

Fereidoon Shahidi and Marian Naczki

Phenolics
in
Food and
Nutraceuticals



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Table of Contents

| | | |
|-------------------|--|-----|
| Chapter 1 | Biosynthesis, Classification, and Nomenclature of Phenolics in Food and Nutraceuticals | 1 |
| Chapter 2 | Cereals, Legumes, and Nuts | 17 |
| Chapter 3 | Phenolic Compounds of Major Oilseeds and Plant Oils | 84 |
| Chapter 4 | Phenolic Compounds in Fruits and Vegetables | 132 |
| Chapter 5 | Phenolic Compounds of Beverages | 238 |
| Chapter 6 | Phenolics in Herbal and Nutraceutical Products | 309 |
| Chapter 7 | Nutritional and Pharmacological Effects of Food Phenolics | 327 |
| Chapter 8 | Antioxidant Properties of Food Phenolics | 397 |
| Chapter 9 | Contribution of Phenolic Compounds to Flavor and Color Characteristics of Foods | 439 |
| Chapter 10 | Methods of Analysis and Quantification of Phenolic Compounds | 480 |
| | Index | 540 |

Preface

Nutraceuticals and functional foods are of considerable interest to scientists, manufacturers and consumers because they assume protective and preventive roles in the pathogenesis of certain types of cancer and several other chronic diseases. The benefits of such products may be based on epidemiological, *in vitro* or *in vivo* cellular, organ, or whole organ assembly assays and could possibly include preclinical and human clinical trials. Thus, control of certain types of cancer, cardiovascular disease, autoimmune disorders and the process of aging may benefit from consumption of foods, ingredients and nutraceuticals capable of neutralizing reactive oxygen species and other oxidants in the body.

Phenolic and polyphenolic compounds in food and nutraceuticals represent the most widely distributed plant secondary metabolites exerting their beneficial effects as free radical scavengers and chelators of pro-oxidant metals and thus preventing low-density lipoprotein oxidation and DNA strand scission or enhancing immune function. This book reports the classification and nomenclature of phenolics as well as their occurrence in food and nutraceuticals, their chemistry and applications, and their nutritional and health effects. Food scientists, nutritionists, chemists, biochemists and health professionals will be interested in this book, which describes methods of analysis and quantification and antioxidant activity of phenolics in food and nutraceuticals.

Phenolics in Food and Nutraceuticals can be used as a text for advanced undergraduate or graduate courses on its subject. Extensive references have been provided to facilitate further reading of the original reports.

Fereidoon Shahidi
Marian Naczka

Authors

Fereidoon Shahidi, Ph.D., FACS, FCIC, FCIYST, FRSC, has reached the highest academic level, university research professor, in the department of biochemistry at Memorial University of Newfoundland (MUN). He is also cross-appointed to the department of biology, ocean sciences centre and the aquaculture program at MUN. Dr. Shahidi is the author of nearly 500 research papers and book chapters and has authored or edited over 30 books and made over 300 presentations at scientific conferences. His research contributions have led to several industrial developments around the globe. Dr. Shahidi's current research interests include different areas of nutraceuticals and functional foods as well as marine foods and natural antioxidants.

Dr. Shahidi serves as the editor-in-chief of the *Journal of Food Lipids* and is an editorial board member of *Food Chemistry*, *Journal of Food Science*, *Journal of Agricultural and Food Chemistry*, *Nutraceuticals and Food*, and the *International Journal of Food Properties*. He was the recipient of the 1996 William J.Eva Award from the Canadian Institute of Food Science and Technology in recognition of his outstanding contributions to food science in Canada through research and service, and also the 1998 Earl P.McFee Award from the Atlantic Fisheries Technological Society in recognition of his exemplary contributions in the seafood area and their global impact. He has also been recognized as one of the most highly cited authors in the world in the discipline of agriculture, plant and animal sciences and was the recipient of the 2002 ADM Award from the American Oil Chemists' Society.

Dr. Shahidi is the current chairperson of the nutraceuticals and functional foods division of the Institute of Food Technologists and the chair of Lipid Oxidation and Quality of the American Oil Chemists' Society. He is also the chair of the agricultural and food chemistry division of the American Chemical Society. Dr. Shahidi serves as a member of the Expert Advisory Panel of Health Canada on Standards of Evidence for Health Claims for Foods, Standards Council of Canada on Fats and Oils, Advisory Group of Agriculture and Agri-Food Canada on Plant Products and the Nutraceutical Network of Canada. He is a member of the Washington-based Council of Agricultural Science and Technology on Nutraceuticals.

Marian Naczk, Ph.D., is professor of food science and chair of the department of human nutrition at St. Francis Xavier University, Antigonish, Nova Scotia, Canada.

Dr. Naczk received his M.Eng. in technology of edible meat products and his Ph.D. in food technology in the department of food preservation and technical microbiology at the Technical University of Gdansk, Gdansk, Poland. He held teaching and research associate and university teacher positions in this department from 1969 to 1982. Dr. Naczk worked as visiting scientist in the department of chemical engineering and applied chemistry in the food engineering group of the University of Toronto between 1982 and 1985. In 1985 he joined the department of food science and nutrition at the University of Minnesota as research specialist. From 1987 to 1989 he worked as a senior research associate in the department of biochemistry of the Memorial University of New

foundland, St. John's. Dr. Naczki joined the department of human nutrition (formerly the department of nutrition and consumer studies) at the St. Francis Xavier University in 1989 as assistant professor and has served as chair since 1998.

Dr. Naczki is the author of over 100 research papers and book chapters and has presented over 50 papers in different national and international conferences. His current research interests include plant phenolic-protein interactions and the antioxidant properties of plant phenolics; these are financed through grants from the Natural Sciences and Engineering Research Council of Canada.

1

Biosynthesis, Classification, and Nomenclature of Phenolics in Food and Nutraceuticals

Phenolic compounds in food and nutraceuticals originate from one of the main classes of secondary metabolites in plants derived from phenylalanine and, to a lesser extent in some plants, also from tyrosine (Figure 1.1) (van Sumere, 1989; Shahidi, 2000, 2002). Chemically, phenolics can be defined as substances possessing an aromatic ring bearing one or more hydroxyl groups, including their functional derivatives. Their occurrence in animal tissues and nonplant materials is generally due to the ingestion of plant foods. Synthetic phenolics may also enter the food system via their intentional incorporation in order to prevent oxidation of their lipid components.

Plants and foods contain a large variety of phenolic derivatives including simple phenols, phenylpropanoids, benzoic acid derivatives, flavonoids, stilbenes, tannins, lignans and lignins. Together with long-chain carboxylic acids, phenolics are also components of suberin and cutin. These rather varied substances are essential for the growth and reproduction of plants and also act as antifeedant and antipathogens (Butler, 1992). The contribution of phenolics to the pigmentation of plant foods is also well recognized. In addition, phenolics function as antibiotics, natural pesticides, signal substances for establishment of symbiosis with rhizobia, attractants for pollinators, protective agents against ultraviolet (UV) light, insulating materials to make cell walls impermeable to gas and water and as structural materials to give plants stability.

Many properties of plant products are associated with the presence, type and content of their phenolic compounds. The astringency of foods, the beneficial health effects of certain phenolics or their potential antinutritional properties when present in large quantities are significant to producers and consumers of foods. Furthermore, anthocyanins are distributed widely in foods, especially in fruits, and also in floral tissues (Harborne and Williams, 2001). They may also be used as nutraceuticals in the dried and powdered form from certain fruit or fruit by-product sources. These anthocyanins are responsible for the red, blue, violet and purple colors of most plant species and their fruits and products. For example, after their extraction, colorants produced from the skin of grapes may be used by the food industry (Francis, 1993). Methods of analysis of different classes of phenolics and polyphenolics have recently appeared (Hurst, 2002).

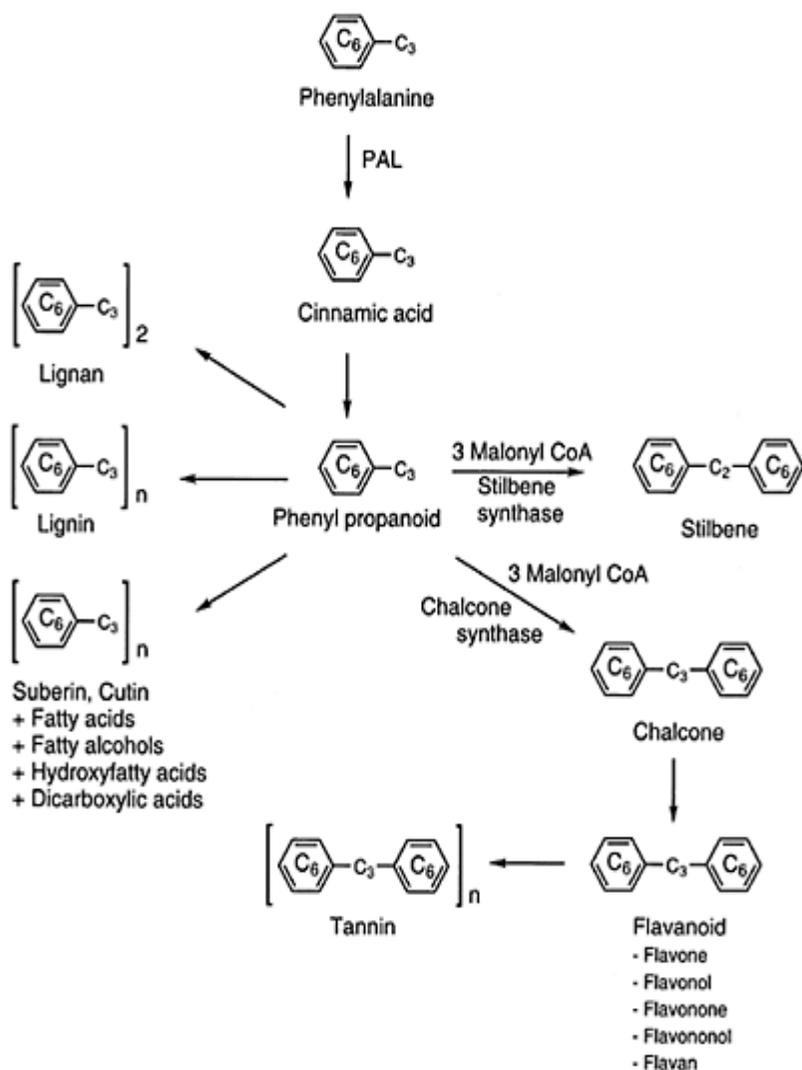


FIGURE 1.1 Production of phenylpropanoids, stilbenes, lignans, lignins, suberins, cutins, flavonoids and tannins from phenylalanine. PAL denotes phenylalanine ammonia lyase.

BIOSYNTHESIS, CLASSIFICATION AND NOMENCLATURE

CINNAMIC AND BENZOIC ACID DERIVATIVES AND SIMPLE PHENOLS

Phenylalanine ammonia lyase (PAL) catalyzes the release of ammonia from phenylalanine and leads to the formation of a carbon-carbon double bond, yielding *trans*-cinnamic acid. In some plants and grasses tyrosine is converted into 4-hydroxycinnamic acid via the action of tyrosine ammonia lyase (TAL). Introduction of a hydroxyl group into the *para* position of the phenyl ring of cinnamic acid proceeds via catalysis by monooxygenase utilizing cytochrome P₄₅₀ as the oxygen binding site. The *p*-coumaric acid formed may be hydroxylated further in positions 3 and 5 by hydroxylases and possibly methylated via O-methyl transferase with S-adenosylmethionine as methyl donor; this leads to the formation of caffeic, ferulic and sinapic acids (Figure 1.2 and Figure 1.3). These compounds possess a phenyl ring (C₆) and a C₃ side chain and are thus collectively termed phenylpropanoids, which serve as precursors for the synthesis of lignins and many other compounds.

Benzoic acid derivatives are produced via the loss of a two-carbon moiety from phenylpropanoids. Salicylic acid is a benzoic acid derivative that acts as a signal substance (Raskin, 1992). After infection or UV irradiation, many plants increase their salicylic acid content, which may induce the biosynthesis of defense substances. Aspirin, the acetyl ester of salicylic acid, was first isolated from the bark of the willow tree. Similar to phenylpropanoid series, hydroxylation and possibly methylation of hydroxybenzoic acid leads to the formation of dihydroxybenzoic acid (protocatechuic acid), vanillic acid, syringic acid and gallic acid (Figure 1.3). Hydroxybenzoic acids are commonly present in the bound form in foods and are often the component of a complex structure like lignins and hydrolyzable tannins. They are also found in the form of organic acids and as sugar derivatives (Schuster and Herrmann, 1985). However, exceptions in which they are present mainly in the free form do exist (Mosel and Herrmann, 1974a, b; Schmidlein and Herrmann, 1975; Stöhr and Herrmann, 1975a, b).

Conventionally, phenylpropanoids (i.e., cinnamic acid family) and benzoic acid derivatives are collectively termed “phenolic acids” in the food science literature. However, it should be noted that this nomenclature is not necessarily correct from a chemical and structural viewpoint. Nonetheless, such compounds are referred to as phenolic acids in the remainder of this book.

Decarboxylation of benzoic acid and phenylpropanoid derivatives leads to the formation of simple phenols (Figure 1.2). Thermal degradation of lignin or microbial transformation may also produce simple phenols in foods (Maga, 1978); thus, vinyl-substituted phenols may be produced by decarboxylation of hydroxycinnamic acids (Pyysalo et al., 1977). However, exposure of 4-vinylguaiacol to oxygen leads to the formation of vanillin (Fiddler et al., 1967). A number of simple phenols, namely, phenol, *o*-cresol, 4-ethylphenol, guaiacol, 4-vinylguaiacol and eugenol, are found in foods of plant origin. Reduction products of phenylpropanoids also yield phenolic compounds such as sinapyl alcohol, coniferyl alcohol, and coumaryl alcohol, among others.

COUMARINS

Coumarins are lactones of *cis-O*-hydroxycinnamic acid derivatives that are present in certain foods of plant origin and exist in the free form or as glycosides. The most important of these are simple coumarins, furanocoumarins (also called psoralens) and pyranocoumarins, which are present in free and glycosidic forms in foods. This group of compounds also acts as phytoalexins in response to disease infection. It is worth noting that most studies on coumarins have been carried out on Umbelliferae and Rataceae families (Murray et al., 1982). Presence of psoralens in different parts of such fruits and

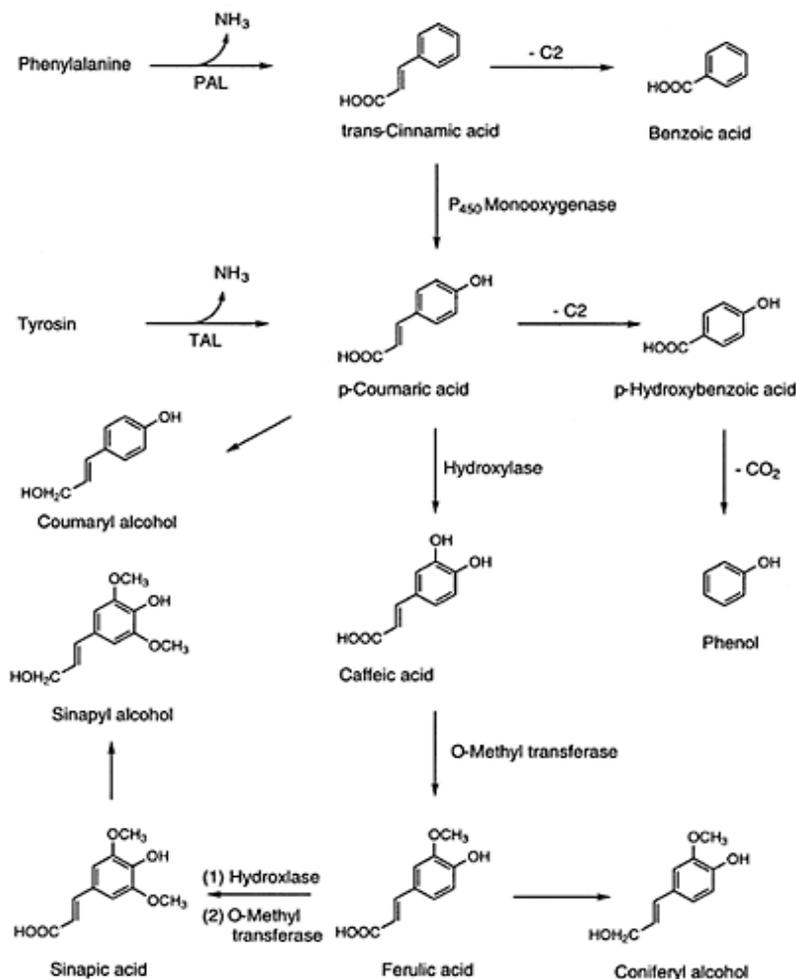
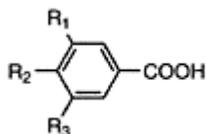
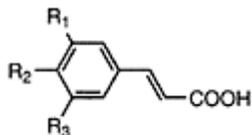


FIGURE 1.2 Formation of phenylpropanoids of cinnamic acid family as well as benzoic acid

derivatives and corresponding alcohols from phenylalanine and tyrosine. PAL denotes phenylalanine ammonia lyase and TAL denotes tyrosine ammonia lyase.



| Acid | | R ₁ | R ₂ | R ₃ |
|------------------|----------------------------|------------------|----------------|------------------|
| p-Hydroxybenzoic | 4-Hydroxybenzoic | H | OH | H |
| Protocatechuic | 3,4-Dihydroxybenzoic | OH | OH | H |
| Vanillic | 4-Hydroxy-3-methoxybenzoic | OCH ₃ | OH | H |
| Syringic | 3, 5-Dimethoxybenzoic | OCH ₃ | OH | OCH ₃ |
| Gallic | 3,4,5-Trihydroxybenzoic | OH | OH | OH |



| Acid | | R ₁ | R ₂ | R ₃ |
|------------|---------------------------------|------------------|----------------|------------------|
| p-Coumaric | 4-Hydroxycinnamic | H | OH | H |
| Caffeic | 3,4-Dihydroxycinnamic | OH | OH | H |
| Ferulic | 4-Hydroxy-3-methoxycinnamic | OCH ₃ | OH | H |
| Sinapic | 4-Hydroxy-3,5-dimethoxycinnamic | OCH ₃ | OH | OCH ₃ |

FIGURE 1.3 Phenolic acids of cinnamic and benzoic acid families found in food and nutraceuticals.

| Chemical structure | Simple coumarins | |
|--------------------|------------------|------------------|
| | R ₁ | R ₂ |
| | H | H |
| | OH | H |
| | OH | OH |
| | OH | OCH ₃ |
| Furanocoumarins | | |
| R ₁ | R ₂ | |
| | H | H |
| | H | OCH ₃ |
| | OCH ₃ | H |
| | OH | H |

FIGURE 1.4 Chemical structures of some coumarins found in food and nutraceuticals.

vegetables has been confined because high levels of it are found only in parsnip roots (Murray et al., 1982). The chemical structures of some naturally occurring coumarins are shown in Figure 1.4.

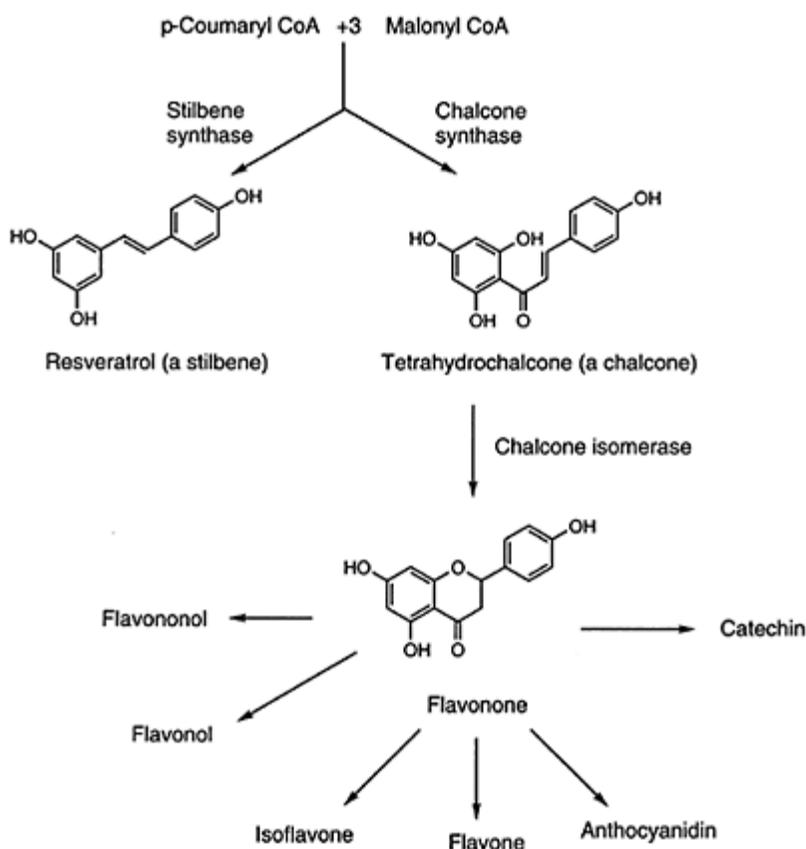


FIGURE 1.5 Production of flavonoids and stilbenes from phenylpropanoid (coumaroyl CoA) and malonyl CoA.

FLAVONOIDS AND STILBENES

The flavonoids, including flavones, isoflavones and anthocyanidins, are formed via condensation of a phenylpropane (C₆-C₃) compound via participation of three molecules of malonyl coenzyme A, which leads to the formation of chalcones that subsequently cyclize under acidic conditions (Figure 1.5). Thus, flavonoids have the basic skeleton of diphenylpropanes (C₆-C₃-C₆) with different oxidation level of the central pyran ring. This also applies to stilbenes, but in this case, after introduction of the second phenyl

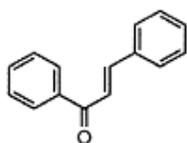
moiety, one carbon atom of the phenylpropane is split off. Stilbenes are potent fungicides in plants, e.g., viniferin of grapevine.

In the case of flavonoids and isoflavonoids, depending on the substitution and unsaturation patterns, flavones, flavonones, flavonols and flavanonols, as well as flavan-3-ols and related compounds, may be formed (Figure 1.6). Flavones and flavonols occur as aglycones in foods; approximately 200 flavonols and some 100 flavones have been identified in plants. These compounds possess a double bond between C-2 and C-3. Flavonols are different from flavones in that they possess a hydroxyl group in the 3-position and can be regarded as 3-hydroxyflavones. On the other hand, flavones may be referred to as 3-deoxyflavonols. Within each class, individual flavonoids may vary in the number and distribution of hydroxyl groups as well as in their degree of alkylation or glycosylation. The formation of flavonol and flavone glycosides depends on the action of light; therefore, they are found mainly in leaves and fruit skins with only trace amounts in parts of plants below the soil surface (Herrmann, 1967). Flavonols are present mainly as mono-, di- and triglycosides. The monoglycosides occur mainly as 3-*O*-glycosides. Glycosylation in positions 5, 7, 3' and 4' is rarely reported in flavonols of fruits and vegetables.

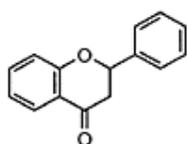
In the case of diglycosides, the two sugar moieties may be linked to the same or two different carbons. Of these, 3-*O*-diglycosides and 3,7-di-*O*-glycosides are found most frequently; 3-rutinosides, with a rhamnose and a glucose molecule joined by a 1→6 bond, are the most widely distributed flavonol diglycosides (Macheix et al., 1990). Rutin is an example of a diglycoside found in a number of fruits and vegetables. Triglycosides, meanwhile, are the least frequently reported flavonol glycosides. The sugar moiety of glycosides is usually composed of D-glucose, D-galactose, L-rhamnose, L-arabinose, D-apose and D-glucuronic acid or their combinations. A number of flavonol glycosides acylated with phenolic acids such as *p*-coumaric ferulic, caffeic, *p*-hydroxybenzoic and gallic acids have been found in fruits. Of these, kaempferol 3-(*p*-coumaryl) glycosides are most widely distributed in plants (Harborne and Williams, 1975, 2001). Meanwhile, flavonones and flavanonols are characterized by the presence of a saturated C₂–C₃ bond and an oxygen atom (carbonyl group) in the 4-position. Thus, flavonones may be referred to as dihydroflavones. Flavononols differ from flavonones by having a hydroxyl group in the 3-position and are often referred to as 3-hydroxyflavonones or dihydroflavonols. Flavonones have one center of asymmetry in the 2-position, while flavononols possess a second center of asymmetry in the 3-position (Figure 1.5 and Figure 1.6). Presence of the phenyl ring in the 3-position instead of the 2-position affords isoflavones. The flavonoids include many phytoalexins, e.g., the isoflavone medicarpin from alfalfa. Flavonoids are usually present as glycosides with a sugar moiety linked through an OH group (known as *O*-glycosylflavonoid) or through a carbon-carbon bond (known as C-glycosylflavonoid).

The flavonones in plants are often glycosylated in the 7-position with disaccharides rutinose and neohesperoside. Both of these disaccharides are made of rhamnose and glucose and differ only in their linkage type: 1→6 for rutinose and 1→2 for neohesperoside. It is worth noting that flavonol glycosides are good fungistatic and fungitoxic substances (Kefford and Chandler, 1970). Meanwhile, flavonol glycosides may also occur in the skin of certain fruits (Trousdale and Singleton, 1983).

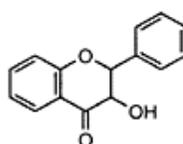
Among flavonoids, anthocyanins (Figure 1.7) and catechins (Figure 1.8), known collectively as flavans because of lack of the carbonyl group in the 3-position, are



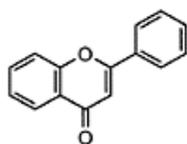
Chalcone



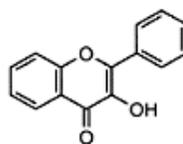
Flavonone



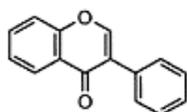
Flavonol



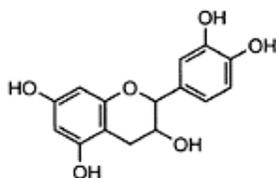
Flavone



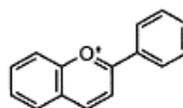
Flavonol



Isoflavone



Catechin



Anthocyanidin
(Flavylium cation)

FIGURE 1.6 Chemical structures of selected C₆-C₃-C₆ compounds found in food and nutraceuticals.

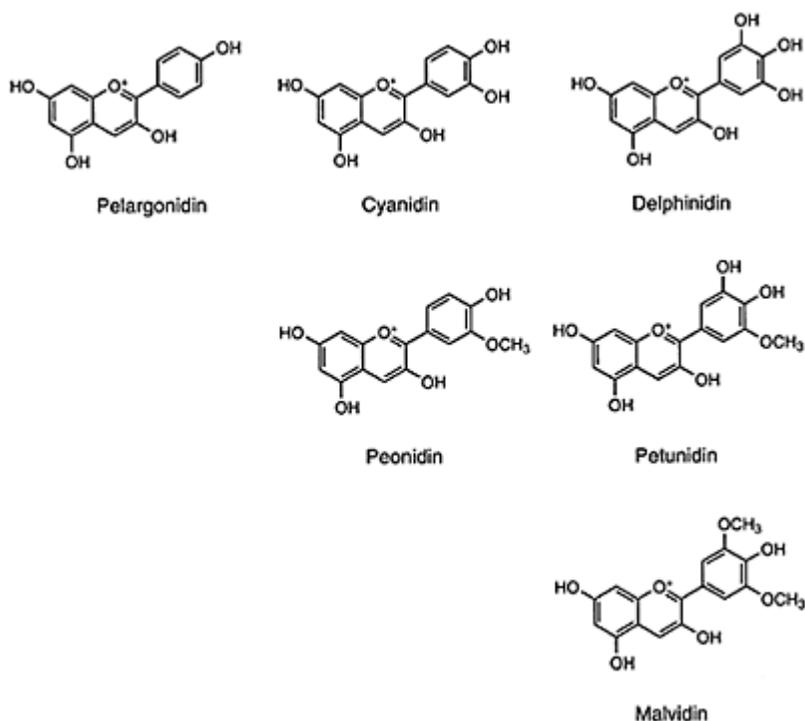


FIGURE 1.7 Chemical structures of anthocyanins.

important; flavan-3-ols and flavan-3,4-diols belong to this category. Anthocyanins are glycosidically bound anthocyanidins present in many flowers and fruits. While chalcones and flavones are yellow, anthocyanins are water-soluble pigments responsible for the bright red, blue and violet colors of fruits and other foods (Mazza and Miniati, 1994). Thus, the bright red skins of radishes, the red skins of potatoes and the dark skins of eggplants are due to the presence of anthocyanins. Blackberries, red and black raspberries, blueberries, cherries, currants, concord and other red grapes, pomegranate, ripe gooseberries, cranberries and plums all contain anthocyanins. Almost 200 different anthocyanins have been identified in plants. The anthocyanin pelargonin contains pelargonidin as its chromophore. Introduction of one or more hydroxyl groups in 3' and 5' positions of the phenyl residue and their successive methylation yields five additional anthocyanidins that differ in color (Figure 1.7). Most anthocyanins occur as monoglycosides and diglycosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Meanwhile, anthocyanins assume different colors when subjected to pH variation in solutions. In addition, catechins and epicatechins, found in different plants and in high amounts in green tea leaves, are similar in their structures to anthocyanidins; however, they are colorless (Figure 1.9).

Examples of some naturally occurring flavonoids in foods and food components are provided in Figure 1.9. The substitution pattern in the ring structure is also given.

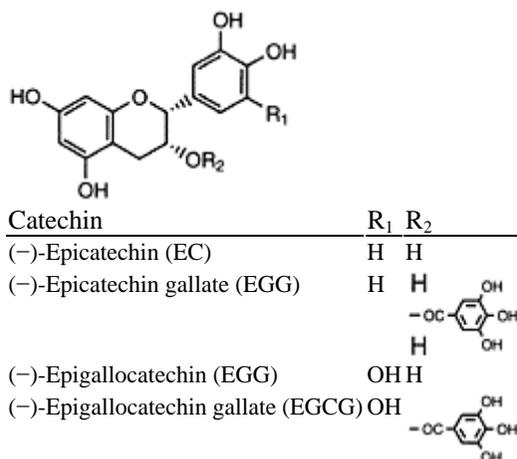


FIGURE 1.8 Chemical structures of catechins.

LIGNANS AND LIGNINS

By definition, lignans are dimers of phenylpropanoid (C₆–C₃) units linked by the central carbons of their side chains. According to their oxygenation, four major groups of linear lignans are found, namely, lignans or butane derivatives, lignolides or butanolide derivatives, monoepoxy lignans or tetrahydrofuran derivatives and biepoxy lignans or cyclolignans or derivatives of 3,7-dioxabicyclo-(3,3,0)-octane. Cyclized lignans or cyclolignans occur as tetrahydronaphthalene or naphthalene derivatives. Structures of some lignans are provided in Figure 1.10.

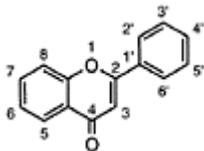
In plants, lignans and their higher oligomers act as defensive substances (Davin and Lewis, 1992). The lignan pinosresinol is formed when the plant is wounded and is toxic to microorganisms. Malagnol inhibits the growth of bacteria and fungi. The pharmacological effects of lignans related to their antiviral activity have also been demonstrated (Davin and Lewis, 1992).

Lignins are formed via polymerization of a mixture of the three monolignols *p*-coumaryl, sinapyl and coniferyl alcohols (Lewis and Yamamoto, 1990; Douglas, 1996). The composition of lignin varies greatly in different plants; lignin of conifers, for example, has a high coniferyl alcohol content. Lignin is covalently bound to cellulose in the cell walls. Liquefied cell walls may be compared to reinforced concrete in which cellulose fibers serve as the steel and lignin the concrete; this makes plants difficult for herbivores to digest. In addition, lignin inhibits the growth of pathogenic microorganisms.

SUBERINS AND CUTINS

Suberins are another group of compounds composed of polymeric phenylpropanoids, long chain fatty acids and fatty alcohols with 18 to 30 carbon atoms (Davin and Lewis, 1992). Hydroxyfatty acids and dicarboxylic acids with 14 to 30 carbon atoms might also

be present. In suberin, as in lignin, phenylpropanoids are partly linked with each other. Suberin is a cell wall constituent that forms gas- and water-tight layers. Cutin is a polymer similar to suberin, but with a relatively small proportion of phenylpropanoids and dicarboxylic acids. Consisting mainly of esterified hydroxyfatty acids with 16 to 18 carbon atoms, cutin impregnates the cell walls of leaves and shoot organs.



| Class | Name | Substitutions | Dietary Source |
|------------------------|-------------------------|----------------------------------|---|
| Chalcone (no 1-2 bond) | Butein | 2, 4, 3', 4'-OH | Miscellaneous |
| | Okanin | 2, 3, 4, 3', 4'-OH | Miscellaneous |
| Flavone | Chrysin | 5, 7-OH | Fruit skins |
| | Apigenin | 5, 7, 4'-OH | Parsley, celery |
| | Rutin | 5, 7, 3', 4'-OH; 3-rutinose | Buckwheat, citrus, red pepper, red wine, tomato skin |
| Flavonone | Naringin | 5, 4',-OH; 7-rhamnoglucose | Citrus, grapefruit |
| | Naringenin | 5, 7, 4'-OH | Citrus |
| | Taxifolin | 3, 5, 7, 3', 4',-OH | Citrus |
| | Eriodictyol | 5, 7, 3', 4'-OH | Lemons |
| | Hesperidin | 3, 5, 3'-OH, 4'-OMe; 7-rutinose | Oranges |
| Flavonol | Isosakuranetin | 5, 7 -OH; 4'-OMe | Citrus |
| | Kaempferol | 3, 5, 7, 4'-OH | Leek, broccoli, endives, grapefruit, black tea |
| | Quercetin | 3, 5, 7, 3', 4'-OH | Onion, lettuce, broccoli, tomato, tea, berries, apples, olive oil |
| Flavononol | Engeletin | 3, 5, 7, 4'-OH; 3-O-rhamnose | White grapeskin |
| | Astilbin | 3, 5, 7, 5', 4'-OH; 3 O-rhamnose | White grapeskin |
| | Genistin | 5, 4'-OH; 7-glucose | Soybean |
| | Taxifolin | 3, 5, 7, 3', 4'-OH | Fruits |
| Isoflavone | Genistein | 5, 7, 4'-OH | Soybean |
| | Daidzin | 4'-OH, 7-glucose | Soybean |
| | Daidzein | 4', 7-OH | Soybean |
| Flavanol | (+)-Catechin | 3, 5, 7, 3', 4'-OH | Tea |
| | (+)-Gallocatechin | 3, 5, 7, 3', 4', 5'-OH | Tea |
| | (-)-Epicatechin | 3, 5, 7, 5', 4'-OH | Tea |
| | (-)-Epigallocatechin | 3, 5, 7, 3', 4'-OH | Tea |
| | (-)-Epicatechin gallate | 3, 5, 7, 3', 4'-OH; 3-gallate | Tea |
| Anthocyanidin | Epigenidin | 5, 7, 4'-OH | Stored fruits |

| | | |
|--------------|------------------------|-------------------------------|
| Cyanidin | 3, 5, 7, 4'-OH; 3-OMe | Cherry, raspberry, strawberry |
| Delphinium | 3, 5, 7, 3', 4', 5'-OH | Dark fruits |
| Pelargonidin | 3, 5, 7, 4'-OH | Dark Fruits |

FIGURE 1.9 Different classes of flavonoids, their substitution patterns and dietary sources.

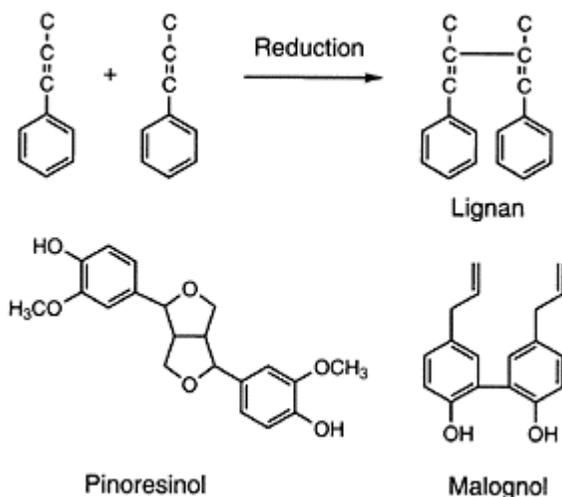
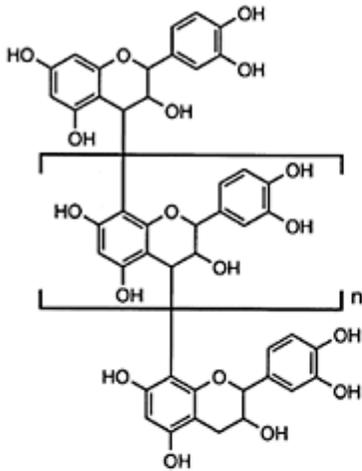


FIGURE 1.10 Chemical structures of selected lignans.

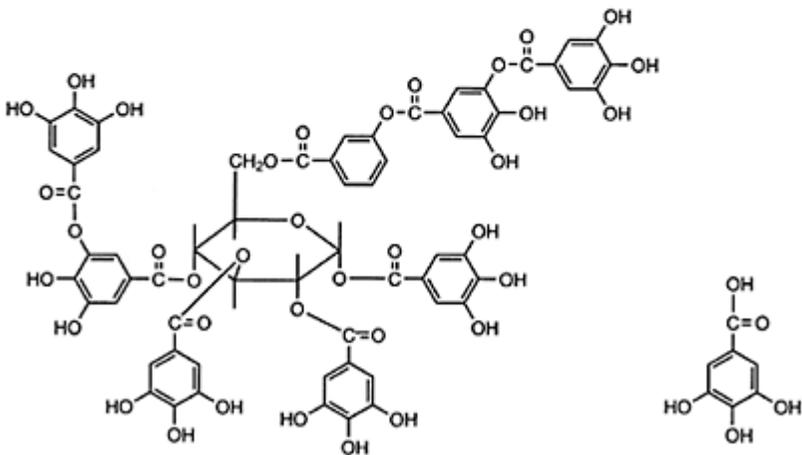
TANNINS

Tannin is a collective term used to describe a variety of plant polyphenols used in tanning raw hides to produce leather. This group of compounds includes oligomeric and polymeric constituents. Tannins are polyphenols capable of precipitating proteins from aqueous solutions and are widely distributed in plants. They are found in especially high amounts in the bark of certain trees such as oak and in galls. Tannins also form complexes with certain types of polysaccharides, nucleic acids and alkaloids (Ozawa et al., 1987). Depending on their structures, tannins are defined as hydrolyzable or condensed, the latter term known also as proanthocyanidin. The condensed tannins are oligomers and polymers of flavonoids, specifically flavan-3-ols, while hydrolyzable tannins are glycosylated gallic acids (Ferreira and Li, 2000; Khanbabaee and van Ree, 2001). Many of these gallic acids are linked to sugar molecules. The molecular weights of hydrolyzable tannins range from 500 to 2800 Da or more (Haddock et al., 1982). Based on their hydrolysis products, hydrolyzable tannins include gallotannins and elagitannins. Condensed tannins include dimers, oligomers and polymers of flavan-3-ols. Approximately 50 procyanidins ranging from dimers to hexamers have been identified

(Hemingway, 1989). Based on the hydroxylation pattern of A- and B-rings, proanthocyanidins can be divided into procyanidins, propelargonidins and prodelphinidins. The consecutive units of condensed tannins are linked through the interflavonoid bond between C-4 and C-8 or C-6 (Hemingway, 1989). The molecular weight of proanthocyanidins isolated from fruits ranges between 2000 and 4000 Da (Macheix et al., 1990) (Figure 1.11).



Condensed Tannin



Hydrolyzable Tannin

Gallic Acid

FIGURE 1.11 Chemical structures of tannins.

The phenolic groups of tannins bind very tightly with the $-NH$ groups of peptides and proteins and prevent their hydrolysis and digestion in the stomach; thus, they are known to be antinutritional in nature. In the tanning process tannins bind to the collagen of the animal hide and thus produce leather that can withstand attack by the degrading microorganisms. Because they inactivate aggressive enzymes, tannins also protect plants against attack by microorganisms.

TOCOPHEROLS AND TOCOTRIENOLS

Another class of plant phenolics often encountered in foods is tocols. These monophenolic and lipophylic compounds occur widely in plant tissues. The eight different tocols with vitamin E activity are divided into two groups known as tocopherols and tocotrienols; each of these is classified into α , β , γ and δ , depending on the number and position of methyl groups on the chromane ring. (See Figure 1.12.) The α -isomer is a 5,7,8-trimethyl; (β , a 5,8-dimethyl; γ , a 7,8-dimethyl; and δ , an

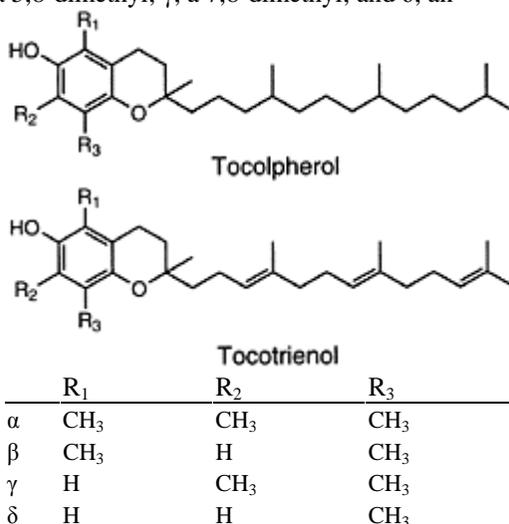


FIGURE 1.12 Chemical structures of tocopherols and tocotrienols.

8-methyl derivative of the parent compound. Furthermore, the side chain in tocopherols is saturated, although it is unsaturated in tocotrienols (Shahidi, 2000).

The occurrence of tocols in vegetable oils is diverse, but animal fats generally contain α -tocopherol and, in some cases, γ -tocopherol. The β -isomer is generally less prevalent. Tocols are by-products of edible oil processing and are isolated as distillates of the deodorization process.

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2

Cereals, Legumes, and Nuts

INTRODUCTION

Phenolic compounds are ubiquitous in cereals, legumes, and nuts. Different phenolics belonging to the benzoic acid, cinnamic acid, flavonoid and tannin class of compounds may be present in free, esterified/etherified or insoluble bound forms (Ribereau-Gayon, 1972; Salunkhe et al., 1982). Thus, ferulic acid may be linked to polysaccharides (Faulds and Williamson, 1999; Hartley et al., 1976; Hatfield et al., 1999; Iiyama et al., 1990; Ishii, 1997; Ishii and Hiroi, 1990; Markwalder and Neukom, 1976), lignins (Higuchi et al., 1967; Iiyama et al., 1990) and suberin (Riley and Kolattukudy, 1975); *p*-coumaric acid may be linked to polysaccharides (Hartley et al., 1976), lignins (Grabber et al., 2000; Nakamura and Higuchi, 1978), and cutin (Kolattukudy et al., 1981). In aleurone layer of cereal grains, ferulic acid is mainly linked to polysaccharides via an ester bond at the *O*-5 position of arabinofuranose (Ishii, 1997). Ferulic acid may also undergo dimerization by oxidative coupling catalyzed by peroxidase (Brett et al., 1999; Geissmann and Neukom, 1973). Dehydrodimers of ferulic acid have been proposed to form cross links between arabinoxylan chains (Markwalder and Neukom, 1976; Waldron et al., 1996). The main dehydrodimers identified in plant material are shown in Figure 2.1.

A number of phenols present in cereal grains, especially roasted products, may be formed as by-products of enzymatic or thermal degradation of substituted benzoic and cinnamic acids. Tressl et al. (1976) studied the thermal degradation of cinnamic and hydroxycinnamic acids under conditions simulating roasting of cereal grains. The 4-vinyl derivatives of these acids were the major thermal degradation products. However, some phenols with extended alkyl side chains, such as 5-*n*-alkyl-resorcinols, occur naturally in cereal grains (Collins, 1986).

The oligomeric flavanols, products of condensation of (+)-catechin, (+)-gallicocatechin and their epimers, are also found in many plant products (Thompson et al., 1972). However, in cereal grains the presence of oligomeric flavanols has been reported only in sorghum (Strumeyer and Malin, 1975) and barley (Pollock et al., 1960); in barley brewers often call them anthocyanogens (Gramshaw, 1968). Simple flavanols such as monomers, dimers and trimers are found in developing sorghum grains (Glennie et al., 1981), while highly polymerized flavanols (tannins) occur in mature sorghum seeds. Simple flavanols are generally considered precursors of haze in beer (Gramshaw, 1969) because they easily undergo acid-catalyzed and autoxidative polymerization (Goldstein and Swain, 1963) to products that may form insoluble complexes with proteins.

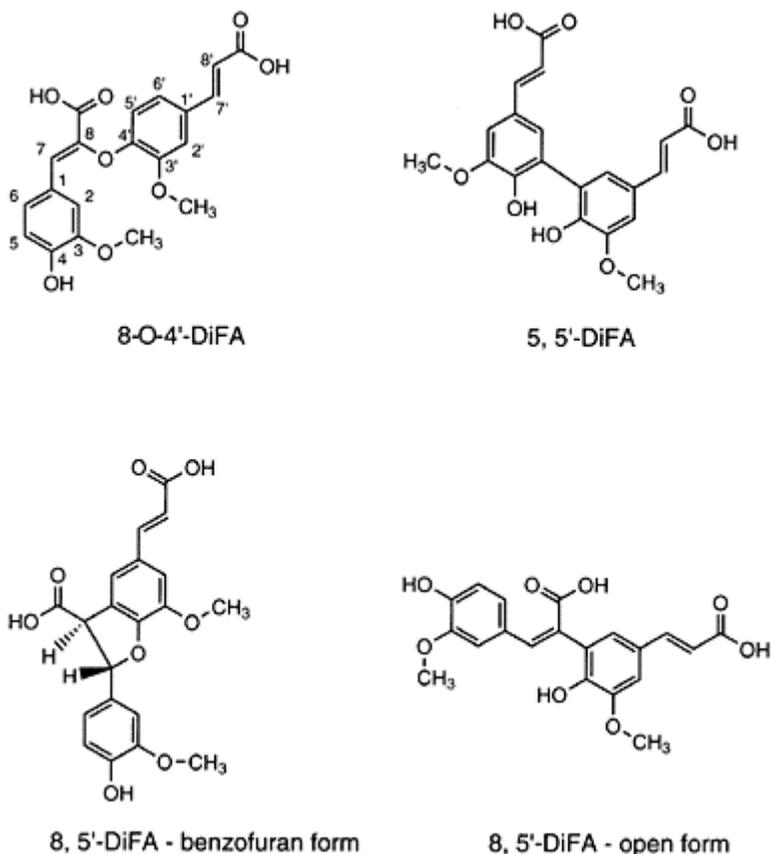


FIGURE 2.1 Chemical structures of some dehydrodimers of ferulic acid (DiFA).

BEANS AND PULSES

Dry beans and peas are important components of traditional diet in many Asian, African, and Central and South American countries. Faba bean, also known as broad bean, has been grown in Egypt, Europe and Asia for centuries. Cowpeas (black-eyed peas) and winged beans are grown in a number of tropical countries. In addition, pigeon peas, lima beans, horse gram, hyacinth beans, moth beans, and black and green gram are also widely harvested for human consumption in Asia and a number of other countries (Duke, 1981). Dry beans and peas serve as a good source of protein and carbohydrate, although they contain a number of antinutritional factors, including phytates, enzyme inhibitors, lectins, and tannins, among others. Flavonol glycosides, tannins and anthocyanins are responsible for the color of seed coat in dry beans (Beninger and Hosfield, 1999; Beninger et al.,

1998, 1999; Takeoka et al, 1997). Tannins in beans and pulses have received considerable attention due to their possible nutritional and physiological implications, biochemical functions, and influence on the aesthetic qualities of foods (Deshpande and Cheryan, 1987; Reddy et al., 1985).

PHENOLIC ACIDS

Sosulski and Dabrowski (1984) reported that defatted flours of 10 legumes contain only soluble esters of *trans*-ferulic, *trans-p*-coumaric and syringic acids. Of these legumes, mung bean, field pea, faba bean, lentil and pigeon pea contain 18 to 31 mg of total phenolic acids per kilogram, while in Navy bean, lupine, lima bean, chickpea and cowpea, the total content of phenolics ranges from 55 to 163 mg/kg. On the other hand, Garcia et al. (1998) reported the presence of caffeic, *p*-coumaric, sinapic and ferulic acids in dehulled soft and hard-to-cook beans (*Phaseolus vulgaris*). The dehulled soft beans contained 45 times more methanol soluble esters of phenolic acids than hard-to-cook beans, while the content of phenolic acids bound to water-soluble fraction of pectins was two times higher in hard-to-cook beans than in dehulled soft beans.

FLAVONOIDS

Hertog et al. (1992) reported that after hydrolysis of all glycosides, French green beans contain 39 mg of quercetin per kilogram and <12 mg of kaempferol per kilogram of sample. Later, Hempel and Bohm (1996) reported that 3-*O*-glucuronides and 3-*O*-rutinosides of quercetin and kaempferol are the main flavonoid glycosides in six varieties of yellow and green French beans. These authors reported that the total content of quercetin- and kaempferol-3-*O*-glycosides was 19.1 to 183.5 and 5.6 to 14.8 mg/kg of sample, respectively. Figure 2.2 shows two new isoflavonoid derivatives, mutabilin (3'-methoxy-5-hydroxy-7-*O*- β -D-glucosylisoflavone) and mutabilein (3'-methoxy-5, 7-dihydroxyisoflavone) that have been identified in seeds of *Lupinus mutabilis* (Fabaceae) (Dini et al., 1998). Beninger et al. (1998) reported that two flavonol glycosides, namely, kaempferol 3-*O*- β -D-glucopyranoside (astragalin; 499 mg/kg of fresh whole bean weight) and kaempferol 3-*O*- β -D-glucopyranoside-(2 \rightarrow 1)-*O*- β -D-xylopyranoside (585 mg/kg of fresh whole bean weight), are responsible for imparting yellow color to the seed coat of 'Prim' variety of Manteca-type dry beans (Figure 2.3).

Later, Beninger and Hosfield (1999) identified three flavonol glycosides contributing to the color of seed coat of commercial dark red bean, cultivar Montcalm astragalin, quercetin 3-*O*- β -D-glucopyranoside-(2 \rightarrow 1)-*O*- β -D-xylopyranoside, and quercetin 3-*O*- β -D-glucopyranoside. In addition, malvidin 3-glucoside, petunidin 3-glucoside, delphinidin 3-glucoside and 3,5-diglucoside have been detected in black-violet beans (Feenstra, 1960). Subsequently, Stanton and Francis (1966) reported that delphinidin 3-glucoside is the major anthocyanin in beans of Canadian Wonder cultivar, while cyanidin 3-diglucoside, 3,5-diglucoside, pelargonidin 3-glucoside and 3,5-diglucoside are the minor anthocyanins. Later, Nozzolillo (1972, 1973) and Nozzolillo and MacNeill (1985) identified only malvidin glucosides in three black bean cultivars, namely, Black Wax, Royalty, and Black Valentine. Recently, Takeoka et al. (1997) reported that delphinidin

3-*O*-glucoside, malvidin 3-*O*-glucoside, and petunidin 3-*O*-glucoside are the main anthocyanins responsible for the color of black and purple seed coat beans.

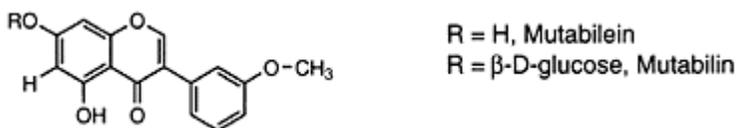


FIGURE 2.2 Chemical structures of isoflavonoid derivatives found in *Lupinus*.

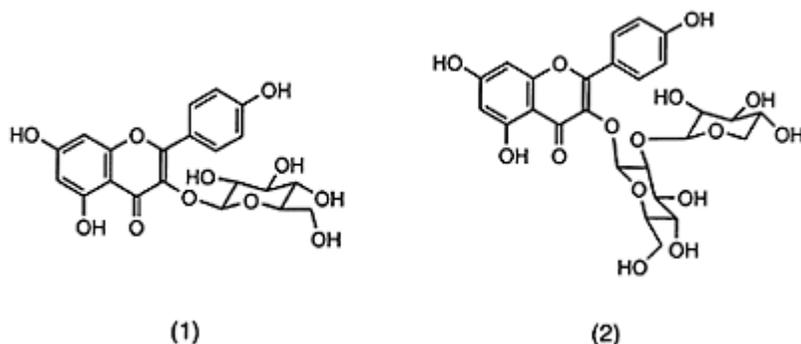


FIGURE 2.3 Chemical structures of (1) kaempferol 3-*O*- β -D-glucoside and (2) kaempferol 3-*O*- β -D-glucosyl-(2 \rightarrow 1)- β -D-xyloside found in dry beans.

CONDENSED TANNINS

Located mainly in the seed coat or testa of beans and peas, condensed tannins are often considered antinutritional factors. The content of tannins in beans and peas is up to 2.0% as expressed in (+)-catechin or tannic acid equivalents (Table 2.1). The average content of tannins in eight faba bean cultivars is 4.3% (Marquardt et al., 1978); the amount in cowpeas ranges from 0 to 0.7% and in chickpeas between 0 and 0.2% (Price et al., 1980). On the other hand, as shown in Table 2.1 and Table 2.2, cotyledons contain fewer tannins than whole seeds do (Bressani and Elias, 1980; Elias et al., 1979). Recently, Wang et al. (1998) estimated the content of condensed tannins in 17 field pea cultivars and 9 grass pea lines grown in western Canada. These authors found barely detectable levels of condensed tannins in peas, but a content of 890 to 5180 mg (+)-catechin equivalent per kilogram in grass peas.

Tannins in beans are linear polymers of flavan-3-ol (catechin and gallic acid) and flavan-3 to 4-diol (leucocyanidin and leucodelphinidin) units. These units are linked

together by carbon-carbon bonds formed between carbon-4 of one unit and carbon-6 or -8 of another. Each chain of tannin polymer contains a flavan-3-ol unit at the terminal ends (Martin-Tanguy et al., 1977). Based on the difference in their solubility in methanol and ether, bean tannins can be fractionated into four groups depending on their degree of polymerization (Cansfield et al., 1980). On the other hand, separation of condensed tannins on a Sephadex LH-20 column yielded two fractions: one containing low molecular weight polyphenolics and another containing soluble condensed tannins (Marquardt et al., 1977).

TABLE 2.1
Tannin Content of Dry Beans^a

| Bean | Whole Bean | Cotyledon |
|--|------------|-----------|
| Tannin Content Expressed as Catechin Equivalents | | |
| Black gram (<i>Phaseolus mungo</i> L.) | 5.40–11.97 | 0.16–0.33 |
| Chick pea (<i>Cicer arietinum</i>) | 0.78–2.72 | 0.16–0.38 |
| Cowpea (<i>Vigna sinensis</i> L.) | 1.75–5.90 | 0.28 |
| Green gram (<i>Phaseolus aureus</i> L.) | 4.37–7.99 | 0.21–0.39 |
| Kidney bean (<i>Dolichos lablab</i>) | 10.24 | 0.73 |
| Pigeon pea (<i>Cajanus cajan</i>) | 3.80–17.10 | 0.22–0.43 |
| Tannin Content Expressed as Tannic Acid Equivalents | | |
| Faba bean (<i>Vicia faba</i> L.) | 7.50–20.00 | 7.40–9.10 |
| Horse gram (<i>Dolichos biflorus</i> L.) | 16.00 | – |
| Lima bean (<i>Phaseolus lunatus</i>) | 6.50–9.30 | – |
| Moth bean (<i>Phaseolus aconitifolius</i>) | 13.00 | – |
| Peas (<i>Pisum sativum</i> L.) | 5.00–10.50 | 4.60–5.60 |
| Winged bean (<i>Psophocarpus tetragonolobus</i> L.) | 1.58–7.50 | 1.08 |

^a Grams per kilogram.

Source: Adapted from Reddy, N.R. et al., 1985, *J. Am. Oil Chem. Soc.*, 62:541–549.

TABLE 2.2
Tannin Content in Bean Samples (*Phaseolus vulgaris*) with Different Seed Coat Color^a

| Sample | Seed Coat Color | Whole Seeds | Cotyledons | Seed Coat | Whole Cooked Seeds |
|--------------|-----------------|-------------|------------|-----------|--------------------|
| NEPI mutant | White | 3.8 | 3.8 | 1.3 | 1.8 |
| Normal | White | 3.9 | 4.5 | 1.3 | 2.1 |
| Normal | Red | 9.3 | 5.0 | 38.0 | 4.1 |
| Normal | Black | 9.2 | 4.6 | 42.0 | 4.7 |
| San Fernando | Black | 6.7 | 5.9 | 43.0 | 5.8 |

^a Grams of tannic acid per kilogram.

Source: Adapted from Elias, L.G. et al., 1979, *J. Food Sci.*, 44:524–527.

There are differences in the content of condensed tannins of beans depending on the color of seed coats. The white varieties of beans usually contain lower concentrations of tannins than those with red, black or bronze seed coats (Table 2.1 and Table 2.2) (Bressani and Elias, 1980; Elias et al., 1979). However, Marquardt et al. (1978) reported that white varieties of faba beans have higher concentrations of tannins than those with dark testa. Moreover, the variability in the content of tannins is much greater in colored varieties of beans than in white-coated beans. Breeding studies show that the genes of low-tannin beans dominate those of high-tannins seeds; thus low-tannin colored beans can be obtained through hybridization techniques (Ma Yu and Bliss, 1978). Furthermore, Ma Yu and Bliss (1978) and Elias et al. (1979) did not find any correlation between the color of seed coat or seed size and tannin content, although a positive correlation exists between tannin concentration in seed coats and trypsin inhibitor activity (Elias et al., 1979).

Tannin content of beans decreases during seed maturation. Mature winged beans (80 to 85 days after flowering) contain about 50% fewer tannins than those harvested 40 days after flowering (Kadam et al., 1982). These changes in tannin content may be attributed to the polymerization of polyphenolic compounds to high molecular weight insoluble polymers.

EFFECT OF PROCESSING

Storing beans at high temperatures and humidity produces “hard-to-cook” seeds, so a longer cooking time is required for cotyledon softening. A number of hypotheses have been proposed to explain the cause of this defect; these include lipid oxidation and polymerization (Muneta, 1964), catabolism of phytin and pectin demethylation with subsequent formation of insoluble pectate (Jones and Boulter, 1983a, b) and formation of protein-polyphenol complexes as well as polymerization of polyphenols (Rozo, 1982). A highly significant negative correlation exists between tannin content and the development of hardness (Mejia, 1979). According to Hincks and Stanley (1986), hardening of beans involves phytate loss during initial storage and phenol metabolism during extended storage.

In addition, Srisuma et al. (1989) reported that storage induced “hard-to-cook” beans that contained higher levels of hydroxycinnamic acids than the control beans. These phenolic acids provide cross-linking sites to pectin in middle lamella and proteins—interactions that may result in textural bean defects. It has been reported that storing dry beans at elevated temperatures and humidity results in lower protein quality (Molina et al., 1975), possibly due to the interaction of proteins with tannins (Bressani, 1975). Sievwright and Shipe (1986) suggested that the adverse effect of tannins on the quality of bean proteins depends on the age of the beans and their storage conditions. They found that poly phenol oxidase enhances protein-tannin interaction and that the incidences of hard-to-cook phenomenon in beans can be reduced by soaking the beans in salt solutions. It has been postulated that salt is involved in breaking down the hydrogen bonds between condensed tannins and proteins.

Heat treatment and cooking of beans improve their texture and acceptability as a result of destruction of their antinutritional factors. Soaking beans before cooking is a common practice. A number of studies have been carried out to determine the effect of cooking on

the antinutritional factors of beans. It has been reported that cooking reduces the content of tannins by 30 to 40% (de Espana, 1977); in this study the cooking water was not discarded. Therefore, it has been suggested that changes

TABLE 2.3
Effects of Cooking on Distribution of Tannins in
***Phaseolus* Beans**

| Cooking Fraction | White Beans | | Black Beans | | Red Beans | |
|------------------|-------------|------|-------------|------|-----------|------|
| | (g/kg) | (%) | (g/kg) | (%) | (g/kg) | (%) |
| Raw seed | 3.6 | 100 | 9.0 | 100 | 14.7 | 100 |
| Cooked bean | 2.4 | 66.7 | 5.44 | 60.4 | 5.5 | 37.4 |
| Cooking water | 0.56 | 15.5 | 1.72 | 19.1 | 13.7 | 11.7 |
| Loss or bound | 0.64 | 17.6 | 1.84 | 20.5 | 7.5 | 50.9 |

Note: Bean to water ratio of 1:3; beans were cooked 4 h at atmospheric pressure.
Source: Adapted from Bressani, R. et al., 1982, *J. Plant Foods*, 4:43-55.

in the polyphenol content may result from binding of phenolics with other organic materials, or alteration of chemical structure of polyphenols, enabling them to give a color reaction with Folin-Denis reagent (Bressani and Elias, 1980). This postulate was later confirmed by the results of studies of Bressani et al. (1982) on partition of polyphenols during cooking of beans. The values shown in Table 2.3 indicate that 37.4 to 66.7% of the total polyphenols of raw beans remained in cooked beans and that the cooking water contained less than 20% of total polyphenols. However, 17.8 to 50.9% of total polyphenols was not accounted for in this study; these polyphenols were probably involved in the formation of insoluble complexes with proteins or other compounds.

The content of tannins of beans may be reduced by the removal of hulls or soaking. Soaking winged beans (*Chimbu* cultivars) in water reduces the content of tannins by 34 and 67.5% after 6 h (de Lumen and Salamat, 1980) and 24 h of treatment, respectively (Sathe and Salunkhe, 1981). Additional removal of seed coat after water soaking further reduces the tannin content in winged beans (Sathe and Salunkhe, 1981) and dry beans (*Phaseolus vulgaris* L.) (Table 2.4) (Deshpande et al., 1982). Substantial reduction in tannin content may be obtained by chemical treatment of beans: soaking winged beans in a sodium hydroxide solution for 6 h reduces their tannin content by 63% (de Lumen and Salamat, 1980), whereas 24-h treatment of beans with 2% aqueous KOH removes approximately 87.2% of their tannins (Sathe and Salunkhe, 1981).

The content of polyphenolic compounds can be reduced substantially during protein isolate production. Aqueous extraction and membrane processes have been considered and are expected to lead to industrial developments. For example, the micellization process developed by Murray et al. (1978) for preparation of protein isolates from faba beans markedly reduced the content of phenolics in the protein isolates produced. This technique consists of salt extraction of proteins followed by precipitation upon dilution with cold water, which affords a protein isolate in which the proteins have a micellar structure. About 46% of phenolics are eliminated during the salt extraction step, possibly due to the removal of phenols associated with hulls as well as low recovery of proteins associated with phenols. Further reduction in the content of

TABLE 2.4
Tannin Content of Whole and Dehulled Dry
Beans (*Phaseolus vulgaris* L.)^a

| Cultivar | Whole Beans | Dehulled Beans | (%) Reduction on Denuding |
|------------------|-------------|----------------|---------------------------|
| Cranberry | 763 | 100 | 86.9 |
| Viva Pink | 1221 | 104 | 91.5 |
| Pinto | 2647 | 142 | 94.6 |
| Light Red Kidney | 1522 | 222 | 85.4 |
| Dark Red Kidney | 1053 | 287 | 72.7 |
| Small Red | 2828 | 190 | 93.3 |
| Black Beauty | 337 | 108 | 68.0 |

Note: Values are mean of triplicate determinations on dry weight basis; dry beans were dehulled after 12 h soaking in water at 21°C.

^a Milligrams of catechin equivalent per kilogram.

Source: Adapted from Deshpande, S.S. et al., 1982, *J. Food Sci.*, 47:1846–1850.

phenolics has been achieved during the dilution steps. The isolate contained only 3.5% of phenolics originally present in the concentrate used for its preparation (Arntfield et al., 1985). According to Murray et al. (1981), the precipitation by dilution favors protein-protein rather than protein-phenolic interactions. The isolate obtained using isoelectric precipitation contains a level of phenolics comparable to that found in the isolate prepared by a micellization technique.

CEREALS

BARLEY

Barley is grown in many parts of the world for food and feed uses. In the U.S., it is mostly a feed crop, as well as a grain for brewing and producing ethanol. Only small quantities of barley crop are used in the form of flour and grit as a component of such food products as soups, dressings, baby foods and specialty items (Shellenberger, 1980). Table 2.5 shows the total content of phenolic compounds present in barley; their content depends on the cultivar and the cultivation location (Bendelow and La Berge, 1979). Phenolics present in barley grains range from tyrosine, tyramine and its derivatives, phenolic acids and their esters and glycosides, and anthocyanins responsible for the blue and red color of barley tissue to lignans and substances related to lignin (Briggs, 1978; Salomonsson et al., 1980).

Barley-malt contributes phenolic and polyphenolic compounds to early stages of the brewing process. A correlation coefficient of 0.90 was found between the content of phenolics in barley and malt (Bendelow and La Berge, 1979). Upon processing, the malt phenolics polymerize to give rise to beer polyphenolics that furnish color, impart astringent taste, serve as a browning substrate, and participate in precipitation of poorly coagulable beer proteins. On the other hand, proanthocyanidins from barley

TABLE 2.5
Content of Polyphenols in Barley and Malt as
Affected by Cultivar^a

| Station in Western Canada | Cultivar | Grain | Malt |
|---------------------------|----------|-------|------|
| Brandon | TR 201 | 3.29 | 3.55 |
| | TR 907 | 3.00 | 2.96 |
| | TR 424 | 2.87 | 2.78 |
| | Betzes | 2.83 | 2.84 |
| | Klages | 2.58 | 2.74 |
| Acme | TR 201 | 3.64 | 3.81 |
| | TR 907 | 3.72 | 3.89 |
| | TR 424 | 3.40 | 3.52 |
| | Betzes | 3.04 | 3.03 |
| | Klages | 3.27 | 3.38 |

^a Grams of gallic acid equivalent per kilogram.

Source: Adapted from Bendelow, V.M. and La Berge, D.E., 1979, *J. Am. Soc. Brew. Chem.*, 37:89–90.

malt influence the development of haze in beer because 80% of these phenolics present in regular beer are derived from barley malt.

Phenolic Acids

The free forms of salicylic, *p*-hydroxybenzoic, vanillic, protocatechuic, *o*-, *m*- and *p*-coumaric, syringic, ferulic and sinapic acids have been identified in barley grains. Recently, Yu et al. (2001) reported the presence of chlorogenic and protocatechuic acids in barley. The highest content of these acids is found in the grains between 19 and 31 days of anthesis. Ferulic acid is the predominant free phenolic acid in barley seeds (Nordkvist et al., 1984) and barley brans (Renger and Steinhart, 2000). The concentration of ferulic acid in 18 barley cultivars ranges from 365 to 605 mg/kg of sample (Zupfer et al., 1998). The content of all phenolic acids in unripe seeds is three to six times higher than that in mature seeds on the day of harvest (Table 2.6). The highest content of ethanol-soluble bound phenolic acids is also detected at 19 to 31 days of anthesis (Slominski, 1980). The content of free chlorogenic and protocatechuic acids in 30 varieties of barley grown in Canada ranges from 3.21 to 16.28 mg/kg of dry matter and from trace to 2.88 mg/kg of dry matter, respectively (Yu et al., 2001).

A number of bound phenolic acids, including ferulic, *p*-coumaric vanillic, sinapic and *p*-hydroxybenzoic acids, have been found in barley after alkaline hydrolysis (Van Sumere et al., 1972). Of these, ferulic and *p*-coumaric acids are the predominant phenolic acids in alkaline hydrolyzates of barley. Recently, Yu et al. (2001) reported the presence of protocatechuic, vanillic, chlorogenic and ferulic acids in acid hydrolyzate of barley. In addition, *p*-hydroxybenzoic, caffeic, and *p*-coumaric acids have been found in the hydrolyzates obtained following treatment of barley

TABLE 2.6

Content of Free Phenolic Acids in Developing Barley Seeds^a

| Phenolic Acid | Development after Anthesis (Days) | | | |
|--------------------------|-----------------------------------|-------|------|--|
| | 19 | 31 | 42 | |
| Salicylic | 0.85 | 0.28 | 0.06 | |
| <i>p</i> -Hydroxybenzoic | 5.65 | 2.76 | 1.82 | |
| Vanillic | 2.11 | 0.56 | 0.11 | |
| <i>o</i> -Coumaric | 0.31 | 0.12 | 0.11 | |
| Protocatechuic | 0.62 | 0.23 | 0.30 | |
| <i>m</i> -Coumaric | 0.76 | 0.21 | 0.12 | |
| Syringic | 0.92 | 1.72 | 0.36 | |
| <i>p</i> -Coumaric | 1.86 | 0.84 | 0.23 | |
| Ferulic | 14.80 | 12.72 | 2.58 | |
| Sinapic | 0.28 | 0.13 | 0.05 | |

^a Grams per kilogram of dry weight.

Source: Adapted from Slominski, B.A., 1980, *J. Sci. Food Agric.*, 31:1007–1010.

TABLE 2.7

Percentage Distribution of Ferulic and *p*-Coumaric Acids and DiFA^a in Barley Grain Fractions Separated Mechanically

| Variety | Grain Fraction | Ferulic Acid ^b | <i>p</i> -Coumaric Acid ^b | DiFA ^{a,b} |
|---------|-----------------|---------------------------|--------------------------------------|---------------------|
| Boira | I ^c | 47.5 | 77.7 | 78.0 |
| | II ^d | 52.5 | 22.3 | 22.0 |
| Iranis | I | 54.4 | 78.7 | 81.4 |
| | II | 45.6 | 21.3 | 18.6 |
| Volga | I | 47.6 | 82.3 | 86.3 |
| | II | 52.4 | 17.7 | 13.7 |

^a Ferulic acid dehydrodimers.

^b Percent=(grams in fraction/gram in grain)×100.

^c Husk and outer layer.

^d Combined intermediate and endosperm fractions.

Source: Adapted from Hernanz, D. et al., 2001, *J. Agric. Food Chem.*, 49:4884–4888.

with acid and α -amylase and also in hydrolyzates obtained after treatment of barley with acid, amylase, and cellulase. The phenolic acids are mainly located in the outer layers of the grain (Nordkvist et al., 1984), which contain 77.7 to 82.3 and 79.2 to 86.8% of the total amounts of ferulic and *p*-coumaric acids in barley grain, respectively (Table 2.7) (Hernanz et al., 2001).

Barley bran contains the same phenolic acids together with syringic and *o*-coumaric acids (Van Sumere et al., 1958). On the other hand, Nordkvist et al. (1984) reported the presence of vanillic, *p*-coumaric, ferulic and diferulic acids in husk, aleurone layer and endosperm of barley. The highest content of total insoluble bound phenolic acids (0.6 to 0.9%) is found in husk, testa and aleurone cells of barley. The endosperm contains only

trace amounts of insoluble bound phenolic acids. Ferulic acid is the predominant insoluble bound phenolic acid found in abraded fraction of barley grain enriched with aleurone layer; *p*-coumaric acid is dominant in the fraction containing a high level of husk. The walls of aleurone cells are rich in arabinoxylans and ferulic acid is known to be linked to this cell wall constituent (Mueller-Harvey et al., 1986; Smith et al., 1981). On the other hand, *p*-coumaric acid is known to form linkages with lignins (Higuchi et al., 1967) and cell walls of husk are considerably more lignified than other parts of the kernel (Salomonsson et al., 1980). Furthermore, *p*-coumaryl conjugate of agmatine (decarboxylated arginine) has been identified in germinated barley grains (Stoessl, 1965).

The content of ferulic acid, as determined in 11 barley varieties, ranges from 359 to 624 mg/kg of dry weight, while the level of *p*-coumaric acid is between 79 and 260 mg/kg of dry weight (Hernanz et al., 2001). Barley bran contains 6401 mg/kg of ferulic acid (free and bound) and 151 mg/kg of *p*-coumaric acid (Renger and Steinhart, 2000).

A number of ferulic acid dehydrodimers (DiFA) have also been identified in barley. These include 8-*O*-4'-DiFA, 8,8'-DiFA, 8,8'-aryl DiFA, 5,5'-DiFA, 8, 5'-DiFA benzofuran form and 8, 5'-DiFA open form (Renger and Steinhart, 2000; Stewart et al., 1994). Of these, 8-*O*-4'-DiFA is most abundant in the grain (73 to 118 mg/kg of dry weight) (Hernanz et al., 2001). The husk and outer layer contain from 79.2 to 86.8% of the total DiFA amount in barley grain (Table 2.7) (Hernanz et al., 2001). According to Renger and Steinhart (2000), 8,8'-aryl DiFA (178 mg/kg), 5,5'-DiFA (197 mg/kg), 8-*O*-4'-DiFA (163 mg/kg) and 8,5'-DiFA (135 mg/kg) are the main DiFA in barley bran (Table 2.8) (Figure 2.1). The distributions of DiFA in barley grain fractions are similar to those reported for wheat (Lempereur et al., 1997; Renger and Steinhart, 2000) and rye (Andreasen et al., 2000a, b; Renger and Steinhart, 2000). In plant material, ferulic acid dehydrodimers provide cross links between cell wall polymers (Ishii and Hiroi, 1990) that may contribute to cell wall extensibility (Kamisaka et al., 1990) and cell-cell adhesion (Waldron et al., 1997). In addition, ether glucosides, namely, *p*-hydroxybenzoic acid 4-*O*- β -glucoside, vanillic acid 4-*O*- β -glycoside, *o*-coumaric acid 2-*O*- β glucoside and ferulic acid 4-*O*- β -glucoside have been found in mature barley grains (Van Sumere et al., 1972).

Other Phenolics

The colored barley grains contain at least eight pigmented compounds that have been indentified as derivatives of cyanidin, delphinidin, and pelargonidin, as well as cyanidin-3-arabinoside and, possibly, cyanidin-3-glucoside (Briggs, 1978). Barley grains also contain a range of flavanols from monomeric, dimeric, and trimeric proanthocyanidins to higher molecular weight condensed tannins. These include (+)-catechin, (-)-epicatechin, dimeric prodelfinidin B3 and procyanidin B3, as well as trimeric procyanidin C2 and three trimeric prodelfinidins (Goupy et al., 1999; McMurrrough et al., 1983, 1996). The oligomeric proanthocyanidins are made of linked units of (+)-catechin and/or (+)-gallocatechin (Delcour and Tuytens, 1984; McMurrrough et al., 1996).

TABLE 2.8

Content of Ferulic Acid (FA) and Its Dehydrodimers (DiFA) in Cereal Brans^{a,b}

| Compound | Barley | Maize | Oats | Rice | Rye | Millet | Wheat |
|---------------|--------|-------|------|------|------|--------|-------|
| FA | 6289 | 9440 | 3282 | 3823 | 9193 | 18047 | 18884 |
| 8-8'aryl DiFA | 178 | 279 | 96 | 57 | 162 | 1313 | 147 |
| 8-8'DiFA | 71 | 67 | 47 | 15 | 188 | 393 | 127 |
| 8-5'DiFA | 135 | 175 | 84 | 32 | 117 | 322 | 146 |
| 8-O-4'DiFA | 163 | 255 | 107 | 41 | 124 | 374 | 182 |
| 5-5'DiFA | 197 | 239 | 132 | 36 | 123 | 281 | 205 |

^a After alkaline treatment with 4 mol/L NaOH (ester+ether linked).

^b Milligrams per kilogram.

Source: Adapted from Renger, A. and Steinhart, H., 2000, *Eur. Food Res. Technol.*, 211:422–428.

TABLE 2.9

Flavanol Content in Three Barley Varieties Grown in Ireland in 1981 (mg/kg)^a

| Barley Variety | (+)-Catechin | Procyanidin B3 | Prodelfinidin B3 | Trimers ^b | Total Polyphenols ^c |
|----------------|--------------|----------------|------------------|----------------------|--------------------------------|
| Ark Royal | 41 | 202 | 281 | 464 | 1460 |
| Emma | 29 | 130 | 218 | 388 | 1220 |
| Triumph | 25 | 138 | 186 | 413 | 1300 |

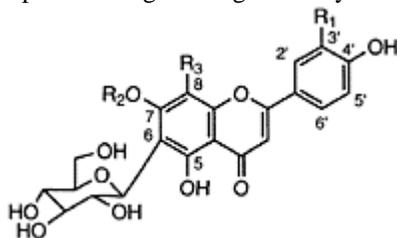
^a Milligrams per kilogram.

^b Sum of four components separated by HPLC.

^c Measured with Folin-Ciocalteu reagent.

Source: Adapted from McMurrough, I. et al., 1983, *J. Sci. Food Agric.*, 34:62–72.

Table 2.9 shows the content of flavanols in three barley varieties grown in Ireland, following their extraction into acetone-water. The monomeric, dimeric and trimeric flavanols collectively account for 58 to 68% of the total polyphenolic content (McMurrough et al., 1983). On the other hand, Griffith and Wayne (1982) reported that the content of water-extracted flavanols in 21 winter and spring barley varieties ranges from 0.11 to 0.17% and also found that prolonged storage of barley grains does not affect the content of flavanols. In addition, Overland et al. (1994) reported no detectable level of proanthocyanidin in the proanthocyanidin-free mutants of barley varieties, although the corresponding parent barley varieties contain from 0.19 to 0.27% of proanthocyanidins as determined by the vanillin-HCl assay. This mutation does not alter the chemical composition of grains significantly.



| Compound | R ₁ | R ₂ | R ₃ |
|--|------------------|----------------|----------------|
| Isoscoparin-7- <i>O</i> -β-D-glucoside | OCH ₃ | β-glucoside | H |
| Carlinoside | OH | H | α-arabinoside |
| Schaftoside | H | H | α-arablnoside |
| Isorientin-7- <i>O</i> -β-D-glucoside | OH | β-glucoside | H |
| Isovitexin-7- <i>O</i> -β-D-glucoside | H | β-glucoside | H |

FIGURE 2.4 Chemical structures of some flavone C-glycosides isolated from barley leaves.

Osawa et al. (1992) and Kitta et al. (1992) isolated a novel flavone C-glycoside, 2''-*O*-glycosylisovitexin, from green barley leaves and reported that its antioxidative activity is similar to that exhibited by α-tocopherol. Later, several more flavone C-glycosides, namely, isoscoparin 7-*O*-β-D-glucoside, carlinoside, shaftoside and 7-*O*-β-D-glucosides of isorientin and isovitexin (Figure 2.4), were identified in young barley leaves (Norbaek et al., 2000). According to Doll et al. (1994) and Christensen et al. (1998), these phenolics may contribute to resistance against leaf diseases.

Effect of Processing

Roasted barley has been used as a component of daily beverage in China for many centuries. Roasting barley for 1 min at 327 to 342°C reduces the content of catechins in the grain by more than 65%. An increase in the roasting temperature from 327 to 342°C does not have any significant effect on the content of catechins (Duh et al., 2001).

A novel pigment, hordeumin, is produced as a secondary product during ethanol fermentation of uncooked barley (Deguchi et al., 2000a; Ohba et al., 1992). Hordeumin is a product of oxidative polymerization of anthocyanins and tannins (Deguchi et al., 1999). According to Ohba et al. (1992), it contains cyanidin and delphinidin. Recently, Deguchi et al. (2000b) demonstrated that hordeumin possesses antimutagenic activity.

Brewer's spent grain, a solid barley residue from the brewing process (after mashing and filtration), contains five times more ferulic and *p*-coumaric acids, as well as from two to five times more ferulic acid dehydrodimers (DiFA), than unprocessed grains. However, the ratio of *p*-coumaric to ferulic acids in spent grain is similar to that of unprocessed barley. The spent grain contains 1860 to 1948 mg ferulic acid and 565 to 794 mg *p*-coumaric acid per kilogram of sample (Hernanz et al., 2001). Also, a marked increase in the ratio of DiFA to ferulic acid has been observed, thus indicating that some monomer forms of ferulic acid are solubilized during the mashing process. Bartolome et al. (1997) evaluated spent grains of barley as a potential source of ferulic acid and reported that the treatment of spent grain with esterase from *Aspergillus niger* (FAE-III) releases only 3.3% of the total ferulic acid present. The addition of xylanase from *Trichoderma viride* to the reaction mixture increases this amount to 30%.

BUCKWHEAT

Buckwheat (*Fagopyrum esculentum* Moench) belongs to the *Polygonaceae* family that includes plants such as rhubarb and Japanese indigo (Watanabe, 1998). Buckwheat is a minor crop compared with other cereals. In western Canada, buckwheat is considered an important alternative crop and is marketed to Japan to be used for production of soba noodles (Mazza, 1993). In the U.S., buckwheat endosperm is used for to manufacture pancake flour. Some buckwheat is also sold in the form of groats for use as breakfast cereal. The production of groats involves dehulling seeds that are adjusted to a moisture content of 22% and then subjected to 10- to 20-min heating at 150 to 164°C. The groats can be additionally roasted at 100 to 150°C for 1 to 2 h (Dietrych-Szostak and Oleszek, 1999).

Buckwheat has been considered a good dietary source of rutin (Ohara et al., 1989; Ohsawa and Tsutumi, 1995). The daily intake of 100 g of buckwheat would provide about 10% of a therapeutic dose of rutin of 180 to 350 mg/day (Schiller et al., 1990). The reported health effects of rutin include antiinflammatory, anti-carcinogenic, estrogen receptor binding, smooth muscle relaxation activities (Pisha and Pezzuto, 1994) and ability to lower the fragility of blood vessels (Iwata et al., 1990; Yildzogle-Ari et al., 1991). According to Deschner (1992), dietary rutin and quercetin, under low dietary fat intake, may significantly suppress colonic neoplasia.

Phenolic Acids

The chemistry of the common phenolics of buckwheat seed and product differs markedly from that of other cereal products. In buckwheat, the content of ferulic and hydroxycinnamic acids is low. Bran-aleurone fraction of buckwheat contains bound syringic, *p*-hydroxybenzoic, vanillic, and *p*-coumaric acids. These acids can be liberated by alkaline or acid hydrolysis, thus indicating the possible presence of phenolic acids in the form of esters and glycosides (Durkee, 1977). Zadernowski et al. (1992) have identified 20 and 14 phenolic acids in buckwheat groats and hulls, respectively. Of these, *p*-coumaric (4.6 mg/100 g), vanillic (1.7 mg/100 g), *p*-hydroxybenzoic (1.7 mg/100 g), and caffeic (1.3 mg/100 g) acids are the predominant phenolic acids in the groats; *p*-coumaric (3.6 mg/100 g), vanillic (1.65 mg/100 g), sinapic (1.4 mg/100 g) and gentisic (1.1 mg/100 g) acids are the major phenolic acids in the hulls. Recently, Watanabe et al. (1997) isolated, by preparative HPLC, and identified protocatechuic acid (13.4 mg/100 g) and 3,4-dihydroxybenzaldehyde (6.1 mg/100 g) in ethanolic extracts of buckwheat hulls.

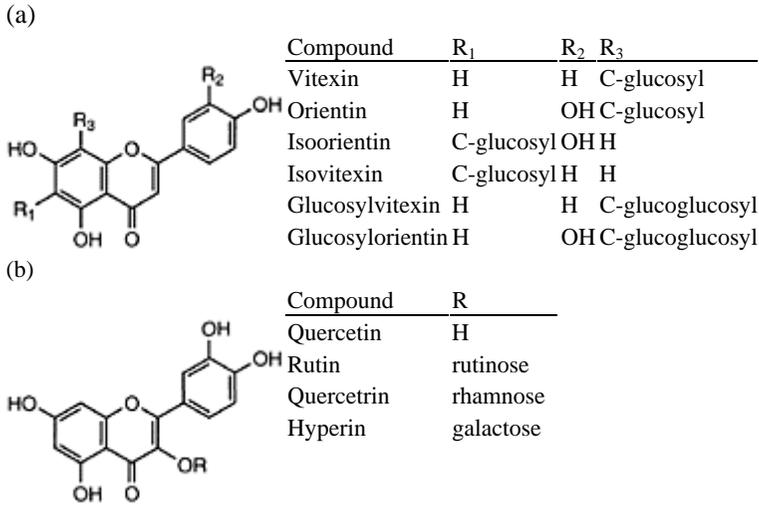


FIGURE 2.5 Chemical structures of (A) flavone and (B) flavonol derivatives.

Flavonoids

Buckwheat seeds serve as a rich source of flavonoids (Ohara et al., 1989; Ohsawa and Tsutsumi, 1995; Oomah and Mazza, 1996; Oomah et al., 1996). The seeds and hulls of Canadian buckwheat varieties contain, on average, 387 and 1314 mg/100 g of flavonoids, respectively (Oomah and Mazza, 1996). On the other hand, the total flavonoid content in the seeds and hulls of Polish buckwheat variety are 18.8 and 74 mg/100 g of dry matter, respectively (Dietrych-Szostak and Oleszek, 1999). Flavonols such as rutin, hyperin, quercitrin and quercetin (Sato and Sakamura, 1975) and flavones such as vitexin, isovitexin, orientin, and isoorientin have been identified in immature buckwheat seeds (Margna and Margna, 1982). Only six flavonoids have been identified in mature seeds and their hulls. Rutin and isovitexin are the only flavonoids of seeds, although the hulls contain rutin, quercetin, orientin, vitexin, isovitexin and isoorientin (Figure 2.5) (Dietrych-Szostak and Oleszek, 1999). In addition, Watanabe (1998) isolated and identified four catechins, namely, (-)-epicatechin, (+)-catechin 7-*O*- β -D-glucopyranoside, (-)-epicatechin 3-*O*-*p*-hydroxybenzoate, and (-)-epicatechin 3-*O*-(3,4-di-*O*-methyl)gallate in the ethanolic extracts of buckwheat groats (Figure 2.6).

The concentration of flavonoids in buckwheat is affected by several factors, including location, growth conditions and variety. According to Oomah and Mazza (1996) and Oomah et al. (1996), geographic location is the major factor responsible for variation in the total flavonoid and rutin contents in seeds; however, growing conditions also have a significant impact on the total content of flavonoids in the hulls. The hull fraction comprises 25.6 to 34% of the seed weight (Oomah and Mazza, 1996). The content of rutin in buckwheat seed may range from 12.6 to 51.1 mg/100 g (Kitabayashi et al., 1995; Oomah and Mazza, 1996), while in the hulls it may range from 4.3 to 85.3 mg/100 g

(Dietrych-Szostak and Oleszek, 1999; Oomah and Mazza, 1996; Watanabe et al., 1997). The content of hyperin and quercetin in buckwheat hulls is 5.0 and 2.5 mg/100 g, respectively (Figure 2.5).

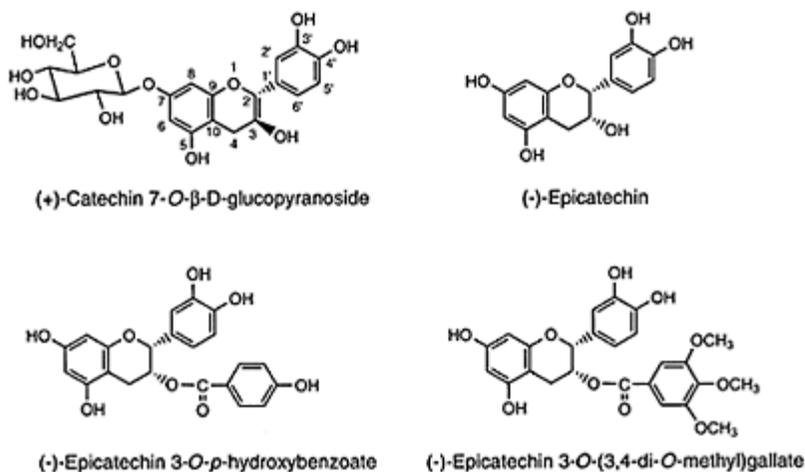


FIGURE 2.6 Chemical structures of catechins found in buckwheat groats.

Soluble condensed tannins of buckwheat are based on pelargonidin and cyanidin structures (Durkee, 1977). The presence of condensed tannins in buckwheat has also been confirmed by Luthar and Kreft (1996). Later, Watanabe et al. (1997) detected the presence of condensed tannins in two fractions of buckwheat phenolics separated on a Sephadex LH-20 column. Gel permeation chromatography of acetyl derivatives of these fractions indicated that buckwheat condensed tannins are a mixture of proanthocyanidins with various degrees of polymerization.

Effect of Processing

Kreft et al. (1999) determined the content of rutin in the husk, bran and flour milling fractions of Siva variety of buckwheat seeds grown in Slovenia. The bran fraction contains 13.1 to 47.6 mg rutin/100 g, the flour fraction 1.9 to 16.8 mg rutin/100 g and the husk fraction 2.9 mg rutin/100 g. Heat treatment of seeds prior to dehulling affects the content of rutin in the groats considerably. Untreated groats contain 18.8 mg rutin/100 g, while the content of rutin in heat-treated groats ranges from 17.76 mg/100 g (10 min at 150°C) to 11.6 mg/100 g (2 h and 10 min at 150°C). Steaming the seeds for 20 min at 164°C, followed by 50-min treatment with steam at 150°C, reduces the rutin content by almost 75% (Dietrych-Szostak and Oleszek, 1999).

CORN

Production of corn in North America constitutes more than half of the world's supply (Benson and Pearce, 1987). In the U.S. and Canada corn is primarily used as a feed

TABLE 2.10

Total Phenolic Acid Content in Some Cereal Flours^a

| Phenolic Acid | Wheat | Rice | Oats | Corn |
|-----------------------------------|-------|-------|-------|-------|
| <i>p</i> -Hydroxybenzoic | Trace | 5.0 | 1.4 | 1.3 |
| (<i>p</i> -Hydroxyphenyl) acetic | – | Trace | 0.4 | 1.1 |
| Vanillic | 3.6 | 2.1 | 4.2 | 3.7 |
| Protocatechuic | Trace | – | 0.5 | 3.0 |
| Syringic | 4.2 | 0.2 | 5.3 | 11.5 |
| <i>Cis-p</i> -Coumaric | – | Trace | – | Trace |
| <i>Trans-p</i> -Coumaric | Trace | 1.3 | 2.0 | 18.9 |
| <i>Cis</i> -Ferulic | Trace | 1.9 | 2.6 | 6.5 |
| <i>Trans</i> -Ferulic | 63.6 | 75.1 | 63.7 | 258.6 |
| Caffeic | Trace | – | 2.6 | 4.5 |
| <i>Cis</i> -Sinapic | Trace | Trace | Trace | – |
| <i>Trans</i> -Sinapic | Trace | Trace | 4.3 | Trace |
| Total | 71.4 | 85.6 | 87.0 | 309.1 |

^a Milligrams per kilogram.

Source: Adapted from Sosulski, F. et al., 1982, *J. Agric. Food Chem.*, 30:337–340.

crop, but it can also be a food crop, especially in the developing countries. A number of food ingredients are produced from corn, among them corn grits, corn meal and corn flour. Corn flour is an ingredient of ready-to-eat breakfast cereals as well as a component in numerous dry mixes such as those for pancakes, muffins and doughnuts (Rooney and Serna-Saldivar, 1987).

Phenolic Acids

Yellow dent corn flour contains 309.1 mg/100 g of phenolic acids (Sosulski et al., 1982). Corn flour contains three times more phenolic acids than cereal flours obtained from rice, wheat and oats (Table 2.10). Phenolic acids of corn are in the free, esterified and insoluble bound forms. Of these, the insoluble bound phenolic acids are the predominant fraction, constituting 69.2% of the total amount of phenolic acids (Grabber et al., 2000; Sosulski et al., 1982).

Some phenolic acids may be linked covalently to amine functionalities through a “pseudo peptide” bond (Figure 2.7). Presence of feruoylputrescine, diferuloylputrescine, *p*-coumarylspermidine, diferuloylspermidine and diferuloylspermine has been reported in the embryo and endosperm of corn (Martin-Tanguy et al., 1982). Furthermore, *p*-coumaryltryptamine and feruloyltryptamine have been isolated from whole corn grains

(Ehmann, 1974). Certain physiological roles have been ascribed to some of these conjugates. According to Martin-Tanguy et al. (1978, 1982), phenolic acids coupled to alkyl amines may exhibit antiviral activity and may also serve as biochemical markers closely associated with pollen and ovule fertility. Three novel ferulic acid glycosides have been isolated from acid hydrolysis of corn hulls. The structures of these feruloylated disaccharides have been identified as *O*-(2'-*O*-trans-feruloyl- α -L-arabinofuranosyl-(1 \rightarrow 3)- β -D-xylopyranose, *O*-[2'-*O*-methoxy-5'-*O*-(*E*)-feruloyl]- α -L-arabino-furanosyl-(1 \rightarrow 3)- β -D-xylopyranose, and *O*-[2'-*O*-methoxy-5'-*O*-(*Z*)-feruloyl]- α -L-arabino-furanosyl-(1 \rightarrow 3)- β -D-xylopyranose (Hosny and Rosazza, 1997).

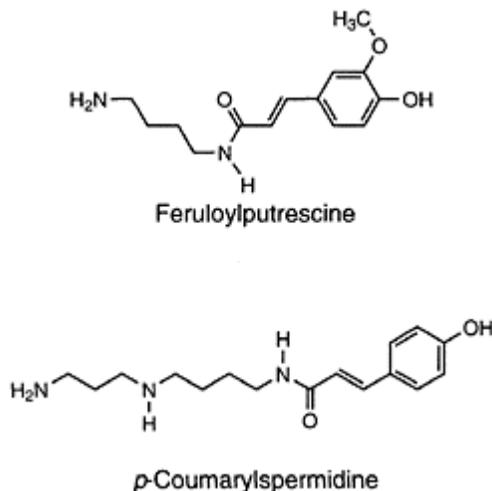


FIGURE 2.7 Chemical structures of some phenolic acid conjugates of amines found in corn.

Stanols and sterols ferulates such as sitostanyl and campestanyl ferulates as well as sitosteryl and campesteryl ferulates have been found in corn; sitostanyl ferulate is the predominant ferulate in corn (Figure 2.8). The total concentration of these ferulates in six samples of corn ranges from 31 to 70 mg/kg. These compounds are mostly located in the inner pericarp (Seitz, 1989). Norton (1994) isolated and identified 16 derivatives of sterols esterified to *p*-coumaric and ferulic acids; the content of these esters in corn bran was between 70 and 540 mg/kg of bran and ranged from 180 to 8600 mg/kg of unrefined corn oil (Norton, 1995).

Other Phenolics

Phlobaphene and anthocyanidin pigments are commonly found in maize (Styles and Ceska, 1975); pelargonidin-3-glucoside and cyanidin-3-glucoside have been found in the aleurone tissue of maize (Harborne and Gavazzi, 1969). On the other hand, maize flowers contain cyanidin 3-glucoside, cyanidin 3-(6''-malonylglucoside), cyanidin 3-(3'',6''-

dimalonylglucoside), peonidin 3-glucoside, peonidin 3-(6"-malonylglucoside), and peonidin 3-(dimalonylglucoside) (Fossen et al, 2001). Phlobaphene pigments are located mostly in the cob and pericarp tissues, whereas anthocyanidins are found in all tissues. Synthesis of phlobaphenes in maize plants is controlled genetically by P locus (Styles and Ceska, 1977). Moreover, monomeric flavan-3,4-diols, namely, leucopelargonidin and leucocyanidin, have been isolated from the aleurone tissue (Reddy, 1964). Presence of two flavonols, kaempferol and quercetin, has also been reported in hydrolyzates of

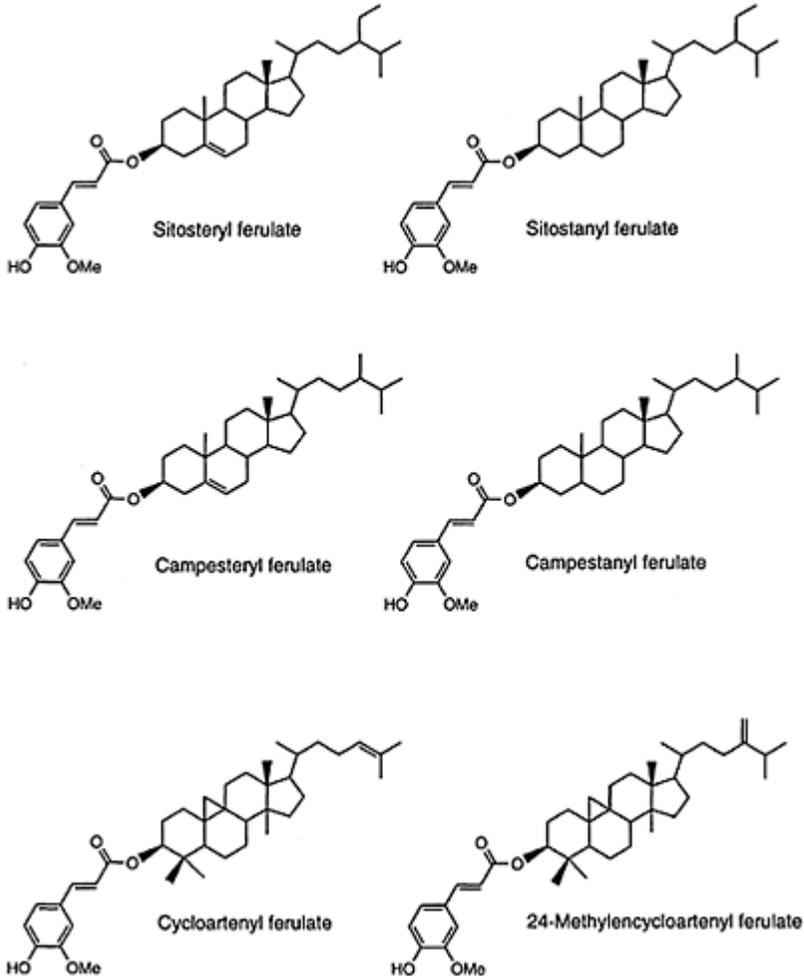


FIGURE 2.8 Chemical structures of some ferulates of cycloartenols, stanols, and sterols.

aleurone tissue of corn (Kirby and Styles, 1970). In addition, 4-vinylguaiacol and 4-vinylphenol have been identified in volatile components from tortilla chips. Of these,

only the 4-vinylguaiacol (1.5 mg/kg) is present at levels exceeding the threshold value, and therefore may contribute to the flavor of tortilla chips (Buttery and Ling, 1998).

MILLETS

The term millet is used for any small-seeded annual grass that is of minor importance in the West but regarded as a staple food in Africa and Asia (Schery, 1963). Five millets, namely, *Setaria italica*, *Pennisetum glaucum* (also known as *Pennisetum typhoideum*), *Eleusine coracana*, *Echinochloa frumentacea*, and *Panicum miliaceum*, are common. *Panicum miliaceum* is also known as Proso millet, *Pennisetum typhoideum* as pearl millet and *Eleusine coracana* as finger millet.

Pearl millet is a major source of protein, essential minerals and calories in many developing countries. It is widely grown in Africa and Asia as a food grain (Desai and Zende, 1979); however, considerable quantities of phytic acid and polyphenols found in millet (Mahajan and Chauhan, 1987) lower the digestibility of millet proteins and starches (Khetarpaul, 1988) as well as the availability of its minerals (Mahajan and Chauhan, 1988). Polyphenols are also responsible for the gray pigmentation of grains, which may be objectionable (Reichert et al., 1980b). The total content of polyphenols in the millet grain may range from 0.5 to about 0.8% (Deokar, 1987; Khetarpaul and Chauhan, 1990). The content of ferulic acid dehydrodimers (DiFA) (Figure 2.1) in millet bran is up to 10 times higher than that of those found in rice, maize, oat, rye and wheat bran. The 8,8'-aryl DiFA (1313 mg/kg) is the most abundant DiFA, comprising about 50% of the total content of DiFA in millet bran (Table 2.8) (Renger and Steinhart, 2000).

Millet pigments are methanol soluble and pH sensitive. The color of millet flour paste can be changed reversibly from gray to yellow-green at alkaline pH and partially and reversibly from gray to creamy white in the presence of acid. Glucosylvitexin, glucosylorientin, and vitexin, a flavonoid compound, (Figure 2.5) have been found to be responsible for the yellow-green discoloration of millet flour paste in the presence of base. These pigments are also known as C-glycosylflavones and are characterized by having a sugar bound via carbon-carbon bond to the flavonoid nucleus. This sugar group is not hydrozylable by normal acid and enzymatic hydrolysis procedures. Depending on the variety, the whole millet may contain from 87 to 259 mg/100 g of C-glycosylflavones expressed as glucosylvitexin equivalents (Reichert et al., 1980b). It has also been found that glucosylvitexin, glucosylorientin and vitexin are present in pearl millet in ratios of 29:11:4, respectively (Reichert, 1979).

Pearl millet varieties also contain substantial quantities of alkali-labile ferulic acid (ALFA) ranging from 133 to 241 mg/100 g. It has been suggested that ferulic acid is esterified to glucose, quinic acid or other sugars or amino acids and it may be responsible for pH-sensitivity of methanol-extracted pearl millet flours (Reichert et al., 1980b).

The concentration of C-glycosylflavones and ALFA not only depends on the pearl millet variety but is also affected by dehulling processes. The greater the degree of abrasive dehulling of millet grain the lower is the content of these phenolics (Figure 2.9) in the dehulled millets (Reichert et al., 1980b).

The gray pigmentation of pearl millet is probably due to more complex interactions involving chelation of phenolics *in vivo* with ions of Cu, Fe, Al, or other metals. In

addition, copigmentation effects that enhance colors may be due to changes in the degree of ionization of the phenolics (Reichert, 1979).

Proso millet may be used as puffed or cooked breakfast cereal or as a replacement for wheat flour in certain baked products (Hinze, 1972). It contains 0.055 to 0.178% tannins, expressed as (+)-catechin equivalents. Dark-colored seeds have the highest content of tannins. The dehulling process reduces the content of tannins by 65 to 80% because the hulls contain 15 to 40 times more tannins than the dehulled grains (Lorenz, 1983).

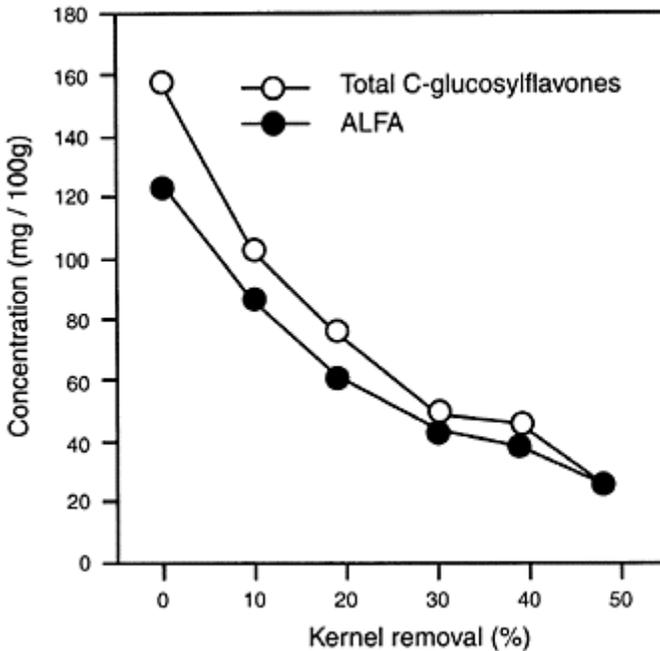


FIGURE 2.9 Concentrations of phenolics in dehulled, methanol-extracted millet flour. (Adapted from Reichert, R.D., 1979, *Cereal Chem.*, 56:291–294.)

Finger millet is an important food crop in India and Africa. Protocatechuic, gallic, and caffeic acids were the predominant free phenolic acids, while ferulic, caffeic, and coumaric acids were the major bound phenolic acids found in finger millets (Subba Rao and Muralikrishna, 2002). This cereal grain contains a much higher level of tannins than proso millets. Of the 19 Indian and 10 African finger millet varieties examined, two Indian cultivars contained approximately 1% tannins while two African cultivars had a tannin content of over 3%. High levels of tannins in finger millets with dark seed coat have been linked with poor protein digestibility; these tannins have been found mostly in the glutelin fraction of finger millet proteins (Ramachandra et al., 1977). The dehulling process removes 88.1 to 100% of tannins originally present in grains, depending on the

seed variety (Ramachandra et al., 1977). On the other hand, 24-h fermentation of finger millet flour with endogenous grain microflora reduces the content of phenols and tannins by 20 and 52%, respectively (Antony and Chandra, 1998); while 96-h of malting of finger millet seeds brought about 3-fold and 2-fold decrease in protocatechuic and bound phenolic acid content, respectively (Subba Rao and Muralikrishna, 2002).

Japanese barnyard millet is a traditional food in colder parts of Japan. It is a rich source of protein, lipid, vitamins B₁ and B₂, and nicotinic acid (Watanabe, 1999). This cereal is low in phenolics and tannins (Suman et al., 1992). Recently, Watanabe (1999) identified three phenolic compounds, namely, N-(*p*-coumaroyl)serotonin, luteolin and tricetin, in the ethanolic extracts of the Japanese barnyard millet. Of these, N-(*p*-coumaroyl)serotonin exhibited antiinflammatory activity (Kawashima et al., 1998).

OATS

Oats are produced worldwide and differ from other cereals in that the bran is not separated from the endosperm during milling and that they are used primarily as breakfast cereal or as feed for animals. Oat products are also used in granola bars, infant foods, and components of breads and cookies, as well as thickening agents in soups, gravies, and sauces. However, despite broad uses of oats and their products in foods, little work has been done to characterize oat grain phenolics. Recently, an excellent review on phenolics in oat groats and hulls reported the presence of derivatives of benzoic and cinnamic acids as well as quinones, flavones, flavonols, chalcones, flavanones, anthocyanidins and amino phenolics (Peterson, 2001; Peterson et al., 2001). The structures and functionality of some conjugated phenolics of oats were summarized by Collins (1986).

Phenolic Acids

Durkee and Thiverge (1977) reported that ferulic acid is a major phenolic acid present in the soluble bound and insoluble bound phenolic acid fractions of oats. These authors also identified traces of free *p*-coumaric, ferulic and vanillic acids in oats. Presence of vanillic, sinapic, *p*-coumaric, *p*-hydroxyphenylacetic, caffeic, protocatechuic, syringic and *p*-hydroxybenzoic acids in bound forms has also been reported (Durkee and Thiverge, 1977; Sosulski et al., 1982). Later, Zadernowski et al. (1992) detected *o*-coumaric and *p*-phenyllactic acids as well as traces of veratric acid. According to Sosulski et al. (1982), oat flour (Harmon variety) contains approximately 87 mg/kg of total phenolic acids (Table 2.10). Of these, 66.3% are in the insoluble bound form and 20.6% as soluble bound phenolic acids.

Bound phenolic acids of oat may be coupled to long chain ω -hydroxy fatty acid, long-chain alcohols, glycerol, sugars, polysaccharides, lignins and amines (Collins, 1986). For example, 30% of phenolic acid diesters found in oat flour are the derivatives of octacosan-1, 28-diol (Daniels and Martin, 1965). Of these, esterified phenolic acids coupled to n-alkanols possess significant antioxidant activities due to their lipophilic properties (Daniels and Martin, 1961). Approximately 36% of a mixture of oat antioxidants was glycerol esters of phenolic acids (Daniels and Martin, 1968). Oat hulls and groats may also contain catechol, coniferyl alcohol, gallic acid, *p*-hydroxy-

phenzaldehyde, salicylic acid, and vanillin (Emmons and Peterson, 1999; Xing and White, 1997).

Avenanthramides

Oat groats contain at least 25 avenanthramides and N-acylanthranilate alkaloids, while oat hull extracts contain only 20 of these compounds (Figure 2.10). These compounds are anionically substituted cinnamic acid conjugates (Collins, 1989; Collins and Mullin, 1988). The complete chemical structures of five avenanthramides have been elucidated and identified as avenanthramides. The content of avenanthramides in dehulled oats is 200 to 800 mg/kg (Collins, 1986). Oats contain from 40 to 132 mg/kg of avenanthramide B (Dimberg et al., 1993); oat groats have 25 to 47 mg/kg of avenanthramide A, 21 to 43 mg/kg of avenanthramide B and 28 to

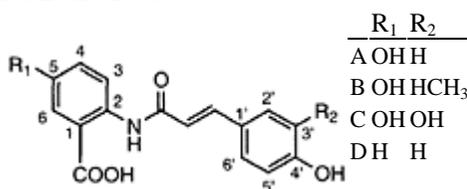


FIGURE 2.10 Chemical structures of some avenanthramides.

62 mg/kg of avenanthramide C (Dimberg et al., 1996). In a review, Peterson (2001) recently reported some unpublished results from his laboratory: oat groats contain 54, 36 and 52 mg/kg and hulls 25, 17 and 25 mg/kg of avenanthramides A, B and C, respectively. Presence of nine novel bound phenolic acids in oats was reported by Collins et al. (1991), three of which were identified as avenalamic acid and its 3'-hydroxy and 3'-methoxy derivatives. These phenolics are ethylenic homologues of *p*-coumaric, caffeic and ferulic acids covalently bound to the amine group of hydroxy-substituted *o*-aminobenzoic acid.

Resistant to acid hydrolysis, avenanthramides are slowly hydrolyzed in the presence of alkali, with slight decomposition, to the corresponding substituted cinnamic and anthranilic acids (Collins, 1989). Two avenanthramides, namely, N-(4'-hydroxy-3'-methoxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (avenanthramide B) and N-(4'-hydroxy-3'-methoxy-(E)-cinnamoyl)-5-hydroxy-4-methoxyanthranilic acid (a novel avenanthramide A₂), have been found to be heat stable (Dimberg et al., 1993). Some N-cinnamoylanthranilic acids have been described in the literature to possess antiallergic, antihistaminic and antiasthmatic activities (Devlin and Hargrave, 1985).

Other Phenolics

American varieties of oats contain from 19 to 30.3 mg of total tocopherols per kilogram (Peterson and Qureshi, 1993), while those grown in Hungary have 15 to 48 mg of total tocopherols per kilogram (Laszti et al., 1980). Alpha-tocopherol and α -tocotrienol are the predominant tocopherols found in oats, contributing 86 to 91% to the total amount (Peterson

and Qureshi, 1993). Apigenin, luteolin, and tricetin have been identified in oat flour based on their UV spectra, co-chromatography with authentic compounds, mass spectroscopy and effects of diagnostic shift reagents (Collins, 1986).

Effect of Processing

Heating oats with hulls (at 100°C for 10 min) significantly increases the content of vanillic acid, vanillin, *p*-coumaric acid, *p*-hydroxybenzaldehyde and coniferyl alcohol in groats. This increase (25 to 900%) in phenolic content may be due to their release from hulls and their diffusion into the groats during processing (Dimberg et al., 1996).

Emmons et al. (1999) evaluated the content of phenolics in various milling fractions of oats. The total content of phenolics in pearlings and dried oat groat flours ranged from 274 to 342 and from 89 to 156 mg/kg, respectively. Pearlaring fractions contained higher amounts of avenanthramides (137 to 185 mg/kg) than oat groat flours (39 to 76 mg/kg). The higher levels of phenolics in pearlaring samples might indicate that more phenolics are located in the seed coat, aleurone and subaleurone layers. The presence of avenanthramides in oat hulls has also been reported (Emmons and Peterson, 1999). On the other hand, extrusion of ground oat grains brings about a 50% loss in their content of soluble phenolic compounds (Zademowski et al, 1999).

RICE

Grown and harvested in more than 100 countries (Luh, 1980), rice is a staple food for over half of the world's population. The milling of rice includes removing hulls to produce brown rice and, following this, removing bran and polishing kernels.

Phenolic Acids

Rice flour contains 856 mg/kg of phenolic acids and in this respect is similar to oat and wheat flour (Table 2.10). Ferulic acid is the major phenolic acid present in rice flour. Approximately 74.2% of the total phenolics in rice are in the insoluble bound form; therefore, phenolic acids have little effect on the color or flavor of aqueous slurries of rice. However, wet processing, especially under alkaline conditions, and cooking or baking may bring about release of some bound phenolics, which will increase their contribution to sensory characteristics of the product (Sosulski et al., 1982). Table 2.8 shows that insoluble bound phenolics have been found to be cell wall components (Harris and Hartley, 1976; Renger and Steinhart, 2000). Moreover, the acids are considered to be present as phenolic-carbohydrate esters because they are released by alkaline solvents. The alkaline extracts of rice endosperm cell walls contain 9.1, 2.5 and 0.56 mg/g of ferulic acid, *p*-coumaric acid and dehydrodimers of ferulic acid, respectively. Dehydrodimers of ferulic acid, a dimeric product of ferulic acid (Figure 2.1), are produced by oxidative coupling (Shibuya, 1984). Rice bran contains a number of sugar-amino acid-phenolic acid conjugates, of which seven contain ferulic acid and at least one an acidic amino acid (Yoshizawa et al., 1970).

Cooked rice has a highly desirable and characteristic odor; however, the specific odor of rice bran is considered an obstacle in its efficient utilization as a food component.

Commercially available polished rice contains trace quantities of rice bran, which may affect the flavor characteristics of cooked rice. This effect depends on the quantity of residual bran in rice before cooking. The volatiles of rice bran contain alcohols, carbonyl compounds, carboxylic acids and phenols. Seven phenols (namely, 4-vinylphenol, 4-vinylguaiacol, guaiacol, phenol, *o*-cresol, *p*-cresol and 3,5-xyleneol) have been identified in steam volatile concentrate of rice bran. Of these, 4-vinylphenol, with a characteristic unpleasant flavor, is the predominant phenolic component present. Products of thermal decarboxylation of ferulic and *p*-coumaric acids, respectively, 4-vinylphenol and 4-vinylguaiacol can be produced during cooking of rice (Fujimaki et al., 1977).

During the traditional production of parboiled rice, paddy grains are soaked in water consecutively for 2 to 3 days, resulting in a considerable loss of dry matter.

TABLE 2.11
Changes in Phenol Content in Rice, Husk and Bran prior to and after Soaking for 72 h^a

| Rice/Component | Before Soaking | After Soaking |
|----------------|----------------|---------------|
| Paddy rice | 0.408 | 0.478 |
| Brown rice | 0.276 | 0.322 |
| Milled rice | 0.148 | 0.418 |
| Husk | 1.284 | 1.361 |
| Bran | 2.722 | 3.792 |

^a Grams of phloroglucinol equivalent per kilogram on dry weight basis.

Source: Adapted from Singaravadel, K. and Raj, S.A., 1979, *J. Food Sci. Technol.*, 16:77–78.

Phenolics are among the components leached out from the grains during soaking. The phenol content of the water in which the grains are soaked increases markedly with the increase in soaking time. This increase is relatively higher in milled and brown rice than in paddy grains. Thus, the intact testa and pericarp might act as barriers for permeation of phenols from whole paddy grains. On the other hand, soaking rice in water also augments the level of phenolic constituents in the grain (Table 2.11) due to their liberation from conjugated phenolic glycosides by the action of β -glucosidase (Singaravadel and Raj, 1979).

Other Phenolics

Isovitexin, a flavonoid compound also known as C-glucosylflavone, has been reported in rice hulls (Figure 2.5). This flavonoid shows good antioxidant activity and plays an important role in postharvest storage of rice by providing a chemical defense for rice germs against oxidative deterioration (Osawa et al., 1985; Ramarathnam et al., 1988, 1989). Moreover, anisole, *m*-hydroxybenzaldehyde, vanillin (4-hydroxy-3-methoxybenzaldehyde), and syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde) have been identified in methanolic extracts of wild rice hulls (Asamarai et al., 1996). Shin and Godberg (1996) reported that rice bran obtained from mixed long-grain varieties (Tebonnet and Lemont) contained 3.1 mg of oryzanol per kilogram of sample and that irradiation of rice bran at 10 kGy reduced its oryzanol content to 2.4 mg/kg.

Rice bran also contains feruloyl esters of triterpene alcohols and sterols (Figure 2.8) (Akihisa et al., 2000; Diack and Saska, 1994; Norton, 1995). According to Norton (1995), the total content of feruloyl esters (ferulates of cycloartenol, 24-methylenecycloartanol, campesterol, and sitosterol) of rice bran is 3.4 g/kg of rice bran. Recently, Akihisa et al. (2000) identified six novel feruloyl esters of triterpene alcohols and sterols in the methanolic extract of rice bran: cycloeucaleanol *trans*-ferulate, 24-methylencholesterol *trans*-ferulate, cycloartenyl *cis*-ferulate, 24-methylenecycloartanyl *cis*-ferulate, sitosteryl *cis*-ferulate and 24-methylcholesterol *cis*-ferulate. These ferulates display good antiinflammatory activities against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear inflammation in mice (Akihisa et al., 2000). Moreover, cycloartenol *trans*-ferulate exhibits a strong inhibitory effect against the tumor-promoting activity of TPA in mice skin carcinogenesis initiated by 7,12-dimethylbenz[a]anthracene (DMBA) (Akihisa et al., 1997; Yasukawa and Akihisa, 1997; Yasukawa et al., 1998).

RYE

Because it can be grown under various conditions, rye (*Secale cereale* L.) is one the most widely distributed cereal grains. Rye plant can be grown in sandy soil of low fertility, at high altitudes and in the cool temperate zones, as well as in semiarid regions near deserts. Germany, Poland and Russia are the main rye-producing countries; the rye crop is used as flour for bread and for animal feed (Bushuk, 1976).

Weidner et al. (1999) reported that grains of Polish rye cultivars contained 14.6 to 15.0 mg/kg of free phenolic acids, 89.9 to 151.9 mg/kg of phenolic acids liberated from esters and 16.9 to 17.4 mg/kg of phenolic acids liberated from soluble glycosides. Ferulic, caffeic, *p*-coumaric and sinapic acids are the predominant phenolic acids in rye grains. Andreasen et al. (1999) also reported that ferulic acid (1000 mg/kg of dry matter) is the most abundant phenolic acid in rye grains (cv. Esprit) followed by sinapic (130 mg/kg of dry matter) and *p*-coumaric acids (60 mg/kg of dry matter). Caffeic, vanillic, protocatechuic and *p*-hydroxybenzoic acids are present in rye in minor quantities (<20 mg/kg of dry matter).

Recently, Andreasen et al. (2000b) measured the content of phenolic acids in the kernels of 17 rye varieties grown in one location in Denmark during 1997 and 1998. Their content of ferulic acid ranged from 900 to 1170 mg/kg, sinapic acid between 70 and 140 mg/kg and *p*-coumaric acid from 40 to 70 mg/kg. Andreasen et al. (2001) have also reported that 8-*O*-4'-DiFA is the predominant ferulic acid dehydrodimer in rye kernel, followed by 8,5'-DiFA benzofuran and 5,5'-DiFA (Figure 2.1). The total content of ferulic acid dehydrodimers in whole rye grain ranges from 241 to 409 mg/kg of dry matter (Andreasen et al., 2000b). Rye bran contains 10 to 15 times more dehydrodimers of ferulic acid than the corresponding flour fraction (Andreasen et al., 2000a). Subsequently, Renger and Steinhart (2000) reported that 8,8'-DiFA is the most abundant dehydrodimer of ferulic acid in rye bran, followed by 8,8' aryl DiFA, 8-*O*-4'-DiFA and 5,5'-DiFA (Table 2.8). In addition, vanillin has been identified as a contributor to the flavor of sour dough rye bread crumbs (Kirchhoff and Schieberle, 2001). Subsequently, Hakala et al. (2002) investigated the distribution of steryl ferulates in rye. The grain used in this study contained 55 to 64 mg/kg of total steryl ferulates, which are predominantly

located in rye bran (150 to 251 mg/kg of total steryl ferulates). Campestanil and sitostanil ferulates are the predominant ferulates in rye (Figure 2.8).

SORGHUM

Sorghum is an important grain for human consumption in parts of Asia and Africa; in the U.S., it is used primarily as a source of animal feed (Strumeyer and Malin,

TABLE 2.12

Distribution of Tannins in Different Protein Fractions of High-Tannin Sorghum Seeds (IS 2825)

| Grain Component | Tannins (%) | Proportion of Total Tannins (%) |
|---|-------------|---------------------------------|
| Albumin and globulin | 0.79 | 22.96 |
| Prolamin | 1.05 | 30.52 |
| Glutelin | 0.50 | 14.54 |
| Total extraction | 2.34 | — |
| Tannin content | 3.44 | — |
| Recovery of tannin in protein fractions | 68.02 | — |

Source: Adapted from Chavan, J.K. et al., 1979, *J. Food Biochem.*, 3:13–20.

1975). Sorghum varieties have been subdivided into three groups according to their level of phenolics, which are primarily proanthocyanidins (Butler and Price, 1977; Cummings and Axtell, 1973). Group I sorghums do not have pigmented testa, but contain a low level of phenols and no tannins. Groups II and III sorghums have pigmented testa. Although tannins of group II sorghums may be extracted into acidic methanol (1% HCl) and not into methanol alone, tannins of group III sorghums may be extracted into methanol and acidic methanol. The color and pigmentation of sorghum pericarp is known to be controlled by R, Y, B₁, B₂, and S genes (Nip and Burns, 1969, 1971; Rooney and Miller, 1982). Nearly all sorghums, regardless of their color, contain pigments or pigment precursors. The content and color of these pigments changes with the previously mentioned genes. The R and Y genes determine the color of the pericarp and the B₁ and B₂ genes control the presence of pigmented testa. Dominant genes R and Y greatly increase the levels of anthocyanidin pigments in groups I and II sorghum (Hahn and Rooney, 1986).

Polyphenolic compounds in sorghum include mainly flavonoids, anthocyanidins, flavanols (tannins) and phenolic acids (Butler, 1989; Hahn et al., 1984; Woodhead, 1981). However, sorghum grains are tannin free (Hoseney et al., 1981) or contain trace quantities of hydrolyzable tannins (Bullard and Elias, 1980). The condensed tannins (also called proanthocyanidins) are found in some bird-resistant varieties of sorghum (Butler, 1982, 1989, 1992).

Sorghum tannins readily associate with sorghum proteins. About 68% of total tannins are recovered from albumins and globulins, prolamins and glutelins fractions (Table 2.12) (Chavan et al., 1979b). However, it remains unknown whether such types of tannin-protein complexes exist *in situ* or are formed only during the extraction process.

Phenolic Acids

Tentative identification of free and insoluble bound phenolic acids of *Sorghum bicolor* (L.) Moench has been reported by Hahn et al. (1983). Free phenolic acids

TABLE 2.13

Free and Bound Phenolic Acid Composition of Several Sorghum Varieties^a

| Phenolic acid | SC0748 | | IS2327 | | SC0719 | |
|--------------------------|--------|-------|--------|-------|--------|-------|
| | Free | Bound | Free | Bound | Free | Bound |
| Gallic | – | 13.2 | – | – | – | 26.1 |
| Protocatechuic | 11.0 | 11.5 | 7.0 | 98.4 | 8.0 | 15.8 |
| <i>p</i> -Hydroxybenzoic | 10.1 | 23.7 | 2.3 | 23.6 | 9.3 | 24.2 |
| Vanillic | 15.5 | – | 126.7 | – | 23.3 | 27.4 |
| Caffeic | 6.0 | 44.6 | 6.3 | 17.0 | 8.7 | 26.8 |
| <i>p</i> -Coumaric | 109.1 | 123.0 | 47.4 | 53.5 | 6.4 | 79.9 |
| Ferulic | 74.0 | 213.0 | 18.2 | 139.0 | 26.0 | 91.9 |
| Cinnamic | 4.7 | – | 2.0 | – | – | 9.4 |

^a Milligrams per kilogram, dry weight basis.

Source: Adapted from Hahn, D.H. et al., 1983, *Cereal Chem.*, 60:255–259.

have been extracted from sorghum grain with methanol and the residue then subjected to acid hydrolysis in order to liberate bound phenolics. Gallic, protocatechuic, *p*-coumaric, *p*-hydroxybenzoic, vanillic, caffeic, ferulic and cinnamic acids have been identified in sorghum extracts (Table 2.13). Moreover, 12 other peaks have been separated, but not identified.

Protocatechuic, *p*-hydroxybenzoic, caffeic, *p*-coumaric, and ferulic acids are found in free and bound forms. However, cinnamic and vanillic acids are found in free and/or bound forms only in some sorghum varieties. Gallic acid is present only in the bound form at an average content of 23.6 mg/kg (Hahn et al., 1983).

Polyphenolic Composition

A number of proanthocyanidins and leucoanthocyanidins have been identified in sorghum grains. Proanthocyanidins are oligomers of flavan-3-ols; flavan-3,4-diols and flavan-4-ols are also known as leucoanthocyanidins (Figure 2.11). Of these, profisetinidins have been isolated and identified in the pericarp of Martin sorghum (Blessin et al., 1963) and propelargonidins in the pericarp of a “commercial” sorghum (Yasumatsu et al., 1965). Later, luteofolorol or proluteolinidin (3',4',5,7-tetrahydroxyflavan-4-ol) was found to be a principal proanthocyanidin of Kafir variety of sorghum (Bate-Smith and Rasper, 1969). Luteolinidin is a major component of red or red-pink pigments as green seeds began to ripen (Figure 2.12) (Bate-Smith and Rasper, 1969; Haslam, 1977). According to Strumeyer and Malin (1975), tannins isolated from Leoti and Georgia varieties are procyanidin in nature. These researchers also showed that

these tannins consist of a series of oligomers of flavan-3-ols that, upon hydrolysis, depolymerize to afford cyanidin.

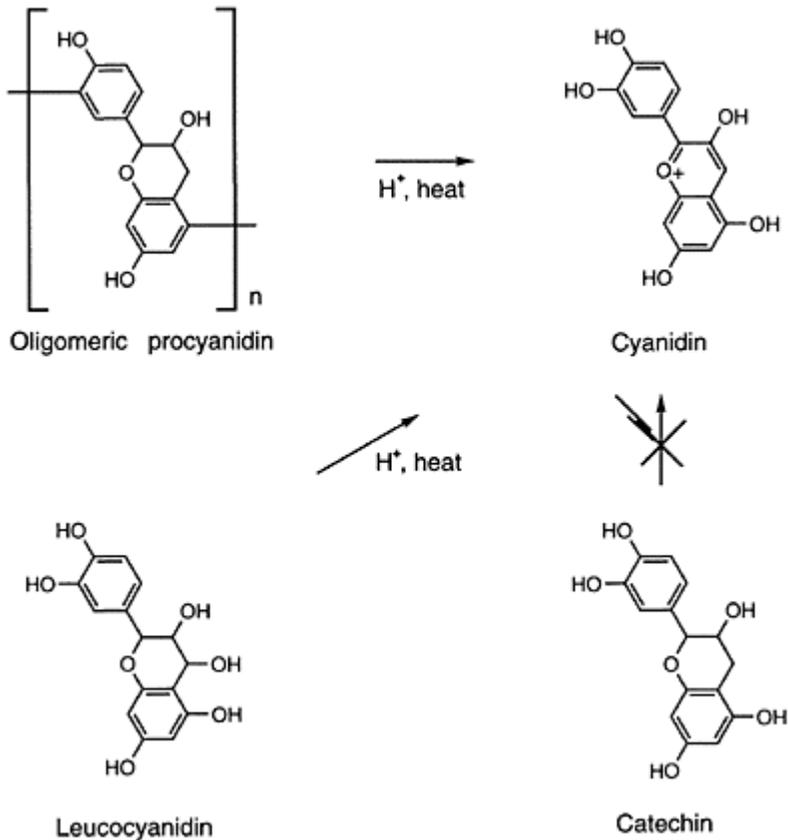


FIGURE 2.11 Conversion oligomeric flavan-3-ols and flavons 3,4-diols to anthocyanidins. (Adapted from Watterson, J.J. and Butler, L.G., 1983, *J. Agric. Food Chem.*, 31:41–45.)

Apiferol (4',5,7- trihydroxyflavan-4-ol) has been tentatively identified in the leaves and seeds of forage sorghum (Figure 2.12) (Watterson and Butler, 1983). This flavan-4-ol is suspected to be a precursor of red coloration observed in leaf tissue as a result of injury or physiological stress. Gupta and Haslam (1978) identified (+)-catechin, procyanidin dimer B-1, and procyanidin polymer corresponding to (–)-epicatechin units linked to (+)-catechin as a chain termination unit in sorghum. On the other hand, methanolic extracts of a Hungarian sorghum (Szegedioerpe) contain four procyanidins with the basic formula epicatechin-(epicatechin)_n-catechin and one procyanidin trimer corresponding to epicatechin-catechin-epicatechin (Figure 2.13) (Gujer et al., 1986).

The pigmentation of sorghum pericarp is mostly due to the presence of anthocyanins and anthocyanidins as well as other flavonoids. Two glucosides of apigenidin (both yellow) and two glucosides of luteolinidin (both orange) were isolated from white pericarp of six sorghum varieties (Nip and Burns, 1971). Eriodictyol (a chalcone) was found to be a predominant pigment in the lemon-yellow pericarp (Kambal and Bate-Smith, 1976), while luteoferol was present in the red pericarp of sorghum (Nip and Burns, 1969). The red sorghum pericarp contained also two yellow pigments identified as apigeninidin-5-glucoside and kaempferol-3-rutinoside-7-glucuronide, one orange pigment only identified as anthocyanin (Nip and Burns, 1969), and two 3-deoxyanthocyanidins: apigeninidin and luteolinidin (Melake-Berhan et al., 1996).

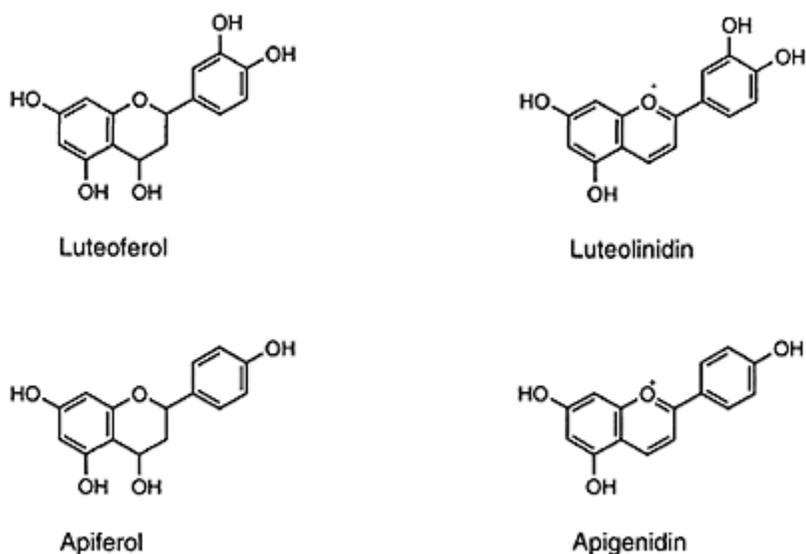


FIGURE 2.12 Chemical structures of some sorghum flavan-4-ols and corresponding anthocyanidins. (Adapted from Watterson, J.J. and Butler, L.G., 1983, *J. Agric. Food Chem.*, 31:41–45.)

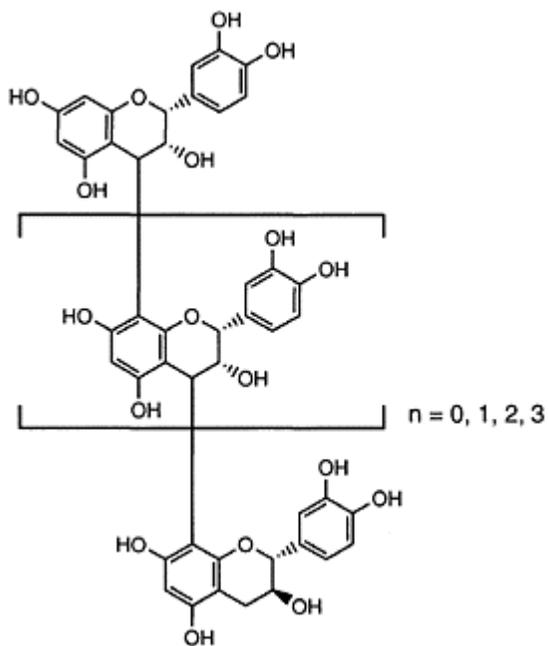


FIGURE 2.13 Chemical structures of procyanidins identified in sorghum grains.

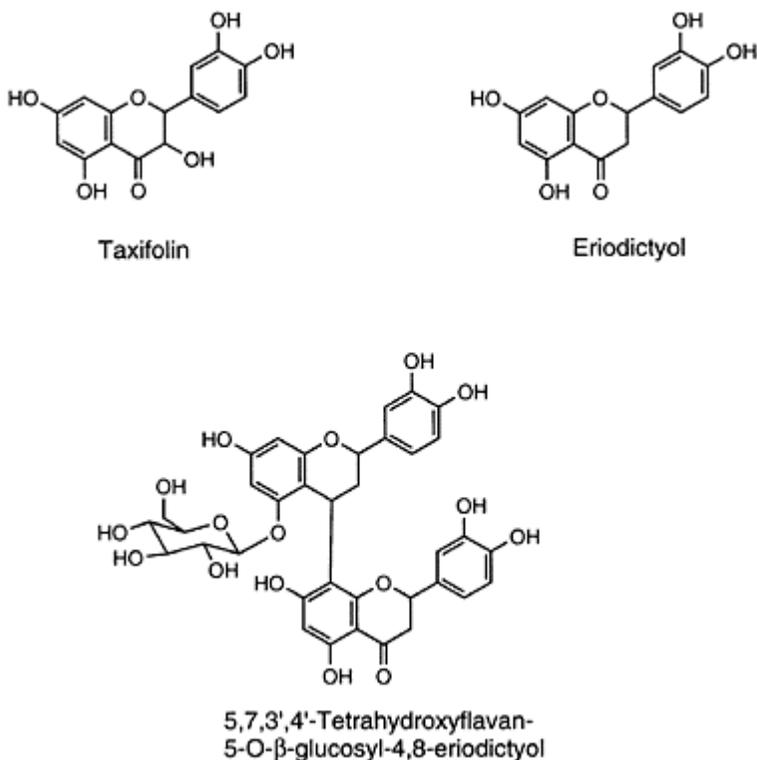


FIGURE 2.14 Chemical structures of some flavonoids isolated from sorghum grains. (Adapted from Gujer, R. et al., 1986, *Prog. Clin. Biol. Res.*, 213:159–161.)

A number of monomeric and oligomeric flavonoids linked by 4,8-interflavan bond have also been found in sorghum grains. The low molecular weight phenolics have been tentatively identified as (+)-catechin, naringenin and eriodictyol (+)-taxifolin, along with three monomeric glycosides (Figure 2.14). Dimeric flavonoids have been identified as 5,7,3',4'-tetrahydroxyflavan-5-O- α -glucosyl-4,8-eriodictyol and 5,7,3',4'-tetrahydroxyflavan-5-O- α -glucosyl-4,8-eriodictyol-5-O- α -glucoside. A trimeric flavonoid has been identified as 5,7,3',4'-tetrahydroxyflavan-5-O- α -glucosyl-4,8-5,7,3',4'-tetrahydroxyflavan-5-O- α -glucosyl-4,8-eriodictyol (Gujer et al., 1986). Recently, Krueger et al. (2003) have detected a number of heteropoly-flavan-3-ols linked by A-type and B-type interflavan bonds in Ruby Red sorghum (*Sorghum bicolor* L.Moench). In addition, a series of glucosylated heteropolyflavans containing a flavanone (eriodictyol or eriodictoyl-5-O- β -glucoside) were also identified.

Polyphenolic Content

The content of polyphenolic compounds in sorghum grains depends on a number of factors, including plant type, cultivar, age of plant or plant part, stage of development and environmental conditions (Table 2.13 and Table 2.14). The content of condensed tannins in sorghum, estimated as (+)-catechin equivalents by different research groups, ranged from 3.6 to 10.2% (Harris and Burns, 1970), 2.69 to 6.88% (Jambunathan and Mertz, 1973), 0.11 to 4.25% (Maxson et al., 1972), 0.1 to 8.0%

TABLE 2.14
Content and Distribution of Tannins in Different Parts of Sorghum Grain

| Grain Component | Content (g/kg) | Proportion of Total Tannins (%) |
|--------------------|----------------|---------------------------------|
| Testa | 224 | 81.4 |
| Pericarp | 42 | 15.1 |
| Endosperm and germ | 0.8 | 3.3 |
| Whole grain | 36.3 | — |

Source: Adapted from Reichert, R.D. et al., 1980b, in *Polyphenols in Cereals and Legumes*, International Development Research Centre, Ottawa, Canada, 50–60.

(McMillan et al., 1972), 0 to 3.88% (Elkin et al., 1996) and 0 to 1.97% (Dicko et al., 2002). However, values expressed as tannic acid equivalents varied from 0.31 to 1.5% (Maxson et al., 1972), 0.20 to 0.76% (Maxson and Rooney, 1972), 0.37 to 1.57% (Rostango et al., 1973b), and 0.33 to 1.41% (Rostango et al., 1973a). Moreover, the mold-resistant sorghum genotypes contain higher levels of proanthocyanidins than those of the susceptible lines (Melake-Berhan et al., 1996).

Maturation of sorghum grains brings about changes in their polyphenolic content and composition. These changes may be due to the metabolism or polymerization of phenolic compounds. The content of condensed tannins increases during the early stages of grain development and then remains constant or declines as the grain approaches harvest time (Bullard and Elias, 1980; Chavan et al., 1979b; Davis and Hoseney, 1979).

The period of time at which the maximum level of polyphenolics is produced in the grain depends on the sorghum variety. The variety GA-615 has the maximum level of (+)-catechin equivalents at 16 days, while for double dwarf Feretita, a significant increase in (+)-catechin equivalents is observed at 32 days (Figure 2.15) (Rooney et al., 1980). Price et al. (1979a) found that, for tannin-containing varieties, the maximum values per seed are reached between 25 and 40 days after half anthesis. The decrease in tannin content is attributed to increased polymerization or association with other cellular components (Bullard et al., 1980; Price et al., 1979a). However, Butler (1982) reported that drying seeds at any stage of maturation has a more pronounced effect on the relative degree of tannin polymerization than maturation by itself. On the other hand, depending on the sorghum cultivar, maturation of grain also brings about different changes in polyphenolic compounds. Thus, the content of assayable tannins of some cultivars from group III sorghum is reduced greatly during seed maturation, while those present in the seeds of some cultivars from group II remain unchanged (Asquith et al., 1983).

Effect of Processing

Various attempts have been made to reduce the detrimental effects of sorghum polyphenolics on digestibility of proteins and enzyme inhibition. Sequential

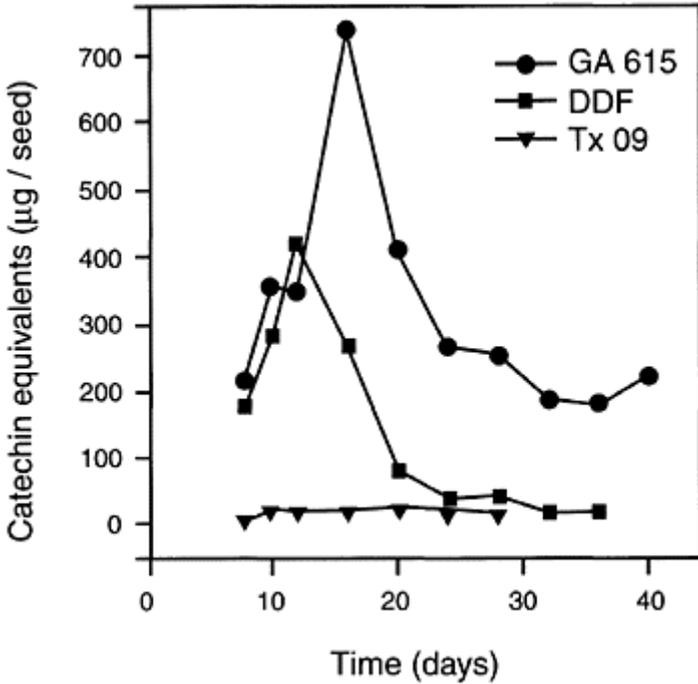


FIGURE 2.15 Variation in catechin content of kernels from three sorghum varieties from anthesis to maturity. The catechin equivalents were determined with the vanillin method on 1% HCl methanol extracts of the grain. Catechin equivalents were calculated subtracting a blank and after subtracting a blank; thus, two curves exist for each variety. (Adapted from Rooney, L.W. et al., 1980, in *Polyphenols in Cereal and Legumes*, International Development Research Centre, Ottawa, Canada, 25–35.)

dehulling of sorghum grains results in the removal of up to 98% of their tannins; however, this process brings about up to 45% protein loss as well as adversely affecting the nutritive value of proteins. The proteins in dehulled grains have a lower content of lysine, histidine and arginine than those in whole grains (Chibber et al., 1978). Dry heat has little effect on sorghum tannins, whereas moist heat decreases the content of assayable tannins from 0.4 to 2.6% (Price et al., 1978). On the other hand, soaking whole seeds in water or alkali also reduces the content of tannins.

Extraction at higher temperatures reduces the time needed for soaking grains. Although 24-h soaking of sorghum grains in distilled water at 30°C is required for 31% removal of tannins, 20 min at 100°C is necessary to achieve a similar removal of tannins. The treatment of sorghum with 0.005 to 0.05 M sodium hydroxide at 30°C for 24 h results in greater tannin removal (from 36 to 84%) than using distilled water. At 100°C, alkali treatment gives similar results after 20 min of soaking. Replacing sodium hydroxide with sodium carbonate or potassium hydroxide produces similar effects on tannins (Chavan et al., 1979a). Steeping sorghum grain in 0.3% NaOH (w/v) solution for 8 h at 25°C is more effective in reducing the tannin level than soaking in water or 0.9% (v/v) HCl solution (Beta et al., 2000). Alkali treatment is expected to convert tannins into insoluble, nutritionally unreactive phlobaphenes (Swain, 1965), which may result in formation of indigestible tannin-protein complexes (Price et al., 1979b; Salunkhe et al., 1982) and promote oxidative polymerization of condensed tannins (Porter, 1992).

WHEAT

Wheat varieties differ from one another with respect to optimal growing conditions, kernel color, hardness and composition. Varieties planted in late summer or fall for maturation the following summer are called winter wheats; these include red and white as well as hard and soft varieties. Wheat varieties planted in the spring for harvesting in late summer are known as spring varieties and include durums, as well as hard red and hard and soft white varieties (Campbell, 1992).

Phenolic Acids

Wheat kernels have been reported to contain free phenolic compounds as well as their derivatives (El-Basyouni and Towers, 1964). The total content of phenolic acids in six wheat cultivars grown at four locations in Canada ranged from 1608 to 2687 mg of ferulic acids equivalents/kg (Abdel-Aal et al., 2001). Weidner et al. (1999) reported that grains of Polish wheat cultivars contain 3.44 to 3.55 mg/kg of free phenolic acids, 42.9 to 69.9 mg/kg of phenolic acids liberated from esters and 3.68 to 9.83 mg/kg of phenolic acids liberated from soluble glycosides. Ferulic, vanillic, gentisic, caffeic, salicylic, syringic, *p*-coumaric and sinapic acids, as well as vanillin and syringaldehyde, have been identified in wheat kernels (Table 2.10 and Table 2.15) (McKeehan et al., 1999; Sosulski et al., 1982; Weidner et al., 1999).

TABLE 2.15
Phenolic Acids Obtained on Alkaline Hydrolysis
of Wheat^{a,b}

| Phenolic Acid | Ungerminated | Germinated (48 h) | |
|-------------------------|-----------------|-------------------|---------|
| | Grains | Grains | Embryos |
| Ferulic acid | | | |
| Ethanol-soluble | 3 | 26 | 310 |
| Ethanol insoluble | ND ^b | 181 | 1200 |
| <i>p</i> -Coumaric acid | | | |
| Ethanol-soluble | 1 | 8 | 261 |
| Ethanol-insoluble | ND ^b | 0 | 0 |
| Sinapic acid | | | |
| Ethanol-soluble | 0 | 7 | 95 |
| Syringic acid | | | |
| Ethanol-soluble | 2 | 8 | 197 |
| Vanillic acid | | | |
| Ethanol-soluble | 14 | 10 | 292 |

^a Milligrams per kilogram, dry weight basis.

^b ND=not determined.

Source: Adapted from El-Basyouni, S. and Towers, G.H.N., 1964, *Can. J. Biochem.*, 42:203–210.

Of these, ferulic acid is the primary phenolic acid in the grain during all stages of development and is present in seeds in free and esterified forms (McCallum and Walker, 1991; McKeehan et al., 1999; Rybka et al., 1993).

Ferulic acid is known to occur in high quantities in the aleurone cell walls of kernels and to a lesser extent in the seed coats and embryos (Fulcher, 1982; Fulcher, et al., 1972; Pussayanawin et al., 1988). This acid is esterified to arabinose in the pentosan fraction (Faurot et al., 1995; Izydorzyc et al., 1991), stanol and sterol (Seitz, 1989) and glucose (Herrmann, 1989). In mature grain the content of ferulic acid may range from 535 to 783 mg/kg of grain (Abdel-Aal et al., 2001). In addition, Weidner et al. (1999) found that, besides ferulic acid, caffeic, *p*-coumaric and sinapic acids are the main phenolic acids in Polish wheat cultivars. Renger and Steinhart (2000) have found the total amounts of ferulic and *p*-coumaric acids in wheat bran subjected to treatment with 4 M NaOH to be 18.8 and 0.34 g/kg, respectively.

Wheat bran also contains 808 mg/kg of total ferulic acid dehydrodimers (DiFA), of which 8-*O*-4'-DiFA is the dominating esterified DiFA and 8,5'-DiFA the most abundant esterified DiFA in wheat bran (Table 2.8) (Figure 2.1) (Garcia-Conesa et al., 1997; Lempereur et al., 1998; Renger and Steinhart, 2000). Ferulic acid and *p*-coumaric acid are the main phenolic acid linked to arabinoxylans, pectic substances or xyloglucans in the cell walls of Gramineae (Hartley et al., 1990; Iiyama et al., 1994; Lam et al., 1992; McDougall, 1993; Scalbert et al., 1985). Régnier and Macheix (1996) reported that bound ferulic acid and bound *p*-coumaric acid reach a maximum level in cell walls of durum wheat during the hydric step (associated with phase II of grain development) and then decrease rapidly during grain dehydration.

Germination of grains significantly increases the content of ethanol-soluble phenolic acids (Table 2.15); the maximum concentration is reached about 9 days after germination. Thereafter, the content of phenolic acids starts to decline and reaches a minimum 4 to 5 weeks later (El-Basyouni and Towers, 1964). McKeehan et al. (1999) reported that the level of ferulic acid decreases by almost 50% during grain ripening. This decrease is thought to be due to

- The increase in rate of endosperm development surpassing the rate of synthesis of the outer grain covering
- The decrease of phenylalanine ammonia-lyase activity (an enzyme that catalyzes the reductive deamination of phenylalanine to cinnamic acid)
- The formation of covalent cross links between grain phenolics and cell wall components

Other Phenolics

Presence of alkylresorcinols in wheat grains has been reported. A series of n-alkylphenols has been found in wheat bran in which n-alkyl side chains containing 17, 19, 21, 23 and 25 carbons are coupled to a resorcinol ring at the 5 position (Wenkert et al., 1964). The total content of these alkylphenols, expressed as 5-n-pentadecylresorcinol equivalents on a dry basis, ranges from 657 to 927 mg/kg for wheat and from 633 to 863 mg/kg for triticale. Wheat bran has the highest content of alkylresorcinols (2110 mg/kg); corresponding flours contain only 380 mg/kg (Musehold, 1978).

TABLE 2.16
Content of Flavonoids in Wheat Bran^a

| Class and Variety | Total Flavonoids |
|--------------------------|-------------------------|
| Hard Red Spring | |
| Len | 149.1 |
| Alex | 245.4 |
| Stoa | 289.0 |
| Hard Red Winter | |
| Roughrider | 288.2 |
| Agassiz | 271.1 |
| Durum Wheat | |
| Rugby | 405.7 |
| Lloyd | 358.4 |
| Vic | 291.2 |

^a Milligrams per kilogram.

Source: Adapted from Feng, Y. and McDonald, C.E., 1989, *Cereal Chem.*, 66:516–518.

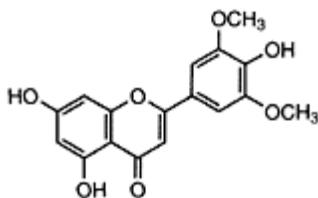


FIGURE 2.16 Chemical structure of triclin, a dominant flavone pigment in wheat.

The bran of hard wheat and commercial wheat germs contains a number of flavonoid pigments. Depending on the wheat class, the content of flavonoids in brans ranges from 149.1 to 405.7 mg/kg (Table 2.16) (Feng and McDonald, 1989). The wheat germs contain the same level of flavonoids as those found in the bran. Tricin (5,7,4'-trihydroxy 3',5'-dimethoxy flavone) is the dominant flavone pigment in cultivated and wild wheats (Figure 2.16). Anderson and Perkin (1931) first isolated this compound from ether extracts of wheat. According to Chen and Geddes (1945), about one third of the total content of flavones in wheat, expressed as triclin equivalents, is contained in the endosperm and two thirds in the bran and embryo. Two other flavonoids have also been isolated from wheat germ and identified as apigenin glycosides (King, 1962). On the other hand, 6-C-pentosyl-8-C-hexosylapigenin and 6-C-hexosyl-8-C-pentosylapigenin have been isolated and identified by spectroscopic methods (Feng et al., 1988; Feng and McDonald, 1989).

Two phenolic acid diacylglycerols (containing two ferulic acids or ferulic acid and *p*-coumaric acid) have also been identified in wheats (Cooper et al., 1978). Phenolic aldehydes such as *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde can be detected in wheat species of *Triticum dicoccoides* and *Aegilops geniculata*. In addition, veratraldehyde and protocatechualdehyde are found in wheat species of *Triticum dicoccoides* (Cooper et al., 1994).

Seitz (1989) identified sitostanyl, campestanyl and campesteryl ferulates in wheat grains (Figure 2.8); of these, campestanyl ferulate and sitostanyl ferulate are the main sterol ferulates. The total content of sterol ferulates in whole wheat grain ranges from 62 to 123 mg/kg (Hakala et al., 2002; Seitz, 1989).

Effect of Processing

Fresh wheat flour (Neepawa wheat) contains 71.4 mg/kg of phenolic acids. Ferulic acid is a major phenolic acid, constituting about 89.1% of total phenolic acids (Table 2.9). Over 84% of phenolic acids are present in the insoluble bound form. Storing wheat flour brings about the loss of free, soluble esters and insoluble bound phenolics due to destructive oxidation reactions (Sosulski et al., 1982).

Ferulic, syringic, caffeic, sinapic, *p*-coumaric, and vanillic acids and phenolic acid dimers have been identified in wheat gluten (Labat et al., 2000; Sosulski et al., 1982), with ferulic acid (49 to 177 mg/kg gluten of dry weight) predominant, followed by

sinapic acid (14 to 35 mg/kg gluten of dry weight) (Labat et al., 2000). Moreover, Hillhorst et al. (1999) found that gluten prepared by hand washing Kolibri flour contains 80 mg of ferulic acid per kilogram of dry weight. The phenolics soluble in an acetone-methanol-water solvent system contain 64 to 85% of total sinapic acid and only 5 to 50% of total ferulic acid detected in wheat gluten (Labat et al., 2000). During gluten mixing, 21 to 66 and 22 to 94% of the initial soluble forms of ferulic and sinapic acids disappear, respectively (Jackson and Hosney, 1986; Labat et al., 2000).

Abdel-Aal et al. (2001) investigated the distribution of phenolic acids in wheat milling fractions. About 73% of grain phenolic acids was found in the shorts and bran, but only 5% in first, second and third break milling fractions. Later, Hakala et al. (2002) studied the distribution of steryl ferulates in wheat milling products and detected only traces of steryl ferulates in low ash wheat flour; wheat brans contained 297 to 390 mg/kg of total steryl ferulates. Enrichment of low ash flour with aleurone layer increases the total ferulates level to 194 to 216 mg/kg.

NUTS

Nuts contain bioactive components; consumption of nuts has been linked with reducing the risk of coronary heart diseases (Hu et al., 1998; Sabate, 1999) and altering serum lipids (Kris-Etherton et al., 1999; Spiller et al., 1998).

ALMONDS

One of the world's most popular tree nuts, almonds (*Prunus amygdalus*) belong to the Rosaceae family (Menninger, 1977) and are used as snack food and as ingredients in many processed foods, including bakery and confectionary products. The U.S. is the largest producer of almonds in the world (Rosengarten, 1984). The almond fruit consists of the edible kernel, brown skin, shell and hull; blanched almonds have their shells and their skins removed. Removed shells, hulls and skins are usually discarded (Menninger, 1977; Rosengarten, 1984); however, they can be used as a source for dietary fiber, sweetener concentrate and antioxidants (Takeoka and Dao, 2003).

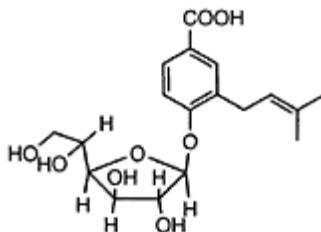


FIGURE 2.17 Chemical structures of prenylated benzoic acid derivative found in almond skins,

The antiradical activity of the almond and its components was recently reported by Siriwardhana and Shahidi (2002). Almond skin serves as a rich source of phenolic compounds (Lou et al., 2001), exhibiting an excellent scavenging activity for reactive oxygen species (Siriwardhana and Shahidi, 2002). Takeoka and Dao (2003) detected chlorogenic acid isomers, namely, chlorogenic acid (42.5 mg/100 g of fresh weight), cryptochlorogenic acid (7.9 mg/100 g of fresh weight) and neochlorogenic acid (3.0 mg/100 g of fresh weight) in almond (*Prunus dulcis* Mill.) hull. Furthermore, Sang et al. (2002a) identified a novel prenylated benzoic acid derivative, 3-prenyl-4-*O*- β -D-glucopyranosyloxy-4-hydroxybenzoic acid (Figure 2.17), as well as (+)-catechin, protocatechuic acid and ursolic acid (triterpenoid) in almond hulls. Later, Sang et al. (2002b) also isolated and identified additional phenolic compounds in almond skins:

- Vanillic acid
- *p*-Hydroxybenzoic acid
- 3'-*O*- β -Methylquercetin 3-*O*- β -D-glucopyranoside
- 3'-*O*- β -Methylquercetin 3-*O*- β -D-galactopyranoside
- 3'-*O*- β -Methylquercetin 3-*O*- α -L-rhamnosylpyranoside-(1 \rightarrow 6)- β -D-glucopyranoside
- Kaempferol 3-*O*- α -L-rhamnosylpyranoside-(1 \rightarrow 6)- β -D-glucopyranoside
- Naringenin 7-*O*- β -D-glucopyranoside

All these compounds, except naringenin derivatives exhibit strong radical scavenging activity. Subsequently, Frison-Norrie and Sporns (2002) reported the presence of isorhamnetin 3-*O*- α -L-rhamnosylpyranoside-(1 \rightarrow 6)- β -D-glucopyranoside, isorhamnetin 3-*O*- β -D-glucopyranoside, and kaempferol 3-*O*- β -D-glucopyranoside in almond skins. Later, Frison and Sporns (2002) also reported that isorhamnetin rutinoside was the most abundant flavonol glucoside in almond skins. Moreover, procyanidins with various degrees of polymerization have also been detected in the flesh and skin of almond fruit (Brieskorn and Betz, 1988; de Pascual-Teresa et al., 1998; Lou et al., 1999).

BETEL NUTS

Betel nut or areca nut (*Areca catechu* L.) is used for human consumption in parts of Southeast Asia and the South Pacific. Used by approximately 10% of the world's population as an important component of their daily diet (Duke, 1985), betel nut also often serves as a breath-sweetening masticatory. In India, whole or broken betel nuts are usually processed by soaking or boiling with various plant extracts to decrease their astringency (Raghavan and Baruah, 1958). The most popular way to consume betel nuts is as "pan," also referred to as "betel quid." The major ingredients of betel quid are betel nut and slaked lime folded together in betel leaves, to which tobacco, catechu, clove, cardamon, anise seed, grated coconut, sugar and other spices or condiments are often added (Stich et al., 1982, 1983).

Betel nuts contain arecoline and other alkaloids responsible for stimulating effects on the nervous system and also have 26% tannins. The predominant class of tannins in betel nuts is gallotannic acid (Duke, 1985; Raghavan and Baruah, 1958). Presence of condensed tannins (proanthocyanidins) and structurally related monomeric and oligomeric flavan-3-ols has also been reported (Nonaka et al., 1981; Stich et al., 1983). Butler and Pushpamma (1991) estimated the content of tannins in some betel nut

varieties; the Chikni variety of betel nut contained 31 and 27% tannins as quantified by proanthocyanidin assay and vanillin assays, respectively. The content of tannins in betel nuts of Vakkalu variety is 8.7% (proanthocyanidin assay) and 20% (vanillin assay); however, a somewhat lower amount of tannin is found in betel quids due to the presence of higher amounts of tannin-free ingredients (Butler and Pushamma, 1991). Later, Wang and Lee (1996) reported that the whole areca fruit contains condensed tannins (92 g/kg of dry weight), hydrolyzable tannins (69 g/kg of dry weight) and a nontannin phenol fraction (56 g/kg of dry weight). Catechin and epicatechin are the main phenolics detected in nontannin phenol fraction.

Betel products may be involved in carcinogenesis; possibly due to their tannin component (Giri et al., 1987; Gothoskar and Pai, 1986; Stich et al., 1982, 1983, 1984; Wenke et al., 1984), consumption of betel nuts has been related to the development of oral cancer deaths each year in Asia. In addition, betel tannins may bring about oral submucous fibrosis, a chronic disabling disease (Paymaster, 1962), and cause antinutritional effects such as diminishing palatability or inhibiting the post-digestive metabolism (Salunkhe et al., 1990). Crude phenolic extracts of whole areca fruit exhibit marked antioxidative activity and antimutagenic effect toward *Salmonella typhimurium* TA98 and TA100 (Wang and Lee, 1996). Betel nut and betel quid tannins are less effective than sorghum tannins in precipitating proteins (Hagerman and Butler, 1978); however, their content is several times higher than that found in high-tannin sorghum varieties (Butler and Pushamma, 1991). Therefore, consumption of betel nut products may result in serious nutritional consequences.

CASHEW NUTS

Cashew nut is the kernel of *Anacardium occidentale* L. The testa of this nut is reddish-brown in color and serves as a good source of catechol-type tannin (Pillai et al., 1963). Main phenolic constituents of cashew kernel testa are (+)-catechin and (-)-epicatechin; accounting for 6 and 7.5% of the dried weight of cashew kernel testa, respectively, these constitute approximately 40% of the total phenolics in cashew nuts. The polymeric

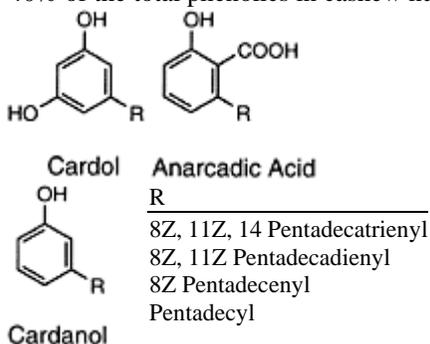


FIGURE 2.18 Chemical structures of anarcadic acids, cardols and cardanols.

proanthocyanidins account for 40% of total testa phenolics; other components of testa phenolics include leucocyanidins and leucopelargonidins (Mathew and Parpia, 1970).

Furthermore, nonylphenol has been identified in cashew apple headspace constituents and cinnamic acid in glycosidically bound components (Bicalho et al., 2000).

During moisture conditioning (a processing step prior to roasting) some nuts develop bluish-black patches on their surfaces—a discoloration that lowers the market value of such cashew nuts. There are a few indications that discoloration of cashew nut testa may be due to formation of complexes between iron and polyphenols. The darkened nuts have a higher level of iron than the good nuts (Fairer, 1935); Mathew and Parpia (1970) reported that polyphenols of cashew nut testa can form dark-colored complexes with iron. These dark pigments can easily be washed away with dilute acidic solutions.

Anacardic acids, cardols and cardanols are the major phenolic constituents of cashew nut shell liquid, a by-product obtained during the production of cashew nuts (Figure 2.18). The side chains of these phenolics comprise fully saturated pentadecyl groups and their unsaturated monoene, diene or triene analogues (Paramashivappa et al., 2001; Spencer et al., 1980). Cashew nut shell liquid and its phenolics are used as components of cement, specialty coatings and primers (Menon et al., 1985). The biological activities of anacardic acids, cardols and cardanols such as antitumor (Kubo et al., 1993) and antiacne (Kubo et al., 1994a) properties, molluscicidal activity (Kubo et al., 1986), prostaglandin synthetase inhibition (Kubo et al., 1987), as well as their antibacterial activity against the Gram-negative bacterium *Helicobacter pylori* (Kubo et al., 1999) and Gram-positive bacteria (Himejima and Kubo, 1991), have been demonstrated.

These acids also exhibit inhibitory effects on urease (Kubo et al., 1999), α -glucosidase and aldose reductase (Toyomizu et al., 1993), tyrosinase (Kubo et al., 1994b), lipoxygenase (Grazzini et al., 1991; Shobba et al., 1994), and prostaglandin endoperoxide synthase (Grazzini et al., 1991). Paramashivappa et al. (2001) proposed a simple procedure for selective isolation of anacardic acids, cardanols and cardols from cashew nut shell liquid. This method involved precipitation of anacardic acid as calcium anacardate, treatment of the shell liquid with ammonia and extraction of cardanols into ethyl acetate/hexane. Subsequently, Kumar et al. (2002) described a method for isolation of cardanol from technical cashew nutshell oil (TCNSO), a mixture of cardanol, cardol and polymeric materials produced by roasting shell. This procedure involves dissolving TCNSO in a mixture of methanol and ammonia (8:5; v/v) and extracting cardanol by hexane. Cardol is then extracted from methanol-ammonia solution with a mixture of ethyl acetate and hexane.

HAZELNUTS

The data on hazelnut (*Corylus* spp.) phenolics are still limited. Senter et al. (1983) reported that protocatechuic acid (0.36 mg/kg of hulls) is the predominant phenolic in hazelnut hulls. The levels of other phenolic acids (gallic acid, caffeic acid, vanillic acid and *p*-hydroxybenzoic acid) do not exceed 10 μ g/kg of testa. Using carbon-13 cross-polarization magic-angle spinning NMR, Preston and Sayer (1992) subsequently detected only condensed tannins in the packing tissue inside shells of hazelnut. Later, Yurttas et al. (2000) isolated and tentatively identified six phenolic aglycones in Turkish and American hazelnuts: gallic acid, *p*-hydroxybenzoic acid, sinapic acid, quercetin, and caffeic acid and epicatechin. Furthermore, nonhydrolyzed extracts of hazelnut phenolics exhibit greater antioxidant activities than corresponding hydrolyzed extracts.

PEANUTS

Nearly two thirds of the world's peanut production is pressed for oil; the peanut meal is used mostly as a feed for livestock. Defatted peanut flour may also serve as an alternative source of functional proteins with potential applications in food product formulations (McWatters and Cherry, 1975; McWatters and Holmes, 1979). However, peanut protein products contain phenolic compounds (Daigle et al., 1983; Maga and Lorenz, 1974) that have been implicated in flavor and color defects (Maga, 1978; Sosulski, 1979). Over half of the U.S. peanut crop is used for production of peanut butter (Potter, 1986).

Peanut protein products contain up to 2000 mg/kg of phenolic acids and up to 120 mg/kg of neutral phenolic compounds. Of these, protein isolates contain the least amount of total phenolic acids. Accounting for 40 to 68% of phenolic acids in all peanut products, *p*-coumaric acid (*trans* and *cis* forms combined) has been identified as the predominant phenolic acid in peanut protein products (Table 2.17) (Seo and Morr, 1985). Vanillic acid, *p*- and *o*-coumaric acids and ferulic acid may contribute significantly to the flavor characteristics of peanut flour because they are present at levels near or above their threshold values (Maga and Lorenz, 1974). Other studies have demonstrated that combinations of phenolic acids show a synergistic effect in lowering the taste threshold values (Maga and Lorenz, 1973).

Different parts of peanuts, including kernel, may contain *trans*-resveratrol, a major stilbene phytoalexin produced in response to exogenous stimuli such as fungal infection (Ingham, 1976). Growing immature peanut kernels contain higher levels of phytoalexins than mature ones (Dorner et al., 1989). The content of resveratrol in four raw shelled peanut samples available in the U.S. market ranges from 0.070 to 0.716 mg/kg in five

TABLE 2.17
Phenolic Acid Composition in Peanut Protein
Products as Determined by HPLC^a

| Phenolic Acid | Laboratory Defatted Meal | Commercial Defatted Flour | Protein Isolate |
|--------------------|--------------------------|---------------------------|-----------------|
| Unknown | 93.2 | 56.3 | 79.9 |
| Unknown | 104.8 | 64.4 | 13.3 |
| Vanillic/Caffeic | 69.6 | 96.5 | 35.4 |
| Unknown | 23.6 | 28.9 | 23.3 |
| Syringic | 14.2 | 12.4 | 13.3 |
| <i>p</i> -Coumaric | 1224.0 | 1193.0 | 537.0 |
| Unknown | 92.0 | 63.6 | 54.6 |
| Ferulic | 143.8 | 83.1 | 34.6 |
| Sinapic | 245.0 | 135.0 | 10.7 |
| Isoferulic | 23.5 | 22.6 | 8.2 |

^a Milligrams per kilogram.

Source: Adapted from Seo, A. and Morr, C.V., 1985, *J. Food Sci.*, 50:262–263.

boiled peanut samples from 1.779 to 7.092 mg/kg and in 15 samples of peanut butter is 0.148 to 0.504 mg/kg. Roasted peanuts contain the lowest amount of resveratrol (Sobolev and Cole, 1999). Sanders et al. (2000) have reported that the content of resveratrol in 15 cultivars of market type peanuts, stored in the cold for up to 3 years, ranges from 0.02 to

1.79 mg/kg. Subsequently, Ibern-Gómez et al. (2000) detected the presence of *trans*-piceid (3- β -glucoside of *trans*-resveratrol) in peanuts.

The content of total stilbenes in peanut butter (natural and blended) is between 0.354 and 0.978 mg/kg. *Trans*-piceid comprises 11.7 to 35.8% of the total stilbenes. Health benefits related to resveratrol are associated with reduced risk of cancer (Jang et al., 1997) and cardiovascular disease (Goldberg, 1995). Some peanut products may also contain vanillin. Sobolev (2001) recently detected vanillin in boiled peanuts (1.61 to 1.71 mg/kg of kernel); peanuts are boiled in hulls high in lignins and vanillin is a product of oxidative hydrolysis of lignins (Fargues et al., 1996).

According to Sanders (1977), the bronze coloration of peanut hulls is due to the presence of tannins. Mature peanut hulls, seed coats, and fruits contain approximately 0.43, 6.04 and 0.04% tannins on a dry weight basis, respectively. Thus, most tannins are located in seed coats and hulls, while fruits (meat nut) are practically tannin free. The content of tannins changes with the maturation of peanuts. In hulls, tannin content increases markedly during the latter stages of maturation, while their content in skins increases rapidly in the early stages of maturation and then begins to decline (Sanders, 1977). The total content of phenolics in peanut hulls increases during the development of peanut from 33.4 mg/kg in hulls of peanuts harvested 74 days after planting (DAP) to 71.3 mg/kg of hulls at 144 DAP. Moreover, the concentration of luteolin in hulls at day 144 (28.6 g/kg) is 15 times that at day 74 (Yen et al., 1993).

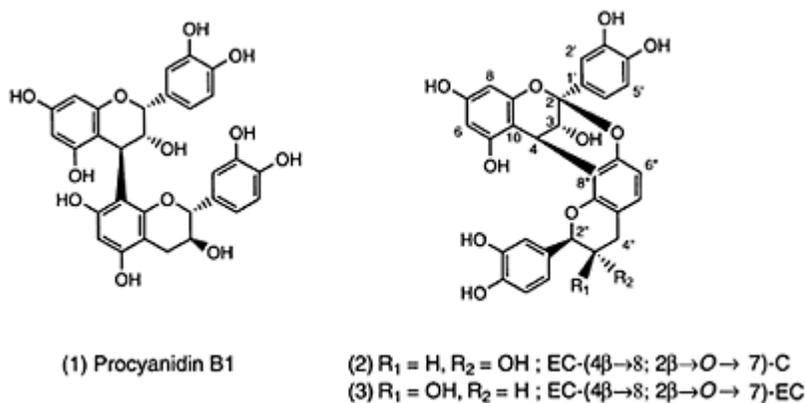


FIGURE 2.19 Chemical structures of some dimeric procyanidins isolated from red peanut skin (EC, (-)-epicatechin; C, (+)-catechin).

Mature peanut skins contain 17% procyanidins by weight. Of these, 55% are low molecular weight oligomers of catechin and epicatechin that are soluble in ethyl acetate. Epicatechin-(4 β →8; 2 β →O-7)-catechin is found to be the predominant dimeric procyanidin in peanut skin, followed by epicatechin-(4 β →8; 2 β →O-7)-epicatechin and epicatechin-(4 β →8)-catechin (procyanidin B1) (Figure 2.19). The higher molecular weight procyanidins soluble in ethyl acetate contain the (4 β →8 or 4 β →6) and (4 β →8;

2 β →O-7) interflavonoid bonds (Karchesy and Hemingway, 1986). However, the nature of flavonoids present in peanuts depends on their degree of maturity: the shells of immature peanuts contain predominantly eriodictyol while mature peanut shells are rich in luteolin (Daigle et al., 1988).

PECANS

Pecan kernels are semiperishable products that must be refrigerated in order to preserve their quality. They contain enough prooxidants and antioxidants to affect their storage stability. Of these, phenolic acids present in pecan kernels contribute to their enhanced storage stability. Senter et al. (1978) found that development of rancidity of pecan kernels closely parallels the content of total phenolics. On the other hand, leucocyanidins and leucodelphinidins participate in the development of red-brown discoloration in the testa of pecan (Senter et al., 1978).

The color of shelled pecan seed coats (testa) is a major criterion in their quality determination. Based on their color, pecans are graded into (1) light (golden color of testa), (2) light amber (light brown), (3) amber (medium brown) and (4) dark amber (dark brown). A light color is indicative of fully mature pecans that have been properly harvested, processed and stored. A darker color is caused by exposure of kernels to adverse conditions. The coloration of pecan kernels depends on growing conditions such as availability of moisture, location of tree within the orchard and horticultural practice (Woodroof, 1967), as well as the time of harvest (Heaton et al.,

TABLE 2.18
Changes in Total Phenols and Flavanols of
Pecan Kernels Stored at 32 C, 50% RH^a

| Storage Time (weeks) | Pecan Variety | | | |
|----------------------|---------------|-----------------|---------------|-----------------|
| | Stuart | | Schley | |
| | Total Phenols | Total Flavanols | Total Phenols | Total Flavanols |
| 0 | 10.65 | 4.43 | 8.30 | 3.63 |
| 6 | 14.20 | 4.15 | 9.30 | 3.65 |
| 12 | 11.15 | 3.88 | 9.10 | 3.70 |
| 15 | 11.55 | 3.58 | 7.80 | 2.78 |

Note: Total phenols determined by Folin-Ciocalteu assay; total flavanols by vanillin assay.

^a Grams per kilogram.

Source: Adapted from Senter, S.D. et al., 1978, *J. Food Sci.*, 43:128–134.

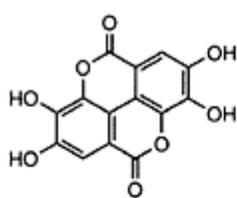
1975). Storage at temperatures below -7°C protects the products against rancidity and discoloration for about 2.5 years.

The total content of phenolics in Stuart and Schley pecan varieties ranges from 7.80 to 14.20 mg/g of kernel (Table 2.18). However, Stuart varieties contain more phenolics than the Schley variety. Total flavanol content is between 2.78 and 4.43 mg/g of kernel (Senter et al., 1978). Thus, significant quantities of other phenolics are present in pecan kernels and may affect their quality.

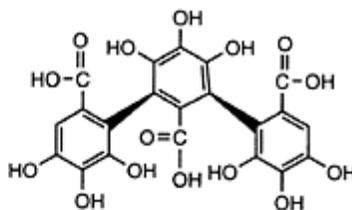
WALNUTS

Walnuts are produced by tree nut cultivars of *Juglans regia* (family Juglandaceae). Production of this crop in the U.S. is mainly concentrated in California (Foreign Agricultural Service, 1998). Consumption of walnuts has a beneficial impact on human serum lipid profile by decreasing total and LDL cholesterol and increasing the level of HDL cholesterol (Chisholm et al., 1998; Lavendrine et al., 1999; Sabate et al., 1993; Zambon et al., 2000). Shelled walnuts contained 16 g/kg of total phenolics expressed as gallic acid equivalents (Anderson et al., 2001). Jurd (1956) identified ellagic acid, methyl gallate, gallic acid and a mixture ellagitannins in the skin of walnuts. Later, Jurd (1958) isolated a novel ellagitannin, isomeric with corilagin, from the walnut skin. Łuczak et al. (1989) identified 10 phenolic acids in the pericarp of walnut: *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric, gallic, ferulic, caffeic and sinapic acids. Bound phenolics (esterified + glycosylated) are the predominant form of phenolic acids and comprise 76% of total phenolic acids in walnuts. Syringic acid is the principal phenolic acid identified, accounting for 40.5 and 33.8% of total free and bound phenolic acids, respectively. Subsequently, Anderson et al. (2001) have reported that ellagitannins are the most abundant phenolics in walnuts. Of these, ellagic acid, valoneic acid dilactone, and pedunculagin have been identified in phenolic extracts from shelled walnuts (Figure 2.21).

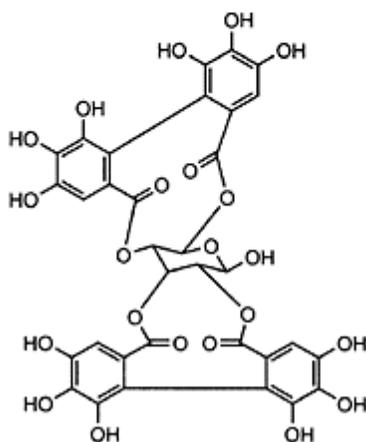
Similar to other nuts, walnuts are prone to contamination with aflatoxins on infection with *Aspergillus flavus* and *Aspergillus parasiticus* (Henry et al., 1999); however, it has been shown that walnut husks are resistant to *A. flavus* growth (Mahoney et al., 1998). Walnuts contain a number of structurally related naphthoquinones, including 1,4-naphthoquinone, juglone (5-hydroxy-1,4-naphthoquinone), 2-methyl-1,4-naphthoquinone, and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (Figure 2.22) (Leistner, 1981; Muller and Leistner, 1978a, b). Higher concentrations of these compounds have been found in husk tissues surrounding the nut (Binder et al., 1989). Recently, Mahoney et al. (2000) reported that naphthoquinones of walnut husk may suppress the growth of *A. flavus*. The growth of *A. flavus* is inhibited by 1,4-naphthoquinone at 170 µg/kg and juglone at 100 µg/kg; however, for plumbagin and 2-methyl-1,4-naphthoquinone, no growth of *A. flavus* has been observed at 50 µg/kg. These authors have also demonstrated that, at levels 10 µg/kg below that required for complete growth inhibition, juglone and plumbagin reduce aflatoxin production by 12 and 39% of the control, respectively.



Ellagic acid



Valoneic Acid



Pedunculagin

FIGURE 2.21 Chemical structures of some phenolic compounds found in walnuts.

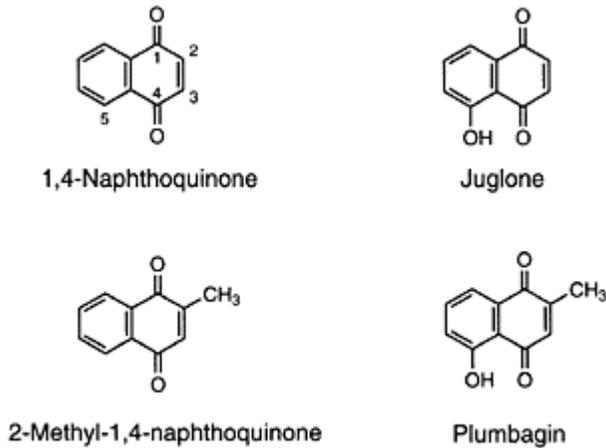


FIGURE 2.22 Chemical structures of some naphthoquinones found in walnuts.

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3

Phenolic Compounds of Major Oilseeds and Plant Oils

INTRODUCTION

The predominant phenolic compounds of oilseed products belong to the phenolic acid, coumarin, flavonoid, tannin and lignin group of compounds (Ribereau-Gayon, 1972). The phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids. Figure 3.1 shows the chemical structures of some phenolic acids and units of condensed tannins of some oilseeds; Table 3.1 gives the phenolic content in some oilseed flours.

RAPSEED AND CANOLA

INTRODUCTION

Rapeseed is conventionally processed to oil and a feed-grade meal. The use of rapeseed meal in human nutrition has been considered for many years; however, its present utilization is limited due to the presence of glucosinolates, phytates, phenolics and hulls. In spite of introduction of double-zero (low glucosinolate and low erucic acid) rapeseed varieties and patenting of a number of dehulling methods (Diosady et al., 1986; Greilsamer, 1983; Kozłowska et al., 1984; Sosulski and Zadernowski, 1981), the use of rapeseed meal as a source of food-grade protein is still limited by the presence of phytic acid and phenolic compounds, among others. Nonetheless, aqueous extraction and membrane processes have more recently been considered and are expected to lead to industrial developments. The phenolic content in rapeseed flour is much higher when compared to that found in flours obtained from other oleaginous seeds, exceeding about 28 times the level of phenolics in soybean flour (Table 3.1 and Table 3.2).

Phenolic compounds may contribute to the dark color, bitter taste and astringency of rapeseed meals (Naczek et al., 1998a, b). Phenolics or their degradation products can also form complexes with essential amino acids, enzymes and other proteins, thus lowering the nutritional value of rapeseed products. Therefore, phenolic compounds are important factors when considering rapeseed meal as a protein source in food formulations. However, the available information on undesirable effects of phenolics on the quality of rapeseed meals is still diverse and fragmentary (Kozłowska et al., 1975; Shahidi and Naczek, 1992; Sosulski, 1979).

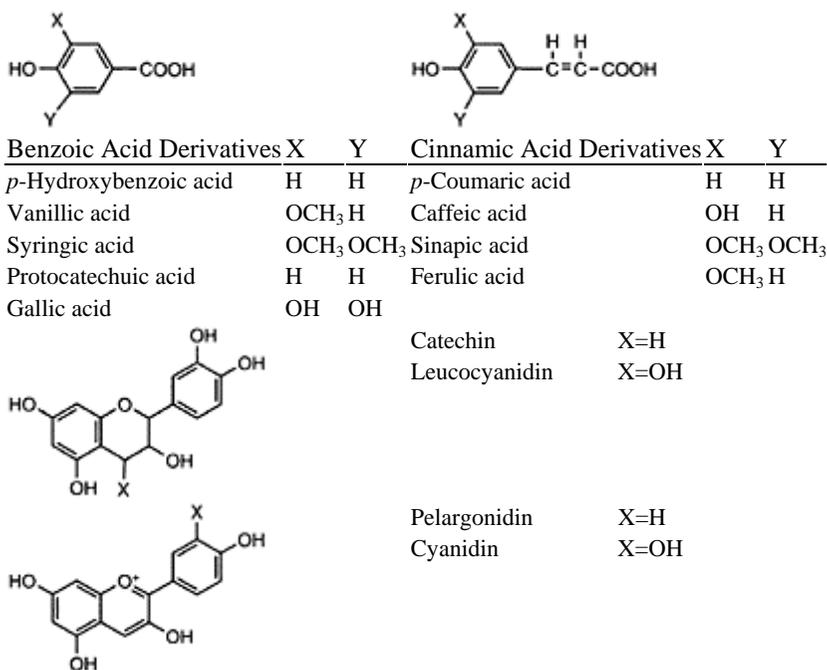


FIGURE 3.1 Chemical structures of basic phenolic acids and units of condensed tannins.

TABLE 3.1

Total Phenolic Acids in Some Oilseed Flours^a

| Phenolic Acid | Cottonseed | Glanded | Sesame | Soybean | Flax | Peanut | Sunflower |
|--------------------------|------------|---------|--------|---------|-------|--------|-----------|
| <i>p</i> -Hydroxybenzoic | | 1.1 | Trace | 13.9 | 2.6 | 2.0 | 7.4 |
| Vanillic | | – | – | – | Trace | – | 0.8 |
| Syringic | | – | – | 28.9 | 0.4 | – | 3.6 |
| <i>trans-p</i> -Coumaric | | 4.3 | 7.2 | 9.4 | 6.1 | 146.4 | 5.6 |
| <i>trans</i> -Ferulic | | 4.5 | 5.7 | 15.7 | 37.6 | 16.2 | 7.2 |
| <i>trans</i> -Caffeic | | 0.5 | 9.8 | 6.0 | 5.3 | 2.8 | 979.1 |
| <i>trans</i> -Sinapic | | – | – | – | 29.1 | 8.1 | – |
| Total | | 10.4 | 22.7 | 73.6 | 81.1 | 175.5 | 1003.7 |

^a Milligram per 100 g of flour.

Source: Adapted from Dabrowski, K. and Sosulski, F., 1984, *J. Agric. Food Chem.*, 32:128–130.

TABLE 3.2
Total Content of Phenolic Acids in Oilseed Meals and Flours^a

| Product | Total Phenolics |
|------------------|------------------------|
| Meals | |
| Evening Primrose | 1040 |
| Borage | 720 |
| Rapeseed | 2570 |
| Soybean | 455 |
| Flours | |
| Cottonseed | 56.7 |
| Peanut | 63.6 |
| Rapeseed | 639.9 |
| Soybean | 23.4 |

^a Milligrams per 100 g.

Source: Meals adapted from Zadernowski, R. et al., 1995, *Acta Acad. Agric. Techn. Olst.*, 27:107–118 (in Polish); soybean adapted from Naczka, M. et al., 1986, *Lebensm.-Wiss. u.- Technol.*, 19:13–16; and flours adapted from Kozłowska, H. and Zadernowski, R., 1988, paper presented at the 3rd North American Chemical Congress, Toronto, ON.

PHENOLIC ACIDS

Commonly cultivated rapeseed varieties, including canola (*Brassica napus* and *Brassica campestris*), have a similar phenolic content (Dabrowski and Sosulski 1984; Kozłowska et al., 1983; Krygier et al., 1982; Naczka and Shahidi, 1989, 1992; Naczka et al., 1986, 1998a, b; Shahidi et al., 1988). These are mainly located in seed cotyledons (Artz et al., 1986) and only trace amounts are found in seed coats. The total content of phenolic acids in various rapeseed protein products ranges from 1324.8 to 1837.0 mg/100 g of defatted meal and from 623.5 to 1280.9 mg/100 g of flour, on a dry weight basis (Table 3.3). On the other hand, rapeseed and canola hulls contain only 60 to 240 mg of sinapine per 100 g of sample, indicating the presence of embryo material in the hulls (Bell and Shires, 1982). Phenolic acids of rapeseed are in the free, esterified, glycosidic and insoluble-bound forms. Phenolic esters and free phenolic acids constitute as much as 80 and 16% of total phenolic compounds of rapeseed meals, respectively (Krygier et al., 1982; Naczka et al., 1986).

Tentative identification of principal phenolic acids of rapeseed has been reported (Durkee and Thivierge, 1975; Fenton et al., 1980; Kozłowska et al., 1975, 1983; Krygier et al., 1982). Sinapic acid is the predominant phenolic acid found in rapeseed (Clandinin, 1961), constituting over 73% of free phenolic acids and about 99% of the phenolic acids released from esters and glycosides (Dabrowski and Sosulski, 1984; Krygier et al., 1982) (Table 3.3). Minor phenolic acids are *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric ferulic and caffeic acids (Figure 3.1). In addition, chlorogenic acid has been found in trace amounts (Kozłowska et al., 1975, 1983; Krygier et al., 1982; Lo and Hill, 1972).

TABLE 3.3
Content of Free, Esterified and Insoluble Bound Phenolic Acids in Some Rapeseed Products

| Product | Phenolic Acids (mg/100 g) | | | Total |
|-----------------|---------------------------|------------|-----------------|--------|
| | Free | Esterified | Insoluble-Bound | |
| Tower meal | 244.0 | 1202.0 | 96.0 | 1542.0 |
| Regent meal | 262.0 | 1470.0 | 105.0 | 1837.0 |
| Altex meal | 248.0 | 1458.0 | 101.0 | 1807.0 |
| Hu You 9 meal | 119.1 | 1182.0 | 38.9 | 1340.0 |
| Midas meal | 143.5 | 1524.0 | 68.7 | 1736.2 |
| Triton meal | 61.5 | 1212.0 | 51.3 | 1324.8 |
| Mustard meal | 108.1 | 1538.0 | 22.4 | 1668.5 |
| Tower flour | 98.2 | 982.0 | – | 1066.5 |
| Candle flour | 84.5 | 1196.4 | – | 1280.9 |
| Gorzanski flour | 60.1 | 574.0 | 4.9 | 639.0 |
| Start flour | 71.8 | 700.0 | 4.7 | 776.5 |

Note: Expressed in sinapic acid equivalents.

Sources: Adapted from Krygier, K. et al., 1982, *J. Agric. Food Chem.*, 30:334–336; Kozłowska, H. et al., 1983, *J. Am. Oil Chem. Soc.*, 60:1119–1123; Naczka, M. and Shahidi, F., 1989, *Food Chem.*, 31:159–164; and Shahidi, F. and Naczka, M., 1990, in *Rapeseed/Canola: Production, Chemistry, Nutrition, and Processing Technology*, Van Nostrand Reinhold, New York, 291–306.

Free Phenolic Acids

Free phenolic acids present in rapeseed may contribute to the taste of rapeseed meals. They constitute 13 to 24% of total phenolic acids present in most rapeseed flours (Kozłowska and Zadernowski, 1988) and about 15% in canola meals (Table 3.3) (Naczka and Shahidi, 1989). However, flour obtained from Yellow Sarson variety contains only trace quantities of free phenolics (Krygier et al., 1982). No detectable amount of free phenolic acids has been found in hulls of Tower rapeseed (Durkee and Thivierge, 1975). Dabrowski and Sosulski (1984) have demonstrated that free phenolic acids are not present in 10 oilseed flours, including rapeseed (*B. campestris L.*).

Sinapic acid is a predominant phenolic acid; *p*-hydroxybenzoic, vanillic, gentisic, protocatechuic, syringic, *p*-coumaric, *cis*- and *trans*-ferulic, caffeic and chlorogenic acids have been found in small quantities (see Table 3.4 and Table 3.5). Both isomeric forms of sinapic acid, *Z* (*cis*) and *E* (*trans*), have been identified by a gas liquid chromatographic technique; these constitute up to 90% of the free phenolic acids present in rapeseed. However, an HPLC analysis did not confirm the presence of *cis*-sinapic acid (Kozłowska and Zadernowski, 1988).

Esterified Phenolic Acids

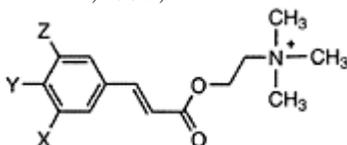
Esterified phenolic acids are the predominant form of phenolics in rapeseed, constituting up to 90% of phenolic acids present in rapeseed and canola (Table 3.3). The total content

of phenolic acids liberated from soluble esters found in flours obtained from Polish rapeseed varieties (Start, Górczanski, and Bronowski), Candle variety of canola and white mustard ranges between 520 and 700 mg/100 g of sample (Kozłowska et al., 1983). However, Krygier et al. (1982) found that Tower and Candle flours contain much higher levels of esterified phenolics, ranging from 982 to 1196.4 mg/100 g of sample. They also reported that Tower hulls contain 110.0 mg of soluble esters per each 100 g of sample. On the other hand, rapeseed meals contained 1182 to 1524 mg of esterified phenolic acids per 100 g sample (Table 3.3) (Naczka and Shahidi, 1989, 1990).

Pokorny and Reblova (1995) have published an excellent review of sinapine, the choline ester of sinapic acid, and the predominant phenolic ester in rapeseed. According to Bouchereau et al. (1991), the composition of phenolic acids in sinapine is genetically controlled, but its content is affected by the cultivar and by growing conditions. The presence of sinapoylcholine (sinapine), feruloylcholine, isoferuloylcholine, coumaroylcholine, 4-hydroxybenzoylcholine and 3,4-dimethoxybenzoylcholine has been reported in rapeseed (Larsen et al., 1983) (Figure 3.2). Mueller et al. (1978) reported that *B. napus* cultivars of rapeseed contain significantly ($P < 0.01$) higher levels of sinapine (1.65 to 2.26%) than *B. campestris* cultivars (1.22 to 1.54% sinapine). Furthermore, Fenwick et al. (1984) demonstrated that *B. napus* meals contain 1.17 to 1.83% sinapine.

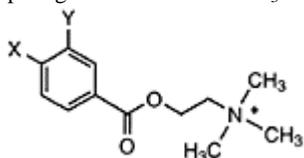
Similarly, a wide range of sinapine content (0.4 to 1.8%) has been observed in several cultivars of cruciferae crops, including the Brassica species (Kerber and Buchloh, 1980). However, Argentine and Polish rapeseed contain only 0.94 and 0.92% of sinapine, respectively (Austin and Wolff, 1968). Furthermore, Blair and Reichert (1984) found that the mean content of sinapine in defatted rapeseed and canola cotyledons is 2.67 and 2.85%, respectively. Later, Clausen et al. (1985) reported that the content of sinapine in *B. napus* cultivars ranges from 0.62 to 1.06% and that of *B. campestris* cultivars is 0.39 to 0.76%. Sinapic acid is the predominant phenolic acid and constitutes from 70.9 to 96.7% of the total content of esterified phenolic fractions (Table 3.4). Small quantities of other phenolic acids, such as *p*-hydroxybenzoic, vanillic, protocatechuic, syringic, *p*-coumaric, *cis*- and *trans*-ferulic, and caffeic acids, are also present in the hydrolyzate of the soluble esters extracted from Tower and Candle flours (Table 3.5) (Krygier et al., 1982). Of these, ferulic and caffeic acids are derived from choline and other ester bonds (Tantawy et al., 1983).

Sinapines are not the only phenolic esters found in rapeseed. Fenton et al. (1980) isolated sinapine and at least seven other compounds from rapeseed meals of Midas and Echo varieties that, upon hydrolysis, yielded sinapic acid. Zadernowski (1987) found that flours obtained from Polish rapeseed varieties contained from 0.16 to 0.65 mmol of phenolic acid glycosides per kilogram of flour. The compound 1-O- β -D glucopyranosyl sinapate is the predominant glycoside in rapeseed and canola varieties (Amarowicz and Shahidi, 1994; Amarowicz et al., 1995; Wanasundara et al., 1994).



| Sinapines | X | Y | Z |
|----------------------------|---|----|------------------|
| <i>p</i> -Coumaroylcholine | H | OH | H |
| Feruloylcholine | H | OH | OCH ₃ |

| | | | |
|--------------------|------------------|------------------|------------------|
| Isoferuloylcholine | H | OCH ₃ | OH |
| Sinapine | OCH ₃ | OH | OCH ₃ |
| Sinapine glucoside | OCH ₃ | O-Glu | OCH ₃ |



| | | |
|-------------------------|------------------|------------------|
| Sinapines | X | Y |
| 4-Hydroxybenzoylcholine | OH | H |
| Hesperalin | OCH ₃ | OCH ₃ |

FIGURE 3.2 Chemical structures of sinapines found in rapeseed.

TABLE 3.4

Contribution of Sinapic Acid in Different Fractions of Phenolic Constituents of Cruciferae Oilseeds

| Meal Sample | Sinapic Acid Contribution (%) in Phenolic Acid Fraction | | | |
|-------------|---|------------|-----------------|-------|
| | Free | Esterified | Insoluble-Bound | Total |
| Hu You 9 | 77.5 | 96.4 | 17.2 | 92.4 |
| Midal | 72.1 | 70.9 | 7.4 | 68.5 |
| Triton | 70.2 | 96.7 | 15.6 | 92.3 |
| Mustard | 85.4 | 72.6 | 32.1 | 72.8 |

Source: Adapted from Shahidi, F. and Naczki, M., 1990, in *Rapeseed/Canola: Production, Chemistry, Nutrition, and Processing Technology*, Van Nostrand Reinhold, New York, 291–306

Insoluble-Bound Phenolic Acids

Rapeseed meals contain 38.9 to 105.0 mg/100-g sample of insoluble phenolic acids expressed as sinapic acid equivalents (Naczki and Shahidi, 1989) (Table 3.3). Kozłowska et al. (1983) have shown that the total content of insoluble-bound phenolics in rapeseed flours ranges from 3.2 in Yellow Sarson to 5.0 mg/100 g in Gorczanski cultivar of Polish rapeseed. Nine phenolic acids have been identified, among which sinapic acid is predominant, followed by *p*-coumaric and *trans*-ferulic acids. Krygier et al. (1982) demonstrated that 24.5 mg/100 g of insoluble-bound phenolic acids are present in Tower hulls and that protocatechuic acid is a predominant phenolic acid. However, they did not find any detectable quantities of bound phenolics in Yellow Sarson, Candle and Tower flours.

TABLE 3.5
Phenolic Acids Liberated from Soluble Esters
Isolated from Rapeseed Flours^a

| Phenolic Acid | Yellow Sarson | Candle | Tower |
|--------------------------|---------------|--------|-------|
| <i>p</i> -Hydroxybenzoic | 0.3 | 1.5 | 1.1 |
| Vanillic | Trace | 0.6 | Trace |
| Protocatechuic | – | 0.5 | 0.2 |
| Syringic | – | Trace | Trace |
| <i>p</i> -Coumaric | – | Trace | 2.2 |
| <i>cis</i> -Ferulic | Trace | – | Trace |
| <i>trans</i> -Ferulic | 5.9 | 5.5 | 8.7 |
| Caffeic | – | – | 1.1 |
| <i>cis</i> -Sinapic | 49.1 | 7.2 | 76.3 |
| <i>trans</i> -Sinapic | 712.3 | 1116.2 | 894.9 |

^a Milligrams per 100 g.

Source: Adapted from Krygier, K. et al., 1982, *J. Agric. Food Chem.*, 30:334–336.

CONDENSED TANNINS

Condensed tannins are complex phenolic compounds widely distributed in foods and feeds of plant origin in soluble and insoluble forms. The total amounts of condensed tannins in canola and rapeseed hulls, calculated as the sum of soluble and insoluble tannin contents, ranges from 1913 to 6213 mg/100 g of oil-free hulls (Naczki et al., 2000). Condensed tannins may form soluble and insoluble complexes with proteins, which could explain the antinutritional effects of tannin-containing ingredients in nonruminant (Martin-Tanguy et al., 1977) and ruminant (Kumar and Singh, 1984) feeds. Condensed tannins are also considered potent enzyme inhibitors due to their complexation with enzymes; for example, condensed tannins in rapeseed may cause tainting of eggs. Tannins appear to block the metabolism of trimethylamine (TMA) by inhibiting TMA oxidase, an enzyme that converts TMA to odorless, water-soluble TMA-oxide (Fenwick et al., 1984).

Soluble Condensed Tannins

Bate-Smith and Ribereau-Gayon (1959) first reported the presence of condensed tannins in rapeseed hulls. This finding was verified by Durkee (1971), who identified cyanidin, pelargonidin (Figure 3.1) and an artifact *n*-butyl derivative of cyanidin in the hydrolytic products of rapeseed hulls. Clandinin and Heard (1968) reported the presence of approximately 3% tannins in rapeseed meal as assayed by the tannin determination method in cloves and allspice (AOCS 1987). Fenwick and Hoggan (1976) showed that this value includes sinapine. Thus, the corrected concentration of tannins in rapeseed meals is 18 to 45% lower than that given by Clandinin and Heard. In addition, Fenwick et al. (1984) demonstrated that whole and dehulled Tower meals contain 2750 and 3910 mg of tannins per 100 g, respectively. Leung et al. (1979) reported that rapeseed hull

contained only 100 mg acetone-extractable condensed tannins per 100 g sample and found that leucocyanidin is the basic unit of the isolated condensed tannins (Figure 3.1).

Mitaru et al. (1982) reported that hulls from rapeseed and canola seeds contain 20 to 220 mg of extractable tannins per 100 g sample. Blair and Reichert (1984) reported the presence of 90 to 390 mg of tannins per 100 g in the defatted rapeseed cotyledons and 230 to 540 mg of tannins per 100 g in the defatted canola cotyledons, as assayed by the modified vanillin method. They also found that sinapine does not interfere with the analysis of tannins. Later, Shahidi and Naczk (1988, 1989a, b) reported that canola varieties contains 682 to 772 mg of condensed tannins per 100 g of oil-free meal as assayed by the modified vanillin method. Only 556 and 426 mg of tannin per 100 g of oil-free meal are present in high glucosinolate Midas rapeseed and Hu You 9 Chinese cultivar of rapeseed, respectively. The latter results are somewhat higher than those reported by Blair and Reichert (1984), which may be due to existing differences in the solvent extraction systems employed for recovery of tannins.

The total content of soluble condensed tannins in hulls of some canola cultivars and Polish rapeseed varieties, as determined by the vanillin and proanthocyanidin assays, is summarized in Table 3.6; the content ranges from 23.4 to 2719 mg per 100 g of hulls. The least tannin content has been found in the sample of Westar hulls contaminated considerably with cotyledons (Naczk et al., 1994, 1998a, b, 2000, 2001a, b). Because seed coats of cereals and legumes are the primary locations of tannins in seeds (Salunkhe et al., 1990), the low tannin content in this sample may be explained as the effect of hull dilution with cotyledons. Thus, canola and rapeseed hulls contain up to 12 times more condensed tannins than those reported previously by Mitaru et al. (1982) and Leung et al. (1979). Up to 15-fold differences in condensed tannin values exist within the Westar, Cyclone and Leo varieties. According to Radhakrishnan and Sivaprasad (1980), the variation in tannin content of sorghum varieties ranges up to eight-fold. The content of tannins also depended on the stage of seed development, e.g., the maximum tannin content in sorghum is found in immature seeds (Price et al., 1979; Butler, 1982). Thus the differences in condensed tannin content within canola and rapeseed varieties may be due to the location and stage of seed development (Naczk et al., 1994, 1998a, b).

The biological and ecological role of tannins is attributed to their ability to bind and precipitate proteins (Bate-Smith, 1973; Hagerman and Butler, 1978; Salunkhe et al., 1990). The available information on the biological activity of canola and rapeseed condensed tannins is still diverse and fragmentary. Mitaru et al. (1982) reported that condensed tannins isolated from rapeseed hulls do not inhibit α -amylase activity *in vitro*, but Leung et al. (1979) found that tannins isolated from rapeseed hulls form a white precipitate after their addition to a 1% gelatin solution. The authors, however, did not attempt to quantify the biological activity of tannins. Later, Naczk et al. (1994) reported that condensed tannins, extracted from canola hull samples, precipitate 23.2 to 58.6 mg of dye-labeled BSA/g of hulls. The complete precipitation of dye-labeled BSA from the solution corresponded to 80 mg BSA/g of hulls.

TABLE 3.6
Content of Condensed Tannins in Canola Hulls
as Determined by Chemical Assays^a

| Variety | Vanillin Assay ^a | Proanthocyanidin Assay ^a |
|-----------------|-----------------------------|-------------------------------------|
| Canola | | |
| Cyclone | | |
| Sample 1 | 1485±63 | 1214±125 |
| Sample 2 | 1705±12 | 1231±35 |
| Sample 3 | 929±43 | 654±77 |
| Sample 4 | 414±5 | 402±42 |
| Ebony | 1335±17 | 910±110 |
| Excel | 83±1 | 175±16 |
| Westar | | |
| Sample 1 | 207±1 | 286±24 |
| Sample 2 | 23±6 | 92±25 |
| Vanguard | 1296±13 | 990±60 |
| Rapeseed | | |
| Leo | | |
| Sample 1 | 60±7 | 69±18 |
| Sample 2 | 2719±30 | 1539±103 |
| Mar | 114±2 | 157±8 |
| Marita | 69±3 | 115±18 |
| Idol | 37±17 | 76±7 |

^a Milligrams per 100 g of hulls.

Source: Adapted from Naczk, M. et al., 2000, *J. Agric. Food Chem.*, 48:1758–1762.

The affinities of canola tannins for proteins have been characterized by the precipitation index (PI) calculated using the equation: $PI=B/C$, where B represents biological activity expressed as milligram of dye-labeled BSA precipitated per 1 g of hulls. The PI values for tannins isolated from samples of high-tannin canola hulls do not exceed 5.0 mg of BSA/mg of tannins but, for low-tannin canola hulls, PI ranges from 17.7 to 40.7 mg of BSA/mg of tannins. Thus, tannins isolated from low-tannin canola hulls show greater affinities for proteins than those from high-tannin canola hulls. This may arise from the existing differences in the molecular weight of tannins isolated from high- and low-tannin samples of canola hulls. According to Porter and Woodruffe (1984), the ability of condensed tannins to precipitate proteins depends on their molecular weight. Subsequently, Naczk et al. (2001b) expressed the protein-precipitating potentials of condensed tannins in tannins from canola and rapeseed hulls as a slope of lines reflecting the amount of insoluble tannin-protein complex formed vs. the amount of tannins added to the reaction mixture. The slope values of lines obtained using the protein precipitation assay of Hagerman and Butler (1978) are 2.96 to 10.91 (absorbance unit at 510 nm/mg of added tannins), and those of lines obtained using the dye-labeled bovine serum albumin assay of Asquith and Butler (1985) range from 28 to 267 (percent of precipitated protein/mg of added tannins). For both assays a statistically significant

($P < 0.001$) correlation exists between the slopes and the condensed tannin contents in canola and rapeseed hulls.

Insoluble Condensed Tannins

The insoluble tannins may comprise 70.0 to 95.8% of the total condensed tannins in canola and rapeseed hulls. The differences in the content of insoluble condensed tannins among and within canola and rapeseed varieties are much lower than those found for soluble condensed tannins. The insolubility of condensed tannins may be the result of polymerization, as well as formation of insoluble complexes with the fiber and protein fractions of canola and rapeseed. The insoluble condensed tannins may be extracted into sodium dodecyl sulfate (SDS) solution. The amounts of SDS-extractable condensed tannins in canola and rapeseed hulls are comparable with those of soluble condensed tannins and comprise 4.7 to 14.7% of insoluble condensed tannins present in the hulls (Naczek et al., 2000).

OTHER PHENOLIC COMPOUNDS

Durkee and Harborne (1973) first observed the presence of flavonoid glycosides containing sinapic acid. These authors also identified a flavonol glycoside, kaempferol-7-glucoside-3-sophoroside, in the Brassica seed extracts. Later, Tantawy et al. (1983) reported the presence of two flavonoid glycosides in rapeseed meal, namely, 3-(*O*-sinapoyl sophoroside)-7-*O*-glucoside of kaempferol and 3-(*O*-sinapoyl glucoside)-7-*O*-sophoroside of kaempferol.

EFFECT OF PROCESSING

Phenolic Acids

Extraction

After oil extraction, meal contains a large amount of phenolic compounds, the content of which in rapeseed and other oilseed meals may be reduced by treatment with ammonia (gaseous or in alkanol solution). McGregor et al. (1983) found that gaseous ammoniation of *Brassica juncea* mustard meal removed up to 74% of sinapine. However, Fenwick et al. (1979, 1984) demonstrated that treatment of *B. napus* meal with ammonia or lime reduces sinapine content by approximately 90%. Similarly, Kirk et al. (1966) reported a drop of up to 90% in the sinapine of Crambe meal after its treatment with gaseous ammonia; however, aqueous ammonia is less effective. The exact mechanism of action of ammonia on sinapine is unknown. However, Austin and Wolff (1968) reported that, under alkaline conditions, sinapine is hydrolyzed to sinapic acid and choline.

TABLE 3.7
Removal of Phenolics and Tannins by Methanol-Ammonia-Water/Hexane Extraction System

| Canola Variety | Removal (%) | |
|----------------|----------------------|----------|
| | Total Phenolic Acids | Tannins |
| Tower | | |
| A | 69.5±2.8 | 70.1±1.7 |
| B | 75.2±2.8 | 78.0±1.7 |
| Regent | | |
| A | 78.1±1.4 | 71.6±2.0 |
| B | 74.6±1.4 | 77.5±2.0 |
| Altex | | |
| A | 71.1±1.8 | 72.6±4.8 |
| B | 72.0±1.8 | 84.9±4.8 |

Note: A—meal extracted with 10% NH₃ in methanol and hexane; B—meal extracted with 10% NH₃, 95% methanol extracted

Source: Adapted from Shahidi, F. and Naczek, M., 1988, in *Proceedings 14th Int. Conf. Group Polyphenols*, Bulletin de Liason, 14:89–92.

Goh et al. (1982) used ethanol containing 0.2 M ammonia to extract Candle and Tower canola meals and removed up to 82 and 39% of their phenolic content, respectively. These authors did not offer any explanation for the difference in the efficiency of removal of phenolics from these meals. In another study, methanol-ammonia-water-hexane is used and results in the removal of 72.4% of the phenolics of canola meals in a laboratory scale process (Table 3.7) (Diosady et al., 1985; Naczek et al., 1986); its efficacy was improved to 80% in a semipilot extraction process (Diosady et al., 1987). The methanol-ammonia-water/hexane extraction system removed 82 and 50% of the esterified and free phenolic acids originally present in the seeds, respectively. However, the concentration of insoluble-bound phenolic acids was not affected (Naczek and Shahidi, 1989; Shahidi and Naczek,

1988). Dabrowski and Siemieniak (1987) found that about 90% of sinapine is removed after 30 min of batch extraction of rapeseed with methanol-ammonia. Dabrowski and Sosulski (1983) reported that processing rapeseed to protein concentrates and isolates by typical industrial procedures reduces the content of phenolics by 60 to 83%. However, preparation of concentrates containing trace amounts of phenolics requires as much as seven extractions with 70% ethanol (Kozłowska and Zadernowski, 1988). Hydrothermal treatment of double-low rapeseed (low glucosinolates and low erucic acid content) of Jantar variety does not affect the phenolic acid content of the resultant flour; this treatment is an alternative to cooking during commercial processing of rapeseed. However, three additional extraction stages with 80% ethanol are required to reduce the content of phenolics to trace levels (Kozłowska and Zadernowski, 1988).

Heating

Cooking may affect the sinapine content in canola and rapeseed meals. Larsen et al. (1983) reported that 30-min toasting of dehulled and defatted Erglu rapeseed meal lowers its sinapine content by 17%—a decrease associated with the formation of oligomeric sinapine derivatives. Jensen et al. (1991) later demonstrated that heating reduces the content of sinapine, but increases the content of lignan-type products in rapeseed meal. Subsequently, by using model systems, Rubino et al. (1995, 1996) showed that sinapic acid readily converts to a lignan, thomasidioic acid (Figure 3.3) under alkaline conditions. These authors envisaged that additional studies would be required to establish whether a similar reaction occurs when canola meal is subjected to alkaline treatment.

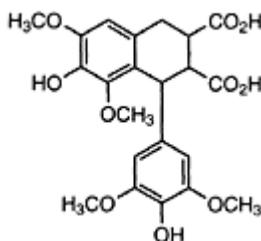


FIGURE 3.3 Chemical structure of thomasidioic acid.

Condensed Tannins

Processing rapeseed with methanol alone extracts 16% of its tannins (Shahidi and Naczki, 1989a, b). Addition of 5% (v/v) water to methanol increases the efficiency of tannin extraction to 36%. Presence of ammonia in absolute or 95% methanol greatly enhances the extraction of tannins from rapeseed. Methanol-ammonia-water/hexane, however, is the most effective system for the removal of tannins (Table 3.7). The resultant meal contains from 4 to 33% of tannins originally present in the meals. This may be due to the extraction of tannins from the seed into the polar phase or decomposition of tannins to products insensitive to vanillin reagent. Gandhi et al. (1975) found that ammonia depolymerized the tannins present in salseed meal so that the processed meal obtained is nontoxic and palatable. Moreover, upon alkali treatment, tannins may form phlobaphenes, which are chemically and nutritionally unreactive (Swain, 1979). On the other hand, Fenwick et al. (1984) reported that treatment of *B. napus* meals with ammonia or lime does not affect their tannin content appreciably.

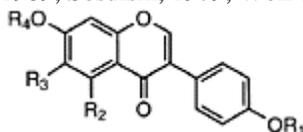
Commercially, crushed rapeseed is generally cooked to 90 to 110°C for 20 to 25 min to facilitate oil release and to inactivate the enzyme myrosinase (Unger, 1990). Under experimental conditions (110°C, 30 min) simulating commercial cooking, heat treatment of canola hulls results in a decrease in the content of tannins as catechin equivalents in hulls from 2374 to 1944 mg/100-g sample. This treatment, however, increased the amount of precipitable phenolics by 18% (Naczki et al., 1996).

SOYBEANS

INTRODUCTION

There are three major species of soybean: *Glycine ussuriensis* (Regel and Maack wild), *Glycine max* (L.) and *Glycine gracilis* (Skvortzow intermediate). Of these, *G. max* (L.) Merr. (Leguminosae) is commonly cultivated throughout the world (Nwokolo and Smartt, 1966; Smith, 1978). Soybean seeds are used for the extraction of edible oil, human food and livestock feed. The highest human consumption of soybean is in Asia, where it has traditionally been processed into fermented (miso, natto, tempeh) and nonfermented (fresh soybean, soybean sprouts, soymilk, tofu) products (Wang and Murphy, 1996). Soybean grits, flours, flakes and protein concentrates and isolates are widely used as a food component in the U.S. and U.K. (Berk, 1992; Wiseman et al., 2002).

Fleury et al. (1991) reported the presence of anthocyanins, flavonols, flavones, isoflavones and chalcones and their derivatives with acetic, *p*-hydroxybenzoic, caffeic, coumaric, ferulic, gallic, malonic, hydroxycinnamic, oxalic, and sinapic acids. Of these, the isoflavones (Figure 3.4) are of much interest because of their estrogenic (Makela et al., 1995), cancer preventive, antimutagenic and antiproliferative (Hirano et al., 1994; Kuntz et al., 1999; Miyazawa et al., 1999, 2001; Wiseman, 1996), and antioxidant (Arora et al., 1998; Wei et al., 1995) activities. Table 3.8 gives the total content and distribution of isoflavone derivatives in some soy foods. Phenolic compounds have also been implicated in the formation of certain undesirable colors and flavors in soybean products (Matsuura et al., 1989; Sosulski, 1979; Wolf and Cowan, 1975).



| Compound | R ₁ | R ₂ | R ₃ | R ₄ |
|-----------------------|-----------------|----------------|------------------|----------------------|
| Daidzein | H | H | H | H |
| Formononetin | CH ₃ | H | H | H |
| Genistein | H | OH | H | H |
| Glycitein | H | H | OCH ₃ | H |
| Daidzin | H | H | H | glucose |
| Genistin | H | OH | H | glucose |
| Glycitin | H | H | OCH ₃ | glucose |
| 6''-O-Acetyldaidzin | H | H | H | 6''-O-acetylglucose |
| 6''-O-Acetylgenistin | H | OH | H | 6''-O-acetylglucose |
| 6''-O-Acetylglycitin | H | H | OCH ₃ | 6''-O-acetylglucose |
| 6''-O-Malonyldaidzin | H | H | H | 6''-O-malonylglucose |
| 6''-O-Malonylgenistin | H | OH | H | 6''-O-malonylglucose |
| 6''-O-Malonylglycitin | H | H | OCH ₃ | 6''-O-malonylglucose |

FIGURE 3.4 Chemical structures of isoflavones found in soybeans.

TABLE 3.8
Range of Isoflavone Content in Some Soy Foods^a

| Soy Food | Glucosides | | | Malonylglucoside | | | Acetylglucoside | | | Aglycon | | | Total |
|---------------|------------|---------|-------|------------------|--------|------|-----------------|-------|-----|---------|--------|-----|---------|
| | DA | GE | GL | DA | GE | GL | DA | GE | GL | D | G | Gn | |
| Soy milk | 20-94 | 25-130 | 4-16 | 6-42 | 14-45 | 0-5 | 0 | 4-10 | 0 | 1-3 | 1-4 | 0- | 80-165 |
| | | | | | | | | | | | | 1 | |
| Tofu | 40-105 | 64-143 | 11-29 | 64-156 | 40-158 | 7-19 | 0-18 | 8-17 | 0 | 3-22 | 4-30 | 2-4 | 202-347 |
| Soy sauce | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0-3 | 1-6 | 0-4 | 0-3 | 1-14 |
| Miso | 62-66 | 89-92 | 10-13 | 14-32 | 17-37 | 0-8 | 8-14 | 20-23 | 0-9 | 25-29 | 29-43 | 12 | 228-235 |
| Tempeh | 93-105 | 206-226 | 14 | 64-66 | 111 | 8 | 35-49 | 46-50 | 0 | 77-85 | 89-103 | 8-9 | 531-538 |
| Cooked burger | 3-10 | 5-23 | 0-6 | 0-14 | 318.7 | 0-6 | 0-20 | 8-31 | 0 | 0-6 | 6-8 | 0-4 | 9-95 |

Note: DA—daidzin; GE—genistin; GL—glycitin; D—daidzein; G—genistein; Gn—glycitein.

^a Milligram per kilogram of wet weight.

Source: Adapted from Murphy, P.A. et al., 1999, *J. Agric. Food Chem.*, 47:2697-2704.

PHENOLIC ACIDS

Maga and Lorenz (1974) reported that soybean flakes contain 25.6 mg of phenolic acid per 100-g sample, whereas Dabrowski and Sosulski (1984) demonstrated that soybean flours contain 73.6 mg of total phenolics per 100-g sample. Later, Naczka et al. (1986) reported the presence of 455 mg of total phenolics per 100 g of 48% commercial soybean flour.

Arai et al. (1966) identified at least seven phenolic acids in ethanolic extracts of defatted soy flour and reported that syringic acid is the predominant phenolic compound present. On the other hand, using an HPLC technique, How and Moor (1982) isolated 40 phenolic substances in the total phenolic fraction of defatted soy flakes. Of these, eight were tentatively identified as gentisic, vanillic, chlorogenic, syringic, *o*- and *p*-coumaric ferulic, and salicylic acids. The compounds *o*- and *p*-coumaric and ferulic acids were predominant. Salicylic acid is the principal phenolic acid in Supra 620 soybean isolates (How and Moor, 1982). Traces of phenolic acids possessing a typical soy flavor have also been identified (Rackis et al., 1970). Maga and Lorenz (1974) reported that ferulic, syringic and vanillic acids are the major phenolic acids in soybean flours. Dabrowski and Sosulski (1984) also found *p*-hydroxybenzoic, *trans*-caffeic, *trans-p*-coumaric, *trans*-femlic, and syringic acids (Figure 3.1) in soybean flours and demonstrated that syringic acid is the major phenolic acid present (Table 3.1).

ISOFLAVONES

Several isoflavones, namely, daidzein (7,4'-dihydroxyisoflavone), glycitein (7,4'-dihydroxy-6-methylisoflavone) and genistein (6,7,4'-trihydroxyisoflavone) (Figure 3.4)

have been isolated and identified in soybean protein products and found to possess an herb-like flavor, bitterness and astringency (Huang et al., 1979). These isoflavones occur in soybean and soy foods in the form of glucosides (daidzin, glycitin, genistin), malonylglucosides (6''-*O*-malonyldaidzin, 6''-*O*-malonylglycitin, 6''-*O*-malonylgenistin), acetylglucosides (6''-*O*-acetyldaidzin, 6''-*O*-acetylglycitin, 6''-*O*-acetylgenistin) and also in the free form (Barnes et al., 1994; Fleury et al., 1991; Hoeck et al., 2000; Kudou et al., 1991). Presence of formononetin (Murthy et al., 1986), isoformononetin (Ingham et al., 1981), and 6,7,4'-trihydroxyisoflavone (Gyorgy et al., 1964) has also been reported. Later, Hosny and Rosazza (1999) identified three novel isoflavones in soybean molasses:

- Glycitein 7-*O*-β-D-(2'',4'',6''-*O*-triacetyl)glucopyranoside
- 8-(γ-Hydroxy-γ,γ-dimethylpropyl)genistein 7-*O*-β-D-glucopyranoside
- 5-Hydroxy-8-(γ-hydroxy-γ,γ-dimethylpropyl)-3',4'-dimethoxyisoflavone-7-*O*-β-D-glucopyranoside

Recently, Hosny and Rosazza (2002) isolated and identified in soybeans two novel isoflavones, dihydrodaidzin and dihydrogenistin, and a new isoflavone, 2'',6''-diacetyloninin. Of these, glucosides were the predominant form of isoflavones in soybean products.

The content of isoflavones in soybean seeds is influenced by varietal and genetic differences, maturity stage, and climate and growing conditions (Dalais et al., 1997; Eldridge and Kwolek, 1983; Simonne et al., 2000; Wang and Murphy, 1994). The total content of isoflavones in soybeans ranges from 47.2 to 420.0 mg per 100 g (Eldridge and Kwolek, 1983; Simonne et al., 2000; Wang and Murphy, 1994, 1996) and, according to Wang and Murphy (1994), may vary from 117.6 to 174.9 mg/100 g among different locations within the same crop year. These authors also reported that Japanese soybean varieties have a higher level of 6''-*O*-malonylglycitin as well as higher ratios of 6''-*O*-malonyldaidzin to daidzin and 6''-*O*-malonylgenistin to daidzin than soybeans grown in the U.S. According to Tsukamoto et al. (1995), isoflavone content in soybean cultivars planted in April at Kyushu, Japan, is significantly lower than in those planted in June and July. Hutabarat et al. (2001) reported that the mean concentration of daidzein and genistein in soybeans grown in Indonesia is 50% higher ($P < 0.05$) than those grown in Australia.

Hoeck et al. (2000) observed varietal and growing location effects on isoflavone content in six soybean cultivars grown at eight locations for 2 years. These authors reported the existence of statistically significant differences ($P < 0.05$) in total and individual contents of isoflavones in soybeans grown at different locations. Furthermore, Fleury et al. (1991) noted that growing location induces a greater difference in isoflavone content (ratio of 4:1) than varietal type (ratio of 2:1). Depending on the genotype, immature soybean seeds harvested at 80% maturity stage may contain higher (Simonne et al., 2000) or lower (Kudou et al., 1991; Simonne et al., 2000) contents of isoflavones than mature samples (Table 3.9). This suggests that the regulation of isoflavone synthesis pathways may be influenced by soybean genotype (Simonne et al., 2000). In the seeds, isoflavones are mostly concentrated in the hypocotyl; cotyledons contain only 20% of the amount of isoflavones found in hypocotyl (Table 3.10).

TABLE 3.9
Content of Isoflavones at Immature^a and Mature Stages of Soybean Development

| Variety | Maturity Stage | Moisture (%) | Total (mg/100 g) |
|-------------|----------------|--------------|------------------|
| NTCPR93-40) | Immature | 65.1 | 720±12.2 |
| | Mature | 13.0 | 225±4.9 |
| Honey brown | Immature | 61.4 | 1283±25.4 |
| | Mature | 13.0 | 644±2.4 |
| NTCPR93-286 | Immature | 60.5 | 810±3.3 |
| | Mature | 13.0 | 377±3.2 |
| Hutcheson | Immature | 51.1 | 2013±53 |
| | Mature | 13.1 | 2545±76.8 |

^a Immature soybeans were harvested at 80% maturity.

Source: Adapted from Simonne, A.H. et al., 2000, *J. Agric. Food Chem.*, 48:6061–6069.

TABLE 3.10
Distribution of Soybean Isoflavones^a

| Variety | Hull | Hypocotyl | Cotyledon |
|---------|------|-----------|-----------|
| Amsoy | 10.6 | 1756.5 | 158.5 |
| Tiger | 20.0 | 1405.2 | 319.2 |

^a Milligrams per 100 g.

Source: Adapted from Eldridge, A.C. and Kwolek, W.F., 1983, *J. Agric. Food Chem.*, 31:394–396.

OTHER PHENOLIC COMPOUNDS

Cyanidin 3-glucoside is the predominant anthocyanin in soybean seed coats (Kuroda and Wade, 1933). Pelargonidin 3-glucoside has also been identified in black-seeded soybean varieties (Yoshikura and Hamaguchi, 1969). Recently, Hosny and Rosazza (1999) identified two glycoside esters in the water-soluble portion of soybean molasses:

- 1-*O*-(*E*)-feruloyl[α -L-arabinofuranosyl-(1→3)][β -D-glucopyranosyl(1→6)] β -D-glucopyranose
- 1-*O*-(*E*)-3,4,5-trimethoxycinnamoyl[α -L-arabinofuranosyl(1→3)-*O*-(β -D-glucopyranosyl(1→6))] β -D-glucopyranose

Soybeans may also contain isoflavonoid phytoalexins such as coumestrol (7,12-dihydroxy-coumestan), a coumestan isoflavone, and a pterocarpan phytoalexins such as glyceollins I, II and III (Figure 3.5). These phytoalexins are accumulated in soybean seeds after their exposure to microorganisms (Boue et al., 2000; Ebel et al., 1976). Knuckles et al. (1976) reported that the content of coumestrol in soybean seed is 0.12 mg/100 g on a dry basis (d.b.), while in soybean sprouts it is 7.11 mg/100 g d.b. Lookhart et al. (1979) found that soybean hulls contain the highest level of coumestrol; its content in seeds increases with increasing germination time. Recently, Hutabarat et al. (2001) reported that soymilk samples purchased in Indonesia contain 8.0 mg of coumestrol per

100 g of wet weight. Glyceollins have been shown to prevent the accumulation of aflatoxin B₁ in cultures of *Aspergillus flavus* (Song and Karr, 1993). Inoculation of living soybean plant with *Aspergillus* species increases the concentrations of coumestrol and glyceollins in the cotyledons (Boue et al., 2000). Recently, using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis, Takahata et al. (2001) detected procyanidins with up to 30 catechin/epicatechin units in brown soybean seed coats.

EFFECT OF PROCESSING

Soaking

Soaking is one of the steps in the production of soymilk, tempeh and tofu; it is used to aid in dehulling and grinding beans. During the soaking process, isoflavone glucosides may be hydrolyzed by β -glucosidases to their aglycones, daidzein and genistein (Figure 3.4). The resulting isoflavone aglycones have more objectionable flavors than their glucoside precursors (Matsuura et al., 1989). The role of β -glucosidases in this reaction was confirmed by Matsuura and Obata (1993) by the addition of glucono- δ -lactone, β -glucosidase competitive inhibitor to the soaking water. The concentration of isoflavone aglycones depends on the conditions of soaking process. Soaking soybeans for 6 h at 50°C in distilled water or in a 0.25% sodium bicarbonate solution results in the formation of substantial amounts of isoflavone aglycones in soybean products (Table 3.11) (Ha et al., 1992). Furthermore, soaking soybeans for 10 to 12 h at room temperature in tap water

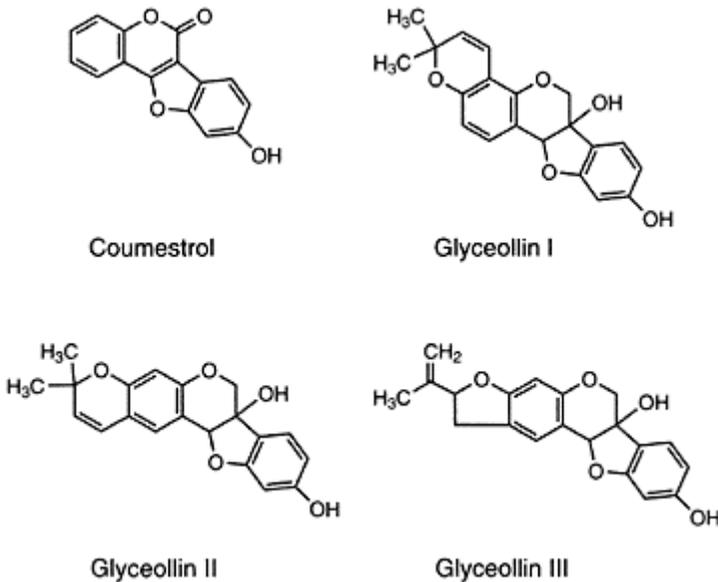


FIGURE 3.5 Chemical structures of soybean phytoalexins.

results in loss of about 10% of isoflavones originally present (Wang and Murphy, 1996). According to these authors, soaking water contained 34% of daidzein and 18% of genistein originally present in the beans.

TABLE 3.11
Concentrations of Isoflavone Aglucones
Daidzein and Genistein in Soaked Defatted
Soybean Flakes^a

| Soaking Conditions | Daidzein | Genistein |
|---|----------|-----------|
| 6 h in 50°C distilled water | 40.1±9.9 | 35.5±6.2 |
| 6 h in 50°C 0.25 NaHO ₃ | 36.2±5.2 | 31.3±6.4 |
| 30 min in boiling 0.25 NaHCO ₃ | 1.8±0.01 | 2.3±0.09 |

^a Milligrams per 100 g, on a dry weight basis.

Source: Adapted from Ha, E.Y.A. et al, 1992, *J. Food Sci.*, 57:414–418.

Heating

Heat treatment of soybean is used to inactivate trypsin inhibitors, kill bacteria, reduce raw and beany flavors, and improve nutritional value of products. Cooking beans is one of the steps employed in the production of tempeh and tofu (Shurtleff and Aoyagi, 1979) because cooking increases leaching of isoflavones to the cooking water. Simmone et al. (2000) reported that blanching immature beans for 1 min in boiling water or boiling the beans for 35 min in water results in similar losses (54 to 56%) of isoflavones originally present in the beans. Furthermore, during production of tempeh most of the isoflavones are leached out into the cooking water (Wang and Murphy, 1996). In addition, it was found that cooking does not affect the retention of isoflavones in tofu because the cooking water is incorporated into the final product.

Heat treatment alters the distribution of individual isoflavones in cooked soy foods. Cooking water slurries of soybean or dehulled soybeans brings about a drastic reduction in the concentration of malonylated isoflavone glucosides, but increases the content of isoflavone glucosides, acetyl isoflavone glucosides and aglycones (Kudou et al., 1991; Simonne et al., 2000; Wang and Murphy, 1996). Kudou et al. (1991) suggested that malonyl glucosides are transformed into acetyl isoflavone glucoside derivatives during heating.

UNFERMENTED SOY PRODUCTS

Soy Protein Isolates

Approximately 53% of isoflavones originally present in raw materials are lost during the processing of soybean into soy protein isolate (Wang and Murphy, 1996). Major losses of isoflavones occur during alkaline extraction of proteins; the alkaline step also modifies the distribution of isoflavone forms. The fraction soluble in alkaline solution contains elevated levels of aglycones compared to those found in raw soybean. Only 25.8%

retention of isoflavones is achieved during processing of soybean to soy protein isolate (Wang et al., 1998). The loss of isoflavones is distributed among the wash water (21.6%), solid waste (19%) and whey (14.3%); however, approximately 19.4% of isoflavones is not accounted for in the mass balance study conducted by Wang et al. (1998).

Soymilk

Soymilk may be produced from soy isolates and whole soybeans. Wang and Murphy (1996) did not observe any loss of isoflavones during soymilk production. The total content of isoflavones in U.S. retail samples of soymilk ranges from 8.0 to 16.5 mg/100 g of wet weight. These isoflavones mostly comprise their glucoside and malonylglucoside derivatives. The contents of daidzin and genistin are between 2.0 to 9.4 and 2.5 to 13.0 mg per 100 g of wet weight, respectively (Murphy et al., 1999). Recently, Hutabarat et al. (2001) demonstrated that starting material used for production of soymilk does not affect the contents of daidzein and genistein on a dry weight basis. However, up to four-fold differences on a dry weight basis have been observed in the content of daidzein and genistein between samples of soymilk brands purchased in Australia and Indonesia. Variations in these levels may be due to different cultivars of soybean used and techniques employed for soymilk production (Hutabarat et al., 2001).

Tofu

The production of tofu involves soaking soybeans in water, washing them, and then grinding them in water. The slurry is subsequently cooked and filtered to remove insoluble matter known as okara from the soymilk. Following this, calcium ion is added to soymilk and proteins are coagulated by heat to form a curd (tofu). Traditional tofu, called momem or cotton tofu, contains only 33% (based on dry matter) of isoflavones originally present in soybean seeds (i.e., 21.7 mg/100 g of seeds). Approximately 43% of the total mass of isoflavones is lost during coagulation of curd, but the distribution of isoflavone forms in tofu is only slightly altered when compared to that of seeds. Isoflavones are not destroyed during tofu production, but rather distributed into soaking water, okara, whey and tofu (Wang and Murphy, 1996). The total content of isoflavones in tofu marketed in the U.S. ranges from 20.2 to 34.7 mg/100 g of wet weight (Murphy et al., 1999). On the other hand, samples of raw tofu purchased in Singapore contained between 25.6 and 31.9 mg of isoflavones per 100 g of wet weight, while those of cooked tofu varied from 23.1 to 28.7 mg/100 g of wet weight (Franke et al., 1999).

FERMENTED SOY PRODUCTS

Fermented Soybean Curd

Fermented soybean curd is a popular Chinese soy food. It is a soft, cheese-like fermented product made of tofu classified into white and red fermented soybean curds (Liu et al., 1988). Chung (2000) identified eight phenolic compounds in volatile flavor components of red fermented soybean curds in concentrations of 0.0033 to 0.22 mg/100-g sample:

eugenol, coumarin, phenol, 4-ethyl-2-methoxyphenol, 4-methylphenol, 4-ethylphenol, ethyl cinnamate, and 2-methoxy-4-vinylphenol.

Soy Pasta

Soybean pasta is an important Oriental soy food known as miso in Japan, tauchu in Indonesia, jang in Korea, taotsi in the Philippines and Jiang or chiang in China (Hutabarat et al., 2001; Lin, 1991). The production of soy pasta involves the preparation of koji (see the next section), which may then be mixed with cereals such as rice or barley (Chiou and Cheng, 2001; Zarkadas et al., 1997). The mash is then cooked after addition of whole soybean mash and salt and inoculated with a mixture of microorganisms and fermented. The total content of isoflavones in miso sold in the U.S. ranges from 22.8 to 23.5 mg/100 g of wet weight. The distribution of isoflavone forms in miso is summarized in Table 3.8 (Murphy et al., 1999). On the other hand, samples of soy pasta purchased in Indonesia have contained 12.8 to 27.6 mg of daidzein per 100 g of wet weight and 10.1 to 23.4 mg of genistein per 100 g of wet weight (Hutabarat et al., 2001).

Soy Sauce

In the production of soy sauce, part of the raw material is first used to prepare a cultured material called koji. During koji production (2 to 3 days), proteins and starches are digested by enzymes produced by *Aspergillus* species and a part of the ferulic acid is converted to vanillin and vanillic acid, while *p*-hydroxycinnamic acid is formed and partially converted to *p*-hydroxybenzoic acid (Yokosuka, 1986). In addition, isoflavone glycosides daidzin and genistin are transformed into daidzein and genistein (Chiou and Cheng, 2001). Koji is then mixed with the remaining raw material and brine to produce a wet mash or moromi; the mixture is finally fermented to afford soy sauce. During the fermentation process, ferulic and *p*-hydroxycinnamic acids are metabolized into 4-ethylguaiacol and *p*-ethylphenol, respectively. These phenolics are considered important components of soy sauce flavor (Yokosuka, 1986) and are mainly derived from wheat components. Because isoflavone glucosides, malonylglucosides, and acetylglucosides are degraded by the microorganisms, soy sauce is considered a poor source of isoflavones (Murphy et al., 1999). On the other hand, novel tartaric acid isoflavone derivatives may be formed. Kinoshita et al. (1997) reported that Japanese soy sauces contain conjugated ethers of tartaric acid with daidzein, genistein, and 8-hydroxygenistein. These authors developed a chemometric technique for differentiating the Japanese soy sauces based on HPLC patterns for these novel isoflavone derivatives.

Tempeh

Tempeh is a fermented product indigenous to Indonesia. Fermentation brought about by genus *Rhizopus* significantly alters distribution of isoflavones in the final product. Tempeh contains 6.5 times higher levels of aglycones and 57% lower levels of glucosides than those in the original raw material (Wang and Murphy, 1994). This increase in aglycones may be due to the hydrolytic action of β -glucosidase from fungi (Murakami et al., 1984). Tempeh purchased in Indonesia and Australia has contained 74.5 and 17.6

mg/100 g of daidzein, respectively, and 85.4 and 33.3 mg/100 g of genistein, respectively, on a dry weight basis. These differences in the isoflavone levels may originate from the soybean cultivar used and processing steps employed (Hutabarat et al., 2001).

OTHER OILSEEDS

BORAGE

The seeds of borage (*Borago officinalis* L.) plant serve as a good source of an oil containing up to 25% of γ -linolenic acid (18:3 ω 6) (Whipkey et al., 1988). Available information on the phenolic compounds of borage seeds is still diverse and fragmentary. Recently, Wettasinghe et al. (2001) fractionated ethanolic extract of borage meal into four fractions using a Sephadex LH-20 column chromatography. Using TLC methodology, these authors located one and two strong antioxidative spots in fractions I and IV, respectively. These compounds were then positively identified as rosmarinic, syringic and sinapic acids.

Recently, Zadernowski et al. (2002) reported that defatted borage meal contains 18.5 mg of total phenolic acids per 100 g of meal. Free phenolics were the predominant form of phenolic acids and comprised 69.3% of the total phenolic acids in borage meal. Ferulic acid was the principal free phenolic acid identified, accounting for 50.2% of total free phenolic acids of borage. Protocatechuic, *p*-hydroxybenzoic, and *p*-hydroxyphenyllactic acids contributed 40.5% to the total free phenolic acids. The total concentration of 2-hydroxy-4-methoxybenzoic, gallic, *p*-coumaric and cinnamic acids did not exceed 1.4 mg/100 g. On the other hand, phenolic acids liberated from soluble esters contributed 40.4% to the total phenolic acids; hydroxycaffeic acid was the major phenolic acid in this fraction. Small quantities (0.1 to 0.3 mg/100 g) of 2-hydroxy-4-methoxybenzoic, caffeic and sinapic acids were also present in borage seeds. In addition, up to eight minor esterified phenolic acids (below 0.1 mg/100 g) were detected in the meal.

COTTONSEED

Cotton is a plant grown in the tropical and subtropical regions of the world for food and fiber use; its hulls and linters comprise about 45% and its kernels 55% of cottonseed weight. Gossypol is the major phenolic component in pigment glands of cottonseed kernels at 1400 to 13,000 mg/kg sample (Heywood et al., 1986; Lawhon et al., 1977; McCarty et al., 1996; Smith, 1970). Other gossypol-like pigments include gossyfulvin, gossypurpurin and gossyaerulin (Chamkasem, 1988). Gossypol [1,1'-,6,6'-,7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-(2,2'-binaphthalene)-8,8'-dicarboxaldehyde] is a reactive triterpenoid aldehyde (Figure 3.6) that exists as (+)- and (-)-enantiomers because of restricted rotation around the binaphthyl ring (Bailey et al., 2000).

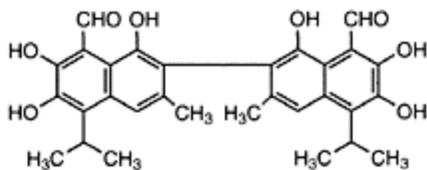


FIGURE 3.6 Chemical structure of gossypol.

Gossypol is present in cottonseed kernels in the free and bound forms. Gossypol and gossypol derivatives that can be extracted into 70% aqueous acetone are defined as free gossypol (AOCS Ba7–58, 1987). On the other hand, total gossypol (free and bound) is defined as gossypol that can be hydrolyzed and complexed with 3-amino-1-propanol in a dimethylformamide solution to form a diamino-1-propanol complex (AOCS Ba 8–78, 1987). Wang and Plhak (2000) have suggested that some gossypol may also be present in cottonseed in oxidized or degraded forms. Oxidation may result in the loss of aldehydic group, but degradation may increase the level of gossypol derivatives.

Gossypol interacts with the ϵ -amino group of lysine and thus may make it unavailable during digestion. In addition, gossypol is toxic to monogastric animals, but does not affect the ruminants (Berardi and Goldblatt, 1980). Recently, Bailey (2000) demonstrated that broilers fed on crushed cottonseed with a high (+)- to (–)-gossypol enantiomer ratio have significantly higher feed-to-weight gain ratios than those fed on crushed cottonseed with a lower (+)- to (–)-gossypol enantiomer ratio. Current U.S. FDA regulations limit the content of free gossypol in edible cottonseed flour to 450 mg/kg (Salunkhe et al., 1992). However, the Protein Advisory Group of the United Nations Food and Agriculture and World Health organizations (FAO/WHO) has limited the content of free gossypol in edible cottonseed flour to 600 mg/kg and total gossypol to 12,000 mg per kilogram (Lusas and Jividen, 1987). Furthermore, *p*-hydroxybenzoic, *trans-p*-coumaric, *trans*-ferulic and *trans*-caffeic acids have been detected in glanded and glandless cottonseed meals (Dabrowski and Sosulski, 1984).

EVENING PRIMROSE

The seeds of evening primrose (*Oenothera biennis* L.) serve as good sources of the essential fatty acid γ -linolenic acid (18:3 ω 6) (Hudson, 1984). This fatty acid is prone to oxidation, but despite this, the oil in the seeds resists oxidation. This suggests that these oleaginous seeds must contain some potent antioxidants (Amarowicz et al., 1999). Lu and Foo (1995), Shahidi et al. (1997a) and Birch et al. (2001) demonstrated that this antioxidative activity in evening primrose may be due to the presence of phenolic compounds.

The total content of phenolic acids extracted from evening primrose cake into water and 70% aqueous acetone is 580 and 180 mg/g of crude extract, respectively. Lu and Foo (1995) reported the presence of procyanidine gallate oligomer evening primrose seeds; later, Shahidi et al. (1997a) demonstrated that they contain catechins as well as dimers and trimers of proanthocyanidins. Furthermore, Balasinska and Troszynska (1998) reported the presence of a phenolic acid in acetone-water extract of evening primrose

betulinic, (2) morolic and (3) oleanolic acids found in evening primrose oil.

Nutritional and health benefits from the consumption of flaxseed and its components have recently been documented (Cunnane and Thompson, 1995; Haumann, 1993; McCord and Rao, 1997; Oomah and Mazza, 1998; Thompson, 1995; Thompson et al., 1996a, b). Flaxseed is gaining popularity as a health food and as an ingredient in muffins, breads and breakfast cereals (Jenkins, 1995; McCord and Rao, 1997).

Flaxseed is a rich source of phenolic acids, containing 800 to 1000 mg of total phenolic acids per 100 g of seeds. The content of esterified and etherified phenolic acids is up to 500 mg/100 g and 300 to 500 mg/100 g, respectively. Esterified phenolic acids comprise 48 to 66% and are not dependent on the cultivar (Oomah et al., 1995). Furthermore, Varga and Diosady (1994) have found that soluble and insoluble phenolic acids constitute 54 and 26 to 29% of total phenolic acids in flaxseed flour, respectively. *Trans*-ferulic and *trans*-sinapic acids are the major phenolic acids and *trans*-caffeic, *p*-coumaric and *p*-hydroxybenzoic are the minor phenolic acids found in dehulled, defatted flaxseed meal (Figure 3.1) (Dabrowski and Sosulski, 1984). However, Harris and Haggerty (1993) reported that the content of ferulic and chlorogenic acids accounted for 84% of total phenolic acids in methanolic extracts of defatted flaxseed meal. In addition, *p*-coumaric acid glucoside (linocinnamarin) and ferulic acid glucoside have been identified in flaxseed (Johnsson et al., 2002; Westcott and Muir, 1996a; Westcott et al., 2000).

Flaxseed also contains flavonoids, coumarins, and lignans (Amarowicz et al., 1994; Bambagiotti-Alberti et al., 1994a, b; Caragay, 1992; Johnsson et al., 2000, 2002; Meagher et al., 1999; Obermeyer et al., 1995; Thompson et al., 1997; Westcott and Muir, 1996b, 2000). The total content of flavonoids in the seeds ranges from 35 to 71 mg/100 g (Oomah et al., 1996) and flavone C- and O-glycosides are the major flavonoids present in flaxseed cotyledons (Ibrahim and Shaw, 1970). Secoisolariciresinol diglucoside (SDG) has been identified as a major lignan of flaxseed (Bakke and Klosterman, 1956; Mazur et al., 1996; Meagher et al., 1999); isolariciresinol, pinoresinol, and matairesinol (Figure 3.8) have been identified as minor lignan components (Meagher et al., 1999). The content of SDG in flaxseed is significantly affected by the cultivar, year of harvest and, to lesser extent, growing location (Westcott and Muir, 1996). Mazur et al. (1996) reported that the content of secoisolariciresinol and matairesinol in flaxseed is 370 and 1.087 mg/100 g, respectively. Secoisolariciresinol diglucoside and matairesinol may be converted into two mammalian lignans, enterodiol and enterolactone, by intestinal microflora (Axelson et al., 1982; Bambagiotti-Alberti et al., 1994a, b; Borriello et al., 1985; Heinonen et al., 2001; Setchell et al., 1983; Thompson et al., 1991; Wanasundara et al., 1997). Flaxseed meal is used as an ingredient in a number of baked goods in which the SDG content may range from 8.9 to 280 mg per bread loaf and from 121 to 1360 mg/kg (dry basis) of flax cookies, bagels, and muffins (Muir and Westcott, 2000)

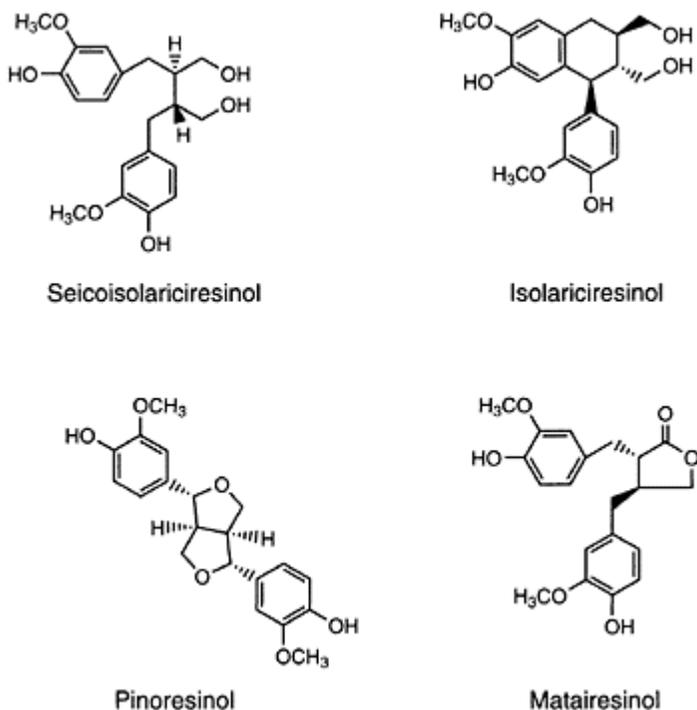


FIGURE 3.8 Chemical structures of lignans found in flaxseed.

Oomah and Mazza (1997) reported that abrasive dehulling of flaxseed concentrated phenolic acids in the dehulled flaxseed and observed that changes in phenolics are highly cultivar dependent ($P \leq 0.0001$). Upon dehulling, the total content of phenolic acids of McGregor seeds changes from 700 to 1980 mg/100 g, but only marginal changes are observed for cultivars NorMan, Omega and Vimy. Furthermore, Varga and Diosady (1994) showed that extraction of flaxseed meal with methanol-ammonia lowers the content of soluble esterified phenolic acids and insoluble bound phenolic acids by 20 and 29%, respectively.

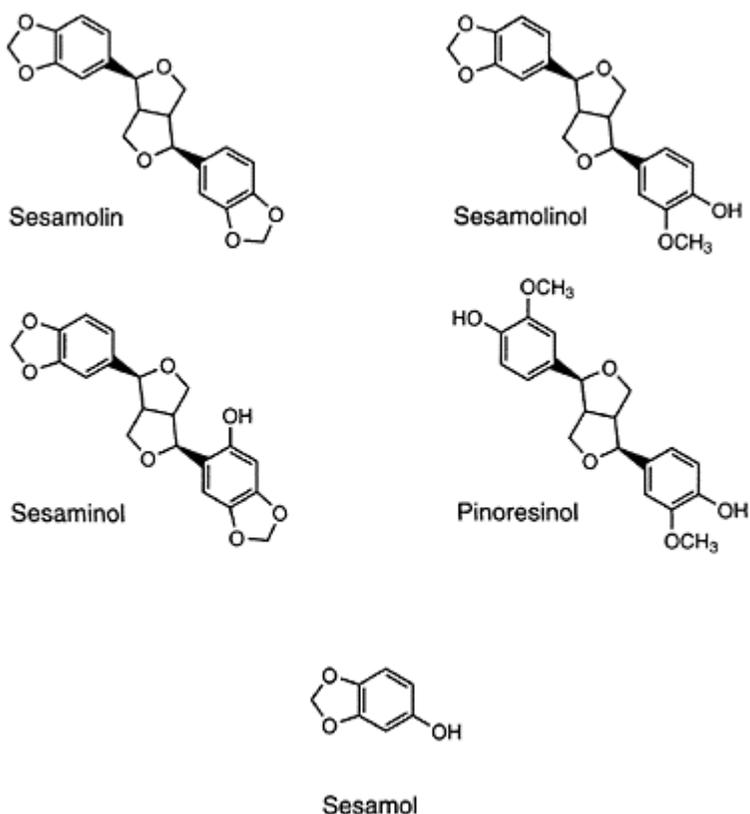


FIGURE 3.9 Chemical structures of some phenolic compounds found in sesame seeds.

SESAME SEEDS

Sesame seeds have been grown for their oil and proteins; defatted sesame seed meals contain up to 50% protein (Sen and Bhattacharyya, 2001). Sesame seed is a popular snack and confectionary ingredient in such products as halva; sesame oil is obtained from unroasted and roasted seeds, usually by expeller pressing (Wanasundara et al., 1997).

Sesame seeds contain lignophenols and carboxyphenols (Budowski, 1950, 1964; Budowski et al., 1950; Fukuda et al., 1981, 1986a, b, c, 1994; Shahidi et al., 1997b; Sirato-Yasumoto et al., 2000). The major lignans of sesame seed are sesamin (200 to 500 mg/100 g) and sesamolin (200 to 300 mg/100 g). In addition, sesamol and sesaminol are found in seeds and oil (Figure 3.9) (Nagata et al., 1987; Osawa et al., 1985). Fresh oils obtained from coated Egyptian and Sudanese sesame seed varieties contain 649.1 and 579.1 mg of sesamin per 100 g of oil and 183.3 and 349.2 mg sesamolin per 100 g of oil, respectively (Shahidi et al., 1997b). A number of pinoresinol glucosides have also been identified in sesame seed, namely (Katsuzaki et al., 1994).

- Pinoresinol 4'-*O*- β -D-glucopyranosyl (1 \rightarrow 6)- β -D-glucopyranoside
- Pinoresinol 4'-*O*- β -D-glucopyranosyl (1 \rightarrow 2)- β -D-glucopyranoside
- Pinoresinol 4'-*O*- β -D-glucopyranosyl (1 \rightarrow 2)- β -D-glucopyranosyl (1 \rightarrow 2)- β -D-glucopyranoside

Sesame seed lignans have shown beneficial physiological effects in experimental animals and humans (Akimoto et al., 1993; Hirata et al., 1996; Hirose et al., 1991; Ogawa et al., 1995; Sirato-Yasumoto et al., 2001).

Purified unroasted sesame oil contains from 50 to 100 mg of sesaminol per 100 g; the refining process brings about an increase in sesaminol level in sesame oil (Wanasundara et al., 1997). According to Fukuda et al. (1986a, b, 1994), sesaminol may be formed from sesamolin through intermolecular group transformation involving formation of oxonium ion and then the carbon-carbon bond. On the other hand, thermal decomposition of sesamolin leads to the formation of sesamol, while its hydrolysis affords sesamol and sesamin (Fukuda et al., 1994). Exposure of sesame lignans to elevated temperatures brings about their degradation. Only 40.5% of sesaminol added to corn oil is detected after 6 h of heating at 180°C, while all added sesamol is decomposed after 4 h heating at 180°C (Fukuda et al., 1994). Shahidi et al. (1997b) also reported that processing sesame oil at 65°C for 35 days has a more drastic effect on sesamolin than on sesamin.

Sesame flour also contains *trans*-caffeic, *trans-p*-coumaric and *trans*-ferulic acids and traces of *p*-hydroxybenzoic acid (Table 3.1) (Dabrowski and Sosulski, 1984). Moreover, the presence of guaiacol and 2-methoxy-5-(1-propenyl)phenol has been detected in the volatile components of roasted sesame seeds (Shimoda et al., 1997). Two potent antioxidants have also been isolated from suspension of sesame callus (Mimura et al., 1994):

- Acteoside (3,4-dihydroxy- β -phenethyl-*O*- α -rhamnopyranosyl (1 \rightarrow 3)-4-*O*-caffeoyl- β -glucopyranoside)
- 3,4-Dihydroxy- β -phenyl-*O*-ethylcarboxyl-*O*- α -rhamnopyranosyl (1 \rightarrow 3)-4-*O*-caffeoyl- β -glucopyranoside (SI-1)

SUNFLOWER

The presence of phenolics in sunflower has restricted its widespread use for human consumption and industrial food applications (Delic et al., 1975). The major phenolics in sunflower seeds are chlorogenic, quinic and caffeic acids (Figure 3.10) (Sosulski, 1979), which occur in the defatted sunflower meal at about 2700, 400 and 200 mg/100 g, respectively (Carter et al., 1972). The minor phenolic acids in sunflower seeds are *trans*-ferulic, *p*-hydroxybenzoic, *trans-p*-coumaric, syringic and vanillic acids (Figure 3.1 and Table 3.1) (Dabrowski and Sosulski, 1984).

The occurrence and the content of chlorogenic acid (Figure 3.10) are responsible for dark discoloration of food products and may also decrease protein availability (Carter et al., 1972; Dryden and Satterlee, 1978; Robertson, 1975). Interaction of phenolics with proteins is a well-known phenomenon (Carter et al., 1972; Sosulski

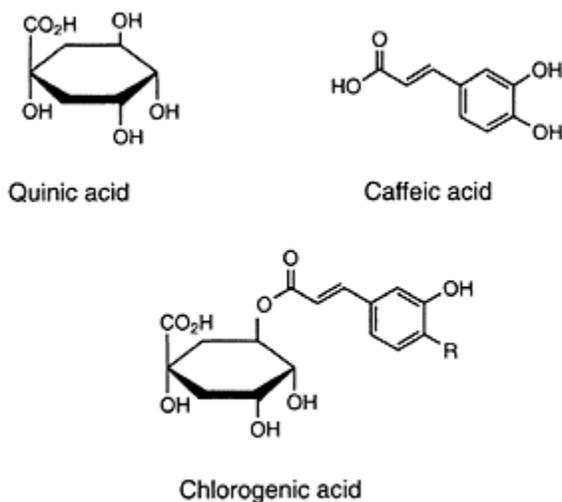


FIGURE 3.10 Chemical structures of chlorogenic, quinic and caffeic acids.

et al., 1973); digestive enzymes such as trypsin, lipase and amylase are affected (Salunkhe et al., 1992). Furthermore, interaction of chlorogenic acid with low molecular weight albumins (<5000 Da) of sunflower is well known (Prasad, 1988; Sabir et al., 1974). These interactions involve sulfhydryl or amino groups of proteins that decrease the nutritional quality of proteins because such condensation products cannot be metabolized by humans (Desphande et al., 1984).

Chlorogenic acid may be removed from sunflower meal by solvent extraction procedures involving water and alcohol (Rutkowski, 1972) or acidic butanol (Sodini and Canella, 1977). Partial removal of chlorogenic acid with water, 40% (v/v) aqueous methanol, 50% (v/v) aqueous isopropanol, ethanol or acetone has also been reported (Gheyasuddin et al., 1970; Sosulski and McCleary, 1972; Sripad and Narasinga Rao, 1987). In addition, dehulling seeds and soaking in citric acid or sodium bisulfite significantly reduces the level of phenolic compounds in sunflower (Bau et al., 1983).

PLANT OILS

OLIVE OIL

The production of virgin olive oil involves mechanical pressing of mesocarp of drupes of olive trees (*Olea europea* L.), washing, decanting, centrifuging and selective filtering—virgin olive oil is consumed without refining (Caponio et al., 2001; Wanasundara et al., 1997). The major olive oil producers are Spain, Italy, and Greece (Brenes et al., 1999). Phenolic compounds affect the sensory characteristics and autoxidative stability of virgin olive oil (Baldioli et al., 1996; Briante et al., 2002; Gutierrez Gonzales-Quijano et al., 1977; Le Tutor and Guedon, 1992; Visioli et al., 1998, 1999). Olive oil phenolics also

possess pharmacological (Le Tutor and Guedon, 1992; Lipworth et al., 1997; Manna et al., 1999; Medina et al., 1999;

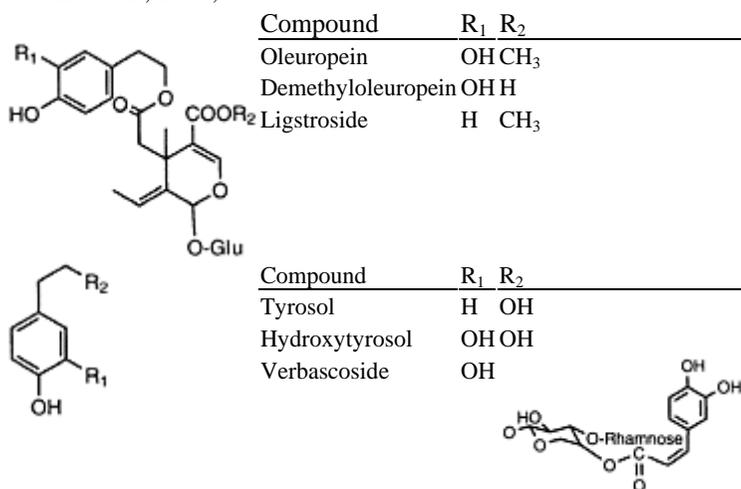


FIGURE 3.11 Chemical structures of some phenolics found in olive oil.

Trichopoulou, 1995; Visioli et al, 2000; Visioli and Galli, 2002) and antimicrobial (Brenes et al., 1992, 1995; Fleming et al., 1973) properties.

Olive fruits may contain up to 80 mg of polyphenols per 100-g sample that are responsible for the unique flavor of virgin olive oil (Visioli et al., 2000). The total phenolic content and the distribution of phenolic components are affected by the cultivar, growing location, and the degree of ripeness (Amiot et al., 1986; Capiono et al., 2001). Oleuropein content decreases as the olive fruits ripen (Amiot et al., 1989; Caponio et al., 2001; Esti et al., 1998), while the content of demethyloleuropein and 2,4-dihydroxyphenylethanol increases (Brenes et al., 1995; Esti et al., 1998).

Secoiridoids, oleuropein, demethyloleuropein, and ligstroside are the main phenolic glucosides and verbascoside (caffeoylrhamnosylglucoside of hydroxytyrosol) is the main hydroxycinnamic acid derivative of olive fruit (Figure 3.11) (Amiot et al., 1986; Angerosa et al., 1995, 1996; Borzillo et al., 2000; Gariboldi et al., 1986; Kuwajima et al., 1988; Maestro-Duran et al., 1994; Ryan and Robards, 1998; Saija and Uccella, 2001; Soler-Rivas et al., 2000; Uccella, 2001). The aglycone of oleuropein is the ester of elenolic acid with 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) (3,4-DHPEA-EA) and the aglycone of ligstroside is the ester of elenolic acid with 2-(4-hydroxyphenyl)ethanol (tyrosol) (Figure 3.12) (Tsimidou, 1998).

Oleuropein is the major phenolic compound responsible for the development of bitterness in olive fruits (Panizzi et al., 1960). Moreover, phenyl alcohols such as hydroxytyrosol (3,4-DHPEA) and tyrosol (p-HEPA), as well as verbascoside (Figure 3.11) and phenolic acids (including hydroxycinnamic, hydroxybenzoic, hydroxycaffeic, and hydroxyphenylacetic acids), have been reported in olive fruits (Bastoni et al., 2001; Bianco and Uccella, 2000; Servilli et al., 1999a, b; Vierhuis et al., 2001). In addition, flavonoids such as quercetin, rutin, luteolin 7-glucoside and apigenin glucosides (Amiot

et al., 1986; Mazza and Miniati, 1993; Rovellini et al., 1997; Vlahov, 1992) and hydroxyisochromans, namely, 1-phenyl-6,7-dihydroxyisochroman and 1-(3'-methoxy-4'-hydroxy)phenyl-6,7-dihydroxyisochroman

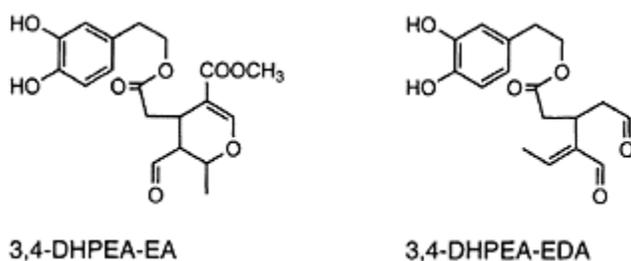


FIGURE 3.12 Chemical structures of aldehydic and dialdehydic forms of oleuropein aglycone (3,4-DHPEA-EA=aldehydic form of oleuropein aglycone; 3,4-DHPEA-EDA=dialdehydic form of decarboxymethyloleuropein aglycone; 3,4-DHPEA=2-(3,4-dihydroxyphenyl)ethanol; EA=elenolic acid; and EDA=dialdehydic form of elenoic acid).

(Bianco et al., 2001), have been identified in olive fruits. Unprocessed olive fruits also contains 3,4-dihydroxyphenylglycol (Bianchi and Pozzi, 1994) and anthocyanins (Macheix et al., 1990). Lactic acid fermentation of table olives brings about complete hydrolysis of oleuropein and luteolin 7-glucoside to hydroxytyrosol, tyrosol and luteolin (Blekas et al., 2002); this is catalyzed by β -glucosidase and esterase from *Lactobacillus plantarum* strains (Ciafardini et al., 1994; Marsilio et al., 1996).

The content of hydroxytyrosol and hydroxytyrosol derivatives (oleuropein, oleuropein aglycone, demethyloleuropein and hydroxytyrosol glucosides) in Greek, Portuguese, Italian and Spanish table olive fruits ranges from 100 to 430 mg/kg and from 3670 to 5610 mg/kg, respectively (Bastoni et al., 2001). Recently, Blekas et al. (2002) detected a high level of hydroxytyrosol (250 to 760 mg/kg) in Kalamata and Spanish-style green olives. The content of verbascoside in olive fruits from Italian cultivars is 160 to 3200 mg/kg (Romani et al., 1999), while the concentrations of rutin and luteolin 7-glucoside, two main flavonoids in olive fruits (Amiot et al., 1986; Vlahov, 1992), are 110 to 660 and 5 to 600 mg/kg, respectively (Esti et al., 1998; Panizzi, 1960). In addition, cyanidin 3-*O*-glucoside (50 to 880 mg/kg) and cyanidin 3-*O*-rutinoside (250 to 3300 mg/kg) are the most abundant anthocyanins in olive fruits (Romani et al., 1999; Vázquez-Roncero and Maestro-Duran, 1970).

The composition of phenolics in some virgin olive oil samples is shown in Table 3.12. The composition of phenolics is affected by the cultivar, degree of ripeness, climate (Amiot et al., 1986; Caponio et al., 2001), type of crushing machine and the conditions used during malaxation (stirring olive fruit paste at elevated temperature) (Catalano and Caponio, 1996; Vierhuis et al., 2001). According to Medina et al. (1999), the total content of phenolics in extra virgin oil is 86 mg of gallic acid equivalents (GAE) per 100 g of oil, whereas refined olive oil contains only 0.8 mg GAE per 100 g of oil.

Simple phenolic compounds of virgin olive oils include hydroxytyrosol, tyrosol, vanillic acid, *p*-coumaric acid and ferulic acid (Montedoro et al., 1992, 1993). Recently, Mateos et al. (2001) detected cinnamic acid and 2-(4-hydroxyphenyl)ethyl acetate in olive oil. In addition, the presence of vanillin and 4-(acetoxy ethyl)-1,2-dihydroxybenzene (Brenes et al., 1999) and flavonoids such as luteolin and apigenin has been reported (Brenes et al., 1999; Rovellini et al., 1997). Luteolin in olive oil

TABLE 3.12
Phenolic Compounds in Some Extra Virgin Oils^a

| | Cultivar | | | |
|-------------------------|----------|--------|-----------|----------|
| | Picual | Picudo | Anbequina | Empeltre |
| Hydroxytyrosol | 17.8 | 7.5 | 11.4 | 1.1 |
| Tyrosol | 10.5 | 10.0 | 8.4 | 3.2 |
| Vanillic acid | 0.1 | 0.2 | 0.1 | 0.1 |
| Vanillin | 0.6 | 0.2 | 0.3 | 0.3 |
| 3,4-DHPEA-AC | 12.8 | 2.4 | 7.4 | 2.8 |
| <i>p</i> -Coumaric acid | 0.3 | 0.1 | 0.1 | 0.1 |
| 3,4-DHPEA-EDA | 82.0 | 14.0 | 28.6 | 14.3 |
| <i>p</i> -HPEA-EDA | 31.0 | 7.3 | 7.5 | 7.0 |
| 1-Acetoxypinosresinol | 4.9 | 6.8 | 36.4 | 31.5 |
| Pinosresinol | 29.5 | 31.2 | 34.0 | 19.0 |
| 3,4-DHPEA-EA | 359.7 | 40.9 | 29.0 | 15.9 |
| <i>p</i> -HPEA-EA | 48.7 | 7.0 | 4.4 | 1.5 |

Note: 3,4-DHPEA-AC: 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA: dialdehydic form of elenolic acid linked to hydroxytyrosol; *p*-HPEA-EDA: dialdehydic form of elenolic acid linked to tyrosol; 3,4-DHPEA-EA: oleuropein aglycone; *p*-HPEA-EA: ligitroside aglycone.

^a Milligrams per kilogram.

Source: Adapted from Brenes, M. et al., 2000, *J. Am. Oil Chem. Soc.*, 77:715–720.

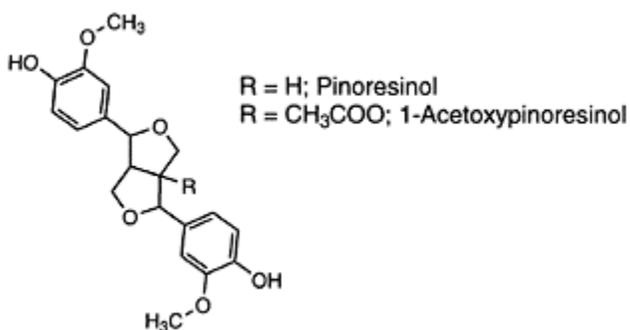


FIGURE 3.13 Chemical structures of lignans found in olive oil.

originates from rutin and from luteolin 7-glucoside while apigenin arises from apigenin glucosides (Brenes et al., 1999). Other phenolics identified in olive oils are oleuropein and ligstroside aglycons and the dialdehydic form of elenolic acid linked to hydroxytyrosyl and tyrosol (Figure 3.12) (Angerosa et al., 1995, 1996; Brenes et al., 1999; Pirisi et al., 2000). Moreover, Brenes et al. (2000) have identified two new phenolic compounds in olive oil, namely, pinoresinol and 1-acetoxypinoresinol (Figure 3.13). The contents of each of these lignans is 5 to 67 mg/kg of oil.

During crushing and malaxing processes, oleuropein and demethyloleuropein are hydrolyzed by endogenous glycosidases to dialdehydic form of decarboxymethyloleuropein aglycone (3,4-DHPEA-EDA) and aldehydic form of oleuropein aglycone (3,4-DHPEA-EA) (Figure 3.12). The aglycons become soluble in the oil phase while the glycosides remain in the wastewater phase (Cortesi et al., 1995). During these processes some isoforms of oleuropein are also generated by ketoenolic tautomeric equilibrium, which involves opening the secoiridoid ring (Angerosa et al., 1995; Gariboldi et al., 1986). Furthermore, during the malaxation process some losses in secoiridoids and phenyl alcohol contents have been observed (Servilli et al., 1999a, b). These phenolic losses may be due to enzymatic oxidation by endogenous oxidoreductases (Sciancalepore, 1985) and nonenzymatic oxidation (Servilli et al., 1998), as well as their complexation with polysaccharides (McManus et al., 1985). Nitrogen flushing as well as addition of cell wall-degrading enzymes during malaxation increases the concentration of phenolics in virgin olive oil (Ranalli and De Mattia, 1997; Servilli et al., 1992; Vierhuis et al., 2001).

Cinquanta et al. (1997) reported increased levels of hydroxytyrosol and tyrosol during olive oil storage and linked this increase to the hydrolysis of complex phenols (secoiridoids). Subsequently, Brenes et al. (2001) noted a rise in the concentration of hydroxytyrosol and tyrosol in virgin olive oil stored in darkness at 30°C and attributed this to the hydrolysis of secoiridoid aglycones to hydroxytyrosol and tyrosol. Moreover, these authors reported that the hydrolysis of secoiridoid aglycones is influenced by the acidity of the oil and the filtration step. Later, Okogeri and Tasioula-Margari (2002) reported a significant decrease in the content of complex phenols (secoiridoids) in virgin

olive oil stored under diffused light or in the dark, but did not detect any increase in the levels of hydroxytyrosol and tyrosol.

Hydroxytyrosol derivatives are most sensitive to thermal oxidation of oil. No hydroxytyrosol derivatives have been detected at peroxide values of 20 to 25 meq/kg of oil. However, tyrosol remains stable even at peroxide value of 70 meq/kg oil (Nissiotis and Tasioula-Margari, 2001). Heating olive oil at 180°C brings about a rapid decrease in polyphenolic compounds due to their thermal destruction or oxidative degradation. On the other hand, boiling olive oil-water mixtures results in the hydrolysis of secoiridoid aglycones to simple phenolics and migration of hydrophilic phenols from oil into the water phase (Brenes et al., 2002; Sacchi et al., 2002).

PALM OIL

Palm oil, an important source of edible oil in many tropical countries, is obtained from the mesocarp of the oil palm fruit (Wanasundara et al., 1997). Because most published studies focus on tocopherols in palm oil, the available information on palm oil phenolics is still fragmentary. Palm oil is a good source of tocopherols such as α -tocopherol, α -tocotrienol, α -tocotrienol, β -tocotrienol, γ -tocotrienol, and δ -tocotrienol (Choo, 1994; Choo et al., 1996; Goh et al., 1985; Strohschein et al., 1999). The content of total tocopherols in palm oil ranges from 800 to 1000 mg/kg (Choo, 1994; Choo et al., 1996). Tocotrienols are the predominant fraction of palm oil tocopherols, comprising up to 78% of total tocopherols (Ab Gapor, 1990; Choo, 1994; Choo et al., 1996).

RICE BRAN OIL

Rice bran, a by-product of rice milling, contains from 15 to 20% of an oil considered a valuable domestic oil in many countries (Salunkhe et al., 1992). The content of unsaponifiable matter in rice bran oil is about 2% (Salunkhe et al., 1992). The unsaponifiables of rice bran oil comprise sterols, higher alcohols and γ -oryzanol (Nicolosi et al., 1994). γ -Oryzanol is a mixture of ferulic acid esters of triterpenoid alcohol and has been shown to lower blood cholesterol (Nicolosi et al., 1994). Xu and Godber (1999) and Xu et al. (2001) identified 10 components of γ -oryzanol (Figure 3.14):

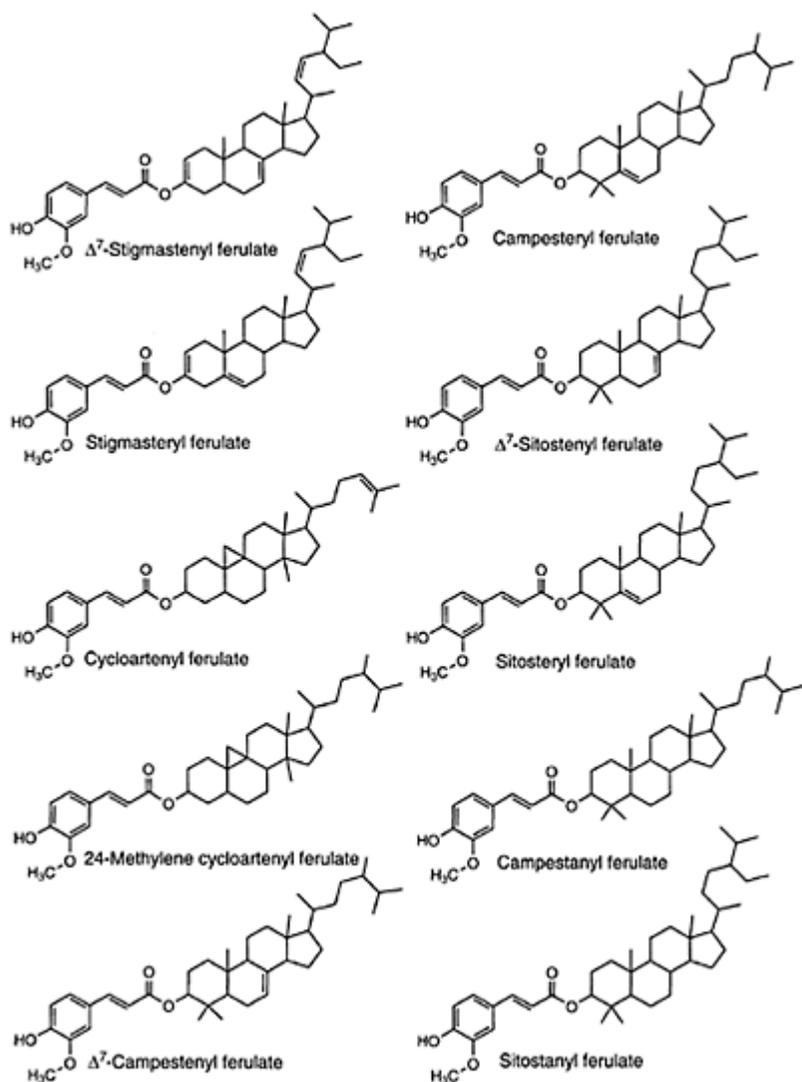


FIGURE 3.14 Chemical structures of γ -oryzanol components.

- Δ^7 -Stigmasteryl ferulate
- Stigmasteryl ferulate
- Cycloartenyl ferulate
- 24-Methylenecycloartanyl ferulate
- Δ^7 -Campestenyl ferulate
- Campesteryl ferulate
- Δ^7 -Sitostenyl ferulate

- Sitosteryl ferulate
- Campestanil ferulate
- Sitostanyl ferulate

Of these, 24-methylenecycloartanyl ferulate, cycloartenyl ferulate and campesteryl ferulate are the major constituents of γ -oryzanol.

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4

Phenolic Compounds in Fruits and Vegetables

INTRODUCTION

Because they exhibit health-promoting effects such as reducing blood pressure and lowering incidences of cancer and cardiovascular diseases, fruits and vegetables are excellent sources of phenolics. (Ames et al., 1993; Ascherio et al., 1992; Block et al., 1992; Cook and Samman, 1996; Facino et al., 1999; Hertog et al., 1993, 1997; Hollman, 2001; Hollman et al., 1996; Huang et al., 1992; Leake, 1997; Middleton and Kandaswami, 1994; Morazzoni and Bombardelli, 1996; Ness and Powless, 1997; Steinmetz and Potter, 1991a, b, 1996; Swanson, 1998; Willet, 1994). Moreover, phenolics and related enzymes affect the quality of fruits and vegetables (Amiot et al., 1997; Brouillard et al., 1997; Clifford, 1997; Crouzet et al., 1997; Lea, 1992; Macheix and Fleuriet, 1993; Nicolas et al., 1993; Parr and Bowell, 2000; Tomás-Barberán and Espin, 2001; Tomás-Barberán and Robins, 1997; Zobel, 1997). Some phenolics are unique for certain fruits: for example, cinnamic esters of tartaric acid in grapes (de Simon et al., 1992), phlorizin in apples (Sanoner et al., 1999) and flavanone glycosides in citrus fruits (Louche et al., 1998; Marini and Balestrieri, 1995; Mouly et al., 1994; Ooghe and Detavernier, 1997). However, published data on the content of phenolics in fruits and vegetables are still incomplete and often restricted to a few cultivars.

FRUITS

BERRIES

Small berries constitute an important source of potential health-promoting phytochemicals; these include fruits of the *Vaccinium*, *Ribes*, *Rubus* and *Fragaria* genera (Fukumoto and Mazza, 2000; Kähkönen et al., 1999; Wang et al., 1996). Lowbush and highbush blueberry, bilberry, cranberry and lingonberry are examples of *Vaccinium* genus and blackberries, red and black raspberries are examples of *Rubus* genus; gooseberries, jostaberries and currants belong to the *Ribes* genus and strawberry to the *Fragaria* genus. These fruits are rich sources of flavonoids and other phenolics that display potential health-promoting effects (Block et al., 1992; Bomser et al., 1996; Feldman, 2001; Landbo and Meyer, 2001; Saito et al., 1998). For example, over 180 *Vaccinium*-based Pharmaceuticals have been introduced to the market (Kalt and Dufour, 1997). Grapes are one of the world's largest berry crops. Approximately 80% of the total crop is utilized for wine making; 13% is consumed as table grapes and 7% processed into juice and raisins. Grapes belonging to the species *Vitis vinifera* L. are predominantly

cultivated in Europe, while those belonging to species *Vitis labrusca* and *Vitis rotundifolia* are grown in North America (Mazza, 1995).

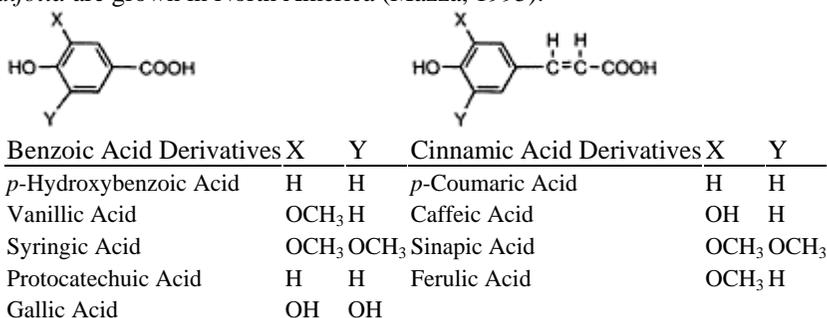


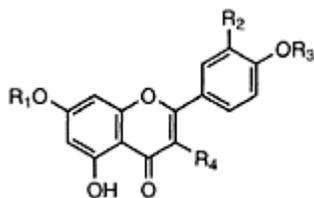
FIGURE 4.1 Chemical structures of phenolic acids.

Bilberries

Bilberry (*Vaccinium myrtillus*) is a rich source of phenolic compounds, namely, phenolic acids and flavonoids; bilberry juice has been used in medicine for its pharmacological properties (Azar et al., 1987). The total content of phenolics in bilberry fruit ranges from 33,000 to 38,200 mg/kg of dry weight as gallic acid equivalents (Kähkönen et al., 2001). The presence of caffeic and chlorogenic acids in bilberry fruit was first reported by Friedrich and Schönert (1973). Later, Brenneisen and Steinegger (1981a, b) found four more phenolic acids in bilberry fruit, namely, *p*-coumaric ferulic and syringic acids, and a derivative of *p*-hydroxybenzoic acid. Subsequently, Azar et al. (1987) confirmed the presence of previously reported phenolic acids, but also found other phenolic acids such as *o*- and *m*-coumaric, gallic, *m*- and *p*-hydroxybenzoic, protocatechuic and vanillic acids in bilberry juice (Figure 4.1). The total content of hydroxycinnamic and hydroxybenzoic acids is 1130 to 2310 mg of chlorogenic acid equivalent/kg of dry weight and 33 to 58 mg gallic acid equivalent/kg of dry weight, respectively (Kähkönen et al., 2001).

Four flavonol glycosides, namely, quercetrin (quercetin 3-rhamnoside), isoquercetrin (quercetin 3-glucoside), hyperin (quercetin 3-galactoside), and astragalín (kaempferol 3-glucoside), have also been found in bilberry fruit and juice (Figure 4.2) (Azar et al., 1987; Brenneisen and Steinegger, 1981a, b; Friedrich and Schönert, 1973). The total content of flavonols in berries is between 540 and 1300 mg of rutin equivalent/kg of dry weight (Kähkönen et al., 2001). In addition, several flavan-3-ols, including (+)catechin and (-)epicatechin (Figure 4.3) (Brenneisen and Steinegger, 1981a, b; Friedrich and Schönert, 1973) and procyanidins B1, B2, B3, and B4 (Figure 4.4) (Brenneisen and Steinegger, 1981a, b), have also been detected. The reported values on the total anthocyanin content range from 3700 to 6980 mg/kg of fresh weight (Kalt et al., 1999a; Nyman and Kumpulainen, 2001; Senchuk and Borukh, 1976) and from 22,980 to 30,900 mg/kg of dry weight (Kähkönen et al., 2001). Ten anthocyanins are found in bilberry fruit powder (Figure 4.5) (Chandra et al., 2001):

- Cyanidin-3-galactoside
- Cyanidin-3-glucoside
- Delphinidin-3-galactoside



| Compound | R ₁ | R ₂ | R ₃ | R ₄ |
|------------------|-----------------|----------------|-----------------|-----------------------|
| <i>Flavones</i> | | | | |
| Apigenin | H | H | H | H |
| Diosmin | rutinosyl | OH | CH ₃ | H |
| Isohoifolin | rutinosyl | H | H | H |
| Luteolin | H | OH | H | H |
| Neodiosmin | neohesperidosyl | OH | CH ₃ | H |
| Rhoifolin | neohesperidosyl | H | H | H |
| <i>Flavonols</i> | | | | |
| Kaempferol | H | H | H | OH |
| Rutin | H | OH | H | <i>O</i> -rutinoside |
| Quercetin | H | OH | H | OH |
| Quercitrin | H | OH | H | <i>O</i> -rhamnoside |
| Isoquercitrin | H | OH | H | <i>O</i> -glucoside |
| Hyperin | H | OH | H | <i>O</i> -galactoside |
| Astragalin | H | H | H | <i>O</i> -glucoside |
| Reynoutrin | H | OH | H | <i>O</i> -xyloside |
| Avicularin | H | OH | H | <i>O</i> -arabinoside |

FIGURE 4.2 Chemical structures of some flavones and flavonols and their glycosylated derivatives.

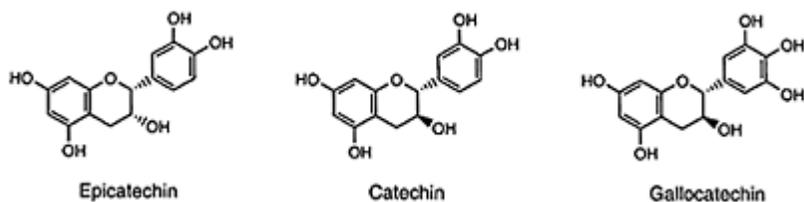


FIGURE 4.3 Chemical structures of catechins.

- Malvidin-3-arabinoside
- Malvidin-3-glucoside
- Malvidin-3-galactoside

- Peonidin-3-galactoside
- Petunidin-3-galactoside

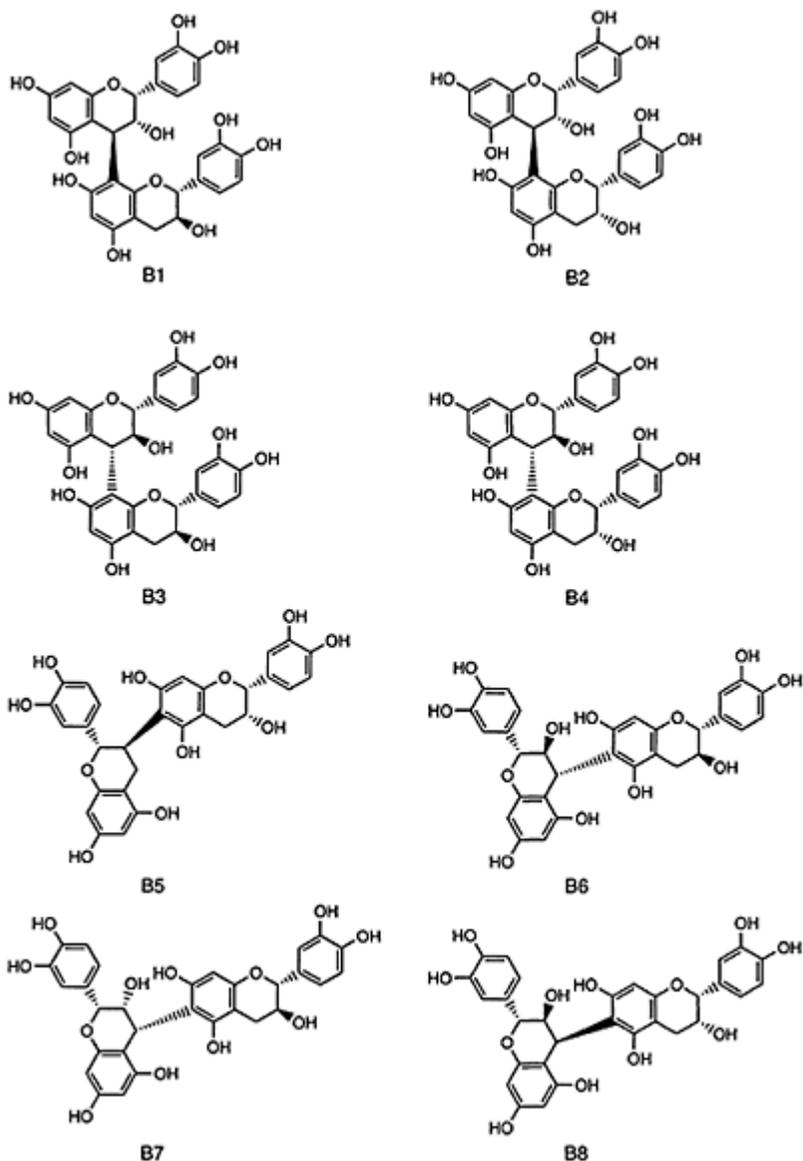
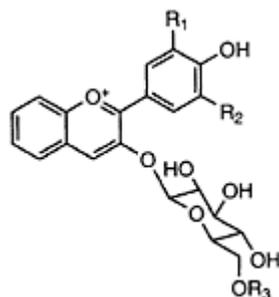


FIGURE 4.4 Chemical structures of procyanidin dimers type B.

- Petunidin-3-glucoside
- Petunidin-3-arabinoside



| Compound | R ₁ | R ₂ | R ₃ |
|--------------------------------------|------------------|------------------|----------------|
| Cyanidin-3- <i>O</i> -glucoside | OH | H | H |
| Cyanidin-3- <i>O</i> -rutinoside | OH | H | rhamnosyl |
| Delphinidin-3- <i>O</i> -glucoside | OH | OH | H |
| Delphinidin-3- <i>O</i> -rutinoside | OH | OH | rhamnosyl |
| Pelargonidin-3- <i>O</i> -glucoside | H | H | H |
| Pelargonidin-3- <i>O</i> -rutinoside | H | H | rhamnosyl |
| Petunidin-3- <i>O</i> -glucoside | OCH ₃ | OH | H |
| Petunidin-3- <i>O</i> -rutinoside | OCH ₃ | OH | rhamnosyl |
| Malvidin-3- <i>O</i> -glucoside | OCH ₃ | OCH ₃ | H |
| Malvidin-3- <i>O</i> -rutinoside | OCH ₃ | OCH ₃ | rhamnosyl |

FIGURE 4.5 Chemical structures of some anthocyanins.

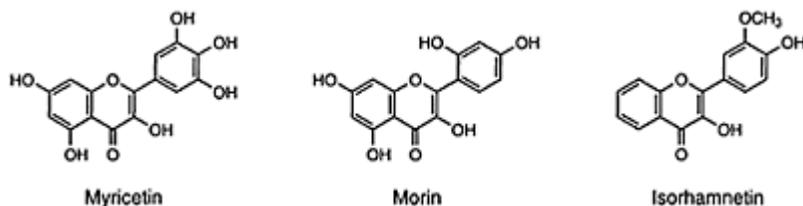


FIGURE 4.6 Chemical structures of myricetin, morin and isorhamnetin.

Blackberries

The total content of phenolics in Georgia-grown blackberries is 4865.3 mg/kg of fresh weight expressed as gallic acid equivalents (Sellappan et al., 2002). Several phenolic acids have been detected in blackberries, namely, gallic, caffeic, ferulic, *p*-coumaric and ellagic acids (Figure 4.1). Of these, ellagic acid is the major phenolic (Sellappan et al., 2002). Ripe fruits of thornless blackberry (*Rubus* sp.) cultivars contain 5 to 35 mg/kg of quercetin and 1 to 3 mg/kg of fresh weight of kaempferol (Bilyk and Sapers, 1986);

however, Sellappan et al. (2002) detected only catechin (2657.5 to 3128.6 mg/kg) and myricetin (99.9 mg/kg) (Figure 4.6) in Georgia-grown blackberries.

The total content of anthocyanins in blackberries ranges from 1165.9 to 1528 mg/kg of fresh weight expressed as cyanidin-3-glucoside equivalents (Sellappan et al., 2002; Wang and Lin, 2000). Several anthocyanins have been identified in blackberry extracts, namely:

- Cyanidin-3-galactoside
- Cyanidin-3-glucoside
- Cyanidin-3-arabinoside
- Cyanidin-3-xyloside
- Malvidin-3-arabinoside
- Pelargonidin-3-glucoside (Dugo et al., 2001)
- Cyanidin-3-rutinoside (Mazza and Miniati, 1993; Nybom, 1968)
- Cyanidin-3-sophoroside
- Cyanidin-3-glucosylrutinoside
- Cyanidin-3-rutinoside (Figure 4.5) (Hong and Wrolstad, 1990; Mazza and Miniati, 1993)

In addition, Sapers et al. (1986) detected two acylated anthocyanins in unripe blackberries and reported that their levels rapidly decrease during the ripening process. Later, Fang-Chiang (2000) detected cyanidin-3-glucoside acylated with malonic acid in blackberry while Stintzing et al. (2002) isolated cyanidin dioxalylglucoside—a novel zwitterionic anthocyanin from evergreen blackberry (*Rubus laciniatus* Wild.). Of these, cyanidin-3-glucoside was the major anthocyanin in blackberry (Garcia-Viguera et al., 1997; Goiffon et al., 1991; Hong and Wrolstad, 1990; Mazza and Miniati, 1993; Sapers et al., 1986; Versari et al., 1997).

Blackcurrants

Blackcurrants are commercially grown for production of juices and jams; these fruits are also a component of fruit-based yogurts. Blackcurrant berries serve as a rich source of phenolic compounds such as anthocyanins, flavonoids, phenolic acids and proanthocyanidins (Constantino et al., 1992, 1993; Demina, 1974; Foo and Porter, 1981; Häkkinen and Auriola, 1998; Koeppen and Herrmann, 1977; Le Lous et al., 1975; Stöhr and Herrmann, 1975). The total content of phenolics in blackcurrant berries is between 6940 and 38,200 mg/kg expressed as gallic acid equivalent (Kähkönen et al., 2001; Moyer et al., 2002). Blackcurrant anthocyanins are located in the skin of berries (Iversen, 1999); their total content ranges from 1560 to 10,640 mg/kg expressed as cyanidin 3-glucoside equivalents (Iversen 1999; Kähkönen et al., 2001; Koeppen and Herrmann, 1977; Moyer et al., 2002). The predominant anthocyanins of blackcurrants are delphinidin-3-rutinoside, cyanidin-3-rutinoside, delphinidin-3-glucoside and cyanidin-3-glucoside (Figure 4.5) (Iversen, 1999; Matsumoto et al., 2001; Mazza and Miniati, 1993; Millet et al., 1984; Renault et al., 1997). These four main anthocyanins make up >97% of total anthocyanins in blackcurrants (Slimstad and Solheim, 2002).

Other anthocyanins detected in blackcurrants include

- Pelargonidin-3-rutinoside
- Cyanidin-3-sophoroside
- Delphinidin-3-sophoroside (Le Lous et al., 1975)
- Peonidin-3-glucoside
- Peonidin-3-rutinoside
- Malvidin-3-glucoside
- Malvidin-3-rutinoside
- Cyanidin-3-arabinoside
- Cyanidin-3-(6''-coumarylglucoside)
- Delphinidin-3-(6''-coumarylglucoside) (Slimstad and Solheim, 2002)

Blackcurrants also serve as an important source of dietary flavonols (Häkkinen and Auriola, 1998; Häkkinen et al., 1999a; Starke and Herrmann, 1976). Flavonols in blackcurrants are present predominantly in the conjugated form and free flavonols contribute only 1.13 to 5.12% to total flavonol contents (Amakura et al., 2000). Recently, Mikkonen et al. (2001) reported great variability in the flavonol content among different blackcurrant cultivars, but did not find any consistent differences within the same cultivar, grown conventionally, and those grown on organic farms. The total content of flavonols in blackcurrant cultivars varies from 157 to 870 mg/kg of fresh weight (Kähkönen et al., 2001; Mikkonen et al., 2001). Myricetin (89 to 245 mg/kg of fresh weight) (Figure 4.6) is the most abundant flavonol in blackcurrants, followed by quercetin (52 to 122 mg/kg of fresh weight) and kaempferol (9 to 23 mg/kg of fresh weight) (Figure 4.2) (Häkkinen et al., 2000a; Mikkonen et al., 2001).

Myricetin is the least stable flavonol in blackcurrant. Postharvest storage temperature has a major affect on the content of flavonols; considerably lower myricetin levels (about 20%) are observed for samples stored 1 day at 22°C in comparison to those stored at 5°C. Myricetin content is also significantly lowered in blackcurrants stored for 6 months at -20°C (Häkkinen et al., 2000a). The content of flavonols in juice is significantly affected by the method of juice extraction. Blackcurrant juice obtained by cold-pressing berries contains twice as many flavonols as that extracted from berries by steam (Häkkinen et al., 2000a). Furthermore, approximately 62% of total anthocyanins are extracted into the raw juice after treatment of blackcurrants with Pectinex™ Ultra SP enzyme, but only 24% of anthocyanins remain after pasteurization of juice (Iversen, 1999).

Blackcurrant seeds are a rich source of potent antioxidant because seed oil containing high levels of polyunsaturated fatty acids, especially γ -linolenic acid (Traitler et al., 1984; Zhao et al., 1994), is stable in the intact seed (Lu et al., 2002; Lu and Foo, 2003). Two novel noncyanogenic nitrile-containing compounds were recently isolated by Lu et al. (2002) from seed residue and identified as nigrumin5-*p*-coumarate and nigrumin-5-ferulate (Figure 4.7). Later, Lu and Foo (2003) identified a number of phenolic acids and their derivatives in blackcurrant seeds, namely, caffeic acid, ferulic acid, *p*-coumaric acid, protocatechuic acid, gallic acid, *p*-hydroxybenzoic acid, 1-cinnamoyl- β -D-glucoside, 1-*p*-coumaroyl- β -D-glucoside. In addition, these authors detected the presence of 3-glucosides and 3-rutinosides of delphinidin and cyanidin and a number of flavonoids such as taxifolin (dihydroquercetin), aureusidin, 3-glucosides of quercetin, kaempferol and myricetin, as well as 3-rutinosides of quercetin and myricetin (Figure 4.2, Figure 4.6, and Figure 4.7).

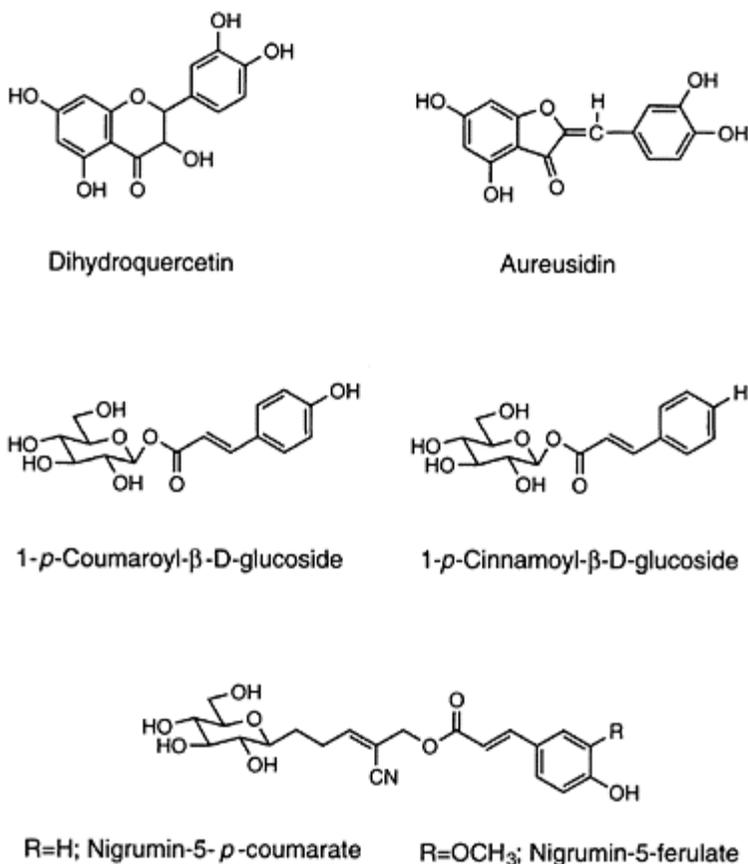


FIGURE 4.7 Chemical structures of some phenolic compounds found in blackcurrant seeds.

Blueberries

Lowbush (wild) and highbush (cultivated) blueberries have been used for many years for production of berries on a commercial scale (Kalt et al., 2001). Blueberries are a rich source of phenolic acids, catechins, flavonols, anthocyanins and proanthocyanidins (de Pascual-Teresa et al., 2000; Gao and Mazza, 1994; Kader et al., 1996; Kalt and Dufour, 1997; Kalt and McDonald, 1996; Prior et al., 1998; Sellappan et al., 2002; Smith et al., 2000; Stöhr and Herrmann, 1976). Blueberry phenolics display inhibitory effects against chemically induced carcinogenesis (Bomser et al., 1996). The content of phenolics in blueberry is affected by the degree of maturity at harvest, genetic differences (cultivar), preharvest environmental conditions, postharvest storage conditions and processing.

Ehlenfeldt and Prior (2001) surveyed the total content of phenolics and anthocyanins in 87 highbush blueberry (*Vaccinium corymbosum* L.) and species-introgressed highbush

blueberry cultivars. The total content of phenolics in these berries ranged from 430 to 1990 (950 on average) mg/kg of fresh weight expressed as gallic acid equivalents, while the total anthocyanin content was between 890 and 3310 (1790 on average) mg/kg of fresh weight expressed as cyanidin-3-glucoside equivalents. Kalt et al. (2000) also surveyed the total phenolic and anthocyanin contents in 100 highbush blueberry varieties and 155 lowbush blueberry clones. The mean total anthocyanin content in highbush and lowbush blueberries was 1180 and 1630mg of cyanidin-3-glucoside equivalents/kg of fresh weight, respectively. On the other hand, the mean total phenolic content in highbush and lowbush blueberries is 1910 and 3760 mg gallic acid equivalents/kg of fresh weight. Thus, lowbush blueberries contain almost 50% more anthocyanins and over 90% more total phenolics than highbush blueberries. Recently, Sellappan et al. (2002) reported that the total content of anthocyanins and phenolics in Georgia-grown rabbiteye blueberries (*Vaccinium ashei* Reade) is between 127 and 1973.4 mg of cyanidin-3-glucoside equivalents/kg of fresh weight and from 2700.2 to 6690.1 mg of gallic acid equivalents/kg of fresh weight, respectively. Subsequently, Moyer et al. (2002) found that the total content of anthocyanins and total phenolics in 29 *Vaccinium* species ranges from 340 to 515 mg of cyanidin 3-glucoside equivalents/kg of fresh weight (on average 2300 mg/kg) and from 2110 to 9610 mg of gallic acid equivalents/kg of fresh weight (on average 5210 mg/kg), respectively.

Several phenolic acids have been identified in rabbiteye blueberries (*V. ashei* Reade) and southern highbush blueberries (*V. corymbosum* L.), namely (Figure 4.1):

- Gallic (0 to 2589 mg/kg of fresh weight)
- Caffeic (to 63.2 mg/kg of fresh weight)
- p*-Coumaric (24 to 157 mg/kg of fresh weight)
- Ferulic (30.2 to 169.7 mg/kg of fresh weight)
- Ellagic acids(2.2 to 66.5 mg/kg of fresh weight)

Rabbiteye blueberries also contain a higher level of catechin (145.3 to 3874.8 mg/kg of fresh weight) than southern highbush blueberries (98.7 to 292.8 mg/kg of fresh weight). The contents of myricetin (Figure 4.6), quercetin, and kaempferol (Figure 4.2) are 67.2 to 99.9, 58.2 to 146, and 25.1 to 37.2 mg/kg of fresh weight, respectively (Sellappan et al., 2002). On the other hand, Bilyk and Sapers (1986) detected only 24 to 28.5 mg quercetin/kg of fresh weight in highbush blueberries.

A number of anthocyanins have been isolated and identified in lowbush (wild), highbush, rabbiteye and Tifblue blueberries, namely, 3-galactosides and 3-arabinosides of cyanidin, delphinidin, peonidin, petunidin and malvidin, and 3-glucosides of cyanidin, delphinidin, peonidin, petunidin and malvidin (Figure 4.5) (Ballington et al., 1987; Francis et al., 1966; Mazza and Miniati, 1993; Prior et al., 2001). Small quantities of 6-acetyl-glucosides of cyanidin, delphinidin, malvidin, peonidin, petunidin, and 6-acetyl-galactoside of malvidin have also been detected in lowbush blueberries (Prior et al., 2001). Of these, malvidin-3-glucoside, malvidin-3-galactoside, cyanidin-3-arabinoside, delphinidin-3-arabinoside, petunidin-3-galactoside, petunidin-3-galactoside and delphinidin-3-galactoside were the major anthocyanins in lowbush and Tifblue blueberries (Ballington et al., 1987; Francis et al., 1966; Mazza and Miniati, 1993; Prior et al., 2001).

Prior et al. (2001) identified oligomeric B-type procyanidins (from monomeric to octamers) with (+)catechin and/or (-)epicatechin units linked through (4→8 or 4→6 bonds) in highbush and lowbush blueberries (Figure 4.8). Later, Gu et al. (2002) reported that lowbush blueberry contains 19,990 mg/kg of dry weight of procyanidins.

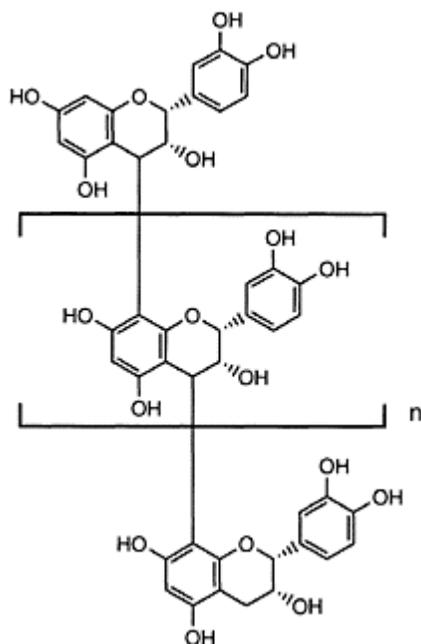


FIGURE 4.8 Chemical structures of oligomeric ($n=1$ to 8) and polymeric ($n>8$) procyanidins.

The polymeric procyanidins comprise over 76% of total procyanidins and are a mixture of polymers with a degree of polymerization ranging from 14.4 to 114.1 as determined by thiolysis (Gu et al., 2002).

Prior et al. (1998) demonstrated that late harvest berries of *V. ashei* Reade cultivars Tifblue and Brightwell exhibit greater antioxidant activities than those from early harvest berries (Prior et al., 1998). On the other hand, Kalt et al. (1999b) and Kalt and McDonald (1996) showed that postharvest storage conditions also affect antioxidant potential of blueberries as determined by ORAC (oxygen radical absorbance capacity) measurements. Almost a 20% increase in anthocyanins was observed in lowbush blueberries (*Vaccinium angustifolium* Ait cultivars) stored for 2 weeks at 1°C (Kalt and McDonald, 1996). However, a similar increase in anthocyanins was noted in highbush blueberries (*V. corybosum* L. cultivar Bluecrop) stored for 8 days at 20°C, but not in berries stored for the same period at 0, 10 and 30°C (Kalt et al., 1999b).

Recently, Connor et al. (2002) evaluated the effect of extended storage (up to 5 weeks at 5°C) on the antioxidant potential of berries harvested from nine blueberry cultivars.

These authors demonstrated that extended storage of blueberries does not significantly decrease their antioxidant potential. The antioxidant activity of blueberry juice is affected by the extraction temperature, pH and addition of oxygen to juice (Kalt et al., 2000). Processing of blueberries to juice has a detrimental effect on their phenolics; only 32, 35, 43 and 53% of anthocyanins, flavonols, procyanidins and chlorogenic acids present remain in their corresponding juices, respectively. These losses are believed to be due to the activity of native polyphenol oxidase during milling and depectinization of berries (Skrede et al., 2000).

Cranberries

The fruits of American cranberries, *Vaccinium macrocarpon*, and European cranberries, *Vaccinium oxycoccus*, possess a distinctive flavor and a bright red color; they are sold fresh or processed into sauce, concentrates and juice. Cranberry fruits serve as an excellent source of anthocyanins (Hong and Wrolstad, 1986; Mazza and Miniati, 1993; Prior et al., 2001; Zapsalis and Francis, 1965), flavonol glycosides (Kandil et al., 2002; Puski and Francis, 1967), proanthocyanidins (Foo and Porter, 1980; Hale et al., 1986; Kandil et al., 2002; Prior et al., 2001) and phenolic acids (Zuo et al., 2002). Cranberry proanthocyanidins display a potent bacterial antiadhesion activity (Howell, 2002), inhibit the sialyllactose specific adhesion of *Helicobacter pylori* to human gastric mucus (Burger et al., 2002), and lower the rate of cardiovascular diseases (Reed, 2002).

Cranberries contain about 1g/kg of fresh weight of phenolic acids (Zuo et al., 2002), predominantly as glycosides and esters (Chen et al., 2001). Only small quantities of phenolic acids are detected in the free form (Zuo et al., 2002). Twelve phenolic acids have been identified in cranberry (Figure 4.1) (Marwan and Nagel, 1982; Zuo et al., 2002):

- *o*-Hydroxybenzoic
- *m*-Hydroxybenzoic
- *p*-Hydroxybenzoic
- *p*-Hydroxyphenyl acetic
- 2,3-Dihydroxybenzoic
- 2,4-Dihydroxybenzoic
- Vanillic
- *o*-Hydroxycinnamic
- Caffeic
- *p*-Coumaric
- Ferulic
- Sinapic

In addition, bound gallic, chlorogenic, *p*-coumaric and *p*-hydroxybenzoic acids have been detected in cranberry pomace by Zheng and Shetty (2000). Sinapic, caffeic and *p*-coumaric acids are the most abundant bound phenolic acids and coumaric, 2,4-dihydroxybenzoic and vanillic acids the predominant free phenolic acids found in cranberry (Zuo et al., 2002).

Resveratrol has also been detected in cranberry fruit (Figure 4.9). The concentration of resveratrol in cranberry juice (0.25 mg/kg) is similar to that found in Concord grape juice

(0.36 mg/kg). Raw cranberry juice contains predominantly *trans*-resveratrol, while processing increases the level of *cis*-resveratrol (Wang et al., 2002a).

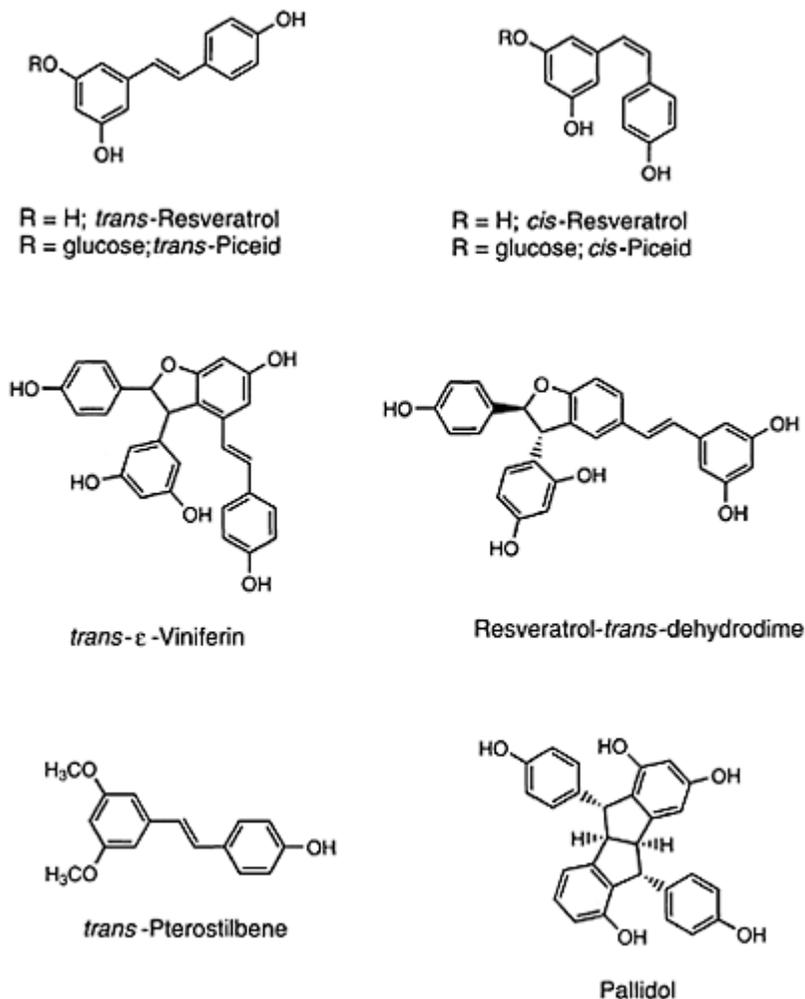


FIGURE 4.9 Chemical structures of stilbens.

Several flavonols have been identified in cranberry fruits, namely quercetin, quercetin 3-*O*-galactoside (hyperin), quercetin 3-*O*-arabinoside, quercetin 3-*O*-rhamnoside (quercitrin) (Figure 4.2), myricetin 3-*O*-arabinoside and 3-*O*-digalactoside (Kandil et al., 2002; Puski and Francis, 1967). The total content of quercetin and myricetin in cranberry fruit ranged from 73 to 250 and from 4.0 to 26.7 mg/kg fresh of weight, respectively. Small quantities of kaempferol (0.6 to 2.7 mg/kg fresh weight) were also detected in some cranberry varieties (Amakura et al., 2000; Bilyk and Sapers, 1986).

The predominant anthocyanins in American cranberries are 3-*O*-galactosides and 3-*O*-arabinosides of cyanidin and peonidin, while European cranberries contain 3-*O*-glucosides of cyanidin and peonidin (Figure 4.5) (Mazza and Miniati, 1993; Sapers and Hargrave, 1987; Zapsalis and Francis, 1965). Recently, Prior et al. (2001) reported the presence of small quantities of petunidin 3-*O*-galactoside and cyanidin 3-*O*-glucoside in American cranberry fruits. The total content of anthocyanins in cranberry fruits ranges from 180 to 656 mg/kg of fresh weight (Bilyk and Sapers, 1986; Wang and Stretch, 2001); these are located under the fruit skin (Francis, 1957; Sapers et al., 1983; Vorsa and Welker, 1985). Cyanidins comprise approximately 55% of total anthocyanins in cranberry (Prior et al., 2001). The content of anthocyanins

TABLE 4.1
Total Anthocyanin Content of Fruit of
Cranberry Cultivars Stored for 3 Months at
Different Storage Temperatures^a

| Cultivar | Storage Temperature, °C | | | |
|-------------|-------------------------|--------|--------|---------|
| | Initial | 0 | 5 | 15 |
| Ben Lear | 250±13 | 460±25 | 546±29 | 766±35 |
| Cropper | 198±13 | 348±16 | 492±30 | 604±21 |
| Crowley | 656±20 | 654±34 | 808±37 | 1092±29 |
| Early Black | 634±15 | 724±52 | 886±41 | 1172±32 |
| Franklin | 541±36 | 604±18 | 696±32 | 936±14 |
| Howes | 235±11 | 330±14 | 494±16 | 710±22 |
| Pilgrim | 207±11 | 308±15 | 386±10 | 600±23 |
| Stevens | 228±13 | 316±12 | 474±25 | 656±18 |
| Wilcox | 243±17 | 548±27 | 646±28 | 724±17 |

^a Milligrams of cyanidin 3-*O*-galactoside per kilogram of fresh weight.

Source: Adapted from Wang, S.Y. and Stretch, A.L., 2001, *J. Agric. Food Chem.*, 49:969–974.

is affected by cultivar, fruit size and pre- and postharvest conditions (Cracker, 1971; Galletta, 1975; Hall and Stark, 1972; Sapers et al., 1983; Vorsa and Welker, 1985; Wang and Stretch, 2001). Smaller berries contain higher levels of anthocyanins than those of larger ones due to the location of anthocyanins in the fruits (Vorsa and Welker, 1985; Wang and Stretch, 2001). Storing cranberries for 3 months at temperatures of up to 15°C significantly increases their total anthocyanin content (Table 4.1); however, this increase is somewhat lower when cranberry fruits are stored at the recommended storage temperature of 2 to 4°C (Hardenburg et al., 1986; Wang and Stretch, 2001).

Whole cranberries contain approximately 17 mg/kg of total proanthocyanidin, while 2.16 to 2.23 mg/L of total procyanidins are found in cranberry juices (Prior et al., 2001). The polymeric proanthocyanidins comprise 63% of total proanthocyanidins in cranberries (Gu et al., 2002). Foo and Porter (1980) reported that the ratio of procyanidins (with units containing two hydroxyl groups on B-ring) to prodelphinidins (containing three hydroxyl groups on B-ring) is 78:22 in European cranberries. Later, Foo et al. (2000) isolated and identified three proanthocyanidin trimers with A-type doubly linked interflavonoid linkages in ripe American cranberry fruits, namely:

- Epicatechin-(4 β →6)-epicatechin-(4 β →8, 2 β →O→7)-epicatechin
- Epicatechin-(4 β →8, 2 β →O→7)-epicatechin-(4 β →6)-epicatechin
- Epicatechin-(4 β →8)-epicatechin-(4 β →8, 2 β →O→7)-epicatechin

Subsequently, Prior et al. (2001) reported that (–)epicatechin and its dimers and A-type trimers (Figure 4.10) are the predominant proanthocyanidins in cranberries.

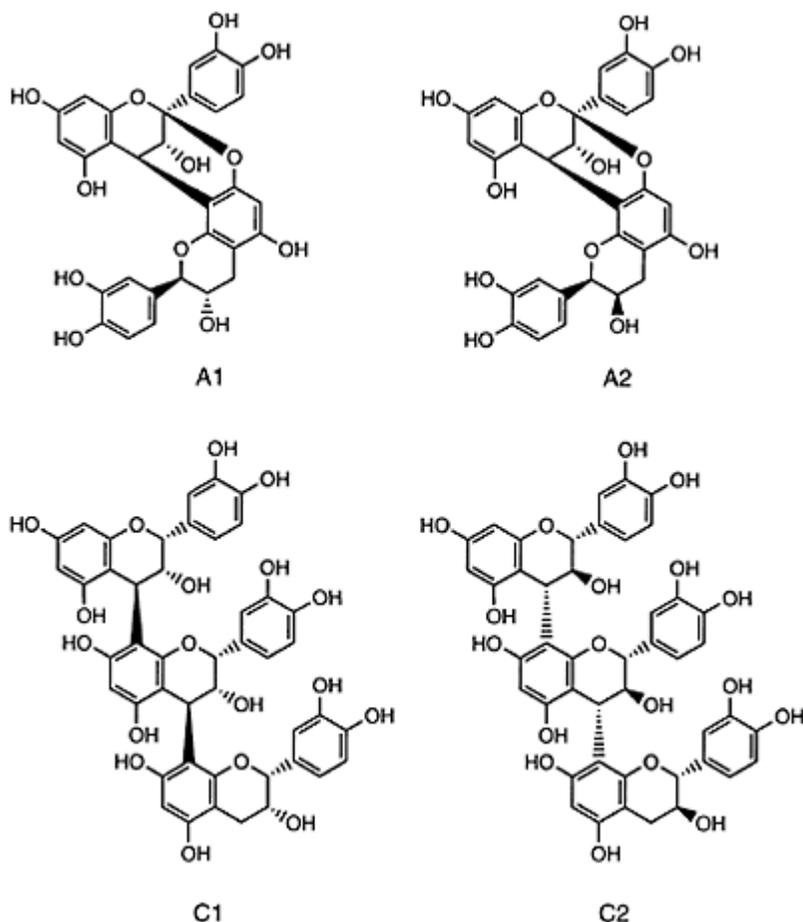


FIGURE 4.10 Chemical structures of some procyanidin dimers type A and trimers type C.

These authors also detected trace amounts of B-type (single linked interflavonoid linkages) and A-type tetramers. Recently, Kandil et al. (2002) detected (–)epicatechin, (+)catechin, gallocatechin and epigallocatechin (Figure 4.3) as well as higher molecular weight proanthocyanidins in the American cranberry.

Grapes

The chemistry of grape phenolics is discussed in Chapter 5 and Mazza (1995) also provides an excellent review on the types of anthocyanins and their distribution in grapes; therefore, only some aspects of the chemistry of grape phenolics are discussed in this section. The main phenolics identified in *Vitis vinifera* berries are listed in Table 4.2 and the total content of phenolics in some table grape varieties is summarized in Table 4.3. Anthocyanins are the predominant phenolics of red table grape varieties, while flavan-3-ols are the main phenolics in white table grape varieties (Cantos et al., 2002; de Simon et al., 1993; Ingalsbe et al., 1963; Mazza, 1995; Spanos and Wrolstad, 1992). These phenolics contribute to the sensory quality of grape products (Jaworski and Lee, 1987).

TABLE 4.2

Main Phenolics Identified in *Vitis Vinera* Berries

Phenolic acids

p-hydroxybenzoic; o-hydroxybenzoic; salicylic; gallic; cinnamic; p-coumaroyl tartaric (=coutaric); caffeoyl tartaric (=caftaric); feruloyl tartaric (=fertaric); p-coumaroyl glucose; feruloyl glucose; glucose ester of coutaric acid

Anthocyanins

Cyanidin 3-glucoside; cy 3-acetylglucoside; cy 3-p-coumaryl-glucoside; peonidin 3-glucoside; pn 3-acetylglucoside; pn 3-p-coumarylglucoside; pn 3-caffeoylglucoside (?); delphinidin 3-glucoside; dp 3-acetylglucoside; dp 3-p-coumarylglucoside; petunidin 3-glucoside; pt 3-p-coumarylglucoside; malvidin 3-glucoside; mv 3-acetylglucoside; mv 3-p-coumarylglucoside; mv 3-caffeoylglucoside

Flavonols

Kaempferol 3-glucoside; k 3-glucuronide; k 3-glucosylarabinoside (?); k 3-galactoside; quercetin 3-glucoside; q 3-glucuronide; q 3-rutinoside; q 3-glucosylgalactoside (?); q 3-glucosylxyloside (?); iso-rhamnetic 3-glucoside

Flavan-3-ols and tannins

(+)catechin; (-)epicatechin; (+)gallocatechin; (-)epigallocatechin; epicatechin-3-O-gallate; procyanidins B1, B2, B3, B4, C1, C2, polymeric forms of condensed tannins

Flavanonols

Dihydroquercetin 3-rhamnoside (=astilbin); dihydrokaempferol 3-rhamnoside (=engeletin)

Source: Adapted from Macheix, J.J. et al., 1990, *Fruit Phenolics*, CRC Press, Boca Raton, FL.

Pomace (skin and seeds), a by-product from processing grape to juice and wine, comprises about 13% of the amount of processed berries (Torres and Bobet, 2001) and may also contain stems when wines are made from nondestemmed crop. Grape seeds, stems and skins are a rich source of health-promoting flavonoids such as proanthocyanidins, flavonols and flavan-3-ols (Cheyner and Rigaud, 1986; Diplock et al., 1998; Roggero et al., 1988; Ruf, 1999; Souquet et al., 1996, 2000; Su and Singleton, 1969; Yang et al., 1997). Of these, proanthocyanidins are the major polyphenols in grape seeds, stems and skins. Procyanidins are the predominant proanthocyanidins in grape seeds, while procyanidins and prodelfinidins are dominant in grape skins and stems (Escribano-Bailón et al., 1992a, b, 1995; Souquet et al., 1996, 2000; Thorngate and Singleton, 1994). The procyanidin dimers identified in grape seeds and their contents are listed in Table 4.4, Table 4.5 and Figure 4.4. In addition, 11 trimers and one tetramer

have also been detected in grape seeds (Boukharta, 1988; da Silva et al., 1991; Escribano-Bailón et al., 1992b; Romeyer et al., 1986; Santos-Buelga et al., 1995). The total contents of dimers, dimer gallates and trimers in seeds from grape cultivars grown in Ontario are 160 to 3750 mg of B2 equivalents, traces of 1080 mg of B2-3'-O-gallate equivalents, and traces of 840 mg of B2 equivalents/kg of seeds (Fuleki and da Silva, 1997).

Approximately 55% of grape seed procyanidins are of polymeric type (degree of polymerization, $DP \geq 5$) (Figure 4.8) (Prieur et al., 1994), while the ratios of polymeric procyanidins ($DP \geq 4$) to monomeric (catechin+epicatechin) are 5.2 to 8.9 (Peng et al., 2001). Grape seeds contain polymeric procyanidins from 33,200 to 50,700 mg/kg in seeds or from 1680 to 3190 mg/kg in berries (Labarbe et al., 1999,

TABLE 4.3
Total Contents of Phenolics in Some White and Red Table Grape Varieties^a

| Total Content of | Red Globe (R) | Flame (R) | Crimson (R) | Napoleon (R) | Superior (W) | Dominga (W) | Moscatel Italica (W) |
|--------------------------------|---------------|-----------|-------------|--------------|--------------|-------------|----------------------|
| Phenols ^b | 225.4 | 361.2 | 131.9 | 135.9 | 135.7 | 114.9 | 145.1 |
| Hydroxycinnamates ^c | 8.4 | 47.6 | 9.5 | 9.5 | 9.0 | 25.0 | 16.3 |
| Flavonols ^d | 61.3 | 53.8 | 12.8 | 32.4 | 64.0 | 32.7 | 47.7 |
| Flavan-3-ols ^e | 40.4 | 109.1 | 41.1 | 18.3 | 62.7 | 57.2 | 81.1 |
| Anthocyanins ^f | 115.3 | 150.7 | 69.5 | 75.7 | | | |

Note: Abbreviations: R=red; W=white.

^a Milligrams per kilogram of fresh weight.

^b Total phenols=total hydroxycinnamates+total flavonols+total flavan-3-ols+total anthocyanins.

^c Total hydroxycinnamates expressed as chlorogenic acid equivalents.

^d Total flavonols expressed as quercetin 3-rutinoside equivalents.

^e Total flavan-3-ols expressed as catechin equivalents.

^f Total anthocyanins expressed as cyanidin 3-rutinoside equivalents.

Source: Adapted from Cantos, E. et al., 2002, *J. Agric. Food Chem.*, 50:5691-5696.

TABLE 4.4
Oligomeric Procyanidin Dimers Identified in Grape Seeds

| Procyanidins | Compound | Reference |
|----------------|-----------------------------------|------------------------------|
| B1 | Ec-(4 β →8)-Cat | Lea, A.G.H. et al. (1979) |
| B2 | Ec-(4 β →8)-Ec | Lea, A.G.H. et al. (1979) |
| B3 | Cat-(4 α →8)-Cat | Lea, A.G.H. et al. (1979) |
| B4 | Cat-(4 α →8)-Ec | Lea, A.G.H. et al. (1979) |
| B5 | Ec-(4 β →6)-Ec | da Silva, J.M. et al. (1991) |
| B6 | Cat-(4 β →6)-Cat | da Silva, J.M. et al. (1991) |
| B7 | Ec-(4 β →6)-Cat | Boukharta, M. (1998) |
| B8 | Cat-(4 β →6)-Ec | da Silva, J.M. et al. (1991) |
| B1-3-O-gallate | Ec-3-O-gallate-(4 β →8)-Cat | da Silva, J.M. et al. (1991) |

| | | |
|----------------------|--|------------------------------------|
| B2-3-O-gallate | Ec-3-O-gallate-(4 β →8)-Ec | da Silva, J.M. et al. (1991) |
| B2-3'-O-gallate | Ec-(4 β →8)-Ec-3-O-gallate | Boukharta, M. (1988) |
| B4-3'-O-gallate | Cat-(4 β →6)-Ec-3-O-gallate | da Silva, J.M. et al. (1991) |
| B7-3-O-gallate | Ec-3-O-gallate-(4 β →6)-Cat | Santos de Buelga, C. et al. (1995) |
| B2-3,3'-di-O-gallate | Ec-3-O-gallate-(4 β →8)- Ec-3-O-gallate | da Silva, J.M. et al. (1991) |

Note: Ec=(-)epicatechin; Cat=(+)catechin.

TABLE 4.5

Content of Catechins and Procyanidin Dimers in Seeds of Different *Vitis Vinifera* Cultivars Grown in Ontario in 1993^a

| Cultivar | Catechins | | Procyanidins | | | |
|----------------|-----------|-------------|--------------|-----|-----|------|
| | Catechin | Epicatechin | B1 | B2 | B3 | B4 |
| Cabernet Franc | 960 | 1360 | 260 | 790 | 210 | 430 |
| Gamay | 1140 | 1140 | 620 | 930 | 710 | 1490 |
| Merlot | 640 | 790 | 200 | 480 | 80 | 210 |
| Pinot Noir | 2440 | 1930 | 560 | 900 | 300 | 590 |
| Riesling | 250 | 240 | 110 | 290 | 20 | 120 |

^a Milligrams per kilogram.

Source: Adapted from Fuleki, T. and da Silva, J.M., 1997, *J. Agric. Food Chem.*, 45:1156-1160.

Souquet et al., 2000). The mean degree of polymerization for proanthocyanidins isolated from the seeds of grapes (cv. Cabernet franc) ranges from 4.7 to 17.4. For those isolated from grape skin, it is between 9.3 and 73.8 (Labarbe et al., 1999; Souquet et al., 2000) and for those extracted from grape stems between 4.9 and 27.6 (Souquet et al., 2000).

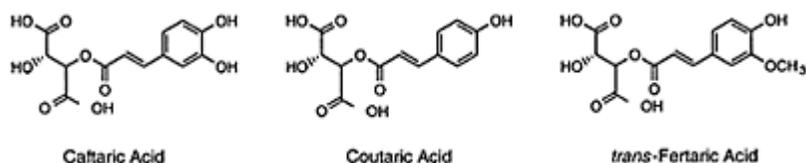


FIGURE 4.11 Chemical structures of caftaric, coutaric and trans-fertaric acids.

Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), Yang and Chien (2000) later observed the presence of procyanidin oligomers of up to nanomers in grape seed extracts. Similar results were obtained by Saucier et al. (2001), who reported that the mean degree of polymerization of proanthocyanidins isolated from seeds of cv. Merlot is 3.1 to 12.2. Furthermore, the galloylation extent of proanthocyanidins isolated from grape seed was much higher (20.4%) than that for proanthocyanidins from grape stems (15.6%) and grape skins (2.7%)

(Labarbe et al., 1999; Souquet et al., 2000). The composition of grape skin proanthocyanidins is influenced by the stage of berry ripening. The change in the color of berry from green to red effects an increase in the mean degree of polymerization in the (–)epigallocatechin extension units and in the level of anthocyanins associated with the proanthocyanidin fraction (Kennedy et al., 2001).

Other phenolics detected in whole grape berries, grape skins and stems include phenolic acids:

- Caftaric acid (*trans*-caffeoyltartaric acid)
- Coutaric acid (*p*-coumaryltartaric acid)
- *trans*-Fertaric acid (Figure 4.11) (Cantos et al., 2002; Singleton et al., 1978; Souquet et al., 2000; Vrhovšek, 1998)

flavonols:

- Quercetin 3-glucuronide
- Quercetin 3-glucoside (Figure 4.2)
- Myricetin 3-glucuronide
- Myricetin 3-glucuronide (Cheynier and Rigaud, 1986; Moskowitz and Hrazdina, 1981; Souquet et al., 2000)

and flavanonols:

- Astilbin (dihydroquercetin 3-rhamnoside)
- Engeletin (dihydrokaempferol 3-rhamnoside) (Figure 4.12) (Lu and Foo, 1999; Souquet et al., 2000; Trousdale and Singleton, 1983).

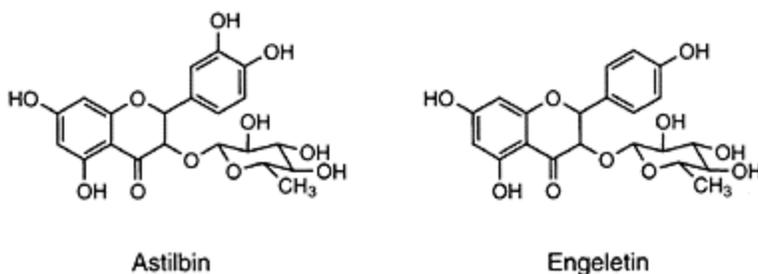


FIGURE 4.12 Chemical structures of some flavanonols.

Grape berries contain from 269 to 467 mg/kg of total hydroxycinnamoyltartaric acids (HCAs). Caftaric acid (117.5 to 369.5 mg/kg) and *trans*-coutaric acid (55.3 to 93 mg/kg) are the predominant HCAs in berries, while *cis*-coutaric acid (11.8 to 21.0) and fertaric acids (1.7 to 16.8 mg/kg) are minor HCAs present (Vrhovšek 1998).

Stilbenes such as *trans*- and *cis*-resveratrols (3,5,4'-trihydroxystilbene), *trans*- and *cis*-piceids (3-*O*- β -D-glucosides of resveratrol), *trans*- and *cis*-astringins (3-*O*- β -D-glucosides of 3'-hydroxyresveratrol), *trans*- and *cis*-resveratrolsides (4'-*O*- β -D-glucosides of resveratrol) pterostilbene (a dimethylated derivative of stilbene) are

grapevine phytoalexins found in grape leaves and berries (Figure 4.9). In berries stilbenes are mostly located in the grape skin (Jaendet et al., 2002; Langcake and Pryce, 1977; Langcake et al., 1979; Versari et al., 2001; Wang et al., 2002a; Waterhouse and Lamuela-Raventós, 1994). Of these, *cis*-piceid is the major stilbene found in berry skins during fruit ripening (39.5 mg/kg of fresh weight at 60 days after véraison), while resveratrol is the predominant stilbene in wilting berries (28 mg/kg at day 74) (Versari et al., 2001). Moreover, *trans*-piceid (3.38 mg/L) is the major resveratrol derivative in red grape juices, while *cis*-piceid (0.26 mg/L) occurs in white grape juices (Romero-Pérez et al., 1999). In grape berries, *p*-coumaroyl-CoA and malonyl-CoA serve as substrates for the synthesis of stilbene catalyzed by stilbene synthase (Fritzsche and Kindl, 1981). The synthesis of stilbenes in grapevine is stimulated by stress factors such as fungal infection (*Botrytis cinerea*), injury, UV radiation, and wilting as well as such factors as grape cultivar, berry development stage, and soil practices (Bavaresco et al., 1997; Cantos et al., 2000, 2001b; Douillet-Breuil et al., 1999; Jaendet et al., 1991; Langcake and Pryce, 1976; Siemann and Creasy, 1992; Versari et al., 2001).

Jaendet et al. (1991) reported that a correlation exists between the accumulation of resveratrol in grape berries at different stages of development and that of grape leaves. Douillet-Breuil et al. (1999) noticed an increase in production of resveratrol and ϵ -viniferin (resveratrol (*E*)-dehydrodimer) in grape berry skins subjected to short UV-C radiation, but not piceid and pterostilbene. Subsequently, Cantos et al. (2000) reported that postharvest treatment of grape berries with UV-B or UV-C radiation increases the level of resveratrol in berries by two- or three-fold, respectively. Meanwhile, Adrian et al. (2000) noticed that UV-C radiation has a more pronounced effect on the production of stilbenes in healthy berries than in berries stressed by fungus. Later, Cantos et al. (2001b) reported an 11-fold increase in accumulation of resveratrol in berries treated with UV radiation pulses (total radiation power of 510W, radiation time of 30 sec, lamp distance of 40 cm from the berries) and then stored for 3 days at 20°C.

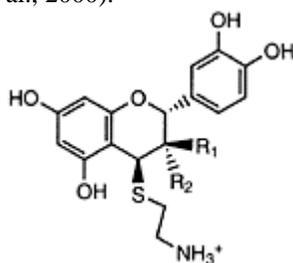
On the other hand, Versari et al. (2001) demonstrated that UV radiation of berries has a more pronounced effect on the synthesis of resveratrol during the early stages of fruit development than during ripening. Extensive fungal infection of grapes brings about biotransformation of stilbenes catalyzed by stilbene oxidase or grapevine peroxidases into ϵ -viniferin (resveratrol (*E*)-dehydrodimer), α -viniferin, pallidol (Figure 4.9), leachinol F and restrytisols A to C (Breuil et al., 1998; Cichewicz et al., 2000; Morales et al., 1997; Versari et al., 2001). Later, Waffo-Teguo et al. (2001) isolated and identified two more products of stilbene oxidative degradation, namely, resveratrol (*E*)-dehydrodimer 11-*O*- β -D-glucopyranoside and resveratrol (*E*)-dehydrodimer 11'-*O*- β -D-glucopyranoside. In addition, they reported that resveratrol (*E*)-dehydrodimer 11-*O*- β -D-glucopyranoside and resveratrol (*E*)-dehydrodimer exhibit a nonspecific inhibitory activity against cyclooxygenase-1 and -2. Furthermore, *trans*- and *cis*-resveratrols display inhibitory effects against tyrosinase kinase (Jayatilake et al., 1993) and platelet aggregation (Chung et al., 1992), while piceids are effective against platelet aggregation (Chung et al., 1992; Orsini et al., 1997) and human LDL oxidation (Frankel et al., 1995).

Chloroanisoles are responsible for a musty off-flavor in stored raisins. These compounds may originate in raisins from chemical reactions and microbial and airborne contaminations (Maarse et al., 1988; Reineccius, 1991). The formation of chloroanisoles involves chlorination of phenols derived from the Shikimic pathway by fungal or host

chloroperoxidases followed by their methylation (Liardon et al., 1991). Aung et al. (1996) demonstrated the formation of chloroanisoles in Thompson seedless, dry-on-the-vine raisins under sterile, nonsterile and $a_w < 0.8$ conditions. The raisins used in this study were sterilized with propylene oxide or hydrogen peroxide.

Torres and Bobet (2001) generated a series of novel flavanol antioxidant from grape by-products by subjecting the extracts of polymeric proanthocyanidins to depolymerization in the presence of cystamine. The terminal flavan-3-ols units were released as such, while the extension units were cleaved as thio-derivatives. Three major aminoethylthio-flavan-3-ols conjugates were isolated and identified in the reaction mixture: 4 β -(2-aminoethylthio)epicatechin, 4 β -(2-aminoethylthio)epicatechin 3-*O*-gallate, and 4 β -(2-aminoethylthio)catechin (Figure 4.13). The antioxidant activities of these novel flavan-3-ol conjugates were similar to those displayed by their nonderivatized counterparts.

Raisins are important processed grape products. Italy, France and the U.S. are the world's largest producers of raisins (Pollack and Perez, 1997). Karadeniz et al. (2000) evaluated the effect on the composition of phenolic in raisins of sun-drying grapes (sun-dried raisins), dipping grapes into hot water (87 to 93°C) for 15 to 20 s before drying at 71°C for 20 to 24 h (dipped raisins), and dipping grapes into hot water followed by 5- to 8-h treatment with sulfur dioxide and then drying at 63°C (golden raisins). Oxidized hydroxycinnamic acids, formed upon the action of polyphenoloxidases, were only found in sun-dried and dipped raisins. According to Aguilera et al. (1987), at least a 2-min dip of berries into water at 93°C is required to inactivate polyphenoloxidases in Sultana grapes. The loss of hydroxycinnamic acids and flavonols during processing of grapes to raisins is in the order of 90 and 62%, respectively; procyanidins are degraded completely (Karadeniz et al., 2000).



| Compound | R ₁ | R ₂ |
|--|----------------|----------------|
| 4 β -(2-Aminoethylthio)-epicatechin | H | OH |
| 4 β -(2-Aminoethylthio)-catechin | OH | H |
| 4 β -(2-Aminoethylthio)-epicatechin-3- <i>O</i> -gallate H | | |

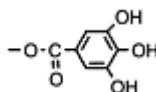


FIGURE 4.13 Chemical structures of 4- β -(2-aminoethylthio)-flavan-3-ols.

Cheynier and da Silva (1991) demonstrated that caftaric acid *o*-quinones may be involved in the oxidation of procyanidins through a coupled oxidation mechanism, leading to the reduction of caftaric acid quinones back to caftaric acid. Drying grape pomace may be an

essential step in the utilization of this by-product for the production of pharmaceuticals. Larrauri et al. (1997) reported that drying red grape pomace at 60°C does not have a detrimental effect on the phenolics and their antioxidant activity. On the other hand, a significant reduction in the extractability of total phenolics and proanthocyanidins and their antioxidant activity was noted when grape pomace was dried at 100 and 140°C.

Raspberries

Raspberries (*Rubus idaeus* L.) are a rich source of phenolic compounds. The content of phenolics in raspberries may be influenced by cultivar, maturity, processing and geographic area of origin (Rommel et al., 1992; Wang and Lin, 2000). The total phenolics in ripe raspberries range from 1137 to 29,900 mg/kg of fresh weight expressed as gallic acid equivalents (de Ancos et al., 2000; Kähkönen et al., 2001; Liu et al., 2002; Moyer et al., 2002; Mullen et al., 2002b; Wang and Lin, 2000). Anthocyanins (Boyles and Wrolstad, 1993), flavonols and their conjugates (Henning, 1981; Rommel and Wrolstad, 1993a; Rommel et al., 1992), and ellagic acid and its derivatives (Rommel and Wrolstad, 1993b) are the major phenolics present in raspberries.

The total content of ellagic acid in fresh raspberry fruits ranges from 172.9 to 244.4 mg/kg of fresh weight (Amakura et al., 2000; de Ancos et al., 2000), while in raspberry juices ellagic acid is present at 5.7 to 80.4 µg/kg (Rommel and Wrolstad, 1993b). According to Häkkinen et al. (1999b), ellagic acid comprises 88% of the total phenolics; de Ancos et al. (2000) have reported that ellagic acid represents 13.8 to 19% of the total phenolic acids. Ellagic acid in raspberries is present in the free form as ellagic acid glycosides and as ellagitannins. Kähkönen et al. (2001) reported that raspberry fruits contain from 16,920 to 17,540 mg of ellagitannins/kg of fresh weight expressed as ellagic acid equivalents. Rommel et al. (1992) and Rommel and Wrolstad (1993b) detected 16 derivatives of ellagic acid in raspberry juices, but did not characterize these compounds any further. These authors suggested that a mixture of ellagic acid with varying methylation, methoxylation and glycolysation patterns, as well as ellagitannins, is present. Later, Zafrilla et al. (2001) and Mullen et al. (2002a) isolated five ellagic acid derivatives, but identified only three derivatives of ellagic acid, namely, 4-arabinoside, 4'(4"-acetyl) arabinoside and 4'-(4"-acetyl) xyloside.

In addition, several ellagitannins have been characterized in raspberries, namely, potentillin and casuarictin and their C-O oxidatively coupled dimeric forms as well as β-1,2,3-tri-*O*-galloyl-4,6-hexahydroxydiphenoyl-D-glucose, pedunculagin, and 2,3-di-*O*-galloyl-4,6-(hexahydroxydiphenoyl)-D-glucose (Figure 4.14) (Haddock et al, 1982; Haslam, 1989; Haslam and Lilley, 1985; Haslam et al, 1982). Subsequently, Mullen et al. (2002a, b) identified and quantified two ellagitannins in Glen Ample raspberries, namely, lambertianin C (18.7 µg/kg of gallic acid equivalent) and sanguin H-6 (69.2 µg/kg of gallic acid equivalent), as major contributors to the antioxidant capacity of raspberries.

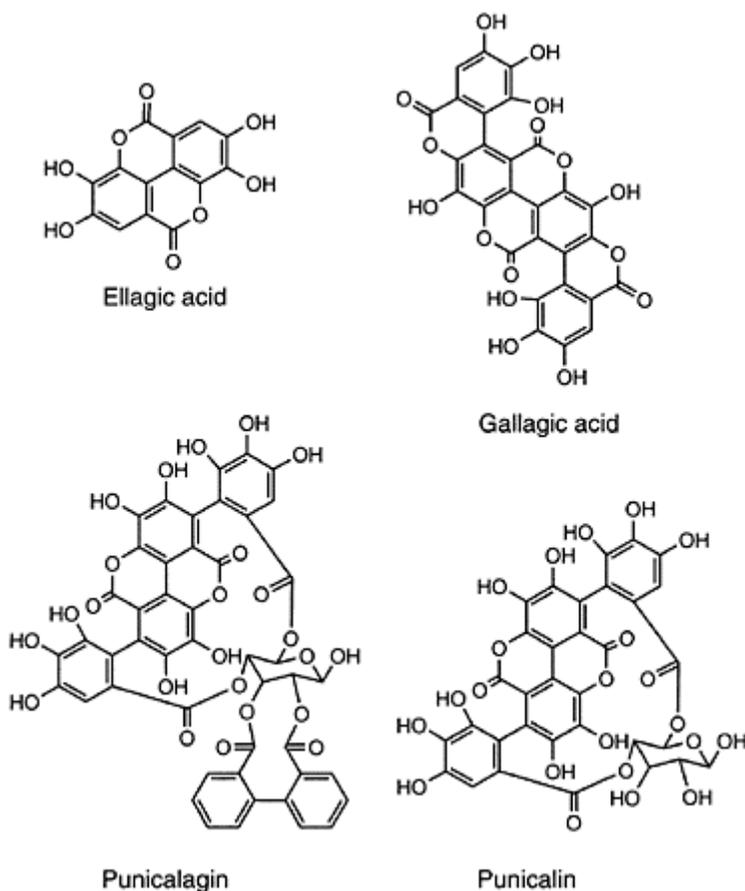


FIGURE 4.14 Chemical structures of some phenolic compounds found in raspberries and pomegranate fruits.

The total flavonoid content in raspberry fruits is between 842 and 1034 mg/kg of fresh weight expressed as (+)catechin equivalents (Liu et al., 2002). Flavonols and their conjugates are the major flavonoids in raspberry. According to Häkkinen et al. (2000a), red raspberries contain 9.5 mg/kg of fresh weight of flavonols expressed as aglycones. Zafrilla et al. (2001) and Kähkönen et al. (2001) have reported values of 70 to 300 mg/kg of fresh weight of quercetin 3-glucoside. On the other hand, Rommel and Wrolstad (1993b) found that total content of flavonols in 55 samples of experimental and commercial red raspberry juices ranges from 15.7 to 286 $\mu\text{g}/\text{kg}$. Several flavonol glycosides have been identified by Ryan and Coffin (1971), Henning (1981) and Mullen et al. (2002a) in fresh raspberry fruits: quercetin 3-glucuronide, quercetin 3-glucoside, quercetin 3-xylosylglucuronide, quercetin 3-galactoside, methylquercetin-pentoside (probably xyloside) and kaempferol 3-glucoside. Later, Rommel and Wrolstad (1993a)

detected more quercetin and kaempferol derivatives, but did not attempt to characterize them. Of these, quercetin 3-glucuronide is the predominant flavonol glycoside in red raspberry juices (Rommel and Wrolstad, 1993a)

Total anthocyanin levels in fruits are between 520 (red raspberry) and 6270 (black raspberry) mg/kg and in juices range from 1.7 (in yellow raspberry) to 1972 mg/kg (black raspberry) (Table 4.6) (Boyles and Wrolstad, 1993; Kähkönen et al., 2001; Liu et al., 2002; Moyer et al., 2002; Rommel et al., 1990; Wang and Lin, 2000; Wrolstad et al., 1993). Rommel et al. (1990) and Wrolstad et al. (1993) evaluated the anthocyanin profiles in selected commercial and laboratory-prepared red raspberry juices. Several anthocyanins were identified, namely, cyanidin-3-sophoroside, cyanidin-3-glucorutinoside, cyanidin-3-glucoside, pelargonidin-3-sophoroside, cyanidin-3-rutinoside and pelargonidin-3-glucorutinoside. In addition, de Ancos et al. (2000) detected malvidin-3-glucoside and delphinidin-3-glucoside in Spanish raspberry cultivars and Mullen et al. (2002a) reported the presence of cyanidin-3,5-diglucoside, cyanidin-3-sambubioside, cyanidin-3-xylosylrutinoside, pelargonidin-3-glucoside and pelargonidin-3-rutinoside in Glen Ample raspberries. Of these, cyanidin-3-sophoroside and cyanidin-3-glucoside are the predominant anthocyanins in raspberry fruits and juices.

Processing red raspberry into jam reduces the content of quercetin 3-glucoside by 6% and kaempferol 3-glucoside by 20%, while storage of jam accelerates these losses. After 6 months of storage, 40 and 50% of quercetin and kaempferol 3-glucosides are lost in the jam, respectively (Zafrilla et al., 2000). Short-term storage (3 days at 4°C or 24 h at 18°C), mimicking the possible routes the fresh fruits may take after harvest before reaching the consumer's table, brings about only an increase in the level of ellagitannins in the fruit (ellagic acid—528%, lambertianin C—145% and sanguin H-6—118% of the values for fresh fruit) (Mullen et al., 2000b). Long-term frozen storage (365 days at -20°C) of raspberry fruits decreases the content of ellagic acid in the fruits by 14 to 21% (de Ancos et al., 2000). de Ancos et al. (2000) also noted that stability of anthocyanins during frozen storage is dependent on the harvesting time and observed a slight increase (5 to 17%) in the total anthocyanin content for early raspberry cultivars, but a slight decrease (4 to 17.5%) in total pigments for late raspberry cultivars. In addition, de Ancos et al. (2000) observed that cyanidin 3-glucoside was the least stable anthocyanin during longterm frozen storage of raspberry fruits. Cyanidin 3-glucoside is the most unstable and cyanidin 3-sophoroside the most stable pigment during fermentation of raspberry pulp; over 50% of total anthocyanins is lost after 2 months' storage of raspberry wine at 20°C (Rommel et al., 1990).

TABLE 4.6
Total Anthocyanin and Phenolics Content of
Juices from Various Cultivars of Raspberry
Fruits at Different Maturity Stages^a

| Cultivar | Maturity Stage | Total Anthocyanins ^b | Total Phenolics ^c |
|------------------------------|----------------|---------------------------------|------------------------------|
| Jewel (black raspberry) | Green | 17±6 | 3380±71 |
| | Pink | 228±54 | 1900±35 |
| | Ripe | 1972±35 | 2670±43 |
| Autumn Bliss (red raspberry) | Green | 16±5 | 1740±37 |
| | Pink | 64±12 | 1040±15 |
| | Ripe | 750±38 | 2450±61 |
| Summit (red raspberry) | Green | 22±6 | 2150±41 |
| | Pink | 159±12 | 1220±21 |
| | Ripe | 995±38 | 2580±56 |

^a Milligrams per kilogram of fresh weight.

^b Expressed as cyanidin 3-glucoside equivalents.

^c Expressed as gallic acid equivalents.

Source: Adapted from Wang, S.Y. and Lin, H.-S., 2000, *J. Agric. Food Chem.*, 48:140–146.

Strawberries

The total content of anthocyanins, the total flavonols and the total phenolics in strawberries grown in Finland ranges from 1840 to 2320 mg as cyanidin 3-glucoside equivalents/kg, 63 to 200 mg as rutin equivalents/kg and 16,000 to 24,100 mg as gallic acid equivalents/kg of fresh weight. On the other hand, the total ellagitaninns, total hydroxybenzoic acid and total hydroxycinnamic acid contents in these strawberries are 810 to 1840 mg of ellagic acid equivalents/kg, 110 to 500 mg of gallic acid equivalents/kg, and 470 to 630 mg of chlorogenic acid equivalents/kg of fresh weight, respectively (Kähkönen et al., 2001).

p-Coumarylglucose, quercetin 3-glucoside, quercetin 3-glucuronide, kaempferol 3-glucoside, kaempferol 3-glucuronide (Macheix et al., 1989), dihydroflavonol (Wang et al., 2002b) and ellagic acid (Maas et al., 1990) have been identified in strawberry fruits (Figure 4.2). Ellagic acid, quercetin and kaempferol derivatives are predominantly located in the external tissues, while a similar concentration of *p*-coumarylglucose has been found in the external and internal tissues of strawberry (Gil et al., 1997) (Table 4.7).

Several anthocyanins have been identified in strawberry fruits, namely, pelargonidin-3-glucoside, cyanidin-3-glucoside, and pelargonidin-3-rutinoside (Gil et al., 1997; Mazza and Miniati, 1993). In addition, Wang et al. (2002b) have detected the presence of pelargonidin-3-glucoside-succinate and cyanidin-3-glucoside-succinate.

TABLE 4.7
The Average Content of Selected Phenolics in
External and Internal Tissues of cv. Selva
Strawberry Fruit^a

| Phenolic | External Tissue | Internal Tissue |
|---------------------------------|-----------------|-----------------|
| Quercetin derivatives | 63.8±5.6 | 3.3±2.1 |
| Kaempferol derivatives | 21.8±2.3 | 3.3±0.6 |
| Ellagic acid | 33.3±3.6 | 8.4±1.1 |
| <i>p</i> -Coumarylglucose | 15.0±5.2 | 17.2±5.1 |
| Total anthocyanins ^b | 195.3±11.3 | 55.1±6.1 |

^a Milligrams per kilogram of fresh weight.

^b Expressed as cyanidin 3-glucoside equivalents.

Source: Adapted from Gil, M.I. et al., 1997, *J. Agric. Food Chem.*, 45:1662–1667.

Of these, pelargonidin-3-glucoside is the predominant anthocyanin in strawberry, comprising approximately 77% of total anthocyanin content in fruit (Gil et al., 1997; Mazza and Miniati, 1993; Wang et al., 2002b). However, pelargonidin-3-glucoside and cyanidin-3-glucoside contribute to the red color of strawberries (Timberlake and Bridle, 1982). Anthocyanins are mainly located in the external tissues of strawberry (Table 4.7) and cyanidin-3-glucoside is found only in the external berry tissues (Gil et al., 1997). Ripe strawberries contain about six times more anthocyanins than berries at their pink color stage (Wang and Lin, 2000).

Processing strawberries by cooking with sugar reduces the content of ellagic acid by 20% (Häkkinen et al., 2000b), and flavonols by 15 to 20% (Häkkinen et al., 2000a). On the other hand, postharvest storage of strawberry fruits for 24 h at 5°C increases their quercetin and kaempferol levels by 10 to 20% in comparison with berries stored for 24 h at 22°C. Prolonged frozen storage (9 months at –20°C) markedly increases the content of quercetin (by 35%), but has a detrimental effect on kaempferol, of which only traces are detected after 6 months of frozen storage. Similar changes in flavonols have been detected in strawberry jams stored up to 9 months at 5 and –20°C. The increase in quercetin level in frozen berries and strawberry jams has been associated with the degradation of cell wall structures (Häkkinen et al., 2000a). Storing strawberries in air increases the content of anthocyanins and flavonols in the external and internal tissues, while storage in CO₂-enriched atmospheres decreases the content of anthocyanins and flavonols, mostly in the internal berry tissues (Gil et al., 1997).

Other Berries

Berries of black chokeberry (*Aronia melanocarpa*) are a rich source of anthocyanins. Methanolic (MeOH +3% formic acid) extract from aronia berries contains 623 g/kg of dry extract of total anthocyanins. Cyanidin 3-galactoside (57%), cyanidin 3-arabinoside (29%), cyanidin 3-xyloside (7%), cyanidin 3-glycoside (4%) and cyanidin 3-glucoside (3%) are the major anthocyanins found in aronia extract. Small quantities of caffeic acid

derivatives (3.22 g/kg) and quercetin derivatives (0.82 g/kg) have also been detected (Espin et al., 2000).

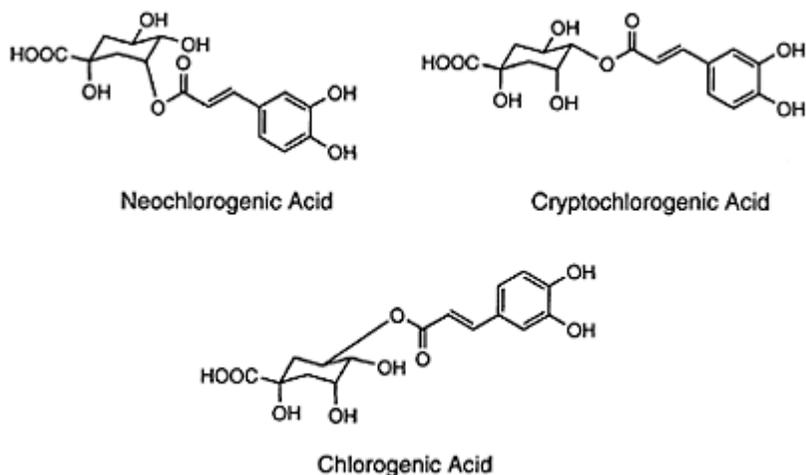


FIGURE 4.15 Chemical structures of chlorogenic acid isomers.

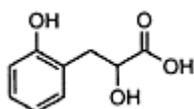
DRUPES

Cherries

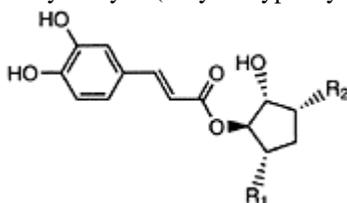
Sweet cherries (*Prunus avium* L.) and tart cherries (*Prunus cerasus* L.) are rich sources of flavonoids. Sweet cherries are grown commercially as table or maraschino cherries as well as ingredients of fruit cocktail (Mazza and Miniati, 1993). Schaller and Von Elbe (1970) identified six isomers of caffeoylquinic acid (Figure 4.15), four isomers of *p*-coumarylquinic acid and two free phenolic acids, in tart cherries cv. Montmorency. Möller and Herrmann (1983) reported that neochlorogenic acid and 3'-*p*-coumarylquinic acid are the major hydroxycinnamoylquinic acids in sweet cherries. Later, Gao and Mazza (1995) confirmed this finding and reported that the content of neochlorogenic acid in 11 cultivars and hybrids of sweet cherries ranges from 240 to 1280 mg/kg, while *p*-coumarylquinic acid ranges from 230 to 1310mg/kg of pitted cherries. Subsequently, Wang et al. (1999a) identified three new phenolic compounds: 2-hydroxy-3-(*o*-hydroxyphenyl) propanoic acid, 1-(3',4'-dihydroxycinnamoyl)-cyclopenta-2,5-diol and 1-(3',4'-dihydroxycinnamoyl)-cyclopenta-2,3-diol (Figure 4.16), as well as chlorogenic acid methyl ester in ethyl acetate extracts of cv. Balaton cherries. The antioxidant activities of these phenolics are similar to those exhibited by commercial antioxidants such as TBHQ and BHT (Wang et al., 1999a).

The total content of anthocyanins in pitted dark cherries ranges from 82 to 298 mg/kg and in light-colored pitted cherries from a few to 41 mg/kg (Gao and Mazza, 1995). Tart cherries cv. Balaton (235.9 mg/kg) contain three times more anthocyanin than does tart

cherry cv. Montmorency (75.3 mg/kg) (Wang et al., 1997). The presence of cyanidin 3-rutinoside and cyanidin 3-glucoside in sweet cherries was first detected by Willstätter and Zollinger (1916) and then confirmed by Robinson and Robinson (1931). Harborne and Hall (1964) detected cyanidin 3-glucosylrutinoside in six out of seven tart cherry varieties. Later, peonidin and its two glycosidic derivatives were also found in cv. Bing cherries (Lynn and Luh, 1964), while Okombi (1979) identified peonidin 3-rutinoside as the dominant anthocyanin in Bigarreau Napoléon. Furthermore, peonidin 3-rutinoside, peonidin, cyanidin, cyanidin 3-sophoroside, cyanidin 3-rutinoside and cyanidin 3-glucoside were found in tart cherry cv. Montmorency (Figure 4.5) (Chandra et al., 1992; Dekazos, 1970; Schaller and Von Elbe, 1968). Subsequently, Gao and Mazza (1995) reported that cyanidin 3-glucoside and cyanidin 3-rutinoside are the main anthocyanins, while peonidin 3-glucoside, peonidin 3-rutinoside and pelargonidin 3-rutinoside are the minor anthocyanins in dark sweet cherries. In addition, tart cherries contain cyanidin 3-glucosylrutinoside, cyanidin 3-rutinoside, and cyanidin 3-glucoside (Chandra et al., 2001; Wang et al., 1997).



2-Hydroxy-3-(*o*-hydroxyphenyl)-propanoic Acid



| Compound | R ₁ | R ₂ |
|--|----------------|----------------|
| 1-(3',4'-Dihydroxycinnamoyl)-cyclopenta-2,5-diol | OH | H |
| 1-(3',4'-Dihydroxycinnamoyl)-cyclopenta-2,3-diol | H | OH |

FIGURE 4.16 Chemical structures of some phenolic compounds isolated from sweet cherries.

The presence of kaempferol 3-glucoside and kaempferol 3-rutinoside in Montmorency cherries was reported by Schaller and Von Elbe (1970). Later, Shrikhande and Francis (1973) identified several more quercetin and kaempferol derivatives, namely, kaempferol-3-rhamnosylglucoside, quercetin-3-rhamnosylglucoside, quercetin-3-glucoside, quercetin-4'-glucoside, and tentatively detected kaempferol-3-rhamnoside-4'-glucoside, and kaempferol-4'-glucoside in sour cherries (*P. cerasus* L. cv. Montmorency). A number of flavonoids have also been detected in the bark of cherry trees (*P. cerasus*) (Geibel, 1995; Geibel and Feucht, 1991; Geibel et al., 1990):

- Pinostrobin
- Naringenin
- Prunin

- Sakuranetin
- Chrysin
- Sakuranin
- Naringenin
- Prunetin
- Genistein
- Tectochrysin
- Tectochrysin 5-glucoside
- Genistein 5-glucoside
- Prunetin 5-glucoside
- Dihydrowogonin 7-glucoside

Recently, Wang et al. (1999b) identified seven flavonoid compounds in Montmorency and Balaton tart cherries, namely, naringenin, genistein, quercetin 3-rhamnoside, rhamnasin, genistein 7-glucoside, kaempferol 6''-O-L-rhamnopyranosyl- β -D-glucopyranoside, and 6,7-dimethoxy-5,8,4'-trihydroxyflavone. Of these, 6,7-dimethoxy-5,8,4'-trihydroxyflavone and quercetin 3-rhamnoside are the most active antioxidants at 10- μ M concentrations.

Nectarines and Peaches

Hydroxycinnamic acid derivatives, anthocyanins, flavonols and flavan-3-ols are the most prevalent phenolics in peaches and nectarines (Bengoechea et al., 1997; Cheng and Crisosto, 1995; Henning and Herrmann, 1980b; Hernandez, 1997; Luh et al., 1967; Mosel and Herrmann, 1974b; Risch and Herrmann, 1988b; Senter and Callaghan, 1990; Senter et al., 1989; Talcott et al., 2000a; Tomás-Barberán et al., 2001). Nectarines and peaches exhibit similar phenolic profiles (Tomás-Barberán et al., 2001). The content of total phenolics in whole peaches ranges from 213 to 1800 mg/kg of fresh weight (Carbonaro et al., 2002; Chang et al., 2000; Imeh and Khokhar, 2002; Van Buren, 1970). In addition, the total phenolics content in clingstone peach flesh is between 432.9 and 768 mg of gallic acid equivalents/kg, but varies in peach peels from 910.9 to 1922.9 mg of gallic acid equivalents/kg (Chang et al., 2000).

Chlorogenic acids, caffeic acid, catechin and procyanidin B3 (catechin-(4 β →8)-catechin) are the major phenolics in peaches (Figure 4.1, Figure 4.3, Figure 4.4, and Figure 4.15) (Lee et al., 1990). More recently, Tomás-Barberán et al. (2001) found that chlorogenic and neochlorogenic acids are the main hydroxycinnamic acid derivatives present, while procyanidin B1 (epicatechin-(4 β →8)-catechin), catechin and epicatechin are the predominant flavan-3-ols in peaches and nectarines. Higher levels of hydroxycinnamic acids, flavan-3-ols, and flavonols have been found in peach peel compared to peach flesh (Chang et al., 2000; Tomás-Barberán et al., 2001) (Table 4.8).

Anthocyanins are predominantly located in the skin of peaches and nectarines (Chang et al., 2000; Tomás-Barberán et al., 2001) (Table 4.8). Small quantities of pigments may also be found in tissues near the stone. Cyanidin 3-glucoside and cyanidin 3-rutinoside are the main pigments in nectarines and peaches (Figure 4.5) (Hsia et al., 1965; Mazza and Miniati, 1993; Tomás-Barberán et al., 2001); some cultivars may also contain cyanidin 3-acetylglucoside and cyanidin 3-galactoside (Tomás-Barberán et al., 2001). Quercetin 3-glucoside and quercetin 3-rutinoside are the major flavonols in nectarines

and peaches and are mainly located in the skin (Table 4.19) (Tomás-Barberán et al., 2001).

TABLE 4.8
Ranges of Phenolic Contents in the Peel and
Flesh of Five Cultivars of Ripe White and
Yellow Flesh Nectarines and Peaches^a

| Phenolic Compounds | White Flesh Cultivars | | | | Yellow Flesh Cultivars | | | |
|------------------------------------|-----------------------|-------|----------|--------|------------------------|--------|---------|-------|
| | Nectarines | | Peaches | | Nectarines | | Peaches | |
| | Peel | Flesh | Peel | Flesh | Peel | Flesh | Peel | Flesh |
| Hydroxycinnamic acids ^b | 77.7– | 50.5– | 76.5– | 76.5– | 137.7– | 66.1– | 153.5– | 59.2– |
| | 727.1 | 460.1 | 498.5 | 321.8 | 429.6 | 186.8 | 347.4 | 170.8 |
| Flavan-3-ols ^c | 133.6– | 23.0– | 296.5– | 117.1– | 128.0– | 30.2– | 225.4– | 93.2– |
| | 1106 | 443.7 | 1166 | 695.8 | 621.2 | 214.6 | 663.0 | 357.0 |
| Flavonols ^d | 0–10.2 | 32.0– | 6.1–47.7 | 0–11.4 | 49.3– | 6.9– | 34.8– | 13.8– |
| | | 73.6 | | | 91.1 | 21.1 | 74.1 | 19.3 |
| Anthocyanins ^e | 99.0– | 0–6.7 | 54.4– | 0–17.6 | 33.5– | 0–31.4 | 85.6– | 0–8.7 |
| | 172.7 | | 142.6 | | 260.9 | | 273.6 | |

^a Milligrams per kilogram of fresh weight.

^b Expressed as chlorogenic acid equivalents.

^c Expressed as catechin equivalents.

^d Expressed as quercetin 3-rutinoside.

^e Expressed as cyanidin 3-rutinoside.

Source: Adapted from Tomás-Barberán, F.A. et al., 2001, *J. Agric. Food Chem.*, 49:4748–4760.

Manufacturing fruit juices consists of processing fruits to more stable products such as concentrates and purees and reconstituting to juices by adding water. Production of purees and concentrates may include removing stones, chemical peeling, enzymatic treatment, and pressing. Thus, juices made from peeled fruits may differ in their phenolic composition from those made from whole fruits. Chlorogenic acid, caffeic acid, protocatechuic acid, catechin, and procyanidin B2 (epicatechin(4 β →8)-epicatechin), B3 (catechin-(4 β →8)-catechin) and B4 (catechin-(4 β →8)-epicatechin) have been found in peach puree (Bengoechea et al., 1997).

Plums and Prunes

Plums include fruits of *Prunus domestica*, *Prunus salicina*, *Prunus spinosa*, *Prunus subcordiata*, and *Prunus insititia*; prune is the dried fruit of some cultivars of *P. domestica* (Pijpers et al., 1986). Phenolic compounds detected in plums include phenolic acid derivatives, coumarins, flavan-3-ols and anthocyanins (Henning and Herrmann, 1980a; Herrmann, 1989; Macheix et al., 1989; Stöhr et al., 1975). Of these, HCA derivatives are the major phenolics, comprising 84 to 90% of total phenolics (Donovan et al., 1998; Herrmann, 1989). Recently, Fang et al. (2002) identified over 30 HCAs in prunes. Of these, six are novel HCA isomers and contain two caffeic acids and quinic

acid in their structures; however, the molecular structures of these compounds are not fully elucidated.

Chlorogenic acids (Figure 4.15) are the major phenolic acid derivatives in plums and prunes. Of these, neochlorogenic acid (3-*O*-caffeoylquinic acid, 3-CQA) is the major Chlorogenic acid in plums and prunes (Donavan et al., 1998; Fang et al., 2002; Herrmann, 1989; Möller and Herrmann, 1983; Nakatani et al., 2000; Raynal et al., 1989; Tomás-Barberán et al., 2001). Möller and Herrmann (1983) detected 88 to 731 mg/kg of 3-CQA, 15 to 129 mg/kg of Chlorogenic acid (5-*O*-caffeoylquinic acid, 5-CQA) and 56 mg/kg of cryptochlorogenic acid (4-*O*-caffeoylquinic acid, 4-CQA) in fresh plums. Following this, Raynal et al. (1989) reported that 3-CQA comprises approximately 50% of total phenolics in exocarp and pulp of fresh plums. Later, Donovan et al. (1998) found 807 and 1306 mg/kg of 3-CQA and 144 and 436 mg/kg of 5-CQA in fresh prune-making plums and pitted prunes, respectively. Subsequently, Nakatani et al. (2000) detected 3-CQA (1228 to 1485 mg/kg), 4-CQA (288 to 351 mg/kg) and 5-CQA (53 to 77 mg/kg) in prunes. The contents of neochlorogenic and chlorogenic acids are 2.4 and 12.4 times higher in exocarp than in the pulp (Raynal et al., 1989).

Recently, Tomás-Barberán et al. (2001) have also reported that hydroxycinnamic acid derivatives are mainly located in the plum peel (Table 4.9). The total HCA content in the peel and the flesh of plums ranges from 115 to 375 mg/kg and from 16.3 to 194 mg/kg of sample, respectively. In addition, a number of minor phenolic acids and their glycosides have been identified in prunes: gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, methoxybenzoic, caffeic, syringic, methoxycinnamic, *p*-coumaric and ferulic acids (Figure 4.1) (Fang et al., 2002; Kayano et al., 2002). Drying plums brings about degradation of HCA linked to the polyphenoloxidase activity in the fruit because more HCAs are degraded when plums are dried at a lower drying temperature. Furthermore, HCAs present in the exocarp disappear more rapidly than those found in the flesh of the fruit and neochlorogenic acid degrades more rapidly during drying than other chlorogenic acid isomers (Raynal et al., 1989).

TABLE 4.9
Content of Hydroxycinnamic Acid Derivatives
and Flavan-3-ols in the Peel and Flesh of Some
Plum Cultivars^a

| Cultivar | Part | 3-CQA ^b | Total HCA ^c | A-type | Procyanidin dimers ^d | | | Total Flavan-3-ols |
|--------------|-------|--------------------|------------------------|--------|---------------------------------|--------|--------|--------------------|
| | | | | | B1 | B2 | B4 | |
| Angeleno | Peel | 40±11 | 115±15 | 477±66 | 81±15 | 340±70 | 52±29 | 1151±208 |
| | Flesh | 17±4 | 17±4 | ND | 31±15 | 120±42 | 154±55 | 377±126 |
| Black Beauty | Peel | 225±0.3 | 282±2 | 257±10 | 292±4 | 137±3 | 668±24 | 1650±20 |
| | Flesh | 103±3 | 113±3 | 103±9 | 136±24 | 88±5 | 137±5 | 566±51 |
| Red Beauty | Peel | 326±20 | 375±25 | 75±10 | 268±62 | 72±19 | 253±52 | 847±171 |
| | Flesh | 184±6 | 194±6 | 37±3 | 41±11 | 41±3 | 42±2 | 182±15 |

| | | | | | | | | |
|---------|-------|-------|--------|------|--------|----|--------|---------|
| Wickson | Peel | 135±7 | 181±11 | 78±6 | 540±13 | nd | 485±28 | 1534±54 |
| | Flesh | 87±4 | 90±4 | ND | 110±17 | nd | 46±6 | 156±23 |

Note: ND=not detected.

^a Milligrams per kilogram of fresh weight.

^b Neochlorogenic acid expressed as chlorogenic acid.

^c Hydroxycinnamic acid derivatives expressed as chlorogenic acid.

^d Expressed as catechin.

Source: Adapted from Tomás-Barberán, F.A. et al., 2001, *J. Agric. Food Chem.*, 49:4748–4760.

The anthocyanins in the ripe fruit of *P. domestica* L. are mainly located in the skin and the outer cells of the flesh (Mazza and Miniati, 1993). The total content of anthocyanins in the peel and the flesh of ripe plums is 129 to 1614 and 0 to 28.4 mg/kg of fresh weight, respectively (Tomás-Barberán et al., 2001). Presence of four anthocyanins, namely, 3-glucosides and 3-rutinosides of cyanidin and peonidin, has been detected in Victoria plums and in sloe (*P. spinosa*) (Figure 4.5) (Van Buren, 1970). Presence of the same anthocyanins in plums was reported by Macheix et al. (1989) and Raynal et al. (1989). Later, Hong and Wrolstad (1990) detected seven anthocyanins in a plum juice concentrate, but fully characterized only two of them: cyanidin 3-glucoside and cyanidin 3-rutinoside. Subsequently, Tomás-Barberán et al. (2001) detected 3-glucoside, 3-rutinoside, 3-galactoside and 3-acetylglucoside of cyanidin, but failed to find peonidin derivatives in plums. Of these, cyanidin 3-glucoside and cyanidin 3-rutinoside are the major anthocyanins in plums.

Anthocyanins degrade rapidly during drying of plums. For example, Raynal et al. (1989) detected only 15% cyanidin 3-rutinoside and 17% peonidin 3-rutinoside in plums dried at 95°C for 1 h. The rate of degradation of anthocyanins increased as the drying temperature increases. No anthocyanins have been found in prunes and prune juices (Fang et al., 2002; Raynal and Moutounet, 1989; Van Gorsel et al., 1992). It has been suggested that loss of anthocyanins involves enzymatic degradation attributed to the presence of quinones and thermal degradation linked with the increase in chalcones (Raynal and Moutounet, 1989).

The total content of flavonol glycosides (Figure 4.3) in a ripe plum ranges from 186 to 352 mg of rutin equivalents/kg of fresh weight (Tomás-Barberán et al., 2001). The fruit of *P. domestica* and *P. salicina* contains 3-arabinoside-7-rhamnosides, 3-rutinosides, 3-glucosides, and 3-galactosides of quercetin and kaempferol, as well as quercetin 3-xyloside. On the other hand, quercetin glycosides have only been detected in the fruit of *P. spinosa* (Macheix et al., 1989). Rutin (quercetin 3-rutinoside) is the predominant flavonol glycoside in plums and prunes (Fang et al., 2002; Henning and Herrmann, 1980a, b; Raynal et al., 1989; Starke and Herrmann, 1976) and is only found in the exocarp (Raynal et al., 1989).

According to Mosel and Herrmann (1974b), plums contain only 20 to 40 mg of flavan-3-ols/kg of fresh weight. Recently, Tomás-Barberán et al. (2001) have reported that the total content of flavan-3-ols in plums ranges from 662 to 1837 and from 138 to 618 mg of catechin equivalents/kg of fresh weight of peel and fruit flesh, respectively. A similar content of flavan-3-ols has been reported by de Pascual-Teresa et al. (2000). No detectable levels of flavan-3-ols have been found in prunes, however (Fang et al., 2002). The discrepancies in the reported values may be due to the differences in the methodologies applied for their quantitation. The major flavan-3-ols identified in plums

are procyanidins B1, B2, B4 as well as A-type dimers (Figure 4.4 and Figure 4.10) (Tomás-Barberán et al., 2001).

POMES

Apples

The content of phenolics in apples is affected by variety, maturity, harvesting season, processing, cultural conditions, crop load, development of infection, fruit position within the canopy, and geographic origin (Awad et al., 2000a, b, 2001; Burda et al., 1990; Escarpa and González, 1998; Ingle and Hyde, 1968; Lattanzio et al., 2001; Lea and Beech, 1978; Lea and Timberlake, 1978; Macheix et al., 1989; Mangas et al., 1999; Mayr et al., 1995; McRae et al., 1990; Mosel and Herrmann, 1974a; Pinicelli et al., 1997; Spanos et al., 1990; Stopar et al., 2002; van der Sluis et al., 2001). Hydroxycinnamic acid derivatives, flavan-3-ols (monomeric and oligomeric), flavonols and their conjugates, and dihydrochalcones are the major phenolics in apples (Table 4.10) (Dick et al., 1987; Duggan, 1969c; Foo and Lu, 1999; Guyot et al., 1997, 1998, 2001; Lea, 1978, 1984, 1990; Lea and Arnold, 1978; Lea and Timberlake, 1974; Lister et al., 1994; Lu and Foo, 1997; McRae et al., 1990; Nicolas et al., 1994; Ohnishi-Kameyama et al., 1997; Oleszek et al., 1988; Pérez-Ilzarbe et al., 1991, 1992; Schmidt and Neukom, 1969; Spanos and Wrolstad, 1992; Tomás-Barberán et al., 1993; Van Buren, 1970; Van Buren et al., 1976; Vallés et al., 1994; Whiting and Coggins, 1975; Wilson, 1981). In addition, anthocyanins are found in the skin of some red apple varieties (Alonso-Salces et al., 2001; Lancaster, 1992; Soji et al., 1999; Sun and Francis, 1967; Timberlake and Bridle, 1971; van der Sluis et al., 2001), but not in the vacuoles of epidermal and subepidermal cells (Strack and Wray, 1994). Of these, only procyanidins are able to bind to cell wall matrix (Renard et al., 2001).

Phenolics are involved in the defense mechanism in apple against fungal pathogens such as *Venturia* sp., *Gloeosporium* sp., *Sclerotinia fructigena*, and *Botrytis cinerea*. Infection of apple tissue brings about an increase in polyphenol oxidase activity leading to acceleration of polyphenol oxidation. Thus, the oxidation products of polyphenols play an important role in apple tissues' resistance to pathogens (Feucht et al., 1992; Ju et al., 1996; Lattanzio et al., 1994, 2001; Nicholson and Hammerschmidt, 1992; Noveroske et al., 1964a, b; Oszmianski and Lee, 1991; Piano et al., 1997; Prusky, 1996; Raa and Overeem, 1968). Recently, Whitaker et al. (2001) identified two novel fatty acid esters of phenolic alcohols in epicuticular wax of apple fruit as *E* and *Z* isomers of *p*-coumaryl alcohol.

The total content of phenolics in epidermis, parenchyma, core, and seeds of apple is above 5000 mg/kg (Burda et al., 1990; Golding et al., 2001; Guyot et al., 1998), but most phenolics found in the entire apple are located in the parenchyma (64.9%) (Table 4.11) (Guyot et al., 1998). The total content of phenolics in most apple varieties is between 1000 and 6000 mg/kg of fresh weight, but in some apple cultivars may be 10,000+ mg/kg of fresh weight (Table 4.10) (Gorinstein et al., 2001; Herrmann, 1973; **Podszędek** et al., 2000; Sanoner et al., 1999; Van Buren, 1970); in the apple peel total phenolics range from 1275 (Granny Smith) to 4036

TABLE 4.10
Content of Phenolic Compounds in Cortex of
Some Apple Varieties^a

| Variety | Total Phenolics ^b | Total Catechins ^b | Procyanidins ^b | Chlorogenic Acid ^c | p-Coumaric Acid ^c | Total Dihydrochalcones ^d |
|------------------|------------------------------|------------------------------|---------------------------|-------------------------------|------------------------------|-------------------------------------|
| Golden Delicious | 1280 | 102 | 761 | 132 | 20 | 26 |
| Judor | 1100 | 106 | 515 | 338 | 59 | 26 |
| Guillevic | 1740 | Traces | 1066 | 465 | 134 | 38 |
| Petit Jaune | 2220 | 177 | 1372 | 415 | 29 | 28 |
| Binet Rouge | 2500 | 241 | 1254 | 601 | 176 | 51 |
| Juliana | 2250 | 154 | 1551 | 522 | 72 | 122 |
| Clozette | 2400 | 351 | 1144 | 832 | 84 | 40 |
| Avrolles | 2560 | Traces | 1528 | 154 | 104 | 80 |
| Dous Moen | 3130 | 418 | 1902 | 967 | 100 | 39 |
| Antoinette | 3350 | 466 | 1796 | 641 | 36 | 55 |
| Bedan | 3210 | 627 | 2417 | 649 | 147 | 42 |
| Jeanne Renard | 6000 | 1464 | 4731 | 666 | 50 | 82 |

^a Milligrams per kilogram of fresh weight.

^b Catechin+epicatechin; expressed as (-)epicatechin equivalents.

^c Assayed according to their own response factors.

^d Phlorizidin+phloretin xyloglucoside; expressed as phlorizidin equivalents.

Source: Adapted from Sanoner, P. et al, 1999, *J. Agric. Food Chem.*, 7:4847-4853.

TABLE 4.11
Distribution of Each Class Phenolic Compounds
among Various Tissues of the Kermerrien Cider
Apple Fruit^a

| | Epidermis Tissue (%) | Parenchyma Tissue (%) | Core (%) | Seeds (%) | Total in Apple Fruit (mg/kg fresh weight) | |
|------------------------------------|----------------------|-----------------------|----------|-----------|---|------|
| Procyanidins | | 27.6 | 65.0 | 7.1 | 0.3 | 3236 |
| Catechins ^b | | 17.8 | 72.1 | 9.9 | 0.01 | 739 |
| Flavonols ^c | | 100.0 | ND | ND | ND | 23 |
| Dihydrochalcones ^d | | 14.2 | 27.9 | 39.6 | 17.8 | 169 |
| Hydroxycinnamic acids ^e | | 14.7 | 67.5 | 17.4 | 0.4 | 837 |

| | | | | | |
|-----------------|------|------|------|-----|------|
| Total phenolics | 23.8 | 64.9 | 10.3 | 1.0 | 5004 |
|-----------------|------|------|------|-----|------|

Note: ND=not detected.

^a Percent of total phenols in apple fruit.

^b Expressed as catechin equivalents.

^c Expressed as hyperoside equivalents.

^d Expressed as phloridzin equivalents.

^e Expressed as chlorogenic acid equivalents.

Source: Adapted from Guyot, S. et al., 1998, *J. Agric. Food Chem.*, 46:1698–1705.

(Lady Williams) mg/kg of fresh weight (Golding et al., 2001). Total content of phenolics is also influenced by crop load of the apple tree. Decrease in crop load for var. Jonagold (*Malus x domestica* Borkh.) from 157 to 30 fruits per crown increased the total content phenolics from 1300 to 1680 mg/kg of fresh weight (cortex+skin) (Stopar et al., 2002). On the other hand, the total content of phenolics in 46 samples of juices made of Spanish cider apple juice cultivars varied from 750 to 2420 (harvest 1995) and from 570 to 20,600 mg/L (harvest 1994) of tannic acid equivalents (Mangas et al., 1999). Furthermore, the level of phenolic compounds varies among individual apples of the same variety. A 10 to 30% variation in the content of chlorogenic acid, phlorizidin, epicatechin and quercetin glycosides between individual Jonagold apples has been detected by van der Sluis et al. (2001).

Hydroxycinnamic acids (HCAs; Figure 4.1) are important substrates for polyphenol oxidase. Their oxidation and condensation products not only contribute to the formation of yellow-brown pigments in apple fruits but may also enhance the oxidation of other phenolics such as flavan-3-ols by coupled mechanism (Amiot et al., 1992, 1993; Brugirard and Tavernier, 1952; Nicolas et al., 1994; Oszmianski and Lee, 1990). On the other hand, chlorogenic acid (Figure 4.15) in combination with phlorizidin (Figure 4.17) and polyphenol oxidase may contribute to quiescence (limited infections with no lesion produced) (Prusky, 1996) of the *Phlyctaena vagabunda* while the fruit is on the tree (Lattanzio et al., 2001). In addition, microbial decomposition of HCAs by bacteria of genus *Lactobacillus* and yeasts of genera *Saccharomyces* and *Brattanomyces* may lead to the formation of volatile phenols,

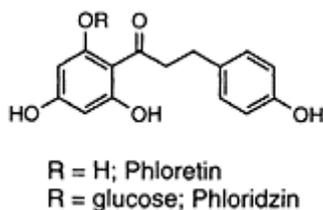


FIGURE 4.17 Chemical structures of dihydrochalcones found in apple fruits.

accumulation of which above their flavor thresholds is responsible for the development of off-flavor in cider juice (Beech and Carr, 1977; Chatonnet et al., 1992, 1993).

HCAs are mainly located in the parenchyma zone of the fruit (Table 4.11). Chlorogenic acid is the major HCA found in the apple fruit and accounts for 79, 76, 79,

and 87% of total HCA in epidermis, parenchyma, core, and seeds, respectively (Guyot et al., 1998). Other isomers of chlorogenic acid and *p*-coumarylquinic acid isomers, as well as *p*-coumaric, ferulic, and caffeic acids, have also been detected in apples (Bengoechea et al., 1997; Durkee and Poapst, 1965; Nicolas et al., 1994). Podszdek et al. (2000) recently reported that the content of chlorogenic acid (CA) in 10 Polish apple varieties ranged from 30 to 430 mg/kg of fresh weight in eight apple varieties grown in the U.S. and varied from 1.5 to 228 mg/kg of fresh weight (Coseteng and Lee, 1987; Lee and Wrolstad, 1988); in 14 French apple varieties (12 cider and 2 juice) it was between 132 and 967 mg/kg of fresh weight (Table 4.10) (Sanoner et al., 1999). Apple purées and concentrates contain 25.8 to 68.2 and 38.9 to 81.3 mg/L of CA (Bengoechea et al., 1997), respectively, while the concentration of CA in juices pressed from 46 apple juice varieties ranges from 21.2 to 350.5 (harvest 1995) and 25.1 to 377.1 (harvest 1994) mg/L (Mangas et al., 1999).

The presence of several anthocyanins, namely, cyanidin 3-galactoside (ideain), cyanidin-3-rutinoside, malvidin-3-glucoside, and malvidin-3,5-diglucoside, has been confirmed in Starking Delicious apple juice. Of these, cyanidin-3-galactoside (ideain) is the predominant anthocyanin in the apple juice and peel (Alonso-Salces et al., 2001; Shoji et al., 1999). Fermentation of Starking Delicious apple juice markedly decreases the concentration of ideain, but induces the formation of anthocyanin polymers. Further, a decrease in cyanidin-3-galactoside content has been noted during maturation of wine (Shoji et al., 1999).

Flavonol glycosides are quantitatively considered to be a minor class of phenolics and are found predominantly in the epidermis tissue of apple fruits (Table 4.11) (Guyot et al., 1998; Lister et al., 1994; Pérez-Illarbe et al., 1991; Price et al., 1999; van der Sluis et al., 2001). These phenolics may be involved in the regulation of apple texture as β -galactosidase inhibitors (Dick and Smith, 1990; Dick et al., 1985; Lidster and McRae, 1985; Lidster et al., 1986). Several quercetin glycosides have been identified in apple fruit: rutin, hyperin (quercetin-3- β -D-galactoside), isoquercitrin (quercetin-3- β -D-glucoside), reynoutrin (quercetin-3- β -D-xyloside), avicularin (quercetin-3- α -L-arabinofuranoside), and quercitrin (quercetin-3- α -L-rhamnoside) (Figure 4.2) (Burda et al., 1990; Coseteng and Lee, 1987; Dick et al., 1987; Golding et al., 2001; Guyot et al., 1998; Lommen et al., 2000; Oleszek et al., 1988; van der Sluis et al., 2001). In addition, Lommen et al. (2000) have detected one quercetin glycoside in apple peel and tentatively identified it as quercetin 3- β -L-arabinopyranoside or 3- β -L-apiofuranoside. More precise identification of this flavonol glycoside is still needed.

Guyot et al. (1998) detected 129 mg of total flavonols/kg of peel, but only 23 mg/kg of fresh weight of the entire Kermerrier cider apple. Subsequently, Lommen et al. (2000) reported that the content of individual flavonol glycosides in apple peel ranges from 200 to 500 mg/kg of peel. Later, van der Sluis et al. (2001) found 60 to 98 mg of total flavonol glycosides/kg of apple (fresh weight), while the level of individual flavonol glycosides in Jonagold apple peel was between 20 (isoquercitrin) to 159 (avicularin) mg/kg of peel. Moreover, Alonso-Salces et al. (2001) reported that flavonol glycosides are one of the main classes of phenolics in the peel of 14 Basque cider apple varieties, comprising 30 to 40% of total polyphenols in the peel. Significant levels of flavonol glycosides have also been found in apple juices produced by diffusion extraction technology (Spanos et al., 1990).

Phloridzin (phloretin 2'- β -D-glucoside) and phloretin 2'- β -D-xylosyl-(1 \rightarrow 6)- β -D-glucoside are the major dihydrochalcones found in apple fruits (Figure 4.17) (Durkee and Poapst, 1965; Lommen et al., 2000). Alonso-Salces et al. (2001) have reported the presence of two additional dihydrochalcones in the apple peel; however, they have not attempted to identify the compounds involved. The orange oxidation products of dihydrochalcones contribute to the color of apple and cider juices (Lea, 1984; Oszmianski and Lee, 1991). Dihydrochalcones are distributed in the entire fruit (Table 4.11); however, in the seeds (3416 mg/kg of seed) they comprise about 66% of total phenols, while in the parenchyma and epidermis tissues they comprise less than 3% of total phenols in each tissue (Guyot et al., 1998).

On the other hand, Alonso-Salces et al. (2001) have reported that dihydrochalcones constitute 17 to 57% and 4 to 13% of total phenolics in the peel and pulp of 15 Basque apple varieties, respectively. The total content of dihydrochalcones in apple cortex ranges from 26 to 122 mg of phloridzin equivalents/kg of fresh weight (Table 4.10) (Guyot et al., 1998; McRae et al., 1990; van der Sluis et al., 2001); in the peel it is between 60 and 500 mg/kg (Burda et al., 1990; Guyot et al., 1998; Lommen et al., 2000). In addition, industrially manufactured apple purées contain 10.4 to 29.0 mg/L of phloridzin, 3 to 7.4 mg/L of phloretin 2'-xyloglucose, and 0.8 to 2.5 mg/L of unknown phloretin derivatives (Bengoechea et al., 1997).

The only catechins detected in apples are (+)catechin and (-)epicatechin (Figure 4.3). Catechins are prone to enzymatic oxidation and together with chlorogenic acid contribute to the development of brown pigments in damaged apples (Goupy et al., 1995; Macheix et al., 1990; Nicolas et al. 1994). The content of catechins is high during early stages of apple fruit development and then drops drastically as the fruit size increases (Mosel and Herrmann, 1974a). Catechins account for 6 to 21% of total phenolics in the cortex of ripened fruits (Table 4.10) (Sanoner et al., 1999). The content of catechin in the cortex of fruit apple ranges from 0 to 154 mg/kg of fresh weight, while epicatechin is present between 0 and 1410 mg/kg of fresh weight (Coseteng and Lee, 1987; Mangas et al., 1999; **Podszędek** et al., 2000; Sanoner et al., 1999; van der Sluis et al., 2001). Moreover, depending on variety, the apple skin may contain up to 10 times more epicatechins than the flesh (Burda et al., 1990).

Procyanidins are found in the entire apple fruit, but their concentration gradually increases from 1232 mg/kg in the seeds to 4964 mg/kg in the epidermal tissue (Guyot et al., 1998). The occurrence of high levels of procyanidins in the epidermis tissue may be due to their protective role against fungi (Feucht et al., 1992). Procyanidins detected in the epidermis and parenchyma tissues account for 65 and 74.5% of the total phenolics in each tissue, respectively. However, globally, the procyanidins found in the parenchyma tissue comprise 65% of the total procyanidins in the entire fruit (Table 4.11) (Guyot et al., 1998). Apple procyanidins are a mixture of oligomers and polymers made of (-)epicatechin and (+)catechin as constitutive units (Figure 4.8) (Lea, 1990). These phenolic compounds are responsible for the bitterness and astringency of cider juice, and partly for the formation of haze in apple juice (Beveridge and Tait, 1992; Beveridge et al., 1997; Lea, 1990; Lea and Arnold, 1978). Lea and Arnold (1978) related the bitterness and astringency of English ciders to the degree of procyanidin polymerization. Low molecular weight procyanidins (up to tetramers) contribute to the bitterness of cider apples; high molecular weight procyanidins affect astringency. In addition, the balance

between astringency and bitterness defines the “mouthfeel” and body of cider apples (Lea, 1990).

Lea and Timberlake (1974) isolated three dimeric procyanidins, B1, B2 and B5 (Figure 4.4), and two unknown trimers in English ciders. Following this, Lea (1978) identified one of the procyanidin trimers as C1 (Figure 4.10) and also detected the presence of more polymerized procyanidins with at least seven-fold degree of polymerization. Subsequently, Vallés et al. (1994) detected two dimeric procyanidins, B1 and B2, one trimer, C1, one tetramer and one unknown procyanidin in juices made from Spanish apple varieties. Bengoechea et al. (1997) identified only two dimeric procyanidins, B1 and B2, in apple purées, but not in apple concentrates.

Later, Guyot et al. (1998) reported that high molecular weight procyanidins represent over 26% of total procyanidins in the apple fruit and those with the highest average degree of polymerization (11.2) are located in the seeds. On the other hand, the average degree of polymerization in apple tissues ranges from 4.4 for procyanidins in core to 6.0 for procyanidins in the epidermis. Subsequently, Sanoner et al. (1999) detected more polymerized procyanidins in the cortex of French apple varieties with the average degree of polymerization varying from 4.5 (var. Judor) to 50.3 (var. Avrolles). Later, Guyot et al. (2001) reported the presence of procyanidins with an average degree of polymerization of up to 190 in the cortex of two cider apple varieties (*Malus domestica*; Kermerrien and Avrolles). These authors reported that only oligomeric procyanidin fractions with average degrees of polymerization of 2 to 8 are eluted using normal-phase HPLC, while more polymeric procyanidin fractions with average degree of polymerization ranging from 7 to 190 are isolated on reversed-phase HPLC.

Pears

The phenolic constituents of pears are hydroxycinnamic acids, arbutin (4-hydroxyphenyl- β -D-glucopyranoside), flavonols, catechins and procyanidins. The average concentration of phenolic compounds in Portuguese pear is 3.7 g/kg of fresh pulp (Ferreira et al., 2002). Presence of chlorogenic acid isomers, *p*-coumarylquinic and dicaffeoylquinic acids in pears has been reported by several researchers (Amiot et al., 1992; Billot et al., 1978; Cartwright et al., 1955; Challice and Williams, 1972; Hulme, 1958; Mosel and Herrmann, 1974a; Sioud and Luh, 1966; Spanos and Wrolstad, 1992; Wald et al., 1989). Oleszek et al. (1994) have identified *p*-coumarylmalic acid while Andrade et al. (1998) found *p*-hydroxybenzoic acid in pears. Of these, 3-*O*-caffeoylquinic and 5-*O*-caffeoylquinic acids are the major phenolic acids of pear puree (Andrade et al., 1998). The content of caffeoylquinic acid in pears ranged from 40 to 140 mg/kg of fresh weight (Amiot et al., 1995; Blankenship and Richardson, 1985; Ferreira et al., 2002).

Pears also contain a mixture of quercetin, isoquercetin, kaempferol and isorhamnetin (3'-methylether of quercetin) glycosides (Figure 4.2) (Andrade et al., 1998; Duggan, 1967, 1969a, b; Herrmann, 1976; Nortje and Koeppen, 1965; Oleszek et al., 1994; Sioud and Luh, 1966; Spanos and Wrolstad, 1992). Flavonols are mostly located in the pear skins; Herrmann (1976) reported that the skin and peel of Williams Christ pears contain 28 mg/kg of quercetin and 12 mg/kg of kaempferol, while the total content of flavonols in remaining pear tissue did not exceed 0.1 mg/kg. Nortje and Koeppen (1965) detected five flavonols in Bon Chretien pears and positively identified four of them: isoquercetin,

isorhamnetin 3-*O*-glucoside, isorhamnetin 3-*O*-rhamnoglucoside, and isorhamnetin 3-*O*-rhamnogalactoside. Later, Sioud and Luh (1966) detected only two flavonols in Barlett pears, identifying one as quercetin 3-*O*-glucoside.

Subsequently, Duggan (1969b) confirmed the presence of quercetin 3-*O*-glucoside in Pakingham pears and detected seven additional flavonols. Of these, four were positively identified as isorhamnetin 3-*O*-glucoside, isorhamnetin 3-*O*-rutinoside, isorhamnetin 3-*O*-galactoside and quercetin 7-xyloside. Oleszek et al. (1994) detected three quercetin and five isorhamnetin glycosides in pears:

- Quercetin 3-*O*-rutinoside
- Quercetin 3-*O*-glucoside
- Quercetin 3-*O*-malonylglucoside
- Isorhamnetin 3-*O*-rutinoside
- Isorhamnetin 3-*O*-galactorhamnoside
- Isorhamnetin 3-*O*-glucoside
- Isorhamnetin 3-*O*-malonylgalactoside
- Isorhamnetin 3-*O*-malonylglucoside

In addition, Andrade et al. (1998) reported the presence of quercetin 3-*O*-galactoside and quercetin 3-*O*-rhamnoside in Rocha pear puree.

Other phenolic compounds found in pears include (+)catechin and (–)epicatechin (Figure 4.3) (Andrade et al., 1998; Mosel and Herrmann, 1974a; Ranadive and Haard, 1971; Sioud and Luh, 1966). The content of monomeric flavan-3-ols in pears is 13 to 92 mg/kg of fresh flesh; (–)epicatechin is the most abundant flavan-3-ol present (Amiot et al., 1995; Ferreira et al., 2002; Mosel and Herrmann, 1974a). Procyanidins B1 and B2 (Figure 4.4) have been detected in Barlett pears (Spanos and Wrolstad, 1992) and procyanidins with a mean degree of polymerization of 13 to 44 detected in Portuguese pears, comprising about 96% of total phenolics (Ferreira et al., 2002). Two anthocyanins have also been found in pears, namely, cyanidin 3-galactoside and cyanidin 3-arabinoside (Francis, 1970; Timberlake and Bridle, 1971).

Quince Fruits

Raw quince fruit (*Cydonia oblonga*) is generally consumed after cooking or processing into jam and jelly. Andrade et al. (1998) detected a number of phenolic compounds in quince fruit puree and identified six of them: *cis*- and *trans*-3-*O*-caffeoylquinic acid, *cis*- and *trans*-5-*O*-caffeoylquinic acid, quercetin 3-rhamnoside, quercetin 3-xyloside, quercetin 3-galactoside and rutin. Later, Silva et al. (2000) identified two additional phenolics, 4-*O*-caffeoylquinic and procyanidin B₃ as well as four unknown procyanidin polymers in quince jam. Subsequently, three more phenolic compounds have been identified—3,5-dicaffeoylquinic acid in the pulp and peel, and kaempferol 3-glucoside and kaempferol 3-rutinoside only in the peel (Silva et al., 2002). These authors also reported that the total content of phenolics in the pulp ranges from 11.7 to 268.3 mg/kg and between 243 and 1738 mg/kg in the peel. Furthermore, rutin and 5-*O*-caffeoylquinic acid are the major phenolics in the peel, whereas 3-*O*- and 5-*O*-caffeoylquinic acids are dominant in the pulp (Silva et al., 2002). Two anthocyanins, cyanidin 3-glucoside and

cyanidin 3,5-diglucoside, have also been identified in quince fruits with red pigmented skin (Markh and Kozenko, 1965).

TROPICAL FRUITS

Banana

Bananas (*Musa* sp.) are popular edible fruits. The ripening stages of banana are scored based on the color of the peel, from 1 for all green to 8 for duffel. Bananas are harvested at ripening stages 1 to 3 (all green to half green), and usually sold at ripening stages 6 to 7 (yellow to star) (Kanazawa and Sakakibara, 2000). The major phenolics of banana include catecholamines, naringin and rutin (Drell, 1970; Griffiths, 1959; Kanazawa and Sakakibara, 2000; Udenfriend et al., 1959). Dopamine and norepinephrine (α -(aminomethyl)-3,4-hydroxybenzyl alcohol) are the predominant catecholamines in banana peel and pulp; phenolics accumulate mainly in the peel (Table 4.12) (Kanazawa and Sakakibara, 2000). Dopamine displays anti-inflammatory activity and protects against intestinal injury by acting as modulator of eicosanoid synthesis (Alanko, et al., 1992; MacNaughton and Wallace, 1989).

During the late stages of ripening, dopamine is converted to salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) (Figure 4.18) (Riggin et al., 1976) via nonenzymatic Pictet-Spengler condensation (Whaley and Govindachari, 1951). The biosynthesis of salsolinol involves complexation of dopamine with acetaldehyde to form an imine, which then cyclizes to salsolinol (Whaley and Govindachari, 1951). The synthesis of salsolinol is accelerated during ripening of banana due to increased formation of acetaldehyde from ethanol produced during the postclimacteric phase (Riggin et al., 1976). Salsolinol has been implicated in the black appearance of overripe bananas (Riggin et al., 1976). Recently, Sojo et al. (2000) demonstrated that salsolinol is oxidized to corresponding quinone by banana pulp polyphenol oxidase.

Tressl and Drawart (1973) identified eugenol methyl ether, eugenol, elimicin, 3,4-dimethoxytoluene and 5-methoxyeugenol in banana volatiles. These compounds are formed in banana from phenylalanine via Shikimic acid pathway. Later, Pérez

TABLE 4.12
Levels of Phenolics in Unripened and Ripened Cavendish Banana^a

| Phenolic | Unripe Banana ^b | | Ripe Banana ^c | |
|----------------|----------------------------|--------|--------------------------|----------|
| | Peel | Pulp | Peel | Pulp |
| Dopamine | 8650–19,400 | 47–100 | 800–560 | 25.0–100 |
| Norepinephrine | 550–1180 | 8–17 | 0–240 | 8.2–16 |
| Naringin | 1200–2600 | 0–650 | 280–950 | 0.0–33 |
| Rutin | 160–230 | 0–48 | 110–160 | 0 |

^a Milligrams per kilogram of fresh weight.

^b Ripening stages 1 to 3 (all green to half green).

^c Ripening stages 6 to 7 (full yellow to star).

Source: Adapted from Kanazawa, K. and Sakakibara, H., 2000, *J. Agric. Food Chem.*, 48:844–848.

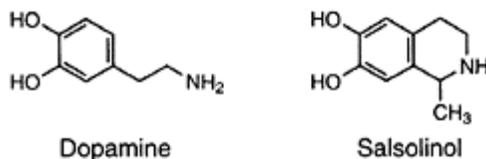


FIGURE 4.18 Chemical structures of some phenolic compounds found in banana fruit.

et al. (1997) reported that these phenolics are present in glycosidically bound form in ripe banana. Glucosides of pelargonidin and cyanidin have been found in *Musa coccinea*, while glucoside of delphinidin and cyanidin is present in *Musa laterita*, *Musa Balbisiانا* and *Musa velutina* (Simmonds, 1954a). In addition, methylated anthocyanidin glucosides of peonidin, petunidin, and malvidin have been detected in *Musa acuminata* (Figure 4.5) (Simmonds, 1954a, b).

Citrus Fruits

Cinnamic acid derivatives, coumarins and flavonoids (flavonones, flavones and flavonols) are the major groups of phenolic compounds occurring in citrus fruits in the free form and/or as glycosides (Horowitz and Gentili, 1977; Maier and Matzler, 1967; Manthey and Grohmann, 2001; Rouseff and Ting, 1979; Wilfried et al., 1994a, b). Flavanone glycosides, phlorin and cinnamic acid derivatives have been proposed for species differentiation and detection of adulterations (Louche et al., 1998; Marini and Balestrieri, 1995; Mouly et al., 1993, 1994, 1995, 1997; Ooghe and Detavernier, 1997; Rouseff, 1988; Rouseff et al., 1987). The peel is the primary waste product from processing citrus fruits. For example, orange peels comprise approximately 45% of the fruit mass.

The various co-products obtained from citrus peel include citrus peel molasses and juices, cold-pressed citrus peel oil, pectin, limonene and dried citrus pulp (Braddock, 1995; Kesterson and Braddock, 1976). For example, peel juice is obtained by liming and then pressing the peel, while molasses is obtained by evaporation of the peel juice to a liquor of at least 35.5°Brix (Grohmann et al., 1999; Manthey and Grohmann, 2001). The highest concentration of flavanones and flavanone glycosides occurs in the peel (Bocco et al., 1998; Horowitz, 1961; Kanes et al., 1993; McIntosh and Mansell, 1997), e.g., orange and grapefruit peels contain between 13.5 and 22.3 g of flavonoids/kg of dry weight (Bocco et al., 1998; Sinclair, 1972). Other groups of phenolic compounds such as hydroxycinnamates, psolarens and polymethoxylated flavones have also been detected in citrus peels (Kanes et al., 1993; Manthey and Grohmann, 2001; Peleg et al., 1991; Risch and Herrmann, 1988a; Stanley and Vannier, 1957; Tatum and Berry, 1979).

Phenolic acids are predominantly located in the flavedo of citrus fruit (Manthey and Grohmann, 2001; Peleg et al., 1991). Caffeic, *p*-coumaric, ferulic and sinapic acids (Figure 4.1) have been detected in citrus fruits and their co-products in the form of esters, amides and glycosides (Bocco et al., 1998; Fallico et al., 1996; Manthey and Grohmann, 2001; Mouly et al., 1997; Peleg et al., 1991; Rapisarda et al., 1998; Risch and Herrmann,

1988a). Only very small amounts of free phenolic acids are present (Manthey and Grohmann, 2001; Peleg et al., 1991). Ferulic acid has been found to be the major phenolic acid in the Shamuti orange fruit (Peleg et al., 1991) and citrus peel and their co-products (Bocco et al., 1998; Manthey and Grohmann, 2001), while coumaric and ferulic acids are present in Italian blood orange juices (Rapisarda et al., 1998).

The total hydroxycinnamic acid content in juices of different Italian blood orange varieties ranges from 53.1 to 158.8 mg/L (Rapisarda et al., 1998), while in citrus peels and seeds it is 144 to 2956 mg/kg of dry matter (Bocco et al., 1998). HCAs have been associated with the formation of off-flavors in citrus fruits and their products. Ferulic and *p*-coumaric acid esters may be enzymatically hydrolyzed during thermal processing and storage and then decarboxylate to unpleasant compounds such as *p*-vinylguaiacol (PVG) and *p*-vinylphenol (Fallico et al., 1996; Klaren-De Wit et al., 1971; Lee and Nagy, 1990; Naim et al., 1988, 1993). Accumulation of PVG can be controlled by storing orange juice under nitrogen, as well as in the presence of cupric ions and L-cysteine (Peleg et al., 1992, Naim et al., 1993).

Mouly et al. (1997) isolated and identified cinnamoyl- β -D-glucopyranoside in blood oranges grown in Florida. Two other glycosides, namely, phlorin (3,5-dihydroxyphenyl β -D-glucopyranoside) (Figure 4.19) and coniferin (coniferyl alcohol 4- β -D-glucopyranoside), have been detected in citrus fruit juices, citrus peels and/or their by-products (Braddock and Bryan, 2001; Hammond et al., 1996; Horowitz and Gentili, 1961; Johnson et al., 1995; Louche et al., 1998; Manthey and Grohmann, 2001). According to Louche et al. (1998), phlorin mainly accumulates in the albedo of the peel (0.8 to 1.1 mg/kg). Based on the level of this phenolic

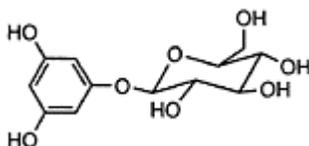


FIGURE 4.19 Chemical structure of phlorin.

compound (1.1 to 25.5 mg/L), Mouly et al. (1997) suggested it as a natural marker for authentication of Florida blood orange juice. On the other hand, Rapisarda et al. (1998) proposed to use hydroxycinnamate profiles as markers for Italian blood orange juices. Subsequently, Louche et al. (1998) suggested phlorin as peel marker in orange juices because a high level of phlorin in juice would indicate adulteration with peels when pulp wash is added to the juice.

Accumulation of certain coumarins, namely, xanthyletin, seselin and scoparone (6,7-dimethoxycoumarin), has been associated with the infection of citrus fruits by pathogens (Figure 4.20) (Afek and Szejnberg, 1988, 1993; Ben-Yehoshua et al., 1988; Khan et al., 1985; Kim et al., 1991; Stange et al., 1993; Vernenghi et al., 1987). Treating tangelo Nova fruits with 0.5% Bromatox, a phyto regulator, significantly enhances the resistance of fruits to fungi. The treated wounded fruits accumulate more scoparone than the untreated ones (Ortuno et al., 1997). Also, treating citrus fruits with UV radiation

stimulates the biosynthesis of coumarins (Ben-Yehoshua et al., 1992; Riov et al., 1971, 1972). Increased accumulation of scoparone has also been noted in uninfected, wounded citrus fruits. For example, 274 mg scoparone/kg of fresh weight has been detected in wounded fruits after 7 days following wounding (Ortuno et al., 1997). Furthermore, auraptene (7-geranyloxycoumarin) (Figure 4.20) has been found in a number of citrus species (Gray and Waterman, 1978; Masuda et al., 1992).

Ogawa et al. (2000) evaluated the auraptene content in 77 citrus species. These authors reported that the peels contain up to 16.6 g of auroptene/kg of dry weight; in juice sacs, up to 10.32 g of auroptene/kg of dry weight is present. The largest quantities of auroptene were detected in *Citrus-Poncirus trifoliata* hybrids. Later, Takahashi et al. (2002) reported that *Citrus natsudaidai* and *Citrus hassaku* peels contained over 0.2 g of auraptene/kg of fresh peel. In addition, several coumarins, including 8-geranyloxypsolaren, 5-geranyloxypsolaren (bergamottin) and 5-geranyloxy-7-methoxycoumarin, have been identified in lemon peels; these are abundantly present in the flavedo of the peel (116, 138 and 83.2 mg/kg of fresh weight, respectively) as well as in a cold-pressed lemon oil (1850, 1700 and 1700 mg/kg, respectively) (Miyake et al., 1999). Recently, Takahashi et al. (2002) developed a rapid method for preparing an auraptene-enriched product from orange peel oils by using SP-70 adsorbent (styrene-divinylbenzene synthetic adsorbent). Auraptene exerts inhibitory effects toward chemical cancerogenesis in some rodent models (Murakami et al., 1997; Tanaka et al., 1997, 1998a, b).

Polymethoxylated flavones (Figure 4.21), a group of phenolic compounds unique for *Citrus* species, are mostly accumulated in the peel (Ortuno et al., 2002) and their profile in citrus fruit is characteristic of each species (Gaydou et al., 1987; Mizuno et al., 1991). Nobiletin (5,6,7,8,3',4'-hexamethoxyflavone) and sinensetin (5,6,7,8,3',4'-pentamethoxyflavone) have been identified in orange peel, while tangeretin (5,6,7,8,4'-pentamethoxyflavone) (Kefford and Chandler, 1970; Ting and Attaway, 1971), tetramethylscutellarein (5,6,7,4'-tetramethoxyflavone), 3,5,6,7,8,3',4'-heptamethoxyflavone, nobiletin and sinensetin have been found in tangerine oil (Gaydou et al., 1987). Tangeretin, 3,5,6,7,8,3',4'-heptamethoxyflavone, 5,7,8,4'-tetramethoxyflavone, and 5,7,8,3',4'-pentamethoxyflavone have been found in grapefruits (Harborne, 1967; Venkataraman, 1975). Later, sinensetin, isosinensetin

(a)

| Compound | R ₁ | R ₂ | R ₃ | R ₄ |
|--------------------------|-----------------|------------------|------------------|-----------------|
| Psolaren | H | H | H | H |
| Bergapten | H | OCH ₃ | H | H |
| Xanthotoxin | H | H | OCH ₃ | H |
| Isopimpenellin | H | OCH ₃ | OCH ₃ | H |
| 4,5',8-Trimethylpsolaren | CH ₃ | H | CH ₃ | CH ₃ |

(b)

| Compound | R ₁ | R ₂ |
|---------------|------------------|------------------|
| Esculetin | OH | OH |
| Scopoletin | OCH ₃ | OH |
| Scoparone | OCH ₃ | OCH ₃ |
| Umbelliferone | H | OH |

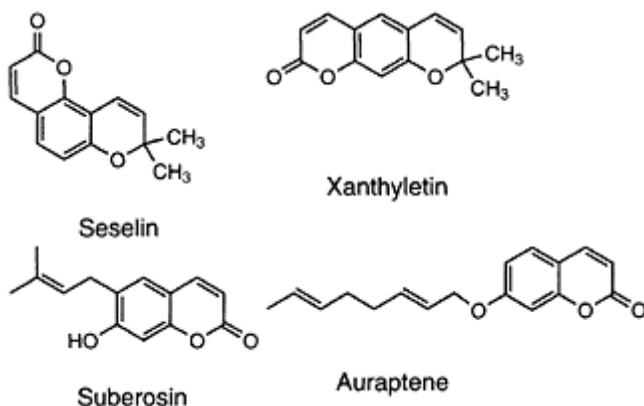
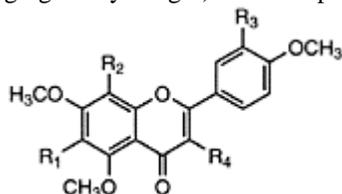


FIGURE 4.20 Chemical structures of furanocoumarins (A) and coumarins (B).

(5,7,8,3',4'-pentamethoxyflavone), hexa-*O*-methylquercetagenin (3,5,6,7,3',4'-hexamethoxyflavone), nobiletin, 3,5,6,7,8,3',4'-heptamethoxyflavone, tetramethylscutellarein, and 5-desmethylnobiletin (5-hydroxy-6,7,8,3',4'-pentamethoxyflavone) were detected in the peel molasses from orange, tangerine and grapefruit (Manthey and Grohmann, 2001). Subsequently, Kawaii et al. (1999c, 2001) detected nobiletin (7 to 173 mg/kg of dry weight), 3,5,6,7,8,3',4'-heptamethoxyflavone (0 to 87 mg/kg of dry weight), natsudaïdain (5,6,7,8,3',4'-hexamethoxyflavone) (to 69 mg/kg of dry weight) and tangeretin 98 to 62 (mg/kg of dry weight) in edible parts of *Citrus* fruits.



| Compound | R ₁ | R ₂ | R ₃ | R ₄ |
|-------------------------|------------------|------------------|------------------|------------------|
| Sinensetin | OCH ₃ | H | OCH ₃ | H |
| Tetramethylscutellarein | OCH ₃ | H | H | H |
| Isosinensetin | H | OCH ₃ | OCH ₃ | H |
| Nobiletin | OCH ₃ | OCH ₃ | OCH ₃ | H |
| Tangeretin | OCH ₃ | OCH ₃ | H | H |
| Heptamethoxyflavone | OCH ₃ | OCH ₃ | OCH ₃ | OCH ₃ |
| Natsudaïdain | OCH ₃ | OCH ₃ | OCH ₃ | OH |

FIGURE 4.21 Chemical structures of some polymethoxylated flavones found in *Citrus* fruits.

The concentration of individual polymethoxylated flavones is affected by the stage of *Citrus* fruit development. In tangelo Nova fruits, the highest concentration of nobiletin, sinensetin and tangeretin is found in immature fruits, while 3,5,6,7,8,3',4'-heptamethoxyflavone is dominant in fruits reaching maturity (Ortuno

TABLE 4.13

Content of Polymethoxylated Flavones in Peel Oils of Various Citrus Fruit Samples^a

| Citrus Fruit | Tangeritin | Nobiletin | Sinensetin | Total |
|-----------------|------------|-----------|------------|-------|
| Orange | 0.70 | 0.50 | 0.30 | 1.88 |
| Common Mandarin | 2.80 | 2.00 | 0.07 | 6.49 |
| King Mandarin | 2.50 | 0.60 | 0.20 | 6.00 |
| Clementine | 1.10 | 0.40 | 0.07 | 3.60 |
| Tangeritin | 1.50 | 1.50 | 0.07 | 3.64 |

^a Grams per liter.

Source: Adapted from Gaydou, E.M. et al., 1987, *J. Agric. Food Chem.*, 35:525–529.

et al., 1999). The accumulation of polymethoxylated flavones in fruits can be modulated by the application of growth regulators during fruit development. It has been shown that 7 days after the treatment of tangelo Nova fruits with 100 mg/kg of benzylaminopurine, a growth cytokinin regulator, the level of polymethoxylated flavones in treated fruits increases by 12% for tangeretin, 20% for nobiletin and sinensetin and 29% for heptamethoxyflavone (Ortuno et al., 2002). The total content of polymethoxylated flavones in peel oil of citrus fruits ranges from 1.88 g/L for orange (*Citrus sinensis*) to 6.49 g/L for common mandarin (Table 4.13) (Gaydou et al., 1987); in peel molasses from orange, tangerine and grapefruit it varies from 0.08 to 1.13% (Manthey and Grohmann, 2001). Polymethoxylated flavones exert potent anticarcinogenic, anti-inflammatory and cardioprotective activities (Benavente-Garcia et al., 1997; Kawaii et al., 1999a, b; Manthey et al., 1999, 2001; Miyazawa et al., 1999).

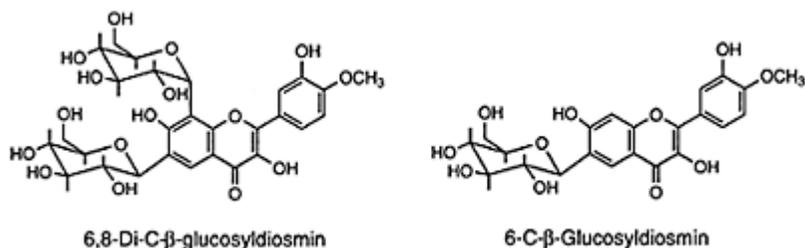


FIGURE 4.22 Chemical structures of C-glycosyldiosmins found in lemon peel.

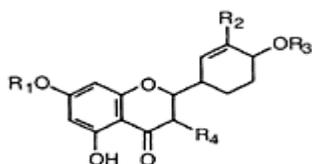
Several glycosylated flavones have been detected in citrus fruits. Of these, diosmin (4'methoxy-5,7,3'-trihydroxyflavone-7-rutinoside) and neodiosmin (4'methoxy5,7,3'-

trihydroxyflavone-7-neohesperidoside) (Figure 4.2) have also been found in immature citrus fruits (Benavente-Garcia et al., 1993; Del Rio et al., 1991; Marin and Del Rio, 2001). The maximum accumulation of these two flavones occurs during the first stages of fruit development (lineal phase) and then their levels gradually decrease (Benavente-Garcia et al., 1993). The concentration of diosmin in immature citrus fruits ranges from 0 for *Citrus aurantium* cultivars to over 30 g/kg of dry weight for *Citrus medica* cv. Buda's fingers and *Citrus limon* cv. Meyer, while in mature fruits it does not exceed 4 g/kg of dry weight.

On the other hand, the content of neodiosmin, found in *C. aurantium* cultivars, is 4.8 to 9 g/kg of dry weight in immature fruits and 0.45 to 1.1 g/kg of dry weight in mature fruits (Marin and Del Rio, 2001). Diosmin and neodiosmin have been found in greater concentrations in flavedo than in albedo of the peel; only small quantities of these flavones are present in the pulp. Application of hormonal treatment with 6-benzylaminopurine and 2,4-dichlorophenoxyacetic acid during the early stages of fruit development greatly increases the content of diosmin and neodiosmin in immature fruits (Marin and Del Rio, 2001). In addition, citrus peel contains small quantities of C-glycosylflavones such as 8-di-C- β -glucosyldiosmin (216 mg/kg) and 6-C- β -glucosyldiosmin (34.8 mg/kg) (Figure 4.22) (Miyake et al., 1997). Diosmin is used in the treatment of circulatory system diseases (Galley and Thiollet, 1993; Tsouderos 1991), in the relief of the symptoms of severe hemorrhoids (Godeberg, 1994; Meyer, 1994) and in inflammatory disorders (Jean and Bodinier, 1994).

Presence of four flavanones, namely, naringenin (5,7,4'-trihydroxy flavanone), eriodictyol (5,7,3',4'-tetrahydroxyflavanone), hesperetin (4'-methoxy-5,7,3'-trihydroxyflavanone), and isosakuranetin (4'-methoxy-5,7-dihydroxyflavanone), in citrus fruits has been reported (Figure 4.23). These aglycones, as well as narirutin 4'-O-glucoside, are glycosylated in the 7-position with disaccharides rutinose or neohesperidose. Both disaccharides are made of rhamnose and glucose and differ only in their linkage type; 1 \rightarrow 6 for rutinose and 1 \rightarrow 2 for neohesperidose.

According to Kanés et al. (1992), flavanone glycosides such as naringin, neoeriodictin and hesperidin comprise 50 to 80% of total flavonoids in *Citrus* fruits. The predominant glycosides in grapefruits are naringin, naringenin 7-neohesperidoside and narirutin, naringenin 7-rutinoside. On the other hand, dominant flavanone glycosides in sweet oranges are 7-rutinosides, namely, narirutin, hesperedin, and hesperetin 7-rutinoside. Sour oranges contain 7-neohesperidosides, namely, naringin and neohesperidin as well as hesperetin 7-neohesperidoside (Table 4.14). Hesperedin, narirutin and didymin (isosakuranetin 7-rutinoside) are the major flavanone glycosides in Navel oranges (Gil-Izquierdo et al., 2002) as well as blood oranges (Mouly et al., 1997). Lemon peel is a rich source of glycosylated flavonones such as neoeriodictin, eriodictyol 7-neohesperidoside (6.1 g/kg), naringin (6.1 g/kg) and neohesperidin (4.4 g/kg) (Bocco et al., 1998). In addition, hesperedin is the predominant glycoside in orange peel and orange peel juice, while narirutin dominates in orange peel molasses (Table 4.15) (Manthey and Grohmann, 1996). Flavanone glycosides contribute to the taste and bitterness of citrus juice; their fungistatic and fungitoxic properties are well established (Kefford and Chandler, 1970). However, flavanone glycosides exert only a weak antiproliferative activity toward cancer cell lines (Kawaii et al., 1999a).



| Compound | R ₁ | R ₂ | R ₃ | R ₄ |
|---------------|-----------------|----------------|-----------------|----------------|
| Eriocitrin | rutinosyl | OH | H | H |
| Hesperidin | rutinosyl | OH | CH ₃ | H |
| Neoeriocitrin | neohesperidosyl | OH | H | H |
| Naringenin | H | H | H | H |
| Naringin | neohesperidosyl | H | H | H |
| Neohesperidin | neohesperidosyl | OH | CH ₃ | H |
| Neoponcirin | rutinosyl | H | CH ₃ | H |
| Narirutin | rutinosyl | H | H | H |
| Poncirin | neohesperidosyl | H | CH ₃ | H |
| Taxifolin | H | OH | H | OH |

FIGURE 4.23 Chemical structures of flavanones and their glycosylated derivatives.

TABLE 4.14

Content of Flavanone Glycosides in Citrus Juices^a

| Fruit | Narirutin | Naringin | Hesperedin | Neohesperedin |
|--------------|-----------|----------|------------|---------------|
| Sour orange | <3 | 133–262 | <3 | 97–209 |
| Sweet orange | 18–65 | – | 122–254 | – |
| Grapefruit | 23–124 | 73–419 | 4–16 | 4–10 |
| Pumello | – | 40–144 | <3 | – |

^a Milligrams per liter.

Source: Adapted from Rouseff, R.I. et al., 1987, *J. Agric. Food Chem.*, 35:1027–1030.

TABLE 4.15

Content of Flavanone Glycosides in Valencia Orange Peel and Peel By-Products^a

| Compound | Peel | Peel Juice | Peel Molasses |
|---------------------------|---------|------------|---------------|
| Eriocitrin | 1010 | 145 | 787 |
| Narirutin | 1998 | 116 | 2050 |
| Narirutin 4'-O-glucoside | 835 | 60 | 933 |
| Hesperedin | 19, 170 | 408 | 961 |
| Isosakuranetin rutinoside | 1858 | 27 | 256 |

^a Milligrams per kilogram.

Source: Adapted from Manthey, J.A. and Grohmann, K., 1996, *J. Agric. Food Chem.*, 44:811–814.

A number of anthocyanins have been identified in blood oranges (Lee, 2002; Maccarone et al., 1983, 1985, 1998; Trifiro et al., 1995). The content of anthocyanins in the blood orange fruit is affected by the variety, stage of fruit ripeness and storage conditions (Rapisarda and Giulfredda, 1992; Rapisarda et al., 2000, 2001). Cyanidin 3-glucoside, cyanidin-3-(6"-malonyl)-glucoside and cyanidin 3-rhamnoside are the major anthocyanins in Italian blood oranges (Maccarone et al., 1983, 1985, 1998; Trifiro et al., 1995), while cyanidin 3-glucoside and cyanidin-3-(6"-malonyl)-glucoside are present in high amounts in Budd blood oranges (Lee, 2002). Cyanidin 3-glucoside comprises 40 to 60% of total anthocyanins in blood oranges grown in Italy (Maccarone et al., 1985) and malonylated anthocyanins account for over 50% of the total anthocyanins in Budd blood oranges (Lee, 2002).

Mango

One of the most popular edible fruits for its tasty, fleshy mesocarp, mango (*Mangifera indica* L.) is widely grown in many tropical and subtropical regions (Arogba, 2000). A number of phenolics have been detected in mango fruits: gallic acid, m-digallic acid, β -glucogallin (β -1-*O*-galloyl-D-glucose), gallotannins, quercetin, isoquercitrin, mangiferin, ellagic acid (Bathia et al., 1967; El Ansari et al., 1971; Saleh and El Ansari, 1975), and peonidin 3-galactoside (Figure 4.24) (Proctor and Creasy, 1969). Volatile components of mango fruits contain a number of phenolic constituents. Adedeji et al. (1992) reported the presence of eugenol, ferulic acid and thymol in glycosidically bound aroma compounds in African mango (*Mangifera indica* L.). Later, Sakho et al. (1997) positively identified two phenolic glucosides in African mango aroma: eugenyl and vanillyl β -D-glucopyranosides.

Phenolic compounds are also involved in allergic contact dermatitis (similar to poison ivy) among workers harvesting green mango fruits from trees. Bandyopadhyay et al. (1985) isolated and identified the mango fruit allegerin as 5-[2(Z)-heptadecenyl] resorcinol. Aqueous decoction of mango stem bark has been used for treatment of diarrhea, menorrhagia, scabies, syphilis, anemia, cutaneous infections and diabetes (Guha et al., 1996; Miura et al., 2001a, b; Napralert Data Base, 2002; Sato et al., 1992; Yoshimi et al., 2001). In Cuba this decoction is carried out on an industrial scale for use as a nutritional supplement and a component of cosmetics and in phytomedicine (Núñez-Selles et al., 2000). The decoction contains 94 g/kg of dry weight of total phenolics expressed as gallic acid equivalents. Seven phenolic constituents have been found in this decoction: gallic acid, 3,4-dihydroxybenzoic acid, methyl gallate, propyl gallate, mangiferin, (+)catechin, and (-)epicatechin. Of these, propyl gallate, mangiferin and (+)catechin are the major phenolics of mango stem bark decoction (Núñez-Selles et al., 2002). Dry mango kernel contains 64 g/kg of dry weight of total phenolics, which are a mixture of tannins, gallic acid and epicatechin. Tannins are the predominant phenolic constituents of mango kernel; hydrolyzable tannins comprise 75% of total tannins present (Arogba, 2000).

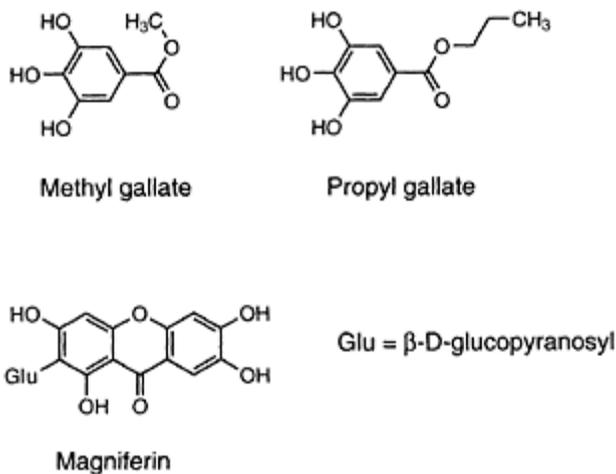


FIGURE 4.24 Chemical structures of some phenolic compounds found in mango fruit.

Passion Fruits

The purple passion fruit (*Passiflora edulis*) and the yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) are two edible passion fruits processed into juice. Several phenolics have been identified in the volatiles of passion fruit. Winter and Kloti (1972) identified eugenol in the aroma compounds of yellow passion fruits. Later, Chassagne et al. (1995) found eugenol and methyl salicylate in the extracts of passion fruits; eugenol was abundant in juice (920 mg/kg) and peels (1720 mg/kg), while methyl salicylate was present only in the juice (Chassagne et al., 1997). Several phenolic glycosides have been characterized in purple and yellow passion fruit volatiles: β -D-glucopyranoside of eugenol and methyl salicylate, as well as 6-*O*- α -L-rhamnopyranosyl- β -D-glucopyranoside of methyl salicylate (Chassagne et al., 1997, 1998). Moreover, several anthocyanins have been detected in the outer cortex of purple passion fruit, namely, pelargonidin 3-diglucoside (Pruthi et al., 1961) delphinidin 3-glucoside (Harborne, 1967), but not in passion fruit juice (Pruthi, 1963).

Pomegranate

Pomegranate (*Punica granatum*) is one of the oldest known edible fruits and is grown in Afghanistan, China, India, Iran, Japan, Mediterranean countries, Russia and the U.S.; it is sold raw or processed to juice and sauce. The total phenolic content in pomegranate juice ranges from 1808 to 2566 mg/L, similar to that found in Cabernet Sauvignon red wine (Gil et al., 2000). Pomegranate is a rich source of hydrolyzable tannins and anthocyanins. The total content of anthocyanins and hydrolyzable tannins in pomegranate juice ranges from 161.9 to 387.4 mg/L and from 417.3 to 556.6 mg/g, respectively (Gil et al., 2000).

Pomegranate juice displays antiatherogenic properties *in vivo* (Aviram et al., 2000); tannins from the bark show antitumoral activity (Kashiwada et al., 1992) and antiviral activity against genital herpes (Zhang et al., 1995).

According to Harborne (1967), delphinidin 3,5-diglucoside is the predominant anthocyanin in pomegranate juice. Later, Du et al. (1975) identified 3-glucosides and 3,5-diglucosides of pelargonidin, delphinidin and cyanidin in the seed coat of pomegranate fruit. Recently, Gil et al. (2000) reported that cyanidin 3-glucoside (59.5 to 128.3 mg/L) is the major anthocyanin in pomegranate juice. Other identified anthocyanins include delphinidin 3-glucoside (23.6 to 95.2 mg/L), cyanidin 3,5-diglucoside (31.4 to 71.4 mg/L), delphinidin 3,5-diglucoside (21.1 to 61.1 mg/L) and pelargonidin 3-glucoside (3.9 to 8.5 mg/L).

A number of hydrolyzable tannins have been isolated and identified in pomegranate leaves, bark and fruits (Gil et al., 2000; Mayer et al., 1977; Nawwar et al., 1994; Tanaka et al., 1985, 1986 a, b). Hydrolyzable tannins of pomegranate include gallotannins, ellagic acid tannins and gallagyl tannins such as punicalagin and punicalin (Figure 4.14). Of these, gallagyl tannins are the major hydrolyzable tannins in commercial pomegranate juices because the juice contains 1500 to 1900 mg/L of punicalagin (Gil et al., 2000). In addition, small quantities of several flavan-3-ols have been detected in pomegranate: catechin (4.0 mg/kg), epicatechin (0.8 mg/kg), galocatechin (1.7 mg/kg), and procyanidins B1 (1.3 mg/kg) and B3 (1.6 mg/kg of fresh weight) (de Pascual-Teresa et al., 2000).

Star Apple

Star apple (*Chrysophyllum cainato*) bears a pear-shaped fruit that is red-purple or pale-green in color. When cut in half, the fruit looks like a star-shaped array of eight segments. The sweet and aromatic flesh of this fruit is edible, but the skin and rind are considered inedible (Morton, 1987). In folk medicine, star apple fruit or its decoction is used for the treatment of laryngitis, pneumonia, diabetes mellitus (Morton, 1987), diarrhea, fever and venereal disease (Coe and Anderson, 1996). Recently, Luo et al. (2002) identified nine phenolic compounds in apple star fruit: (+)catechin, (-)epicatechin, (+)galocatechin, (-)epigallocatechin, quercetin, quercitrin, isoquercitrin, myricitrin and gallic acid. The content of these phenolic compounds ranges from 0.5 (quercetin) to 7.3 ((-)epicatechin) mg/kg of fresh weight.

VEGETABLES

BULBS, ROOTS, AND TUBERS

Carrots

The total content of soluble phenolics in carrots ranges from 5088 to 7699 mg/kg of dry weight (Talcott and Howard, 1999a). Phenolic acids and isocoumarins, predominant phenolics in carrots, contribute to plant tissue's defense mechanism against infections or injuries (Babic et al., 1993). Phenolic acid content in carrot peels may increase up to

seven-fold when carrots are subjected to abiotic stress during post-harvest handling and storage (Sarker and Phan, 1979). Phenolics have also been associated with imparting bitter and sour tastes to stored and processed carrots (Cantwell et al., 1989; Sarker and Phan, 1979) and may result in color loss in processed strained carrots (Talcott and Howard, 1999c; Talcott et al., 2000b).

The total content of phenolic acids in fresh carrots ranges from 77.2 mg/kg of fresh weight for yellow varieties to 746.4 mg/kg of fresh weight for purple varieties (Alasalvar et al., 2002); in processed strained carrots the amount is between 24.9 and 156.2 mg/kg of dry weight (Talcott and Howard, 1999a). Major phenolic acids in carrots include various hydroxycinnamic acid derivatives, *p*-hydroxybenzoic, and syringic acids (Figure 4.1) (Babic et al., 1993; Talcott and Howard, 1999a). However, Babic et al. (1993) did not find *p*-hydroxybenzoic acid and its esters in freshly shredded carrots, but observed an increase in their contents during storage at 4°C. Moreover, Sarker and Phan (1979) identified only caffeic, isochlorogenic and chlorogenic acids in orange carrot roots (Figure 4.15). Recently, Alasalvar et al. (2002) reported that orange, purple, yellow and white carrot varieties contain mainly hydroxycinnamic acid derivatives:

- 3'-Caffeoylquinic acid (neochlorogenic acid)
- 5'-Caffeoylquinic acid (chlorogenic acid)
- 3', 4'- and 5'-Feruloylquinic acids
- 3'- and 5'-*p*-Coumaroylquinic acids
- 3',4'- and 3',5'-Dicafeoylquinic acids
- 3',4'- and 3',5'-Diferuloylquinic acids

The total content of phenolic acids esterified to cell wall material of carrots is between 324.8 mg/kg of cell wall carbohydrate in mature, and 661.1 mg/kg in stored, carrots (Parr et al., 1997; Ng et al., 1998a). Ten phenolic acids have been identified in alkaline hydrolyzates from carrot cell wall material. Of these, *p*-hydroxybenzoic acid is the predominant phenolic acid in cell wall material. Over 30% of total ferulic acid exists in the dehydrodimer form as 5,8'-(benzofuran form), 8,8'-(aryltetralin form) and 8-*O*-4'-diferulic (β -[4-(2-carboxyvinyl)-2-methoxyphenoxy]-4-hydroxy-3-methoxycinnamic) acids; 8-*O*-4'-diferulic acid is the major dehydrodimer in mature carrots (Figure 4.25) (Hartley and Harris, 1981; Ng et al., 1998a; Parr et al., 1997). Cell wall material also contains small amounts of vanillic, *p*-coumaric, *trans*- and *cis*-ferulic acids as well as *p*-hydroxybenzaldehyde and vanillin (Massiot et al., 1988; Ng et al., 1998a; Parr et al., 1997).

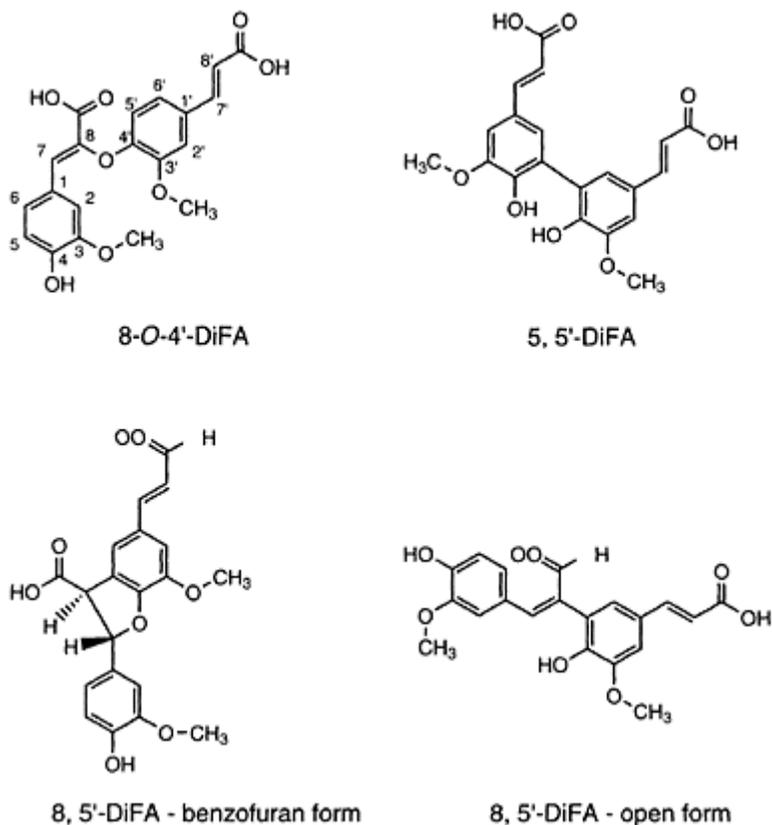


FIGURE 4.25 Chemical structures of ferulic acid dehydromers.

Two isocoumarins, namely, 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (6-methoxymellein) and 3-methyl-6,8-dihydroxy-3,4-dihydroisocoumarin (6-hydroxymellein), have been detected in carrots (Figure 4.26) (Coxon et al., 1973; Harding and Heale, 1980; Marinelli et al., 1990; Sondheimer, 1957). The presence of furanocoumarins has also been reported in diseased carrot tissues (Ceska et al., 1986; Harborne, 1985). The reported levels of 6-methoxymellein in whole carrots range from 0 to 400 mg/kg of dry weight (Talcott and Howard, 1999a). Isocoumarins are predominantly accumulated in the periderm tissue of carrot root and their concentrations incrementally decrease from the peel to vascular tissues (Talcott and Howard, 1999b). Both 6-hydroxymellein and 6-methoxymellein exhibit toxic effects on animal cells, microorganisms and plant cells. The toxic effect exerted by 6-methoxymellein on Chinese hamster cells ($EC_{50}=0.46$ mM) (Superchi et al., 1993) are much lower than that exerted on microorganisms and plant cells ($EC_{50}=0.04$ to 0.05 mM) (Kurosaki et al., 1984; Harding and Heale, 1980; Hoffman and Heale, 1987; Marinelli et al., 1989).

The accumulation of 6-methoxymellein has been associated with the development of bitterness in carrots (Lafuente et al., 1989; Sarker and Phan, 1979; Sondheimer, 1957; Talcott and Howard, 1999a). The formation of 6-methoxymellein in carrots is induced by their exposure to ethylene (Lafuente et al., 1989; Sarker and Phan, 1979; Sondheimer, 1957; Talcott and Howard, 1999b) and to UV light (Mercier

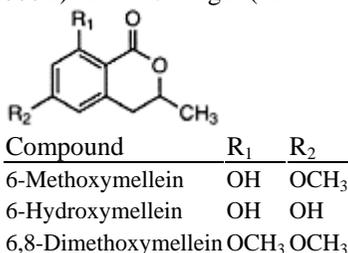


FIGURE 4.26 Chemical structures of some dihydroisocoumarins.

et al., 1994), by microbial infection (Kurosaki and Nishi, 1983; Hoffman et al., 1988), by wounding (Talcott and Howard, 1999b), by elevated storage temperatures (Lafuente et al., 1989), and by pectinolytic enzymes (Marinelli et al., 1990, 1994; Movahedi and Heale, 1990). Whole carrots exposed to ethylene contain 15 to 21% more 6-methoxymellein in the root tip than in the other root sections. Moreover, removal of the tip also elevates the content of 6-methoxymellein in the tip section of carrot root, while the removal of crown significantly increases the concentration of 6-methoxymellein in the root section next to the crown (Talcott and Howard, 1999b).

Onions

One of the most widely consumed vegetables, onions are classified based on their color into yellow, red, and white; based on their taste, they are divided into sweet and nonsweet products. Onions are rich in flavonoids (Bilyk et al., 1984; Crozier et al., 1997; Price and Rhodes, 1997; Rhodes and Price, 1996) and serve as one of the major sources of flavonols such as quercetin, isorhamnetin, myricetin and kaempferol conjugates in the diet (Leighton et al., 1992; Tsushida and Suzuki, 1995). Of these, quercetin is the major flavonol in onions (Rhodes and Price, 1996; Sellappan and Akoh, 2002). Levels of quercetin glucosides are much higher in onions than those in other vegetables (Table 4.16) (Crozier et al., 1997; Hertog et al., 1992a; Mizuno et al., 1992). The content of quercetin conjugates in bulbs of red onion cultivars ranges from 110 to 295 mg of quercetin equivalents/kg and between 119 and 286 mg of quercetin equivalents/kg in bulbs of yellow onion cultivars (Bilyk et al., 1984; Patil and Pike, 1995; Patil et al., 1995; Tsushida and Suzuki, 1996), and varies from 185 to 634 mg of quercetin equivalents/kg in bulbs of white onion cultivars (Crozier et al., 1997). The total content of myricetin and kaempferol in *Vidalia* onions ranges from 27.7 to 41.3 and from 15.4 to 19.8 mg/kg of fresh weight, respectively (Sellappan and Akoh, 2002).

Leighton et al. (1992) identified four flavonoids in onions: quercetin diglucoside (quercetin 3,4'-*O*-diglucoside), quercetin monoglucoside (quercetin 4'-*O*-glucoside),

isorhamnetin monoglucoside and quercetin. Later, Park and Lee (1996) detected the presence of rutin, isorhamnetin and kaempferol (Figure 4.2) and identified isorhamnetin 4'-*O*-glucoside. Subsequently, Price and Rhodes (1997) reported that quercetin 3,4'-*O*-diglucoside and quercetin 4'-*O*-glucoside are the major flavonols in edible portions of onions. In addition, 20 minor quercetin derivatives have been detected (Rhodes and Price, 1996).

TABLE 4.16
Total Content of Quercetin Conjugates in
Lettuce, White Onions and Tomatoes^a

| Vegetable | Content |
|---------------------------------------|----------|
| “Round” lettuce var. Cortina | 11 |
| “Green Salad Bowl” lettuce | 147 |
| White onions | 185–634 |
| Spanish tomatoes var. Assun | 3.5–4.4 |
| Spanish tomatoes var. Daniella | 2–8.7 |
| Scottish tomatoes var. Spectra | 4.6–11.2 |
| Dutch beef tomatoes var. Trust | 2.2–6.8 |
| Spanish cherry tomatoes var. Paloma | 23–203 |
| English cherry tomatoes var. Favorita | 17–77 |

^a Milligrams per kilogram of fresh weight.

Source: Adapted from Crozier, A. et al., 1997, *J. Agric. Food Chem.*, 45:590–595.

The flavonols are mostly concentrated in the skin, which contains from 5.3 to 34.15 g of quercetin equivalents/kg of fresh weight; of these, 23 to 53% of total quercetin is present as aglycone. In addition, the skin contains 6 to 677 mg of kaempferol/kg of fresh weight (Bilyk et al., 1984). The tissue and spatial distribution of quercetin and its two major glucosides, quercetin 3,4'-diglucoside (Q_{DG}) and quercetin 4'-monoglucoside (Q_{MG}), have been evaluated by Hirota et al. (1998). The highest level of flavonols is detected in abaxial epidermis of scales while the lowest amount is found in the mesophyl. Moreover, approximately 50% of flavonols accumulate in the top quarter part of the scales. The mole ratios of Q_{DG}/Q_{MG} in the outer onion scales are lower than those in the inner onion scales. The outer scales are more aged than those of the inner scales (Barden et al., 1987); thus, the aging of scales may promote more rapid synthesis of Q_{MG} (Hirota et al., 1998) or induce enzymatic hydrolysis of Q_{DG} to Q_{MG} (Tsushida and Suzuki, 1996).

Onions also contain small quantities of phenolic acids bound to cell walls. Of these, protocatechuic acid has been detected only in the papery scales, while *p*-hydroxybenzaldehyde and vanillin have been identified only in the fleshy scales. In addition, ferulic, *p*-hydroxybenzoic, vanillic and coumaric acids have been found in the papery and fleshy scales (Ng et al., 2000). Moreover, *p*-hydroxybenzoic acid-*O*- β -D-glucoside (9 mg/kg), vanillic acid-*O*- β -D-glucoside (3 mg/kg) and protocatechuic acid-4-*O*- β -D-glucoside (91 mg/kg) have been detected in onion peels (Herrmann, 1989).

Red onions contain a number of anthocyanins that are mostly concentrated in the skin and the outer fleshy layer (Table 4.17) (Gennaro et al., 2002). Fuleki (1971) and Herrmann (1976) identified cyanidin 3-glucoside, peonidin 3-glucoside and cyanidin 3-

laminariobioside in the outer layer of onions. Later, Ferreres et al. (1996) detected cyanidin 3-glucoside and cyanidin 3-arabinoside and their malonylated

TABLE 4.17

Distribution of Main Anthocyanins in Different Parts of Tropea Red Onions^a

| Anthocyanin | Dry Skin | Outer Fleshy Layer | Edible Portion |
|-----------------------------------|-----------------|---------------------------|-----------------------|
| Delphinidin 3-glucosylglucoside | 7831±75 | 582±29 | 815±37 |
| Cyanidin 3-(6"-malonylglucoside) | 7340±154 | 1250±51 | 188±8 |
| Cyanidin 3-(6"-glucosylglucoside) | 4565±84 | 625±17 | 125±4 |

^a Milligrams per kilogram of dry matter.

Source: Adapted from Gennaro, L. et al., 2002, *J. Agric. Food Chem.*, 50:1904–1910.

TABLE 4.18

Effect of Storing and Processing on Total Quercetin Conjugate Losses in Onions

| Onion | Method of Processing | Loss of Quercetin (%) | Reference |
|----------------|-----------------------------|------------------------------|--------------------------------------|
| White-skinned | Frying, 3 min | 21 | Crozier, A. et al. (1997) |
| | Boiling, 15 min | 75 | |
| | Microwaving, 1.3 min | 64 | |
| Brown-skinned | Storage, 24 weeks | 16 | Price, K.R. . et al. (1997) |
| | Boiling, 20 min | 21.9 | |
| | Frying, 15 min | 29 | |
| Red-skinned | Storage, 24 weeks | 35.4 | |
| | Boiling, 20 min | 14.3 | |
| | Frying, 15 min | 23 | |
| Orange-skinned | Chopping | 1 | Makris, D. and Rossiter, J.T. (2001) |
| | Boiling, 60 min | 20.6 | |
| Red-skinned | Peeling | 21 | Gennaro, L. et al. (2002) |

derivatives in red onions. Subsequently, Donner et al. (1997) found peonidin 3-malonylglucoside; Gennaro et al. (2002) recently found delphinidin and petunidin derivatives in red onions. According to Fossen et al. (1996) and Donner et al. (1997), cyanidin 3-(6"-malonylglucoside), cyanidin 3-(6"-malonylglucoside-3"-glucosylglucoside) and cyanidin 3-glucoside comprise over 95% of total anthocyanins in whole red onions. On the other hand, Gennaro et al. (2002) have demonstrated that cyanidin derivatives constitute over 50% of total anthocyanins and delphinidin derivatives comprise about 30% of total anthocyanins in whole red onions.

Common domestic storing and processing, such as peeling, chopping, boiling, microwaving, and frying, reduce the total content of quercetin conjugates from 1% in the case of chopping onions to up to 75% for boiling them in water (Table 4.18). Price et al. (1997) reported an overall loss of 25% in quercetin glycosides in onions boiled for 20 min; however, no preferential leaching of quercetin 3,4'-*O*-diglucoside (QDG), quercetin,

4'-*O*-glucoside (Q_{MG}) and quercetin (Q) into boiling water was noted. Subsequently, Hirota et al. (1998) found over 50% decrease in the content of Q_{DG} and Q_{MG} after 25 and 15 min of boiling, respectively. Later, Makris and Rossiter (2001) reported an overall loss of 20.5% of total flavonols after boiling bulbs in water for 60 min. These authors demonstrated that 8.4, 37.6 and 43.2% of Q_{DG} , Q_{MG} , and Q, respectively, leached into the cooking water. Blanching may also yield higher loss of flavonols; according to Ewald et al. (1999), blanching onions decreases the content of quercetin and kaempferol by 39 and 64%, respectively.

Parsnip

Five furanocoumarins, angelicin, psolaren, bergapten (5-methoxypsolaren), xanthotoxin (8-methoxypsolaren) and isopimpinellin (5,8-dimethoxypsolaren), have been detected in parsnip roots (Figure 4.20) (Alley, 1987; Ostertag et al., 2002). Of these, angelicin and xanthotoxin are the predominant furanocoumarins in parsnips. Ivie et al. (1981) reported that raw parsnip roots contain up to 40 mg/kg of total furanocoumarins. Later, Ostertag et al. (2002) reported that the total content of furanocoumarins in freshly harvested parsnips did not exceed 2.5 mg/kg of fresh weight; however, in parsnips purchased from the market the amount reached 49 mg/kg of fresh weight. Peeling parsnip roots removes only 30% of the total furanocoumarins present, while boiling and microwaving do not reduce the level of furanocoumarins in the roots to any great extent (Ivie et al., 1981). Storing whole parsnips at +4°C for 7 days effects an increase in furanocoumarins from 1 mg/kg of fresh weight to 33 mg/kg of fresh weight; however, storing them at -18°C for up to 50 days does not markedly affect the content of furanocoumarins (Ostertag et al., 2002).

Potato

Friedman (1997) has published an excellent review on the chemistry, biochemistry, and dietary role of potato polyphenols; some aspects of the chemistry of potato phenolics is discussed here. Chlorogenic acid is the predominant phenolic in potato tuber, constituting up to 90% of total phenolics (Friedman, 1997; Malmberg and Theander, 1985). Chlorogenic acids in potato tuber contribute to the defense mechanism of the plant against insects and microbial pathogens (Friedman, 1997). In addition, Chlorogenic acids are linked with after-cooking blackening of potato tubers. A colorless iron-chlorogenic chelate, formed during cooking of susceptible potato cultivars, oxidizes to produce a bluish-gray ferric complex upon subsequent exposure of cooked potato to air (Griffiths et al., 1995). The after-cooking blackening of potato tubers is influenced by fertilization, soil, and climatic conditions as well as storage period and exposure to light (Griffiths and Bain, 1997; Silva et al., 1991). Approximately 50% of Chlorogenic acid isomers are found in potato skin and adjoining tissues, while in the tuber cortex the level of Chlorogenic acids gradually decreases from outside toward the center of potato tuber (Friedman, 1997; Hasegawa et al., 1966; Reeve et al., 1969).

The total content of monomeric anthocyanins in different red-fleshed potato cultivars (*Solanum tuberosum* and *Solanum stenotum*) ranges from 24 to 403 mg/kg of tuber (Rodriguez-Saona et al., 1998). Higher levels of anthocyanins have been detected in the skin of purple potato cv. Urenika (5078 mg/kg) than in the flesh (1836 mg/kg) (Lewis,

1996). On the other hand, a somewhat higher concentration of anthocyanin has been detected in the flesh than in the skin of red-fleshed potatoes. Pelargonidin 3-rutinoside-5-glucoside acylated with *p*-coumaric acid is the predominant anthocyanin (70% of total anthocyanins) (Rodríguez-Saona et al., 1998).

Wounding and/or challenge of potato with phytopathogens brings about a rapid modification of cell wall in periderm, wound periderm, and endo- and exodermal cells known as suberization. In potato, suberized cells become an effective defensive barrier against dehydration and pathogen penetration (Kolattukudy, 1987; Lulai and Orr, 1994). The suberin is composed of polyphenolic and aliphatic domains. The poly phenolic domain is linked to the cell wall; the aliphatic domain is covalently linked to the polyphenolic domain (Kolattukudy, 1987; Lulai and Corsini, 1998; Razem and Bernards, 2002), which makes up 50 to 75% of suberized cell wall (Dean and Kolattukudy, 1977). The polyphenolic domains contain not only lignins but also small amounts of monolignols (coniferyl and sinapyl alcohols) as well as significant amounts of nonlignin precursors such as hydroxycinnamic acids and their derivatives (Figure 4.27) (Bernards et al., 1995; Negrel et al., 1996; Razem and Bernards, 2002). Kolattukudy (1980) has suggested that polymerization of phenolics during the suberization process occurs via a peroxidases-hydrogen peroxide-mediated free radical coupling. Recently, Razem and Bernards (2002) demonstrated the involvement of hydrogen peroxide in the development of polyphenolic domain in suberized cell wall.

Fresh-cutting and subsequent cold storage of potatoes at 4°C induces the biosynthesis of chlorogenic acids and flavonols. Three flavonols, namely, quercetin 3-rutinoside, quercetin diglucoside and quercetin 3-glucosylrutinoside, have been detected in potato after a lag period of 3 days of cold storage. A similar lag period has also been observed for chlorogenic acid. Depending on the cultivar, the total contents of flavonols and chlorogenic acids in fresh-cut potatoes after 6 days of cold storage are 60 to 140 and 70 to 300 mg/kg of fresh weight, respectively. Cold storage of fresh-cut potatoes under light enhances the accumulation of flavonols (Tudela et al., 2002); prolonged cold storage of potato tubers at 5°C decreases the level of chlorogenic acids (Percival and Baird, 2000).

The light-induced accumulation of chlorogenic acid is also affected by the cold-storage period in the dark. The rate of chlorogenic acids accumulation is three to four times greater in potato tubers exposed to light after 2 weeks of cold storage in the dark than in tubers exposed to light after 3 months of dark cold storage (Percival and Baird, 2000). Boiling, steam-cooking, frying and microwaving significantly reduce the level of phenolics in potato tubers. Cooked potato strips retain about 50% of flavonols, while the retention of chlorogenic acids is influenced by the method of cooking (Tudela et al., 2002). About 55% of the initial chlorogenic acid is detected in microwaved potato strips while only 24% is retained in fried potato strips (Tudela et al., 2002).

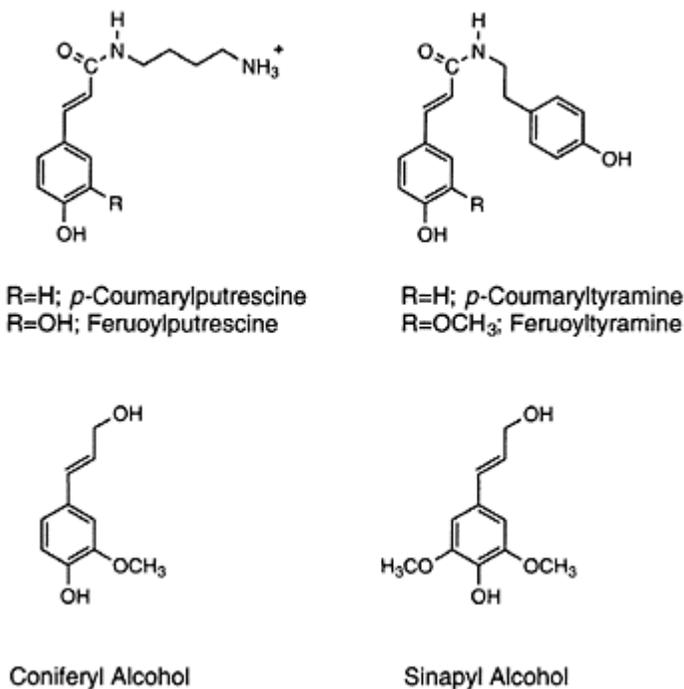


FIGURE 4.27 Chemical structures of some phenolic compounds detected in suberizing potato tuber.

Red Beetroot

The content of phenolics in various red beetroot (*Beta vulgaris*) parts decreases in the order of peel, crown and flesh. Betanin is the predominant phenolic compound in red beetroot (Figure 4.28) and β -D-fructofuranosyl- α -D-(6-*O*-(*E*)-feruloyl)glucopyranoside (FAE) is the main known ferulic acid ester found in the peel. Beet root cell wall material also contains high levels of dehydrodimers of ferulic acids such as 5,5'-, 8-*O*-4'-, and 5,8'-diferulic acids (Ng et al, 1998b; Steglich and Strack, 1991; Waldron et al., 1997). A two-fold increase in the content of dehydrodimers of ferulic acid has been noted in samples of beetroot treated with hydrogen peroxide (Ng et al., 1998b). In addition, *p*-hydroxybenzoic, *cis*- and *trans*-ferulic, *trans*-coumaric, and vanillic acids, as well as *p*-hydroxybenzaldehyde and vanillin, have been detected in beetroot. Of these, ferulic acids (156.6 mg/kg of fresh weight) are found to be the predominant phenolic acids in red beetroot (Ng et al., 1998b). Cold storage of red beetroot for 9 months decreases the content of betanin from 38.7 to 30.7 g/kg of dry weight, but has a minor effect on the total content of phenolics. On the other hand, cold storage has resulted in an increase in the content of FAE in peels from trace to 357.7 mg/kg of dry weight (Kujala et al., 2000). Furthermore, pasteurization of red beetroot nectar reduces the content of betanin by 50% (Patkai et al., 1997).

Sweet Potato

The total content of phenolics in seven sweet potato tubers (*Ipomoea batatas*) ranges from 117 to 467 mg of chlorogenic acid equivalents/kg of fresh weight (Walter and

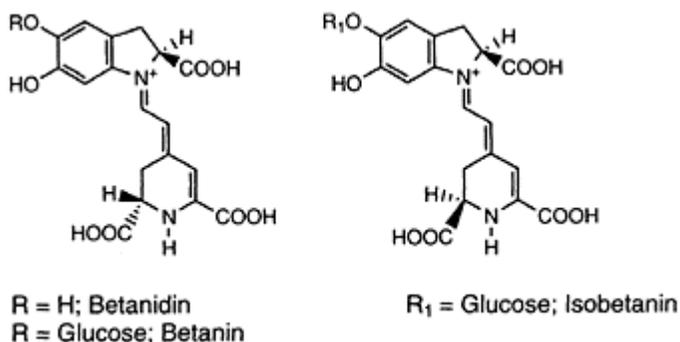
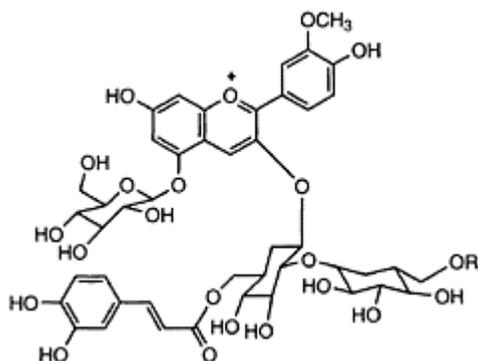


FIGURE 4.28 Chemical structures of some betanins.



| Anthocyanin | R |
|---|-------------------------------|
| Peonidin-3-(dicaffeoyl)-sophoroside-5-glucoside | caffeic acid |
| Peonidin-3-(<i>p</i> -hydroxybenzylcaffeoyl)-sophoroside-5-glucoside | <i>p</i> -hydroxybenzoic acid |
| Peonidin-3-caffeoylsophoroside-5-glucoside | H |
| Peonidin-3-(ferulylcaffeoyl)-sophoroside-5-glucoside | ferulic acid |

FIGURE 4.29 Chemical structures of some anthocyanins isolated from purple-fleshed sweet potato.

Purcell, 1979). Anthocyanins and chlorogenic acids are the major phenolics found in sweet potato tubers (Suda et al., 2002; Walter and Purcell, 1979).

The purple-fleshed sweet potato tuber is a good source of anthocyanins; the major anthocyanin pigments identified in sweet potato include mono- and diacetylated forms of cyanidin and peonidin (Goda et al., 1997; Odake et al., 1992; Suda et al., 2002; Terahara

et al., 1999). Figure 4.29 shows the chemical structures of some anthocyanins detected in purple-fleshed sweet potato. The total content of anthocyanins in sweet potato is 590 mg of peonidin 3-caffeoylsophoroside-5-glucoside equivalent/kg (Furuta et al., 1998). Acylated peonidin and acylated cyanidin constitute 74 and 19% of anthocyanin pigments in sweet potato cv. Ayamurasaki, respectively (Tsukui et al., 1999).

The greens of sweet potato (*Ipomoea batatas* (L) Lam) cultivars are used in many parts of the world as fresh vegetables (Nwinyi, 1992; Villareal et al., 1982; Woolfe, 1992). These greens are a rich source of antioxidants, proteins, vitamin B and minerals such as iron, calcium and zinc (Islam et al., 2002a; Woolfe, 1992). The total phenolic content in sweet potato greens ranges from 14.2 to 171 g of chlorogenic acid equivalents/kg of dry matter. The leaves of sweet potato are the richest source of phenolics (61.9 g/kg of dry matter), followed by petioles (29.7 g/kg of dry matter) and stems (18.8 g/kg of dry matter) (Islam et al., 2002b). Six phenolic acids have been identified in sweet potato leaves: 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, chlorogenic acid, 3,4-di-*O*-caffeoylquinic acid, 3,4,5-tri-*O*-caffeoylquinic acid and caffeic acid. Of these, 3,4,5-tri-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid are the predominant phenolics in sweet potato greens (Islam et al., 2002b).

LEAVES AND STEMS

Asparagus

Ferulic and *p*-coumaric acids have been detected only in the cell wall material of white asparagus and are predominantly concentrated in the basal region of the spear. No phenolic acid dimers are present, suggesting that cross linking polysaccharides via phenolic acid dimers is not an important component of cell wall structure. A slight increase in the phenolic acid levels is detected after 21-day storage of asparagus at 4°C (Rodriguez et al., 1999). Later, Rodriguez-Arcos et al. (2002) detected the presence of ferulic acid dehydrodimers, namely, 5,5'-, 8,5'- and 8,8'-diferulic acid, 8-*O*-4'diferulic acid, 8,5'-diferulic acid benzofuran form and 8,8'-diferulic acid aryltetralyn form, in green asparagus (Figure 4.25). The ferulic acid dehydrodimers comprise >60% of total ferulic acid and >70% after 3 days of storage at 21°C. These authors also noticed a three-fold increase in the total content of ferulic acids during storage, particularly in the cell walls located in the basal section of the stem.

Eleven phenolic compounds have been detected in the aroma components of cooked asparagus: phenol, *o*-, *m*- and *p*-cresols, guaiacol, acetovanillone, 4-hydroxybenzaldehyde, vanillin, veratrol, 4-vinylphenol and 4-vinylguaiacol. These phenols are products of thermal fragmentation of free *p*-coumaric acid and ferulic acid released from cell wall materials by enzymes (Tressl et al., 1977).

Rutin is the major flavonoid found in asparagus spears, but eight more flavonoid (quercetin and kaempferol) glycosides have also been detected (Figure 4.2) (Billau et al., 1985; Dame et al., 1957; Makris and Rossiter, 2001; Stevenson, 1950; Woldecke and Herrmann, 1974c). The content of rutin in green asparagus ranges from 250 to 1000 mg/kg of fresh weight (Makris and Rossiter, 2001; Stevenson, 1950), while in white asparagus it is between 1 and 20 µg/kg of fresh weight (Billau et al., 1985; Woldecke and Herrmann, 1974c). Chopping and maceration of asparagus spears decreases rutin content

by 18.5%; boiling for 60 min in water results in a 43.9% decrease (Makris and Rossiter, 2001).

Rutin is also involved in the development of objectionable black discoloration on the surface of canned green asparagus within a few minutes to 3 h after the can is opened (Lueck, 1970) and in the formation of highly visible white and grayish deposits on the surface of pickled green asparagus after 6 months of storage (Fuleki, 1999). The black discoloration is caused by the formation of a rutin-ferric ion complex, while crystalline deposits on the surface of pickled green asparagus are the result of lower solubility of rutin in acidic solutions.

Celery

The major phenolic components of celery include linear furanocoumarins (psolarens), psolaren, bergapten (5-methoxypsolaren), xanthotoxin (8-methoxypsolaren) and isopimpinellin (5,8-dimethoxypsolaren) as shown in Figure 4.20 (Beier et al., 1983; Diawara et al., 1995). Their presence was first reported by Scheel et al. (1963), who isolated and identified two psolarens, 4,5',8-trimethylpsolaren and 8-methoxypsolaren, in diseased celery. Subsequently, Wu et al. (1972) reported the presence of xanthotoxin and bergapten in diseased celery. Later, Tang et al. (1990) identified psolaren, 9-methoxypsolaren, 4-methoxypsolaren and 4,9-dimethoxypsolaren in volatiles, while Trumble et al. (1992) and Nigg et al. (1997) detected several psolarens in stems, leaves and petioles of healthy fresh celery (*Apium graveolens* L.). Synthesis of psolarens in plants may be induced by a number of environmental stresses such as pathogens, cold temperature (Beier and Oertli, 1983), acidic atmospheric pollution (Dercks et al., 1990), and even fungicide treatment (Nigg et al., 1997).

Except for isopimpinellin (Ashwood-Smith et al., 1992; Hudson et al., 1987), these phenolic compounds have been linked to acute (Austad and Kavli, 1983) and chronic (Seligman et al., 1987) dermatitis in celery handlers. The total content of furanocoumarins at levels of 7 to 9 mg/kg and ≥ 18 mg/kg of fresh weight may cause chronic (Seligman et al., 1987) and acute (Austad and Kavli, 1983) dermatitis responses, respectively. Furthermore, ingestion of celeriac may cause a serious phototoxic burn in humans (Ljunggren, 1990). Psolarens have also been implicated in photocarcinogenicity in mice and humans (Young, 1990), as well as initiation of skin cancer (Berenbaum, 1991; Musajo and Rodighiero, 1962; Stern et al., 1979;). The World Health Organization (International Agency for Research on Skin Cancer, 1983) has listed psolarens as causative agents of skin cancer.

The total content of furanocoumarins in six Florida celery cultivars processed similar to kitchen preparation ranged between 11.7 and 49.6 mg/kg. Of these, the Florida 296 cultivar contained the highest level of total furanocoumarins; the contents of furanocoumarins in remaining cultivars did not differ from one another (Nigg et al., 1997). Similar levels of furanocoumarins have also been reported by others (Dercks et al., 1990; Diawara et al., 1993; Trumble et al., 1992). Celery leaves contain 7 to 15 times more furanocoumarins than stalks (Diawara et al., 1993; Trumble et al. 1992). The total content of furanocoumarins in the outer leaves and inner mature leaves of celery ranges between 40 and 49.8 mg/kg of fresh weight and between 8 and 12 mg/kg of fresh weight, respectively; however, in the heart leaf and different parts of petioles (outer, inner and

heart), the amounts do not exceed 3.8 mg/kg of fresh weight (Table 4.19) (Diawara et al., 1995). In addition, seasonal concentrations of bergapten in celery leaves and petioles are much higher than those of xanthotoxin and psolaren. However, the concentration of bergapten in celery petioles declines by 55 to 75% as the plant matures (Diawara et al., 1995).

TABLE 4.19
Distribution of Main Psolarens^a in Different
Parts of Celery Variety Tall Utah 5270R (*A.*
***graveolens* L. var. dulce)**

| Plant Part | Bergapten | Psolaren | Xanthotoxin |
|------------|------------|-----------|-------------|
| Outer leaf | 28.00±4.55 | 3.94±1.21 | 17.90±3.66 |
| Inner leaf | 4.80±0.57 | 0.17±0.13 | 3.00±0.55 |
| Heart leaf | 2.42±0.43 | 0.02±0.02 | 1.29±0.24 |
| Petiole | 2.69±0.18 | 0.01±0.01 | 1.06±0.15 |
| Roots | 0.57±0.12 | 0.04±0.01 | 0.49±0.12 |

^a Milligrams per kilogram of fresh weight.

Source: Adapted from Diawara, M.M., et al., 1995, *J. Agric. Food Chem.*, 43:723–727.

Beier and Oertli (1983) reported a significant increase in the contents of psolaren, bergapten and xanthotoxin in celery treated with copper sulfate and stored for 3 days at 4°C. Later, Trumble et al. (1992) reported that treatment of celery with pesticides (prometryn, naled, methomyl) did not significantly affect the concentration of coumarins. Subsequently, Nigg et al. (1997) demonstrated that treatment of celery with fungicides such as Bravo 500, Manzate-D or Kocide 101 effects a two- to four-fold increase in the content of bergapten in leaves and stalk, two- to three-fold increase of xanthotoxin in stalk, and two- to three-fold increase of isopimpinellin in leaves. Acidic pollution may also increase the content of furanocoumarins in leaves and petioles of celery up to 540% (to 135 mg/kg of fresh weight) and 440% (to 55.56 mg/kg of fresh weight), respectively. Moreover, a 10-fold increase in the level of furanocoumarins has been noted in celery infected with *Erwinia caratovora* cv. Caratovora (Surico et al., 1987), *Fusarium oxysporum* (Heath-Pagliuso et al.1992) and *Sclerotinia sclerotiorum* (Chaudhary et al., 1985). However, growth of celery under elevated atmospheric carbon dioxide conditions does not significantly increase the content of furanocoumarins in celery (Reitz et al., 1997).

Apigenin and luteolin have been identified in acidic extracts of celery (Figure 4.2). Celery consumed in the Netherlands contains 22 and 108 mg/kg of fresh weight of luteolin and apigenin, respectively (Hertog et al., 1992a, b). Similar levels of luteolin and apigenin have been found in celery purchased in Glasgow, U.K., (Table 4.20) (Crozier et al., 1997) and in Malaysia (Miean and Muhamed, 2001).

Endive

The total content of flavonoids in various cultivars of endive (*Cichorium endivia*) may range from <2 to 246 mg/kg of fresh sample weight (DuPont et al., 2000; Hertog et al.,

1992a). Flavonoids found in endive are a mixture of kaempferol and quercetin conjugates, namely, kaempferol 3-glucuronide, kaempferol 3-*O*-(6-*O*-malonyl)-glucoside, kaempferol 3-*O*-glucoside (DuPont et al., 2000; Herrmann, 1976), quercetin 3-rhamnoside and quercetin 3-galactoside (Goupy et al., 1990; Hertog et al., 1992a;

TABLE 4.20

Content of Luteolin and Apigenin in Commercial Celery^a

| Celery | Apigenin | Luteolin |
|---------------|-----------------|-----------------|
| White stalks | 0–104 | 0–40 |
| White hearts | 17 | 6.6 |
| Green hearts | 191 | 35 |

^a Milligrams per kilogram of fresh weight.

Source: Adapted from Crozier, A. et al., 1997, *J. Agric. Food Chem.*, 45:590–595.

Rees and Harborne, 1984). Of these, kaempferol 3-glucuronide comprises over 70% of total flavonoids (DuPont et al., 2000). Small quantities of hydroxycinnamoyl derivatives such as dicaffeoyl tartaric esters and caffeoyl esters (DuPont et al., 2000; Goupy et al., 1990; Winter and Herrmann, 1986; Woldecke and Herrmann, 1974a) have also been detected in endive extracts. Foliar parenchyma contain higher levels of phenolics than veins; phenolics are located in the foliar parenchyma in the vacuoles, preferentially in the green over the etiolated foliar parenchyma (Goupy et al., 1990). Exposure of shredded endives, sealed in polyethylene bags, to light at 22°C for 48 h reduces the total content of flavonoids and also alters their composition (DuPont et al., 2000). Presence of phenol and 2-ethylphenol in neutral volatiles from blended endive has also been confirmed (Gotz-Schmidt and Schreier, 1986).

Lettuce

Caffeoyltartaric, chlorogenic, dicaffeoyltartaric and 3',5'-dicaffeoylquinic acids have been identified in red lettuce (cv. Lollo Rosso) (Ferrerres et al., 1997a), as well as in iceberg and romaine lettuce (Cantos et al., 2001a; Hyodo et al., 1978; Ke and Saltveit, 1989; Winter and Herrmann, 1986). A number of quercetin conjugates have also been detected in red pigmented and green leaf lettuce. These include quercetin 3-(6-malonylglucoside), quercetin 3-glucoside, quercetin 3-glucuronide, and quercetin 3-rhamnoside (DuPont et al., 2000; Ferreres et al., 1997a). In addition, green leaf lettuce contains quercetin 3-galactoside (DuPont et al., 2000), while quercetin 3-(6-malonylglucoside)-7-glucoside, and cyanidin 3-*O*-glucoside cyanidin 3-*O*-[(6-*O*-malonyl)glucoside] are found in red pigmented lettuce (DuPont et al., 2000; Ferreres et al., 1997a; Yamaguchi et al., 1996).

The total content of phenolic acids in whole red pigmented lettuce ranges from 65 to 270 mg/kg of fresh weight (Winter and Herrmann, 1986), while romaine, iceberg and butter leaf lettuce contain between 2.83 and 45 mg of phenolic acids/kg of fresh weight (Cantos et al., 2001a; Tomás-Barberán et al., 1997). Red lettuce is a good source of flavonoids (Table 4.21) (Crozier et al., 1997; DuPont et al., 2000; Gil et al., 1998a).

According to Bilyk and Sapers (1985), green leaf and head lettuce varieties contain 2 to 54 and 1 to 28 mg quercetin/kg of fresh weight, respectively. The outer and inner leaves of Lollo Rosso red lettuce contain 911 and 450 mg of quercetin/kg of fresh weight, respectively (Crozier et al., 1997); its red tissues

TABLE 4.21**Total Content of Flavonoids in Whole Lettuce^a**

| Variety | Total Flavonoids | Reference |
|------------------|------------------|----------------------------|
| Round | 11±0.5 | Crozier, A. et al. (1997) |
| Green salad bowl | 147±5.2 | |
| Lollo biondo | 94±4.6 | |
| Iceberg | 0.3±0.05 | DuPont, M.S. et al. (2000) |
| Green salad bowl | 19.9±2.0 | |
| Lollo biondo | 95.7±4.2 | |
| Lollo rosso | 207.0±13.0 | |
| Red oak leaf | 76.2±4.0 | |

^aMilligrams per kilogram of fresh weight.

TABLE 4.22**Total Phenolic Acids and Flavonoids Content in Various Tissues of Lollo Rosso Lettuce^a**

| Tissue | Phenolic Acids | Flavonoids |
|--------|----------------|------------|
| White | 213±60 | 43±9 |
| Green | 570±54 | 244±20 |
| Red | 1696±166 | 1384±197 |

^aMilligrams per kilogram of fresh weight.

Source: Adapted from DuPont, M.S. et al., 2000, *J. Agric. Food Chem.*, 48:3957–3964.

contain three to eight times more phenolics and 5 to 30 times more flavonoids than its white and green tissues (Table 4.22) (DuPont et al., 2000).

Exposure of shredded green lettuce leaves to fluorescent light for 48 h at 22°C affects the total flavonoid content and the distribution of quercetin conjugates. The changes in quercetin conjugates are brought about by their demalonylation (DuPont et al., 2000; Gil et al., 1998a). Gil et al. (1998a) have suggested that demalonylation leads first to glucosides and then to formation of acetyl derivatives. However, according to Horowitz and Asen (1989), changes in the flavonoid malonylglycosides may be initiated by their decarboxylation. Wounding lettuce leaves increases the content of phenolic acids two-fold in the stems of red pigmented lettuce leaf (Ferrerres et al., 1997a); in stems of iceberg lettuce leaves the increase is 10-fold (Cantos et al., 2001b; Tomás-Barberán et al., 1997). Chlorogenic acid is the predominant phenolic acid accumulated in processed midribs of lettuce leaves (Cantos et al., 2001a; Ferrerres et al., 1997a). In addition, a marked increase in the activity of phenylalanine ammonia-lyase (PAL) in wounded lettuce leaves has been observed (Cantos et al.,

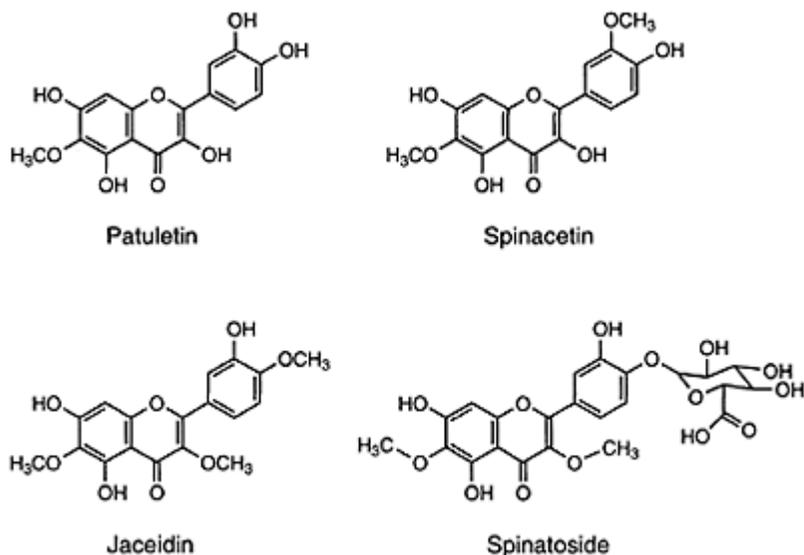


FIGURE 4.30 Chemical structures of some phenolic compounds found in spinach.

2001a; Tomás-Barberán et al., 1997). However, no correlation exists between browning of lettuce leaves and the increase in phenolic acid and PAL activities (Cantos et al., 2001a).

Spinach

Total phenol content in fresh spinach (*Spinacia oleracea*) leaves is 1629 to 4835 mg of chlorogenic acid equivalents/kg of fresh weight. Moreover, spring-grown spinach contains a higher level of total phenols than that of spinach grown in the fall (Howard et al., 2002). Spinach leaves are a rich source of flavonoids; fresh-cut spinach contains from 807 to 2241 mg of flavonoids/kg of fresh weight (Gil et al., 1999; Howard et al., 2002). Spinach flavonoids exhibit antioxidant (Gil et al., 1999), antiinflammatory (Lomnitski et al., 2000a, b), antimutagenic (Edenharder et al., 2001) and anticarcinogenic (Nyska et al., 2001) properties. The spinach flavonoids include

- Patuletin (quercetagenin 6-methyl ether)
- Spinacetin (quercetagenin 6,3'-dimethyl ether) (Zane and Wender, 1961)
- Spinatoside (3,6-dimethylquercetagenin 4'-O-glucuronide) (Figure 4.30) (Wagner et al., 1977)
- Jaceidin 4'-glucuronide
- Patuletin 3-gentobioside
- Patuletin 3-glycosyl-(1→6)-[apiosyl](1→2)]-glucoside
- Spinacetin 3-glycosyl(1→6)-[apiosyl(1→2)]-glucoside

- Patuletin 3-(2''-feruloylglycosyl)(1→6)-[apiosyl(1→2)]-glucoside
- Spinacetin 3-(2''-feruloylglycosyl)(1→6)-[apiosyl(1→2)]-glucoside
- Spinacetin 3-(2''-*p*-coumaroylglycosyl)(1→6)-[apiosyl(1→2)]-glucoside
- Spinacetin 3-gentiobioside (Aritomi et al., 1984, 1986; Ferreres et al., 1997b; Howard et al., 2002)

In addition, Edenharder et al. (2001) and Howard et al. (2002) have identified the 4'-glucuronides of

- 5,7,4'-Trihydroxy-3,6,3'-trimethoxyflavone
- 5,3',4'-Trihydroxy-3-methoxy-6,7-methylenedioxyflavone
- 5,4'-Dihydroxy-3,3'-dimethoxy-6,7-methylenedioxyflavone (Aritomi and Kawasaki, 1984)
- 5,3'-Dihydroxy-4'-methoxy-6,7-methylenedioxyflavonol 3-*O*-β-glucuronide
- 5,2',3'-Trihydroxy-4'-methoxy-6,7-methylenedioxyflavonol 3-*O*-β-glucuronide
- 5-Hydroxy-3',4'-dimethoxy-6,7-methylenedioxyflavonol 3-*O*-β-glucuronide
- 5,6,3'-Trihydroxy-7,4'-dimethoxyflavonol 3-*O*-β-glucuronide
- 5,6-Dihydroxy-7,3',4'-trimethoxyflavonol 3-*O*-β-glucuronide
- 5,6,4'-Trihydroxy-7,3'-dimethoxyflavonol 3-*O*-disaccharide
- 5,6,3'4'-Tetrahydroxy-7-methoxyflavonol 3-*O*-disaccharide
- 5,8,4'-Trihydroxyflavanone
- 7,8,4'-Trihydroxyflavanone

Patuletin derivatives, spinacetin derivatives, jaceidin and flavones account for 21.8, 32.4, 6.3, and 39.5% of the total flavonoids in spinach leaves, respectively (Howard et al., 2002). Storage of spinach up to 7 days under modified atmosphere at 10°C does not affect the total flavonoid content. After 10 min of cooking in water (spinach-to-water ratio of 1:4) at 90°C, the total content of flavonoids in spinach is reduced by 50%. Flavonoid glucuronides have been extracted into cooking water more than other flavonoid compounds (Gil et al., 1999).

Swiss Chard

The total content of flavonoids in leaves of green and yellow Swiss chard cultivars ranges from 2.4 to 3.0 g and from 2.1 to 2.3 g of flavonoid/kg of fresh weight, respectively. Five flavonoids isolated from green cultivars are identified as 2''-xyloside vitexin, 6''-malonyl-2''-xyloside vitexin, 3-gentiobioside kaempferol, 3-gentiobioside isorhamnetin and 3-vicianoside isorhamnetin. Only 2''-xyloside vitexin and its 6''-malonylated derivative have been detected in the yellow cultivar. Of these flavonoids, 2''-xyloside vitexin and 6''-malonyl-2''-xyloside vitexin comprise over 60% of total flavonoids in green and yellow cultivars (Gil et al., 1998b). Boiling the leaves of Swiss chard in water for 10 min removes about 30 and 50% of flavonoids initially present in green and yellow cultivars, respectively (Gil et al., 1998b). On the other hand, storing them under modified atmosphere (7% O₂ and 10% CO₂) does not affect the total flavonoid content to any great extent (Gil et al., 1998b).

OTHER VEGETABLES

Avocado

Golan et al. (1977) reported that mesocarp (edible portion of avocado) of Fuerte cultivar of avocado contains 287 mg of phenolics/kg of fresh weight expressed as chlorogenic acid equivalents; they identified four phenolic acids, namely, *p*-coumaric, caffeic, ferulic and protocatechuic acids. Later, Torres et al. (1987) found that the total content of phenolics in mesocarps of four avocado cultivars ranged from 1100 to 1800 mg/kg of fresh weight expressed as gallic acid equivalents. These authors identified 16 phenolic acids in avocado mesocarp: *p*-hydroxybenzoic, *o*-pyrocatechuic, protocatechuic, α - β - and γ -resorcylic, caffeic, *o*-, *m*- and *p*-coumaric, and syringic, ferulic, sinapic, isovanillic and vanillic acids. Two anthocyanins, cyanidin 3-galactoside and cyanidin 3,5-diglucoside acylated with *p*-coumaric acid, have also been detected (Prabha et al., 1980).

Pepper

Consumed as fresh or pickled fruits, as well as spices and seasonings, over 200 *Capsicum* cultivars are cultivated around the world (Contreras-Padilla and Yahia, 1998). The quality of peppers is affected by their color, aroma and pungency. Capsaicinoids are responsible for the development of pungency in pepper (Kirschbaum-Titze et al., 2002a, b).

Total content of soluble phenolics in pepper is between 1180 and 3849 mg of chlorogenic acid equivalents/kg of fresh weight (Lee et al., 1995; Racchi et al., 2002). Flavonoids and capsaicinoids are the predominant phenolics found in pepper. Small amounts of phenolic acids such as protocatechuic, chlorogenic, coumaric and ferulic acids are also detected. Of these, protocatechuic, chlorogenic and coumaric acids are the major phenolic acids in the fruit of pepper (Figure 4.1 and Figure 4.15) (Contreras-Padilla and Yahia, 1998; Estrada et al., 2000). Various phenolic acid glycosides have been identified; these include *cis-p*-coumaric acid 4-*O*- β -D-glucoside, *trans*-sinapoyl β -D-glucoside and vanilloyl β -D-glucoside (Iorizzi et al., 2001). The total content of phenolic acids declines during fruit ripening from 215 μ g/kg (14 days after flowering) to 73 μ g/kg (42 days after flowering) of dry weight (Estrada et al., 2000). This decrease in phenolic acids has been linked to increase in capsaicinoid content (Contreras-Padilla and Yahia, 1998).

Quercetin and luteolin (Figure 4.2), two major flavonoids found in pepper fruits (*Capsicum* species), are present in fruits in the form of glycosidic conjugates (Iorizzi et al., 2001; Sukrasno and Yeoman, 1993). The total content of flavonoids in pepper cultivars ranges from 1.75 mg/kg of fresh weight in *Capsicum Chinese* cv. Red Savina to 851.53 mg/kg of fresh weight in yellow wax (*Capsicum annum* L.) cv. Hungarian Yellow (Howard et al., 2000; Lee et al., 1995). Recently, Iorizzi et al. (2001) reported the occurrence of lignin glycoside icaraside E₅, a potential antioxidant containing a vanillyl moiety, in *Capsicum* species.

Genus *Capsicum* contains a unique group of phenolic compounds, known as capsaicinoids, responsible for the development of pungency in the pepper fruit (Figure 4.31) (Bennett and Kirby, 1968; Leete and Loudon, 1968). These phenolics

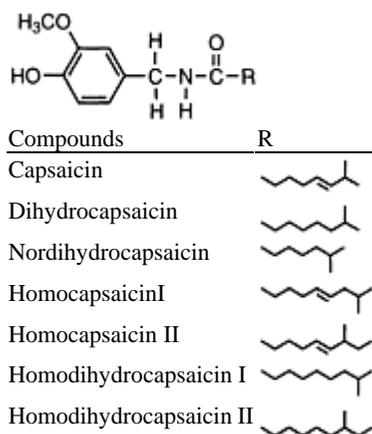


FIGURE 4.31 Chemical structures of some capsacinoids.

are amides of vanillylamine and C_8 and C_{13} branched fatty acids and their synthesis occurs on the membrane of vacuoles in the placenta of the fruit (Fujikawe et al., 1982; Gassner, 1973; Suzuki and Iwai, 1984) by condensation of vanillylamine with fatty acids. The fatty acid moiety of the capsacinoid is derived from L-valine, L-leucine, and L-isoleucine; the vanillylamine is formed from L-phenylalanine via phenylpropanoid pathway (Bennett and Kirby, 1968; Curry et al., 1999; Estrada et al., 1999a; Fujikawe et al., 1982; Leete and Loudon, 1968; Sukrasno and Yeoman, 1993; Suzuki and Iwai, 1984). Furthermore, Sukrasno and Yeoman (1993) suggested that, in *Capsicum frutescens*, glycosylated phenolics may act as a source of intermediates for capsacinoids synthesis. Later, Sakamoto et al. (1994) found no correlation between the accumulation of free phenolics and capsacinoids in *Capsicum annuum*. Furthermore, individual capsacinoids are not transformed into one another in the pepper fruit (Kopp and Jurenitsch, 1982), but their levels are controlled by the pool of CoA derivatives of fatty acids (Fujikawe et al., 1980; Suzuki and Iwai, 1984).

Capsacinoids are accumulated predominantly in the epidermal tissue of the placenta (Ishikawa et al., 1998; Iwai et al., 1979; Rowland et al., 1983; Suzuki et al., 1980). Over 15 capsacinoids have been isolated and identified (Bennett and Kirby, 1968; Constant and Cordell, 1996; Suzuki and Iwai, 1984). Of these, capsaicin (8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin contribute about 90% to the total pungency. Nordihydrocapsaicin is considered a third major pungent principle in the fruit of pepper (Iwai et al., 1979; Kosuge and Furata, 1970). The fruits of nonpungent cultivars of *Capsicum*, on the other hand, contain nonpungent capsacinoid-like substances (CLS) that are esters of vanillyl alcohol and fatty acids (Yazawa et al., 1989). Kobata et al. (1999) proposed to name this group of compounds “capsinoids” (Figure 4.32). Capsilate (4-hydroxy-3-methoxybenzyl (*E*)-8-methyl-6-nonenoate), dihydrocapsilate (4-hydroxy-3-methoxybenzyl-8-methyl-6-nonanoate) and nordihydrocapsilate (4-hydroxy-3-methoxybenzyl-7-methyloctanoate) are the predominant capsinoids in nonpungent pepper fruits,

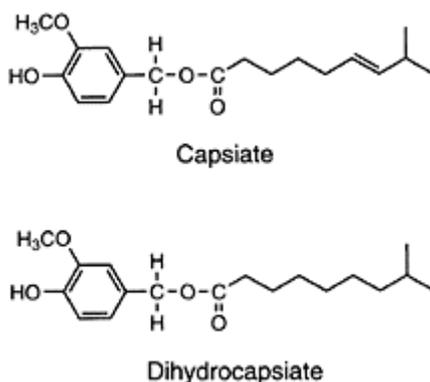


FIGURE 4.32 Chemical structures of some capsaicinoid-like substances.

but several minor CLS compounds have also been detected (Kobata et al., 1998, 1999).

The total content of capsaicinoids in the fruits of pepper ranges from 189 to 778 mg/kg of fresh weight (Contreras-Padilla and Yahia, 1998; Kirschbaum-Titze et al., 2002a, b; Rowland et al., 1983) and is affected by the species, growing season, ripening, and environmental factors such as light, temperature, fertilizer supplementation, as well as processing after harvesting (Contreras-Padilla and Yahia, 1998; Estrada et al., 1997, 1998, 1999a, b, 2000; Govindarajan, 1986; Iwai et al., 1979; Jurenitsch et al., 1979). *C. frutescens* varieties produce more pungent fruits compared to those of *C. annuum* varieties (Jurenitsch et al., 1979). The first signs of capsaicinoid accumulation in fruits are detected 15 to 20 days after flowering.

As *Capsicum* fruits begin developing, the content of capsaicinoids levels off between 25 and 50 days after flowering (Estrada et al., 1997, 1998, 1999b, 2000; Iwai et al., 1979; Suzuki et al., 1981) and then declines (Contreras-Padilla and Yahia, 1998). Oxidative degradation of capsaicinoids and/or phenolic precursors of capsaicin biosynthesis by peroxidase isoenzyme B₆, located in the placental epidermal cells (Bernal et al., 1993a, b, c, 1994, 1995; Contreras-Padilla and Yahia, 1998), have been implicated in the loss of pungency in later stages of fruit development. Bernal et al. (1995) demonstrated involvement of hot pepper peroxidase in oxidation of capsaicin precursors, caffeic and ferulic acids, leading to insolubilization of phenylpropanoid precursors. Later, Bernal and Ros Barcelo (1996) also identified 5,5'-dicapsaicin, 4'-*O*-5-dicapsaicin ether and dehydrogenation polymers with high molecular weights as products of capsaicin oxidation by peroxidase.

The fruits of pepper harvested between June and September contain a higher level of capsaicinoids compared to those harvested in October (Estrada et al., 1999b). Variations in capsaicinoid levels have also been found among fruits harvested at the same time from the same plant as well as plants subjected to identical growing conditions (Kirschbaum-Titze et al., 2002b). The content of capsaicinoids does not correlate with color (Kirschbaum-Titze et al., 2002a), but is affected by the location of fruit on the plant. The apical fruits contain higher levels of capsaicinoids (1090 mg/kg of dry weight) compared

to those harvested from the middle and basal (780 mg/kg of dry weight) segments of the plant (Estrada et al., 2002).

Upon heating, capsaicin decomposes to three major products: vanillin, 8-methyl-6-nonenamide and 8-methyl-6-nonenic acid. Other minor products of thermal decomposition include heptane, 2-methoxyphenol, 2-methoxy-4-methylphenol, methylvanillin, n-vanillyl-di(8-methyl-6-nonen)imide, iso- and nonanamide, pentamide and nonanic acid (Henderson and Henderson, 1992). Storing halved fruits at 4°C for 15 days does not have any detrimental effect on the capsaicinoid content in fruits, but storing minced fruits at 4°C results in the loss of 90% of the original amount of capsaicinoids after several days. Much lower losses of capsaicinoids are observed when minced fruits are stored under nitrogen (Kirschbaum-Titze et al., 2002a). Enzymatic metabolism has been found responsible for the reduction of capsaicinoids in minced fruits; this was affected by temperature and water activity (Titze and Petz, 1999).

Tomato

The total flavonol content of tomatoes grown in different countries ranges from 1.3 to 36.4 mg of quercetin/kg of fresh weight (Dewanto et al., 2002; Stewart et al., 2000) but, according to Hertog et al. (1992a) and Crozier et al. (1997), the total quercetin content is between 1.3 and 203 mg quercetin/kg of fresh weight. Flavonols are located mostly in the tomato skin and only small quantities are found in the flesh and seeds (Table 4.23) (Stewart et al., 2000).

The content of flavonols in tomatoes is affected by the cultivar, harvesting season (Crozier et al., 1997; Stewart et al., 2000) and environmental stresses such as the amount of daylight and temperature, nutritional status of soil, application of plant growth regulators, and invasion with pathogens (Bongue-Bartelsman and Phillips, 1995; Christie et al., 1994; Dixon and Paiva, 1995; Flors et al., 2001; Landry et al., 1995). Cherry tomatoes contain a much higher level of flavonols (17 to 203 mg/kg of fresh weight) than larger size tomato cultivars (2.2 and 11.2 mg/kg of fresh weight) (Crozier et al., 1997).

TABLE 4.23

Distribution of Flavonols in Spanish Cherry Tomatoes cv. Paloma

| Sample | (% Total Flavonols) | | | Total Flavonols (mg/kg f.w. ^a) |
|--------|---------------------|----------------------|-----------------------|--|
| | Free | Conjugated Quercetin | Conjugated Kaempferol | |
| Whole | 2.8 | 92.5 | 4.7 | 25.3±1.3 |
| Skin | 0.8 | 96.2 | 3.0 | 143.3±5.8 |
| Flesh | 8.3 | 75.0 | 17.7 | 1.2±0.1 |
| Seed | 20.0 | 66.7 | 13.3 | 1.5±0.1 |

^a f.w.=fresh weight.

Source: Adapted from Stewart, A.J. et al., 2000, *J. Agric. Food Chem.*, 48:2663–2669.

Furthermore, marked seasonal variations in the flavonol content have been observed. The content of flavonols in Spanish cherry tomatoes var. Paloma, harvested in February

1997 and February 1998, varied between 10.2 and 27.8 mg of quercetin/kg of fresh weight (Stewart et al., 2000), while that of English cherry tomatoes var. Favorita, harvested between February 1995 and August 1995, ranged from 17 to 77 mg of quercetin/kg of fresh weight (Crozier et al., 1997). Exogenous application of a plant growth regulator (mixture of 10 mM adipic acid monoethyl ester, 7.5 mM 1,2,3,4-tetra-O-acetyl- β -D-glucopyranose, and 2.5 mM furfuralamine) during the vegetative development cycle of the tomato plant (at a 0.2% dose) effects an increase in the content of soluble phenolics and flavonoids by over 100% (Flors et al., 2001).

Quercetin conjugates are the predominant form of flavonols found in tomatoes, but smaller quantities of kaempferol conjugates and traces of free aglycons have also been detected (Crozier et al., 1997; Stewart et al., 2000). Flavonols of tomatoes are a mixture of quercetin 3-rhamnosylglucoside (rutin), quercetin 3-rhamnosyldiglucoside, kaempferol 3-rhamnosylglucoside and kaempferol 3-rhamnosyldiglucoside (Woldecke and Herrmann, 1974b). Presence of *p*-coumaric acid conjugate of rutin has also been reported (Buta and Spaulding, 1997). Of these, rutin is the major flavonol in tomatoes (Stewart et al., 2000; Woldecke and Herrmann, 1974b).

Immature green tomato fruits contain a high level of chlorogenic acid in the pericarp and pulp. The level of chlorogenic acid rapidly declines as the color of the fruit changes from green to pink and then to red. The content of rutin also reaches a maximum in green tomato and then decreases during successive stages of fruit development (Buta and Spaulding, 1997; Fleuriet and Macheix, 1981). On the other hand, *p*-coumaric acid glucoside has been found only in the pulp and reaches a maximum level at the breaker stage (i.e., when the fruit color changes from green to pink). However, no rapid decline in *p*-coumaric acid glucoside content has been noted at successive stages of fruit development (Buta and Spaulding, 1997). Changes in chlorogenic acid and rutin are similar to those observed for auxin (indole-3-acetic acid) metabolism. Therefore, rutin (Figure 4.20) and chlorogenic acid (Figure 4.15) have been suggested as regulators of auxin metabolism during ripening of tomato fruit (Buta and Spaulding, 1994; Hoche et al., 1992; Jacobs and Rubery, 1988). Cohen (1996) demonstrated that supplying tomato fruits with exogenous indole-3-acetic acid delayed their ripening.

Frying, boiling or microwaving removes 35 to 78% of quercetin conjugates originally present in tomatoes. These losses may be due to the degradation or extraction of flavonols from tomato by water (Crozier et al., 1997). Tomato juice and puree are a rich source of flavonols. Processing tomatoes to juice and puree increases the content of free quercetin by up to 30%, an increase that may be brought about by enzymatic hydrolysis of quercetin conjugates. Tomato juice and puree contain 15.2 to 16.9 mg/L and 16.6 to 72.2 mg/kg of fresh weight of flavonols, respectively. On the other hand, canned tomatoes are a poor source of flavonols (Stewart et al., 2000).

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5

Phenolic Compounds of Beverages

INTRODUCTION

Popular beverages in the world include tea, coffee, cocoa, beer, wine and fruit juices; all of these beverages contain phenolic compounds. The content of phenolics in beverages depends on species, degree of maturity and processing and climatic factors of the starting materials.

TEA

Tea has been cultivated and consumed in China and Asia for over 2000 years. Tea beverage is prepared by brewing the leaves of the plant *Camellia sinensis* (Balentine et al., 1997; Harbowy and Balentine, 1997; Ukers, 1994; Wilson and Clifford, 1992). Approximately 20% of world tea production is in the form of green tea—a product obtained by rapid inactivation (dry heating or steaming) of the phenol oxidase (phenolase) present in tea leaves. Lack of enzymatic oxidation of phenolics gives the green tea its light color and characteristic astringency. Red and yellow teas are semifermented products with some of the characteristics of green tea and black tea. Phenolics of oolong and Pouchong teas are 70 and 30% oxidized, respectively (Balentine et al., 1997).

Black tea is one of the most popular beverages in the world and is traditionally made from flush that comprises the apical bud with two or three leaves. Production of black tea involves six stages:

1. Flush is plucked at different intervals, depending on the season, to obtain the best possible chemical composition (Sanderson, 1964). Plucking may be done by hand or using harvesting machines.
2. Flush is dried out in a process called withering. During this period a number of chemical changes takes place (Graham, 1983).
3. Dried tea leaves are rolled to disrupt the tissue, which brings about a cellular decompartmentation and provides conditions required for oxidation of polyphenols in the cell vacuole by the tea oxidative enzymes.
4. Broken tea leaves are fermented at room temperature in the presence of oxygen and high humidity. During this process polyphenols in tea undergo nonenzymatic and enzymatic oxidation, leading to the formation of theaflavins and thearubigins (Robertson, 1992).

TABLE 5.1
Content of Polyphenols in Various Types of
Green and Black Tea^a

| Tea | Content |
|------------------------|---------|
| Green Tea | |
| Finest grade (Japan) | 132 |
| Popular grade (Japan) | 229 |
| Popular grade (China) | 258 |
| Black Tea | |
| High grown (Sri Lanka) | 280 |
| Low grown (Sri Lanka) | 302 |

^a Grams per kilogram of dry weight.

Source: Adapted from Wickremasinghe, R.L. 1978. Tea, *Adv. Food Res.*, 24:229–286.

5. The fermentation process is terminated by firing with hot air, during which time and temperature are manipulated to obtain a final moisture content of about 3 to 5%.
6. Fermented tea leaves are graded (Graham, 1983).

COMPOSITION OF POLYPHENOLICS

Polyphenolic compounds constitute up to 35% of dry weight of tea (Table 5.1). The major constituents of tea polyphenolics are flavanols, such as (–) epicatechin gallate, (–) epigallocatechin, (–) epigallocatechin gallate, (–) epicatechin, (+) catechin (Figure 5.1), and their derivatives (Table 5.2), flavonols (quercetin, kaempferol and their glycosides), flavones (vitexin, isovitexin), phenolic acids, and depsides (gallic acid, chlorogenic acid, theogallin) (Balentine et al., 1997; Finger et al., 1991; Harbowy and Balentine, 1997; Wickremasinghe, 1978). The composition of unprocessed and black tea polyphenols is summarized in Table 5.3. According to Haslam (1989), tea flush (the immature vegetative portions of the tea plant) may contain up to 30% polyphenols, of which the major components are flavan-3-ols.

This finding was later confirmed by Yamanishi et al. (1995), who reported that the total content of polyphenols in tea flush is between 20 and 35% and that flavanols comprise up to 90% of total polyphenols. Soluble polyphenols constitute about 15% (w/w) of black tea. However, their composition may vary depending on the variety of tea, its geographical origin, and environmental conditions, as well as agronomic situation. According to Chen et al. (2001), the total contents of epicatechin derivatives in Chinese black teas is between 2.4 and 5.1 g/kg; in Chinese oolong tea it ranges from 41.4 and 46.3 g/kg and in Chinese green tea between 80 and 144.4 g/kg. Epigallocatechin gallate is the most abundant epicatechin derivative in teas.

Recently, Sano et al. (1999) isolated two novel catechin derivatives with a potent antiallergic activity from Taiwanese oolong tea and elucidated their structures as

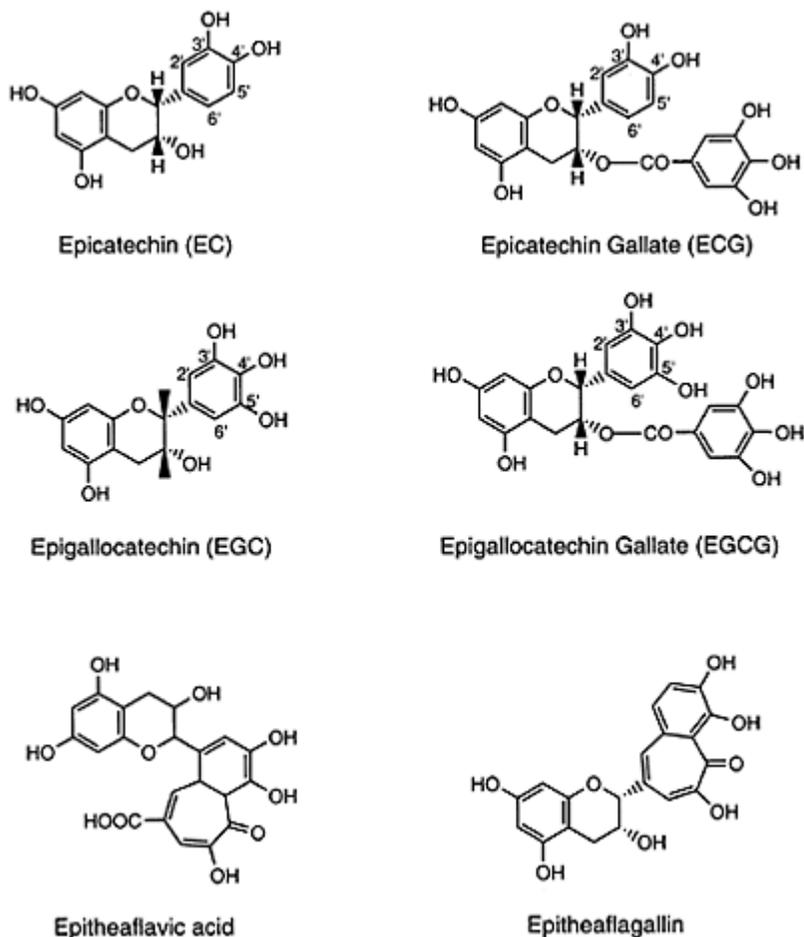


FIGURE 5.1 Structures of catechins, epitheaflavonic acid and epitheaflagallin found in tea.

(-)-epigallocatechin 3-*O*-(3-*O*-methyl)gallate (C-1) and (-)-epigallocatechin 3-*O*-(4-*O*-methyl)gallate (C-2). Of the green tea leaves tested, 13 of 15 contain from 0.2 to 8.2 g of C-1/kg; C-2 is detected only in one cultivar. On the other hand, oolong tea cultivar, Tong ting, contains 3.6 and 2.0 g/kg of C-1 and C-2, respectively. These authors demonstrated that oral doses between 5 and 50 mg/kg of C-1 and C-2 show greater inhibition of type I allergic (anaphylactic) reaction in mice, sensitized with ovalbumin than the major tea catechin (-)-epigallocatechin 3-*O*-gallate. Recently, Lakenbrink et al. (1999) isolated two novel proanthocyanidins from Chinese green tea (Lung Ching from Zhejiang) and elucidated their structures as epiafzelechingallate(4 β →8)-epicatechingallate and epiafzelechingallate-(4 β →6)-epicatechin-gallate.

FERMENTATION AND POLYPHENOLICS

A critical process for production of good quality tea, fermentation leads to the formation of orange-yellow to red-brown pigments and volatile flavor compounds

TABLE 5.2
Content of Some Flavanols in Fresh Tea Flush^a

| Compound | Content^b |
|------------------------------|----------------------------|
| (-) Epicatechin | 10–30 |
| (-) Epicatechin gallate | 30–60 |
| (-) Epigallocatechin | 30–60 |
| (-) Epigallocatechin gallate | 90–130 |
| (+) Catechin | 10–20 |
| (+) Gallocatechin | 30–40 |

^a Grams per kilogram of dry weight.

Source: Adapted from Wickremasinghe, R.L. 1978. Tea, *Adv. Food Res.*, 24:229–286.

TABLE 5.3
Polyphenolic Composition of Tea (Assam Variety)

| Phenolic Compounds | Content^a |
|--------------------------------------|----------------------------|
| Unprocessed tea | |
| Flavanols (catechins+gallocatechins) | 170–300 |
| Flavonols+flavonol glycosides | 30–40 |
| Flavandiols (leucoanthocyanidins) | 20–30 |
| Phenolic acids+depsides | 50 |
| Black tea | |
| Dialysable thearubigins+bisflavanols | 20–40 |
| Other soluble thearubigins | 15 |
| Theaflavins | 10–20 |
| Phenolic acids and depsides | 40 |
| Unchanged flavanols | 10–30 |
| Flavonols+flavonol glycosides | 20–30 |

^a Grams per kilogram of dry weight.

Source: Adapted from Stagg, G.V. and Millin, D.J., 1975, *J. Sci. Food Agric.*, 26:1439–1459.

(Balentine et al., 1997; Harbowy and Balentine, 1997). During this process polyphenolases catalyze the oxidation of polyphenolic compounds present in tea leaves to their corresponding *o*-quinones (Figure 5.2). Oxidation of flavan-3-ols, leading to the formation of theaflavins and thearubigins responsible for the formation of the characteristic color and flavor of fermented tea, is catalyzed by catechol oxidase (Robertson, 1992). Therefore, control of the fermentation process has important effects on the flavor and color of tea, which depend on the degree of oxidation of

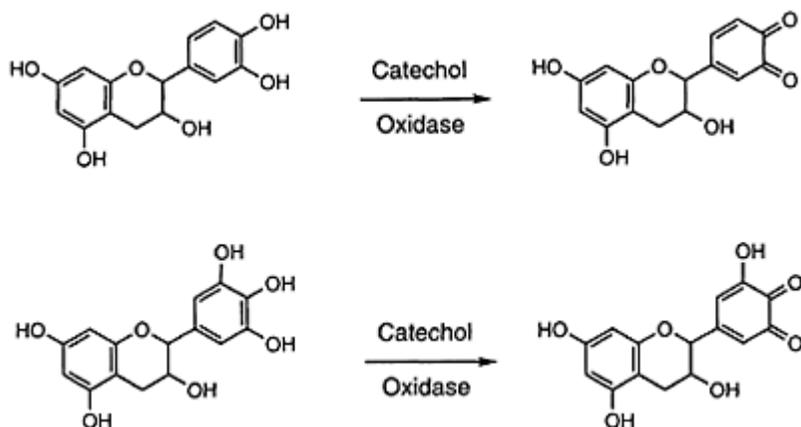


FIGURE 5.2 Oxidation of catechin (top) and epigallocatechin (bottom) to o-quinones.

tea phenolics. The rate and degree of polyphenolic oxidation depends on the composition, distribution, and content of flavanols in fresh tea shoot, activity of oxidizing enzymes, degree of tissue damage and cellular disruption, and temperature and oxygen content of the fermenting tea leaves. The polyphenolase is a copper-containing enzyme with a molecular weight of 140,000 Da located in the leaf microsomes (Balentine, 1992; Graham, 1983). A correlation exists between the flavor and activity of tea polyphenolases as reported by Roberts (1952).

Flavanols such as catechins and their derivatives, as well as flavonols such as quercetin, myricetin and kaempferol, constitute 15 to 25% of dry weight of tea. The content of some flavanols found in fresh tea flush is given in Table 5.2 and Table 5.3. Epigallocatechin gallate and epigallocatechin constitute the major catechins in green tea leaves. During fermentation, oxidation of flavanols to quinones is followed by a Michael addition of gallocatechin quinone to catechin quinone, carbonyl addition across the ring and decarboxylation (Balentine, 1992). This leads to the formation of theaflavins and finally to polymeric compounds referred to as thearubigins (Figure 5.3). It has also been suggested that thearubigins are formed from theaflavins via coupled oxidation with other polyphenolic compounds (Roberts, 1962). In addition, during the initial stages of fermentation, thearubigins may also be formed directly from flavanols by oxidative transformation (Brown et al., 1969). Therefore, reactions leading to the formation of theaflavins and thearubigins are competitive.

As the fermentation progresses, fewer theaflavins and more thearubigins are found in fermented tea leaves. Small quantities of other oxidation products have also been identified in black tea, namely, theaflavic acids and theaflagallins (Figure 5.1). Theaflavic acids are products of the reaction of gallic acid and catechin quinones, while theaflagallins are products of the reaction of gallic acid with gallocatechin quinones (Robertson, 1992). The composition of phenolic compounds in black tea is given in Table 5.4.

The values provided in Table 5.3 indicate that fermentation significantly reduces the total content of flavanols in processed tea; however, other polyphenolic sub-stances in their monomeric form (flavanols and flavanol glycosides) remain unchanged throughout the fermentation process and thus coexist with oxidized

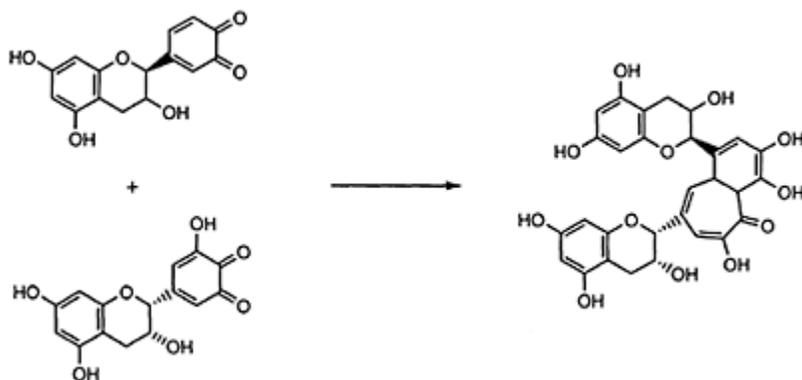


FIGURE 5.3 Formation of theaflavin from oxidized flavan-3-ols.

TABLE 5.4

Effect of Fermentation on Distribution of Catechin Derivatives in Taiwanese Teas^a

| Tea Variety | EGCG | EGC | ECG | EC | C | GC | C-1 | Total |
|-------------|-------|------|------|------|-----|-----|-----|-------|
| Benihomare | | | | | | | | |
| Green tea | 97.4 | 45.8 | 22.7 | 11.1 | 1.8 | 2.4 | 5.8 | 187.0 |
| Oolong tea | 92.0 | 35.2 | 20.2 | 9.0 | 2.1 | 2.2 | 4.7 | 165.4 |
| Black tea | 3.4 | 1.3 | 0.5 | 0.8 | 0.2 | 0.7 | – | 6.9 |
| Izumi | | | | | | | | |
| Green tea | 131.1 | 47.6 | 29.1 | 10.6 | 3.6 | 3.6 | 0.4 | 226.0 |
| Oolong tea | 109.9 | 38.3 | 25.1 | 8.6 | 3.3 | 3.9 | 0.2 | 189.3 |
| Black tea | 4.8 | 0.9 | 1.0 | 1.0 | 0.5 | 0.7 | 0.2 | 9.1 |

Note: EGCG=epigallocatechin gallate; EGC=epigallocatechin; ECG=epicatechin gallate; EC=epicatechin; C=catechin; GC=gallocatechin; C-1=epigallocatechin 3-*O*-(3-*O*-methyl)gallate.

^a Grams per kilogram of dry weight.

Source: Adapted from Sano, M. et al., 1999, J. Agric. Food Chem., 47:1906–1910.

polyphenols in black tea. Changes brought about by fermentation in the distribution of catechins in some Chinese tea cultivars are shown in Table 5.4. Theaflavins and thearubigins are considered primary and secondary products of polyphenol oxidation, respectively (Haslam, 1989). The relative content of theaflavins and thearubigins is determined by the fermentation temperature, pH and plucking intervals.

Low-temperature fermentation enhances the production of theaflavins during the initial stages and reduces the chance of conversion of theaflavins to thearubigins during

the latter stages. On the other hand, tea produced at higher fermentation temperatures contains more thearubigin pigments, including a nondialyzable polymeric fraction (Cloughley, 1980). This nondialyzable material is composed largely of an ill-defined polyphenols-protein complex. It contains a polyphenolic component formed by excessive polymerization during the fermentation process (Millin et al., 1969; Sanderson et al., 1972). Subramanian et al. (1999) showed that lowering the pH of macerated tea leaves from 5.5 (the normal pH of tea leaves) to 4.5 increases the content of theaflavins up to 40%. These researchers also demonstrated, *in vitro*, that hydrogen peroxide is generated during oxidation of catechins by polyphenol oxidase. This hydrogen peroxide is utilized for oxidation of theaflavins to thearubigins. The content of theaflavins also depends on plucking intervals. A shorter plucking interval produces tea with a higher content of theaflavins (Owuor, 1990).

Sanderson et al. (1972) studied the biochemistry of tea fermentation in order to determine the role of various flavanols as precursors of polyphenolic compounds found in black tea. A number of flavanols isolated from fresh tea leaves were subjected to oxidation catalyzed by tea enzymes. Results of this study indicate that theaflavins are formed according to the following oxidative condensation reactions:

- (-)Epicatechin+(-)epigallocatechin+3/2 O₂→theaflavin+CO₂
- (-)Epicatechin+(-)epigallocatechin-3-gallate+3/2 O₂→theaflavin gallate A+CO₂
- (-)Epicatechin-3-gallate+(-)epigallocatechin+3/2 O₂→theaflavin gallate B+CO₂
- (-)Epicatechin-gallate+(-)epigallocatechin-3-gallate+3/2 O₂→theaflavin digallate+CO₂

During the fermentation of flavanols some bisflavanols (colorless, reactive substances also known as theasinensins) are also formed (Figure 5.4). Their rearrangement leads to the formation of undefined black tea flavonoids (Hashimoto et al., 1988; Nonaka et al., 1983). Reactions leading to the formation of bisflavanols are given below:

- 2(-)epigallocatechin-3-gallate+1/2 O₂→bisflavanol A+H₂O
- (-)epigallocatechin-3-gallate+(-)epigallocatechin+1/2 O₂→bisflavanol B+H₂O
- 2(-)epigallocatechin+1/2 O₂→bisflavanol C+H₂O

Theaflavins are yellow-orange in color and contribute to the astringency as well as another flavor characteristic of black tea known as “briskness” (Mcdowell et al., 1995; Sanderson and Graham, 1973). Hilton and Ellis (1972) reported a correlation between the content of theaflavins and taste sensory scores of tea from Central Africa. Oxidized flavanols may also react with amino acids and carotenoids. In addition, Strecker degradation of amino acids may lead to the formation of various aldehydes, and ionones and their derivatives may be formed from carotenoids. These reaction products contribute to the aroma of fermented tea (Sanderson et al., 1971).

Thearubigins, the polymeric form of oxidized flavanols, are dominant phenolics of black tea (Table 5.5). These heterogenous oxidation products may be derived not

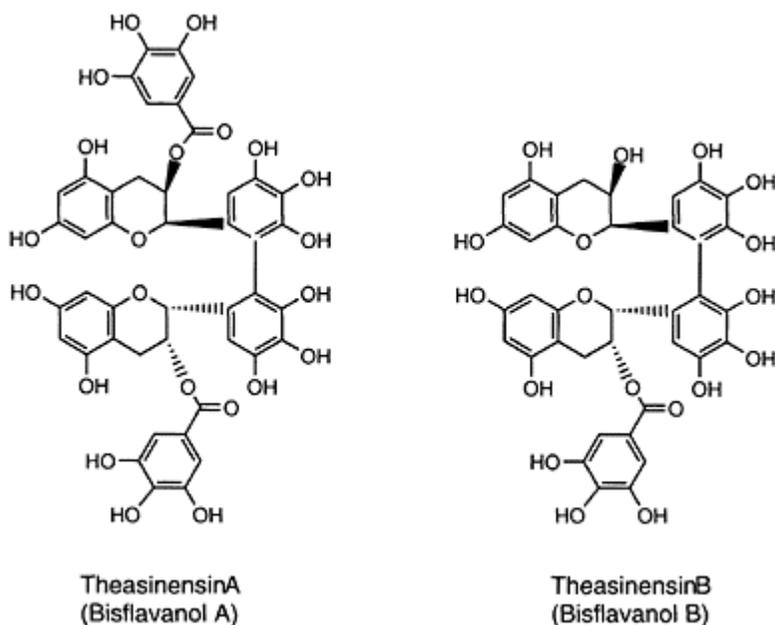


FIGURE 5.4 Chemical structure of bisflavanol A, also known as theasinensin A.

only from theaflavins but also from oxidative condensation of all tea flavanols (Sanderson et al., 1972). Recently, Tanaka et al. (2001) demonstrated two types of oxidative dimerization of theaflavins to thearubigins. One reaction leads to the formation of bistheaflavin A via self-association of theaflavin into antiparallel dimer at the benzotropolone rings, followed by their oxidative C–C coupling. Another reaction leads to the formation of bistheaflavin B via autoxidation of theaflavins. According to these authors, bistheaflavin B is probably formed by intermolecular cyclization between dehydrotheaflavin and dehydrotheanaphthoquinone.

Thearubigins are black-brown in color and contribute to the color, acidity, body and astringency of tea. Thearubigins found in hot water extracts of tea are mixtures of polymers with a wide molecular weight range. While some thearubigins contain up to 100 flavonoid subunits, on average, four to five subunits of flavonoid thearubigins are found in hot water extracts of tea. These compounds are heterogenous polymers of catechin and flavan-3-ol gallates linked at C4, C6, C8, C2', C5' and C6' positions in flavon-3-ol units (Ozawa et al., 1996). Two fractions have been isolated from black tea thearubigins, namely, theafulvin and oolongtheanin (Bailey et al., 1992; Hashimoto et al., 1988). Acid hydrolysis of thearubigins results in the formation of cyanidin and delphinidin (Balentine, 1992; Sanderson, 1972).

BREWING

Tea beverage is commonly prepared by brewing tea in hot water in a proportion of 1g of tea leaf to 100 mL of water. The concentration of solids in tea infusion after 3 min of brewing ranges from 2.5 to 3.5 g/L, but black and green tea infusions differ in their composition of phenolics as affected by the manufacturing process (Balentine et al., 1997).

TABLE 5.5
Composition of Phenolics in Black Tea Leaves^a

| Polyphenols | Content |
|--------------------------|---------|
| Catechin | 2.3 |
| Epicatechin | 4.1 |
| Epicatechin gallate | 8.0 |
| Epigallocatechin | 10.5 |
| Epigallocatechin gallate | 16.5 |
| Flavonol glycosides | 0.5 |
| Theaflavin | 2.5 |
| Theaflavin gallate | 6.6 |
| Thearubigins | 59.5 |

^a Grams per kilogram of dry weight.

Source: Adapted from Balentine, D., 1992, in *Phenolic Compounds in Food and Their Effects on Health*, Ho, C.-T., Lee, C.Y., and Huang, M.T., Eds., American Chemical Society, Washington, D.C., 102–117.

Green tea infusions have a clear, greenish-yellow color and a balanced taste of bitterness, astringency, and sweet aftertaste (Sanderson et al., 1976; Wickremasinghe, 1978). The distinct sensory quality of green tea extract is affected by the addition of sugar, milk, lemon and heat processing as well as storage (Wang et al., 2000). Therefore, production of a high-quality canned green tea beverage is a difficult task. Recently, Wang et al. (2000) evaluated the effect of 1-min heat treatment at 121°C and accelerated 12-day storage at 50°C (storage conditions equivalent to 70-day storage at 20°C) on flavanols and sensory qualities of green tea extracts. Green tea infusions used in this study were prepared by extraction of green tea with hot water (80°C) at a 1:160 ratio (tea leaves to water, w/w). Epigallocatechin gallate (EGCG) and epigallocatechin (EGC) accounted for 80% of total epicatechins of green tea infusion. After processing and accelerated storage, 86% of EGCG and 79% of EGC were not accounted for in the tea infusion. Therefore, according to these authors, changes in EGCG and EGC may be responsible for changes in color and taste of green tea brought about by processing and storage. Chen et al. (2001) observed epimerization of EGCG to gallic catechin gallate when pure EGCG is subjected to autoclaving at 120°C for 20 min.

The stability of green tea catechins is pH dependent. Green tea catechins are very unstable at pH values of >7.0 and degrade in a few minutes, but very stable at pH < 4.0 for at least 18 h. Moreover, boiling green tea catechins in water (pH = 4.9) for 7 h reduces the

total content of catechins by only 15% (Zhu et al., 1997). The mechanism of catechin breakdown under alkaline conditions is still unknown. However, it has been demonstrated that at pH>7.0, catechins tend to form semi-quinone free radicals (Guo et al., 1996; Yoshioka et al., 1991). Later, Chen et al. (1998) observed that addition of ascorbic acid at a level of 0.2g/L significantly

TABLE 5.6
Content of Polyphenols in Consumer Tea Leaves and Brews

| Tea Source | Polyphenols | Tea Leaves ^a | Tea Brew ^b | |
|------------|-----------------|-------------------------|-----------------------|------|
| | | | 40–60 | 120 |
| U.K. | Total phenolics | 160.0 | 591–694 | 843 |
| U.S. | | 162.9 | 533 | 967 |
| U.K. | Catechins | 13.4 | 39.1–48.1 | 56.2 |
| U.S. | | 36.5 | 18.5 | 37.1 |
| U.K. | Theaflavins | 15.4 | 55.3–70.2 | 77.4 |
| U.S. | | 11.7 | 24.4 | 39.4 |
| U.K. | Flavonols | 8.6 | 50.2–54.0 | 63.0 |
| U.S. | | | 37.8 | 67.2 |

^a Grams per kilogram.

^b Milligram per liter.

Source: Adapted from Lakenbrink, C. et al., 2000, *J. Agric. Food Agric. Chem.*, 48:2848–2852.

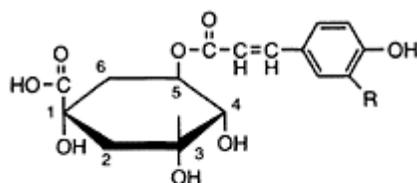
improves the stability of green tea catechins at pH>7.0. These authors suggested that ascorbic acid might protect catechins by recycling the catechin free radical forms or by slowing down their oxidation by removing oxygen from the solution.

Liebert et al. (1999) evaluated the effect of different brewing times and conditions on the extraction of total phenolics from black tea. All tea infusions were prepared at tea leaves-to-water ratio of 1:77. The total content of phenolics in tea infusion ranges from 338 mg/L after 0.5 min to 684 mg/L after 10 min of brewing time. Stirring black tea during infusion increased the total phenolic content in tea extract up to 40%. The content of flavonol glycosides in tea infusions is also affected by brewing conditions and varies from 36.5 to 88.3 mg/L (Price et al., 1998). Arts et al. (2000) later reported that the maximum catechin concentration in tea infusion is nearly reached after 5 min of brewing. Lakenbrink et al. (2000) subsequently reported that only 35 to 55% of total phenolics, total flavonoids, catechins and theaflavins originally present in tea leaves are extracted at brew times of up to 2 min (Table 5.6).

COFFEE

Coffee beans are seeds of red cherries of evergreen shrubs belonging to the *Rubiaceae* subfamily *Cinchonoideae* tribe *Coffeae*. Two genera containing numerous species and varieties of coffee, namely, *Coffea* and *Psilanthus*, belong to this tribe. An example is *Coffea* subgenera *Coffea*, which contains 85 species of coffee. Of these, only two species,

known as *Coffea arabica* and *Coffea canephora* (commonly referred to as arabicas and robustas, respectively), are of commercial importance (Flament, 1995).



R = H 5-*p*-Coumaroylquinic Acid

R = OH 5-Caffeoylquinic Acid

R = OCH₃ 5-Feruloylquinic Acid

FIGURE 5.5 Structure of chlorogenic acid and its derivatives.

The cherry usually has one or two seeds (beans) that account for 55% of the weight of fruit dry matter. The pericarp pulp, a by-product obtained during wet-processing of fruit, accounts for 29% of the cherry. Most of the pulp solids are currently discarded. Because this may bring about serious pollution problems, attempts have been made to utilize coffee pulp as feed for cattle, swine and poultry. However, the presence of antinutritional factors such as caffeine and polyphenols in the pulp used in animal feed impairs the growth rate of experimental animals when used in excess of 10% of rations. Elucidation of the exact chemical nature of compounds responsible for these effects requires further investigation (Bressani and Graham, 1980; Clifford and Ramirez-Martinez, 1991).

GREEN COFFEE BEANS

Green coffee beans may contain at least five major groups of chlorogenic acid isomers: caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA), feruloylquinic acids (FQA), *p*-coumaroylquinic acids (CoQA) and caffeoylferuloylquinic acids (CFQA). Figure 5.5 depicts the general structure of chlorogenic acid. The contents of total chlorogenic, caffeoylquinic, and feruloylquinic acids for four varieties of green coffee beans are summarized in Table 5.7 and Table 5.8. Data presented in these tables indicate that Robusta coffee has a substantially higher content of chlorogenic acids. The total content of chlorogenic acids in *C. arabica* and *C. canephora* is 5 to 8% and 7 to 10% on a dry weight basis, respectively (Clifford, 1985), and varies with maturity (Clifford and Kazi, 1987). The content of chlorogenic acids in *C. canephora* accessions grown in Colombia is even higher and ranges from 12 to 13.5% (dry basis) (Guerrero et al., 2001). Vanillin, 4-ethylguaiacol and 4-vinyl-guaiacol have been identified among the 16 potent odorants in raw Arabica coffee beans. However, only the concentration of 4-vinylguaiacol exceeds the odor threshold (Czerny and Grosch, 2000).

Many attempts have been made to find a correlation between the level of total, or any specific isomer of, chlorogenic acid and any quality characteristic of beverages such as astringency. However, data available in the literature to indicate such

TABLE 5.7
Content of Chlorogenic Acid Isomers in Some Green Coffee Beans

| Isomer | Samples ^a | | | | |
|-------------------------|----------------------|------|------|------|------|
| | B-1 | B-2 | B-3 | B-4 | B-5 |
| 3-CQA | 3.0 | 2.6 | 2.5 | 2.3 | 2.4 |
| 4-CQA | 4.9 | 4.8 | 5.4 | 4.8 | 5.2 |
| 5-CQA | 45.6 | 39.7 | 47.2 | 39.2 | 40.0 |
| Total CQA | 53.3 | 47.1 | 55.1 | 46.3 | 47.7 |
| 5-FQA | 7.9 | 3.3 | 3.5 | 3.3 | 3.4 |
| 3,4-diCQA | 4.4 | 0.9 | 0.8 | 1.0 | 0.9 |
| 3,5-diCQA | 5.6 | 2.6 | 2.2 | 3.0 | 2.5 |
| 4,5-diCQA | 0.5 | 2.3 | 1.6 | 2.6 | 2.1 |
| Total diCQA | 10.5 | 5.8 | 4.5 | 6.6 | 5.6 |
| Total chlorogenic acids | 71.7 | 56.2 | 63.1 | 56.2 | 56.7 |

Note: Results are average of duplicate determinations for dry matter; CQA=caffeoylquinic acids; FQA=feruloylquinic acids; diCQA=dicaffeoylquinic acids; B-1=*Coffea canephora*; B-2=Timor hybrid (*C. arabica* x *C. robusta*); B-3=catimor (Timor hybrid x *C. arabica* var. caturra); B-4=*Coffea arabica* var. caturra vermelho; B-5=*Coffea arabica* var. bourbon vermelho.

^a Grams per kilogram of dry weight.

Source: Adapted from Clifford, M.N. and Ramirez-Martinez, J.R., 1991, *Food Chem.*, 40:35–42.

TABLE 5.8
Content of Feruloylquinic Acids, Caffeoylquinic Acids, and Total Chlorogenic Acids in Green Coffee Beans^a

| Phenolic Acids | Arabica | | Robusta | |
|-------------------------|-----------|-----------|-----------|-----------|
| | Santos | Sao Paulo | Ghana | Uganda |
| Feruloylquinic acids | | | | |
| Range | 2.3–3.3 | 0–2.1 | 11.6–12.0 | 5.4–6.8 |
| Mean | 2.8 | 0.6 | 11.8 | 6.1 |
| Caffeoylquinic acids | | | | |
| Range | 60.8–62.6 | 56.2–58.2 | 79.2–84.3 | 77.1–80.9 |
| Mean | 61.7 | 57.2 | 81.8 | 79.1 |
| Total chlorogenic acids | | | | |
| Range | 64.2–64.8 | 56.5–59.1 | 92.6–94.7 | 83.9–86.6 |
| Mean | 64.5 | 57.8 | 93.6 | 85.2 |

^a Grams per kilogram of dry weight.

Source: Adapted from Clifford, M.N. and Wright, J., 1976, *J. Sci. Food Agric.*, 27:73–84.

correlations are scarce. Nonetheless, it is commonly recognized that Arabica coffee with a lower content of chlorogenic acid is superior to Robusta coffee. Recently, Guerrero et al. (2001) developed a multivariate model for prediction of coffee genotype that correlates HPLC chromatographic profiles of 80% aqueous ethanolic extracts of coffee with chlorogenic acid contents.

ROASTED COFFEE BEANS

Phenols play an important role in the formation of coffee flavor. From over 800 volatile compounds found in roasted coffee aroma, only 42 have been identified as phenols (Flament, 1989, 1995; Nijssen et al., 1996). Phenolic compounds are indigenous or are produced from thermal degradation of chlorogenic acid and lignins. Time and temperature of roasting influence the phenols produced and their composition. Roasting results in a significant decline in the content of chlorogenic acid in green coffee beans; the content of chlorogenic acid decreases rapidly as temperature increases throughout the roasting cycle (Merrit and Proctor, 1959a). A longer roasting time enhances the decomposition of chlorogenic acids in coffee. During a 7-min roasting, 77 and 60.9% of chlorogenic acids present in Robusta green beans of Tanzania and Arabica green beans of Guatemala are decomposed, respectively (Table 5.9). Light roasting produces a higher level of caffeoylquinic, dicaffeoylquinic, and feruloylquinic acids in Robusta coffee than in Arabica coffee.

The rates of degradation of chlorogenic acids in these two types of coffee may change significantly as the roasting process continues. Darker Arabica coffee contains more caffeoylquinic acids than Robusta coffee; however, the total content of dicaffeoylquinic and feruloylquinic acids is higher in darker Robusta coffee (Table 5.10). Thus, the generally accepted inferior quality of Robusta as compared with Arabica coffee may be due to the presence of a higher concentration of dicaffeoylquinic and feruloylquinic acids (Trugo and Macrae, 1984a). The dicaffeoylquinic acid isomers have a “peculiar lingering metallic taste” that may play a role in the acceptability of coffee (Clifford and Ohiokpehai, 1983). Figure 5.6 outlines the degradation of quinic and caffeic acids to phenolics found in coffee aroma; the content of some phenols found in coffee aroma is provided in Table 5.11. Catechol is the predominant volatile phenolic compound found in coffee aroma, followed by 4-ethylguaiacol, 4-ethylcatechol, pyrogallol, quinol, and 4-vinylcatechol. A higher guaiacol content in the roasted Robusta compared to Arabica may be associated with greater destruction of feruloylquinic acid during the roasting process. According to Czerny et al. (1999), 4-vinylguaiacol is one of the character impact odorants of roasted Arabica coffee. The compounds 4-vinylguaiacol, 4-ethylguaiacol and vanillin are the predominant volatiles in roasted Arabica beans (Czerny and Grosch, 2000).

The ratio of 5-caffeoylquinic acid to caffeine has been proposed as an indicator for monitoring the roasting process of coffee. During roasting, the content of chlorogenic acid declines while caffeine remains virtually unchanged. Because of this, caffeine may be used as an internal standard, thus allowing measurement of the level of chlorogenic acid in roasted coffee bean independent of weight losses due to formation of volatiles during roasting. This ratio may be useful for measuring the compositional changes of roasted coffee, especially those produced by fast roasting

TABLE 5.9
The Decline in Chlorogenic Acid Contents
during Roasting of Coffee Beans^a

| Sample | Roasting | | Chlorogenic Acid Isomers | | | Total |
|-----------|----------|---------------|--------------------------|-------|-----|-------|
| | Time | Loss | CQA | diCQA | FQA | |
| Arabicas | | | | | | |
| Colombia | 0 | 0 | 42.8 | 9.1 | 3.3 | 55.2 |
| | | not specified | 16.9 | 2.5 | 0.5 | 19.9 |
| Guatemala | 0 | 0 | 57.6 | 8.7 | 2.5 | 68.8 |
| | 420 | – | 23.8 | 2.2 | 0.9 | 26.9 |
| | 600 | – | 19.8 | 1.5 | 0.8 | 22.2 |
| | 780 | – | 7.1 | 0.3 | 0.3 | 7.7 |
| | 1140 | – | 2.2 | 0.1 | 0.1 | 2.4 |
| Tanzania | 0 | 0 | 32.6 | 12.0 | 2.9 | 51.9 |
| | – | 7 | 21.1 | 5.9 | 1.8 | 31.0 |
| | – | 9 | 13.7 | 4.8 | 1.8 | 22.1 |
| | – | 10 | 11.9 | 4.6 | 1.8 | 20.0 |
| | – | 11 | 8.8 | Nd | 0.3 | 9.1 |
| Robusta | | | | | | |
| Uganda | 0 | – | 68.3 | 13.8 | 6.0 | 88.1 |
| | 300 | – | 30.2 | 2.9 | 2.4 | 35.4 |
| | 420 | – | 17.8 | 1.4 | 1.5 | 20.7 |
| | 840 | – | 5.2 | 0.5 | 0.5 | 6.2 |
| | 960 | – | 1.4 | 0.2 | 0.1 | 1.8 |

Note: CQA caffeoylquinic acids; diCQA dicaffeoylquinic acids; FQA feruoylquinic acids.

^a Grams per kilogram of dry weight.

Source: Adapted from Smith, A.W., 1985, in *Coffee, Vol. 1. Chemistry*, Clark, R.J. and Macrae, R., Eds., Elsevier Applied Science, London, 1–41.

(3 to 5 min of roaster residence time). Shorter roasting times promote the so-called “case roasting,” i.e., uneven roasting from the surface to the center of the individual bean (Purdon and McCamey, 1987).

INSTANT COFFEE

The content of Chlorogenic acids in instant coffee depends on the nature of roasting and extraction conditions; Table 5.12 shows the content of Chlorogenic acid isomers in five selected commercial instant coffees. The major isomer found in all the samples is 5-caffeoylquinic acid, which represents about 30% of the total, whereas the sum of the caffeoylquinic acid isomers accounts for approximately 70% of the phenolic compounds. The feruloylquinic and dicaffeoylquinic acids accounted for about 20 and 10% of the instant coffee phenolics, respectively. The decaffeination process brings about considerable loss in the chlorogenic-acid content of coffee. Decaffeinated coffee may

contain approximately three times less chlorogenic acid (3.6%) than that found in “mild” coffee (10.7%) (Trugo and Macrae, 1984b).

TABLE 5.10
Effect of Roasting of Coffee Beans on Content of Chlorogenic Acids^a

| Coffee | Chlorogenic Acid | Green Coffee | Type of Roasting | | | |
|------------------------|------------------|--------------|------------------|--------|------|-----------|
| | | | Light | Medium | Dark | Very Dark |
| Arabica (Guatemala) | Total CQA | 57.61 | 23.78 | 19.84 | 7.10 | 2.22 |
| | 5-FQA | 2.49 | 0.86 | 0.84 | 0.30 | 0.08 |
| | Total diCQA | 8.67 | 2.24 | 1.53 | 0.31 | 0.12 |
| | Total | 68.77 | 26.88 | 22.21 | 7.71 | 2.42 |
| Robusta (Uganda) | Total CQA | 68.23 | 30.20 | 17.82 | 5.17 | 1.41 |
| | 5-FQA | 6.04 | 2.39 | 1.50 | 0.46 | 0.11 |
| | Total diCQA | 13.77 | 2.85 | 1.42 | 0.52 | 0.24 |
| | Total | 88.04 | 35.44 | 20.74 | 6.15 | 1.76 |

Notes: CQA=caffeoylquinic acids; diCQA=dicaffeoylquinic acids; 5-FQA=5-feruloylquinic acid. Roasting times at 205°C: light=7 min; dark=13 min; very dark=19 min.

^a Grams per kilogram of dry weight.

Source: Adapted from Trugo, L.Z. and Macrae, R., 1984b., *Analyst*, 109:263–266.

BREWING METHOD

The extraction of Chlorogenic acids into the coffee beverage depends on a number of factors, including the proportion of ground coffee to water, freshness of roasted coffee, grind, method of brewing, temperature of water and length of time that water is in contact with the grounds.

Higher brewing temperatures result in greater extraction of Chlorogenic acid from the coffee grounds into the brew. The extraction rate increases over the 10 min of brewing generally employed, although this increase is less pronounced after the first 2 min of brewing. About 69% of the Chlorogenic acid is extracted into the coffee brew under conditions normally encountered during coffee brewing, i.e., 2-min contact time at 93°C (Table 5.13) (Merrit and Proctor, 1959). Holding the coffee brew at elevated temperatures results in loss of Chlorogenic acid, which depends on temperature and time. About 15% of Chlorogenic acid is lost after 24 h of holding at 83°C. The decrease in chlorogenic acid may play an important role in flavor changes in the coffee brew (Segall and Proctor, 1959).

Table 5.14 summarizes the amount of chlorogenic acid extracted by pouring 150 mL of boiling water over 6, 9 and 12 g of coffee. The brew obtained using 6 g of coffee contains 1.34 to 1.67 g of chlorogenic acid per liter. When 12 g of coffee is used, the amount of chlorogenic acid ranges between 2.62 and 3.65 g per liter. The pH of coffee is not significantly affected by the content of chlorogenic acid (Hamboyan and Pink, 1990).

FEATHERING OF COFFEE CREAM

Polyphenolic compounds present in coffee, notably chlorogenic and caffeic acids, are implicated in curdling of unstabilized coffee cream added to hot coffee, also

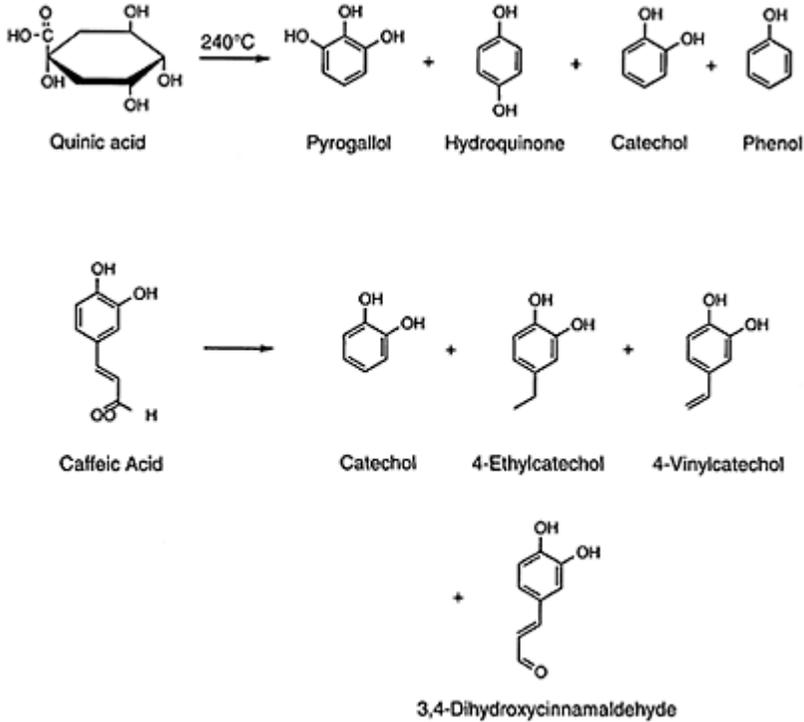


FIGURE 5.6 Degradation of quinic acid and caffeic acid during roasting.

known as feathering (Charley, 1982). The extent of curdling, however, is not uniquely correlated with the content of chlorogenic acid in the coffee. The concentration of chlorogenic acid together with a parameter describing the shape of UV spectrum in the 230- to 340-nm region may serve as a reliable criterion for predicting the occurrence of feathering in hot coffee (Hamboyan et al., 1989).

COCOA

Cocoa beans are seeds found in woody plants (shrubs or small trees) belonging to the genus *Theobroma*. Only two species of this genus, *Criollo*, bearing warty fruits containing white or faintly purple seeds, and *Forastero*, bearing smoother fruits containing seeds of a deeper purple shade, are recognized from a commercial standpoint. Numerous hybrids containing genes of both species have recently been developed (Flament, 1989).

Manufacturing cocoa involves fermentation, drying, cleaning, roasting, and milling steps. The fermentation process reduces the astringency, acidity and bitterness of cocoa (Bonvehi and Coll, 1997a, b; Luna et al., 2002). The precursors of cocoa aroma are formed during the fermentation and drying/roasting steps. Drying/roasting

TABLE 5.11
Content of Phenols in Coffee Aroma^a

| Compound | Arabica | Arabusta | Robusta |
|-----------------------------|---------|----------|---------|
| Phenol | 13.0 | 9.5 | 17.0 |
| 2-Methylphenol | 1.2 | 0.7 | 1.1 |
| 4-Methylphenol | 1.3 | 0.3 | 1.0 |
| 3-Methylphenol | 0.7 | 1.0 | 1.2 |
| 3-Ethylphenol | – | 0.4 | – |
| 4-Vinylphenol | 0.2 | 0.2 | 0.2 |
| Guaiacol | 2.7 | 3.9 | 8.4 |
| 4-Ethylguaiacol | 0.3 | 1.2 | 5.6 |
| 4-Vinylguaiacol | 9.5 | 18.4 | 19.5 |
| Vanillin | 5.2 | 4.4 | 5.0 |
| Catechol | 80.0 | 95.0 | 120.0 |
| 4-Methylcatechol | 16.0 | 10.0 | 13.0 |
| Quinol | 40.0 | 25.0 | 30.0 |
| 4-Ethylcatechol | 37.0 | 20.0 | 80.0 |
| 4-Vinylcatechol | 25.0 | 15.0 | 25.0 |
| Pyrogallol | 45.0 | 25.0 | 35.0 |
| 1,2,4-Trihydroxybenzene | 20.0 | 6.0 | 13.0 |
| 3,4-Dihydroxycinnamaldehyde | 10.0 | 5.0 | 12.0 |
| 3,4-Dihydroxybenzaldehyde | 20.0 | 8.0 | 9.0 |

^a Milligrams per kilogram of dry weight.

Source: Adapted from Smith, A.W., 1985, in *Coffee*, Vol. 1. *Chemistry*, Clark, R.J. and Macrae, R., Eds., Elsevier Applied Science, London, 1–41.

contributes significantly to changes in the composition of cocoa beans and is responsible for the development of the specific cocoa aroma due to partial degradation of cocoa components and their subsequent interactions (Cros et al., 1999; Rohan, 1969; Ziegleder and Biehl, 1988). After fermentation cocoa beans contain about 50 g of water per 100 g; therefore, drying is also necessary to preserve beans (Hor et al., 1984). Cocoa beans that contain more than 6 to 7 g of water per 100-g sample are prone to microbial (fungi) and enzymatic (lipoxygenase, lipase, peroxidase and polyphenol oxidase) degradation (Villeneuve et al., 1985). Cocoa liquor, a component of all chocolate products, is prepared by finely grinding the nib of the cocoa bean, while cocoa powder is prepared by removing part of the cocoa butter from the liquor (Apgar and Tarka, 1998; Vinson et al., 1999).

COMPOSITION OF POLYPHENOLICS

Polyphenolic compounds comprise approximately 2% of fresh unfermented cocoa beans (*Theobroma cacao*) (Porter et al., 1991). Epicatechin, catechin, epigallocatechin and epicatechin-based procyanidins have been identified as the predominant phenolics in the flesh of fresh unfermented cocoa seeds (Porter et al., 1991);

TABLE 5.12
Chlorogenic Acid Isomers Contents in Some Commercial Instant Coffees

| Isomer | Samples ^a | | | | |
|-------------------------|----------------------|------|-------|------|------|
| | C-1 | C-3 | C-5 | C-7 | C-9 |
| 3-CQA | 13.8 | 9.0 | 18.9 | 7.5 | 10.0 |
| 4-CQA | 16.5 | 10.4 | 22.5 | 8.7 | 11.6 |
| 5-CQA | 22.5 | 13.8 | 35.0 | 10.2 | 16.9 |
| Total CQA | 52.8 | 33.2 | 76.4 | 26.4 | 38.5 |
| 3-FQA | 2.5 | 1.7 | 3.5 | 1.5 | 2.1 |
| 4-FQA | 5.9 | 4.4 | 7.6 | 3.2 | 4.9 |
| 5-FQA | 5.8 | 3.2 | 8.2 | 2.7 | 4.1 |
| Total FQA | 14.2 | 9.3 | 19.3 | 7.4 | 11.1 |
| 3,4-diCQA | 2.1 | 1.0 | 3.9 | 0.9 | 1.2 |
| 3,5-diCQA | 1.6 | 0.7 | 2.9 | 0.7 | 0.8 |
| 4,5-diCQA | 0.3 | 1.4 | 4.8 | 0.7 | 1.0 |
| Total diCQA | 4.0 | 3.8 | 11.6 | 2.3 | 3.0 |
| Total chlorogenic acids | 71.0 | 45.6 | 107.3 | 36.1 | 52.6 |

Notes: Results are average of duplicate determination for dry matter; CQA=caffeoylquinic acids; FQA =feruloylquinic acids; diCQA=dicafeoylquinic acids. C-1 to C-9 samples are commercial instant coffees.

^a Grams per kilogram of dry weight.

Source: Adapted from Trugo, L.Z. and Macrae, R., 1984, Analyst, 109:263–266.

TABLE 5.13
Effect of Brewing Conditions (Temperature and Time) on Extraction of Chlorogenic Acid from Coffee^a

| Brewing Temperature | Brewing Time (min) | | | | |
|---------------------|--------------------|------|------|------|------|
| | 0.5 | 1.0 | 2.0 | 5.0 | 10.0 |
| 100 | 15.8 | 16.9 | 22.6 | 26.8 | 29.8 |
| 120 | 20.8 | 22.2 | 26.3 | 29.8 | 34.9 |
| 140 | 22.3 | 23.0 | 26.7 | 33.9 | 37.3 |
| 160 | 23.6 | 25.1 | 29.6 | 37.2 | 38.4 |
| 180 | 35.8 | 28.2 | 33.3 | 39.2 | 41.6 |
| 200 | 26.9 | 30.0 | 35.4 | 41.5 | 43.7 |

Note: Roasted ground coffee contained 51.3 (g/kg) chlorogenic acid.

^a Grams per kilogram of dry weight.

Source: Adapted from Merrit, M.C. and Proctor, B.E., 1959b, Food Res., 24:735–743.

TABLE 5.14
The Content of Total Chlorogenic Acid and pH
of Coffee Beverage as Affected by Portion of
Coffee Grinds Used

| Coffee Sample | Portion of Grinds Used (g) | Chlorogenic Acid (g/L) | pH |
|---------------|----------------------------|------------------------|------|
| CD | 12 | 3.65 | 4.99 |
| | 6 | 1.79 | 5.01 |
| CS | 12 | 2.67 | 5.58 |
| | 9 | 1.92 | 5.70 |
| | 6 | 1.34 | 5.79 |
| IG | 12 | 2.86 | 5.32 |
| | 9 | 1.95 | 5.31 |
| | 6 | 1.37 | 5.32 |
| TC | 12 | 2.62 | 5.23 |
| | 9 | 2.17 | 5.00 |
| NS | 12 | 2.80 | 5.07 |
| | 9 | 2.39 | 5.31 |
| | 6 | 1.67 | 5.16 |

Source: Adapted from Hamboyan, L. and Pink, D., 1990, J. Dairy Sci., 57:227–232.

Thompson et al., 1972; Villeneuve et al., 1989). The following have been identified as the major flavanoids in fresh cocoa beans:

- Epicatechin-(4 β →8)-catechin (procyanidin B-1)
- Epicatechin-(4 β →8)-epicatechin (procyanidin B-2)
- [Epicatechin-(4 β →8)]₂-epicatechin (procyanidin C-1) (Thompson et al., 1972)
- Epicatechin-(2 β →5, 4 β →6)-epicatechin
- 3T-*O*- β -D-galactopyranosyl-ent-epicatechin-(2 α →7, 4 α →8)-epicatechin
- 3T-*O*- β -L-arabinopyranosyl-(2 α →7, 4 α →8)-epicatechin (Porter et al., 1991)

Presence of higher molecular weight proanthocyanidin oligomers with up to 9 monomer units (nonamers) in raw cocoa and up to 10 units (decamers) in chocolate have been reported (Hammerstone et al., 1999; Porter et al., 1991). In addition, two quercetin glycosides, the 3-*O*-glucoside and 3-*O*-galactoside, have been identified in cotyledons of fresh cocoa beans (Jalal and Collin, 1977).

Polyphenolic compounds comprise 12 to 18% of the weight of whole dry bean and are implicated in the formation of the characteristic flavor and color of fermented cocoa (Bracco et al., 1969). Approximately 35% of polyphenol content of unfermented *Forastero* cocoa beans is (-)-epicatechin (Forsyth, 1955, 1963). On the other hand, the content of (-)-epicatechin in freshly harvested *Catongo* and *Forastero* cocoa beans of verified genetic origin ranges from 34.65 to 43.27 mg/g of defatted sample as determined by high-performance liquid chromatography (Table 5.15 and

TABLE 5.15

Concentration of (-) Epicatechin in Defatted Cocoa Beans Unaffected by Postharvest Variables^a

| Clone | Variety | Content |
|----------|------------------|------------|
| NA-22-23 | Nacional | 37.63±0.22 |
| N-22-A3 | Nacional | 34.65±0.20 |
| UP-667 | Trinitario | 41.33±0.54 |
| UF-11 | Trinitario | 39.33±0.71 |
| UF-296 | Trinitario | 36.33±1.30 |
| EEG-29 | Amazon Forastero | 35.33±1.18 |
| SIC-250 | Amazon Forastero | 43.27±0.44 |

Notes: Pods opened, testa removed, and cotyledons freeze-dehydrated.

^a Grams per kilogram of dry weight.

Source: Adapted from Kim, H. and Keeney, P.G., 1984, *J. Food Sci.*, 49:1090-1092.

TABLE 5.16

Concentration of (-)Epicatechin in Fermented Defatted Cocoa Beans from Shipments Representing Several Countries of Production^a

| Bean Source | Content |
|-----------------------|---------|
| Ivory Coast | 6.22 |
| Maracaibo (Venezuela) | 3.62 |
| Samoa | 10.64 |
| Trinidad | 4.68 |
| Bahia (Brazil) | 8.23 |
| Ghana | 4.52 |
| Lagos (Nigeria) | 4.68 |
| Costa Rica | 16.52 |
| Arriba (Ecuador) | 8.08 |
| Jamaica | 2.66 |

Note: Results are mean of duplicate injections of a single extract.

^a Grams per kilogram of dry weight.

Source: Adapted from Kim, H. and Keeney, P.G., 1984, *J. Food Sci.*, 49:1090-1092.

Table 5.16) (Kim and Keeney, 1984). Moreover, clovamide (N-(3',4'-dihydroxy-*trans*-cinnamoyl)-3-(3,4-dihydroxyphenyl)-L-alanine) and deoxyclovamide (N-(4'-hydroxy-*trans*-cinnamoyl)-3-(3-hydroxyphenyl)-L-alanine), as well as quercetin 3-*O*-β-D-glucopyranoside and quercetin 3-*O*-α-D-arabinopyranoside, have been isolated and identified in cocoa liquor (Sanbongi et al., 1998).

The purple color of unfermented cocoa beans is due to the presence of anthocyanins, which constitute about 0.5% of the weight of the defatted beans. Two main components of anthocyanin fraction of cocoa beans are 3-β-D-galactosidyl- and 3-α-L-arabinosyl-cyanidins (Forsyth and Quesnel, 1957). Polyphenols present in cocoa and chocolate are

thought to be anticariogenic, but some may also have toxic and carcinogenic effects (Singleton and Kratzer, 1973; Singleton, 1981).

According to Bonvehi and Coll (1998), the total content of low molecular weight phenolics in cocoa powder, such as phenol, 2-methoxyphenol, 3-methylphenol, 4-methylphenol, 2,3-dimethylphenol, 3-ethylphenol, 4-ethylphenol, 3,4-dimethylphenol and 3,5-dimethylphenol, should not exceed 9.6 mg/kg. Increased levels of these phenolics indicate contamination with smoke and contribute to the development of smoky taste in cocoa powder. Poor drying and storage conditions are responsible for the contamination of cocoa powder with smoke. A sensorially acceptable cocoa powder should contain no more, per kilogram of sample, than 2 mg of phenol, 0.9 mg of 3-methylphenol, 0.55 mg of 2,3-dimethylphenol, 0.9 mg of 3-ethylphenol, and 0.7 mg of 4-ethylphenol.

EFFECT OF PROCESSING

Fermentation is a very complex process involving external microbiological processes and internal structural changes as well as enzymic reactions. Lactic acid- and acetic acid-producing bacteria and yeast are predominant microorganisms found in the fermented mass. Microbial processes such as formation of acetic acid bring about an increase in the temperature of fermented mass that often exceeds 45°C. The combination of increased temperature and ingress of acids inhibits germination of seeds and also contributes to structural changes in fermented beans, resulting in removal of the compartmentation of enzymes and substrates (Biehl and Adomako, 1983). Accumulation of acetic acid during fermentation lowers the pH of cocoa beans. This modifies biochemical processes leading to the development of cocoa flavor (Jinap, 1994), such as formation of amino acid and peptides (Biehl et al., 1993), reduction of sugars (Rohan and Stewart, 1966a, b) and loss of solubility by phenolics (Villeneuve et al., 1989).

Commercially, the degree of bean fermentation is determined by cutting 100 beans and recording the color of their cotyledons. Presence of brown color indicates that beans are fully fermented. During fermentation and subsequent drying, flavor precursors responsible for the development of the characteristic flavor of cocoa are formed (Rohan, 1963, 1964, 1967; Rohan and Connell, 1964; Rohan and Stewart, 1967a, b). These precursors include flavonoids (catechin, epicatechin and galocatechin) (Kharlamova, 1964), amino acids and sugars; however, unfermented cocoa beans do not contain these aroma precursors (Knapp, 1937). Recently, Luna et al. (2002) demonstrated the existence of a positive correlation between polyphenol levels and bitterness and astringency of cocoa liquors and also showed that polyphenols are essential contributors to the overall sensory characteristics of Ecuadorian cocoa liquors.

Fermentation and drying bring about complex chemical changes in cocoa polyphenolic compounds. Kim and Keeney (1984) reported a 90% drop in the concentration of epicatechin. Later, Porter et al. (1991) reported a similar drop in the concentration of tannins, but a large increase in the relative content of procyanidin B-1. Structural changes in cocoa phenolics generally occur after 24 to 48 h of bean fermentation. At this point, (-)epicatechin molecules diffuse from their storage cells, undergo oxidation in the presence of polyphenol oxidase, and polymerize with one another or with anthocyanidins to form complex phenolics (tannins) (Forsyth, 1963; Wong et al., 1990). The content of (-)epicatechin decreases sharply after 2 or 3 days of

fermentation (Figure 5.7), indicating the diffusion of (-)epicatechin from storage cells and the subsequent oxidation. The enzymic oxidation may continue during the drying stage, although some published data demonstrate a decline in the activity of cocoa polyphenol oxidase as the fermentation process progresses (Villeneuve et al., 1985). Thus, an increase in the oxidation rate may be due to greater penetration of oxygen into the cocoa bean mass. The enzymic oxidation of (-)epicatechins and leucocyanidins results in the formation of a brown color characteristic of chocolate caused by the production of melanin and melanoproteins (Griffiths, 1957). Eventually, when the moisture content in beans is insufficient, the enzymic oxidation reaction is inhibited. Another pathway for the loss of (-)epicatechin is its spontaneous nonenzymic oxidation.

During the fermentation process, anthocyanins are hydrolyzed to anthocyanidins that polymerize along with simple catechins to form complex tannins (Forsyth, 1963). Anthocyanins usually disappear rapidly during the fermentation process (Forsyth, 1952). After 4 days of fermentation, the content of anthocyanins decreases by up to 7% of its initial value (Figure 5.8). Underfermented beans are purple or slaty due to the presence of anthocyanin pigments, whereas fully fermented beans are quite brown. Thus, the content of anthocyanin may be considered a good index for determining the degree of cocoa bean fermentation. Further reduction in anthocyanin content is brought about by the drying process. Depending on the length of fermentation time, drying reduces the content of anthocyanins of beans by 13 to 44%. However, although drying unfermented beans results in loss of 79% of their anthocyanin, they still contain seven times more anthocyanin pigments than those of fermented products. A 97% loss of phenolics results from 4-day fermentation of beans followed by drying (Pettipher, 1986).

The fermentation and drying processes alter the content and composition of polyphenolic compounds. Fermented cocoa beans contain a lower amount of low molecular weight phenolics and an enhanced content of condensed phenolics. The latter compounds are involved in protein-phenol interactions and may contribute to the low digestibility and poor biological value of cocoa proteins (Chatt, 1953). Formation of the protein-phenol complex also reduces the bitterness and astringency associated with the presence of polyphenolics and reduces the unpleasant flavors and odors in roasted beans (Griffiths, 1957). On the other hand, the low molecular weight polyphenols still present in chocolate may be responsible for its astringent and bitter taste.

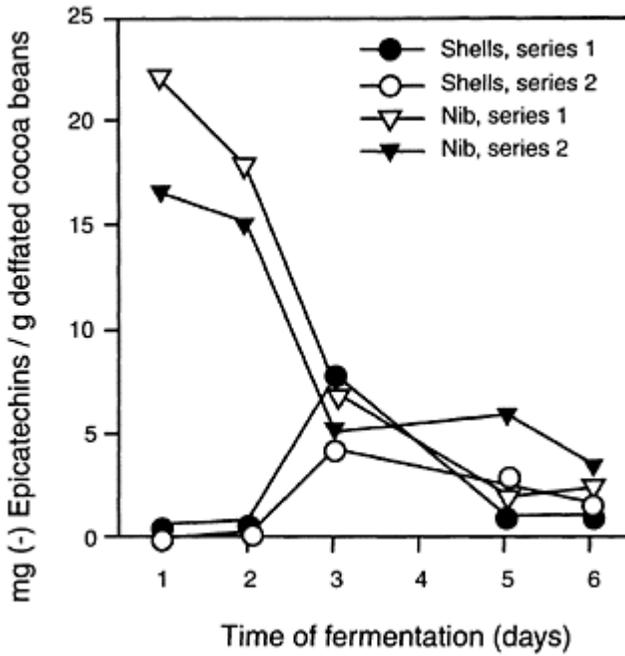


FIGURE 5.7 Changes in (-)epicatechin of Trinidad-Jamaica hybrid of cocoa beans during fermentation. (Adapted from Kim, H. and Keeney, P.G., 1984, *J. Food Sci.*, 49:1090–1092.)

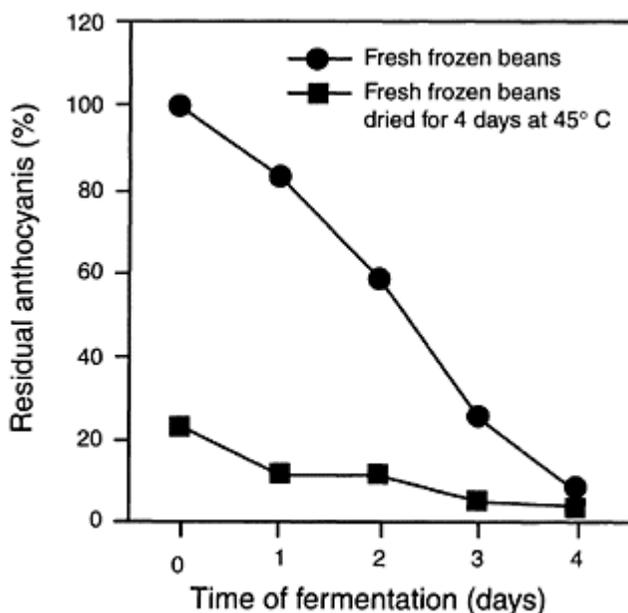


FIGURE 5.8 The effect of fermentation with or without drying on the anthocyanin content of cocoa beans. (Adapted from Pettipher, G.L., 1986, *J. Sci. Food Agric.*, 37:289–296.)

BEER

The characteristic flavor of beer arises from malt, the predominant source of carbohydrates, and hops, a source of bitter compounds. The production of beer involves malting, mashing, fermentation and storage. The malting and mashing produce a wort, an aqueous extract of malted barley and hops. Yeasts are added to this wort and utilize nutrients present in it to produce ethanol, carbon dioxide, and other by-products. The final product after removal of yeast is beer, which contains some malt and hops constituents as well as substances produced by metabolism of yeast.

COMPOSITION OF POLYPHENOLICS

Brewing material such as barley malt and other raw or malted grains (rice, corn, wheat) used as adjuncts, hops and other aromatic materials contribute phenolic and polyphenolic compounds to the early stages of the brewing process. Further polymerization of phenolics and formation of polyphenols can occur during wort boiling, and possibly

during fermentation and storage of beer. Beer polyphenolics furnish color, impart astringent taste, serve as a reservoir for oxygen reduction and substrate browning, and participate in precipitation of poorly coagulable beer proteins.

Phenolic compounds of beer contribute to its flavor quality and are also implicated in nonbiological haze formation (Charalambous, 1974; Dadic, 1974a, b; Gramshaw, 1970). Phenolic substances in beer are present in various forms; their volatility depends on their molecular weight (Table 5.17). Beer contains 283 mg/L of nontannin and nonflavonoid phenolic compounds (Fantozzi et al., 1998). Phenolic compounds of beer are classified as beneficial, harmful, or neutral in so far as their influence on beer stability and sensory properties is concerned (Dadic, 1971). Some beer phenolics may also act as antioxidants or may contribute to the formation of carbonyls in beer. It has been found that ferulic acid markedly increases the formation of carbonyls in beer aging under high and low air conditions. Moreover, ferulic acid and quercetin have been found to be active promoters of diacetyl formation in aging beer (Dadic, 1974b). Some 67 different phenolic compounds have been identified in beer. Simple phenolics, aromatic carboxylic- and phenol carboxylic acids, hydroxycoumarins, catechins, leucoanthocyanidins, anthocyanidins, flavonols, flavonones, flavones, prenylated flavonoids and phenolic glycosides are included in this list (Bohm, 1989; Drawert et al., 1977; Stevens et al., 1999a, b).

Hops serve as the main source of prenylated flavonoids (predominantly of chalcone type) in beer (Figure 5.9). Xanthohumol, desmethylxanthohumol and 3'-geranylchalconaringenin comprise over 95% of the prenylated flavonoid fraction of hops. In addition, hops contain small quantities of prenylated flavonones that are probably formed in hops upon drying and storage (Stevens et al., 1997). Total content of prenylated flavonoids in hops is between 2000 and 6000 mg/kg (Stevens et al., 1998, 1999b). During the boiling of hops with wort, prenylated chalcones undergo

TABLE 5.17

Classification of Beer Phenolic According to Volatility

Monomeric Monophenols

Monomeric Polyphenols

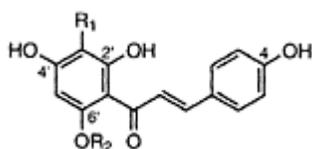
Polymeric Polyphenols

Volatility decreases→

Phenols, cresols, chlorophenols, guaiacols, aromatic alcohols, amines, phenolic and cinnamic acids, lactones, flavonols and glycosides, catechins, anthocyanogens, anthocyanidins, anthocyanins, polymers (tannins)

Source: Adapted from Dadic, M., 1974a, *MBAA Tech. Q.*, 11:140–145.

(a)



(b)

| | R ₁ | R ₂ |
|----------------------------|----------------|-----------------|
| Xanthohumol | isoprenyl | CH ₃ |
| Desmethylxanthohumol | isoprenyl | H |
| 3'-Geranylchalconaringenin | geranyl | H |

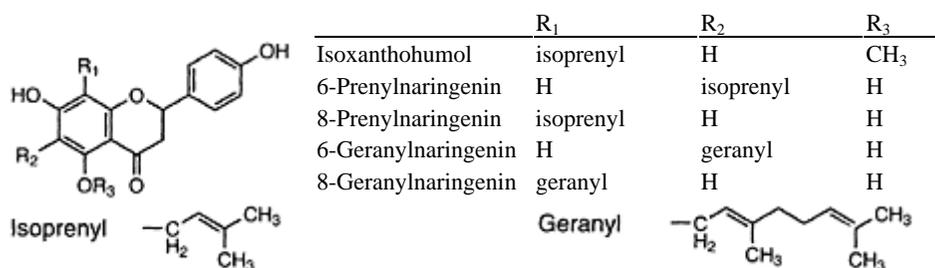


FIGURE 5.9 Chemical structures of some hops flavonoids (A) and their conversion products formed during processing (B).

cyclization into their isomeric flavanones. The presence of free 2'- or 6'-hydroxyl groups on A-ring of prenylated chalcone is a prerequisite for ring closure. Therefore, upon cyclization, xanthohumol yields only one flavanone, namely, isoxanthohumol; cyclization of desmethoxyxanthohumol leads to the formation of 6-prenylnaringenin and 8-prenylnaringenin. Also, 3'-geranylchalconaringenin converts during processing into 6-geranylnaringenin and 8-geranylnaringenin (Figure 5.9) (Stevens et al., 1999a, b).

According to Stevens et al. (1999a), the content of isoxanthohumol in beer accounts for only 22 to 30% of hops' xanthohumol; only 10% of the hops'

TABLE 5.18

Content of Phenolic Acids in Selected Beers^a

| Phenolic Acid | British Export A | American Malt Liquor | German Rauchbier | Irish Ale |
|-------------------------------|---------------------|-------------------------|---------------------|--------------|
| Benzole Acid Derivatives | | | | |
| Gallic acid | 1.8 | 1.4 | 3.5 | 0.3 |
| Protocatechuic acid | 1.0 | 0.5 | 1.2 | 0.2 |
| <i>p</i> -Hydroxybenzoic acid | 1.8 | 1.1 | 0.4 | 0.3 |
| Vanillic acid | 5.4 | 2.5 | 12.7 | 1.5 |
| Syringic acid | 1.2 | 0.8 | 2.2 | 0.7 |
| Cinnamic Acid Derivatives | | | | |
| Caffeic acid | trace | 0.1 | 1.0 | 0.1 |
| <i>p</i> -Coumaric acid | 1.1 | 1.9 | 4.6 | 1.0 |
| Ferulic acid | 2.7 | 4.2 | 10.8 | 1.1 |
| Sinapic acid | 1.4 | 1.1 | 3.0 | 0.2 |
| Total | 16.4 | 16.6 | 39.4 | 5.4 |

Note: Results are mean values of three samples measured by HPLC on ethyl acetate extracts.

^a Milligrams per liter.

Source: Adapted from McMurrough, I. et al., 1984, *J. Inst. Brew.*, 90:181–187.

desmethylxanthohumol is completely converted into prenylnaringenin during the brewing process. Therefore, these authors suggest that these prenylated chalcones may also be involved in chemical reactions other than isomerization. Milligan et al. (1999) demonstrated that 8-prenylnaringenin exhibits a greater estrogenic activity than known phytoestrogens such as coumestrol and genistein. Beer may contain up to 20 µg of 8-prenylnaringenin per liter (Tekel et al., 1999). Recently, Promberger et al. (2001) found that average estrogenic activity of beer equals that exerted by 43 ng of 17β-estradiol per liter of beer. Moreover, these authors suggested a negligible human health hazard from drinking beer containing compounds showing activity on the estrogen receptor *α*. *In vitro* studies also indicate that prenylchalcones and prenylflavones show greater protection for human low-density lipoprotein (LDL) from oxidation than *α*-tocopherol and genistein (Miranda et al., 2000).

The content of phenolic acids in selected British, American, German and Irish beers is shown in Table 5.18. The lowest content of phenolic acids is found in Irish beer, while German beer contains approximately five times more phenolics. The content of benzoic acid derivatives in the finished beer is higher than that of cinnamic acid derivatives (McMurrough et al., 1984). Italian lager beers contain only 1.187 mg of free phenolic acids per liter (Montanari et al., 1999), while samples of Spanish, Danish and German commercial brands contain 1.759 mg of free phenolic acids per liter (Bartolome et al., 2000). The major free phenolic acids in Italian beers are *m*-, *p*- and *o*-coumaric, and ferulic acids (Montanari et al., 1999), while vanillic, ferulic and *p*-coumaric acids are the predominant free phenolic acids in Spanish, German and Danish brands (Bartolome et al., 2000). Sinapic, vanillic, chlorogenic, homovanillic,

TABLE 5.19
Phenolic Acid Contents of Samples Taken at
Stages during Production of Commercial Lager^a

| Phenolic Acid | Unboiled Wort | Fermented Wort | Finished Beer |
|----------------------------------|---------------|----------------|---------------|
| Benzoic Acid Derivatives | | | |
| Gallic acid | 0.1 | 0.2 | 0.2 |
| Protocatechuic acid | 0.5 | 0.4 | 0.3 |
| 4-Hydroxybenzoic acid | 0.6 | 1.1 | 1.1 |
| Vanillic acid | 1.4 | 1.6 | 0.9 |
| Syringic acid | 0.6 | 0.4 | 0.3 |
| Cinnamic Acid Derivatives | | | |
| Caffeic acid | 0.1 | 0.4 | 0.3 |
| <i>p</i> -Coumaric acid | 0.6 | 0.4 | 0.3 |
| Ferulic acid | 1.3 | 1.7 | 1.8 |
| Sinapic acid | 0.4 | 0.5 | 0.4 |
| Total | 5.6 | 8.0 | 6.7 |

Note: Results are mean values of three samples in ethyl acetate extracts measured by HPLC.

^a Milligrams per liter.

Source: Adapted from McMurrough, I. et al., 1984, *J. Inst. Brew.*, 90:181–187.

p-hydroxybenzole, 2,6- and 3,5-dihydroxybenzoic, syringic, gallic, protocatechuic and caffeic acids are present in small quantities (Bartolome et al., 2000; Montanari et al., 1999). Other monomeric phenolics identified in beer include tyrosol, catechin, 2,3-dihydroxy-1-guaiacylpropan-1-one and vanillin (Bartolome et al., 2000). Monomeric phenolics account for 10 to 20% of the total content of beer phenolics (Sogawa, 1972). On the other hand, different batches of the same beers may differ in their content and composition of polyphenols (Table 5.19). This difference may be significant for the storage stability of beers (McMurrough et al., 1984).

The contents of catechin, epicatechin and galocatechin do not generally exceed 15 mg/L (Bellmer, 1977; Drawert et al., 1977; McMurrough et al., 1984); the total content of catechins found in beer is approximately 40 mg/L (Bellmer, 1977). Beer contains less than 1 mg/L hydroxycoumarins (umbelliferon, scopoletin, and daphnetin) as well as anthocyanins and anthocyanidins (cyanin, cyanidin, pelargonin, pelargonidin, and delphinidin) (Drawert et al., 1977). The normal concentration of dimeric procyanidins in beer does not exceed 4 mg/L (Bate-Smith, 1973; Haslam, 1974). The contents of leucocyanidin (5,7,3',4'-tetrahydroxyflavan-3,4-diols) and quercetin are less than 10 mg/L of beer. Moreover, flavones and flavonols such as vitexin, isoquercetin, quercetin, rutin, kaempferol, myricetin and myricitrin have also been found in beer at less than 1 mg/L (Drawert et al., 1977).

Phenolic compounds contribute to bitterness and astringency of beer, play a role in its harshness and intensify its color. Polymerization of phenolics during the mashing and postfermentation periods heightens these latter effects. The flavor thresholds of polyphenols in beer have been found to be 10 to 50 mg/L. The major flavors imparted to beer by pure phenolics, listed in the order of incidence, are sour-acidic, bitter, harsh, bitter-sweet, astringent and aromatic. On the other hand, the oxidation of phenolics during aging brings about the following major notes to beer: harsh, bitter, astringent, sour-acidic, dry and bitter-sweet. Harsh and bitter notes become more pronounced upon oxidation of phenolics due to simultaneous lowering of flavor thresholds (Dadic and Belleau, 1973). Cinnamic acid, after oxidation, gives an old aged taste to beer and *p*-coumaric acid produces a sharp bitter note. Oxidized gallic acid produces a lingering, bitter and astringent note along with depression of the normal flavor characteristics (Charalambous, 1974) while decarboxylation of hydroxycinnamic acids resulting from the formation of 4-vinylguaiacol and 4-vinylphenol gives off-flavor to beer (Madigan et al., 1994).

Tyrosine-derived phenolic amines such as hordenine, N-methyltyramine, and tyrosol possess a bitter taste that is particularly pronounced in lightly hopped beer. These phenolic amines have a taste threshold of 20 mg/L and, according to Charalambous (1974), also have a distinctive influence on the taste and aroma of beer. Presence of some tannin is also necessary to impart desirable qualities to beer. Sometimes an off-flavor described as "smoky" or "apothecary-like" is developed in beer; such beverages contain a higher than usual level of steam-volatile phenols. It is assumed that most of these compounds pass into beer from malt.

The malt obtained from directly fired kilns contains higher levels of steam-volatile phenols than that produced from indirectly heated kilns (Kieninger et al., 1977). A taste panel can detect 25 mg/L of tannins added to beer (Owades et al., 1958). Beer phenolics are also suspected as a common source of some flavor-active carbonyls such as

salicylaldehyde and vanillin (Palamand et al., 1971; Dadic et al., 1974). Average content of tannins in malt is 1.1 g/kg compared with 50 to 67 g/kg in hops. However, malt contributes twice as many tannins to beer as hops (Owades et al., 1958).

EFFECT OF PROCESSING

The content of phenolic acids in finished beer depends largely on the extent of their extraction during mashing. Mashing especially increases the content of hydroxycinnamic acid derivatives as a result of their release from previously unextractable combinations. McMurrough et al. (1984) found that mashing malt increases the total content of its phenolic acids from 65 mg/kg before mashing to 191 mg/kg after.

The extraction of phenolics from malt into the wort depends markedly on malt to water ratios. Wort and beer produced from thinner mashes (malt to water ratio of 1:4 and 1:5, respectively) contain higher levels of phenolics than those obtained from more concentrated mashes (1:3). An increase of mashing time from 105 min to 180 and 360 min reduces the level of phenolics in beer, but also adversely affects the taste of the beer (Von Narzib et al., 1979). On the other hand, infusion and decoction-mashing systems produce worts of very different polyphenol compositions with higher levels of oxidizable phenols. The quantities of extracted oxidized and oxidizable poly phenols, however, affect the degree of polyphenol loss during wort boiling and fermentation (Kirby et al., 1977).

The contribution of hops polyphenolics to boiled wort depends on the time of addition to hops; late addition in decoction wort boiling allows inclusion of phenolics but not subsequent removal of oxidizable polyphenols. The late addition of hops to boiling wort may even result in a need for extensive treatment to produce haze-stable beer (Kirby et al., 1977). In total, the water-soluble polyphenols derived from hops account for 20 to 30% of malt polyphenols. Hops polyphenols have a greater affinity to beer proteins; therefore, they tend to precipitate more completely during wort boiling than those extracted from malt (Bellmer, 1981).

The content of polyphenols may decrease during the fermentation process as a consequence of protein precipitation. Sugars present in wort are capable of hydrogen bond formation and compete with polyphenols for available sites. As the fermentation process progresses, the sugar concentration in wort decreases, resulting in increased formation of hydrogen bonding between polyphenols and beer proteins (Kirby et al., 1977).

The content of phenolic acids in the finished beer differs only slightly from that of unboiled wort. Unboiled wort of commercial lager is somewhat deficient in caffeic acid. Boiling wort with hops increases the content of hydroxybenzoic and *p*-coumaric acids. Subsequent unit operations, such as fermentation, cold storage, treatment with adsorbents and filtration, bring about a slight loss of phenolic acids, with the exception of vanillic acid content, which decreases by more than 40% (Table 5.20) (Gardner, 1967; McMurrough et al., 1984).

Nonbrewing strains of *Saccharomyces* yeast often produce undesirable off-flavors referred to as "phenolic," "herbal phenolic," or "clove-like" flavor notes in beer. Decarboxylation of phenol carboxylic acids (*p*-coumaric, ferulic and sinapic acids) may be responsible for production of such flavor notes. Beers with phenolic taste contain

significant amounts of 4-vinylguaiacol that may be produced by decarboxylation of ferulic acid present in wort during fermentation (Thurston and Tubb, 1981). Worts fermented with nonbrewing wild-type yeast strains contain 10 times higher

TABLE 5.20
Polyphenol Content in Lager Beer^a

| Beer Sample | Catechin | Epicatechin | Total Monomeric | Total Dimeric |
|-------------|----------|-------------|-----------------|---------------|
| 1A | 6.3 | 1.6 | 7.9 | 4.3 |
| 1B | 3.5 | 1.7 | 5.2 | 2.4 |
| 2A | 3.4 | 0.9 | 4.3 | 3.3 |
| 2B | 4.9 | 1.9 | 6.8 | 5.0 |

^a Milligrams per liter.

Source: Adapted from Kirby, W. and Wheeler, R.E., 1980, *J. Inst. Brew.*, 86:15–17.

TABLE 5.21
Levels of 4-Vinyl Guaiacol in Beers Fermented with Brewing Yeasts and Wild Type Strains^a

| Yeast Strain | Content of 4-guaiacol |
|--------------------------------------|-----------------------|
| Wild yeast | 1154 |
| <i>Saccharomyces diastaticus</i> | 1914 |
| <i>Saccharomyces pastorianus</i> | 1035 |
| <i>Saccharomyces willianus</i> | 1333 |
| <i>Saccharomyces uvarum</i> , J-3015 | 132 |
| <i>Saccharomyces uvarum</i> , J-2270 | 150 |
| <i>Saccharomyces uvarum</i> , J-2036 | 115 |
| <i>Saccharomyces uvarum</i> , J-28C4 | 132 |

^a Micrograms per liter.

Source: Adapted from Villarreal, R. et al., 1986, *ASBC J.*, 44:114–117.

levels of 4-vinylguaiacol compared to those found in normal beer (Table 5.21) (Villarreal et al., 1986).

A number of procedures have been developed for production of alcohol-free and low-alcohol beers, including removal of alcohol by (Bartolome et al., 2000):

- Distillation
- Vacuum distillation
- Dialysis
- Reverse osmosis
- Restricted fermentation
- Use of special yeasts
- Use of spent grain
- Carbon dioxide extraction

Alcohol-free beers contain lower levels of phenolics in comparison to the phenolic concentrations found in standard beer. These changes in phenolic contents, according to

Bartolome et al. (2000), may be brought about mainly by the differences in fermentation times, the yeast strains employed, and the dealcoholization process employed.

FORMATION OF HAZE

Polyphenols in beer may contribute to the formation of haze (Bamforth, 1999; Siebert, 1999; Siebert and Lynn, 1998; Siebert et al., 1996), which may contain up to 55% polyphenols (Table 5.22). According to Siebert (1999), polyphenolics should have at least two binding sites in order to participate in the formation of beer haze, each of which should possess at least two hydroxyl groups on an aromatic ring. The largest amount of haze is formed when the number of polyphenol and protein binding sites are nearly equal (Siebert, 1999). Eleven phenolic acids and two flavonols

TABLE 5.22
Gross Chemical Composition of Beer Haze (%)

| Protein | Polyphenol | Carbohydrate | Ash | Reference |
|---------|------------|--------------|------|----------------------------|
| 40 | 45 | 2–4 | 1–3 | Bengough and Harris (1995) |
| 58–77 | 17–55 | 2–12.4 | 2–14 | Gramshaw, J.W. (1970) |
| 40–76 | 17–55 | 3–13 | | Clark, A.G. (1960) |
| 15–45 | 1–3 | 50–80 | | Martin et al. (1962) |

Source: Adapted from Dadic, M., 1976, *MBAA Tech. Q.*, 13:182–189.

(quercetin and kaempferol), as well as (+)-catechin and (–)-epicatechin, have been identified in ether extracts of acid- and alkali-treated haze (Dadic and Belleau, 1980). The most abundant types of beer haze are chill haze and permanent haze. Chill haze is defined as the haze formed at 0°C and is redissolved upon warming of beer; the haze that remains in beer at or above 20°C is called permanent haze. The chill haze is probably formed through weak intermolecular bonding, while the permanent haze ensues after the formation of a certain number of covalent bonds between the units of the haze particles (Dadic, 1976).

The haze particles are thought to be built from units with a molecular weight of 10,000 to 100,000 Da (Claesson and Sandegren, 1969). Most of the polysaccharides and polypeptides present in beer have the postulated size, while polyphenols reach it through a polymerization process. About 90% of polyphenolics found in aged beer have a molecular weight of 500 to 10,000 Da; only trace quantities of phenolics have a molecular weight over 10,000 Da (Sogawa, 1972). Due to their chemical character and reactivity, polyphenols may undergo oxidative and acid-catalyzed polymerization in beer upon storage (Dadic et al., 1970; Gramshaw, 1968, 1970). Of the phenolics present in beer, catechins in the free form are most sensitive to oxidative changes during the initial stages of aging (Bellmer, 1977). The catalyzed polymerization of phenolics occurs in the later stages of beer storage when most of the air in the headspace has been depleted. Complexation of polymerized polyphenolics with polypeptides, first through hydrogen bonds, produces sufficient hydro-phobicity. The insolubility of this complex and consequent formation of haze are largely determined by the nature and distribution of polyphenols in the complex (Figure 5.10). Monomeric phenolics are also found in beer

haze; however, their role in haze formation is still disputed because they have usually been identified after alkaline hydrolysis of haze or in beer poly amide adsorbates (Dadic et al., 1971; Gardner and McGuinness, 1977).

The rate of haze formation depends on the degree of polyphenolic polymerization and increases from monomeric to dimeric and finally to polymeric catechin units. The structure of polyphenols also plays an important role in haze formation because procyanidin-B3 (catechin-catechin) increases haze formation more quickly than procyanidin-B4 (catechin-epicatechin) (Gardner and McGuinness, 1977). The threshold values for tanning of beer proteins by procyanidins have been found to be 150 mg/L for the trimeric procyanidin and about 850 mg/L for procyanidin B3. On the other hand, the threshold value for tanning by tannic acid is

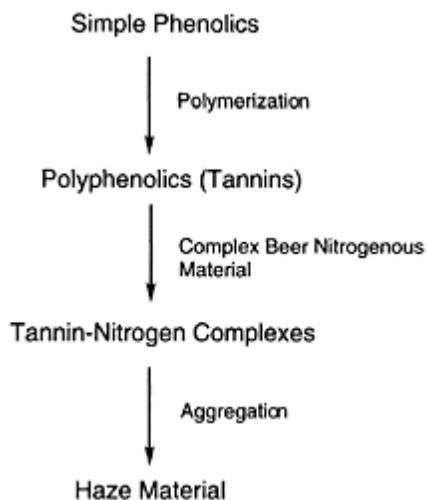


FIGURE 5.10 Participation of phenolics and tannins in haze formation in beverages.

25 mg/L; half of the protein material of this polyphenolic is precipitated at 140 mg/L and total precipitation has been observed at 650 mg/L (Delcour et al., 1983). The normal concentration of dimeric procyanidins in beer does not exceed 4 mg/L (Bate-Smith, 1973; Haslam, 1974); therefore, their contribution to haze formation in beer may be negligible.

Proanthocyanidins from hops and malt have a tremendous influence on haze development. Equal concentrations of hops and malt proanthocyanidins produce similar haze value, but their impact on beer stability depends on their relative concentrations in the beverage (Delcour et al., 1985). Approximately 80% of proanthocyanidins present in regular beer is derived from malt and only 20% from hops (Bellmer, 1981). Thus, malt proanthocyanidins are the main contributors to beer haze.

The permanent and chill haze developed during aging of beer can be effectively reduced by treatment of wort with tannase or laccase before boiling. The action of the

enzymes results in some discoloration of wort, but this is totally lost during fermentation and aging of beer (Rossi et al., 1988).

WINE

Phenolic compounds are important components of wine. They contribute to sensory characteristics such as color, flavor, astringency and hardness of wine directly or by interaction with proteins, polysaccharides, or other phenolic compounds (Lee and Jaworski, 1987; Garcia-Viguera et al., 1997). The sensory characteristics of wine are influenced by the grape variety, the vinification process and degradation and polymerization of phenolics during the aging of wine (Auw et al., 1996; Zoecklein et al., 1997). These reactions are influenced by storage temperature (Gomez-Plaza et al., 2000; Somers and Evans, 1986; Somers and Pocock, 1990) and duration (Dallas and Laurenro, 1994a, b).

COMPOSITION OF POLYPHENOLICS

The composition of phenolics in wine depends on the types of fruits used (usually grapes) for vinification and their extraction, procedures employed for wine making and chemical reactions that occur during the aging of wine (Macheix et al., 1990). The wood barrel's contact with must and wine also accounts for the presence of some phenols in wine. Thus, some simple phenols, certain flavonoids and hydrolyzable tannins can be leached out from wood to wine (Maga, 1984). The composition of phenolics is also modified by yeasts as a result of conversion of nonphenolic substances and solubilization and extraction of phenolics by the ethanol produced during fermentation (Singleton and Noble, 1976). Based on a multivariate analysis of midinfrared spectra of wine phenolic extracts, Edelmann et al. (2001) recently developed a method for discrimination of Austrian red wines obtained from different *Vitis vinifera* cultivars.

Phenolics of wine and grapes usually include derivatives of hydroxybenzoic and hydroxycinnamic acids, the trihydroxystilbenes such as *cis*- and *trans*-resveratrol as well as *cis*- and *trans*-piceid, flavonoids such as flavan-3-ols, flavan-3,4-diols, anthocyanins and anthocyanidins, flavonols, flavones and condensed tannins (Burns et al., 2002; Escribano-Bailón et al., 1992; Jaendet et al., 1993; Lamuela-Raventós and Waterhouse, 1993; Lea et al., 1979; Mazza et al., 1999; Siemann and Creasy, 1992; Singleton and Esau, 1969; Soleas et al., 1997; Somers, 1966). The main phenolics identified in *Vitis vinifera* berries are given in Table 5.23. White wines tend to be low in their content of phenolics compared to red wines. The proportion of different classes of phenolic substances in wines is influenced by grape variety and harvesting season (Table 5.24) as well as the age of wine (Table 5.25). Malvidin-3-monoglu-

TABLE 5.23**Main Phenolics Identified in *Vitis Vinera* Berries**

Phenolic acids

p-Hydroxybenzoic; *o*-hydroxybenzoic; salicylic; gallic; cinnamic; *p*-coumaroyl tartaric (=coutaric); caffeoyl tartaric (=caftaric); feruloyl tartaric (=fertaric); *p*-coumaroyl glucose; feruloyl glucose; glucose ester of coutaric acid

Anthocyanins

Cyanidin 3-glucoside; cy 3-acetylglucoside; cy 3-*p*-coumaryl-glucoside; peonidin 3-glucoside; pn 3-acetylglucoside; pn 3-*p*-coumarylglucoside; pn 3-caffeoylglucoside (?); delphinidin 3-glucoside; dp 3-acetylglucoside; dp 3-*p*-coumarylglucoside; petunidin 3-glucoside; pt 3-*p*-coumarylglucoside; malvidin 3-glucoside; mv 3-acetylglucoside; mv 3-*p*-coumarylglucoside; mv 3-caffeoylglucoside

Flavonols

Kaempferol 3-glucoside; k 3-glucuronide; k 3-glucosylarabinoside (?); k 3-galactoside; quercetin 3-glucoside; q 3-glucuronide; q 3-rutinoside; q 3-glucosylgalactoside (?); q 3-glucosylxyloside (?); iso-rhamnetic 3-glucoside

Flavan-3-ols and tannins

(+)Catechin; (-)epicatechin; (+)gallocatechin; (-)epigallocatechin; epicatechin-3-O-gallate; procyanidins B1, B2, B3, B4, C1, C2, polymeric forms of condensed tannins

Flavanonols

Dihydroquercetin 3-rhamnoside (=astilbin); dihydrokaempferol 3-rhamnoside (=engeletin)

Source: Adapted from Macheix, J.-J. et al., 1990, *Fruit Phenolics*, CRC Press, Boca Raton, FL.

TABLE 5.24**Effect of Season and Grape Variety on Total Phenolics, Flavonols, and Anthocyanins in Skin, Must, and Wine from Okanagan Valley (B.C., Canada)**

| Grape Variety Units | Total Phenolics ^a | | Flavonol ^b | | Anthocyanins ^c | |
|---------------------|------------------------------|-----------|-----------------------|---------|---------------------------|----------|
| | 1996 | 1997 | 1996 | 1997 | 1996 | 1997 |
| Cabernet Franc | | | | | | |
| Skin | mg/kg 548–793 | 462–1009 | 82–134 | 93–200 | 530–826 | 289–1048 |
| Must | mg/L 163 | 326–965 | 45 | 96–181 | 8 | 42–644 |
| Wine | mg/L 863–982 | 1095–1193 | 98–128 | 121–157 | 232–420 | 37–469 |
| Merlot | | | | | | |
| Skin | mg/kg 777–1128 | 633–1028 | 111–150 | 118–182 | 708–1137 | 485–1094 |
| Must | mg/L 130 | 196–851 | 31 | 47–147 | 32 | 81–644 |
| Wine | mg/L 912–974 | 907–921 | 128–130 | 110–113 | 279–412 | 338–455 |
| Pinot Noir | | | | | | |
| Skin | mg/kg 806–915 | 955–1074 | 109–151 | 167–172 | 665–803 | 715–794 |
| Must | mg/L 209 | 152–650 | 47 | 45–109 | 78 | 25–314 |
| Wine | mg/L 723–748 | 913–965 | 88–94 | 76–91 | 223–340 | 166–219 |

^a Expressed as gallic acid equivalents.

^b Expressed as quercetin equivalents.

^c Expressed as malvidin-3-glucoside equivalents.

Source: Adapted from Mazza, G. et al., 1999, *J. Agric. Food Chem.*, 47:4009–4017.

TABLE 5.25
Gross Phenol Composition for Typical Wines
from *Vitis Vinifera* Grapes^a

| Phenol Class | Source | White Wine | | Red Wine | |
|---------------------------|----------|------------|---------|----------|----------|
| | | Young | Aged | Young | Aged |
| Total nonflavonoids | | 170 | 160–260 | 235 | 240–500 |
| Cinnamates deriv. | G,D | 154 | 130 | 165 | 150 |
| Low volat. benzene deriv. | D,M,G,E | 10 | 15 | 50 | 60 |
| Tyrosol | M | 10 | 10 | 15 | 15 |
| Volatile phenols | M,D,E,E, | 1 | 5 | 5 | 15 |
| Hydrozylable tannins etc. | | 0 | 0–100 | 0 | 0–260 |
| Macromolecular complexes | | | | | |
| Protein-tannin | G,D,E, | 10 | 5 | 5 | 10 |
| Total flavonoids | | 30 | 25 | 1060 | 705 |
| Catechins | G | 25 | 15 | 200 | 150 |
| Flavanols | G,D | tr | tr | 50 | 10 |
| Anthocyanins | G | 0 | 0 | 200 | 20 |
| Soluble tannins, deriv. | G,D | 5 | 10 | 550 | 450 |
| Other flavonoids, deriv. | G,D,E,M, | ? | ? | 60? | 75? |
| Total phenols | | 210 | 190–290 | 1300 | 955–1215 |

Note: D=degradation products; E=environment; G=grapes; M=microbes.

^a Milligrams of gallic acid equivalents per liter.

Source: Adapted from Singleton, V.L., 1980, *Grape Phenolics: Background and Prospects*, Centenaire, Paris.

coside is the major anthocyanin of Cabernet Franc, Merlot and Pinot Noir wines from British Columbia (Canada). These wines also contain 3-monoglucosides of delphinidin, cyanidin, petunidin and peonidin. In addition, 3-monoglucoside-acetates and 3-monoglucoside-coumarates of anthocyanidins have been found in Cabernet Franc and Merlot wines (Gao et al., 1997; Mazza, 1995; Mazza et al., 1999).

Condensed tannins contribute to astringency, browning and turbidity in wines and also participate in the aging processes of wine (Haslam and Lilley, 1988). According to Somers (1966), the molecular weight of condensed tannins isolated from wine ranges from 2000 to 4000 Da. Later, Souquet et al. (1996) separated condensed tannins extracted from grape skin (*Vitis vinifera* var. Merlot) into six fractions using normal phase HPLC. The mean degree of polymerization of tannins present in different fractions ranged from 3 (fraction I) to 80 (fraction VI). Catechin comprised 67% of terminal tannin units while 60% of extension units in tannin polymer consisted of epicatechin. Sarni-Manchado et al. (1999) reported that the mean degree of polymerization of tannins precipitated by gelatin from wine is six. The wine used in this study was made from *Vitis vinifera* var. Merlot (50%) and var. Carignan (50%). Epicatechin is the major component of precipitable condensed tannins as well as the predominant phenolic of extension units in tannins.

Young wines contain mainly low to medium molecular weight phenolics while aged wines are relatively higher in polymerized phenolics. Of the low molecular weight phenolics found in wines, catechin, epicatechin, procyanidin B1, B2, B3, B4, C1 and T2, quercetin, gallic acid (Carando et al., 1999; Singleton, 1982; Van Dam and Daniel, 1980) and hydroxycinnamates, i.e., *trans*-caftaric, *trans*- and *cis*-coumaric acids (Betes-Saura et al., 1996; Karagiannis et al., 2000), are predominant. Smaller quantities of 2-S-glutathionylcaftaric, protocatechuic, *cis*- and *trans*-caffeic, syringic, fertaric, *trans*-coumaric and *trans*-ferulic acids have also been reported (Betes-Saura et al., 1996).

Figure 5.11 shows the structures of some procyanidin dimers and one of the procyanidin trimers commonly found in wine (Lunte et al., 1988). Typical Italian red and white wines contain 203 to 805 and 11 to 49 mg/L of flavanols, respectively. In addition, appreciable amounts of quercetin and rutin have been found in red wines, but only traces are present in white wine (Simonetti et al., 1997). Furthermore, the concentration of (+)catechin and (–)epicatechin in French wines ranges from 32.8 to 209.8 mg/L and from 22.1 to 130.7 mg/L, respectively. The level of procyanidins in these wines is between 7.8 and 39.1 mg/L for B1, 18.3 to 93 mg/L for B2, 21.4 to 215.6 mg/L for B3, 20.2 to 107.2 mg/L for B4, 8.6 to 36.9 mg/L for C1 and 26.7 to 79.3 mg/L for T2 (Carando et al., 1999; Landrault et al., 2001). McDonald et al. (1998) surveyed the flavonol contents of 65 red wines from different geographical origins. The total content in red wines was between 4.6 and 41.6 mg/L, while free flavonols comprised 3 to 67% of total flavonols. The high total flavonol levels were detected in wines produced from thick-skinned grape varieties such as Cabernet Sauvignon.

The total content of phenols, expressed in milligrams of gallic acid equivalents, may range from 50 mg/L in some white wines to 6500 mg/L in certain types of red wine. A typical white wine contains about 250 mg phenols per liter, although some wines may contain even 2000 mg/L. On the other hand, the total content of phenolics in typical red wine ranges from 700 to 4000 mg/L (Landrault et al., 2001; Salunkhe et al., 1990; Sato et al., 1996; Simonetti et al., 1997). The total content of phenolics in wine depends on a number of factors, for example:

- Variety of grapes (red or white)
- Nature of crushing
- Possible inclusion or elimination of crushed grape skins, pulp, and seeds (especially from red grape varieties) prior to fermentation
- Heating of grape skins
- Vinification mode (temperature and maceration time)
- Aging

A longer fermentation period of skins, pulps and seeds allows more phenolics to be extracted into the wine because the ethanol produced acts as an extraction medium for phenolics.

Table 5.26 gives the content of low molecular weight phenolics extractable in ethyl acetate from some young red Bordeaux wines produced from different vine cultivars. Ethyl acetate enables catechins, procyanidins, flavonols, aromatic alcohols and phenolic acids to be extracted from wine. Data presented in Table 5.26 indicate a significant variation in the content of low molecular weight phenolics, depending

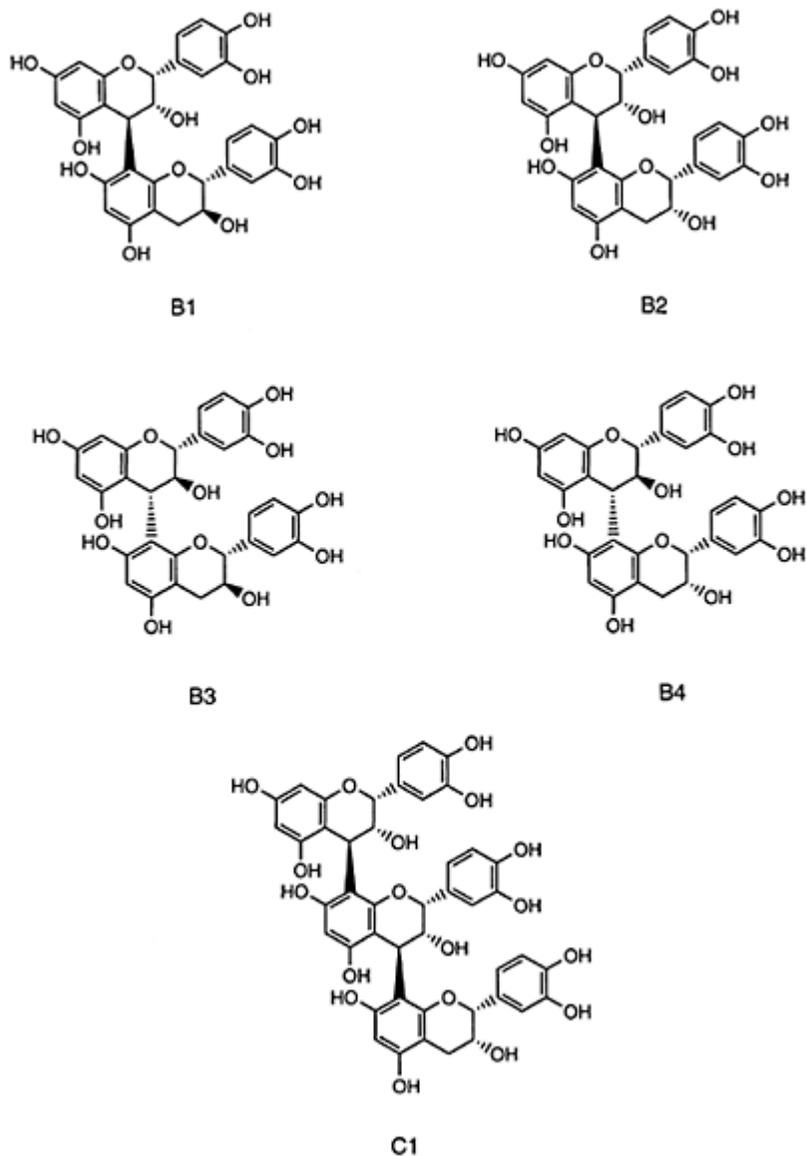


FIGURE 5.11 Some procyanidins found in wine. (Adapted from Lunte, S.M. et al., 1988, *Analyst*, 113:99–102.)

on the vine cultivar. Therefore, Salagoity-Auguste and Bertrand (1984) proposed to use the analysis of low molecular weight phenolics as a fingerprinting method for

identification of the cultivar from which red wine is produced. The total content of low molecular weight phenolics ranges from 100 to 200 mg/L, which is low when compared to the total content of phenolics present. Thus, most of the phenolic

TABLE 5.26
Average Concentrations of Phenolic Compounds
Extracted from Red Wines of Three Bordeaux
Cultivars^a

| Compound | Cultivar | | |
|-------------------------------|--------------------|--------|--------|
| | Cabernet Sauvignon | Merlot | Malbec |
| Tyrosol | 3.7 | 3.1 | 1.7 |
| Tryptophol | 1.1 | 0.9 | 1.2 |
| Catechin | 7.6 | 11.1 | 16.3 |
| Epicatechin | 11.6 | 11.3 | 6.3 |
| Procyanidin A ₂ | 1.5 | 11.2 | 1.8 |
| Procyanidin B ₂ | 9.2 | 6.1 | 2.4 |
| Procyanidin B ₃ | 38.5 | 2.9 | 1.6 |
| Procyanidin B ₄ | 15.7 | 19.1 | 5.4 |
| Myricetin | 2.6 | 6.1 | 3.2 |
| Quercetin | 10.4 | 14.8 | 7.8 |
| Gallic acid | 5.8 | 3.9 | 2.7 |
| Protocatechuic acid | 0.4 | 0.2 | 0.4 |
| <i>p</i> -Hydroxybenzoic acid | 2.2 | 0.9 | 0.4 |
| Vanillic acid | 0.3 | 0.7 | 0.6 |
| Caffeic acid | 0.9 | 0.5 | 0.3 |
| Syringic acid | 3.8 | 1.3 | 3.0 |
| <i>p</i> -Coumaric acid | 1.3 | 0.4 | 0.8 |
| Ferulic acid | 0.1 | 0.1 | 0.1 |

^a Milligrams per liter.

Source: Adapted from Salagoity-Auguste, M.-H. and Bertrand, A., 1984, *J. Sci. Food Agric.*, 35:1241–1247.

compounds present in red wine, including anthocyanins and polymerized pigments, are not extracted into ethyl acetate.

Six phenolic acids have been identified in Seyval white wines, including gallic (1.8/L), caffeic (1.1 mg/L), and *p*-coumaric acids (0.6 mg/L) (Jindra and Gallander, 1987). In Vidal white wines, eight acidic phenolics have been identified: gallic acid (1.45 mg/L), *p*-hydroxybenzoic acid (0.17 mg/L), *m*-hydroxybenzoic acid (0.84 mg/L), caffeic acid (12.97 mg/L), salicylic acid (0.66 mg/L), and *p*-coumaric acid (1.94 mg/L) (Woodring et al., 1990). More recently, Soleas et al. (1997) determined the content of 15 polyphenols in a wide range of wine produced in Ontario, Canada. These authors reported that ferulic acid is the predominant phenolic acid in Riesling wines, caffeic acid predominates in Vidal and Chardonnay, and *p*-coumaric acid in Seyval Blanc, while gallic acid is predominant in all red wines.

Baderschneider and Winterhalter (2001) have identified a number of novel phenolic acid derivatives, phenylpropanoids, flavonoids and lignans in Riesling wine. These include 15 new benzoic and cinnamic acid derivatives, two phenylpropanoids, namely, 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one and 2,3-dihydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one, six flavonoids, i.e., dihydrokaempferol, dihydroquercetin and four dihydroflavonol glycosides, three neolignans and six lignans, i.e., derivatives of lariciresinol, isolariciresinol and secoisolariciresinol.

Numerous volatiles present in small quantities in wines are responsible for their specific flavors (Schreier, 1979; Singleton and Noble, 1976). Twelve volatile phenols have been identified in wines stored without contact with oak barrel (Etievant, 1981). Wine aged in wooden barrels contains vanillin, as well as coniferylaldehyde, syringaldehyde, sinapaldehyde, vanillic acid, and ethyl vanillate, most probably extracted from wood by ethanol (Singleton and Noble, 1976). However, some of these compounds may have also been produced from the degradation of hydroxycinnamic acid esters present in juice extracted from grapes. Decarboxylation of *p*-coumaric and ferulic acids by yeasts and bacteria produces 4-vinylphenol and 4-vinylguaiacol, which are volatile and contribute to off-flavors in wine (Schreier, 1979).

POLYPHENOLICS AND VINIFICATION

The vinification process brings about qualitative and quantitative changes in wine phenolics. The extraction of phenolics is minimal from grape solids in making white wine, but plays an important role in making red wine. The quantities of pigments and other phenolics may vary greatly with grape variety, region, and cultural practices. The level of phenolics retained during the vinification process determines the type of wine and also has a potential influence on wine quality (Burns et al., 2001; Somers and Pocock, 1986).

Crushing is the first operation in winemaking, enhancing the level of polyphenolics in wine due to the contact of juice with the solid parts of grapes. Crushing increases the surface of the solid parts in contact with the juice. In red wines, it facilitates maceration during the fermentation process and thus increases the extraction of phenolic substances from grape solids. However, excessive crushing with tearing of the solid parts and skins increases the extraction of astringent phenolics into the juice and thus may affect wine quality. Red grapes are usually destemmed prior to crushing, which prevents stems running out into the fermentation vessel and reducing the content of phenolics in wine (Macheix et al., 1991). The crushing of white grape is done without destemming to reduce damage to the stems, which release fewer polyphenols into the juice. Also, tearing solid parts of the fruits and crushing the seeds must be avoided because high levels of phenolics may have a profound and subtle effect on the quality of white wines. Polyphenols are the major contributors to bitterness and astringency in white wines and act as substrates in oxidation processes (Hartnell, 1987; Robichaud and Noble, 1990).

Longer contact periods of skins, pulp, and seeds during fermentation allow more phenolics to be extracted into wine because the ethanol produced enhances the extraction of phenols. Wines with higher levels of phenolics are more astringent and therefore require longer maturation than those with lower phenolic content (Somers and Pocock, 1986). During the initial period of fermentation, phenolic compounds steadily diffuse

from skin into the must (Bourzeix, 1971). Thus, 4 to 5 days' maceration is sufficient to produce a colored, low-tannin wine for early drinking, such as Bordeaux wines. However, in the case of wines subjected to aging processes, the skins should be allowed to remain for a longer period in the mixture during the fermentation process to increase the level of tannins (Ribereau-Gayon and Glories, 1987). In addition, Gil-Munoz et al. (1999) demonstrated that cooling the grapes to 10°C prior to crushing decreases the rate of polyphenol extraction from the skin, but only during the first 3 to 4 days of alcoholic fermentation.

The fermentation process brings about qualitative and quantitative changes in phenolic composition of wines. At the beginning, a rapid decrease in the content of hydroxycinnamic acid derivatives occurs, but the rate of degradation of *p*-coumaroyltartaric acid (coutaric acid) is greater than that of caffeoyltartaric acid (caftaric acid) (Macheix et al., 1990). During fermentation, hydroxycinnamic acid derivatives are hydrolyzed to free hydroxycinnamic acid, which may then be oxidized (Cilliers and Singleton, 1989), converted into volatile phenols by yeast decarboxylase (Chatonnet et al., 1993; Dugelay et al., 1993), or adsorbed by yeast (Schreier, 1979; Somers et al., 1987). About 27.6% of hydroxycinnamates are lost in the vinification process (Betes-Saura et al., 1996). Caffeic acid has been found to be the most abundant acidic phenol in Vidal Blanc and white wines from Penedes that are made from grapes high in *trans*-caffeoyl tartrate (Betes-Saura et al., 1996; Woodring et al., 1990). As the fermentation process progresses, due to tannin-anthocyanin polymerization, the level of anthocyanins decreases (Macheix et al., 1990).

POLYPHENOLICS AND AGING OF WINE

After fermentation, wine usually is matured first in barrels or tanks in the presence of air. This maturation process from end of vinification to bottling typically lasts 12 to 24 months. Following this, the wine is further aged in bottles and thus is protected from air to a considerable extent. During a year of storage in barrels, wine can absorb up to 40 mg of oxygen/L and evidence suggests that oxygen may even penetrate to wine stored in corked bottles (Ribereau-Gayon, 1974; Riberau-Gayon et al., 1983). The presence of oxygen induces the chemical transformation of phenolics and affects the flavor and color of wine. The extent of these changes depends on a number of factors including pH, temperature, presence of oxygen, ethanol content, nature and composition of phenolics, and bisulfite concentration, de Freitas et al. (1998) investigated the oxidative stability of grape seed procyanidins in a model wine solution and found the order of oxidative decomposition for procyanidin dimmers to be B3~B4>B7~B6>B1~B2>B8. These authors also demonstrated that the oxidative decomposition of trimers is affected by their conformation because trimer C1 is more oxidizable than catechins, while trimers (+)catechin-(4a→8)-(+)catechin-(4→8)-(-)epicatechin and (+)catechin-(4a→8)-(-)epicatechin-(4b→6)-(+)catechin are less oxidizable than catechins.

During barrel aging, composition of phenolics of wine undergoes changes due to an increase in the content of phenols extracted from the wood. Gallic acid, initially low in the control wine (1.8 mg/L), increases to 4.0 mg/L after 12 weeks of aging in American-oak barrels. On the other hand, the content of procatechuic, vanillic, caffeic, syringic, and *p*-coumaric acids remains relatively constant. Overall, gallic acid represents only 7%

of the total increase in nonflavonoid phenolics. However, the remaining 93% increase in phenolic level of aged wine is unaccounted for (Jindra and Gallander, 1987). The increase in gallic acid may be attributed in some cases to the hydrolysis of catechin and epicatechin gallates naturally occurring in wines (Singleton and Essau, 1969). The amount of phenolics extracted into wine depends on aging time, oak type, size and the possibility of the barrel having been used previously (Rous and Alderson, 1983; Singleton, 1974). Some phenols extracted from wood include lignins, hydrolyzable tannins, gallic acid, ellagic acid, aromatic carboxylic acids and aldehydes. Of these, ellagitannins and ellagic acid account for 10% of the total phenolic content of Riesling wines treated with oak chips (Quinn and Singleton, 1985).

During the aging of red wine, the content of anthocyanins decreases progressively and irreversibly as a result of formation of polymeric pigments. The color of these pigments is less sensitive to changes of pH (between 2.2 and 5.5) and is quite resistant to decolorization by sulfur dioxide (at a concentration of up to 200 mg/L); however, some of these pigments may be more prone to degradation in aqueous solutions than anthocyanins (Escribano-Bailon et al., 2001). The polymeric forms of pigments account for about 50% of the color density of wine during the first year of aging; this value averages 85% for 10-year-old wines (Somers, 1971). The formation of these pigments has been evaluated in model wine systems. Based on the results of these studies, various mechanisms have been proposed for the formation of polymeric pigments.

According to Mistry et al. (1991) and Figueiredo et al. (1996), the polymeric pigments are the products of intramolecular copigmentation of anthocyanins. Several other researchers (Liao et al., 1992; Santos-Buelga et al., 1996; Somers, 1971) have suggested that the formation of these pigments is the result of direct condensation between anthocyanins and flavanols. Reactions leading to the formation of pigments from anthocyanins are depicted in Figure 5.12. Initially a labile flavone (II) is formed from electrophilic substitution of a carbonium ion (I) into a wine component, RH. The R substituent is most likely a dimeric proanthocyanidin. The flavone is readily oxidized by air or a redox system to condensed anthocyanin species (III); this deprotonates to a more stable quinonoid structure (IV) designated to the chromophores of the polymeric pigment that is then formed. The enhanced stability of the latter pigment is believed to be due to aryl substitution of the anthocyanin at 4-position (Haslam, 1989). Another plausible mechanism suggests that the reactions between anthocyanidins and flavanols are mediated by acetaldehyde (Bakker et al., 1993; Dallas et al., 1996; Escribano-Bailon et al., 2001; Francia-Aricha et al., 1997). This mediation leads to the formation of ethyl bridges between C-8 positions of their phloroglucinol rings (Francia-Aricha et al., 1997). This reaction is favored at more acidic conditions, which promote the formation of the acetaldehyde cation required for mediation of condensation between flavanols and anthocyanins (Garcia-Viguera et al., 1994).

Acetaldehyde is a precursor of ethanol during primary oxidation, but can also be formed during aging as a result of oxidation of ethanol. The latter reaction involves oxidation of *o*-diphenol groups leading to intermediate formation of hydrogen peroxide and subsequent coupled oxidation of ethanol to acetaldehyde (Wildenradt and Singleton, 1974). Francia-Aricha et al. (1997) reported the formation of three novel pigments from malvidin-3-*O*-glucoside (Mv-3-glu) and procyanidin B2 in the

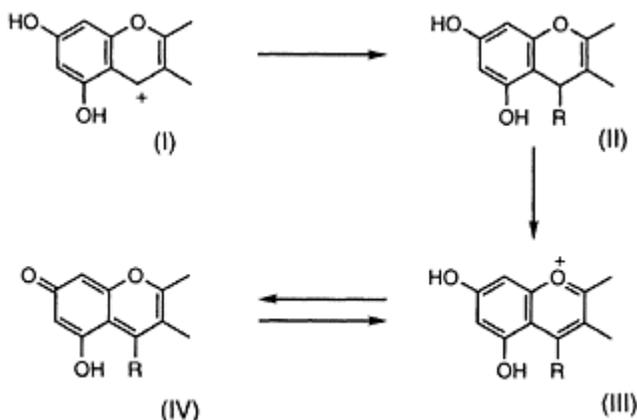


FIGURE 5.12 Reactions involved in the formation of polymeric pigments in red wine. (Adapted from Somers, T.C., 1971, *Phytochemistry*, 10:2175–2186.)

presence of acetaldehyde. Two of these pigments, reddish-blue in color, are identified as enantiomers of Mv-3-glu and procyanidin B2 linked at their C-8 by ethyl bridges. The third pigment is orange-red in color and possibly made of two flavylum mesomeric forms corresponding to malvidin and pelargonidin. This latter pigment does not contain ethyl bridge, but the presence of acetaldehyde is needed for its formation. The mechanism involved in the formation of this pigment remains unknown. Condensed anthocyanin species can be further substituted with additional oligomeric proanthocyanidins. Finally, the polymerization reaction leads to precipitation of polymeric pigments and loss of astringency (Ribereau-Gayon et al., 1983; Timberlake and Bridle, 1976).

Another possible mechanism of the evolution of phenolics in aging red wine involves electrophilic condensation reactions between oligomeric proanthocyanidins and acetaldehyde (Figure 5.13) (Haslam, 1989) as well as between flavanols and acetaldehyde (Es-Safi et al., 1999). Catechin ethyl-bridged dimers and trimers have been identified in red wine samples (Saucier et al., 1997) and it is thought that these reactions may lead to the formation of insoluble polymers, bringing about a decrease in astringency (Tanaka et al., 1994) and changes in color. In addition, the formation of adducts between anthocyanins and pyruvic acid has been reported. Such compounds have been isolated from Port wines (Fulcrand et al., 1998; Mateus and de Freitas, 2001; Mateus et al., 2001; Romero and Bakker, 1999). Using a winelike model system, Vidal et al. (2002) recently investigated possible reactions leading to the loss of astringency in wine. These authors demonstrated that cleavage of the proanthocyanidin interflavanic bond takes place during wine aging. In the absence of (–)epicatechin, the carbocations released upon this cleavage form unknown species (phlobaphenes). However, in the presence of (–)epicatechin, a decrease in mDP (mean degree of polymerization) of proanthocyanidins from 36 to 25

occurred after 53 days of reaction and accumulation of oligomeric proanthocyanidins in the winelike solution was noted.

Storage of wine in contact with yeasts (aging *sur Lie*) alters the flavor and aroma of certain white and red wines and also leads to the production of sparkling wines.

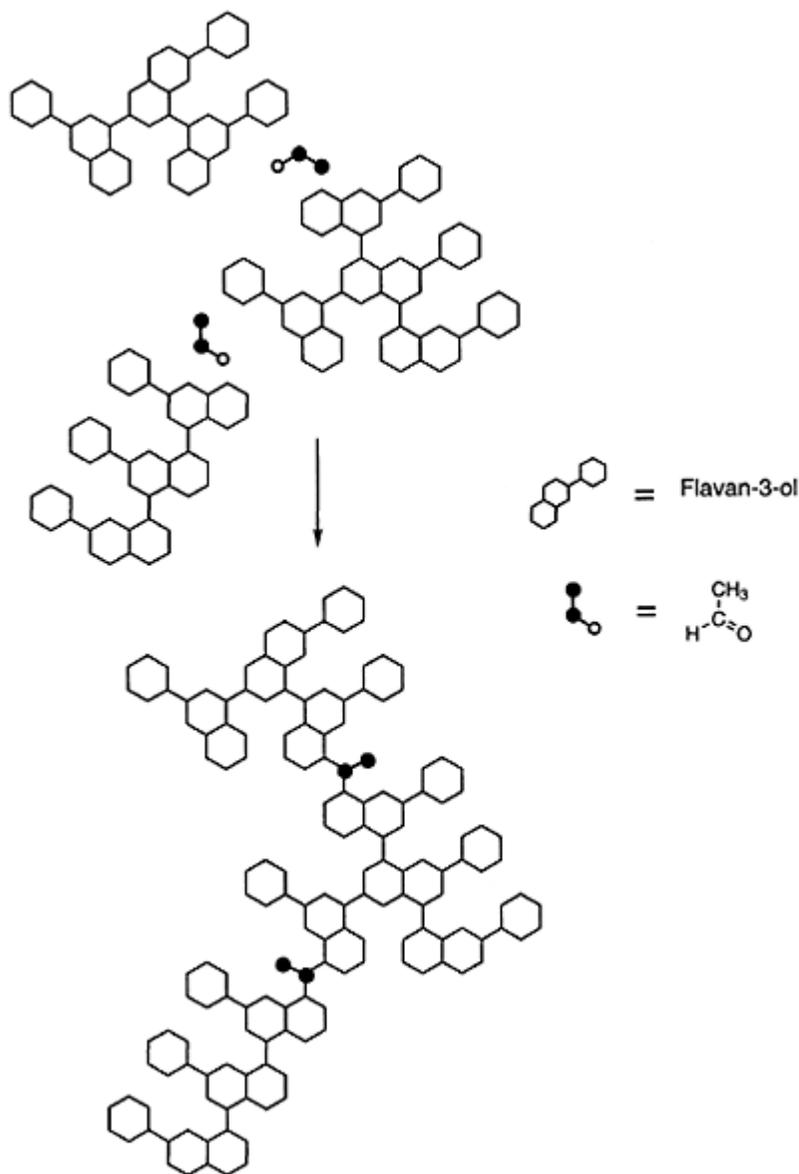


FIGURE 5.13 Proanthocyanidin polymerization in wine via reaction

with acetaldehyde. (Adapted from Haslam, E., 1989, *Plant Polyphenols*, Cambridge University Press, Cambridge, U.K.)

Grape-derived glycosides are considered to be the primary precursors of wine aroma (Baltenweck-Guyot et al., 2000; Zoecklein et al., 1997). Aging *sur Lie* decreases the total content of glycosides in Riesling wines by 73.4% (Zoecklein et al., 1997). Vasserot et al. (1997) demonstrated that the yeast lees also have the capacity to adsorb anthocyanins.

POLYPHENOLICS AND BROWNING REACTIONS IN WINES

Phenolic compounds may contribute to oxidative browning of white musts and wines. *Trans*-caffeoyl tartaric acid and other hydroxycinnamic acid derivatives are major phenolic compounds in grape juice (Singleton et al., 1984, 1985) and good substrates for grape polyphenol oxidase (Gunata et al., 1987). The oxidation of phenolics brings about a modification of initial wine phenolics and may result in the formation of condensed brown substances that may negatively affect the color and flavor of white wines. The rate of browning depends on a number of factors; of these, availability of oxygen, the nature and concentration of phenolics, and activity of polyphenol oxidases during the initial phases of wine making are the most important parameters (Macheix et al., 1991).

Susceptibility of grape phenolics to oxidation can be described by the so-called browning potential. This potential is calculated by taking into account the browning index of each phenolic, i.e., phenolic acids, catechin, epicatechin, procyanidins, and catechin-catechin gallate and their concentration in grape juice (Lee and Jaworski, 1987, 1988). However, the browning potential of grapes is not a function of their polyphenol oxidase because the activity of this enzyme is not a limiting factor for browning reactions. The level of oxidizable substrates may be the limiting factor for browning reactions because polyphenol oxidase is present in grapes in excess (Sapis et al., 1983).

The oxidative polymerization leading to the formation of brown pigments depends on the nature and relative concentration of the phenolic compounds present (Cheynier et al., 1988). However, no correlation exists between the level of hydroxycinnamic acid esters and browning potential of grape varieties (Romeyer et al., 1985). Therefore, it has been suggested that caftaric acid/glutathione ratio, which determines the quantities of untrapped quinones, may serve as an important factor to help explain the varietal differences in browning potential (Salgues et al., 1986). On the other hand, a significant correlation has been observed between catechin level and wine browning (Simpson, 1982). However, catechin shows poor affinity towards polyphenol oxidase and is found at low concentrations in wine (Gunata et al., 1987; Singleton, 1982, 1987). Therefore, catechin is probably not much affected by the oxidation process, but may participate in coupled oxidation reactions as demonstrated by Cheynier et al. (1988).

In model systems containing grape polyphenol oxidase, caftaric acid is degraded faster than catechin, epicatechin and epicatechin gallate, while procyanidins B1, B2, B3, B4 and 2-S-glutathionyl caftaric acid (referred to as grape reaction product, GRP) are oxidized slowly (Cheynier et al., 1988). The degradation process is considerably faster for mixtures of monomeric and dimeric flavan-3-ols and gallic acid in proportions

naturally found in grape seeds (Oszmianski et al., 1985). Presence of caffeoyl tartaric acids increases the degradation of all flavanols, probably due to a coupled oxidation mechanism. This mechanism involves enzymatic oxidation of tartaric acid followed by chemical oxidation of the second compound by caftaric acid quinone with regeneration of caftaric acid as follows (Cheynier et al., 1988):

- Caftaric acid + $1/2 \text{ O}_2 \rightarrow$ caftaric acid quinone + H_2O
- Caftaric acid quinone + o-diphenol \rightarrow caftaric acid + o-quinone

The rate of coupled oxidation induced by caftaric acid is influenced by the redox potentials of phenolic compounds involved. The order of redox potential for grape phenolics is: epicatechin gallate < procyanidins < GRP < epicatechin, catechin < caffeoyl tartaric acid (Cheynier et al., 1988). Caftaric acid quinone may also react with hydroquinone to yield a condensation product for the reaction of two semi-quinone free radicals (Singleton, 1982, 1987), or by a mechanism similar to a 1,4-Michael addition (Gramshaw, 1970; Singleton, 1982, 1987).

Under normal processing conditions, caftaric acid is oxidized very fast by grape polyphenol oxidase to corresponding orthoquinone, which then reacts primarily with the available glutathione to form the so-called GRP (Cheynier et al., 1986; Singleton et al., 1984). It is thought that formation of GRP limits browning of musts by trapping caftaric acid quinones into a colorless product with a lower affinity for the enzyme as a substrate and thus prevents them from proceeding to brown polymerized products (Singleton et al., 1985). However, capacity of GRP to control browning of grape juice and must depends on the amount of glutathione available, the contribution to coupled oxidation and its oxidation by laccase (Macheix et al., 1991).

The 2-S-glutathionyl caftaric acid may, however, be oxidized by *Botrytis cinerea* laccase if grape juice is prepared from infected fruits. Laccase is an enzyme that attacks a wider range of substrates than polyphenol oxidase (Dubernet and Ribereau-Gayon, 1973). Thus, the oxidation process may contribute to the browning of some white grape juices and wines made from infected grapes. The sequence of reactions involved in the oxidation of 2-S-glutathionyl caftaric acid is depicted in Figure 5.14. First, the laccase catalyzes the oxidation of 2-S-glutathionyl caftaric acid to the corresponding quinone. Following this, in the presence of surplus glutathione, a second thioether linkage is formed. Thus, the vicinal dihydroxyphenol structure is regenerated and 2,5-di-S-glutathionyl caftaric acid is produced. Otherwise, the orthoquinone formed may rapidly proceed to brown polymers. However, glutathionyl derivatives are also oxidizable and therefore can further contribute to the browning reaction (Salgues et al., 1986).

Flavanols may play a more prominent role in the formation of brown pigments than caftaric acid (Cheynier et al., 1989; Fernandez-Zurbano et al., 1995). Results of studies carried out using model systems demonstrate that the formation of catechin oligomers is more rapid than that of caftaric acid oligomers (Cheynier et al., 1989). Fernandez-Zurbano et al. (1998) measured changes in the concentration of hydroxycinnamic acids, hydroxycinnamic acid esters and flavanols brought about by the oxidation process; only the flavanol contents correlated with the degree of wine browning measured as the absorbance change at 420 nm.

OTHER ALCOHOLIC BEVERAGES

Processes involved in production of distilled spirits are specific to each alcoholic beverage (Lembeck, 1983). The composition of phenolic components in these

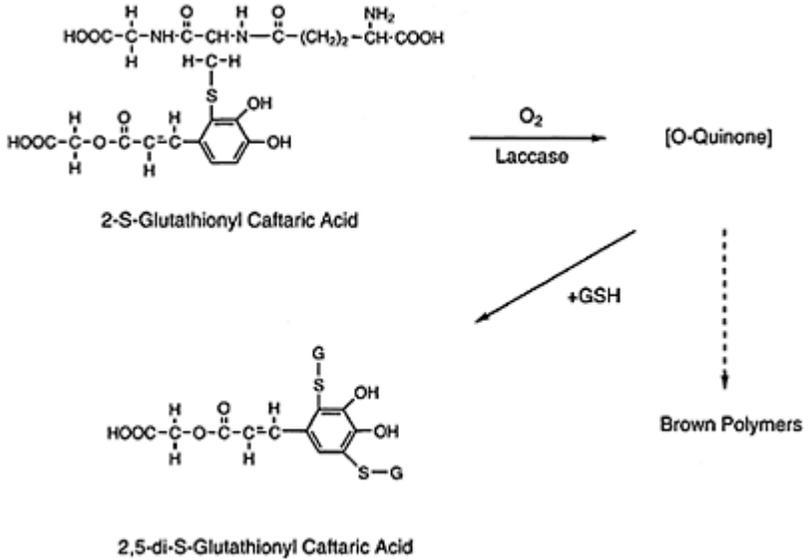


FIGURE 5.14 The proposed reaction sequence for the oxidation of 2-S-glutathionyl caftaric acid (top) by laccase and formation of 2,5-di-S-glutathionyl caftaric acid (bottom); GSH, glutathione. (Adapted from Salgues, M. et al., 1986, *J. Food Sci.*, 51:1191–1194.)

beverages is affected by the starting material and the aging process, if used. Volatile components of alcoholic beverages such as cognac, rum, and whisky contain minute quantities of phenolic compounds; these are also identified in fusel oils obtained by fractional distillation of the beverage. Otsuka et al. (1965) reported the presence of phenolic acids in cognac and whisky stored for a number of years in oak barrels. On the other hand, 4-methylguaiacol and eugenol were first identified in Jamaican rum (Moarse and ten Noever de Brauw, 1966). Later, Timmer et al. (1971) found phenol, *p*-ethylphenol, *o*-cresol, *p*-cresol, guaiacol, *p*-ethylguaiacol and dihydroeugenol. Furthermore, guaiacol, phenol, *o*-cresol, *p*-cresol, 4-methylguaiacol, *o*-ethylphenol and 4-ethylguaiacol have been found in whisky (Kahn, 1969). The presence of 6-methylguaiacol, *o*-isopropylphenol, *p*-ethylphenol, 2,6-xyleneol, eugenol, *o*-hydroxyacetophenone and 2-hydroxy-5-methylacetophenone in Japanese and Scotch

whisky varieties was reported by Nishimura and Masuda (1971). The content of these compounds ranges from 0.001 to 0.035 mg/L for *p*-ethylguaiacol in Scotch whisky.

Polyphenols represent up to one third of cognac extracts; the total content of polyphenols in aged cognacs ranges from 92 (after 1 year of aging) to 833 mg/L (after 30 years of aging) (Viriot et al., 1993). Distilled spirits aged in oak barrels contain low molecular weight phenolic compounds such as vanillin, syringaldehyde, sinapaldehyde, coniferaldehyde, and scopoletin, as well as vanillic, syringic, gallic, trimethoxyphenylacetic, 3,4-dihydroxybenzoic and ellagic acids (Delgado and Gomez-Cordoves, 1987; Goldberg et al., 1999; Guymon and Crowell, 1968; Panossian et al., 2001; Puech, 1988; Salagoity-Auguste et al., 1987; Viriot et al., 1993). The presence of these phenolics in beverages is attributed to the lignin and ellagitannin fractions of wood (Guymon and Crowell, 1968; Viriot et al., 1993). The concentration of gallic acid in aged cognacs is of the same magnitude as that of ellagic acid (Viriot et al., 1993). However, Goldberg et al. (1999) found that the content of gallic acid in seven samples of American whiskies (single-malt Scotch, blended Scotch, Canadian rye, American bourbon) and brandies (cognac, armagnac, regular brandy) is 3 to 10 times lower than that of ellagic acid. Panossian et al. (2001) reported that the content of sinapaldehyde in Armenian brandies is between 0.511 and 1.618 mg/L; coniferaldehyde is between 0.497 and 2.544 mg/L, syringaldehyde from 2.32 to 34.2 mg/L and vanillin from 1.13 to 12.83 mg/L.

Distilled spirits also contain soluble oligomers of lignins and ellagitannins. Viriot et al. (1993) evaluated the changes in ellagitannins and ellagic acid content during the aging of cognacs in oak barrels. These authors observed that the ellagitannins present in wood are quickly solubilized and degraded, but ellagic acid is released into the beverage at a slower rate. The presence of ellagic acid in cognacs may arise from solubilization of free ellagic acid as well as from the hydrolysis of soluble and insoluble ellagitannins present in woods. Not only ellagitannins but also lignins are chemically altered to soluble forms during aging of distilled spirits in oak barrels. Viriot et al. (1993) have suggested that the solubilization of lignins is brought about by the cleavage of alkyl aryl ether linkages. The content of soluble lignins in distilled spirits increases with duration of aging; one-year-old cognacs contain only 12 mg of lignins per liter while those stored for 30 years in oak barrels have a total lignin content of 219 mg/L (Viriot et al., 1993).

According to Steinke and Paulson (1964), guaiacol and its alkyl derivatives (noted earlier) as well as *p*-ethylphenol and *p*-vinylphenol are produced from coumaric and ferulic acids in grains at the mashing process by heating or by the action of fungi. However, presence of guaiacol, phenol and *p*-cresol may be the result of further degradation of those phenolics. On the other hand, *o*-cresol and 2,6-xylenol may be derived from lignin-like materials (Nishimura and Masuda, 1971).

Tequila is an alcoholic beverage obtained by distillation of fermented juice of cultivated variety of *Agave tequilana* (Valenzuela-Zapata, 1985). The process involves harvesting the stems of the agave plant, cooking them in an oven to hydrolyze inulins to fructose and glucose, and milling and pressing the cooked stems. The juice obtained is first fermented with yeast and then doubly distilled; after adjusting the alcohol content, it is stored in oak barrels (Benn and Peppard, 1996). Guaiacol, cresol, 4-ethylguaiacol, eugenol, vanillin, syringaldehyde and coniferaldehyde have been identified as flavor components of tequila. Of these, vanillin is considered one of the most powerful odorants

present; other powerful odorants of tequila are isovalerylaldehyde, 2-phenylethanol and β -damascenone (Benn and Peppard, 1996).

Cider brandy is an alcoholic beverage obtained by distillation of cider followed by a maturation process in American or French oak casks for different time periods, depending on traditional practices (Cabranes et al., 1990). Aging of distilled cider brandies in oak casks increases the total content of phenolics from 12.7 to 25.9 mg/L (Mangas et al., 1997). This increase may be brought about by the extraction and acid ethanolsysis of lignin followed by oxidation (Puech, 1988). Syringaldehyde, vanillin and gallic, vanillic and syringic acids are the predominant phenolics present in freshly distilled and aged cider brandies. Small quantities of *p*-hydroxybenzaldehyde and caffeic and protocatechuic acids have also been detected (Mangas et al., 1997).

CIDER AND APPLE JUICE

The traditional method of juice extraction from apples involves crushing and milling the fruit to pulp. The pulp may then be built up in alternate layers with cloths and wooden racks to form a "cheese" from which the juice is squeezed by vertical pack press or extracted by a continuous extraction system such as hot water diffusion. In order to improve pressing characteristics and yield, the pulp may be mixed with pectinolytic enzymes.

Phenolic compounds of apples have aroused considerable interest because of their influence on the sensory characteristics such as color and clarity of juice. Flavons and chlorogenic acid may contribute to the browning of juice because these compounds are good substrates for apple polyphenol oxidase (Stelzig et al., 1972). On the other hand, quercetin glycosides are considered very poor substrates for this enzyme. The content of quercetin glycosides and flavons declines during the storage of juice, but chlorogenic acid is quite stable (Tanner and Rentschler, 1956). Thus, changes in the phenolic composition may play an important role in the overall quality and acceptability of apple juice.

COMPOSITION OF POLYPHENOLICS

The composition of phenolic compounds in apple juice and cider depends on their initial composition in the apple cultivar, cultural conditions, and the extraction of phenolics during pressing. The composition of phenolics in fruits may vary from one cultivar to another and also depend on the degree of fruit maturity. The contents and relative proportions of phenolics in juices and ciders may also be changed as a result of enzymatic browning reactions and formation of haze. Phenolics have an important role in providing a bitter and astringent flavor to apple juice or cider. Phenolic acids (primarily chlorogenic and *p*-coumaric acids), dihydrochalcones (phloretin xyloglucoside and phloridizin), catechins (epicatechin and catechin) and procyanidins are major groups of phenolic compounds found in apple juice or cider (Lea, 1978; Wilson, 1981). Figure 5.15 depicts the chemical structure of major classes of cider phenolics. Of the phenolics found in apple juice and cider, the polymeric procyanidins are particularly responsible for their bitter/astringent characteristics. Phloridizin is another bitter phenolic found in apple juice

and cider; however, due to its low concentration, phloridzin may make only a small contribution to the overall bitterness of these products (Lea and Arnold, 1978; Lea and Timberlake, 1974).

The content of monomeric phenols in ciders, in the order of decreasing concentration, is 5-chlorogenic acid, *p*-coumarylquinic acid, catechin, phloridzin (phloretin glucoside), and phloretin xyloglucoside (Table 5.27) (Whiting and Coggins, 1975). Versari et al. (1997) identified phloridzin and phloretin xyloglucoside as quality indicators of fruit-based beverages. These two compounds are characteristic of apples

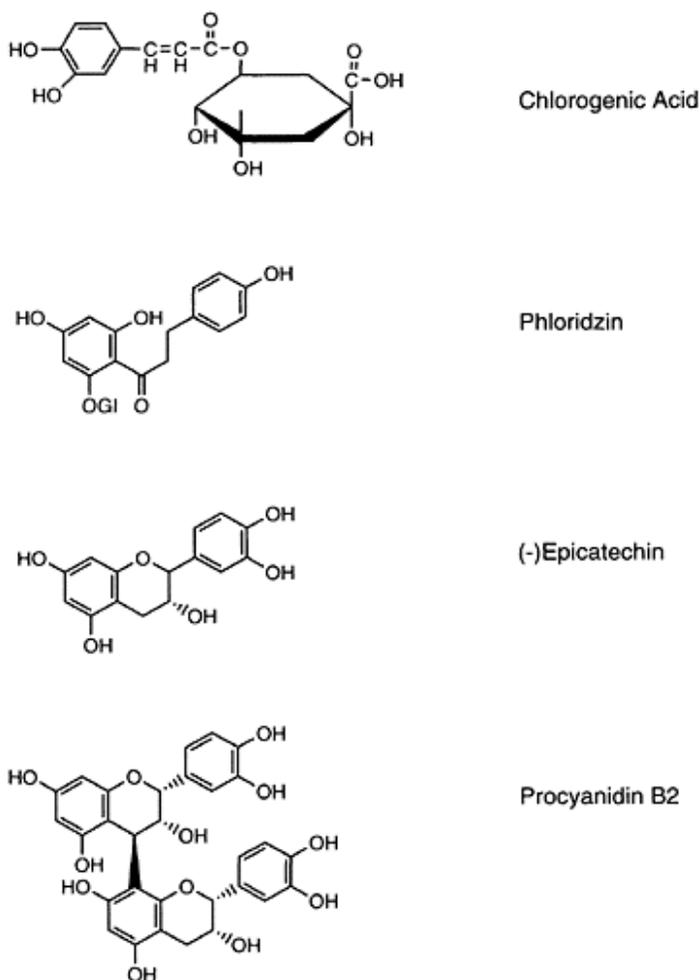


FIGURE 5.15 Phenolic compounds found in cider. (Adapted from Lea, A.G.H., 1978, *J. Sci. Food Agric.*, 29:471–477.)

and have not been detected in any other fruits. The addition of 50 to 60 mL/L of commercial apple juice to pear juice has been detected by the presence of one of these two dihydrochalcones (Versari et al., 1997). According to Prabha and Patwardhan (1985), the major monomeric phenols of apple pulp are *cis*-chlorogenic acid followed by epicatechin and *trans*-chlorogenic acid (Table 5.28). However, the contribution of chlorogenic acid, analyzed by HPLC, is 6.2 to 10.7% of the total polyphenols expressed as chlorogenic acid equivalents (Cilliers et al., 1990). The content of monomeric phenolics is also affected by cultural conditions. Cider produced from fruits harvested from trees under reduced supply of nutrients contains 17% higher levels of phenolic compounds (Lea and Beech, 1978).

Chlorogenic acid, a principal component of monomeric phenol fraction in apples, is generally considered a major browning substrate (Hulme and Rhodes, 1971;

TABLE 5.27
Concentration of Phenolics in Ciders from a Range of Cultivars^a

| Cultivar | Chlorogenic Acid | <i>p</i> -Coumarylquinic | Catechins | Phloridzin | Phloretin Xyloglucoside |
|------------|------------------|--------------------------|-----------|------------|-------------------------|
| Frederick | 0.42 | 0.07 | ND | 0.07 | 0.1 |
| Dabinett | 0.4–0.57 | 0.03 | 0.3–0.5 | 0.15–0.16 | 0.04–0.12 |
| Michelin | 0.51 | 0.06 | 0.25 | 0.07 | 0.04 |
| Vilberie | 0.73 | 0.43 | 0.4 | 0.03 | 0.03 |
| Yarlington | 0.68–1.5 | 6.2–7.9 | 0.6 | 0.03 | 0.02 |
| Kingston | 0.25 | 0.03 | 0.1 | 0.05 | 0.06 |

^a Grams per liter.

Source: Adapted from Whiting, G.C. and Coggins, R.A., 1975, *J. Sci. Food Agric.*, 26:1833–1838.

TABLE 5.28
Concentration of Polyphenols in Different Varieties of Apple Pulp^a

| Polyphenol | Aumburi | Royal Delicious | Kesari | Golden Delicious |
|--------------------------------|---------|-----------------|--------|------------------|
| Epicatechin | 250 | 190 | 130 | 70 |
| Quercetin glycoside | 38 | 20 | traces | traces |
| D-Catechin | 200 | 95 | 80 | 58 |
| Leucoanthocyanidins | 730 | 340 | 460 | 195 |
| Gallocatechin | 60 | 20 | traces | 10 |
| <i>cis</i> -Chlorogenic acid | 370 | 260 | 160 | 140 |
| <i>trans</i> -Chlorogenic acid | 180 | 80 | 188 | 50 |
| <i>p</i> -Coumaryl quinic acid | 55 | 12 | – | 8 |

^a Milligrams per kilogram.

Source: Adapted from Prabha, T.N. and Patwardhan, M.V., 1985, *J. Food Sci. Technol.*, 22:404–407.

Stelzig et al., 1972). Thus, changes in chlorogenic acid may contribute to the quality of apple juice. The content of chlorogenic acid in juice obtained from eight apple cultivars grown in Michigan, Washington, Argentina, Mexico and New Zealand ranges from 1.41 mg/L in juice obtained from Granny Smith apples to 214 mg/L in juice from McIntosh apples (Lee and Wrolstad, 1988). Van Buren et al. (1973) reported that the content of chlorogenic acid in juice obtained from different apple varieties ranges from 120 mg/L for juice from Golden Delicious variety to 310 mg/L for Carrel variety juice. Juice obtained from crab apples contains 390 to 1350 mg/L, while fresh fruits have 460 to 2050 mg of chlorogenic acid per kilogram (Kuusi and Pajunen, 1971). However, the pulp obtained from some Indian apple varieties contains from 190 mg of total chlorogenic acid per kilogram for Golden Delicious to

TABLE 5.29
Distribution of Cider Procyanidins

| Phenolic Type | Apple Cultivar | |
|--------------------------|-------------------|----------|
| | Tremlett's Bitter | Vilberie |
| Polymeric procyanidins | 1.06 | 1.23 |
| Pentameric procyanidins | 0.51 | 0.47 |
| Tetrameric procyanidins | 0.47 | 0.42 |
| Trimeric procyanidins C1 | 0.46 | 0.39 |
| Dimeric procyanidins B2 | 0.70 | 0.61 |
| Epicatechin | 0.51 | 0.40 |
| Total oligomers | 2.65 | 2.29 |
| Total polymers | 1.06 | 1.23 |
| Total procyanidins | 3.71 | 3.52 |

^a Grams per liter.

Source: Adapted from Lea, A.G.H. and Arnold, G.M., 1978, *J. Sci. Food Agric.*, 29:478–483.

550 mg/kg for Aumburi variety (Prabha and Patwardhan, 1985) (Table 5.28). Other researchers have reported values of 250 mg chlorogenic acid per kilogram (Macheix, 1968) to 890 mg/kg of fresh mature fruits (Walker, 1963) for different apple varieties.

Cider prepared from true bittersweet apples typically contains 2 to 3 g/L of procyanidins (Lea, 1978; Lea and Timberlake, 1974). Characterization of cider procyanidins shows a range of procyanidin oligomers with varying degrees of polymerization. The main series of procyanidins in cider is based on epicatechin (e.g., procyanidin B2, B5, C1). However, small amounts of a mixed series of catechin-epicatechin (e.g., procyanidin B1) are also present (Lea, 1978; Wilson, 1981). The content of oligomeric and polymeric procyanidins in cider is shown in Table 5.29. The degree of procyanidin polymerization influences the organoleptic characteristics of cider. Thus, cider with a lower ratio of oligomeric to polymeric procyanidins is more astringent while that with a higher ratio is more bitter (Lea and Arnold, 1978).

EFFECT OF PROCESSING

The major loss of phenolics during processing of apples to cider is brought about by oxidation during the milling process and before pressing by incomplete extraction of apple tissues and during clarification of juice. Longer juice contact with air and pomace before pressing results in larger amounts of polyphenolics—in particular, polymeric procyanidins—responsible for sensory characteristics of apple juice and cider. This is probably due to the oxidation of procyanidins to their corresponding quinones, which are then irreversibly bound to the pomace constituents. On the other hand, enzymatically induced oxidative loss of phenolics is much less pronounced after extraction of the juice because the content of soluble polyphenol oxidase is very low in the juice (Lea and Timberlake, 1978).

TABLE 5.30
Content of Polyphenolic in Ciders Obtained
from Diffuser and Pack Press Techniques^a

| Phenolics | Diffuser | Pack Press | Pack Press Corrected to Diffuser Dilution |
|-------------------------|----------|------------|---|
| Phenolic acids | 850 | 1170 | 860 |
| Phloretin xyloglucoside | 90 | 140 | 100 |
| Epicatechin | 200 | 260 | 190 |
| Phloridzin | 200 | 150 | 110 |
| Procyanidin dimers | 340 | 500 | 360 |
| Procyanidin trimers | 100 | 220 | 160 |
| Procyanidin tetramers | 210 | 260 | 190 |
| Procyanidin polymers | 850 | 580 | 420 |

^a Milligrams per liter.

Source: Adapted from Lea, A.G.H. and Timberlake, C.F., 1978, *J. Sci. Food Agric.*, 29:484–492.

Extraction of apple juice by diffusion techniques almost doubles the content of phloridzin and polymeric procyanidins compared to the levels found in the juice obtained by pressing (Table 5.30). Simple warm incubation under nonoxidative conditions also increases the content of polymeric procyanidins and phloridzin. On the other hand, use of pectolytic enzymes to improve the yield of juice extraction does not have any detrimental effect on polyphenols. Clarification of juice with gelatin leads to a significant loss of tetraneric and polymeric procyanidins (Lea and Timberlake, 1978).

Table 5.31 shows the effect of different treatments on the content of polyphenolics in apple juice obtained from Golden Delicious variety. Holding the pulp for up to 3 h under oxidative conditions produces juice with low contents of chlorogenic acid and flavonoids (mainly monomeric and polymeric flavans), but with higher levels of flavonol glycosides. Holding pulp under nonoxidative conditions, however, improves the extraction of all phenolic compounds from pulp into the juice (Van Buren et al., 1976).

OTHER FRUIT JUICES

ORANGE JUICE

Oranges are a good source of flavanones (mainly hesperidin and narirutin) (Albach and Redman, 1969), polymethoxyflavones (e.g., nobiletin, tangeretin and sinensetin) (Park et al., 1983) and hydroxycinnamic acid derivatives (ferulic, *p*-coumaric, sinapic and caffeic acid derivatives) (Clifford, 1999; Risch and Herrmann, 1988). Cinnamoyl- β -D-glucose has been detected only in blood orange juices (Mouly et al., 1997). The total content of phenolics in several juices obtained from five different *Citrus sinensis* (L.)

TABLE 5.31

Phenolic Compounds in Apple Juice^a

| Method of Juice Preparation | Flavonol Glycosides | Other Flavonoids | Chlorogenic Acid | Other Nonflavonoids |
|-----------------------------|---------------------|------------------|------------------|---------------------|
| Conventional | 4 | 200 | 140 | 170 |
| Rapidly pasteurized | 2 | 710 | 230 | 80 |
| Oxidized pulp, 1 h | 7 | 90 | <10 | 150 |
| Oxidized pulp, 3 h | 60 | 10 | <10 | 210 |
| Held and nonoxidized pulp | 80 | 1010 | 320 | 200 |

Notes: Flavonol glycosides are expressed in rutin equivalents, other flavonoids are expressed in catechin equivalents, and other nonflavonoids are expressed in chlorogenic acid equivalents.

^a Milligrams per liter.

Source: Adapted from Van Buren, J. et al., 1976, *J. Agric. Food Chem.*, 24:448–451.

TABLE 5.32

Composition of Phenolics in Orange Juice^a

| Orange Variety | Total Anthocyanins | Total Flavonones | Total Hydroxycinnamic Acid | Total Phenols |
|----------------|--------------------|------------------|----------------------------|---------------|
| Moro | 97.5–278.4 | 266.1–444.5 | 60.1–140.2 | 673.9–1147.2 |
| Tarocco | 1.2–99.4 | 150.1–179.4 | 38.0–91.2 | 387.3–1090.5 |
| Saquinello | 6.4–52.6 | 185.7–300.2 | 46.4–92.1 | 382.5–602.9 |

^a Milligrams per liter.

Sources: Adapted from Rapisarda et al., 1998, *J. Agric. Food Chem.*, 46:464–470 and 2000, *J. Agric. Food Chem.*, 48:2249–2252.

varieties ranges from 361 (Washington navel variety) to 1147 mg of ferulic acid equivalents per liter (Moro variety) (Table 5.32) (Rapisarda et al., 1998, 2000). According to Gil-Izquierdo et al. (2001), hand-squeezed navel orange juice contains 839 mg of phenolics per liter. Citrus flavonoids possess health-promoting activities; these

compounds show activity against myeloid leukemia (Kawaii et al., 1999) and possess antiinflammatory, antianalgesic (Galati et al., 1994), anticarcinogenic (Bracke et al., 1994; Stavric, 1994), antihypertensive, diuretic (Galati et al., 1996) and hypolipidemic activities (Monforte et al., 1995).

Blood oranges have traditionally been consumed as fresh fruit because processing them into juice is unsatisfactory due to unstable pigments. This problem has been circumvented to a great extent by acidification or addition of antioxidants (Maccarone et al., 1985). Another way of utilizing blood orange juice may be to mix it with other juices for color enhancement (Lee et al., 1990). California and

TABLE 5.33
Effect of Processing on Phenolic Content in Soluble and Cloud Fractions of Orange Juices^a

| Type of Processing | Flavonones | | C-glycosylflavone | | Total Phenolics |
|--------------------|------------|-------------|-------------------|---------|-----------------|
| | Soluble | Cloud | Soluble | Cloud | |
| 70°C; 30 | 91.7–116.0 | 247.0–644.0 | 338.0–53.3 | 2.9–8.1 | 416.5–867.4 |
| 92–95°C; 30 | 80.9–127.6 | 80.9–127.6 | 32.1–51.1 | – | 511.0–595.6 |

^a Milligrams per liter.

Source: Adapted from Gil-Izquierdo, A. et al., 2001, *J. Agric. Food Chem.*, 49:1035–1041.

Italian blood oranges contain red anthocyanin pigments. The content of anthocyanin pigments in the juice depends on the season and varies from 96 to 166 mg/L of juice obtained from California oranges (Lee et al., 1990) and from 1.2 to 278 mg/L of juice obtained from Italian oranges (Rapisarda et al., 1998, 2000). Comprising over 90% of total anthocyanin pigments, cyanidin is the predominant substance, followed by delphinidin with about 6%, peonidin at 1.2 to 1.7%, and traces of petunidin and pelargonidin. On the other hand, Florida blood oranges contain only 3.7 mg/L of total anthocyanidins, but their profile is similar to those found in California blood oranges (Lee et al., 1990).

Flavanones are the predominant flavonoids found in citrus juices (Table 5.32); of these, hesperidin is the major flavanone glycoside found in orange juices. Other flavanone glycosides identified in citrus fruits include narirutin and didymin (Mouly et al., 1997). Hesperidin is tasteless and therefore does not contribute to the taste of orange juice (Horowitz and Gentili, 1977); its content in orange juice is 350 to 7000 mg/L (Mears and Shenton, 1973). However, much lower amounts of hesperidin have been found in 14 common sweet oranges (Rouseff et al., 1987). Depending on season, California blood oranges contain from 457 to 657 mg of hesperidin per liter of juice (Lee et al., 1990). Narirutin is the other major flavanone glycoside found in orange juices; Rouseff et al. (1987) reported that Florida citrus cultivars contain 18 to 65 mg/L and California blood oranges have 108 to 180 mg/L of narirutin (Lee et al., 1990).

Small quantities of fully methoxylated flavones have also been found in orange juices. According to data reported by Veldhuis et al. (1970), the total content of fully methoxylated flavones in orange juice is less than 7 mg/L. Nobiletin, sinensetin, heptamethoxyflavone, tetra-*o*-methylscutellarein and tangeretin have been identified in blood orange juices, with nobiletin and heptamethoxyflavone the predominant

substances. Commercial orange juices contain only 0.5 mg/L of tangeretin juice, a major fully methoxylated flavone found in tangerine juice (Veldhuis et al., 1970).

Orange juices have a tendency to form cloud upon storage. It has been demonstrated that flavanones are incorporated into the cloud because of their limited solubility in acidic solutions (Table 5.33). The extent of flavanone precipitation is affected by the citrus fruit cultivar, pressure used during juice extraction, holding time before pasteurization, and pasteurization conditions (Baker and Cameron, 1999; Cameron et al., 1997). Recently, Gil-Izquierdo et al. (2001) demonstrated that thermal treatment of citrus juice has a limited effect on precipitation of flavanones.

PEAR JUICE

Pear juice concentrate is used in the production of wine, vinegar and beverages, and as a syrup base for fruit canning. During storage, however, it may become dark brown, which reduces its versatility and commercial value. Discoloration of pear juice is predominantly due to Maillard reactions. Cornwell and Wrolstad (1981) demonstrated that pear juice concentrates with phenolics removed brown at the same rate as those where phenolics are not removed. Thus, oxidation of phenolics does not appear to be a major cause of browning in pear juice. Nonetheless, Anderson (1970) reported the occurrence of oxidation and polymerization of phenolics during storage of fruit juices. Probably the pH of pear juice (pH=4.2) does not favor oxidation of phenolics that occur at above pH 6 (Gregory and Bendall, 1966). On the other hand, the amber-to-brown color developed during the processing is ascribed to the action of polyphenol oxidase (Sioud and Luh, 1966). Considerable loss of cinnamic acids and total disappearance of procyanidins occur in pear juices processed without addition of SO₂. Arbutin and flavonol glycosides are less affected by SO₂ and processing, but storing juice concentrates for 9 months at 25°C results in extensive degradation of cinnamics and flavonol glycosides and total loss of procyanidins (Spanos and Wrolstad, 1990).

Blending pear juice with anthocyanin-pigmented juices may create a greater market for this type of product, but may also bring about the development of haze and color instability. Anthocyanins degrade during storage and may subsequently condense and polymerize to produce polymeric pigments and sediments during storage. At 25°C, the turbidity and polymeric pigments of juices increase, while anthocyanin levels decrease, during 48 weeks of storing pear juices blended with 5, 10 and 20% of pigmented juices such as Concord grape and black raspberry juice. The changes are more pronounced in blends containing a larger amount of pigmented juice (Spayd et al., 1984).

STRAWBERRY JUICE

Using strawberry juice concentrate as a natural flavorant in food and beverages is still hindered by its susceptibility to color and flavor degradation after a short period of storage at room temperature or longer periods at refrigeration temperatures. Astringent taste, musty or moldy and pungent aromas, and brown colors develop rapidly after 6 days of storage at 20°C (Lundahl et al., 1989). The content of anthocyanins decreases while the level of brown-red pigments increases as the browning process progresses (Wrolstad et al., 1980). The rate of color degradation increases with reduced water activity

(Erlandson and Wrolstad, 1972), increased storage temperature (Hassanein, 1982), and lowered concentration of antioxidants in the system (Poei-Langston and Wrolstad, 1981). Phenolics present in the juice may also contribute to nonenzymatic and enzymatic browning reactions. Oxidation of ascorbic acid through a free radical chain reaction produces hydrogen peroxide that, in turn, may react with anthocyanins to form undesirable breakdown products (Sondheimer and Kertesz, 1953; Starr and Francis, 1968).

The flavonols that always accompany anthocyanins in fruits have a protective effect on anthocyanins in fruits by retarding the oxidation of ascorbic acid. Quercetin and quercetin in concentrations of 30 to 60 and 90 mg/L, respectively, retard the oxidation of ascorbic acid by an average of 20% over 72 h. On the other hand, anthocyanins in the absence of flavonols accelerate oxidation of ascorbic acid by an average of 34% over a pure solution of ascorbic acid without anthocyanins (Shrikhande and Francis, 1974). Color degradation can also be accelerated in strawberry juice by polyphenol oxidase. This reaction involves orthoquinones, ascorbic acid and anthocyanins (Wrolstad et al., 1980). However, complexation of anthocyanins with proteins, modified by oxidase or peroxidase, cannot be excluded (Matheis and Whitaker, 1984).

The increase in astringency of strawberry juice can be detected after only 1 day of storage at 20°C (Lundahl et al., 1989). This may be due to polymerization of anthocyanins and other phenolics because this taste attribute has been associated with polymerization of phenolics (Bate-Smith, 1973; Hagerman and Butler, 1981; Lea and Arnold, 1978; Lea and Timberlake, 1974). The formation of protein-phenolic complexes that can elicit an astringent taste cannot be ruled out (Lea and Timberlake, 1974; Lundahl et al., 1989; Matheis and Whitaker, 1984).

KIWIFRUIT JUICE

The total content of phenolics in kiwifruit is much lower than that found in other fruits. One month after polination the fruit contains from 1800 to 2200 mg of total phenolics per kilogram; this level declines by 50% during fruit growth (Fuke and Matsuoka, 1984). Kiwifruit juice concentrate contains only 380 to 440 mg of total phenolics per liter (Wong and Stanton, 1993), but in clarified juice the total content of phenolics is between <1 and 7 mg/L. Webby (1990) identified glucosides, rhamnosides and rutinoides of kaempferol and quercetin. Later, Dawes and Keene (1999) found chlorogenic and protocatechuic acids, derivatives of coumaric and caffeic acids, a derivative of 3,4-dihydroxybenzoic acid, catechin, epicatechin, and procyanidins B2, B3, B4 and oligomers in kiwifruit juices.

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6

Phenolics in Herbal and Nutraceutical Products

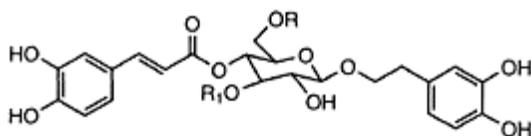
The fact that phytochemicals occurring in food and natural health products play a significant role in disease prevention and health promotion was first recognized as a result of epidemiological studies using both animal and human subjects. This led to an ever-growing interest in herbs and botanical and nutraceutical products. Bioactives in herbal and nutraceutical products constitute a myriad of chemical compounds, among which phenolic substances often play a primary or a synergistic function. Herbal products are used to enhance immunity, for antiinflammatory, anticancer, and antidepressant effects and for treatment of heart disease, urinary tract infections and prostate conditions, among others. The herbal products of interest include, but are not limited to, echinacea, ginseng, ginkgo biloba, St. John's wort, valerian, kava kava, saw palmetto, black cohosh, devil's claw, goldenseal, hawthorn, ginger, licorice, and milk thistle (Blumenthal, 2000; Lawson and Bauer, 1998). These products serve as a source of the phenolic compounds summarized in this chapter.

ECHINACEA

Echinacea products serve as popular herbal immunostimulants, particularly in North America and Europe (Grunwald and Büttel, 1996). Roots and aerial parts, as well as their tinctures and extracts, are used, mainly in encapsulated forms. Echinacea genus comprises nine species and two varieties (McGregor, 1968), of which only *E. purpurea* (L.), *E. angustifolia* (DC.) and *E. pallida* (Nutt.) are used medicinally. These herbs were used initially by the American Indians for treatment of respiratory ailments, wounds, snake bites, common cold, and headache. Their current use is as antiseptic, antiviral, peripheral vasodilation, healing for skin conditions, carbuncles, boils, wounds, ulcers, burns, bedsores, bites, stings, and poisonous insects, to build the immune system when taken orally and as supportive therapy for cold and influenza (Tyler, 1998).

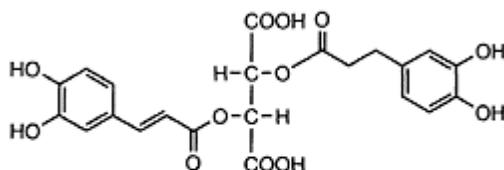
The active components of Echinacea include polysaccharides, glycoproteins, caffeic acid derivatives, flavonoids, poly acetylenes, and alkylamides. Caffeic acid derivatives are a major group of compounds present (Becker et al., 1982; Facino et al., 1993). Echinacoside is the major polar constituent of the roots of *E. angustifolia* present at 0.3 to 1.7% (Schenk and Franke, 1996); cichoric acid (2,3-*O*-dicaffeoyltartaric acid) occurs in especially high concentrations in the flowerheads and roots (up to 3.1 and 2.1%, respectively). The content of cichoric acid depends on the season and stage of development of the plant (Alhorn, 1992; Bauer and Von Hagen-Plettenberg, 1999). In

addition, 1,3-*O*-dicaffeoylquinic acid (cynarine) and 2-*O*-caffeoyltartaric acid (cichoric acid) as well as chlorogenic acid and ferulates



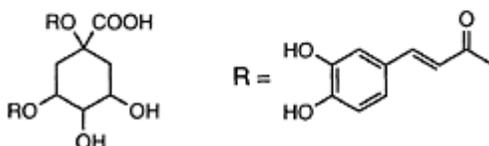
Echinacoside

R = glucose (1, 6-) R₁ = rhamnose (1, 3-)



Cichoric Acid

(2,3-dicaffeoyltartaric acid)



Cynarine

(1,3-dicaffeoylquinic acid)

FIGURE 6.1 Chemical structures of caffeic acid derivatives in Echinacea species.

and tartaric acid may be present (Bisset, 1994; D'Amelio, 1999; Newall, 1996). The chemical structures of some caffeic acid derivatives found in Echinacea products are given in Figure 6.1. Cichoric acid possesses phagocytosis stimulatory activity *in vitro* and *in vivo* (Bauer et al., 1989) and inhibits hyaluronidase (Facino et al., 1993) and free radical degradation of type III collagen (Facino et al., 1995). Cichoric acid also inhibits human immunodeficiency virus type 1 (HIV-1) integrase (Robinson, 1998). Other constituents found in Echinacea include 15 lipophilic alkalamides, most of which contain one or two acetylenic bonds. In addition, a number of ketoalkynes have been identified in Echinacea.

GINSENG

Ginseng represents perennial herbs derived from the genus *Panax* grown in China, India, Japan, Korea, Vietnam and North America. The Siberian ginseng is a different plant, referred to as *Eleutherococcus senticosus* ginseng. The beneficial health effects of ginseng relate to its transient regulatory effects on carbohydrates, lipids, and nucleic acid metabolism (Nagasawa et al., 1977; Oura and Hiai, 1973; Sotaniemi et al., 1995). It also has a prophylactic effect that reduces susceptibility to illness and may act as an aphrodisiac. The main active components of ginseng are triterpenoid ginsenosides, and steroidal saponins (panaxosides), which are present at a 2 to 3% level in ginseng roots (Bisset, 1994; D'Amelio, 1999; Newall, 1996). These constituents are linked to different sugar molecules and, together with flavonoids, serve as the main bioactive components of ginseng (Zhang et al., 1979a, b). Presence of caffeic acid, kaempferol, and vanillic acid has also been reported (Kitts, 2000). Ginseng products protect against plasmid DNA scissions and scavenge hydroxyl radicals (Kitts et al., 1999).

GINKGO BILOBA

Ginkgo (*Ginkgo biloba* L.) leaf extracts have served as one of the oldest natural therapeutics; ginkgo's fruits were used as medicinal agents 5000 years ago (Michel, 1986). Its seeds have been used extensively as a remedy for asthma, cough, bladder inflammation and alcohol abuse, among others. The leaves have also been used to alleviate cardiovascular disorders and asthma as well as for the treatment of disorders associated with aging and peripheral circulation insufficiencies (Foster and Chongdi, 1992). The antiinflammatory and antiallergenic effects of ginkgo and its vasodilatory activity have been reported in the literature (D'Amelio, 1999; Juretzek, 1997). The physiological benefits of ginkgo are due to the antioxidive, vasoregulatory, and neuroprotective activities of its constituents. Active components of ginkgo with documented biological activity include ginkgolides, bilobalide, flavonoids, and ginkgolic acids (Tyler, 1998).

Phenolics in ginkgo are aglycones of flavone glycosides. The flavonols present include quercetin, kaempferol, rutin, and isorhamnetin whose glycosidic constituents are mono-, di- or triglycosides of glucose and rhamnose. Thus, dimeric flavonoids, (bilobetin, ginkgetin, isoginkgetin, and sciadopitysin), flavonols, flavonol glycosides, and coumaric esters of flavonol glycosides, as well as proanthocyanidins, have been isolated from ginkgo biloba leaves (Van Beek et al., 1998; D'Amelio, 1999; Newall, 1996). Quercetin and kaempferol coumaryl glycosides are essential for the efficacy of extracts of ginkgo. Rutin and quercetin as well as flavonoid mixtures from ginkgo biloba have been found to protect cerebellar cells from oxidative damage and apoptosis by scavenging hydroxyl radicals (Chen et al., 1999). Ginkgo leaves also contain ginkgolic acids, which are 6-alkyl salicylic acids and 3-alkyl phenols, known as ginkgols, as well as 5-alkyl resorcins represented by bilobol and hydrobilobol, known as cardols. These latter compounds may cause gastrointestinal disturbance following consumption of ginkgo biloba fruits (Becker and Skipworth, 1975) and may also possess antimicrobial and

antitumoral activity (Jaggy and Koch, 1997). Chemical structures of ginkgolic acids, cardanols and cardols, are given in Figure 6.2.

ST. JOHN'S WORT

St. John's wort (*Hypericum perforatum* L.) is a shrubby aromatic perennial herb. It is native to Asia, North Africa, Azores and other parts of Europe and has also been naturalized in Australia and North America. St. John's wort has a long history as a valuable treatment for depression and sleep-related problems (Linde et al., 1996;

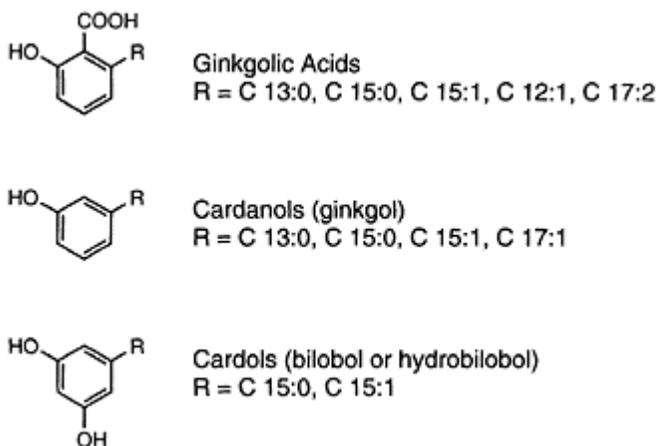
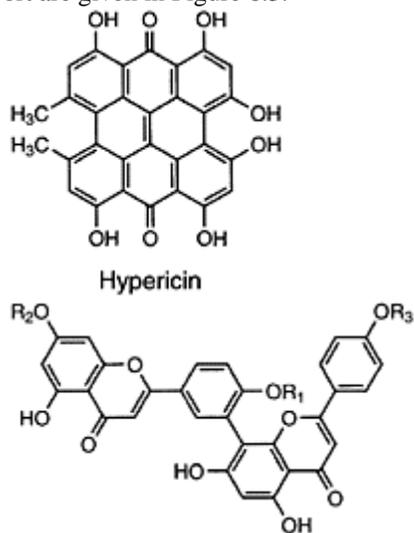


FIGURE 6.2 Chemical structures of ginkgolic acid, cardanols and cardols of ginkgo biloba.

Hippius, 1998; Giese, 1999). It has been found very helpful in easing emotional and stress symptoms and has also been used for the treatment of burns, wounds, insomnia, shocks, concussions, hysteria, gastritis, hemorrhoids, and kidney disorders (Hobbs, 1989, Chavez and Chavez, 1997; Upton, 1997). The main healing qualities of St. John's wort originate from tiny glands on the plant. In addition, extracts of flowering tops of *Hypericum* are known to possess antiinflammatory, wound- and burn-healing effects, and antibacterial, antifungal, antibiotic, and antiviral activities, among others (Hobbs, 1989; Chavez and Chavez, 1997).

St. John's wort contains a complex mixture of bioactive anthraquinones derivatives, mainly 0.05 to 0.3% of hypericin, isohypericin, and protolypericin (Bisset, 1994; Newall, 1996); flavonols (kaempferol, myricetin, and quercetin) (Newall, 1996); flavones (luteolin and rutin) (Newall, 1996); glycosides (hyperoside (hyperin), isoquercitrin, quercitrin and rutin) (Bisset, 1994; Newall, 1996); biflavonoids (biagigenin and amentoflavone) (Bisset, 1994; Newall, 1996) as well as catechins associated with condensed tannins (Newall, 1996). In addition, caffeic, chlorogenic, *p*-coumaric, ferulic,

isoferulic, *p*-hydroxybenzoic, genistic, and vanillic acids (Newall, 1996; Hobbs, 1989; Upton, 1997) as well as perenylated phloroglucinol derivatives (Bisset, 1994; Newall, 1996) have been found in St. John's wort. Hyperforin (2 to 4% in the dried herb; *H. perforatum*) has been shown to inhibit the reuptake of serotonin, dopamine and norepinephrine and thus has been identified as the leading candidate for a single antidepressant compound in *Hypericum* (Muller et al., 1998). Flavonoids constitute 11.7 and 7.4% of the leaves and stalks of St. John's wort (Upton, 1997). Proanthocyanidins or condensed tannins are found in large amounts in the areal parts of the plant and are oligomers and polymers of catechin and epicatechin, all of which possess antioxidant, antimicrobial, and cardioprotective effects (Cook and Samman, 1996; Hollman et al., 1996; Baureimel et al., 1997; Kim et al., 1998). The chemical structures of some phenolics of St. John's wort are given in Figure 6.3.



| Compound | R ₁ | R ₂ | R ₃ |
|---------------|-----------------|-----------------|-----------------|
| Amentoflavone | H | H | H |
| Bilobetin | CH ₃ | H | H |
| Ginkgetin | CH ₃ | CH ₃ | H |
| Isoginkgetin | CH ₃ | H | CH ₃ |
| Sciadopitysin | CH ₃ | CH ₃ | CH ₃ |

FIGURE 6.3 Chemical structures of hypericin and bioflavonoids of St. John's wort.

VALERIAN

There are approximately 300 species of valeriana, also called valerian, three of which are commonly used for medicinal purposes. These are *Valeriana officinalis* L. or valerian, a Eurasian member of the Valerianaceae, *V. wallichii* of India and Pakistan and *V. edulis* of

Mexico. The *V. officinalis* is the species of interest in most applications. Roots and rhizomes of valerian have been used for over 2000 years as a sedative and are now used for treating tension, nervous excitability, hysterical state, insomnia, hypochondriasis, migraines, cramps, rheumatic pain, and irritability with falling asleep (Blumenthal et al., 1998; D'Amelio, 1999; Cott, 1995; Houghton, 1988; Newall, 1996).

Active constituents of valerian include pyridine type alkaloids, namely, actinidine, chatinine, shyanthine, valerianine, and valerine (D'Amelio, 1999; Newall, 1996), some of which are phenolic in nature (see Figure 6.4). In addition, they contain a number of iridoids, namely, valtrates and isovaltrates, as well as terpenes and sesquiterpenes. Caffeic and chorogenic acids as well as tannins and other polyphenolics have been identified in roots and rhizomes of valerian (Newall, 1996).

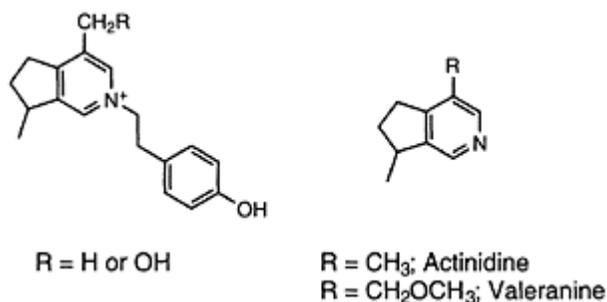


FIGURE 6.4 Chemical structures of pyridine alkaloids of valeriana.

KAVA KAVA

Kava kava (*Piper methysticum*) is an herb originating from Oceania Polynesia, where it has been used as a ceremonial beverage. It is gaining popularity in the United States and Europe as a relatively safe remedy for anxiety and compares favorably with benzodiazepines (Blumenthal et al., 1998). Dried rhizomes are used; these also have a relaxing effect on the central muscular system and are effective in the treatment of urinary tract infections, asthma, anxiety disorders, menopause symptoms, and mental function, as well as acting as anticonvulsant and antispasmodic agents (Backhausß and Krieglstein, 1992; Blumenthal, 2000; Woelk et al., 1993). Kavalactones present at 3.5% or more are the main active components and include methysticin, dihydromethysticin, kavain, 7,8-dihydrokavain, 5,6-dehydrokavain, 5,6-dehydromethysticin and yangonin (Blumenthal, 2000). Chalcones, namely, flavokavains A, B and C, are also present (Blumenthal, 2000)

SAW PALMETTO

Saw palmetto (*Serenoa repens* or *Serenoa serrulata*) is a small shrubby palm grown in the U.S. The dark brown or black single-seeded fruit or berry of saw palmetto looks like an olive in shape and size and turns yellow, orange or purplish-black in color when ripe. Saw palmetto berries are accepted botanical medicine (USP, 1999) and are used for treating chronic and subacute cystitis, laryngitis, bronchitis and, most importantly, benign prostatic hyperplasia (BPH) and enlarged prostate. Saw palmetto stimulates the genitourinary tract and mucous membranes (Blinker, 1994; Bennett and Hicklin, 1998; Wilt et al., 1998), promotes the growth of new flesh and has estrogenic activity (D'Amelio, 1999). The constituents of saw palmetto include steroids, flavonoids and tannins as well as carotenoids and other pigments (Blumenthal, 2000).

BLACK COHOSH

The roots and rhizomes of black cohosh, *Cimicifuga racemosa*, are used for the treatment of depression and hot flashes and is known to possess estrogenic activity (Blumenthal, 2000; D'Amelio, 1999). It has been suggested for treating peripheral arterial diseases because it possesses vasoactive effects. Black cohosh also exhibits antimalarial activity and is effective in relieving the symptoms of menopause. The active components of black cohosh include cimicifugic acids A to E, fukinolic acid and fukiic acid. Cimicifugic acids A to E and fukinolic acid are esters between cinnamic acids and the hydroxyl group of benzyltartaric acids (Noguchi et al., 1998). Tannins are also present in saw palmetto berries (D'Amelio, 1999; Newall, 1996).

DEVIL'S CLAW

Devil's claw (*Harpagophytum procumbens* DC.) is the root (especially its secondary tubers) marketed in Canada and in Europe as a home remedy for the relief of arthritic disease and as an antiinflammatory agent (Bisset, 1994; Blumenthal, 2000; D'Amelio, 1999). When applied externally, it is also effective in the treatment of sores, ulcers, boils and skin lesions (Blumenthal, 2000). The constituents of the plant are primarily sucrose and its galactosides, namely, stachyose, present at 46%, and raffinose. The active constituents present at 0.1 to 2% are primarily iridoiglycosides: harpagoside, harpagide and procumbide (Bisset, 1994; Blumenthal, 2000; D'Amelio, 1999; Newall, 1996) (Figure 6.5). In addition, chlorogenic acid and quinones (D'Amelio, 1999) as well as flavonoids kaempferol and luteolin (Blumenthal, 2000; D'Amelio, 1999; Newall, 1996) are present. Preparations also include verbascosides, acetoside, isoacetoside and abisoide, and glycosylated phytosterols along with oleanic and ursolic acids (Bisset, 1994; Blumenthal, 2000; D'Amelio, 1999; Newall, 1996).

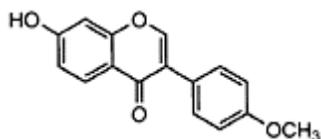


FIGURE 6.5 Chemical structure of bioactive flavonoid of devil's claw,

GOLDENSEAL

Goldenseal (*Hydrastis canadensis* L.) is another medicinal herbaceous perennial that grows in shady woods in Western Ontario and the Eastern U.S. The rhizomes and roots of goldenseal have been used since ancient times in China and India as an antidiarrheal medication. Its liquid extracts may also be used for treatment of colds, flu, sinus infections, and wounds and as an antiinflammatory preparation (Foster, 1991). Duke (1985) has reported its use as a diuretic, echarotic or stimulant and as a cure for boils, hemorrhoids, ulcers, asthma, jaundice, liver ailments and other diseases. These preparations have a bright yellow color due to the presence of the yellow alkaloid berberine and have therefore been used as a dye for clothing (Thornton, 1998). Goldenseal has also been referred to as Indian paint, Indian turmeric or jaundice root (Snow, 1998).

The active components of goldenseal are a number of isoquinoline alkaloids, including berberine, hydrastin, hydrostidine, isohydrastidine, berberostine, canadine, canadoline, corypalmine, and isocorypalmine (Messana et al., 1980; Leone et al., 1996). At recommended doses berberine and berberine-containing plants are considered nontoxic, but at 2- to 3-g doses goldenseal can lower heartbeat; at higher doses, it can paralyze the central nervous system (Flynn and Roest, 1995).

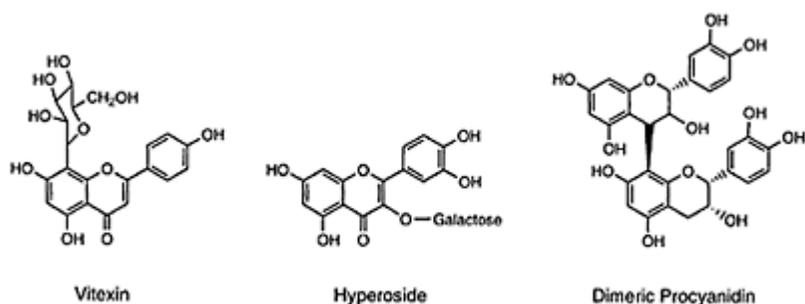


FIGURE 6.6 Chemical structures of some bioactive polyphenolics of hawthorn.

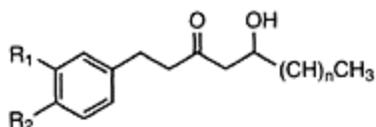
HAWTHORN

The flowers, leaves and berries of hawthorn, *crataegus*, are used for the treatment of a variety of cardiac diseases (Bisset, 1994; D'Amelio 1999; Newall, 1996), coronary and myocardial circulation, tachycardia, arteriosclerosis and as an antispasmodic and a sedative agent (Bisset, 1994; Blumenthal, 2000; Blumenthal et al., 1998; D'Amelio 1999). The constituents of hawthorn include 1 to 3% oligomeric procyanidins, 1 to 2% flavonoids, hyperosides, vilexins, vilexin glucosides, quercetin and its glucoside. In addition, phenolic acids and catechin are present (Bisset, 1994; Blumenthal, 2000; D'Amelio 1999; Newall, 1996). Figure 6.6 shows structures of some bioactives of hawthorn.

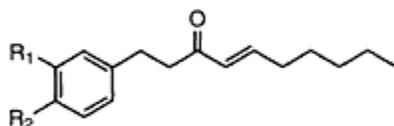
GINGER

An important folk medicine as well as a spice used in Western and Middle Eastern cooking, ginger (*Zingiber officinals*) has been cultivated and used for over 3000 years in India and China (Schulick, 1993). Different parts of the plant, including flower, bud, leaves, fruit and, particularly, rhizome, have been used for treatment of a number of ailments and discomforts. These include stomachache, cough, anorexia, eczema, diarrhea, rheumatism, jaundice, anemia, constipation, asthma, gastritis and can also function as an anticonvulsant and antiemetic agent. The fresh rhizomes of *Zingiber officinale* are dried and used as digestive stimulants and antiinflammatory agents in China and tropical Asia (Kikuzaki et al., 1994).

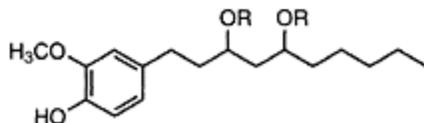
The bioactive components of ginger include gingerols, which are the main pungent component of the rhizome, and shogaol, a monohydrated gingerol, as well as other gingerol-related compounds formed from phenylalanine via ferulic acid, and diarylheptanoids and terpenoids (Kawakishi et al., 1994; Kikuzaki et al., 1992; 1994; Kikuzaki and Nakatani, 1996; Wu et al., 1998). The antioxidant activity of phenolic constituents of ginger has been documented (Kikuzaki et al., 1994). The compounds involved also served as good antimicrobial agents (Galal, 1996; Yamada et al., 1992). Figure 6.7 and Figure 6.8 summarize the chemical structures of gingerol-related compounds. Figure 6.9 shows the chemical structures of other ginger phenolics.



$R_1 = \text{OCH}_3, R_2 = \text{OH}, n=4, 6, 8$; [n+2]-gingerol
 $R_1 = R_2 = \text{OCH}_3, n = 4$; [6]-methylgingerol
 $R_1 = \text{H}, R_2 = \text{OH}, n = 4$; [6]-demethoxygingerol



$R_1 = \text{OCH}_3, R_2 = \text{OH}$; [6]-shogaol
 $R_1 = R_2 = \text{OCH}_3$; [6]-methylshogaol
 $R_1 = \text{H}, R_2 = \text{OH}$; [6]-demethoxyshogaol,



$R = \text{H}$; [6]-gingerdiol
 $R = \text{Ac}$; [6]-gingerdiacetate

FIGURE 6.7 Chemical structures of some gingerol-related compounds of ginger.

LICORICE

Licorice is the name used for roots and stolous of some *Glycyrrhiza* species. It is grown in Spain, Italy, Greece, Russia, Turkey, Iran, Iraq, Syria and China, and also cultivated in South Africa, Australia, New Zealand and the U.S. One of the oldest Chinese medicines, licorice root was also discovered in a Pharaoh's tomb in Egypt. It is used as a flavoring and sweetening agent in chewing gums, candies and beverages. Licorice is also used in certain pharmaceutical products, including antiulcer drugs (Farina et al., 1998), and is reported to have antimutagenic, anticarcinogenic, antiviral and antiinflammatory properties (Kelloff et al., 1994; Wang et al., 1991). In addition, antihepatitis, anti-HIV, and antiatherogenic effects have been attributed to licorice (Vodovozova et al., 1996). Several triterpenoid saponins, namely, glycyrrhizin (3.63 to 13.06% in dried roots) and glycyrrhetic acid (α - and β -) have been found in licorice. These are known to inhibit

tumorigenesis in mouse lung and liver (Nishino, 1992). Polyphenols found in licorice (Figure 6.10) are also known to serve as important factors responsible for the antiinflammatory, antimutagenic, anticarcinogenic and antiatherogenic effects of licorice (Fuhrman et al., 1997).

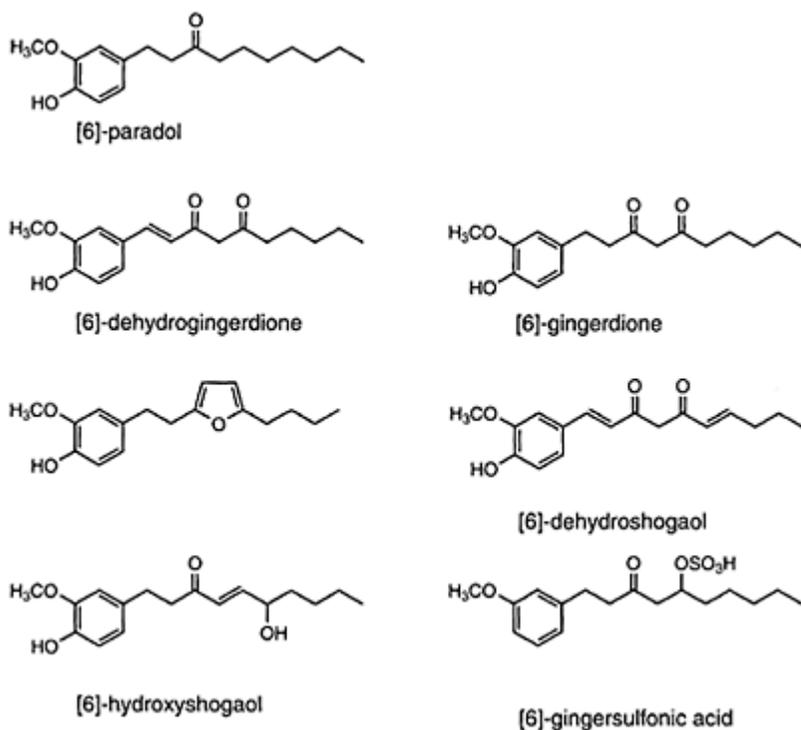
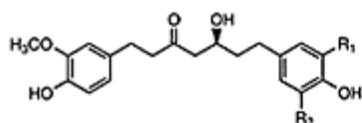


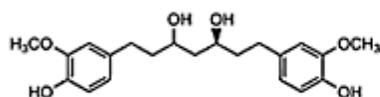
FIGURE 6.8 Chemical structures of other gingerol-related compounds of ginger.

MILK THISTLE

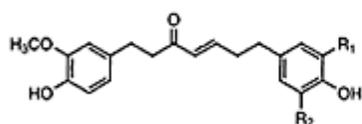
Milk thistle has been used since antiquity for the treatment of liver disease. Wagner et al. (1971) isolated and identified its active principles as silymarin, a mixture of flavonolignans, with hepatoprotective effect in laboratory animals exposed to a variety of toxic compounds. It has also been shown to be effective in treating liver cirrhosis in alcoholic subjects and hepatitis (Sonnenbichler et al., 1998). Silibinin makes up approximately 60% of the main active components (Lawson and Bauer, 1998) of milk thistle. Silymarin constituents of milk thistle are shown in Figure 6.11.



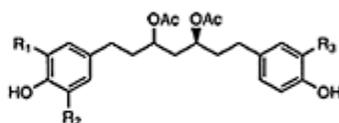
$R_1 = R_2 = H$
 $R_1 = R_2 = OCH_3$
 $R_1 = OCH_3, R_2 = H$; Hexahydrocurcumin



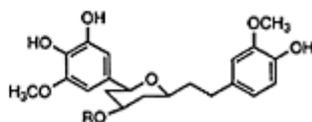
3R or 3S



$R_1 = OCH_3, R_2 = H$, GingerenoneA
 $R_1 = R_2 = OCH_3$
 $R_1 = R_2 = H$



$R_1 = R_3 = OCH_3, R_2 = H$; (3R, 5S)
 $R_1 = R_2 = R_3 = OCH_3$; (rel RS)
 $R_1 = R_2 = R_3 = OCH_3$; (3S, 5S)
 $R_1 = OCH_3, R_2 = H, R_3 = OH$; (rel RS)
 $R_1 = R_3 = OH, R_2 = H$; (3S 5S)



$R = H$
 $R = Ac$

FIGURE 6.9 Chemical structures of diarylheptanoids of ginger.

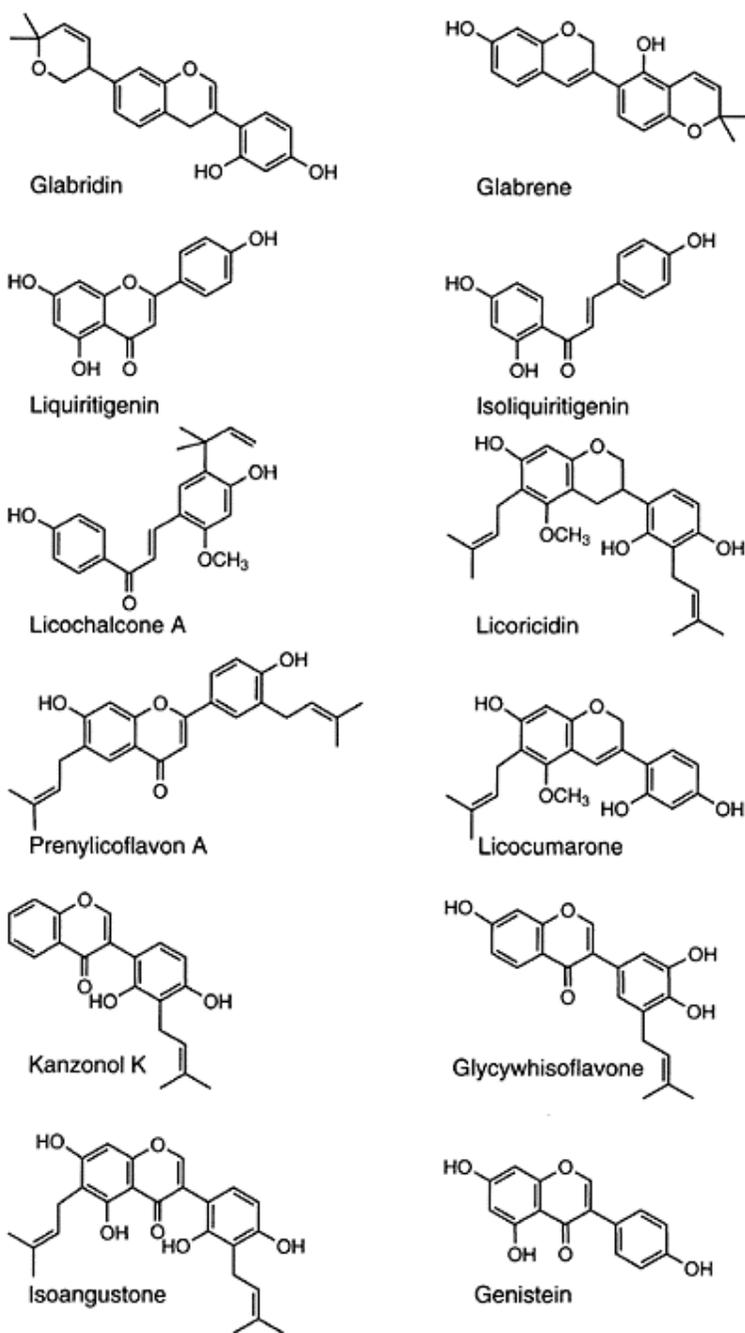


FIGURE 6.10 Chemical structures of polyphenolic compounds of licorice.

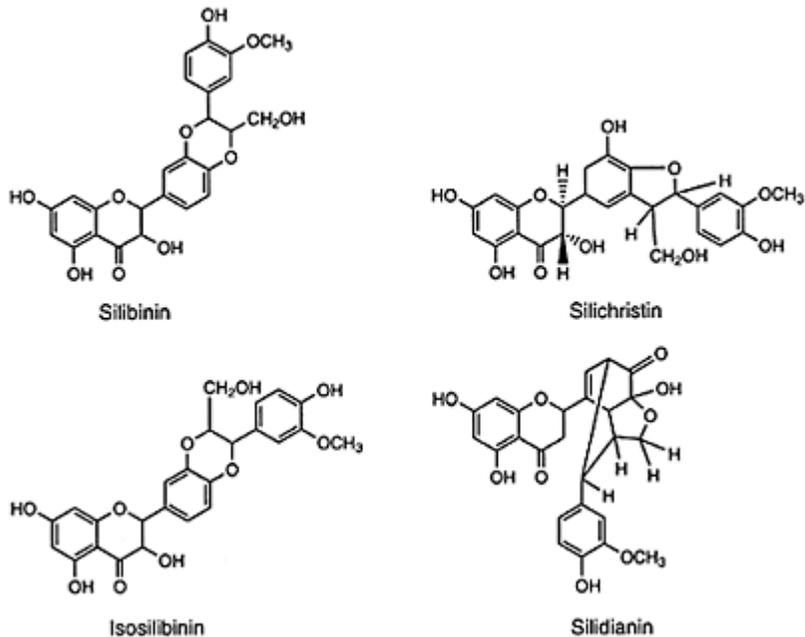


FIGURE 6.11 Chemical structures of silymarin constituents of milk thistle.

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Nutritional and Pharmacological Effects of Food Phenolics

Antinutritional effects of polyphenolics have been demonstrated using laboratory animals (Glick and Joslyn, 1970; Myer and Gorbet, 1985; Rostagno et al., 1973) and humans (Hussein and Abbas, 1985; Stavric and Matula, 1992). These effects can be manifested as a decrease in growth rate or feed conversion (Mehansho et al., 1985; Myer and Gorbet, 1985; Rostagno et al., 1973), inhibition of enzymes (Ahmed et al., 1991; Guyot et al., 1996; Oh and Hoff, 1986), and lower egg production (Sell et al., 1983). Diets containing high levels of tannins have been reported to interfere with pancreatic digestion (Driedger and Hatfield, 1972) as well as to cause methionine deficiency by requiring active methyl groups derived from methionine in the detoxification process (Singleton and Kratzer, 1969). A number of reviews have been published on dietary intake and bioavailability of polyphenols (Bravo, 1998; Clifford, 2000; Heider and Fuchs, 1997; Heim et al., 2002; Hollman, 1997; Hollman et al., 1997; Hollman and Katan, 1997, 1999; Peterson and Dwyer, 1998; Remesy et al., 1996; Ren et al., 2001; Santos-Buelga and Scalbert, 2000; Scalbert and Williamson, 2000; Scalbert et al., 2002; Stavric and Matula, 1992; Teissedre and Landrault, 2000; Waterhouse and Walzem, 1998). Therefore, this chapter discusses only topics related to dietary intake and metabolism of polyphenols.

DIETARY INTAKE

Dietary intake of phenolics is greatly affected by the eating habits and preferences of individuals. The average daily intake of dietary polyphenols is about 1 g per person; the main sources are beverages, fruits and, to a lesser extent, vegetables and legumes (Scalbert and Williamson, 2000). Furthermore, factors such as structural diversity of phenolics, lack of standardized and reliable methods for quantification of various classes of phenolics, variations in phenolic content within each food, uneven distribution of phenolics in plant food, and food processing make accurate assessment of dietary intake of food phenolics cumbersome and difficult (Scalbert and Williamson, 2000). Thus, more precise data on the dietary intake of food phenolics could be obtained by carrying out a comprehensive measurement of phenolic contents in a wide range of foods.

Hertog et al. (1993a) reported that the total daily intake of flavonoids by men participating in the Zutphen Elderly Study ranged from 12 to 41.9 mg/day; however, according to Linseisen et al. (1997), the daily Western diet provides approximately 50 mg of different flavonoids. Black tea is the richest source of flavonoids, contributing 61% of total dietary intake, while onions and apples comprise only 13 and 10% of total dietary flavonoid intake, respectively. On the other hand, 2 and 21 mg of flavones and flavonols are consumed daily by the Dutch population (Hertog et al., 1993b).

According to Deschner et al. (1991), the daily intake of quercetin with food is 50 to 500 mg. Kühnau (1976) estimated the average daily intake of anthocyanins in the U.S. population to be 185 to 215 mg, while Hollman and Katan (1999) and Scalbert and Williamson (2000) have found the average daily intake of anthocyanins by humans to range from 25 to 1000 mg. Subsequently, using a representative sample of the Dutch population (Dutch National Food Consumption Survey), Arts et al. (1999) estimated the contribution of tea and chocolate to the daily intake of catechins. These authors reported that chocolate provides 20% of the daily intake of catechins and that about 55% of daily catechin intake is contributed by tea. The highest amount of tea consumption per person (3.16 kg/year) is in Northern Ireland, followed by the U.K. (2.53 kg/year) and Kuwait (2.52 kg/year) (Weisburger, 1999). The daily intake of isoflavones by Japanese ranges from 30 to 40 mg (Kimira et al., 1998; Wakai et al., 1999) compared to a maximum of 5 mg/day in the U.S. (Ho et al., 2000).

ABSORPTION AND METABOLISM

Information on the bioavailability and absorption of food phenolics is diverse, fragmentary and controversial. Figure 7.1 shows possible routes for metabolism of polyphenols ingested by humans; enzymes, phenol sulfotransferases, catechol-*O*-transferases, β -glucosidases, lactase-phloridzin oxidases, and UDP glucuronosyl transferases are involved in polyphenol metabolism (Scalbert and Williamson, 2000). Absorption and bioavailability of polyphenols in the body depend on their metabolism in the small intestine (Gee et al., 2000; Piskula and Terao, 1998; Ren et al., 2001; Sfakianos et al., 1997; Spencer et al., 1999; Walle et al., 1999). Polyphenol metabolism is influenced by factors such as molecular size, lipophilicity, solubility, and pK_a , as well as gastric and intestinal transit time, membrane permeability, lumen pH, and first-pass metabolism (Higuchi et al., 1981; Lin et al., 1999). Only polyphenols not absorbed in the stomach and small intestine and polyphenol metabolites excreted back to the small intestine are degraded by gut microflora (Scalbert and Williamson, 2000; Setchell, 2000).

Glycosylated polyphenols may be absorbed as such or after hydrolysis by intestinal enzymes (Scalbert and Williamson, 2000). Hawksworth et al. (1971) demonstrated β -glucosidase activity by *Lactobacilli*, *Bacteroides* and *Bifidobacteria* of human gut. Using an *in-situ* perfusion model, Crespy et al. (1999) showed that quercetin is absorbed, metabolized and partially reexcreted by the small intestine. Donovan et al. (2001) have reported that after absorption into intestinal wall, catechins are extensively metabolized. Only glucuronide conjugates of catechin and 3-*O*-methylcatechin (no intact catechin) have been detected in mesenteric plasma. Additional metabolism of polyphenols involving their conjugation by glucuronidation, *O*-methylation, sulfation, or a combination may occur in the liver (Donovan et al., 2001; Ren et al., 2001; Scalbert and Williamson, 2000). The conjugated metabolites are then transferred to tissues or excreted in urine or bile (Scalbert and Williamson, 2000; Zhang et al., 1999b).

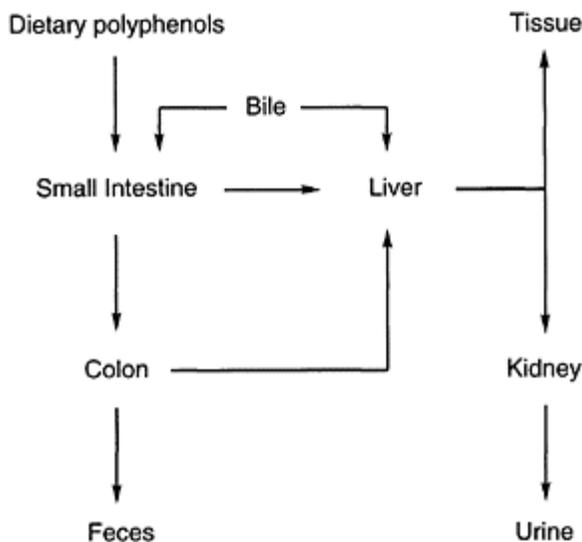


FIGURE 7.1 Possible routes for dietary polyphenols in humans. (Adapted from Scalbert, A. and Williamson, G., 2000, *J. Nutr.*, 130:2073S–2085S.)

PHENOLIC ACIDS

Olthof et al. (2001) investigated the absorption of chlorogenic and caffeic acids in ileostomy human subjects, in which the degradation of phenolics by gut microflora is minimal. The absorption of chlorogenic and caffeic acids in the small intestine was 33% for chlorogenic acid and 95% for caffeic acid. Only traces of chlorogenic acid and about 11% of ingested caffeic acid were excreted in the urine. According to these authors, chlorogenic acid is absorbed in the intact form and subsequently metabolized in the liver. On the other hand, the absorption of caffeic acid may have occurred by passive nonionic diffusion in the stomach as well as via an active Na^+ -dependent transport mechanism in the small intestine. Subsequently, Simonetti et al. (2001) examined the bioavailability of caffeic acid in plasma following the intake of red wine. The concentration of caffeic acid in plasma was dose dependent and the maximum level of caffeic acid was reached approximately 60 min after red wine intake. Recently, Nardini et al. (2002) evaluated the absorption of phenolic acids in humans after the consumption of 200 mL of freshly brewed coffee. Only caffeic acid was detected in plasma, with an absorption peak at 60 min following the intake of coffee.

Gallic acid is one the main phenolics of black tea. Shahrzad and Bitsch (1998) reported that 4-*O*-methylgallic acid is a major metabolite of gallic acid in humans. Later, Hodgson et al. (2000) identified the metabolites of gallic acid in human urine 24 h after

4-week ingestion of five cups of black tea per day. They identified three metabolites in human urine: 4-*O*-methylgallic acid, 3-*O*-methylgallic acid and



FIGURE 7.2 Chemical structures of some metabolites of gallic acid.

3,4-*O*-dimethylgallic acid (Figure 7.2). Total excretion of these metabolites was 1.5 mg/day, accounting for only 7.5% of ingested free gallic acid and 1.5% of ingested total gallic acid. According to these authors, the phenolics examined may be used as potential markers of black tea phenolic intake.

ANTHOCYANINS

Little is known about the metabolic fate of anthocyanins; however, published data suggest that they are poorly absorbed. Tsuda et al. (1996) reported that protocatechuic acid may be considered an oxidative degradation product of cyanidin-3-glucoside. Later, Tsuda (1999) also suggested that protocatechuic acid may contribute to the *in vivo* antioxidative activity of cyanidin-3-glucoside. Morazzoni et al. (1991) investigated the pharmacokinetics of anthocyanins in rats fed with a mixture of 15 anthocyanins at a level of 400 mg/kg of body weight. Only 2.5 mg/L of anthocyanins were transported into plasma 15 min after ingestion and about 5% of the administered anthocyanins were found intact in urine within 24 h. Later, Lapidot et al. (1998) detected only 1.5 to 5.1% of ingested red wine anthocyanins in the urine of human subjects within 12 h of wine consumption. These authors suggested that anthocyanins absorbed through the guts undergo molecular modification brought about by their antioxidative action.

Yamasaki et al. (1996) demonstrated bleaching of anthocyanins by superoxide ($O_2^{\bullet-}$) in *in vitro* studies. Furthermore, Cao and Prior (1999) reported poor absorption of cyanidin glucosides from elderberry extract into human plasma and Miyazawa et al. (1999) demonstrated that intact cyanidin glucosides are absorbed across the intestinal wall into plasma and liver of rats as well as into human plasma. The amounts of cyanidins incorporated into plasma were similar to those estimated by Marazzoni et al. (1991). Subsequently, Youdim et al. (2000) demonstrated an incorporation of elderberry anthocyanins into the membrane and cytosol of vascular endothelial cells. Later, Bub et al. (2001) investigated the absorption of malvidin-3-glucoside from red wine, dealcoholized red wine and grape juice; only small amounts of malvidin-3-glucoside were detected in the plasma and urine. Matsumoto et al. (2001) also reported poor absorption of blackcurrant anthocyanins—only 0.11% of anthocyanin intake was

excreted 0 to 8 h after ingestion in the intact form. Recently, Milbury et al. (2002) examined the bioavailability of elderberry anthocyanins in humans. These researchers detected anthocyanins in their unchanged glycosylated forms in plasma and urine samples and demonstrated that elimination

TABLE 7.1
Concentrations of Catechin Metabolites^a in Mesenteric Plasma, Abdominal Aortic Plasma and Bile of Rats after Perfusion with 30 μ mol Catechin/L

| Catechin Metabolite | Mesenteric Plasma | Aortic Plasma | Bile |
|---|--------------------------|----------------------|----------------|
| Catechin glucuronide | 0.7 \pm 0.2 | 0.2 \pm 0.1 | 0.2 \pm 0.0 |
| Catechin glucuronide+sulfate | – | – | 0.8 \pm 0.2 |
| 3'- <i>O</i> -methyl catechin glucuronide | 0.6 \pm 0.2 | 0.3 \pm 0.2 | 9.0 \pm 2.2 |
| 3'- <i>O</i> -methyl catechin glucuronide+sulfate | – | – | 16.8 \pm 0.4 |
| Total metabolites | 1.2 \pm 0.1 | 0.5 \pm 0.1 | 26.8 \pm 2.6 |

Note: Values are means \pm standard deviations; n=4.

^a Micromoles per liter.

Source: Adapted from Donovan et al., 2001, *J. Nutr.*, 130:1753–1757.

of anthocyanins in plasma follows first-order kinetics. Thus, more research is needed to investigate the metabolic fate of anthocyanins in the human body.

FLAVANOLS AND PROANTHOCYANIDINS

Kuhnle et al. (2000a) demonstrated that epicatechin and catechin are extensively metabolized and conjugated during transfer from the gut lumen to the serosal surface. In the serosal fluid, glucuronidated, *O*-methylated, and *O*-methylglucuronidated flavanol derivatives account for 45, 30 and 20% of the total flavanols consumed, respectively. Furthermore, Donovan et al. (1999) reported that catechin metabolites are predominantly found in the plasma of human subjects after consumption of red wine. Later, Rein et al. (2000a) detected a 12-fold increase in the plasma epicatechin, from 22 to 257 nmol/L, after consumption of 80 g of procyanidin-rich chocolate containing 557 mg of procyanidin per meal. Unmethylated glucuronide, glucuronide sulfate, unmethylated sulfate of epicatechin and free epicatechin contributed to the increase in plasma epicatechin. Subsequently, using an *in situ* perfusion rat model, Donovan et al. (2001) showed that approximately 35% of catechin is absorbed into the intestinal wall during the first 30-min period. Only glucuronidated and *O*-methylated metabolites of catechin were detected in the mesenteric plasma and abdominal aortic plasma. On the other hand, glucuronidates or sulfates of *O*-methylated catechin metabolites were identified in the bile (Table 7.1).

Recently, Warden et al. (2001) found that only 1.68% of catechins ingested from black tea are detected in the plasma, urine and feces in the free form. Rapid metabolism of catechins by gut bacteria, quick absorption and metabolism of catechins in the gastrointestinal tract or their quick distribution in the tissues may be responsible for a low

while the presence of eriodictyol and 3,4-dihydroxycinnamic acid was detected after 6 and 9 h of incubation, respectively.

FLAVONES AND FLAVONOLS

Olthof et al. (2000) demonstrated that quercetin-3-glucoside and quercetin-4'-glucoside are rapidly absorbed by healthy human subjects. The peak concentrations of

TABLE 7.2

Kinetics of Quercetin Absorption and Elimination in Human Plasma after One-Time Ingestion of Quercetin-3-Glucoside or Quercetin-4-Glucoside

| Property | Quercetin-3-glucoside | Quercetin-4-glucoside |
|--|------------------------------|------------------------------|
| Peak concentration ($\mu\text{g/L}$) | 1526 \pm 315 | 1345 \pm 212 |
| Time to reach peak concentration (min) | 37 \pm 12 | 21 \pm 5 |
| Elimination half-life (h) | 18.5 \pm 0.8 | 17.7 \pm 0.9 |

Note: Values are means \pm standard deviations; n=9.

Source: Adapted from Olthof, M.R. et al., 2000, *J. Nutr.*, 130:1200–1203.

quercetin in the blood were reached 37 \pm 12 and 27 \pm 5 min after ingestion of quercetin-3-glucoside and quercetin-4'-glucoside, respectively. The half-life of elimination of quercetin from the blood was 18.5 \pm 0.8 h and 17.7 \pm 0.9 h after ingestion of quercetin-3-glucoside and quercetin-4'-glucoside, respectively (Table 7.2). According to Walle et al. (2000), quercetin glucosides are completely hydrolyzed in ileostomy patients before absorption. Only 19.5 to 35.2% of total ingested quercetin glucosides was detected in the ileostomy fluid. Using intestinal Caco-2 cell monolayers as a model of human intestinal absorption, Walgren et al. (1998, 2000a, b) demonstrated that quercetin is absorbed more rapidly than its glycosides. These data are in contrast to those reported by Gee et al. (2000) and Hollman et al. (1995, 1996), who demonstrated that quercetin monoglucosides are transported more rapidly than quercetin aglycone in the small intestine.

It has been proposed that sugar moiety may be involved in transporting flavonoid glucoside by the sodium-dependent glucose transporter-1 (SGLT1) (Lostao et al., 1994; Panayotova-Heiermann et al., 1996). Gee et al. (2000) observed rapid deglycosylation and then glucuronidation of flavonoid glycosides during passage across the rat small intestine epithelium. However, because the location of hydrolysis is still unknown, several possible locations have been suggested. Recently, de Vries et al. (2001) investigated the absorption of quercetin in humans after consumption of similar quantities of quercetin from red wine, yellow onions or tea. The absorption of quercetin was estimated based on its concentration in the plasma and urine. Results suggest that the bioavailability of quercetin from red wine is lower than that from onions, but slightly higher than that from tea.

Booth et al. (1956) identified hydroxyphenylacetic and phenylpropionic acids in the urine of rats fed with rutin (quercetin-3-O-rhamnoglucoside). Later, Bokken-heuser et al.

(1987) and Winter et al. (1989) reported that rutin is metabolized by the intestinal microflora to quercetin, phloroglucinol and 3,4-dihydroxyphenylacetic acid. Schneider et al. (1999) estimated that 10^7 to 10^9 fecal bacteria per gram of dry mass are able to degrade rutin and suggested that the degradation of quercetin by *Eubacterium ramulus* requires the presence of sugar liberated from rutin. Justesen et al. (2000) investigated the metabolism of rutin and quercetin by human fecal microflora using an *in vitro* model. Rutin was almost completely degraded within 48 h of culturing and only 5% of rutin originally present in fermenting medium was detected. On the other hand, quercetin was completely metabolized into six metabolites after 8 h of incubation. Five of these metabolites were identified: 3,4-dihydroxytoluol, 3,4-dihydroxybenzaldehyde, 3- or 4-hydroxyphenylacetic acid, 3- or 4-hydroxypropionic acid and 3,4-dihydroxyphenylacetic acid.

ISOFLAVONES

Joannou et al. (1995) postulated that isoflavone β -glycosides are hydrolyzed by β -glucosidase present in the jejunum and then further metabolized in the distal intestine. Meanwhile, Lundh (1995) reported that isoflavones are predominantly (95%) conjugated in the liver with glucuronic acid. Isoflavone aglycones and their bacterial metabolites undergo mainly glucuronidation during uptake by the enterocyte (Axelson and Setchell, 1981; Setchell, 2000; Sfakianos et al., 1997; Zhang et al., 1999a). The extent of this metabolism is highly variable among individuals and affected by dietary factors as well (Setchell and Cassidy, 1999). Xu et al. (1995) reported that isoflavone bioavailability varies from 13 to 35% depending on the ability of individual gut microflora to degrade isoflavones. According to Andlauer et al. (2000), the bioavailability of isoflavones is significantly affected by the food matrix. Subsequently, Izumi et al. (2000) observed that isoflavone aglycones in fermented soy food products are absorbed more rapidly than their corresponding glycosides.

Piskula et al. (1999) examined the absorption of orally administered isoflavone aglycones and glucosides in surgically treated rats with absorption site restricted to the stomach. The absorption was measured by monitoring the level of isoflavone metabolites in blood plasma for 30 min. Results indicated that isoflavones are rapidly absorbed in the rat stomach. A few minutes' delay in the detection of metabolites of isoflavone glucosides in plasma compared to those of aglycones was observed. This, according to the authors, indicates that isoflavone aglycones, but not their glucosides, are absorbed from the rat stomach.

Using the simulator of the human intestinal microbial ecosystem, de Boever et al. (2000) demonstrated the cleavage of β -glycosidic bond of conjugated isoflavones under the conditions prevailing in the large intestine. Hur et al. (2000) isolated two strains of human microflora, *Escherichia coli* HGH21 and an unidentified Gram-positive strain HGH6, metabolizing daidzin and genistin to their respective aglycones. Administration of antibiotics significantly blocks the metabolism of isoflavonones. Also, limited isoflavone metabolism has been observed in infants of up to 4 months old, when gut microflora is underdeveloped. These data suggest that gut microflora plays an important role in isoflavone metabolism (Axelson and Setchell, 1981; Setchell et al., 1997, 1998).

Setchell et al. (2001) have suggested that, based on pK_a values, isoflavone aglycones are absorbed from jejunum by the nonionic passive diffusion mechanism. A maximum concentration of isoflavones in blood plasma is observed between 4 and 7 h and 8 to 11 h after ingesting isoflavone aglycones and their β -glycosides, respectively (Setchell et al., 2001; Watanabe et al., 1998). Presence of isoflavones

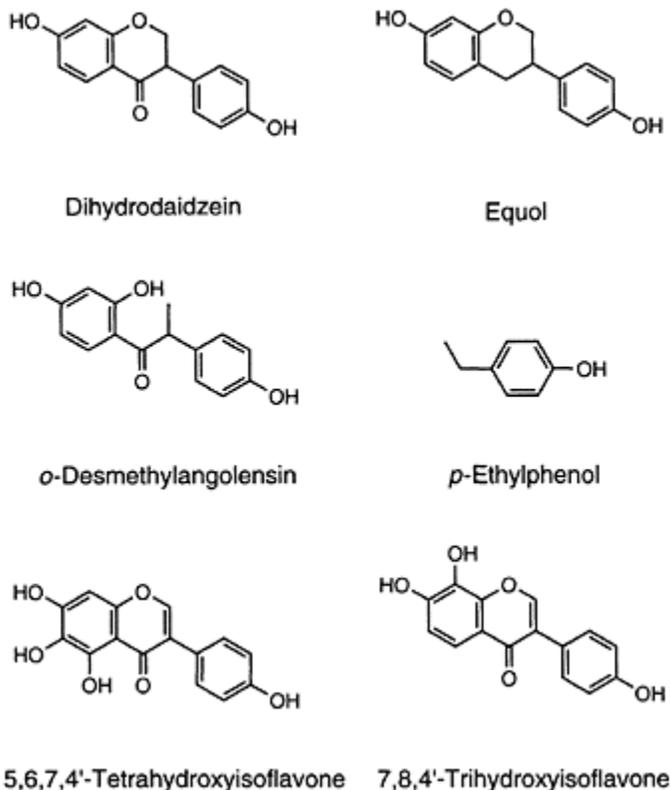


FIGURE 7.4 Chemical structures of some metabolites of isoflavones.

in plasma has been detected up to 24 h after consumption (Setchell, 2000; Zhang et al., 1999b).

Figure 7.4 shows that aglycones, genistein and daidzein may be further metabolized to equol, 7-hydroxy-3-(4-hydroxyphenyl)chroman, dihydrodaidzein, *O*-desmethylangolensin and *p*-ethylphenol (Chang and Nair, 1995; Setchell and Cassidy, 1999). Daidzein is first transformed by gut microflora to dihydrodaidzein, which is then metabolized to equol and *O*-desmethylangolensin. On the other hand, genistein is first transformed by gut microflora to dihydrogenistein and then converted to 6'-hydroxy-*O*-desmethylangolensin, which is degraded to 4-hydroxyphenyl-2-propionic acid. Decarboxylation of this acid leads to the formation of *p*-ethylphenol (Coldham et al.,

1999; Joannou et al., 1995; Kurzer and Xu, 1997). The amounts of aglycones and their metabolites recovered in urine and feces are summarized in Table 7.3. Of the isoflavone metabolites, equol possesses an estrogenic activity of an order higher than that displayed by daidzein (Axelson et al., 1982a; Shutt and Cox, 1972). Higher conversion of daidzein to equol has been observed with diets higher in carbohydrates (Lampe et al., 1998; Shutt and Cox, 1972).

Kulling et al. (2001) studied the oxidative metabolites of isoflavones excreted in the urine of human subjects ingesting soya products for 2 days. The following compounds were identified as oxidative metabolites of daidzein:

TABLE 7.3
Recovery of Daidzein, Genistein, O-Desmethylangolensin and Equol in Urine and Feces of Men for 3 Days after Ingestion of 60 g of Kinako (Baked Soybean Powder)^a

| | Urine | | Feces | |
|-----------------------|-------------|-------------|------------|-----------|
| | Range | Mean±SD | Range | Mean±SD |
| Daidzein | 27.28–64.56 | 36.97±13.90 | 1.07–12.59 | 4.57±4.04 |
| Genistein | 9.68–47.91 | 19.71±14.03 | 0.20–7.14 | 2.85±2.82 |
| O-desmethylangolensin | 0.59–11.70 | 4.80±4.10 | 0.08–6.12 | 1.97±1.97 |
| Equol | 0.07–33.51 | 7.60±13.37 | 0.08–8.12 | 1.67±3.07 |

Note: SD=standard; n=7.

^a Micromoles.

Source: Adapted from Watanabe, S. et al., 1998, *J. Nutr.*, 128:1710–1715.

- 6,7,4'-Trihydroxyisoflavone
- 7,3',4'-Trihydroxy isoflavone
- 7,8,4'-Trihydroxy isoflavone
- 7,8,3',4'-Tetrahydroxyisoflavone
- 6,7,8,4'-Tetrahydroxyisoflavone
- 6,7,3',4'-Tetrahydroxyisoflavone

Of these, only 6,7,8,4'-tetrahydroxyisoflavone is detected in trace amounts. On the other hand, the main oxidative metabolites of genistein are 5,6,7,4'-tetrahydroxyisoflavone, 5,7,8,4'-tetrahydroxy isoflavone, and 5,7,3',4'-tetrahydroxyisoflavone; 5,7,8,3',4'-pentahydroxyisoflavone and 5,6,7,3',4'-pentahydroxyisoflavone are identified as minor metabolites.

Fang et al. (2002) identified 17 metabolites of isoflavones in the urine of female rats fed on a diet containing soy protein isolates. The detected isoflavone metabolites indicated that any of the hydroxyl groups of the isoflavone molecule may be involved in the formation of glucuronides. In addition, glucuronides of equol and O-desmethylangolensin were also identified in rat urine.

OTHER PHENOLICS

Kuhnle et al. (2000b) used an *in vitro* model system to investigate the absorption and metabolism of resveratrol. These authors noted that only small amounts unmetabolized resveratrol are absorbed across the enterocytes of jejunum and ileum and identified the glucuronide conjugate of resveratrol as a major metabolite on the serosal side. Based on mass spectrometry data, these authors suggest that the 4'-hydroxyl site of resveratrol serves as the preferential site for glucuronidation.

Malathi and Crane (1969) detected lactase-phloridzin hydrolase activity in the brush border of hamster small intestine. This enzyme hydrolyzes phloridzin (phloretin 2'-*O*-glucose) to phloretin and glucose. Later, Monge et al. (1984) demonstrated that phloretin is metabolized by fecal microflora to phloretic acid and phloroglucinol. Recently, Crespy et al. (2002) reported that phloretin is absorbed more rapidly than phloridzin. However, only free phloretin and its glucuronidated or sulfated conjugates have been detected in plasma up to 24 h after ingesting meal containing phloretin or phloridzin and only 10.4% of the ingested phloretin and phloridzin recovered in the urine after 24 h.

Nakazawa and Ohsawa (1998) investigated the metabolism of rosmarinic acid administered orally to rats. They found metabolites attributed to this acid only in the urine and plasma of rats, detecting four in the plasma: *trans*-caffeic acid 4-*O*-sulfate, *trans-m*-coumaric acid 3-*O*-sulfate, *trans*-ferulic acid 4-*O*-sulfate, *m*-hydroxypropionic acid and *trans-m*-coumaric acid. In addition, unchanged rosmarinic acid and *trans*-caffeic acid were identified in the urine. The total amount of rosmarinic acid and its metabolites excreted in the urine 48 h after the oral administration accounted for 31.8% of the administered dose.

For many years, plant lignans matairesinol and secoisolariciresinol were considered the only precursors of mammalian lignans enterodiol and enterolactone (Axelson et al., 1982b; Borriello et al., 1985). Recently, Heinonen et al. (2001) demonstrated that lariciresinol and pinoresinol are also effectively converted by gut microflora to mammalian lignans. It has been suggested that the microbial metabolism of pinoresinol involves the formation of secoisolariciresinol via lariciresinol. Other known plant lignans are not effectively converted to mammalian lignans.

Only limited data have been published on the metabolism of hydroxytyrosol, the principal phenolic component in olive oil. Manna et al. (2000) reported that, in Caco-2-cells, hydroxytyrosol is converted to homovanillic alcohol. Subsequently, while investigating the metabolism of hydroxytyrosol after oral consumption of olive oil by human subjects, Visioli et al. (2000) identified only unmetabolized hydroxytyrosol and its glucuronide conjugate in the urine. Reexamination of these urine samples led to the identification of homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid) and homovanillic alcohol (Figure 7.5) (Caruso et al., 2001). Following this, Tuck et al. (2001) observed extensive absorption of hydroxytyrosol administered to rats in the form of olive oil solution. Five unidentified hydroxytyrosol metabolites were detected in the urine, three of which were later identified: a monosulfate conjugate, monoglucuronide conjugate and 3-hydroxy-4-methoxyphenylacetic acid (Figure 7.5) (Tuck et al., 2002).

Because a number of coumarin metabolites have been identified, namely, 3-,4-, 5-,6-,7-, and 8-hydroxycoumarins, *o*-hydroxyphenylacetylaldehyde (*o*-HPA) and *o*-

hydroxyphenylacetic acid (*o*-HPAA) (Fentem and Fry, 1993; Pelkonen et al., 1997), different pathways may be involved in the metabolism of coumarins in various tissues (Pelkonen et al., 1997). Of these, 8-hydroxycoumarin, *o*-HPAA, and *o*-HPA are the major metabolites of coumarin formed by rat esophageal microsomes and cytochrome P450 enzymes (von Weyarn and Murphy, 2001). According to Born et al. (1997, 2000), *o*-HPA is formed from the unstable coumarin 3,4-epoxide, while *o*-HPAA is a product of *o*-HPA oxidation.

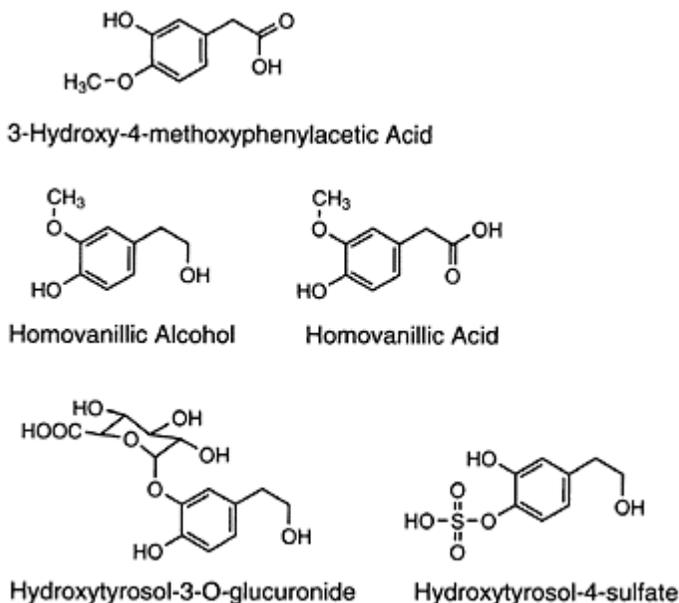


FIGURE 7.5 Chemical structures of some metabolites of hydroxytyrosol.

NUTRITIONAL IMPLICATIONS

Tannin-protein complexes may account for the antinutritional effects of tannin-containing feeds in nonruminants (Clandinin and Robblee, 1981; Martin-Tanguy et al., 1977) and ruminants (Kumar and Singh, 1984). Detrimental effects, including a decrease in food palatability, voluntary intake and growth of experimental animals and a decrease in fiber and protein digestibility (Barry and Duncan, 1984; Barry and Manley, 1984; Barry et al., 1986; Jung and Fahey, 1983; Mitaru et al., 1984a), have been associated with ingestion of polyphenols (Glick, 1981; Reddy et al., 1985). Recently, Carbonaro et al. (2001) demonstrated partial binding of catechins and tannic acid by endogenous proteins in the intestinal lumen. These authors postulated that this binding is responsible for antinutritional effects displayed by tannic acid because it may interfere with the function of gut.

Mitjavila et al. (1977) reported increased fecal calcium loss by rats fed 3% tannic acid in their diet as a result of increased endogenous gut secretion. Marquardt and Ward (1979) observed that tannins account for about 50% of the growth depression of chicks fed a faba bean diet. The tannin content was highly correlated with decreased weight gain, decreased feed intake, and decreased protein and increased fat retention. Utilization of barley proteins by rats has also been negatively correlated with the level of tannins (0.55% to 1.23%) in samples (Eggum and Christensen, 1975). The reduction in utilization of proteins may be due to the binding of tannins to digestive enzymes or to dietary proteins. Condensed tannins may also bind to methionine, thus making it unavailable and, in turn, lowering the utilization of dietary proteins (Armstrong et al., 1974; Ford and Hewitt, 1974).

Tannins in rapeseed meal are reported to cause tainting of eggs. It has been postulated that they block the metabolism of trimethylamine (TMA) by inhibiting TMA oxidase (Butler et al., 1982; Fenwick et al., 1981, 1984). This enzyme converts TMA to odorless, water-soluble TMA oxide. Addition of tannins extracted from rapeseed meal to soybean-containing diets for chicks results in a reduction of their metabolizable energy; however, it has no apparent effect on the absorption of proteins by chicks (Yapar and Clandinin, 1972). In another study, Mitaru et al. (1982) reported that condensed tannins isolated from rapeseed hulls do not inhibit the activity of α -amylase *in vitro*. However, other *in vitro* studies have indicated that tannins may show stimulatory, rather than inhibitory, effects on protein digestion (Mole and Waterman, 1985; Oh and Hoff, 1986), perhaps due to partial denaturation of protein substrates.

On the other hand, it has been shown that water extracts of field bean testa inhibit the *in vitro* activity of α -amylase, lipase, and trypsin (Griffith, 1979). Reporting that sorghum tannins inhibit the activity of α -amylase, Davis and Hosney (1979) found a near-linear inhibition of enzymes that is quite sensitive to tannin concentration in the range between 0.0115 and 0.014 mg of tannins/mL (Figure 7.6). Later, Griffith and Moseley (1980) found that trypsin and α -amylase activities in the intestine of rats fed on a diet containing high levels of tannins (field bean testa) are significantly reduced. However, trypsin activity is recovered when guts were extracted with a polyvinylpyrrolidone-saline solution. These results indicate that tannins of field bean testa are responsible for the inhibition of trypsin activity.

Moreover, under *in vivo* conditions, digestive enzymes are less susceptible to inhibition by tannins due to competition of other proteins, such as salivary proteins for binding (Mehansho et al., 1987; Robbins et al., 1987). Phospholipids and bile acids in the intestine may also cause breakdown of some tannin-protein complexes (Blytt et al., 1988).

INTERACTIONS WITH PROTEINS

The complexation of polyphenols and their enzymatic and nonenzymatic oxidation products with proteins in seeds, meals or flours may reduce the nutritive value of the proteins involved. Oxidized phenolics may react with amino acids and proteins and inhibit the activity of enzymes such as trypsin and lipase (Milic et al., 1968). The ability of polyphenols to form insoluble complexes with proteins interferes with utilization of

dietary proteins (Butler et al., 1986). In addition, a decrease in the antioxidant activity of phenolic-protein systems has also been associated with phenolic-protein interactions (Hainonen et al., 1998).

FORMATION OF PHENOLIC ACID-PROTEIN COMPLEXES

Phenolic acids can form complexes with food proteins, thus lowering nutritional value of proteins. Loomis and Battaile (1966) suggested that phenols may reversibly complex with proteins by a hydrogen-bonding mechanism or irreversibly by oxidation to quinones that combine with reactive groups of protein molecules. Wade et al. (1969) found that the binding of serum albumin correlated well with the pK_a of simple phenols. Thus, the hydrogen bond from phenol to protein is stronger for more acidic phenols. Products of enzymatic and nonenzymatic oxidation of phenolics in seeds, meals or flours may readily react with the ϵ -NH₂ group of lysine and CH₃S group of methionine of enzymes and other proteins to form complexes, thus rendering them nutritionally unavailable to monogastric animals (Rutkowski et al., 1977).

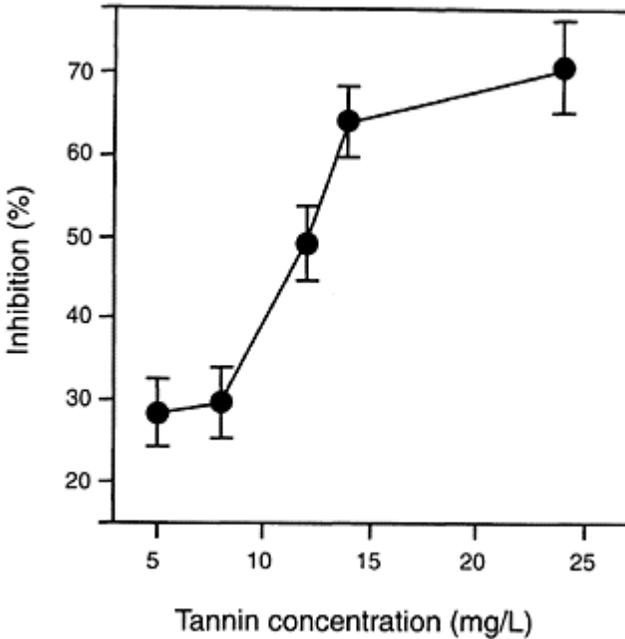


FIGURE 7.6 Percent α -amylase inhibition vs. the concentration of condensed tannins (mg/mL). Standard deviation at 0.012 mg tannins/mL=4.3. (Adapted from Davis, A.B. and

Hoseney, R.C., 1979, *Cereal Chem.*,
56:310–314.)

Bartolome et al. (2000) used model systems to study interactions between a mixture of low molecular weight phenolics (*p*-hydroxybenzoic, protocatechuic, *p*-coumaric and caffeic acids, and catechin) and bovine serum albumin (BSA). This mixture was separated using Sephadex G-50. Change in the elution profile was used as an indicator of affinity of phenolics for BSA. Protocatechuic and caffeic acids exhibited the strongest affinity for BSA while *p*-hydroxybenzoic acid did not interact with it at all. Crude extracts of lentil phenolics displayed interactions with BSA similar to those observed in the model systems.

The possibility of phenolic-protein complex formation has also been indirectly evidenced from the amount of soluble matter extracted into 80% ethanol. Kozłowska and Zadernowski (1988) reported that the quantity of extracted matter increases with increasing pH of the 80% ethanol used for its extraction (Figure 7.7). The formation of these complexes has also been investigated in model systems consisting of sinapic acid and BSA by using a fluorescence spectrophotometric technique (Figure 7.8). These studies indicated that complex formation is favored under neutral and basic conditions (Smyk and Drabent, 1989).

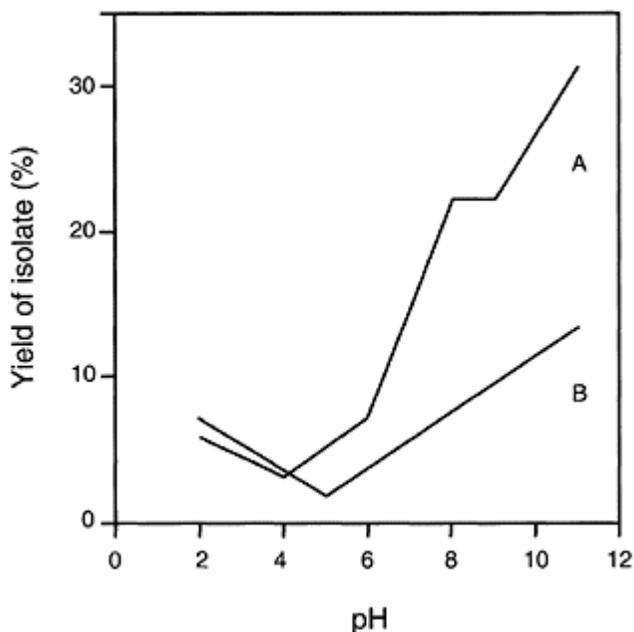


FIGURE 7.7 Effect of pH on the yield of isolate (A) and on the amount of substances extracted from rapeseed with 80% ethanol (B). (Adapted from

Kozłowska, H. et al., 1990, in *Rapeseed and Canola: Production, Chemistry, Nutrition and Processing Technology*, Shahidi, F., Ed., Van Nostrand Reinhold, New York, 193–210.)

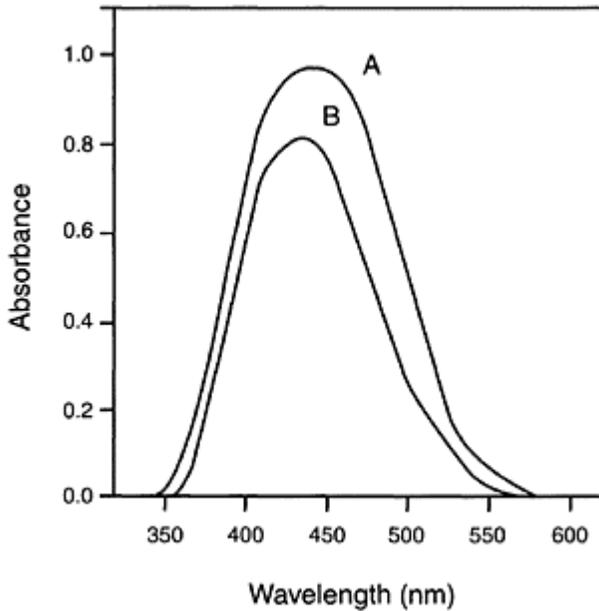


FIGURE 7.8 The fluorescence spectra of sinopic acid (A) and sinopic acid-bovine serum albumin. (Adapted from Kozłowska, H. et al., 1990, in *Rapeseed and Canola: Production, Chemistry, Nutrition and Processing Technology*, Shahidi, F., Ed., Van Nostrand Reinhold, New York, 193–210.)

Sunflower protein solutions under neutral and alkaline conditions develop darkgreen and brown colors, presumably due to the formation of complexes between oxidized polyphenols and proteins. Sabir et al. (1973) reported bonding of chlorogenic acid to low molecular weight sunflower proteins, which constitute about 15% of the salt-extractable proteins in sunflower flour. Later, Sabir et al. (1974) reported that 30% of chlorogenic acid is nondialyzable and remains bound to the flour constituents. These authors also

found that approximately 50% of the polyphenolic compounds is extracted with the fraction of sunflower proteins soluble in neutral salt solutions. Fractionation of the neutral salt soluble protein on Sephadex G-200 revealed that soluble chlorogenic acid is associated with low molecular weight proteins (MW<5000 Da). In addition, these researchers found that 68% of the soluble chlorogenic acid is hydrogen bonded and 32% covalent bonded to the saltsoluble sunflower proteins.

It has been reported that polyphenols found in beans and peas inhibit the activity of amylases *in vitro* and may also be responsible for diminishing the overall digestibility of carbohydrates in the intestinal tract of rats (Griffith, 1979, 1981). This may be due to phenol-enzyme interaction as well as starch-phenol association.

Dick and Bearne (1988) studied the inhibition of purified β -glucosidase from "Spartan" apples by flavonoids, flavonoid glycosides, phenolic acids and polyphenols. They found that quercetin, fustin, rutin, *p*-coumaric acid, and catechol cause classical noncompetitive inhibition, while keampferol and chlorogenic acid do not. The *trans*-3-(4-hydroxyphenyl)-propenoyl group common to 4'-hydroxyflavones and *p*-hydroxyphenolic acids is an important structural feature for effective inhibition of β -glucosidase activity that has been implicated in the softening process of fruits (Hartley, 1974).

FORMATION OF TANNIN-PROTEIN COMPLEX

Tannins may form soluble or insoluble complexes with proteins (Calderon et al., 1968; Hagerman, 1989, 1992; Mole and Waterman, 1982; Siebert, 1999). The specificity of tannin-protein interactions depends on the size, conformation, and charge of the protein molecules (Hagerman and Butler, 1981; Hagerman, 1989, 1992). Relative affinity of proteins for tannins, estimated by using a competitive binding assay, may vary by four orders of magnitude or more (Table 7.4). Thus, proteins with a high affinity for tannins may be selectively bound out of a large excess of proteins with a lesser affinity for tannins (Butler, 1989a, b). Proteins with compact globular structure, such as ribonuclease, lysozyme or cytochrome C, exhibit low affinity for tannins, whereas conformationally open proteins such as gelatin and polyproline readily form complexes with tannins.

Precipitation of tannin-protein complex is due to the formation of a sufficient hydrophobic surface on the complex (McManus et al., 1981). Haslam (1989) suggested nonspecific coating of the protein surface with hydrophobic polyphenols. Later, Kawamoto et al. (1996) and Luck et al. (1994) postulated that the formation of polyphenol-protein precipitate is a two-stage process: (1) soluble polyphenol-protein complexes are formed and (2) subsequent interaction between these complexes leads to the formation of insoluble high molecular weight aggregates. Binding of polyphenols by proteins does not affect their abilities to self-associate, i.e., to form cross links with the protein and other polyphenol molecules. Therefore, aggregation of soluble tannin-protein complexes can be accomplished by noncovalent cross linking mediated by polyphenols (Baxter et al., 1997; Charlton et al., 2002).

TABLE 7.4
Relative Affinity of Proteins for Sorghum
Tannins

| Protein | Relative Affinity |
|---------------------------|--------------------------|
| Gelatin | 14.0 |
| Proline-rich salivary | 6.8 |
| Pepsin | 1.1 |
| Bovine serum albumin | 1.0 |
| Bovine hemoglobin | 0.068 |
| Ovalbumin | 0.016 |
| α -Lactoglobulin | 0.0087 |
| Lysozyme | 0.0048 |
| Soybean trypsin inhibitor | <0.001 |

Sources: Adapted from Butler, L.G., 1989a, in Food Products, AOCS, Champaign, IL and Hagerman, A.E. and Butler, L.G., 1981, *J. Biol. Chem.*, 256:4494–4497.,

According to Kawamoto and Nakatsubo (1997) pH, temperature, and ionic strength have a greater effect on the formation of insoluble than soluble tannin-protein complexes. These authors also suggested that the absence of precipitation does not indicate lack of binding between tannins and proteins. Subsequently, Silber et al. (1998) postulated that the precipitation of tannin-protein occurs when a critical number of tannin molecules are associated with a protein molecule. At low concentrations of proteins, the precipitation is due to the formation of a hydrophobic monolayer of polyphenols on the protein surface. However, at higher concentrations, the hydrophobic surface results from complexing of polyphenols on the protein surface and cross linking of different protein molecules with polyphenols. Thus, the stoichiometry of protein-phenol complex depends on the protein concentration in the solution.

In the presence of an excess amount of tannins, precipitates with a fixed ratio of tannin to protein are formed (20 mol of polyphenol to 1 mol of protein for epicatechin (4→8) catechin (EC₁₆-C) and 40 mol of polyphenol to 1 mol of protein for pentagalloylglucose) (Hagerman et al., 1998). The formation of insoluble protein-phenol complexes may be reversed by the addition of an excess amount of protein (Haslam, 1989). Binding a tannin to a protein molecule may also bring about changes in the conformational structure of both molecules, thus lowering their solubility (Asquith and Butler, 1986).

The lowest solubility of tannin-protein complex occurs at a pH near the isoelectric point of the protein (Hagerman and Butler, 1981; Van Buren and Robinson, 1969). Figure 7.9 shows the effect of pH on the amount of canola tannins precipitating with selected proteins. Bovine serum albumin (BSA), fetuin, gelatin and pepsin were precipitated at pH values between 3.0 and 5.0, but the maximum precipitation of lysozyme occurred at pH>8.0. The precipitation of proteins by tannins depends on the availability of unionized phenolic groups for hydrogen bonding (Naczki et al., 1996).

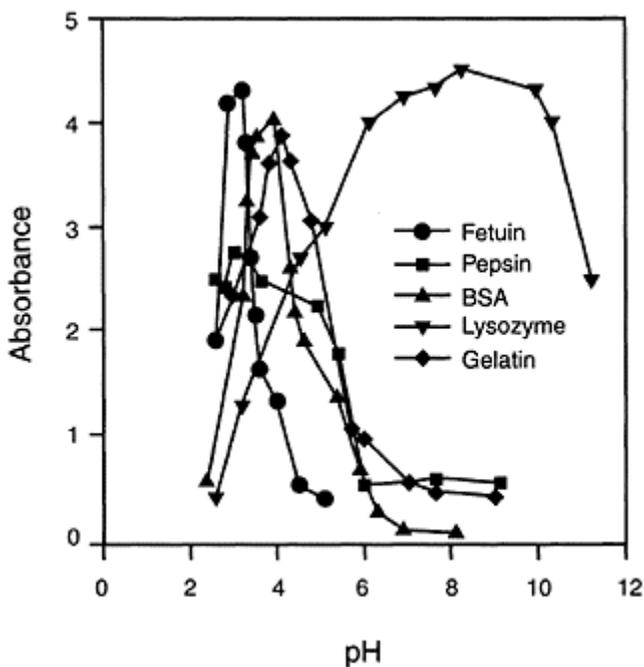


FIGURE 7.9 pH dependence of complex formation between crude condensed tannins of canola hulls and several proteins. (Adapted from Naczki, M. et al., 1996, *J. Agric. Food Chem.*, 44:2144–2148.)

The tannin-protein interaction also depends on the initial concentration of tannins and proteins. All proteins are precipitated in the presence of excess tannins (Hagerman and Robbins, 1987); however, an excess of protein leads to the formation of soluble protein-tannin complexes (Calderon et al., 1968; Hagerman and Robbins, 1987; McManus et al., 1981). The relationship between the amount of tannin-protein complex precipitated and the quantity of protein added to the reaction mixture is described by a bell-shaped curve (Hagerman and Robinson, 1987; Naczki et al., 1996; Silber et al., 1998). Silber et al. (1998) developed a quantitative model describing the dependence of the yield of protein-tannic acid precipitate as a function of protein and tannic acid concentrations. This model was derived based on the assumption that the random binding of tannic acid to proteins follows a Poisson-type distribution and that the protein-binding sites are identical and noninteractive. According to these authors, the proposed model described reasonably well the experimental data obtained for BSA and gelatin.

According to Hagerman (1980) and Hagerman and Butler (1981), proteins with a high affinity for tannins usually have a high molecular weight and open and loose conformational structure, and are rich in proline and other hydrophobic amino acids.

Siebert (1999) also indicated that haze-forming activity of polypeptides in beer is affected by the content of tannins present. Asquith et al. (1987) reported that the glycosylated salivary proteins have enhanced affinity and selectivity towards binding of tannins. Removing the carbohydrate portion from a salivary proline-rich protein (GP66sm) results in reduction of the affinity of deglycosylated protein for sorghum tannins by a four-fold factor and for quebracho tannins by an eight-fold factor (Asquith et al., 1985; Mehensho et al., 1987). The enhanced affinity of glycosylated proteins for tannins may be due to the ability of the oligosaccharide portion of the proteins to maintain the protein structure in a relatively open conformation. Thus, this kind of protein structure would more readily form hydrogen bonds with tannins.

Subsequently, Lu and Bennick (1998) demonstrated that the fraction of basic proline-rich salivary proteins displays a greater affinity for condensed and hydrolyzable tannins than the acidic and glycolysated proline-rich salivary protein fractions. Later, Naurato et al. (1999) reported that human salivary histatins are more effective tannin precipitants than human salivary proline-rich proteins. Histatins are a proline-free group of peptides that are high in histidine and exhibit antibacterial and antifungal activities (Azen et al., 1978; Yan and Bennick, 1995). Perez-Madonaldo (1994) observed that soluble complexes between salivary proteins from sheep and goats and tannic acid and condensed tannins are formed only at 38°C. The formation of insoluble complexes was noticed after the reaction mixture was kept for 12 h at 5°C. Similar observations have been reported for salivary proteins of cattle (Jones and Mangon, 1977) and cattle and sheep (Austin et al., 1989).

Binding of proteins to tannins is also affected by the presence of surfactants. Martin and Martin (1984) observed that higher concentrations of tannins are required for precipitation of rubilose-1,5-bisphosphate carboxylase/oxygenase (RuBPC) from *Menduca sexta* gut fluid adjusted to pH 6.5. These authors suggested that interference of surfactants present in the insect gut fluids might be responsible for this observation. Later, Martin et al. (1985) demonstrated that, between pH 6.15 and 7.55, lysolecithin (0.06%), a surfactant detected in the gut fluids of *Pierris brassicae* (Turunen and Kastari, 1979), prevents the precipitation of RuBPC by tannic acid. However, at pH 8.3 and 9.3, lysolecithin decreases the amount of RuBPC precipitated from 49 to 16% and from 54 to 21%, respectively.

Binding of proteins to tannins may be due to the formation of multiple hydrogen bonds between the hydroxyl groups of tannins and the carbonyl functionalities of peptide bonds of proteins (Gustavson, 1954; Haslam, 1974; Loomis and Battaile, 1966; Spencer et al., 1988). The tannin-protein complex may also be stabilized by other types of molecular interactions such as ionic bonds between the phenolate anion and the cationic site of protein molecules (Loomis, 1974) or covalent links formed as a result of condensation of oxidized phenolic groups of tannins with nucleophilic groups (OH, NH₂) of proteins (Haslam, 1974; Loomis, 1974; Pierpoint, 1969). Figure 7.10 shows the hydrophobic interactions between the aromatic rings of tannins and hydrophobic regions of proteins that may also be involved (Goldstein and Swain, 1965; Hagerman and Butler, 1980; Hagerman et al., 1998; Loomis, 1974; Naczck et al., 2001; Oh et al., 1980; Spencer et al., 1988).

The 1,2-di- (or 1,2,3-tri-) hydroxyphenyl residue is considered the prime binding site of tannins. It is believed, however, that tannin-protein complexation is usually the result

of formation of hydrogen bonds and hydrophobic interactions (Hagerman and Butler, 1981; Oh et al., 1980), particularly under acidic conditions (McManus et al., 1985). Hagerman and Butler (1978) did not observe any precipitation of proteins at pH values above pK_a of the phenolic groups. Based on these results, it has been suggested that the ionic bonds between protein and tannin moieties are less important.

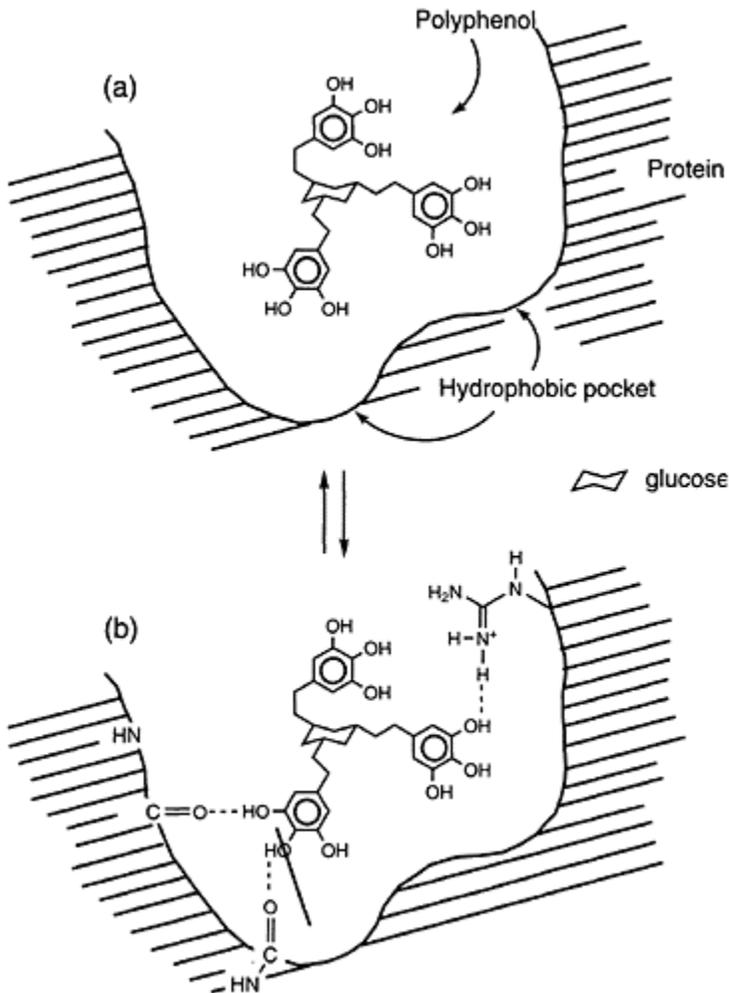


FIGURE 7.10 Polyphenol-protein complexation: (A) docking of the polyphenol; (B) hydrogen bonding to the protein surface. (Adapted from Spencer, C.M. et al., 1988, *Phytochemistry*, 27:2397–2409.)

Hoff and Singleton (1977) demonstrated that BSA immobilized on Sepharose binds tannins selectively at pH 4. Monomeric polyphenolic substances such as gallic acid, chlorogenic acid and catechol do not complex with proteins under these conditions. They also reported that protein-tannin complexes can be dissociated by organic solvents such as methanol and dimethylformamide. Later, Serafini et al. (1997) postulated that ethanol is responsible for the increase in bioavailability of polyphenols in red wine by inhibiting the interactions between proteins and polyphenols.

The formation of tannin-protein complex is affected not only by the composition and structure of proteins but also by the size, length and flexibility of tannin molecules and the number and stereospecificity of binding sites on the tannin molecule. Haslam et al. (1992) suggested the existence of an inverse correlation between the solubility of polyphenols in water and their ability to precipitate proteins. It has been demonstrated that tannins should possess at least three flavonol subunits to be effective protein-precipitating agents. Dimers precipitate proteins, but are much less effective; simple flavonols do not precipitate proteins at all (Artz et al., 1987; Bate-Smith, 1973; Roux, 1972). Similarly, Porter and Woodruffe (1984) found that the ability of condensed tannins to form insoluble complexes with proteins (hemoglobin) depends more on the molecular weight of tannins than on the configuration and number of hydroxyl groups on the B-ring of tannins. Later, Sarni-Manchado et al. (1999) reported that high molecular weight procyanidins (average degree of polymerization of 7.4) are selectively precipitated by salivary proteins from solutions that also contain dimers and trimers.

The configuration and number of hydroxyl groups on the B-ring may also affect the ability of tannins to precipitate proteins. It has been demonstrated that flavonols with three orthohydroxyl groups, like prodelphinidins, bind proteins more tightly than those with two orthohydroxyl groups on their B-ring, such as procyanidins (Asano et al., 1984; Bate-Smith, 1975). Later, Bacon and Rhodes (1998) found a relationship between the degree of galloylation of flavan-3-ol monomers from tea and their affinity to human parotid saliva proteins. The compounds were ranked in the order of their ability to bind to salivary proteins:

(-)-epigallocatechin gallate > (-)-gallocatechin gallate > (-)-epicatechin gallate > (-)-epigallocatechin = (-)-epicatechin = (+)-catechin

Subsequently, de Freitas and Mateus (2001) showed that tannin-protein interactions are affected by the stereochemistry of interflavonoid linkage. Procyanidins with a 4→8 interflavonoid linkage have a greater affinity for proteins than those with a 4→6 linkage. In addition, esterification of (-)-epicatechin or (-)-epicatechin moiety of procyanidin dimer with gallic acid at C3 carbon enhances the affinity of these polyphenols for proteins.

The protein-precipitating capacity of hydrolyzable tannins is influenced by the degree of galloylation, hexahydroxydiphenol esterification, and polymerization (Bacon and Rhodes, 2000; Hagerman et al., 1992; Kawamoto et al., 1996), as well as the flexibility of tannin molecules (Haslam et al., 1992). Beart et al. (1985) observed that the affinity of galloyl esters of D-glucose for bovine serum albumin is enhanced with the addition of every galloyl ester group. Similarly, Ozawa et al. (1987) reported that the binding of β -glucosidase is affected by the degree of glucose galloylation. Subsequently, based on analysis of the galloylglucose-protein co-precipitates, Kawamoto et al. (1995, 1996)

found a positive linear relationship between the degree of galloylation and the affinity of tannins for BSA. The compounds were ranked in order of their ability to bind to BSA:

penta->tetra->2,3,6-tri->2,3,4-tri->>di->>monogalloylglucose

Furthermore, Baxter et al. (1997) reported that more than one galloyl ester group of hydrolyzable tannins may be involved in association of polyphenol with proline-rich peptide, while Haslam (1998) postulated that the ability of polyphenols to form multiple bonds with salivary proteins is affected by their molecular weights. Thus, tannins are able to form numerous bonds with adjacent proline residues of protein molecules but simple phenolics may only bind to a single proline residue.

Based on evaluation of protein-precipitating activities exhibited by pentagalloylglucose and procyanidin (epicatechin₁₆ (4→8) catechin; EC₁₆-C), Hagerman et al. (1998) recently reported that tannin-protein interactions are strongly influenced by the structure of tannins. Hydrogen bonds are primarily involved in interactions between EC₁₆-C and protein, while hydrophobic interactions are the predominant mode of interaction between pentagalloylglucose and proteins. Hydrophobic interactions also play an important role in complexation of proteins with condensed tannins isolated from grape (Oh et al., 1980) and canola hulls (Naczka et al., 2001), and in the formation of haze in beer (Siebert et al., 1996).

INTERACTIONS WITH CARBOHYDRATES

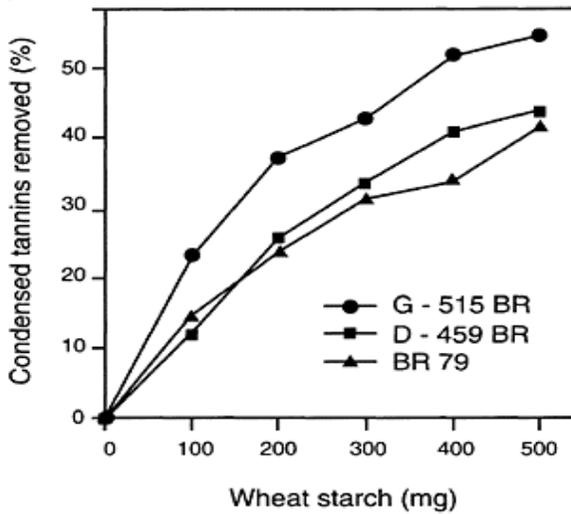
Polyphenols may complex with carbohydrates; the affinity of polyphenols for polysaccharides depends strongly on molecular size, conformational mobility and shape, as well as solubility of polyphenols in water. Thus, an increase in the molecular size and conformational flexibility of tannins enhances their affinity for carbohydrates (Cai et al., 1989).

The affinity of phenolics for carbohydrates is well represented by their association with Sephadex chromatographic gels. The complexation may be due to inclusion of phenolics within the pores of Sephadex, or interaction between oxygen atoms from ether groups that cross link gels and phenolic hydroxyl groups or between a phenyl ring acting as an electron donor and the hydroxyl groups of gels (Brook and Housley, 1969; deLigny, 1979; Haglund, 1978). According to Cai et al. (1989), even subtle changes in the structure of polyphenols may drastically affect the retention

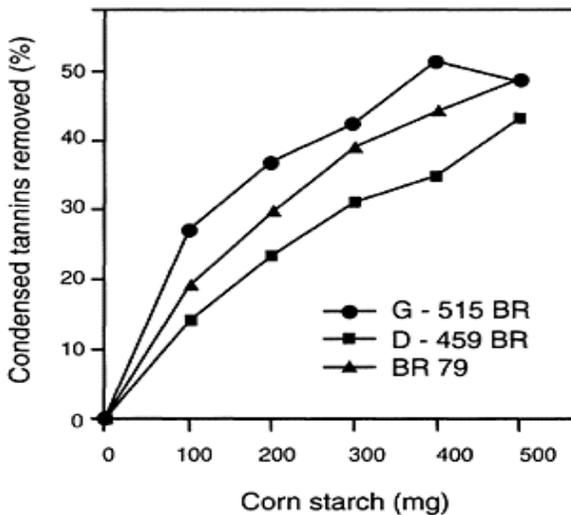
of polyphenols by Sephadex gel. Polyphenolics show a strong affinity toward cyclodextrins. Ya et al. (1989) suggested that a "key and lock" type matching is probably responsible for the association of phenolics within the cavity of a cyclodextrin molecule. This association depends on the size of the cavity, as well as the configuration of the phenolic compound involved. Later, Bianco et al. (1997) applied NMR methodology to investigate the interactions of 3,4-dihydroxyphenylacetic acid (DHPA) with β-cyclodextrin. The thermodynamic data indicated that these interactions are controlled by enthalpy with an opposing entropy contribution.

Davis and Harbers (1974) found that starch prepared by wet milling of birdresistant sorghum is less susceptible to attack by enzymes than other starches. They suggested that absorption and retention of condensed tannins on starch might be responsible for this phenomenon. Later, Davis and Hosney (1979) reported that, depending on the source of

tannins as well as the original type of starch, 40 to 60% of tannins are bound to starch (Figure 7.11). These researchers also found two α -amylase-inhibiting fractions; one is absorbed on starch and the other one is not.



(a)



(b)

FIGURE 7.11 Percent condensed tannins removed by certain amounts of

wheat (A) and corn (B) starch.
(Adapted from Davis, A.B. and
Hoseney, R.C., 1979, *Cereal Chem.*,
56:310–314.)

TABLE 7.5
Binding of Tannic Acid to Different
Starch/Starch Fractions^a

| Conditions | Starch/Starch Fraction | | | | |
|----------------|------------------------|----------------|--------|---------|-------------|
| | Split Yellow Pea | Small Red Bean | Potato | Amylose | Amylopectin |
| 21°C for 4 h | 522 | 261 | 358 | 652 | 587 |
| 95°C for 30min | 394 | 278 | 267 | 186 | 214 |

^a Milligrams of catechin equivalents per 100 g of starch.

Source: Adapted from Deshpande, S.S. and Salunkhe, D.K., 1982, *J. Food Sci.*, 47:2080–2081, 2083.

Deshpande and Salunkhe (1982) studied the interaction of tannic acid and catechin with different legume and potato starches in model systems. Starches associated up to 652 µg of tannic acid and up to 586 µg of catechin per 100-mg sample. A 30-min heating at 95°C substantially decreased the ability of starch molecules to associate with tannic acid and catechin (Table 7.5).

It has been reported that some phenolic acids may have flatulence-inhibiting properties. Rackis et al. (1970) found that phenolic acids such as syringic and ferulic acids inhibit flatulence activity of soybean meals in *in vitro* and *in vivo* studies.

INTERACTIONS WITH MINERALS AND VITAMINS

Tannins containing *o*-dihydroxyphenyl groups may form a stable complex with a wide range of essential minerals, thus lowering their bioavailability (Conrad, 1970; Faithful, 1984; Kennedy and Powell, 1985; Powell and Rate, 1987; Slabbert, 1991). Scalbert (1991) and Mila and Scalbert (1994) suggested that the formation of tannin-metal complexes may play a similar role for plant defense against microorganisms as the complexation of tannins with proteins does. Furthermore, many industrial applications of tannins involve their complexation with metal ions. These include using tannins as modifiers of rheological properties of minerals and clays (Chang and Anderson, 1968), chelators for metal recovery from waste waters (Randall et al., 1974), components of anticorrosive primers (Seavell, 1978), and writing inks (Grimshaw, 1976).

McDonald et al. (1996) used model systems for studying the precipitation of copper (II) and zinc (II) by low molecular weight phenols, penta-*O*-galloyl-β-D-glucose, and commercial tannin at pH 5. They found that the precipitation of polyphenol-metal complexes is affected by initial concentrations of the metal ion and phenol in the reaction mixture and the control of acidification of the reaction mixture brought about by this complexation.

Low availability of iron in the diet is considered an important factor contributing to the high prevalence of iron deficiency anemia in developing countries (Tatala et al., 1998). Phenolic compounds in tea (Disler et al., 1975a; Hallberg and Rossander, 1982; Hurrell et al., 1998, 1999; South et al., 1997), vegetables (Gillooly et al., 1983), wines (Bezwodna et al., 1985; Cook et al., 1995), sorghum (Gillooly et al., 1984) and coffee (Brune et al., 1989; Derman et al., 1977; Hallberg and Rossander, 1982; Hurrell et al., 1998; Morck et al., 1983) have been identified as possible inhibitors of iron absorption through the gut barrier. This inhibition may be due to formation of insoluble iron (III)-phenol complexes, thus making the iron unavailable for absorption in the gastrointestinal tract (Gust and Suwalski, 1994; Porter, 1992; Slabbert, 1992). Phenolic compounds bearing galloyl and catechol groups are mainly responsible for inhibition of iron absorption (Brune et al., 1989, 1991; Gust and Suwalski, 1994; Slabbert, 1992; Zijp et al., 2000).

Gallooly et al. (1983) found an inverse relationship between the content of polyphenols in foods and the degree of inhibition of iron absorption. Later, Brune et al. (1989) reported that the inhibition of iron absorption by tannic acid is a function of its dose. Gallic acid decreases the iron absorption to the same extent that tannic acid does, but no inhibition is observed in the presence of catechin. On the other hand, addition of ascorbic acid increases the absorption of nonheme iron in the presence of polyphenols due to the reduction of Fe(III) to Fe(II) (Siengeberg et al., 1991). Germination of finger millet also improves the accessible iron due to a reduction in phytate and tannin contents (Mbithi-Mwikya et al., 2000; Sriprya et al., 1997; Udayasekhara and Deostahale, 1988). In addition, Matuschek et al. (2001) have demonstrated that reduction of phytate in high-tannin cereals (sorghum and finger millet), followed by oxidation of polyphenols with polyphenol oxidase (mushroom tyrosinase), increases the amount of iron available for absorption approximately 1.5 and 3 times in finger millet and sorghum, respectively.

A number of researchers have reported that adding tea to test meals for a period of 1 to 2 weeks lowers the retention of iron in the blood of human subjects and that chronic tea drinking is related to the incidence of anemia. It has been suggested that flavonoids present in tea may be responsible for decreasing iron absorption from test meal by 63 to 91%, thus lowering its incorporation into blood cells (Brune et al., 1989; Derman et al., 1977; Disler et al., 1975a, b; Morck et al., 1983; Reddy et al., 1991; Rossander et al., 1979). The results of these studies are quite diversified: the consumption of a cup of tea with bread as a test meal (Disler et al., 1975a) and a cup of tea dextrimaltose as a test meal (Reddy and Cook, 1991) bring about inhibition of iron absorption by 64 and 91%, respectively. Later, Zijp et al. (2000) reported that strength of the tea consumed does not have a major impact on iron absorption and that the consumption of black tea infusion with iron-containing foods decreases iron absorption by 60 to 70%. However, drinking black tea between meals inhibits iron absorption only by about 20%.

Caffeic acid and tea flavonoids have been reported to possess an antithiamine effect. Formation of thiamine oxidation products is probably implicated because addition of ascorbic acid prevents this antithiamine effect (Rungruangsak et al., 1977; Somogyi, 1978). Tannic acid has been found to precipitate vitamin B₁₂, which makes it unavailable and results in anemia (Carrera et al., 1973).

REMEDIES FOR ANTINUTRITIONAL EFFECTS OF FOOD PHENOLICS

Several methods for lowering the potential antinutritional effect of phenolics in the diet have been evaluated. These include supplementing the diet with phenol-binding

TABLE 7.6

Effects of Dehulling on Removal of Tannins and Proteins from Sorghum Seeds

| Variety | Dehulled Fraction (% of Grain) | Tannins (%) | |
|---------------------|--------------------------------|---------------------------|------|
| | | In Dehulled Grain Removed | |
| BR 64 (high tannin) | 0 | 4.54 | — |
| | 12.3 | 3.99 | 33.0 |
| | 24.3 | 1.58 | 73.6 |
| | 37.0 | 0.17 | 97.6 |
| BR 626 (low tannin) | 0 | 0.54 | — |
| | 11.3 | 0.35 | 42.5 |
| | 23.4 | 0.30 | 57.5 |
| | 36.0 | 0.19 | 77.5 |

Source: Adapted from Chibber, B.A.K. et al, 1978, *J. Agric. Food Chem.*, 26:679–683.

materials, dehulling, cooking, soaking in water and chemical solutions, and promoting metabolic detoxification (Butler, 1989b; Salunkhe et al., 1989).

The antinutritional effects of tannins for poultry can be overcome by supplementing diet with methionine and choline (Chang and Fuller, 1964). Methionine may participate as a methyl group donor in the detoxification methylation of tannin breakdown products (Salunkhe et al., 1989). The growth-depressing effect of tannins can also be alleviated by increasing the level of proteins in the diet. The supplementary proteins, such as gelatin, serve as tannin-binding agents and not as a source of amino acids (Rogler et al., 1985; Schaffert et al., 1974). Weight gain and feed-to-weight ratio can be significantly improved by adding tannin-binding polymers such as polyvinylpyrrolidone to the diet (Armstrong et al., 1973; Marquardt et al., 1977).

Because tannins are mostly located in the pericarp and testa of seed coats, tannin content in cereals and legumes can be significantly lowered by dehulling grains. According to Chibber et al. (1978), the efficiency of the dehulling process in the removal of tannins depends on the amount of dry matter removed from seeds. The tannin content of high-tannin sorghum can also be reduced with cooking (Mitaru et al., 1984b), steaming (Ekpenyong, 1985), soaking and germination (Elmaki et al., 1999; Iwuoha and Aina, 1997; Mukuru et al., 1992; Obizoba and Atii, 1991).

Approximately 37% of dry matter of BR64 sorghum must be removed in order to lower its tannin content by 98% (Table 7.6). A poor recovery of sorghum grains dehulled with a mechanized milling process has been noted (Reichert et al., 1988); however, wet and dry milling techniques are commonly used in Asia for commercial dehulling of grain legumes. The dehulling removes from 24.4 to 98.2% of tannins initially present in soybean and black gram beans, respectively (Salunkhe et al., 1989).

Heat processing seeds or their meals may lower their level of assayable tannins, perhaps because of the formation of insoluble complexes between tannins and other seed components (Butler, 1989a). The amount of assayable tannins and loss of bound

TABLE 7.7
Effects of Soaking on Content of Tannins in Dry
***Phaseolus* Beans^a**

| Treatment | Pinto Beans | Cranberry Beans | Viva Pink Beans |
|----------------------------------|-------------|-----------------|-----------------|
| Raw beans | 264.7 | 76.3 | 122.1 |
| Beans soaked in Distilled water | | | |
| 6h | 208.6 | 67.0 | 100.0 |
| 12 h | 96.1 | 57.0 | 80.0 |
| 18 h | 69.9 | 51.0 | 73.0 |
| Sodium bicarbonate (2%) 12 h | 24.1 | 25.0 | 27.0 |
| Mixed salt solution ^b | 18.0 | 20.0 | 20.0 |

^a Milligrams of catechin equivalents per 100 g of starch.

^b Mixed aqueous salt solution contained 2.5% (w/v) sodium chloride, 1% (w/v) sodium tripolyphosphate, and 1.5% (w/v) sodium carbonate.

Source: Adapted from Deshpande, S.S. and Cheryan, M., 1983, *Nutr. Rep. Int.*, 27:371–377.

tannins in cooked black beans account for 60.4% compared to 20.5% in raw black beans (Bressani et al., 1982). Furthermore, Price et al. (1980) have reported that cooking high-tannin sorghum grains does not improve their nutritional value, but instead depresses the growth rate of rats fed on such diets.

Several chemical treatments have been proposed to improve the nutritional value of cereals and legumes. These include ammoniation, treatment with alkaline solutions, acids and formaldehyde (Butler, 1989b). Ammoniation of high-tannin sorghum grains at 80 psi for 1 h significantly decreases the content of assayable tannins and improves the growth of chickens fed on such grains; however, similar treatment of low-tannin sorghum has a detrimental effect on its nutritional value. The mechanism responsible for this detrimental effect in low-tannin sorghum has not been elucidated (Price et al., 1978, 1979). On the other hand, soaking cowpea seeds in an alkaline or acidic solution decreases the content of assayable tannins by approximately 84%. Soaking in a 0.5-M solution of NaOH, KOH or acetic acid is most effective in removing polyphenols from cowpeas (Laurena et al., 1986). Similarly, as shown in Table 7.7, up to 93% of tannins are removed from dry *Phaseolus* beans after 12 h of soaking in a 2% sodium bicarbonate or a mixed-salt solution (Deshpande and Cheryan, 1983). Makkar and Becker (1996) have also reported that tannins are effectively inactivated in the presence oxidizing agents under alkaline conditions.

TOXICITY OF ABSORBED POLYPHENOLS

Morton (1978) attributed esophageal cancer to the use of tannin-rich beverages such as tea. A significant correlation exists between the incidence of cancer in some parts of the

world and excessive drinking of tea (Morton, 1989) and apple-based drinks. Apple ciders are made of fruits rich in tannins, which give the beverage its bitterness and astringency (Haslam, 1989). According to Haslam (1989), frequently drinking beverages with high tannin content may damage the esophageal epithelium, thus increasing its susceptibility to known carcinogens. Singleton (1981) attributed the effect of tannins to irritation and cellular damage to DNA mutagenic-type cancer. Salunkhe et al. (1989) have also associated the possibility of high risk of cancer incidence to the chronic ingestion of high quantities of tannins in the diet. However, they suggested that regular consumption of tannins may induce the development of a defensive mechanism by animals and human beings to lower the risk of cancer.

When Singleton and Kratzer (1969) studied the acute toxicity of tannins, they found that LD₅₀ values for rats, mice and rabbits for a single dose of orally administered tannins range from 2.25 to 6.00 g/kg of body weight. They suggested that phenols may become toxic if natural barriers or detoxification mechanisms are overloaded by the amount of ingested phenols. The toxicity level of phenols depends also on the manner of their administration and is affected by the presence of substances containing diether or isopropenoid structures. According to Singleton (1981), small- and large-size phenolics may cause nutritional implications as a result of consuming the metabolized energy in their detoxification process or by lowering the contribution of methyls or glucuronic acid to more useful metabolisms. Phenolic compounds can produce a long-term toxic effect at the magnitude of common phenol intakes ranging from 1 to 5% in the diet. These values are much higher for insoluble phenolics such as lignin or ellagic acid. General agreement is that phenolics from common foodstuffs at the regular intake have very low toxicities. This is attributed to their low absorption and rapid metabolism, as well as the presence of an efficient defense mechanism in mammals (Lake, 1999; Singleton, 1981; Williams et al., 1999).

Wren et al. (2002) assessed the safety of grape extract containing less than 5.5% catechin monomers. The grape seed extract was administered to rats in their feed at levels of 0, 0.5, 1.0, and 2.0% for 90 days. A significant increase in body weight in rats consuming grape seed extract compared to that of the control rats was observed. No significant toxicological effects in rats were noticed during this study. Furthermore, potential adverse effects of excessive intake of flavonoids should not be overlooked as high intakes of food phenolics may potentially lead to mutagenesis, prooxidant and inhibition of key enzymes (Galati et al., 2002; Skibola and Smith, 2000). More research is still required to establish toxicological effects associated with high intake of food phenolics.

PHARMACOLOGICAL EFFECTS

ANTIMICROBIAL PROPERTIES

Many preservatives are added to foods as antioxidants or antimicrobial agents. Phenolic compounds are known to possess both of these properties (Atroshi et al., 2003; Haslam, 1989; Nychas, 1995; Nychas et al., 1990; Scalbert, 1991; Schmitz et al., 1993; Takenaka

et al., 1997); however, the lipophilic nature of phenols may reduce their antimicrobial properties (Baranowski and Nagel, 1984).

Antimicrobial action of phenolic compounds was first related to the inactivation of cellular enzymes, which depended on the rate of penetration of the substance into the cell (Fogg and Lodge, 1945). Later, Judis (1963) suggested that inhibition of

TABLE 7.8
Effect of BHA on Aflatoxin Production of
***Aspergillus parasiticus* Spores^a**

| Treatment | Aflatoxins ^b | | | |
|------------------|-------------------------|----------------|----------------|-------|
| | B ₁ | B ₂ | G ₁ | Total |
| Control | 3.56 | 0.54 | 2.02 | 6.12 |
| BHA (0.1 mg/kg) | 2.34 | 0.62 | 1.76 | 4.72 |
| BHA (10 mg/kg) | 10.5 | 0.84 | 4.34 | 15.68 |
| BHA (1000 mg/kg) | ND | ND | ND | ND |

Note: ND=no toxin detected.

^a Molds were grown in 50 mL of glucose-salts medium in 125-mL flask for 7 days at room temperature.

^b Milligrams per liter of broth.

Source: Adapted from Chang, H.C. and Branen, A.L., 1975, *J. Food Sci.*, 40:349–351.

microbial growth is due to weakening or destruction of the permeability of cell membranes. Subsequently, Juven et al. (1972) demonstrated that phenolics cause membrane permeability changes. They showed that oleuropein, a phenolic glycoside found in olives, causes leakage of the radioactively labeled glutamate, potassium, and phosphorus from *Lactobacillus plantarum*. According to Bernheim (1972), changes in the permeability of cell membrane may be due to the interaction of phenols with phospholipid components of membranes. However, using intracellular UV-absorbing materials and C¹⁴ labeled compounds, Davidson and Branen (1980a, b) proposed that disruption of cytoplasmic membranes is at least partially responsible for inhibitory action of butylated hydroxyanisole (BHA) on *Pseudomonas fluorescens* and *Pseudomonas fragi*. Moreover, they speculated that BHA may also affect the activity of cell-bound enzyme systems.

Chang and Branen (1975) found that BHA at 1000 mg/kg totally prevents the growth and production of aflatoxin by *Aspergillus parasiticus* (Table 7.8). They also demonstrated that BHA has a profound antimicrobial effect against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium*. Later, Davidson and Branen (1980a, b) examined the antimicrobial activity of BHA against psychrotropic bacteria, namely, *P. fluorescens* and *P. fragi*. They found that 100 mg/kg of BHA delays the growth of *P. fluorescens* at 22°C and totally inhibits it at 7°C. On the other hand, *P. fragi* is more resistant to BHA: delayed growth has been observed at ≥300 mg/kg. According to Shelef and Liang (1982), BHA acts as an effective bacteriostatic agent against *Bacillus cereus*, *Bacillus subtilis* and *Bacillus megaterium* at ≤200 mg/kg in laboratory media and at ≤10,000 mg/kg in food systems. They also suggested that including BHA to control food microorganisms is premature because BHA is fat soluble; thus its inhibitory effects

in lipid-containing foods may be influenced by the partition coefficient between the lipid and the bacteria.

Klindworth et al. (1979) observed reduced antibacterial activity for BHA against *Clostridium perfringens* in the presence of lipids. On the other hand, Davidson et al. (1981) reported that potassium sorbate and BHA have little or no delaying effect in initiating growth of *S. aureus* or *S. typhimurium*. However, potassium sorbate (500 to 1000 mg/kg) and BHA (50 to 100 mg/kg) show a synergistic effect in suppressing the growth of both microorganisms, thus allowing a reduced level of potassium sorbate to be used when BHA is present.

Pierson and Reddy (1982) examined the activity of some phenolic antioxidants against the growth and toxin production of *Clostridium* types A and B. They found that inoculation with 8000 spores/g of sample of meat-containing esters of gallic acid, *p*-hydroxybenzoic acid, and related phenolics delays the time of first toxic swell for 3 days beyond the control. The inhibitory activity of phenolics increases as the spore level per gram of meat decreases.

Kubo et al. (2002) have examined a series of synthetic alkyl gallates as potential antifungal agents against *Saccharomyces cerevisiae*, which is responsible for spoilage of sauerkraut, syrups, honey, fruit juices, meats, wine and beer (Fleet, 1992). Of these synthetic alkyl gallates, nonyl gallate, followed by octyl gallate, is the most effective antifungal agent tested. The nonyl and octyl gallates display fungicidal activities at a minimum concentration of 12.5 and 25 mg/L, respectively. The antifungal activity of these gallates is not affected by pH and the growing stage of *S. cerevisiae*; the antifungicidal activity of nonyl gallate is 64 times greater than that of sorbate (Kubo et al., 2002).

Widely distributed as secondary metabolites in plants, hydroxycinnamates exhibit considerable antimicrobial effect under appropriate conditions. Gupta et al. (1976) found that naturally occurring ethyl *p*-methoxycinnamate inhibits growth of selected molds (*Trichophyton rubrum*, *Aspergillus niger*, *S. cerevisiae*, *Epidermophyton floccosum*, *Aspergillus fumigatus*, *Penicillium purpurogenum*, *Trigonopsis variabilis*, *Microsporium gypseum*, *Sclerotium rolfsii*, *Geotrichum candidae*, *Fusarium oxysporum*, *Helminthosporium oryzae*, *Candida cruseum* and *Trichophyton mentagrophytes*) at concentrations of 10 to 50 mg/kg. Later, Baranowski et al. (1980) reported that *p*-coumaric acid at a 100 mg/kg-level increases the lag phase of *S. cerevisiae*; at concentrations >250 mg/kg the inhibition after 72 h of growth is proportional to its concentration. They also found that ferulic acid at 50 mg/kg brings about an increase in the lag phase and that as little as 250 mg/kg results in complete inhibition.

Baranowski and Nagel (1982) examined the effectiveness of alkyl esters of six hydroxycinnamic acids and cinnamic acid (so-called alkacins), as well as free acids, in inhibiting the growth of *P. fluorescens* (a bacterium commonly implicated in food spoilage). They found that free caffeic, *p*-coumaric, ferulic, sinapic, cinnamic, *p*-methoxycinnamic and 3,4-dimethoxycinnamic acids are largely ineffective at 400 mg/kg. On the other hand, alkyl esters of *p*-coumaric and caffeic acids have a more inhibitory effect than parabens (alkyl esters of *p*-hydroxybenzoic acid) at 125 mg/kg (Table 7.9). Later, Baranowski and Nagel (1984) demonstrated the inhibitory effect of alkacins in a wide range of bacteria in model systems and showed it to be comparable to the effects of methyl and propyl parabens. They also found that, although these compounds have fair to

very good antimicrobial activity in model systems, their relative effect in foods depends on the system under investigation.

Marvan and Nagel (1986) demonstrated that ethanolic extracts of cranberry exert a significant antimicrobial effect on *Saccharomyces bay anus* and *P. fluorescens*. They

TABLE 7.9
Relative Effectiveness of Alkacins and Parabens
against *Pseudomonas Fluorescent* in Trypticase
Soy Broth^a

| Compound | Concentration ^b (mg/kg) | | | |
|-----------------------------------|------------------------------------|------|------|------|
| | 25 | 50 | 125 | 250 |
| Methyl caffeoate | 1.00 | 1.06 | 1.71 | 2.84 |
| Ethyl caffeoate | 1.03 | 1.07 | 1.66 | 2.24 |
| Propyl caffeoate | 1.00 | 1.24 | 1.84 | 2.84 |
| Methyl cinnamate | 0.96 | 1.03 | 1.28 | 2.46 |
| Methyl <i>p</i> -coumarate | 1.03 | 1.15 | 1.72 | 2.11 |
| Methyl ferulate | 1.02 | 1.00 | 1.02 | 1.20 |
| Methyl sinapate | 1.02 | 1.01 | 1.02 | 1.06 |
| Methyl <i>p</i> -methoxycinnamate | 1.02 | 1.02 | 1.02 | 1.02 |
| Methyl 3,4-dimethoxycinnamate | 1.00 | 0.99 | 1.03 | 1.02 |
| Methyl paraben | 1.00 | 1.07 | 1.31 | 2.18 |
| Propyl paraben | 1.05 | 1.09 | 1.38 | 2.40 |

^a pH 7.0 at 20°C.

^b Milligram per kilogram.

Source: Adapted from Baranowski, J.D. and Nagel, C.W., 1982, *J. Food Sci.*, 47:1587–1589.

showed that proanthocyanidins account for 21.3% of total cranberry inhibition, while the inhibitory effects of flavonols and benzoic acid are 18.5 and 15.6%, respectively, when tested at pH 4.0. A synergistic effect between benzoic acid and proanthocyanidins or flavonols was also observed, but the activities of proanthocyanidins and flavonols are additive. At higher pH values, the dominant inhibitors are proanthocyanidins. The proanthocyanidin fraction completely inhibits the growth of microorganisms at pH 5.0 and 6.0 for 1 month. This may be brought about by stronger interaction between proanthocyanidins and microorganisms' cell membrane proteins or oxidation and polymerization of proanthocyanidins. Waage et al. (1984) showed that procyanidin of higher molecular weight (MW) has a broader activity than that of those with lower MW.

Cranberry proanthocyanidins have been linked to the maintenance of urinary tract health (Gibson et al., 1991; Howell, 2002; Papas et al., 1996). These compounds may be responsible for inhibition of cellular adherence of uropathogenic strains of P-type (mannose-resistant) *E. coli* to mucosal cells in the urinary tract (Howell, 2002; Howell et al., 1998; Ofek et al., 1991; Zafriiri et al., 1989) because the urinary tract infection is initiated by bacteria adhering to the mucosal cells (Beachey, 1981). Foo et al. (2000a, b) demonstrated that cranberry proanthocyanidins with A-type linkage exhibit greater antiadhesion activities than those with B-type linkage. Howell (2002) suggested that

cranberry proanthocyanidins competitively inhibit the adhesion of *E. coli* to mucosal cells through receptor-ligand interactions.

Oleuropein, a phenolic compound found in olive, and its hydrolysis products, namely, aglycone and elenolic acids, possess antimicrobial properties (Fleming et al., 1973; Juven and Henis, 1970; Juven et al., 1972). Pradhan et al. (1999) evaluated the antimicrobial properties of two major phenolic compounds present in green pepper berries (*Piper nigrum* L.): 3,4-dihydroxyphenylethanol glucoside and 3,4-dihydroxy-6-(N-ethylamino) benzamide. These phenolics are not detected in black pepper because of their oxidation to black pigment by polyphenoloxidase during sun drying of green pepper berries (Bondopadhyay et al., 1990; Variyar et al., 1988). Of these phenolic compounds, 3,4-dihydroxyphenyl ethanol glucoside displays greater bacteriostatic activity against *S. typhimurium*, *S. aureus*, *Bacillus aureus* and *E. coli* than 3,4-dihydroxy-6-(N-ethylamino) benzamide (Pradhan et al., 1999).

Norton (1999) examined the inhibitory effects of anthocyanidins (cyanidin, pelargonidin, malvidin, peonidin, apigenidin, and luteolinidin) and their mono- and diglucosides against the growth and biosynthesis of aflatoxin B₁ by *Aspergillus flavus*. All anthocyanidins tested inhibited the synthesis of aflatoxin B₁; those with a 3-hydroxyl group (peonidin, delphinidin, pelargonidin, and cyanidin) displayed approximately three times greater inhibitory effect than the corresponding 3-deoxy anthocyanidins. Kaempferol and naringenin also display strong inhibitory effects on the growth of *A. flavus* and formation of aflatoxin (Mallozzi et al., 1996), while catechin and luteolin are weak inhibitors (Norton, 1999). On the other hand, the steryl ferulate and *p*-coumarate ester fraction isolated from corn does not inhibit the growth of *A. flavus*, but at a concentration range of 0.33 to 1.0 g/L, displays a stimulatory effect on the biosynthesis of aflatoxin B₁ (Norton and Dowd, 1996).

Kubo et al. (1999) characterized antibacterial activity of several anarcadic acids against *Helicobacter pylori*, an infectious bacterium responsible for gastric ulcer and other gastrointestinal diseases. Adhesion of bacteria to epithelial cells is an essential step in the development of these diseases (Dorell et al., 1998; Dunn et al., 1997; Lingwood et al., 1992). Maximum antibacterial activity is displayed by anarcadic acid with saturated C_{12:0} and unsaturated C_{15:3} and C_{15:2} alkyl side chains, while anarcadic acid with saturated C_{15:0} alkyl side chain is inactive against *H. pylori* (Kubo et al., 1999). The antiulcer activity of anarcadic acids may be from their action as detergents (Kubo et al., 1995) and urease inhibitors (Kubo et al., 1999). Urease neutralizes the acid environment of the stomach by hydrolyzing urea to ammonia and carbon dioxide (Blaser, 1993) and thus is thought to help in colonization and survival of *H. pylori*. According to Kubo et al. (1999), regular consumption of fresh cashew apple and its processed products may effectively control *H. pylori*. Burger et al. (2002) have demonstrated the inhibitory effect of high molecular weight constituents of cranberry (proanthocyanidins) on a specific adhesion of *H. pylori* human gastric cell lines *in vitro*. Cranberry phenolics are thought to act on sialyllactose specific adhesion of *H. pylori*; in turn, this prevents adhesion of pathogen to the stomach (Burger et al., 2002).

Tea polyphenols substantially inhibit the growth of cariogenic bacteria, namely, *Streptococcus mutans* and *Streptococcus sobrinus*, and the synthesis of extracellular glucans from sucrose by the action of their glucosyltransferases (Hamilton-Miller, 2001; Hattori et al., 1990; Matsumoto et al., 1999; Ooshima et al., 1998; Sakanaka and Kim,

1998; Sakanaka et al., 1992). The glucans are important cariogenic factors mediating the aggregation of cariogenic bacteria on tooth surfaces—an accumulation that leads to formation of dental plaque and caries (Freedman and Tanzer, 1974;

TABLE 7.10

Comparison of 50% Inhibitory Doses (ID₅₀) of Selected Phenolics on Glucosyltransferase Activity^a

| Compound | Glucosyltransferase from | |
|--------------------------------|--------------------------|--------------------|
| | <i>S. mutans</i> | <i>S. sobrinus</i> |
| Crude apple polyphenol extract | 120 | 25 |
| Caffeic acid | >1000 | >1000 |
| Chlorogenic acid | >1000 | >1000 |
| Epicatechin | >1000 | >1000 |
| Procyanidin B2 | >1000 | >1000 |
| Apple condensed tannins | 5 | 1.5 |
| Phlorizidin | >1000 | >1000 |
| Epigallocatechin gallate | >1000 | >1000 |
| Quercetin | ND | 120 |

Note: ND=no data.

^a Milligrams per liter.

Source: Adapted from Yanagida, A. et al., 2000, *J. Agric. Food Chem.*, 48:5666–5671.

Hamada and Torii, 1978). Bactericidal activity for anarcadic acid against *S. mutans* has also been reported (Muroi and Kubo, 1993). Moreover, apple polyphenols (Matsudaira et al., 1998) and cranberry juice constituents (Weiss et al., 1999) effectively inhibit the formation of dental plaque in human subjects. Yanagida et al. (2000) have demonstrated that apple polyphenols are effective in inhibiting the glucosyltransferase activity (Table 7.10), but have no significant effect on *in vitro* growth of cariogenic bacteria. Recently, Daglia et al. (2002) observed the inhibition of adsorption of *S. mutans* to hydroxyapatite beads coated with saliva by a roasted coffee solution. The antiadhesive property of the roasted coffee is thought to be due to absorption of coffee components (trigonelline, nicotinic acid, chlorogenic acids) on tooth surfaces, thus preventing adhesion of bacteria to these surfaces.

ANTIVIRAL PROPERTIES

A number of flavonoids present in foods of plant origin possess antiviral activity. Quercetin, taxifolin and flavonoids from red wine are able to inactivate a wide range of viruses and prevent their infectivity (Bakay et al., 1968; Konowalchuk and Speirs, 1976a). Tannins from strawberry are able to inactivate polio, enteric, and herpes viruses (Konowalchuk and Speirs, 1976a, b). Quercetin, a flavonol aglycone found in a number of fruits such as apple, apricot, fig, plum, strawberry, and tomato, shows antiviral activities against herpes simplex virus type 1, parainfluenza virus type 3 and polio virus

type 1 in *in vivo* and *in vitro* studies (Middleton, 1986; Musci, 1986; Veckenstedt et al., 1986).

There have also been reports that quercetin synergistically enhances the beneficial effect of interferon (Musci, 1986). Tea polyphenols are able to inhibit influenza A and B infections by preventing the virus from adsorbing to cells (Nakamaya et al., 1993), while hesperetin inhibits the infectivity of herpes simplex type viruses, polio viruses, and parainfluenza viruses (Kaul et al., 1985). Furthermore, curcumin and its analogs exhibit inhibitory activities against human immunodeficiency virus type 1 (HIV-1) integrase by preventing binding of the enzyme to viral DNA (Mazumder et al., 1995, 1997), and tannins from pericarp of pomegranate display antiviral activity against the genital herpes virus (Zhang et al., 1995). King et al. (1999) examined 34 analogues of dicaffeoylquinic and dicaffeoyltartaric acids as potential inhibitors of HIV-1 integrase and replication. Biscatechol group was found to be essential for the inhibition of integrase while at least one free carboxyl group was required for anti-HIV activity. Of the evaluated phenolic acid analogues, D-cichoric, *meso*-cichoric *bis*(3,4-dihydroxybenzoyl)-L-tartaric acid, *bis*(3,4-dihydroxydihydrocinnamoyl)-L-tartaric acid, digalloyl-L-tartaric acid, dicaffeoylglyceric acid and *bis*(3,4-dihydroxyphenylacetyl)-L-tartaric acid were the most potent inhibitors of HIV-1 integrase.

OTHER PHARMACOLOGICAL POTENTIALS

A number of books and reviews have been published to describe the pharmacological potentials of food phenolics (Adams and Lewis, 1977; Bagchi et al., 2000; BenaventeGarcia et al., 1997; Bidlack et al., 2000; Block et al., 1992; Blot et al., 1996; Böhm et al., 1998; Brandi, 1997; Butrum, 1996; Cody et al., 1986; Cook and Samman, 1996; De Bruyne et al., 1999; Dubnick and Omaye, 2001; Dugas et al., 2000; Facino et al., 1998; Gilman et al., 1995; González de Mejía et al., 1999; Habtemariam, 1997; Haslam et al., 1989; Haslam, 1996; Hertog et al., 1997a, b, 1993a, b; Hertog, 1996; Hollman and Katan, 1998; Huang et al., 1994a, b; Ielpo et al., 2000; Ito and Hirose, 1989; Jang et al., 1997; Katiyar and Mukhtar, 1996; Keli et al., 1996; Knekt et al., 1997; Konneh and Caen, 1996; Kuntz et al., 1999; Kuo, 1997; Langseth, 1995; Leake, 1997; Mabry and Ulubelen, 1980; Messina et al., 1994; Meyers et al., 1996; Middleton and Kandaswami, 1993; Morton et al., 2000; Murakami et al., 1996; Nehlig and Debry et al., 1994; Noguchi, 2002; Pathak et al., 1991; Reed, 2002; Rice-Evans and Packer, 1997; Stavric and Matula, 1988, 1992; Steinmetz and Potter, 1996; Stoner and Mukhtar, 1995; Suschetet et al., 1996; Tapiero et al., 2002; Temple, 2000; Tijburg et al., 1997; Ursini et al., 1999; Verstraeten et al., 2003; Waladkhani and Clemens, 1998; Willet, 2002; Wollgast and Anklam, 2000; Yang and Wang, 1993; Yochum et al., 1999). Therefore, this section provides a discussion of only some aspects of pharmacological potentials of food phenolics.

Saito et al. (1998) used rats to examine the antiulcer property of grape seed extracts. Rich in flavanol, these extracts strongly suppress the stomach mucosal injury caused by acidified ethanol. Active oxygen species have been implicated in the development of gastric mucosal damage caused by acidified ethanol (Itoh and Guth, 1985; Matsumoto et al., 1993; Pihan et al., 1987). It has been suggested that the antiulcer property of grape procyanidins may be due to their radical scavenging activity and their ability to bind to

proteins. However, the exact antiulcer mechanism of procyanidins against stomach mucosa injury is still not well understood (Saito et al., 1998). Procyanidin-rich extracts from grape seeds also display anticataract activity in hereditary cataractous (ICR/f) rats fed a standard diet containing 0.213% (w/w) of grape seed extract (Yamakoshi et al., 2002), as well as in streptozotocin-induced diabetic rats fed a diet containing 0.5% (w/w) (Nguyen et al., 1999).

According to Spector (1995) and Taylor et al. (1995), oxidative stress is responsible for cataractogenesis; therefore, the retardation of cataract progression may be due to the antioxidant activity of grape seed procyanidins and their metabolites (Yamakoshi et al., 2002). Other reported biological activities of grape seed extracts include antiatherosclerotic (Yamakoshi et al., 1999), antidiabetic (Nguyen et al., 1999), and anticarcinogenic (Alam et al., 2002; Arai et al., 1998; Bagchi et al., 2000; Krohn et al., 1999; Ye et al., 1999; Zhao et al., 1999) properties, production of vasorelaxation by inducing nitric oxide release from endothelium (Fitzpatrick et al., 2000), control of LDL-cholesterol level in high cholesterol-fed rats (Tebib et al., 1994a, b), and prevention of plasma postprandial oxidative stress in humans (Natella et al., 2002).

Cocoa procyanidins are also effective scavengers of reactive oxygen and nitrogen species (Arteel and Sies, 1999; Kondo et al., 1996; Steinberg et al., 2002; Waterhouse et al., 1996; Wollgast and Anklam, 2000) and have the ability to modulate immune function and platelet aggregation (Mao et al., 1999; Rein et al., 2000b; Sanbongi et al., 1997; Wollgast and Anklam, 2000). Yamagishi et al. (2000) demonstrated that cacao liquor polyphenols exhibit a strong antimutagenic activity on the action of heterocyclic amines (HCA) *in vitro* and *ex vivo*. The suppression of antimutagenic activity of HCA may involve not only inhibition of the metabolic activation of HCA in the liver but also adsorption of HCA by some phenolic component of cacao liquor such as procyanidins. Pomegranate polyphenols exhibit strong antiatherogenic (Aviram et al., 2000; Kaplan et al., 2001) and antitumoral (Kashiwada et al., 1992) activities. Moreover, pomegranate juice has been recommended in the treatment of acquired immune deficiency syndrome (AIDS) due to pomegranate bioflavonoids' inhibitory effect on lipoxigenase (Lee and Watson, 1998).

Phenolic compounds extracted from bilberry (*Vaccinium myrtillus*) juice after fermentation constitute the active principle of a drug used for vascular protection. Anthocyanins present in the extract act on capillary permeability and fragility (Azar et al., 1987; Wagner, 1985). Pool-Zobel et al. (1999) have demonstrated that the anthocyanins from *Aronia melanocarpa*, elderberry, and Macqui and Tintorera fruits exhibit stronger antioxidant activities *in vitro* than those displayed by Trolox and vitamin C in a ferric-reducing ability assay. Anthocyanins are not able to prevent endogenous oxidative DNA damage in human colon cells; thus, according to Pool-Zobel et al. (1999), the cancer preventive potential of anthocyanins within specific tissue is questionable. On the other hand, anthocyanins exhibit inhibitory effects on the formation of nitric oxide in lipopolysaccharide (LPS)- and interferon- γ (INF- γ)-activated mouse macrophage cell line RAW 264.7 (American type culture collection).

Anthocyanins at a concentration ≥ 250 mg/L have a stimulatory effect on secretion of tumor necrosis factor- α (TNF- α) in macrophages (Wang and Mazza, 2002a, b), which display cytostatic and cytotoxic activities on malignant cells (Camussi et al., 1991; Wadsworth and Koop, 1999). Frank et al. (2002) have reported that cyanidin-3-glucoside

and anthocyanin mixtures from elderberry and blackcurrant, when consumed at normal dietary levels, have little effect on the cholesterol and liver fatty acid profiles in healthy rats. However, cyanidin-3-glucoside elevates the level of tocopherols in the liver and lungs.

Tea polyphenols exhibit a very broad spectrum of biological activities (Erland et al., 2001; Gao et al., 1994; Katiyar and Mukhtar, 1996; Stensvold et al., 1992; Trevisanato and Kim, 2000; Yang and Chung, 2000; Yang and Wang, 1993; Yang et al., 1996). Green tea polyphenols inhibit intestinal uptake of glucose through the sodium-dependent glucose transporter (SGLT1) of the rabbit intestinal epithelial cells. Presence of galloyl group enhances the inhibitory effect of tea polyphenols. It has been suggested that tea polyphenols act as antagonist-like molecules (Kobayashi et al., 2000) and thus may contribute to the reduction of blood glucose level. Aqueous extracts of green, oolong, pouchong, and black tea inhibit over 90% of mutagenicity of the 2-amino-3-methylimidazo(4,5-f)quinoline, 3-amino-1,4-dimethyl-5H-pyridyl(4,3-b)indole, 2-amino-6-methylpyrido(1,2-a:3',2'-d)imidazole, benzo[a]pyrene and aflatoxin B1 toward *S. typhimurium* TA 98 and TA100 (Yen and Chen, 1994).

Imai et al. (1997) and Nakachi et al. (1998) have reported that increased consumption of green tea is responsible for lowering the risk for breast cancer metastasis and recurrence among Saitama, Japan, women. The (-)-epigallocatechin-3-gallate (EGCG), a polyphenolic component of green tea, has been found to reduce the incidence of spontaneously and chemically induced tumors in experimental animals, as observed for tumors of liver, stomach, skin, lungs and esophagus (Huang et al., 1992). EGCG causes cell cycle dysregulation and apoptosis of cancer cells mediated through nuclear factor- κ B (NF- κ B). The inhibition of NF- κ B constitutive expression and activation in cancer cells occurs at lower concentrations of EGCG compared to those of normal cells (Ahmad et al., 2000a, b). Pan et al. (2000) have reported that, isolated from oolong tea, theasinensin A and mixtures of theaflavin-3-gallate and theaflavin-3'-gallate exhibit strong growth inhibitory effects against human histolytic lymphoma U937, but are less effective against human acute T cell leukemia Jurkat. Induced by tea phenolics, the apoptosis of these cells involves the caspase3 mediated mechanism.

Yang and Chung (2000) demonstrated that effective levels of tea polyphenols required for imparting signal transduction pathways, inhibiting cell proliferation and inducing apoptosis of cancer cells are higher than those detected in blood and tissues. In addition, Maeda-Yamamoto (1999) reported that tea polyphenols, namely, epicatechin gallate, epigallocatechin gallate, and theaflavin, strongly prevent the invasion of highly metastatic human fibrosarcoma HT1080 cells into the monolayer of human umbilical vein endothelial cells/gelatin membrane *in vitro*. Of these phenolics, only epicatechin gallate inhibits invasion in the absence of cytotoxicity. Thus, more studies are needed to determine the mechanisms involved in prevention of cancer by tea phenolics.

Low incidence of coronary heart disease among populations consuming red wine has been linked to the presence of resveratrol in wine (Das et al., 1999; Goldberg, 1995; Goldberg et al., 1995; Gronbaek, 1999; Hegsted and Ausman, 1988; Renaud and de Lorgeril, 1992; Sharpe et al., 1995; Waterhouse et al., 1999). Animal studies indicated that resveratrol and piceid lower the accumulation of cholesterol and synthesis of triacylglycerols in rat liver (Arichi et al., 1982). Waffo-Teguo et al. (2001) have reported that two products of oxidative degradation of stilbene, namely, resveratrol (*E*)-

dehydrodimer 11-*O*- β -D-glucopyranoside and resveratrol (*E*)-dehydrodimer, exhibit a nonspecific inhibitory activity against cyclooxygenase-1 and -2. These enzymes play an important role in the development of tumorigenesis and inflammation (Subbaramaiah et al., 1998).

Pterostilbenes display moderate inhibitory activity against cyclooxygenase-1 and weak inhibition of cyclooxygenase-2 (Rimando et al., 2002). Furthermore, *trans*- and *cis*-resveratrols display inhibitory effects against kinases (Jayatilake et al., 1993), platelet aggregation (Chung et al., 1992), and LDL oxidation (Belguendouz et al., 1997; Fauconneau et al., 1997; Stivala et al., 2001), while piceids are effective against platelet aggregation (Chung et al., 1992; Orsini et al., 1997) and LDL oxidation (Frankel et al., 1995; Fremont et al., 1999).

Resveratrol also prevents cell proliferation in tumor cell lines *in vitro* (Jang et al., 1997; Hsieh and Wu, 1999) and decreased tumor growth in a rat tumor model (Carbo et al., 1999). In addition, resveratrol and its oxidation product are a potent inhibitor of dioxygenase activity of lipoxygenase, but not its hydroperoxidase activity (Papatheofanis and Land, 1985; Pinto et al., 1999). However, by its hydroperoxidase activity, lipoxygenase is involved in the oxidation of resveratrol in the presence of hydroperoxy derivatives of polyunsaturated fatty acids or hydrogen peroxide (Pinto et al., 1999). Lipoxygenase has been implicated in inflammatory responses in allergy, asthma, and arthritis (Kulkarni et al., 1990), and in the formation of atherosclerotic lesions (Prigge et al., 1997) and carcinogenic processes (Furstenberger et al., 1991; Kamitani et al., 1998).

Frankel et al. (1993) suggested that the beneficial effect of red wine may be explained by the inhibition of oxidation of low-density lipoproteins (LDL) by wine phenolics. Later, Satué-Gracia et al. (1999) reported that phenolics present in Spanish sparkling wines (cava) also inhibit oxidation of LDL. This activity correlates positively with the total phenolic content, quercetin 3-glucuronide, *trans*-caffeic acid, protocatechuic acid, and coumaric acid. Subsequently, Gómez-Cordovés et al. (2001) demonstrated that all fractions of wine phenolics (anthocyanins, other flavonoids, phenolic acids) show potential as therapeutic agents in the treatment of melanoma.

Citrus flavonoids display a broad spectrum of biological activities (Rouseff and Nagy, 1994). Of these, auraptene (7-geranyloxycoumarin) found in citrus fruit peel exhibits chemopreventive activity in mouse skin (Murakami et al., 1997), rat colon (Tanaka et al., 1997, 1998a) and rat tongue (Tanaka et al., 1998b) carcinogenesis models; 8-geranyloxypsolaren, bergamottin, and 5-geranyloxy-7-methoxycoumarin are effective inhibitors of tumor promoter 12-*O*-tetradecanoyl-13-phorbol acetate-induced Epstein-Barr virus activation in Raji cells (Miyake et al., 1999). Flavanones display protective effects against cancer (Koyuncu et al., 1999), as well as rendering antiallergic and antiinflammatory properties (Gabor, 1986; Noguchi et al., 1999) and a cholesterol-lowering activity in the rat (Bok et al., 1999; Galati et al., 1994). A number of polymethoxylated flavones suppress the production of tumor necrosis factor- α in culture of human monocytes (Manthey et al., 1999). These flavones are also effective inducers of HL-60 (human acute promyelocytic leukemia cells) differentiation (Kawai et al., 1999a, b; Sugiyama et al., 1993) and therefore may have great therapeutic potential (Koeffler, 1983). Diosmin is an active component of some drugs used for the treatment of circulatory system illnesses (Galley and Thiollet, 1993; Tsouderos, 1991), inflammatory disorders (Jean and Bodinier, 1994) and severe hemorrhoidal disease (Godeberg, 1994).

Furthermore, diosmin and its aglycone (diosmetin) prevent the activation of carcinogenesis by heterocyclic amines (Ciolino et al., 1998).

Isoflavones exert a broad spectrum of biological activities. Besides estrogenic activities, isoflavones protect against several chronic diseases. Results of epidemiological studies indicate that consumption of soybean isoflavones lowers the incidence of breast, prostate, urinary tract and colon cancers; they also provide protection against coronary heart diseases and osteoporosis (Brandi, 1997; Clarkson et al., 1995; Cline and Hughes, 1998; Miyazawa et al., 2001; Moyad, 1999; Peterson and Dwyer, 1991; Sheu et al., 2001; Su et al., 2000). Peterson and Dwyer (1991) examined the effect of isoflavones on the growth of breast carcinoma cell lines, MDA468 (estrogen receptor negative), MCF-7 and MCF-7-D-40 (estrogen receptor positive). Genistein is the most potent inhibitor of cancer cell growth, while daidzein and biochanin A display weaker inhibitory activity. However, isoflavone glucosides, genistin and daidzin have little effect on the growth of breast cancer cells (Peterson and Dwyer, 1991). In addition, isoflavones exhibit marked inhibitory activity against oxidation of lipoprotein in serum (Hodgson et al., 1996; Kerry and Abbey, 1998). Sheu et al. (2001) have reported that genistein, daidzein and glycitein suppress the formation of nitric oxide in RAW 264.7 macrophages by inhibiting activity and expression of the inducible nitric oxide synthase in lipopolysaccharide-activated macrophages.

The pharmacological effects of anarcadic acids, cardols and cardanols, the major phenolics of cashew nuts, include cytotoxic activity against BT-20 breast carcinoma cells (Kubo et al., 1993) and antiacne (Kubo et al., 1994a) and antibacterial (Gellerman et al., 1969; Himejima and Kubo, 1991; Kubo et al., 2002) activity. Anarcadic acids also exhibit inhibitory effects against a number of enzymes, including aldose reductase (Toyomizu et al., 1993), glycerol-3-phosphate dehydrogenase (Irie et al., 1996), lipoxygenase (Grazzini et al., 1991; Shobha et al., 1994), prostaglandin synthase (Bhattacharya et al., 1987; Grazzini et al., 1991; Kubo et al., 1987) and tyrosinase (Kubo et al., 1994b).

Curcuminoids, namely, curcumin, demethoxycurcumin and bisdemethoxycurcumin, are the major phenolics found in turmeric (*Curcuma longa* L.) (Figure 7.12) (Govindarajan, 1980; Jayaprakasha et al., 2002). Of these, curcumin is the predominant phenolic constituent of turmeric (Ashan et al., 1999; Govindarayan, 1980). The total content of curcuminoids in four samples of commercially available varieties of turmeric ranged between 23.4 and 91.8 g/kg (Jayaprakasha et al., 2002). Curcuminoids display antioxidant (Ashan et al., 1999; Kim et al., 2001; Osawa et al., 1994; Reddy and Lokesh, 1992; Schaich et al., 1994; Wright, 2002), anticancer, antimutagenic, anti-inflammatory (Brouet and Ohshima, 1995; Chang and Fong, 1994; Lin et al., 1994; Srimal, 1997), and antiviral (Mazumder et al., 1995, 1997) activities. Simon et al. (1998) evaluated curcumin, demethylcurcumin and bisdemethylcurcumin as potential inhibitors of proliferation of MCF-7 human breast cancer cells. Of these, demethylcurcumin displayed the best inhibitory effect, followed by curcumin and bisdemethylcurcumin. Lin et al. (1994) demonstrated that curcumin is an effective inhibitor of tumor promotion induced by 12-*O*-tetradecanoylphorbol13-acetate (TPA). Curcumin suppresses the TPA-induced synthesis of protein kinase C in mouse fibroblast cells and 8-hydroxydeoxyguanosine in cellular DNA. In addition, curcumin displays immunomodulating activities by enhancing the production of interleukin-4 in T helper-2 cells (Chang and Fong, 1994).

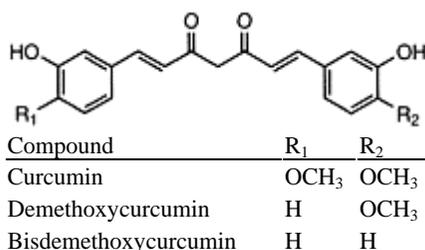


FIGURE 7.12 Chemical structures of some curcuminoids.

The ellagic, protocatechuic and chlorogenic acids present in fruits and vegetables have been found to serve as potential chemopreventers against several carcinogens (Nakamura et al., 2001; Tanaka et al., 1992). Repeated oral administration of protocatechuic acid at a 100 µg/kg-level inhibits the growth of colon and oral cancers in rats (Tanaka et al. 1993; Ueda et al., 1996), while topical administration of protocatechuic acid at doses less than 1/1000 of toxic level effectively inhibits growth of tumor in mouse skin (Nakamura et al., 2000). However, topical pretreatment of mouse skin with a high dose (>150 mg) of protocatechuic acid enhances tumor promotion and contact hypersensitivity in mouse skin (Nakamura et al., 2000). Meanwhile a toxic oral dose of protocatechuic acid (500 mg/kg) causes depletion of glutathione, the major cellular antioxidant, in mouse liver and kidney (Nakamura et al., 2001).

The colonic neoplasia induced by azoxymethanol can be inhibited by quercetin and rutin found in vegetables and fruits (Deschner, 1992; Deschner et al., 1991). Quercetin is also known for its vasoactive properties (Alarcon de la Lastra et al., 1994; Formica and Regelson, 1995) and gastroprotective effect (Kahraman et al., 2003; Martin et al., 1998). In addition, quercetin displays an inhibitory effect on the mutagenic activity of heterocyclic amines (HCA). Several mutagenic HCAs have been isolated from cooked foods (Ohgaki et al., 1991; Sugimura, 1988; Wakabayashi et al., 1993). The suppression of HCA activity is due to inhibition of metabolic activation of HCA in the liver (Alldrick et al., 1986, 1989). On the other hand, quercetin may also act as a tumor promoter and carcinogenic agent (Formica and Regelson, 1995; Stavric, 1994). According to Nagabhushan (1990), catechin from sorghum and faba beans acts as an antimutagen of benzo[α]pyrene and dimethyl [a] anthracene in *Salmonella*.

Pearson et al. (1997) demonstrated that carnosic acid, carnosol, and rosmarinic acid present in rosemary extract and thymol, carvacrol, and zingerone (Figure 7.13) found in thyme, oregano, and ginger display inhibitory effects on endothelial cell-mediated oxidation of LDL. Their inhibitory activities decrease as follows:

carnosol > carnosic acid ≈ rosmarinic acid >>> thymol > carvacrol > zingerone

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8

Antioxidant Properties of Food Phenolics

INTRODUCTION

Antioxidants markedly delay or prevent oxidation of the substrate (Halliwell, 1999; Shahidi, 2000), when they are present in foods or in the body at low concentrations compared to that of an oxidizable substrate. Food manufacturers have used food-grade antioxidants, mainly of a phenolic nature, to prevent quality deterioration of products and to maintain their nutritional value. Antioxidants have also been of interest to health professionals because they help the body to protect itself against damage caused by reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) and reactive chlorine species (RCS) associated with degenerative diseases (Figure 8.1).

Antioxidants act at different levels in the oxidative sequence involving lipid molecules. They may decrease oxygen concentration, intercept singlet oxygen, prevent first-chain initiation by scavenging initial radicals such as hydroxyl radicals, bind metal ion catalysts, decompose primary products of oxidation to nonradical species and break chains to prevent continued hydrogen abstraction from substrates (Shahidi, 2000, 2002).

Natural antioxidants from dietary sources include phenolic and polyphenolic compounds, among others. The mechanism by which these antioxidants exert their effects may vary depending on the compositional characteristics of the food, including its minor components. Furthermore, the beneficial health effects of consuming plant foods have been ascribed, in part, to the presence of phenolics, which are associated with counteracting the risk of cardiovascular diseases, cancer and cataract as well as a number of other degenerative diseases. This is achieved by preventing lipid oxidation, protein cross linking and DNA mutation and, at later stages, tissue damage (Figure 8.2).

Although phenolic compounds and some of their derivatives are very efficient in preventing autoxidation, only a few phenolic compounds are currently allowed as food antioxidants. The major considerations for acceptability of such antioxidants are their activity and potential toxicity and/or carcinogenicity. The approved phenolic antioxidants have been extensively studied, but the toxicology of their degradation products still is not clear.

The process of autoxidation of polyunsaturated lipids in food involves a free radical chain reaction that is generally initiated by exposure of lipids to light, heat, ionizing radiation, metal ions or metalloprotein catalysts. Enzyme lipoxygenase can also initiate oxidation. The classical route of autoxidation includes initiation (production of lipid free radicals), propagation and termination (production of nonradical products) reactions (Reaction 8.1 to Reaction 8.4). Figure 8.3 represents a general scheme for autoxidation of polyunsaturated lipids and their consequence in quality deterioration of food:

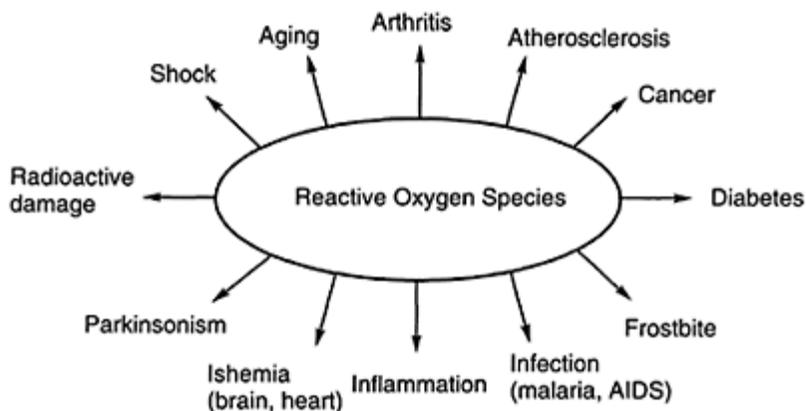


FIGURE 8.1 Diseases and damages caused by reactive oxygen species.

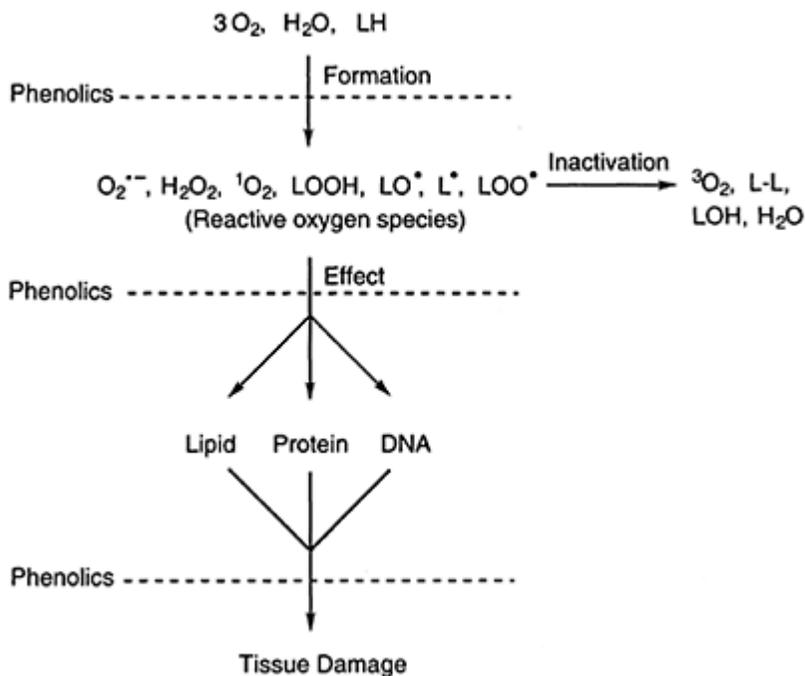


FIGURE 8.2 Consequences of reactive oxygen species in diseases and preventive role of phenolics.

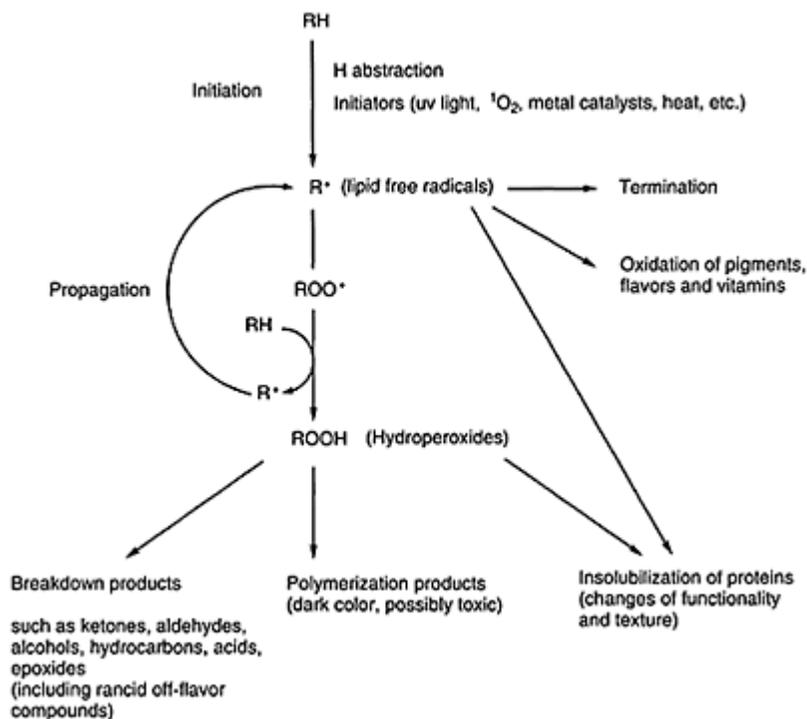
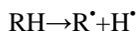


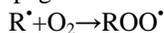
FIGURE 8.3 General scheme for autoxidation of lipids containing polyunsaturated fatty acids (RH) and their consequences.

Initiation

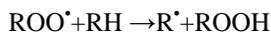


(8.1)

Propagation

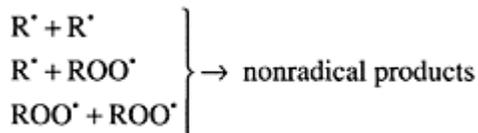


(8.2)



(8.3)

Termination



(8.4)

Some commercially produced plant phenolic compounds have recently been considered as antioxidants. Flavonoids, the most potent antioxidative compounds of plant phenolics, need further investigation in order to explore the feasibility of their use in foods and to determine their toxicological implications.

PREVENTION OF AUTOXIDATION AND USE OF ANTIOXIDANTS

Vacuum (or *sous vide*) packaging or packaging under an inert gas (i.e., modified atmosphere packaging) to exclude oxygen, as well as refrigeration or freezing, can reduce the rate of autoxidation (Dziedzic and Hudson, 1983a). However, these means are not always practical because very little oxygen is needed to initiate and maintain the oxidation process. It is neither economical nor practical to remove traces of oxygen from foods; therefore, it is quite common to combine such methods with the use of antioxidants. The main justification for using an antioxidant is to extend the shelf life of foodstuffs and to reduce waste and nutritional losses by inhibiting and delaying oxidation. However, antioxidants cannot improve the quality of an already oxidized food product (Sherwin, 1978; Coppen, 1983).

Antioxidants are regarded as compounds capable of delaying, retarding or preventing autoxidation processes. According to the USDA Code of Federal Regulations, "antioxidants are substances used to preserve food by retarding deterioration, rancidity or discoloration due to oxidation" (21, CFR 170.3 (0) (3); Dziezak, 1986). Synergists are substances that enhance the activity of antioxidants without possessing antioxidant activity of their own (Nawar, 1996).

It has been suggested that an ideal food-grade antioxidant should be safe, not impart color, odor or flavor, be effective at low concentrations, be easy to incorporate, survive after processing, and be stable in the finished product (carry through) as well as available at a low cost (Coppen, 1983).

ESTIMATION OF ANTIOXIDANT ACTIVITY

The activity of an antioxidant can be estimated by quantitatively determining primary or secondary products of autoxidation of lipids or by monitoring other variables. Generally, the delay in hydroperoxide formation or production of secondary products of autoxidation by chemical or sensory methods can be used. These procedures can be applied to intact foods, their extracts or to model systems. Studies on foods can be performed under normal storage conditions or under accelerated oxidation such as active oxygen method (AOM), Schaal oven test, oxygen uptake/absorption, and oxygen bomb calorimetry, or by using a fully automated oxidative stability instrument (OSI), a Rancimat apparatus, or an oxidograph, among others (Kahl and Hildebrandt, 1986; Kochhar and Rossell, 1990). The extension of the induction period by addition of an antioxidant has been related to antioxidant efficacy, which is sometimes expressed as antioxidant index or protection factor. It is also possible to use a luminescence apparatus, also known as PHOTOCHEM (Analytik Jena, Delaware, OH), which measures

antioxidant activity of hydrophilic and lipophilic compounds (Amarowicz et al., 2003). ORAC (oxygen radical absorbance capacity) and TEAC (Trolox equivalent antioxidant capacity) tests have also been used in the recent literature; artificial radicals such as DPPH (2,2-diphenyl-1-picrylhydrazyl) radical have been employed. All of these offer means of evaluating antioxidant activity of food phenolics and other constituents.

The ORAC test measures the scavenging capacity at 37°C of peroxy radical induced by 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) using fluorescence at a wavelength of 565 nm with excitation at 540 nm (Cao et al., 1993). The results are calculated and expressed as millimoles of Trolox equivalents per gram. The TEAC assay determines the capacity of an antioxidant, antioxidant mixtures or an extract to scavenge ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) radical and thus disappearance of the blue/green color of this radical upon the action of antioxidants (Arts et al., 2003). Rice-Evans et al. (1995, 1996) have reported TEAC values for selected flavonoids. In addition, DPPH assay measures the scavenging of this radical with antioxidants using spectrophotometric or EPR (electron paramagnetic resonance) techniques (Wettasinghe and Shahidi, 2000; Yu et al., 2002).

Formation of hydroperoxides is measured by an iodometric titration of released iodine by hydroperoxides and is generally expressed as the peroxide value (PV). The 2-thiobarbituric acid (TBA) test or para-anisidine test, measurement of total carbonyls, or a selected carbonyl compound and assessment of off-flavors and off-odors due to the formation of volatile decomposition products of hydroperoxides by objective and subjective means are used extensively (Shahidi, 1998) to measure hydroperoxide decomposition products and determine aldehydic compounds (e.g., spectrophotometric determination of malonaldehyde and related compounds) collectively referred to as malonaldehyde equivalents. Sensory evaluation provides information on the overall acceptability of foods in addition to the results provided by chemical determinations (Kahl and Hildebrandt, 1986). Application of Fourier transform infrared (FT-IR) spectroscopy (van de Voort et al., 1994) and nuclear magnetic resonance (NMR) spectroscopy (Wanasundara and Shahidi, 1993) for the evaluation of the oxidative state of food lipids have recently been reported (van de Voort et al., 1994). These techniques reflect the overall changes in primary and secondary oxidation products.

Model systems for testing the antioxidant activity of food components and additives have been used extensively. Peroxidation catalyzed by metmyoglobin or a Fe^{2+} -EDTA system, as well as AOM tests, may be performed on linoleic acid instead of food in evaluation of the activity of antioxidants. However, model systems have a disadvantage in that intact foods also contain natural compounds that may possess antioxidant or synergistic properties.

TYPES OF ANTIOXIDANTS

According to their mode of action, antioxidants may be classified as free radical terminators, chelators of metal ions, or oxygen scavengers that react with oxygen in closed systems. Thus, primary antioxidants react with high-energy lipid radicals to convert them to thermodynamically more stable products. Secondary antioxidants, also known as preventive antioxidants, function by retarding the rate of chain initiation by

breaking down hydroperoxides. Examples of these secondary antioxidants include dilauryl thiodipropionate and thiodipropionic acid (Dziedzic, 1986; Gordon, 1990); phenolic antioxidants are included in the category of free radical terminators. Because the main emphasis is on phenolic antioxidants, the mechanism of the action of free radical terminators will be discussed in detail.

MECHANISM OF ACTION OF PHENOLIC ANTIOXIDANTS

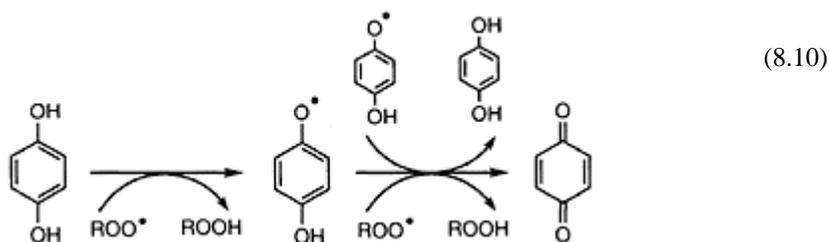
The first detailed kinetic study of antioxidant activity was conducted by Boland and ten-Have (1947) who postulated Reaction 8.5 and Reaction 8.6 for free radical terminators. Phenolic antioxidants (AH) interfere with lipid oxidation by rapid donation of a hydrogen atom to lipid radicals (Reaction 8.5 and Reaction 8.6). The latter reactions compete with chain propagation Reaction 8.3 and Reaction 8.9:



These reactions are exothermic in nature. The activation energy increases with increasing A-H and R-H bond dissociation energy. Therefore, the efficiency of the antioxidants (AH) increases with decreasing A-H bond strength. The resulting phenoxy radical must not initiate a new free radical reaction or be subject to rapid oxidation by a chain reaction. In this regard, phenolic antioxidants are excellent hydrogen or electron donors; in addition, their radical intermediates are relatively stable due to resonance delocalization and lack of suitable sites for attack by molecular oxygen (Sherwin, 1978; Nawar, 1986; Belitz and Grosch, 1987).

In the body, free radicals may be involved in a number of diseases and tissue injuries such as those of the lung, heart, cardiovascular system, kidneys, liver, eye, skin, muscle and brain, as well as the process of ageing. Oxidants and radicals are known to mediate such disorders, but are generally neutralized by antioxidant enzymes in healthy individuals. However, with age and in individuals with certain ailments, the endogenous antioxidants may require exogenous assistance from dietary antioxidants in order to maintain the integrity of cell membranes.

The phenoxy radical formed by reaction of a phenol with a lipid radical is stabilized by delocalization of unpaired electrons around the aromatic ring as indicated by the valence bond isomers in Reaction 8.10.



However, phenol is inactive as an antioxidant. Substitution of the hydrogen atoms in the ortho and para positions with alkyl groups increases the electron density of the OH moiety by an inductive effect and thus enhances its reactivity toward lipid radicals. Substitution at the para position with an ethyl or *n*-butyl group rather than a methyl group improves the activity of the phenolic antioxidant; however, presence of chain or branched alkyl groups in this position decreases the antioxidant activity (Gordon, 1990).

The stability of the phenoxy radical is increased by bulky groups at the ortho positions as in 2,6-di-*tert*-butyl, 4-methoxyphenol or butylated hydroxyanisole (BHA) (Miller and Quackenbush, 1957). Because these substituents increase the steric hindrance in the region of the radicals, they further reduce the rate of possible propagation reactions that may occur, as in Reaction 8.11 to Reaction 8.13, involving antioxidant free radicals (Gordon, 1990):

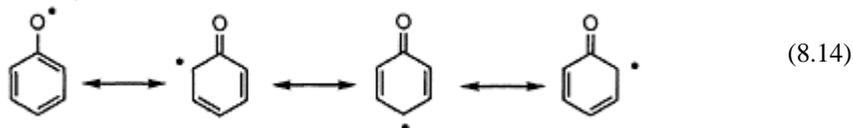


The introduction of a second hydroxy group at the ortho or para position of the hydroxy group of a phenol increases its antioxidant activity. The effectiveness of a 1,2-dihydroxybenzene derivative is increased by the stabilization of the phenoxy radical through intramolecular hydrogen bond. Thus, catechol and hydroquinone are much more effective in their peroxy radical scavenging activity than phenol (Heigen et al., 2001). Similarly, flavonols containing a catechol moiety (3'- and 4'-OH) in ring B (rutin and monohydroxyethyl rutinoside) or an AC-ring with three OH groups (3-, 5-, and 7-OH) are potent scavengers. The 3-OH group is found to be the active center, its activity influenced by electron-donating groups at the 5- and 7-positions (galangin, kaempferol, and trihydroxyethyl quercetin). In another study, Heim et al. (2002) found that multiple hydroxyl groups conferred substantial antioxidant, chelating, and, in some cases, pro-oxidant activity to the molecule. Methoxy groups introduce unfavorable steric effects, but presence of a double bond and a carbonyl functionality in the C ring increases the activity by affording a more stable flavonoid radical through conjugation and electron delocalization.

Finally, the antioxidant activity of hydroxyflavones is influenced by pH. The antioxidant potential increases, as determined by the TEAC assay, upon deprotonation of the hydroxyl group. This indicates that the mechanism of action of flavonoids is variable

and, although abstraction of the hydrogen atom is involved for underprotonated species, electron (not hydrogen) atom donation is involved in the deprotonated species (Lemanska et al., 2001). Furthermore, the hydroxyl radical scavenging activity of phenolics involves multiple mechanisms, including hydroxyl bond strength, electron donating ability, enthalpy of single electron transfer and spin distribution of the phenoxy radical after hydrogen abstraction (Cheng et al., 2002). More recently, Arts et al. (2003) have reported a critical evolution of the use of antioxidant capacity in defining optimal antioxidant structures.

The antioxidant activity of dihydroxybenzene derivatives is partly due to the fact that the semiquinoid radical produced initially can be further oxidized to a quinone by reaction with another lipid radical. It can also form into a quinone or hydroquinone molecule, as in Reaction 8.14:



The activity of 2-methoxyphenol is, in general, much lower than that of catechol, which possesses two free hydroxy groups (Rosenwald and Chenicek, 1951), because 2-methoxyphenols are unable to stabilize the phenoxy radical by hydrogen bonding as in Reaction 8.14 (Gordon, 1990). The effect of antioxidant concentration on autoxidation rates depends on many factors, including the structure of the antioxidant, oxidation conditions, and nature of the sample oxidized. Often phenolic antioxidants lose their activity at high concentrations and behave as pro-oxidants (Cillard et al., 1980) by involvement in initiation reactions such as those in Reaction 8.11 to Reaction 8.13 (Gordon, 1990). Antioxidant activity by donation of a hydrogen atom is unlikely to be limited to phenols. Endo et al. (1985) have suggested that the antioxidant effect of chlorophyll in the dark occurs by the same mechanism as phenolic antioxidants.

Phenolic antioxidants are more effective in extending the induction period when added to an oil that has not deteriorated to any great extent. However, they are ineffective in retarding decomposition of already deteriorated lipids (Mabrouk and Dugan, 1961). Thus, antioxidants should be added to foodstuffs as early as possible to achieve maximum protection against oxidation (Coppen, 1983).

SYNTHETIC PHENOLIC ANTIOXIDANTS

The application of antioxidants in foods is governed by federal regulations. FDA regulations require that the ingredient labels of products declare antioxidants and their carriers followed by an explanation of their intended purpose (Dziezak, 1986). Table 8.1 and Table 8.2 summarize the permitted food phenolic antioxidants, some of their properties and amounts of their allowable usage. Synthetic food antioxidants currently permitted for use in foods are butylated hydroxytoluene (BHT), butylated

TABLE 8.1
Synthetic Food Antioxidants and Their Properties

| Characteristic | BHA | BHT | Gallates | | TBHQ |
|--------------------------|------------------|--------------|----------|-----------|---------|
| | | | Propyl | Dodecyl | |
| Melting point °C | 50–52 | 69–70 | 146–148 | 95–98 | 126–128 |
| Carry-through properties | Very good | Fair-good | Poor | Fair-good | Good |
| Synergism | BHT and gallates | BHA | BHA | BHA | – |
| Solubility (w/w%) | | | | | |
| Water | 0 | 0 | <1 | <1 | <1 |
| Propylene glycol | 50 | 0 | 6.5 | 4 | 30 |
| Lard | 30–40 | 50 | 1 | – | 05–10 |
| Corn oil | 30 | 40 | 0 | 0 | 10 |
| Glycerol | 1 | 0 | 25 | – | <1 |
| Methyl linoleate | Very soluble | Very soluble | 1 | 1 | >10 |

Sources: Adapted from Coppen, P.P., 1983, in *Rancidity in Foods*, Allen, J.C. and Hamilton, R.J., Eds., Applied Science Publishing Company, London, 67–87 and Dziezak, J.D., 1986, *Food Technol.*, 9, 94–102.

hydroxyanisole (BHA), propyl gallate (PG), dodecyl gallate (DG) and tertiary-butylhydroquinone (TBHQ).

BHA and BHT are monohydric phenolic antioxidants (Figure 8.4) originally used to protect petroleum from oxidative degumming before their introduction and acceptance in the food industry (Porter, 1980). Chemically, BHA is a mixture of 3-tertiary-butyl-4-hydroxyanisole (90%) and 2-tertiary-butyl-4-hydroxyanisole (10%). BHA is commercially available as white waxy flakes and BHT is available as a white crystalline compound; both are extremely soluble in fats and insoluble in water (Table 8.2). Furthermore, both assert a good carry-through effect; however, BHA is slightly better than BHT in this respect (Dziezak, 1986).

BHT is more effective in suppressing oxidation of animal fats than vegetable oils. Among its multiple applications, BHA is particularly useful in protecting the flavor and color of essential oils and is considered the most effective of all food-approved antioxidants for this application (Stuckey, 1972). BHA is particularly effective in controlling the oxidation of short-chain fatty acids such as those found in the coconut and palm kernel oils typically used in cereal and confectionary products (Dziezak, 1986).

As a monophenol, BHT can produce radical intermediates with moderate resonance delocalization. The tertiary butyl groups of BHT do not generally allow involvement of the radical formed from it after hydrogen abstraction in other reactions. Thus, a lipid peroxy radical may join the molecule of BHT in the para position to the phenoxy group (Dziezak, 1986).

The volatile nature of BHA and BHT makes them important additives in packaging materials because they can migrate into foods. For this purpose, these antioxidants are

TABLE 8.2

**Maximum Usage Levels Permitted by U.S. FDA
in Specific Application of Antioxidants (from
Code of Federal Regulations)**

| Food | Maximum Usage Levels ^a | | | | |
|--------------------------------------|-----------------------------------|--------------------|-----------------|-------------------|-----|
| | BHA ^{b,c} | BHT ^{c,d} | PG ^e | TBHQ ^b | |
| Dehydrated potato shreds | 50 | | 50 | — | — |
| Active dry yeast | 1000 ^g | | — | — | — |
| Beverages prepared from dry mixes | 2 ^g | | — | — | — |
| Dry breakfast cereals | 50 ^g | | 50 | — | — |
| Dry diced glazed fruits | 32 ^g | | — | — | — |
| Dry mixes for beverages and desserts | 50 | | — | — | — |
| Emulsion stabilizers for shortenings | 200 | | 200 | — | — |
| Potato flakes | 50 | | 50 | — | — |
| Potato granules | 10 | | 10 | — | — |
| Sweet potato flakes | 50 | | 50 | — | — |
| Poultry products ^h | 100 ^g | 100 ^j | 100 | 100 | 100 |
| Dry sausage ⁱ | 30 ^g | 30 ^j | 30 | 30 | 30 |
| Fresh sausage ⁱ | 100 ^g | 100 ^j | 100 | 100 | 100 |
| Dried meat ⁱ | 100 ^g | 100 ^j | 100 | 100 | 100 |

^a Milligrams per kilogram.^b 21 CFR 172. 110.^c Given levels are for total BHT and BHA.^d 21 CFR 172. 115.^e 21 CFR 184. 1660.^f 21CFR 172. 185.^g BHA only.^h 9 CFR 381. 147(f) (3).ⁱ 9 CFR 318.7 © (4).^j BHT only.

Source: Adapted from Dziezak, J.D., 1986, *Food Technol.*, 9, 94–102.

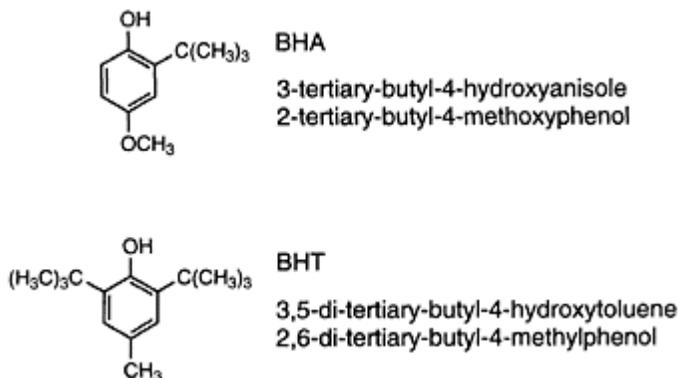


FIGURE 8.4 Chemical structures of butylated hydroxyanisole (BHA) and butylated hydroxy-toluene (BHT).

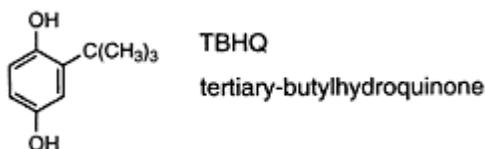


FIGURE 8.5 Chemical structure of tertiary-butylhydroquinone (TBHQ).

added directly to the wax used in making inner liners or applied to the packaging board as an emulsion (Porter, 1980; Dziezak, 1986). A synergistic effect has been shown to exist when BHT and BHA are used in combination. The oxidative reactions of nut and nut products are very responsive to the combination of these two antioxidants (Dziezak, 1986).

Tertiary-butylhydroquinone, shown in Figure 8.5, is regarded as the best antioxidant for protecting frying oils against oxidation (Khan and Shahidi, 2001) and provides good carry-through protection to fried products similar to those of BHA and BHT. TBHQ may be considered as an alternative to hydrogenation for increasing oxidative stability (Dziezak, 1986). TBHQ is adequately soluble in fats and does not complex with iron or copper; therefore, it does not discolor the treated products. TBHQ is available as a beige-colored powder to be used alone or in combination with BHA or BHT at a maximum amount of 0.02% or 200 mg/kg, based on the fat content of foods, including essential oils. TBHQ is not permitted in combination with propyl gallate (Dziezak, 1986). Coppen (1983) has reported that TBHQ shows good performance in stabilizing crude oils.

Chelating agents such as citric acid and monoacylglycerol citrate can further enhance lipid-stabilizing properties of TBHQ. This combination is primarily used in vegetable oils and shortenings but not extensively for animal fats. Confectioneries, including nuts and candies, also benefit from the use of TBHQ or its mixtures (Buck, 1984). As a diphenolic antioxidant, TBHQ reacts with peroxy radicals to form a semiquinone resonance hybrid. The semiquinone radical intermediates may undergo different reactions to form more stable products. They can also react with one another to produce dimers or react with one another to produce a quinone and a hydroquinone molecule or add to a lipid peroxy radical to produce a semiquinone.

Propyl gallate is commercially prepared by esterification of gallic acid with propyl alcohol followed by distillation to remove the excess alcohol (Figure 8.6). PG is available as a white crystalline powder and is sparingly soluble in water; it functions particularly well in stabilizing animal fats and vegetable oils. With a melting point of 148°C, PG loses its effectiveness during heat processing and is therefore not suitable in frying applications that involve temperatures exceeding 190°C. PG chelates iron ions and forms an unappealing blue-black complex. Hence, PG is always used with chelators such as citric acid to eliminate the pro-oxidative iron and copper catalysts. Good synergism is obtained with BHA and BHT; however, their coapplication with TBHQ is not permitted (Buck, 1984).

PG may be used to inhibit the oxidation of vegetable oils, animal fats and meat products, including fresh and frozen sausages and snacks. Its usage has been permitted in chewing gum base at <0.1% and with BHA and/or BHT at a total concentration of

<0.1%. Moreover, the amphiphilic nature of PG makes it a very effective antioxidant for dry vegetable oils. Gallates have lower volatility and thus

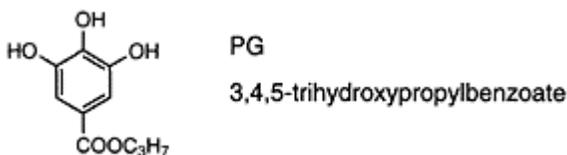


FIGURE 8.6 Chemical structure of propyl gallate (PG).

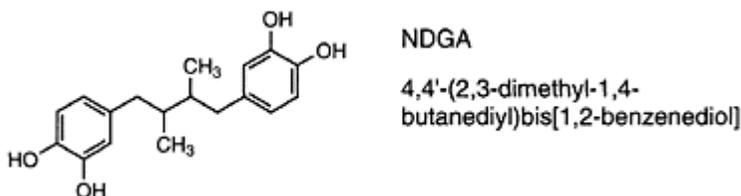


FIGURE 8.7 Chemical structure of nordihydroguaiaretic acid (NDGA).

have less phenolic odor than monohydric phenols such as BHA and BHT (Dziezak, 1986).

Nordihydroguaiaretic acid (NDGA) is a grayish-white crystalline compound that was widely used as an antioxidant in animal fats in the 1950s and 1960s (Figure 8.7). It possesses phenolic properties similar to gallates, including their advantages and disadvantages. Besides the isolation of natural material (resinous exudate of creosote bush), NDGA has also been chemically synthesized. Due to unfavorable toxicological findings, NDGA is no longer of practical importance in the food industry (Gordon, 1990; Schuler, 1990).

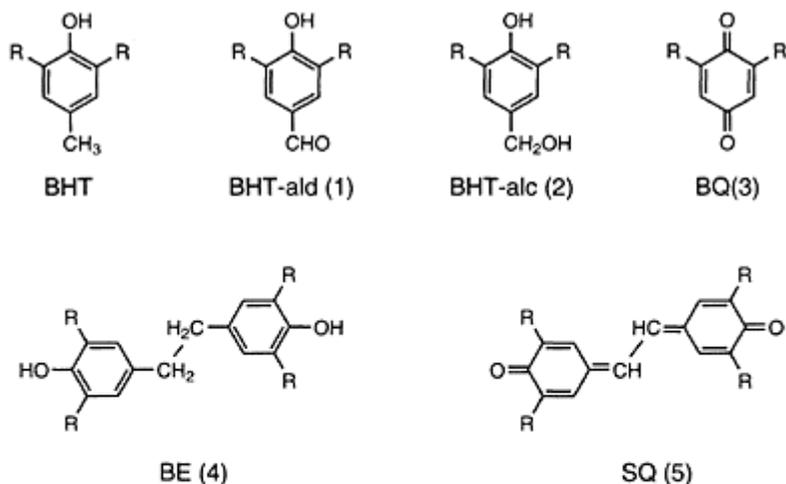
DEGRADATION PRODUCTS OF PHENOLIC ANTIOXIDANTS

Phenolic antioxidants generally undergo degradation during the course of oxidation of fats and oils and produce a range of products, especially dimers of the antioxidants. Most oxidation products of antioxidants retain some antioxidant activity. Among the breakdown products of BHT shown in Figure 8.8, products (2) through (4) possess antioxidant properties while products (1) and (5) are not effective as antioxidants. The degradation products of BHA shown in Figure 8.9 are less effective than BHA itself, in the order of BHA >(8)>(7), as reported by Kikugawa et al. (1990).

The mechanism and breakdown products of TBHQ due to irradiation have been reviewed by Kikugawa et al. (1990). All the oxidation products of TBHQ retain some antioxidant effect, but compounds (10) and (12) exhibit antioxidant activities greater than

that of TBHQ (Figure 8.10). As shown in Figure 8.11, irradiation of propyl gallate in ethanol produces ellagic acid with excellent antioxidant activity (Kikugawa et al., 1990).

Degradation of mixtures of BHA, BHT and PG produced heterodimers between different antioxidant components (Figure 8.12). Although product (15) was new, the heterodimer (16) and heterodimer (17) exhibited activities comparable to that of PG (Kikugawa et al., 1990).



R = tertiary-butyl

(1) 3,5-ditertiary-butyl-4-hydroxybenzaldehyde

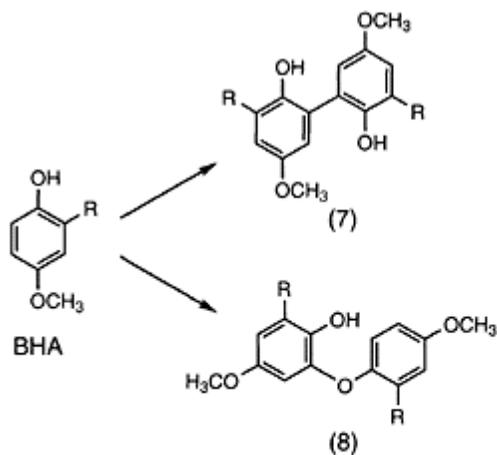
(2) 3,5-ditertiary-butyl-4-hydroxybenzylalcohol

(3) 2,6-ditertiary-butyl-benzoquinone

(4) 3,5,3',5'-tetra-tertiary-butyl-4,4'-dihydroxy-1,2-diphenylethane

(5) 3,5,3',5'-tetra-tertiary-butyl-stilbenequinone

FIGURE 8.8 Degradation products of BHT. (Adapted from Kikugawa, K. et al., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier, London, 65–98.)

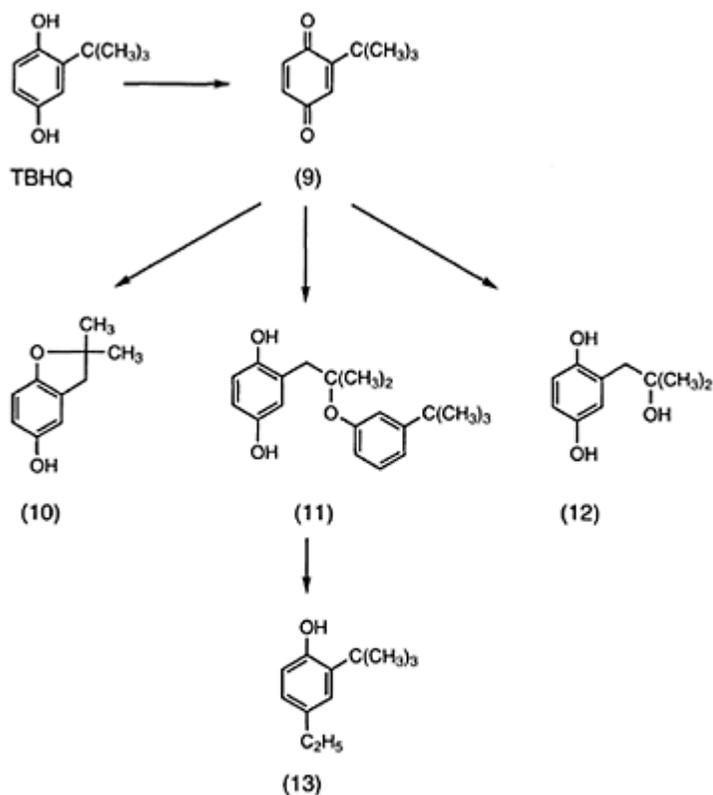


R = tertiary butyl

(7) 2,2'-dihydroxy-5,5'-dimethoxy-3,3'-di-tert-butyl biphenyl

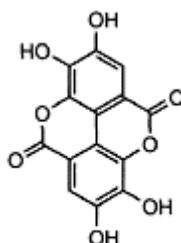
(8) 2',3-ditertiary-butyl-2-hydroxy-4',5-dimethoxy biphenyl ether

FIGURE 8.9 Degradation products of BHA. (Adapted from Kikugawa, K. et al., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier, London, 65–98.)



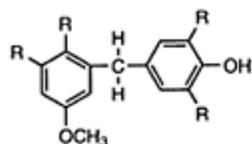
- (9) 2-tertiary-butyl- *p*-benzoquinone
 (10) 2,2'-dimethyl-5-hydroxy-2,3-dihydrobenzo[6]furan
 (11) 2-[2-(3'-tertiary-butyl-4'-hydroxy-phenoxy)-2-methyl-1-propyl]hydroquinone
 (12) 2-(2-hydroxy-2-methyl-1-propyl)hydroquinone
 (13) 2-tertiary-butyl-4-ethoxyphenol

FIGURE 8.10 Degradation products of TBHQ. (Adapted from Kikugawa, K. et al., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier, London, 65–98.)



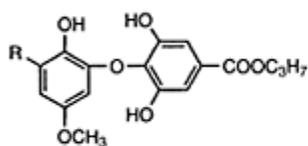
(14) ellagic acid

FIGURE 8.11 Degradation of PG and production of ellagic acid. (Adapted from Kikugawa, K. et al., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier, London, 65–98.)



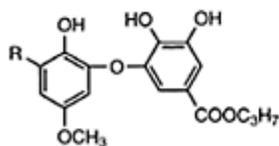
(15)

3,3',5'-tri-tertiary-butyl-5-methoxy-2,4'-dihydroxy diphenylmethane



(16)

propyl-3,5-dihydroxy-4-(2'-hydroxy-5-methoxy-3'-tertiary-butyl phenoxy) benzoate



(17)

propyl-3,4-dihydroxy-5-(2'-hydroxy-5'-methoxy-3'-tertiary-butyl phenoxy) benzoate

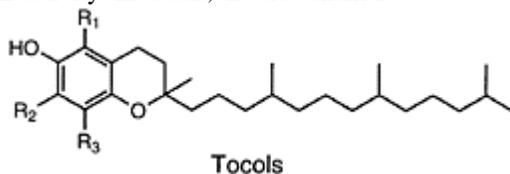
FIGURE 8.12 Degradation products of a mixture of BHA and PG. (Adapted from Kikugawa, K. et al., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier, London, 65–98.)

NATURAL ANTIOXIDANTS

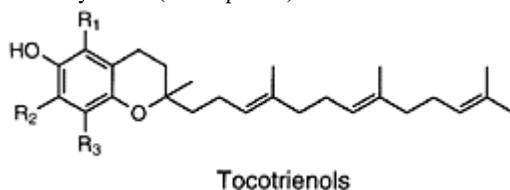
Antioxidants in foods may originate from compounds that occur naturally in the foodstuff or from substances formed during its processing (Shahidi, 1997; Shahidi and Wanasundara, 1992). Natural antioxidants are primarily plant-phenolic and polyphenolic compounds that may occur in all parts of the plant. Plant phenolics are multifunctional and can act as reducing agents (free radical terminators), metal chelators, and singlet oxygen quenchers. Examples of common plant phenolic antioxidants include flavonoid compounds, cinnamic acid derivatives, coumarins, tocopherols and polyfunctional organic acids (Löfliger, 1983; Pratt and Hudson, 1990). Several studies have been carried out in order to identify natural phenolics that possess antioxidant activity. Some natural antioxidants have already been extracted from plant sources and are produced commercially (Schuler, 1990).

TOCOPHEROLS AND THEIR ANTIOXIDANT ACTIVITY

Monophenolic antioxidants that help to stabilize most vegetable oils, tocopherols occur widely in nature. Tocopherols are composed of eight different compounds belonging to two families, namely, tocots and tocotrienols, referred to as α , β , γ , or δ , depending on the number and position of methyl groups attached to the chromane rings. Tocopherols also possess vitamin E activity. In tocots, the side chain is



| Compound | R ₁ | R ₂ | R ₃ |
|---|-----------------|-----------------|-----------------|
| 5,7,8-Trimethyl tocol (α -tocopherol) | CH ₃ | CH ₃ | CH ₃ |
| 7,8-Dimethyl tocol (β -tocopherol) | CH ₃ | H | CH ₃ |
| 5,8-Dimethyl tocol (γ -tocopherol) | H | CH ₃ | CH ₃ |
| 8-Methyl tocol (δ -tocopherol) | H | H | CH ₃ |



| Compound | R ₁ | R ₂ | R ₃ |
|--|-----------------|-----------------|-----------------|
| 5,7,8-Trimethyl tocotrienol (α -tocotrienol) | CH ₃ | CH ₃ | CH ₃ |
| 7,8-Dimethyl tocotrienol (β -tocotrienol) | CH ₃ | H | CH ₃ |
| 5,8-Dimethyl tocotrienol (γ -tocotrienol) | H | CH ₃ | CH ₃ |
| 8-Methyl tocotrienol (δ -tocotrienol) | H | H | CH ₃ |

FIGURE 8.13 Chemical structures of vitamin E and related compounds.

saturated, while in tocotrienols it is unsaturated (Figure 8.13). With regard to vitamin E activity, α -tocopherol is the most potent member of this family. The antioxidant activity decreases from δ to α (Dziezak, 1986).

Vegetable foods contain considerable amounts of different tocopherols and tocotrienols in their lipid fraction (Table 8.3). Cereals and cereal products, oilseeds, nuts and vegetables are rich sources of tocopherols (Table 8.4); however, in the animal kingdom, tocopherols are only found in trace quantities. During manufacturing of oils, 30 to 40% of tocols and tocotrienols are lost (Dziezak, 1986; Schuler, 1990).

Tocopherols are important biological antioxidants. Alpha-tocopherol, or vitamin E, prevents oxidation of body lipids including polyunsaturated fatty acids and lipid components of cells and organelle membranes. Tocopherols are produced commercially and used as food antioxidants. The antioxidant activity of tocopherol is based mainly on the tocopherol-tocopheryl quinone redox system (Figure 8.14).

TABLE 8.3
Approximate Content of Tocopherol and Tocotrienol Found in Vegetable Oils^a

| Oil | Tocopherols | | | | Tocotrienols | | | |
|-----------------|-------------|---------|----------|----------|--------------|---------|----------|----------|
| | α | β | γ | δ | α | β | γ | δ |
| Coconut | 5–10 | – | 5 | 5 | 5 | Trace | 1–20 | – |
| Cottonseed | 40–560 | – | 270–410 | 0 | – | – | – | – |
| Maize, grain | 60–260 | 0 | 400–900 | 1–50 | – | 0 | 0–240 | 0 |
| Maize, germ | 300–130 | 1–20 | 450–790 | 5–60 | – | – | – | – |
| Olive | 1–240 | 0 | 0 | 0 | – | – | – | – |
| Palm | 180–260 | Trace | 320 | 70 | 120–150 | 20–40 | 260–300 | 70 |
| Peanut | 80–330 | – | 130–590 | 10–20 | – | – | – | – |
| Rapeseed/canola | 180–280 | – | 380 | 10–20 | – | – | – | – |
| Safflower | 340–450 | – | 70–190 | 230–240 | – | – | – | – |
| Soybean | 30–120 | 0–20 | 250–930 | 50–450 | 0 | 0 | 0 | – |
| Sunflower | 350–700 | – | 10–50 | 1–10 | – | – | – | – |
| Walnut | 560 | 20–40 | 590 | 450 | – | – | – | – |
| Wheat germ | 560–1200 | 660–810 | 260 | 270 | 20–90 | 80–190 | – | – |

^a Milligrams per kilogram.

Source: Adapted from Schuler, P., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier, London, 99–170.

TABLE 8.4
Tocopherol Content of Cereal Grains

| Product | Tocopherols (mg/100 g) | | | α -Tocotrienol |
|-------------------|------------------------|---------|----------|-----------------------|
| | α | β | γ | |
| Whole yellow corn | 1.5 | – | 5.1 | 0.5 |
| Yellow corn meal | 0.4 | – | 0.9 | – |
| Whole wheat | 0.9 | 2.1 | – | 0.1 |
| Wheat flour | 0.1 | 1.2 | – | – |

| | | | | |
|-------------|-----|---|------|-----|
| Whole oats | 1.5 | – | 0.05 | 0.3 |
| Oat meal | 1.3 | – | 0.2 | 0.5 |
| Whole rice | 0.4 | – | 0.4 | – |
| Milled rice | 0.1 | – | 0.3 | – |

Source: Adapted from Herting, D.C. and Durry, E.J.E., 1969, *J. Agric. Food Chem.*, 17, 785–790.

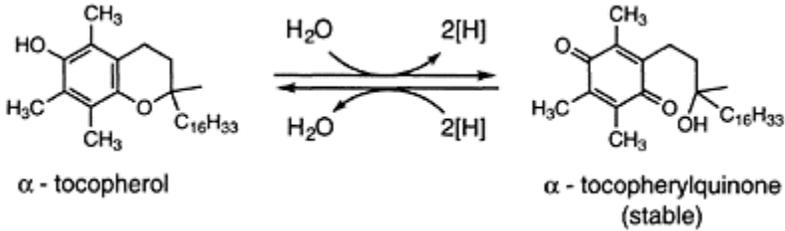


FIGURE 8.14 Alpha-tocopherol/alpha-tocopheryl quinone redox system. (Adapted from Schuler, P., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier, London, 99–170.)

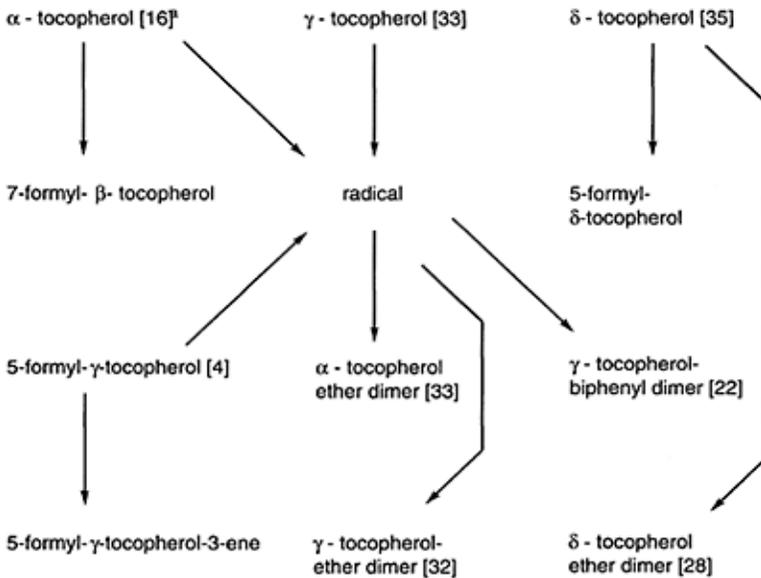


FIGURE 8.15 Antioxidant activity of selected tocopherols and their decomposition products. Length of

induction period is given in days in brackets. Control without antioxidants had an induction period of 2 days.

(Adapted from Ishikawa, Y. and Yuki, E., 1975, *Agric. Biol. Chem.*, 39, 851–857.)

Tocopherols (AH₂) are radical scavengers and quench lipid radicals (R[•]), thus regenerating RH molecules as well as producing a tocopheryl semiquinone radical. Two tocopheryl semiquinone radicals (AH[•]; Figure 8.15) may form a molecule of tocopheryl quinone (A) and a regenerated molecule of tocopherol, as can be seen in Reaction 8.20 and Reaction 8.21:



The mechanism of oxidation of α -tocopherol with linoleate hydroperoxides has been studied in detail (Tappel, 1972). After releasing one H atom, the α -tocopheryl radical formed releases another H atom to produce methyl tocopheryl quinone, which is unstable and gives rise to α -tocopheryl quinone as its main product. The reaction between two semiquinoid radicals may also lead to the formation of α -tocopherol dimer, which possesses antioxidant properties (Schuler, 1990). Ishikawa and Yuki (1975) described the antioxidant effect of the products of tocopherols as oxidized α -, γ -, and δ -tocopherol with trimethylamine oxide. Some of the oxidation products formed were isolated and tested for their antioxidant activity; the results are summarized in Figure 8.15.

Tocopherols are commercially extracted from deodorizer sludge obtained in the deodorization of vegetable oils. Various tocopherols and tocotrienols of such extracts contain sterols, esters of sterols, free fatty acids and triacylglycerols. The separation of tocopherols from other compounds is possible by esterification with lower alcohol, washing and vacuum distillation or by saponification or fractional liquid-liquid extraction. Further purification may be achieved using molecular distillation, extraction, crystallization or a combination of these processes (Schuler, 1990). The total tocopherol content of the extracts is usually between 30 and 80%, but higher in γ - and δ -tocopherols.

To obtain stable α -tocopheryl acetate, the mixture is methylated and subsequently acetylated. The α -tocopheryl acetate is the commercially available form of vitamin E, which is not an antioxidant because its active OH group is blocked. However, under acidic aqueous conditions, tocopherol is released by hydrolysis and the released tocopherol may then act as an antioxidant (Schuler, 1990).

The commercial synthesis of α -tocopherol involves condensation of 2,3,5-trimethyl hydroquinone with phytol, isophytol or phytol halogenides. The crude tocopherol product is purified by vacuum distillation. Because use of isophytol is preferred, the distillation produces a racemic mixture of all possible tocopherol isomers. Products intended for antioxidant applications are generally marketed in oil forms. Pure, all-racemic α -tocopherol, mixed tocopherols having various contents of α -, γ - and/or δ -tocopherols;

synergistic mixtures composed of tocopherols, ascorbyl palmitate or other antioxidants, and synergists such as lecithin, citric acid and carriers are also available (Schuler, 1990).

PHENOLIC ACIDS, FLAVONOIDS AND THEIR ANTIOXIDANT ACTIVITY

Flavonoids usually occur in living cells as glycosides and may break down to their respective aglycone and sugar by enzymes or acid-heat treatments. Pratt and Watts (1964) and Pratt (1965, 1972) have considered flavonoids as primary antioxidants. Many of the flavonoid and related phenolic acids have shown marked antioxidant characteristics (see Table 8.5 through Table 8.7) (Mehta and Seshadri, 1959). Structures of these flavonoid and related compounds are given in Chapter 1.

Flavonoids and cinnamic acids are known as primary antioxidants and act as free radical acceptors and chain breakers. Flavonols are known to chelate metal ions at the 3-hydroxy-4-keto group and/or 5-hydroxy-4-keto group (when the A-ring is hydroxylated at the fifth position). An *o*-quinol group at the B-ring can also demonstrate metal chelating activity (Pratt and Hudson, 1990).

TABLE 8.5
Antioxidant Activity of Flavones

| Compound | Time to Reach Peroxide Value of 50 (h) ^a | Induction Period by Rancimat (h) ^b |
|---|---|---|
| Control | | |
| Stripped corn oil | 105 | — |
| Lard | — | 1.4 |
| Aglycones | | |
| Quercetin (3,5,7,3'-4'-pentahydroxy) | 475 | 7.1 |
| Fisetin (3,7,3',4'-tetrahydroxy) | 450 | 8.5 |
| Lueteolin (5,7,3',4'-tetrahydroxy) | — | 4.3 |
| Myricetin (3,5,7,3',4',5'-hexahydroxy) | 552 | — |
| Robinetin (3,7,3',4',5'-pentahydroxy) | 750 | — |
| Rhamnnetin (3,5,3',4'-tetrahydroxy-7-methoxy) | 375 | — |
| Glycosides | | |
| Quercetrin (3-rhamnoside) | 475 | 1.9 |
| Rutin (3-rhamnoglucoside) | 195 | — |

^a 5×10^{-4} M in stripped corn oil.

^b 2.3×10^{-4} M in lard.

Source: Adapted from Pratt, D.E. and Hudson, B.J.F., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier Applied Science, London, 171–192.

It has been established that the position and degree of hydroxylation are of primary importance in determining antioxidant activity of flavonoids. The *o*-dihydroxylation of

the B-ring contributes to the antioxidant activity. The *p*-quinol structure of the B-ring has been shown to impart an even greater activity than *o*-quinol; however, para and meta hydroxylation of the B-ring do not occur naturally (Pratt and Hudson, 1990). All flavonoids with 3',4'-dihydroxy configuration possess antioxidant activity (Dziedzic and Hudson, 1983b).

Robinetin and myricetin have an additional hydroxy group at their 5' position, thus leading to enhanced antioxidant activities over those of their corresponding flavones that do not possess the 5' hydroxy group, namely, fisetin and quercetin (Figure 1.9 and Table 8.5). Two flavanones (naringenin and hesperitin) have only one hydroxy group on the B-ring and possess little antioxidant activity (Figure 1.9 and Table 8.5). Hydroxylation of the B-ring is the major consideration for antioxidant activity (Pratt and Hudson, 1990). Other important features include a carbonyl group at position 4 and a free hydroxy group at position 3 and/or 5 (Dziedzic and Hudson, 1983b).

Uri (1961) has investigated the importance of other sites of hydroxylation. It has been shown that the *o*-dihydroxy grouping on one ring and *p*-dihydroxy grouping on the other (e.g., 3,5,8,3',4'- and 3,7,8,2',5'-pentahydroxy flavones) produce very potent antioxidants, while 5,7 hydroxylation of the A-ring apparently has little

TABLE 8.6
Antioxidant Activity of Flavanones

| Compound | Time to Reach Peroxide Value of 50 (h) ^a | Induction Period by Rancimat (h) ^b |
|--|---|---|
| Control | | |
| Stripped corn oil | 105 | – |
| Lard | – | 1.4 |
| Aglycones | | |
| Taxifolin (dihydroquercetin) | 470 | 8.2 |
| (3,5,7,3',4'-pentahydroxy) | – | 6.7 |
| Fustin (3,7,3',4'-tetrahydroxy) | – | 6.7 |
| Eriodictyol (3,7,3',4'-tetrahydroxy) | – | – |
| Naringenin (5,7,4'-tetrahydroxy) | 198 | – |
| Hesperitin (5,7,3'-trihydroxy-4-methoxy) | 125 | – |
| Glycosides | | |
| Hesperidin (7-rhamnoglucoside) | 125 | – |
| Neohesperidin (7-glucoside) | 135 | – |

^a 5×10^{-4} M in stripped corn oil.

^b 2.3×10^{-4} M in lard.

Source: Adapted from Pratt D.E. and Hudson, B.J.F., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier Applied Science, London, 171–192. With permission.

influence on the antioxidant activity of the compounds (Pratt and Hudson, 1990). Thus, quercetin and fisetin have almost the same activity, while myricetin possesses an activity similar to that of robinetin (Figure 1.9 and Table 8.5). The 3-glycosylation of flavonoids

with monosaccharides/disaccharides reduces their activity compared with that of the corresponding aglycones (e.g., rutin is less active than quercetin; Figure 1.9).

The ability of flavonoids to form complexes with cupric ion (Figure 8.16) has also been demonstrated by UV spectral studies. Such complexations may contribute to the antioxidative action of flavonoids (Hudson and Lewis, 1983). Chelation of metal ions renders them catalytically inactive.

Chalcones, the natural precursors of flavones and flavanones, are readily cyclized under acidic conditions (see Chapter 1) and have been shown to possess potent antioxidant activity. The 3,4-dihydroxychalcones are particularly effective and chalcones are more effective than their corresponding flavanones. Effectiveness of 3,4-dihydroxychalcones, namely, butein and okanin (Figure 1.9 and Table 8.7), depends on the formation of resonance-stabilized free radicals (Dziedzic and Hudson, 1983b) (Figure 8.17).

In the isoflavone, it is clear that both hydroxyl groups in 4' and 5 positions are needed for significant antioxidant activity as genistein (Figure 8.18). Even 6,7,4'-trihydroxyisoflavone is marginally active when compared to analogous flavone

TABLE 8.7
Antioxidant Activity of Some Flavonoid-Related Compounds

| Compound | Time to Reach Peroxide Value of 50 (h)^a | Induction Period by Rancimat (h)^b | |
|--|---|---|----------|
| | | A | B |
| Control | | | |
| Stripped corn oil | 110 | A | B |
| Lard | – | 1.3 | 0.35 |
| Isoflavones | | | |
| Daidzein (7,4'-dihydroxy) | – | 1.4 | – |
| Genistein (5,7,4'-trihydroxy) | – | 2.6 | – |
| Chalcones | | | |
| Butein (2',4',3,4-tetrahydroxy) | – | 94.0 | – |
| Okanin (2',3',4',3,4-pentahydroxy) | – | 97.0 | – |
| Phenolic acids | | | |
| Protocatechuic acid (3,4-dihydroxybenzoic acid) | – | – | 4.8 |
| Gallic acid (3,4,5-trihydroxybenzoic acid) | – | – | 28.6 |
| Coumaric acid (<i>p</i> -hydroxycinnamic acid) | – | 120 | 0.8 |
| Ferulic acid (4-hydroxy-3-methoxycinnamic acid) | – | 145 | 2.0 |
| Caffeic acid (3,4-dihydroxycinnamic acid) | – | 495 | 23.3 |
| Dihydrocaffeic acid (3,4-dihydroxyphenyl-propionic acid) | – | – | 31.4 |
| Chlorogenic acid (caffeoyl quinic ester) | – | 505 | – |

| | | | |
|-----------------------------------|---|-----|------|
| Quinic acid | – | 105 | – |
| Phenolic ester | | | |
| Propyl gallate | – | 435 | 21.8 |
| Miscellaneous | | | |
| D-Catechin | – | 410 | – |
| Hesperidin methychalcone | – | 135 | – |
| Asculetin (6,7-dihydroxycoumarin) | – | – | 15.5 |

Note: A and B denote different batches of oil.

^a 5×10^{-4} M in stripped corn oil.

^b 2.3×10^{-4} M in lard.

Source: Adapted from Pratt D.E. and Hudson, B.J.F., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier Applied Science, London, 171–192.

apigenin, which is inactive as an antioxidant (Figure 1.9). Genistein is particularly active. The resonance-stabilized quinoid structures show that for isoflavone the carbonyl group at position four remains intact and can interact with the 5-hydroxy group, if present; however, in flavone, the carbonyl group at position four loses its functionality. This may explain the superior antioxidant activity of genistein compared with that of apigenin (Dziedzic and Hudson, 1983a).

Naturally occurring isoflavones are found mainly in the Leguminosae family and are much less widespread than flavones. Pronounced synergism was evidenced

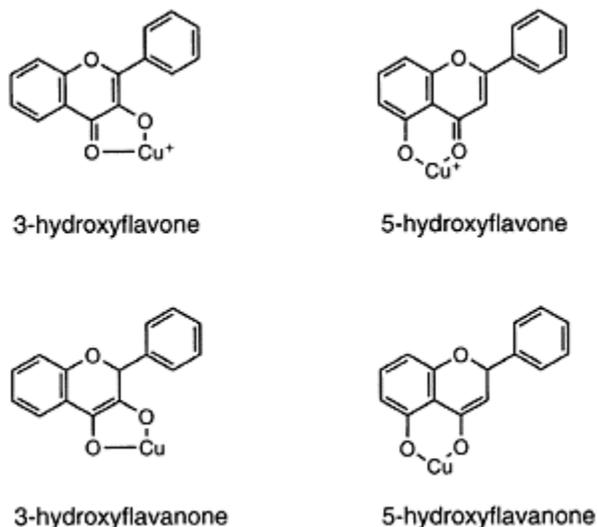


FIGURE 8.16 Possible copper complexes of flavones and flavonones. (Adapted from Hudson, B.J.F. and Lewis, I.F., 1983, *Food Chem.*, 10, 47–55.)

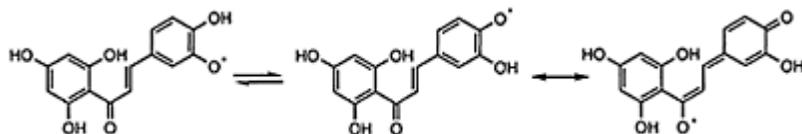
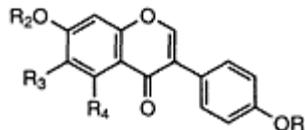


FIGURE 8.17 Resonance-stabilized free radicals of 3',4'-dihydroxychalcones. (Adapted from Dziedzic, S.Z. and Hudson, B.J.F., 1983, *Food Chem.*, 12, 205–212.)



| Compound | R ₁ | R ₂ | R ₃ | R ₄ |
|-------------------------------|-----------------|-----------------|------------------|----------------|
| Daidzein | H | H | H | H |
| Formononetin | CH ₃ | H | H | H |
| Genestein | H | H | H | OH |
| Prunetin | H | CH ₃ | H | OH |
| 4',6',7'-trihydroxyisoflavone | H | H | OH | H |
| glycitein (7-O-glucoside) | H | Gluc | OCH ₃ | H |

FIGURE 8.18 Chemical structures of isoflavone and isoflavone glycosides of soybean.

when isoflavones were supplemented with phospholipids such as phosphatidyl ethanolamine (Dziedzic and Hudson, 1983a).

The antioxidant activity of phenolic acids and their esters depends on the number of hydroxy groups in the molecule; this will be strengthened by steric hindrance (Dziedzic and Hudson, 1983b). Hydroxylated cinnamic acids have been found to be more effective than their benzoic acid counterparts. It has been mentioned that at least two and, even better, three neighboring phenolic hydroxy groups (catechol or pyrogallol structure) and a carbonyl group in the form of an aromatic ester or lactone or a chalcone, flavanone or flavone are the essential molecular features required to achieve a high level of antioxidant activity (Dziedzic and Hudson, 1983b).

NATURAL SOURCES OF PLANT ANTIOXIDANTS

Although plant-based antioxidants are found in different plant parts, including fruits, seeds and leaves among others, this chapter provides examples of selected oilseeds, teas, several herbs and spices, and wood smoke only.

SOYBEAN

Soybean (*Glycine max* L.) flour has shown antioxidant property in various food products (Table 8.8). In soybean oil, the active antioxidant is tocopherol (mainly α -tocopherol)

and, to a lesser extent, δ -tocopherol (Schuler, 1990). Soybean flour and other soybean derivatives are sources of a large variety of antioxidant compounds (Table 8.9) belonging to the family of isoflavone glycosides and their derivatives, phospholipids, tocopherols, amino acids and peptides (Gyorgy et al., 1964; Naim et al., 1973, 1974; Herrmann, 1976; Hayes et al., 1977; Murakani et al., 1984). Isolated isoflavone glycosides from soy flour are genistein, daidzein and glycitein; 7,4'-dihydroxy-6-methylisoflavone (Figure 8.18; Rackis, 1972; Naim et al., 1973).

It has been reported that 99% of the isoflavones are present as glycosides, of which 64% are genistein, 23% daidzein and 13% glycitein 7-*O*- β -glycoside (Naim et al., 1973). Murakani et al. (1984) have shown that, in tempeh (a fermented soybean product), the isoflavones are liberated from glycosides by acids formed during the fermentation. The main antioxidants in tempeh were determined to be daidzein and genistein; glycitein had only a slight antioxidant activity. The reported isoflavone and isoflavone glycosides in soybean meal, soy protein products and soy isoflavones are summarized in Table 8.8 through Table 8.10, respectively. The antioxidant activity of aglycones is superior compared to the parent isoflavone glycoside (Hayes et al., 1977). As discussed earlier, the antioxidant activity is not as pronounced as that of their corresponding flavones possessing dihydroxy substitutes in the A- or the B-ring.

Phenolic acids, namely, syringic, vanillic, caffeic, ferulic, *p*-coumaric and *p*-hydroxybenzoic acids, possessing antioxidative activity have also been found in soybean and soybean products (Arai et al., 1966; Hammerschmidt and Pratt, 1978; Pratt and Birac, 1979). Among polyphenolic antioxidants extracted by methanol or water from dried and fresh soybean, chlorogenic, isochlorogenic, caffeic and ferulic acids were identified in addition to isoflavones (Pratt and Birac, 1979). The antioxidant activity of soy isoflavones in a β -carotene/linoleate model system in the increasing order was glycitein, daidzein, genistein, quercetin and 6,7,4'-trihydroxy isoflavones, respectively. Meanwhile, for the phenolic acids, the increasing order of antioxidant activity was *p*-coumaric, ferulic, chlorogenic and caffeic acids, respectively.

TABLE 8.8
Effective Antioxidant Levels of Soybean in Various Products

| Product | Effective level (% of flour) | Comment |
|------------------------------------|------------------------------|---|
| Lard | 5–10 | – |
| Premier jus | 2–6 (unspecified type) | Original condition of fat strongly influenced soy effectiveness |
| Ghee (buffalo butter) | 0.5–1.0 (full-fat) | About equally effective |
| Frozen pastry | 5–20 (full-fat) | All concentrations were about equally effective |
| Raw pastry mixes and baked pastry | 10 (low-fat) | 50°C storage |
| Ration biscuits | 4–20 (defatted) | Progressively lower peroxide values with increasing cone. |
| Dehydrated pork-corn meal scrapple | 2.8 (full-fat) | – |

| | | |
|--|--|---|
| Frozen, raw ground pork and frozen, precooked ground pork | 2.5–7.5 (full-fat) | No significant difference in peroxide values with cone. |
| Degermed, uncooked corn meal-soy flour blend | 15, 20 (toasted, defatted) or (commercial-process full-fat) or (extrusion-cooked full-fat) | No flavor difference between 15 and 20% products |
| Degermed, uncooked corn meal-soy flour blend plus ferrous sulfate | 15–25 (toasted, defatted) | No rancidity after storage |
| Instant, fully cooked corn meal-soy flour-milk blend (plus 5% soybean oil) | 27.5 (toasted, defatted) | Low peroxide values after storage |

Source: Adapted from Pratt D.E. and Hudson, B.J.F., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier Applied Science, London, 171–192.

PEANUT AND COTTONSEED

The methanolic extracts of protein ingredients of peanut (*Arachis hypogea*, variety: Spanish) and glandless cottonseed (*Gossypium hirsutum*, variety: McNair) have demonstrated antioxidant activity in lipid peroxidation model systems catalyzed by metmyoglobin and Fe²⁺-EDTA, and also in fresh beef homogenate (Rhee et al., 1979). The same protein ingredients in beef patties (replaced beef up to 10%) were able to retard rancidity development when cooked and refrigerated (Ziprin et al., 1981). Cottonseed protein ingredients have been shown to have a higher activity in retarding development of rancidity than peanut meals.

Pratt and Miller (1984) have identified dihydroquercetin taxifolin as an antioxidant in hot methanolic extracts of peanut (variety: Spanish; Figure 1.9). Quercetin and rutin are identified as the major flavonoids in the methanolic extracts of delinted

TABLE 8.9

Probable Principal Antioxidants Compounds of Soybean Products and Derivatives

| Derivative | Antioxidant Component |
|---------------------------------|--|
| Aqueous extract | Isoflavone glycosides, their aglycones, phenolic acids |
| Organic solvent extract | Flavonoids, tocopherols, phospholipids |
| Soy protein concentrate/isolate | Isoflavone glycosides, their aglycones |
| Protein hydrolysate | Amino acids and peptides |
| Textured vegetable proteins | Phospholipids |

Sources: Adapted from Pratt, D.E., 1972, *J. Food Sci.*, 37, 322–323; Hammerschmidt, P.A. and Pratt, D.E., 1978, *J. Food Sci.*, 43, 556–559; Hayes, R.E. et al., 1977, *J. Food Sci.*, 42, 1527–1532; Pratt, D.E. and Birac, P., 1979, *J. Food Sci.*, 44, 1720–1722; Pratt, D.E. et al., 1981; Murakani, H. et al., 1984, *Agric. Biol. Chem.*, 48, 2971–2975.

TABLE 8.10
Isoflavone and Isoflavone Glycosides Content of Soybean Meal^a

| Component | Full-Fat Flakes (fat-free basis) | Defatted Flakes |
|--------------------------------|---|------------------------|
| Daidzin | 118.5 | 114.5 |
| Glycitin-7- β -glucoside | 0.9 | 0.8 |
| Genistin | 204.1 | 188.5 |
| Daidzein | 2.0 | 2.5 |
| Glycitein | 1.0 | 1.2 |
| Genistin | 4.4 | 4.4 |
| Total | 330.9 | 311.4 |

^a Milligrams per 100 grams.

Source: Adapted from Hudson, B.J.F. and Lewis, J.I., 1983, *J. Food Chem.*, 10, 47–55.

cottonseed (Whiltern et al., 1984). Both possess potent antioxidant activity; however, rutin is comparatively inferior (Yousseff and Rahman, 1985).

The antioxidant activity of phenolics of rapeseed and mustard has been reported in model systems of β -carotene/linoleate and muscle foods (Takagi and Iida, 1980; Ramanathan and Das, 1992; Shahidi et al., 1992). Canola and mustard flour, or their extracts, possess strong antioxidative activity. The effectiveness in retarding meat flavor deterioration of an enzyme-deactivated mustard flour, referred to as deheated mustard flour (DMF), at 1 to 2% level of addition is superior to that of BHT at 200 mg/kg. Extracts of DMF, however, are less effective. Furthermore, the activity of these extracts is proportional to their total content of phenolics (Shahidi et al., 1992).

BORAGE, EVENING PRIMROSE, AND BLACKCURRANT

The low molecular weight phenolics of borage (*Borago officinalis* L.) meal have been recently identified (Wettasinghe et al., 2001). Syringic acid, sinapic acid and rosmarinic acid are important phenolics found in defatted borage meal. Following chromatographic separation on Sephadex LH-20, the crude extracts of borage as well as their fractions possess antioxidant activity by quenching free radicals or by chelation (Wettasinghe and Shahidi, 1999a, 2002).

In evening primrose (*Oenothera biennis* L.), Wettasinghe et al. (2002) identified gallic acid as well as (+)-catechin and (–)-epicatechin in the extracts. In addition, a substantial amount of high molecular weight compounds, including oenothin B, are tentatively identified in the extracts of evening primrose. The latter compound is an oligomeric hydrolyzable tannin first reported by Okuda et al. (1993) as present in green parts of the evening primrose plant. Other polymeric phenolics have also been found in evening primrose extracts (Lu and Foo, 1995). The extracts of evening primrose, or their fractions, are antioxidative in nature, as exhibited by their effective quenching of free radicals as well as chelation of metal ions (Wettasinghe and Shahidi, 1999b, 2002).

Blackcurrant (*Ribes nigrum*) seeds provide a good source of an array of polyphenols with potentially excellent antioxidant activity. The polyphenolics present are dominated

by rutinoides and glucosides of delphinidin and cyanidin, as well as 1-cinnamoyl- and 1-*p*-coumaroyl- β -D-glycosides, as shown in Figure 8.19 (Lu and Foo, 2003).

RICE

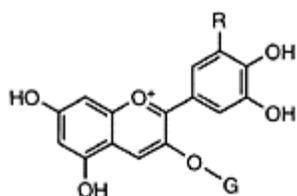
The methanolic extracts of rice hulls (*Oryza saliva* Linn.) from long life (Katakutara) and short life (Kusabue) varieties exhibit superior antioxidant activity compared with α -tocopherol (Ramarathnam et al., 1989). The HPLC separation of methanol-water (50:50, v/v) extracts of both varieties showed that the short-life variety includes compounds similar to α -tocopherol. The long-life variety had a stronger antioxidant activity and contained compounds other than α -tocopherol. The active compound has been characterized as isovitexin, a C-glycosyl flavonoid (Ramarathnam et al., 1989; Figure 8.20).

It has been observed and established that storability and longevity of rice grains is related to the antioxidative activity of the seed coat or husk (Osawa et al., 1985a; Ramarathnam et al., 1986). Presence of isovitexin has confirmed the antioxidative activity of long-life rice (Ramarathnam et al., 1989).

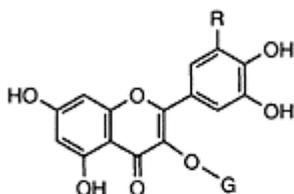
SESAME SEED

The oil from sesame seed (*Sesamum indicum* L.) has a superior oxidative stability compared to other vegetable oils; this special property has been attributed to the presence of sesamol in the seeds (Lyon, 1972). Sesamol is usually present in traces, but may also be released from sesamol by hydrogenation, bleaching earth, or other conditions of processing. Sesame seed contains 0.4 to 1.1% sesamin, 0.3 to 0.65% sesamol and traces of sesamol (Figure 8.21). Sesamol is a free 3,4-methylene-diphenoxy phenol; sesamol is an acetal type derivative of sesamol and sesamin. The 3,4-methylenediphenoxy phenol is attached to the 2,7-dioxabicyclo-(3,3,0)-octane nucleus directly.

Sesamol is as effective as BHT and BHA and more effective than PG (Lyon, 1972). Fukuda et al. (1985) have reported that acetone extract of sesame seed produces a strong antioxidant activity. The active compounds were identified as bisepoxylignan or sesamolol (Osawa et al., 1985b), as given in Figure 8.22. Sesamole is readily oxidized to sesamol dimers and then to sesamol dimer semi-quinone by treatment with H_2O_2 /horseradish peroxidase (Kikugawa et al., 1990).



R = H or OH; G = glucose or rutinose



R = H or OH; G = glucose or rutinose

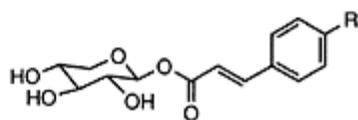
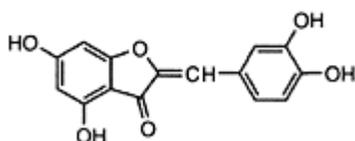
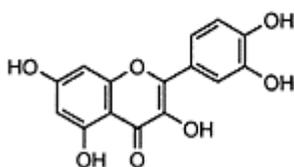
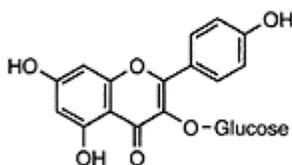
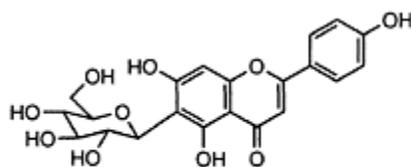
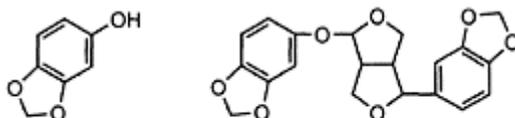


FIGURE 8.19 Chemical structures of polyphenols of blackcurrant seeds.



Isovitexin

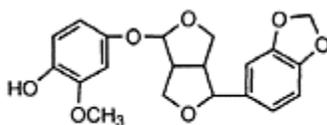
FIGURE 8.20 Chemical structure of isovitexin.



Sesamol

Sesamolin

FIGURE 8.21 Chemical structures of sesamol and sesamolin.



Sesamolinol

FIGURE 8.22 Chemical structures of sesamolinol.

CANARY SEEDS

Canary seeds (*Phalaris canariensis*) are a popular bird feed. Takagi and Iida (1980) have shown that extracts (i.e., successive extraction of seeds with hexane, ether and methanol) of canary seeds exhibit excellent antioxidant activity in lard and sardine oil. The active components are identified by gas chromatography to be esters of caffeic acid as well as cycloartenol, gramisterol, sitosterol and campesterol, and lesser quantities of 24-methylenecycloartanol, obtusifoliol, brassicasterol and Δ , 7-stigmasterol (Takagi and Iida, 1980). These sterols and triterpene alcohol esters of caffeic acid are lipid soluble and have higher melting points. Lipid solubility and lack of bitter taste may enhance the potential use of this source of natural antioxidant.

TEA

Presence of catechin and its derivatives in tea has been well documented. Matsuzaki and Hara (1985) have reported the antioxidative efficiency of isolated catechins from green tea leaves. The extracts included (-)-epicatechin (EC), (-)-epigallocatechin

TABLE 8.11
Antioxidant Activity of Spices in Oils and Fats^a

| Spice/Antioxidant 30 mg/kg | Soybean | Linseed | Olive | Sesame | |
|----------------------------|---------|---------|-------|--------|-----|
| Fennel | | 2.3 | 2.2 | 8.0 | 5.1 |
| Ginger | | 1.9 | 2.6 | 7.8 | 3.8 |
| Capsicum | | 2.0 | 2.6 | 7.3 | 5.4 |
| Clove | | 5.1 | 6.0 | 23.8 | 8.5 |
| Garlic | | 2.3 | 2.2 | 6.6 | 7.0 |
| Turmeric | | 2.0 | 2.1 | 8.5 | 3.3 |
| BHA (20 mg/100 g) | | 1.1 | 1.1 | 2.5 | 1.1 |
| Control | | 9.5 | 3.7 | 16.0 | 8.7 |



Note:

^a Active oxygen method (AOM) h.

Sources: Adapted from Gordon, M.H., 1990, in *Food Antioxidants*, Hudson, B.J.F., Ed., Elsevier, London, 1–18 and Gyorgy, P. et al., 1964., *Nature* (London), 203, 870–872.

(EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG) (Figure 1.7). The activity of catechins in model systems is in the following order:

EC<ECG<EGC<EGCG

At similar molar concentrations, the activity of these compounds is superior to those of BHA and di- α -tocopherol in lard (Matsuzaki and Hara, 1985; Namiki, 1990).

HERBS AND SPICES

Chipault et al. (1952, 1956) investigated antioxidant activity of spices in various fats. In general, alcoholic and ether extracts of spices are less active than native spices (Table 8.11 and Table 8.12). Allspice, clove, sage, oregano, rosemary, and thyme possess antioxidant activity in all types of fats examined. Clove appears to be the most active antioxidant in vegetable oils; however, extracts of rosemary and sage are the most effective (Chipault et al., 1952, 1956). Spice extracts have attracted a lot of interest in recent years because they can be easily added to fats and oils in bulk. Many extracts possess a strong odor and bitter taste and thus are of limited use in many food products. Rosemary and sage provide the most potent antioxidant spice extracts with little odor of the original spice.

Chang et al. (1977) were able to prepare an odorless and flavorless natural antioxidant from rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.). These antioxidants can be successfully extracted into different organic solvents such as benzene,

chloroform, diethyl ether and methanol. The diethyl ether extract of rosemary was purified and evaluated for its antioxidant activity (peroxide value) at 0.02% (w/w) in potato chips, sunflower oil and corn oil. It showed a very low

TABLE 8.12

**Antioxidative Activity of Ground Spices,
Distilled Water Soluble Fraction and Ethanol
Soluble Fraction from Spices in Lard^a**

| Spice | Ground Spice (0.25%) | Distilled Water Soluble Fraction ^b | Ethanol Soluble Fraction ^b |
|-----------------|-------------------------|--|--|
| Control | 5.7 | 5.7, 21.9 | 5.7, 21.9 |
| Allspice | 11.8 | 9.7, 32.8 | 8.7, 15.5 |
| Black pepper | 7.7 | 5.8, 28.9 | 6.4, 24.7 |
| Capsicum | 8.0 | 6.8, 31.7 | 7.0, 21.8 |
| Clove | 19.6 | 13.9, 33.4 | 18.9, 27.5 |
| Ginger | 14.7 | 6.7, 30.8 | 11.7, 24.7 |
| Mace | 24.0 | 6.3, 27.1 | 21.7, 34.6 |
| Nutmeg | 21.7 | 6.1, 27.3 | 18.5, 21.7 |
| Rosemary | 67.0 | 8.2, 29.7 | 58.5, 58.1 |
| Sage | 54.8 | 6.9, 26.6 | 42.1, 42.7 |
| Turmeric | 16.8 | 7.0, 27.7 | 12.4, 24.5 |

^a Active oxygen method (AOM) h.

^b First entry=0.25% ground spice equivalent; second entry=soluble fraction+100 mg/kg of α -tocopherol.

Source: Adapted from Watanabe Y. and Ayano, Y., 1974, *J. Jpn. Soc. Food Nutr.*, 27, 181–188.

peroxide value and provided excellent flavor stability to the products tested (Chang et al., 1977).

The extracts of rosemary leaves contain a phenolic diterpene, namely, carnosol (Houlihan et al., 1984). Furthermore, rosmanol, another phenolic diterpene that has a closely related structure to carnosol, and rosmaridiphenol have also been identified in rosemary leaves (Figure 8.23). Rosmaridiphenol (at 0.02%) has shown antioxidant activity similar to BHT at the same level in prime steam lard. Carnosic acid and rosmaric acid are the most active antioxidant constituents of rosemary and rosmaric acid possesses an activity comparable to that of caffeic acid (Schuler, 1990). In animal fats, carnosic acid has been described as the most active antioxidative constituent of rosemary (Schuler, 1990). Commercial antioxidant extracts (molecular or vacuum distilled) from rosemary are available as a fine powder. Depending on their content of active antioxidants, they are recommended for use at concentrations ranging between 200 and 1000 mg/kg of the processed product. However, pure carnosic acid is not available as a food antioxidant (Schuler, 1990). It has also been observed that ascorbic acid (500 mg/kg of ascorbic acid+200 mg/kg of rosemary extract) enhances the antioxidant activity of rosemary extract in lard (Chang et al., 1977).

The extracts of dried leaves of oregano (*Origanum vulgare* L.) in dichloromethane and methanol have been isolated as reported for rosemary. The main antioxidative compound of oregano is identified as a phenolic glycoside (Nakatani

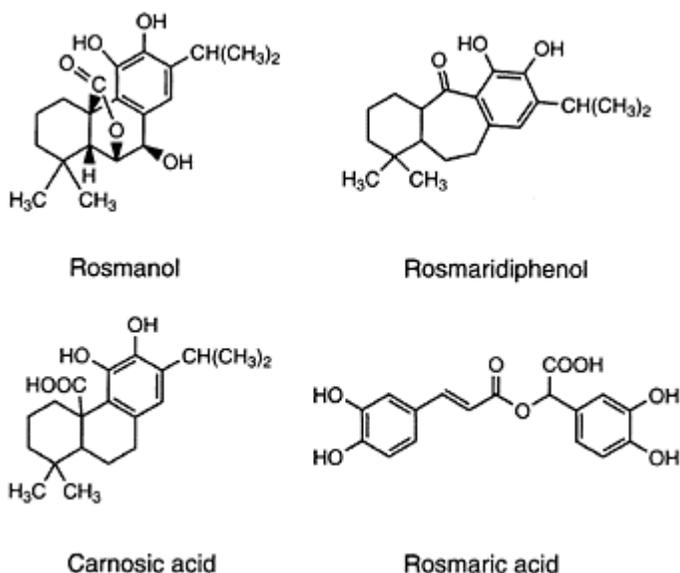
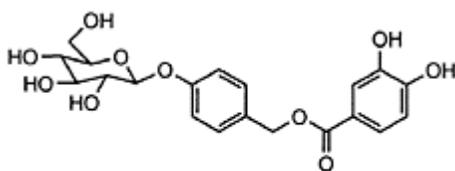


FIGURE 8.23 Chemical structures of selected antioxidant compounds in rosemary.



Antioxidative glycoside from Oregano

FIGURE 8.24 Chemical structure of an antioxidative compound in oregano.

and Kikuzaki, 1987). Further investigations have suggested that the compound is 2-caffeoyloxy-3-[2-(4-hydroxybenzoyl)-4,5-dihydroxy]phenyl propionic acid (Kikuzaki and Nakatani, 1989; Figure 8.24).

The strong antioxidant activity of Papua mace (*Myristica argented*) is related to the presence of 2-allylphenols and a number of lignans (Nakatani and Ikeda, 1984). Capsicin, a pungent antioxidant component of *Capsicum frutescens*, and a new strong antioxidant (Figure 8.25) with no pungency have also been isolated from this spice (Nakatani et al.,

1988). Meanwhile, a ferulic acid amide of tyramine (Figure 8.25) and piperine-related compounds with an open methylenedioxy ring found in black pepper (*Riper nigung*) have stronger antioxidant activity than tocopherol. These compounds are fat soluble, odorless and tasteless (Nakatani et al., 1986). In addition, tetrahydrocurcumin, a colorless, heat-resistant, antioxidative compound, has been found in turmeric (*Curama longa L.*) (Figure 8.25; Osawa et al., 1989).

OTHER SOURCES OF ANTIOXIDANTS

Olives and their leaves contain polyphenolic compounds that may be extracted into methanol and ethyl acetate, successively. Fractions of this polyphenolic extract were

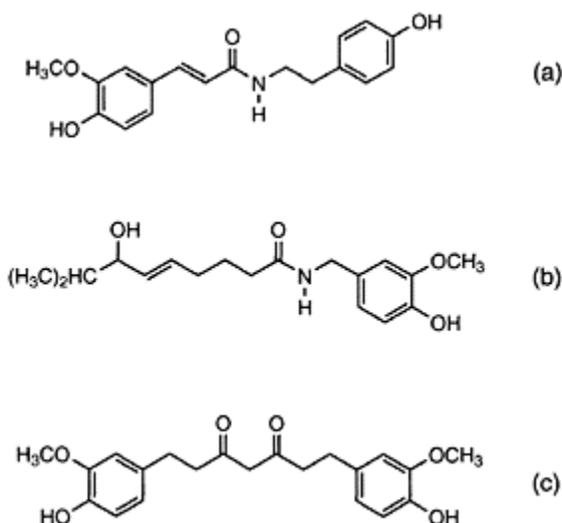


FIGURE 8.25 Chemical structure of antioxidative compounds in (a) black pepper, (b) capsicum and (c) turmeric.

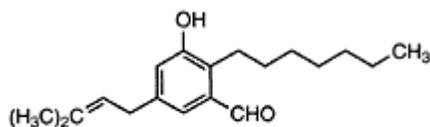
eluted on TLC and the compounds with the highest *o*-diphenol concentration separated and evaluated for their inhibition of oxidative deterioration of vegetable oils. Oxidative deterioration of soybean and olive oils was inhibited when the extracts were used at a concentration of 100 mg/kg. Nergiz and Unal (1991) have reported that virgin olive oil contains high amounts of *p*-coumaric acid (0.73 to 10.37 mg/kg) compared to the syringic, vanillic, and ferulic acids that are also present. The active antioxidant compounds in olive have not been studied in detail.

Varieties of onion (*Allium cepa L.*) with colored skin have exceptionally high flavonol content, mainly 2.5 to 6.5% quercetin (as aglycone) (Herrmann, 1976). Akaranta and Odozi (1986) have shown that acetone extracts of red onion skin (0.3%) reduce the peroxide value of oils as effectively as the commercial antioxidant 1,2-dihydro-2,2,4-

trimethyl-quinorine at a 0.1% level of addition. Meanwhile, various phenolic compounds of waste fluid from the starch and ethanol manufacturing process of sweet potato (*Iopomea batatas*) have been determined (Hayes and Kato, 1984). The antioxidative activity of a 70% methanol extract of sweet potato was evaluated in a linoleate aqueous system and its strong antioxidant activity was attributed to the presence of chlorogenic and isochlorogenic acids. Caffeic and 4-O-caffeoyl quinic acids were also present. The synergistic effect of these phenolic compounds with amino acids has also been contemplated (Hayes and Kato, 1984).

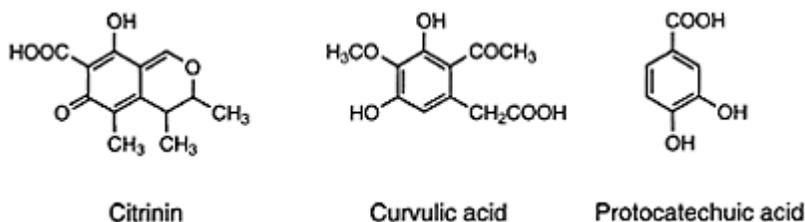
Oat (*Avena sativa* L.) products have also been suggested as potential food antioxidants. Their antioxidant activity is supposedly based on their high content of dihydrocaffeic acid and phospholipids (Schuler, 1990).

Flavoglaucin, a phenolic compound isolated from the mycelial mat of *Eurotium chevalieri*, has been found to be an excellent antioxidant in vegetable oils at a 0.05% (w/w) level of addition (Figure 8.26; Ishikawa et al., 1984). It stabilized lard when used in combination with α -tocopherol at a 0.04% concentration. However, this fungal antioxidative compound has not shown mutagenic activity to *Salmonella*



Flavoglaucin

FIGURE 8.26 Chemical structure of flavoglaucin.



Citrinin

Curvulic acid

Protocatechuic acid

FIGURE 8.27 Chemical structures of citrinin, curvulic acid and protocatechuic acid.

typhimurium, TA 100 and TA 98, and seems to have limited potential use in foods (Ishikawa et al., 1984). Aoyama et al. (1982) screened 750 strains of filamentous fungi from soil for their antioxidant activity against linoleate. Fourteen strains were positive for the test and the two superior strains were studied for their microbial products. The curvulic acid produced by these two fungi strains possess significant antioxidant activity (Figure 8.27). Furthermore, they were able to find protocatechuic acid and citrinin as

other antioxidative components present (Figure 8.28). The anti-oxidative activity of these compounds was compared with BHA and α -tocopherol. It was concluded that citrinin was as effective as α -tocopherol, while curvulic acid and protocatechuic acid showed an activity between α -tocopherol and BHA. Citrinin is a known mycotoxin and has limited potential in foods; protocatechuic acid is a known antioxidant and curvulic acid has shown low toxicity at 150 mg/kg in rats (Aoyama et al., 1982).

WOOD SMOKE

Wood smoke is a complex system consisting of dispersed and particulate phases. Absorption of vapor (dispersed phase) by the foodstuff results in the characteristic color, flavor and preservative properties of smoked foods. It has been shown that certain levels and types of specific wood smoke have antioxidative properties (Maga, 1988). Wood smoke can retard oxidative rancidity in smoked foods. The neutral fraction of wood smoke, which contains most of the phenols, has been shown to possess the highest antioxidative properties while the basic fraction actually promotes lipid oxidation (Maga, 1988). During pyrolysis of wood, its lignins produce the most important compounds in smoke, namely, phenols and phenol ethers, typified by guaiacol (2-methoxyphenol) and syringol (2,6-dimethoxyphenol) and their homologous derivatives. Soft woods form guaiacols predominantly, and hard woods

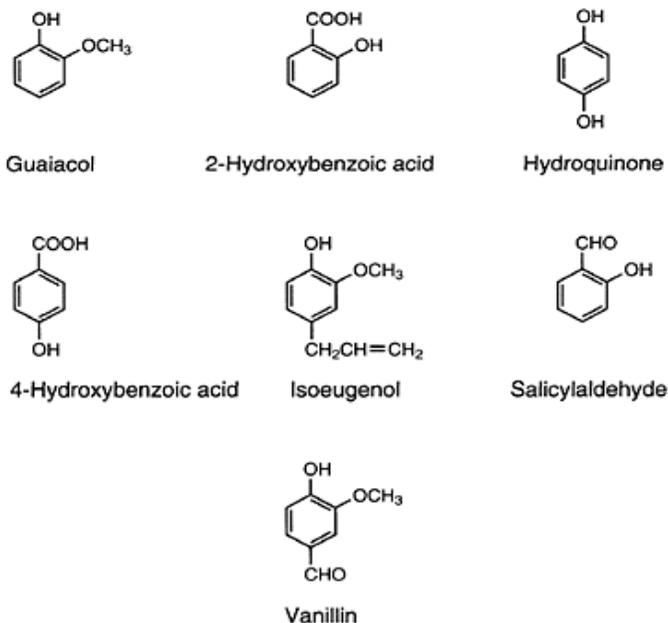


FIGURE 8.28 Selected phenolic compounds of wood smoke.

produce a mixture of guaiacols and syringols (Maga, 1988). It is interesting to note that lignin has antioxidant properties; inclusion of 1 to 10% lignin in the rat diet results in increased deposition of retinal as compared to cellulose-fed controls (Catignani and Carter, 1982). Studies on lignin have shown the presence of phenolic compounds such as ferulic, vanillic, syringic, and *p*-hydroxybenzoic acids (Catignani and Carter, 1982)—compounds that may contribute to the antioxidant properties of lignin.

It has been demonstrated that phenols are the primary antioxidant-related components associated with smoke (Miller and Quackenbush, 1957). Phenols with a high boiling point are the key compounds responsible for antioxidant properties, while low-boiling phenols show weak antioxidant activity (Barylko-Pikielna, 1977; Maga, 1988). The 2,6-dimethoxyphenols exhibit strong antioxidant effect (Kjällstrand and Petersson, 2001). The chemical structures of some antioxidative phenolics of wood smoke are given in Figure 8.28.

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9

Contribution of Phenolic Compounds to Flavor and Color Characteristics of Foods

CONTRIBUTION TO FLAVOR

In general, phenolic compounds contribute to the aroma and taste of numerous food products of plant and animal origin (Crouzet et al., 1997; Teranishi et al., 1989). According to Arai et al. (1966), the objectionable flavor of some oilseeds is due to the presence of phenolics that possess sour, bitter, astringent and/or phenolic-like flavor characteristics. Huang and Zayas (1991) found that the presence of ferulic and coumaric acids together may allow prediction of the intensity of sourness of corn germ protein flour products (Table 9.1). On the other hand, a number of alkylphenols contribute to the sheep/mutton flavor of ovine fat and aged cheese (Ha and Lindsay, 1991a, b). A mixture of pure alkylphenols in concentrations equivalent to those found in ram depot fat results in a sheep-like aroma (Ha and Lindsay, 1990).

The detection thresholds and odor description of some volatile phenols are given in Table 9.2 and Table 9.3, respectively. Taste thresholds for some individual phenolic acids present in foods ranges from 30 mg/kg (protocatechuic acid) to 240 mg/kg (syringic acid). The taste threshold for sinapic acid has not been determined due to its insolubility in water at concentrations required for testing. Combining phenolic acids results in much more sensitive detection thresholds than in the individual acids (Maga and Lorenz, 1973). On the other hand, the recognition threshold of the acid taste of p-coumaric as bitter, astringent and unpleasant was perceived at 48 mg/kg. Ferulic acid can be perceived as having a sour taste at 90 mg/kg. The combination of p-coumaric and ferulic acids results in a sour-bitter taste at 20 mg/kg (Huang and Zayas, 1991). The lower taste thresholds for combined phenolics are due to a synergistic effect. Studies, therefore, have indicated that free phenolic acids contribute to the taste of foods.

Sinapine is a bitter phenolic compound present in rapeseed meals at quite high concentrations (0.39 to 2.85%) (Naczka et al., 1998); therefore, it also contributes to the reported unpleasant and bitter flavor of glucosinolate-free rapeseed flour (Sosulski et al., 1977). Moreover, it may have adverse effects on the palatability of rapeseed products (Appelqvist, 1972). Sinapine is also linked to a crabby or fishy taint noted in eggs from some brown-egg laying hens (Butler et al., 1982; Fenwick et al., 1984; Hobson-Frohock et al., 1973) and is responsible for production of trimethylamine (TMA) (Pearson et al., 1980) at low concentrations of about 1 mg/kg (Hobson-Frohock et al., 1973).

TABLE 9.1
Regression Models of Free Phenolic Acids for
Sensory Attributes in Tested Corn Germ Protein
Products

| Equation of Model | R ² |
|---|----------------|
| S.I.=1.18+0.083 (Ferulic)-0.019 (<i>o</i> -Coumaric) | 0.989 |
| B.I.=0.326-1.638 (Syringic)+0.353 (<i>p</i> -Coumaric) | 0.514 |
| A.I.=0.428+0.069 (Vanillic)+0.112 (<i>p</i> -Coumaric) | 0.750 |

Note: S.I., sourness intensity; B.I., bitterness intensity; A.I., astringency intensity.

Source: Adapted from Huang, C.A. and Zayas, J.F., 1991, *J. Food Sci.*, 56:1308-1313; 1315.

TABLE 9.2
Effect of Phenolic Acid Combinations on Flavor
Thresholds^a

| Compounds | Individual Thresholds | Combination Thresholds |
|--|-------------------------|------------------------|
| Salicylic+ <i>p</i> -hydroxybenzoic | (90); (40) | 35 |
| Salicylic+ <i>p</i> -hydroxybenzoic+gentisic | (90); (40); (90) | 40 |
| Vanillic+ <i>p</i> -hydroxybenzoic | (30); (40) | 10 |
| Vanillic+syringic | (30); (240) | 90 |
| Ferulic+ <i>p</i> -coumaric | (90); (40) | 25 |
| Ferulic+gentisic | (90); (90) | 80 |
| Ferulic+gentisic+caffeic | (90); (90); (90) | 60 |
| Ferulic+gentisic+caffeic+syringic | (90); (90); (90); (240) | 95 |

^a Milligrams per kilogram.

Source: Adapted from Maga, J.A. and Lorenz, K., 1973, *Cereal Sci. Today*, 18:326-329.

Singleton and Nobel (1976) related the presence of chlorogenic acid, other hydroxycinnamates and, in particular, oligomeric proanthocyanidins to the bitterness and astringency of wine and cider. Similarly, Dacic and Bellau (1973) associated bitterness of beer to the presence of phenolics. They found bitterness threshold values of chlorogenic acid to be 20 mg/L in beer and 10 mg/L in 5% ethanol. On the other hand, Nagel et al. (1987) reported that chlorogenic acid even at 100 mg/L in aqueous 2% potassium acid tartrate solution is not bitter when its acid character is masked. It has also been postulated that hydroxycinnamic acids and their derivatives are responsible for the sour-bitter taste of cranberries (Marwan and Nagel, 1982).

Phenolic compounds are responsible for the bitterness and astringency of potatoes. The cortex tissue of potatoes cooked with peel is more bitter than that of potatoes cooked without the peel (Mondy and Gosselin, 1988). Mondy et al. (1971) found a high positive correlation between phenol content and bitterness (+0.72) and astringency (+0.82) of potatoes. Sinden et al. (1976) reported similar results. Increased bitterness of potatoes with peel may be due to the migration of phenolics from peel into the cortex tissue.

TABLE 9.3
Odor Description of Some Volatile Phenolic Compounds

| Compounds | Aroma Description |
|---|---|
| <i>p</i> -, <i>m</i> -, and/or <i>o</i> -Thiocresol | Sulfurous, stench, burnt rubber, medicinal, meaty, old fruit, cooked meat, brothy |
| Phenol | Phenolic, medicinal, smoky |
| 2,6-Diisopropylphenol | Phenolic, medicinal, chemical |
| <i>o</i> -Cresol | Phenolic, indol-like, medicinal |
| <i>p</i> -Cresol | Phenolic, wood preservative-like smoky, very animal-like, sheep wool-like |
| <i>m</i> -Cresol | Phenolic, wood preservative-like, smoky |
| 3,4-Dimethylphenol | Phenolic, medicinal, animal-like |
| 2-Isopropylphenol | Must, medicinal, wood cedar-like, licorice-like |
| Thymol | Phenolic, medicinal, musty |
| Carvacrol | Phenolic, medicinal |
| 3-and/or 4-Isopropylphenol | Phenolic, medicinal |

Source: Adapted from Ha, J.K. and Lindsay, R.C., 1991b, *J. Food Sci.*, 56:1197–1202.

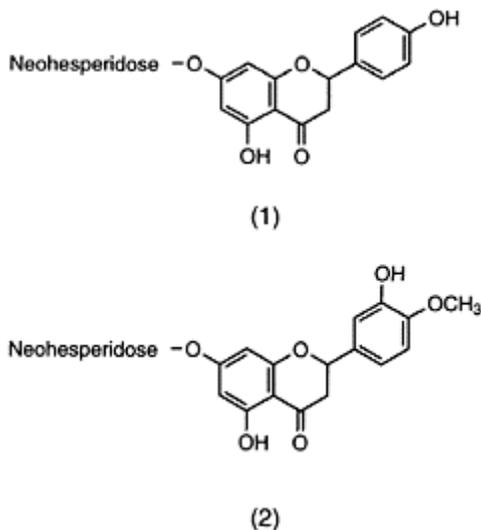


FIGURE 9.1 Structures of naringin (1) and neohesperidin (2).

Citrus fruits contain a number of flavanone glycosides that contribute to their bitter taste. Naringin (naringenin 7-neohesperidoside) is the predominant bitter flavanone found in grapefruits; naringin and neohesperidin (hesperetin 7-neohesperidoside) are dominant in sour oranges (Figure 9.1) (Rouseff et al., 1987). The sugar moiety of these glycosides

is linked to the 7-hydroxy group of aglycone and is a disaccharide neohesperidose composed of L-rhamnose linked (1→2) to D-glucose. The bitterness of flavanone glycosides depends on the structure of sugar moiety because the point of attachment of rhamnose to glucose affects the taste. Thus, a 1→2 linkage between L-rhamnose and D-glucose is associated with bitterness. The bitter taste of pure naringin can be detected at 10^{-4} to 10^{-5} M in aqueous solutions (Horowitz and Gentili, 1969); however, narirutin (naringenin 7-rutinoside) with a 1→6 linkage between L-rhamnose and D-glucose is not bitter. Free ferulic acid is a precursor of the objectionable flavor in stored orange juice caused by the presence of *p*-vinylguaiacol produced by decarboxylation of ferulic acid (Naim et al., 1988). This phenol is also present in processed alcoholic beverages, cooked beef, and dried mushroom (Maga, 1978); however, its presence in popcorn is considered desirable (Walradt et al., 1970).

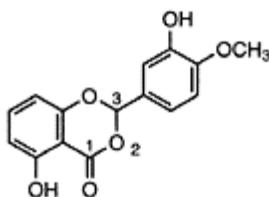
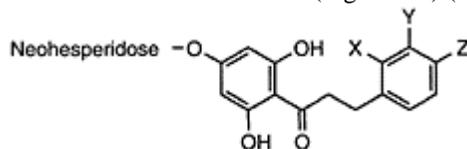


FIGURE 9.2 Structure of phyllodulcin.

Phyllodulcin, a 3,4-dihydroisocoumarin compound, is reported to be 200 to 300 times sweeter than sucrose (Figure 9.2). This sweet-tasting phenol was first isolated in 1916 by Asahina and Ueno from the *Hydrangea Macrophylla Seringe* and structurally identified by Arkawa and Nakazari in 1959 (Crosby, 1976). In comparison to sucrose, the phyllodulcin gives a delayed onset of sweetness and a lingering licorice-like aftertaste. The bitter flavonones in citrus, naringin and neohesperidin can be hydrolyzed to chalcones and then converted by simple hydrogenation into the corresponding dihydrochalcones; chalcones and dihydrochalcones are sweet (Horwitz and Gentili, 1969 and 1974). Some B-ring-substituted neohesperidin dihydrochalcones are reported to possess up to 2000 times the sweetness of sucrose (Figure 9.3) (Inglett, 1969). The



| Sweetness (Sucrose=1) | X | Y | Z |
|-----------------------|------|------------------|----|
| | 0H | H | H |
| | 0OH | H | H |
| | 110H | OH | H |
| | 110H | H | OH |
| | 0H | OH | OH |
| | 0H | OCH ₃ | OH |

| | | |
|--------|----|--------------------------------|
| 950 H | OH | OCH ₃ |
| 1100 H | OH | OC ₂ H ₅ |
| 2000 H | OH | OC ₃ H ₇ |

FIGURE 9.3 Sweetness of neohesperidin dihydrochalcone modifies with various substituents in ring B. (Adapted from Inglett, G.E., 1969, *J. Food Sci.*, 34:101–103.)

glycoside portion of dihydrochalcone is unnecessary for sweetness, but its removal lowers the water solubility of the resultant aglycone. On the other hand, replacement of the glycoside portion by carboxymethyl or carboxypropyl groups produces dihydrochalcone derivatives sweeter than saccharin and cyclamate. However, all dihydrochalcone sweeteners are notably slow in onset of sweetness and have a considerable lingering effect that may render them suitable for many food uses (DuBois et al., 1977).

Phenolic substances contribute to the flavor of vanilla pod and vanilla extracts. Vanillin, *p*-hydroxybenzaldehyde and *p*-hydroxybenzyl methyl ester are the most abundant volatiles present. Simple phenolics such as *p*-cresol, eugenol, guaiacol, *p*-vinylguaiacol and *p*-vinylphenol, as well as aromatic acids such as vanillic and salicylic acids, are also identified as minor components (Purseglove et al., 1981).

Ripe banana contains volatile phenolics such as eugenol, methyleugenol, elimicin, and vanillin. Strawberry volatiles contain esters of some phenolic acids such as ethylsalicylic, methylcinnamic and ethylbenzoic acids (Nurster, 1970); thymol, eugenol and carvacrol are found in plum mirabelle (Walker, 1975a). Thymol is also a major contributor to the flavor of essential oils from tangerine and mandarin orange (Wilson and Shaw, 1981). Moreover, several coumarins such as citral, nooketone and citropten are found in the oil of lime and thymol in the peel oil of mandarin orange. A number of synthetic fruits and chocolate flavorings contain vanillin and ethylvanillin (Table 9.4).

Phenolic substances may be responsible for the flavor of a number of spices and herbs. Anethole, estragole, eugenol, thymol and carvacrol are common constituents of many herbs and spices, thus contributing to their overall sensory characteristics.

TABLE 9.4
Composition of Some Synthetic Flavoring Formulations

| Flavoring | Component | Amount (mg) |
|-----------|---|-------------|
| Raspberry | Vanillin | 20 |
| | Ethylvanillin | 8 |
| | α -Ionone | 1 |
| | Maltol | 30 |
| | 1-(<i>p</i> -hydroxyphenol)-3-butanone | 100 |
| | Dimethyl sulfide | 1 |
| | 2,5-Dimethyl-N-(2-pyrazinyl) pyrrole | 1 |

| | | |
|-----------|----------------------|------|
| Chocolate | Dimethyltrisulphide | 1 |
| | 2,6-Dimethylpyrazine | 3324 |
| | Ethylvanillin | 143 |
| | Isovaleraldehyde | 100 |

Note: Carriers and solvents are not included.

Source: Adapted from Coultate, T.P., 1984, *Food—the Chemistry of its Components*, The Royal Society of Chemistry, London, using U.S. Patents 3886289 and 3619210.

Eugenol is one of the main flavor compounds found in essential oils obtained by distillation of bark or leaf oil from cinnamon. Presence of thymol is reported in the headspace volatiles of living and picked thyme plant, *Thymus vulgaris*, as well as in the commercial oil produced from this herb. Thymol constitutes 15.7, 9.0, and 39.0% of the headspace volatiles found in the living plant, picked plant and commercial oil of thyme, respectively (Mookherjee et al., 1989; Russell and Olson, 1972). Wyllie et al. (1990) identified thymol and carvacrol in the volatile components of the fruit of *Pistacia Lentiscus*, a fruit used to aromatize olives, vinegar, and salad dressings.

Some vegetables and spices contain derivatives of phenolics that cause a characteristic hot, sharp and stinging sensation in the mouth referred to as pungency. The pungent compounds found in *Capsicum* species such as red and green chilies are known as capsaicinoids. These substances are vanillylamides of monocarboxylic acids with varying degrees of unsaturation and chain length (Figure 9.4) and are ascribed mainly to alkaloids. The pungent characteristic of fresh ginger, as well as ginger oleoresin, is brought about by phenylalkyl ketones that are derivatives of vanillin. This group of compounds is known as gingerols. The predominant gingerol homologues are those with $n=4$ (Figure 9.5); Govindarajan (1979) reported that (6)-gingerol is the most pungent active member of these compounds. Processing of ginger, such as drying, can result in the loss of a water molecule from gingerols, producing shogols that have a greater pungency than the gingerols (Figure 9.6). The essential pungent substance found in cloves is eugenol.

Millin et al. (1969) examined the effect of nonvolatile compounds of black tea on its flavor. They studied the taste of pure compounds as well as the nonvolatile fractions isolated from tea. Based on this study, they concluded that monomeric phenolics (flavanols, flavonols, theogallin, chlorogenic acid, *p*-coumarylquinic acid, and caffeic acid) do not contribute significantly to the taste of tea.

A number of polyphenols are found in the methanolic extracts of cocoa beans that produce chocolate aroma. Rohan and Connell (1964) identified two flavonols (quercetin and quercetrin) and three phenolic acids (*p*-coumaric, caffeic and chlorogenic) in cocoa beans. Stewart (1970) studied the development of chocolate aroma in model systems and found that phenolics enhanced the production of chocolate aroma. Phenolics may also contribute to the quality defects found in some cocoa beans and chocolate liquors, thus imparting a smoke- or ham-like flavor note. This type of flavor defect has been associated with the method used to dry beans (Lehrian

et al., 1978). Phenolic compounds are known to contribute to the flavor of fermented soy sauce. Of these, *p*-ethylguaiaicol and *p*-ethylphenol are considered the most important flavor components of soy sauce; a difference of 0.5% in *p*-ethylguaiaicol content is easily

detectable. Compounds *p*-ethylguaiacol and *p*-ethylphenol are products of metabolism of ferulic and *p*-hydroxycinnamic acids during the fermentation process (Yokotsuka, 1986).

Nine phenolics have been identified in the volatile constituents of yellow passion fruit juice: 2-,3-, and 4-methylphenols, 4-ethyl and 4-allylphenol, and 2,4-dimethylphenol, 3,4,5-trimethylphenol, 2-methoxyphenol and 4-allyl-2-methoxyphenol. These phenolics are found in quantities below 0.01 mg/kg (Casimir et al., 1981).

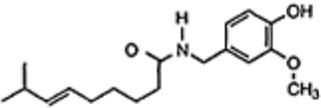
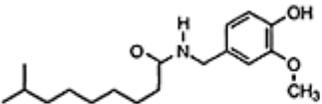
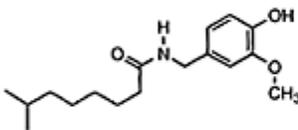
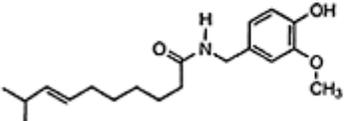
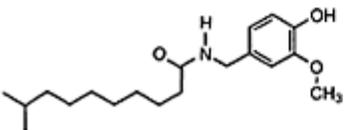
| | Pungency Threshold |
|---|---------------------------------|
|  | Capsaicin 16.1 ppm |
|  | Dihydrocapsaicin 16.1 ppm |
|  | Nordihydrocapsaicin 9.3 ppm |
|  | Homocapsaicin 6.9 ppm |
|  | Homodihydrocapsaicin 8.1 ppm |

FIGURE 9.4 Structure of capsaicinoids and their pungency thresholds. (Adapted from Wong, D.W.S., 1989, in *Mechanism and Theory in Food Chemistry*, AVI Publishing Co., Westport, CT, 147–189.)

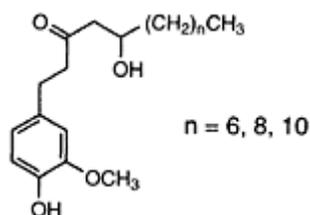


FIGURE 9.5 Chemical structures of gingerols.

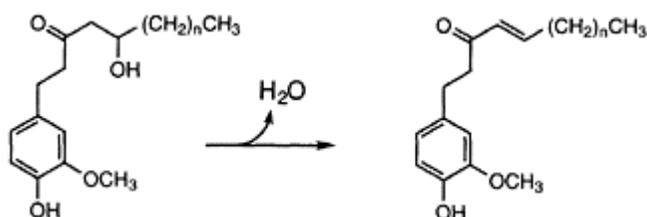


FIGURE 9.6 Reaction of gingerol dehydration. (Adapted from Wong, D.W.S., 1989, in *Mechanism and Theory in Food Chemistry*, AVI Publishing Co., Westport, CT, 147–189.)

TABLE 9.5

Content of Volatile Phenolics in Some Animal Fats^a

| Phenols | Caprine Male Goat | Ovine | | |
|------------------------------|-------------------|-------|------|------|
| | | Ram | Lamb | Beef |
| Thiophenol | 5 | 1766 | 8 | – |
| Phenol | 3 | 7 | 0.6 | 109 |
| <i>o</i> -Cresol | 1 | 901 | 5 | 14 |
| 2-Ethylphenol | 0.6 | 200 | 26 | – |
| <i>p</i> -Cresol | 5 | 173 | 33 | 25 |
| <i>m</i> -Cresol | 0.5 | 5 | 2 | 68 |
| 2-Isopropylphenol | – | 1493 | 156 | – |
| 3,4-Dimethylphenol | 3 | 65 | 16 | – |
| 3- and(or) 4-Ethylphenol | – | – | – | 2 |
| Thymol | – | 1139 | 170 | – |
| Carvacrol | 0.4 | 480 | 36 | – |
| 3- and(or) 4-Isopropylphenol | – | 1065 | 70 | – |

| | | | | |
|-----------------------|---|----|-----|-----|
| 3,5-Diisopropylphenol | - | 26 | 0.7 | - |
| 2,6-Diisopropylphenol | - | - | - | 0.3 |

^a Parts per billion (ppb).

Source: Adapted from Ha, J.K. and Lindsay, R.C., 1991b, *J. Food Sci.*, 56:1197–1202.

Alkylphenols such as *p*-cresol, 2-isopropylphenol, 3,4-dimethylphenol, thymol, carvacrol, 3-isopropylphenol and 4-isopropylphenol contribute distinguishing phenolic-sheep odors to ovine fat. On the other hand, *p*-cresol and 3- and/or 4-ethylphenol combined with 3-methylbutanoic acid are possibly responsible for a pork-like flavor of swine fat. Presence of cresols and, particularly, *m*-cresol may have an influence on the beefy flavor note of fats (Ha and Lindsay, 1991b). The content of volatile phenolic compounds in animal fats is summarized in Table 9.5. Presence of phenolics in animal muscles may be derived from plant phenolics ingested from feeds, as well as from phenolics derived from microbial intestinal fermentation of tyrosine (Brot et al., 1965; Enei et al., 1972). A schematic representation for the formation of *p*-cresol, *p*-ethylphenol, and phenol from tyrosine by microbial metabolism is shown in Figure 9.7.

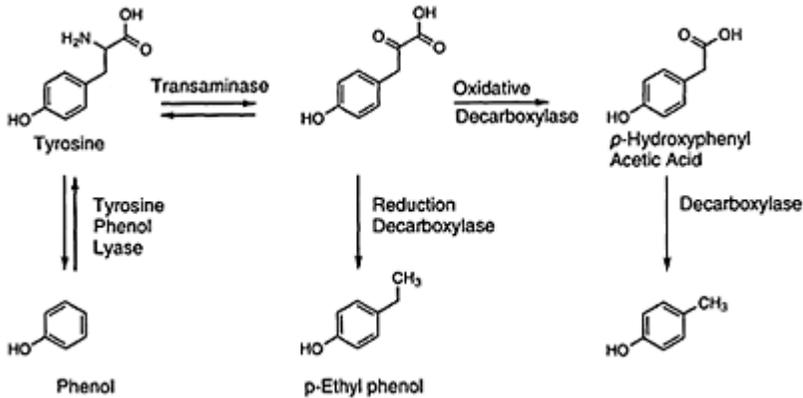


FIGURE 9.7 Schematic for the formation of *p*-cresol, 4-ethylphenol and phenol from tyrosine by microbial metabolism. (Adapted from Ha, J.K. and Lindsay, R.C., 1991a, *J. Food Sci.*, 56:1241–1247.)

Phenolic compounds also contribute to the flavor of dairy products. Presence of flavor notes such as phenolic, medicinal, cow and sheep has been noticed in varieties of aged Italian cheese such as provolone, Romano peccorino, and parmesan. This is attributed to the presence of phenol, *m*-cresol, *p*-cresol, *p*-ethylphenol, *p*-ethylphenol, 3,4-dimethylphenol, *o*-isopropylphenol, thymol and carvacrol (Ha and Lindsay, 1991a; Ney, 1973). Phenol and *o*-cresol have also been found as flavor components of butter (Urbach

et al., 1972) and have been included as a part of flavoring mix used to simulate a cheese flavor (Urbach et al., 1972).

Tilgner (1977) estimated that smoke used in meat and fish curing may contain up to 10,000 different compounds; of these, probably about 500 are responsible for a smoky flavor note. A number of studies have indicated that phenolic compounds present in the vapor phase of smoke may contribute to imparting a smoky flavor to foods (Baryłko-Pikielna, 1977; Bratzler et al., 1969; Daun, 1972; Hamm, 1977). Some 85 different phenolics have been identified in smoke condensate, but only 20 phenols in smoked food product (Toth and Potthast, 1984). The flavor characteristics of some simple phenols found in wood smoke are summarized in Table 9.6. Kurko (1963) reported that only phenolics with a boiling point of 76 to 89°C at 5.33 hPa (4 mm Hg) carry a smoke-like flavor. Syringol, guaiacol, 4-methylguaiacol, 4-methylsyringol and eugenol may be the dominant contributors to the pleasant smoke-like flavor (Fiddler et al., 1970). Guaiacols contribute to a smoky taste and syringols impart a smoky odor; however, a much more complex mixture of compounds is responsible for the characteristic aroma of smoked products (Daun, 1972).

Presence of simple bromophenols has been detected in Australian ocean prawns (Whitfield et al., 1988), North American crustaceans and molluscs (Boyle et al., 1992a), Pacific salmon, molluscs (Boyle et al., 1992a), and North Atlantic shrimp (Anthoni et al., 1990). Of these, 2- and 4-bromophenols, 2,4- and 2,6-dibromophenols and 2,4,6-tribromophenol have been identified as key flavor components in

TABLE 9.6
Sensory Characteristics of Phenols from Smoke

| Phenol | Optimum Concentration (mg/100 mL H ₂ O) | Smell | Taste |
|------------------|--|---|---|
| Guaiacol | 3.75 | Phenolic, smoky aromatic, hot, sweet | Phenolic, hot spicy, flavor of smoked ham, sweet, dry |
| Isoeugenol | 9.80 | Sweet, fruity, vanilla-like, phenolic | Sweet-fruity, mild smoke flavor, dry, hot |
| <i>o</i> -Cresol | 7.50 | Phenolic, sweet, fruity, aromatic caramel-like, like smoked ham | Sweet, hot, unpleasant smoky, burning |

Source: Adapted from Toth, L. and Potthast, K., 1984, *Adv. Food Res.*, 29:87–158.

seafoods (Boyle et al., 1992a, b; Whitfield et al., 1988, 1997, 1998). Flavor thresholds for 2-bromophenol, 2,6-dibromophenol and 2,4,6-tribromophenol in water are 3×10^{-2} , 5×10^{-4} and 0.6 $\mu\text{g}/\text{kg}$, respectively. The flavor of 2,4,6-tribromophenol at 0.6 $\mu\text{g}/\text{kg}$ is described as iodoform-like while that of 2,6-dibromophenol at its threshold level is phenolic/iodine-like (Whitfield et al., 1998). It has been postulated that bromophenols at concentrations below their flavor thresholds contribute the marine/ocean-like flavor to seafoods and may enhance existing seafood flavors (Boyle et al., 1992a; Whitfield et al., 1997, 1998). Polychaetes and bryozoans have been implicated as the main sources of bromophenols in seafoods (Whitfield et al., 1999).

CONTRIBUTION OF FOOD PHENOLICS TO ASTRINGENCY

Some phenolic substances present in foods are able to bring about a puckering and drying sensation over the whole surface of the tongue and the buccal mucosa (Lea and Arnold, 1978). This sensation is referred to as astringency and is related to the ability of the substance to precipitate salivary proteins (Bate-Smith, 1973; Clifford, 1997). The presence of astringent phenols may affect the acceptability of a number of foods. The most significant examples of astringent foods are cider apples, Japanese persimmons, sloes, perry pears, cranberries, and wines.

Astringent phenols are substances of moderate molecular size whose phenolic groups are oriented into 1,2-dihydroxy or 1,2,3-trihydroxy configurations. At least two such orientations of phenolic groups are required to impart an astringent characteristic to a phenolic compound (Haslam, 1981). These phenolic substances bind to proteins more strongly than phenols with isolated hydroxyl groups (McManus et al., 1981). The phenol-protein complex can only precipitate when the complex becomes sufficiently hydrophobic. Molecular interpretation of chemical reactions responsible for the formation of astringency has been reported (Haslam et al., 1986; Haslam and Lilley, 1988).

Delcour et al. (1984) determined the astringency thresholds for tannic acid, (+)-catechin, procyanidin B-3, and mixtures of trimeric and tetrameric proanthocyanidins dissolved in deionized water. These threshold values range from 4.1 to 46.1 mg/L; greater molecular weight substances have lower threshold values. The recognition taste threshold of *p*-coumaric acid perceived as astringent is 48 mg/kg (Huang and Zayas, 1991). Good correlations exist between the astringent note of corn-germ, protein-flour products and the content of free *p*-coumaric acid ($r=0.850$) and *p*-hydroxybenzoic acid ($r=0.815$).

Millin et al. (1969) examined the effect of nonvolatile compounds on the flavor of black tea. Monomeric phenolics, such as flavanols, flavonols, theogallin, chlorogenic acid, *p*-coumarylquinic acid, and caffeic acid do not significantly contribute to the taste of tea. However, intermediate molecular weight oxidation products of flavanols formed during the fermentation process, such as theaflavin, are astringent. Later, Sanderson et al. (1976) reported that astringency of tea is related to the quantity of polyphenols and the degree of oxidation of flavanols as well as the degree of substitution of flavanols with galloyl groups. The astringency of theaflavins in tea is attenuated by the formation of complexes with caffeine (Stagg and Millin, 1975).

The astringency of unripened fruits is related to the presence of condensed tannins. According to Haslam (1975), only tannins with molecular weights ranging from 500 to 3000 Da may bring about the astringency sensation. The process of fruit ripening brings about changes in the composition and quantity of tannins, thus lowering astringency and improving palatability of fruits. Tannin polymerization is thought to be responsible for reduced astringency of ripened fruits (Goldstein and Swain, 1963). Increase in the molecular size of tannins is a factor that inhibits the interaction of tannins with salivary proteins (Mehansho et al., 1987). On the other hand, according to Ozawa et al. (1987), loss of astringency during ripening of fruits may also be due, in part, to the interaction of the involved polyphenols and proteins. However, the interaction of tannins with acetaldehyde is attributed to the loss of astringency upon ripening of the persimmon fruit (Matsuo and Ito, 1982).

Polyphenolic compounds also contribute to the remarkably astringent taste of semidried banana products obtained by dehydration of ripe fruits following their blanching or sulfiting. This is due to the diffusion of tannin-like materials from the latex cells to the surrounding tissues. Light microscopy observations show that blanching and sulfiting disrupt the cell walls and result in extensive leakage of active tannins (Ramirez-Martinez et al., 1977).

PHENOLICS AS NATURAL FOOD PIGMENTS

A large and diversified group of phenolic substances, known as flavonoids, are responsible for the color and flavor of foods. Over 4000 flavonoids have been identified in vascular plants (Harborne, 1988a, b). Among them, the anthocyanins are responsible for the pink, scarlet, red, mauve, blue and violet colors of vegetables, fruits, fruit juices and wines. Other flavonoids may contribute to the ivory and pale yellow colors or act as copigments to anthocyanins (Harborne, 1967, 1988b). Most flavonoids are present in plant cells in the form of glycosides. The sugar part usually consists of mono-, di-, and trisaccharides of glucose, rhamnose, galactose, xylose and arabinose. The sugar moiety can be additionally acylated with phenolic acids such as *p*-coumaric, caffeic, or ferulic acids. The role of flavonoids in foods has been extensively reported and several comprehensive reviews have been published (Brouillard et al., 1997; Harborne et al., 1975, 1988a, b; Jackman et al., 1987; Kuhnau, 1976; Markakis, 1974, 1982; Timberlake, 1980). Foods of plant origin may contain up to several grams of flavonoids per kilogram of fresh weight. It is estimated that daily average consumption of flavonoids may range from 50 mg to about 1 g per person. Nearly half of the total flavonoid intake is anthocyanins, catechins and 4-oxoflavonoids (Kuhnau, 1976).

ANTHOCYANINS

Anthocyanins are water-soluble pigments found in most species in the plant kingdom; they are responsible for the bright red skin of radishes, the red skin of potatoes, and the dark skin of eggplants. Blackberries, red and black raspberries, nectarines, peaches, blueberries, bilberries, cherries, currants, grapes, pomegranates, cranberries, and ripe gooseberries contain anthocyanins. The visual detection thresholds for selected anthocyanins are shown in Table 9.7. The total content of anthocyanins varies among fruits and in different cultivars of the same fruit and is also affected by genetic make-up, light, temperature, and agronomic factors (Delgado-Vargas et al., 2000; Francis, 1989; Harborne and Grayer, 1988; Harborne and Williams, 2001; Ju et al., 1995; Mazza and Miniati, 1993; Wrolstad, 2000). The exposure of apples (Faragher, 1983), cranberries (Hall and Stark, 1972), and nectarines (Basiouny and Buchanan, 1977) to low temperature promotes the formation of anthocyanins. However, blackberries ripened at low temperatures contained fewer anthocyanins compared to those ripened at warm weather conditions (Naumann and Wittemberg, 1980).

The content of anthocyanins is also affected by the intensity and quality of light. For example, light is the predominant factor affecting the content of anthocyanins in olives;

olive fruits ripened in the dark contain 10 times fewer anthocyanins than those ripened under normal conditions (Vazquez-Roncero et al., 1970). In apples,

TABLE 9.7
Visual Detection Thresholds (VDT) for Selected Anthocyanins

| Anthocyanin | VDT (mg/L) |
|---|------------|
| Cyanidin 3-glucoside | 1.3 |
| Cyanidin 3-xylosyl-galactoside | 0.9 |
| Cyanidin 3-xylosyl-glucosyl-galactoside | 2.4 |
| Cyanidin 3-sinapoyl-xylosyl-glucosyl-galactoside | 0.9 |
| Cyanidin 3-feruoyl-xylosyl-glucosyl-galactoside | 0.4 |
| Cyanidin 3-sophoroside-5-glucoside | 3.6 |
| Cyanidin 3-coumaroyl-sinapoyl-sophoroside-5-glucoside | 2.0 |

Source: Adapted from Stintzing, F.C. et al., 2002, *J. Agric. Food Chem.*, 50:6172–6182.

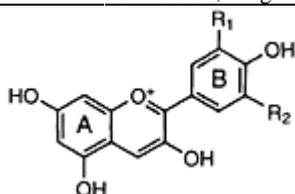
the production of anthocyanins is stimulated by light in the blue-violet region (Siegelman and Hendricks, 1958) as well as light in the UV region (Chalmers and Faragher, 1977). The presence of sucrose, glucose, fructose, lactose, and maltose also stimulates the formation of anthocyanins (Vestheim, 1970). Endogenous growth promoters such as auxins, cytokinins and gibberellins affect the synthesis of anthocyanins (Mazza and Miniati, 1993). Moreover, treating plants with excess nitrogen reduces the level of anthocyanins in fruits. Diversion of products of photosynthesis toward the formation of amino acids and proteins may be contemplated (Macheix et al., 1989).

Over 250 naturally occurring anthocyanins have been identified (Strack and Wray, 1993); of these, approximately 70 have been reported in fruits. The composition and content of anthocyanins allow differentiation of fruits from one another (Clifford, 2000; Delgado-Vargas et al., 2000; Jackman and Smith, 1996; Mazza and Miniati, 1993; Strack and Wray, 1993). These pigments usually are found in nature in the form of 3-monoglycosides, 3,5-diglycosides and acylglycosides of anthocyanidins; however, glycosylated anthocyanins in positions 7, 3', 4' or 5' may also occur (Brouillard, 1982). Presence of a second glycosyl group in anthocyanins brings about a characteristic bathochromic shift in their absorption maxima. This is used as a means for spectral differentiation of anthocyanins with a glycosyl group at position C-3 from those with glycosyl groups at position C-3 and position C-5 (Harborne, 1958). The sugar moiety can be glucose, arabinose, xylose, galactose, rhamnose, and fructose as well as rutinose (6-*O*- α -L-rhamnosyl-D-glucose), sophorose (2-*O*- β -D-xylosyl-D-glucose), gentobiose (6-*O*- β -D-glucosyl-D-glucose), sambubiose (2-*O*- β -D-xylosyl-D-glucose), xylosylrutinose and glycosylrutinose. Common names of some naturally occurring anthocyanins are listed in Table 9.8. The aglycone remaining after hydrolysis of anthocyanins is known as anthocyanidin.

Most anthocyanins found in nature are the glycosides of pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Figure 9.8 shows structures of some important anthocyanidins such as pelargonidin found in strawberry and radish, as well as cyanidin present in apple and red cabbage, and malvidin and delphinidin in

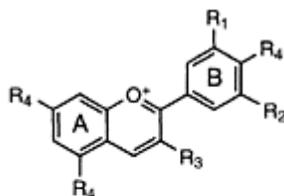
TABLE 9.8
Common Names of Major Anthocyanins Found in Fruits and Vegetables

| Common Names | Compound |
|--------------|------------------------------|
| Kuromanin | Cyanidin-3-glucoside |
| Keracyanin | Cyanidin-3-rhamnoglucoside |
| Ideain | Cyanidin-3-galactoside |
| Cyanin | Cyanidin-3,5-diglucoside |
| Callistephin | Pelargonidin-3-glucoside |
| Pelargonin | Pelargonidin-3,5-diglucoside |
| Oenin | Malvidin-3-glucoside |
| Malvin | Malvidin-3,5-diglucoside |



| Compound | R ₁ | R ₂ |
|--------------|------------------|------------------|
| Cyanidin | OH | H |
| Delphinidin | OH | OH |
| Malvidin | OCH ₃ | OCH ₃ |
| Pelargonidin | HH | |

FIGURE 9.8 Chemical structures of some anthocyanidins found in foods.



| | |
|----------------|---------------------------|
| R ₁ | H, OH or OCH ₃ |
| R ₂ | H, OH or OCH ₃ |
| R ₃ | H or Glycosyl |
| R ₄ | OH or Glycosyl |

FIGURE 9.9 Chemical structure of flavylium cation.

grapes. The basic structure of these anthocyanidins is the flavylum (2-phenylbenzopyrilium) cation substituted with a number of hydroxyl and methoxyl groups (Figure 9.9). The patterns of hydroxylation and methoxylation affect the color of anthocyanidins (Table 9.9). Presence of larger numbers of hydroxyl groups in the molecule tends to deepen the blue hue, while an increase in the number of methoxyl groups enhances the redness (Braverman, 1963; Delgado-Vargas et al., 2000; Francis, 1989; Iacobucci and Sweeny, 1983; Mazza and Miniati, 1993). The intensity of color, however, depends also on the pH, presence of metal ions, self-association of anthocyanins (Figuerido et al., 1996a, b; Hoshino et al., 1980; Mazza and Miniati, 1993; Wrolsdal, 2000), pigment mixtures and copigments such as other colorless phenolic compounds (Brouillard, 1983; Dangles et al., 1993; Mazza and Miniati, 1993; Ohta et al., 1980), as well as processing and storage conditions (temperature, sugar content, presence of ascorbic acid, and presence of oxygen, among others) (Dao et al., 1998; Debicki-Pospisil et al., 1982; Delgado-Vargas et al., 2000; Francis, 1989; Markakis, 1974). Depending on the conditions, ascorbic acid may have positive or detrimental effects on anthocyanin stability (Sarma et al., 1997).

TABLE 9.9
Naturally Occurring Anthocyanidins

| Name | Substitution Pattern ^a | | | | | | | Color |
|--------------|-----------------------------------|-----|----|-----|-----|-----|-----|------------|
| | 3 | 5 | 6 | 7 | 3' | 4' | 5' | |
| Apigenidin | H | OH | H | OH | H | OH | H | Orange |
| Aurantinidin | OH | OH | OH | OH | H | OH | H | Orange |
| Cyanidin | OH | OH | H | OH | OH | OH | H | Orange-red |
| Delphinidin | OH | OH | H | OH | OH | OH | OH | Bluish-red |
| Europinidin | OH | OMe | H | OH | OMe | OH | OH | Bluish-red |
| Hirsutidin | OH | OH | H | OMe | OMe | OH | OMe | Bluish-red |
| Luteolinidin | H | OH | H | OH | OH | OH | H | Orange |
| Malvidin | OH | OH | H | OH | OMe | OMe | OMe | Bluish-red |
| Pelargonidin | OH | OH | H | OH | H | OH | H | Orange |
| Peonidin | OH | OH | H | OH | OMe | OH | H | Orange-red |
| Petunidin | OH | OH | H | OH | OMe | OH | OH | Bluish-red |
| Puchellidin | OH | OMe | H | OH | OH | OH | OH | Bluish-red |
| Rosinidin | OH | OH | H | OMe | OMe | OH | H | Red |
| Tricetinidin | H | OH | H | OH | OH | OH | OH | Red |

^a For substitution patterns see Figure 9.9.

Source: Adapted from Mazza, G. and Miniati, E., 1993, *Anthocyanins in Fruits, Vegetables and Grains*, CRC Press, Boca Raton, FL.

Acylation of the sugar moiety of anthocyanins contributes to their color stability. Acylation usually occurs at the C-3 position of the sugar (Harborne, 1964). Presence of two or more acyl groups also increases the color stability of anthocyanins in aqueous solutions (Brouillard, 1982). For example, stabilization of anthocyanins containing two acyl groups is brought about by the formation of sandwich-type stacking due to hydrophobic interaction between the anthocyanidin ring and the acyl groups

(intramolecular interactions) (Brouillard, 1982; Mazza and Miniati, 1993; Wrolstad, 2000). The sugar is usually acylated with *p*-coumaric, caffeic, ferulic or sinapic acids (Francis and Harborne, 1966; Harborne, 1964; Somers, 1966). The stability of anthocyanins in plants is also associated with their ability to form complexes with other phenolics, nucleic acids, sugars, amino acids and metal ions such as calcium, magnesium and potassium (Brouillard, 1983). The colored forms of anthocyanidins are stabilized by the presence of hydroxyl groups at the C-5 position and substitution at the C-4 position. This inhibits the addition of water and subsequent formation of colorless species (Mazza and Miniati, 1993). Moreover, the addition of a methyl or phenyl group at the C-4 position (Timber-lake and Bridle, 1968) or the conversion to deoxyanthocyanidins (Sweeny and Iacobucci, 1983) improves the stability of anthocyanidins in the presence of sulfur dioxide or ascorbic acid.

Anthocyanins are very sensitive to changes in pH (Dao et al., 1998; Mazza and Brouillard, 1987; Ohta et al., 1980; Sweeny and Iacobucci, 1980). In solution, they exist in four different forms: neutral and ionized quinonoidal base, flavylium cation

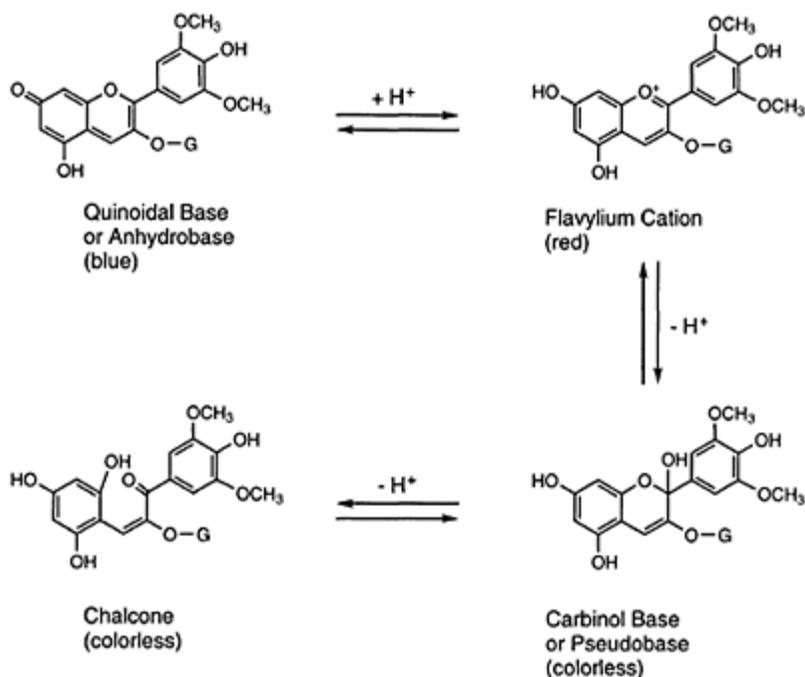


FIGURE 9.10 Chemical structures of anthocyanin chromophores. (Adapted from Timber-lake, C.F., 1980, *Food Chem.*, 5:69–90 and Wong, D.W.S., 1989, in *Mechanism and Theory in*

Food Chemistry, AVI Publishing Co.,
Westport, CT, 147–189.)

or oxonium salt, the colorless pseudobase and chalcone. The flavylium cation is weak acid, and a neutral quinoidal base behaves as a weak acid and a weak base (Brouillard, 1983; Mazza and Miniati, 1993). The distribution of these forms at equilibrium depends on pH and structure of anthocyanidin. The effect of pH on the predominant forms of anthocyanin chromophores is given in Figure 9.10. Due to the equilibrium between flavylium cation and colorless carbinol structures, most intense red coloration of anthocyanins occurs in the pH range of 1 to 3 (Hrazdina, 1981).

In slightly acidic solutions (pH 4 to 6.5), anthocyanins undergo color fading. In this range of pH, anthocyanins rapidly transform to red or blue quinoidal bases as a result of rapid proton loss of any hydroxyl groups at C-4', C-5 or C-7. Following this, nucleophilic addition of water to anthocyanins at C-2 or C-4 results in the formation of colorless carbinol structures and this equilibrates to the open, colorless chalcone forms. The reaction involves transferring the proton as well as breaking or forming the C–O bond (Brouillard, 1982, 1988; Brouillard and Dangles, 1994; Dao et al., 1998; Mazza and Miniati, 1993; Timberlake and Bridle, 1967a; Wrolsdad, 2000). Anthocyanins also show a minimum coloration at their isoelectric point (Markakis, 1960). Shifting the pH of anthocyanins to higher values may even bring about complete loss of color. Thus, alkaline conditions, in particular at high temperatures, should be avoided in the processing of anthocyanin-containing foods (Brouillard, 1982).

The change in coloration of anthocyanins may be due not only to pH but also to the formation of a weak complex with another anthocyanin molecule (self-association) or with a colorless phenolic compound such as gallotannin, quercetin, rutin or hydroxycinnamic acid derivatives (intermolecular copigmentation). Self-association brings about a greater than linear increase in the color intensity with increasing pigment concentration. The formation of anthocyanin-anthocyanin complex is primarily due to the hydrophobic interaction between aromatic nuclei of the molecules involved (Goto, 1987). The intermolecular copigmentation process brings about a shift from red to blue and increases the absorbance of the visible band, thus enhancing the color (Asen et al., 1972; Brouillard, 1982; Markakis, 1974; Mazza and Miniati, 1993; Osawa, 1982; Wrolsdad, 2000).

Anthocyanins are very unstable compounds. Loss of their color can occur during thawing of frozen vegetables and fruits as well as during processing and storage of plant products. This loss of color may be due to the hydrolysis of unstable aglycones (Shrikhande, 1976). On the hand, high temperature and pH bring about the formation of an unstable quinoidal base, carbinol pseudobase and chalcones (Brouillard, 1982, 1988; Brouillard and Dangles, 1994; Dao et al., 1998; Timberlake, 1981; Wrolsdad, 2000). Anthocyanins may also degrade by intermediate products of nonenzymatic browning reactions and by formation of copolymers with melanins (Debicki-Pospisil et al., 1982); they can form complexes with flavonoids, heavy metals and other compounds present in foods (Asen et al., 1972; Markakis, 1974; Mazza and Miniati, 1993).

Stability of anthocyanins during processing depends on the composition of food (anthocyanins, enzymatic systems, etc.) as well as temperature, pH, level of sugar, light, organic chemicals (ascorbic acid, tartaric acid, etc.) and contaminations with metal ions

(de Ancos et al., 2000; Francis, 1989; Gil et al., 1997; Macheix et al., 1989; Markakis, 1982; Wong, 1989; Wrolstad et al., 1990; Wrolstad, 2000; Zabetakis et al., 2000). The mechanism of thermal degradation of anthocyanin is not understood well, although a number of pathways have been proposed. In the presence of oxygen a brown-colored degradation product is formed. Degradation of anthocyanins depends on the temperature and duration of heat treatment. Application of HTST (high temperature-short time) processes improves the retention of anthocyanins. It has been demonstrated that loss of anthocyanins in red fruit juices is negligible when heat processing is carried out for less than 12 min at 100°C (Markakis, 1974, 1982). However, thermal treatments such as pasteurization, sterilization and concentration bring about degradation of anthocyanins (Mazza and Miniati, 1993).

The rate of anthocyanin degradation is also affected by storage temperature. A linear relationship was found between the logarithm of the rate of color loss of anthocyanins in strawberry preserves and storage temperature (Meschter, 1953). Thus, the half-life of anthocyanin color in strawberry preserves is 1 h, 240 h, 1300 h and 11 months when stored at 100, 38, 20, and 0°C. Similarly, higher storage temperatures also increased the loss of anthocyanin color in orange and blackberry juices (Figure 9.11). On the other hand, storing apples at 2°C and 70 to 80% humidity slows the loss of anthocyanins (Lin et al., 1989). Thus, the retention of anthocyanin color may be enhanced by lowering storage temperature of foods.

The loss of anthocyanins can also be minimized by storing fruits under a modified atmosphere (Gil et al., 1997; Kalitka and Skrypnik, 1983; Lin et al., 1989), high hydrostatic pressure treatment (Zabetakis et al., 2000) or freezing (de Ancos et al., 2000; Polessello and Bonzini, 1977; Wrolstad et al., 1990). Gil et al. (1997) reported that moderate carbon dioxide concentrations have little effect on the external color of strawberries; however, changes in the color of strawberry skin have been noted at carbon dioxide levels greater than 40% (Gil et al., 1997; Kader, 1986; Ke et al., 1991; Li and Kader, 1989). Furthermore, Zabetakis et al. (2000) have demonstrated that high hydrostatic pressure treatment (800 Mpa) of strawberries for 15 min reduces the loss of anthocyanins in fruits stored for 8 days at 4°C. Drying food at high temperatures brings about rapid degradation of anthocyanins (Macheix et al., 1989). Anthocyanins can be bleached by sulfur dioxide; the colorless sulfonic acid derivatives are formed as a result of addition of sulfur dioxide to the 4-position of the anthocyanin molecule, but this reaction is reversible (Timberlake and Bridle, 1967b).

OTHER FLAVONOIDS

Other flavonoids may also contribute to the color of food products. The group of yellow or ivory flavonoid pigments that exists in plants includes flavonols, flavones, chalcones, aurones, flavanones, isoflavanones and biflavonols. Although many of these compounds are colorless, under conditions of food handling and processing they can be converted to colored products.

Flavones are characterized by the presence of a double bond between C-2 and C-3, whereas flavonols are flavones with a hydroxy group in the 3-position (Figure 9.12). Many plants contain appreciable quantities of flavones or flavonols in the form of

glycosides. These phenolics may act in plant as copigments to modify the color of anthocyanins. Figure 9.13 shows four flavonol aglycons commonly found in fruits: kaempferol, quercetin, myricetin and isorhamnetin (Macheix et al., 1990). The flavonols kaempferol, quercetin and myricetin also contribute to the color of green tea (Roberts et al., 1956). Flavones and flavonols do not contribute much to the coloration of plants unless they are present in high concentrations such as in onion skins. Outer dry skin of onions contains 2.5 to 6.5% quercetin, mostly in the free aglycone form (Herrmann, 1976). Flavones and flavonols possessing orthodihydroxy groups (quercetin, luteolin) may be responsible for discoloration of foods because they are able to chelate metal ions. Iron chelates are dark in color while aluminum chelates are bright yellow or brown (Bate-Smith, 1954; Swain, 1962). In the form of bright yellow crystals, rutin, a 3-rhamnoglucoside of quercetin, occasionally precipitates on asparagus packed in glass containers (Dame et al., 1959). This is due to its limited solubility in water at room temperature. Rutin may also complex with iron to give a dark discoloration in canned asparagus.

Flavan-3-ols and flavan-3,4-diol are commonly found in plant foods (Figure 9.14). Flavan-3-ols participate in the structure of protoanthocyanidins as their monomers. Although they are colorless, under certain conditions they may cause discoloration of fruits and vegetables. They may also form dark-colored chelates with metals as well as participate in enzymatic browning reactions. Bate-Smith (1954) suggested that proanthocyanidins might participate in the formation of pinkish discoloration sometimes observed in overcooked stewed pears. Joslyn and Peterson (1956) demonstrated the presence of proanthocyanidins in Bartlett pears that yielded cyanidin. A pinkish color is often observed in pears with a higher proanthocyanidin content, especially at lower pH values (Luh et al., 1960).

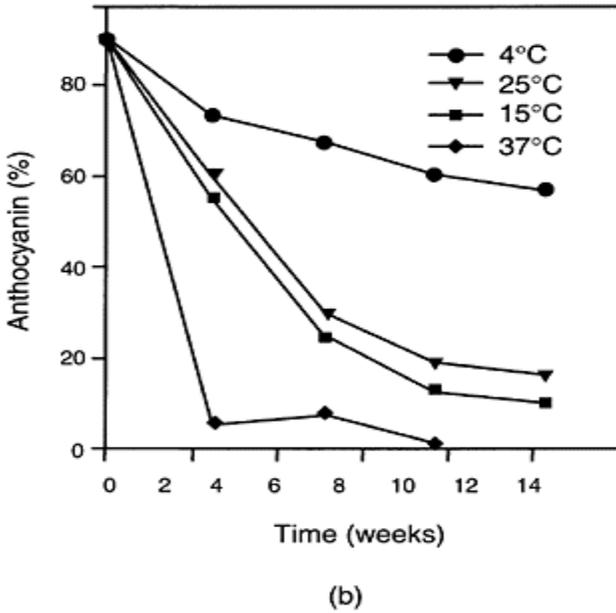
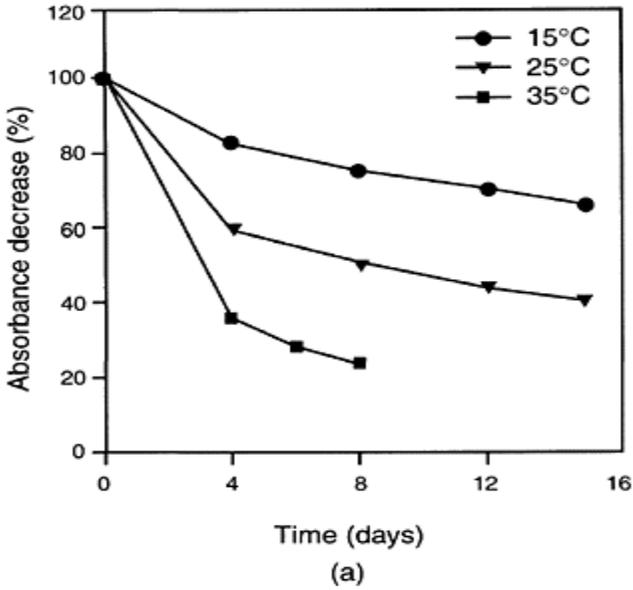
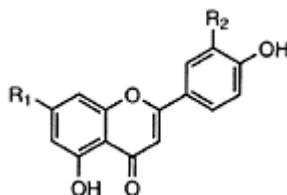


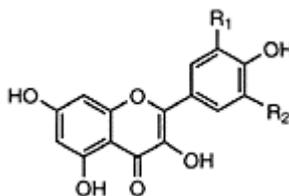
FIGURE 9.11 Effect of storage temperature on the color degradation of orange juice (a) and blackcurrant

juice (b). (Adapted from Macheix, J.-J. et al., 1989, *Fruit Phenolics*, CRC Press, Boca Raton, FL.)



| Compound | R ₁ | R ₂ |
|------------|----------------|------------------|
| Apigenin | OH | H |
| Luteolin | OH | OH |
| Tangeretin | H | OCH ₃ |

FIGURE 9.12 Chemical structures of some flavones.



| Compound | R ₁ | R ₂ |
|--------------|------------------|----------------|
| Kaempferol | H | H |
| Myricetin | OH | OH |
| Quercetin | OH | H |
| Isorhamnetin | OCH ₃ | H |

FIGURE 9.13 Chemical structures of some flavonols.

ENZYMATIC REACTION OF DISCOLORATION

A major concern to food processors and researchers working with plant foods is the presence of polyphenol oxidase (monophenol monooxygenase or tyrosinase: EC 1.14.18.1). Diphenol oxidase or catechol oxidase (EC 1.10.3.2) and laccase (EC 1.10.3.1) are also involved in enzymatic browning reactions. Producing insoluble polymers that serve as a barrier to spread of infection in living plants, these enzymes play an important role in resistance to infection by viruses, fungi, bacteria or mechanical damage (Eskin, 1990). In food products polyphenol oxidase is mainly

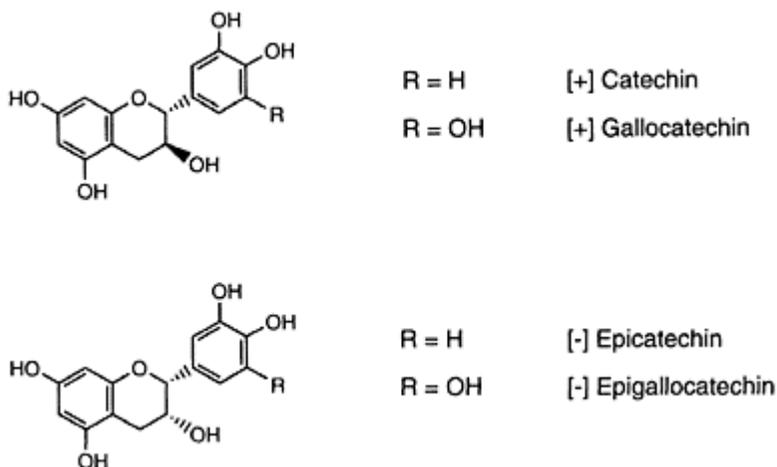


FIGURE 9.14 Chemical structures of catechins.

responsible for spoilage because it catalyzes oxidation of phenols to highly active quinones. Subsequently, quinones may react with amino and sulfhydryl groups of proteins and enzymes, as well as with anthocyanins. These secondary reactions may bring about changes in physical, chemical and nutritional characteristics of food proteins and may also affect sensory properties of food products (Mayer and Harel, 1979). Quinones may also contribute to the formation of brown pigments due to participation in polymerization and condensation reactions with proteins (Mathew and Parpia, 1971).

ENZYMATIC BROWNING REACTIONS

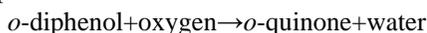
Some enzymes present in fruits and vegetables have the ability to catalyze oxidation of phenols to quinones with subsequent rapid nonenzymatic polymerization. The *o*-quinones may also modify proteins as a result of reaction with their amino and sulfhydryl groups (Matheis and Whitaker, 1984). Moreover, *o*-quinones may oxidize compounds of lower oxidation-reduction potential such as anthocyanins to colorless products (Friedman, 1996; Mathew and Parpia, 1971; Peng and Markikis, 1963). The enzyme-catalyzed oxidation of phenols leads to browning of foods such as potatoes, peaches, bananas, tea, and coffee. The enzymatic browning reaction is considered wholesome in the processing of coffee, leaf tea and dates.

The enzymatic browning reaction occurs during the ageing or senescence of fruits and vegetables or as a result of injury to plant product. Polyphenol oxidase is activated as a result of disruption of cell integrity and when the contents of plastid and vacuole are mixed (Amiot et al., 1997; Eskin, 1990). The enzymatic oxidation of flavonols plays an important role in the formation of color of fermented tea. Oxidized flavonols readily condense to theaflavins and subsequently to thearubigins. Theaflavins are yellow-orange in color whereas thearubigins, the polymeric form of oxidized flavonols, are black-brown

in color and participate in the formation of bulk color of tea (Sanderson and Graham, 1973).

The enzymatic browning reaction is considered harmful when it causes degradation of food quality due to browning discoloration or development of off-flavor and off-odor (Burnette, 1977; Friedman, 1996). A number of technological treatments, such as crushing, slicing, cutting, extraction, handling, storage conditions, low temperature, and thawing, may cause unwanted browning discoloration in fruits and plants. The “black ring” defect in canned beet root slices is associated with endogenous polyphenol oxidase activity. According to Im et al. (1990), the initiation of this discoloration requires exposure of beet slices to oxygen for a minimum time (20 min) under high-temperature treatment. Thus, thermal processing may bring about quinone participation in secondary reactions to yield dark pigmented products. Enzymes may also be responsible for dark discoloration or melanosis of shrimp and other crustacean species, thus reducing the market value of these products (Savagaon and Sreenivasan, 1978; Simpson et al., 1988).

The enzymes involved in catalyzing the reaction of *o*-diphenol oxidation are copper-containing enzymes called phenolases, polyphenolases, polyphenol oxidases, or phenol oxidases (Mathew and Parpia, 1976; Sanchez-Ferrer et al., 1995). The reaction can be represented as



Depending on the type of substrate, this enzyme can show cresolase (monophenolase) activity when monophenol is converted to *o*-diphenol and catecholase (diphenolase) activity when *o*-diphenol is oxidized to *o*-quinone (Espin et al., 1997; Itoh et al., 2001; Sanchez-Ferrer et al., 1995) (Figure 9.15). It has been demonstrated that monophenolase activity of polyphenol oxidase displays a pH-dependent lag period prior to reaching a steady state rate (Escribano et al., 1997; Espin et al., 1997). In many vegetables and fruits, polyphenol oxidase exists in the inactive or latent form (Fraignier et al., 1995; Laveda et al., 2001; Nunez-Delicado et al., 1996; Sanchez-Ferrer et al., 1993). *In vitro* activation of the latent enzyme can be brought about by treatments with surfactants (Moore and Flurkey, 1990; Nunez-Delicado et al., 1996; Sanchez-Ferrer et al., 1993), fatty acids (Golbeck and Cammarata, 1981) and proteases (Golbeck and Cammarata, 1981; Harel et al., 1973; King and Flurkey, 1987; Laveda et al., 2001), as well as exposure to low pH (Kenten, 1957). It has been postulated that the activation of the latent polyphenol oxidase may be due to its solubilization (Mayer and Harel, 1979), limited conformational changes in enzyme (Swain et al., 1966) or dissociation of an enzyme-inhibitor complex (Kenten, 1958).

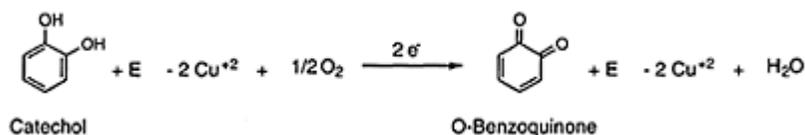


FIGURE 9.15 Enzymatic oxidation of *O*-diphenol by catecholase.

The oxidation of food phenolics may also be catalyzed by another group of enzymes in foods called peroxidases (Burnette, 1977; Nicolas et al., 1994; Williams et al., 1985). These enzymes may use polyphenols as hydrogen donors and are able to oxidize hydroxycinnamic derivatives and flavans (Nicolas et al., 1994; Robinson, 1991). Richard-Forget and Gaillard (1997) reported that in the presence of polyphenol oxidase, peroxidase enhances the oxidation of polyphenol. This enhancement may be due to the formation of hydrogen peroxide during oxidation of phenolics by polyphenol oxidase. The hydrogen peroxide may then be used by peroxidase as electron acceptor to oxidize the phenolic compounds further. The possible use of quinonoid forms by peroxidase as peroxide substrate has also been postulated.

The rate of enzymatic browning reaction depends on the nature and content of phenolic compounds, activity of phenol oxidases present in foods, presence of oxygen-reducing substances and metal ions, pH and temperature. The enzymatic browning reaction can be controlled by inactivating or inhibiting phenol oxidases, excluding oxygen, modifying or lowering the phenolic content, adding reducing substances, and interacting with the copper prosthetic group, as well as reducing or trapping quinones and even removing products of browning reactions (Friedman, 1996).

Some phenolic compounds do not serve as substrates of enzymatic browning reactions but may act as synergists or inhibitors of polyphenol oxidases (PPO) from certain sources. Shannon and Pratt (1967) reported that *p*-coumaric acid is a non-competitive inhibitor of apple PPO that catalyzes the oxidation of chlorogenic acid. Protocatechuic acid completely inhibits the browning reaction in broad beans (*Vicia faba* L.) (Robb et al., 1966), but resorcinol and phloroglucinol act as synergists of PPO-mediated browning reactions (Pratt, 1972; Shannon and Pratt, 1967). Similarly, Kahn (1976) reported that *p*-coumaric acid, *p*-cresol and protocatechuic acid arrest browning of 4-methylcatechol by avocado PPO, while phloroglucinol, orcinol and resorcinol markedly increase the browning reaction rate.

Polyphenol oxidase can be inactivated by application of heat for a sufficient time period to denature the protein. However, thermal treatment may have detrimental effects on sensorial and nutritional quality of vegetables and fruits (Lund, 1977; Sapers, 1993). Development of bitter off-flavors has been reported in avocados heated to inactivate the enzyme (Benet et al., 1973). Dimick et al. (1951) found that a temperature of 90°C is necessary for almost total inactivation of polyphenolase activity in pear puree. Arslan et al. (1998) reported approximately 50% loss in enzymatic activity by polyphenol oxidase isolated from Malaya apricot after heating the enzyme for 47 and 16 min at 60 and 80°C, respectively. Hot water or steam blanching are usually used as pretreatment of plant foods prior to freezing, canning and drying. This process retards enzymatic browning but results in the leaching of soluble materials. The time and temperature used for blanching should be carefully selected in order to avoid undesirable changes in flavor and texture (Table 9.10). Weemaes et al. (1998) investigated the effect of high hydrostatic pressures and thermal treatments on inactivation of avocado polyphenol oxidase. Inactivation of enzyme followed first-order kinetics in the pH range between 5 and 8, but displayed a deviation from first order at pH 4. The minimum pressure required to inactivate PPO ranged from 450 to 850 MPa at pH 4 and 8, respectively.

TABLE 9.10
Blanching Time in Water at 100°C for
Vegetables for Freezing

| Vegetable | Blanching Time (min) |
|--|----------------------|
| Asparagus | |
| Small (5/16 in. diam. or less at butt) | 2 |
| Medium (6/16 to 9/16 in. diam. at butt) | 3 |
| Large (10/16 in. diam. or larger at butt) | 4 |
| Peas | 1–1.5 |
| Spinach | 1.5 |
| Swiss chard | 2 |
| Beets | |
| Small, whole (1.25 in. diam. or less) | 3–5 |
| Beans | |
| Small (less than 5/16 in. diam. or sieve no. 2 or smaller) | 1–1.5 |
| Medium (5/16–6/16 in. diam. or sieve no. 3 and 4) | 2–3 |
| Large (6/16 in. diam. and larger or sieve no. 5 or larger) | 3–4 |

Source: Adapted from Potter, N.N., 1986, *Food Science*, 4th ed., Van Nostrand Reinhold, New York.

Another method of retarding enzymatic browning reaction is to lower the pH of plant food tissue because the optimum pH for most polyphenol oxidases ranges from 4.0 to 7.0 (Eskin, 1990). However, polyphenol oxidase isolated from Malaya apricot (*Prunus armeniaca* L.) toward catechol, L-dopa and gallic acid displays a maximum activity at pH 8.5 (Arslan et al., 1998). Citric, ascorbic, malic and phosphoric acids are usually employed. Recently, Soliva-Fortuny et al. (2001) reported a 62% depletion of polyphenol oxidase activity in ready-to-eat apples stored under a modified atmosphere (90.5% nitrogen+7% carbon dioxide+2.5% oxygen), which extended their shelf-life by several weeks.

Numerous chemical species have been identified as effective inhibitors of browning reaction, including

- Ascorbic acid and its derivatives (Sapers, 1993)
- 2-Mercaptoethanol, sodium metabisulfite, and thiourea (Arslan et al., 1998)
- Cysteine and cysteine derivatives (Molnar-Perl and Friedman, 1990a, b)
- Honey (Chen et al., 2000; Oszmianski and Lee, 1990)
- 4-Hexylresorcinol (Dawley and Flurkey, 1993; Gonzalez-Aguilar et al., 2000; Monsalve-Gonzalez et al., 1995)
- Oxalic acid (Son et al., 2000)
- Kojic acid (Chen et al., 1991a, b)
- Tropolone (Kahn and Andrawis, 1985)
- β -Cyclodextrin (Hicks et al., 1996)
- Protein hydrolyzates (Kahn and Andrawis, 1985)
- Proteases (Labuza et al., 1992)
- Calcium chloride (Tomas-Barberan et al., 1997)

- 2,4-Dichlorophenoxyacetic acid (Tomas-Barberan et al., 1997)
- Acetic acid (Tomas-Barberan et al., 1997)

Inhibitors may prevent browning by directly inhibiting polyphenol oxidase activity, removing phenolic substrate or reducing *o*-quinones back to *o*-diphenols (Hicks et al., 1996).

Irwin et al. (1994) demonstrated that cyclodextrins protect chlorogenic acid from oxidation by forming a 1:1 inclusion complex with the acid. Later, Hicks et al. (1996) showed that in the presence of 1 to 1.5% of soluble β -cyclodextrin, browning of apple juice held at room temperature is efficiently inhibited for 6 h. Addition of 0.25 to 0.5% of sodium hexametaphosphate inhibits the browning of apple juice for 1 day at room temperature or for up to 3 weeks at 4°C. Furthermore, 10-min stirring of apple, pear, green grape and celery juice with an insoluble β -cyclodextrin or elution of juices through the column filled up with insoluble β -cyclodextrin results in a product that is β -cyclodextrin free and also resistant to browning (Irwin et al., 1994).

Walker (1975b, 1977) found that cinnamic and *p*-coumaric acids are potent inhibitors of browning reactions in fruit juices. Less than 0.01% of cinnamic acid is required to prevent the browning reaction. Addition of sodium chloride also effectively inhibits the formation of browning reaction products in apples (Rouet-Mayer and Philippon, 1986), cherries (Benjamin and Montgomery, 1973) and grapes (Interesse et al., 1984). Sapers et al. (1990) investigated the use of vacuum and pressure infiltration as a means of applying ascorbate- and erythorbate- based enzymatic browning inhibitors to apple and potato surfaces. They found that pressure treatment brings about a more uniform uptake of solution and less extensive water logging than vacuum treatment. The apple cuts treated by pressure infiltration gained 3 to 7 days storage life, while potato plugs gained 2 to 4 days, compared to dipping.

Enzymatic browning reactions in foods of plant origin as well as discoloration (melanosis) of shrimp and other crustacean species can be prevented by sulfiting agents. Sulfites are also used to delay browning reactions in salad bar items and in prepeeled potatoes, cut apples and other fruits supplied to the baking industry (Ponting et al., 1971). The mechanism of sulfite action may be due to direct inhibition of enzymes by sulfite, or interaction of sulfites with intermediates of enzymatic browning reactions, or action of sulfite as a reducing agent, thus converting quinones back to the original phenolic compounds. The level of sulfite addition to foods depends on the nature of the phenolics present and the length of time that browning reaction should be inhibited (Taylor et al., 1986). However, increased consumer concern about the safety of sulfites has brought about a search for suitable alternatives. So far the potential substitutes are less effective and more costly (Otwell and Marshall, 1986; Taylor et al., 1986).

OTHER ENZYMATIC DISCOLORATIONS

Anthocyanins in food products are poor substrates for oxidation reactions catalyzed by phenolases. However, they may undergo other types of enzymatic degradation resulting in discoloration. Degradation of anthocyanins may be due to their hydrolysis to anthocyanidins by anthocyanases (Forsyth and Quensel, 1957; Huang, 1955, 1956).

Phenolases may indirectly accelerate degradation of anthocyanins as a result of contribution of *o*-quinones to colorless products. Peng and Markikis (1963) studied

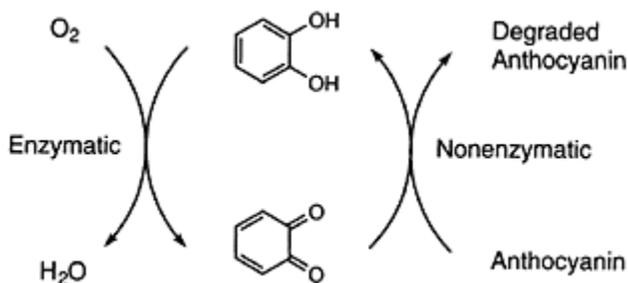


FIGURE 9.16 Possible mechanism of anthocyanin degradation by *O*-diphenol peroxidase. (Adapted from Pifferi, P.G. and Cultrera, R., 1974, *J. Food Sci.*, 39:786–791.)

the degradation of anthocyanins isolated from red tart cherries by mushroom phenolase and found that the presence of catechol accelerates their degradation. Based on the results of their study, these researchers proposed a mechanism of sequential degradation of anthocyanins involving phenolases and catechol (Figure 9.16). This reaction scheme was also confirmed by Sakamura et al. (1965), who found that eggplant anthocyanins are rapidly degraded by phenolases in the presence of catechin and chlorogenic acid. Degradation of anthocyanins may be due to their reaction with *o*-quinones or polymeric products of *o*-quinones condensation. Wesche-Ebeling and Montgomery (1990) studied the degradation of anthocyanins by strawberry polyphenolase in model systems containing D-catechin alone or in combination with pelargonidin or cyanidin. Polyphenol oxidase and D-catechin together caused the loss of 50 to 60% of anthocyanin pigments after 24 h at room temperature with the formation of a precipitate.

Similarly, flavone and flavonol glycosides remain unoxidized or are oxidized relatively slowly in the presence of oxidative enzymes such as *o*-polyphenoloxidase. However, they undergo a more rapid oxidization in the presence of transfer substances such as chlorogenic acid and catechin (Baruah and Swain, 1959).

NONENZYMATIC DISCOLORATION REACTIONS

Alkaline treatment of sunflower seeds brings about the development of a distinct color that progresses from a cream yellow to light green, dark green, and eventually brown. Thus, the conventional process of producing protein isolates from sunflower would result in dark and discolored products; this discoloration is due to the complexation of sunflower proteins with chlorogenic acid (Cater et al., 1972; Smith and Johnsen, 1948).

Addition of sunflower meal to baked products also results in the formation of gray-colored crumbs (Clandinin, 1958).

Phenols are involved in discoloration of certain potato cultivars shortly after cooking (Table 9.11). This discoloration is often referred to as “after-cooking darkening.” This is due to the formation of phenol-ferrous complexes that, upon exposure to air, turn to bluish-gray phenol-ferric complexes as cooking eliminates reducing conditions in the tuber (Heisler et al., 1964; Hughes et al., 1962; Hughes and Swain, 1962). The discoloration occurs more often in the stem end of cooked potatoes where phenols are more concentrated (Reeve et al., 1969). Mondy and Gosselin (1988)

TABLE 9.11

Effect of Irradiation^a on Relative Tendency of Certain Potato Cultivars to Darken after Cooking

| Cultivar | Type of Flesh Discoloration | |
|----------------|-----------------------------|---|
| | Soon after Boiling | After 4-h Exposure to Air |
| Kufri Alankar | Pale gray | Dark gray |
| Kufri Deva | White | Pale gray |
| Kufri Kuber | Straw yellow | Straw yellow with pale gray background |
| Kufri Sheetman | Pale gray | Medium gray |
| Kufri Sinduri | Straw yellow | Straw yellow with pale medium-gray background |

^a kRad.

Source: Adapted from Thomas, P., 1981, *J. Food Sci.*, 46:1620–1621.

found that cortex tissue of potatoes cooked with the peel discolor significantly more (< 0.05) than cortex tissues cooked without peel.

The appearance of a blue discoloration on the surface of the meat or in the coagulated blood released from the meat can greatly decrease the quality of canned or cooked crab. Discolorations ranging from blue-gray to black have been observed in canned Dungeness crab (Babbit et al., 1973a, b; Elliot and Harvey, 1951). Phenolics (Babbit et al., 1973b), iron (Waters, 1971) and copper (Babbit et al., 1975; Elliot and Harvey, 1951) are involved in the process of blueing canned crab meat. The discoloration is not considered purely enzymatic because the thermal processing of canned crab denatures the enzymes present. However, phenol oxidases present in live crab may participate in the initiation of blueing because precooking freshly caught crab greatly improves the overall quality of canned crabs (Babbit et al., 1975). According to Babbit et al. (1973b), the mechanism of blueing may involve initiation of phenol oxidation by enzymes followed by further oxidation and polymerization of phenols to colored melanins; this may proceed nonenzymatically, particularly in the presence of metals (Cu, Fe) and under alkaline conditions.

Canned green asparagus is often subjected to greenish-black discoloration of the spears and the liquid in the can—a phenomenon of great economic significance. The color change usually occurs from a few minutes to 3 hours after opening the can and is due to the formation of a pigmented rutin-ferric ion complex. The ferric ion is formed via oxidation and dissolution of iron from the can. The black discoloration is pH dependent

and does not occur when sufficient amounts of stannic ions are present in the solution (Hernandez and Vosti, 1963; Lueck, 1970).

OTHER UNDESIRABLE EFFECTS

The reaction between polyphenolic compounds and proteins may produce haze and precipitates in stored beverages such as wine, beer and fruit juice. Ferulic acid is also considered a potential off-flavor precursor in stored orange juice. Under mild acidic conditions, ferulic acid may decarboxylate to *p*-vinylguaiacol (PVG), which is considered the most detrimental component of "old-fruit" or "rotten" flavor of the juice. Ferulic acid is generally present in orange juice in the bound form (feruoylglucose, feruloyl putrescine) (Reschke and Herrmann, 1981; Wheaton and Stewart, 1965). However, the juice contains up to 200 µg/L of free ferulic acid and pasteurization may increase its content to approximately 300 µg/L. It is also possible that some free ferulic acid may be generated from the bound forms during juice storage. These small amounts of free ferulic acid are sufficient to bring about off-flavor development in the juice (Naim et al., 1988).

Phenolic acids, notably chlorogenic and caffeic acids, are also implicated in the curdling of unstabilized coffee cream in hot coffee known as feathering (Charley, 1982). Hamboyan et al. (1989) reported that the extent of feathering is not uniquely determined by the content of chlorogenic acid. However, when the amount of chlorogenic acid is considered together with a parameter that described the shape of ultraviolet spectrum in the 230- to 340-nm range, a reliable criterion for the occurrence of feathering can be obtained.

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10

Methods of Analysis and Quantification of Phenolic Compounds

INTRODUCTION

Nutritional and sensory effects of food phenolics have become the center of attention in recent years. Although these compounds generally possess beneficial health effects, they may also lower the availability of dietary proteins and minerals and affect the flavor and color of foods. No reliable method for quantification of phenolic compounds that could be applied to all food products exists; moreover, not all phenolics present in foods are of nutritional or sensory concern. Therefore, determining their total content in food cannot be considered a reliable index for phenolics'

nutritional and sensory qualities. Various assay methods for phenolic compounds have been developed and a number of reviews on the analysis of polyphenolics published (Antolovich et al., 2000; Deshpande et al., 1986; Hagerman et al., 1997; Jackman et al., 1987; Makkar, 1989; Markham, 1975; Porter, 1989; Scalbert et al., 1989; Scalbert, 1992; Tempel, 1982). These assays can be classified as those that determine total phenolic content or those quantifying a specific group or class of phenolic compounds. Folin-Denis assay and Prussian blue test are examples of methods used for total phenol determination (Salunkhe et al., 1989). The vanillin test is used as an assay method for catechins and proanthocyanidins (Price et al., 1978) while protein precipitation methods are employed for biologically active phenols (Makkar et al., 1988). A number of chromatographic techniques have also been developed to identify and quantify specific phenolics. However, only extractable polyphenolics under selected conditions are measured by all the methods described (Antolovich et al., 2000). Hillis and Swain (1959) found that, after exhaustive extraction, some additional phenolics could be extracted with change of solvent. The content of phenolic compounds, determined by various assay methods, is summarized in Table 10.1 and Table 10.2.

Analysis of polyphenolic compounds is influenced by their chemical nature, the extraction method employed, sample particle size, and storage time and conditions, as well as assay method, selection of standards and presence of interfering substances such as waxes, fats, terpenes and chlorophylls. Therefore, no completely satisfactory extraction procedure is suitable for extraction of all phenolics or a specific class of phenolic substances in foods. Solubility of polyphenolics is governed by the type of solvent (polarity) used, degree of polymerization of phenolics, interaction of phenolics with other food constituents and formation of insoluble complexes. Solvents frequently used for the extraction of polyphenolic compounds

TABLE 10.1
Phenolic Compound^a Content in Some Foods and Beverages as Determined by Different Assays

| Foods | FAS Assay^b | F-C Assay^c | Vanillin Assay^d | |
|----------------|------------------------------|------------------------------|-----------------------------------|-------|
| Spinach | | 0.15 | 0.43 | 0.15 |
| Tomato | | ND | 0.23 | 0.03 |
| Peanut | | ND | 4.3 | ND |
| Oregano | | 20.5 | 70.5 | 1.2 |
| Cinnamon | | 38.7 | 27.5 | 27.0 |
| Black tea leaf | | 105.0 | 67.7 | 92.0 |
| Green tea leaf | | 84.0 | 62.3 | 70.0 |
| Coffee powder | | 5.6 | 33.2 | 33.0 |
| Red wine | | 87.0 | 1345.0 | 990.0 |
| White wine | | ND | 181.0 | 35.0 |

Note: ND=not detected.

^a Milligrams per fresh sample or milligrams per liter.

^b FAS assay=assay for determination of iron-binding phenolic groups according to Brune, M. et al., 1991.

^c F-C assay=Folin-Ciocalteu assay according to Hoff, J.F. and Singleton, K.I., 1977.

^d Vanillin assay determined according to Price, M.L. et al., 1978.

Source: Adapted from Brune, M. et al., 1991, *J. Food Sci.*, 56:128–131; 167.

TABLE 10.2
Phenolic Content of Dry Beans as Measured by Prussian Blue and 0.5% Vanillin Methods^a

| Legume Variety | Prussian Blue | Vanillin |
|-----------------------|----------------------|-----------------|
| Cranberry | | 106 94 |
| Viva pink | | 114 181 |
| Pinto | | 153 291 |
| Small red | | 136 239 |
| Black beauty | | 113 26 |
| Dark red kidney | | 136 143 |
| Light red kidney | | 112 191 |

^a Milligrams of catechin equivalents per 100 g of beans.

Source: Adapted from Deshpande, S.S. and Cheryan, M., 1987, *J. Food Sci.*, 52:332–334.

include methanol, ethanol, acetone, water, ethyl acetate, propanol, dimethylformamide and their combinations (Antolovich et al., 2000). It is difficult to find a specific and suitable standard for quantification of polyphenolics due to the complexity of food phenolic substances as well as existing differences in the reactivity of phenols toward reagents used for their assay.

EXTRACTION PROCEDURES

Because of the chemical nature of food phenolics, no satisfactory solvent extraction systems are suitable for isolation of all classes of food phenolics or even a specific class of phenolics. These vary from simple to very highly polymerized substances that include varying quantities of phenolic acids, phenylpropanoids, anthocyanins and tannins, and possible interactions of phenolics with carbohydrates, proteins and other food components. Because some high molecular weight phenolics and their complexes are quite insoluble, the extracts always contain a mixture of different classes of phenolic substances soluble in the solvent system of choice. Usually, additional steps are required in order to purify the isolates and to remove unwanted phenolics and nonphenolic substances.

Krygier et al. (1982) extracted free and esterified phenolic acids from oilseeds using a mixture of methanol-acetone-water (7:7:6, v/v/v) at room temperature. First, the free phenolics were extracted with diethyl ether, then the extract was treated with 4M NaOH under nitrogen. The hydrolyzate was acidified and the liberated phenolic acids were extracted with diethyl ether. After exhaustive extraction with a mixture of methanol-acetone-water, the left-over sample was treated with 4M NaOH under nitrogen to liberate insoluble bound phenolic acids. On the other hand, Kozłowska et al. (1983) extracted phenolic acids with hot 80% methanol (See Figure 10.1). Other solvents, such as ethanol and acetone, have also been used for the extraction of phenolics, often with different proportions of water (Antolovich et al., 2000; Naczek et al., 1992a).

Anthocyanins are usually extracted with an acidified organic solvent, most commonly methanol. This solvent system destroys cell membranes and simultaneously dissolves the anthocyanins. The acid also provides favorable conditions for formation of flavylium chloride salt, thus stabilizing the anthocyanins. However, according to Moore et al. (1982a, b), the acid may change the native form of anthocyanins by breaking down their complexes with metals and co-pigments. The acidic extracts of anthocyanins are first concentrated under vacuum and then extracted with petroleum ether, ethyl acetate or diethyl ether in order to remove lipids and unwanted polyphenols (Fuleki and Francis, 1968a, b). The pigment can also be partially purified using ion exchange resins, as described by Fuleki and Francis (1968c).

Concentration of extracts of pigments in acidic solutions before purification, however, may bring about losses of labile acyl and sugar residues (Adams, 1972). In order to avoid this detrimental effect, several researchers have proposed reducing the acid contact (Du and Francis, 1975), using neutral organic solvents or boiling water (Adams, 1972), or using weak organic acids such as formic or acetic acid to acidify the solvents (Antolovich et al., 2000; Moore et al., 1982a, b). Anthocyanins

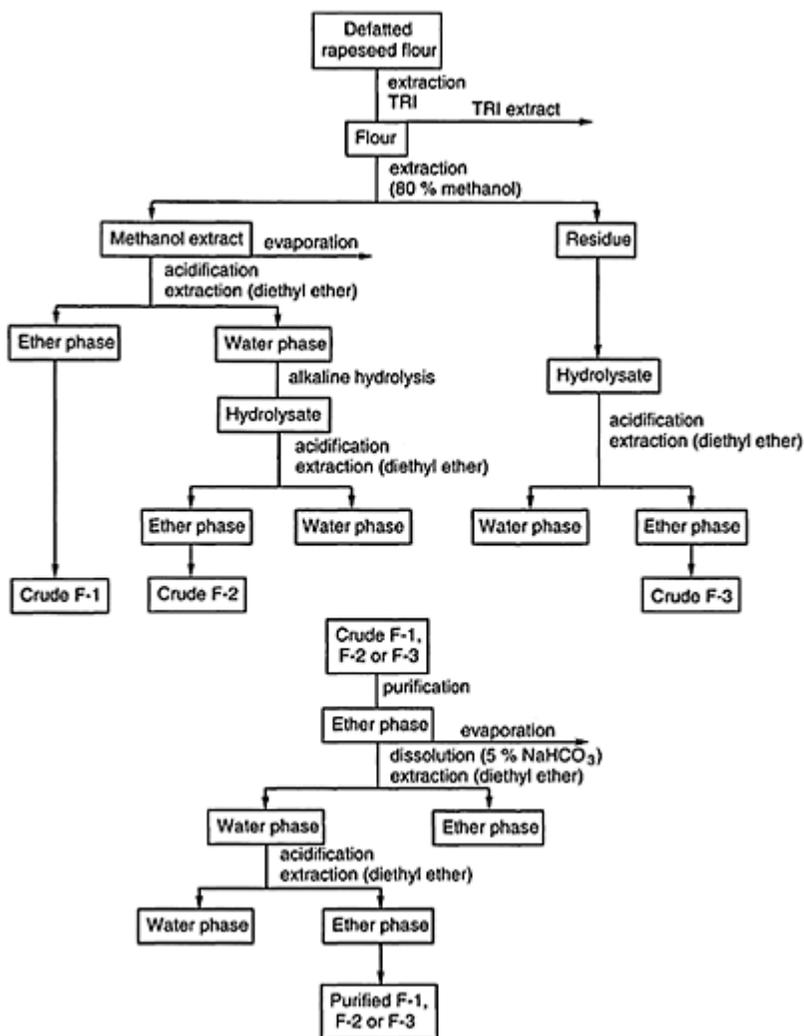


FIGURE 10.1 A simplified flow diagram for fractionation of free, esterified and bound phenolic compounds and their hydrolysis to free phenolic acids. TRI=trichloroethylene; F-1 = free phenolic acids; F-2=esterified phenolic acids; and F-3=insoluble phenolic acids. (Adapted from Krygier, K. et al., 1982, *J. Agric. Food Chem.*, 30:330–333.)

may also be recovered using solid-phase extraction (SPE) or solid-phase micro-extraction (SPME) on C₁₈ cartridges. Hong and Wrolstad (1990) fractionated anthocyanins by elution through an SPE cartridge with an alkaline borate solution. Only anthocyanins with o-dihydroxy groups (cyanidin, delphinidin and petunidin) were

TABLE 10.3

Effect of HCl Addition and Boiling on Recovering Total Phenolics and Tannins from Canola Meal

| Solvent System | Total Phenolics | Tannins |
|--|-----------------|---------|
| 70% Methanol | 847.0 | 241.3 |
| 1% HCl in methanol | 892.1 | 73.0 |
| 1% HCl in 70% methanol | 1079.8 | 225.9 |
| 1% HCl in methanol+4 min of boiling | 1053.3 | 112.5 |
| 70% Acetone | 805.8 | 321.3 |
| 1% HCl in 70% acetone | 1010.7 | 216.9 |
| 1% HCl in 70% acetone+4 min of boiling | 1051.6 | 338.7 |

Notes: Total phenolics are expressed as milligrams of sinapic acid equivalents per 100 g of meal; tannin content is expressed as milligrams of catechin equivalents per 100 g of meal.

Source: Adapted from Naczk, M. and Shahidi, F., 1991, presented at 8th International Rapeseed Congress, Saskatoon, Canada, July 9–11, 1991.

preferentially eluted from the SPE cartridge with borate solution due to the formation of hydrophilic borate-anthocyanin complex. On the other hand, Wang and Sporns (1999) isolated anthocyanins from fruit juices and wine using an SPE cartridge and elutions with a methanol-formic acid-water (70:2:28, v/v/v) solvent system.

For the extraction of condensed tannins, several solvent systems have been used. Absolute methanol, ethanol, acidified methanol, acetone, water, and their combinations are among the most commonly used solvent systems. For example, 1% HCl in methanol has been used for the extraction of tannins from sorghum (Price et al., 1978) and dry beans (Deshpande and Cheryan, 1985). Acetone-water (70:30, v/v) solution is the best solvent system for the extraction of tannins from rapeseed and canola (Table 10.3) (Leung et al., 1979; Shahidi and Naczk, 1989, Naczk and Shahidi, 1991), beach pea (*Lathyrus maritimus* L.) (Chavan et al., 2001; Shahidi et al., 2001) and blueberries (Prior et al., 2001). Acetone-water (60:40, v/v) solution is used for extraction of tannins from cider apple (Guyot et al., 2001) and grape skins (Labarbe et al., 1999). Tannins of cloves and allspice have been extracted with boiling water over a 2-h period (AOAC, 1980), a method also used to determine the content of tannins in rapeseed meal (Kozłowska et al., 1990).

The extraction period is another factor that affects the recovery of polyphenolics. Extraction periods varying from 1 min (Price and Butler, 1977) to 24 h (Burns, 1971; Maxson and Rooney, 1972) have been reported. However, longer extraction times increase the possibility of oxidation of phenolics unless reducing agents are added to the solvent system (Khanna et al., 1968). Price et al. (1978) reported that rotating the test tube containing suspensions of samples in a given solvent system is sufficient to extract

tannins from sorghum. However, Deshpande (1985) has demonstrated that the optimum extraction time required for dry bean phenolics is 50 to 60 min. On the other hand, Naczek and Shahidi (1991) and Naczek et al. (1992a) found that a two-stage extraction with 70% (v/v) acetone, 1 min each at 10,000 rpm using a

TABLE 10.4

Effect of Number of Extraction Steps on Recovery of Total Phenolics and Tannins

| Number of Extraction Steps | 70% Acetone | | 70% Methanol | |
|----------------------------|-----------------|---------|-----------------|---------|
| | Total Phenolics | Tannins | Total Phenolics | Tannins |
| 1 | 720.0 | 268.1 | 837.4 | 127.7 |
| 2 | 805.8 | 321.3 | 847.0 | 241.3 |
| 4 | 972.1 | 328.0 | 1025.7 | 243.8 |
| 6 | 1075.0 | 331.3 | 1081.0 | 235.5 |

Notes: Total phenolics are expressed as milligrams of sinapic acid equivalents per 100 g of meal; tannin content is expressed as milligrams of catechin equivalents per 100 g of meal.

Source: Adapted from Naczek, M. and Shahidi, F., 1991, presented at 8th International Rapeseed Congress, Saskatoon, Canada, July 9–11, 1991.

TABLE 10.5

Effect of the Canola Meal-to-Solvent Ratio (R) on Recovery of Total Phenolics and Tannins

| R | 70% Acetone | | 70% Methanol | |
|------|-----------------|---------|-----------------|---------|
| | Total Phenolics | Tannins | Total Phenolics | Tannins |
| 1:5 | 773.5 | 257.3 | 844.3 | 188.3 |
| 1:0 | 805.8 | 321.3 | 874.0 | 241.3 |
| 1:20 | 948.0 | 324.6 | 886.7 | 275.4 |

Note: Total phenolics are expressed as milligrams of sinapic acid equivalents per 100 g of meal; tannin content is expressed as milligrams of catechin equivalents per 100 g of meal.

Source: Adapted from Naczek, M. and Shahidi, F., 1991, presented at 8th International Rapeseed Congress, Saskatoon, Canada, July 9–11, 1991.

Polytron homogenizer, is sufficient for extraction of tannins from commercial canola meals. Further extraction (up to six stages) only marginally enhances the yield of extraction of other phenolic compounds (Table 10.4).

The recovery of polyphenols from food products is also influenced by the ratio of sample to solvent (R) (Table 10.5). Naczek and Shahidi (1991) and Naczek et al. (1992a) found that changing R from 1:5 to 1:10 increases the extraction of condensed tannins from commercial canola meals from 257.3 to 321.3 mg/100 g of meal and total phenolics from 773.5 to 805.8/100 g of meal when using 70% acetone.

Deshpande and Cheryan (1985) demonstrated that the yield of tannin recovery from dry beans is strongly influenced by variations in the sample particle size. They found that 0.5% vanillin-assayable tannins (Price et al., 1978) decrease by about 25 to 49% as the minimum size is reduced from 820 to 250 μ (Table 10.6).

TABLE 10.6
Effects of Particle Size on Tannin Analysis of
Dry Beans Using 0.5% Vanillin Assay^a

| Minimum Particle Size (μ) | Bean Variety | | | |
|---------------------------------|--------------|-----------|-----------|--------------|
| | Pinto | Small Red | Viva Pink | Black Beauty |
| 820 | 337 | 304 | 169 | 154 |
| 420 | 269 | 262 | 111 | 172 |
| 350 | 245 | 250 | 105 | 213 |
| 250 | 223 | 227 | 87 | 258 |

^a Milligrams of catechin equivalents per 100 g of beans, on dry basis.

Source: Adapted from Deshpande, S.S. and Cheryan, M., 1985, *J. Food Sci.*, 50:905–910.

SPECTROPHOTOMETRIC ASSAYS

A number of spectrophotometric methods for quantification of phenolic compounds in plant materials have been developed. Based on different principles, these assays are used to determine various structural groups present in phenolic compounds. Spectrophotometric methods may quantify all extractable phenolics as a group (Earp et al., 1981; Price and Butler, 1977; Swain and Hillis, 1959) or may determine a specific phenolic substance such as sinapine (Tzagoloff, 1963), or sinapic acid (Naczki et al., 1992b), or a given class of phenolics such as phenolic acids (Brune et al., 1991; Mole and Waterman, 1987a, b; Naczki and Shahidi, 1989; Price et al., 1978). The modified vanillin test (Price et al., 1978), Folin-Denis assay (Swain and Hillis, 1959), Prussian blue test (Price and Butler, 1977) and Folin-Ciocalteu assay (Deshpande and Cheryan, 1987; Earp et al., 1981; Hoff and Singleton, 1977; Maxson and Rooney, 1972) are most frequently used.

DETERMINATION OF TOTAL PHENOLICS

Folin-Denis Assay

The Folin-Denis assay is the most widely used procedure for quantification of total phenolics in plant materials and beverages. Reduction of phosphomolybdic-phosphotungstic acid (Folin-Denis) reagent to a blue-colored complex in an alkaline solution occurs in the presence of phenolic compounds (Folin and Denis, 1912). Swain and Hillis (1959) modified this method for routine analysis of a large number of samples. The Folin-Denis assay is also used for determination of total phenolics using the AOAC method (1980). This procedure is outlined in Figure 10.3.

Total phenolics are assayed colorimetrically by the method of Swain and Hillis (1959): to 0.5 to 1.0 mL of phenolics solution, 7 mL of distilled water are added and mixed well, followed by the addition of 0.5 mL of Folin-Denis reagent. After 3 min standing, 1 mL of saturated sodium bicarbonate solution is added and the total volume is made up to 10 mL with distilled water. After thorough mixing, the

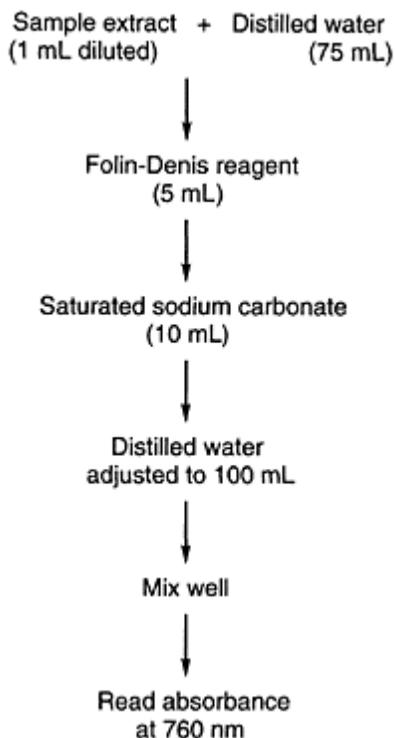


FIGURE 10.2 Procedure for tannin analysis by the Folin-Denis method. (Adapted from Salunkhe, O.K. et al., 1989, *Dietary Tannins: Consequences and Remedies*, CRC Press, Boca Raton, FL.)

absorbance is read at 725 nm after 1 h standing at room temperature. A mixture of water and reagents is used as a blank.

Folin-Ciocalteu Assay

The Folin-Ciocalteu assay is often used to determine the total content of food phenolics (Brune et al., 1991; Deshpande and Cheryan, 1987; Earp et al., 1981; Hoff and Singleton, 1977; Maxson and Rooney, 1972). Folin-Ciocalteu reagent is not specific and detects all phenolic groups found in extracts including those found in the extractable proteins. A disadvantage of this assay is the interference of reducing substances such as ascorbic acid with the determinations.

The total phenolics are assayed colorimetrically as modified by Singleton and Rossi (1965) and Hoff and Singleton (1977): 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent,

2 mL of a 7.5% solution of sodium carbonate, and 0.5 mL of phenolics solution are mixed well. The absorbance is measured at 765 nm after a 15-min heating at 45°C; a mixture of water and reagents is used as a blank. The content of phenolics is expressed as gallic acid or catechin equivalents.

Prussian Blue Assay

The Prussian blue method was suggested by Price and Butler (1977) for determination of phenolics from sorghum grain and was later reevaluated by Deshpande and Cheryan (1987) for dry beans. Reduction of ferric to ferrous ion by polyphenolic compounds and formation of ferricyanide-ferrous ion complex, also called Prussian blue, is involved. The ability of polyphenolic compounds to reduce ferric ion depends on their hydroxylation pattern and the degree of polymerization. According to Deshpande and Cheryan (1987), tannic acid is about 25% more effective reducing ferric ion than catechin because tannic acid has more phenolic hydroxyl groups than catechin does. This method's sensitivity towards flavonoids is sufficient to measure concentrations of less than 10^{-4} M. The possibility of interference from sulfides, aromatic amines, unsaturated aliphatics, glucose and ascorbic acid that may reduce ferricyanide (Snell and Snell, 1953), as well as organic solvents (Price and Butler, 1977), should be considered.

Price and Butler (1977) and Deshpande and Cheryan (1987) reported that the content of polyphenolics obtained by the Prussian test is always somewhat lower than that obtained by the vanillin assay (Table 10.2). These authors suggested that catechin used as a standard in the vanillin test may lead to overestimation of results. Furthermore, the Prussian blue test may also underestimate the results if polyphenolics are more oxidized than catechin or less reactive than the standard monomer.

The Prussian blue procedure involves a 1-min rapid extraction of phenolic compounds with water (Price and Butler, 1977). Subsequently, an aliquot of the extract is diluted with distilled water in order to eliminate any interfering color. Then an excess of ferric chloride in 0.1 M HCl (added to increase stability) is added to ensure rapid and complete reaction. Due to inherent instability of diluted ferric chloride solution, ferricyanide should be added immediately.

The polyphenolic compounds are assayed colorimetrically by the method of Price and Butler (1977): to 6 mL of aqueous solution of phenolics, 50 mL of distilled water is added followed by mixing. To this, 3 mL 0.1 M of ferric chloride is added followed immediately by timed addition of 3 mL of 0.008 M potassium ferricyanide solution. The absorbance at 720 nm is read after 10 min standing at room temperature. Distilled water is used as a blank.

DETERMINATION OF CONDENSED TANNINS

Vanillin Assay

The vanillin method is widely used for quantification of proanthocyanins (condensed tannins) in fruits (Goldstein and Swain, 1963a) and grains (Burns, 1971). This assay has been critically reevaluated for detection of tannins in sorghum (Price et al., 1978), dry beans (Deshpande and Cheryan, 1985), forage legumes (Broadhurst and Jones, 1978) and

canola meals (Naczek and Shahidi, 1991), as well as catechins and proanthocyanidins (Sun et al., 1998a). The vanillin test is specific for flavan-3-ols, dihydrochalcones and proanthocyanins, which have a single bond at the 2,3-position and possess free meta-hydroxy groups on the B-ring (Sarkar and Howarth, 1976; Gupta and Haslam, 1980). Catechin, a monomeric flavan-3-ol, is often used as a standard in the vanillin assay. According to Price et al. (1978) and Gupta and Haslam (1980), this may lead to overestimation of tannin contents.

TABLE 10.7
Response of Some Flavonoid and Chromone
Compounds to Vanillin Test

| Class | Compound | Reaction with Vanillin |
|------------------|--|------------------------|
| Flavonols | Catechin (5,7,3',4'-tetrahydroxyflavan-e-ol) | +++ |
| Dihydrochalcones | Phoretin (4,2',4',6'-tetrahydroxydihydrochalcone) | +++ |
| Chalcone | Butein (3,4,2',4'-tetrahydroxychalcone) | - |
| Flavanones | Naringenin (5,7,4'-trihydroxyflavanones) | - |
| Flavones | 7-Hydroxyflavone | - |
| Flavanonol | Dihydroxyquercetin | + |
| Flavanols | Kaempferol (5,7,4'-trihydroxyflavanol) Quercetin (5,7,3',4'-tetrahydroxyflavanol) | - |
| Chromones | Eugenin (2-methyl-5-hydroxy-7-methoxychromone) | - |

Notes: +++=very positive; +=positive; and -=negative.

Source: Adapted from Sarkar, S.K. and Howarth, R.E., 1976, *J. Agric. Food Chem.*, 24:317-320. With permission.

Methanol, a usual solvent used for vanillin test, may affect the kinetics of catechin and tannin reactions with vanillin differently. The vanillin assay in methanol is more sensitive to polymeric tannins than monomeric flavan-3-ols; due to its simplicity, sensitivity and specificity, it is generally recognized as a useful method for the detection and quantification of condensed tannins in plant materials. The method can be used for quantifying condensed tannins in the range of 5 to 500 µg with precision and accuracy of greater than 1 µg when the optimum concentrations of reactants and solvents are selected (Broadhurst and Jones, 1978).

The possibility of interference with dihydrochalcones and anthocyanins (Sarkar and Howarth, 1976) when determining tannins, as well as ascorbic acid or ascorbate and chlorophylls (Broadhurst and Jones, 1978; Sun et al., 1998a), should be considered (Table 10.7). At usual concentrations present in plant extracts, anthocyanins do not react with vanillin (Broadhurst and Jones, 1978; Sun et al., 1998a). However, their presence in phenolic extract may lead to overestimation of tannins because maximum absorption of tannin-vanillin adducts coincides with that of anthocyanins. This interference can be eliminated by the use of an appropriate blank (Broadhurst and Jones, 1978).

Ascorbic acid or ascorbate interferes with the vanillin assay in the presence of sulfuric acid, which may be due to the oxidative nature of sulfuric acid (Broadhurst and Jones, 1978). Sun et al. (1998b) described a simple procedure for the removal of ascorbic acid

and ascorbates from wine and grape juice by fractionation with C₁₈ Sep-Pak cartridges. Chlorophyll-like anthocyanins also absorb in the region that coincides with the maximum absorption displayed by tannin-vanillin pigment. Therefore, presence of chlorophylls in plant phenolic extracts may lead to overestimation of tannins. This interference may be eliminated by hexane extraction of chlorophylls from the aqueous phenolic solution (Sun et al., 1998a).

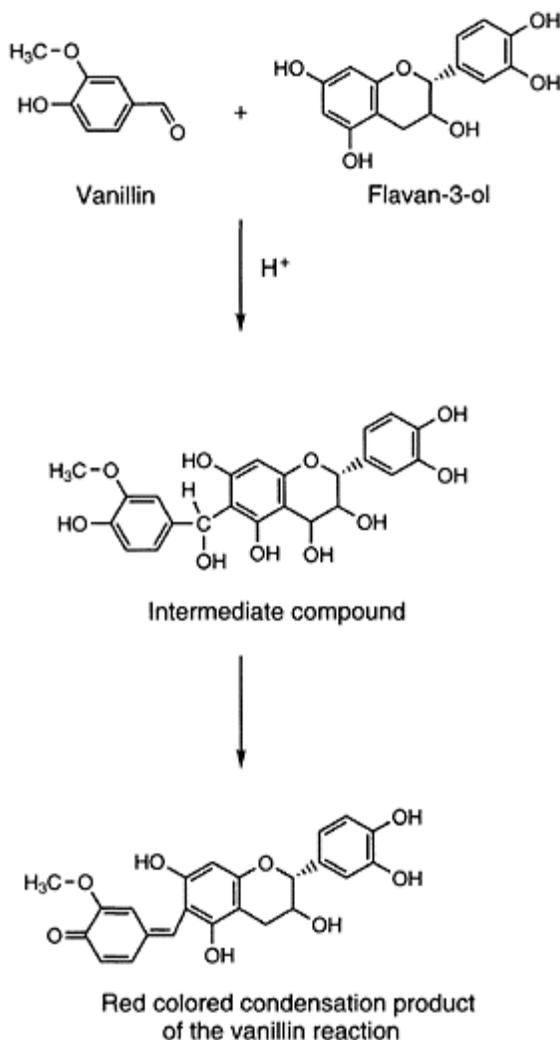


FIGURE 10.3 Condensation reactions of vanillin with leucocyanidin. (Adapted from Salunkhe, O.K. et al., 1989, *Dietary Tannins: Consequences*

and Remedies, CRC Press, Boca Raton, FL.)

The vanillin method is based on condensation of the vanillin reagent with proanthocyanins in acidic solutions. Protonated vanillin, a weak electrophilic radical, reacts with the flavonoid ring at the 6- or 8-position. The intermediate product of this reaction dehydrates readily to give a light pink to deep red cherry-colored product (Figure 10.2). The vanillin reaction is affected by the acidic nature and concentrations of substrate, the reaction time, the temperature, the vanillin concentration and water content (Sun et al., 1998a). Improvement in carbonium ion stability may be responsible for an increase in sensitivity (Scalbert, 1992) of vanillin assay when sulfuric acid is used rather than hydrochloric acid (Scalbert et al., 1989; Sun et al., 1998a). However, high acid concentrations should be avoided because they may promote self-reaction of vanillin and decomposition of proanthocyanidins (Broadhurst and Jones, 1978; Beart et al., 1985; Scalbert, 1992). According to Sun et al. (1998a), the

TABLE 10.8
Molar Extinction Coefficient of Phenolic Complexes with Vanillin

| Compound | $\epsilon_{500} \times 10^{-3}$ in Methanol | $\epsilon_{500} \times 10^{-3}$ in Acetic Acid |
|--------------------------------|---|--|
| Monomers: | | |
| Epicatechin | 0.43 | 9.8 |
| Catechin | 0.30 | 8.5 |
| Dimers: | | |
| B-2 (epicatechin) ₂ | 2.52 | 11.2 |
| B-3 (catechin) ₂ | 1.82 | 12.3 |
| Trimers: | | |
| B-9 (epicatechin) ₃ | 4.16 | 12.2 |

Source: Adapted from Butler, L.G. et al., 1982, *J. Agric. Food Chem.*, 30:1087–1089.

vanillin concentration used in the vanillin assay should be between 10 and 12 g/L. At higher vanillin concentrations self-reaction of vanillin may lead to the formation of colored products (Broadhurst and Jones, 1978). Furthermore, the color stability of vanillin-tannin adducts may increase when light is excluded and the temperature of the reaction is controlled (Broadhurst and Jones, 1978). Thus, to obtain accurate and reproducible results, the vanillin assay must be conducted at a fixed temperature (Gupta and Haslam, 1980; Sun et al., 1998a).

The reaction of vanillin with phenolics containing meta-oriented di- and trihydroxy groups on the benzene rings is approximately stoichiometric (Goldstein and Swain, 1963b). The solvent used in the vanillin assay may also affect the reaction kinetics. In methanol, the time course of the vanillin reaction with catechin is different from that with tannins; however, use of glacial acetic acid in place of methanol affords similar kinetics for the monomers and polymers. This, according to Butler et al. (1982), suggests that vanillin reacts only with the terminal groups in glacial acetic acid and thus the

concentration of oligomeric molecules rather than the total content of flavan-3-ols is measured. Table 10.8 depicts the molar extinction coefficients of vanillin monomer and vanillin oligomer adducts.

The aforementioned authors proposed that vanillin assay in glacial acetic acid may be used for estimating the degree of polymerization of condensed tannins. The procedure involves extraction of phenolic compounds with methanol (Burns, 1971), 1% concentrated HCl in methanol (Price et al., 1978), and 70% acetone (Broadhurst and Jones, 1978; Shahidi and Naczki, 1989). The sample should be extracted within 1 day after grinding. Extracts are directly assayed or the supernatants are first evaporated to dryness at 30°C under vacuum and the residues then dissolved in methanol. The vanillin assay is performed at 30°C; vanillin reagent must be prepared freshly each day.

The condensed tannins are assayed colorimetrically by the method of Price et al. (1978): to 1 mL of methanolic solution of condensed tannins, 5 mL of freshly prepared 0.5% vanillin solution in methanol containing 4% concentrated HCl (sample) or 5 mL of 4% concentrated HCl in methanol (blank) are added and mixed well. The absorbance of the sample or the blank is then read at 500 nm, after 20 min standing at 30°C. The condensed tannins are expressed as milligrams of catechin equivalents per 100 g of sample.

DMCA (4-(Dimethylamino)-Cinnamaldehyde) Assay

The formation of a green chromophore between catechin and 4-(dimethylamino)cinnamaldehyde (DMCA) was first reported by Thies and Fisher (1971). Subsequently, McMurrugh and McDowell (1978), Delcour and de Varebeke (1985) and Treutter (1989) demonstrated that DMCA does not react with a wide range of flavonoids including dihydrochalcones, flavanones and flavononols, and phenolic acids. Weak responses are detected for resorcinol, orcinol, naphtoresorcinol, and phloretin. Treutter (1989) also reported that DMCA reacts with indoles and terpenes. Later, Li et al. (1996) reexamined the use of the DMCA assay for determination of proanthocyanidins in plants. According to these authors, on a molar basis, the sensitivity of DMCA toward proanthocyanidins is 4 and 30,000 times greater than that of indole and thymol, respectively.

In comparison to the vanillin reaction carried out in methanol, the DMCA reagent reacts only with the terminal groups of condensed tannins. The presence of methanol, acetone, ethyl acetate, and dimethylformamide does not have any detrimental effect on the rate and intensity of the color development. However, the DMCA reagent is sensitive to monomeric and to polymeric units (McMurrugh and McDowell, 1978), which may lead to overestimation of condensed tannin content.

The colorimetric assay of condensed tannins using the DMCA reagent described by McMurrugh and McDowell (1978) and Thies and Fisher (1971) is: 1 mL of diluted (up to 1:10, v/v) methanolic solution of condensed tannins is mixed with 5 mL of DMCA reagent. The absorbance of the sample is read at 635 nm, after 15 min standing at room temperature, using an appropriate blank. The DMCA reagent is prepared by dissolving 1 g of DMCA in a precooled solution containing 250 mL of concentrated HCl and 700 mL of methanol. Following this, the solution is made up to 1 L with methanol. The DMCA reagent is stable for at least 1 week when stored in the dark.

Proanthocyanidin Assay

The proanthocyanidin assay is carried out in a solution of butanol-concentrated hydrochloric acid (95:5, v/v). In the presence of this hot HCl, proanthocyanidins (condensed tannins) are converted to anthocyanidins that are products of autoxidation of carbocations formed by cleavage of interflavanoid bonds (Porter et al., 1986). The yield of reaction depends on the content of HCl and water, temperature and length of the reaction period, presence of transition metals, and length of the proanthocyanidin chain (Porter et al., 1986; Scalbert et al., 1989). Govindarajan and Mathew (1965) reported that water content of up to 20% does not affect the yield of antho-cyanidins. Later, Porter et al. (1986) demonstrated that the maximum yield of antho-

cyanidins is obtained at a 6% (v/v) level of water in the solvent. Both lower and higher contents of water have a detrimental effect on the formation of anthocyanidins. The reproducibility and the yield of conversion of proanthocyanidin to anthocyanidins are enhanced in the presence of transition metals. Porter et al. (1986) investigated the effect of several transition metals on the formation of anthocyanidins. These authors found that Fe^{+2} and Fe^{+3} are the most effective catalysts. The maximum yield of anthocyanidin is achieved at Fe^{+3} concentration of 7×10^{-4} M. Scalbert et al. (1989) reported that the reproducibility of the method is significantly improved if the reaction temperature is strictly controlled.

The colorimetric assay of condensed tannins by the proanthocyanidin procedure described by Mole and Waterman (1987a) is: to 1 mL of methanolic solution of condensed tannins, add 10 mL of 1-butanol-HCl reagent. This mixture is heated in sealed ampules for 2 h in a boiling water bath and then allowed to cool in an ice-water bath. The absorbance of the solution is read at 550 nm against a reagent blank. For $A > 0.75$, the reaction mixture is diluted with 1-butanol. The 1-butanol-HCl reagent is prepared by dissolving 0.7 g of ferrous chloride heptahydrate in 25 mL of concentrated HCl containing a small amount of 1-butanol; this solution is then made up to 1 L with 1-butanol.

DETERMINATION OF INSOLUBLE CONDENSED TANNINS

Insoluble condensed tannins were first identified in herbs by Bate-Smith (1973a). Subsequently, Terrill et al. (1992) fractionated condensed tannins into tannins soluble in organic solvents, tannins soluble in solution of sodium dodecyl sulfate (SDS), and insoluble tannins. The procedure proposed by these authors included the extraction of soluble tannins with a mixture of acetone-water-diethyl ether (4.7:2.0:3.3, v/v/v), followed by the extraction of SDS-soluble tannins with a boiling aqueous solution of SDS containing 2-mercaptoethanol. The insoluble tannins were then determined directly on the remaining residue after extraction of SDS-soluble tannins by using the proanthocyanidin assay. The residue was subjected to one-step treatment with butanol-HCl; this treatment with butanol-HCl was also used by Makkar et al. (1997), Degen et al. (1995) and Makkar and Singh (1991) for determining insoluble condensed tannins in forages. Later, Makkar et al. (1999) reported incomplete recovery of insoluble condensed tannins after one-step treatment of sample with butanol-HCl. These authors used solid state ^{13}C NMR as a probe for the measurement of bound condensed tannins. Recently, Naczek et al. (2000) investigated a multistep treatment of canola hulls with butanol-HCl

and reported that, with butanol-HCl, a six-step extraction of hulls free of soluble tannins removes most of the insoluble condensed tannins as anthocyanidins.

DETERMINATION OF HYDROLYZABLE TANNINS

Various approaches have been used for convenient screening of a large number of plant samples for their hydrolyzable tannins. Of these, the most widely used method is based on the reaction between potassium iodate and hydrolyzable tannins. This reaction was first described by Haslam (1965) and later utilized by Bate-Smith (1977) for the development of an analytical assay for estimation of hydrolyzable tannins in plant materials. The original assay included mixing the sample and potassium iodate, cooling the mixture in an ice-water bath for 40 min (Haslam, 1965) or 90 min (Bate-Smith, 1977) and measuring the absorbance at 550 nm. Later, Willis and Allen (1998) demonstrated that chilling the samples after addition of potassium iodate is unnecessary. These authors recommended carrying out the reaction at 25°C for a time unique for each type of plant material.

Several limitations have been reported for the original assay, including variability in the reaction time required for different tannins for maximum color development, variability in spectral properties of chromophores formed from different hydrolyzable tannins, formation of yellow oxidation products by nontannin phenolics, the possibility of precipitate formation and difficulties in reproducibility of the results (Inoue and Hagerman, 1988; Hartzfeld et al., 2002; Waterman and Mole, 1994).

Recently, Hartzfeld et al. (2002) modified this assay by including a methanolysis step followed by oxidation with potassium iodate. The assay is based on gallic acid, which is a common structural component found in gallotannins and ellagitannins. Methyl gallate, formed upon methanolysis of hydrolyzable tannins in the presence of strong acids, reacts with potassium iodate to produce a red chromophore with a maximum absorbance at 525 nm. The oxidation reaction is carried out at 30°C for 50 min at pH 5.5. The detection limit of the method is 1.5 µg of methyl gallate. Methanol and acetone stabilize the chromophore, while the presence of water accelerates degradation of the resultant pigment. According to Hartzfeld et al. (2002), the modified potassium iodate method provides a good estimate for gallotannins, but underestimates the content of ellagitannins.

Other analytical assays proposed for quantification of hydrolyzable tannins in plant materials include the rhodanine (Inoue and Hagerman, 1988) and the sodium nitrate (Wilson and Hagerman, 1990) methods. The rhodanine assay may be used for estimating gallotannins and is based on determination of gallic acid in a sample subjected to acid hydrolysis under anaerobic conditions. The sodium nitrate assay was developed for quantitative determination of ellagic acid in sample hydrolysates, but requires large quantities of pyridine as a solvent.

DETERMINATION OF SINAPINE

Sinapine, a choline ester of sinapic acid, has been reported in seeds of *Brassica napus*, *Brassica campestris* and *Crambe abyssinica* (Austin and Wolff, 1968; Clandinin, 1961). Methods for estimating sinapine are based on the formation of a water-insoluble complex between Reinecke salt and quaternary nitrogen base (Austin and Wolff, 1968; Tzagoloff,

1963) and formation of a colored complex between sinapine and titanium chloride (Ismail and Eskin, 1979; Eskin, 1980).

Reinecke Salt Assay

This procedure involves three extractions with hot methanol (25, 15, and 15 mL per extraction, respectively) of 1 g of ground, defatted meal. The extracts are then combined, the solvent evaporated under reduced pressure, and the residue suspended in water and filtered through analytical grade Celite (Austin and Wolff, 1968). A 5-mL clear aliquot of the extract is chilled in an ice bath for a few minutes, then 1 mL of a 50% saturated aqueous solution of Reinecke salt (not more than 2 days old) is added and the mixture is allowed to stand in an ice bath for 1 h. The precipitate is then centrifuged at 1750×g for 15 min. Following this, the precipitate is dissolved in 5 mL of slightly acidified methanol. This methanol solution is alkalinized with 1 mL of 0.2 M NaOH and its absorbance read immediately (within 5 to 7 sec after addition of alkali because the color fades quickly) at 400 nm (Austin and Wolff, 1968; Tzagoloff, 1963).

Titanium Tetrachloride Assay

This procedure involves extracting 5 g of defatted product with acetone-water (60:40, v/v; acidified to pH 3 with trichloroacetic acid) three times for 30 min under reflux conditions. The combined acidified extracts are evaporated under reduced pressure to remove acetone, and washed with diethyl ether to remove the trichloroacetic acid. Following this, an aqueous extract is passed through a neutral aluminum oxide column to remove phenols, and sinapine is eluted with water. Subsequently, the aliquot (0.2 to 2.0 mL) is dried (50°C and 700 mm pressure). The dried extract is then dissolved in 5 mL of concentrated HCl containing 0.25 mL of titanium tetrachloride; the absorbance of the solution is read at 485 nm. The concentrated HCl is the best medium for the formation of titanium tetrachloride complex with sinapine, whereas acetone is best for other phenolic compounds (Ismail and Eskin, 1979).

DETERMINATION OF CHLOROGENIC ACID

A number of UV spectrophotometric methods have been used for determination of chlorogenic acid in foodstuff (AOAC, 1980; Corse et al., 1962; Merrit and Proctor, 1959; Moores et al., 1948). A colorimetric method for quantification of chlorogenic acid in green coffee beans was proposed by Clifford and Wright (1976), who found that metaperiodate reagent provides a rapid and simple means of measuring the total chlorogenic acid. On the other hand, successive use of molybdate and metaperiodate reagents enables the estimation of total content of feruloylquinic acids as well as accurate measurement of caffeoylquinic acids and total chlorogenic acids. Molybdate reagent is specific for orthodihydroxyphenols while metaperiodate reagent reacts with *ortho*- and *para*-dihydroxyphenols and their monomethyl ethers to produce a yellow-orange color (Clifford and Wright, 1973).

The molybdate reagent can be prepared by dissolving the following in 800 mL of distilled water, one by one: sodium molybdate dihydrate (16.5 g), disodium hydrogen

phosphate dihydrate (8.04 g) and sodium dihydrogen phosphate dihydrate (7.93 g). Following this, the solution is made up of 1000 mL with distilled water (Swain and Hillis, 1959); the phenolics are assayed by adding 10 mL of molybdate reagent to 0.1 mL aliquot of phenolic extract and subsequent mixing. This solution is then examined spectrophotometrically against reagent blank at 370 nm for caffeoylquinic acid and 353 nm for caffeic acid (Clifford and Wright, 1976).

The determination of phenolics with metaperiodate reagent (0.25% aqueous solution of sodium metaperiodate) involves adding 1 mL of aliquot of phenolic solution to 10 mL of metaperiodate reagent, mixing thoroughly and allowing to stand for 10 min at room temperature. Following this the absorbance of the solution is read at 406 nm for chlorogenic acids and 423 nm for caffeic and ferulic acids (Clifford and Wright, 1976).

DETERMINATION OF ANTHOCYANINS

Quantification of anthocyanins takes advantage of their characteristic behavior in acidic media; anthocyanins exist in these media as an equilibrium between the colored oxonium ion and the colorless pseudobase form. The oxonium form comprises about 15% of the equilibrium mixture at pH 3.9 and 100% at pH 1 (Timberlake and Bridle, 1967). Sondheimer and Kertesz (1948) first developed the analytical procedure for quantification of anthocyanins. Swain and Hillis (1959) later modified this procedure by suggesting expression of the concentration of pigments in terms of the change in the absorbance at λ_{\max} between pH 3.5 and pH ≤ 1.0 . Fuleki and Francis (1968a-c) suggested extending the pH differential to between pH 4.5 and 1.0. They proposed to determine the optical density of the two samples at 515 nm for aliquots buffered to the preceding pH values. Using an average extinction coefficient (Moskowitz and Hrazdina, 1981), the total content of anthocyanins may also be estimated from the absorption of the total extracts at 520 nm.

Anthocyanins may also be assayed colorimetrically by the differential method of Fuleki and Francis (1968a, c). In this method, the anthocyanins are extracted twice with acidified ethanol (95% v/v ethanol:1.5M HCl=85:15; v/v); 1 mL of extract is then diluted in a buffer pH 1.0 (0.2 M KCl:0.2 M HCl=25:67; v/v) until an absorbance reading of 0.6 to 0.8 is obtained. Subsequently, the same amount of extract is diluted in a buffer pH 4.5 (1 M sodium acetate: 1 M HCl:water=100:60:90; v/v/v). The diluted samples are allowed to equilibrate in the dark for 2 h and then the absorbance of sample is read at its λ_{\max} . The difference in the absorbance is then calculated by subtracting the reading at pH 4.5 from that at pH 1.0 and dividing it by the average extinction coefficients for the four major anthocyanins or by the extinction coefficient of the principal anthocyanin of the sample in order to yield the total anthocyanin content. However, Little (1977) reported that downward and upward pH manipulations offer no advantage for products such as wine, with a natural pH of around 3.4. The use of transreflectometric thin layer measurements at two pH levels, natural (about 3.5) and pH 1.0, was suggested in order to obtain the content of anthocyanins.

DETERMINATION OF THEAFLAVINS

Theaflavins in black tea can be determined spectrophotometrically using the Flavognost method described by Hilton (1973). Flavognost reagent is believed to form a green complex with *cis*-2,2-dihydrobenzene rings associated with theaflavins, so the color intensity may be used to calculate the total theaflavin content (Figure 10.4) (Spiro and Price, 1986). The procedure consists of three critical stages: (1) sample preparation, (2) infusion, and (3) partitioning and color development. According to Reeves et al. (1985), control of infusion temperature is of prime importance in

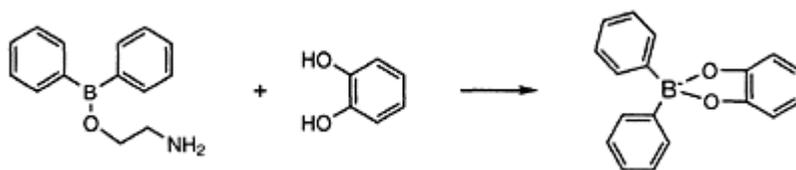


FIGURE 10.4 Reaction of complexation of the Flavognost reagent with theaflavin. (Adapted from Spiro, M. and Price, W.E., 1986, *Analyst*, 3:331–333.)

theaflavin analysis. However, the results of critical investigation into this method by Robertson and Hall (1989) indicate that failure to control conditions at each of these critical stages can give a four-fold variation in the results.

Extraction of theaflavins is influenced by the particle size of ground sample and the type of grinder used to produce it. The temperature of water used and time over which the infusion is carried out are also very significant. The extraction of theaflavins decreases rapidly after 12.5 min of infusion, which may be due to the oxidation of theaflavins to thearubigins. Moreover, it has been found that shaking the infused leaf may affect the extraction of theaflavins. Thus, occasional but regular inversion of flask by hand gives results similar to those obtained using a horizontal shaker. On the other hand, the use of vibratory shakers may result in poor extraction of theaflavins.

The theaflavin analysis according to the Flavognost method is: the black tea must be first ground to particles of less than 0.5-mm diameter. Next, 9 g of ground tea is infused in 175 g of boiling water; the infusion temperature should be kept close to boiling. The infusion is carried out in a thermos flask with continuous mechanical shaking. After infusion, the hot liquor is filtered and quickly cooled in cold water. Then, a 10-mL aliquot of the infusion is shaken for 10 min with 10 mL of isobutylmethyl ketone and the two layers are allowed to separate. Next, 2 mL of aliquot of the upper layer, 4 mL of ethanol and 2 mL of Flavognost reagent (2% w/v diphenylboric acid-2-aminoethyl ester in ethanol) are mixed well. The absorbance is then read at 625 nm after 15 min standing at room temperature; a mixture of isobutylmethyl ketone and ethanol (1:1, v/v) is used as a blank. The content of theaflavins in black tea is calculated from the following formula:

$$\text{Theaflavin } (\mu\text{mol/g}) = E_{625} \times 47,900 / \text{DM}$$

Where E_{625} is optical density and DM the dry matter of tea sample.

DETERMINATION OF IRON-BINDING PHENOLIC GROUPS

Brune et al. (1991) developed a spectrophotometric method for determination and evaluation of iron-binding phenolic groups in foods. This method is based on the findings of Mejbaum-Katzenellenbogen and Kudrewicz-Hubica (1966), who observed that Fe-galloyl and Fe-catechol complexes are colored differently. Ferric ions react with phenolic compounds containing three adjacent hydroxyl groups (galloyl groups) to form a blue-colored complex, while phenolics with two adjacent

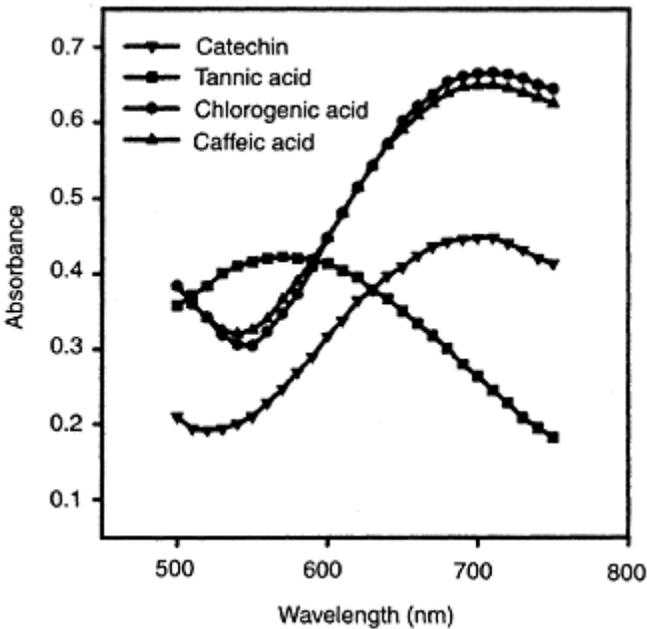


FIGURE 10.5 Absorbance spectra for equimolar (1.378 $\mu\text{mol/mL}$) concentrations of chlorogenic acid 500 $\mu\text{g/mL}$ (\bullet — \bullet); caffeic acid 248 $\mu\text{g/mL}$ (\blacktriangle — \blacktriangle); catechin 400 $\mu\text{g/mL}$ (\blacktriangledown — \blacktriangledown); and tannic acid 200 $\mu\text{g/mL}$ (\blacksquare — \blacksquare). (Adapted from Brune, M. et al., 1991, *J. Food Sci.*, 56:128–131; 167.)

hydroxyl groups (catechol groups) produce a green-colored complex. The absorption maximum for iron-tannin complex is around 580 nm, while that for complexes of iron with catechin, pyrocatechol, caffeic acid, and chlorogenic acid is around 680 nm (Figure 10.5). The iron-binding reagent does not lend itself for determination of monohydroxy phenolics and dihydroxy phenolics with a meta- or parahydroxylation pattern.

This procedure involves a 16-h extraction of phenolic compounds in the dark from dry or freeze-dried food samples with a 50% dimethylformamide solution in 0.1 M acetate buffer (pH 4.4) at room temperature. A ferric ammonium sulfate (FAS) reagent consisting of 1 M of HCl solution containing 5% ferric ammonium sulfate and 1% gum Arabic is added to the filtered extract. The resulting color is read spectrophotometrically against the blank at 578 and 680 nm, which corresponds to the absorption maxima of iron-galloyl (blue color) and iron-catechol (green color) complexes, respectively. The absorbances of the blank are also read and subtracted from those of the food sample at the two given wavelengths. Following this, the contents of catechol groups (expressed as catechin equivalents) and galloyl groups (expressed as tannic acid equivalents) are calculated from four standard curves obtained by using tannic acid and catechin at the two specified wavelengths values.

The iron-binding phenolic groups are assayed colorimetrically. To 2.0 mL of the extract, 8 mL of fresh FAS reagent is added and mixed well. The absorbance is then read at 578 and 680 nm after 15 min standing at room temperature. The mixture of extraction solvent and FAS reagent is used as a blank. A food blank is prepared by mixing 2 mL of the extract with 8 mL of the food blank reagent (obtained by mixing 89 parts of 50% dimethylformamide solution in 0.1 M acetate buffer, pH of 4.4, 10 parts of 1% gum Arabic solution, and 1 part of 1 M HCl). The absorbances of the food blank at 578 and 680 nm are read vs. those of the blank prepared by mixing 2 mL of the extraction solvent and 8 mL of food blank reagent. These absorbances (measurement of food sample base color) are subtracted from those of the food sample. The content of iron-binding phenolics is expressed as tannic acid or catechin equivalents.

UV SPECTROPHOTOMETRIC ASSAY

A number of approaches have been used to develop a simple and satisfactory UV spectrophotometric assay. Each group of phenolic compounds is characterized by one or several UV absorption maxima (Table 10.9). Simple phenolics have absorption maxima between 220 and 280 nm (Owades et al., 1958b); however, closely related phenolics show quite wide variations in their molecular absorptivity. Absorption is affected by the nature of solvent and pH; moreover, the possibility of interference by UV-absorbing substances such as proteins, nucleic acids and amino acids should be considered—a rather cumbersome and difficult task. Therefore, suitability of the UV assay depends on the material to be analyzed. Phenolics in tea and beer (Owades et al., 1958a, b), tea, coffee and other beverages (Hoff and Singleton, 1977), as well as those in cereals, legumes (Sharp et al., 1977; Davis, 1982) and oilseeds

TABLE 10.9
UV Absorption Patterns of Various Phenolic Compounds

| Class of Compounds | UV Absorption, λ_{\max} |
|-------------------------|-------------------------------------|
| Benzole acids | 270–280 |
| Hydroxycinnamic acids | 290–300 ^a ; 305–330 |
| Anthocyanic pigments | 270–280, 315–325 ^b |
| Flavonols | 250–270; 330 ^b ; 350–380 |
| Flavan-3-ols | 270–280 |
| Coumarins | 220–230; 310–350 |
| Flavones | 250–270; 330–350 |
| Flavanones, Flavanonols | 270–295; 300–330 ^a |
| Chalcones | 220–270; 300–320; 340–390 |
| Aurones | 240–270 |
| Isoflavones | 245–270; 300–350 |

Note: Solvent is methanol, except for anthocyanin pigments in which methanol containing HCl 0.01% is used.

^a Shoulder.

^b In the case of acylation by hydroxycinnamic acids.

Source: Adapted from Macheix, J.-J. et al., 1990, *Fruit Phenolics*, CRC Press Inc., Boca Raton, FL.

such as canola meals (Blair and Reichert, 1984; Naczk et al., 1992b; Shahidi and Naczk, 1990), have been estimated by this method. Determination of the absorbancy at 325 nm has also been proposed for estimating the hydroxycinnamic acid derivatives (chlorogenic acid) in pears (Billot et al., 1978) and coffee (Moore et al., 1948).

UV and visible spectroscopic techniques are often used for identification of isolated phenolic compounds, particularly flavonoids (Mabry et al., 1970). The spectra of phenolics in methanol after addition of Schiff reagents are recorded. For example, the addition of sodium hydroxide or hydrochloric acid is helpful in determining the phenolic and carboxylic acid groups present in the molecules (Jurd, 1957). On the other hand, the absorption spectra of total phenolic extracts can be used to identify the presence of groups of predominant phenolic compounds (Macheix et al., 1990).

CHEMOMETRIC METHODS

Traditional spectroscopic assays may lead to overestimation of polyphenol contents of crude extracts from plant materials due to overlapping spectral responses; however, these problems can be overcome by using a chemometric technique to analyze the spectra. Partial least squares (PLS) or principal component analysis (PCA) is commonly used to resolve these types of problems (Kramer, 1998; van der Voort, 1992). Chemometric technique uses information (such as a spectrum) and chemical indices (such as concentration of a component) to establish a mathematical relation between the two. It assumes that the chemical index (concentration) is correct and attributes weightings of

the spectral information accordingly. The set-up of the model, correlating the information with a chemical index, is known as calibration (Beebe and Kowalski, 1987).

Monedero et al. (1999) developed a chemometric technique for controlling the content of phenolic aldehydes and acids during production of wine subjected to accelerated aging. The wine was aged by adding oak wood extracts obtained by maceration of charred oak shavings. Charring time and the interactions between charring temperature and time were essential factors to control the content of 10 of the 11 phenolic compounds studied. Edelmann et al. (2001) developed a rapid method of discrimination of Austrian red wines based on midinfrared spectroscopy of phenolic extracts of wine. The samples of wine were cleaned up by solid-phase extraction (SPE) before collection of spectra using FTIR spectrophotometry. These authors also reported that the use of UV-vis spectroscopy was limited to the authentication of the Burgundy species Pinot Noir. Subsequently, Brenna and Pagliarini (2001) employed a multivariate analysis for establishing a correlation between polyphenolic composition and antioxidant power of red wines.

Briandet et al. (1996) applied PCA to differentiate between Arabica and Robusta in instant coffees based on their FTIR spectra. Spectra used in this study were obtained by using the diffuse reflection infrared Fourier transform and attenuated total reflection sampling techniques. According to these authors, the discrimination between species of coffees is based on the different chlorogenic acid and caffeine contents. Later, Downey et al. (1997) successfully applied factorial discriminant analysis and PLS to develop a mathematical model for varietal authentication of lyophilized samples of coffee based on near- and midinfrared spectra.

Schulz et al. (1999) used a near-infrared reflectance (NIR) spectroscopic method for prediction of polyphenols in the leaves of green tea (*Camelia sinensis* (L.) O.Kuntze). The contents of gallic acid and catechins were determined using a reversed-phase HPLC methodology. The PLS method was used to calibrate NIR spectra with the contents of gallic acid and catechins in tea. The models predicted the contents of catechins and gallic acid with good accuracy. On the other hand, Mangas et al. (1999) used the linear discrimination analysis for discrimination of bitter and nonbitter cider apple varieties. The most discriminant variables were chlorogenic acid, phloretin 2-xyloglucoside and an unidentified phenolic acid derivative with a maximum absorption at 316.7 nm. Using this model, 91.3 and 85.7% correct classification was obtained for internal and external evaluation of the model, respectively.

Recently, Naczek et al. (2001c) employed a multivariate model for the prediction of soluble condensed tannins in crude extracts of polyphenols from canola and rapeseed hulls. PLS was used to correlate the spectral data of the crude polyphenols in methanol between 265 and 295 nm with the tannin content in hulls. The tannin contents were determined by using proanthocyanidin and vanillin assays. The ratio of the standard deviation of the data to the standard error of calibration indicated that the predictive ability of the models was good. Subsequently, Naczek et al. (2002) used UV spectrophotometry to develop a chemometric procedure for the prediction of total phenolic acids in crude polyphenol extracts from defatted rapeseed and canola meals. The phenolic acids were isolated from meals as described by Krygier et al. (1982) and quantified using the Folin-Denis assay. PLS was employed to correlate the spectral data of the crude extract of phenolics in methanol with the total phenolic acid contents. The

best correlation was obtained in the 320 to 355 nm region that includes the absorption maxima specific for canola and rapeseed phenolic acids.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Various nuclear magnetic resonance (NMR) spectroscopic techniques have been employed for structural elucidation of complex phenolics isolated from foods (Porter, 1989). These include ^1H NMR and ^{13}C NMR, two-dimensional homonuclear (2^1H - ^1H) correlated NMR spectroscopy (COSY), heteronuclear chemical shift correlation NMR (C-H HECTOR), totally correlated NMR spectroscopy (TOCSY), nuclear Overhauser effect in the laboratory frame (NOESY) and rotating frame of reference (ROESY) (Bax, 1985; Bax and Grzesiek, 1996; Belton et al., 1995; Derome, 1987; Ferreira and Brandt, 1989; Kolodziej, 1992; Hemingway et al., 1992; Newman and Porter, 1992). Combinations of high-resolution spectroscopic techniques with novel mathematical treatments of data now provide greater insight into structural elucidation of mixtures of compounds without their prior separation into individual components (Gerothanassis et al., 1998; Limiroli et al., 1996; Sacchi et al., 1996).

TITRATION METHODS

Titration is one of the official AOAC (1980) methods for determination of tannins in tea, cloves and spices. The assay is based on the oxidation of water extracts of phenolics using potassium permanganate in the presence of indigo as an indicator. Permanganate is not specific for phenolics and also readily oxidizes other substances present in the extract. Indigo not only serves as an end point indicator, but also slows down the oxidation reaction because it is less oxidizable than polyphenols.

Tannins in clove and allspice are assayed according to the AOAC (1980) method: a 2-g sample is extracted with anhydrous diethyl ether for 20 h. The residue is then boiled with 300 mL of water for 2 h, cooled, diluted to 500 mL and filtered. A 25-mL sample of this infusion is transferred to a porcelain dish and 20 mL of indigo solution and 750 mL of water added to it. Following this, a standardized solution of potassium permanganate is added, 1 mL at a time, until the blue color of the solution changes to green; then a few drops are added at a time until the solution becomes golden yellow. Similarly, a mixture of 20 mL of indigo solution and 750 mL of water is titrated. The difference between these two titrations is multiplied by a constant factor to obtain tannic acid equivalents or the amount of oxygen absorbed. The potassium permanganate solution is prepared by dissolving 1.333 g of KMnO_4 in 1 L of water and standardizing it against a 0.1 M oxalic acid solution. Indigo solution is prepared by dissolving 6 g of sodium indigotin disulfonate in 500 mL of water by heating. After cooling, 50 mL of sulfuric acid is added and the solution diluted to 1 L and then filtered.

PROTEIN PRECIPITATION ASSAYS

Evaluation of biological activity of polyphenolics is based on their ability to form insoluble complexes with proteins as well as bind certain essential minerals. A number of methods have been developed for quantification of biological activity of phenolics. Most of these methods are based on the ability of phenolics to precipitate proteins such as gelatin, bovine serum albumin, and hemoglobin as well as different enzymes (Table 10.10). Protein precipitation methods are highly correlated with the biological value of high tannin-content foods and feeds (Hahn et al., 1984).

Phenolic compounds have been identified as potent inhibitors of iron absorption, presumably by forming insoluble complexes with iron ions in the gastrointestinal lumen and thus making the iron unavailable for absorption (Brune et al., 1989). Therefore, evaluation of biological activity of food phenolics should also include measurement of the content of iron-binding phenolic groups.

BOVINE SERUM ALBUMIN ASSAYS

Several methods have been published that use bovine serum albumin to determine the ability of polyphenols to precipitate proteins (Asquith and Butler, 1985; Hagerman and Butler, 1978; Hoff and Singleton, 1977; Makkar et al., 1987, 1988; Marks et al., 1987; Naczek et al., 2001a; Silber et al., 1998; Verzele et al., 1986). The first step in these procedures is the formation of a protein-polyphenolic complex.

Complete precipitation of this complex depends on the structure and quantity of polyphenols and may take from 15 min to 24 h (Hagerman and Robbins, 1987; Makkar, 1989); therefore, the precipitation time should be standardized. The formation of a protein-polyphenol complex is affected by the presence of small amounts of residual organic solvents such as acetone used for the extraction of polyphenols (Dawra et al., 1988). Other factors that may influence the assay include the concentration and nature of proteins used in the test mixture as well as pH and ionic strength of the reaction mixture (Makkar, 1989).

TABLE 10.10

Some Methods to Determine Tannins by Protein Precipitation

| Tannins Measured | Remarks | Reference |
|--------------------------|--|---|
| Hemoglobin precipitation | Requires fresh blood; interference by plant pigments, saponins etc. | Bate-Smith, E.C. (1973b); Schultz, J.C. et al. (1981) |
| AOAC | Accuracy $\pm 10\%$; not suitable for sorghum grains; suffers from disadvantages associated with FD, FC, PB methods | AOAC (1980) |

| | | |
|-------------------------------|--|---|
| β-Glucosidase inhibition | Relationship between enzyme activity and insoluble complex formation not known; cumbersome, expensive | Goldstein, G.L. and Swain, T. (1965) |
| Immobilized protein | Time-consuming, expensive, difficult to handle large number of samples at a time | Hoff and Singleton (1977) |
| Dye-labeled BSA precipitation | Simple but rather insensitive; preferentially forms soluble complexes | Asquith, T.N. and Butler, L.G. (1985) |
| Radial diffusion | Insensitive to acetone; simple but involves an element of subjectivity | Hagerman, A.E. (1987) |
| BSA precipitation | Simple method; measures phenolics in complex; nonspecific binding of phenols to complex can introduce error; not suitable for comparing tannins from different sources | Hagerman, A.E. and Butler, L.G. (1978) |
| | Indirect method; protein estimations by Lowry or Bradford assays; takes longer time; less sensitive | Martin, J.S. and Martin, M.M. (1982, 1983) |
| | Indirect method; protein determination by Kjeldahl method; less sensitive | Amory, A.M. and Schubert, C.L. (1987) |
| | Indirect method; protein determination by HPLC; more sensitive compared to indirect methods above | Verzele, M. et al. (1986) |
| | Ninhydrin method used to measure protein in complex after its alkaline hydrolysis; takes only 20 min for hydrolysis; assay a bit messy because of ninhydrin reagent | Makkar, H.P.S. et al. (1987) |
| | Same as in method of Makkar, H.P.S. et al. (1987) except that hydrolysis of the complex is under acidic conditions and takes longer (22 h) | Marks, D. et al. (1987) |
| | Measures both tannins and proteins Highly sensitive; requires microquantity of tannins; allows quantitation of hydrolyzable and condensed tannins in plant extract | Makkar, H.P.S., et al. (1988) Dawra, R.K. et al. (1988) |

Source: Adapted from Makkar, H.P.S., 1989, *J. Agric. Food Chem.*, 37:1197–1202.

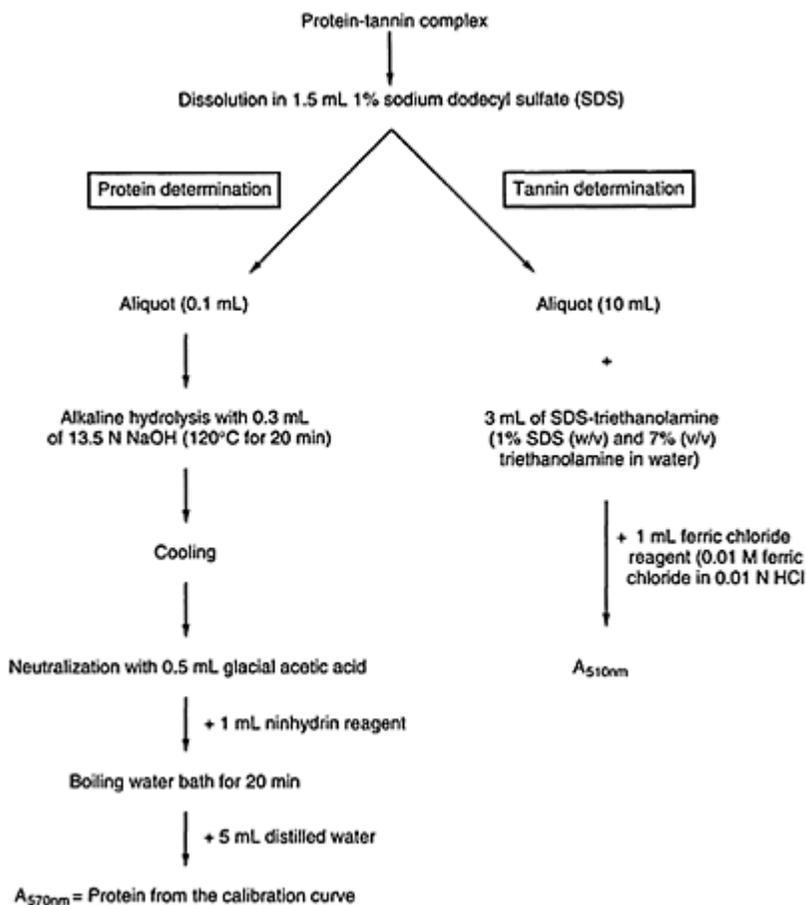


FIGURE 10.6 Estimation of protein and tannin in a tannin-protein complex. (Adapted from Makkar, H.P.S. et al., 1988, *J. Agric. Food Chem.*, 36:523–525.)

Some methods only determine the quantity of precipitable phenolics (Hagerman and Butler, 1978) while others may also determine the precipitation capacity of phenolics (Figure 10.6) (Asquith and Butler, 1985; Dawra et al., 1988; Hagerman and Butler, 1980; Makkar et al., 1987, 1988; Marks et al., 1987). Naczek et al. (2001a) proposed to express protein precipitating potential of crude tannin extracts as a slope of lines (titration curves) reflecting the amount of tannin-protein precipitated as a function of the amount of tannins added to the reaction mixture. The titration curves are obtained using the protein precipitation assay (Hagerman and Butler, 1978) and the dye-labeled protein assay (Asquith and Butler, 1985). The determination of slope values is based on the statistical analysis (linear regression) of experimental data involving the precipitation of tannin-

protein complexes at a minimum of four different tannin levels. Determination of the slopes under standardized conditions (type and concentration of protein, pH and temperature) affords meaningful differences in the slope values for crude tannin extracts from various canola and rapeseed hulls (Naczek et al., 2001a), as well as for condensed tannins of beach pea, evening primrose and faba bean (Naczek et al., 2001b).

Asquith and Butler (1985) developed a simple method to determine the amount of protein precipitated by tannins. In this assay, a bovine serum albumin (BSA) covalently bound with Remazol brilliant R was used as a substrate. The protein-labeling procedure was adapted from Rinderknecht et al. (1968). The dye reacts irreversibly with BSA under mild conditions without apparent denaturation. The blue BSA can be stored at 6°C for several months without loss of precipitating activity. In the presence of excess blue BSA, the amount of precipitated protein is proportional to the amount of tannin added. The plant pigments do not interfere with the assay because the maximum absorption of dye-labeled BSA is at longer wavelengths. Because the assay detects all protein precipitants, it should be corroborated by assays for protein-precipitable phenols before the formation of precipitate can be interpreted as indicative of the presence of tannins.

Recently, Silber et al. (1998) described a novel dye-labeled protein assay based on utilization of a Coomassie brilliant blue (G-250)—BSA complex. Coomassie blue pigment exists in a cation form (red with maximum absorbance at 470 nm), an anionic form (blue with maximum absorbance at 595 nm) and a neutral form (green with maximum absorbance at 650 nm) (Matejovicova et al., 1997). Binding Coomassie blue to protein markedly enhances the absorbance of light (Matejovicova et al., 1997; Silber et al., 1998). The complex absorbs light intensely at 600 to 620 nm, but the mechanism of its binding is still not well understood (Atherton et al., 1996; Silber et al., 1998). It takes 3 h of standing at refrigerated temperature to completely precipitate the dye-labeled protein-tannin complexes.

Hagerman (1987) developed a radial diffusion assay for tannin analysis in which tannins were quantified by forming a visible ring precipitation in BSA-containing agarose gel. The tannin-containing solution is placed in a well in a BSA-containing agarose slab. Tannins readily diffuse through the gel to form precipitable BSA—tannin complexes. The carbohydrate medium of the gel does not form precipitable complexes. The acetone extracts of tannins can be analyzed directly because the acetone does not affect this assay. The area of the visible ring of precipitation is linearly correlated to the amount of tannin placed in a well. The results obtained with this assay are highly correlated with the results of the protein precipitation method of Hagerman and Butler (1978). The detection limit of the assay is 0.025 mg of tannin and depends on the concentration of protein in the plates. Nontannin phenolics such as flavonoids, hydroxybenzoic, and hydroxycinnamic acids do not interfere with the assay.

Hoff and Singleton (1977) developed a method for determination of tannins in foods by employing immobilized bovine serum albumin on a Sepharose matrix. They found that immobilized proteins bind tannins selectively at pH 4. Monomeric polyphenolic substances such as chlorogenic acid, catechol and gallic acid do not form complexes with the immobilized proteins under these conditions. Thus, this method may be used as a means for separating tannins from nontannin materials. Tannin—protein complexes are then dissociated in organic solvents such as methanol or dimethylformamide and the content of tannins determined spectrophotometrically. However, this method is

cumbersome and time-consuming, and does not allow handling a large number of samples at a time.

The polyphenols precipitating BSA are assayed as described by Hagerman and Butler (1978): to 2 mL of a standard solution of BSA (1 mg/mL) 1.0 mL of crude extract of polyphenols is added, followed by mixing and standing at room temperature for 15 min. The mixture is then centrifuged at 5000×g for 15 min and the supernatant is discarded. The pellet is rinsed with 0.2 M acetate buffer (pH 5.0) and then dissolved in 4 mL of an SDS-triethanolamine solution containing 1% SDS and 5% triethanolamine in distilled water. Then 1 mL of a 0.01 M ferric chloride in 0.01 M HCl solution is added and the solution mixed immediately. After 15 to 30 min standing at room temperature, the absorbance of the solution is read at 510 nm. A blank consisting of SDS-triethanolamine and ferric chloride solution is used and the results expressed as A_{510} per gram of tannins.

The assay developed by Asquith and Butler (1985) is: to 4 mL of a standard solution of blue BSA (2 mg/mL) 1.0 mL of a methanolic solution of crude polyphenol extract is added. After 5 min of vigorous mixing at room temperature, the solution is centrifuged at 5000×g for 15 min and the supernatant discarded. The pellet is then dissolved in 3.5 mL of an SDS-triethanolamine solution containing 1% SDS, 5% triethanolamine and 20% isopropanol in distilled water. The absorbance of the solution is read at 590 nm against a blank consisting of SDS-triethanolamine-isopropanol solution and methanol. The results are expressed as percent of blue BSA precipitated.

COLLAGEN ASSAY

The collagen assay is one of the official AOAC (1980) methods for determination of tannins. This method is based on the precipitation of tannins with gelatin, hide powder or kaoline, and subsequent oxidation of tannins in an acidic solution with potassium permanganate in the presence of indigo as an indicator. Polyphenols are slowly oxidized in acid solutions by permanganate. Therefore, a large excess of indigo is used to determine the end point of the titration and limit the rate of oxidation reaction in order to avoid oxidation of nonphenolic substances. However, the method has a limitation because different phenols at the same concentration do not reduce the same quantity of permanganate.

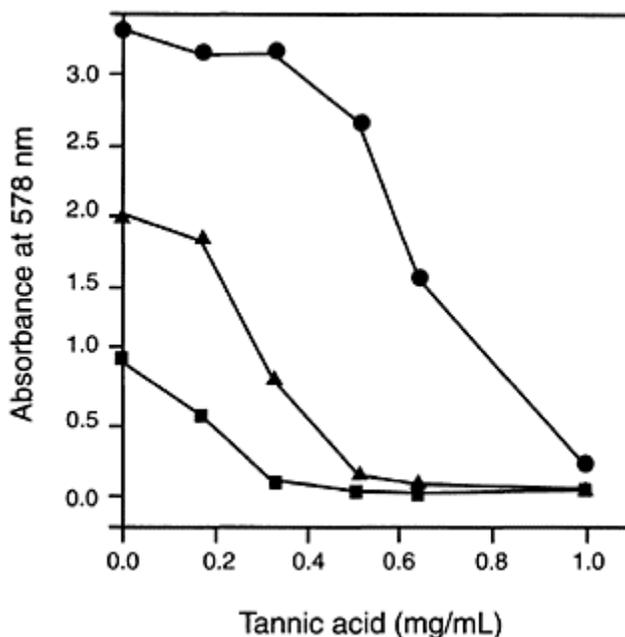


FIGURE 10.7 Optical density of the supernatant at 578 nm after precipitation with tannic acid. Three initial hemoglobin concentrations are shown. (Adapted from Schultz, J.C. et al., 1981, *J. Agric. Food Chem.*, 29:823–829.)

HEMOGLOBIN ASSAY

The hemoglobin assay is based on the use of hemoglobin as a binding substrate for polyphenols (Bate-Smith, 1973b). This assay requires freshly drawn blood because commercial preparations do not give satisfactory results (Asquith and Butler, 1985). The residual hemoglobin can be measured directly by spectrophotometry. Schultz et al. (1981) found that the relationship between hemoglobin absorbancy and tannin concentration is linear over a large range of tannic acid concentration; however, the linearity range depends on the concentration of hemoglobin (Figure 10.7). At higher initial concentrations of hemoglobin, a specific threshold level of tannin is necessary to initiate precipitation. Thus, the initial concentration of hemoglobin must be adjusted for optimal sensitivity over the range of tannin content to be assayed. Saponins and some enzymes present in plant materials may interfere with this assay, however (Bate-Smith, 1977).

The assay given by Bate-Smith (1977) is: a 1.0-mL sample of freshly drawn blood is diluted with 50 mL of distilled water. To 1 mL of this aliquot, 1 mL of phenolic extract is added; the mixture is rapidly mixed and centrifuged and the content of residual hemoglobin measured at 578 nm (Bate-Smith, 1977). The percentage of hemoglobin precipitation is calculated from the difference between the absorption of the sample and the control consisting of 1 mL of diluted blood with 1 mL of water. The content of phenolics is expressed as tannic acid equivalents and calculated from the relationship describing tannic acid content as a function of precipitation percentage. According to Bate-Smith (1977), tannic acid equivalent contents obtained this way serve as a measure of effective astringency.

COMPETITIVE BINDING ASSAYS

Interactions between tannins and proteins are often measured using assays based on competition of a test protein and labeled protein for binding and coprecipitation with tannins (Asquith and Butler, 1985; Bacon and Rhodes, 1998; Hagerman and Butler, 1981). These assays permit a direct comparison of protein affinities for condensed tannins.

Hagerman and Butler (1981) developed a competition assay based on the assumption that proanthocyanidin-tannin and antigen-antibody interactions are similar. The similarities include comparable sizes of ligand and binding agent that are multivalently bound to form soluble and insoluble complexes. This procedure utilizes an iodine 125-labeled bovine serum albumine prepared, as described by Hagerman and Butler (1980), as the labeled ligand. In this assay, the proanthocyanidins are simultaneously exposed to a mixture of the competitor (unlabeled protein) and labeled protein. The insoluble tannin-protein complex is separated by centrifugation and the amount of unprecipitated labeled protein is determined in supernatant by counting using the Gamma 300 gamma counter.

Asquith and Butler (1985) developed a competition assay based on the dye-labeled protein assay. A dye-labeled protein for this assay was prepared by covalently binding BSA with Remazol brilliant blue R as described by Rinderknecht et al. (1968). The competition procedure used in this assay is similar to that described by Hagerman and Butler (1981). A standard solution of dye-labeled BSA is mixed with a methanolic solution containing enough tannins to precipitate 70 to 80% of dye-labeled proteins. Varying amounts of competitor protein are mixed with a standard solution of dye-labeled BSA before tannins are added and the amount of precipitated dye-labeled BSA measured spectrophotometrically. Results are presented on a semi-log plot. Following this, the amount of competitor required to inhibit 50% precipitation of dye-labeled protein due to tannin is established and proposed as a measure of relative affinity of competitor protein for tannins.

Bacon and Rhodes (1998) developed a novel competitive tannin-salivary protein-binding assay. This assay was developed based on the principle of a competitive ELISA A and competition between an enzyme-labeled tannin (HPR-EGC) and a tested tannin to bind to parotid salivary protein immobilized in the well of microtiter plate. (-)-Epigallocatechin (EGC) conjugated to horseradish peroxidase via a linker molecule, 1,4-butanediol diglycidyl ether, is used as an enzyme-labeled tannin. Coated with parotid salivary proteins and loaded with a known quantity of HPR-EGC, different amounts of

the tannin under test are added to the wells. The plates are incubated for 2 h at 37°C and then the peroxidase activity in each well is determined. Following this the amount of tannin required to displace 50% of HRP-EGC bound to the protein coating on microtiter plate is calculated and proposed as a measure of tannin affinity for proteins. A control sample (no test tannin added) is used to measure the amount of HRP-EGC conjugated to proteins coating the microtiter plate.

ENZYMATIC ASSAYS

Enzymatic methods are based on the ability of tannins to form complexes with enzymes, thus inhibiting their activities. Phenolic compounds can affect enzymes by forming (1) insoluble protein-phenolic complexes and lowering the concentration of enzymes in the reaction mixture or (2) inactive soluble protein-phenolic complexes. Thus, competitive and noncompetitive reaction kinetics as well as mixed kinetics for the inhibition of enzymes of phenolic compounds must be considered. Hagerman and Butler (1978) have suggested, however, that the results of enzymatic assay are difficult to interpret because the relationship between enzymatic activity and formation of insoluble complexes is not fully understood.

Later, Dick and Bearne (1988) studied the inhibition of β -galactosidase activity by polyphenolic compounds. They demonstrated that *trans*-3-(4-hydroxyphenyl)-propenoyl group, common to 4'-hydroxyflavones and *p*-hydroxyphenolic acid, is important for effective inhibition of enzymatic activity. Mole and Waterman (1987c) found that adding tannic acid to a standard trypsin solution leads to formation of an insoluble complex. Trypsin remaining in the solution has an enhanced rate of autolysis, however; conformational changes in enzyme brought about by the formation of soluble complexes with tannic acid might be responsible. This phenomenon has not been observed when excess bovine serum albumin is added to the system containing trypsin and tannic acid.

The inhibition of α -amylase by sorghum polyphenolics has been assayed according to Earp et al. (1981): ground sorghum grains are extracted with water and filtered. A Phadebas tablet, which is a water-insoluble, cross-linked starch polymer with a blue dye bound to it, is added to 4 mL of the extract. Upon hydrolysis the dye is released; 1 mL of α -amylase (0.17 units/mL) is added and the sample incubated for 10 min at 37°C. Adding 1 mL of 0.5 M NaOH to the mixture stops the reaction. After addition of 4 mL of distilled water and filtration, the absorbance of the solution is read at 620 nm. The standard curve of enzyme units of activity vs. absorbance is prepared in order to calculate the percentage inhibition of enzyme activity.

Ittah (1991) developed an enzymatic assay for quantitation of tannins. The assay utilizes the ability of tannins to form complexes with more than one protein and is based on determining the activity of bound alkaline phosphatase. Tannin in this assay is sandwiched between two BSA molecules and alkaline phosphatase. A linear relationship between tannin concentrations and the activity of bound alkaline phosphatase has been found. The procedure involves immobilization of BSA on polystyrene microplates; the quantity of immobilized BSA is low in comparison with the amount of tannin applied. Unbound tannins are washed out and then the microplates are incubated with alkaline phosphate solutions. Following this the unbound enzyme is washed out and the activity of

the bound alkaline phosphatase is determined using *p*-nitrophenylphosphate as a substrate.

ELECTROCHEMICAL METHODS

Electrochemical methods such as cyclic voltammetry and liquid chromatography/electrochemistry are very useful tools for the analysis of food phenolics. Most phenolics are electrochemically active at modest oxidation potentials. These methods can be used to determine redox potentials of phenolics, identify the mechanism of oxidation, identify a flavonoid based on comparison with a standard and determine redox potentials for unknown phenolics. The knowledge of redox potential is of great interest for the food industry because oxidized phenolics may have detrimental effects on the quality of wine and beer, grape juice and other beverages (Lunte and Lunte, 1988).

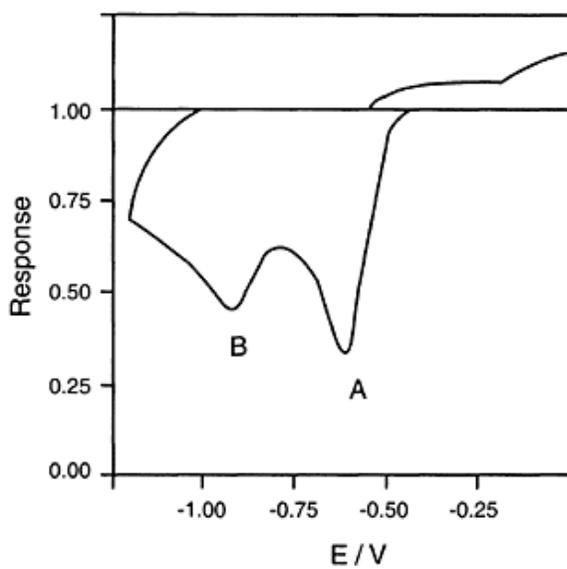
Cyclic voltammetry is used to determine the redox potential as well as the mechanism of oxidation for a pure phenolic compound. The voltammogram (Figure 10.8) is obtained by scanning the potential of the working electrode as a function of time in positive and negative directions. The peak potential for a specific phenolic compound is defined at the point at which the current is diffusion limited. The greater the positive peak potential, the more difficulty a phenolic substance has in oxidizing (Kissinger and Heineman, 1984). Thus, knowledge of peak potentials enables one to estimate the ease of oxidation of food phenolics. Oxidation potentials for some phenolics are given in Table 10.11.

Voltammetry also enables one to estimate the stability of oxidized phenolics. Presence of stable quinone species formed during a voltammetric scanning of potential in the positive direction can be detected when the potential is scanned in the negative direction. This is due to the production of a cathodic current as a result of reduction of quinones to corresponding phenols (Lunte and Lunte, 1988). Voltammetric information also makes it possible to choose the potential that will provide the optimum information for a chromatographic component of interest (Lunte et al., 1988a).

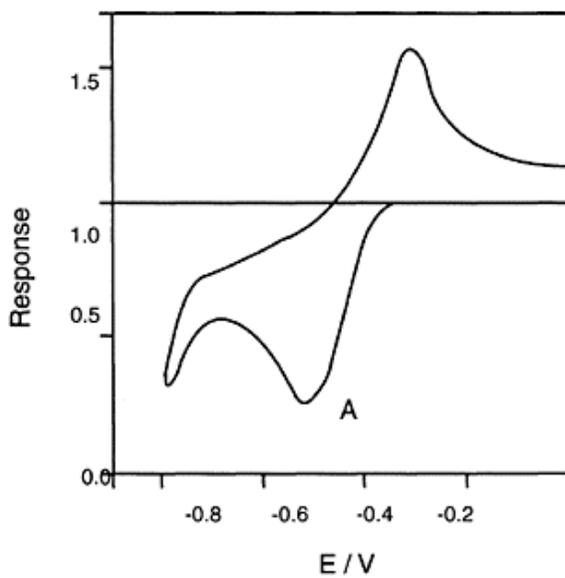
Fernando and Plambeck (1988) demonstrated the ability to measure theaflavin content in tea liquor using a voltammetric method. Theaflavins undergo a completely irreversible reduction during acyclic voltammetry on a hanging mercury drop electrode. The theaflavin content in tea liquor was simultaneously determined by Flavognost method (Hilton, 1973) and by differential pulse polarography. A very highly correlated relationship between the peak currents and the Flavognost absorbance was found with a correlation coefficient equal to 1.

Liquid chromatography/electrochemistry also serves as a useful tool to detect, classify, and identify phenolics in complex samples. It provides information that cannot be obtained using liquid chromatography and a UV detector. According to Chaviari et al. (1988), electrochemistry is a useful alternative to UV detection owing to its better selectivity. The phenolics eluted from the column are detected using a dual electrode detector in the series or parallel configuration.

In the series configuration the upstream electrode is set up at a potential where the flavonoid of interest is oxidized, while a downstream electrode is set at the reducing potential. In cases of reversible oxidation the quinone produced at the upstream electrode may be detected at the downstream electrode. The combination of dual electrodes with a



(a)



(b)

FIGURE 10.8 Cyclic voltammograms of epicatechin; (a) oxidation of the

phenolic groups on ring B; and (b) oxidation of the phenolic groups on ring A. (Adapted from Lunte, S.M. et al., 1988b, *Analyst*, 113:99–102.)

TABLE 10.11
Peak Potentials for Oxidation of Selected Phenolic Compounds at Glass Carbon Electrode

| No. | Compound | V ^a |
|-----|------------------------------------|----------------|
| 1 | Eriodictyol | +0.51; +1.20 |
| 2 | Catechin | +0.53; +0.88 |
| 3 | Caffeic acid | +0.53 |
| 4 | Gallic acid | +0.54; +0.87 |
| 5 | Hydroquinone | +0.57 |
| 6 | Pyrocatechin | +0.61 |
| 7 | Syringic acid | +0.75 |
| 8 | Guaiacol | +0.77 |
| 9 | Syringaldehyde | +0.81 |
| 10 | Ferulic acid | +0.82 |
| 11 | <i>p</i> -Coumaric acid | +0.85 |
| 12 | Vanillic acid | +0.88 |
| 13 | Acetovanillone | +0.89 |
| 14 | Vanillin | +0.92 |
| 15 | Resorcinol | +0.94 |
| 16 | Phenol | +0.98 |
| 17 | <i>o</i> -Hydroxyphenylacetic acid | +0.98 |
| 18 | <i>m</i> -Hydroxyphenylacetic acid | +1.02 |
| 19 | <i>p</i> -Hydroxyphenylacetic acid | +1.02 |
| 20 | <i>p</i> -Hydroxybenzoic acid | +1.15 |
| 21 | 2,4-Dihydroxyacetophenone | +1.15 |
| 22 | 2-Hydroxyacetophenone | +1.25 |

Note: All samples are 0.5-mM solutions in methanol—0.1 % perchloric acid in water (15+85).

^a Volts vs. saturated calomel electrode at 20 mVs⁻¹.

Source: Adapted from Chiavari, G. et al., 1988, *Analyst*, 113:91–94.

diode array detector can be used to classify phenolics based on their conjugation pattern and hydroxyl substitution (Lunte et al., 1988). In the parallel configuration one of the electrodes is set at the peak potential for the phenolic of interest whereas the other is set at the potential where the current vs. potential responses are changing rapidly. The ratio of these two responses is specific for a particular phenolic; thus, the comparison of retention time and current ratios of an unknown with the standard allows one to identify a compound with greater certainty (Lunte and Lunte, 1988; Lunte et al., 1988a).

A combination of HPLC technique and voltammetry has been successfully applied for detection, identification, and quantification of flavonoid and nonflavonoid phenolics in

wine. Positive identification may be obtained by comparing the capacity factor (k') and electrochemical behavior of wine phenols with those of standard solutions containing pure phenolics (Lunte et al., 1988a; Mahler et al., 1988; Woodring et al., 1990).

The voltammetric-amperometric detector has also been applied to detect food phenolics; it operates by scanning the potential of the upstream (voltammetric) electrode and detecting the electrolysis product (amperometric) electrode at a constant potential downstream. This allows one to obtain voltammograms without the charging current problems typical of a single voltammetric detector. Voltammetric-amperometric detection results in a three-dimensional data array of current response vs. time and potential. The voltammetric-amperometric detector also has the capability of detecting each component of phenolic mixture at its optimum potential because each chromatovoltammogram contains the current responses for a range of potentials. Moreover, the chromatographic-voltammetric data array obtained in the course of analysis can also be used for postexperimental optimization of detection potentials (Lunte et al., 1988a).

CHROMATOGRAPHIC TECHNIQUES

Various chromatographic techniques have been employed for separation, preparative isolation, purification and identification of phenolic compounds (Karchesy et al., 1989; Jackman et al., 1987; Merken and Beecher, 2000). Chromatographic procedures have also been used to study the interaction of phenolics with other food components (Oh et al., 1985).

LIQUID CHROMATOGRAPHY

Many liquid chromatographic methodologies have been described in the literature for fractionation of tannins (proanthocyanidins) using Sephadex G-25 (McMurrough and McDowell, 1978; Michaud and Margail, 1977; Somers, 1966), Sephadex LH-20 (Asquith et al., 1983; Boukharta et al., 1988; Davis and Hosene, 1979; Lea and Timberlake, 1974; Strumeyer and Malin, 1975; Thompson et al., 1972); Sepharose CL-4B (Hoff and Singleton, 1977), Fractogel (Toyopearl) TSK-HW 40(s) (Derdelinckx and Jerumanis, 1984; Mateus et al., 2001; Ricardo da Silva et al., 1991), Fractogel (Toyopearl) TSK 50(f) (Labarbe et al., 1999; Meirelles et al., 1992), inert glass microparticles (Labarbe et al., 1999) and C₁₈ Sep-Pak cartridge (Guyot et al., 2001; Jaworski and Lee, 1987; Salagoity-Auguste and Bertrand, 1984; Sun et al., 1998b).

Hoff and Singleton (1977) developed a chromatographic procedure for separating tannins from nontannin materials using bovine serum albumin immobilized on Sepharose CL-4B as a column packing material. Nontannin polyphenolics are first separated by washing the column with an acetate buffer. The protein-tannin complexes are then dissociated by elution of the column with organic solvents such as methanol or dimethylformamide to release and recover tannin substances.

The preparative isolation of proanthocyanins is, however, most commonly achieved by employing Sephadex LH-20 column chromatography (Thompson et al., 1972; Strumeyer and Malin, 1975; Davis and Hosene, 1978; Asquith et al., 1983). The crude

extract is applied to the column, which is then washed with ethanol to elute the nontannin substances. Following this, proanthocyanins are eluted with acetone-water or alcohol-water. For purification of proanthocyanins, the crude extract of phenolic substances is applied to a Sephadex LH-20 column that is then eluted with water containing increasing proportions of methanol (Nonaka et al., 1983).

Derdelinckx and Jerumanis (1984) employed Fractogel (Toyopearl TSK HW-40(s) gel) to separate malt and hop proanthocyanidin dimers and trimers after chromatography of polyphenols on Sephadex LH-20 with methanol. The proanthocyanidin fractions are applied to the Fractogel column, which is then eluted with methanol. The four major peaks of hops polyphenolics correspond to the B3 and B4 procyanidin dimers and two unidentified procyanidin oligomers. On the other hand, malt polyphenolics are separated into a mixture of four proanthocyanidin trimers, two procyanidin trimers and an unknown procyanidin oligomer. According to Derdelinckx and Jerumanis (1984), Fractogel (Toyopearl TSK HW-40(s) gel) allows one to obtain the proanthocyanidins in an advanced state of purity.

Recently, Labarbe et al. (1999) applied an inert glass powder (Pyrex microparticles, 200 to 400 μm) for the fractionation of grape (seed or skin) proanthocyanidins according to their degree of polymerization. Proanthocyanidins are extracted from grape with acetone-water (60:40, v/v). Following this, the nontannin phenolics and proanthocyanidins are fractionated on a Fractogel (Toyoperl TSK HW-50(f) gel) column (35 \times 8 cm) as described by Souquet et al. (1996). The nontannin phenolics are washed out from the column with 2 bed volume, of water followed by 5 bed volumes of ethanol-water-trifluoroacetic acid (55:44.05:0.05, v/v/v). Subsequently, proanthocyanidins are washed from the column with 3 bed volumes of acetone-water (60:40, v/v). Purified proanthocyanidins are dissolved in methanol and then applied onto the column filled with glass microparticles and equilibrated with methanol-chloroform (25:75, v/v) and massively precipitated on top of the column with chloroform. Proanthocyanidins are sequentially eluted from the column by increasing proportions of methanol in a methanol-chloroform solvent system.

Fulcrand et al. (1999) fractionated wine phenolics into simple (phenolic acids, anthocyanins, flavonols and flavanols) and polymeric phenolics using a Fractogel (Toyopearl) HW-50(f) column (bed 12 \times 120 mm). Simple phenolics are eluted from the column with ethanol-water-trifluoroacetic acid (55:45:0.005, v/v/v) while polymeric phenolics are recovered with 60% (v/v) acetone. Later, Mateus et al. (2001) employed a Fractogel (Toyopearl) HW-40(s) column for fractionation of anthocyanin-derived pigments in red wines. Two liters of wine are directly applied onto the Toyopearl gel column (200 \times 16 mm i.d.) at a flow rate of 0.8 mL/min. The anthocyanins are subsequently eluted from the column with water-ethanol (20%, v/v). The elution of wine phenolics from Toyopearl column yields malvidin 3-glucoside and three derived pigments, namely, malvidin 3-glucoside pyruvic adduct, malvidin 3-acetylglucoside pyruvic adduct and malvidin 3-coumarylglucoside pyruvic adduct. These glucosides account for 60% of total monoglucoside content.

Oh et al. (1980, 1985) employed a gel chromatographic technique to study tannin-protein interaction. Tannins are immobilized on Sepharose-4B via epoxy activation. Protein is then applied to the column at pH 4 and the elution of protein from the column achieved using a pH gradient or by anionic and nonionic detergents.

Salogoity-Auguste and Bertrand (1984) as well as Jaworski and Lee (1987) demonstrated that a C₁₈ Sep-Pak cartridge can be used to separate grape phenolics into acidic and neutral fractions. Later, Sun et al. (1998b) successfully used a C₁₈ Sep-Pak cartridge for fractionation of grape proanthocyanidins according to their polymerization degree. The procedure involves passing the extract of grape phenolics through two preconditioned neutral C₁₈ Sep-Pak cartridges connected in series. The phenolic acids are then washed out with water; catechins and oligomeric proanthocyanidins are subsequently eluted with ethyl acetate and anthocyanidins and polymeric proanthocyanidins with methanol. The ethyl acetate fraction is redeposited on the same C₁₈ Sep-Pak cartridges; catechins are first eluted with diethyl ether and then oligomeric proanthocyanidins with methanol.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatographic (HPLC) techniques are now widely used for separation and quantitation of phenolic compounds. Various supports and mobile phases are available for the analysis of anthocyanins, procyanidins, flavonones and flavonols, flavan-3-ols, procyanidins, flavones and phenolic acids (Senter et al., 1989). Introduction of reversed phase columns has considerably enhanced the HPLC separation of different classes of phenolic compounds (Hostettmann and Hostettmann, 1982) and several reviews have been published on the application of HPLC methodology for the analysis of phenolics (Daigle and Conkerton, 1983, 1988; Karchesy et al., 1989; Robards and Antolovitch, 1997; Merken and Beecher, 2000).

Table 10.12 through Table 10.17 summarizes some modern HPLC procedures applied for the analysis of various classes of food phenolics. Food phenolics are commonly detected using UV-Vis and photodiode array (DAD) detectors (Carando et al., 1999; Edenharter et al., 2001; Siess et al., 1996; Tomas-Barberan et al., 2001; Wang et al., 2000a, b; Zafrilla et al., 2001). Other methods used for the detection of phenolics include electrochemical coulometric array detector (EC) (Mattila et al., 2000; Sano et al., 1999), chemical reaction detection technique (de Pascual-Teresa et al., 1998), and fluorimetric detector (Arts and Hollman, 1998; Carando et al., 1999).

Mass spectrometry (MS) detectors coupled to high-performance liquid chromatography (HPLC-MS tandem) have been commonly employed for structural characterization of phenolics. Electrospray ionization mass spectrometry (ESIMS) has been employed for structural confirmation of phenolics in plums, peaches, nectarines (Tomas-Barberan et al., 2001), grape seeds (Peng et al., 2001), soyfoods (Barnes et al., 1998) and cocoa (Hammerstone et al., 1999). Satterfield and Brodbelt (2000) demonstrated that complexation of flavonoids with Cu⁺² enhances the detection of flavonoids by ESIMS. Mass spectra obtained for metal-flavonoid complexes are more intense and simpler for interpretation than those of corresponding flavonoids. Identification of phenolics collected after HPLC analysis has also been carried out using fast atom bombardment mass spectrometry (FABMS) (Bakker et al., 1992; Edenharter et al., 2001; Sano et al., 1999) and electron impact mass spectrometry (Edenharter et al., 2001). Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been employed for qualitative and quantitative analysis of anthocyanins in foods (Wang and Sporns, 1999).

HIGH-SPEED COUNTERCURRENT CHROMATOGRAPHY

High-speed countercurrent chromatography (centrifugal partitioning chromatography; HSCCC) is an all-liquid chromatographic technique very suitable for preparative isolations of pure compounds (Degenhart et al., 2000a, b). Separation of compounds is based on their partitioning between two immiscible liquids (Conway and Petrovski, 1995).

TABLE 10.12**Some HPLC Procedures to Determine Isoflavones in Soy Foods**

| Food | Sample Preparation | Stationary Phase | Mobile Phase | Reference |
|------------------|---|--|---|-----------------------------|
| Seed | Extraction with 80% MeOH; centrifugation | Aquapore C ₈ ; (220×4.6 mm) | A: 10% acetonitrile in H ₂ O with 0.1% TFA; B: 90% acetonitrile in H ₂ O with 0.1% TFA; linear gradient: 100% A, 0% B, 0 min; 70% A, 30% B, 30 min; step gradient to 0% A, 100% B | Simonne, A.H. et al. (2000) |
| Soy foods | Extraction with 80% MeOH; centrifugation, evaporation of MeOH, extraction of lipids with hexane | Brownlee Aquapore C ₈ reverse phase; (300×4.5 mm) | A: 0.1% TFA in H ₂ O; B: acetonitrile; linear gradient: 100% A, 0% B, 0 min; 53.6% A, 46.4% B, 20.6 min | Coward, L. et al. (1993) |
| Seeds, soy foods | Extraction with 0.1% HCl-acetonitrile(1:5), filtration | YMC-pack ODS-AM-303 (250×4.6 mm, 5µm) | A: 0.1% acetic acid in water; B: 0.1% acetic acid in acetonitrile; 85% A, 15% B, 0 min; 85% A, 15% B, 5 min; 71% A, 29% B, 31 min; 65% A, 35% B, 8 min; 85% A, 15% B, 3 min | Murphy, P.A. et al. (1997) |
| Seeds | Extraction with 80% MeOH | YMC-Pack ODS-AQ 303 (250×4.6 mm; 5 µm) | A: 0.1% acetic acid in H ₂ O; B: 0.1% acetic acid in acetonitrile; modified gradient: 85% A, 15% B, 0 min; 69% A, 31% B, 45 min | Wang, J. et al. (2000a) |
| Soy sauce | Direct injection | Wakosil II 5C18 HG (250×4.6 mm) fitted with a precolumn (30×4.6 mm) packed with the same material | A: 0.05% TFA in H ₂ O; B: 90% acetonitrile with 0.05% TFA; 100% A, 0% B, 0 min; 100% A, 0% B, 20 min; 75% A, 25% B, 270 min, linear gradient | Kinoshita, E. et al. (1997) |
| Soy foods | Extraction with 80% MeOH, centrifugation | NovaPak C ₁₈ reverse phase (150×3.9 mm, 4 µm) coupled to Adsorbosphere C ₁₈ column guard | A: 10% acetic acid in H ₂ O; B: MeOH-acetonitrile-dichloromethane (10:5:1 v/v/v). 95% A, 5% B, 0 min; 95% A, 5% B, 5 min; linear gradient: 45% A, 55% B, 20 min; 30% A, 70% B, 6 min; 95% A, 5% B, 3 min | Franke, A.A. et al. (1999) |

| | | | | |
|-----------|--|--|--|-------------------------------|
| Soy foods | Extraction with 96% EtOH, centrifugation, filtration | NovaPak C ₁₈ (150×3.9 mm; 4 µm) | Isocratic: acetonitrile-water (33:67, v/v) | Hutabarat, L.S. et al. (2001) |
|-----------|--|--|--|-------------------------------|

Source: Adapted from Merken, H.M. and Beecher, G.R., 2000, *J. Agric. Food Chem.*, 48:577–599.

TABLE 10.13
Some HPLC Procedures to Determine Catechins and Proanthocyanidins (PA) in Selected Foods

| Food | Sample Preparation | Stationary Phase | Mobile Phase | Reference |
|-----------------------|---|--|---|---|
| Grape seed | Extraction with EtOH; fractionation of PA using Sephadex LH-20 | Exsil 100 ODS C ₁₈ , reverse phase (250×4.6 mm, 5 µm) coupled to C ₁₈ column guard | A: 0.2% phosphoric acid (v/v); B: 82% acetonitrile with 0.4% phosphoric acid; gradient: 100% A, 0% B, 0 min; 85% A, 15% B, 15 min; 84% A, 16% B, 25 min; 83% A, 17% B, 5 min; 57% A, 43% B, 3 min; 48% A, 52% B, 1 min; isocratic 52% B, 7 min; 57% A, 43% B, 1 min; 83% A, 17% B, 1 min; 100% A, 0% B, 2 min | Peng, Z. et al. (2001) |
| Beverages | Direct injection | Spherisorb ODS2 (150×46 mm, 3 µm) | A: H ₂ O; B: MeOH; C: 4.5% aqueous de formic acid; D: 4.5% aqueous formic acid-MeOH (90:10, v/v); gradient: 0–10 min, 100% A to 100% C; 10–20 min, 0–15% D in C; 20–30 min, 15% D in C, isocratic; 30–40 min, 15–35% D in C; 40–45 min, 35% D in C, isocratic; 45–60 min, 35–45% D in C; 60–75 min, 45–100% D in C; 75–175 min, 0–50% B in D; 175–180 min, 50–80% B in D | Pascual-Teresa, S. et al. (1998) |
| Apples, grapes, beans | Extraction with 90% MeOH (apples, grapes) or 70% MeOH (beans); filtration | Inertsil ODS-2 (150×4.6 mm, 5 µm) coupled with OptiGuard PR C 18 Violet A guard | A: 5% acetonitrile in 0.025 M phosphate buffer pH 2.4; B: 25% acetonitrile in 0.025 M phosphate buffer pH 2.4; isocratic, 0–5 min, 10% B; 5–20 min, linear gradient: 5–20 min, 10–80% B; 20–22 min, 80–90% B; isocratic 22–25 min, 90% B; linear gradient, 25–28 min, 10% B; isocratic, 28–37 min, 10% B | Arts, I.C.W. and Hollman, P.C.H. (1998) |
| Wine | Dealcoholized under vacuum; fractionation of polymeric fraction using Tyopearl TSK gel HW-50 (f), thiolysis | Nucleosil 120 (125×4 mm, 3 µm) | A: 2% HCOOH in H ₂ O; B: CH ₃ CN–H ₂ O–HCOOH (80:18:2, v/v/v); linear gradient: 15–75% B, 0–15 min; 75–100% B, 15–20 min | Fulcrand, H. et al. (1999) |
| Wine | Dealcoholized under vacuum; fractionation of procyanidins and | Superspher 100 RP18 (250×4 mm; 4 µm) | A: H ₂ O; B: H ₂ O-acetic acid (90:10, v/v); catechins: 10–80% B, 0–5 min; 80–100% B, 5–29 | Sun, B.S. et al. (1998b) |

| | | | | |
|------------|---|--|---|------------------------------------|
| | catechins using two C ₁₈ Sep Pak cartridges in series | | min; 100% B, 29–45 min; procyanidins: 10–70% B, 0–40 min; 70–85% B, 40–55 min; 85–100% B, 55–74 min | |
| Wine | Filtration, direct injection | Nucleosil 100 C ₁₈ , (250×4 mm, 5 µm) coupled to C ₁₈ column guard | A: 2 mM NH ₄ H ₂ PO ₄ , adjusted to pH 2.6 with H ₃ PO ₄ ; B: 20% A with acetonitrile; C: 0.2 M H ₃ PO ₄ adjusted to pH 1.5 with ammonia; gradient: 100% A, 0–5 min; 0–4% B, 5–15 min; 4–8% B, 15–25 min; 8% B, 92% C, 25.1 min; 8–20% B, 25.1–45 min; 20–30% B, 45–50 min; 30–40%, 50–55 min; 40–80% B, 55–60 min | Carando, S. et al. (1999) |
| Cocoa | Extraction of defatted seeds with 70% acetone (v/v), followed by extraction with 70% MeOH (v/v), fractionation of phenolic from nonphenolics on column packed with Baker octadecyl for flash chromatography | Phenomenex Luna (250×4.6 mm, 5 µm) | A: dichloromethane; B: MeOH; C: acetic acid-H ₂ O (1:1,v/v); gradient: 0 min, 14% B in A; 0–30 min, 14–28.4% B in A; 28.4–50% B in A, 30–60 min; 50–86% B in A, 60–65 min; isocratic 65–70 min | Hammerstone, (1999) |
| Oolong tea | Extraction with acetonitrile-water (1:1, v/v) | Devolosil PhA-5 (250×4.6 mm) | 0.1 M NaH ₂ PO ₄ buffer (pH 2.5)-acetonitrile (85:15, v/v) with 0.1 mM EDTA2 Na, isocratic | Sano, M. et al. (1999) J.F. et al. |

| | | Stationary Phase | Mobile Phase | Reference |
|--------------------|--|---|--|---------------------------|
| Food | Sample Preparation | | | |
| Green tea beverage | Washed with chloroform; extraction with ethyl acetate, ethyl acetate layers combined, evaporated, residue dissolved in 50% acetonitrile | µ-Bondapak C ₁₈ (300×3.9 mm) | Acetonitrile-ethyl acetate-0.05% phosphoric acid (12:286, v/v/v), isocratic | Wang, J. et al. (2000b) |
| Green tea | Extraction with hot water, filtration, washing with chloroform, extraction of water layer with ethyl acetate, ethyl acetate layers combined and evaporated, residue dissolved in water | Hypersil ODS (250×4.6 mm, 5 µm) | 0.05% H ₂ SO ₄ aqueous-acetonitrile-ethyl acetate (86:12:12, v/v/v), isocratic | Chen, Z.-Y. et al. (1998) |

Source: Adapted from Merken, H.M. and Beecher, G.R., 2000, *J. Agric. Food Chem.*, 48:577–599.

TABLE 10.14
Some HPLC Procedures to Determine Anthocyanins and Anthocyanidins in Selected Foods

| Food | Sample Preparation | Stationary Phase | Mobile Phase | Reference |
|-----------------------------|---|---|---|------------------------------|
| Red onions | Extraction with MeOH containing 0.1% HCl, filtration | Prodigy ODS2 (250×4.6 mm, 5 μm) | A: 10% formic acid in H ₂ O (v/v); B: MeOH-H ₂ O-formic acid (50:40:10, v/v/v); isocratic: 80% A, 20% B, 0–4 min; linear regression: 20–80% B in A, 4–26 min | Gennaro, L. et al. (2002) |
| Red wine fruit juices | Dealcoholization of wine under vacuum; dilution of fruit juice with water; separation of anthocyanins using C ₁₈ Sep-Pak | SPLC-18DB (250×10 mm, 5 μm) preparative reverse phase coupled with preinjection C ₁₈ saturator with silica-based packing (75×4.5 mm, 12 μm) and guard with Supelco LC-18 reverse-phase packing (50×4.6 mm, 20–40 μm) | A: 5% formic acid in H ₂ O; B: formic acid-H ₂ O-MeOH (5:5:90, v/v/v); linear gradient: 5–20% B in A, 0–1 min; 20–25% B in A, 1–12 min; 25–32% B in A, 12–32 min; 32–55% B in A, 32–38 min; 55–100% B in A, 38–44 min; 100% B in A, 44–46 min; 100–5% B in A, 46–47 min | Wang, J. and Spoms, P (1999) |
| Red wine | Direct injection | Ultrasphere (C18) ODS (250×4.6 mm) | A: H ₂ O-formic acid (9:1, v/v); B: CH ₃ CN-H ₂ O-formic acid (3:6:1, v/v/v); gradient: 20–85% B in A, 0–70 min; 85–100% B in A, 70–75 min; isocratic: 100% B, 75–85 min | Mateus, N. et al. (2001) |
| Red Wine | Centrifugation, addition of formic acid to 1.5%, filtration | Supelcosik LC-18, (250×2.1 mm), reverse-phase | A: 5% formic acid in H ₂ O (v/v); B: MeOH; gradient: 5% B in A, 0–5 min; 5–65% B in A, 5–55 min; 65–100% B in A, 55–58 min; 100–5% B in A, 58–60 min; 5% B in A, 60–64 min | Mazza, G. et al. (1999) |
| Red blood orange juice | Homogenization with (acetone-EtOH-hexane, 25:25:50, v/v/v), centrifugation, concentration of acetone-EtOH layer, separation of anthocyanins using C ₁₈ Sep-Pak | Prodigy ODS3 (150×4.6 mm, 5 μm) | A: 0.1% phosphoric acid in H ₂ O; B: 0.1% phosphoric acid in acetonitrile; gradient: 10% B in A, 0–2 min; 10–50% B in A, 2–32 min; 50% B in A, 32–37 min; 50–70% B in A, 37–57 min | Lee, H.S. (2002) |

Source: Adapted from Merken, H.M. and Beecher, G.R., 2000, *J. Agric. Food Chem.*, 48:577–599.

TABLE 10.15
Some HPLC Procedures to Determine Flavones
and Flavonols in Selected Foods

| Food | Sample Preparation | Stationary Phase | Mobile Phase | Reference |
|-----------------------------------|--|---|---|--|
| Red onions | Extraction with MeOH stabilized with BHT; dilution with MeOH | Supelcosil LC-18 (250×4.6 mm, 5 μm) coupled with a Spherisorb Supelguard LC-18 | A: 0.01 M sodium phosphate adjusted to pH 2.5 with H ₃ PO ₄ ; B: MeOH; linear gradient: 87–60% A in B, 0–13.5 min; 60–10% A in B, 13.5–39 min; 10–0% A in B, 39–42 min; 0–87% A in B, 42–46 min | Gennaro, L. et al. (2002) |
| Yellow and green french beans | Extraction with chloroform to remove chlorophyll and carotenoids, drying, extraction with 70% MeOH, evaporation of MeOH, purification of phenolics using polyamide cartridge, filtration | LiChrospher 100 RP-18 (250×4, 5 μm) coupled with guard (4×4 mm) packed with the same stationary phase | A: acetonitrile; B: 2% acetic acid in H ₂ O; gradient: 10–30% A in B, 0–35 min; 30–45% A in B, 35–37 min; 45% A in B, 37–42 min; 45–10% A in B, 42–44 min | Hempel, J. and Bohm, H. (1996) |
| Red bean seed coats | Extraction with MeOH, separation of tannins using Sephadex LH-20, flavonoid fraction rechromatographed on Sephadex LH-20 | Shiseido Capcell Pak C ₁₈ , preparative reverse phase (250×10 mm, 5 μm) | Isocratic: acetonitrile-H ₂ O (30:70, v/v) | Beninger, C.W. and Hosfield, G.L. (1999) |
| Buckwheat | Extraction with 80% MeOH, filtration, evaporation, dissolving in MeOH-H ₂ O-oxalic acid (13:36:1, v/v/v) filtration | Capcell Pak C ₁₈ -SG 120, (100 ×4.6 mm, 3 μm) | A: MeOH-H ₂ O-acetic acid (13:36:1, v/v/v); B: MeOH-H ₂ O-acetic acid (73:25:2, v/v/v); gradient: 10–50% B in A, 0–20 min; 50% B in A, 20–25 min; 50–10% B in A, 25–30 min | Oomah, B.D. and Mazza, G. (1996) |
| Tomatoes, onions, lettuce, celery | Extraction with 1.2M HCl in 50% MeOH for 2 h at 90°C; extract adjusted to pH 2.5 with TFA, filtration | C ₁₈ Symmetry (150×3.9 mm, 5 μm) reversed-phase, coupled with C ₁₈ symmetry guard | A: acetonitrile; B: H ₂ O adjusted to pH 2.5 with TFA; gradient: 15–35% A in B, 0–20 min | Crozier, A. et al. (1997) |
| Edible tropical plants | Extraction with 1.2 M HCl in 50% MeOH for 2 h at 90°C, filtration | Nova PakC ₁₈ (150×3.9; 4 μm) | Isocratic: MeOH-H ₂ O (1:1, v/v) adjusted to pH 2.5 with TFA | Miean, K.H. and Mohamed, S. (2001) |

Source: Adapted from Merken, H.M. and Beecher, G.R., 2000, *J. Agric. Food Chem.*, 48:577–599.

TABLE 10.16
Some HPLC Procedures to Determine Other
Classes of Phenolics in Selected Foods

| Food | Phenolics | Sample Preparation | Stationary Phase | Mobile Phase | Reference |
|--------------------------|---------------------|---|---|--|--|
| Finger millet | Free phenolic acids | Extraction with 70% EtOH, centrifugation, concentration, adjusting pH to 2–3, extraction with ethyl acetate, evaporation, dilution in MeOH | Shimpak C ₁₈ (250×4.6 mm) reverse phase | Isocratic: H ₂ O:acetic acid-MeOH (80:5:15, v/v/v) | Subra Rao, M.V.S.S.T. and Muralikrishna, G. (2002) |
| Barley | Phenolic acids | Extractions: hot H ₂ O; acid hydrolysis; acid and α -amylase hydrolysis; acid, α -amylase and cellulase hydrolysis; centrifugation | Supelcosil LC-18 (150×4.6 mm, 5 μ m) | A: 0.01 M citrate buffer pH 5.4 adjusted with 50% acetic acid; B: MeOH; gradient: 2–4% B in A, 0–12 min; 4–13% B in A, 12–20 min; 13% B in A, 20–26 min; 13–2% B in A, 26–30 min | Vasanthan, J.Y.T. and Temelli, F. (2001) |
| Rice bran oil | Coumarins | Extraction with acetone, filtration, evaporation dissolving in MeOH-acetone (1:1, v/v), filtration | Hypersil ODS (125×4 mm, 5 μ m) | Isocratic: MeOH-H ₂ O (75:25, v/v) | Ogawa, K. et al. (2000) |
| Citrus fruits | γ -Oryzanol | Solubilization of oil in hexane-ethyl acetone (9:1, v/v), removal of lipids using silica column (250×25 mm) | Microsorb-MV C ₁₈ (250×4.6 mm) | Isocratic: MeOH-acetonitrile-dichloromethane-acetic acid (50:44:3:3, v/v/v/v) | Xu, Z. and Golber, J.S. (1999) |
| Flaxseed flour, defatted | Lignans | Extraction with 1,4-dioxane-95%EtOH (1:1,v/v), centrifugation, evaporation, alkaline hydrolysis, | Econosil RP C ₁₈ (250×4.6 mm, 5 μ m) | A: 5% acetonitrile in 0.01 M phosphate buffer, pH 2.8; B: acetonitrile; gradient: 100–70% A in B, 0–30 min; 70–30% A in B, 30–32 min | Johnsson, P. et al. (2000) |

acidification to
pH 3, removal of
salt using C 18
reversed SPE

| | | | | | |
|---------|-------------|--|--|---|-----------------------------|
| Peanuts | Resveratrol | Extraction with 80% EtOH, centrifugation, semipurification Al ₂ O ₃ silica gel 60R ₁₈ (1:1) | Vydac C ₁₈ (150×4.5 mm) reverse phase | A: acetonitrile; B: 0.1% TFA; gradient: 0% A in B, 0–1 min; 0–15% A in B, 1–3 min; 15–27% A in B, 3–23 min; 27–100% A in B, 23–28 min | Sanders, T.H. et al. (2000) |
|---------|-------------|--|--|---|-----------------------------|

Source: Adapted from Merken, H.M. and Beecher, G.R., 2000, *J. Agric. Food Chem.*, 48:577–599.

TABLE 10.17

Some HPLC Procedures to Determine Multiple Classes of Phenolics in Selected Foods

| Food | Phenolics | Sample Preparation | Stationary Phase | Mobile Phase | Reference |
|---|--|--|--|--|-------------------------------|
| Lingoberry, cranberry, onions, broccoli | Catechins, flavanones, flavones, flavonols | Extraction with 1.2 M HCl in 50% MeOH for 2 h at 90°C; filtration | Inertsil ODS (150×4 mm; 3 µm) coupled with C-18 guard | A: 50 mM H ₃ PO ₄ pH 2.5; B: acetonitrile; catechins: 86% A in B, isocratic; other flavonoids: gradient: 95% A in B, 0–5 min; 95–50% A in B, 5–55 min; 50% A in B, 55–65 min; 50–95% A in B, 65–67 min | Mattila, P. et al. (2000) |
| Nectarines, peaches, plums | Phenolic acids, catechins, flavonols, procyanidins | Extraction with 80% MeOH containing 2 mM NaF; centrifugation, filtration | Nucleosil C ₁₈ (150×4.6 mm, 5 µm) reverse phase coupled with guard containing the same stationary phase | A: 5% MeOH in H ₂ O; B: 12% MeOH in H ₂ O; C: 80% MeOH in H ₂ O; EA. et al. D: MeOH; gradient: 100% A, 0–5 min; 100% B in A, 5–10 min; 100% B, 10–13 min; 100–75% B in C, 13–35 min; 75–50% B in C, 35–50 min; 50–0% B in C, 50–52 min; 100% C, 52–57 min; 100% D 57–60 min | Tomas-Barberan, et al. (2001) |
| Red raspberry | Ellagic acids, flavones | Extraction with MeOH, filtration, addition of H ₂ O, evaporation, semipurification of phenolics using Sep-Pak C18, filtration | Lichrocart 100 RP-18 (250×4 mm, 5 µm) reverse phase | A: 5% formic acid in H ₂ O; B: MeOH; gradient: 10–15% B in A, 0–5 min; 15–30% B in A, 5–20 min; 30–50% B in A, 20–35 min; 50–90% B in A, 35–38 min | Zafrilla, P. et al. (2001) |
| Propolis | Phenolic | Dilution with EtOH, | Lichrosorb | A: H ₂ O adjusted to pH | Siess, M.-H. |

| | | | | |
|--|---|--|--|------------------------------------|
| acids, flavones, flavonols flavonones | alkaline hydrolysis, acidification, extraction of phenolic with ethyl acetate, evaporation, dissolving in EtOH | RP18 (200× 3, 7 pm) coupled with C-18 guard | 2.6 with H ₃ PO ₄ ; B: acetonitrile; gradient: 0–9% B in A, 0–12 min; 9–13% B in A, 12–20 min; 13–40% B in A, 20–40 min; 40– 70% B in A, 40–60 min; 70% B in A, 60– 85 min | et al. (1996) |
| Spinach Flavonols, flavanones | Extraction with 70% MeOH, removal of carotenoids and chlorophyll using ODS-C ₁₈ packing material, centrifugation, concentration | YMC ODS- AQ (250× 4.6, 5 μm) | A: H ₂ O containing 0.01% TFA; B: acetonitrile containing 0.01% TFA; gradient: 100% A, 0–10 min; 100–50% A in B, 10– 40 min; 50–0% A in B, 40–50 min | Edenharder, R. et al. (2000) |

Source: Adapted from Merken, H.M. and Beecher, G.R., 2000, *J. Agric. Food Chem.*, 48:577–599.

Degenhart et al. (2000c) used HSCCC for preparative isolation of anthocyanins from red wines and grape skins. Anthocyanins were fractionated based on their polarities into four solvent systems:

- Solvent I, consisting of *tert-butyl* methyl ether-*n*-butanol-acetonitrile-water (2:2:1:5, v/v/v/v) containing 0.1% trifluoroacetic acid (TFA), was used as a medium for fractionation of monoglucosides and acylated diglucosides.
- Solvent II, consisting of ethyl acetate-*n*-butanol-water (2:3:5, v/v/v) and 0.1% TFA, was used as a medium for separation of visitins and diglucosides.
- Solvent III, consisting of ethyl acetate-water (1:1, v/v) and 0.1% TFA, was used as a medium for extraction of coumaryl and caffeoyl mono-glucosides.
- Solvent IV, consisting of ethyl acetate-*n*-butanol-water (4:1:5, v/v/v) was employed as a medium for fractionation of acetylated anthocyanins.

Vitrac et al. (2001) have also applied HSCCC for fractionation of red wine phenolics. Phenolics are extracted first from red wine into ethyl acetate. Subsequently the phenolic extract is chromatographed using a 1.5-×60-cm cation-exchange Dowex (Sigma) column. Nonphenolic constituents are washed out from the column with water and then phenolics are eluted with aqueous methanol (75%, v/v). Afterwards, the phenolic extract is fractionated using centrifugal partition chromatography in ascendant and descendant modes. The solvent system's water-ethanol-hexane-ethyl acetate in ratios of 3:3:4:5 (v/v/v/v) and 7:2:1:8 (v/v/v/v) are used, at a flow rate of 3 mL/min, for elution of phenolics in ascendent and descendent modes, respectively.

Degenhart et al. (2000d) demonstrated that HSCCC can be used for isolation of theaflavins, epitheaflavic acids, and thearubigins from black tea using hexane-ethyl acetate-methanol-water (2:5:2:5 and 1.5:5:1.5:5, v/v/v/v). Prior to HSCCC, theaflavins were extracted from tea infusion with ethyl acetate and then cleaned up using a Sephadex LH-20 column to avoid coelution of catechins and theaflavins. On the other hand, isolation of thearubigins required cleaning up tea infusion on an Amberlite XAD-7 column prior to HSCCC to remove all nonphenolic compounds.

Baumann et al. (2001) have developed a simple and efficient method for separating catechin gallates from spray-dried tea extract. Tea phenolic extract is first subjected to liquid-liquid partitioning between ethyl acetate and water. The organic layer containing catechins is then submitted to high-speed centrifugal countercurrent chromatography operating in an ascending mode. Using n-hexane-ethyl acetate-water (1:5:5, v/v/v) or ethyl acetate-methanol-water (5:1:5 and 5:2:5, v/v/v) achieves favorable partitioning. Sephadex LH-20 column with methanol as a mobile phase is used for final purification of catechin gallates.

OTHER CHROMATOGRAPHIC TECHNIQUES

Other chromatographic techniques have also been employed for purification and separation of food phenolics. Of these, paper chromatographic (PC) and thin-layer chromatographic (TLC) techniques are still widely used for purification and isolation of anthocyanins, flavonols, condensed tannins and phenolic acids using different solvent systems (Chu et al., 1973; Durkee and Harborne, 1973; Fenton et al., 1980; Forsyth, 1955; Francis et al., 1966; Francis, 1967; Harborne, 1958, 1967; Jackman et al., 1987; Leung et al., 1979; Mabry et al., 1970).

PC on Whatman no. 3 has been employed for separation of anthocyanins using butanol-acetic acid-water, chloroform-acetic acid-water, or butanol-formic acid-water as possible mobile phases (Jackman et al., 1987). On the other hand, two-dimensional PC has been used for the analysis of procyanidin oligomers. Chromatograms are developed using 6% acetic acid as a mobile phase in the first direction and 2-butanol-acetic acid-water as mobile phase in the second direction (Haslam, 1966; Thompson et al., 1972).

Azar et al. (1987) identified phenolics of bilberry juice (*Vaccinium myrtillus*) using a two-dimensional TLC. Phenolic acids were chromatographed on a 0.1 mm cellulose layer with solvent I: acetic acid-water (2:98, v/v), and solvent II: benzene-acetic acid-water (60:22:1.2, v/v/v). However, TLC analysis of flavonols was carried out on silica gel plates using ethyl acetate-ethyl methylketone-formic acid-water (5:3:1:1, v/v/v/v) or on cellulose plates using solvent I: t-butanol-acetic acid-water (3:1:1, v/v/v) and solvent II: acetic acid-water (15:85, v/v). The phenolic acids were detected by first spraying the chromatograms with deoxidized *p*-nitroaniline and then with a 15% solution of sodium carbonate in water; flavonols were detected by spraying with a 5% aluminum chloride solution in methanol.

Two-dimensional cellulose TLC plates have also been employed to separate procyanidins. t-Butanol-acetic acid-water (3:1:1, v/v/v) was used for development in the first direction while 6% acetic acid was used for development in the second direction. Detection of polyphenols on TLC was carried out using ferric chloride, potassium ferricyanide or vanillin-HCl solutions (Karchesy et al., 1989). TLC on silica using ethyl acetate-formic acid-water (90:5:5, v/v/v) or toluene-acetone-formic acid (3:3:1, v/v/v) has been used for monitoring the isolation of procyanidins by column chromatography (Karchesy and Hemingway, 1986) and by HPLC (Carando et al., 1999), respectively. On the other hand, phenolic acids have been separated on silica TLC plates using n-butanol-acetic acid-water (40:7:32, v/v/v) as a mobile phase (Dabrowski and Sosulski, 1984).

Various gas chromatographic (GC) methodologies have been employed for separation and quantitation of phenolic acids (Dabrowski and Sosulski, 1984; Krygier et al., 1982),

isoflavones (Liggins et al., 1998), capsaicinoids (Thomas et al., 1998), phenolic aldehydes (Friedman et al., 2000), and condensed tannin monomers (Hemes and Hedges, 2000; Matthews et al., 1997). Novel high-temperature gas chromatographic columns, electronic pressure controllers and detectors have significantly improved the resolution and have also led to an increase in the upper range of molecular weights of substances that could be analyzed by GC. Preparation of samples for GC may include removing lipids from the extract, liberating phenolics from ester and glycosidic bonds by alkali (Dabrowski and Sosulski, 1984; Krygier et al., 1982), acid (Zadernowski, 1987) and enzymatic hydrolysis (Liggins et al., 1998) or depolymerizing tannin acid in the presence of nucleophiles such as phloroglucinol (Hemes and Hedges, 2000; Matthews et al., 1997) or benzyl mercaptan (Guyot et al., 2001; Labarbe et al., 1999; Matthews et al., 1997). Prior to chromatography, phenolics were usually transformed to more volatile derivatives by methylation (Jurenitsch et al., 1979; Jurenitsch and Leinmuller, 1980; Kosuge and Furuta, 1970), trifluoroacetylation (Chassagne et al., 1998; Sweeley et al., 1963), conversion to trimethylsilyl derivatives (Dabrowski and Sosulski, 1984; Krygier et al., 1982) or derivatization with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (Hemes and Hedges, 2000).

CAPILLARY ELECTROPHORESIS

Capillary electrophoresis is a novel analytical tool for separating many classes of compounds (Cikalo et al., 1998; Zeece, 1992) based on the electrophoretic migration of charged analytes. Small internal diameter capillary columns minimize the ohmic heating problems that may have an adverse effect on bandwidths. In addition, small sample sizes can be used and separations require little or no organic solvents (Cikalo et al., 1998).

Hall et al. (1994) used capillary electrophoresis for separation of food antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Later, Andrade et al. (1998) utilized capillary zone electrophoresis to evaluate the effect of grape variety and wine aging on the composition of noncolored phenolics in port wine. Noncolored phenolics were extracted from wine into diethyl ether, then concentrated to dryness and redissolved in methanol. Subsequently, phenolics were separated on a fused-silica capillary column with 0.1 M sodium borate (pH 9.5) at 30°C and voltage of 20 kV producing a current of 90 μ A.

Chu et al. (1998) separated pure forms of *cis*- and *trans*-resveratrol isomers from wine using capillary electrophoresis in micellar mode. Direct separation of resveratrols in wine samples was performed with fused silica capillaries in 25 mM sodium borate, 25 mM sodium phosphate and 75 mM SDS (pH 9.3) at 16 kV and 20°C using a UV detector set at 310 nm. The detection limit of the method was 1.25 mM. To determine rutin content in different fractions of buckwheat flour and bran, Kreft et al. (1999) utilized capillary electrophoresis with a UV detector. The extraction of rutin from buckwheat fractions was carried out using 60% ethanol containing 5% ammonia in water. Identification of resveratrol and rutin was confirmed by spiking the samples with standards.

Moane et al. (1998) utilized capillary electrophoresis for direct detection of phenolic acids in beer. Separation of phenolic acids was performed with fused silica capillary in 25 mM phosphate buffer, pH 7.2 at 25 kV. The sample was injected to capillaries using a

reversed-polarity injection technique to remove nonphenolic cationic and neutral compounds. (These substances interfere with electrochemical detection of phenolic acids by passivation the electrode surface.) Recently, Pan et al. (2001) developed a method for determining protocatechuic aldehyde and protocatechuic acid by capillary electrophoresis with amperometric detection. Under optimum conditions these two analytes were completely separated in 8 min with detection limits of 0.10 g/mL for protocatechuic aldehyde and 0.25 µg/mL for protocatechuic acid.

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Index

A

- absorption of
 - caffeic acid, 333
 - food phenolics, 332
 - Quercetin, 337
- abrasive dehulling of flaxseed, 107
- acetaldehyde, 281
- acetoside, 319
- acetovanillone, 190
- acetoxypinoresinol, 113
- acetylaidzin, 97
- acetylgenistin, 97
- acetylglycitin, 97
- acid(s)
 - anarcadic, 56, 362, 363, 368
 - benzoic, 1
 - benzyltartaric, 319
 - caffeic, 5, 19, 37, 38, 42, 44, 50, 57, 61, 85, 105, 106, 109, 110, 132, 135, 141, 158, 162, 166, 172, 181, 190, 255, 267, 278, 292, 313, 315, 336, 344, 355, 426, 431
 - caffeoylferuloylquinic, 251
 - caffeoylquinic, 156, 170, 190, 251, 254
 - caftaric, 148, 151, 284, 313
 - caftaric, *trans*-, 276
 - caffeic, *trans*-, 84
 - carnosic, 369, 433
 - chlorogenic, 25, 54, 86, 97, 109, 110, 158, 160, 164–168, 181, 186, 187, 188, 190, 193–195, 197, 201, 242, 251, 253, 255, 288, 289, 333, 346, 369, 370, 426, 444, 503
 - cichoric, 313
 - cimicifugic, 319
 - cinnamic, 44, 104, 268
 - cinnamic, *trans*-, 3
 - citric, 413
 - coumaric, 37, 60, 97, 104, 105, 109, 113, 135, 141, 184, 188, 197, 344
 - coumaric, *p*-, 3, 5, 19, 25, 30, 38, 42, 44, 50, 57, 61, 85, 86, 106, 109, 132, 141, 166, 190, 267, 268, 269, 278, 288, 316, 426
 - courmaric, *trans-p*-, 84
 - coumarylquinic, 164, 166
 - coumaroylquinic, *p*-, 168, 251
 - coutaric, 148, 276
 - cryptochlorogenic, 54, 160
 - curvulic, 436
 - dicaffeoylquinic, 168, 193, 251, 254, 364
 - dicaffeoyltartaric, 193, 364

- digallic, 178
- dihydroxybenzoic, 2, 4-, 141
- elenolic, 112
- ellagic, 61, 151–154, 178, 180, 286, 369
- ferulic, 5, 18, 19, 25, 37, 40, 44, 50, 51, 57, 61, 85, 86, 97, 105, 106, 109, 132, 135, 166, 184, 188, 190, 197, 264, 267, 269, 276, 278, 292, 316, 426, 437
- fertaric, *trans*-, 148
- ferulic, *trans*-, 84
- feruloylquinic, 251, 254
- free phenolic, 26, 86
- fukiic, 319
- fukinolic, 319
- gallagic, 152
- gallic, 3, 5, 12, 38, 44, 57, 60, 61, 104, 105, 132, 135, 162, 178, 197, 242, 267, 268, 276, 278, 280, 281, 286, 333, 351, 370, 429, 504
- gallotannic, 55
- genistic, 50, 60, 85, 316
- gingkolic, 315
- glucuronic, 338
- glutathionylcaftaric, 2-*S*-, 276
- glycyrrhetic, 321
- hippuric, 336
- hydroxy-4-methoxybenzoic, 104, 105
- hydroxybenzoic, 42, 57, 84, 104, 105, 106, 109, 111, 132, 184, 269, 273, 437
- hydroxybenzoic, 4-, 267
- hydroxybenzoic, *p*-, 5, 25, 30, 38, 44, 60, 61, 85, 86, 109, 162, 181, 188, 197, 278, 316, 426
- hydroxycaffeic, 111
- hydroxycinnamic, 111, 132, 154, 158, 159, 163, 165, 168, 172, 181, 187, 268, 292
- hydroxycinnamic, 4-, 3
- hydroxycinnamoyltartaric, 148
- hydroxyphenylacetic, 111
- hydroxyphenylacetic, *p*-, 38, 60
- hydroxyphenyllactic, 104
- insoluble phenolic, 88
- isochlorogenic, 181, 426
- isoferulic, 316
- isovanillic, 197
- lactic, 261
- linolenic γ -, 103, 105, 137
- methoxybenzoic, 162
- methoxycinnamic, 162
- neochlorogenic, 54, 156, 160, 181
- nordihydroguaiaretic, 414
- phenolic(s), 19, 25, 38, 43, 50, 57, 84, 85, 97, 104, 106, 132, 135, 136, 138, 141, 172, 181, 188, 193, 194, 197, 242, 266, 268, 270, 273, 284, 288, 320, 333, 343, 346, 421, 426, 444, 448, 485, 504
- phloretic, 341
- protocatechuic, 3, 5, 25, 30, 37, 38, 42, 44, 54, 60, 85, 104, 105, 132, 162, 184, 197, 267, 276, 278, 344, 369, 436
- quinic, 109, 110
- resorcylic, 197
- rosmarinic, 103, 341, 369

- salicylic, 3, 25, 38, 50, 97, 105, 447
- sinapic, 5, 19, 25, 38, 42, 50, 57, 85, 86, 88, 103, 106, 132, 141, 197, 267, 269, 292, 497
- sinapic, *trans*-, 84
- syringic, 3, 5, 19, 25, 30, 38, 50, 60, 61, 84, 85, 86, 97, 103, 109, 132, 162, 181, 197, 267, 276, 278, 286, 426, 437
- tannic, 20, 271, 342, 355
- tartaric, 284
- theaflavic, 245
- thiobarbituric, 2-, 407
- thomasidioic, 94
- trimetoxyphenylacetic, 286
- ursolic, 54, 319
- vanillic, 3, 5, 25, 30, 38, 41, 42, 44, 50, 52, 57, 60, 61, 84, 85, 97, 109, 112, 113, 132, 162, 184, 188, 197, 251, 253, 267, 268, 278, 286, 315, 316, 370, 426, 437, 447
- acteoside, 109
- actinidine, 317, 318
- active oxygen method, 406
- acylation, 457
- aesculetin, 5
- affinity of tannins, 351
- affinity of polyphenols, 352
- aflatoxin, 359
- aged cognacs, 286
- aging of red wine, 281, 282
- aging of wine, 273, 280
- alcohol, 270
 - coniferyl, 3, 38
 - coumaryl, 3, 163
 - homo vanillic, 341
 - phenyl, 111
 - sinapyl, 3
 - triterpene, 41
- alcoholic beverages, 285
- alcohol-free beers, 270
- alkacins, 360, 361
- alkaline hydrolysis of wheat, 50
- alkaline phosphatase, 512
- alkaloids, 317
- alkyl gallates, 360
- alkylphenols, 51, 450
- alkylresorcinols, 51
- allspice, 433, 487
- almond, 43, 54
- almond skins, 54
- amentoflavone, 317
- amides, 198
- anethole, 447
- angelicin, 186
- anthocyanin(s), 7, 95, 132, 135, 136, 138, 139, 142, 146, 154, 155, 156, 158, 160, 162, 166, 178, 179, 180, 184, 187, 197, 261, 273, 281, 294, 296, 334, 453–455, 457, 485
 - chromophores, 457
 - color of, 460

- color stability of, 457
- coloration of, 459
- degradation of, 459
- determination of, 499
- loss of, 460
- metabolic fate of, 334
- oxidative polymerization of, 29
- quantification of, 499
- self-association of, 456
- stability of, 457, 459
- sugar moiety of, 457
- anthocyanidin(s), 6, 43, 171, 264, 273, 456
 - pigments, 34
- anthocyanin-anthocyanin complex, 459
- anthraquinones, 316
- antibacterial activity, 359, 362
- antifungal agents, 360
- antiinflammatory properties, 367
- antimicrobial agent, 358
- antimutagenic activity, 365
- antinutritional, 342
- antinutritional effects, 89, 342, 355
- antinutritional factors of beans, 22
- antioxidant activity, 406, 417, 428, 431
 - of spices, 432
- antioxidants, 358, 406
- antiulcer property, 364
- antiviral properties, 363
- apiferol, 44
- apigenidin, 45
- apigenin, 11, 39, 52, 112, 133, 192, 424
- apple(s), 163
 - fruit, 166, 167
 - peel, 167
 - juice, 288, 292, 293
 - polyphenols, 363
 - pulp, 290
 - ciders, 357
- Arabica, 252, 257
- Arabica coffee, 253
- Arabusta, 257
- arbutin, 168
- aroma, 443
- asparagus, 190
- astilbin, 11, 148, 149
- astragalin, 19, 132, 133
- astringency, 452
 - of theaflavins, 453
 - of tea, 453
 - thresholds, 453
 - sensation, 453
- astringent phenols, 452

astringins, 149
auraptene, 173, 174, 367
aureusidin, 137
autoxidation, 403
autoxidative stability, 110
avenanthramides, 38
avicularin, 133, 166, 167
avocado, 197

B

bacteria, 261
banana, 170, 447
Barlett pears, 169
barley, 24
barley bran, 27
barley malt, 24, 264
barnyard millet, 37
barrel aging, 280
beach pea, 487
bean fermentation, 261
beans, 18, 22
beefy flavor note, 450
beer, 264
beer haze, 271
berberine, 319
bergamottin, 173, 367
bergapten, 5, 174, 186, 191
bergaptol, 5
berries, 131
betanidin, 189
betanin, 189
betel nut, 54, 55
beverages, 241
biagigenin, 316
biflavonoids, 316
bilberry (bilberries), 131, 365, 454
bilobalide, 315
bilobetin, 315, 317
bilobol, 315
binding of catechins, 342
binding of proteins, 349
bing cherries, 157
bioavailability
 of polyphenols, 332
 of caffeic acid, 333
 of elderberry, 334
 of quercetin, 337
biological activity, 91
biologically active phenols, 483
bisdemethoxycurcumin, 368
bisflavanol(s), 247, 248

bistheafavin A, 248
bitter flavor, 443
bitter flavonones, 446
bittersweet apples, 291
black raspberry juice, 295
black chokeberry, 155
black cohosh, 318
black pepper, 362, 433
black tea, 241, 366
black discoloration, 469
black raspberry, 154
black gram beans, 356
blackberries, 135, 454
blackcurrant, 136, 365, 429
blood orange, 172, 177, 293, 294
blue discoloration, 469
blueberries, 138, 454, 487
borage, 85, 103, 429
bovine serum albumin assays, 505
brandy, 287
brassicasterol, 431
Brew's spent grain, 29
brewing method, 255
brewing, 248
brewing tea in hot water, 248
bromophenols, 452
brown rice, 40
bryozoans, 452
buckwheat, 30
butein, 11, 423
butylated hydroxyanisole, 359, 409
butylated hydroxytoluene, 410

C

caffeine, 251
caffeoyltartaric, 193
Camellia sinensis, 241
campesterol, 431
canadaline, 319
canary seeds, 431
canned tomatoes, 201
canola, 83, 487
biological activity of, 90
hulls, 85, 91, 94, 348
tannins, 91
capillary electrophoresis, 530
capsaicin, 198
capsaicinoid, 197
capsaicinoid-like substances, 198
capsiate, 198
capsicum, 197, 199, 433

- carbohydrates, 352
- carbonyls in beer, 264
- carboxyphenols, 108
- cardanols, 56, 368
- cardols, 56, 315
- carlinoside, 29
- carnosol, 369, 433
- carotenoids, 318
- carrots, 181
- carvacrol, 369, 447
- cashew nut, 55, 56, 368
- cashew nutshell oil, 56
- casuarictin, 152
- catechin(s), 6, 7, 11, 20, 27, 47, 54, 55, 105, 132, 133, 135, 138, 180, 242, 249, 257, 264, 267, 271, 276, 278, 284, 288, 429, 431, 504
- catechin-glutathione, 336
- catechin metabolites, 335
- catechol oxidase, 244, 462
- catechol, 38, 346, 409
- catecholamines, 170
- catecholase, 464
- celery, 191
- cell proliferation, 366
- cereals, 17
- chalcone, 6, 8, 95, 423, 446
- chalcone isomerase, 6
- chalcone synthase, 6
- chatinine, 317
- cheese flavor, 450
- chemometric methods, 503
- chemopreventive activity, 367
- cherries, 454
- cherry varieties, 157
- cherry tomatoes, 200
- chiang, 102
- chickpea, 21
- chickpeas, 20
- chill haze, 271
- chloroanisoles, 150
- chocolate liquors, 448
- chocolate aroma, 448
- chromatographic techniques, 516
- chromatography of polyphenols, 517
- chrysin, 11, 158
- cider apple, 165, 487, 504
- cider apple juice, 165
- cider brandy, 287
- cider, 288
- cinnamon, 448
- citral, 447
- citrimin, 436
- citropten, 447

- citrus flavonoids, 367
- citrus fruit, 171, 172, 173, 175, 294, 367, 445
- citrus peel oil, 171
- clovamide, 261
- clove, 433, 448, 487
- cocoa, 256
 - powder, 261
 - proteins, 262
 - procyanidins, 365
 - bean fermentation, 262
 - seeds, 257
 - liquor, 261
 - polyphenol oxidase, 262
 - beans, 256, 259, 261, 448
- coffee, 250
 - flavor, 253
 - beverage, 255
 - beans, 250
 - cream, 255
 - aroma, 257
- cognac, 286
- cold-pressed lemon oil, 173
- collagen assay, 509
- color, 453
- color of green tea 460
- color stability, 457
- colorimetric assay of condensed tannins, 496
- competitive binding assays, 511
- competitive inhibitor, 100
- competitive tannin-salivary protein-binding assay, 511
- complexation of tannins, 354
- complexation of polyphenols, 343
- condensed tannin(s), 13, 20, 55, 89, 90, 94, 273, 275, 348, 352, 453, 492, 495,
 - of beach pea, 508
 - of buckwheat, 32
- coniferaldehyde, 286
- coniferin, 172
- conjugate of resveratrol, 340
- Coomassie brilliant, 508
- copigmentation, 281, 459
- copigments, 453, 456
- corn, 32–34
- corpylpalmine, 319
- cottonseed, 84, 85, 104, 427
- coumarin, 5, 106, 160, 173, 174
- coumarin metabolites, 341
- coumaroylcholine, 87
- coumaroylcholine, *p*-, 88
- coumarylglucose, 154
- coumarylspemidine, 33
- coumaryltryptamine, *p*-, 33
- coumestan, 99

coumestrol, 99, 266
cowpea, 20, 21
crab apples, 290
crambe meal, 93
cranberry (cranberries), 131, 141, 360, 361, 454
 juice, 141
 fruits, 141
 proanthocyanidins, 141
 beans, 357
cresolase, 464
cresol(s), 40, 190, 447
curcumin, 364, 368
curcuminoids, 368
curdling, 255
cutin, 1, 10
cyanidin, 9, 11, 27, 135, 171, 275, 429
cycloartenol, 431
cyclodextrin, 352
cynarine, 313

D

daidzein, 11, 95, 97, 339, 426
daidzin, 11, 95, 97
daily intake of flavonoids, 331
daily intake of quercetin, 332
daphnetin, 267
decarboxylation of ferulic acid, 446
decomposition of proanthocyanidins, 493
dehulled millets, 36
dehulling, 49, 356
dehydrodimers of ferulic acid, 188
dehydrokavain, 318
delicious apple juice, 166
delphinidin, 9, 19, 27, 135, 171, 275, 429
delphinium, 11
demethoxycurcumin, 368
demethylleuropein, 110, 111
deoxyclovamide, 261
deoxyflavonols 3-, 7
depsides, 242
desmethylangolensin, 339
desmethyloxanthohumol, 264, 265, 266
detection thresholds, 443
determination of
 condensed tannins, 491
 hydrolyzable tannins, 496
 chlorogenic acid, 498
 sinapine, 497
 theaflavins, 499
 insoluble condensed tannins, 496
Devil's claw, 319

didymin, 177
 diferuloylputrescine, 33
 diferuloylspermine, 33
 difeuloylspermidine, 33
 dihydrocapsaicin, 198
 dihydrocapsiate, 199
 dihydrochalcones, 164, 166, 167, 446
 dihydrodaidzein, 339
 dihydrodaidzin, 97
 dihydroflavonol, 154
 dihydrogenistin, 97
 dihidrokavain, 318
 dihydromethysticin, 318
 dimeric proanthocyanidin, 281
 dimeric procyanidin(s), 168, 320
 dimethoxybenzoylcholine 3, 4-, 87
 dimethylphenol 2,3-, 261
 diosmin, 133, 176
 diphenol oxidase, 462
 diphenylpropanes, 6
 discoloration, 462, 464
 distilled spirits, 285, 287
 DMCA assay, 495
 dodecyl gallate, 411
 dopamine, 170
 double-low rapeseed, 93
 double-zero rapeseed, 83
 DPPH, 407
 drying plums, 162
 dye-labeled protein assay, 508

E

echinacea, 313
 echinacoside, 313
 effect of processing, 32, 39, 48, 53, 92, 99, 261, 291, 268
 eggplants, dark skin of, 454
 elderberry, 334, 365
 extract, 334
 electrochemical methods, 512
 elimicin, 170, 447
 ellagitannins, 12, 151, 154, 287
 endive, 192
 engeletin, 11, 148, 149
 enzymatic, 343
 enzymatic assays, 512
 enzymatic browning reactions, 462, 463, 464
 enzymatic methods, 512
 enzymic oxidation, 262
 epicatechin(s), 10, 11, 27, 55, 57, 105, 132, 133, 165, 180, 242, 249, 257, 275, 276, 278, 282, 284, 289, 429, 431
 epicatechin gallate, 10, 11, 242, 249, 366, 432

epicatechin-based procyanidins, 257
epidermis, 163
epigallocatechin gallate, 10, 242, 249, 366, 432
epigallocatechin, 10, 11, 180, 242, 249, 257, 431
epigenidin, 11
equol, 339
eriodictrin, 177, 336
eriodictyol, 11, 45, 47, 59, 176, 336
esculatin, 174
essential oils, 447, 448
estragole, 447
estrogenic activity, 266, 339
ethylcatechol 4-, 253
ethylguaiacol 4-, 251, 253
ethylphenol, 3, 261, 339
eugenol, 3, 170, 179, 447, 448
evening primrose, 85, 105, 429
excess tannins, 348
extraction of tannins, 487

F

faba bean, 21
feathering of coffee cream, 255
fermentation process, 269
fermented cocoa beans, 262
fermented tea, 247
fermented soy products, 102
ferulic acid dehydrodimers, 27, 36, 42, 51, 190
feruloyl esters, 41
feruloylcholine, 87, 88
feruloyltryptamine, 33
feruoylputrescine, 33
field pea, 20
finger millet, 37, 355
fisetin, 422
flavan-3-ol(s), 6, 7, 20, 132, 144, 146, 158, 159, 160, 163, 165, 244, 273, 460, 491,
flavan-4-ols, 44
flavan-3,4-diols, 7, 273, 460
flavanols, 285, 335
flavanone glycosides, 445
flavanone(s), 177, 264, 265, 292, 294, 336
 antioxidant activity of, 423
flavanonol(s), 148, 273, 336
flavoglucanin, 435, 436
flavognost reagent, 499
flavokavains, 318
flavone glycosides, 315
flavone C-glycoside, 29
flavone(s), 6, 31, 95, 133, 242, 264, 273, 336, 370, 460
 antioxidant activity of 422
flavonoid(s), 1, 6, 19, 31, 43, 106, 112, 132, 136, 194, 278, 315, 337, 346.

flavonoid glycosides, 346
flavonoid pigments, 460
flavonol(s), 6, 31, 95, 133, 137, 138, 146, 158, 160, 168, 183, 187, 200, 201, 242, 245, 264, 273, 296, 315, 336, 370, 409,
flavonol glycosides, 132, 153, 162, 249
flavonolignans, 322
flavonols in tomatoes, 200
flavononol(s), 6
flavor, 453
flavor of
 black tea, 453
 cocoa, 261
 vanilla, 447
flavor thresholds, 268, 444, 452
flavor defect, 448
flavylium cation, 8, 456
flax, 84
flaxseed, 105
Florida blood orange juice, 173
Folin-Ciocalteu assay, 490
Folin-Denis assay, 489, 504
food-grade antioxidant, 406
formation of haze, 270
formononetin, 95, 97
French wines, 276
fresh tea shoot, 245
fruit juices, 160
fruit(s), 131, 453
furanocoumarins, 174, 182, 186, 191

G

galactosidase activity, 512
gallocatechin, 11, 133, 180
gallotannins, 12, 178, 180
gas chromatographic, 529
gaseous ammonia, 93
genistein, 11, 95, 97, 158, 266, 339, 424, 426,
genistin, 11, 95, 97,
geranylchalconaringenin, 264
geranyloxy-7-methoxycoumarin, 5-, 173
geranyloxypsolaren, 173
germination of grains, 51
ginger, 320, 369, 433
ginger oleoresin, 448
gingerol(s), 320, 448
ginkgetin, 317
ginkgo biloba, 315
ginkgolides, 315
ginkgols, 315
ginseng, 314
glucogallin, 178

glucopyranosyl sinapate, 87
 glucosinolate-free rapeseed, 443
 glycosyldiosmins, C-, 176
 glucosylflavone, C-, 36, 41, 176
 glucosylorientin, 36
 glucosylvitexin, 36
 glucuronidation, 332, 337
 glucuronide conjugates, 332
 glyceollins, 99
 glycitein, 95, 97, 426
 glycitin, 95, 97
 glycosides, 177
 glycosylated polyphenols, 332
 glycyrrhizin, 321
 goldenseal, 319
 gooseberries, 454
 gossyaerulin, 104
 gossyfulvin, 104
 gossypol, 104
 gossypurpurin, 104
 gramisterol, 431
 grape(s), 144, 273, 295
 cultivars, 145
 juice(s), 149, 284, 285
 berries, 149
 seed procyanidins, 280
 varieties, 144
 seed(s), 145, 364, 365
 skin(s), 145, 147, 275
 grapefruit, 174, 177
 grass pea, 20
 green barley leaves, 29
 green coffee beans, 251, 252
 green gram, 21
 green lettuce, 194
 green pepper berries, 362
 green tea, 241–248, 431, 504
 green tea catechins, 249
 greenish-black discoloration, 469
 growth-depressing effect of tannins, 356
 guaiacol, 3, 40, 109, 190, 436, 447

H

ham-like flavor note, 448
 hard-to-cook beans, 19
 harpagide, 319
 harpagoside, 319
 hawthorn, 320
 haze formation, 271
 hazelnut, 57
 head lettuce, 193

heat treatment, 22, 101
hemoglobin assay, 510
heptamethoxyflavone, 175, 294
herbs, 432, 447
hesperidin, 11, 177, 292, 294
heterocyclic amines, 365, 369
high-performance liquid chromatography, 518
high-speed countercurrent chromatography, 518
high-tannin sorghum, 357
highbush blueberry, 131, 138
histatins, 349
homocapsaicin, 198
homodihydrocapsaicin, 198
hops polyphenols, 269
hordenine, 268
hordeumin, 29
horse gram, 21
hydrastin, 319
hydrobilobol, 315
hydrolysis of haze, 271
hydrolyzable tannin(s), 13, 43, 180, 273, 281, 351, 496
hydrolyzates of barley, 25
hydroperoxide formation, 406
hydrophobic, 349
hydrostidine, 319
hydroxybenzaldehyde, 38, 39, 41, 52, 188, 190
hydroxybenzoylcholine 4-, 87
hydroxycinnamates, 146, 276, 360
hydroxycoumarins, 264
hydroxyflavones 3-, 7
hydroxyisochromans, 111
hydroxyl radicals, 403
hydroxyl radical scavenging activity, 410
hydroxytyrosol, 110, 111, 113, 341
hyperforin, 316
hyperin, 31, 132, 133, 141, 166, 316
hyperoside(s), 316, 320

I

ideain, 166
ingestion of polyphenols, 342
inhibition of iron absorption, 355
initiation, 405
insoluble tannins, 92, 496
insoluble tannin-protein complex, 511
instant coffee, 254, 503
interflavonoid linkage, 351
iridoids, 317
iridoiglycosides, 319
iron absorption, 355
isoacetoside, 319

isobetanin, 189
isocorpylpalmine, 319
isocoumarins, 181, 182
isoferuloylcholine, 87, 88
isoflavone(s), 6, 95, 96, 97, 338, 368
isoflavone aglycones, 100, 338
isoflavone bioavailability, 338
isoformononetin, 97
isogingetin, 315, 317
isohofolin, 133
isolarieiresinol, 107
isolation of proanthocyanins, 516
isoorientin, 29, 31
isopimpenellin, 174, 186, 191
isoquercetin, 169, 267
isoquercetrin, 132, 133, 167, 178, 180, 316
isoquinoline alkaloids, 319
isorhamnetin, 135, 169, 183, 196, 315, 460
isosakuranetin, 11, 176
isosinensetin, 173, 175
isovaltrates, 317
isovitexin, 29, 31, 41, 242, 429
isoxanthohumol, 265
Italian blood orange juice, 173

J

jaceidin, 195
jang, 102
jiang, 102
juglone, 61

K

kaempferol, 11, 19, 34, 92, 133, 135, 137, 153, 154, 157, 158, 169, 183, 192, 242, 245, 267, 296, 315, 316, 346, 370, 460
kava kava, 318
kavain, 318
kavalactones, 318
kidney bean, 21
kiwifruit juice, 296
kiwifruit, 296
koji, 102

L

laccase, 272, 285
lactic acid fermentation, 112
lambertianin, 152, 153
leaching of isoflavones, 101
leachinol, 150
legumes, 17
lettuce, 184, 193
leucoanthocyanidins, 44, 264

leucocyanidin(s), 34, 55, 59, 60
leucodelphinidin(s), 59, 60
leucopelargonidin(s), 34, 55
licorice, 321
lignans, 1, 10, 106, 108, 113, 278
lignins, 1, 10, 287
lignophenols, 108
ligstroside, 110, 111, 113
lima bean, 21
limonene, 171
lingonberry, 131
lipid oxidation, 403
liquid chromatography, 516
low-tannin bean, 22
low-tannin sorghum, 357
lowbush blueberry, 139
luteoferol, 45
luteoforolor, 44
luteolin, 37, 39, 58, 111, 112, 133, 192, 197, 316
luteolinidin, 45

M

mace, 433, 434
magniferin, 179
Maillard reaction, 295
malognol, 12
malonaldehyde, 407
malonyl coenzyme A, 6
malt, 24, 264
malt polyphenols, 269
malvidin, 9, 19, 133, 135, 171, 282
mammalian lignans, 341
mandarin orange, 447
mangiferin, 178
mango, 178
manoyldaidzin, 97
manoylgenistin, 97
manoylglycitin, 97
matairesinol, 107, 341
maturation of peanuts, 58
maturation process, 280
maturation of sorghum grains, 48
metabolism of
 trimethylamine, 342
 coumarins, 341
 eriocitrin, 336
 catechins, 335
 resveratrol, 340
 rosmarinic acid, 341
metabolite of gallic acid, 333
metabolites of catechins, 335

methoxy-5-(1-propenyl) phenol, 109
methoxylated flavones, 294
methoxymellein, 6-, 182
methoxyphenol, 2-, 261
methyl salicylate, 179
methyl gallate, 179
methylenecycloartanol 2,4-, 431
methyleugenol, 447
methylphenol, 261
methylscutellarein, 294
methysticin, 318
micellization, 23
milk thistle, 322
millet(s), 35, 36
milling fractions of oats, 39
miso, 102
monolayer of polyphenols, 347
monophenolic antioxidants, 417
morin, 135
moromi, 103
moth bean, 21
mustard flour, 428
mutabilein, 19
mutabilin, 19
myricetin, 135, 137, 148, 183, 245, 267, 278, 316, 422, 460
myricitrin, 267

N

N-acylantranilate alkaloids, 38
N-methyltyramine, 268
naphthoquinones, 61
naringenin, 11, 158, 176, 177
naringin, 11, 170, 177, 445, 446
narirutin, 177, 292, 294
natsudaïdain, 174, 175
natural antioxidants, 403, 417
natural food pigments, 453
natural health products, 313
Naval oranges, 177
NDGA, 414
near-infrared reflectance, 504
nectarines, 158, 454
neodiosmin, 133, 176
neoeriocitrin, 177
neohesperidin, 177, 370, 445, 446
neoponcirin, 177
nobiletin, 173, 175, 292, 294
non-competitive inhibitor, 465
nonenzymatic polymerization, 463
nonenzymatic discoloration, 468
nonenzymic oxidation, 262

nooketone, 447
nordihydrocapsaicin, 198
norepinephrine, 170
nuclear magnetic resonance, 504
nutmeg, 433
nutraceutical, 313
nuts, 17

O

cresol, *o*-, 3, 40
oak barrels, 280, 287
oak chips, 281
oat(s), 38, 48, 435
obtusifoliol, 431
octacosan-1, 28-diol, 38
odor description, 443, 445
oilseeds, 83
okanin, 11, 423
okara, 102
oleanic, 319
oleuropein, 110, 111, 113, 359, 361
oligomeric proanthocyanidins, 444
olives, 434
olive oil, 110–114
onion(s), 183, 435
oolong tea, 242, 366
ORAC (oxygen radical absorbance capacity), 407
orange, 174
orange juice, 172, 292, 293
oregano, 433
orientin, 31
oryzanol, 41, 115
oxidation, 343
oxidation of phenols, 463
oxidizable polyphenols, 269
oxygen radical absorbance capacity, 140

P

pallidol, 142, 150
palm oil, 114
palm fruit, 114
panaxosides, 315
paper chromatography, 529
para-anisidine, 407
parabens, 360, 361
parboiled rice, 40
parsnip, 186
passion fruit, 179
patuletin, 195
peaches, 158, 454
peanut butter, 58

- peanut(s), 57, 84, 85, 427
- pearl millet flours, 36
- pearl millet, 35
- pear(s), 168, 289, 295
- pea(s), 18, 21
- pecan kernels, 59
- pecan, 59
- pectin, 171
- pedunculagin, 152
- pelargonidin, 9, 11, 27, 135, 171, 282
- peonidin, 9, 133, 171, 189, 275
- pepper, 197
- peroxidases, 465
- persimmon, 453
- petunidin, 9, 19, 133, 135, 171, 275
- pharmacological effects, 358
- pharmacological potentials, 364
- phenol, 3, 40, 190, 261
- phenol-binding, 355
- phenol-enzyme interaction, 346
- phenol-ferrous complexes, 468
- phenol-protein complex, 452
- phenolases, 464, 467
- phenolic(s)
 - acid glycosides, 87
 - acid-protein complexes, 343
 - antioxidants, 360, 408
 - association of, 352
 - beer, 264
 - bilberry juice of, 529
 - black tea of, 333
 - bound, 61
 - degradation of, 333
 - determination of total, 489
 - dietary intake of, 331
 - extracts of wine, 503
 - food, 364, 403, 452, 465, 483
 - glycosides, 264
 - grape, 144, 284
 - iron-binding, 500, 501
 - nonenzymatic oxidation of, 343
 - pharmacological effects of food, 331
 - phenolic-like flavor, 443
 - phenolic-protein complex, 344
 - plant, 406
 - precipitable, 507
 - soluble protein-phenolic complexes, 512
 - synthetic phenolic antioxidants, 410
 - total, 489, 490
 - volatile, 447, 450
 - wine in, 279
- phenols, 1, 146

- phenoxy radical, 408, 409
- phenylalanine, 2
- phenylalanine ammonia lyase, 2
- phenylpropane, 6
- phenylpropanoids, 1, 278
- phlobaphene(s), 34, 49, 60, 282
- phloretin, 166, 167, 288, 341
- phloridzin, 165, 166, 167, 288, 289, 341
- phlorin, 172
- phloroglucinol, 336, 341
- phyllodulcin, 446
- phytoalexins, 4, 99, 149
- phytochemicals, 313
- phytoestrogens, 266
- piceid(s), 142, 149, 273, 366
- pigeon pea, 21
- pigment, 456
- pinoresinol, 12, 107, 113, 341
- pinostrobin, 158
- pinto beans, 357
- plant antioxidants, 426
- plant lignans, 341
- plasma epicatechin, 335
- pleasant smoke-like flavor, 450
- plum(s), 160, 162
- plumbagin, 62
- polished rice, 40
- polychaetes, 452
- polymehtoxylated flavones, 173
- polymeric procyanidins, 140, 145
- polymerization of tannins, 275
- polymethoxyflavones, 292
- polymethoxylated flavones, 367
- polyphenol metabolism, 332
- polyphenol oxidase(s), 163, 262, 284, 295, 462, 464, 465
- polyphenol oxidation, 163
- polyphenol-metal complexes, 354
- polyphenolases, 244, 464
- polyphenoloxidase(s), 150, 362
- polyphenolic(s), 20, 43, 242
 - beer, 264
 - fermentation and polyphenolics, 243
 - quantification of, 485
 - recovery of, 487
 - tea, 242
- polyphenols, 12, 25, 145, 271, 346, 347
- polyphenols-protein complex, 247
- polyunsaturated fatty acids, 367
- pomace, 145
- pomegranate(s), 180, 454
- poncirin, 177
- potassium sorbate, 359

- potato, 186
- potato polyphenols, 186
- potent bacterial antiadhesion activity, 141
- potentillin, 152
- pouchong teas, 241, 366
- precipitation of tannin-protein complex, 346
- precipitation index, 91
- prenylated benzoic acid, 54
- prenylated chalcones, 264
- prenylated flavonoids, 264
- prenylnaringenin, 265
- primary antioxidants, 407
- proanthocyanidin(s), 12, 27, 32, 43, 44, 55, 136, 138, 143, 145, 259, 272, 283, 315, 335, 361, 462, 495
- proanthocyanidin assay, 495
- processing strawberries, 155
- processing of soybean, 101
- procyanidin(s), 12, 27, 28, 59, 132, 139, 143, 145, 163, 164, 169, 170, 180, 271, 276, 278, 284, 289, 291, 295
- procyanidin dimer(s), 45, 145, 158, 276
- procyanidin polymer, 45
- procyanidine gallate oligomer, 105
- prodelphinidin(s), 12, 27, 28, 143, 145, 351
- proflisetinidins, 44
- proluteolinidin, 44
- prooxidant activity, 409
- propagation, 405
- propelargonidins, 12, 44
- propyl gallate, 179, 411
- proso millet(s), 36, 37
- protein precipitation assays, 505
- protein-phenol interactions, 262
- protein-phenol complex, 347
- protein-polyphenol, 22
- protein-tannin complexes, 350, 516
- protein-tannic acid interaction, 348
- prunes, 160
- prunetin, 158
- prunin, 158
- Prussian blue assay, 490
- psolaren(s), 4, 5, 174, 186, 191
- pterocarpan, 99
- pterostilbene(s), 142, 149, 367
- pulses, 18
- pumello, 177
- pungency, 448
- punicalagin, 152, 180
- punicalin, 152, 180
- purple potato, 187
- purple passion fruit, 179
- purple-fleshed sweet potato, 189
- pyrogallol, 253

Q

- quantification of flavonoid, 515
- quebracho tannins, 348
- quercetin, 11, 19, 31, 34, 57, 111, 133, 135, 137, 148, 153, 154, 157, 158, 165, 169, 180, 183, 187, 192, 193, 197, 242, 245, 267, 276, 278, 296, 315, 316, 320, 337, 363, 369, 460, 422, 426, 427
- quercetin conjugates, 194, 201
- quercetin diglucoside, 187
- quercetin glycosides, 166
- quercetrin, 132
- quercitrin, 31, 141, 133, 166, 180, 316
- quince fruit, 170
- quinol, 253

R

- rabbiteye, 139
- radical scavenging activity, 105
- raisins, 150
- rapeseed, 83, 85, 487
 - meal, 83, 487
 - hulls, 508
- raspberry (raspberries), 151, 153, 454
- reactive chlorine species, 403
- reactive nitrogen species, 403
- reactive oxygen species, 403
- red wine(s), 273, 275, 276, 279, 363, 366
- red skin of potatoes, 454
- red grapes, 279
- red pigmented lettuce, 193
- red beetroot, 188
- red raspberry, 153, 154
- red lettuce, 193
- red onion skin, 435
- red skin of radishes, 454
- red-fleshed potatoes, 187
- reinecke salt assay, 497
- removal of hulls, 23
- restrytisol(s) A to C, 150
- resveratrol(s), 6, 141, 142, 149, 190, 273, 340, 366, 367,
- resveratrol, *trans*-, 57
- retinal, 437
- reynoutrin, 133, 166
- rhamnasin, 158, 370
- rhoifolin, 133
- rice, 40, 429
 - bran, 40, 115
 - bran oil, 115
 - flour, 40
- ripened fruit(s), 167, 453
- roasted barley, 29
- roasted beans, 264

roasted coffee beans, 253
robinetin, 422
Robusta, 257
Robusta coffee, 253
roots, 181
rosemary, 432, 433
rosemary extract, 369, 433
rosmanol, 433
rosmaridiphenol, 433
rum, 286
rutin, 11, 30, 31, 111, 133, 170, 183, 190, 267, 315, 316, 337, 370, 369, 427
rutinoside, 112
rye, 42

S

sage, 432, 433
sakuranetin, 158
sakuranin, 158
salicylaldehyde, 268
salsolinol, 170
sanguiin, 152, 153
saw palmetto, 318
schaftoside, 29
sciadopitysin, 315, 317
scopanone, 173, 174
scopoletin, 5, 174, 267, 286
secoiridoid(s), 110, 111, 114
secoiridoid aglycones, 114
secoisolariciresinol, 341
secoisolariciresinol diglucoside (SDG), 107
secondary antioxidants, 407
seed maturation, 22
seeds of apple, 163
sensory attributes, 444
serotonin, 37
sesame, 84
sesame seeds, 108, 429
sesamin, 108, 429
sesaminol, 108
sesamol, 429
sesamolin, 108, 431
seselin, 173, 174
shogaol(s), 320, 448
shyanthine, 317
Siberian ginseng, 314
silibinin, 322
silmaryn, 322
sinapaldehyde, 286
sinapine, 85, 87, 88, 92, 94, 443, 497
sinapine glucoside, 88
sinapoylcholine, 87

sinensetin, 173, 174, 175, 294
singlet oxygen, 403
sitosterol, 431
smoky flavor note, 450
solid-phase microextraction, 485
solid-phase extraction, 485
solubility of polyphenols, 352
soluble tannins, 496
soluble condensed tannins, 89, 90
solvent extraction, 485
sorghum, 42, 352, 356, 487
sorghum tannins, 347
sour orange, 177
soybean, 84, 85, 95, 356, 426
 cultivars, 98
 flour, 426
 pasta, 102
 seeds, 98, 102
soy protein isolate, 101
soy sauce, 103, 448
soymilk, 99, 101
spectrophotometric methods, 489
spent grain, 29
spices, 432, 447
spinacetin, 195
spinach, 195
spinatoside, 195
St. John's wort, 315
standard beer, 270
stanol(s), 34, 51
star apple, 180
steroids, 318
sterol, 51
sterols ferulates, 34
steryl ferulates, 42, 53
stigmasterol, 431
stilbene(s), 1, 6, 58, 149
stilbene oxidase, 150
stilbene synthase, 6
storage stability of beers, 267
strawberries, 131, 154, 447
strawberry fruits, 154
strawberry juice, 295, 296
suberin(s), 1, 10, 187
suberosin, 174
sulfation, 332
sultana grapes, 150
sunflower, 84, 109
sunflower protein, 344
sweet cherries, 156
sweet orange, 177
sweet potato, 188

sweet-tasting phenol, 446
sweetness of neohesperidin, 446
swiss chard, 196
syringaldehyde, 41, 52, 286
syringol, 436

T

tangelo Nova fruits, 173
tangeretin, 174, 175
tangerine oil, 173
tangerine, 174, 294, 447
tannase, 272
tannin(s), 1, 12, 43, 273, 346, 347, 354, 357
tannin analysis, 489
tannin-metal complex, 354
tannin-protein, 348
tannin-protein interaction, 517
tannin-protein complex(es), 346, 342, 349, 350, 508
tannin-vanillin adducts, 492
tanning of beer proteins, 271
tart cherry, 156
taste threshold(s), 443, 453
taucho, 102
taxifolin, 11, 47, 137, 177, 363, 427
tea, 241, 431
tea leaves, 245
tea polyphenols, 362, 363, 366
TEAC (Trolox equivalent antioxidant capacity), 407, 409
tempeh, 99, 101, 103
tequila, 287
termination, 405
tertiary-butylhydroquinone, 411
tetrahydrochalcone, 6
tetrahydroxyisoflavone, 339
tetramethylscutellarein, 175
theaflagallins, 245
theaflavin(s), 244–249, 366, 463, 499
theaflavin analysis, 500
thearubigins, 245, 246, 247, 463
theasinensin, 247, 248
theflavin gallates, 249
theogallin, 242
thermal oxidation of oil, 114
thick-skinned grape, 276
thin-layer chromatographic, 529
thornless blackberry, 135
thyme, 369
thymol, 369, 447
titanium tetrachloride assay, 498
titration methods, 505
tocols, 39, 114, 417

tocopherols, 13, 366, 417
tocotrienols, 13, 114, 417
tofu, 99, 101, 102
tomato, 200
tomato juice, 201
tortilla chips, 34
tricin, 37, 39, 52
trihydroxyisoflavone, 339
trihydroxystilbenes, 273
trimethylpsolaren, 174
tryptophol, 278
tubers, 181
tumor cell lines, 367
turmeric, 368, 433
tyrosinase kinase, 150
tyrosine ammonia lyase, 3
tyrosol, 110, 111, 113, 267, 268, 278

U

umbelliferone, 5, 174, 267
unbound tannins, 512
unfermented cocoa beans, 257
unfermented soy products, 101
unprocessed barley, 29
unprocessed tea, 244
unripened fruits, 453
UV spectrophotometric assay, 502

V

valeranine, 317, 318
valeriana, 317
valerian, 317
vanilla extracts, 447
vanillin, 370, 447
 assay, 489, 491, 494
vanillylamine, 198
vegetables, 131, 181
verbascoside(s), 110, 111, 112, 319
vilexins, 320
vine cultivar, 277
viniferin, 142, 150
vinification, 273, 279
vinylcatechol, 253
vinylguaiaicol, 3, 34, 40, 172, 190, 251, 253, 268, 269, 446, 447
vinylphenol, 34, 40, 172, 190, 268, 447
virgin olive oil, 110, 112, 435
visual detection thresholds, 454
vitexin, 31, 36, 196, 242, 267, 320
viva pink beans, 357
volatile phenols, 443

W

walnuts, 61
water-soluble pigments, 454
wheat, 50
wheat bran, 52
wheat milling fractions, 53
whisky, 286
white wine(s), 273, 275, 276, 279
white onion, 184
wine, 272, 273
 aroma, 283
 phenols, 515
winged bean(s), 21, 23
wood smoke, 436

X

xanthaxin, 5
xanthohumol, 264, 265
xanthotoxin, 174, 186, 191
xanthyletin, 173, 174
xylenol, 3,5-, 40
yellow passion fruit, 179, 448

Z

zingerone, 369