1 Dietary Flavonoids and Phenolic Compounds

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CONTENTS

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INTRODUCTION

Plants synthesize a vast range of secondary metabolites with a significant portion consisting of phenolic compounds and flavonoid compounds [Crozier et al., 2006a]. These phytochemicals are structurally diverse, and many are distributed among a very limited number of species within the plant kingdom. This character allows them to act as biodiagnostic markers in chemotaxonomic studies. Phenolic compounds and flavonoids accumulate in relatively high amounts in plants and appear to have a myriad of supplemental functions in a plant's life cycle. These include structural roles in different supporting or protective tissues, involvement in defense strategies, as attractants for pollinators and seed-dispersing animals, and as allelopathic agents, ultra violet (UV) protectants and signal molecules in the interactions between plants and their environment. One of the most versatile classes of flavonoids, the anthocyanins, protect chloroplasts from photodegradation by absorbing high-energy quanta, while scavenging free radicals and reactive oxygen species (ROS) [Gould, 2004]. Flavonols, as well as providing protection against the damaging effects of UV-B light, are also involved in promoting the growth of pollen tubes down the style to facilitate fertilization. In addition, isoflavonoids play important defense roles against pathogen and insect attack and are key signal molecules in the formation of nitrogen-fixing root nodules in legumes. After the death of plants, phenolic compounds may persist for weeks or months and affect decomposer organisms and decomposition processes in soils. Therefore, their effects are not restricted to only the plant itself but may extend to the functioning of whole ecosystems [Horner et al., 1988].

Secondary metabolites, other than providing plants with unique survival or adaptive strategies, are of commercial significance to humankind. They have been used as dyes, fibers, glues, oils, waxes, flavoring agents, drugs, and perfumes and are viewed as potential sources of new natural drugs, antibiotics, insecticides, and herbicides [Croteau et al., 2000; Dewick, 2002]. In recent years the role of phenolic compounds and flavonoids as protective dietary constituents has become an increasingly important area of human nutrition research. Unlike the traditional vitamins, they are not essential for short-term well-being, but there is increasing evidence that modest long-term intakes may exhibit a potential for modulating human metabolism in a manner favorable for the prevention or reduction in the risk of degenerative diseases such as cardiovascular diseases, diabetes, obesity, and cancer [Anderson et al., 1999].

HEALTH BENEFITS AND MODE OF ACTION OF FLAVONOIDS AND PHENOLIC COMPOUNDS

The rapid rise of degenerative diseases worldwide is threatening economic and social development as well as the lives and health of millions of people. It represents a major health challenge to global development in the coming century.

It is estimated that up to 80% of cardiovascular disease, 90% of Type II diabetes, and one third of cancers can be avoided by changing lifestyle, including diet [WHO, 2003]. Diet-related high cholesterol, high blood pressure, obesity, and insufficient consumption of fruits and vegetables have been cited as significant interlinking risk factors that cause the majority of these diseases. There is, therefore, increasing interest in the role of nutrition and specific dietary constituents in the prevention of such diseases. Flavonoids and phenolic compounds are prominent among dietary constituents that are the focus of such interest.

Since the 1990s a number of epidemiological studies have been carried out attempting to correlate high dietary phenolic compounds and flavonoid intake, through the consumption of fruits and vegetables, with reduced risk of degenerative diseases. Many, but not all, of these studies have indicated some degree of inverse associations between high dietary phenolic/flavonoid intake and reduction of degenerative diseases [Steinmetz and Potter, 1996; Law and Morris, 1998; Riboli and Norat, 2003]. Since oxidative stress imposed by ROS is known to play a crucial role in the pathophysiology associated with neoplasia, atherosclerosis, and neurodegenerative diseases, the potential mechanism of the protective effects of phenolic compounds and flavonoids were thought to be due to direct scavenging of free radicals [see Heim et al., 2002].

Accumulating evidence now indicates the importance of interactions between various phytochemicals in reducing the risk of various degenerative diseases [Chan et al., 2000; Mouria et al., 2002; Mertens-Talcott et al., 2003]. The combination of antioxidative agents with different modes of action is thought to increase efficacy and minimize toxicity. In a recent review by Lee and Lee [2006], the abilities of phenolic-based antioxidant therapies to decrease ROS levels was shown to produce the best health benefits through a diet rich in multiple antioxidants rather than a high dosage of a single supplement. Evidence of the potential benefits of food synergy was provided by Liu et al. [2000] when they demonstrated that a combination of fruits, such as orange, apple, grape, and blueberry, displayed a synergistic effect on antioxidant activity in vitro. The median effective dose (EC_{50}) of each fruit in combination was five times lower than the EC_{50} of each fruit alone, suggesting synergistic effects due to the combination of the four fruits. In another study, Sakamoto [2000] emphasized the importance of consuming black tea together with soybean products as commonly occurs in a typical Japanese diet. In this study, thearubigen in black tea did not alter the in vitro growth of human prostate cancer cells. However, a small amount of thearubigen (0.5 $\mu g\ mL^{-1}$) administered with genistein (20 $\mu g\ mL^{-1}$), the major isoflavone in soybean, synergistically inhibited cell growth and increased the DNA distribution at the G2 M phase of the cell division cycle by 34% compared with genistein alone [Sakamoto, 2000]. Similar conclusions were reached by Temple and Gladwin [2003] when they reviewed 200 cohort and case–control studies that provided risk ratios concerning intake of fruits

and vegetables and risk of cancer. Their studies showed that the cancer-preventing action of fruits and vegetables is most probably due to the many bioactive compounds that act in concert to prevent cancer rather than being due to one or two potent anticarcinogens.

Nutrients generally have very specific functions such as being an enzyme cofactor. In contrast, in addition to their additive and synergistic effects, phenolic compounds and flavonoids, often exhibit pleiotropic effects that in combination may reduce the risk of chronic disease. For instance, curcumin, the active constituent of turmeric (*Curcuma longa*), a root vegetable, has been shown to be beneficial in all three stages of carcinogenesis [Thangapazham et al., 2006]. Isoflavones, the bioactive ingredient in leguminous vegetables, not only cause a small reduction in blood cholesterol but also reduce blood pressure, arterial dimensions, and oxidative stress [Anderson et al., 1999]. This combined effect may cause a reduction in the risk of coronary heart disease [Kris-Etherton et al., 2004].

In addition to the complexity mentioned above, the health implications of dietary phenolic compounds and flavonoids are also dependent on the composition of the components of the diet and the bioavailability of the individual compounds under study. Accumulating evidence on the absorption and bioavailability of phenolic compounds and flavonoids in humans reveals that most of these phytochemicals are modified during absorption from the small intestine, through conjugation and metabolism, and by the large intestine, mainly through the actions of the colonic microflora, and by subsequent hepatic metabolism [Graefe et al., 2001; Manach et al., 2004; Mullen et al., 2004, 2006, 2008; Jaganath et al., 2006]. Thus, metabolites that reach the cells and tissues are chemically, and, in many instances, functionally distinct from the dietary form, and such features underlie their bioactivity [Kroon et al., 2004]. This, in addition to the fact that in most instances very low levels of dietary phenolic compounds and flavonoids are actually absorbed and appear in the bloodstream (<10 μM), implies that the concept of these compounds functioning as hydrogen-donating antioxidants in vivo appear to be an over-simplified view of their mode of action [Williams et al., 2004; Williamson and Manach, 2005; Fraga, 2007].

It has been hypothesized that cells respond to phytochemicals through direct interactions with receptors or enzymes involved in signal transduction, or through modifying gene expressions that may result in alteration of the redox status of the cell that may trigger a series of redox-dependent reactions [Williams et al., 2004]. There is now emerging evidence that flavonoids may play an important role in molecular processes especially as modulators of intracellular signaling cascades, which are vital to cellular function [Williams et al., 2004]. For example, in a recent study carried out by Mackenzie and associates (2008), a naturally occurring phenolic compound, curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione,1] was found to deregulate signaling cascades, such as NF-κB, leading to a decreased expression of proteins involved in cell proliferation and apoptosis. In another study on Caco-2 cells, hexameric

procyanidins was found to inhibit TNFα-induced NF-κB activation, which is belived to play a central role in inflammation including human intestinal bowel disease [Erlejman et al., 2008].

There is growing evidence from human feeding studies that the absorption and bioavailability and thus bioactivity of phenolic compounds and flavonoids are very much dependent on the nature of their chemical structure. Their chemical classification and dietary occurrence is briefly discussed in the following section.

FLAVONOIDS—STRUCTURE AND THEIR DIETARY OCCURRENCE

To date, more than 6000 different flavonoids have been described and the number continues to increase [Harborne and Williams, 2000]. Flavonoids are polyphenolic compounds comprising of 15 carbons, with 2 aromatic rings connected by a 3-carbon bridge. According to the modifications of the central C-ring, they can be divided into different structural classes including flavonols, flavones, flavan-3-ols, flavanones, isoflavones, and anthocyanidins (Fig. 1.1). In a few cases, the 6-membered heterocyclic ring C occurs in an isomeric open form or is replaced by a 5-membered ring as in the case of chalcone. Other flavonoid groups, which quantitatively are relatively minor dietary components, are dihydroflavones, flavan-3,4-diols, coumarins, and aurones.

Flavonols

The flavonols are the most widespread of the flavonoids in plant food. They vary in color from white to yellow and are closely related in structure to the flavones. They are represented mainly by quercetin, kaempferol, and myricetin while the methylated derivative isorhamnetin is also quite common (Fig. 1.2). Of the various flavonols found in the diet, quercetin is the most ubiquitous. It is present in various fruits and vegetables, with especially high concentrations, 200–1000 μg g^{-1} fresh weight, occurring in onions (*Allium cepa*) [Hertog et al., 1992; Crozier et al. 1997]. In a recent study by Sultana and Anwar [2008], flavonol levels were determined in 22 plant materials (9 vegetables, 5 fruits, and 8 medicinal plants). The highest concentrations were detected in the medicinal plant, moringa (*Moringa oleifera*) (68 μg g^{-1} fresh weight), followed by strawberry (*Fragaria* spp.) (40 μg g^{-1}), peepal (*Ficus religious*) (12 μg g^{-1}), spinach *(Spinaceae oleraceae)* (19 μg g^{-1}), and cauliflower *(Brassica oleraceae)* (18 μg g^{-1}).

Flavonols that accumulate in plant tissues are almost always in the form of glycosylated conjugates. The main flavonols in onions are quercetin- 4′-*O*-glucoside and quercetin-3,4′-*O*-,diglucoside with smaller amounts of isorhamnetin-4′-*O*-glucoside (Fig. 1.3) [Mullen et al., 2004].

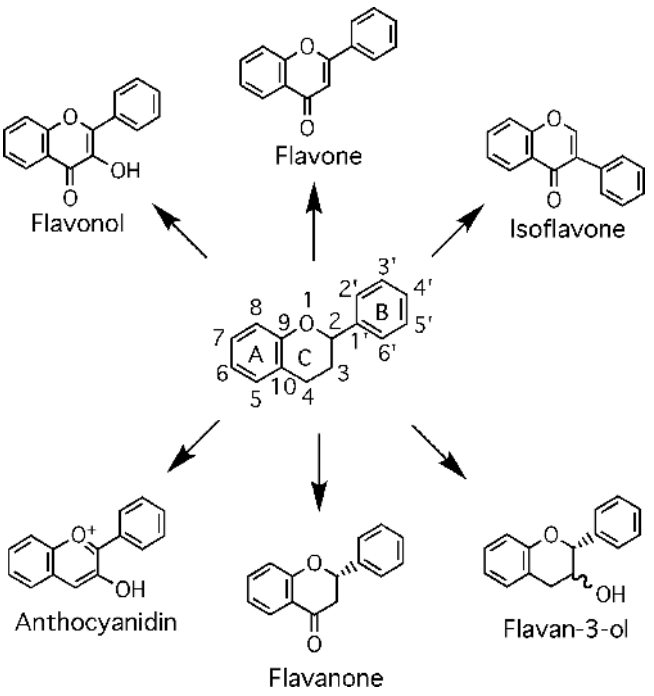


Figure 1.1 Structures of the main flavonoid subgroups.

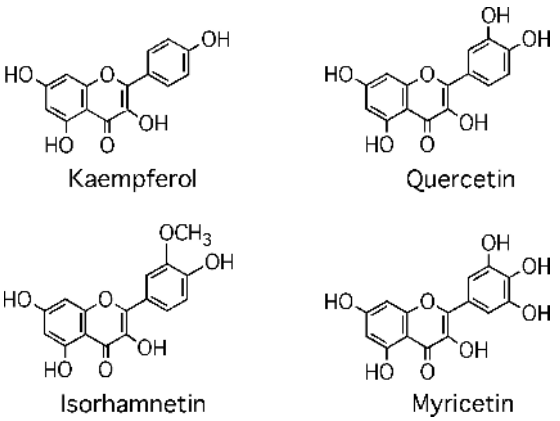


Figure 1.2 Structures of common flavonol aglycones.

A whole range of other quercetin conjugates such as quercetin-3-*O*-galactoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-arabinopyranoside, and quercetin-3-*O*-arabinofuranoside are found in apples (*Malus* x *domestica*) (Fig. 1.4) [Marks et al., 2008].

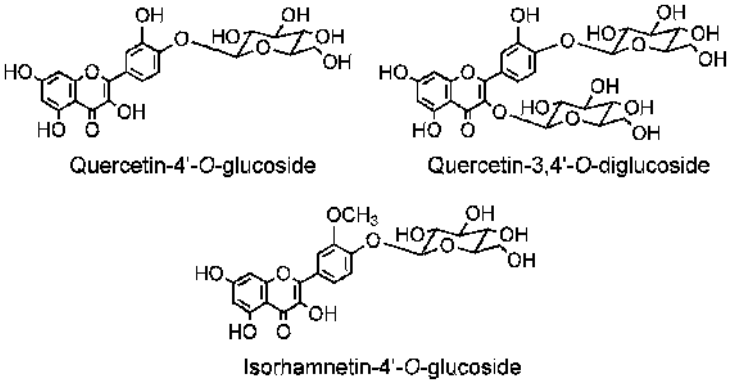


Figure 1.3 Main flavonol glucosides in onion.

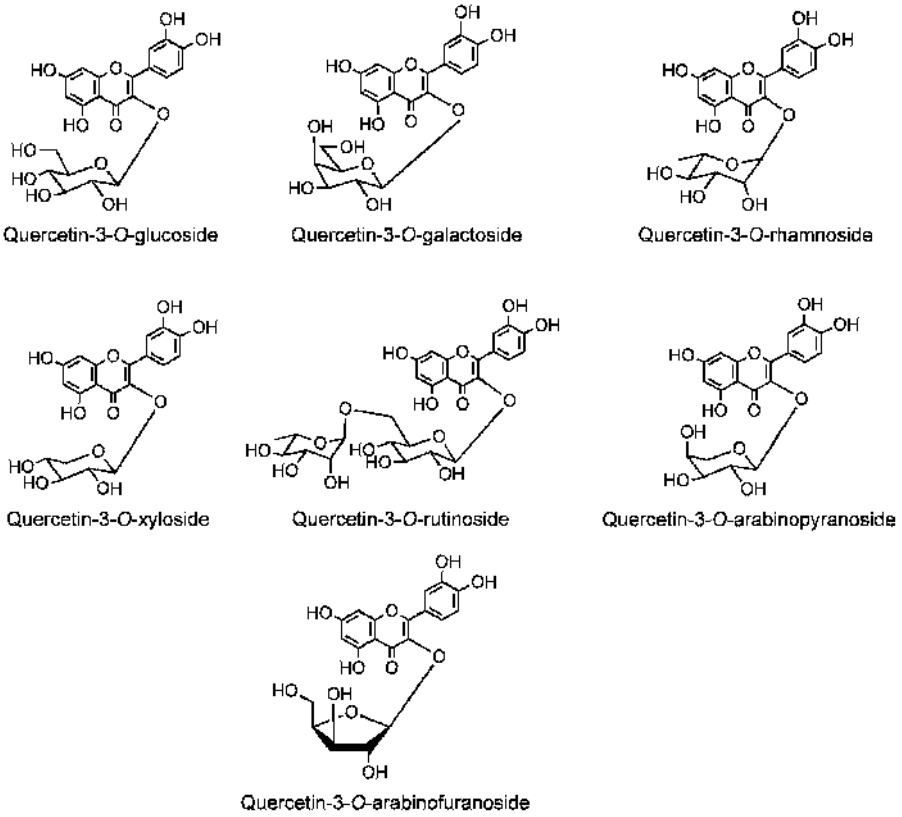


Figure 1.4 Principal flavonol glucosides in apples.

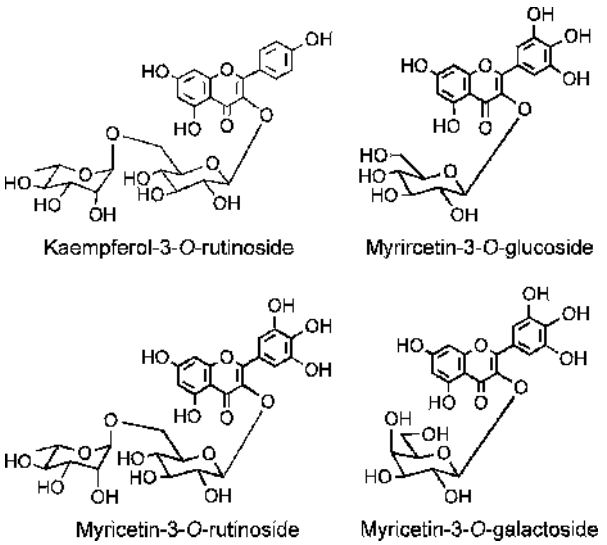


Figure 1.5 Flavonol conjugates found in berries.

Quercetin-3-*O*-rutinoside, on the other hand, is the main flavonol in tomatoes *(Lycopersicon esculentum)*, asparagus *(Asparagus officinalis)*, peaches *(Prunus persica)*, and nectarines *(Prunus persica* var. nectarina*)* [Makris and Rossiter, 2001; Crozier et al., 2006c]. Quercetin-3-*O*-glycoside, quercetin-3-galactoside, and aquercetin arabinoside has also been detected in mangos *(Mangifera indica)* [Schieber et al., 2000]. Other flavonols in the diet include kaempferol-3-*O*-rutinoside in kiwi fruit *(Actinidia deliciosa)* and conjugates of myricetin in berries (Fig. 1.5) (Peterson and Dwyer, 1998].

Grapes of *Vitis vinifera*, grape products, and wines contain a wide range of flavonols such as quercetin, myricetin, kaempferol, isorhamnetin, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside, kaempferol-3-*O*-glucoside, and kaempferol-3-*O*-galactoside [Makris et al., 2006]. Tea *(Camellia sinensis)* infusions also contain a diverse spectrum of flavonols linked to mono-, di- and tri-saccharides [Del Rio et al., 2004].

Flavones

Flavones are structurally very similar to flavonols and differ only in the absence of hydroxylation at the 3-position on the C-ring. Flavones are mainly represented in the diet by apigenin and luteolin. Unlike flavonols, they are not widely distributed with significant concentrations being reported in only celery (*Apium graveolens*), parsley (*Petroselinum crispum*), and artichoke (*Cynara scolymus*) [Crozier et al., 2006a]. As a consequence their dietary intake

is very low. Flavone conjugates such as the 7-*O*-(2″-*O*-apiosyl)glucosides of apigenin, luteolin, and chrysoeriol (Fig. 1.6) are found in celery [Herrmann, 1976], while artichoke contains luteolin-7-*O*-glucoside, luteolin-7-*O*-rutinoside, and apigenin-7-*O*-rutinoside (Fig. 1.7) [Wang et al., 2003].

Substantial quantities of luteolin-7-*O*-glucuronide, luteolin-7-*O*-glucoside, and luteolin-7-*O*-rutinoside occur in Red Oak Leaf and Lollo Rosso, two red-leaved varieties of lettuce (*Lactuca sativa*) [Llorach et al., 2008]. Polymethoxylated flavones such as nobiletin, scutellarein, sinensetin, and tangeretin (Fig. 1.8) are found exclusively in citrus species [Crozier et al., 2006c], while diosmetin-7-*O*-glucuronide has been isolated from the fruits of a Chinese herb, *Luffa cylindrical*.

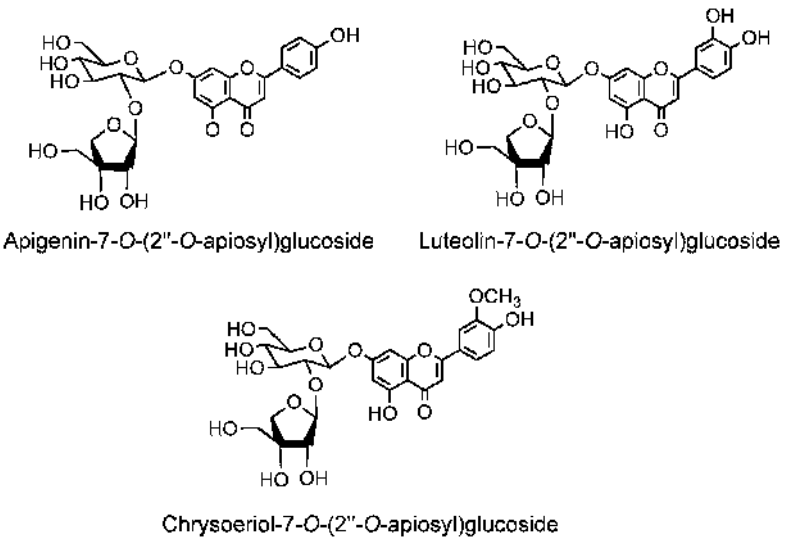


Figure 1.6 Flavone conjugates occurring in celery.

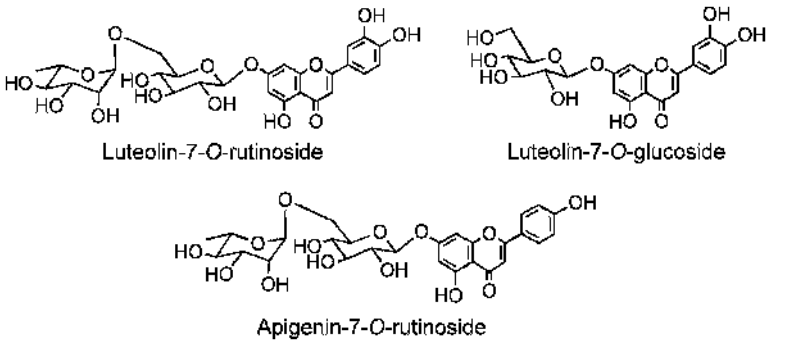


Figure 1.7 Flavone conjugates found in artichoke.

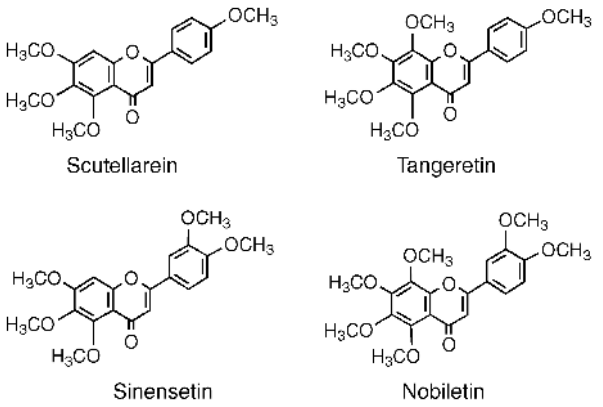


Figure 1.8 Polymethoxylated flavones found in citrus species.

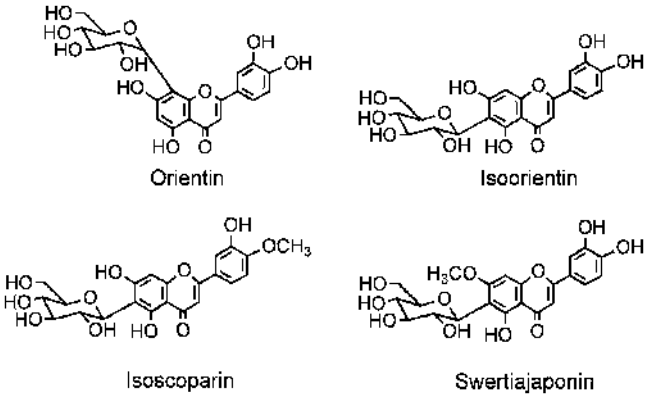


Figure 1.9 Flavones found in rooibos tea.

Red bush or rooibos tea, made from infusions of young leaves and shoots of the South African shrub *Aspalathus linearis,* and popularized by the *The No. 1 Ladies' Detective Agency* novels of the Edinburgh University Emeritus Professor of Medical Law, Alexander McColl Smith [1999, 2000], contains a number of compounds including *C*-flavone glycosides in the form of isoorientin (luteolin-6-*C*-glucoside) and orientin (luteolin-8-*C*-glucoside) [Bramati et al., 2003]. Orientin and isoorientin also occur in lemongrass (*Cymbopogon citrata*) along with two other flavone *C*-glucosides, chrysoeriol-6-*C*-glucoside (isoscoparin) and 7-*O*-methyl-luteolin-6-*C*-glucoside (swertiajaponin) (Fig. 1.9) [Cheel et al., 2005].

Recent observations reveal that when flavones are methoxylated, metabolic stability and membrane transport in the intestine/liver dramatically increases,

thus improving oral bioavailability. In addition, methoxyflavones also show increased cancer chemopreventive properties when compared to the more common unmethylated flavones [Walle, 2007].

Flavan-3-ols

Flavan-3-ols represent the most common flavonoid consumed in the American and, most probably, the Western diet and are regarded as functional ingredients in various beverages, whole and processed foods, herbal remedies, and supplements. Their presence in food affects quality parameters such as astringency, bitterness, sourness, sweetness, salivary viscosity, aroma, and color formation [Aron and Kennedy, 2007]. Flavan-3-ols are structurally the most complex subclass of flavonoids ranging from the simple monomers (+)-catechin and its isomer (−)-epicatechin to the oligomeric and polymeric proanthocyanidins (Fig. 1.10), which are also known as condensed tannins [Crozier et al., 2006b].

The most abundant type of proanthocyanidins in plants are the procyanidins, which consist exclusively of (epi)catechin units. The less common proanthocyanidins containing (epi)afzelechin (Fig. 1.11) and (epi)gallocatechin (Fig. 1.10) subunits are called propelargonidins and prodelphinidins, respectively [Balentine et al., 1997].

Flavan-3-ols are found abundantly in fruits such as apricots (*Prunus armeniaca*), sour cherries (*Prunus cerasus*), grapes and blackberries (*Rubus* spp.) [Porter, 1988]. The seeds of grapes contain substantial quantities of (+)-catechin, (−)-epicatechin, procyanidin oligomers, and polymers [Gu et al., 2004]. Apples, on the other hand, are a good source of (−)-epicatechin and procyanidin dimers B_1 and B_2 (Fig. 1.12), while peaches and nectarines contain (+)-catechin, (−)-epicatechin, and proanthocyanidins including procyanidin B_1 [Hong et al., 2004]. Barley, seemingly, is the only common cereal with a significant proanthocyanidin content (0.6–1.3 g kg^{-1}) [Santos-Buelga and Scalbert, 2000].

(+)-Catechin and the proanthocyanidin prodelphinidin B_3 are, respectively, the major monomeric and dimeric flavan-3-ols found in barley and malt where prodelphinidin B_3 is the main contributor for the radical scavenging activity [Dvoráková et al., 2007]. Proanthocyanidins have also been detected in nuts. Hazelnuts *(Corylus avellana)* and pecans *(Carya illinoensis)* are particularly rich in proanthocyanidins containing ca. 5 g kg^{-1}, whereas almonds *(Prunus dulcis)* and pistachios *(Pistachio vera)* contain 1.8–2.4 mg kg^{-1}, walnuts (*Juglans* spp.) ca. 0.67 g kg^{-1}, roasted peanuts *(Arachis hypgaea)* 0.16 g kg^{-1}, and cashews *(Anarcardium occidentale)* 0.09 g kg^{-1} [Crozier et al., 2006c]. Dark chocolate derived from the roasted seeds of cocoa (*Theobroma cacao*) is also a rich source of procyanidins [Gu et al., 2004]. Monomeric flavan-3-ols and the proanthocyanidin B_2, B_5 dimers, and C_1 trimer are found in fresh cocoa beans (Fig. 1.13). Flavan-3-ols have also been detected in mint

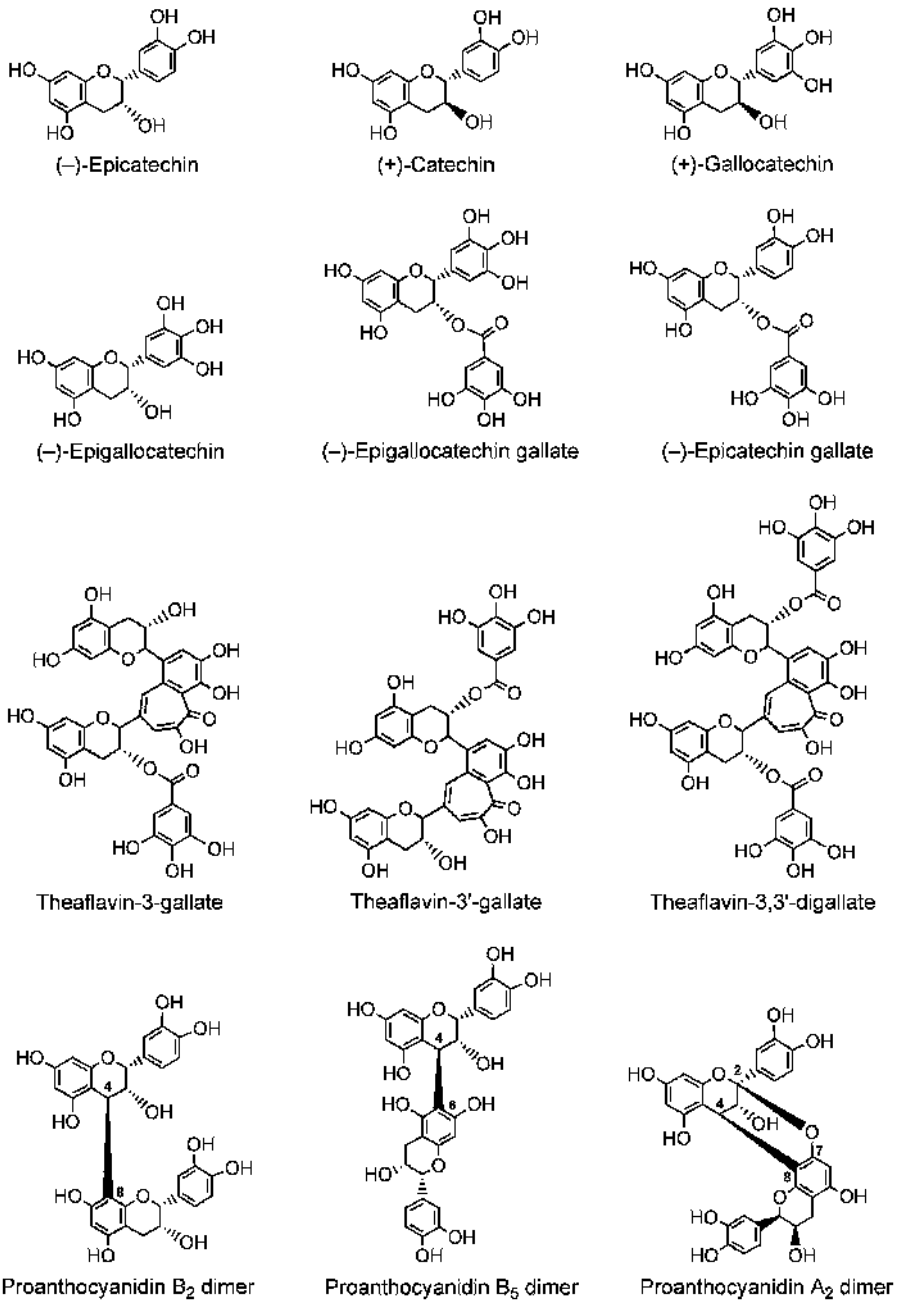


Figure 1.10 Flavan-3-ol structures.

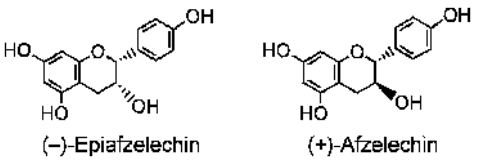


Figure 1.11 Less common flavan-3-ol monomers: (−)-epiafzelechin and (+)-afzelechin.

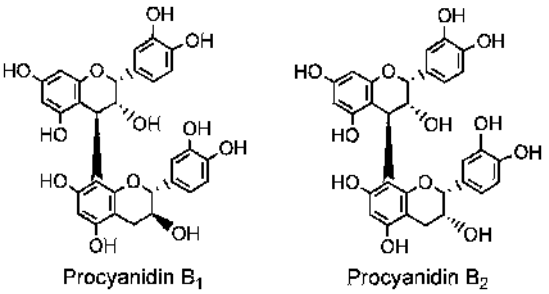


Figure 1.12 Procyanidin dimers occurring in apples.

(Mentha rotundifolia), basil *(Ocimum basilicum),* rosemary *(Rosemarinus officinalis),* sage *(Salvia officinalis)*, and dill (*Anethum graveolens*) [Shan et al., 2005].

Flavan-3-ols can undergo esterification with gallic acid to form catechin gallates, and hydroxylation reactions to form gallocatechins (Fig. 1.10). Gallocatechins such as (−)-epigallocatechin, (−)-epigallocatechin gallate, and (−)-epicatechin gallate are abundant in green tea infusions [Stewart et al., 2005]. During fermentation to produce black tea, these compounds polymerize, giving rise to theaflavins and high-molecular-weight thearubigins (Fig. 1.14) [Crozier et al., 2006c]. Other beverages such as red wine and beer are also rich in flavan-3-ols. Red wines contain oligomeric procyanidins and prodelphinidins, originating mainly from the seeds of red grapes [Auger et al., 2004]. Flavan-3-ols such as (+)-catechin and (−)-epicatechin, and the dimers prodelphinidin B_3 and procyanidin B_3 have been detected in beer [Crozier et al., 2006c].

Flavanones and Chalcones

Flavanones are mainly represented by naringenin, hesperetin, and eriodictyol, while a number of minor compounds, including sakuranetin and isosakuranetin, also occur (Fig. 1.15). Two structural features—the absence of a $\Delta^{2,3}$ double

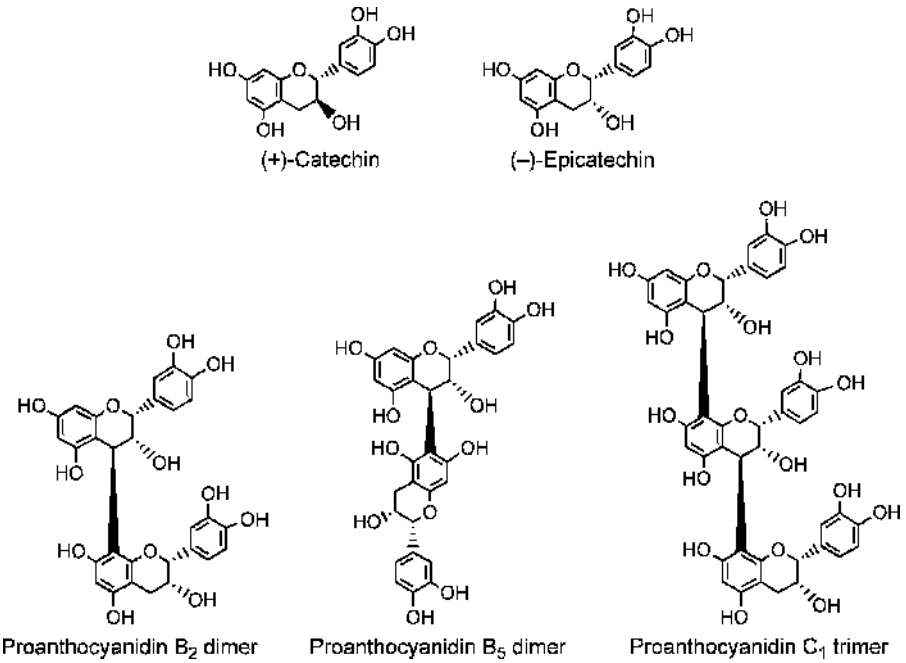


Figure 1.13 Monomeric flavan-3-ols and proanthocyanidin B_2, B_5 dimers, and C_1 trimer found in fresh cocoa beans.

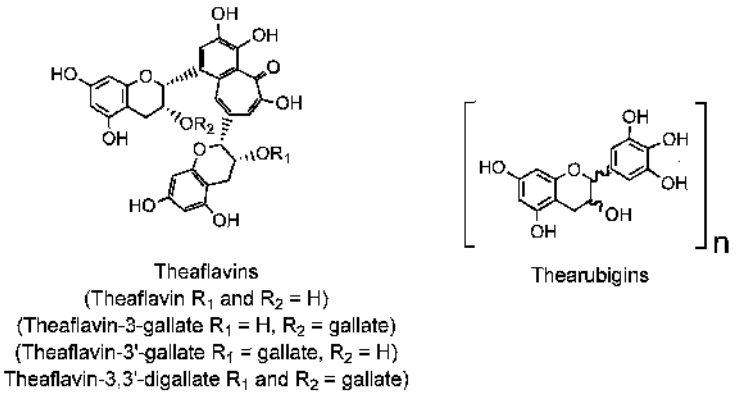


Figure 1.14 Theaflavins and thearubigins present in black tea.

bond and the presence of a chiral center at the carbon-2—characterize flavanones [Iwashina, 2000]. In the majority of naturally occurring flavanones, the C-ring is attached to the B-ring at C_2 in the α configuration.

The flavanone structure is highly reactive and has been reported to undergo hydroxylation, glycosylation, and *O*-methylation reactions. Flavanones are

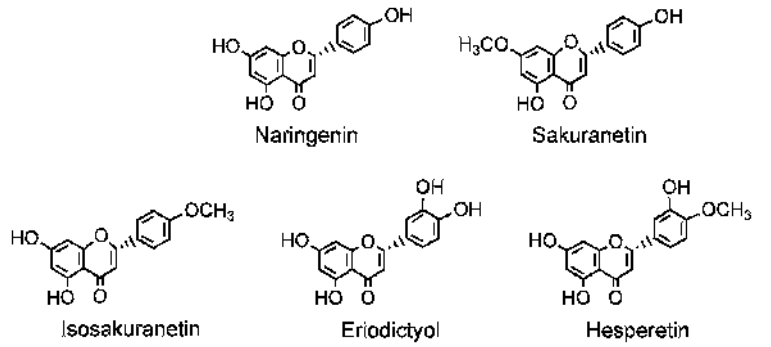


Figure 1.15 Structures of common flavanone aglycones.

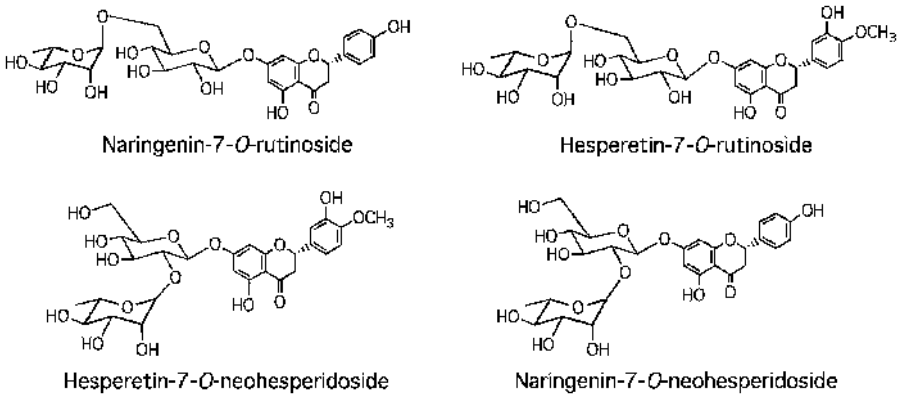


Figure 1.16 Flavanone conjugates in citrus fruit.

exclusively found in citrus fruits in their glycosidic forms. Grapefruit *(Citrus paradisi)* juice contains up to 377 mg L^{-1} of naringin (naringenin-7-*O*-neohesperidoside) and orange juice, 16–84 mg L^{-1} of narirutin (naringenin-7-*O*-rutinoside) [Manach et al., 2004; Tomás-Barberán and Clifford, 2000]. The peel is by far the richest part of citrus fruit in terms of its flavanone content. Substantial quantities of eriodictyol-7-*O*-rutinoside have been reported in lemon (*Citrus limon)* and lime *(Citrus aurantifolia)* [Peterson et al., 2006]. Flavanone rutinosides are tasteless, while neohesperidoside conjugates such as hesperetin-7-*O*-neohesperidoside (neohesperidin) from bitter orange (*Citrus aurantium*) and naringenin-7-*O*-neohesperidoside (naringin) from grapefruit peel (*Citrus paradisi*) have an intensely bitter taste (Fig. 1.16). Naringenin is also found in tomatoes and tomato-based products. Fresh tomatoes, especially the skin, also contain naringenin chalcone, which is converted to naringenin during the manufacture of tomato ketchup [Krause and Galensa, 1992]. Hesperetin-7-*O*-rutinoside has also been detected in kiwi fruit, while

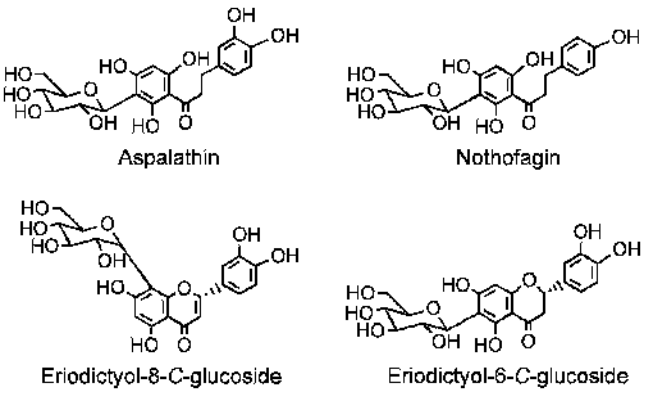


Figure 1.17 Hydroxychalcones occurring in unfermented rooibos tea and flavanone *C*-glycosides that accumulate during fermentation.

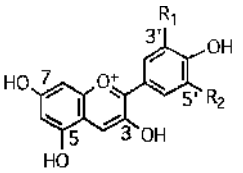
hesperetin-7-*O*-neohesperidoside was reported in bananas (*Musa cavendishii*) [Dégenéve 2004; Kanazawa and Sakakibara, 2000].

As mention earlier, rooibos tea, which is claimed to have a number of medicinal properties [Joubert and Ferreira, 1996; McKay and Blumberg, 2007], contains the flavone *C*-glucosides orientin and isoorientin. It also contains a number of rare dihydrochalcone *C*-glycosides, the main components being 2′,3′,4,4′,6′-pentahydroxy-dihydrochalcone-3-*C*-glucoside (aspalathin) and 2′,4,4′,6′-tetrahydroxy-dihydrochalcone-3-*C*-glucoside (nothofagin). During fermentation aspalathin is oxidized to the flavanone *C*-glycosides eriodictyol-6-*C*-glucoside eriodictyol-8-*C*-glucoside (Fig. 1.17) [Krafczyk and Glomb 2008].

Anthocyanidins/Anthocyanins

Anthocyanins are water-soluble plant pigments and are particularly evident in fruit and flower tissue where they are responsible for a diverse range of red, blue, and purple colors. They occur primarily as glycosides of their respective aglycone anthocyanidin-chromophores (Fig. 1.18), with the sugar moiety typically attached at the 3-position on the C-ring or the 5-position on the A-ring [Prior and Wu, 2006]. They are involved in the protection of plants against excessive light by shading leaf mesophyll cells and also have an important role to play in attracting pollinating insects.

There are about 17 anthocyanidins found in nature, but only 6 — cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin—are ubiquitously distributed and of dietary importance. The variation of anthocyanins are due to: (i) the number and position of hydroxyl and methoxy groups on the basic anthocyanidin skeleton; (ii) the identity, number, and positions at which sugars are attached; and (iii) the extent of sugar acylation and the identity of the



Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 1.18 Structures of major anthocyanins.

acylating agent [Prior and Wu, 2006]. Unlike other subgroups of flavonoids with the same C_6–C_3–C_6 skeleton, anthocyanins have a positive charge in their structure at acidic pH.

The most widespread anthocyanin in fruits is cyanidin-3-glucoside [Kong et al., 2003]. However, malvidin glycosides are the characteristic anthocyanins in red grapes and their derived products [Mazza and Miniati, 1993]. Other anthocyanins that occur in grapes include petunidin-3-*O*-glucoside, malvidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside, malvidin-3-*O*-(6″-*O*-acetyl)glucoside, delphinidin-3-*O*-glucoside, and malvidin-3,5-*O*-diglucoside (Fig. 1.19) [Burns et al., 2001, 2002a].

Purple grape juice, from Concord grapes, a native American cultivar *Vitis labrusca,* which have a thicker skin and larger seeds than grapes of *Vitis vinifera*, is a rich source of more than 20 anthocyanins. The main components being 3-*O*-glucosides and 3,5-*O*-diglucosides of cyanidin, peonidin, delphinidin, and malvidin, delphinidin-3-*O*-(6″-*O*-acetyl)glucoside, delphinidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside, and delphinidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside (Fig. 1.20) [Wang et al., 2003; Mullen et al., 2007]

Anthocyanins occur in abundance in berries where they provide the fruits with their distinctive and vibrant palate of colors. Cranberry (*Vaccinium macrocarpon*), blackberry, and elderberry (*Sambucus nigra*) contain derivatives of only one type of anthocyanin (i.e., cyanidin), while a wide array of anthocyanins is found in blueberry (*Vaccinium corymbosum*) and blackcurrant (*Ribes nigrum*) (Fig. 1.21).

Anthocyanins such as cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside, and peonidin-3-*O*-rutinoside (Fig. 1.22) have also been reported in sweet cherries (*Prunus avium*) and sour cherries *(Prunus cerasus)* [Wu et al., 2004].

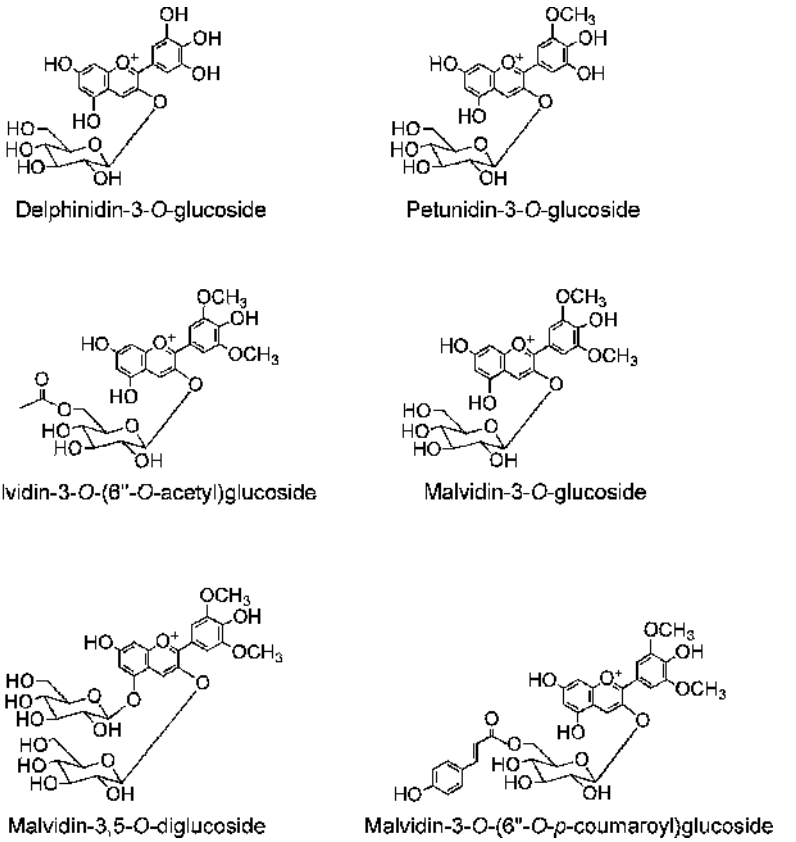


Figure 1.19 Anthocyanins found in red grapes.

Plums *(Prunus domestica)* and peaches are also a rich source of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside [Crozier et al., 2006c].

Red onions contain up to 250 mg kg^{-1} anthocyanins [Clifford, 2000]; among the major components are cyanidin-3-*O*-(6″-malonyl)glucoside and cyanidin-3-*O*-(6″-malonyl)laminaribioside (Fig. 1.23) [Donner et al., 1997]. Cyanidin-3-*O*-(6″-malonyl)glucoside is also a component of the red-leaved Lollo Rosso lettuce [Ferreres et al., 1997], while 3-*O*-glucosides and 3,5-*O*-diglucosides of cyanidin and delphinidin have also been detected in pomegranate (*Punica granatum*) juice [Gil et al., 2000].

Isoflavones

In contrast to most other flavonoids, isoflavones are characterized by having the B-ring attached at C3 rather than the C2 position. They have a

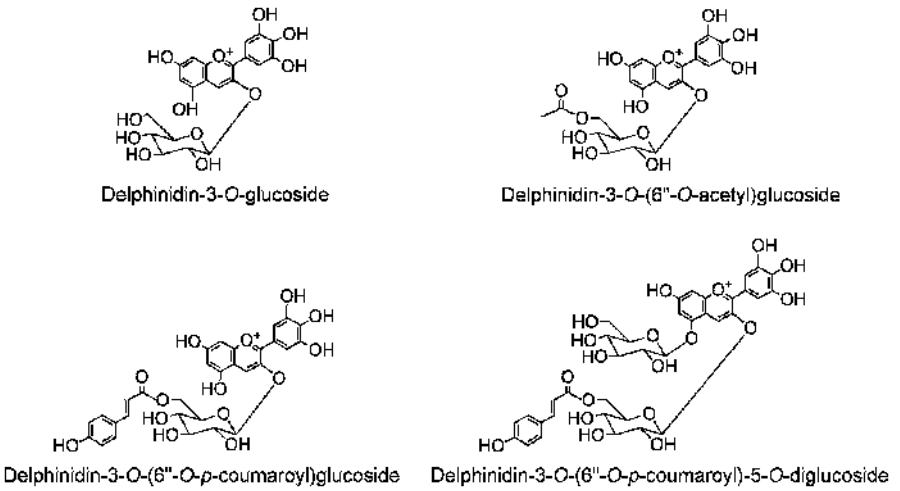


Figure 1.20 Purple grape juice anthocyanins.

very limited distribution in the plant kingdom with substantial quantities being found only in leguminous species [Graham, 1991; Dixon and Steele, 1999]. Isoflavones are known for their estrogenic activity due to their ability to bind to estrogen receptor and have received much attention due to their putative role in the prevention of breast cancer and osteoporosis [Barnes, 2003].

Worldwide, soybeans (*Glycine max*) are almost the sole dietary source of isoflavones. Common isoflavones such as genistein, daidzein, and glycitein (Fig. 1.24), also occur, albeit in low levels, in black beans (*Phaseolus vulgaris*) and green peas (*Pisum sativum*). In plants isoflavones occur predominantly as β-glucosides (genistin, daidzin, glycitin), or as acetyl-β-glucosides and malonyl-β-glucosides, and are therefore polar, water-soluble compounds [Coward et al., 1998]. Isoflavones also undergo various modifications, such as methylation, hydroxylation, or polymerization, and these modifications lead to simple isoflavonoids, such as isoflavanones, isoflavans, and isoflavanols, as well as more complex structures including rotenoids, pterocarpans, and coumestans [Dewick, 1993].

Isoflavones such as diadzein-7-*O*-(6″-*O*-malonyl)glucoside and diadzein-7-*O*-(6″-*O*-acetyl) glucoside (Fig. 1.25) occur in high concentrations in soybean [Barnes et al., 1994]. Formononetin and biochanin A (Fig. 1.24), present as 6″-*O*-malonyl-7-*O*-glucosides, 7-*O*-glucosides, and aglycones, are the most abundant isoflavones in red clover (*Trifolium pretense*), which is one of the ingredients used to extract isoflavones for dietary supplements [Delmonte

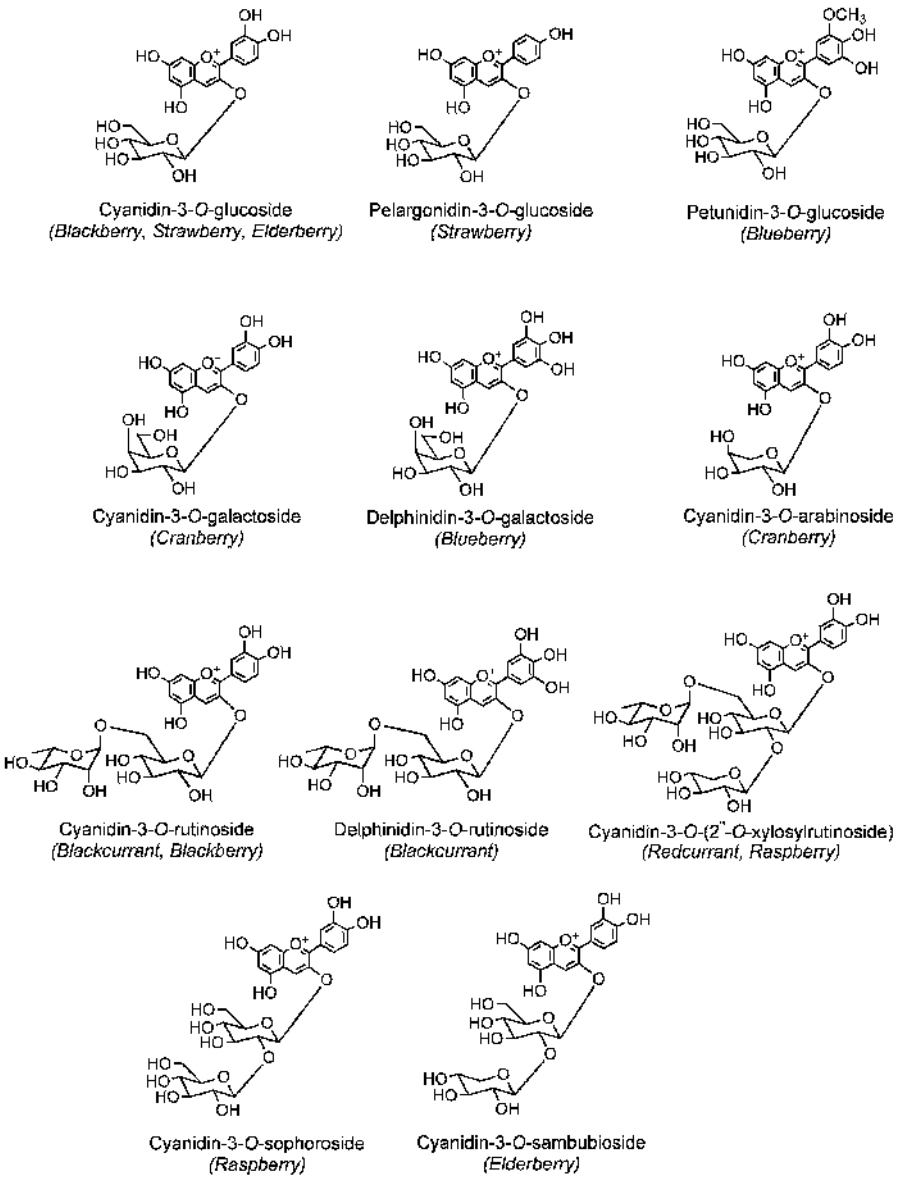


Figure 1.21 Major anthocyanins in berries.

et al., 2006]. Other than soya, *Pueraria lobata* (common name kudzu), a perennial vine native to Japan and China that also grows in the southeastern United States, is another commercial source of isoflavones for dietary supplements. Puerarin (daidzein-7-*C*-glucoside), daidzin (daidzein-7-*O*-glucoside), and daidzein are the main isoflavones in kudzu [Delmonte et al., 2006].

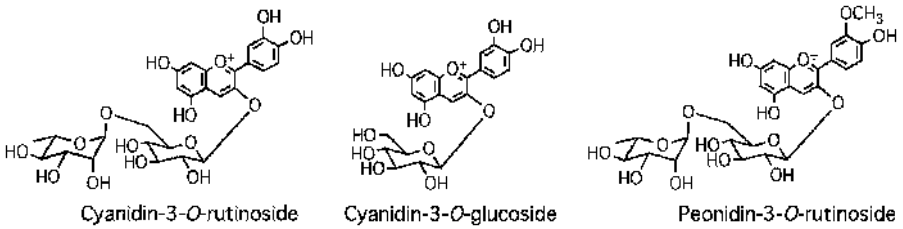


Figure 1.22 Anthocyanins present in cherries.

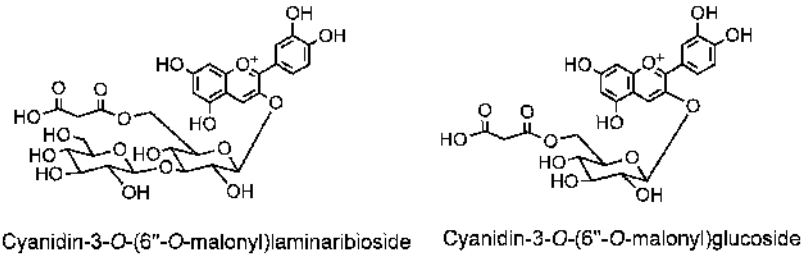


Figure 1.23 Main anthocyanins in red onions.

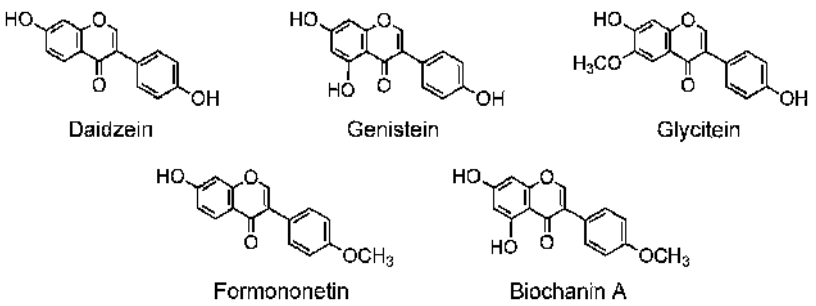


Figure 1.24 Structure of common isoflavone aglycones.

NONFLAVONOID PHENOLIC COMPOUNDS—STRUCTURE AND THEIR DIETARY OCCURRENCE

Phenolics are defined as compounds possessing one or more aromatic rings to which is attached at least one hydroxyl group. Phenolic compounds can be categorized as flavonoids and nonflavonoid phenolic compounds. The main nonflavonoid phenolic compounds of dietary significance are the C_6–C_1

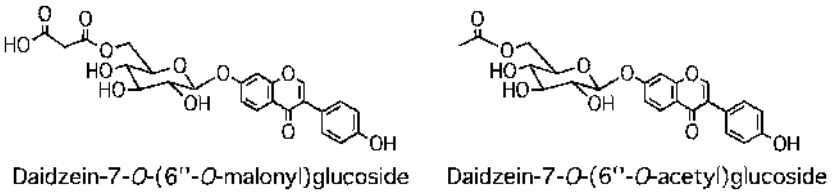


Figure 1.25 Legume isoflavone conjugates.

phenolic acids, the C_6–C_3 hydroxycinammates and their conjugated derivatives, and the polyphenolic C_6–C_2–C_6 stilbenes.

Phenolic Acids

Phenolic acids are also known as hydroxybenzoates, and they are commonly represented by gallic, *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids. Phenolic acids are usually present in the bound form and are typically components of complex structures such as lignins and hydrolyzable tannins. They can also be found as derivatives of sugars and organic acids in plant foods. Gallic acid is the base unit of gallotannins, whereas gallic acid and hexahydroxydiphenoyl moieties are both subunits of the ellagitannins, which are classified as hydrolsable tannins.

Ellagic acid has been reported to be present in berries, particularly raspberries (*Rubus idaeus*), strawberries, and blackberries [Amakura et al., 2000]. However, free ellagic acid is normally present in low levels in berries that more commonly contain ellagitannins, such as sanguiin H-6 and lambertianin C, which release ellagic and gallic acid when treated with acid (Fig. 1.26) [Mullen et al., 2002].

Pomegranate juice is increasing in popularity and some, but far from all, commercial juices/drinks have a high ellagitannin and antioxidant content [Mullen et al., 2008]. Pomegranate juice contains gallagic acid, an analog of ellagic acid containing four gallic acid residues, and punicalagin, the principal monomeric, hydrolysable tannin, in which gallagic acid is bound to glucose (Fig. 1.27) [Gil et al., 2000]. Dates (*Phoenix dactylifera*), one of the oldest cultivated fruit, contain protocatechuic acid, vanillic acid, and syringic acid (Fig. 1.28) [Al-Farsi et al., 2005].

Free and bounded phenolic acids are also found in cereals. Different grains such as sorghum *(Sorghum bicolor)*, millet *(Pennisetum americanum)*, barley *(Hordeum vulgare)*, wheat *(Triticum vulgare)*, rice *(Oryza sativa)*, oat *(Avena sativa)*, and rye *(Secale cereale*) contain diverse phenolic acids such as gallic, protocatechuic, *p*-hydroxybenzoic, gentisic, salicylic, vanillic, and syringic acids [see Dykes and Rooney, 2007]. Hydroxybenzoic acid glycosides are also characteristic of some herbs and spices [Tomás-Barberán and Clifford, 2000]. After hydrolysis, protocatechuic acid is the dominant hydroxybenzoate in

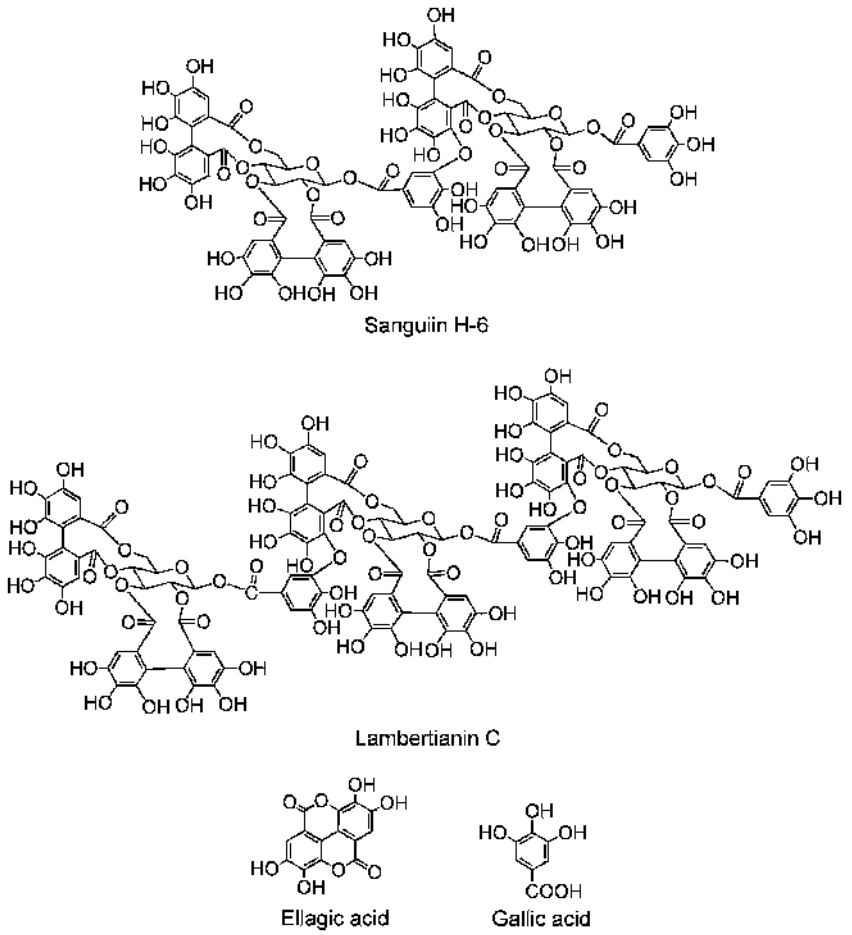


Figure 1.26 Ellagitannins and trace amounts of gallic acid and ellagic acid occur in raspberries.

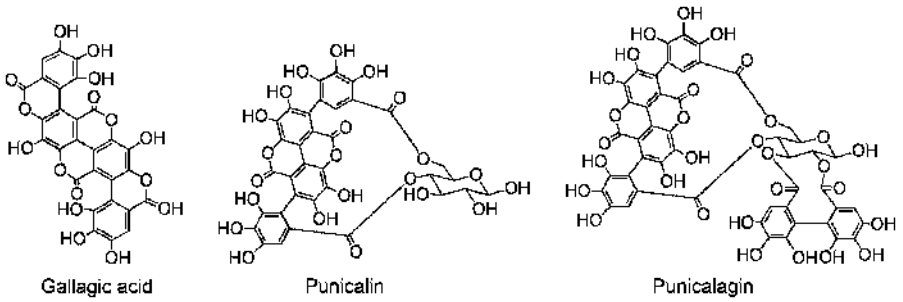


Figure 1.27 Ellagitannins detected in pomegranate juice.

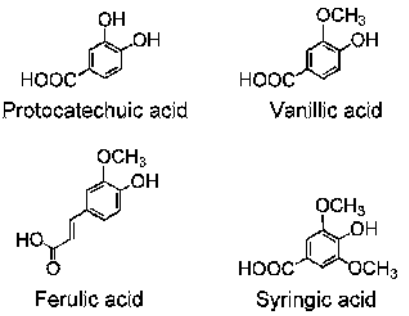


Figure 1.28 Phenolic acids in dates.

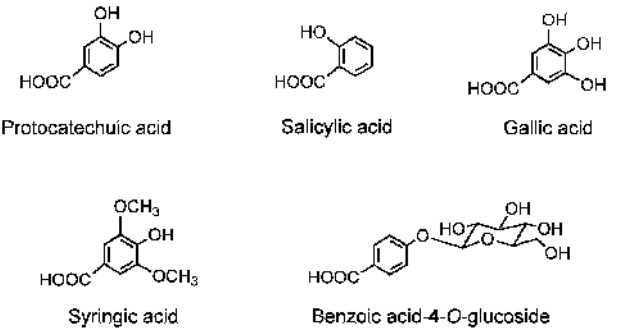


Figure 1.29 Some hydroxybenzoates derivative compounds found in herbs.

cinnamon bark accompanied by salicylic and syringic acid. Gallic acid occurs in clove buds (*Eugenia caryophyllata* Thunb.) along with protocatechuic and syringic acid (Fig. 1.29). Benzoic acid-4-*O*-glucoside is the common phenolic acid in many herbs such as in anise (*Pimpinella anisum*), star anise (*Illicium verum*), dill (*Anethum graveolens*), fennel (*Foeniculum vulgare*), caraway (*Carum carvi*), and parsley (*Petroselinum crispum)* (Fig. 1.25) [Crozier et al., 2006c].

Hydroxycinammates

The most common hydroxycinnamates, *p*-coumaric, caffeic, and ferulic acids, frequently accumulate as their respective tartrate esters, coutaric, caftaric, and fertaric acids. Quinic acid conjugates of caffeic acid, namely 3-, 4-, and 5-*O*-caffeoylquinic acid, which belong to a family of hydroxycinnmate-quinic acid conjugates known as chlorogenic acids, are commonly found in fruits and vegetables. Fruits such as apples and dates (*Phoenix dactylifera*) are a good source of diverse phenolic compounds. 5-*O*-Caffeoylquinic acid, 4-*O*-*p*-coumaroylquinic acid, and caffeic acid have been detected in apples [Clifford et al., 2003; Kahle et al., 2005], while dates contain ferulic acid [Crozier et al., 2006c].

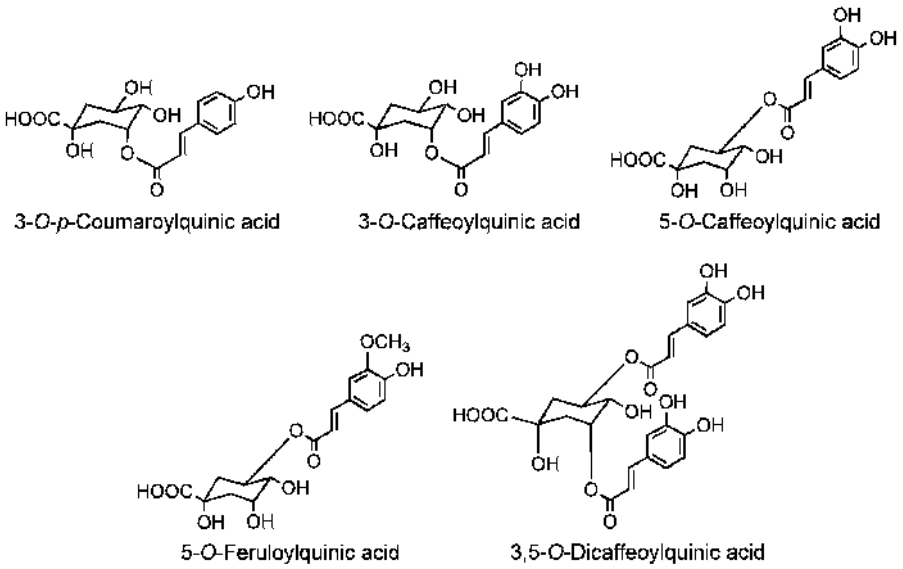


Figure 1.30 Chlorogenic acids in carrots.

Carrots *(Duacus carota)* contain a range of chlorogenic acids including 3-*O*- and 5-*O*-caffeoylquinic acids, 3-*O*-*p*-coumaroylquinic acid, 5-*O*-feruloylquinic acid, and 3,5-*O*-dicaffeoylquinic acids (Fig. 1.30). These chlorogenic acids are found in almost all varieties of carrot with a 10-fold higher level of 5-*O*-caffeoylquinic acid in purple carrots [Alasalvar et al., 2001].

The red-leaved lettuce Lollo Rosso contains the hydroxycinammates caffeoyltartaric acid, dicaffeoyltartaric acid, 5-*O*-caffeoylquinic acid, and 3,5-*O*-dicaffeoylquinic acid (Fig. 1.31) [Ferreres et al., 1997]. 5-*O*-Caffeoylquinic acid has also been detected in tomatoes [Paganga et al., 1999].

Green coffee beans *(Coffea arabica)* are one of the richest dietary sources of chlorogenic acids. 5-*O*-Caffeoylquinic acid is the dominant chlorogenic acid accounting for 50% of the total. This is followed by 3-*O*- and 4-*O*-caffeoylquinic acid, the three analogous feruloylquinic acids and 3,4-*O*-, 3,5-*O*- and 4,5-*O*-dicaffeoylquinic acids (Fig. 1.32) [Clifford, 1999]. Levels decline ca. 80% during the roasting of coffee beans, but sizable amounts with substantial antioxidant activity are still found in the typical cup of coffee.

There is also dietary interest in the curcuminoids, which are cinnamoylmethanes (diaryl-heptenoids), are characteristic of ginger (*Zingiber officinale*), cardamon (*Elettara cardamonum*), and turmeric (*Curcuma longa*). Curcumin is a diferuloylmethane. Three curcuminoids, curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Fig. 1.33), are the principal components in tumeric, and all three impart the yellow pigmentation that is a hallmark of the spice [Jayaprakasha et al., 2005].

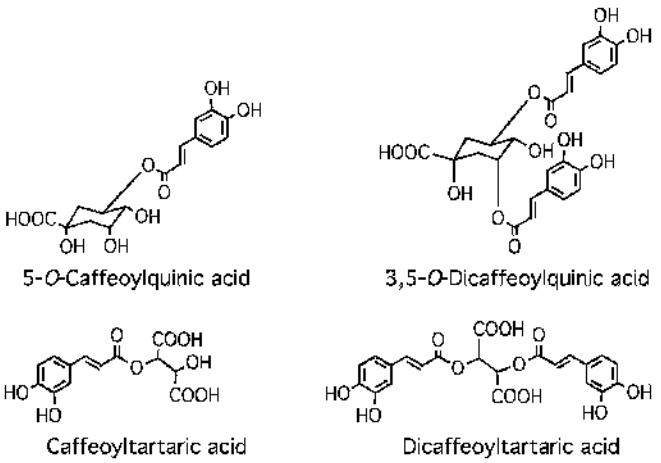


Figure 1.31 Hydroxycinammates in Lollo Rosso lettuce.

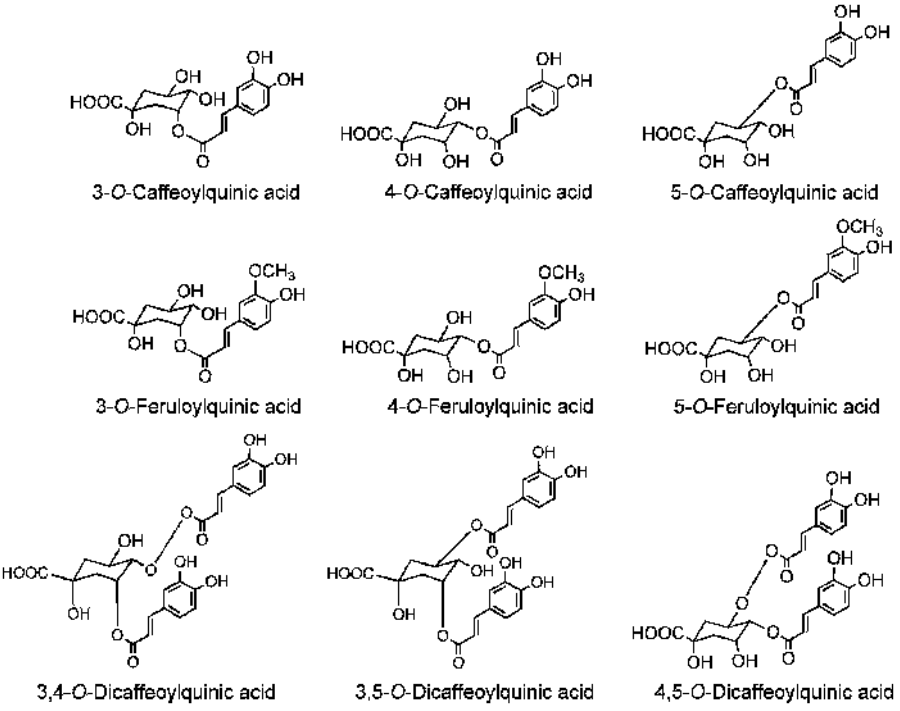
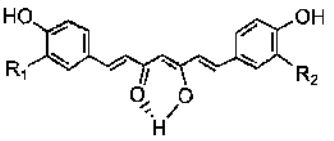


Figure 1.32 Main chlorogenic acids in green coffee beans.



Curcumin (R_1, R_2 = OCH_3)
Demethoxycurcumin (R_1 = H, R_2 = OCH_3)
Bisdemethoxycurcumin (R_1, R_2 = H)

Figure 1.33 Curcuminoids accumulate in the rhizomes of turmeric.

Stilbenes

Members of the stilbene family have the C_6–C_2–C_6 structure and are phytoalexins produced by plants in response to disease, injury, and stress (Fig. 1.34) [Langcake and Pryce, 1977]. The main dietary source of stilbenes is resveratrol (3,5,4′-trihdroxystilbene) from red wine and peanuts (*Arachis hypogaea*) [Burns et al., 2002b] with lesser amounts found in berries, red cabbage (*Brassica oleraceae*), spinach, and certain herbs. Resveratrol occurs as *cis* and *trans* isomers and *trans*-resveratrol and *trans*-resveratrol-3-*O*-glucoside (*trans*-piceid) have recently been detected in pistachio nuts (*Pistacia vera* L.) [Grippi et al., 2008].

The woody root of the noxious weed *Polygonum cuspidatum* (Japanese knotweed or Mexican bamboo) has been shown to contain very high levels of *trans*-resveratrol and its glucosides with concentrations of up to 377 mg 100 g^{-1} dry weight [Vastano et al., 2000]. As well as resveratrol, Brazilian red wines have been shown to contain *trans*-piceatannol (3,3′,4,5′-tetrahydroxystilbene) and *trans*-astringin, its 3-*O*-glucoside [Vitrac et al., 2005] *trans*-resveratrol is transformed by *Botrytis cinerea*, a fungal grapevine pathogen, to pallidol and resveratrol *trans*-dehydrodimer, and both these compounds have been detected in grape cell cultures along with the 11-*O*- and 11′-*O*-glucosides of resveratrol *trans*-dehydrodimer [Waffo-Téguo et al., 2001]. Viniferins are another family of oxidized resveratrol dimers [Langcake and Pryce, 1977], and δ-viniferin and smaller amounts of its isomer δ-viniferin have been detected in *Vitis vinifera* leaves infected with *Plasmopara viticola* (downy mildew) [Pezet et al., 2003].

trans-Resveratrol that has gained significant worldwide attention because of its ability to inhibit or retard a wide variety of animal diseases [Baur and Sinclair, 2006] that include cardiovascular disease [Bradamante et al., 2004] and cancer [Jang et al., 1997]. It has also been reported to increase stress resistance and enhance longevity [Baur et al., 2006; Valenzano et al., 2006]. The protective effects of red wine consumption are regularly attributed to resveratrol [Kaeberlein and Rabinovitch, 2006]. However, this is highly unlikely as the levels of resveratrol in red wines are low, and for humans to ingest the quantity of resveratrol that affords protective effects in animals they would have to drink in excess of 100 L of red wine per day [Corder et al., 2003].

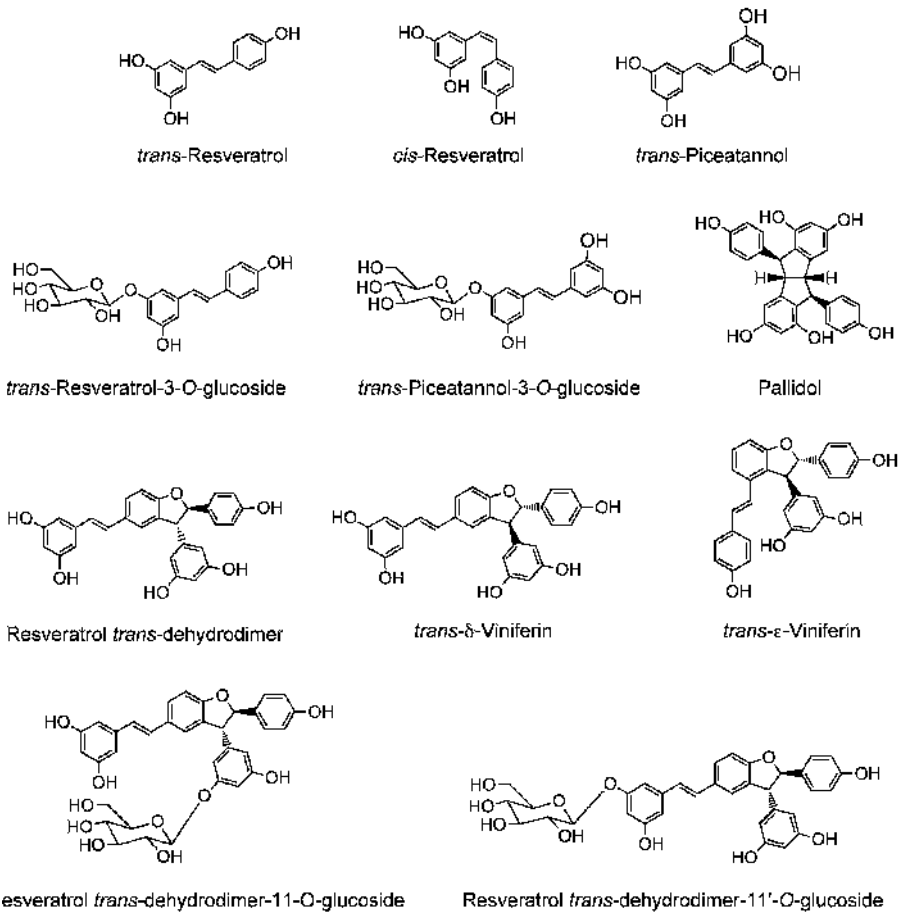


Figure 1.34 Structures of the *trans*- and *cis*-resveratrol and other stilbenes.

OVERVIEW OF FLAVONOID AND PHENOLIC BIOSYNTHETIC PATHWAYS

The biosynthesis of flavonoids, stilbenes, hydroxycinnamates, and phenolic acids involves a complex network of routes based principally on the shikimate, phenylpropanoid, and flavonoid pathways (Figs. 1.35 and 1.36). These biosynthetic pathways constitute a complex biological regulatory network that has evolved in vascular plants during their successful transition on land and that ultimately is essential for their growth, development, and survival [Costa et al., 2003].

From the 1970s to the 1990s, there was a rapid and substantial progress in the research on the phenylpropanoid pathway, focusing mainly on a broad understanding of the metabolic pathway [Hahlbrock and Grisebach, 1975; Ebel and Hahlbrock, 1982; Heller and Forkmann, 1988]. However, in more recent

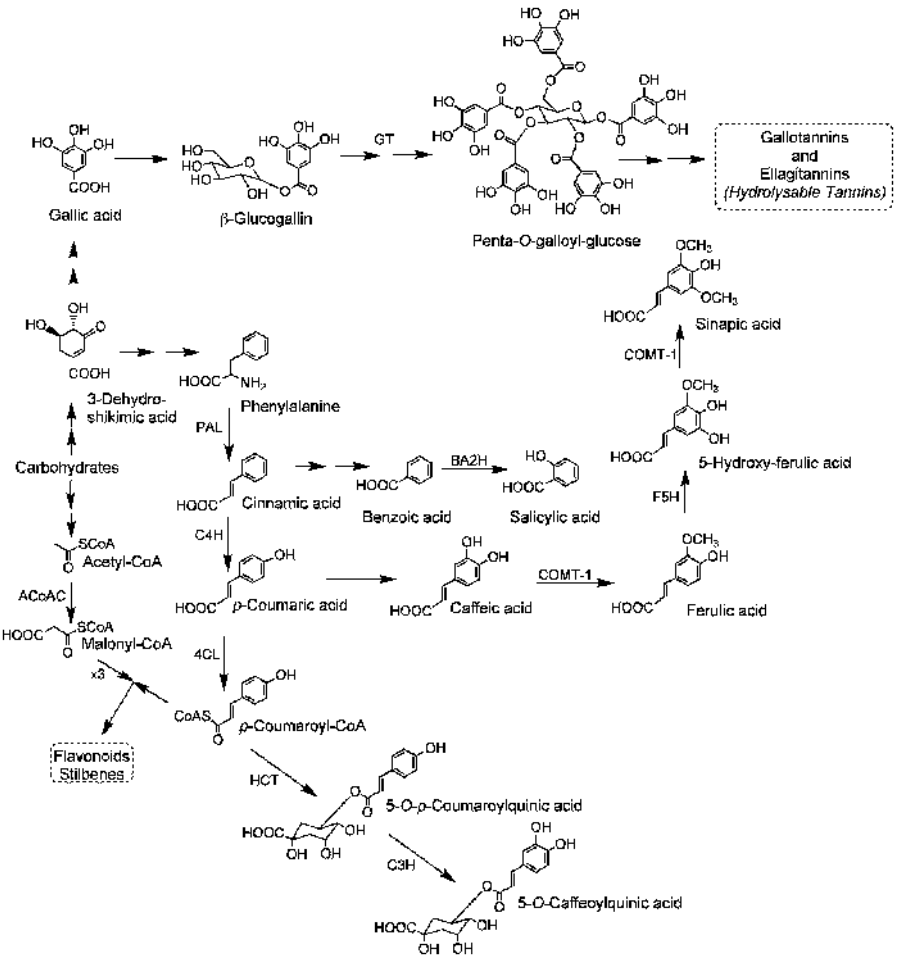


Figure 1.35 Schematic diagram of the phenolic biosynthetic pathway accompanied by the key enzymes involved. Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; BA2H, benzoic acid 2-hydroxylase; C4H, cinnamate 4-hydroxylase; COMT-1, caffeic/5-hydroxyferulic acid *O*-methyltransferase; 4CL, *p*-coumarate:CoA ligase; F5H, ferulate 5-hydroxylase; GT, galloyltransferase; ACoAC, acetylCoA carboxylase.

years, much effort has been directed at elucidating the phenylpropanoid biosynthetic pathway from a biochemical and a molecular point of view by using approaches such as transposon tagging, positional cloning, co-immunoprecipitation, affinity chromatography, and two-hybrid experiments mainly utilizing *Arabidopsis thaliana* as a test system [Winkel-Shirley, 2001]. New information is also emerging regarding the regulation of the phenylpropanoid pathway. In the last few years, a great deal has been learned from studies in a variety of plant species, primarily about transcriptional regulation. A number of these studies were carried out using flavonoid mutants generated by

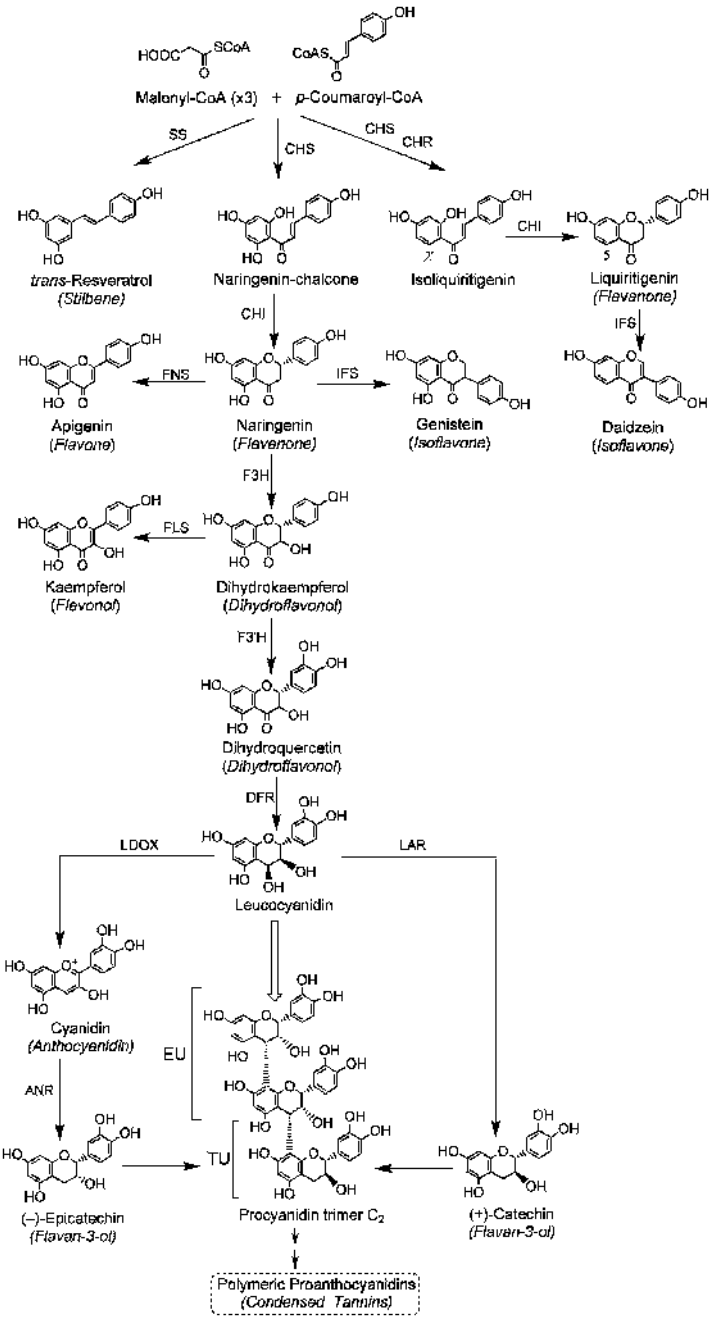


Figure 1.36 Schematic diagram of the stilbene and flavonoid biosynthetic pathway. Enzyme abbreviations: SS, stilbene synthase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; IFS, isoflavone synthase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; F3′H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin 4-reductase; LDOX, leucocyanidin deoxygenase; ANR, anthocyanidin reductase; EU, extension units; TU, terminal unit.

activation tagging [Borevitz et al., 2000; Mathews et al., 2003]. Characterization of flavonoid mutants in a variety of plant species has led to the identification of a number of novel regulatory proteins that are beginning to fill in the void between signals that induce the pathway and well-known flavonoid regulators such as the myb domain and basic helix–loop–helix transcription factors [Winkel-Shirley, 2001]. In addition, increasing evidence is being generated demonstrating that as well as inducing the phenylpropanoid pathway, these transcriptional regulators also influence the modification, transport, and deposition of metabolites in the vacuole [Broun, 2004].

In addition to the molecular techniques, technical advances both in chromatographic techniques and in identification tools, particularly the diverse forms of mass spectrometry, has allowed successful challenges to the separation and characterization of compounds of increasing complexity, poor stability, and low abundance [Whiting, 2001]. Information generated utilizing these techniques has resulted in characterization of a plethora of complex secondary metabolites that, in conjunction with the characterization of the enzymatic steps, has permitted the complete or partial elucidation of the flavonoid and the phenolic pathways present in many plants (Figs. 1.35 and 1.36).

Comprehensive information on the network of pathways responsible for the synthesis of numerous secondary metabolites can be found in Chapter 21. In addition, information on this aspect is also available in articles by Shimada et al. [2003], Toshiaki [2003], Tanner et al. [2003], Boatright et al. [2004], Hoffmann et al. [2004], Dixon et al. [2005], Niemetz and Gross [2005], Xie and Dixon [2005], and Ferrer et al. [2008]. Nonetheless, the complete dissection of phenolic metabolic pathway is far from being complete. For example, recent reports underline that important questions still remain to be answered in the field of protoanthocyanidins and tannins [Xie and Dixon, 2005], and that the exact nature of the biosynthetic pathway(s) leading to lignin monomers has not been fully elucidated [Boudet, 2007].

An example of the phenolic pathway, which produces secondary metabolites that have health benefiting effects, is the biosynthesis of curcuminoids. The initial investigations into the biosynthesis of curcuminoids were carried over 25 years ago [Denniff and Whiting, 1976; Macleod and Whiting, 1979; Denniff et al., 1980], although little has been done subsequently to elucidate fully the routes involved. The proposed biosynthetic pathway is presented in Figure 1.37. The curcuminoids are thought to be formed from condensation of two molecules of *p*-coumaroyl-CoA with one molecule of malonyl-CoA via the action of possibly a polyketide synthase. The resulting bisdemethoxycurcumin would then be transformed through demethoxycurcumin into curcumin via two sequential rounds of hydroxylation followed by *O*-methylation. Alternatively, it is possible that the curcuminoid synthase enzyme may utilize the CoA esters of both *p*-coumaric acid and ferulic acid as substrates [Ramirez-Ahumada et al., 2006].

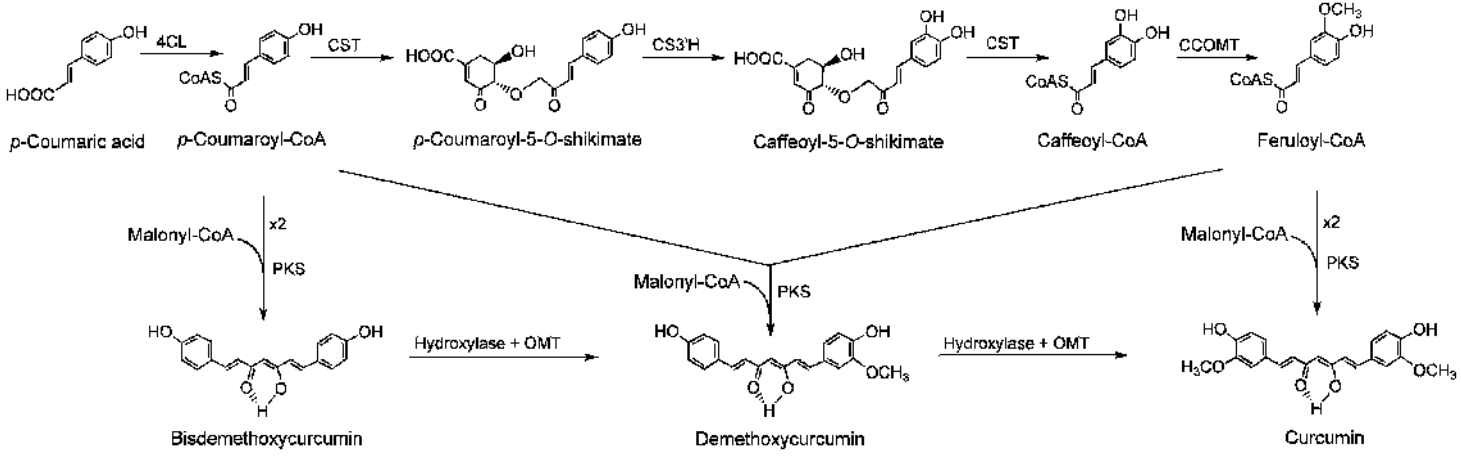


Figure 1.37 Proposed biosynthetic pathway of curcuminoids in tumeric. Enzyme abbreviations: CCOMT, caffeoyl-CoA *O*-methyltransferase; 4CL, 4-coumarate:CoA ligase; CST, shikimate transferase; CS3′H, *p*-coumaroyl 5-*O*-shikimate 3′-hydroxylase; OMT, *O*-methyltransferase; PKS, polyketide synthase. [Adapted from Ramirez-Ahumada et al. (2006)]

OPTIMIZATION OF THE FLAVONOID AND PHENOLIC PROFILES IN CROP PLANTS

The recent increase in consumer awareness on the health benefits of dietary phytochemicals accompanied by the rapid progress in the field of molecular biology have provided the means and incentive to enhance the functional value of plant material. This enhancement of health-promoting compounds is being tackled using a variety of approaches, which are discussed in the ensuing sections.

Agronomical and Physiological Modifications

Abiotic and biotic stresses are known to induce the accumulation of phenolic and flavonoid compounds in many higher plants. As sessile organisms, plants rely on the accumulation of such chemicals for defense, protection, cell-to-cell signaling, and other stress adaptations. As such, wild-type berries from harsh environmental growing regimes were found to be among the most biologically potent in terms of antioxidant content compared to their commercially grown counterparts [Deighton et al., 2000; Reyes-Carmona et al., 2005]. Hence agronomic manipulation by the application of mild stress at defined points during the growing season may have generic effects on phenolic and flavonoid accumulation. This is why deliberate stress on the target plants that are specifically cultivated for their health-benefiting compounds is now becoming a popular research strategy. Environmental deprivation, such as exposure of plants to low temperatures, as well as heavy metals, wounding, desiccation, and high irradiance are typical triggers that switch on a biochemical pathway cascade leading to increased secondary product accumulation [Lila, 2006].

High temperature is known to reduce and low temperature to enhance anthocyanin synthesis [Saure, 1990; Leng et al., 2000]. This was observed in Starkrimson and Golden Delicious apples where there was rapid anthocyanin accumulation in the skin in cooler habitats compared to warmer climates [Li et al., 2004]. Similarly, when grapes were grown under low night temperatures, anthocyanin synthesis, L-phenylalanine ammonia-lyase activity and *chalcone synthase 3* transcript levels were all markedly higher [Mori et al., 2005]. Apart from temperature, it has long been known that UV radiation, specifically the UV-B, can up-regulate key genes such as the *phenylalaline ammonia-lyase* [Kuhn et al., 1984] and *chalcone synthase* [Christie and Jenkins, 1996]. This up-regulation of the genes in the phenylpropanoid pathway is part of the plant's ability to offset the absorption of excessive UV radiation by accumulating UV-filtering secondary metabolites such as flavonols [Cuadra et al., 1997] and anthocyanins [Oelmüller and Mohr, 1985]. Similarly, investigations with apples have shown that covering the orchard floor with metallic foil in an effort to reflect increased light into the canopy resulted in an increase in anthocyanin concentration in the skin of the fruit [Ju et al., 1999].

Agronomical manipulation has also been employed to improve the phytochemical content in plants. In a number of investigations, anthocyanins were observed to accumulate in plants deficient in nutrients such as phosphorus and nitrogen [Cobbina and Miller, 1987; Hodges and Nozzolillo, 1996; Close et al., 2000]. In another study, it was found that production of the isoflavones, daidzein and genistein, could be modulated by changing the ammonia/nitrate ratio. In bean plants cultured on phosphorus-deficient media, higher concentrations of anthocyanins were found in the leaves, and this may play a role in protecting the plant against oxidative stress [Juszczuk et al., 2004]. Substantial variability in the levels of caffeoylquinic, sinapic, and ferulic acid derivates in eight broccoli *(Brassica oleracea)* cultivars grown under different agronomic conditions has also been reported by Vallejo et al. [2003]. In tomato, in addition to increasing flavonoid content, nitrogen stress also produces differential effects on expression of genes encoding anthocyanin biosynthetic enzymes [Bongue-Bartelsman and Phillips, 1995].

Another popular way to enhance the production of bioactive compounds is through elicitation. In this process, target plants are deliberately challenged with chemicals that trigger physiological responses that mimic the parallel environmental challenges. This in turn results in the accumulation of specific phytochemicals. This may include abiotic elicitors, such as metal ions and inorganic compounds, and biotic elicitors including fungi, bacteria, viruses or herbivores, plant cell wall components, as well as chemicals that are released by plants when they are subjected to pathogen or herbivore attack [Zhao et al., 2005]. Two well-known elicitors are salicylic acid and jasmonic acid, and these compounds have frequently been added to cell cultures to induce the accumulation of compounds with potential health benefits including flavonoids and phenylpropanoids [Zhao et al., 2005]. Other natural elicitors such as fish protein hydrolysates and lactoferrin have also been used. These elicitors stimulate the phenylpropanoid pathway in mung bean sprouts, probably through the pentose phosphate and shikimate pathways. This resulted in significant improvement of the phenolic content and antioxidant and antimicrobial properties of mung bean sprouts [Randhir et al., 2004]. In another study, preharvest treatment with benzothiadiazole increased *trans*-resveratol and anthocyanin levels in grapevine [Iriti et al., 2004]. Further investigations revealed that five monoglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin, accompanied by the corresponding acetylated and *p*-coumaroyl derivatives, were enhanced by benzothiadiazole treatment [Fumagalli et al., 2006]. Plant hormones can also affect the phenolic and flavonoid content of plants as revealed in a number studies [Jeong et al., 2004; Peppi et al., 2007; Kondo and Inoue, 1997].

Genetic Manipulation—Conventional Breeding and Genetic Engineering

The roles that dietary flavonoids and phenolic compounds play in promoting human health have stimulated intense interest in genetically manipulating their

accumulation in plants, through either conventional or molecular means. Genetic variability is one of the key factors in determining the amount of functional metabolites that accumulate in plants. Conventional breeding and cross-varietal screening tests have repeatedly revealed that genotypes within a plant species can have widely divergent levels of phytochemicals. For example, a number of different cultivars and species of blueberries exhibited varying levels of anthocyanins and proanthocyanidins, which were tightly correlated with the antioxidant capacity of fruit extracts [Kalt et al., 2001]. Enhanced lycopene and flavonoid levels have also been reported in some varieties of tomato and these lines are currently the preferred hosts for further genetic manipulation through conventional and molecular breeding [Long et al., 2006]. However, it is important to note that even though these genotypes are known to be capable of accumulating enhanced levels of specific phytochemicals, the final content is dependent on the selective pressure imposed by the environment. Genes are not always expressed, but instead can be triggered by environmental signals that may ultimately become the principal determinant for the accumulation of key secondary products. It is, therefore, important to note that gene–environment interactions are inherent as plants grow, which makes it difficult to predict phytochemical responses based on heritable traits and distinguish them from environmental influences [Lila, 2007]. The influences of the genome and the environment can be resolved through rigorous comparative tests of the identical plant genotypes in multiple environments followed by gene sequencing and phytochemical profiling of selected candidate plants [Taylor et al., 2002, Lim et al., 2005; Mpofu et al., 2006].

Due to the rapid speed at which knowledge of the genetic control of plant secondary metabolism has grown, it is hypothesized that over the next 25 years the most significant changes in the productivity and quality of crops will come about by applying genetic engineering tools. Normally, genetic engineering of a secondary metabolic pathway aims to increase the quantity of an individual or a group of specific compounds in the normal producing plant species or to transfer a pathway, or part of a pathway, to other plant species [Verpoorte and Memelink, 2002]. To increase the production of the compound(s) of interest, two general approaches have been followed. First, the structural genes encoding enzymes that participate directly in the formation of the compound of interest can be overexpressed. This is to enable the genetically modified plant to overcome specific rate-limiting steps in the pathway, to shut down competitive pathways, and to decrease catabolism of the product of interest. Secondly, attempts have been made to change the expression of regulatory genes that control the expression of the structural genes [Verpoorte and Memelink, 2002]. Regulatory genes control the expression of structural genes though the production of proteins called transcriptional factors. Transcriptional factors are believed to play an important role in regulating secondary metabolism pathways. Since transcriptional factors are able to control multiple steps within a pathway, they are potentially more powerful than structural genes that

control only a single step, when attempting to manipulate metabolic pathways in plants [Broun, 2004].

The best studied route at the genetic level is the flavonoid biosynthesis pathway leading to the formation of anthocyanins. Most of the structural and several regulatory genes involved in this pathway have now been cloned. The use of structural genes in metabolic engineering was used by Jung et al. [2000] who introduced the *isofavone synthase* gene into the nonlegume arabidopsis *(Arabidopsis thaliana)* in order to convert naringenin, which is ubiquitous in higher plants, to the isoflavone genistein. In another study, chalcone isomerase (CHI), the key enzyme to increased flavonol production, was overexpressed in tomato. Results revealed a 78-fold increase of flavonol levels in the skin of tomatoes [Muir et al., 2001]. To date, several *leucoanthocyanidin reductase (LAR)* and/or *anthocyanidin reductase (ANR)* genes have been cloned and characterized from different plant species [Tanner et al., 2003; Xie et al., 2003; Bogs et al., 2005; Pang et al., 2007; Paolocci et al., 2007]. When the *ANR* gene was overexpressed in barrel clover (*Medicago truncatula)* and tobacco *(Nicotiana tabacum)*, accumulation of proanthocyanidins was observed with a corresponding reduction of anthocyanin levels [Bogs et al., 2005; Xie et al., 2006]. Beyond the modified expression of one gene, more sophisticated strategies have been adopted such as the simultaneous introduction by cotransformation of a sense and an antisense construct to simultaneously up-regulate one enzyme and down-regulate another. For example, aspen trees (*Populus tremuloides*), expressing both antisense 4-coumarate-CoA ligase and sense coniferaldehyde 5-hydroxylase, had 52% reduced lignin content and a 64% higher syringyl/guaiacyl ratio [Li et al., 2003].

To control expression of structural genes, regulatory genes such as *LC*, *C1*, *MYB*, *HLH*, and the like are used. In an investigation where *LC* and *C1* genes were overexpressed in tomatoes, an increase in flavonols in the flesh of the fruit was observed. The total flavonol content of these overexpressed ripe transgenic tomatoes were ca. 20-fold higher than that of the controls where flavonol production occurred only in the skin [Bovy et al., 2002; Le Gall et al., 2003]. Similarly, when the *LC* gene was introduced into apple, both anthocyanin and proanthocyanidin accumulation was observed, and this was accompanied by induction of both the anthocyanin pathway genes and proanthocyanidin-specific pathway genes such as *LAR* and *ANR* [Li et al., 2007]. MYB and bHLH transcription factor is envisaged to be central to the control of proanthocyanidin biosynthesis. When two MYB transcription factors, AtTT2 and PAP1, together with one bHLH transcription factor, were introduced into Arabidopsis, the *ANR* gene was induced, which resulted in anthocyanin and proanthocyanidin accumulation [Sharma and Dixon, 2006].

Engineering of novel natural products by enzymatic modifications of core skeletons is another method where molecular tools are used to produce a range of novel products with enhanced/modified bioactivity. This is mainly carried out because the distribution of many of these compounds are either restricted or they accumulate at low levels, which is insufficient for large-scale extraction

[Tian et al., 2008]. Modifications to the ring structure and/or acyclic side structure of aglycones through the use of specific modification enzymes can be carried out to cause oxidation, *C*- or *O*-methylation, *C*- or *O*-glycosylation, and *C*- or *O*-prenylation to produce a range of phytochemical derivatives. Genes encoding some of the modification enzymes have been cloned and characterized in recent years using genetic, genomic, and biochemical approaches. The use of this method to produce a range of novel isoflavonoids has been reviewed in detail by Tian et al. [2008].

One of the major drawbacks of targeted induced modifications of key enzymes of phenolic and flavonoid metabolism, aiming to increase or decrease a specific phytochemical, is the observation of unexpected effects. This may be due partly to the effect of combined outcomes of a complex interplay of various metabolic pathways and variation between plant species. Other than the internetworking and regulation of endogenous pathways, the final result of metabolic engineering is also dependent on a number of factors such as the approach used, the encoded function of the introduced gene, and the type of promoter [Lessard et al., 2001; Broun, 2004]. Besseau et al. [2007] recently exhibited how network complexity and pathway interactions observed between different branches of phenolic biosynthesis resulted in an unexpected array of events. In arabidopsis plants silenced for hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) expression lignin repression lead to an increase in chalcone synthase activity, which resulted in a metabolic flux into flavonoid pathway. Correlated with this was a prominant reduction in plant growth. When this process was reversed through the repression of *chalcone synthase* expression in HCT-silenced plants, the wild-type plant growth was restored. The results suggest that the dwarf phenotype may be due to an indirect effect of ectopic flavonoid accumulation altering auxin transport [Boudet, 2007].

In several cases, studies on overexpression of genes have resulted in the production of unexpected products, as revealed by Bovy et al. [2002] when C1 and R transcriptional factors were cloned into tomato. Although several flavonoid genes were induced, they were not sufficient to induce flavonoid-3′,5′-hydroxylase activity to enhance anthocyanin production by the fruit. Alternatively, the introduction of a new branch point into an existing pathway may interfere with endogenous flavonoid or phenolic biosynthesis and/or the transgenic enzyme may fail to compete with the native enzymes for the common substrate. This could, in part, be due to compartmentalization and metabolic channeling of substrates that may further complicate metabolic engineering strategies by limiting the access of substrates to introduced enzymes. This occurred when soybean-derived isoflavone synthase (IFS) was introduced into arabidopsis and tomato [Jaganath, 2005]. The nonleguminous species did not synthesize genistein despite expression of the IFS protein.

Based on these studies on biosynthetic pathways and metabolic engineering, it can be envisaged that once the plant cell factory has been assembled, the important determinants controlling the fluxes through the pathways are the

posttranslational regulation of enzyme activity, enzyme, and metabolite compartmentation and transport [Verpoorte and Memelink, 2002].

FUTURE TRENDS AND PROSPECTS

Phenolic compounds and flavonoids are a unique category of plant phytochemicals especially in terms of their vast po ential health-benefiting properties. They represent the most abundant and the most widely represented class of plant natural products. A substantial amount of research has been carried out over the past two decades yet large information gaps still exist. For example, the inventory of these compounds is still incomplete, although there is continuous effort to provide new structures. In addition the dissection of the metabolic pathways for certain phenolic compounds remains to be resolved. Recent reports underline that important questions that still need to be answered in the field of proanthocyanidin and tannin biosynthesis [Xie and Dixon, 2005], and even the exact nature of the biosynthetic pathway(s) leading to lignin monomers is not fully elucidated.

Phenolic compounds and flavonoids are widely present in plant foods, and research in the last decade has increased dramatically. Two major objectives have been targeted: (i) to rationalize the potential health benefits of these phytochemicals and (ii) to redesign plants to enhance their production. The existing literature on biological activities suggests that polyphenol-rich products such as soya, teas, berries, red wine, and cocoa products may have positive effects on human health, especially by reducing the incidence of cardiovascular diseases and some types of cancer. Additional research is needed to substantiate whether it is a specific class of phenolic compounds and flavonoids present in plant foods that contributes to the observed bioactivities in man, or whether it is the consumption of a broad spectrum of phytochemicals that is more important. The exact mode of action of these phytochemicals still remains to be answered. Many earlier studies suggested that phenolic compounds and flavonoids protect cell constituents through direct scavenging of free radicals due to their antioxidant properties. However, recent data indicate that the protective effect of flavonoids and phenolic compounds may extend beyond their antioxidant activity. However, research in this field is still at its infancy as it has been carried out only on specific phytochemicals. Future research needs to focus on methods to better evaluate and optimize the in vivo effects of health-promoting compounds in biological system. Many promising results have been obtained to engineer or breed plants with enhanced levels of phenolic compounds and flavonoids. However, metabolomics and microarray analysis of global gene expression patterns have revealed that playing with a piece of the jigsaw may induce unfavorable changes in the fragile equilibrium of the interconnected pathways. Accurate controls should be envisaged in future studies to check for potential pitfalls. Once these setbacks are overcome there

remains the possibility to develop super crop varieties containing enhanced health-promoting flavonoid and phenolic compounds.

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