglobin (Mb $Fe^2 \pm NO^{\bullet}$) is only about $10^{-6}\ M^{-1}s^{-1}$ (Traylor *et al.*, 1979). If $NO^{\bullet}$ is liganded to ferrous ion complexes, this affects the metal-catalyzed reaction and the products developed during the interaction with oxygen and oxygen-active species. Nitric oxide liganded to ferrous complexes changes its interaction with O_2, H_2O_2 or LOOH without forming $O_2^{\bullet-}$, H_2O_2, ferryl or $LO^{\bullet}$ radicals by the following reactions:

$$X\text{–}Fe^2 \pm NO^{\bullet} + O_2 \longrightarrow X\text{–}Fe^{3+} + NO_3^- \quad 2.27$$

$$X\text{–}Fe^2 \pm NO^{\bullet} + H_2O_2 \longrightarrow X\text{–}Fe^{3+} + NO_2^- + HO^- \quad 2.28$$

$$X\text{–}Fe^2 \pm NO^{\bullet} + LOOH \longrightarrow X\text{–}Fe^{3+} + LONO + HO^- \quad 2.29$$

where X is a chelator such EDTA, citrate or hemeproteins such myoglobin or hemoglobin.

$NO^{\bullet}$ and its interaction with hemeproteins (Kanner *et al.*, 1980) and non-heme iron (Kanner *et al.*, 1984) was earlier suggested by us as the active factor in preventing the development of oxidative rancidity in nitrite-cured meat. We have investigated the isolated effect of $MbFe^{2+}NO^{\bullet}$ in oxidative processes in a carotene-linoleate model system, and it was found that 2–10 μM of $MbFe^{3+}$ or $MbFe^2$–O_2 were pro-oxidative, while $MbFe^{2+}NO^{\bullet}$ at all investigated concentrations was not pro-oxidative, and $MbFe^{2+}NO$ could even inhibit the pro-oxidative effect of 2 μM $MbFe^{3+}$ (Kanner *et al.*, 1980). It has further been proved that $MbFe^{2+}NO$ in the presence of excess Mb Fe^{3+} completely inhibited oxygen consumption in a peroxidizing lipid system (Kanner *et al.*, 1980; Carlsen *et al.*, 2005). Oxo-ferryl myoglobin produced by interaction of metmyoglobin with H_2O_2 was reduced by brief exposure of ferrylmyoglobin to $^{\bullet}NO$ generating metmyoglobin and nitrite by the following reaction (Kanner *et al.*, 1991a):

$$^{\bullet}X\text{–}Fe^{4+}{=}O + 2^{\bullet}NO + HO^- \longrightarrow X\text{–}Fe^{3+} + 2NO_2^- \quad 2.30$$

Most recently it has also been found that $NO^{\bullet}$ inactivates ferrylmyoglobin $X\text{–}Fe^{4+}{=}O$. The reaction mechanism includes two steps, with a rapid initiation of an intermediate, $X\text{–}Fe^{3+}\text{–}O\text{–}N{=}O$, which subsequently decay on a longer

time scale to $X–Fe^{3+}$ and nitrite, NO_2^- (Herold and Rehmann, 2001; Beckman *et al.*, 1990).

We believe that nitric oxide acted to modulate oxidative reactions and especially the generation of active oxygen species, lipid free radicals and activated hemeproteins to higher oxidation states. These reactions include: (a) modulation of the reactivity of iron-heme and iron non-heme in forms that prevent or decrease its potential to generate reactive oxygen species and free radicals; and (b) scavenging of free radicals. Generally, the reactions of $^{\bullet}NO$ might act to protect tissues from oxidative stress, but $^{\bullet}NO$ might also act as a cytotoxic compound (Beckman *et al.*, 1990; Trujillo *et al.*, 2008). The overall effects of $^{\bullet}NO$ as activator, modulator or inhibitor are dictated by its site of synthesis, its concentration and the presence and concentration of transition metals.

2.4.3 Control of oxygen

The level of available oxygen for oxidative reactions in food and model systems could be controlled by specific processing such as de-aeration or nitrogen flushing in package. It may be controlled by oxygen scavengers such as the glucose/glucose oxidase/catalase system, which reduces oxygen to water (Kanner, 1994). Direct reduction of oxygen to water could be achieved by enzymes such as ascorbic acid oxidase and caeruloplasmin. These enzymes transfer four electrons to oxygen molecules which split to water.

2.4.4 Control of active oxygen species

The main protective mechanism used by cells *in vivo* against the toxic effects of transition metals which activate oxygen to oxygen reactive species are enzymes such as superoxide dismutase (SOD), catalase, glutathione and ascorbate peroxidases, peroxidases and thioredoxin/ thioredoxin reductase. There are three families of SOD depending on the metal cofactor: Cu/Zn (which bind Cu/Zn in the active center) Fe and Mn types and the nickel type which bind nickel. The Cu/Zn-SOD most commonly used by eukaryotes, is present in cytosol of all cells. Fe-SOD was only found in bacteria and plants, mostly in plastids.

The Mn-SOD is the main scavenger of superoxide in mitochondria and Ni-SOD was only found in prokaryotic. In humans, three forms of SOD are present: SOD1 is located in cytoplasm, SOD2 in mitochondria and SOD3 is extracellular. SOD1 and SOD3 contain Cu/Zn and SOD2 manganese in its reactive center. The main reactions of SOD are the following:

$$SOD–Cu^{2+} + O_2^{\bullet -} \longrightarrow SOD–Cu^{+} + O_2 \qquad 2.31$$

$$SOD–Cu^{+} + O_2^{\bullet -} \longrightarrow SOD–Cu^{2+} + H_2O_2 \qquad 2.32$$

$$\text{net } 2O_2^{\bullet -} \xrightarrow{SOD} H_2O_2 + O_2 \qquad 2.33$$

The rate constant of SOD with $O_2^{\bullet -}$ is about $7 \cdot 10^9\ M^{-1}\,s^{-1}$ high enough to prevent its reaction with other free radicals such as $^{\bullet}NO$. Although SOD

enzymes are protective, they produce H_2O_2. Since H_2O_2 inhibits SOD, is the precursor of $HO^{\bullet}$ radicals and activation of hemeproteins to oxoferryl radicals, H_2O_2 molecules must be rapidly eliminated from tissues. In animal cells, two enzymes exist to rapidly remove H_2O_2: catalase and glutathione peroxidase. In plants, there are catalase and several peroxidases including ascorbate peroxidase. Catalase by one cycle degradates two H_2O_2 molecules to water and oxygen, but peroxidases by one cycle degrades only one molecule of H_2O_2 (Halliwell and Gutteridge, 2007).

$$\text{Catalase–Fe}^{3+} + H_2O_2 \longrightarrow \text{Catalase–Fe}^{4+}\text{=O} + H_2O \qquad 2.34$$

$$\text{Catalase–Fe}^{4+}\text{=O} + H_2O_2 \longrightarrow \text{Catalase–Fe}^{3+} + H_2O + O_2 \qquad 2.35$$

Glutathione peroxidases are enzymes which reduce H_2O_2 to water and lipid hydroperoxides to alcohols. There are several isozymes such as glutathione peroxidase (GPX1), which is found in cytoplasm whose preferred substrate is H_2O_2. GPX4 has a high preference for lipid hydroperoxides, GPX2 is an intenstinal and extracellular enzyme, while GPX3 is extracellular, especially abounded in plasma. So far, eight different isoforms of glutathione peroxidase have been identified (Brigelius-Flohe, 1999; Halliwell and Gutteridge, 2007). Glutathione peroxidases have been shown to be with a selenium-cysteine active center acting by the following reactions:

$$2GSH + H_2O_2 \xrightarrow{GPX} 2GS\text{–}SG + 2H_2O \qquad 2.36$$

$$2GSH + LOOH \xrightarrow{GPX} 2GS\text{–}SG + LOH + H_2O \qquad 2.37$$

where GSH represents reduced glutathione and GS-SG glutathione disulfide, LOOH lipid hydroperoxide and LOH, hydroxy-fatty acid. Glutathione reductase then reduces the oxidized glutathione to complete the cycle.

2.5 Metal catalyzed oxidation in beverages

Citrus juices, especially orange and grapefruit, are popular breakfast beverages and a good source of vitamin C and folic acid in human nutrition. The juices also contain flavonoids that are believed to have beneficial health effects. However, all those vitamins and flavonoids are sensitive to heat-treated process and storage temperatures, due to aerobic and anaerobic reactions of non-enzymic nature.

Ascorbic acid destruction rates were directly proportional to initial concentration of dissolved oxygen and presence of transition metals in model systems and products (Kanner and Shapira, 1989). Storage studies on the loss of ascorbic acid potency in canned orange juices have shown an initial period of rapid loss of ascorbic acid that was caused by the presence of free oxygen from the head space or this penetrate through packaging material.

The involvement of ascorbic acid in non-enzymic browning of citrus products is well known (Lee and Nagy, 1988; Kanner and Shapira, 1989; Johnson *et al.*,

1995). Degradation of ascorbic acid produces dehydro-ascorbic acid, which further degrades to form carbonyls such as acetaldehyde, furfural and others that subsequently reacted via interaction with amino acids (Maillard reaction, Strecker degradation) and aldol condensation, polymerize to give brown pigments (Kanner and Shapira, 1989; Johnson *et al.*, 1995). The non-enzymic browning and ascorbic acid oxidation of grapefruit juice was enhanced by ascorbic acid oxidase and decreased significantly by EDTA, at pH 3.0. The effect of EDTA is complicated. EDTA is known to accelerate ascorbic acid oxidation by ferric ions at neutral pH (Kanner and Shapira, 1989). It is well known that the redox potential of the Fe^{3+}/Fe^{2+} pair can vary by complexing ligands (Miller *et al.*, 1990; Harel, 1994). EDTA reduces the redox potential of Fe^{2+}; however, at pH 3–4, EDTA was found to also reduce the potential of Fe^{3+}, decreasing its effectively reduction by ascorbic acid (Kanner and Shapira, 1989). In addition, ascorbic acid at low pH adopts a non ionic form, which decreases its redox potential and oxidation rate (Khan and Martell, 1967).

All plant extracted beverages, such as tea, coffee, cocoa, apple juice, cranberry juice, red wine, etc., contain trace amounts of metal 'free' ions, such as iron, copper and other transition metals, which could increase oxidation of redox compounds, such as polyphenols, ascorbic acid, cysteine and others. This reaction is very much dependent on oxygen concentration, pH, temperature, water activity (powders) and the presence of compounds which affect the redox potential of the catalysts. Canned and bottled tea, cocoa and fruit juices, which contain a high concentration of polyphenols, are becoming popular worldwide, because it is believed that they are a great benefit to human health. The stability of all these products is dependent on the presence of free oxygen in the headspace, which penetrates through the packaging material and is very much enhanced after opening the package to air and humidity.

2.6 Metal catalyzed oxidation in dehydrated foods

The poor stability of carotenoids in dehydrated foods, and especially in paprika powder, constitutes a serious economic problem. In our studies we have used an aqueous model system to investigate water soluble factors present in the pepper fruit that affect carotene-lipid oxidation (Kanner *et al.*, 1976, 1977a, 1977b, 1978b, 1979; Kanner and Budowsky, 1978).

Subsequently, these studies were extended to a solid model based on powdered cellulose containing absorbed carotene and linoleic acid (Kanner and Budowsky, 1978), and finally we have developed a model system to simulate more closely the natural product, paprika powder, by using cellulose powder containing oleoresin, i.e., the lipid extract from paprika (Kanner *et al.*, 1978). It was found that the main catalyzer in paprika powder is a peroxidase-like protein (hemeprotein) as well as copper and iron ions. These catalyzers of lipid peroxidation are relatively not affected by the water activity (a_w) of the powder. The main antioxidants in paprika were found to be α-tocopherol and ascorbic acid, both at a concentration of

0.9 mg/g and 17.6 mg/g dry matter, respectively. The stability of the carotenoids in paprika is very much affected by the water activity being the best at 0.64 a_w. When the a_w in powdered paprika exceeded 0.32, carotenoid bleaching proceeded in a sigmoidal three stages. After an initial bleaching, there was a prolonged period of stability after which oxidation is resumed.

The intermediate period of stability was absent in the dry products. Results obtained with the oleoresin-cellulose model showed that the three-stage carotenoid bleaching at high a_w value was characteristic of the combined action of all the catalyzers only after addition of ascorbic acid. It is concluded that ascorbic acid, becoming soluble only at high water activities, only then plays its important antioxidant part in the stabilization of the carotenoids in powdered paprika or in a model system. Under conditions of high a_w, the pro-oxidant effect of the peroxidase-like protein (hemeprotein) and the metal ions are overcome by the marked antioxidant action of ascorbic acid present at very high concentration in this product. In order to increase the stability of paprika powder by activation of ascorbic acid it is very much recommended to keep it at a_w of 0.64 or ~10% moisture, at 4 ºC. Metal affected oxidation in many other dehydrated food systems (Delgado-Vargas *et al.*, 2000).

2.7 Metal catalyzed oxidation in muscle foods

Oxidative degradation is a major cause of quality deterioration in muscle foods, following slaughtering, processing and storage (Kanner, 1994). A critical question in the control of oxidation in muscle foods concerns the source of the primordial active species or free radicals that initiate the oxidative deterioration. When cells are injured, such as in muscle foods after slaughtering, oxidation and peroxidation are favored and traces of $O_2^{\bullet -}$, H_2O_2 and hydroperoxides (LOOH) are formed. The stability of a muscle product will depend on the 'tone' of these peroxides and especially from the involvement of metal ions in the process. The cytosol contains not only pro-oxidants, but also antioxidants and the tone of both affects the overall oxidation. Lipid peroxidation is one of the primary mechanisms of quality deterioration in meat products. The change in quality is mainly manifested by deterioration in color, flavor and nutritive value, including production of cytotoxic compounds (Kanner, 1994; Gorelik *et al.*, 2005).

2.7.1 Muscle food color

Oxymyoglobin is the main pigment in fresh muscle food, responsible for the bright red color. The redox state of the heme-iron and the presence or nature of the ligand bound to iron account for the meat color. Oxidation of heme-iron from ferrous state in oxymyoglobin or deoxymyoglobin to the ferric state, produces the brownish color of fresh meat, which consumers find undesirable (Lin and Hultin, 1977; Faustman and Cassens, 1990; Kanner, 1994). Heme-proteins such as oxymyoglobin and oxy-hemoglobin are particularly prone to

oxidation and autoxidation, and are affected by a wide spectrum of active oxygen species and metal ions, such as $O_2^{\bullet -}$, H_2O_2, $HO^{\bullet}$, HOCl, $NO_2^{\bullet}$, lipid oxy-radicals, ferrous/O_2 and ferryl.

Several authors have postulated that oxymyoglobin oxidation and lipid peroxidation in muscle tissue are interrelated (Greene, 1969; Lin and Hultin, 1977; Yin and Faustman, 1993). Free radicals generated during lipid peroxidation promote the accumulation of metmyoglobin (Kanner, 1994). The use of hemichrome accumulation from the oxidation of hemeproteins has been recommended as a method for the evaluation of tissue oxidation and autoxidation *in vivo* (Tappel, 1999). We have developed a model system containing oxymyoglobin and muscle membranes oxidized by an iron redox cycle to elucidate the mechanism of oxymyoglobin oxidation (Gorelik and Kanner, 2001a, 2001b).

The model system demonstrated that MbO_2 oxidation is a process that is affected by two pathways; the first pathway generates active oxygen species such as $O_2^{\bullet -}$ and H_2O_2; the second generates lipid peroxides and lipid free radicals. Maximum inhibition was achieved only by introducing inhibitors of both pathways, such as SOD, catalase, conalbumin (chelator) and catechin, into the system. The membrane lipid peroxidation in this model was affected mainly by iron 'redox cycle'. In order to elucidate the interrelated effects of MbO_2 oxidation and lipid peroxidation in *in-situ* muscle tissues, a study was developed to examine the effects of high PUFA and vitamin E supplementation in the feed on calf muscle lipid peroxidation and fresh muscle color retention, following NaCl addition and storage at 4ºC.

Meat tissue from calves fed a ration rich in PUFA was significantly more susceptible to lipid peroxidation and MbO_2 oxidation following salting. However, vitamin E supplementation in the diet prevented these changes, and the fresh meat retained its high sensory properties (color, flavor) and nutritive value (vitamin E) (Granit *et al.*, 2001). In raw muscle tissue Mb acts mostly as a pseudo-peroxidase and inhibit lipid peroxidation and propagation of color loss, (Harel and Kanner, 1985b; Kanner *et al.*, 1991c; Ahn *et al.*, 1995; Gorelik and Kanner, 2001a).

2.7.2 Muscle food flavor

The term 'warmed-over flavor' was first introduced (Tims and Watts, 1958), to describe the rapid onset of rancidity in cooked meat during storage. Heating could affect many factors involved in lipid peroxidation of muscle foods. The level of 'free' iron in muscle tissue greatly increases during cooking (Kanner *et al.*, 1988a, 1991a, 1991b) and the iron 'redox cycle' is more pronounced and propagation of lipid peroxidation increases. This pro-oxidant effect is inhibited by relatively low concentration of EDTA, which indicate that most of the metal catalyzed lipid peroxidation in cooked muscle tissues is affected by 'free' iron redox cycle (Kanner *et al.*, 1988a). Heating inactivates many antioxidant enzymes, myoglobin release hemin (Grunwald and Richards, 2006) and loses its solubility and antioxidant effect. Heating of muscle food very much disturbs the

balance between pro-oxidants and antioxidants, and the pro-oxidative effects greatly increase. In cooked muscle foods, lipid peroxidation propogate mostly by the following reactions:

$$Fe^{2+} + LOOH \longrightarrow Fe^{3+} + LO^{\bullet} + HO^{-}$$

$$Fe^{3+} + AH_2 \longrightarrow Fe^{2+} + AH^{\bullet}$$

where AH_2 and $AH^{\bullet}$ are ascorbic acid or other reducing agents and the ascorbyl radical.

2.7.3 Muscle food nutritive value

Partially oxidized food, for example, heated red meat, undergoes further oxidation by metal catalysis under simulated and *in vivo* stomach conditions yielding deleterious compounds such as hydroperoxides and reactive carbonyls; for example, malondialdehyde (MDA), a lipotoxin (Kanner and Lapidot, 2001). The cross-reaction between free radicals produced during this reaction in stomach conditions also co-oxidized vitamin E, β-carotene and vitamin C (Gorelik *et al.*, 2008a, 2008b). The lipotoxin MDA level in rat and human plasma increase significantly following the consumption of red turkey meat (Gorelik *et al.*, 2008a, 2008b). In human volunteers baseline plasma levels of MDA were 50 ± 20 nM and after a meal of turkey meat cutlets, plasma MDA levels in volunteers increased by 160 nM. The elevation of plasma MDA in rats and humans was completely prevented by integration of red-wine polyphenols in the diet (Gorelik *et al.*, 2008a, 2008b). The findings explain the potential harmful effects of metal catalysis of lipid peroxidation and lipotoxins found in foods, further lipid peroxidation in stomach medium and the important benefit of consumption of dietary polyphenols during the meal.

2.8 Future trends

Current research and development studies should emphasize their direction towards functional, health promoting foods and dietary recommendation for health maintenance and well-being throughout life. Epidemiological studies and experimental data suggest that diets high in fat and red meat are risk factors contributing to the development of atherogenesis and several kinds of cancer. The Western diet contains large quantities of oxidized fatty acids, oxidized cholesterol and cytotoxic carbonyls because a large proportion of the food in the diet is often consumed in a fried, heated or processed form.

Consumption of partially oxidized food enhanced lipid peroxidation in the stomach and consequent production and absorption of cytotoxic lipid peroxidation end products into the plasma. Repeated consumption of oxidized fat in the diet poses a chronic threat to human health. In order to prevent the risk factors from oxidized foods on human health, action should be considered 'from the farm to the fork' and more.

- As muscle tissue starts its oxidation process just after slaughtering, increasing the stability of muscle food by elevation of α-tocopherol in muscles prior to slaughtering should be adopted by all farmers and industries.
- Genetic manipulation should be adopted in order to increase absorption of α-tocopherol from the gut in animals, and especially in those, such as turkey, which suffer from very low efficiency absorption of the vitamin.
- Developing methods for better utilization of antioxidants after harvesting or slaughtering to increase the shelf-life of foods.
- Developing better methods of processing the raw material and storage which prevent food oxidation.
- Prevention of food oxidation by cocktail antioxidants working synergistically in foods during storage.
- Developing packaging with active O_2 destroyers to non-toxic compounds.
- Identification of natural chelators which reverse iron and other metals from catalysis of oxidation to active antioxidants. Such compounds should not be affected by high temperature, be non-toxic and working as mimic SOD or catalase to destroy $O_2^{\bullet -}$ and H_2O_2 without reducing agents. Some synthetic metallo-porphyrins as catalytic antioxidants are in the market for research (Patel and Day, 1999).
- Identification of natural chelators which will prevent the interaction between transition metals and oxygen. Such chelators will prevent activation of oxygen to ROS. (Ferrozine is a chelator which stabilizes the ferrous state, most probably at a low spin, preventing oxidation of ferrous to ferric ion and the generation of $O_2^{\bullet -}$.)
- Identification of non-toxic natural chelators, which will mimic glutathione peroxidase activity, but utilize ascorbic acid as the reducing agent for decomposition of hydroperoxides in emulsions and membranes.
- Developing special antioxidants to suppress further lipid peroxidation in the stomach and prevent absorption of advanced lipid peroxidation end products.

2.9 References

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3

The impact of singlet oxygen on lipid oxidation in foods

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Abstract: Singlet oxygen has specific properties that result in a distinctively different reactivity towards food components compared to triplet oxygen. These molecular properties have a major impact on the reactivity of singlet oxygen towards lipids. Furthermore, a detailed description is given for a variety of different methods that can be used to detect molecular oxygen and its reaction products. This background information is then used to discuss the influence of singlet oxidation on a variety of food products in more detail. Also the influence of several micro ingredients on the photooxidation process in foods is illustrated.

Key words: photooxidation, singlet oxygen, photosensitizer, oxygen quencher, food oxidation.

3.1 Introduction

Oxidation plays a fundamental role in the reduction of the quality of fats, oils and many other basic ingredients in food or animal feed. A significant amount of research data is available on the impact of autoxidation caused by the most abundant and stable form of oxygen: *triplet oxygen* ($^3O_2$). However, an alternative oxidation process occurs when food components are exposed to a light source, even at low temperatures. Many molecules are able to absorb energy from light after which this energy is transferred to an oxygen molecule. This process leads to the conversion of the most abundant triplet oxygen into the more reactive *singlet oxygen* ($^1O_2$). Several analytical techniques that can be used to specifically measure singlet oxygen will be discussed.

Both types of oxygen react very differently with lipids and other nutrients due to the dissimilarity in their fundamental chemical structure. In this chapter the differences between the reaction mechanisms will be discussed. This will help to predict which breakdown products of oxidation can be used as markers to identify the type of oxygen (singlet or triplet) that is the principal cause of an observed oxidation problem.

The impact of singlet oxygen on the reduction of nutritional and sensory quality will be reviewed for a wide variety of food products such as fats and oils, dairy products and meat in which the oxidation of lipids and proteins plays a prominent role. Afterwards examples will be shown of the role that specific micronutrients can have the singlet oxidation process. Also the negative effect of singlet oxygen on the level of essential vitamins in food products will be illustrated.

The end of this chapter will focus on the prevention of singlet oxidation in foods and on the future research that will be required for a better understanding of the impact and prevention of singlet oxidation in the food and feed industry.

3.2 Properties of singlet oxygen

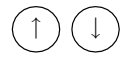
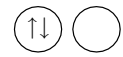
3.2.1 Discovery of singlet oxygen

Joseph Priestley discovered oxygen in 1775, but it took several decades before the scientific world found out about its diamagnetic properties. It was Faraday who took the credit for discovering this major difference between oxygen and the other permanent gasses such as nitrogen. In the twentieth century the oxygen molecule was completely exposed. The paramagnetic properties of oxygen (triplet oxygen) could finally be explained at a molecular level and in 1934 a higher energy state of oxygen was also discovered by spectroscopy and reported as singlet oxygen. The importance of singlet oxygen in chemical reactions was not recognized for almost 30 years until the 1960s. Singlet oxygen was rediscovered by a group of chemists interested in photooxidation experiments in organic chemistry (Corey and Taylor, 1964). The insight into singlet oxygen chemistry has progressed from that point in time.

3.2.2 Properties and reactivity of singlet oxygen

It is important to specify which of the specific forms of the oxygen molecule is present under certain conditions in order to understand its diverse reactive properties towards food components. Therefore, it is necessary to take a look at the molecular structure in more detail and more specifically the distribution of the outer shell electrons. The distribution of the electrons of the individual oxygen atoms in the molecular orbital diagram reveals many chemical properties of both singlet and triplet oxygen. Filling the molecular orbitals with electrons in the diagram for the oxygen molecule specifically results in a

Table 3.1 Properties of triplet and singlet oxygen

Property	Type of oxygen	
	$^3O_2$ (triplet)	$^1O_2$ (singlet)
π^*-orbitals		
Energy level	0	22.5 Kcal/mole
Nature	Diradical	Non-radical
Reacts with	Radicals	Electron-rich compounds

situation where the last two electrons need to be distributed over the outer two orbitals (π^*-orbitals). The most stable form of oxygen (triplet oxygen) is obtained when the electrons are placed in different orbitals according to the Hund's rule, while the exited and less common form (singlet oxygen) is obtained when the two electrons are present in one orbital (Table 3.1). These names for the different forms of molecular oxygen are derived from the nomenclature of the spin multiplicity of the molecule which is defined a 2S+1, where S is the total spin quantum number. One spin is designated as (+1/2). In case of ground state of oxygen, S is 1 for two electrons with the same spin in two individual orbitals. Therefore the molecular spin is ½ + ½ = 1. The spin multiplicity of the grounds state of the oxygen molecule then becomes 3 ($= 2 * 1 + 1$), which is referred to as triplet oxygen. For the excited form of the oxygen molecule there are two electrons with an opposite spin present in one orbital which leads to a molecular spin of $-½ + ½ = 0$. The spin multiplicity of the excited state of the oxygen molecule then becomes 1 ($= 2 * 0 + 1$), which is referred to as singlet oxygen.

In singlet oxygen the electronic repulsion between the two negatively charged electrons that are forced in one orbital brings about a highly energetic and reactive molecule. The energy difference of 22.5 Kcal/mole is the main driver for the higher reactivity of singlet oxygen (Korycka-Dahl and Richardson, 1978). However, the electron distribution also generates a very different chemical reactivity for both compounds. Triplet oxygen is a biradical because it contains two free electrons and therefore it will only react with other radicals. Singlet oxygen, on the other hand, has an electron pair instead of two free radicals. As a consequence singlet oxygen will be looking for a 'tenant' to fill its empty molecular orbital in order to reach an energetically more favorable state. Because an electron pair would fit nicely into the highest vacant molecular orbital, singlet oxygen will react rapidly with all electron rich compounds.

3.2.3 Reaction of singlet oxygen with lipids

Oxidation plays a fundamental role in the reduction of the quality of fats and oils and therefore this matrix is also very useful to further discuss oxidation by singlet oxygen *versus* oxidation by triplet oxygen. A significant amount of research data is available on the impact of the so-called autoxidation in which triplet oxygen is involved. Initially a lipid radical is formed in this oxidation process, which requires a significant amount of energy. Therefore, autoxidation is strongly accelerated by increased temperatures. Once the lipid radical is formed it will react with other radicals such as triplet oxygen. In polyunsaturated lipids with several double bonds present, the radical can be stabilized via different resonance forms (Frankel, 2005). The observation that polyunsaturated lipids have a higher relative oxidation rate confirms the role of radical formation in the reaction mechanism of autoxidation (Table 3.2).

For singlet oxidation the reaction does not proceed via a radical type reaction. The singlet state oxygen reacts directly with the unsaturated fatty acid via a concerted 'ene' addition mechanism (Fig. 3.1). For this reaction mechanism the effect of the level of unsaturation of the lipids on the oxidation rate is only limited because oxidation by singlet oxygen will not proceed via a radical mechanism. The influence of temperature on the reaction rate is also negligible

Table 3.2 Relative oxidation rates of singlet and triplet oxygen with unsaturated lipids

Type of oxygen	Degree of unsaturation		
	C18:1	C18:2	C18:3
Triplet	1	27	77
Singlet	3×10^4	4×10^4	7×10^4

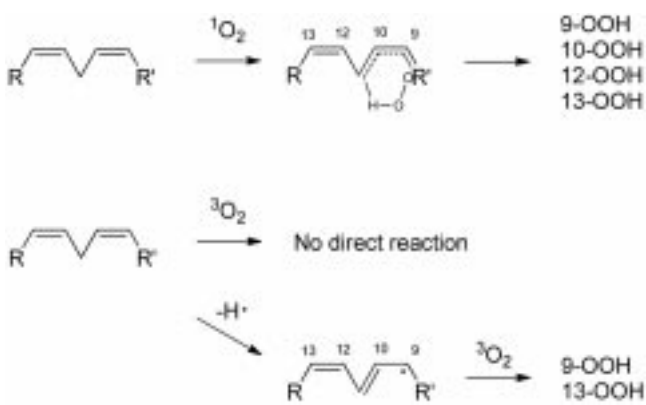


Fig. 3.1 Difference between lipid oxidation mechanism singlet and triplet oxygen.

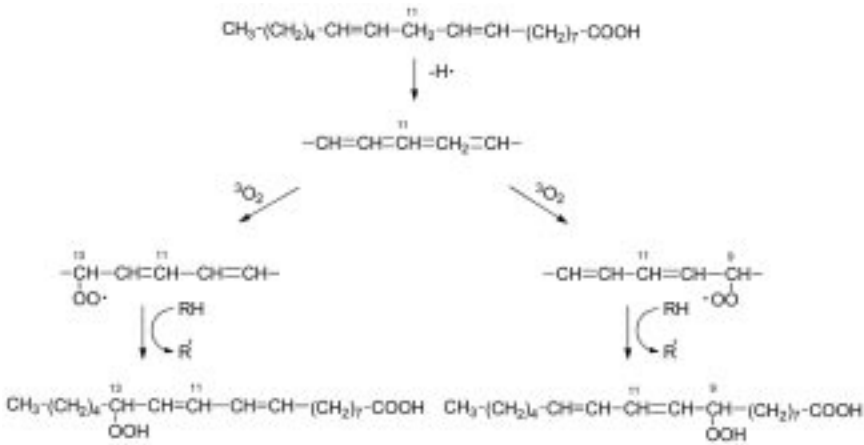


Fig. 3.2 Reaction products formed by autoxidation of linoleic acid.

since much lower activation energies are required for oxidation reactions that involve singlet oxygen.

The main importance of the difference in reaction mechanism is the generation of different oxidation breakdown products. In lipid oxidation the two types of oxygen will react differently with the lipid matrix generating distinctive hydroperoxides which decompose further in specific aldehydes and other breakdown products.

In the case of linoleic acid oxidation with triplet oxygen, two hydroperoxides will be generated (Frankel, 2005). Triplet oxygen does not react directly with the double bonds. Due to its molecular structure (biradical) the reaction needs to proceed via an initial formation of a lipid radical. Therefore, the first step in the triplet oxidation of linoleic acid is hydrogen abstraction at carbon 11, which is the most easily removed as it is a bis-allylic methylene group. The radical produced is resonance stabilized and two resonance forms are particularly stable because they contain conjugated double bonds. Therefore the radical will be present preferentially on carbon 9 and 13, and triplet oxygen will react with these alkyls radicals to form a hydroperoxide (Fig. 3.2).

Because singlet oxygen does react via a concerted mechanism there will be a direct reaction with the double bond without any preference for a specific carbon. Consequently hydroperoxides can be formed on carbon 9, 10, 11 and 12 in equal quantities.

A similar reasoning can be applied on other fatty acids (Table 3.3). There is always a preferential formation of conjugated hydroperoxides for poly-unsaturated fatty acids that are oxidized with triplet oxygen. For singlet oxidation the peroxides will be introduced at either end carbon of all double bonds. Owing to the difference in the oxidation mechanism between singlet and triplet oxygen the hydroperoxide distribution will be different. Consequently also the breakdown product of the hydroperoxides will be different. Chemical

Table 3.3 Hydroperoxides formed by oxidation of fatty acids with singlet and triplet oxygen

	Fatty acid		
	Oleate	Linoleate	Linolenate
Singlet oxygen			
Saturated hydroperoxides	9-OOH 10-OOH		
Conjugated hydroperoxides		9-OOH 13-OOH	9-OOH 12-OOH 13-OOH 16-OOH
Non-conjugated hydroperoxides		10-OOH 12-OOH	10-OOH 15-OOH
Triplet oxygen			
Saturate hydroperoxides	8-OOH 9-OOH 10-OOH 11-OOH		
Conjugated hydroperoxides		9-OOH 13-OOH	9-OOH 12-OOH 13-OOH 16-OOH

From Frankel *et al.* (1979).

analysis of the oxidation breakdown products therefore allows differentiating between the types of oxygen that may cause the oxidation.

3.2.4 Methods to study singlet oxygen in foods

Detection and evaluation of singlet oxygen in foods

Singlet oxygen detection in food oxidation of foods is difficult due to the short lifetime of the excited molecule. Several analytical techniques have been developed for the detection of singlet oxygen.

Spectrophotometric methods can be used to measure singlet oxygen indirectly. These methods use a compound of which absorption at a suitable wavelength decreases after reaction with singlet oxygen. In organic solvents the molecule 1,3-diphenylisobenzofuran can be used since it reacts readily with singlet oxygen. This reaction results in decreased absorbance at 410 nm (Kochevar and Redmond, 2000). In aqueous systems para-nitrosodimethyl-alanine can be monitored at 440 nm absorbance as the molecule reacts with an imidazole intermediate.

Another interesting molecule for indirect detection of singlet oxygen is cholesterol as it reacts with singlet oxygen to form specific oxidation products, more specifically hydroperoxides. The specificity even allows differentiation of

oxidation originating from triplet oxygen, as this results in hydroperoxides on position 7a and 7b, while singlet oxygen introduces the hydroperoxide on 6a and 6b (Girotti and Korytowski, 2000).

The more advanced technique of electron spin resonance spectroscopy (ESR) can also be used to detect singlet oxygen. This technique specifically detects free radicals, often by reaction of a radical with a spin-trapping agent, thus forming a stable radical adduct. Consequently ESR seems more suitable to study the reactions in which free radicals play a role such as the oxidation of food products with triplet oxygen. However, one of the spin-trapping agents called TMPD (2,2,6,6-tetramethly-4-piperidone) reacts very specifically with singlet oxygen to form a stable nitroxide radical adduct TAN (2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl). No other reactive oxygen species have been found to convert TMPD to TAN (Ando *et al.*, 1997). ESR detected the formation of singlet oxygen in meat (Whang and Peng, 1988) and milk (Bradley, 1991).

Another suitable analytical technique to detect singlet oxygen is detection of its chemiluminescence at 1270 nm. A photon can be released at 1270 nm which corresponds to the specific energy differential between excited singlet oxygen and ground-state triplet oxygen. Detection of singlet oxygen by measuring the emission at 1270 nm has been successful in biological systems (Kanofsky, 2000).

Methods of studying oxidation by singlet oxygen

Because of the different chemical nature of $^1O_2$ and $^3O_2$ their reaction with lipids proceeds via very dissimilar oxidation pathways. Both types of oxygen will attack the lipid skeleton at different locations. When these oxidized lipids then decompose further, distinct breakdown products are formed specific for the type of reactive oxygen that lies at the basis of the oxidation process. The occurrence of these products can therefore be used to study oxidation pathways.

Most of the oxidation breakdown products are very volatile and also cause the rancid flavor of products that contain oxidized fats or oils. It is possible to sample these volatiles in the headspace above an oxidized lipid. All components in the headspace can then be separated (via gas chromatography, GC) and these individual constituents of the total flavor can be identified (via mass spectrometry, MS).

If one wants to find out whether the rancidity was caused by singlet or triplet oxygen, the profile of the flavor molecules can reveal some valuable information. In order to be able to pinpoint which flavor molecules are produced from singlet oxidation and which from the more common triplet oxidation, it is helpful to design an experiment that looks at only one of the two possible oxidation pathways. Triplet oxygen-borne lipid oxidation can be promoted by working at high temperatures combined with the use of a singlet oxygen quencher. Alternatively singlet oxygen-borne lipid oxidation is promoted by the addition of a photosensitizer that is able to transfer energy from light to the oxygen molecule, which leads to the formation of high levels of singlet oxygen. By working simultaneously at low temperatures the reactivity of remaining triplet oxygen can be slowed down.

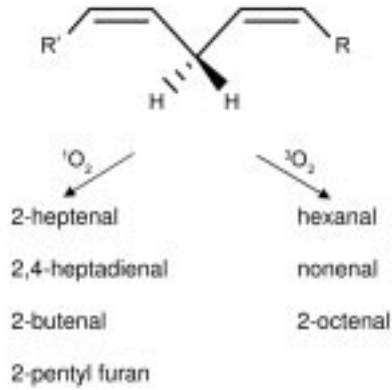


Fig. 3.3 Reaction products of singlet and triplet oxygen with lipids results in different breakdown products.

In rapeseed oil, the light-induced promotion of singlet oxidation leads to a strong increase in the concentration of volatile components such as butenal, heptenal and several other specific lipid oxidation products (Fig. 3.3). The headspace analyses of samples subjected to conditions that promote lipid oxidation by triplet oxygen reveal the presence of a distinctly different set of volatile oxidation products. Compounds such as hexanal and nonenal appear which therefore can be considered as markers for triplet oxidation (Van Dyck, 2007).

3.3 Impact of singlet oxygen on quality reduction in foods

Many quality changes in food products are caused by singlet oxidation. Of course this means that the food product should be packed in a way that contact with sunlight remains possible. For several food products the flavor change after storage in sunlight has been known for a long time, but the mechanism behind this often fast deterioration of food products was only unraveled in the last two decades. In most cases the molecules that were causing the strange 'sunlight' taste were identified well before the impact of singlet oxygen on the formation of these compounds was understood.

Probably the two most notorious products sensitive to storage in sunlight are soybean oil and milk, but also other food products may suffer significant quality losses when exposed to singlet oxygen.

3.3.1 Vegetable oils

Smouse and Chang (1967) identified that the molecule 2-pentylfuran was responsible for the 'reversion flavor' that appears when soybean oil is stored in light. Many years later Callison (2001) discovered that a natural photosensitizer played a role in this process by means of a relatively simple experimental protocol. Addition of chlorophyll to the oil and subsequent storage in sunlight

increased the level of the undesired 2-pentylfuran. On the other hand, when the same samples were stored in the dark, the level of the reversion flavor did not change. In another experiment a purification technique was used to completely remove all chlorophyll from the soybean oil and then the oil was stored in light again. Remarkably it was found that in this case the level of 2-pentylfuran did not increase. These experiments are a clear proof of the importance of singlet oxidation and the critical role of the natural photosensitizer chlorophyll. Furthermore a complete reaction mechanism that leads to the formation of 2-pentylfuran could be proposed.

3.3.2 Animal fats

Lard is a very suitable lipid matrix to study singlet oxidation. The photosensitizer, myoglobin which is naturally present in pork has a very low solubility in the fat (Baron and Andersen, 2002). Consequently lard is not 'contaminated' with a photosensitizer during meat processing. A GC-analysis of lard spiked with 5 ppm chlorophyll and stored under light was found to be different both quantitatively and qualitatively from lard with 0 ppm chlorophyll stored under the same conditions (Lee, 2002). The total volatile compounds in lard with 0 ppm chlorophyll stored under light were not different from those in lard stored in the dark, which indicates that lard with 0 ppm chlorophyll was not sensitive to photooxidation. However, the concentration of volatile oxidation products in lard spiked with 5 ppm chlorophyll stored under light increased dramatically, which shows that chlorophyll accelerated lipid oxidation in lard.

3.3.3 Marine oils

Marine oils can be used to investigate the effects of photosensitized oxidation and free radical oxidation. More specifically cod liver oil, a typical fish oil rich in polyunsaturated fatty acids, has been used by Pan (2005a) to study photosensitized oxidation initiated by water-soluble food colorants that can exhibit photosensitizing and generate singlet oxygen. Because in food systems fats and oils usually exist in the form of an emulsion, cod liver oil-in-water emulsions were used as a model. Oxidation products were detected with GC-MS which lead to the observation that singlet oxidation by the photosensitizer Rose Bengal occurred much faster than ferrous chloride accelerated triplet oxidation. The n-9 family of fatty acids accounted for up to 28.35% of total fatty acids present in the cod oil. When monounsaturated n-9 family fatty acids are oxidized predominately two hydroperoxide isomers are expected in the concerted reaction mechanism for oxidation with singlet oxygen. In contrast, four hydroperoxides are expected for the autoxidation in which hydrogen atom abstraction occurs on the methylene group adjacent to the double bond (at each side of the double bond) which leads to a resonance form where the radical can reside on four carbon atoms. Therefore this would lead to the generation of four possible hydroperoxides in total.

When n-9 family fatty acids were oxidized to reach a similar degree of oxidation (similar hydroperoxide levels), more n-9- hydroperoxides and n-10-hydroperoxides, which are the precursors of nonanal and 2-decenal, should be generated by photosensitized oxidation compared to autoxidation. The research of Pan (2005a) shows that more nonanal and 2-decenal were formed in an o/w emulsion with added Rose Bengal (1.78% of nonanal and 6.01% of 2-decenal) compared to ferrous chloride accelerated autoxidation (0.73% of nonanal and 3.12% of 2-decenal).

3.3.4 Dairy milk

Another light-sensitive food product is milk. Chlorophyll is an important photosensitizer in vegetable oils, but quite unexpectedly does not play any role in the oxidative stability of milk. This is because cows do not transfer this molecule from the grass they eat into the milk. However, another natural photosensitizer called riboflavin causes the oxidation problems in milk. Riboflavin is a member of the vitamin B family which is synthesized in green plants. The vitamin is absorbed during digestion and is deposited in the milk by ruminants.

The reduction potential of triplet riboflavin was found to be about 1.7 V at pH 7 (Lu *et al.*, 1999). This is sufficiently high to remove an electron or hydrogen atom from food components such as polyunsaturated fatty acids, ascorbic acid, and tocopherol (Decker, 1998).

The role of singlet oxygen in light-induced off-flavor was first suggested by Foote (1976), but the actual presence of singlet oxygen in milk exposed to sunlight or fluorescent light has been detected only very recently by Bradley and Min (1992) with the use of ESR spectroscopy. Riboflavin induced photosensitization has been identified to play a major role in the formation of light-induced off-flavor in dairy products (Skibsted, 2000). The lipid and protein fraction in milk can react with singlet oxygen. The major compounds identified to be responsible for the flavor defects in light-exposed milk are dimethyl disulfides and short chain aldehydes.

A study looking at the light-induced off-flavor in solutions of the amino acids cysteine, methionine, or valine revealed a hydrogen sulfide odor in the light-exposed cystein sample (Jung *et al.*, 1998). Dimethyl disulfide was identified in the methionine sample that was exposed to light. The valine solution produced no odors that were specific for singlet oxidation. Neither hydrogen sulfide, nor dimethyl disulfide were found in the absence of light and riboflavin. This observation supported the hypothesis that light and riboflavin are required for the development of light-induced off-flavor. When riboflavin was removed from milk, no sunlight flavor developed. The role of singlet oxygen could be confirmed by the addition of the singlet oxygen quencher ascorbic acid to the milk. The addition of the quencher led to an expected reduction of dimethyl disulfide in the milk. A study with a trained sensory panel showed that sulfurous off-flavors could be detected in milk that was exposed to sunlight for only

15 min. On the other hand, no sunlight flavor was detected in milk after a period of 8 hours when the samples were stored in the dark. Sensory panel evaluations concluded that dimethyl disulfide was a key compound for the light-induced sunlight flavor in milk.

About two decades earlier Forss (1979) had already proposed that methanethiol, dimethyl sulfide, and dimethyl disulfide were responsible for sunlight flavor in milk. Later it was identified that prolonged light-exposure altered the methional flavor to a methyl mercaptan-like flavor (Dimick and Kilara, 1983) and supported that methional decomposed to methyl mercaptan and dimethyl disulfide. It was identified that methionine sulfoxide, is derived from methionine in the presence of light, and that riboflavin, protein, and oxygen were required for the development of light-induced sunlight flavor in milk.

Other important compounds contributing to the sunlight flavor of milk are lipid oxidation products such as pentanal, hexanal and heptanal (Kim and Morr, 1996; Marsili, 1999). Lee (2002) investigated the role of fat content, riboflavin content, singlet oxygen quencher or free radical scavengers on the light-induced volatile compounds in milk. In this study milk with a basal level of 1.5 ppm riboflavin was spiked with different levels of riboflavin. Already after two hours a clear effect was observed for riboflavin on the on the formation of pentanal, dimethyl disulfide, hexanal, and heptanal in milk stored at 4 ºC under light. As the concentration of added riboflavin increased from 0 to 50 ppm the peak area of pentanal and hexanal increased by 35 and 150%. Methyl disulfide and heptanal were not detected in milk with 0 and 5 ppm riboflavin added. In contrast the milk spiked with 10 or 50 ppm riboflavin did contain both off-flavors.

Also the effect of fat content was investigated on the formation of pentanal, hexanal, heptanal, and dimethyl disulfide in milk stored at 4 ºC under light for 8 h. As the fat content in milk increased from 0.5 to 3.4%, pentanal increased by 560%, heptanal by 130% and hexanal by 120%. The concentration of dimethyl disulfide did not change significantly as the fat content increased. Also Cadwallader and Howard (1998) reported that when the fat level of the milk increased, light-induced flavor increased. The variability for the increase in relative reaction rates between different aldehydes has also been reported by Marsili (1999) and was assigned to the availability of substrates for pentanal and hexanal.

3.3.5 Soy milk

Soy milk is an important traditional beverage originating from oriental countries. The consumption of soymilk is increasing in Western countries because of rising customer awareness of the health beneficial functions of soy foods (Savitry and Prakash, 2004). Soy milk contains 2.86% protein, 1.53% fat, 0.27% ash, 1.53% carbohydrate, 93.81% moisture, and about 3 ppm riboflavin (Huang *et al.,* 2004). Addition of riboflavin is also a good way to fortify soy drinks and foods.

The presence in soy milk of riboflavin, or added for fortification in could induce undesired changes in sensory and nutritional values if the product comes

in contact with light of the presence of riboflavin affects the concentration of hexanal and pentane in soy milk. Also the degradation of the beneficial soy isoflavones daidzein and genistein has been reported by Yang *et al.* (2008) for a model system exposed to riboflavin photosensitization.

The impact of singlet oxidation on soy isoflavones was further studied by Lee *et al.* (2008). High levels of riboflavin were added to soymilk and stored in transparent airtight sealed bottles. Samples were stored in a light box at 25 ºC and a second set of samples was stored under dark conditions. Riboflavin photosensitization decreased the stability of isoflavones in soymilk significantly. Compared to the previously mentioned model system, the photodegradation rate of isoflavones in soymilk was lower. This difference was explained by possible matrix effects such as interference of colloidal suspension, turbidity of samples, and other compounds. Daidzein showed better stability than genistin in riboflavin spiked soymilk.

3.3.6 Meat products

Hemoproteins such as myoglobin are known to be the primary photosensitizers that influence singlet oxidation in meats (Whang and Peng, 1988). Peroxide values for samples of pork and turkey meat exposed to light were significantly higher than for samples stored in the dark. Furthermore, it was found that myoglobin and its derivatives functioned as photosensitizers in model systems leading to the formation of singlet oxygen. Certain colorants for meat products have also been shown to exert photosensitizing effects (Usuki *et al.*, 1984).

Besides lipid oxidation also protein oxidation plays a major role in meats. Singlet oxygen reacts primarily with five amino acids: tryptophan, histidine, tyrosine, methionine, and cystein to form peroxides (Michaeli and Feitelson, 1994, 1997). The reaction rate of proteins and singlet oxygen depends strongly on the type of amino acids that are the building blocks of the protein. Most reactive are the amino acids which contain double bonds or a electron-rich sulfur atom.

3.4 Micro-ingredients in food and singlet oxygen

3.4.1 Natural photosensitizers

In plant material the two most abundant photosensitizers are chlorophyll and riboflvavin. Although these compounds are only present in low amounts in final food products such as vegetable oils (chlorophyll) and milk (riboflavin) the foregoing discussion shows the important impact on the quality characteristics of these products when stored in light. Furthermore these natural photosensitizers have been widely used to study singlet oxidation by spiking a food matrix or model system. In animal tissues hemoproteins are the most abundant photosensitizers that influence photooxidation.

3.4.2 α-tocopherol

Vitamin E or α-tocopherol is generally considered to be a very potent natural antioxidant. However, the antioxidant activity of tocopherols decreases at higher levels and becomes prooxidant above the optimum concentration (Jung and Min, 1990). Oxidation products from α-tocopherol have been shown to generate singlet oxygen (Barclay *et al.*, 1989).

Kim *et al.* (2007) oxidized α-tocopherol in methanol containing methylene blue for 30 hours under light. Afterwards the effect of different concentrations of oxidized α-tocopherol on the oxidative stability of purified soybean oil was studied in the absence of light. A mechanism was proposed for the reaction of singlet oxygen with α-tocopherol. Two moles of α-tocopherol peroxy radical form dimerized α-tocopherol peroxide and singlet oxygen. The prooxidant effect of α-tocopherol at high concentration could be due to high amounts of peroxy radical and oxy radical of α-tocopherol, hydroxyl radical, and singlet oxygen formed from tocopherol during storage. This shows that that reaction of vitamin E with singlet oxygen generated during storage in light can even lead to the formation of singlet oxygen and free radicals during storage in the dark.

3.4.3 Food colorants

Many food colorants were found to be potent photosensitizers (Pan, 2005b). The effect of a wide variety of food colorants on the photosensitized oxidation of methyl linoleate has been investigated. Rose Bengal, erythrosine B and phloxine B accelerated oxidation of methyl linoleate under light exposure and their prooxidative effects were concentration-dependent. Light exposure of methyl linoleate with added colorants induced the generation of hydroperoxide isomers, including 10-*cis,trans*- and 12-*cis,trans*-methyl linoleate hydroperoxide. Based on the different reactivity of singlet and triplet oxygen, the introduction of oxygen at these positions suggests that the food colorants served as photosensitizers. The addition of the singlet oxygen quenchers α-tocopherol or β-carotene effectively suppressed the oxidation of methyl linoleate, and their antioxidative effects were concentration-dependent. The study also revealed that the presence of a xanthene skeleton with an increased number of halogen substituents increased the potential as photosensitizer. Because agricultural food products and seafood products often contain these food colorants, photosensitized lipid oxidation can shorten shelf life significantly if packaged in transparent material.

3.4.4 Emulsifiers

Phosphatidylcholine (PC), a principal component of the food emulsifier lecithin has been shown to quench singlet oxygen in two model systems (Lee and Choe, 2008). In a non-food microemulsion it could be shown that PC added at different concentrations clearly quenched singlet oxygen. Also a food emulsion model was prepared with sunflower oil, distilled water, and xanthan gum. Also PC was

added and 4 ppm of the photosensitizer chlorophyll. The emulsion was stored in light and the oxidation was measured using a variety of end points. The chlorophyll-photosensitized oxidation of the oil in the food model was also significantly reduced by the addition of PC and was assigned to possible quenching of singlet oxygen.

3.4.5 Natural antioxidants

Carotenoids

The influence of carotenoids on oxidation has been studied widely in diverse food systems (Dondeena and Kilara, 1992). β-carotene is considered to be the most powerful physical singlet oxygen quenching agent in foods and is a particularly effective quencher of singlet oxygen. One molecule of β-carotene can quench 250 to 1000 molecules of singlet oxygen (Foote, 1976). The rate of singlet oxygen quenching by carotenes is highly dependent on the number of conjugate double bonds in the carotenoid. Also the type and number of functional groups on the ring portion of the molecule play an important role. The importance of the functional groups is strongly linked to the solubility of the carotenoids (Kobayashi and Sakamoto, 1999). Besides the solubility, the effectiveness of carotenoids also depends on the number of double bonds in the skeleton. A total of five carotenoids have been evaluated for their singlet oxygen quenching efficacy in a chlorophyll-sensitized photooxidation of soybean oil (Lee and Min, 1990). The results showed that the effectiveness of the carotenoids increased with the number of double bonds. Those carotenoids with seven or fewer double bonds are ineffective as quenchers, being unable to accept the energy from singlet oxygen. A comparison of quenching rates of several polyenes and carotenoids has been reported (Beutner *et al.*, 2000).

Polyphenols

Polyphenols have been generally known as quenchers of triplet oxygen. However, several studies shown that some polyphenols can also inhibit singlet oxidation. Tea catechins have been found to quench singlet oxygen in an ethanol solution (Mukai *et al.*, 2005) and other plant extracts such as from Piper Betel have been shown to delay light induced photooxidation (Bhattacharya *et al.*, 2007).

3.5 Vitamin loss in foods due to singlet oxygen

3.5.1 Vitamin D

Vitamin D plays a key role in the normal mineralization and growth of bones. Its mode of action is linked to the stimulation of calcium absorption in the intestine. Milk is frequently fortified with vitamin D because it is an important source of calcium. Unfortunately, this vitamin is rapidly destroyed under light storage (United States Public Health Services/Food and Drug Administration, 1993).

King and Min (1998) reported that the oxidation of vitamin D in a milk model system did not occur in the absence of either riboflavin nor light. The reaction rate of vitamin D with singlet oxygen was found to be $2.23 \times 10^7\,M^{-1}\,s^{-1}$. The rate of headspace oxygen depletion was dependent on the amount of riboflavin or vitamin D (Li and Min, 1998). The addition of riboflavin had a significant effect on the loss of vitamin D under light. Alternatively, the absence of either riboflavin or light resulted in no vitamin D loss in the milk system.

3.5.2 Vitamin C

Exposure of milk to sunlight can result in a loss of 80% to100% of ascorbic acid (vitamin C) within one hour (Satter and deMan, 1975). Yang (1994) studied the effect of singlet oxygen on the stability of ascorbic acid in a model system. The loss of ascorbic acid increased under light storage as the riboflavin content in milk was increased. All ascorbic acid was lost after only 12 minutes of light exposure when 6 ppm riboflavin was added. In contrast, only 2% of the ascorbic acid was destroyed in the sample to which no riboflavin was added (Jung and Kim, 1995). Addition of a singlet oxygen quencher (sodium azide) also reduced the loss of ascorbic acid significantly.

3.5.3 Vitamin B_2

Riboflavin or Vitamin B_2 was discussed abundantly in this chapter mainly due to its strong photosensitizing characteristics which leads to the formation of singlet oxygen. Riboflavin is also sensitive towards destruction by the singlet oxygen that it generates because it has a triene structure with many reactive double bonds (Bradley and Min, 1992). The riboflavin content in milk stored in a gas-tight bottle under light decreased as the headspace oxygen decreased in the bottle. This observation suggests that the riboflavin decrease occurred due to oxidation. When the milk was purged with nitrogen to remove the oxygen, the riboflavin content of the milk remained much higher. However, the samples with and without nitrogen purging stored in the dark for prolonged periods showed no reduction of the vitamin.

3.6 Prevention of singlet oxidation

The deleterious effect of (sun)light on flavor stability during shelf storage requires an evaluation of suitable preventive measures. Appropriate procedures could include the selection of a packaging material that is not transparent in order to exclude light. Also removal of oxygen and traces of unnecessary photosensitizers can significantly reduce the speed of the singlet oxidation process.

Unfortunately, for marketing purposes these options are often not preferred. Some products need transparent packaging so that consumers can actually see

the product before they buy. Furthermore, in some cases natural photosensitizers can not be removed, or sometimes photosensitizers are added to fortify the product.

Therefore, an alternative approach to maintain product quality is to use antioxidants that specifically interact with singlet oxygen. Conventional antioxidants such as BHA and BHT are typical radical quenchers and therefore will only interfere with radical compounds which mainly appear in the process of triplet oxidation. In order to prevent singlet oxidation a molecule is required that is able to absorb the energy that is stored in the 'excited' singlet oxygen molecule. Such compounds are generally referred to as singlet oxygen quenchers. Typical singlet oxygen quenchers are ascorbic acid, carotenoids and tocopherols. These molecules are able to transform excited singlet oxygen into ground-state triplet oxygen while the antioxidant itself will shift from the triplet state into the singlet state.

3.7 Future trends

Singlet oxygen can have a dramatic impact on the quality and the shelf life of food products. Because of its very different chemical nature compared to triplet oxygen distinctive oxidation breakdown products will be formed. Simultaneously a different set of antioxidant molecules will be required in all attempts to reduce the level of singlet oxidation.

The results from the quantification of volatile breakdown products obtained with GC-MS analyses helps to understand the influence of oxygen quenching antioxidants on the formation of rancid flavors. As a result, the information obtained from these analyses will be valuable to optimize antioxidant formulations for specific food and feed applications (Van Dyck, 2007). Owing to the complexity and interaction between all stages of the oxidation process it is often very difficult to develop an antioxidant formulation that is suitable for all food and feed matrices and for all storage conditions. Some antioxidants may become pro-oxidant when the main oxygen species responsible for the oxidation changes due to a change in storage conditions or packaging. Therefore it is of extreme importance to carefully balance antioxidant formulations for specific applications and to assess the efficacy of products under a variety of different relevant storage conditions.

Also the study of the effects of singlet oxidation will need to be developed further. Many studies make use of models to identify the parameters that play a role in the oxidation. Unfortunately the insights from the simpler model systems are not always verified against real food systems. However, this is a crucial step that is required to give the food and feed industry the required information on the complete food matrix in order to develop suitable formulations or strategies to inhibit singlet oxidation.

Although formulation of products with a focus on oxidative stability is still an area that needs further research, further exploration of new packaging strategies

could also offer valuable solutions. Incorporation of singlet quenchers in the packaging materials or in a transparent window or foil would be valuable innovations for the food industry.

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4

Heme proteins and oxidation in fresh and processed meats

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Abstract: This chapter describes mechanisms of heme protein-mediated discoloration and rancidity development in raw muscle as well as processed meat products. Protein crystallography and site-directed mutagenesis efforts have improved our understanding of the mechanisms involved. Steric displacement of bound ligands, weak anchoring of the heme propionates to the globin, and larger channels for solvent entry into the heme crevice explain why certain heme proteins are more reactive than others. The interplay between pH, O_2 partial pressure, light, metals, and other factors that promote pigment and lipid oxidation are described. Muscle-based products that are particularly vulnerable to oxidation are noted. Strategies to inhibit discoloration and off-flavor formation mediated by heme proteins are also covered briefly.

Keywords: rancidity, discoloration, blood, hemoglobin, myoglobin, quality deterioration.

4.1 Introduction

The main heme proteins in muscle tissue are hemoglobin (Hb) and myoglobin (Mb). Heme proteins dictate the appearance (e.g., color) of muscle foods. In addition, Hb and Mb have the capacity to promote lipid oxidation during storage which leads to off-odors and off-flavors. Hemoglobin is located within erythrocytes (e.g., red blood cells). Myoglobin is located within muscle cells (e.g., myocytes). Bleeding is often believed to remove hemoglobin from the muscle; however substantial quantities of blood remain in the muscle after bleeding (Porter *et al.*, 1992, Warriss, 1977). The primary function of Hb in the

living animal is to transport O_2 throughout the vasculature for delivery to different tissues. Mb may facilitate O_2 transport from erythrocytes to mitochondria in order to maintain cellular respiration during periods of high O_2 demand. However, mutated mice without Mb were found to be fertile, exhibited normal exercise capacity, and normal response to hypoxia (Garry *et al.*, 1998). This brings into question the necessity for Mb as an O_2 transporter in mice and it may indicate that Mb serves other functional roles in the animal.

There are other functionally distinct heme proteins in biological tissues including cytochrome c, catalase and cytochrome P450 reductase. This chapter will focus mainly on Hb and Mb in regards to reactants, enzymes, and conditions that affect the reactivity of these heme proteins in raw and thermally processed muscle foods. Specifically, mechanisms of heme protein oxidation (discoloration) and heme protein-mediated lipid oxidation (off-flavor) will be discussed. Methods to inhibit discoloration and off-flavor formation will also be covered. Relative Hb and Mb concentrations in muscle from fish, poultry, swine, and cattle are also briefly reviewed.

4.2 Quality implications of oxidation in fresh and processed meats

Immediately after death, Mb and Hb are mostly in their 'reduced' forms in which the iron atom of the heme group is in the +2 (ferrous) oxidation state. This provides red pigments to the muscle that are often desirable. With increased storage time, the iron atom in the heme ring becomes oxidized to the +3 (ferric) oxidation state resulting in brown color which is often undesirable. The oxidized pigments are termed metHb and metMb. The process of met formation is termed 'autooxidation'. Only reduced Hb and Mb can bind and release O_2. Sodium nitrite and reductants are used to 'cure' processed muscle foods (Cassens, 1997). The nitric oxide (NO) produced during curing binds to the ferrous iron atom of the heme ring in Hb and Mb which results in a pink appearance after thermal processing. Thermal processing denatures Hb and Mb but the NO ligand remains bound to the metal. The presence of light and traces of O_2 cause NO-heme pigment to become oxidized resulting in brown pigments. Thus, Hb and Mb are responsible for both desirable and undesirable color attributes in muscle foods.

Hb and Mb are capable of stimulating lipid oxidation processes during storage of raw and cooked muscle foods (Johns *et al.*, 1989). Lipid oxidation causes the formation of off-odors and off-flavors (e.g., rancidity) during storage of raw muscle. The term warmed over flavor (WOF) is used to describe off-flavor due to lipid oxidation that develops during storage of cooked meat.

Lipid oxidation can cause the oxidation of vitamins endogenously present in the muscle. Rancidity developed in mackerel fillets at around the time that ascorbic acid (vitamin C) was depleted during iced storage (Petillo *et al.*, 1998). Beta-carotene concentrations decreased as lipid oxidation products increased

during chilled storage (2 ºC) of mechanically separated turkey (Kathirvel *et al.*, 2008).

Heme protein-mediated lipid oxidation can negatively affect textural attributes of muscle foods (Kanner, 1994). Lipid derived free radicals can oxidize protein sulfhydryl groups resulting in protein cross-links via disulfide bond formation, which decreases protein solubility. Soluble proteins are more functional (e.g., enhanced water-holding capacity) compared to insoluble proteins. The ability of metMb to react with hydrogen peroxide resulted in ferryl Mb that caused cross-links in myosin, the main myofibrillar protein present in muscle (Lund *et al.*, 2008).

The ability of oxidized lipids in meat to affect human health should also be considered as it relates broadly to quality implications. In the event that lipid oxidation products from a meat meal enter the bloodstream, those products have the potential to exacerbate reactions associated with the onset of vascular disease (Bochkov *et al.*, 2002; Spiteller, 2005). Drinking red wine (200 ml) with three meals of cooked turkey cutlets (compared to drinking water) suppressed malondialdehyde formation 75% in human plasma obtained after eating (Gorelik *et al.*, 2008). Grape seed extract and butylated hydroxytoluene (BHT) effectively inhibited lipid oxidation in a simulated stomach containing cooked turkey meat; addition of these antioxidants to cooked turkey meat also decreased the concentration of conjugated dienes around 11% in pig chylomicrons obtained after eating (Kuffa *et al.*, 2009).

4.3 Meat products particularly affected by oxidation

4.3.1 Certain fish species

Many species of fish are particularly susceptible to discoloration and lipid oxidation. This is partly due to the ability of fish Hbs to incur rapid lipid oxidation compared to Hbs from beef and poultry (Richards *et al.*, 2002). The mechanism by which fish Hbs promote lipid oxidation is related to rates of metHb formation and rates of hemin release from the globin. Rainbow trout Hb and perch Hb rapidly autooxidized and also rapidly released their porphyrin hemin moiety compared to bovine Hb (see Sections 4.5.3 and 4.6.4). Lipids in muscle from cold-water fish contain relatively high amounts of polyunsaturated fatty acids including 22:6 (DHA) and 20:5 (EPA) (Richards *et al.*, 2007). These highly unsaturated fatty acids are generally more prone to lipid oxidation than less unsaturated fatty acids. Muscle from Atlantic cod (*Gadus morhua*) contains substantial amounts of EPA and DHA yet lipid oxidation is relatively slow in cod muscle. This can be partly attributed to the high antioxidant capacity that was characterized in the aqueous fraction of cod muscle (Undeland *et al.*, 2003). pH values are also generally higher in muscle from white-fleshed species such as cod compared to dark-fleshed species (Shimizu *et al.*, 1992). Elevated pH decreases the reactivity of Hb and Mb (see Sections 4.5.1 and 4.6.5). Hb and Mb concentrations are also relatively low in cod muscle which limits the amount of reactant for lipid oxidation.

4.3.2 Turkey muscle

Lipid oxidation occurred more rapidly in turkey dark muscle compared to chicken dark muscle (Kanner *et al.*, 1988). Turkey muscle is more deficient in tocopherol (vitamin E) compared to chicken muscle (Mecchi *et al.*, 1956). Other factors that may cause lipids in turkey muscle to rapidly oxidize include lower post mortem pH in turkey compared to chicken, more unsaturated fatty acids in turkey compared to chicken, and a deficiency of antioxidants in the aqueous phase of turkey muscle compared to duck muscle (Gong *et al.*, 2010). A particularly rapid discoloration of turkey muscle was observed compared to pork muscle (Trout, 1990). Mechanically separated turkey is also much more susceptible to lipid oxidation compared to mechanically separated chicken (unpublished observation).

4.3.3 Beef muscle

Met heme protein formation occurred rapidly in beef compared to pork (Trout, 1990). This may be partly due to the high content of Mb in beef muscle compared to pork. Mb autooxidizes more rapidly compared to Hb (Richards *et al.*, 2005). Mb levels were 2.7 and 0.9 mg/g in beef and pork, respectively, while the Hb levels were 1.2 and 0.8 mg/g, respectively (Rhee and Ziprin, 1987). Bovine Mb was also more susceptible to adduction by 4-hydroxy-2-nonenal (HNE), a lipid oxidation product, compared to porcine Mb (Suman *et al.*, 2006). HNE adduction accelerates conversion of oxyMb to metMb. It turns out that bovine Mb contains more histidine residues (that can be adducted by HNE) compared to porcine Mb and this may explain the greater HNE adduction rate in bovine Mb.

4.3.4 Cooked meats

There is a growing demand for pre-cooked meat products since modern consumers spend less time preparing meals at home. Lipid oxidation in cooked meats causes warmed-over flavor to develop rapidly during refrigerated and frozen storage which is objectionable (Sato and Hegarty, 1971). Warmed-over flavor seems to occur even in muscles that are relatively stable when stored raw (e.g., chicken muscle) (Igene *et al.*, 1985). This may be related to the ability of heating to displace iron atoms from the porphyrin of Hb and Mb (Fig. 4.1). However other forms of Mb that form during thermal processing (hemin, metMb, heat denatured Mb) were considered more likely to incur the bulk of lipid oxidation observed compared to released iron atoms (Kristensen and Andersen, 1997). Hemichrome formation occurred at temperatures well above the thermal denaturation temperature of Mb (Kristensen and Andersen, 1997). Hemichromes are considered to be poor promoters of lipid oxidation (Baron *et al.*, 2000). Hemichrome formation occurs when a nitrogen base (often the distal histidine) covalently binds to the sixth coordination site of the iron atom in the porphyrin ring (Fig. 4.1).

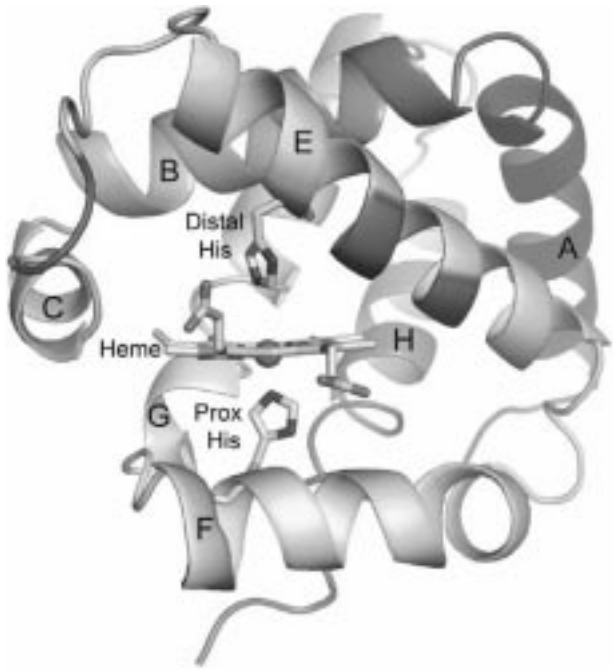

Fig. 4.1 Structural representation of trout IV hemoglobin alpha chain at pH 6.3. The heme, distal histidine, and proximal histidine are labeled. Each helix is also labeled. The iron atom in the center of the heme ring is shown as a sphere. Note the absence of a D-helix in Hb alpha chains. The PDB structure 2R1H was used to prepare the image shown using PyMOL software.

4.3.5 Uncured processed meats

There is demand for uncured, roast type products for consumers that object to added nitrites. There has been little success in this area due to the reactive nature of Hb and Mb after thermal processing in the absence of curing agents. The nitric oxide produced from sodium nitrite during curing inhibits Hb and Mb-mediated lipid oxidation. (Igene *et al.*, 1979, Ohshima *et al.*, 1988). The binding of nitric oxide to the sixth coordination site of the iron atom in the heme ring (Fig. 4.1) causes the heme proteins to become weakly reactive. Nitric oxide also is an effective free radical scavenger at low concentrations (Hummel *et al.*, 2006).

4.4 Quantities of hemoglobin (Hb) and myoglobin (Mb) in muscle and structural characteristics

Hb concentrations were higher in extracts from certain bled fish compared to certain unbled fish although the mean Hb values were around 30% greater in extracts from the unbled fish (Richards and Hultin, 2002). This indicated that a substantial amount of Hb remained in the muscle after bleeding. Hb was the only

detectable heme pigment in whole muscle from trout while dark muscle from mackerel contained roughly equal amounts of Hb and Mb (Richards and Hultin, 2002). Hb was the only detectable heme pigment in chicken breast muscle while in thigh muscle there was 86% Hb and 14% Mb on a weight basis (Kranen *et al.*, 1999). In various pork muscles, Hb comprised 23–30% of the total heme protein (Pisula, 1975). In pork *Longissimus dorsi*, Hb comprised 39–54% of the total heme pigment (Rhee and Ziprin, 1987). In minced beef, Hb comprised 9% of the total heme protein (Oellingrath *et al.*, 1990). *Longissimus dorsi* from beef contained 15–42% Hb while in the *semimembranosus*, Hb comprised 32–34% of the total heme pigment (Rhee and Ziprin, 1987).

Hb concentrations can be underestimated relative to Mb due to the greater tendency of Hb to release its porphyrin moiety during extraction (Gattoni *et al.*, 1998). Optical density of Hb and Mb between 700 and 400 nm is often used to quantify the heme protein concentration. The extinction coefficients of Hb and Mb containing its porphyrin moiety are much greater than the extinction coefficients of either the apoglobin or free porphyrin. In addition, multiple hemoglobins (e.g., Hb isoforms) can be present especially in fish muscle (Rizzotti and Gioppato, 1999) so that one Hb component can be mistakenly identified as Mb.

Hb is a tetramer consisting of four globins and four porphyrin moieties while Mb is a monomer (one globin and one associated porphyrin group). The porphyrin moiety is termed 'heme' when the iron atom of the porphyrin is in the reduced (+2) oxidation state and 'hemin' when in the oxidized (+3) oxidation state.

In Hb, there are two α-chains (chain A and C) and two β-chains (chain B and D). The amino acid composition of the α-chains is substantially different compared to the β-chains which causes functional differences between the chains. The central part of the porphyrin is the iron atom that has six coordination sites (Fig. 4.1). Four of the sites are occupied by nitrogen atoms of the protoporphyrin ring and one is attached to the proximal imidazole group of a histidine in the globin portion. The sixth site forms a complex with ligands such as O_2. Water is weakly coordinated to the iron atom of the porphyrin in deoxyMb and metMb. The ligand and oxidation state of the iron atom in the porphyrin ring dictates the color of the flesh.

There is a distal histidine residue that coordinates different ligands in the heme crevice while the proximal histidine covalently links the globin to the porphyrin (Fig. 4.1). The heme-6-propionate and heme-7-propionate extend out of the globin towards solvent. In Fig. 4.1 the heme-7-propionate is to the right. The distal histidine is at site E7; this is the 7th residue along the E-helix. E7 is the 64th residue in sperm whale Mb, the 58th residue in bovine Hb α chains, and the 62nd residue in bovine Hb β chains. The proximal histidine is at site F8; this is the 8th residue along the F-helix. Mbs and Hb β chains have eight helices (A through H) while Hb α chains have 7 helices. The D-helix is missing from Hb α chains (Fig. 4.1). Thus there is a CD corner as a random coil between helix C and D in Mb and Hb β chains and a CE corner between the C and E-helix in Hb α chains. Acetylation of Rainbow trout Hb has been reported at the N-terminus

of the α chains (Jolly and Taketa, 1984). Acetylation at the N-terminus can make it difficult to determine the amino acid sequence of the protein subunit.

4.5 Discoloration in meat products

Color is a critical factor that consumers use to decide which raw and processed meat products to purchase. This section deals primarily with mechanisms by which heme protein oxidation (e.g., formation of brown pigments) occurs during refrigerated and frozen storage. Autooxidation rate (k_{ox}) is a term used to describe Hb and Mb oxidation rates (e.g., metHb and metMb formation). Sodium chloride (e.g., table salt) accelerated Mb oxidation at post mortem pH values (Andersen *et al.*, 1988). Pink color in uncured, fully-cooked poultry and beef is a separate defect that has been described (Cornforth *et al.*, 1986; Seyfert *et al.*, 2004).

4.5.1 Effect of pH

Soon after death, the pH of muscle drops from around pH 7.4 (physiological pH) to values ranging from 5.5 to 6.8 (post-mortem pH). This is especially noteworthy because Hb and Mb oxidation rates increase as pH is decreased in this pH range (Shikama, 1998; Yin and Faustman, 1993). Rapid Hb and Mb oxidation at low pH is related to the ability of protons (H^+) to enter the heme crevice and protonate liganded O_2. The neutral superoxide radical ($^{\bullet}OOH$) then dissociates from the heme crevice resulting in met formation (Fig. 4.2, top). An

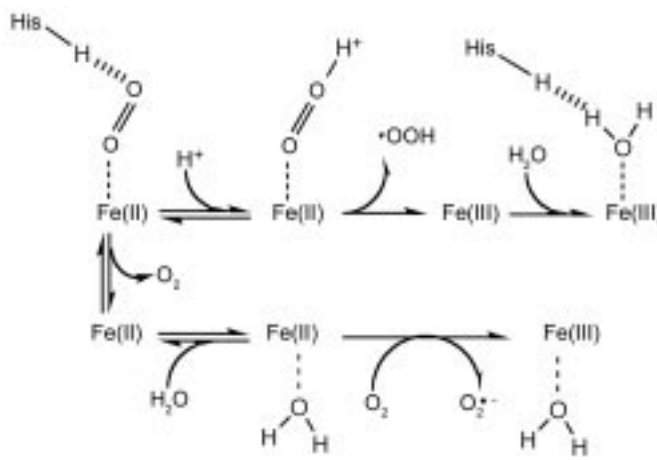

Fig. 4.2 Pathways by which hemoglobin and myoglobin autooxidize. Protons facilitate release of neutral superoxide radical ($^{\bullet}OOH$) from OxyHb. O_2 and a weakly coordinated water molecule facilitate metHb formation from deoxyHb; the superoxide anion radical ($O_2^{\bullet-}$) is also produced in this reaction. The unprotonated distal histidine (E7) provides hydrogen bonds with liganded O_2 in OxyHb and water in metHb. Adapted from Brantley *et al.* (1993).

illustration of how dramatically pH can alter access of protons to the heme crevice is shown in Fig. 4.3. pH reduction from 8.5 to 5.7 caused one of the histidine residues at site CE3 in bovine Hb to swing outward exposing the heme crevice to solvent. Protonation of the distal histidine (E7) at low pH likely causes His(CE3) to swing out away from the porphyrin group. This creates a channel for solvent (e.g., protons and water) to enter the heme crevice accelerating autooxidation (Fig. 4.2). pH reduction from 8.0 to 6.3 also caused structural changes in perch Hb β-chains at site CD3 that exposed more of the heme crevice to solvent at the lower pH (Richards *et al.*, 2009).

Lowering the pH from 7.4 to 6.0 dramatically decreases the oxygen affinity of certain fish Hbs (Binotti *et al.*, 1971). This phenomenon in general is termed the 'Bohr Effect' which is a decrease in oxygen affinity as the hydrogen ion concentration increases. The Bohr Effect occurs in mammalian Hbs to a slight extent but when the effect is exaggerated (as with trout IV Hb) the term 'Root

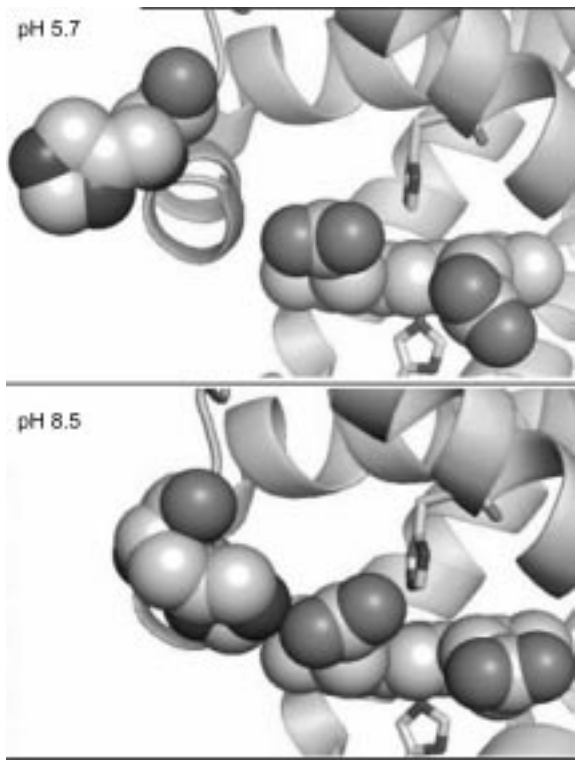

Fig. 4.3 Gap for solvent entry into a heme crevice of bovine Hb at pH 5.7 and pH 8.5. The varying sized gap is located between CE3 of the alpha A-chain and the heme-6-propionate. Lowering pH increases exposure of the heme crevice to solvent molecules including protons and water. His(CE3) and the heme group are shown as spheres. The proximal histidine below the heme and the distal histidine above the heme are shown in stick representation. The PDB structures 2QSP and 1G0A were used to prepare the image shown using PyMOL software.

Effect' is used. This creates a large pool of deoxyHb that is in equilibrium with a small pool of oxyHb and ample O_2 reactant. These conditions facilitate rapid Hb oxidation at pH values near 6 because the relatively high concentration of deoxyHb reacts with copious amounts of O_2 to produce metHb and superoxide anion radical ($^{\bullet-}O_2$) (Fig. 4.2, bottom).

4.5.2 Effect of oxygen partial pressure

If the Root Effect Hbs are disregarded for a moment, low O_2 partial pressures (PO_2) are required for substantial amounts of deoxyMb and deoxyHb to be present in post-mortem muscle. Ground beef turned brown rapidly in O_2-depleted atmospheres (PO_2 of 7 mm Hg) compared to normal atmospheric pressure (PO_2 of 160 mmHg) (Ledward, 1970). Apparently the O_2-depleted atmosphere creates an environment in which a substantial fraction of the Hb and Mb present exist as deoxygenated heme proteins and there still is enough O_2 available to promote met formation as described in the lower half of Fig. 4.2.

O_2-depleted atmospheres (e.g., low PO_2 values) can occur just below the surface of intact muscle since O_2 from the atmosphere only penetrates 1–4 mm into the tissue (Lawrie, 1974). Consequently it is sometimes observed that the interior of a beef steak contains high levels of met heme protein (brown pigments) while the surface and deep interior contains only reduced heme proteins. The surface will be red in color due to oxy-heme proteins while the deep interior will have purple hues due to the presence of only deoxygenated heme proteins. The deep interior of post mortem muscle is anaerobic.

O_2-depleted atmospheres can also occur at the interface of sliced products that are 'shingled' and when multiple pieces of muscle are pressed against each other. Stacking of slices or pressing of muscle pieces causes there to be substantial amounts of deoxyHb and deoxyMb that react with the small amount of O_2 present at the interfaces which facilitates met formation.

Sodium ascorbate (vitamin C) and sodium erythorbate (a synthetic isomer of vitamin C) have O_2 scavenging properties. Thus the potential of ascorbate to lower the PO_2 to a point that accelerates Hb and Mb oxidation should be kept in mind. If all the O_2 were to be scavenged by ascorbate, this should inhibit browning since met formation-mediated by deoxygenated heme proteins requires O_2 as a reactant (Fig. 4.2).

Respiring mitochondria at the surface of muscle can also compete for atmospheric O_2 which can increase deoxyHb and deoxyMb concentrations at the surface of the muscle. Consumption of O_2 by mitochondria occurs more readily at elevated pH. This is the reason that beef at elevated pH can appear dark (with purple hues) due to deoxygenation of the heme pigments at the surface.

4.5.3 Autooxidation of different Hbs

Perch Hb and trout IV Hb autooxidized 26-fold and 19-fold faster compared to bovine Hb, respectively at pH 6.3 (Aranda *et al.*, 2009). This was mostly attributed to variation in amino acids surrounding the porphyrin group when

comparing the fish and bovine Hbs. The fish Hbs contain isoleucine at site E11 while bovine Hb contains valine. Ile (E11) is closer to the coordinated ligand in the heme pocket compared to valine (Aranda *et al.*, 2009). The larger isoleucine residue displaces ligands (e.g., O_2 and protonated O_2) which increases k_{ox} (Fig. 4.2). Mutation studies in sperm whale Mb indicate that Ile at E11 causes very rapid autooxidation compared to the native Val at site E11. The autooxidation rate (k_{ox}) for the Val(E11)Ile mutant Mb was 15-fold greater than in the wild-type Mb containing valine at site E11 (Brantley *et al.*, 1993). Fish Hbs also contain threonine at site E10 while bovine Hb contains lysine at E10. The larger lysine can displace solvent from the heme crevice thereby decreasing k_{ox}. Fish Hbs also contain glycine at site E14 while bovine Hb contains larger residues. Larger residues at site E14 should decrease access of solvent to the heme crevice. We observed that k_{ox} was significantly greater in a Ala(E14)Gly Mb mutant compared to wild type (unpublished observation). Finally, at site CD3 of the β-chains, the gap for solvent entry at pH 5.7 was lowest in bovine Hb (~4 Å), intermediate in trout IV (~6 Å), and highest in perch Hb (~8 Å) which correlated with the k_{ox} rates (Aranda *et al.*, 2009).

Certain fish Hbs have low oxygen affinity at post mortem pH values even when exposed to normal PO_2 (around 160 mmHg). This is termed exaggerated Root and Bohr effects. Trout IV Hb for example is highly oxygenated at pH values near 7.4 while at pH 6.3 (and lower) it is a mixture of mostly deoxyHb and some oxyHb (Binotti *et al.*, 1971). This combination of deoxyHb in the presence of O_2 promotes rapid autooxidation (Fig. 4.2). Mammalian Hbs have relatively low equilibrium O_2 dissociation constants at post mortem pH values which is one reason mammalian Hbs are resistant to k_{ox} compared to fish Hbs (Aranda *et al.*, 2009).

Trout IV Hb autooxidized more rapidly compared to trout I Hb at pH 7 and 30 ºC (Fedeli *et al.*, 2001). Trout IV also autooxidized more rapidly compared to trout I at pH 6.3 and 2 ºC (Richards *et al.*, 2005). The rapid k_{ox} in trout IV Hb is partly due to the fact that trout IV Hb (a Root Effect Hb) is less oxygenated at these pH values compared to trout I Hb (a non-Root Effect Hb) (Binotti *et al.*, 1971). The ability of residues that can enhance or hinder access of solvent to the heme crevice in each Hb should also be considered.

Rates of autooxidation were around 10-fold higher in Hbs from cold-adapted fish compared to warm-adapted fish while hydrostatic pressure (e.g., deep or shallow dwelling species) did not seem to affect k_{ox} (Wilson and Knowles, 1987). A sampling of 27 coastal bottlenose dolphins (*Turiops truncatus*) exhibited different Hb oxidation rates (Remington *et al.*, 2007). This was attributed to the observation that some dolphins had Hb isoforms that differed compared to the other dolphins examined.

4.5.4 Autooxidation of different Mbs

Oxygen dissociation rates and autooxidation rates were found to be higher in fish Mbs (yellowfin tuna, mackerel and Antarctic teleost) compared to

mammalian Mbs (sperm whale and horse) (Cashon *et al.*, 1997). Mackerel Mb had low oxygen affinity and high oxygen dissociation rates compared to zebrafish, yellowfin tuna, and Antarctic teleost (Madden *et al.*, 2004). However Antarctic teleost had the highest k_{ox} rate among the four fish species indicating factors other than equilibrium oxygen dissociation constants affect autooxidation rates. Mackerel Mb had the most flexible structure based on dynamic simulations. It is interesting to note that aldehydic lipid oxidation products (4-hydroxy-2-nonenal) have been shown to covalently bind to histidine residues of bovine Mb which accelerated metMb formation (Suman *et al.*, 2006).

4.6 Lipid oxidation in meat products

Any factor that accelerates Mb and Hb oxidation (see Section 4.5) should also accelerate Mb and Hb-mediated lipid oxidation. One reason is because the porphyrin moiety in oxidized Mb and Hb is weakly anchored (60-fold less) in the globin compared to the reduced forms (Tang *et al.*, 1998). Released hemin is a potent promoter of lipid oxidation as described in detail below. MetHb and metMb are also readily converted to ferryl states which have the capacity to initiate lipid oxidation (Kanner and Harel, 1985). OxyMb promoted lipid oxidation more effectively compared to metMb in liposomes which suggested that the superoxide radicals released during autooxidation (Fig. 4.2) enhanced the ability of Mb to promote lipid oxidation (Chan *et al.*, 1997).

Physical aspects in addition to chemical aspects should be considered when addressing Hb and Mb-mediated lipid oxidation. For example, Hb maintained inside erythrocytes was less capable of promoting lipid oxidation in washed cod muscle at pH 6.3 compared to erythrocytes that were lysed (Richards and Hultin, 2002). Rupture of the erythrocyte membrane will dilute Hb which increases the amount of Hb subunits that are especially prone to autooxidation and hemin loss compared to Hb tetramers (Manning *et al.*, 1998). MetHb reductase in the erythrocyte will be less effective once the erythrocyte membrane is ruptured (Scott and Harrington, 1985). The pH will decrease as Hb exits the erythrocyte upon hemolysis in post mortem muscle which increases oxidative degradation of Hb (see Sections 4.5.1 and 4.6.5).

4.6.1 Interactions of low molecular weight metals and heme proteins

Low molecular weight metals should be considered in the context of heme protein-mediated lipid oxidation. Copper ions were found to accelerate Mb oxidation (Moiseeva and Postnikova, 2001). The reaction schemes below provide examples of how low molecular weight metals can enhance the ability of heme proteins to stimulate lipid oxidation. An appropriate chelator (e.g., adenosine diphosphate) chelates trace metal (e.g., iron and copper) present in the tissue. The trace metal could be endogenous to the muscle or incorporated from processing equipment. Reduced (ferrous) iron converts O_2 into superoxide anion radical

($^{\bullet -}O_2$) (reaction 1). This is activation of oxygen. It should be noted that certain chelators will facilitate reaction 1 while other chelators will not. The experimental conditions (e.g., pH, metal to chelator ratio) will also dictate rates of reaction.

$$Fe + \text{chelator} \longrightarrow Fe\ (\text{chelate})$$

$$Fe^{2+}(\text{chelate}) + O_2 \longrightarrow {}^{\bullet -}O_2 + Fe^{3+}(\text{chelate}) \qquad \text{(reaction 1)}$$

At post-mortem pH values, there are appreciable amounts of the conjugate acid form of superoxide ($^{\bullet}OOH$, neutral superoxide radical) in equilibrium with the conjugate base form ($^{\bullet -}O_2$, superoxide anion radical) (pKa ~ 5) which leads to rapid production of hydrogen peroxide (reactions 2–4) (Halliwell and Gutteridge, 1999).

$$^{\bullet -}O_2 + {}^{\bullet -}O_2 + 2H^+ \longrightarrow HOOH + O_2\ (k < 0.3\,M^{-1}\,s^{-1}) \qquad \text{(reaction 2)}$$

$$^{\bullet -}O_2 + {}^{\bullet}OOH + H^+ \longrightarrow HOOH + O_2\ (k = 9.7 \times 10^5\,M^{-1}\,s^{-1}) \qquad \text{(reaction 3)}$$

$$^{\bullet}OOH + {}^{\bullet}OOH \longrightarrow HOOH + O_2\ (k = 8.3 \times 10^5\,M^{-1}\,s^{-1}) \qquad \text{(reaction 4)}$$

Hydrogen peroxide converts oxyHb to metHb (Weiss, 1982) (reaction 5).

$$HOOH + 2\text{oxyHb}(2+) + 2H^+ \longrightarrow 2\text{metHb}(3+) + 2HOH + O_2 \qquad \text{(reaction 5)}$$

Hydrogen peroxide is especially efficient in converting deoxyMb to metMb (Wazawa *et al.*, 1992, Yusa and Shikama, 1987) (reactions 6 and 7).

$$HOOH + \text{deoxyHb}(2+) \longrightarrow \text{ferrylHb}(4+) + 2OH^- \qquad \text{(reaction 6)}$$

$$\text{ferrylHb}(4+) + \text{deoxyHb}(2+) \longrightarrow 2\text{metHb}(3+) \qquad \text{(reaction 7)}$$

Hydrogen peroxide reacts with metHb and Fe^{2+}(chelates) to form the ferryl heme protein radical and hydroxyl radical, respectively (reactions 8 and 9) (Reeder *et al.*, 2004).

$$HOOH + \text{metHb}(3+) \longrightarrow \text{ferrylHb}^{(\bullet +)}(4+){=}O + H_2O \qquad \text{(reaction 8)}$$

$$HOOH + Fe^{2+}(\text{chelate}) \longrightarrow {}^{\bullet}OH + {}^{-}OH + Fe^{3+}(\text{chelate}) \qquad \text{(reaction 9)}$$

Both the hydroxyl radical and ferryl Hb radical can abstract hydrogen atoms from polyunsaturated fatty acids (reactions 10 and 11) resulting in peroxyl radical and lipid hydroperoxide accumulation (reactions 12 and 13). The hypervalent Hb produced in reaction 11 can also abstract hydrogen atoms from polyunsaturated fatty acids (reaction 14) and decompose LOOH to peroxyl radicals (reaction 15) (Kanner and Harel, 1985, Reeder *et al.*, 2004). Note that metHb is regenerated in reactions 14 and 15.

$$^{\bullet}OH + LH \longrightarrow L^{\bullet} + HOH \qquad \text{(reaction 10)}$$

$$\text{ferrylHb}^{(\bullet +)}(4+){=}O + LH \longrightarrow L^{\bullet} + \text{ferrylHb}(4+){=}O \qquad \text{(reaction 11)}$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \qquad \text{(reaction 12)}$$

$$LOO^{\bullet} + LH \longrightarrow LOOH + L^{\bullet} \quad \text{(reaction 13)}$$

$$\text{ferrylHb}(4+)=O + LH \longrightarrow L^{\bullet} + \text{metHb}(3+) + HOH \quad \text{(reaction 14)}$$

$$\text{ferrylHb}(4+)=O + LOOH \longrightarrow LOO^{\bullet} + \text{metHb}(3+) \quad \text{(reaction 15)}$$

Lipid hydroperoxides react with metHb, certain Fe2+(chelates), and certain Fe3+(chelates) to form ferryl Hb radical, alkoxyl, and peroxyl radicals, respectively (reactions 16–18). Alkoxyl radicals can also form due to reaction of metHb with LOOH (reaction 19) (Reeder *et al.*, 2004). Alkoxyl radicals more readily abstract hydrogen atoms from polyunsaturated fatty acids compared to peroxyl radicals based on their one-electron reduction potentials (Buettner, 1993). It should be noted that the ferryl cation radical in reactions 8 and 16 reacts with O_2 to form peroxyl radical (Reeder *et al.*, 2004).

$$\text{metHb}(3+) + LOOH \longrightarrow \text{ferrylHb}^{(\bullet+)}(4+) =O + LOH \quad \text{(reaction 16)}$$

$$Fe^{2+}(\text{chelate}) + LOOH \longrightarrow LO^{\bullet} + OH^{-} + Fe^{3+}(\text{chelate}) \quad \text{(reaction 17)}$$

$$Fe^{3+}(\text{chelate}) + LOOH \longrightarrow LOO^{\bullet} + H^{+}\ Fe^{2+}(\text{chelate}) \quad \text{(reaction 18)}$$

$$\text{metHb}(3+) + LOOH \longrightarrow \text{ferrylHb}(4+)\text{–OH} + LO^{\bullet} \quad \text{(reaction 19)}$$

Lipid hydroperoxides also accelerate met formation (Nagy *et al.*, 2005) as does hydrogen peroxide (reactions 5–7). Porphyin release occurs around 60-fold faster from metMb compared to ferrous forms of Mb (e.g., oxyMb and deoxyMb) (Tang *et al.*, 1998). Released hemin porphyrin from metHb reacts with LOOH to form alkoxyl and peroxyl radicals (reactions 20 and 21) that stimulate lipid oxidation.

$$\text{Hemin}^{(3+)} + LOOH \longrightarrow LO^{\bullet} + \text{hemin}^{(4+)}\text{–OH} \quad \text{(reaction 20)}$$

$$\text{Hemin}^{(4+)}\text{–OH} + LOOH \longrightarrow LOO^{\bullet} + \text{hemin}^{(3+)} + HOH \quad \text{(reaction 21)}$$

The reaction schemes above suggest a continuous cycle of free radical and lipid hydroperoxide production yet often a small amount of the lipid substrate becomes oxidized in even rancid muscle (Xing *et al.*, 1993). The reason only a small fraction of the total lipids become oxidized can be due to termination reactions of free radicals with other free radicals when their concentration becomes high (reaction 22).

$$LO^{\bullet} + LO^{\bullet} \longrightarrow LOOL \quad \text{(reaction 22)}$$

It should also be noted that lipophilic free radicals ($LO^{\bullet}$ and $LOO^{\bullet}$) (see reactions 12, 15 and 17–21) can randomly attack the carbon methene bridges of the tetrapyrrole rings, producing various pyrrole products in addition to releasing iron (Nagababu and Rifkind, 2004). This destruction of the porphyrin will prevent hemin and ferryl Mb-mediated lipid oxidation. The ability of low molecular weight metals, heme protein autooxidation, released hemin, and ferryl heme proteins to promote lipid oxidation and termination reactions are illustrated in a schematic representation (Fig. 4.4).

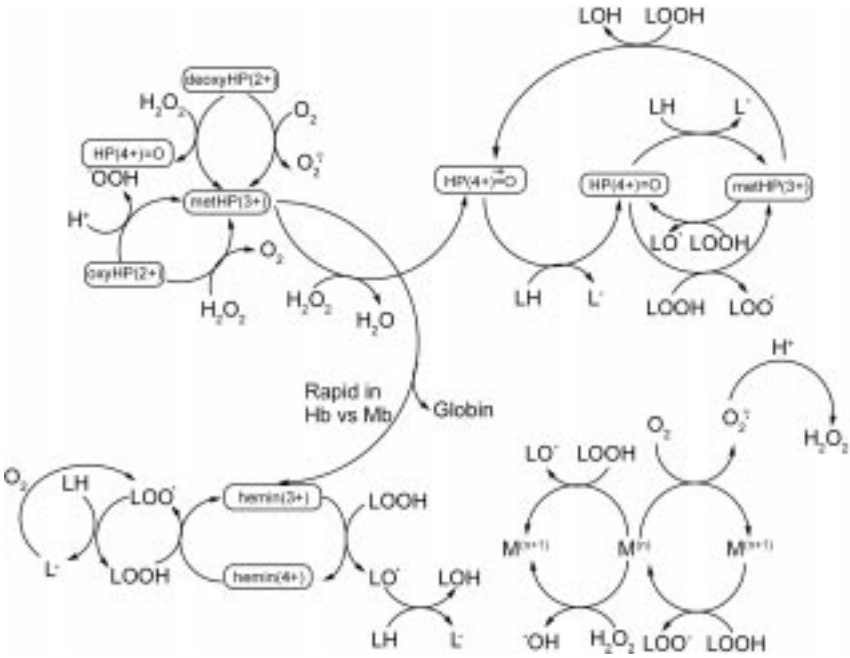

Fig. 4.4 Heme protein (HP) oxidation and HP-mediated lipid oxidation can occur by multiple pathways. Metals (M), oxyHP, and deoxyHP are sources of $^{\bullet-}O_2$ and H_2O_2 that accelerate HP oxidation. MetHP readily releases hemin. Hemin decomposes LOOH stimulating free radical-mediated lipid oxidation. H_2O_2 and LOOH produce ferryl(+4)HP forms that can initiate lipid oxidation. Reactions involving M are facilitated or prevented depending on the type of chelator bound to the metal.

4.6.2 Relative ability of Hb and Mb to autooxidize and promote lipid oxidation

Human Hb is known to release its ferric hemin moiety much more rapidly compared to human Mb (Bunn and Jandl, 1968; Gattoni *et al.*, 1998). In fact apoMb can completely extract hemin from Hb (Banerjee, 1962).This is relevant because released hemin is capable of stimulating extensive lipid oxidation through decomposition of pre-formed lipid hydroperoxides (Tappel, 1955, Van der Zee *et al.*, 1996) (reactions 20 and 21). Release of hemin from trout Hb occurred much more rapidly compared to trout Mb (Richards *et al.*, 2005). On the other hand, trout Mb autooxidized to metMb much more rapidly compared to trout Hb (Richards *et al.*, 2005). Ferrous, trout Hb was a more effective promoter of lipid oxidation in washed fish muscle at pH 6.3 compared to ferrous, trout Mb (Richards *et al.*, 2005). This suggested that hemin loss from metHb was the primary factor that promoted lipid oxidation in washed fish muscle at pH 6.3 while metMb and the rapid burst of superoxide that formed during Mb autooxidation were relatively weak reactants. Hb promoted lipid oxidation more effectively compared to Mb in lipoproteins which was also attributed to the lower hemin affinity of Hb (Grinshtein *et al.*, 2003).

Ser(F7) and His(FG3)in Mbs interact with the heme-7-propionate while Leu(F7) and Leu(FG3) in Hb do not so that the porphyrin group is more 'anchored' in Mbs. Evidence for this can be seen when examining Mb mutants in which the native residues at F7 and FG3 were replaced with residues identical or similar to those found in Hbs at the same site. For example, the Ser(F7)Leu Mb mutant released its hemin moiety 20-fold faster compared to WT Mb (Smerdon *et al.*, 1993). The His(FG3)Val mutant released its hemin moiety 23-fold more rapidly compared to WT Mb (Hargrove *et al.*, 1996).

It should be noted that the tendency of Hb tetramers to dissociate to monomers and dimers accelerates hemin loss and Hb oxidation (Griffon *et al.*, 1998; Hargrove *et al.*, 1997). Enhanced ionic strength and dilution of Hb promotes dissociation of Hb tetramers to subunits (Antoni and Brunoni, 1971; Manning *et al.*, 1998). There have been reports that decreasing pH (in the pH range of 7 to 5) enhances Hb subunit formation but this may be due to effects from using acetate buffer. An Hb mutant (rHb 0.1) with decreased ability to form subunits was a weaker promoter of lipid oxidation compared to wild type Hb (Grunwald and Richards, 2006a).

The reaction of nitrite with oxyMb resulted in the formation of a ferryl Mb radical while reaction of nitrite with oxyHb did not (Keszler *et al.*, 2006). Formation of the Mb radical was inhibited by catalase indicating involvement of hydrogen peroxide in Mb radical formation. The ferryl heme protein radical is capable of abstracting hydrogen atoms from polyunsaturated fatty acids which can initiate lipid oxidation (reaction 11).

4.6.3 Role of released hemin compared to other oxidative forms of Hb and Mb

The challenge in understanding the pathway by which heme proteins promote lipid oxidation is that heme protein autooxidation, ferryl radical formation, hemin release, heme protein crosslinking, hemichrome formation, and iron release can all occur in a very short time sequence (and simultaneously) so that the most relevant step related to lipid oxidation is obscured. However, amino acid substitutions of native Hb and Mb can be used to manipulate various properties of the heme proteins. For example, the ability of the hemin porphyrin to remain attached to the globin can be varied 975-fold by comparing wild-type sperm whale Mb with V68T and H97A (Hargrove *et al.*, 1996). Substitution of valine at site E11 with threonine increases hemin affinity 25-fold. Thr(E11) will hydrogen bond with liganded water increasing hemin affinity while the native valine cannot hydrogen bond with liganded water (Fig. 4.5). Substituting histidine at site FG3 with alanine decreases hemin affinity 39-fold. The smaller alanine at FG3 allows water to rapidly enter into the heme crevice which hydrates the proximal histidine lowering hemin affinity; the native histidine at FG3 sterically blocks water from the proximal side of the heme crevice and chemically bonds with the heme-7-propionate (Fig. 4.6). The different mutants are separately added to washed fish muscle at post-mortem pH to assess the

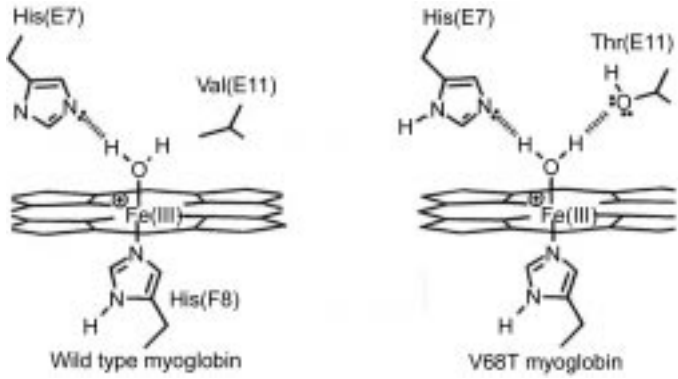

Fig. 4.5 Valine at site E11 (68th residue) in wild-type sperm whale metMb cannot hydrogen bond with water that is liganded to the iron atom of the porphyrin. Threonine at E11 does form a hydrogen bond with liganded water increasing hemin affinity 25-fold. Adapted from Hargrove *et al.*, 1996.

ability of each Mb mutants to promote lipid oxidation. H97A readily promoted lipid oxidation in washed cod at pH 5.7 while WT Mb was intermediate and V68T was the weakest promoter of lipid oxidation (Grunwald and Richards, 2006b). These results suggest that hemin release is the primary mechanism by which heme proteins promote lipid oxidation in washed fish muscle at pH 5.7. Hemin readily decomposes preformed lipid hydroperoxides producing alkoxyl

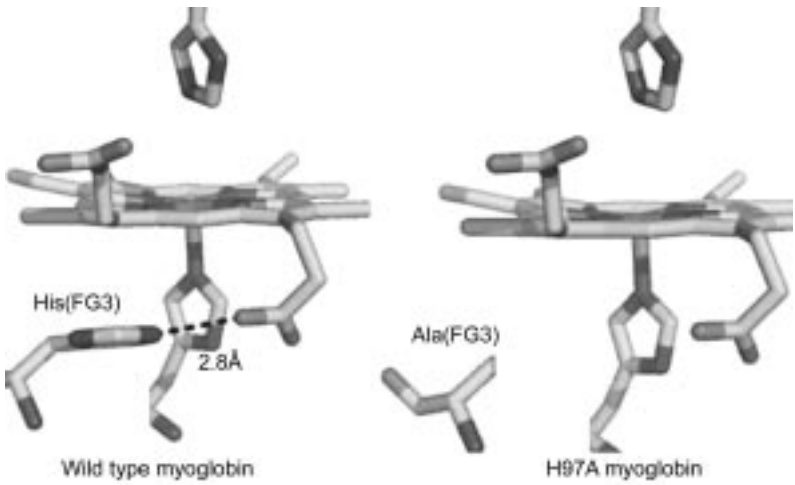

Fig. 4.6 Histidine at site FG3 (97th residue) in wild type sperm whale Mb excludes solvent from the heme crevice and also interacts with the heme-7-propionate. An alanine substitution at site FG3 allows water to hydrate the proximal histidine decreasing hemin affinity. Ala(FG3) cannot interact with the heme-7-propionate group which also decreases hemin affinity. The proximal histidine is shown below the heme group. The distal histidine is shown above the heme group. The PDB structure 1A6K was used to prepare the image shown using PyMOL software.

and peroxyl radicals that propagate lipid oxidation (Van der Zee *et al.*, 1996) (reactions 20 and 21).

L29F/H64Q is susceptible to porphyrin destruction in the presence of hydrogen peroxide compared to WT Mb (Alayash *et al.*, 1999). Porphyrin destruction releases the iron in the heme ring and produces biliverdin. L29F/H64Q was a weaker promoter of lipid oxidation in washed cod muscle compared to WT Mb (Grunwald and Richards, 2006b). This may be partly due to the antioxidant action of biliverdin (Baranano *et al.*, 2002). It also indicates that liberated iron atoms from the porphyrin were not pro-oxidative. It should be noted hydrogen peroxide is water soluble so that heme destruction likely occurred in the aqueous phase. Iron atoms in the aqueous phase may be less reactive than iron atoms that incorporate into the membrane. Hemin is noted as a molecule that transports reactive iron atoms to lipid sites (Balla *et al.*, 1991; Grinshtein *et al.*, 2003).

Ferrous V68T and ferrous WT Mb were compared to assess the ability of autooxidation relative to hemin affinity to promote lipid oxidation. V68T autooxidizes rapidly compared to WT while WT has lower hemin affinity. Ferrous WT Mb was a better promoter of lipid oxidation in washed cod compared to ferrous V68T (Grunwald and Richards, 2006a). This suggested that hemin affinity was more critical in promoting lipid oxidation compared to Mb autooxidation rate in washed cod.

The fact that animal tissues contain hemopexin and heme oxygenase further implicates hemin as an oxidant that must be removed for cells to avoid oxidative effects associated with unbound hemin. Hemopexin binds hemin and is then detoxified in the liver (Paoli *et al.*, 1999). Heme oxygenase degrades the heme ring releasing iron atoms and forming biliverdin that is reduced by biliverdin reductase to bilirubin, a potent antioxidant (Halliwell and Gutteridge, 1990; Kumar and Bandyopadhyay, 2005)

Tyrosine at site G4 is considered a critical residue that facilitates ferryl Mb formation from hydrogen peroxide and metMb. Ferryl Mb can stimulate lipid oxidation (Baron *et al.*, 1997). Substituting Tyr with Phe decreased ferryl Mb radical formation 1.4-fold (Witting *et al.*, 2002). The Tyr(G4)Phe human Mb mutant promoted lipid oxidation about half as effectively as wild-type Mb when examining linoleic acid with added hydrogen peroxide at pH 7.4 (Rayner *et al.*, 2004). This suggested that decreasing ferrylMb radical formation through mutagenesis decreased the ability of Mb to promote lipid oxidation in linoleic acid.

Hydrogen peroxide caused the heme moiety of horse heart Mb to be covalently cross-linked to the globin at pH values near 7 (Vuletich *et al.*, 2000). This cross-linked Mb promoted lipid oxidation in low density lipoproteins more readily compared to native Mb. Thus under certain conditions, cross-linked Mb promotes lipid oxidation more effectively than Mb that is not cross-linked. There is also evidence that cross-linked Mb formation is favored at acidic pH values (Reeder *et al.*, 2002). Hydrogen peroxide was also found to degrade the heme ring of Hb (Nagababu and Rifkind, 1998). This indicates hydrogen

peroxide can have varying effects depending on whether Mb or Hb is examined. More studies are needed to compare the relative ability of dissociated hemin and cross-linked Mb (and Hb) to promote lipid oxidation.

4.6.4 Ability of mammalian and fish Hbs to promote lipid oxidation

Perch Hb was found to promote lipid oxidation in washed cod muscle rapidly compared to trout Hb at pH 6.3 (Richards and Dettmann, 2003). Bovine Hb was a remarkably poor promoter of lipid oxidation in washed cod compared to trout Hb at pH 6.3 (Richards *et al.*, 2002). These findings can be partly attributed to the rapid autooxidation of the fish Hbs compared to the bovine Hb (Aranda *et al.*, 2009). The hemin affinity of the met forms of fish and mammalian Hbs should also be considered based on the ability of released hemin to promote lipid oxidation (Tappel, 1955).

Perch Hb and trout IV Hb released hemin 55-fold and 26-fold faster compared to bovine Hb, respectively at pH 6.3 (Aranda *et al.*, 2009). These dramatic differences can be attributed to steric and amino acid differences around the heme moiety when comparing fish and mammalian hemoglobins. The gap for solvent entry (water and protons) into the heme crevice at CD3 was 8 Å in perch Hb, around 6 Å in trout IV Hb, and around 4 Å in bovine Hb (Aranda *et al.*, 2009). Hydration of the proximal histidine will decrease hemin affinity (Hargrove *et al.*, 1996). Lysine at site E10 in bovine Hb formed favorable electrostatic and hydrogen bond interactions with the heme-7-propionate group while the smaller threonine at site E10 in the fish Hbs did not (Aranda *et al.*, 2009). The interaction of Lys(E10) with the heme-7-propionate group increases hemin affinity in the bovine Hb. Perch Hb and trout IV Hb have glycine at site E14 while bovine Hb has the larger alanine (α chains) and serine (β chains) at this site. We have found that the Ala(E14)Gly Mb mutant rapidly autooxidized and had lower hemin affinity compared to WT Mb (unpublished observation). Gly(E14) creates a channel for solvent entry into the heme crevice and may affect stability of the E-helix.

4.6.5 Effect of pH on Mb and Hb-mediated lipid oxidation

There are numerous reasons that decreasing pH increases the ability of Hb and Mb to promote lipid oxidation. First, protonation of the heme propionates at low pH will decrease hydrogen bonding and electrostatic interactions of the heme propionates with neighboring amino acids of the globin. Loss of these interactions decreases hemin affinity which promotes lipid oxidation. Second, protonation of the proximal histidine at low pH weakens the covalent linkage between the proximal histidine and the iron atom of the porphyrin. Hemin affinity of sperm whale Mb decreased 200-fold when decreasing the pH from 6.0 to 5.0 (Hargrove *et al.*, 1994). Third, protonation of the distal histidine prevents hydrogen bonding with liganded water in metMb (Fig. 4.5) which decreases hemin affinity (Hargrove *et al.*, 1996). Fourth, formation of hydrogen peroxide

(e.g., from superoxide that is released from Hb and Mb during autooxidation) occurs more rapidly at reduced pH (reactions 2–4). Hydrogen peroxide converts reduced Hb and Mb to more oxidative forms (reactions 5–8). Fifth and sixth, protonation of liganded O_2 and the distal histidine as it relates to autooxidation (Fig. 4.2) and solvent exposure to the heme crevice (Fig. 4.3) has been addressed (see Section 4.5.1). Seventh, low molecular weight metals also have enhanced solubility and thus enhanced reactivity at low pH (see Section 4.6.1).

4.6.6 Protein oxidation

Protein oxidation due to Hb and Mb can negatively impact textural attributes of muscle foods (see Section 4.2). Addition of hydrogen peroxide to metMb in the presence of albumin resulted in formation of ferryl forms of Mb that caused cross-linking of Mb to albumin and formation of dityrosine (Østdal *et al.*, 2001). MetMb and hydrogen peroxide caused the formation of thiyl, tyrosyl, and unidentified radical species on myosin as well as cross-links between myosin molecules (Lund *et al.*, 2008). Blocking of sulfhydryls with N-acetylmaleimide prevented formation of myosin radicals and myosin cross-links when exposing porcine myosin to metMb and hydrogen peroxide (Frederiksen *et al.*, 2008). This demonstrated that thiol groups of myosin were important in the formation of cross-links due to protein oxidation. Protein oxidation in a myofibrillar protein isolate was generally highest when using a hydroxyl radical generating system ($FeCl_3$ and ascorbate) compared to a metMb oxidizing system (0.05–0.5 mM metMb) and a lipid oxidizing system (linoleic acid and lipoxidase) (Park *et al.*, 2006).

4.7 Inhibition of Hb- and Mb-mediated quality deterioration in meat products

Multiple strategies can be used to inhibit discoloration as well as Hb and Mb-mediated lipid oxidation. These include:

- free radical scavengers
- reducing met and ferryl forms of Hb and Mb with reductants
- removal of all O_2 (often difficult to do in a commercial setting)
- maintaining erythrocyte integrity which limits Hb reactivity
- curing with sodium nitrite which stabilizes Hb and Mb by producing nitric oxide that binds to the iron atom of the heme
- modestly elevating the pH which decreases Hb and Mb reactivity, and
- choice of metal chelators that inactivate metal-mediated lipid oxidation.

Section 4.6.1 describes the ability of low molecular weight metals to increase the ability of Hb and Mb to promote lipid oxidation. It is noteworthy that an 'antioxidant' at one condition can be a 'pro-oxidant' at another condition. For example, ascorbate can scavenge free radicals (antioxidant properties) but also

reduce ferric low molecular weight iron to ferrous iron (pro-oxidant property) (Suh *et al.*, 2003). Factors such as ascorbate concentration, metal concentrations, pH, O_2 partial pressure, lipid hydroperoxide content, the presence or absence of metal chelators, chelator-to-metal ratio, and type of metal chelator will determine whether ascorbate inhibits or accelerates heme protein-mediated lipid oxidation (Boyer *et al.*, 1988; Lee *et al.*, 2001; Tampo *et al.*, 1994). It is noteworthy that ascorbate can scavenge O_2. This can be anti-oxidative if *all* the O_2 is removed. It can be pro-oxidative if ascorbate lowers the oxygen concentration to a level that accelerates Hb and Mb-mediated autooxidation (Ledward, 1970). Ascorbate can also decompose lipid hydroperoxides at certain conditions which can be pro-oxidative (Kanner and Mendel, 1977).

Tocopherol can inhibit Hb and Mb-mediated lipid by scavenging free radicals that are produced by both heme proteins (Fig. 4.4). The ability of tocopherols to physically modify cell membranes may be an additional mechanism that inhibits lipid oxidation (Atkinson *et al.*, 2008). Exogenous addition of tocopherol to muscle (during meat processing) is often less effective at inhibiting lipid oxidation compared to dietary addition. The poor ability of exogenous tocopherol to inhibit lipid oxidation may be attributed to different orientations of the molecule in cellular membranes compared to when tocopherol is metabolically incorporated into cellular membranes through dietary addition.

Rosemary extracts delayed color and flavor deterioration when added exogenously to fresh and cooked sausages (Sebranek *et al.*, 2005). The mechanisms involved include free radical scavenging and possibly inactivation of low molecular weight metals through chelation. An advantage of using rosemary is that it can be labeled as a natural flavor. Synthetic free radical scavengers such as butylated hydroxyanisole (BHA), butylated hydroxytolune (BHT) and propyl gallate are less expensive and more effective than natural extracts (on an added weight basis) yet some consumers object to having synthetic antioxidants in food.

Temperature control is critical in delaying quality deterioration in muscle foods. Muscle contains an extensive pool of endogenous antioxidants that degrade more slowly at lower temperatures (Halliwell and Gutteridge, 1999; Petillo *et al.*, 1998). Added antioxidants will also be more effective if elevated temperatures are avoided.

Sodium tripolyphosphate (STPP) and other polyphosphates are added to increase water binding in meats but also can inactivate pro-oxidant metals via chelation. STPP effectively inhibited met heme protein formation in ground beef muscle containing 1.5% sodium chloride at pH 5.5 and 6.0 but not at pH 6.5 and 7.0 (Trout, 1990). Metals have enhanced solubility (and thus reactivity) at reduced pH which may explain why STPP was effective only at the lower pH values.

4.7.1 Inhibiting pigment and lipid oxidation in a commercial product

Many cured and processed meat products (e.g., wieners and fermented sausages) are exposed to light during retail display because consumers want to visually

assess the quality of the product. The combination of light and trace amounts of O_2 cause color fading (e.g., Hb and Mb autooxidation) in the cured product. An oxygen-impermeable film is used to package the wieners although trace amounts of O_2 are still present after vacuum stuffing and packaging. One function of added sodium ascorbate in a wiener formulation is to deplete the residual O_2 in the product after packaging, but this must be done in the dark to prevent the color fading. Thus, packaged wieners are stored in the dark for a few days prior to displaying the product in stores. This allows adequate time for the traces of O_2 to be depleted by ascorbate while in the dark so that color fading does not occur during display when the product is exposed to light.

4.8 Future trends

4.8.1 Natural antioxidants

Natural antioxidants continue to be held in high regard because many consumers do not want to purchase products containing synthetic antioxidants. Developing a cost-effective natural extract with efficacy similar to or better than current rosemary extracts could draw a substantial market share. It is important to avoid using extraction procedures that cause the extract to no longer be 'natural' even though the starting material was from a natural plant or fruit tissue. Discoloration of muscle food products due to pigments from fruit or plant extracts can prevent application even if the extract is highly effective at inhibiting lipid oxidation.

4.8.2 Packaging innovations

Novel packaging strategies using various gas mixtures in the headspace can be utilized to decrease pigment and lipid oxidation in meats and fish. Use of carbon monoxide (CO) gas can inhibit discoloration and lipid oxidation in meats and fish (Mantilla *et al.*, 2008). CO binds to the iron atom of the heme moiety which decreases reactivity of Hb and Mb analogous to how nitric oxide binds the iron atom of the heme group. O_2 should be excluded when using CO gas packaging since the equilibrium constant for CO binding to the iron atom of Mb is only 27-fold greater compared to O_2; the equilibrium constant of nitric oxide binding to the iron atom of Mb is around 8000-fold greater compared to CO (Olson and Phillips Jr, 1997). Consumer concerns regarding the use of CO gas remains a major hurdle to acceptance. A review on recent advances in meat packaging is available (Belcher, 2006).

4.8.3 The role of sodium nitrite in meat curing and human health

The use of sodium nitrite in meat products is often perceived negatively due to the concern of nitrosamine formation when nitrite reacts with amine groups of meat proteins. The meat industry has carefully developed procedures to virtually eliminate the presence of nitrosamines in cured products. For example, in bacon,

sodium nitrite is limited to 120 ppm, 550 ppm sodium ascorbate must be utilized to ensure that nearly all of the sodium nitrite is converted to nitric oxide, sodium nitrate cannot be used, and the bacon should be fried at a temperature not greater than 340 ºF for 3 min per side to ensure that nitrosamines do not form during frying (Code of Federal Regulations 9 424.22). To date there has been no link between the use of nitrite in meats and cancer development in humans. Most of the sodium nitrite is converted to nitric oxide during meat processing which decreases reactivity of Hb and Mb during storage as noted earlier (see Sections 4.2 and 4.7). The meat industry continues to use sodium nitrite not only because it inhibits pigment and lipid oxidation but also because sodium nitrite is an excellent antimicrobial that limits food-borne illness outbreaks. The pink color that results from curing with sodium nitrite is also desirable in cured products. A new trend is to add celery powder to meat formulations in order to cure the product without adding the pure chemical form of sodium nitrite. Celery is a rich source of sodium nitrate (Walker, 1990). A bacterial culture (that is added to the meat formulation) reduces the nitrate to nitrite (and the nitrite is then converted to nitric oxide which fixes to the iron of Hb and Mb) so that the meat is cured during processing. This type of product is more 'label-friendly' since sodium nitrite does not appear as an added ingredient.

The perception of sodium nitrite may change from a liability to that of a beneficial ingredient in cured meats. There is recent evidence that dietary nitrite at low doses (50 mg/L drinking water) actually decreases processes associated with the development of vascular disease (Stokes *et al.*, 2009). Sodium nitrite is converted to nitric oxide by bacteria in human saliva (Bryan, 2006). Nitric oxide dilates blood vessels, decreases inflammation and protects biomolecules from oxidative events (Bryan *et al.*, 2007).

4.9 Sources of further information and advice

The National Center for Biotechnology Information (NCBI) can be used to obtain amino acid sequences and three dimensional structures for different hemoglobins and myoglobins. Software programs such as PyMOL are available to measure distances between different atoms in a structure and to hide parts of the molecule so that specific interactions can be observed. Other useful references are:

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5

Lipoxygenase and lipid oxidation in foods

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Abstract: Lipoxygenases (LOXs) are enzymes found in plants, animals and microorganisms that catalyze the oxidation of polyunsaturated fatty acids and lipids containing a *cis,cis*-1,4-pentadiene structure. This reaction produces lipid hydroperoxides, which decompose and form secondary oxidation products that give strong undesirable flavors, causing food deterioration. LOXs are also involved in development and self-defense of plants and the production hormone-like eicosonoids in animals. In this chapter, the mechanism of this enzyme-catalyzed reaction and its implication in quality changes in soybeans and soy foods, in rice bran, in meats and fish, in dairy products, in fruits and vegetables, and in nuts are reviewed. Several means of prevention of this enzyme-catalyzed oxidation are also discussed.

Key words: lipoxygenase, isoenzymes, lipid oxidation, soybeans, rice bran, fish, nuts, prevention of enzyme catalyzed oxidation.

Lipoxygenases (LOXs) are known as linoleate:oxygen oxidoreductases (EC 1.13.11.12). They are a family of iron-containing enzymes that catalyze the oxidation of polyunsaturated fatty acids and lipids containing a *cis,cis*-1,4-pentadiene structure. This reaction produces lipid hydroperoxides (ROOH), which can decompose and form secondary oxidation products that often have strong flavors. LOXs are found in plants, animals and microorganisms. They are common in plants where they may be involved in growth and development, pest resistance, and senescence or responses to cellular damage. In animals, LOXs are involved in the production of eicosonoids such as prostaglandins and leukotrienes; however, these biological reactions are not the main focus of this

chapter, which is primarily concerned with the role of LOXs in lipid oxidation and their effect on food quality.

5.1 The lipoxygenase (LOX) enzymes

Soybean LOX isoenzymes (LOX-1, 2 and 3) were first reported in seed in 1970 and 1972 by researchers in Axelrod's laboratory. LOX, also referred to as lipoxidase, was first crystallized in 1947 (Gardner *et al.*, 1996). Soybean plants also have multiple LOX isoenzymes that are different from those in the seeds. Certain molecules that are able to stimulate plant growth, such as jasmonic acid, are produced by an initial LOX-catalyzed oxidation of fatty acids and finished by further enzymatic reactions. Jasmonic acid is a phytohormone that leads to the expression of a vegetative storage protein, and it is typically present in vegetative tissues at low concentration. The biological functions of the aldehydes and other lipid oxidation products produced by LOXs are more speculative than that of jasmonic acid. These aldehydes may be responsible for inhibiting the growth of pathogenic organisms (Gardner *et al.*, 1996). These biologically active compounds are not expected to contribute to the typical oxidized flavor in plant food systems.

Although there are various forms of LOXs in plant and animal tissues, they all have a single peptide chain. The three-dimentional structure of the catalytic domain is primarily an α-helix with a single atom of non-heme iron near its center. The center of the domain consists of two long helices, which donate four iron-binding histidine groups. The fifth group that coordinates the non-heme catalytic iron is the carboxyl group of the C-terminal isoleucine.

The animal-source enzyme has a size of about 550–600 amino acids, which is shorter and more compact than the plant LOXs, which have about 650–750 amino acids. Therefore, LOXs in animals have a molecular mass of 75–80 kDa, and in plants the mass is about 94–104 kDa (Brash, 1999). A more detailed description of LOX structure and active sites has been presented by Prigge and co-workers (1996).

LOXs can place hydroperoxide groups at various positions on a fatty acid chain after attacking the 1,4-*cis,cis*-pentadiene group. This position may be designated by placing the number of the position on which the enzyme acts before the enzyme name. For example, a LOX that oxidizes arachidonic acid (AA, 20:4) at the C12 position, counting from the carboxyl end of the chain, would be called a 12-LOX. The 13-LOX, found in plants, oxidizes linoleic (18:2) and linolenic (18:3) acids at the 13th carbon from the carboxyl end or the ω-6 position, counting from the methyl end of the chain. Mutagenesis studies on LOX have suggested that its positional specificity depends on the enzyme binding of the terminal methyl group of a fatty acid and correctly aligning the substrate relative to the catalytic site. Therefore, the relative distance of the 1, 4-pentadine group from the methyl terminus plays a significant role in determining the enzyme's point of attack (Prigge *et al.*, 1996). Kato and co-workers (1992)

showed that soybean LOX-2 and LOX-3 (a number place after the enzyme name is used to designate various isozymes) exhibited a strong preference for the free acid form of 18:3 as a substrate over the free acid form of 18:2. LOX-1 showed a higher activity toward the 18:2 fatty acid.

The soybean plant produces at least eight identified LOXs (Brash, 1999), and its LOXs are the best characterized among the plant LOXs. However, mature seed cotyledons contain primarily three LOX isozymes designated LOX-1, LOX-2, LOX-3 (Axelrod *et al.*, 1981) and a minor isoenzyme, LOX-4 (Brash, 1999). The LOX-4, 5 and 6 are produced or start to appear during seed germination (Wang *et al.*, 1999). The first three isoenzymes differ in their substrate specificity and pH optimum. LOX-1 catalyzes oxygen addition on C13 of 18:2 and 18:3. LOX-1 prefers free fatty acids over esters, and it has an optimum activity at pH 9. LOX-2, or a mixture of LOX-2 and LOX-3, have an optimum activity at pH 7 and does not exhibit a strong positional specificity, oxygenating both the C9 and C13 positions. Both LOX-2 and LOX-3 are said to be equally activity toward 18:2 and 18:3 (Axelrod *et al.*, 1981), and attack both the free fatty acid and esterified forms quite well (Brash, 1999). However, Fukushige and co-workers (2005) reported that LOX-2 gave hydroperoxides at positions 13 and 9 in a ratio of 4:1. LOX-3 produced 65% 13-ROOH and 35% 9-ROOH, but the proportion is said to be highly dependent on the reaction conditions (Christopher *et al.*, 1972). The type and quantity of the ROOHs will affect the composition of their decomposition products and the flavor profile of the oxidized foods may be different with the different LOXs or different reaction conditions. A most recent study by Iassonova and co-workers (2009) showed evidence of another possible isoenzyme in a LOX-null soybean which has substrate specificity toward phosphatidylcholin, as discussed later in the chapter.

LOX isoenzymes are localized in the cytoplasm of the cotyledon cells (Wang *et al.*, 1999) and their physiological roles are not yet fully understood. No harmful consequences have been observed in soybean mutants lacking specific LOX isozymes (Siedow, 1991). LOXs are believed to play a role in the defense of the plant, and presumably they might play such a role during seed germination and maturation. During the germination of commodity soybeans, LOX-1, 2, and 3 activities decreased (Song *et al.*, 1990; Kato *et al.*, 1992; Wang *et al.*, 1999) and isoenzymes LOX-4, 5, and 6 started to appear. This may explain why triple-null soybeans (seeds with the three main LOX isoenzymes removed) do not suffer from poor agronomic performance. The effect of seed LOX elimination (triple-null) on the resistance to plant pathogens, such as stem canker, frogeye leaf spot, and powdery mildew was studied, and in general LOX removal did not affect the resistance of soybeans to these diseases (Martins *et al.*, 2002).

LOXs are also involved in lipid oxidation in animal tissues and they play an important role in controlling cell functions. The 18:2 and 18:3 fatty acids in animal tissues can be elongated and oxidized to produce the essential eicosanoids, which are a class of physiologically important fatty acid derivatives that include prostaglandins, thromboxanes, and leukotrienes. Two pathways or

types of enzymes are involved in producing these molecules, the cyclic and linear pathways. The cyclo-oxygenase of the cyclic pathway produces prostaglandins and thromboxanes while the linear LOX pathway produces leukotrienes. These 20 carbon molecules are not known to affect food flavor and quality because seemingly, they do not lead to the secondary scission products responsible for oxidized flavor.

5.2 Mechanism of lipoxygenase (LOX)-catalyzed oxidation

The LOX enzymes are usually in the inactive ferrous (Fe^{2+}) form when isolated. Oxidation to the active ferric (Fe^{3+}) enzyme is required for catalysis. LOX's Fe^{2+} is oxidized to Fe^{3+} by hydroperoxides, and it is this form that catalyzes the reaction that produces either 9(S)- or 13(S)-hydroperoxides or a mixture of them both from 18:2 and 18:3 acids. The reaction starts with a proton abstraction from the methylene carbon between the double bonds of the *cis,cis*-1,4-pentadiene group of the substrate, followed by addition of a molecular oxygen molecule to either end of the 1,4-pentadiene structure. The enzyme catalyzed reaction is illustrated in Fig. 5.1 (Brash, 1999).

In some instances the LOX is more active on the free fatty acid form of the substrate, and lipase hydrolysis of the triacylglycerides (TAG) produces free fatty acid substrates with a 1,4-pentadiene group for such reaction. Oxylipin is a generic name for the family of oxidized lipids produced by the LOX reactions and their secondary decomposition products. In animal tissue 20:4 is a typical substrate, and in plants 18:2 and 18:3 are the typical substrates for these enzymes.

Aside from LOX, biological systems also may contain LOX-like enzymes and molecules containing iron in a porphyrin ring which also can catalyze lipid oxidation (Rao *et al.*, 1994), for example heme (Fe^{2+}) and hemin (Fe^{3+}). In these molecules iron is bound covalently to the four nitrogen atoms of the porphyrin structure, and in myoglobin and hemoglobin, the iron is also bound to a histidine molecule of the protein chain and the 6th bonding orbital of Fe interacts with O_2 or water. Enzymes based on these molecules are especially important in the flavors of animal products. Important enzymes based on heme and hemin include peroxidase and catalase and the myoglobin of muscle. These molecules catalyze lipid oxidation similarly to LOX but the details of their mechanism is

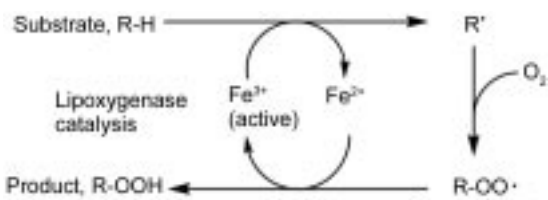

Fig. 5.1 Lipid hydroperoxide formation catalyzed by a lipoxygenase.

still unclear. These hemes are typically more reactive than the ferrous and ferric ions they contain in catalyzing the breakdown of the lipid hydroperoxides.

Heating deactivates LOXs and myoglobins and provides an important control technique; however, after denaturation of the proteins, the non-enzymatic catalysis can then play a major role in lipid oxidation via the heme or free iron ions, and the rate of such oxidation may be increased compared to before heating. Copper ions also can be released from a chelated state and catalyze lipid free radical auto-oxidation.

5.3 Sources of lipoxygenase (LOX) and products particularly affected

5.3.1 In soybeans and soy foods

The undesirable 'green' and 'beany' flavors of soybeans and soy protein are major factors limiting their acceptability and use in foods. In raw soybeans, these flavors can be generated very rapidly once the cellular structure is broken, as illustrated by the rapid appearance of strong beany flavor when chewing raw soybeans. In industrial processing such as oil and protein separation the seed is first cracked, conditioned, and flaked, and then extracted with a solvent. During the process, lipid oxidation by LOX and free radical auto-oxidation is inevitable. The volatile secondary oxidation compounds, such as aldehydes and ketones, have low flavor thresholds and strong affinities for the soy protein. Thus, developing soybeans without or with reduced LOX contents has been a very desirable goal to increase soybean and soy ingredient consumption in the Western countries.

Mutant soybean lines lacking LOX isozymes have been developed (Davies and Nielsen, 1986; Narvel *et al.*, 1998). LOX-null seeds with unaltered yields, seed weights, and protein contents were developed by Narvel and co-workers (1998). Low 18:3 (<5%) and triple-null soybeans have been developed to improve the oxidative stability of soybean oil and reduce off-flavors. The study of agronomic performance showed that such modifications did not cause any obvious detrimental effects on agronomic traits including yield (Reinprecht *et al.*, 2006), and it might be possible to use this novel germplasm to develop competitive soybean cultivars with improved oxidative stability. A new soybean variety, Zhonghuang 31, was developed in the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences through years of biochemical marker assisted selection for null trypsin inhibitor and LOX-null (Han *et al.*, 2006). This line has a high and stable yield and good quality in general. In an triple-null soybean, the degradation of antioxidative compounds, such as vitamin E, vitamin C, and lutein in aqueous homogenate was much less than in commodity soybean (Nishiba and Suda, 1998). This suggests that the LOX-null soybean could become a superior food ingredient which not only improves the flavor of soy products but also its nutritional quality.

The oxidative stability of the extracted and purified oil from LOX-null beans are not expected to be different than the traditional oil if the fatty acid

composition is unchanged. Numerous research publications have validated this prediction (Engeseth *et al.*, 1987; King *et al.*, 1998; Shen *et al.*, 1996). LOX-null soybean lines that lack LOX-2, or LOX-2 and 3, and contain normal (8.0–8.6%) or low (2.0–2.8%) 18:3 were evaluated for their oil qualities and storage stabilities (Shen *et al.*, 1996). In general, the absence of LOX-2 or LOX-2 and 3, although having a small effect on lipid oxidation, was not as important to oil quality as was the 18:3 content. Frankel and co-workers (1988) found that in a direct comparison of oil products from the LOX-1 null and commodity soybeans, there was no significant differences in either flavor quality or in flavor stability based on total volatiles, and in the analysis for 2,4-decadienal. Therefore, factors other than LOX appear to control the food quality of soybean oils and meals.

Soy flour and purified soy proteins from the LOX-null varieties are expected to have improved flavor quality because of the less oxidation thus less binding of the flavor compounds to proteins during seed processing and protein preparation. As expected, soymilk and tofu made from LOX-null soybeans were less beany than products made from commodity beans as demonstrated by many researchers and reviewed by Wilson (1996). Yuan and Chang (2005) compared the beany flavor profiles of soymilk made from two LOX-null soybean types with three other varieties. Beany flavor compounds, including hexanal, hexanol, 2-nonenal, 1-octen-3-ol, and 2,4-decadienal were quantified. The results showed that the LOX-null varieties produced significantly lower levels of the beany flavor than the two low-18:3 varieties and a commodity soybean. However, the flavor of bread, meat patties, and beverage products made with LOX-null soybeans was not improved (King *et al.*, 2001).

Soybean oil is a 18:2 type of oil, and it naturally oxidizes rapidly by autoxidation. This may be the reason that the LOX-null soybeans are not showing great promise in effectively and significantly improving the flavor quality of protein products. Oil composition modifications such as high oleic and mid-oleic together with low or ultra-low 18:3 have been developed to replace hydrogenated soybean oil for many purposes. These may results in much more improved soybean products than LOX-null varieties. These new protein products need to be tested systematically and compared with the LOX-null type of proteins.

A most recent publication showed that there may be another LOX enzyme present in LOX-null soybean (triple-null) and this enzyme may be responsible for the off-flavors in the LOX-null soybeans (Iassonova *et al.*, 2009). Volatiles production in triple LOX-null soybean could be terminated by heat treatment, which suggests an enzymatic cause to the off-flavors. The source is LOX-like in that the volatile compounds produced are similar to LOX-generated products of polyunsaturated fatty acids. Oxygen was consumed when a LOX-null protein solution was incubated with crude soybean oil suggesting that the enzyme-catalyzed oxygen consuming reactions. The generation of flavor compounds was inhibited by the typical LOX inhibitors. The enzyme was more active with phosphatidylcholine than with soybean oil or its free fatty acids as substrates.

This is the first recent report on another LOX enzyme in soybeans which has a unique substrate specificity. Further research is needed to fully characterize this enzyme.

It is logical to suspect that soybeans with triple-null LOXs may be more vulnerable to insect attack on the seeds because oxylipins are important molecules in plant defense. Defense against insect attack was investigated for the lipid oxidation products produced by soybean seed LOXs, to study the physiological role of the enzymes in seeds. Repellent effects against bean bugs, a major soybean pest, were observed in products of LOX oxidation of 18:2 such as hydroperoxides and hexanal (Mohri *et al.*, 1990). Bean bugs preferred LOX-null (triple-null) and LOX-2-null seeds, more than commodity soybean seeds. However, no significant difference was seen in feeding preference at the ripening period of the seed. The lipid oxidation products of LOXs, 18:2-hydroperoxide and hexanal, also repelled two species of leaf beetles that do not usually feed on soybean seeds. These results suggested that the lipid oxidation products repelled the tested insects and that the soybean seed LOX could act defensively, although the effect of the products on the pest insects was not great.

LOX catalyzed lipid oxidation also affects the functional properties of soy proteins. Native soy flour's three main LOX isoenzymes can improve dough characteristics by oxidizing unsaturated fatty acids to hydroperoxides. The hydroperoxides can oxidize proteins, which can lead to improved rheological properties. The hydroperoxides also can bleach carotenoids leading to a whiter product (Roozen *et al.*, 1993). However, the fatty acid hydroperoxides can also form volatile compounds that detract from the flavor of bland-flavored products.

Oxidation of lipids leads to protein structural change in soybeans, thus soy protein functionality can be altered. The primary lipid oxidation product by the LOX-catalyzed reaction in soy protein is the 13-hydroperoxide of 18:2 (Wu *et al.*, 2009). Incubation of soy protein with increasing concentration of this hydroperoxide resulted in generation of protein carbonyl derivatives, loss of protein sulfhydryl groups and loss of α-helix structure. Surface hydrophobicity of the protein also decreased, indicating that aggregation had occurred. The extent of protein aggregation increased with exposure to the hydroperoxide in a dose-dependent manner that was quantified by size exclusion chromatogram. β-Conglycinin was more vulnerable to hydroperoxide than glycinin. Aggregation of soybean proteins induced by lipid oxidation was also investigated by Huang and co-workers (2006). Soybean proteins obtained from the model systems with various levels of linoleic acid and LOX showed increased turbidity, protein oxidation, surface hydrophobicity, and decreased sulfhydryl content.

Besides developing LOX-null soybeans, other approaches can be used to reduce the beany flavor resulting from LOX activity. These measures inactivate the LOX during processing and minimize or mask the flavors, and they include heating of the soybean seeds or grinding the seeds at an acidic pH, with hydrogen peroxide, with calcium ion, in hot water, with added antioxidants, using steam or vacuum distillation or supercritical extraction to remove the volatiles, and adding other flavor compounds to mask the undesirable flavor

(Wilson, 1996). The enzymes can be readily deactivated by heating and this is commonly done in the industry to stop LOX initiated oxidation. LOX-2 and 3 are more susceptible to heat treatment than LOX-1. LOX-2 and 3 can be inactivated by heating to 70 ºC for 20 min, whereas LOX-1 required 120 min at 70 ºC to be inactivated (Hildebrand and Kito, 1984).

5.3.2 In rice bran

Rice bran is a by-product from the milling of rice caryopsis. It contains significant quantity of oil, and it has been an important source of vegetable oil in Asian countries. The presence of lipid oxidative and hydrolytic enzymes is a significant problem for a successful processing and utilization of this by-product and oil. LOX activity in rice seeds is localized in the bran fraction, and LOX, together with lipases, plays critical role in product quality.

Shastry and Rao (1975) showed that rice bran extract contained three distinct protein bands with LOX activity, and the major band which was 3 to 5 times more intense on SDS-PAGE gel was the smallest molecule and it still contained five proteins. The purification of this major band, enzyme stability, optimum pH for activity, enzyme activity and kinetics, activators and inhibitors of this band were further studied. Cupric ion at 1mM concentration was found to completely inhibit the enzyme activity. John and co-workers (1981) also isolated and purified rice caryopsis LOX. The molecular weight was about 100KDa, and its pI was 4.8. The optimum activity for lipid oxidation was at pH 6.8–7.0 and 30 ºC. The enzyme was relatively stable at neutral pH and room temperature but was markedly inactivated by incubation at temperature of greater than 50 ºC. The heat inactivation of rice bran LOX, as for other enzymes, requires high moisture content. Rice LOX was not deacitivated at 21% moisture content, microwave oven heated (at 850 W) for 3 min with internal temperature reaching 107 ºC (Ramezanzadeh *et al.*, 1999), and it was suggested that much higher moisture content is needed for full inactivation. The fact of rice bran LOX showing 90% activity inhibition by free radical quencher, such as BHA, demonstrates the involvement of free radicals in this enzyme catalyzed oxidation (Jhon and Lee, 1985).

LOX-3 is the major isoenzyme component in rice bran (Shirasawa *et al.*, 2008) and the LOX-3 null rice has less stale flavor during storage than normal rice. Thus, introduction of the LOX-3 null phenotype would facilitate the retention of high quality during storage. A molecular genetic tool was developed by Shirasawa and co-workers for breeding and screening LOX-3 null rice lines. Using the Thai rice variety Daw Dam which lacks LOX-3 in its seed, and the Japanese rice varieties Koshihikari and Koganemochi, which have normal LOX-3 activity, the oxidative stability of lipids in rice bran fractions was studied during storage at 4 and 37ºC (Suzuki *et al.*, 1996). FFA content in lipids differed between storage temperature and peroxide value differed among varieties. The results suggest that lipid oxidation occurred at lower levels in the Daw Dam bran fraction than in the varieties with LOX-3 in their seeds.

Many studies have suggested that the main reason for rice bran deterioration is the effect of lipase, but information about LOX activity has been relatively limited. The relationship between LOX activity (LOX1, 2, and 3) and rice bran deterioration was studied with an accelerated-aging experiment (Zhang *et al.*, 2009) using white or red coat rice from near isogenic lines. The results seem to indicate that the activity of some LOX and lipase is negatively correlated. In this study, several rice bran materials with different activities of LOX isoenzyme within the seed coat color group were studied for their LOX activity and FFA content. Red coat with high LOX-1, 2 and low LOX-3 activity gave low FFA value, and white coat with low LOX-1, 2 and high LOX-3 activity gave low FFA value, and these samples had the least deterioration. Therefore, the results indicate that high LOX isoenzyme activity may be beneficial to delay rice bran deterioration, since rice LOX may mainly act on FFA and there is a shortage of the substrate in high-LOX activity rice.

5.3.3 In meats

Rao and co-workers (1994) suggested that myoglobin has LOX-like activity because it forms ferryl myoglobin when treated with hydrogen peroxide, and ferryl myoglobin is able to initiate lipid oxidation by abstracting a hydrogen atom from a methylene group of the 1,4-pentadiene system in a fatty acid chain to start the lipid oxidation process (Rao *et al.*, 1994; Baron and Andersen 2002). Spectroscopic studies showed that the binding kinetics of linoleic acid to myoglobin is similar to that of LOX-oxidation and that linoleic acid reduced the ferryl species to the ferric state. The stereochemical results ruled out any role of singlet oxygen in myoglobin-catalyzed and hydrogen peroxide-dependent oxidation of 18:2. The myoglobin protein radical formed with hydrogen peroxide also played no role in the reaction because the rate of formation of the 9-ROOH was not affected if the protein radical was allowed to decay before the substrate was added. The 18:2 was oxidized within the heme crevice by reacting with the ferryl oxygen in an enzymatic fashion (Rao *et al.*, 1994).

To examine if myoglobin acts as a LOX-like catalyst, various meat homogenates preparations were analyzed to identify the factors in raw chicken breast and beef loin that result in lipid oxidation (Min and Ahn, 2009). Chicken breast showed greater oxidative stability than beef loin during 10-day storage. All fractions (meat homogenate, precipitate, and supernatant) from chicken breast showed lower amounts of free ionic iron and myoglobin and higher total antioxidant capacity than those from beef loin. Therefore, myoglobin was responsible for the high LOX-like activity and lipid oxidation potential of beef loin. In a similar study, the susceptibility to oxidation of meats from various animal species was reported (Min *et al.*, 2008). Oxidation of raw beef increased significantly compared to chichen and pork during 7-day storage as a result of beef's high heme iron content and high LOX-like activities.

The LOX-like activity of myoglobin on an 18:2 emulsion was 8.39 units per mg myoglobin protein (Min and Ahn, 2009). This was derived by measuring the

double bond conjugation in an 18:2 emulsion treated with meat extract. To fully understand the enzymatic nature of myoglobin, a more in-depth characterization is needed that fully describes the effects of temperature, pH, response to antioxidants, and substrate specificity of the 'enzyme'.

5.3.4 In fish

The 12- and 15-LOXs have been identified in fish gill tissue that have properties similar to the plant LOXs. LOXs of Atlantic mackerel muscle had two prominent molecular weights of 119 and 125 kDa (Saeed and Howell, 2001). The presence of LOXs indicates the possibility that lipid oxidation is initiated enzymatically in chilled and frozen mackerel fillets, and that this oxidative deterioration could be inhibited by antioxidants (BHT, ascorbic acid and tocopherols) and heating which are used widely in the food industry.

Fu and co-workers (2009) used a model system of minced silver carp to examine the effect of LOX and hemoglobin on the kinetics of lipid oxidation and fishy-odor formation. The major LOX in silver carp muscle was a 12-isoenzyme. Compared to hemoglobin, LOX caused faster lipid oxidation in the initial phase but lower lipid hydroperoxides and less thiobarbituric acid test response (TBARS). The LOX was affiliated with a strong fishy odor, while hemoglobin resulted in a severe oxidized odor.

LOXs are also present in fish skin tissue, and skin tissue LOX initiated lipid oxidation in trout skin and affected the conversion of docosahexenoic acid (DHA) and AA into polar products (German and Kinsella, 1985). A novel LOX also was found in sardine skin (Mohri *et al.*, 1992) that oxidized 18:2 more efficiently than AA or eicospentenoic acid (EPA). Esterified fatty acids such as the methyl ester of 18:2 and glyceryltrilinoleate also were oxidized. This enzyme is believed to participate in the initiation of lipid oxidation in fish.

The role of LOX in causing lipid oxidation (measured as TBARS) in lake herring was studied by Wang and co-workers (1991). LOX activity was correlated to phospholipids content, which is highest in light muscle, lowest in skin and intermediate in dark muscle. This may be because of the enrichment of EPA and DHA in phospholipids. Heat could be used to inactivate this LOX (80 ºC for 1.5–2.0 min); however, when heating for too long (80 ºC for more than 5.0 min), non-enzymatic oxidation was accelerated.

In fish muscle, both enzymatic and non-enzymatic oxidation can take place, with low temperature promoting an enzymatic mechanism and high temperature a non-enzymatic mechanism (Frankel, 1998). As mentioned previoussly, LOXs occur mainly in fish gill and skin tissues, and they can be inactivated at above 60 ºC. But such thermal treatment increases the non-enzymatic reaction rate since the denatured metallo-proteins are also active catalyst for lipid oxidation, and the breakdown of the resulting hydroperoxides can cause appreciable flavor generation. Such heat generated non-enzymatic oxidation may exceed the rate of oxidation catalyzed by LOX in unheated tissue. A post-mortem reduction in the natural reducing agents of fish, such as ascorbate, NADH and glutathione may

affect lipid oxidation by changing the balance of concentration and activity between heme iron and free iron.

Although lipid oxidation is considered a deteriorative process responsible for generating off-flavors, specific oxidation products are desirable flavor compounds particularly when formed in more precise reactions, such as the generation of fresh fish flavor (German *et al.*, 1992). The specificity and stability of endogenous LOX and the oxidation volatiles generated by the enzymes in the gills of marine and freshwater fish were important in the generation of fresh fish flavors. Understanding these enzyme systems may facilitate the development of methods for the generation of desirable flavors in seafood products.

5.3.5 In dairy products

Many enzymes in milk are known to initiate lipid oxidation. Xanthine oxidase is a flavin enzyme found in milk fat globule membranes that generate superoxide radical ($O_2^{-\bullet}$), a reaction by an O_2 taking up one electron. Superoxide dismutase catalyzes the dismutation of this radical to produce hydrogen peroxide, thus superoxide dismutase serves as an antioxidant in the presence of catalase which can remove hydrogen peroxide from the system. In the presence of Fe^{2+}, H_2O_2 produces very reactive $HO^{\bullet}$ by the Fenton reaction. This active free radical can react non-selectively with all organic molecules. Milk neutral lipid has little polyunsaturated fatty acid and inherently stable to oxidation. Copper and iron exposure and photo-oxidation have been the major causes of oxidation. Riboflavin generated singlet oxygen oxidation may play important role in milk lipid oxidation. Cupric catalyzed oxidation of milk fat seems to give rise to 1-octene-3-one or possibly 1,5-octadiene-3-one from n-3 fatty acids. These compounds play a minor role in the oxidation of vegetable oil but they are the dominant flavor in milk fat.

5.3.6 In fruits and vegetables

Lipid derived flavors are produced in tomatoes during ripening via the action of endogenous enzymes such as LOX. They may be further influenced by non-enzymatic oxidative decomposition reactions which occur during processing and storage (Karmas *et al.*, 1994). The generation of lipid oxidation compounds in fresh tomatoes during ripening by LOX activity is considered a desirable reaction.

Undesirable LOX catalyzed oxidation is observed during potato processing. The impact of processing on lipid oxidation in potato flakes was investigated by Gosset and co-workers (2008). LOX activity was mostly related to lipid oxidation and flavor generation. However, non-enzymatic lipid oxidation products were also found during processing, and these auto-oxidative processes cannot be inactivated by the main endogenous non-enzymatic antioxidants in potato tubers, such as ascorbic acid, phenolic compounds and carotenoids, because these antioxidants are degraded during processing. Therefore, efficient exogenous antioxidants as well as adequate storage conditions should be used.

5.3.8 In tree nuts

Almond seeds age rapidly during storage at high relative humidity (80%) and moderate temperature (20 ºC). The content of unsaturated fatty acids (18:2 and 18:3) decreased during accelerated aging, and the aged seeds contain high levels of malondialdehyde (Zacheo *et al.*, 1998). LOX was believed to cause this oxidation because increased activity of this enzyme was observed during the aging experiment. In hazelnut, significant genotypic variability was found in total fat, fatty acid and alpha-tocopherol contents, as well as LOX activity in various varieties of the nuts (Pershern *et al.*, 1995). Relationships were found between shelf-lives for hazlenuts and their polyunsaturated fatty acids, α-tocopherol and LOX activity levels.

5.4 Prevention of lipoxygenase (LOX)-initiated reactions

5.4.1 Enzyme inhibitors

A natural LOX inhibitor, sclerotiorin, purified from the fermented broth of *Penicillium frequentans*, can be used as a potent, reversible, uncompetitive inhibitor against soybean LOX-1 (Chidananda and Sattur, 2007). The inhibitor also showed an antioxidant property by scavenging free radical. Therefore, sclerotiorin may inhibit LOX in two ways: by interacting with the enzyme-substrate complex, and as an antioxidant by quenching or trapping the free radical intermediates formed in the enzyme reaction. Sclerotiorin compared well with other known natural and synthetic LOX inhibitors.

Esculetin, a known inhibitor of LOX, inhibited the production of 12-hydroxyeicosatetraenoic acid from the reaction of LOX with AA, and the hydroperoxide level also was further reduced by the presence of antioxidants, namely BHT, ascorbic acid and tocopherols (Saeed and Howell, 2001).

LOXs can be inactivated by LOX substrate analogs. The substrate analogs include 18:1 fatty acid, and 'suicide' inhibitors such as 5, 8, 11, 14 eicosatetraynoic acid and colneleic acid (Zhuang *et al.*, 2002). Some nonsteroidal anti-inflammatory drugs also can be inhibitory to LOXs.

5.4.2 Heating

Heating can effectively inactivate LOX enzymes and prevent undesirable reactions to some degree. For heat denaturation of soybean LOX, steam treatment for 2 min can inactivate LOX-1 and LOX-2, and the defatted flour thus will have a better flavor score than the unheated beans. Toasting of soybeans at 100–110 ºC also inactivates these isozymes to produce good quality soy protein products. In the conventional solvent extraction of soybean oil, the slight heating conditioning (65–75 ºC) of the cracked seeds before flaking is not sufficient to inactivate these enzymes. Once deactivated, LOX may also act as non-enzymatic catalysts for lipid oxidation, as discussed earlier.

Lipid nutrients can be preserved by heat deactivation of the LOXs. For example, less than 10% of the vitamin E in wholemeal and white wheat flours

was retained after drum-drying. This extensive destruction of vitamin E may be due to lipid degradation catalyzed by LOXs. When LOX was heat-inactivated by a low moisture processes, vitamin E retention was greatly improved (Hakansson and Jagerstad, 1990).

Cereal products such as wheat flour undergo fast oxidation and generate bitter and rancid flavors once the raw materials are mixed with water. Heat processing can easily deactivate the LOX, especially in the presence of a high moisture content (64 ºC at 12% moisture and 103 ºC at 8% moisture) (Frankel, 1998). The endogenous tocopherols in plant materials play an important role in preventing lipid oxidation. Synthetic BHA and TBHQ and natural phenolic extracts are effective in extending the shelf life of dry cereal products.

Inactivation of enzymes by heat generated from radiofrequency (RF) electric fields was also investigated for inactivating LOX (Manzocco *et al.*, 2008). The effect of increasing RF on the activity of polyphenoloxidase and LOX was tested in model systems and in apple products. The treatments efficiently inactivated both enzymes because RF generated thermal effect that denatured the enzymes. RF also allowed apples to be adequately blanched and produced a puree quality equivalent to that obtained by traditional blanching.

5.4.3 Irradiation

Gamma-irradiation of buckwheat seeds at 4–6 kGy resulted in a significant reduction in LOX activity, achieving 22–43% activity of the untreated control (Henderson and Eskin, 1990). The authors suggested that heating is more efficient and effective way to eliminate LOX than irradiation.

5.4.4 Pressure change

LOX inactivation by pressurization-depressurization may be possible. In an Indian patent (Mukhopadhyay and Chakraborty, 2007), supercritical CO_2 treatment of legumes and cereals was shown to inactivate LOX, lipase and trypsin inhibitor by using a sequential processing protocol that involves a pressurization-depressurization at temperature of 35–60 ºC, pressure of 7–15 MPa, and treatment time of 15–90 min. This process produced final products without beany flavor, trypsin inhibitor, and lipase activity.

5.4.5 Endogenous antioxidant systems

The endogenous antioxidants in muscle include α-tocopherol, ubiquinone, ascorbate, histidine-dipeptide, and enzymes such as superoxide dismutase (converts superoxide anion to hydrogen peroxide), catalase (convert hydrogen peroxide to water and oxygen), glutathione peroxidase (converts hydroperoxides to alcohols). Glutathion can reduce the lipid oxidation initiator ferryl myoglobin to metmyoglobin, reducing its LOX-like activity. Lipid oxidation in meat can be accelerated by the depletion of glutathione peroxidase and glutathione transferase, which decompose hydroperoxides.

Carnosine (beta-alanyl-L-histadine) is an endogenously formed dipeptide in various meat products (0–70 mM concentration). It acts as antioxidant by chelating ions and scavenges free radicals. High cost of carnosine makes its applications as feed additives impractical.

5.4.6 Exogenous antioxidants

Antioxidants effectively used in meats include phenolic compounds from spice extracts, and other additives used as processing aids, such as curing agent nitrite. Other common synthetic antioxidants are used in combination such as BHA and TBHQ. LOXs can be inactivated by natural phenolic antioxidants (Zhuang *et al.*, 2002). Citric, ascorbic and phosphoric acids are also successfully used as metal ion chelating agents to retard meat oxidation. These measures stabilize the free radicals and reduce or chelating Fe^{3+} to Fe^{2+}, so they are not true enzyme inhibitors.

Rosemary extracts containing carnosol, carnosic acid and ursolic acid have strong inhibitory effects on lipid oxidation and soybean LOX activity (Chen *et al.*, 1992). Phospholipases could inhibit lipid oxidation by liberating free fatty acid from membrane, which form ion complexes by chelating mechanism. Exogenous antioxidants as mentioned above can also be used to slow oxidation in fish (Frankel 1998). EDTA and antioxidants inhibit enzyme-catalyzed oxidation by removing iron and reducing hydrogen peroxide, and these antioxidants are more effective in minced meat because of the easier and more thorough mixing than in whole fish.

5.5 Future trends

The discovery of a manganese LOX and its unique ability to oxidize the fatty acid carbon between the two *cis* double bonds (C-11 of linoleic acid) provides many new questions and issues related to catalysis and reaction mechanisms. It remains to be seen if this represents a new class of enzyme or is related to the LOX family.

A better understanding of myoglobin and hemoglobin acting as catalysts for lipid auto-oxidation versus acting as LOX-like catalysts under various conditions (pH, protein denaturation, redox potential, etc.) need to be made. The effect of other metallo-proteins on lipid oxidation and their mechanisms should also be investigated.

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6

Understanding and reducing oxidative flavour deterioration in foods

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Abstract: Lipid oxidation gives rise to undesirable off-flavours as well as formation of unhealthy compounds. Moreover, oxidative deterioration of proteins and pigments may affect texture and colours of foods. This chapter discusses the sensory impact of lipid oxidation on oils, mayonnaise, dairy products, spreads, cereals, meat products and fish and examples on how oxidative flavour deterioration can be reduced in some of these food products are given. In addition, the volatile oxidation products responsible for off-flavours are discussed.

Key words: sensory analysis, oxidative flavour deterioration, mayonnaise, dairy products, cereals, nuts, meat products, fish.

6.1 Introduction

Lipid oxidation is one of the most important factors limiting shelf life in many food products. Lipid oxidation gives rise to the formation of unhealthy compounds such as free radicals and reactive aldehydes. Moreover, lipid oxidation reduces the nutritional value of lipids. These consequences of lipid oxidation are not immediately detected by the consumer. However, lipid oxidation will also lead to significant changes in the sensory properties including odour, flavour, colour and texture, which are easily detected by the consumer and which may determine the shelf life of the product. In products containing both lipids and protein, such as meat and fish, lipid oxidation will go hand in hand with oxidation of the proteins and this may particularly have a large impact on the texture changes of the product as will be discussed later. This chapter will

discuss different methods to evaluate sensory changes due to oxidation and will subsequently summarize the sensory impact of lipid oxidation in a range of different products. The impact of lipid oxidation on odour and flavour changes will receive most attention, but examples on colour and texture changes due to lipid oxidation will also be given. For some food products, examples on how oxidative degradation of the foods products can be avoided by e.g. optimization of the packaging method will be given.

6.2 Evaluation of oxidative flavour deterioration of foods

Different methods to chemically evaluate lipid oxidation are described in Chapter 8 and this chapter will only discuss different methods to evaluate the impact of lipid oxidation on the sensory properties of foods. However, correlations between data from selected chemical and instrumental methods and sensory analysis will also be touched upon.

6.2.1 Sensory methods

Sensory evaluation of an oil or other food stuff is the most reliable method to determine whether the product is acceptable for consumption, at least when it concerns evaluation of off-flavour formation due to lipid oxidation. Therefore, the results obtained from chemical/instrumental methods should always be compared to sensory data at some stage. Sensory evaluations are time consuming and costly and sometimes the results obtained are not reproducible. Therefore, there is a need to develop methodologies that can replace sensory evaluations. Today, the current recommendation is to use sensitive chemical and instrumental methods to complement the sensory analyses (Frankel 2005). Sensory evaluations can be performed by different types of sensory panels: the expert panel, the analytical panel and the consumer panel (Meilgaard *et al.* 1991). The expert panel consists of a relatively low number of trained panel members (3–5) and is mainly used for routine control (Meilgaard *et al.* 1991).

The analytical panel consists of a larger number of assessors (at least five persons), which are trained to perform different types of tests such as difference tests (e.g., triangle tests) and quantitative descriptive tests such as flavour profiling. The latter method includes a rating of the different sensory attributes evaluated by the panel. This kind of test is often used in product development before consumer tests are performed. In lipid oxidation studies, the quantitative descriptive test can be used to correlate certain important off-flavours with chemical data as will be discussed later. Sensory attributes such as bland, green, cucumber, rancid and painty are used to describe the progress of oxidation in vegetable oils or products thereof, whereas attributes such as train oil, fishy and metallic are often used in foods rich in long chain omega-3 lipids. The intensity of a given attribute is ranked on a continuous intensity scale, e.g. from 0 to 15. It is generally not recommended to use the analytical panel to evaluate the

acceptability of the product as the assessors are trained and use their senses in a different way than the normal consumer. The consumer panel consists of a large number of untrained consumers, who are believed to be potential customers of a product. This type of test is used to evaluate the preference and acceptance of the consumers for a product or a range of products.

Traditionally, sensory data have been calculated by determining the overall mean scores for intensity or quality by dividing the total points by the number of panelists for each session. Subsequently, the significance of the overall mean scores have been calculated statistically by two-way analysis of variance (ANOVA). This methodology does not, however, take into account the differences in the sensory score levels between the different assessors that will always exist no matter how much the panel is trained. This is particularly a problem when sensory evaluations on the same product are carried out in a storage experiment over several weeks. In this case, it will inevitable happen that one or more assessors are not present at all the sensory evaluations. If these assessors are inclined to use, e.g. the higher end of the intensity scale, the absence of the assessors will significantly reduce the overall mean score for all samples on that day. Recent developments in multivariate data analytical techniques make it possible to project away these differences between the assessor score levels and thereby only the difference between samples are evaluated (Martens and Martens 1986). The projection is done by making a so-called ANOVA partial least squares regression using the assessor design variables as X-data and sensory data as the Y-data. The residuals from this model can then be used for further calculations.

6.2.2 Accelerated methods versus traditional storage experiments

Evaluation of the oxidative flavour deterioration of neat lipids can either be carried out at ambient temperatures or at elevated temperatures to accelerate oxidation. When it comes to emulsified foods such as dressing and mayonnaise it is often difficult to increase temperature much above 30 ºC as the emulsion may break at higher temperatures. For other food products such as raw meat and fish products it may be difficult to increase temperature to higher than 5 ºC as higher temperatures would lead to microbial spoilage. For neat oils and emulsions storage experiments at ambient temperatures may be accelerated by light or by the addition of metals such as iron or copper, whereas for fish muscle models oxidation is accelerated by addition of heme iron (Jacobsen *et al.* 2008). Storage experiments accelerated by light should only be carried out if the commercial product under real circumstances is stored under light. This is due to the fact that light induced oxidation will give rise to other types of secondary oxidation products and thereby other off-flavours than those produced by autoxidation.

6.2.3 Correlations between sensory data and data from chemical or instrumental measurements of lipid oxidation

Measurement of lipid oxidation can be carried out by a wide range of methods such as peroxide value (PV), anisidine value (AV), thiobarbituric acids reacting

substances (TBARS) as well as instrumental methods such as HPLC, GC-MS, NIR, FTIR and DSC. In lipid oxidation studies on meat products there seems to be a fairly good correlation between TBARS and sensory data, although in many cases no attempts have been made to statistically evaluate the correlations (Eckert *et al.* 1997; Murano *et al.* 1998; Winne and Dirinck 1997). However, newer data indicate that even in meat, TBARS may be an unreliable method (see further discussion in Section 6.3.6 below; Summo *et al.* 2010).

PV and AV are often used to evaluate the lipid oxidation in oils and the author's experience is that AV, at least to some extent, correlates with sensory data in oxidized oils. In contrast, findings from fish oil enriched spreads clearly showed that the correlation between sensory and PV and AV data was very poor (Jacobsen 1999b) (Fig. 6.1(a) and (b)). It is not surprising that PV did not correlate with sensory data since lipid hydroperoxides are not responsible for off-flavour formation. Moreover, since lipid hydroperoxides are broken down to secondary oxidation products during the later stages of oxidation a low PV does not necessarily indicate that the lipids are not oxidized. However, it was surprising that AV in some samples was high despite a high flavour acceptability score. This finding could indicate that the sensitivity and specificity of this method is too low to detect the changes in concentrations of volatiles responsible for the off-flavour formation. Therefore, AV cannot be recommended for measurement of lipid oxidation in complex food systems.

Good correlations between sensory data and data from headspace GC analysis on a range of different oxidized products such as boiled potatoes (Blanda *et al.* 2010), fish (Milo and Grosch 1995), fish oil enriched milk (Venkateshwarlu *et al.* 2004a), mayonnaise and dressing (Hartvigsen *et al.* 2000; Let *et al.* 2007) have been extensively reported in the literature and headspace GC analysis can therefore be recommended as one of the best methods to chemically assess

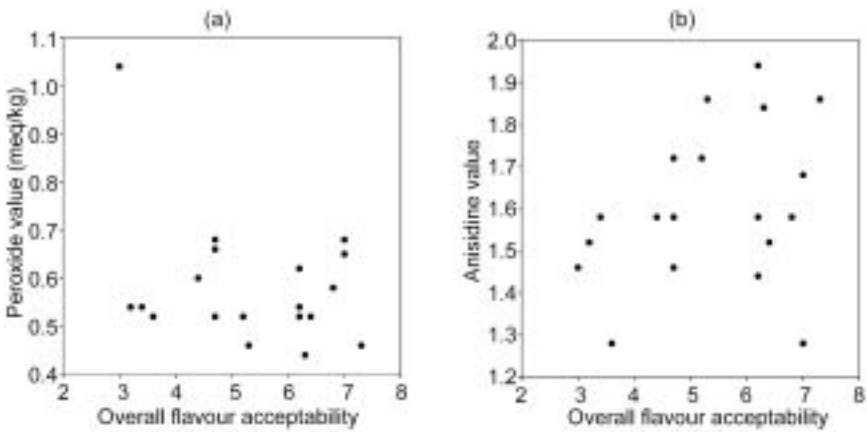

Fig. 6.1 (a) Peroxide values, and (b) Anisidine values versus overall flavour acceptability obtained from storage experiment with fish oil enriched spreads. Reprinted from Jacobsen, C. (1999b). Sensory impact of lipid oxidation in complex food systems. *Fett/Lipid 12*, 484–492 with permission from Wiley.

oxidative flavour deterioration. Different headspace methods are available for collection of the volatile oxidation products including static headspace, dynamic headspace and solid phase microextraction. It is beyond the scope of this chapter to discuss these methods in further details, but the reader may refer to Chapter 8 for more information.

6.3 Oxidation and food flavour

During the lipid oxidation process free radicals, lipid hyroperoxides and secondary oxidation products (aldehydes, ketones, alcohols, hydrocarbons, core aldehydes) are formed. Only the secondary oxidation products are responsible for the undesirable changes in the aroma and flavour properties of foods caused by lipid oxidation. Among these compounds the vinyl ketones and the *trans*, *cis*-alkadienals have the lowest flavour thresholds in oils (Table 6.1). In contrast, hydrocarbons (alkanes and alkenes) have the highest flavour thresholds (Frankel 2005).

As will be descussed in the following, lipid oxidation products can give rise to different sensory impressions in different food products. The term 'rancid' is often used to describe the off-odours and off-flavours caused by lipid oxidation. However, this term may also be used to describe off-flavours that develop through the hydrolytic liberation of short-chain fatty acids in dairy products (Jacobsen 1999b).

6.3.1 Oils

Lipid oxidation of neat oils will give rise to off-flavours ranging from nutty through green, grass cucumber to rancid and synthetic. For fish oils, the term train oil is frequently used. In cases where lipid oxidation is extremely advanced

Table 6.1 Flavour thresholds of compounds formed from oxidized oils

Compounds	Thresholds (mg/kg)
Hydrocarbons	90–2150
Substituted furans	2–27
Vinyl alcohols	0.5–3
1-alkenes	0.02–9
2-alkenals	0.04–2.5
Alkanals	0.04–1.0
Trans,trans-2,4-alkadienals	0.04–0.3
Isolated alkadienals	0.002–0.3
Isolated *cis*-alkenals	0.0003–0.1
Trans,cis-alkadienals	0.002–0.006
Vinyl ketones	0.00002–0.007

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Table 6.2 Odour threshold values of selected aldehydes produced by lipid oxidation

Aldehydes	Odour threshold in oil (ppm)		Description
	Nasal	Retronasal	
Hexanal	320	75	Tallowy, green, leafy
Heptanal	3200	50	Oily, fatty
Octanal	320	50	Oily, fatty, soapy
Nonanal	13500	260	Tallowy, soapy-fruity
Decanal	6700	850	Orange peel-like
2-t-pentenal	2300	600	Pungent, apple
2-t-hexenal	10000	400	Apple
3-c-hexenal	14	3	Green, leafy
2-t-heptenal	14000	400	Fatty, bitter almond
2-t-nonenal	900	65	Tallowy, cucumber
3-c-nonenal	250	35	Cucumber
2-t-decenal	33800	150	Tallowy, orange
2,4-t,c-heptadienal	4000	50	Frying odour, tallowy
2,4-t,t-heptadienal	10000	30	Fatty, oily
2,6-t,t-nonadienal	2500	460	Fatty, oily
2,6-t,c-nonadienal	4	1.5	Cucumber-like
2,4-t,c-decadienal	10	–	Frying odour
2,4-t,t-decadienal	180	40	Frying odour
2,4,7-t,t,c-decatrienal	–	24	Cut beans
4,5,2-t-epoxy-t-decenal	1.3	3	Metallic

Source: Belitz H.D. and Grosch W. (1999). *Food Chemistry*. Springer-Verlag, Heidelberg, Berlin, with permission.

the oil may develop a painty odour. A number of different volatile oxidation compounds have been suggested to contribute to these off-flavours. Table 6.2 shows some of these volatiles and the odour that have been ascribed to them.

Oxidized oil will contain a mixture of different volatiles and the relationship between the concentration of these volatiles and their sensory impact is poorly understood. To the author's knowledge only one model describing the quantitative relationship between volatiles and an important off-flavour in oxidized oils has been reported. This model describes the intensity of the fishy taste in fish oil as a function of the concentration of three volatiles, 2,6-nonadienal, 4-heptenal, and 3,6-nonadienal oil (MacFarlane *et al.* 2001).

6.3.2 Mayonnaise

Hartvigsen *et al.* (2000) identified and quantified the levels of volatile oxidation products in stored mayonnaise with or without fish oil by dynamic headspace GC-MS. Subsequently, they used GC-olfactometry to identify the most potent odorants, i.e. the compounds that most likely are responsible for the fishy off-flavour observed in the oxidized product. More than 140 volatiles were found in the fish oil enriched mayonnaise and slightly fewer volatiles were found in the

traditional mayonnaise made only with rapeseed oil. Among the 140 volatiles were 16 alcohols, 39 aldehydes, 7 furans, 20 ketones, 40 non-cyclic and 15 cyclic hydrocarbons and 11 miscellaneous compounds. Most of the identified compounds have previously been identified as lipid oxidation compounds in vegetable oils or fish oils. In a GC-olfactory analysis the odour characteristics ranged from sour, cheese-like, through glue, grassy, burnt, fatty to fishy, cucumber and deep-fried. Interestingly, (Z)-4-heptenal and (E,Z)-2,4-heptadienal were detected with distinct fishy notes. Multivariate analysis of the volatiles data from the different mayonnaises during storage suggested that the following volatiles could be responsible for the fishy off-flavour in oxidized fish oil enriched mayonnaise; 2-methyl-propanal, 1-penten-3-one, (Z)-3-hexenal, (Z)-4-heptenal, 1,5-octadien-3-one, and (E,Z)-2,6-nonadienal. Means to avoid oxidative flavour degradation in fish oil enriched mayonnaise are discussed in Chapter 5 of Volume 2.

On the basis of a comparison of the sensory impact of the same level of lipid oxidation in fish oil and fish oil enriched mayonnaise Jacobsen (1999a) previously discussed why the same volatile oxidation products may have different sensory impact in different food products. Firstly, it was shown that fish oil enriched mayonnaise developed fishy and rancid off-flavours much earlier than bulk oil mixtures of fish oil and rapeseed oil in the same ratio as that used in the fish oil enriched mayonnaise. This fast development of off-flavour was found even though chemical oxidation data did not indicate that the mayonnaise was more oxidized than the bulk oil mixtures (Jacobsen 1999a). Based on identification and quantification of volatiles in mayonnaise as well as an evaluation of the partitioning properties of selected volatiles in mayonnaise it was hypothesized that those oxidation products originating from EPA and DHA, which have a low oil-water partitioning coefficient will partition into the aqueous phase of the mayonnaise. Thereby, their volatility will increase and their flavour threshold will be reduced compared to in bulk oil mixtures. Therefore, these compounds may significantly affect the sensory perception of fish oil enriched mayonnaise even when present in low concentrations.

6.3.3 Dairy products and spreads

As mentioned earlier two types of rancidity exists, namely oxidative and hydrolytic rancidity and both reactions are important in non-pasteurized milk, where lipolytic enzymes will give rise to hydrolytic rancidity and oxidation of milk lipids will lead to oxidative rancidity. In milk and cream, flavour defects caused by lipid oxidation are usually referred to as ‘oxidized flavour’ and in butter they are referred to as ‘metallic’ or ‘tallowy’ (Frankel 2005). Oxidized flavours are very easily detected in liquid milk even at very low oxidation levels (PV < 1 meq/kg) (Frankel 2005). This is partly due to the fact that milk has a mild flavour, which cannot easily mask the off-flavour of the oxidation products. Importantly, exposure of milk or butterfat to sunlight produces off-flavours that have been described as burnt, light-activated or sunlight flavour (Frankel 2005).

This flavour is due to the formation of carbonyls and methional formed via photoxidative degradation of sulfur containing amino acids including methionine. Riboflavin, which is present in substantial amounts in milk, plays an important role as a photosensitizer in this process.

Dairy products will not only develop off-flavours when exposed to sunlight, but also exposure to light in the grocery stores will lead to oxidative flavour degradation if the product is not properly packaged. It is possible to protect dairy products against such flavour deterioration by excluding all kinds of light exposure, e.g. by using black or non-transparent packaging material/materials with light barrier.

Larsen *et al.* (2009) investigated photooxidation of sour cream packaged in cups with different light barrier properties. The sour cream was light exposed for 36 h with a standard fluorescent light tube simulating storage conditions in grocery stores. Three different cups were evaluated: a white cup, a cup with medium light barrier and a cup with high light barrier. As expected the cup with the high light barrier resulted in the best protection against photooxidation. A sensory panel could not distinguish between sour cream stored in the high light barrier cup and sour cream stored in the dark, whereas the intensities of off-odours/flavours (sunlight, rancid, acidic) were significantly higher in sour cream stored in white cups or in cups with medium light barrier. Hence, only the cup with the high light barrier protected the sour cream sufficiently. Fluorescence measurements of the sour cream showed that photosensitizers were intact in the sour cream stored in the high light barrier cup, whereas they were somewhat degraded in the sour creams stored in the white cup or in the medium light barrier cup.

In a recent study on sweet cream butter (non-cultured), changes in the sensory profile and volatiles profile during storage at 4 ºC and −20 ºC were followed (Lozano *et al.* 2007). After approximately 6 months at refrigerated temperature the development of a 'stale' flavour was reported by the sensory panel. This flavour increased in intensity in refrigerated butters between 6 and 12 months of storage and it could also be detected after 12 months of storage at −20 ºC in butters packaged in wax paper, but not in butters packaged in foil. Moreover, during storage positive descriptors for cooked/nutty and milkfat flavour decreased. During storage the development of volatile lipid oxidation products (E)-2-nonenal, 2-heptanone, (Z)-4-heptenal and (E,Z)-2,6-nonadienal was observed. Further, after 12 months of refrigerated storage the concentration of hexanal also began to increase. Indications of hydrolytic activity in the butters were also found. Thus, δ-octalactone and δ-decalactone increased during storage. These two compounds were suggested to be formed via hydrolysis of 4-/5-hydroxy fatty acids or via oxygen attack on fatty acids at positions 4 or 5 followed by hydrolysis.

Recent attempts have been made to improve the nutritional value of butter by mixing butterfats with lipids with a more healthy profile. Kristensen *et al.* (2006) evaluated the oxidative stability and sensory properties of butter blends in which 40% of the fat was replaced by either rapeseed oil, sunflower oil or

diacylglycerols (DAG) produced from sunflower oil or rapeseed oil. The interest in DAG is due to the finding that DAG has been able to reduce body fat mass in animals (Kristensen *et al.* 2006). Moreover, a DAG cooking oil has won large market shares in Japan (Kristensen *et al.* 2006). Butter blends with sunflower oil or DAG made from sunflower oil had a lower oxidative stability and more rancid odour and flavour than butter blends made with rapeseed oil or DAG produced from rapeseed oil. This was expected since sunflower oil has a higher content of polyunsaturated fatty acids than rapeseed oil. For the sunflower oil butter blends the DAG version tended to be more rancid throughout the storage period, whereas no difference was observed between the butter blends made with rapeseed oil or DAG produced from rapeseed oil (Fig. 6.2). Interestingly, the biggest difference between the butter blends made with DAG or the traditional oils was that the DAG butter blends had a significantly lower intensity of butter and salty flavours (Fig. 6.2). This was despite the fact that all four butter blends had the same salt content. However, the difference in butter aroma between the butter blends with DAG and the traditional oils was much less pronounced. Dynamic headspace analysis of the volatiles in the butter blend showed significantly higher levels of 2,3-butanedione (diacetyl) in the blends with the traditional oils than in the blends with DAG. 2,3-butanedione is one of the most important compounds in butter flavour (Lund and Hølmer 2001). Moreover, butyric acid has been suggested to play a very important role for the butter odour, but this compound was found in similar concentrations in all four butter blends. Hence, differences and similarities in the intensities of butter odour and flavour in the four butter blends could at least partly be explained by their contents of these two volatile compounds. The physical structure of the butter blends was evaluated by droplet size measurements and microscopy. Both analyses clearly showed that the DAG butter blends had smaller droplet sizes indicating a high physical stability of the droplets. A good flavour release is obtained when the water-in-oil emulsion is broken down easily in the mouth. Hence, the perception of saltiness is influenced by the stability of the emulsion. The smaller droplet size and higher stability of the DAG butter blends was therefore suggested to be responsible for their less intense salty flavor (Kristensen *et al.* 2006). The small droplet size of the DAG butter blends was suggested to be due to the emulsifying properties of DAG and also to the low levels of monoacylglycerides present in the DAG oil. It is interesting to note that physical structure of the butter blends mainly seemed to influence the saltiness and to a lesser extent rancid aroma and flavour. At least the physical structure was not able to prevent the perception of the DAG sunflower oil blend as being the most rancid.

Several studies have also been carried out on the enrichment of milk with omega-3 fatty acids (Let *et al.* 2003; 2005; Venkateshwarlu *et al.* 2004b). Lipid oxidation gave not only rise to an intense fishy off-flavour, but also to a strong metallic off-flavour. These off-flavours were found even when fish oil of a relatively good quality was used. Thus, even when a fish oil with a PV of 1 meq/kg was used a sensory panel could clearly distinguish the fish oil enriched milk

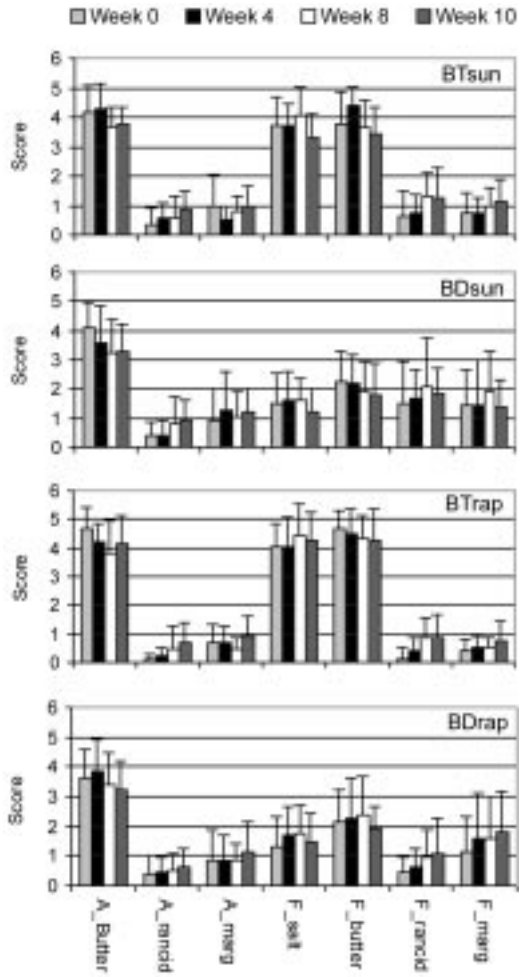

Fig. 6.2 Sensory data for butter blends after 0, 4, 8, and 10 weeks of storage at 5 ºC in the dark. A = aroma and F = flavour. Butter blends were produced from sunflower oil (BTSun), DAG oil made from sunflower oil (BDSun), rapeseed oil (BTRap), or DAG oil made from rapeseed oil (BDRap) Error bars represent SD. Reprinted from Kristensen, J.B., Nielsen, N.S., Jacobsen, C., Mu, H. (2006) Oxidative stability of diacylglycerol oil and butter blends containing diacylglycerols. *Eur J Lipid Sci Technol.* 108, 336–350 with permission from Wiley.

from milk without fish oil, but if a fish oil with a PV of 0.1 meq/kg was used, the fish oil enriched milk did not have a significantly higher fishy off-flavour than milk without fish oil (Let *et al.* 2005).

Venkateshwarlu *et al.* (2004a) used dynamic headspace GC-MS to analyze the volatiles profiles in conventional milk and in fish oil enriched milk after storage at 2 ºC for 14 days. A total of 16 volatiles were identified in the pure milk, whereas 62 volatiles were identified in the fish oil enriched milk (Fig. 6.3).

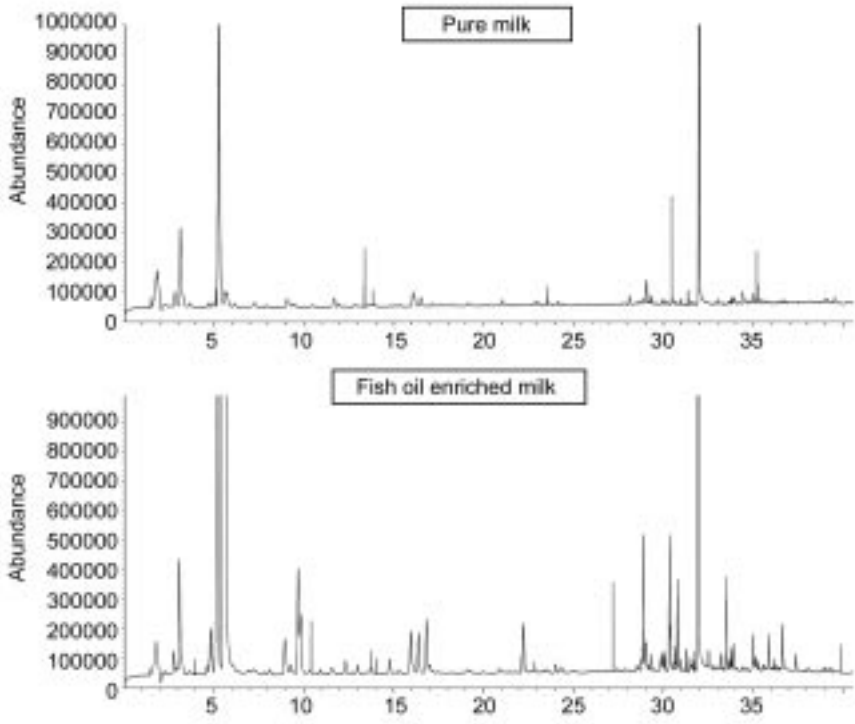

Fig. 6.3 Total ion chromatograms of volatiles collected from pure milk and fish oil enriched milk both stored at 2 ºC for 14 days. Reprinted from Venkateshwarlu, G., Let, M.B., Meyer, A.S., Jacobsen, C. (2004a) Chemical and olfactometric characterization of volatile flavor compounds in a fish oil enriched milk emulsion. *J. Agric. Food Chem.* 52, 311–317 with permission from ACS Publications.

Most of the compounds isolated from pure milk belonged to the classes of ketones, especially methyl ketones, straight chain aldehydes and n-alcohols. The methyl ketones are a characteristic feature of volatile profile of pasteurized milk (Venkateshwarlu *et al.* 2004a). The following ketones were found 2-propanone, 2-butanone, 2-heptanone and 2-nonanone. The volatiles identified in fish oil enriched milk were mostly carbonyl compounds encompassing alkenals, alkadienals, alkatrienals and vinyl ketones. Venkateshwarlu *et al.* (2004a) concluded that despite their potency, none of the separated individual volatiles gave rise to the same fishy or metallic odour that was observed in oxidized fish oil enriched milk. Subsequently, a sensory panel evaluated the sensory properties of milk to which different combinations and concentrations of these 4 volatiles had been added (Venkateshwarlu *et al.* 2004b). Mathematical modelling of the obtained sensory responses was then performed and it was concluded that the combination of (E,Z)-2,6-nonadienal and 1-penten-3-one could be a useful marker for fishy and metallic off-flavours in fish oil and fish oil enriched foods.

6.3.4 Cereals

Cereals are in general more stable than other foods because they are low in total fat (2–5%) and contain relatively high levels of natural tocopherols (20–50 ppm α-, β-, and γ-tocopherol (Frankel 2005). Furthermore, antioxidant compounds formed during baking by browning or the Maillard reaction contribute to the good oxidative stability of cereals (Frankel 2005). Cereal products from whole grain or those containing bran or germ components are more easily oxidized owing to the fact that they contain more lipoxygenases and more unsaturated lipids. Moreover, lipase activity is found mainly in the bran components and can result in the formation of free fatty acids, i.e. hydrolytic rancidity. Lipid oxidation is particularly a problem in extruded cereals such as corn flakes. Important factors affecting oxidative flavour deterioration in this product are: low moisture, high surface/volume ratio due to expansion and flaking, high iron levels due to wearing of the extruder screw and barrel (Paradiso *et al.* 2009). The following descriptors have been used to describe the sensory changes due to lipid oxidation in corn flakes: rancid odour/flavour, pungent odour and stale odour. In addition, in the fresh product odour and flavour were evaluated together with the overall acceptability (Paradiso *et al.* 2009). In this study, volatiles were analysed by SPME-GC-MS after 1 year of storage and the following groups of volatiles were found: 2 hydrocarbones, 6 alcohols, 14 aldehydes, 2 free fatty acids, 4 ketones, 2 furans and 4 lactones. Multivariate data analysis on the sensory and volatiles data showed that no specific relationships between individual compounds and particular off-flavours appeared. However, a close relationship between undesired sensory properties and the pattern of volatile oxidation products was found. The following compounds seemed to contribute to the formation of the undesirable off-flavours: pentanoic and hexanoic acid, γ- and δ-hexalactone, 2-decenal, 1-heptanol, 1-pentanol, 1-hexanol, 1-octanol, heptanal, octanal, nonanal, 1-octen-3-ol, (E)-2-octenal, (E-)-2-nonenal and (E,E)-2,4-nonadienal. Paradiso *et al.* (2009) also investigated the effect of different antioxidants (rosemary extract, tocopherol alone, or in combination with ascorbic acid) on the formation of volatile oxidation products and off-flavours. They found that tocopherol conferred better antioxidative activity than rosemary and that addition of ascorbic acid to tocopherol further improved the effect of tocopherol in a synergistic manner.

6.3.5 Nuts

Nuts are a rich source of unsaturated fatty acids and although most nuts also contain high levels of antioxidants they are highly susceptible to lipid oxidation, which has been reported to result in oxidized and card board like flavours (Olmedo *et al.* 2009). A recent study showed that the oxidative flavour deterioration in fried-salted peanuts could be reduced by flavouring with oregano essential oil due to its antioxidative properties.

Nuts are susceptible to infestation by molds, insects and larvae. Chemical fumigation has been banned or being phased out due to its negative impact on

human health and the environment. γ-irradiation has been suggested as an alternative technology, but irradiation leads to formation of free radicals which promote lipid oxidation (Mexis and Kontominas 2009a; 2009b). Two recent studies by Mexis and Kontominas (2009a; 2009b) on cashew nuts and walnuts, respectively, showed that if the irradiation dose is kept below 3.0 kGy, taste and odour scores indicated that the nuts were of acceptable quality, i.e. that irradiation did not lead to unacceptable oxidative flavour deterioration.

6.3.6 Meat products

Many meat products especially sausages have a high fat content and the lipid fraction significantly influences the sensory properties of these products. Hence, the lipid fraction affects structure, juiciness, colour, odour and flavour. Moreover, oxidative and hydrolytic degradation of the lipid fraction can lead to significant changes in the flavour profile as will be illustrated in the following. This may particularly be a problem in sausages and other minced products where the structure of the meat has been disrupted leading to an increased lipid surface that can be exposed to oxidation.

Both primary and secondary lipid oxidation products will interact with proteins and amino acids and this interaction will have significant impact on the flavour of meat products during processing, cooking and storage. For example, hydroperoxide radicals will react with sulfur, amine and amino acids and secondary oxidation products will react with thiols from cysteine. Moreover, Schiff bases formed from the interactions between aldehydes and proteins are unstable and produce a wide range of volatile compounds that affect the sensory profile of meat products (Frankel 2005). Table 6.3 shows some of the volatile compounds that have been identified in beef fat as well as their flavour threshold. From the table it is evident that (Z)-4-heptenal and (E,E)-2,6-nonadienal were the most flavour significant compounds (Frankel 2005).

In meat products, the term warmed over flavour (WOF) is often used to describe oxidized flavours in precooked, stored and reheated meat. WOF is mainly caused by oxidation of lipids, particularly the formation of hexanal, 1-octen-3-one, (E)- and (Z)-2-octenal, (Z)-2-nonenal, (E,E)-2,4-nonadienal and trans-4,5-epoxy-(E)-2-decenal (Konopka and Grosch 1991). WOF becomes apparent within a short period of time whereas common rancidity develops during lengthy storage (Sarraga *et al.* 2006; Byrne *et al.* 1999). WOF is mainly believed to be the result of oxidation of membrane phospholipids, a process triggered by hemoproteins and other iron species during cooking (Gray and Pearson 1987). However, degradation of proteins and some minor components such as Maillard-derived aroma volatiles associated with the aroma of freshly cooked meat may also contribute to formation of WOF. Degradation of these proteins will result in the disappearance of desirable meat flavour notes present in freshly cooked meat (Byrne *et al.* 1999; Spanier *et al.* 1988). The term rancid as well as a range of other descriptors are therefore also being used to describe changes in the sensory profile due to oxidative degradation as will be illustrated in the following.

Table 6.3 Volatile compounds (carbonyls) from beef-fat

Carbonyls (in order of concentration)	Flavour threshold (ppm)	Flavour significance
2-decenal	5.5	12
2-undecenal	4.2	11
2-nonenal	0.1	5
2-octenal	1.0	10
Hexanal	0.15	6
2-heptenal	0.63	9
Heptanal	0.042	3
Nonanal	0.32	8
Octanal	0.068	4
2,4-decadienal	0.28	7
Cis-4-heptenal	0.001	1
Trans-4-heptenal	0.32	8
Trans,trans-2,6-nonadienal	0.001	1
Trans,cis-2,6-nonadienal	0.002	2

Source: Frankel, E.N. 1984. Recent advances in the chemistry of rancidity of fats. In: A.J. Bailey (ed.) *Recent advances in the chemistry of meat.* The Royal Society of Chemistry, London, pp. 87–118 with permission.

Summo *et al.* (2010) investigated oxidative and hydrolytic changes in vacuum-packed ripened sausages during long term storage (5 months) using both sensory analysis and HPSEC analysis of TAG oligopolymers, oxidized TAG, DAG as well as analysis of free fatty acids and TBARS. They used the following descriptors to describe defects occurring during storage due to lipid oxidation and hydrolysis: rancid smell/flavour, pungent smell/flavour and acid taste, whereas spiciness was used as a 'positive' descriptor. Moreover, the panel also evaluated the overall acceptability of the product. Despite the fact that the sausages were vacuum packed the sensory panel was clearly able to pick up undesirable changes in the flavour profile due to lipid oxidation and hydrolysis. Thus, acid flavour increased significantly throughout the storage, whereas pungent smell/flavour and rancid smell/flavour increased significantly up to 3 months whereafter they decreased. Rancid smell/flavour and acid taste correlated well with HPSEC determination of oxidized TAG and determination of free fatty acid, respectively. In contrast, TBARS neither increased significantly during storage nor did it correlate to sensory data. The authors therefore concluded that TBARS was not a reliable method for description of oxidation and hydrolysis products during storage (Summo *et al.* 2010). This is most likely due to the fact that the method underestimates malonaldehyde, which is the main compound determined by the TBARS test, due to its reactions with amino acids, sugars and nitrites.

In a recent study by Clausen *et al.* (2009) the eating quality of beef was investigated in response to different types of modified atmosphere packaging (MAP). MAP is increasingly being used by the food industry as it offers the opportunity to extend the shelf life of the product. An important parameter is the

desirable red colour of fresh meat. To maintain the red colour, O_2 is applied. However, the composition of the gas mixture (ratio between O_2, CO_2 and N_2) will significantly affect the rate of oxidative flavour degradation. In the study by Clausen *et al.* (2009) the following parameters were used to evaluate the eating quality: tenderness, juiciness, meat flavour and warmed over flavour (WOF). In agreement with other studies, Clausen *et al.* (2009) found that packaging in high O_2 MAP decreased the tenderness. Their data suggested that the high O_2 levels lead to increased oxidative modification of proteins that at least to some extent could be responsible for the decreased tenderness. They also suggested that the decreased tenderness was partly due to decreased proteolysis, which may be caused by inactivation of proteolytic enzymes in an oxidative environment. Packaging in high O_2 MAP resulted in increased WOF and TBARS levels. Hence, in contrast to the studies by Clausen *et al.* (2009) and Summo *et al.* (2010) a good correlation between TBARS and rancidity was found in this study. On the basis of this study, Clausen *et al.* (2009) concluded that packaging in O_2 concentrations compromises several aspects of meat eating quality and cannot be recommended to the meat industry or for retail distribution of meat.

Sarraga *et al.* (2006) investigated whether addition of antioxidants (α-tocopheryl acetate and β-carotene) in the diet of turkey could improve the oxidative status and sensory properties of turkey breast and leg meat. A sensory panel evaluated the rancid flavour and WOF as well as juiciness, hardness, pastiness, stringiness and tooth adhesion on meat that had been cooked in a 80 ºC water bath, stored for 1 day and reheated at 65 ºC in an electric oven. The rancid flavour was described as the flavour perception similar to that of an old olive oil odour, and WOF as the oxidized flavour of reheated meat. Only small effects of the antioxidant addition to the diet were observed. Hence, no significant effects of the diet were observed on the rancid flavour, WOF nor on any of the texture parameters in the breast meat. However, the pastiness (pasty feeling inside the mouth) and stringiness (perception of long and parallel coarse particles in the meat during chewing) were significantly affected in leg muscles. The stringiness was lower in turkeys fed with antioxidant-supplemented diet, whereas pastiness was higher. These findings were suggested to be due to the ability of vitamin E supplementation to increase cathepsin B and L activities. Hence, a higher protelytic activity could have produced greater pastiness as a result of the protein break down and lower stringiness caused by the myofibrillar structure breakdown.

Recently, Tikk *et al.* (2008) monitored WOF development in cooked, cold stored (4ºC) and re-heated meatballs from pork muscles (*M. longissimus (LD)* and *M. Semimembranosus (SM)*) using a gas sensory system (electronic nose), headspace GC-MS, TBARS and sensory analysis. The meatballs were prepared from pig fed either palm oil or rapeseed oil. The following attributes were used to evaluate the meat balls: roasted meat odour and taste, boiled meat odour and taste, piggy odour and taste, sweet odur, sourish odour and taste, warmed-over odour and taste, metal taste, bitter taste, rancid taste, sourish after-taste, metallic

after-taste, warmed-over after-taste and juiciness after 3–4 chews. The aim of the study was to substantiate the potential of electronic noses as future quality control systems in the meat industry, i.e. to investigate whether electronic nose can replace sensory analysis, TBARS and/or headspace GC-MS methods. The authors concluded that significant positive correlations were found between the gas sensor signals and the WOF associated sensory attributes and the levels of secondary lipid oxidation products and that this confirms that the electronic nose can be a valuable quality control tool in the meat industry. They also found that WOF-associated attributes correlated to the development of TBARS, hexanal, pentanal, pentanol and nonanal.

6.3.7 Fish

The most important flavour change during frozen storage of fatty fish such as salmon is the formation of train oil, bitterness and metal taste (Refsgaard *et al.* 1998). Likewise, Milo and Grosch (1995) observed the development of a train oil flavour during prolonged frozen storage in a lean fish species such as cod. On the basis of GC-olfactometry, Milo and Grosch (1995) suggested that the increased concentration of 1-octen-3-one, (Z)-1,5-octadien-3-one, hexanal, (Z)-3-hexenal, (Z)-4-heptenal, (Z,Z)-2,6-nonadienal and (E,Z)-2,6-nonadienal observed during storage most likely were important contributors to the train oil odour in the stored cod sample. In contrast, Refsgaard *et al.* (1998) suggested that the formation of volatile oxidation products was not the cause of the most pronounced sensory changes found during frozen storage of salmon. Rather, they proposed that compounds of low volatility contributed to the increased intensity of train oil taste, bitterness and metal taste. Subsequently they hypothesized that the lipid hydrolysis that occurred parallel to lipid oxidation and which will give rise to free fatty acid formation contributed significantly to the sensory deterioration of salmon during frozen storage (Refsgaard *et al.* 2000). This hypothesis was corroborated by data showing that addition of each of the unsaturated fatty acids: palmitoleic acid (16:1, *n*-7), linoleic acid (C18:2, *n*-6), eicosapentaenoic acid (EPA; C20:5, *n*-3) and docosahexaenoic acid (DHA; C22:6, *n*-3) to fresh minced salmon changed the sensory perception and increased the intensity of train oil taste, bitterness, and metal taste. The added level of each fatty acid (1 mg/g salmon meat) was equivalent to the concentration of the fatty acids determined in salmon stored as fillet at −10 ºC for 6 months. The effect of addition of the fatty acids on the intensity of train oil taste, bitterness and metal taste was in the order: DHA > palmitoleic acid > linoleic acid > EPA. Taken together these findings suggest that the formation of unpleasant train oil, bitter and metallic off-flavours may not be solely ascribed to lipid oxidation, but may be a due to combination of lipid oxidation and lipid hydrolysis.

In a recent study on frozen stored rainbow trout, formation of volatile oxidation products and free fatty acids increased in parallel to the formation of rancid off-flavours (Baron *et al.* 2009). Moreover, an increased grainy, firm and

fibrous texture was observed in the rainbow trout, which were most rancid. These changes in texture were most likely due to protein oxidation. The impact of protein oxidation on myofibrillar protein functionality and on muscle food quality has recently received more attention (Martinaud *et al.* 1997; Saeed *et al.* 1999), and protein oxidation has been shown to affect protein solubility, decrease gel elasticity, and affect water distribution in muscle foods (Ooizumi and Xiong 2004; Bertram *et al.* 2007; Rowe *et al.* 2004) and this may have severe impact on fish texture.

Apart from affecting odour and flavour, lipid oxidation may also affect colour. Thus, autoxidation of heme iron may decrease the red colour of fish meat (Sannaveerappa *et al.* 2007), whereas lipid oxidation of the lipids will lead to an increase in its yellow colour.

6.4 Conclusions

As illustrated in this chapter, oxidative deterioration of sensory properties is a major problem in many food products, even in low-fat products, so precautions should therefore be taken to avoid lipid oxidation in all steps from catch/slaughter/harvesting of raw materials through processing, packaging, storage and transportation to the consumer.

Lipid oxidation will lead to the formation of many different oxidation products that will impart different off-odours and off-flavours in different food products. The term rancid is often used to describe oxidative flavor deterioration, but in some products other terms such as train oil, metallic and WOF are equally important. Aldehydes such as hexanal, heptadienal, nonadienal and ketones like 1-penten-3-one, 1-octen-3-one and 1,5-octadien-3-one seem to contribute to oxidative flavour deterioration in many different products. More research is, however, needed to fully understand the quantitative relationship between off-flavour formation and the concentrations of specific volatile oxidation compounds.

In muscle foods lipid oxidation often go hand in hand with oxidation of proteins and pigments and this leads to texture and colour changes, which in some cases may be prevented by the same means as lipid oxidation. However, in other cases other precautions than those which prevents lipid oxidation, are necessary to reduce oxidation of proteins and pigments.

6.5 Future trends

Owing to the economic losses caused by lipid oxidation, means to protect food products against oxidative flavour deterioration will continue to be an important area of research. It is expected that continued research in the following areas will help to reduce the oxidative flavour deterioration problem in the future:

- developments of new packaging technologies including increased use of MAP;
- development of new and more efficient antioxidants based on natural ingredients including modification of antioxidant compounds by, e.g. enzymatic esterification; and
- development of better delivery systems for sensitive oils such omega-3 fatty acids. This may involve the use of new technologies such as nanotechnology.

6.6 Sources of further information and advice

http://www.aocs.org/member/division/lipidox/

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7

Health aspects of oxidized dietary fats

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Abstract: This chapter addresses health-related effects associated with the ingestion of oxidized fats. The chapter first reviews the knowledge about nutritional quality changes and formation of lipid peroxidation products in oxidized fats. The chapter then presents the current knowledge about effects of oxidized fats on blood lipid profile, fatty liver development, carnitine homeostasis, thyroid function and thyroid hormones, endogenous and exogenous antioxidant defense mechanisms, glucose tolerance and insulin sensitivity as well as inflammation.

Key words: oxidized fat; deep-fat frying; lipid peroxidation; PPARα.

7.1 Introduction

In recent years the contribution of oxidized fats to total energy intake has markedly increased in industrialized countries (Guthrie *et al.*, 2002). This is mainly due to the rising consumption of deep-fried products, which are very popular because of their desirable flavor, color, and crispy texture. For instance, in a Spanish cohort from the European Prospective Investigation into Cancer and Nutrition-Study, the percentage of energy intake from fried food was 26.4% (men) and 22.8% (women) in the highest quintile of consumption (Guallar-Castillón *et al.*, 2007). Deep-frying is a process of immersing food in hot oil, which acts as a heat transfer medium and contributes to the texture and flavor of the fried food. During deep-frying several chemical reactions occur within the oil resulting in the formation of a heterogeneous mixture of chemically distinct substances. In addition, deep-frying causes losses of nutritive and non-nutritive

compounds, thereby leading to alterations in the nutritional quality of the fats. Large quantities of the frying oil are absorbed into the fried food during deep-frying and therefore ingested during their consumption. For instance, the fat contents of potato crisps, doughnuts, and French fries are 35–40%, 20–25%, and 10–15% (Moreira *et al.*, 1999), respectively. Based on these amounts of heated fat in fried foods and the frequent consumption of such foods in our population (Guallar-Castillón *et al.*, 2007), the daily amount of heated fat consumed can be 50 grams and more.

Feeding experiments using rats, mice, and pigs revealed that ingestion of heated, oxidized fats, compared with fresh fats, provokes a wide array of biological effects in mammals. Although most of these effects of heated fats are considered to be detrimental to health, recent evidence suggests that heated fats also exert effects in the organism which might be beneficial to health. The present chapter summarizes the current knowledge about nutritional quality changes and formation of lipid peroxidation products in oxidized fats as well as health-related effects of oxidized fats.

7.2 Nutritional quality loss of dietary fats during oxidation

7.2.1 Loss of polyunsaturated fatty acids (PUFA)

The quantitatively most important components of dietary fats are triacylglycerols which consist of a single molecule of glycerol combined with three fatty acids on each of the hydroxy groups. It is well known that the fatty acids, in particular the PUFA, are prone to oxidation by a free radical chain mechanism. This process catalyzed by light, heat or free radicals is initiated by the abstraction of a hydrogen atom from the fatty acid molecules (initiation reaction) producing an alkyl radical ($R^{\bullet}$) which further reacts rapidly with molecular oxygen to produce a peroxyl radical ($ROO^{\bullet}$). The peroxyl radicals propagate the oxidation reaction by abstracting hydrogen from other fatty acids thereby producing hydroperoxide (ROOH) and other alkyl radicals (free radical chain reaction or propagation step). The susceptibility of a fatty acid to hydrogen abstraction is dependent on the dissociation energy of the carbon-hydrogen bond within the fatty acid molecule. In general, the dissociation energy of the carbon-hydrogen bond at bis-allylic methylene groups is lower than at allylic methylene groups (50 kcal/mole vs. 75 kcal/mole), whereas the dissociation energy of the carbon-hydrogen bond at methylene groups without adjacent double bonds is markedly higher (approximately 100 kcal/mole) (Min and Boff, 2002). Hence, the various strengths of the carbon-hydrogen bond of different fatty acids explain that PUFA (e.g., linoleic acid, linolenic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid) are more easily oxidized than monounsaturated fatty acids (oleic acid), and saturated fatty acids (palmitic acid, stearic acid) are less easily oxidized than unsaturated fatty acids. The relative rates of autoxidation of stearic acid, oleic acid, linoleic acid, and linolenic acid were shown to be 1, 11, 114, and 179. Within the group of PUFA, the

oxidizability increases with increasing degree of unsaturation. Cosgrove *et al.* (1987) demonstrated that the oxidizabilities of PUFA are linearly dependent on the number of bis-allylic positions present in the molecule; e.g., the measured oxidizability of linoleic acid, linolenic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid was found to increase about two-fold for each bis-allylic methylene group.

The increased susceptibility of unsaturated fatty to oxidation is also shown during heating of dietary fats. For instance, deep-fat frying experiments of potato chips in refined, bleached, and deodorized soybean oil and vanaspati (partially hydrogenated vegetable oil blend) at different frying temperatures (170, 180, and 190 ºC) revealed a decrease in iodine values (an indicator of the amount of unsaturated fatty acids within a fat) and a significant loss of PUFA during frying (Tyagi and Vasishtha, 1996). The iodine value of soybean oil and vanaspati decreased from their initial values of 129.8 and 74.7 to 96.2 and 59.6, respectively, after 70 h of frying. Moreover, these experiments clearly showed that the extent of PUFA loss is directly correlated with frying time and temperature: The longer the frying time and the higher the frying temperature the greater the loss of PUFA. The authors of that study (Tyagi and Vasishtha, 1996) observed the highest losses of PUFA in soybean oil with a 79% decrease in trienoic acids and a 60% decrease in dienoic acids.

Trace amounts of heavy metals, such as iron and copper, decreases the oxidative stability of fats even at low temperatures and have a marked accelerating effect on the rates of lipid oxidation, because transition metals promote hydrogen proton abstraction from fatty acids due to single electron transfer which occurs during the change of oxidation states (Velasco and Dobarganes, 2002). Hence, even trace amounts of heavy metals are considered to be detrimental for fat quality (Rossell, 1998).

7.2.2 Loss of amino acids

Nutrient losses during deep-fat frying also occur due to the reaction of lipid oxidation products with amines, amino acids, and proteins in fried foods (Pokorny, 1981; Gardner *et al.*, 1992). Aldehydes, dialdehydes, and epoxides derived from the decomposition of hydroperoxides can react with amino groups to produce imino *Schiff* bases, which themselves polymerize by aldol condensation to dimers and complex high-molecular weight brown macromolecules known as melanoidins (Frankel, 1998). This complex reaction known as *Maillard reaction* or as *non-enzymatic browning*, leads to browning of the fried food. Although this reaction significantly contributes to the development of the typical flavor of fried foods, it also causes significant losses of labile amino acids, such as lysine, histidine, tryptophane, cysteine, cystine, methionine and tyrosine, and therefore reduces the nutritive value of food proteins. Interestingly, the intensity of browning is correlated with the loss of these amino acids. Loss of amino acids can also occur due to oxidation of amino acids by lipid radical compounds. For instance, methionine is oxidized to sulfoxide, and cystine can

be oxidized to thiosulfinate. Besides changes in the flavor and nutritional quality of foods, the reaction of lipid oxidation products with proteins is responsible for changes in the rheological properties of foods. For instance, hydroperoxides can react with proteins to form protein radicals which cause polymerization of the peptide chain to form protein-protein crosslinks (Frankel, 1998).

In addition to the abovementioned reactions, a loss of amino acids occurs due to the reaction of carbonyl compounds such as acrolein formed during lipid oxidation with amino acids, in particular asparagine. This reaction leads to the formation of acrylamide, which not only decreases the nutritional value but also the safety of the fried foods. Acrylamide formation requires heating temperatures of above 100 ºC and the extent of acrylamide formation increases with increasing temperature (Becalski *et al.*, 2003; Kim *et al.*, 2004; Pedreschi *et al.*, 2005). Although acrolein and carbonyls formed during deep-fat frying are not the main precursors of acrylamide (Tareke *et al.*, 2002; Zhang *et al.*, 2005), acrylamide formation from the reaction of acrolein with asparagine during deep-fat frying has been clearly demonstrated (Yasahura *et al.*, 2003).

7.2.3 Loss of tocopherols

Besides PUFA and amino acids, antioxidants are typically reduced in the fats during deep-fat frying. The predominant antioxidants in virgin vegetable oils are tocopherols (Huang *et al.*, 1995; Wagner and Elmadfa, 2000; Warner, 2005). For instance, soybean oil contains about 1000 to 1500 mg/kg of tocopherols (Jung and Min, 1990). Tocopherols efficiently protect lipids, in particular membrane phospholipids, from oxidation by donating hydrogen from their phenolic group on the chromanol ring to peroxy radicals formed during the propagation step (Kamal-Eldin and Appelqvist, 1996; Nawar, 1996). This reaction occurs at a very high rate (Niki *et al.*, 1984; Choe and Min, 2005) and is markedly faster than the reaction of unsaturated lipids with lipid peroxy radicals (Niki *et al.*, 1984; Naumow and Vasil'ev, 2003). As a result of donating hydrogen from tocopherols to peroxy radicals, the tocopherols form resonance stabilized tocopherol radicals (called tocopheryl-semiquinone radicals), which do not propagate oxidation but combine with other lipid peroxy radicals in a termination reaction yielding non-radical tocopherol oxidation products, such as tocopherolquinone, tocopherol-hydroquinone, 4a,5-epoxy-tocopherolquinone, and 7,8-epoxy-tocopherolquinone (Mukai *et al.*, 1993; Verleyen *et al.*, 2001b; Liebler *et al.*, 1996; Faustman *et al.*, 1999; Verleyen *et al.*, 2001a). In addition, tocopherol radicals also dimerize with other tocopherol radicals to form dimers (tocopheroldiphenylether dimer, tocopherolbiphenyl dimer) and/or trimers (Kamal-Eldin and Appelqvist, 1996). Because tocopherol oxidation products such as tocoquinones are red colored, heat treatment of tocopherol-rich fats such as soybean oil can result in dark color formation. This can be prevented by removing a significant proportion of the tocopherols by either vacuum steam distillation or deodorization.

Owing to the formation of tocopherol oxidation products, which easily decompose (Verleyen *et al.*, 2001b), the tocopherol concentrations markedly

decline in vegetable oils under deep-frying conditions, as demonstrated in frying experiments with different fats (Miyagawa *et al.*, 1991; Marquez-Ruiz *et al.*, 1999). The rate of tocopherol decomposition during deep-fat frying depends amongst others on the type of fat and the tocopherol isomer examined: During oxidation at 100 ºC and during simulated frying at 180 ºC, tocopherols were shown to be more slowly depleted in PUFA-rich fats such as soybean oil, corn oil and safflower oil than in SFA-rich fats such as palm oil and coconut oil or partially hydrogenated fats (Frankel *et al.*, 1959; Yuki and Ishikawa, 1976). This effect has been explained by the more rapid decomposition of PUFA hydroperoxides before they react with tocopherols (Frankel *et al.*, 1959). The rate of decomposition of γ-tocopherol during deep-fat frying of potatoes in a mixture of soybean and rapeseed oil at 180 ºC was faster than that of δ-tocopherol followed by α-tocopherol (Miyagawa *et al.*, 1991).

7.3 Loss of non-nutritive components during oxidation

Fats and oils contain varying amounts (1–2%) of non-glyceride components. To this fraction several non-nutritive components such as carotenoids, polyphenols, phytosterols, squalene, and chlorophylls belong. For most edible oils, carotenoids are largely removed and destroyed during conventional processing by deodorization at elevated temperatures. In other oils, such as virgin olive oil and corn oil, the carotenoids are desirable for optical reasons, and maintained by partial or full omission of the bleaching step or by deodorizing at lower temperatures. Squalene and phytosterols have little effects on lipid oxidation, but some types of phytosterols are claimed to have protective effects on oxidative stability of the oils during heating. Chlorophylls a and b and its degradation products pheophytins a and b are very active promoters of photosensitized oxidation, and are therefore removed as completely as possible in the bleaching step.

7.3.1 Loss of carotenoids

Other antioxidants that are contained in virgin vegetable oils in significant amounts (i.e., olive oil: 3.1 to 9.2 mg/kg; Gandul-Rojas and Minguez-Mosquera, 1996) are carotenoids, such as β-carotene, lutein, neoxanthin, violaxanthin, luteoxanthin, atheraxanthin, mutatoxanthin, and β-cryptoxanthin.

Frying experiments with red palm oil, which has a high native carotene content, revealed a complete loss of β-carotene during the frying process (Manorama and Rukimi, 1992). Carotenoids, in particular β-carotene, are very efficient singlet oxygen quenchers and prominent antioxidants in the light (Burton and Ingold, 1984). However, due to their highly conjugated double bonds, carotenoids add radicals, resulting in unstable radical adducts which initiate new lipid oxidation reactions. Hence, to be effective for inhibition of photosensitized oxidation of PUFA, β-carotene must be protected by an

antioxidant like vitamin E. In soybean oil containing natural tocopherols, β-carotene was demonstrated to be effective in protecting against light oxidation (Frankel, 1998). During frying, β-carotene was not effective in protecting oils from thermooxidation (Kajimoto *et al.*, 1992).

7.3.2 Loss of polyphenols

Besides tocopherols, polyphenols are further important phenolic compounds acting as antioxidants in vegetable oils. Polyphenols, like tocopherols, act as chain breakers by donating a hydrogen radical to alkylperoxyl radicals, which are formed during the propagation step of lipid oxidation, thereby forming a resonance stabilized radical. In contrast to tocopherols, polyphenols are only present in significant amounts in virgin oils because they are largely lost during oil refining. The most prominent representatives of polyphenols are tyrosol, hydroxytyrosol, simple phenolic acids and esterified derivatives of tyrosol and hydroxytyrosol (Tsimidou, 1998).

Similar to tocopherols, polyphenols rapidly decompose at frying temperatures (160–190 ºC) as demonstrated by Beltran-Maza *et al.* (1998) and Pellegrini *et al.* (2001). However, polyphenols are effective stabilizers of α-tocopherol during frying as shown in frying experiments where oil samples with a similar α-tocopherol content but increasing contents of polyphenols were investigated (Pellegrini *et al.*, 2001).

7.3.3 Loss of phytosterols

Phytosterols are the major constituents in the nonsaponifiable fraction of vegetable oils and are found mainly in either their free form or as steryl-fatty acid esters (Moreau *et al.*, 2002; Piironen *et al.*, 2000). The amount of phytosterols in vegetable oils varies greatly, from 70 mg/100 g in palm oil to over 1000 mg/100 g in evening primrose oil (Phillips *et al.* 2002; Verleyen *et al.*, 2002). Although more than 100 different phytosterol structures have been identified, the most predominant phytosterols in vegetable oils are campesterol, β-sitosterol, stigmasterol and D5-avenasterol (Moreau *et al.*, 2002; Piironen *et al.*, 2000; Phillips *et al.* 2002). Brassicasterol is also a predominant phytosterol in oils from plants in the Brassicaceae family, such as canola. The saturated stanols, sitostanol and campestanol are found in corn oil and oils from grains such as wheat and rice.

It is well known that phytosterols can undergo oxidation reactions when heated, leading to a variety of polar and nonpolar compounds (Dutta and Savage, 2002). Heating experiments revealed that the phytosterol loss is extreme when oils are heated without frying (Lampi *et al.*, 2000; Boskou and Morton, 1975). In contrast, the loss of phytosterols is markedly lower during deep-fat frying (Ghavami and Morton, 1984; Winkler *et al.*, 2007; Dutta and Appelqvist, 1996; Dutta and Appelqvist, 1997; Dutta, 1997). For instance, Winkler *et al.* (2007) demonstrated that the reduction of phytosterol content ranged between 4 and 6%

in most oils during continuous frying, and between 1 and 15% during batch (intermittent) frying. Although several heating studies have demonstrated that phytosterol loss is higher in fats with high amounts of saturated fatty acids (Soupas *et al.*, 2004; Ghavami and Morton, 1984), suggesting that unsaturated fatty acids are oxidized preferentially to sterols, no relation between fatty acid composition and phytosterol loss was found in the study from Winkler *et al.* (2007). From their findings the authors proposed that in vegetable oils low in saturated fatty acids, factors other than fatty acid composition of the medium are responsible for the increased phytosterol destruction during frying. One such factor that may increase phytosterol stability in vegetable oils has been suggested to be the concentration of tocopherols (Winkler *et al.*, 2007), which is generally high in PUFA-rich vegetable oils such as soybean, corn, and sunflower oils (Gunstone *et al.*, 1986). Winkler *et al.* (2007) proposed that in PUFA-rich oils tocopherols are preferentially oxidized over phytosterols, and thus phytosterols are less oxidized until most or all of the tocopherols in the oils are lost. Interestingly, phytosterols were also reported to have protective effects on the stability of oils during frying (Frankel, 1998). Phytosterols isolated from the unsaponifiable fraction of certain vegetable oils and oat oil were shown to retard oxidative polymerization of vegetable oils heated at elevated/frying temperature (180 ºC) (Frankel, 1998; Sims *et al.*, 1971; Gordon and Magos, 1983; White and Armstrong, 1986; Lampi *et al.*, 1999). This antioxidant and anti-polymerization activity has been attributed to the ethylidene group in the side-chain, present in D5- and D7-avenasterol and citrostadienol. The antioxidant activity of the ethylidene group is based on the formation of an allylic free radical at carbon atom 29 followed by isomerization to a relatively stable tertiary free radical at carbon atom 24. Other phytosterols without the ethylidene side-chain such as sitosterol, stigmasterol, and campesterol had either no effect or a slightly prooxidant effect (Sims *et al.*, 1971; Gordon and Magos, 1983; White and Armstrong, 1986; Lampi *et al.*, 1999).

7.3.4 Loss of squalene

A further non-nutritive component which is quite prominent in some edible oils is squalene. Squalene is a triterpene and an intermediate in the biosynthesis of sterols in plants and animals (Psomiadou and Tsimidou, 1999). The native concentrations found in vegetable oils vary greatly; whereas squalene is not detected in flaxseed oil, grape seed oil and soybean oil, relatively high concentrations are found in peanut oil (1.28 g/kg), pumpkin oil (3.53 g/kg), and olive oil (5.99 g/kg) (Amarowicz, 2009). Squalene has antioxidant activities (Malecka, 1991), although the antioxidative mechanism is only poorly understood and its antioxidant properties depend on the model system employed for the study (Psomiadou and Tsimidou, 1999; Finotti *et al.*, 2000; Manzi *et al.*, 1998; Dessi *et al.*, 2002).

During storage and processing, squalene exhibits great stability. In photo-oxidation studies, the squalene content showed only marginal losses of 4 to 12%

(Psomiadou and Tsimidou, 2002). The losses of squalene during a half year of storage of virgin olive oil in the dark at room temperature were in the range between 26 and 47% (Manzi *et al.*, 1998). Under accelerated storage conditions (60 °C), the loss of squalene in extra virgin olive oil was below 20% (Hrncirik and Fritsche, 2005). During roasting (20 min at 150 °C), the squalene loss in amaranth was about 12%. In addition, it has been reported that squalene is remarkably stable during domestic and commercial frying. During pan-frying of French fries in different edible oils, squalene concentration in the fat showed only marginal losses (Chiou *et al.*, 2009). Similarly, during domestic pan-frying and deep-frying of potatoes, the squalene content of the frying oils was only slightly reduced (Kalogeropoulos and Andrikopoulos, 2004), a finding that was also confirmed by Chiou *et al.* (2009). Because a considerable amount of squalene is absorbed by the fried foods, squalene becomes part of the diet (Kalogeropoulos and Andrikopoulos, 2004).

7.4 Formation of lipid peroxides in dietary fats during oxidation

During heat treatment of fats (e.g., deep-frying, baking, roasting) several chemical reactions occur such as hydrolysis, oxidation, and polymerization of the oil. Hydrolysis increases the amount of free fatty acids, mono- and diacylglycerols, and glycerols in oils (Choe and Min, 2007; Chung *et al.*, 2004). The thermal oxidation, in particular of unsaturated fatty acids, occurs at a greater rate than hydrolysis during deep-fat frying (Choe and Min, 2007). In the initial step of fatty acid oxidation, an alkyl radical is formed by removing hydrogen. These alkyl radicals further react rapidly with oxygen to produce a peroxy-radical, which abstracts hydrogen from other fatty acids and produces hydroperoxide (free radical chain reaction or propagation step). The hydroperoxides are relatively unstable and are decomposed to alkoxy radicals and hydroxy radicals by homolysis of the peroxide bond. Typical hydroxyl derivatives identified in heated fats rich in linoleic acid are 9-hydroxy- and 13-hydroxyoctadecadienoic acids (9-HODE, 13-HODE), which are formed from decomposition of the respective hydroperoxides (Toschi *et al.*, 1997).

Besides fatty acid radicals several low molecular volatile compounds such as aldehydes, ketones, carboxylic acids, and short-chain alkanes and alkenes are formed during oxidation. The alkyl radicals can also react with other alkyl radicals, alkoxy radicals, and peroxy radicals to form dimers, trimers, and polymers which are linked by –C–C–, –C–O–C–, and –C–O–O–C– bonds (Stevenson *et al.*, 1984; Choe and Min, 2005). Fatty acid radicals also can react with other radicals within one fatty acid leading to cyclic fatty acid monomers (CFAM). The formation of polymerization products in general depends on the type of fat (e.g., degree of unsaturation of fatty acids) and the heating temperature. As the heating temperature increases the amounts of polymers increase (Cuesta *et al.*, 1993; Sanchez-Muniz *et al.*, 1993; Takeoka *et al.*, 1997).

CFAM in specific are only formed to a significant extent at temperatures exceeding 200 ºC. Oils rich in linoleic acid or linolenic acid are more easily polymerized during deep-fat frying than oils rich in oleic acid (Takeoka *et al.*, 1997; Tompkins and Perkins, 2000; Bastida and Sanchez-Muniz, 2001). Similarly, the formation of CFAM increases as the amount of linoleic acid and linolenic acid increases (Tompkins and Perkins, 2000; Rojo and Perkins, 1987; Christopoulou and Perkins, 1989). Hence, heated vegetable oils contain significant amounts of CFAM accounting for up to 0.66% of total fatty acids (Sebedio and Grandgirard, 1989; Sebedio and Juaneda, 2007).

7.5 Bioavailability of lipid oxidation products

Numerous studies in both, humans and animals provided indication that lipid oxidation products are readily absorbed from the diet. This indication is based on the observation that lipid peroxidation products (TBARS, conjugated dienes) were found at increased levels in plasma, lipoproteins, tissues, and urine of humans and animals following ingestion of thermally treated fats or foods (Brown *et al.*, 1995; Naruszewicz *et al.*, 1987; Staprans *et al.*, 1994; 1996a; 1996b; 1999; Suomela *et al.*, 2004; 2005a; 2005b).

Strong evidence for the absorption of lipid oxidation products from the diet was provided from Wilson *et al.* (2002). These authors observed an increase in the plasma concentrations of [U-^{13}C]-labeled hydroxy and dihydroxy fatty acids in healthy women following consumption of 30 g fat containing 15 mg [U-^{13}C]-labeled hydroxy and dihydroxy triglycerides. Noteworthy, the authors of this study noticed differences in the absorption rates of monohydroxy and dihydroxy fatty acids. The increase in plasma concentrations of [U-^{13}C]-labeled hydroxy fatty acids was much higher that of labeled dihydroxy fatty acids, and the calculated absorption rate of labeled monhydroxy fatty acids was 21%, whereas that of labeled dihydroxy fatty acids was less than 4.5% (Wilson *et al.*, 2002). This indicates that substantial differences exist in the absorption rates of certain lipid oxidation products.

Whereas certain lipid oxidation products such as dihydroxy fatty acids are obviously only poorly absorbed in the intestine, other lipid oxidation are not absorbed at all. Namely, several studies observed that, in contrast to hydroxides, epoxides, ketones and aldehydes, hydroperoxides were not found in lipoproteins and tissues of animals fed oxidized fat (Suomela *et al.*, 2004, 2005a; 2005b). This is, however, probably not due to an inability of enterocytes to take up hydroperoxides – the uptake of 13-HPODE by cultured enterocytes has been demonstrated (Penumetcha *et al.*, 2000) – but rather due to an effective reduction or degradation of hydroperoxides in the gastrointestinal tract. It was demonstrated that hydroperoxides are efficiently reduced to hydroxides by reducing agents such as glutathione and detoxifying enzymes present in the mucus layer of small intestine (Glavind, 1970; Bergan and Draper, 1970; Kowalski *et al.*, 1990; Aw *et al.*, 1998; Kanazawa and Ashida, 1998; Mohr *et al.*,

1999; Samiec *et al.*, 2000) or decomposed to other secondary oxidation products such as epoxides or ketones (Kanazawa and Ashida, 1998).

Noteworthy, the absorption of lipid peroxidation products can be inhibited by other food components. According to a recent study (Gorlik *et al.*, 2008), polyphenols inhibit the absorption of malondialdehyde (MDA), which is considered to be one of the most abundant lipid peroxidation products in thermally treated foods (Kanner and Harel, 1985). It has been suggested that the inhibition of MDA absorption by polyphenols is due to adduct formation between polyphenols and MDA, which cannot be taken up by the enterocyte (Gorlik *et al.*, 2008).

7.6 Health-related effects of oxidized fats

7.6.1 Effects on blood lipid profile

Feeding experiments in rats have consistently demonstrated that oxidized fats cause a reduction in the concentrations of triacylglycerols and cholesterol in liver, plasma, and very low-density lipoproteins (VLDL) (Huang *et al.*, 1988; Eder and Kirchgessner, 1998; Eder *et al.*, 2003b; Sülzle *et al.*, 2004). The reason for the oxidized fat-induced lowering of triacylglycerol concentrations became clear only recently when it was shown that oxidized fats are strong activators of hepatic peroxisome proliferator-activated receptor α (PPARα) (Chao *et al.*, 2001) – an effect that could be confirmed in subsequent studies (Sülzle *et al.*, 2004; Chao *et al.*, 2004; 2005; Ringseis *et al.*, 2007a; 2007b). PPARα as well as other PPAR subtypes, PPARβ/δ and PPARγ, are transcription factors belonging to the superfamily of nuclear receptors that can be activated by fatty acids and their metabolic derivatives. They are implicated in the regulation of lipid and lipoprotein metabolism, glucose homeostasis, cellular differentiation, and inflammation (Desvergne and Wahli, 1999; Chinetti *et al.*, 2000; Duval *et al.*, 2002). Since PPARα is highly expressed in tissues with high rates of fatty acid catabolism such as liver, where it controls a comprehensive set of genes that regulate most aspects of lipid catabolism (cellular fatty acid uptake, intracellular fatty acid transport, mitochondrial fatty acid uptake, and fatty acid oxidation) (Mandard *et al.*, 2004), activation of PPARα results in decreased triacylglycerol concentrations in plasma, liver and VLDL. This suggests that activation of hepatic PPARα is probably largely responsible for the triacylglycerol lowering effect of oxidized fats.

The components of oxidized fats which are supposed to be responsible for PPARα activation are oxidized fatty acids such as hydroxy and hydroperoxy fatty acids (e.g., 9-HODE, 13-HODE, 13-HPODE) (König and Eder, 2006; Mishra *et al.*, 2004; Muga *et al.*, 2000; Delerive *et al.*, 2000). Fats heated at moderate temperatures of below 100 °C for a long period (several days or weeks) usually contain high amounts of these primary lipid peroxidation products, whereas fats heated at high temperatures have low levels of these compounds because hydroperoxides are relatively unstable and easily decompose at these

temperatures. In addition, the studies from Martin *et al.* (2000) and Bretillon *et al.* (2003) suggest that CFAM, which are also characteristic substances of oxidized fats, are activators of PPARα too. CFAM are only significantly formed from the unsaturated 18-carbon fatty acids (C18:1n-9, C18:2n-6, C18:3n-3) in vegetable oils heated at temperatures above 200 ºC (Sebedio and Grandgirard, 1989; Rojo and Perkins, 1987; Tompkins and Perkins, 2000; Christopoulou and Perkins, 1989).

The cholesterol-lowering effect of oxidized fat is likely due to inhibition of transcription of genes involved in cholesterol homeostasis as suggested from a recent study (Koch *et al.*, 2007b). Cholesterol homeostasis in mammalian cells is mainly regulated by sterol regulatory element-binding protein (SREBP)-2. This transcription factor preferentially activates genes involved in cellular cholesterol uptake (e.g., LDL receptor) and cholesterol synthesis (e.g., 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase) (McPherson and Gauthier, 2004; Horton, 2002). Koch *et al.* (2007b) demonstrated in rats that administration of oxidized fat inhibits activation of hepatic SREBP-2 and reduces hepatic transcript levels of LDL receptor and HMG-CoA reductase genes, both of which are important for cellular cholesterol uptake and synthesis, respectively. As a consequence of the reduced expression of these genes in the liver, the concentrations of cholesterol in liver and plasma are lowered, which explains the reduction of cholesterol concentrations in liver and plasma by oxidized fats.

Inhibition of SREBP-2 activation by oxidized fat is likely mediated by an up-regulation of insulin-induced gene (Insig)-1 in the liver which was observed in rats fed oxidized fat (Koch *et al.*, 2007b). Koch *et al.* (2007b) proposed that the effect on Insig-1 is due to activation of PPARα because it was recently shown that synthetic PPARα activators also cause an up-regulation of Insig-1 in the liver (König *et al.*, 2007). Insig are membrane proteins that reside in the endoplasmic reticulum and play a central role in the regulation of SREBP activation, because they prevent the translocation of SREBP from the endoplasmic reticulum to the Golgi, where proteolytic activation of SREBP and subsequent release of transcriptionally active forms of SREBP occur (Yang *et al.*, 2002; Yabe *et al.*, 2002). As a result, the synthesis of cholesterol declines in response to PPARα activation. Therefore, up-regulation of Insig by oxidized fat inhibits proteolytic activation of SREBP-2 in the Golgi, thereby lowering hepatic cholesterol synthesis and liver and plasma cholesterol concentrations. However, whether the stimulatory effect of oxidized fat on Insig-1 expression is indeed mediated by PPARα is uncertain, because lowering of mature SREBP-2 concentration and inhibition of cholesterol synthesis was also reported for PPARγ activators (Klopotek *et al.*, 2006), and hydroxy and hydroperoxy fatty acids from oxidized fats are able to activate PPARγ (Nagy *et al.*, 1998; Krey *et al.*, 1997). Since the oxidized fat in the study from Koch et al. (2007b) was prepared by heating the oil for a long period (25 days) at a moderate temperature (60 ºC), which typically leads to the formation of hydroxy and hydroperoxy fatty acids, it is not unlikely that the stimulatory effect of oxidized fat on Insig-1 expression is indeed mediated by activation of PPARγ.

Similar to SREBP-2 activation, administration of oxidized fat is also supposed to inhibit activation of SREBP-1c in the liver. In contrast to SREBP-2, this SREBP isoform is mainly responsible for the transcriptional activation of genes involved in fatty acid synthesis, the so-called lipogenic enzymes such as fatty acid synthase, acetyl CoA-carboxylase, glucose-6-phosphate dehydrogenase, malic enzyme, and ATP citrate lyase. In a previous study from Eder and Kirchgessner (1998), it could be shown that oxidized fats reduce the activities of these lipogenic enzymes in rats. These findings could be confirmed in a further study from Eder *et al.* (2003b). In addition, inhibition of other lipid-synthesizing enzymes, namely Δ9-desaturase and phosphatidate phosphohydrolase, could be demonstrated in the liver of rats administered CFAM (Martin *et al.*, 2001). The study from Eder *et al.* (2003b), moreover, showed that gene expression of lipogenic enzymes in the liver is reduced by oxidized fats. Due to the fundamental role of SREBP-1c in the transcriptional regulation of lipogenic enzymes, Eder *et al.* (2003b) proposed that reduction of gene transcription of lipogenic enzymes by oxidized fats involves inhibition of SREBP-1 maturation. This assumption is strongly supported by a recent study from König *et al.* (2009) demonstrating a link between activation of PPARα or PPARγ and reduction of nuclear SREBP-1 and fatty acid synthesis – an effect which probably involves up-regulation of Insig-1 and Insig-2a. Due to its PPAR activating properties it is therefore very likely that oxidized fat inhibits fatty acid synthesis through the activation of either PPARα or PPARγ or both of them, and subsequent reduction of SREBP-1 maturation. This indicates that the triacylglycerol-lowering effect of oxidized fats is not only mediated by stimulating fatty acid catabolism but also by inhibiting fatty acid synthesis.

7.6.2 Effects on fatty liver development

Chronic alcohol abuse is the most common reason for the development of fatty liver in humans. Fatty liver development is the result of both impaired fatty acid catabolism due to blockade of PPARα and increased lipogenesis in the liver due to activation of the lipogenic SREBP-1 pathway (You and Crabb, 2004; You *et al.*, 2002; Fischer *et al.*, 2003). A central role of the disturbed PPARα function in the pathogenesis of alcoholic fatty liver has been evidenced by the observation that administration of pharmacological PPARα agonists to ethanol-fed animals prevented fatty liver by reversing PPARα dysfunction, and stimulating the rate of fatty acid β-oxidation in the liver (Tsutsumi and Takase, 2001; Crabb *et al.*, 2004).

Based on the knowledge that oxidized fats markedly activate hepatic PPARα and PPARα-regulated genes (Chao *et al.*, 2001; Sülzle *et al.*, 2004) and reduce hepatic triacylglycerol concentrations (Sülzle *et al.*, 2004; Eder *et al.*, 2003b), a study has recently been conducted investigating whether dietary oxidized fat is useful in the prevention of alcoholic fatty liver (Ringseis *et al.*, 2007b). In this study, like in many other studies dealing with ethanol (You *et al.*, 2002; Tsutsumi and Takase, 2001; Tomita *et al.*, 2004; Hong *et al.*, 2004; Kharbanda

et al., 2005) the rat was used as an animal model. For oral administration of the ethanol, the Lieber-DeCarli liquid diet was used, which provides an excellent means for reproducing early lesions of alcoholic liver disease such as steatosis (Hall *et al.*, 2001). This study clearly demonstrated that triacylglycerol accumulation in response to ethanol-feeding is markedly reduced in rats by simultaneous administration of dietary oxidized fat when compared to a fresh fat. The observation that dietary oxidized fat resulted in similar hepatic triacylglycerol levels during ethanol-feeding as observed in rats fed fresh fat in the absence of ethanol suggests that dietary oxidized fat is indeed capable of preventing alcoholic fatty liver disease.

To elucidate the molecular mechanisms underlying these beneficial effects of oxidized fat, the transcript levels of PPARα and several genes involved in fatty acid catabolism, that are known to be reduced by ethanol administration, were determined in this study (Ringseis *et al.*, 2007b). These investigations showed that, in agreement with previous studies (Fischer *et al.*, 2003; Crabb *et al.*, 2004), the transcript levels of PPARα and its target genes were reduced by ethanol-feeding (Ringseis *et al.*, 2007b). However, administration of the oxidized fat, but not fresh fat, during ethanol-feeding resulted in the induction of PPARα target genes involved in fatty acid oxidation, which is indicative of PPARα activation, even in the presence of ethanol. Based on these results, it has been concluded that the oxidized fat-induced expression of PPARα target genes enhanced the capacity of the liver to oxidize fatty acids and, thus, counteracted the elevated levels of triacylglycerols and the diminished PPARα function during ethanol-feeding (Ringseis *et al.*, 2007b).

Similar observations as with oxidized fat have been made using synthetic PPARα agonists such as WY-14,643 and fibrates (Fischer *et al.*, 2003; Tsutsumi and Takase, 2001; Spritz and Lieber, 1966). Treatment with WY-14,643 restored the ability of the PPARα/RXR complex to bind its specific PPAR response element and induce transcript levels of many PPARα target genes resulting in a higher rate of fatty acid β-oxidation (Fischer *et al.*, 2003). Consequently, excessive accumulation of triacylglycerols in the liver during ethanol-feeding is prevented by these agents (Fischer *et al.*, 2003; Tsutsumi and Takase, 2001; Spritz and Lieber, 1966). Thus, the results of the study dealing with oxidized fat (Ringseis *et al.*, 2007b) suggest that dietary oxidized fat prevents fatty liver development by similar mechanisms as reported for synthetic PPARα activators.

7.6.3 Effects of oxidized fats on carnitine homeostasis

Many years ago it was shown that starvation or treatment of rats with PPARα agonist clofibrate increases the concentration of carnitine in the liver (McGarry *et al.*, 1975; Brass and Hoppel, 1978; Paul and Adibi, 1979). Carnitine is an essential metabolite, which has a number of indispensable functions in intermediary metabolism, like the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix where β-oxidation takes place

(McGarry and Brown, 1997; Brass, 2002; Steiber *et al.*, 2004). As both starvation and clofibrate treatment lead to an activation of PPARα, a transcription factor belonging to the nuclear hormone receptor superfamily (Schoonjans *et al.*, 1996), we have recently raised the hypothesis that activation of this nuclear receptor is responsible for the increased liver carnitine concentrations observed in those studies. Indeed, a study in our group revealed for the first time a marked, about 8-fold increase in the hepatic mRNA content of novel organic cation transporter 2 (OCTN2) in the liver of rats treated with the PPARα agonist clofibrate (Luci *et al.*, 2006b). OCTN2 is the physiologically most important carnitine transporter, operating for the reabsorption of carnitine from the urine as well as playing a major role in tissue distribution. Subsequent studies in PPARα-knockout mice further demonstrated that transcriptional up-regulation of hepatic OCTN2 by fasting or treatment with PPARα agonist WY-14,643 is dependent on PPARα (van Vlies *et al.*, 2007; Koch *et al.*, 2008). In addition, studies in rats and pigs showed that OCTN2 is induced by fasting or clofibrate also in several other tissues with abundant PPARα expression including kidney, skeletal muscle, heart, and small intestine (Ringseis *et al.*, 2007d; 2008a; 2008b; 2008c; Luci *et al.*, 2008).

Besides cellular carnitine uptake, evidence has been provided to suggest that carnitine biosynthesis is also regulated by PPARα; e.g., studies in PPARα-knockout mice and corresponding wild-type mice showed that treatment with the PPARα agonist WY-14,643 stimulates the transcription of enzymes involved in carnitine biosynthesis, trimethyllysine dioxygenase (TMLD), 4-N-trimethyl-aminobutyraldehyde dehydrogenase (TMABA-DH) and γ-butyrobetaine dioxygenase (BBD) only in the liver of wild-type (van Vlies *et al.*, 2007; Koch *et al.*, 2008). This indicates that transcriptional regulation of those genes is also mediated by PPARα. Other studies revealed that PPARα-knockout mice had markedly lower plasma and tissue levels of methionine and α-ketoglutarate, which serve as biosynthetic precursors and enzymatic cofactors (Vaz and Wanders, 2002), respectively, for carnitine synthesis, when compared to wild-type mice (Makowski *et al.*, 2009). Collectively, these observations in PPARα-null mice clearly show that genes encoding proteins involved in carnitine uptake and carnitine biosynthesis are transcriptionally regulated by PPARα.

Since oxidized fats are also capable of activating PPARα, a recent study has investigated the effect of oxidized fat on tissue carnitine concentrations and on expression of genes involved in carnitine homeostasis (Koch *et al.*, 2007a). The oxidized fat used in this study (Koch *et al.*, 2007a) was prepared by heating sunflower oil at a relatively low temperature (60 ºC) for a long period. Such fats usually contain relatively high concentrations of primary lipid peroxidation products including 9-HODE, 13-HODE and 13-HPODE, which are very potent PPARα agonists (König and Eder, 2006; Delerive *et al.*, 2000; Mishra *et al.*, 2004; Sülzle *et al.*, 2004). The study from Koch *et al.* (2007a) showed that treatment of rats with such an oxidized fat causes the same alterations as observed with the administration of the PPARα agonist clofibrate (Luci *et al.*, 2006b; Ringseis *et al.*, 2007d; 2008b), namely increased hepatic mRNA

concentrations of OCTN1 and OCTN2 and an increased hepatic carnitine concentration. Based on similar effects of pharmacological or starvation-induced activation of PPARα on carnitine homeostasis, the authors proposed that the up-regulation of OCTN in the liver by the oxidized fat was also mediated by PPARα activation (Koch *et al.*, 2007a). Considering that OCTN2 has a higher carnitine transport activity than OCTN1 (Tamai *et al.*, 2000), and that OCTN2 was more strongly induced by the oxidized fat than OCTN1, the authors suggested that the increased hepatic and the reduced plasma concentrations of carnitine in rats treated with oxidized fat were caused mainly by an increased uptake of carnitine from plasma into the liver via OCTN2.

Interestingly, it was also shown that dietary oxidized fat leads to an up-regulation of OCTN2 in the small intestine (Koch *et al.*, 2007a) – an effect which was also observed with PPARα activator clofibrate (Ringseis *et al.*, 2007d, 2008b). Since clofibrate was recently demonstrated to markedly increase the absorption rate of carnitine in small intestine (Ringseis *et al.*, 2008b), it is not unlikely that oxidized fats also increase intestinal carnitine absorption.

With respect to hepatic carnitine biosynthesis, the study from Koch *et al.* (2007a) revealed that oxidized fat does not up-regulate rate-limiting enzymes involved in carnitine biosynthesis (Vaz and Wanders, 2002). Nevertheless, these observations do not definitely exclude the possibility that hepatic carnitine biosynthesis was increased in rats treated with oxidized fat, because the liver has a high capacity to convert γ-butyrobetaine, which is a good substrate for OCTN (Tamai *et al.*, 1998; 2000), into carnitine (Vaz and Wanders, 2002). Thus, it is likely that an increased expression of OCTN2 by oxidized fat may have led to an increased uptake of γ-butyrobetaine from plasma into the liver which in turn may have stimulated synthesis of carnitine in the liver.

7.6.4 Effects on thyroid function and thyroid hormones

Only few studies have been carried out to investigate the effect of oxidized fat on thyroid function (Eder and Stangl, 2000; Eder *et al.*, 2002; Skufca *et al.*, 2003). Nevertheless, these studies performed with either rats or pigs consistently demonstrated that dietary oxidized fats increase the concentrations of free and total thyroxine (T_4) in plasma.

In rats, moreover, it was shown that dietary oxidized fat causes alterations in the morphology of the thyroid gland (Skufca *et al.*, 2003). Rats fed oxidized fat exhibited an increased height of thyroidal epithelial cells while the diameter of follicle lumen was reduced. This finding suggests that dietary oxidized fat stimulates the thyroid function because the function of the thyroid gland is well reflected by its morphology, particularly by the height of epithelial cells and the diameter of follicle lumen (Bidey and Cowin, 1995). Furthermore, the study from Skufca *et al.* (2003) revealed that dietary oxidized fat alters the expression of genes involved in thyroid hormone synthesis (Skufca *et al.*, 2003); i.e. gene expression of sodium iodide symporter (NIS), which plays a key role in the formation of thyroid hormones by mediating the uptake of iodide from blood

into the thyrocyte (Dai *et al.*, 1996), was reduced, whereas that of thyroid peroxidase (TPO) increased in the thyroid glands of these rats (Skufca *et al.*, 2003). TPO catalyzes the transformation of iodide to iodine and is involved in the iodination of thyroglobulin and the coupling reaction leading to thyroxine formation (Ekholm, 1981). Although NIS was down-regulated by oxidized fat, the increased gene expression of TPO is also indicative of a stimulatory effect of oxidized fat on thyroid function, because TPO but also NIS are regulated on the transcriptional level by thyrotropin (TSH), which is the most important regulator of thyroid hormone function (Schröder-van der Elst *et al.*, 2001; Riedel *et al.*, 2001; Damante *et al.*, 1989). However, because the concentration of TSH was not different between rats fed oxidized fat and those fed fresh fat (Skufca *et al.*, 2003), it is likely that the effect of the oxidized fat on the thyroid gland was not mediated by TSH. Since the study from Skufca *et al.* (2003) clearly showed that the alteration of thyroid function by oxidized fat is not due to an interaction with the metabolism of iodine, which is a key nutrient for the formation of thyroid hormones (Riesco *et al.*, 1976), it has been suggested that lipid peroxidation products of the diet directly influence the expression of genes involved in thyroid hormone synthesis.

In an attempt to identify the components responsible for the effects of oxidized fat on thyroid function and the mechanisms underlying the alterations in thyroid function by oxidized fat, a recent study investigated the effect of a primary lipid peroxidation product, namely 13-HPODE – the quantitatively most important primary oxidation product of linoleic acid (Niki *et al.*, 2005), and a characteristic compound of fats heated at moderate temperatures (< 100 ºC) – on the function of primary porcine thyrocytes (Luci *et al.*, 2006a). Unexpectedly, the study from Luci *et al.* (2006a) failed to demonstrate an important role of 13-HPODE as a mediator of the alterations of thyroid function observed with oxidized fats. For instance, Luci et al. (2006a) showed that incubation of porcine thyrocytes with 13-HPODE did not lead to alterations in gene expression of NIS and TPO or iodide uptake, even in non-physiologic high concentrations of 100 μM. In addition, gene expression of the TSH receptor was also not influenced by 13-HPODE suggesting that it also does not influence the effect of TSH on the function of porcine thyrocytes.

Luci *et al.* (2006a), moreover, showed that 13-HPODE leads to a down-regulation of dual oxidase 2 (DUOX2), which, like NIS and TPO, is involved in thyroid hormone synthesis by acting as a subunit of NADPH-oxidase (the major generator of H_2O_2 in thyrocytes (Moreno *et al.*, 2002)), and to a reduced release of hydrogen peroxide (Corvilain *et al.*, 1991). Although the authors of this study did not measure the activity of NADPH oxidase, they suggested that the reduced gene expression of DUOX2 might be associated with a reduced activity of this enzyme. The authors, moreover, suggested that the reduced concentrations of H_2O_2 in the cells and in the cell medium could be due to an increased activity of GPx observed in cells treated with 13-HPODE. GPx protects thyrocytes against a high intracellular concentration of H_2O_2, which for instance can lead to apoptosis (Demelash *et al.*, 2004). It has been postulated that GPx in thyrocytes

acts as a regulator of thyroid hormone biosynthesis by controlling the concentration of H_2O_2 available for thyroid hormone synthesis (Howie *et al.*, 1995). As the concentration of H_2O_2 in thyrocytes is the rate-limiting factor of thyroid hormone synthesis (Corvilain *et al.*, 1991), Luci *et al.* (2006a) suggested that high concentrations of 13-HPODE could have led to a reduced formation of thyroid hormones. Since it has been shown that generation of ROS inhibit the formation of thyroid hormones in cultured thyroid cells (Sugawara *et al.*, 2002), the authors pointed out the possibility that the effects observed on DUOX2 expression and release of H_2O_2 by 13-HPODE were due, at least in part, to ROS produced or to lipid oxidation products formed during incubation in the cell. Furthermore, the authors pointed out to the possibility that a reduction of the release of H_2O_2 could result in a reduced formation of thyroid hormones in thyrocytes.

It has also been noted (Eder and Stangl, 2000) that the hormonal pattern observed in pigs fed oxidized fat (increased concentrations of total and free T_4, and an increased ratio of T_4 to T_3) is similar to that observed in selenium-deficient rats (Ruz *et al.*, 1999). Selenium is an integral part of type I, 5-deiodinase (Arthur *et al.*, 1990), but also of GPx, which is involved in the degradation of hydroperoxides. Since some studies reported an increased GPx activity in response to dietary sources of highly unsaturated fatty acids such as fish oil (Bellisola *et al.*, 1992; Olivieri *et al.*, 1988) and reduced selenium concentrations in plasma and some tissues (Bellisola *et al.*, 1992; Smith and Isopenko, 1997), it is conceivable that dietary oxidized oils, which also promote oxidative stress by reducing the vitamin E status, could affect the selenium status of tissues and thus interfere with the metabolism of thyroid hormones. Therefore, it is possible that the induction of oxidative stress is responsible for the alterations of thyroid hormone status induced by oxidized fat.

7.6.5 Effects on endogenous and exogenous antioxidant defense mechanisms

It has long been suggested that the pathophysiologic effects of dietary oxidized fats are mainly due to oxidative stress induced by lipid peroxidation products present in the oxidized fats (Gabriel and Alexander, 1977). This was based on the knowledge that the primary and secondary lipid peroxidation products are partially absorbed from the oxidized fat (Kanazawa *et al.*, 1985; Oarada *et al.*, 1986). Since oxidized fats are also known to induce microsomal cytochrome P-450 enzymes (Huang *et al.*, 1988; Chao *et al.*, 2001; Sülzle *et al.*, 2004), which causes generation of ROS (Terelius and Ingelman-Sundberg, 1988), it is also possible that the induction of oxidative stress by oxidized fat is due to induction of microsomal enzymes. Indeed, several studies indicated that ingestion of thermally oxidized oil resulted in increased lipid peroxidation as evidenced by increased tissue concentrations of TBARS (Izaki *et al.*, 1984; Kok *et al.*, 1988). In addition, these studies in rats also revealed that the vitamin E concentration in liver and serum decreased following ingestion of oxidized oil (Izaki *et al.*, 1984;

Kok *et al.*, 1988). Moreover, Huang *et al.* (1988) noted that administration of oxidized fat to rats was accompanied by an increased hemolysis of red blood cells which also implies that the vitamin E status was compromised by the oxidized fat. These findings provided strong evidence to suggest that ingestion of oxidized fat induces oxidative stress and causes a depletion of antioxidants.

Subsequent studies aimed to investigate whether the oxidative stress induced by oxidized fat could be alleviated by supplementation with antioxidants. For instance, the study from Liu and Huang (1995) demonstrated that reduced concentrations of α-tocopherol and increased concentrations of TBARS in various tissues of rats fed oxidized fat could be alleviated by supplementation with a high concentration of dietary vitamin E. These authors, however, postulated that the reduction of α-tocopherol concentrations in plasma and tissues of rats fed oxidized fat is not only due to an increased consumption of α-tocopherol by lipid hydroperoxides ingested from the oxidized fat or formed *in vivo* but also due to a reduced absorption of vitamin E from the diet. Nevertheless, a subsequent study from the same group (Liu and Huang, 1996) clearly established that ingestion of oxidized fat is accompanied by an increased α-tocopherol catabolism and/or turnover due to the following reasons:

- The α-tocopherol concentrations in tissues were lower in rats receiving a vitamin E-devoid diet containing oxidized fat compared to rats receiving a vitamin E-devoid diet containing fresh fat during a 9-week depletion period.
- The response of the oxidized fat group to α-tocopherol repletion by intraperitoneal injection of all-*rac*-α-tocopherol was less than that of the control group as evidenced by reduced α-tocopherol concentrations in tissues of rats fed the oxidized fat diet during the repletion period compared to those fed the fresh fat diet.

Another study in Sprague-Dawley rats demonstrated that the susceptibility of LDL to lipid peroxidation is increased by feeding oxidized fats (Eder *et al.*, 2003a). The susceptibility of LDL to lipid peroxidation depends mainly on their PUFA contents and their concentrations of antioxidants (Esterbauer *et al.*, 1989). Since the percentages of PUFA in LDL total lipids were not different between rats fed the oxidized fat and those fed the fresh fat (Eder *et al.*, 2003a), it has been suggested that the increased susceptibility of LDL to lipid peroxidation of rats fed oxidized fat was due to their lower vitamin E concentrations. As expected, supplementing the oxidized fat diets with a high vitamin E concentration prolonged the lag time before onset of lipid peroxidation during incubation of LDL with copper ions (Eder *et al.*, 2003a), which is indicative of a decreased susceptibility of LDL to lipid peroxidation.

Similar to vitamin E, the vitamin C status is also impaired by feeding oxidized fat as demonstrated in guinea pigs (Liu and Lee, 1998), which, like humans, lack an ascorbate synthetic pathway. Guinea pigs fed oxidized fat had lower vitamin C and vitamin E concentrations in plasma and all tissues investigated than those fed oxidized fat (Liu and Lee, 1998). However, the vitamin C and vitamin E status of the guinea pigs significantly improved with

increasing dietary vitamin C levels in the diet. Guinea pigs fed the oxidized fat diet supplemented with 1500 mg vitamin C per kg diet had essentially the same plasma and tissue vitamin E concentrations as guinea pigs fed the fresh fat diet supplemented with 300 mg vitamin C/kg diet. In this study, moreover, levels of TBARS were lowered in tissues of guinea pigs fed oxidized fat with increasing levels of vitamin C in the diet. These findings from Liu and Lee (1998) suggest that vitamin C reduces lipid peroxidation and has a vitamin E sparing action during oxidative stress induced by oxidized fat. The vitamin E sparing action of vitamin C is based on the ability of ascorbic acid to reduce tocopheroxyl radicals generated during oxidation of tocopherol to regenerate vitamin E (Niki *et al.*, 1984; Niki, 1987). In another study with guinea pigs the lowering effect of oxidized fat on the vitamin E status and the vitamin E sparing effect of vitamin C could be confirmed (Keller *et al.*, 2004). In addition, this study demonstrated that vitamin E is also able to spare ascorbic acid under conditions of oxidative stress induced by oxidized fat, because supplemental vitamin E increased plasma concentrations of ascorbic acid in guinea pigs fed an oxidized fat.

Besides exogenous antioxidants such as vitamin E and vitamin C, endogenous antioxidants such as glutathione (GSH) contribute to the antioxidant defense mechanisms. GSH in the reduced form is known as a free, non-protein thiol compound that exerts its antioxidant function by providing an hydrogen atom to electrophilic compounds and hydroperoxides (Ziegler, 1985). Studies in male Wistar rats revealed that administration of oxidized fats significantly decreases reduced GSH content in the liver (Saka *et al.*, 2002). The decreased GSH content is likely explained by the reduction of lipid peroxides by reduced GSH catalyzed by GPx which leads to the formation of primary alcohols and oxidized GSH. This assumption is supported by the findings of a study in guinea pigs demonstrating an increase in the concentrations of oxidized GSH following administration of oxidized fat (Keller *et al.*, 2004). The observation, however, that supplemental vitamin E and vitamin C did not reduce concentrations of oxidized GSH in the liver of guinea pigs fed the oxidized fat, suggests that the vitamins E and C do not interfere with GSH metabolism. Besides oxidation of reduced GSH, it has also been suggested (Saka *et al.*, 2002) that the decreased reduced GSH concentration is due to conjugation of reduced GSH with compounds absorbed from the oxidized fat, such as CFAM, through enzymatic catalyzes by GSH-S-transferase, leading to non-toxic mercapturic acids.

7.6.6 Effects on glucose tolerance and insulin sensitivity

Whilst the effect of oxidized fat on lipid metabolism has been extensively studied, only a limited number of studies have addressed the effect of oxidized fat on glucose metabolism and insulin sensitivity. So far, only two studies dealing with this topic in rodents have been reported (Chao *et al.*, 2007; Liao *et al.*, 2008). The first study from Chao *et al.* (2007) revealed that rats fed an oxidized fat prepared by simulating a deep-frying process had increased fasting glucose levels and an impaired glucose tolerance as shown by an increased

$AUC_{Glucose}$ (area under the curve for blood glucose) following an oral glucose load. These effects of oxidized fats were unexpected because rats fed the oxidized fat had a significantly smaller visceral adipose tissue mass (epididymal and retroperitoneal fat) and adipocyte cell size, and reduced leptin levels. It is generally accepted that a reduction in visceral adipose tissue mass is accompanied by an improved insulin sensitivity due to decreased plasma levels of white adipose tissue-secreted factors, e.g. leptin, resistin or TNFα, which are known to mediate insulin resistance. Pharmacological PPARα activators of the fibrate class, for instance, reduce visceral adiposity and improve insulin sensitivity and glucose tolerance in rodents (Guerre-Millo *et al.*, 2000; Mancini *et al.*, 2001; Ye *et al.*, 2001; Lee *et al.*, 2002). Since oxidized fats exhibit significant PPARα activating properties, which should improve insulin sensitivity, the findings from Chao *et al.* (2007) suggest that the glucose intolerance induced by oxidized fat did not develop from insulin resistance. In line with this assumption is the finding that rats fed oxidized fat showed hypoinsulinemia rather than hyperinsulinemia (Chao *et al.*, 2007). This suggests that the glucose intolerance induced by oxidized fat is due to insulin deficiency and not peripheral insulin resistance. Insulin deficiency leads to a decreased glucose uptake into skeletal muscle and white adipose tissue and an impaired glucose tolerance.

In contrast to the first study (Chao *et al.*, 2007), mice of the C57BL/6J strain were used as model objects in the second study (Liao *et al.*, 2008). The oxidized fat fed to the mice was prepared under the same conditions (deep-frying of dough sheets at 205 ºC for four 6-hourperiods) as in the rat study. The results of the mouse study (Liao *et al.*, 2008) are largely confirmatory of those of the rat study in that administration of oxidized fat caused an impaired glucose tolerance despite reduced fasting and feeding insulin levels as evidenced by significantly higher $AUC_{Glucose}$ but significantly reduced $AUC_{Insulin}$ and $AUC_{C\text{-}peptide}$ in mice fed the oxidized fat (Liao *et al.*, 2008). Also in agreement with the rat study, visceral adipose tissue masses (epididymal and retroperitoneal fat) but also subcutaneous fat mass were significantly smaller in mice fed the oxidized fat compared to those fed the fresh fat. Again, these findings in mice indicate that the glucose intolerance induced by oxidized fat is due to an impaired insulin secretion by pancreatic β-cells.

Regarding the mechanisms underlying the hypoinsulinemic effect of oxidized fat, the authors of both studies suspected that the oxidized fat might have provoked oxidative stress, which might have caused oxidative damage to the pancreatic β-cells, and, thereby, an impaired insulin secretion (Chao *et al.*, 2007; Liao *et al.*, 2008). Evidence for the induction of oxidative stress by oxidized fat has been clearly provided in one of those studies by increased levels of TBARS and reduced levels of α-tocopherol in the liver (Liao *et al.*, 2008). Although the authors of both studies did not directly study markers of oxidative stress in the pancreatic islets, it is very likely that the oxidized fat also caused oxidative stress in this tissue because several former studies dealing with oxidized fats have consistently demonstrated a depletion of antioxidants and induction of

oxidative stress in every tissue investigated (see Section 1.4.5). In addition, it has been shown that among many tissues studied, the pancreatic islets are highly susceptible to antioxidant depletion (Asayama *et al.*, 1986). Furthermore, it is well documented that the glucose-stimulated insulin-releasing capacity of pancreatic β-cells is impaired by the induction of oxidative stress (Ammon *et al.*, 1984). This also explains the diabetogenic actions of alloxan and streptozotocin which are mediated by reactive oxygen species (Heikkila *et al.*, 1976; Sandler and Andersson, 1982). Induction of oxidative stress, therefore, might also explain that diet-derived hydroperoxides, which are also contained in heated fats, were shown to contribute to the loss of insulin secretion activity in pancreatic β-cells (Tsujinaka *et al.*, 2005). Tsujinaka *et al.* (2005) reported that a diet high in lipid hydroperoxides due to a lack of vitamin E resulted in glucose intolerance in rats and that this was associated with the development of insulin resistance and an inability to secrete insulin. In response to the oxidative stress caused to β-cells, activation of nuclear factor-κB (NF-κB) signaling pathway in islet cells from hydroperoxide-fed rats was also noticed (Tsujinaka *et al.*, 2005). NF-κB is known to be activated by ROS generated during oxidative stress, and, thus, activation of NF-κB can be used as an indicator of oxidative stress.

As a further mechanism explaining the hypoinsulinemia induced by the oxidized fat, the authors of the first study raised the hypothesis that alterations in prostaglandin metabolism might be associated with the hypoinsulinemia (Liao *et al.*, 2008). This assumption was based on the observation that oxidized fat was shown to increase prostaglandin E_2 levels in plasma and urine of rats (Huang, 2003), and that transgenic induction of prostaglandin E_2 was reported to cause a destruction of pancreatic β-cells (Oshima *et al.*, 2006).

7.6.7 Effects on inflammation

Several reports in the literature demonstrate that oxidized fats strongly induce oxidative stress (see Section 1.4.5). Although a link between oxidative stress and inflammation has been clearly established (Schreck *et al.* 1991; Sen and Packer, 1996), only one study so far has investigated the effect of oxidized fat on inflammatory processes (Ringseis *et al.*, 2007c). In that study, two groups of pigs were fed two different diets containing either fresh fat or oxidized fat prepared by heating at 200 ºC for 24 h. After 4 weeks on the diets, the pigs were sacrificed and intestinal epithelial cells were isolated and markers of inflammation (NF-κB transactivation and NF-κB target gene expression) determined. NF-κB plays a key role in inflammatory diseases due to its ability to bind specifically to NF-κB-response elements in the promoters of key inflammatory genes (e.g., COX-2, iNOS, TNFα, and IL-6) and induce their gene transcription (Barnes and Karin, 1997).

The results of the pig study (Ringseis *et al.*, 2007c) show that markers of inflammation in intestinal epithelial cells were not altered by dietary oxidized fat indicating that oxidized fat does not induce an inflammatory response in the intestine. These findings were unexpected because in that study an increased

lipid peroxidation and a depletion of antioxidants, both of which are indicative of the induction of oxidative stress, in the intestinal cells were clearly shown (Ringseis *et al.*, 2007c). In addition, reduced activities of antioxidant enzymes in intestinal cells were observed in that study. This is also indicative of the induction of oxidative stress, because oxidative stress in the intestine is accompanied by reduced activities of enzymatic antioxidants in enterocytes (Mehta *et al.*, 1998; Thomas *et al.*, 2005). However, the authors of the pig study observed only a comparatively (Mehta *et al.*, 1998; Thomas *et al.*, 2005) slight impairment of the antioxidant defense mechanisms, suggesting that the oxidative stress induced by the oxidized fat was only moderate. Hence, it is not unlikely that the antioxidant defense system of the intestinal epithelial cells of the pigs was still sufficient to cope with the oxidative burden of the ingested lipid peroxides and the oxidative stress induced was not strong enough to induce proinflammatory gene transcription. In contrast, strong induction of oxidative stress in intestinal cells leads to a marked activation of proinflammatory transcription factors and destabilization of cell integrity (Bernotti *et al.*, 2003). Thus, the authors of the pig study proposed that extracellular detoxifying enzymes in the mucus layer of the intestine, which are involved in the protection of enterocytes from direct contact with diet-derived oxidants such as lipid peroxides in the gut lumen, might have limited the oxidative burden to the epithelial cell, and are therefore responsible for the slight impairment of the antioxidant defense system observed (Samiec *et al.*, 2000). Thus, future studies have to investigate whether oxidized fats which cause a stronger induction of oxidative stress are able to induce inflammatory processes.

Inflammatory processes in various tissues, including the intestine (Straus *et al.*, 2000) were shown to be attenuated by activation of PPARγ (Su *et al.*, 1999; Tanaka *et al.*, 2001; Sanchez-Hidalgo *et al.*, 2005). This effect is mediated by inhibition of the NF-κB pathway and other signalling pathways involved in inflammatory processes by PPARγ (Takagi *et al.*, 2002). This transrepression activity likely constitutes the mechanistic basis for the anti-inflammatory properties of PPARγ and probably also explains why pharmacological PPARγ-ligands markedly reduce inflammation in animal models of inflammatory colitis (Takagi *et al.*, 2002; Su *et al.*, 1999; Tanaka *et al.*, 2001; Sanchez-Hidalgo *et al.*, 2005). Because oxidized fats also contain agonists of PPARγ such as oxidized fatty acids (Bull *et al.*, 2003; Grisham *et al.*, 1990), it has been hypothesized that oxidized fats inhibit inflammatory processes through activation of PPARγ. This hypothesis, however, could not be verified, although the oxidized fat caused a moderate PPARγ activation in the intestinal epithelial cells (Ringseis *et al.*, 2007c). Presumably, the lack of effect of oxidized fat on markers of inflammation is due to an insufficient transrepression of NF-κB by the oxidized fat due to the moderate PPARγ activation. Alternatively, the lack of effect on inflammatory gene transcription is due to the fact that the basal inflammatory state in the intestinal epithelium of the pigs was rather low, and a reduction of inflammatory indices in normal healthy animals, as used in that study, is expected to be only marginal. Therefore, future studies should investigate whether oxidized fats

exert a different effect on inflammatory gene transcription during states of acute intestinal inflammation, e.g. in animal models of experimental colitis.

Whether the impairment of the antioxidant defense system by oxidized fat is of significance for the integrity of the intestinal epithelium (barrier function) has to be investigated in future studies. It has been suggested that a progressive fall in enzymatic and nonenzymatic antioxidants as observed in the present study precedes the occurrence of damage to intestinal cell constituents (Bernotti *et al.*, 2003). Since the intestinal epithelial cells are primarily responsible for the antioxidant defense of the epithelium against luminal oxidants (Grisham *et al.*, 1990), it is likely that in the presence of stronger irritants, such as toxins or pathogens, the already impaired defense mechanisms of the intestinal epithelial cells in response to oxidized fat are not sufficient to cope with this additional challenge nor to preserve cellular integrity and homeostasis of the intestinal epithelium.

7.7 Future trends

Although oxidized fats are widely considered to be detrimental on health, feeding experiments in rats, mice and pigs have also demonstrated several beneficial effects of oxidized fat such as lowering of blood lipids and prevention of ethanol-induced fatty liver development. The reduction of blood and liver lipids by oxidized fat might be of particular significance for human health, because elevated plasma concentrations of both cholesterol and triacylglycerols are known risk factors for cardiovascular disorders such as atherosclerosis, and atherosclerosis is the principle cause of coronary heart disease and stroke, both being responsible for more than 40% of all deaths in Europe and the US. Thus, future studies employing animal models of experimental atherosclerosis have to demonstrate whether oxidized fat might be useful in the prevention from atherosclerosis.

The finding that dietary oxidized fat prevents from ethanol-induced fatty liver development in rats might be also of great interest for health prevention in humans. Chronic alcohol abuse is the most common reason for the development of fatty liver in humans. Since fat accumulation in the liver makes this organ more prone to injury by various agents such as drugs and toxins (Bhagwandeen *et al.*, 1987), which are involved in the pathogenesis of alcoholic hepatitis and fibrosis (Yang *et al.*, 1997; Diehl *et al.*, 2001), approaches to prevent or even treat fatty liver are of great importance. However, whether oxidized fat is indeed a suitable nutritional approach for the prevention of alcoholic fatty liver development in humans remains to be established, because humans, unlike rats and mice, have a lower expression of PPARα in the liver and show a weaker response to treatment with PPARα agonists (Cheon *et al.*, 2005). Nevertheless, as PPARα agonists retain a triacylglycerol-lowering effect also in non-proliferating species (Holden and Tugwood, 1999), it is not unlikely that oxidized fat counteracts the development of an alcoholic fatty liver in humans.

At least one pilot study in humans investigating the effect of a PPARα activator (200 mg fenofibrate per day for four weeks) on alcoholic fatty liver in humans (Tsutsumi and Takase, 2001) indicates that PPARα activators may be effective for the treatment of hypertriglyceridemia in alcoholics and promising agents to prevent alcoholic fatty liver.

Also of great interest is the observation that oxidized fat markedly stimulates the expression of OCTN2 in small intestine. As intestinal OCTN localised in the apical membrane of intestinal mucosa cells are able to transport carnitine from the diet into the cell (Taylor, 2001; Wu *et al.*, 1999), these observations indicate that an increased expression of these transporters in response to oxidized fat enhances their capacity to absorb carnitine from the diet. However, whether or not dietary oxidized fat increases carnitine absorption from the intestine has to be explored in future studies. Regardless of this, the observed up-regulation of intestinal OCTN may be relevant because they are polyspecific and do not only transport carnitine from the intestinal lumen into the mucosa cell but are also able to bind various drugs such as verapamil, spironolactone or mildronate and other monovalent cations (Kato *et al.*, 2006; Koepsell and Endou, 2004; Lahjouji *et al.*, 2001; Grube *et al.*, 2006; Hirano *et al.*, 2006). Thus, it is possible that oxidized fats increase the absorption of various drugs from the intestine by stimulating gene expression of OCTN in the small intestine.

7.8 Sources of further information and advice

As a source of further information and advice, it is recommended to visit the electronic Lipid Library (URL: http://www.lipidlibrary.co.uk/). These web pages produced by Dr William W. Christie, retired head of the Chemistry Department at The Scottish Crop Research Institute, provide excellent overviews about:

- definitions, structures, composition, occurrence, biochemistry and functions of most types of fatty acids and lipids
- practical and theoretical descriptions of the many techniques used for the analysis of lipids, both chromatographic and spectroscopic
- chemistry, nutrition and analysis of frying oils and their degradation products, and the major oils and fats as commodities (this chapter has been produced by leading experts in this field who have been long-term collaborators in most aspects of frying with vegetable oils).

As a further source of information and advice, the book *Lipid Oxidation* by Edwin N. Frankel is recommended. This book integrates a large body of interdisciplinary information on the oxidation of unsaturated lipids in order to develop the basic principles involved in the methodology and mechanisms of free radical oxidation. *Lipid Oxidation* is particularly recommended not only for graduate students but also academic and industrial scientists concerned with the many phases of the complex series of lipid oxidation reactions.

7.9 References and further reading

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8

Methods to determine the extent of lipid oxidation in foods

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Abstract: Several methods are available to measure the same or different indicators of the progress of lipid oxidation. The researcher needs to define the measurement strategy and select the indicators to follow depending on the purpose of the research especially since lipid oxidation is a multistep reaction with a wide range of products and product groups. Conventional measurements include assessment of total hydroperoxide levels and a selection of indicators of secondary oxidation (hydroperoxide decomposition) products usually by simple titremetric or spectroscopic techniques. Chromatographic techniques (gas chromatography and/or high performance liquid chromatography together with different detectors including mass spectroscopy) are useful for more detailed description of the reaction products.

Key words: lipid oxidation, hydroperoxides, secondary oxidation products, analysis.

8.1 Introduction

When studying the extent of lipid oxidation, the researcher is driven by one of two motives, either to follow changes in a given lipid mixture for which the initial composition can be identified or to assess the oxidation status in a sample of an unknown provenance. In the first case, comparison will be between some sort of control (presumably zero oxidation) and sample(s) at different oxidation stages after being subjected to certain oxidation conditions. The oxidation is often induced and the aim is to compare the susceptibility of the original lipids

and/or to investigate the effects of other chemicals (e.g., pro- and antioxidants) and oxidation conditions (light, heat, containers, etc.). In this case, certain assumptions and approximations can be made still leading to sound conclusions. The other case, i.e. the assessment of oxidation status of a sample of unknown history, is indeed tricky and might lead to erroneous conclusions. Studies on lipid oxidation are generally difficult because lipids are usually complex mixtures of different molecular species that give rise to different oxidation products. The variability of molecular species, of both substrates and products, presents an analytical challenge that might be serious in some cases. In addition, despite the availability of a wide range of analytical methods to assess oxidation, each method has advantages and limitations depending on the dominance of different products.

The primary products of lipid oxidation are the hydroperoxides (ROOH), which subsequently decompose to secondary products such as hydrocarbons, alcohols, aldehydes, and ketones (Gardner, 1989). The classical analytical methods, titrimetric and colorimetric, used for monitoring the oxidation state of lipids include the peroxide value (PV), anisidine value (AV), 2-thiobarbituric acid reactive substances (TBARS), carbonyl value, and total polar compounds (Fritsch, 1981). These methods are rather simple and are reasonably sensitive, reliable, and reproducible when carried out under standardized conditions. However, they are highly empirical as they measure complex mixtures of oxidized molecules. In addition, they are generally labor-intensive and use large amounts of solvents and reagents that might be hazardous.

8.2 Volumetric methods

The peroxide value (PV) is a very important characteristic of lipid quality. The assessment of hydroperoxides provides an estimate of the overall oxidation status for lipids and lipid-containing foods especially in the primary phase of oxidation, generally known as the induction period. In the official AOCS methods (1997), method Cd 8b-90, hydroperoxides are reacted with iodide ions to form iodine and the PV is determined by titration of the liberated iodine with thiosulphate using starch as an indicator:

$$ROOH + 2I^- + H_2O \longrightarrow ROH + 2OH^- + I_2$$

$$2S_2O_3^{2-} + I_2 \longrightarrow S_4O_6^{2-} + 2I^-$$

PV is usually expressed as the milliequalivalants of peroxides per kilogram of lipids but can also be divided by 2 and expressed as millimoles of active oxygen per kilogram lipids (SI units).

The extent of lipid oxidation can also be determined by other titrations leading to the estimation of the acid value (AOCS method Ca 5a-40), the epoxide or oxirane value (AOCS method Cd 9-57), and the iodine value (AOCS method Cd 1d-92). The amount of free fatty acids, also formed during lipid oxidation as a result of secondary oxidation of unsaturated aldehydes and other

degradation products of hydroperoxides, can be assessed by titration against sodium or potassium hydroxide, using phenolphthalein as an indicator, and expressed as the acid value (AV). Epoxy derivatives (oxiranes), which are formed by reaction of hydroperoxide groups with reactive double bonds, react with excess acids to open the epoxy ring and form hydroxyl derivatives. The excess acid can be determined by titration against alkali and the epoxide value can be calculated and expressed as mmol epoxide groups per kg of lipids. Finally, the degree of unsaturation of lipids can be assessed as the iodine value (IV), which will be useful when studying oxidation of a substrate. Lipids are reacted with a halogenation agent, e.g. iodine trichloride (ICl_3) or iodine monobromide (IBr), and the unreacted halogen is converted by reaction with a potassium bromide solution into free iodine, which is titrated with a sodium thiosulfate. In general, the volumetric methods suffer from some problems related to the large amount of lipids required and the compromised efficacy of the reaction of lipid oxidation products with the different reagents and the sensitivity of these reactions to temperature, pH, solvents, oxygen, and other co-existing chemicals. In addition, the carbonyl value provides an estimate of the aldehydes and ketones that are formed as secondary oxidation products during lipid oxidation. The carbonyl groups react with hydroxylamine hydrochloride to liberate hydrochloric acid in equivalent amounts, which are assessed by titration with potassium hydroxide using bromophenol blue or bromocresol green as an indicator (Bhalerao *et al*., 1961).

8.3 Spectroscopic methods

The products of lipid oxidation contain different functional groups that allow their collective estimation using spectroscopic techniques. The advantage of using spectroscopic methods is mainly related to the fact that in most assays determinations can be made with no or minimal sample treatment for transparent oils and that the results obtained provide a collective measure pertinent to certain chemical transformation. Depending on the goal of the assessment, more than one spectroscopic assay can be combined to provide a sufficient description of oxidative status. The choice and use of spectroscopic methods should be based on a sound hypothesis, since mistaken choice may lead to erroneous results. A typical example is the use of peroxide value to assess oxidation of conjugated linoleic acids, for which the oligomeric peroxides rather than hydroperoxides are the dominant primary oxidation products (Privett, 1959, Brimberg and Kamal-Eldin, 2003).

8.3.1 UV-visible spectroscopy

Several colorimetric and UV absorption methods can be used to assess lipid hydroperoxides and other oxidation products (Gray, 1978). Mono-hydroperoxides resulting from linoleic and higher polyunsaturated fatty acids have conjugated double bond systems, e.g. –CH=CH–CH=CH–CH(OOH)–CH_2–.

Lipid oxidation can be roughly estimated by measuring the UV absorption at 234 nm (conjugated dienes) and 268 nm (conjugated trienes) (Vieira and Regitano-d'Arce, 1999). The concentration of linoleic acid hydroperoxides can be estimated roughly using a molar extinction coefficient at 234 nm in methanol (molar absorptivity of $E \approx 28\,000\,M^{-1}\,cm^{-1}$).

Hydroperoxides in lipids can also be reacted with potassium iodide under acidic conditions to quantitatively form iodine, which is combined with starch to form the blue complex that can be assayed at 550 nm using ultraviolet–visible spectrometry (Lea, 1931). As with the titration methods, the accuracy of this method is affected by a number of experimental factors such as reaction time, temperature, and light. In highly unsaturated samples, the liberated iodine can react with the unsaturated lipids and cause erroneous results. To avoid these problems, excess potassium iodide is allowed to react with the liberated iodine to form a triiodide anion, which can be measured at 353 nm at a molar absorptivity of $E = 23\,000\,M^{-1}\,cm^{-1}$ (Cramer *et al.*, 1991).

Another spectroscopic method for the determination of hydroperoxides is based on the fast oxidation of ferrous to ferric salts under acidic conditions. The most commonly used method is based on the reaction of ferric salts with potassium thiocyanate to form a red complex whose colorimetric intensity can be measured at 505 nm. Another method uses the ferric ions oxidation of xylenol orange (FOX) to forms a Fe(3+)-xylenol orange complex, which can be measured spectrophotometrically at 560 nm (Bou *et al.*, 2008). Alternatively, the ferric ions can react and form colored complexes with N,N′-dimethyl-p-phenylene diamine or N,N′-di(2-naphthyl)phenylene-1,4-diamine.

The carbonyl compounds formed by decomposition of lipid hydroperoxides, mainly aldehydes and ketones, react easily with p-anisidine to form intensively colored Schiff bases with absorption maxima at 350 nm. The p-anisidine value (p-AV) is calculated as 100 times the optical density measured at 350 nm in a 1 cm cell of a solution containing 1 g of lipids in 100 ml of a mixture of solvents and reagents. Because p-anisidine reacts weakly with hydroperoxides, the p-AV is especially useful for samples not dominated by hydroperoxides, e.g. thermally-oxidized and frying oils.

Thiobarbituric acid reactive substances (TBARS) is a well known method for both screening and monitoring of lipid oxidation status. In this assay, malondialdehyde (MDA), which is formed by the decomposition of certain primary and secondary lipid peroxidation products, reacts with thiobarbituric acid at low pH and elevated temperatures by nucleophilic addition to form the $MDA(TBA)_2$ adduct that can be measured colorimetrically at 533 nm. The TBA assay (Fig. 8.1)

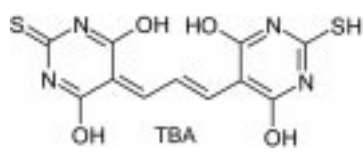

Fig. 8.1 The TBA assay.

is rapid and easy but it is intrinsically nonspecific for MDA and reacts variably with other lipid oxidation products (Janero, 1990).

8.3.2 Fluorescence spectroscopy

Synchronous fluorescence (SyF), in which the excitation and emission wavelengths are scanned simultaneously under a constant wavelength interval ($\Delta\lambda$), is useful in assessing the extent of lipid oxidation. SyF spectra of oxidized olive oil were collected in the excitation wavelength range 250–720 nm and varying the wavelength interval from 20 to 120 nm in 20 nm intervals (Poulli *et al*., 2009). Total synchronous fluorescence three-dimensional spectra, obtained by plotting fluorescence intensity as a combined function of the excitation wavelength and the wavelength interval, increases spectral selectivity leading to better characterisation of multi-component alterations. In other applications, BODIPY (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid), which reacts with peroxyl radicals to yield strong fluorescence (ex. 510 nm, em. 520 nm), is used as a probe to assess lipid peroxidation in biological samples such as plasma (Itoh *et al.*, 2007).

8.3.3 Infra-red spectroscopy

The PV can be measured by Fourier transform infra-red spectroscopy (FT-IR) with reference to the characteristic –OO–H stretching vibration band of hydroperoxide moieties between 3600 and 3400 cm^{-1} (van de Voort *et al*., 1994) and two overtones at 1417–1500 nm and 954–1011 nm (Moh *et al*., 1999). These analysts found that the NIR spectral region 1350–1480 nm, with single-point baseline at 1514 nm, was the best region for predicting hydroperoxides. A better method for predicting PV employs triphenylphosphine (TPP), which can be added as a 'signal-transducing reagent'. TPP reacts stoichiometrically with hydroperoxides and gets converted into triphenylphosphine oxide (TPPO), which has a strong and distinct absorption band in a spectral region (4710 to 4540 cm^{-1}) that is largely devoid of spectral interferences (Ma *et al*., 1997).

FT-IR can also be used to determine the level of FFAs in oxidized oils. As the direct measurement of the carboxyl absorption, ν(C=O), is noisy due to overlap with the overwhelmingly strong ester ν(C=O) band of triacylglycerols, reacting the FFAs with base to convert them to carboxylate salts and measuring the absorption of the COO^- group would minimize spectral interferences and improves sensitivity (Ismail *et al*., 1993). Since the use of a strong base like NaOH caused some saponification of the sample and cloudiness, it was replaced with a much weaker base, sodium hydrogen cyanamide (NaHNC=N) in methanol (Al-Alawi *et al*., 2004). Later, the method based on the extraction of the FFA into a low-viscosity, oil-immiscible solvent and carrying out signal transduction in the extraction solvent using potassium phthalimide as a signal-transducing reagent was used to facilitate the automated determination of FFA content in edible oils (Al-Alawi *et al*., 2005). Moreover, FT-IR can be used to

determine the degree of unsaturation in lipids from the –CH stretch in *cis* HC=CH and *cis* C=C stretch regions at around 3006 and 1650 cm^{-1}, respectively (Guillèn and Cabo, 1999; Che Mana *et al*., 1999).

8.3.4 Chemiluminescence

Lipid oxidation is accompanied by the emission of ultra-weak chemiluminescence (CL). CL originates from photon emission accompanying relaxation of excited states of certain molecules, e.g. the tetroxodie (ROOOOR) generated by the coupling of two peroxy radicals ($ROO^{\bullet}$) by the Russel mechanism, alkoxy radicals ($RO^{\bullet}$) produced by the decomposition of hydroperoxides (ROOH) (Russell, 1957). Owing to the very low quantum yield ($<10^{-4}$), the detection of CL intensity requires the use of light amplifiers such as luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). The luminol-enhanced chemiluminescence involves oxidation of luminol with hydroperoxides in basic solution to form a luminol-derived product in excited state which eventually relaxes to ground state emitting strong blue light at 430 nm. Addition of hemin to the reaction mixture catalyzes the decomposition of hydroperoxides to hydroxyl radicals:

$$\text{LOOH} + \text{luminol (hemin)} \longrightarrow \text{LO}^{\bullet} + \text{LO}^{\bullet} + \text{Lum}^{2-}$$

Lum^{2-} anions react with $LO^{\bullet}$ radicals leading to formation of luminol-derived free radical (diazamiquinone radical) $Lum^{\bullet}$ and LOH. Interaction of the luminol radical with oxygen leads to the formation of a transient luminol endoperoxide, $Lum–O–O^{\bullet}$, which decomposes to give aminophthalate and N_2 and chemiluminescence light as shown in Fig. 8.2 (Rolewski *et al*., 2009).

8.3.5 Nuclear magnetic resonance spectroscopy

Both ^{1}H NMR and ^{13}C NMR can be used to study the presence of hydroperoxy groups as well as of hydrogen and carbon atoms associated in conjugated-dieoic systems, carbonyl groups, and epidioxides (Claxson *et al*., 1994; Haywood *et al*., 1995; Guillén and Ruiz, 2004, 2005a,b,c, 2006; Guillén and Goicoechea, 2007). As with other spectroscopic techniques, NMR measurements can easily be applied to lipid solutions in chloroform or to extracts of lipids and these measurements are especially useful to follow molecular changes during the oxidation cascade. The interpretation of the NMR spectra is not trivial and requires special skills but it offers a possibility to simultaneously evaluate oxidation products and follow processes. When molecular species are isolated from the reaction mixture, NMR is the technique most useful in illustrating their structures providing necessary information in understanding mechanistic details.

8.3.6 ESR spectrometry

Direct detection of radical species, e.g. peroxyl radicals deriving from hydroperoxides, is only possible at very low temperatures where lipid-derived radicals

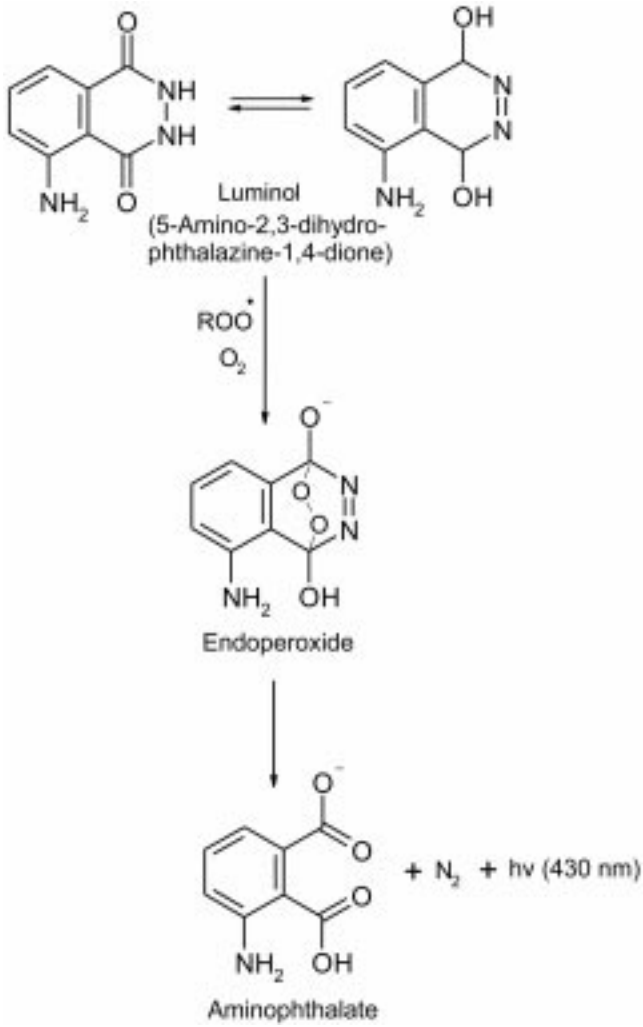

Fig. 8.2 Chemistry of the chemiluminescence resulting from the oxidation of luminol by hydroperoxides.

have reasonably long lives. A better approach is to use spin trapping techniques that are based on the reaction of radicals with diamagnetic Nitroso and nitrone compounds (spin traps) to form stable radicals (spin adducts) that are easily detectable by ESR. In addition, ESR can be used to monitor oxygen consumption during autoxidation via the use of a relatively stable radicals (spin probes), such as 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO), added to the samples at low concentrations. There is a vast potential for developments in the use of ESR in studying lipid oxidation since this application has been in use for only about ten years and is still relatively new (Andersen *et al*., 2005).

8.4 Chromatographic methods

Chromatographic methods are useful for the specific identification and quantification of individual oxidation products, which will allow understanding of reaction mechanisms and follow-up of certain products with undesirable sensory or nutritional effects. Chromatographic analysis of lipid oxidation in samples of unknown history will also allow certain predictions about sample initial composition and storage and environmental condition. Despite these advantages, the analysis of specific compounds is cumbersome and time consuming and relies on the correctness of estimations due to lack of standards.

8.4.1 High performance liquid chromatography (HPLC) methods

HPLC coupled to electron spin resonance spectroscopy (HPLC-ESR) and high performance liquid chromatography-electron spin resonance-mass spectrometry (HPLC/EPR/MS) have been used for detection and identification of the radical adducts resulting from lipid oxidation, for example, as α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) (Iwahashi *et al*., 1991a,b, 1992, 1996) or DMPO adducts (Guo *et al*., 2003).

Various lipid hydroperoxides can be separated from reaction mixtures using normal-phase or reversed-phase HPLC coupled to UV, post-column derivatization-UV/visible, chemiluminescence, electrochemical or mass spectrometry detectors. Post-column derivatization can be performed using luminol chemiluminescence (Yamamoto, 1994), ferrous (II)/xylenol orange (FeXO) reagent (592 nm) (Sugina, 1999). As hydroperoxides are electro-active, they can be detected with high sensitivity and specificity, e.g. using HPLC with reductive mode electrochemical detection on a mercury drop cathode (Korytowski *et al*., 1999).

HPLC with UV detection can be used to separate and detect MDA based on their reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNPH derivatives are strongly absorbing in the region of 300–380 nm but derivatization procedures are complicated (Korchazhkina *et al*., 2003).

8.4.2 High performance size exclusion spectroscopy (HPSEC) methods

At late stages of lipid oxidation and under thermo-oxidation conditions, the monomeric molecular species of lipids undergo various degrees of dimerization, oligomerization and polymerization. HPSEC provides a valuable tool to assess the degree of such alterations and is the most useful technique to study oxidative polymerization at high temperatures, e.g. in frying oils (Dobarganes, 1998). Separations (100–20 000 Da) are performed on co-polymers of styrene divinyl benzenes of pore sizes 50, 100 and 500 Å using a single solvent, commonly tetrahydrofuran. Detection is performed on non-selective mass sensitive detectors such as refractive index (RI) and evaporative light scattering detector (ELSD). HPSEC separations can be performed for the oxidation products of fatty acid methyl esters as well as triacylglycerols, either on the overall

oxidation material or on fractions obtained by adsorption chromatography. The simplest separation is to separate non-polar from polar lipids, which will allow differentiating alkyl-chain dimers/polymers from oxygenated dimers/polymers through the action of alkoxyl or peroxyl radicals. In addition to frying oils, HPSEC has been successfully applied to the study of oxidation of crude and refined oils, dispersed lipids and dried microencapsulated oils (Márquez-Ruiz and Dobarganes, 2005).

8.4.3 Gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-MS)

GC and GC-MS can be used for the analysis of lipid hydroperoxides and other oxidation products. Since hydroperoxides are labile at high temperatures, they may need to be reduced to hydroxy derivatives by sodium borohydride or catalytic hydrogenation (Turnipseed *et al*., 1993; Grechkin *et al.*, 2005). For GC separation, the hydroxyl groups need to be acetylated or trisilylated to increase their volatility. Double bonds in the hydroperoxide molecules are usually hydrogenated and fatty acid moities are converted to methyl esters. GC-MS is useful in revealing structural information related to other substituents on the hydroperoxides such as epoxy or hydroxyl groups, identifying the specific hydroperoxide structures, and increasing the sensitivity of the analysis. The drawback of GC is related to lengthy time of analysis because of the need for reduction and derivatization of the hydroperoxides before analysis.

GC-MS is the preferred method for the analysis of volatile secondary oxidation products, whose analysis would reveal information not only about the degree of oxidation in different samples but also about the identity of unsaturated fatty acids in a sample of unknown history. For example, volatile oxidation products hexanal, heptanal, 2-butanone, 2-propanone, 1-pentanol, 1-hexanol, pentane and toluene are known to derive mainly from the oxidation of linoleic acid while octanal, 1-octanol and heptane derive mainly from the oxidation of oleic acid (Frankel, 1982). Analysis of volatile oxidation products in oxidized samples is performed by static or dynamic headspace analyses or by solid-phase microextraction.

In static headspace analysis, the oxidized is placed in a closed container and the volatile compounds in the sample are allowed time to equilibrate in the headspace above the sample before an aliquot is injected into a gas (e.g., Snyder *et al*., 1985; Girard and Nakai, 1991). While this method is useful to compare samples of the same matrix, it can not be used to compare different samples, since the volatile compounds have different solublities/attachment to the matrix. Dynamic headspace, also called purge and trap or direct thermal desorption, volatile components are extracted by purge and trap, adsorbed onto a suitable material, and then rapidly desorbed, e.g. by means of microwave heating and injected into GC-MS (Vercellotti *et al*., 1987).

A newer technique is the solid-phase microextraction (SPME), where a piece of fused silica fiber, *ca.* 1 cm coated with an adsorbent such as poly(dimethyl-

siloxane), is immersed in a liquid or gas sample causing partitioning of compounds between the sample and the fiber surface. The adsorbed compounds are then thermally desorbed in the GC injection port to enter the column for separation (Yang and Peppard, 1994). SPME has the same sensitivity as dynamic headspace but is less expensive and have better precision and accuracy (Marsili, 1999).

8.5 Sensory analysis and correlation between sensory and instrumental analyses

While there are a number of options to study oxidation in pure lipids and lipid extracts, evaluation of the degree of alterations in complex foods at low oxidation level is still a tricky subject. No analytical method has, to date, proved reliable in describing low degrees of oxidation in complex foods, typical examples being off-flavor in milk/milk powders or warmed off-flavor in meats. Sensory analysis of volatile compounds is the method of choice in most of these and other complex cases but it suffers from difficulties and high costs of performance. Sensory assessment of lipid oxidation is generally performed by trained panels, who assess flavors such as grassy, painty, rancid, buttery, fishy, beany, etc., commonly on a 10-cm flavor score 10-cm line scale ranging from 'not detectable' to 'intense'.

Comparisons between sensory and instrumental analyses have been routinely performed in studies, and good agreements were generally found between the sensory analysis and the levels of secondary oxidation products in oxidized lipid mixtures. Good correlations were, for example, obtained between sensory descriptors, TBARS, and volatile compounds (Kulshrestha and Rhee, 1996; Salih *et al.*, 1987; Shahidi and Pegg, 1994; Tikk *et al.*, 2008). Sensory descriptors of rancid odor (typically oxidized, painty, and grassy) correlate with volatile oxidation products such as hexanal, 2-hexanol, heptanol, 2-octenal, and 2,4-decadienal and TBARS correlate better with sensory scores than PV (Maisuthisakul *et al.*, 2007). Correlations between grassy and painty scores and volatile oxidation products in whole milk powder (Lloyd *et al.*, 2009) are given as examples:

$$\begin{aligned}\text{Grassy score} = {} & 0.92 + 0.00058\text{*hexanal} + 0.024\text{*2-heptanone} + \\ & 0.073\text{*1-octen-3-ol} - 0.083\text{*nonanal} \\ & (R^2 = 0.38,\ p < 0.0001),\ \text{and}\end{aligned}$$

$$\begin{aligned}\text{Painty score} = {} & 0.47 + 0.00073\text{*hexanal} + 0.018\text{*Octanal} + \\ & 0.07\text{*2-methylbutanal} - 0.086\text{*3-methylbutanal} - \\ & 0.022\text{*2-octen-2-one}\ (R^2 = 0.61,\ p < 0.0001)\end{aligned}$$

Sometimes, the correlations are not linear. For example, an exponential relationship was found between sensory score and log TBARS in cooked pork (Nissen *et al.*, 2004).

8.6 Research methods to study oxidation in foods and beverages

Most of the analytical method development in the area of evaluating oxidation status has been performed and evaluated for the study of neat lipids or lipids extracted from foods. The extent of oxidation in fats and oils can be evaluated by different complementary methods, and research questions can be answered fairly convincingly. On the other hand, methods to study the extent of lipid oxidation in complex foods and beverages are still in their infancy. Extraction of lipids from these samples is not only tedious but it also introduces analytical errors because of the instability of the oxidation products, particularly hydroperoxides, or co-extractability of other molecules (Velasco *et al*., 2008).

The two most commonly used methods to extract lipids from complex matrices are the Folch method (Folch *et al*., 1957), the Hara and Radin method (Hara and Radin, 1978), and their modifications. In general, increased extraction solvent polarity is needed for extracting highly oxidized lipids or oxidized lipids in difficult matrices because of the binding of lipid oxidation products. Specifically, the extractability of the volatile oxidation products may be significantly affected by the food matrix. Moreover, lipid oxidation products are highly unstable and the reaction mixture is significantly dynamic. Studies on the effect of extraction conditions and other sample treatments on the oxidation status are highly warranted.

8.7 Future trends

With the current advances in statistical tools, it is possible to process complex data such as that generated by analysing lipid oxidation by NMR, IR, or mass spectrometry, alone or coupled to chromatography. Certain extractions and/or chemical modifications can be performed to signal particular molecules and the comprehensive NMR, IR, MS or both can be repeated before chemometrics can be applied to extract information pertinent to the evolution of molecular species and the progress of the reaction.

8.8 Sources of further information and advice

Detailed chapters dedicated to the use of common techniques in the analysis of lipid oxidation can be found in A. Kamal-Eldin and J. Pokorny, eds. 2005, *Analysis of Lipid Oxidation*, AOCS Press, Champaign, IL.

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9

Methods for food shelf life determination and prediction

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Abstract: Oxidation is the most frequent event leading to the end of shelf life of microbiologically stable foods. The evaluation/prediction of shelf life is crucial to verify how long the product will last before it oxidized to a level that makes the product unsuitable for consumption. Shelf life assessment strategies of foods and beverages suffering oxidation will be discussed, focusing on definition of the acceptability limit, described by proper oxidative indicators, and methodologies for shelf life testing. In this regard, methods of shelf life evaluation under actual storage conditions and methods of shelf life prediction based on exploitation of proper accelerating environmental factors will be illustrated.

Key words: shelf life, oxidative reactions, acceptability limit, kinetic modelling, accelerated shelf life tests.

9.1 Introduction

Shelf life is defined as the time, under defined storage conditions, during which food remains safe, retains desired sensory, chemical, physical and biological characteristics as well as complies with any label declaration.

Oxidative reactions are the most frequent event leading to the end of the shelf life of microbiologically stable foods, such as ambient stable and frozen foods. Since quality is a dynamic condition, continuously moving to lower levels, the shelf life of these products depends on the oxidation rate and the acceptability limit. The latter is the oxidation level above which the product becomes unsuitable for consumption.

In this chapter, shelf life assessment strategies of foods and beverages susceptible to oxidation during their life will be discussed. We will focus on the definition of the acceptability limit described by the proper oxidative indicator as well as possible methodologies for shelf life testing. In particular, methods of shelf life evaluation under actual storage conditions and methods of shelf life prediction based on exploitation of proper environmental factors able to accelerate oxidation will be illustrated with reference to uncertainties and possible risks arising during shelf life assessment.

9.2 Shelf life assessment strategies

The adoption of a systematic approach for shelf life determination is necessary to obtain a cost-effective experimental design. Figure 9.1 schematically shows the possible strategies that can be pursued to assess the shelf life of foods undergoing oxidative reactions. The first step implies the definition of the *acceptability limit*, defined as the quality level discriminating products that are still acceptable for consumption from the no more acceptable ones. Following this, the proper oxidative indicator should be identified before proceeding to shelf life testing, which allows one to compute/predict the product shelf life through kinetic modelling. Shelf life testing can be performed under actual or accelerated storage conditions. The former is based on the direct measure of shelf life under storage conditions simulating those actually experienced by the product. This approach can be easily used when quality decay occurs in rather short times (i.e., perishable foods). Generally, this is not the case of foods undergoing oxidation. The prediction of long-term shelf life is traditionally obtained by accelerating shelf life experiments performing stability tests under environmental conditions able to speed up quality deterioration. After the identification of the most suitable accelerating factor, the oxidation kinetics

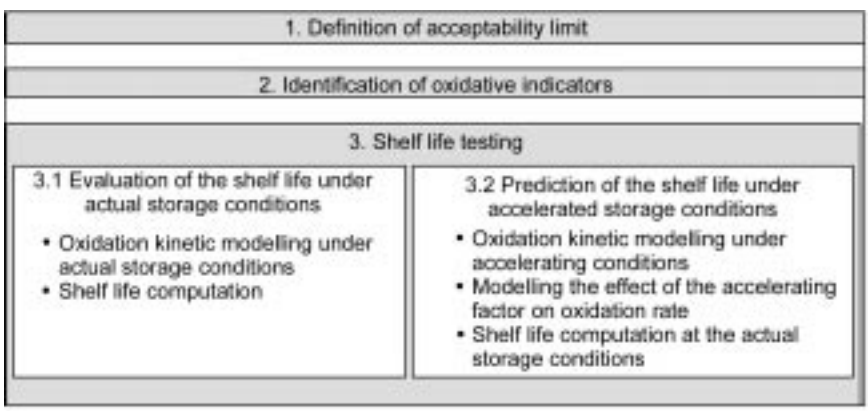

Fig. 9.1 Strategies for shelf life testing of foods undergoing oxidative reactions.

should be evaluated under accelerated conditions to identify mathematical models that can be used to extrapolate kinetic parameters at milder conditions, usually experienced by the product (Labuza and Riboh, 1982; Labuza and Schmidl, 1985; Fu and Labuza, 1993).

9.3 Definition of acceptability limit

The acceptability limit of products whose quality deterioration during storage is mainly attributable to oxidative reactions corresponds to a certain level of oxidation/rancidity. Its definition can be the result of the application of different criteria depending on product criticism (Table 9.1).

The simplest case is when the acceptability limit is compulsory since it is specified by national or international regulations. It is compulsory that this limit is respected by the producer in order to market the product. For instance, the characteristics of olive oil and olive-residue oil are dealt with in Commission Directive 1991/2568/EC (Corrigendum OJ L 248, 5.9.1991). Based on this directive, olive oils are subdivided in different categories according to their quality requirements, such as oxidation indices (e.g., peroxide value, acidity, conjugated dienes) and sensory attributes. The regulation also reports the methodologies to assess the values of these indicators. Considering that conformity to regulatory indications must be guaranteed throughout the product life, such values can be considered as acceptability limits to calculate olive oil shelf life. Even if not compulsory, recommendations on the oxidation/rancidity level

Table 9.1 Criteria for definition of shelf life acceptability limits for foods undergoing oxidative reactions

Product life end	Acceptability limit nature	Subject deciding the acceptability limit	Acceptability limit
Legal requirements default	Compulsory	Authority	Value of the quality indicators indicated by current regulation
Label claims default	Compulsory	Producer	Concentration of nutrients voluntarily claimed by the producer
Company standard default	Voluntary	Producer	Oxidation/rancidity level considered critical for product marketing
Excessive consumer rejection	Voluntary	Producer	Oxidation/rancidity level corresponding to a certain percentage of consumer rejecting/not buying the product

corresponding to a certain quality standard for other fats and oils can be found in the relevant Official Codex Standards.

Compulsory shelf life limits can also derive from voluntary label claims. In fact, according to the regulation, producers must guarantee product conformity to any claim reported on the label. For instance, the amount of bioactive compounds claimed on the label can be regarded as a shelf life acceptability limit. The latter is thus the result of marketing considerations merging actual product functionality, product positioning on the market and consumer perception of the claim.

The most complex and common case is the one which is not supported by any regulatory indication about specific constraints to be adopted as acceptability limits. The producer is thus completely free to choose the acceptability limit according to its own policy and quality targets. The acceptability limit may be simply chosen by company management on the basis of the experience of the product performance on the market or on the emulation of competitors' products. Such procedures are obviously fraught with the risk of critical over-estimation or disadvantageous underestimation of the shelf life. This hazard is much more probable in the case of new foods, for which no previous experience is available. One other approach to define the acceptability limit is based on the application of sensory analysis, since sensory perception is often the earliest indicator of product failure in food undergoing oxidation. In such case, the company may decide that the product reaches the acceptability limit when it is recognized as significantly different from the fresh one.

In some companies, descriptive sensory analysis carried out with expert panels is applied to describe the evolution of sensory attributes potentially responsible for consumer rejection. These data can be used to support the acceptability limit choice by converting oxidation attribute scores into binary data (acceptable/not acceptable) on the basis of an arbitrary predefined cut-off value. Unfortunately, results achieved with the expert panel could be far away from those obtained with consumers. This is due to the fact that the cut-off of the oxidation attribute, arbitrarily chosen by the company, could have no relation to that applied by the consumer when deciding to eat/buy the product or not. In other words, any choice of a cut-off value for an oxidative index without clear knowledge of its relation to consumer acceptability could induce mistakes in shelf life guessing. For this reason, the hazard should not be focused on the properties of the product undergoing oxidation, rather on the attitude of consumer to accept or reject it (Hough *et al.*, 2006).

An interesting approach is to consider the percentage of consumers rejecting the product due to unacceptable oxidation/rancidity as an indicator of the acceptability limit. For instance, at a given storage time, the product is certainly still acceptable to some consumers, despite being rejected by others. The food company can choose to be exposed to more or less risk of product failure by selecting, as an acceptability limit, the proper percentage of consumers rejecting the product. In other words, the acceptability limit becomes the maximum percentage of consumers that the company can tolerate to dissatisfy. Table 9.2

Table 9.2 Percentage of consumer rejection and relevant proposed risk level

Acceptability limit (% consumer rejection)	Risk level
0	Negligible
10	Very low
25	Low
50	Medium
75	High

shows the relation between acceptability limit and the proposed risk level. In most shelf life studies a medium risk level (50% consumer rejection) is chosen as a reasonable acceptability limit but it has been suggested that lower percentages of consumer rejection could be much more reliable. According to Guerra *et al.* (2008), the choice of the risk level can strongly affect the final shelf life value from 20 to 100%.

Following this approach, the acceptability limit can be identified using survival analysis methodology (Gacula and Kubala, 1975; Gacula and Singh, 1984; Hough *et al.*, 2003). In particular, the product is analyzed during storage by asking the consumers a response of acceptability/unacceptability and data are elaborated by survival analysis. This powerful methodology has only rarely been applied to products suffering oxidative reactions. An example of plot of probability of consumer rejection of raw minced beef versus storage time at 5 ºC is reported in Fig. 9.2 (Hough *et al.*, 2006).

It is noteworthy that such methodology allows by itself the estimation of product shelf life, which is exactly the time needed to reach the chosen percentage of consumers rejecting the product. Despite being powerful and

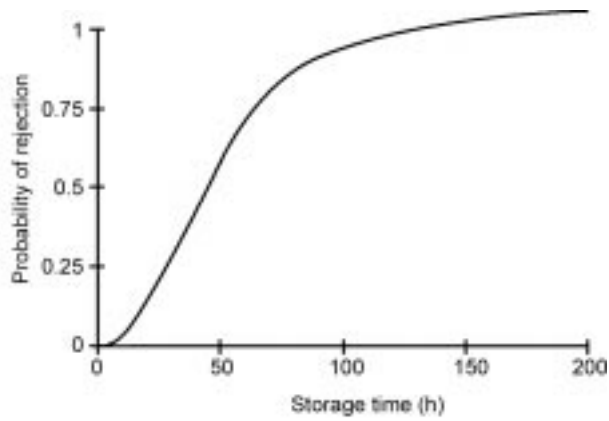

Fig. 9.2 Probability of consumer rejection of raw minced beef versus storage time at 5 ºC (Hough *et al.*, 2006 with permission).

accurate, survival analysis of consumer data is not yet part of the quality management systems of food companies since it suffers the basic criticism of requiring assembling consumer panels. This is well known to be expensive, not routinely performable, and definitely not consistent with industrial needs.

It should be noted that the definition of any volunteer acceptability limit is a slow procedure, since it requires continuous monitoring of the product during its life on the shelf. To our knowledge, there is no literature indication of procedures able to speed up the process of acceptability limit definition. In fact, there is no evidence that oxidative index limits can be successfully assessed under accelerated storage conditions.

9.4 Identification of oxidative indicators

Owing to the difficulty of routine exploitation of consumer panels in the definition of the acceptability limit, it would be easier to continuously monitor instrumental or sensory oxidative indices whose evolution could be correlated to the evolution of the percentage of consumer rejection (Garitta *et al.*, 2004a; Calligaris *et al.*, 2007). Figure 9.3 summarizes a possible methodology for the identification of oxidative index values corresponding to the consumer acceptability limit.

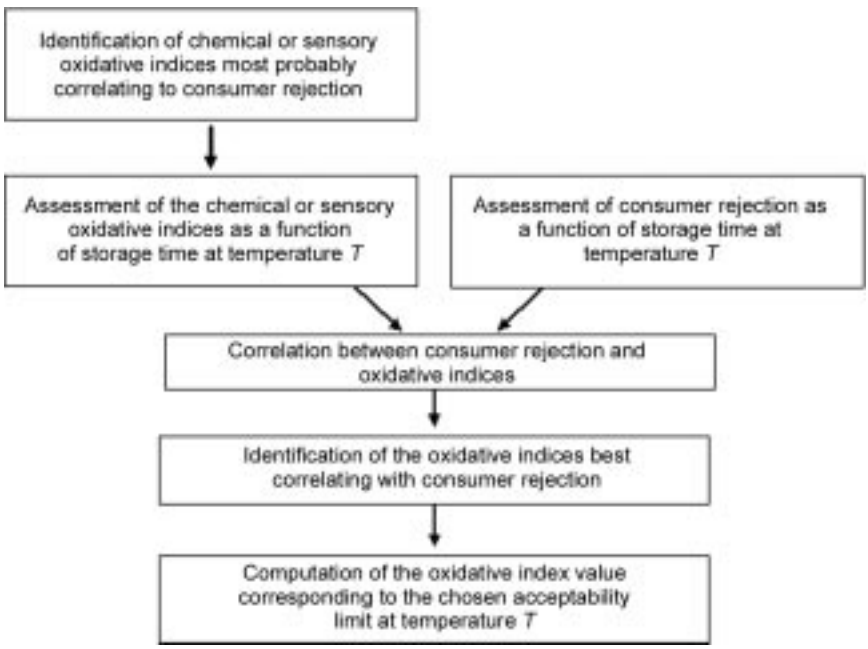

Fig. 9.3 Methodology for the identification of oxidative index values corresponding to the consumer acceptability limit (elaborated from Nicoli *et al.*, 2009).

Table 9.3 Main indicators of oxidative reactions potentially correlating with consumer rejection

Compounds undergoing oxidation	Indicator
Unsaturated fatty acid	Peroxide value Conjugated dienes (CD) Volatile carbonyl compounds Anisidine value Thiobarbituric acid index (TBA) Hydrocarbons and fluorescent products Sensory attributes (off-flavour, off-odours)
Pigments	Colour Selected compounds (i.e., β-carotene, lycopene) Sensory attributes (colour fading, off-colour)

In order to follow this approach, the first critical step implies the identification of oxidative indices that are easily measurable and potentially correlated to sensory perception of oxidation. Different indicators can be suggested. Table 9.3 summarizes the main indicators proposed in the literature to continuously monitor the development of oxidation during food storage.

The methods to monitor lipid oxidation allow the evaluation of either primary and/or secondary oxidation products. Among others, aldehydes, ketones, hydrocarbons and alcohols are odour-active oxidation products which could be good predictors of consumer acceptability. Beside these chemical indicators, sensory attributes have been also applied to detect the formation of off-flavour (Shahidi *et al.*, 1987; Frankel, 1993; Fernandez *et al.*, 1997; Brewer *et al.*, 1999; Grosso and Resurreccion, 2002, Kanavouras *et al.*, 2004; Purcaro *et al.*, 2008).

Consumer rejection could also be the result of oxidative reactions involving compounds other than fat and oil, such as pigments. Although the change in the pigment oxidative status may represent a good indicator, food colour, as assessed by instrumental or sensory methodologies, is reasonably the quickest and easiest indicator of consumer acceptability.

Once the oxidative index most likely correlating with consumer perception have been identified, the issues to be addressed are:

- How do these oxidative indices and consumer acceptability evolve during food storage?
- Which are the oxidative indices best correlated with consumer acceptability during food storage?
- What value of the oxidative index corresponds to the acceptability limit?

The identification of this relationship is a key step in developing a simple and ready to use methodology for shelf life estimation minimizing the routine need for expensive and complex consumer tests. Such methodology was applied to identify the oxidative index values corresponding to the consumer acceptability limit of biscuits. In this case, the peroxide number was found to be the best

Table 9.4 Mean peroxide value corresponding to different percentages of consumer rejecting the biscuits stored at 20 °C

Consumers rejecting the sample (%)	Peroxide value ($meqO_2/kg_{fat}$)		
	95% lower limit	Estimated	95% upper limit
10	4.9	6.4	8.9
30	8.6	10.9	14.4
50	12.9	15.6	18.1
70	18.4	21.7	25.7

From Calligaris *et al.* (2007a) with permission.

indicator of consumer acceptability. Peroxide values corresponding to different acceptability limit were thus calculated (Table 9.4).

Further examples demonstrating the possible application of this approach have been reported by Hough *et al.* (2002), Gambaro *et al.* (2004), Garitta *et al.* (2004) and Manzocco and Lagazio (2009) with reference to different food products.

It must be noted that this approach only leads to oxidative index values corresponding to consumer acceptability limits at a specific storage temperature, which is chosen to apply the methodology reported in Fig. 9.3. At the moment there is no literature indication that an oxidative index determined at one temperature can be successfully used at temperatures other than those used in the initial experiment.

9.5 Shelf life testing

As shown in Fig. 9.1, once an appropriate oxidative indicator has been identified, it is necessary to estimate the length of time needed to reach some critical value. This step, generally defined as shelf life testing, implies the continuous monitoring of the changes of the oxidative indicator during storage of food under controlled environmental conditions. Data relevant to the evolution of oxidation during storage are then modelled to obtain proper parameters describing/predicting the oxidation kinetics. The latter are necessary to obtain the shelf life once knowing the acceptability limit.

Two strategies of shelf life testing can be pursued depending on the expected product life time:

1. shelf life testing under actual storage conditions for perishable foods;
2. shelf life testing under accelerated storage conditions for shelf-stable products.

While the former is based on the direct measure of product shelf life according to its usual storage conditions, the latter implies stability tests to be carried out under environmental conditions able to speed up quality loss.

9.5.1 Evaluation of shelf life under actual storage conditions

When there is no necessity to speed up shelf life testing, the latter can be carried out under conditions simulating as closely as possible those actually experienced by the product on the shelves. The basic requirement is that storage conditions (e.g., temperature, moisture, oxygen concentration, light) during shelf life testing are kept constant and equal to the conventional storage temperature of the product. Data describing the changes of the oxidative indicator under conditions simulating actual storage are submitted to modelling according to the fundamental kinetic principles or by exploiting descriptive mathematical models.

According to the well-known fundamental kinetic principles, the rate of changes of an oxidative indicator (I_{ox}) can be calculated by integrating the general kinetic equation:

$$\int_{I_{ox_0}}^{I_{ox}} \frac{dI_{ox}}{I_{ox}^n} = \pm \int_0^t k \, dt \qquad 9.1$$

where k is the rate constant and n the reaction order. The general rate law can be integrated to obtain the equations of the pseudo zero, first, second or n order (Table 9.5). In experimental kinetic studies I_{ox}, $\ln(I_{ox})$, $1/I_{ox}$, $1/{I_{ox}}^{n-1}$ values are plotted versus time to estimate the reaction rate (k) by linear regression analysis. A comprehensive discussion on kinetic modelling of reactions in food is reported in the textbook *Kinetics Modelling of Reactions in Foods* (van Boekel, 2009).

By solving the integrated forms of equation 9.1 as a function of time, shelf life at the actual storage conditions can be calculated:

$$SL = \frac{\int_{I_{ox_0}}^{I_{ox}} \frac{dI_{ox}}{I_{ox}^n}}{\pm k} \qquad T = \text{const} \qquad 9.2$$

Table 9.5 Zero, first, second and n order integrated kinetic and shelf life equations. Kinetic rate constants have positive or negative values if the quality indicator increases or decreases vs time

Reaction order	Integrated rate law	Shelf life equation
$n = 0$	$I_{ox} = \pm kt + I_{ox_o}$	$SL = \frac{I_{ox} - I_{ox_0}}{\pm k}$
$n = 1$	$\ln I_{ox} = \pm kt + \ln I_{ox_0}$	$SL = \frac{\ln I_{ox} - \ln I_{ox_0}}{\pm k}$
$n = 2$	$\frac{1}{I_{ox}} = \pm kt + \frac{1}{I_{ox_0}}$	$SL = \frac{\frac{1}{I_{ox}} - \frac{1}{I_{ox_0}}}{\pm k}$
$n \neq 1$	$\frac{1}{{I_{ox}}^{n-1}} - \frac{1}{{I_{ox_0}}^{n-1}} = \pm(n-1)\,kt$	$SL = \frac{\frac{1}{{I_{ox}}^{n-1}} - \frac{1}{{I_{ox_0}}^{n-1}}}{\pm k}$

where I_{ox0} is the value of the food quality indicator just after production, I_{ox} is the quality indicator value corresponding to the acceptability limit.

Since oxidation reactions are highly complex and there are a huge number of factors potentially affecting the reaction rate, it should be stressed that the evolution of any oxidation indicator versus time can be frequently the result of different reactions taking place simultaneously or consecutively. Thus, the reaction order n does not give any indications on the true reaction mechanisms involved and k is therefore considered as an 'apparent' rate constant. A number of papers dealing with this well-recognized procedure have produced huge amounts of data on the oxidation rates of several foods in different environmental conditions. Table 9.6 reports some examples of recent literature data relevant to the reaction order of selected oxidation indices in different food products. Due to the huge number of literature data available on the kinetics of oxidative reactions, Table 9.6 should not be considered exhaustive.

Table 9.6 shows that zero and first order are frequently applied to describe the changes of oxidation indicators. However, due to the complex pathway of

Table 9.6 Examples of apparent reaction orders used to describe the evolution of oxidation indicators

Apparent reaction order (n)	Index	Product
Zero	Peroxide value	Potato chips (Houhoula and Oreopolou, 2004); Biscuits (Calligaris *et al.*, 2007a); extra virgin olive oil (Calligaris *et al.*, 2006; Mancebo-Campos *et al.*, 2008); salmon oil (Huang and Sathivel, 2008)
	K232	Extra virgin olive oil (Mancebo-Campos *et al.*, 2008)
First	Vitamin C degradation	Frozen vegetables (Giannakourou and Taoukis, 2003); orange juice (Polyedra *et al.*, 2003; Tiwari *et al.*, 2009); fresh-cut strawberries (Odriozola-Serrano *et al.*, 2009)
	K270	Extra virgin olive oil (Gutierrez and Fernandez, 2002; Mancebo-Campos *et al.*, 2008)
	Oxygen consumption	Soybean oil (Colakoglu, 2007)
Other	Peroxide value	Bread sticks (Calligaris *et al.*, 2008); bulk oils (Aragao *et al.*, 2009)
	Anthocyanins	Fresh-cut strawberries (Odriozola-Serrano *et al.*, 2009)
	Volatile compounds	Olive oil (Kanavouras *et al.*, 2004)
	Carotenoids	Frozen tomato puree (Calligaris *et al.*, 2004)
	Vitamin C	Orange juice (Manso *et al.*, 2001)

oxidative reactions, fitting data with these models may be precluded and other models have to be found. For instance, the evolution of peroxide index is a case in point. As is well known, peroxide values as a function of storage time follow typical bell-shaped curves. After the induction period, during which no significant changes of peroxide value are observed, the progressive increase of peroxide value occurs until a maximum is reached, indicating that the oxidative reactions approaches the termination step. Traditionally, the kinetics of peroxide formation and decomposition has been modelled by separately considering each reaction step. The assumption is that all the intermediate reactions follow fixed order reaction kinetics, each having its characteristic apparent rate constant.

It should be noted that, depending on the environmental and compositional factors, the induction period could be negligible or even very long. In the latter case the estimation of the lag time (*lag*) may be critical for the final product dating. For this reason, the lag time should be carefully estimated and included in the shelf life equation as shown in the following example referred to a zero order kinetic:

$$SL = \frac{I_{ox} - I_{ox_0}}{k} + lag \qquad T = \text{const} \tag{9.3}$$

The second approach is to consider the entire evolution of the peroxide value during the overall product life. In this case, the fundamental kinetic concepts are skipped and empirical mathematical model can be achieved by best fitting the peroxide value curves. Different mathematical models have been applied. For instance, Aragao *et al.* (2009) proposed a phenomenological mathematical model, comprising a decay factor superimposed on an accumulation term, to describe peroxide changes during lipid oxidation. Additional models (i.e., sigmoidal model, Weibull distribution function, logistic model) are also frequently used (Özilgen and Özilgen, 1990; Cunha *et al.*, 1998; Corradini and Peleg, 2007; Calligaris *et al.*, 2008; Imai *et al.*, 2008; Odriozola-Serrano *et al.*, 2009).

As an example, equation 9.4 shows the mathematical model, obtained by best fitting approach, applied to describe peroxide value (*PV*) changes as a function of storage time:

$$PV = PV_0 + \frac{PV_{max} - PV_0}{1 + e^{\left(\frac{4k_{max}(\lambda - t)}{PV_{max} - PV_0} + 2\right)}} \tag{9.4}$$

where PV_0 is the initial peroxide value, PV_{max} is the peroxide value at the maximum of the curve, λ is the lag phase defined as the crossing point between the tangent through the inflection and k_{max} is the maximum reaction rate. By solving equation 9.4 as a function of time, a shelf life prediction model can be built up:

$$SL = -\frac{PV_{max} - PV_0}{4k_{max}} \ln\left(\frac{PV_{max} - PV_{lim}}{PV_{lim} - PV_0}\right) + \lambda + 2\left(\frac{PV_{max} - PV_0}{4k_{max}}\right) \tag{9.5}$$

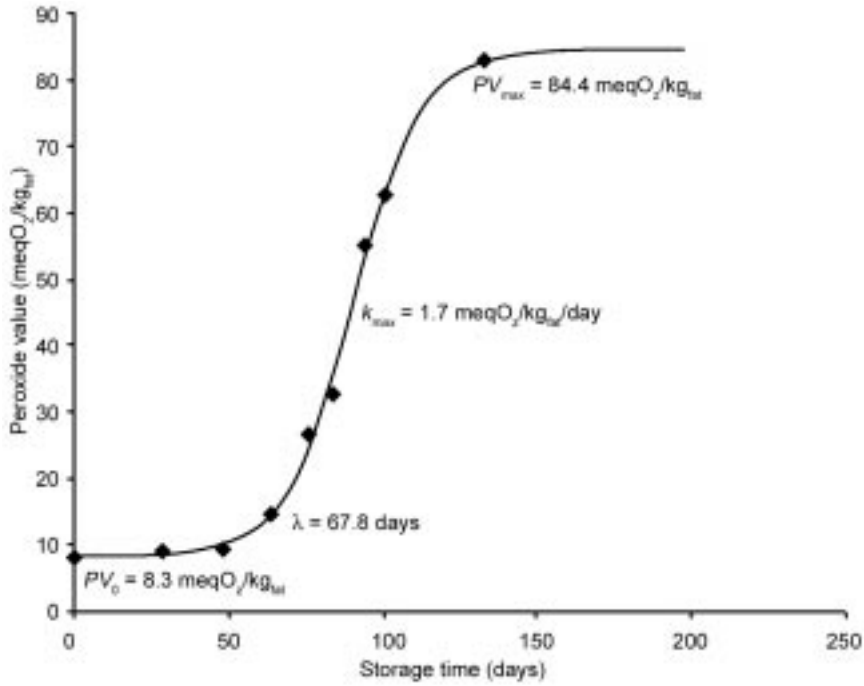

Fig. 9.4 Peroxide value of bread sticks as a function of storage time at 20 ºC. The line is the result of the regression analysis performed applying equation 9.4 (elaborated from Calligaris *et al.*, 2008).

where *SL* is the shelf life, expressed as days, PV_{lim} is the peroxide value corresponding to the acceptability limit. Figure 9.4 shows the experimental data of peroxide changes as a function of storage time at 20 ºC of bread sticks and the relevant regression results of the lag phase length, maximum rate and maximum level of peroxide formation. Such parameters were integrated in equation 9.5 to predict shelf life of bread sticks.

9.5.2 Prediction of shelf life under accelerated storage conditions

As previously mentioned, oxidation often proceeds fairy slowly under actual storage conditions. For this reason, it is convenient to accelerate shelf life experiments by testing food under environmental conditions that speed up food quality deterioration and then extrapolating the results to milder conditions usually experienced by the product (Mizrahi, 2000). This kind of test is generally called accelerated shelf life test (ASLT). The basic premises for the ASLT application are:

- the oxidation rate varies only as a function of the accelerating factor, while other environmental and compositional variables are kept constant;

- an accurate kinetic/descriptive model of oxidation during food storage is available;
- the relationship between the accelerating factor and oxidation rate is known.

In the case of oxidative reactions, temperature is certainly the most used accelerating factor. However, other factors, such as oxygen concentration, moisture, pro-oxidants and light can also find application (Frankel, 2005).

Although the study of food oxidative stability as a function of different environmental factors is extensive, results are difficult to interpret in terms of shelf life data because of variations in test conditions and acceptability limit definitions. It should be pointed out that in most cases the lipid stability tests conducted under extreme environmental conditions (i.e., high temperatures, high oxygen concentration) have a different finality with respect to a shelf life study. The former are generally set up to evaluate the susceptibility of a sample to oxidation and the aim is to predict its stability as a function of different variables. However, a shelf life predictive study has the objective to correctly estimate the product shelf life under actual storage conditions. Thus, the mathematical model adopted should have the power to predict the shelf life with defined estimation errors.

9.5.3 Temperature as accelerating factor

Among the potential factors accelerating oxidation reactions, temperature is the only one commonly exploited in ASLT. This is due not only to the fact that temperature is one of the most critical factors for many products but also to the availability of a theoretical basis for the development of a mathematical description of the temperature sensitivity of chemical reaction rates. In fact, the Arrhenius equation 9.6 (Arrhenius, 1901), developed theoretically on the molecular basis for reversible chemical reactions, has been shown to hold empirically for a wide range of complex chemical and physical phenomena occurring in foods (Labuza and Riboh, 1982):

$$k = k_0 \cdot e^{-E_a/RT} \qquad 9.6$$

where k is the reaction rate constant; R is the molar gas constant (8.31 J/K/mol), T is the absolute temperature (K); E_a is the activation energy (J/mol) and k_0 is the frequency factor. The possibility to apply the Arrhenius equation to describe the temperature dependence of oxidation rates can be easily and quickly assessed by plotting $\ln k$ as a function of the reciprocal of temperature. A linear relation between these variables can be observed when Arrhenius behaviour is fulfilled. Hence, by measuring the rate of oxidation at least at three different temperatures, the oxidation rate at a desired temperature can be extrapolated. In this context, the application of a rigorous statistical approach is essential to evaluate the accuracy of the extrapolated data. The methodology for the statistical application of Arrhenius equation to model the temperature dependence of food quality is well described by van Boekel (2008).

Integrating shelf life (equation 9.2) and Arrhenius equations (equation 9.6), a general shelf life predictive model can be obtained as follows:

$$SL = \frac{\int_{I_{ox_0}}^{I_{ox}} \frac{dI_{ox}}{I_{ox}^n}}{\pm k_0 e^{-E_a/RT}} \qquad 9.7$$

Table 9.7 reports some examples of literature data relevant to the application of the Arrhenius equation to describe the temperature dependence of oxidative reactions. The Arrhenius equation was successfully used to estimate the temperature dependence of oxidation rate not only for food lipids, but also for bioactive compounds. As can be observed, the E_a values reported in the literature for different oxidative indicators greatly varies. This can be attributed to the difference in compositional and environmental factors taken into account in these studies.

The successful application of ASLT based on temperature as the accelerating factor of oxidative reactions is strictly dependent on the choice of the temperature range to be adopted in the ASLT. In fact, deviations from linearity of the Arrhenius equation can often be detected when the temperature range of shelf life testing is not properly selected, potentially causing significant shelf life prediction errors. As stated by Frankel (2005) for food lipids, the use of temperatures higher than 100 ºC is questionable, because samples develop

Table 9.7 Examples of the application of the Arrhenius equation to describe the temperature dependence of oxidative reactions. Estimated values of E_a are also reported

Index	E_a (kJ/mol)	Product
Peroxide value	24–240	Sunflower oil (Calligaris *et al.*, 2004); encapsulated rapeseed oil (Orlien *et al.*, 2006); extra virgin olive oil (Calligaris *et al.*, 2006); salmon oil (Huang and Sathivel, 2008); bread sticks (Calligaris *et al.*, 2008)
Conjugated dienes	65–114	Almond-based products (Tazi *et al.*, 2009)
TBARS	61–100	Almond-based products (Tazi *et al.*, 2009)
Volatiles	34–194	Sunflower oil (Calligaris *et al.*, 2004); extra virgin olive oil (Calligaris *et al.* 2006); milk powder (Thomsen *et al.*, 2005); paprika (Topuz, 2008); tomato derivatives (Manzocco *et al.*, 2006)
Carotenoids	9–125	Osmo-dehydrofrozen tomatoes (Dermesonlouoglou *et al.*, 2007)
Lycopene	25–49	Frozen vegetables (Giannakourou and Taoukis, 2003)
Vitamin C	71–112	Orange juice (Manso *et al.*, 2001); osmo-dehydrofrozen tomatoes (Dermesonlouoglou *et al.*, 2007)

excessive levels of rancidity, which are not relevant to normal storage conditions. For instance, Kaya *et al.* (1993) evidenced an overestimation of the induction periods at room temperature when performing the tests at temperatures >100 ºC. In addition, a kinetic study of olive oil triacylglycerol oxidation indicates that the Arrhenius equation could be employed to describe the temperature dependence of primary and secondary oxidation product formation only between 25 and 75 ºC (Gomes-Alonso *et al.*, 2004). According to Frankel (2005), the more polyunsaturated the oils, the lower the temperatures that should be used to test their oxidative stability; for instance, vegetable oils should be tested at temperatures lower than 60 ºC while fish oils only below 40 ºC. Beside the rancidity level reached in ASLT, the eventual thermal degradation of minor compounds with pro- or antioxidant activity could become critical since they can modify the temperature dependence of the overall oxidation rate of lipids.

Additional and even more intricate complications arise in multi-component foods, in which lipids, carbohydrates and proteins could react at high temperatures producing new compounds. The latter could affect the oxidation rate by acting as pro- or antioxidants, e.g., Maillard reaction products. Moreover, it is evident that the successful application of the Arrhenius model is that food is able to withstand the increase in temperature without leading to dramatic development of phenomena other than oxidative reactions responsible for product unacceptability at usual storage temperatures. For instance, at high temperature, pigment bleaching upon oxidation can be masked by the concomitant

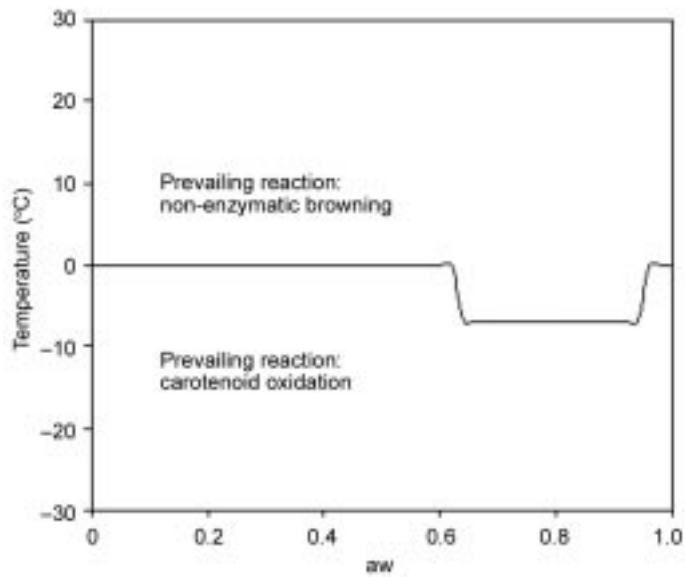

Fig. 9.5 Temperature and water activity combination discriminating conditions associated to the prevalence of carotenoid oxidation or non-enzymatic browning in tomato derivatives.

development of other colour-effective reactions, such as non-enzymatic browning. An example relevant to tomato derivatives is reported in Fig. 9.5, which shows the combinations of storage temperatures and product water activities discriminating conditions associated to the prevalence of carotenoid oxidation or non-enzymatic browning. For instance, in the case of shelf life accelerated tests of frozen foods containing tomato, storage temperatures above −10 ºC should be avoided.

Finally, modifications in the physical structure (e.g. phase transitions or viscosity changes) upon temperature increase could unexpectedly modify the temperature dependence of the oxidation rate. For these reasons, it is evident that extrapolation of oxidation rates at usual storage temperatures from accelerated data must be performed only within the temperature range experimentally proven to conform to the Arrhenius model. In other words, the Arrhenius methodology requires being adapted to the specific circumstances of the product being considered.

Unfortunately, deviation from linearity of Arrhenius equations may also be detected not only at accelerated storage conditions but also near the actual storage temperature. For instance, Arrhenius deviations for peroxide formation in biscuits were observed at their usual storage temperature (20 ºC) (Fig. 9.6(a)). Similar deviations were also detected for carotenoid decolouration in frozen tomato powders below 0 ºC (Fig. 9.6(b)). In both cases, the existence of a critical temperature below which the Arrhenius equation is not fulfilled can be attributed to the occurrence of phase transitions of lipids and/or water as a consequence of temperature changes (Parker and Ring, 1995; Kristott, 2000; Calligaris *et al.*, 2004, 2006, 2007b, 2008; Manzocco *et al.*, 2007).

When deviations are observed, the availability of other models should be verified. In this context, the Williams–Landel–Ferry (WLF) model (1955), based on the variation of the viscosity in the temperature range above the glass transition temperature (T_g), has been applied to describe the rate–temperature relation in foods and biological systems. However, the application of this model is often precluded to oxidative reactions due to the limited significance of T_g for lipid components. When a feasible model correctly predicting the temperature dependence of the oxidation rate is not available, new models must be identified. Such models may be simply descriptive ones (Waterman and Adami, 2005) or built up starting from the understanding of the physicochemical phenomena leading to the Arrhenius deviation.

In the case of lipid oxidation, deviations from the Arrhenius behaviour of oxidation rate may be the result of the occurrence of phase transitions. In fact, crystallizations could induce a cascade of temperature-dependent events, such as solute concentration and changes in physicochemical properties (i.e., reactant solubility, pH, ionic strength, water activity, viscosity) in the liquid phases surrounding crystals (Parker and Ring 1995; Fennema 1996; Champion *et al.*, 1997). These compositional modifications could counterbalance and/or even oppose the direct effect of temperature on the oxidation rate, giving reason to the observed deviations.

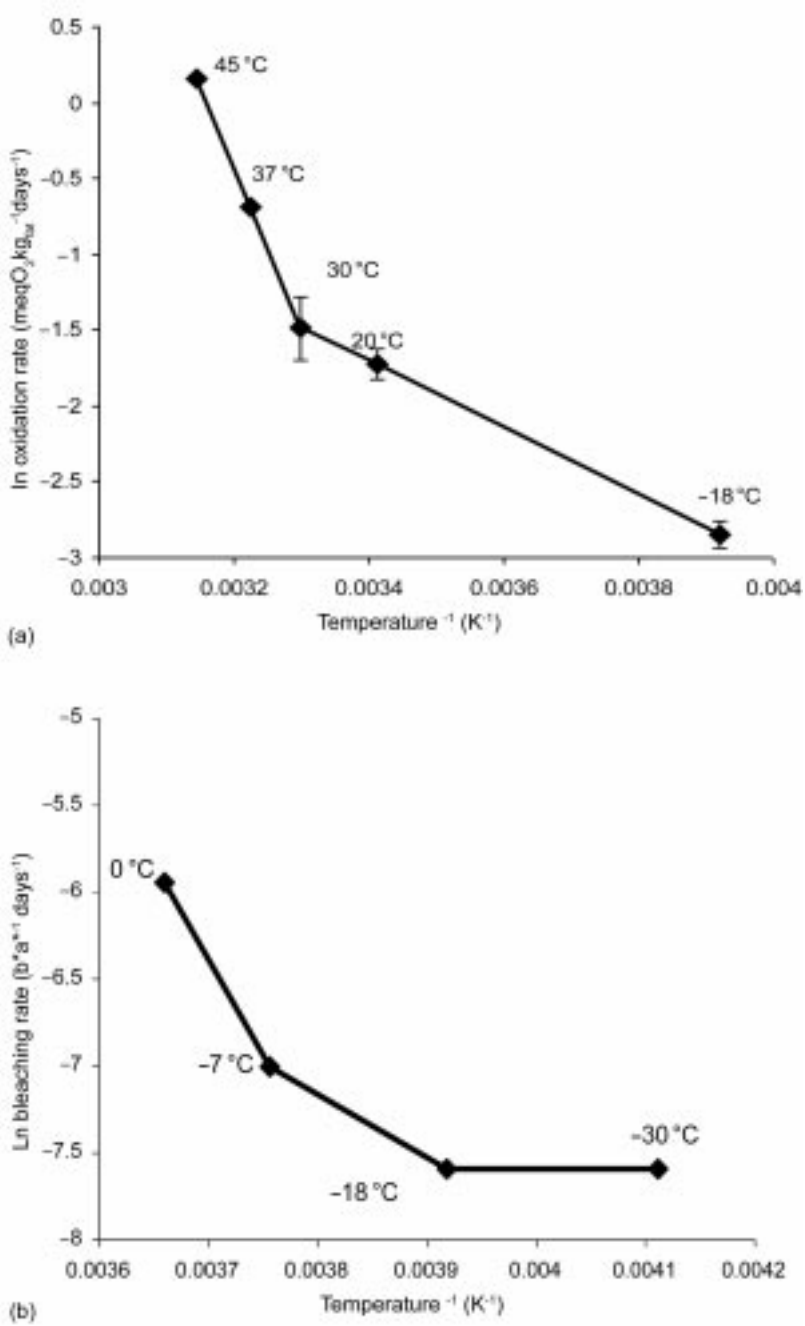

Fig. 9.6 Arrhenius plots of apparent oxidation rate of biscuits (a) and tomato powders (b) (elaborated from Calligaris *et al.*, 2007 and Manzocco *et al.*, 2006).

In order to find out a feasible predictive model, Calligaris *et al.* (2004) proposed a modified Arrhenius equation that can be used to predict the temperature dependence of the oxidation rate in partially crystallised matrices:

$$k = k_0 \cdot \Delta k \cdot e^{-E_a/RT} \qquad 9.8$$

where Δk is a corrective factor included into the Arrhenius equation to take into account the influence of variables, other than temperature, which significantly affect the reaction rate in the partially crystallized matrix. Since at a given temperature the rate at which any reaction develops can be considered as the result of the ratio between driving forces and resistances, Δk can be defined by the identification of the proper forces and resistances involved in the Arrhenius deviation.

Starting from the simplest case, involving only lipid phase transition, the changes in the relative reactant concentration has been indicated as the main driving force affecting the oxidation rate (Calligaris *et al.*, 2004). In fact, the progressive separation of fat crystals gradually leads to an increase in the reactant concentration (i.e., unsaturated TAGs, O_2, antioxidants and pro-oxidants) in the liquid phase surrounding fat crystals causing an unexpected acceleration of the oxidation rate. In such context it should be remembered that the phase transition behaviour of triacylglycerols is peculiar due to their compositional complexity. As temperature decreases, a number of crystallisation events could take place: the higher saturated fractions firstly crystallize followed by the more unsaturated ones. In addition, the geometry of the crystals could greatly differ depending on heating/cooling profile and storage conditions according to lipid polymorphic properties (Sato, 1999).

In order to quantify the effect of the changes in the reactant concentration as a consequence of crystallization and build up a predictive model, a concentration factor (C), defined as the ratio between the liquid fraction (LF) originally present in the sample and the liquid fraction at selected temperatures, has been proposed. C value indicates how many times compounds involved in the oxidative process concentrate in the liquid phase as a consequence of crystallization. A C value equal to 1 indicates that, at the given temperature, crystallization did not occur and sample concentration remained unchanged. By contrast, C values higher than 1 indicate that lipid/water partially crystallized at the given temperature leading to a C-times increase in the concentration of the liquid phase.

The liquid fraction (LF) of the oil/fat at selected temperatures could be easily determined by the partial integration of the melting curve obtained via differential scanning calorimetry. In such way the enthalpy of the mass melted at the selected temperature can be computed and compared with the total peak enthalpy (Fig. 9.7). Table 9.8 shows, as example, the liquid fraction and the relevant C factor of the fat extracted from biscuits at different temperatures.

After the computation of the C factor at different temperatures, such value can be integrated in the modified Arrhenius equation (9.8). Figure 9.8 graphically shows the temperature dependence of the new dependent variable

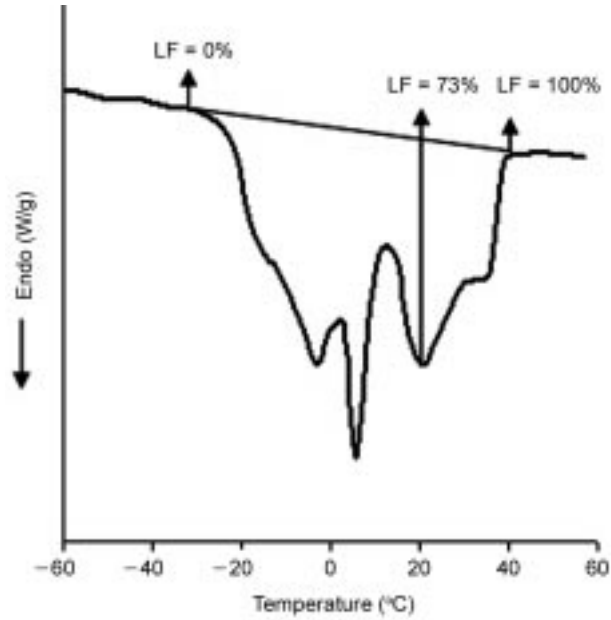

Fig. 9.7 Melting thermogram of fat extracted from biscuits (elaborated from Calligaris *et al.*, 2007a).

($k_{ox}C^{-1}$) in an Arrhenius plot. The linear relationship between this variable and the reciprocal of temperature is evident, indicating that the modified Arrhenius equation efficiently predicts oxidation rate of biscuits over a wide temperature range.

A similar approach has been adopted to obtain linearism of deviating Arrhenius plots and to develop accurate predictive models to estimate shelf life of other products such as sunflower oil and bread sticks (Calligaris *et al.*, 2004, 2008).

Table 9.8 Liquid fraction (LF, % w/w) and corresponding concentration factor (C) of oil extracted from biscuits as a function of temperature

Temperature (ºC)	LF (% w/w)	Concentration factor (C)
−18	3.21	31.1
20	73.4	1.36
30	90.6	1.10
37	98.5	1.01
45	100	1.00

From Calligaris *et al.* (2007a) with permission.

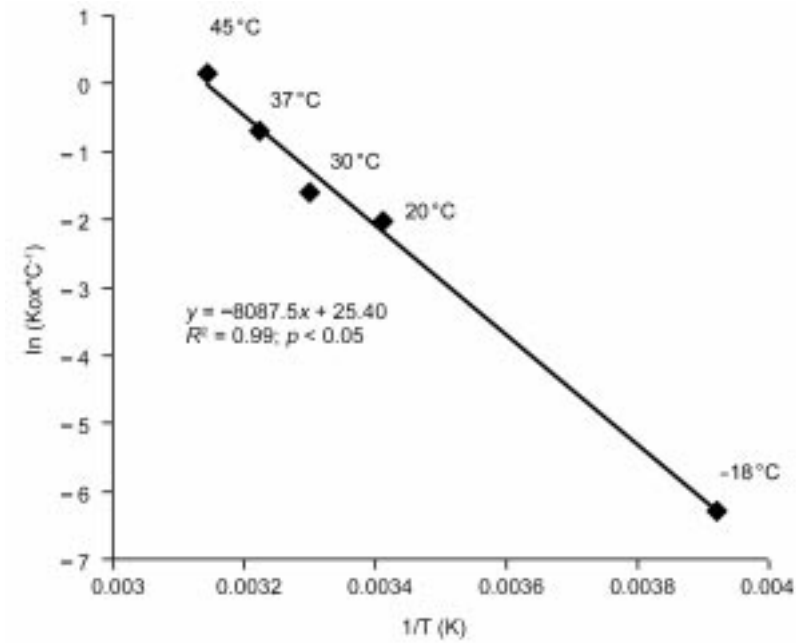

Fig. 9.8 Modified Arrhenius plots of apparent oxidation rate of biscuits (Calligaris *et al.*, 2007a with permission).

By applying a similar approach, modified Arrhenius equations can be identified for foods containing water undergoing crystallization below 0 °C. For instance, in tomato derivatives undergoing carotenoid oxidation, the concentration of reactants in the unfrozen aqueous solution was assessed by calorimetric analysis (Manzocco *et al.*, 2006). However, in this case, to obtain an effective predictive model, beside the *C*-factor relevant to the water phase, an additional factor has to be considered in the modified Arrhenius equation. Since oxygen concentration in the aqueous solution is well-known to increase as temperature decreases affecting the reaction rate, Δk has been defined as follows:

$$\Delta k = C \cdot O \qquad 9.9$$

where O is the oxygen solubility factor defined as the ratio between oxygen solubility at temperature T and oxygen solubility at 0 °C. Figure 9.9 shows the goodness of the fitting of the new dependent variable $kC^{-1}O^{-1}$ versus temperature. A similar approach was found to work well even for tomato derivatives having different water activity.

9.5.4 Other accelerating factors

The Arrhenius model performs well only if temperature really affects the reaction rate in reasonable times, which is not always the case. For instance, in the case of

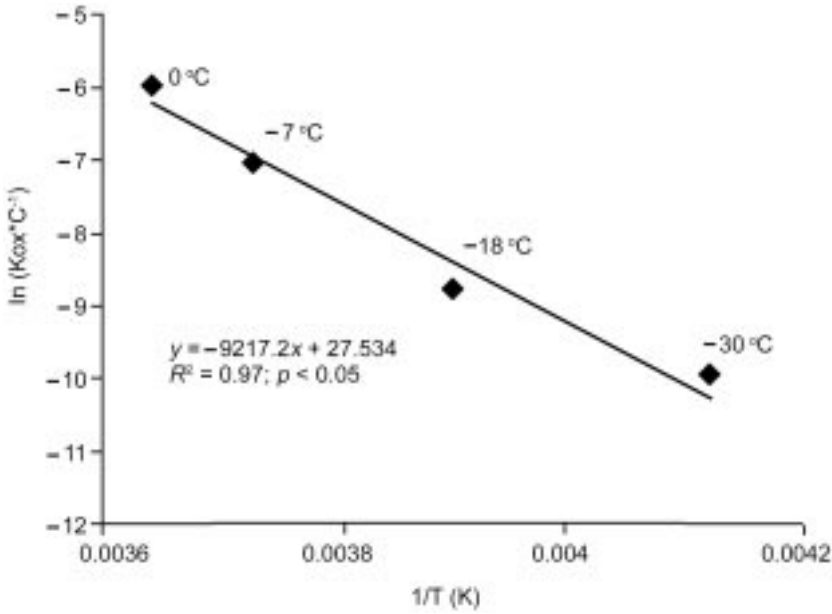

Fig. 9.9 Modified Arrhenius plots of apparent oxidation rate, expressed as $kC^{-1}O^{-1}$, of tomato puree as a function of temperature (elaborated from Manzocco *et al.*, 2006).

coffee derivatives packed under modified atmosphere, temperature may not be a proper accelerating factor for ASLT. In this regard, it has been reported that the change in temperature from 4 to 35 ºC of roasted coffee having less than 3% oxygen concentration in the head space did not allow reducing their shelf life testing times below 30 weeks (Cardelli and Labuza, 2001). On the basis of these considerations, it is evident that accelerating factors other than temperature should be identified and used to develop ASLT methodologies allowing correct extrapolation of shelf life data at usual storage conditions. Since the oxidative reaction is strongly dependent on oxygen concentration, this parameter could represent a potentially exploitable accelerating factor to develop proper shelf life assessment methodologies and relevant predictive models.

Similarly, light-induced oxidative reactions may be scarcely temperature dependent events. In this regard, Kristensen *et al.* (2001) showed that riboflavin was degraded on light exposure independently of storage temperature. More recently, Manzocco *et al.* (2008) demonstrated that carotenoid oxidation in beverages was strongly temperature dependent (from 20 to 40 ºC) under dark ($E_a = 29\,\text{kJ mol}^{-1}$) but, at increasing light intensity, temperature had a much smaller effect leading to negligible acceleration of the oxidation rate ($E_a = 8\,\text{kJ mol}^{-1}$).

The common practice is for most photosensitive foods to be packed in see-through materials and exposed on very well-lit shelves. For this reason, the extent of oxidative degradation is likely to be more related to the intensity of the

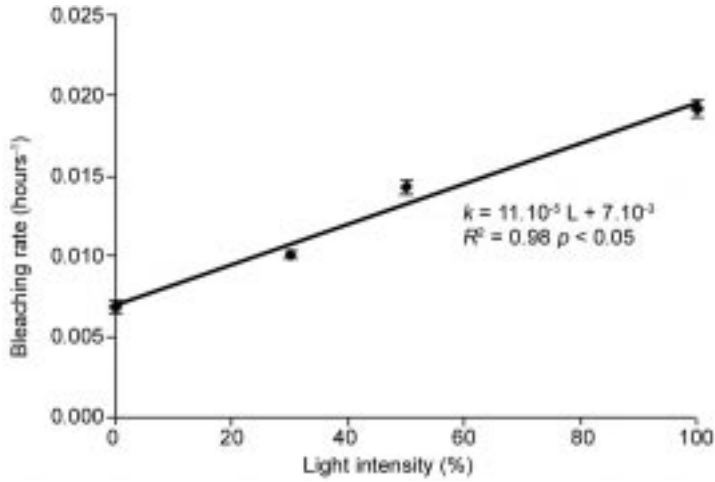

Fig. 9.10 Bleaching rate constants (k) of a saffron-containing beverage stored at 20 ºC under increasing light intensity (L). Results of linear regression analysis are also shown (Manzocco *et al.*, 2008 with permission).

light the food is exposed to rather than to the environmental temperature. In this regard, Fig. 9.10 shows the light dependence at 20 ºC of colour fading of a saffron-containing beverage upon pigment oxidation under increasing light conditions.

It is thus evident that light can speed up the oxidation rate of photosensitive foods thus strongly affecting their shelf life. At constant temperature, a linear relation between bleaching rate (k_L) and light intensity (L) was obtained:

$$k_L = m \cdot L + n \qquad 9.10$$

where m and n are experimental parameters. Light could thus be easily used as a non-conventional accelerating factor in shelf life testing by measuring bleaching rate under increasing light intensity and then extrapolating the rate to the milder conditions usually experienced by the product on the retail shelves.

It is also possible to integrate into a single model the effect of both light and temperature:

$$k_L = (m \cdot L + n) e^{\left(\frac{p \cdot L + q}{R}\right) \cdot \left(\frac{1}{T} - \frac{1}{T_{ref}}\right)} \qquad 9.11$$

where p and q are experimentally determined parameters. Such a model, which was validated for a saffron-containing beverage (Manzocco *et al.*, 2008), is particularly useful and versatile since it allows the prediction of the bleaching rate at any combination of temperature and light intensity within the experimental range considered. In fact, if the beverage is stored in the dark ($L = 0$) at increasing temperatures, the model is brought back to the Arrhenius equation. By contrast, if the beverage is stored at room temperature under increasing light intensity, the model returns to equation 9.10.

This approach, based on exploitation of the accelerating effect of light, is certainly interesting, since it may allow solving the difficult task of predicting shelf life of photosensitive food usually marketed in the presence of light. In addition, another advantage could be represented by the possibility to use light instead of temperature to quickly predict shelf life of foods that cannot withstand high temperature during shelf life testing.

9.6 Future trends

Shelf life assessment of food undergoing oxidation has always represented a challenge for food scientists and industry managers. Since these products are relatively shelf stable, real-time shelf life testing is generally precluded. In fact, the actual industrial timescale for new product development is generally so short that there is little or no opportunity to establish how long the product quality is maintained in storage.

For this reason, food industries need reliable shelf life tests to be carried out in times as short as possible. To shorten the shelf-life assessment process, accelerated shelf-life tests should be applied and proper predictive models developed. However, the generation of effective predictive models for product suffering oxidative reactions could be an arduous task due to the huge number of environmental and compositional factors affecting the oxidation rate. In addition, an established and rational procedure to support the choice of acceptability limits should be clearly defined during the food dating process.

To achieve the goal of setting up reliable shelf life assessment strategies, more research has to be done in order to:

- develop criteria and protocols to support the choice of acceptability limits really accounting for consumer response;
- improve the understanding of the relation between product oxidation level and consumer acceptability;
- provide a deeper insight of the influence of food matrix properties on temperature dependence of oxidation rate;
- identify environmental accelerating factors other that temperature and develop proper shelf life predictive models.

The availability of this information will allow producers to manage shelf life labelling by the adoption of rigorous, systematic and well-designed shelf life assessment that are consumer driven, company tailored and routinely performable in a short time.

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10

Understanding antioxidant mechanisms in preventing oxidation in foods

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Abstract: Many foods are becoming more susceptible to oxidative rancidity due to attempts to make foods healthier by increasing polyunsaturated fatty acids, and more sustainable by introducing light weight oxygen-permeable and light-penetrating packaging. Unfortunately, very few new food antioxidants have been made available over the past several decades and the use of synthetic antioxidants is disfavored by many consumers. Thus, in order to make natural, more sustainable and healthier foods, the food technologist must find ways to use existing antioxidants more effectively. This requires a strong understanding of antioxidant chemistry. This chapter reviews the chemistry of free radical scavengers, metal chelators, singlet oxygen quenchers and antioxidant enzymes. Interactions between antioxidants to enhance activity are also discussed.

Key words: antioxidants, free radical scavengers, chain breaking antioxidants, metal chelators, singlet oxygen quenchers, synergistic antioxidant activity, tocopherol, ascorbic acid, carotenoids, BHA, BHT, TBHQ, propyl gallate, EDTA, polyphosphates, citric acid, antioxidant interactions.

10.1 Introduction

The oxidative susceptibility of foods is increasing due to factors such as removal of hydrogenated oils, use of light weight oxygen-permeable and light-penetrating packaging and inclusion of bioactive polyunsaturated fatty acids. Therefore, new antioxidant technologies are needed to make lipid-containing

foods healthier and more environmentally friendly. Traditionally, antioxidants were thought of as compounds that scavenge free radicals. However, as the food industry struggles to find ways to inhibit oxidative deterioration, it is often more useful to broadly define antioxidants as any compounds which inhibit the oxidative processes that deteriorate lipids and, therefore, food quality. Antioxidant mechanisms which fit this definition include free radical scavenging, control of hydroperoxides and other reactive oxygen species, chelation of metals, and techniques to physically encapsulate lipids to inhibit their oxidation.

Biological tissues have an exquisite system for inhibiting lipid oxidation. This is because atmospheric oxygen is itself a radical and the metabolism of oxygen can produce other reactive oxygen species such as superoxide anion and hydrogen peroxide, thus placing biological tissues under constant oxidative stress. Since we obtain most foods from biological tissues, we can expect them to contain multiple endogenous antioxidant systems. These endogenous antioxidants include several distinctively different mechanisms in order to protect against different prooxidative compounds including transition metals, heme-containing proteins, enzymes, photosensitizers and numerous sources of free radicals. Since prooxidants and free radicals are both water- and lipid-soluble, endogenous antioxidant systems are found in essentially all phases of the food. These multi-component and multi-phase antioxidants represent nature's own hurdle technology antioxidant system, which can be a guide to developing novel antioxidant systems for foods.

Unfortunately, many food processing operations can destroy endogenous antioxidant systems. These can include operations such as particle size reductions that can destroy the physical organization of the cell; heating, which can inactivate antioxidant enzymes; and oil refining operations that remove naturally occurring antioxidants. In addition, food processing operations can alter cellular components to increase oxidative stress by increasing oxygen concentrations and releasing protein-bound metals. Therefore it is often necessary to add exogenous antioxidants to processed foods. These antioxidants can be obtained from natural sources or chemically synthesized. Since consumers are concerned about synthetic food additives, including antioxidants, the search for additional natural antioxidants has been the subject of significant research over the past several decades.

This chapter will cover the basic mechanisms by which antioxidants influence oxidative reactions including: inactivation of free radicals, metal chelation, singlet oxygen quenching, inactivation of oxidation intermediates and the impact of physical structures on antioxidant activity.

10.2 Inactivation of free radicals

Antioxidants can inhibit the initiation and propagation steps of lipid oxidation by scavenging and reducing the energy of free radicals. Free radical scavengers (FRS) or chain breaking antioxidants accept a radical from oxidizing lipids

species such as fatty acid peroxyl ($LOO^{\bullet}$) or alkoxyl ($LO^{\bullet}$) radicals by the following reactions (Bolland, 1947).

$$LOO^{\bullet} \text{ or } LO^{\bullet} + FRS \longrightarrow LOOH \text{ or } LOH + FRS^{\bullet}$$

Peroxyl radicals are thought to be the radicals most likely to interact with FRS since the lower energy of peroxyl radicals limits them to interacting with compounds with a labile hydrogen such as an unsaturated fatty acids or FRS. Higher energy free radicals such as alkoxyl radicals and hydroxyl radicals can abstract hydrogen from antioxidants but they will also undergo competing reactions such the involvement of alkoxyl radicals in β-scission reactions and the interaction of high energy hydroxyl radicals in their immediate vicinity (Buettner, 1993; Liebler, 1993a).

The ability of a FRS to inhibit lipid oxidation is linked to its ability to interact with free radicals more efficiently than unsaturated fatty acids, and to produce lower energy antioxidant radicals that do not readily promote further oxidation. Chemical properties that are important to the effectiveness of a FRS include reduction potential, hydrogen bond energies, resonance delocalization and susceptibility to autoxidation. Initially, antioxidant efficiency is dependent on the ability of the FRS to donate hydrogen to the free radical. As the bond energy of the donating hydrogen on the FRS decreases, the transfer of the hydrogen to the free radical is more energetically favorable and thus more rapid. One way to predict the ability of a FRS to donate a hydrogen to a free radical is from standard one electron reduction potentials (Buettner, 1993). Any compound which has a reduction potential lower than the reduction potential of a free radical (or oxidized species) is capable of donating a hydrogen to that free radical unless the reaction is kinetically unfeasible (Table 10.1). Examples of compounds with reduction potentials lower than peroxyl radicals ($E^{\circ\prime} = 1000$ mV) include epigallocatechin 3-gallate ($E^{\circ\prime} = 530$ mV), α-tocopherol ($E^{\circ\prime} = 480$ mV), quercetin ($E^{\circ\prime} = 330$ mV), and ascorbate ($E^{\circ\prime} = 282$ mV). These lower reduction potentials mean that these compounds can donate a hydrogen to peroxyl radicals to form a hydroperoxide. Standard reduction potentials can also be used to predict the rate by which a compound can donate a hydrogen to the peroxyl radical. For instance, the above antioxidants (282–530 mV) have lower reduction potentials than the methylene interrupted hydrogen of a polyunsaturated fatty acid ($E^{\circ\prime} = 600$ mV),allowing them to react more rapidly with peroxyl radicals ($E^{\circ\prime} = 1000$ mV) and, thus, being preferentially oxidized before unsaturated fatty acids.

The ability of a FRS to inhibit lipid oxidation is also dependent on the energy of the resulting free radical scavenger radical ($FRS^{\bullet}$). If the $FRS^{\bullet}$ is a low energy radical, than the likelihood of the $FRS^{\bullet}$ catalyzing the oxidation of other molecules decreases. The most efficient FRS have low energy radicals due to resonance delocalization (Fig. 10.1; Nawar, 1996; Shahidi, 1992). The low energy of the $FRS^{\bullet}$ can also be shown by standard reduction potentials where FRS such as α-tocopherol and catechol (480 and 530 mV respectively) have lower reduction potentials than polyunsaturated fatty acids (600 mV) and

Table 10.1 The one electron reduction potential (E') of the radical form of reactive oxygen species, phenolic antioxidants, carotenoids, and flavonoids

Compounds	$E^{\circ\prime}$ (mV)	Ref.	Compounds	$E^{\circ\prime}$ (mV)	Ref.
H_2O	2310	1	Uric acid	590	9
CH_3CH_3	1900	1	pyrogallol	575	10
ROH (aliphatic alkoxyl radical)	1800	1	Catechin	570	7
H_2O_2 ($HOO^{\bullet}$, H^+)	1060	1	3,4-dihydroxyphenylalanine (DOPA)	570	7
β-carotene in TX-100	1060	2	EC	570	9
Canthaxanthin in TX-100	1041	2	Gallic acid	560	11
Zeaxanthin in TX-100	1031	2	Methyl gallate	560	11
Astaxanthin in TX-100	1030	2	Sinapic acid	556	8
β-carotene in TX-405/ TX-100	1028	2	Chlorogenic acid	550	8
ROOH (alkyl peroxyl radical)	1000	1	ECG	550	9
Lycopene in TX-405/ TX-100	980	2	3,4-dihydroxylcinnamic acid	540	10
Astaxanthin	970	3	Theaflavin digallate	540	12
Canthaxanthin	950	3	Caffeic acid	534	8
H_2O_2 ($O_2^{\bullet -}$, $2H^+$)	940	1	Catechol	530	1
Glutathione	920	4	4-Methylcatechin	520	6
Zeaxanthin	850	3	4-methylcatehol	520	11
β-carotene	840	3	Theaflavin	510	12
Resorcinol	810	10	Taxifolin	500	5
Kaempferol	750	5	α-tocopherol	500	1
Hesperidin	720	6	Trolox	480	1
Galangin	620	5	EGC	430	6
3,4-dihydroxylbenzoic acid	600	7	EGCG	430	11
Luteolin	600	5	Myricetin	360	5
Morin	600	5	Fisetin	330	10
PUFA (*bis*-allylic-H)	600	1	Quercetagetin	330	5
Rutin	600	7	Quercetin	330	6
o-Coumaric acid	596	8	Ascorbic acid	282	1
Ferulic acid	595	8	Fe(II) EDTA	120	1
b-coumaric acid	590	8	Fe(II) (aqueous)	110	1
p-Coumaric acid	590	8	Fe(II) citrate	100	1
			Ubiquinone	−36	1
			Dehydroascorbic acid	−174	1

1. Buettner, 1993; 2. Burke *et al*., 2001; 3. Han *et al*., 2010; 4. Sharma and Buettner, 1993; 5. Jovanovic and Simic, 2000; 6. Jovanovic *et al*., 1996; 7. Jovanovic *et al*., 1994; 8. Foley *et al*., 1999; 9. Luczaj and Skrzydlewska, 2005; 10. Rice-Evans and Packer, 2003; 11. Jovanovic *et al*., 1995; 12. Jovanovic *et al*., 1997).

therefore do not efficiently abstract a hydrogen from unsaturated fatty acids (Table 10.1, 2). Effective FRS also have $FRS^{\bullet}$ that do not readily reactive with triplet oxygen to form hydroperoxides. If a FRS forms a hydroperoxide, it could decompose into higher energy free radicals species that could oxidize unsaturated fatty acids (Liebler, 1993a).

Another way to look at the potential activity of antioxidants is through their bond dissociation enthalpy, also called bond dissociation energy (BDE). BDE is the standard enthalpy change when a chemical bond is broken by homolytic or

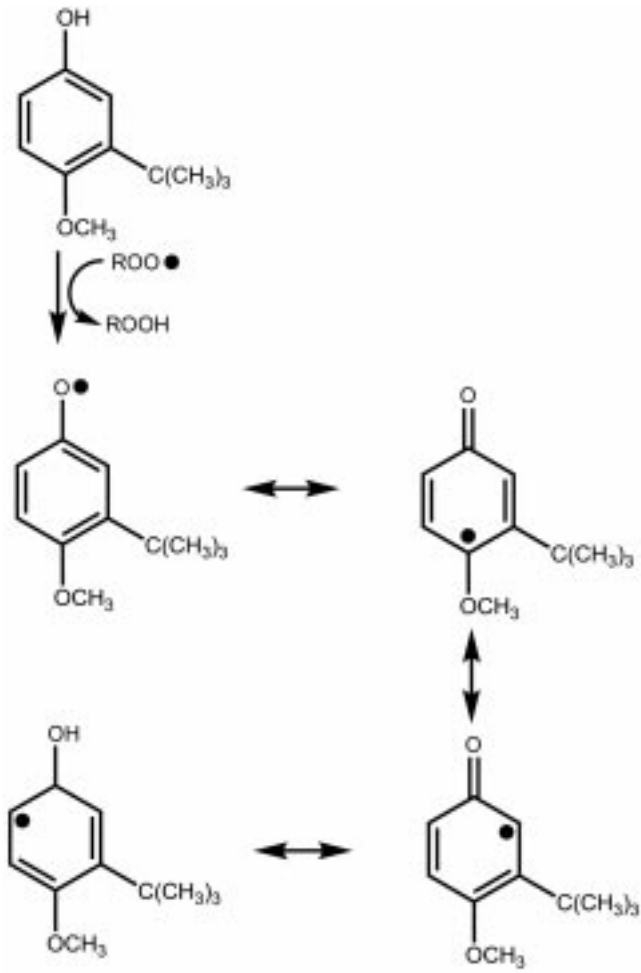

Fig. 10.1 Resonance stabilization of a free radical by a phenolic.

hemolytic cleavage (Luo, 2007). The BDE is also considered an important thermodynamic quantity that determines the stability of a radical and thus its propensity to promote the oxidation of fatty acids (Berkowitz, 1994; Menon, 2007). The minimum O–H bond dissociation enthalpy (OH–BDE_{min}) of phenolic antioxidants is usually correlated to the rate constant of the ability of an antioxidant to inhibit chain propagation (Amiæ, 2009; Foti, 2007; Takeuchi, 2007; Thavasi, 2009; Zhu, 1997). The experimental BDE values of hydroxyl groups in various antioxidants and other compounds of interest are shown in Table 10.2.

By the nature of the reaction, FRS by themselves are not able to scavenge an unlimited number of free radicals. At a minimum, a FRS is capable of inactivating at least two free radicals, the first being inactivated when the FRS interacts with a peroxyl or alkoxyl radical and the second when the $FRS^{\bullet}$ enters

Table 10.2 Bond dissociation enthalpies (BDEs) of antioxidants and related compounds

Compounds	BDE (kcal/mol)	Compounds	BDE (kcal/mol)
Ascorbic acid[1,2]	69.00***, 83.00***	Catechol[9,7]	81.80, 82.82*
Dehydroascorbic acid[2]	71.00***	Luteolin[7]	81.91
β-carotene[3]	73.85**	Quercetin[7]	81.98
α-tocopherol[4,8,5]	77.10, 78.87, 79.30	Gallic acid[7, 9]	83.03*, 82.00
BHA[4]	77.20	Mesityl alcohol[5]	82.10
3,5-di-tert-butylcatechol[6]	79.30	Dihydroquercetin[7]	82.12
6,7-Dihydroxyflavone[7]	79.42*	Rutin[7]	82.15*
7,8-Dihydroxyflavone[7]	79.59*	Octyl gallic acid[6]	82.50
Propyl gallic acid[8]	79.97*	Propyl gallic acid[6]	82.60
BHT[4]	80.00	Hesperidin[7]	82.65
β-Glugalline[7]	80.07	Fisetin[7]	82.77
γ-tocopherol[7,5]	80.04, 80.10	Epicatechol[7]	82.84*, 83.13
β-tocopherol[5]	80.20	ρ-hydroquinone[8]	84.13
Ubiquinol-10[5,7]	80.50, 82.53	tert-Butyl hydroperoxide[5]	84.20
3,5-di-tert-butylcatechol[6]	80.70	Hesperidin[7]	84.56
Epigallocatechol[7]	80.71	Chrysin[7]	85.35
2,5-di-tert-pentylhydroquinone[6]	80.80	o-Polyphenol (1,1-biphenyl-2-ol)[8]	86.50
Tannic acid[7]	80.81	Galangin[7]	86.78
Epicatecholgallate (pH7, 9)[7]	81.64, 80.88	Morin[7]	86.90
Pyrocatechol[8]	81.24	Hydroperoxides[5]	87.49
EC[9,7]	81.20, 82.03	ρ-Polyphenol (1,1-biphenyl-4-ol)[8]	88.12
Caffeic acid[7]	81.21*	Resorcinol[8]	88.19
Tocol[7]	81.29	Phenol[5]	90.40
Propyl gallate[7]	81.45	Methanol[5]	105.20
Myricetin[7]	81.48*	Ethanol[5]	105.40
δ-tocopherol[7,9]	81.62, 82.20	H_2O[5]	118.00

* indicated the BDEs observed in micelles, ** indicated the estimated C-H BDE, and *** indicated the BDEs from the DFT methods. Besides the techniques, the different BDEs of the same compounds are dependent on the solvent used and pH of the systems.

1. Wright, 2002; 2. Wang *et al*., 2009; 3. Mortensen and Skibsted, 1998; 4. Foti, 2007; 5. Luo, 2007; 6. Lucarini *et al*., 2002; 7. Denisova and Denisov, 2008; 8. Denisov and Denisova, 2000; 9. Pazos *et al*., 2007

a termination reaction with another radical to form nonradical species (Fig. 10.2). However, some FRS can scavenge more than two free radicals. This can occur when the oxidized FRS still possess a susceptible hydrogen that can be donated to free radicals. In addition, a $FRS^{\bullet}$ can be reduced by another compound to regenerate and reactivate the original FRS. Such a relationship exists between ascorbate and α-tocopherol. This will be discussed in more detail later in the chapter.

It should be re-emphasized that standard reduction potentials and bond dissociation energy cannot solely predict the ability of a compound to scavenge free radicals. As mentioned above, kinetics factors can also impact the rate at which FRS and radicals can interact. For instance, the susceptible hydrogen on a compound could be surrounded by bulky side groups that sterically hinders interactions with free radicals. In addition, the solubility of a compound will

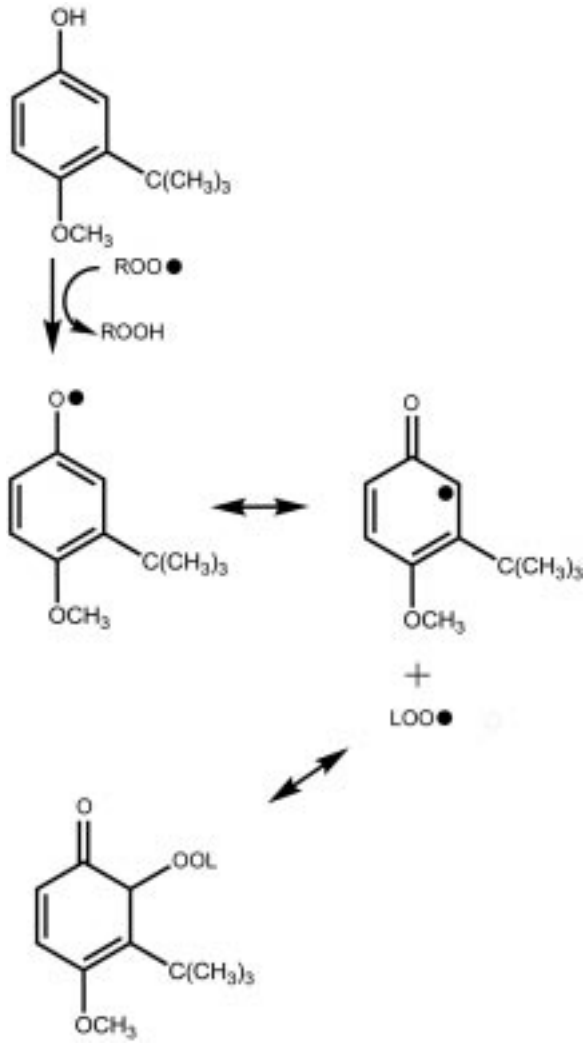

Fig. 10.2 Mechanism by which one phenolic free radical scavenger can inactivate two peroxyl radicals.

impact its ability to interact with free radicals. For example, if a compound is lipid soluble it will not readily react with a water-soluble free radical regardless of its reduction potential or bond dissociation energy. In foods, the efficiency of phenolic FRS is dependent on additional factors besides chemical reactivity. Factors such as volatility, pH sensitivity, interaction with other food components and heat stability can influence the retention and activity of the FRS in stored and processed foods (Nawar, 1996).

Many of the most effective FRS contains a phenol ring somewhere in their structure. Hydrogen donation in phenolics generally occurs through the hydroxyl

group and the subsequent radical is stabilized by resonance delocalization throughout the benzene ring structure. The effectiveness of phenolic FRS can be increased by substitution groups. Alkyl groups in the *ortho*- and *para*-positions enhance the reactivity of the hydroxyl hydrogen towards lipid radicals, bulky groups at the *ortho*-position increase the stability of phenoxy radicals and a second hydroxy group at the *ortho*- or *para*-position stabilizes the phenoxy radical through an intramolecular hydrogen bond (Shahidi, 1992). Below are examples of some of the most common FRS in foods.

10.2.1 Naturally occurring phenolic free radical scavengers

Tocopherols

Tocopherols are a group of 8 different homologs that have a hydroxylated ring system (chromanol ring) with a phytol chain. Differences in tocopherols are due to different degrees of methylation on the chromanol ring with α being trimethylated, β (positions 5 and 8) and γ (positions 7 and 8) being dimethylated and δ being monomethylated (position 8). Tocotrienols have 3 double bonds in the phytol chain while the phytol chain of tocopherols is saturated. Tocopherols have 3 asymmetric carbons on the phytol chain and thus each homolog can have 8 possible steroisomers. Natural tocopherols are found in the all *rac* or *RRR* configuration. Tocopherols originate in plants and eventually ending up in animal foods via the diet (Parker, 1989).

The initial interaction of tocopherol with a free radical results in the formation of tocopheroxyl radicals. Two tocopheroxyl radicals can react to form tocopherylquinone and a regenerated tocopherol (Nawar, 1996). Formation of tocopherylquinone is also thought to occur by the transfer of an electron from a tocopheroxyl radical to a phospholipid peroxyl radical to form a phospholipid peroxyl anion and a tocopherol cation. The tocopherol cation hydrolyzes to 8a-hydroxytocopherone which rearranges to tocopherylquinone (Liebler, 1992). Under condition of extensive oxidation, high concentrations of peroxyl radicals can favor the formation of tocopherol–peroxyl complexes via radical–radical termination reactions. These complexes can hydrolyze to tocopherylquinone. Of less importance are interactions between tocopheroxyl and peroxyl radicals which form an addition product *ortho* to the phenoxyl oxygen followed by elimination of an alkoxyl radical, addition of oxygen and abstraction of a hydrogen to form two isomers of epoxy-8a-hydroperoxytocopherones. Subsequent hydrolysis leads to the formation of epoxyquinones (Liebler, 1990, 1991). Formation of epoxide derivatives of tocopherol represents no net reduction of radicals (due to the formation of an alkoxyl radical) and a loss of tocopherol from the system whereas formation of tocopherylquinone can be regenerated back to tocopherol in the presence of reducing agents (e.g., ascorbic acid and glutathione). An additional reaction which can occur is the interaction of two tocopheroxyl radicals to form tocopherol dimers (Draper, 1967).

Generally, α-tocopherol is the most reactive and less stable form of tocopherol followed by β-, γ-, and δ-tocopherols. This is related to their bond

dissociation energies of 79.1, 80.2, 80.1, and 82.2 kcal/mol, respectively which show that α-tocopherol has the most easily abstracted hydrogen on its hydroxyl group.

Plant phenolics

Besides tocopherols, plants contain a diverse group of phenolic compounds including simple phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives and flavonoids originating from foods such as fruits, spices, tea, coffee, seeds and grains (see Fig. 10.3 for several examples). Many plant phenolics have numerous hydroxyl groups suggesting that they could scavenge multiple free radicals. However, in phenolics such as flavonoids, only the OH group at 1 and 3 position in the B-ring are active, while the OH group at 2 position in the A-ring of flavonoids does not scavenge free radicals (Thavasi, 2009).

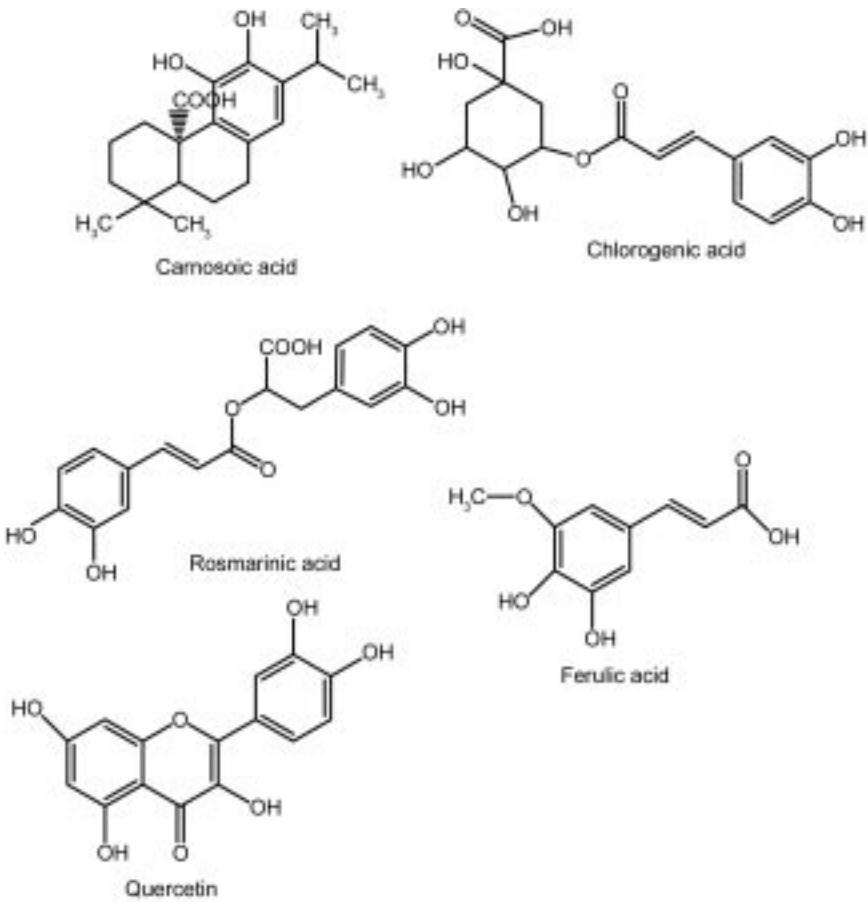

Fig. 10.3 Structures of several important phenolic free radical scavengers found naturally in plants.

There is a large variation in the antioxidant effectiveness of these compounds as they can have multiple properties such as metal reduction and chelation that influence lipid oxidation reactions in addition to their ability to scavenge free radicals. Phenolics that can participate in redox reactions with iron result in the formation of ferrous ions which are stronger prooxidants than their oxidized counterpart, ferric ions (Chen, 1991; Tsuge, 1991; Yamaguchi, 1975). Thus iron reduction by phenolics can increase oxidation rates which is why plant phenolics are sometimes observed to be prooxidative. Some phenolic compounds can chelate iron while others like ferulic acid which do not have a galloyl moiety do not bind iron (Alamed, 2008; Rhee, 1983). Metal chelating by phenolics could inhibit oxidation if the bound metal becomes inactivated but in some cases chelated metals have increased prooxidant activity due to their increased solubility and accessibility to lipids and hydroperoxides.

Literally hundreds of manuscripts have been published on the antioxidant potential of different plant extracts. Unfortunately, many of these papers only evaluated antioxidant potential by simple chemical assays that are not accurate predictors of antioxidant activity in foods (Alamed, 2008; Decker, 2005). Rosemary extracts are the most effective source of phenolic plant antioxidants that is available as a commercial food additive. Carnosic acid, carnosol and rosmarinic acid are the major antioxidant phenolics in rosemary extracts. Rosemary extracts have been found to inhibit lipid oxidation in a wide variety of foods such as meats, bulk oils and lipid emulsions (Aruoma, 1992; Frankel, 1996; Mielche, 1994). Utilization rosemary extracts is limited by the presence of highly flavorful monoterpenes that may not be compatible with all foods. Use of more purified forms of rosemary or other herbal phenolics is restricted by both economic and regulatory hurdles.

Grape seed extract also represents a potential commercially available source of plant phenolics for use as an antioxidant additive. Grape seed extract contains polyphenols that can both scavenge free radicals and chelate metals. The utilization of grape seed extract in foods is limited by the high astringency of polyphenols.

Ubiquinone

Ubiquinone or coenzyme Q, is a phenolic conjugated to an isoprenoid chain found mainly in mitochondria (Zubay, 1983). Reduced ubiquinone is capable inactivating peroxyl radicals; however, its radical scavenging activity is less than α-tocopherol even though its reduction potential is lower (Ingold, 1993). The lower free radical scavenging activity of reduced ubiquinone has been postulated to be due to internal hydrogen bonding, which makes hydrogen abstraction more difficult (Ingold, 1993). Despite the lower radical scavenging activity of reduced ubiquinone, it has been found to inhibit lipid oxidation in liposomes (Frei, 1990) and low density lipoprotein (Stocker, 1991) and presumably could be an important endogenous antioxidant in many foods including red meats that contain large amounts of mitochondria.

10.2.2 Synthetic phenolic antioxidants

The major synthetic antioxidants are substituted phenols of varying polarity (Fig. 10.4). These include butylated hydroxytoluene (nonpolar), butylated hydroxyanisole (nonpolar), tertiary butylhydroquinone (intermediate polarity) and propyl gallate (most polar). Synthetic phenolics can be extremely effective in food systems but their utilization by the food industry is declining due to consumer demand for all natural products.

The antioxidant mechanism of the synthetic phenolics involves the formation of a phenolic radical which neither rapidly catalyzes the oxidation of other unsaturated fatty acids nor reacts with oxygen to form antioxidant hydroperoxides which decompose into free radicals (Shahidi, 1992). Synthetic phenolic radicals can potentially react with each other in mechanisms similar to α-tocopherol resulting in the formation of hydroquinones and phenolic dimers. The phenolic radicals can also react with other peroxyl radicals in termination reactions resulting in formation of phenolic-peroxyl species adducts. In addition, oxidized synthetic phenolic undergo numerous degradation reactions (for a review, see Shahidi, 1992) to form compounds that still contain active hydroxyl groups and thus can further scavenge free radicals. Therefore, the net antioxidant activity of synthetic phenolics in food actually represents the activity of the original phenolic plus some of its degradation products.

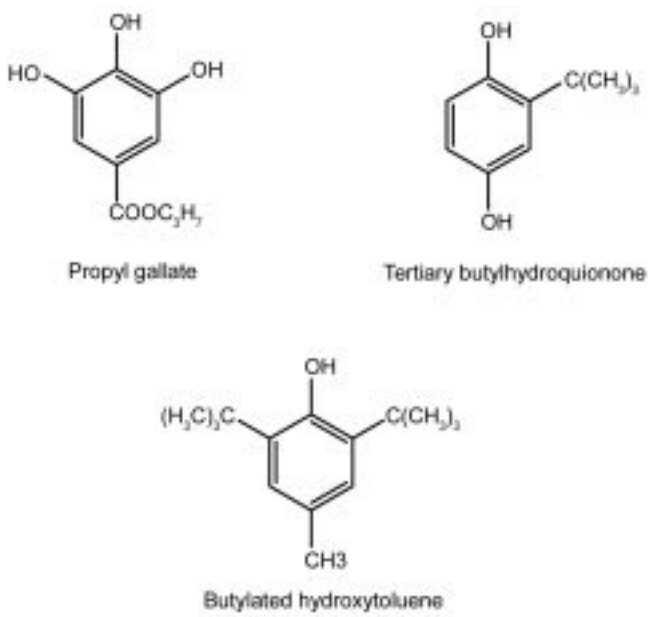

Fig. 10.4 Structures of several important synthetic free radical scavengers approved for use in foods.

10.2.3 Other naturally occurring free radical scavengers

Carotenoids

Carotenoids contain 3-13 conjugated double bonds and in some cases 6 carbon hydroxylated ring structures at one or both ends of the molecule (Olson, 1993). The carotenoid, β-carotene, is the major dietary source of vitamin A. Vitamin A is often referred to as an antioxidant vitamin but its effectiveness along with the effectiveness of other carotenoids is extremely dependent on environmental conditions and the nature of oxidation catalyst. For example, carotenoids can be effective antioxidants in the presence of singlet oxygen (discussed in later sections) and can inhibit free radical promoted oxidation under conditions of low oxygen concentrations.

The free radical scavenging mechanism of β-carotene involves its reaction with lipid peroxyl radicals to form a carotenoid radical. Under conditions of high oxygen tension, the antioxidant activity of β-carotene is diminished due to the increased formation of carotenoid peroxyl radicals that favor autoxidation over inactivation of lipid peroxyl radicals. Under conditions of low oxygen tension, the lifetime of the carotenoid radical is long enough so that it may react with another peroxyl radical thus forming a nonradical species and effectively inhibiting oxidation by eliminating radicals from the system (Burton, 1984).

Incubation of β-carotene with peroxyl radical generators in organic solvents at high (atmospheric) oxygen tensions leads to additional reactions to form carotenoid-peroxyl adducts. Addition of a peroxyl radical to the cyclic end group or the polyene chain followed by loss of alkoxyl radicals leads to the formation of 5, 6 and 15, 15′ epoxides. Elimination of the alkoxyl radical from the 15, 15′ positions can also cause cleavage of the polyene chain resulting in formation of aldehydes. Since the formation of β-carotene epoxides from the addition of peroxyl radicals results in the formation of an alkoxyl radical, the net change in radical number is zero and thus an antioxidant effect is not expected (Liebler, 1993b).

β-Carotene can also inactivate peroxyl radicals by donation of an electron to produce a β-carotene cation radical and a peroxyl anion. The resulting cation radical is less likely to react with oxygen to form hydroperoxides due to its resonance stabilization over the conjugated double bond system. However, the β-carotene cation radical has sufficient energy to oxidize other lipophilic hydrogen donors, including tocopherols and ubiquinone (Liebler, 1993b). Overall, carotenoids are not very effective free radical scavengers in food systems. In addition, carotenoids can be prooxidative through their ability to reduce metals (Boon, 2009). Finally, even if conditions in a food product were favorable to allow effective free radical scavenging, these reactions lead to the bleaching of the carotenoid and thus result in a change in the color of the food during storage.

Ascorbic acid

Biological systems contain both lipid- and water-soluble free radicals. Water soluble free radicals are generated by processes such as the Fenton reaction

which produces hydroxyl radicals from hydrogen peroxide (Dunford, 1987; Kanner, 1987). Ascorbic acid is one of the major water-soluble free radical scavengers found in biological tissues. Ascorbic acid has a low reduction potential so it is effective at scavenging free radicals and forming low energy radicals (Buettner, 1993). However, other reactions strongly influence whether ascorbate will act as an antioxidant in food. Ascorbate will rapidly reduce transition metals thus increasing their ability to decompose hydrogen and lipid hydroperoxides into free radicals (Decker, 1992; Kanner, 1992). Ascorbate can also increase the concentration of reactive iron by promoting its release from proteins such as ferritin and phosvitin (Halliwell, 1986; Jacobsen, 2001). Because of this potential prooxidant activity, ascorbate is often not an effective antioxidant in food (Alamed, 2009; Decker, 1992).

Thiols

Thiols such as cysteine and glutathione are capable of scavenging free radicals; however the energy of the resulting thiol radical is high suggesting that it may promote oxidation (Buettner, 1993). Thioctic acid is another thiol which can inactivate peroxyl radicals (Kagan, 1992). However, as with ascorbate, the reduced state of thioctic acid, dihydrolipoic acid (Bast, 1988) and cysteine (Kanner, 1986) can be prooxidative due to their strong reducing potential and thus their ability to stimulate metal-catalyzed oxidation. Cysteine in proteins and peptides is a more effective antioxidant than free cysteine. More information on the antioxidant activity of amino acids and proteins can be found in Chapter 11.

10.3 Metal chelation

Transition metals maybe the most important prooxidant in foods due to their existence in essentially all biological tissues and their ability to directly abstract hydrogen from fatty acids to form alkyl radicals and to decompose hydroperoxides into free radicals (Kanner, 1987). In many foods, iron promoted oxidation can be decreased by removal of metals from raw materials (e.g., refined oils and water) and avoiding metal contamination from sources such as processing equipment, food ingredients and packaging. When prooxidant cannot be removed from foods, their activity is controlled by chelators or sequestering agents. Simple chelation of transition metals does not guarantee that they will not promote lipid oxidation. This is because transition metals such as iron exhibit low water solubility at pHs near neutrality (Dunford, 1987). If the metal is not soluble its ability to promote oxidation is diminished. Some metal chelators will increase metal solubility and thus increase reactivity. The prooxidant activity of metals is also influenced by their redox state. In general, the reduced state of a metal is more reactive and more soluble (Mahoney, 1986). Once a reduced metal promotes oxidation it is oxidized, meaning that factors that promote metal redox cycling are also very prooxidative. Chelators can influence the redox cycling of metals by increasing their solubility and potential

to interact with reducing agents or by binding them in a manner that reduces their redox potential thus making redox cycling easier.

Chelators which exhibit antioxidative properties inhibit metal catalyzed reactions by one or more of the following properties: prevention of metal redox cycling; occupation of all metal coordination sites; formation of insoluble metal complexes; and steric hindrance between metals and lipids or oxidation intermediates (e.g., peroxides) (Graf, 1990). The prooxidative/antioxidative properties can be dependent on both metal and chelator concentrations. For instance, ethylenediaminetetraacetic acid (EDTA) is prooxidative when EDTA : iron ratios are < 1 and antioxidative when EDTA : iron is > 1 (Mahoney, 1986). This is because at low EDTA:iron ratios, iron is still redox active.

Chelators are typically water soluble but some will exhibit solubility in lipids (e.g., citric acid) thus allowing for inactivation of metals in the lipid phase (Lindsay, 1996). Chelator activity is dependent on pH since the chelator must be ionized to be active. Chelator activity can also be decreased by the presence of other chelatable ions (e.g., calcium) which will compete with prooxidative metals for binding sites.

10.3.1 EDTA

Ferdinand Munz was the first to describe EDTA in 1935. EDTA preferentially binds ferric iron. In most food systems, EDTA is the most effective metal chelator to inhibit lipid oxidation when the EDTA concentration is greater than the concentration of prooxidant metals. The effectiveness EDTA is due to it high iron stability constant (1.2×10^{25}) and the broad range of pK_as of EDTA (1.7, 2.6, 6.3, 10.6) insuring that it is charged and capable of binding iron at the pH values of most foods. EDTA is generally used a sodium or calcium salt that have high water solubility. EDTA is also attractive to the food industry due to its low cost. Many food companies are looking for effective EDTA replacements due to consumer concerns for synthetic food additives.

10.3.2 Organic acids

Organic acids such as citric and lactic acid can chelate prooxidative metals. Citric acid is commonly used in foods especially in refined oils where it is added after the deodorization step. Citric acid is not as effective as EDTA due to its lower iron stability constant (1.5×10^{11}) and higher pK_as (3.1, 4.7 and 5.4).

10.3.3 Phosphates

Phosphates can also chelate metals with polyphosphates being stronger chelators and antioxidants than mono- and diphosphates (Sofos, 1986). Sodium tripolyphosphate, a commonly used food ingredient, has a stability constant for iron of 7.2×10^{22} and pK_as of 0.8 and 2.0. However, in some foods polyphosphates were not found to be effective antioxidants (Hu, 2004). One

potential reason for this is the susceptibility of polyphosphates to hydrolysis producing less effective mono- and diphosphates. This can be observed in muscle foods, where polyphosphates are relatively ineffective in raw meats which contain high levels of phosphatase activity (Li, 1993) but are highly effective in cooked meats where the phosphatases have been inactivated (Trout, 1990). Phytate is another form of phosphate that can chelate metals and inhibit lipid oxidation. However, phytate is not allowed as a food additive due to its ability to decrease iron, calcium and zinc absorption (Miller, 1996).

10.4 Control of reactive oxygen species

10.4.1 Singlet oxygen

Singlet oxygen is an important prooxidant in foods because it is able to add directly across the double bonds of unsaturated fatty acids, resulting in the formation of lipid hydroperoxides which can then be decomposed into free radicals by light, metals and high temperatures. Singlet oxygen differs from triplet oxygen in that it has two electrons in the outer orbitals that have opposite spin directions. This excited state of oxygen is highly electrophilic allowing it to interact with double bonds (Bradley, 1992). In foods, singlet oxygen is typically formed by photosensitizers (e.g., riboflavin and chlorophyll) in the presence of light.

Both chemical and physical quenching pathways exist for the inactivation of singlet oxygen. Chemical quenching of singlet oxygen typically occurs by compounds with double bonds such as tocopherols and carotenoids. Singlet oxygen interactions with β-carotene will lead to the formation of β-carotene-5,8-endoperoxide and carotenoid breakdown products containing aldehyde and ketone groups. β-Carotene-5,8-endoperoxide mainly forms upon the oxidation of β-carotene by singlet oxygen and therefore may provide a unique marker which may be used to monitor singlet oxygen/carotenoid interactions in foods and biological systems (Stratton, 1993). Interactions between tocopherols and singlet oxygen lead to the formation of tocopherol hydroperoxides and epoxides (Bradley, 1992). Other compounds including amino acids, peptides, proteins, phenolics, urate and ascorbate can chemically quench singlet oxygen; however, much less is known about the resulting oxidation products (Bradley, 1992; Dahl, 1988; Kanofsky, 1990). Chemical quenching of singlet oxygen leads to destruction of the antioxidant and loss of activity.

A more effective pathway for singlet oxygen inactivation is by physical quenching, which primarily occurs through interactions between singlet oxygen and carotenoids. As mentioned previously, singlet oxygen is in an excited state with the energy of its two main forms being 22.4 and 37.5 kcal above the ground state (Bradley, 1992). Carotenoids can physically quench singlet oxygen through the transfer of this energy to the carotenoid to produce an excited state of the carotenoid and ground state, triplet oxygen. The excited carotenoids can then dissipate their energy to the surrounding environment through vibrational and

rotational interactions to return the carotenoid to the ground state allowing it to interact with another molecule of singlet oxygen (Stahl, 1993). Nine or more conjugated double bonds on the carotenoids are necessary for physical quenching (Di Mascio, 1989) and the presence of oxygenated ring structures at the end the molecules increases the effectiveness of carotenoids (Di Mascio, 1989). While the physical quenching of singlet oxygen by carotenoids does not cause destruction of the molecules, it can result in *trans*- or *cis*-isomer conversions (Stahl, 1993).

Other reactive oxygen species that are of interest in the lipid oxidation pathway are superoxide anion, hydrogen peroxide and lipid hydroperoxides. These reactive oxygen species are primarily controlled by antioxidant enzymes that are discussed in Chapter 11. The food additives, thiodipropionic acid and dilauryl thiodiproprionate are capable of decomposing peroxides and peracids; however, at their allowable concentrations (≤ 200 ppm) they are not very effective and therefore rarely used (Lindsay, 1996). Methionine, which has been found to antioxidative in some lipid systems, is thought to decompose peroxides by mechanisms similar to thiodipropionic acid and dilauryl thiodiproprionate (Lindsay, 1996).

10.5 Control of other prooxidative factors

10.5.1 Photoactivated sensitizers

As mentioned earlier, foods contain photosensitizers such as chlorophyll, riboflavin and heme-containing proteins that can produce singlet oxygen in the presence of light. Besides their ability to inactivate singlet oxygen, carotenoids can also inhibit the activity of photoactivated sensitizers. This occurs by the transfer of energy of the excited photosensitizers to the carotenoid to form the excited state of the carotenoid which then returns to the ground state by transfer of energy into the surrounding solvent (Palozza, 1992; Stahl, 1993). Through this pathway the photosensitizer is inactivated before it can produce singlet oxygen.

10.5.2 Lipoxygenases

Numerous plant tissues and selective animal tissues contain enzymes, known as lipoxygenases, which produce lipid hydroperoxides. Plant lipoxygenases are cytoplasmic enzymes that contain a non-heme iron. Lipoxygenase activity can be controlled by heat inactivation and plant breeding programs which decreases their concentrations. Phenolics such as anthocyanins are capable of inhibiting lipoxygenase by an unknown noncompetitive pathway (Knaup, 2009).

10.6 Antioxidant interactions

The biological systems from which food originates contain multi-component antioxidant systems to maximize protection against oxidative damage. These

multi-component antioxidant defense systems protect cells by controlling pro-oxidants (metals, reactive oxygen species and enzymes); activating and repairing oxidative damage (e.g., glutathione peroxidase) and scavenging free radicals. The antioxidant protection systems exist in different physical locations (aqueous, interfacial and lipid phases) and controls oxidative factors at different stages of the reactions (e.g., initiating species ($^{\bullet}$OH) and propagating species (hydroperoxides)).

An additional benefit to multi-component antioxidant systems is that combinations of antioxidants can produce synergistic interactions that maximize protection and allow for conservation of the antioxidants. Combinations of chelators and FRS often result in synergistic inhibition of lipid oxidation (Nawar, 1996) because the chelator 'spares' free radical scavengers from rapid oxidation. This occurs because the chelator will decrease metal-promoted hydroperoxide decomposition thereby decreasing free radical generation. Less free radical generation decreases the eventual inactivation of the FRS through reactions such as termination making the free radical scavenger concentration greater at any given time.

Another antioxidant interaction that can result in synergistic activity is due to radical transfer mechanisms, or so-called redox recycling. To predict the direction of electron transfer, and the rate constant of the regeneration, it is necessary to understand the thermodynamics of radical reactions and structure-related bond energies by determining the one-electron reduction potential ($\Delta E^{\circ\prime}$) and OH-bond dissociation enthalpies (BDEs). As illustrated in Table 10.1, the $E^{\circ\prime}$ values are listed in order from high to low. The higher the reduction potential, the higher the ability to steal the electron (or hydrogen atom) from those with the lower reduction potential and visa versa (Buettner, 1993). For example, α-tocopherol (480 mV) and ascorbic acid (280 mV) have lower reduction potentials than PUFA (600 mV) and alkoxyl ($\sim$1000 mV) radicals, so than the radical scavenging activity of these antioxidants are thermodynamically feasible and generally effective. In addition, ascorbic acid also can regenerate α-tocopherol and/or other phenolic compounds that have higher reduction potentials. Synergistic interaction of these antioxidants by redox recycling have been reported *in vivo* and *in vitro* model systems espectially focusing on the interations among α-tocopherol, ascorbic acid, flavonoids and carotenoids (Amorati, 2002; Bohm, 1997; Iglesias, 2009; Jorgensen, 1999; Luczaj, 2005; Mukai, 1991; Palozza, 1992; Zhou, 2005).

However, the use of the reduction potential to predict antioxidant interactions has its limitation, since the prediction is based on only electron transfer reactions and in some cases, thermodynamic feasible reactions are not kinetically favored due to physical and chemical barriers (Buettner, 1993; Girotti, 1985). Interstingly, thermodynamically unfeasible reactions can be pulled forwared by the dissappearance of their product (Amorati, 2002; Buettner, 1993; Iglesias, 2009). For instance, the thermodynamically disfavored regeneration of α-tocopherol by antioxidants such as caffeic acid (534 mV), gallic acid (560 mV), and catechin (570 mV) have been reported (Table 10.1). However, the efficiency

of α-tocopherol regeneration by these antioxidants is low at 1.4×10^{-4}, 4.3×10^{-4}, and 4.5×10^{-4} mole of α-tocoperoxyl radical reduced per mole of antioxidant, respectively (Pazos, 2007) compared to the regenerating efficiency by ascorbyl palmitate at 0.93 mole of α-tocoperoxyl radical reduced per mole of antioxidant (Iglesias, 2009).

Since multi-component antioxidant systems can inhibit oxidation at many different phases of oxidation the resulting antioxidant activity can be more effective than when using a single antioxidant. This suggests that the most effective antioxidants systems for foods would contain antioxidants with different mechanisms of action and/or physical properties. Determining which antioxidants would be most effective depends on factors such as type of oxidation catalysts, physical state of lipid (bulk vs. emulsified), and factors which influence the activity of the antioxidants themselves (e.g., pH, temperature and ability to interact with other compounds in the foods).

Improved control of lipid oxidation in foods can also been seen by physical structures that impact the location of the antioxidant. Bulk oil contains numerous minor components that are amphiphilic, such as mono- and diacylglycerols, phospholipids, sterols, free fatty acids, and polar products arising from lipid oxidation, such as lipid hydroperoxides, aldehydes, ketones, and epoxides (Chaiyasit, 2007a). These surface active components, individually or in the combination, possess the ability to associate in to physical structures in the bulk oils in the presence of the small quantities of water (~300 ppm) (Chaiyasit, 2007b). These structures are known as association colloids which includes reverse micelles and lamellar structures. Reverse micelles are efficient nano-reactors that allow increased interactions between lipid- and water-soluble components and can greatly alter chemical reaction rates so it is possible that association colloids are one of the sites of oxidation reaction in bulk oils. Koga and Terao (Koga, 1995) observed that the presence of phospholipids enhanced the antioxidant activity of α-tocopherol in model bulk oil systems containing a trace amount of water (1% v/v). In the presence of 2,2′-azobis(2-amidinopropyl) dihydrochloride (AAPH), a water-soluble peroxyl radical generator, the presence of phospholipid increased the degradation of α-tocopherol more than in the absence of phospholipids. Degradation of α-tocopherol by the water-soluble free radicals decreased as the phospholipid's hydrocarbon tail group size was decreased, and thus the ability of the phospholipid to form association colloids was lost. The investigators suggested that the presence of association colloids produced by phospholipids increased the accessibility of α-tocopherol to the site where free radical concentrations were greatest. In addition, the reduction potential of α-tocopherol in polar environments have been suggested to be lower, which could make tocopherol a more effcient free radical scavenger (Laranjinha, 2001). Koga and Terao (Koga, 1994) also found that the antioxidant activity of α-tocopherol could be increased when it was conjugated to the polar head group of phosphatidylcholine [1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′,8′-tetramethyl-6′hydroxychroman]. This increase in activity was again thought to be due to the increased partitioning of the reactive

portion of α-tocopherol into the water phase of the association colloids in bulk oil (Koga, 1994). Overall, this work suggests that the presence of physical structure can also impact the activity of free radical scavengers.

10.7 Conclusions

Unfortunately, the number of antioxidants available to food manufacturers is limited and the approval of new antioxidants is unlikely due to economic barriers including expensive government approval for new food additives. Therefore if the food industry is to better control oxidation reactions, existing antioxidants must be used more efficiently. In order to accomplish this goal, a better understanding of the mechanisms by which lipids oxidize and antioxidants inhibit oxidation is needed. More research in lipid oxidation chemistry in complex food systems will lead to the development of novel antioxidant technologies that substantially increase the ability of food manufacturers to inhibit oxidative rancidity. Overcoming this challenge will allow food manufacturers to produce healthier foods will elevated levels of polyunsaturated fatty acids that can be delivered to the consumer without risk of quality deterioration.

10.8 References

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11

Protein antioxidants for the stabilization of lipid foods: current and potential applications

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Abstract: Fats and oils that are high in polyunsaturated fatty acids are generally regarded as beneficial to human health yet are extremely liable to oxidative deterioration. The number of antioxidant intervention strategies available for foods is limited and will likely decline as the consumer demand for 'all natural' products increases. New antioxidant technologies are clearly needed, especially those based on non-synthetic compounds. Proteins and peptides have the potential to fill this niche, as they are capable of inhibiting oxidative rancidity by a number of mechanisms, including radical scavenging, metal chelation, two-electron peroxide reduction, and aldehyde quenching. This chapter focuses on the demonstrated and potential use of food proteins as antioxidants in lipid foods, as well as their limitations.

Key words: protein, peptide, oxidation, antioxidants, radical scavenging, chelation.

11.1 Introduction

Lipid oxidation is one of the most important spoilage mechanisms in foods, resulting in enormous economic losses each year. Fats and oils that are high in polyunsaturated fatty acids are generally regarded as beneficial to human health yet are extremely labile to oxidative deterioration. The protection of these lipids with regard to oxidation presents a major challenge to the food industry. As consumer demand for polyunsaturated bioactive lipids increases, food chemists

are tasked with designing novel chemical stabilization methods that often include the use of antioxidants. The number of antioxidant intervention strategies available for foods is limited and will likely decline as the consumer demand for 'all natural' products increases. New antioxidant technologies are clearly needed, especially those based on non-synthetic compounds. This chapter focuses on the demonstrated and potential use of food proteins as antioxidants.

Proteins are able to inhibit lipid oxidation, which makes them an important component of the antioxidant defense of biological tissues from which foods are derived. Antioxidant enzymes, as well as specialized metal-binding proteins, are often the first examples of antioxidant proteins to come to mind; however, virtually all proteins have the potential to inhibit lipid oxidation reactions in food. As such, an attractive strategy for increasing the oxidative stability of lipid foods without the use of synthetic antioxidant additives could involve protecting endogenous antioxidant enzymes, enhancing the activity of proteins found naturally in foods by altering protein structure, and/or the use of antioxidant proteins or peptides as food additives. However, the use of proteins to inhibit lipid oxidation could be limited by their potentially deleterious effect on food product color (e.g., scattering light; formation of Maillard browning products), texture (e.g., unwanted gelation; increased viscosity), and flavor (e.g., co-reactants with critical flavor components; increased bitterness, particularly in the case of small peptides).

11.1.1 Role of proteins in modulating oxidative stress

The stability of foods with respect to oxidation is a function of the balance between antioxidant and prooxidant factors. The biological tissues from which foods are derived are host to a complex system of antioxidant and prooxidant factors. Living organisms must maintain the balance between these factors in order to do work while also protecting their constituent components (e.g., lipids, proteins, DNA) from oxidation. The antioxidant systems responsible for this protection include free radical scavengers (e.g., tocopherols, glutathione, ascorbic acid), metal complexing compounds (e.g., transferrin, ferritin, many organic acids), and enzymes capable of inactivating reactive oxygen species (e.g., catalase, superoxide dismutase). Non-specialized proteins also contribute to the antioxidant capacity of biological tissues. For example, it is estimated that blood proteins provide 10–50% of the peroxyl radical trapping activity of plasma (Frei *et al.*, 1988; Wayner *et al.*, 1987).

While the antioxidant–prooxidant balance in healthy biological tissues is normally controlled to provide an antioxidative environment, this balance can easily be disrupted when these materials are converted to foods. Many food processing operations lead to oxidative stress by introducing oxygen (e.g., mixing, grinding, and homogenizing operations), destruction of endogenous antioxidants (e.g., heat inactivation of antioxidant enzymes), removal of natural antioxidants (e.g., physical and chemical separation steps of oil refining), and

increasing prooxidative factors (e.g., light exposure leading to singlet oxygen production, heat treatments that liberate protein-bound transition metal catalysts). Finally, the industry trend towards incorporating higher concentrations of unsaturated oils (e.g., oils high in ω-3 fatty acids) within processed foods results in products that are considerably more labile to oxidative deterioration.

11.1.2 Classification

The generic term antioxidant refers to a broad class of compounds capable of inhibiting oxidation reactions. From the food scientist's perspective, antioxidants function either by disrupting the initiation or propagation step of free radical reactions involving lipids (i.e., primary antioxidants), or by inactivating prooxidants (i.e., secondary antioxidants). As is the case with either type, the net result of their activity is a delay in the onset of volatile organic compounds that are perceived as off-flavors or off-aromas. It should be noted that some antioxidants are capable of behaving as both primary and secondary antioxidants, and are referred to as multiple-function antioxidants (Reische *et al.*, 2002). Many proteins and polyphenols found in food exhibit multiple-function antioxidant activity, as will be discussed in the following sections.

Primary, or chain-breaking antioxidants interfere with the initiation or propagation steps of oxidation reactions by readily donating hydrogen atoms (i.e., hydrogen atom transfer reactions) to lipid peroxyl radicals (Frankel, 1998); however, some antioxidants in foods exert their activity through single electron transfer reactions. Hydrogen abstraction from chain-breaking antioxidants yields a radical antioxidant molecule; however, this radical species is of insufficient energy to promote further oxidation reactions. The radical antioxidant that results is a relatively lower energy species, often due to resonance delocalization of the unpaired electron around a conjugated ring system to form stable resonance hybrids (Reische *et al.*, 2002). Thus, many primary antioxidants comprise phenolic moieties with bulky alkyl substituents, a classic example being tocopherols and tocotrienols (Fig. 11.1). It is also important to note that some chain-breaking antioxidants are prooxidative under certain conditions. For example, at elevated concentrations and temperatures, α-tocopherol can become prooxidative chain-carriers by regenerating peroxyl radicals (Frankel, 1998).

A second class of antioxidants is referred to as preventative or secondary antioxidants. Unlike chain-breaking antioxidants, preventative antioxidants do not inactivate free radical species. In fact, the term preventative antioxidant denotes a very broad class of antioxidants that all function by slowing the rate of lipid oxidation reactions, but do so by a variety of mechanisms. Two of the most common types of preventative antioxidants suitable for food systems are metal chelators and hydroperoxide destroyers. A brief discussion of these antioxidant classes follows.

Many redox-active, multivalent transition metals (e.g., Fe, Cu, Mn, Cr, V, Zn, Al) are capable of increasing lipid oxidation rates by promoting the

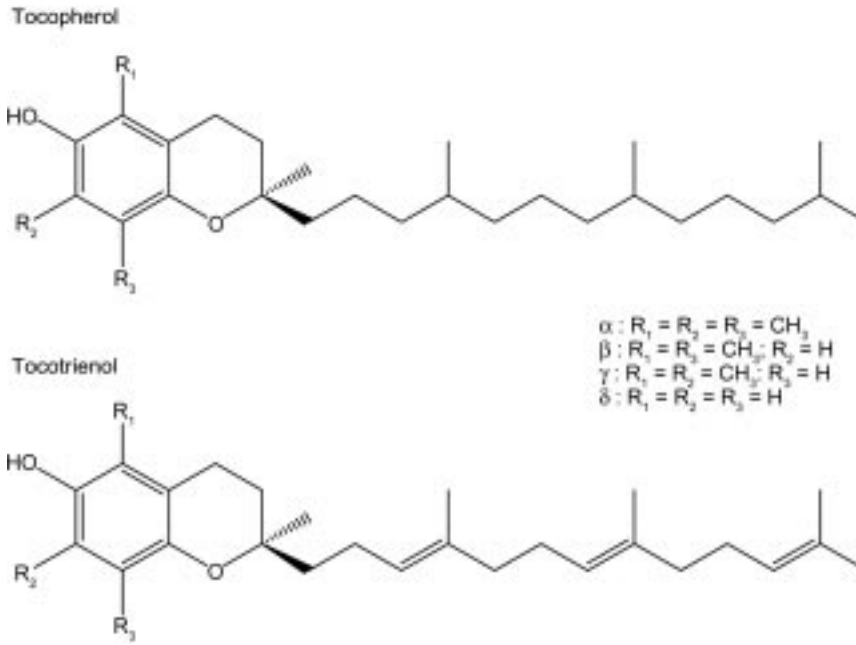

Fig. 11.1 Tocopherol and tocotrienol structures.

decomposition of lipid hydroperoxides (Decker and McClements, 2001; Frankel, 1998; McClements and Decker, 2000; Min and Boff, 2002; Nawar, 1996). Due to the fact that all food products contain trace amounts of metals, and that it is not practical or feasible to remove metals from food ingredients, metal-catalyzed decomposition of hydroperoxides is an important reaction in the oxidation of food lipids. Two metal-hydroperoxide reactions, which follow classical Fenton chemistry, are typically observed.

The application of metal inactivators, or chelators, to food lipids is often an effective strategy for inhibiting lipid oxidation. In the presence of metals, chelating compounds can form chelates, or complexes resulting from the combination of a metal ion and a multidentate ligand such that the ligand forms two or more bonds with the metal, resulting in a ring structure that includes the metal ion (Miller, 1996). By complexing with transition metals in such a manner, the Fenton-mediated decomposition of lipid hydroperoxides is effectively disrupted. Common inactivating chelating compounds include citric acid, phosphoric acid, ethylenediaminetetraacetic acid (EDTA), and 8-hydroxy-quinoline (Frankel, 1998). Other compounds are capable of strongly binding metals without rendering them redox inactive. Such compounds can alter the physical location of metal-catalyzed reactions, thus inhibiting lipid oxidation by preferentially partitioning metals to regions of low-lipid concentration.

Substances that safely destroy peroxides inhibit oxidation reactions by converting lipid hydroperoxides into non-reactive species. The inactivation of

hydroperoxides is a non-radical (i.e., two-electron) reaction, and is frequently accomplished by reduction or hydrogen donation to yield stable lipid alcohols or other inactive products (Frankel, 1998). Some common examples of hydroperoxide destroyers in foods include α-tocopherol, Trolox (a water-soluble carboxylic acid analog of α-tocopherol), sulfur and selenium compounds, phosphites, and a host of reducing agents (Frankel, 1998).

The evaluation and 'scoring' of naturally occurring antioxidants has become popular in recent years, which are often based on one or more *in vitro* antioxidant capacity assays. Whereas many of these assays are based on a test compound to donate hydrogen atoms to controlled amounts of radical species (e.g., the oxygen radical absorbance capacity assay, ORAC), other assays are specific for single electron transfer mechanisms (e.g., the ferric reducing ability of plasma, FRAP). Prior *et al.* (2005) have reviewed several common antioxidant capacity assays, with a comprehensive discussion of their respective advantages and disadvantages.

11.2 Important antioxidant enzymes

11.2.1 Superoxide dismutases

The one electron reduction of ground-state oxygen ($^3O_2$) at physiological pH gives the relatively reactive superoxide radical ($^{\bullet}O_2^-$). This species is far less reactive than the hydroxyl radical ($^{\bullet}OH$), or even its protonated form the hydroperoxyl radical ($^{\bullet}O_2H$), and as such is incapable of reacting with most biological molecules in aqueous solution (Halliwell and Gutteridge, 2007). Superoxide can promote the peroxidation of unsaturated fatty acids, however, so controlling this species is important in minimizing oxidative damage *in vivo*. The prooxidative nature of superoxide is also due to its ability to reduce transition metals, as well as liberate transition metal catalysts from proteins (e.g., ferritin).

Because of the potential damage that this species can cause, many organisms have specialized systems that enzymatically reduce superoxide to hydrogen peroxide. These enzymes are known as superoxide dismutases (SOD), of which there are two major isoforms, which catalyze the following disproportionation reaction:

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

Isoforms of SOD contain either copper plus zinc in the active site (CuZnSOD) or manganese (MnSOD), with CuZnSOD being the most common. The CuZnSODs are relatively stable metalloenzymes, and retain their activity even after exposure to harsh treatments. Most CuZnSODs are resistant to some organic solvents (e.g., chloroform, ethanol), thermal treatment, enzymatic proteolysis (e.g., proteinases), and denaturation by a range of common reagents (e.g., guanidium chloride, sodium dodecyl sulfate, urea) (Halliwell and Gutteridge, 2007).

11.2.2 Catalase

Hydrogen peroxide results from the two-electron reduction of O_2, and is ubiquitous in biological systems. A key process leading to hydrogen peroxide production *in vivo* is by the reaction between superoxide and SOD, as described above. In food systems, hydrogen peroxide can be produced non-enzymatically as a result of polyphenol oxidation (Halliwell, 2008; Long *et al.*, 1999). While hydrogen peroxide is a non-radical species and is not a particularly potent oxidant, it is easily reduced by metal catalysis or ultraviolet light to highly reactive $^{\bullet}OH$ radicals.

One of nature's answers to this problem is catalase (CAT), a heme-containing enzyme found in many biological systems that catalyzes the reduction of hydrogen peroxide to water by the following pathway:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

In plants, hydrogen peroxide can also be removed by ascorbate peroxidase via the following mechanism:

$$2 \text{ ascorbate} + H_2O_2 \longrightarrow 2 \text{ monodehydroascorbate} + 2H_2O$$

11.2.3 Glutathione peroxidase

Most biological tissues also contain the enzyme glutathione peroxidase (GSH-Px). GSH-Px is capable of deactivating both hydrogen and lipid peroxides. GSH-Px contains a selenium ion within its active site and reduced glutathione (GSH) to reduce hydrogen peroxide or lipid hydroperoxide to water:

$$H_2O_2 + 2GSH \longrightarrow 2H_2O + GSSG$$

or

$$LOOH + 2GSH \longrightarrow LOH + H_2O + GSSG$$

where GSSG is oxidized glutathione and LOH is a fatty acid alcohol.

11.3 Metal chelation

11.3.1 Specialized iron-binding proteins

Redox active transition metals such as iron and copper are capable of catalyzing the decomposition of hydroperoxides and are important prooxidants in food lipids. There are several important proteins found in food whose sole biological function is to chelate, store and/or transport metals (e.g., iron). Many of these proteins play a critical role *in vivo* by maintaining a near zero steady-state concentration of free iron that would otherwise catalyze unwanted oxidation reactions. Some examples of specialized iron-binding proteins include transferrin, lactoferrin, ovotransferrin (conalbumin), phosvitin, and ferritin. Transferrin (blood) and lactoferrin (milk) bind two ferric ions each, while ovotransferrin binds three ferric ions. Haptoglobins and hemopexins are also

known extracellular antioxidants, capable of binding hemoglobulin and heme, respectively (Symons and Gutteridge, 1998).

Ferritin serves as a good example of how metal-binding proteins control oxidation *in vivo* and, potentially, in food products. Ferritin is a large globular protein composed of 24 polypeptide subunits arranged as a sphere, and is capable of storing up to 4500 atoms of iron within its hollow cavity (Fennema, 1996; Weinberg, 1990). Its primary function is the storage of iron. In fact, the majority of intracellular iron is stored in ferritin (Halliwell and Gutteridge, 2007). Owing to its high affinity and large storage capacity for iron, ferritin is thought to function as a cellular iron reserve that can be mobilized when the metal is needed for the synthesis of hemoglobin, myoglobin, or other iron-containing proteins (Fennema, 1996). Ferritin's effectiveness in preventing metal-catalyzed oxidation reaction stems from its ability to interfere with iron redox cycling. Ferrous (Fe^{+2}) ions are oxidized to the ferric (Fe^{+3}) state after binding to a specific site located inside the subunit helical fold (i.e., the ferroxidase center) of ferritin (Arosio and Levi, 2002). Once oxidized, iron is moved to within the protein's cavity, where it nucleates and aggregates to form a ferric hydroxide core (Arosio and Levi, 2002; Carrondo, 2003). In this way, iron is sequestered and maintained in its ferric state, and is therefore unable to catalyze deleterious oxidation reaction (e.g., reduction of lipid hydroperoxides and hydrogen peroxides to lipid alkoxyl radicals and hydroxyl radicals, respectively). However, it should be noted that ferritin is prooxidative under some conditions, especially when it is present at high concentrations, or when its pool of stored iron is liberated following reduction by superoxide, ascorbic acid or other reducing agents (McCord, 2002).

Transferrin is another major iron-binding protein, which binds virtually all circulating iron *in vivo*. Two monomeric forms of the protein are found, with binding domains at either C- or N-terminals (Halliwell and Gutteridge, 2007). Transferrin has been demonstrated to inhibit lipid oxidation in foods, especially at neutral pH where its affinity for iron is greatest. For example, Mancuso *et al.* (1999) reported that a 31 μM concentration of apotransferrin (iron-free transferrin) was capable of inhibiting lipid oxidation in a Tween 20-stabilized salmon oil-in-water emulsion at pH 7 versus a non transferrin-containing control. Not only do these data suggest that transferrin inhibits the reactivity of iron, but they offer strong evidence that iron is the most important prooxidant in oil-in-water emulsions, since transferrin has a very high binding specificity for iron.

Lactoferrin is another glycoprotein that is closely related to transferrin, and may serve as a useful endogenous antioxidant in dairy foods, and as an effective antioxidant additive in processed lipid foods. For example, lactoferrin has been shown to inhibit lipid oxidation in infant formula (Satue-Gracia *et al.*, 2000), milk, and mayonnaise (Nielsen *et al.*, 2004).

11.3.2 Non-specific metal binding

Proteins have been shown to chelate aqueous transition metals in dispersed lipid systems, resulting in an inhibition of lipid oxidation reactions (Diaz and Decker,

2004; Diaz *et al.*, 2003; Faraji *et al.*, 2004; Tong *et al.*, 2000). Protein chelators can inhibit oxidation reactions leading to rancidity by changing the physical location of transition metals (e.g., partioning metals away from oxidatively labile dispersed lipids), forming insoluble metal complexes, reducing the chemical reactivity of transition metals, and sterically hindering the interaction of metals and dispersed lipids (Diaz *et al.*, 2003).

Aside from the specialized metal binding proteins discussed in the preceding section, many 'non-specialized' proteins have been shown to complex transition metals. Examples include casein (Diaz and Decker, 2004; Diaz *et al.*, 2003), whey proteins (Faraji *et al.*, 2004; Elias *et al.*, 2006; Tong *et al.*, 2000), soy proteins (Faraji *et al.*, 2004), bovine serum albumin (Villiere *et al.*, 2005), zein (Kong and Xiong, 2006), and potato protein (Wang and Xiong, 2005). Several amino acid residues such as histidine, cysteine, and methionine are known to bind metals, and may serve to localize Fenton-type reactions (the one-electron reduction of peroxides by reduced transition metals) and other metal-catalyzed oxidations at certain sites on a protein (Davies and Dean, 1997). Iron binding by proteins can result in site-specific oxidation reactions that can increase the effectiveness of aqueous proteins with respect to free radical scavenging by directing oxidation reactions away from dispersed lipids. Faraji and coworkers reported that whey protein isolate, soy protein isolate, and sodium caseinate (10 mg/mL) were capable of binding 185, 405, and 980 μmoles or iron, respectively (Faraji *et al.*, 2004). These proteins have been observed to inhibit lipid oxidation in oil-in-water emulsions and meats.

As is also the case with some specialized metal-binding proteins, non-specialized proteins have also been shown to alter the redox state of transition metals, thus reducing their reactivity with lipid hydroperoxides. Ferrous ions are far more effective than ferric ions catalysts in Fenton type reactions (Decker and McClements, 2001). Furthermore, ferrous ions are 10^{17} and 10^{13} times more water soluble than ferric ions at pH 7 and 3, respectively (Decker and McClements, 2001). Some proteins have been shown to preferentially bind ferric ions, thereby maintaining iron is its less reactive state. For example, the polar domains of caseins (α_{s1}, α_{s2}, and β) that contain phosphorylated serine residues are capable of forming complexes with iron, and have been shown to tightly bind ferric ions (Diaz *et al.*, 2003; Vegarud *et al.*, 2000). These proteins and their phosphorylated derivatives (e.g. caseinophosphopeptides) are effective antioxidants in oil-in-water emulsions (Diaz *et al.*, 2003), which may be due to their ability to retard lipid hydroperoxide decomposition by keeping iron in its oxidized state.

While iron is thought to be the most important transition metal catalyst in foods, copper is also capable of reducing lipid hydroperoxides and hydrogen peroxide to reactive radical species. In fact, copper is a more effective catalyst than iron in peroxide decomposition reactions (Halliwell and Gutteridge, 1990), but this metal is often ignored because it is typically present at much lower concentrations than iron. As is the case with iron, proteins are capable of forming strong complexes with copper which may impact lipid oxidation

reactions. In biological tissues, copper is bound by proteins such as serum albumin (one cupric ion per protein) and ceruloplasmin (six cupric ions per protein). These proteins can inhibit lipid oxidation as can been seen in the copper-catalyzed oxidation of low-density lipoprotein (LDL), where oxidation rates are inversely related to the concentration of BSA (Bourdon *et al.*, 1999; Schnitzer *et al.*, 1997).

11.4 Non-specific protein antioxidant mechanisms

11.4.1 Scavenging of free radicals and reactive oxygen species

Proteins derived from both animal and plant sources have been shown to inhibit lipid oxidation reactions in food lipid systems (Taylor and Richardson, 1980; Tong *et al.*, 2000; Faraji *et al.*, 2004; Diaz *et al.*, 2003; Elias *et al.*, 2006). It has been reported that a major contributor to the antioxidant activity of these proteins and polypeptides is free radical scavenging by certain amino acid side groups, thus allowing them to behave act as radical trapping devices (Neuzil *et al.*, 1993; Østdal *et al.*, 2002). In reality, all 20 amino acids can be oxidized assuming the radical insult is sufficiently powerful, as is the case with the highly oxidizing $^{\bullet}$OH radical. However, as is the case with all chain-breaking antioxidants in food lipid systems, protein antioxidants are only effective if they do not initiate or propagate further oxidation reactions. Protein- and peptide-based radical scavengers disrupt peroxidation chain reactions by quenching lipid radicals, usually by hydrogen donation. This reaction yields a protein radical which, ideally, is of insufficient strength to propagate lipid oxidation (i.e., it is incapable of abstracting a hydrogen atom from a methylene interrupted double bond). For example, tyrosine radicals formed on bovine serum albumin are significantly longer lived and are therefore less reactive than free tyrosine radicals (Østdal *et al.*, 1999). The long half-lives of tyrosinyl radicals in bovine serum albumin is likely due to the ability of the protein to transfer radicals on surface exposed amino acid residues to tyrosine residues buried in the protein's hydrophobic core. It is conceivable that a protein's antioxidant activity is at least partially attributable to the lower reactivity of protein radicals if those radicals are transferred to the interior of the protein where they are unable physically interact with lipids. However, it should be noted that it is possible that not all protein radicals are transferred to within a protein's interior, as bovine serum albumin radicals have been found to promote the oxidation of linoleic acid emulsions (Østdal *et al.*, 2002).

Methionine has been proposed to be an important free radical scavenger in proteins in biological systems (Levine *et al.*, 1996, 1999, 2000; Stadtman *et al.*, 2003). This is because methionine residues are very labile to oxidation and can potentially scavenge radicals before they are able to attack other amino acid residues that are critical to protein structure or function. For example, it has been proposed that the high concentration of methionine residues at the active site of bacterial glutamine synthetase serves as a 'last chance' antioxidant defense

system by oxidizing preferentially to other amino acids that are critical to enzyme function (Levine *et al.*, 2000). The role of methionine as an important free radical scavenger in biological systems can also be argued based on the ubiquity of methionine sulfoxide reductases (enzymes capable of repairing oxidized methionine residues) in virtually all forms of life. Thus methionine could act as a free radical scavenger that can be regenerated in biological tissues similar to α-tocopherol, which is thought to be regenerated by ascorbic acid (ascorbic acid is subsequently regenerated by NADPH). Regeneration of a free radical scavenger would obviously increase its role as an antioxidant since it would not be lost in the early stages of oxidation as are many other free radical scavengers. Whether oxidized methionine residues can be regenerated in foods has not been determined.

In a study of the role of β-lactoglobulin as an antioxidant in a model oil-in-water emulsion system, continuous phase protein was found to inhibit lipid oxidation (Elias *et al.*, 2005). Evaluation of the oxidative stability of cysteine, tryptophan, and methionine residues in continuous phase β-lactoglobulin found that tryptophan and cysteine, but not methionine, oxidized prior to lipid oxidation, thus indicating that these amino acids were oxidized preferentially to unsaturated fatty acids. Methionine was not found to oxidize in this system, despite its reported high susceptibility to oxidation. The fact that β-lactoglobulin's methionine residues are buried within its hydrophobic core, and therefore possibly inaccessible to aqueous phase oxidants, could explain this observation.

Proteins have been shown to scavenge free radicals in a range of antioxidant assays. These studies have typically employed the use of free radical generators, with antioxidant status conferred to the test protein if it can competitively quench the generated radicals. For instance, β-lactoglobulin (Elias *et al.*, 2006) and casein (Diaz and Decker, 2004), along with their hydrolysates, were shown to scavenge peroxyl radicals by 2,2′-azo-bis (2-methylpropionamidine) dihydrochloride (AAPH) in the ORAC assay. The radical scavenging capacity of zein (Kong and Xiong, 2006) and potato proteins (Wang and Xiong, 2005) was also demonstrated using free radicals generated by 2,2′-azinobis (3-ethylbenzothiazoline-6sulfonic acid) (ABTS). Furthermore, egg yolk proteins were observed to scavenge 1,1-diphenyl-2-picrylhyrazyl (DPPH) radicals (Sakanaka *et al.*, 2004).

The radical scavenging capacity of proteins can also be effectively measured by electron paramagnetic resonance (EPR) (Davies and Hawkins, 2004). Using EPR, bovine serum albumin, β-lactoglobulin, and lactoferrin were shown to scavenge free radicals generated from cumene hydroperoxide and iron (Pazos *et al.*, 2006). EPR is also useful for observing amino acid radicals in proteins, as it is the only method that allows the direct detection of paramagnetic species (e.g., organic radicals, transition metals). Examples of radicals observed in food systems include tyrosinyl radicals in bovine serum albumin (Østdal *et al.*, 1999) and thiyl radicals in porcine myosin (Frederiksen *et al.*, 2008).

11.4.2 Two-electron reduction of lipid hydroperoxides

Proteins have been shown to reduce lipid hydroperoxides to non-reactive lipid hydroxides by two-electron (non-radical) processes (Garner *et al.*, 1998a, 1998b; Pryor *et al.*, 1994; Pryor and Squadrito, 1995), which may contribute significantly to their overall antioxidant activity. Methionine residues are thought to be central to this process, as it has been observed that canine high density lipoprotein (HDL) showed weaker lipid hydroperoxide reducing activity than human HDL, which has two additional methionine residues (Met112, Met148) (Garner *et al.*, 1998a). The proposed mechanism for the reduction of lipid hydroperoxides to lipid hydroxides (Fig. 11.2) involves a direct two-electron transfer from the sulfide of thioethers of oxidatively labile methionine residues, resulting in the oxidation of methionine to methionine sulfoxide (MetO) (Panzenbock and Stocker, 2005; Garner *et al.*, 1998b).

Under this scenario, methionine residues exert their activity by competing for lipid hydroperoxides with transition metal catalysts, thereby inhibiting lipid peroxidation. In processed foods, hydroperoxide scavenging would not be a significant antioxidant mechanism once all available methionine residues have been oxidized. However, in some foods that are physiologically active under postharvest or postslaughter conditions (e.g., fruits, vegetables, minimally processed muscle foods), methionine residues could be vastly more important antioxidants if methionine sulfoxide reductases – enzymes capable of reducing oxidized methionine – remain active.

11.4.3 Quenching of volatile aldehydes

Oxidized lipids are not perceived as rancid until lipid alkoxyl radicals decompose via β-scission reactions into lower molecular weight, volatile compounds that are perceived as off-flavors and off-aromas. These compounds are aldehydic species that have considerably longer half-lives than the hydroperoxides or lipid alkoxyl radicals from which they derive (Bruenner *et al.*, 1995; Davies and Dean, 1997) and have a deleterious effect on the sensory, functional, and nutritive quality of lipid-containing foods (Stangelo *et al.*, 1987, Zhou and Decker, 1999b). However, aldehydes are known to react with the side chains of certain amino acid residues by either Schiff base reactions or 1,4- (Michael

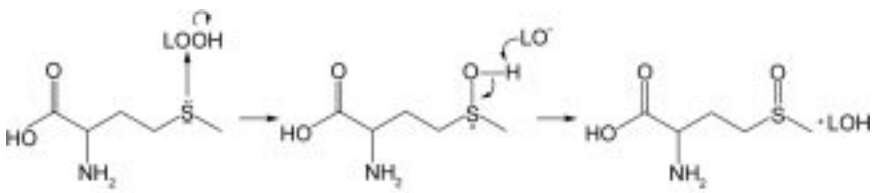

Fig. 11.2 Two-electron reduction of lipid hydroperoxide (LOOH) by thioether-containing side chain of methionine (Garner *et al.*, 1998a, 1998b; Panzenbock and Stocker, 2005).

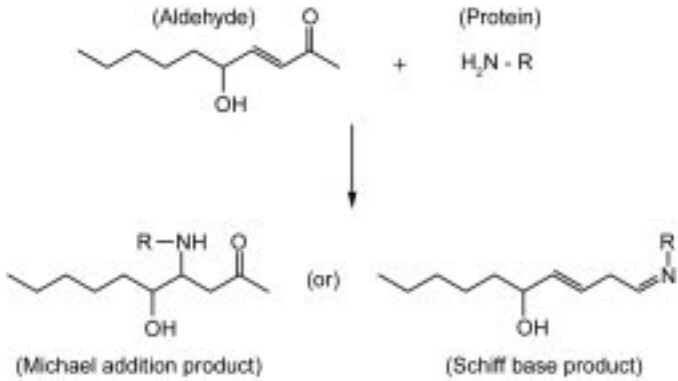

Fig. 11.3 Protein modification by Michael addition or Schiff base formation.

additions) to yield aldehydic adducts (Fig. 11.3). These reactions are non-radical, two-electron processes. The resulting aldehyde adduct is non-volatile and, therefore, would have a minimal effect on the food's aroma profile.

Schiff bases are imines that form in complex food systems when the carbonyl groups of aldehydic lipids react with the nucleophilic side chains of amino acid residues (e.g., cysteine). Depending on the characteristics of the lipid oxidation products, Schiff base formation can be accompanied or dominated by competing reactions, such as 1,4 (Michael) addition reactions. Bruenner *et al.* reported that greater than 99% of β-lactoglobulin B and human hemoglobin was modified via Michael addition reactions in the presence of 4-hydroxy-2-nonenal (4-HNE; an α,β-unsaturated aldehydic product often observed in oxidizing ω-6 poly-unsaturated fatty acids) (Bruenner *et al.*, 1995). Thus, it appears as though Schiff base reactions dominate when proteins are in the presence of saturated fatty acid oxidation products, whereas Michael addition reactions dominate when unsaturated fatty acid oxidation products are present.

Given that volatile lipid oxidation products greatly impact the sensory attributes of foods, any pathway that leads to a net reduction in the volatility of aldehydes can positively affect quality. Strictly speaking, this pathway is not an antioxidant mechanism, as it does not inhibit lipid oxidation *per se*, but the perception of oxidative rancidity is inhibited through the transformation of lipid oxidation productions into non-volatile, high molecular weight covalent adducts. However, it should be mentioned that while proteins can, in fact, decrease rancidity, these reactions can be deleterious to other food quality attributes, most notably the development of undesirable colors. Several studies have shown that aldehydic lipid oxidation products react with myoglobin, which adversely affects the color of myoglobin-containing meat products (Alderton *et al.*, 2003; Faustman *et al.*, 1999; Lynch and Faustman, 2000). Myoglobin (Mb) is the major heme-containing sarcoplasmic protein in skeletal muscle that can exist in three forms depending on the redox state of its heme iron: ferrous deoxymyoglobin (deoxyMb) and ferrous oxymyoglobin (oxyMb) or oxidized ferric metmyoglobin (metMb) (Alderton *et al.*, 2003). OxyMb is an important

form because it is responsible for the characteristic cherry red appearance of fresh meat. The oxidized form of oxyMb is known as metMb, and results in discoloration of the meat, turning it from red to brown, which is generally unacceptable to consumers. Chan and others have shown that the rate of oxyMb oxidation to metMb increased in the presence of reactive aldehydic oxidation products (Chan *et al.*, 1997). Moreover, Lynch and Faustman reported that 4-hydroxy-2-nonenal adducts render metMb a less suitable substrate for metmyoglobin reductase (Lynch and Faustman, 2000), thereby decreasing its ability to be reduced back to oxyMb by the enzyme. Mb modification by 4-hydroxy-2-nonenal also resulted in increased rates of lipid oxidation, indicating that these adducts confer a structural change upon metMb that enables its heme group to more readily catalyze oxidation reactions. It is clear that not all interactions between aldehydic lipid oxidation products are beneficial to food quality.

11.5 Food applications of protein antioxidant methods

11.5.1 Efficacy of protein-based antioxidants in foods

Proteins originating from milk, blood plasma, and soy protein all have been shown to exhibit antioxidant activity in muscle foods (Table 11.1). Porcine blood plasma contains antioxidant proteins such as serum albumin and transferrin, and can retard the formation of thiobarbituric acid reactive substances (TBARS) in both salted ground pork (Faraji *et al.*, 1991) and cooked ground beef (Shantha *et al.*, 1994). Whey protein concentrate is antioxidative in cooked beef, and whey and soy proteins inhibit lipid oxidation in cooked pork patties containing 2% protein (Pena-Ramos and Xiong, 2003), with soy protein isolate being more effective than whey proteins. Whey proteins have also been found to inhibit lipid oxidation in oil-in-water emulsions (Taylor and Richardson, 1980; Allen and Wrieden, 1982a, 1982b; Elias *et al.*, 2005, 2006). Park and coworkers found that soy protein inhibited the oxidation of ethyl esters of eicosapentaenoic acid dried in a maltodextrin-stabilized, freeze-dried emulsion powder system (Park *et al.*, 2005).

Peptides can also inhibit lipid oxidation in foods. Whey, casein, soy, and egg yolk hydrolysates have been shown to inhibit lipid oxidation in various muscle foods, such as beef, pork, and tuna (Sakanaka and Tachibana, 2006; Pena-Ramos and Xiong, 2003; Sakanaka *et al.*, 2005). Endogenous peptides found in foods can also act as antioxidants. Carnosine and anserine are histidine-containing dipeptides found in skeletal muscle that exhibit antioxidant properties (Chan *et al.*, 1994). Addition of carnosine to muscle foods can inhibit lipid oxidation and inhibit myoglobin discoloration (Decker and Crum, 1991). Park *et al.* (2005) found that soy peptide, gelatin peptide, and carnosine all inhibited the oxidation of freeze-dried ethyl esters of eicosapentaenoic acid encapsulated in maltodextrin.

Table 11.1 Protein sources, specific proteins and protein hydrolysates or peptides with demonstrated antioxidant activity

Type	Reference
Protein source	
Dairy	Taylor and Richardson, 1980
Soybean	Pena-Ramos and Xiong, 2003
	Park *et al.*, 2005
	Faraji *et al.*, 2004
Egg yolk	Sakanaka and Tachibana, 2006
Blood plasma	Faraji *et al.*, 1991
Potato	Shantha and Decker, 1995
Gelatin	Wang and Xiong, 2005
	Park *et al.*, 2005
Specific protein	
Casein	Diaz *et al.*, 2003
	Diaz *et al.*, 2004
β-Lactoglobulin	Elias *et al.*, 2005
Transferrin	Mancuso *et al.*, 1999
Lactoferrin	Satue-Gracia *et al.*, 2000
	Nielsen *et al.*, 2004
Zein	Kong and Xiong, 2006
Peptides	
Hydrolyzed egg proteins	Sakanaka and Tachibana, 2006
Hydrolyzed gelatin	Park *et al.*, 2005
Hydrolyzed soy proteins	Pena-Ramos and Xiong, 2003
	Park *et al.*, 2005
Hydrolyzed whey proteins	Elias *et al.*, 2005
	Pena-Ramos and Xiong, 2003
	Hernandez-Ledesma *et al.*, 2005
	Sakanka *et al.*, 2005
Hydrolyzed casein	Rival *et al.*, 2001
	Diaz *et al.*, 2003
	Diaz *et al.*, 2004
Hydrolyzed potato	Wang and Xiong, 2005
Carnosine	Chan and Decker, 1994
Anserine	Chan and Decker, 1994

Adapted from Elias *et al.* (2008).

11.5.2 Augmenting protein antioxidant activity

Just as protein function is ultimately dictated by primary structure, protein antioxidant activity is dependent on amino acid composition and sequence. The ability of proteins to chelate transition metals, scavenge free radicals species, reduce lipid hydroperoxides and hydrogen peroxide, and quench aldehydic secondary products is determined by the composition and distribution of several key amino acids. However, the antioxidant activity of these amino acids residues appears to be limited by the tertiary structure. This is due to the fact that many

Table 11.2 The solvent accessible surface area (SASA) of β-Lg's oxidatively labile amino acid residues calculated using the program GETAREA (Fraczkiewicz and Braun, 1998). The ratio of side-chain surface area to random coil value of each amino acid residue is listed in the 4th column. The 'random coil' value of a residue X is the average solvent-accessible surface area of X in the tripeptide Gly-X-Gly in an ensemble of 30 random conformations. Residues are considered to be solvent exposed if the side-chain surface area to random coil value ratio value exceeds 50% and to be buried if the ratio is less than 20% (Fraczkiewicz and Braun, 1998). Buried residues are denoted with an 'i'. Cys residues in bold typeface represent residues involved in disulfide bonds (i.e. cystine). A probe radius of 1.4 Å was used

Residue	Sidechain	Random coil	Ratio (%)	Buried
Met 7	25.58	158.3	16.2	X
Trp 19	0.00	224.6	0.0	X
Tyr 20	53.55	193.1	27.7	
Met 24	0.00	158.3	0.0	X
Tyr 42	16.58	193.1	8.6	X
Trp 61	93.04	224.6	41.4	
Cys 66	10.51	102.3	10.3	X
Phe 82	1.58	180.1	0.9	X
Tyr 99	35.28	193.1	18.3	X
Tyr 102	13.81	193.1	7.2	X
Phe 105	16.40	180.1	9.1	X
Cys 106	2.45	102.3	2.4	X
Met 107	7.34	158.3	4.6	X
Cys 119	0.15	102.3	0.1	X
Cys 121	0.00	102.3	0.0	X
Phe 136	0.10	180.1	0.1	X
Met 145	15.17	158.3	9.6	X
His 146	43.60	154.6	28.2	
Phe 151	12.67	180.1	7.0	X
Cys 160	12.38	102.3	12.1	X

Adapted from Elias *et al.* (2008).

amino acids with antioxidant potential may be buried within the protein's hydrophobic core where they are inaccessible to aqueous prooxidants and radical species. For example, in native β-lactoglobulin (β-Lg), the majority of free radical scavenging amino acids are located in the protein interior and therefore may not be able to contribute to the protein's overall antioxidant activity. The solvent accessibility of the major free radical scavenging amino acids in native β-Lg are shown in (Table 11.2), which was calculated using an algorithm for determining solvent accessible surface area based on the protein's crystal structure (Elias *et al.*, 2007; Fraczkiewicz and Braun, 1998). Based on this native structure, 17 of β-Lg's most oxidatively labile amino acids are not solvent accessible, whereas histidine 146, tyrosine 20, and tryptophan 61 are partially exposed. On this basis, it can be argued that these amino acids are unlikely to contribute to β-Lg's overall antioxidant activity when the protein is in its native state.

A potential means for increasing a protein's overall antioxidant activity is by disrupting it tertiary structure, for example by partial denaturation, which may make its buried amino acid residues more accessible to the aqueous phase. Taylor and Richardson found that heat treatment (70 to 130 ºC for up to 30 min) increased the antioxidant capacity of skim milk in the presence of hemoglobin, which they attributed, in part, to increased exposure of sulfhydryl groups (Taylor and Richardson, 1980). The authors' heat treatment yielded an increase in reactive sulfhydryls, from a starting concentration of 48 μM to a final concentration of 71 μM. It was also shown that the optimum heat treatment to increase reactive sulfhydryl concentration in whey, without decreasing total sulfhydryl levels, was 80 ºC. In a separate study, it was shown that heated β-Lg only (95 ºC for 30 min) increased the protein's antioxidant activity in a menhaden oil-in-water emulsion (Elias *et al.*, 2007). This effect was likely due to increased solvent exposure of some key free radical scavenging amino acid residues, since heating increased sulfhydryl exposure and peroxyl radical scavenging capacity but lowered iron chelation capacity. It should also be noted that heating proteins in the presence of sugars, as was the case for lactose-containing skim milk systems, can yield Maillard reaction products with good antioxidant activity.

The enzymatic hydrolysis of proteins is another potential means for increasing the solvent accessibility of amino acids. Antioxidant activity of hydrolysates has be reported for dairy (Hernández-Ledesma *et al.*, 2005; Sakanaka *et al.*, 2005; Diaz and Decker, 2004; Elias *et al.*, 2005), soy (Pena-Ramos and Xiong, 2003; Park *et al.*, 2005), zein (Kong and Xiong, 2006), potato (Wang and Xiong, 2005), gelatin (Park *et al.*, 2005), and egg yolk (Sakanaka and Tachibana, 2006) proteins. The observed increase in activity due to hydrolysis may stem from increased solvent exposure of amino acids. This exposure leads to increased metal chelation capacity and free radical scavenging activity (Rival *et al.*, 2001; Wang and Xiong, 2005; Elias *et al.*, 2006).

In many cases, extensive proteolysis has a negative effect on antioxidant activity, as free amino acids are typically poor antioxidants. For example, the proteolytic digestion of bovine serum albumin yields protein radicals with longer half-lives as compared to native bovine serum albumin, whereas extensive proteolysis yielded BSA radicals with relatively shorter half-lives (Østdal *et al.*, 1999). Moveover, it has been shown that tyrosyl radicals can be detected in tyrosine-containing peptides after treatment with peroxynitrite only if the tyrosine residue is located in a central position of a relatively long peptide sequence (Pietraforte and Minetti, 1997). Thus, the greater antioxidant activity of some peptides may be related to their unique, intrinsic properties, which enable them to better participate in free radical scavenging reactions, complex transition metal catalysts, and quench aldehydic oxidation products (Chan *et al.*, 1994; Zhou and Decker, 1999a, 1999b).

Proteins are, along with reducing sugars, key substrates in nonenzymatic browning (Maillard) reactions in foods. The products that result from these reactions are complex and often difficult to characterize, although the antioxidant activity of many of these products has been demonstrated (Bailey

and Um, 1992; Zamora and Hidalgo, 2005). Maillard reaction products represent a wide range of structures, which include furans, reductones, Schiff bases, and aldehydes. Many of these products have been observed to inhibit lipid oxidation reactions through radical scavenging mechanisms and metal chelation. Protein-sugar Maillard reaction products are often water soluble or produced on the surface of protein-stabilized emulsion droplets. Augustin *et al.* (2006) found that heating casein-stabilized fish oil-in-water emulsions in the presence of glucose yielded Maillard reaction products with good antioxidant activity.

11.5.3 Potential problems with protein antioxidants in foods

Despite their proven antioxidant activity in many food systems, proteins and peptides are not suitable replacements for traditional, or synthetic, antioxidants in all food applications. For example, proteins and peptides are potential allergens, which should be avoided among consumers with pre-established hypersensitivy. Dairy, soy, nut, and egg protein pose the greatest risk. From a sensory and consumer acceptability perspective, small peptides are often perceived as bitter, and can be perceived as such even at the concentrations used to inhibit lipid oxidation reactions. Furthermore, the incorporation of proteins and peptides to foods may cause unwanted rheological changes (e.g., increased viscosity of aqueous solutions or gelation) under some conditions. Maillard reaction products that form between proteins and sugars may have good antioxidant activity in lipid foods, but the inevitable color changes (e.g., browning reactions) that occur may not be desirable in all foods.

11.6 Consequences of protein oxidation

Protein oxidation most often results in amino acid R-group modification. Protein backbone (i.e., peptide bond) fragmentation is also possible with some oxidants, as are polymerization reactions. Dissolved oxygen concentration has also been observed to affect the course of protein oxidation in model systems. For instance, the hydroxyl radical-mediated oxidation of BSA results in extensive cross-linking under anoxic conditions; however, only limited cross-linking is seen in the presence of oxygen (Dean *et al.*, 1997).

The chemistry of free amino acid R-groups oxidation is similar to that which occurs on peptide and protein residues (Davies and Dean, 1997). However, a key difference is that radical transfer reactions (i.e., the transfer of free radicals from one amino acid to another) are known to occur in proteins, meaning that the initial site of protein oxidation may be difficult to assess by conventional methods in some cases. The intramolecular transfer of radicals within a protein is affected by its physical structure. For example, little evidence of radical transfer is observed between Trp25 and Tyr29 on the neurotoxin erabutoxin *b*, despite the fact that these two amino acids are a mere 1.3 nm apart (Butler *et al.*, 1982; Prutz *et al.*, 1982). This appears to be a

consequence of the protein's tertiary structure, which is made especially rigid by the presence of four disulfide bonds (Davies and Dean, 1997). When these disulfide bridges are reduced, however, radical exchange between Trp25 and Tyr29 was observed, presumably due to the protein's more flexible conformation (Prutz *et al.*, 1982).

There are various ways to measure protein oxidation in foods. A common method for determining general protein oxidation is the spectrophotometric analysis of protein carbonyls (Requena *et al.*, 2003), which can be derivatized to hydrazones with 2,4-dinitrophenylhydrazine. The resulting hydrazones are stable (compared to the carbonyls from which they derive) and have high extinction coefficients. Proteins carbonyls are known to increase with oxidative stress and age *in vivo*, but these compounds can also be readily detected in meat and dairy products, indicating that protein oxidation occurs during food processing and storage (Fedele and Bergamo, 2001; Stagsted *et al.*, 2004; Salminen *et al.*, 2006). EPR can also be used for the direct detection of stable protein radicals, as well as spin adducts resulting from the reaction between a spin trap (e.g., 5,5-dimethylpyrroline-N-oxide, DMPO) and a protein radical (Gomez-Mejiba *et al.*, 2009). General, or non-specific, oxidation can also be assessed by measuring protein fragmentation and polymerization rates and changes in proteolytic susceptibility (Stadtman *et al.*, 2003). Amino acid R-group oxidation is commonly measured by reverse phase HPLC following acid hydrolysis of the protein, although this approach cannot determine which particular amino acid residue has been modified. Site-specific oxidation of food proteins can be determined by MS/MS (Elias *et al.*, 2006), however, which can offer important mechanistic insight.

11.7 Future trends

Proteins are unique antioxidants in that their mode of action often encompasses multiple pathways, including inactivation of reactive oxygen species, free radical scavenging, decreasing the catalytic capacity of prooxidative transition metals, reduction of lipid hydroperoxides to lipid hydroxides, and alteration of the physical properties of food systems. These attributes make peptide and proteins potentially attractive replacements for synthetic antioxidants in foods.

Studies have shown that the antioxidant activity of native food proteins can be augmented by manipulating tertiary structure by heating or enzymatic hydrolysis, or through Maillard chemistry. In some cases, peptides give greater activity than the native proteins from which they derive; however, more work needs to be done in this area, particularly with respect to correlating peptide activity with amino acid composition and sequence. Progress in this area will inevitably lead to the development of a new class of multifunctional, 'natural' antioxidants that can be used to produce oxidatively stable foods and clean product labels.

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12

Synthetic and natural antioxidant additives in food stabilization: current applications and future research

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Abstract: This chapter reviews the use of synthetic and natural antioxidants in the stabilization of food products. It categorizes both natural and synthetic antioxidants in terms of how they interfere with the oxidation process and suggests new areas for research in the development of more effective natural food antioxidants by examining and contrasting the latest antioxidant technology now in use in the related field of polymer science.

Key words: natural and synthetic antioxidant food additives, polymer stabilization technology, antioxidant activity in plants and spices, managing food oxidation, novel antioxidant strategies.

12.1 Introduction

We would like to accomplish two things in this chapter. First, we would like to provide a general review of the use of natural and synthetic antioxidants in foods in a manner as wide-ranging as limited space will allow. Since a large number of excellent reviews are available on this subject, we will begin by referring readers to these comprehensive sources of information. Second, we would like to suggest new areas for research in food antioxidants by examining and contrasting the latest antioxidant technology in the related field of polymer science. By exploring the structures and mechanisms of action of antioxidants currently finding use in the plastics industry, we hope to challenge food scientists to broaden their search for naturally available substances that will

provide building blocks for safe, more effective antioxidant formulations for foods. We are not suggesting that compounds currently used in polymers be applied directly to foods. But we are suggesting that an assessment of the unique antioxidant materials in use in specific polymer applications may stimulate new thinking leading to the development of more effective food antioxidants. Such thinking is required because the food industry faces enormous food preservation challenges. Consumers are demanding more convenience and better nutrition. More precooked meals, the elimination of hydrogenated fats and the incorporation of oxidatively unstable nutritional ingredients such as omega-3 fatty acids and vitamins will require more powerful strategies for controlling oxidation in foods. The state of the art in polymer stabilization involves blends of antioxidants that operate on different components of the oxidation mechanism and act synergistically to provide extraordinary increases in stability. The food industry needs to reach a similar state to meet the challenge of providing extraordinary stability in today's complex foods. While other technologies, including new packaging methods and improved food processing techniques will play an important role, the development of new, more effective and more targeted antioxidant additives will be a key element in any progress that is made.

12.2 Reviews of antioxidant use in foods

A number of excellent reviews of antioxidants in foods have been published and continue to be sources of relevant information. Although it is somewhat older, Johnson (1975) provides a comprehensive overview of the synthesis and application of antioxidants, extensively covering the patent literature. The book concentrates on polymer applications, but does include a chapter on antioxidants for foods, including interesting early references to natural extracts. The book, *Food Antioxidants* contains chapters by Madhavi and Kulkarni (1996) and Rajalakshmi and Narasimhan (1996) that are wonderfully complete reviews covering various technical aspects of food antioxidants. Nanditha and Prabhasankar's (2009) newly published review is focused on the use of antioxidants in bakery products. Choe and Min (2006) review edible oils, concentrating on mechanisms and the effect of endogenous antioxidants. Other reviews worthy of attention include: Coppen (1989), Hall III and Cuppett (1997), Löliger (1989), Pokorný (1990), Reische *et al.* (2008) and Stuckey (1973).

12.3 Background

12.3.1 History of use

Natural preservatives, including constituents that are now recognized as antioxidants, have been used in foods since prehistoric times. Smoking meats and fish and treating foods with spices have long been known as techniques for

preserving foods, albeit, without an understanding of the underlying preservation mechanisms that are now known to include the antioxidant function. At the beginning of the last century food stabilization was limited to these long-practiced techniques. With the rise of an increasingly capable chemical industry and early investigations into the mechanism of oxidation in foods, the development of antioxidants as intentional food additives began in earnest in the 1920s and became a full-fledged industry in the 1940s and 1950s. Gum guaiac was approved as an antioxidant additive for the stabilization of lard in the 1930s (Grettie, 1933), and lecithin and tocopherols followed in the early 1940s (Higgins and Black, 1944). Great strides were made in the middle of the 20th century with the rise of the synthetic plastics and synthetic chemicals industries. While many of the antioxidants used in the plastics industry are toxic or have other properties that render them unsuitable for use in foods, a few of the compounds developed originally for polymers have become staples in the food industry. The development and use of synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) was seen as just another aspect of the progress of technology. The middle to latter part of the 20th century, however, saw the rise of a more skeptical attitude toward the use of synthetic chemicals, in all walks of life. This shift spurred efforts to better understand and utilize antioxidant substances found in nature. While significant progress to this end occurred mainly in the 1970s and 1980s, early work on the antioxidative effects of spices and endogenous antioxidants in oils can be traced back to the 1920s and 1930s.

The future will bring more challenges to the food industry as demands for quality increase at the same time distribution channels lengthen. The demand for an increasingly natural approach to food stabilization will require an increased understanding of the underlying chemistry of the oxidation of complex substances in complex matrices, and the development of more powerful and targeted, naturally derived antioxidant formulations that work better than the options available today. There is much work to do.

12.3.2 The lipid oxidation mechanism

Oxidation mechanisms are reviewed in detail in other chapters of this book. In general, the mechanism of lipid oxidation involves a free radical chain reaction that can be separated into three phases: initiation, propagation and termination (Fig. 12.1). In the initiation phase, free radicals are generated by incompletely understood processes involving hydrogen abstraction reactions mediated by metal ions, light, radiation or other means. The key intermediate formed is a highly reactive carbon-centered lipid free radical. Oxygen, if present, reacts with these radicals at near diffusion-controlled limits, generating hydroperoxyl radicals. In turn, these radicals can abstract a hydrogen from a lipid, generating yet another carbon-centered radical which propagates the reaction. In this process, the hydroperoxyl radical is converted to a hydroperoxide, which can become the source of additional reactive radicals under the action of metal ions

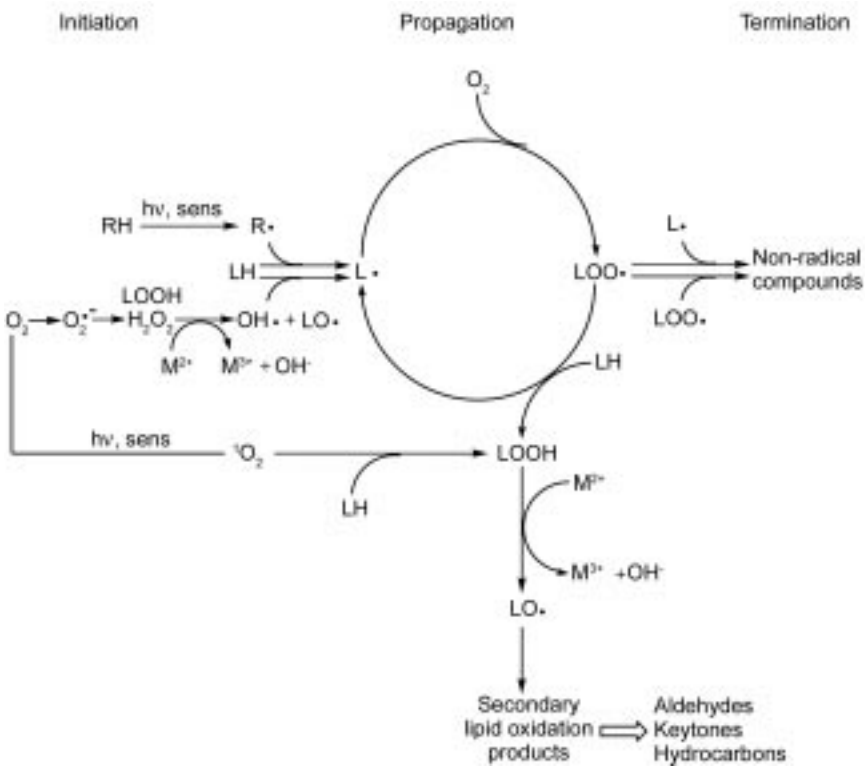


Fig. 12.1 The lipid oxidation mechanism.

via chemistry akin to the Fenton reaction. The alkoxyl and hydroxyl radicals produced in this way can continue the propagation phase of the reaction by abstracting a hydrogen from a lipid and forming another carbon-centered radical. Hydroperoxides also undergo a variety of decomposition reactions generating compounds that result in off, rancid or stale aromas. The oxidation process ends as substrates become depleted and radical recombination reactions begin to dominate. Non-lipid food constituents can also undergo oxidative changes. Pigment degradation, oxidative vitamin loss and protein oxidation are just a few examples.

12.3.3 How antioxidants work

Antioxidants function in different ways, depending upon which part of the lipid oxidation pathway they interfere with. Antioxidants are commonly classified as primary antioxidants, secondary antioxidants, chelators, quenchers, oxygen scavengers, and antioxidant regenerators. The role of these antioxidants in interfering with various stages of the antioxidant mechanism is shown in Fig. 12.2. Some antioxidants function in more than one of these categories.

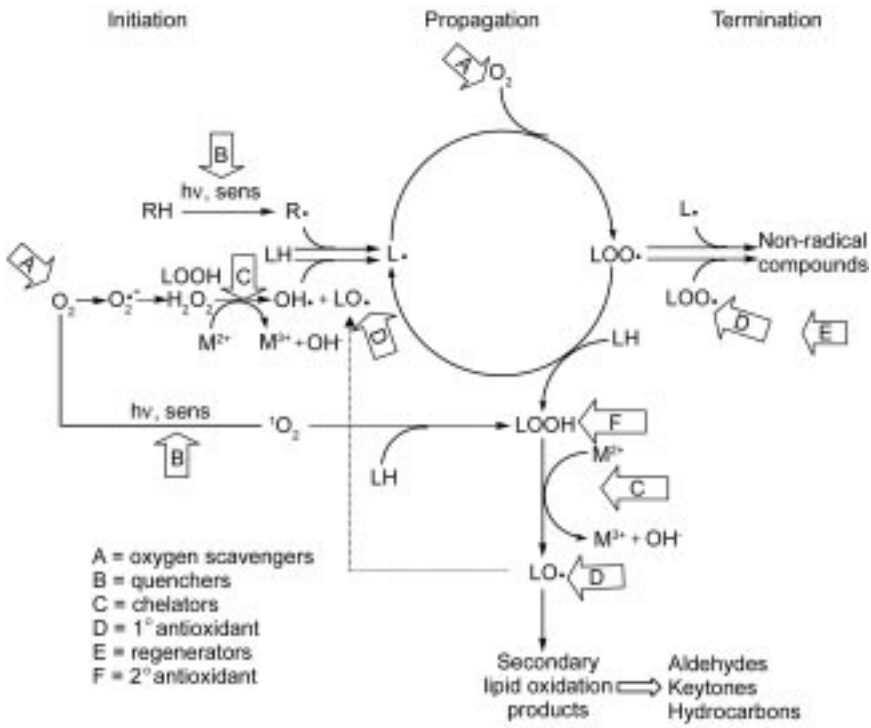


Fig. 12.2 Antioxidant function.

Primary antioxidants

Primary antioxidants are those substances that can donate a hydrogen atom or an electron to a radical, and thereby disrupt its ability to continue the free radical chain propagation process. By donating a hydrogen atom or an electron, the oxidized antioxidant becomes a radical species. It is important that the antioxidant radical that is formed be sufficiently stable (low in energy) so as to be unable to continue the free radical propagation. Phenolic antioxidants, such as BHT, propyl gallate, tocopherol, or carnosic acid, function predominantly as primary antioxidants. Hydrogen donation from primary antioxidants to carbon-centered radicals is usually not rapid enough to compete with the nearly diffusion-controlled rate of reaction of these radicals with molecular oxygen. This means that the effect of primary antioxidants is mainly manifested in hydrogen or electron donating interactions with oxygen-centered radicals.

Under certain circumstances, scavenging of carbon-centered radicals can occur. Compounds that provide this function are well known in the plastics industry, and include hindered amine light stabilizers (HALS), hydroxyl amines and benzofuranones. Some antioxidant effects noted in frying oil applications may be due to carbon-centered radical scavenging (see Section 12.6.2).

Secondary antioxidants
Secondary antioxidants are those substances that react with hydroperoxides, converting them to more stable, non-radical products. Hydroperoxides react with metals to form hydroxyl and alkoxyl radicals which can in turn abstract hydrogen from lipids, generating carbon-centered radicals that further the propagation cycle. It is important that hydroperoxides be removed from the system. Examples of secondary antioxidants include phosphites, used extensively in the plastics industry and sulfur compounds, used in plastics and to a much smaller extent, in foods.

Chelators
Chelators are substances that bind with metals and prevent them from initiating radical formation. Examples include ethylenediamine tetraacetic acid (EDTA), citric acid, phytic acid, and phosphoric acid.

Quenchers
Quenchers are substances that deactivate high energy species such as singlet oxygen or other photoactivated states and divert that energy into less detrimental paths. Phenols, carotenoids and nickel salts (used only in the plastics industry) are examples.

Oxygen scavengers
Oxygen scavengers are substances that react with and remove oxygen from the system being stabilized. Ascorbic acid and metallic powdered iron are examples of antioxidants that work in this manner.

Antioxidant regenerators (synergists)
Antioxidant regenerators are substances that reduce the radicals that are formed when a primary antioxidant donates a hydrogen atom or electron to a free radical. Tocopherol, an active primary antioxidant, is regenerated when tocopheryl radical is reduced by ascorbic acid.

12.4 Synthetic antioxidants and food applications

Natural antioxidants, such as guaiaretic acid or nordihydroguaiaretic acid (NDGA) (Fig. 12.3), were used in foods before the advent of synthetics. They were replaced by synthetics for a number of reasons, including low cost, high purity and constant activity. Synthetics were tested extensively for toxicity and food safety.

12.4.1 Food approval

BHA and propyl gallate (PG) predated tert-butyl hydroquinone (TBHQ) in their approval for use as food additives. They have been used for over 60 years in the

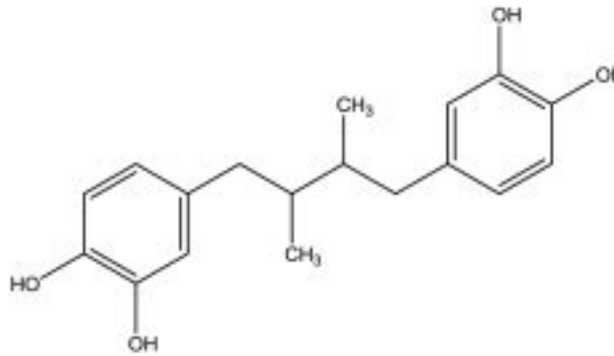


Fig. 12.3 Chemical structure nordihydroguaiaretic acid (NDGA).

stabilization of vegetable oils, potato chips, animal fats (Kraybill *et al.*, 1949; Magoffin and Bentz, 1949; Sherwin and Luckadoo, 1970). BHA was approved for food use as an antioxidant in the late 1940s, while BHT and PG were approved later. BHT was first patented in 1940 as a petroleum oil antioxidant and was widely used in the gasoline field and later patented for use in animal fats and dry breakfast cereals in 1947 (Kraybill *et al.*, 1949). TBHQ was approved for food use in the United States in 1972, with targeted applications being polyunsaturated oils (Sherwin, 1989). Regulations governing the use of synthetic antioxidants vary from country to country and depend upon the food application in question. It has only been in the last ten years that TBHQ has been authorized for use in Canada, though its use is restricted to the stabilizing of fats, oil and lard. Recent amendments found in the European Directive 2006/52/EC have allowed a somewhat broader use of TBHQ in Europe. Because of studies that link high doses of BHA with cancer of the forestomachs of rats, BHA finds itself on the California Proposition 65 list of substances known to the State of California to cause cancer. Readers are encouraged to consult applicable regulations for each country of interest for more information.

12.4.2 Properties

While BHA and BHT are highly oil soluble and water insoluble, PG is very slightly soluble in water and only slightly soluble in animal and vegetable oils. BHA and propyl gallate are quite soluble in propylene glycol, while BHT is insoluble in this solvent. BHT is slightly less soluble in animal and vegetable oils than BHA and TBHQ.

BHA in its pure form has a phenolic odor, while BHT and PG have practically no odor. BHA can develop a slight pink color in the presence of alkaline metals (Joyner and McIntyre, 1938). BHT forms a yellow color in the presence of iron in foods. TBHQ does not form a complex with iron or copper. PG forms purple or violet complexes with iron, and as such, is best used with metal chelators such as citric acid. TBHQ can interact with free amines to

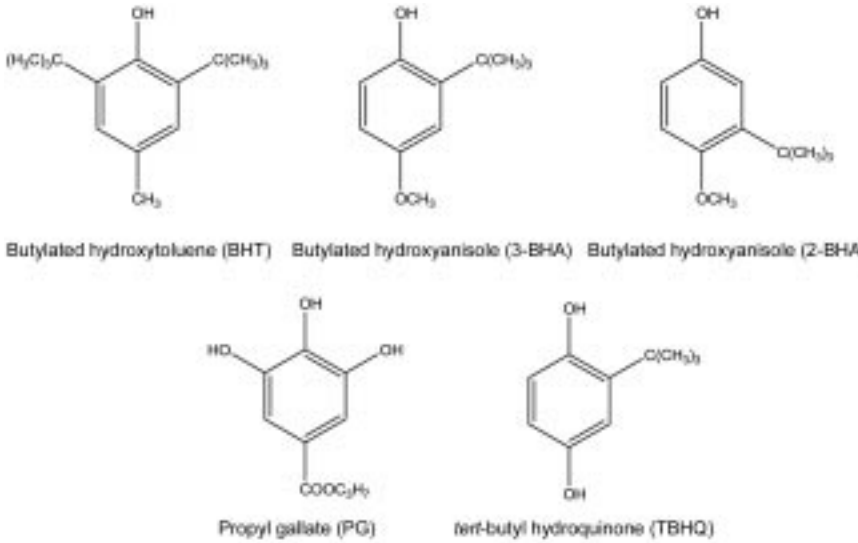


Fig. 12.4 Chemical Structures of BHT, 3-BHA and 2-BHA, PG and TBHQ.

produce a red colored material (Sherwin, 1989). Commercial BHA contains a higher proportion of the 3-isomer (90%) than the 2-isomer (see Fig. 12.4).

BHA, BHT, PG and TBHQ are all hydroxy substituted phenols (see Fig. 12.4), and as such, act as radical scavengers by donating hydrogen atoms, thereby forming more stable phenoxy radicals that do not contribute to the lipid oxidation mechanism. They can react further to form color bodies, but they do not otherwise further destabilize the lipid system.

BHT is not as effective as BHA, in general, due to the presence of the two *t*-butyl groups, which offer greater steric hindrance. BHT is also more volatile than BHA and is more readily lost in baking and frying applications. The antioxidant activity of BHA has been found to increase up to 0.02% and remains constant after that (Thompson and Sherwin, 1966). BHT, on the other hand, does not appear to plateau in this manner.

BHA has been shown to have antimicrobial (Chang and Branen, 1975), antifungal (Ahmad and Branen, 1981), and antibacterial properties (Shelef and Liang, 1982). TBHQ also has antimicrobial properties (Nanditha and Prabhasankar, 2009).

12.4.3 Food applications

Oils play an important role in our food systems and have been the basis for many studies involving oxidation. More is known about the oxidative mechanism of lipids than is known about the oxidation food components such as proteins and carbohydrates. Synthetic antioxidants have been used to stabilize many different types of lipidic systems. They have been effective in vegetable oils, fish oils,

animal fats and oils and low-fat snack foods, to name a few examples. Antioxidant efficacy is due to many factors, but their structure, solubility and the properties of the matrix that they are operating in, are three very important factors. The sections below will deal with these three factors for synthetic antioxidants and the effects they have on stabilization.

Protection of vegetable fats and oils

The treatise on synthetic antioxidants that follows is meant to be a short survey providing leading references for the reader to follow up on for more detailed information. BHA and BHT are less effective in vegetable oils than they are in animal fats (Chipault, 1962), and they have no significant effect on the stability or margarine (Tollenaar and Vos, 1958). The effect of synthetic antioxidants on virgin olive oil aged at 65 ºC and 100 ºC provide results that are fairly typical for other vegetable oils. In this work, the efficacy of the antioxidants was found to be in the order of TBHQ = BHA > BHT. Similar tests done at 50 ºC had the efficacy order: TBHQ > BHA > BHT. The efficacy was measured using peroxide values, conjugated diene levels test and thiobarbituric acid (TBA) absorption values (Kiritsakis *et al.*, 1983).

Oils containing omega-3 fatty acids, such as linolenic acid, have high nutritive value, but undergo levels of lipid oxidation that dramatically lower the nutritive value (Addis and Warner, 1991). Synthetic antioxidants including BHA, BHT and PG have been used to protect these unstable fatty acids, but the safety of these synthetic materials has been questioned (Barlow, 1990).

PG has been widely used in fats and oils, meat products, confectioneries, nuts, milk products, fish products, margarine, baked goods at levels between 0.001 and 0.04% (Madhavi and Kulkarni, 1996, Nanditha and Prabhasankar, 2009).

Protection of animal fats

Kraybill *et al.* (1949) reported that BHT was effective in protecting animal fats from oxidation and that BHT was slightly more effective in lard than BHA as measured by the AOM stability test, but not as effective as BHA when measured in Schaal oven tests. The same researchers also reported synergism between BHA and BHT in lard (Kraybill and Dugan, 1954). Gearhart and Stuckey (1955) demonstrated the stabilizing effect of BHT, BHA, and PG on lard, potato chips, crackers, and pastry. PG alone or in combination with BHT or BHA produced the highest stability in these tests.

In the vast majority of tests, the activity of TBHQ is equivalent to or greater than that of BHA, BHT or PG. It is most efficacious in vegetable and animal fats and oils and has the advantage that it does not contribute to the color and odor of the fats and oils. TBHQ is equivalent to BHA and more effective than BHT or PG in increasing the stability of lard and is more effective than all three in stabilizing rendered poultry fat. TBHQ is also more effective than BHT or PG in stabilizing crude whale oil (Chahine and MacNeill, 1974) and mackerel skin lipids (Ke *et al.*, 1977).

The activity of TBHQ is improved when used in conjunction with synergists such as citric acid, particularly in vegetable oils, shortenings and animal fats. It performs synergistically with BHT, BHA, PG, tocopherols, ascorbyl palmitate, and EDTA. TBHQ is also less steam volatile than BHT or BHA. BHA functions synergistically with other primary antioxidants such as gallates, tocopherols, BHT, TBHQ, or secondary antioxidants, like thiodipropionic acid, or chelators, like citric acid and phosphoric acid (Madhavi and Kulkarni, 1996).

BHA is less effective in animal fats and shortenings when used alone than BHT or gallates, but its effectiveness increases with added synergists. As an example, BHA alone increased the oxidative stability of lard from 4 to 16 hours, but increased to 36 hours when used with citric acid (Madhavi and Kulkarni, 1996). BHA has been reported to very effective in stabilizing lard when used in combination with thiodipropionic acid, citric acid, phosphoric acid, triethyl phosphate, ethyl acid phosphate, methionine, and lecithin (Kraybill *et al.*, 1949).

Frying oils

BHA works better in frying or baking applications when mixed with gallates. BHT is better in these applications when mixed with a chelator. Typically, BHT or BHA, alone, provide minimal protection in frying applications, but they can have a carry-through effect in the fried foods (Sims and Stahl, 1970). TBHQ has greater carry-through than either BHT or BHA in fryed potato chips (Sherwin and Thompson, 1967). Similar effects have been observed in other fried products such as deep-fried noodles (Park *et al.*, 1989) and (Rho *et al.*, 1986), fish crackers (Ahmad and Augustin, 1985) and tapioca chips (Augustin and Berry, 1984).

Protection of low fat foods

BHA has the ability to remain active in baked or fried foods. It is used in applications such as cereal products, cake mixes, crackers, and pastries (Madhavi and Kulkarni, 1996; Nanditha and Prabhasankar, 2009). While the volatility of BHT and BHA are a disadvantage in frying oils, it can be advantageous in low fat foods. Antioxidants can be added to foods prior to drying or cooking, and the volatilization that occurs during processing disperses the antioxidant throughout the food, and acting to protect the food during the processing and later in storage. BHT and BHA can also be carried through into the finished food from a frying application, and this is an advantageous aspect of these antioxidants (Madhavi and Kulkarni, 1996).

BHA typically outperforms BHT in carry through into baked or fried foods, but in low-fat foods and cereal products, BHT is as effective as BHA. BHA is widely used in low-fat products such as cereals, dehydrated mashed potatoes (Sapers *et al.*, 1975). Combinations of BHA, BHT and PG increase the stability of wheat germ meal, brown rice, rice bran, and dry breakfast cereals (Stuckey, 1955). BHT, BHA and TBHQ have all been used in packaging liners to help protect food freshness (Sims and Fioriti, 1980).

12.4.4 Synthetic chelators and sequesterants

EDTA

Calcium disodium ethylenediaminetetraacetic acid is a food grade, FDA-approved metal sequestering agent (Ash and Ash, 2004). EDTA is widely used in the USA to slow down color loss, as well as rancidity and off-flavor development in lipid containing foods. In spite of its relatively wide-spread acceptance in the USA, it is more restricted in Europe (limited applications) and Japan (low dose). EDTA is not particularly useful in bulk oils due to its insolubility, but it is very effective in beverages and in water-phase containing emulsions, where it forms stable metal complexes and hence prevent transition metal ions from initiating the Fenton reaction that generates hydroxyl radicals in the initiation stage of the lipid peroxidation cycle.

EDTA is particularly useful in mayonnaise, salad dressings, sandwich spreads and margarines. However, EDTA is also useful in a variety of fruit and vegetable products, vitamin stabilization, dairy products, beverages and meat products (Madhavi and Kulkarni, 1996).

12.4.5 Synthetic secondary antioxidants

Thiodipropionic acid and thiodipropionates

Dilauryl thiodipropioate (DLTDP) has been approved for use in food since the 1950s. Thiodipropionic acid (TDPA) and its dilauryl and distearyl diesters can function as chelators and synergists. They act to decompose peroxy acids and hydroperoxides into relatively harmless compounds such as carboxylic acids and alcohols (see Fig. 12.5), and provide superior performance when used in conjunction with primary radical scavengers. Thiodipropionic acid derivatives are allowed in foods in the United States, but their use is rather limited in practice. This may in part be due to the potential formation of sulfur-derived aromas that would be unacceptable in certain food applications. Their limited use might also be due to some dispute as to their effectiveness.

Karahadian and Lindsay (1988) found that extremely high concentrations of dilauryl thiodipropionate (50 000 ppm), far above the regulatory limits, were required to see a significant reduction of hydroperoxide concentration in heat-

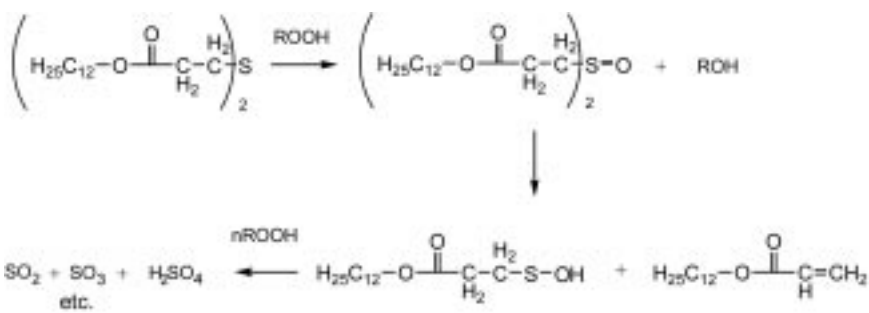


Fig. 12.5 Structures of thiodipropionic acid and its dilauryl and distearyl esters.

rendered siscowet lake trout oil at 50 ºC. These authors found that dilauryl thiodipropionate also failed to act synergistically with BHT, but did react rapidly with peracids. The lackluster performance in these studies is in contrast to other studies that show this kind of sulfur derivative to be an effective antioxidant in many systems. Schwab *et al.* (1953) found that both thiodiacetic acid and thiodipropionic acid were excellent antioxidants in the stabilization of soybean oil and postulated that the activity was due primarily to chelation. Cort (1974) reported that 0.02% dilaurylthiodipropionate doubled the stability of soy oil at 45 ºC and that its activity was synergistic with ascorbyl palmitate. Hill *et al.* (1969) describing an organoleptic study of the effectiveness of antioxidants in milk fat, found that thiodipropionic acid functioned as an antioxidant. In contrast, thiodipropionic acid and dilauryl thiodipropionate were found to be among the least effective antioxidants in a study of flavor stability of foam-dried whole milk (Tamsma *et al.*, 1963). Other antioxidants studied in this test were, in decreasing order of effectiveness, lauryl gallate, propyl gallate, nordihydroguaiaretic acid, ascorbyl palmitate, BHA, ascorbic acid dihydroquercitin, and diethylthiodicarbamate.

Wright (1975) showed that dilauryl thiodipropionate was more effective, alone, than BHA or BHT in the stabilization of soybean oil at room temperature. Dilauryl thiodipropionate in combination with BHA or BHT gave additively better performance, but nothing that could be described as synergistic. Synergistic effects of a combination of DLTDP and BHA were observed in the stabilization of lard (Kraybill *et al.*, 1949). Shaheen (1984) in a study that bridges the disciplines of polymer and food chemistry, found that a combination of vitamin E and dilauryl thiodipropionate provided an excellent stabilizer system for a styrene butadiene rubber chewing gum base. The author states that in the absence of an antioxidant, the chewing gum base is prone to catch fire during processing. BHT is an excellent antioxidant in this system, but there is consumer demand that it be replaced. Neither dilauryl thiodipropionate nor vitamin E, alone, makes a suitable antioxidant. When dilauryl thiodipropionate, alone, is used, the gum base experiences an increase in viscosity as measured using the Mooney torque test. When solely vitamin E is used, the gum base experiences a decrease in Mooney torque (BHA, alone, shows constant Mooney torque during processing). A combination of dilauryl thiodipropionate and vitamin E in the right ratio provides a fully functional replacement for BHT, with the opposite effects on torque cancelling each other out. Finnan (1985) reported that blends of dithiopropionate esters and tocopherol effectively stabilize oil solutions of carotenoid pigments during frying. Rubin (1991) describe a process for restoring rancid marine oil that involves treating the oil with dilauryl thiodipropionate, followed by separation of the oil degradation products using chromatography.

Ascorbyl palmitate

Ascorbyl palmitate (Fig. 12.6) is the palmitate ester of ascorbic acid. The long hydrocarbon tail of the fatty acid helps improve the oil solubility of the ascorbic

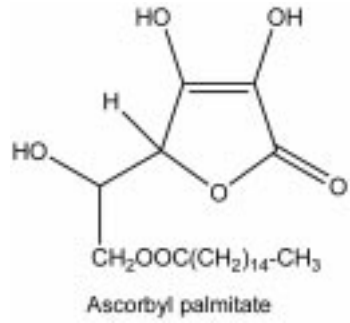


Fig. 12.6 Chemical structure of ascorbyl palmitate.

acid moiety. The ester functions as an oxygen scavenger and a synergist, just like free ascorbic acid. It is, therefore, no surprise that the ester acts synergistically with tocopherols to regenerate them after they have been oxidized to tocopheryl radicals. Combinations of ascorbyl palmitate with tocopherols, lecithin, PG, and TDPA, have been used to stabilize animal fats (Cort, 1974; Park and Addis, 1986), vegetable oils (Cort, 1974) and frying fats (Gwo *et al.*, 1985).

12.4.6 Ethoxyquin

Ethoxyquin (EQ; 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) (Fig. 12.7) is a synthetic antioxidant used widely in the feed industry and as a stabilizer for carotenoids, fish meal, and ground paprika. It forms a stable nitroxide free radical upon oxidation, which is a more effective antioxidant than EQ itself (Lin and Olcott, 1975; Weil *et al.*, 1968). For a more thorough discussion on the mechanism of action of ethoxyquin, and its relationship to hindered amine light stabilizers, see Section 12.6.4.

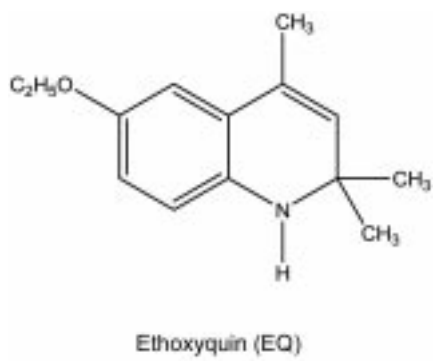


Fig. 12.7 Chemical structure of ethoxyquin.

12.4.7 US governmental regulations

The use of antioxidants in foods, drugs, cosmetics, oils is regulated in the United States by the Federal Food, Drug and Cosmetic Act. Table 12.1 lists various antioxidants, their Generally Recognized As Safe (GRAS) status along with their FDA usage limitations based primarily on fat content. Table 12.2 provides

Table 12.1 Antioxidants, GRAS status, and FDA food-addition limits

Antioxidant	GRAS (yes/no)	FDA usage limitation
BHA	Yes	0.02% of fat or oil content including essential oil content of the food
BHT	Yes	0.02% of fat or oil content including essential oil content of the food
Propyl gallate	Yes	0.02% of fat or oil content including essential oil content of the food
Dilauryl thiodipropionate	Yes	0.02% of fat or oil content including essential oil content of the food
Thiodipropionic acid	Yes	0.02% of fat or oil content including essential oil content of the food
Gum guaiac	Yes	0.1% in edible fats and oils
Tocopherols	Yes	No listed limits
Ethoxyquin	No	0.01% to preserve color in paprika, chili powder, and ground chili
4-Hydroxy-2,6-di-*tert*-butylphenol	No	0.02% of fat or oil content, including essential oil content of the food

Table 12.2 US antioxidant addition limits to foods based on total food weight (ppm)

Food	BHA	BHT	Propyl gallate	Total permissible[a]
Beverages and desserts prepared from dry mixes	2	–	–	2
Cereals, dry breakfast	50	50		50
Chewing-gum base	1000	1000	1000	1000
Dry mixes for beverages and desserts	90			90
Emulsion stabilizers for shortenings	200	200		200
Fruit, dry, glaceed	32			32
Meats, dried	100	100	100	100
Potato flakes	50	50		50
Potato granules	10	10		10
Potato shreds, dehydrated	50	50		50
Rice, enriched		33		33
Sausage, dry	30	30	30	30
Sausage, pork, fresh	100	100	100	200[b]
Sweet potato flakes	50	50		50
Yeast, active dry	1000			1000

[a] Combination of lawful antioxidants
[b] Based on fat content of sausage

Table 12.3 Antioxidant demand (million dollars) by type

Item	1997	2002	2007	2012	2017
Food and beverage shipments (billion dollars)	461.2	482.0	514.5	551.0	586.0
lb antioxidant demand/$000 food and beverage shipments	0.11	0.12	0.14	0.16	0.18
Antioxidant demand (million lbs)	52	58	74	88	103
$/lb	3.08	3.31	3.36	3.47	3.54
Antioxidant demand totals	160	192	249	305	365
Hindered phenols	55	66	85	100	115
Acids and other antioxidants	105	126	164	205	250
Preservative demand totals	310	379	486	605	740
% Antioxidants	51.6	50.7	51.2	50.4	49.3

Source: the Freedonia Group, Inc. Table reprinted with authorization from Freedonia Group, Inc., Cleveland, OH

usage limits based on the total weight of the food application. The usage limits greatly limit the performance levels that can be achieved using synthetic antioxidants.

12.4.8 Usage

The usage in percent sales value for various synthetic and natural antioxidants is given in Table 12.3. It is of interest to note that overall over the span of years from 2002 to 2006, the values did not changed much. The table also shows that

Table 12.4 US and European antioxidants market by type, 2002–2006

	% Sales value	
Year	2002	2006
Synthetics		
BHA	23.4	23.2
TBHQ	11.2	11.1
BHT	3.0	2.9
Propyl gallate	2.4	2.4
Total synthetics	40.0	39.6
Naturals		
Tocopherols	5.5	5.8
Ascorbates and erythorbates	42.6	42.2
Herb extracts	11.9	12.4
Total naturals	60.0	60.4
Grand total	100.0	100.0

Source: Leatherhead Food International. Table reprinted with authorization from Leatherhead Food International

the food and beverage market for antioxidants is a $500 billion industry and is growing at 5–7% per year, and is expected to grow at that rate through the year 2017. Table 12.4 reveals that the market is 50% larger for naturals than it is for synthetics, with the market for ascorbates and erythorbates being as large as the entire synthetic market.

12.5 Natural antioxidants and food applications

Although synthetic antioxidants are demonstrated to be very effective in delaying oxidation and consequently prolonging shelf-life and maintaining freshness, the development of alternative natural antioxidants holds considerable potential in terms of consumer acceptance. After all, petroleum-based synthetic antioxidants like BHA and BHT were used to protect industrial polymers against oxidation prior to their introduction to the world of foods and have been in the human diet for only about 60 years. Natural antioxidant preservatives have been used for thousands of years in the human diet.

Synthetic antioxidants are much cheaper and readily produced in large amounts and high purity but the introduction of new ones requires extensive safety testing in order to fulfill regulatory obligations. This is not the case with natural antioxidants. In the United States for instance, the use of natural antioxidants from herbs and spices that are considered GRAS (Generally Recognized As Safe) is automatically granted and no safety testing is required.

Regulations also limit the usage level of synthetic antioxidants such as BHA, BHT, TBHQ and EDTA in each type of food application, which is not the case of extracts derived from plants, where the limitation is generally determined by the flavor threshold in each type of food. Combinations of natural and synthetic antioxidants can be very helpful when synthetic antioxidants, alone, used at their regulatory limit, fail to give the needed level of protection. Adding a natural antioxidant under these circumstances allows the formulator to further improve the stability of the food while still taking advantage of the cost savings that a base-level of synthetics provide. There may be other advantages of such combinations. Romano *et al.* (2009) recently reported synergistic antioxidant and antibacterial activity of rosemary in combination with BHA or BHT.

The use of natural antioxidants has its own set of limitations and disadvantages. One common issue is effectiveness, although on the molecular level natural antioxidants can be as potent as their synthetic counterparts. The active components usually exist amongst other extract constituents that are not active in managing oxidation, resulting in a need to use much higher levels of the overall extract in order to deliver the required amount of antioxidant active ingredient to the system. This may impair the flavor, aroma and sometimes the color of the food when the natural antioxidant is used at a high level. With high usage levels also comes higher cost and natural antioxidants are generally more expensive to start with, coming from botanical sources with limited availability.

12.5.1 Natural phenolics

According to Boskou, plant phenols are probably the most intriguing plant pytochemicals (Boskou, 2006). Researchers have been studying their natural abundance, chemical structures, potential role in preventing diseases caused by oxidative stress, biological ability to interact with enzymes and proteins and their ability to stabilize foods.

Natural phenolic antioxidants occur in various chemical forms, such as phenolic acids (rosmarinic and carnosic acid), hydroxybenzoic acids (vanillic acid), hydroxycinnamic acids (ferulic and chlorogenic acid), flavonoids (quercetin, catechin and rutin), anthocyanins (delphinidin), tannins (procyanidin, ellagic acid and tannic acid), lignans (sesaminol), stilbenes (resveratol), coumarins (*o*-coumarine) and essential oils (carvacrol, eugenol) (Pokorný, 2007). Many, such as the pyrogallol or pyrocathecol groups contain more then one phenolic OH group.

*Rosemary (*Rosmarinus officinalis*)*

Among the different spice extract antioxidants in commerce today, rosemary (*Rosmarinus officinalis* L.) extract is the dominant natural antioxidant in the market, both in Europe and in the United States. It possesses high efficacy in many food systems, due to the high potency of several of its predominantly lipid

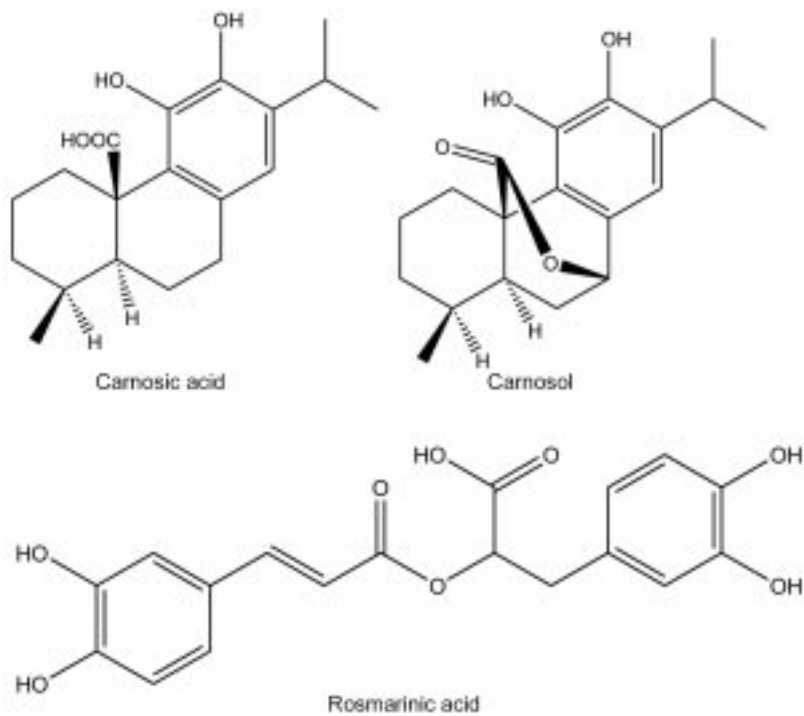


Fig. 12.8 Active phenolics in rosemary.

soluble (carnosic acid and carnosol) and water soluble (rosmarinic acid) active phenolics (Fig. 12.8).

Like most phenolic antioxidants, rosemary's active ingredients function as chain-breaking radical scavengers by donating hydrogen to free radical intermediates in the oxidation process. Carnosic acid and carnosol are peroxyl radical scavengers. They are more effective against lipid peroxidation than propyl gallate in microsomal and liposomal systems (Aruoma *et al.*, 1992). In addition, rosmarinic acid is a better superoxide scavenger than ascorbic acid (Nakamura *et al.*, 1998).

Carnosic acid has super-stoichiometric radical scavenging capabilities that stem from its ability to regenerate four new and active phenolic antioxidant structures through a series of reactions and rearrangements first outlined by (Masuda *et al.*, 2001). This reaction scheme is shown in Fig. 12.9.

Rosemary has been used in culinary applications since ancient times and reports on its antioxidant activity date back to the 1950s (Chipault *et al.*, 1956; Rac and Ostric, 1955). In a review by Nakatani (2000), it was stated that the extract of rosemary leaves exhibited the most effective activity among a variety of herbs that were tested. This might explain why natural rosemary products have found their way into a multitude of food applications, including vegetable oils, fish oils, frying oils, lard, butter, cereals, baked goods, potato chips, fried

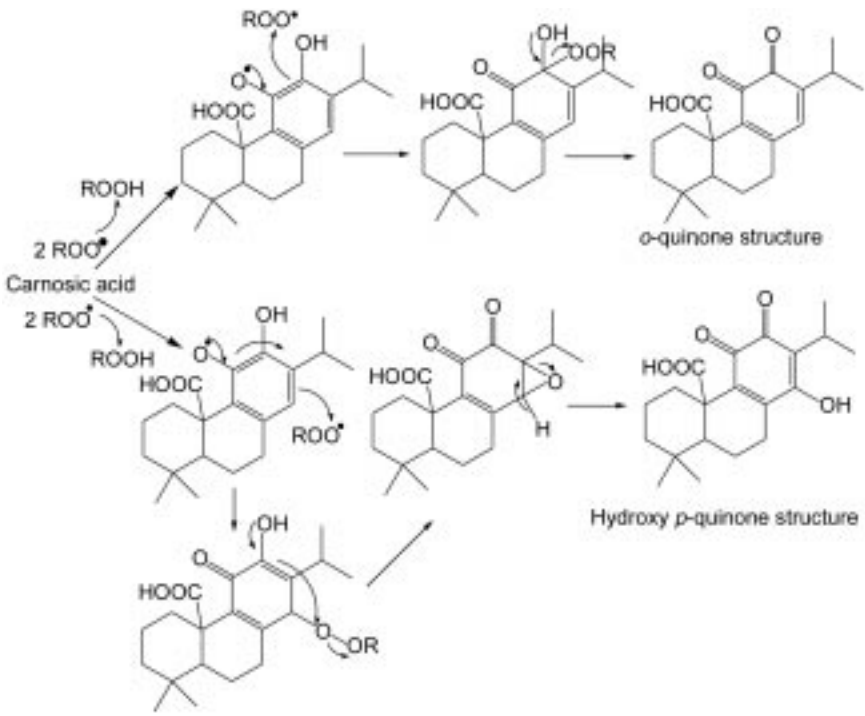


Fig. 12.9 Proposed antioxidant mechanism of carnosic acid.

foods, beef, pork, chicken and beverages. Etter (2004) highlighted over 250 reports on the potency of dry rosemary, rosemary oleoresin, rosemary extracts and the individual active antioxidant molecules in rosemary (carnosic acid, carnosol, epirosmaniol, isorosmanol, methy-epirosmanol, rosmanol, rosmanidiphenol, rosmarinic acid, rosmariquinone and ursolic acid) in various food systems (Etter, 2004). There are additional studies summarized in Table 12.5 that prove the efficacy of commercially available rosemary products in ground chicken, irradiated beef, cooked beef patties, high oxygen modified atmosphere packaged fresh meat, raw beef and pork, fish fillets, frying oils and colors.

Synergistic effects have been demonstrated between rosemary extracts and other antioxidants. Rosemary preparations are often used with tocopherols to protect oil from developing rancidity (Pokorný *et al.*, 2001). Rosemary and citric acid can also work synergistically (Irwandi *et al.*, 2004).

Antioxidant properties extend to other members of the *Labiatae* family and to other spices. Nakatani (1994) found that the family *Labiatae* possesses antioxidant activity above that found in other families such as *zingiberaceae*, *myristicaceae*, *lauraceae*, *myrtacea* and *umbelliferae*. Sage and Oregano were among the *Labiatae* spices that showed potency in a widely acknowledged early study (Chipault *et al.*, 1956).

*Sage (*Salvia officinalis L.*)*

Although there are many antioxidant compounds isolated from sage, including 9-ethylrosmanol ether, luteolin-7-O-β-glucopyranoside, 6-O-caffeoyl-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside and 1-O-caffeoyl-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside, the most active antioxidants are carnosic acid, carnosol and rosmarinic acid (Yanishlieva *et al.*, 2006). In a study by Cuvelier *et al.* (1996), various pilot plant and commercial extracts of sage and rosemary were analyzed and found to consist of phenolic acids, carnosol derivatives, and flavonoids. The most effective ones were carnosic acid, carnosol and rosmarinic acid and to a lesser extent rosmanol, rosmadial, genkawin and cirsimaritin (Cuvelier *et al.*, 1996).

Large-scale processing methods were developed in the late 1970s and early 1980s that produced decolorized and deflavorized sage extracts suitable for use in foods (Adegoke *et al.*, 1998). With many similaritites to rosemary, sage extract is an effective antioxidant for various oils such as sunflower, rapeseed, palm and bergamot in addition to chicken fat and lard (Adegoke *et al.*, 1998; Banias *et al.*, 1992; Che Man and Tan, 1999; Pokorný, 2008), meat (McCarthy *et al.*, 2001) and fried foods (Karpinska *et al.*, 2001). Sage is less commercially attractive than rosemary because of its cost and inferior activity compared to the latter (Löliger *et al.*, 1996).

*Oregano (*Origanum*)*

Oregano is another *Labiatae* family member similar to rosemary and sage in extract chemical composition, showing efficacy as a potential antioxidant for various foods.

Table 12.5 Studies on commercially available rosemary extracts

Food system	Method	Effect	Reference
Cooked ground chicken (high-oxygen atmosphere)	TBA, Sensory, Hexanal	Significant improvement on oxidative stability, color stability and sensory	Keokamnerd *et al.* (2008)
Chicken breakfast sausage	TBA	Effect similar to synthetic BHA-BHT mixture	Lee *et al.* (1997)
Irradiated beef myoglobin	Colorimetric	Significantly preserved color, better than citric acid	Stetzer *et al.* (2009)
Fried beef patties	Formation of heterocyclic amines	Reduced formation of heterocyclic amines by 49–77%	Tsen *et al.* (2006)
Cooked ground beef	TBA, Sensory, Colorimetric	TBA reduced by 92% Significantly reduced hexanal Slight color improvement	Ahn *et al.* (2007)
Vitamin E supplemented beef, MAP packaged	TBA	Significantly better than synthetic BHA/BHT mixture	Formanek *et al.* (2001)
Frozen vacuum-packaged beef and pork	TBA, Colorimetric, Sensory	Slight improvement	Rojas and Brewer (2008)
Fish (fillets) supplemented with antioxidants during feeding	TBA	Notable improvement over control	Sant'Ana and Mancini-Filho (2000)
Frying flaxseed oil	Retained fatty acids	Significant improvement	Irwandi *et al.* (2004)
Deep-fat frying palm oil	Response surface methodology	Rosemary was the most important factor among sage extract and citric acid for sensory acceptability	Irwandi and Che-Man (1999)
Palm olein	PV and TBA, Fatty acids, Polymers, Viscosity, Color	Rosemary > BHA > Sage > BHT > control	Che Man and Tan (1999)
Fish oil	PV, Anisidine, GC	Effective	Kendrick and Macfarlane (2003)
Soybean oil	OSI	Significant improvement	Reynhout (1991)
Food oil systems	Differential scanning calorimetry	Protective effect	Irwandi *et al.* (2000)
Fragrances and aromas	Color	Effective	Christopher and Pisano (2003)

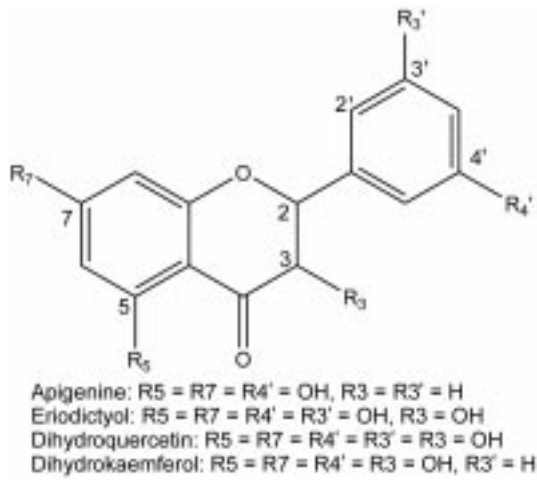


Fig. 12.10 Less polar compounds isolated from oregano.

While the rosmarinic acid content of rosemary and oregano is similar, carnosic acid and carnosol levels are present at a much lower levels in oregano than in rosemary (Hernández-Hernández *et al.*, 2009). Oregano seems to be rich in other, less polar, antioxidants identified as apigenin (flavone), eriodictyol (flavanone), dihydroquercetin (dihydroflavonol) and dihydrokaemferol (dihydroflavonol). These showed high efficacy in protecting lard and vegetable oil against lipid oxidation (Vekiari *et al.*, 1993) (Fig. 12.10). Nakatani (1994) reported the presence of a water soluble glycoside, rosmarinic acid and a rosmarinic acid congener, in addition to several polyhydroxy benzoic and cinnamic acids in oregano (Fig. 12.11).

The essential oil of the oregano is also very active due to the presence of carvacrol and thymol (Botsoglou *et al.*, 2002). As with any of the essential oils, the high flavor levels of these ingredients greatly limits their utility.

Some of the foods that have been preserved with oregano include vegetable oils (Pokorný *et al.*, 2001), lard (Banias *et al.*, 1992), oil-in-water fish oil emulsions (Jimenez-Alvarez *et al.*, 2008). Oregano is effective in meat. Hernández-Hernández *et al.* (2009) showed that oregano slowed color loss and lipid oxidation in raw pork batters. Supplementing oregano oil in the diet was shown to improve the quality of the meat in chicken (Botsoglou *et al.*, 2002), lamb (Simitzis *et al.*, 2008) and turkey (Govaris *et al.*, 2004).

*Tea (*Thea sinensis L.*)*

It is commonly recognized that the main active phenolics in fresh tea are polyphenols known as catechins. Catechins are enzymatically oxidized during fermentation to yield theaflavins and thearubigens (dark compounds in tea). The structures of major catechins and theaflavins are elucidated as: epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate

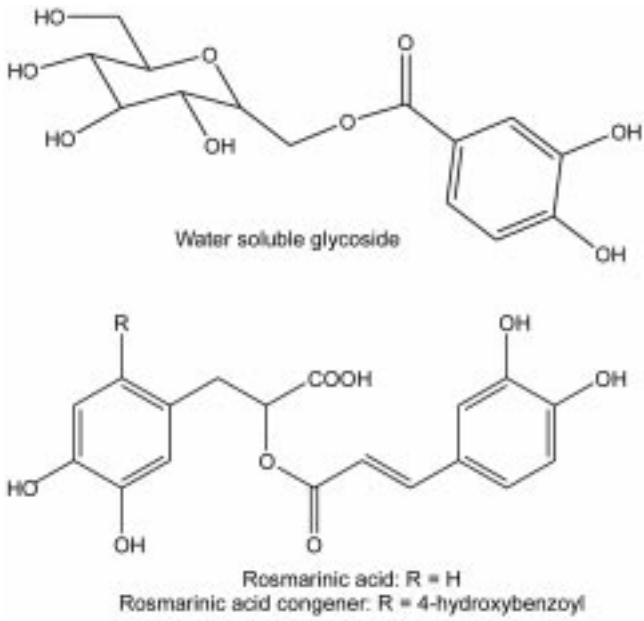


Fig. 12.11 Polar compounds isolated from oregano.

(EGCG) (the predominant catechin in green tea) theaflavin (TF), theaflavin monogallate A (TH-1A), theaflavin monogallate B (TH-1B) and theaflavin digallate (TH-2). However, the thearubigens are not fully characterized and studied (Ho *et al.*, 1997) (Figs 12.12 and 12.13).

Tea extracts exhibit marked antioxidant activity with the main mode of its action resulting from oxygen-centered free radical scavenging (Yen *et al.*, 1997). It is also suggested that metal chelation (Gramza and Korczak, 2005) and lipoxygenase enzyme inhibition (Xie *et al.*, 1993) may play a role. Benzie and Szeto (1999) noted that the antioxidant power of tea is highly correlated to its phenolic content. Within the scope of stabilizing food against lipid oxidation, tea extracts have shown good potential, accompanied by an increase in commercial interest. In a study by Tang *et al.* (2000) tea catechins were found to reduce the thiobarbituric acid reactive substance (TBARS) values in various meats and tissues (chicken meat, liver and heart) if the chickens were supplemented with tea catechins (40% EGCG, 24% EGC, 12% ECG and 10% EC). Koketsu and Satoh (2007) showed that a slightly different green tea catechin composition (27.1% EGCG, 19.3% GC, 16.7% GCG, 16.1% EGC, 8.1% ECG 7.5% EC, and 5.2% (+)-catechin) was a more effective antioxidant than tocopherols in lard and soybean oil, and better than tocopherols and BHA in fish oil. Noodles fried in lard, in presence of the latter catechin mixture, showed oxidative stability proportional to the concentration of polyphenols. Green tea extracts have been reported to have prooxidant effects in seal blubber oil and menhaden oil (Wanasundara and Shahidi, 1998) and in a deoxyribose oxidation system

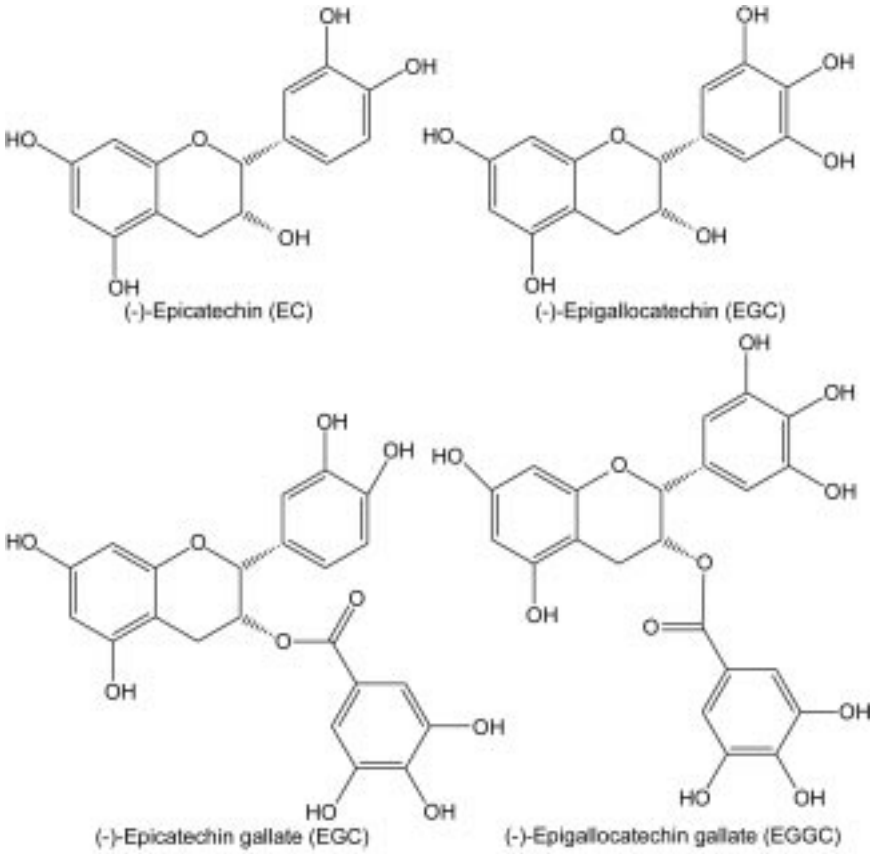


Fig. 12.12 Tea catechins.

initiated by Fe^{3+} and H_2O_2 (Yen *et al.*, 1997). Huang and Frankel attributed that prooxidant effect to variations within the stability, reducing potentials, and partitions between different phases of a lipid system of the various tea catechins (Huang and Frankel, 1997).

Tocopherols

The family of tocopherols consists of four different congeners known as α-tocopherol (vitamin E), β-tocopherol, δ-tocopherol and γ-tocopherol (Fig. 12.14).

Discovery of vitamin E (α-tocopherol) was documented in 1922 in a *Science* paper entitled 'On the existence of a hitherto unrecognized dietary factor essential for reproduction' (Evans and Bishop, 1922). The structure was elucidated years later and tocopherols derived from various vegetable oils and grain sources became known and allowed as additives in foods in the early 1940s (Higgins and Black, 1944). Tocopherols are stable, very effective, lipid soluble antioxidants available in large, economical scale. They are commonly used in fats, oils, meat and baked goods.

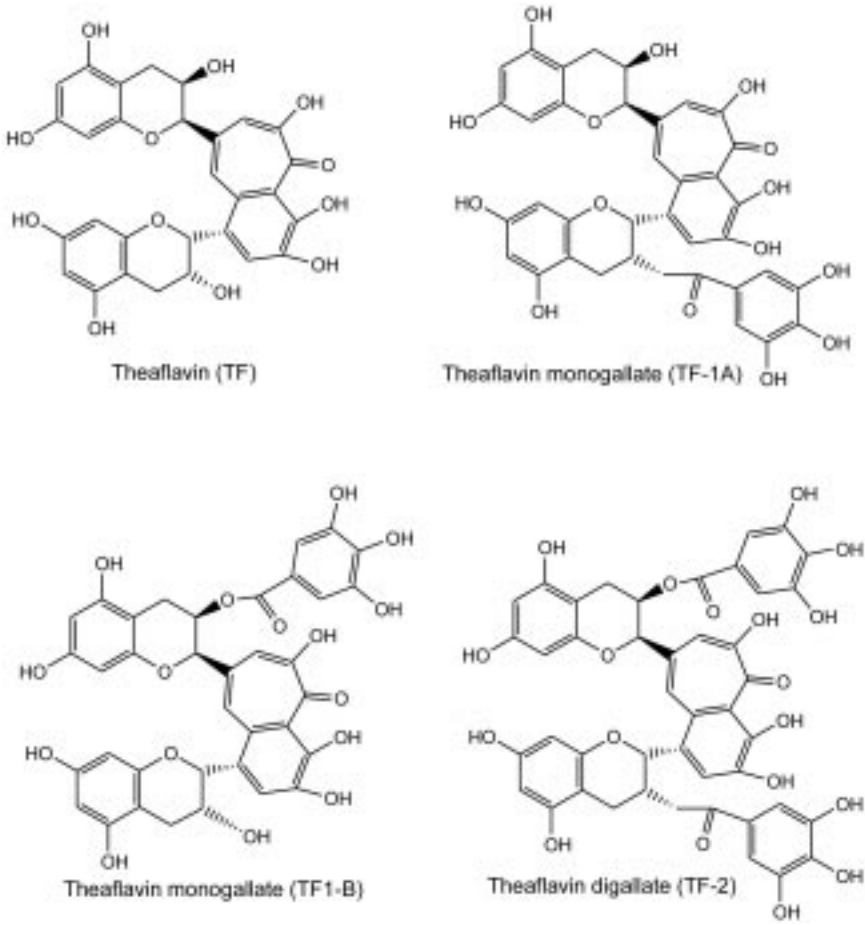


Fig. 12.13 Tea theaflavins.

Owing to their liposolubility, tocopherols are used for both vegetable oils-where they naturally occur-and animal fats. During heating, δ-tocopherol was found to be the most stable followed by β-tocopherol, γ-tocopherol and finally α-tocopherol. On the other hand, δ-tocopherol and γ-tocopherol appear to be the most potent (in lard at 100 ºC) while α-tocopherol is almost inactive (Sims and Foioriti, 2008) with the principal mode of antioxidant action being radical scavenging of both peroxyl and alkoxyl radicals (Frankel, 1996). It is also reported that tocopherols are good singlet oxygen quenchers through a charge transfer mechanism (Kim and Min, 2008) (Fig. 12.15).

In a review by Kamal-Eldin and Appelqvist, it was mentioned that the mode of action of chromanols (chemical class of tocopherols) varies significantly as the physico-chemical parameters of the system change (Kamal-Eldin and Appleqvist, 1996). Hence, it is not surprising to see reports of prooxidant effects

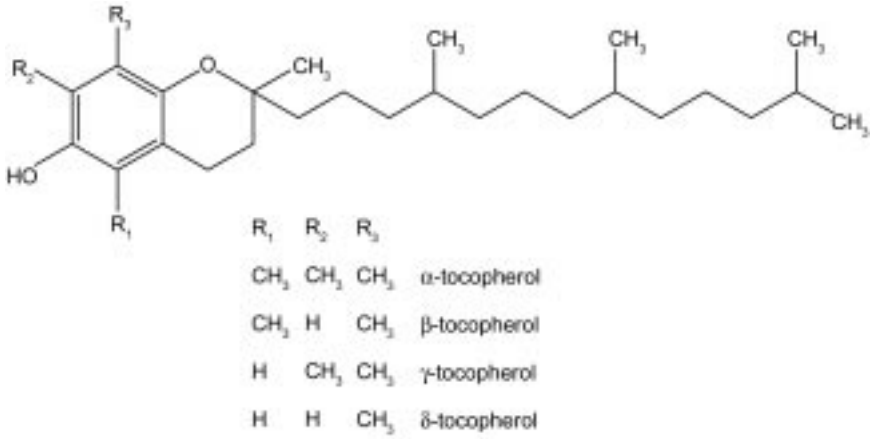


Fig. 12.14 Structure of tocopherols.

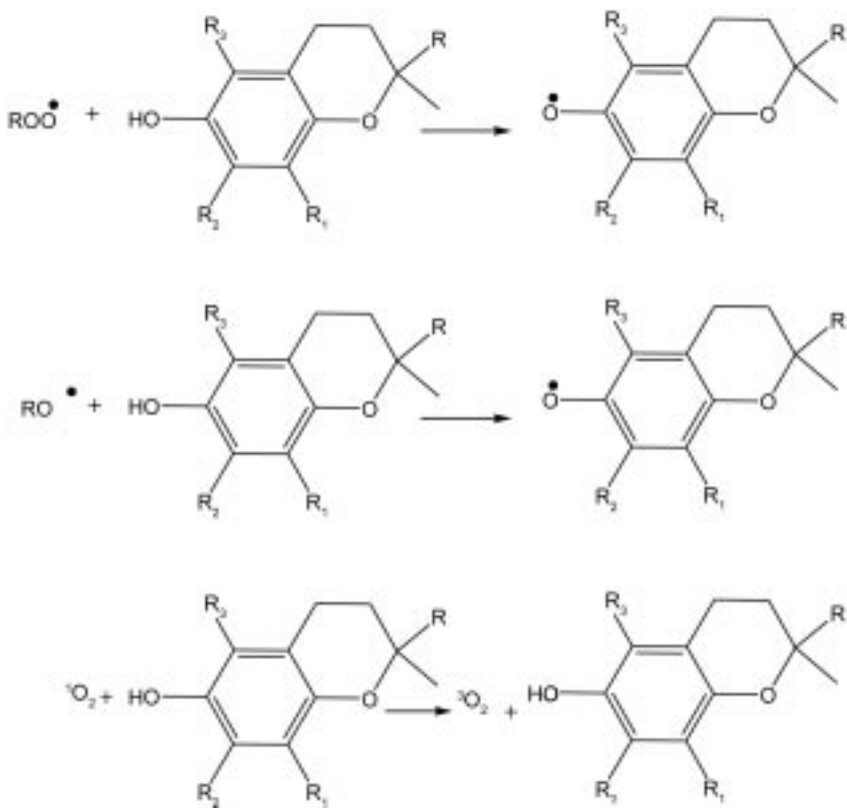


Fig. 12.15 Modes of action of tocopherols.

of tocopherols (Frankel, 1996) depending on the test model, assay method, or even food being studied. Sims and Foioriti (2008) attributed the prooxidant effect to a hydroperoxide radical species generated during the oxidation of tocopherol. This testing system variability might explain some of the discrepancies between reports, some of which conclude that tocopherols are not effective for vegetable oils, and therefore only effective for animal fats. When it comes to vegetable oils, tocopherols do extend the shelf-life and retard lipid oxidation. Lampi and Kamal-Eldin (1998) found γ-tocopherol to be very effective in inhibiting polymerization in sunflower, rapeseed and high-oleic sunflower frying oils due to its higher stability and lower oxidizability (Lampi *et al.*, 1999).

Studies have shown that butterfat containing foods are applications where tocopherols can be very effective (Dougherty, 1993). Tocopherols appear to be a useful antioxidant when added directly in both raw and cooked meat (McCarthy *et al.*, 2001) as well as when it is supplemented with the feed (Formanek *et al.*, 2001; Lavelle *et al.*, 1995). Tocopherols are also effective in slowing lipid oxidation in fish oil-enriched energy bars if prooxidative concentrations are avoided (Jacobsen *et al.*, 2008) and fish fillets (Sant'Ana and Mancini-Filho, 2000).

Alpha-tocopherol and its acetate have been chemically synthesized and these are designated as *dl*-α-tocopherol and *dl*-α-tocopheryl acetate, which are mixtures of the four racemates. As such, these synthetic compounds are called 'nature-identical', since they have the same structure as the natural material, but come from synthetic sources. However, the acetate version lacks the phenol group functionality and therefore lacks the functionality of acting as a food antioxidant. On the other hand, the tocopherol acetate gets hydrolyzed *in vivo*; hence it is a useful antioxidant in feeding experiments.

12.5.2 Ascorbic acid

This ubiquitous sugar acid was discovered early in the 20th century and its structure was determined in the 1930s. L-ascorbic acid is vitamin C (Fig. 12.16).

Ascorbic acid is a natural, water-soluble food antioxidant, distinguished because of its complexity of modes of action. It can act as a metal chelator, an oxygen scavenger and reducing agent and can cause prooxidant or antioxidant effects depending on the system and the circumstances in which it is used

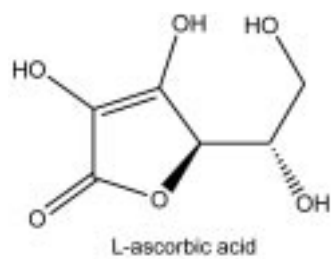


Fig. 12.16 Structure of ascorbic acid.

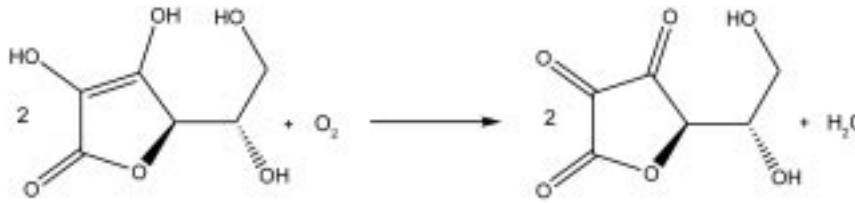


Fig. 12.17 Oxygen scavenging mechanism of ascorbic acid.

(Frankel, 1996). Cort (1974) proposed an oxygen scavenging mechanism where ascorbic acid consumes oxygen, produces water and in the process is converted to dehydroascorbic acid (Fig. 12.17).

Ascorbic acid is widely used as an oxygen scavenger and synergist in numerous food applications. Since ascorbic acid has a higher oxidation potential (greater reducing capacity) than most phenolic antioxidants, it is used to regenerate phenolic antioxidants that have already donated one or more hydrogens to a more reactive free radical. It acts synergistically with tocopherols in this manner, and allows for lower levels of tocopherols to be used, since they are regenerated by the synergist.

Being a reducing agent, ascorbic acid can reduce transition metal salts to their lower valence states that are more active in catalyzing the Fenton reaction. This could explain some of the cases where ascorbic acid has been found to act as a prooxidant. Another important mechanistic aspect of ascorbic acid is its ability to work synergistically with tocopherols. According to Frankel (1996), ascorbic acid interferes with the metal-catalyzed degradation of tocopherols. Another synergistic mode of action is the regeneration of tocopherols by the reduction of the tocopheryl radical.

Many food applications rely on its water solubility and oxygen scavenging properties. Its use was recommended in the stabilization of beer (Wales, 1956) and other food applications where it can serve to reduce the oxygen from the headspace of a closed system (Cort, 1974).

Nowadays, and in spite of its insolubility in oil – which prompted reviewers to exclude classifying it as an oil stabilizer – ascorbic acid has proven useful in stabilizing oils and lipid-containing foods, especially when its combined with other natural antioxidants that function synergistically with it. Nevertheless, prooxidant effects are still observed in some systems. Ascorbic acid showed a prooxidative effect in mayonnaise and energy bars, a weak prooxidative effect in milk and dressings which could be due both to its metal reducing power and to the release of iron from the egg yolk in the case of mayonnaise and dressings (Jacobsen *et al.*, 2008).

12.5.3 Carotenoids

Lycopene, β-carotene, lutein, zeaxanthin and astaxanthin are some of over 600 naturally occurring carotenoids. These are lipid soluble color pigments in fruits

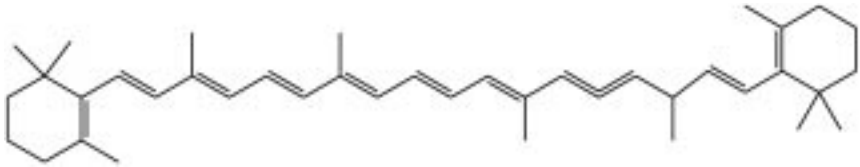


Fig. 12.18 Chemical structure of β-carotene.

and vegetables whose orange, yellow or red coloration stems from their extensively conjugated double bond systems. A main interest in the use of carotenoids – besides being natural food colorants – is due to possible connections between consumption and health. The health effects are thought to be associated with their antioxidant properties. Epidemiological studies have suggested a relationship between the ingestion of carotenoids and good health (Paiva and Russel, 1999).

The extended electron delocalization in carotenoids is the driving force behind the radical scavenging ability of these natural antioxidants. Rajalakshmi and Narasimhan (1996) suggest that carotenoids act as singlet oxygen quenchers and hydrogen peroxide scavengers at high oxygen pressure, and chain-breaking primary antioxidants at low oxygen pressure when singlet oxygen is not present.

β-Carotene (Fig. 12.18) contributes to the oxidative stability of food systems where they are naturally present, such as palm oil (500–3000 ppm) and carrots (Thyrion, 1999). Carotenoids contribute to the oxidative stability of foods when they are used as additives, including, water-in-oil emulsions and margarines (Pokorný *et al.*, 2001), butter, butterfat, coconut oil and corn oil (Nanditha and Prabhasankar, 2009). Additionally, β-carotene exhibited synergistic effects in oil-in-water emulsions when combined with with other carotenoids, such as bixin from annatto (Kiokias and Gordon, 2003) and when combined with α-tocopherol (Li *et al.*, 1995).

In spite of their antioxidant benefits in foods, carotenoids are very sensitive to light and heat and other food properties such as pH and the presence of metal ions (Thyrion, 1999).

12.5.4 Natural chelators

Most of the previously mentioned natural antioxidants function principally as primary antioxidants by trapping the generated radicals and eliminating them. There are only few natural antioxidants that work by sequestering metal ions and hence are called ‘preventative antioxidants’.

While it is usually assumed that many plant phenolics with multiple phenolic groups can act both as radical scavengers and metal chelators, there are some natural molecules that can stabilize food with metal chelating being their main mode of action. The most common ones are citric acid, phospholipids and amino acid/peptides/proteins.

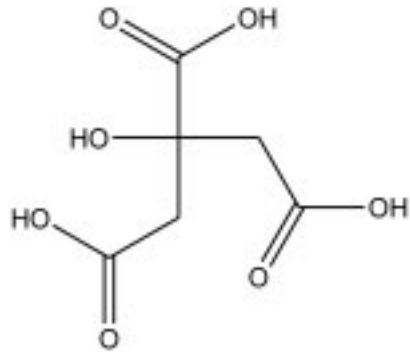


Fig. 12.19 Chemical structure of citric acid.

Citric acid

This naturally occurring acid can be found in a variety of fruits and vegetable, with lemons being the richest source (Madhavi and Kulkarni, 1996). Citric acid is probably one of the most widely used chelators in foods, but it is certainly not the most potent (Fig. 12.19).

Citric acid was studied in the 1940s and 1950s as a food antioxidant useful in butterfat, vegetable oils, shortenings, lard, fats, and the phospholipid portion of milk (Stull *et al.*, 1951). Its use has been studied in combination with primary antioxidants such as mixed tocopherols (Lea, 1944) and BHA (Magoffin and Bentz, 1949). In all these studies, citric acid was referred to as 'synergist' mainly because of its metal chelating potential in oil. The other chelators such as EDTA and phosphates are not readily dispersible in oils.

Madhavi and Kulkarni (1996) list a variety of applications such as fats and oils, fruits and vegetable products and meat products where citric acid is being used in combination with BHA, BHT, PG, tocopherols, ascorbic acid, and ascorbyl palmitate in order to retard lipid oxidation, browning, discoloration of fruits, and the formation of nitric-oxide hemoglobin in cured meats as well as rancidity in ground beef and pork sausage. In addition, it is reported that citric acid can work synergistically with natural antioxidants from spices such as rosemary and sage (Irwandi *et al.*, 2004).

Phospholipids

Lecithin is a broad term used to describe crude fractions of oil, fat, or egg phospholipids. Although nowadays the term lecithin can be used as a reference to phosphatidylcholine; crude lecithin contains other phospholipids, such as phosphatidyl ethanolamine, phosphatidyl inositol, and phosphatidyl serine (Fig. 12.20).

Commercial lecithin, mostly coming from soybeans, is a widely used surfactant for food emulsions. Although there are contradicting reports on its antioxidant capabilities and the mechanism of action of its active ingredients; lecithin is still classified as a food preservative, especially when used with primary antioxidants.

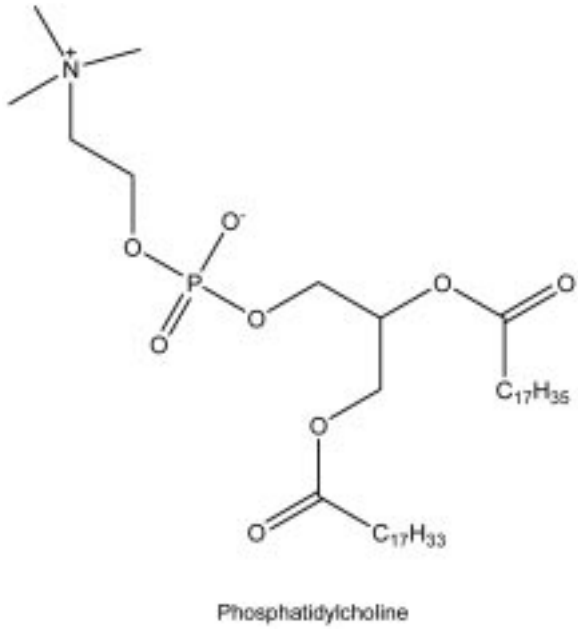


Fig. 12.20 Chemical structure of phosphatidylcholine.

In 1932, Olcott and Mattill assessed the antioxidant activity of lecithin in one of the earliest studies on its potential use to stabilize oils. Using the oxygen absorption method in lard, cottonseed and lard-cod liver oil mixtures, they studied crude lecithin, as well as its alcohol-soluble fraction (the fraction referred to as containing 'the real *lecithin*') and the alcohol-insoluble fraction. It was found that most of the activity is in the alcohol-insoluble fraction, with the active ingredient not decisively determined (Olcott and Mattill, 2002). Sims and Foioriti (2008) found that the alcohol-soluble fraction is particularly active in refined sunflower oil and lard. It is now acknowledged that crude lecithin, pure phosphatidylcholine and phosphatidylethanolamine function as chelator synergists (Madhavi and Kulkarni, 1996).

Two additional possible modes of action were explored by Löliger *et al.* in an attempt to determine whether lecithin actively participates as an antioxidant with vitamin E and vitamin C, or whether it is useful because of its emulsifying property – allowing the two primary antioxidants to be in contact with each other. They found that phospholipids participate actively in shielding vitamin E through the antioxidant activity of the nitroxide radical that forms during the oxidation of the phosphatides (Löliger *et al.*, 1996). For more detail on nitroxide antioxidant effects, see Section 12.6.4.

Soy lecithin is a better antioxidant than several other synthetic and natural antioxidants in bread, the day after baking (Kaur *et al.*, 2007). In addition, soybean lecithin can be used for lard, sunflower oil and butter fat (Sims and Foioriti, 2008).

Proteins and peptides

Proteins from vegetable sources have been investigated in the last decade as a rich source of antioxidant peptide sequences. Although none of the studied peptides has yet resulted in a commercialized product on the market, these materials hold considerable potential as natural metal chelating and radical scavenging compounds.

Milk protein has been extensively assessed. In addition to antioxidant compounds such as vitamin E, β-carotene, and antioxidant catalytic enzymes, milk is a rich source of protein based metal chelators such as glycoprotein, serum albumin, casein and lactoferrin (Pihlanto, 2006).

Casein-phosphopeptides are another milk derived, promising antioxidant. They result from the enzymatic hydrolysis of casein and it is believed that they possess metal chelating abilities due to the presence of phosphate groups originating from serine amino acids in the sequence. These polar functional groups create a medium favorable for chelating metals such as calcium, zinc, copper, manganese and iron (Kitts, 2005). Diaz and Decker (2004) showed that both casein hydrolysates and purified casein-phosphopeptides are effective in inhibiting oxidation in a liposome model system as well as in cooked ground beef. In another study, it was shown that casein hydrolysates can provide antioxidant protection to oil-in-water emulsions (Diaz *et al.*, 2003). Whey is another milk derived protein, shown to possess antioxidant activity through its Bovine Serum Albumin (BSA) portion. Almajano and Gordon (2004) found BSA to be a synergistic antioxidant with caffeic acid and EGCG in oil-in-water emulsions and the antioxidant effect was attributed to a protein-antioxidant adduct that forms during storage. However, Tong *et al.* (2000) attributed the antioxidant activity of a high molecular weight fraction of whey in salmon oil-in-water emulsion to a mechanism involving the sulfhydryl residues availability in addition to radical scavenging through other amino acids and chelation of iron. On a similar note, Taylor and Richardson (1980) studied 15 amino acids and native proteins and found that only cysteine possesses antioxidant activity in addition to proteins with sulfhydryl groups.

Soybean protein and its hydrolysates also showed antioxidant potential by inhibiting iron induced oxidation in a liposomal system through TBAR measurements (Pena-Ramos and Xiong, 2002). Other antioxidant proteins and protein hydrolysis products can be obtained from various sources such as potato (Hou *et al.*, 2005; Wang and Xiong, 2008), chickpeas and white beans (Arkan and Yemenicioglu, 2007), rapeseed (Yoshie-Stark *et al.*, 2008) and Sundakai shrub (Sivapriya and Srinivas, 2007).

Maillard reaction antioxidants

The Maillard reaction is probably the most important reaction that takes place in food during processing. The heating of amino acids and reducing sugars results in a cascade of complicated reactions resulting in pigments responsible for food coloring as well as chemical structures that constitute what we perceive as processed, or cooked food taste and aroma. Besides the Maillard reaction

products' (MRPs) organoleptic notes that characterize baked, broiled and roasted foods, MRPs cause nutritional variations, one of which is the appearance of antioxidant activity.

Numerous studies have demonstrated the antioxidant effectiveness of MRP either during food processing, or in model systems. Devshand and De Muelenaere (1996) assessed the effect of reducing sugars and available lysine during extrusion by determining the antioxidant activity of the extracted lipids and found that MRP are a source of antioxidants. In a review by Namiki, several examples of MRP antioxidant contribution were reported in actual foods such as processed cereals, cookie dough and powdered milk (Namiki, 1988).

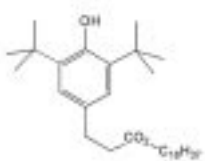
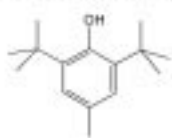
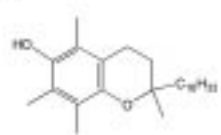
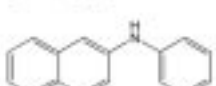
The antioxidant capacity of MRP was also evaluated in model systems such as heated soybean oil and pregelatinized starch containing a Maillard reaction reducing agent and amino acid, where the extent of browning correlated with the antioxidant protection (Mastrocola and Munari, 2000).

Although the antioxidant effects of MRP have been confirmed in a multitude of studies, the mechanism of action is not well defined. It could be related to radical scavenging activity, or metal chelating potential (or both) or to the ability of some of the intermediates to act as reductases. The specific compounds responsible for the activity have not been isolated nor identified due to the complexity of the Maillard cascade of reactions (Reische *et al.*, 2008).

12.6 What can we learn from the plastics industry?

The types of plastics now on the market were made possible only through the development and use of antioxidants. Without them, polymers we rely on would not have the properties necessary to fill the many applications we now take for granted. Just as with foods, antioxidants of various kinds and functions protect polymers during their synthesis, processing, fabrication and long-term use. Both polymers and foods are subject to oxidation during preparation processes that can involve exposure to heating, shear, light and metals. Both polymers and foods suffer oxidation during ambient storage or use. The mechanism of oxidation of these two materials share many features in common, including a radical chain mechanism, free radical intermediates, and types and actions of initiators. Just as polymer antioxidants function and are classed as primary and secondary antioxidants, chelators, quenchers, and so on, so too are food antioxidants. Table 12.6 lists representative examples of various types of antioxidants for polymers and foods. There are many empty spaces in the column for food antioxidants, where no known representatives of a given chemical class exist. It is one purpose of this chapter to highlight these gaps and encourage researchers to search for naturally occurring substances from GRAS plants that have the appropriate chemical functionality to fill these gaps and provide new and better ways of stabilizing foods. Powerful combinations of antioxidants (including primary, secondary, chelators, quenchers, and carbon radical scavengers) have transformed the plastics industry – each component of which

Table 12.6 Various types of antioxidants in the polymer industry

AO Type	Representative polymer examples	Representative food examples synthetic	Representative food examples natural
Primary antioxidant Phenolics	Stearyl-3-(3',5'-di-t-butyl-4-hydroxyphenyl) propionate (Irganox 1076) 	2,6-di-t-butyl-4-methylphenol (BHT) 	Tocopherol 
Diaryl amines	N-Phenyl-2-naphthylamine 	None	None

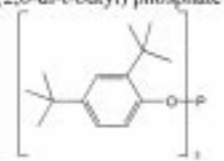
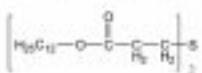
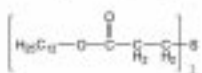
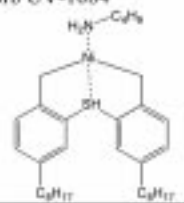
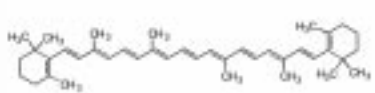
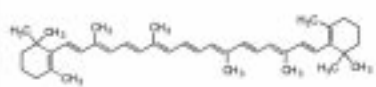
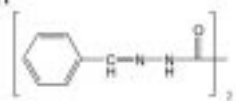
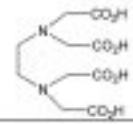
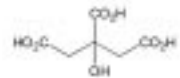
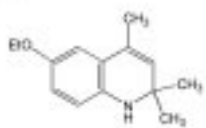
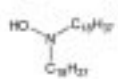
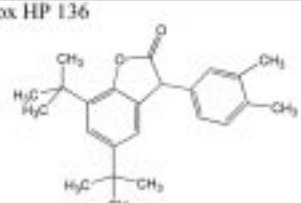
Secondary antioxidants (hydroperoxide decomposers) Phosphites	Tris-(2,6-di-t-butyl) phosphate 	None	None
Sulfur compounds	Dilaurylthiodipropionate 	Dilaurylthiodipropionate 	Naturally occurring sulfur derivatives?
Quenchers	Cyasorb UV-1084 	Synthetic β-carotene 	β-Carotene 

Table 12.6 *Continued*

Chelators	Oxalic Acid Bis-(Benzylidine Hydrazide) OABH 	EDTA Ethylenediamine tetraacetic acid 	Citric acid 
Carbon radical scavengers Hindered amine stabilizers	Tinuvin 770 	Ethoxyquin 	None
Carbon radical scavengers Hydroxylamines	N-hydroxy distearylamine 	None	None
Carbon radical scavengers Benzofuranones	Irganox HP 136 	None	None

operates on a different part of the radical chain oxidation mechanism. Simple polymer systems, like polyethylene and polypropylene, whose uses were once severely limited by stability issues, now compete with low-end engineering thermoplastics because highly functional and synergistic antioxidant combinations now allow them to withstand more abusive environments and have much longer lifetimes. But it has taken more than simple phenolic antioxidants to make such a change possible. This transformation required the use of combinations of highly effective antioxidants taking advantage of synergistic effects.

12.6.1 Primary antioxidant comparisons

There are literally hundreds of phenolic compounds that have been synthesized for use as antioxidants in polymer systems. Some of these compounds such as BHA, BHT, TBHQ and propyl gallate, after rigorous toxicological studies, have proven very useful in the stabilization of foods. There are literally hundreds of naturally occurring phenolic compounds that have shown antioxidant activity. Recently, the 'distinction' between natural and synthetic phenolic antioxidants narrowed a bit with the finding of a fresh water strain of phytoplankton that produces BHT naturally (Babu and Wu, 2008). Both naturally and synthetically derived phenolic compounds demonstrate high antioxidant activity and both classes of materials provide food formulators with benefits. It is not likely that major advances in the practice of food stabilization will come from the discovery of additional natural phenolic substances.

12.6.2 Carbon-centered radical scavenging – a special case

Carbon radical scavengers play an incredibly important role in polymer stabilization. Phenolic compounds do not act as carbon-centered radical scavengers in either polymers or foods. Hydrogen transfer from a phenolic compound to a carbon-centered radical cannot compete with the rapid, near diffusion controlled rate of the reaction of a carbon-centered radical with oxygen. The compounds that act as carbon-centered radical scavengers in plastics include the hindered amine light stabilizers (HALS), hydroxylamines and certain benzofuranone derivatives. HALS and hydroxylamines will be discussed in a separate section, below.

Benzofuranones are a new class of carbon-centered radical scavenger being used to improve polymer stability. The mechanism by which they act is shown in Fig. 12.21 (Schwarzenbach *et al.*, 2001). The 'active' carbon-hydrogen bond is tertiary and doubly benzylic, and the radical formed upon abstraction of the hydrogen is stabilized by resonance into both of the aromatic rings. The benzofuranones seem to be active only at high temperatures and are most effective under low oxygen partial pressure conditions. They appear to have no antioxidant effect under ambient conditions. Given these properties, benzofuranones find their major use in the polymer extrusion process, where oxygen concentrations are low and high temperatures and shear forces create carbon-centered radical species. Benzofuranones are not used in food applications, but

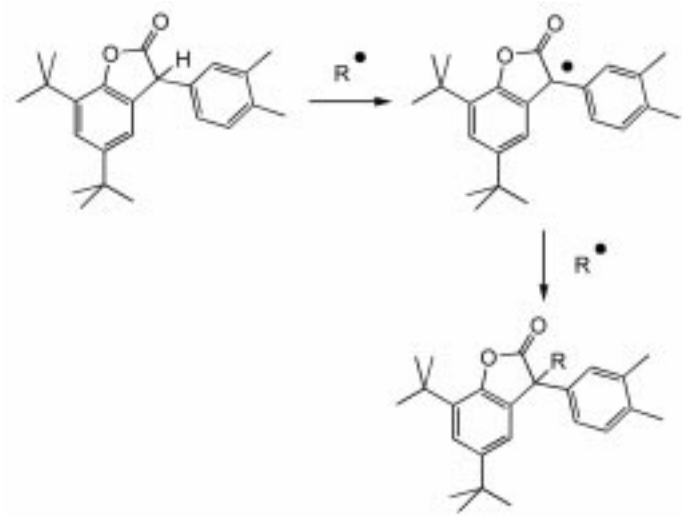


Fig. 12.21 Benzofuranones mode of action.

there are perhaps two circumstances in the processing of food where compounds with benzofuranone-like reactivity might prove beneficial. The first instance, strictly analogous to its use in polymers would be in food extrusion. Although the temperatures encountered in food extrusion are dwarfed by those encountered in polymer extrusion, low oxygen levels and shear might compromise stability in a way that could be countered through addition of a compound that reacted like a benzofuranone. The second circumstance where benzofuranone-like reactivity might prove beneficial is in deep fat frying. Frying oil at temperature (around 190 ºC) and subject to vaporization of water from the food being prepared has a very low oxygen content. Benzofuranone-like compounds may serve as carbon-centered or oxygen-centered radical scavengers under these conditions. Perhaps this kind of reactivity that relies on the formation of a relatively stable carbon-centered radical, is what is behind the finding that certain plant sterols, when added to frying oils, reduce polymer formation.

Polymerization is the result of coupling of lipidic free radical intermediates. Musher (1942) reported the stabilization of a number of organic materials (linseed oil, cod liver oil, soap, heated cottonseed oil, and lard with water extracts of cereals and grains). Sims *et al.* reported that sterols containing the isofucosterol side chain are active, whereas other sterols are inactive or slightly prooxidant (Sims *et al.*, 1972). This work was confirmed by other authors. Boskou and Morton (1976) found that cottonseed oils treated with olive oil-derived sterols (containing Δ^5-avenasterol) were stabilized, whereas the same oil treated with β-sitosterol was unprotected. White and Armstrong (1986) found similar results with oat sterols in soybean oil. Gordon and Magos (1983) postulated that lipid free radicals react with sterols at unhindered allylic sites.

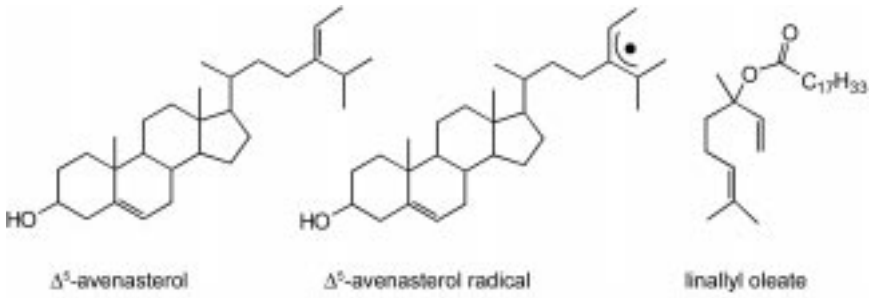


Fig. 12.22 The structures of Δ^5-avenasterol and linallyl oleate.

Tian and White (1994) found that oil extracted from bread cubes fried in soybean and cottonseed oils containing a sterol fraction extracted from oats had significantly lower peroxide values and a higher linoleic/palmitic acid ratio than did control oils or oils containing polydimethylsiloxane stabilizers. Yan and White (2002) and Onal-Ulusoy *et al.* (2005) stabilized oil systems using structurally related linalool derivatives. Both the plant sterols and linallyl esters contain active allylic sites that might form relatively stable carbon-centered free radicals with stability and reactivity reminiscent of the chemistry of benzofuranones used in polymers. This mechanism is highly speculative and has not been proved. The structures of Δ^5-avenasterol, an allylically stabilized Δ^5-avenasterol radical and linallyl oleate are shown in Fig. 12.22.

12.6.3 Secondary antioxidant comparisons

In the plastics industry, highly reactive phosphite stabilizers are routinely used as secondary antioxidants, where they perform particularly well in helping to control viscosity during melt processing. They react stoichiometrically with hydroperoxides and therefore play a smaller role in enhancing the long-term stability of the polymer (Schwarzenbach *et al.*, 2001). Because of their toxicity and reactivity, phosphites cannot be used in foods. Some sulfur compounds, though overtly less reactive than phosphites, can serve a hydroperoxide-decomposing function in both non-food and food applications. Denison (1944) studied the oxidative stability of crude oil-derived lubricating oils and found that the hydrocarbon base 'possessed negligible resistance to oxidation' after removal of the 2–20% sulfur compounds present in the typical commercial oils. In plastics, thiodipropionic acid and its long-chain esters are antioxidant common additives. Each thiodipropionic acid molecule can consume multiple hydroperoxides as shown in Fig. 12.5. Initial oxidation leads to the sulfoxide which can decompose into a sulfenic acid, which can continue to consume hydroperoxides in a series of further oxidation steps, leading ultimately to low molecular weight oxidized sulfur species.

Diesters of thiodipropionic acid are less effective than phosphites at controlling melt viscosity in polymers, but the cascade of chemistry that allows the

sulfur compounds to consume over-stoichiometric amounts of hydroperoxides makes them more effective than phosphites in the long-term thermal aging of plastics. We have already discussed the use of thiodipropionic acid derivatives in foods (see Section 12.4.5). What sorts of naturally occurring sulfur compounds have attracted interest as potential secondary antioxidants in foods? Sulfur compounds present in garlic and onions have been studied in this regard. Allicin is a thiosulfinate found in garlic. Okada *et al.* (2006) have shown that the addition of allicin to chlorobenzene solutions, separately, of cumene and methyl linoleate slows the rate of oxidation of these substrates. The authors provide experimental evidence that allicin is acting as a primary antioxidant, however, not a hydroperoxide decomposer, and that the mechanism involves the transfer of an allylic hydrogen from a position alpha to the unoxidized sulfur to the respective cumene or methyl linoleate hydroperoxyl radicals. This conclusion has been disputed, however (Amorati and Pedulli, 2008). The latter authors question the conclusions of the kinetic data and interpret separate studies on allyl methyl sulfide and diallyl disulfide (major constituents in distilled garlic and onion oil), by concluding that these sulfides do not scavenge peroxyl radicals. Clearly, more work needs to be done in this area. The role of sulfur compounds as secondary antioxidants in foods is not nearly as well established as it is in the polymer industry.

12.6.4 Hindered amine stabilizers

HALS serve primarily to protect polymers from photooxidation and long-term degradation (Gensler *et al.*, 2000), although they have also been reported to serve as thermal antioxidants (Step *et al.*, 1995). Sterically hindered amines are easily converted to nitroxyl radicals under the action of a number of oxidizing agents, including oxygen or hydroperoxides, via mechanisms that are not completely understood. Kumar *et al.* (2007) describe the reaction in which an aminyl radical oxidizes a hydroperoxyl radical to a ketone, via an intermediate alkoxyl radical.

Nitroxyl radicals are postulated as the key intermediates responsible for this class of compounds' antioxidative effects. Nitroxyl radicals react rapidly with carbon-centered or oxygen-centered radicals and are regenerated as shown in Fig. 12.23 (Step *et al.*, 1995). The catalytic nature of their activity may explain their extraordinary effectiveness and is in contrast to the self-sacrificial activity of phenols. Perhaps the antioxidative activity of the phospholipids in foods involves nitroxide and nitroxyl radical chemistry (see Section 12.5.4, phospholipids). We are unaware of any natural products that incorporate the tetramethyl piperidine structure. This is somewhat surprising since this structure is prepared industrially from the simple components, acetone and ammonia, and one might think that Mother Nature armed with these two compounds and several billions of years might have derived this functionality. While no HALS are known to exist naturally, a synthetic compound, ethoxyquin (6-ethoxy-2,2,4-trimethyl-1,2-dihydroquinoline) has certain structural features somewhat reminiscent of a

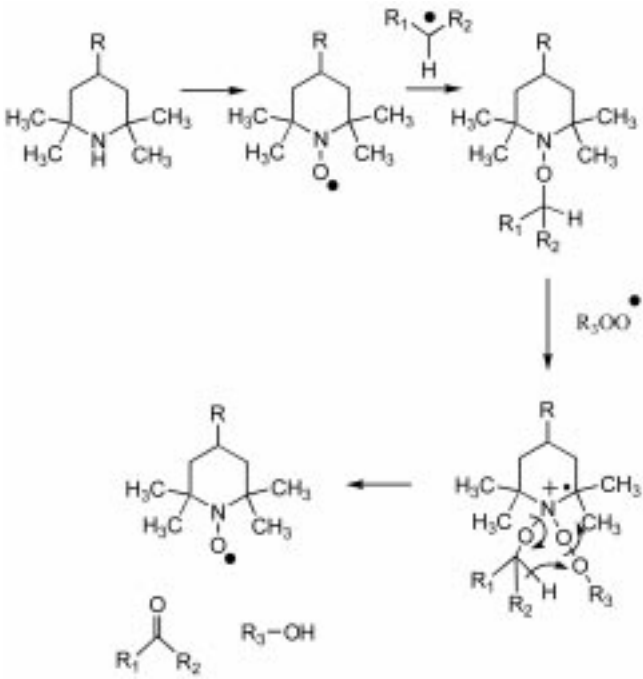


Fig. 12.23 Reaction of nitroxyl radicals with carbon- or oxygen-centered radicals.

HALS. While it is only 'half' hindered, it is a very effective antioxidant. See Section 12.4.6, above. Its use in foods, however, is very highly restricted. In the United States, ethoxyquin can be used to stabilize fish meal and to stabilize the color of ground paprika. In the absence of ethoxyquin, highly unstable fish meal is known to spontaneously combust and the incorporation of this antioxidant is necessary for its safe transport and storage (de Koning, 2002). Ethoxyquin is an extraordinarily effective antioxidant for polyunsaturated fats. The mechanism of action of ethoxyquin has been the subject of much research and debate. In mechanistic thinking analogous to that involving HALS, Lin and Olcott (1975) described the formation of the nitroxyl radical of ethoxyquin, and suggested it was responsible for ethoxyquin's antioxidative effect. That work was supported by Gunstone *et al.* (1991) who showed that the nitroxyl was formed upon oxidation of an initially formed aminyl radical. Kříž and Taimr (1993) however suggested that the antioxidative effect of ethoxyquin was due in some measure to hydrogen donation from the 4-methyl group, and isolated a dimeric product that retained some antioxidative activity. Taimr (1994) further stated that the nitrosyl radical of ethoxyquin 'acts as an efficient antioxidant which, however, does not participate in the cyclic mechanism that is employed to explain the action of antioxidants of the HALS type.' de Koning (2002) returned to the idea that ethoxyquin is an effective antioxidant because two oxidation products, 1,8′-di-(1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline and 2,6-dihydro-2,2,4-

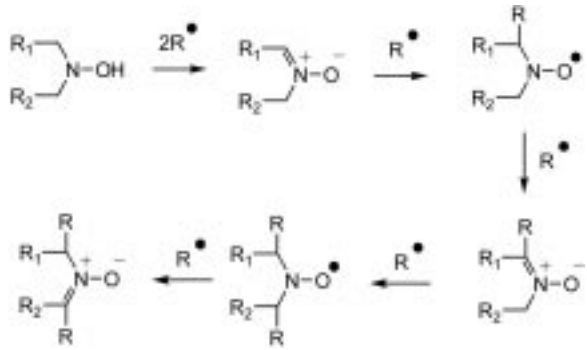


Fig. 12.24 Hydroxyl amines mode of action.

trimethyl-6-quinolone, have antioxidant activity (de Koning, 2002). Kumar *et al.*, however, found evidence for the formation of a substance that could trap alkyl radicals, and postulated that this was the nitroxyl radical (Kumar *et al.*, 2007). Such thinking is very reminiscent of the cyclic mechanism postulated to explain the carbon-centered radical trapping behavior of HALS.

While hydroxyl amines are also efficient carbon-centered radical scavengers in polymer systems, they differ from HALS both in their lack of steric hinderance and in their mechanism of action. Nitrones are thought to be the intermediates responsible for the stabilization observed as shown in Fig. 12.24 Schwarzenbach *et al.* (2001). The authors are not aware of any naturally occurring compounds in foods that are acting in this manner, but it is interesting to note that the polyamine, spermine, present in all eukaryotic cells has been postulated to serve as a free radical scavenger protecting DNA from free radical attack. A primary N-hydroxylated compound was suggested as an intermediate (Ha *et al.*, 1998).

12.7 Conclusion

Synthetic and natural antioxidant additives play a significant role in the modern food industry. While the progress in controlling unwanted oxidation in foods has been enormous, significant advances in our capabilities will be required to meet the challenges of the future. For those consumers who reject the use of synthetic antioxidants in foods, many effective natural alternatives are available. It is our belief that even better natural antioxidant formulations are possible and will come about by combining the highly effective natural phenolics now available with natural, synergistic chelators, secondary antioxidants, carbon-centered radical scavengers and quenchers. The polymer industry shows us what kinds of chemistries will be needed. Truly effective control of oxidation in foods will rely on complex mixtures of antioxidant components capable of attacking the lipid oxidation pathway at multiple points. Such mixtures will also need to be tailored with respect to their physical properties. It will be necessary to formulate

combinations of water and oil-soluble components in such a way that each component can be efficiently delivered to that phase of the food where it is needed. Given the level or research being done in this area, such formulations are near at hand.

12.8 References

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13

Effects of food structure and ingredient interactions on antioxidant capacity

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Abstract: Foods are complex biological materials, and the lipids within the food are susceptible to lipid oxidation, which is retarded by antioxidants. The precise structure and composition of the food may affect the antioxidant activity quite strongly in some cases. Solubility of the antioxidant in the phases present is one of the main parameters that affect the variation in antioxidant activity with phase composition of food. Polar antioxidants are more effective in oils, whereas non-polar antioxidants are more effective in oil-in-water emulsions. Antioxidant activity has been reported in a range of different media, including oils, emulsions, liposomes, microemulsions, fish and meat muscles, and the antioxidant activity may vary from one medium to another. Synergy between antioxidants may also vary from one medium to another. Interactions with metals and with proteins affect antioxidant activity and these interactions are also dependent on the phases present.

Key words: antioxidant, emulsion, liposome, metals, oil, proteins, structure, synergy.

13.1 Introduction

Foods are complex biological materials that may contain some or all of the classes: fat, protein, carbohydrates, lignin, water and air as major components. A wide range of minor components may also be present, and the structure of the food may be dependent on both major and minor components. The precise structure and composition of the food may affect the antioxidant activity quite strongly in some cases, either by affecting the location of the antioxidant within the food or by interactions of the antioxidant with some of the food components.

13.2 Food structure

As a consequence of their composition, and the method of preparation, the physical structure of foods and the state of the lipids vary widely. Some foods are homogeneous such as beverages with all minor components dissolved in water, or vegetable oils with minor components dissolved or dispersed in the oil. However, most foods are not homogeneous and these may contain a variety of distinct phases. The phases present may include solid particles (varying in polarity from polar sugar particles to non-polar fat crystals), liquid phases that may be oil- or water-based droplets and the continuous phase, which may be oil or water. Gas bubbles may also be present. Surface active molecules may be distributed in micelles or reverse micelles, or they may be adsorbed at solid surfaces, or present at interfaces between immiscible liquids or between liquid and gas phases, and these surface active compounds may have a significant effect on the location of antioxidants in foods. In fruits, vegetables and meat products, antioxidants may be present in lipid droplets, which comprise mainly triacylglycerols, or in the cell membranes which contain a phospholipid bilayer, and the activity of antioxidant molecules may vary as a consequence.

The importance of the chemical composition and physical structure of the lipid phase on antioxidant activity has been demonstrated frequently. Solubility of the antioxidant in the phases present is one of the main parameters that affects the variation in antioxidant activity with phase composition of food. Alpha-tocopherol showed a strong synergistic effect with quercetin in an emulsion, but the effect was reduced in liposomes and a clear antagonistic effect was observed in the neat oil (Becker *et al.*, 2007).

Most studies of antioxidant activity have taken place in oil systems. Porter (1980) classified these as low surface to volume ratio systems. Antioxidants, which are more water-soluble including propyl gallate, Trolox, and tertiary butyl hydroquinone are more effective in oil media than less polar antioxidants including tocopherols, BHA and BHT. Porter suggested that the antioxidant action was concentrated at the surface of the lipid. Effective chelation of metals by polar antioxidants represents another mechanism that may contribute to the effectiveness of the antioxidants in an oil system (Fig. 13.1). In contrast, in foods with a high surface to volume ratio, including whole tissue foods, intracellular colloidal micelles of neutral lipids, and the micelles of emulsified oils including salad dressings, non-polar antioxidants such as tocopherols, alkyl gallates, BHA and BHT are much more effective than polar antioxidants. This is due to non-polar antioxidants being concentrated at the interface between the lipid and the polar phase, so that the antioxidant is ideally situated to protect the lipids from radicals generated in the aqueous phase (Fig. 13.2). Recent reports have shown that the formation of derivatives with higher surface activity may lead to reduced antioxidant activity in oil in water emulsions (Yuji *et al.*, 2007), and this indicates that other factors including changes in antioxidant structure that affect the stability of antioxidant-derived radicals, interactions with metals or emulsifiers are also important.

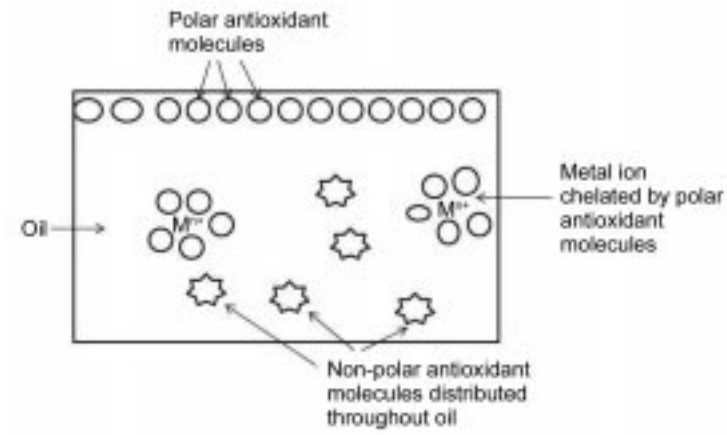

Fig. 13.1 Distribution of polar and non-polar antioxidant molecules in an oil.

The rate of lipid oxidation in an emulsion is strongly influenced by a range of variables including emulsifier, droplet size and pH. Lipid oxidation is accelerated by reactions that take place at the surface of o/w emulsion droplets. This causes the rate of lipid oxidation to increase as the droplet size decreases, because of the increased surface area that is exposed to the aqueous phase (Nakaya *et al.*, 2005). The rate of oxidation of emulsions also depends on the surfactant. Oil-in-water emulsions prepared with a polymeric emulsifier were oxidized more slowly than similar emulsions prepared with a low molecular weight emulsifier (Schwarz *et al.*, 2000). Proteins that are adsorbed at the oil-

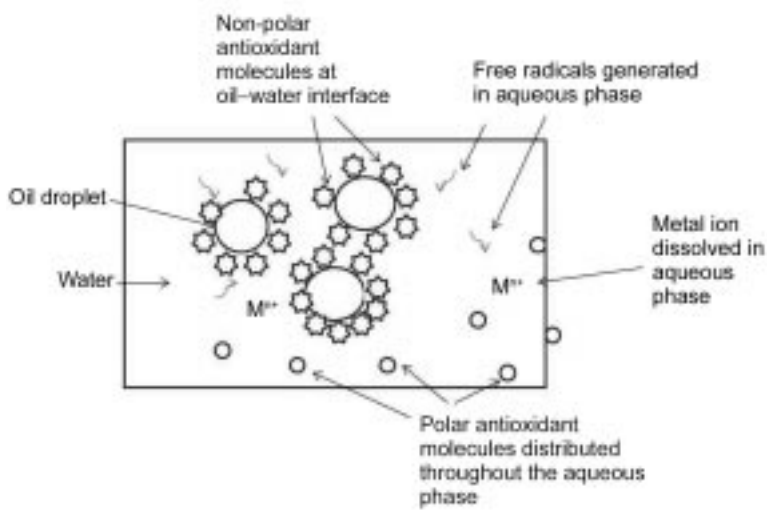

Fig. 13.2 Distribution of polar and non-polar antioxidant molecules in an oil-in-water emulsion.

water interface of an emulsion may also retard lipid oxidation (Lethuaut *et al.*, 2002; Kiokias *et al.*, 2006). Hydroperoxide contents were lower but residual oxygen content was also lower for oil-in-water emulsions of smaller droplet size in the study of Kiokias *et al.* (2006). The pH of an emulsion influences the rate of lipid oxidation by the repulsion of metal ions by positively charged oil droplets at low pH (Hu *et al.*, 2003) as well as affecting the degree of ionization and solubility of antioxidants in the emulsion and the charge on the proteins, which may interact with antioxidants. Studies of polyphenols in aqueous solution at pH 7.4 showed that a catechol or galloyl structure is needed for antioxidant activity at this pH. The order of antioxidant activity was protocatechuic acid < hydroxytyrosol < gallic acid < caffeic acid < chlorogenic acid (Andjelkovic *et al.*, 2006). Vanillic acid, syringic acid and ferulic acid, which do not contain a catechol or galloyl structure did not show any iron complex formation at this pH.

The relative rates of lipid oxidation reactions which convert lipids to hydroperoxides and those that cause hydroperoxide decomposition may vary depending on the lipid medium, and the effects of antioxidants on hydroperoxide formation and decomposition may also vary depending on the medium. Methyl carnosate was more active than Trolox in retarding the rate of formation of hexanal in oil or in water-in-oil emulsions but the order of activity was reversed in oil-in-water emulsions (Schwarz *et al.*, 2000). Methyl carnosate is an ortho-dihydroxy aromatic ester, which has metal chelating ability. The effect of the medium on metal chelation by polyphenols is likely to be an important mechanism by which the medium affects the relative ability of antioxidants to reduce the rate of hydroperoxide decomposition relative to the rate of hydroperoxide formation. The stability of metal-polyphenol complexes varies depending on the medium, and polar solvents also provide ionic pathways by which the complexes can degrade.

In many fatty foods, e.g. chocolate and margarine, the fat phase is partly solidified. According to Porter (1980), amphiphilic antioxidants may be incorporated into lipid droplets which are then solidified, but if solid fats are cut to reveal new surfaces, it would not be possible for antioxidant molecules to diffuse to the new surface. Only non-polar antioxidants, which are homogeneously distributed in the oil before solidification, can be effective in preventing oxidation at these surfaces. When oils solidify on cooling, the antioxidant capacity can change significantly. Cooling olive oil from the liquid state at 25 ºC to the solid state at 3 ºC did not improve the oxidative stability of the oil, and this represented a discontinuity in the effect of temperature on oil stability from the behavior predicted from data in the range 25 ºC to 60 ºC. This effect was attributed to the increase of unsaturated triacylglycerols and decrease of polyphenols in the liquid phase as solid fat crystals were formed (Calligaris *et al.*, 2006). Rapid cooling which generates lipid crystals in less ordered polymorphs may allow impurities such as antioxidant molecules to be incorporated into the crystal structure.

The incorporation of air into lipid containing foods such as aerated creams clearly increases the surface area of the lipid system exposed to the air and is

expected to accelerate lipid oxidation. This phenomenon has been exploited in the Active Oxygen method and the Rancimat method for assessing oil stability. However, the dynamic passage of air through the oil is very different from the situation in stored foods. Volatile antioxidants and lipid oxidation products are swept from the sample in these assays, and this can have dramatic effects on oxidative stability.

13.3 Effect of nature of the lipids and the medium

The nature of the lipids has an important effect on the effect of antioxidants on the susceptibility of foods to oxidation. In products such as meat and fish, oxidation of phospholipids is an important source of off-flavours (Jittrepotch *et al.*, 2006). Simpler iron species originating from degradation of heme proteins and other sources bind to negatively charged phospholipids in membranes and catalyze the cleavage of preformed lipid hydroperoxides. Heme initiated lipid peroxidation, is important for quality deterioration of muscle-based foods (Carlsen *et al.*, 2005). The α-tocopherol content of membranes is an important variable that affects the oxidative stability of the membranes, and the importance of α-tocopherol in ensuring the stability of muscle membranes is particularly high in muscles that are richer in polyunsaturated fatty acids (Yang *et al.*, 2003).

Liposomes are often used as models for phospholipid membranes. Liposomes may be either unilamellar or multilamellar in structure, although the unilamellar structure is a better model of a biological membrane. The multilamellar liposome contains successive shells of phospholipid bilayers separated by aqueous compartments, whereas the unilamellar liposome contains a single phospholipid bilayer with an internal aqueous compartment (Fig. 13.3). Both

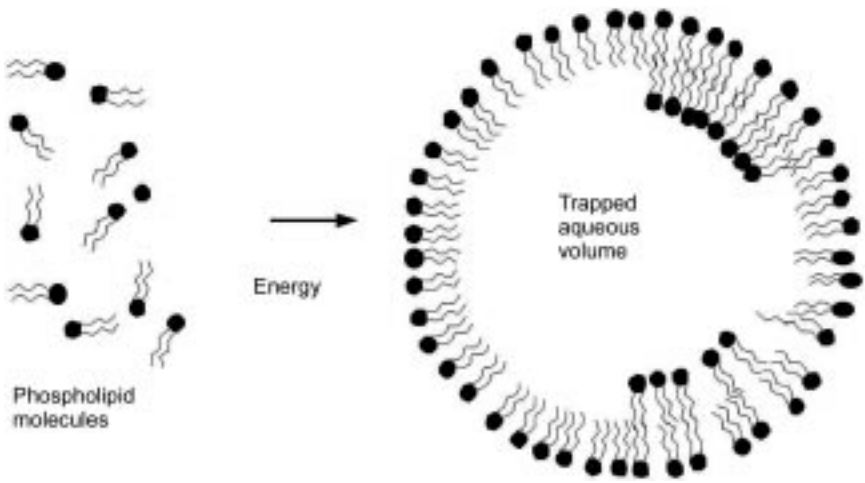

Fig. 13.3 Structure of a unilamellar liposome particle.

multilamellar and unilamellar liposomes have been used to investigate antioxidant effects and synergy between antioxidants. Iron and copper have been used to initiate peroxidation in some studies of antioxidant- containing liposomes, but the majority of studies have used 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (AMVN), which are water-soluble and lipid-soluble thermo labile azo initiators respectively. These azo compounds form peroxyl radicals after initially decomposing to give carbon radicals and a nitrogen molecule. The peroxyl radical can attack the lipids and induce lipid peroxidation (13.2).

$$R–N=N–R \longrightarrow R^{\bullet} + N_2 + R^{\bullet} \qquad 13.1$$

$$R^{\bullet} + O_2 \longrightarrow RO_2^{\bullet} \longrightarrow \qquad 13.2$$

Liposomes are very useful structures for investigating synergy in the activity of antioxidants. Lipophilic antioxidants may be incorporated into the liposome membrane whereas polar antioxidants are in the external aqueous phase. Synergy in the antioxidant effects of mixtures of carotenoids has been effectively demonstrated using both unilamellar (Liang *et al.*, 2009) and multilamellar vesicles (Stahl *et al.*, 1998). Synergy between vitamin C and α-tocopherol (Roberts and Gordon, 2003), and between vitamin A and α-tocopherol (Tesoriere *et al.*, 1996) has been effectively demonstrated using unilamellar liposomes.

Synergy between antioxidants has been reported in a range of different media, including oils, emulsions, liposomes, microemulsions, fish and meat muscles. In some reports, the antioxidants used in a combination only have additive effects, but the term synergy should be restricted to situations where the combination of antioxidants has a greater effect than the sum of the effects contributed by each antioxidant when studied separately. Synergy between antioxidants may vary both with the medium and the nature of the lipids. Caffeic acid was effective in protecting α-tocopherol in fish muscle but not in an oil-in-water emulsion, and this was important in the synergistic effect of a mixture of caffeic acid and α-tocopherol in retarding lipid oxidation in the fish muscle (Iglesias *et al.*, 2009). α-Tocopherol showed a strong synergistic effect with quercetin in a methyl oleate in water emulsion, but the effect was reduced in phospholipid liposomes and the combination of α-tocopherol and quercetin had a shorter induction time than quercetin alone, when the oxidative stability was assessed in an oil by the Rancimat test (Becker *et al.*, 2007).

13.4 Interactions of antioxidants with other components

13.4.1 Interactions with metals

Iron and copper ions induce peroxidation of lipids, but many antioxidants have the ability to chelate metal ions and to thereby reduce the pro-oxidant effect of these metals. Some plant polyphenols possess a remarkable transition metal chelating activity and form redox stable complexes with transition metal ions

(Brown *et al.*, 1998; Hider *et al.*, 2001; Psotova *et al.*, 2003). The kinetics and mechanisms of reactions of polyphenols with ferric ions (El Hajji *et al.*, 2006; Hynes and O'Coinceanainn, 2004), and the binding constants of complexes have been reported (Andjelkovic *et al.*, 2006), although the interactions of plant polyphenols with transition metal ions require further study. Ortho-dihydroxyphenols are effective at chelating metal ions, and phenolic acids containing the ortho-dihydroxy (catechol) substitution in an aromatic ring (caffeic acid, chlorogenic acid, protocatechuic acid, gallic acid, catechol and methylgallate) were shown to form complexes with ferric ions (Chvátalová *et al.*, 2008).

However, the effectiveness of antioxidants by metal chelation depends on food structure, with chelating agents being more effective in oils than in emulsions. This can be due to the complex between an antioxidant and a metal having a lower stability constant or it may be due to the complex undergoing ionic reactions in an aqueous medium. It was clearly demonstrated that the amount of complex formed by chelation of Al(III) by three mono-site ligands: 3-hydroxyflavone, 5-hydroxyflavone and 3′,4′-dihydroxyflavone was reduced by the presence of water molecules in the medium (Dangleterre *et al.*, 2008).

Metals catalyse decomposition of hydroperoxides, but although metal chelating agents may prevent this or reduce the rate of decomposition, effects of polyphenols in aqueous foods may be more complex. Many molecules with antioxidant activity are also able to reduce metals, and this may have a strong effect on the oxidative stability of foods (Perron and Brumaghim, 2009). The reduced form of iron is a much more effective catalyst of lipid oxidation than the oxidized form. Fe(II) is much more effective at catalysing hydroperoxide decomposition than Fe(III). Consequently polyphenols that reduce Fe(III) to Fe(II) may catalyse lipid oxidation rather than retarding it (Paiva-Martins and Gordon, 2002). This effect occurs mainly in aqueous systems, where electron transfer reactions are favoured due to the polarity of the medium.

Hydroperoxide stability assessed by the ratio of peroxide value to thio-barbituric acid reacting substances was higher for oil samples containing the effective chelating agents citric acid or chlorogenic acid than for α-tocopherol in the presence of added ferric chloride (Maisuthisakul *et al.*, 2006). However, the ratio of peroxide value to thiobarbituric acid reacting substances for the samples containing antioxidants fell rapidly to lower values in a soybean oil-in-water emulsion than in the soybean oil. This was due to increased hydroperoxide decomposition in the emulsion at the same peroxide value. The complex formed between caffeic acid and Fe(III) has been shown to decompose by an electron transfer reaction in aqueous solution (Hynes and O'Coinceanainn, 2001).

13.4.2 Interactions with proteins

Enzymes may remove antioxidants from food systems. This occurs particularly in plant tissues, where the action of the enzyme polyphenoloxidase may remove phenolic components by enzymic browning. The reaction is usually associated

with damage to fruit and vegetables, although it also occurs in seafood. Polyphenoloxidase is present in the plastids and chloroplasts of plants whereas phenolic compounds are present in the cytoplasm. However, tissue damage brings them into contact together with molecular oxygen. Diphenol oxidase activity leads to losses of *o*-diphenols including chlorogenic acid and caffeic acid. Many polyphenoloxidase enzymes also have monophenol oxidase activity, and this leads to losses of antioxidants with a monophenol structure including coumaric acid.

Some proteins such as β-lactoglobulin have antioxidant properties. As well as acting as antioxidant enzymes in some foods such as fruit and vegetables, proteins may inhibit lipid oxidation by various mechanisms including scavenging free radicals, inactivation of reactive oxygen species, chelation of prooxidant transition metals, reduction of hydroperoxides, and changes in the physical properties of food systems, such as changes in the droplet size or charge of the disperse phase in food emulsions (Elias *et al.*, 2008; Kellerby *et al.*, 2006).

Some proteins which are relatively weak antioxidants themselves are highly effective in combination with polyphenols. Synergistic interactions between albumin and various antioxidants including virgin olive oil phenolic compounds, green tea catechins, and various water-soluble antioxidants have been reported (Almajano and Gordon, 2004; Almajano *et al.*, 2007; Bonoli-Carbognin *et al.*, 2008). The mechanism for this action has not been fully identified, although it has been noted that reaction of the phenol with the protein occurs during storage with an increase in the antioxidant capacity of the protein fraction. This is most likely due to oxidization of the antioxidant to a quinone which subsequently reacts with a free amine group of a lysine or arginine residue in the protein. However, it is not clear that the product is the main cause of the synergistic increase in oxidative stability.

The interactions between Fe(III) and the hydroxycinnamic acids caffeic, chlorogenic, sinapic and ferulic acids and the flavonoid naringin have been investigated in aqueous acid solution. The mechanisms for caffeic and chlorogenic acid are generally consistent with the formation of a 1:1 complex that subsequently decays through an electron transfer reaction (Hynes and O'Coinceanainn, 2004). For gallic acid and methyl gallate at pH 1-3, a protonated complex is in equilibrium with an unprotonated complex (Hynes and O'Coinceanainn, 2001).

On reaction with iron(III), ferulic and sinapic acids undergo an electron transfer without the prior formation of any complex, and this confirms that a mono-hydroxyphenol structure is not sufficient for complex formation under acid conditions (Hynes and O'Coinceanainn 2004).

13.5 Implications

The use of a wide range of processing techniques to produce foods with varying textures and compositions necessitates the selection of antioxidants and mixtures

of antioxidants that are effective in the range of phases that are present. The desire to incorporate highly unsaturated lipids, such as those in fish oil, in foods for nutritional reasons increases the oxidative challenge that must be met by the combination of antioxidants present. Knowledge of the partition coefficients, reducing properties and chelating activity of antioxidants helps selection of antioxidants for foods of particular structure. As a consequence of the different properties of antioxidants and the different mechanisms by which they act, mixtures of antioxidants may be preferred for the processing and storage of foods where raw materials of different polarity are combined during the processing operation. During these operations, one antioxidant may be more effective at one stage of the processing operation, e.g. in an oil-continuous mixture, whereas it may be less effective in the final product, e.g. an oil-in-water emulsion, whereas a second antioxidant may be more effective in the final product.

13.6 Future trends

Important considerations that will continue to place demands on the need for effective antioxidants are the recommendations from nutritionists that consumption of long chain polyunsaturated fatty acids should be increased, and the desire of consumers for new products with novel textures. Highly unsaturated fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid which occur in fish oil, increase the need for highly effective antioxidants in the food. The development in food processing techniques and the continuous drive to develop new products of novel structures using techniques such as nanotechnology is likely to increase the need for the application of novel mixtures of antioxidants which should be selected based on an understanding of the properties of the antioxidants, and the structure and composition of the food in which they are applied.

13.7 Sources of further information and advice

Further information about the subject can be found in the following books and reviews:

ELIAS RJ, KELLERBY SS, DECKER EA (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition*, 48(5), 430–441.

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Assessing the activity of natural food antioxidants

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Abstract: In the present chapter a working plan for the assessment of antioxidant activity of individual phenolic compounds or of their mixtures (plant extracts, beverages) is presented considering the current trends and advances in methodology. A series of decisive factors that should be taken into consideration when planning antioxidant activity assessment are highlighted. Shortcomings and improvements of available tools for the antioxidant activity assessment are critically presented. Reference is made on computational methods to predict the radical scavenging potential of phenolic compounds and complement experimental findings. Regarding the experimental protocols, emphasis is given to those employing biologically relevant reactive species. Comments are also made on the popular assays $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ as they are highly appreciated by researchers in the fields of food and life sciences. Since lipids are often the source of radicals in foods, assays based on lipid-derived radicals during oxidation of appropriate models (bulk oils, dispersed systems or complex matrices) are presented. The multifaceted character of antioxidant activity is stressed with regard to methodology choice. High resolution screening assays to obtain information on both chemical composition and antioxidant activity of individual constituents of mixtures of antioxidants is acknowledged. As scientists are aware of limitations and drawbacks of existing methods a consensus among experts is needed in order to produce data under standardized protocols, even if methodologies are under scrutiny. The accumulated information can, thus, be transformed to a useful databank for future revisions in this scientific field.

Key words: antioxidants, food lipid oxidation, DFT calculations, radical scavenging, metal reduction, metal chelation, lipoxygenase activity inhibition.

14.1 Overview

The interest in antioxidants in the field of food chemistry decades ago had, as a target, retardation or prevention of lipid oxidation. The examined compounds, which were mostly synthetic, were added to food or tested in the laboratory at levels corresponding to good manufacturing practices (i.e., ~0.02% of the fat or oil content of the food matrix). Later on, the introduction of the biological oxidative stress concept and increased evidence of the contribution of dietary antioxidants, such as polyphenolic compounds, vitamins C and E, and carotenoids, to support the body's defense mechanism against *in vivo* oxidants (reactive oxygen and nitrogen species) renewed the interest and concern in antioxidants. This resulted in a large number of published works in the field of food chemistry, as well as in the life sciences. Some studies showing the possible mutagenic activity of synthetic food antioxidants, together with the increasing demand of consumers for natural ingredients, have also raised interest in natural antioxidants. All these urged the need to develop suitable methodologies for the study of individual constituents and/or extracts from innumerable dietary or novel sources. To this day, various methods for the assessment of antioxidant activity have been reported in the literature. The characteristics of the most frequently employed methods have been critically presented in peer review articles over the years. The various methodologies employed so far are broadly classified into those evaluating changes in a lipid substrate and those measuring free radical scavenging activity, either directly or using a suitable probe. Focus on radical scavenging activity has been given, as this is considered to be the main mechanism of action of phenolic compounds (AH), which are the most widely distributed natural antioxidants and constitute the major part of those consumed on a daily basis.

The large amount of effort invested in developing tests and in executing studies on natural antioxidants resulted in a great diversity of methods, as well as the accumulation of a tremendous amount of data which have created much confusion in the field. The data presented are often conflicting or difficult to compare, since the protocols applied differed significantly between methods despite the fact that the same chemical reagents were used. Almost a decade ago, the complexity of antioxidant activity was highlighted, the multitude of factors affecting the behavior of antioxidants in a system, as well as the different mechanisms of action reported for antioxidants were extensively discussed. The impact of Professor Edwin Frankel's contributions to this evolution is unquestionable. Since then, investigators have refined methodology and introduced more than one tests in their experimental part. Still, the main concern is the critical need for standardized protocols. This is emphasized in all reviews on antioxidant methodology since 2000, thus, motivating related scientific societies to be engaged with the issue. Owing to the interest in antioxidants beyond the application as food additives, the American Chemical Society (ACS) organized the first International Congress on Antioxidant Methods in Orlando in 2004, in which 140 scientists from 18 countries participated. The discussions had as an

objective to extract useful guidelines that should be followed in the near future for publication in journals, protocols for submission to validation by the Association of Official Analytical Chemists (AOAC) collaborative assays and the current status of standard methods for antioxidants of the American Oil Chemists' Society (AOCS). During the meeting focus was on chemical methods for antioxidant content and activity in model systems and foods, measurement of antioxidant activity (reactivity and/or capacity) in food and biological systems, and the *in vitro* and *in vivo* methods to estimate effectiveness in animals and humans. There were interesting and often strong debates on methodologies. The general outcomes were:

- there is no universal assay for the efficient measurement of antioxidant activity,
- only certain protocols can be carried out in both hydrophilic and lipophilic environments, and
- an array of methods should be employed instead of one.

As a consequence of the sound impact of the discussions, in the following years numerous review articles were published referring to the chemistry and the protocols of existed assays. Considering all of the above, in the present chapter an effort is made to present a working plan for the assessment of antioxidant activity of individual phenolic compounds or of their mixtures (plant extracts, beverages). It has to be emphasized that when pure compounds are tested, the ultimate goal is to understand the contribution of structural characteristics, whereas in the case of extracts their antioxidant potential is assessed in relation to total content of active compounds (e.g., total phenol content, total flavonoid content) and to the presence and level of individual constituents. Sections are developed unevenly focusing more on aspects less emphasized in recent book chapters or review articles.

14.2 Assessment of individual compounds

A number of decisive factors should be taken into consideration in the working plan for activity assessment of individual compounds, in particular the molecular characteristics of the compound of interest. Given that antioxidants may have significant differences in size due to the presence of side chains and sugar moieties, it should be stressed that the practice to compare the activity on the same weight basis may give misleading results, especially if structure-activity relationships (SAR) are sought. This is due to the fact that for larger molecules, fewer active hydroxyl groups will be available to react with target species. Therefore, it is recommended to carry out studies on the same molar basis. Another important parameter is the hydro/lipophilicity of the antioxidants with regard to the characteristics of the available methods for activity testing. Solubilization of tested compounds is of paramount significance in order to allow contact with the oxidizing species employed in a specific protocol. As

described later in this chapter, a variety of reaction environments can be employed in certain assays, whereas in others there are limitations. Improper solubilization of the antioxidant could result in a significant underestimation of activity. It is therefore necessary to define limits for the applicability of existing protocols. Such a descriptor is the partition coefficient (log *P*) values of compounds to be tested. These values can be either calculated using software or determined experimentally. Table 14.1 summarizes descriptors for the prioritization of compounds with regard to hydro/lipophilicity presented in the last decade.

Solvent effect is also critical to the ability of antioxidants to scavenge free radicals. Formation of hydrogen bonds between the solvent and the substituents of antioxidants may interfere with the reaction mechanism and consequently affect activity. Other important factors to consider are the physical properties of the compounds, such as color and volatility. The former may cause interferences when measuring the efficiency by means of visible spectrometry, while the latter affects temperature selection in accelerated methods of oxidation. The stability of some test compounds (e.g., phenols) may also be a factor, as are possible interactions with other components of the test system. Temperature affects the rate of reaction, a significant factor bearing in mind that incubation at selected temperatures is a feature of most methods. Analytical parameters including monitoring period, result expression, selection of proper reference compounds, as well as the efficiency of the protocols regarding the number of compounds that can be tested within a working day with sufficient repeatability of the measurements, are matters of concern for either the newcomer or the experienced experimentalist.

14.3 Radical scavenging

As radical scavenging is considered to be the main mechanism of action of phenolic compounds through hydrogen atom (HAT) or single electron donation (SET)[24] (R14.1 and R14.2, respectively), assessment of the ability to scavenge individual oxidizing species is the first goal.

$$AH + ROO^{\bullet} \longrightarrow A^{\bullet} + ROOH \qquad \text{(R14.1)}$$

$$AH + ROO^{\bullet} \longrightarrow ROO^{-}\text{–}AH^{\bullet +} \longrightarrow ROOH + A^{\bullet} \qquad \text{(R14.2)}$$

Published review articles cover a series of methods that are classified on the basis of mechanism of reaction of radical species with antioxidants, in terms of substrate type (synthetic probe or lipid substrate) or in terms of commonly used protocols as indicated in Table 14.2.

In the present chapter, the usefulness of theoretical methods is presented along with experimental approaches. Emphasis is given to protocols assessing scavenging of biologically relevant radical species.

Table 14.1 Descriptors for the prioritization of antioxidants with regard to hydro/lipophilicity

Descriptor	Method/Tool	Antioxidant compounds	Reference
Theoretical approach			
log P[a]	COSMO model	Eugenol related compounds	1
		Hydroxycinnamic acids and halogenated derivatives	2–7
		Phenolic, ascorbic, uric acids	
	Chem Draw program	Oleuropein and related compounds	
log P	simulation of partition in water/*n*-octanol	Simple phenols and phenolic acids	
	(1:1, v/v mixture)	Ferulic acid and related compounds	
		Caffeic acid amide and ester analogues	
Dipole moment	Gaussian Inc program	Caffeic acid and related monopherols	8
Experimental approach			
log P	Mixtures of water or buffer/*n*-octanol (1:1, v/v),	Gallic acid derivatives	9–13
	UV-Vis, room temperature or incubation	Echinochrome	
		Caffeic acid and related monophenols	
		Natural and synthetic phenolic antioxidants	
		Hydroxytyrosol acetate and olive phenols	
K p[b]	Phospholipid/water (varying liposome quantity),	7-Hydroxyflavone	14–16
	incubation 30 °C, fluorescence	Catechin and derivatives, norlignans, hydroxytyrosol, oleuropein	
		Galloylated catechins	
log P	Liposome/buffer,	Hydroxycinnamic acid derivatives	17, 18
	derivative spectroscopy (2^d derivative)	Dietary flavonoids	
log P	HPLC, retention times (calibration with		
	compounds of known log P values)	Caffeic acid, ferulic acid, Trolox, myricetin	19
% distribution	Water/methyl linoleate (9:1 w/w, 1% Tween 20),		
	HPLC, ultrafiltration	Hydroxybenzoic and hydroxycinnamic acids	20
R_f[c]	TLC	Phenolic acids and flavonoids	21
% in the aqueous	20% w/w olive oil in water emulsion	Gallic acid, quercetin, catechin	22
phase	(thawing, Folin-Ciocalteu in the aqueous phase)		
	Tributyrin/Brij 30/water emulsions		
P_W and P_O[d]	(derivatization method with arenediazonium ion,	α-Tocopherol	23
	without physical isolation of phases)		

[a]Logarithm of partition coefficient in water/*n*-octanol (1:1, v/v) mixture, [b]phospholipid/water partition coefficient; [c]retention factor, [d]partition constants in water (w) and oil (o) phase.

Table 14.2 Review articles on antioxidant activity methodology presented in literature since 2000

Method presentation focused on	Reference
Problems regarding assessment in lipid substrates or commonly used free radical scavenging protocols (DPPH$^{\bullet}$, TRAP, $O_2^{\bullet-}$, TEAC, ORAC, FRAP)	25
Free radical scavenging activity assays in both food and biological systems ($O_2^{\bullet-}$, H_2O_2, HOCl, $^{\bullet}OH$, $ROO^{\bullet}$, TRAP, ORAC, TEAC, DPPH$^{\bullet}$, DMPD$^{\bullet+}$)	26
Accelerated stability tests /free radical scavenging assays (ESR, ABTS$^{\bullet+}$, DPPH$^{\bullet}$, FRAP, ORAC, TRAP)	27
Methodological aspects in food quality control or health effect studies	28
Assays involving hydrogen atom transfer (ORAC, TRAP, CBA) /electron transfer (Folin-Ciocalteu, TEAC, FRAP, Copper reduction, DPPH$^{\bullet}$) assays and protocols regarding reactive oxygen species causing oxidative damage in the human body ($O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$, $^1O_2$, $ONOO^-$)	29
Candidate methods involving hydrogen atom transfer (ORAC, TRAP, CL, TOSC, PCL, CBA, LDL oxidation) /electron transfer (FRAP, CUPRAC, Folin Ciocalteu) or mixed mechanism (TEAC, DPPH$^{\bullet}$) for standardization, with regard to the general chemistry and the advantages/disadvantages	30
Assays determine chain-breaking antioxidant activity in food classified as direct (ORAC, CBA, β-carotene bleaching, KI competition assay) or indirect (ABTS$^{\bullet+}$, DPPH$^{\bullet}$, DMPD$^{\bullet+}$) assays	31
Assays measuring activity in lipid model substrates	32
Model lipids, including dietary and biological lipid substrates used in oxidation	33
Currently available methods for the measurement of antioxidant activity in foods (ABTS$^{\bullet+}$, DPPH$^{\bullet}$, ORAC, FRAP) with regard to their validity	34
Critical evaluation of existing antioxidant assays applied to phenolics (ABTS$^{\bullet+}$, DPPH$^{\bullet}$, Folin-Ciocalteu, FRAP, ORAC) in comparison to CUPRAC	35
Various *in vitro* methods used for the determination of antioxidant activity (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, DMPD$^{\bullet+}$, ORAC, FRAP, TRAP, PCL, Phosphomolybdate, TOSC, cyclic voltametry, etc) and their merits and limitations	36
The chemical principles of *in vitro* methods based either on biological ($ROO^{\bullet}$, $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$, HOCl, $^1O_2$, $NO^{\bullet}$, $ONOO^-$) or non biological (TEAC, DPPH$^{\bullet}$, FRAP, Folin-Ciocalteu, Electrochemical reduction) oxidants	37
On the protocol evolution including advantages/disadvantages of most widely used antioxidant capacity assays (ORAC, TRAP, β-carotene/CBA, Folin-Ciocalteu, TEAC, DPPH$^{\bullet}$, FRAP, CUPRAC, lipid oxidation, CL, TOSC, other reactive oxygen/nitrogen species)	38
On the mechanisms underlying assays measuring lipid peroxidation/radical scavenging (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, FRAP, Ferric Thiocyanate, Aldehyde/ Carboxylic Acid) and its application to various plants and foods	39

ABTS$^{\bullet+}$: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation; CBA: Crocin bleaching assay; CL: Chemiluminescence; CUPRAC: Cupric ion reducing antioxidant capacity; DMPD$^{\bullet+}$: N,N-dimethyl-p-phenylenediamine radical cation; DPPH: 1,1-diphenyl-2-picrylhydrazyl radical; ESR: Electron spin resonance; FRAP: Ferric reducing antioxidant power; H_2O_2: Hydrogen peroxide; HO$^{\bullet}$: Hydroxyl radical; HOCl: Hypochlorous acid; KI: Potassium iodide; LDL: Low density lipoprotein; NO$^{\bullet}$: Nitric oxide radical; $^1O_2$: Singlet oxygen; $O_2^{\bullet-}$: Superoxide anion radical; $ONOO^-$: Peroxynitrite; ORAC: Oxygen radical absorbance capacity; PCL: Photochemiluminescence; ROO$^{\bullet}$: Peroxyl radical; TEAC: Trolox equivalent antioxidant capacity; TOSC: Total oxidant scavenging capacity; TRAP: Total radical trapping antioxidant parameter

14.4 Theoretical prediction

Owing to great advances in computer science over the last decade, theoretical methods have been recognized as a potentially useful tool in studies regarding antioxidant activity.[24] In contrast to experimental studies, wherein the evaluation of activity is often based on monitoring substrate changes or probe decay, theoretical studies can predict radical scavenging activity of a compound of interest as a function of various physicochemical parameters computed for an optimized structure.

Various molecular descriptors have been utilized in antioxidant activity studies of phenols. These include bond length values and order, the eigenvalue of the highest occupied molecular orbital (HOMO), dipole moment values, differences in heat of formation (ΔHOF), phenolic O–H bond dissociation enthalpy (BDE), and the vertical (VIP) or the adiabatic ionization potential (IP).[6,8,40–43] Of these, the most frequently employed are BDE and IP values. The former is calculated according to the formula: BDE = $H_r + H_h - H_p$, where H_r is the enthalpy of phenoxyl radical generated after H atom abstraction, H_h is the enthalpy of hydrogen atom and H_p is the enthalpy of the parent molecule; adiabatic ionization potential value is given by the formula: IP = $E_c - E_p$, where E_c is the energy of the cation radical generated after electron transfer and E_p is the energy of the parent molecule. These two descriptors may well characterize the ability of phenols to scavenge free radicals via HAT and/or SET mechanism previously described (R14.1 and R14.2).[42] Low BDE and IP values predict high antioxidant activity. Nevertheless, very low IP values may also indicate a possible prooxidant activity.[42]

BDE and IP values can be computed using various quantum chemical methods. So far, there is no defined method that is both accurate and efficient. Semiempirical quantum chemical methods[44,45] that do not require the user to have a strong theoretical background and training are adequate for experimental researchers as a high-throughput means to screen and rank antioxidants.[24] Nevertheless, with semi-empirical methods, the theoretical values are typically not consistent with experimentally determined ones, as the computational approach utilized is simplified (e.g., the core electrons are not included and some integrals are omitted). In addition, the solvent effect cannot be considered. In order to take into account the latter and obtain more accurate data (with an error of ~1–2 kcal/mole compared with experimental methods), density functional theory (DFT) is suggested.[46–48] DFT, which has been developed more recently than other advanced computational methods, usually employing B3LYP (Becke 3 term with Lee, Yang, Parr exchange), is the most widely used approach in molecular calculations. DFT takes into account all electrons, is less computationally expensive than other methods (e.g., Møller-Plesset, MP2) with similar accuracy.[46] An alternative for saving time withought a significant compromise in accuracy is the combination of AM1 (structure optimization) and DFT (single point energy calculation) methods.[4,6,49]

BDE and IP values have been successfully applied to structure-activity relationship studies of various classes of natural phenolic compounds such as the

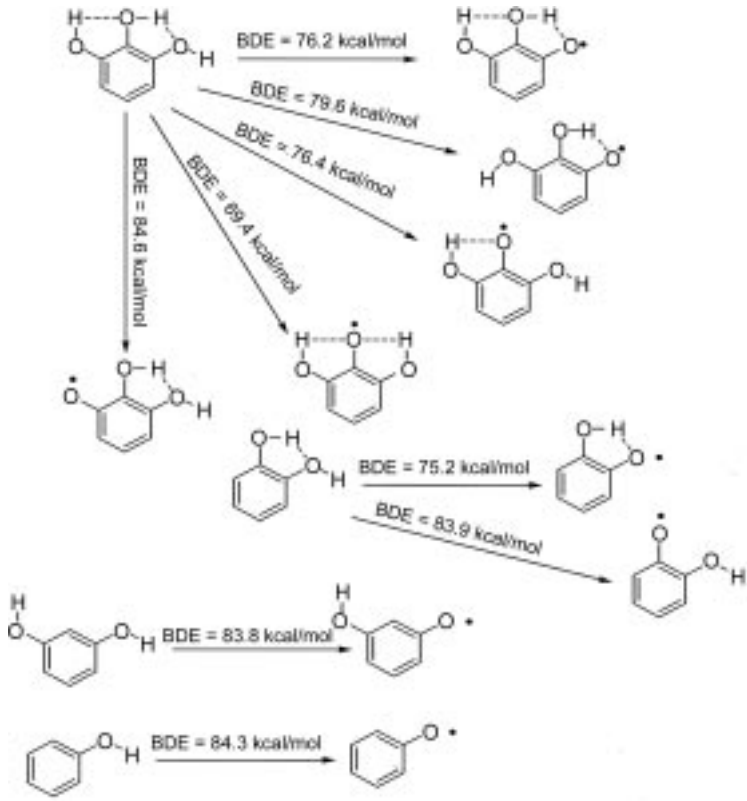

Fig. 14.1 Bond dissociation enthalpy values of O-H bonds of selected simple phenols at the level B3LYP/6-311++G(2d,2p)//B3LYP/6-31G, $T = 298.15$ K (based on Nenadis and Sigalas[50]).

phenolic acids or more complex ones like flavonoids.[24] As an example,[50] the beneficial effect of a pyrogallol or catechol moiety to the radical scavenging activity, known to be important according to experimental findings, is easily reflected in BDE values as shown in the case of simple phenols (Fig. 14.1). In the same figure the contribution of intramolecular hydrogen bonds to the stability of derived phenoxy radicals, and, therefore, to the predicted activity according to BDE values is also illustrated.

Theoretical calculations can be very helpful when a great number of phenols is identified in an extract but, due to oxidative instability, difficulties in synthesis or isolation of individual compounds experimental studies are not easy to carry out.[4] Furthermore, comparison of such data with experimental findings can aid in the understanding of mechanistic aspects or, in certain cases, the necessity to revise established views. More specific BDE and IP values have been found useful in studies related to the effect of pH, and consequently, of compound ionization, to the scavenging of free radicals observed experimentally.[51,52] Moreover, these values aided in the understanding of the

reactivity of certain phenolics (catechins, gallic acid derivatives, gentistein, chalcones) under certain experimental procedures or in the presence of specific radicals,[49,53–55] the effect of conjugation or of the electronic phenomena induced by characteristic groups in the side chain of certain hydroxycinnamates.[6,8] It should be noted that most of the aforementioned published works have been carried out in the gas phase, since such calculations are less computationally expensive and, consequently, less time demanding. Yet, gas-phase BDE and IP values can be valuable primary indices for the antioxidant potential of compounds.[56] Consideration of solute–solvent interaction is of importance as reactions take place in solution and may provide better information than those in the gas phase.[50] A clear limitation at present is extrapolation of such results to predict activity in heterophasic systems. Nonetheless, the theoretical approach that can be tailored to the needs and background of experimental researchers can add to the battery of methods in the field of antioxidants and can be introduced to SAR studies before conducting experimental work.

14.5 Assays using radicals of biological relevance

Numerous procedures have been developed in order to assess the radical scavenging activity of individual compounds towards various radical species (e.g., $ROO^{\bullet}$, $O_2^{\bullet -}$, $NO^{\bullet}$, $^{\bullet}OH$). Characteristic protocols are subsequently presented.

14.5.1 Peroxyl radicals ($ROO^{\bullet}$)

Peroxyl radicals ($ROO^{\bullet}$) are the most common reactive oxygen species (ROS) formed in foods containing lipids resulting in nutritional loss and quality deterioration.[57] In the protocols developed so far, peroxyl radicals are produced via thermal decomposition of thermolabile azo-compounds (R–N=N–R′) that provide a constant flux of hydrophilic or lipophilic $ROO^{\bullet}$. Methods with an impact in recent literature are the crocin bleaching and the oxygen radical absorbance capacity assays.

Crocin bleaching assay (CBA)

The protocol introduced by Bors and co-workers[58] and modified by Tubaro *et al.*[59] assesses the ability of phenolic antioxidants to protect crocin, a naturally occurring carotenoid derivative, from bleaching due to competitive reactions with radicals. Bleaching is observed as a consequence of the attack of the hydrophilic $ROO^{\bullet}$ generated by 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH) radicals, which abstracts a hydrogen atom resulting in the disruption of the crocin conjugated system. Scavengers compete with crocin for the reaction with free radicals. Efficient competitors are consumed preferably so that crocin bleaching takes place at a lower rate. The reaction is carried out at pH 7.0 at 40 ºC, and the progress is monitored by recording absorbance decrease (ΔA or

ΔA_0) at 443 nm for a short period of time (usually 10 min) in the presence (V) or absence (V_0) of antioxidants. Quantitative information expressed as relative rate constants is derived from the equation:

$$\frac{V_0}{V} = 1 + \frac{k_{AH}}{k_C} \times \frac{[AH]}{[C]}$$

where [AH] and [C] are the concentrations of the tested antioxidant and crocin ($\epsilon = 1.33 \times 10^5\ M^{-1}\ cm^{-1}$) and k_C and k_{AH} are rate constants for the reaction of the radicals with crocin and AH, respectively. By determining the V_0/V value at a known ratio [AH]/[C], k_{AH}/k_C could then be calculated. Recently, a step by step examination of CBA led authors[60] to propose a simpler expression of results in the form of percent inhibition. CBA has been used in SAR studies of flavonoids[61] as well as simple phenols[5,62] and phenolic acids[5,63] and the derived order generally obeyed the principles of physical organic chemistry. Nevertheless, SARs among compounds belonging to different classes should be interpreted with caution.[5] Validation data with regard to probe, test compound characteristics, conditions for peroxyl radical generation and reaction monitoring period showed the robustness of the assay.[60] Though the assay is considered to be applicable to 'water-soluble' radical scavengers and related compounds, a modification of the protocol using either canthaxanthin as a probe[58] or a lipophilic initiator in organic solvent (e.g., toluene)[59,64] has been proposed when 'lipid-soluble' compounds are to be tested. Automated versions of the assay using microplates have been reported for plasma testing.[65]

Oxygen radical absorbance capacity (ORAC)

Initially introduced by Glazer *et al.*,[66] the oxygen radical absorbance capacity (ORAC) assay was soon accepted as the 'most valuable' alternative to the DPPH$^\bullet$ assay (see below). Peroxyl radicals produced by the decomposition of AAPH at 37ºC react with a fluorescent probe (fluorescein) at pH 7.0 to form a non-fluorescent product.[67] Hydrogen atom abstraction from the probe result in a decrease in intensity of fluorescence, which is recorded until fluorescein is completely destroyed. In the presence of an antioxidant; however, the probe decay is inhibited as the antioxidant competes for peroxyl radicals. Evaluation of the antioxidant capacity is based on the difference of integrated areas under the decay curves (AUC) obtained for the fluorescein solution in the presence or absence of the compound of interest. In this way it is stated that lag time, rate, and total inhibition is taken into consideration in a single value. Therefore, the assay estimates the 'capacity'* and not just the 'reactivity'* of the tested antioxidant. ORAC values are usually reported as Trolox equivalents. Lipophilic antioxidants can also be evaluated using a solution of acetone/water (1:1, v/v) containing 7% randomly methylated β-cyclodextrin with the same probe.[68] However, fluorescein is not sufficiently lipid soluble, and its fluorescence

* Capacity: estimate of the duration of antioxidative action; reactivity: characterizes the starting dynamics of an antioxidant at a certain concentration.[31]

intensity in non-polar organic solvents is rather low. For this reason the use of a suitable alternative is the pair of the fluorescent fatty acid 4,4-difluoro-3,5-bis(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene known as BODIPY$^{665/676}$ and 2,2′-azobis-2,4-dimethylvaleronitrile (AMVN) as a lipid peroxyl radical generator.[69] The method is of increasing popularity among researchers and has been proposed for standardization by Prior *et al.*, during the first international congress on antioxidant methods.[30] The merits of this protocol are:

- applicability to both hydrophilic and lipophilic antioxidants,
- automation potential (multichannel liquid handling system) that assist parallel analysis of numerous samples (high-throughput),[67,70,71]
- good linearity of AUC vs antioxidant concentration,[70]
- good precision (CV% < 3.0).[71]

Though the extended use of ORAC for testing food extracts and beverages, its application to the assessment of individual compounds activity is rather limited.[3,71–75] It should be stressed that under ORAC assay conditions gallic acid, which is known to be a very efficient antioxidant, is estimated to be of low potency,[3,71] while compounds such as tyrosine and tryptophan that are not strong antioxidants were found to be very efficient and presented high ORAC values.[76]

Activity order of phenolic compounds based on the parallel use of CBA and ORAC data

Data on the activity of AHs can be found in the literature regarding the use of either CBA or ORAC assay. Those presented by Ordoudi *et al.*,[5,73] (Table 14.3) indicated both similarities (e.g., quercetin) or deviations (e.g., protocatechuic/homoprotocatechuic acids) in the order of activity. On physical organic chemistry grounds, ORAC values based order deviated more than expected.

Table 14.3 Antioxidant activity of selected AHs using CBA and ORAC assay by the same analyst

AH	CBA TEV_{krel}* ($n = 3$)	Order of activity	ORAC TEV* ($n = 3$)	Order of activity	Reference
Protocatechuic acid	0.71	4	1.51	8	5
o-Pyrocatechuic acid	0.18	8	2.84	7	
Homoprotocatechuic acid	0.54	7	4.48	4	73,
Dihydrocaffeic acid	0.63	5	3.34	6	Ordoudi and Tsimidou
Caffeic acid	3.51	3	5.20	2	(unpublished)
Catechol	0.53	6	3.76	5	
Rosmarinic acid	4.53	2	4.68	3	5
Quercetin	5.03	1	6.50	1	

* Data given as Trolox equivalent values (TEV)

14.5.2 Superoxide anion radical ($O_2^{\bullet -}$)

Superoxide radical anion ($O_2^{\bullet -}$) is produced as a result of the donation of one electron to oxygen. In foods it is formed when xanthine oxidase (XOD) acts on xanthine or hypoxanthine in the presence of molecular oxygen or by photo-activation of hematoporphyrin.[77] It is considered to be a rather weak oxidant; however, it can lead to the formation of more active species such as hydroxyl radical and peroxynitrite.[77,78] The most common analytical protocol developed for the determination of $O_2^{\bullet -}$ is based on the system XOD/hypoxanthine or xanthine at pH 7.4 to generate the anion radical.[79] Nevertheless, since phenolic compounds may inhibit the radical formation by interfering with the enzyme, a non-enzymatic reaction of phenazine methosulphate (PMS) in the presence of nicotinamide adenine dinucleotide (NADH) is also reported for assessing only the radical scavenging ability.[79] In both cases the detection of the radical is achieved via reduction of nitroblue tetrazolium (NBT) into formazan, which absorbs at 560 nm.[79] The aforementioned protocols are the most preferable ones, though other detection methods of the anion radical through reduction of ferricytochrome C instead of NBT,[80] nitrite formation after reaction with hydroxylamonnium and detection at 530 nm[81] or by GC determination of ethylene produced after reaction with α-keto-γ-methiolbutyric acid (KMBA) have been reported.[82] Methods based on electron spin resonance (ESR) spectrometry and chemiluminescence have also been reported in the literature.[83,84] However, ESR requires expensive equipment and skills for carrying out such tests. Superoxide radical anion has been employed in the assessment of activity of various phenolic antioxidants.[79–81,83–86] The findings, indicate the importance of the number and position of hydroxyl groups to antioxidant activity. Detection of the superoxide anion radical using NBT or chemiluminescence have significant drawbacks due to solubility problems of colored end product and interference with the generated radical.[87] To overcome this, a novel high-throughput assay has been introduced, which is referred to as the superoxide radical absorbance capacity (SORAC) assay. The particular protocol is based on radical production using XOD/xanthine but detection is made via fluorescence. Specifically, a non-fluorescent probe (hydroethidine) is used which is converted to 2-hydroxyethidium (a strongly fluorescenct compound) when oxidized ($\lambda_{exc} = 480$ nm and $\lambda_{emis} = 567$ nm).[87] Unlike other probes, hydroethidine does not interfere with the system, nor does it yield water-insoluble products. The method has been validated relative to linearity, precision, accuracy, and robustness, and then tested using a series of catechin derivatives.[87] Although the SORAC assay is a promising method for SAR studies, a wider diversity of compounds should be tested.

14.5.3 Nitric oxide radical ($NO^{\bullet}$)

Nitric oxide is an important free radical formed *in vivo*, which may participate in both physiological and pathological processes.[88] Nevertheless, $NO^{\bullet}$ scavenging is also of importance from the point of view of food chemistry, as its formation is

possible in muscle foods due to the activity of NO synthase.[89] $NO^{\bullet}$ can react with superoxide to form peroxynitrite, which promotes the oxidation of lipids.[78,90] Van Acker *et al.*,[91] testing a series of flavonoids in aqueous solution (pH 7.4), used a NO-meter to follow $NO^{\bullet}$ consumption over time in the presence of antioxidants. The $NO^{\bullet}$ solution was prepared by commercial NO gas dissolved to a desirable concentration in water that was previously deoxygenated with gaseous nitrogen. The reaction showed pseudo-first order kinetics, as the tested compound was present in excess. By dividing the pseudo-first order rate constants with the concentration of the antioxidant, the scavenging rate constant was obtained.

The chemical production of $NO^{\bullet}$ and its photometric determination via reaction with Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthyl-ethylenediamine dihydrochloride) is another protocol worthy of mention. Specifically, $NO^{\bullet}$ is produced when sodium nitroprusside spontaneously decomposes in aqueous solution at physiological pH (7.2). In the presence of oxygen, $NO^{\bullet}$ reacts with it to produce stable products (nitrate and nitrite), which can be determined quantitatively using the aforementioned reagent by measuring the absorbance of the chromophore formed at 550 nm. The latter is produced by diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride. The amount of nitrite is calculated based on a standard curve using sodium nitrite, with results expressed as either percent inhibition or as IC_{50} values (the concentration of the tested compound needed to reduce 50% of the nitrite formation). The respective protocol has been utilized in the past for the testing of curcumin and related compounds.[92]

ESR spectroscopy can be employed for $NO^{\bullet}$ scavenging testing as described for a series of phenolic compounds isolated from *Agrimonia pilosa*.[93] Fluorescence as a means of detection of $NO^{\bullet}$ or other reactive nitrogen species is promising as various probes that are sensitive and specific have been developed for this purpose.[94]

14.5.4 Hydroxyl radical ($^{\bullet}OH$)

Hydroxyl radical is the most reactive ROS. It can be formed via γ-ray irradiation of water, ultraviolet-induced homolytic fission of hydrogen peroxide, and by the decomposition of hydrogen peroxide or lipid hydroperoxides by reduced transition metals.[77] The radical is extremely short-lived, resulting a low diffusion path, which suggests that it reacts at its site of formation.[33] The deoxyribose method is the most commonly used method for assessing $^{\bullet}OH$ scavenging activity. The radical is generated via a Fenton reaction using a Fe^{3+}-EDTA/ascorbic acid/H_2O_2 system at pH 7.4.[95] Malondialdehyde-like products formed from the degradation of 2-deoxyribose, are measured after the reaction using thiobarbituric acid with detection at 532 nm. As reported in the literature, this particular assay, though simple, has several shortcomings. For example, it is pH sensitive and not compatible with organic solvents.[96] Novel assays that make use of fluorescein as a probe to monitor reactions with the radical were claimed to be effective.[96,97] The proposed approaches are similar to the one described in

ORAC, suggesting that the same equipment and probe can be exploited for testing both peroxyl and hydroxyl radical scavenging. In such protocols cobalt (II) or ferric (III) ions are mixed with H_2O_2 to generate the radical. In both cases a plate reader equipped with a fluorescence detector can assist in high-throughput measurements. A recent method presented by Moore *et al.*[96] was the result of a meticulous study aiming at the development of a reliable protocol. Nevertheless, interaction between the test compound and metal ions cannot be avoided as they are added to the test solution prior to the reaction. Therefore, chelating effects may influence the produced results. Zhu *et al.*[98] introduced a metal-independent, organic Fenton reaction by mixing tetrachlorohydroquinone and H_2O_2. The $^{\bullet}OH$ formed, unless scavenged, resulted in hydroxylation of salicylic acid. The inhibition of this reaction is assessed by HPLC coupled with an electrochemical detector with quantification based on measuring the 2,3 and 2,5 dihydroxybenzoic acids formed. The reaction was not affected by several iron chelators, nevertheless the halogenated hydroquinone is a major metabolite of the biocide pentachlorophenol. Chemiluminescence assays have also been described to evaluate the $^{\bullet}OH$ scavenging capacity of phenolic compounds as a simple protocol instead of assays comprising expensive equipment (HPLC, ESR, pulse radiolysis). The Fenton reaction is used to generate hydroxyl radicals and luminol is used as a marker compound to trap oxidants and convert weak light emissions into intense, prolonged, and stable ones.[99]

14.6 Assays using synthetic radicals

Despite the frequent objection on the use of model synthetic radicals not directly associated with food or biological systems, literature survey reveals that still in most studies model synthetic radicals are employed. This is generally due to protocol simplicity, acceptable repeatability and low cost. The most preferable ones are the $DPPH^{\bullet}$ and the $ABTS^{\bullet+}$.

14.6.1 1,1-Diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$)

1,1-Diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$) is the most common synthetic radical used to study the contribution of structural characteristics to the radical scavenging activity of phenolic compounds.[3,6,7,11,20,55,62,73,100–107] Researchers in the fields of food and life sciences commonly employ this assay for a number of reasons. For example, $DPPH^{\bullet}$ does not dimerize in either its solid state or in solution, as demonstrated by ESR studies;[108,109] it can accept electrons or hydrogen atoms but it is difficult to be oxidized;[100] $DPPH^{\bullet}$ solutions are colored and relatively stable ($\lambda_{max} = 515$ in methanol);[100] the $DPPH^{\bullet}$ assay is simple as it is based on measurement of the loss of $DPPH^{\bullet}$ absorbance at 515 nm after its reaction with the test compounds, which means that only a low-cost spectrophotometer is required. Various versions of the $DPPH^{\bullet}$ test can be found in the literature, regarding the reaction conditions as well as expression of results.

Typically, the test is carried out in methanol or ethanol at room temperature, and results are expressed as percent radical scavenging activity [(%RSA) = [($Abs_{t=0}$ − Abs_t)/$Abs_{t=0}$] × 100], or in terms of antioxidant concentration that causes a decrease in the initial $DPPH^{\bullet}$ concentration by 50% (EC_{50}). The % RSA is usually preferred for rapid protocols (e.g., 10–30 min) because EC_{50} values are based on kinetic data. The latter can be time-consuming as a plateau is reached in seconds, minutes, or even many hours.[101] Calculation of reaction rate constants of phenolic compounds have been also used,[102,107] as well as a parameter proposed by Sánchez-Moréno and co-workers[105] called 'antiradical efficiency (AE)', which is defined as [1/(EC_{50} × T_{EC50})], where T_{EC50} is the time needed to reach the steady state when antioxidant concentration equals that of EC_{50}. The order of activity obtained with the $DPPH^{\bullet}$ assay, in general, corresponds to the number and position of hydroxyl groups in the aromatic ring of the test compounds. Nevertheless, the stoichiometry of the reaction calculated for phenolic compounds is higher than the available hydroxyl groups in many cases,[11,101] as some side reactions have been proposed to occur.[101] Aside from not being a biologically relevant radical, the assay has been reported to have other limitations. $DPPH^{\bullet}$ is not water soluble, though a mixture of ethanol and water (1:1 v/v) can be used.[110] The steric effects imposed by the radical may affect its reactivity, as small compounds may have better access to the radical's active site compared with larger compounds.[37] Colored compounds or turbid samples affect spectrometric measurements. Adaptation of the assay to HPLC[111] or amperometric reduction of the radical at a glassy carbon electrode[112] eliminates such sources of interference. Furthermore, reactivity of the radical with phenols is affected by the solvent, which may determine the magnitude or even the order of activity of the tested compounds.[113–116] Solvent effect is a parameter that should always be considered in all radical scavenging assays. Despite these issues, the $DPPH^{\bullet}$ assay remains a popular method among researchers for obtaining information on the activity of antioxidants, or for use with advanced instrumentation for the investigation of complex phenolic mixtures (*vide infra*).

14.6.2 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$)

The assay involves monitoring the decay of the radical cation of 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) at 734 nm, in the presence of a test compound. Scavenging has been proposed to take place via electron donation.[29] The results are expressed as Trolox equivalents, that is, the concentration of Trolox solution (mM) with an antioxidant capacity equivalent to that observed for 1 mM of the test compound. For this reason, the assay is also known as 'Trolox equivalent antioxidant capacity (TEAC) assay'. It was initially introduced by Miller *et al.*,[117] and after several revisions aiming at preventing the interference of test compounds with radical formation, it was one of the methods recommended for further standardization in the first international congress on antioxidant methods.[30] Its selection was based on its simplicity,

stability of the radical cation, low cost, short assay time, absorption maxima of the probe at 414, 645, 734, and 815 nm (advantageous in the case of colored compounds), and applicability to testing both hydrophilic and lipophilic compounds as the radical is soluble in both water and certain organic solvents.[118,119] A commonly used protocol is described by Re *et al.*,[118] wherein the generation of the radical is obtained by oxidizing ABTS with potassium persulfate, and its reaction with a test antioxidant is monitored up to 6 minutes. The assay has been employed in SAR studies of numerous flavonoids and phenolic acids, as described in the review of Rice-Evans and co-workers,[120] as well as in studying the effect of pH on the antioxidant mechanism of benzoic acids and certain flavonoids.[51,52] It should be noted that the reaction with $ABTS^{\bullet+}$ is not complete with various compounds within the 6-minute window, suggesting that an underestimation of activity is possible.[30] It is also important to note that certain compounds that are known to have poor activity are occasionally found to have higher activity than known antioxidants. These compounds usually bear a resorcinol moiety, which has been related to formation of side products in the course of the reaction that can further scavenge the radical.[121,122] Nevertheless, such a finding cannot justify why, for example, *p*-coumaric acid have been found to be twice as active as caffeic acid.[122] In addition, tyrosine and cysteine, which demonstrate virtually no activity in the $DPPH^{\bullet}$ assay, are efficient scavengers of $ABTS^{\bullet+}$.[76] The data obtained by this assay may be questionable, as an overestimation of activity may occur; therefore, this particular method needs further refinement.

14.6.3 Activity order of phenolic compounds based on the parallel use of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ data

$DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays are commonly used to assess antioxidant *in vitro*.[3,11,51,52,73,75,118,119] Representative studies published by the same group on selected AHs (Table 14.4) highlight further the views expressed in Sections 14.6.1 and 14.6.2.

Using $DPPH^{\bullet}$, the order of activity within the same group of compounds is related to the number and position of hydroxyl groups in the aromatic ring, which is consistent with the principles of SAR. With $ABTS^{\bullet+}$, some discrepancies were observed. For example, among hydroxycinnamic acids, the monohydroxyl compounds were more active than caffeic and chlorogenic acids. Similarly, resorcinol and resorcilic acid demonstrated virtually no activity towards $DPPH^{\bullet}$, but were efficient $ABTS^{\bullet+}$ scavengers.

14.7 Assays using radicals derived from lipid oxidation

Lipids are often the source of radicals in foods; therefore it can be argued that antioxidant assays based on lipid-derived radicals have more relevance to real food systems, and thus are of more practical use to the food industry.

Table 14.4 Antioxidant activity of selected AHs using $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays by the same analyst

AHs	$DPPH^{\bullet}$ (%RSA)*	Order of activity within groups	$ABTS^{\bullet+}$ (TEAC)	Order of activity within groups	Reference
Hydroxycinnamic acid					
Rosmarinic acid	88.4	1	2.13	1	115, 122
Caffeic acid	76.6	2	1.01	5	
Chlorogenic acid	52.0	4	0.95	6	
Sinapic acid	56.1	3	1.27	4	
Ferulic acid	30.9	5	1.32	3	
p-Coumaric acid	3.6	6	2.00	2	
Simple phenols					
Catechol	65.1	1	0.97	2	122
Resorcinol	2.7	3	1.14	1	
Hydroquinone	48.6	2	0.68	3	
Benzoic acids					
Gallic acid	96.3	1	2.18	1	122
Protocatechuic acid	68.7	2	0.84	2	
Resorcilic acid	2.9	3	0.63	3	
Flavonoids					
Quercetin	68.2	1	1.85	1	122
Morin	43.2	2	1.20	2	

* Values obtained at the ratio $[AH]/[DPPH^{\bullet}] = 0.25$; $t = 20$ min

The models usually employed in lipid oxidation studies are bulk oils and dispersed systems (e.g., micelles, emulsions, liposomes). Other more complex matrices are less frequently met. Variability in the type of the substrates found in the literature is remarkable. It seems that choices for substrates are influenced by prevailing views.

14.7.1 Bulk oils

The oxidation of bulk oils is a common approach to test the efficacy of antioxidants. The lipid substrate (e.g., free fatty acid, fatty acid methyl ester, mixtures of triacylglycerols), which has been stripped of pro-/anti-oxidant factors,[123] is often oxidized under accelerated conditions in the absence or the presence of AHs at various concentrations. The accumulation of primary and secondary oxidation products is followed periodically.

Oxidation of bulk oils is usually carried out through stability tests, such as the Schaal oven test (40–60 ºC), the Active Oxygen Method (AOM) (100 ºC, purging air through the sample), or the Rancimat apparatus ($T > 100$ ºC, purging air through the sample). The two latter procedures have been criticized

and some have advised that they should be abandoned.[57] This is because high temperatures give rise to reactions that are not expected to occur under typical processing and storage conditions.[57] Furthermore, oxygen solubility decreases at such high temperature, and the stability of the test antioxidant compounds may be compromised,[103] which may result in an over- or under-estimation of activity, respectively. Therefore, an oven test at ambient temperatures is currently recommended.

In a typical oven test, lipids are left exposed to air in open vessels. The active surface area is an important factor that has to be specified in the experimental part of all publications. It is suggested that a large quantity of the substrate should be prepared with the test antioxidant, which is then distributed in equal quantities to identical sample vials. Withdrawal of vials periodically to measure oxidation products is preferable instead of taking aliquots from a single large vessel. The combination of ambient temperature and substrates with low concentrations of unsaturated fatty acids leads to lengthy experiments and low accumulation in oxidation products.[123]

In bulk oil accelerated oxidation tests, evaluation of antioxidant activity is made by measuring primary and/or secondary oxidation products. Primary oxidation products may be measured by the iodometric determination of lipid hydroperoxide, expressed as peroxide values (PV),[124] or by the ultraviolet determination of conjugated dienes at 232 nm.[124] Determination of secondary oxidation products is possible by measuring carbonyls at 268 nm, unsaturated aldehydes (anisidine values);[124] malonaldehyde (thiobarbituric acid test);[125] and analysis of volatile compounds by headspace GC.[125] Improved determination of malonaldehyde can be achieved using HPLC or GC as recently described.[39] Determination of the extent of oxidation of oil matrices may also be achieved by derivative spectrophotometry and infra-red spectroscopy with Fourier transformation.[126–128]

When testing the activity of phenolic compounds in bulk oils, it is imperative that the initial substrate is not oxidized (PV ~ 0) in order the results of the antioxidant activity to be meaningful.[32] Furthermore, it should be mentioned that both primary and secondary oxidation products should be measured, as the trend in activity may not be the same on the basis of the two indices.[125] The types of secondary products depend on fatty acid composition.

14.7.2 Dispersed systems

Food lipids are often encountered in the form of dispersions systems. The models used to simulate these systems are emulsions and liposomes, though others can be also found in literature. Emulsions are mixtures of oil and water, where one liquid is dispersed within the other as small droplets. Depending on the composition of the dispersed liquid, either an oil-in-water or water-in-oil emulsion is formed. As emulsions are thermodynamically unstable, surface-active compounds that adsorb to the surface of droplets during homogenization can prevent droplet aggregation. Some common emulsifiers used in food

emulsions include surface-active proteins, phospholipids, Spans and Tweens.[57] Liposomes are composed of phospholipids which, when dispersed in water, tend to form closed spherical structures consisting of bilayers organized between aqueous compartments and a monolayer next to the oil phase.[57]

In such models, oxidation is typically carried out at ambient conditions ($T \leq 60$ ºC) and under constant agitation at a defined pH value.[57] Addition of transition metal ions,[3,11,129] thermally labile initiators[130] or oxidative enzymes (lipoxygenase)[131] combined with heating are also common practices to induce lipid oxidation. The products of oxidation are monitored using the above-mentioned assays but with some adaptations. The ferric thiocyanate assay[129] is preferred when lipid quantities are small. Hydroperoxide accumulation can be also monitored by measuring the formation of conjugated dienes in the lipid phase.[132]

The importance of the type of substrate and its oxidative status, droplet size, charge, type and concentration of emulsifier, pH, on the stability of the model during the course of the experiment has been thoroughly investigated by several research groups and reviewed by Frankel,[57] and McClements and Decker.[133] It has been observed that polar antioxidants are more effective in bulk oils, whereas nonpolar antioxidants are more effective in lipid dispersions. This finding was initially presented by Porter *et al.*[134] and is referred to as the antioxidant polar paradox. Therefore, it is proposed that the effectiveness of a given antioxidant depends on its physical location, specifically its ability to partition to air/oil (bulk oils) or water/oil (dispersed systems) interfaces. In the case of bulk oils, polar antioxidants may also aggregate at reverse micelles, which result from the stabilization of small amounts of water by relatively surface active contaminants (e.g., free fatty acids, mono- and diacylglycerols, phospholipids). Theoretical and experimental procedures designed to assess partitioning behavior are given in Table 14.1. In the case of liposomes, apart from polarity, the size and conformation of the molecule affect antioxidant activity. For this reason, fluorometric techniques that can measure the ability of AHs to penetrate membranes have been reported.[135]

14.7.3 Complex matrices

Complex food matrices are also used as a model to study antioxidant activity of compounds with the goal of creating model system that more closely resemble actual foods. Dispersed lipid systems, where AHs are tested in the presence of other food components, as well as true food matrices, can be used to this end.

There are few studies that report interactions of proteins with AHs. Proteins can significantly affect the antioxidant activity through, i.e., hydrogen bonding and ionic interactions with phenols.[57] Studies carried out in the presence or absence of bovine serum albumin (BSA) have shown that the protein may increase the efficiency of some antioxidants in an o/w emulsion (epigallocatechin gallate, caffeic acid) or liposomes (ferulic acid, malvidin, rutin).[136,137] The improvement in activity has been attributed to the enhanced adsorption of

phenolic compounds to the interface where oxidation takes place. Except for the importance of the structural features of the tested compounds, the nature and conformation of the added protein is also crucial. This was evidenced by Estévez *et al.*[138] for various AHs tested in dispersed rapeseed oil emulsified with either 1% BSA or 1% skeletal muscle myofibrillar proteins.

Muscle foods are a good example of a complex food system. Muscle tissue is a matrix containing various antioxidants (e.g., ascorbate) as well as pro-oxidant (e.g., transition metals, heme proteins) factors affecting its nutritional quality, which may change when the food is processed and stored. Hemoglobin is often used as an initiator of oxidation. In order to achieve fast oxidation rates, muscle foods containing high concentrations of polyunsaturated fatty acids (e.g., fish) are preferred. An extensive presentation of complex food models is covered by Decker and collaborators.[32]

Monitoring of oxidation is accomplished by measuring lipid hydroperoxide accumulation using the ferric thiocyanate method, or secondary oxidation products formation employing the thiobarbituric acid test and by volatile analysis by headspace-GC.[139,140]

Activity order of phenolic compounds based on the parallel use of radical scavenging assays and food models

Recently the comparative testing of selected AHs using ORAC and $DPPH^{\bullet}$ assays and cooked ground beef,[140] proved the complexity of antioxidant activity assessment. Two groups of compounds were tested. Using the ORAC assay for hydrophilic compounds, the order of activity was ferulic acid > coumaric acid > propyl gallate > gallic acid > ascorbic acid, while the order of nonpolar compounds tested by the $DPPH^{\bullet}$ assay was rosmarinic acid > butylated hydroxytoluene > *tert*-butylhydroquinone (TBHQ) > α-tocopherol. In cooked ground beef propyl gallate and TBHQ were found to inhibit lipid oxidation as determined by thiobarbituric acid reactive substances. The rest of the compounds showed moderate to no activity.

14.8 Other methods

The antioxidant activity of phenolics is multifaceted, therefore its useful to study different mechanisms of antioxidant activity (e.g., metal binding, inhibition of prooxidant enzymes).

14.8.1 Studies using transition metal ions

Trace levels of iron and copper ions found in lipid systems may significantly accelerate oxidation. This is attributed to the production of free radicals through a direct electron transfer to fatty acids (R14.3) or by catalyzing the homolytic decomposition of preformed hydroperoxides (R14.4–14.5).[57]

$$M^{(n+1)+} + RH \rightarrow M^{n+} + R^{\bullet} + H^{+} \qquad \text{(R14.3)}$$

$$M^{n+} + ROOH \rightarrow M^{(n+1)+} + RO^{\bullet} + OH^{-} \quad \text{(R14.4)}$$

$$M^{(n+1)+} + ROOH \rightarrow M^{n+} + ROO^{\bullet} + H^{+} \quad \text{(R14.5)}$$

Phenolic compounds may participate in redox reactions with transition metal ions and/or form coordination complexes.[141] Owing to such interactions, some phenolic compounds (e.g., flavonoids) may either promote or retard oxidation. An observed prooxidant activity is postulated to be due to reduction of metal ions by phenols to more prooxidative species. On the other hand, a protective effect may be due to metal chelating ability, thus hindering the participation of metal ions in the abovementioned reactions (R14.3–14.5). Information on pro/antioxidant activity of phenols is obtained by the following techniques.

Metal reducing assays

The reducing ability of phenols towards iron or copper ions can be estimated spectrophotometricaly with the ferric reducing antioxidant power (FRAP) and the cupric ion reducing antioxidant capacity (CUPRAC) assays respectively. In brief, the former is based on the ability of phenolics to reduce ferric tripyridyltriazine complex (Fe(III)-TPTZ) to ferrous complex (Fe(II)-TPTZ) at pH = 3.6 (in order to maintain iron solubility), as described by Benzie *et al.*[142] A second technique is based on the reduction of Cu(II)-neocuproine complex to Cu(I) at pH = 7, as described by Apak *et al.*[143] These two assays are often employed in order to estimate the radical scavenging activity by single electron transfer reactions.[3,11,143–146] However, this practice is often criticized as compounds that reduce transition metals do not necessarily reduce free radicals.[147] Nevertheless, the application of the two methods gives evidence for the possible prooxidant activity of phenols in lipid systems in the presence of Fe(III) or Cu(II).

Metal chelating studies

A straightforward way to screen compounds for chelating ability is to record their aqueous spectra in a selected pH prior to, and after, the addition of variable concentrations of metal ions. The chelating capability is influenced by pH value as ionization of hydroxyl groups of phenols assist metal binding.[148] Chelation is evidenced by significant spectral changes as in the case of caffeic acid (Fig. 14.2), wherein a bathochromic shifting of absorption was observed after Cu(II) addition.[63] EDTA is typically added afterwards to investigate the stability of the formed complex. The spectra of phenolic solution is restored if the metal ion is chelated by the added EDTA.[63,149]

While the aforementioned assay provides qualitative data, chelating ability may also be quantitatively assessed. An approach is the one adopted by Andjelkovic *et al.*,[149] where UV-Vis spectroscopy was used to study the Fe(II) chelating effect of a series of phenolic acids by calculation of binding constants*

* Binding constants are defined as the ratio intercept/slope, where intercept and slope are parameters of a linear relationship between 1/(total ferrous ion concentration) and 1/(absorbance of complexed ion).

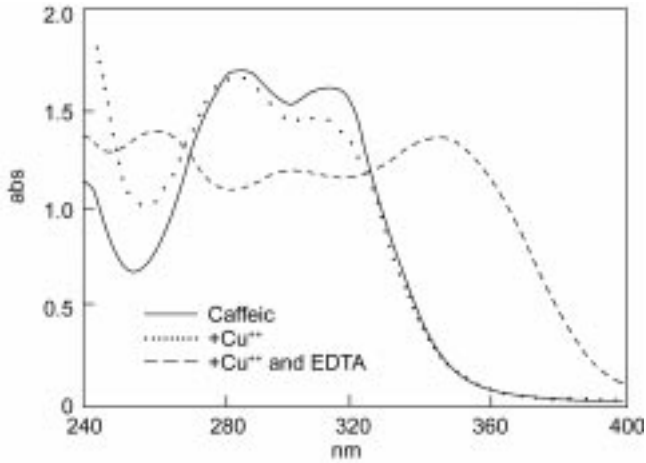

Fig. 14.2 Chelating effect of caffeic acid (100 μM) in 10 mM phosphate buffer saline (pH 7.4) in the absence/presence of $CuCl_2$ (100 μM) or Cu(II) and EDTA, EDTA (1 mg/mL final concentration) (abstracted from Fig. 5 of ref 63).

for the respective compounds. The authors noted that interferences due to buffer constituents (e.g., phosphates) were possible, however. Furthermore, a ferrozine based colorimetric procedure has been reported for the same reason. Results were expressed either as percent chelated iron or in EDTA equivalents.[150]

14.8.2 Studies using oxidative enzymes (lipoxygenases)

Lipoxygenases are oxidative enzymes bearing ferrous ions in their active sites, and can selectively oxidize fatty acids containing *cis,cis*-1,4-pentadiene moieties, resulting in off-flavors and off-aromas. For this reason, testing the ability of antioxidants to inhibit enzymic activity is gaining interest. To date, SAR studies have been carried out for a number of flavonoids.[151] Assessment of flavonoid capacity to inhibit lipoxygenase activity is achieved by addition of the test compound and enzyme to a linolenic acid solution and recording accumulation of conjugated dienes at 234 nm.

14.9 Assessment of mixtures of compounds

Antioxidant activity can be assessed for mixtures of antioxidants in model systems or in natural products. Artificial mixtures of individual compounds can be tested for synergism or antagonism. Such studies can be monitored using many of the previously mentioned procedures. The molar ratio among tested compounds varies with regard to the aim of the study, or is carried out at levels expected in natural products. In such studies, the number of the tested compounds is often limited (e.g., 2–5 compounds). However, natural products,

commonly in the form of extracts, can potentially contain a large number of structurally related antioxidants which are difficult to isolate and identify without using sophisticated procedures or instrumentation. Recently reported methodologies that combine on-line separation and radical scavenging activity of antioxidants are emphasized in this section. Analytical parameters such as sample preparation procedures (drying, milling), extraction methods (agitation, ultrasonic or microwave assisted), extraction temperature, solvent systems (aqueous or organic), and sample clean-up or fractionation all influence the composition and, consequently, the activity of the tested extracts. The formation of artifacts cannot be excluded (characteristic is the formation of carnosol or other diterpenes from carnosic acid upon storage and extraction of rosemary) in certain cases, and therefore have to be considered together with interferences due to the presence of other components (e.g., pigments, ascorbic acid). It has been argued that kinetic parameters (e.g., EC_{50}, T_{EC50}, AE) offer a more comprehensive assessment of antioxidant capacity versus percent inhibition.[152] The selection of suitable reference compounds, where necessary, is not often straightforward. However, in general, a reference compound should be structurally related to the antioxidants of interest. The widespread use of Trolox is understandable because it is inexpensive and stable, but is not always justified.

14.10 On-line chemical characterization and assessment of antioxidants present in complex mixtures

A number of the so-called 'high resolution screening assays' (HRS) were developed during the last decade.[153,154] Such assays combine HPLC with a fast post-column reaction, often with a solution of a chromogen free radical.[154] The radical scavengers present in an extract are then identified by means of a UV detector as negative peaks. The instrumentation employed for the reaction with $DPPH^{\bullet}$ is depicted in Fig. 14.3.

Integration of the respective negative peaks is used to calculate the % RSA of each of the individual components that could scavenge the free radical. The $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays[155,156] have been successfully adapted to HRS systems, and have been optimized in order to increase sensitivity.[153]

Recent advances in this field include in-line coupling of diode-array (DAD) or ultra violet detectors (UV) with mass spectrometers (MS), or sample preparation with solid phase extraction (SPE) followed by characterization with nuclear magnetic resonance (NMR) spectrometers. LC-ESI/MS has been successfully applied on line with $DPPH^{\bullet}$ assay to simultaneously separate, characterize and assess scavenging activity of complex natural phenolic mixtures.[157] Such procedures offer information about the reactivity of individual compounds with the radical; however, the kinetics of these reactions are overlooked, which means that AE values cannot be obtained.

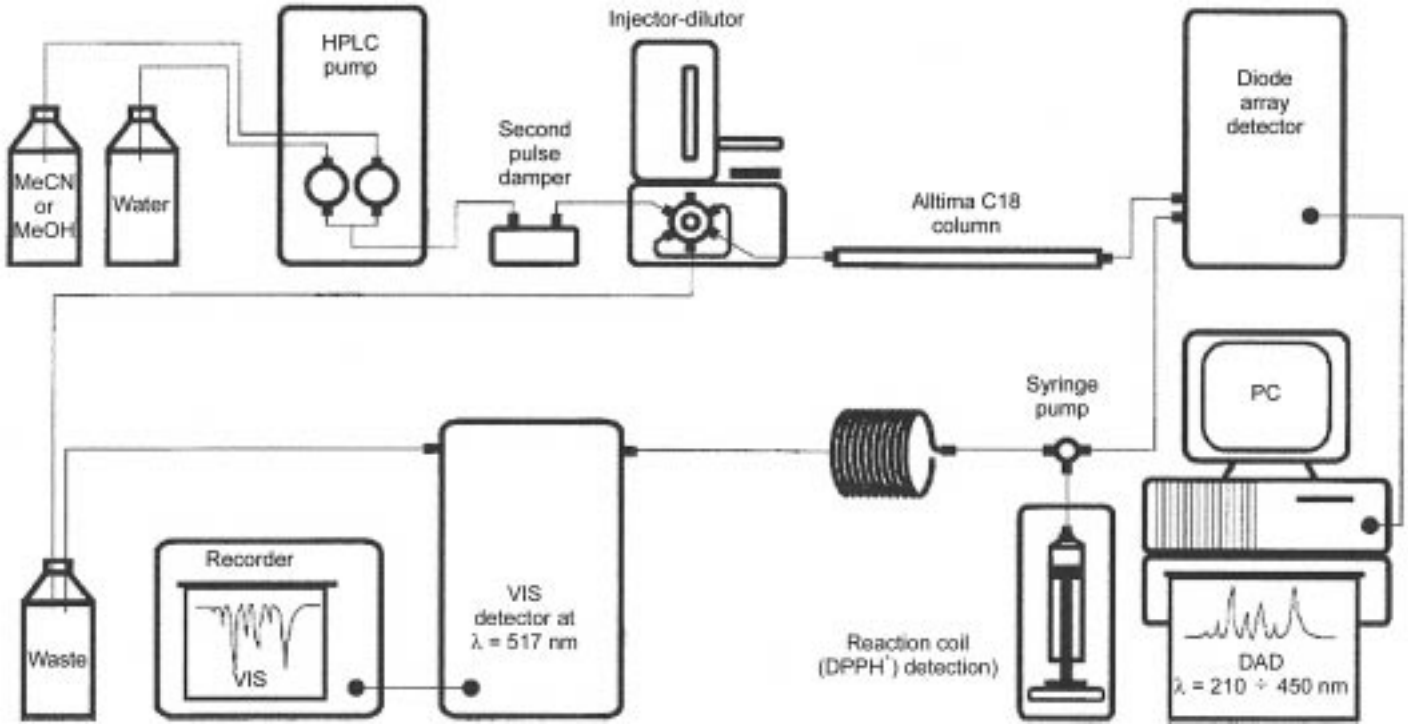

Fig. 14.3 Layout of high-resolution screening assay for DPPH$^{\bullet}$ scavengers in complex mixtures (reprinted from the open accessed paper of Van Beek and co-workers[154]).

14.11 Epilogue

The era of 'innocence' regarding antioxidant activity assessment is over. Scientists are aware of limitations and drawbacks of each and every method described in this chapter. A consensus among experts is needed in order to produce data under standardized protocols, even if methodologies are under scrutiny. The accumulated information can, thus, be transformed to a useful databank for future use and revisions in this scientific field.

> No doubt it is a grand thing also to be a tactician, since there is all the difference in the world between an army properly handled in the field and the same in disorder; just as stones and bricks, woodwork and tiles, tumbled together in a heap are of no use at all, but arrange them in a certain order – at bottom and atop materials which will not crumble or rot, such as stones and earthen tiles, and in the middle between the two put bricks and woodwork, with an eye to architectural principle, and finally you get a valuable possession – to wit, a dwelling-place.
> [Xenophon memoirs of Socrates (English translation by H. G. Dakyns)]

14.12 Sources of further information and advice

The following book is recommended for consultation: *Antioxidant Measurement and Applications*; Shahidi, F., Ho, C.-T., Eds. ACS Symposium Series 956; Washington, DC: American Chemical Society, 2007.

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14.14 List of abbreviations

AAPH: 2,2′-Azobis(2-aminopropane) dihydrochloride
Abs: Absorbance
$ABTS^{\bullet+}$: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation
AE: Antiradical efficiency
AH: Antioxidant
AM1: Austin Model 1
AMVN: 2,2′-azobis-2,4-dimethylvaleronitrile
AOM: Active oxygen method
AUC: Areas under curve
B3LYP: Becke 3 term with Lee, Yang, Parr exchange
BDE: Bond dissociation enthalpy
$BODIPY^{665/676}$: 4,4-difluoro-3,5-bis(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene
BSA: Bovine serum albumin
C: Concentration
CBA: Crocin bleaching assay
CL: Chemiluminescence
CUPRAC: Cupric ion reducing antioxidant capacity
DFT: Density functional theory
$DMPD^{\bullet+}$: N,N-dimethyl-p-phenylenediamine radical cation
$DPPH^{\bullet}$: 1,1-diphenyl-2-picrylhydrazyl radical

ΔA: Decrease in absorbance
ϵ: Extinction coefficient
EC_{50}: Efficient concentration of antioxidant causing decrease of radical concentration by 50%
EDTA: Ethylenediaminetetraacetic acid
ESR: Electron spin resonance
FRAP: Ferric reducing antioxidant power
GC: Gas chromatography
H_2O_2: Hydrogen peroxide
HAT: Hydrogen atom transfer
$HO^{\bullet}$: Hydroxyl radical
HOCl: Hypochlorous acid
HOMO: Highest occupied molecular orbital
HPLC: High performance liquid chromatography
IP: Adiabatic ionization potential
k: Rate constant
KI: Potassium iodide
KMBA: α-keto-γ-methiolbutyric acid
K p: Phospholipid/water partition coefficient
k_{rel}: Relative rate constant
LDL: Low density lipoprotein
λ_{exc}: Excitation wavelength
λ_{emis}: Emission wavelength
λ_{max}: Wavelength of maximum absorbance
Log P: Logarithm of partition coefficient in water/*n*-octanol (1:1, v/v) mixture
Lox: Lipoxygenase
MP: Møller-Plesset
MS: Mass spectrometer
NADH: Nicotinamide adenine dinucleotide
NBT: Nitroblue tetrazolium
NMR: Nuclear magnetic resonance
$NO^{\bullet}$: Nitric oxide radical
$^1O_2$: Singlet oxygen
$O_2^{\bullet -}$: Superoxide anion radical
$ONOO^-$: Peroxynitrite
ORAC: Oxygen radical absorbance capacity
PCL: Photochemiluminescence
PMS: Phenazine methosulphate
Pw/Po: Partition constants in water (w) and oil (o) phase
%RSA: % Radical scavenging activity
R-N=N-R′: Thermolabile azo-compound
$ROO^{\bullet}$: Peroxyl radical
ROS: Reactive oxygen species
R_f: Retention factor
SAR: Structure–activity relationship

SET: Single electron transfer
SPE: Solid phase extraction
SORAC: Superoxide radical absorbance capacity
TBHQ: *tert*-Butylhydroquinone
TEAC: Trolox equivalent antioxidant capacity
TEV: Trolox equivalent values
TOSC: Total oxidant scavenging capacity
TRAP: Total radical trapping antioxidant parameter
T_{EC50}: Time needed for the reaction to reach the steady state with EC_{50} concentration
TPTZ: Tripyridyltriazine
VIP: Vertical ionization potential
Vis: Visible
XOD: Xanthine oxidase
ΔHOF: Difference in heat of formation

15

Effects of processing and storage on antioxidant efficacy in foods

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Abstract: Antioxidants are affected mainly by oxidation, less by pyrolysis and hydrolysis under seed and crude oil processing and during culinary operations. Chemical changes of antioxidants under those conditions are known only in some simpler cases and in model systems. They do not often correlate with changes of antioxidant functionality. Changes during seed storage and oil processing are briefly discussed. Main attention is paid to changes during storage, emulsification and culinary operations under heating, such as boiling, baking, roasting, microwave cooking, and frying. Natural and synthetic antioxidants and the original compounds and their degradation products are compared.

Key words: oxidation of synthetic and natural antioxidants, effect of oxidation and pyrolysis on antioxidant functionality, comparison of synthetic and natural antioxidants.

15.1 Introduction

The chemical structures of synthetic antioxidants and their functionality in model experiments are well known, and an enormous amount of special literature is available. The case of natural antioxidants from food products is more complicated, as they are present mostly in mixtures with similar compounds and accompanied by various impurities. Every year, many papers on antioxidants obtained from exotic foods and nonfood plant matrerials are available, often of local interest only, possessing antioxidant activity. Much less,

however, is known about their changes during food storage and meal preparation by several culinary procedures.

The reasons for this lack of precise information about antioxidant changes are as follows: variable material composition, processing technology, and possibilities of concurrent reactions of antioxidants and their primary reaction products. The published literature mostly reports only on losses of original antioxidants or their antioxidant capacity.

In this chapter, we shall start by explaining how antioxidants and synergists can react in foods. Many food components may influence the capacity of antioxidants and their products, which often possess their own antioxidant activity. Changes of antioxidants during processing of seeds and crude oils are briefly discussed.

The main part of the chapter is devoted to antioxidant changes during storage and different culinary operations, either at ambient temperature or under heating, such as boiling, baking, roasting, microwave cooking, frying, and drying. Tocopherols and their changes are emphasized as they are widely distributed in almost all foods, and are biologically important. Flavonoids and catechins are also considered as they are strong antioxidants.

As it is mostly not possible to explain the changes of antioxidants in food properly, a few typical examples only are given in such a case.

Synthetic and natural antioxidants were compared, and it was observed they were very similar. Differences between antioxidant activities of originally present antioxidants and their changes were much greater. Hydrolytical reactions and reactions of phenolic antioxidants bound to protein or starch were relatively unimportant. The tendency to replace synthetic antioxidants with natural products will continue in the near future. The stability of foods will be improved by fast decomposition of enzymes, appropriate packaging and storage conditions, so that the use of antioxidants should be unnecessary in many cases.

15.2 Chemistry of changes of antioxidants

15.2.1 Phenolic antioxidants

In the proper sense, antioxidants are compounds which act with free radicals in foods, inactivating them. Many food scientists denote as antioxidants all substances that could inhibit the oxidation, even by non-radical reactions. They should be regarded as oxidation inhibitors. For example, tryptophane, cysteine, cystine, methionine and glutathione, both free or bound in proteins, reduce lipid hydroperoxides. Similarly, Maillard non-enzymic browning products inhibit the oxidation in other ways. The transient-valency metals, which are efficient pro-oxidants, are converted into insoluble inactive complexes so that they contribute to the inhibition of lipid oxidation. In order to avoid confusion, we shall use the broader term, and call both antioxidants and other inhibitors antioxidants.

The best known are synthetic antioxidants, mainly derivatives of pyrocatechol or hydroquinol, but only a few have been permitted for food

stabilization: tert. butylhydroxytoluene (BHT), tert.butylhydroxyanisole (BHA), polar, very efficient antioxidant propyl gallate (PG), less polar, but efficient dodecyl gallate (DG) and tert. butylhydroquinone (TBHQ). They belong to the pyrocatechol and hydroquinol series, which react with free radicals in nearly the same way.

The first step of the antioxidant action is the reaction with lipid-derived free radical, which is transformed into less reactive lipid hydroxylated derivative. The antioxidant is converted into a free radical, which is less reactive than lipid free radicals. An example of a pyrocatechol derivative is shown in Fig. 15.1. Two antioxidant radicals are in equilibrium. The free radical antioxidant shown in Fig. 15.1 can be transformed in a semiquinoid free radical, and again, the two isomers are in equilibrium (Fig. 15.2).

A hydroquinol derivative behaves in a similar way (Fig. 15.3(a)), and it is slightly less reactive. It may react with another lipid radical to form a biradical, which is isomerized into a *para*-quinone (Fig. 15.3(b)). A pyrocatechol derivative can form an *ortho*-quinone in a similar way.

Another possibility is the reaction with a lipidic free radical. A copolymer with a peroxide structure is produced (Fig. 15.4). Analogous reaction can take place with pyrocatechol free radicals, which are more frequent among natural antioxidants. The co-polymerization may be produced with a lipid peroxy radical, accompanied with a loss of oxygen (Fig. 15.5).

The last possibility is the polymerization by interaction of two antioxidant free radicals (Fig. 15.6). The dimer again have antioxidant activity so that they

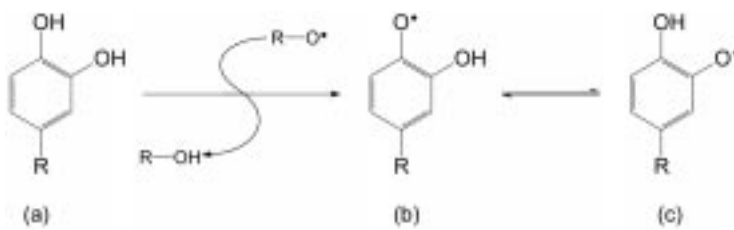

Fig. 15.1 Formation of antioxidant free radical: free radical activity located on an oxygen atom: (a) substituted pyrocatecol, (b) and (c) isomeric free radicals.

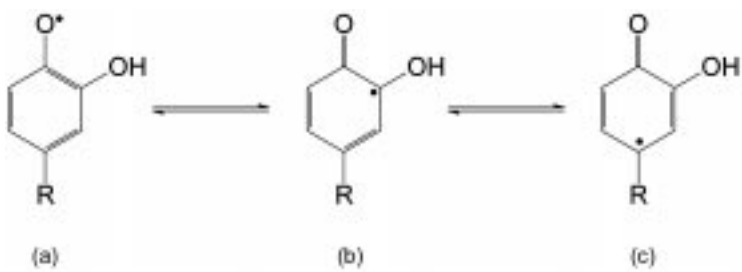

Fig. 15.2 Formation of antioxidant free radical: free radical activity located in benzene ring: (a) substituted pyrocatecol, (b) and (c) isomeric free radicals.

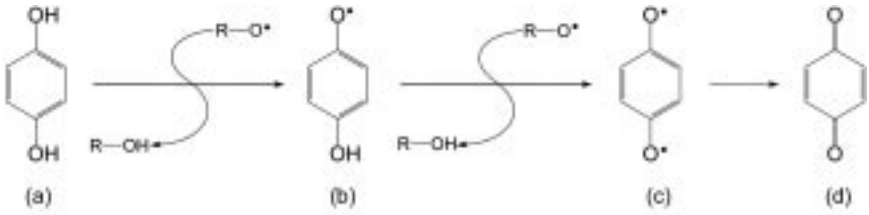

Fig. 15.3 Transformation of an antioxidant into a quinone: (a) hydroquinol, (b) free radical, (c) free biradical, (d) *para*-quinone.

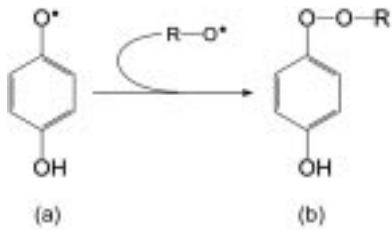

Fig. 15.4 Formation of peroxy copolymer: (a) hydroquinol, (b) *para*-lipoperoxyphenol.

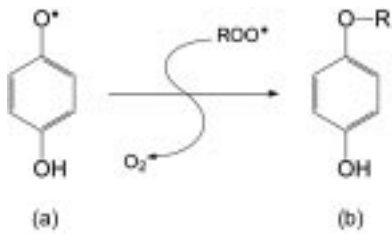

Fig. 15.5 Formation of an antioxidant lipidoxy molecule: (a) hydroquinol, (b) *para*-lipoxyphenol.

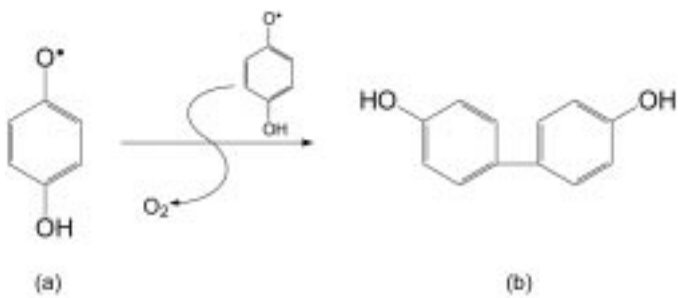

Fig. 15.6 Dimerization of an antioxidant: (a) hydroquinol free radical, (b) C–C dimeric product.

contribute to the total antioxidant functionality. Polymers could dimerize during slow oxidation, which explains the free radical scavenging activity of polyphenols (Hotta *et al.*, 2002).

If oxidized lipid free radicals do not react with antioxidants, the lipid hydroperoxides decompose with formation of both nonvolatile and volatile products, mostly aldehydes, the flavour of which is unpleasant for human consumers in most cases. Formation of objectionable volatile oxidation products was inhibited by antioxidants – TBHQ, thiodipropionic acid, and thiourea, the first mentioned being the most powerful one (Kajimoto and Murakami, 1998). Minced jack mackerel was stored at −20 ºC with addition of polar inhibitors, and the best stability and sensorial properties were obtained with erythrosorbate (an isomer of ascorbic acid), and with sodium phosphate and their mixtures. Sodium polyphosphate and its mixtures with some synthetic antioxidants were also active (Maldonaldo *et al.*, 2006). Their activity resides in their metal complexing capacity.

Substituted phenolic compounds may contain another active group. An example of caffeic acid is given in Fig. 15.7. Free radicals formed from caffeic acid easily polymerize with formation of a dimer, again possessing the antioxidant activity. Caffeic acid reacted with cysteine forming 2,5-cysteinylcaffeic acid, which has higher antioxidant activity than caffeic acid (Bassil *et al.*, 2005).

Quinones formed by oxidation of antioxidants can react with free amine groups of proteins (bound lysine – Fig. 15.8) or phospholipids (phosphatidylethanolamine or phosphatidylcholine) with formation of an imine (Fig. 15.9) – a very reactive derivative. A simplified review of antioxidant reactions was published earlier (Pokorný, 2006).

Green tea antioxidants contain (−)-epigallocatechin gallate and (−)-epigallocatechin as the most active components (Fig. 15.10). The triphenyl-B-ring is the

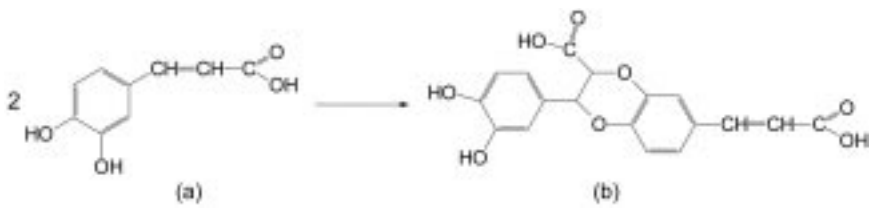

Fig. 15.7 Dimerization of caffeic acid: (a) Caffeic acid, (b) tricyclic dimer.

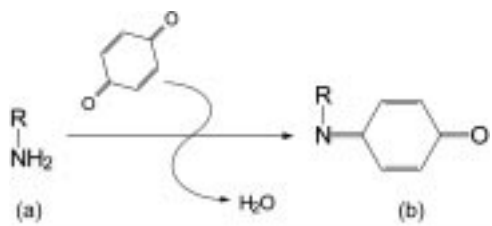

Fig. 15.8 Reaction of amine with para-quinone: (a) primary amine, (b) *para*-quinimine.

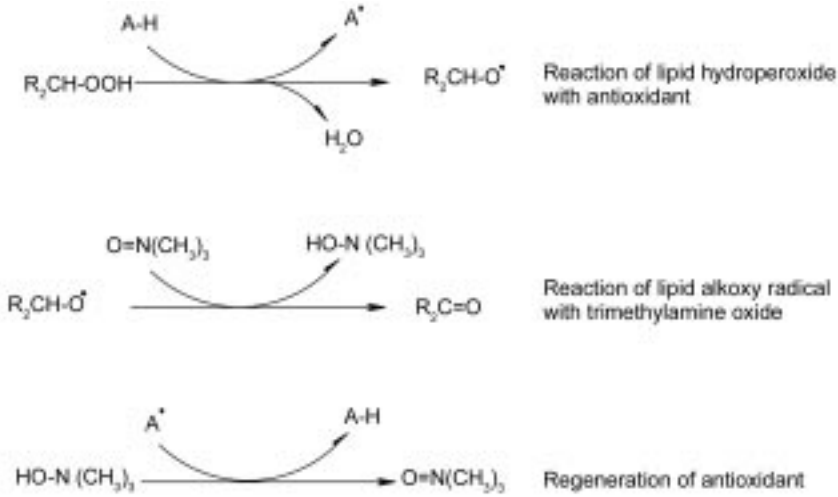

Fig. 15.9 Reaction of antioxidant free radical with degradation product of phosphatidylcholine.

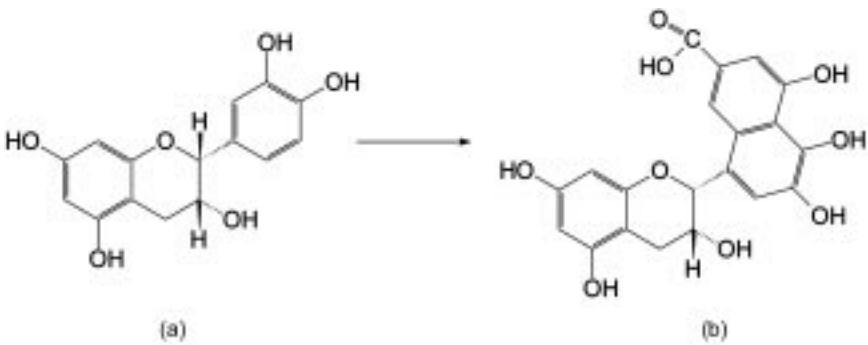

Fig. 15.10 Transformation of a tricyclic compound into a tetracyclic adduct by reaction with antioxidant free radical.

major active site of antioxidant action, rather than the galloyl moiety (Nanqun *et al.*, 2000). They easily polymerize with formation of products possessing an antioxidant activity. Hirose *et al.* (1991) reported on another chemical structure of a dimer of (+)-catechin produced by oxidative dimerization. Cyanidins (anthocyanidin, delfinidin, protisin, and their glycosides) were studied (Acevedo *et al.*, 2010), and the activity also resided in the ring B.

15.2.2 Synergists

Synergists are substances that do not possess, or own only negligible antioxidant activity, but they increase the activity of antioxidants. However, as the synergistic activity is often named the phenomenon, the activity of two

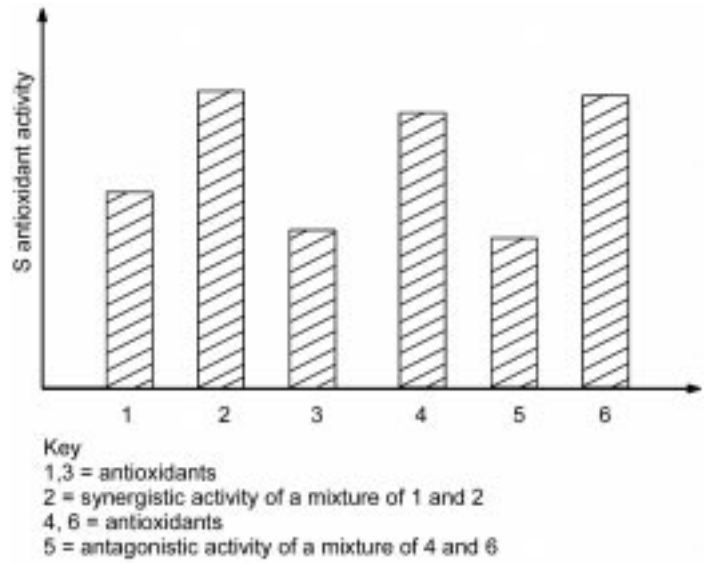

Fig. 15.11 Synergism and antagonism of antioxidants.

antioxidants is higher than those of the same antioxidants present alone. In the text, we shall use the broader term in order to avoid confusion.

Synergists increase the antioxidant activity to higher values than each component (Fig. 15.11). On the contrary, in the presence of antagonists, the resulting antioxidant activity of a mixture is lower than would be expected. A few examples of synergism follow.

Among various tomato cultivars, the highest content of antioxidants, the highest activity and high lycopene content were found in cherry tomatoes. Lipophilic extracts were observed to contain the highest antioxidant activity and high carotenoid content. Synergism was found in mixtures of lycopene and β-carotene or lutein, and lutein and β-carotene (Zanfini *et al.*, 2010). A positive correlation existed between lycopene content and the antioxidant activity.

15.3 Changes of antioxidant functionality during isolation from seeds

15.3.1 Preparation operations

Oil is located either in seeds of plants, or in some cases in the pericarp, too. Seeds have only 6–10% water, thus are relatively stable on storage. After seed cropping, they are usually stored in silos (elevators) at ambient temperature until they are processed in an oil factory. They should remain dry, otherwise various prooxidative enzymes could get activated. They contain natural antioxidants, mainly tocopherols, carotenes, decomposition products of lignin, etc., which are located in different parts of the cell than enzymes to minimize interaction.

Pericarp contains more water, therefore it is unstable, and the present pro-oxidative enzymes are active. For this reason, it should be processed as quickly as possible. The material contains similar antioxidants as oilseeds. If the processing is careful, oils obtained by gentle pressing have agreeable flavour, and can be directly used, without refining. They are called virgin oils, or extra virgin oils in the case that the quality is extraordinary. The more widely used virgin oil is produced from olives. The virgin and extra virgin olive oil contains a group of related phenolic antioxidants.

Seeds are converted into meal prior to processing, allowing enzymes to come in contact with oil. They are treated with steam in order to inactivate enzymes and to cleave the lipoprotein complexes, so that antioxidants remain without any perceptible change.

15.3.2 Expeller pressing and solvent extraction

Oil is isolated from the oilseed meal, usually by a combination of expeller pressing and solvent extraction, most often with hexane. Nonpolar natural antioxidants remain in the oil phase, while polar antioxidants remain in the expeller cakes (Amarowicz *et al.*, 2001) or in extracted meals (Schmidt and Pokorný, 2005). Natural antioxidants of rice brans (tocopherols, γ-oryzanol) were decomposed during storage and solvent extraction. The decrease could be minimized by optimizing technological factors, such as heating time and temperature (Yan and Gu, 2003).

15.4 Changes of antioxidant functionality during oil processing

15.4.1 Changes during oil refining

The taste and flavour of crude oil as obtained by pressing and extraction, are unacceptable for most consumers, and should be refined before consumption. The classical process consists of several steps (Tailor, 2005); treating with acids precipitates phospholipids; it is called degumming. Degummed oils are mixed with an aqueous solution of sodium hydroxide or sodium carbonate to remove fatty acids as water-soluble salts. Natural pigments are removed by bleaching with a bleaching earth.

Impurities are removed by deodorization, i.e. oil is heated under very low pressure and high temperature so that volatiles are distilled off. A part of tocopherols is co-distilled. The losses of tocopherols may be as high as 30%, but the stability may be improved by adding citric acid before the deodorization (Greyt and Kellens, 2005).

Loss of tocopherols means loss of vitamin activity as well, therefore, α-tocopherol acetate is added to deodorized oil. The acetate is used instead of free tocopherols as it is more oxidation-resistant than tocopherol, but has the same biological activity *in vivo*. The disadvantage is that α-tocopherol acetate has no

antioxidant activity *in vitro*. Tocopherols lost in the deodorization may be recovered from distillation sludges (Kamal-Eldin, 2003). They possess high antioxidant activity. It is suitable for nutrition, as the biological potency of the natural (+)-α-tocopherol is many times higher than that of the synthetic racemic tocopherol (Pagani and Baltanas, 2010).

If the acid value (measure of the content of free fatty acids) in crude oil is low, it is possible to proceed with physical refining, which was found convenient for zero erucic rapeseed oil (Čmolík *et al.*, 1995). In the process of physical refining, alkali is not used therefore it is cheaper and more environmentally acceptable than the classical procedure. Free fatty acids are distilled off during the deodorization at low pressure. Tocopherol losses are higher (temperature of 200–240 ºC, depending on the equipment) than in alkali refining, and they are extracted from the distillate with more difficulty because of the excess free fatty acids present.

Both types of refined oils are equivalent in their stability against oxidation (more than a year in a refrigerator).

15.4.2 Production of lecithin and its antioxidant activity

Phospholipids removed by degumming, are used as feed. In case of soybean oil, their quality is sufficient for human consumption. The purified phospholipid fraction is called lecithin, which is used as an emulsifier in products where the price has no significant role. It possesses certain antioxidant activity (Fig. 15.9) as it can react with free lipid radicals. The activity was studied in a model containing 15% methyl linoleate and 85% methyl laurate. Phospholipids retarded the decomposition of vitamin E, and prolonged the induction period, due to free-radical scavenging activity (Koga and Terao, 1995).

15.4.3 Changes of antioxidant activity during hydrogenation

Oils are liquid, but solid fats, such as butter or lard, are necessary in traditional culinary practice for the preparation of bakery products and some meals. Oils become solid by addition of hydrogen to double bonds. Nickel acts as a catalyst. The content of tocopherols is only slightly changed. Hydrogenated oils are very resistant against oxidation because of low content of polyunsaturated fatty acids (Farr, 2005). Partially hydrogenated fats have the consistency of lard, but contain *trans*-fatty acids, which are objectionable in human nutrition. Therefore, oils are fully hydrogenated (*trans*-double bonds included) and transformed into plastic fats by interesterification with liquid oils. Another way is to replace hydrogenated oils with palm stearin, a fraction of palm oil. Margarine and most cooking fats (shortenings) do not now contain more than 1% *trans*-fatty acids, which is less than in milk butter (Brát and Pokorný, 2000).

15.4.4 Changes during the production of margarine and mayonnaise

Margarine is a water-in-oil emulsion and contains the same amount of fat and water as milk butter. Similarly to butter, margarines are now produced with

lower fat content but such products are not allowed to be labelled as butter. They are marketed under special commercial names. The most widely used emulsifiers are monoacylglycerols and diacylglycerols. Carotenes are added as colouring agents, and tocopherols may be added, too. The oily phase of margarines need not be very stable as they should be stored in a refrigerator and for a restricted time, and only some products are stabilized with preservatives, such as *para*-hydroxyphenolic acid.

Mayonnaises are oil-in-water emulsions. They can be stored for only limited time and in a refrigerator so that antioxidants have no special importance.

15.5 Changes during culinary operations at ambient and low temperatures

15.5.1 Changes in bulk fats and oils at ambient temperature

In bulk fats and oils, more polar antioxidants are usually more active than less polar phenolic antioxidants (Cuvellier *et al.*, 2000). Propyl gallate (PG) and di-tert. butylhydroxytoluene (BHT), are typical examples. In food materials or in model oil-in-water emulsions (Frankel *et al.*, 1993, 1996), the activities may be quite different, as shown on the example of tocopherol and Trolox (Frankel, 2007). Trolox has the same active group as tocopherols, but it is more polar as it lacks the hydrophobic hydrocarbon chain of tocopherols. Therefore, it has the same antioxidant activity as tocopherols in bulk oils. On the contrary, in emulsions Trolox has more affinity towards water than tocopherols, and it concentrates on the oil/water interface (Frankel, 1995). The same occurs in the case of synthetic antioxidants, as polar antioxidants, like PG, accumulate at the W/O interface while BHT remains in the oil droplet. Therefore, the data obtained in model experiments in the systems free of water with polar natural antioxidants, such as caffeic acid or catechin, cannot be applied to their activity in real foods, which regularly contain water. Turkey meat lipids were oxidized on storage under evolution of off-odour volatiles, especially on irradiation. Antioxidants, such as gallates, sesamol, tocopherols reduced the off-odour intensity (Lee and Ahn, 2002).

Antioxidant enzymes of broccoli were stored at different temperatures (Zhang *et al.*, 2009), and the temperature of 10 ºC was found the most suitable for storage. No effect of dietary antioxidants and free fatty acids was observed in fresh cooked pork, but lipoprotein oxidation rose in prefrozen uncured cooked meat under aerobic conditions (Haak *et al.*, 2006).

Ground beef was irradiated at 84 ºC for up to seven days. On subsequent storage, sesamol and tocopherols were more efficient than other antioxidants in reducing the oxidation accompanied by evolution of off-odour volatiles (volatile sulfur compounds appeared during the treatment and again disappeared) (Nam *et al.*, 2003).

Tryptophan bound in proteins reacts with phenolic aldehydes with formation of tetrahydro-β-carbolins. Trolox showed better protection than a mixture of Trolox and ascorbic acid (Herrais *et al.*, 2003).

15.5.2 Changes in oil emulsions

In bulk fats, antioxidants and emulsifiers are distributed by chance (Fig. 15.12(c)). In water-in-oil emulsion, emusifiers are oriented with their hydrocarbon chains in oil, and with their polar groups in the water phase. Nonpolar antioxidants remain in the oil phase, being not affected by the nonpolar end of emulsifer molecules (Fig. 15.12(a)). On the contrary, the structure is inversed in oil-in-water emulsions. Polar groups of emulsifiers are oriented to continuous water phase and hydrocarbon, nonpolar chains towards oil droplets. Nonpolar antioxidants remain in the oil phase (Fig. 15.12(b)).

In deep coloured fruit juices (elderberry, blackberry, sour cherry) processed by conventional technology, about 30–50% anthocyanins, phenols, chlorogenic acid and cyanidin-3-glucosides were partially lost, too. They were lost without particular discolouration. Antioxidants were lost in all juices, but to a different degree (Dietrich *et al.*, 2003).

An emulsion of linoleic acid with Fe(III) salts and ascorbic acid as catalysts was stored at 30 ºC. Phenolic antioxidants could not prevent the formation of primary hydroperoxides, but stopped the propagation reaction with free lipidic radicals. BHA, BHT, α-tocopherol and isoeugenol acted as strong antioxidants, but rosmarinic and caffeic acids were only weak antioxidants in this model system. Linoleic acid emulsion was stabilized with caseins and whey proteins. The oxidation of linoleic acid was lower in proteins in the continuous phase by binding trace metals and increasing the free radical scavenging activity, e.g. by reacting with bound cysteine or cystine (Ries *et al.*, 2010).

Ascorbic acid and β-carotene acted as synergists of casein in hydrolyzate in model systems (Bzducha and Wolosiak, 2006). The antioxidant activity in emulsions depends on the composition of emulsifiers. Sodium caseinate and lactose emulsions were more stable against oxidation than Tween-20 emulsions, stabilized by antioxidants of low polarity – α-tocopherol and dodecylgallate. In comparison, the protection by polar antioxidants – Trolox and gallic acid – was less effective (Velasco *et al.*, 2004). Meat was smoked for up to 23 h with

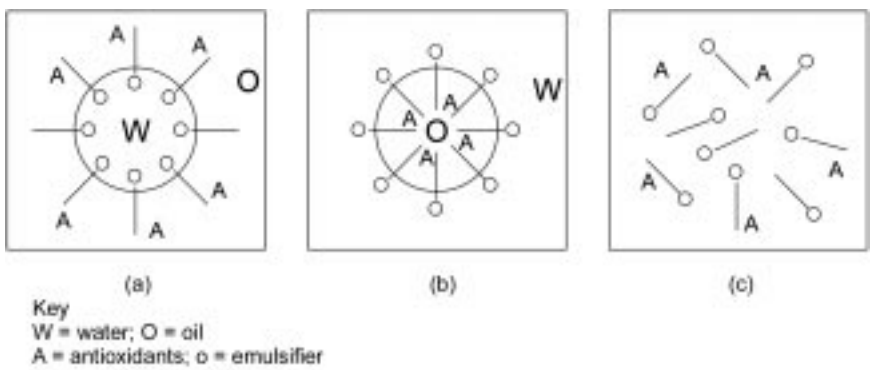

Fig. 15.12 Distribution of a hydrophobic antioxidant in emulsions: (a) water-in-oil emulsion, (b) oil-in-water emulsion, (c) bulk oil.

addition of antioxidants. Various antioxidants have different dynamic activities. The presence of ferrous (II) salts reduced the antioxidant activity (Bystricki *et al.*, 2004).

The objectionable changes of irradiated turkey breasts and products can be reduced not only by antioxidants, but also by vacuum packaging or by a combination of appropriate antioxidants and packaging (Lee and Ahn, 2002).

Milk containing the addition of Trolox and riboflavin was stored at 27 ºC in the light in closed packages. Both additives competed for singlet oxygen produced in the headspace. During 24 h, riboflavin was completely lost (Hall *et al.*, 2010).

15.5.3 Changes during seed germination

During the seed germination, lipids are converted into other products, such as cellulose or hemicelluloses. Vitamin C, tocopherols, carotenoids and phenolic antioxidants are synthesized, as they are necessary for growing organism.

Wheat grains were germinated at 16.5 ºC and 98% relative humidity for nine days. The maximum reached in case of ferulic acid was 9.3 mg/kg, that of vanillic acid 0.92 mg/kg, respectively (Yang *et al.*, 2001). Concentrations of vitamin C and vitamin E increased steadily for nine days. During the germination of 13 edible seeds, seeds were dipped in water and then germinated for seven days. Great differences were observed among seeds in antioxidant formation (Ceballos-Casals and Cisneros-Zevallos, 2010). Broccoli and radish were fermented, dried, and either illuminated or subjected to chilling. In both cases the phenolic acids content in sprouts rose (Myung-Min and Radjashekar, 2009).

15.5.4 Changes under refrigeration temperature

During refrigeration (0–10 ºC), the oxidation is rather slow because of low temperature, and losses of antioxidant activity are negligible, if lipoxygenases and other oxidative enzymes have been inactivated. Of course, slow hydrolysis could take place. The lipolytic enzymes should be inactivated prior to the storage, because they would act slowly even under refrigeration. Proteins and antioxidants combined with lipoproteins are similarly active as free antioxidants. Insertion of antioxidants on the backbone of polysaccharides does not decrease the antioxidant activity, either (Spizzirri *et al.*, 2010). Natural plant extracts containing polyphenolic compounds were incorporated in cold ground beef. Commercial antioxidants, such as BHA, BHT, α-tocopherol or rosemary resins, reduced the formation of hexanal on storage as hexanal is an indicator of lipid oxidation by 73–97%.

Mandarin and satsuma fruits were minimally processed, and stored at 4 ºC for up to 15 days. The antioxidant activity was due to phenolic antioxidants, while ascorbic acid acted only as a minor antioxidant (Piga *et al.*, 2002).

15.5.5 Changes under frozen storage

During frozen storage (below 0 ºC), water present in food is frozen. Air can thus penetrate to the lipid components easily through channels left in food after water freezing. Oxidation is often faster in spite of low temperature, than during refrigeration, and losses of phenolic antioxidants become higher than on chilling. Frozen food may be protected against losses of antioxidant activity by application of phenolic antioxidants on the surface. The natural antioxidants, mainly tocopherols, are then protected against losses caused by oxidation. Polyunsaturated fatty acids, e.g. in fish, are protected against oxidation, too. Hydrolytical changes are negligible if water is turned into ice. Fresh beef meat was packed in a polypropylene film under frozen conditions. An addition of rosemary resins enhanced the stability against oxidation (Nerin *et al.*, 2006). Red raspberries were frozen at −30 ºC, and then stored at 4 ºC for three days and one day at +18 ºC. Antioxidants remained stable, and the antioxidant activity was unaffected, too (Mullen *et al.*, 2002).

15.6 Changes of antioxidant functionality during heating

15.6.1 Changes during boiling

During boiling, food material is dipped in water, which is the medium of heat transfer (Pokorný and Schmidt, 2001, 2003). The effect of phenolic antioxidants depends on the access of oxygen. The concentration of oxygen in boiling water is very low, and the temperature is also rather low compared to other culinary operations at high temperature. Some synergists, such as ascorbic acid and phosphates, are dissolved in the water phase, where the access of oxygen is slow, but considerably easier than in boiled food. The degree of oxidation of less polar or nonpolar antioxidants may be considered as negligible. However, some polyphenolic antioxidants may be bound to food proteins during boiling, so that the concentration of free phenolic antioxidants becomes lower. A small part of polar antioxidants is dissolved in the water phase.

During steam cooking of broccoli, the contents of flavonoids and phenolic compounds rose as they were liberated from insoluble complexes, while vitamin C was not affected. By water boiling and steam cooking, the contents of β-carotene, lutein, and α-tocopherol and γ-tocopherol increased, too. The cooking has similar effect on the antioxidant activity, as well (Gliszczyńska-Świglo *et al.*, 2006). Changes of antioxidants during cooking depend very much on the composition of cooked food, e.g. they are different in case for pasta (Fares *et al.*, 2010) or beef muscle (Ahn *et al.*, 2002).

Storage and pasteurization of green or black teas increased the optical density, due to the formation of polymers, but decreased the content of catechins (Manzocco *et al.*, 1998). Phenolics are also bound to lipoproteins, but they still exhibited the antioxidant activity corresponding to catechins (Vinson and Dabbagh, 1998). Red cabbage may be prepared by conventional cooking. During the process, 35.5–67.3% ascorbic acid and 33.1–54.3% of total phenols

were lost. During cabbage cooking by steaming, losses are smaller, i.e. 21–23% ascorbic acid and 10% total phenols. The Trolox antioxidant activity decreased by 5–20% (Podsedek *et al.*, 2008). Phenolic compounds enhanced the antioxidant capacity of peas, due to phenol–protein interaction. Hydroxycinnamic acids (such as ferulic, coumaric and caffeic acids), were the most active ingredients (Pi-Jen and Chen-Hue, 2006).

Hydrolysis of phenolic antioxidants bound as glycosides could proceed during boiling, but aglycones usually have almost the same molar activity as the respective glycosides so that the antioxidant functionality is not significantly affected.

15.6.2 Changes during baking

In the course of baking, food materials are heated to temperatures higher than 150–200 ºC. Such a high temperature is only on the surface, but less than 100 ºC inside the material. Therefore, the decomposition of phenolic antioxidants is much more intensive in the surface layer than in the core. Oxidized products of phenolic substances can react with amine and thiol groups of proteins present in baked foods. Both types of oxidation products react with proteins. During baking, Maillard reactions proceed (Manzocco *et al.*, 2001). Phenolic antioxidants oxidize into free radicals and partially further to quinones. The basis for Maillard reaction can be either sugars or ascorbic acid and proteins, or oxidized lipids and proteins. The resulting brown products act as synergists of phenolic substances by forming free radicals, or partially by binding the prooxidative metals, such as iron and copper, into undissociated inactive complexes.

Garlic juice heated to 130 ºC for two hours showed strong antioxidant activity because the formation of thiacremonone (2,4-dihydro-2,5-dimethyl-thiophen-3-one) by secondary decomposition of garlic sulfur compounds. Thiacremonone has stronger antioxidant properties than α-tocopherol, ascorbic acid, and BHA (Guk *et al.*, 2007).

15.6.3 Changes during microwave heating

Microwave cooking is a modern, frequently used procedure for food preparation. In the case of microwave heating, lipids are oxidized rapidly because of close contact with oxygen so that the oxidation in a microwave oven is faster than in case of a hot-air oven. The time necessary for microwave cooking to reach the desired temperature, and the time of heating is, however, much shorter than in a hot-air oven. Therefore, the changes of lipid by oxidation processes and antioxidant destruction are not much different from the conventional hot-air oven, at least under conditions of food cooking (Table 15.1) or may be even lower. Phenolic antioxidants offer satisfactory protection of the lipid fraction (Dostálová *et al.*, 2005). Almost no changes took place after 8–10 min heating of canola oil stabilized with BHA, BHT or citric acid (Vieira and Reghitano-d'Arce, 2001).

Table 15.1 Comparison of changes of antioxidant capacity during microwave heating and hot air oven heating (Dostálová J and Pokorný J, unpublished results)

Conditions	Heating time (min)	Reducing power	DPPH (%)	Ferulic acid (%)
Original oil	0	0.87	52.9	61.5
Hot air oven	30	0.05	40.6	45.1
Original oil	0	0.87	52.9	61.5
Microwave oven	30	0.04	44.8	53.7

A system of glucose and glycine was microwaved, and Maillard browning products were studied in the bulk oil and in emulsion. High concentrations of water soluble antioxidants decreased the browning intensity but increased the fluorescence (Porter *et al.*, 2006).

In the presence of 50–1000 mg/kg tocopherols in tocopherol-stripped oil, microwave heating accelerated the tocopherol oxidation (Yoshida *et al.*, 1993). The tocopherol stability corresponds to the stability differences after heating generally: δ-tocopherol > β-tocopherol > γ-tocopherol > α-tocopherol.

Vegetable oil always contain multiple tocopherol species. Tocopherols and sesamol were tested in tocopherol-stripped soybean, rapeseed, and safflower oils during microwave heating. Sesamol and α-tocopherol protected γ-tocopherol against oxidation (Yoshida and Takagi, 1999). Tocopherols were oxidized only moderately (by 20%) after 5 min, but γ-tocopherol was completely destroyed after 20 min.

Sunflower seeds were roasted in a microwave oven (2450 MHz) for 6–30 min. Only minor losses of antioxidants were observed, and 92% tocopherols still remained unchanged after 30 min microwave heating (Yoshida *et al.*, 2002), probably because of protection of seeds by seed hulls.

15.6.4 Changes during grilling, extruder cooking and roasting

Grilling is similar to roasting, but the temperature is higher and the heating time shorter. The very hot surface layer is thus only very thin, and the decomposition of phenolic antioxidants in inner layers is low.

Extrusion usually occurs at temperatures above 100 ºC, but the residence time is very short, therefore, the decomposition of antioxidants is relatively small. Buckwheat groats were extruded at 120–200 ºC. The tocopherol loss was 62%, but content of phenolic acids rose by liberation from their bonds with protein. A sum of polyphenols decreased (Zieliński *et al.*, 2006).

Roasting of nuts, beans (e.g., coffee beans) or similar low-moisture foods proceeds at high temperatures (220–170 ºC) for a short time (usually 15–30 min) under free access of air oxygen. The oxidation is then rather fast, but nonenzymic reactions between sugars and amino acids or proteins (Maillard reactions) take place at the same time. Maillard products have certain anti-

oxidants activities. Additions of BHA, BHT or TBHQ increased the fluorescence (absorption at 412 nm, emission at 507 nm), due to Maillard reaction products (Liu *et al.*, 2007). Similar behaviour was observed in model experiments of asparagin and glucose, heated at 160 ºC.

In case of buckwheat products preparation, the content of flavonoids decreased from 188 mg/kg to 40 mg/kg, measured in dark brown groats (Dietrich-Szostak and Oleszek, 2001). The content of flavonoids was lower to a lesser degree, only from 740 to 660 mg/kg. Buckwheat (*Fagopyrum tartaricum* Gärtn.) was subjected to pressure and steaming, and then roasted. The content of antioxidants decreased, and a positive regression was observed between total phenolics and the antioxidant activity (Min *et al.*, 2010).

Roasting of pulses (chickpea), cashew nuts, and seeds increased antioxidant capacity through Maillard reaction, but at the same time, antioxidants were thermally decomposed, so that the final balance was negative and the antioxidant activity decreased (Acar *et al.*, 2004).

Therefore the oxidation degree of lipids in roasted foods, such as in coffee beans, is not as high as could be anticipated. At relatively high roasting temperatures the oxidation may be quite moderate, because of relatively short heating times, especially in the inner layers, where the access of air is limited. The antioxidant capacity of robusta coffee decreased to 50% of the original value, and a further 10% was lost on subsequent storage (Votavová *et al.*, 2004). Roasted coffee residues contain 0.8–2.4% antioxidants, such as caffeic or chlorogenic acids, flavonoids and polyphenolics, which is why they are a good source of antioxidants. Maillard products formed at such a high temperature also exhibit certain antioxidant activity (Wen-Jye *et al.*, 2005). The antioxidant capacity of barley grain was maximum during roasting to 250 ºC for 63.5 min in a 1.5 grain layer (Omwamba and Quihui, 2009).

Cocoa beans are very rich in flavanols, and the free radical scavenging activity is high (Hall, 2001). During traditional roasting, only a slight decease of phenolics was observed and the antioxidant capacity remained high. Dutch processing was introduced to obtain darker products and stronger flavour. For this purpose, beans are alkalized before roasting. At higher pH value, flavanols and other polyphenols, including procyanidins, are rapidly destroyed (Miller *et al.*, 2008).

Deep fat frying of nuts, particularly peanuts or almonds, is often called roasting, but the technology is similar to frying, as frying oil acts as the heat tranfer medium. The degree or oxidation corresponds more to that of fried products. Hydrolysis is very limited because of low moisture content on the surface, and fast inactivation of lipases.

15.6.5 Changes during frying

Two types of frying procedures should be distinguished, namely pan frying on a thin layer of fats and oils, and deep fat frying, where the food product is dipped into a deep layer of fat, e.g. in a fryer.

Pan frying is the traditional way of frying. A thin layer of fat is heated to about 180–200 ºC for 2–10 min. The fried material is heated only on the surface so that the antioxidants losses, expressed in the whole food mass, are only moderate. Phenolic substances present in frying oil are, however, oxidized to a high degree as air oxygen has easy access not only to the surface, but also to thin lower layers. The technology of deep fat frying is discussed in more detail in another chapter.

The impact of air oxygen is lower in deep fat frying as the layer of frying oil is thick (100–200 mm), and oxygen can enter only through the interface of frying oil with air. Therefore, the rate of oxidation in frying oil is relatively low, and in comparison with pan frying, antioxidants are more important for their inhibition of lipid polymer formation (Réblová *et al.*, 1999), and of course, antioxidants do polymerize, too. The stability of synthetic or natural antioxidants depends on their non-volatility and activity/temperature dependence.

Special antioxidants were reviewed from the standpoint of their application in frying oil (Boskou and Elmafa, 1999). The access of oxygen may be prevented by addition of small amounts of polysiloxane. It forms a thin layer on the interface between air and frying oil so that the diffusion of air oxygen to polymeric acid and antioxidants in frying oil is inhibited.

Fish balls (made of comminuting fish meat) were coated with zein and stored at 4 ºC. An addition of BHA, BHT, and PG to the coating improved the stability. The last mentioned antioxidant was most effective in this system (Lian-Sun *et al.*, 2009).

Losses of phenolic antioxidants in frying oil are higher in materials containing more lipids, such as meat or fish, as they are partially lost by diffusion in frying oil. Losses of antioxidants are much lower in the case of frying potatoes or vegetables as another technological procedure is applied. Potatoes have a high percentage of water, which is evaporated in hot frying oil, and replaced by frying oil during the process. Therefore, losses of frying oils by absorption into the fried material are relatively high, and the absorbed oil should be often replaced by fresh oil (Pokorný, 2002), so that its degree of oxidation is lower than in case of meat. The concentration of antioxidants in frying oil is thus not so much affected by frying, and in frying French fries, the frequently replenished frying oil is used for a very long time.

Frying is often performed without any oil to reduce the energy content. Its flavour is different from products fried in oil. Such processes are more similar to roasting. The lipid fraction (Dostálová *et al.* 2005) is relatively stable, nevertheless, tocopherols and other natural antioxidants suffer great losses.

15.7 Changes of antioxidant functionality during drying

Drying can proceed under ambient temperature (air drying) or more often, under heating. Vacuum drying is more expensive. Water present in food is converted to steam, and is replaced by air so that the lipids are oxidized, and natural

antioxidants are decomposed more or less thoroughly by reaction with free lipid radicals.

Lycopene and other natural antioxidants are decomposed during drying of tomatoes at the temperature up to 120 ºC. The retention of antioxidants attained the minimum at 40–80 ºC (Chang and Liu, 2007). Processing of blackberries can cause antioxidant losses up to 65% (Hager *et al.*, 2008), but no significant losses of antioxidants were observed during the subsequent storage at −20 ºC. Freeze dried apples were stored at 40 ºC under different water activities (a_w). At $a_w < 0.316$ the stability of antioxidants was acceptable, but at $a_w > 0.316$, antioxidants were rapidly destroyed (Lavelli and Vantaggi, 2009). Tomatoes were dried to 2% moisture at 40 ºC (Veillet *et al.*, 2009). Lycopene content increased from 9.4 to 12.6 mg/kg, but the content of total phenols from 25.7 to 21.4 gallic acid equivalents per 100 g. The final antioxidant activity increased to 2373 Trolox equivalents, expressed in μmol/100 g DM.

15.8 Safety aspects of antioxidant changes in foods

15.8.1 Comparison of natural and synthetic antioxidants

Only a few synthetic antioxidants have been permitted in the European Union, and further few in the United States (Míková, 2001). Their permissions are based on long, very sophisticated tests on several species of laboratory animals. The permitted antioxidant concentration (usually the maximum of 0.02%) is 100–200 times lower than the concentration found still safe in the laboratory experiments. The reaction products of these synthetic antioxidants are also safe, even when their effect was not as thoroughly tested as in original antioxidants (Frankel, 2007).

In the case of natural antioxidants, the criteria are not as strict as in the case of synthetic antioxidants. Experts assume that natural antioxidative compounds are usually food components, and the consumption for several thousand years is regarded as a guarantee of their safety (Shahidi, 1997). There is no scientific proof for such a belief. Acute toxicity is most probably absent, but chronic low toxicity cannot be excluded. There are many papers published every year on extracts with an antioxidant activity from other natural sources than food. The application of those extracts cannot be recommended without experimental tests.

Moreover, natural antioxidants are not absolutely pure compounds, but they usually possess very complicated composition (Pokorný and Korczak, 2001). They contain not only active components (often, a mixture of substances of similar structure and antioxidant activity), but also co-extracted materials, the safety of which is only assumed, but not thoroughly proven. Purified compounds would be too expensive, and they, being pure substances, should be tested after similar criteria as synthetic antioxidants. Changes of natural antioxidants during food processing are very similar to those of synthetic antioxidants, only the possible presence of synergists or prooxidants should be accounted for (Yanishlieva and Marinova, 1992).

Table 15.2 Advantages and disadvantages of synthetic and natural antioxidants, respectively

Synthetic antioxidants	Natural antioxidants
Pure compounds	No defined composition
Defined and constant quality	Variable antioxidant activity
Reproducible antioxidant activity	Safety mostly only expected
Tested as safe compounds	Unknown effect of impurities
Regarded as suspect by consumers and environmental experts	Readily accepted by consumers as natural substances

Maillard reaction products can be formed either by reactions between sugars and amino acids or proteins, of the reaction between oxidized lipids and proteins. The products are formed slowly at ambient temperature and rapidly under heating, The intermediary products are colourless, but they further polymerize into brown products. The browning is undesirable in many technologies. They have also certain antioxidant activity. Cysteine and sulfites act as prooxidants at lower concentrations, but have an antioxidant activity in high concentrations (Sawamura *et al.*, 2000).

In case of natural antioxidants, not only the antioxidant activity, but also their potential beneficial effect for the prevention of chronic diseases should be examined It could also increase or decrease during the processes of food preparation.

15.8.2 Comparison of between functionality of antioxidants and their degradation products

The safety of synthetic antioxidants is well known, but in the case of natural antioxidants, safety even of those most frequently used, is only guessed and expected. During storage and food preparation, the antioxidants originally present are oxidized, co-polymerized or polymerized or changed in many other ways, both synthetic and natural antioxidants similarly. Some reaction products have their own activity or act as promotors or inhibitors of further oxidation, in some cases as metal-complexing agents. The oxidation may be prevented by microencapsulation, e.g. in case of sunflower oil (Velasco *et al.*, 2009).

The products of oxidation or polymerization of phenolic antioxidants have also positive or negative impact on other biological activities, which might change during food preparation (Table 15.2).

15.9 Future trends

Consumers and environmental experts have an emotionally based aversion against all synthetic additives, even synthetic antioxidants, despite their safety having been tested. Therefore, it may be assumed, there would be a trend to

eliminate them from food products. On the contrary, antioxidants would be more intensively applied in the Third Word as a cheap way to improve food stability.

Nature-identical antioxidants are synthetized in the industry, but they have the same chemical structure as the respective natural antioxidants, e.g. α-tocopherols, β-carotene, ascorbic acid or citric acid. They will probably be tolerated even in future, but their content will be limited in the same way as that of synthetic antioxidants (mostly to 0.02%) as they are pure compounds.

Another group of antioxidants has been isolated from non-food plants, and they should be regarded under the same conditions as synthetic antioxidants, especially, if they have been isolated from tropical plants, not common for our consumers from the temperate zone. They have not got used to their consumption. The majority of spices possess a certain antioxidant activity, such as rosemary, sage, black pepper, ginger, garlic, onion, etc.

Natural antioxidants, which have been consumed in foods for several thousand years, may be considered as free from acute toxicity. They might still possess weak chronical toxicity, especially when they are taken at higher concentrations (e.g., spices, such as black pepper) (Wilson, 2003). There exists some hope that their health aspects could be more intensively studied. The disadvantage is that natural antioxidants are present in very different concentrations, depending on the cultivar, climatic conditions or time of cropping. Raw plant materials with higher content of natural antioxidants will probably be developed.

Reaction products of antioxidants during food preparation and storage also often have antioxidant activity, which has not yet been sufficiently studied. Their mutual synergism is only very rarely known.The same can be stated on the production of antioxidants from inactive precursors, e.g., in the course of Maillard reactions. Their safety is not sufficiently known but they have been consumed for thousands of years.

The prevailing tendency will be to eliminate the necessity for the use of antioxidants at all. More and more food products will be stored in a refrigerator or a freezer. The activity of enzymes catalyzing the lipid oxidation will decrease at low temperature. Other enzymes destroying antioxidants will be rapidly inactivated by blanching to an increasing degree so that their antioxidating activity will not be impaired.

Suitable packaging is an efficient way to prolong the storability and to reduce the rate of food oxidation. Foods could be packed under vacuum or inert gas in impermeable material, such as certain plasts or steel, or other packaging material impregnated with antioxidants. They might have almost unlimited storage time, at least from the standpoint of oxidative deterioration. Storage under inert medium would not prevent browning reactions. Modification of technology of food preparation will decrease the rate of oxidation or of pyrolysis.

More emphasis on the sensorial value is expected. Increasingly, it will be necessary to optimize the technological procedures to satisfy consumers to the maximum degree.

Naturally, trend forecasting is always accompanied by uncertainty, as new results of research can lead to unexpected conclusions.

15.10 Literature for further study

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