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# Novel Bioconversions for the Production of Designer Antioxidant and Colourant Flavonoids using Polyphenol Oxidases

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## Introduction

There is increasingly clear evidence for a relationship between diet and health, and this may be responsible for the trend towards the use of 'natural' additives, in particular colourants, by the food industry (MAFF, 1987). Many plant secondary metabolites, including the flavonoids, possess antioxidant activity (Bors and Saran, 1987, Rice-Evans *et al.*, 1995; Yoshiki *et al.*, 1995; Cook and Samman, 1996; Rice-Evans *et al.*, 1996; Wiseman, 1996a). Flavonoids as additives could thus provide technological functions, including the prevention of rancidity of fats and the stabilisation of colours. The antioxidant properties of such flavonoid-derived additives could potentially provide health benefits, by protecting against the oxidative damage implicated in many disease states (Wiseman, 1996a; Wiseman and Halliwell, 1996). Indeed, a number of scientific developments, including a greater understanding of the role of specific nutrients in foods in disease prevention and treatment, together with the technologies of biotechnology (and in particular genetic engineering) have led to the availability of increasing numbers of potential nutritional products with medical and health benefits termed functional foods (also described as 'designer foods', 'pharmafoods' and 'nutraceuticals') (Goldberg, 1994). There is evidence that consumption of food and beverages such as apples and tea, rich in antioxidant flavonoids (including quercetin) may be inversely correlated with death from heart disease in the elderly (Hertog *et al.*, 1993; Hertog and Hollman, 1996). New nature-identical food additives, particularly flavonoid derivatives with both health beneficial antioxidant and colourant properties, could, therefore, have potential as components of functional foods.

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Abbreviations: PAL, Phenylalanine ammonium lyase; CHS, Chalcone synthase; BHT, Butylated hydroxytoluene; LDL, Low density lipoproteins; PSII, Photosystem II; TBA, Thiobarbituric acid; TBARS, Thiobarbituric acid reactive substances; ROS, Reactive oxygen species; RNS, Reactive nitrogen species.

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The bioconversion of phloridzin to a number of derivatives, by the enzyme polyphenol oxidase, involves phenolic oxidation. This process is involved in many aspects of food processing, including the production of black teas and cocoa products. However, phenolic oxidation, particularly in fruit and vegetables, is generally regarded as an undesirable spoiling reaction. Prospects for inhibition of phenolic oxidation, including the use of the genetic engineering of plants are considered in this chapter. Genetic transformation of plants provides an opportunity to improve and modify the flavonoid content of plants, by providing increased yields of parent compounds for bioconversions, and by increasing the levels of specific desirable flavonoids and this is discussed principally with reference to apple and to the flavonoids phloridzin and quercetin. This chapter then considers the prospects for the production of the dimerised coloured oxidation product of phloridzin by the enzyme polyphenol oxidase, and of 3-hydoxyphloridzin (see *Figure 1*), an intermediate in this bioconversion. To achieve these bioconversions there is evidently a requirement for the extraction of both phloridzin and an appropriate polyphenol oxidase, and this is discussed. The antioxidant properties of these compounds in model membranes (Ridgway *et al.*, 1996, 1997) are evaluated, and the importance of these potential dietary antioxidants in protection against free radical-mediated oxidative damage is also considered and assessed in relation to environmental and food-processing applications.

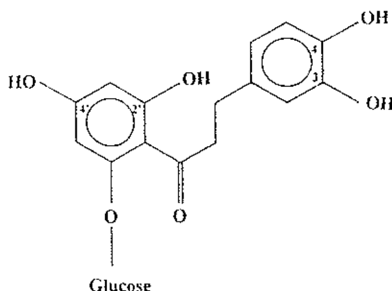


**Figure 1.** The chemical structure of the phloridzin derivative, 3-hydroxyphloridzin.

## Phloridzin and its extraction from apple tissue

Phloridzin is a flavonoid (dihydrochalcone) largely restricted to apple (*Malus sp.*). Phloridzin is found in limited quantities in the mature apple fruit, but in young apple leaves and twigs accounts for up to 10% of dry weight. Although it is generally accepted that aromatic compounds in plants are usually synthesised by one of the three major pathways (acetate-mevalonate, acetate-malonate or shikimic acid pathways), some aromatic compounds such as the flavonoids, the stilbenes and the xanthones are biosynthesised by a route that involves both the acetate-malonate and the shikimic acid pathways and are thus considered to have a mixed biogenesis. The A ring of the flavonoid ring-structure is derived from three condensed acetate units. The B-ring and three carbons of the central ring are, by contrast, derived from cinnamic acid, which results from the deamination of phenylalanine (not tyrosine) by phenylalanine ammonium lyase (PAL). The enzyme chalcone synthase (CHS) ca-

talyses the addition of the malonyl Co A units to the cinnamic acid derivative p-coumaric acid to form chalcones, for example, naringenin chalcone. All naturally occurring chalcone derivatives are hydroxylated and the hydroxyl groups are frequently glucosylated or methylated. The pattern of hydroxylation at least in the A-ring is largely determined at the point of cyclisation. Chalcones are coloured compounds and reduction of the α, β-unsaturated bond produces colourless dihydrochalcones (note: terminology then reverses the A and B rings) such as phloridzin. These are in fact quite rare, and phloridzin and its aglycone phloretin are the best characterised of these compounds (Mann, 1992).

Polyphenol levels, in particular of flavonoids, may be useful as taxonomic markers for the systematic biochemical analysis of plants (Denford, 1984). In this context, the concentrations of the major flavonoids and phenolic acids in the peel and cortex of fruit of eight commercial apple cultivars grown commercially in Eastern Canada has been determined by HPLC (McRae *et al.*, 1990). The resulting polyphenol profiles were analysed by the multivariate statistical technique of correspondence analysis in order to describe distinctive groups of cultivars and of polyphenols and their joint correspondence (McRae *et al.*, 1990). Chlorogenic acid (generally the principle polyphenol in the cortex), phloridzin and catechins were present in both cortex and skin, whereas quercetin glycosides were detectable only in the peel. The Red Delicious cultivar had the most distinctive cortex tissue with a low level of chlorogenic acid and a high level of phloridzin. The next most distinctive was Gravenstein with a high level of chlorogenic acid and low levels of phloridzin and catechins; Jerseymac had a high level of chlorogenic acid but a low level of phloridzin and Cortland had high levels of phloridzin and catechins but a low level of chlorogenic acid. Other cultivars had less distinctive cortex profiles (McRae *et al.*, 1990). HPLC analysis of the polyphenolic profiles of apple cultivars with correspondence analysis of polyphenol profile may be useful in the classification and identification of apple cultivars, which can sometimes be difficult to identify from samples of fruit. Indeed, this approach could be particularly useful in the future in identifying apple cultivars with highly-favourable profiles of antioxidant-flavonoids.

Phloridzin is currently available commercially, but at high price. To enable its potential use as a parent compound for food additive production, cheap alternative purification methods have been developed (Ridgway and Tucker, 1997a). Examples of these simple effective methods of purification of phloridzin from apple tissues, are considered below. The actual methods utilised would depend on a number of factors, including the value of the derivatives, wage costs and whether on-site or centralised initial extraction was performed.

For on- or near-site extraction, crystallisation of phloridzin from hot water (>80°C) extracts is possible, if tissue disruption is minimal i.e., whole leaves and not too small twig segments (>0.25 mm) are used (Ridgway and Tucker, 1997a). Concentration of filtered extract (by open or reduced pressure evaporation) is then necessary to take the phloridzin concentration below the supersaturation limit of phloridzin (1 g/240 ml) and to compensate for the impurity impedance of crystallisation. Under these conditions, a 100 g mid-season leaf extract would require concentration to 40ml from an initial extraction volume of 600 ml to achieve crystallisation, a process that may take over 24 h to complete. Phloridzin purification following solubilisation into ethyl acetate containing 5% methanol by volume can then proceed as described above. An alternative

hot water extraction scheme can be used deliberately to prevent crystallisation (Ridgway and Tucker, 1997a). This is achieved by grinding the tissue in hot water to maximise impurity impedance. Following filtration of the bulk material and concentration down to very low volumes (for example 20 ml for a 100 g extract) an almost black phloridzin solution or crude 'apple oil' is produced. Phloridzin can then be extracted by partition against ethyl acetate and purification can proceed (see above): although to achieve full purification, additional organic steps may be required. Typical mid-season leaf yields using initial hot water extractions of Bramley's Seedling, and 10:1 initial water:tissue extraction ratio are 0.8 g and 0.6 g respectively for the two hot-water-extraction methods described above (Ridgway and Tucker, 1997a).

A particularly effective method, for relatively small scale laboratory use or the production of high value derivatives, was found to involve the drying of apple leaves followed by the extraction of phloridzin into ethyl acetate (containing 5% methanol by volume), filtration and crystallisation *via* the addition of 0.7 volumes of chloroform and 0.02 volumes of water (Ridgway and Tucker, 1997a). Pure crystals of phloridzin were then obtained by recrystallisation (typically twice) from water (1 g of phloridzin in 100 ml water is a useful working ratio). Although freeze-drying aids laboratory procedures, slow atmospheric drying results in little or no loss of phloridzin yield, providing that the temperature is kept moderate (< 12°C) and exposure to light low. A typical mid-season (July–early August UK) yield from leaves of the cultivar Bramley's Seedling and a 10:1 ethyl acetate: dry leaf extraction ratio and two water recrystallisations was 0.9g phloridzin from 100g leaves (Ridgway and Tucker, 1997a).

**Table 1.** Variation in phloridzin content of tissue according to season

| | g Phloridzin/g tissue (wet weight) | |
|---|---|---|
| | Leaf | Twig |
| Early-season (18/5/92) | 0.0214 ± 0.0033 | 0.0164 ± 0.0018 |
| Mid-season (13/7/92) | 0.0177 ± 0.0006 | 0.0112 ± 0.0004 |
| Late-season (14/9/92) | 0.0138 ± 0.0012 | 0.0093 ± 0.0008 |
| Late-season (24/9/93) | 0.0130 ± 0.0008 | 0.0093 ± 0.009 |

Results for Bramley's seedling cultivars (Sutton Bonington Experimental Orchard, University of Nottingham). Samples (10g), obtained from the central third of shoots, including 10 twigs from 10 separate trees, were freeze-dried, ground and extracted in 100ml of methanol overnight before filtration, dilution and assay for phloridzin content by HPLC. A C18 column was used with a 10–70% methanol gradient, containing 2.5% acetic acid. The results show the average values ± the 95% confidence limits for 3, 10g tissue replicates with duplicate HPLC analysis for each.

The most important determinant in a given tissue of phloridzin content is its period of development and this is demonstrated by the twig and leaf yields from Bramley's seedling cultivar (see *Table 1*) (Ridgway and Tucker, 1997a). Cultivar, including rootstock type, was found to have a much less pronounced effect. However, the effect of cultivar can be dramatic in relation to total tissue growth. This gives wide variations in total phloridzin production by given cultivars, illustrated by the results obtained for different rootstocks (see *Table 2*) (Ridgway and Tucker, 1997a). Production of phloridzin is then best carried out by the coppicing of fast growing rootstocks, such as M25 or MM106. This is comparable to the production of willow or poplar for biomass, for which mechanical harvesting equipment has already been developed. Yields of approximately 250 kg of phloridzin/hectare should then be readily obtained. Note that although tissue yields may be increased by early harvesting, the total phloridzin produced will then decline.

**Table 2.** Phloridzin content of individual rootstock shoots (leaves and twigs)

| | Phloridzin yield/shoot (g) |
|---|---|
| M25 | 0.860 ± 0.109 |
| MM106 | 0.755 ± 0.0644 |
| M26 | 0.677 ± 0.0211 |
| M27 | 0.387 ± 0.0495 |

The rootstocks were supplied by Matthew's Ltd. Tenbury Wells, U.K. Samples were taken from 10 shoot batches (used to derive the mean shoot weights used in the calculations), corresponding to tissue assay samples, and were freeze-dried, ground and extracted in 100ml of methanol overnight before filtration, dilution and assay for phloridzin content by HPLC. A C18 column was used with a 10–70% methanol gradient containing 2.5% acetic acid. The results show the average values ± the 95% confidence limits for 3, 10 shoot samples with duplicate HPLC analysis for each.

With the development of simple purification methods and the availability of fast growing rootstock, large scale, and economic production of phloridzin could be carried out. Indeed it is possible that phloridzin production could lead to the development of the apple as a new agrochemical crop (Ridgway and Tucker 1997a), there being much current interest in such crops in the context of maintenance of the rural economy, 'land set aside', and the development of renewable technologies. In the future, it may be possible to further improve yields of phloridzin *via* the use of genetic engineering (see below).

## Plant polyphenol oxidases and food processing bioconversions

Before the use of plant polyphenol oxidases in bioconversions is assessed, it is important to consider their occurrence, effect on food production and potential means for their purification. The term bioconversion (or biotransformation) is used to describe the use of enzyme-catalysed reactions in organic synthesis (Roberts *et al.*, 1995). Interest in the field of bioconversions increased greatly in the 1980s because of the realisation that enzyme families will transform a wide range of unnatural compounds as well as their natural substrates, and also because of the increased availability of a wide variety of enzymes (Roberts *et al.*, 1995). It appears likely that, in the future, enzyme-catalysed reactions and whole-cell-mediated bioconversions will contribute even more to synthetic organic chemistry and its many applications. There are many advantages in using enzymatic steps in organic synthesis (Roberts *et al.*, 1995). These include the ability of enzymes to catalyse reactions under mild conditions of temperature (around 37°C), pressure (1 atm) and pH (around 7): and the transformations are usually very energy-efficient compared to the comparable chemical process. Enzymes can achieve reactions that are very difficult by other means and indeed bioconversions can generate new series of chiral synthons. It may also be possible to exploit non-natural enzyme-catalysed reactions to produce optically pure compounds for pharmaceutical use and thus to reduce unwanted toxicity caused by undesirable enantiomers. Bioconversions can be carried out with either pure or partially purified enzymes, or whole cells can be used (Roberts *et al.*, 1995). The types of bioconversions that can be carried include oxidoreductase, transferase, hydrolase, lyase, isomerase and ligase reactions. The advantages of using isolated enzymes include the fact that a particular enzyme may be specific for a selected reaction, co-solvents are better tolerated, and only simple apparatus and work-ups are required. The disadvantages include the expense involved in isolating highly purified enzymes

and the possible requirements for the addition of enzyme co-factors (or enzyme co-factor recycling). In contrast, whole cells are inexpensive and the required enzyme co-factors will be present. However, the work-up required involves large-scale glassware and side reactions can interfere with or dominate the substrate and/or the product. Furthermore, use of a co-solvent may disrupt membrane-bound enzymes, or even the cytoplasmic membrane of whole-cell preparation. Increased permeability of the cytoplasmic membrane in immobilised whole-cell preparations may however be essential for the process to work. Growth *in situ* of whole-cell preparations may fortuitously provide a renewal biocatalyst in some cases. Green algae of the *Chlorella sp* have been used in the bioconversion of progesterone: hydroxylation and side-chain degradation reactions were observed (Pollio *et al.*, 1996).

Polyphenol oxidase is a plastid-associated (in particular with the thylakoid membrane and PSII (Photosystem II): Vaughan *et al.*, 1988), ubiquitous copper containing plant enzyme, which catalyses the hydroxylation of monophenols to *o*-diphenols (monophenol monooxygenase or tyrosinase activity, EC 1.14.18.1) and the oxidation of *o*-diphenols to *o*-quinones by utilizing molecular oxygen (catechol oxidase or diphenol oxidase or diphenol oxygen oxidoreductase activity, EC 1.10.3.1). The laccases, found in fungi and higher plants (E.C.1.10.3.2) oxidise *p*-diphenols in addition to *o*-diphenols to their corresponding quinones (Mayer, 1987). The organisation of the polyphenol oxidase gene family has been described for tomato (Newman *et al.*, 1993). Polyphenol oxidase in potato (*Solanum tuberosum*) has been reported to be inducible by systemic wounding: only those tissues which were developmentally competent to express polyphenol oxidase mRNA were capable of responding to the systemic wound signal by increased accumulation of polyphenol oxidase mRNA (Thipyapong *et al.*, 1995). Furthermore, an apple polyphenol oxidase cDNA has been shown to be up-regulated in wounded tissues and this again suggests transcriptional control of polyphenol oxidase after wounding (Boss *et al.*, 1995) and a potential role in defence mechanisms.

Polyphenol oxidase has been implicated in enzymatic browning in a number of plant tissues including potato tubers, bananas, grapes, pears, green olives, kiwis, strawberries, plums and apples (Nicolas *et al.*, 1994). Apple is one of the most common fruits in which enzymatic browning, generally considered to be an undesirable reaction because of the unpleasant appearance and development of an off-flavour, is important from the consumer and food industry viewpoint (Nicolas *et al.*, 1994). This enzymatic browning occurs when plant tissues are damaged, because polyphenol oxidase then catalyses the oxidation of phenolic compounds to quinones (in the presence of oxygen), which condense to form darkened pigments (Mayer, 1987). This is an important economic problem. Considerable effort has been devoted to inhibiting this reaction by using a number of different strategies (Walker and Ferrar, 1995). The most widespread method used in the food industry for the control of browning is the addition of sulphiting agents, although health concerns over sulphites have led to a search for alternatives to such additives. Chelating agents such as EDTA may be used as inhibitors of polyphenoloxidase and are thought to either bind to the active site copper or reduce the level of copper available for incorporation into the holoenzyme. A copper-binding metallothionein from *Aspergillus niger* has also been reported to inhibit polyphenol oxidase (mushroom tyrosinase) activity (Goetghebeur and Kermasha, 1996). The most successful approach to the prevention of browning may

be the use of antisense inhibition of polyphenol oxidase gene expression. Using constitutive promoters (e.g., CaMV 35S) to express antisense polyphenol oxidase RNA, melanin formation is then specifically inhibited in the potato tuber (Bachem *et al.*, 1994). The lack of bruising sensitivity achieved in transgenic potatoes (and the absence of any apparent detrimental side effects), suggests that this is a new possibility for the prevention of enzymatic browning in a wide variety of food crops (see above).

Although it is usually desirable to inhibit the browning effect of polyphenol oxidase in plant food produce, in some circumstances this reaction may be of use: black tea is produced by the controlled use of polyphenol oxidases in the plant tissue. This process is misleadingly termed 'fermentation', as it is in fact phenolic oxidation. The dark colour and much of the characteristic flavour of cocoa products are also the result of the action of endogenous polyphenol oxidases. Similarly, the positive use of polyphenol oxidase in the production of antioxidant and colourant products is worthy of discussion.

Ironically, polyphenol oxidase is generally considered to be a difficult enzyme to purify because of the presence of phenolics with which it reacts resulting in the modification and inactivation of the protein molecule (Mayer, 1987). In addition, solubilization of particulate polyphenol oxidase is a difficult procedure. However a number of methods for its extraction and purification from apple have been reported.

Partial purification of polyphenol oxidase has been achieved (by ammonium sulphate precipitation and hydrophobic chromatography on Phenyl Sepharose CL4B) from the fruit of 12 cultivars grown in France and analysed for polyphenol oxidase activity in both the cortex and peel (Janovitz-Klapp *et al.*, 1989. Enzyme activity ranged from 0.62 to 3.1 mkat/kg in the cortex and from 0.3 to 3mkat/kg in the peel; it is of interest that polyphenol oxidase activity was always equivalent or lower in the peel than in the cortex (Janovitz-Klapp *et al.*, 1989). The optimum conditions for polyphenol oxidase extraction from apple fruit were achieved using a buffer with a pH > 7.0 and containing 15 mM ascorbic acid and 0.5% Triton X100. The Red Delicious apple cultivar showed the highest polyphenol oxidase activity and the Elstar cultivar the lowest. Polyphenol oxidase has been purified 120-fold from the cortex of Red Delicious with a yield of around 40% (Janovitz-Klapp *et al.*, 1989). The optimum pH for maximum activity was 4.5–5 for the substrates methylcatechol, chlorogenic acid and (+)catechin and the $K_m$ values were around 5 mM for the three substrates and were independent of pH on the acid side of the pH optimum (Janovitz-Klapp *et al.*, 1989). Chlorogenic acid was a better substrate for this apple polyphenol oxidase than catechin at pH 4, which is closer to the natural pH of apple vacuoles (Janovitz-Klapp *et al.*, 1989). It is of interest that the polyphenol oxidase activity declined steadily in the Red Delicious apples in the 8 weeks leading up to the commercial harvest date, resulting in an overall decrease in enzyme activity of around 30% (Janovitz-Klapp I., 1989).

Furthermore, by ammonium sulphate precipitation and dialysis, partial purification has been achieved from the Amaysa apple (grown in Turkey), which has one of the highest rates of enzymatic browning among several apple cultivars; indicating high levels of polyphenol oxidase (Oktay *et al.*, 1995). The optimum pH for the substrates catechol, 4-methyl catechol, pyrogallol and L-dopa were 7.0, 9.0, 8.6 and 6.6 respectively. Catechol was found to be the most suitable substrate for Amaysa apple

polyphenol oxidase and 18°C was the optimum temperature for maximum polyphenol oxidase activity with catechol as substrate. Electrophoretic separation enabled the detection of three isoenzymes with catechol and L-dopa substrates. In relation to control of browning a number of inhibitors were tested and their order of effectiveness was L-cysteine > sodium metabisulphite > ascorbic acid > sodium cyanide > mercaptoethanol > glutathione > thiourea (Oktay *et al.*, 1995).

Polyphenol oxidase has been extensively purified from apple flesh (*Malus pumila* cv Fuji); it was purified 470-fold from the plastid fraction by ammonium sulphate precipitation, gel filtration and ion-exchange chromatography with a total yield of around 70% (Murata *et al.*, 1992). The $M_r$ was found to be around 65,000 by both gel filtration chromatography and sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and the N-terminal amino acid sequence was N-Asp-Pro-Leu-Ala-Pro-Pro (Murata *et al.*, 1992). The optimum pH for enzyme activity was around pH 4 and the enzyme was stable in the pH range 6–8 (Murata *et al.*, 1992). The $K_m$ of the enzyme for the substrate, chlorogenic acid, was 0.122 mM and indeed the rate of reaction of the purified enzyme was much greater for chlorogenic acid than for other *o*-diphenols such as (+)catechin, (−)epicatechin and 4-methylcatechol. Furthermore, the enzyme lacked both monophenol mono-oxygenase and *p*-diphenol oxidase activity (Murata *et al.*, 1992). Interestingly, the purified enzyme was found to be much less thermally stable than the enzyme of the plastid fraction (Murata *et al.*, 1992).

Apples (*Pyrus malus* L. cv Granny Smith) have been used in the purification of an active proteolysed isoform of apple pulp polyphenol oxidase by a rapid three-step method based on the resistance of polyphenol oxidase to further sodium dodecyl sulphate-proteinase K digestion (Marques *et al.*, 1994). Extraction from the thylakoid membrane pellet and pre-purification by temperature-induced phase partitioning was followed by sodium dodecyl sulphate-proteinase K digestion and then purification to 388-fold homogeneity by DEAE-cellulose column chromatography (Marques *et al.*, 1994). A yield of greater than 40% was achieved and this active polyphenol oxidase isoform was used to raise polyclonal antibodies resulting in the production of high titre specific serum used to perform immunoblots to detect active and latent forms of the enzyme (Marques *et al.*, 1994). The sodium dodecyl sulphate-proteinase K digestion had no effect on the apparent $K_m$ at pH 4.6, which was 7 mM for 4-methylcatechol before digestion and 6.7 mM after (Marques *et al.*, 1994). These results are comparable to those obtained with polyphenol oxidase from Red Delicious apples, at the same pH (Janovitz-Klapp *et al.*, 1989).

In addition to the isolation and purification of apple-derived polyphenol oxidase, considerable effort has been put into the purification of potato tuber (*Solanum tuberosum* cv Cara) polyphenol oxidase free from the storage protein patatin (Partington and Bolwell, 1996). In potato, patatin is the major storage protein in the tuber and often contaminates preparations. However, purification of polyphenol oxidase from the potato tuber has been achieved by using as an important step hydrophobic chromatography on Octyl-Sepharose to remove patatin completely (Partington and Bolwell, 1996). The resulting purified polyphenol oxidase had a $K_m$ of 4.3 ± 0.3 mM for L-dihydroxyphenylalanine and was shown to be a doublet of $M_r$ 60,000 and 69,000 when analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (both bands had similar N-termini corresponding to polyphenol oxidase isoforms when sequenced) (Partington and Bolwell, 1996). Purification of

polyphenol oxidase from carrot ($M_r$ 59,000) has also been reported (Soderhall, 1995).

Although polyphenol oxidase is bound to the thylakoid membranes of the chloroplast in healthy leaves, it is not an intrinsic membrane protein and can be released from the thylakoids by sonication, mild detergent treatment or protease treatment. Anionic detergents such as sodium dodecyl sulphate and proteases such as trypsin can activate the latent activity of polyphenol oxidase, and the effect of sodium dodecyl sulphate on polyphenol oxidase (from broad bean leaf *Vicia faba*) has been investigated (Jimenez and Garcia-Carmona, 1996). This particular polyphenol oxidase was enzymatically inactive in aqueous buffers at neutral pH and active at acid pH (pH 3–4). In the presence of sodium dodecyl sulphate, however, the activity at acid pH was eliminated and the monophenol monooxygenase and catechol oxidase activities at neutral pH were activated (Jimenez and Garcia-Carmona, 1996). This activation was rapid and dependent on the concentration of sodium dodecyl sulphate used. The relationship demonstrated between sodium dodecyl sulphate concentrations and proton concentrations may be related to a displacement (dependent on the binding of sodium dodecyl sulphate to a specific centre with a dissociation constant of 0.52 mM) of the sensitive pKs of the enzyme by interaction with sodium dodecyl sulphate molecules (Jimenez and Garcia-Carmona, 1996).

Alternative, potentially economic methods have been developed for the purification of apple polyphenol oxidase (Ridgway and Tucker unpublished results; commercial confidence). The yields of this polyphenol oxidase were sufficiently high that alternative potential production methods, such as expression of the enzyme in micro-organisms, would not be an economically viable alternative. This apple polyphenol oxidase was used then to produce the oxidation products of phloridzin described below.

## Production of phenolic oxidation products *in vitro*

The 3-hydroxylation of phloridzin has been achieved by using L-ascorbic acid partially to block a polyphenol oxidase reaction. Apple-derived polyphenol oxidase is particularly effective at catalysing this hydroxylation step (Ridgway and Tucker unpublished results). This is a reaction which many forms of polyphenol oxidase, such as commercial fungal tyrosinase, do not carry out effectively. The L-ascorbic acid acts by blocking the formation of quinones, which it achieves by continually reducing them to the *o*-diphenol form as they are formed. Recovery of the 3-hydroxyphloridzin was by partition against ethyl acetate and precipitation with chloroform, followed by water recrystallisation, i.e., essentially the organic phloridzin purification procedure described above.

If the reaction is allowed to proceed in the absence of L-ascorbic acid quinones are produced, which after shifting the pH to pH 8 (from the enzyme optimum of pH 5), form two isomeric dimers: the structure of these has not yet been fully characterised. Interestingly, high enzyme activity/substrate concentration ratios produced poorer product yields in terms of specific absorbance (Ridgway and Tucker, unpublished results). This effect varied according to the source and purity of the enzyme: it could be induced by peroxidase type activity as low levels of hydrogen peroxide may potentially be produced by the cycling reactions that take place amongst quinone products.

The apple polyphenol oxidase used shows optimum activity at approximately 30% oxygen saturation of water. The design of the reaction vessel used to produce the phloridzin oxidation products took this into account in addition to its function in replenishing the oxygen consumed in the reaction. With respect to the desirability for relatively slow reaction rates, the optimum design was a 'balanced oxygen type'. This essentially consisted of a stirred tank in which oxygen uptake by stirring, was balanced at 30% saturation by the oxygen used as part of the reaction process. In practice (because the reaction rate is determined by oxygen uptake), excess enzyme may be added and the tank stirred at a defined rate. Polyphenol oxidase is inactivated as a result of its own activity, with the result that fixing it to a support material would be uneconomic for this procedure, especially considering the loss of activity then incurred (the development of a suitably protective antioxidant support might be useful here).

The specific example of hydroxylating and dimerising phloridzin has been considered. Many other flavonoids may give useful products if subject to similar bioconversions. Taylor and Clydesdale (1987), for example, studied the potential for generating useful coloured products by the oxidation of phenolics. Monophenolic flavonoids and soy isoflavonoids such as daidzein and genistein, which have a relatively poor antioxidant activity (Wiseman and O'Reilly, 1997a), could also have their antioxidant activity enhanced by hydroxylation to form *o*-diphenolics (Ridgway and Tucker, 1997c). Work is currently in progress to investigate this.

Another potential *in vitro* application using polyphenol oxidases could be in the development of new tea products. Ethanolic extracts of teas, in particular of green teas, have been shown to have better antioxidant properties than the usual hot water infusions (Wiseman *et al.*, 1996). Furthermore, aqueous extracts of green and black teas have been shown to have antioxidant activity *in vivo* in man (Serafini *et al.*, 1996). Addition of polyphenol oxidase to a suitably diluted solution (<20% ethanol) may then lead to the production of the desired black tea taste. According to the quantity of polyphenol oxidase added this could range from a light 'Oolong' type flavour to a strong 'Assam' type flavour. There could also be advantages in the development of more water soluble derivatives by this approach, limiting the potential for tanning type reactions. However, the effective removal of the potential for lipoxygenase reactions (Lea, 1995) important in the formation of the hexenal that contributes to tea aroma, should also be taken into consideration.

There is currently some concern about the presence of phyto-oestrogens in foodstuffs and in formula milk given to infants. During processing it is possible that added or endogenous polyphenol oxidases may aid their removal or modify them so that they become beneficial. This is because flavonoids and isoflavonoids, many of which demonstrate oestrogenic activity, for example phloretin and daidzein (Miksicek, 1993, 1994, 1995), may have their oestrogenic functionality decreased or even removed by *o*-diphenol formation (Miksicek, 1995), which concomitantly provides enhanced antioxidant ability, as shown for 3-hydroxyphloretin (Ridgway *et al.*, 1996, 1997). This would evidently only be possible if high levels of reducing agents, such as L-ascorbic acid were present. In the absence of reducing agents, the *o*-diphenols formed would be likely to undergo tannin-type reactions, rendering them biologically non-functional.

Endogenous polyphenol oxidase is already used in producing clear ciders and

wines, where a high degree of phenolic oxidation causes tanning reactions and hence a clearing of the beverages (Lea, 1995). This 'hyperoxidative' effect leads to a light, relatively astringent-free taste. Interest has been expressed by commercial sources in using added polyphenol oxidases for this purpose: apple polyphenol oxidase, especially considering its acceptable source, would seem particularly suited for this purpose.

It is possible that 3-hydroxyphloridzin could also be manufactured *in situ* in apple juice by adding L-ascorbic acid followed by stirring with the pressed apple pulp, which contains endogenous polyphenol oxidase (Ridgway and Tucker, 1997c).

Increasing flavonoid levels in apple products by the use of cider apples, which have a much higher flavonoid content than dessert and culinary types (Lea, 1984), could be another improvement. A potential problem with this strategy, however, is that it may lead to an unacceptably astringent and bitter taste. Flavonoid levels may also be increased by harvesting fruit at an early stage of development: with the apple cultivar Bramley's Seedling this leads to an increase in the proportion of phloridzin from 10% to 35% of total phenolic content (Ridgway and Tucker, unpublished results).

The 3-hydroxylation and *o*-diphenol bioconversions of phenolics, particularly the improvement of the antioxidant properties of flavonoids, may thus have an important impact on processes ranging from food processing to human health (see below).

## Antioxidant properties of phloridzin and its derivatives

The structure-function relationships of a wide-range of flavonoids in relation to antioxidant activity is well characterised (Bors and Saran, 1987; Rice-Evans *et al.*, 1995, 1996; Yoshiki *et al.*, 1995; Cook and Samman, 1996; Wiseman, 1996a). One of the principle features is the formation of *o*-diphenolic structures which readily take part in redox coupled reactions. Phloridzin and its derivatives have been studied as antioxidants in lard (Dziedzic *et al.*, 1983, 1985), as inhibitors of lipid peroxidation model membrane systems (Ridgway *et al.*, 1996a,b) and as protectants of human low-density lipoproteins against oxidative damage (Wiseman et al., unpublished results).

Lipid peroxidation is a free radical-mediated chain-reaction, which can be initiated by the hydroxyl radical and attacks polyunsaturated fatty acids in membranes and plasma lipoprotein particles, resulting in oxidative damage (Halliwell and Gutteridge, 1989; Halliwell, 1995 a,b). Free radicals are any species capable of an independent existence that contain one or more unpaired electrons (Halliwell and Gutteridge, 1989; Halliwell, 1995 a,b). Reactive oxygen species (ROS) is a collective term and refers not only to oxygen-centred radicals such as superoxide and the hydroxyl radical but also to hydrogen peroxide, singlet oxygen, hypochlorous acid and ozone. This includes, therefore, some potentially dangerous, non-radical derivatives of oxygen (Wiseman and Halliwell, 1996). Reactive nitrogen species (RNS) can be derived from nitric oxide and include the damaging species peroxynitrite (Darley-Usmar *et al.*, 1995). Free radicals are produced as the by-products of normal metabolism. Thus superoxide, the hydroxyl radical and nitric oxide and ROS such as hydrogen peroxide are all formed *in vivo*. Free radicals have been implicated in over 100 diseases including cardiovascular disease and cancer (Halliwell and Gutteridge, 1989, 1994; Diplock *et al.*, 1994; Cerutti, 1994; Witzum, 1994). However, in many cases they may not be the major cause of disease but rather a consequence and complicating

component of the underlying disease pathology leading to lipid peroxidation, as a consequence – rather than a cause – of cell injury (Halliwell and Gutteridge, 1989; Halliwell, 1994). In cancer, ROS/RNS-mediated damage to DNA bases is implicated in mutation and tumorigenesis (Cerutti, 1994; Wiseman and Halliwell, 1996; Wiseman, 1996): oxidative damage to low-density lipoproteins is implicated in atherosclerosis (Witzum, 1994; Darley-Usmar *et al.*, 1995). Protein modifications include oxidation of thiol groups and especially the generation of carbonyl derivatives of amino acid residues (Oliver *et al.*, 1987). The formation of nitrosothiols and nitrotyrosine residues may occur as a result of RNS attack (Darley-Usmar *et al.*, 1995).

Membrane lipid peroxidation will also damage membrane proteins through consequent free radical attack (Dean *et al.*, 1993; Stadtman, 1993). In addition, lipid hydroperoxides can be readily decomposed by traces of transition metal ions to produce the free radical intermediates of lipid peroxidation capable of propagating the chain reaction. Oxidative DNA damage includes ·OH-mediated modification of DNA bases. ROS/RNS induced mutations could result not only from direct DNA damage but also indirectly as a consequence of oxidative damage to membranes (Wiseman and Halliwell, 1996; Wiseman 1996). This attack on lipids in membranes can initiate the process of lipid peroxidation and the lipid peroxides formed as a result of this oxidative membrane damage can subsequently decompose to mutagenic carbonyl products (Cheeseman, 1993). Lipid peroxidation has been suggested to have a role in human breast cancer risk: urinary excretion of the mutagen malondialdehyde has been shown to be approximately double in women with mammographic displasia (high risk) than in women without these changes (Boyd and McGuire, 1991).

Membrane lipid peroxidation can be measured by a number of different methods, each with its advantages and disadvantages (Halliwell and Chirico, 1993). Membrane lipid peroxidation is often measured in microsomes or liposomes. Microsomes are a heterogenous mixture of vesicles derived from both endoplasmic reticulum and plasma membranes and are used as an *in vitro* test system to assess the ability of a wide range of drugs and dietary components to protect (as antioxidants) against membrane lipid peroxidation (Halliwell and Gutteridge, 1989). Liposomes are used extensively as a model membrane system for studying the influence of dietary components and drugs on membrane lipid peroxidation *in vitro* (Wiseman, 1996a). Liposomes are artificial lipid structures, made by shaking or sonicating phospholipids in aqueous suspension (New, 1992). The most extensively used method is probably the thiobarbituric acid (TBA) test. The test sample is heated with TBA at low pH and the absorbance of a pink chromogen presumed to be a $(TBA)_2$-malondialdehyde adduct (although the term TBARS is frequently used) is measured at 532 nm. Although the TBA test is adequate for measuring lipid peroxidation in defined membrane systems such as microsomes and liposomes, its application to body fluids has many problems relating to its lack of specificity (Halliwell and Chirico, 1993). A modified TBA test has been developed that avoids many of the artefacts resulting from the reaction of TBA with other body-fluid constituents to give different chromogens and uses HPLC to separate the authentic $(TBA)_2$-MDA adduct from other chromogens absorbing at 532 nm (Halliwell and Chirico, 1993).

In healthy individuals the generation of ROS (and RNS) should be in balance with antioxidant defences. When imbalance occurs between generation of free radicals and antioxidant defences then this is referred to as oxidative stress (Halliwell and

Gutteridge, 1989). Oxidative stress can be caused by (1) increased ROS formation e.g., caused by toxic chemicals and drugs (including cytochrome P-450-dependent futile-cycling resulting in the formation of superoxide radicals as byproducts) and at sites of inflammation (as a result of the phagocyte oxidative burst); (2) depletion of antioxidant levels e.g., malnutrition lowers antioxidant vitamin and glutathione levels. A slow general accumulation of oxidative damage is thought to contribute to the ageing process and age-related diseases such as cancer (Ames, 1989). Mitochondria are exposed to a high level of oxidative stress because the mitochondrial energy supply is associated with the production of ROS. Oxidative damage to mitochondria using molecular oxygen, including damage to the mitochondrial membrane (increased levels of lipid peroxidation products have been found in mitochondria exposed to oxidative stress), has been implicated in neurodegenerative disorders such as Parkinson's disease and ageing (Shigenaga *et al.*, 1994; Jenner, 1994).

Cancer can be considered to be a degenerative disease of old age and in many cases may relate to the effects of continuous damage to macromolecules, especially DNA, over a lifespan by ROS and RNS (Ames, 1989). ROS/RNS can cause DNA base changes, strand breaks, damage to tumour suppressor genes and enhanced expression of proto-oncogenes (Cerutti, 1994; Wiseman and Halliwell, 1996). However, the development of human cancer depends on many other factors, including the extent of DNA damage, antioxidant defences, DNA repair systems, efficiency of removal of oxidized nucleosides before they are incorporated into DNA and the cytotoxic effects of ROS in large amounts as well as their growth-promoting effects in small amounts (Cerutti, 1994; Wiseman and Halliwell, 1996). Although the link between cancer and inflammation is by no means a simple one, there is considerable evidence that ROS/RNS are involved in the link between chronic inflammation and malignant progression to cancer (Rosin *et al.*, 1994; Wiseman and Halliwell, 1996).

An antioxidant is 'any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate' (Halliwell, 1995a). 'Oxidizable substrates' include DNA, lipids, proteins (including of membranes and lipoproteins) and carbohydrates (Halliwell, 1995a). Flavonoids such as quercetin and myrecetin (found in red wine, tea, apples and onions) have been widely reported to inhibit membrane lipid peroxidation (Laughton *et al.*, 1989; Terao *et al.*, 1994; Ioka, 1995). Isoflavonoid phyto-oestrogens (found in soy products), such as genistein and daidzein, also protected microsomal membranes against lipid peroxidation (Jha *et al.*, 1985; Wiseman and O'Reilly, 1996a, b) and liposomal membranes against lipid peroxidation (Record *et al.*, 1995; Wiseman and O'Reilly, 1996). This action is observed also for endogenous oestrogens such as 17β-oestradiol (Sugioka *et al.*, 1987; Wiseman *et al.*, 1990; Wiseman 1994a, b; Ruiz-Larrea, 1994) catechol oestrogens (Lacort *et al.*, 1995) and tamoxifen (Wiseman *et al*, .1990; Wiseman 1994a, b).

Pulse radiolysis of aqueous solutions has been used to study the spectral, acid-base and redox properties of phenoxyl radical derived from 3, 4,-dihydroxybenzene derivatives and selected flavonoids: favourable reduction potentials of the phenoxyl radicals suggests that flavonoids may act as efficient antioxidants of alkylperoxyl and superoxide/hydroperoxyl radicals (Jovanovic *et al.*, 1994). Furthermore the relative antioxidant activities of a number of flavonoids, including the catechins, as both scavengers of radicals in the aqueous phase and against lipid peroxyl radicals have

been measured (Salah *et al.*, 1995). The order of potency, measured as chemiluminescence intensity, of flavonoids such as the benzoic and cinnamic acids (in the presence of hydrogen peroxide or hydroxyl radicals) has been shown to correlate with their radical scavenging abilities (Yoshiki *et al.*, 1995). Kaempferol-3-O-galactoside protects mice against bromobenzene-induced hepatic lipid peroxidation and flavonoids fed to mice have protective effects against gamma-ray induced oxidative damage, with luteolin being the most effective (Shimoi *et al.*, 1994). Flavonoids have been reported to be potent scavengers of nitric oxide: the anthocyanidins were found to be more effective than the hydroxyethylrutosides (van Acker *et al.*, 1995) and further investigations of this are in progress. Some flavonoids and tannins have been reported to have xanthine oxidase inhibitory action (Schmeda-Hirschmann *et al.*, 1996).

An antioxidant index has been compiled to assess the effectiveness of the flavonoids and other phenols in red wines compared to white wines (Vinson and Hontz, 1995). Furthermore, the total antioxidant capacity of fruits and fruit juices (rich in flavonoids and antioxidant vitamins) has been measured (Miller *et al.*, 1995; Wang *et al.*, 1996). On the basis of wet weight of fruits (edible portion), strawberry had the greatest antioxidant capacity and the overall order of potency was strawberry > plum > orange > red grape > kiwi fruit > pink grapefruit > white grape > banana > **apple** > tomato > pear > melon (Wang *et al.*, 1996). On the basis of dry weight the order was strawberry > plum > orange > pink grapefruit tomato > kiwi fruit > red grape > white grape > **apple** > melon > pear > banana (Wang *et al.*, 1996). The antioxidant capacity of these fruits was mostly provided by the juice fractions with the fruit pulp contributing less than 10%. The commercial fruit juices had an order of potency of grape juice > grapefruit juice > tomato juice > orange juice > **apple** juice (Wang *et al.*, 1996). The results indicate that there is potential for improving the antioxidant capacity of apple fruit and apple juice by modifying levels both of antioxidant flavonoids such as quercetin and antioxidant vitamins such as vitamin C (see on). Furthermore, the total antioxidant activity of apple juice has been used as a marker of the deterioration of apple juice on storage (Miller *et al.*, 1995). In 'longlife apple' juice vitamin C represented a minor fraction of the total antioxidant capacity compared to chlorogenic acid and phloridzin, which were the major antioxidants (Miller *et al.*, 1995), suggesting that enhancement of antioxidant activity by improving the antioxidant properties of phloridzin would be desirable (see above and on).

Studies of antioxidant applicability in lard (i.e., prevention of rancidity) showed that an *o*-diphenolic structure as part of the A-ring of a dihydrochalcone (e.g., 2′, 4′, 6′, 3, 4-pentahydroxydihydrochalcone: 3-hydroxyphloretin) gives a compound that is more effective as an antioxidant than when the *o*-diphenolic is attached to either a pyran ring (e.g., as in flavanones, flavones or flavanols) or to an unsaturated α-β bond (e.g., as in chalcones) (Dziedzic *et al.*, 1983, 1985). 3-Hydroxyphloretin, for example, has been demonstrated to be extremely effective in preventing fat rancidity: at least 6 times more effective than the food antioxidant butylated hydroxytoluene (BHT), which is used commercially (Dziedzic *et al.*, 1983, 1985).

3-Hydroxyphloretin was a particularly effective inhibitor of liposomal lipid peroxidation (Ridgway *et al.*, 1996, 1997). It displayed an $IC_{50}$ value (concentration required to inhibit lipid peroxidation by 50%) in the low μM range, which was similar to that of quercetin, the most potent flavonoid tested in this system (Wiseman *et al.*, unpublished results). Phloretin and the aglycone dimer were also good inhibi-

tors of lipid peroxidation. 3-Hydroxyphloridzin was an effective inhibitor of lipid peroxidation. Phloridzin and the glycone dimer were poor antioxidants in this system. The overall order of potency of the phloridzin derivatives in this system was 3-hydroxyphloretin > aglycone dimer > 3-hydroxyphloridzin > phloretin > phoridzin = glycone dimer (Ridgway *et al.*, 1996a). These results indicate that, although phloridzin itself is not a good inhibitor of lipid peroxidation in the liposomal model membrane system, its derivatives are much more effective. Phloridzin has been reported to have antioxidant properties in an aqueous-based system for measurement of antioxidant capacity (Miller *et al.*, 1995). Its lack of ability in the ox-brain phospholipid liposomal system is presumably because of the influence of its glucose group, resulting in decreased lipophilicity and thus influencing its uptake and orientation within the liposomal membrane (Ridgway *et al.*, 1996a). This hypothesis is supported by the much improved ability to inhibit lipid peroxidation demonstrated by the aglycone forms.

Bioconversion of phloridzin to 3-hydroxyphloridzin greatly enhanced its ability to inhibit lipid peroxidation. 3-Hydroxylation of phloretin enhanced its ability to inhibit lipid peroxidation by 10-fold, making it of comparable effectiveness to quercetin (Ridgway *et al.*, 1996). In addition, the effectiveness of the aglycone dimer as an inhibitor of lipid peroxidation was 4-fold greater than that of phloretin.

**Table 3.** Antioxidant properties of phloridzin and derivatives

| Compound | Systems | |
|---|---|---|
| | Liposomal $IC_{50}$ (μM) | Microsomal $IC_{50}$ (μM) |
| Phloridzin | NR | NR |
| 3-Hydroxyphloridzin | 7 | 17 |
| Phloretin | 27 | 12 |
| 3-Hydroxyphloretin | 3 | 1.5 |
| 17 β-Oestradiol | 14 | 13 |

$IC_{50}$ values are shown for the inhibition of liposomal and microsomal lipid peroxidation by phloridzin, phloretin, 3-hydroxyphloridzin and 3-hydroxyphloretin compared to oestrogen. Values were determined from graphs of concentration-dependent inhibition of lipid peroxidation in which each point on the graph represented the mean ± S.D. of 6 separate assays (data not shown).

The antioxidant action of oestrogens such as 17β-oestradiol may contribute to their cardioprotective effects (Wiseman and O'Reilly, 1997b). Phloretin, the aglycone form of phloridzin, has been reported to be oestrogenic (Miksicek, 1993) and its 3-hydroxy derivative may have greatly diminished oestrogenic activity that, together with its enhanced antioxidant properties, could enable it to act in a similar manner to the antioxidant antioestrogen/weak oestrogen drug tamoxifen (Ridgway *et al.*, 1997). The results shown in *Table 3* (Ridgway *et al.*, 1997) indicate that in the microsomal model membrane system only 3-hydroxyphloretin was more effective than 17 β-oestradiol and the extent of enhanced potency increased from ~5-fold to ~9-fold. In the liposomal model membrane system, only the hydroxylated phloridzin derivatives, 3-hydroxyphloridzin and 3-hydroxyphloretin were more potent as inhibitors of lipid peroxidation than 17β-oestradiol (by 2-fold and ~5-fold respectively). Phloretin was ~2-fold less potent than 17 β-oestradiol in the liposomal system and was approxi-

mately equipotent in the microsomal system. In contrast an $IC_{50}$ value for phloridzin was not reached in either system. Although phloridzin itself was not an effective inhibitor of lipid peroxidation in the systems tested, its aglycone form phloretin was as effective as 17β-oestradiol in the microsomal system and only 2-fold less potent in the liposomal system. Dietary phloridzin/phloretin may thus confer similar antioxidant cardioprotective effects to 17 β-oestradiol, and further exploration of this possibility is in progress. 3-Hydroxyphloretin is markedly more potent as an inhibitor of lipid peroxidation than 17 β-oestradiol, in both liposomal and microsomal systems and it may, therefore, have a use as a food additive (or therapeutic agent). Functional foods (nutraceuticals) are being sought, and promoted, worldwide: phloridzin derivatives may be candidates for such use.

A large number of dietary components have been reported to protect human low-density lipoproteins (LDL) against oxidative damage. This may be of importance because oxidative damage to LDL (particularly to the apoprotein B molecule) is an important stage in the development of atherosclerosis: it is a prerequisite for macrophage uptake and cellular accumulation of cholesterol leading to the formation of the atheromal fatty streak (Witzum, 1994). The importance of the transfer of LDL into the arterial wall to the process of atherosclerosis has also been considered (Nielsen, 1996). Lipid peroxidation starts in the polyunsaturated fatty acids of the phopholipids on the surface of LDL and then propagate to core lipids resulting in modification of the cholesterol, phospholipids and the apolipoprotein B molecule, and also to the polyunsaturated fatty acids (Witzum, 1994; Halliwell, 1995b). This oxidative hypothesis of atherosclerosis and the likely effectiveness of dietary antioxidants is supported by evidence from recent clinical trials (Nyyssonen *et al.*, 1994, Hoffman and Garewell, 1995). LDL is usually either isolated following a dietary study or dietary components are added to isolated LDL *in vitro*. In most of these studies on the action of dietary components on oxidative damage to LDL, human LDL is stimulated to undergo lipid peroxidation by the addition of Cu(II) ions: a widely used experimental system that is relevant to events occurring within the atherosclerotic lesion (Smith *et al.*, 1992; Halliwell, 1995b) and it appears to involve pre-exisiting lipid hydroperoxides (Thomas *et al.*, 1994; Halliwell, 1995b). Epidemiological evidence from the Netherlands, the Zutphen elderly study, suggests that flavonoid consumption is associated with a lower risk of coronary heart disease (Hertog *et al.*, 1993; Hertog and Hollman, 1996). Flavonoids in red wine have been reported to protect LDL against oxidative damage (Furhrman *et al.*, 1995; Whitehead *et al.*, 1995) and the antioxidant properties of flavonoids may contribute to the reduced risk of coronary heart disease in wine drinkers (despite their high-fat diet and smoking habits): this is the so-called French paradox (Renaud and De Lorgeril, 1992). Flavonoids (morin, quercetin, fisetin, galangin and chrysin) have been reported to inhibit to a characteristic extent the oxidative modification of low-density lipoproteins by macrophages (de Whalley *et al.*, 1990). Dietary phenolic acids from each of the three groups present in the human diet such as caffeic and chlorogenic acids (hydroxycinnamic acid derivatives), ellagic acid (a tannic compound) and protocatechuic acid (a hydroxybenzoic acid derivative) all protect isolated LDL against oxidative damage (Laranjinha *et al.*, 1994), . Furthermore (+)-catechin (Mangiapane *et al.*, 1992) and the flavonoid myrigalone B (in combination with ascorbic acid) (Mathiesen *et al.*, 1996) also protect LDL against oxidative damage.

Phloridzin and its derivatives were able to protect LDL against oxidative damage and for some of these compounds this effect was greater than in model membrane systems (Wiseman *et al.*, unpublished results): such a promising outlook for the application of apple-derived phloridzin derivatives would increase the need for an efficient bioconversion procedure (see above).

Bioavailability is of fundamental significance to the efficacy of a dietary component and the glycone forms of flavonoids have been found to be present in the body in greater quantities than their aglycone forms (Hollman *et al.*, 1996). Thus the use of 3-hydroxyphloridzin as a food additive/health supplement may therefore be preferential to 3-hydroxyphloretin, even though the latter is a less effective antioxidant *in vitro*. It is interesting to speculate whether bioavailability could help to explain the potential health benefits of red wines, which have a high flavonoid content. Flavonoids have a low water solubility, but become increasingly soluble with increasing alcohol content and thus those in the red wine may be better absorbed as a result the alcohol present. Phloridzin, for example has a water solubility of 1 g/920 ml, but in water containing 10% alcohol by volume (comparable to the alcohol content of around 12% of many red wines) this rises to 1 g/360 ml (Ridgway, and Tucker, unpublished results). Increased solubility of flavonoids in alcohol could help further to explain the 'French Paradox' (Renaud and De Lorgeril, 1992) and would be a most interesting example of the dietary benefit in the context of the mode of consumption of the dietary component.

Aqueous solubility could also be of assistance in a physiological setting *via* flavonoid-mediated redox-coupled transfer of lipid damage to the plasma and subsequent quenching by glutathione/vitamin C systems. The potential importance of combined water/lipid antioxidant systems is in relation to the underlying mechanism of the hydrogen donating chain-breaking antioxidant action of flavonoids. In contrast to the regeneration of vitamin E, which only occurs at the lipid surface, it is likely that flavonoids could flip between lipid and water phases as a result of the observed dramatic changes in polarity on reduction and oxidation; hence the term 'transfer antioxidants' would seem appropriate (Ridgway and Tucker, 1996b). Deliberate manipulation of flavonoid aqueous-lipid partition coefficients would allow the development of a whole new range of antioxidants for particular food-processing, environmental and dietary utilisation.

Antioxidant action may not always be beneficial: apoptosis (programmed cell death) can be initiated by oxidative damage, and when cancer cells are exhibiting oxidative stress, but are unable to achieve an apoptotic event (Toyokuni *et al.*, 1995), insufficient pro-oxidant activity may be available for self-destruction of the tumour. Antioxidant potential could thus hinder potentially important destructive cellular processes (indeed it may have contributed to the reported increase in lung cancer among smokers given antioxidant β-carotene supplements). Flavonoids, however, may overcome this apparent paradox as they both exhibit antioxidant properties and yet can limit cell division and possibly destroy tumours (Ridgway and Tucker, 1997b). A possible mechanism for this is 'phenolisation' of cyclin proteins, which is in contrast to the usual regulatory phosphorylation mechanism. This process could occur by $\pi$ $\pi$ binding or covalently *via* the formation of oxidised flavonoid-derived semiquinones (Ridgway and Tucker, 1997b). One possibility is the 'phenolisation' of exposed tyr-15 on $p34^{cdc2}$ which would lead to arrest at the G2-M phase of mitosis;

phosphorylation at this point is a checkpoint control (Jacobs' 1995). G2-M Phase arrest is observed in the breast cancer cell line MDA-MB468 when exposed to quercetin (Avila *et al.*, 1994). The isoflavonoid genistein, in addition, has the potential to interfere directly in the process of phosphorylation as it can inhibit tyrosine kinase activity (Akiyama *et al.*, 1987).

Antioxidant activity can be regarded as susceptibility to oxidation by the compound itself. Readily oxidised flavonoids such as 3-hydroxyphloretin are, therefore, more likely to interact with target sites, such as exposed tyrosine residues, than non-antioxidant compounds. In the future, it may be possible to enhance such effects by the use of polyphenol oxidase active fragments linked to tumour-specific antibodies (Ridgway and Tucker, 1997b). In high concentrations, it is likely that oxidised phenolics will produce a potentially useful direct toxic effect: redox-cycling to generate toxic free radicals is the basis of the current use quinonic pharmaceuticals in chemotherapy (Powis, 1989). The potential success of this strategy would again appear to depend on the differential response of cells: cancer cells are unable to cope with high oxidative stress. The redox potential of many existing (and new) biochemicals will thus emerge as a design feature of antioxidants (and pro-oxidants).

## Food technology applications of phloridzin derivatives

Recent work on colourants has concentrated on the extraction and processing of existing types (Spears, 1988) or the synthetic production of 'nature identical' compounds, developments with are likely to have little or no difficulty in gaining permission for use (and 'E' listing in the EU) and require no additional toxicity testing to be carried out. Substances such as novel anthocyanins may also be expected to experience little difficulty due to their similarity to existing products. Entirely new dyes or antioxidants would, however, have to pass toxicity tests and food manufacturer acceptance. An intermediate position may apply to one group of potential new compounds, the dimerised oxidation products of phenolics, which may be produced as a result of traditional food processing. Many phenolic oxidation productions were investigated by Taylor and Clydesdale (1987), showing that a range of potentially useful colours was possible. Similarly, Lea (1982) and Goodenough *et al.* (1983) had shown that a large percentage of the colour of apple juice is due to the oxidation product of phloridzin, this compound having a hue similar to that of the synthetic colourant tartrazine (E 102). Furthermore, yellow dimers have been produced from the grape polyphenol oxidase-mediated coupling of (+)-catechin (Guyot *et al.*, 1996).

The yellow/orange properties of the dimerised oxidation product of phloridzin suggests its use as a food colourant. This hue is, in fact, very similar to that shown by tartrazine (E102), a synthetic additive over which there has been much consumer concern because of reports of hyperactivity in children. Hunter Lab colorimetric profiles have confirmed the similarity at soft drink pH values (2.7–4.0), specific absorbance at peak wavelength (420 nm) has been found to be greater than that of tartrazine, and stability trials in produce have shown the product to be more stable than tartrazine, particularly in light (Ridgway and Tucker, 1996b). In addition, the phloridzin dimerisation product has greater light stability than tartrazine in actual products (lemonade and cider) (Ridgway and Tucker, unpublished results) thus showing greater technical performance in addition to greater potential marketability

as a 'nature identical' substance (Spears, 1988). As the phloridzin dimerised oxidation product has been shown to make up a large proportion of the colour of apple juices and ciders (Lea, 1982) it represents a potential natural or 'nature identical' product of superior performance to synthetic tartrazine (Ridgway and Tucker, 1996).

The antioxidant properties of 3-hydroxyphloretin (see above) suggest its potential use as a technological food antioxidant. Butein, for example, which is a tetrahydroxychalcone and hence a less potent antioxidant than 3-hydroxyphloretin, which is a pentahydroxydihydrochalcone, was found to be 6 times more effective in preventing fat rancidity than the commercial food antioxidant BHT (Dziedzic, 1983). Thus 3-hydroxyphloretin would be expected to be at least 6 times more potent than BHT (see above).

3-Hydroxyphloridzin, with its greater water solubility than 3-hydroxyphloretin, could find application in aqueous systems. A particular application could be in the protection of the colourant properties of flavonoids. Depending on the concentration used, 3-hydroxyphloridzin itself is able to show coloured properties upon the formation of dimerised oxidation products. Combinations of 3-hydroxyphloridzin and the dimerised oxidation product of phloridzin could then provide a highly stable mixture: loss of colour of the dimerised oxidation product could be compensated for by the colour formation that occurs upon oxidation of the 3-hydroxyphloridzin.

## Genetic transformation of plants: prospects for modification of flavonoid content

Flavonoids may potentially be beneficial to human health, and the genetic transformation of plants may greatly contribute to this aim. The prospects for modifying the flavonoid content of plants is discussed (see below), with particular reference to the apple, as the requisite transformation and regeneration systems for apple have been developed (James *et al.*, 1994). Interestingly, flavonoids may aid the actual process of transformation: a number of chalcones are most effective in *vir* gene induction in *Agrobacterium*-mediated gene transfer (Joubert *et al.*, 1995).

Apple has been considered as a potential source of phenolics such as phloridzin. Evidently, the economics of phloridzin production would be more favourable if the tissue yields of phloridzin could be improved. Potential schemes for this include the use of regulatory elements, for example, maize R genes, which may co-ordinately regulate flavonoid biosynthesis, or alternatively the up-regulation (expression of non-endogenous forms under a constitutive promoter) of the genes for PAL and CHI, which form potential regulatory points in flavonoid biosynthesis.

*o*-Diphenols undergo greater rates of auto-oxidation than mono-phenols: the lower levels of stress associated with monophenols may possibly be one of the reasons that apple is able to accumulate large quantities of the monophenolic phloridzin. To increase the accumulation of flavonoids in plants, it may be necessary to up-regulate regulatory antioxidant systems such as the glutathione system. This could be carried out by the transformation of plants with bacterial genes such as *gor*, *gsh I* and *II*, coding for glutathione reductase, γ-glutamylcysteine synthetase and glutathione synthetase respectively (Foyer *et al.*, 1994). In addition, the down-regulation of genes coding for L-ascorbic acid oxidase could help to maintain a high

antioxidant level. Similarly, the down-regulation of the gene(s) coding for polyphenol oxidase, as successfully carried out in potato (Bachem *et al.*, 1994), may lead to greater flavonoid stability and accumulation of these health-promoting components.

It is interesting to note that regulatory elements both *cis* and *trans*, may be affected by redox status (Garcia-Olmedo *et al.*, 1994). Increasing antioxidant ability may then be a prerequisite for boosting levels of secondary metabolites, in particular flavonoids. Flavonoids (especially oxidised flavonoids) may interact with cyclin proteins (see above). After a critical level of flavonoid accumulation, cell division may cease and cell expansion initiated thus limiting the flavonoid content of the tissue. A requirement for flavonoid auto-oxidation is possible and this would again emphasise the importance of high cellular antioxidant activity. Evidently, there are many possibilities and much further work needs to be done in elucidating appropriate mechanisms for increasing flavonoid levels in plants. Indeed, most work on flavonoids has concentrated on anthocyanin production in flowers, which principally relates to the elucidation of pathways and the switching-on and off of genetic elements (Mullineaux and Creissen, 1996).

In addition to increasing general levels of flavonoids in plants, there is potential to increase levels of specific flavonoids such as quercetin, which have good antioxidant properties (see above). Quercetin is known to be present in apple, particularly in the skin of the fruit. This flavonoid is synthesised from dihydroquercetin and represents the end product in this particular biosynthetic pathway. Levels of quercetin may potentially be increased by antisensing the gene for dihydroflavonol reductase. This enzyme competes for available dihydroquercetin with the enzyme responsible for quercetin production and down-regulation of dihydroflavonol reductase may thus lead to a diversion of metabolites into quercetin. Fortunately for the potential success of the scheme, the gene for dihydroflavonol reductase appears to be highly conserved and in the species examined so far, exists as a single copy.

## Bioconversion of flavonoids: the future

A wide range of applications of flavonoid oxidation have been discussed, from *in vitro* bioconversions (mediated by polyphenol oxidase) for the production of new food additives, to bioconversions in plant material during food processing, and even transformation in the body, both as part as a redox-couple to prevent oxidative damage and oxidation to help destroy tumours. The effectiveness of phloridzin derivatives as inhibitors of lipid peroxidation suggests that they could be of use as functional foods to protect the health of the consumer against the oxidative damage implicated in many disease states. The antioxidant activity of 3-hydroxyphloretin (Ridgway *et al.*, 1996, 1997), clearly shows its potential for use as a therapeutic antioxidant. Its use is likely to prove most effective in combination with other types of antioxidant, not just aqueous phase types such as L-ascorbic acid (Vitamin C with added bioflavonoids is already to be found on sale as a food supplement), but perhaps also with membrane fluidity modifiers such as tamoxifen (Wiseman, 1994a). Degrees of potential synergy of antioxidant types *in vitro* are currently being investigated.

The weak oestrogenic properties of phloridzin and its derivatives suggest that they could act in a similar way to soy-derived isoflavonoids to block the mitogenic effects

of oestrogen on breast cells and thus act as dietary chemopreventative agents against breast cancer (Ridgway *et al.*, 1996, 1997; Wiseman, 1996b). Oestrogenic properties of the 3-hydroxy derivatives of phloridzin have yet to be reported. It is likely, however, that the 3-hydroxy derivatives will have greatly reduced oestrogenic activity (Miksicek, 1993, 1994, 1995). Other potentially useful direct bioconversions of phloretin include methylation, for example, the use of naringenin 7-*o*-methyltransferase (Rakwal *et al.*, 1996). This would perhaps relate to improving oestrogen antagonist and hence anticancer activity.

These novel flavonoids could provide the standard technological antioxidant function of preventing rancidity in fatty foods. Furthermore, the colorimetric and colour stability properties of the glycone and aglycone forms of the coloured dimer would suggest their use as natural alternatives to the synthetic additive tartrazine (E102), which is now unacceptable to the consumer (Ridgway and Tucker, 1997b).

Biochemically phloretin is only one reverse dehydrogenase (i.e., NADPH dependent) step away from naringenin, a central precursor in flavonoid biosynthesis. As a large proportion of flavonoid biosynthesis has been characterised it is possible that phloridzin could be used in the future as the starting point for the production of a wide range of 'nature identical' and designer flavonoid products. It is also possible that phloridzin could feed into the general chemical intermediate market. For example, an enzyme has been identified (Barz and Koster, 1981), which splits phloretin into phloroglucinol and *p*-hydroxydihydrocinnamic acid, both of which retail as relatively valuable intermediates. Production of further derivatives could then perhaps be mediated by algal phenolic halogenating enzymes. Full degradation, potentially by bacterial enzymes, should lead to a mixture which could be fractionated as with the products of combinatorial chemical synthesis. Certainly there is plenty of scope in the future for the development of additional flavonoid bioconversion technology if the scope of the product utilization, preferably on a large scale was adequate – and the price was right!

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