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Antioxidants in food

Practical applications

Edited by
Jan Pokorny, Nedyalka Yanishlieva and Michael Gordon

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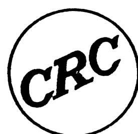

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1

Introduction

Professor Jan Pokorný, Prague Institute of Chemical Technology

Fats, oils and lipid-based foods deteriorate through several degradation reactions both on heating and on long term storage. The main deterioration processes are oxidation reactions and the decomposition of oxidation products which result in decreased nutritional value and sensory quality. The retardation of these oxidation processes is important for the food producer and, indeed, for all persons involved in the entire food chain from the factory to the consumer. Oxidation may be inhibited by various methods including prevention of oxygen access, use of lower temperature, inactivation of enzymes catalysing oxidation, reduction of oxygen pressure, and the use of suitable packaging.

Another method of protection against oxidation is to use specific additives which inhibit oxidation. These are correctly called oxidation inhibitors, but nowadays are mostly called antioxidants. These inhibitors represent a class of substances that vary widely in chemical structure, and have diverse mechanisms of action (Table 1.1). The most important mechanism is their reaction with lipid free radicals, forming inactive products. Additives with this mechanism are antioxidants in the proper sense. Usually, they react with peroxy or alkoxy free radicals, formed by decomposition of lipid hydroperoxides. Other inhibitors stabilise lipid hydroperoxides, preventing their decomposition into free radicals. Decomposition of hydroperoxides is catalysed by heavy metals, and consequently metal chelating agents also inhibit oxidation. Some substances called synergists demonstrate no antioxidant activity in themselves, but they may increase the activity of true antioxidants. Another group of substances decompose lipid hydroperoxides by a non-radical pathway, thereby reducing free-radical content. Finally, singlet oxygen oxidises lipids many times faster than

Table 1.1 Mechanisms of antioxidant activity

Antioxidant class	Mechanism of antioxidant activity	Examples of antioxidants
Proper antioxidants	Inactivating lipid free radicals	Phenolic compounds
Hydroperoxide stabilisers	Preventing decomposition of hydroperoxides into free radicals	Phenolic compounds
Synergists	Promoting activity of proper antioxidants	Citric acid, ascorbic acid
Metal chelators	Binding heavy metals into inactive compounds	Phosphoric acid, Maillard compounds, citric acid
Singlet oxygen quenchers	Transforming singlet oxygen into triplet oxygen	Carotenes
Substances reducing hydroperoxides	Reducing hydroperoxides in a non-radical way	Proteins, amino acids

the common triplet oxygen, and consequently singlet oxygen quenchers also have an important inhibitory effect on lipid oxidation. In this book, we shall discuss all the above groups of inhibitors, calling all of them antioxidants.

Antioxidant activity depends on many factors such as the lipid composition, antioxidant concentration, temperature, oxygen pressure, and the presence of other antioxidants and many common food components, e.g. proteins and water. Antioxidants were first used before World War II for food preservation. These early antioxidants were natural substances. They were, however, soon replaced by synthetic substances, which were cheaper, of more consistent purity, and possessed more uniform antioxidant properties. They were tested for toxicity by a range of methods in concentrations at 100–200 times the level actually consumed, to confirm their safe use as additives. The increased use of various synthetic food additives was then challenged by consumer groups. Consumers wished to have these additives replaced by natural materials, which were considered to be more acceptable as dietary components. Industrial producers have tried to comply with consumers' wishes, and have moved to increased use of natural antioxidants. Most natural antioxidants are common food components, and have been used in the diet for many thousands of years so that humans have adapted to their consumption. In our book we place more emphasis on natural antioxidants than on synthetic antioxidants.

In the first part of this book we discuss the oxidation of lipids and its inhibition by antioxidants generally. The second part is devoted to health aspects and current research on the role of antioxidants in inhibiting the development of cardiovascular diseases and of cancer. In the third part, sources and properties of natural antioxidants are discussed. The

emphasis is on substances present in plant foods which are frequently consumed in the human diet. In the last part of the book, practical aspects of antioxidant use in food processing are discussed, such as their regulation, preparation, changes during food processing, and application in food products.

Part 1

Antioxidants and food stability

2

The development of oxidative rancidity in foods

Dr Michael H. Gordon, The University of Reading

2.1 Introduction

2.1.1 Lipids in foods

Lipids occur in nearly all food raw materials with the major classes being triglycerides (also known as triacylglycerols), which occur in fat storage cells of plants and animals, and phospholipids, which occur in biological membranes. In the processing of a wide range of foods, fats may be added as part of the food formulation. The added fats are a major component of many foods including mayonnaise, margarine, and frying oils. These fats are almost completely triglycerides, and it is these components that are of most significance as potential sources of oxidative off-flavours in these foods. In plant or animal tissues used as foods, the phospholipids present in all biological membranes may be an important substrate for oxidative deterioration.

2.1.2 Pathways to lipid oxidation

The spontaneous reaction of atmospheric oxygen with lipids, known as autoxidation, is the most common process leading to oxidative deterioration. Polyunsaturated fatty acids have the potential for decomposing by this process, whether they are present as free fatty acids or whether they are present in triglycerides (or diglycerides or monoglycerides) or phospholipids. When light and a sensitiser such as chlorophyll are present, activation of oxygen to singlet oxygen may play a role in the initiation of oxidative deterioration. Alternatively, metals including iron or copper, or the enzyme lipoxygenase, may play a role in the process by which oxidative deteriora-

tion is initiated. Lipoxygenase is present in plant tissues including those of soybean, pea and tomato. The enzyme may cause oxidative deterioration of lipids during isolation of oils from oilseeds, but it also plays a role in the formation of positive flavours in vegetables during mastication.

2.1.3 Lipid oxidation products

The components formed in the initial stage of autoxidation are the hydroperoxides, and these are also the products formed in lipoxygenase-catalysed oxidation. Although hydroperoxides are involatile and odourless, they are relatively unstable compounds and they decompose either spontaneously or in catalysed reactions to form volatile aroma compounds, which are perceived as off-flavours. The nature of the off-flavour detected depends mainly on the fatty acid composition of the substrate and the extent of oxidation, although oxidation conditions may also affect the volatiles produced and the sensory properties of the oxidised oil. Examples of oxidative off-flavours are the beany flavours that commonly develop in soybean oil, the fishy flavours that develop in fish oil, and creamy or metallic flavours that may develop in milk fat. Aldehydes commonly contribute to the off-flavours that develop during lipid oxidation (Table 2.1).

Besides the development of rancid flavours, oxidative deterioration of lipids may cause the bleaching of foods due to the reaction of pigments, especially carotenoids, with the reactive intermediates, termed free radicals, which are formed during lipid oxidation. Free radicals may also lead to a reduction of nutritional quality by reaction with vitamins, especially vitamin E, which is lost from foods during its action as an antioxidant.

Table 2.1 Flavours of aliphatic aldehydes[1]

No. of carbon atoms	Saturated	Homologous series	
		2-Enals	2,4-Dienals
C2	Fresh, pungent		
C3	Fresh, milky		
C4	↓	Sweet, pungent	
C5		Sweet, green	
C6	Fresh, green		
	↓	↓	
C7			Sweet, oily
C8	Fresh, citrus		
C9	↓	Sweet, fatty, green	
		↓	↓
C10			
C11	Fatty	Sweet, fatty	
C12	↓	↓	

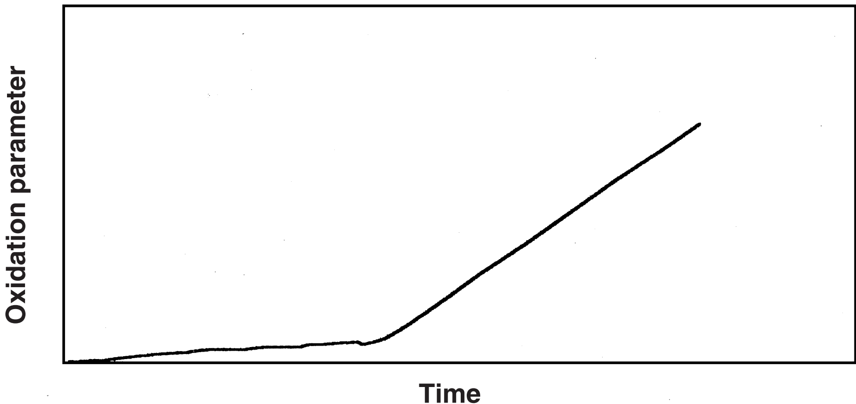
In frying oils, the concentration of free radicals increases to a much higher level than in foods stored or processed at moderate temperatures. At the elevated temperatures used in frying, which are normally about 180 °C, free radicals reach concentrations where combination to form dimers becomes significant. This causes an increase in the viscosity of the oil. Formation of free fatty acids, darkening of the oil and an increase in foaming and smoking also occur during frying. According to the recommendations of the German Society for Fat Research (DGF), a frying fat should be regarded as deteriorated if it contains more than 24 % polar material or 12 % polymeric material.[2] By this time a considerable proportion of the tocopherols together with part of the polyunsaturated fatty acids present in the oil will have been lost.

The off-flavours that develop during lipid oxidation normally act as a warning that a food is no longer edible, although this does not apply to polyunsaturated lipid supplements taken in capsule form. There is some concern that excessive intake of lipid hydroperoxides may lead to adverse health effects. In theory, if hydroperoxides are absorbed they are a potential source of radicals, which may cause damaging effects *in vivo.* Free radicals produced by hydroperoxide decomposition may cause damage to proteins, including enzymes, or to DNA and may also generate carcinogens. Concern has been expressed about the effects of cholesterol oxides, which are partly absorbed and which cause arterial disease in rabbits.[3] However, many sources of free radicals occur *in vivo*, and the presence of dietary or endogenous antioxidants normally ensures that human tissues remain healthy.

2.2 Types and effects of rancidity

Understanding of the mechanisms by which lipids deteriorate developed rapidly during the twentieth century. Autoxidation reactions commonly show an induction period, which is a period during which very little change occurs in the lipids. After the end of the induction period, oxidative deterioration of the lipids occurs much more rapidly, as shown in Fig. 2.1. Off-flavours become most noticeable after the end of the induction period. One consequence of the sharp rise in the concentration of off-flavour components after the end of the induction period is that the rate of deterioration of foods is relatively insensitive to the precise fat content of the food.

The induction period (IP) is very sensitive to small concentrations of components that shorten the IP, the pro-oxidants, or lengthen the IP, which are the antioxidants. Metal ions are the most important pro-oxidants in foods, whereas antioxidants include compounds that act by radical scavenging, metal chelation or other mechanisms. The presence of an induction period is characteristic of chemical reactions that proceed by a free-radical



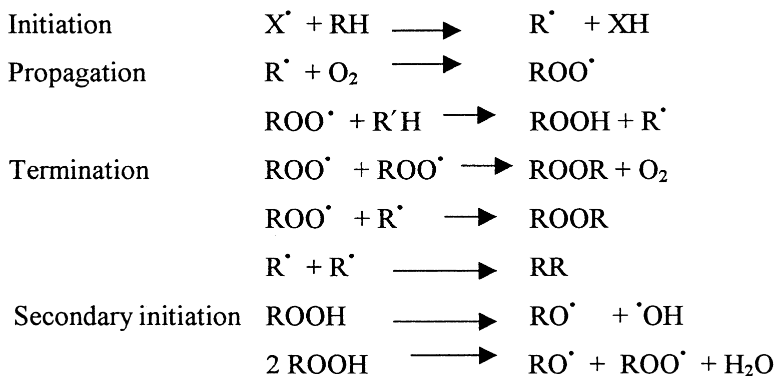
2.1 A typical induction period in fat deterioration.

mechanism. The level of free radicals in oils is generally low, but in frying the rapid formation of free radicals can lead to the combination of free radicals to form triglyceride dimers.

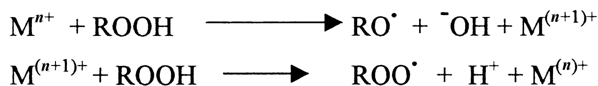
Hydroperoxides may form by autoxidation, but an alternative pathway is by the action of the enzyme lipoxygenase on polyunsaturated fatty acids. Lipoxygenase occurs in various plants including soybeans, corn, potato, tomatoes, cucumber, oat seed and barley seed. It is of significance in the development of flavour in vegetables, but in oilseed crops the action of lipoxygenase before and during oil extraction may lead to the hydroperoxides that subsequently decompose to form off-flavours in the oil. Hydroperoxides may also form by photo-oxidation if light acts on a fat in the presence of a sensitiser. However, the decomposition of hydroperoxides is a low energy reaction for the initiation of autoxidation, and the composition of the volatile off-flavours that are formed are normally characteristic of autoxidation products, no matter how the initial hydroperoxides are formed.

2.3 Mechanism of autoxidation

As a free-radical reaction, autoxidation proceeds in three distinct steps (Fig. 2.2). The first step is initiation in which lipid radicals are formed from lipid molecules. Abstraction of a hydrogen atom by a reactive species such as a hydroxyl radical may lead to initiation of lipid oxidation. However, in oils there is often a trace of hydroperoxides, which may have been formed by lipoxygenase action in the plant prior to and during extraction of the oil. Secondary initiation by homolytic cleavage of hydroperoxides is a relatively low energy reaction, and it is normally the main initiation reaction in edible oils. This reaction is commonly catalysed by metal ions. After initiation,



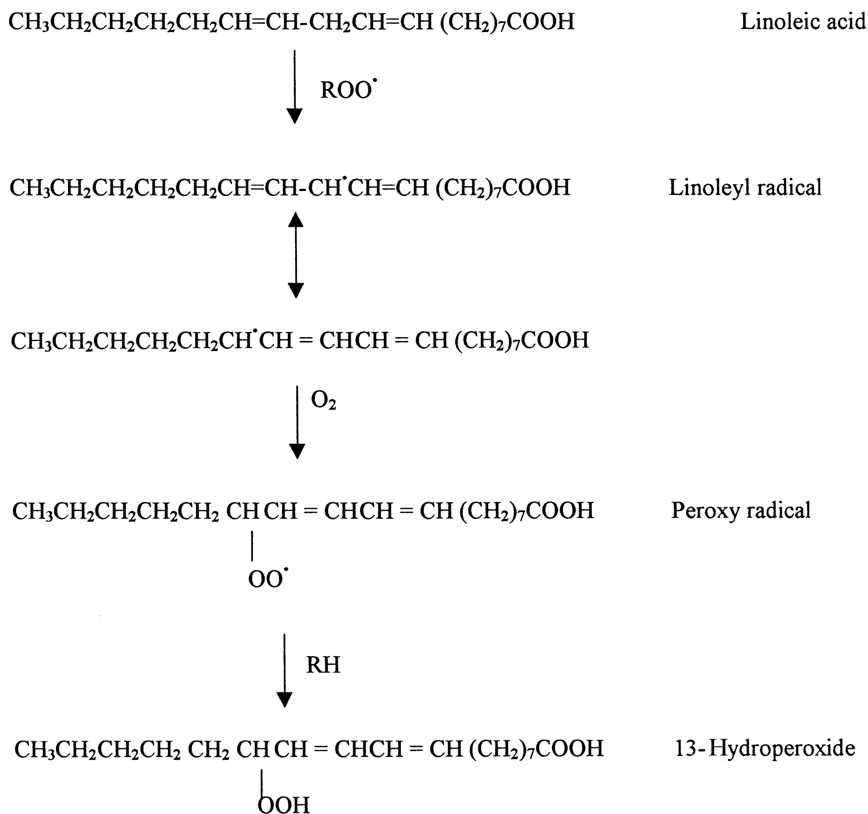
Metal-catalysed initiation:



2.2 Mechanism of lipid autoxidation.

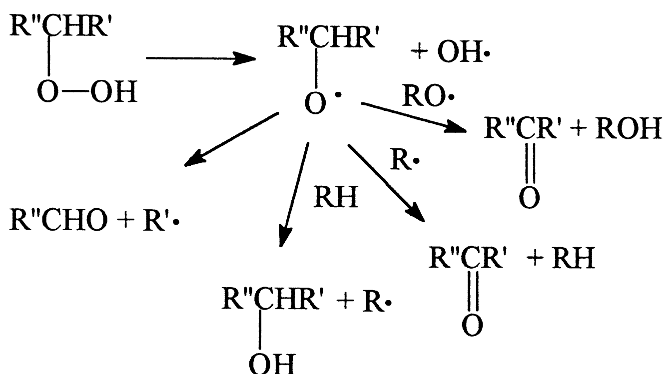
propagation reactions occur in which one lipid radical is converted into a different lipid radical. These reactions commonly involve abstraction of a hydrogen atom from a lipid molecule or addition of oxygen to an alkyl radical. The enthalpy of reaction is relatively low compared with that of the initiation reactions, so propagation reactions occur rapidly compared with initiation reactions. At normal atmospheric pressure of oxygen, the reaction of alkyl radicals with oxygen is very rapid, and the peroxy radicals are present at much higher concentrations than the alkyl radicals. Abstraction of hydrogen takes place preferentially at carbon atoms where the bond dissociation energy is low. Since the bond dissociation energy of the C–H bond is reduced by neighbouring alkene functionality, abstraction of hydrogen takes place most rapidly at the methylene group between two alkene groups in a polyunsaturated fatty acid (PUFA). The radical formed initially from a PUFA is delocalised across five carbon atoms of the 1,4-pentadienyl moiety, and reaction with oxygen occurs preferentially by addition at one of the end carbons of this structure. This leads to the formation of the 9- and 13-hydroperoxides from linoleic acid as shown in Fig. 2.3. Termination reactions in which free radicals combine to form molecules with a full complement of electrons are low energy reactions but are limited by the low concentration of radicals and by the requirement for radicals with the correct orientation for reaction to collide. However, in frying oils termination reactions are important, with dimers and higher polymers contributing to the increased viscosity of the oil.

Alkoxy radicals formed by hydroperoxide decomposition can decom-

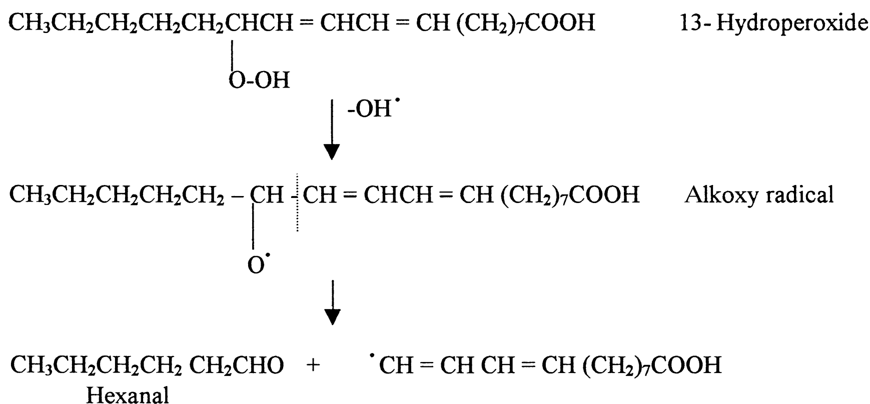


2.3 Formation of 13-hydroperoxide from linoleic acid (9-hydroperoxide is the other major product formed by a similar route).

pose to release volatile hydrocarbons, alcohols or aldehydes that are no longer bound to the glycerol backbone when the fatty acid is present as a glyceride. Non-volatile alcohols and ketones may also be formed as shown in Fig. 2.4. Volatile aldehydes are particularly important as contributors to the aroma of oxidised oils, and hexanal is commonly monitored in assessing the formation of secondary oxidation products during lipid oxidation. Hexanal is normally formed in relatively large amounts during the oxidation of lipids via the 13-hydroperoxide (Fig. 2.5), although it is not one of the aldehydes to which the palate is most sensitive. Consequently, other volatile products may contribute more than hexanal to the off-flavour perceived in the sensory assessment of oxidised oils. The flavour thresholds of some aldehydes formed in the autoxidation of linoleic acid are shown in Table 2.2. The concentration required for a volatile component to be detected as a contributor to the flavour depends on the medium. Normally



2.4 Formation of secondary products by hydroperoxide decomposition.



2.5 Decomposition of 13-hydroperoxide from linoleic acid to form hexanal.

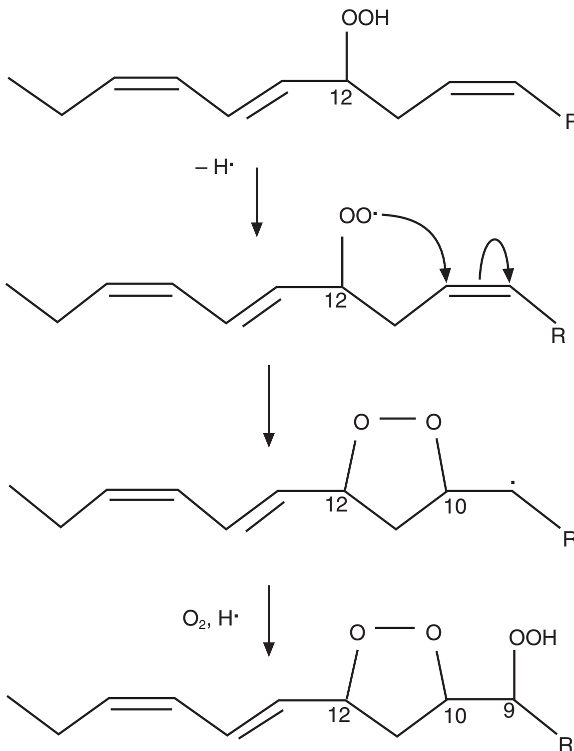
Table 2.2 Flavour thresholds of possible linoleic acid oxidation products in paraffin oil[4]

Compound	Threshold (mg. kg^{-1})
Hexanal	0.08–0.6
Heptanal	0.04–0.055
Octanal	0.04–0.6
trans-2-Nonenal	0.04–0.4
cis-2-Decenal	0.1
trans,trans-2,4-Nonadienal	0.46
trans,cis-2,4-Decadienal	0.02

non-polar components have a higher flavour threshold in non-polar media such as edible oils than they have in water.

The phases present in the food will also affect the rate of oxidation by affecting the activity of the antioxidants present, and by partitioning of pro- and antioxidants between oil and aqueous phases. The term polar paradox has been applied to the phenomenon whereby polar antioxidants are most effective in oils, whereas non-polar antioxidants are more effective in emulsions. Normally, metal chelation is less effective as an antioxidant mechanism in water-containing foods than in oils.

As well as decomposition to form secondary oxidation products, hydroperoxides formed from polyunsaturated fatty acids may undergo further oxidation reactions to form dihydroperoxides and molecules which have oxygen-containing rings including hydroperoxy epidioxides and bicycloendoperoxides. The mechanism for the formation of hydroperoxy epidioxides from α-linolenic acid is shown in Fig. 2.6.



2.6 Reaction of 12-hydroperoxide from α-linolenic acid to form 9-hydroperoxy endoperoxide.[5]

2.4 Photo-oxidation

An alternative route leading to the formation of hydroperoxides instead of the free radical mechanism is via a *photo-oxidation route*. In this case, excitation of lipids (type I photo-oxidation) or excitation of oxygen (type II photo-oxidation) may occur in the presence of light and a sensitiser. There is no induction period in the photo-oxidation route.

2.4.1 Type I photo-oxidation

In the presence of some sensitisers, such as riboflavin, type I photo-oxidation occurs. Type I photo-oxidation is characterised by hydrogen atom transfer or electron transfer between an excited triplet sensitiser and a substrate, such as a polyunsaturated fatty acid, producing free radicals or free radical ions. Autoxidation may then proceed. The mechanism for type I photo-oxidation can be summarised as follows:

$$^{1}\text{Sens} + h\nu \rightarrow {}^{1}\text{Sens}^{*} \rightarrow {}^{3}\text{Sens}^{*} \qquad [2.1]$$

$$^{3}\text{Sens}^{*} + \text{X (acceptor)} \rightarrow \text{[intermediate I]} \qquad [2.2]$$

$$\text{[intermediate I]} + {}^{3}\text{O}_2 \rightarrow {}^{1}\text{Sens} + \text{XO}_2 \qquad [2.3]$$

2.4.2 Type II photo-oxidation

Oxygen in the environment is normally in the triplet electronic state, $^{3}O_2$. This is the lowest energy state, in which the two highest energy electrons have parallel spins and are in two different degenerate molecular orbitals. The direct reaction of a $^{3}O_2$ molecule with a lipid molecule, RH, would contravene the principle of conservation of spin angular momentum, since the lipid molecule and a hydroperoxide molecule produced would both be in the singlet electronic state. Hence the reaction of oxygen with lipid molecule according to reaction [2.4] would result in a change in spin angular momentum.

$$\begin{array}{lccccc} & {}^{3}\text{O}_2 & + & \text{R—H} & \longrightarrow & \text{R—O—O—H} \\ \text{Electron spins} & \uparrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow \end{array} \qquad [2.4]$$

However, triplet oxygen can be excited by light to singlet oxygen in the presence of a sensitiser, such as chlorophyll, according to reaction [2.5]. Singlet oxygen reacts much faster than triplet oxygen with the unsaturated lipid via the 'ene' reaction producing an allylic hydroperoxide via a shift of a double bond.[6]

$$^{1}\text{Sens} \xrightarrow{h\nu} {}^{1}\text{Sens (excited)} \xrightarrow{\text{intersystem crossing}}$$
$$^{3}\text{Sens (excited)} \xrightarrow{^{3}\text{O}_2} {}^{1}\text{Sens} + {}^{1}\text{O}_2 \qquad [2.5]$$

The singlet oxygen formed may then react with a polyunsaturated fatty acid to form a hydroperoxide. No change in spin occurs during this reaction

which is therefore allowed, and it occurs more than 1500 times faster than the reaction between triplet oxygen and a polyunsaturated fatty acid.

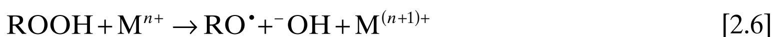
2.5 Ketonic rancidity

Ketonic rancidity is a problem that can be encountered with some products such as desiccated coconut which contain short-chain saturated fatty acids. Moulds such as *Eurotium amstelodami* degrade triglycerides in the presence of limited amounts of air and water. Free fatty acids are liberated initially and these subsequently suffer β-oxidation with the formation of methyl ketones and aliphatic alcohols. A musty, stale note in the product is characteristic of ketonic rancidity.[7]

2.6 Metal-catalysed lipid oxidation

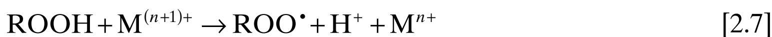
All materials of biological origin contain small amounts of transition metals, which cannot be completely removed by normal food processing. Transition metals, e.g. Fe, Cu, Co, which possess two or more valence states with a suitable oxidation–reduction potential affect both the speed of autoxidation and the direction of hydroperoxide breakdown to volatile compounds.[8]

Transition metal ions in their lower valence state (M^{n+}) react very quickly with hydroperoxides. They act as one-electron donors to form an alkoxy radical and this can be considered as the branching of the propagation step:



$$ROOH + M^{n+} \rightarrow RO^{\bullet} + {}^{-}OH + M^{(n+1)+} \qquad [2.6]$$

In a slow consecutive reaction the reduced state of the metal ion may be regenerated by hydroperoxide molecules:



$$ROOH + M^{(n+1)+} \rightarrow ROO^{\bullet} + H^{+} + M^{n+} \qquad [2.7]$$

Owing to the presence of water, or metal complexing with chain termination products, the regeneration is not complete. The radicals produced in equations [2.6] and [2.7] enter the propagation sequence and decrease the induction period. The catalytic activity of heavy metals depends in reality not only on the ion species and its redox potential but also on the ligands attached to it, the solvent system, the presence of electron donors such as ascorbate and cysteine which keep the metal ion in its lower valance state, and the pH. Maximum degradation of peroxides occurs in the pH region 5.0–5.5 and the activity at catalysing degradation decreases from $Fe^{2+} > Fe^{3+} > Cu^{2+}$, as detailed by O'Brien.[9]

Metals can abstract a hydrogen atom from the fatty acids themselves according to equation [2.8], but the ubiquitous presence of traces of

hydroperoxides in oils is likely to ensure that hydroperoxide decomposition is the normal initiation reaction.

$$RH + M^{(n+1)+} \rightarrow R^{\bullet} + H^{+} + M^{n+} \quad [2.8]$$

2.7 Antioxidant effects

Antioxidants in food may be defined as any substance which is capable of delaying, retarding or preventing the development in food of rancidity or other flavour deterioration due to oxidation. Antioxidants delay the development of off-flavours by extending the induction period. Addition of antioxidants after the end of this period tends to be ineffective in retarding rancidity development.

Antioxidants can inhibit or retard oxidation in two ways: either by scavenging free radicals, in which case the compound is described as a *primary antioxidant*, or by a mechanism that does not involve direct scavenging of free radicals, in which case the compound is a *secondary antioxidant*. Primary antioxidants include phenolic compounds such as vitamin E (α-tocopherol). These components are consumed during the induction period. Secondary antioxidants operate by a variety of mechanisms including binding of metal ions, scavenging oxygen, converting hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen. Normally, secondary antioxidants only show antioxidant activity when a second minor component is present. This can be seen in the case of sequestering agents such as citric acid which are effective only in the presence of metal ions, and reducing agents such as ascorbic acid which are effective in the presence of tocopherols or other primary antioxidants.

2.8 Other relevant reactions

2.8.1 Loss of fat-soluble vitamins and pigments

The vitamins A, D, E and K, and chlorophyll and carotenoids are fat-soluble and loss of these food components by radical-catalysed reactions may often accompany lipid oxidation in foods containing these components. The bleaching of β-carotene in lipid solutions is sometimes used as a method of monitoring lipid oxidation. Vitamin E acts as an antioxidant and is consumed during the induction period of autoxidation.

2.8.2 Reaction of oxidised lipids with other food components

Lipid oxidation products may react with proteins or with nucleic acids in food. Carbonyl compounds derived from oxidation of phospholipids may react with proteins to lead to flavour compounds in roasted meat.[10]

2.9 Mechanism of lipoxygenase-catalysed oxidation

The enzyme lipoxygenase (linoleate oxygen oxidoreductase, EC 1.13.11.12) is present in a wide variety of plant and animal tissues.[11] The enzyme in oil-bearing seeds, e.g. soybeans, can be an important source of hydroperoxides formed in the oil during extraction. In vegetables, oxidative changes due to the enzyme may lead to off-flavours during storage. The enzyme does, however, contribute to flavour formation in some plant foods including tomatoes and cucumbers.

Lipoxygenase activity requires the presence of free polyunsaturated fatty acids. Linoleic acid is the most common substrate in plant foods. The enzyme occurs in a variety of isozymes, which often vary in optimum pH, as well as product and substrate specificity. Four isozymes have been isolated from soybeans. Soy isozyme 1 has an optimum pH of 9.0. It only acts on free polyunsaturated fatty acids and it forms 9- and 13-hydroperoxides in the ratio of 1:9 at room temperature. Soy isozyme 2 has an optimum pH of 6.8, it acts on triglycerides as well as free polyunsaturated fatty acids and it forms 9- and 13-hydroperoxide in the ratio of about 1:1 at room temperature. Soy isozyme 3 is similar to isozyme 2, but its activity is inhibited by calcium ions, whereas lipoxygenase 2 is stimulated by the metal. Lipoxygenase 4 is very similar to isozyme 3, but can be separated by gel chromatography or electrophoresis. Lipoxygenase isozymes are commonly classified as type 1, which have an optimum pH in the alkaline region and are specific for free fatty acids, and type 2, which has optimum activity at neutral pH and causes co-oxidation of carotenoids. The ability of lipoxygenase type 2 to bleach carotenoids has found practical application in the addition of soya flour to wheat flour in order to bleach the flour in the manufacture of white bread.

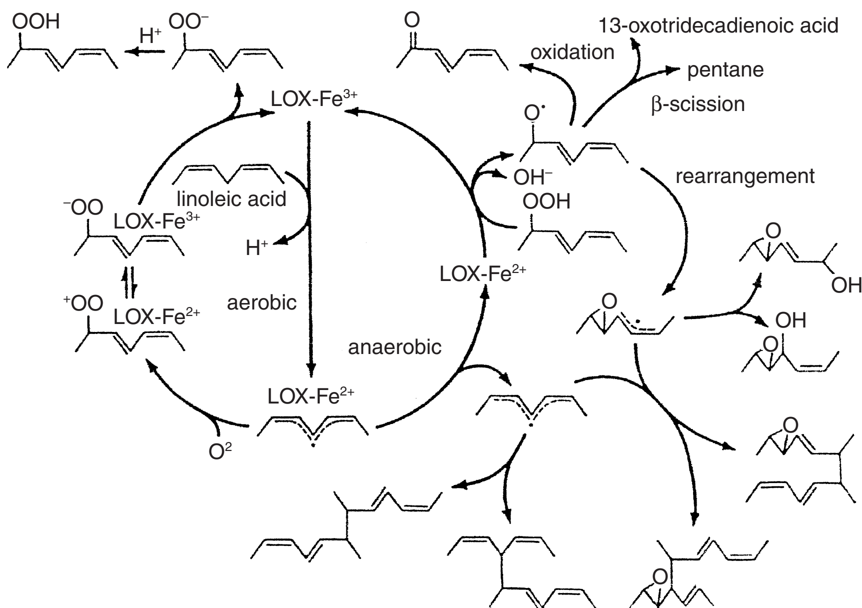
In plant tissues, various enzymes occur that cause the conversion of hydroperoxides to other products, some of which are important as flavour compounds. These enzymes include hydroperoxide lyase which catalyses the formation of aldehydes and oxo acids, hydroperoxide-dependent peroxygenase and epoxygenase, which catalyse the formation of epoxy and hydroxy fatty acids, and hydroperoxide isomerase, which catalyses the formation of epoxyhydroxy fatty acids and trihydroxy fatty acids. Lipoxygenase produces similar flavour volatiles to those produced during autoxidation, although the relative proportions of the products may vary widely depending on the specificity of the enzyme and the reaction conditions (Table 2.3).

Lipoxygenase molecules contain one atom of iron. The iron atom is in the high spin Fe(II) state in the native resting form of lipoxygenase, and it must be oxidised to Fe(III) by the reaction product, fatty acid hydroperoxides or hydrogen peroxide before it is active as an oxidation catalyst. As a consequence of this requirement for oxidation of the iron in the enzyme, a lag period is observed, when the enzyme is used with pure fatty acid sub-

Table 2.3 Composition of the volatile fraction produced by (a) pea lipoxygenase[12] and (b) autoxidation[13]

Linoleic acid substrate			Linolenic acid substrate		
Product	% (a)	% (b)	Product	% (a)	% (b)
n-pentanal	7	0.7	Acetaldehyde	5	
n-hexanal	41	67	Propanal	27	*
n-hept-2-enal	15	6	*n*-but-2-enal	3	1
n-oct-2-enal	10	18	*n*-pent-2-enal	22	10
n-nona-2,4-dienal	5	0.4	*n*-hex-2-enal	2	1
n-deca-2,4-dienal	20	5	*n*-hex-3-enal	–	14
			n-hepta-2,4-dienal	38	50

* Not detected by Badings,[13] but detected by other workers as a major product of linolenic acid autoxidation.[14]
The concentrations of *cis* and *trans* isomers are summed in this table.



2.7 Pathway of lipoxygenase-catalysed oxidation (reproduced from Gardner[15] with permission).

strates. The active enzyme abstracts a hydrogen atom stereospecifically from the intervening methylene group of a polyunsaturated fatty acid in a rate limiting step, with the iron being reduced to Fe(II) (Fig. 2.7). The enzyme–alkyl radical complex is then oxidised by molecular oxygen to an enzyme–peroxy radical complex under aerobic conditions, before electron

transfer from the ferrous atom to the peroxy group occurs. Protonation and dissociation from the enzyme allow the formation of the hydroperoxide. Under anaerobic conditions, the alkyl radical dissociates from the enzyme–alkyl radical complex, and a mixture of products including dimers, ketones and epoxides is produced by radical reactions.

2.10 Future trends

Increasing appreciation of the nutritional effects of highly unsaturated fatty acids including eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) are likely to encourage the development of food products that are particularly susceptible to autoxidation. This is likely to increase the search for more effective antioxidant combinations, and physical methods of preventing oxidative deterioration, especially by microencapsulation, are likely to become more widely used. The careful processing of oils prior to incorporation into food in order to avoid, or minimise, the formation of hydroperoxides is also likely to become more important for highly unsaturated oils. Highly unsaturated hydroperoxides are relatively labile, and decomposition of these components is a ready pathway for the initiation of autoxidation.

Increasing understanding of the importance of free-radical reactions in the development of a range of human disease states is likely to encourage studies of oxidative reactions in multiphase systems, and the interaction of lipid oxidation products with proteins and nucleic acids. There is currently enormous effort being applied to studies of the effects of plant components in reducing oxidative deterioration, and this area will continue to be intensively studied.

2.11 Sources of further information and advice

There have been a number of books written in recent years about autoxidation and oxidative deterioration in foods or other biological systems. The following books are recommended for consultation:

Allen J C and Hamilton R J (eds), *Rancidity in Foods*, 3rd ed., London, Chapman & Hall, 1994.

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

Scott G (ed.), *Atmospheric oxidation and antioxidants*, Amsterdam, Elsevier Science Publishers BV, 1993.

2.12 References

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12. Grosch W, 'Linol-und Linolensäure als Substrate für die enzymatische Bildung flüchtiger Carbonylverbindungen in Erbsen (*Pisum sativum*)', *Lebensm. Unters. Forsch.*, 1968 **137** 216–23.
13. Badings H T, 'Cold storage defects in butter and their relation to the autoxidation of unsaturated fatty acids', *Ned. Melk-Zuiveltijdschr.*, 1970 **24** 147–256.
14. Ellis R, Gaddis A M, Currie G T and Powell S L, 'Carbonyls in oxidising fat: XII. The isolation of free aldehydes from autoxidised triolein, trilinolein, and trilinolenin', *J. Am. Oil Chem. Soc.*, 1968 **45** 552–59.
15. Gardner H W, 'Lipoxygenase pathways in cereals', in *Advances in Cereal Science and Technology*, Y Pomeranz (ed.), Volume IX, American Association of Cereal Chemists, St Paul, MN, 1988, 161–215.

3

Inhibiting oxidation

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3.1 Critical points of oxidation

Oxidation is generally treated as the most frequently occurring form of lipid deterioration, which leads to the development of rancidity, off-flavour compounds, polymerisation, reversion, and other reactions causing reduction of shelf life and nutritive value of the food product. Lipids occur in almost all foodstuffs, and most of them (more than 90 %) are in the form of triacylglycerols, which are esters of fatty acids and glycerol. Two major components involved in lipid oxidation are unsaturated fatty acids and oxygen. Oxidative degradation of lipids may be initiated by active oxygen and related species (Table 3.1), which are more active than triplet oxygen molecules present in air,[1,2] as well as by exogenous agents (UV, ionisation radiation, heat).

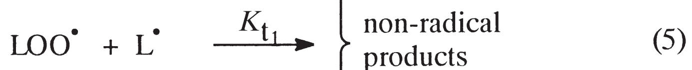
3.1.1 Types of lipid oxidation

One of the major concerns in food technology is the autoxidation of lipids which occurs autocatalytically through free-radical intermediates and is generally initiated by trace metals and peroxides present as ubiquitous impurities in food systems. Several factors such as ultraviolet or ionising radiation are known to bring about initiation.

Molecular oxygen behaves as a biradical by having two unpaired electrons (•O–O•) in the ground state and is said to be in a triplet state. The free-radical chain mechanism of autoxidation can be described by initiation, propagation and termination steps (Scheme 3.1). The direct reaction of unsaturated lipids with triplet oxygen ($^3O_2$) is spin forbidden.[3] The spin

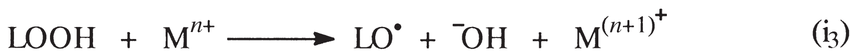
Table 3.1 Active oxygen and related species[1,2]

Radicals	Non-radicals
$O_2^{\bullet -}$ superoxide	H_2O_2 hydrogen peroxide
$HO^\bullet$ hydroxyl radical	$^1O_2$ singlet oxygen
$HO_2^\bullet$ hydroperoxyl radical	O_3 ozone
$L^\bullet$ lipid radical	LOOH lipid hydroperoxide
$LO_2^\bullet$ lipid peroxyl radical	Fe=O iron–oxygen complexes
$LO^\bullet$ lipid alkoxyl radical	HOCl hypochlorite
$NO_2^\bullet$ nitrogen dioxide	
$^\bullet NO$ nitric oxide	
$RS^\bullet$ thiyl radical	
$P^\bullet$ protein radical	

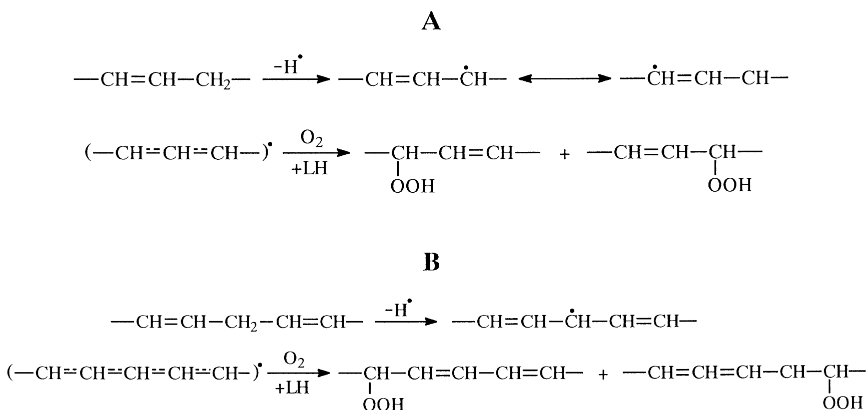


Scheme 3.1 Main reactions of non-inhibited lipid autoxidation during the initial stage of the process.

barrier between lipids and oxygen can be overcome by a number of initiating mechanisms, including singlet oxygen, partially reduced activated oxygen species (H_2O_2, $O_2^{\bullet -}$, HO•), cleavage of the hydroperoxides (in the absence and in the presence of redox metals M), described also as chain branching (Scheme 3.2). The alkoxy radical LO• can also abstract a hydrogen atom from a lipid molecule, in effect starting a new chain reaction and contributing further to the propagation phase. The rate of hydrogen abstraction by the alkoxy radical LO• is in the order of 10^4–10^6 times faster than by the peroxyl radicals LOO• generated in the propagation phase. At atmospheric oxygen pressure, reaction (2) is very rapid [>10^6-fold greater than



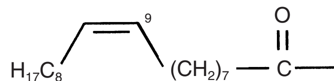
Scheme 3.2 Reactions of chain branching.

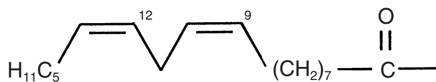


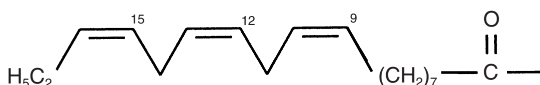
Scheme 3.3 Formation of hydroperoxides during autoxidation of molecules with one isolated double bond (**A**) and 'methylene-interrupted' double bonds (**B**).

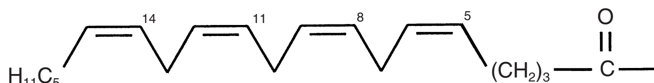
reaction (3)] which means that the concentrations of alkyl radicals $L^{\bullet}$ are very low compared to the concentrations of peroxyl radicals. This reaction (3) is the rate limiting one in the propagation phase.

During the initiation and propagation steps the hydrogen atom in the neighbourhood of the single double bond (allylic hydrogen) is abstracted, and the alkyl radical formed is stabilised by resonance (Scheme 3.3, **A**), the so-called 'α-methylene mechanism'.[4] In fatty acid molecules having 'methylene interrupted' double bonds, such as linoleate, linolenate and arachidonate (Scheme 3.4), only bis-allylic methylene groups are attacked. The hybrid pentadienyl radical that is formed reacts with oxygen at the end carbons to produce a mixture of two conjugated diene hydroperoxides (Scheme 3.3, **B**). Lower bond energies for bis-allylic and allylic hydrogens versus methylene hydrogens (75 and 88 *vs* 100 kcal mol^{-1}, respectively), as well as resonance stabilisation of the radical intermediate, contribute to the ease of abstraction from unsaturated fatty acids.[5]

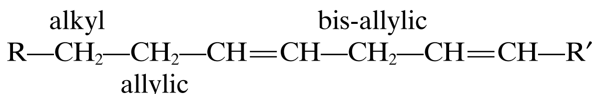








Scheme 3.4 Structures of unsaturated fatty acid esters.



The main products of lipid autoxidation during the initial stage of the process are the hydroperoxides, whereas epoxides, cyclic peroxides and polyperoxides are initially formed during autoxidation of other types of olefins.[6] The initially formed hydroperoxides are without smell and taste. The reaction products, including alcohols, carbonyl and carboxyl compounds give rise to the unpleasant taste and smell of oxidised food.[7]

At oxygen pressures greater than 100 mm Hg, the reaction of alkyl radical L• with oxygen is so rapid that the concentration of the resulting peroxy radicals LOO• is much higher than the concentration of L•. Applying stationary state conditions, by assuming the concentrations of L• and LOO• do not change,[3] the following expression can be derived for the rate of hydroperoxide formation:

$$d[LOOH]/dt = K_p(R_i/2K_t)^{1/2}[LH] \qquad [3.1]$$

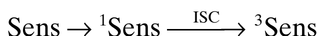
and the rate of oxidation is independent of oxygen pressure. The ratio of the rate constants $K_p/(2K_t)^{1/2}$ is referred as 'oxidisability' and used as a measure of the reactivity of unsaturated lipids to undergo autoxidation. When oxygen is limited and the partial oxygen pressure (p_{O_2}) is below 100 mm Hg, assuming a constant rate of initiation and amount of lipid substrate, the rate equation can be simplified as follows:

$$\mathrm{d[LOOH]}/\mathrm{d}t = A[p_{o_2}/(p_{o_2} + B)] \qquad [3.2]$$

where A and B are constants and the rate becomes oxygen dependent.[3] The autoxidation process can be retarded by chain-breaking inhibitors, as well as by addition of preventive antioxidants (see Section 3.2.2).

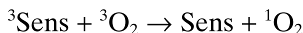
In another type of oxidation, photosensitised oxidation, the active oxygen species is the singlet oxygen, which may be formed by chemical and enzymatic methods, as well as by photosensitisation.[8,9]

In the presence of a sensitiser (Sens), such as chlorophylls, porphyrins, bilirubin, riboflavin, pheophytins,[10] and under light, singlet oxygen can be generated according to the following scheme: Sens is excited to its first excited state (^{1}Sens) and then undergoes intersystem crossing (ISC) to a metastable triplet state (^{3}Sens):



$$\mathrm{Sens} \rightarrow {}^1\mathrm{Sens} \xrightarrow{\mathrm{ISC}} {}^3\mathrm{Sens}$$

Triplet state ^{3}Sens can initiate a reaction with ground state triplet oxygen $^3O_2$ to form singlet oxygen $^1O_2$:



$$^3\mathrm{Sens} + {}^3\mathrm{O}_2 \rightarrow \mathrm{Sens} + {}^1\mathrm{O}_2$$

The photosensitised oxidation is not autocatalytic. In contrast to autoxidation, it is not inhibited by chain-breaking antioxidants, but by singlet oxygen quenchers, e.g. β-carotene.[11,12] The structures of the products formed are a result of the direct reaction of the dienophilic singlet oxygen $^1O_2$ with the double bonds of the unsaturated fatty acids,[13,14] the so-called 'ene addition' mechanism. Oxygen is thus inserted at the carbon atoms of the double bond, which is shifted to yield an allylic hydroperoxide in *trans*-configuration.[15]

In contrast to autoxidation, the hydroperoxides formed during singlet oxygen oxidation of linoleate may be unconjugated.[14,16,17] The rate of photosensitised oxidation is much higher than that of autoxidation, e.g. linoleate reacts with singlet oxygen about 1450 times faster than with the triplet oxygen.[14] Various enzymes, mainly lipoxygenases, catalyse the oxidation of linoleic, linolenic and related fatty acids.[18] These enzymes (recognised as catalysts of lipid oxidation in plant tissues) contain an iron atom in their active centre and are activated by hydroperoxides.[3] Enzymes such as cytochrom b_5 accelerate lipid oxidation in raw meat and seafoods.[19,20] Milk globule membranes contain a flavine enzyme, xanthine oxidase, that generates superoxide radicals $O_2\cdot^-$.

3.1.2 Relative rate of autoxidation of fatty acids

The influence of the different type and degree of unsaturation on the oxidisability of lipids was clarified after intensive study on the oxidation of various fatty acid methyl or ethyl esters, as well as of their mixtures, under different conditions. The following relative rates of autoxidation were determined: oleate : linoleate : linolenate = 1 : 12 : 25,[21] stearate : oleate : linoleate : linolenate = 1 : 11 : 114 : 179,[22] stearate : oleate : linoleate = 1 : 100 : 1000.[23] Holman and Elmer[24] have found that linolenate was 2.4 times more active than linoleate, and arachidonate was two times more reactive than linolenate.

The oxidisability of linoleate, linolenate and arachidonate, determined at 37 °C, were 0.020, 0.045 and 0.058 $M^{-0.5} s^{-0.5}$, respectively.[25] Minor amounts of linoleate in oleate increased the oxidation rate considerably,[21,26,27] and this has been proved to be due to the initiating role of linoleate in the process.[28] It has also been pointed out that the oxidisability of the lipids depended not only on the degree of their unsaturation, but also on the intramolecular arrangement of the double bonds:[29] mixtures of linoleate and stearate oxidised much faster than oleate that has the same degree of unsaturation (the same iodine value).[22]

The results obtained from the autoxidation of fatty acid methyl or ethyl esters and their mixtures, however, cannot be applied directly to the triacylglycerols (TG) with the same fatty acid composition. It has been established that the position of more easily oxidised fatty acid in the glyceride molecule influenced the rate of the process,[30] and the concentration of unsaturated fatty acids in the 2-position stabilised the fat towards autoxidation. Numerous authors investigated the role of the triacylglycerol structure and position of the fatty acids in lipid oxidation.[31–35]

It has been also reported that the oxidation rate of trilinolein was one order of magnitude smaller than the rate of methyl linoleate in a dry microcrystalline system at 37 °C.[36] The same effect was observed when comparing bulk phase autoxidation kinetics of methyl and triacylglyceryl esters of olive and sunflower oils at different temperatures (60–90 °C): triacylglycerols oxidised more slowly than methyl esters.[37] Obviously, the chain reaction propagates more rapidly in the mobile single ester than in the larger triacylglycerol.[36,37] Free fatty acids have been shown to oxidise faster than their triacylglycerols,[36,38] and faster than ethyl[39] and methyl[40] esters.

3.1.3 Factors affecting the oxidation rate

The extent to which oxidation of fatty acids and their esters occur in food depends on the chemical structure of the fatty acids (Section 3.1.2) and minor constituents present in the oxidising system, e.g. on the substrate factors, as well as on the conditions of food processing and storage.[41] Oxygen pressure, surface area with oxygen, temperature and irradiation are included in the physical factors.

Basic to any discussion of oxidation is the concentration of oxygen in the

affected system, which depends on the oxygen pressure (Section 3.1.1). If the surface area with the oxidising agent is 'small', even at sufficiently high oxygen pressure the diffusion rate of oxygen alone will be significant in starting oxidation. In this case one can speak about a diffusion controlled oxidation. Performing the process in a kinetic regime (in thin layers, or by blowing oxygen or air through the sample) will ensure a maximum rate for the process.

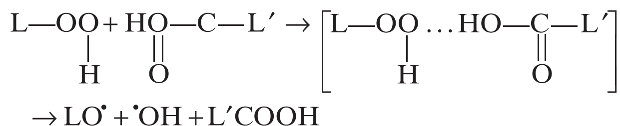
The mechanism and kinetics of bulk phase autoxidation are different from those of the oxidation in the monomolecular layers.[42–45] The oxidation rate of linoleate in the monomolecular layer, e.g. on silica gel surface, is much higher,[42,43,45] and the activation energy of the process is lower[45] than in bulk phase oxidation. The oxidation in bulk phase has an autocatalytic character whereas in the monomolecular layer on silica gel it proceeds with a continuously decreasing rate.[45]

Increasing the temperature is undesirable because of its accelerating effect on lipid oxidation and in fact on every stage of the process. That is why storage at low temperatures may be recommended. Nevertheless, there is a danger that when water freezes, all water present becomes frozen so that the food material becomes dry under these conditions, the protective layer of hydrated proteins is damaged and the lipid fraction leaks from the natural emulsions or liposomes, so that lipids become exposed to air oxygen.[18]

Irradiation increases the initiation rate (formation of free radicals), which is due to the high energy of the light quanta, α-, β-, γ- and X-rays. The shorter the wavelength, the higher the energy and the more detrimental the irradiation effect. The initiation rate R_i is directly proportional to the irradiation intensity, I, and the autoxidation rate W is proportional to the square root of I, e.g. $W \approx I^{1/2}$.

Numerous microcomponents may influence the rate and products of lipid oxidation.[46,47] The primary oxidation products, the hydroperoxides, are chain initiators and their presence in the lipid system causes an acceleration of the process.[48]

The free fatty acids also exert a pro-oxidative effect.[40,46] This effect is due to a complex formation between hydroperoxides and carboxyl groups through a hydrogen bond which results in an accelerated decomposition of hydroperoxides into free radicals:[40,46]



$$\rightarrow LO^{\bullet} + {}^{\bullet}OH + L'COOH$$

A series of lipid hydroxy compounds such as higher fatty alcohols, sterols, mono- and diacylglycerols are components of natural lipids and may influence the rate of their oxidation.[49–53] The effect depends on the type of the lipid substrate and on the oxidation conditions. Franzke et al.[54] found

that mono- and diacylglycerols exercised no influence on the process, whereas other authors have observed a pronounced pro-oxidative effect.[49,55,56]

Yanishlieva and Kortenska[51] reported that the extent of the pro-oxidative effect of fatty alcohols depended on the type of lipid system, its hydroperoxide content, and the chain length and concentration of the alcohols. The pro-oxidative action of the lipid hydroxy compounds in non-inhibited lipid oxidation has been proved to be due to acceleration of hydroperoxide decomposition into free radicals.[57] The thermally oxidised lipid compounds also showed a pro-oxidant effect on both refined and purified soybean oils.[58]

Transient-valency metal ions catalyse the hydroperoxide decomposition (Scheme 3.2), producing free radicals which initiate further reaction chains.[41] Therefore, minute traces of copper and iron, and to a lesser degree manganese and cobalt, are important promotors of lipid oxidation.

The relationship between rates of lipid oxidation and water is complex. The amount of water, the water activity and the state of water in food along with other factors must be considered.[3,47,59,60] Pigments may strongly influence the rate of lipid oxidation.[41] Among them chlorophylls and pheophytins are photosensitisers of the process under light[10] (Section 3.1.1), whereas in the dark they show antioxidant activity.[61] Depending on the conditions, carotenoids may promote the oxidation of oils under light[62–65] or inhibit it.[66–69] The non-lipidic components present in foods, such as proteins, sugars and minerals, mostly in the presence of water, may also have a strong influence on the rate and mechanism of lipid oxidation.[70]

There is conflicting information concerning the effect of sugars on the autoxidation of fats,[47,71] depending on the type of the oxidising system. Sims et al.[71] established that the stability to oxidation of emulsions containing safflower oil increased by the addition of sugar and sugar alcohols.

The role of the inhibitors in lipid oxidation is discussed in Sections 3.2 and 3.3.

3.2 Inhibiting oxidation

3.2.1 Minimising the influence of the physical factors

Optimum oxidative stability can be achieved by minimising exposure of lipids and lipid-containing food products to air, light and higher temperatures during processing and storage. Theoretically, the most elegant way of preserving fatty foods from oxidative spoilage is to remove all oxygen from the food during manufacture and from the packaging container. Modern packaging material and equipment allows inert-gas vacuum packaging, but residual oxygen levels of less than 1 % are extremely difficult to obtain in a production environment.[72]

Liquid oils were traditionally packaged in clear glass containers and

brown bottles were sometimes used to protect unstable oils from light oxidation. Glass bottles are now generally replaced by plastic containers. Polyvinyl chloride is preferred because it is superior to polyethylene, which is more permeable to oxygen.

3.2.2 Inhibiting autoxidation

The free radical chain process of autoxidation can be retarded by two categories of inhibitors: chain-breaking inhibitors (or antioxidants) and preventive inhibitors.

The chain-breaking antioxidants AH scavenge the free radicals (LOO•, LO•) interupting the propagation step [reactions (7) and (7′) in Scheme 3.5] and forming an antioxidant radical A• of such a low reactivity that no further reaction with lipids can occur.

$$LOO^{\bullet} + AH \xrightarrow{K_{inh}} LOOH + A^{\bullet} \qquad (7)$$

$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet} \qquad (7')$$

Radical scavengers usually donate one electron to the unpaired electron of the free radical and thus reduce it. Polyphenols are very active in this respect and the radical-scavenging activities of gallates, nordihydroguairetic

$$LOO^{\bullet} + AH \longrightarrow LOOH + A^{\bullet} \qquad (7)$$

$$LO^{\bullet} + AH \longrightarrow LOH + A^{\bullet} \qquad (7')$$

$$A^{\bullet} + LOOH \longrightarrow AH + LOO^{\bullet} \qquad (-7)$$

$$A^{\bullet} + LOO^{\bullet} \longrightarrow AOOL \qquad (8)$$

$$A^{\bullet} + A^{\bullet} \longrightarrow \text{non-radical products} \qquad (9)$$

$$A^{\bullet} + LH \longrightarrow AH + L^{\bullet} \qquad (10)$$

$$AH + LOOH \longrightarrow A^{\bullet} + LO^{\bullet} + H_2O \qquad (11)$$

$$AH + O_2 \longrightarrow A^{\bullet} + HO_2^{\bullet} \qquad (12)$$

$$AOOL \longrightarrow AO^{\bullet} + LO^{\bullet} \qquad (13)$$

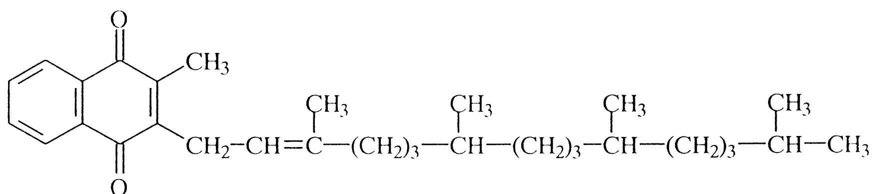
$$A^{\bullet} + O_2 \longrightarrow AOO^{\bullet} \qquad (14)$$

Scheme 3.5 Inhibited lipid autoxidation reactions.

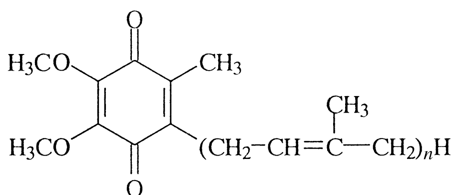
acid and flavonoids (Section 3.4) arise from this process. Aromatic amines inhibit the autoxidation via the same electron-transfer mechanism.

Quinones (vitamin K **1**, ubiquinone **2**, α-tocopheryl quinone **3**) are also chain-breaking inhibitors (or antioxidants) of autoxidation[73] acting as electron-acceptor antioxidants by competing with oxygen for alkyl radicals. This competitive reaction would only become important at low oxygen pressures (e.g. elevated temperatures), because alkyl radicals react extremely rapidly with oxygen under atmospheric conditions.

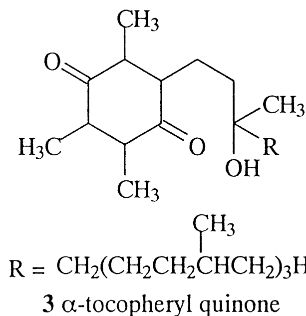
The preventive inhibitors deactivate the active species and possible precursors of free radicals and thereby suppress the generation of free radicals and reduce the rate of oxidation. Peroxide decomposers such as thioethers, methionine and thiodipropionic acid and its esters prevent the formation of free radicals for initiation of new chain reactions. The specific compounds which chelate metal ions and reduce the rate of reactions (i_3) and (i_4), as depicted in Scheme 3.2, are also considered as preventive antioxidants. For example, transferrin and albumin bind iron and copper. Ceruloplasmin oxidises ferrous ion, Fe^{2+}, to the less reactive ferric ion, Fe^{3+}. Glutathione



1 vitamin K



2 ubiquinone



3 α-tocopheryl quinone

peroxidase and catalase decompose hydroperoxides and hydrogen peroxide respectively without generation of free radicals and thereby reduce the generation of free radicals. Catechins and theaflavins possess an excellent superoxide radical-scavenging activity.[74] Singlet oxygen quenchers such as β-carotene also function as preventive inhibitors.

3.2.3 Inhibiting (quenching) photosensitised oxidation

The physical transfer of energy from the primary excited molecule to the quencher results in energy dissipation by light emission or as heat and thus avoids single electron transfer reaction of the primarily excited molecule. Tocopherol and carotenoids are physical quenchers of the excited states of pigment molecules, as well as of singlet oxygen; it is only after every 20th interaction with an excited-state molecule that a chemical change, i.e. an oxidation of the quencher, may statistically occur.[75]

3.2.4 Inhibiting and inactivation of enzymes

Flavonoids,[76–78] phenolic acids and gallates[78] (see Section 3.4.2) have been shown to inhibit the lipoxygenase. Theaflavin monogallate B and theaflavin digallate (see Section 10.42 [49] and [50]) appeared also to be active in inhibiting soybean lipoxygenase.[79] Lipoxygenases can be thermally inactivated above 60 °C with a resulting improvement in the shelf-life of foods.[80] However, heating also increases non-enzymatic oxidation and thus may exceed the oxidation due to lipoxygenase.

3.3 Types of inhibitors

All substances that protect foods against autoxidation should be called inhibitors of oxidation, and only substances that inhibit oxidation by reaction with free radicals should be called antioxidants.[18] The preventive inhibitors acting in the first defence line suppress the formation of free radicals and active oxygen species, and the radical scavenging antioxidants are responsible in the second defence line and inhibit chain initiation and/or break the chain propagation.[2]

3.3.1 Chain-breaking antioxidants

The most widely used inhibitors in foods are able to compete with the substrate for the chain-carrying species normally present in highest concentration in the system, the peroxyl radicals LOO•; reaction (7) in Scheme 3.5. The efficient inhibitors are well known to terminate free-radical chain oxidation by trapping two peroxyl radicals according to reactions (7) and (8).

$$LOO\cdot + A\cdot \rightarrow A{-}OOL(\text{non-radical combination products}) \quad (8)$$

The stoichiometric inhibition factor n (the number of kinetic chains broken per molecule of antioxidant) is normally two or fewer.[73] A characteristic action of antioxidants of this type is that, at least in *in vitro* reactions, they produce a lag period, the so-called induction period IP, which usually is proportional in duration (or 'length') to their concentration, and which continues until about 90 % of the antioxidant has been destroyed. During this lag period, lipid peroxidation proceeds at a very low rate, but at the end, oxidation continues at a rate equal to that of the unprotected lipid, or even greater.

Phenolics are not active antioxidants unless substitution at either the *ortho*- or *para*-position has increased the electron density at the hydroxy group and lowered the oxygen–hydrogen bond energy, in effect increasing the reactivity towards the lipid free radicals. Substitution in phenolic compounds at the *meta*-position has a rather limited effect. Steric and electronic effects are responsible for the antioxidant activities and stoichiometric factors of the chain-breaking phenolic antioxidants.[81] Satisfactory inhibition is obtained only if the concentration of antioxidant becomes higher than a critical value, e.g. 0.003 %.[41] For elucidation of the hydrogen abstraction mechanism of phenolic antioxidants in the chain process of autoxidation an *ab initio* molecular orbital theory has been applied.[82]

The effectiveness of an antioxidant is estimated on the basis of the IP, usually determined (in time units) by the method of tangents to two parts of the kinetic curve.[83,84] The effectiveness represents the possibility of blocking the radical chain process by interaction with peroxyl radicals, as in reaction (7) of Scheme 3.5.

From a kinetic point of view, the antioxidant has an inhibiting effect in the lipid system when during the IP the following inequality is observed:[85]

$$nK_{\text{inh}}[\text{AH}] >> (K_{\text{t}}R_{\text{i}})^{0.5} \quad [3.3]$$

The end of the IP is characterised by the transition from a quasistationary state to an autocatalytic oxidation regime. During the IP the inhibitor exhibits its activity. The transition corresponds in practice to the time of inhibitor consumption. The introduction of an inhibitor into the oxidising system leads to a change in the mechanism of the process (Schemes 3.1 and 3.5), and as a result, in the process kinetics. The stabilising effect of the antioxidant depends on the participation of its molecule and radicals in a series of reactions, presented in Scheme 3.5.[86,87]

Chimi et al.[88] reported that for sterically hindered phenols, the rates of reactions (8) and (9) (Scheme 3.5), which produce non-radical products, exceed the rates of reactions (–7) and (10), which produce free radicals, resulting in an overall inhibition of lipid oxidation. Lack of hindrance favours reactions (–7) and (10), which produce free radicals thus decreasing the overall antioxidant activity of the phenol.

Irrespective of the fact that the reactions where the inhibitor moieties participate can be many in number, the mechanism of the process is determined only by some of them. Depending on the structure of the antioxidant, on the oxidising substrate, and on the oxidation conditions, different side reactions can play the main role in the process.[87,89–97]

The oxidation rate during the IP, i.e. the rate of inhibited oxidation (W_{inh}), is another kinetic parameter characterising the antioxidant action. In a kinetic regime of oxidation (sufficiently high oxygen concentration) W_{inh} can be given by the expression

$$W_{inh} = K_p [LH] R_i / K_{inh} n[AH] \quad [3.4]$$

where K_p is the propagation rate constant of the chain reaction, K_{inh} is the rate constant of inhibition, R_i is the rate of chain initiation, and n is the stoichiometric factor. W_{inh} depends on the possibility of the inhibitor moieties participating in the reactions of chain initiation (11), (12), (13), and propagation (-7), (10), (14) (Scheme 3.5). W_{inh} characterises the strength of the antioxidant.

The participation of an antioxidant in the side reactions of chain initiation and propagation may also result in decreasing its effectiveness. For example, peroxydienones A (Scheme 3.6), resulting from reaction (8), limit the usefulness of butylated hydroxytoluene (BHT) at high temperature or under UV light, because they produce new free radicals that continue the kinetic chain reaction.

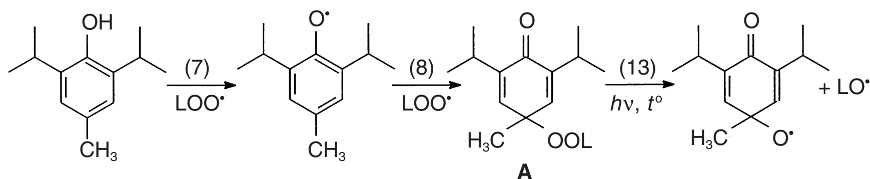
To compare the action of various antioxidants in different lipid systems and under different oxidation conditions, the relative parameters stabilisation factor F, and oxidation rate ratio ORR are used:

$$F = IP_{inh} / IP_o \quad [3.5]$$

where IP_{inh} is the induction period in the presence of an inhibitor, and IP_o is the induction period of the non-inhibited system.

$$ORR = W_{inh} / W_o \quad [3.6]$$

where W_{inh} is the oxidation rate in the presence of an inhibitor, and W_o is the rate of non-inhibited oxidation. F is a measure of the effectiveness and ORR is an inverse measure of the strength of the antioxidant. The lower



Scheme 3.6 Reaction products from a hindered phenol (BHT) during autoxidation.

the ORR, the stronger the inhibitor. When the ORR is larger than one then the oxidation proceeds faster in the presence of an inhibitor than in its absence, which, for example is observed at high tocopherol concentrations.[89]

Taking into account the complicated changes in the kinetic parameters of inhibited oxidation and the fact that the estimation of the antioxidative effect on the basis of IP or on the process rate may lead in many cases to different results, Yanishlieva and Marinova[87] proposed a general kinetic parameter, antioxidant activity A. This parameter, A unifies the effectiveness of an inhibitor in termination of the autoxidation chain, on the one hand, and its ability to decrease the oxidation rate during the IP, on the other:

$$A = F/ORR \quad [3.7]$$

In Table 3.2 the activity A of some natural antioxidants during autoxidation of triacylglycerols of lard (TGL), triacylglycerols of olive oil (TGOO) and triacylglycerols of sunflower oil (TGSO) at different temperatures are presented.

3.3.2 Preventive inhibitors

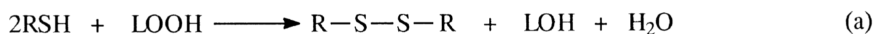
The hydroperoxide decomposers inhibit oxidation by induced decomposition of hydroperoxides by forming either stable alcohols or inactive products by non-radical processes, e.g. by reduction or hydrogen donating.

Elemental sulphur, thiols, sulphides and disulphides are active inhibitors of autoxidation. The last two compounds are more active than polysulphides.[41] Thiols, such as cystein and glutathione, are oxidised into disulphides [Scheme 3.7, reaction (a)]. Sulphides, such as methionine and 3,3′-thiodipropionic acid, are oxidised into sulphoxides [Scheme 3.7, reaction (b)]. The disulphides may be further oxidised into sulphinic acid [Scheme 3.7, reaction (c)], and sulphoxides may react with other hydroperoxide molecules resulting in the respective sulphones [Scheme 3.7, reaction (d)]. Onion and garlic contain sulphur compounds with hydroperoxide-decomposing activity.[18] Recently, Kim et al.[98] have investigated the stabilising effect of allicin, diallyl disulphide, and diallyl trisulphide, isolated from garlic, in lard autoxidation. Hydroperoxides may react with free amine groups of protein [Scheme 3.7, reaction (e)], resulting in imine formation by subsequent dehydration.

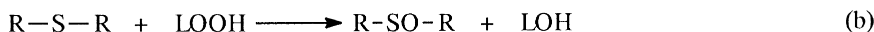
Selenium and its compounds can also destroy peroxides by changing them into inactive products. Selenium compounds are essential micronutrients and possess an important biological activity.[99] This is often discussed in relation to their antioxidant properties in lipid oxidation in membranes *in vitro* and *in vivo*.[100] They are powerful inhibitors, significantly decreasing the concentration of lipid peroxides and increasing the oxidation lag time.[101,102] Some selenium-containing compounds display an interesting

Table 3.2 Activity *A* of some natural antioxidants during autoxidation of triacylglycerols of lard (TGL), triacylglycerols of olive oil (TGOO), and triacylglycerols of sunflower oil (TGSO) at different temperatures

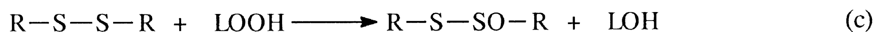
Antioxidant	Lipid system	Antioxidant concentration, $M \times 10^4$	Temperature, °C	*A*	Ref
Ferulic acid	TGL	10.3	100	5.2	90
Caffeic acid	TGL	11.1	100	10350	90
Ferulic acid	TGOO	10.3	100	20	94
Caffeic acid	TGOO	11.1	100	4867	94
Ferulic acid	TGSO	10.3	100	4.3	93
Caffeic acid	TGSO	11.1	100	448	93
α-Tocopherol	TGL	2.33	22	24.6	95
α-Tocopherol	TGL	2.33	90	221.4	95
α-Tocopherol	TGSO	2.33	22	6.9	96
α-Tocopherol	TGSO	2.33	90	167.5	96
Esculetin	TGL	5.6	100	324	91
Fraxetin	TGL	4.8	100	764	91
Esculetin	TGSO	5.6	100	97	91
Fraxetin	TGSO	4.8	100	88	91
Quercetin	TGL	4.44	22	86.7	95
Morin	TGL	4.44	22	84.2	95
Quercetin	TGL	4.44	90	22.1	95
Morin	TGL	4.44	90	195.7	95
Quercetin	TGSO	4.44	22	21.9	96
Morin	TGSO	4.44	22	14.4	96
Quercetin	TGSO	4.44	90	310	96
Morin	TGSO	4.44	90	48.6	96
Thymol	TGL	13.3	22	7.1	97
Carvacrol	TGL	13.3	22	5.5	97
Thymol	TGSO	13.3	22	75.0	97
Carvacrol	TGSO	13.3	22	2.8	97



$$2RSH + LOOH \longrightarrow R{-}S{-}S{-}R + LOH + H_2O \quad (a)$$



$$R{-}S{-}R + LOOH \longrightarrow R{-}SO{-}R + LOH \quad (b)$$



$$R{-}S{-}S{-}R + LOOH \longrightarrow R{-}S{-}SO{-}R + LOH \quad (c)$$

$$R{-}SO{-}R + LOOH \longrightarrow R{-}SO_2{-}R + LOH \quad (d)$$

$$R{-}NH_2 + LOOH \longrightarrow R{-}N(OH)L + H_2O \quad (e)$$

Scheme 3.7 Deactivation of lipid hydroperoxides by sulphur and nitrogen compounds.

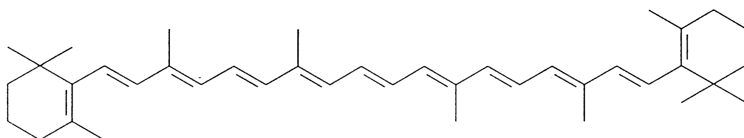
antioxidant capacity because they catalyse the disproportion of the superoxide ion.[103] Selenium has more importance as a part of the antioxidative enzyme selenoglutathione oxidase, inactivating free radicals and other oxidants, particularly hydrogen peroxide.[18]

Phospholipids also decompose the hydroperoxides by a non-radical mechanism and thus have a positive influence on autoxidation retardation[104,105] and enhance the activity of antioxidants in lipids.[106,107] Phospholipids improve the oxidative stability of edible oils both by decomposing hydroperoxides via a non-radical pathway and chelating traces of heavy metals.[108]

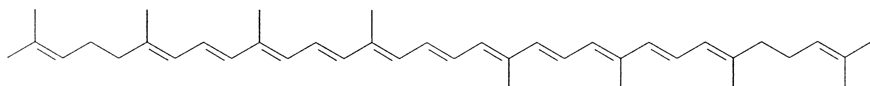
Metal chelating agents may have a dramatic effect on increasing the oxidation stability through blocking the pro-oxidant metal ions, and thus limiting the formation of chain initiators by preventing metal-assisted homolysis of hydroperoxides [Scheme 3.2, reactions (i_3) and (i_4)]. Many metal chelating substances are present in foods, especially in plant materials. The salts of phytic acid, phospholipids, and oxalates are the most common representatives of this group. Phosphoric, citric, tartaric, malic and ascorbic acids also possess pronounced chelating activities. Polyphosphates are added to inactivate iron, for example, in meat products.[109]

Amino acids and peptides are typical metal chelating agents.[41,110,111] Chen et al.[112] established that the characteristic amino acid sequences of peptides were required for them to manifest inhibiting effects. The antioxidant activity of histidine-containing peptides is thought to be related to their metal-chelating ability, as well as to lipid-radical trapping potential of the imidazole ring.[113,114] The antioxidant role of histidine-containing dipeptides carnosine and anserine seems to involve not only singlet oxygen and free-radical scavenging, but also metal chelation.[115] The metal chelating characteristics of natural phenolics, such as flavonoids, are also an important factor in their antioxidant activities.[116–119] Water-soluble metal chelators, such as ethylenediamine tetraacetic acid (EDTA) and its salts, phosphates and ascorbic acid (Section 3.3.3), are effective in improving the oxidative stability of aqueous food emulsion systems, e.g. salad dressing, mayonnaise and margarine.[3]

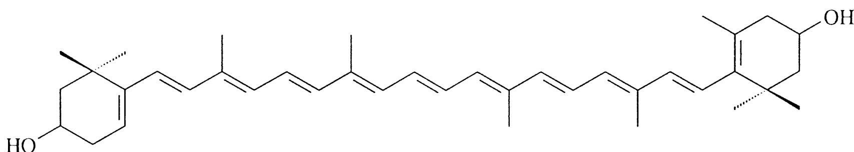
The discovery by Foote and Denny[8] that carotenoids, such as β-carotene **4**, lycopene **5**, zeaxanthin **6**, lutein **7** and canthaxanthin **8**, could quench the singlet oxygen $^1O_2$ was an important advance in understanding the effectiveness of carotenoid pigments in preventing damage in photobiological systems.[120] The singlet oxygen quenching can involve either a route whereby the quencher Q, e.g. a carotenoid Car, undergoes no ultimate chemical change (physical quenching), or another route, which involves a chemical reaction resulting in new products.[121] The deactivation of $^1O_2$ by carotenoids results predominantly from physical quenching, a process involving transfer of excited energy from $^1O_2$ to the carotenoids and resulting in the formation of ground state oxygen $^3O_2$ and triplet excited carotenoid $^3Car^*$:[122]



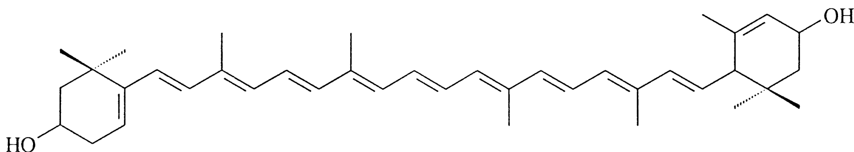
4 β-carotene



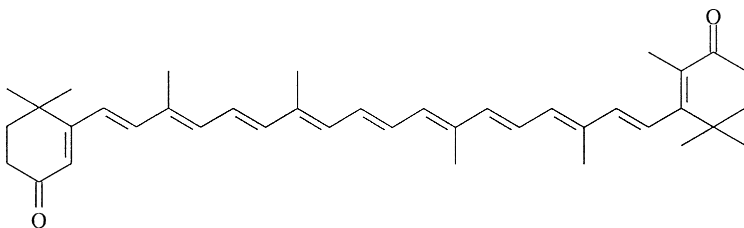
5 lycopene



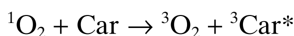
6 zeaxanthin



7 lutein

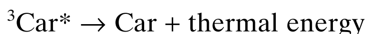


8 canthaxanthin



$$^1O_2 + Car \rightarrow {^3O_2} + {^3Car^*}$$

The energy is dissipated through rotational and vibrational interactions between $^3Car^*$ and the solvent to recover the ground state of the carotenoid:[122]



$$^3Car^* \rightarrow Car + \text{thermal energy}$$

One molecule of β-carotene is estimated to quench up to 1000 molecules of singlet oxygen.[120]

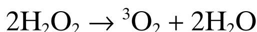
In a manner that does not involve hydrogen abstraction, carotenoids are postulated to scavenge peroxyl radicals through addition of the radical to the conjugated system such that the resulting carbon-centred radical is stabilised by resonance.[123] When oxygen concentrations are low, a second peroxyl radical is added to the carbon-centred radical to produce a non-

radical product.[124] At high oxygen pressures, however, carotenoids act as pro-oxidants.[125]

The inhibition of photosensitised oxidation by carotenoids is complicated because they are very susceptible to autoxidation, and are quickly destroyed during the free-radical oxidation process. To be effective in unsaturated lipids exposed to light irradiation, carotenoids must be protected by an antioxidant.[62,64,65,126,127] Without antioxidant protection, e.g. in pure triacylglycerols of rapeseed[64,65] and of sunflower[126,127] oils, they exert a pro-oxidative effect. Photosensitised oxidation can be also quenched by tocopherols[128,129] and flavonoid substances,[130] the rate of physical quenching being several orders of magnitude higher than the rates of chemical reactions.[130,131]

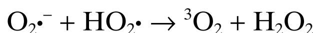
Enzymes have been evaluated as new types of natural antioxidants in some food applications.[3] They can be used beneficially to remove oxygen and reactive oxygen species and to reduce lipid hydroperoxides.

Catalase destroys H_2O_2 via disproportional reaction:



$$2H_2O_2 \rightarrow {}^3O_2 + 2H_2O$$

Superoxide dismutase (SOD) catalyses the dismutation of superoxide:



$$O_2{\bullet}^- + HO_2{\bullet} \rightarrow {}^3O_2 + H_2O_2$$

The suggestion that SOD should be more effective in conjunction with catalase in destroying the H_2O_2[59] was not confirmed by Lingnert[132] and Lingnert et al.[133]

The seleno enzyme glutathione peroxidase (GSH) catalyses the reduction of peroxides by reduced glutathione:

$$2GSH + LOOH \rightarrow LOH + H_2O + GSSG$$

Glucose oxidase coupled with catalase is the best known commercially available system to remove oxygen from food.[3] Glucose oxidase utilises oxygen by catalysing the oxidation of β-D-glucose to produce 2-δ-gluconolactone and hydrogen peroxide. The gluconolactone is spontaneously hydrolysed to D-gluconic acid and the hydrogen peroxide can be removed by catalase.

3.3.3 Synergism

The cooperative effects of the inhibitors during oxidation which results by their reinforcing each other is known as synergism. Usually, the synergistic action is represented by the parameter S:

$$S = IP_{1,2} - (IP_1 + IP_2) > 0 \qquad [3.8]$$

where $IP_{1,2}$ is the induction period in the presence of both inhibitors, and IP_1 and IP_2 are the induction periods in the presence of every inhibitor alone. The percent synergism is calculated as follows:[3]

$$\% \text{ synergism} = \frac{IP_{1,2} - (IP_1 + IP_2)}{IP_1 + IP_2} \times 100 \qquad [3.9]$$

Significant synergism is generally observed when chain-breaking antioxidants are used together with preventive inhibitors, e.g. hydroperoxide decomposers, metal chelators and singlet oxygen quenchers (Section 3.3.2). Most synergists act in several different ways.

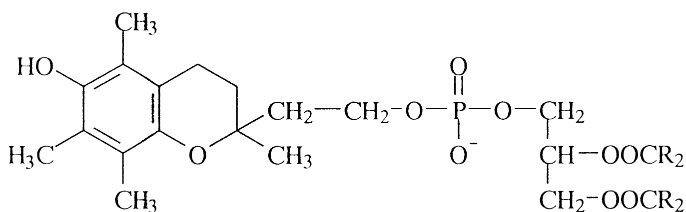
Phospholipids enhance the activity of tocopherols.[106,134–138] The mechanism of the synergistic effect between vitamin E and phospholipids is still the subject of argument, because it depends on the oxidation conditions and phospholipid classes:[139] decomposition of hydroperoxides,[104] regeneration of vitamin E from its radical,[138] or chelating of pro-oxidant metal ions.[140] Weng and Gordon[141] also established that phosphatidyl ethanolamine reduced α-tocopheryl quinone into α-tocopherol. Phospholipids are also synergists for flavonoid antioxidants.[142]

Koga and Terao[143] investigated the antioxidative properties of a novel phosphatidyl derivative of vitamin E, 1,2-diacyl-*sn*-glycero-3-phospho-2′-(hydroxyethyl)-2′,5′,7′,8′-tetramethyl-6′-hydroxychroman (PCh) **9**. They found that during lard autoxidation at 60 °C the IP with PCh at a level of 0.4 μmol kg^{-1} was longer than that with the mixture of vitamin E and phosphatidylcholine. Thus, it was demonstrated that PCh possessed a property of synergism of phospholipids and vitamin E, because their structures are included in its molecule.

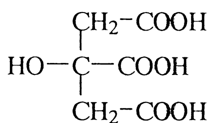
The synergistic effect of citric acid **10** is attributed to metal chelation.[3,144] Other polyvalent acids such as tartaric **11**, malic, gluconic, oxalic, succinic and hydroxyglutaric acids, as well as sodium triphosphate and pyrophosphate also possess synergistic properties similar to those of citric acid. Another chelator, phytic acid (inositol hexaphosphate), has been also reported to be a synergist in lipid oxidation.[145]

Ascorbic acid **12** can act as a synergist with tocopherols by regenerating or restoring their antioxidant properties.[146] Ascorbic acid and its esterified derivatives may also function as oxygen scavengers. Ascorbic acid has been demonstrated to be an effective radical scavenger of superoxide, hydrogen peroxide, hydrochlorite, hydroxyl radical, peroxyl radical, and singlet oxygen. Ascorbic acid can protect against lipid peroxidation by trapping the peroxyl radical in the aqueous phase before it can get into the lipid membrane of lipoproteins.[147] The lipophilic ascorbyl palmitate **13** has been proved not only to be synergist for α-tocopherol, but also to act as a radical trapping inhibitor.[148]

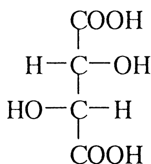
5-Aminosalicylic acid reveals an important cooperative effect with α-tocopherol during phosphatidyl choline peroxidation either affording an efficient protection to the antioxidant, when free radicals are generated in the aqueous site, or potentiating its activity when oxidation is initiated inside the lipid bilayer.[149] A combination of carotenoids, e.g. β-carotene, with tocopherols[150–153] and other phenolic antioxidants such as *tert*-butylated



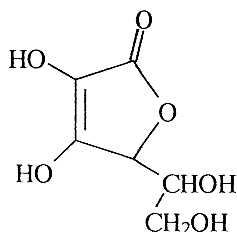
9 PCh (see the text)



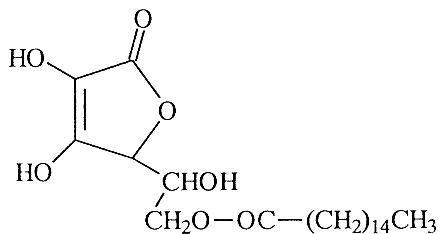
10 citric acid



11 tartaric acid



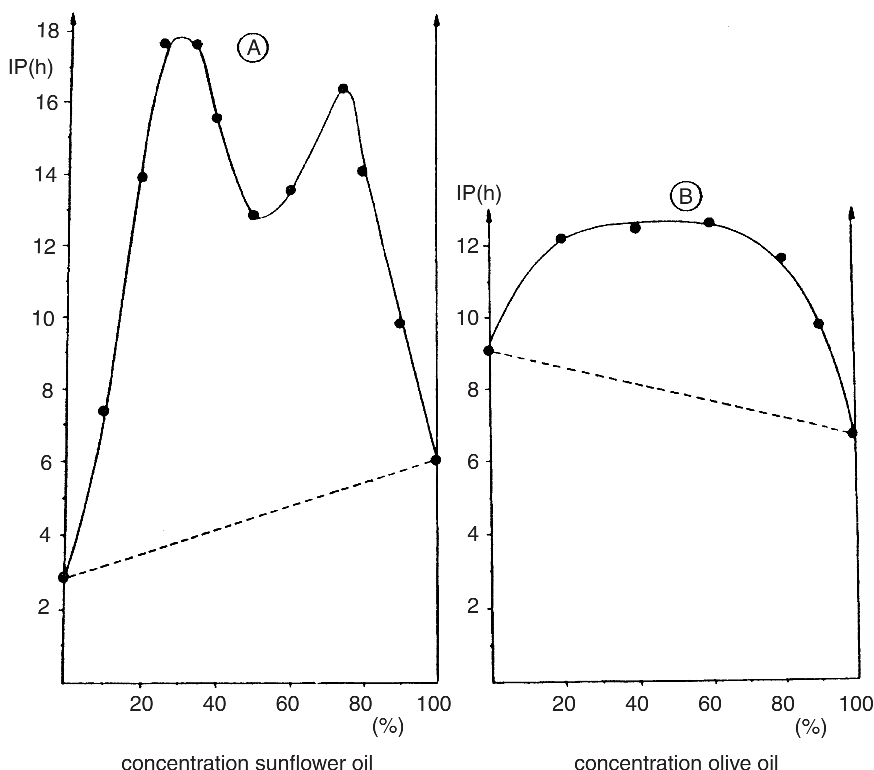
12 ascorbic acid



13 ascorbyl palmitate

hydroquinone (TBHQ)[154] and quercitrin[155] synergistically inhibited lipid peroxidation. In exploitation of the synergism between phenolic antioxidants and carotenoids the reaction of carotenoids with phenoxyl radicals is clearly of importance.[156] Mortensen and Skibsted[157] found that the rate of this reaction increased with the number of conjugated double bonds in the carotenoids and decreased in the presence of hydroxy and especially keto groups. A remarkable synergistic effect of carotenoids and vitamin C (ascorbic acid) is found in lard and in rapeseed oil.[158]

Strong synergistic effects have been observed as a result of mixing different types of lipid systems (different fatty acid and microcomponent composition), such as lard and sunflower oil or tallow and sunflower oil.[159] Synergism was also established in the systems olive oil–sunflower oil, lard–olive oil, and tallow–olive oil.[159] Figure 3.1 shows the dependence of the IP on the composition of the mixtures lard–sunflower oil and tallow–olive oil during oxidation at 100 °C.



3.1 Dependence of the IP (h) on the concentration (%) of sunflower oil in the mixture lard—sunflower oil (A) and on the concentration of olive oil in the mixture tallow—olive oil (B).[159] Oxidation temperature 100 °C (permission to reproduce granted by Verlag für chemische Industrie H. Ziolkowsky GmbH).

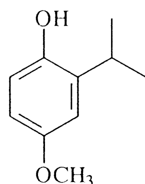
A negative synergism (or antagonism) is observed with the combination of BHT and propyl gallate,[160] and with the combination of ellagic acid and catechin.[161] Meyer et al.[161] proposed that the mechanism behind the antagonistic interaction is due to hydrogen bonding between carbonyls in ellagic acid and *o*-dihydroxyl groups in catechin.

3.4 Types of antioxidants

To prevent or retard the oxidative deterioration of foods, antioxidants have been widely used as additives in fats and oils, and in food processing.

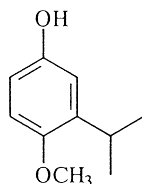
3.4.1 Synthetic antioxidants

Some of the more popular synthetic antioxidants used are phenolic compounds such as butylated hydroxyanisol (BHA) **14**, butylated hydroxy-



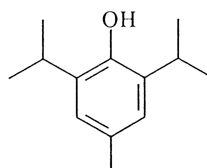
4-methoxy-2-*tert*-butyl phenol (2-BHA)

and

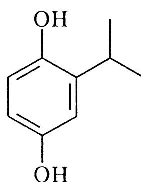


4-methoxy-3-*tert*-butyl phenol (3-BHA)

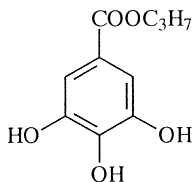
14 butylated hydroxyanisol (BHA)



15 2,6-di-*tert*-butyl-*p*-hydroxytoluene (BHT)



16 *tert* butyl hydroquinone (TBHQ)



17 propyl gallate (PG)

toluene (BHT) **15**, tertiary butylhydroquinone (TBHQ) **16**, and esters of gallic acid, e.g. propyl gallate (PG) **17**. Synthetic phenolic antioxidants are always substituted by alkyls to improve their solubility in fats and oils.[162] The four major synthetic antioxidants in use are subjected to a 'good manufacturing practice' limit of 0.02 % of the fat or oil content of the food.[163]

The most suitable antioxidant for vegetable oils is TBHQ. BHA and BHT are fairly stable to heat and are often used for stabilisation of fats in baked and fried products. Antioxidants that are heat stable have the property referred to as 'carry-through'. The disadvantages of gallates lie in their tendency to form dark participates with the iron ions and their heat sensitivity. Some antioxidants, such as BHA and BHT, are used in combination with resulting synergistic effects.[164,165] BHA is also synergistic with PG.[160]

The synthetic antioxidants have been very thoroughly tested for their toxicological behaviours, but some of them are coming, after a long period of use, under heavy pressure as new toxicologocal data impose some caution in their use.[166] In this context, natural products appear as healthier and safer than synthetic antioxidants.[167] Since about 1980 natural antioxidants have appeared as an alternative to synthetic antioxidants.

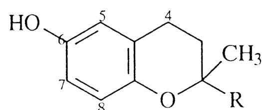
3.4.2 Natural antioxidants

The empirical use of natural compounds as antioxidants is very old. The popularity of smoking and spicing in the home for preservation of meat,

fish, cheese and other fat-rich foods may be due, at least partly, to recognition of the rancidity-retarding effect of these treatments. It is dangerous to try to define natural antioxidants, but generally the term alludes to substances which occur in and can be extracted from plant or animal tissues and those which may be formed as a consequence of cooking or processing plant or animal components for food.[163] Natural antioxidants are found in almost all plants, microorganisms, fungi, and even in animal tissues.[18] The majority of natural antioxidants are phenolic compounds and the most important groups of natural antioxidants are the tocopherols, flavonoids and phenolic acids.

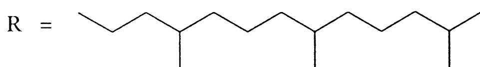
Tocopherols are the best known and most widely used antioxidants.[41] They can be classified as tocopherols (Toc) and tocotrienols (Toc-3) and within each of these two classes there are four isomers (α-, β-, γ- and δ-) making a total of eight tocopherol isomers **18**, **19**, **20**, **21**, **22**, **23**, **24**, **25**. They are present, at least in traces, in nearly all food materials. The most important antioxidant of this group is α-tocopherol **18**, which has lower antioxidant activity in edible oils than other tocopherols.

Tocopherols work as antioxidants by donating the hydrogen of the hydroxyl group to the lipid peroxyl radical. The radical formed from α-tocopherol is stabilised through delocalisation of the solitary electron over the aromatic ring structure (Scheme 3.8). This radical forms non-radical



Tocopherols

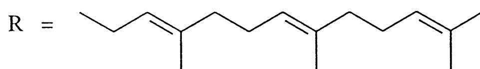
R =



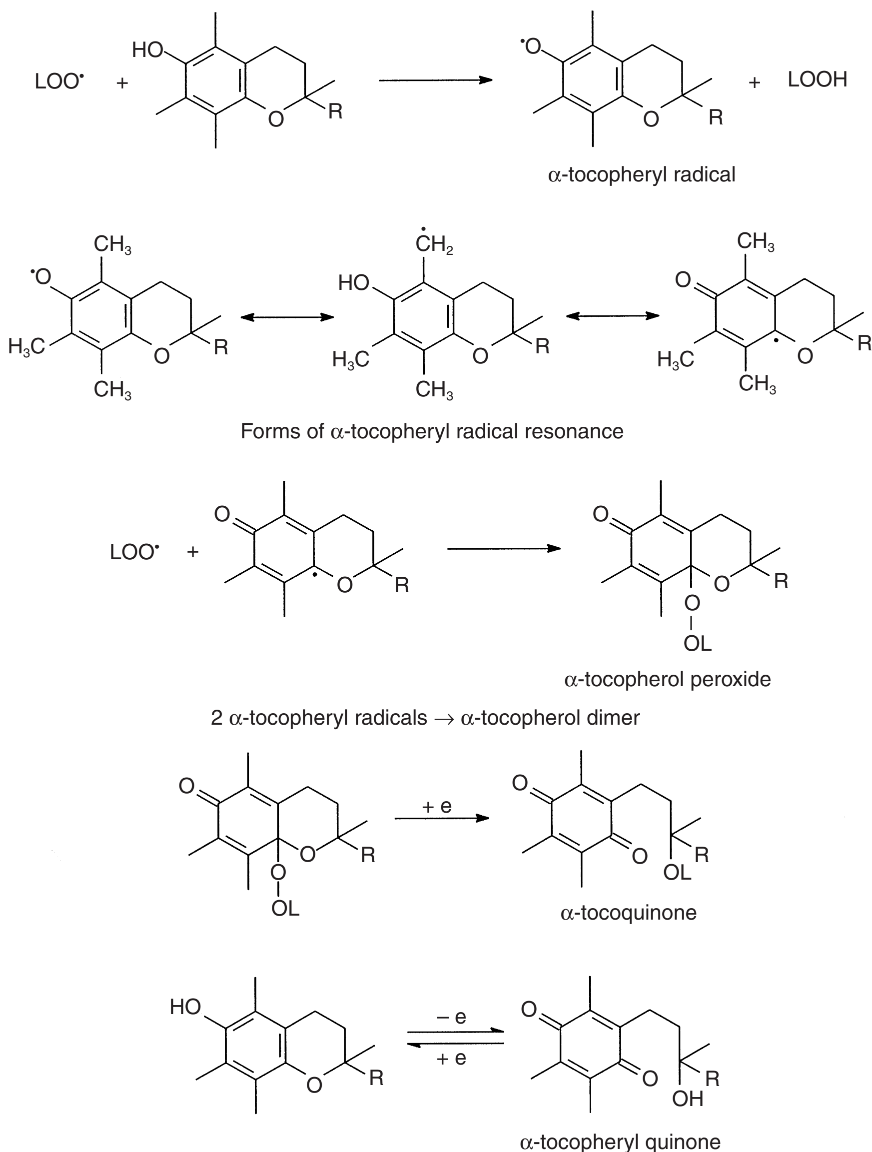
5, 7, 8 - Trimethyl **18** α-Tocopherol
5, 8 - Dimethyl **19** β-Tocopherol
7, 8 - Dimethyl **20** γ-Tocopherol
8 - Methyl **21** δ-Tocopherol

Tocotrienols

R =



5, 7, 8 - Trimethyl **22** α-Tocotrienol
5, 8 - Dimethyl **23** β- Tocotrienol
7, 8 - Dimethyl **24** γ- Tocotrienol
8 - Methyl **25** δ- Tocotrienol



Scheme 3.8 Mechanism of α-tocopherol action.

products, including stable peroxides, which can be reduced to tocoquinones and to tocopherol dimers. α-Tocopherol has also been associated with retarding the decomposition of hydroperoxides.[168]

The hydrogen-donating power of tocopherols in fats, oils and lipoproteins is in the order $\delta > \beta \cong \gamma > \alpha$.[169] It has been found that the antioxidant

strength of α-tocopherol **18** in butter oil triacylglycerols was less than that of γ-tocopherol **20**.[170]

The antioxidant power of tocopherols is strongly concentration dependent: α-tocopherol in rapeseed oil triacylglycerols was a more effective antioxidant than γ-tocopherol at low levels ($\leq 50\,\mu g\,g^{-1}$), and at high levels ($>100\,\mu g\,g^{-1}$) γ-tocopherol was more effective than α-tocopherol.[171] Analogical effects were observed with sunflower oil triacylglycerols.[172] It has been found that γ-tocopherol was more effective as an antipolymerisation inhibitor than α-tocopherol.[173]

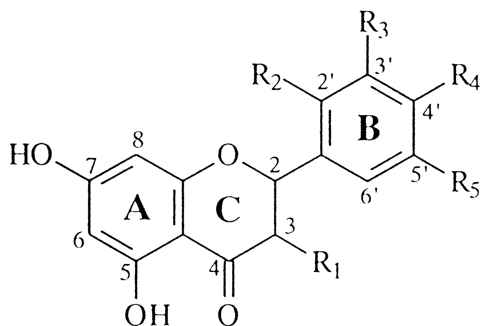
The antioxidant activity of tocopherols was established mainly in fats containing a small amount of antioxidants. At high concentrations a pro-oxidative effect can be observed. α-Tocopherol has been shown to have a pro-oxidative action at concentrations greater than 0.01 % during the early autoxidation stages in lard,[89] corn[174] and olive oil triacylglycerols.[175] The pro-oxidative effect of α-tocopherol was related to the reactions of tocopheroxyl radical with lipids,[176,177] e.g. reactions (–7) and (10) in Scheme 3.5.

Tocopherols are very stable with respect to heat and have an excellent carry-through effect, e.g. in crackers, pastry and potato chips.[178] It has been established[89] that α-tocopherol was more effective in increasing the oxidative stability (stabilisation factor *F*) and in reducing the relative rate of oxidation (ORR) as the temperature increased.

In foods, tocopherols act as relatively weak antioxidants; by reaction with free radicals they are converted into quinones, spirodimers and various other compounds,[128] as well as into copolymers with oxidised lipids.[18]

It has been also reported that α-Toc-3 possessed slightly higher antioxidative activity than that of α-Toc in lard[179] and in sunflower oil methyl esters.[180] In a heterogeneous system, a phospholipid membrane, the antioxidative activity of Toc-3 was greater than that of Toc when Toc and Toc-3 were distributed from water to the model membrane, while both were the same when the compounds were in the membrane from the beginning.[181] Tocopherols can also function as inhibitors of lipid oxidation by scavenging singlet oxygen molecules.[128,129,182] The mechanism was proposed to occur either by a quenching process or by an irreversible reaction.[182]

Flavonoids constitute a large group of naturally occurring plant phenolics. They are characterised by the carbon skeleton C_6–C_3–C_6. The basic structure of these compounds consists of two aromatic rings linked by a three-carbon aliphatic chain which normally has been condensed to form a pyran or, less commonly, a furan ring. Flavonoids, including flavones, flavonols, isoflavones, flavonones and chalcones occur in all types of higher plant tissues.[183,184] Flavones and flavonols are found in almost every plant, particularly in the leaves and petals, with flavonols occurring more frequently than flavones.[185] Some of the common flavonoids are apigenin **26**, chrysin **27**, luteolin **28**, datiscetin **29**, quercetin **30**, myricetin **31**, morin **32** and kaemferol **33**. Approximately 90 % of the flavonoids in plants occur as



	R_1	R_2	R_3	R_4	R_5
Flavones					
26 Apigenin	H	H	H	OH	H
27 Chrysin	H	H	H	H	H
28 Luteolin	H	H	OH	OH	H
Flavonols					
29 Datiscetin	OH	H	OH	OH	H
30 Quercetin	OH	H	OH	OH	H
31 Myricetin	OH	H	OH	OH	OH
32 Morin	OH	OH	H	OH	H
33 Kaemferol	OH	H	H	OH	H

glycosides.[186] Usually the aglucones are more active in bulk phase,[187,188] as well as in phospholipid bilayers oxidation.[189]

The ability of flavonoids to inhibit lipid oxidation is well documented, both for natural lipid products and for model lipids.[190–194] Flavonoids may act as antioxidants by scavenging radicals that include superoxide anions,[195,196] lipid peroxyl radicals,[197] and hydroxyl radicals.[198] Other mechanisms of action of selected flavonoids include singlet oxygen quenching,[130,199] metal chelation,[200,201] as well as lipoxygenases inhibition.[76–78,202] The glycosides are less effective as antioxidants than are the aglycones.[203] It is concluded that for maximal radical scavenging activity a flavonoid molecule needs to meet the following criteria: (i) 3′,4′-dihydroxy structure in the B-ring, (ii) 2,3-double bond in conjunction with a 4-oxo group in the C-ring, and (iii) presence of a 3-hydroxyl group in the C-ring and a 5-hydroxyl group in the A-ring.[204–208]

Flavonoids with free hydroxyl groups act as free-radical scavengers, and multiple hydroxyl groups, especially in the B-ring, enhance their antioxi-

dant activity.[209] The hydroxyls in ring B are the primary active sites in interrupting the oxidation chain. Quercetin was much more effective than kaemferol in retarding autoxidation of lard at 100 °C as described by Yanishlieva et al.,[210] and of methyl linoleate at 40 °C.[188] Myricetin better stabilised sunflower oil at 60 °C than quercetin,[211] and was a stronger antioxidant than quercetin in liposome oxidation at 30 °C.[212]

The relative reactivities, as well as the stoichiometric coefficients for a number of flavonoids, catechols and standard phenolic antioxidants have been determined by Roginsky et al.[213] The authors have analysed the kinetics of oxygen consumption in organic and micellar systems, with peroxidation initiated by lipid- and water-soluble initiators. The results obtained demonstrated that the flavonoids did not behave as classic phenolic antioxidants such as α-tocopherol, but showed only moderate chain-breaking activities.[213]

The antioxidant activity of flavonoids, such as anthocyanins, is greatly affected by the system used as a substrate and the conditions used to catalyse oxidation.[214]

The structure–activity relationship for the antioxidant activities of flavonoids continues to be discussed.[215–218]

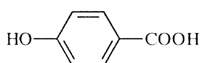
Phenolic acids, such as *p*-hydroxybenzoic **34**, 3,4-dihydroxybenzoic **35**, vanillic **36**, syringic **37**, *p*-coumaric **38**, caffeic **39**, ferulic **40**, sinapic **41**, chlorogenic **42**, and rosmarinic **43** acids are widely distributed in the plant kingdom. They usually exist as esters of organic acids or glycosides.[219–222]

The derivatives of cinnamic acid are more active antioxidants than the derivatives of benzoic acid.[90,92,223–225] Figure 3.2 illustrates, by way of example, the dependences of the stabilisation factor F and of the activity A of syringic and sinapic acids on their concentration during oxidation of lard at 100 °C.[90] It is to be seen that sinapic acid is a much more effective antioxidant than syringic acid, and the activity A of sinapic acid is one order of magnitude greater than that of syringic acid.

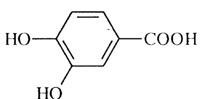
The position and the degree of hydroxylation are of primary importance in determining antioxidant activity.[90,92,223,224,226] Cuvelier et al.[223] established a relationship between the structure of many phenolic acids and their antioxidant activity. Monophenols were less efficient than polyphenols. The introduction of a second hydroxy group in the *ortho* or *para* position increased the antioxidative activity. The inhibiting effectiveness of monophenols was increased substantially by one or two methoxy substitutions. The combination of two acid phenols increased the efficiency, e.g. rosmarinic acid is a better antioxidant than caffeic acid. Esterification of caffeic acid by sugar moiety decreased its activity, e.g. chlorogenic acid was less effective than caffeic acid.[223,224]

Pekkarinen et al.[227] have examined the effect of selected hydroxybenzoic and hydroxycinnamic acids on both bulk and emulsified methyl linoleate oxidation in the dark at 40 °C. It has been established that specific interactions of the antioxidant with other compounds, for example, the emulsifier,

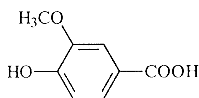
Benzoic acid derivatives



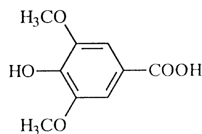
34 *p*-hydroxybenzoic acid



35 3,4-dihydroxy-benzoic acid

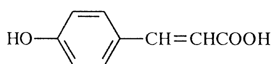


36 vanillic acid

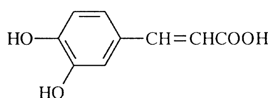


37 syringic acid

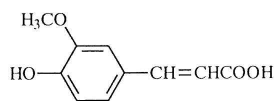
Cinnamic acid derivatives



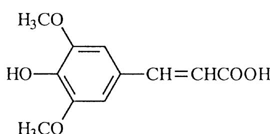
38 *p*-coumaric acid



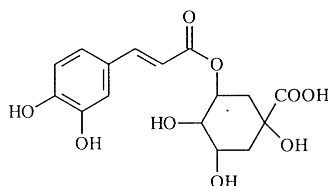
39 caffeic acid



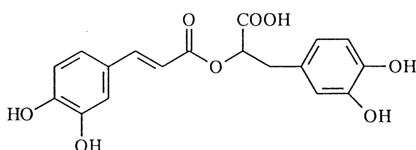
40 ferulic acid



41 sinapic acid



42 chlorogenic acid

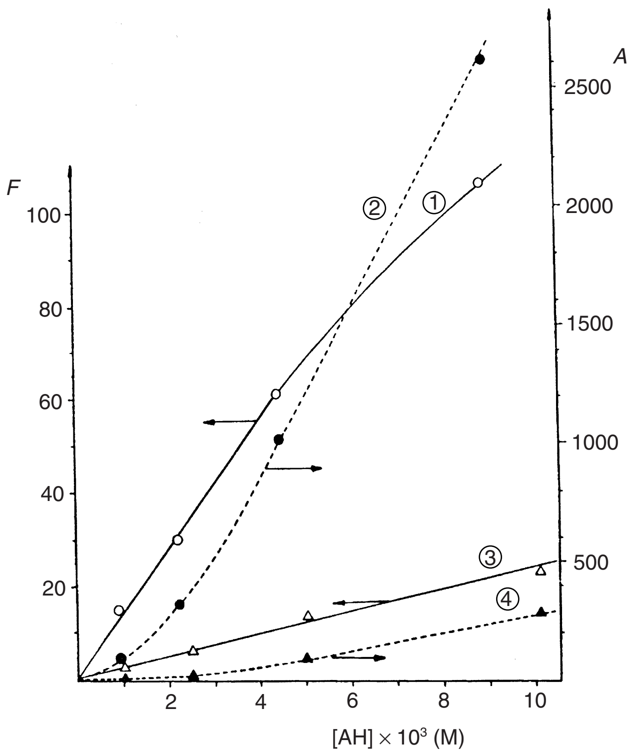


43 rosmarinic acid

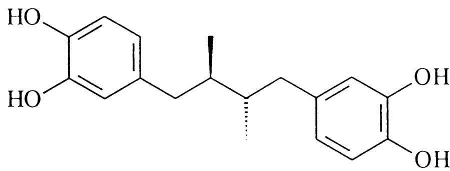
and intramolecular hydrogen bonds may play an important role in reducing the antioxidant activity.

The major constituent of the resinous exudate from creosote bush *Larrea divaricata* is nordihydroguaiaretic acid (NDGA) **44**, which was one of the earliest of the phenolic antioxidants permitted for stabilisation of edible fats and oils.[41]

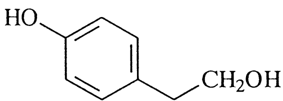
Tyrosol **45** and hydroxytyrosol **46** are among the most important phenolic substances in olive oil.[228,229] Papadopoulos and Boskou[230] studied the antioxidative effect of the polar fraction and individual phenolics present in virgin olive oil during bulk phase oxidation of refined olive oil. The



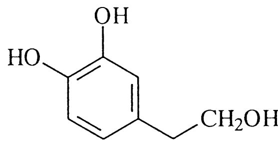
3.2 Dependences of the stabilisation factor *F* (1,3) and of the activity *A* (2,4) on the concentration of sinapic (1,2) and syringic (3,4) acids in lard autoxidation at 100 °C[90] (adapted).



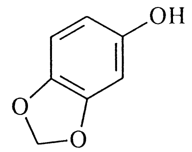
44 nordihydroguaiaretic acid (NDGA)



45 tyrosol



46 hydroxytyrosol



47 sesamol

authors found that hydroxytyrosol and caffeic acid had greater protection factors than BHT. Chimi et al.[88] established that in a micellar substrate, composed of linoleic acid, the antioxidant activity of the inhibitors at a level of 10^{-4} M increased in the order tyrosol < caffeic acid < hydroxytyrosol. At a concentration of 6×10^{-3} M these phenolics also scavenged hydroxyl radicals, with an efficiency which increased in the order tyrosol < hydroxytyrosol < caffeic acid. Sesame oil contains several natural antioxidants, such as sesamol **47**.[231]

Additional information on natural antioxidants it can be found in Chapters 8, 9 and 10.

3.5 Key influences on antioxidant activity

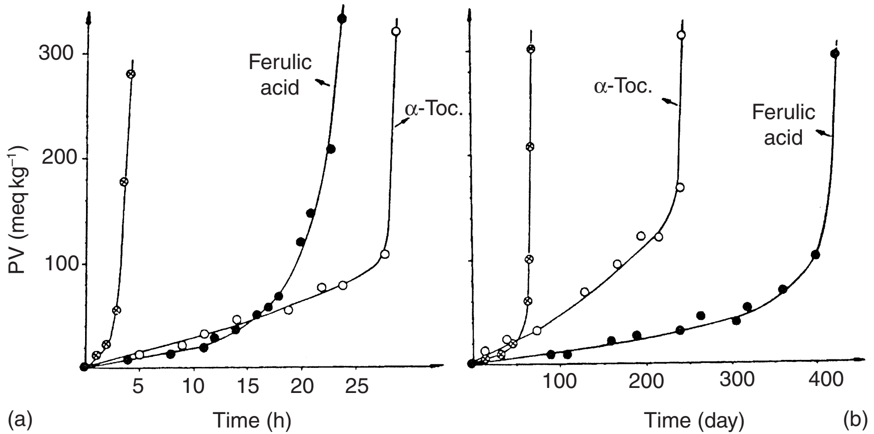
The activities of antioxidants depend not only on their structural features, for instance, on their chemical reactivities towards peroxyl and other active species, but also on many other factors, such as concentration, temperature, light, type of substrate, physical state of the system, as well as on the numerous microcomponents acting as pro-oxidants or synergists.[128]

3.5.1 Physical factors

A high oxygen pressure, a greater surface area with oxygen, heating or irradiation cause an acceleration of the chain initiation and propagation of the oxidation process (Section 3.1.3), and hence a decrease in the oxidation stability, or in the activity of the present or added antioxidant. The detrimental role of sunlight may be illustrated by the following results: the IP of sunflower oil during oxidation at ambient temperature under sunlight had the same IP as the oil oxidised at 80 °C in the dark.[232] This is why, to ensure a better shelf-life, foods are often stored in light-impermeable packaging, in vacuum or in nitrogen, as well as at lower temperatures.

The variation in temperature may change the mechanism of action of some antioxidants, and as a result the order of their effectiveness.[89] By way of example, Figure 3.3 illustrates the oxidation kinetics of TGL in the presence of 2.4×10^{-3} M α-tocopherol and ferulic acid at 100 °C and at room temperature. It is to be seen that at room temperature ferulic acid is more effective, and at 100 °C α-tocopherol exhibits greater effectiveness. Moreover, in the presence of α-tocopherol at room temperature, the oxidation rate during the IP is greater than in the case of non-inhibited system, which is not observed at 100 °C.

The effectiveness, or the stabilisation factor F, of the antioxidants usually increases with their concentration. The character of the dependence of F on antioxidant concentration is influenced strongly on the type of inhibiting system, as well as on the oxidation conditions. It may have a linear character, e.g. for syringic, ferulic and sinapic acids in lard oxidation at



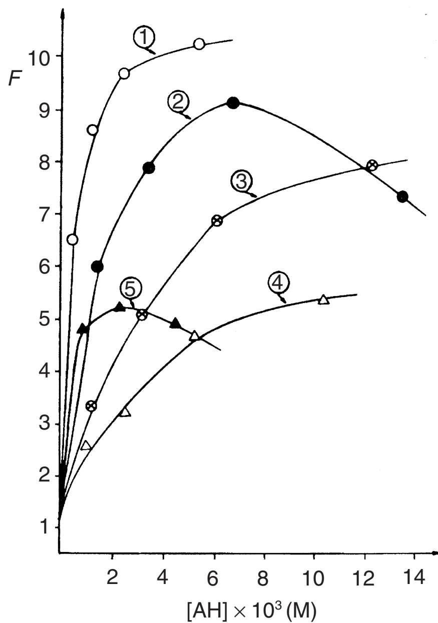
3.3 Kinetic curves of peroxide accumulation (peroxidevalue, pv) during inhibited oxidation of TGL in the presence of equal molar concentration (2.4×10^{-3} M) of α-tocopherol and ferulic acid at 100 °C (a) and at room temperature (b). The curves which have no label represent non-inhibited oxidation of TGL at the same temperatures[89] (permission to reproduce granted by John Wiley & Sons Ltd on behalf of the SCI).

100 °C.[90] Sometimes an absence of linearity of the dependences in question can be observed (Fig. 3.4). The absence of linearity is due to the participation of the antioxidant molecules and radicals formed from them in the side reactions of inhibited oxidation (Scheme 3.5).[87,89–94,97]

3.5.2 Substrate factors

The antioxidant behaviour in lipids and lipid-containing foods is system-dependent. The main problem in this respect is the oxidisability of the lipid system, followed by other substrate factors, such as different microcomponents (Section 3.1.3). The rate of initiation and propagation being a function of the type and degree of lipid unsaturation significantly affects antioxidant activity.[91–94] Yanishlieva and Popov[83] studied the effect of methyl linoleate concentration on the IP of the system methyl oleate–methyl linoleate, inhibited with 1×10^{-3} M α-naphthol. It was found that methyl linoleate had the more pronounced effect in decreasing the antioxidant effectiveness up to 10 %. As established, methylene-interrupted fatty acids act as initiators of the lipid autoxidation chain process.[28] Table 3.2 illustrates the influence of the lipid substrate, as well as of temperature, on the activity of some antioxidants.

Lampi and Kamal-Eldin[173] pointed out that for the stability of frying oils the fatty acid composition is more important than the presence of minor antioxidants, in contrast to oxidation at lower temperatures, where antiox-



3.4 Dependence of the stabilisation factor F on the antioxidant concentration [AH] during autoxidation of various lipid systems: 1-α-tocopherol, lard, 100 °C[87]; 2 thymol, triacylglycerols of sunflower oil (TGSO), 22 °C[97]; 3 *p*-coumaric acid, triacylglycerols of olive (TGOO), 100 °C[94]; 4 ferulic acid, TGSO, 100 °C[93]; 5 BHT, lard, 100 °C[87] (adapted).

idant components may sometimes be more important than fatty acid composition. The stabilising effect of an antioxidant depends also on whether the fatty acid moieties are bound in the triacylglycerol molecules or are esters of monohydric alcohols.[37,93,94] The effect is influenced also by the fatty acid composition of the lipid system: the activity A of the phenolic acids was greater in TGSO than in methylesters of sunflower oil (MESO),[93] and A was greater in methylesters of olive oil (MEOO) than in TGOO.[94]

An illustration of the complex influence of the microcomponents present in a lipid system on the effectiveness of the antioxidant are the different stabilisation factors F, obtained for 0.01 % quercetin added to eight commercial samples of lard, oxidised at 100 °C,[233] and are given as 9.1, 13.2, 3.5, 10.8, 3.1, 4.1, 13.9 and 6.9. This example shows that it is very difficult to predict the inhibiting effectiveness of an antioxidant in a natural lipid or food system because of the complex participation of the numerous microcomponents present in the oxidation process.[46]

The initial concentration of the primary products of autoxidation, the hydroperoxides in the lipids may reduce strongly the efficiency of the antioxidant added to the system being stabilised. The influence of the initial hydroperoxide concentration on decreasing the effectiveness of the model

Table 3.3 How the initial peroxide concentration [LOOH] influences the induction period IP (h) during inhibited autoxidation of different lipid systems in the presence of hydroquinone[48]

$[LOOH] \times 10^2$ (M)	Oxidizing lipid system: antioxidant concentration (M); temperature (°C)		
	Methyl oleate; 1.6×10^{-4} M; 100 °C	Methyl linoleate; 1×10^{-4} M; 70 °C	TGSO; 1×10^{-4} M; 70 °C
0	14.3	23.7	26.5
0.7	8.6	19.8	11.3
1.0	6.9	15.2	7.7
2.0	4.3	12.5	6.0
3.5	2.5	10.0	4.5
6.8	0.6	6.4	2.7
8.0	0	5.3	1.8
9.8	–	4.5	1.5

inhibitor hydroquinone is presented in Table 3.3.[48] As chain initiators, hydroperoxides reduce the time for inhibitor consumption.

Free fatty acids also decrease the oxidative stability.[40,46,234] Other lipid microcomponents, such as fatty alcohols and mono- and diacylglycerols, have been also shown to decrease the effectiveness of the phenolic antioxidants.[234–237] The effects observed are due to blocking of the antioxidant action as a result of formation of a complex based on a hydrogen bond between the phenolic group of the antioxidant and the hydroxy group of the alcohols respectively, of the partial acylglycerols.

The relative activities of metals on decreasing the oxidation stability of lipids depend on several factors, concentration being important.[41] At high concentrations, iron (2–50 mg kg^{-1}) was found to be more active than copper (2–10 mg kg^{-1}) in herring oil stored at 27 °C because of reversion of copper activity.[238] Copper was substantially more active than iron at low concentrations. Generally, the more unsaturated the lipid system, e.g. the higher the rate of initiation and propagation, the less pronounced the effects of the heavy metal addition.[239] The order of different metals also changes with temperature.[240]

3.5.3 Physicochemical state

Throughout the food and biological systems, there recur patterns of seemingly contradictory antioxidant behaviour. Prominent among these is the contrast in relative effectiveness of phenolic antioxidants in bulk *vs* dispersed systems, dependent on their hydrophilic/lipophilic balance.[241]

Frankel et al.[242] showed that α-tocopherol was more effective in an oil-in-water emulsion system than in bulk oil, whereas the opposite trend was

found for Trolox C, a hydrophilic carboxylic derivative of α-tocopherol. Yanishlieva et al.[243] established that the sequence of efficiencies of α-tocopherol and caffeic acid was reversed when passing from bulk phase to liposome oxidation. In the first case caffeic acid was more effective than tocopherol, while in the second case α-tocopherol showed higher efficiency than did caffeic acid. Frankel et al.[244] found that the more polar rosmarinic and carnosic acids (containing a carboxy group) were most effective antioxidants in bulk corn oil, whereas the antioxidant activity of carnosol was only limited in this oil. However, in an oil-in-water emulsion a high antioxidative activity was seen for carnosol, while only slight antioxidative activity was found for rosmarinic acid.

The observation that the more polar antioxidants are more active in pure lipids, and the non-polar antioxidants are more active in a polar substrate, e.g. emulsions, liposomes, has been described by the term 'polar paradox'.[245] In the bulk oil the hydrophilic antioxidants are oriented in the oil–air interface providing optimal protection of the lipids against oxygen radicals, while hydrophobic antioxidants dissolve in the homogeneous lipid phase. The opposite situation with hydrophobic antioxidant concentration in the oil–water interface is encountered in the emulsion system, where the hydrophobic antioxidants are more efficient. The understanding of the antioxidant mechanism is further compounded by complex interfacial phenomena in heterogeneous systems where hydrophilic polyphenolic compounds vary in their partition behaviour between the water and oil phases and their interface.[246–248]

3.6 Future trends

The inhibitors are important not only to protect foods but may also be required in their own right for protection against several diseased conditions. Because of a growing concern about the potential health hazard of synthetic antioxidants, there is renewed interest in the increased use of naturally occurring antioxidants. Because they occur in nature and in many cases are derived from plant sources, natural antioxidants are presumed to be safe. The permitted antioxidants and synergists are restricted to a few compounds: propyl- and dodecylgallate, BHT, BHA, TBHQ, ascorbyl palmitate, tocopherols, citric acid and its esters, thiodipropionic acid, lecithin, carotene, and silicone oil (for frying fats). The permitted antioxidants are different in different countries. The need for the development of novel natural antioxidants which are effective at frying temperatures is obvious.[249]

The suitability of an antioxidant for a particular application has at the present time to be determined on a case-by-case basis, as it is difficult to predict the efficiency of a particular antioxidant in a given food.[250] A fundamental understanding in the mechanism of antioxidant activity, obtained,

for example, from electron spin resonance (ESR) studies, could be the first step in determining the optimum use of antioxidants.

For practical reasons, it is suitable to add mixtures of antioxidants which usually have higher activities than single compounds, and which guarantee that the limits for single compounds have not been exceeded.

Recently, the unification of different inhibiting functions in one molecule has been developed, such as in vitamin E and phospholipids,[143] selenium-containing moieties and carotenoids,[251] vitamin E and carotenoids.[252] The binding of various types of inhibitors in a molecule should provide unique compounds, eliminating the need for physical mixtures. Such analogues of natural substances are expected to manifest intramolecular synergism of constituent fragments.

3.7 Sources of further information and advice

More information on lipid oxidation and inhibiting of the process in fats, oils and lipid-containing foods is to be found in the several books and reviews as listed in the references.[1,2,3,7,9,18,41,59,60,72,75,86,124,128,146,160,162,167,168,194,241,249,250,253–266] It is difficult to summarise guidelines for the avoidance of lipid oxidation in foods. Each product has its own special susceptibility, depending on its composition, presentation and packaging and the processing conditions used.

Natural antioxidants are generally preferred by consumers, and may gain legislative approval more easily than synthetic additives do. However, the fact that a substance is commonly found in a food is no guarantee that it is entirely nontoxic.[267] Synthetic antioxidants are tested for carcinogenic or mutagenic effects, but many natural food compounds have not yet been tested.

The advantages and disadvantages of synthetic and natural antioxidants are summarised in Table 3.4.[167] No rational scientific or technical argument can be given for natural antioxidants: they are more acceptable to consumers mainly on emotional grounds. While it is important for manufac-

Table 3.4 Advantages and disadvantages of natural and synthetic antioxidants[167]

Synthetic antioxidants	Natural antioxidants
Inexpensive	Expensive
Widely applied	Use restricted to some products
Medium to high antioxidant activity	Wide ranging antioxidant activity
Increasing safety concern	Perceived as innocuous substances
Use banned for some of them	Increasing use and expanding applications
Low water-solubility	Broad range of solubilities
Decreasing interest	Increasing interest

Table 3.5 Methods of producing stable foods without adding antioxidants[255]

Technique	Methods
Elimination of oxygen	Packaging under nitrogen; packaging in vacuum; packaging with an oxygen scavenger
Elimination of sensitive substances	Replacement of polyunsaturated oils with oils which are less unsaturated and more stable, such as olive oil or palm oil
Decreasing the rate of oxidation	Storage at low temperatures; storage in the dark; use of fats and oils that contain low levels of oxidation promoters (e.g. oxidised products and heavy metals); use of ingredients that are naturally rich in antioxidants

turers to meet the requirements of consumers, it is imperative that the safety of additives that are not 'generally recognised as safe (GRAS)' be tested before use.

The interaction between food additives and nutrient components within the food matrix is an area of future interest. A majority of antioxidants present or added to foods (e.g. propyl gallate, flavonoids, α-tocopherol, carnosic acid, carnosol, catechins, vitamin C) are capable of stimulating free-radical damage to nonlipid components, carbohydrates and DNA in foods.[260] Perhaps the safest approach is to avoid the use of both synthetic and natural antioxidants by using appropriate packaging and storage methods, or by avoiding the use of ingredients that are readily oxidised, as summarised in Table 3.5.[255] However, the benefits of using antioxidants outweigh the risk. Without antioxidants in foods, oxidation products are formed, and these cause a greater risk to health than the possible hazardous effects of antioxidants.

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4

Measuring antioxidant activity

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4.1 Introduction

Antioxidants are used in a wide variety of food products, and their activity may vary depending on the temperature, food composition, food structure and availability of oxygen. Temperatures at which antioxidant activity may be required range from 180–200 °C for frying oils, to about 5 °C for products such as margarine or mayonnaise that are stored in the fridge. Besides the processing and storage temperatures to which these products are exposed, the accompanying constituents including water, proteins, carbohydrates, vitamins, minerals and other food components vary, and the physical structure of the food also varies. This can cause big changes in the activity of the antioxidant in different food systems. It is commonly observed that a non-polar antioxidant such as α-tocopherol is relatively ineffective in oil but is strongly effective in an oil-in-water emulsion. In contrast, a polar antioxidant such as ascorbic acid or trolox (a water-soluble derivative of α-tocopherol) is more effective in an oil than in an emulsion. This has been described as the polar paradox.[1,2]

Normally, a more rapid measurement of antioxidant activity is required than would be obtained by making the food product, storing it at ambient temperature and then measuring the oxidative state of the food. Consequently, there are three decisions to be made:

(a) What model food system should be used for the test?
Most assessments of antioxidant activity have been performed in oil. This commonly gives sensible predictions for the activity in oil or water-in-oil emulsions such as margarine, but the data may be misleading for oil-in-water emulsions as discussed above. Some information may be gained by

the use of a radical-scavenging test in an organic solvent. Would this be useful?

(b) How should oxidation be accelerated?
The most common methods of accelerating oxidation are to raise the temperature and to increase the supply of oxygen. The combination of these effects can reduce the oxidative stability by a large amount. Thus, whereas a sample of refined olive oil required 103 days for significant deterioration at 20 °C, the assessment time was reduced to 20.4 h when the oil was assessed in the Rancimat at 100 °C. Other factors affecting the oxidation rate include the content of metal ions in the test sample, the oxidative state of the test sample before the addition of antioxidant and exposure to UV light.

Frankel summarised several problems with using elevated temperatures of 100 °C or higher to predict antioxidant activity at lower temperatures.[3] Although an increase in temperature accelerates oxidation by a large factor, the temperature may affect the mechanism of autoxidation, the stability and volatility of the antioxidant and oxidation products, and the partition of the antioxidant between different phases present in the food. At high temperatures where reaction rates are fast, transport of oxygen may become rate-limiting. The mechanism of autoxidation is complex involving several steps and competing reactions that differ in enthalpy and entropy of activation. Ragnarsson and Labuza claimed that antioxidants were normally less effective at elevated temperatures than at ambient temperature.[4] Therefore, it can be difficult to predict antioxidant effects at low temperatures from measurements at elevated temperatures.

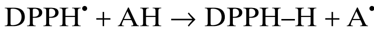
(c) How should oxidation be monitored?
In principle, one could consider measuring the loss of lipid starting material, i.e. fatty acids or triglycerides, or the formation of oxidation products as a method of monitoring oxidative deterioration or antioxidant activity. In practice, the formation of oxidation products is a much more sensitive method of monitoring oxidation. However, the assessment of antioxidant activity by monitoring the formation of oxidation products is not a trivial task. Since a complex mixture of oxidation products is formed and the relative amounts of these products depend on a variety of variables including temperature, metal ion content, and other components present such as water, deciding which components to monitor is an important decision. Monitoring antioxidant activity under frying conditions may well require other products to be monitored than if the activity is to be assessed under ambient conditions. Thus, hexanal formation can be used to monitor oxidative deterioration in ambient stored products, but not in used frying oils.

4.2 Radical-scavenging methods

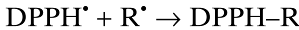
Radical scavenging is the main mechanism by which antioxidants act in foods. Several methods have been developed in which the antioxidant activ-

ity is assessed by the scavenging of synthetic radicals in polar organic solvents, e.g. methanol, at room temperature. Those used include 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzthiazoline-sulphonic acid) (ABTS) radicals.

In the DPPH test, the scavenging of DPPH radicals is followed by monitoring the decrease in absorbance at 515 nm which occurs due to reduction by the antioxidant (AH) or reaction with a radical species (R˙).[5]



$$\mathrm{DPPH^{\bullet} + AH \rightarrow DPPH{-}H + A^{\bullet}}$$



$$\mathrm{DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH{-}R}$$

Fast reaction of DPPH radicals occurs with some phenols e.g. α-tocopherol, but slow secondary reactions may cause a progressive decrease in absorbance, so that the steady state may not be reached for several hours. Most papers in which the DPPH method has been used report the scavenging after 15 or 30 min reaction time. The data is commonly reported as EC_{50}, which is the concentration of antioxidant required for 50 % scavenging of DPPH radicals in the specified time period.

The ABTS radical cation is more reactive than the DPPH radical, and reaction of the ABTS radical cation with an antioxidant is taken as complete within 1 min.[6] The method of generation of the radical cation has changed several times since the method was first described. The most recent method describes the use of potassium persulphate to oxidise ABTS to the radical cation.[7] The radical scavenging activity assessed by the ABTS method has been expressed as the TEAC (trolox equivalent antioxidant capacity) value in most papers employing this method.

These methods may be useful for screening antioxidants, but antioxidant effectiveness in foods must always be studied by other methods because their activity in foods is dependent on a variety of factors including polarity, solubility, and metal-chelating activity.

4.3 Methods for measuring the current state of an oil or food sample

Some methods can be applied to assessing the current state of an oil or food sample. In order to be applied in assessment of antioxidant effectiveness, an experiment must be designed in which the antioxidant is incorporated into the food and the food is stored under controlled conditions. The principles of these methods are described below.

4.3.1 Sensory analysis

For the food industry, the detection of oxidative off-flavours by taste or smell is the main method of deciding when a lipid-containing food is no

longer fit for consumption. Consequently, any antioxidant used in the food will ultimately be evaluated by its potential for extending the time before this off-flavour can be detected. The ability of individuals to describe the nature of the aroma is useful, and the sensitivity of a trained panel to oxidative off-flavours may allow detection of oxidative deterioration at a stage when common chemical methods, e.g. peroxide value measurements, are unable to detect any deterioration. The main problems with sensory evaluation are that different individuals vary in their sensitivity to these off-flavours, and their performance may vary depending on their state of health and other variables. Trained panellists are much more reliable than untrained panellists, but the reproducibility of sensory analysis is normally worse than that of chemical or instrumental methods.

4.3.2 Headspace analysis

Although they only represent a small proportion of the oxidation products, volatile lipid decomposition products are those that are perceived by the consumer as off-flavours. Consequently, it is tempting to monitor these volatile oxidation products in order to have an instrumental method that correlates well with consumer perception of the extent of deterioration of an oil. The application is normally a correlation between an individual aroma component or the total volatile concentration and sensory assessment of oil deterioration. However, it should be remembered that the aroma of a sample includes contributions from many different compounds. Individual aroma compounds vary in their contributions to the aroma with different flavour thresholds and concentration dependence of the aroma. Nevertheless, volatile analysis has been widely used as a method of monitoring oxidative deterioration of oil samples. Several procedures have been developed.

4.3.2.1 Static headspace analysis

The mass of each component in the headspace of a sample in a sealed vial depends on the vapour pressure of the pure component, the sample temperature and the concentration of the component in the sample. Although it is possible to sample the headspace with a gas syringe and inject it onto a gas chromatography (GC) column, it is difficult to avoid problems due to adsorption of trace components, condensation of volatiles in the syringe and leakage from the syringe during transfer to the GC. Consequently, automated headspace injectors are normally used. In a typical commercial headspace analyser, the sample is sealed in a vial closed with a septum and crimped aluminium cap. Vials are held in a temperature-controlled autosampler for equilibration. A sample needle penetrates the septum when the vial is in the position for analysis, and the vial is pressurised for a time before injection in order to raise the pressure within the vial to that of the column head. The carrier gas supply to the column is then switched

off by a solenoid valve, the pressure at the head of the column falls and sample vapour flows via the sample needle onto the column. After the injection period, which is typically 5 s, the carrier gas flow to the column is restored and injection of vapour ceases, after which time the injection needle is withdrawn.

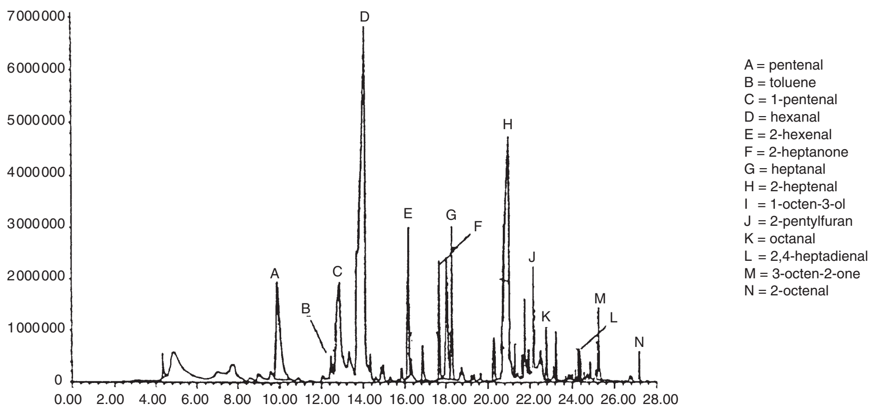
The temperature used for analysis of headspace volatiles in an edible oil may vary between 40 and 180 °C. Above 150 °C, hydroperoxides are unstable and the measured headspace volatiles are due to the volatiles in the sample when inserted into the vial and those formed by hydroperoxide decomposition. Even at temperatures as low as 90 °C, partial decomposition of hydroperoxides will occur during the equilibration time. The time required for equilibration is typically 10–20 min. Static headspace analysis is a relatively quick and simple procedure, and no solvents are used. It is less sensitive than dynamic headspace analysis and the procedure mainly detects the very volatile components, while dynamic headspace analysis detects components with a wider range of volatility. When monitoring the oxidative deterioration of edible oils, either the hexanal, pentane or total volatile concentration is normally monitored for oil containing linoleic acid or other polyunsaturated fatty acids with an *n*-6 structure. Propanal, 2-hexenal, 3-hexenal and 2,4-heptadienal are formed from α-linolenic acid or other polyunsaturated fatty acids with an *n*-3 structure.

Solid phase microextraction (SPME) is an alternative technique for static headspace analysis. SPME uses a special syringe with a short length of fused silica optical fibre externally coated with a polymeric GC stationary phase (e.g. polydimethylsiloxane). The coated fibre is shielded by an adjustable needle guide as it is pushed through a septum into the headspace above a sample in a sealed sample vial. After penetrating the septum, the needle guide is withdrawn to allow the coated fibre to be exposed to the headspace. After allowing equilibrium to be achieved, or after a defined time, the fibre is again shielded by the needle guide. The syringe is withdrawn, and transferred to a GC injection port. The fibre is exposed after puncturing the septum, and the analytes are thermally desorbed to introduce them onto the GC column.

SPME has the advantage that the technique is easy to use and quick, requiring about 30 min for trapping the volatiles. No special injector is required, so there are no capital costs involved, and no artifacts are introduced. However, the technique is less sensitive than other headspace techniques, and the fibres are fragile and require periodic replacement.

4.3.2.2 Dynamic headspace analysis

An alternative method for the analysis of volatile components is dynamic headspace analysis, which involves purging the sample with nitrogen or helium for a period whilst continuously trapping the volatiles. The volatiles are trapped on a porous polymer trap (often Tenax™) held at room temperature. The most common method of transferring the volatiles onto a GC



4.1 Gas chromatogram of volatiles from oxidised sunflower oil determined by dynamic headspace analysis.

column involves placing the trap in the inlet of a gas chromatograph, then heating the trap to desorb the volatiles. Solvent extraction of volatiles from the trap and injection of a solution may be used as an alternative transfer procedure, but this is much less sensitive than thermal desorption. Dynamic headspace analysis allows components with a wider range of volatility to be detected than in the case of static headspace analysis, but poorly adsorbed components may be lost by passing through the trap (breakthrough) before the trapping period is complete. A typical chromatogram is shown in Fig. 4.1.

4.3.2.3 Direct injection method

The direct injection method involves applying a sample at the inlet of a GC column, and then passing carrier gas through the sample to sweep the volatiles onto the column, which is often cooled to allow efficient trapping as a narrow band. The method allows the collection of volatiles covering a wide range of volatility. However, a much smaller sample size is used than in dynamic headspace analysis. The method can only be applied directly to oils whereas static and dynamic headspace analysis can be applied to more complex foods.

4.3.3 Peroxide value (PV)

The PV is still the most common chemical method of measuring oxidative deterioration of oils. Although hydroperoxides decompose to a mixture of volatile and non-volatile products and they also react further to endoperoxides and other products, the PV measurement is a useful method of monitoring oxidative deterioration of oils, although it should normally be combined with a method of monitoring secondary oxidation products to

provide a fuller picture of the progress of oxidation. Huang et al.[8] showed that increased addition of α-tocopherol to an oil may increase the PV whilst reducing hexanal formation. This suggests that a high PV value may reflect either increased formation of hydroperoxides or reduced decomposition. Consequently, antioxidants may improve the flavour stability of an oil without it being evident from PV measurements.

The traditional method of determining PV involves a titration of the oil containing potassium iodide in a chloroform–acetic acid mixture. The hydroperoxides oxidise the iodide to iodine, which is determined by titration with sodium thiosulphate. In order to avoid the use of chloroform, the AOCS has developed an alternative method which uses isooctane as solvent, although the method is limited to PV < 70 meq kg^{-1}, as described in the AOCS guidelines.[9]

The PV determination should not be used as a method of assessing the deterioration of oils used for frying, since hydroperoxides decompose spontaneously above 150 °C, and the measured PV can be more an indication of the cooling and storage conditions after frying than of oxidation products formed at frying temperatures. Even at temperatures of 80 to 90 °C, formation of hydroperoxides is accompanied by decomposition at a significant rate.

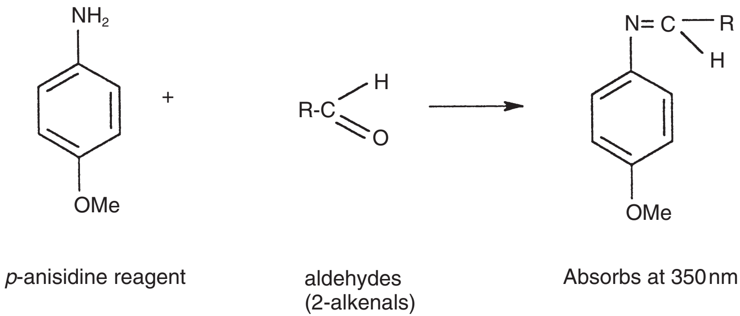
The PV at which oxidation of oils can be detected as an off-flavour varies widely depending on the nature of the oil. Samples of olive oil may not be perceived as rancid till the PV reaches 20 meq kg^{-1} whereas fish oil may develop off-flavours at PV < 1 meq kg^{-1}.

4.3.4 Conjugated dienes

Formation of hydroperoxides from polyunsaturated fatty acids (PUFA) leads to conjugation of the pentadiene structure. This causes absorption of UV radiation at 233–234 nm. This represents a simple and rapid method of monitoring oxidative deterioration of an oil. Although the absorbance is mainly a measure of hydroperoxide content, some products formed following hydroperoxide decomposition such as 9-hydroxyoctadeca-10,12-dienoic acid and 13-hydroxyoctadeca-9,11-dienoic acid retain this conjugated structure and will contribute to the absorbance. The method is therefore less specific than PV measurement.

4.3.5 *Para*-anisidine value

Para-anisidine is a reagent that reacts with aldehydes to give products that absorb at 350 nm (Fig. 4.2). The *p*-anisidine value is defined as the absorbance of a solution resulting from the reaction of 1 g fat in isooctane solution (100 ml) with *p*-anisidine (0.25 % in glacial acetic acid). The products formed by reaction with unsaturated aldehydes (2-alkenals) absorb more strongly at this wavelength, and consequently the test is particularly



4.2 The reaction of *p*-anisidine with alkenals.

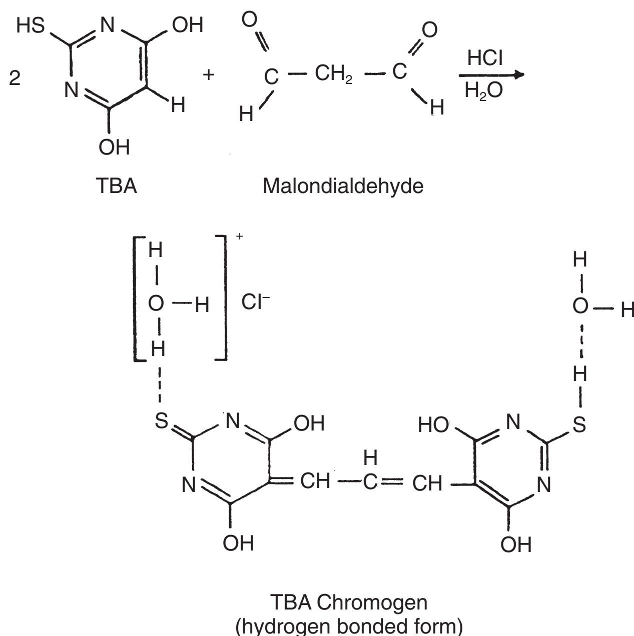
sensitive to these oxidation products. Although the test does not distinguish between volatile and non-volatile products, the palate is generally more sensitive to unsaturated volatile aldehydes than to saturated volatile aldehydes, so the test is a reasonable way to assess secondary oxidation products. Measurements of *p*-anisidine value are commonly used together with peroxide value measurements in describing the total extent of oxidation by the Totox value, which equals the sum of the *p*-anisidine value plus twice the peroxide value. However, the Totox value is an empirical parameter since it corresponds to the addition of two parameters with different units.

4.3.6 Thiobarbituric acid value (TBA)

Malonaldehyde may be formed from polyunsaturated fatty acids with at least three double bonds. The concentration of this product may be assessed by reaction with thiobarbituric acid which reacts with malonaldehyde to form red condensation products (Fig. 4.3) that absorb at 532–535 nm with molar absorptivity of 27.5 absorbance units/µmol. However, the reaction is not specific, and reaction with a wide variety of other products may contribute to the absorbance. 2,4-Alkadienals such as 2,4-decadienal also react with TBA to show strong absorption at 532 nm. Saturated aldehydes normally absorb at lower wavelengths after reaction with TBA. Several food components including proteins, Maillard browning products and sugar degradation products affect the determination. In order to emphasise the lack of specificity, the values obtained in the test are commonly described as TBARS (TBA reactive substances). The TBA test has recently been reviewed.[10]

4.3.7 Octanoate value

The octanoate value is a measure of the bound octanoate present in an oil. Octanoate is formed from the decomposition of linoleic acid 9-hydroper-



4.3 Formation of a chromogen by reaction of TBA with malondialdehyde.

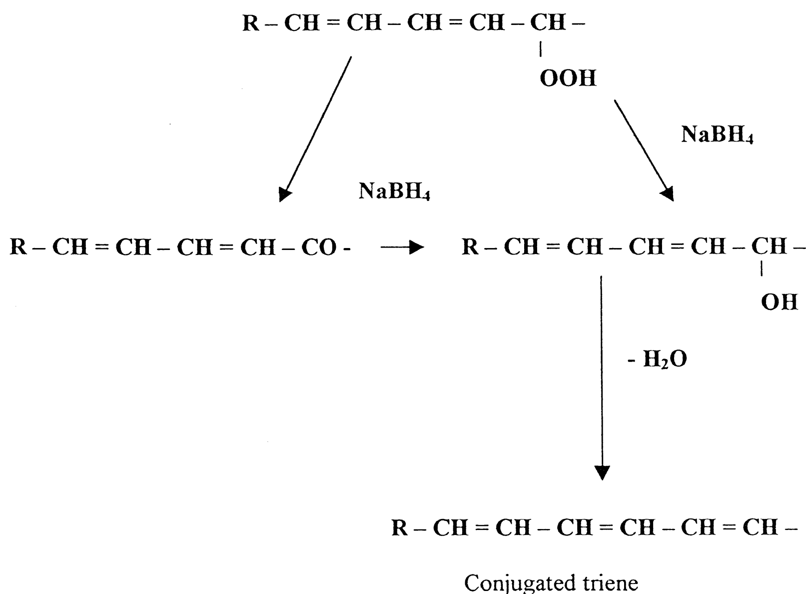
oxide.[11] The method involves *trans*-methylation of an oil with a base such as sodium methoxide, and GC analysis of the methyl octanoate formed.

4.3.8 Conjugable oxidation products

Analysis of conjugable oxidation products is based on the fact that hydroperoxides from polyunsaturated fatty acids and some decomposition products may be reduced with sodium borohydride and dehydrated to give conjugated trienes and tetraenes (Fig. 4.4).[12] Triene and tetraene concentrations are determined from the absorbance values at 268 nm and 301 nm respectively.

4.3.9 Infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) has considerable potential for the analysis of hydroperoxides in oils. Some progress was made in analysing the content of hydroperoxides and other oxidation products by direct determination in oils.[13] The method involved calibration with known standards, and it is an attractive approach due to its speed, once calibrated, and avoidance of chemicals for the analysis. However, improved specificity and precision can be achieved by addition of triphenylphosphine (TPP) to an oil containing hydroperoxides.[14] This causes the formation of



4.4 Formation of conjugable oxidation products.

triphenylphosphine oxide (TPPO) which has an intense absorption band at 542 cm^{-1}. In the paper describing the method, PV was determined in the range of 0–15 meq kg^{-1} by calibrating with TPPO as the standard. The resulting calibration was linear over the analytical range. The analytical procedure involved addition of a 33% (w/w) stock solution of TPP in hexanol (0.2 g) to melted fat or oil (30 g). The mixture was shaken and transferred to a 100-nm IR transmission cell maintained at 80 °C. The reaction and FTIR analysis required about 2 min per sample. The method was validated by comparing the analytical results of the AOCS PV method to those of the automated FTIR procedure by using both oxidised oils and oils spiked with *tert*-butyl hydroperoxide. The reproducibility of the FTIR method is superior to that of the standard chemical method.

4.4 Methods to monitor changes in oxidation

All of the above methods may be applied to assess the state of oxidation of oils, but other methods can only be used to monitor changes in oils. These include the following:

4.4.1 Loss of polyunsaturated fatty acids

Analysis of changes in fatty acid composition is always an insensitive way of assessing oxidative deterioration. This is in line with the general scien-

tific principle that it is much more difficult to measure a small change in a large number than the same change in a very small number. This can clearly be demonstrated by a simple calculation. If a change in linoleic acid content is determined by gas chromatographic analysis of fatty acid methyl esters, a change from 50.0 ± 0.1 % to 49.6 ± 0.1 % might be detectable by a careful analyst. This represents a change of 0.4 % in the sample composition. For comparison, oxidation of 0.4 % PUFA to monohydroperoxides would represent a change in peroxide value of 16 meq kg^{-1}, whereas a change of <1.0 meq kg^{-1} could readily be detected by measuring the PV.

4.4.2 Weight gain

Edible oils increase in weight during the early stages of lipid oxidation as fatty acids combine with oxygen during the formation of hydroperoxides. The increase in weight of a heated sample during storage can be used to determine the induction time of the fat. Rapid weight gain occurs after the induction period, or the time for a certain weight increase can be determined. However, decomposition of hydroperoxides leads to a weight reduction, and the fat is severely oxidised at the end of the induction time.

4.5 Predictive methods

Predictive methods are methods in which samples are continuously monitored during accelerated oxidation conditions.

4.5.1 Differential scanning calorimetry (DSC)

DSC is an instrumental method that monitors exothermic or endothermic changes due to phase changes or chemical reactions in samples. The end of the induction time is marked by an increased heat of reaction due to more rapid reaction of unsaturated lipids reacting with oxygen.[15] However, the reproducibility of the DSC induction time is poor unless measurement is performed at temperatures below 155 °C, and this reduces the value of the method for assessing antioxidant activity.

4.5.2 Oil stability index (OSI)

The OSI is an automated development of the AOM (active oxygen method). In the AOM, the time for an oil to reach a PV of 100 meq kg^{-1} during oxidation at 97.8 °C, with an air flow of 2.33 ml per tube per second is determined. Instruments for determining the OSI are the Rancimat™, manufactured by Metrohm, Basel or the Oxidative Stability Instrument™, manufactured by Omnion, Rockland, USA. These instruments depend on the increase in electrical conductivity, when effluent from oxidising oils is

passed through water. Volatile carboxylic acids are generated in the oxidising oil and these cause the increase in electrical conductivity. The samples, assessed by the OSI methods, are held at 100 °C, 110 °C, 120 °C, 130 °C, or 140 °C. The temperature may be adjusted to allow the oxidation time to fall within the range of 4–15 h. The sample size is 2.5 g or 5 g depending on the instrument used. Although these instruments are useful for quality control of oils, they are not recommended for the assessment of antioxidant effectiveness for several reasons. The high temperatures used do not allow reliable predictions of antioxidant effectiveness at lower temperatures. Volatile antioxidants may be swept out of the oil by the air flow under the test conditions, and also the oils are severely deteriorated when the end-point is reached.

4.5.3 Oxipres

The Oxipres™, manufactured by Mikrolab Aarhus, is a method for examining the oxidative stability of heterogeneous products such as potato crisps, margarine or mayonnaise. Oxidation is accelerated by heating and by the use of oxygen under pressure. The pressure drop in a glass pressure vessel, containing the sample (up to 100 ml), and filled with oxygen at pressures up to 10 bar (1 MPa), is monitored. The instrument consists of a control unit, a block heater, which can heat two samples at temperatures up to 150 °C, and a bomb into which the sample is introduced in a glass bottle. The pressure in the bomb is measured electronically and recorded on a multichannel recorder or transferred to a PC.

4.5.4 Oxidograph

The Oxidograph™, manufactured by Mikrolab Aarhus, is an instrument based on the FIRA-Astell apparatus which employs the principle of the Sylvester test. The sample of oil or fat is exposed to oxygen or air at an elevated temperature, with stirring, to accelerate the test. As the sample absorbs oxygen, the pressure drop is measured electronically by means of pressure transducers. The aluminium heating block has spaces for six sample tubes. An analogue signal is recorded for each sample on a six-channel recorder. The sample tubes are glass, and designed to be leak proof, when connected, with no grease required.

4.6 Applications to particular foods

Most of the methods mentioned above can be applied to a wide range of foods. Additional steps, e.g. isolation of fat, may be required in some cases. The standard PV determination and some other analyses cannot be applied to samples with significant water contents. One approach is to separate the

fat phase and perform the standard tests. For butter this can be achieved by melting the sample, and for model emulsions freezing overnight at –70 °C may allow the oil to separate due to its lower density than water. However, for commercial emulsions, e.g. margarine and mayonnaise, addition of solvent, normally hexane, is required to extract the oil and the standard tests can then be performed after evaporation of the solvent. Confectionery products and biscuits also commonly require solvent extraction of the fat before analysis.

For oils used for frying, the PV is not a good method of assessing oxidative deterioration, and the analysis of polar components is a more common method. This involves column chromatography to separate the oil into polar and non-polar components. The method is described in AOCS method Cd 20-91.

Sensory evaluation is a good method for monitoring deterioration of meat and fish products. For fish oil in particular, the human olfactory system is a very sensitive detector of off-flavours. Off-flavours are normally detected at very low PV values. Headspace analysis represents a useful instrumental method, and the TBA method is also commonly applied as an objective method of detecting deterioration in these foods.

4.7 Future trends

Instrumental methods of assessing the state of oxidation of oils are likely to become more important in the future. Methods that do not require the use of chemical reagents or solvents reduce waste disposal problems. Headspace analysis and FTIR spectroscopy in particular are likely to become more widespread. The trend towards miniaturisation of chromatographic equipment is likely to allow the development of small scale GC equipment that can be transported for headspace analysis of samples to be carried out with reduced space requirements.

The use of autosamplers allows many samples to be analysed by headspace analysis with minimal operator attention. Developments in robotics are likely to allow some of the chemical assays to be automated too.

4.8 Sources of further information and advice

There are many commercial sources of food antioxidants. Details of the chemical properties of food antioxidants and their suppliers are given in *The Index of Antioxidants and Antiozonants*.[16]

The following books are recommended for consultation:

Allen J C and Hamilton R J (eds) *Rancidity in Foods*, 3rd ed., London, Chapman & Hall, 1994.

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

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Part 2

Antioxidants and health

5

Cardiovascular disease and nutritional phenolics

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5.1 Introduction

Arteriosclerosis is a chronic pathogenic inflammatory-fibro-proliferative process of large and medium-sized arteries that results in the progressive formation of fibrous plaques, which in turn impair the blood flow of the vessel. These lesions can either promote an occlusive thrombosis in the affected artery or produce a gradual but relentless stenosis of the arterial lumen. In the first case, an infarction of the organ supplied by the afflicted vessel occurs, such as in a heart attack, when a coronary artery is affected, and in a thrombotic stroke when a cerebral artery is suddenly blocked. In the second case, the stenosis of the vessel leads to a progressive and gradual damage of the affected organ part.

A number of subtle dysfunctions occur at the cellular and molecular levels in the early stages of disease progression associated with the loss of cellular homeostatic functions of endothelial cells, smooth muscle cells and macrophages which constitute the major cell types in the atheroma environment. These events include the modification of the pattern of gene expression, cell proliferation and apoptosis.

In the last few decades, several epidemiological studies have shown that a dietary intake of foods rich in natural antioxidants correlates with reduced risk of coronary heart disease;[1,2] particularly, a negative association between consumption of polyphenol-rich foods and cardiovascular diseases has been demonstrated. This association has been partially explained on the basis of the fact that that polyphenols interrupt lipid peroxidation induced by reactive oxygen species (ROS). A large body of studies has shown that oxidative modification of the low-density fraction of lipoprotein (LDL) is

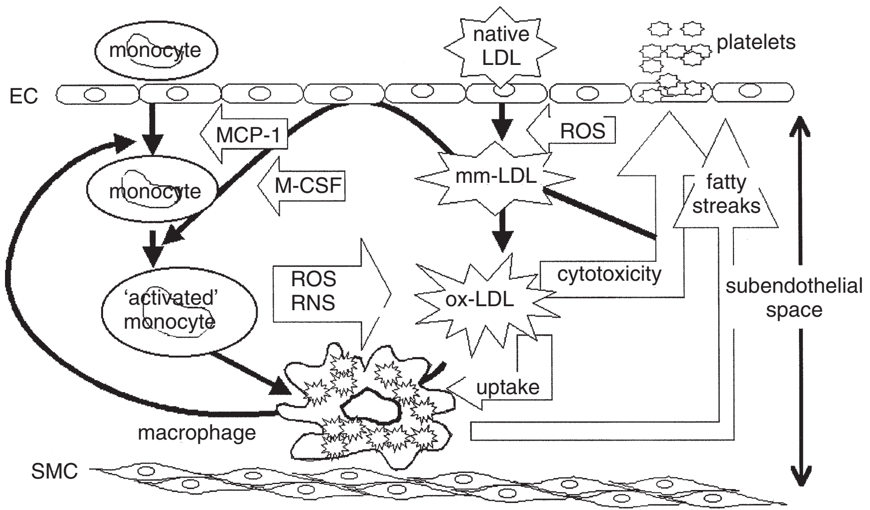
implicated in the initiation of arteriosclerosis. More recently, alternative mechanisms have been proposed for the activity of antioxidants in cardiovascular disease, which are different from the 'simple' shielding of LDL from ROS-induced damage. Several polyphenols recognised for their antioxidant properties might significantly affect cellular response to different stimuli, including cytokines and growth factors.

5.2 LDL oxidation and atherogenesis

At cellular level each stage of atheroma development is accompanied by the expression of specific glycoproteins by endothelial cells which mediate the adhesion of monocytes and T-lymphocytes.[3,4] Their recruitment and migration is triggered by various cytokines released by leukocytes and possibly by smooth muscle cells.[5] Atheroma development continues with the activation of macrophages, which accumulate lipids and become, together with lymphocytes, so-called fatty streaks.[3,4,6] The continuous influx, differeniation and proliferation finally leads to more advanced lesion and to the formation of the fibrous plaque.[6]

It is accepted that oxidation of LDL is a key event in endothelial injury and dysfunction.[7] Oxidised LDL (oxLDL) may directly injure the endothelium and trigger the expression of migration and adhesion molecules.[8–10] Monocytes and lymphocytes interact with oxLDL and the phagocytosis which follows leads to the formation of foam cells, which in turn are associated with the alteration of the expression pattern of growth regulatory molecules, cytokines and pro-inflammatory signals.[6] The proposed role of oxLDL in atherogenesis, based on studies *in vitro*, is shown in Fig. 5.1.

LDL, modified by oxidation, glycation and aggregation, is considered a major cause of injury to the endothelium and underlying smooth muscle. LDL, entrapped in the subendothelial space, can undergo progressive oxidation (minimally modified-LDL, mm-LDL).[11] Once modified, LDL activates the expression of molecules entitled for the recruitment of monocytes and for the stimulation of the formation of monocyte colonies (monocyte chemotactic protein, MCP-1; monocyte colony stimulating factor M-CSF) in the endothelium.[12–14] These molecules promote the entry and maturation of monocytes to macrophages, which further oxidise LDL. Modified LDL is also able to induce endothelial dysfunction, which is associated with changes of the adhesiveness to leukocytes or platelets and the wall permeability.[14,15] Dysfunctional endothelium also displays pro-coagulant properties and the expression of a variety of vaso-active molecules, cytokines, and growth factors.[16,17] LDL, oxidised *in vitro* by several cell systems or by cell-free systems (transition metal ions or azo-initiators), is recognised by the scavenger receptor of macrophages.[18] The increasing affinity of LDL for the scavenger receptor is associated with changes in its structural and biochemical properties, such as the formation of lipid hydroperoxides, oxida-



5.1 Sequence of events in atherogenesis and role of low-density lipoprotein. Native LDL, in the subendothelial space, undergoes progressive oxidation (mmLDL) and activates the expression of MCP-1 and M-CSF in the endothelium (EC). MCP-1 and M-CSF promote the entry and maturation of monocytes to macrophages, which further oxidise LDL (oxLDL). Ox-LDL is specifically recognised by the scavenger receptor of macrophages and, once internalised, formation of foam cells occurs. Both mmLDL and oxLDL induce endothelial dysfunction, associated with changes of the adhesiveness to leukocytes or platelets and to wall permeability.

tive modification and fragmentation of apoprotein B-100 and an increase of negative charge.[19] The exact mechanism of LDL oxidation *in vivo* is still unknown, but transition metal ions, myeloperoxidase, lipoxygenase, and nitric oxide are thought to be involved.[7]

5.3 Polyphenols and cell response

Plants produce a variety of secondary products containing a phenol group, i.e. a hydroxyl group on an aromatic ring. These compounds are of a chemically heterogeneous group that includes simple phenols, flavonoids, lignin and condensed tannins. About four thousand plant substances belong to the flavonoid class, of which about 900 are present in the human diet. The daily intake of flavonoids in Western countries has been estimated to be about 23 mg per day.[1] No analogous calculation has been done for phenolic acids but it is likely to be quite similar in the western diet.

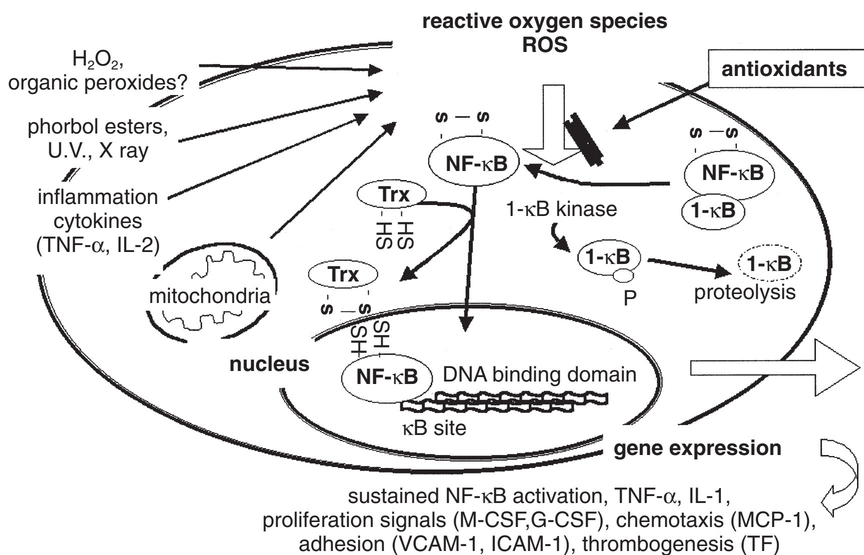
Many studies have been undertaken to establish the structural criteria for the activity of polyhydroxy flavonoids in enhancing the stability of fatty

acid dispersions, lipids, oils, and LDL.[20,21] As for phenolic acids, the inhibition of oxidation by flavonoids is related to the chelation of metal ions via the *ortho*-dihydroxy phenolic structure, the scavenging of alkoxyl and peroxyl radicals, and the regeneration of α-tocopherol through reduction of the tocopheryl radical.[20] The contribution of flavonoids and phenolic acids to the prevention and possibly to the therapy of cardiovascular disease can also be found on metabolic pathways other than the antioxidant capacity. As previously mentioned, arteriosclerosis is characterised by early cellular events and by the dysregulation of the normal cellular homeostasis.[17] Molecular mechanisms, by which polyphenols may play a role either in the etiopathology or in the pathophysiology of arteriosclerosis, will be discussed here, with particular regard to the modulation of gene expression regulated by the transcription factor nuclear factor-kappa B (NF-κB), and to the induction of either apoptotic or proliferative responses.

5.4 Polyphenols and activated NF-κB

The transcription factors of the nuclear factor-κB/Rel family control the expression of a spectrum of different genes involved in inflammatory and proliferation responses. The typical NF-κB dimer is composed of the subunits p50 and p65, and it is present as its inactive form in the cytosol bound to the inhibitory proteins IκB. Following activation by various stimuli, including inflammatory or hyperproliferative cytokines, ROS, oxidised LDL and bacterial wall components, the phosphorylation and proteolytic removal of IκB from the complex occurs. The activated NF-κB immediately enters the nucleus where it interacts with regulatory κB elements in the promoter and enhancer regions, thereby controlling the transcription of inducible genes.[22,23] A spectrum of different genes expressed in arteriosclerosis have been shown to be regulated by NF-κB, including those encoding TNF-α, IL-1, the macrophage or granulocyte colony stimulating factor (M/G-CSF), MCP-1, c-myc and the adhesion molecules VCAM-1 and ICAM-1.[24] In the early stages of an atherosclerotic lesion, different types of cells (macrophages, smooth muscle cells and endothelial cells) interplay to cause a shift from the normal homeostasis and a vicious circle may be triggered, exacerbating dysfunction. Figure 5.2 shows a sketch of the regulation of NF-κB activation by oxidants/antioxidants. Some of major genes involved in the atherogenesis are also listed.

Several lines of evidence, including the inhibition by various antioxidants, suggest that NF-κB is subject to redox regulation. Because of its pivotal role in inflammatory response, a significant effort has focused on developing therapeutic agents that regulate NF-κB activity. In this scenario polyphenols may play an important role, either by directly affecting key steps in the activation pathway of NF-κB, or by modulating the intracellular redox status, which is, in turn, one of the major determinants of NF-κB



5.2 Simplified scheme of oxidant/antioxidant regulation of NF-κB activation. Different *stimuli*, leading to an increase of ROS generation inside the cell, activate the phosphorylation of IκB inhibitory protein and the subsequent proteolysis. Thioredoxin (Trx) may reduce activated NF-κB proteins facilitating nuclear translocation. Once released from IκB, the NF-κB complex translocates into the nucleus and the binding to DNA domain in the promoters and enhancers of genes such as TNF-α, IL-1, proliferation and chemotactic factors, adhesion molecule. Some of these genes, in turn, may further induce NF-κB activation, leading to a vicious circle if the regulatory cellular system escapes from control.

activation.[6,25] Consistently, experimental data are accumulating regarding polyphenolic compounds as natural phytochemical antioxidants that possess anti-inflammatory properties by downregulating NF-κB. Some of the most relevant findings about this aspect are summarised in Table 5.1.

5.5 Other aspects of polyphenols as modulators of signal transduction

Several studies have demonstrated that depending on their structure, flavonoids may be inhibitors of several kinases involved in signal transduction, mainly protein kinase C (PKC) and tyrosine kinases.[26–29] Agullo et al.[30] tested 14 flavonoids of different chemical classes and reported that myricetin, luteolin and apigenin were efficient inhibitors of phosphatidylinositol 3-kinase, PKC and tyrosine kinase activity. The authors also observed a structure–function in that the position, number and substitution of hydroxyl groups on the B ring and the saturation of C_2–C_3 bonds affect

Table 5.1 Flavonoids and flavonoid-related compounds suppressing NF-κB activity in cell culture studies

Name	Concentration (duration)	Inducers	Cell lines	Ref.
Apigenin	25 μM (4 h)	TNFα, TNFα + IFNγ	HUVEC	[Gerritsen et al., 1995]
Caffeic acid phenetyl ester (*propolis*)	25 μg/ml (2 h)	TNFα PMA Ceramide-C8 Okadaic acid H_2O_2	U937	[Natarajan et al., 1996]
Epigallocatechin-3-gallate (green tea)	15 μM (Co-incubation with inducer)	LPS	Mouse peritoneal macrophages	[Lin et al., 1997]
	100 μM (2 h)	LPS	RAW 264.7	[Yang et al., 1998]
Genistein (soy, clover)	148 μM (1–2 h)	TNFα,	U937, Jurkat, HeLa	[Natarajan et al., 1998]
		Okadaic acid	U937	
Ginkgo biloba extract	100–400 μg/ml (18 h)	H_2O_2	PAEC	[Wey et al., 1999]
Quercetin (wine, onion)	265 μM (1 h)	TNFα	U937	[Natarajan et al., 1998]
	10 μM (co-incubation with inducer)	H_2O_2	HepG2	[Musonda and Chipman, 1981]
Silymarin (*Silybum marianum*)	12.5 μg/ml (24 h)	Ultraviolet	HaCaT	[Saliou et al., 1999]
		Okadaic acid LPS	HepG2	[Saliou et al., 1998]
		TNFα	Würzburg	
		TNFα	U937, HeLa, Jurkat	[Manna et al., 1999]
Taxifolin (pine bark)	303 μM (24 h)	IFNγ	RAW 264.7	Park et al., 2000
Theaflavin-3,3′-digallate (black tea)	10 μM (co-incubation with inducer for 1 h)	LPS	RAW 264.7	[Lin et al., 1999]

M. E. Gerritsen, W. W. Carley, G. E. Ranges, C. P. Shen, S. A. Phan, G. F. Ligon and C. A. Perry (1995) *Am. J. Pathol.* **147**, 278.

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C. Saliou, B. Rihn, J. Cillard, T. Okamoto and L. Packer (1998) *FEBS Lett.* **440**, 8.

S. K. Manna, A. Mukhopadhyay, N. T. Van and B. B. Aggarwal (1999) *J. Immunol.* **163**, 6800.

Y. C. Park, G. Rimbach, C. Saliou, G. Valacchi and L. Packer (2000) *FEBS Lett* **465**, 93.

Y. L. Lin, S. H. Tsai, S. Y. Lin-Shiau, C. T. Ho and J. K. Lin (1999) *Eur. J. Pharmacol.* **367**, 379.

flavonoid activity on different kinases. Wolle et al.[31] examined the effect of flavonoids on endothelial cell expression of adhesion molecules. A synthetic flavonoid, 2-(3-amino-phenyl)-8-methoxy-chromene-4-one, an analog of apigenin, markedly inhibited TNF-α-induced VCAM-1 cell surface expression in a concentration-dependent fashion, but had no effect on ICAM-1 expression. The inhibition correlated with decreases in steady state mRNA levels, resulting in a reduction in the rate of gene transcription rather than changes in mRNA stability. No effects on NF-κB activation were observed either by mobility shift assay or by reporter gene assay, indicating that the modulation of VCAM-1 gene expression is due to a NF-κB-independent mechanism. More recently, Nardini et al. reported that both caffeic acid and the procyanidin-rich extract from the bark of *Pinus maritima* inhibit *in vitro* the activity of phosphorylase kinase, protein kinase A and protein kinase C.[32] Taken together, these studies opened an important issue in the ability of polyphenols to modulate the expression of genes responsible for pro-atherogenic processes with or without altering the activity of NF-κB, which can be considered fundamental for other cellular functions.

Hu et al.[33] reported that oncogene expression (c-myc, c-raf and c-H-ras) *in vivo*, induced by nitrosamine treatment, is inhibited in mouse lung by tea drinking. The same authors also reported that topical pre-treatment with the tea flavonoid (–)-epigallocatechin gallate significantly inhibits oncogene expression induced by PMA in mouse skin.[33] Similarly, c-fos expression, cell growth and PKC activity induced by PMA in NIH3T3 cells were inhibited by the natural flavonoid apigenin, as reported by Huang et al.[34] Green tea polyphenol extract stimulates the expression of detoxifying enzymes through antioxidant responsive element in the cultured human hepatoma cell line HepG2.[35] This activity seems to be mediated by potentiation of the mitogen activated protein kinases (MAPKs) signalling pathway, suggesting an indirect activity of polyphenols in the regulation of cellular responses to oxidative injury. Lin et al.[36] reported that both curcumin and apigenin inhibit PKC activity induced by PMA treatment in mouse skin. The same inhibitory effect can be observed in mouse isolated fibroblasts pretreated with curcumin. Apigenin, kaempferol and genistein reverted the transformation of the morphology of the v-H-ras transformed NIH3T3 line. The authors suggest that both PKC activity and oncogene expression may be the mechanism by which polyphenols exert their anti-tumor activity.[36] The flavonoid silymarin inhibits the expression of TNF-α mRNA induced by either 7,12-dimethylbenz(a)anthracene or okadaic acid in the SENCAR mouse skin model.[37] This inhibitory activity, which is associated with a complete protection of mouse epidermis from tumour promotion by OA and results in a significant reduction (up to 85 %) of tumour incidence induced by 7,12-dimethylbenz(a)anthracene,[26] may also be relevant in the atherogenesis, since TNF-α plays a central role in the vicious circle of macrophage-endothelial cell dysfunction.[24,38]

The cell-to-cell interaction following the expression of adhesion molecules (ICAM-1, VCAM-1 and selectin) in endothelial cells induced by cytokines treatment has been reported to be blocked by hydroflavones and flavanols.[39] Apigenin, the most potent flavone tested in this study, inhibited the expression of adhesion molecules, the expression of both interleukin-6 and interleukin-8 induced by TNF-α and interleukin-1-induced prostaglandin synthesis. Apigenin was found to have no effect on the nuclear translocation of NF-κB, but significantly inhibited the expression of the reporter gene β-galactosidase driven by NF-κB elements in SW480 cells induced by TNF-α, suggesting that NF-κB transcriptional activation was affected.[39] Also the adhesion of cytokine treated lymphocytes to endothelial cells was blocked by pretreatment of endothelial cells with apigenin.[39] Finally, the same study reports apigenin to have a strong anti-inflammatory activity *in vivo* on carragenin-induced rat paw edema and on delayed type hypersensitivity in the mouse. Taken together, these data suggest that both flavonoids and phenolic acids may have important effects in diseases involving leukocyte adhesion and trafficking and oxidant-induced gene expression.

5.6 Indirect evidence for polyphenol activity in atherogenesis

An indirect effect of flavonoids and phenolic acids on NF-κB activation, and therefore on NF-κB-driven gene expression, may be inferred from two kinds of study: one addressing the modulation of NF-κB activity by other antioxidant molecules (α-tocopherol, thiolic antioxidants such as *N*-acetyl-cysteine, lipoic acid, pyrrolidinedithiocarbamate), and others addressing the role of flavonoids and phenolic acids in the antioxidant network. α-Tocopherol and lipoic acid inhibit NF-κB in different cellular models,[40–42] and several studies describe the ability of flavonoids and phenolic acids to exert a significant tocopherol and glutathione sparing effect either under basal homeostatic conditions or following oxidative challenge.

Roy and co-workers demonstrated that the adhesion of lymphocyte to endothelial cells is regulated by the thiolic antioxidant α-lipoic acid and by α-tocopherol.[43] Similarly, an enhancement of the endogenous levels and a protective effect on α-tocopherol after peroxynitrite treatment by the procyanidin-containing extract from pine bark was reported by Virgili et al.[44] The same complex mixture of procyanidins has been reported to enhance the activity of the enzymatic machinery which regulates the GSH redox status in endothelial cells.[45,46] In fact, a significant increase in GSH (reduced glutathione) levels, an increased activity of the GSH redox enzymes (GSH reductase and GSH peroxidases) and an increase in the enzymatic activity of both SOD (superoxide dismutase) and catalase have been reported and proposed by Wei and collaborators to be mediated by

an increase of protein synthesis.[46] The important role of GSH in the antioxidant network usually results in a greater resistance to pro-oxidant cytotoxicity and, in general, leads to a greater resistance of cells to dysfunction.[17]

Proliferation of vascular smooth muscle cells is one of the most important features of arteriosclerosis.[6] Vascular smooth muscle cells display a unique susceptibility to antioxidants which indicates that they respond differently from other types to changes in the redox status. In fact, hydrogen peroxide has been demonstrated to stimulate the proliferation of vascular smooth muscle cells while inhibiting the proliferation of vascular endothelial cells.[47] However, the effect of antioxidants on smooth muscle cell proliferation is still unclear. α-Tocopherol inhibits the proliferation of smooth muscle cells by preventing the activation of PKC.[48] Two structurally different thiol-containing compounds, *N*-acetylcysteine (NAC) and pyrrolidinedithiocarbamate (PDTC) have been reported to induce apoptosis in cultured vascular smooth muscle cells in a dose- and time-dependent fashion.[49] In the same report the overexpression of the proto-oncogene *bcl-2* was observed to counter PDTC and NAC-induced apoptosis, suggesting that thiol oxidation status in the cell plays an important role in switching on the apoptotic program.

5.7 Conclusions and future trends

Dietary consumption of polyphenols is associated with a lower risk of degenerative diseases. In particular, protection of serum lipids from oxidation, which is a major step in the development of arteriosclerosis, has been demonstrated. More recently, new avenues have been explored in the capacity of polyphenols to interact with the expression of the human genetic potential. The understanding of the interaction between this heterogeneous class of compounds and cellular responses, due either to their ability to interplay in the cellular antioxidant network or directly to affect gene expression has increased.

One main line of future research could be in the inhibitory/activating effect on key enzymes involved in the pathogenesis of arteriosclerosis. In particular, enzymes regulating signal transduction involved in phosphorylation of proteins, such as PKC and tyrosine protein kinase, seems to be somehow modulated by different polyphenols and may represent a possible target for polyphenol activity.

The ability of polyphenols to modulate redox-sensitive pathways of cellular response in endothelial cells, lymphocytes and smooth muscle cells has also been observed. Although some data is already available on NF-κB, AP-1 and other transcription factors sensitive to the cellular redox status in response to oxidatively modified LDL, the cellular response to lipoproteins modified by the exposure to reactive nitrogen species, is still largely unknown. The unravelling of the mechanisms of the regulation of tran-

scriptional control of gene expression will possibly be a promising future line of investigation.

In conclusion, polyphenols seem to be able to affect the expression of genes involved in the pathogenesis of atherogenesis. Cytokines and adhesion molecules appear to be among the most important genes expressed during the pro-inflammatory situation which precedes the formation of the atheroma, and have also been reported to be affected, at least in part, by phenolics. We can therefore foresee that a considerable effort will be addressed to the study of the mechanisms through which polyphenols affect the control of the espression of these genes. These studies will give a solid background for the understanding of the molecular mechanisms of the beneficial effects of polyphenols on human health.

5.8 List of abbreviations:

- IκB: inhibitory protein kappa B
- ICAM-1: intercellular adhesion molecule 1
- IL-1: interleukin-1
- LDL: low density lipoprotein
- MAPKs: mitogen activated protein kinases
- MCP-1: macrophage chemotactic protein 1
- M-CSF: macrophage colony stimulating factor
- mmLDL: minimally modified LDL
- NAC: *N*-acetylcysteine
- NF-κB: nuclear factor-kappa B
- oxLDL: oxidised LDL
- PKC: protein kinase C
- PMA: phobol myristate acetate
- ROS: reactive oxygen species
- TNF-α: tumour necrosis factor alpha
- AM-1: vascular cell adhesion molecule 1

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6

Antioxidants and antitumour properties

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6.1 Introduction

Cancer is as old as the human race but what little evidence is available suggests that it was probably a relatively rare disease in the ancient world. In most populations, the principal causes of death are infant mortality, infectious disease and the chronic conditions of old age – principally cancer, heart disease and stroke. Deaths from the first two causes tend to decline dramatically with increasing prosperity, so cancer and cardiovascular disease inevitably cause a larger proportion of deaths in industrialised countries than they do in the developing world. Nevertheless, the precise reasons for the high levels of death from cancer experienced today in developed countries are controversial. Since cancer is largely a disease of old age, its prevalence will inevitably rise with the average longevity of the population but other factors seem to be at work in prosperous countries. Even in the nineteenth century it was possible for Tanchou to propose that increasing rates of cancer were a characteristic of urban societies,[1] and careful international studies of age-corrected rates for cancer continue to support this view.[2] A classic illustration of the historical association between increased industrialisation and cancer rates is provided by Japan, where until quite recently rates of breast and colorectal cancer were four to five times lower than in the USA and many countries of Northern Europe, whereas stomach cancer was several times more common. Since 1970 rates of breast and bowel cancer have risen steeply in Japan, but stomach cancer, as in many other industrialised countries, has declined. The explanation for these changes must lie in some aspect of environment or lifestyle, but despite decades of epidemiological and laboratory investigation we are still far

from understanding the factors that determine the risk of cancer at sites other than the lung.

In their classic epidemiological analysis of this issue, Doll and Peto[3] estimated that diet was responsible for approximately 35% of cancers in the West; however, the uncertainty attached to this estimate was very high, and the precise causes virtually unknown. More recently, in an encyclopaedic report on nutrition and cancer, the World Cancer Research Fund[4] has confirmed the central importance of diet as a major determinant of many forms of cancer across the globe, and stated that the sharp increase in cancer rates in the developing world should be regarded as a global public health emergency.

The interactions between diet and the biological processes leading to the development of cancer are extremely complex but one can envisage three general factors that are potentially important in any human population. The first is the presence in food of carcinogenic compounds which play an active role in damaging cells and inducing tumours. This topic is largely beyond the scope of this chapter, but in any case its relevance to Western industrialised societies is questionable. Although there are many proven carcinogens in our diets, the human body is equipped with efficient defences, and the level of exposure is usually far too low to be of relevance to health. One obvious exception to this rule is the chronic exposure of many of us to ethanol from alcoholic drinks, but the decision to drink alcohol lies in the hands of the consumer, and there is evidence that there are protective effects of alcohol against heart disease,[5] and these may well outweigh the adverse effects on cancer.

The second issue is the adequacy of nutrient intake, and the possibility that certain deficiencies might influence an individual's susceptibility to cancer. The risks of cancer tend to be greater at the lower end of the socio-economic scale in many western countries. The reasons for this are complex; part of the reason may be that though there is little evidence that this is due to malnutrition in the classical sense, it remains possible that optimal levels of certain nutrients may be higher than is currently accepted. Finally, susceptibility to cancer may be increased by an inadequate intake of biologically active food components that exert anticarcinogenic effects, but which are not currently classified as nutrients in the conventional sense. Since the early 1980s a large body of epidemiological evidence in favour of a protective effect of plant foods has appeared and become generally accepted by nutritionists and regulatory bodies.[4,6,7] The widespread promulgation of public health measures to encourage the consumption of five 80g portions of fruits and vegetables per day has been one outcome of this consensus, and another has been a remarkable growth of interest in the possibility that the active principles in fruits, vegetables and cereals might be incorporated into functional foods. The purpose of this chapter is to review the nature of cancer, and to explore the various ways in which antioxidants and some other

dietary factors can influence the initiation and development of this group of diseases.

6.2 The nature of tumour growth

The existence of cancer and the distinction between benign and malignant tumours were recognised by the early Greek physicians, who coined the term 'carcinoma', derived from the Greek *karkinos*, meaning 'crab', alluding to the creeping crab-like behaviour of a spreading tumour. The development of microscopy eventually led to the recognition that tumours contained cells that differed fundamentally in appearance and behaviour from those of the surrounding tissue. Oncology, the scientific investigation and clinical treatment of tumours, was founded in the early years of the twentieth century but it is only since about 1980 that the development of the cell and molecular sciences has enabled biologists to begin to acquire a deeper understanding of tumour biology. Much of this insight has been gained through the use of isolated tumour cells grown *in vitro*, and of animal models of carcinogenesis, which enable tumours to be studied within the complex environment of living tissue. Both of these approaches have their limitations and we are still far from a full understanding of cancer in human beings.

All cancers are diseases of abnormal cell proliferation, development and death. During the earliest stages of human life all of the embryonic cells divide constantly, and differentiate to form the specialised tissues and organs. Throughout infancy and childhood cell proliferation continues at whatever rates are necessary to fulfil the requirements of growth, but as maturity is reached organs such as the central nervous system, muscles and skeletal tissues cease to grow, and cell division becomes minimal. However, certain tissues continue to proliferate throughout life. These include the blood-forming tissues, the epithelia which line the surfaces of the body exposed to the environment, the glandular tissues which produce secretions, and the sexual organs which produce new reproductive cells. Cancer can affect virtually any organ of the body but tissues such as those of the lungs and gut, which have characteristically high rates of cell division and chronic exposure to the external environment, are particularly vulnerable.

6.2.1 Tumour cell biology

A tumour can be defined as any focal accumulation of cells beyond the numbers required for the development, repair or function of a tissue. Tumours may be benign or malignant. The former are usually relatively slow growing, but more importantly the cells tend to retain much of the specialisation and spatial localisation of the tissue from which they are derived. In contrast, malignant cells are characterised by a loss of differentiation,

faster growth and a tendency to invade surrounding tissues and migrate to other organs to form secondary tumours or metastases. Thus cancer may be defined as the development, growth and metastatic spread of a malignant neoplasm. Malignant tumours derived from epithelial cells are called carcinomas, and those derived from connective or mesenchymal cells are called sarcomas. It is usually the secondary tumour that is lethal, so the early diagnosis of malignant primary tumours is essential for effective treatment.

6.2.2 Molecular biology

Regardless of their function in the body, all cells carry a complete set of genetic instructions for the development and function of the whole organism. The subset of genes which is expressed by any particular cell type determines its *phenotype*, the precise details of the structure, specialised functions and life cycle of the cell which enable it to exist in harmony with other cells as part of a tissue. The events that occur during the early stages of cancer development usually involve damage to the DNA coding for such crucial genes.

With the exception of certain cancers of childhood which often affect growing tissues such as the brain or bones, *carcinogenesis* – the development of cancer from normal cells – is usually a relatively slow process which occupies a substantial proportion of the lifetime of an individual. Tumour cells invariably contain a number of mutations affecting genes controlling the rate at which cells divide, differentiate or die, or the efficiency with which DNA damage is repaired.[8,9] Such mutations may be inherited though the germ-line, and these form the basis for a number of recognised familial cancer syndromes, but most of the genetic abnormalities detectable in sporadic cancers, which are far more common, are somatic mutations acquired during carcinogenesis. Such damage may result from exposure to radiation or chemical mutagens, or through the effects of molecular species such as oxygen free radicals generated by the normal metabolism of the body. Whatever the source of the DNA damage, however, the defining characteristic of a pro-carcinogenic mutation is that it favours the proliferation and survival of an abnormal population of cells that have the potential for further evolution towards the malignant state.[10] Chemical carcinogens such as those present in tobacco smoke tend to be electrophiles – substances that can react easily with electron-rich regions of cellular proteins and DNA. The products formed by such interactions with DNA are called adducts. These are stable compounds which disrupt the synthesis of new DNA when the cell next divides, so that the sequence of genetic code in that region is damaged and the new cell carries a mutation. Many chemical carcinogens must be activated to an electrophilic form before they can act and, ironically, this often occurs as part of the sequence of events employed by the cell to detoxify the parent molecule or pro-carcinogen.

Many of the target genes that undergo mutation during carcinogenesis have been identified, and their functions and interactions with other genes are at least partially understood.[11] The proto-oncogenes were first identified through their near-homology to the critical DNA sequences present in certain cancer-causing viruses which, when inserted into mammalian cells, would transform them into tumours. These so-called viral oncogenes have evolved through the 'capture' and exploitation of mammalian genes by viruses. In their original form such genes are essential components of normal mammalian cellular physiology and are expressed, usually to facilitate increased cellular proliferation, only at critical stages in the development or function of a tissue. When such 'proto-oncogenes' are activated inappropriately within the mammalian genome, without the intervention of a virus, they are termed 'oncogenes'. This can occur because of a mutation to the control sequence for the gene, causing overexpression of the normal product, or a mutation in the coding sequence itself, giving rise to a product that functions normally but which cannot be broken down. For example, the K-*ras* gene, which codes for a protein-regulating cell proliferation, is mutated and hence abnormally expressed early in the development of approximately 40% of human colorectal carcinomas.[12]

In contrast to the proto-oncogenes, overexpression of which creates conditions that favour tumour growth, it is the loss of expression of a tumour-suppressor gene that facilitates the development of malignant characteristics in a cell. The *p53* gene is a good example.[13] The *p53* product is a protein of molecular weight 53kD, which functions as a regulator of cell proliferation, and as a mediator of programmed cell death in response to unrepaired DNA damage. The absence of *p53*, or its presence in a mutated and therefore non-functional form, allows cells bearing other forms of DNA damage to continue dividing rather than undergoing apoptosis.[14,15] There are familial forms of cancer caused by an inherited *p53* defect, and acquired mutations of this gene are among the most common genomic abnormalities found in a variety of human cancers.

According to the 'two hit hypothesis' for the functional role of tumour-suppressor genes, mutations at both alleles are required fully to inactivate the tumour suppressor activity of such genes.[16] However, another important mechanism for the induction of genetic abnormalities has attracted attention in recent years. Cytosine bases in the DNA backbone can acquire a methyl group which, if they lie within the promoter region of a gene, can cause it to be 'silenced' or, in effect, switched off.[17] This is a normal mechanism for the regulation of gene expression but it is becoming clear that abnormal DNA methylation can also occur and be transmitted across successive cell divisions. This provides a so-called 'epigenetic' mechanism for the inactivation of genes regulating tumour suppression or DNA repair, which can contribute to the complex series of events leading to the development of a tumour.[18]

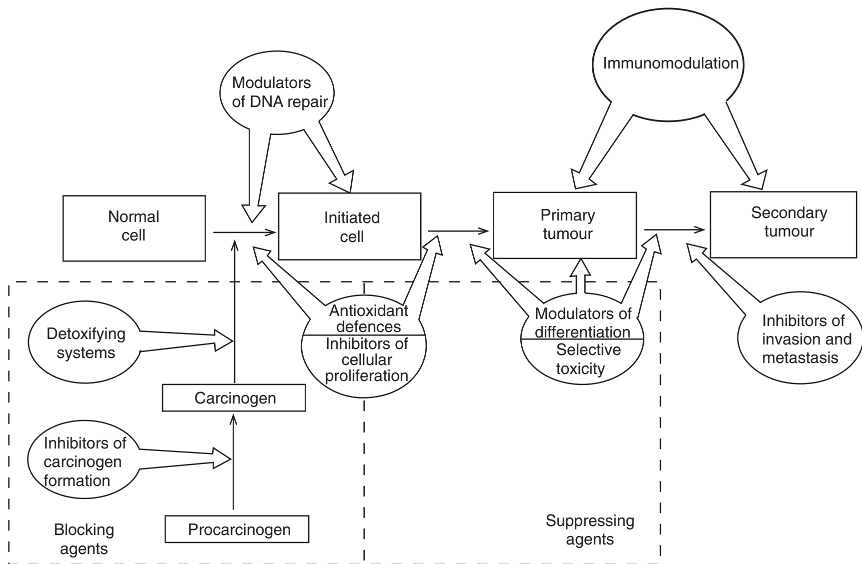
6.3 Models of carcinogenesis

The simplest experimental model of carcinogenesis is the three-stage model consisting of initiation, promotion and progression.[19] At the initiation stage, a single cell is thought to acquire a mutation and then divide repeatedly so that the mutation is passed on to a clone of daughter cells, thus forming a focal lesion that can survive and grow at the expense of neighbouring cells. During promotion, the normal constraints on proliferation and spatial organisation within the affected tissue are disrupted further, and the appearance of further mutations to proto-oncogenes and tumour suppressor genes leads to a progressive loss of differentiation and orderly growth. The genes involved in this transition to cancer, and the functions they perform, are under intensive investigation and are particularly well characterised in the intestinal epithelium.[20]

At the progression stage the lesion has made the transition to malignancy and can give rise to secondary tumours at remote sites. Animal models have been used to identify specific carcinogenic substances which can act as mutagens at the intiation stage but do induce malignancy on their own, promoters which cannot initiate tumours but do accelerate tumour development after initiation, and complete carcinogens, which can do both. As we shall see later, this approach has also been used to identify inhibitors of carcinogenesis and to delineate their mode of action. The difficulty with animal models of carcinogenesis is that they usually require the application of large doses of carcinogens and promoters to groups of rodents, so that a high tumour yield is obtained during the course of the experiment. Such techniques are a poor model for induction of human cancers because these are usually caused by very prolonged exposure to a complex array of unknown carcinogenic stimuli over the course of a lifetime. However, there is no doubt that much of the fundamental understanding of tumour biology that has been gained from animal studies applies also to human disease.

6.4 Diet and gene interactions

As we have seen, carcinogenesis is a prolonged multi-stage process which usually occurs over many years. Because of its complexity there are, in principle, many critical steps at which food-related substances or metabolic processes may interact with the sequence of events so as to accelerate, delay or even reverse it. Diet-related anti-carcinogenesis can usefully be classified into *blocking mechanisms*, which operate during the initiation phase of carcinogenesis, and *suppressing mechanisms*, which delay or reverse tumour promotion at a later stage.[21,22] A schematic illustration of these concepts and a summary of the mechanisms through which they may act is given in Fig. 6.1.



6.1 Hypothetical sites of interaction between anti-carcinogenic substances in the diet and the progressive stages of carcinogenesis. Blocking agents are those acting to prevent initiation, whereas suppressing agents act to inhibit the development of tumours from initiated cells. (Reproduced from Johnson et al., 1994.[21])

The principal blocking mechanism through which dietary constituents are thought to act is modulation of the Phase I and Phase II biotransformation enzymes which are expressed strongly in the gastrointestinal mucosa and in the liver and act as a first line of defence against toxic substances in the environment.[23] Phase I enzymes such as the cytochrome P450 complex catalyse oxidation, reduction and hydrolytic reactions, thereby increasing the solubility of potentially toxic compounds. However, this phase may also create electrophilic intermediates and hence activate procarcinogens. Phase II enzymes such as glutathione *S*-transferase act on the products of Phase I metabolism to form conjugates, which generally reduces their reactivity and increases their excretion. Thus the biological activity of a carcinogen will often depend upon the relative activities of the Phase I and II enzymes involved in its metabolism. Pharmacological and dietary treatments can be used to block Phase I enzymes and enhance Phase II activity, so as to minimise the activation of carcinogens and increase their excretion. There is good evidence from experimental animal studies that this strategy can reduce DNA damage and tumour yield.[24]

Experimental animal studies have also shown that some substances can inhibit the appearance of tumours, even when given days or weeks after exposure to a chemical carcinogen.[25] Hence the mechanism of action cannot involve protection against DNA damage, but can instead be due to some

reduction in the rate at which initiated cells develop into tumours (Fig. 6.1). Suppression of carcinogenesis may involve inhibition of mitosis and increased expression of the differentiated phenotype, which serves to reduce the clonal expansion of initiated cells, or an increased susceptibility to undergo programmed cell death or apoptosis, which can eliminate precancerous cells from the tissue.[26,27]

6.5 Mechanisms of action: nutrients

6.5.1 General nutrition

Although prolonged energy, protein or micronutrient malnutrition may increase an individual's risk of developing cancer, perhaps by reducing the effectiveness of the immune system, life expectancy in societies with large malnourished populations is low, and infectious diseases are more likely to be the principal causes of illness and mortality. In prosperous Western societies, over-consumption of energy, coupled with inadequate exercise, appears to be a major risk factor for cancer. The World Cancer Research Fund report on diet and cancer[4] made some general recommendations on food supply, eating and related factors. For individuals, the general advice was to consume nutritionally adequate and varied diets based predominantly on fruits, vegetables, pulses and minimally processed starchy foods. Overweight, defined as body mass index (BMI: weight in kg/[height in metre]^2) in excess of 25 is associated with a rise in the relative risk of most cancers, and frank obesity is particularly associated with cancers of the breast and endometrium. For these reasons the report recommended that BMI should be maintained between 18.5 and 25. The committee did not consider that fat consumption was directly associated with cancer risk, but it did recommend that fat should contribute no more than 30 % of total energy consumption, so as to reduce the risk of weight gain.

The general recommendations on energy and fat intake are similar to those for the avoidance of heart disease and are not in themselves very relevant to the concept of functional foods. However, the recommendation to consume a variety of fruits and vegetables is based partly on the putative presence of diverse protective factors in plant foods. This concept does provide, at least in principle, a rationale for the development of functional products with desirable biological effects beyond the simple provision of nutrients at a level that prevents symptoms of deficiency.

6.5.2 Antioxidant nutrients

As mentioned earlier, mutations can occur as a result of oxidative damage to DNA caused by free radicals generated as a damaging side-effect of aerobic metabolism.[28] Superoxide radicals are formed by the addition of an

electron to molecular oxygen. These highly reactive species can then acquire a further electron and combine with protons to form hydrogen peroxide. In the presence of transition metal ions such as Fe^{2+} and Cu^{2+}, hydrogen peroxide can break down to give even more highly reactive hydroxy radicals which can damage DNA directly, or participate in self-propagating chain reactions with membrane lipids. Plant and animal cells defend themselves against these effects by deploying so-called antioxidant compounds to trap or quench free radicals and hence arrest their damaging reactions. A variety of defence systems based on both water- and lipid-soluble antioxidant species and on antioxidant enzymes are deployed throughout the intra- and extracellular environment, at the sites most vulnerable to pro-oxidant damage. Many of those in the human body are dependent upon antioxidants derived from the diet. The theory that free radicals are a major cause of human cancer and that the risk of disease can be reduced by increased consumption of food-borne antioxidants has prompted an enormous growth of interest in antioxidant nutrients and other antioxidant substances in food.[29] It is worth noting, however, that the role of mutagenesis due to oxygen free radicals in the pathogenesis of human cancers remains largely hypothetical,[28] and attempts to prevent cancer by intervention with high doses of antioxidant vitamins have been largely unsuccessful.[30,31]

6.5.2.1 Vitamin E

The major lipid-soluble antioxidant is vitamin E, first isolated from wheatgerm oil and obtained principally from nuts, seed oils and cereals. Vitamin E is actually a collective term for eight compounds: α-, β-, γ- and δ- tocopherol, and α-, β-, γ- and δ- tocotrienol, but RRR-α-tocopherol, accounts for 90 % of endogenous vitamin E activity in humans. All the tocopherols and tocotrienols contain a hydroxyl-bearing aromatic ring structure, which enables them to donate hydrogen to free radicals, and thus act as biological antioxidants. The unpaired electron which results from hydrogen donation is delocalised into the ring structure of the tocopherol, rendering it relatively stable and unreactive. Chain reactions initiated by hydroxy radicals can be broken by the formation of a stable radical as a result of interaction with vitamin E.[32] Vitamin E is readily incorporated into cell membranes, which, being rich in polyunsaturated fatty acids, are highly susceptible to damage by free radicals derived from metabolic activity. In humans, frank symptoms of vitamin E deficiency are only seen in premature infants or malabsorption states, but intakes higher than are required to protect against deficiency may provide additional protection against free-radical mediated DNA damage. Epidemiological studies show a strong inverse correlation between risk of cancer and vitamin E intake at the population level, but the association is not corroborated by studies of individuals taking supplements.[33] Moreover, a well-controlled investigation designed to test the hypothesis that dietary supplementation with vitamins

C and E would reduce the recurrence of adenomas in patients who had undergone polypectomy showed no evidence of a protective effect.[31] Similarly, a prolonged placebo-controlled intervention with vitamin E or vitamin E and beta-carotene failed to prevent the development of lung cancer in smokers.[30]

6.5.2.2 Carotenoids

Approximately 500 carotenoids have been identified in vegetables and fruits used as human foods, but the vast majority of these compounds occur at low concentrations and are probably of little nutritional importance. By far the most well-known and intensively studied of the carotenoids is beta-carotene,[34] which is a precursor for vitamin A, but the increased interest in dietary antioxidants in recent years has focused attention on other carotenoids such as lycopene and lutein which are abundant in tomatoes and coloured vegetables.[35,36] The molecular structure of the carotenoids includes an extended chain of double bonds which enables them to function as antioxidants. Carotenoids are released from plant foods in the small intestine and absorbed in conjunction with dietary fat. Beta-carotene is converted into vitamin A by enzymes in the intestinal mucosa but it is detectable in human plasma, at levels that are related positively to the dietary intake of fruits and vegetables, and at least ten other carotenoids have also been recorded in human blood.

There is good epidemiological evidence for an inverse association between intake of carotenoids and lung cancer, and weaker evidence for protective effects against cancers of the alimentary tract.[37] The possibility that carotenoids might express antioxidant activity in human tissues, thereby protecting cell membranes, proteins and DNA against damage by free radicals, provides a plausible rationale for these associations, but once again the causal link has not been proven and the possibility remains that carotenoids are acting as markers for fruit and vegetable intake which may be beneficial for other reasons.[36] Intervention trials with beta-carotene have proved disappointing. The alpha-tocopherol beta-carotene (ATBC) study, which involved over 29000 male smokers, and included a cohort given 20mg betacarotene daily for up to eight years, produced no evidence for a protective effect against cancer at any site. On the contrary, there was a higher incidence of lung, prostate and stomach cancer in the beta-carotene group.[30] Similarly, the CARET study, in which subjects received 30mg beta-carotene and 25000 international units of retinol per day, was terminated because of an increase in deaths from lung cancer in the treatment group.[38] There is no suggestion that beta-carotene is toxic in any other circumstances, even when given at pharmacological doses for long periods to treat photosensitivity disorders,[39] but the evidence suggests that it may act as a tumour promoter when taken by subjects already harbouring precancerous lesions induced by chronic exposure to tobacco smoke. Under these circumstances it is obviously inappropriate to encourage the devel-

opment and consumption of functional foods designed to provide consumers with high doses of carotenoids, but the general advice to increase fruit and vegetable consumption remains valid.

6.5.2.3 Vitamin C

Vitamin C occurs as L-ascorbic acid and dehydroascorbic acid in fruits, vegetables and potatoes, as well as processed foods to which it has been added as an antioxidant. The only wholly undisputed function of vitamin C is the prevention of scurvy. Although this is the physiological rationale for the currently recommended intake levels, there is growing evidence that vitamin C may provide additional protective effects against other diseases including cancer, and the RDA may be increased in the near future. Scurvy develops in adults whose habitual intake of vitamin C falls below 1 mg per day, and under experimental conditions 10 mg per day is sufficient to prevent or alleviate symptoms.[40] The recommended dietary allowance (RDA) is 60 mg per day in the USA, but plasma levels of ascorbate do not achieve saturation until daily intakes reach around 100 mg.[41] Ascorbate is probably the most effective water-soluble antioxidant in the plasma. It scavenges and reduces nitrite, thus inhibiting the formation of carcinogenic *N*-nitroso compounds in the stomach, and *in vitro* studies suggest that it plays a protective role against oxidative damage to cell constituents and circulating lipoproteins.[42] The epidemiological evidence is consistent with a protective effect of vitamin C against cancers of the stomach, pharynx and oesophagus in particular,[43] but the evidence for causality remains inconclusive because of the sheer complexity of the composition of fruits and vegetables, which are the main source of the vitamin in the unsupplemented diet. Byers and Guerrero[33] considered the collective evidence from a large series of case-control and cohort studies in which intakes of fruits and vegetables, and of vitamins C and E from food or from supplements, were determined. There was a strong and consistent protective effect of fruits and vegetables against cancers of the alimentary tract and lung and a correlation with estimated vitamin C intake based on fruit and vegetable composition. However, there were considerable confounding effects of other dietary constituents and the evidence for a protective effect of vitamin C from supplements was less convincing. Most of the ascorbate in human diets is derived from natural sources, and consumers who eat five portions, or about 400–500 g, of fruits and vegetables per day could obtain as much as 200 mg of ascorbate. Nevertheless, given the low cost and toxicity of ascorbate, it seems likely that there will be a continuing trend towards supplementation of foods.

6.5.2.4 Selenium

Selenium is a trace mineral, widely distributed at relatively low concentrations in the human food chain. It is essential to human nutrition because of the role played by the amino acid selenocysteine as a component of the

mammalian selenoproteins. Over 30 of these have now been identified,[44] the best characterised and widely studied of which are the glutathione peroxidases. Cytosolic glutathione peroxidase was the first of the selenoproteins to be identified,[45] but three other tissue-specific glutathione peroxidases have been identified more recently. All of the glutathione peroxidases catalyse the reduction of hydrogen peroxide and other reactive oxygen species, including lipid peroxides, at the expense of the tripeptide glutathione, which is synthesised within the cell. This system is the major regulator of intracellular redox status throughout the body, but tissue-specific glutathione peroxidases such as that of the gastrointestinal mucosa probably play a more particular role in the detoxification of dietary lipid peroxides.[46] Biosynthesis of the selenoproteins is rate-limited by the supply of selenium in the diet, and selenium is therefore classed as an antioxidant nutrient. It is interesting to note that when the supply of dietary selenium is limited, the mineral is preferentially used for the biosynthesis of certain glutathione peroxidases at the expense of others. This hierarchy is thought to reflect their relative importance in the maintenance of antioxidant defences in key tissues.[44]

Selenium supplementation has been reported to have a variety of positive effects on the cardiovascular system, the immune system, and on general health, not all of which necessarily reflect its antioxidant role.[47] However, it is reasonable to propose that adequate selenium status is required to minimise the risk of cancer because maintenance of cellular redox balance is probably crucial to the prevention of free-radical mediated DNA damage. The design of epidemiological studies to test this hypothesis has been complicated by the difficulty of assessing accurately the exposure of individuals to dietary selenium, but there is evidence of an inverse relationship between risk of cancer and dietary intake of selenium at the population level.[48] Prospective studies in which the incidence of cancer has been correlated with selenium status assessed by analysis of toenails have provided evidence of protective effects against a variety of cancers, but such studies have usually been relatively small. In one recent study, however, a significant three-fold inverse association between prostate cancer and toenail selenium was observed in a cohort of 33 737 men.[49] Perhaps the strongest evidence for the protective effect of selenium comes from the Nutritional Prevention of Cancer Trial, an intervention study in which a selenium-rich yeast product was used in an attempt to reduce the recurrence of non-melanoma skin cancer. Although there was no effect of supplementation on the primary endpoint, there were significant reductions of around 50 % in the incidence of other cancers, including those of the lung, colon and prostate.[50] Further intervention trials with large cohorts designed to explore the implications of these findings are planned for both Europe and the USA.[47]

Dietary provision of selenium is problematic in a number of countries, including many in Europe, because of the low availability of soil selenium

to plants used as human food. Although there are a few rich sources of selenium in the human food chain, notably Brazil nuts and some types of offal, these are usually only eaten in small quantities. Wheat is one of the most important sources of selenium in western diets, but European cereals are relatively low in selenium compared to North American wheat, and this has led to fears that populations may be at increased risk of cancer because of sub-optimal selenium intakes.[51] Unfortunately, selenium is potentially toxic at relatively low levels of intake, and food fortification carries a real risk of harm to individuals who might be tempted to overconsume. Another strategy is the addition of selenium to agricultural fertilisers, so as to raise the selenium content of staple crops. This approach has been pursued successfully in Finland. Both public health policy in relation to selenium, and the commercial development of selenium-enriched products seem likely to continue to evolve as further information about the potential health benefits of selenium becomes available.

6.5.3 Folate

In historical terms, folates are among the most recently identified of the vitamins. Wills was the first to describe a form of anaemia associated with pregnancy and malnutrition which could be cured by yeast or liver extract.[52,53] The active constituent of these dietary supplements was eventually isolated as folic acid (pteroylglutamic acid), a water-soluble substance containing a pteridine ring linked to *para*-aminobenzoic acid and glutamic acid. Naturally occurring folates originate from green plants and yeast cells, and are plentiful in liver and kidney. They are usually reduced and substituted in the pteridene moiety, and contain up to seven glutamate residues. Dietary folates are deconjugated to the monoglutamic form at the surface of the intestinal mucosa, actively transported and mostly metabolised by the epithelial cells to the main circulating form which is 5-methyltetrahydrofolic acid. The principal metabolic role of folates and their derivatives is to act as coenzymes in reactions involving transfer of single carbon groups during the synthesis of amino acids and DNA. This accounts for their vital role in the support of growth, pregnancy and the production of blood cells. It has been conclusively established that an inadequate supply of folates during the early stages of embryonic development increases the risk of neural tube defects,[54] and a number of foods, including breakfast cereals and bread, are now routinely enriched with folic acid. Growing interest in the relationship between human folate status an the long-term risk of disease will probably ensure that this trend continues.

It is well established that folate-deficient diets are associated with increased risk of hepatic cancer in animal models.[55] Rats fed diets deficient in methyl donating groups have higher rates of cell proliferation, increased DNA damage and a higher susceptibility to experimentally induced cancers, which appears to result from changes in gene expression associated

with abnormalities of DNA synthesis.[56] The precise relationship between folate metabolism and carcinogenesis is unclear, but the link may lie in the role that folate coenzymes play in the control of DNA methylation. In mammals and many other organisms the cytosine nucleotides in the DNA backbone frequently become methylated by the enzyme DNA-methyltransferase (DNA-MTase) after replication. As mentioned earlier, the methylation pattern of the cytosine residues is now believed to be an important determinant of gene expression. Much remains to be learned about this topic, but in general, loss of methylation could cause abnormal expression of oncogenes controlling cell proliferation, whereas inappropriate methylation of cytosine-rich regions of DNA in the promoter regions of tumour suppressor genes could cause loss of function.[57]

Issa et al.[58] demonstrated that methylation of CpG islands in the oestrogen receptor gene (*ER*) occurs in a very high proportion of colorectal tumours, and that the same site-specific abnormality occurs progressively with age in the otherwise normal colorectal mucosa of human subjects with no colorectal neoplasia. The same authors have also shown that expression of *ER* in tumour cells slows mitosis and should perhaps be regarded as a tumour suppressor gene, the silencing of which may be an early 'field' event inducing hyperproliferation and predisposing the colorectal mucosa to induction of neoplasia. There is no direct evidence that human folate metabolism is involved with these phenomena, but there is circumstantial evidence that inadequate folate nutrition is a risk factor for cancer, particularly of the bowel and cervix.[59] Recently Ma et al.[60] explored the relationship between risk of colorectal carcinoma and a common mutation affecting the activity of the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) in a large cohort study. The presence of a homozygous mutation was shown to reduce the risk of colerectal cancer in men with adequate folate levels, but the protection was absent in men with low overall folate status. One possible explanation for this effect is that low levels of MTHFR expression shunt folates into DNA synthesis, thereby helping to maintain normal patterns of DNA methylation. Low levels of folate might favour hypomethylation of cytosines, and possibly cause a compensatory upregulation of DNA-MTase, leading to hypermethylation of CpG islands, but this remains highly speculative. Nevertheless, the growing epidemiological evidence that inadequate folate nutrition increases the risk of cancer whereas long-term use of folate supplements reduces risk[61] will ensure that interest in the preventive role of folate-supplemented foods will continue.

6.6 Mechanisms of action: phytochemicals

The discovery that in industrialised societies diets that are deficient in fruits and vegetables can effectively double the risk of developing many differ-

ent types of cancer has focused renewed attention on the beneficial properties of these foods.[62] As we have seen, plant foods are rich in micronutrients, but they also contain an immense variety of biologically active secondary metabolites providing colour, flavour and natural toxicity to pests and sometimes humans.[21] The chemistry and classification of such substances is still a matter for much research and debate, but this has not prevented attempts to isolate and exploit substances that have variously been termed 'protective factors', 'phytoprotectants' and 'nutraceuticals'. Commercial applications tend to be confined to the health food market at the present time. The non-nutrient carotenoids mentioned earlier fall into the present category, as do a host of compounds containing phenol rings, phytosterols, sulphur-containing compounds found in onions and their relatives, and another group of sulphur compounds, the glucosinolates from brassica vegetables. Only a few of the more important examples will be discussed here.

6.6.1 Phenolic compounds

A huge variety of biologically active phenolic compounds containing one or more aromatic rings are found naturally in plant foods, where they provide much of the flavour, colour and texture. The simpler phenolic substances include monophenols with a single benzene ring, such as 3-ethylphenol and 3,4-dimethylphenol found in fruits and seeds, the hydroxycinnamic acid group which contains caffeic and ferulic acid, and the flavonoids and their glycosides which include catechins, proanthocyanins, anthocyanidins and flavonols. The tannins are a complex and poorly defined group of water-soluble phenolics with high molecular weights. The daily intake of phenolic substances may be as high as 1 g per day, but the quantity of defined flavonoids in the diet probably amounts to no more than a few tens of milligrams per day.

6.6.1.1 Flavonoids

As long ago as 1936, Rusznyák and Szent-Györgi[63] proposed that the flavonols were an essential dietary factor contributing to the maintenance of capilliary permeability. This is no longer thought to be true, but recent interest in dietary antioxidants and metabolically active phytochemicals has focused renewed attention on the possible beneficial effects of flavonoids.[64,65] Flavonoids are very effective antioxidants and it has been proposed that they protect against cardiovascular disease by reducing the oxidation of low density lipoproteins. There is some epidemiological evidence for this, but flavonoids are generally poorly absorbed from food, and their effects on the overall antioxidant capacity of the plasma remains to be established. Nevertheless, flavonoids and other phenolic substances may exert local anti-carcinogenic effects in the intestine where, in addition to acting as intraluminal antioxidants, they may induce Phase II xenobiotic

metabolising enzymes, suppress the production of biologically active prostaglandins by inhibiting the arachidonic acid cascade,[66] and inhibit mitosis by inhibiting intracellular protein kinases.[67]

Although briefly under suspicion as a natural carcinogen,[68] the ubiquitous flavonol quercetin is now regarded as a possible protective factor against cancers of the alimentary tract.[69]

6.6.1.2 Phytoestrogens

The phytoestrogens are diphenolic compounds derived from plant foods and which bear a structural similarity to mammalian oestrogens.[70] The glycosides genistin and daidzin, and their methylated derivatives biochanin A and formononetin, which are found principally in soya products, are broken down by the intestinal microflora to yield genistein, daidzein, and in some individuals, equol, all of which are absorbed into the circulation, and they or their breakdown products can be detected in human urine.[71] The lignan precursors matairesinol and secoisolariciresinol occur more commonly in cereal seeds such as flax. They are also degraded in the gut to yield the active lignans enterolactone and enterodiol. These compounds exert weak hormone-like activity and may bind to oestrogen receptors *in vivo*, thereby effectively blocking the more potent activity of endogenous oestrogens. In human feeding trials with soy products, isoflavones have been shown to modify the menstrual cycle, and there is much interest in the possibility that these compounds could suppress the growth of hormone-dependent tumours of the breast and reproductive organs.[70] There are also epidemiological associations suggesting a protective effect of soy-based diets against prostate cancer in males,[72] but once again the causal mechanisms have not been proven and there is a strong possibility of confounding by other dietary factors.

Genistein may also suppress tumour growth by other non-oestrogenic mechanisms including suppression of cell turnover by inhibition of protein kinases involved in the regulation of mitosis. On the other hand, it is less widely recognised that genistein is an inhibitor of topoisomerase II, an enzyme that helps to maintain the structure of DNA during mitosis. Both synthetic topoisomerase poisons and genistein are known to be mutagenic *in vitro*, but the biological significance of this is unclear.[73] There is no epidemiological evidence to suggest any adverse effect of soy products in humans, but caution is obviously necessary when considering the incorporation of such biologically active compounds into functional foods.

6.6.2 Glucosinolates

Interest in glucosinolates stems from epidemiological and experimental evidence showing that brassica vegetables such as cabbage, sprouts, kale and

broccoli seem to offer particularly strong protection against cancer of the lung and gastrointestinal tract.[7,74] The brassicas, and a few other edible plants drawn from the order *Capparales*, are the source of all the glucosinolates in the human diet. Around 100 different compounds have been identified, all of which possess the same fundamental structure comprising a β-D-thioglucose group, a sulphonated oxime moiety and a variable side-chain.[75] Glucosinolates occur throughout the plant, although the concentration varies between tissues, and they are stable under normal conditions. However, when the plant tissue is physically damaged, for example by food preparation or chewing, they come into contact with an enzyme – myrosinase – which is released from intracellular vacuoles. Myrosinase hydrolyses the glucosinolates to release glucose and an unstable product which then undergoes further degradation to release a complex variety of breakdown products. The most important from the nutritional point of view are the isothiocyanates, a group of hot and bitter compounds, commonly termed 'mustard oils'. These compounds, which are often volatile with an acrid smell, are the principal source of flavour in mustard, radishes and the milder vegetables.[75] High levels of glucosinolates reduce the palatability of plant tissues for generalist herbivores such as birds and molluscs, but specialist invertebrate herbivores have adapted to their presence and may be attracted specifically to feed on plants containing particular compounds.[76] Glucosinolates with an aliphatic side chain containing a β-hydroxy group yield isothiocyanates which spontaneously cyclise to form stable oxazolidine-2-thiones. These compounds are goitrogenic to domestic livestock, and this is an important limiting factor in the commercial exploitation of brassica feedstuffs.[77]

There is ample evidence from both animal experiments and tissue cultures studies to show that brassica vegetables and their constituents selectively induce Phase II enzymes. Evidence for the induction of Phase II enzymes by two classes of glucosinolate breakdown products, the isothiocyanates and indole-3-carbinole, has been systematically reviewed recently by Verhoeven et al.[78] Particular attention has been paid to induction of Phase II enzymes by sulphorophane, an isothiocyanate derived from broccoli,[79] but other isothiocyanates derived from other common brassica vegetables probably exert comparable levels of biological activity.[80]

Wattenberg[25] showed that both cruciferous vegetables and benzyl isothiocyanate could inhibit the appearance of tumours in experimental animals long after the initial exposure to a carcinogen. Suppressing mechanisms are still poorly understood but one possibility is that glucosinolate breakdown products modulate the level of apoptosis in target tissues. Isothiocyanates have been shown recently to induce apoptosis in tissue culture, and in the colorectal crypts of the rat after treatment with the carcinogen dimethylhydrazine, an effect which is associated with a reduction in pre-cancerous lesions.[27]

6.7 Conclusion: the role of functional foods

Great progress has been made towards a better understanding of the relationship between diet and cancer since Doll and Peto published their study on the causes of human cancer in 1981,[3] but the practical application of this knowledge in the fight against human disease remains frustratingly limited. As should now be clear, cancer is not a single disease arising from one causal event. In most cases the victim acquires the disease only after years of exposure to a host of environmental factors which will have interacted with his or her unique genome, throughout a large fraction of their lifespan. Even in the case of carcinoma of the lung, which is the most frequent cause of death from cancer in most Western countries, and which has a known and avoidable cause, it has required many years of patient epidemiological investigation to establish this relationship beyond doubt, and it is still not possible to predict any individual smoker's particular level of risk with certainty. The problem of diet and cancer is immensely more difficult because of the variety of diseases involved and the complexity of human diets, and because the task requires the recognition and understanding of an array of protective factors rather than any single source of carcinogens.

As we have seen, some of the most compelling evidence for a protective effect of diets against cancer to emerge in recent years is that for fruit and vegetables.[6,81] Despite the difficulties of disentangling the effects of diet from other aspects of lifestyle such as smoking, exercise and alcohol consumption, most authorities agree that, compared to those at the other end of the scale, the highest consumers of fruits and vegetables in most populations have about half the risk of developing most types of cancer.

In an age of convenience foods and pre-cooked meals, many consumers find a high consumption of fresh vegetables difficult to achieve. At first sight this seems to provide an excellent opportunity for the development of functional food products which could provide the protective effects of fresh vegetables without the need for greatly increased bulk or frequency of consumption. The difficulty lies in the sheer complexity of plants and the bewildering variety of diseases to which the protective effects seem to apply. There have been brave attempts to confront this problem with the development of a unifying hypothesis such as the dietary fibre model, or more recently the antioxidant theory, but attempts to prove these hypotheses have failed. Diet is inescapably complex, and food often seems to exert biological effects greater than the sum of its parts. No doubt the anti-carcinogenic mechanisms that underlie the protective effects of plant foods are susceptible to experimental investigation but we seem far from the isolation of a single substance or group of substances that can be incorporated into functional food products with any confidence.

6.8 Future trends

What of the future? First, there is ample room for optimism that the expansion of basic knowledge in the field of human cancers and their causes will continue, and probably accelerate. It follows that our understanding of the relationship between conventional nutrition, other protective substances in food, and cancer will continue to develop and provide greater insight into the role of diet across the lifetime of the individual. With this knowledge we will be better able to assess the role of individual foods within the diet, and hence to optimise the composition of such foods to increase their impact.

The second major factor is the rapid progress that is being achieved in the related fields of human genomics and the genetic manipulation of organisms used for human food. In time, our increasing knowledge of the human genome will shed more light on the interplay of genes and environment which determines an individual's risk of disease, and this should lead to an increasing degree of 'personalisation' of dietary advice. At the same time our ability to manipulate the genome of other organisms will give food producers and manufacturers the power to enhance the composition of plant foods to maximise their protective effects. This is, of course, an optimistic assessment of future trends; the inherent difficulties of this approach to human diets in terms of consumer confidence must by now be obvious to all. Already it is possible to predict much of a person's genetically determined risk of disease, but such knowledge can be an emotional burden to the individual concerned, and an excuse for damaging discrimination by institutions and potential employers. At the time of writing, the issue of genetic modification of plant foods has gripped the public imagination, and products containing such ingredients are rapidly disappearing from the marketplace. It remains to be seen whether a second or third generation of products carrying proven benefits to health will, given time, become acceptable to public opinion. In any event we must try to ensure that the premature release of commercial products that prove to be ineffectual or, far worse, actually harmful, does not lead to a general debasement of the whole concept of dietary strategies for the avoidance of cancer.

6.9 Sources of further information and advice

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7

Predicting the bioavailability of antioxidants in food: the case of carotenoids

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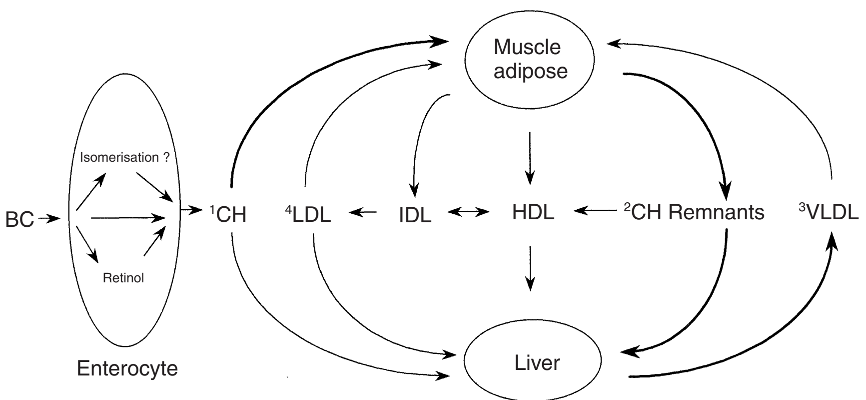
7.1 Introduction

With the advent of present-day analytical techniques and instrumentation, it is possible to describe the complex chemical nature of our foods with an ever-increasing degree of accuracy and sensitivity. However, the types and quantities of either the nutrient, or non-nutrient, components of foods may have very little bearing on their potential contribution to our nutrient or 'health' status. This is particularly true of many of the proven, or purported, antioxidant compounds. The reason for this is that only a proportion (sometimes a highly variable proportion depending upon the food matrix, processing and storage) of these food components can be absorbed and utilised. Understanding this concept of bioavailability is essential to all involved in food production, nutritional assessment and determining diet:health relationships. The term bioavailability means many things to many people. For the purposes of this chapter, bioavailability is defined as the proportion of a nutrient (food component) that is digested, absorbed and utilised in normal metabolism; however, measurement of bioavailability relies heavily upon estimates of amounts absorbed.

The absorption and transport processes of many of the potentially bioactive components of foods (including those with antioxidant capacity) are complex and not fully understood; thus, prediction of their bioavaiability is problematic. This is particularly true of the lipid-soluble antioxidants. In this chapter carotenoids have been chosen for special focus, because they serve as an excellent example of food components where too little understanding of complex behaviour within the food and within human tissues can lead to misinterpretation of study results.

Carotenoids and related compounds are the colours of nature. They consist of a group of over 600 naturally occurring coloured pigments that are widespread in plants, but only about 24 commonly occur in human foodstuffs. In the plant, they serve two essential functions: as accessory pigments in photosynthesis and in photoprotection. These functions are achieved through the polyene structure of carotenoids (Fig. 7.1), which allows the molecules to absorb light and to quench, or inactivate, singlet oxygen and free radicals. In most cases, the carotenoid (normally present as the all *trans* form) is associated intimately with the light-harvesting complex in the thylakoid membranes of the chloroplast where it is found as an ordered structure in association with binding protein. In the case of the carrot root and tomato fruit the β-carotene and lycopene, respectively, occur as membrane bounded semi-crystalline structures derived from chromoplast or chloroplast structures. It might be expected that in plants the lipophilic carotenoids would be found in association with sub-cellular lipid structures but it is also known that there are associated binding proteins.[1] Such a complex environment has implications for their extraction, analysis and behaviour during digestion.

The hypothesis, based on epidemiological evidence, that health benefits arising from the consumption of coloured fresh fruit and vegetables are at least in part due to their carotenoid content has led to interest in the non-provitamin, as well as the provitamin, A carotenoids. At present, there are no quantifiable biochemical or physiological markers of carotenoid 'status' (other than in relation to the vitamin A activity). Neither carotenoid 'deficiency' nor 'toxicity' is recognised. However, low plasma



7.1 Lipoprotein carriers.

carotenoid concentration is used as an indicator of those 'at risk' of chronic disease based on the direct association between the intake of carotenoid containing vegetables and fruit, plasma and tissue concentrations of carotenoids[2–4] and the development of chronic disease states, particularly cardiovascular disease[5] and cancer of various organs.[6] A possible negative impact on health, at least for β-carotene, is indicated by the ATBC (α-tocopherol: β-carotene) trial where increased risk of lung cancer was reported in smokers receiving relatively high dose β-carotene supplements for several years.[7] Epidemiological and experimental evidence from cell culture and human studies points to a 'window of wellness' with respect to β-carotene intake, with beneficial responses being associated with amounts available from diets rich in coloured fruits and vegetables.[8] Most recently, it has been shown in a prospective cohort study that there is a strong negative correlation between the intake of tomato and tomato products, but not tomato juice, and the incidence of prostate cancer and it is concluded that the effective agent is lycopene.[9] The difference in response to different tomato products has been ascribed to differences in lycopene bioavailability.

Since it is recognised that the carotenoids are not actively absorbed by the gut but are passively absorbed along with lipids, the efficiency of absorption of the carotenoids is dependent on getting the lipophilic molecules and crystals to dissolve into dietary lipids both during processing or domestic preparation and during the digestive processes. It is now recognised that this is a key process in absorption and may well be the single most important factor governing the rate and limit of absorption. It is not surprising therefore to find greater bioavailability from heat-treated foods that have also been comminuted, or co-processed, with oils.[10,11]

Whilst emphasis in this chapter is placed on carotenoids, many of the approaches presented and discussed have relevance in determining the bioavailability of a much wider range of food components.

The chapter will cover aspects of:

- Carotenoid metabolism – the knowledge required for prediction of absorption.
- Experimental systems for measuring absorption – their strengths and weaknesses.
- Maximising bioavailability – food processing and dietary interactions.
- Sources of further information – a current European initiative.

7.2 Metabolism

7.2.1 Digestion

The carotenoids must be disassociated from their native environment in the plant tissue and, since the carotenoids are lipophilic, they must also be dissolved in dietary lipids before they can be absorbed. The physical processes

involve disruption of the physical structure of the food and the dissolution of the carotenoid into the bulk lipid phase, lipid emulsion or mixed micelles. The mass transfer, therefore, involves both the dietary lipid and its hydrolysis products (free fatty acids, mono- and diacyl glycerols), phospholipids and the bile salts needed to emulsify the lipid to form mixed micelles.

7.2.2 Gastric and lumenal events

The lumenal environment and the form of the carotenoid play a crucial role in determining how much of the carotenoid is presented for absorption. Both the hydrocarbon (e.g. β-carotene) and hydroxy carotenoids (e.g. zeaxanthin) have very limited solubility in bulk lipid (0.112–0.141 % and 0.022–0.088 %, respectively). Additionally, the hydrocarbon carotenoids are found mainly in the core of the lipid droplets whereas the hydroxy carotenoids are preferentially located at the surface[12] because of their different polarities. This has implications for the absolute amount and type of carotenoid that can be carried by emulsified lipid droplets and in the much more highly structured micelles. These differences in physical properties and their preferred lipid domains will also control the possible transfer of carotenoid between lipid structures both in the gut lumen and post absorption.

7.2.3 Absorption

Carotenoids are passively absorbed from the micellar phase. However, it is not known if all the carotenoid present in a mixed micelle is absorbed, or whether some is left behind in association with unabsorbed bile salts and cholesterol, to be absorbed perhaps more distally or lost to the large intestine. Factors that increase the thickness of the unstirred layer on the surface of the gut, for example soluble dietary fibre, act to attenuate the absorption of dietary fats and may, therefore, also inhibit the absorption of carotenoids.[13,14]

Disease states which impair lipid absorption, for example cystic fibrosis and coeliac disease also lead to low plasma carotenoid levels, although in some cases persistent inflammation may be a significant factor in reducing plasma levels of carotenoids.[15] The mass transfer of the carotenoids from digesta to absorbable species is clearly a limiting step in their bioavailability on the basis that free carotenoids given orally (as supplements, solution or suspension in oil) are much better absorbed[16] than those from foods and there is evidence that homogenisation of the food and heat treatment enhance absorption.

7.2.4 Transport

As already stated, the carotenoids are passively absorbed from mixed micelles at the brush border along with dietary lipids, lipid hydrolysis prod-

ucts, sterols and bile salts. The absorbed carotenoids are transported through the enterocyte from the lumenal side to the serosal side, where they are re-excreted in chylomicrons into the thoracic duct and hence find their way into the circulating blood. The cleavage of some of the retinol precursor (provitamin A) carotenoids by 15,15′dioxygenase occurs in the enterocyte. The resulting retinal is reduced to retinol and subsequently esterified to the retinyl ester (mainly palmitate) which also enters the mesenteric lymph with the chylomicrons.[17,65] It is probable that some of the retinol produced in the enterocyte is excreted into the portal blood in association with retinol binding protein. It is not known if any of the non-provitamin A carotenoids are competitive inhibitors of 15,15′dioxygenase but the *cis*-isomers of β-carotene can give rise to the corresponding *cis*-retinol.

The chylomicrons have a biologically-controlled composition with regard to protein (2 %, consisting of apoproteins A-1, A-2, A-4 and B-48) and lipid (dietary derived), and appear to carry the carotenoids (and other lipid soluble components) passively in the mesenteric lymph. The chylomicrons are acted upon by endothelial lipoprotein lipase in the extrahepatic capillary bed. Some of the lipid is absorbed as free fatty acids and the glycerol is metabolised. It is not known if any of the carotenoid is also absorbed at this point, although clearance kinetics[16] would imply that some is absorbed, perhaps by adipocytes. The chylomicron remnants, including residual carotenoids, are then cleared from the circulation by passage through the liver. However, lycopene in the rat and monkey model does appear to concentrate in the liver and gut[19] and in a range of tissues in humans[20–22] indicating that it disperses to tissues differently from the way that β-carotene does. This may help to explain why it is so difficult to increase plasma concentration of lycopene with chronic supplementation of 15 mg lycopene per day.[23]

The liver re-exports lipid in the form of very low density lipoproteins (VLDL) and these contain carotenoids dissolved in the lipid portion. No specific carrier proteins have been identified. The VLDL is acted upon by endothelial lipoprotein lipases in the extrahepatic capillary bed and this removes lipid and glycerol to produce intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). The IDL is converted to LDL by receptor-mediated endocytosis in the liver and the LDL is cleared from the plasma by receptor-mediated endocytosis in both liver and other tissues. The hydrocarbon carotenoids appear to remain associated with these lipoproteins, such that around 80 % of β-carotene and lycopene found in fasting plasma are carried by LDL.[17,24,25] How much of the carotenoid is distributed to the tissues by these processes is unknown, nor is it known how quickly the process occurs, but endocytosis of LDL particles by adipose tissues is likely to be a major carotenoid 'sink'.

The hydroxy carotenoids (e.g. lutein, zeaxanthin) are found almost equally distributed between the LDL and the high density lipoproteins (HDL) in fasting subjects.[24] HDL particles are produced in the liver and

intestine and are secreted into the blood and mesenteric lymph, respectively. Other HDL particles may arise from chylomicrons as the triacylglycerol is removed by lipoprotein lipase. The carotenoid associated with HDL may, therefore, be derived from the liver, or directly from the gut, or by exchange between lipoproteins. Unlike LDL, where the bulk of the particle is cholesterol esters, HDL contains much more protein and phospholipid. However, both particles have a hydrophobic core in which the hydrocarbon carotenoids may be carried. It is unclear, therefore, why the polar hydroxy carotenoids partition almost equally between LDL and HDL unless both particles have similar interfacial characteristics.

If the currently accepted model of carotenoid absorption and transport is correct, then dietary carotenoids should first appear in the chylomicrons and then VLDL, LDL and HDL, depending upon the amounts of each lipoprotein and their residence time (half life) in the plasma. It would be expected that the chylomicrons would show a peak at around 4–6 h post ingestion, as the meal passes through the ileum, and would be clear at about 12 h. Studies of β-carotene absorption, using thoracic duct cannulation,[26,27] found this to be the case. Studies of the absorption of α-carotene, lycopene and lutein in the triglyceride rich lipoprotein (TRL) fraction of plasma also show a peak plasma concentration around 4–6 h which returns to baseline by 12 h post dose.[28] Appearance in the TRL would be followed by peaks in VLDL then LDL as the hepatically sequestered carotenoid is re-excreted into the circulation. Figure 7.1 (see Section 7.1) illustrates the main features of carotenoid distribution in the plasma carriers.

7.2.5 Isomerisation and *cis*-isomers

Human blood plasma contains mainly the all *trans* forms of the common dietary carotenoids but 5*Z*-lycopene (up to 50% of the total plasma lycopene) and 9*Z*- and 9′*Z*-lutein and 9*Z*-β-carotene[29] are also commonly found in human blood plasma. In some cases 5*Z*-lycopene appears in plasma in a much greater proportion than in the food.[30] This could suggest that the 5*Z*-lycopene is preferentially absorbed, or less rapidly cleared from the plasma, or that all *trans* lycopene undergoes isomerisation as a result of some biochemical interaction since simulated digestion *in vitro* does not cause significant acid-catalysed isomerisation.

It has been reported that 9*Z*-β-carotene preferentially accumulates in the lipoprotein carriers[31] but Gaziano et al.[32] found that there was a marked preferential absorption of the all *trans*-β-carotene in man. Supplementation of female volunteers with 15 mg per day of palm oil carotenoids (a mixture of *trans*- and *cis*-β-carotene) elicited a plasma response where the ratio of *cis*:*trans* forms was much lower than in the supplement.[18] This would indicate that the *trans* form is better absorbed than the *cis* form, or that the *cis* form is cleared from the plasma more rapidly. However, in ileostomists given an acute oral dose of all-*trans*-β-carotene and 9*Z*-β-carotene, both

isomers appeared to be equally well absorbed from the gut and *cis–trans* isomerisation did not occur during passage of the β-carotene through the GI tract.[16] Currently it is unclear at what stage of absorption (mass transfer from food, dissolution in the lumenal lipid structures, absorption from the micelle, transport within the enterocyte and incorporation into chylomicrons) these effects occur, although simulated gastric digestion of lycopene does not produce *cis* isomers.

7.3 Systems for predicting carotenoid absorption

Various approaches have been used to assess carotenoid absorption and in each case assumptions are made in order to estimate values, either absolute or comparative. The limitations imposed by such assumptions should be borne in mind when considering data evaluation.

7.3.1 Mass balance methods

7.3.1.1 Faecal mass balance

Such studies are normally carried out over a period (5–8 d) known as the balance period. The chronic method is dependent on establishing an equilibrium between intake and excretion. This approach requires faecal markers at the beginning and end of the balance period to determine the 'measurement' period and, because absorption is the difference between intake and excretion, both values need to be determined with great accuracy. The use of food tables is insufficient and foods and supplements need to be assayed accurately.

If this method is applied to measuring absorption of a carotenoid from a single acute dose, the diet needs to be carotenoid free for 5 days before and during the test period and faecal collections need to be continued until no further carotenoid from the test dose is lost in the faeces. The collection period is usually 3–5 days giving a total study period of 6–10 days during which there will almost certainly be perturbation of the 'normal' plasma concentration of carotenoids due to the modification of the diet.

A major assumption when using the faecal balance technique to assess carotenoid absorption is that faeces are the only significant excretory mechanism of unabsorbed carotenoids, that there is no enterohepatic recycling, that the carotenoids recovered from the faeces are of dietary origin and that none of the unabsorbed carotenoid has undergone biotransformation, or otherwise been lost, due to the presence of the colonic microflora. The latter assumption gives cause for concern. Along with many other organic food components, the carotenoids are likely to be susceptible both to microbial degradation in the large bowel and to oxidative degradation. Thus, it is unlikely that unabsorbed carotenoids are quantitatively recov-

ered from the faeces. Unfortunately, much of the carotenoid absorption data from foods and isolates are based on either acute or chronic faecal mass balance methods[33] and show great variability.

7.3.1.2 Gastrointestinal lavage technique

With this technique the entire gastrointestinal track is washed out by consuming a large volume (1 gallon/4.5 l) of 'Colyte' containing polyethylene glycol and electrolyte salts. Washout is complete with the production of clear rectal effluent (2.5–3.5 h) and the volunteers then consume the test meal and are permitted only water or 'diet' soft drinks (non-caloric) for the next 24 h. All the effluent is collected and pooled with the effluent collected on the following day when another dose of 'Colyte' is given to wash out the remainder of the test meal. The carotenoid recovered in the stool is subtracted from that fed to obtain an absorption figure.[34] Difficulties associated with the method is that it is relatively time consuming, can only be applied to healthy individuals, and may give an underestimation of absorption if absorption is compromised or normal transit time is reduced due to the use of Colyte. In addition, as with the faecal mass balance, the method depends upon there being no degradation or loss of unabsorbed carotenoids. On the other hand, it has the advantage of at least standardising the residence time of carotenoids in the GI tract.

7.3.1.3 Ileostomy studies

In individuals who have undergone ileostomy, the colon has been surgically removed and the terminal ileum brought to a stoma on the abdominal wall. Ingested food passes through the stomach and ileum in around 6 h as it would in the intact individual. The digesta (ileal effluent) can be recovered at regular intervals (2 h) and all the residue from a test breakfast can be recovered in 12 h if the volunteers are given carotenoid-free midday and evening meals. Test meals of either an isolated carotenoid or food can, therefore, be given to an overnight fasted volunteer at breakfast (without dietary modification) and the unabsorbed carotenoid recovered from the ileal effluent in real time without the delay of the colon and rectum, or the confounding influence of the colonic microflora. The model has the added advantage that an excretion profile can be obtained, the timing of which gives a time span for the absorption, which can in turn be compared to changes in plasma concentration over the 12 h test period. Using this approach, absorption of all-*trans*- and 9-*cis*-β-carotene dispersed in a milk shake has been estimated at 75–90 %.[16]

7.3.2 Whole plasma methods

Measurements of absorption are usually carried out by the administration of an acute or chronic oral dose of isolated carotenoid, or carotenoid-containing food, and following the changes in plasma concentration of the

carotenoid of interest. Changes in plasma concentration are then interpreted as measures of absorption. Comparisons between plasma concentration excursions for different carotenoids should not be made without a knowledge of absorption and clearance kinetics or the form of the dose response curve. If assumptions are made these should be clearly stated and supported.

Absorption, although it comprises a major element of bioavailability, is only part of the story in that it take no account of the metabolic fate of the carotenoids. In assessing absolute absorption from plasma responses it is necessary to use a 'disposal' term. This disposal term takes account of the distribution of carotenoids to body 'compartments' other than plasma. The term does not identify these normally inaccessible body 'compartments' or 'pools' into which the carotenoids enter after leaving the plasma, but it does acknowledge their existence and influence. Lack of information results frequently in data obtained from whole plasma based methods being misinterpreted.

7.3.2.1 Acute doses

Such tests are usually carried out in fasted individuals who have restricted their dietary intake of the carotenoid of interest (and other carotenoids) for several days before the test day and for a period of days following. Blood samples are drawn at various time intervals after the test meal and the plasma/serum analysed for the carotenoid(s) of interest. Plasma concentration is then plotted as a function of time and the area under the curve (AUC) calculated as concentration × time (*Ct*) This method cannot determine absolute absorption but it is possible to compare different doses and foods and derive some information as to the relative absorption by comparison to a standard dose, normally the isolated carotenoid. Such studies cannot normally be carried out blind because of the problem of disguising the treatment. A crossover design, with an adequate period of washout between treatments, is the most suitable approach so that each individual can act as their own control and data can be compared using a paired t-test. Each volunteer acting as their own control is essential since the AUC for the same dose in different individuals will be very variable, and such variability does not only depend upon the amount absorbed but on the absorption and clearance kinetics which may vary widely between individuals.

The measurement of absolute absorption of a carotenoid, calculated from the changes in plasma concentration following a single acute dose, is difficult and frequently misunderstood. The first point to deal with is the form and duration of the plasma response curve. Peak plasma concentration occurs at between 6h and 48h, depending upon the dose and the frequency of making the measurements. Since it is evident that the dose passes through the ileum in about 6h, the advent of plasma peaks found beyond this time can only result from delayed passage of carotenoid to the serosal

side after absorption into the enterocyte, or rapid absorption of carotenoid into the body, sequestration from the circulation, and then re-exportation to the plasma.

Evidence cited for the first case is a frequently found second plasma peak occurring following the meal. However, this is countered by lack of evidence for temporary storage in the enterocyte. There is no known storage mechanism, no 'tailing' of ileal loss in ileostomy patients[16] and radiolabelled β-carotene absorption appears complete in less than 12 h.[26,27] The second peak could simply result from an increase in the plasma lipids following a meal, providing the lipoprotein and triglyceride needed to transport carotenoid into the plasma. Alternatively, and most probably, the first peak in plasma concentration is due to the carotenoid present in the newly absorbed chylomicrons and the second peak, or prolonged duration of the first peak, results from hepatically re-exported carotenoid in VLDL and LDL.

The transfer of carotenoids from the short lived chylomicrons to the longer lived LDL and HDL (which appear to carry most of the carotenoid in fasting subjects) would also explain why the plasma concentration may remain elevated for up to and beyond 10 days post dose.[35,36] Under such circumstances, the plasma AUC approach is not appropriate for the calculation of absolute absorption because the kinetics of absorption, disposal and re-exportation are not known. Even comparative studies of two sources within a single individual may not be valid unless the equation of the dose response curve is known and attenuated delivery or absorption caused by different physical characteristics of the meal is known not to occur.

7.3.2.2 Chronic doses

Chronic dosing with supplements or foods needs to be carried out until the plasma concentration reaches a plateau. This normally takes a period of weeks when supplementing with amounts of carotenoids in the region of 10–15 mg per day, and may increase the plasma concentration of β-carotene up to 10-fold; with other common carotenoids, particularly lycopene, showing smaller increases. Again, absolute absorption cannot be measured but the data may allow comparisons between isolated compounds and foods, and between different foods provided dose response is linear. As with the acute dose studies, it is essential that each study volunteer acts as their own control since the plasma response for the same dose in different individuals can be highly variable. Decay curves of falling plasma concentration of carotenoids, when supplementation is discontinued, may also provide some data on the half-life of the body carotenoid pool.[37,38]

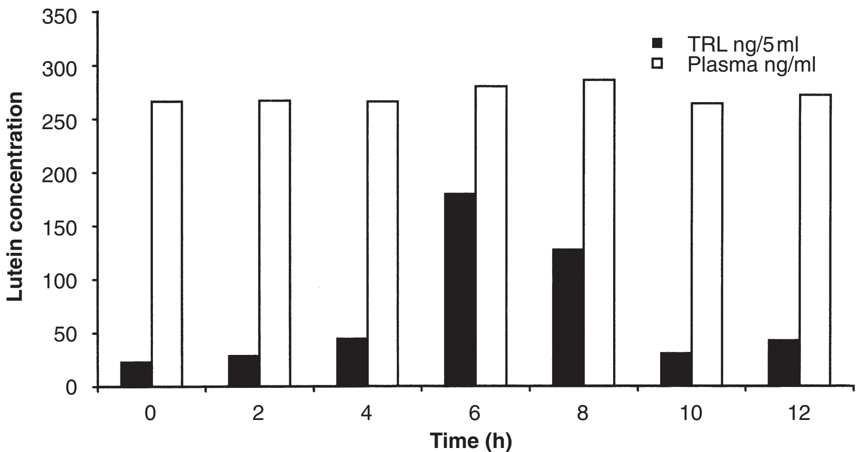
7.3.3 Plasma triglyceride rich lipoprotein (TRL) fraction approach

Newly absorbed carotenoids are initially present in plasma chylomicrons before they are sequestered by body tissues and re-exported in, or transferred to, other lipoprotein fractions (Fig. 7.1); see Section 7.1. Thus, mea-

surement of carotenoids in this fraction, and a knowledge of the rate of clearance of the chylomicrons, should permit the calculation of rates of absorption, disposal and overall absorption based on AUC measurement.

This method has the advantage that chylomicrons present in fasting plasma are few and they are almost devoid of carotenoids. Thus, response following a carotenoid-meal is more clearly defined (Fig. 7.2). The disadvantage is that the plasma has to be ultracentrifuged to separate the lipoprotein classes. Ultracentrifugation, however, does not normally permit the separation of the chylomicron fraction free of other low density lipoproteins, particularly the VLDL, which may be the primary vehicle for the hepatic re-export of absorbed carotenoids. Recent work[39] has shown that chylomicrons and VLDL can be separated by less vigorous centrifugal treatment.

In addition, absorption data based on oral AUC in TRL, and the theoretical AUC that would be obtained if the dose had been administered intravenously (using plasma volume and chylomicron clearance half life), give results that differ. For β-carotene Van Vliet et al.,[40] calculated an absorption figure of 11 % (central cleavage) or 17 % (eccentric cleavage), whereas O'Neill and Thurnham[41] calculated 3.9 % and 2.5 % absorption in males and females, respectively, assuming only central cleavage. Both authors assume a cited chylomicron remnant half life of 11.5 min. However, a true clearance rate of carotenoid in the TRL fraction can also be obtained from the graph of TRL carotenoid concentration vs time and this could also be used to provide a carotenoid half-life term which would be independent of assumptions based on lipid kinetics. It is worth noting that much shorter half lives (2.5–7.9 min) have been reported for the clearance of chylomicron triglyceride[42] and use of these values,[16] rather than those of chylomicron



7.2 Whole plasma and TRL fraction lutein concentrations over 12 h following a spinach meal (150 g).

remnant clearance,[43] has the effect of proportionally increasing the apparent absorption (percent absorption doubles every time the half life is halved). The plasma chylomicron concentration will depend both on the lipid load and the ability of the individual to clear the chylomicrons from the plasma and unless this is known it will introduce errors by the use of inappropriate half-life values. The calculation of absorption, using a theoretical plasma concentration excursion based on plasma pool size and a theoretical intravenous dose,[40,41] must therefore be treated with caution unless exact clearance kinetics of the carotenoids are known.

Some difficulties in explaining carotenoid kinetics may arise from:

- The observations that the triglyceride in the TRL peaks at 2 h whereas the β-carotene peaks at 5–6 h.[40]
- Individuals who are highly variable in their plasma and TRL response (or not) to oral β-carotene.[35,39,44]
- Individuals with a high concentration of plasma β-carotene appear to be those that show the greatest increment in plasma carotenoid concentration on supplementation.[18]

7.3.4 Isotope methods

The use of radioactive tracers in human volunteers to determine the bioavailability of the carotenoids is not now possible because of ethical constraints. There are, however, two studies[26,27] in males using ^{14}C and ^{3}H. These studies provide useful information on the duration and extent of absorption of β-carotene and the degree of conversion to retinol. Absorption of radiolabelled β-carotene was found to be in the range 8.7–16.8 % but most of this was recovered as the retinyl esters. This indicated that β-carotene absorbed by this route was largely converted to retinol. Peak absorption was found to be at 3–4 h and 6–7 h for each of two volunteers, respectively, and this time coincided with maximum lactescence in the lymph as assessed visually. In both cases, despite the relatively low absorption, no further radiolabel was found in the lymph after 12 h. Transitory storage in the enterocytes, prior to transfer to the serosal side, would probably have been detected as a tailing of the absorption curve and the high level of conversion may explain why elevation of plasma β-carotene is not always seen in volunteers given small acute doses.

The use of stable isotopes is more ethically acceptable. Highly labelled β-[^{13}C] carotene has been used to study the metabolism of β-carotene in man.[45,46] The single acute oral dose used in these studies was 1–2 mg of purified labelled (>95 % ^{13}C), dissolved in tricaprylin or safflower oil and given with a standard meal. Blood samples were drawn at intervals and the β-carotene, retinol and retinyl esters separated, quantified and purified by HPLC (high performance liquid chromatography). The β-carotene (converted to the perhydro derivative by hydrogenation over platinum oxide),

and the retinol and retinol derived from retinol esters, were subjected to gas chromatography-combustion-isotope ratio mass spectrometry (GCC-IRMS). The method was sufficiently sensitive to track the ^{13}C in retinol esters up to 2 days and β-carotene and retinol up to 25 days.

Potentially, the use of [^{13}C] carotenoids, either as an isolate or within a food, should permit the measurement of absolute absorption and the kinetics of disposal and conversion to other metabolites. An alternative to the use of β-[^{13}C] carotene is octadeuterated β-carotene (β-carotene-d_8), an isotopomer that can be separated from natural abundance β-carotene by HPLC, thus, avoiding the use of mass spectrometry.[47] The retinol-d_4 derived from β-carotene-d_8, has to be separated from the plasma using a solid phase system[48] and derivatised to the *tert*-butyldimethylsilyl ether[49] before measurement by gas-chromatography-mass spectrometry. The method has been applied successfully to the tracking of both β-carotene-d_8 and retinol-d_4 in human volunteers for up to 24 days after an oral dose of 73 μM (40 mg).[47] Application of a compartmental model Novotny et al.[50] indicated that 22 % of the carotenoid dose was absorbed; 17.8 % as carotenoid and 4.2 % as retinoid. This result is close to the 11 % absorption of β-carotene found by Van Vliet[40] but indicates much lower percentage conversion to retinol than that found using very small oral doses of β-[^{13}C] carotene.[46] It is worth noting that the percentage conversion to retinol is dose and retinol status dependent and that retinol palmitate measurements need to be made to allow for this. Table 7.1 summarises the range of absorptions that may be found in the literature.

7.4 Maximising the bioavailability of carotenoids

Because the carotenoids are lipophilic they need to be transferred from their aqueous environment in vegetable tissue to the lipid phase of the food or digesta before they can be effectively absorbed. Barriers to this process are the binding proteins with which the carotenoids are associated in photosynthetic structures and the physical architecture and strength of the plant tissue. Besides these physical constraints the thermodynamics of dissolution in the absorbable lipid domains from protein–carotenoid complexes, carotenoid-rich lipid droplets and membrane-bounded crystalline structures need to be considered.

Mass transfer can only occur where the lipid structure (bulk lipid, lipid emulsion, micelle) is contiguous with the carotenoid and this intimate contact can only occur once the structure has been disrupted. The interfacial characteristics of the lipid structures are also important because they will control aggregation of food particles, a prerequisite for the close physical contact needed for mass transfer. Interfacial characteristics will be dependent on pH, salt concentration, surfactant (bile salt, phospholipid) and peptides in the food or generated by digestion of dietary proteins.

Table 7.1 Absorption of carotenoids

Carotenoid	% Absorption	Source	Reference
β-Carotene[a]	40–98	Isolate in oil	33
β-Carotene[a]	1–87	Raw carrot	33
β-Carotene[a]	4–22	Raw carrot, grated	33
β-Carotene[a]	1–48	Cooked carrot	33
β-Carotene[a]	25–56	Carrot puree	33
β-Carotene[a]	9–45	Raw spinach	33
β-Carotene[a]	6–88	Cooked spinach	33
β-Carotene[b]	75–98	Isolate in milk shake	16
β-Carotene[c]	17–52	Isolate	34
β-Carotene[d]	11–17*	Beadlets	40
β-Carotene[d]	3.4	Isolate, capsule	41
Lutein[d]	2.7	Isolate, capsule	41
Lycopene[d]	2.6	Isolate, capsule	41
β-Carotene[e]	22	Deuterated isolate	50
β-Carotene[f]	9–17	Isolate, radiolabelled	27

[a] faecal mass balance
[b] mass balance in ileostomy volunteers
[c] gastrointestinal lavage mass balance
[d] calculated from plasma triacylglycerol rich fraction carotenoid excursion and hypothetical clearance kinetics
[e] compartmental model based on plasma concentration excursion
[f] based on recovery of radiolabel from thoracic duct
* assuming central (11 %) or eccentric cleavage (17 %)

Cooking and mechanical comminution are the most common method of increasing availability, but other methods, e.g. co-processing with lipid as in prepared recipes, or enzyme treatments which cause cell separation or disruption (juice production) may be used. During digestion, any disease state that compromises fat absorption will reduce the absorption of lipid-soluble micronutrients. The consumption of fat replacers, e.g. Olestra®, or cholesterol absorption inhibitors, e.g. plant phytosterols, will also have a negative impact on the absorption of carotenoids[51,52] and perhaps of other lipid-soluble micronutrients.

7.4.1 Processing and storage

Carotenoids are normally present in fruits and vegetables as predominantly the all-*trans* (*E*) form although in some cases there may be a considerable proportion of *cis* isomers (*Z*) particularly in algae. During processing there are a number of physical and chemical changes that need to be considered for their possible impact on bioavailability.

Thermal processing is normally undertaken to render the product edible, to eliminate any spoilage/pathogenic organisms and to inactivate enzymes. Cooking therefore softens the cell walls so that they are easily separated or

broken mechanically, all cellular membranes are destroyed and proteins denatured. The carotenoids, normally stable within the original structure, are then exposed to the external environment where they may be subject to light, atmospheric oxygen and oxidised or reactive products of other components. Lycopene (tomatoes) and lutein and β-carotene (green leaves) appear to be quite stable in the fresh tomato and green leaf even when exposed to intense sunlight. During processing the protection of the native environment is lost and the carotenoids are readily oxidised and photobleached. This is particularly true if the product is dried and exposed to the air and light. It has been shown that excessive thermal processing may also create *cis* (*Z*) isomers, particularly 5*Z*-lycopene although isomerisation can also occur at positions 9,13 and 15.[30,53] *Cis* isomers with their kinked structure tend to be more soluble in organic solvents and this change in physical properties may have an influence on the ease with which they are absorbed by the gut, their partitioning between the various lipoprotein carriers and half life in the plasma.[30,53]

Losses of carotenoid that occur after thermal processing and storage in anaerobic and light-free conditions (e.g. canning) are slight and may be as a result of oxidation by compounds formed enzymically or thermally during processing. Processing, however, increases the availability of lycopene for absorption, particularly if processed in the presence of lipid.[10,11] It is also recognised that dietary fat itself improves carotenoid bioavailability.[37,54–56]

7.4.2 Interactions

Single acute oral doses of lutein and β-carotene have shown that the two carotenoids interact to reduce the apparent absorption of lutein as measured by the plasma area under the curve (AUC), and in some instances lutein has been shown to reduce the AUC for β-carotene.[57] Similarly, a combined dose of β-carotene and lycopene does not appear to affect the absorption of β-carotene but enhances the absorption of lycopene.[58] Short term supplementation of volunteers with β-carotene, either as a pure compound or as the major constituent of a natural β-carotene source, has also been found to reduce the plasma concentration of lutein.[38,23,18] However, in a long term study (4 years) of β-carotene supplementation, although there was a trend, the reduction of plasma lutein was not significant.[59] The other carotenoids, therefore, do not appear to affect the apparent absorption of lycopene, possibly because only β-carotene and lycopene are the main hydrocarbon carotenoids. Although they use the same carriers both in the gut lumen and *in vivo* (LDL) carrier capacity is probably not limiting.[60]

With respect to other carotenoids and vitamin E, β-carotene supplementation of volunteers with colorectal adenomas for 2 years resulted in highly significant increases in plasma lycopene and α-carotene in both men and women.[61] However, short-term supplementation (15 mg per day

β-carotene for 35 days) of apparently healthy volunteers had no effect on the plasma concentration of lycopene, or on plasma vitamin E.[18]

In humans, concurrent feeding of acute doses of β-carotene and canthaxanthin was found to inhibit the plasma appearance of canthaxanthin but there was no converse interaction.[62] The observation that the carotenoid profile in the TRL fraction is not the same as in a supplement[63] clearly indicates that in order to assess bioavailability of any one carotenoid the carotenoid profile of the supplement or food needs to be defined, as does the amount and type of fat in the test meal.[64] These findings suggest that β-carotene has a sparing effect on lycopene and that if this is the case other 'interactions' *in vivo* may also occur which will need to be considered when looking at plasma carotenoid profiles and dietary habits.

7.5 Future trends

It is becoming increasingly clear that all the major food carotenoids behave differently with regard to the plasma responses they provoke when given orally and that the differences are due to (a) the amount absorbed, (b) kinetic behaviour, (c) degree of conversion to retinol and (d) partitioning between lipoprotein carriers. In order to determine the exposure of tissues to levels of carotenoids that may be efficacious in reducing the incidence of disease it is essential that a clear picture of absorption and plasma persistence is defined and linked with known mechanisms of action (immune function, DNA damage (antioxidant) and repair (gene regulation), cell–cell communication and lipid oxidation). Such data will form the basis of recommendations as to which carotenoids are most efficacious, level of intake and processing methods that enhance absorption.

7.6 Sources of further information and advice

Understanding factors controlling antioxidant nutrient availability is a necessary step for providing informed food choice and designing commercial processes that provide desired levels of bioavailability in food products. A multicentre European project is focused on providing the experimental tools required to define the bioavailability of bioactive lipid-soluble components of the diet (using carotenoids as model components).

Model systems *in vitro* and *in vivo* have been developed as part of this project. The *in vitro* systems offer the advantages of providing rapid results, produced in strictly controlled environments, at low cost. Such methods are, however, useless without validation. This project incorporates validation of several *in vitro* model systems against several *in vivo* (human) models. The majority of nutrient bioavailability studies *in vivo* have used isolated materials to overcome problems associated with the complexity of the food

matrix. This is a valid experimental tool in the early stages of research but progression to more complex systems of whole foods and mixed diets is essential to characterise absorption and utilisation in the 'real-life' situation. Thus, studies in this project focus on carotenoid bioavailability from both raw and processed foods (fruits and vegetables). Experimental approaches *in vitro* include consideration of the importance of key factors of food structure, the extent of release of carotenoids from the food structure; transfer of carotenoids to emulsion and micellar phases during digestion; and development of a computerised, dynamic, model gut system for prediction of the carotenoid absorbability. Experiments *in vivo* include different approaches to measuring carotenoid absorption and metabolism in human volunteers, using labelled (C^{13}) and unlabelled foods. Tissue targeting of carotenoids and bioactivity of their metabolic products have also been investigated. (Model systems, *in vitro* and *in vivo*, for predicting the bioavailability of lipid soluble components of food fair shared-cost contract CT97-3100; contact the coordinator at sue.southon@bbsrc.ac.uk for further information).

7.7 References

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Part 3

Natural antioxidants

8

Introducing natural antioxidants

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8.1 Introduction

The importance of the antioxidants contained in foods is well appreciated for both preserving the foods themselves and supplying essential antioxidants *in vivo*. With increasing experimental, clinical and epidemiological data which show the beneficial effects of antioxidants against oxidative stress-induced degenerative and age-related diseases, cancer and ageing, the importance and role of antioxidants have received renewed attention.

We are protected from oxidative stress by various antioxidants which have different functions. Some are enzymes and proteins and others are small molecule antioxidants. Foods are important as an essential source of such antioxidants, components and trace elements. In addition, numerous synthetic antioxidants have been developed and some of them have been used in practice as, for example, food additives, supplements and drugs. The phenolic compounds such as vitamin E and flavonoids are typical antioxidants. Numerous phenolic compounds have been also synthesised: 2,6-di-*tert*-butyl-4-methylphenol known as BHT is one of the most popular synthetic antioxidants. It is generally accepted, however, that natural antioxidants are more potent, efficient and safer than synthetic antioxidants. For example, α-tocopherol is the most active form of vitamin E and natural 2*R*,4'*R*,8'*R*-α-tocopherol is more potent than synthetic racemic α-tocopherol primarily because α-tocopherol transfer protein selectively recognises natural α-tocopherol. As such, natural antioxidants are more favourably accepted than synthetic antioxidants.

8.2 Categorising natural antioxidants

Table 8.1 shows the antioxidants which constitute the defence system *in vivo*. As shown, there are several lines of defence. The first defence line is to inhibit the formation of active oxygen species and free radicals by sequestering metal ions, reducing hydroperoxides and hydrogen peroxide and to quench superoxide and singlet oxygen. The radical-scavenging antioxidants function as the second line defence. Vitamin E and vitamin C are major lipophilic and hydrophilic radical-scavenging antioxidants. They scavenge radicals and inhibit chain initiation or break chain propagation. Polyphenolic compounds may also work as important radical-scavenging antioxi-

Table 8.1 Defence systems *in vivo* against oxidative damage

1. Preventive antioxidants: suppress the formation of free radicals.	
(a) Non-radical decomposition of hydroperoxides and hydrogen peroxide:	
catalase	decomposition of hydrogen peroxide $2H_2O_2 \rightarrow 2H_2O + O_2$
glutathione peroxidase (cellular)	decomposition of hydrogen peroxide and free fatty acid hydroperoxides $H_2O_2 + 2GSH \rightarrow 2\ H_2O + GSSG$ $LOOH + 2GSH \rightarrow LOH + H_2O + GSSG$
glutathione peroxidase (plasma)	decomposition of hydrogen peroxide and phospholipid hydroperoxides $PLOOH + 2GSH \rightarrow PLOH + H_2O + GSSG$
phospholipid hydroperoxide glutathione peroxidase	decomposition of phospholipid hydroperoxides
peroxidase	decomposition of hydrogen peroxide and lipid hydroperoxides $LOOH + AH_2 \rightarrow LOH + H_2O + A$ $H_2O_2 + AH_2 \rightarrow 2H_2O + A$
glutathione-*S*-transferase	decomposition of lipid hydroperoxides
(b) Sequestration of metal by chelation:	
transferrin, lactoferrin	sequestration of iron
haptoglobin	sequestration of haemoglobin
haemopexin	stabilisation of haem
ceruloplasmin, albumin	sequestration of copper
(c) Quenching of active oxygens:	
superoxide dismutase (SOD)	disproportionation of superoxide $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
carotenoids, vitamin E	quenching of singlet oxygen
2. Radical-scavenging antioxidants: scavenge radicals to inhibit chain initiation and break chain propagation.	
hydrophilic: vitamin C, uric acid, bilirubin, albumin	
lipophilic: vitamin E, ubiquinol, carotenoids, flavonoids	
3. Repair and *de novo* enzymes: repair the damage and reconstitute membranes lipase, protease, DNA repair enzymes, transferase.	
4. Adaptation: generate appropriate antioxidant enzymes and transfer them to the correct site at the correct time and in the correct concentration.	

dants. The third-line of defence is the repair, *de novo* and clearance of oxidatively damaged lipids, proteins and DNA. Various enzymes such as lipases, proteases and DNA repair enzymes are responsible for such defence. There is another defence mechanism in which appropriate antioxidants are produced and transferred to the correct place at the correct time and in the correct amounts.

Foods are essential for supplying the above mentioned antioxidants, components and trace elements. For example, glutathione peroxidases play a pivotal role in reducing hydroperoxides and hydrogen peroxide. Various types of glutathione peroxidases have been found and they and selenoprotein contain selenium as an essential metal ion. We obtain selenium mostly from vegetables and any soil which contains insufficient selenium causes selenium deficiency, such as in Keshan disease. We take phenolic antioxidants from foods, fruits and drinks such as tea.

8.3 Potency of natural antioxidants

As endogenous antioxidants synthesised by aerobes (e.g. SOD, catalase, GSH) do not completely prevent damage by reactive species *in vivo*,[1] efficient repair systems are needed to reduce the damage and humans must also obtain antioxidants from the diet. There is currently a considerable amount of interest in dietary antioxidants as bioactive components of food. The physiological role of some of these, such as vitamin E and vitamin C, is well established. The interest in flavonoids has increased in recent years because of their ubiquitous presence as antioxidants in food.

Flavonoids are diphenylpropanes that commonly occur in plants and are frequently components of human diet. They are consumed in relatively high quantities in our daily food. The main source of flavonoids is vegetables, fruits and beverages. For example, the content of quercetin glycoside in outer leaves of lettuce could be as high as 237 mg/kg fresh weight, and the content of kaemferol glycoside in kale could be 250 mg/kg fresh weight.[2,3] It has been found that flavonoids and other polyphenols possess antitumoral, anti-allergic, anti-platelet, anti-ischemic, and anti-inflammatory activities. Epidemiological evidence for the importance of flavonoids in reducing mortality from coronary heart disease was provided by the Zutphen Elderly Study.[4] The role of dietary phenolic antioxidants *in vivo*, protecting against cancer, has also been underlined by some epidemiological studies.[5,6] In these studies, flavonoids and other dietary compounds have been mentioned as statistically beneficial and protective against carcinogenesis.[4] Most of these biological effects are believed to come from their antioxidant properties. Flavonoids can exert their antioxidant activity by inhibiting the activities of enzymes including xanthine oxidase, myeloperoxidase, lipoxygenase and cyclooxygenase,[7,8] by chelating metal ions,[7,9–12] by interacting with other antioxidant such as ascorbate,[13] and most importantly,

by scavenging free radicals. This chapter introduces the free radical scavenging potency of flavonoids in food, their scavenging effect on reactive oxygen species and reactive nitrogen species, the inhibiting effect on lipid peroxidation, assessment of their potency as hydrogen-donating antioxidants, and their activity–structure relationships.

8.3.1 Ability to scavenge reactive oxygen species

Because of the importance of reactive oxygen species such as the superoxide anion and the hydroxyl radical in biological environments and in human health and disease, the reactivity of flavonoids toward these radicals has been extensively studied. There are generally two superoxide anion sources used: the enzymatic (hypo)xanthine-xanthine oxidase (X/XO) system and non-enzymatic sources such as the phenazine methosulphate-NADH system or potassium superoxide. Quercetin, myricetin and rutin are the flavonoids that were tested in the earlier studies. They quenched superoxide anions generated from either the X/XO system or from non-enzymatic sources.[14–16] Besides pure substances, an extract from *Ginkgo biloba* (GBE) showed SOD activity and scavenges superoxide anions produced from a non-enzymatic system. Further research showed that the scavenging ability depends on the chemical structures of flavonoids.[17,18] When the enzymatic system is used in the study, the scavenging effect may come directly from the radical-quenching effect or/and the enzyme-inhibiting effect. The structure–activity relationship of flavonoids as inhibitors of xanthine oxidase and as scavengers of the superoxide radical was recently reported by Cos et al.[19] It was found that the hydroxyl groups at C-5 and C-7 and the double bond between C-2 and C-3 was essential for a high inhibitory activity on xanthine oxidase. For a high superoxide scavenging activity, on the other hand, a hydroxyl group at C-3′ in ring B and at C-3 was essential. According to their effect on xanthine oxidase and as superoxide scavengers, flavonoids are classified into six groups: 1, superoxide scavengers without inhibitory activity on xanthine oxidase; 2, xanthine oxidase inhibitors without any additional superoxide scavenging activity; 3, xanthine oxidase inhibitors with an additional superoxide scavenging activity; 4, xanthine oxidase inhibitors with an additional pro-oxidant effect on the production of superoxide; 5, flavonoids with a marginal effect on xanthine oxidase but with a pro-oxidant effect on the production of superoxide, and finally; 6, flavonoids with no effect on xanthine oxidase or superoxide.

The hydroxyl radical is more reactive than the superoxide anion and is therefore more harmful to biological samples. Most flavonoids possess a high reactivity with the hydroxyl radical. For instance, (+)-catechin, (−)-epicatechin, 7,8-dihydroxy flavone, and rutin scavenge the hydroxyl radical at 100–300 times more than mannitol, a typical hydroxyl radical scavenger.[17] The reactivity of flavonoids toward hydroxyl radical is generally much higher than that toward superoxide anion. The reaction rate constants of

quercetin and kaempferol with the hydroxyl radical are $4.3 \times 10^9 M^{-1} s^{-1}$ and $4.6 \times 10^9 M^{-1} s^{-1}$, respectively, which are about four orders higher than those with the superoxide anion.[20] Some of the compounds from Indian medicinal plants behaved as scavengers of the hydroxyl radical in the deoxyribose degradation assay, with a calculated rate constant for kaempferol-3-*O*-galactoside of $1.55 \times 10^{10} M^{-1} s^{-1}$.[21] Baicalin reacts with the hydroxyl radical and the superoxide anion at the rate constants $7.7 \times 10^{11} M^{-1} s^{-1}$ and $3.2 \times 10^6 M^{-1} s^{-1}$, respectively.[22]

Flavonoids also react with singlet oxygen ($^1O_2$). Flavonoids in green tea such as (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin (EGC) and (–)-epicatechin (EC)) have been shown to scavenge $^1O_2$.[23] Tournaire et al.[24] studied the reactivity of 13 selected flavonoids (from the flavonol, flavone, flavanone and flavane families) with $^1O_2$ and tried to establish a structure–activity relationship. They found that the efficiency of the physical quenching is mainly controlled by the presence of a catechol moiety on ring B, whereas the structure of ring C (particularly the presence of a hydroxyl group activating the double bond) is the main factor determining the efficiency of the chemical reactivity of these compounds with $^1O_2$.

Flavonoids inhibit the production of reactive oxygen species in cells. Certain flavonoids slow down O_2 consumption (respiratory burst) of stimulated human neutrophils (PMNs) by its inhibitory action on NADPH-oxidase, the enzyme responsible for the reduction of O_2 to the superoxide anion. Consequently, superoxide anions and hydrogen peroxide production are significantly decreased when the PMNs stimulation is carried out in the presence of flavonoids. Some flavonoids are able to reduce significantly the activity of myeloperoxidase contained in neutrophils. This enzyme, secreted into the intra- and extracellular medium, catalyses the oxidation of chloride (Cl^-) by H_2O_2 to yield strong oxidants such as HOCl.

8.3.2 Ability to interact with reactive nitrogen species

Although nitric oxide (NO) is relatively unreactive *per se*, it may become potentially harmful once its concentration overwhelms its neurotransmitter and second messenger function and in particular when it reacts with the superoxide anion generating peroxynitrite ($ONOO^-$), which is a very reactive oxidant for most of the biological molecules. Some studies have demonstrated that flavonoids can efficiently quench reactive nitrogen species. The $ONOO^-$ scavenging activity of some of the flavonoids was found to be 10 times higher than that of ebselen, an efficient peroxynitrite scavenger.[25–27] Anthocyanidins are potent scavengers of NO and $ONOO^-$ as described by van Acker et al.[28] and by Haenen et al.[25] respectively. Pycnogenol, a procyanidin-rich extract from pine bark, significantly decreased in a dose-dependent fashion the accumulation of nitrite after spontaneous decomposition of sodium nitroprusside, thus acting as a nitric oxide radical scavenger.[29]

Morin, quercetin, or catechin may attenuate SIN-1 induced cytotoxic to cultured porcine aortic endothelial cells.[30] On the other hand, flavonoids can modulate the NO production level by regulating the expression of inducible nitric oxide synthase (iNOS).[31,32] Certain flavonoids have been reported to inhibit NO production in lipopolysaccharide-activated RAW 264.7 cells, and their inhibitory activity was suggested to be due to reduction of iNOS enzyme expression. Quercetin at 0.1 mM inhibited lipopolysaccharide-dependent production of iNOS mRNA and decreased NO release.

8.3.3 Effect on the lipid peroxyl radical and lipid peroxidation

As hydrogen-donating antioxidants, flavonoids directly scavenge lipid peroxyl radicals.[23,33] As clinical and biochemical evidence shows that low-density lipoprotein (LDL) oxidation is a crucial event in the pathogenesis of atheriosclerosis, the protective effect of flavonoids on LDL oxidation has been an interesting subject. De Whalley et al. have shown that quercetin, morin, fisetin and gossypetin with the IC_{50} at 1–2 μM inhibited the oxidative modification of human LDL induced by macrophages and delayed the depletion of endogenous α-tocopherol.[34] It has been known that red wine contains a considerable amount of flavonoids and experimental results showed that it could inhibit porcine LDL oxidation induced by copper ion or AAPH.[35] Wine diluted 1000-fold containing 10 μM total phenolics inhibited LDL oxidation significantly more than α-tocopherol.[36] It was suggested that wine polyphenols seem to act by their antioxidant activity rather than by metal chelating, and it has been reported that the antioxidant activity of human serum was elevated for several hours after wine consumption.[37] Studies in humans provide direct evidence that regular and long-term consumption of red wine, but not of ethanol, inhibited LDL oxidation *in vivo*.[38,39] It is suggested that red wine intake may reduce atherosclerosis and morbidity and mortality from coronary heart disease. These studies have provided a plausible explanation for the 'French paradox'.[40]

8.3.4 Assessment of antioxidant potential of flavonoids

For measuring the antioxidant potentials of flavonoids, either the total antioxidant activity (TAA), or the trolox equivalent antioxidant activity (TEAC) has been extensively used.[41–44] The TEAC method is to compare the ability of a hydrogen-donating antioxidant to scavenge a radical generated from the reaction of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) with a ferrylmyoglobin radical species[41–44] or potassium persulphate,[45] with that of trolox. The value of TEAC may be considered as a stoichiometric number because TEAC for trolox was set at 1.0. To test the efficacy of a certain substance as a radical scavenger, clear evidence may come from the determination of reaction rate constants with specific radicals. Although the stoichiometric number determines the duration of the

inhibition period or lag time, the rate constant can give the extent of inhibition in oxidation. Two methods including both inhibition period and extent of inhibition in oxidation are used in studying the potential of natural antioxidants such as flavonoids. Descriptions of them follow.

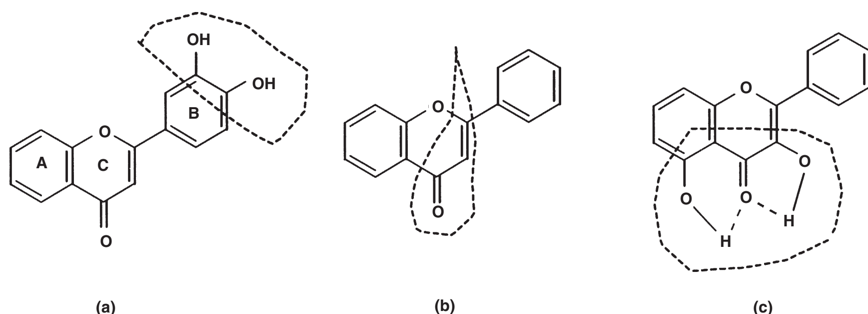
8.3.4.1 ORAC method

The oxygen radical absorbance capacity (ORAC) assay developed by Cao and co-workers[46,47] provides a new way of evaluating potential antioxidant activity. The method uses an area-under-curve technique and thus combines both inhibition time and inhibition degree of free radical action by an antioxidant into a single quantity, while other similar methods use either the inhibition time at a fixed inhibition degree or the inhibition degree at a fixed time as the basis for quantifying the results.[41,48–50] In addition, different free radical (lipid peroxyl radical, hydroxyl radical, superoxide anion etc.) generators can be used in the ORAC assay.

8.3.4.2 Galvinoxyl method[51]

Galvinoxyl is a rather stable radical and, while it accepts an electron or hydrogen radical to become a stable and diamagnetic molecule, can be oxidised irreversibly. Because of its odd electron, galvinoxyl shows a strong absorption band at 428 nm (in ethanol), its solutions appearing yellow in colour (at low concentration). As the electron is paired off, the absorption vanishes and the resulting decolorisation is stoichiometric with respect to the number of electrons taken up. Taking advantage of the colour change of galvinoxyl in the presence of an antioxidant, the dynamics of the antioxidant activity, hydrogen-donating activity, can be easily measured in a simple and similar manner. Galvinoxyl can be used not only to measure the stoichiometric number of active phenolic hydrogens of a substance, but also to determine the rate constant for related antioxidants. Furthermore, the method can be used to determine and compare the antioxidative activity of hydrogen-donating compounds, either pure substances or mixtures. The second-order rate constant of GBE has been compared with other pure substances on the molar basis of active hydroxyl groups.

Some results from the three methods, TEAC, ORAC and galvinoxyl, are listed in Table 8.2. Although the radical sources are different, the efficacies of flavonoids are comparable. The stoichiometric number in the reaction with galvinoxyl decreases in the following order: myricetin > quercetin > catechin > kaempferol > α-tocopherol. The total reactive rate constant decreases in the order of myricetin > quercetin > α-tocopherol > kaempferol > catechin. The average reactive potential of active hydroxyl groups studied decreased in the order myricetin > α-tocopherol > quercetin > GBE = kaempferol > catechin. The $ORAC_{LOO\cdot}$ gives the order myrecetin > quercetin > kaempferol. The TEAC order is quercetin > myricetin > catechin > kaempferol > α-tocopherol. In the ORAC and galvinoxyl study, myricetin was the strongest antioxidant among the substances tested, while the TEAC



8.1 Antioxidant activity–structure relationship of flavonoids.

value of myricetin was less than that of quercetin. This indicates that the TEAC value may not completely reflect the potential of an antioxidant.

8.3.5 The structure–activity relationships

As mentioned above, the antioxidant activity of flavonoids depends on their chemical structure. Generally, there are three structure groups in the determination, the free radical scavenging and/or antioxidative potential of flavonoids (Fig. 8.1): (a) a catechol moiety of the B-ring, (b) the 2,3-double bond in conjugation with a 4-oxofunction of a carbonyl group in the C-ring and (c) presence of hydroxyl groups at the 3 and 5 positions. Quercetin possesses all the three structure groups and thus usually gives a higher antioxidant potential than kaempferol, which has no catechol moiety in the B-ring. From our study (Table 8.2) and others, the presence of a hydroxyl group at 5′ in the B-ring increases the antioxidant potential significantly.

Table 8.2 Comparison of antioxidant potential determined by three methods, galvinoxyl,[51] ORAC[52] and TEAC[42]

	Galvinoxyl			$ORAC_{LOO\bullet}$	TEAC
	n	$k_2{}^{b}$	$k_2{}^{1b}$		
myricetin	4.5	1.1×10^6	2.4×10^5	4.3	3.1
quercetin	4.0	5.9×10^3	1.5×10^3	3.3	4.7
kaempferol	1.9	2.1×10^3	1.1×10^3	2.7	1.3
catechin	3.0	1.5×10^3	5.0×10^2	–	2.2
α-tocopherol	1.0	2.4×10^3	2.4×10^3	–	1.0
GBE	1.1×10^{-4} [a]	0.13[c]	1.2×10^3	–	–

[a] mol active hydroxyl groups/g
[b] second-order rate constant for molecule (k_2) and per active hydrogen (k_2') in $M^{-1} s^{-1}$
[c] $(g\ l^{-1})^{-1} s^{-1}$

The stoichiometric number of myrecetin is only 0.5 greater than that of quercetin but the total reactive potential (k_2) and the average reactive potential (k_2') are two orders higher than those of quercetin. This is in agreement with other reports indicating that the antioxidant activity of myricetin is more potent than quercetin in emulsions, oils, liposome and LDL.

8.4 Future trends

There are now increasing reports which suggest the non-classical role and function of antioxidants which are not explained by the inhibition of oxidation alone. For example, there is a general consensus that vitamin E exerts a broad range of effects that promote vascular homeostasis,[53] by, for example, inhibiting protein kinase C activity, proliferation of smooth muscle cells and adhesion of inflammatory cells to be endothelial cells. Furthermore, it has been shown that some antioxidants are capable of inducing phase II defence enzymes such as quinone reductase and glutathione *S*-transferase. The physiological role of such functions is a matter of future study.

8.5 Sources of further information

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9

Sources of natural antioxidants: oilseeds, nuts, cereals, legumes, animal products and microbial sources

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9.1 Introduction

The mere mention of natural antioxidants (NAOs) brings about an association with spices and herbs, in that product developers utilise spice and herb extracts as replacements for synthetic antioxidants. However, other natural products such as oilseeds, nuts, cereals, legumes, animal products, and microbial products can serve as sources of NAO. Although the aforementioned natural products are not touted as sources of antioxidants, these materials can provide product developers with an alternative source of NAO. This chapter will highlight the general classes or categories of antioxidants found in the above mentioned alternative NAO sources.

Simple phenols, polyphenolics and phenolic acid derivatives are the antioxidants that are common to all plant sources of NAO in this chapter. In addition, modified proteins and amino acids are antioxidants derived from animal and microbial products. This chapter will provide information regarding the structural components of the active antioxidants, concentrations of the NAO in specific natural products, and factors that affect antioxidant activity. In addition, the antimicrobial activity of natural antioxidants will be presented. Methods for the isolation and characterisation of NAOs, from the above sources, will not be presented in detail in this chapter. The author suggests that the review of the referenced literature will be of value in this regard.

This chapter will first provide characteristics of NAOs, followed by the presentation of antioxidants from: 1, legumes, nuts and oilseeds; 2, cereals; 3, animal products; and 4, microbial sources. Preservation activity of the NAO and future trends will complete the chapter.

9.2 Characteristics of natural antioxidants

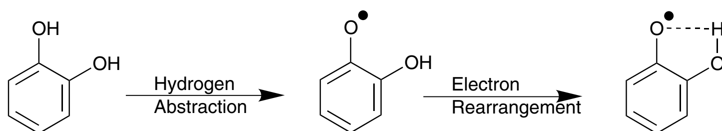
9.2.1 Structural components – general

The overall effectiveness of the NAO is dependent on the involvement of the phenolic hydrogen in radical reactions, the stability of the NAO radical formed during radical reactions, and chemical substitutions present on the structure. The substitutions on the structure are probably the most significant contribution to the ability of an NAO to participate in the control of radical reactions, and the formation of resonance stabilised NAO radicals. The effect of substituting functional groups to synthetic phenols has been known for more than 40 years since Miller and Quackenbush found that alkyl substitutions could enhance antioxidant activity (AOA).[1,2]

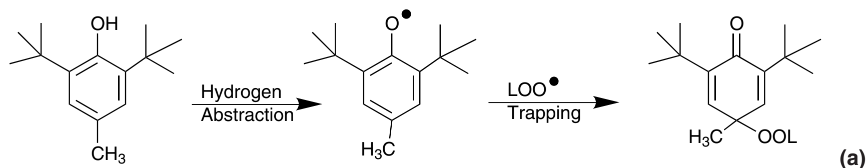
The electron-donating capability of methyl, ethyl, and tertiary butyl substitutions at positions *ortho* and *para* to the hydroxyl groups greatly enhance the AOA of phenol. In addition, hydroxyl substitutions at these positions will enhance AOA. *Ortho* substituted phenols, e.g. 1,2-dihydroxybenzene, tend to form intramolecular hydrogen bonds during radical reactions (Fig. 9.1), which enhance the stability of the phenoxy radical.[3] The presence of a methoxy (OCH_3) substitution *ortho* to the hydroxy group is unable to undergo hydrogen bonding resulting in a weaker AOA. Similar AOA would be expected for NAOs having structural characteristics similar to synthetic phenols.

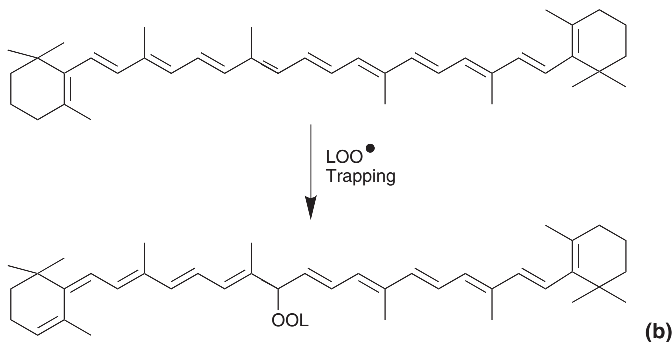
NAOs would be expected to participate in radical trapping and singlet oxygen quenching mechanisms. Radical trapping mechanisms can occur via interactions between radical species such as an antioxidant radical and lipid peroxyl radical (Fig. 9.2a). Alternatively, lipid peroxy radicals can interact with electron dense regions of a molecule. For example, the conjugated polyene system of carotenoids has been found to interact with peroxy radicals (Fig. 9.2b and c).[4]

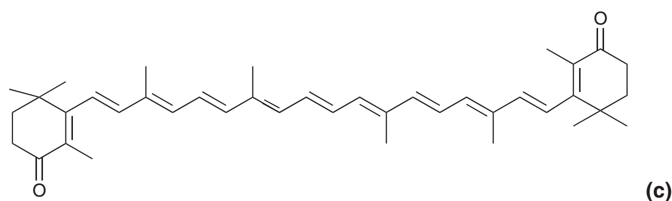
Metal chelating is an example of a secondary antioxidant mechanism by which many NAOs can influence the oxidation process. Metal chelators can stabilise the oxide forms of metals, that is, reduce redox potentials, thus preventing metals from promoting oxidation. In addition, the metal chelators form complexes with the metals making them unavailable to promote oxidation. The NAOs pertinent to this chapter have metal-chelating proper-



9.1 Intramolecular hydrogen bonding of *ortho* substituted phenols proposed by Baum and Perun.[3]





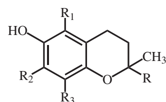


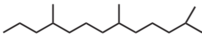
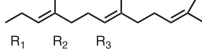
9.2 Radical trapping mechanism for phenolic antioxidants (a) and carotenoids (b) and (c).

ties as well as hydrogen donating, radical scavenging, and singlet oxygen quenching activities.

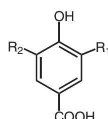
9.2.2 Structural components – specific groups of antioxidants

Monophenols and phenolic acids (Fig. 9.3) participate in hydrogen donating and radical scavenging reactions. Tocopherols and tocotrienols have been widely documented as having antioxidant activity, due primarily to the phenolic hydrogen at the C_6 position. Also, the AOA of phenolic acids is due to the phenolic hydrogens. Pratt and Birac reported that caffeic acid was a better antioxidant than ferulic acid and *p*-coumaric acid.[5] The presence of a second hydroxyl group enhances the AOA, mainly via intramolecular hydrogen bonding, of caffeic over *p*-coumaric and ferulic. The *ortho* methoxy substitution in ferulic acid may provide a stabilising effect on the



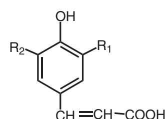
	R
Tocopherol	
Tocotrienol	

	R_1	R_2	R_3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3



Benzoic Acid Derivatives

Compound	R_1	R_2
p-Hydroxy benzoic acid	H	H
Vanillic acid	H	OCH_3
Syringic acid	OCH_3	OCH_3
Dihydroxybenzoic acid	OH	H
Gallic acid	OH	OH



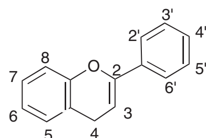
Cinnamic Acid Derivatives

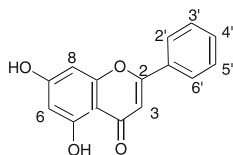
Compound	R_1	R_2
p-Coumaric acid	H	H
Ferulic acid	H	OCH_3
Sinapic acid	OCH_3	OCH_3
Caffeic acid	OH	H

9.3 Monophenol (e.g. tocopherols and tocotrienols) and phenolic acids as examples of common natural antioxidants.

phenoxyl radical thus explaining the better AOA of ferulic over *p*-coumaric acid. The presence of three hydroxyl groups gives added protection as seen by the improved AOA of trihydroxybenzoic acid (i.e. gallic acid) over 3,4-dihydroxybenzoic acid (i.e. protocatechuic acid). The acid proton appears to have little impact on the AOA. The acid proton of caffeic acid is replaced with a quinic acid via an ester bond in chlorogenic acid (Fig. 9.3), yet both compounds were equally effective in controlling lipid oxidation.[5] The allylic group, as found in cinnamic acid derivatives (Fig. 9.3), appears to improve AOA when compared to benzoic acid derivatives. Pratt and Hudson reported that caffeic acid (3,4-dihydroxycinnamic acid) was a better antioxidant than protocatechuic acid (3,4-dihydroxybenzoic acid) in a lard system.[6] One plausible explanation could be that the allylic group enhances the resonance stability of the phenoxyl radical.[7]

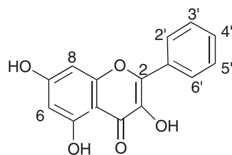
Flavonoids are a group of compounds characterised by a C_6–C_3–C_6 configuration (Fig. 9.4) and can participate in hydrogen donating, radical scavenging, and metal chelating mechanisms.[8–17] As in other phenolic antioxidants, the position and number of hydroxyl groups dictates the AOA of flavonoids. Effective hydrogen donation activity is due primarily to the *ortho*-dihydroxylation on the B ring (Fig. 9.4).[8,16] The additional hydroxyl (OH) group at the 5′ position enhances the AOA whereas one OH group dramatically reduces the AOA.[8] For example, myricetin with three OH groups is more active than quercetin (two OH) and hesperitin (one OH). The presence of a C-3 OH (aglycone) group enhances AOA when compared to the glycosylated form.[12,13] In addition, ring A OH substitutions at the 5,8 or 7,8 but not 5,7 improved AOA while the C-4 carbonyl and the C-2:C-3 double bond did not influence AOA.[18]





Flavones

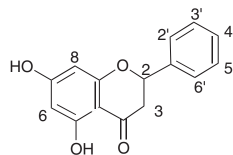
	3'	4'
Apigenin	H	OH
Chrysin	H	OH
Luteolin	OH	OH



Flavonols

	2'	3'	4'	5'
Datiscetin	OH	H	H	H
Quercetin	H	OH	OH	H
Dihydroquercetin*	H	OH	OH	H
Myricetin	H	OH	OH	OH
Morin	OH	H	OH	H
Kaempferol	H	H	OH	H
Rutin**	H	OH	OH	H
Hesperidin***	H	OH	OCH_3	H

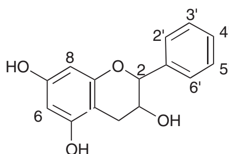
*Dihydroquercetin has an additional H at the C-3 position due to the loss of the double bond at the C-2:C-3 position.
**Rutin is a glycoside in which the C-3 position contains an *o*-rutinose.
***Hesperidin contains an *o*-rutinose at the C-7 position.



Flavanones

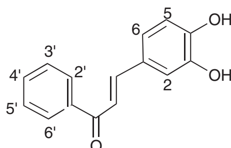
	3	3'	4'
Naringenin	H,H	H	OH
Naringin*	ORh	H	OH
Taxifolin	OH	OH	OH
Fustin**	OH	OH	OH
Eriodictyol	H,H	OH	OH

*Naringin is a glycoside in which the C-3 position contains a rhamnoglucose unit.
**Fustin lacks a C-5 OH.



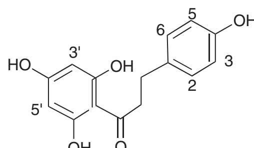
Flavans

	3'	4'
Catechin	OH	OH



Chalcones

	2'	3'	4'
Butein	OH	H	OH
Okanin	OH	OH	OH



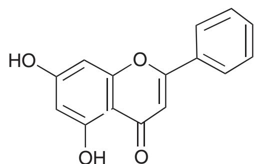
Dihydrochalcone (Phloretin)

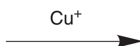
9.4 Structure of flavonoids and related compounds isolated from plant materials.

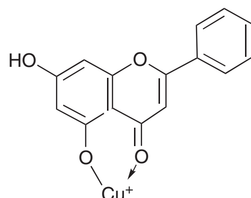
The *ortho* substitution in ring B as well as three OH groups enhance radical scavenging activity.[10,11,15,17] Unlike hydrogen donating, the C-4 carbonyl can enhance scavenging activity while the C-3 OH has no effect.[10] Conflicting findings have been reported for the role of the C ring C-2:C-3 double bond on AOA. Husain et al.[10] reported that the double bond did not influence scavenging activity. However, Foti et al.[15] and Bors et al.[11] proposed that the double bond in conjunction with the C-4 carbonyl could stabilise the radical via electron rearrangements. The metal-chelating activity of flavonoids requires the presence of the 3′,4′-dihydroxy configuration and

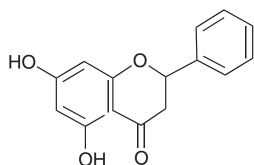
5-Hydroxy flavones and flavanones

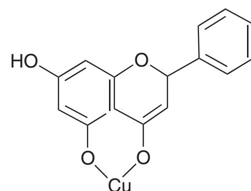
Metal complexes of 5-hydroxy flavonoids





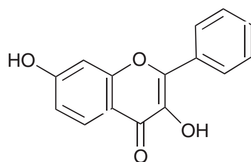


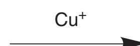


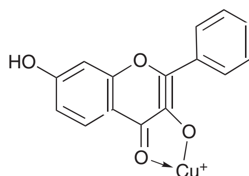


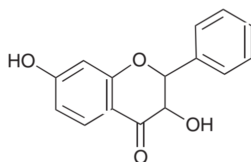
3-Hydroxy flavones and flavanones

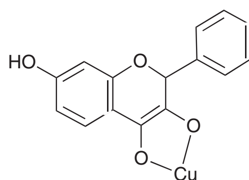
Metal complexes of 3-hydroxy flavonoids







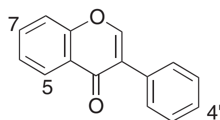




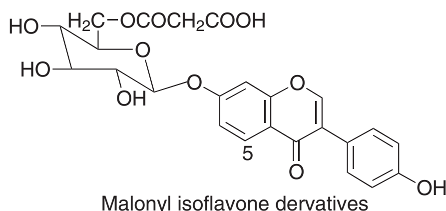
9.5 Metal-chelating mechanism of flavonoids as proposed by Hudson and Lewis.[21]

more importantly the C-4 quinone and a C-3 or C-5 OH.[19–21] The hydrogenation of the C2:C3 double bond resulted in the loss of metal-chelating activity, presumably due to the lack of electron rearrangement that occurs during the formation of the metal–flavonoid complex (Fig. 9.5).[19,20]

Isoflavones (Fig. 9.6) are structurally similar to the flavonoids and found most often in the *Leguminoseae* family. Genistein and the 7-β-glucoside, genistin, were found to have the highest AOA of the isoflavones followed by daidzein and daidzin, formononetin and Biochanin A.[22] The C-7 location has little influence on AOA as noted by the similar AOA of the aglycone and glycoside forms of isoflavones. The OH at the C-4′ position is key to the antioxidant activity which is further enhanced by an OH at the C-5



	4'	5	7
Genistein	OH	OH	OH
Genistin	OH	OH	O-Glucose
Daidzein	OH	H	OH
Daidzin	OH	H	O-Glucose
Biochanin A	OCH_3	OH	OH
Formononetin	OCH_3	H	OH



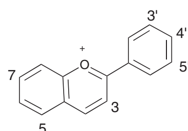
	5	6
6"-O-Malonylgenistin	OH	H
6"-O-Malonyldaidzin	H	H
6"-O-Malonylglycitin	H	OCH_3

9.6 Structure of isoflavones common to the *Leguminoseae* family.

location.[22,23] The loss of the C-2:C-3 double bond in combination with the C-4 carbonyl had a slight enhancement of the AOA.[23]

Anthocyanins and anthocyanidins (Fig. 9.7) are metabolic products of flavanones and are placed in the flavonoid group. The lack of the C-4 carbonyl suggests that metal-chelating activity is due to the *ortho* OH substitutions at the C-3′ and C-4′ position in ring B. Compared with flavones, anthocyanidins are less active, and this is attributed to the lack of the C-4 carbonyl that, in conjunction with the C-2:C-3 double bond, plays an important role in AO.[17,24] Radical scavenging activity of anthocyanidins is also dependent on the *ortho* OH configuration.[16,24,25] However, the addition of a third OH at the 5′ position did not enhance activity, contrary to other flavonoids.[16,24] Radical scavenging activity of the anthocyanins (glycoside form) in general was better than that of the anthocyanidins.[24,26] The sugar moiety also influences activity, which may account for the differences in the radical scavenging activity observed by researchers.[16,24,26] The addition of glucose enhanced activity to a greater degree than rhamnoglucose and galactose did.[24] The difference in molecular structure of the sugars may cause structural configurations in the anthocyanins that enhance or diminish their ability to form a stable radical.[11,27]

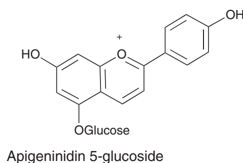
The condensation of anthocyanins to other flavonoids through the C-4 position results in the formation of proanthocyanidin polymers (i.e. condensed tannins).[28] The antioxidant activity of these compounds would be expected to be similar to their parent flavonoid compounds, with the activity dependent on the C-3′ and C-4′ OH groups. Salah et al.[14] showed that the activity of a group of condensed tannins composed of gallic acid and epicatechin were effective antioxidants. They note that as the numbers of OH groups increased, so did AOA. Hydrolysable tannins consist of a glucose linked via ester bonds to a number of gallic acid and/or hexahydroxydiphenic acid units and can be degraded by acid or alkaline conditions to release



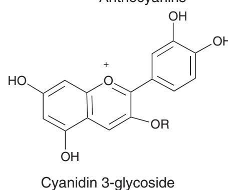
Anthocyanidins

	3	3'	4'	5'	5	7
Apigeninidin	H	H	OH	H	OH	OH
Cyanidin	OH	OH	OH	H	OH	OH
Delphinidin	OH	OH	OH	OH	OH	OH
Luteolinidin	H	OH	OH	H	OH	OH
Malvidin	OH	OMe	OMe	OMe	OH	OH
Pelargonidin	OH	H	OH	H	OH	OH

Anthocyanins

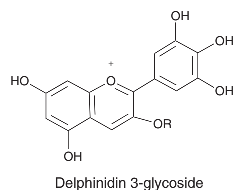


Apigeninidin 5-glucoside



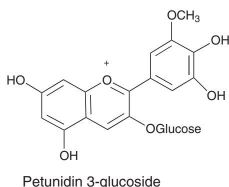
Cyanidin 3-glycoside

	R
Cyanidin 3-glucoside	glucose
Cyanidin 3-galactose	galactose

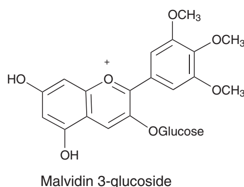


Delphinidin 3-glycoside

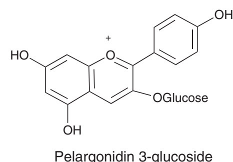
	R
Delphinidin 3-glucose	glucose
Delphinidin 3-rutinoside	rutinose



Petunidin 3-glucoside



Malvidin 3-glucoside



Pelargonidin 3-glucoside

9.7 Example structures of anthocyanidins and anthocyanins isolated from plant materials.

glucose and the phenolic unit. The large numbers of OH groups would provide AOA, thus under proper conditions the tannins can provide AOA.

Carotenoids constitute a final category of NAO. The conjugated polyene system contributes to the singlet oxygen quenching characteristics of carotenoids.[4,29,30] The presence of nine or more double bonds in the carotenoid structure greatly enhanced the singlet oxygen quenching activity. In addition, the oxo groups at the 4(4′) position in the β-ionone ring improved AOA (Fig. 9.2c).[30] The carbonyl present on the ring enhanced the stability of trapped radicals and therefore reduced the tendency of carotenoids to promote radical reactions. The polyene system can also trap radicals, thus providing additional protective activity.

The natural antioxidants in this chapter fall under one of the category of compounds described above and thus, predicting AOA can be based on the general rules for each category of compounds. The effect of processing on a specific compound can be used as a predictor for a category of compounds having similar characteristics.

9.2.3 Factors affecting antioxidants

The loss of the NAO during the processing of a commodity is of the utmost concern. Although not considered a source of NAO, soybean oil can be mixed into formulas to provide tocopherols. The loss of tocopherol during the processing of soybean oil is an example of how processing may affect the NAO content in a product.[31] The deodorisation step in the process caused a 20% reduction in tocopherol content while degumming through the bleaching process resulted in a 12% reduction. In addition, the crude oil was more stable than the refined oil suggesting that the removal of the antioxidant (AO) played a key role and that processing methods should be considered when preparing or using NAO in food formulas.

The trend towards eating a healthier diet has led to an increase in consumption of grain products. Wang et al.[32] reported that the pearling process more efficiently concentrates the tocotrienols and tocopherols than traditional milling processes. If barley is utilised in food formulas, the pearling flour could contribute more to the oxidative stability than a fraction resulting from traditional milling operations. Similar observations have been made for the milling operations in wheat and oats in that the concentration of the AO could be completed by fractioning the grain into bran and flour, for example.[33,34]

The pH of a system will also dictate AOA. Anthocyanins were less effective as metal chelators as the solution pH became more acidic.[26] However, the hydrogen-donating activity of the anthocyanins increases the more acidic the solution. The ionisation of the OH groups under more basic conditions may promote metal complexing.[26,35]

Other factors that influence the activity of NAO include heat, fermentation, and the presence of metals. These factors will be addressed in the sections that follow that are appropriate for the specific NAO. The usefulness of NAO will be dependent on the fractions used in the food item or whether a crude extract can provide sufficient antioxidant activity. The intent for the NAO listed below is that a product would be added to a food formula as a flour or extract rather than by preparing isolates.

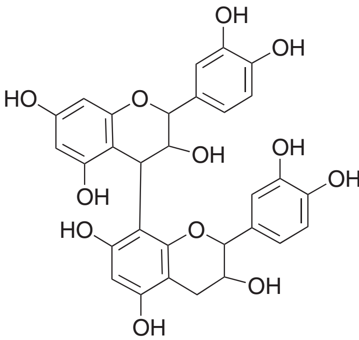
9.3 Antioxidants from legumes, nuts and oilseeds

9.3.1 Legume (including pulses) antioxidants

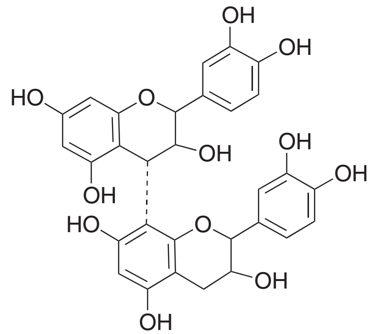
Peanuts, peas, and edible beans will be highlighted with a focus on polyphenolics and phenolic acids. Red peanut skins were found to contain 7–9% procyanidins with 50% of these as low molecular weight phenolic oligomers.[36] The water soluble 2,3-*cis*-procyanidins were found to be the predominant procyanidins and are characterised by a bond linked by a 4β→8 or 4β→6 arrangement that terminates with a 2,3-*trans*-flavan-3-ol

catechin (Fig. 9.8). The low molecular weight phenolics were mainly flavan-3-ols of catechin and epicatechin. Epicatechin-(4β→8;2β→O7)-catechin was the predominant species with epicatechin-(4β→8;2β→O7)-epicatechin and epicatechin-(4β→8)-catechin making up smaller concentrations of flavonoids.

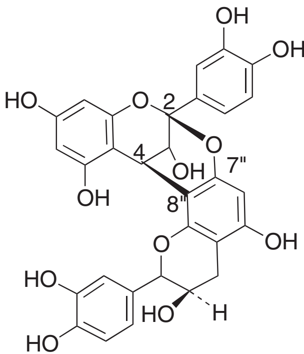
Methanol, ethanol, and acetone extracts of peanut hulls were significantly better antioxidants than chloroform and hexane extracts.[37] Fractionation of the methanol extract showed that luteolin was a major component of one fraction with AOA. A total phenolic content of 1.67 $mg\,ml^{-1}$ provided the maximum protection (93–95 %) against the oxidation of linoleic acid.[38] The predominant flavonoid, luteolin, was found to increase by a factor of 15 between days 74 to 144 of the growing season. However, the AOA of extracts prepared each day was not significantly dif-



Procyanidin B-1



Procyanidin B-3



Epicatechin-(4β→8;2β→O7)-catechin

9.8 Condensed tannins isolated from the skins of mature peanuts (*Arachis hypogea* L.), as characterised by Karchesy and Hemingway.[36]

ferent, suggesting that a low level of luteolin and total phenolic content could effectively control oxidation. Yen and Duh attributed the radical scavenging to the total phenolic components while metal-chelating activity was due to luteolin, which made up 34 % of the phenolics in a methanol extract of peanut hulls (MEPH).[39] These authors noted that the scavenging activity was concentration dependent. The addition of MEPH at 0.06 mg ml^{-1} gave 1 % inhibition while 1.2 mg ml^{-1} resulted in an 89 % inhibition of radical activity.

The effectiveness of the MEPH decreased as the pH went from 3 to 9. At pH 7.0 80 % of the AOA remained but was completely lost at pH 9.0.[40] Alterations in the structure of the phenolics may be responsible for the loss of the hydrogen-donating activity. When MEPH was heated at 185 °C for 20 min prior to addition to linoleic acid, a 5 % decrease in the oxidative inhibition was found.[40] In addition, storage of the MEPH extract at 30 °C resulted in a 7 % reduction of AOA while the storage under nitrogen prevented the loss of activity.

Phenolic acids also contribute to the AOA of peanut products. Seo and Morr found that a defatted peanut meal and commercial flour contained around 2000 and 1750 μg g^{-1} phenolic acids, respectively.[41] Peanut isolates had a phenolic acid content of 800 μg g^{-1} while ion exchange and carbon-treated products contained 92 and 82 % less phenolic acid than the defatted meals. The application of peanut meal and flour to food formulas can contribute to the oxidative stability while highly purified products, i.e. isolates, would have less of an impact. Dabrowski and Sosulski found that 91.4 and 8.6 % of the phenolic acids were in the ester and insoluble forms, respectively.[42] *p*-Coumaric acid was the predominant phenolic acid (84 %) followed by ferulic (8.7 %), sinapic (5.0 %), caffeic (1 %) and *p*-hydroxybenzoic (1 %).

Tocopherols have a long tradition as being hydrogen-donating antioxidants. Peanut oil was found to contain between 350 and 650 ppm tocopherols.[43,44] Gamma (γ) accounts for 55 % of the tocopherols while alpha and delta make up 40 and 5 %, respectively. Although not natural, roasting of peanuts enhanced the AOA due mainly to the development of Maillard browning reaction compounds.[45] The combination of NAO and antioxidants developed via roasting provide food product developers with an alternative antioxidant source.

9.3.1.1 Pea bean

Methanol extracts of the pea bean (*Phaseolus vulgaris* L.) had strong AOA.[46] When it was further fractioned into *n*-butanol and water extracts it was shown that the water extract was the predominant source of AOA and contained 0.31 mg g^{-1} total phenolics. In addition, the water extract was synergistic with α-tocopherol. Heating the water extract for 1 h at 100 °C did not affect the AOA of the extract. Tsuda et al.[47] assessed the AOA of white,

red and black bean seeds (*Phaseolus vulgaris* L). They found that the seed coat and germ of the white varieties had no AOA whereas the red and black seed coats had good AOA. The germ of the black seed had no AOA and only minimal AOA was reported for the germ of the red bean. The germ was not a significant source of tocopherol thus explaining the lack of AOA in the germ. Further extraction of the red and black seed coats using 80 % ethanol with 0.5 % trifluoroacetic acid resulted in the identification of three anthocyanins.[47] Cyanidin-3-*O*-β-D-glucoside (C3G) and pelargonidin-3-*O*-β-D-glucoside (Fig. 9.7) were found in the red beans while delphinidin-3-*O*-β-D-glucoside was found in the black bean. Takeoka et al.[48] found that delphinidin-3-*O*-β-D-glucoside, petunidin-*O*-β-D-glucoside, and malvidin-3-*O*-β-D-glucoside accounted for 56, 26, and 18 %, respectively, of the anthocyanins in black bean. Pelargonidin-3-*O*-β-D-glucoside and delphinidin-3-*O*-β-D-glucoside had no AOA in linoleic acid liposomes at pH 7.0 but were strong antioxidants at pH values of 3 and 5. The stabilised flavylium cation at pH 3 and 5 may contribute to the AOA. C3G was found to be a strong antioxidant at pH 7.0.[47] The disappearance of C3G after 10 days did not diminish the AOA. Tsuda et al.[49] found that cyanidin, aglycone form of C3G, was a better antioxidant than C3G in liposomes. They proposed that the AOA of C3G after 10 days was due to the formation of the cyanidin.

9.3.1.2 Miscellaneous edible beans

Edible beans and pulses have been used widely as a food stable. Tsuda et al.[50] evaluated the AOA of methanol extracts of 35 pulses and found that 11 species had strong AOA in a linoleic–ethanol–phosphate system (pH 7.0). Kidney bean, guar and tamarind had strong AOA while azuki, black soybean, cowpea, lentil and faba had low AOA when added at $1\,mg\,ml^{-1}$. Tocopherol contents ranged from 13 to 152 ppm in the extracts. Pulses with the highest AOA also had the highest tocopherol concentrations. However, the tocopherol alone may not be responsible for all AOA due to the intense colour of the pulses which indicates the presence of flavonoids.

Other edible beans can be a source of NAO if the beans themselves do not affect the sensory quality of a product. Ariga and Hamano reported that procyanidins B-1 and B-3 from azuki beans had radical scavenging activity.[51] A total of eight radicals could potentially be trapped by the procyanidins. Rings A and B could each trap up to four radicals. The presence of procyanidins in other bean or pulses would be expected to have similar radical trapping activity.

Onyeneho found that freeze-dried extracts of the navy, garbanzo, and pinto bean hulls were effective at controlling the oxidation of soybean oil.[52] Navy bean hulls were most effective followed by pinto and garbanzo bean

hulls. Subsequent investigations of navy bean hulls showed a phenolic acid content of 191 mg/100 g hulls. Protocatechuic acid represented 49 % of the total phenolic acids while syringic and salicylic acids accounted for 12 % each. *p*-Coumaric acid, *p*-hydroxybenzoic acid, caffeic acid, gentisic acid, gallic acid and vanillic acids were other phenolic acids which contribute to AOA.

Carbonaro et al.[53] found that tannins in faba beans could provide hydroxyl radical scavenging activity; thus, other polyphenolics such as tannins may play a significant role in the AOA of navy bean hulls. The flour of faba beans was also shown to have AOA, although steaming of the flour caused a reduction in the AOA.[54] Methanol extracts of the mung bean were found to be both metal chelators and radical scavenging suggesting that flavonoids were present in the extract.[55] For additional information regarding the anthocyanins in legumes see Mazza and Miniati.[56]

9.3.2 Nut antioxidants

Antioxidants from nuts are generally localised in the seed coat with lower amounts in the cotyledons. Expressed nut oil from macadamia nuts shows this trend in which the kernel oil obtained from the cotyledons had a significantly lower phenolic content (49 $\mu g\, g^{-1}$) than the 838 $\mu g\, g^{-1}$ in the shell.[57] The phenolic compounds identified were 2,6-dihydroxybenzoic acid, 2′-hydroxy-4′-methoxyacetophenone, 3′,5′-dimethoxy-4′-hydroxyacetophenone, and 3,5-dimethoxy-4-hydroxycinnamic acid. Refining of the macadamia nut oil significantly reduced the oxidative stability of the oil. Quinn and Tang[57] postulated that the cold pressing of the nuts co-extracts the phenolics and that the refining process removes important phenols. This was supported by the observed increase in oxidative stability of macadamia nut oil supplemented with the ground shell of the macadamia nut. The AOA of a refined macadamia nut oil containing 0.01 % 3′,5′-dimethoxy-4′-hydroxyacetophenone was significantly better than the oils containing other purified antioxidants. However, a commercial cold-pressed product had good stability indicating that a combination of components contributed to the oxidative stability.

9.3.2.1 Hazelnut

Pershern et al.[58] reported that the stability of hazelnuts was correlated to α-tocopherol content. Samples containing 210 ppm tocopherol were significantly more stable than nuts with lower tocopherol levels (80 ppm). Storage of the hazelnuts at a water activity of 0.6 was more stable than at low water activities, which is consistent with the relationship between AOA and water activity models.

9.3.2.2 Pecan and cashew

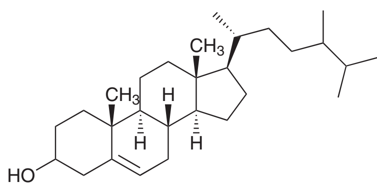
Pecan and cashew nut quality is inversely dependent on phenolic content.[59,60] In pecans, leucocyanidins and leucodelphinidins are converted to cyanidin and delphinidin, respectively. Oxidative processes in the nuts promote polymerisation of the flavonoid, causing discoloration. In addition, the phenolic acids gallic, gentisic, and vanillic were reduced by 39, 36 and 28 %, respectively, which related to the poor sensory characteristics.[61] The successful application of pecan nut meals in food formulas will require the inactivation of enzyme and protection from oxidative stability; thus, this information represents a case where phenolic materials do not provide sufficient protection and should not be used as a source of NAO. Similarly, cashew nuts with a high iron content were found to develop dark pigments.[60] However, washing the cashew nut with dilute acid diminishes the discoloration and represents a viable application of ground cashew meals in food systems having a weak acid environment. The antioxidants present in cashews include catechins, epicatechins, leucocyanidins and leucopelargonidins.[60]

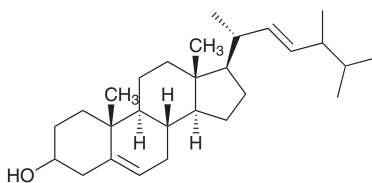
9.3.3 Oilseed antioxidants

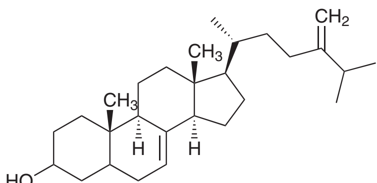
Flaxseed, sunflowers, soybean, cottonseed, and canola antioxidants typify the antioxidants from oilseeds. An important group of antioxidants not previously mentioned includes the sterols (Fig. 9.9). These compounds have been shown to prevent thermal oxidative degradation of oils.[62–64] Furthermore, sterols with ethylidene side chains (e.g. Δ^5-avenasterol) were most effective.[62,63] Gordon and Magos proposed that the non-lipid radicals react rapidly with the unhindered allylic carbons of the sterols.[64] Subsequent electron rearrangement results in a stable allylic tertiary free radical that reacts slowly with the lipid thus disrupting the autoxidation process. Other antioxidants common to oilseeds include tocopherols and tocotrienols (Table 9.1).

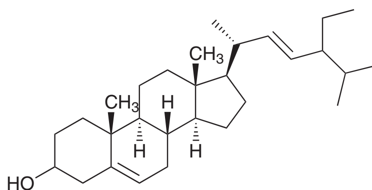
9.3.3.1 Flaxseed

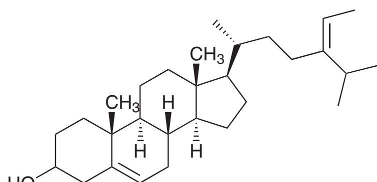
Amarowicz et al.[65] reported that fractionation of flaxseed could produce a fraction with high AOA. Extraction with 95 % ethanol followed by separation of a Sephadex LH20 column resulted in four predominant fractions. Fraction IV had the highest phenolic content at 156 mg g^{-1} while fraction I contained only 66 mg g^{-1} flax. Interestingly, fraction I had the highest AOA of all fractions suggesting that the total phenolic content is not the most important factor to consider when using flaxseed extracts as a source of NAO. Shahidi et al.[66] suggested that lignans (Fig. 9.10) in flaxseed were responsible for the AOA, which was supported by the radical scavenging property of secoisolariciresinol diglucoside.[67] Meagher et al.[68] characterised isolariciresinol and pinoresinol (Fig. 9.10) from flaxseed, which may

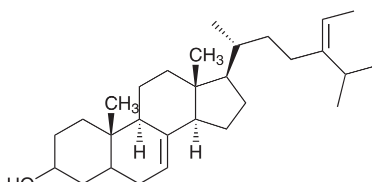












9.9 Common plant sterols (i.e. phytosterols)

contribute to the AOA. Other components such as phenolic acids and tannins may provide AOA. Dabrowski and Sosulski reported a phenolic acid content of *c*. 740 ppm with 89 % in the ester form.[42] *Trans*-ferulic accounted for 45 % of the phenolic acids while *trans*-sinapic, *trans*-*p*-coumaric, *trans*-caffeic, and *p*-hydroxybenzoic acids made up 39, 7, 5, and 3.5 %, respectively, of the phenolic acids. The insoluble form of phenolic acids included *trans*-ferulic (60 %), *trans*-caffeic (24 %), and *trans*-*p*-coumaric (16 %). Wanasundara and Shahidi reported similar phenolic acid levels and found condensed tannins level near 136 mg/100 g of flax.[69] Thus, the combination of lignans, phenolic acids and tannins contribute to the AOA of flaxseed. The incorporation of flaxseed into bakery products can provide a health benefit as well as giving AOA. Malcolmson et al.[70] noted that stored ground flaxseed was stable for 128 days as indicated by the low level of oxidation. Chen et al.[71] reported a loss in α-linolenic acid after heating 1.5 h at 178 °C. These authors also noted that oxygen consumption increased as the ground flaxseed particle size decreased but noted that α-linolenic degradation was not significant and again attributed the stability to the lignans.

Table 9.1 Tocopherol and tocotrienol content of cereals and oilseeds

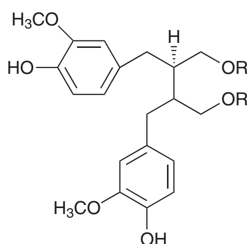
	Source of antioxidants				
	Canola[1]	Corn[2]	Cottonseed[3]	Soybean[2]	Sunflower[2]
Average total Tocopherol (ppm)	686	840	790	1099	683
Tocopherol range (ppm)	580–850	550–1148	776–802	900–1200	596–835
Contribute (%)[4]					
Tocopherols					
α	32	28	50	12	94
β	–	–	–	1	3
γ	66	57	48	60	2
δ	2	3	1	27	1
Tocotrienols					
α	–	5	–	–	–
β	–	1	1	–	–
γ	–	6	–	–	–

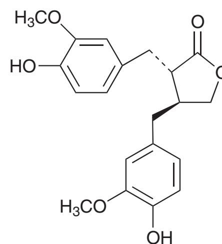
[1] Tocopherol and tocotrienol data summarised from Tan,[43] Clough,[44] and Warner and Mounts[75]
[2] Tocopherol and tocotrienol data summarised from Tan,[43] Clough,[44] Van Niekerk and Burger,[73] Speek et al.[74] and Warner and Mounts[75]
[3] Tocopherol and tocotrienol data summarised from Tan[43] and van Niekerk and Burger[73]
[4] Percent distribution of specific tocopherols and tocotrienols based on the average tocopherol content

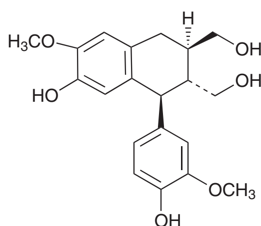
9.3.3.2 Sunflower

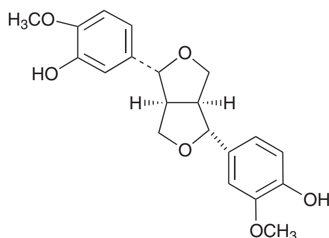
The antioxidants of confectionery and oil sunflowers include phenolic acids, tocopherols and sterols while purple hulled varieties contain significant concentrations of anthocyanins.[72] The average tocopherol content, including tocotrienols, in sunflowers is 683 ppm (Table 9.1) with 94 % as α-tocopherol, and β- and γ-tocopherols each accounting for 3 % of the total.[43,44,73–75] Sunflower sterol content ranged from 1900 to 3200 ppm with β-sitosterol accounting for 62 to 78 % of the total sterols (Table 9.2).[72,74] Other sterols include campesterol, stigmasterol, Δ^7-stigmasterol, and Δ^5- and Δ^7-avenasterol. The high concentrations of sterols would play a significant role in frying applications as a means of preventing oil polymerisation and thermal degradation. In addition, tocopherols serve as hydrogen-donating and radical-trapping antioxidants, which benefit fat-based food systems.

Phenolic acids represent the final group of antioxidants from sunflowers. The content of phenolic acids, as esters, is 981 mg/100 g with 98 % as *trans*-caffeic acid.[42] Chlorogenic acid contributes to the caffeic acid content but is responsible for the brown discoloration in foods which may be unwanted. Other phenolic acids include *trans*-*p*-coumaric, *trans*-ferulic, and









9.10 Flaxseed lignans as potential antioxidants.

Table 9.2 Sterol content of cereals and oilseeds

	Source of antioxidants				
	Canola[1]	Corn[2]	Cottonseed[3]	Soybean[2]	Sunflower[2]
Average total sterol (ppm)	673	8668	3745	2359	2587
Specific sterols (%)[4]					
Brassicasterol	10	–	–	–	–
Campesterol	33	19	7	21	8
β-Sitosterol	57	67	89	21	7
Stigmasterol	–	4	1	49	61
Δ^5-Avenasterol	–	5	2	2	5
Δ^7-Stigmasterol	–	1	–	3	9
Δ^7-Avenasterol	–	1	1	2	3
Methylene-cycloartenol	–	3	–	2	6

[1] Sterol data determined by Warner and Mounts[75]
[2] Sterol data summarised from Van Niekerk and Burger[73] and Warner and Mounts[75]
[3] Sterol data summarised from Van Niekerk and Burger[73]
[4] Percent distribution of sterols based on the average sterol content

p-hydroxybenzoic acids. Flours containing reduced chlorogenic acids may provide AOA in baking applications.

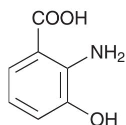
Peonidin-3-divanillyl-sambubioside and cyanidin-3-vanillyl-sambubioside are two major anthocyanins identified in purple hulled cultivars, with cyanidin-3-vanillyl-glucosylarabinoside and acylated diglucoside of malvidin as minor components.[76] Bullard et al.[77] and Holm[78] reported the presence of cyanidin-3-glucoside, acetylated forms of cyanidin-3-glucoside, cyanidin-3-xyloside, and acetylated forms of cyanidin-3-xyloside from the Neagra de Cluj cultivar of sunflower. Mok and Hettiarachchy found that the anthocyanins were most stable at a pH of 3.0 during the heating process.[79] The conversion to a brown pigmentation during this heating may limit the use of the anthocyanins, as antioxidants, to baking applications since the brown coloration would not be detrimental to the final product.

9.3.3.3 Soybean

Soybean health claims on food labels have recently been approved by the US Food and Drug Administration. Soy concentrates and aqueous extracts of soybeans contain isoflavones and phenolic acids as the main antioxidants. Organic solvent extracts contain tocopherols, sterols, phospholipids and other flavonoids while protein hydrolysates contain antioxidant amino acids and peptides.

Isoflavones are the predominant flavonoid in soybean and have been promoted for their oestrogenic activity. The glucoside form of isoflavones represent 99 % of the total isoflavones in soybeans.[80] Owing to the polar nature of these compounds, soybean oil has no isoflavones while small amounts (4 %) remain in soy flour. Recently, a survey of 210 varieties of soybeans grown in South Dakota showed that the total isoflavone content ranged from 1161 to 2743 $\mu g\, g^{-1}$.[81] Malonyl genistin and malonyl daidzin were the predominant isoflavones and accounted for 44 and 23 % of the isoflavones, respectively. Genistin (11 %), daidzin (7 %) and acetyl daidzin (5 %) made up the majority of the remaining isoflavones while glycitin, malonyl glycitin, daidzein, acetyl genistin and genistein accounted for 10 % of the isoflavones. The observations of Wang et al.[81] support those of Kudou et al.[82] who identified 6″-*o*-malonyl daidzin, 6″-*o*-malonyl glycitin, and 6″-*o*-malonyl genistin as isoflavones in soybean. The malonyl isoflavones were reported to give an undesirable taste described as bitter or astringent. However, these isoflavones are thermally unstable and convert to their respective isoflavone glucosides, which have a lower sensory impact.[82]

Isoflavone glucosides can be converted to the aglycone form during the fermentation process. Murakami et al.[83] noted that well-fermented tempeh lacked isoflavone glucosides but retained high concentrations of isoflavones as the aglycone form. Daidzein, glycitein, and



9.11 3-Hydroxyanthranilic acid isolated from tempeh and characterised by Esaki et al.[85]

genistein were the predominant isoflavones present in tempeh while daidzein and genistein were hypothesised as the active antioxidants.[83] Murata considered 6,7,4′-trihydroxyisoflavone and an unidentified compound as the active components in tempeh.[84] However, the AOA was system dependent in that 6,7,4′-trihydroxyisoflavone was active in aqueous suspensions of linoleic acid but not in bulk oils. Recently, 3-hydroxyanthranilic acid (Fig. 9.11) has been identified in tempeh and has a strong AOA in soybean oil and soybean powders.[85] By comparison, genistein lacked AOA. 3-Hydroxyanthranilic acid reached a maximum level (50 mg/100 g dry matter) at day 2 of the fermentation process, which corresponds well to the maximum AOA.

Protein hydrolysates have been found to contain predominantly isoflavone aglycones and phenolic acid as well as antioxidant peptides.[86,87] The hydrolysis of β-conglycinin resulted in the formation of six antioxidant peptides. The peptides had an amino acid sequence that was 5 to 16 amino acids in length and had valine or leucine at the N-terminal. The sequence of amino acids was critical as the mixing of individual amino acids at the concentration found in the peptide, in the emulsion system did not have AOA.[87] Three peptides were found to have good AOA while the other peptides had marginal activity. Peptide one (Leu-Leu-Pro-His-His) had an AOA equal to BHA (butylated hydroxyanisole) in an emulsion system while peptide two (Val-Asn-Pro-His-Asp-His-Gln-Asn) and three (Leu-Val-Asn-Pro-His-Asp-His-Gln-Asn) had slightly lower AOA. The authors proposed that the radical trapping activity of the imidazole ring of histidine might be responsible for the AOA. In addition, the hydrophobicity of the N-terminal amino acids may provide a better contact point between the peptide and the fatty acid, thus promoting AOA. The presence of tyrosine in the less active peptides may promote hydrogen-donating activity.[87] An alternative hypothesis postulates that the peptides could orient to the water–linoleic acid interface providing a barrier to oxygen, thus retarding the oxidation process. It is well known that enzyme hydrolysis can improve the emulsification activity of soy protein, thus lending credence to the alternative hypothesis. Further investigation would clarify this issue. The addition of peptides to emulsion-based food systems for AOA has potential, considering the need to stabilise these products against oxidation.

Dabrowski and Sosulski reported a phenolic acid content of 69 mg/100 g soybean.[42] They noted that 93 % of the phenolic acids were in the ester form

of which syringic made up 39 % of the phenolic acids. *trans*-Ferulic, *p*-hydroxybenzoic, and *p*-coumaric were equally present at 21, 19 and 14 %, respectively with *trans*-caffeic representing 7 % of the phenolic acids. Tocopherols are commercially removed from soybean oil for the pharmaceutical industry and are abundant in soybeans. A range of tocopherols between 900 and 1200 ppm has been reported (Table 9.1).[43,44,73–75] Gamma(γ) tocopherol accounts for 60 % of the total tocopherol while δ-, α-, and β-tocopherols make up 27 %, 12 % and 1 %, respectively. β-Sitosterol, stigmasterol, and campesterol account for 91 % of the sterol present in soybeans.[73] Δ^5- and Δ^7-avenasterol account for 4 % (109 $mg\,kg^{-1}$ soybean oil) of the sterols. Gordon and Magos reported that 0.1 % Δ^5-avenasterol could effectively control thermal oxidation, thus a minimum concentration of 0.1 % Δ^5-avenasterol would be required if sterols are used to promote oxidative stability of food products and frying oils.[64]

The trend to incorporate more soy into foods for nutritional benefit may be accomplished by the addition of soy flours, and the soy flour can also contribute to oxidative stability which is an added benefit. Soy flour addition to bakery products has been shown to inhibit the oxidative process.[88,89] Hot water extracts of soy protein concentrates and soy flours proved to be effective antioxidants, with soy flour extracts being more active.[88] Rhee et al.[90] reported that soy flour inhibited oxidation of bulk safflower oil and a metal-catalysed oxidation significantly better than soy concentrates and isolates. Utilising the less refined soy flour rather than the highly refined isolates or concentrates is more beneficial from an AOA perspective. This in turn provides an economical alternative to the use of highly purified NAO extracts.

9.3.3.4 Cottonseed

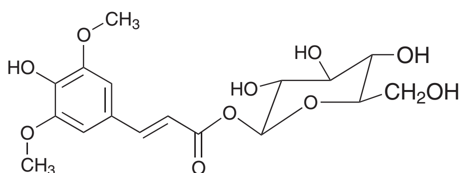
Cottonseed is not traditionally considered a source of NAO due to the presence of gossypol, an anti-nutrient and toxicant with very good AOA.[91,92] To utilise successfully cottonseed as a source of NAO, glandless varieties should be selected due to the lack of gossypol in these. Methanol extracts of glandless cottonseed flours had higher AOA in safflower oils than methanol extracts of cottonseed concentrates and isolates.[90] Similar findings were observed in the metmyoglobin-catalysed system. However, in the iron–EDTA oxidative system, the AOA of cottonseed flours and concentrates were not significantly different but were better than the isolates. Interestingly, the phenolic content was greatest for the isolates and lowest for the flours. This demonstrates the importance of phenolic type versus total phenolic content.[66] Ziprin et al.[93] found that ground beef extended with cottonseed protein ingredients was protected against rancidity. The presence of phenolic acids and flavonoids may be responsible for the AOA of cottonseed.

Dabrowski and Sosulski reported that the soluble phenolic acids were predominantly in the ester form and that there was no significant difference between gland and glandless cottonseeds.[42] The phenolic acid distribution was also similar between cottonseed varieties in which *trans*-ferulic (43 %), *trans-p*-coumaric (40 %), *trans*-caffeic (5 %), and *p*-hydroxybenzoic acids (11 %) were the predominant phenolic acids. In the glandless variety, *trans-p*-coumaric was slightly lower (33 %) and *trans*-caffeic (15 %) slightly higher compared to the gland cottonseed. Whittern et al.[94] reported that quercetin and rutin were the major flavonoids in cottonseed and responsible for the AOA.

An average tocopherol content for cottonseed is around 800 ppm (Table 9.1).[43,73] Gamma(γ) and α-tocopherol account for 48 % and 50 % of the total tocopherols, respectively. In addition, δ-tocopherol and β-tocotrienol each represent about 1 % of the tocopherol species. The sterols β-sitosterol and campesterol account for 96 % of the sterol present in cottonseed while Δ^5-avenasterol accounts for 2 % (85 mg kg^{-1} oil). All the other sterols make up the remainder.[73] The hydrogen-donating activity of tocopherols and the fact that cottonseed products could be used in meat systems warrants further investigation of glandless cottonseed in other food products.

9.3.3.5 Canola/rapeseed

Double zero rapeseed varieties (i.e. canola) are used for the production of food grade oils due to the low glucosinolates and erucic acid content.[95] Rapeseed has 30 times more phenolics than soybean of which 80 % are in the ester form and 16 % in the free form. Rapeseed phenolic content ranges from 1500 to 1800 mg/100 g in the meal and 640 to 1100 mg/100 g in the flour.[95,96] Krygier et al.[96] and Dabrowski and Sosulski[42] reported that sinapic acid was the predominant phenolic acid in rapeseed. Yellow Sarson, Candle, and Tower varieties contained 61, 87, and 91 % sinapic acid, respectively.[96] Sinapine, a choline ester of sinapic acid, occurs at levels of 1 to 2.5 % in meals and 1–2 % in flours, and is characterised by a fishy odour.[96] The Yellow Sarson variety contained 16 % syringic and 13 % ferulic while the minor phenolic acids in the Candle and Tower varieties, respectively, included 8 and 3 % ferulic acid and 1 and 3 % *p*-coumaric acid. The high concentration of sinapic acid was further supported by Naczk et al.[97] who reported that free, esterified, or insoluble bound forms of sinapic acid accounted for 65–86 %, 71–97 %, and 7–32 % of the phenolic acids in each category, respectively. Condensed tannins in rapeseed hull are polymers of leucocyanidin.[98] Cyanidin, pelargonidin, and malvidin were reported as breakdown products of rapeseed hull tannins.[99] Naczk et al.[100] found that the condensed tannins ranged from 14 to 1556 mg catechin equivalence/100 g hulls. Environment and cultivar were responsible for the wide range of condensed tannins. In theory, rapeseed hulls should have good AOA due to the high number of hydroxyl groups available to



9.12 1-*O*-β-D-Glucopyranosyl sinapate isolated from canola meal and characterised as an antioxidant by Wanasundara et al.[103]

participate in radical reactions. However, product discoloration should be considered an important factor when using rapeseed hulls as a source of natural antioxidant. In addition, the range of tannin concentrations would need to be standardised.

Nowak et al.[101] reported that sinapic acid had good AOA. Ethanolic extracts of canola, at 500 and 1000 ppm, were more effective than synthetic antioxidants with the exception of TBHQ (tertiary butylated hydroguinone).[102] Further investigation led to the identification of 1-*O*-β-D-glucopyranosyl 3,5-dimethoxy-4-hydroxycinnamate (1-*O*-β-D-glucopyranosyl sinapate; Fig. 9.12).[103] The data suggest that sinapic acid and analogues contribute significantly to AOA. Shahidi et al.[66] fractionated canola and found that the total phenolic content was not the critical factor in determining AOA. Fraction IV had a lower phenolic content and outperformed fractions with higher phenolic content. In addition, the chemical nature of the phenolic compounds in fraction IV was more complex with nine compounds representing eight types of phenolic material present in the fraction. Mono-, di-, and tri-hydroxy groups were identified by TLC (thin layer chromatography) with sinapic acid, *p*-hydroxybenzoic acid, flavonoids and 1-*O*-β-D-glucopyranosyl sinapate being present in the fraction.[66]

Tocopherols are also antioxidants in canola. Tocopherol contents ranged from 580 to 850 ppm with γ representing 66 % of the tocopherols and α- and δ-tocopherols accounting for 32 and 2 %, respectively.[43,44,75] The sterol content is high at 6700 ppm but the predominant sterols, β-sitosterol, campesterol and brassicasterol have minimal AOA. A diversity of phenolic compounds is present in canola or rapeseed flours, meals or extracts indicating that these products can protect a food against rancidity by several mechanisms. The high AOA of the canola fraction containing several groups of phenolics demonstrates the protection via multiple mechanisms.

9.4 Antioxidants from cereals

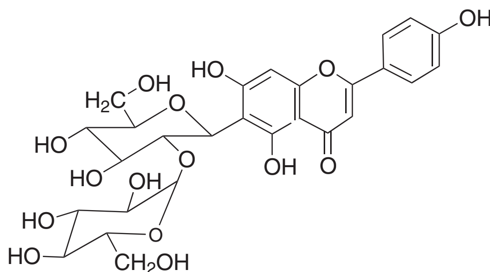
Cereals are not considered a significant source of NAO but the recent trend to consume more grains may give the product developer a secondary source

of antioxidants. The following cereals are commonly consumed and for some, the identification of new health benefits has enhanced consumption. For example, barley and oat β-glucans in the fight against heart disease has triggered a revival in the consumption of these grains.

9.4.1 Barley

The separation of the barley kernel and subsequent processing can produce a fraction with significant concentrations of antioxidants. Hand dissection of barley kernels showed that the germ was a significant source of tocopherol and tocotrienols (i.e. Tocols) at 206 $mg\,kg^{-1}$ of germ. The whole kernel (40 $mg\,kg^{-1}$), hull (29 $mg\,kg^{-1}$), and endosperm (33 $mg\,kg^{-1}$) had dramatically lower concentrations of Tocols.[104] However, α-tocotrienol accounted for 96, 83, and 63% of the Tocols in the endosperm, whole and hull, respectively. In contrast, α-tocopherol represented 90% of the total Tocols. The malting and brewing processes had a positive impact on the total Tocols. Malting caused a slight but non-significant reduction in total Tocols from 56.7 $mg\,kg^{-1}$ in the whole grain to 52 $mg\,kg^{-1}$ after malting whereas the spent grain remaining after brewing had a significantly higher Tocols content at 152.9 $mg\,kg^{-1}$.[104] The tocopherol content increased from 12.8 $mg\,kg^{-1}$ in whole grain to 25.4 $mg\,kg^{-1}$ in the spent grain and tocotrienols increased from 43.9 to 127.5 $mg\,kg^{-1}$. The spent grain is also rich in B vitamins which means that the application of spent grain has potential to enhance NAO concentrations and nutrition quality. Δ^5-Avenasterol accounts for 23 and 21% of the sterols in free and bound lipids. The concentration of Δ^5-avenasterol is 5.61 $mg\,g^{-1}$ of free lipids and 2.71 $mg\,g^{-1}$ of bound lipids which is significantly higher than the concentrations found in oilseeds.[105] The high level of Δ^5-avenasterol suggests possible applications in fried oil systems.

The isoflavonoid 2″-*o*-glycosyl isovitexin (Fig. 9.13) found in the leaves of barley has comparable AOA to α-tocopherol.[106] Other flavonoids include anthocyanins, proanthocyanins and flavonols. The anthocyanins include



9.13 2″-*O*-β-D-Glucosylisovitexin isolated from barley and characterised as an antioxidant by Nishiyama et al.[106]

pelargonidin, pelargonidin glycosides, cyanidin, cyanidin 3-arabinoside, delphinidin, and delphinidin glycoside, which are located in the pericarp and aleurone layers of the barley kernel whereas proanthocyanins are primarily in the aleurone cells and consist of procyanidin B-3 dimer, (+)-catechin, and leucodelphinidin.[56,107] Ten phenolic acids and four phenol glucosides have been isolated from the whole grain of barley.[108,109] The phenolic acids include sinapic, ferulic, *p*-, *m*-, and *o*-coumaric, syringic, vanillic, protocatechuic, salicylic and *p*-hydroxybenzoic acids. Phenolic glucosides include *p*-hydroxybenzoic acid 4-*O*-β-glucoside, vanillic acid 4-*O*-β-glucoside, ferulic acid 4-*O*-β-glucoside, and *o*-coumaric acid 2-*O*-β-glucoside and can be found throughout the whole grain.[108]

Antioxidant screening of plant extracts in a methyl oleate system showed that pearl barley and barley grain extracts had low AOA at a 500 ppm level.[110] An increase to 5000 ppm resulted in a reduction in activity suggesting that additional research is needed fully to characterise the AOA of barley. Kajimoto et al.[111] found that an extract of roasted barley had significantly better AOA than non-roasted barley. In addition, the AOA increased as roasting time increased suggesting that Maillard browning reaction products were being created and were probably responsible for the AOA.

9.4.2 Buckwheat

Commercially, buckwheat is used as a component in pancake mixes, noodle and pasta formulations, porridge and soups. The fact that buckwheat is already used in many products and that antioxidant activity has been found shows that a potential new source of NAO can be derived from buckwheat.[112–115] Buckwheat contains 387 and 1314 mg/100 g flavonoids and 47 and 77 mg/100 g rutin in the seeds and hulls, respectively.[112] However, Dietrych-Szostak and Oleszek[116] reported 18.8 and 74 mg/100 g total flavonoids for the seed and hull, respectively while Watanabe et al.[113] reported *c.* 36 mg/100 g for the hull based on the summation of concentration of purified flavonoids.

Oomah and Mazza[112] reported that flavonoids were weakly correlated with AOA whereas rutin had no correlation with AOA. On the contrary, Watanabe et al.[113] found that purified flavonoid fractions had radical scavenging activity, although crude extracts had little AOA. The active components of the hull included protocatechuic acid (13.4 mg/100 g dried hull), 3,4-dihydroxybenzaldehyde (6.1 mg/100 g), hyperin (5 mg/100 g), rutin (4.3 mg/100 g) and quercetin (2.5 mg/100 g). Proanthocyanins also contributed to the radical-scavenging activity while vitexin (4.6 mg/100 g) and isovitexin (3.3 mg/100 g) were not active. The fractionation of buckwheat groat extracts resulted in four catechin compounds, namely (−)-epicatechin, (−)-epicatechin 3-*O*-*p*-hydroxybenzoate, (−)-epicatechin 3-*O*-(3,4-di-

O-methyl)-gallate, and (+)-catechin 7-*O*-β-D-glucopyranoside.[115] All catechins had better radical scavenging activity than rutin.

Przybylski et al.[114] showed that methanol extracts of the groats were more effective than the methanol extracts of the hulls. The methanol was the best solvent for extracting phenolic materials. The methanol extracts were found to provide the best protection against canola oil oxidation followed by acetone, diethyl ether=ethyl acetate and hexane. However, ethyl acetate and acetone extracts were better DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavengers followed by methanol, hexane and diethyl ether extracts. Methanol extracts were useful in extracting phenolic acids and flavonoids but did not remove tocopherol while the opposite was true for hexane extractions.[114] Phenolic acids identified in buckwheat include caffeic, *o*-coumaric, *p*-coumaric, ferulic, gallic, *p*-hydroxybenzoic, syringic and vanillic.[114,117] Vanillic accounted for 57 % of the phenolic acids while caffeic was next at 17 %. Tocopherol content obtained from the hexane extraction was 539 ppm with α-tocopherol constituting 70 % of the total followed by γ (20 %), δ (7 %) and β (3 %).

Kreft et al.[118] analysed the rutin content of ten bran and ten flour samples from the buckwheat milling operation. Bran fractions contained 131–476 ppm rutin while flour samples contained between 19 and 168 ppm rutin. The rutin composition follows the same pattern observed by Durkee[117] for phenolic acid in which the bran/aleurone layers had higher phenolic acids levels than the flour. Dietrych-Szostak and Oleszek assessed the effects of processing on the flavonoids in buckwheat.[116] They found that dehulling at different temperatures caused a 75 % reduction in flavonoids in the grain while a 20 % reduction was found in the hulls. Avoiding heat processes can produce buckwheat products that have good AOA. The incorporation of buckwheat extracts to lipid-containing systems has promise that is not currently exploited.

9.4.3 Corn

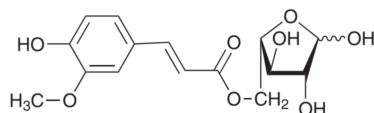
The antioxidants of corn are unique in the fact that carotenoids make up part of the antioxidants, which is not the case in most cereals. Chen and Yang reported carotene and xanthophyll levels of 2.7 and 19.9 ppm respectively in yellow corn.[119] Kurilich and Juvik assessed the carotenoid and tocopherol concentrations of 44 varieties of sweet and dent corn lines.[120] The carotenes identified were α- and β-carotene and the xanthophylls included β-cryptoxanthin, lutein, and zeaxanthin. The mean carotenoid level for all 44 varieties was 10.4 ppm and ranged from 0.15 to 33 ppm. Lutein accounted for 57 % of the carotenoids while zeaxanthin and β-cryptoxanthin made up 21 and 5 % of the carotenoids, respectively. Carotenes accounted for 8 % of the total carotenoids. The total tocopherol content of 30 ppm was found for all varieties with a range of 7–86 ppm. The α-, γ-, and δ-

tocopherols were identified in all 44 varieties and averaged 8, 20, and 1 ppm, respectively for them all. The tocopherols of the varieties tested were low while corn oils typically have around 880 ppm tocopherols (Table 9.1).[43,44,73–75,121] γ-Tocopherol accounted for 57 % of the tocols followed by α-tocopherol (28 %), γ-tocotrienol (6 %), α-tocotrienol (5 %), δ-tocopherol (3 %), and β-tocotrienol (1 %). Grams et al.[121] reported that the tocols are mainly distributed in the germ (up to 90 %) with the pericarp and endosperm accounting for *c.* 5 % each. Nearly all (96 %) of the α- and γ-tocopherols were found in the germ while the tocotrienols were predominantly found in the endosperm. Also, corn germ oil contains 682 ppm or 5 % Δ^5-avenasterol.[73]

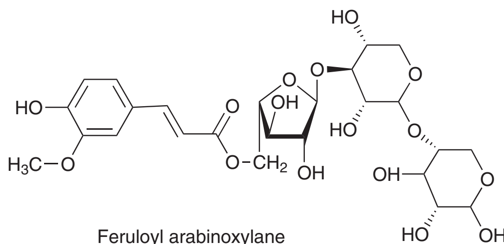
Wang et al.[122] found that vitamin C addition to the steeping water of the wet milling process of corn significantly protected the tocols. This was evidenced by the increased concentration of tocols above that of traditional steeping methods containing an SO_2 additive. The most dramatic protection was found for γ-tocopherol and α-tocotrienol.

Classen et al.[123] used a base hydrolysis method to optimise the extraction of phenol acids from corn. They found that (*E*)-ferulic and (*Z*)-ferulic acids accounted for 57 and 33 % of the total (1143 ppm) phenolic acids, respectively. Sinapic acid was the only other significant phenolic acid and accounted for *c.* 10 % of the phenolic acids. Ohta et al.[124] reported that corn bran had good AOA and identified that 5-*O*-feruloyl-L-arabinofuranose (FAA), *O*-(5-*O*-feruloyl-α-L-arabinofurnosyl)-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX), and diferulic acid (Fig. 9.14) were the main contributors to the AOA. In addition, ester forms of ferulic acid were more active than the free form in liposomes.[124,125]

Di Mascio et al.[126] reported that α-carotene had the highest singlet oxygen quenching activity followed by β-carotene, zeaxanthin, lutein, and



Feruloyl arabinose



Feruloyl arabinoxylane

9.14 Feruloyl glycosides isolated from corn and characterised as antioxidants by Ohta et al.[124]

β-cryptoxanthin. The hydrogen-donating capability of tocopherols complements the activity of the carotenoids, thus extracts or concentrates of corn antioxidants would serve as a good source of NAO.

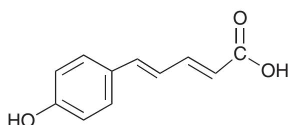
9.4.4 Millet

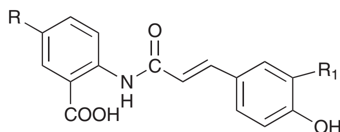
Millet is predominantly a cattle feed in the United States but in Japan and India millet is used as human food. Watanabe identified three phenolic antioxidants from Japanese barnyard millet (*Echinochloa utilis*).[127] The three antioxidants are tricin, luteolin, and *N*-(*p*-coumaroyl)serotonin (NCS). All antioxidants had radical-scavenging activity with NCS being the most active, equivalent to BHA, followed by luteolin and tricin. In addition, luteolin was more active than quercetin. Sripriya et al.[128] reported free-radical quenching activity of finger millet (*Eleusine coracana*). They noted that non-processed brown finger millet had the highest radical quenching activity and postulated that tannins and phytic acid were responsible for the activity. The phenolic content increased during the fermentation and the combined germination and fermentation processes. However, the radical scavenging activity decreased so that total phenolics did not dictate AOA. The loss of phytate and hydrolysis of tannins may account for the loss of radical-scavenging activity.[128] More research is needed to characterise fully the AOA of millet.

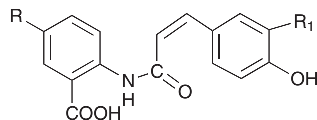
9.4.5 Oats

Daniels and Martin identified eight monoesters of caffeic and ferulic acids.[129] They reported the presence of *n*-hexacosyl caffeate, octacosyl caffeate, 26-*O*-caffeoyl-26-hydroxyhexacosanoic acid, 28-*O*-caffeoyl-28-hydroxyoctacosanoic acid, *n*-hexacosyl ferulate, 26-*O*-feruloyl-26-hydroxyhexacosanoic acid and hexacosane-1,2-diol monoferulate. The caffeic acid ester had higher AOA than ferulic acid esters due to the number of available hydroxyl groups. A follow-up study revealed the presence of glyceryl esters of caffeic and ferulic acids.[130] Additional studies are needed to confirm the proposed structure. The esterification of two caffeic acids or a caffeic and ferulic acid to the glycerol backbone is interesting because an amphiphilic compound would be created. Thus, the possibility for stabilising emulsions against oxidation may be significantly better than that provided by the addition of hydrophilic or hydrophobic antioxidants alone due to the ability of an amphiphilic compound to orient to the oil–water interface.

Avenanthramides (Fig. 9.15) are a unique group of antioxidants found almost exclusively in oats and are predominantly cinnamic acid conjugates in which 25 and 20 are exclusive to the groat and hull, respectively.[131] An additional 15 can be found in both groats and hulls. Collins et al.[132] isolated







	R	R_1
N-(4'-hydroxycinnamoyl)-5-hydroxyanthranilic acid	OH	H
N-(4'-hydroxy-3'-methoxycinnamoyl)-5-hydroxyanthranilic acid	OH	OCH_3
N-(3',4'-dihydroxycinnamoyl)-5-hydroxyanthranilic acid	OH	OH
N-(4'-hydroxycinnamoyl)anthranilic acid	H	H
N-(4'-hydroxy-3'-methoxycinnamoyl)anthranilic acid	H	OCH_3

9.15 Avenanthramides isolated from oats and characterised by Collins.[131]

avenalumic (Fig. 9.15) and the 3′-hydroxy and 3′-methoxy analogues, which would be expected to have similar AOA to caffeic and ferulic acid, respectively. Emmons et al.[33] reported that the avenanthramides, caffeic acid, ferulic derivative and vanillic acid correlated well with AOA. Avenanthramide A, avenanthramide C, and avenanthramide K were the predominant phenolic compounds in oat fractions.[33] The pearling fractions had the highest as well as the best total phenolic and tocols content. Dimberg et al.[133] reported that the avenanthramide, *N*-(4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid, had very good AOA and was located predominantly in the bran, which was in agreement with Collins.[131] Steam treatment of the oat did not promote degradation of the avenanthramide and so these compounds may contribute to the AOA of oat extracts in frying applications. In general, the avenanthramide content decreases away from the aleurone layer.[33,131,133]

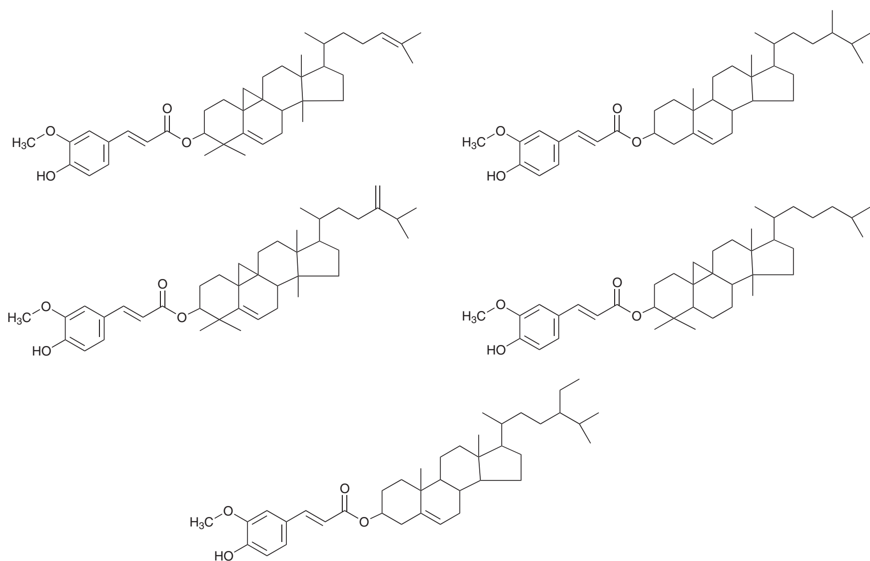
Handelman et al.[134] reported that the methanol extract of the aleurone layer had the greatest AOA while heat treatment of the pearling fractions resulted in a reduction of AOA. Dimberg et al.[135] reported that less than 20 % of the phenolics degrade during heat processing, thus the loss of these 20 % may account for the diminished AOA reported by Handelman et al.[134] Methanol extracts of the oat hull and groat were better antioxidants than petroleum ether extracts at low storage temperatures. However, petroleum

ether extracts were found to be significantly better antioxidants at high temperatures.[136] The presence of Δ^7-avenasterol and other sterols with an ethylidene group may be the reason for the protective effect of the petroleum ether extract.[137] Other components may include caffeic and ferulic acid esters and the avenanthramide.[129–131]

In order to use oats as an NAO source, the antioxidants must be concentrated. The phenolic content of a 0.01 % extract is equivalent to 3.3 parts oat to 1 part oil.[138] This ratio may be acceptable if bakery products are to be made using oats. In addition, processing of oats can influence the antioxidant content. Peterson reported that dried groats had the highest tocols content (40 ppm) while rolled oats and flour have the lowest content at 28 ppm.[139] However, storage at room temperature for seven months resulted in a dramatic reduction in tocols in all samples except the undried groat. Oats have a variety of unique antioxidants that appear to be stable to harsh processing conditions (e.g. steaming, frying), and therefore oat antioxidants should be investigated further as a means to develop oats as a source of NAO.

9.4.6 Rice

Rice bran oil is a rich source of antioxidant components. Rice bran oil contains 608 ppm tocols (343 ppm tocopherol and 265 ppm tocotrienols), or 157 ppm based on the dried rice bran, and 2847 ppm oryzanols (Fig. 9.16)



9.16 Oryzanols isolated from rice bran oil.

in dried rice bran.[140] Isopropyl alcohol (IPA) extraction increased the tocols to 171 ppm and the oryzanols to 2930 ppm on a dry weight basis. Thus, in this case no significant differences were found between hexane and IPA extracted antioxidants. The data were in agreement with the results of Zhao et al.[141] Rogers et al.[142] evaluated commercially available rice bran oils and found a wide range in antioxidant concentrations. Oryzanol contents ranged from 115 to 787 ppm while tocopherols (16 to 452 ppm) and tocotrienols (72 to 1157 ppm) also showed a significant variation. The brand with a low oryzanol content also had a low tocols level. Cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campesteryl ferulate, β-sitosteryl ferulate and cycloartanyl ferulate were the oryzanols identified in all commercial oils and are predominant in the germ/bran fraction of the rice kernel. Ramarathnam et al.[143] found that the hull, bran/germ and whole grain extracts were better antioxidants than tocopherols.

Ramarathnam et al.[144] found that a 50 % aqueous methanol was optimal for extracting rice hull antioxidants. Subsequent fractionation led to the identification of isovitexin, which they hypothesised was the active component in rice hull extracts.[145] However, Watanabe et al.[113] reported that isovitexin had minimal radical scavenging activity, thus demonstrating the importance of the oxidative system when evaluating AOA.

The inactivation of lipase is a critical factor if rice bran is to be used as an edible oil. Ramarathnam et al.[146] reported that treatment of rice with 15 kGy γ-irradiation resulted in a complete loss of tocols and a reduction in oryzanols from 170 to 153 ppm. Treatment of rice with 5 kGy γ-irradiation promoted the reduction of tocols by 46 % and a higher reduction (76 %) was found when the hulls were removed. Hall (unpublished data) found that microwaving rice bran did not significantly cause a reduction in tocols or oryzanols.[147] Similar findings were observed by Rhee and Yoon,[148] Vetrimani et al.[149] and Tao et al.[150] However, the opposite was found in the reduction of vitamin E during the microwaving of oils.[151] Rice bran incorporation into food products could serve as a valuable source of NAO provided that the bran is stabilised immediately after milling to prevent the formation of free fatty acids caused by cell wall lipases.

9.4.7 Wheat

The antioxidant activity of wheat kernel is very low. Kähkönen et al.[110] found that wheat grain had minimal AOA while the bran fraction had low AOA. Ferulic acid and *p*-coumaric acid were predominant phenolic acids in hard red spring (HRS) wheat hulls while ferulic was the predominant phenolic acid in HRS wheat and flour.[34] These authors reported that 50 and 500 ppm of ferulic acid was found in the flour and ground wholewheat, respectively. Durum wheat varieties protected oil oxidation equally well and were found to contain similar phenolic acid profiles.[152] The wheat bran

extract was composed of protocatechuic acid (226 ppm), *p*-hydroxybenzoic acid (124 ppm), gentisic acid (108 ppm), caffeic acid (116 ppm), vanillic acid (637 ppm), chlorogenic acid (84 ppm), syringic acid (130 ppm), *p*-coumaric acid (580 ppm), and ferulic acid (764 ppm). The individual phenolic acids were tested along with the durum wheat bran extract and a simulated extract made by mixing the identified phenolic acids in the proportion found in the original extract. The durum wheat extract was a significantly better antioxidant than the individual phenolic acids except protocatechuic acid and chlorogenic acid. The simulated extract had slightly less AOA than the durum wheat extract. The data supports the concept that synergistic activity occurs between the phenolic materials and therefore provides a better antioxidant than purified components.

Dolde et al.[153] evaluated 18 wheat lines for tocopherol and found that the tocopherol content in wheat germ oil ranges from 1947 to 4082 ppm. The range of individual tocopherols was 601–1396 ppm α, 229–562 ppm β, and 1135–2232 ppm γ. The 4α-methyl-sterols were found to protect against frying oils oxidation[62] and the ethylidene group is believed to be responsible for the protective effect. It is not surprising that the sterols from wheat germ oil could protect against oxidation considering that Δ^5-avenasterol and Δ^7-avenasterol are present at 1600 and 900 ppm, respectively.

To use plant sources as NAO their components must not affect the sensory quality of foods. Phenolic acids have long been associated with bitter and astringent perceptions,[154] and as low as 5 ppm can contribute to these tastes. Sinapine, a choline ester of sinapic acid is an example of a product that can contribute a fishy odor.[96] Cater et al. reported that chlorogenic acid contributes a brown and grey discoloration in products while caffeic acid can contribute a pink colour, which changes to grey.[155]

9.5 Antioxidants from animal products

Peptides, amino acids, and carotenoids are three animal products that could potentially serve as NAOs. Enzymes such as glutathione peroxidase, superoxide dismutase and catalase are antioxidant enzymes present in muscle systems. However, the cost of isolating these enzymes is prohibitive, thus limiting their use as food antioxidants. Carnosine, anserine, and ophidine are histidine-containing dipeptides reported to chelate metals and scavenge radicals.[156] For a complete review of the histidine-containing dipeptides, see Cuppett.[157]

Uchida and Kawakishi identified the N-terminal amino acid sequence of Asp-Arg-Val-Tyr as being important for interaction with copper/ascorbate systems.[158] This implies that peptides with this N-terminal sequence could terminate metal-catalysed oxidation. However, in biological systems, this may not be preferred but for food applications additional research is

needed. L-Histidine in the free form or as part of a small peptide or protein can scavenge hydroxyl radicals and quench singlet oxygen.[159,160] In addition to singlet oxygen quenching of L-histidine, singlet oxygen can react with the double bond of L-histidine to form a peroxyl radical.[159] L-Histidine has singlet oxygen quenching three-fold higher than that of tryptophan and five-fold higher than methionine.[161]

Carotenoids are typically associated with the colour of fruits and vegetables but are also found in many animals. Crustacea demonstrate significantly the multitude of carotenoid pigments found in nature. Zagalsky et al.[162] observed that carotenoids of invertebrates were associated with the protein in a complex defined as carotenoprotein. The carotenoids of the exoskeleton of crustaceans may provide the best opportunity to develop NAO from animal sources. Astaxanthin (Fig. 9.17) is the most common xanthophyll, with lutein, zeaxanthin and tunaxanthin being important pigments.[163,164] Red crabs contain β-carotene and astaxanthin while blue crabs contain canthaxanthin, 4-hydroxyechinenone, 3-hydroxycanthaxanthin, echinenone, isocryptoxanthin, β-carotene and astaxanthin.[165,166] The previous examples demonstrate the complexity of the carotenoids in crustacea. Limited research has been completed on the AOA of crustacea carotenoids. However, their activity would be expected to be similar to plant carotenoids due to the structural similarities between plant and animal carotenoids.

Seymour et al.[167] isolated 1,2-diamino-1-(*o*-hydroxyphenyl)propene (Fig. 9.18) from shrimp shells. At a concentration of 0.18 mg/100 g shrimp shell and 13.5 million kg (30 million pounds) of shrimp waste go unused annually, 24.3 kg (54 lbs) of 1,2-diamino-1-(*o*-hydroxyphenyl)propene would be generated annually from a waste product. Although 24.3 kg is not a significant amount, the shrimp waste represents an available source of NAO. The production of NAO from animal products will require the development of extraction or concentration processes, thus adding cost factors to the production of animal NAO.

9.6 Antioxidants from microbial sources

Microorganisms are one of the most abundant and diverse species found on earth. The exploitation of microorganisms to produce food ingredients has been going on since antiquity. However, the isolation of microbial antioxidants did not become a focus of research until the early 1980s, although Forbes et al.[168] and Meisinger et al.[169] established a relationship between antioxidants and microorganisms. Since this early work, a vast number of compounds and microorganisms have been characterised. The intent of the following discussion is to highlight some of the studies that demonstrate the antioxidant activity of microorganisms.

The AOA of ethyl acetate extracts of several *Penicillium* and *Aspergillus* species, including *Rhizopus oryzae*, were evaluated using the thiocyanate

Astaxanthin

Canthaxanthin

4-Hydroxyechinenone

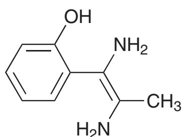
Echinenone

Isocrytoxanthin

β-Carotene

Tunaxanthin

9.17 Examples of carotenoids isolated from seafood.



9.18 1,2-Diamino-1-(*o*-hydroxyphenyl) propene isolated from shrimp shell waste by Seymour et al.[167]

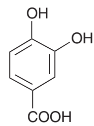
method.[170] Extracts of two *Penicillium* and four *Aspergillus* species protected linoleic acid better than the control. One species, *Aspergillus candidus* CCRC 31543, protected the oil as well as BHA. In a subsequent study, Yen and Chang found that sucrose or lactose and ammonium sulphate in the culture media enhanced the *A. candidus* CCRC 31543 production of antioxidants.[171] Ethyl acetate extraction of the broth and of the mycelium produced extracts with similar activity.

Aoyama et al.[172] screened 750 filamentous fungi isolated from soil. Although the organisms were not identified, two antioxidants were identified as citrinin and protocatechuic acid (Fig. 9.19). A third compound, curvulic acid, isolated from an unidentified *Penicillium* was also evaluated for antioxidant activity in linoleic acid. All three compounds, including the methyl ester of curvulic acid, had good AOA: the curvulic acid had the largest AOA followed by the curvulic acid methyl ester, protocatechuic acid, and citrinin. BHA was a stronger antioxidant than all the compounds tested while α-tocopherol had AOA equal to citrinin, but weaker than curvulic acid, curvulic acid methyl ester and protocatechuic acid.[172]

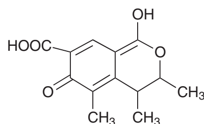
Aspergillus species are effective producers of AOA compounds. Esaki et al.[173] evaluated the AOA of 30 strains of *Aspergillus* and found that methanol extracts of fermented soybeans (MEFS) prevented oxidation of methyl linoleate. The MEFS of 28 strains had better AOA than the non-fermented soybean while all strains were better than the control. The MEFS obtained from *A. saitoi* had the best AOA, whereas the MEFS of a 4 day incubation gave higher AOA than MEFS of shorter incubations. Separation of the MEFS revealed 2,3-dihydroxybenzoic acid (Fig. 9.19) as a component of the most active fraction, with the highest concentration being found in the 4 day incubated samples. Hayashi et al.[174] also identified this compound in *Penicillium roquefortii* IFO 5956 cultures.

Esaki et al.[173] evaluated the AOA of methanol extracts (ME) of miso, natto, and tempeh and found that tempeh was the most effective followed by miso. The AOA of the natto ME was less than that of other fermented products but was equivalent to that of unfermented soybeans; this suggests that fermentation by mould cultures are more active than bacterial (*Bacillus natto*) ones in producing antioxidants. Recently, 3-hydroxyanthranilic acid (Fig. 9.11) has been identified in tempeh and has a strong AOA in soybean oil and soybean powders.[85] Furthermore, Hoppe et al.[175] identified 5-(δ-tocopheroxy)-δ-tocopherol (Fig. 9.19) as an antioxidant obtained from tempeh fermented by *Rhizopus oligosporus*.

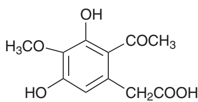
Gallic acid is a phenolic acid found in many natural sources, including microbial products. Gallic acid has been isolated from cultures of *Penicillium* and *Aspergillus*.[176–178] *Aspergillus terreus* S-4 produced the highest gallic acid of 98 strains of soil organisms tested.[177] Media containing sake



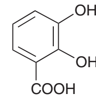
Protocatechuic acid



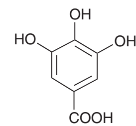
Citrinin



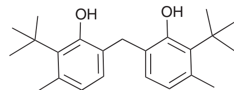
Curvulic acid



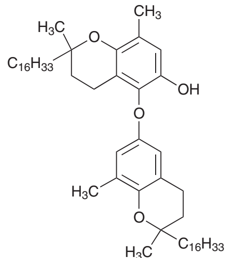
2,3-Dihydroxybenzoic acid



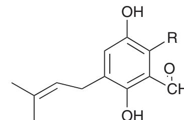
Gallic acid



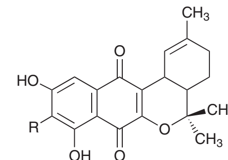
2,2'-Methylenebis(5-methyl-6-*tert*-butylphenol)



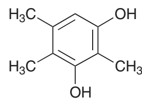
5-(δ-Tocopheroxy)-δ-tocopherol



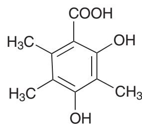
	R
Dihydroauroglaucin	
Auroglaucin	
Flavoglaucin	



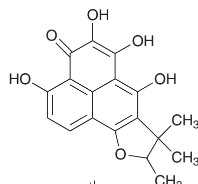
	R
Naphterpin	CH3
7-Demethylnaphterpin	H

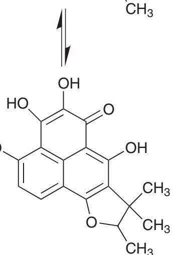


2,4-Dihydroxy-3,5,6-trimethylbenzene

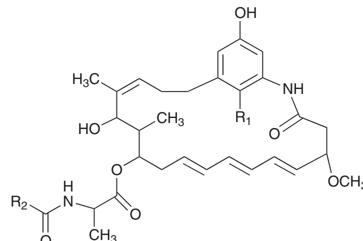


2,4-Dihydroxy-3,5,6-trimethylbenzoic acid

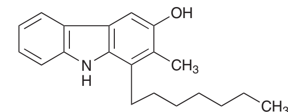




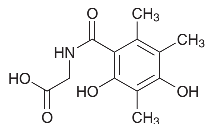
Enantiomers of atrovenetin



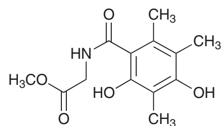
	R1	R2
Mycotrienin II	OH	
Trienomycin A	H	
Trienomycin B	H	CH2CH(CH3)2



Carazostatin



N-(4,6-Dihydroxy-2,3,5-trimethylbenzoyl)glycine



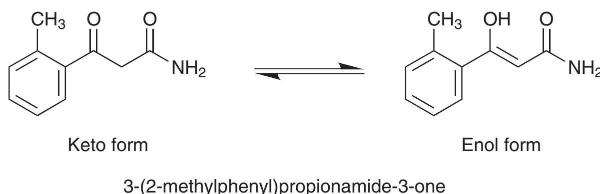
N-(4,6-Dihydroxy-2,3,5,-trimethylbenzoylamino)acetic acid methyl ester

9.19 Antioxidants isolated from microbial sources.

cake significantly improved the organisms' ability to produce gallic acid compared to basal medium and Czapek-Dox medium. Protocatechuic acid was also produced by *Aspergillus terreus* S-4. 2,2′-Methylenebis(5-methyl-6-*tert*-butyl-phenol) has been identified as an antioxidant from *Penicillium janthinellum*.[179]

Eurotium species have been found to produce several antioxidants.[180,181] Three of the seven metabolites were found to have AOA and were identified as dihydroauroglaucin, auroglaucin, and flavoglaucin (Fig. 9.19). *E. chevalieri* IFO 4086, and *E.repens* IFO 4041 produced all three antioxidants, but *E. echinulatum* IFO 5862 did not do so. However, *Aspergillus chevalieri* produced all three antioxidants while *Penicillium charlesii* produced flavoglaucin.

Streptomyces sp. USF-319 produces three radical scavenging antioxidants, of which one inhibits 5-lipoxygenase.[182] The antioxidants include mycotrienin II, trienomycin A, and trienomycin B (Fig. 9.19), which are ansamycin antibiotics. Mycotrienin II was the most active compound of the three, but was considered a moderate antioxidant compared to BHT (butylated hydroxytoluene). However, mycotrienin II was found to inhibit 5-lipoxygenase.[182] *Streptomyces* sp. USF-142 produced 3-(2-methylphenyl)propionamide-3-one, a compound identified as having AOA based on the reduction of 2,6-dichlorophenol indophenol to leuco indophenol (Fig. 9.20).[183] Although the reducing activity was similar to that of ascorbic acid, 3-(2-methylphenyl)propionamide-3-one was not found to have AOA in linolenic acid. The authors attribute the lack of AOA to the fact that the molecule would exist as the keto form, thus no OH would be available to participate in the AOA. In light of these findings, testing of the AOA in liposomes and metal-containing systems would be worth investigating to establish the true activity of this compound. Atroventin was isolated from *Penicillium paraherquei* and found to have good antioxidant activity.[184] Carazostatin and 7-demethylnaphterpin are free radical scavengers isolated from *Streptomyces chromofucus* and *S. prunicolor*, respectively.[185,186] Hirota et al.[187] isolated 2,4-dihydroxy-3,5,6-trimethylben-



9.20 3-(2-Methylphenyl)propionamide-3-one isolated from *Streptomyces* sp. USF-142 and reported to have reducing activity similar to that of ascorbic acid.[183]

zene (DHTMB), 2,4-dihydroxy-3,5,6-trimethylbenzoic acid (DHTMBA), *N*-(4,6-dihydroxy-2,3,5-trimethylbenzoyl)glycine (DHTMBG) and its methyl ester (DHTMBE) (Fig. 9.19) from an unidentified *Mortierella* species. The scavenging of DPPH radicals by DHTMB and DHTMBA was greater than that by BHT or α-tocopherol while DHTMBG and DHTMBE were less active. All compounds had similar AOA in linoleate emulsion system.

Carotenoids are the final group of antioxidants that can be synthesised by microorganisms. Nelis and Leenheer reported that β-carotene from *Blakeslea trispora* and *Duniella salina*, and lycopene from *B. trispora* and *Streptomyces chrestomyceticus*, subsp. *rubescens* were approved for human foods as colourants.[188] Astaxanthin from microbial sources, e.g. *Xanthophyllomyces dendrorhous*, has been approved for use in fish foods. Astaxanthin and lycopene were found to have excellent singlet oxygen quenching activity.[189] Miki reported that the AOA of astaxanthin was 10 times greater than that of lutein, β-carotene, zeazanthin, and canthaxanthan.[190] Recently, the activity of several carotenoids including lutein, β-carotene, and astaxanthin were confirmed using a new fluorometric assay.[191]

B. trispora and *X. dendrorhous* (formerly *Phaffia rhodozyma*) are two organisms with the most promise for microbial production of carotenoids. The inclusion of Span 20 in *B. trispora* culture broths enhanced the production of β-carotene from $0.15\,g\,l^{-1}$ to $2.16\,g\,l^{-1}$.[192,193] In addition, alkaline conditions (pH 10) significantly improved *B. trispora* culture ability to produce β-carotene.[192] Gavrilov et al.[194] found that the addition of tobacco dust could enhance lycopene production by preventing the cyclisation of the lycopene to β-carotene. Alternatively, pyridine derivatives were found to enhance lycopene synthesis with 2-amino-5-methylpyridine having a greater stimulating effect than 2-amino-6-methylpyridine.[195] *X. dendrorhous* produced more carotenoid when cultured on corn wet milling co-products compared to culturing on yeast–malt extracts.[196] In addition, hydrolysed wood was a good substrate for *X. dendrorhous*.[197,198] Both total carotenoid and astaxanthin levels increased by a factor of four. The use of microbial fermentation as a method for producing NAO has promise. More work is needed to optimise production conditions.

9.7 Antioxidants as preserving agents

Numerous reports of antimicrobial activity of phenolic antioxidants have been made. For antimicrobial activity of plant and animal antioxidants, see the review articles of Raccach,[199] Fung et al.[200] and Nakatani.[201] Gailani and

Fung found that phenolic antioxidants (e.g. BHA) had antimicrobial activity against a number of Gram positive and Gram negative bacteria.[202] In addition, the phenolic antioxidants inhibited the growth of psychrotrophs, coliforms and faecal coliforms in a ground pork system. Ogunrinola et al.[203] found that phenolic antioxidants were bactericidal against *Escherichia coli* O157:H7. These authors also noted that the combination of phenolic antioxidants acted synergistically at 4 °C to inhibit *E. coli* O157:H7 growth; a phenomenon similar to the synergism in the control of oxidation. In an attempt to promote natural antimicrobial agents, many authors have investigated the antimicrobial activity of natural antioxidants from many plant species. The following discussion highlights specific compounds identified as the active antioxidants from the plant and animal sources presented in this chapter.

A number of flavonoids have antimicrobial activity. Bohm summarised the antibacterial and antifungal properties of a variety of plant sources.[204] Although the plant sources were varied, isoflavanones, isoflavones, dihydroflavonol, chalcones, dihydrochalcones, and flavans were among the active components. Naim et al.[80] found that concentrations as low as 0.005 % of free isoflavones were active against plant pathogens whereas bound isoflavones were less so. Soybean isoflavones containing methoxy groups were found to have significantly greater antifungal activity than non-methylated isoflavones. Nowak et al.[101] assessed the antimicrobial activity of extracts prepared from rapeseed flour. They found that sinapic acid (SA) had high antimicrobial activity against both Gram-negative and Gram-positive organisms. A free phenolic acid (FPA) fraction also had antimicrobial activity, although not as strong as SA. However, the choline ester sinapine was ineffective against all organisms tested. In solid media, a 0.3 % SA concentration completely inhibited the growth of the organisms while a concentration of 0.6 % SA in liquid media reduced the population of organisms by 98 %, with some organisms completely inhibited.[101] Using similar concentrations of FPA, the range of antimicrobial activity was between 19 and 100 % on the solid media and 70 and 97 % in the liquid media.

Saxena et al.[205] reported the antibacterial compounds of *Rhus glabra* were methyl gallate, gallic acid, and 4-methoxy-3,5-dihydroxybenzoic acid and the minimum inhibitor concentrations for these compound were 12.5, 25, and >1000 μg ml^{-1}, respectively. The presence of a methoxy unit decreased the polarity of the compound and improved the antibacterial action. Other phenolic acids and analogues having antibacterial activity against *E. coli* and *Bacillus cereus* include caffeic and protocatechuic acids at concentrations of 300 and 500 μg ml^{-1} respectively.[206] Syringic acid also inhibited *B. cereus* at 500 μg ml^{-1} while *p*-hydroxybenzoic, vanillic, and *p*-coumaric acids were found to be antibacterial against *E. coli* and *B. cereus* at the 400 μg ml^{-1} concentration. Caffeic and vanillic acids completely inhibited the growth and aflatoxin production of *Aspergillus flavus* and A.

parasiticus at 200 μg ml^{-1} while syringic, *p*-hydroxybenzoic, protocatechuic, and *p*-coumaric acids and quercetin inhibited the growth of these organisms at 300 μg ml^{-1}. The presence of phenolics in extracts of plant, animal, or microbial origins have the capacity to act as an antibacterial agent. The previous discussion highlights the flavonoids and phenolic acids as being NAO with antibacterial activity.

9.8 Concluding remarks – future trends and sources of further information

Similar to the concept of microbial fermentations, plant cell culturing has provided a method for producing antioxidants in a controlled environment. Rosmarinic acid production via cell cultures has been well documented.[207–215] Kirikae et al.[216] found that alfalfa cell cultures treated with yeast extract could produce isoflavones (daidzein) and 7,4′-dihydroxyflavone. Ferulic acid and anthocyanins production by *Ajuga pyramidalis* has been completed by Madhavi et al.[217] Although cell culture techniques for the production of antioxidants have been around since about 1990, the industry has not fully embraced the process for food antioxidant production, which is not the case in such industries as the pharmaceutical. Reasons for this may include the value of the natural antioxidant as opposed to a pharmaceutical compound, the lack of optimal production techniques, the expense of cell culturing when compared with traditional agricultural and extraction methodologies and competition from synthetic antioxidant sources.

Genetically modified organisms (GMO) can be used as a way to enhance the production of specific compounds or to promote resistance within a commodity to a specific chemical. However, the poor public image of GMOs is a steep hurdle to overcome and will limit the use of transgenic modification, in addition to the factors that have limited the use of cell culture techniques. In light of the positive and negative attributes of GMO a thorough risk-benefit assessment should be completed before developing GMO products. Although GMO has a poor public image, the author believes that as scientists we have the responsibility to investigate GMO products and to assess the risk benefits of these products. The insertion of a Car B, Car R or Car S genes from mutant *Rhodotorula* or *Phycomuces blakesleeanus* yeast into other organisms such as vegetables or cereals would enhance carotenoid levels thus providing a means to produce NAO. The Car S gene is unique in mutant yeast strains because it inactivates the negative feedback control, thus β-carotene production continues even after high levels of carotenoids have been reached.[218,219] Normal carotenoid-producing yeasts lack the Car S gene and will only produce a set level of carotenoids before the negative feedback control is switched on. The application of cell culture and GMO techniques to produce natural

antioxidants is a direction that will shape the field of antioxidant chemistry well into the twenty first century.

This chapter describes antioxidants from oilseeds, nuts, cereals, legumes, animal products, and microbial sources. Throughout the text several references have been made to book chapters or review articles and they should be a valuable source of information for the reader. The number of books published on antioxidants is ever increasing but four books that were helpful in the preparation of this chapter include: *Antioxidant Methodology: In vivo and in vitro Concepts*,[220] *Food Antioxidants*,[221] *Food Phenolics: Sources, Chemistry, Effects, Applications*,[222] and *Anthocyanins in Fruits, Vegetables, and Grains*.[56] Several review articles[223–225] were sources of valuable reference material for information on antioxidants pertinent to this chapter. The American Oil Chemists' Society, Institute of Food Technologist, and American Chemical Society are three organisations that publish significant numbers of manuscripts on NAOs.

9.9 References

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10

Sources of natural antioxidants: vegetables, fruits, herbs, spices and teas

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10.1 Introduction

This chapter presents the results of the stabilisation of lipids and lipid-containing food against oxidation, by various vegetables, fruits, herbs, spices and teas. The antioxidative activity of different extracts obtained from the plant material, as well as of individual antioxidants isolated from them is discussed. Usually the effectiveness (or the stabilisation factor, F) of the substrates added is established on the basis of the determination of the ratio between the oxidation stability (or induction period, IP) of the lipid or food system in the presence of the additive, IP_{add}, and in its absence, IP_0, e.g. $F = IP_{add}/IP_0$. Oxidation stability is expressed in time units, and is determined from the kinetic curves of peroxide accumulation, or of oxygen absorption, or/and of conjugated dienes accumulation. Apart from following the lipid oxidation in bulk phase, results from measuring radical-scavenging activities and peroxidation in chemical and biological systems are also discussed. The chemical structures of individual antioxidants isolated from vegetables, fruits, herbs, spices and teas are also given.

10.2 Antioxidants from vegetables

Vegetable and fruit consumption has been shown in epidemiological studies to be related to reduced risk of cancer and cardiovascular disease.[1,2] Vegetables such as root and tuberous crops (carrots, potatoes, sweet potatoes, red beets etc.), cruciferous vegetables (cabbage, Brussels sprouts, broccoli etc.), green leafy vegetables (lettuce, spinach etc.), onions, toma-

toes and other vegetables have been screened for antioxidant activity using different oxidation systems.[3–14] In addition to the differences in methodologies, different extraction methods used to release antioxidative constituents result in variation of the antioxidant activities reported for vegetables.

In early studies Pratt and Watts[3] and Pratt[4] found that green onion tops were twice as potent as antioxidants than potato peel, green pepper and green onion and four times more potent than potatoes in inhibiting the coupled oxidation of β-carotene and linoleic acid. Using the same oxidation model Gazzani et al.[10] reported that when prepared at 2 °C, most vegetable juices showed initial pro-oxidant activity. This pro-oxidant activity was very high for eggplant, tomato, and yellow bell pepper. In the cases of carrot, celery, garlic, mushroom, zucchini, tomato, and particularly eggplant juice, it was reported that the antioxidant activity of the vegetables was increased by boiling. This suggests that the pro-oxidant activity was due to peroxidases which were inactivated at high temperature. Kähkönen et al.[13] studied the effect of plant extracts on oxidation of pure methyl linoleate at 40 °C. The results showed that at the level of 5000 ppm on the basis of the plant dry weight the order of antioxidant activity was as follows: pea, legume (37 % inhibition) > cucumber, leaf (35 %) > pea (28 %) > onion (11 %) > carrot (10 %). Compared to the poor activity of these vegetables, the peel extracts of beetroot, sugar beet, and potato showed remarkable antioxidant activity ranging from 86 to 99 % inhibition. By measuring the oxygen radical absorbance capacity (ORAC), Cao et al.[6] reported that the antioxidant score decreased in the following order: kale > garlic > spinach > Brussels sprouts > alfalfa sprouts > broccoli flowers > beets > red bell pepper > onion > corn > eggplant > cauliflower > potato > sweet potato > cabbage > leaf lettuce > string bean > carrot > yellow squash > iceberg lettuce > celery > cucumber. Results on spiking plasma with vegetable extracts showed that beans, garlic, onions, asparagus, beet, potato and broccoli ranked highest in inhibiting the oxidation of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) fractions.[11]

However, little is known about the activity of antioxidant components isolated from these vegetables. Research has focused more on the activity of commercial antioxidant compounds that are also present in these vegetables such as flavonoids and phenolic acids,[15] tocopherols,[16] carotenoids,[17] ascorbic acid,[18] and sulphur-containing compounds.[7] Table 10.1 illustrates the sparse literature on antioxidant compounds identified in different vegetables.

10.2.1 Root and tuberous vegetables

Carrot (*Daucus carota*) has been reported to exert low antioxidant activity compared to other vegetables.[5,6,9,11,13,14] Extracts of carrot leaves and peel

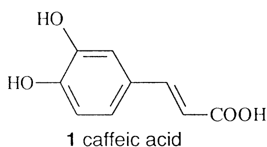
Table 10.1 Antioxidant compounds identified in different vegetables

Vegetables	Antioxidative compounds	References
Bell peppers	quercetin	3, 4
Cruciferous vegetables	phenolic compounds	7, 19
Onions	quercetin, allicin	3, 4, 20
Potato	caffeic acid derivatives, chlorogenic acid, patatin	5, 21, 22
Purple sweet potatoes	peonidin glycoside	23
Spinach	phenolic compounds	11, 24

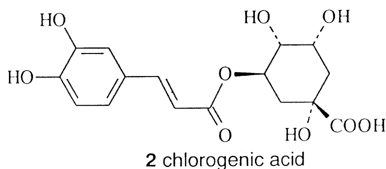
showed antioxidant activity towards oxidation of pure methyl linoleate at 40 °C while the carrot flesh was inactive.[13] Boiling carrots for 30 min significantly improved their antioxidant activity towards coupled oxidation of β-carotene and linoleic acid.[10] In addition, the most polar fraction of carrots was found to be pro-oxidative.

Potato (*Solanum tuberosum*) is considered a good source of antioxidants such as ascorbic acid, α-tocopherol and polyphenolic compounds. However, most studies have been focused on the antioxidant activity of phenolic compounds in potato.[5,12,21,25] According to Lugasi et al.[25] ethanolic extracts of potato tubers showed marked hydrogen-donating activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and had reducing power in the Fe(III) → Fe (II) reaction. Potato peelings especially have been reported to show high antioxidant activity.[3,4,13,22,26] The active compounds isolated from potatoes,[21,22] especially potato peelings, and other root crops such as the Japanese vegetable, burdock (*Arctium lappa* L),[27] are derivatives of caffeic acid **1** such as chlorogenic acid **2** or caffeoylquinic acid derivatives with sugar moiety. According to Hayase and Kato[28] these phenolic compounds are responsible for enzymatic browning and act as antioxidants in sweet potatoes (*Iopomea batatas*). Burdock[9] and sweet potato[9,11] extracts were also reported to be highly active towards lipid oxidation.

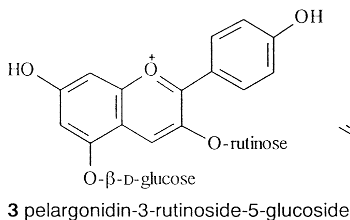
Purple potatoes and peel have been shown to exhibit greater antioxidant activities than the white and yellow varieties.[12,13] This difference in antioxidant activity may result partly from the presence of anthocyanins such as pelargonidin-3-rutinoside-5-glucoside **3** identified as the dominant anthocyanin in red-fleshed potato varieties.[29] Also an anthocyanin, peonidin glycoside, isolated from purple sweet potatoes was reported to exhibit strong antioxidant activity.[23] According to Al-Saikhan et al.[5] patatin, a water-soluble glycoprotein, appeared to be the major water-soluble compound that showed antioxidant activity while ascorbic acid promoted bleaching of the β-carotene emulsion and carotenoid pigments were probably not responsible for much of the antioxidant activity of potatoes.



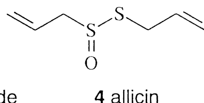
1 caffeic acid



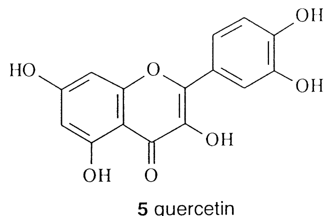
2 chlorogenic acid



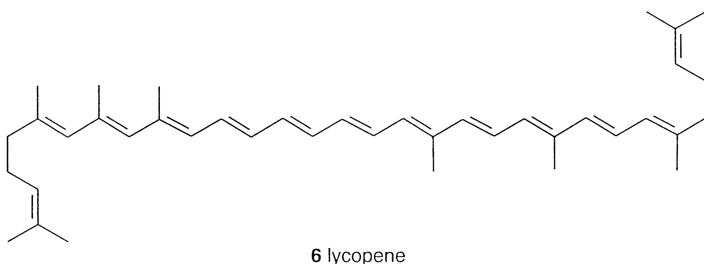
3 pelargonidin-3-rutinoside-5-glucoside



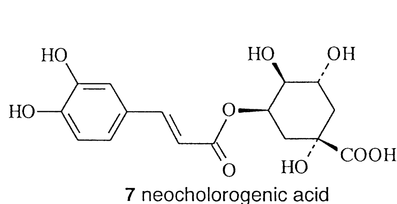
4 allicin



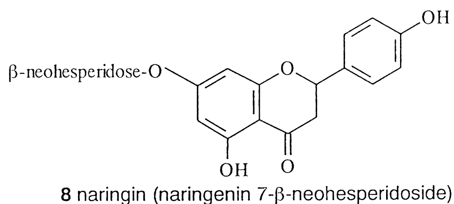
5 quercetin



6 lycopene



7 neocholorogenic acid



8 naringin (naringenin 7-β-neohesperidoside)

Similarly to carrot and potato peel, beetroot peel (*Beta vulgaris L*) and sugar beet peel (*Beta vulgaris esculenta*) showed remarkably high antioxidant activities.[13] Beet ranked eighth among 23 vegetables assayed for inhibition of LDL oxidation.[11]

10.2.2 Cruciferous vegetables

One major group of bioactive components of cruciferous vegetables is that of the glucosinolates and their breakdown products.[19] According to Plumb et al.[7] extracts from broccoli (*Brassica olearacea* L cv Italica L), Brussels sprouts (*B olearacea* L Gemmifera), red cabbage (*B olearacea* L cv Rubra), white cabbage (*B olearacea* L cv Alba) and cauliflower (*B olearacea* L cv Botrytis) show significant antioxidant properties against lipid peroxidation.

However, most of the direct antioxidant action of the crucifers is not due to the glucosinolate content. It is suggested that the total antioxidant activity of the crucifers probably involves the hydroxylated phenol and polyphenol content, as has been identified in broccoli.[30] In contrast, cabbage, cauliflower and Brussels sprouts were pro-oxidants towards lipid peroxidation in microsomes containing specific cytochrome P450s.[8]

Kale (*B olearacea L cv Acephala*), Brussels sprouts and broccoli were found to exert higher antioxidant activity than cauliflower and other vegetables.[5,6,9,11] White cabbage was reported to show more than 80% inhibition of coupled oxidation of β-carotene and linoleic acid[10] and it was also an active hydroxyl radical scavenger.[7] Boiled (15 min) Brussels sprouts were found to promote peroxidation of human liver microsomes and of phospholipid liposomes,[7] while boiled (5 min) broccoli exhibited 96% inhibition of oxidation of β-carotene linoleic acid emulsion.[5] Swede peel (*Brassica napus rapifera*) was inactive towards oxidation of methyl linoleate.[13]

10.2.3 Green leafy vegetables

Contradictory results have been reported using different oxidation model systems to assess antioxidant activity of green leafy vegetables, especially spinach. The antioxidant activity of green leafy vegetables has been reported to be low: spinach (*Spinacia olearacea* L) ranked 18th and lettuce (head) (*Lactuca sativa* L cv Capita) 22nd among 23 vegetables assayed for inhibition of LDL.[11] Yet, according to Vinson et al.[11] the phenols in spinach were able to enrich the lipoproteins by binding with them and subsequently protect them from oxidation. The ORAC activity of spinach was very high while that of leaf lettuce and iceberg lettuce was poor.[6] Moderate antioxidant activity of spinach was reported towards oxidation of linoleic acid.[9] Differently processed spinach samples were also found to inhibit formation of lipid hydroperoxides but to act as pro-oxidants in cooked meat.[24] Blends of two to four vegetables including spinach increased the inhibitiory effect on lipid peroxidation, mainly due to the high levels of antioxidants in spinach.[14]

10.2.4 Onions

The antioxidant activity of onion (*Allium cepa*) and onion scales has been studied in lipid oxidation models[3,4,5,9,10,13] and in radical scavenging assays.[6,11] Both yellow and red onion were poor antioxidants towards oxidation of methyl linoleate[13] in contrast to their high antioxidant activity towards oxidation of LDL.[11] Onion had also a poor antioxidant score in the ORAC activity test while garlic (*Allium sativum* L) gave a score that was four times higher.[6] Yin and Cheng[31] reported that the presence of garlic bulb, garlic

greens, Chinese leek, scallion, onion bulb, and shallot bulb significantly delayed lipid oxidation of phosphatidylcholine liposomes. While allicin **4** is responsible for the antioxidant activity of garlic bulb[20] compounds other than allicin are involved in determininig the antioxidant effect of other *Allium* members. According to Velioglu et al.[12] anthocyanin-rich vegetables including red onion scales generally showed very strong activities towards oxidation of β-carotene linoleic acid model system. Similarly, green onion tops were reported to be twice as active as green onions with quercetin **5** included in the antioxidant substances.[3,4]

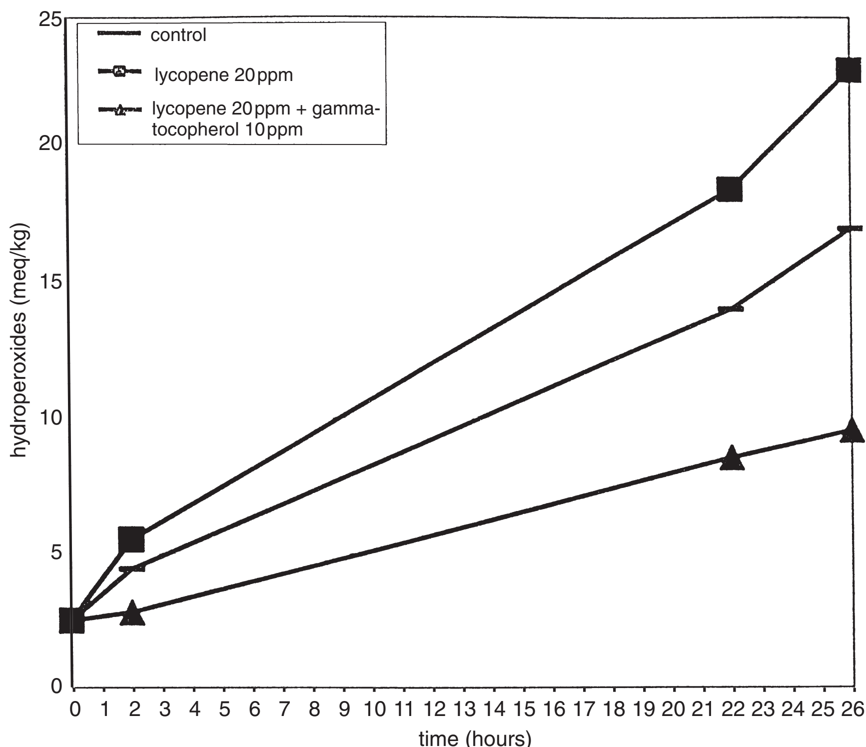
10.2.5 Other vegetables

The interest in tomato (*Lycopersicon esculentum*) is due to its high concentration of lycopene **6** as well as phenolic compounds present. Tomato was reported to exert antioxidant activity in some studies[11,13] while in other experiments it showed no antioxidant activity[32] or acted as pro-oxidant.[10] Among commercial juices tested, tomato juice has a higher oxygen radical absorbance capacity than orange and apple juices.[33] In this study, tomato juice had much higher ORAC than the acetone extract of fresh tomatoes, which may be due to differences in the varieties of tomatoes used. In addition, it was not clear whether vitamin C was added to the commercial tomato juice. Antioxidant activity of tomato juice decreased after initial 2–5 h of heating but was restored after prolonged heating.[34] In beef homogenates, tomato significantly inhibited lipid peroxidation.[14] The antioxidant effect of tomato is most probably due to synergism between several compounds and it is not due to lycopene content alone as pure lycopene and several other carotenoids act as pro-oxidants in a lipid environment.[5,35] Figure 10.1 illustrates the pro-oxidant effect of lycopene compared to the antioxidant effect of a combination of lycopene and γ-tocopherol on oxidation of rapeseed oil triglycerides under light.[35]

Bell peppers have been shown to exert low antioxidant activity[5,6,11] or pro-oxidant activity.[10] Other vegetables investigated for antioxidant activity include asparagus,[11] celery,[6,10,11] corn,[6,11] cucumber,[10,11,13] eggplant,[6,10] pea,[13] and zucchini.[10]

10.3 Antioxidants from fruits and berries

Data on the antioxidant activity of fruits and berries, their juices and wines vary widely partly due to the use of different oxidation systems and methods to analyse antioxidant compounds. The recent literature has focused to a large degree on the antioxidant effect of flavonoids and phenolic acids isolated from fruits and berries although ascorbic acid,



10.1 Pro-oxidant effect of lycopene and antioxidant effect of the combination of lycopene and γ-tocopherol on hydroperoxide formation in rapeseed oil triacylglycerols oxidised under light at 25 °C[35] (reprinted with permission from *J Agric Food Sci*, 1996 **44** 2096–100).

carotenoids and tocopherols also contribute to the antioxidant activity of fruits and berries. By using the ORAC method, the extract of fresh strawberries had the highest total antioxidant capacity compared with the extracts of plum, orange, red grape, kiwi fruit, pink grapefruit, white grape, banana, apple, tomato, pear and honeydew melon.[33] However, in lipid oxidation models (methyl linoleate, LDL) phenolic extracts from strawberries ranked among the least active antioxidants compared with the activities of other berries.[13,36]

The difference in antioxidant results due to differences in methodologies is also shown by Hopia et al.[37] by comparing different methods for measuring lipid oxidation or radical scavenging using berry wines as test material. A comparison of the antioxidant activities of fruits and berries, their juices and wines using one and the same methodology is shown in Table 10.2. The inhibition of human LDL oxidation *in vitro* in a

Table 10.2 Inhibition (%) of human low-density lipoprotein (LDL) oxidation *in vitro* in a copper-catalysed system of selected fruits, berries, their juices and wine tested at the level of 10 µM

Fruits, berries, their juices or wines	% Inhibition	References
Red and blush table grapes	22–49	38
Red wine grapes	39–60	38
Red grape juice (Concord)	68–70	39
Red wine	37–65	40
White table grapes	30	38
White wine grapes	44–46	38
White grape juice	71–75	39
White wine	25–46	40
Peaches, fresh	64–87	41
Peaches, canned	56–85	41
Prunes	82	42
Prune juice	62	42
Blackberries	84	36
Blueberries	65	36
Red raspberries	79	36
Strawberries	54	36
Sweet cherries	71	36

copper-catalysed system was measured at a level of 10 µM of the samples. From the results presented in Table 10.2 it is obvious that the antioxidant activity of fruits and berries is comparable to that of their juices and wines. The antioxidant compounds identified in fruits and berries are listed in Table 10.3.

10.3.1 Stone fruits

Prunes and prune juice (*Prunus domestica*) as well as neocholorogenic acid **7** and chlorogenic acid, the two predominant phenolic compounds in prunes, were antioxidants toward oxidation of human LDL.[42] According to the ORAC test prunes rank highest with more than twice the level of antioxidants than other high-scoring fruits such as raisins and blueberries.[60] In this study, the antioxidant score of plums was seven times less than that of prunes, which may be explained by the difference in their dry weight. Using the same method Wang et al.[33] reported earlier that plum ranked second among 12 fruits tested for antioxidant activity.

The inhibition of LDL oxidation by peach (*Prunus persica*) extracts, including raw and canned peaches, ranged between 56–87 % with the antioxidant activity mainly attributed to the presence of hydroxycinnamic acids, chlorogenic and neochlorogenic acids**,** but not to carotenoids such as β-carotene and β-cryptoxanthin present.[41] Lower activities were obtained for peach peel. On the contrary, Plumb et al.[46] reported that

Table 10.3 Antioxidant compounds identified in different fruits and berries

Fruits and berries	Antioxidative compounds	References
Apple juice	chlorogenic acid, phloretin glycosides, ascorbic acid	43, 44
Apple pomace	epicatechin, its dimer (procyanidin B_2), trimer, tetramer, oligomer, quercetin glucosides, chlorogenic acid, phloridzin, 3-hydroxyphloridzin	45
Apple	chlorogenic acid	45
Grapefruit	naringin (naringenin 7-β-neohesperidoside)	46
Grapes	total phenolics, anthocyanins, flavonols, malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside	38, 47, 48
Wild grapes	malvidin-3,5-diglucoside	49
Red grape juice	total phenolics, anthocyanins	39
White grape juice	hydroxycinnamates, flavan-3-ols	39
Grape seeds	procyanidin B_2 3′-*O*-gallate	50
Red wine	anthocyanins, catechin, gallic acid, resveratrol	40, 51, 52
Peach	chlorogenic acid, neochlorogenic acid	41, 46
Pear	chlorogenic acid	46
Orange juice	hesperidin, narirutin	43
Prunes, prune juice	chlorogenic acid, neochlorogenic acid	42
Tart cherries	cyanidin, 6,7-dimethoxy-5,8,4′-trihydroxyflavone, genistein, chlorogenic acid, naringenin, genistin, 2-hydroxy-3-(*o*-hydroxyphenyl) propanoic acid, 1-(3′,4′-dihydroxycinnamoyl)-cyclopenta-2,5-diol, 1-(3′,4′-dihydroxycinnamoyl)-cyclopenta-2,3-diol	53, 54, 55
Berries	anthocyanins, hydroxycinnamates, flavonols	36, 56, 57, 58, 59

hydroxycinnamic acids do not contribute to the inhibition of lipid peroxidation of liver and cell microsomes by fruit extracts including plum and peach although these fruits had an ability weakly to scavenge hydroxyl radicals.

10.3.2 Citrus fruits

Grapefruit (*Citrus paradisi*) extracts inhibited ascorbate–iron-induced lipid peroxidation of liver microsomes in a dose-dependent way, but were less effective antioxidant towards an NADH–iron induced system.[46] Naringin (naringenin 7-β-neohesperidoside) **8**, a major component in grapefruit, was reported not to contribute to the lipid peroxidation but to be responsible for most of the hydroxyl radical scavenging activity of grapefruit. Grapefruit was also effective towards ascorbate–iron-induced lipid per-

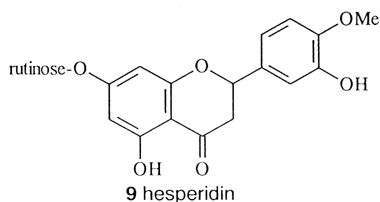
oxidation of P450-containing microsomes.[8] According to Wang et al.[33] orange (*Citrus sinensis*) was more active than pink grapefruit in scavenging peroxyl radicals (ORAC assay) while grapefruit juice was more active than orange juice.

In a study by Scarlata and Ebeler[61] citrus juices from orange, tangerine and grapefruit did not have any antioxidative effect towards oxidation on lipoproteins isolated after plasma spiking. In this study, hesperetin and hesperidin, two of the major phenolic compounds in citrus fruits, did not show any activity. On the contrary, Miller and Rice-Evans[43] showed that in orange juice the total antioxidant activity could be accounted for by hesperidin **9** and narirutin **10**. Bocco et al.[62] studied the antioxidant effect of by-products of the citrus juice industry and found that, in general, the seeds of lemon, bergamot, sour orange, sweet orange, mandarin, pummelo and lime possessed greater antioxidative activity than the peels.

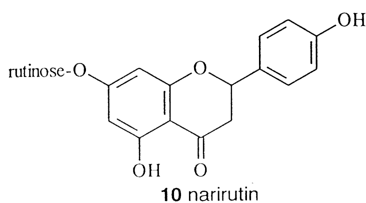
10.3.3 Grapes and wines

Antioxidants in grapes (*Vitis vinifera*) and grape juices have been recently reviewed by Frankel and Meyer.[47] Both fresh grapes and commercial grape juices are a significant source of phenolic antioxidants. Extracts of fresh grapes inhibited human LDL oxidation from 22 to 60 % and commercial grape juices from 68 to 75 % when standardised at 10 μM gallic acid equivalents (GAE).[38,39] The antioxidant activities of grapes and grape juices were comparable to those found for wines (Table 10.2).[40] The LDL antioxidant activity correlated highly with the concentration of total phenolics for both grape extracts and commercial grape juices, with the level of anthocyanins and flavonols for grape extracts, with the levels of anthocyanins for Concord grape juices, and with the levels of hydroxycinnamates and flavan-3-ols with the white grape juice samples.[39] Vitamin C had no significant effect on the antioxidant activity of grape juices.[39] Grape extracts were also shown to inhibit formation of both hydroperoxides and hexanal in lecithin liposomes.[63] According to Wang et al.[33] grapes and grape juices also had high ORAC activities. A major anthocyanin pigment, malvidin-3,5-diglucoside **11**, with antioxidant activity, was isolated from wild grapes (*Vitis coignetiae*).[49] Anthocyanins with malvidin nucleus, especially malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside **12**, isolated from Muscat Bailey A grape proved to be more effective than (+)-catechin and α-tocopherol.[48]

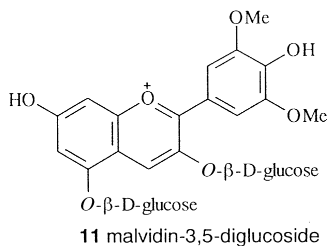
According to Meyer et al.[64] phenolic antioxidants that were released from grape pomace using enzymes significantly retarded human LDL oxidation. Oxygen radical scavenger ability of procyanidins for superoxide and hydroxyl radicals was evaluated by da Silva et al.[50] In this study, procyanidin B_2 3′-*O*-gallate **13**, isolated from grape seeds was found to be the most



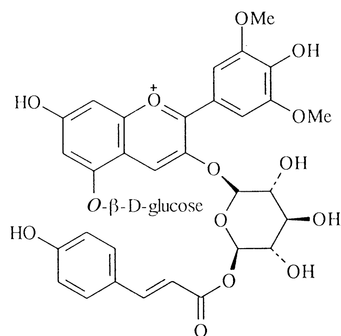
9 hesperidin



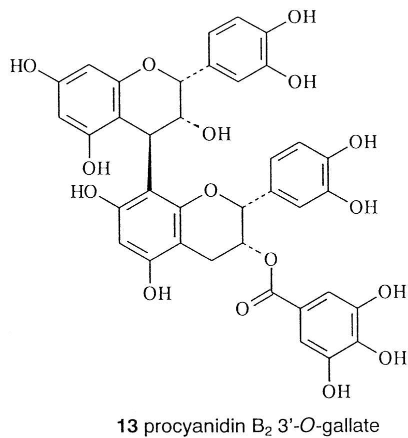
10 narirutin



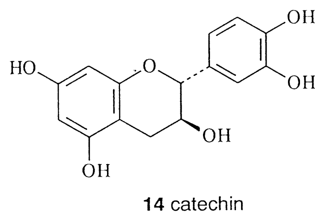
11 malvidin-3,5-diglucoside



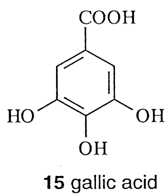
12 malvidin 3-*O*-(6-*O*-*p*-coumaroylglucosido)-5-glucoside



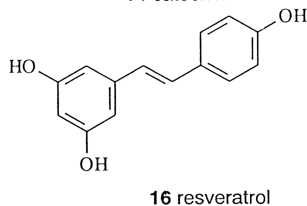
13 procyanidin B_2 3'-*O*-gallate



14 catechin



15 gallic acid



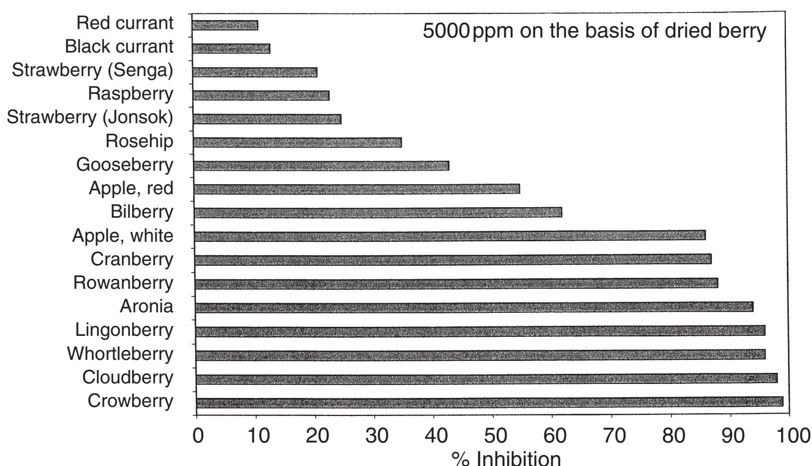
16 resveratrol

effective compound. Grape seed extract could also reduce radical species measured by electron spin resonance (ESR) spectroscopy.[65]

The potent antioxidant activity of wines, notably red wines, was first reported by Frankel et al.[66] by showing that wine phenolics were more effective than tocopherol in reducing LDL lipid oxidation as evidenced by carotene-bleaching, a sensitive index of free radical-induced oxidation. This result has been confirmed by several other research groups.[51,67–72] The phenolic compounds responsible for antioxidant activity in red wine include catechin **14**, anthocyanins, gallic acid **15** and resveratrol **16**.[51,52,61] According to Ghiselli et al.[51] the anthocyanin fraction was the most effective both in scavenging reactive oxygen species and in inhibiting lipoprotein oxidation compared to two other red wine fractions of phenolic acids + quercetin-3-glucuronide and catechins + quercetin-3-glucoside. Gardner et al.[73] reported the findings made by using ESR spectroscopy that quercetin and related flavonols are minor antioxidants in red wine.

10.3.4 Berries

Berries constitute a significant source of antioxidants such as ascorbic acid, tocopherols, carotenoids, flavonoids and phenolic acids. Figure 10.2 shows the antioxidant activity of extracts from berries on the oxidation of methyl linoleate at 40 °C at a level of 5000 ppm on the basis of the plant dry weight.[13] It is to be seen that the most potent berries were crowberry (*Empetrum nigrum*), cloudberry (*Rubus chamaemorus*), whortleberry (*Vaccinium uligonosum*), lingonberry (*Vaccinium vitis-idaea*), aronia



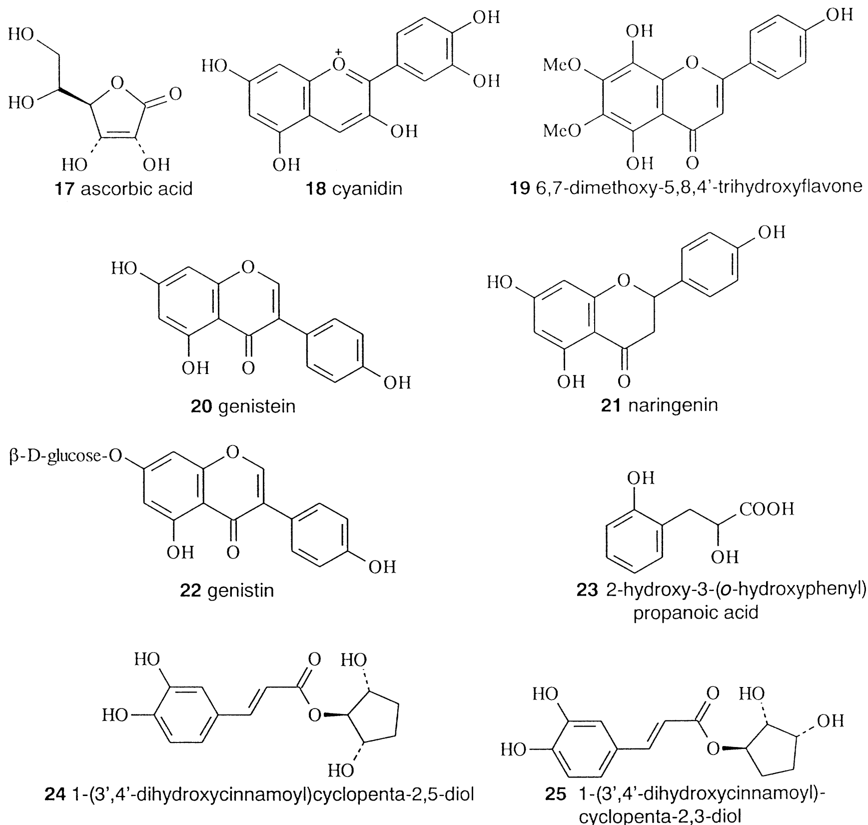
10.2 Antioxidant activity of 70 % acetone extracts from berries on the oxidation of methyl linoleate at 40 °C at a level of 5000 ppm on the basis of plant dry weight[13] (reprinted with permission from *J Agric Food Sci*, 1999 **47** 3954–62).

(*Aronia melanocarpa*), cranberry (*Vaccinium oxycoccus*) and rowanberry (*Sorbus aucuparia*), all being wild berries, while the cultivated berries such as strawberry (*Fragaria ananassa*), redcurrant (*Ribes rubrum*), blackcurrant (*Ribes nigrum*), and raspberry (*Rubus idaeus*) exerted low antioxidant activity. Somewhat surprisingly blackcurrant was not among the most active berries as blackcurrant along with crowberries and bilberries (*Vaccinium myrtillus*) were earlier[74] evaluated as the most active raw materials for berry wines. It may be that the wine making procedure more effectively extracts the active phenolic compounds from the berries as compared to the solvent extraction of the berries. Berry extracts inhibited LDL oxidation in the order: blackberries (*Rubus fructicosus*) > red raspberries > sweet cherries (*Prunus avium*) > blueberries (*Vaccinium corymbosum*) > strawberries.[36] In the same study, sweet cherries were the most active towards oxidation of lecithin liposomes followed by blueberries, red raspberries, blackberries and strawberries. Different blueberries and bilberries were reported to exhibit good antioxidant capacity in the ORAC assay.[56,59] The antioxidant capacity of blueberries was about three-fold higher than either strawberries or raspberries with only a small contribution of ascorbic acid **17** to the total antioxidant capacity compared to total phenolics and anthocyanins.[59]

The antioxidant activity for LDL was associated directly with anthocyanins and indirectly with flavonols, and for liposomes it correlated with the hydroxycinnamate content.[36] However, according to Costantino et al.[57] the activities of black raspberries, blackcurrants, highbush blueberries, blackberries, redcurrants and red raspberries toward chemically generated superoxide radicals were greater than those expected on the basis of anthocyanins and polyphenols present in the berries. According to Miller and Rice-Evans[43] it is possible that ascorbic acid significantly contributes to the antioxidant activity of berries and berry juices, as it was reported that blackcurrant juice has an ascorbate sparing effect.

Spray-dried elderberry (*Sambucus nigra*) juice, containing high amounts of anthocyanin glucosides, inhibited copper-induced oxidation of LDL.[58] It has been found also that the anthocyanins were able to reduce α-tocopheroxyl radical to α-tocopherol.[58] According to Velioglu et al.[12] the carotenoid-rich sea buckthorn berry (*Hippophae rhamnoides* L cv Indian-Summer) had a high antioxidant activity of 94 % in a β-carotene bleaching method.

Tart cherries (*Prunus cerasus*) were reported to exhibit antioxidant activity.[53–55] According to Haibo et al.[53] anthocyanidin and its aglycone, cyanidin **18** isolated from tart cherries were responsible for the antioxidant action. In a further study,[54] the antioxidant assays revealed that 6,7-dimethoxy-5,8,4′-trihydroxyflavone **19** is the most active, followed by genistein **20**, chlorogenic acid, naringenin **21** and genistin **22**. In an Fe(II)-induced liposome peroxidation bioassay, the ethylacetate extract of tart cherries was found to have strong antioxidant activity with active components identified

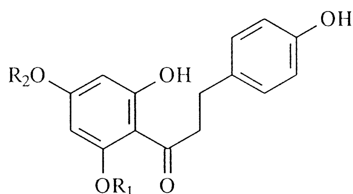


17 ascorbic acid
18 cyanidin
19 6,7-dimethoxy-5,8,4'-trihydroxyflavone
20 genistein
21 naringenin
22 genistin
23 2-hydroxy-3-(*o*-hydroxyphenyl) propanoic acid
24 1-(3',4'-dihydroxycinnamoyl)cyclopenta-2,5-diol
25 1-(3',4'-dihydroxycinnamoyl)-cyclopenta-2,3-diol

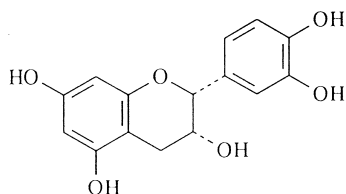
as chlorogenic acid methyl ester and three novel compounds: 2-hydroxy-3-(*o*-hydroxyphenyl) propanoic acid **23**, 1-(3′,4′-dihydroxycinnamoyl)-cyclopenta-2,5-diol **24** and 1-(3′,4′-dihydroxycinnamoyl)-cyclopenta-2,3-diol **25**.[55]

10.3.5 Other fruits

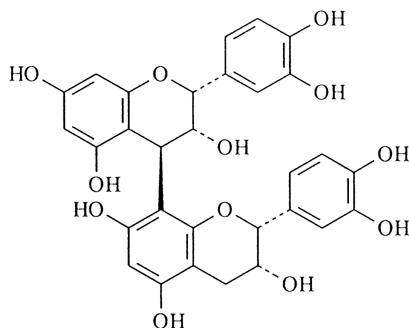
Apple extracts with 70 % acetone tested on the basis on their dry weight showed strong antioxidant activites towards oxidation of methyl linoleate although apples were low in total phenolics.[13] In apple juice, vitamin C activity represented a minor fraction of the total antioxidant activity with chlorogenic acid and phloretin glycosides **26** as the major identifiable antioxidants.[43,44] According to Plumb et al.[46] chlorogenic acid contributes about 27 % of the total activity of apple extract to scavenge hydroxyl radicals. Apple polyphenols isolated from gala apple pomace such as epicatechin **27**, its dimer (procyanidin B_2) **28**, trimer, tetramer and oligomer,



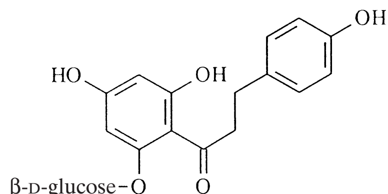
a R_1 = β-D-glucose, R_2 = H
b R_1 = H, R_2 = β-D-glucose
26 phloretin glycosides



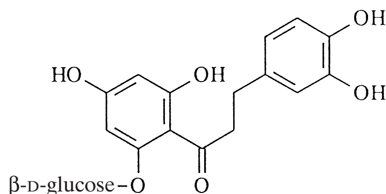
27 epicatechin



28 procyanidin B_2



29 phloridzin



30 3-hydroxyphloridzin

guercetin glucosides, chlorogenic acid, phloridzin **29** and 3-hydroxy-phloridzin **30** showed strong antioxidant activities in β-carotene linoleic acid system and DPPH radical scavenging activities.[46]

Other fruits studied include banana, pear, honeydew melon and kiwi fruit.[33,46,60] Also, the antioxidant activity of olives has been recently reported.[75]

10.4 Antioxidants from herbs, spices and teas

Herbs, spices and teas are one of the most important targets in the search for natural antioxidants from the point of view of safety. Man has used them not only for flavouring foods but also for antiseptic and medical properties

since the prehistoric era. Table 10.4 presents the taxonomical classification of spices.[76]

Since the early work of Chipault et al.[77–79] which examined more than 70 spices, herbs and teas, interest in the antioxidative activity of spices has increased and led to an increase in information about the compounds and mechanisms involved.

In the evaluation of spices, some investigations have been carried out using the whole spice.[80–85] Table 10.5 illustrates the importance of the substrate used for the antioxidative activity of the additives. Rosemary and sage were the most effective antioxidants in lard[77,86] and both spices were found to have a low redox potential in sausages indicating antioxidative activity.[89,90] However, in an oil-in-water emulsion, clove was the most effective spice.[78,87,88] In general, the stabilisation factors obtained for the spices in the emulsions were several times greater than those in lard, indicating a higher efficiency against oxidation in the emulsion.[92] The antioxidant activity of 17 different spices was investigated in mayonnaise and French dressing, and oregano was found to display the highest activity.[79]

Shahidi et al.[93] reported that the antioxidative activity of ground clove, ginger, oregano, sage and thyme in meat lipids was concentration depen-

Table 10.4 Taxonomic classification of spices

ANGIOSPERMAE			Tubiflorae	*Libiatae*	basil, balm, dittany, marjoram, mint, oregano, perilla, rosemary, sage, savory, thyme
				Solanaceae	chili, paprika, red pepper
	Dicotyledoneae			*Pedaliaceae*	sesame
		Sympetalae	Campalunatae	*Compositae*	camomile, chicory, tarragon
		Archichlamydaeae	Piperales	*Piperaceae*	cubeba, long pepper, pepper
			Ranales	*Myristicaceae*	mace, nutmeg
				Lauraceae	bay leaf, cassia, cinnamon
				Magnoliaceae	star-anise
			Rhoeadales	*Cruciferae*	mustard, wasabi
			Myrtiflorae	*Myrtaceae*	allspice, clove
			Umbelliflorae	*Umbelliferae*	anise, caraway, celery, chervil, coriander, cumin, dill, fennel parsley
	Monocotyledoneae		Liliiflorae	*Liliaceae*	garlic, onion
				Iridiceae	saffron
			Scitamineae	*Zingiberaceae*	cardamon, ginger, turmeric
			Orchidales	*Orchidaceae*	vanilla

Table 10.5 Relative antioxidative effectiveness (RAE) of spices, herbs and teas, evaluated as whole plant material in different substrates

Spice, herb, tea	Substrate	RAE	References
Marjoram, nutmeg, white pepper, rosemary, sage, coriander, black pepper	Lard	rosemary>sage>nutmeg >white pepper >marjoram	86
32 different plant materials	Lard	rosemary>sage>oregano> nutmeg>thyme	77
19 different plant materials	Oil-in-water-emulsion	clove>cinnamon> sage>mace>oregano	87
32 different plant materials	Oil-in-water-emulsion	clove>tumeric>allspice> mace>rosemary	78
10 different plant materials	Oil-in-water-emulsion	clove>allspice>cinnamon> nutmeg>ginger	88
Allspice, red paprika, savory, marjoram, black pepper, white pepper, coriander	Sausage, water	allspice>red paprika >savory>marjoram >black pepper	89
15 different plant materials	Sausage, water	sage>rosemary>paprika> marjoram>aniseed	90
12 different plant materials	Ground chicken meat	marjoram>caraway> peppermint>clove	91

dent, but clove was most effective, followed by sage and then rosemary. Ginger and thyme exerted the weakest effect. It was also established that the addition of sage to pork sausages treated with sodium chloride was able to inhibit the oxidative effect of salt.[94]

Dried leaves of rosemary added to cooked minced pork meat balls retarded the development of warmed over flavour (WOF) during cold storage.[95] Pulverised summer savory and rosemary (0.05 %) significantly improved the oxidative stability of minced, cooked pork meat balls in two accelerated model systems with the rosemary showing a slightly higher antioxidative activity.[96] Tsimidou et al.[97] found that 1 % oregano was equivalent to 200 ppm butylated hydroxyanisole (BHA) in controlling oxidation of mackerel oil. For an oil-in-water emulsion dressing, addition of 0.15 % of dried leaves of summer savory or more significantly of rosemary resulted in a significantly better antioxidative protection than the addition of 80 ppm propyl gallate.[98]

Table 10.6 Stabilisation factor *F* of different herbs, spices and teas in four foods[99]

Substrate	Lard	Oil-in-water emulsion	Minced pork meat	Mayonnaise
Storage temperature, °C	99	63	–5	20
Spice, concentration in fat, %	0.2	0.1	0.25	0.2
Clove	1.8	16.7	<5.3	1.4
Rosemary	17.6	10.2	<5.3	2.2
Sage	14.2	7.8	<5.3	2.4
Oregano	3.8	7.9	<7.2	8.5
Summer savory	1.6	7.9	1.0	1.5
Thyme	3.0	6.8	6.0	1.8
Ginger	1.8	8.8	1.3	1.0
Curcuma	2.9	15.9	4.5	0.9
Nutmeg	3.1	9.2	5.3	0.9

The influence of the type of food system on the stabilisation factor of different spices is presented in Table 10.6.[99]

10.4.1 Rosemary (*Rosmarinus officinalis* L) and sage (*Salvia officinalis* L)

Rosemary is one of the most effective spices widely used in food processing. It is the only spice commercially available for use as an antioxidant in Europe and United States. One of its main potential uses is the suppression of WOF.[100] However, because of their prime use as flavouring agents, rosemary extract products are not technically listed as natural preservatives or antioxidants.

The first use of an extract of rosemary leaves as an antioxidant was reported by Rac and Ostric in 1955.[101] Berner and Jacobson[102] obtained a patent in 1973 for production of an antioxidant extract from rosemary using oil as a solvent. Chang et al.[103] reported a process for the extraction of rosemary and sage, followed by vacuum steam distillation in an edible oil or fat to obtain a colourless, odourless natural antioxidant. Bracco et al.[104] described an extraction process using peanut oil, followed by micronisation, heat treatment, and molecular distillation. Inahata et al.[105] obtained a patent in 1996 for production of odourless and safe antioxidants from rosemary by repeated extraction, evaporation, purification and dissolving procedures. More recently, another technique, supercritical carbon dioxide extraction, has been used to produce extracts of rosemary and sage.[106,107]

Antioxidant properties of rosemary have been well documented.[95,108–113] Rosemary was considered to be both a lipid antioxidant and a metal chelator.[110] Rosemary extracts were also found to scavenge superoxide radicals.[113] The application of rosemary extracts in food has given a variety of results and these depend on the test model being used.

Many different solvents have been used for the extraction of the antioxidative compounds.[103,114–117] Chang et al.[103]extracted rosemary leaves with hexane, benzene, ethyl ether, chloroform, ethylene dichloride, dioxane and methanol. The extracts (0.02 %) were tested during oxidation of lard at 60 °C in the dark. It was established that the greatest antioxidant activity was located in the methanol extract. This extract was further purified, and the resultant fraction showed an outstanding activity in potato chips fried in sunflower oil and held at 60 °C in the dark for 60 days.

Marinova et al.,[115] Chen et al.,[116] and Pokorny et al.[117] found that the hexane extracts from rosemary were better antioxidants for lard,[115,116] rapeseed and sunflower oils,[117] than methanol[116] or ethanol[117] extracts. Hexane extract (0.05 %) caused a 35-fold increase of the oxidation stability of lard determined at 100 °C, and the use of 0.05 % ethanol extract resulted in a 20-fold increase.[115] In bulk rapeseed oil hexane extracts from rosemary and sage were also more efficient than ethylacetate or acetone extracts.[118] It was established that rosemary extracts were more active than sage extracts,[117,118] and that rapeseed oil was more efficiently stabilised than was sunflower oil.[117] The antioxidative effect of rosemary ethanol extract on butter,[119,120] as well as on filleted and minced fish during frozen storage has been studied.[121]

Rosemary antioxidants were found suitable for deep frying in edible oils,[122] especially in the presence of ascorbyl palmitate.[123] Réblova et al.[124] investigated the effect of acetone and ethyl acetate extracts on the changes in rapeseed oil and in an oil containing polysiloxanes during frying of potatoes. The authors established that the rosemary extracts inhibited the formation of polar substances, polymers and decomposition of polyunsaturated triacylglycerols, especially in the case of rapeseed oil, and improved the sensory attributes of french fries.

Barbut et al.[109] studied the effectiveness of rosemary oleoresin (RO) in turkey breakfast sausages. The authors found that RO was as effective as the combination of BHA or butylated hydroxytoluene (BHT) with citric acid in suppressing oxidative rancidity. A standardised RO has many different phenolic components. It is thought that they act in synergy to provide antioxidant activity.

Results from the oxidation of stripped soybean oil exposed to fluorescent light, in the presence of rosmariquinone (RQ) and RO[125] indicated that RO contained compounds, such as chlorophyll, pheophytin and mono- and diglycerides, which under light interfere with the antioxidant components, thus reducing the antioxidant activity. This was confirmed by the highest level of antioxidant activity exhibited by the RQ in comparison to RO.

Lai et al.[126] and Murphy et al.[127] investigated the antioxidant properties of RO alone or in combination with sodium tripolyphosphate (STPP) in controlling lipid oxidation in restructured chicken nuggets[126] and in precooked roast beef slices[127] during refrigerated and frozen storage. Stoick

et al.[128] studied the oxidative stability of restructed beef steaks processed with RO, tertiary butylhydroxyquinone (TBHQ) and STPP. They found that the addition of RO gave no benefit over STPP. The RO/STPP combination was equivalent to TBHQ/STPP treatment in preventing oxidation.

Wada and Fang[129] observed a strong synergistic effect between rosemary extract (0.02 %) and α-tocopherol (0.05 %) in sardine oil at 30 °C and in frozen-crushed fish meat models. The authors suggested that rosemary extract functions as a hydrogen atom donor regenerating the α-tocopheroxyl radical to α-tocopherol. Synergistic effects were also found between rosemary and sage extracts, and tocopherols or soybean meal hydrolysates in a linoleic acid emulsion.[130] Basaga et al.[113] reported that rosemary extract and BHT, when added as mixtures of 75:25, 50:50 and 25:75 had a synergistic effect on preventing oxidation of soybean oil. A very pronounced synergistic effect was seen between citric acid and rosemary extract.[77]

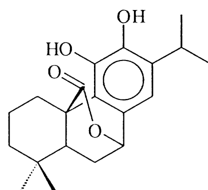
Concurrent with the evaluation of rosemary extracts as antioxidants to inhibit lipid oxidation in food systems, research was also focused on isolation, identification and testing of the active compounds contained in the extracts. In a study of 16 compounds isolated from rosemary, Bracco et al.[104] concluded that the antioxidant activity of rosemary extracts is primarily related to two phenolic diterpenes, carnosol **31** and carnosic acid **32**. This conclusion was confirmed by other investigators.[116,131] Nakatani and Inatani[132] identified rosmanol **33** and carnosol and found that both were more effective than α-tocopherol, BHT and BHA. The same authors[133] isolated also rosmadial **34** from rosemary.

Several other antioxidative diterpenes such as epirosmanol **35** and isorosmanol **36**,[134] rosmaridiphenol **37**[135] and rosmariquinone **38**[136] have been reported to contribute to the antioxidant activity of rosemary extracts. During the storage and extraction of rosemary, carnosic acid is partially converted either into carnosol or into other diterpenes such as rosmanol.[104,137–139]

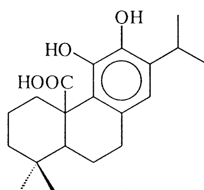
Rosmarinic acid (RA) **39** was reported by Gerhardt and Schröter[140] to be the second most frequently occurring caffeic acid ester, following chlorogenic acid, and to have antioxidant activity equivalent to that of caffeic acid. The authors detected RA in rosemary, balm, sage, thyme, oregano, marjoram, savory, peppermint, and for the first time in basil.

There are many data in the literature concerning the antioxidative properties of the individual compounds isolated from rosemary. Brieskorn and Domling[141] showed that carnosic acid and carnosol were as effective as BHT and that their effectiveness was concentration dependent. The authors noted that the activity of both compounds was due to the cooperation of their *ortho* phenolic groups with their isopropyl group.

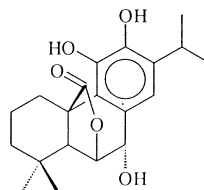
It was also reported that rosmanol had greater antioxidant activity than carnosol,[132] with carnosic acid being more potent than carnosol.[138,142] In soybean oil carnosic acid was found to be more active than BHT and BHA,



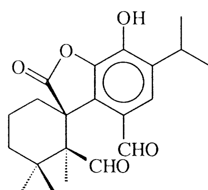
31 carnosol



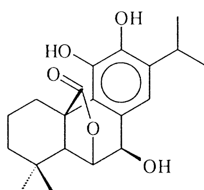
32 carnosic acid



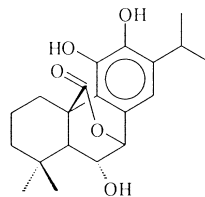
33 rosmanol



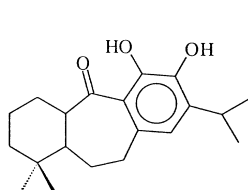
34 rosmadial



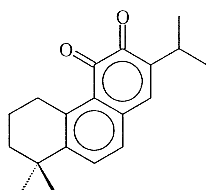
35 epirosmanol



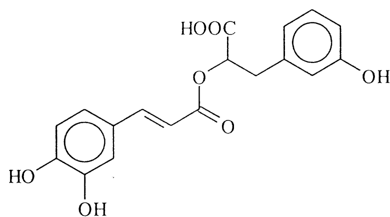
36 isorosmanol



37 rosmaridiphenol



38 rosmariquinone



39 rosmarinic acid

but less active than TBHQ. Carnosic acid and carnosol showed the ability to chelate iron and were effective radical scavengers of peroxyl radicals.[131] It has been established[115] that the molecules of carnosol and the radicals formed from them participate in the reactions of chain initiation and propagation to a much lower degree than is the case with most natural and synthetic antioxidants.

Houlihan et al.[135] found rosmaridiphenol to be more active than BHA in lard and equivalent to BHT in this test system. They reported also that RQ was superior to BHA and equivalent to BHT in controlling the oxidation of lard.[136] RQ has been shown to have good antioxidant activity also in soybean oil.[125] Hall et al.[143] proved that RQ acted as a hydrogen-donating antioxidant. Isorosmanol and epirosmanol showed high activity in both lard and linoleic acid;[134] in lard they were four times more active than BHA and BHT. Nakamura et al.[144] reported that RA exhibited a significantly higher superoxide scavenging activity than ascorbic acid.

As far as complex food systems are concerned, it is important to clarify the antioxidative behaviour not only in bulk oil but also in oil-in-water emulsions,[145–147] as well as in microsomal and liposomal systems.[131] Frankel et al.[145] reported that in bulk corn oil rosemary extract, carnosic and rosmarinic acids were significantly more active than carnosol. In contrast, in corn oil-in-water emulsion, the rosemary compounds were less active than in bulk oil, and the rosemary extract, carnosic acid and carnosol were more active than rosmarinic acid. The decreased antioxidant activity of the polar hydrophilic rosemary compounds in the emulsion system may be explained by their interfacial partitioning into water, thus becoming less protective than in the bulk oil system.[145] Carnosol and carnosic acid were powerful inhibitors of lipid peroxidation in microsomal and liposomal systems.[131]

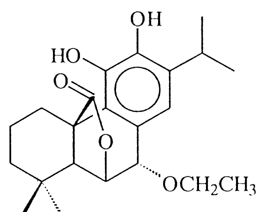
Cuvelier et al.[148] found no correlation between the antioxidative effectiveness of the rosemary extracts from different pilot-plant or commercial sources and their composition in 20 specific phenols: this finding is an illustration of the complex influence of the various factors on the lipid oxidation stability (see also Chapter 3, Section 5).

Salvia officinalis L, commonly known as sage (Dalmatian sage), is used in foods for flavouring and seasoning. It was found that, along with rosemary, it had the best antioxidant activity among the numerous herbs, spices and teas tested.[77,78] Its extracts are also well known as efficient antioxidants.[77,78,148–150] Because rosemary and sage belong to the Labiatae family, it is not suprising to find the same antioxidants in both plants: carnosol,[148,151] carnosic acid,[138,141,152–154] rosmanol,[148,154] rosmadial,[148] rosmarinic acid.[153] Various methyl and ethyl esters of carnosol, rosmanol, and carnosic acid can be found in sage, as well as in other Labiatae plant extracts, in most cases the compounds are believed to be artifacts from the extraction procedures.[137,138,141] The main antioxidative effect of sage was reported to relate to the presence of carnosic acid, carnosol, and rosmarinic acid.[148,153] The list of the antioxidants isolated from sage is growing, e.g. 9-ethylrosmanol ether **40**,[149] luteolin-7-*O*-β-glucopyranoside **41**,[155] 6-*O*-caffeoyl-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside **42** and 1-*O*-caffeoyl-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside **43**.[156]

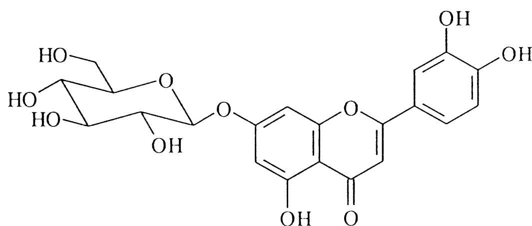
Since methanol and ethanol were found to be the most suitable solvents for extraction of antioxidants from the plant materials, a number of publications have dealt with further purification of the alcohol extracts. Vacuum steam distillation[103] or molecular distillation[104] are recommended for use on production scale.

10.4.2 Teas

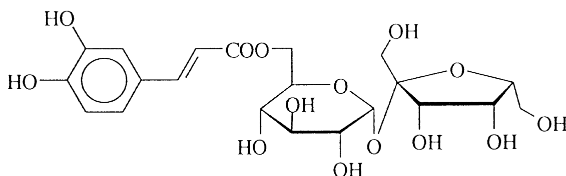
Tea, a beverage originating from a single species of a plant, *Camellia sinensis*, is widely cultivated around the world in both tropical and subtropical



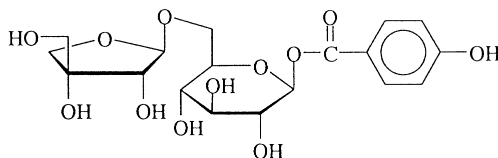
40 9-ethylrosmanol ether



41 luteolin-7-*O*-β-glucopyranoside



42 6-*O*-caffeoyl-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside



43 1-*O*-caffeoyl-β-D-apiofuranosyl-(1→6)-β-D-glucopyranoside

regions. There are three major forms of manufactured teas: green tea (non-fermented), oolong tea (semifermented), and black tea (fermented). The antioxidant activity of the water extracts isolated from them decreased in the order semifermented tea > nonfermented tea > fermented tea.[157] It was also found[158] that ethanol extracts of green, yellow and white teas strongly inhibited oxidation of canola oil compared to BHT, and oolong teas exibited only moderate activity because of the partial destruction of natural polyphenols by semifermentation. The ethanol extracts from black, dark-green, and ginseng teas showed little or no protection to canola oil, due to the complete destruction of natural polyphenols by fermentation during manufacturing processes.

Zandi and Gordon[159] reviewed the main literature published prior to 1995 on tea as a source of natural antioxidants. Many authors have recently published results on the antioxidative role of green[160–167] and of black tea[160–162] extracts in different oxidising systems. Frankel et al.[165] observed an improved antioxidant activity for green teas in lecithin liposomes compared

to corn oil emulsions that was explained by the greater affinity of the polar tea catechin gallates for the polar surface of the lecithin bilayers, thus affording better protection against oxidation.

Zandi and Gordon[168] established that the methanol extract of old tea leaves was effective in retarding rapeseed oil deterioration at 60 °C dose dependent in the range 0.02–0.25 %. The authors concluded that old tea leaves, which often are considered as a waste, contain antioxidants that may usefully be extracted and added to foods.

There are three major polyphenol groups in teas: catechins, theaflavins, and thearubigins. The chemical structures of catechins and theaflavins have been identified[169] as (–)-epicatechin (EC) (see Section 10.3.5), (–)-epicatechin gallate (ECG) **44**, (–)-epigallocatechin (EGC) **45**, (–)-epigallocatechin gallate (EGCG) **46**, theaflavin (TF) **47**, theaflavin monogallate A (TF-1A) **48**, theaflavin monogallate B (TF-1B) **49**, theaflavin digallate (TF-2) **50**. The structure and chemistry of thearubigins have not been well characterised.[169] Catechins are major costituents of green tea, EGCG being the major polyphenol. It was established that the antioxidant capacity of tea was strongly correlated ($r = 0.956$) with its total phenolic content.[170]

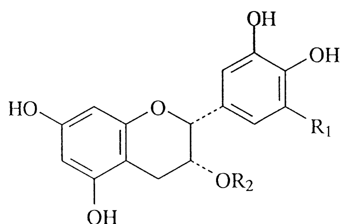
Koketsu and Satoh[171] fried noodles in lard containing green tea polyphenols. They found that the oxidation stability of the noodles was proportional to the green tea polyphenols in lard. Crude tea catechin powder reduced the formation of peroxides in lard at 100 °C far more effectively than α-tocopherol or BHA.[172] Green tea catechin extract was much more effective than the rosemary extract against lipid oxidation in canola oil, lard, and chicken fat at 100 °C.[173]

Recent studies have shown that phenolic compounds isolated from green and black tea had strong antioxidant activity in different lipids and lipid-containing products.[169,174] Green tea polyphenols, namely the catechins EGCG, ECG, EGC, and EC exhibited good superoxide-, lipoxygenase-, as well as lipid oxidation-inhibition abilities, but all the theaflavins showed little effect on the inhibition of lipid peroxidation.[172]

The order of the relative antioxidant activity of catechins depends on the lipid system, the presence of metal catalysts, the temperature, the antioxidant concentration, the oxidation stage, and the method used to evaluate lipid oxidation.[164,175–178]

Amarowicz and Shahidi[175] have shown that in a β-carotene–linoleate model system ECG possessed the strongest and EGC the weakest antioxidative effect. As a result of a head-space analysis of pentane levels after 30 days storage of a sunflower oil-in-water emulsion at pH 5.5 Roeding-Penman and Gordon[164] found the following order of antioxidant activity myricetin > EGCG > ECG. In a lipoprotein oxidation model the order was EGCG > EGC > ECG > catechin.[177]

Huang and Frankel[178] tested the catechins, propyl gallate (PG) and gallic acid (GA) during oxidation of different systems. The authors established


OH
OH
HO
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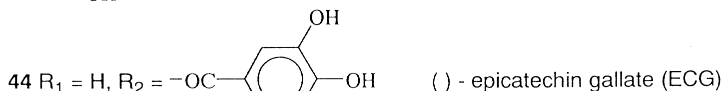
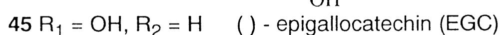
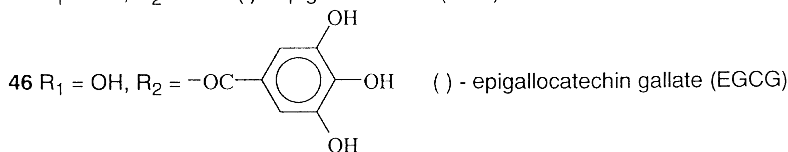
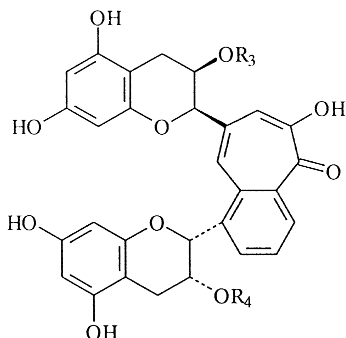
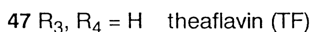
R1
OR2
OH

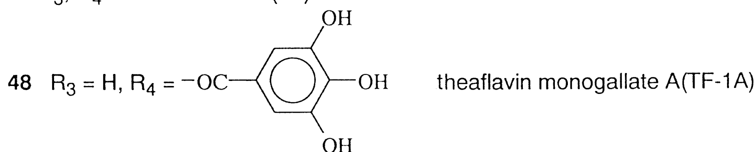

OH
44 R1 = H, R2 = −OC
OH
( ) - epicatechin gallate (ECG)
OH

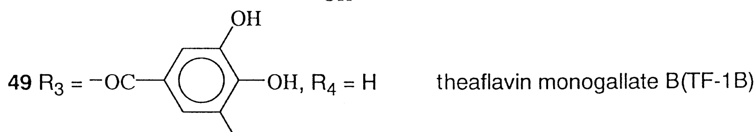

45 R1 = OH, R2 = H ( ) - epigallocatechin (EGC)

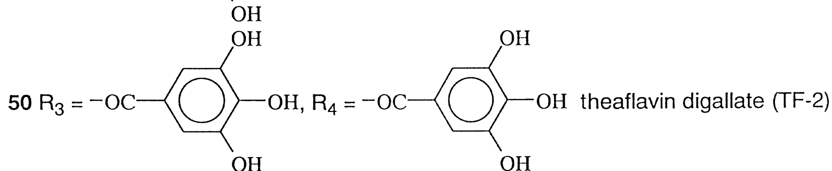

OH
46 R1 = OH, R2 = −OC
OH
( ) - epigallocatechin gallate (EGCG)
OH


OH
OR3
OH
HO
O
O
HO
O
OR4
OH


47 R3, R4 = H theaflavin (TF)


OH
48 R3 = H, R4 = −OC
OH
theaflavin monogallate A(TF-1A)
OH


OH
49 R3 = −OC
OH, R4 = H
theaflavin monogallate B(TF-1B)
OH


OH
OH
50 R3 = −OC
OH, R4 = −OC
OH theaflavin digallate (TF-2)
OH
OH

that in bulk corn oil oxidation at 50 °C the rate of hydroperoxide formation decreased in the order GA > ECG > EGCG > EGC. In corn oil-in-water emulsions the polyphenols worked as pro-oxidants, and the order of inhibiting hydroperoxide formation in soy lecithin liposomes was EGCG > EC ≈ PG > catechin ≈ ECG > EGC ≈ GA. Huang and Frankel[178] emphasised that for clarifying the antioxidant or pro-oxidant mechanism of tea catechins in different lipid systems, more information is needed on their redox potentials, stability, metal chelation, and partition properties. Jia et al.[179] reported on the antioxidant synergism of tea polyphenols and α-tocopherol.

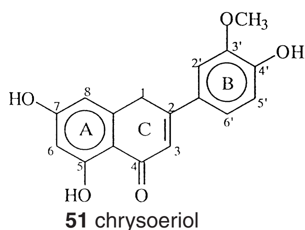
The antioxidant compounds in hot water extract from barley grain (referred to as barley tea) were identified and their antioxidant activity was determined.[180] The structures of catechol, GA, gentisic acid, GC and EGCG were established.

The many health-giving properties of rooibos tea, made from the leaves and fine stones of *Aspalathus linearis*, grown in South Africa, are thought to be attributed to the plant's antioxidant properties. In this connection, the influence of the fermentation process[181] as well as the effect of extraction time and additional heating[182] on the antioxidant activity of rooibos tea was studied. It was concluded that rooibos tea can be added to the list of plants with known antioxidant activity due to its flavonoid compounds **5, 51–60**.[183]

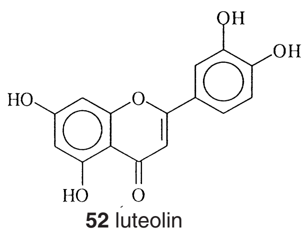
10.4.3 Oregano (*Origanum vulgare* L)

Oregano is very often used as a spice and its flavour is very popular with consumers all over the world. It is valued also for its antimicrobial and antioxidant properties. Dry oregano as well as extracts obtained by using solvents of different polarity (hexane, dichlormethane, methanol) have been tested as retarders of lipid oxidation in model systems or in real food products.[77,79,97,184] Abdalla and Roozen[150] reported that oregano acetone extract was more active in sunflower oil than in its 20 % oil-in-water emulsion during oxidation in the dark at 60 °C. Other species of oregano and its close relatives, e.g. *Origanum onites*, *Satureja thymbra*, *Coridothymus capitatus*, *Origanum dictamnus* were also investigated.[185–187]

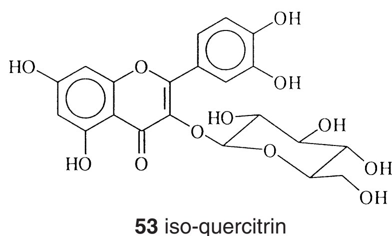
The water-soluble fraction of the methanol extract of oregano leaves was purified with polyamide chromatography to give five polar compounds[188,189] – rosmarinic acid, caffeic acid, protocatechuic acid **61**, a new glucoside of protocatechuic acid **62**, and a derivative of rosmarinic acid, 2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxy]phenylpropionic acid **63**. In the ethyl ether layer from the ethanol extract Vekiari et al.[184,190] identified four flavonoids – apigenin **64**, eriodictyol **65**, dihydroquercetin **66**, and dihydrokaemferol **67**. In the hexane extract α-, β-, γ- and δ-tocopherols were found.[186]



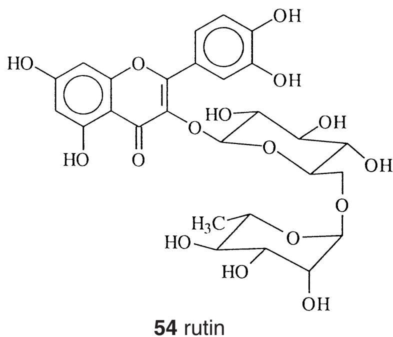
51 chrysoeriol



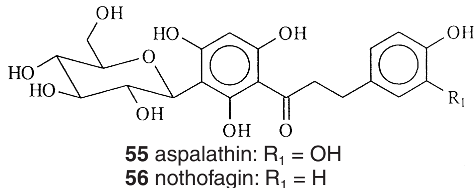
52 luteolin



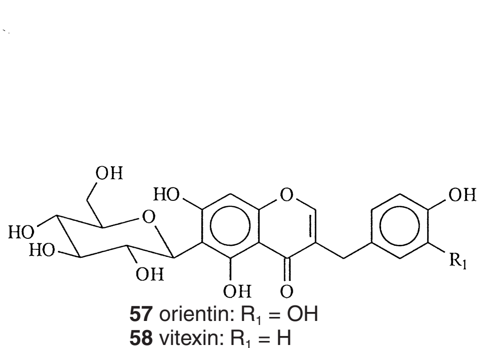
53 iso-quercitrin



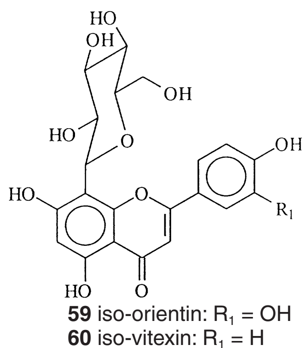
54 rutin



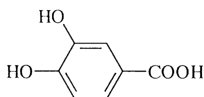
55 aspalathin: R_1 = OH
56 nothofagin: R_1 = H



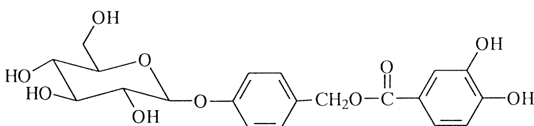
57 orientin: R_1 = OH
58 vitexin: R_1 = H



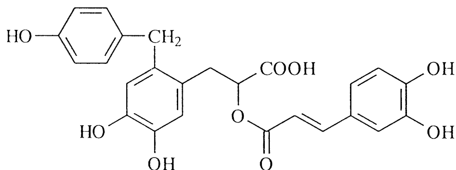
59 iso-orientin: R_1 = OH
60 iso-vitexin: R_1 = H



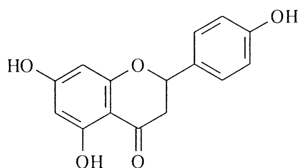
61 protocatechuic acid



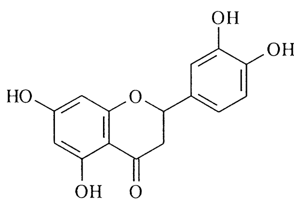
62 glucoside of protocatechuic acid



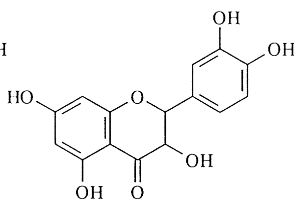
63 2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxy] phenylpropionic acid



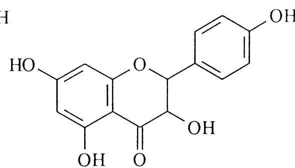
64 apigenin



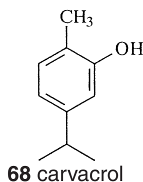
65 eriodictyol



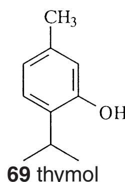
66 dihydroquercetion



67 dihydrokaemferol



68 carvacrol

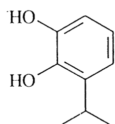


69 thymol

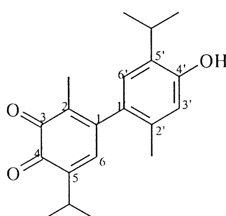
The essential oil obtained from oregano contained carvacrol **68** and thymol **69**.[185,191] According to Lagouri et al.[185] the antioxidative effect of oregano may be related to the presence of these isomers. The authors found that they are equally effective on the autoxidation of lard at 37 °C. Yanishlieva et al.[192] established that thymol and carvacrol differed in the mechanism of their inhibiting action at room temperature which depended on the character of the lipid medium. Thymol was a better antioxidant in triacylglycerols of sunflower oil (TGSO) than in triacylglycerols of lard (TGL).

10.4.4 Thyme (*Thymus vulgaris* L)

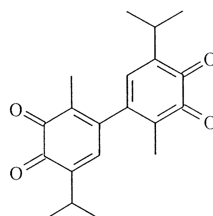
Thyme comes originally from the regions around the Mediterranean and is used as cough medicine. It has also been commonly used as one of the culinary herb spices for adding flavour and deodorising. The phenolic monoterpenes in thyme, thymol and carvacrol, are the primary compounds



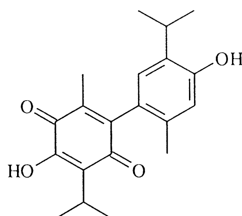
70 *p*-cumene-2, 3 -diol



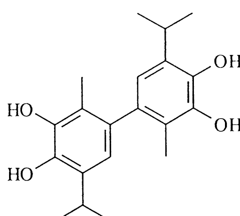
71 4'-hydroxy-5,5'-diisopropyl-2, 2'-dimethylbiphenyl-3,4-dione



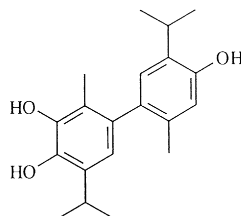
72 5,5'-diisopropyl-2,2'-dimethyl biphenyl-3,4,3',4'-tetraone



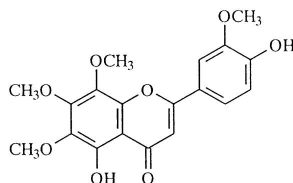
73 4,4'-dihydroxy-5,5'-diisopropyl-2,2'-dimethylbiphenyl-3,6-dione



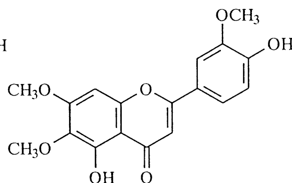
74 3,4,3',4'-tetrahydroxy-5,5'-diisopropyl-2,2'-dimethylbiphenyl



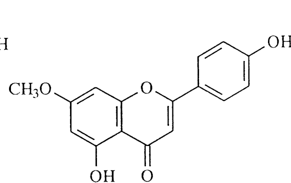
75 3,4,4'-trihydroxy-5,5'-diisopropyl-2,2'-dimethyl-3,6-biphenyl



76 5,4'-dihydroxy-6,7,8,3'-tetramethoxyflavone



77 5,4'-dihydroxy-6,7,3'-trimethoxyflavone



78 5,4'-dihydroxy-7-methoxyflavone

which contribute to the characteristic aroma of its essential oil.[76] They are also known to inhibit lipid peroxidation.[191–194] *p*-Cumene-2,3-diol **70** isolated from thyme is also a strong antioxidant.

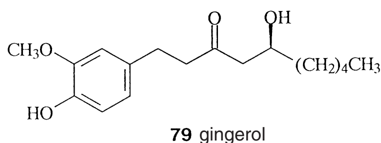
Antioxidative activity was found in the weakly acidic fraction, which was repeatedly purified by chromatography to give five new biphenyl-dimers of thymol and carvacrol **71–75**,[195] and highly methoxylated flavonoids **76–78**.[196] The biphenyls also possessed significant deodorant properties.[197]

10.4.5 Ginger (*Zingiber officinale*) and turmeric (*Curcuma domestica* L)

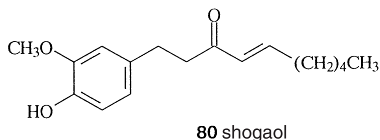
The rhizome of the popular ginger species, *Zingiber officinale* is currently widely used as a spice and food seasoning due to its sweet aroma and pungent taste. It has been known to have antioxidant activity.[198,199] The nonvolatile fraction of dichloromethane extract of dried ginger was purified by CC (column chromatography) and HPLC (high performance liquid chromatography) to yield more than 30 compounds, 16 of which were new.[200] These com-

pounds were structurally classified into gingerol-related compounds and diarylheptanoids (compounds **79–92**), and their structure–antioxidant activity relationship in an aqueous ethanolic solution of linoleic acid was examined.[200,201] The pungent components, gingerol **79**, shogaol **80** and zingerone **81** were reported to show a high activity.[202]

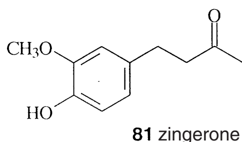
The dried rhizome of turmeric is widely used as a spice, as a colouring agent and as a folk medicine. The yellow pigment curcumin **92** and



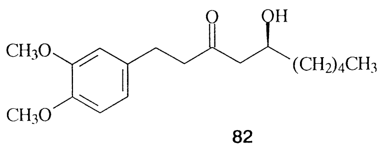
79 gingerol



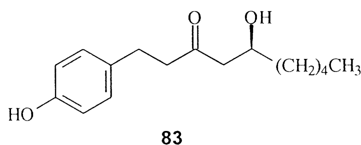
80 shogaol



81 zingerone



82



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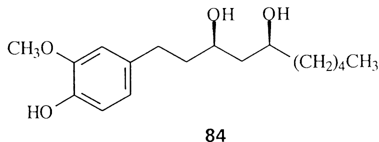

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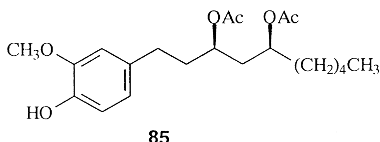

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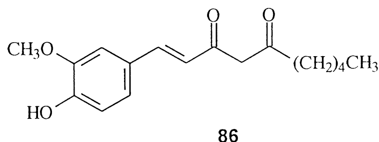

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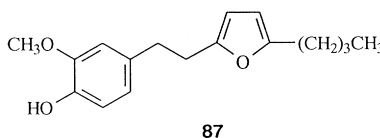

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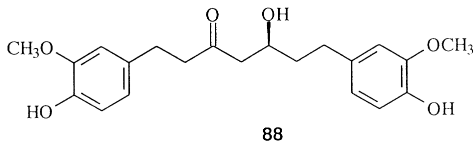

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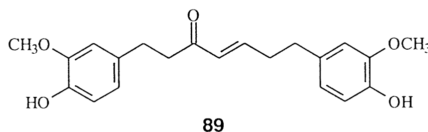

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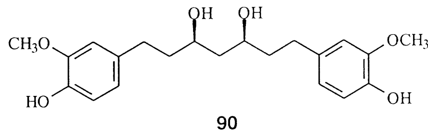

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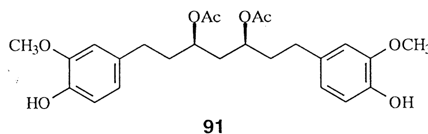

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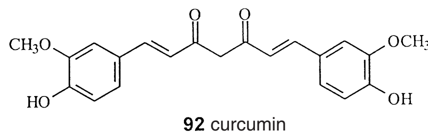
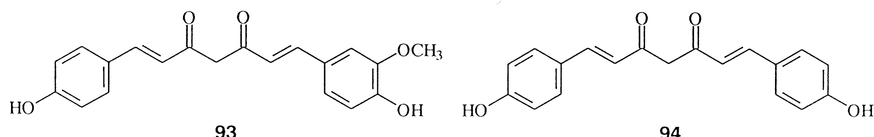
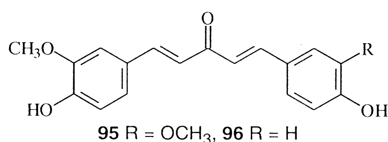


92 curcumin

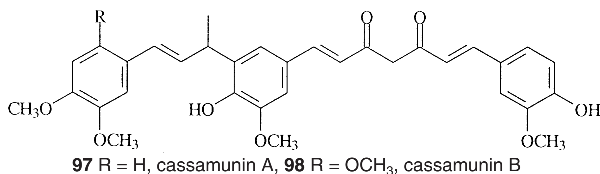
demethoxylated curcumins **93–102** are known to possess potent antioxidant activity.[200,201] Curcumin suppressed the oxidation of methyl linoleate in organic homogeneous solution and aqueous emulsions, soybean phosphatidylcholine liposomal membranes and rat liver homogenate induced by free radicals.[203] A mechanism for the dimer production is proposed and its relation to curcumin's antioxidant activity is discussed.[204] The results



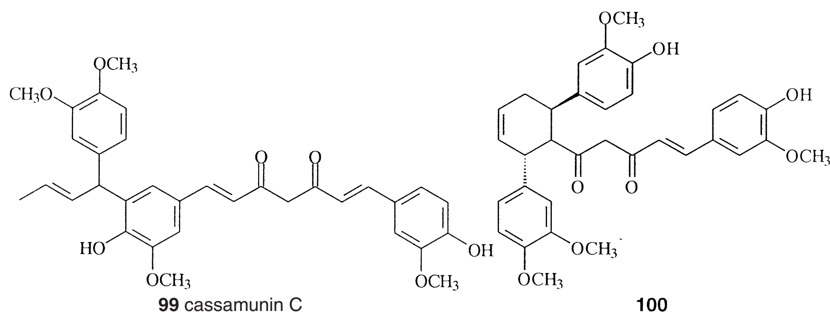
93 **94**



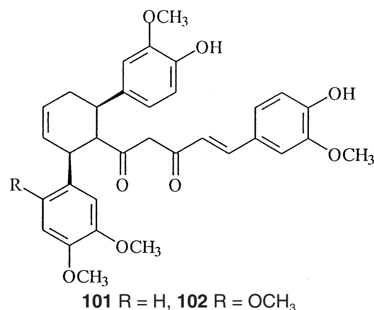
95 R = OCH_3, **96** R = H



97 R = H, cassamunin A, **98** R = OCH_3, cassamunin B



99 cassamunin C **100**



101 R = H, **102** R = OCH_3

indicated that the dimer is a radical-terminated product formed during the initial stage of the process.[204]

Masuda and Jitoe[205] reported that the antioxidant activity of cassmunins A–C was stronger than that of curcumin as was their anti-inflammatory activity. Jitoe et al.[199] studied the relationship between antioxidant activity of nine tropical ginger acetone extracts and their curcuminoid quantities in a linoleic acid–ethanol system. The data indicated that the antioxidant activities of the ginger extracts were greater than that estimated from the quantity of curcuminoids found in the extracts. The extracts of fresh ginger showed higher activity than those of stored ginger.[206]

10.4.6 Summer savory (*Satureja hortensis* L)

Summer savory is an annual culinary herb widely used in the food industry.

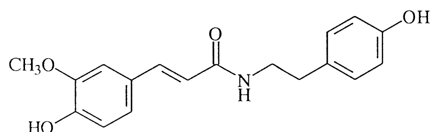
Yanishlieva and Marinova[207] established that the ethanol extract of the spice exhibited a good antioxidative effect in TGSO at 100 °C, as well as in commercial sunflower oil.[208] The effect of the extract in lard is stronger at room temperature than at 100 °C.[209] Addition of 0.1–0.5 % of the ethanol extract to sunflower oil decreased the oxidative and thermal changes in it during simulated deep fat frying.[210] The effect of *c.* 200 ppm of freeze-dried methanol extract of summer savory, added to an oil-in-water emulsion dressing, was comparable to the effect of PG.[98] The antioxidative effect found for dark storage changed to a pro-oxidative effect during light exposure (850 lx). The chlorophyll present in summer savory is believed to have acted as an efficient sensitiser causing an acceleration of the oxidation process.[98]

Antioxidative compounds isolated from summer savory are rosmarinic acid,[211,212] carnosol, and carnosic acid,[213] carvacrol and thymol in the essential oil.[191] Portugese savory oil does not contain thymol.[214] Carvacrol and thymol were also found in the essential oils of two other species of *Satureja*: *S. montana*[215] and *S. spicigera*.[216] Carvacrol was isolated from the leaf oil of *Satureja odora* and *Satureja parvifolia*.[217]

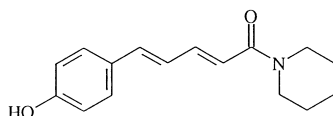
10.4.7 Other herbs and spices

Supercritical carbon dioxide extracts of ground black pepper (*Piper nigrum* L) have been found to be superior in reducing lipid oxidation of cooked ground pork.[218] The antioxidative activity of black pepper can, at least partially, be ascribed to the presence of glycosides of the flavonoids kaemferol, rhamnetin and quercetin,[219] as well as to the phenolic amides **103–107**.[220]

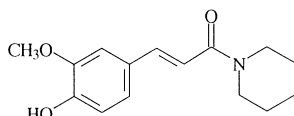
Chilli pepper (*Capsicum frutescence* L) and red pepper (*C. annum* L) contain capsaicin **108**, a punget principle showing significant antioxidative properties. A new antioxidant, capsaicinol **109**, was isolated from chilli



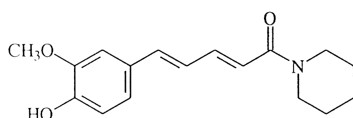
103 *N*-feruloyl tyramine

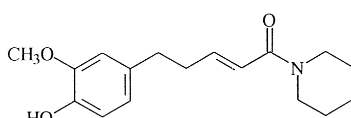


104 coumaperine



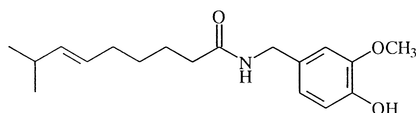
105 *N-trans*-feruloyl piperidine



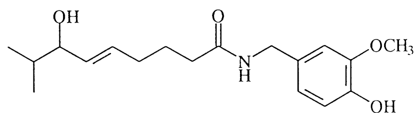


106 *N*-5-(4-hydroxy-3-methoxyphenyl)-2*E*, 4*E*-pentadienoyl piperidine

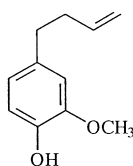
107 *N*-5-(4-hydroxy-3-methoxyphenyl)-2*E*-pentenoyl piperidine



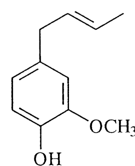
108 capsaicin



109 capsaicinol



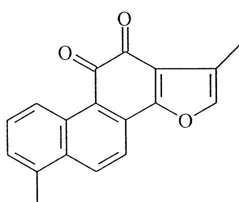
110 eugenol



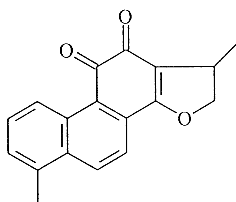
111 isoeugenol

pepper.[76] Marcus et al.[221] reported recently the change in antioxidant content in red pepper (paprika) as a function of ripening and some technological factors.

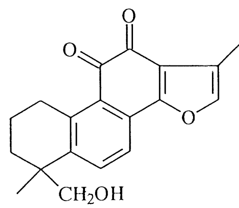
Gallic acid and eugenol **110** have been identified as the major active components in clove (*Eugenia caryophyllata*).[222] It has been established that isoeugenol **111**, more rarely found in nature, exhibited higher antioxidative efficiency than eugenol during methyl oleate,[223] and lard and sunflower oil[224] oxidation. Eugenol and isoeugenol also have an inhibiting effect on the peroxidation of lecithin induced by the Fe^{2+}-H_2O_2 system.[225]



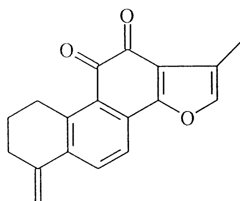
112 tanshinone I



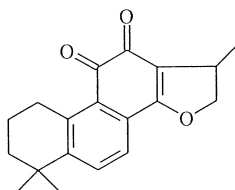
113 dihydrotanshinone I



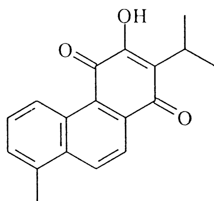
114 tanshinone II_B



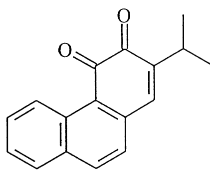
115 methylene tanshinquinone



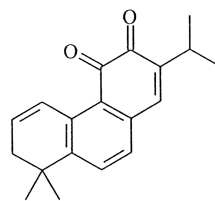
116 cryptotanshinone



117 danshenxinkun B



118 miltirone I



119 dehydrorosmariquinone

Part of the clinical effect of tanshen (*Salvia miltiorrhiza* Bunge), a widely used medical plant in China, could be related to its antioxidative properties. Powdered tanshen has strong antioxidative activity in lard, the hexane extract being very effective both at 100 °C and 180 °C[226] showing a synergistic effect with citric acid.

The antioxidative principles of tanshen are the compounds tanshinone I **112**, dihydrotanshinone I **113**, tanshinone II_B **114**, methylene tanshinquinone **115**, cryptotanshinone **116**, danshenxinkun B **117**, miltirone I **118**, rosmariquinone, dehydrorosmariquinone **119**.[227,228] Tanshen quinones are thought to capture lipid radicals to form stable radicals, thus interrupting the autoxidation chain process.[228]

Extracts of licorice (*Glycyrrhirra glabra* L) also possess antioxidant activity.[229,230] The antioxidant effect of licorice phenolics is well known.[231] Gordon and An[230] isolated eight purified flavonoids from the solvent extracts of licorice and studied their antioxidant effectiveness in lard at 100 °C. The authors supposed a synergistic effect of the flavonoid mixture may be responsible for the high activity observed. Different flavonoids were also

isolated from the extract of the sage *Salvia nemorosa* L,[232] an aromatic perennial herb of the southern European region, and from the extracts of *Anthriscus sylvestris*,[233] a Serbian plant species used in folk medicine and in salad dressing. The antioxidant extracts prepared from the African spice *Aframomum danielli* were more effective than BHT and α-tocopherol in stabilising refined peanut oil.[234]

Numerous other herbs and spices, or spice vegetables, have been shown to possess antioxidative properties, e.g. marjoram,[82,92,235–241] basil,[140,207,208,237,239,242] dittany,[238] peppermint,[191,207,208,236,238] spearmint,[207,208,237,243] common balm,[207,208,244] allspice,[92] fennel,[245–248] nutmeg,[92,238] caraway,[240] cinnamon,[225,238,240,248,249] bay,[238] dill,[238] parsley,[238] coriander,[238,243,250] cumin,[251] garlic,[92,252,253] hyssop,[235,241] and juniper.[254]

The main antioxidative compounds and types of compound isolated from herbs, spices and teas are listed in Table 10.7. The flavonoids, and especially flavonol glycosides, are to be found in nearly all herbs and spices tested.[255]

Even though strong antioxidant activities of many plant extracts have been reported, the need for novel natural antioxidants is obvious, and industry continues to look for them. Kim et al.[256] studied the antioxidant activities of methanol extracts of 180 oriental herbs during linoleic acid storage at 50 °C. Strong antioxidant activities in methyl linoleate emulsion at 40 °C were shown by 44 species. It was also established that the antioxidative properties of most herb extracts were greatly dependent on the extraction solvent used.

Recently, more than 700 Chinese medicines, herbs and spices were screened for natural antioxidants.[257] Among them, 64 were found to possess obvious antioxidant effectiveness ($F = 2$–4), and 24 to have strong antioxidant effectiveness ($F > 4$).

10.4.8 Comparison of the antioxidative effects of various herbs and spices

A large number of reports concerned with the antioxidative activity of herbs, spices and teas have been published. Comparison of the results is complicated by several factors. Different activities are found for whole plant material and the extracts. Antioxidant activity varies according to the country in which the plant was grown.[92] The early research also recognised that the antioxidative activity of herbs, spices and teas, or of their extracts, depends on the substrate used in the evaluation. Chipault et al.[77,78] reported that rosemary and sage were remarkably effective antioxidants and that oregano, thyme, nutmeg, mace and turmeric also retarded the oxidation of lard. In an oil-in-water emulsion, clove showed extremely high antioxidant activity, and to a lesser degree so did turmeric, allspice, mace, rosemary, nutmeg, ginger, cassia, cinnamon, oregano, savory and sage. Some of the results, obtained later, on the relative antioxidant activity of various herbs, teas and spices in different substrates and at different oxidation conditions are summarised in Table 10.8.

Table 10.7 Antioxidants isolated from herbs, spices and teas

Species	Systematic names	Substances and type of substances	References
Rosemary	*Rosemarinus officinalis*	Carnosic acid, carnosol, rosmarinic acid, rosmanol	104, 132, 140
Sage	*Salvia officinalis*	Carnosol, carnosic acid, rosmanol, rosmarinic acid	141, 148, 152 183
Green tea	*Camelia sinensis*	Catechins	169
Black tea (fermented tea)	*Camelia assamica*	Theaflavins, thearubigins	169
Oregano	*Origanum vulgare*	Derivatives of phenolic acids, flavonoids, tocopherols	184, 186, 189
Thyme	*Thymus vulgaris*	Thymol, carvacrol, *p*-cumene- 2,3-diol, biphenyls, flavonoids	195, 196
Ginger	*Zingiber officinale*	Gingerol-related compounds, diarylheptanoids	200
Tumeric	*Curcuma domestica*	Curcumins	205
Summer savory	*Satureja hortensis*	Rosmarinic acid, carnosol, carvacrol, thymol	191, 212, 213
Black pepper	*Piper nigrum*	Phenolic amides, flavonoids	219, 220
Red pepper	*Capsicum annum*	Capsaicin	76
Chili pepper	*Capsicum frutescence*	Capsaicin, capsaicinol	76
Clove	*Eugenia caryophyllata*	Eugenol, gallates	222
Tanshen	*Salvia miltiorrhiza*	Tanshenquinones	227, 228
Marjoram	*Majorana hortensis*	Flavonoids	235
Common balm	*Melissa officinalis*	Flavonoids	244
Licorice	*Glycyrrhiza glabra*	Flavonoids, licorice phenolics	230, 231

The observation that the more polar antioxidants are more active in pure lipids, and non-polar antioxidants most active in a polar substrate, e.g. oil-in-water emulsion, and for which the term 'polar paradox' has been introduced,[145] may at least partially explain the variation of antioxidative activity for different herbs and spices in different foods. It is therefore necessary to emphasize the evaluation of antioxidative activity in heterogenous model systems and in the actual food products prior to practical use. A three-step procedure for evaluation of spices was suggested: (a) determination of radical scavenging using ESR spectroscopy, (b) test in model systems, (c) final test in food storage experiments.[261]

Table 10.8 Relative antioxidative effectiveness (RAE) of spice extracts

Extraction method	Substrate, conditions	Analytical method	RAE	References
Commercial product	Lecithin emulsion, daylight, room temperature, 26 days	PV, TBARS, AV	rosemary >sage>nutmeg	258
Commercial product	Lard, 50 °C	PV, AV	rosemary>sage>marjoram> mace >black pepper	259
Suspension in peanut oil, followed by molecular distillation	Chicken fat, 90 °C	O_2 uptake	sage>rosemary	104
Oleoresin – commercial product	Methyl linoleate, 100 °C	Gas chromatography	sage>deodorized rosemary>untreated rosemary	260
Methanol	Lard, 75 °C	PV	oregano>thyme>dittany>majoram> spearmint>lavender>basil	237
Ethanol	TGSO, 100 °C	PV	summer savory>peppermint> common balm>spearmint >oregano>common basil	207
Ethanol	Low-erucic rapeseed oil, 60 °C, 23 days	PV	sage>thyme>oregano>juniper	254
Methanol	Methanol	Scavenging effect of DPPH radical, H_2O_2 and O_2^-	clove>oregano> cinnamon=marjoram>caraway	240
Dichloromethane	Lard	PV, AV	ginger>clove>pepper> cinnamon>fennel	248
Dichloromethane	Peanut oil	PV, AV	ginger>cinnamon>clove >pepper>fennel	248
Ethanol	Minced chicken meat, 4 ° and –18 °C	TBARS	caraway>wild majoram >cinnamon	91
Ethanol	Raw pork meats, pretreated with NaCl, 4 °C and –18 °C	TBARS	sage>basil>thyme>ginger	91
Ethanol	Microwave cooked pork patties treated with NaCl, –18 °C	PV	ginger>basil=thyme	91

PV = peroxide value, AV = aldehyde value, TBARS = thiobarbituric reactive substances

It was also found that there was a reduced antioxidant activity in extracts prepared from an equvalent amount of spice as opposed to that prepared from the whole spice, confirming that a wide range of compounds acting together are important as antioxidants in the plant material, which further may act synergistically.[77,98]

The ESR method based on the hydroxyl-generating system showed that the presence of spice extracts (basil, marjoram, hyssop, summer savory, oregano, sage) diminished the ESR signal, indicating that compounds in the extracts compete efficiently for the hydroxyl radicals.[239] The relatively high activity and hence small selectivity for all of the spices could be due to the fact that the hydroxyl radicals are very agressive.

Leafy spices like thyme, marjoram, basil, sage, summer savory all showed pro-oxidative activity for foods exposed to light,[98,262,263] while the same food stored in the dark confirmed the antioxidative effect of the spices. The effect of photosensitisation of chlorophyll present in spices may be more important than the effect of the antioxidants for food exposed to light. However, the balance between photosensitisation and antioxidative effect is very delicate and may depend on co-extraction of carotenoids, which may act as singlet oxygen quenchers.[264]

The essential oils from a number of herbs and spices were also studied for antioxidative activity, e.g. oregano,[185,238,250] rosemary,[238,250,265,266,269] sage,[250,265,266] clove,[238,265,266] coriander,[238,250] cumin,[238,265–267] fennel,[238,268] thyme,[265–268] marjoram,[238,269] laurel,[250,268] caraway,[238,265,266] peppermint, basil, cinnamon, nutmeg, dill, black pepper.[238] Although the compounds in the essential oils such as carvone from caraway, eugenol from clove, thymol from thyme and thujone from sage possess antioxidant activity, the aromatic character of these compounds limits the use of the essential oils as antioxidants in foods.[92]

The synergistic effect of various herbs and spices with synthetic antioxidants,[87] with citric acid,[77] and with α-tocopherol[111,129,179] has been investigated. Synergism has been observed between different spices and BHA,[270] the effect being most pronounced with sage, rosemary and mace. A very pronounced synergistic effect was seen between citric acid and rosemary extract.[77] No synergism has been detected between different spices apart from a few exceptions. The combination of thyme, marjoram, spearmint, lavender or basil with oregano revealed no synergism. Only a combination of thyme and marjoram or thyme and spearmint showed a slight synergism.[188]

10.5 Future trends

It is emphasised that the use of spices and herbs as antioxidants is a promising alternative to the use of synthetic antioxidants. In spite of scientific documentation of the antioxidative effect of many spices, herbs and teas, today it is mainly the extracts from leaves of rosemary and sage that are used

as antioxidative spice additives. A range of commercial products containing extracts of rosemary is available; some of the products are water dispersible, others are oil soluble and, in order to exploit the synergistic effect, some of them are combined with tocopherols. The results support the hypothesis that rosemary antioxidants regenerate oxidised α-tocopherol.

A determination of the reduction potential of key compounds would be of interest for determining which of the spice antioxidants can regenerate the tocopherols. The problems encountered because photosensitising chlorophyll co-extraction from the spice makes the food product sensitive to light also needs further investigation.

Although rosemary extracts and tocopherols are the most popular natural antioxidants on the market it is believed that tea extracts will become even more promising. Thyme and oregano extracts may also be used in the future.

More data on the active components in the plant materials will soon be available as the isolation of the antioxidant compounds from vegetables, fruits, berries, herbs, spices and teas is currently under way by several research groups.

10.6 Sources of further information and advice

Additional information on the antioxidative properties of various fruits, vegetables, herbs, spices and teas, as well as of the extracts and antioxidants isolated from them may be found in some recent reviews and books.[92,139,169,202,270–278]

The activities of spice and herb extracts could be increased by synergistic activity of other harmless antioxidants like soy, rapeseed or sunflower lecithin, ascorbyl palmitate, ammonium or amino acid salts of phosphatic acids, amino acids, lower peptides. The activities of the extracts are greater in food containing protein as the sulphur and amino groups of the polypeptide chain interact with hydroperoxides thus decreasing the free radical level.[278]

The stabilisation effect of the additives depends strongly on the composition of the complex lipid system and of the lipid-containing foods, as well as on the conditions of processing and storage (temperature, irradiation, partial oxygen pressure). That is why, prior to practical use in the food industry, any spice or spice extract should accordingly be tested in the actual food under realistic conditions. In experiments with foods, spices should be evaluated at concentrations which are accepted by the senses and with all interfering compounds present.

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Part 4

Practical applications

11

The regulation of antioxidants in food

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11.1 Introduction

In countries like the United States, Canada, Australia, many European countries and certain others food regulations have existed for many decades. Many countries have yet to formulate national food policies, responding appropriately to their health situation and economy, or, where these policies have been formulated, they often do not reflect appropriately the true nature and extent of current or emerging food safety problems. The advances in science and technology are important stimuli for modification of the laws.[1]

Because food is essential to life and can be improperly prepared or handled, it can threaten life. The purveyor of food therefore has a duty to provide safe and wholesome products to every customer. Given the fundamental importance of food, it is appropriate for any government to define and enforce this ethical obligation and thereby protect what many would consider the right of every individual to safe and wholesome food.

From the legal point of view, antioxidants are substances which prolong the shelf-life of foodstuffs by protecting them against deterioration caused by oxidation, such as fat rancidity, colour changes and loss of nutrient value. Hundreds of compounds, both natural and synthesised, have been reported to possess antioxidant properties. Their use in food, however, is limited by certain obvious requirements, not the least of which is adequate proof of safety.

Owing to differences in their molecular structure, the various antioxidants exhibit substantial differences in effectiveness when used with different types of foodstuffs and when used under different processing and

handling conditions.[2] The problem of selecting the optimum antioxidant or combination of antioxidants is further complicated by the difficulty of predicting how the added antioxidant will function in the presence of prooxidants and antioxidants already present in the food or produced in the course of processing.

11.2 Toxicological aspects

Since food additives are subjected to the most stringent toxicological testing procedures, only a few synthetic antioxidants have been used in foods for any length of time. Antioxidants are extensively tested for the absence of carcinogenity and other toxic effects in themselves, in their oxidised forms, and in their reaction products with food constituents, for their effectiveness at low concentrations, and for the absence of the ability to impart an unpleasant flavour to the food in which they are used.

Table 11.1 presents the most common antioxidants permitted for use in food products.

The use of antioxidants in food products is governed by regulatory laws of the individual country or by internal standards. Even though many natural and synthetic compounds have antioxidant properties, only a few of them have been accepted as 'generally recognised as safe (GRAS)' substances for use in food products by international bodies such as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Community's Scientific Committee for Food (SCF).

Toxicological studies are crucial in determining the safety of an antioxidant and also in determining the acceptable daily intake (ADI) levels. ADIs for widely used antioxidants such as BHA, BHT and gallates have changed over the years mainly because of their toxicological effects in various species.[3] Table 11.2 presents the ADIs allocated by JECFA.

Table 11.1 Antioxidants conventionally permitted in foods

ascorbic acid, sodium, calcium salts	glycine
ascorbyl palmitate and stearate	gum guaiac
anoxomer	lecithin
butylated hydroxyanisole (BHA)	ionox-100
butylated hydroxytoluene (BHT)	polyphosphates
tert-butyl hydroquinone[a] (TBHQ)	propyl, octyl, and dodecyl gallates
citric acid, stearyl, and isopropyl esters	tartaric acid
erythorbic acid and sodium salt	thiodipropionic acid, dilauryl and distearyl esters
ethoxyquin	tocopherols
ethylenediaminetetraacetic acid (EDTA) and calcium disodium salt	trihydroxy butyrophenone

[a] Not permitted for use in European Economic Community countries

Table 11.2 ADIs of some antioxidants permitted in foods

Antioxidant	ADI (mg/kg bw)
propyl gallate	0–2.5
BHA	0–0.5
BHT	0–0.125
TBHQ	0–0.2
tocopherols	0.15–2.0
gum guaiac	0–2.5
ethoxyquin	0–0.06
phosphates	0–70.0
EDTA	2.5
tartaric acid	0–30.0
citric acid	not limited
lecithin	not limited
ascorbic acid	not limited
sulphites (as sulphur dioxide)	0–0.7
ascorbyl palmitate or ascorbyl stearate (or the sum of both)	0–1.25

bw = body weight

The safety of the antioxidant must be established. According to Lehman et al.,[4] an antioxidant is considered safe if it fulfils two conditions: its LD_{50} must not be less than 1000 mg/kg body weight, and the antioxidant should not have any significant effect on the growth of the experimental animal in long-term studies at a level 100 times greater than that proposed for human consumption. Approval of an antioxidant for food use also requires extensive toxicological studies of its possible mutagenic, teratogenic and carcinogenic effects.

New toxicological data on some of the synthetic antioxidants cautioned against their use. In the recent past, natural antioxidants attracted the attention of many food manufacturers as a result of the necessity to produce healthy foods. Numerous antioxidative efficacious compounds that are found in animal or plant tissues and that are also available as synthetic molecules are used in several food applications. Herbs and spices occupy a special position in foods as traditional food ingredients and hence are appropriately used directly for their antioxidant characteristics. If they are applied to foods, they do not need to be declared as antioxidants.

Antioxidants should satisfy several requirements before being accepted for incorporation into food products.[5] The antioxidant should be soluble in fats; it should not impart a foreign colour, odour or flavour to the fat even on long storage; it should be effective for a least one year at a temperature of between 25 and 30 °C; it should be stable to heat processing and protect the finished product (carry-through effect); it should be easy to incorporate and it should be effective at low concentrations.

Antioxidants can be added directly to vegetable oils, melted animal fats or other fat-containing or polyphenol-containing systems. In some cases,

however, better results are achieved when the antioxidant is administered in a diluent (e.g. propylene glycol or volatile solvents). Food products can also be sprayed with, or dipped in solutions or suspensions of, antioxidants, or they can be packed in films containing antioxidants. The use of antioxidants is possible only if it is technologically substantiated and indispensable.

11.3 The *Codex Alimentarius*

The *Codex Alimentarius* is a collection of internationally adopted food standards presented in an uniform manner.[6] These food standards aim to protect the consumer's health and ensuring fair practices in the food trade. *The Codex Alimentarius* also includes provisions of an advisory nature in the form of codes of practice, guidelines and other recommended measures. Codex standards contain requirements for food including provisions for food additives.

The standards and limits adopted by the *Codex Alimentarius* Commission are intended for formal acceptance by governments in accordance with its general principles. *Codex Alimentarius* permits only those antioxidants which have been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for use in foods. Antioxidants may be used only in foods standardised by Codex. The antioxidant provisions of Codex Commodity Standards are included in and superseded by the provision of this Standard. Food categories or individual foods where the use of additives are not allowed or are restricted are defined by this Standard. The primary objective of establishing permitted levels of use of antioxidants in various food groups is to ensure that the intake does not exceed the acceptable daily intake (ADI).

The antioxidants covered by the Standard and their maximum levels of use are based in part on the food additive provisions of previously established Codex Commodity Standards, or upon the request of governments after subjecting the requested maximum levels to an appropriate method which would verify the compatibility of a proposed maximum level with the ADI.

Adequate information should be given about the manner in which the antioxidant is to be used in food. This information may be given on the label or in the documents relating to the sale. All antioxidants must be characterised by the name. The name should be specific and not generic and should indicate the true nature of the food additive. Where a name has been established for a food additive in a Codex list of additives, that name should be used. In other cases, the common or usual name should be listed or, where none exists, an appropriate descriptive name should be used. As an alternative to the declaration of the specific names the International Numbering System for Food Additives (INS) has been prepared by the Codex

Committee on Food Additives and Contaminants for the purpose of providing an agreed international numerical system for identifying food additives. It has been based on the restricted system already introduced successfully within the EEC. The INS is intended as an identification system approved for use in the member countries. The antioxidants reviewed in *Codex Alimentarius* are listed in Table 11.3.

In the *Codex Alimentarius* a number of further sequestrants and antioxidant synergists are issued which also pertain to the group of antioxidants. The main representatives of sequestrants are acetates, citrates, tartrates, phosphates, orthophosphates, diphosphates, triphosphates, and polyphosphates, esters of glycerol, sorbitol and sorbitol syrup, gluconates, and 1,4-heptonolactone. Lactates and tartaric acid are considered as antioxidant synergists. The spectrum of antioxidants cited in the *Codex Alimentarius* is very comprehensive. Individual countries set down their own diversified regulation of antioxidants with regard to local customs and habits, agricultural practices and potentials, economic considerations and technological advances.

11.4 The regulation of antioxidants in the European Union (EU)

EU regulation of antioxidants is stipulated by European Parliament and Council Directive No. 95/2/EC of 20 February 1995 on food additives other than colours and sweeteners.[7]

Only antioxidants which satisfy the requirements laid down by the Scientific Committee for Food may be used in foodstuffs. Having regard to the most recent scientific and toxicological information on these substances, some of them are to be permitted only for certain foodstuffs and under certain conditions of use. On the other hand, a wide range of natural antioxidants may be added to all foodstuffs, with the exception of formulae for special purposes (such as for infants), following the *quantum satis* principle.

The substances which can be used generally as antioxidants in food processing are listed in Table 11.4.

Antioxidants listed in Table 11.4 are permitted in foodstuffs with the exception of unprocessed foodstuffs, butter, pasteurised and sterilised milk, unflavoured live fermented milk products, and natural unflavoured buttermilk. Within the meaning of the directive, the term 'unprocessed' means not having undergone any treatment resulting in a substantial change in the original state of the foodstuffs (except, for instance, cutting, dividing, cleaning, peeling). In some foodstuffs only a limited amount of antioxidants from Table 11.4 may be used. These are listed in Table 11.5. Conditionally permitted antioxidants and related foodstuffs are listed in Table 11.6.

Table 11.3 List of antioxidants

INS	Name
220	sulphur dioxide
221	sodium sulphite
222	sodium hydrogen sulphite
223	sodium metabisulphite
224	potassium metabisulphite
225	potassium sulphite
226	calcium sulphite
227	calcium hydrogen sulphite
228	potassium bisulphite
300	ascorbic acid (L-)
301	sodium ascorbate
302	calcium ascorbate
303	potassium ascorbate
304	ascorbyl palmitate
305	ascorbyl stearate
306	mixed tocopherols concentrate
307	alpha-tocopherol
308	synthetic gamma-tocopherol
309	synthetic delta-tocopherol
310	propyl gallate
311	octyl gallate
312	dodecyl gallate
313	ethyl gallate
314	guaiac resin (gum guaiac)
315	isoascorbic acid (erythorbic acid)
316	sodium isoascorbate
317	potassium isoascorbate
318	calcium isoascorbate
319	tertiary butylhydroquinone (TBHQ)
320	butylated hydroxyanisole (BHA)
321	butylated hydroxytoluene (BHT)
322	lecithins
323	anoxomer
324	ethoxyquin
330	citric acid
384	isopropyl citrates
385	calcium disodium ethylenediaminetetraacetate
386	disodium ethylenediaminetetraacetate
387	oxystearin
388	thiodipropionic acid
389	dilauryl thiodipropionate
390	distearyl thiodipropionate
391	phytic acid
512	stannous chloride
539	sodium thiosulphate
1102	glucose oxidase

Table 11.4 Antioxidants generally permitted in foods

E number	Name	Maximum level
E 300	ascorbic acid	
E 304	esters of fatty acids with ascorbic acid	*quantum satis*
	(i) ascorbyl palmitate	
	(ii) ascorbyl stearate	
E 306	tocopherol-rich extract	
E 307	α-tocopherol	
E 308	γ-tocopherol	
E 309	β-tocopherol	
E 322	lecithins	
E 330	citric acid	
E 334	tartaric acid	

Table 11.5 Foodstuffs in which a limited amount of antioxidants generally permitted in foods may be used

Foodstuff	Antioxidant	Maximum level
Cocoa and chocolate products	E 330 citric acid	0.5 %
	E 334 tartaric acid	0.5 %
Non-emulsified oils and fats of animal or vegetable origin (except virgin oils and olive oils)	E 322 lecithins	$30\,g\,l^{-1}$
Refined olive oil, including olive pomace oil	E 307 α-tocopherol	$200\,mg\,l^{-1}$

There is a special position for sulphur dioxide and sulphites (Table 11.7), which are classified as preservatives but may be used as antioxidants when the risk of oxidation is greater than risk of microbial spoilage. Maximum levels are expressed as sulphur dioxide in mg/kg or mg/l as appropriate and relate to the total quantity available from all sources. Maximum levels range from 15 mg SO_2/kg to 2000 mg SO_2/kg depending on the foodstuff. Sulphur dioxide and sulphites may be used only for a limited number of foods and these are stipulated in the directive, e.g. dried vegetables and fruits, peeled and processed potatoes and white vegetables, sugars, fruit juices and concentrates, beer, wines, mustard, jams, jellies, pectin, gelatine, starches, toppings, dry biscuits etc. If the sulphur dioxide content is no more than 10 mg/kg or 10 mg/l it is not considered to be present.

The application of antioxidants in foods for infants and young children and for particular nutritional needs is regulated by Directive 89/398/EEC. Antioxidants permitted in weaning foods for infants and young children in good health are listed in Table 11.8.

Antioxidants should be labelled on the retail package with the specific chemical name or with the EEC number. The legislation of member states

Table 11.6 Antioxidants which are conditionally permitted in foods

E Number	Name	Food	Maximum level ($mg\ kg^{-1}$)
E 310 E 311 E 312 E 320 E 321	propyl gallate octyl gallate dodecylgallate BHA BHT	fats and oils (for heat treated foodstuffs)	200* (gallates and BHA individually or in combination)
		frying oil and frying fat (excluding olive pomace oil) lard; fish oil; beef, poultry and sheep fat	100* (BHT) both expressed on fat
		cake mixes cereal-based snack foods milk powder dehydrated soups and broths sauces dehydrated meat processed nuts seasonings and condiments pre-cooked cereals	200 (gallates and BHA, individually or in combination) expressed on fat
		dehydrated potatoes	25 (gallates and BHA, individually or in combination)
		chewing gum dietary supplements	400 (gallates, BHT and BHA, individually or in combination)
E 315 E 316	erythorbic acid sodium erythorbate	semi-preserved and preserved meat products	500 expressed as erythorbic acid
		preserved and semi-preserved fish products frozen and deep frozen fish	1500 expressed as erythorbic acid
E 385	calcium disodium ethylene diamine tetra-acetate (EDTA)	emulsified sauces	75
		canned and bottled pulses, legumes, mushrooms and artichokes	250
		canned and bottled fish, crustaceans and molluscs	75
		frozen crustaceans	75
		minarine	100
E 512	stannous chloride	canned and bottled white asparagus	25 as Sn

* When a combination of gallates, BHA and BHT are used, the individual levels must be reduced proportionally

Table 11.7 Sulphur dioxide and sulphites

E Number	Name
E 220	sulphur dioxide
E 221	sodium sulphite
E 222	sodium hydrogen sulphite
E 223	sodium metabisulphite
E 224	potassium metabisulphite
E 226	calcium sulphite
E 227	calcium hydrogen sulphite
E 228	potassium hydrogen sulphite

Table 11.8 Antioxidants permitted in foodstuffs for infants and young children

E Number	Name	Foodstuff	Maximum level
E 330	citric acid	weaning foods	*quantum satis*
E 300 E 301	L-ascorbic acid sodium L-ascorbate	fruit- and vegetable-based drinks, juices and baby foods	$0.3\,g\,kg^{-1}$
E 302	calcium L-ascorbate	fat-containing cereal-based foods including biscuits	$0.2\,g\,kg^{-1}$
E 304 E 306 E 307 E 308 E 309	L-ascorbyl palmitate tocopherol-rich extract α-tocopherol γ-tocopherol β-tocopherol	fat-containing cereals, biscuits, rusks, and baby foods	*$100\,mg\,kg^{-1}$ individually or in combination
E 322	lecithins	biscuits and rusks, cereal-based foods, baby foods	$10\,g\,kg^{-1}$

* 10 mg/kg for follow-on formulae for infants in good health

of the EU is influenced by the decision taken within the EEC. Some food standards are fully based on EEC directives and some still based on national considerations. There may be differences between European states, for instance, the utilisation of ascorbic acid as antioxidant for egg products is permitted in France but prohibited in Germany. These differences concern usually the utilisation of antioxidants in various food commodities. The specification of antioxidants mentioned in EEC directives are respected by all member states. But it is still generally required that individual countries of the European Community as well as the central organisation should be approached. The requirements appearing in the EEC directives on additives must be applied by the member states. This means in the first place that for those categories of additives for which a Commu-

nity positive list exists, member states may not authorise any additives which do not appear on the positive list.

These demands directly or indirectly affect other countries wishing to trade in Europe. In recent years also other European countries have assumed the regulation of additives from the EEC directive to be compatible in multinational markets. The countries of the Eastern block have developed new guidelines too, through association with the EU.

The use of food additives is restricted in all European countries by national order concerning food additives, only those antioxidants specially mentioned are allowed to be used. The antioxidants may only be used with the foods mentioned and in the amounts specified. The veterinary service and national food agencies or another national authorities have supervisory powers.

11.5 The regulation of antioxidants in the United States

In the United States antioxidant use is subject to regulation under the Federal Food, Drug and Cosmetic Act.[8] Antioxidants for food products are also regulated under the Meat Inspection Act, the Poultry Inspection Act, and various state laws. Antioxidants permitted for use in foods are divided into two groups:

1) The following antioxidants are restricted to use in the foodstuffs indicated:
   BHA
   BHT
   Ethoxyquin
   Gallates, dodecyl-, propyl-, octyl-
   Glycine
   Lecithin
   Resin guaiac
   TBHQ
   Tocopherols, α-tocopherol
2) Compositional standards where these exist may also restrict the use of antioxidants. Where no standard exists, antioxidants listed under 'non-standardised products' may be used subject to the conditions imposed. These include:
   Anoxomer
   Ascorbic acid, calcium ascorbate, sodium ascorbate
   Ascorbyl palmitate, ascorbyl stearate
   BHA
   BHT
   Erythorbic acid
   4-Hydroxymethyl-2, 6-di-*tert*-butylphenol

Propyl gallate
Stannous chloride
TBHQ (tertiarybutylhydroquinone)
THBP (trihydroxybutyrophenone)
Thiodipropionic acid, dilaurylthiodipropionic acid
Tocopherols, tocopherol acetate

In general, the total concentration of authorised antioxidants added singly or in combination must not exceed 0.02% by weight based on the fat content of the food. Certain exceptions exist in the case of standardised foods and products covered by special regulations. Under the Meat Inspection Act, concentrations up to 0.01% are permitted for single antioxidants based on fat content, with a combined total of no more than 0.02%. The tocopherols and the major acid synergists are unregulated.

Antioxidants that may be used in foods that are subject to a standard of identity are laid down in the relevant standard (Table 11.9). In the case of food that is not subject to a standard of identity, additives must be used in accordance with the conditions or limits of use specified in regulations.[9] For some types of foodstuffs which have standards of identity, the use of antioxidants is not permitted. No antioxidants may be used, for instance, in dairy products, ices, and egg products.

For non-standardised products tocopherols, tocopherol acetate, ascorbic acid and its sodium and calcium salts, ascorbyl palmitate, ascorbyl stearate, erythorbic acid and stannous chloride (max. 0.0015% calculated as tin) may be used according to good manufacturing practice.

Propyl gallate, BHA, BHT, TBHQ, THBP, 4-hydroxymethyl-2,6-di-*tert*-butylphenol, thiodipropionic acid and dilaurylthiodipropionic acid may be used provided that the total antioxidant content does not exceed 200 $mg\,kg^{-1}$ of the fat or oil content when used according to good manufacturing practice. Anoxomer may be used provided that the total antioxidant content does not exceed 5000 $mg\,kg^{-1}$ of the fat or oil content of the food.

Petitions for the use of a new food additive may be submitted to the Food and Drug Administration (FDA) in accordance with the form specified in the regulations.

There are some differences in the regulation of US and EU antioxidants. The restrictions on synthetic antioxidants are more strict in EU, e.g. TBHQ, THBP, anoxomer, ethoxyquin, guaiac resin and derivates of thiodipropionic acid are not permitted there. On the other hand, sulphur dioxide and sulphites, citric and tartaric acid and their salts and salts of EDTA are not listed as permitted antioxidants in the US.

11.6 The regulation of antioxidants in Australia

In Australia each State and Territory used to have their own food legislation.[8] However, in 1987 the National Health and Medical Research Council

Table 11.9 Standardised products in which a limited number of antioxidants may be used

Foodstuff	Antioxidant	Maximum level (mg kg^{-1})
non-alcoholic beverages (from dry mixes)	BHA BHT	2 prohibited
dry mixes for beverages	BHA BHT	90 prohibited
chewing gum	BHA BHT propyl gallate	1000
animal fat, rendered animal fat plus vegetable fat, rendered	BHA BHT glycine propyl gallate guaiac resin TBHQ	100 singly or 200 combined TBHQ should not be used in combination with glycine, propyl gallate or guaiac resin
	tocopherols	300
margarine	propyl-, octyl-, or dodecyl gallates	200
	BHA BHT ascorbyl palmitate ascorbyl stearate TBHQ	TBHQ should not be used in combination with gallates, ascorbyl palmitate or stearate
shrimp, frozen raw breaded	BHA BHT ascorbic acid erythorbic acid ascorbyl palmitate calcium and sodium ascorbates tocopherols	200 (total content)
fruit butters, jams, jellies, preserves	ascorbic acid	1000
fruit, glazed, diced, dry	BHA	32
fruit nectars	ascorbic acid	150
potato granules potato flakes	BHA BHT	10 50
bacon, pump-cured	α-tocopherol	500
meats, dried	BHA BHT propyl gallate TBHQ	100 singly or combined TBHQ and propyl gallate should not be used in combination
	tocopherols	30 not to be used in combination with other antioxidants

Table 11.9 *Continued*

Foodstuff	Antioxidant	Maximum level ($mg\,kg^{-1}$)
meats, restructured	tocopherols	30
sausage, dry	BHA BHT propyl gallate TBHQ	30 singly 60 combined TBHQ and propyl gallate should not be used in combination
	tocopherols	30 not to be used with other antioxidants
sausages, fresh Italian sausage products, fresh beef patties pizza topping, raw and cooked meatballs, raw and cooked poultry and poultry products	NHA BHT propyl gallate TBHQ	100 singly 200 combined TBHQ and propyl gallate should not be used in combination
poultry and poultry products	tocopherols	300 (200 combined with other antioxidants except TBHQ)
	lecithins	*quantum satis*
breakfast cereal, dry	BHA BHT	50
chilli powder, paprika	ethoxyquin	100
desserts (from dry mix)	BHA	2
emulsion stabilisers	BHA BHT	200
flavouring substances	BHA	5000 based on oil content
mixes for desserts	BHA	90
yeast (active, dry)	BHA	1000

produced the Food Standards Code to bring harmonisation of food legislation throughout the country. The Imported Food Control Act requires that all imported foods comply with the Food Standards Code. In August 1991, the new National Food Authority (NFA) was established. NFA is now responsible for setting all food standards in Australia.

The following are considered as permitted antioxidants in all States:

Gallates (propyl-, octyl-, and dodecyl-, or any mixture thereof)
BHA
TBHQ
Lecithins (including phospholipids from natural sources)

Table 11.10 Permitted antioxidants for fats and oils

Group	Name	Maximum level (%)
essential oils	gallates	0.1
	BHA	0.1
	TBHQ	0.1
	tocopherols	
	lecithin	
	ascorbyl palmitate	
fats and oils, other table spreads	gallates	0.01
	BHA	0.02
	TBHQ	0.02
	tocopherol	
	lecithin	
	ascorbyl palmitate	

Tocopherols – tocopherols can be used with or without citric acid, malic acid, tartaric acid, lactic acid (singly or in combination)
Ascorbic acid and its sodium salt
Erythorbic acid and its sodium salt
Ascorbyl palmitate
BHT (only for walnut kernels, pecan nut kernels, vitamins A and D).

For the group of fats and oils the antioxidants listed in Table 11.10 are used. For fish and fish products (including prawns and shrimps), fruit and vegetable products (including raw peeled potatoes) and meat and meat products (corned, cured, pickled or salted and cooked) only ascorbic acid, erythorbic acid and their sodium salts may be used.

A mixed food containing one or more foods in which antioxidants are permitted may contain antioxidants in not greater amounts than is specifically allowed in the quantity of food or foods containing the antioxidant used in the preparation of the mixed food.

11.7 The regulation of antioxidants in Japan

There are certain legal requirements and standards which cover the manufacture, processing, storage and quality of some foodstuffs.[10] In general, these requirements do not cover composition in any detail except in the case of milk products.

There exists a list of permitted additives. This list is concerned only with chemical synthetics (substances obtained by a chemical reaction other than degradation). It means that the substances on the list are those which either

Table 11.11 Japanese restrictions on the use of antioxidants

Antioxidant	Limitation or restriction	Maximum permitted level ($mg\ kg^{-1}$)
butylated hydroxyanisole (BHA)[a]	butter	200
	fats and oils	200
	frozen fish, shellfish, and whale meat (for dipping solution)	1000
	mashed potato (dried)	200
	salted fish and shellfish	200
	dried fish and shellfish	200
butylated hydroxytoluene (BHT)[b]	butter	200
	chewing gum	750
	fats and oils	200
	frozen fish, shellfish, and whale meat (for dipping solution)	1000
	mashed potato (dried)	200
	salted fish and shellfish	200
	dried fish and shellfish	200
isopropyl citrate (as monopropyl citrate)	fats and oils	100
EDTA $CaNa_2$, EDTA Na_2 (as EDTA $CaNa_2$)[c]	canned or bottled soft drinks	35
	canned or bottled food (except for soft drinks)	250
erythorbic acid	only for antioxidant use	
sodium erythorbate	only for antioxidant use	
nordihydroguaiaretic acid	butter	100
	fats and oils	100
propyl gallate	butter	100
	fats and oils	100
resin guaiac	butter	1000
	fats and oils	1000
(±) – α-tocopherol	only for antioxidant use	

[a] if used in combination with BHT, total amount of both antioxidants must not exceed permitted level
[b] if used in combination with BHA, total amount of both antioxidants, except for chewing gum, must not exceed permitted level
[c] to be chelated with calcium ions before addition to the food

do not occur naturally or are not obtained from natural sources. Of the substances which are not on the list it is not always possible to decide whether these may be used in food. The antioxidants and foodstuffs in which a limited amount of antioxidant is permitted are given in Table 11.11. Only

the above-mentioned foods may contain antioxidants, except α-tocopherol which may be generally used in foods as an antioxidant.

11.8 Future trends

Synthetic antioxidants such as BHA, BHT and gallates were introduced in the 1940s. In recent years, there has been an enormous demand for natural antioxidants mainly because of adverse toxicological reports on many synthetic compounds. Thus, most of the recent investigations have been targeted towards identification of novel antioxidants from natural sources. Plant phenolic compounds such as flavonoids, sterols, lignanphenols, and various terpene-related compounds are potent antioxidants. Since the antioxidant activities of natural extracts and compounds have been determined by a wide range of methods and varying endpoints, it has also become increasingly difficult to make a realistic assessment of the efficacy of various natural antioxidants. There is an urgent need for standardisation of evaluation methods in order to obtain meaningful information.[3]

Considerable efforts have also been made toward the development of novel compounds with superior antioxidant properties. Some attempts were also made to introduce new synthetic polymeric compounds which are nonabsorbable and nontoxic. These are generally hydroxyaromatic polymers with various alkyl and alkoxyl substitutions. Such compounds are usually very large molecules and their absorption from the intestinal tract is practically nil. In addition to their reportedly high antioxidant activity, they are nonvolatile under deep-fat frying conditions, which result in nearly quantitative carry-through to the fried items, but they have not yet received FDA approval.

Synthetic analogues or derivatives of α-tocopherol which have better antioxidant properties can be introduced. Many natural antioxidants such as flavonols, flavones, tea leaf catechins, rosemary antioxidants and spice extracts have been reported to be more active than BHA, BHT or the tocopherols in model systems. The food applications of these compounds need to be explored further.

The toxicological effects of food antioxidants have been the focus of controversy in recent years. Toxicological studies are mainly carried out to establish the no-effect level for an ADI for humans.

The laws must be modified to ensure the integration of these issues in determining the criteria for safety. Food safety will not only be an issue of protection, but also one of the maintenance and promotion of health. It also means that the regulatory and judicial system must be prepared to accept this expanded role for food in the matrix of national life and the international market. Major efforts are being made at the international level to obtain maximum coordination in regulation of principles for control and acceptance of new additives.

11.9 Sources of further information and advice

The selection of the most suitable antioxidant depends on the character of food and the targets which should be attained. Naturally occuring fats and oils contain indigenous antioxidants that protect the unsaturated lipids from free-radical destruction in their native vegetable and animal sources. On the other hand, fats and oils exist in a commingled fashion with reactive substances which cause their rapid decomposition. Intensity of oxidative alterations is also influenced by the shelf-life of products and storage conditions. All these facts should be considered when deciding whether any and if so what antioxidant will be used.

Further restriction is made by legislative regulation of antioxidants in foods. The most detailed information about permitted and recommended antioxidants may be seen in *Codex Alimentarius*. It is modernised on the basis of the actual knowledge of WHO. However various countries regulate the use of antioxidants by their own national legislation (see above). The divergences result from tradition, the composition of local diet and the boarding custom practices. The national regulations are usually established by the Ministry of Health or another state authority.

Lists of permitted antioxidants and foods in which antioxidants may be used are presented in national directives dealing with additives and contaminants and in duty tariffs. They are published in the special bulletins issued by the Ministry, European Parliament, FDA or analogous institutions and are publicly available. Nowadays they are often published on the Internet. These national regulations must be respected in international trade. They can be used or misused in the restraint on food exports and imports.

The food producer has full responsibility for the choice of suitable antioxidant and should obtain all information about the antioxidant from the data sheet (product information) that declares its safety. In most countries the antioxidants used in the product must be labelled on the package. Customers prefer foods with a minimum of additives as indicated by E number, and so the trend is directed towards mixtures of spices that contain antioxidants that do not need to be declared.

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12

The use of natural antioxidants in food products of animal origin

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12.1 Introduction

In this introduction, I will break my own cardinal rule of no first person referencing. However, when first asked to prepare the following review, I thought 'why not?' After all, lipid oxidation in meats had provided me with a doctorate dissertation and it was an area with which I had lost contact, other than at surface level. I needed to reacquaint myself with the literature and this was a perfect opportunity. What I found were some very exciting approaches to the problem that will have greater ramifications than just stabilising the meat lipids in the food system; not that this is not a good and noble goal. The greatest problem I faced was preparing this document, without exceeding the designated page limit, while presenting as broad a picture as possible. Therefore, in the meat area I stayed primarily with the most recent findings, i.e. since 1990, except for a historical perspective. This research has been divided into two categories. The first looks at how altering the meat's concentration of inherent/endogenous antioxidants (tocopherol and the cartenoids) can affect the meat's stability. In the second place, I wanted to update the role of exogenous natural antioxidants in preventing lipid oxidation in meat systems. In each of these two sections there is overlap and some work has been done to define any possible synergies between natural antioxidants in the meat system. Mechanisms/interactions as they have been defined have been presented.

Relative to the remainder of this document, i.e. dairy products, eggs and cholesterol, there was much less literature with which to work, since the addition of antioxidants is neither traditionally accepted nor needed in many of these foods. Again, I tried to stay with the most recent findings

while still presenting a view of what has been done with natural antioxidants in preventing lipid oxidation. Again, there has been some work done in defining the role of inherent compounds and on ways to increase their levels as a means of reducing the level of oxidative rancidity.

12.2 Control of lipid oxidation in meat products

12.2.1 Background

Since its naming by Timms and Watts in 1958, warmed over flavor (WOF), now also called meat flavour deterioration (MFD), has been a primary research issue for food scientists and the meat industry. There are many endogenous and exogenous factors that have been shown to affect WOF. Early research showed that this was a phenomenon related to the meat's phospholipids as opposed to its triacylglycerides.[1] In addition it also found that a primary catalyst to WOF was iron[2,3] and that during cooking there was a release of (free) iron from the heme compounds.[4]

Since these early findings, researchers have begun to establish the role of endogenous factors in the development of WOF. Some endogenous factors help to control oxidation and include the presence of compounds that are active antioxidants, i.e. dipeptides, tocopherols, etc. while others are enzymes capable of deactivating active oxygen species.[5] In contrast, pro-oxidants are also present in the cell; these can include free iron, ascorbic acid and active oxygen species. The final stability of the system is therefore dependent on the balance between these factors.[5] The balance or lack of balance translates into the fact that there are differences in oxidative stability between animal species and between muscle types within a species. For example, generally between species differences are that beef is the most stable, followed by pork, chicken, turkey and finally fish, and within a species such as poultry, the dark (thigh) meat is more susceptible to oxidation than the white (breast) meat.

Exogenous factors affecting the oxidative stability of meats include the level of processing, cooking technique/time, pre- and post-cooking storage time and temperature, packaging system, and/or use of antioxidants. When meat is processed the balance of the system becomes altered. Grinding disrupts the muscle tissue and allows mixing of the cell contents with oxygen; it also allows pro-oxidants access to the more unsaturated fatty acids in the membranal phospholipids. Increases in free iron have been found to result from cooking and during storage. During storage reducing agents (ascorbate and superoxide anion) can act to release the iron from its chelated state. Myoglobin is a major source of this released iron while ferritin has been shown to be less susceptible to releasing iron when denatured. The released iron is very active and in close proximity to the lipid substrate. Finally cooking also acts to destabilise the unsaturated fatty acids in the membranal phospholipids.

Control of oxidation in meat systems can occur in the raw or cooked meat system. In the raw meat system, factors affecting the levels of endogenous antioxidants have been receiving a great deal of attention in the literature in the 1990s. Prior to this research, most of the activity was focused on the application of exogenous antioxidants during processing. The remainder of this section will be divided to discuss most recent findings on the role of endogenous and exogenous antioxidants and combinations of both in controlling lipid oxidation in meat systems. Antioxidants are either lipid soluble (tocopherols and carotenoids) or water-soluble (ascorbic acid, dipeptides, and plant phenolics or polyphenolics; raw meat will also contain antioxidant enzymes).

12.2.2 Endogenous antioxidants

Since about 1990, increasing the levels of tocopherols and carotenoids in muscle tissue via dietary supplementation has shown strong promise for increasing the oxidative stability of muscle foods.

12.2.2.1 Beef

Because cattle are ruminants, it is difficult to alter their body composition through diet unless the ingredient is encapsulated to protect it from metabolism in the rumen. However, it is possible to increase the tocopherol content of beef muscle by feeding higher levels of tocopheryl acetate. Researchers[6–9] have shown that feeding 2000–3000 mg per day tocopheryl acetate to cattle for up to 135 days increased the tocopherol content of the resultant meat. Liu et al.[6] reported that after 126 days there was a six-fold increase in the tocopherol content of *gluteus medius* from supplemented *vs* non-supplemented animals. Galvin et al.[7] showed that muscle type responded differently relative to level of tocopherol content. In the *longissimus dorsi* (LM) muscle the level went from (basal diet) 0.84 to 2.45 $\mu g\,g^{-1}$ muscle, while in the *psoas major* (PM) muscle the level went from 2.45 to 5.78 $\mu g\,g^{-1}$ muscle. Higher levels fed longer times gave the greatest increase in muscle tocopherol levels. Lynch et al.[8] noted the threshold level of tocopherol needed in beef tissue to increase its stability was 3.5 $\mu g\,g^{-1}$ muscle.

When tocopherol-supplemented meat was cooked and evaluated for WOF, as reflected by TBARS (thiobarbituric reactive substances) values, studies[6,8] have shown there was a significant increase in oxidative stability. O'Grady et al.[9] compared fed tocopherol to tocopherol added during processing and found that dietary supplementation was more effective in controlling the stability of meat. Faustman et al.[10] showed that during oxidation in beef tissue microsomes, there was a decrease of α-tocopherol with an increase of its oxidation products, i.e. α-tocopherolquinone (TQ) and 2,3-epoxy-α-tocopherolquinone (TQE_2). This conversion of α-tocopherol to its respective oxidation products was consistent with it acting as a peroxy-radical scavenging compound.

Galvin et al.[7] also found that although tocopherol supplementation significantly reduced lipid oxidation, stabilising cholesterol against oxidation appeared to be influenced by muscle type. There was a supplementation-attributable reduction of cholesterol oxidation, i.e. 7-ketocholesterol production, in the PM but not in the LM samples. In vacuum packag meat, dietary tocopherol-treated meat showed less colour change, i.e. there was less metmyoglobin formation. However, O'Grady et al.[9] also found that in meat stored at different levels of oxygen, tocopherol-supplemented meat showed no differences in colour stability.

12.2.2.2 Pork

The stability of meat from monogastric animals is more easily affected by diet. Researchers have shown that increasing dietary tocopherol levels results in increased tocopherols in the meat tissue. Onibi et al.[11] fed pigs 35 day-diets with two levels of tocopheryl acetate (basal and basal +200 mg kg^{-1} diet). Two basal diets were used, i.e. soy-based *vs* full-fat rapeseed meal. The authors[11] found that there was a two–three-fold increase in muscle tocopherol content in the *longtissimus dorsi* muscle depending on the diet fed, i.e. larger differences were found in the soy-fed pigs. This was partially explained by the fact that there was about twice as much (40 *vs* 16 mg kg^{-1} diet) natural tocopherol in the unsupplemented rapeseed diet than in the unsupplemented soy diet.

Buckley et al.[12] investigated the effect of dietary vitamin E either short term (4 weeks prior to slaughter) or long term (10 weeks prior to slaughter) for its effects on membrane stability and meat quality. In addition, long term feeding of α-tocopherol alone *vs* mixed tocopherols was investigated. α-Tocopherol alone was much more effective than was feeding the mixed tocopherols, which essentially had no effect. It was felt that this was because the body absorbs the tocopherol isomers at different levels with $\alpha > \beta > \gamma > \delta$, resulting in less tocopherol in the meat when the mixture was fed. Tocopherol supplementation long term was more effective than short term in stabilising the lipids in pork microsomes and mitochondria to metmyoglobin/hydrogen peroxide mediated oxidation. In pork patties processed with and without salt and held at 4 °C in dark or in light, tocopherol supplementation, either short term or long term, had no effect on the stability of meat patties made without salt. However, when salt was used tocopherol supplementation acted to increase the oxidative stability of the meat patties.[12]

Monahan et al.[13] found a reduction in microsomal free radical production and lipid oxidation in tissue from pigs fed α-tocopherol (200 mg kg^{-1}) *vs* control pigs (10 mg kg^{-1}). Pork chops from the supplemented animals had lower susceptibility to oxidation during refrigerated storage. It was shown that tocopherol supplementation resulted in higher membrane levels of the vitamin and that there was suppression in the production of free radicals capable of initiating and/or propagating lipid oxidation.

Kingston et al.[14] evaluated the individual and combined effects of muscle vitamin E levels, cooking rates and final temperature and packaging on lipid oxidation in refrigerated cooked pork from pigs fed extra dietary vitamin E. Individual effects showed that higher muscle tocopherol levels ($4.24\,\mu g\,g^{-1}$ *vs* $0.97\,\mu g\,g^{-1}$) increased pork oxidative stability. Cooking at a faster rate ($2\,°C\,min^{-1}$ *vs* $0.3\,°C\,min^{-1}$) and to a lower final temperature (72 *vs* 82 °C) both reduced oxidation and vacuum packaging reduced oxidation. When tested for interactions it was found that any combination of these factors were more effective than any single factor.

Feeding oxidised corn oil (peroxide value PV = 300) at 2% of the diet for 10 weeks resulted in increased susceptibility of cellular microsomes and mitochondria to lipid oxidation as evidenced by TBARS being higher than the control.[14] Consuming oxidised oil might have provided a source of free radicals capable of destabilising subcellular membranal lipids. In contrast, Monahan et al.[13] fed oxidised lipids to pigs and evaluated their effect on iron-induced free radical production in the meat. In this case, including oxidised ($150\,meq\,kg^{-1}$ PV) corn oil had no effect on the lipid stability of the muscle lipids.

12.2.2.3 Poultry

The effect(s) of diet has been widely investigated in poultry; feeding different fats, tocopherols and/or carotenoids has been shown to have benefit in the final consumer-ready product. Feeding more saturated fats, such as coconut and olive oil, increases the oxidative stability of both thigh and breast meat from broilers.[15] Ruiz et al.[16] showed feeding broilers more unsaturated fat resulted in less stability in the meat. Lin et al.[15] noted that feeding different levels of saturated/unsaturated fat affected the meat's triglyceride more than phospholipid composition.

Renerre et al.[17] reported that feeding highly unsaturated fats (tallow *vs* soybean *vs* linseed oils) to turkeys increased the levels of antioxidant enzymes (catalase, superoxide dismutase, and glutathione reductase) in the thigh and breast meat, with the higher levels found in the thigh meat. Maraschiello et al.[18] fed broilers different fat sources (lard, sunflower and olive oil) and analysed for glutathione peroxidase (GSHPx) activity. Dietary fat affected the GSHPx activity, in that between sunflower and olive oil the more unsaturated sunflower oil resulted in the higher GSHPx activity. However, feeding lard produced the highest GSHPx activity.

Other dietary treatments, i.e. tocopherol and/or carotenoid supplementation have been studied for increasing the oxidative stability of poultry meat. When tocopherol is added via the diet it becomes incorporated in the subcellular membrane and is in position to be more effective in intercepting free radicals as they are formed. Lin et al.[15] showed that tocopherol supplementation increased the level of tocopherol in the meat and improved the lipid stability of both the dark and white broiler meat. Ahn et al.[19] found up to a three-fold increase in tocopherol content in turkey meat when the

dietary tocopherol content increased from 25 to 200 IU kg^{-1} diet. The tocopherol content of the thigh meat was about 50 % greater than the breast meat.

Ahn et al.[20] fed α-linolenic acid and mixed tocopherols alone or in combination to broilers. It was found that the α-linolenic acid supplementation increased the level of fatty acid unsaturation in the phospholipid fraction. Birds fed α-linolenic acid and tocopherol had the highest meat tocopherol levels with the greatest amount in the thigh tissue. α-Linolenic acid fed alone reduced tissue tocopherol content and this meat was most susceptible to oxidation. The authors[20] noted that it was possible to increase the ω-3 fatty acids content of poultry meat, but it may be hard to incorporate sufficient tocopherol to control the oxidation of the meat.

Ahn et al.[19] showed that supplementing turkey diets with vitamin E, above basal levels of 25 IU kg^{-1}, to 200, 400 or 600 IU kg^{-1} diet was effective in reducing oxidative stability in patties made from the thigh or breast meat. The patties were stored at 4 °C for up to 14 days, under vacuum or open to air, and in each case the tocopherol-supplemented meat was more stable. These authors also showed tocopherol supplementation reduced oxidation when the patties were irradiated with 0 or 2.5 kGy prior to cold storage. The best combination for longer shelf-life was tocopherol supplementation and vacuum packaging.

Addition of carotenoids to poultry diets has become popular since they give the finished carcass a 'golden' tone that is considered desirable by the consumer. This practice can have some stability implications. Work by Ruiz et al.[16] showed that adding β-carotene alone to broiler diet resulted in pro-oxidant activity in the meat. They further showed that some level of tocopherol was necessary for β-carotene to act as an antioxidant in the raw and cooked meat. The authors felt that work is needed to determine the optimum ratio of tocopherol to β-carotene for controlling lipid oxidation in poultry meats.

Canthaxanthin is another carotenoid that has been studied for its effect on poultry meat stability. Chickens were fed diets containing full-fat flaxseed and supplemented with mixed tocopherol and canthaxanthin.[21] Both tocopherol- and canthaxanthin-supplemented cooked meat was more stable during refrigerated storage. The best system was the combination of the two compounds. Tocopherol is known to act as a hydrogen donor; however, canthaxanthin can stop peroxy free radical chain propagation by trapping the radical in its conjugated polyene system.[16]

12.2.2.4 Fish

As indicated earlier, fish muscle is probably the most susceptible to oxidation, primarily because of the high level of unsaturation found in its lipids. Feeding sources of antioxidant compounds have also been studied as a means of increasing the oxidative stability of fish muscle. Canthaxanthins are naturally occurring carotenoids found in red fish tissue, such as salmon

and rainbow trout, and in cultivated fish achieving a colour similar to that found in the wild is important to consumer acceptance of the cultivated product. Sigurgisladottir et al.[22] fed salmon mixed tocopherols and found that there was an equilibrium in muscle tocopherol levels after 15 weeks of supplementation. It was also found that the fish incorporated more of the δ- and β-tocopherol isomers than they did the α- and γ-isomers. Akhtar et al.[23] reported that feeding $500\,mg\,kg^{-1}$ α-tocopherol in the diet resulted in a five-fold increase of tocopherol in rainbow trout muscle, and the muscle with the higher levels of tocopherol were more stable to oxidation.

Akhtar et al.[23] also reported that it was possible by using a surface application of a rosemary oleoresin on muscle from fish supplemented with tocopherol to enhance further the stability of rainbow trout muscle. Sant'Ana and Mancini-Filho[24] fed 100 ppm α-tocopheryl acetate, 100 ppm butylated hydroxytoluene (BHT) or 1.4 g rosemary extract (Herbalox®)/kg diet to freshwater fish and found differences in the lipid stability of the fish muscle in the order of rosemary > tocopherol > BHT > control. When the muscle was irradiated, the order of efficacy changed to BHT> tocopherol = rosemary > control.

Akhtar et al.[25] also fed tocopherol, canthaxanthin or oleoresin paprika alone or in combination. When vitamin E and canthaxanthin were both included in the diet, a strong antioxidant effect was found and this was attributed to the concept that these two compounds use different mechanisms to control lipid oxidation. α-Tocopherol is a hydrogen donor and can donate the hydrogen from its C-6 carbon while canthaxanthin acts to trap the peroxyl free radical in its conjugated polyene system. The oleoresin of paprika would also contain cartenoids and this treatment appeared to give equal protection as found with the canthaxanthin.[25] These authors also noted that canthaxanthin's effect could be due to its being converted to β-carotene, which has been shown to occur metabolically in rainbow trout.

Clark et al.[26] supplemented rainbow trout with canthaxanthin prior to processing into patties and showed that the amount of canthaxanthin deposited was critical if lipid stability was to be achieved. In a liposome system canthaxanthin delayed the formation of TBARS in a concentration-dependent manner.[23]

12.2.3 Exogenous antioxidants

Adding antioxidants during processing has been the more traditional technique used to control lipid oxidation in meats. Some of the added compounds are found at low levels in meats, i.e. ascorbic acid and carnosine, while others are derived from plants, i.e. phenolics/polyphenolics.

12.2.3.1 Carnosine

Meat tissue contains several dipeptides, i.e. carnosine, anserine and ophinine, that have been shown to have antioxidant activity.[27] Carnosine has

received the most attention relative to its ability to control lipid oxidation in meats. Carnosine (β-alanyl-L-histidine) was first identified in 1900 by Gulewitsch and Amiradzibi.[28] However, its antioxidant activity was not heavily investigated until the early 1990s. Since then carnosine has been shown to have many mechanisms by which it can affect the rate of lipid oxidation in meats.

Carnosine, added to salted and unsalted frozen and cooked ground pork, at high levels (1.5 %) was more effective in inhibiting WOF than 0.5 % sodium tripolyphosphate (STPP), α-tocopherol and BHT; the last two were added at 0.02 % of total fat. Its superiority to α-tocopherol and BHT indicated that carnosine was a better hydrogen donor than these two known donors and its superiority to STPP indicated that it could chelate metals.[29,30] However, a lower level (0.5 %) of carnosine added to these same systems was ineffective in the salted meats, indicating that salt interfered with its ability to act as an antioxidant and that carnosine's activity as a metal chelator was concentration dependent. Salt has been shown to be a powerful prooxidant in meat systems; however, exactly how salt acts to increase oxidation in these systems has not been totally defined. Studies have shown that salt can stimulate lipid oxidation through iron activation.[31] Chloride ions were reported to be involved in iron activation in mackerel muscle, while the sodium ions may act to displace the iron from the myoglobin creating free iron, which can catalyse lipid oxidation.[32]

The role of transition metal ions as lipid oxidation accelerators is well documented in the literature. Carnosine can act to inhibit iron and copper accelerated oxidation.[32] One of carnosine's roles in controlling iron -catalysed oxidation is by scavenging hydroxyl groups generated by the Fenton reaction. In addition, iron has been shown to catalyse lipid oxidation in muscle in the presence of ADP and NADPH and it has been theorised that this occurs via a reduction of the ferric into the ferrous ions. Ferrous ions are more powerful than ferric ions in catalysing the decomposition of peroxides into free radicals.[33]

Relative to copper-catalysed oxidation, carnosine can chelate copper but cannot chelate iron. Carnosine forms a tetramer with copper when its concentration is 100–1000 times more than that of the metal ion.[33] The formed tetramer involves binding of four molecules of carnosine to copper through the N-3 of the imidazole ring.[33] Although carnosine does form a complex with copper that appears to be unreactive, the complex may in fact still be catalytically active and able to form peroxy radicals.[33] The ability or lack of ability of carnosine to form a complex with copper and iron, respectively, is not affected by the oxidation state of the metal.[33] Lee et al. showed that carnosine was able to inhibit copper (II)-catalysed ascorbate oxidation.[34] In addition carnosine has been shown to inhibit lipid peroxidation catalysts including hydrogen peroxide activated haemoglobin, photoactivated riboflavin and lipoxygenase.[34] Lee and Hendricks[35] evaluated the antioxidant activity of carnosine in model systems. In the presence of ascorbic acid,

carnosine inhibited the iron-catalysed deoxyribose degradation in a dose-dependent manner. This effect indicates that carnosine can scavenge the hydroxyl radical.

Carnosine has been tested for its ability in the presence of other antioxidants. In a salted and unsalted ground turkey dark muscle, Calvert and Decker[36] tested carnosine (0.5 %) alone and in combination with α-tocopherol (0.05 %), ascorbyl palmitate (0.05 %), sodium tripolyphosphate (0.5 %), or citrate (0.01 or 0.05 %). An additive effect was found with the combination of tocopherol and carnosine in both salted and unsalted meat systems. The combination of carnosine and ascorbyl palmitate was effective, as evidenced by reduced TBARS, in the salted meat but not in the unsalted meat. When carnosine was combined with either the STPP or citrate, they did not enhance its activity.

A series of studies with phytic acid with and without carnosine have been reported. Lee et al.[37] evaluated carnosine and phytic acid for their effect on pH, meat colour stability, glycogen and oxidation levels in a fresh prerigor meat model system held at 4 °C for 30 days. They found that during the first 7.5 h of storage, carnosine- and phytic-treated systems had a more rapid decline in muscle pH and glycogen contents, with phytic acid having the greatest effect. During storage, the authors found that carnosine and phytic acid inhibited metmyoglobin production; carnosine kept the meat bright red while phytic acid maintained a purple-red color.[37] Both carnosine and phytic acid inhibited TBARS in the test systems in a dose-dependent manner with phytic acid having the greatest effect. Lee et al.[37] felt both of these compounds were chelating transition metals and preventing free radical production, which prevented myoglobin oxidation and kept TBARS low.

In another work, Lee and Hendricks[38] added phytic acid (0 to 2 mM) to beef homogenate and tested for lipid oxidation. They also evaluated the effect of pH on the activity of phytic acid and found that as the pH increased so did the antioxidant activity with the best being at neutral or higher pH. Phytic acid complexes metals in a dose-dependent manner and these complexes are insoluble over a wide pH range. Phytic acid can bind with six divalent molecules and the metal can bridge between two or more phytate molecules, creating a complex that is unable to participate in the Fenton reaction and thus reducing the production of hydroxyl radicals. A molar ratio of 0.25 phytate to 1 of iron is minimal for controlling the superoxide generation of hydroxyl radicals. Lee and Hendricks[39] also reported that phytate acted in a dose-dependent manner in controlling TBARS production in metal-catalysed model systems. It facilitated the conversion of Fe^{2+} to Fe^{3+} and prevented the reverse conversion of Fe^{3+} to Fe^{2+} by ascorbic acid.

Lee et al.[34] showed that carnosine was more effective than ascorbic acid in controlling lipid oxidation, as measured by TBARS, in refrigerated beef patties. When the patties were cooked, carnosine increased cook yield and

salt-soluble proteins more than ascorbic acid. This was attributed to the fact that carnosine increased the pH of the meat which subsequently increased the water-holding capacity and solubility of the salt-soluble proteins. Ascorbic acid levels (low *vs* high) have different effects on lipid peroxidation. Low levels accelerate oxidation while high levels do not. At low levels ascorbic acid could be facilitating the formation of a Fe^{2+}-O_2-Fe^{3+} complex which could initiate oxidation, while at high levels of ascorbic acid, it can reduce the ferric ions to ferrous ions and reduce lipid oxidation.[39] Muscle foods do not contain appreciable amounts of ascorbic acid; therefore, its addition to meat could be beneficial. However, ascorbic acid can be readily oxidised in the presence of copper and/or iron with copper (II) being about 80 times more reactive than iron (III). With carnosine being able to inhibit the copper (III)-catalysed ascorbate oxidation, the application of these two compounds to ground beef could add some strong benefits in maintaining the quality of fresh ground beef.

Lipid stability and cholesterol oxidation were monitored in salted chicken thigh meat from chickens fed tocopherol 200 mg kg^{-1} α-tocopheryl acetate and then treated with carnosine.[32,40] The meat was processed into patties to which carnosine (1.5 %), salt (1 %) or a mixture of both was added. The patties were held raw or cooked and then held refrigerated under fluorescent light. Including tocopherol into the diet or adding carnosine to the meat did reduce the oxidation of both the lipids and cholesterol. The combination showed the greatest level of protection.

12.2.3.2 Plant phenolics

Man has always relied on plant materials as part of his diet. Some plants such as herbs and spices were traditionally used to increase the palatability of the food. That these plant materials were also contributing to the safety and shelf-life of the food was not recognised to any great extent in the scientific literature until the late 1950s. The findings reported by Chipault and co-workers in 1957 and 1958 were hallmarks in what has now become the basis for the development of a more natural, healthier food supply. Plants from the family Labiatae, which includes rosemary, sage, thyme, oregano and the mints, to name a few, have shown strong promise for providing compounds capable of inhibiting lipid oxidation. Rosemary and sage have received the greatest amount of attention as potential inhibitors of WOF in meats and are usually found to be more effective than plants such as savory,[41] marjoram[42] and oregano.[43]

Rosemary in the 1970s was originally processed as an oleoresin that was sold to provide flavour to foods and 'by the way' it had antioxidant activity. The problem with this product was that it contained strong colour and strong flavour that was only acceptable in a limited number of foods, one of which was meats. However, since that time, new rosemary-based systems have been developed which are colourless and odourless; this expands their potential use in a wider variety of foods. But since the focus of this chapter

is animal-based foods, the following will only discuss findings in meat systems. Rosemary oleoresin has been shown to be as effective as polyphosphate and a combination of BHT/BHT/citric acid in mechanically deboned poultry meat[44] and in a breakfast sausage made from mechanically deboned poultry meat.[45] When combined with sodium tripolyphosphate (STPP), rosemary oleoresin (RO) was effective in controlling oxidation in restructured chicken nuggets.[46] However, Stoick et al.[47] reported that the combination of STPP/OR was not effective in restructured beef steaks. Liu et al.[48] also found that neither an oil-soluble RO nor a water-soluble RO was as effective as STP in controlling oxidation in restructured pork steaks. In cooked frozen fish flakes, Boyd et al.[49] found a rosemary extract to be as effective as TBHQ/ascorbic acid treatment in controlling lipid oxidation.

Lee et al.[50] used mechanically deboned Leghorn spent hen meat to make breakfast sausages and evaluated the effect of two rosemary oleoresins (Herbalox® Type O extract and Colorlife® powdered concentrate) on the products' oxidative stability during refrigerated (10 days) and frozen (6 months) storage. In the refrigerated samples there was no difference in TBARS relative to the control; all levels were in the range 0.24–0.25 mg malondialdehyde (MDA)/kg sample. In the frozen samples, by 6 months the Colorlife® treated samples had the lowest TBARS values (3.92 mg MDA/kg sample); the Herbalox® did not exhibit antioxidant activity.

Vareltzis et al.[51] studied the stability of filleted and minced horse mackerel (*Trachurus trachurus*) and Mediterranean hake (*Merluccius mediterraneus*) that had been treated with rosemary extract prior to being held frozen (−18 °C) for 120 days. Mackerel is a high fat fish while the hake is a low fat fish. In both systems, rosemary-treated samples had lower rates of lipid oxidation during the study. In addition, there was reduced loss of polyunsaturated fatty acids (PUFA).

Wada and Fang[52] showed that the mixture of α-tocopherol (AT) (0.05 %) and rosemary extract (RE) (0.02 %) had the strongest activity (as measured by TBA and PV) among the antioxidants tested (rosemary, AT and BHA) in a sardine oil model system and in a frozen-crushed fish meat. Fang and Wada[53] tested the activity of a combination of tocopherol and rosemary in a sardine oil model system and in the dark muscle of bonito fish. It was found that in the sardine oil system catalysed by Fe^{2+} or haemoprotein, the mixture showed a stronger antioxidant activity than the individual antioxidants. They theorised that the synergistic mechanism between RE and AT was that AT acts as a primary antioxidant/hydrogen donor and RE acts to regenerate the AT by H donation. Once RE was depleted AT began to oxidise and was lost from the system. It was also believed that RE was able to chelate metals which contributed to the synergy in the Fe^{2+} catalysed system.

More recently, Wong et al.[54] studied lipid oxidation and its inhibition by rosemary and sage extracts and tocopherol in a cooked beef homogenate

held 5 days at 5 °C. The addition of vitamin E at increasing levels (25–100 $\mu g\,g^{-1}$) showed a reciprocal reduction in TBARS. Addition of the rosemary and sage extracts (30 $\mu g\,g^{-1}$) reduced TBARS by 53 and 62%, respectively. When equal parts (15 $\mu g\,g^{-1}$) of tocopherol and each plant extract were tested in combination, no synergism was found. This could have been due to using a more purified rosemary system than that used by Fang and Wada;[53] the earlier extracts carried many other compounds (flavonoids) which could have contributed to the synergy observed in the earlier work. Rosemary has been shown to contain diterpenes, which have antioxidant activity, including carnosic acid (most active), carnosol, rosmanol, and rosmarinic acid.

Many other natural antioxidant systems and pure compounds have been evaluated for activity in animal-based systems. Jurdi-Haldemann et al.[55] studied the antioxidant activity of onion (20 %) and garlic (4.8 %) juice *vs* controls containing salt (1 %) or water in ground lamb patties that were cooked, cooled, vacuum packaged and stored in the light at 5 °C for 3–7 days or stored at –20 °C for 15–30 days. Onion juice was more effective than garlic juice on the TBARS and sensory analysis. Some of the effect, as measured by sensory analysis, could have been due to the flavour effect of the treatment as in masking WOF. Karastogiannidou[56] treated chicken thigh meat with onion or quercetin prior to cooking, packaging and storing at 5 °C for 6 days. The addition of dried onion reduced lipid oxidation by 84 % while equivalent levels of quercetin reduced TBARS by 59 %. This indicated that quercetin was an important onion antioxidant but it did not account for the total activity of the onion. Onion was tested at two levels in the thigh meat, i.e. 1.6 and 3.0 %, both levels reduced the TBARS levels, i.e. control at 5 days was at 19.9 mg MDA/kg *vs* 3.2 and 0.5, respectively for the 1.6 and 3 % onion. Sensory analysis showed the higher level of onion to be offensive while the lower level was pleasant.

Ramanathan and Das[57] evaluated the antioxidant activity of some polyphenolics, including rutin, quercetin, morin, myricetin, kaemferol, tannic acid, ellagic acid, ascorbic acid and α-tocopherol in raw and cooked fish samples. It was found that quercetin, myricetin, tannic acid and ellagic acid were excellent antioxidants, but ascorbic acid was the pro-oxidant in the cooked fish. Ramanathan and Das[58] also tested seven natural systems, including fresh spices – ginger, turmeric, onion, and garlic; dry spices – cloves, cinnamon, cumin, black pepper, fennel, and fenugreek; and polyphenols – ellagic acid, tannic acid, myricetin and quercetin. The polyphenols were most active followed by the dry spices and then the fresh spices. Within each group it was found that ellagic acid> tannic acid> myricetin>quercetin; cloves>cinnamon>cumin=black pepper> fennel=fenugreek; ginger and tumeric were more potent that the onion and garlic.

Ahn et al.[59] treated ground turkey meat patties with a variety of natural antioxidants alone or in combination with tocopherol or ascorbate. The natural systems included tripolyphosphate (TPP), citrate, EDTA, cysteine,

histidine, ascorbate, BHA, or egg white. The meat was either hot or cold vacuum packaged before refrigerated storage. Hot packaged meat had lower TBARS values and by day 7 of storage all treatments had lower TBARS that the untreated control. When vacuum packaging was used in combination with tocopherol or ascorbate there was a reduction in TBARS with no difference between those treated with tocopherol or ascorbate. When the system was challenged with salt, ferrous chloride or both, it was found that all treatments reduced TBARS.

He and Shahidi[60] using a model system made from blended white mackerel tissue tested the antioxidant effectiveness of green tea, extracts of green tea and individual catechins found in green tea. After addition of each treatment the fish was cooked and held at 4 °C for 7 days. All treatments had good activity and within the catechins the activity was decreasing in the order of EGCG (epigallocatechin gallate) ≈ ECG (epicatechin gallate) > EGC (epigallocatechin) >> EC (epicatechin). Catechins, flavonoids found in many plants, have been shown to have excellent antioxidant activity in many systems and act by scavenging free radicals and chelating metals. Green tea has been receiving wide attention for its potential health benefits, most of which has been attributed to the catechins.

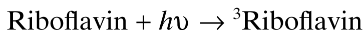
Black pepper extract from supercritical carbon dioxide extractions was effective in inhibiting lipid oxidation in ground pork. Black pepper antioxidant activity has been attributed to its piperine and piperine isomers and some of its monoterpenes. Piperine isomers include chavicine, isopiperine and isochavicine.[61]

12.3 Dairy products

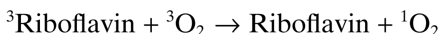
12.3.1 Fluid milk

Milk is a very interesting and very perishable food system with a shelf-life affected by many factors including microbial load, processing factors (such as agitation, temperature of processing and/or storage prior to processing, exposure to light). In addition, milk contains components that affect its lipid stability, i.e. ascorbic acid, copper ions, tocopherols, etc. The inherent compounds in milk will be the focus of the following discussion.

Riboflavin in milk has positive and negative effects; it contributes to the high nutritional quality of milk but it also aggravates the light oxidation of milk fat. Under light, as found in dairy cases, riboflavin produces superoxide anions and singlet oxygen which are responsible for accelerated lipid oxidation. Allen and Joseph[62] reported that riboflavin acts to initiate lipid oxidation in milk by two mechanisms; these can be shown as follows:



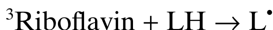
$$\text{Riboflavin} + h\upsilon \rightarrow {}^3\text{Riboflavin}$$



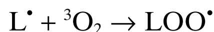
$${}^3\text{Riboflavin} + {}^3\text{O}_2 \rightarrow \text{Riboflavin} + {}^1\text{O}_2$$

$${}^1\text{O}_2 + \text{LH} \rightarrow \text{LOO}^{\bullet}$$

and



$$^{3}\text{Riboflavin} + \text{LH} \rightarrow \text{L}^{\bullet}$$



$$\text{L}^{\bullet} + {}^{3}\text{O}_2 \rightarrow \text{LOO}^{\bullet}$$

In both cases the lipid peroxy radical ($LOO^{\bullet}$) is available for the propagation of lipid oxidation.

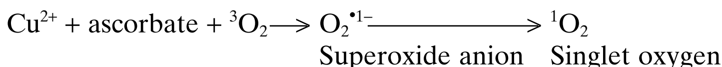
The actual mechanism producing 'light oxidised' flavour in milk starts with the oxidation of methionine and what happens next has been debated in the literature. Allen and Parks in 1975 reported finding methional in light-oxidised milk and postulated it was formed through a Strecker-like degradation mechanism.[63] However, other researchers found evidence that methional decomposed to methyl mercaptan and dimethyl sulphide and these were the active flavour components.[63] In addition, Dimick[64] stated that three components were needed for generation of the light-oxidised flavour in milk, i.e. riboflavin, oxygen and protein-containing sulphur amino acids. Jung et al.[63] conducted a study on the flavour impact in model milk systems exposed to light. Milk was prepared with and without riboflavin and one of three amino acids (methionine, cysteine or valine). Results showed that riboflavin alone did not contribute to off-flavour production nor did valine. However, in the cysteine/riboflavin sample a strong hydrogen sulphide-like odour was found and in the methionine/riboflavin sample a dimethyl disulphide-like odour was found.

As noted earlier, milk is an important source of riboflavin and its degradation represents a major dietary loss of an essential vitamin. The light-induced loss of riboflavin has been shown to follow first-order kinetics and is affected by many factors including the intensity and wavelength of the light, exposure time, and package system used; in addition the surface area relative to volume plays a role.[65]

Ascorbic acid is another compound that plays a major role in the oxidative stability of milk lipids. It has strong singlet oxygen and superoxide anion quenching ability and has been shown to protect riboflavin loss in milk and in a dose-dependent manner. The type of milk also plays a role; 0.1 % ascorbic acid treatment reduced riboflavin loss by 25.5 and 50 % in skim and whole milk, respectively.[65] Jung et al.[63] reported similar results when they evaluated the effect of ascorbic acid on the development of light-oxidised flavour. They showed that the addition of ascorbic acid (200, 500 and 1000 ppm) reduced the formation of dimethyl disulphide by acting as a singlet oxygen quencher.

However, ascorbic acid can also act as a strong pro-oxidant and its effect has been related to concentration and to the presence of copper ions. Allen and Joseph[62] noted that at low concentrations (20–100 $mg\,l^{-1}$) ascorbic acid was a pro-oxidant but at higher levels (>500 $mg\,l^{-1}$) it acted as an antioxidant; milk generally contains between 12 and 25 $mg\,l^{-1}$ ascorbic acid. The

low level of ascorbic acid in combination with the presence of Cu^{2+} ions has been shown to play a major role in milk lipid oxidation. Copper ions are known to be strong promoters of the propagation stage of oxidation. Their presence in milk has been attributable to the fact that during processing the fat globule membrane is disrupted resulting in the release of protein bound copper. Aurand et al.[66] presented a mechanism to explain the interaction between ascorbic acid, copper and milk lipid oxidation, i.e.



$$Cu^{2+} + \text{ascorbate} + {}^3O_2 \longrightarrow \underset{\text{Superoxide anion}}{O_2^{\bullet 1-}} \longrightarrow \underset{\text{Singlet oxygen}}{{}^1O_2}$$

Both of these oxygen products are known to be promoters/initiators of lipid oxidation.

At present, the addition of antioxidants (synthetic) is not allowed in dairy products and so their stability must rely on components present in the milk. Milk does contain tocopherol and β-carotene that are known to act as antioxidants. Tocopherol has been shown to act as a hydrogen donor and to serve as a singlet oxygen quencher, while β-carotene acts as a singlet oxygen quencher. Several studies have looked at the effect of diet on increasing the stability of milk lipids. In 1991 Nicholson and St. Laurent[67] reported that supplementing Holstein cows with 7000 IU day^{-1} of α-tocopherol acetate totally eliminated oxidised milk flavour. In 1998, Focant et al.[68] found that with dietary tocopherol supplementation, they were able to increase significantly the level of tocopherol in the milk and subsequently increase the stability of the milk fat. Fearon et al.[69] fed dairy cattle diets supplemented with naked oats and found oat supplementation increased the oxidative stability of the milk. The milk lipid was found to contain higher levels of monounsaturated fatty acids which contributed to this effect, but it was felt that other factors might also be pertinent. Oats have been shown to contain many antioxidant phenolic compounds.

The role of amino acids in stabilising milk lipids has also been investigated. Chen and Nawar,[70] using oxygen uptake, studied the effect of a series of amino acids, their non-amino acid counterparts, or the amino acid with its amine group blocked on the rate of oxidation in milk fat held at 95 °C. Of the amino acids tested, cysteine, tryptophan and lysine were most protective. Using the acid counterpart it was determined that the primary amino group was important in the antioxidant activity of amino acids. Blocking the epsilon amino group showed it plays a role but was not as important as the primary amino group. In addition it was found that the indole group was not important in the antioxidant activity of amino acids. Chen and Nawar[70] also showed that dissolution of alanine in water or 0.1 N HCl reduced its antioxidant activity. It was thought that in water, activity was lost due to possible hydrogen bonding of the amino acid with the water. The effect of the acid on activity was thought to be due to the

state of the —COOH group. In acid the —COOH would exist which is less effective than the $—COO^-$ group.

Chen and Nawar[71] also treated milk with the phospholipids dipalmitoylphosphatidyl-ethanolamine (DPE) or dipalmitoylphosphotidyl-choline (DPC) in a dry or wet (dissolved in water) system and stored it at 50 and 95 °C. Samples were monitored for oxidation using oxygen uptake. In the dry system it was found that DPE and DPC both exhibited antioxidant activity, with DPE being the best. The authors felt DPE's antioxidant activity was due to the free amino group of the PE. The protonated amino group (NH_3^+) accelerates oxidation while the non-protonated (NH_2) group inhibits lipid oxidation. This was thought to be due to the free amino group possibly reacting with free radicals from lipid oxidation forming Schiff's base reaction products, which could also have antioxidant activity. However, in the wet system both DPC and DPE were pro-oxidant with DPE having the greatest impact. It is theorised that DPE could form a more dispersed system, which could allow more oxygen accessibility and enhance oxidation.

12.3.2 Milk protein stability

Although antioxidants are not used in milk, two studies were found involving the addition of phenolic compounds and evaluating their effects on the heat stability of the milk proteins. The stability of milk proteins to denaturation by heat, known as heat coagulation time (HCT), is affected by various factors found in the milk. A primary factor is pH and in typical milk heated to 140 °C, HCT increases as pH increases from 6.4 to 6.7 after which there is a sharp decrease and it is at its lowest at pH 6.9. After 6.9 there is a gradual increase in HCT. Typical milk is referred to as 'type A' milk. However, some milk, referred to a 'type B', is not typical and exhibits a HCT curve less affected by pH; the HCT increases with pH.[72] Sweetsur and White[73] reported that the type A milk coagulates in two steps, while the type B milk coagulates in one.

O'Connell and Fox[74,75] evaluated the effect of polyphenolic compounds on milk protein heat stability. Chlorogenic acid, guaiacol, thymol, vanillin, BHT, PG, BHT and 2,5-dimethoxycinnamic acid and 3,4-dimethoxycinnamic did not affect the heat stability of the milk while quinic acid reduced it. Pyrogallol, catechol, tannic acid, ellagic acid, phloroglucinol, and gallate caused the heat coagulation time-pH profile to change from an A to a B type. Ferulic and vanillic acid increased heat stability in the region of the maximum, which did not recover with increased pH; however they had little effect on the minimum heat stability of the milk. Caffeic acid increased the heat stability of the milk protein.

These authors[75] went on to study how caffeic acid increases the heat stability of milk. They found that several factors affected the ability of caffeic acid to alter the heat stability of milk:

1 Caffeic acid had to be converted to a more active quinone form and this conversion required the presence of oxygen and when in oxygen it was temperature dependent.
2 Caffeic acid was capable of chelating Ca^{2+} which is important since Ca^{2+} ions reduce the heat stability of milk proteins.
3 Caffeic acid can block the ε-amino groups of lysine and inhibit the production of hydroxymethylfuraldehyde (HMF), which is indicative of the Maillard reaction. The authors felt that blocking lysine also prevented the dissociation of κ-casein-rich proteins from the casein micelles.
4 Caffeic acid reduced the level of sulphhydryl in the heated milk by being able to interact with the cysteine group of the β-lactoglobulin and other whey proteins.

The final concept elucidated by O'Connell and Fox[75] postulated the nature of the structural components responsible for the heat stabilisation effect of caffeic acid. Using a series of caffeic acid derivatives, they found that the hydroxyl groups played a role and they had to be in either the *ortho*- or *para*-position on the benzene ring; the chain on C-1 was important, i.e. replacing the C-1 chain with an aldehyde group increased the heat stabilising effect; but the double bond in the C-1 chain had no effect; saturation of the ring eliminated the activity; and it appeared that the carboxylic group was important in the calcium-chelating role.

12.3.3 Butter

The susceptibility of butter to oxidative reactions has been investigated. Emmons et al.[76] showed that butter held frozen (–18 °C) and in the dark showed no evidence of oxidation after 1 year of storage, but there was some loss of butter quality after 14 weeks storage in the dark at 5 °C. However, when Luby et al.[77,78] stored butter in light, either in fluorescent in the cold (5 °C) or daylight at 22 °C, evidence of lipid oxidation (cholesterol oxide production) was found. Their data showed that both singlet and free radical oxidation was occurring.

Other researchers have looked at the potential for natural antioxidants to prevent lipid oxidation in butter. Zegarska et al.[79] showed that an ethanolic extract of rosemary increased the stability of butter against oxidation and that the effect was concentration dependent. This study also evaluated the effectiveness of the rosemary extract in inhibition of copper-catalysed oxidation and found evidence that the extract was able to chelate metals. Farag et al.[80] showed that thyme and cumin essential oils could prevent oxidation in butter stored at room temperature, and at 200 ppm the essential oils were more effective than BHT in inhibiting lipid oxidation in the butter. Farag et al.[80] felt the preservative effect of the essential oils from thyme and cumin was due to the phenols found in the oils. The phenolic hydroxy group would be able to donate hydrogen to lipid.

12.4 Eggs and egg products

Eggs, like milk, contain many naturally occurring antioxidant compounds. Because of the number, concentrations and location of the antioxidant compounds in eggs, the in-shell system is very stable to oxidation. Eggs contain two proteins, phosvitin and conalbumin that have been reported to have antioxidant activity. Phosvitin, a yolk protein, has been shown to inhibit Fe^{2+} and Cu^{2+} catalysed oxidation;[81] it was more effective against iron than copper catalysis. Lu and Baker[81] also showed that pasteurisation did not affect phosvitin's antioxidant activity. Conalbumin (ovotransferrin) found in the albumin, can also bind di- and trivalent ions, including Fe^{3+} and Cu^{2+}.[82] Work by Froning et al.[83] showed that conalbumin was capable of reducing oxidation in cooked turkey thigh meat.

Egg yolk contains a wide range of fatty acids ranging in length from 8 carbons to 20 carbons; of these around 11 % are saturated with palmitic (7 %) and stearic (3 %) predominant; 14–15 % are monounsaturated with oleic (13 %) predominant and the remaining 4–5 % are polyunsaturated with linoleic (3.8 %) predominant. The yolk contains relatively high levels (7 %) of phosphotidylethanolamine (lecithin) which has been shown to have antioxidant activity. In addition, the yolk contains α-tocopherol, again a proven antioxidant, and xanthophylls, including lutein, zeaxanthin and cryptoxanthins; lutein has been shown to have antioxidant activity.

As in the case of milk, addition of antioxidants to eggs is not allowed. However, several studies have reported the effects of dietary supplementation as a means of increasing the antioxidant content of eggs. Increasing the level of dietary tocopherol(s) has been shown to increase the lipid stability of eggs during storage.[84,85] When menhaden oil was fed to hens, the eggs had higher levels of 20:5 n-3 and 22:6 n-3 fatty acids; dietary tocopherol supplementation was effective in reducing oxidation in these eggs during 40 days of storage at 4 °C.[84] Tocopherol-supplemented eggs had higher (three–four-fold) levels of tocopherol. During storage there was a gradual loss of tocopherol across treatments, especially the δ-tocopherol, but the supplemented eggs retained the higher levels.[84]

Two studies were found that had a novel twist; herbs known to contain antioxidant compounds were fed to hens. Laying hens were fed one of three diets, i.e. control, control +0.28 % rosemary extract or control +0.57 % rosemary extract.[86] Eggs from the hens were collected approximately every 4 days for 28 days and analysed for carnosic acid content. It was found that the hen deposited carnosic acid into the yolk and deposition increased with increased dietary levels of rosemary extract; a maximum level of deposition occurred between day 12 and day 20. Botsoglou et al.[87] showed that adding thyme to layer diets produced eggs which were more stable against oxidation. To explain some of their findings the authors also tested the effectiveness of added thyme, thymol (an active ingredient from thyme), a thyme

extract found to be 98 % thymol, and ascorbic acid on the stability of the egg yolk. It was found that the thyme extract was a better inhibitor of oxidation. Ascorbic acid exhibited a pro-oxidant effect.

12.5 Cholesterol

Cholesterol oxidation has been of great interest due to its implication in heart-related diseases. Specifically, cholesterol oxidation products (COPS) have been shown to have potential as cytotoxic and mutagenic compounds. Cholesterol is found in the cell membrane and is associated with polyunsaturated fatty acids of the membranal phospholipids. Therefore, cholesterol oxidation is accelerated by the same factors affecting lipid oxidation and the level of cholesterol oxidation is closely related to the degree of unsaturation in the neighbouring fatty acids. Therefore, antioxidants effective in reducing lipid oxidation should have a strong impact on the oxidation of cholesterol.

Rankin and Pike[88] used an aqueous meat model system to evaluate the effect of rosemary oleoresin (Herblox®), quercetin, myricetin, tocopherols (α-, γ-, and δ-isomers) alone and mixed and BHA (control) on cholesterol oxidation at pH 5.5 and at 80 °C. Antioxidant activity was determined by measuring the induction period for the production of 7-ketocholesterol. It was found that rosemary oleoresin, quercetin, myricetin, or BHA were ineffective in reducing cholesterol oxidation. The tocopherols were able to inhibit cholesterol oxidation with the γ- and δ-isomers being most active; no synergism was found with blends of the tocopherols. Dietary supplementation of tocopherol has also been shown to reduce the oxidation of cholesterol in chicken meat[32,89] and tocopherol supplementation with or without surface application of a rosemary extract reduced cholesterol oxidation in rainbow trout muscle.[90]

Dairy products are generally resistant to cholesterol oxidation, which is attributed to their low level of transition metals, low cholesterol content and a fat system that contains high levels of saturated fatty acids. However, drying milk can contribute to the production of COPS and since dry milk is usually stored at room temperature COPS production will occur. Angulo et al.[91] studied the effect of factors including time, temperature and packaging atmosphere (air or nitrogen) on the production of cholesterol oxides (COPS) in whole and skim milk powders. They found a direct relationship between the level of COPS and storage time (1 year in the dark) at two different temperatures (32 °C and 55 °C). The primary COPS formed was 7-ketocholesterol. In samples stored in air, 7-ketocholesterol and 7β-hydroxycholesterol were found. These are formed via autoxidation involving triplet oxygen ($^3O_2$). When stored in nitrogen two additional COPS were found, i.e. cholestanetriol and α-epoxide, indicating a double-oxidation which occurs through a ground-state dioxygen and hydroperoxy-

induced free radical mechanism. The samples held at 55 °C developed a brown colour, which indicated that the Maillard reaction initiated by free radicals was also occurring.

Although in-shell eggs are relatively stable against oxidation, dried eggs have been shown to be subject to lipid oxidation, specifically cholesterol oxidation. Several methods can be used to produce dried eggs, but the most common process is spray-drying. Many studies have reported the presence of COPS in spray-dried eggs and the data shows that COPS are formed at a higher level in direct fire spray-dryers as opposed to the indirect heat dryers. This difference in COPS production has been attributed to the formation of nitrogen oxides (NO and NO_2) that are formed in the natural gas flame.

Fontana et al.[92] showed that egg powders stored at room temperature developed COPS, with production of the 7α and β-hydroxycholesterols, the β-cholesterol epoxide being predominant. Some work has been reported on the use of natural antioxidants in helping to control COPS production in spray-dried eggs. As in controlling lipid oxidation in meat, meat and dairy systems, increasing the inherent level of tocopherol in the egg via dietary supplementation has shown promise in reducing COPS in the final product.[93] Li et al. also showed that the dried products from supplemented eggs had higher tocopherol levels and were therefore able to maintain their stability during four months of room temperature storage.

12.6 Summary and future trends

Natural antioxidants have great impact on the safety and acceptability of the food system and will continue to do so. Not only do they keep the food stable against oxidation but can also be effective in controlling microbial growth. By increasing the inherent levels of the antioxidants in animal products through dietary supplementation, we are providing a more consumer-acceptable product. This area of research is exploding in the literature. Since the first writing of this document, many articles have been published evidencing the viability of this practice in meat animal systems. That references, albeit lesser in number, were found for dairy produce and eggs shows that this avenue has just started being used to improve these food systems. However, as shown in this paper, the traditional practice of adding antioxidants during processing can still play a very important role since the added compounds have the potential for enhancing the activity of the inherent antioxidants systems. The literature cited shows that more work is needed to define the optimum dietary combinations and/or the minimum levels of the compound in the food necessary for obtaining the greatest stability in the resultant product. This may involve defining interactions of dietary components on the uptake on the desired compounds; this will eventually require more sophisticated feed formulations and a better understanding

of the nutrient impact of the by-products that are traditionally used as animal feeds. One of the biggest problems for this approach is the cost of the feed, especially if plant phenolics are to be used. Sources need to be identified other than the traditional ones; for instance, rosemary may not always be the best source for its active compounds. Finding less valuable plants for feed formulations may be necessary.

12.7 Sources of further information

To learn more about the systems discussed the following books are recommended

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13

Preparation of natural antioxidants

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13.1 Introduction

There is a big difference between the preparation of synthetic antioxidants and natural antioxidants for application in food products and processing. Synthetic antioxidants are produced as pure substances of constant composition, and are applied as such or in well defined mixtures with other pure substances. Application is thus relatively easy, requiring no substantial modifications of the recipe and processing conditions.

On the contrary, natural antioxidants are available from raw materials of variable composition. Both the content of active substances (usually a mixture of several compounds) and the content of various other compounds, either inactive or possessing negligible activities, depend on the plant variety, agrotechnology, climatic conditions, degree of ripeness, and many other factors. Their composition should be determined in every batch, and if necessary, the procedure of their preparation or application, and the amount added to food products should be adapted according to analytical results.

Most widely used natural antioxidants are not exactly purely natural, but nature identical. This means that their structure is the same as that of natural substances, but they have been prepared by synthesis. They are supplied in a relatively pure state, like other synthetic antioxidants. Tocopherol, ascorbic acid and citric acid belong to this group. From the standpoint of preparation for their application, they may be regarded as pure synthetic substances, requiring no preliminary preparation.

13.2 Direct application of active food ingredients

Some nature-identical antioxidants, such as α-tocopherol or β-carotene, are available on the market in a pure form or in defined solutions so that they can be added very easily in the amount desired. Solutions of these compounds are prepared in the industry in order to improve the solubilisation of the preparation in the food to be stabilised.

Many other food components possessing antioxidant activities are used in their natural form, such as spices. The preliminary processing of such food components may be drying (in case of leaves or stems), milling of dried material (such as seeds), or some other mechanical treatment. Several ground spices (added in the amount of 5 %) were found to be active in sunflower oil, especially, sage, sumac and thyme.[1]

Spices are aromatic and pungent food ingredients, therefore, their direct use as antioxidants is limited to foods that are usually seasoned. Precooked comminuted meat systems are stabilised by ground spices during refrigeration and frozen storage. Clove, rosemary and sage are most effective in inhibition of oxidation of meat lipids.[2] Better flavouring effect is found when rosemary and sage are used in a mixture with sodium glutamate, protein hydrolysate, garlic, and onion, than as a single spice.[3]

An alternative is to prepare a paste from soft, water-containing substances, and to add it as a part of the recipe. These preparations are added to the mass of components before processing, or they may be applied on the surface of food products as it is exposed to heat and oxygen more than the inner layers. Rosemary oleoresin extract is found to be efficient on application on the surface of muscle tissue from rainbow trout.[4] Sometimes, these ingredients are added after thermal processing, such as roasting, just to prevent the destruction of antioxidants during the processing.

Some natural antioxidants have lower volatility and better thermal stability than butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) at higher temperatures, such as those used in deep-fat frying. Therefore, they can be used as an antioxidant in fried food or their extract can be added to frying oil. Rosemary and sage applied in both ways decreased the rate of autoxidation of oil in fried potato products (french fries, chips and crisps).[5–8]

Some natural food antioxidants are concentrated in products of food processing, essentially they are wastes from another primary process in the food industry. The processing of oilseeds is a classic example. Oil is extracted from oilseeds by expeller pressing and/or solvent extraction and the residual extracted meal, which contains amino acids, proteins, flavonoids and phenolic acids, may then be used as an additive to various meat or fish products. During the degumming of crude edible oils, lecithin is isolated from crude oil. Lecithin or its concentrates may also be used as a food additive with an antioxidant activity (see Section 13.6).

Table 13.1 Antioxidant activity of essential oils and residues after distillation of rosemary and sage tested in lard using Schaal Oven Test at 60 °C (IP = induction period expressed as the time (days) to reach the peroxide value of 20 meq kg^{-1}; PF = protection factor expressed as the ratio of induction periods of the stabilised sample and the control sample)

Antioxidant	PF in case of rosemary	PF in case of sage
Spice	11.27	8.75
Essential oil	0.97	0.99
Residue after distillation	10.25	6.12

During the refining of edible oils, the last step is deodorisation, when the deacidified and bleached oil is treated with superheated steam at 220–250 °C. At such a high temperature, not only are objectionable volatile lipid oxidation products removed, but also a part of tocopherols and phytosterols. The vapours are cooled and collected, and the deodorisation condensate may then be used as a food additive (see also Section 13.4). Tocopherols are efficient antioxidants and phytosterols are valuable because of their ability to decrease the cholesterol level in the blood serum. An advantage of deodorisation concentrates, containing natural tocopherols, in comparison with nature-identical synthetic α-tocopherol is that they are natural mixtures of antioxidants, which are optically active, which proves their natural origin.

Another example is tea leaves, used for the preparation of tea brew. Some antioxidants are extracted into the tea infusion but a part remains in the tea leaves and can be used after drying as a relatively rich source of antioxidants. Green tea is more efficient than fermented (black) tea because of a higher concentration of catechins.

Essential oils are prepared by steam distillation of spices, such as rosemary or sage oils. After the removal of volatile essential oils leaves may be dried, milled and used as a source of natural antioxidants, either directly or after extraction.

Volatiles of essential oils are responsible for the characteristic aroma of the spice, therefore, rosemary and sage can be used at higher concentration after removing those components. The antioxidant activity moderately decreases during the distillation and drying processes, but remaining antioxidant activity is still satisfactory as was found in experiments using rosemary and sage[9] (see Table 13.1).

13.3 Preparation of antioxidants by extraction of food ingredients

The content of active antioxidants in natural materials is usually rather low so that large additions would be necessary to obtain a significant

improvement in stability against oxidation. However, such large additions could have a negative effect on the flavour or functional properties of the product. It is often useful to prepare more concentrated materials. The easiest way is to remove water by a suitable drying procedure and the next optimal procedure is extraction. The choice of solvent is of crucial importance.

Three procedures may be used, extraction using fats and oils, extraction using organic solvents, and supercritical fluid carbon dioxide extraction.

13.3.1 Extraction of antioxidants with fats and oils

Extraction using edible oil or fat is a very simple method. Natural material containing antioxidants, such as herbs and spices, is mixed with fats and/or oils, and the mixture is left at room temperature or at a moderately increased temperature (in case of solid fats, such as pork, lard, beef tallow or cooking fats) for a defined time, for example overnight, with or without stirring. The mixture is then filtered and the fat or oil containing dissolved antioxidants is used directly in food preparation. Rosemary, sage, paprika, nutmeg or cocoa shells have been powdered and extracted with edible oil.

The extraction of antioxidants from rosemary and sage with edible vegetable oil has been patented.[10] The ground spice was combined with oil in a proportion of 15–20 %, and heated at 120–125 °C for two hours with continuous agitation. The extract was separated by centrifuging, and deodorised by heating to 175–185 °C for 30 min, with simultaneous sparging with steam. The antioxidant activity of sage extract, prepared according to this process in lard[10] is presented in Table 13.2.

The procedure of extraction and purification of antioxidants from spices was improved,[11] using a new method based on the following steps: (a) micronising spice in an edible oil; (b) cleaning the lipid phase by centrifuging, molecular distillation on falling film, or using a centrifugal system. The micronisation allowed mechanical transfer of the antioxidant to the lipid phase, and the use of molecular distillation allowed deodorisation or partially cleaning the lipid phase. Monoacylglycerols can be used as co-distillants. The activity of rosemary, sage and cocoa hull extracts, prepared using this procedure,[12] was found to be comparable with that of synthetic antioxidants (see Table 13.3).

Acceptable yields (34–88 %) were obtained by extraction of rosemary and other spices with oil.[13] Antioxidants may also be extracted with monoacylglycerol concentrates, which are more polar than oil.[14]

The advantage of this procedure is its simplicity and safety, as no organic solvents are used for the extraction therefore no residual solvents are present. The procedure is, however, suitable only in rare cases, especially when large amounts of fat are added to the recipe. In most cases, the

Table 13.2 Antioxidant activity of sage extract prepared by extraction with cottonseed oil and tested in lard, using the active oxygen method at 110 °C (IP = induction period; PF = protection factors; see Table 13.1)

Antioxidant	Induction period (h)	Protection factor
Control	20	1.0
1.25% extract	480	24.0
2.50% extract	1050	52.5
5.00% extract	1110	55.5
7.50% extract	1620	64.5
0.02% BHA	570	28.5
0.02% PG (propyl gallate)	940	47.0

Table 13.3 Activity of rosemary, sage and cocoa hull antioxidants in chicken fat (evaluated using the Astell method at 90 °C)

Antioxidant	Induction period (h)
Control	4
0.1% rosemary	25
0.1% rosemary with monoglyceride as co-distillant	20
0.1% sage	30
0.1% cocoa hulls	20
0.01% BHA + BHA (1:1 m/m)	20–25

content of antioxidants dissolved in fat is too low to guarantee satisfactory stabilisation.

13.3.2 Extraction of antioxidants with organic solvents

Extraction with organic solvents is another possibility. The choice of a solvent depends on the particular material and on the stabilised substrate. Some examples are given in Table 13.4, showing that even in such closely related substances as rosemary and sage leaves, the optimum solvent may be different.

Hexane, acetone, ethyl acetate, and methanol were compared, and the solvents of intermediary polarity seemed to be preferable to either non-polar or highly polar solvents.[15] Ethanol would probably be better than methanol as eventual solvent residues would be less toxic. Mixtures of organic solvent, such as acetone, methanol or ethanol with water (8:2 v/v) were tested on the example of lentil seeds, and aqueous acetone was found to be best.[16]

Methanol was used for extraction of phenolic antioxidants from peanut hulls.[17] The same solvent was used for extraction of spices[18] but our experience showed less polar solvents to be more suitable (e.g. acetone or ethyl

Table 13.4 Relative activities of 0.05 % rosemary and sage extracts, when extracted with different organic solvents
(PF = protection factor; determined using Schaal Over Test at 40 °C)

Extracted material	Extraction solvent	PF in sunflower oil	PF in rapeseed oil
Rosemary	hexane	2.4	3.9
	ethyl acetate	3.2	3.1
	acetone	2.6	3.3
	methanol	2.2	2.3
Sage	hexane	1.5	2.3
	ethyl acetate	2.4	2.2
	acetone	1.8	2.6
	methanol	1.3	1.7

Table 13.5 Effect of essential oil removal on the antioxidant efficiency of 0.05 % plant extracts in rapeseed oil
(PF = protection factor; determined using the Schaal Oven Test at 40 °C)

Extracted meterial	PF of the extract	PF of deodorised extract
Sage	2.0	1.6
Chamomile	1.4	1.3
Sweetgrass	1.6	1.6
Dragonhead	1.3	1.1
Savory	1.3	1.2
Perilla	1.4	1.2

acetate). Chloroform and ethyl acetate were found to be favourable for extraction of tea leaf catechins.[19]

The extracts obtained using organic solvents may be further concentrated, for instance, by molecular distillation.[5,12] The extracts prepared with organic solvents have a strong odour, bitter taste, and undesirable colour, therefore, many procedures for removing these impurities have been proposed.

Essential oils present in spice extracts, are responsible for the characteristic aroma of the spices, and may be objectionable in the stabilised product, especially in relatively neutral foodstuffs, such as edible oils. Volatiles of essential oils can be removed by steam distillation at normal atmospheric pressure or in a vacuum. Steam distillation can be used as a step preceding or following the extraction with a non-polar or polar organic solvents.[20] When essential oils are removed by the steam deodorisation, essential oils are almost quantitatively removed. The antioxidant activity is, however, partially lost (see Table 13.5 and Table 13.1) as even essential oils possess some (usually not very high) antioxidant activity. Essential oils from thyme and cumin prevented rancidification of butter.[21]

Table 13.6 Antioxidant activity of 0.1 % purified antioxidant extracts in lard (determined by active oxygen method; expressed as the peroxide value [meq kg^{-1}] after the time (h); A = starting material; B = water soluble fraction of ethanolic extract; C = water insoluble fraction of ethanolic extract)

Antioxidant	Fraction	17 h	45 h	71 h	97 h	117 h
Sage	A	2.7	5.8	12.2	259.0	–
	B	3.3	10.5	76.8	–	–
	C	2.8	6.8	11.9	14.2	22.9
Rosemary	A	2.6	3.9	9.3	23.6	436.3
	B	4.4	11.8	40.8	741.3	–
	C	3.3	6.0	10.6	14.8	18.2
Thyme	A	4.9	25.0	647.4	–	–
	B	3.1	10.8	50.9	722.0	–
	C	2.6	7.0	11.1	13.4	39.3
Marjoram	A	5.6	31.9	859.5	–	–
	B	5.0	16.1	385.0	–	–
	C	4.5	15.1	87.1	–	–
BHA (0.02 %)	–	5.0	20.3	75.2	–	–
Control		13.0	33.0	653.8	–	–

Another procedure for removing volatiles is the use of extraction with carbon dioxide under supercritical conditions. The residual material is extracted with organic solvents.[22] This way was used for deodorisation of crude extract.[23]

The oleoresin, which is the non-volatile extract, is responsible for the typical taste and pungency of the spice. Another disadvantage of extracts from plant leaves is the presence of chlorophyll pigments,[24] which impart dark colour to the stabilised fat, and act as pro-oxidants in the light, especially when present at higher concentrations. Ethanol or methanol extracts may be purified from chlorophyll pigments by further fractionation.[25] Activated carbon is proposed for bleaching the crude extracts prepared with polar or non-polar solvents.[22,26,27]

Kimura proposed the washing of extracts prepared with organic solvents with cold or hot water to remove bitter substances from crude extracts. The water soluble fraction possesses weak antioxidant activity[27] as compared to starting material and water insoluble fraction (see Table 13.6).

The yield of active substances depends very much on the type of extracted materials and on the extraction process. The yields of purified extracts presented in Table 13.6 vary from 2.1 % for marjoram to 11.2 % for rosemary.

Chang et al.[5] investigated the activity of rosemary and sage extracts, prepared with different solvents and treated by the procedure of bleaching with activated carbon, washing with water, and separation by molecular distillation (see Table 13.7). Extracts from sage, prepared with non-polar sol-

Table 13.7 Antioxidant activity of 0.02 % purified antioxidants from extraction of rosemary and sage with different solvents in lard (aging at 60°C; expressed as the peroxide value [meq/kg]; rosemary extracts after 11 days, sage extracts after 12 days)

Extraction solvent	Rosemary extract	Sage extract
Hexane	2.6	61.9
Benzene	2.2	13.7
Diethyl ether	1.6	5.2
Chloroform	2.9	4.4
Methanol	1.8	4.4
Control	38.8	56.4

vents, were less effective as an antioxidant. The benzene extract showed lower activity, and the hexane extract showed no activity at all.

Tea leaves were extracted with methanol or acetone in six different ways, and the yields were compared. It was observed that using refluxing with methanol resulted in higher yields of tannins and catechins than other methods.[28] Green tea extracts under Schaal Oven Test conditions at 65 °C exhibited a pro-oxidant effect in marine oils, perhaps due to the catalytic effect of the chlorophyll constituents. A green tea extract, dechlorophyllised by the column chromatographic technique, possessed excellent antioxidant activity[29] and its efficacy was higher than those of BHA or BHT, but less than of *tert*-butyl hydroquinone (TBHQ).

Extracts, obtained with application of organic solvents, may be concentrated by a subsequent extraction with water to remove sugars and other undesirable water-soluble inactive substances. Some efficient antioxidants can also be removed in this step (see Table 13.6), therefore, the increase in the activity of re-extracted materials is not necessarily always pronounced. The removal of interfering components sometimes compensates for this disadvantage, for example sugars could initiate Maillard reactions, imparting foreign flavours to the product and causing the colour to deteriorate.

Some antioxidants from rosemary and sage leaves can be extracted with aqueous alkaline solution.[30] Alternatively crude extract prepared from non-polar organic solvent can be washed with alkaline solution for the recovery of the active acidic fraction.[20] Antioxidant constituents from rosemary and sage can also be extracted during the process of aromatising vinegar with these spices. Aromatised vinegar inhibited lipid oxidation, and extended the shelf-life of mayonnaise.[31] Aeschbach and Rossi[32] proposed a method of extraction of hydrosoluble or polar antioxidants from herbs, spices, tea, coffee, fruit and vegetable peel or cereals. The active constituents were extracted with propylene glycol as a polar carrier by a purely mechani-

cal procedure. The authors recommended them for direct application in food systems.

The yield of active substances depends very much on the extraction process. Tea leaves were extracted with methanol or acetone in six different ways and the yields compared. It was observed that using refluxing with methanol resulted in higher yields of tannins and catechins than other methods.[28]

Extracts obtained with application of organic solvents may be concentrated by a subsequent extraction with water to remove sugars and other water-soluble inactive substances. Some efficient antioxidants can also be removed in this step, therefore, the increase in the activities of re-extracted materials are not necessarily always pronounced. The removal of interfering components sometimes compensates for this disadvantage, for example, sugars could initiate Maillard reactions, imparting foreign flavours to the product, and causing the colour to deteriorate. Oregano leaves were extracted with organic solvents, and the ethanol extract was again re-extracted with petroleum ether, diethyl ether, ethyl acetate, and butanol. The diethyl ether extract was found to be very efficient in lard.[33]

Extraction costs are rather high, sometimes increased by a subsequent re-extraction so that the usefulness of the extraction should be always estimated for every case of industrial application. The extraction is very useful, however, in the research into antioxidants present. In some cases the extracts may not be considered as natural food materials (not limited by regulations) but extracts from spices would probably be acceptable as they have already been used as food ingredients for other purposes.

13.3.3 Extraction of antioxidants with supercritical fluid carbon dioxide

A modern method is extraction with gases, usually carbon dioxide, under supercritical conditions. The method and its application for fats and oils were reviewed by King and List.[34] Propane/butane, methanol, ethanol and other substances may be used as co-solvents, improving yield or selectivity. Extraction with carbon dioxide is relatively selective, generally better than that of organic solvents. As mentioned above, this treatment was proposed for removal of volatiles, preceding extraction with organic solvents (both polar and non-polar). The safety aspect should be considered because of the high pressure used although carbon dioxide, being a gas at atmospheric pressure, is easily removed so that solvent residues present no risk factor.

A big disadvantage of supercritical extraction is the high operation pressure, which requires expensive equipment. Several suitable devices have been proposed and critical reviews are available. The cost of the process is high making it unsuitable for the extraction of main food components, such as lipids. Antioxidants are, of course, a more expensive group of food prepa-

rations so that price would not play a crucial role if it is compensated by other advantages, such as high purity of extracts and great efficiency of the process.

The application of supercritical solvent extraction to the preparation of natural antioxidants has, until now, been limited. A few examples of application of supercritical carbon dioxide to lipid and oilseed extraction are reviewed.[34] The procedure was used for the extraction of rosemary[35] and sage[36] leaves. Phenolic substances can be removed from sunflower extracted meals using supercritical fluid extraction with carbon dioxide.[37] Phenolics may be used as natural antioxidants and the residual protein possesses a higher nutritional value.

It can be hoped that applications will be more frequent in the near future, when the procedure becomes better investigated and the equipment becomes cheaper.

13.3.4 Purification and modification of extracts

Extracts from natural materials are mixtures of many components and the content of substances with an antioxidant activity could be rather low. The content of active substances does not depend only on the raw material, but also on processing conditions,[38] which should be optimised in each case.

Commercial antioxidant extracts from spices, usually from rosemary, are available in powder form or as oily oleoresins. They are lipid soluble, oil dispersable, water soluble, or water dispersable. Depending on their content of active substances, it is recommended that they be used at levels between 200 and 1000 mg per 1 kg of the stabilised product. The composition of 28 commercial rosemary and sage extracts was found[39] to possess different antioxidant activities, and showed great variation in their high performance liquid chromatography (HPLC) profiles, containing 20 identified phenolic compounds. The authors found no correlation between the antioxidative efficiency of the extracts and their composition of phenolics. The data indicated only that the most effective components were carnosol, rosmarinic acid, and carnosic acid, followed by caffeic acid, rosmanol, rosmandial, genkwanin, and cirsimaritin. Synergism and antagonism are very important in a mixture such as that of plant extracts. From this example it is evident how difficult the quality control of extracts is.

Theoretically, it is possible to prepare nearly pure or entirely pure substances by liquid chromatography, repeated crystallisation or other procedures, but further purification is not recommended. The resulting pure antioxidants would be very expensive in comparison with synthetic antioxidants.

The activity of pure natural antioxidants can be improved through their chemical modification. Rosemariquinone substituted with a tertiary butyl group exhibited better antioxidant activity than compounds not sub-

stituted.[40] Veldsink et al.[41] studied the modification of natural phenolic antioxidants (cumaric acid and tyrosol) based on enzymatic transesterification with fatty acids. Their study demonstrates the possibility of improvement of the lipid solubilisation and thermal stability of natural antioxidants.

As new pure substances, antioxidants would not be considered as natural food ingredients. It would be necessary to have the application approved by the authorities on the basis of sophisticated and very expensive tests similar to those that are obligatory for synthetic antioxidants. Most natural substances would not pass such tests in order to obtain permission for use as food additives, because natural antioxidants (being *ortho*-disubstituted pyrocatechol or pyrogallol derivatives) are usually more toxic than synthetic antioxidants of comparable activity, which are mainly *para*-disubstituted compounds.

13.4 Commercial production of tocopherols from natural sources

Tocopherols exploited commercially as natural antioxidants are either obtained by extraction from natural sources or by chemical synthesis. The last procedure is relatively simple and cheap, but only racemic α-tocopherol is produced.

The most important natural raw materials for production of tocopherols by extraction are deodoriser sludges, which are distillates obtained in the deodorisation of vegetable oils. Such distillates contain sterols, sterol esters and triacylglycerols, as well as tocopherols and tocotrienols. The concentration of tocopherols depends on the deodorisation parameters[42] (temperature, vacuum, quantity of injected steam and equipment) but their amount is lower than 10 %, usually 8–9 %, of unsaponifiable matter present. Separation of tocopherol from the other distilled compounds is possible by several methods: (1) by esterification with a lower alcohol, washing and vacuum distillation; (2) by saponification, or (3) by fractional liquid–liquid extraction. The concentrates obtained in this way may be purified further by molecular distillation, extraction, crystallisation, or combinations of these procedures.

The tocopherol concentrates recommended as antioxidants are mixtures with relatively high contents of γ-tocopherol and δ-tocopherol (being obtained from soybean oil), but α-tocopherol is also present. The total tocopherol concentration usually lies between 30 and 80 %. The rest is constituted of triacylglycerols.

Mixed tocopherols are available on the market diluted in vegetable oils, or prepared as synergistic mixtures of tocopherols with rosemary extracts and mixtures of tocopherols, ascorbyl palmitate, other antioxidants and synergists (lecithin, citric acid), and carriers.

13.5 Preparation and application of amino acids as antioxidants

Amino acids, peptides, such as carnosine and anserine, and proteins are common food components. Amino acids were found to be efficient antioxidants in model experiments. Their application is advantageous in mixtures with other inhibitors as they often act as synergists of phenolic antioxidants and as chelating agents. Amino acids convert hydroperoxides into imines, and sulphur containing amino acids reduce hydroperoxides into the respective inactive hydroxylic derivatives. Methionine and selenomethionine were found to be more active than α-tocopherol in olive oil.[43]

An addition of pure amino acids would be the best choice, but they are too expensive. The application of protein hydrolysates will thus be preferable. In addition to free amino acids, they also contain peptides, nonenzymatic browning products, stable antioxidants from the starting material, like phenolic acids, salt, and many other impurities. Therefore, the sphere of their mechanism of antioxidant action is wide.[44,45] They may be prepared by enzymic or acid-catalysed hydrolysis of cheap proteins, such as oilseed extracted meals (most often, soybean meal), wheat gluten, keratin, and many other food materials and wastes. Protein hydrolysates from aquatic species contain both antioxidative and pro-oxidative components and their final effect depends on their concentration.[46] Enzymatic hydrolysates from egg albumin and fish proteins were found to be more efficient than soy or casein hydrolysates.[47] All hydrolysates showed metalchelating activities. Several amino acids were isolated from krill proteolysates, and were found to be active in lard mixed with phenolic antioxidants.[48] They are usually directly applied to food material, as they are only sparingly soluble in the lipid phase. Protein hydrolysates are used as food seasoning ingredients and can be used in meat products to inhibit lipid oxidation.[49] They are also suitable as an additive to dried beef or chicken soup, stew, ground meat, fish or other foods where the flavour of protein hydrolysates is not an objection.

The antioxidant activity of amino acids is due to the reaction of amine or sulphur groups present with lipid hydroperoxides with formation of imines, sulphides, thiosulphinates and sulphoxides, respectively (see Table 13.8). The reaction proceeds in a non-radical way.

Proteins react in a similar way to amino acids, but the content of free active amine groups is lower, mainly 6-amine groups of bound lysine molecules. Proteins are cheap and present in nearly all foods, so their antioxidative activity should be accounted for.

For example, skim milk powder added to margarine increases the activity of added antioxidants. Soy protein concentrates and soybean isolates are used in a range of food products and exhibit the inhibition of lipid oxidation. Their activity is connected not only with phenolic antioxidants, but also with proteins, amino acids and peptides.

Table 13.8 Reactions of amino acids with lipid hydroperoxides

Functional group	Formula	Reaction product	Formula
Amine, primary	R-NH_2	imine	=N-R
Amine, secondary	R-N-R	imine oxide	-NR_2=O
Thiol	R-SH	disulphide	R-S-S-R
Disulphide	R-S-S-R	thiosulphinate	R-S-SO-R
Sulphide	R-S-CH_3	sulphoxide	R-SO-CH_3
Selenide	R-Se-CH_3	selenoxide	R-SeO-CH_3

Proteins, peptides and amino acids are involved in Maillard reactions. Many observations were made suggesting that heat treatment at high temperatures increased the storage stability of foods. Products of the interaction of protein and carbohydrates exert an antioxidant activity (see Chapter 12). Maillard reaction products as natural antioxidants have not been commercialised, but many preparations are available as flavour concentrates produced on the basis of Maillard reactions. They exhibit antioxidant activity.[50,51] Application of Maillard products as food antioxidants is limited due to their brown colour, but a process for their decolourisation was proposed.[52]

13.6 Preparation and application of phospholipids as antioxidants

Phospholipids or phospholipid concentrates have antioxidant activity which has been observed in model experiments for a long time. Pure antioxidants would be too expensive as food additives, but phospholipid concentrates are available at a relatively acceptable price. They are active following different mechanisms (see Table 13.9). They may react with lipid hydroperoxides in a non-radical way. This procedure is applicable to all phospholipids possessing an amine group, such as phosphatidylethanolamine and phosphatidylserine. Nitrogen-containing phospholipids are more active than other phospholipid classes.[53] Phosphatidylcholine is attacked by lipid hydroperoxides after a cleavage mechanism, with formation of a nitroxyl

Table 13.9 Mechanisms of antioxidant activity of phospholipids

Mechanism	Reacting phospholipid	Products
Synergism	different phospholipids	regenerated antioxidant
Non-radical reduction	phosphatidylethanolamine	imine
Non-radical reduction	phosphatidylcholine	trimethylamine oxide
Metal scavenging	phosphatidic acids	undissociated salts

derivative. Phospholipids may also act as active synergists and heavy metal scavengers, especially acidic phospholipids, such as phosphatidic acids, act in this way.

Soybean lecithin is the most widely used phospholipid concentrate. It contains about 50–70 % phospholipids, the rest are triacylglycerols, free fatty acids and various other lipidic and non-lipidic minor substances, such as tocopherol, sucrose and α-galactosides.

Dietetic oils can be stabilised with the use of soybean lecithin.[54] The contribution of contaminating substances present in commercial lecithin may be very important.[55] Preparations containing nearly 100 % phospholipids are available as they are easily prepared by fractionation of lecithin with cold acetone at refrigeration temperatures (among other procedures). Lecithin is sometimes enriched in phosphatidylcholine, mostly by transesterification in presence of phospholipases, or by supercritical fluid carbon dioxide extraction. Lecithin is sometimes hydrolysed by other phospholipases with production of lysophospholipids. Their antioxidant activities are similar to those of original phospholipids, but they possess higher surface activity, useful in some food applications.

Even oxidation products of phospholipids have substantial antioxidative efficiency, such as trimethylamine oxide, cleaved from phosphatidylcholine by reaction with a hydroperoxide. It is very active as a synergist of tocopherols.[56]

Soybean lecithin is used almost exclusively, but sometimes it is mixed with ammonium salts of phosphatidic acids.[57,58] The use of other plant lecithins is also possible, however, their quality is generally lower than that of soybean lecithin. Egg yolk is a rich source of phospholipids which could find application despite their higher price compared to plant lecithins.

13.7 Organic polyvalent carboxylic acids as food antioxidants

Polyvalent organic acids such as citric, tartaric, uvic, malic, succinic, and ascorbic acids, are used as synergists. Citric acid is produced by microbial synthesis on a large scale and is applied in pure crystalline form. Tartaric acid is isolated from grapes or residues after wine production and is also supplied in a crystalline form. Ascorbic acid is most often synthesised from glucose and is available as crystalline acid or its sodium salt. Isomeric isoascorbic acid has no vitamin activity but is still active as a synergist. The application of organic acids to foods offers no specific problems and no preliminary preparation is necessary. Mixtures of citric or ascorbic acids with phenolic antioxidants and monoacylglycerols, glycols or any other solubilising agents are available on the market.

Citric acid was first suggested by Taussky for the stabilisation of edible oils, where it acts as a synergist of tocopherols. Citric acid is usually added to oil before its deodorisation. During the deodorisation, it is transformed

into itaconic, citraconic or aconitin acids, which are less active synergists.[59] Citric acid also has some metal-chelating activity.

Ascorbic acid may exert a beneficial influence as an antioxidant on the stability of beverages, especially soft drinks. Theoretically 1 mg of dissolved oxygen is eliminated by 11 mg of ascorbic acid.

Ascorbic acid (often applied as a sodium salt) is a powerful synergist of tocopherols and other phenolic antioxidants. It is often esterified on an industrial scale to improve the solubility in fats and oils. It is esterified with sulphuric acid, and then re-esterified with palmitic acid. Ascorbyl palmitate is subsequently purified by recrystallisation. Its poor solubility in edible oils and fats and its insolubility in cold water cause problems in direct application. Ascorbyl palmitate is available on the market either in pure form or as a mixture with phenolic antioxidants and monoacylglycerols, propylene glycol or other solubilising solvents.

13.8 Chelating agents as substances improving the stability of lipids against oxidation

Heavy metals are important promoters of lipid oxidation as they catalyse the decomposition of lipid hydroperoxides into free radicals. Chelating heavy metal ions into inactive complexes improved the stability of fats, oils and food lipids. Ethylene diaminotetraacetic acid (EDTA) was the first widely used metal-chelating agent, but it is a synthetic substance, and its application could raise objections among some consumers.

Phosphoric acid and sodium phosphate also possess metal-chelating activity and are available at a low price. Their disadvantage is low solubility in lipids, but in lipid foods they are active on the lipid–water interface. The most widely known natural metal chelator is phytic acid (*myo*inositol hexaphosphoric acid), which occurs in many plant materials. It was considered to be a negative factor from the standpoint of nutrition as it reduces the availability of calcium and iron. On the other hand, the chelating activity is sometimes appreciated as it reduces the pro-oxidant activity of iron and other heavy metal ions. Phytic acid is usually not isolated from plant materials, but food components rich in phytic acid, such as legumes, could be added.

Other substances already mentioned as inhibitors of lipid oxidation or synergists also possess a pronounced metal-chelating activity, such as phospholipids, especially phosphatidic acids, polyvalent organic acids, e. g. citric acid, flavonoids and other phenolics. Maillard products, especially melanoidins, can also bind iron and copper ions into inactive macromolecular complexes (see Chapter 14).

The deleterious effect of heavy metals on the stability of foods is still undervaluated. Even when oils and fats added to food products in the course of their preparation are essentially free of heavy metals, other food

components contain iron and copper at concentrations about 20–100 times higher than fats and oils. If only a very small fraction of this natural content of metal ions enters the lipid phase, the pro-oxidising activity should not be neglected. It should be borne in mind that not only ionic forms, but also undissociated salts, such as salts of fatty acids, and even some complexes (like haeme derivatives) are active.

13.9 Future trends

The greater acceptability of natural antioxidants in comparison to synthetic compounds will probably continue for the next 10–15 years. Only slight progress may be expected in the preparation procedures for isolation of natural antioxidants, excepting the use of supercritical carbon dioxide extraction of raw materials. There will be a tendency for the application of whole plant materials without previous fractionation by extraction or other methods. In case of extraction, supercritical carbon dioxide will be preferred for environmental reasons in spite of higher costs, which will be paid by the consumer. Subfractionation will probably not be looked for.

Scientists will look for new and/or less well known sources of antioxidants present in food raw materials that have been used only locally or occasionally, such as sweetgrass (*Hierochloe odorata*), which contains potent antioxidants (Miškusová et al., 2000).[60] Details will probably only be introduced in the preparation of extracts optimised for a particular material.

There is a tendency to utilise herbs, used for a long time in local medicine, such as gingko leaves or various herbs used in the Far East.[61] Dry roots of *Scutellaria baicalensis*, a plant from the *Labiatae* family, were studied as a potential source of natural antioxidants for use in processed foods. Chen et al.[62] found that acetone extract at the 100 mg kg^{-1} level was more effective than BHT at 200 mg kg^{-1} level in protecting canola oil from oxidation. Plants which may have medical activities should not be used as food additives without previous detailed proof of their safety. If they are used in medicine only small amounts are ingested for a short time which may be harmless. When applied to food products much higher amounts are ingested – often for long time periods – which may become harmful. The hepatotoxicity or carcinogenicity of some Far Eastern herbal drugs is known. Any preparation of extracts would hardly result in acceptable food antioxidants from these sources.

Research will be orientated to the optimisation of antioxidant content as it depends on plant variety, agrotechnology and climatic conditions. Transgenic plants with a higher antioxidant content will be developed and used only when the fear of consumers about transgenic products ceases. High flavonoid tomatoes have already been developed. Preparation of highly efficient antioxidants from such plants would be relatively

easy using any conventional method of supercritical carbon dioxide extraction.

As the application even of natural antioxidants will not be the priority, food producers will look for synergistic combinations of natural antioxidants with superior activities so that the total amount of natural antioxidants added to food can be minimised. Modern food processing technologies, such as good packaging in an oxygen-free atmosphere, refrigerated storage, and a reduction in time between food production and food consumption, will probably make the use of antioxidants less necessary.

In contrast, novel foods with a high antioxidant content will probably be developed for people suffering from atherosclerosis or similar diseases. However, these antioxidants should be easily resorbable and their activity in scavenging free radicals in blood plasma will be tested.

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14

Natural antioxidant functionality during food processing

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14.1 Introduction

Raw materials used for food preparation already contain various components which are able to inhibit lipid oxidation, and other natural antioxidants may be added before processing. Much interest has developed during the last few decades in naturally occurring antioxidants because of the adverse attention received by synthetic antioxidants and because of the world wide trend to avoid or minimise the use of artificial food additives.[1] One disadvantage of natural antioxidants is their low resistance against oxygen, particularly under exposure to light, high temperature and drying. Antioxidant changes continue during storage of food products.

Food products available on the market are usually further treated in catering plants and in households during meal preparation. The most important processing technologies are listed in Table 14.1. Despite the great importance of the course of these processes, relatively little has been published on changes in antioxidants, their interactions with other food components, and the effect of these changes on food resistance against oxidation. In general, the activity of natural antioxidants is greatly affected by complex interfacial phenomena in emulsions and multicomponent foods.

14.2 Types of changes in antioxidants during food processing and storage

Antioxidants present in foods change during the processing, in a similar way to other food components. Although many studies deal with estimated

Table 14.1 Types of food processing which affect antioxidants and oxidative stability of foods

Temperature	Type of process	Examples
Elevated temperature	Water as heat transfer medium	Pasteurisation Sterilisation Blanching Evaporation Extrusion
	Air as heat transfer medium	Drying Roasting, baking
	Oil as heat transfer medium	Frying Microwave heating
	Waves giving energy	Infrared heating
Ambient temperature	Effect of enzymes	Fermentation
	Effect of chemicals	Curing, smoking
	Effect of time	Storage (also cold and frozen storage)

Table 14.2 Changes in antioxidant properties of foods during processing and storage

Resulting resistance against oxidation	Examples of changes in foods, affecting the antioxidant activity
No effect	In case of moderately intensive processes positive and negative influences are counterbalanced
Increased resistance against oxidation	Transformation of antioxidants into more active compounds, such as glycosides into aglycones, formation of novel compounds, such as Maillard products, destruction of pro-oxidants, especially photosensitizers or heavy metals Inhibition of oxygen access, e.g. encapsulation
Decreased resistance against oxidation	Destruction of antioxidants by oxidation or interactions with other food components Losses of antioxidants by evaporation Improved access of oxygen, e.g. caused by drying Formation of pro-oxidants or their liberation from inactive complexes

losses of food nutrients, including antioxidants, through different operations of food processing, only the residual concentration of antioxidants has been determined in most cases, rather than total antioxidant capacity of foods.[2] Very different, and sometimes even opposite, effects on the intrinsic antioxidant properties of foods can occur during processing and storage,[3] as is evident from Table 14.2.

Table 14.3 Oxidative destruction of antioxidants in foods

Reaction type	Examples
Oxidation with lipid oxidation products	Oxidation with lipidic free radicals ROO* or RO* Oxidation with lipid hydroperoxides ROOH Oxidation with lipid dioxolanes
Oxidation with singlet oxygen	In presence of chlorophyll pigments
Oxidation with triplet oxygen	Oxidation of antioxidant free radicals A* Formation of quinones from phenolics
Oxidation with heavy metals	Metal ions in higher oxidation state

The most important losses of antioxidant activity occur as a result of chemical changes in antioxidants present in food materials.

Naturally, the most pronounced changes result from oxidation reactions occurring rapidly on heating or slowly in storage (Table 14.3). Antioxidants are oxidised either by lipid oxidation products (mainly hydroperoxides) or directly by oxygen, either dissolved in lipidic and aqueous phases or absorbed from the air. Tocopherols have been oxidised by ferric ions and hydroperoxides even in absence of oxygen with formation of tocopherones and their mixed dimers with the polyunsaturated acid residue.[4] Reaction products of antioxidants may retain antioxidant activity, for example *tert*-butylated hydroquinone (TBHQ) is oxidised either in an inactive quinone or in 2,2-dimethyl-5-hydroxy-2,3-dihydrobenzo(*b*) furan, which is a strong antioxidant.[5] Other changes are mostly neglected even when they affect food resistance more than oxidation processes, such as removal of water or evaporation of volatile antioxidants or pro-oxidants.

Modification of a recipe during preparation of foods or ready meals improves the stability against oxidation especially the addition of spices. Soy sauce improved the resistance against oxidation due to its metal chelating activity.[6]

Some examples of different changes and their effect on the resulting stability will be given in the following discussion of thermal processes, packaging, storage, etc.

14.3 Changes under heating when water is the heat transfer medium

Exposure of food components to temperatures above ambient conditions (during heat processing) is a major cause of detectable changes, not only of

Table 14.4 Changes in antioxidants during treatment of food with hot water or steam

Type of process	Type of precursors	Type of products
Enzyme denaturation	Oxidoreductases	Inactive enzymes
Hydrolysis	Heteroglycosides	Aglycones
Pyrolysis	Ascorbic acid	Degradation products
Extraction	Vitamins, phenolics	Loss in cooking water

nutritional quality, but also of antioxidant activity. Although some processes involving higher temperatures are used in order to produce positive changes, especially of the sensory value, they often result in loss of nutritional quality, and in some cases, in losses of their resistance against lipid oxidation.

The application of moderate temperatures, up to 100 °C, reduces the negative changes of nutritional quality. Various changes under these conditions are listed in Table 14.4. Food processing by application of such temperatures results in protein denaturation and aggregation reactions. Killing micro-organisms is the main reason for moderate heating. The denaturation of enzymes, which are also proteins, is often desirable. However, various changes occur in parallel, including changes in flavour, texture and colour as well as destruction of heat-sensitive nutrients. These factors have to be considered, and thermal processes must be carefully designed, to avoid overprocessing and unnecessary reductions in product quality.

Pasteurisation and blanching are similar thermal processes utilising relatively mild thermal treatments to achieve the desired stability of food products during subsequent storage. The pasteurisation is most often associated with liquid foods, while the more complex blanching is associated with solid foods. It is generally recognised that application of a higher temperature for a shorter time will lead to improved quality retention in pasteurisation and blanching.[7]

Boiling is often used for processing vegetables, fruit, meat and fish. Boiling temperatures are 100 °C (or slightly higher or lower, depending on the atmospheric pressure). They are not very different from the two industrial processes described above.

14.3.1 Changes during pasteurisation

Losses of vitamins are a good marker of negative changes due to thermal destruction. Transformations of tocopherols (vitamin E) are the best known changes in antioxidants during thermal food processing,[8] but they are only moderate during pasteurisation. Losses of ascorbic acid (an important inhibitor of oxidation) are used as an indicator of food quality and therefore the severity of pasteurisation, blanching, or the length of cooking.

These changes are due mainly to thermal destruction, and to a lesser extent, to oxidation. In fruit juices the main cause of colour deterioration is enzymatic browning of polyphenolics, catalysed by polyphenoloxidases in the presence of dissolved oxygen. Polyphenoloxidases destroy phenolic antioxidants so their rapid inactivation is desirable when the preservation of phenolics is important. Losses of ascorbic acid and carotenes are minimised by de-aeration as well. Moreover, an addition of these antioxidants before or after processing is quite common.

14.3.2 Changes during blanching

Commercial methods of blanching involve passing solid foods through an atmosphere of saturated steam or a bath of hot water, so that only the water in both physical states is the carrier of heat. Rapid heating of the food material deactivates enzymes, such as lipoxygenases, which would otherwise catalyse lipid oxidation. The primary products of lipoxygenase-catalysed oxidation – lipid hydroperoxides – would partially destroy natural antioxidants. The deactivation of polyphenoloxidases is also very useful for the protection of phenolics against enzyme-catalysed oxidation into the respective quinones, the antioxidant activity of which is very low or non-existent.

14.3.3 Changes during sterilisation

Another more severe thermal food process is referred to as a commercial sterilisation, which proceeds at higher temperatures than pasteurisation. Traditionally, this process has been used to achieve long-term shelf stability of canned foods, but it is now used for a broad range of food products. The intensity of the commercial sterilisation process is such that it results in significant changes in the quality characteristics of the product. Excepting the elimination of micro-organisms, however, these changes are usually more detrimental than positive. In canned fruits and vegetables substantial vitamin losses may occur in all water-soluble vitamins, particularly ascorbic acid, which is the most important antioxidant in these foods. Therefore, the presence of residual oxygen in the medium has to be minimised. In some foods, ascorbic acid or other antioxidants are added into the brine or syrup. The effect of processing conditions on vitamin changes in sterilised milk was discussed in detail.[9] Although traditional sterilisation processes result in losses of sensory and nutritional quality attributes, the processes are still widely used, and could be optimised to improve quality retention regarding the specificity of any particular commodity.

Lipoxygenases were deactivated during treatments of fruit juices at 70–90 °C, depending on the type of juice, for instance, the lipoxygenase activity did not substantially change in apricot and apple juices, but decreased in carrot, green bean and zucchini juices.[10]

14.3.4 Changes during boiling

Boiling is a very common procedure for food preparation. In this case boiling water transfers heat. It is useful to add food to hot water to shorten the time for enzyme deactivation, especially the deactivation of oxidoreductases. During boiling, the antioxidant activity of proteins is affected because of their denaturation. The effect on antioxidants is similar to that occurring during sterilisation. The heat denaturation of haeme pigments in foods of animal origin could increase the pro-oxidative effect of iron and thus reduce the activity of antioxidants. During boiling, antioxidants are partially extracted and remain in the boiling water. If the boiling water is not used but discarded these antioxidants are lost.

14.3.5 Changes during evaporation

Evaporation has historically been the primary technology for liquid concentration in the food industry. Evaporation proceeds at higher temperature, which is in contrast with other methods of concentration (such as membrane filtration or freeze concentration) where the main aim is to reduce heat damage. The evaporation darkens the colour of food, for example milk, partly because of the increase in concentration of solids, but also because the reduction in water activity promotes chemical changes, such as caramelisation of sugars, Maillard browning reactions or lipid–protein interactions. The evaporation temperature also destroys some types of heat-labile vitamins, reduces the biological value of proteins, and promotes lipid oxidation.

The effect of heat on natural antioxidants during evaporation is also obviously considerable, reducing antioxidant efficiency through thermal decomposition. To minimise the degradation due to prolonged heating during evaporation, it is necessary to minimise the residence time of food products at elevated temperature and the time needed to reach the evaporation temperature. Requirements for optimal evaporation include rapid rate of heat transfer, low-temperature operation through application of reduced pressure and efficient vapour–liquid separation.

14.3.6 Changes during the extrusion cooking

Extrusion is a process which combines several unit operations, including mixing, cooking, kneading, shearing, shaping and forming. If the food is heated, the process is known as hot extrusion or extrusion cooking. Water under pressure is the medium of heat transfer. For hot extrusion processes, high temperature (most often, 120–160 °C), high pressure and short time duration (less than 1 min) are typical conditions. The effect of these conditions results in products with moderate heat treatments. Therefore, nearly all of the heat-sensitive compounds remain in the final product. This high-temperature–short-time process still reduces some negative factors,

such as undesirable enzymes, micro-organisms and labile anti-nutritional components.

Nevertheless, vitamins are potentially lost during the hot extrusion process. Ascorbic acid, an extremely heat-sensitive vitamin and antioxidant, can be significantly reduced during extrusion, especially at higher temperatures. Losses of ascorbic acid and vitamin A are up to 50 % depending on the time and moisture content of the food held at elevated temperatures.[11] Losses of antioxidants rise moderately with increasing temperature and water content in the barrel. They may be prevented by addition of rosemary extract and by other antioxidants.[12] In contrast to hot extrusion, losses in cold extrusion are minimal.

14.4 Changes in functionality of antioxidants during processes when hot air is the medium of heat transfer

Heat is transferred more slowly by hot air than by hot water, therefore, higher temperatures and/or longer processing times are often used. Changes are much more intensive on the surface than in inner layers, so that antioxidants are formed or destroyed mostly at or near the surface.

14.4.1 Changes during roasting and baking

Roasting and baking belong to common, frequently used culinary operations. Heat is applied through hot air. During these operations, outer layers of the material are heated to 120–200 °C or even more, while in inner layers, the temperature does not exceed 10 °C. Changes in antioxidants and other food components are about the same in the inner layers as in the case of boiling; but in outer layers, pyrolysis, caramelisation and Maillard reactions proceed. The most important changes are summarised in Table 14.5. They may transform antioxidants into less active, or exceptionally into more active products.

Table 14.5 Changes in antioxidants during roasting and baking

Type of process	Type of precursors	Type of products
Caramelisation	Sugars, ascorbic acid	Chelating macromolecules
Maillard reaction	Sugars, amino acids	Chelating macromolecules
Strecker degradation	Dicarbonyls, amino acids	Dihydroheterocycles
Oxidation	Phenolics	Quinones
Hydrolysis	Glycosides, esters	Aglycones, phenolic acids

Table 14.6 Inhibitory activity of Maillard reaction products

Type of compounds	Precursors	Effect on food stability
Imines (Schiff bases)	Sugars, amino acids	Hydroperoxide reduction
Amino deoxy sugars	Schiff bases	Hydroperoxide reduction
Amadori, Heyns products	Amino deoxy sugars	Hydroperoxide reduction
Melanoidins	Premelanoidins	Metal chelation
Dihydrocyclic derivatives	Strecker compounds	Hydroperoxide reduction
Reductones	Dideoxytriulose	Free radical scavenging

If reducing sugars and free amino acids are present, Maillard reactions start slowly from 100–120 °C, and become very rapid at temperatures above 150 °C. Inhibitory activity of Maillard products is summarised in Table 14.6. Maillard products are formed at the maximum rate in media of intermediate humidity. An interaction product of glucose and lysine inhibits the peroxide formation in fats.[13] Some intermediary Maillard products, such as reductones, have high antioxidant activities in aqueous solutions or emulsions. Volatile products originating in various side reactions, such as dihydrofuran, dihydropyridine or dihydropyrazine derivatives, are further oxidised into substituted furans, pyridines and pyrazines, respectively. Oxygen present in the system is thus consumed, and the oxidation of lipids or antioxidants is prevented in this way.

Dehydroascorbic acid, produced by oxidation of ascorbic acid, can also participate in Maillard reactions, for example a pyrone derivative is formed by reaction with aspartame which shows moderate but significant antioxidant activity.[14] Analogous reactions may proceed with other peptides or amino acids.

Maillard products also possess some chelating activity. By binding heavy metal ions or active complexes into complexes possessing no oxidation-promoting activity, they partially protect other antioxidants against destruction.[15] An antimicrobial activity of Maillard products may be partially explained by the chelation of metal ions, essential for microorganisms.[16] The antioxidant activity of Maillard products is stronger in emulsions than in dry systems.[17] The mechanism of browning is very complex and until now is not yet fully understood. Some intermediary products may also act as weak pro-oxidants.

Aldehydic lipid oxidation products react with amino acids such as lysine, forming substituted pyrole derivatives possessing a moderate antioxidant activity.[18]

Maillard products were found to be active as oxidation inhibitors in biscuits, confectionery, sausages[19] and extrusion products.[20]

Many active phytochemicals are phenolics in their nature, which undergo various reactions during thermal processing, thus affecting the flavour of

foods. Chlorogenic and caffeic acids were found substantially to modify the flavour compounds formed in browning reactions between sugars and amino acids. Pyrazines, which are formed during roasting and baking, are characterised by their nutty and roasted flavours. The presence of chlorogenic and caffeic acid significantly reduced the amount of pyrazines. It also changed the relative distribution of different pyrazines and their ratios. These processes altered the total sensory quality.

Losses of natural antioxidants in relation to processing conditions and the formation of Maillard reaction products have been studied in coffee brews and tomato puree.[21] The antioxidant activities of both these evaluated food commodities were enhanced as the roasting time and temperature increased. In conclusion, although natural antioxidants are partially lost during heating, the overall antioxidant properties of heated foods can be maintained or even enhanced by the development of new antioxidants, such as Maillard reaction products.

Tocopherols and tocotrienols present in flour are partially destroyed (by about 25 % in the case of α-tocopherol) during cooking and baking. In rye bread, prepared by traditional technology, a loss of 50 % α-tocopherol was observed.[22]

14.4.2 Changes during drying

During evaporation the water content of the material is reduced to about 30–60 %. The drying is an operation when the water content is reduced to about 6–12 %, so that a solid product results. In original foods, lipid droplets, liposomes or membranes are protected by layers of hydrated proteins against oxygen access from the air. Owing to the dehydration, this protective layer is damaged so that lipids are exposed to the free access of oxygen, being transformed into a thin film on the surface of non-lipidic particles. Therefore, the oxidation of lipids (including sterols) is much more rapid in dry foods than in original water-rich foods, even at ambient temperature or under refrigerated storage. High concentration of free lipidic radicals involves heavy losses of antioxidants, and their low efficiency. Relative antioxidant activities are changed by water removal as they are often different in bulk oil and in an emulsion.[23] These oxidation reactions occur only during storage of dry foods. During the drying process, lipid oxidation is restricted because of short drying time (only a few minutes or still less in modern drying equipments), and the presence of water vapour in the atmosphere. Antioxidants are usually not damaged during drying, and their evaporation is only moderate.

Freezing is a process similar to drying in that water is being removed in the form of ice crystals. Lipids are again distributed as a thin film or in droplets, exposed to the access of air. Oxidative changes in lipids and antioxidants may be greater in frozen foods than in refrigerated foods.

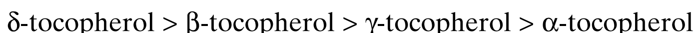
14.5 Processes where the energy is transferred as waves

Microwave and infrared energy are both transmitted as waves which penetrate food, and are then converted to heat. Nowadays, microwave cooking is the most versatile method all over the world. It is more energy efficient and reduces the cooking time compared to conventional heating. For industrial applications, microwave heating can be used for many processes, including tempering, dehydration, blanching, cooking, pasteurisation, and sterilisation.

Microwaves induce molecular friction in water molecules to produce heat, whereas infrared energy is simply absorbed and converted to heat. Heating by microwaves is determined in part by the moisture content of the food, whereas the extent of heating by infrared energy depends on the surface characters and colour of food. Some studies were published on the effect of microwaves on food constituents[24] and on the nutrient retention in food. Several publications dealing with certain aspects related to edible fats, such as olive oil[25] or other fats[26] or α-tocopherol[27] also appeared.

The effect of microwaves and air-drying of grape pomace on the physical and chemical parameters of grapeseed oil were investigated.[28] The results of microwave conditioning demonstrate the impact in producing oil from grapeseeds. Microwave treatment improved oil yield and increased viscosity, peroxide value, saponification value, and content of conjugated dienes. The content of γ-tocotrienol (the major representative of this class in grape-seed oil) increased, while concentrations of δ-tocopherol with high antioxidant activity decreased on heat treatment of grapeseed.

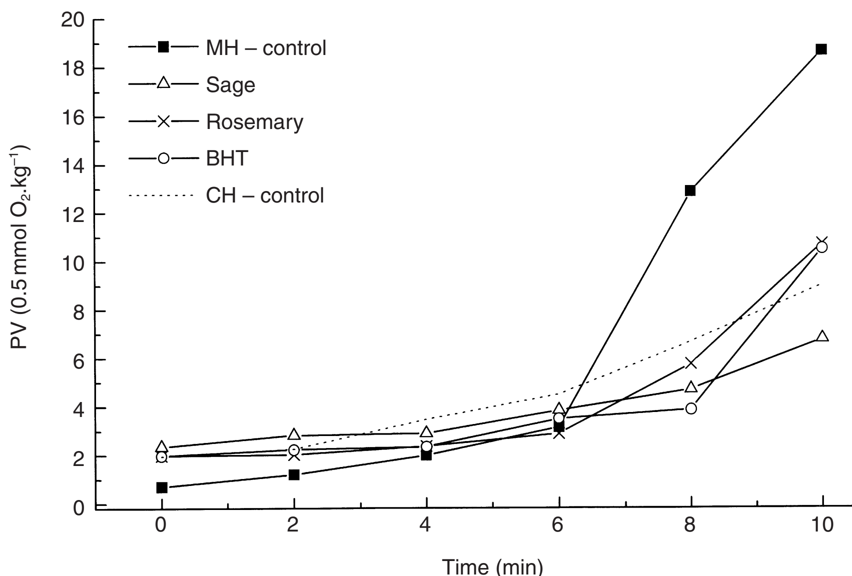
Tocopherols and other antioxidants were partially destroyed during microwave cooking of oilseeds.[29] During the microwave heating, tocopherols are destroyed[30] in the order of:


δ-tocopherol > β-tocopherol > γ-tocopherol > α-tocopherol

About 10 % were decomposed during the first 6 min of microwave heating, which is the optimum time. During this short period, the lipid fraction was not affected. The degree of tocopherol decomposition increased to 40 % during the next 6 min of microwave heating.[31]

The influence of microwave and conventional heating on the quality of lipids in refined cottonseed and hydrogenated palm oils was studied.[32] Exposing the oil samples to microwaves caused some hydrolysis of triacylglycerols into free fatty acids and accelerated the formation of hydroperoxides and secondary oxidation products. In general, the peroxide values of lipids heated by microwaves were nearly twice as high as those produced by conventional heating.

The oxidation stability of lard, rapeseed and sunflower oils was investigated during 10 min microwave and conventional heating experiments.[33] It was observed that the microwave heating accelerates their oxidation two or three times faster than conventional heating. This effect started when the



14.1 Oxidation stability (peroxide value) of lard in the presence of antioxidants at 0.1 % (w/w); MH = microwave heating; CH = conventional heating.

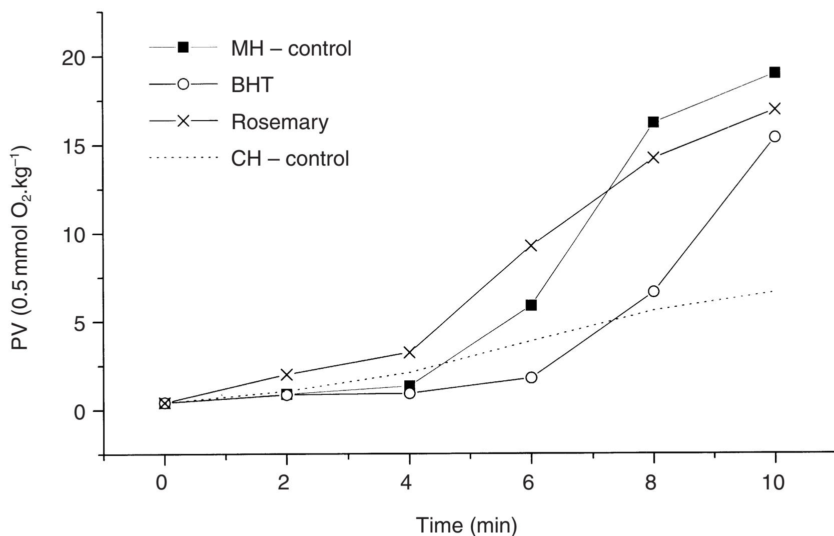
temperature of examined fat and oils rose over 100 °C. Below 100 °C, the rate of oxidation was relatively slow.

Some attempts were made to minimise the undesirable effect of microwave heating by the use of BHT and natural antioxidants, such as sage and rosemary extracts.[34] Addition of antioxidants was only effective for lard (Fig. 14.1), which contains no natural antioxidants in its native form. For sunflower oil the effect was only slight (Fig. 14.2), no significant effect was observed for rapeseed oil.

The maximum temperature of fat samples was about 155 °C after 10 min of microwave heating, and the same value was adjusted for conventional heating. No undesirable effect was monitored below the temperature of 100 °C.

14.6 Changes during processes where oil is the heat transfer medium

Deep fat frying is one of the most commonly used procedures for the preparation and production of foods. The presence of food moisture and atmospheric oxygen cause various chemical changes at high temperatures, such as polymerisation and degradation, for example. These reactions lead to changes in functional, sensory and nutritional quality of fried foods.



14.2 Oxidation stability of sunflower oil in the presence of antioxidants at 0.1 % (w/w); MH = microwave heating; CH = conventional heating.

Table 14.7 Changes in antioxidants during deep frying

Type of change	Precursors	Effect on stability
Evaporation	Water in fried food	Steam distillation of antioxidants
Oxidation	Polyunsaturated acids	Destruction of phenolics
Interchange of frying oil with fried substance	Proteins, lipids	Formation of chelating lipoproteins, reduction in protein activity
Reaction with fried material	Original antioxidants,	Decrease in their pro-oxidative activity
	Lipid oxidation products, Antioxidants, lipids	Decrease in pro-oxidative activity of oxidation products, but decrease in antioxidant activity
Polymerisation	Frying oil oxidation products, antioxidants	Activity decrease

During deep fat frying, food is added to on oil bath preheated at 130–200 °C for 2–10 min. Heat is transferred by frying oil. Changes in antioxidants during frying are listed in Table 14.7. At high frying temperatures antioxidants may be partially lost by evaporation, especially in combination with water vapour originating from water present in fried material. Steam enhances losses of nonpolar volatile material. Synthetic antioxidants, such as BHT or BHA, are lost relatively easily, for example 80 % BHT escaped

during french frying, and BHA almost entirely disappeared during frying of four batches.[35] BHA does partially dimerise during frying, forming dimers with carbon-to-carbon and ether linkages.[36] The dimers retain certain antioxidant activity. Because of volatility of common synthetic antioxidants, less volatile synthetic antioxidants suitable as additives to frying oils have been developed. They are more polar, containing hydroxyl groups in side chains, such as a methylol group – CH_2OH instead of a methyl group, or are dimers or trimers of common antioxidants. Following our opinion, they are not used in industry or are only used at a very limited extent. Losses of natural antioxidants are only small as their volatility is much lower than that of common synthetic antioxidants. They are also changed by oxidation reactions, for example carnosol is converted into miltirone (a quinone) and dehydrorosmariquinone.[37]

Another characteristic of deep frying is the low level of oxygen in frying oil. All oxygen originally dissolved in frying oil has already been consumed by oxidation in the time taken to heat frying oil to frying temperatures. Additional oxygen can enter frying oil only by diffusion from air, which is sometimes prevented by special metal cover sheets on the frying bath. Under these conditions autoxidation chains are short so that antioxidants are used up relatively rapidly. While carotene is rapidly oxidised with full access of oxygen, at low oxygen pressure, such as occurs during deep frying, carotenes act as free radical scavengers. Tocopherols in frying oils are decomposed by both direct oxidation with oxygen and by reaction with oxidised fatty acids.[38] The more unsaturated the frying oil, the more rapid the destruction of tocopherols under frying conditions. The concentration of free lipidic radicals is high in frying oils so that polymerisation is easy between two lipidic free radicals, for example:

$$2\ \text{R-O}^* \Rightarrow \text{R-O-O-R} \qquad [14.1]$$

or

$$\text{R-O}^* + \text{R}^* \Rightarrow \text{R-O-R} \qquad [14.2]$$

and also between a lipidic free radical and an antioxidant free radical, such as:

$$\text{R-O}^* + \text{A}^* \Rightarrow \text{R-O-A} \qquad [14.3]$$

which results in higher formation of mixed dimers. The tocopheryl free radicals reacted in three ways under deep frying conditions[39]:

1 They were oxidised with molecular oxygen into the respective free peroxy radicals.

$$\text{A}^* + \text{O}_2 \Rightarrow \text{A-OO}^* \qquad [14.4]$$

2 They could abstract hydrogen from lipids, forming lipid radicals (this reaction is very slow).

$$A^* + R\text{-}H \Rightarrow A\text{-}H + R^* \quad [14.5]$$

3 Two tocopheryl radicals could react with one another forming a dimer (reaction [14.6]).

If the concentration of antioxidants is high (it is more common in the case of natural antioxidants), like those of tocopherols in soybean or rapeseed oils used as frying oils, the concentration of antioxidant free radicals increases so that the chance of dimeric compound products being formed by interaction of antioxidant free radicals rises in spite of their low reactivity:

$$A^* + A^* \Rightarrow A\text{-}A \quad [14.6]$$

Spirodimers and trimers of tocopherol radicals have been detected and these dimers still possess moderate antioxidant activity.

The stabilisation of frying oils with flavonoids or phenolic acids is less efficient as they are only sparingly soluble in oil and their antioxidant activity is thus evident only on interfaces of frying oil and fried material. Destruction of tocopherols was retarded by addition of rosemary extract to frying oil because carnosic acid decomposed faster than tocopherols.[40]

Substituted polysiloxanes are often used as a permitted non-toxic additive to frying oils as they prevent the access of air oxygen into frying oil by diffusion. Silicones were used earlier for the same purpose. The concentration of non-oxidised lipid free radicals R* increases because of lack of oxygen and the possibility of their interaction with one another or with an antioxidant free radical increases. Non-polar dimers were detected in frying oils.

During foodservice frying of french fries, tocopherols are oxidised in frying oil, in parallel with oxidation of fatty acids.[41] An addition of antioxidants to frying oil reduced losses of tocopherols. The content of ascorbic acid in french fries decreased with decreasing tocopherol content. Citric acid had no synergistic effect on the inhibition of tocopherol decomposition. Catechins were decomposed during the destruction of tocopherols.[42] Catechins were destroyed more easily than gallocatechins and their gallates.

In the case of frying fish, polyunsaturated fish lipids are partially released into frying oil, which thus becomes more unsaturated and more easily oxidised. The consumption of both native tocopherols and added synthetic or natural antioxidants thus increases. The sensory value of frying oil and fried products thus deteriorates. Therefore, it is dangerous, with respect to quality, to use frying oil for frying chicken or potatoes immediately after frying fish.

Fried food contains heavy metals in far higher concentrations than frying oil. During deep frying, heavy metal derivatives are partially released into frying oil, where they enhance the deterioration. Fortunately, their activity is only low under frying conditions. Nevertheless, it is advisable to add some metal scavengers to frying oil, preferably citric acid.

In a similar way, an interchange between frying oil and fried food occurs

Table 14.8 Tocopherol losses during deodorisation (% of the original amount)

Tocopherol	Alkali refining	Physical refining
α-tocopherol	67	67
γ-tocopherol	61	59
δ-tocopherol	50	50
Total tocopherols	64	62

in the case of antioxidants. Many food components, such as proteins and phenolics, stabilise food under frying conditions.[43] Fried food rich in lipids and antioxidants (e.g. tocopherols) loses a part of its lipid fraction into frying oil together with antioxidants dissolved in the lipid phase. These antioxidants improve the resistance of frying oil against oxidation, but the stability of fried food on further storage decreases by loss of antioxidants.

Quite a different operation proceeds at extremely high temperatures, i.e. the deodorisation of edible oils. Deodorisation is the last step in oil refining, used for removal of volatile sensory objectionable substances. Fortunately, the process takes place in superheated steam free of oxygen and under substantially reduced pressure, so that the oxidation reactions are excluded or remain negligible. At deodorisation temperatures of 220–250 °C, tocopherols are partially evaporated and are collected in deodorisation condensate. Tocopherol losses depend on the temperature,[44] and may amount to 30 % of the original content. Relative tocopherol losses depend on the degree of methylation of the chroman ring of tocopherols, and on the resulting volatility of tocopherols (Table 14.8).

14.7 Changes during processes not requiring heat application

Under very common processing conditions, the reaction proceeds at ambient temperature or at a temperature not substantially different, without or with only negligible heat application.

14.7.1 Effect of smoking and curing meat products

Smoke contains numerous phenolic compounds, produced by pyrolysis of phenolics and lignin. They are mainly bound to meat proteins, but nevertheless, they increase the resistance to oxidation and protect tocopherols against oxidation. Lipoxygenases are partially deactivated in the process.

Curing consists of application of nitrates, nitrites and salt to protect meat against bacterial spoilage, and to prevent browning changes of the natural colour. Nitric oxide is an inhibitor of oxidases, such as lipoxygenases and cyclo-oxygenases, and deactivate haemoglobin by its conversion into

nitroxylhaemoglobin.[45] In presence of ascorbic acid (added to protect meat against discoloration), nitrite is reduced to NO, which reacts with ferricytochrome c, forming ferrocytochrome c nitroso derivatives.[46]

Nitrite can be regarded as an antioxidant, reducing the oxidation rate of polyenoic fatty acids, but its activity decreases by long cooking or heating to high temperatures.[47] Cooked ground pork and beef, treated with nitrite was stored at 4 °C, and the antioxidant activity was found to be 1.5–3 times better than in untreated meats.[48]

14.7.2 Changes in antioxidants during fermentation processes

Fermentation processes are enzymatic reactions taking place for a relatively long time at temperatures close to the ambient temperature so that lipids are damaged by oxidation processes to a negligible degree, and thus, antioxidants are not damaged by oxidation as well. Oxygen is partially replaced by carbon dioxide in most fermentation processes.

Hydrolytic processes could cause cleavage of esters or glycosides of phenolic antioxidants into the respective acids or aglycones. They are usually more active as antioxidants than the original compounds, such as quercetin and myricetin, which are more active than the respective glycosides.[49] Polyvalent organic acids with a synergistic activity may be formed.

Proteins are partially hydrolysed into free amino acids, which contribute as synergists to the stability of fermented products against oxidation. Soy, fish proteins, egg albumin and casein were hydrolysed by proteases into products inhibiting lipid oxidation, being synergists of tocopherols.[50] Soybean fermentation can release potent antioxidants from their inactive precursors, for example during the preparation of tempeh[51] or natto.[52]

In food processing, technical enzymes are used to reduce processing costs, to increase yields of extracts from raw materials and to improve the shelf life and sensory characteristics of foods. From the point of view of food deterioration through oxidation there are important oxidases. Glucose oxidase transforms glucose into gluconic acid in the presence of oxygen. It is used to remove sugar, to stabilise egg products, and to increase the shelf life of bottled beer, soft drinks, and other oxygen-sensitive foods. It has an advantage over chemical antioxidants because it does not lose its activity with time, as it is not itself oxidised.[53] Catalase decomposes hydrogen peroxide to form water and oxygen. It is used to provide oxygen for desugaring egg products by glucose oxidase.

14.7.3 Changes in antioxidant functionality during storage

The main reason for the application of antioxidants is to prolong the shelf life of food products by inhibiting the rancidification and other deterioration processes. Numerous papers deal with this operation.[54–56]

Several methods exist for food storage. Their advantages and disadvan-

Table 14.9 Methods of food storage and their effect on antioxidant functionalities

Process	Advantages	Disadvantages
Simple storage with free access of air	Low price, simplicity	Easy destruction of antioxidants
Vacuum packaging	Good protection, when low residual oxygen	High price
Packaging under inert gas	Good protection, when low residual oxygen	High price
Use of oxygen scavengers	Satisfactory, but no complete protection	Higher price, regulations should be observed
Addition of antioxidants	Satisfactory, but no complete protection	Higher price, regulations should be observed

tages from the standpoint of antioxidant preservation are compared in Table 14.9.

The most frequent application is to improve the oxidative stability of meat products. The use of antioxidant additives is best combined with adequate packaging techniques. Although muscle contains a multicomponent antioxidant defence system, processing operations alter the oxidative balance of muscle foods. The denaturation of muscle proteins is the main reason for this imbalance. Cooking meat releases iron, and salting changes its distribution. The deleterious effect of salt was recognised early, and encapsulated salt was suggested as a solution. To maintain a balance between pro-oxidants and antioxidants in muscle foods, modified atmosphere packaging, vacuum packaging or oxygen scavengers (such as addition of glucose and glucose oxidase) can help to control oxygen content. The most important step before packaging is to remove oxygen quickly. Additives can decrease the catalytic activity of iron. Decreasing the iron release from proteins is another good method of control. Protecting endogenous antioxidants, and increasing their content through dietary supplements and additives also help to minimise rancidity in meat products.

Foods of both animal and plant origin contain phospholipids which have antioxidant activity in lard or in sunflower oil.[57] The more polar, ethanol-soluble fraction is more active than the ethanol-insoluble fraction. A synergism exists between α-tocopherol and phospholipids, especially phosphatidylethanolamine, on storage of sardines and mackerel.[58] When tocopherol becomes almost exhausted, rapid oxidation of lipids starts. A possible explanation is a reaction between phosphatidylethanolamine with α-tocopheryl quinone.[59] Phospholipids decompose lipid hydroperoxides, thus enhancing the stability of stored foods.[60] Phosphatidylcholine is oxidised with formation of trimethylamine oxide, which reacts with another lipid hydroperoxide to form a keto derivative.[61] Both phosphatidylcholine and phosphatidylethanolamine decompose lipid hydroperoxides in a non-radical way.[62]

Under such conditions, natural antioxidants are rapidly destroyed unless metal chelators are present. In vacuum or inert gas packaging, oxidation processes, and thus destruction of antioxidants is minimised so that their content may be very low. The presence of spices is usually quite sufficient to keep oxidative processes under control. Lipid–protein complexes, formed on storage of meat products, possess moderate antioxidant activity.[63]

Mincing, cooking and maturing expose meat products to oxidative stress for a long time so that larger additions of antioxidants are desirable. In a study on this subject,[64] antioxidant properties of some compounds from vegetable sources, such as catechin, phytic acid, and sesamol were evaluated in two samples of minced, cured high-fat pork products, and compared with antioxidant properties of sodium ascorbate. The results indicated that the natural plant antioxidants slowed down lipid oxidation while sodium ascorbate seemed to have a rather pro-oxidative effect under experimental conditions. Its activity could be influenced by concentration. The conclusion was that the antioxidants tested acted in different ways on lipid and colour stability.

Onion and garlic juices were added to ground lamb and after cooking the mixture was stored. Onion juice was found more efficient than garlic juice in inhibiting the warmed-over flavour, due to slight oxidation and rancidity of the product.[65]

The effects of addition of antioxidants (α-tocopherol acetate and sodium ascorbate) on physical, sensory and microbial properties of buffalo meat nuggets were examined.[66] With increasing duration of storage, the quality of control products decreased, but that of products containing added antioxidants was stable and the stabilised nuggets were acceptable during 30 days of refrigerated storage. This is surprising as α-tocopherol acetate has no antioxidant activity. Therefore, it may be assumed that its cleavage with formation of active free α-tocopherol takes place. The additional use of vacuum packaging further improves the quality of refrigerated storage. In vacuum packaging the oxygen level is very low so that even the low addition of an antioxidant or the presence of weak antioxidants gives satisfactory results. Even carotenes may become active under such conditions.

Another important field of application is the stabilisation of fried products on storage. In fried materials a thin film of frying oil covers the fried product which is exposed to free access of oxygen on storage. The content of antioxidants is usually very low in frying oil as antioxidants have been consumed during frying. Even when fried products have been packed under nitrogen or in vacuum, they are still not sufficiently stable on storage, being attacked by residual oxygen in the atmosphere or dissolved in the fried material. The oxidation is much faster after the packaging has been opened. It is advisable to add antioxidants to the surface of fried food, for instance, to spike fried snacks with ground spices possessing antioxidant activity.

Vegetable products contain natural antioxidants[67] so that further additions of antioxidants are mainly unnecessary. Other examples of antioxidant application in products of vegetable origin are given in Chapter 15. The effects of packaging in a modified atmosphere and of cooking were evaluated in fresh-cut spinach,[68] which contains both flavonoids and vitamin C. The content of total flavonoids remained fairly constant during storage both in air and in a modified atmosphere, while vitamin C was better preserved in a modified atmosphere. A decrease in total antioxidant activity was observed during storage, particularly in spinach stored under modified atmosphere, which proves that antioxidants were gradually consumed by free radical scavenging. Boiling extracted 50 % total flavonoids and 60 % vitamin C in the cooking water, which could be used for further processing. Both ascorbic acid and total polyphenols were significantly destroyed during cold storage at +5 °C for 6 months.[69]

Wine flavonoids and anthocyanins are relatively stable in closed bottles but are easily oxidised once the bottle has been opened. The oxidation products easily polymerise. The flavour of wine, especially of red wine, is very much influenced by these processes so that wine should be consumed on the same day as the bottle is opened. Ascorbic acid added to extracts of elderberries and red grape skin caused higher degradation of anthocyanins during their storage for 6 months due to their reduction to colourless leucoanthocyanidins.[69]

Changes in antioxidant properties of green and black tea infusions as a result of processing and storage were evaluated by measuring their chain-breaking activity, oxygen-scavenging activity and redox potential.[70] The results showed that pasteurisation, storage and forced oxygenation caused increased browning in both green and black tea extracts. These changes were accompanied by increasing colour intensity. Green tea catechins and residual catechins of fermented black tea were obviously oxidised into the respective quinones, which were then dimerised. The dimers possessed similar chemical structures as black tea pigments – theaflavin and thearubigin.

Not only lipids, but also other lipophilic components are oxidised on storage, such as essential oils. The composition of products of their oxidation, including the ratio of *cis* and *trans* isomers, is influenced by natural antioxidants in food material, such as rosemary extracts.[71] Similarly to oxidised lipids, oxidation products of essential oils also react with food proteins,[72] and special sensory active reaction products are formed. The relative activity of natural antioxidants (expressed as protection factors or antioxidant indices) is only moderate in mixtures of lipids with proteins, and similarly in mixtures of essential oils with proteins, being much lower than in systems containing no proteins.

Although the loss of nutrients as a consequence of food processing and storing has been widely documented, relatively few data have been available up until now on the potential interactions of natural (and of course,

synthetic) antioxidants with other food components during industrial food processing, household cooking, and storage both in distribution and in households.

14.8 Future trends

Changes in synthetic antioxidants during food processing and storage are relatively well known, as are the interactions of their oxidation products with other food components. However, modern consumers ask for natural products, free of synthetic additives. Therefore, the application of natural antioxidants will probably continue even in future,[73] and it will be necessary to study their changes and interactions in more detail. The best known and most widely used compounds with antioxidant activity will remain tocopherols, carotenoids and ascorbic acid. They have been the object of numerous studies, but usually only their changes in edible fats and oils, and in model systems were investigated. Their behaviour in such complex mixtures as foods and ready meals is far less well understood. Plants such as rosemary, sage, thyme, savory, marjoram, nutmeg and ginger contain different phenolic compounds possessing antioxidant activities. Their structures and antioxidant activities were studied in detail, but the course of their oxidation and their interactions with other food components should also be studied in future.

Vitamin E and carotenoids belong to natural antioxidants, which are deposited in adipose and other tissues of farm animals so that their fat and meat become more stable. The economics of this process should be evaluated from complex aspects, as until now it seems that the application of antioxidants after slaughtering and during processing of food materials is cheaper and more efficient.

Consumers believe that foods rich in antioxidants may afford a degree of protection against free radical damage not only in foods, but also in the human body, protecting against cardiovascular diseases, damage of nucleic acids, and other deteriorative processes. The absorption of tocopherols and carotenoids into the blood stream is well known, but much less has been published on the fate of other antioxidants and their reaction products. Some antioxidants may not be resorbed in the intestinal tract at all, even when they are active in foods.

A concerted effort should be made to eat a well-balanced diet, which includes a range of foods and beverages rich in natural antioxidants, such as fruit, vegetables, cereals, nuts, tea or wine. It is a very serious challenge for the food industry to produce a new generation of food products with enriched content of natural antioxidants. The question is open, however, as to how much such food would make people really healthier and happier. But this is probably not the task of food scientists and technologists.

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15

The use of natural antioxidants in food products of plant origin

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15.1 Introduction

Foods of vegetable (plant) origin are stabilised by addition of antioxidants less frequently than foods of animal origin, perhaps with the exception of edible and essential oils.

In contrast to animal foods, foods of vegetable origin usually contain natural antioxidants, such as tocopherols, carotenoids or flavonoids in sufficient amounts. The pro-oxidative activity of iron and other heavy metals is less dangerous in plant materials than that of haeme derivatives in animal products, as plant materials usually also contain metal-chelating agents. The only important oxidation catalyst in raw materials and foods of vegetable origin is a group of lipoxygenases and related enzymes.

Synthetic antioxidants prevailed for the stabilisation of foods of plant origin in earlier applications, but in the last decade or two, natural antioxidants have been intensively applied, following consumers' wishes.

Lipids in foods of vegetable origin are usually more unsaturated than lipids of foods of animal origin, therefore, the initiation rate of oxidation reactions is higher and natural antioxidants, originally present in foods are more rapidly consumed than in lard or tallow and other animal fats. The stabilisation of products of vegetable origin against autoxidation is thus less efficient than the stabilisation of animal products. Protection factors of comparable antioxidants are several times higher in lard than in edible oils.

The initial concentration of natural antioxidants in plant foods is already near the optimum so that a further addition of antioxidants has only a small effect, but it is useful for those cases when rapid decomposition of

antioxidants is expected. For instance, both synthetic and natural antioxidants can be added to foods heated to high temperature or stored for a long time.[1]

15.2 Application of antioxidants in edible oils

15.2.1 General remarks

Edible oils are mostly refined before their consumption so that the resulting taste is sensorially neutral. Even very small traces of volatile products are easily perceived by the consumer. In the USA and many European countries, consumers prefer oils without any flavour, while in some European, Asian and African countries, weak natural oily, nutty or buttery flavour notes are tolerable, and often even required.

Oils produced from seeds where lipids were attacked by lipoxygenases contain traces of oxidised products and are refined only with difficulty. They also easily turn rancid on storage. In contrast to refined oils, virgin olive oils and some other virgin oils contain volatile oxidation products in small amounts, which impart a characteristic herbal flavour to olive oil or a weak walnut flavour to sunflower oil.

Edible oils become rancid on storage, the type of rancid off-flavour depending on their fatty acid composition (for example, it may become painty and fishy in oils containing linolenic acid, such as rapeseed oil) and the presence of minor components (for example, in flavour-reverted soybean oil). Edible oil producers try to prolong the shelf life of edible oils by different techniques, including the addition of antioxidants. The presence of natural antioxidants should always be taken into account, when appropriate levels of added antioxidants are considered.

All edible oils contain tocopherols in different total amounts and different ratios of α-, β-, γ- and δ-tocopherols. Tocopherols are partially removed during the deodorisation process, which is the last step in refining edible oil. They are often restored by adding nature-identical preparations or natural tocopherol concentrates. Instead of tocopherol concentrates, some speciality oils rich in tocopherols may be added or mixed with edible oils, for example, oil from grapefruit seeds,[2] which are wastes of grapefruit juice production.

Soybean and rapeseed oils are rich in tocopherols, especially γ-tocopherol. Both these oils contain linolenic acid, which is easily oxidised and requires more tocopherols for their stabilisation. γ-tocopherol is more active in bulk oils than α-tocopherol.

α-tocopherol acetate is often added to refined oils. Tocopherol acetate is a good vitamin source, but has no antioxidant activity in edible oils unless it is hydrolysed. After consumption, it is hydrolysed in the body, the hydrolysis product is absorbed, passes through the intestinal wall and may then act as an antioxidant in the blood serum.

Citric acid is often added to edible oils before their deodorisation and its decomposition products are also efficient as synergists of tocopherols. Taussky first applied this method for soybean oil refining[3] .

15.2.2 Stabilisation of sunflower oil

Refined sunflower oil belongs to edible oils with a high sensory and nutritional value, but it is rather unstable in storage, due to the high content of linoleic acid (between 60 and 70%) and relatively low content of γ- and δ-tocopherols, which are more active antioxidants in oil than α-tocopherol – the main tocopherol in sunflower oil.

In addition to tocopherols and citric acids, more active antioxidants are often required, such as different phenolic compounds. Until recently, synthetic antioxidants were mostly used, but recently many authors have proposed natural plant extracts. Antioxidant properties of many herbs and spices have been reported to be effective in retarding the development of rancidity in oils. It is known that a number of natural extracts from selected herbs, spices and some vegetables, are stable to autoxidation due to the presence of natural phenolic compounds. The antioxidant activity of these extracts depends on the type and polarity of extraction solvent (see Chapter 13), isolation procedures and purity and identity of active components of extracts from these raw materials. Only a few examples of the many references can be given here.

Rosemary oleoresin is the first choice, as it is commercially available, but applications of other spice extracts are possible. Antioxidant activities of six plant extracts (catnip, hyssop, lemon balm, oregano, sage and thyme) were evaluated in sunflower oil at 60 °C in the dark. Sage extracts (600 and 1200 $mg\,kg^{-1}$) showed the highest antioxidative activities during both the first and second stages of oxidation. Thyme and lemon balm extracts inhibited the generation of hexanal and pentanal more than the formation of conjugated dienes and oregano extract was also active.[4] In contrast, catnip extract showed pro-oxidative effects during incubation. Many extracts from Turkish spices had antioxidant activities in sunflower oil at 70 °C. The most active extracts were those prepared from plants of the *Labiatae* family, such as rosemary, sage, marjoram, thyme, savory and oregano.[5]

The antioxidative effect of an ethanol extract from savory (*Satureja hortensis* L.) in sunflower oil was investigated during high temperature treatment (at 180 °C). The extract improved the oxidative stability of sunflower oil even after 50 h at 180 °C and inhibited the oxidative processes more than the thermal processes under these conditions.[6] Main components of the extract are thymol and carvacrol,[7] the former being more active than the latter.

It has been known for many years that sesame oil is highly resistant to oxidative deterioration compared with other edible oils. Unsaponifiable

matter from both roasted and unroasted white sesame seeds had antioxidant activity in sunflower oil, which increased with increasing concentration.[8] It is not necessary to isolate the unsaponifiables. Mixtures of sunflower and other edible oils with sesame oil also improve stability against rancidification.

Another example concerns a less common oil, produced from industrial wastes. Antioxidant activities of a grapefruit seed extract, which contains high levels of tocopherols, citric acid and ascorbic acid, were investigated in a mixture of soybean and sunflower oils.[9] The product is recommended for fats and foods stored for a long time at room temperature, but it is not suitable for frying.

More examples can be found in Chapter 10, where potential natural antioxidants are discussed. Until now, the application of natural antioxidants has been very limited, mostly because of their high price and variable activity.

15.2.3 Stabilisation of soybean oil against rancidification

Soybean oil contains a high percentage of linoleic acid (about 50 % total fatty acids), but also a small amount of linolenic acid. Soybean oil suffers from flavour reversion, which is due to specific trace fatty acids, this can hardly be prevented by antioxidants.

Butylated hydroquinone (TBHQ) may support tocopherols in stabilising edible oils against rancidification. Stabilisation with chlorogenic acids – a very common food phenolic component – is significant, but metal chelators (phytic or citric acids) have no synergistic effect.[10] Extracts from waste materials, such as peanut hulls[11] or mung bean hulls[12] were found to be efficient, especially at higher concentrations. Phenolics isolated from Thompson grape bagasse (in the amount of 0.3 or 0.5 % total phenolics) are equally as active in soybean oil as TBHQ and more active than butylated hydroxyanisole (BHA) under the same conditions.[13] Gallic acid was found to be more active than δ-tocopherol both in bulk soybean oil and in emulsions.[14] Natural antioxidants as additives are less important for this purpose, with the exception of rosemary extracts, which are commercially available and have synergistic activity in the presence of tocopherols and other antioxidants.

Antioxidant properties of tocopherols and sesamol or their mixtures at various concentrations were evaluated in tocopherol-stripped soybean oil, when heated in a microwave oven.[15] The concentrations of 200 or 400 $mg\,kg^{-1}$ for γ-tocopherol, and 50, 200 or 400 $mg\,kg^{-1}$ for sesamol were found to be the most effective.

15.2.4 Stabilisation of rapeseed oil

Rapeseed oil is stabilised with difficulty, in spite of its relatively low linoleic acid content, as it contains higher amounts of extremely oxylabile

linolenic acid. Nevertheless, it could be stabilised efficiently by 0.02–0.10% rosemary or sage extracts[16] or 0.5% ethanolic allspice extract.[17] Antioxidative effects of ethanolic extracts from thyme, allspice, clove and nutmeg were detected in rapeseed oil.[17–19] Tea extracts are powerful antioxidants owing mainly to the presence of flavonols,[20] as tested during heating at 60°C.

15.2.5 Stabilisation of other vegetable oils

Sesame oil has medium unsaturation, but contains several natural antioxidants of the lignan structure, such as sesamol, so that it is not necessary to stabilise it. Peanut oil also has medium linoleic acid content but it is less stable as it contains only a relatively low amount of tocopherols, and no other native antioxidants. Its stability can be significantly improved by the addition of catechin, followed by rosemary oleoresin and phospholipids, while tocopherols were found to be less active.[21]

Another edible oil of comparable unsaturation is corn oil. Green tea catechins were tested at 50°C. Epigallocatechin, epigallocatechin gallate and epicatechin gallate were better antioxidants than epicatechin and catechin at the concentration level of 140 μmol kg^{-1}. Used as the reference compound, gallic acid was more active than propyl gallate and both were more efficient than epicatechin and catechin.[22] Antioxidant activities of caffeic acid and the related hydroxycinnamic acid were tested in corn oil in comparison with other naturally occurring phenolics.[23] Rosmarinic acid had the highest activity, followed by caffeic acid.

Olive oil is relatively stable as it is mainly monounsaturated and contains several natural antioxidants of the tyrosol series, so that its stabilisation is mostly unnecessary, even during thermal treatments such as cooking processes.[24] Dry powdered oregano and rosemary were tested at a concentration of 2%. Stabilised oils showed good shelf life and were sensory acceptable.

Liquid vegetable shortenings are still less unsaturated than edible oils, but are often stored for several months. A liquid canola shortening was stabilised[25] more efficiently with TBHQ than with butylated hydroxytoluene (BHT) and BHA.

15.3 Application of emulsified fat products

While edible oils do not contain more than minute traces of dissolved water, various edible products are emulsions of the oil-in-water or water-in-oil type. Even more complicated systems of multiple emulsions may be present. The aqueous phase influences the antioxidant activity, so that emulsions should always be tested and the results obtained for edible oils cannot be used for efficiency predictions.

15.3.1 Stabilisation of margarines

Margarines are water-in-oil emulsions (similar to butter), containing β-carotene as a nature-identical additive, or related substances, imparting yellow colour to the product. Carotene contributes to stability against oxidation. The oil phase contains tocopherols, as they were already present in edible oils used for the production of emulsified products. In the presence of water, some more polar antioxidants lose their antioxidant activity as they are partially dissolved in the aqueous phase or are accumulated at the oil–water interface. Non-polar synthetic antioxidants, such as TBHQ, BHT, BHA or higher gallates, may be added to protect the oil phase.[26] The combination of 0.1 % ascorbyl palmitate and 0.01 % propyl gallate was the most efficient antioxidant in a dietetic margarine rich in (n-3) fatty acids,[27] followed by TBHQ, while quercetin and catechin were less efficient. Among many natural antioxidants, rosemary extracts are also useful. Lecithin is sometimes added as a synergist.

Betaine contains a tetramethylammonium group, similar to phosphatidylethanolamine, so that a similar reaction mechanism may be expected (see Chapter 13). Its addition to margarine retarded autoxidation and improved the sensory value of the margarine,[28] stored for 3 months at 4–6 °C.

All emulsified fat products have a shorter shelf life than edible oils, because of their lower resistance to microbial spoilage. For this reason, they are stored under refrigeration so that the degree of autoxidation is low and naturally present tocopherols are usually sufficient to stabilise them. Sunflower oil may be less resistant because of high polyunsaturation, often requiring an addition of antioxidants.

15.3.2 Stabilisation of mayonnaise

In contrast to margarine, mayonnaise is an oil-in-water emulsion. The oil phase (about 70 % w/w) usually consists of edible oil. The antioxidant activity of antioxidants in mayonnaise may differ greatly from that of the edible oil used for its preparation. For instance, green tea extracts were active as antioxidants in corn oil or in liposomes,[29] but were pro-oxidative in oil-in-water emulsions. Black tea extracts were also proposed. Oleoresin preparations from spices were found to be fairly efficient.[30] In a sample of mayonnaise stabilised by the addition of a mixture of BHA and BHT, stability against oxidation was affected more by salty substances (sodium chloride substitutes) than by antioxidants.[31]

A new type of product was introduced for the prevention of cardiovascular diseases: mayonnaise enriched with (n-3) polyunsaturated fatty acids by the addition of fish oil. Such products require effective stabilisation against oxidation, as fish oils are very oxylabile and their rancidity is extremely objectionable. Tocopherol was compared with oil soluble (Toco 70) and water soluble (Grindox 1032) preparations. The latter mixture

better protected against sensory deterioration than the oil soluble preparation.[32] It is interesting that the antioxidant preparations also affected rheological properties of the product. Addition of TBHQ increased the safe storage time.[33] The addition of antioxidants and metal-chelating agents was found to be useful even in nitrogen-treated mayonnaise.[34]

15.3.3 Stabilisation of salad dressings

Salad dressings are oil-in-water emulsions, analogous to mayonnaise, therefore, their stabilisation shows great similarity. Several types of salad dressings are often stored after opening for a long time in a refrigerator so stabilisation against rancidification is useful. The advantage of salad dressings is their characteristic flavour. This allows various spices or spice extracts to be used for their stabilisation. Spices of the *Labiatae* family are active, especially rosemary and sage and to a lesser degree, summer savory and oregano.[30,31] Ground nutmeg belongs to those spices with antioxidative activity, the flavour type of which allows the application to salad dressings,[35] but the activity depends on the grinding procedure.

Phenolic antioxidants can also be used. A salad dressing based on soybean oil was efficiently stabilised by addition of BHA and a metal chelator,[36] a dressing enriched by (n-3) unsaturated fatty acids was efficiently stabilised[33] by TBHQ. The application of BHA, BHT, TBHQ and their mixtures has been patented, with improved shelf life of fish oil salad dressing.[37] For an oil-in-water emulsion dressing (50 % rapeseed oil, 3 % vinegar, 2 % sugar, 1 % mustard, 0.7 % salt, 0.1 % citric acid, 0.1 % potassium sorbate, and 41.1 % water) of dried leaves of summer savory (*Satureja hortensis* L.) or, more significantly, of rosemary (*Rosmarinus officinalis* L.), resulted in substantially better protection against oxidation than the addition of 80 mg propyl gallate per kg (the standard concentration for this type of product) during dark storage at 19 °C for up to 24 weeks.[38]

15.4 Stabilisation of frying oils and fried foods

Frying oils must contain non-volatile antioxidants, otherwise the antioxidants are gradually lost by evaporation at high frying temperatures, usually in the range of 160–180 °C and in a stream of water vapour formed from water present in the fried material. Several non-volatile synthetic antioxidants have been developed, but they are not on the list of chemicals permitted as food additives.

Tocopherols are already present in original edible oils used for deep frying, and citric acid may be useful as a synergist. Other prospective antioxidants should be tested to find out whether their activities remain satisfactory at frying temperature.

Many antioxidants with high inhibitory activity at storage temperatures rapidly lose their efficiency with increasing temperature. Sesame oil, especially oil from roasted sesame seeds, was found to be very efficient for frying croutons, probably due to the high sesaminol content.[39] Such oil could be used in a mixture with other less stable oils.

In soybean oil used for deep frying, BHA was found to be more efficient than sesamol or α-tocopherol.[40] Oil for frying potato chips is stabilised with 0.2–1.0 % distillate from deodorisation of olive oil,[41] the activity increasing with the increasing concentration of the antioxidant. Rosemary and sage extracts remain relatively efficient. During the frying of potato chips in rapeseed oil, additions of BHA, BHT and TBHQ were compared with those of δ-tocopherol, lecithin, ascorbyl palmitate and rosemary extract,[42] TBHQ being more efficient than other antioxidants, followed by lecithin.

An addition of 0.1 % rosemary extract and 0.02 % ascorbyl palmitate prevented tocopherols from decomposing on deep frying and reduced the rate of oxidation.[43] Even smaller additions of rosemary extracts were efficient in our experiments with french frying to inhibit both oxidation and polymerisation.[44] Oxygen is rapidly consumed at frying temperatures so that the oxygen level is very low in frying oils. Carotenoids show some activity under such conditions because carotenoids are active antioxidants at low oxygen levels (see Chapter 13).

The stability of frying oils against oxidation is substantially improved by substituted polysiloxanes.

Other properties of inhibitors are required for stored fried foods. They contain a thin film of frying oil on their surface, which is exposed to free access of air oxygen. The autoxidation is thus rapid even at low temperatures, such as under refrigeration. The frying oil film contains only traces of tocopherols as they have been decomposed during frying, but may contain residues of antioxidants, added previously to the frying oil. Frying oil thus shows only low resistance against oxygen attack.

Frying oil stability is moderately improved by several types of antioxidants. Peanut oil used for frying potato chips, was stabilised by ascorbyl palmitate, either alone or in a mixture with synthetic antioxidants, such as BHA, BHT and propyl gallate.[45] Antioxidant salts were used for stabilising potato chips (2 % on the salt basis),[46] TBHQ being better than either BHA, or BHT. Rosemary and sage extracts improved the shelf life of potato cisps.[47] Interesting flavour notes are imparted by onion and garlic powders. They extended the shelf life of potato chips to 3 months.[48]

Palm olein received great attention as a novel frying oil because of its relatively low degree of unsaturation. This improves the shelf life of fried products. Rosemary oleoresin or sage extracts were efficient in a mixture with BHA and BHT for deep frying and storage of potato chips.[49] A mixture of oleoresin rosemary and sage with citric acid produced potato chips with a good shelf life and acceptable sensory properties.[50] They can also be used separately, but a combination was found to be more efficient.[51]

Citric acid helps to neutralise alkaline contaminants produced in frying oil, which contribute to a rapid deterioration in quality.[52] Ground oregano was found to be suitable for stabilising potato chips fried in palm olein, and other edible oils, such as cottonseed, soybean and olive oils.[53]

Banana chips were fried in refined palm olein and then stored at 65 °C. An addition of BHT improved the stability more than butylated hydroxyanisole.[54] In tapioca chips fried in the same medium, TBHQ was found more active than either BHT or BHA.[55]

Noodles deep fried in rice bran oil or palm oil were stabilised with 0.02 % TBHQ more efficiently than with tocopherol, BHA, or ascorbyl palmitate with citric acid.[56] After frying in a mixture of 70 % palm oil and 30 % rapeseed oil, instant noodles were stabilised much more efficiently with TBHQ, followed by a mixture of ascorbyl palmitate and citric acid, while BHA and tocopherol were almost inefficient.[57] When stabilising fried instant noodles with tocopherols, a much better effect was observed with δ-tocopherol than with α-tocopherol, which was still better than BHA or BHT for noodles fried in palm oil. Ascorbyl palmitate was a better synergist than citric acid.[58]

Antioxidants may be applied on the surface of fried food after frying. It is difficult to stabilise french fries or potato chips, which are rather sensory neutral and antioxidants could produce a negative effect on their sensory value. It is, however, possible to stabilise fried snacks in this way, as the application of spices on the surface would not decrease the sensory acceptance in spite of a sharp taste. The application of rosemary or sage extracts was found to be more suitable when they were added to frying oil then on application to the surface of fried potato crisps.[47]

15.5 Application in products from nuts and oilseeds

Nuts such as walnuts, hazelnuts, almonds and peanuts are best stored in their shells, where they are sufficiently stable for a year. After shelling, dehulling and roasting they may rapidly deteriorate. Dry roasted nuts are difficult to stabilise other than by applying spices on their surface. It is thus better to store them in a vacuum or in an inert atmosphere.[59] In the case of roasting in hot oil, about 2–3 % oil remains on the surface of peanuts. Such products can be stabilised similarly to fried products.

The best way is to add antioxidants into frying oils before the operation. Additions of rice bran oil containing natural antioxidants improved the shelf life of nuts roasted in soybean or rapeseed oils.[60] Salt mixed with TBHQ can be used for the stabilisation.[61] The same may be said of soybeans (used in the Far East), poppyseed (used in Central Europe) or flaxseed and hempseed (used in Baltic countries).

When seeds are crushed or ground to produce a paste, like peanut butter, addition of antioxidants may be useful to improve stability during storage.

Tocomix D (a mixture of α- and δ-tocopherols, citrate esters of monoacylglycerols) and Embanox 10 (a mixture of BHT and BHA) were used with equal success.[62] Rosemary oleoresins were also active.[63]

15.6 Application in cereal products

Numerous cereal products are on the market, but only durable products have to be stabilised by the addition of antioxidants. Ascorbic acid and other antioxidants are added to dough for other purposes.

15.6.1 Application in cereal grains, flour, breakfast cereals

Cereal products such as dehulled rice, white flour or grits, are not usually stabilised. In whole grain flours, enzymes have to be inactivated to increase shelf life. After heating, natural antioxidants from brans are sufficient for lipid stabilisation.

Antioxidants may be added to breakfast cereals, the shelf life of which should be long. Rice bran, stabilised by extrusion, has a high antioxidant content, and thus it was found suitable as a component for breakfast cereals with high stability.[64] Aqueous extracts from other whole grains or brans, tea extracts and fruit extracts may be used with good results.[65] The catalytic effect of iron is eliminated by phytic acid.[66] Natural amino acids – methionine and cystine, phospholipids and uric acid – were active as antioxidants in breakfast cereals.[67] The nutritional value of breakfast cereals is increased by the addition of flavonoids and related plant antioxidants, which extend shelf life.[68] Breakfast cereals, fortified with vitamin A (a very unstable compound) were efficiently stabilised with commercial synthetic phenolic antioxidants.[69] Browning products, often present to improve the flavour, may also help their stabilisation.

15.6.2 Application in extruded products

Another kind of durable cereal products are extruded products, such as flat bread. Antioxidants are best added with flour and other additives to the extruder barrel. They are thus uniformly distributed in the extruded product. Spices are useful for stabilisation as they impart interesting flavour notes to the final product.

The agreeable colour of extruded snack products, due mainly to carotenoids, rapidly disappears on storage. Therefore, it needs to be stabilised. An oil-soluble liquid rosemary extract (4942 Rosmanox) and its mixture with tocopherols (4993 Rosmanox E) preserved the natural coloration for more than 7 months.[70] Various snacks are easily stabilised with salt-containing antioxidants.[46]

Mongra – a popular snack in India – is prepared from legume flour and oil. The product containing cottonseed oil was efficiently stabilised by butylated hydroxyanisole.[71] Fried snacks based on corn flour were prepared using 0.01 % TBHQ as an antioxidant.[72]

15.6.3 Application in cakes, crackers, cookies and similar durable bakery products

Some cereal products contain added fat, mostly hydrogenated edible oil and/or fillings also rich in fat. Even when hydrogenated oils are rather stable against oxidation, off-flavours may arise on storage. Application of antioxidants may be useful in such products as the shelf life should be very long. Both synthetic and natural antioxidants or mixtures of both additives are available for these speciality products. Certain spices, Maillard products and essential oils could be tested.

Sugar-snap cookies may be stabilised by BHA, but efforts were made to replace it with natural antioxidants. Ferulic acid and sodium phytate were found to be suitable as antioxidants.[73] Cookies containing phytate were sensorially fully acceptable.

In sugar cookies, BHT may be replaced by casein, whey proteins or Maillard reaction products without any loss of storage stability.[74] The antioxidant activity of proteins is discussed in Chapter 12. Active antioxidants were formed during Maillard reactions in butter cookies.[75] Coffee bean components, such as chlorogenic acid, caffeic acid and roasted coffee bean powder or extract, have been added to cookies. The last two additives are more active than tocopherol.[76] Ascorbic and erythorbic acids, citric acid and its isopropyl ester act as synergists of tocopherols.[77]

The addition of spices, such as extracts from lemongrass, clove leaves, black pepper leaves and turmeric increased the shelf life of cakes and also contributed to their characteristic flavour.[78] The keeping quality of crackers and cookies is of great economic importance since these products are often stored for extended periods before they are consumed (sometimes after opening the packaging) and they are not protected from oxidation. A soda cracker biscuit was processed using a fine powder of marjoram, spearmint, peppermint and basil, and their purified diethyl ether extracts as natural antioxidants. Addition of ether extract from each of the above four plant materials gave an excellent antioxidative effect compared with the effect of BHA at concentrations of 0.01, 0.02 and 0.03 %. Addition of fine powder of all plant materials at 0.5 % level gave an antioxidant effect compared to the control sample. Addition of a 1 % mixture of equal amounts of the four plant powders caused a pro-oxidant effect.[79]

Carotene in bread and crackers is stabilised against oxidative bleaching by α-tocopherol and ascorbyl palmitate.[80] Large losses of coloration, otherwise observed during baking, were thus reduced.

15.7 Application in fruits and vegetables

The shelf life of fruits and vegetables is limited by factors other than lipid oxidation, for example antioxidants are added to fruit and mushrooms to prevent oxidation of polyphenols, resulting in the enzymic browning.[81] The lipid content in fruit and vegetables is about 1 % or less, so that the effect of their rancidification may be masked by other, sensorily more active substances.

Fruits contain natural essential oils, which possess antioxidant activities but are also easily oxidised. They are protected by similar antioxidants as glyceridic oils. If lipoxygenases are deactivated by blanching, the content of natural antioxidants (mostly flavonoids) would be sufficient to protect the lipid fraction against oxidation. Natural antioxidants are applied only exceptionally, for example to protect carotenoids or anthocyanins against oxidation. Pigmented orange juice was stabilised with ascorbic acid and phenolic acids and pasteurised.[82]

The use of antioxidants is more justified for the stabilisation of dried products. The stability of dehydrated mashed potatoes was achieved with ascorbyl palmitate or with Prolong P (a mixture of rosemary, thyme and marjoram),[83] with more success than with α-tocopherol or with TBHQ.

15.8 Application in flavouring agents, spices and essential oils

Flavouring agents, spices and essential oils are most often added to improve not only the flavour, but also the shelf life, as many of them are potent antioxidants. They are usually added in small amounts, about 1 %, so that the addition of the antioxidant fraction is much lower. Nevertheless, their effect should not be ignored, as they act in combination with various native antioxidants or synergists. Sometimes, these additives require stabilisation, for instance, essential oils from citrus fruits.

Non-volatile fractions of essential oils may possess antioxidant activities, such as from oranges,[84] which could be used for the stabilisation of the volatile fractions. Essential oils from red pigmented fruits, such as a special variety of mandarins, contain anthocyanins, which act as antioxidants or synergists.[85]

Lemon oil was stabilised with α-tocopherol and citric acid,[86] and mandarin oil with NDGA (nordihydroguaiaretic acid), which is not permitted nowadays.[87] Essential oil from *Citrus hystrix* from Indonesia[88] and bergamot oil[89] were stabilised with rosemary extract. The activity of antioxidants is weaker but still persists in mixtures with proteins or polysaccharides,[90] simulating real flavoured foods. A combination of BHA or tocopherols with citric acid has been proposed for the stabilisation of citrus essential oil.[91]

Synthetic antioxidants are more common than natural antioxidants in this case because of their lower price, but natural antioxidants may be preferable in special cases. Hydroxycitronellal and cyclamen aldehyde (important components of some essential oils) were successfully stabilised with nature-identical 2,6-diisobornyl-4-methylphenol and 2-methyl-4,6-norbornylphenol.[92] Lemon oil can be stabilised by the addition of surface active agents, such as sorbitan esters, as it is not oxidised in oil-in-water emulsions.[93] Limonene is the major component of most essential oils from citrus fruits but contributes only a little to their agreeable aroma. In contrast, it is very unstable against attack by air oxygen. Rosemary oleoresin and extracts from other spices such as anise, caraway, and dill were found to be equally as active as BHA and/or BHT for the prevention of limonene oxidation.[94]

15.9 Application in eco (bio) agrotechnology products

Eco (bio) agrotechnology products are produced by special agriculturists' plants, without the application of industrial fertilisers and synthetic pesticides. Because of less favourable conditions, they usually contain more phenolics and similar stress factors and less fat than conventional products, but sometimes their stabilisation may become necessary. No synthetic antioxidants (even nature-identical compounds) should be added, but high-tocopherol virgin edible oils, herbs and spices with an antioxidant activity would be eligible.

15.10 Future trends

It may be expected that the introduction of modified raw materials and new technologies will improve the stability of foods and thus eliminate the necessity of using antioxidants. On the other hand, steadily growing requirements to extend shelf life will create new possibilities for the application of antioxidants.

The application of synthetic antioxidants will probably be reduced further. They could be replaced by natural or nature-identical antioxidants. We believe, however, that only a few new natural antioxidants will be introduced to the market in the near future in addition to the rosemary extract currently being used. Green tea extracts (prepared from dust, old leaves, and other tea wastes) have a fair prospect of market success. Tocopherols and β-carotene will probably be increasingly used.

Prolongation of the shelf life of complex foods will be achieved mainly by modifying recipes, introducing herbs and spices with a high concentration of antioxidants, using high-oleic edible oils requiring lower antioxidant levels and using protein hydrolysates, which are good synergists, etc.

The biggest problem is likely to arise from innovations in high-stability crackers and similar cereal foods. There will be a tendency to replace hydrogenated oil in these products with fats containing no *trans*-unsaturated fatty acids. Its replacement will require intensive research activity.

Consumption of fried foods is increasing steadily and novel frying oils such as high-oleic oils will be introduced to the market. Their use will necessitate study into their stability under frying conditions and the stabilisation of foods fried in such oils. It may be assumed that the need for application of antioxidants in frying oils will be reduced.

New raw materials, technologies and products are being developed so rapidly that reality may overtake estimated progress, which may not meet expectations. It may be assumed that in future, foods of vegetable origin will be safer, will possess better sensory properties and will have an appropriate shelf life. Antioxidants will surely play their role in these improvements.

15.11 References

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Antioxidants are an increasingly important ingredient in food processing. Their traditional role is, as their name suggests, in inhibiting the development of oxidative rancidity in fat-based foods, particularly meat and dairy products and fried foods. However, more recent research has suggested a new role in inhibiting cardiovascular disease and cancer. *Antioxidants in food* provides a review of the functional role of antioxidants and discusses how they can be effectively exploited by the food industry.

The first part of the book looks at antioxidants and food stability with chapters on the development of oxidative rancidity in foods, methods for inhibiting oxidation and ways of measuring antioxidant activity. Part 2 looks at antioxidants and health, including chapters on antioxidants and cardiovascular disease, their antitumour properties and bioavailability.

A major trend in the food industry, driven by consumer concerns, has been the shift from the use of synthetic to natural ingredients in food products. Part 3 looks at the range of natural antioxidants available to the food manufacturer. The final section of the book looks at how these natural antioxidants can be effectively exploited, covering such issues as regulation, preparation, antioxidant processing functionality and their use in a range of food products from meat and dairy products, frying oils and fried products, to fruit and vegetables and cereal products.

Antioxidants in food will be an essential resource for the food industry in making the best use of this important ingredient.

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