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Phenolic compounds and their role in oxidative processes in fruits

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Abstract

Phenolic compounds occur in all fruits as a diverse group of secondary metabolites. Hence, they are a component of the human diet although data for dietary intakes and metabolic fate are limited. Their role in oxidation processes, as either antioxidants or substrates in browning reactions, is examined. They are characterised by high chemical reactivity and this complicates their analysis.

1. Introduction

Phenolic compounds are extremely diverse. For example, phenolic diterpenes such as carnosol and rosmanol have been identified in herbs and spices (Houlihan, Ho & Chang, 1984) while the main phenolic constituents of soybean are isoflavone glycosides (Eldridge & Kwolek, 1983; Naim, Gestetner, Zilkah, Birk & Bondi, 1973) plus several phenolic acids (Pratt & Birac, 1979) including chlorogenic, caffeic and ferulic acids. These same phenolic compounds are found in most fruits and vegetables. Phenolic compounds (Fig. 1) form one of the main classes of secondary metabolites with a large range of structures and functions, but generally possessing an aromatic ring bearing one or more hydroxy substituents. This definition is not entirely satisfactory, however, since it inevitably includes compounds such as oestrone, the female sex hormone that is principally terpenoid in origin. For this reason, a definition based on metabolic origin is preferable, the plant phenols being regarded as those substances derived from the shikimate pathway and phenylpropanoid metabolism. Some members are characterised as ''polyphenols'', an unfortunate term since not all are polyhydroxy derivatives. In particular, a number of compounds, for example, cinnamic acid, elenolic acid, shikimic acid and quinic acid are treated in the present discussion as phenolics because of metabolic considerations although they lack a phenolic group or even an aromatic ring. Plant phenols (Table 1) have been classified into major groupings distinguished by the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton. The most widespread and diverse of the phenolics are the flavonoids which are built upon a C_6–C_3–C_6 flavone skeleton in which the three-carbon bridge between the phenyl groups is commonly cyclised with oxygen. Several classes of flavonoid are differentiated on the degree of unsaturation and degree of oxidation of the three-carbon segment. Within the various classes, further differentiation is possible based on the number and nature of substituent groups attached to the rings. The range of known phenolics is thus vast and also includes polymeric lignins and condensed tannins although these species are not considered in the present review.

Secondary plant metabolites refer to compounds that are not essential to survival of the whole plant or certain parts of the plant. They are characterised by a number of features, including structural diversity and synthesis, from a limited pool of biosynthetic precursors, typically phosphoenolpyruvate, pyruvate, acetate and a few amino acids, acetyl CoA and malonyl CoA. Various hypotheses have been proposed to account for the production of secondary metabolites but none is entirely satisfactory. Meanwhile, information on their

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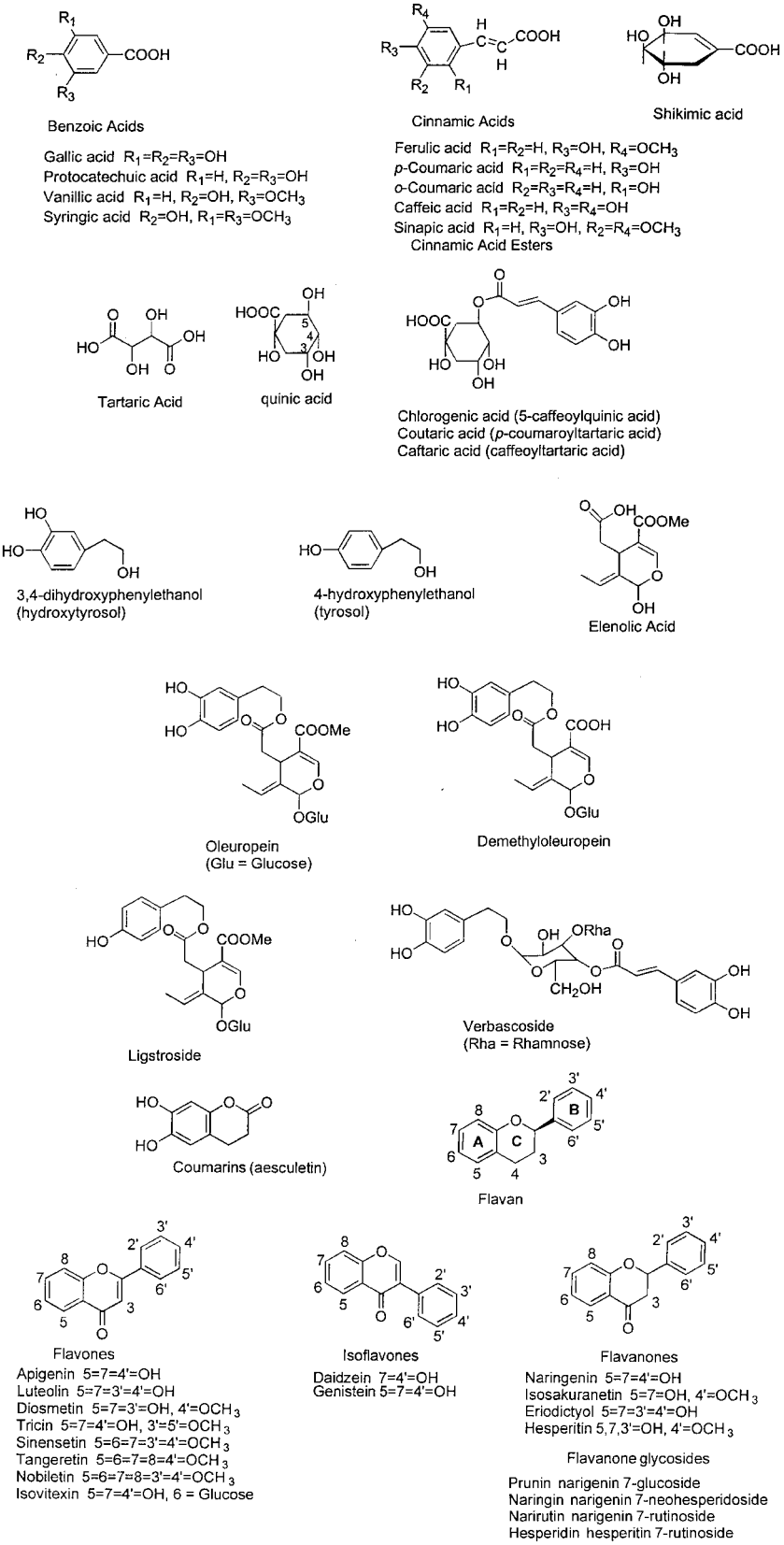
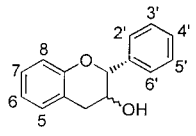
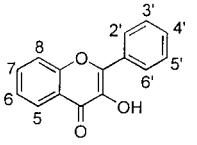


Fig. 1. Structure of selected phenolic compounds. The nomenclature of the flavonoid compounds is confusing and based on trivial names often derived from the species in which a compound was originally isolated. The statement made by Swain (1976) in the 1970s: "In some cases different names are still being used for the same substance" remains as valid as when originally written. In other instances, the same name is used to refer to two structures, morin being a case in point. Note that flavanones, flavanonols, and flavan-3,4-diols may exhibit enantiomers.



Flavanols

Catechin (2R, 3S) 5=7=3'=4'=OH
Epicatechin (2R, 3R) 5=7=3'=4'=OH
Epigallocatechin (2R, 3R) 5=7=3'=4'=5'=OH
Epicatechin gallate (2R, 3R) 5=7=3'=4'=OH, 3-gallic acid ester
Epigallocatechin gallate (2R, 3R) 5=7=3'=4'=5'=OH, 3-gallic acid ester

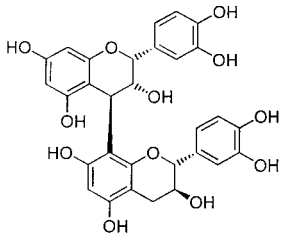


Flavonols

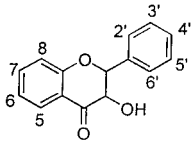
Fisetin 7=3'=4'=OH
Kaempferol 5=7=4'=OH
Morin 5=7=2'=4'=OH
Herbacetin 5=7=8=4'=OH
Quercetin 5=7=3'=4'=OH
Robinetin 7=3'=4'=5'=OH
Isorhamnetin 5=7=4'=OH, 3'=OCH_3
Myricetin 5=7=3'=4'=5'=OH
Gossypetin 5=7=8=3'=4'=OH

Flavonol glycosides

Rutin quercetin 3-*O*-rhamnosylglucoside
Hyperin quercetin 3-*O*-β-D-galactopyranoside

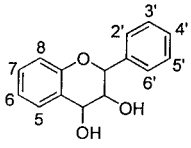


Procyanidin B-1

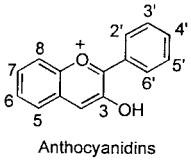


Flavanonols

Taxifolin 5=7=3'=4'=OH (dihydroquercetin)



Flavan-3,4-diols (leucoanthocyanidins)

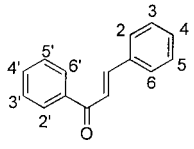


Anthocyanidins

Pelargonidin 5=7=4'=OH
Cyanidin 5=7=3'=4'=OH
Peonidin 5=7=4'=OH, 3'=OCH_3
Delphinidin 5=7=3'=4'=5'=OH
Petunidin 5=7=4'=5'=OH, 3'=OCH
Malvidin 5=7=4'=OH, 3'=5'=OCH_3

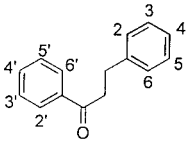
Anthocyanins

Cyanidin 3-glucoside
Cyanidin 3-galactoside
Cyanidin 3-arabinoside
Cyanidin 3-rutinoside
Malvidin 3-glucoside
Malvidin 3,5-diglucoside



Chalcones

Butein 2'=4'=3=4=OH
Licochalcone B 4'=3=4=OH, 2=OCH_3
Okanin 2'=3'=4'=3=4=OH
Chalconarigenin 2'=4'=6'=4=OH



Dihydrochalcones

Phloridzin 4'=6'=4=OH, 2'= *O*-glucosyl

Fig. 1. (cont.)

biosynthesis is essential to understanding the interaction between plants and the environment.

The phenolic composition of fruits is determined by genetic and environmental factors but may be modified by oxidative reactions during processing and storage. Two of the more important processes involve the antioxidant activity of the phenols and oxidative browning. Many phenolic compounds (e.g. caffeic esters, catechins) are

Table 1
Different classes of phenolic compounds and characteristic examples in various fruit

Basic skeleton	Class	Common fruit source	Examples	References
C_6	Simple phenols		Catechol, hydroquinone, resorcinol	
	Benzoquinones			
C_6-C_1	Phenolic acids	Widely distributed	*p*-Hydroxybenzoic acid, Salicylic acid	
C_6-C_2	Phenylacetic acids		*p*-Hydroxyphenylacetic acid	
C_6-C_3	Cinnamic acids	Widely distributed	Caffeic acid, Ferulic acid	Baldioli et al., 1996; Fallico et al., 1996; Nergiz and Unal, 1991a,b; Peleg et al., 1991; Rapisarda et al., 1998; Spanos and Wrolstad, 1990a,b; Torres et al., 1987
	Phenylpropenes		Eugenol, myristicin	
	Coumarins	Citrus	Umbelliferone, aesculetin, scopolin	
	Chromones		Eugenin	
C_6-C_4	Naphthoquinones	Walnut	Juglone	
C_6-C_1-C_6	Xanthones	Mango	Mangostin, mangiferin	
C_6-C_2-C_6	Stilbenes	Grape	Resveratrol	
	Anthraquinones		Emodin	
C_6-C_3-C_6	Flavonoids			
	Flavones	Sweet orange	Sinensetin, nobiletin, tangeretin, isosinensitin, various polymethoxylated flavones	Nogata et al., 1994
		Grapefruit	Tangeretin, various polymethoxylated flavones	Robards et al., 1997
		Lemon	Diosmin, luteolin-7-rutinoside	Miyake, Yamamoto, Morimitsu et al., 1997b; Miyake, Yamamoto, and Osawa, 1997a; Park et al., 1983
	Flavonols	Apple	quercetin, kaempferol	Nicolas et al., 1994
		Pear	quercetin, kaempferol	Spanos and Wrolstad, 1990b
	Flavonol glycosides	Widely distributed	Rutin	
	Flavanonols	Grape	Dihydroquercetin and dihydrokaempferol glycosides	Macheix et al., 1991
	Flavanones	Usually found in citrus fruits such as grapefruit, oranges and lemons	Hesperitin, naringenin	Park et al., 1983; Robards et al., 1997
		Tomato	Naringenin	Hunt and Baker, 1980.
	Flavanone glycosides	Citrus	hesperidin, neohesperidin, narirutin, naringin, eriocitrin	Coll, Coll Laencina, and Tomás-Barberán, 1998; Mouly, Gaydou, and Auffray, 1998; Pupin, Dennis, and Toledo, 1998.
		Strawberry	naringin	Guo et al., 1997
	Anthocyanins	Apple	Cyanidin glycosides including acylated derivatives	Van Gorsel, Li, Kerbel, Smits, & Kader, 1992; Nicolas et al., 1994.
		Sweet orange	Glycosides of pelargonidin, peonidin, delphinidin, petunidin	
		Grape	Glycosides of cyanidin, peonidin, Delphinidin, petunidin, malvidin including acylated forms	Macheix et al., 1991; Van Gorsel et al., 1992.
		Pear	Cyanidin glycosides	
		Cherry	Cyanidin 3-glucoside and 3-rutinoside	Van Gorsel et al., 1992.
		Peach	Cyanidin glycosides	Van Gorsel et al., 1992.
		Plum	Glycosides of cyanidin, peonidin	Raynal and Moutounet, 1989; Van Gorsel et al., 1992
		Sweet cherry	Cyanidin glycosides	Van Gorsel et al., 1992
	Flavanols (catechins)	Apple	(+)-catechin, (−)-epicatechin	Amiot et al., 1992; Nicolas et al., 1994; Spanos et al., 1990; Versari et al., 1997.
		Grape	(+)-catechin, (−)-epicatechin, (+)-gallocatechin, (−)-epigallocatechin	Macheix et al., 1991.
		Pear	(+)-catechin, (−)-epicatechin	Spanos and Wrolstad, 1990b.
		Peach	(+)-catechin, (−)-epicatechin	Cheng and Crisosto, 1995.
	Chalcones	Apple	phloretin derivatives notably phloridzin	
		Pear	Arbutin, phloretin glucoside	
		Tomato	Chalconaringenin	
$(C_6$-$C_3)_2$	Lignins		pinoresinol	
$(C_6$-C_3-$C_6)_2$	Biflavonoids		Agathisflavone	

both good browning substrates and good antioxidants. They are functional as antioxidants at relatively low concentrations while, at higher concentrations, since they themselves are susceptible to oxidation, they can behave as pro-oxidants due to their involvement in initiation reactions. The present review examines the dual role of phenolic compounds as antioxidants and as substrates for oxidative browning reactions. Interest in these compounds is related to their involvement in such processes, their role in plant growth and metabolism and impact on organoleptic and nutritional qualities of fruits and vegetables and, more recently, their demonstrated physiological activity in humans. In both roles, the key process is oxidation. The action of phenolics as antioxidants is viewed as beneficial in both foods and the body where phenolics are oxidised in preference to other food constituents or cellular components and tissues. On the other hand, their role as substrates for oxidative browning is probably restricted to foods and is invariably detrimental although, in some instances (e.g. dates, currants, sultanas), it is intentional and essential to the character of the product. In both systems, the potential oxidising agents are important. In physiological systems, they appear to be reactive oxygen species while polyphenol oxidases and free radicals are the main oxidants in foods when the phenolics act as substrates and antioxidants, respectively.

Knowledge of such compounds offers a number of opportunities including a better understanding of the relationships that may exist between these substances and the physiology and organoleptic qualities of the fruit which, in turn, can be translated into a more solid basis for processing techniques. Furthermore, such knowledge will provide the basis to manipulate the level of these compounds in the fruit to enable dietary modulation or prevention of disease. Alternatively, genetically manipulated plants may provide an environmentally sustainable source of feedstock for the petrochemical industry. The identification of natural antioxidants will provide alternatives to synthetic materials such as butylated hydroxyanisole (BHA) for use in foods (Camire & Dougherty, 1998).

The role of fruit phenolics as antioxidants and as substrates for oxidation reactions is reviewed. Their isolation and identification are the initial steps to understanding their significance and action and are also examined. Dietary intake in humans and metabolic fate are considered in relation to in vivo antioxidant activity.

1.1. Isolation and identification

Methods of characterisation and identification of fruit phenolics follow those in general use for natural substances. Hence, preparation of an extract, biological screening, bioguided fractionation, isolation and structure elucidation is the usual approach. For the latter, physical methods based on spectral characteristics feature prominently. Preliminary fractionation of the phenolic compound remains as the first step in characterisation. Various aspects have been reviewed (Cook & Samman, 1996; Pino, 1997; Robards & Antolovich, 1997; Ryan & Robards, 1998; Tsimidou, 1998) and only the salient features are presented.

Traditional methods for the determination of the phenolic component relied on measurement of total phenols or, in some instances, 1,2-diphenols (Nergiz & Unal, 1991a) because of their association with browning reactions. The need for profiling and identifying individual phenolic compounds has seen traditional methods based on colorimetry replaced by high performance chromatographic analyses. Gas chromatography (GC) has traditionally been regarded as marginal for the separation of biological phenols. However, with suitable derivatisation they are amenable to GC and a recent procedure has described (Soleas, Diamandis, Karumanchiri, & Goldberg, 1997) the recovery of phenolic compounds from wine by solid phase extraction, preparation of the trimethylsilyl derivatives and analysis by GC with mass spectrometric detection. Nevertheless, reverse phase HPLC (RPLC) currently represents the most popular and reliable technique for phenolic analysis. Compound elution is typical of RPLC, that is, polar compounds (e.g. phenolic acids) elute first, followed by those of decreasing polarity. Hence, an elution order can be developed as phenolic acids < cinnamic acids < flavonoids (Nogata, Ohta, Yoza, Berhow & Hasegawa, 1994; Shahrzad & Bitsch, 1996; Torres, Mau-Lastovicka & Rezaaiyan, 1987) although overlap of the individual members of different classes is inevitable because of the diversity of compounds. The elution pattern for flavonoids containing equivalent substitution patterns (Park, Avery, Byers & Nelson, 1983) is flavanone glycoside followed by flavonol and flavone glycosides and then the free aglycones in the same order. In cinnamic and phenolic acids, polarity is increased most by hydroxy groups at the 4-position, followed by those at the 3- and 2-positions. Methoxy and acrylic groups reduce polarity and hence increase retention times. In one of the early reports (Wulf & Nagel, 1976) on the RPLC of phenolic compounds, different mixtures of acetic acid, water and methanol were used to separate members of several classes of phenols, and the effects of organic modifier on selectivity were deduced. Since then, numerous mobile phases have been employed with different modifiers (usually methanol, acetonitrile or tetrahydrofuran), acids (acetic, formic or trifluoroacetic acid) and/or salts (ammonium phosphate).

Detection is usually based on absorption of ultraviolet or, less commonly, visible radiation at various wavelengths (Table 2) characteristic of the class of phenolic compound (Bengoechea, Hernandez et al., 1995). Most

Table 2
Absorption spectra of the various classes of phenolic compound

Class of compounds	UV Band II[a]	UV Band I[a]	Visible[a]
Benzoic acids	270–280		
Cinnamic acids	(290–300)[b]	305–330	
Anthocyanic pigments	240–280	(315–325)[c]	450–560
Flavonols	250–270	(300)[b] 350–380	
Flavanols	270–280		
Coumarins	220–230	310–350	
Flavones	250–270	330–350	
Flavanones, Flavanonols	270–295	(300–330)[b]	
Chalcones	220–270	(300–320)[b] 340–390	
Aurones	240–270		340–370
Isoflavones	245–270	300–340	

[a] Usual solvent is methanol except methanol-HCl for anthocyanins; changes in mobile phase composition will cause solvent shifts.
[b] Shoulder.
[c] In the case of acylation by cinnamic acids.

flavonoids exhibit two major absorption bands; Band I in the 320–385 nm range, representing the B-ring absorption and Band II, in the 250–285 nm range, representing A-ring absorption. A red shift is induced by increasing the numbers of hydroxyl groups, for instance, from 367 nm in kaempferol, to 371 nm in quercetin and 374 nm in myricetin. For this reason, Band I in flavones is always at a shorter wavelength by 20–30 nm than that in the equivalent flavonols. Hypsochromic shifts are produced by glycosylation and *O*-methylation. Other methods of detection have been developed and the introduction of soft ionisation sources has facilitated the coupling of LC and mass spectrometry which has been used to advantage in a number of analyses (He, Lian, Lin & Bernart, 1997; Robards, Li, Antolovich & Boyd, 1997; Ryan, Robards & Lavee, 1999). The coulometric array detector is also a powerful tool for phenolic detection (Achilli, Cellerino & Gamache, 1993; Chiavari, Concialin & Galetti, 1988; Gamache, Ryan & Acworth, 1993) that provides data on the redox potential of the eluted species. The linearity, precision and limits of detection have been compared for HPLC of benzoic and cinnamic acids using UV, electrochemical and mass spectrometric detection (Bocchi et al., 1996).

Phenolic compounds are highly reactive species and this complicates their extraction and (quantitative) recovery becomes particularly problematic. Methods of protecting the compounds from oxidation and other deteriorative processes have included the addition of antioxidants during the extraction and the use of inert atmospheres. Extraction is further complicated by the uneven distribution of the phenolics in various forms. For instance, methanolic extracts from orange peel were rich in flavones and glycosylated flavanones whereas hydrolysed extracts comprised mainly phenolic acids and flavonols (Bocco, Cuvelier, Richard, & Berset, 1998a). Artefactual changes, for example, isomerisation (Gao & Mazza, 1995) during the extraction process are a constant concern.

At the subcellular level, the phenolics are located mainly in the vacuoles (Yamaki, 1984) with small amounts in free space and none in the cytoplasm. The seeming homogeneity of the subcellular distribution is perhaps misleading as lignin and certain simple molecules (flavonoids and ferulic acid esters) accumulate in the cell wall while soluble phenolic compounds are stored in the vacuoles. The occurrence of phenolics in soluble, suspended and colloidal forms, and in combination with cell wall components (Lichtenthaler & Schweiger, 1998), may have significant impact on their extraction. At the tissue level, there are significant qualitative and quantitative differences between the phenolic content of seeds, epidermal and subepidermal layers (peel) and the internal tissue (cortex). This is easily demonstrated using suitable staining reagents (Hawker, Buttrose, Soeffhy & Possingham, 1972). Accumulation of soluble phenolics is greater in the outer tissues (epidermal and subepidermal layers) than in the inner tissues (mesocarp and pulp) (Bengoechea, Sancho et al., 1997). For instance, in many fruits, flavonol glycosides are chiefly located in the outer portion or in the epicarp. This is seen in the greater abundance of glucosides and rutinosides in the peel than the flesh of passionfruit (Chassagne, Crouzet, Bayonove, & Baumes, 1997). Anthocyanins are located primarily in the skin of grapes but are present throughout the fruit in strawberry and blueberry. The situation with the anthocyanins is further complicated by pH-dependent equilibria and, in the inner cells in the skin, anthocyanins are mainly in the neutral quinonoidal base form whereas, in the outer cell vacuoles, they are found essentially in the flavylium cationic form. The outcome is that the phenolic profile will vary between those fruits consumed or processed whole and those from which skin or peel has been removed.

NMR is a powerful technique for structural assignment but the spectra of the phenolics are frequently complex and identification of the isolated compounds is complicated by the absence of suitable reference standards which requires time-consuming syntheses of the relevant materials. Although 2D NMR can be used for structural analysis without a reference compound, the technique requires relatively large amounts of the compound. Limited sensitivity (and the need to isolate relatively large quantities of sample) is currently the greatest drawback of NMR.

1.2. Dietary intakes and metabolic fate

Accurate data on population-wide intakes of phenolic compounds are not available. The intake of flavonols and flavones around 1960 in a variety of countries ranged

from 6 mg day^{-1} in Finland to 64 mg day^{-1} in Japan (Hertog, Van Poppel & Verhoeven, 1997). More recently, the average daily intake of flavonoids in the United States was estimated in 1976 as high as 170 mg (expressed as aglycones, or 1 g expressed as glycosides) consisting of flavonols, flavanones and flavones (Kühnau, 1976). These values, although often quoted, were based on techniques now considered inappropriate and probably overestimate the average dietary intake. Kühnau drew attention to the several limitations of the data. His comment regarding analytical methods is appropriate: "Only during the last four years reliable procedures for the assay of the particular flavonoid subgroups have been developed". The same comment would be appropriate here and will sadly no doubt be equally valid in another 20 years.

The average dietary intake in The Netherlands was estimated as 23 mg day^{-1} (as aglycones) based on analysis of commonly consumed fruits, vegetables and beverages plus data from the Dutch National Food Consumption Survey 1987–1988 (Hertog, Hollman Katan & Kromhout, 1993). Apples were the most important fruit source while quercetin, with a mean intake of 16 mg day^{-1} was quantitatively the most important flavonoid. Flavonoid analyses were performed by HPLC and confidence in the data is further enhanced by peak purity testing based on photodiode array detection. However, the analyses were restricted to selected flavonols and flavones following hydrolysis and therefore represent an underestimate of total intake. The Dutch data are substantiated by results from a study (Pietta et al., 1996) of villagers of a southern Italian agricultural region. Fruit was the main dietary source of flavonoids and quercetin was the main aglycone, while apigenin was the predominant flavone with lesser amounts of kaempferol and luteolin. However, the available data are limited to these few studies in which the authors targeted a few specific compounds following hydrolysis. The need for more extensive work is indicated to identify the flavonoid content of foods consumed in other countries. Data from a recent German study (Linseisen, Radtke & Wolfram, 1997; Radtke, Linseisen & Wolfram, 1998) suggest that dietary intakes may be much higher in countries where coffee is consumed.

The absorption and bioavailability of phenolics in humans is also controversial. Data on these aspects of phenolics are scarce and merely highlight the need for extensive investigations of the handling of phenolics by the GI tract and their subsequent absorption and metabolism. Studies must distinguish between the fate of phenolics at levels of dietary intake from pharmacological doses. A number of investigators have studied the urinary excretion of orally administered phenolics as a measure of absorption. Recent studies have shown the presence of several dietary phenolics including cinnamates and flavonoids in urine (Bourne & Rice-Evans, 1998) and phloridzin, quercetin glucosides and rutinosides in human plasma (Paganga & Rice-Evans, 1997) of nonsupplemented subjects. The methodology used in the latter study would not have distinguished between a flavonol glycoside and a flavonol glucuronide produced endogenously from the aglycone. Nevertheless, there is clear evidence that some dietary phenols, such as quercetin, are absorbed but the extent is unclear (Hollman & Katan, 1997). It was originally believed that the glycosidically-linked phenolics were non-absorbable and that only the free aglycones were able to pass through the gut wall. However, Hollman and Katan demonstrated that quercetin glycosides from onions were absorbed far better than the pure aglycone. Subsequent pharmacokinetic studies with dietary quercetin glycosides showed marked differences in absorption rate and bioavailability. Absorbed quercetin was eliminated only slowly from the blood.

The sugar moiety in glycosidically-linked phenols played an important role in their absorption (Hollman et al., 1997) but there was no difference in absorption of quercetin glucosides where attachment of the sugar moiety was to the A- or C-ring of the aglycone (Olthof, Hollman & Katan, 1998). The results of these studies showed that quercetin glucosides were rapidly and well absorbed whereas quercetin rutinoside and aglycone were more slowly and less well absorbed. A recent study (Gee, Dupont, Rhodes & Johnson, 1998) has demonstrated that quercetin glucosides can interact with the sodium-dependent glucose transport receptors in the mucosal epithelium and may therefore be absorbed by the small intestine.

Free quercetin was not detected in the plasma of subjects fed a diet rich in plant products and containing an estimated 87 mg quercetin (Manach, Morand, Crespy, Demigne & Remesy, 1998). On the other hand, within three hours, a significant increase in conjugated forms of quercetin was observed which returned to basal levels by 20 h. The liver and the colonic flora (Miyake, Yamamoto & Osawa, 1997) are major sites of flavonoid metabolism and there is evidence in animals for *O*-methylation, sulphation and glucuronidation of hydroxyl groups in the liver. Bacterial ring fission of flavonoids occurs in the colon and the subsequent degradation products, phenolic acids, can be absorbed and are found in urine of animals. Tangeretin is one of the predominant polymethoxylated flavones found in citrus and it is metabolised in the presence of human liver microsomes to at least three derivatives (Bracke et al., 1994; Cavenc-Lavier, Guenot, Salles, Siess & Suschetet, 1998). Nevertheless, data on phenolic absorption, metabolism and excretion in humans is very limited (Hertog et al., 1997) and until this is remedied it will remain unclear whether the phenolics remain in the body in the appropriate chemical form for sufficient time to provide in vivo protection.

1.3. Fruit as a source of phenolic compounds

Phenolic compounds are ubiquitous in the plant kingdom, being found in all fruits and vegetables in

virtually all parts of the plant (Table 3) but with quantitative distributions that vary between different organs of the plant and within different populations of the same plant species (Van Buren, 1970). The phenolic components of plants constitute a complex mixture, and only a small number of fruits have been examined systematically for their phenolic content. Qualitative and quantitative knowledge of the phenolics consumed by humans is therefore incomplete.

Plant phenols may be classified as in Table 1. Four phenolic acids are said to be universal—-*p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids (Torres et al., 1987). Fruit represents a significant source of cinnamic acids, largely comprised of ferulic, sinapic, p-coumaric and caffeic acids (Meyer, Donovan, Pearson, Waterhouse & Frankel, 1998). The cinnamic acids exhibit geometric isomerism but in the case of the four-named acids they exist in plants exclusively as the *trans*-isomer (Graf, 1992). They are rarely present as the free acid but occur in the fruit predominantly in esterified form with quinic acid, glucose or tartaric acid (Herrmann, 1989) but are not glycosylated at the phenolic hydroxyl groups. Chlorogenic acid (5-caffeoylquinic acid) is the most important cinnamic acid derivative in fruits where it is sometimes the predominant single phenolic compound. Other important esters are coutaric acid (*p*-coumaroyltartaric acid) and caftaric acid (caffeoyltartaric acid). The universal distribution and high concentration of cinnamic acids in fruits may be due, in part, to the key role they play in the biosynthesis of other, more complicated phenolics.

The flavonoids (Fig. 1) represent the most widespread and structurally diverse of the phenolics. Here, also, geometric isomerism is possible due to restricted rotation in the C-ring with the additional possibility of C-2, C-3 and C-4 being chiral. Three of the numerous classes of flavonoids are widespread and quantitatively dominant: flavanols, flavonols and anthocyanins. The monomeric flavan-3,4-diols, referred to as leucoanthocyanidins, are not major compounds in fruits. On the other hand, the flavanols are important constituents of fruits in oligomeric or polymeric forms as proanthocyanidins or condensed tannins. However, the monomers are also important natural products in their own right (Amiot, Tacchini, Aubert, & Nicolas, 1992). Among these, (+)-catechin, (−)-epicatechin, (+)-gallocatechin and (−)-epigallocatechin (Fig. 1) are found in fruits generally in free rather than glycosylated forms which distinguishes them from other flavonoids.

Table 3
Relative concentration of phenolic compounds in plant tissue (Pratt & Hudson, 1990)

Tissue	Relative concentrations
Fruit	Cinnamic acids > catechins≅ leucoanthocyanins (flavan-3,4-diols) > flavonols
Leaf	Flavonols≅cinnamic acids > catechins≅leucoanthocyanins
Wood	Catechins≅leucoanthocyanins > flavanols > cinnamic acids
Bark	As for wood but greater concentrations

The flavonols are also widely distributed in fruits with substitution patterns commonly involving A- and/or B-ring hydroxylation in the 5- and 7-positions or 3′- and 4′-positions, respectively (Fig. 1) (Herrmann, 1976). For example, the flavonols, quercetin, kaempferol, myricetin and isorhamnetin, are common in fruits. Flavones including apigenin, luteolin and tricin (Fig. 1) are encountered much less commonly than flavonols and then only in trace amounts. The quercetin content of fruits tested by Hertog, Hollman and Katan, (1992) averaged 15 mg kg^{-1} (dry mass basis) following hydrolysis of glycosides. However, myricetin, luteolin and apigenin were below the limit of detection of 1 mg kg^{-1}.

Flavonols and flavones occur in living cells almost exclusively as *O*-glycosides or, less commonly, as C-glycosides in which one or more of the phenolic hydroxyl groups is bound to a sugar or sugars by an acid-labile hemiacetal bond. Glucose is the most commonly encountered sugar with rhamnose and the disaccharide, rutinose (6-*O*-α-*L*-rhamnosyl-*D*-glucose) also encountered. The preferred bonding site of the sugar moiety to the aglycone is the 3-position and much less frequently the 7-position and, only in rare cases, the 4′-, 3′- or 5-position. Acylation of the glycosides occurs in which one or more of the sugar hydroxyls is derivatized with cinnamic and phenolic acids.

Apart from the flavanols and flavonols, the anthocyanins (anthocyanidin glycosides) are the next most abundant and widely distributed flavonoids. These compounds give most fruits their red, violet and blue colour (Hong & Wrolstad, 1986; Rommel, Wrolstad & Heatherbell, 1992) although the red colour of some fruits (e.g. orange, tomato) is caused by carotenoid pigments rather than anthocyanins. The anthocyanidin aglycones exist in cationic form in acidic medium with numerous mesomeric forms. Six anthocyanidins are widespread and commonly contribute to the pigmentation of fruits. Cyanidin is the most common and, in terms of frequency of occurrence (Ishikura & Sugahara, 1979), is followed in decreasing order by delphinidin, peonidin, pelargonidin, petunidin and malvidin. There are few fruits which do not contain cyanidin and, in a number of fruits, for example, peach and pear, it is the single dominant anthocyanidin. In other fruits, two aglycones are characteristic, for example, cyanidin and pelargonidin in raspberry cultivars.

Glycosylation of anthocyanidins almost always occurs at the 3-position with glucose, arabinose and galactose the most common sugar moieties. Hence, the most common anthocyanins in fruit consist of the

3-monoglucosides of cyanidin, delphinidin, peonidin, pelargonidin and petunidin, cyanidin 3-galactoside, cyanidin 3-arabinoside plus the single diglycoside, cyanidin 3-rutinoside. Among these pigments, cyanidin 3-glucoside is the most abundant. In addition to glycosylation, acylated anthocyanins are found fairly often in fruits, the situation being particularly complex in grapes (Goldy, Ballinger & Maness, 1986; Lamuela-Raventos & Waterhouse, 1994; Spanos & Wrolstad, 1990a) where the 3-monoglucosides corresponding to the five aglycones, delphinidin, cyanidin, petunidin, peonidin, and malvidin can all be acylated by acetic or *p*-coumaric acid.

The other classes of flavonoids, notably flavanones, flavanonols, chalcones and dihydrochalcones, are important only in particular fruits. For example, the most important flavonoids in citrus are the flavanones. Isoflavones are quite rare in fruits. While flavanones and flavones are found together in some fruits, notably citrus, there is a certain mutual exclusion between flavones and flavonols. Moreover, a limited number of flavonoids are usually characteristic of most fruits. This raises an important and interesting feature of flavonoid distribution, namely, the strong tendency for taxonomically related plants to produce similar types of flavonoids. Fernández de Simón, Pèrez-Ilzarbe, Hernández, Gómez-Cordovès and Estrella (1990, 1992) concluded that cinnamic acid esters with tartaric acid are typical of grape, phloridzin is typical of apple, and isorhamnetin glycosides are typical of pear. Myricetin is only found in peach, and luteolin and apigenin glucosides are found only in orange. Apricot could be detected by the presence of two coumarins and pineapple by the presence of sinapic acid and the general absence of phenolics (benzoic acids and aldehydes, flavanols, flavonols, chalcones, cinnamic acids, and their derivatives) although *p*-hydroxybenzoic acid is present in pineapple in our experience.

Grape (Vitis spp.): The phenolic content of grape and wine has probably been more extensively studied than any other fruit with the possible exception of apple (Goldberg, Karumanchiri, Tsang & Soleas, 1998; Goldberg, Tsang, Karumanchiri & Soleas, 1998; Macheix, Sapis & Fleuriet, 1991; Soleas, Dam, Carey & Goldberg, 1997; Soleas, Diamandis & Goldberg, 1997; Soleas, Diamandis, Karumanchiri & Goldberg, 1997; Soleas, Tomlinson, Diamindis & Goldberg, 1997). Five classes of phenolics, namely, phenolic acids, flavonols, flavanols, flavanonols and anthocyanins are well represented in the ripe berries. Grape differs, for example, from apple and pear in that the grape cinnamic acids are esterified with tartaric acid rather than quinic acid to give caftaric and coutaric derivatives.

Anthocyanins were the most abundant phenolic compounds in red grapes, and flavonols were most abundant in white grapes (Yi, Meyer & Frankel, 1997). However, the grape berry comprises the skin, pulp and seeds and the phenolic content, particularly the anthocyanins, differs significantly between the three regions. This is particularly important in grape because of the large-scale production of red and white wines using techniques that result in differential exposure to the skin (McDonald et al., 1998). Anthocyanins are found in the skin of most cultivars where they exhibit extremely complex distributions based on five aglycones (as noted above) and numerous glycosides of which many are acylated (Goldy et al., 1986; Lamuela-Raventos & Waterhouse, 1994; Spanos & Wrolstad, 1990a). A number of studies (Bakker & Timberlake, 1997; Goldberg, Karumanchiri et al., 1998; Goldberg, Tsang et al., 1998) have examined the phenolic composition of wines. Phenolics reported in these studies include a number of novel malvidin glycosides which are believed to be formed in wines during maturation. These particular phenolics are interesting because they are resistant to bleaching by sulfur dioxide. Quercetin concentrations were highest in wines from warmer climates notable for high sunshine. (Goldberg, Tsang et al. 1998) The distribution of *p*-coumaric acid in wines seemed to be more random than that of quercetin and genetically determined metabolic set-points may be more relevant for this key phenolic acid that acts as a precursor of trihydroxystilbenes as well as of flavonoids and flavanols.

Citrus: Cinnamic acids constitute a major part of the phenolic content of citrus but very little occurs as the free acids (Peleg, Naim, Rouseff & Zehavi, 1991). However, free acids are readily liberated from conjugated forms by alkaline hydrolysis. Ferulic acid is generally the major cinnamic acid with lesser amounts of coumaric, sinapic and caffeic acids (Fallico, Lanza, Maccarone, Asmundo & Rapisarda, 1996; Rapisarda, Carollo, Fallico, Tomaselli & Maccarone, 1998).

In most of the plant kingdom, flavanones occur in small amounts compared to other flavonoids, yet they are the predominant flavonoid in citrus (Nogata et al., 1994). In terms of its flavonoid composition, citrus is exceptional, some citrus flavanoids being found nowhere else. Four aglycones are common, namely, naringenin, eriodictyol, isosakuranetin and hesperetin. Furthermore, citrus flavanones usually occur as glycosides whereas in other plants, flavanones are seldom found in glycosidic form (Rouseff, 1980). Glycosylation occurs at position 7 either by rutinose or neohesperidose, disaccharides formed by a glucose and rhamnose molecule differing only in the type of linkage: 1→6 or 1→2. This has formed the basis for classification of citrus. Thus, most commercial citrus cultivars (Park et al., 1983) contain only the non-bitter rutinosides whereas sour orange and pummelo have only bitter flavanone neohesperidosides (Albach & Redman, 1969; Rouseff, Martin & Youtsey, 1987). Grapefruit are considered as hybrids because they contain both flavanone rutinosides and neohesperidosides (Wallrauch, 1995).

It is now accepted that naringin is absent from sweet orange varieties. Nevertheless, the evidence is contradictory (Robards et al., 1997) and a recent publication (Gamache et al., 1993) reported concentration data for naringin in a number of sweet orange varieties. In our experience, naringin is generally absent but in some sweet oranges a peak co-elutes with naringin using typical reversed phase conditions, a fact noted also by Ooghe, Ooghe, Detavernier and Huyghebaert (1994a) but the true identity of the peak remains unknown. Nevertheless, it does account for the confusion about the presence of naringin in sweet orange. Resolution of this issue is important as naringin has been used as a chemotaxonomic marker in distinguishing sweet orange from other citrus cultivars.

Flavones are not common in fruits and are never predominant. Citrus is again a special case containing a number of polymethoxylated flavones as minor flavonoids. Some of these, for example, nobiletin and sinensetin (sweet orange peel) and tangeretin (tangerine oil) have been known for some time. Concentrations are very high in the flavedo and they are readily isolated from the essential oil of citrus fruits but are also identifiable in the juice (Ooghe et al., 1994b).

Apple (Malus pumila): The phenolic content of apple has been studied extensively (Spanos & Wrolstad, 1992; Spanos, Wrolstad & Heatherbell, 1990; Nicolas, Richard-Forget, Goupy, Amiot & Aubert, 1994). Cinnamic acid derivatives and flavanols (Amiot et al., 1992) represent about 90% of the phenolic content of the cortex of apple with wide variations among cultivars. Among the cinnamic derivatives, the main compound is generally chlorogenic acid with trace amounts of its isomers and significant amounts of 4-coumaroylquinic acid. The flavanols, epicatechin and procyanidin B2, are present in high concentrations. Flavonols and dihydrochalcones (phloridzin) are present only in minor quantities but distinguish apple from a number of other fruits (Versari, Biesenbruch, Barbanti & Farnell, 1997). The characteristic flavonol glycosides of apple (Spanos et al., 1990) include quercetin-3-*O*-*β*-*D*-galactopyranoside (hyperin) as the most significant flavonol with quercitrin and rutin. The major anthocyanin is cyanidin-3-galactoside.

Apricots, plums (Prunus domestica) *and peaches* (Prunus persica): Apricots, plums and peaches contain (Bengoechea et al., 1997; Garcia-Viguera, Bridle, Ferreres & Tomás-Barberán, 1994; Henning & Herrmann, 1980a,b) significant amounts of kaempferol and quercetin glycosides. The main glycosides in peaches were 3-glucosides and 3-galactosides of kaempferol and quercetin. Chlorogenic acid was the dominant phenol in apricots (Garcia-Viguera et al.; Radi et al., 1997) while rutin was the dominant flavonoid. Other phenolics in apricots included 3-coumaroylquinic (neochlorogenic) acid, (+)-catechin, (−)-epicatechin and procyanidins. The major portion of the flavonoids was located in apricot peel whereas chlorogenic acid was located primarily in the flesh.

Tomato: A large proportion of tomato phenolics occurs in combination with the cuticle. Of the phenolics identified in the fruit cuticles of tomato cultivars (Hunt & Baker, 1980), coumaric acids, naringenin, naringenin 7-glucoside (prunin) and the corresponding chalcone, chalconaringenin were abundant. These were synthesised mainly during the climacteric and were largely bound to the cutin matrix. The composition of the flavonoid fraction was controlled by the spectral quality of incident radiation, red light favouring the formation of chalconaringenin. Quercetin levels varied greatly between cherry and normal-sized cultivars (Crozier, Lean, McDonald & Black, 1997) and were lowered by various methods of cooking.

Cherry: Anthocyanins were the major phenolics in cherry (Gao & Mazza, 1995) with total anthocyanin content ranging from 82 to 297 and 2 to 41 mg/100 g of pitted cherry for the dark-coloured and light-coloured cherries, respectively. The 3-rutinoside and 3-glucoside of cyanidin were the major anthocyanins with the same glycosides of peonidin as minor anthocyanins. Another minor anthocyanin was pelargonidin 3-rutinoside. The major non-anthocyanic phenolics were 3-coumaroylquinic acid (24 to 128 mg/100 g) (noted also by Van Buren, 1970) and *p*-coumaroylquinic acid (23 to 131 mg/100 g). Sour cherries (*Prunus cerasus*) contain more phenolic compounds than sweet cherries (*Prunus avium*), mainly due to a large amount of phenolic acids (Friedrich & Lee, 1998). Compounds common to both varieties included chlorogenic acid, caffeoyltartaric acid and coumaroyltartaric acid with (−)epicatechin as the dominant flavanol in both. Sweet cherries were characterised by two dominant phenolics, caffeoyltartaric acid and 3-*p*-coumaroylquinic acid, while procyanidin B2 was dominant in sour cherry but absent in sweet cherry. High concentrations of chlorogenic acid have been reported in cherry juice (Shahrzad & Bitsch, 1996). The observation of 3-coumaroylquinic acid versus chlorogenic acid may be varietal but may also be artefactual and arise from isomerisation during extraction.

Pear (Pyrus cummunis): Cinnamic acids, flavonols and flavanols are well represented in pears (Oleszek, Amiot & Aubert, 1994; Spanos & Wrolstad, 1990b). Pear flesh is enriched in cinnamic acids and flavanols while the peel is rich in flavanols and flavonols (Amiot, Tacchini, Aubert & Oleszek, 1995). The most abundant phenolics are chlorogenic acid (range 20—140 mg kg^{-1} fresh weight) and (−)-epicatechin (5–90 mg kg^{-1} fresh weight). The presence of (+)-catechin has also been reported (Risch & Herrmann, 1988). Apart from these flavanol monomers, oligomer forms were also present (Amiot, Aubert & Nicolas, 1993). Pear flavonols have been characterised as glycosides or malonyl glycosides of quercetin and isorhamnetin (Amiot et al., 1995; Oleszek et al., 1994).

Berries (Rubus): The anthocyanins are responsible for the characteristic colours of various berry fruits. For example, the principal red pigment of strawberries is pelargonidin-3-glucoside (Lopez-Serrano & Barcelo, 1996). The anthocyanin profiles, which are highly characteristic, are complex mixtures of galactosides and glucosides of the five common aglycones, together with acetylated forms and various arabinosides (Gao & Mazza, 1994). The cinnamic acids (*p*-coumaric, caffeic and ferulic acids) represent a major proportion of the phenolic content of strawberries and black currant (Häkkinen, Mykkänen, Kärenlampi, Heinonen & Törrönen, 1996). Significant amounts of flavanols and the flavonols, myricetin, quercetin and kaempferol, are also present. The flavonol glycoside profile does not differ greatly between the various Rosaceae fruits, for example, strawberry, raspberry and blackberry (Boyles & Wrolstad, 1993; Rommel & Wrolstad, 1993; Withy, Nguyen, Wrolstad & Heatherbell, 1993) and is dominated by quercetin and kaempferol and their glycosides.

Olive (Olea): Various flavones, flavonols (Amiot, Fleuriet & Macheix, 1986; Vlahov, 1992), vanillic acid and cinnamic acids such as caffeic and *p*-coumaric acids have been reported in olives (Brenes-Balbuena, Garcia & Garrido, 1992). The predominant phenolic acid in virgin olive oil (Nergiz & Unal, 1991b) is *p*-coumaric acid (1–10 μg g^{-1}) with lesser amounts of syringic and vanillic acids. However, olives also contain a number of distinctive compounds (Fig. 1), including tyrosol and hydroxytyrosol (3,4-dihydroxyphenylethanol), whilst the most significant olive phenolic is oleuropein, a heterosidic ester of elenolic acid with hydroxytyrosol. Other glycosides found in the fruit are ligstroside and verbascoside (Ryan & Robards, 1998; Tsimidou, 1998). Recently, verbascoside isomers (Ryan et al., 1998) and an oleuropein analogue with additional glucose moieties (De Nino et al., 1997) have been reported.

Other fruits: Data are reported in Macheix, Fleuriet, and Billot (1990) for a variety of fruits including persimmon (*Diospyros*), date (*Phoenix dactylifera*), kiwi fruit, passion fruit (*Passiflora edulis*), mango, pineapple, banana (*Musa*) but are limited and date mainly from the 1970s and earlier (Van Buren, 1970). This is surprising, particularly in the case of banana and mango, because of their commercial significance and propensity for browning.

2. Phenolic compounds as antioxidants

Flavonoid phenoxy radicals exhibit reduction potentials in the range 540–700 mV and the corresponding parent flavonoids are expected to efficiently inactivate various reactive oxygen species with higher potentials (2000–950 mV) (Lin, Navaratnam, Yao & Lin, 1998; Pietta et al., 1996). This process is important in two situations: in the food sciences, where the term antioxidant is often implicitly restricted to chain-breaking inhibitors of lipid peroxidation and, in humans, where free radicals, generated in vivo, damage many other targets besides lipids. It is this latter role that has driven research in recent years.

Many of the chronic diseases that affect humans have an uneven geographic distribution. Global epidemiological studies link the prevalence of certain diseases to dietary patterns and show an inverse relationship between the consumption of fruits and vegetables and the incidence of many diseases, particularly cardiovascular diseases and cancer. Such studies are hampered by at least three possible sources of error as noted by Deshpande, Deshpande and Salunkhe (1996). Nevertheless, there is now convincing evidence that the development of these diseases is associated with the damaging effects of reactive oxygen species such as the superoxide free radical anion, hydrogen peroxide, hydroxyl radical and singlet oxygen (Cheeseman & Slater, 1993) and further that the effects can be ameliorated by dietary constituents.

A number of chemical and physical phenomena can initiate peroxidation which proceeds continuously in the presence of a suitable substrate until a blocking defence mechanism occurs. Target substances include oxygen itself, polyunsaturated fatty acids, phospholipids, cholesterol and DNA, but it is the lipids, as constituents of cellular membranes, which are most susceptible because of the high probability of rapidly progressing, destructive chain reactions being initiated (Gey, 1986). However, in studying antioxidant activity, the source of reactive oxygen species and the damage target must always be considered. An antioxidant may protect lipids against oxidative damage whilst accelerating damage to other biological molecules (Aruoma, Spencer et al., 1997). Thus, Aruoma, Spencer et al. used several measures of antioxidant activity and posed a series of questions which serve as a guide in evaluating antioxidant efficacy.

The use of biomarkers for in vivo testing provides a composite index of damage to cell constituents such as protein, lipid, or DNA (Bowen & Mobarhan, 1995). Oxidation of the guanine base is the most prominent and mutagenic oxidative modification and its repair results in urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) (Hertog, DeVries et al., 1997; Prieme, Loft, Nyyssonen, Salonen & Poulsen, 1997) which therefore functions as a urinary marker for oxidative DNA damage. However, excretion of oxidised bases may reflect both endogenous oxidation and ingestion of oxidised bases in foods and interpretation of the data can be a problem. Tissue levels of oxidised bases are determined by the balance between oxidation and repair but the rate at which the oxidation occurs is presumably predictive for the risk of mutation. ELISA

kits are commercially available for 8-oxodG but, in a comparison with multidimensional HPLC (Prieme, Loft, Cutler & Poulsen, 1996), the ELISA assay gave about eight-fold higher results. This was attributed to the poor specificity of the antibodies employed in the system. The compound 5-hydroxymethyluracil has also been suggested as a biomarker (Bianchini & Cadet, 1996).

Further discussion will be limited largely to the role of phenolic antioxidants in restricting lipid peroxidation. Coronary heart disease is a major cause of death in western society. A heart attack is usually the consequence of two events—the narrowing of coronary arteries by atherosclerosis and the formation of a thrombus in a narrowed artery which blocks it completely. Atherosclerosis first appears in arteries as fatty streaks comprising slightly raised yellow, narrow areas of abnormality on the arterial wall that contain lipid-laden foam cells. The fatty streaks may develop into fibrous plaques which are rounded raised lesions typically with a fibrous cap covering an area rich in macrophages, lymphocytes and muscle cells. The rupture of the cap and release of its noxious contents often triggers the thrombosis that finally blocks an artery and produces ischaemia.

The origin of atherosclerosis is uncertain but the most popular theory at present is that it may be due to oxidised low density lipoprotein (LDL) (Steinberg & Workshop Participants, 1992). LDL is a large spherical particle, with a molecular weight of about 2.5 million, comprising a central core of about 1600 cholesteryl ester molecules and 200 molecules of triacylglycerols; this is surrounded by an outer monolayer shell composed of about 700 phospholipid molecules and 600 molecules of free cholesterol (Esterbauer, Wag & Puhl, 1993). The function of LDL is to transport the cholesterol to cells. A large protein termed apolipoprotein B is embedded in the outer monolayer which, in native LDL, is recognised by the LDL receptors. LDL enters the arterial wall from the plasma and is believed to be oxidised locally within the wall due to a localised oxidative stress. The mechanisms by which this occurs are highly controversial (Leake, 1997). The oxidised LDL is no longer recognised by the LDL receptors but is taken up readily by macrophages via the scavenger receptor to ultimately form foam cells. LDL oxidation by cells usually requires a source of iron or copper ions with copper being more potent than iron in catalysing LDL oxidation. LDL particles are protected against oxidation by several antioxidants.

The so-called lipid hypothesis is contrary to the French paradox which relates to the paradoxical association of a diet high in saturated fat and cholesterol with low coronary heart disease mortality. However, the Mediterranean regions with low heart disease mortality have a high consumption of vegetables and fruit, notably olives, olive oil and red wine. It is hypothesised that the antioxidants in these commodities protect against the effects of the high lipid diet (Williams & Marmot, 1997) as supported by Oliver who concludes (Oliver, 1997) "that people who eat a relatively large quantity of vegetables, fruits and grains have low rates of cardiovascular disease. This may be due to high intakes of antioxidant vitamins or to the myriad of other substances in the plants ... which need to be examined for preventive properties". Participants at the Saas Fee conference on free radical and antioxidant biology and preventive medicine were in no doubt of the value of antioxidants. The Saas Fee Declaration (Packer, 1996), that has been signed by many hundreds of researchers, states inter alia that "There is now general agreement that there is a need for further work at the fundamental scientific level ..... The major objective ... is the prevention of disease. This may be achieved by use of antioxidants which are natural physiological substances".

The plant phenols, because of their diversity and extensive distribution, are the most important group of natural antioxidants. They possess several common biological and chemical properties, namely, antioxidant activity, the ability to scavenge both active oxygen species and electrophiles, the ability to inhibit nitrosation (Helser & Hotchkiss, 1994) and to chelate metal ions, the potential for autoxidation, and the capability to modulate certain cellular enzyme activities.

2.1. Measurement of antioxidant activity

Lipid peroxidation involves generation of free radicals. Hence, one approach to assessing antioxidant activity is to examine, directly, free radical production and its inhibition by antioxidants. Electron spin resonance spectrometry is the only analytical technique that directly measures free radicals (Holley & Cheeseman, 1993). However, it is relatively insensitive. Spin trapping involves addition to samples of a compound which reacts with the free radicals to form radical-adducts (Noda et al., 1997) that are very much more stable and longer-lived than the original species. Nevertheless, the technique has had limited application. In the more usual approach, various indirect measurements are used to assess the effectiveness of pure phenolic compounds, individual fruit constituents or the total antioxidant potential of a fruit in preventing oxidative damage. The literature describing methods of assessing antioxidant behaviour falls into two groups reflecting the focus on activity in fruit (or, more generally, foods) or bioactivity in humans. In the case of food systems, the need is to assess the efficacy of an antioxidant(s) in providing protection for the food against oxidative spoilage. Oxidative stress in humans arises from an imbalance in the antioxidant status (reactive oxygen species versus defence and repair mechanisms). Among the endogenous

defences are enzymes such as superoxide dismutase, catalase and glutathione peroxidase, plus vitamin E, uric acid and serum albumins. Besides these defences, consumption of dietary antioxidants is also important and the analytical requirement is to determine the ability of consumed antioxidants to contribute to the antioxidant capacity of the biological system, hence relieving oxidative stress. This is the more demanding test that will ultimately require synthesis of radiolabelled phenolics targeted to specific organs to demonstrate specific activity. An important distinction from food-based systems is the absence of a single, definable substrate in many instances in vivo.

Antioxidant effectiveness is measured by monitoring the inhibition of oxidation of a suitable substrate. After the substrate is oxidised under standard conditions, the extent of oxidation (an end point) is measured by chemical, instrumental or sensory methods. Hence, the essential features of any test are a suitable substrate, an oxidation initiator and an appropriate measure of the end point (Table 4). In rare instances, an initiator has been omitted and the scavenging of endogenous preformed hydroperoxides has been studied (Salah et al., 1995). The combinations of substrate, initiation and, end point which have been used are numerous and even with the same reagents, several analytical strategies are possible (Rice-Evans & Miller, 1994). These include (1) Inhibition assay, measuring at a fixed time point; (2) Inhibition assay, measuring reaction rate, (3) Lag phase measurement, and (4) Decoloration assay. In systems 1 and 2, the reagents are mixed and the end point is measured after a pre-determined time interval in 1, while in 2, the rate of the reaction is monitored. In both cases, the presence of antioxidant in the reaction mixture reduces the change in end point parameter. In system 3, the length of the lag time to end point change is measured; samples with higher antioxidant activity suppress the change far longer than those with less activity. System 4 is restricted to methods using a coloured initiator. The initiator is generated until a stable absorbance has been achieved, and then the addition of an antioxidant mixture decolorises the reaction mixture and either the colour lost or colour remaining is used as an index of activity.

The extent of lipid peroxidation is monitored by various chemical and physicochemical measurements of the products of autoxidation. The primary products, hydroperoxides are frequently determined by iodometry (Marinova & Yanishlieva, 1996) or iron thiocyanate colorimetry and the result expressed as the peroxide value (PV). Several measures of the more or less stable final products of oxidation are used. Lipid peroxidation results in diene conjugation (Heinonen, Lehtonen & Hopia, 1998) that can be measured by ultraviolet absorbance at 230—235 nm corresponding to absorption by a diene-conjugated bond sequence (Dormandy and Wickens, 1987). Total or selected carbonyl compounds are determined spectrophotometrically following reaction with reagents of greater or lesser specificity. For example, total carbonyls are measured after reaction with 2,4-dinitrophenylhydrazine whereas malonaldehyde, the split product of an endoperoxide of unsaturated fatty acids, is determined spectrophotometrically at 532 nm following reaction with 2-thiobarbituric acid (TBARS) (Guillensans & Guzmanchozas, 1998). This reaction is widely used even though the reaction is not very specific and reaction conditions have a significant effect on colour development (Asakawa & Matsusshita, 1980; Slater, 1984). Selectivity of the TBARS procedure is improved by the use of HPLC to characterise the individual species (Chirico, Smith, Marchant, Mitchinson & Halliwell, 1993; Shih & Hu, 1999). Alternative measurements include anisidine value (Sheabar & Neeman, 1988) which measures 2-alkenals and volatile decomposition products as determined by either sensory or physicochemical methods. For instance, the oxidation of refined olive and soybean oils was followed by measurement of both PV and anisidine values (Sheabar & Neeman). Phenolic compounds, extracted from the rape (aqueous by-product) of olive oil with acetone, inhibited oxidative deterioration of the oils when stored in the dark at elevated temperature.

The simplest tests of antioxidant activity involve addition of the antioxidant to an oil, fat (Dziedzic & Hudson, 1983a) or model substrate such as methyl linoleate (Le Tutour & Guedon, 1992). Such tests are often performed under accelerated conditions involving elevated temperatures and air bubbling as, for example, the Rancimat test (Dziedzic & Hudson, 1984) or the Active Oxygen Method. Stability tests and their limitations have been reviewed by Frankel (1993) who summarised some of the published literature on the methods used in the evaluation of various natural antioxidants.

Data on induction times, obtained from the Rancimat test by measurement of conductivity, demonstrated that total hydrophilic phenols and the oleosidic forms of 3,4-dihydroxyphenylethanol (hydroxytyrosol) were correlated ($r = 0.97$) with the oxidative stability of virgin olive oil (Baldioli, Servili, Perretti & Montedoro, 1996) whereas tocopherols showed low correlation ($r = 0.05$). More specifically, the activity of tyrosol (in refined tallow) was lower than that of the synthetic BHT whereas oleuropein showed a stronger activity although the best protective effect was obtained with gallic acid esters and hydroxytyrosol (Castera-Rossignol & Bosque, 1994). Using milder test conditions (lower temperature, no active aeration), antioxidant activity in refined olive oil decreased (Papadopoulos & Boskou, 1991) in the series hydroxytyrosol, caffeic acid > butylated hydroxytoluene (BHT) > protocatechuic acid, syringic acid. Tyrosol, *p*-hydroxyphenylacetic acid, *o*-coumaric acid,

Table 4
Analytical methods used to assess antioxidant activity[a]

Substrate	Initiator	Species tested	End point measurement	Analytical strategy	References
LDL	Copper sulfate	Cinnamic acids	Hexanal production		Meyer, Donovan et al., 1998; Meyer, Heinonen et al., 1998.
Methyl linoleate	40°C	Wines and liquors	Diene absorption at 234 nM	System I[b]	Heinonen, Lehtonen et al., 1998
ABTS	Metmyoglobin, hydrogen peroxide	Fruit juices, phenols	Absorbance at 734 nM; TEAC	System I	Miller and Rice-Evans, 1997a,b
a? Linoleic acid a? Liposomes b? LDL	50°C b? AAPH c? Copper sulfate	Lemon peel phenolics	a? Ferric thiocyanate b? TBARS c? Diene formation	c. System III	Miyake, Yamamoto, Morimitsu et al., 1997; Miyake, Yamamoto, and Osawa, 1997
Phycoerythrin	AAPH	Fruits, phenols	Fluorescence quenching curves; ORAC	System III	Guo et al., 1997
Microsomes	Ascorbate, ferric chloride	Fruits	TBARS		Plumb et al., 1997.
Linoleic acid	40°C	Pineapple shell	Ferric thiocyanate		Larrauri et al., 1997.
2,7-Dichlorofluorescin-diacetate	AAPH	Serum	Absorbance and fluorescence; TRAP		Valkonen and Kuusi, 1997.
Liposomes	Iron(III), ascorbate	Apple phenolics (phloridzin)	TBARS	System II	Ridgway et al., 1996.
LDL	Copper(II)	Wine	Hexanal production		Frankel, Kanner et al., 1993
ABTS	Peroxidase, hydrogen peroxide	Fruit juices	Absorbance at 414 nm	System II + III	Arnao et al., 1996.
Olive oil	120°C, air flow in Rancimat	Phenolics	Conductivity	System I (Induction time)	Baldioli et al., 1996.
Phycoerythrin	AAPH	Fruits	Fluorescence quenching curves; ORAC	System III?	Wang et al., 1996.
Triacylglycerols and methyl esters of olive oil	100°C	Ferulic, caffeic and *p*-coumaric acids	Peroxide value	System II	Marinova and Yanishlieva, 1996.
LDL	Copper(II)	Flavonoids and wines	TBARS fluorescence; IC_{50} data.		Vinson and Hontz, 1995; Vinson, Dabbagh et al., 1995; Vinson, Jang et al., 1995
1. ABTS 2. LDL	1? Metmyoglobin, hydrogen peroxide 2? Nil	Flavonoids	1? absorbance at 734 nm, TEAC 2? TBARS and diene formation		Salah et al., 1995
ABTS	Metmyoglobin, hydrogen peroxide	Flavonoids	Absorbance at 734 nm; TEAC	System I	Rice-Evans et al., 1995.
•NO		Flavonoids		System II	van Acker et al., 1995.
Phycoerythrin	AAPH	Plasma	Fluorescence quenching curves; TRAP	System III	Ghiselli et al., 1995.
LDL	Copper(II)	Wines	Hexanal production	System I	Frankel et al., 1995.
a. DPPH b. linoleic acid	b. Xanthine oxidase	Chlorogenic acid and related phenolics	a? Absorbance decrease b? Conjugated diene formation	System I	Ohnishi et al., 1994.
LDL	Copper(II)	Resveratrol, wines	Hexanal production	System I	Frankel, Kanner et al., 1993; Frankel, Waterhouse et al., 1993.
Methyl linoleate	60°C	Olive leaf extract, oleuropein, tyrosol, hydroxytyrosol	Oxygen absorption	System II	Le Tutour and Guedon, 1992

continued

Table 4 (*continued*)

Substrate	Initiator	Species tested	End point measurement	Analytical strategy	References
LDL	Copper chloride	Olive oil	TBARS and diene production; TRAP		Scaccini et al., 1992.
Methyl linoleate hydroperoxide	Cytochrome c; luminol	Fruits	Chemiluminescence reduction		Ashida et al., 1991.
Olive oil	63°C	Phenols	Peroxide value	System I	Papadopoulos and Boskou, 1991.
Phycoerythrin	AAPH	Ascorbate, vitamin E	Fluorescence quenching curves		DeLange and Glazer, 1989
Soybean or olive oils	100°C	Olive extract	Peroxide and anisidine values	System I/II (Induction time)	Sheabar and Neeman, 1988.
Xanthine	Xanthine oxidase	Flavonoids	Superoxide anion scavenging		Robak and Gryglewski, 1988
Lipid	AAPH	Plasma	Oxygen consumption; TRAP		Wayner et al., 1985
Linoleic acid	β-carotene	Chia extracts, extracted phenols	Reduction in absorbance at 470 nm	System II	Taga et al., 1984.
Lard	100–140°C, air flow in Rancimat	Flavonoids	Conductivity of volatile acids	System I (induction time)	Hudson and Lewis, 1983.
Rendered lard	Heat, air flow in Rancimat	Flavones, isoflavones, chalcones and flavanones	Conductivity	System I (Induction time)	Dziedzic and Hudson 1983a,b

[a] DPPH, 1,1-diphenyl-2-picrylhydrazyl; AAPH, 2,2′-azobis-(2-amidinopropane) dihydrochloride; TRAP, total radical-trapping antioxidant parameter, ORAC, oxygen radical absorbance parameter; LDL, low density lipoprotein; TBARS, thiobarbituric acid-reactive substances.

[b] System I: Inhibition assay, measuring at a fixed time point; II: Inhibition assay, measuring reaction rate; III: Lag phase measurement; and IV: Decoloration assay.

p-coumaric acid, *p*-hydroxybenzoic acid, and vanillic acid had very little or no antioxidant activity, and their contribution to the stability of the oil, was negligible under the test conditions. However, using refined sunflower oil thin films in an accelerated oven-test (Castera-Rossignol & Bosque, 1994), the activity of hydroxytyrosol was lower than that of gallic acid esters. Similarly, the trends in antioxidant activity of phenolics differed (Satue, Huang & Frankel, 1995) according to whether hydroperoxide formation (PV) or decomposition (hexanal and volatiles) was measured in accelerated stability tests on olive oil.

Taga, Miller and Pratt (1984) described a procedure for determining antioxidant activity in which an aqueous emulsion of the antioxidant, carotene and lipid were prepared. The oxidative destruction of carotene by degradation products of linoleic acid in the system was measured spectrophotometrically at 470 nm.

Other substrates have included citronellal (Bocco, Cuvelier, Richard & Berset, 1998a, b) that was recently used in an accelerated test based on measurement of its degradation product by gas chromatography. Linoleic acid has also been used as a model substrate in various studies (Larrauri, Ruperez, Bravo & Calixto, 1996; Larrauri, Ruperez & Calixto, 1997; Miyake, Yamamoto, Morimitsu & Osawa, 1997). The linoleic acid and antioxidant were incubated at 40–50°C for 7 days in the dark, following which time the hydroperoxides from linoleic acid oxidation were determined, for example, by the iron thiocyanate method. Antioxidant activity was expressed as a reduction in peroxidation relative to a blank determination. Using this approach, lime peel fibre showed greater antioxidant capacity than orange peel fibre (Larrauri, Ruperez et al., 1996) and a plausible explanation was the presence in lime peel fibre of ellagic acid, quercetin and kaempferol. Alternatively, methyl linoleate has been used as substrate (Heinonen et al., 1998) to compare the antioxidant activity of 44 different berry and fruit wines and liquors with total phenolic contents between 91 and 1820 mg/L, expressed as gallic acid equivalents (GAE). In this case, oxidation was followed by conjugated diene measurement. Removal of sugars from the samples was a necessary step to prevent interference during oxidation of the methyl linoleate. Juices and raw materials including apple, arctic bramble, cowberries, cranberries, red currants, or rowanberries possessed antioxidant activity. However, the total phenolic content did not correlate with the antioxidant activity of the berry and fruit wines and liquors, therefore highlighting the importance of further characterization of the phenolic antioxidants present in berry and fruit wines.

These results emphasize the need to exercise caution in the interpretation of data and to measure a number of oxidation parameters to better evaluate antioxidant activity. Results obtained at elevated temperatures may involve some loss of volatile phenolic substances. Furthermore, oxidation mechanisms can change as temperatures are raised (Dziedzic & Hudson, 1984) while substrate effects (Marinova & Yanishlieva, 1996) and analytical technique also influence the results. The activity of an antioxidant on β-carotene will not be the same as on vegetable oil (Dziedzic & Hudson, 1983b). Moreover, water may have a pronounced effect on the efficacy of an antioxidant (Labuza, Silver, Cohn, Heidelbaugh & Karel. 1971). The effect of substrate can be attributed to the strong influence of the unsaturation type and degree of the lipid system (Hamilton, Kalu, Prisk, Padley & Pierce, 1997) on the kinetics and mechanism of the antioxidative action of the phenols.

The need for caution is further illustrated by studies on carnosine, a dipeptide, which is a useful antioxidant in food systems. Its activity has been carefully examined with large differences in the results in model systems (Kansci, Genot, Meynier & Gandemer, 1997). On the basis of malonaldehyde release in a liposome system, carnosine exhibits good antioxidant activity during methylene blue-photosensitised oxidation, weak antioxidant activity during riboflavin 5′-phosphate-sensitised oxidation and even a pro-oxidant effect during copper(II)-catalysed oxidation. The antioxidant effect in liposomes decreases according to the catalyst in the following order: copper/ascorbate, iron/ascorbate, hydrogen peroxide activated haemoglobin, photoactivated riboflavin and lipoxygenase. In the case of rosemary extracts, antioxidant effectiveness was significantly influenced by the type of system tested (bulk oils vs oil-in-water emulsions), by the oil substrates, the methods used to follow oxidation and the concentrations of test compounds (Frankel, Huang, Prior & Aeschbach, 1996).

Strategies have been developed for measuring the total antioxidant activity (TAA) of phenols or of the fruits containing them as their ability to scavenge free radicals generated in the aqueous and lipophilic phases. The first approach involves generation of a free radical species and direct measurement of its inhibition due to addition of antioxidant(s) (Arnao, Cano, Hernández-Ruiz, Garcia-Cánovas & Acosta, 1996). Alternatively, the generation of a radical is coupled to oxidation of a substrate in which case measurement of the inhibitory effect of an antioxidant is based on detection of either the radical or the products of oxidation. In either case, the ability to scavenge specific radicals may be targeted as, for example, hydroxyl radical (Aruoma et al., 1997) or nitric oxide radical (van Acker, Tromp, Haenen, Van der Vijgh & Bast, 1995).

The radical that is generated varies and systems have been described using horseradish peroxidase/H_2O_2 (Arnao et al., 1996), *o*-phenylenediamine/H_2O_2, copper(II)/cumene hydroperoxide, trichloromethyl peroxyl radical (Aruoma et al., 1997) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Brand-Williams, Cuvelier & Berset,

1995; Ohnishi et al., 1994). The use of azo compounds (e.g. 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) (ABTS) (Miller & Rice-Evans, 1997a) for the controlled initiation of oxidation is common. End point detection also varies and has been based on measurement of fluorescence inhibition, chemiluminescence (Ashida, Okazaki, Tsuzuki & Suzuki, 1991; Whitehead, Robinson, Allaway, Syms & Hale, 1995), oxygen uptake and absorbance (Rice-Evans & Miller, 1994).

The chromogenic redox indicator ABTS (absorption maximum 342 nm) has high water solubility and chemical stability. It is a peroxidase substrate which, when oxidised in the presence of H_2O_2 generates a metastable radical with a characteristic absorption spectrum and high molar absorptivity at 414 nm (Arnao et al., 1996; Miller, 1996a). However, there are secondary absorption maxima in the wavelength regions of 645, 734 and 815 nm. Its use, as described by Rice-Evans and Miller (1994), is based on the formation of the ferrylmyoglobin radical (from reaction of metmyoglobin with hydrogen peroxide) which is then free to react (at a higher reaction rate) with ABTS to produce the ABTS radical cation. The formation of this radical was measured in the near infrared at 734 nm which minimised interference from other absorbing components and from sample turbidity. The accumulation of the ABTS radical can be inhibited by the presence of an antioxidant in the reaction medium, to an extent and on a time scale dependent on the antioxidant activity.

The method of Arnao et al. (1996) is similar to that of Rice-Evans and Miller (1994) but differs in a number of important aspects. Unlike the Rice-Evans method that used the metmyoglobin peroxidase activity, a commercial peroxidase was used by Arnao. There were no interferences in the Arnao method at the optimal wavelength of 414 nm and this translated to better detection limits. Miller and Rice-Evans (1997b) found that results of the myoglobin/ABTS assay and direct reduction of the ABTS radical cation were very similar, establishing that the action of the antioxidants studied was via scavenging of the ABTS radical cation and not by inhibition of its formation through reduction of ferrylmyoglobin or reaction with hydrogen peroxide.

The production of peroxyl free radicals by the thermal decomposition of 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) can be followed in a number of ways. For instance, 2,7-dichlorofluorescin is oxidised by the free radicals to the fluorescent 2,7-dichlorofluorescein and the effect of added antioxidant is seen as an increase in the lag phase (Valkonen & Kuusi, 1997). Alternatively, the highly fluorescent protein phycoerythrin (PE) has been used as the target of free radical damage (De Lange & Glazer, 1989; Wang, Coa & Prior, 1996). Peroxyl radicals quench the fluorescence of phycoerythrin while addition of an antioxidant that reacts rapidly with peroxyl radicals inhibits the loss of fluorescence intensity and this inhibition is proportional to the antioxidant activity. The initial procedure has been modified to overcome interference by plasma proteins.

Methods of expressing results are as varied as the approaches to measurement. For instance, Arnao et al. (1996) reported the TAA of orange and grapefruit juices as 4.3 and 6.1 mM L-ascorbic acid equivalents, respectively. Alternatively, the EC_{50} has been determined (Brand-Williams et al., 1995; Ohnishi et al., 1994) as the amount of antioxidant necessary to decrease, by 50% the initial DPPH concentration by monitoring the decrease of its absorbance at 517 nm. The time taken to reach the steady state to EC_{50} concentration (T_{EC50}) was also calculated. In recognition of the effect of both parameters on antiradical capacity, a new parameter, namely, Antiradical Efficiency, which combined both factors, was defined (Sanchez-Moreno, Larrauri & Saura-Calixto, 1998). It is too early to comment on the acceptance of this new term. Olive oil phenolics, namely hydroxytyrosol and oleuropein, showed low EC_{50}s indicating that both compounds are potent scavengers of superoxide radicals and inhibitors of neutrophils respiratory burst (Visioli, Bellomo & Galli, 1998), properties which may account for the health benefits of olive oil.

The TRAP (Total Radical-trapping Antioxidant Parameter) assay of Wayner, Burton, Ingold, and Locke (1985) has been widely used to determine TAA, based on measuring oxygen consumption during a controlled lipid peroxidation reaction induced by thermal decomposition of AAPH. The acronym TRAP, meaning Total Radical-trapping Antioxidant Parameter, expresses results as the number of μmoles of peroxyl radicals trapped by 1 litre of plasma. Results can be standardised by addition of Trolox (water-soluble vitamin E analogue) to the sample after consumption of natural antioxidants to produce a second induction period. The method is time-consuming and suffers a number of problems (Ghiselli, Serafini, Maiani, Azzini & Ferro-Luzzi, 1995; Rice-Evans & Miller, 1994) although the concept has been very useful for quantifying and comparing antioxidant capacity (Serafini, Maiani & Ferroluzzi, 1998).

A more useful method of expressing results employs the Trolox Equivalent Antioxidant Activity (TEAC) (Rice-Evans & Miller, 1994) which is also standardised against Trolox. This measures the concentration of Trolox solution with an equivalent antioxidant potential to a 1.0 mM solution of the substance under investigation. As used by Rice-Evans and Miller, the TEAC reflects the relative ability of hydrogen- or electron-donating antioxidants to scavenge the ABTS radical cation compared with that of Trolox. Miller and Rice-Evans (1997a) reported the TEAC of orange and apple juices and blackcurrant drink (Ribena) as well as the contribution of individual phenolic antioxidants

(Table 5). The bulk of the TAA of apple juice could be accounted for by chlorogenic acid and the phloretins, while in both orange juice and Ribena, vitamin C was the major antioxidant. However, in the case of orange juice, HPLC required preliminary filtration and the measured composition reflects the soluble flavonoid portion only (Rouseff et al., 1987). The authors concluded that the phenolic antioxidants protect vitamin C against oxidative decomposition, with those in blackcurrant having the greatest vitamin C-sparing activity. However, the situation is complex and winemakers add ascorbic acid during fermentation as an anti-browning agent, presumably to protect the phenolics against oxidation.

In another approach, the TAA of several juices and fruits (Table 6) has been reported (Wang et al., 1996) as the automated oxygen radical absorbance capacity (ORAC). This value combined both inhibition time and the extent of inhibition into a single quantity whereas other methods use either the inhibition time at a fixed inhibition degree or the inhibition degree at a fixed time as the basis for quantifying results. Results, which were reported as the ORAC value in μmoles of Trolox equivalents, showed significant variation in the TAA between fruits with strawberry having the highest ORAC activity on the basis of both wet and dry weight of fruit. The contribution of vitamin C to the TAA was less than 15% except for kiwi fruit and honey dew melon. Most of the antioxidant capacity of these fruits was from the juice fractions. The contribution of the fruit pulp fraction (extracted with acetone) to the total ORAC activity of a fruit was usually less than 10%.

ORAC values showed a significant positive linear correlation with electrochemical data (Table 7) obtained by HPLC with coulometric array detection (Guo, Cao, Sofic & Prior, 1997). Phenolic acids, in general, had lower antioxidant activities against peroxyl radicals than flavonoids that contained multiple hydroxyl groups. However, the flavonoid glycosides (including rutin, naringin and hesperidin) usually had low ORAC activities. A number of factors determine antioxidant activity including reactivity as a hydrogen- or electron-donating agent and this aspect relates to its reduction potential. Indeed, there is broad agreement between the half-peak reduction potential and the TAA (Table 7) as measured by TEAC (Rice-Evans, Miller & Paganga, 1997). This was rationalised on the basis that both electrochemical oxidation and hydrogen-donating free radical scavenging involve the rupture of the same phenolic bond. Thus, with the exception of kaempferol, flavonoids with efficient scavenging properties have a TEAC value exceeding 1.9 mM and a half-peak reduction potential below 0.2 mV.

The bioactivity of phenolic antioxidants has been tested by in vitro measurements in systems that attempt to simulate biological conditions but are otherwise based on the same principles as for food testing, namely, the inhibition

Table 5
Antioxidant activity of apple and orange juice and black-currant drink with relative contributions of juice components (Miller & Rice-Evans, 1997a)

Constituent	TEAC[a]	Concentration (μM)	Activity (=conc.×TEAC) (μM)
Apple juice			
Chlorogenic acid	1.24	274	340
p-Coumaroylquinic acid	2.22	74	164
Phloridzin	2.38	23	55
Phloretin Xyloglucoside	2.38	35	83
Epicatechin	2.50	< 14	35
Ascorbic acid	1.00	51	51
Calculated TAA			728
Measured TAA			840
TAA unaccounted for			112
Orange juice			
Hesperidin	1.08	141	152
Narirutin	0.76	62	47
Ascorbic acid	1.00	2270	2270
Calculated TAA			2469
Measured TAA			2610
TAA unaccounted for			141
Ribena black-currant drink			
Delphinidin-3-glucoside	2.47	5.9	150
Delphinidin-3-rutinoside	3.25	19.9	65
Cyanidin-3-glucoside	2.47	2.4	6
Cyanidin-3-rutinoside	3.25	15.0	49
Gallic acid	3.01	Undetectable	
Ascorbic acid	1.00	3726	3726
Calculated TAA			3861
Measured TAA			5070
TAA unaccounted for			1209

[a] TEAC, millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation. For purposes of clarity, data are reported as mean values only.

Table 6
Antioxidant activity of various fruits and fruit juices (Wang et al., 1996)[a]

Fruit	Total ORAC (as is basis)	Total ORAC (dry-matter basis)	ORAC juice extract (as-is basis)
Strawberry	15	150	12
Plum	9	80	8
Orange	7	50	7
Grape, red	7	36	4
Kiwi fruit	6	36	6
Grapefruit, pink	5	48	5
Grape, white	5	26	3
Banana	2	9	2
Apple	2	13	2
Tomato	2	38	2
Pear	1	10	1
Melon	1	13	1

[a] Data are quoted as means and expressed as micromoles of Trolox equivalents per gram of fruit (as is or dry basis).

Table 7
Antioxidant activity, dominant potential and half peak reduction potential of phenolic compounds (Guo et al., 1997; Rice-Evans and Miller, 1996; Rice-Evans et al., 1995, 1996, 1997)

Compound	ORAC	Dominant potential (mV)	TEAC (mM)	Half-peak reduction potential (mV)
Hydroxybenzoic acids				
4-hydroxybenzoic acid	0.17	700		
Protocatechuic acid	2.06	210		
Gallic acid	1.74	140	3.0	
2,5-dihydroxybenzoic acid	1.20	0–70		
Vanillic acid	1.11	420		
Syringic acid	1.27	350		
Hydroxyphenylacetic acids				
4-hydroxyphenylacetic acid	0.41	560		
Cinnamic acids				
Caffeic acid	2.23	140	1.3	
p-Coumaric acid	1.09	490	2.2	
Ferulic acid	1.33	350	1.9	
Sinapic acid	1.66	210		
Flavanols				
Catechin	2.49	490	2.4	0.16
Epicatechin	2.36	490	2.4	
Gallocatechin gallate	2.43	490		
Flavanones				
Fustin	3.91	770		
Taxifolin	3.59	700	1.9	0.15
Naringin	0.47	700	0.2	
Narirutin			0.8	
Naringenin			1.5	0.6
Hesperidin	0.04	440	1.0	
Hesperetin			1.4	0.4
Eriodictyol	3.41	770		
Flavonols				
Rutin	0.56	700	2.4	0.18
Quercetin	3.29	770	4.7	0.03
Kaempferol	2.67	700–770	1.3	0.12
Flavones				
Luteolin			2.1	0.18
Apigenin			1.5	>1
Anthocyanidins				
Cyanidin			4.4	
Pelargonidin	0.96	420	1.3	
Isoflavone				
Genistein	2.38	700		

of the accumulation of oxidised products. Caution is necessary when extrapolating from in vitro test systems using food components, or especially ill-defined extracts, to the human in vivo situation, as antioxidant activity is a complex interplay of several related factors. Moreover, there is a distinction between antioxidant activity and the antioxidant capacity that this confers on the blood plasma and the effect on oxidative susceptibility, for example, of LDL. In this context, the morphology of the LDL particle is important and differences in antioxidant activity can often be rationalised in terms of partition coefficients and accessibility to the lipid peroxyl radicals (Miura et al., 1995). A considerable amount of evidence is accumulating to suggest that synergism between aqueous and lipophilic systems is the important factor (Harats et al., 1998) and this shift in attitude is reflected in a holistic approach to the Mediterranean diet (Ghiselli, Damicis & Giacosa, 1997). For this reason, where the interest is in the relative bioactivity of an antioxidant, tests should be performed in both aqueous and lipophilic phase systems (Salah et al., 1995). Antioxidant activity in the lipophilic phase is a composite response to partitioning behaviour and rates of reaction with the relevant radical species. The kinetics of the various reactions need to be considered as most radicals are highly reactive species and can diffuse only very short distances (Slater, 1991). Data on the lipophilic phase derive from studies on fatty acids, liposomes, which have been used extensively as in vitro cellular models for investigating antioxidant activity, and especially LDL. Rice-Evans, Miller and Paganaga (1996) have presented a detailed discussion of structure–activity effects in both lipophilic and aqueous phases, the latter based on measurement of TEAC.

Peroxidation of LDL can be initiated in vitro by incubating it with macrophages, endothelial cells, lymphocytes or in cell-free systems utilising a variety of prooxidants. For instance, in membrane systems, such as liposomes or microsomes, LDL peroxidation can be initiated by oxidation of iron(II) with hydrogen peroxide, or the reduction of iron(III) by ascorbate (Aruoma, Halliwell, Laughton, Quinlan & Gutteridge, 1989; Minotti & Aust, 1987; Ridgway, O'Reilly, West, Tucker & Wiseman, 1996). However, the efficiency of a phenolic or fruit extract in preventing lipid peroxidation will vary according to the catalyst (Plumb et al., 1996). For instance, rutin had minimal effect on lipid peroxidation in normal microsomes but inhibited it by 75% in iron-overloaded microsomes (Osato, Afanas'ev & Korkina, 1996). Grapefruit extract was a potent inhibitor of iron/ascorbate-induced lipid peroxidation of microsomes (Plumb, Chambers, Lambert, Wanigatunga & Williamson, 1997) enriched with cytochrome P450 isoenzymes (1A1 and 3A4) and control microsomes containing negligible amounts of cytochrome P450. Apple and tomato extracts produced a differential effect between the classes of microsomes while pear demonstrated no effect. The degree of peroxidation was a function of the total amount of P450 present. The antioxidant activity of grapefruit was not dependent on the presence of P450 isoenzymes. On the other hand, the effects of apple and tomato must be related to the action of compounds derived from these fruits on the P450s since the levels of peroxidation in the control microsomes were unaffected.

LDL oxidation by macrophages or copper was inhibited by a variety of flavonoids (Vinson, Dabbagh, Serry & Jang, 1995; Vinson, Jang, Dabbagh, Serry & Cai, 1995) including quercetin, morin and fisetin (de Whalley, Rankin, Hoult, Jessup & Leake, 1990) and by cinnamates in prunes (Donovan, Meyer & Waterhouse, 1998). Quercetin was also capable of protecting cells from the toxic effects of oxidised LDL (Negre-Salvayre & Salvayre, 1992) while rutin acted synergistically to protect endothelial cells (Negre-Salvayre, Mabile, Delchambre & Salvayre, 1995). There has been intense interest in the bioactivity of wine and grape juice (Carbonneau, Leger, Descomps, Michel & Monnier, 1998; Frankel, Bosanek, Meyer, Silliman & Kirk, 1998; Frankel, Waterhouse & Kinsella, 1993; Frankel, Waterhouse & Teissedre, 1995; Ghiselli, Nardini, Baldi & Scaccini, 1998; Heinonen et al., 1998) which also inhibited copper-catalysed LDL oxidation (Frankel, Kanner, German, Parks & Kinsella, 1993). The phenol concentration (largely flavonoids) of red wines varied from 4 to 10 mM and, in white wines, from 0.4 to 0.7 mM (Vinson & Hontz, 1995) and Frankel, Waterhouse & Teissedre correlated the inhibition of LDL oxidation with individual phenols. The antioxidant activity of white wine and juices was related to their flavanol and cinnamate contents while that of red juices was related to their anthocyanin levels (Frankel, Bosanek et al., 1998). In contrast, Plumb et al. (1996) observed that cinnamic acids and naringin did not contribute to the inhibition of lipid peroxidation by fruit extracts but that they inhibited deoxyribose degradation which has been used as a measure of hydroxyl radical scavenging ability.

The antioxidant effects of an extract of the fruit exudate of *Myrica gale* L., and isolated dihydrochalcones (Myrigalone B, MyB) were studied (Mathiesen, Malterud & Sund, 1995) in hepatocytes and mitochondria incubated with *tert*-butyl hydroperoxide. Lipid peroxidation was measured by TBARS and the antioxidant activity expressed as IC_{50} values (compare EC_{50}) which, for MyB, were 23 μM in hepatocytes and 5.2 μM in mitochondria. Both MyB and the fruit extract caused radical scavenging of the diphenylpicrylhydrazyl radical with IC_{50} values of 32 and 14 μM (as MyB), respectively. MyB was also an effective antioxidant in copper(II)-induced oxidation of human LDL (Mathiesen, Wang, Halvorsen, Malterud & Sund 1996) and LDL from cholesterol-fed rabbits (Mathiesen, Malterud, Nenseter & Sund, 1996) as shown by its ability to dose-dependently prolong the lag time for the formation of conjugated dienes. MyB also dose-dependently reduced the maximum rate of formation of conjugated dienes but it had no influence on the maximal amount of conjugated dienes formed. Future studies should examine carefully the need to measure both rate and extent of oxidation (see also Wang et al., 1996). The action of MyB was synergistic with ascorbic acid in improving the antioxidant status of LDL particles (Mathiesen, Wang et al., 1996).

The antioxidant activities of the flavonoids, eriocitrin, diosmin, hesperidin, and narirutin extracted from lemon fruit were examined using linoleic acid autoxidation (as previously described), and both a liposome and an LDL oxidation system (Miyake et al., 1997b). In the liposome system, lipid peroxidation was induced by AAPH and the extent of inhibition by added antioxidant was determined as TBARS at 532 nm. For the LDL system, the effect of antioxidant on lag time of the copper(II)-mediated oxidative modification of LDL was measured by monitoring conjugated diene formation at 234 nm. Flavonoid glycosides generally exhibited weaker activity than the corresponding aglycones. Eriocitrin exhibited the highest activity of all lemon constituents, as measured by all three methods. Its metabolites by intestinal bacteria (the aglycone eriodictyol, 3,4-dihydroxyhydrocinnamic acid, and phloroglucinol) exhibited weaker antioxidative activity but nevertheless exhibited greater activity than α-tocopherol in the LDL oxidation system and had approximately the same activity as (−)-epigallocatechin gallate.

2.2. Structure–activity relationships

There are approximately 5000 known plant phenolics and model studies have demonstrated that many have antioxidant activity. However, there is a wide degree of variation between different phenolic compounds in their effectiveness as antioxidants. Furthermore, there are a number of different mechanisms by which phenolics may act as antioxidants: via free radical scavenging, hydrogen donation, singlet oxygen quenching, metal-ion chelation, or as substrates for attack by superoxide (Hamilton et al., 1997; Robak and Gryglewski, 1988). Additional mechanisms may be involved in vivo where phenolics may protect the α-tocopherol from oxidation by being preferentially oxidised themselves; or they may regenerate α-tocopherol by donating a hydrogen atom to the α-tocopherol radical (Miura, Watanabe, Tomita, Sano & Tomita, 1994), thereby extending the lag phase before lipid hydroperoxides build up in LDL. Phenolics may also inhibit oxidation by chelating divalent metal ions and thus reducing the formation of free radicals induced by Fenton reactions. This leaves open the question, what makes a phenolic compound a good antioxidant?

Lipid autoxidation is a free radical chain reaction that, like all chain reactions, involves initiation during which free radicals are formed. The major initiation process is likely to involve the decomposition of hydroperoxides

$$ROOH^{*} \rightarrow ROO^{\bullet} + H^{\bullet}$$

$$ROOH \rightarrow RO^{\bullet} + {}^{\bullet}OH$$

$$2\ ROOH \rightarrow RO^{\bullet} + H_2O + ROO^{\bullet}$$

since direct reaction of a lipid molecule (singlet electronic state) with molecular oxygen (triplet electronic state) is unlikely (Privett & Blank, 1962). However, triplet oxygen can be converted to the 1000-fold more active singlet oxygen in the presence of photosensitizers such as phytin pigments and FD and C red No. 3 (Koryckа-Dahl & Richardson, 1978; Hamilton et al., 1997). The radicals formed in the above reactions, including peroxyl, $ROO^{\bullet}$ and alkoxyl, $RO^{\bullet}$, participate in further reactions.

Phenolic antioxidants function primarily as terminators of the free radical reactions, depending for their activity on the ability to interfere with the chain propagation reactions (Boland and ten-Have, 1947) by rapid donation of a hydrogen atom to lipid radicals:

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

$$RO^{\bullet} + AH \rightarrow ROH + A^{\bullet}$$

Alternative mechanisms only become important at very low oxygen pressures, very low rates of chain initiation or very high concentrations of antioxidant. This can have important implications for comparing results from model systems with those from in vivo studies since the oxygen pressure in healthy biological tissues (2.7×10^2 Pa) is much lower than in the atmosphere. Hence, tests for bioactivity should be performed in conditions of low oxygen partial pressure. The UV-initiated generation of stable phenoxy radicals leading to termination of other radical chain reactions or, in the case of cinnamic acids, isomerisation, plays only a minor antioxidant role in aqueous solutions. However, this UV-catalysed radical-scavenging mechanism may become very important in biological materials of limited light permeability (Graf, 1992).

To be successful as an antioxidant, the phenoxy radical formed in the above reactions must not initiate formation of further radicals but must rather be a relatively stable species. The effectiveness of phenolic antioxidants is related to the stabilisation of the phenoxy radical by delocalisation and the absence of suitable sites for attack by molecular oxygen (Belitz & Grosch 1986). Based on this mechanism, a number of generalisations are possible. The efficiency of an antioxidant will increase with decreasing A–H bond strength and stability of the resulting phenoxy radical. For example, activity is enhanced by substitution of the phenol with bulky substituents or groups exerting electron-donating inductive effects. In the case of bulky substituents, steric hindrance in the region of the radical reduces the rate of propagation reactions involving the antioxidant radical itself. As an illustration, the efficiency of 1,2-dihydroxybenzene derivatives is related to the stabilisation of the phenoxy radical via an intramolecular hydrogen bond (Gordon, 1990) and to the fact that the initially-produced semiquinoid radical can be further oxidised to a quinone or hydroquinone molecule.

Flavonoids and cinnamic acids act as free radical acceptors and chain breakers whilst the action of flavonols is potentially bimodal. Chelation can occur at the 3-hydroxy-4-keto group and/or the 5-hydroxy-4-keto group of the flavonol (when the A-ring is hydroxylated in the 5-position (Hudson & Lewis, 1983). Metal-complexing ability is also possible with an o-quinol grouping on the B-ring. However, hesperitin (5,7,3′-trihydroxy-4′-methoxyflavone) possesses an active chelating site and yet displays negligible activity. Based on this observation and other evidence, it has been concluded that the main action of all flavonoids is via a free radical acceptor mechanism. Recent evidence (George, Figueiredo & Brouillard, 1998), however, indicates the existence of an open-cavity complex formed selectively between iron(III) and certain anthocyanins. The authors suggest that the role of anthocyanins as antioxidants in living systems may be related to their regulation of iron uptake. In another recent study (Arora, Nair & Strasburg, 1998), several flavonoids exhibited higher antioxidant efficacies against metal ion-induced peroxidations than peroxyl radical-induced peroxidation, suggesting a more important role for metal chelation (Halliwell, 1994) than has been supposed (Bors, Michel & Saran, 1994; Morel, Lescoat, Cillard & Cillard, 1994). A number of structure–activity relationships were observed. Data from electron paramagnetic resonance spectrometry provided evidence for the phenolic-mediated redox coupling between ascorbate and peroxidase (Yamasaki & Grace, 1998). The results imply that metal ions may influence the nature of plant phenolics in vivo by altering the lifetime of phenoxyl radicals as the oxidised products.

The relation between chemical structure of phenolics and their antioxidant activity has been of considerable interest. Apart from the general observations made above, more specific effects have been noted. The activity of phenolic acids increases when additional hydroxy groups are present. Steric hindrance would also enhance the activity, as observed with the synthetic antioxidant, butylated hydroxytoluene. The hydroxylated cinnamic acids are more effective than their benzoic acid counterparts and activity becomes marked in caffeic and chlorogenic acids (Hudson & Mahgoub, 1980). Ortho-substitution with electron-donating alkyl or methoxy groups increases the stability of the aryloxyl radical and hence its antioxidant potential (Rice-Evans et al., 1996).

The alkoxyl radical scavenging ability in a lipid peroxidation system increased in the order salicyclic < vanillic < chlorogenic < caffeic < gallic acids (Milic, Dijlas & Canadonovic-Brunet, 1998). The free cinnamic acids and their esterified derivatives also exerted considerable antioxidant potency toward copper-catalysed

human LDL oxidation in vitro (Meyer, Donovan, et al., 1998). The differences in activity between the different cinnamates can primarily be ascribed to variations in the hydroxylation and methylation pattern of the aromatic ring. For example, methylation of one of the o-hydroxy groups decreased antioxidant activity toward LDL, as seen in the reduced activity of ferulic and fertaric acids compared to caffeic acid and its derivatives. The reduced activity of caffeic acid, when esterified to quinic acid as in chlorogenic and 3-coumaroylquinic acid, agrees with data from other systems (Chen & Ho, 1997). These differences may in part be due to changes in solubility and partition of caffeic acid in the LDL system. Alternatively, Meyer, Donovan, et al. (1998) hypothesised that the antioxidant mechanism may involve sterical blockage of the copper access to apolipoprotein B tryptophans, via binding of antioxidants to apolipoprotein B.

Antioxidant activity of flavonoids is closely related to the position and degree of hydroxylation of the molecule (Table 7). The major consideration for activity is hydroxylation of the B-ring (Herrmann, 1976; Miller, 1996b). A single hydroxy substituent generates little or no antioxidant activity. For instance, the flavanones, naringenin and hesperitin, with only one hydroxy group on the B-ring, possess negligible antioxidant activity. In contrast, all flavonoids with 3′,4′-dihydroxy substitution possess antioxidant activity (Dziedzic & Hudson, 1983a) illustrating the overriding importance of the catechol grouping. Quercetin and cyanidin, with 3′,4′-dihydroxy substituents in the B ring and conjugation between the A-and B-rings, have antioxidant potentials four times that of Trolox (Rice-Evans, Miller, Bolwell, Bramley & Pridham, 1995). The hydroxyl radical scavenging activity of flavonoids increases with the number of hydroxyl groups substituted on the B-ring, especially at C-3′ (Ratty & Das, 1988).

The effect of glycosylation awaits clarification. In some tests, the glycoside and aglycone possess approximately the same antioxidant activity but, in other tests, the glycoside has lower activity. The glycoside rutin, with disaccharide substitution, has reduced activity to the corresponding aglycone, quercetin (Kelley & Watts, 1957) which had the same protective effect as quercitrin (quercetin 3–rhamnoside) tested in a carotene–lard system. Most observations relating to structure–activity relationships are based on relative antioxidant efficiencies. Quantitative structure-activity relationships of antioxidant properties of phenols will require absolute rate constants for the various reactions (Bors et al., 1994).

The influence of chemical structure on antioxidant activity of catechin, quercetin, cyanidin, caffeic acid, and ellagic acid was evaluated by measuring inhibition of copper-catalysed human LDL oxidation in vitro (Meyer, Heinonen & Frankel, 1998). These compounds, which all possess a similar *o*-dihydroxy moiety, showed decreasing antioxidant activity in the sequence catechin > cyanidin ≅ caffeic acid > quercetin > ellagic acid. Structural effects on activity are complex and often subtle which means that comparisons must be made between members of particular classes of phenolic. Both aqueous and lipid phase antioxidants are needed to protect LDL from lipid peroxidation (Belguendouz, Fremont & Gozzelino, 1998; Valkonen & Kuusi, 1997). However, synergism has been reported between both groups of substances and flavonoids and cinnamates are efficient antioxidants in both lipid and aqueous phases. The latter has been demonstrated (Plumb, Price, Garcia-Conesa & Williamson, 1998) by measuring the TEAC value using scavenging of the ABTS radical (indicative of aqueous phase capacity) and the inhibition of lipid peroxidation of phosphatidyl choline vesicles (antioxidant potential in the lipid phase) (Williamson, Plumb, Uda, Price & Rhodes, 1997).

The bioactivity of the phenolics has been controversial. This can be related, in part, to the limited knowledge and understanding of the intestinal absorption and bioavailability of phenolics. Masquelier (1986) stressed a double prerequisite for the metabolic efficiency of flavonoids as their intestinal absorbability and their compatibility with the plasma pH. A model comprising erythrocyte membrane damage consisting of lipid peroxidation and increase in passive potassium ion permeability after blocking superoxide dismutase has been used (Maridonneau-Parini, Braquet & Garay, 1986) to classify the various flavonoids into four groups:

- those decreasing the oxygen free radical-stimulated potassium ion permeability, e.g. kaempferol, naringenin, apigenin, naringin;
- those increasing the deleterious effect of oxygen-free radicals, e.g. delphinidin, myricetin, quercetin;
- those characterised by opposite effects depending on the concentration, e.g. catechin, cyanin, morin, phloretin;
- those remaining inactive, e.g. phloridzin, rutin.

The distinction is not always so unequivocal and the results depend on the oxidation system considered and the model used. For example, using cytochrome P450-mediated reaction in rat liver microsomes (Sousa & Marletta, 1985), quercetin was a potent uncoupler of P450 reactions, elevating the rates of hydrogen peroxide formation. On the other hand, quercetin inhibited the generation of hydrogen peroxide (Ogasawara, Fujitani, Drzewiecki & Middleton, 1986) in the peroxide-induced histamine release from human basophils. An inhibitory effect of flavonoids was observed (Koch & Löffler, 1985) by assaying the malonaldehyde formed in a model using human platelets submitted to peroxidation. The following order of decreasing potency was reported (Hodnick, Kung, Roettger, Bohmont & Pardini, 1986)

for 14 flavonoids in beef heart mitochondria: chalcone > flavone > flavonol > dihydroflavonol > anthocyanidin.

For a fuller discussion of this aspect the reader is referred to Rice-Evans et al. (1996).

2.3. Degradation products of antioxidants

The extensive but diminishing use of synthetic compounds such as butylatedhydroxyanisole and gallates has generated numerous studies of their toxicology and degradation products. In general, the most common breakdown products are dimers produced by the formation of phenoxy radicals followed by rearrangement and coupling reaction with a second radical (Kurechi, Aizawa & Kunugi, 1983). The naturally-occurring phenolics have traditionally not been commercially exploited and there is considerably less information on their degradation. However, by analogy with the synthetic materials and consistent with their free radical chemistry, dimers and higher oligomers plus, in the case of some phenolics, quinone and hydroquinone derivatives are expected to feature prominently amongst the products. Being electron-richer than the A-ring, organic peroxyl radicals selectively attack the B-ring of flavonoids (Jovanovic, Steenken, Tosic, Marjanovic & Simic, 1994). In the case of dihydroxyflavonoids, a consecutive two-electron oxidation reaction produces the flavonoid phenoxyl radical which subsequently scavenges another peroxyl radical to form a quinone.

3. Phenolic compounds as substrates for oxidation reactions

Phenolic compounds act as substrates for a number of oxidoreductases, namely, polyphenoloxidases (PPO) and peroxidases (POD). These enzymes are very widely distributed (Mayer & Harel, 1987) and, although there is much similarity between the oxidases from plants and animals, the discussion is confined to oxidation reactions occurring in fruit. The main oxidative phenomenon (usually deteriorative) of this type is enzymatic browning which involves an initial enzymatic oxidation of phenolic compounds located predominantly in the vacuole by polyphenoloxidases located in cytoplasm to form slightly coloured quinones. Although the oxidation of phenolic compounds leads to discoloration, the products are considered as antioxidants. Situations that give rise to browning are physiological during maturation, various disorders (Abdallah, Gil, Biasi & Mitcham, 1997) and technological processes involving crushing or wounding. Nevertheless, these situations alone are not sufficient for browning which also requires the appropriate enzyme activities and substrate content. Many authors have attempted to correlate browning intensity with these factors, often with contradictory results (Goupy et al., 1995). Unfortunately both substrate content and enzyme activity change during fruit development and the susceptibility to browning illustrates the complex interactions involved.

One of the biggest problems is associated with the difficulty of analytical measurement of the substrate concentration and the measurement of browning which can be followed either by absorption spectrometry or reflectance methods (Goupy et al., 1995; Sapers et al., 1989). Absorption techniques commonly involve spectrophotometric measurement on solutions obtained after crushing the tissues and removal of solids. Such measurements estimate the soluble pigments and are usually performed near 400 nm corresponding to the absorption maxima of catechol or 4-methylcatechol quinones (Waite, 1976; Weemaes, Ludikhuyze, Van den Broeck, Hendrickx & Tobback, 1998) but make no allowance for the diverse spectral characteristics of the absorbing species whose absorption maxima may vary between 360 and 500 nm. Measurement of the insoluble polymerised pigments bound to membranes can be evaluated by reflectance measurements on the insoluble fraction.

The role of peroxidases (E.C. 1.11.1.7) in enzymatic browning is limited by the availability of hydrogen peroxide and it has been assumed that, with few exceptions (e.g. browning in *Litchi*, internal browning in pineapple) (Underhill & Critchley, 1995) peroxidase oxidation is rarely important. There is now unequivocal evidence that POD has no oxidative (oxygen-dependent) activity. Nevertheless, POD does appear to play a role in enzymatic browning (Richard-Forget & Gauillard, 1997) and, in the presence of PPO, enhanced the degradation of phenols. Two mechanisms were proposed to explain this additional consumption. First, whatever the substrate used, PPO oxidation generated hydrogen peroxide, the amount of which varied with the phenolic structure. Second, quinonic forms were used by POD as peroxide substrate.

The extensive literature on browning reactions in fruits emphasises two aspects, namely, the role of the various enzyme systems and the prevention of browning (Lee & Jaworski, 1988; Martinez & Whitaker, 1995). There is high heterogeneity in the expression of PPO concerning enzymatic activity (optimum pH, latency, substrate specificity, etc.) between species and within the same species at different stages of development (Amiot, Fleuriet, Cheynier & Nicolas, 1997) although some of the differences may be artefactual and arise during PPO extraction and purification. Emphasis in the current work is on the phenols as substrates and the enzyme systems will not be discussed further except where relevant to substrate behaviour.

Of commercially important crops, apples and apple juice are very sensitive to enzymatic browning due to the

high concentrations of polyphenols and polyphenol oxidases (Nicolas et al., 1994). Enzymatic browning may also be a significant cause of quality loss in grapes and wines (Singleton, 1987). Other fruits particularly susceptible to oxidative browning are pears (Sapers & Miller, 1998), *litchi* (lychee) (Holcroft & Mitcham, 1996), bananas, rambutan (Landrigan, Morris & McGlasson, 1996), and plantain (Giami & Alu, 1994). There is very little information on the enzymatic browning of orange (juices) which is presumably related to the low PPO activity of citrus, and hence enzymatic browning is not a major contributor in the browning of oranges or other citrus.

3.1. Initial step in enzymatic oxidation

Much attention has been devoted to determining the exact pathway by which enzymatic browning may occur. It is now generally accepted (Nicolas et al., 1994) that two reaction steps are involved. The first reaction consists of the hydroxylation of monophenols into o-diphenols and *o*-diphenols into quinones (Fig. 2). The quinones resulting as the primary products of enzymatic oxidation have different spectral characteristics with colours dependent on the phenol from which they originate and on the pH (Taylor & Clydesdale, 1987). For instance, after oxidation, chlorogenic acid is a dull orange-yellow with an absorption maximum at 420 nm, catechin is bright yellow with a maximum at 380 nm and o-dihydroxyphenylalanine is pink with a maximum at 480 nm (Rouet-Mayer, Ralambosoa & Philippon, 1990; Waite, 1976).

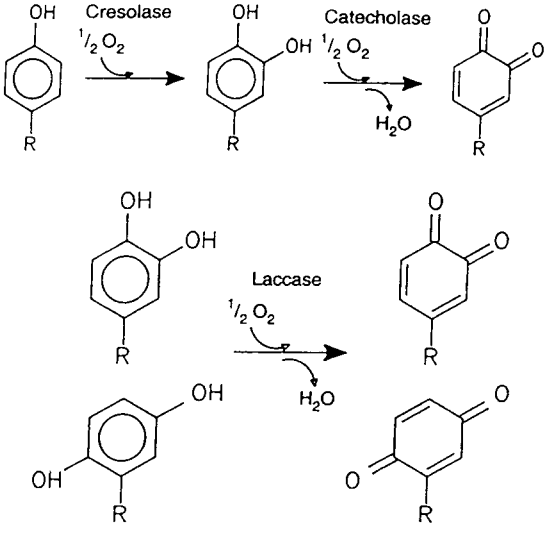


Fig. 2. Reactions catalysed by polyphenoloxidases (cresolase, E.C. 1.14.18.1 and catecholase, E.C. 1.10.3.1) and by laccases (E.C. 1.10.3.2). E.C. 1.10.3.1 is a copper-containing enzyme also known as catechol oxidase, diphenol oxidase, *o*-diphenolase, phenolase and tyrosinase.

3.1.1. Substrate specificity

Data on the substrate specificity of PPO show inconsistencies in the apparent K_m values between species, cultivars and experimental conditions (Macheix et al., 1991; Nicolas et al., 1994). These variations could have different origins as outlined by Nicolas et al. Michaelis–Menten kinetics for monohydroxy compounds determined by HPLC ranged from a K_m of 1.8 for tyrosine to 31.5 mM for *p*-hydroxyphenylacetic acid (Wichers, Peesma, Malingre & Huizing, 1984). The rate-determining step in the overall conversion to dopaquinone was the initial hydroxylation of *L*-tyrosine to *L*-dihydroxyphenylalanine. It was noted that this may not accurately reflect the in vivo situation. Apple PPO showed apparent K_m values for hydroxylation of phloridzin and p-coumaric acid of 1.5 and 4 mM, respectively (Goodenough, Kessel, Lea & Loeffler, 1983). However, the subsequent oxidation of 3-hydroxyphloridzin or caffeic acid had an apparent K_m of 200 nM. The apparent K_m of chlorogenic acid, (−)-epicatechin and (+)-catechin and procyanidins B2 and C1, the main phenolics of the apple cortex, were in the 2 to 6 mM range, indicating that the affinity of apple PPO for these substrates is relatively low. These values are typical although higher affinities have been reported (Mayer & Harel, 1979) for substrates from some sources.

Copper is essential for PPO activity and complexation of copper, situated at the active site of the enzyme, results in enzyme inhibition (Ferrar & Walker, 1996). Optimal pH values around 5.0–7.0 are frequently reported for PPO (Weemaes et al., 1998) whereas PPO isolated from blueberry fruit had an observed optimum pH at 4.0, followed by a shoulder at pH 5.0 (Kader, Rovel, Giradin & Metche, 1997a). In this instance, caffeic acid was the best substrate (100%), followed by chlorogenic acid (60%) and pyrocatechol (32.5%). No activity was detected towards catechin, protocatechuic acid, resorcinol, *p*-coumaric acid and tyrosine. Although caffeic acid had the highest activity, the results do not suggest that it is the best substrate for this enzyme, for which purpose the ratio V_m/K_m could be a better parameter to measure the enzyme efficiency at low substrate concentration. Moreover, caffeic acid has not been detected in Highbush blueberry which contains chlorogenic acid as the main cinnamate. Simple phenolic compounds such as catechol and caffeic acid containing ortho-dihydroxy groups reacted in vitro with PPO (E.C. 1.14.18.1) (Taylor & Clydesdale, 1987) whereas quercetin and hyperoside were not oxidised, despite the ortho-dihydroxy groups. On the other hand, dihydroquercetin was readily oxidised to a yellow compound, suggesting that the double bond in the C-ring affects the reactivity of these molecules with regard to PPO. On this basis, anthocyanins are not expected to react as was observed during tissue browning in peach peel (Arakawa & Ogawa, 1994). However, the precise

role of anthocyanins in oxidative browning is still debatable (Underhill & Critchley, 1993; Underhill & Critchley 1994) although it probably involves coupled oxidation (Raynal & Moutounet, 1989).

In the case of blueberry, browning reactions occurred rapidly after crushing of the fresh fruit (Kader et al., 1997b). The dominant role of PPO activity in the browning process was confirmed whereas peroxidase did not contribute to the brown colour formation. The loss of 29% of anthocyanin colour in a model system containing PPO and blueberry anthocyanins indicated that PPO could act directly on these pigments but they remain fairly poor substrates. The rate of anthocyanin degradation was stimulated by the addition of chlorogenic acid which was the major substrate for oxidation. The resulting quinone stimulated degradation of anthocyanins. Anthocyanins with *o*-dihydroxy substitution in the B-ring were oxidised via the enzymatically generated *o*-quinone by a coupled oxidation mechanism, whereas anthocyanins lacking this substitution pattern formed adducts with the same *o*-quinone (Sarni, Fulcrand, Souillol, Souquet & Cheynier, 1995).

The degree of browning of apple was related to the amounts of degraded phenolics (Goupy et al., 1995); Amiot et al. (1992) found that all phenolic compounds were affected during the browning process but that flavonols and dihydrochalcones were less degraded than cinnamics and flavanols. None of the phenolic contents correlated well with browning susceptibility as measured by absorption and reflectance. However, for a selected group of cultivars, a strong correlation was found between browning and the initial chlorogenic acid concentration. Soluble brown pigments derived from cinnamics, predominantly chlorogenic acid (Oszmianski & Lee, 1991), whereas the insoluble brown pigments were mainly formed from flavanols. The latter compounds were probably polymerised and remained bound to the cell membranes. The pigments derived from flavanols are more intensely coloured than those of chlorogenic acid (Amiot et al., 1992). Moreover, their degradation rates are enhanced by chlorogenic acid, mainly by coupled oxidation mechanisms. Coupled oxidation by *o*-quinones enzymatically formed mainly from chlorogenic acid could account for the degradation of other phenolics (Goupy et al.).

Browning in peach and nectarine extracts showed a significant positive correlation with chlorogenic acid content (Cheng & Crisosto, 1995). The association of chlorogenic acid content and browning in the first hour but not the following 4 h incubation, suggests that chlorogenic acid oxidation proceeded rapidly. Browning occurring in the latter period was related to an unidentified peak in the HPLC chromatogram. Chlorogenic acid is also implicated as an important factor in browning of coconut fruit (Jiang, Liu, Zhang, Chang & Li, 1995) whilst data on pears agree with that on apples and chlorogenic acid and (−)-epicatechin, the two major compounds found in pears, are the best endogenous substrates for pear polyphenol oxidases (Amiot et al., 1995). Flavanols were degraded during oxidation and contributed to a large extent in the development of brown pigments in pears. Some oligomers (procyanidins) have also been implicated in enzymatic browning of pears.

Flavonoids (Lea, 1992) are not attacked or are only relatively slowly attacked by oxidative enzymes such as *o*-phenoloxidases. In the presence of transfer substances, such as catechins and chlorogenic acids they are oxidised more rapidly with the probable formation of dimers as a first step (Herrmann, 1976). Ju, Yuan, Liu, Zhan and Wang (1996) concluded that simple phenols initiated the oxidation process in apples but the flavonoids produced brown colours by polymerisation and further that anthocyanins may play a protective antioxidant role.

The substrates most rapidly oxidised by grape PPO (effectively catecholase activity in grape) (Macheix et al., 1991) were (+)-catechin, (−)-epicatechin, caffeic acid and catechol. Activity was enhanced by esterification of caffeic and *p*-coumaric acids with tartaric acid, these compounds being the major phenolic species of grape. However, significant differences were observed between cultivars in the relative activity of PPO for these substrates. K_m values were in the usual range of 1–2 mM.

3.2. Non-enzymatic browning

Several different mechanisms of non-enzymatic browning have been identified including metal ion interaction with phenolics, ascorbic acid degradation and Maillard reactions. Of these, only the first involves direct intervention of phenols as substrates. The exact mechanism, in which browning via metal ion interaction occurs is not well understood (Oszmianski, Cheynier & Moutounet, 1996) but presumably involves the ability of phenolic compounds to form metal complexes (Cheng & Crisosto, 1997).

Compounds formed between polyphenols and metal ions are of particular interest in wine where several different metal ions such as copper, iron, tin, magnesium, calcium and potassium have been implicated in polyphenol interactions (Beveridge, 1997; Mathew & Parpia, 1971). Copper has been linked to an increased rate of phenolic oxidation in wines where the main phenolic studied was catechin (unpublished data). Studies by Oszmianski et al. (1996) indicate that the degradation of catechin in wine-like solutions increased in the presence of iron. Thus, the presence of these two metal ions is associated with increased rates of oxidation of catechin and other phenolics to their brown products. One possible mechanism involves copper oxidation of phenolics to quinone by-products which are then thought to undergo polymerisation

reactions to form brown pigments. However, the mechanism remains controversial as Fulcrand, Cheynier, Oszmianski, and Moutounet (1997) have suggested another pathway involving iron oxidation of tartaric acid.

Nonenzymatic browning due to metal–phenolic complexing, may also occur in fruit juices. Several polyvalent cations are capable of forming complexes with phenolics and combining with carbohydrates and proteins (Beveridge, 1997). Early studies of apple juice components in isolated systems suggest that the presence of copper is a main factor in deposit formation and that copper catalyses the degradation of juice phenolic substances, particularly of leucoanthocyanins and epicatechin, leading to their precipitation (Kieser, Pollard & Timberlake, 1957). Iron has also been implicated in such browning reactions in apple juices but not to the same extent as copper.

Metal ions are ubiquitous in the environment and enter fruits from soils, agricultural chemicals and processing equipment. Data for the copper and iron content of fruits are widely available. For example, the copper concentration in several fruits ranged between 0.03 and 0.15 mg/100 g fruit (as consumed) with the corresponding values for iron being 0.06 and 0.4 mg/100 g (Miller-Ihli, 1996). These data are supported by other sources (Pennington et al., 1995a,b). The concentrations of metal ions in fruit juices are rather low but, even at these relatively low concentrations, copper promotes browning and leads to sediment formation (Kieser et al., 1957). Analysis of apple juice sediment showed high concentrations of copper and iron (Johnson, Donnelly & Johnson, 1968) indicating that both metals had been concentrated in the sediment and thus that copper and iron play a role in oxidation and sedimentation in apple juices. Recent data showed that oxidation (browning) of apple juice was closely related to the soil type of the orchard (Kashimura et al., 1994). Data on the trace metal content of the juices would be informative.

3.3. Polymerisation reactions of quinones

In some instances, e.g. wines, it is necessary to perform browning tests under conditions which accelerate the phenomenon. This is generally acceptable but one must be aware of the potential differences in mechanism that may arise (Macheix et al., 1991). Model systems containing one or two phenolics have been particularly useful in combination with different methods of analysis for establishing the products of oxidation (Kader, Haluk, Nicolas & Metche, 1998). Both chemical (Fulcrand, Cheminat, Brouillard & Cheynier, 1994) and enzymatic oxidation have been used in these systems in an attempt to elucidate the mechanism of oxidation.

HPLC has been used to separate the products of enzyme activity, many of which arise by secondary reactions (Cheng & Crisosto, 1995; Goodenough et al., 1983; Wichers et al., 1984). This is illustrated by the enzymatic oxidation of phloretin glucoside in model systems (Oszmianski & Lee, 1991) which showed a lag time corresponding to the initial oxidation step. The quinones formed during this time as the primary products of oxidation are relatively unstable and undergo further reaction (Taylor & Clydesdale, 1987). These reactions, which may lead to pigment formation, are similar, regardless of whether the quinone was formed by enzymatic or nonenzymatic oxidation (Singleton, 1987). The only real difference in the two reaction pathways is the rate at which the browning may occur; enzymatic browning is usually faster.

Nicolas and Potus (1994) have highlighted the importance of coupled oxidation reactions showing the primary intervention of PPO in a first phase, leading to the formation of unstable products. In a second phase, mainly non enzymatic, these quinone products are able to react with a wide range of other compounds. The fate and stability of quinones, and hence the hue and intensity of any resulting colour, vary widely, depending both on the phenolic precursor and on the chemical environment (Guyot, Cheynier, Souquet & Moutounet, 1995).

Reactions of the quinones have been classified into those involving phenolic compounds (Fig. 3) and non-phenolic compounds (Fig. 4). Coupled oxidations of the quinone with another phenol molecule can be very rapid and are dependent on the respective reduction potentials of the different quinone/phenol couples present (Cheynier, Osse & Rigaud 1988; Nicolas et al., 1994). These reactions lead to formation of dimers of the original phenol or regeneration of the phenol (Fig. 3). Of course, the products themselves are subject to further oxidation, either enzymatically or by another *o*-quinone, resulting in formation of larger oligomers.

The existence of such coupled oxidations has been demonstrated (Cheynier, Basire & Rigaud, 1989; Sarni-Manchado, Cheynier & Moutounet, 1997) in caftaric acid/flavanol mixtures with grape PPO (Cheynier & Ricardo da Silva, 1991) and chlorogenic acid/anthocyanin mixtures with blueberry and plum PPO (Kader et al., 1998; Raynal & Moutounet, 1989). The mechanisms responsible for phenolic oxidation and browning in white musts have been thoroughly studied. The first reaction is the enzymatic oxidation of caffeoyltartaric acid to the corresponding *o*-quinone, followed by trapping of the latter by the available glutathione. Excess caffeoyltartaric quinones may then enter coupled oxidations with flavanols and anthocyanins (Cheynier, Souquet, Kontek & Moutounet, 1994).

Model systems containing purified anthocyanins, chlorogenic acid, and blueberry PPO were analysed by HPLC and polarographic methods (Kader et al., 1998). Cyanidin 3-glucoside was not oxidized by PPO since no molecular oxygen was consumed. However, the presence of chlorogenic acid induced pigment degradation. The chlorogenic acid quinone, formed enzymatically,

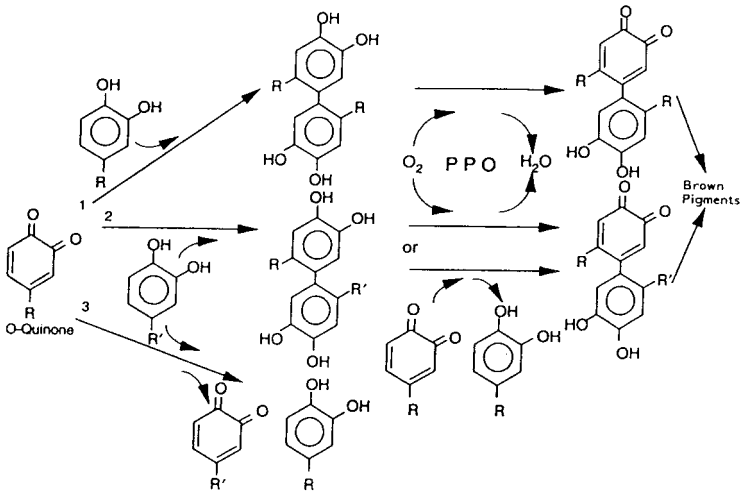


Fig. 3. Reactions of *o*-quinones with phenolic species (Rouet-Mayer, Philippon, & Nicolas, 1993).

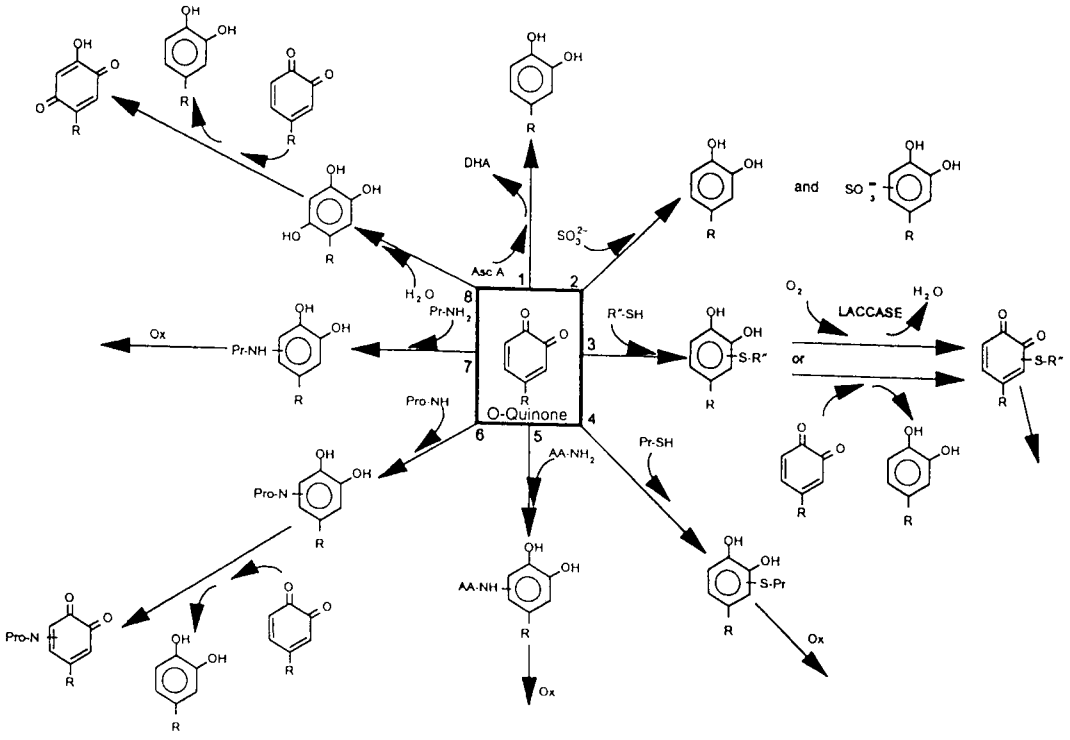
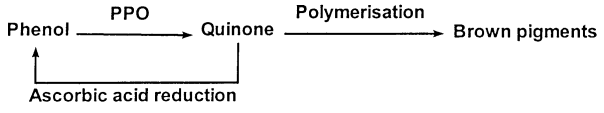


Fig. 4. Reactions of *o*-quinones with nonphenolic species (Rouet-Mayer et al., 1993).

was involved in the degradation of cyanidin 3-glucoside by coupled oxidation mechanisms with partial regeneration of chlorogenic acid. There are now a number of instances recognised in which phenolics such as glycosides of flavonols and anthocyanins are not substrates of PPO but are degraded (Amiot, Tacchini, Aubert & Nicolas, 1992; Amiot, Tacchini, Aubert & Oleszek, 1995), nevertheless, by coupled reactions. The mechanism proposed for the caftaric acid/flavanol system (Cheynier et al., 1988) involved enzymatic oxidation of both phenols, followed by chemical oxidation of the flavanol by the caftaric quinone with regeneration of the caftaric acid. The quinones formed via either route can also react with another phenolic molecule to yield condensation products. Based on these studies, the *o*-quinones of caftaric and chlorogenic acids were the most efficient carrier for coupled oxidation and appear to play a determinant role in degradation of other phenolics.

The second scheme (Fig. 4) comprises the reactions of *o*-quinones with non-phenolic compounds, i.e. ascorbic acid, sulfites, thiols, primary and secondary amines (either free or present in proteins) and water (Rouet-Mayer,

Ralambosoa et al., 1990), leading also to the formation of pigments. Cross-linked polymers formed by reactions with protein functional groups (Friedman, 1996) have been implicated as the browning agents. Coupled oxidations are again important and have been observed with ascorbic acid. The quinones formed by oxidation can react with ascorbic acid, which results in the regeneration of the phenol. Since the phenol is regenerated, browning is inhibited (Rouet-Mayer, Ralambosoa et al., 1990) until ascorbic acid is depleted, whereupon the formation of brown pigments will occur. Nevertheless, the action of ascorbic acid is unclear and may even be actively involved in oxidative browning (Kacem, Cornell, Marshall, Shireman & Mathews, 1987; Kennedy, Rivera, Lloyd, Warner & Jumel, 1990). Ascorbic acid oxidation enhances anthocyanin degradation (Holcroft & Mitcham, 1996) via a mechanism that is not well understood. There has been interest recently in a peroxidase/phenolics/ascorbic acid system in plant vacuoles as a defence mechanism for hydrogen peroxide scavenging in plants (Takahama & Oniki, 1997; Yamasaki, Sakihama & Ikehara, 1997; Yamasaki & Grace, 1998).



Polarography, spectrophotometry and HPLC were used to follow the oxidation of several phenols catalysed by apple PPO (Richard-Forget, Rouet-Mayer, Goupy, Philippon & Nicolas, 1992) between pH 4 and 5. The reactivities of the *o*-quinone products varied greatly from one phenol to another. Two pathways were proposed for the degradation of 4-methylcatechol *o*-quinones. The first, favoured by acid pH, corresponded to a hydroxylation followed by a coupled oxidation of another molecule or *o*-quinone and leading to regeneration of 4-methylcatechol. The second pathway involved polymerisation reactions which were favoured by higher pH values. The same pathways were observed with chlorogenic acid although the polymerisation reactions seemed to be dominant. The *o*-quinones of (+)-catechin and (−)-epicatechin are much less stable than that of chlorogenic acid (Rouet-Mayer et al., 1990) and were not examined by Richard-Forget et al. The reaction products become quite complex with as few as two phenols (*o*-quinones) in admixture (Oszmianski & Lee, 1990). In summary, the *o*-quinones will enter along the different pathways according to their oxidative and electrophilic properties. As oxidants, the *o*-quinones will oxidise any other substances with lower reduction potentials. This will include other phenols, ascorbic acid and sulfur dioxide. In this process the quinones are themselves reduced to the original phenol. They will react as electrophiles with nucleophilic substances, including amino derivatives and water. In the absence of other substrates, condensation and polymerisation will occur via reaction with the corresponding hydroquinone. The products formed in such reactions are pH-dependent (Guyot et al., 1995). Mostly colourless products were formed during the PPO-catalysed oxidation of (+)-catechin in aqueous buffers at pH below 4, whereas yellow compounds, less polar than the colourless ones, predominated at higher pH values. The yellow products were identified as dimers and possible mechanisms for their formation were proposed as a Michael type addition or through a semiquinone radical intermediate. Michael (1→4) addition could be favoured by high pH, which increases the nucleophilic character of (+)-catechin whereas low pH could favour radical mechanisms by increasing the reactivity of semi-quinone radicals.

4. Conclusions

This review has taken a novel approach to the chemistry of phenolic compounds by linking what many may see as disparate areas, namely the potential antioxidant activity of these compounds in the human diet and their role in oxidative processes ("browning") in fruit. The common thread is the redox chemistry of the phenolics, which may be beneficially oxidised in preference to cellular subrates in humans, or detrimentally oxidised during fruit spoilage. Despite being approached from two distinct areas, both with large volumes of literature, there are many unanswered questions, in particular with regard to the *mechanism* of the redox activity. Enzymatic browning in foods is reasonably well understood, but does not account for all browning observed. The picture is even less clear in human physiology; however, more data on dietary burden and metabolic fate would help this situation.

One of the difficulties with data acquisition, however, is confidence in methodology. There appears to be no discussion of how phenolic recovery may be influenced by temperature, presence of oxygen, solvent, etc. There are no standard procedures in the literature for isolation of phenolics; therefore any quantitative data on phenolic concentration must be evaluated in this light. Qualitative data, too, suffer from lack of rigour in analytical techniques.

The range of tests used for antioxidant activity is also a testimony to the uncertainty surrounding the chemistry of phenolic compounds. For example, in tests where free radical oxidation is induced by a metal ion (e.g. Cu^{2+}), does the test measure the ability of a phenolic to interact with a free radical or its ability to bind the metal ion? As noted earlier, the subject of metal ion chemistry in both dietary antioxidant studies and food chemistry is also controversial. There would appear to be enough evidence in the literature to warrant further investigations in this area.

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