Analysis of
Antioxidant-Rich
Phytochemicals

EDITED BY • ZHIMIN XU • LUKE R. HOWARD

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Preface

Antioxidant-rich phytochemicals in plants and agricultural food products have recently become an attractive subject for food, biomedical and nutrition scientists, and food producers. Thousands of articles in this area are published each year and numerous related research projects are being carried out in institutes and companies. Compared to synthetic food antioxidants, the antioxidants in natural sources are generally recognized as safe for food applications. Also, most of the phytochemicals have been confirmed to have health-promoting functions in preventing various human epidemiological diseases, such as cardiovascular diseases, cancers, obesity, and diabetes. In addition to antioxidant function, many phytochemicals have been found to alter cell signalling pathways and gene expression, and thus have the ability to regulate numerous physiological functions involved in the pathogenesis of various chronic diseases.

Antioxidant-rich phytochemicals are micro-constituents in plants and agricultural food products. They differ from proteins, carbohydrates, and lipids, which are macro-nutrients that are abundant in plants and food products. The type and quantity of antioxidant-rich phytochemicals vary significantly from source to source. Different types of the antioxidant-rich phytochemicals may have different antioxidant and other biological activities and bioavailability. Although most phytochemicals have UV absorption, using traditional spectrophotometeric methods to quantify the antioxidants is not practical as they could be significantly masked or interfered with by many other compounds in the sources. Thus, the analysis methods for antioxidant-rich phytochemicals are more complicated and sophisticated than the methods used for macro-nutrient compounds.

Chromatography techniques with different detectors followed by skillful sample preparation are usually applied to quantify these antioxidants in natural sources. These techniques offer sensitive and specific analysis methods for most of the antioxidants. This is the first book that particularly covers and summarizes the details of sample preparation procedures and methods developed to identify and quantify various types of natural antioxidants in plants and food products. In the book, the principle of quantification methods for natural antioxidant-rich phytochemicals is introduced and current methods used in the determination of antioxidants in different sources are reviewed and summarized by experts in the field. As a handbook of analysis of natural antioxidant-rich phytochemicals, the book provides useful information for many researchers in this area to learn ideal analysis methods for the antioxidants they are examining. The book may also serve as a lecture resource for courses in food analysis, functional foods, and nutrition.

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Chapter 1

Important Antioxidant Phytochemicals in Agricultural Food Products

Zhimin Xu

Abstract

Antioxidant phytochemicals are secondary plant metabolites widely present in the plant kingdom. Most of the phytochemicals are phenolic derivatives with monohydric or polyhydric phenols. Numerous clinical studies have confirmed that antioxidant phytochemicals can prevent some cholesterol-related and oxidation-induced chronic diseases. The antioxidant properties and health benefits of phytochemicals in different agricultural food plants have been intensively studied in recent years. This chapter will discuss the phytochemicals in common fruits, vegetables, and grains and their potential to reduce the risk of epidemiological disease. As more and more consumers are becoming concerned about health functions of foods, the information of this chapter will be useful for scientists in the food, plant breeding and physiology, medicine, and epidemiology areas to understand and utilize natural antioxidant phytochemicals in health-promoting food and other products.

Keywords: antioxidant; phytochemical; phenolic; polyphenolic; antioxidation; plants

1.1 Introduction

Antioxidant phytochemicals generally possess one or more hydroxylated aromatic or phenolic rings, which contribute to their antioxidant

activity. They are widely distributed in plants and synthesized by shikimate and chorismate biosynthesis from the essential precursor L-phenylalanine (Haslam, 1993; Herrmann, 1995). The synthesis reaction involves a series of deamination, enzymatic conversion, and enzymatic hydroxylation reactions, and generates a variety of the phytochemicals with one or more phenolic rings (Herrmann, 1989). Based on the number and pattern of phenolic rings in the structure, antioxidant phytochemicals may be simple structures such as phenolic acids, which have only one phenolic ring, or complicated polyphenolics and tannins, which have two or more than two phenolic rings, respectively. The phenolic phytochemicals can also link with different moieties, such as sugars, long carbon chains, and phytosterols to form complex phenolic derivatives. Thousands of phenolic derivative phytochemicals have been found in the plant kingdom. The major physiological function of antioxidant phytochemicals in plants is to defend against oxidative and environmental stress, such as UV radiation, microbes, pathogens, parasites, etc. (Croteau et al., 2000). Most phytochemicals are also responsible for the colors of plants. For example, anthocyanins, one class of polyphenolics are purple, black, or red pigments. In edible plants such as fruits and vegetables, phytochemicals also contribute to attributes like astringency, bitterness, or a spicy taste (Lule and Xia, 2005).

Phenolic acids, derivatives of hydroxybenzoic and hydroxycinnamic acids, are divided into subgroups and may be found in many different types of agricultural products. Protocatechuic, caffeic, coumaric and chlorogenic acids are phenolic acids found in abundance in fruits and vegetables. Ferulic acid is a phenolic acid commonly found in grains, especially in grain bran. Polyphenolics are a group of flavonoids, which are divided into anthocyanins, isoflavones, flavones, flavonols, flavanols, and flavanones. Anthocyanins are present in high levels in berries, and isoflavones are abundant in beans. The flavonol quercetin is largely present in apples, while catechin, a flavanol, is found in teas and coffees. Grapefruits are rich in flavanones, such as naringenin. Tannins are a group of polymerized polyphenolics present in berries and red wines. Some tannins existing in fruit juice and wine have a molecular weight over 2000 daltons and are not water-soluble (Khanbabaee and van Ree, 2001). Also, some antioxidant phytochemicals in grain germ and bran, such as tocols and oryzanols, are lipid-soluble.

Antioxidant phytochemicals have recently become an attractive subject for scientists in many different research areas. The scope of research in phytochemicals is no longer limited to the functionality associated with plants and organoleptic properties, but has been expanded to include their functionality in human health, which is linked to various epidemiological diseases and micro-nutrition. Most phytochemicals in natural agricultural sources have been generally recognized as bioactive or health-promoting compounds, which play an important role in preventing cardiovascular diseases, cancers, obesity and diabetes, lowering blood cholesterol level, and reducing inflammatory action (Halliwell, 1996).

1.2 Lipid Oxidation and Antioxidant Property of Phenolic Derivative Phytochemicals

Antioxidants are substances that inhibit the generation and reduce the number of oxidation-initiating free radicals, which eventually helps to prevent or delay oxidation reactions, such as lipid auto-oxidation. Lipids, essential components in animal and human cell membranes, are very vulnerable substances that are readily oxidized when exposed to free radicals, light, oxygen, pro-oxidants, and high temperatures (Frankel, 1999). In food systems, lipid oxidation causes serious deterioration in food quality during the storage of lipid-containing foods (Decker and Xu, 1998). The oxidation of lipids produces undesirable rancid odors and oxidation products, and decreases the sensory and nutritional quality of food. The primary products of lipid oxidation are hydroperoxides, which are unstable and further decompose into various secondary compounds such as alkanes, alkenes, aldehydes, ketones, alcohols, esters, acids, and hydrocarbons. Also, some lipid oxidation products are harmful to a variety of mammalian cells. They can affect cell division and proliferation, resulting in cell inflammation, and increase the risk of developing chronic diseases (Berliner et al., 1995). Lipid auto-oxidation is a major type of lipid oxidation, in which lipids react with oxygen through a free radical mechanism (Asghar et al., 1988). Below are the steps of free radical lipid auto-oxidation (RH indicates a fatty acid; ROOH indicates a hydroperoxy fatty acid; $^{\bullet}$ denotes a free radical):

Radical initiation:

$$RH + O_2 \rightarrow R^\bullet + {}^\bullet OH$$

Radical propagation:

$$R^\bullet + O_2 \rightarrow ROO^\bullet$$
$$ROO^\bullet + RH \rightarrow R^\bullet + ROOH$$
$$ROOH \rightarrow RO^\bullet + HO^\bullet$$
$$RO^\bullet + RH \rightarrow R^\bullet + ROH \rightarrow \text{Oxidation products}$$

Radical termination:

$$R^\bullet + R^\bullet \rightarrow RR$$
$$R^\bullet + ROO^\bullet \rightarrow ROOR$$
$$ROO^\bullet + ROO \rightarrow ROOR + O_2$$
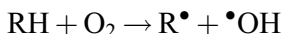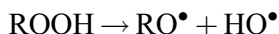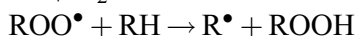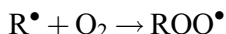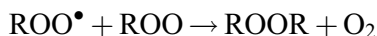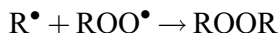
Lipid oxidation is not restricted to the fatty acids and triglycerides of foods. Another important food lipid, cholesterol, can also be oxidized by free radicals (Maerker, 1987; Xu et al., 2001). Cholesterol is present at significant levels in food from animal sources, such as egg yolk, meat, and milk products. It is an essential molecule for humans as a component of cell membranes and as the precursor of steroid hormones and bile acids. Most cholesterol in the human bloodstream is carried by low-density lipoprotein (LDL) particles. High levels of LDL cholesterol, known as "bad cholesterol," is directly associated with various cardiovascular diseases. Cholesterol oxidation products from foods, or those produced by human metabolism, are toxic and harmful to blood vessel tissue cells (Kumar and Singhal, 1991; Lyons and Brown, 1999). They can trigger the development of a progressive thickening of the artery wall due to the accumulation of the oxidation products in LDL particles. Eventually, this can lead to the formation of plaque, which results in cardiovascular diseases and the formation of certain types of cancers (Morel and Lin, 1996; Wilson et al., 1997). Lipid oxidation reactions that occur in the cell membrane may also lead to various types of cancer, as these reactions result in damage to the membrane due to mutations that arise during the cell-duplication process (Jadhav et al., 1996).

Phenolic antioxidants can quench and terminate the free radicals without being transformed to new free radicals in the system.

The hydroxyl groups on the phenolic ring contribute to the antioxidant function by donating electrons to eliminate free radicals in a system. As the phenolic radical intermediates are relatively stable due to resonance occurring on the phenolic ring, they do not initiate a new free radical chain reaction. Furthermore, the phenolic radical intermediates can react with other free radicals to terminate the chain reaction. Phenolics may also suppress reactive oxygen and nitrogen species formation by deactivating related enzymes and chelating free radical-producing metal ions. The number of free hydroxyl groups on phenolic rings is correlated to the antioxidant activity of a phenolic compound (Cotelle, 2001). Hydroxycinnamic acid derivatives were found to have higher antioxidant activity than their corresponding hydroxybenzoic acid derivatives because the –CH=CH-COOH linked to the phenyl ring may enhance the stability of the resonance (Rice-Evans et al., 1996). The number and position of hydroxyl groups in phenolic rings are also important to the antioxidant activity of flavonoids (Bors and Michel, 2002).

Unlike natural antioxidant phytochemicals, which are accumulated and excreted in biological systems, there is a group of antioxidants that are artificially synthesized. The most common synthetic antioxidants used in foods are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG). They are all phenolic derivatives and have either a mono- or polyhydroxyl group on the phenolic ring. The use of synthetic antioxidants is restricted because of food safety concerns, which are increasing around the world. Even though only a small amount of these artificial antioxidants are used, they are still a concern owing to of potential harmful health problems from long-term consumption (Kotsonis et al., 2001). They could be the promoting agents that target liver, lung, and stomach tissues to alter their gene expression (Pitot and Dragon, 2001). However, the antioxidant phytochemicals from agricultural products are usually considered to be GRAS (generally recognized as safe). With this advantage, more and more consumers and food developers prefer using phytochemicals as natural antioxidants to replace synthetic antioxidants in food products. Foods labeled "all natural" or "no artificial" ingredients are becoming common in the markets. Furthermore, the benefits of reducing the risk of chronic diseases by a higher daily consumption of fruits, vegetables and grains have been confirmed in a number of studies. Many studies have suggested that antioxidant phytochemicals in these agricultural food

plants play an important role in preventing chronic diseases (Block et al., 1992; Boyer and Liu, 2004; Delgado-Vargas and Paredes-Lopez, 2003; Harborne and Williams, 2000; Ness and Powles, 1997; Steinmetz and Potter, 1996).

1.3   Antioxidant Phytochemicals in Fruits

1.3.1   *Berries*

The most important antioxidant phytochemicals in berries are anthocyanins, a group of polyphenolics responsible for the black, purple, or red colors of these fruits. The profiles of anthocyanins and health benefits of different berry varieties – such as blackberries, raspberries, blueberries, cranberries, bilberries, and strawberries – have been studied extensively (Seeram, 2009). Compared to other berries, bilberries have more than 15 different types of anthocyanins (Lätti et al., 2008; Yue and Xu, 2008), and the content and composition of anthocyanins in berries is largely dependent on the growth environment. There is large variation of total anthocyanin content in bilberries harvested in different geographic areas, ranging from 19.3 to 38.7 mg/g dry weight (Lätti et al., 2008). Delphinidin and cyanidin sugar-conjugated derivatives dominated in those bilberry samples. Also, the total anthocyanin content in the concentrated bilberry extracts could be up to 24% (Zhang et al., 2004). Besides anthocyanins, berries also have significant quantities of flavan-3-ols, hydroxybenzoic and hydroxycinnamic acid derivatives, condensed and hydrolyzable tannins, and other antioxidant phytochemicals (Howard and Hager, 2007). These compounds and anthocyanins directly contribute to the antioxidant capacity of berries. The order of antioxidant activity from high to low of different berries using a LDL oxidation model was blackberries, raspberries, blueberries, and strawberries (Heinonen et al., 1998). This study also suggested that the bioavailability and bioactivity of different anthocyanins was variable. The health function of anthocyanins in preventing obesity and diabetes was also reported. Mice that were fed cyanidin-3-O-glucoside in their diet had significantly lower body weight gain typically induced by the high-fat diet, and white and brown adipose tissue weights were also significantly lower after 12 weeks (Tsuda et al., 2003). In another study, mice fed a high-fat (35%) diet plus purified anthocyanins from blueberries also had lower body weight gains and less body fat than the

high-fat controls (Prior et al., 2008). In general, the antioxidant activity of wild berries, such as crowberries, cloudberries, whortleberries, lingonberries, rowanberries, and cranberries, was higher than that of cultivated berries, such as strawberries and raspberries (Määttä-Riihinen et al., 2004). In a thermal stability study, degradation of the ten anthocyanins, delphinidin, cyanidin, petunidin, peonidin, and malvidin derivatives with different conjugated sugars at heating temperatures of 80, 100, and 125 °C were similar at the same heating temperature (Yue and Xu, 2008). However, the degradation of each compound increased drastically when the heating temperature was increased to 125 °C, with half-lives of all anthocyanins reported to be less than 8 min.

1.3.2 Grapes

The major antioxidant phytochemicals in grapes are anthocyanins, catechin, gallic acid, and resveratrol (Carle et al., 2004). The antioxidant capacity of grape extract or red grape wine in inhibiting lipid oxidation has been studied intensively. Both commercial grape juices and fresh grape extracts were reported to have the ability to lower human LDL oxidation, which was significantly correlated with the level of antioxidant phytochemicals in the juices and extracts (Frankel et al., 1998). Resveratrol is a polyphenolic present in much higher quantities in grapes than in other fruits. It is found in the vines, roots, seeds, and stalks, but its highest concentration is in the grape skin (Carle et al., 2004). The contents of polyphenolic antioxidants in red wine are variable and depend on the grape variety, vineyard location, cultivation system, climate, soil type, vine practices, harvesting time, and enological practices (Ribéreau-Gayon, 2006). Resveratrol and catechin in red wine are in ranges of 0.2–5.8 and 10–250 mg/L, respectively (Gu et al., 1999). Malvidin-3,5-diglucoside, an anthocyanin, was identified and isolated from wild grapes and was found to have higher antioxidant activity than alpha-tocopherol (Tamura and Yamagami, 1994). Red wines that contain both types of phenolics demonstrated greater efficacy in preventing LDL lipid oxidation than tocopherol alone (Frankel et al., 1993). In red wine, the anthocyanin-containing fraction had greater activity in inhibiting LDL oxidation than other phenolic fractions without anthocyanins (Ghiselli et al., 1998). Recently, the capacities of red and white wines to inhibit cholesterol oxidation were compared (Tian et al., 2011), and it was found that the ability of red wine was

50 times higher than that of white wine in preventing cholesterol oxidation induced by free radicals. These studies support the notion that daily intake of the appropriate amount of red wine may lower the risk of cardiovascular diseases.

1.3.3   Grapefruits

Flavanone, naritutin, naringin, hesperetin, and hesperidin are the major antioxidants in grapefruit and grapefruit juices (Miller and Rice-Evans, 1997). Naringin was present in a range of 407–1548 mg/ 100 g fresh weight while naritutin was present in amounts from 28 to 2059 mg/100 g fresh weight in different varieties of grapefruit (Ortuno et al., 1995). White grapefruit had higher concentrations of naringin and furanocoumarins located in the albedo and flavedo than red grapefruit varieties (Castro et al., 2006). The juice of grapefruit exhibited higher antioxidant activity than the juice from orange in a free radical scavenging model. In a LDL model, juices from orange, tangerine, and grapefruit did not show any antioxidant capability in reducing the lipid oxidation of LDL (Wang et al., 1996). Also, a diet supplemented with fresh red grapefruit positively influenced serum lipid levels, especially serum triglycerides and serum antioxidant activity (Gorinstein et al., 2006). However, it was found that naritutin, hesperetin, and hesperidin may have lower antioxidant activity when compared with the phenolics in berries or grapes (Scarlata and Ebeler, 1999).

1.3.4   Apples

A variety of antioxidant phytochemicals in apples include catechin, procyanidins, hydroxycinnamates, flavonols, anthocyanins, and dihydrochalcones. The consumption of apple contributes to the reduced risk of diseases such as cardiovascular disease and some forms of cancer (Boyer and Liu, 2004). In a recent study, the phenolic phytochemical composition and antioxidant activity of 67 varieties of apple cultivars were examined by Wojdylo et al. (2008). The total content of these phytochemicals varied from 0.5 to 2.7% of dry weight. In apple juice, the total phenolic content ranged from 0.02 to 0.1% of juice. Catechin and procyanidins are the major classes of apple phenolics, representing more than 80% of the total content. A small amount of anthocyanin was also found in red apples. The results of this study demonstrated that new varieties of apple, such as Ozark Gold, Julyred, and Jester, had the same

or higher values of bioactive compounds in comparison with the old varieties, such as Golden Delicious, Idared, and Jonagold. Antioxidant phytochemicals in apple peel were reported to have the capabilities of preventing macro- and microscopic damage and of barrier dysfunction along the gastrointestinal tract in an animal study (Carrasco-Pozo et al., 2011). A recent study investigated the effect of apple polyphenolics on the lifespan of fruit flies and their interaction with gene expressions of superoxide dismutase, catalase, and cytochrome-c oxidase (CcO) subunits III and VIb (Peng et al., 2011). The results showed that the mean lifespan was significantly extended by 10% in fruit flies fed an apple polyphenolics diet.

1.4 Antioxidant Phytochemicals in Vegetables

1.4.1 Tomato

Tomatoes have been recognized as a rich source of beta-carotene, provitamin A, ascorbic acid and vitamin C (Hanson et al., 2004). In recent years, another important carotenoid in tomatoes, lycopene, has received considerable attention. Lycopene is responsible for the red color of tomatoes and watermelons (Rao and Agarwai, 2000). Lycopene consists of eight units of isoprene to form a long carbon chain, which has eleven conjugated double bonds and two non-conjugated double bonds. Although the beta-carotene and ascorbic acid in tomatoes have been confirmed as being free radical scavenging antioxidants, lycopene has been reported to quench free radicals twice as efficiently as beta-carotene, making it one of the most potent antioxidants of the carotenoids (Breeman et al., 2002). The antioxidant capacity of lycopene has led to promising results in decreasing the risk of some illnesses and cancers. Several studies showed that lycopene is able to prevent the oxidation of LDL, which causes the atherogenic process and heart disease (Delgado-Vargas and Paredes-Lopez 2003). In fresh tomatoes, the content of lycopene was reported to range from 2.5 to 200 mg/100 g of raw tomato (Takeoka et al., 2001; Dewanto et al., 2002; Seybold et al., 2004). Although a decrease in lycopene content has been observed during cooking processing in some studies, an increase was found in other studies. This may be because cooking temperatures below 80 °C increase free lycopene by disrupting cell walls or hydrolyzing lycopene

derivatives rather than degrading the lycopene (Thompson et al., 2000). In a high temperature thermal stability study using pure lycopene, 50% of lycopene was degraded at $100\,°C$ after 60 min, $125\,°C$ after 20 min and $150\,°C$ after less than 10 min. Only 64.1 and 51.5% lycopene was retained when the tomato slurry was baked at 177 and $218\,°C$ for 15 min, respectively. At these temperatures, only 37.3 and 25.1% of lycopene was retained after baking for 45 min. In 1 min of high power microwave heating, 64.4% of lycopene still remained. However, greater degradation of lycopene in the slurry was found with frying. Only 36.6 and 35.5% of lycopene was retained after frying at 145 and $165\,°C$ for 1 min, respectively. Thus, different cooking conditions could have various impacts on the stability of lycopene in tomato (Mayeaux et al., 2006).

1.4.2   Root Vegetables

Carrot, potato, and sweet potato are the most common root vegetables in our daily diet. Generally, carotenoids are the most abundant phytochemicals in these root vegetables. However, anthocyanins are also largely present in carrot and sweet potato varieties having intense purple colors. Although raw carrot has been reported to have lower antioxidant activity than other vegetables (Kähkönen et al., 1999), boiling significantly improved their antioxidant activity (Gazzani et al., 1998). Potato and sweet potato, especially in the peels, contain significant amounts of antioxidant phytochemicals, such as chlorogenic, gallic, protocatechuic, and caffeic acids (Rodriquez De Sotillo et al., 1994). Purple potatoes and peels have exhibited greater antioxidant activities than the white and yellow varieties due to the contribution of high levels of anthocyanins (Kähkönen et al., 1999). Pelargonidin glucoside and peonidin glucoside were identified as the major anthocyanins in red-flesh sweet potatoes (Rodriquez-Saona et al., 1998). Anthocyanins from purple sweet potato were reported to suppress the development of atherosclerotic lesions and oxidative stress in mice (Miyazaki et al., 2008).

1.4.3   Peppers

Peppers are rich in vitamins C and E, provitamin A, and carotenoids (Materska and Perucka, 2005). Peppers also contain various antioxidant phytochemicals, such as ferulic and sinapic acids, quercetins, luteolins, and apigenins (Marin et al., 2004; Materska and Perucka, 2005).

These compounds were reported to have the capability of reducing harmful oxidation reactions in the human body and preventing various diseases associated with free radical oxidation, such as cardiovascular disease, cancer, and neurological disorders (Doll, 1990; Hollman and Katan, 1999; Harborne and Williams, 2000; Delgado-Vargas and Paredes-Lopez, 2003; Shetty, 2004). Green, yellow, orange, and red sweet bell peppers are commonly available in markets, with green bell pepper being the most produced and consumed (Frank et al., 2001). Carotenoids and flavonoids are the colorants that impart the orange and red colors of peppers (Delgado-Vargas and Paredes-Lopez, 2003). The yellow–orange colors of peppers are formed by alpha- and beta-carotene, zeaxanthin, lutein, and cryptoxanthin (Howard, 2001). The red color of peppers is due to the presence of carotenoid pigments capsanthin, capsorubin, and capsanthin 5,6-epoxide. All four colored peppers exhibited significant capability in preventing the oxidation of cholesterol or polyunsaturated fatty acid (docosahexaenoic acid – DHA C22:6) during heating (Sun et al., 2007).

1.4.4 Culinary Herbs

Culinary herbs have been used to enhance and complement the flavors of various foods for hundreds of years. Recently, culinary herbs were found to have functionality not only with their unique flavor characteristics, but also in their medicinal benefits such as antioxidant activity, and anti-inflammatory and antimicrobial capability (Shan et al., 2005). A number of studies have confirmed that rosemary, sage, oregano, basil, ginger, turmeric, and thyme show strong antioxidant activity. Unique antioxidant phytochemicals, including carnosic acid, carnosol, rosmarinic acid, curcumin, eugenol, and other common phenolic compounds, are present in the culinary herbs (Hirasa and Takemasa, 1998; Hinneburg et al., 2006; Kikuzaki and Nakatani, 1993; Frankel, 1999). The antioxidant capacity of the phenolic compounds in these herbs is responsible for their beneficial health effect of preventing some chronic diseases (Kähkönen et al., 1999; Shan et al., 2005). Their antioxidant activity also helps to retard or prevent lipid oxidation or rancidity in a variety of food products mixed with the culinary herbs (Birch et al., 2001; Rababah et al., 2004). Ginger and turmeric extracts have higher antioxidant activity than synthetic alpha-tocopherol (Kikuzaki and Nakatani, 1993). Bhale et al. (2007) found that rosemary and oregano

demonstrated their capability in inhibiting oxidation of susceptible long-chain unsaturated fatty acids in menhaden oil, and they also found that the amount of the herb extracts used in foods has to be carefully examined, as the extracts might contain some pro-oxidants. The ability of oregano extract to prevent lipid oxidation was increased with a higher extract concentration in menhaden oil. However, the capacity for rosemary extract to prevent lipid oxidation was highest at an extract concentration of 2.5% and decreased when the concentration was increased to 5% in both heating and room temperature incubation studies. At room temperature, the antioxidant capacity of rosemary extract is much higher than that of oregano extract. Thus, for food preservation purposes, rosemary extract may be more effective than oregano extract. However, at higher cooking temperatures, the anti-oxidants in oregano extract are more stable and stronger than those in rosemary extract in retarding fish oil oxidation. This study provided useful information relative to the natural herb antioxidants used in stabilizing lipid-rich foods during cooking and storage.

1.5   Antioxidant Phytochemicals in Grains

1.5.1   *Soybean*

Soybean is one of the major protein and lipid sources in the diets of most developing countries. Soy-containing foods have received considerable attention for their potential role in reducing the formation and progres-sion of certain types of cancers and some chronic diseases such as cardiovascular disease, Alzheimer disease, and osteoporosis (Messina, 1999; Zhao et al., 2002). Also, soy has the capability of lowering oxidative stress, stimulating or inhibiting estrogen activity and pre-venting the harmful proliferation of cells (Mitchell et al., 1998; Hwang et al., 2000; Maggiolini et al., 2001). The major antioxidant phyto-chemicals in beans are isoflavones, of which soybean is the richest source among beans. The total level of isoflavones in soybean is up to 0.5% (Liu, 1997). The hydroxyl groups on the two phenolic rings of isoflavones provide excellent antioxidant activity (Meng et al., 1999). The various types of isoflavones are distinguished by the different substitute groups and sugar moieties on the two phenolic rings. As isoflavones are not lipid-soluble, the level of isoflavones in soybean oil

is much lower. In defatted soy flour extract, soy isoflavones could be more than 100 times higher than in soybean oil (Yue et al., 2008). This study also found that the overall antioxidant activity of soy oil was lower than the defatted soy flour, although soy oil contained higher levels of vitamin E. The chemical structures of soy isoflavones are not consistent during food processing. The isoflavones with beta-glucoside, daidzin, glycitin, and genistin – which are the major isoflavones in unprocessed soy flour — could release the beta-glucoside and become their aglycone forms during thermal processing (Xu et al., 2002). At the same time, they could also be produced by the de-esterification from malonyl and acetyl beta-glucoside soy isoflavones in the soy flour. Factors induced in soy food processing, such as enzymes in raw soy flour, heating and additives, could affect the stabilities of soy isoflavones (Kudou et al., 1991; Mahungu et al., 1999, Tipkanon et al., 2010; Wang and Murphy, 1996; Yue and Xu, 2010).

1.5.2 Wheat

Wheat bran possesses various natural antioxidant phytochemicals that contribute toward preventing cardiovascular disease and certain cancers (Halliwell, 1996; Truswell, 2002). Phenolics, tocopherols, and fibre in wheat bran are generally believed to be primarily responsible for its positive effects on cardiovascular disease. Undesirable lipid oxidation reactions in the body contribute to these disease conditions (Moller et al., 1988; Alabaster et al., 1997; Andreasen et al., 2001). Many studies have found that these compounds of wheat bran exhibit significant capabilities in scavenging free radicals, chelating metal ion oxidants, and reducing lipid oxidation at different conditions (Yu et al., 2002; Zhou and Yu, 2004; Adom and Liu, 2005). Similar to other cereal grains, wheat bran contains many different types of antioxidant phytochemicals, such as ferulic, vanillic, caffeic, coumaric, and syringic acids (Li et al., 2005; Kim et al., 2006), as well as relatively high levels of carotenoids, tocopherols, and phytosterols (Nystrom et al., 2005; Zhou et al., 2005). A recent study identifying the most important antioxidant fractions of wheat grain found that the aleurone content in the fractions was highly correlated with the antioxidant capacity of the fractions (Anson et al., 2008). Ferulic acid was considered to be the major contributor to the antioxidant capacity in fractions with higher antioxidant capacity.

1.5.3   Rice

Rice is one of the most important commodities in most Asian countries. Its edible part, white rice kernel, is produced during rice mill processing, which removes rice hull and rice bran from the harvested rough rice. Although rice bran accounts for up to 10% of the rice grain, it is considered a waste product of rice milling to be discarded or used as animal feed. However, it was found that rice bran contains a much higher level of antioxidant phytochemicals than rice kernel (Godber and Juliano, 2004). Rice bran lipid fraction consists of unsaponifiable material that seems to present a positive health function, mainly because of its high levels of alpha- and gamma-tocopherol, alpha- and gamma-tocotrienol, and gamma-oryzanol (Xu and Godber, 1999). Tocopherols and tocotrienols, known as vitamin E homologues, are recognized as antioxidant compounds that are able to prevent chronic degenerative illness, cardiovascular diseases, and tumors (Bramley et al., 2000; Qureshi et al., 2001). Gamma-oryzanol is a mixture of compounds derived from ferulic acid with sterols or triterpene alcohols (Xu and Godber, 1999). The levels of tocopherols, tocotrienols, and gamma-oryzanol in rice bran are variable and depend on factors such as cropping areas and varieties (Bergman and Xu, 2003). Many studies have demonstrated that gamma-oryzanol compounds could reduce serum cholesterol level, the risk of tumors incidence and inflammatory action (Rong et al., 1997; Wilson et al., 2002; Tsuji et al., 2003). Gamma-oryzanol in rice bran exhibited significant antioxidant activity in the inhibition of cholesterol oxidation, compared with the four vitamin E components. Because the amount of gamma-oryzanol could be up to ten times higher than vitamin E in rice bran, it may be the more important antioxidant of rice bran in reducing cholesterol oxidation, even though vitamin E has been traditionally considered the major antioxidant in rice bran. The higher antioxidant activities of gamma-oryzanol components may be due to their structure, which is very similar to cholesterol. The analogous structure of gamma-oryzanol components and cholesterol leads to similar chemical characteristics in a system. The gamma-oryzanol components may have a greater ability to associate with cholesterol in the small droplets of an emulsion system and become more efficient in protecting cholesterol against free radical attack (Xu et al., 2001). In addition to those lipophilic antioxidant phytochemicals, black- or purple-colored rice bran or rice contain

significant amounts of the hydrophilic antioxidant phytochemicals known as anthocyanins (Jang and Xu, 2009).

1.5.4 Oat

The soluble fiber compound of oat, beta-glucan, is generally believed to be responsible for its beneficial effects on cardiovascular disease and certain cancers (Handelman et al., 1999; Gray et al., 2002). Some studies have suggested that some of the health-promoting capabilities of oat are due not only to its antioxidant phytochemicals, but also its beta-glucan gum (White and Armstrong, 1986; Peterson and Qureshi, 1993; Wood et al., 2000). Similar to other cereal grains, oat contains relatively high levels of tocopherols, tocotrienols and phytosterols (Peterson, 2001). It is also a good source of a variety of phenolic antioxidants such as avenanthramides, *p*-hydroxybenzoic acid, and vanillic acid (Shahidi and Naczk, 1995; Peterson et al., 2001). Oat extracts, along with highly concentrated antioxidants, could be used as a natural preservative in preventing food oxidation during cooking and storage, especially for foods rich in unsaturated long-chain fatty acids and cholesterol (White and Armstrong, 1986; Sun et al., 2006). The oat extract showed the greatest capability of preventing cholesterol and DHA oxidation during heating in a model study (Sun et al., 2006). It significantly reduced cholesterol decomposition and DHA degradation, and prevented the production of harmful toxic cholesterol oxidation products. Therefore, oat extract has the potential to maintain the stability of cholesterol- and fatty acid-rich foods during cooking or storage.

1.5.5 Corn

Corn is also recognized as an excellent source of phytochemicals, such as tocopherol, phytosterols, and carotenoids, which generally possess the ability to prevent oxidation (Truswell, 2002; Martinez-Tome et al., 2004). Corn is a rich source of lutein, which is a non-provitamin A carotenoid and acts as a yellowish pigment (Johnson, 2004). Lutein is predominately transported by high-density lipoprotein (HDL) of plasma because of its relatively higher polarity. One major health function of lutein is to prevent age-related macular degeneration (AMD) and cataracts (Johnson, 2000). Lutein was also reported to have the capability of reducing the risk of certain cancers such as colon cancer (Slattery et al., 1988), and this may be due to its antioxidant function,

which makes lutein an effective free radical scavenger to prevent cell mutation (Schunemann et al., 2002). In addition to lutein, corn also contains other carotenoids, such as alpha- and beta-carotene, beta-cryptoxanthin, and zeaxanthin, which are not found at a significant level in most other cereals. Corn germ, a common source for producing vegetable oil, has 95% of the total vitamin E content in raw corn (Grams et al., 1970; Wang et al., 1998) and corn oil has even higher levels of vitamin E, up to 900 ppm. The major vitamin E homologues found in corn are alpha- and gamma-tocopherol. Although less than 5% of the vitamin E in corn is distributed in the corn endosperm, the major vitamin E homologues present in the endosperm were gamma-tocopherol and gamma-tocotrienol (Grams et al., 1970), which is similar to rice bran. Adom and Liu (2005) found that the total antioxidant activity of corn was the highest compared with wheat, oat, and rice. It was approximately three times higher than wheat or oat and twice as high as rice.

Traditionally, the benefits of grains, vegetables, and fruit have been associated with their nutrients, proteins, lipids, carbohydrates, and vitamins. However, many evidences suggest that, in fact, the antioxidant phytochemicals in these edible agricultural plants are the true beneficial components of these plants. Phytochemicals are not only responsible for preventing oxidation reactions but also play an important role in maintaining human health. Additional studies in this area are still needed for discovering new phytochemicals that are effective as antioxidants, and for utilizing these antioxidants in food systems and nutrition supplements. Also, further studies for comprehensively understanding their bioactive mechanisms of health-promoting functions are necessary. The information obtained from these studies could be used to expand the market of agricultural products, which would appeal to consumers who recognize the importance of diet as a means of promoting health. In turn, this would increase economic benefits for producers and manufacturers, as well as expand the utilization of agricultural commodities and their by-products as value-added materials.

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Chapter 2

The Procedure, Principle, and Instrumentation of Antioxidant Phytochemical Analysis

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Abstract

Interest in potential health-promoting properties of phytochemicals has led to a wealth of information about the various extraction, purification, and identification techniques for these compounds. Pressurized liquid extraction, supercritical fluid extraction, ultrasound-assisted extraction, microwave-assisted extraction, and matrix solid-phase extraction in addition to conventional homogenization techniques can be used to extract target compounds from plant matrices. Purification methods including liquid–liquid extraction, solid-phase extraction, column chromatography, countercurrent chromatography, and semi-preparative high-performance liquid chromatography can then be used to further prepare samples for identification. High-performance liquid chromatography and gas chromatography coupled with various detection systems and mass spectrometers has improved the capability to identify individual compounds. The principles of these techniques are discussed in the chapter and examples of their applications are also provided.

Keywords: Pressurized liquid extraction; supercritical fluid extraction; ultrasound-assisted extraction; microwave-assisted extraction; matrix solid-phase extraction; high-performance liquid chromatography; gas chromatography; liquid–liquid extraction; solid phase extraction; column chromatography

2.1   Introduction

Epidemiological studies have provided considerable evidence that consumption of fruits and vegetables is associated with reduced risk of several chronic diseases including cancer and cardiovascular disease (Riboli and Norat, 2003; Dauchet and Dallongeville, 2008). This has been attributed to the presence of naturally occurring phytochemicals in foods of plant origin. Phytochemicals are secondary metabolites in plants that play important roles in their metabolism, defense mechanisms, and disease resistance. They are also widely recognized for their bioactive and health-promoting properties, which include antioxidant, anti-inflammatory, anticarcinogenic, and anti-atherosclerotic properties among others (Riboli and Norat, 2003).

More than 5000 different phytochemicals have been identified, but it is believed that thousands more have yet to be discovered (Liu, 2003). Based on their structural characteristics, they may be classified into several groups including polyphenols, terpenoids, and alkaloids (Manach et al., 2009). As evidence continues to emerge linking phytochemicals to health promotion and disease prevention, there is increased interest in extraction, isolation, and analysis of these compounds from a variety of plant sources. These procedures have been optimized for several different types of phytochemicals and are used in the discovery of compounds from new sources.

Analysis of antioxidant phytochemicals generally consists of four main steps (Figure 2.1). The first – the sample preparation – which may include obtaining a representative sample, drying, and particle size reduction. This is followed by extraction of the analytes of interest from the plant material using appropriate solvents or mixtures of solvents. Several extraction techniques have been developed for a variety of applications, and these will be discussed in this chapter. The next step is purification of the extract to remove compounds that might interfere with further analysis and concentration of the analytes of interest. Purification is often achieved using techniques such as liquid–liquid extraction, solid phase extraction (SPE), column chromatography, countercurrent chromatography, or semi-preparatory high-performance liquid chromatography (HPLC). The sample resulting from the preceding steps is then ready for qualitative and/or quantitative analysis using liquid chromatography, gas chromatography, and mass spectrophotometric techniques. This chapter will discuss in detail the procedures,
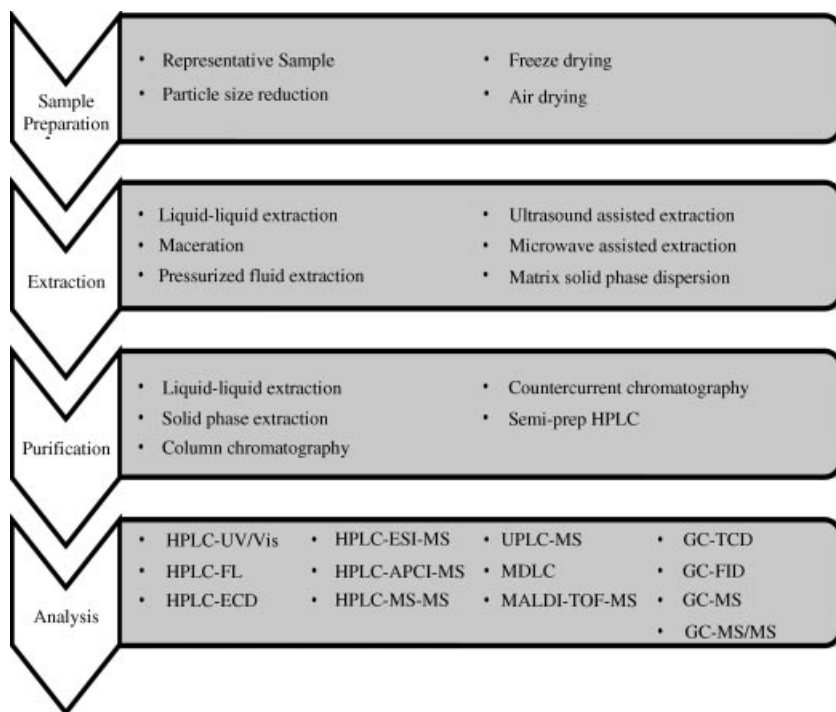
Figure 2.1 Sample preparation, extraction, purification, and analysis of antioxidant phytochemicals.

principles, and instrumentation necessary for the analysis of several common antioxidant phytochemicals.

2.2 Sample Preparation

The first step in the analysis of antioxidant phytochemicals is extraction of the compounds from the plant matrix. The means by which the samples are prepared for extraction is very important because, if it is not carefully performed, compounds can be chemically altered or degraded during the process. In general, sample preparation conditions should avoid oxidation, excessive exposure to high temperatures, enzymatic reactions, and other chemical changes to the target compounds. The first step is to select a representative sample of plant material that has been collected as recently as possible. If extraction cannot be performed immediately, it is recommended that samples be stored at refrigeration

or frozen temperatures and in the absence of light. Compounds may be extracted from fresh or frozen plant material, however it is often desirable to dry the samples prior to extraction to increase stability and reduce enzymatic activity. Generally, freeze drying is preferred over oven or air drying because it tends to preserve higher levels of the target compounds (Pérez-Jiménez et al., 2008; Chan et al., 2009). Novel drying technologies such as radiant zone drying and vacuum microwave drying have also been explored and show similar retentions to freeze drying (Chakraborty et al., 2010; Leusink et al., 2010). If the phyto-chemicals are to be extracted from fresh material, care should be taken not to disrupt the tissue prior to addition of extraction solvent otherwise, upon disruption of the cells, endogenous enzymes such as polyphenol oxidase or glycosidases (which are stored in plastids) come into contact with phenolic compounds released from the vacuole, resulting in degradation or alteration of their native structures (Renard et al., 2011).

Particle size reduction is another important step in sample prepara-tion that can affect extraction efficiency. It has been observed that reducing the particle size of the material prior to extraction leads to a greater extraction of antioxidants because the distance in which the compounds have to travel to reach the surface of the particle is reduced (Landbo and Meyer, 2001). Additionally, the food matrix is disrupted by particle size reduction which may aid in the extraction of bound antioxidants (Pérez-Jiménez et al., 2008). Care should be taken, how-ever, to minimize heating of the sample and the time it is exposed to oxygen during the grinding procedure as these could result in significant losses of antioxidant phytochemicals. Powdering of fresh samples in a Waring blender using liquid nitrogen has been done to minimize losses prior to extraction (Garzón and Wrolstad, 2009).

2.3   Solvent Extraction

The selection of an extraction procedure depends primarily on the class of compounds of interest as well as the type and physical state of the sample. Liquid samples generally do not require extraction prior to analysis; however liquid–liquid extraction may be performed to isolate a certain class of compounds. Extraction of antioxidants from solid plant materials is most commonly done using aqueous mixtures of organic solvents. This is based on the principle that, upon extraction,

antioxidant compounds are transferred from the solid material to the liquid phase according to their affinity to the selected solvent. The relative efficiency of solid–liquid extraction depends upon several factors, including solvent polarity, sample particle size, the solid-to-liquid ratio, and other extraction conditions such as time and temperature. To achieve successful extraction, target compounds must be dissolved in the solvent until an equilibrium concentration is reached. Therefore, increasing the solid-to-liquid ratio can improve extraction yields. Antioxidant phytochemicals are structurally diverse compounds that range in polarities, with most being only moderately soluble in water, therefore organic solvents are often required. The choice of solvent is a critical step in selecting an extraction procedure as different classes of compounds require solvents with different polarities. Typically, aqueous mixtures of methanol, ethanol, and acetone, are used to extract more polar antioxidants, while less polar compounds generally require the use of ethyl acetate or chloroform. Aqueous mixtures are often preferred over pure solvents because of the ability of water to hydrate the food matrix and facilitate extraction. No single solvent system is suitable for extraction of all antioxidant phytochemicals from a food matrix, so for total antioxidant capacity assays, it is often recommended that at least two extractions be performed using solvents with different polarities (Pérez-Jiménez et al., 2008). To prevent degradation during solvent extraction, antioxidants such as TBHQ, BHT, ascorbic acid, and sulfites or enzyme inhibitors may be added to the extraction solvent (Tomas-Barberan et al., 2001; Escribano-Bailon and Santos-Buelga, 2003). However, as the addition of ascorbic acid can readily degrade anthocyanins in the presence of metals, it should not be used when these are the target compounds. Additional precautions include nitrogen purging of the extraction vessel and extraction over ice or using cold solvents (Tomas-Barberan et al., 2001; Monrad et al., 2010a).

A variety of solvent extraction techniques have been used to extract antioxidants from food matrices. The most commonly used is maceration or homogenization of the sample with an extraction solvent; however, alternative procedures have been developed including pressurized fluid extraction (PFE), ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and matrix solid-phase dispersion (MSPD), among others. The principles of each extraction technique will be briefly discussed.

Extraction of phytochemicals by maceration or homogenization with organic solvent has been recognized as the conventional extraction method and is widely used to recover bioactive compounds from food matrices. In these extraction methods, solvent is mixed with a solid sample and macerated or homogenized to facilitate migration of the target compounds to the solvent. The resulting mixture is generally filtered or centrifuged, the supernatant collected, and extraction repeated on the resulting residue. Aside from the choice of solvent, recovery of compounds using this extraction technique is largely dependent upon particle size, solvent to solid ratio, and extraction time with smaller particles, higher solvent to solid ratios, and longer extraction times resulting in higher yields. One advantage of this extraction method is that it does not require any specialized equipment; however, it is relatively time consuming compared to other methods and requires large amounts of organic solvent which presents potential for negative environmental impact.

Alternatively, PFE extraction includes several extraction techniques including pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), and subcritical water extraction (SWE). PLE is a technology that can be used to extract phytochemicals using solvents under conditions of high temperature and pressure, and is most commonly performed using a commercial accelerated solvent extractor (ASE) made by Dionex Corporation. Increasing temperatures have been shown to increase extraction efficiency; however, antioxidant degradation may occur simultaneously at high temperatures. Therefore, high-temperature short-time extraction procedures have been employed to improve extraction efficiency while minimizing thermal degradation. PLE allows the solvent to remain a liquid at temperatures higher than their boiling point by increasing the pressure in the extraction chamber. Extractions are relatively rapid (3–20 min) and are typically performed at 40–200 °C under pressures of 3.3–20.3 MPa. This increases the speed and efficiency of extraction by aiding the solvent penetration of the matrix, increasing the solute diffusion rate, accelerating mass transfer, and reducing solute–matrix interactions (Richter et al., 1996). A sample dispersant such as sea sand or diatomaceous earth is often used to reduce the amount of solvent needed and avoid compaction of the sample in the extraction cell. When the solvent is pure water at temperatures above its boiling point (100 °C) but below its critical point (374 °C), the process is known as SWE. Pressurized liquid extraction has been used to

efficiently extract several classes of antioxidant phytochemicals from a variety of materials including flavonoids from *Houttuynia cordata* (Zhang et al., 2008), grapes (Palma et al., 2001), apples (Alonso-Salces et al., 2001), and spinach (Howard and Pandjaitan, 2008), as well as isoflavones from soybeans (Rostagno et al., 2004), phenolic acids from black cohosh (Mukhopadhyay et al., 2006), and procyanidins from malt (Papagiannopoulos et al., 2002). PLE was more effective than Soxhlet in extracting anthocyanins and total phenolics from red grape skin using acidified water and aqueous acidified methanol (Ju and Howard, 2003). Additionally, PLE using ethanol:water mixtures extracted similar levels of anthocyanins and more oligomeric procyanidins than homogenization with acidified aqueous acetone, but it was less effective in extracting procyanidin polymers (Monrad et al., 2010a; 2010b). PLE was also more effective in extracting chlorogenic acid and total phenolics from egg-plant than shaking, vortexing, sonication, and Soxhlet (Luthria and Mukhopadhyay 2006; Luthria et al., 2007). A summary of studies using PLE for analysis of phytochemicals is presented in Table 2.1.

SFE also uses compressed fluids, but at temperatures and pressures above their critical points, which allows the fluid to retain the properties of a liquid and a gas. The density of the fluid is similar to a liquid while its viscosity is more like a gas, which allows the fluid to diffuse easily through solid substances to facilitate extraction (Herrero et al., 2006). Several fluids have been used for SFE; however, carbon dioxide is the most commonly used because it is cheap, environmentally friendly, nontoxic, nonflammable, generally recognized as safe by the FDA and EFSA, and has an easily attainable critical temperature ($31.1\,°C$) and pressure ($7.39\,MPa$). Additionally, since CO_2 is a gas at room temperature and pressure, resulting extracts are free of solvent and easily recoverable. However, supercritical CO_2 is relatively nonpolar, so highly polar modifiers (co-solvents) such as ethanol, methanol, or isopropyl alcohol must be added if polar antioxidants are to be extracted (Herrero et al., 2006). SFE has been used to extract antioxidant phytochemicals from various fruits, vegetables, and by-products. It is important that extraction conditions such as time, temperature, pressure, sample size, and presence/amount of modifiers be optimized for each matrix to maximize the recovery of target compounds. Optimization is generally achieved using some form of designed statistical optimization such as a simplex centroid design or response surface methodology, which reduce the number of data points but are still

Table 2.1 Examples of extraction of antioxidant phytochemicals from various plant materials by pressurized liquid extraction (PLE).

Plant Material	Compound(s) of Interest	Extraction Solvent	Optimal Extraction Conditions	Reference
Houttuynia cordata	Flavonoids	50% ethanol	1.8 mL/min, 70 °C, 8 MPa	Zhang et al., 2008
Apples	Catechins, flavonols glycosides, hydroxycinnamic acids	methanol	5 min preheat, 60% flush volume, 1 cycle, 90 s nitrogen purge, 40 °C, 5 min static, 6.8 MPa	Alonso-Salces et al., 2001
Spinach	Flavonoids	70% ethanol	3 × 5 min cycles, 70% flush volume, 90 s nitrogen purge, 170 °C, 13.8 MPa	Howard and Pandjaitan, 2008
Soybeans	Isoflavones	70% ethanol	3 × 7 min cycles, 100% flush volume, 300 s nitrogen purge, 100 °C, 10 MPa	Rostagno et al., 2004
Eggplant	Chlorogenic acid, total phenolics	80% methanol	4 × 5 min cycles, 5 min equilibration 90 s nitrogen purge, 100 °C, 6.8 MPa	Luthria and Mukhopadhyay, 2006
Black cohosh	Total phenolics, phenolic acids	60% methanol	2 × 5 min cycles, 50% flush volume, 90 s nitrogen purge, 40 °C, 6.8 MPa	Mukhopadhyay et al., 2006
Malt	Procyanidins	80% acetone	5 min preheat, 10 min static, 60 s nitrogen purge, 50% flush volume, 60 °C, 100 MPa	Papagiannopoulos et al., 2002
Grape pomace	Anthocyanins	50–70% ethanol	1 cycle, 70% flush volume, 90 s nitrogen purge, 0 static time, 0 preheat time, 80 – 120 °C, 6.8 MPa	Monrad et al., 2010a
Grape pomace	Procyanidins	50% ethanol	1 cycle, 70% flush volume, 90 s nitrogen purge, 0 static time, 0 preheat time, 80 – 140 °C, 6.8 MPa	Monrad et al., 2010b

effective in determining optimum conditions. Lycopene was extracted from tomato skins using supercritical CO_2 at various temperatures (40–100 °C) and pressures (20–40 MPa), and lycopene yield increased with increasing temperature and pressure (Yi et al., 2009). Similarly, SFE parameters were optimized to extract total phenolics and anthocyanins from grape peels, and extraction yields were influenced by temperature and pressure, with higher temperatures and higher pressures producing higher levels of bioactive compounds (Ghafoor et al., 2010). SFE has been used to extract a variety of compounds including carotenoids (Machmudah et al., 2008), phytosterols (Lu et al., 2007), anthocyanins (Seabra et al., 2010; Xu et al., 2010), catechins and procyanidins (Lazze et al., 2009), tocopherols (Gelmez et al., 2009), among others. A summary of studies using the SFE of phytochemicals from various plant materials is presented in Table 2.2.

Another relatively recent extraction technique known as ultrasound-assisted extraction (UAE) uses acoustic waves in the kilohertz range to disrupt biological membranes, which facilitates the release of target compounds from the solid matrix into the solvent. Tiny cavitation bubbles form and subsequently implode on the surface of particles to produce a shear force capable of inducing physical and chemical changes. This enhances solvent penetration and improves overall mass transfer, and can aid in swelling and hydration of the cell wall (Chemat et al., 2009). Extraction time and temperature appear to be the most important factors determining extraction efficiency. When compared to Soxhlet and conventional solid–liquid extraction, UAE with aqueous acetone and ethanol improved the extraction (>54%) of phenolic compounds from soybeans (Chung et al., 2010). Recently, UAE has been used to extract phytochemicals from grape seeds (Ghafoor et al., 2009), mandarin peel (Ma et al., 2008), and orange peel (Khan et al., 2010). Table 2.3 presents several studies involving the UAE of various phytochemicals from plant materials.

Microwave-assisted extraction (MAE) is also growing in popularity for use in the extraction of phytochemicals. In the electromagnetic spectrum, microwaves exist on frequencies ranging from 300 MHz to 300 GHz. These waves have potential in creating more efficient extractions of plant metabolites. Much of the differences in efficiency between this method and other methods result from differences in the heating mechanism. Instead of typical conduction heating, in which much energy is lost to the surroundings, heat generation from

Table 2.2 Examples of extraction of antioxidant phytochemicals from various plant materials by supercritical fluid extraction (SFE).

Plant Material	Compound(s) of Interest	Extraction Solvent	Modifier	Optimal Extraction Conditions	Reference
Tomato skins	Lycopene	CO_2	none	90 min, 100 °C, 40 MPa, 1.5 mL/min	Yi et al., 2009
Grape peels	Total phenolics, anthocyanins	CO_2	6–7% ethanol	30 min, 45–46 °C, 15.6–16.1 MPa, 2 mL/min	Ghafoor et al., 2010
Rosehip	Carotenoids	CO_2	none	150 min, 80 °C, 45 MPa, 4 mL/min	Machmudah et al., 2008
Corn, sesame, oat, peanut	Phytosterols	CO_2	none	2 h, 55 °C, 45 MPa, 8 L/min	Lu et al., 2007
Red cabbage	Anthocyanins	CO_2	none	5-15 min, 60 °C, 10 MPa	Xu et al., 2010
Elderberry pomace	Anthocyanins	CO_2	ethanol, water	55 min, 40°C, 21 MPa	Seabra et al., 2010
Grape waste	Catechins, procyanidins	CO_2	ethanol, water	50°C, 9 MPa, 100 g/min	Lazze et al., 2009
Wheat germ	Total phenolics, tocopherols	CO_2	none	10 min, 58 °C, 34 MPa, 2 g/min	Gelmez et al., 2009

Table 2.3 Examples of extraction of antioxidant phytochemicals from various plant materials by ultrasound-assisted extraction (UAE).

Plant Material	Compound(s) of Interest	Extraction Solvent	Optimal Extraction Conditions	Reference
Grape by-products	Anthocyanins	Ethanol/water (50:50 v/v)	35 kHz, 70 °C, 1 h	Corrales et al., 2008
Wheat bran	Phenolic compounds	64% ethanol	40 kHz, 60 °C, 25 min	Wang et al., 2010
Soybeans	Total Phenolics	50 or 80% acetone, 50 or 70% ethanol, 80% methanol	40 W, 3 min (1 min intervals)	Chung et al., 2010
Grape seeds	Total Phenolics, Anthocyanins	53% ethanol	40 kHz, 56 °C, 29 min	Ghafoor et al., 2009
Orange peel	Flavanone glycosides	Ethanol/water (4:1 v/v)	25 kHz, 150 W, 40 °C, 30 min	Khan et al., 2010
Olive drupes and leaves	Phytosterols	Dichloromethane/ hexane (2:1 v/v)	20 kHz, 50 W, 20 °C, 10 min	Orozco-Solano et al., 2010

microwaves is based on dipole rotation and ionic conduction. These phenomena agitate molecules, which creates friction and heat (Letellier and Budzinski, 1999; Letellier et al., 1999; Mandal et al., 2007). The polar water molecules present in plant samples make the sample vulnerable to microwave-energy-generated heat, which can lead to cell disruption and promotion of metabolite extraction (Ganzler et al., 1990; Kaufmann et al., 2001; Wang and Weller, 2006). The efficacy of metabolite extraction is dependent on the dielectric constant of the selected solvent because this is an important component of the ability of solvents to absorb microwave energy.

Yang and Zhai (2010) optimized MAE conditions for extracting anthocyanins from purple corn. Using an ethanol-based solvent (1.5M HCl: 95% ethanol (15:85)), the highest anthocyanin yield resulted from 19 minutes in a 1:20 solid-to-liquid ratio when the microwave was set to 555 W. This demonstrates the primary advantages of MAE, which are reduced extraction time compared to conventional Soxhlet extraction, reduced solvent usage, and improved extraction yield (Wang and Weller, 2006; Mandal et al., 2007). However, one must also consider that an additional clean-up step is necessary to rid the sample of solid residue. Additionally, the method is somewhat limited to compounds and solvents with polar properties because nonpolar entities are not readily heated by microwaves (Wang and Weller, 2006). Optimized conditions for the extraction of anthocyanins from red raspberries were 1:4 solid-to-liquid ratio (g/mL) for 12 minutes at 355 W, which differed from those of purple corn, but the anthocyanin profile also differed (Sun et al., 2007). Proanthocyanidins have also been extracted with mixtures of methanol and water using MAE at 150 W for 200 s (Mayer et al., 2008). The optimized conditions for MAE of phenolic acids from citrus peels were: microwave power of 152 W, 49 s extraction time, 16:1 solid-to-liquid ratio, and 66% methanol extraction (Hayat et al., 2009), while the highest yield of flavonoids from *Radix puerariae* (a Chinese herb) were: 35 mL of solvent (70% ethanol), 6.5 min extraction time, and 225 W microwave power (Wang et al., 2010). In this study, Wang et al. (2010) avoided methanol due to its toxicity even though its higher dissipation factor can help a sample achieve higher energy absorption. The authors also observed that while an increase in solvent initially improved extraction, an excess of solvent had a detrimental effect on extraction possibly due to excess swelling that may have occured with increased levels of solvent. Xiao et al. (2008) also found that excess

solvent eventually had a negative effect on extraction yields. The differing optimization parameters for different food products demonstrate that optimizations for MAE in industry need to be tailored to specific products. Table 2.4 outlines experimental conditions for MAE of phytochemicals from plant materials.

Some studies have shown that MAE may be superior to other extraction methods. Total phenolics and antioxidant activity were found to be higher in spice samples extracted by MAE than by UAE (Gallo et al., 2010). In extracting anthraquinones from *Morinda citrifolia*, commonly known as noni, optimized MAE conditions were found to be 80% ethanol, 60 °C extraction temperature, and 30 min extraction time. MAE was shown to require less extraction time while achieving higher yields when compared to Soxhlet, maceration and UAE methods (Hemwimon et al., 2007).

Matrix solid-phase dispersion involves incorporating silica- or polymer-based materials that have a surface-bound phase with semi-solid or solid samples to promote particular isolation. The irregularly shaped particles promote grinding, which works together with the bound phase to create sample disruption and dispersion (Barker et al., 1993). Dispersion increases the surface area of the solvent, which facilitates better extraction. The resulting mixture is placed in a column and washed with solvents (Karasova et al., 2003). The selection of solvents depends on the analytes present. Žiaková et al. (2003) found that methanol-acidified water achieved the highest yields for some phenolic acids in *Melissa officinalis* because polar phenolic acids are more soluble in water than in organic elution agents. In the recovery of anthocyanins from blackcurrant juice, methanol/water (1:1) at pH 2 and tetrahydrofuran/water (1:1) were equally effective among the solvents tested (Manhita et al., 2006). Xiao et al. (2004) compared MSPD with Soxhlet extraction and UAE in their ability to extract isoflavonoids from *Radix astragali*, a herb used in Chinese medicine. Though the Soxhlet method was better at extracting glycosides, overall, MSPD achieved higher extraction levels of formononetin and calycosin, and consumed less time, solvent, and sample than Soxhlet. A method that combines MSPD and liquid chromatography (LC) was developed to measure β-carotene (Chase et al., 1999). C_{18} and isopropyl palmitate were mixed with the prepared sample, and then the mixture was added to a tube and sandwiched between two frits. The contents were compressed, washed with hexane with 0.5% isopropyl alcohol, and then washed with

Table 2.4 Examples of extraction of antioxidant phytochemicals from various plant materials by microwave-assisted extraction (MAE).

Plant Material	Compound(s) of Interest	Microwave Power	Solvent	Extraction Time	Solid to liquid ratio	Reference
Purple corn	Anthocyanins	555 W	1.5 M HCl: 95% ethanol (15:85)	19 min	1:20	Yang and Zhai, 2010*
Red raspberries	Anthocyanins	355 W	1.5 M HCl: 95% ethanol (15:85)	12 min	1:4	Sun et al., 2007*
Citrus peels	Phenolic acids	152 W	66% methanol	49 s	1:16	Hayat et al., 2009*
Grape seeds	Proanthocyanindins	150 W	50% methanol	200 s	1:7.14 to 1:16.66	Mayer et al., 2008*
Radix puerariae (aka Chinese herb)	Flavonoids	225 W	70% ethanol	6.5 min	1:70	Wang et al., 2010*
Morinda citrifolia (aka noni)	Anthraquinones	60% (1200 W)	80% ethanol	30 min	1:100	Hemwimon et al., 2007*
Spices	Bioactive compounds	200 W	50% ethanol	18 min	1:20	Gallo et al., 2010

*In the case of optimization studies, the most successful combination of operation parameters is shown.

methylene chloride-ethyl acetate-hexane $(3 + 3 + 4;$ v/v/v/$)$. The collected extract was dried, and the residue was diluted with hexane prior to HPLC analysis. The procedures for MSPD of several phytochemicals is shown in Table 2.5. In all of the examples, the initial sample is combined with sorbent material, washed with solvent, dried, and then diluted or dissolved with solvent.

2.4 Purification

Antioxidant capacity assays are generally performed on crude extracts that contain a mixture of antioxidant compounds extracted from a food matrix so that a complete picture of antioxidant capacity can be obtained. However, further purification of extracts is often required if characterization and quantification of specific compounds by GC or HPLC is desired. The most commonly used preparative purification method is liquid–liquid extraction, which uses two immiscible phases to crudely remove certain constituents. The most common separation systems use various ratios of ethyl acetate/water, diethyl ether/water, dichloromethane/water, and chloroform/water. Liquid–liquid extraction generally requires large volumes of solvent that must be evaporated prior to analysis, and the formation of emulsions between the two phases makes complete separation difficult. Additionally, the use of multiple solvent systems in sequence is required if separation of several classes of compounds is desired. As an alternative, solid phase extraction (SPE) was developed and is widely used for purification of extracts for analytical purposes. SPE involves adsorption of components of a liquid sample onto the surface of a solid sorbent material. Pre-packed cartridges are often available or columns may be hand-packed with pre-hydrated sorbent material. Compounds of interest are eluted using an appropriate solvent while interfering compounds are retained on the packing material, or vice versa. The resulting extracts can be analyzed directly, concentrated, or evaporated to dryness for resuspension in another solvent. Care should be taken when using SPE for quantification purposes as incomplete recovery can occur, depending on the method. SPE has been shown to be superior with regards to recovery and efficiency compared to liquid–liquid extraction in the separation of lutein and β-carotene (Shen et al., 2009). Anthocyanins have been isolated in quantities suitable for feeding trials by SPE using Amberlite

Table 2.5 Examples of extraction of antioxidant phytochemicals from various plant materials by matrix solid phase dispersion. (MSPD).

Plant Material	Compound(s) of Interest	Initial Aliquot Sample Mass	Initial Mixture Solvent (Volume)	Initial Mixture Sorbent Mass	Wash	Drying Time	Dissolving/ Diluting Solvent	Reference
Melissa officinalis	Phenolic acids	0.5 g	*n*-hexane (1 mL)	2 g	10 mL *n*-hexane (10 mL), dichloromethane (10 mL)	5 min under vacuum	methanol/ water, pH 2.5 (80:20)	Žiaková et al., 2003
Radix astragali	Isoflavonoids	0.5 g	water (0.5 mL)	1.0 g	water (10 mL)	To dryness	methanol/ water (90:10, v/v)	Xiao et al., 2004
Vegetables	Anthocyanins	0.1 g	N/A	0.4 g	Methanol/water (1:1, v/v) at pH 2 (5 mL)	N/A	water	Manhita et al., 2006
Medical Food	β-carotene	0.5 g	isopropyl palmitate (0.1 mL)	2 g	hexane with 0.5% isopropyl alcohol, methlene chloride-ethyl acetate hexane (3 + 3 + 4) containing 0.5% isopropyl alcohol	to near dryness	hexane	Chase et al., 1999

XAD-7 resin as the column material. Additionally, procyanidins have been separated from sugars and other interfering phenolic compounds by SPE using Sephadex LH-20 as the sorbent (Prior et al., 2001). SPE has also been used to isolate phytoestrogens – including isoflavones, coumestans, and lignins – from foods prior to further separation by liquid chromatography–mass spectrometry (LC-MS) (Kuhnle et al., 2007).

Similarly, column chromatography is used to separate a mixture of compounds in an extract into individual fractions using a solvent gradient. It has been used to prepare quantities of purified antioxidant compounds in the microgram to kilogram range using columns with diameters ranging from 5 to 50 mm. Compounds are eluted sequentially with gradual changes in solvent concentration or polarity. Column chromatography has been used to prepare 91–100% pure standards of six classes of carotenoids extracted from leafy vegetables (Kimura and Rodriguez-Amaya, 2002). This process is relatively low cost and provides high purity standards, including those that are not commercially available. Seeram et al. (2005) used column chromatography with Amberlite XAD-16 as a rapid method to purify large amounts of ellagitannins from pomegranate husks. Procyanidins have also been fractionated, based on the degree of polymerization using column chromatography (Lea et al., 1979; Gu et al., 2002).

Countercurrent chromatography (CCC) is a liquid–liquid chromatographic method that is dependent upon two immiscible solvents to promote separation (Pan and Lu, 2007). This approach offers two advantages: lowered cost (due to reduced solvent consumption and less costly instrumentation) and elimination of the solid support matrix. The solid support matrix is sometimes problematic owing to the possibility of contamination and adsorption (Pan and Lu, 2007). During the 1980s, high speed countercurrent chromatography (HSCCC) was created to improve the efficiency of the technique through the addition of centrifugal force (Stalikas, 2007). One of the most important aspects of optimizing this technique's potential is choosing the appropriate solvent to use with the selected analytes. There are two basic approaches to this question. One, HPLC can be used to measure the distribution ratio of the chosen analytes. The K_D of the analyte should be between 0.5 and 2.0, while the selectivity factor ($\alpha = K_{D2}/K_{D1}$) between two analytes should be at least 1.5. The second method involves running a low mass sample on an analytical CCC column, which requires only a minimum amount

of solvent. This procedure is done with several solvent systems, and the best one is chosen for the actual analysis (Hu et al., 2010). A crude Chinese rhubarb extract was successfully separated into six fractions using elution–extrusion countercurrent chromatography (EECCC) with a solvent system consisting of *n*-hexane, ethyl acetate, methanol and water at a ratio of 1:1:1:1 (v/v/v/v). The fractions were analyzed with liquid chromatography mass spectrometry (LC-MS), which identified 50 compounds. The first fraction contained many large hydrophilic compounds including phenolic acids and tannins and had the highest antioxidant capacity according to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Hu et al., 2010).

HSCCC has been applied to the separation of polyphenols in apple pomace. The solvent system used was hexane-ethyl-acetate-1% acetic acid in which hexane was much more nonpolar than ethyl acetate. The differing polarities of the molecules allow the polyphenols to elute at different times. The resulting fractions are then analyzed by HPLC (Cao et al., 2009). To separate anthocyanins from bilberry, the solvent system was methyl *tert*-butyl ether-*n*-butanol-acetonitrile-water-trifluoroacetic acid (TFA) (1:4:1:5:0.01, v/v). HSCCC was particularly suited to the separation of anthocyanins because these compounds were much less polar than other compounds present in the matrix, namely proanthocyanidins. The latter elute more quickly than anthocyanins because of their lower affinity for the stationary phase (Dua et al., 2004). Yanagida et al. (2006) used *tert*-butyl methyl ether acetonitrile-0.1% aqueous TFA to isolate catechins, procyanidins, phenolic acids, and flavonol glucosides. Countercurrent chromatography has been used in conjunction with microwave-assisted extraction to prepare samples for HPLC analysis. Deng et al. (2010) extracted, purified, and identified bergenin from *Ardisia crenata* and *Rodgersia sambucifolia*. First, MAE was optimized for both plant samples; better extraction for *Ardisia crenata* was achieved with 60% aqueous methanol as the solvent, 10:1 liquid-to-solid ratio, 500 W microwave power, and an extraction time of 15 min at 60 °C, while better extraction for *Rodgersia sambucifolia* was achieved with the same solvent as *Ardisia crenata* but used a liquid-to-solid ratio of 15:1 and an extraction time of 5 min at 50 °C. The yields were approximately the same as in the Soxhlet method but extraction time was greatly reduced, which made MAE the better extraction method in this example. As for the purification step with HSCCC, the solvent that achieved the highest retention of the stationary phase was

ethyl acetate-*n*-butanol-water (3:2:5 v/v/v) and the highest acceptable sample loading size was 160 mg.

Semi-preparative HPLC utilizes the separation power of the HPLC to improve the purity of compounds. Though analytical HPLC can achieve higher separation due to its smaller sample size, preparative HPLC can separate a larger amount; thus, preparative HPLC is a purification technique as opposed to an analytical tool. To ultimately conserve sample, the process is often optimized with the analytical procedure before being scaled up to the preparative procedure (Huber, 2000). Generating higher volumes of purified compounds has potential for use as analytical standards in biological research. For example, Kelm et al. (2006) tested a diol stationary phase and a binary mobile phase to separate procyanidins from cacao. The diol phase is advantageous because it can be used under a wider selection of solvents, and the binary phase is more easily adapted by a wider range of researchers due to tertiary and quaternary pumps being unnecessary. Semi-preparative HPLC has also been used to purify procyanidins from grape seeds (Prieur et al., 1994).

2.5 High-Performance Liquid Chromatography

HPLC is currently the most widely used method to separate, detect, and quantify antioxidant phytochemicals from plant extracts. Various combinations of columns and mobile phases have been developed for the analysis of several classes of compounds including phenolic acids, carotenoids, anthocyanins, flavonols, and ellagitannins, among others. A typical HPLC instrument consists of a stationary phase, a pump to facilitate movement of the mobile phase and analyte through the column, and a detector. The packing material in HPLC columns is a smaller particle size and more densely packed compared to conventional column chromatography, which allows for better separation on shorter columns. The most common separation strategies employed are based on the differences in analyte polarity. Normal phase HPLC (NP-HPLC) was the first polarity-based separation strategy developed and uses a polar stationary phase with a relatively nonpolar mobile phase. Compounds are eluted from the column in order of increasing polarity. Although NP-HPLC is no longer favored for the analysis of antioxidant phytochemicals, it is used for the separation of procyanidins because it

provides better separation of higher oligomers (Adamson et al., 1999). Additionally, due to their nonpolar nature, carotenoids, tocopherols, and tocotrienols are better separated using NP-HPLC methods (Khachik et al., 1997; Panfili et al., 2003). RP-HPLC has since become the most widely used LC technique because of its enhanced separation abilities. Compounds are separated by either isocratic or gradient elution in order of decreasing polarity. RP C_{18} bonded silica columns are most commonly employed with lengths ranging from 100 to 250 mm and internal diameters of 3.9 to 4.6 mm (Stalikas, 2007). Some columns contain a polar end-cap to improve separation of polar molecules. Mobile phases typically consist of aqueous mixtures of methanol or acetonitrile, and low concentrations of acid may be added to the mobile phases to prevent ionization of hydroxyl groups and minimize peak tailing. Due to ionization of hydroxyl groups of many compounds above pH 4, it is recommended that the pH of the mobile phases be maintained between 2 and 4. For this purpose, the most commonly used acids are acetic, formic, and phosphoric (Stalikas, 2007). Analysis is usually performed with the column at ambient temperature; however, a column oven might be desired to maintain a constant temperature at or above ambient temperature for improved reproducibility.

A relatively new analytical separation technique known as Ultra Performance HPLC or Ultra Performance Liquid Chromatography[TM] (UPLC[TM]) is similar in principle to HPLC except that it uses smaller particle sizes (<2.5 μm) and higher flow rates to increase the speed of separation and peak capacity, or number of peaks resolved per unit time in gradient separations (Swartz, 2005). UPLC was used to separate and quantify six major polyphenolic compounds in cocoa with a 50 mm by 2.1 mm column with a 1.7 μm particles size and a run time of only 3 min (Cooper et al., 2007). UPLC-MS/MS was also used to identify and quantify phenolics in extracts from different cocoa sources (Ortega et al., 2008). The column dimensions were 100 mm by 2.1 mm i.d. with a particle size of 1.8 μm, and a flow rate of 0.4 mL min^{-1} was employed. Using two different gradients, the researchers were able to identify and quantify 37 different compounds in the cocoa extracts with a run time of 20 min. A method was also developed to separate 17 phenolic acids in beverages in 9.5 min using reverse-phased UPLC (Gruz et al., 2008). The system operated at 4000–8000 psi throughout the run and was coupled with a PDA detector and an electrospray source for MS analysis. Additionally, researchers developed

a UPLC-DAD-MS/MS method to separate glucosinolates and phenolic compounds from vegetables of the Brassicaceae family (Gratacós-Cubarsí et al., 2010).

Multi-dimensional liquid chromatography (MDLC) is an emerging technique used for the analysis of complex samples with enhanced peak capacity compared to traditional one-dimensional chromatography (Herrero et al., 2009). This technique involves combination of two or more separation steps to enhance the power of separation using different approaches. The off-line approach is the oldest form of MDLC and involves manually collecting fractions and subsequently evaporating them and injecting into an LC separation system. Similarly, on-line MDLC uses an interface that is able to automatically collect and transfer selected fractions from one separation system into a second. Comprehensive LC is the most powerful of the MDLC methods and involves continuously collecting the entire eluate from the first separation system and injecting it into a second system through an interface, which is usually a switching valve. Although MDLC offers opportunities for enhanced separations unattainable by one-dimensional analyses, there are also problems associated with its widespread use, including solvent immiscibility issues. However, comprehensive LC has been used to separate 40 carotenoids from red orange essential oil (Dugo et al., 2008). To overcome the problem of solvent immiscibility, a low flow rate (10 µL/min) was used in the first dimension, and a monolithic column with a flow rate of 5 mL/min was used in the second dimension, which effectively diluted out the solvent from the first dimension. Comprehensive LC has also been used to separate phenolic acids from herbal extracts (Kivilompolo and Hyötyläinen, 2007), phenolics in beer and wines (Cacciola et al., 2007), and phenolic acids and flavonoids in red orange essential oil (Jandera et al., 2008).

Post-column detection of phytochemicals is usually achieved using ultraviolet/visible (UV/VIS), photodiode array (PDA), or fluorescence detectors. Many phytochemicals, particularly phenolics, absorb in the UV range; however, there is no single wavelength that can detect all classes of compounds. For example, anthocyanins are typically detected in the range 515–535 nm (Giusti and Wrolstad, 2001), phenolic acids at 280 nm (Robbins, 2003), phytosterols at 200–210 nm (Lagarda et al., 2006), and flavonols at 360–370 nm (Merken and Beecher, 2000). Furthermore, individual compounds within a certain class of phytochemicals often have varying $\lambda_{max\text{-}vis}$ values, as is the case with

carotenoids (Khachik et al., 1997). To combat this, PDA detection is often used because it allows for simultaneous scanning of a range of wavelengths for all analytes that pass through the detector. Additionally, absorption patterns at multiple wavelengths can aid in the identification of individual compounds and provide insight to their purities (Stalikas, 2007).

Fluorescence detection is based on the ability of a molecule to emit light when its electrons are excited by a beam of light of higher energy. It is not too frequently employed in phytochemical analysis, but is particularly useful for compounds with specific functional groups and substitution patterns. For example, the fluorescence detection limit of procyanidins is approximately 100 times lower than that of UV detection (Gu et al., 2002). Fluorescence is also much more sensitive than UV for the detection of tocopherols (Hoehler et al., 1998). Establishing proper excitation and emission wavelengths is necessary for proper detection, so rapid scanning fluorescence detectors are often preferred.

Electrochemical detection (ECD) is useful for compounds that can be oxidized or reduced. An oxidation or reduction reaction occurs at an electrode resulting in an electron flow at the surface of the electrode. As the reaction proceeds and analyte is depleted, a concentration gradient is formed between the surface of the electrode and the bulk of the solution, which is proportional to the concentration of the analyte. Similarly, coulometric detection is based on the same principle, except that the oxidation or reduction reaction proceeds to completion and the total charge generated is proportional to the mass of material in the reaction. ECD and coulometric detection are generally much more sensitive than other methods, allowing for quantification of low concentrations of analytes, which is particularly useful for plasma or urine samples (Wolfender, 2009). HPLC with ECD in the oxidative mode has been used for the quantification of catechin and epicatechin in cacao, and was far more sensitive than UV detection at 280 nm (Subagio et al., 2001). Isoflavones from soy-based products were analyzed using HPLC with coulometric electrode array detection, which was also more sensitive than UV or PDA detection (Nurmi et al., 2002). HPLC-ECD has also been used for analysis of flavonoids in red grape skins (Novak et al., 2008), carotenoids and tocopherols in tomatoes (Rozzi et al., 2002), and various phenolic compounds including anthocyanins, flavonols, and ellagitannins in strawberries (Aaby et al., 2007).

2.6 HPLC-MS and HPLC-MS/MS

HPLC coupled with mass spectrometry (MS) has become a valuable tool for compound identification beyond retention time comparison. Various ionization techniques and mass analyzers have been applied to the analysis of phytochemicals. Early ionization methods such as electron impact, thermospray, fast atom bombardment, and particle beam ionization have, for the most part, been replaced by newer soft ionization techniques (Ryan et al., 1999). Currently, atmospheric pressure ionization (API) is the most widely used ionization technique applied to the analysis of phytochemicals because the lack of vacuum allows for easier coupling with HPLC. Specifically, two sources of API, which include atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), are frequently used either in the positive- or negative-ion mode. In ESI, a liquid sample is nebulized and dispersed into tiny electrically charged droplets at atmospheric pressure. As the solvent evaporates, the droplets are forced apart into smaller droplets by coulombic repulsion until each droplet contains only one molecule, which is charged (Ryan et al., 1999). The resultant ions, which are either positively charged due to the addition of a proton or cation, or negatively charged due to the removal of a proton, then travel to the mass analyzer. Large molecules, such as procyandins and ellagitannins, may be multiply charged which allows for accurate determination of their molecular weights by producing a smaller *m/z* ratio, which is in the instrument range (Hammerstone et al., 1999; Hager et al., 2008). ESI is considered to be a "soft" ionization technique because minimal fragmentation occurs during ionization unless the potential difference between the analyzer and the transfer capillary is increased. APCI is similar to ESI except that ionization occurs in the gas phase instead of the liquid phase, which makes it less suitable for polar phytochemicals that are thermally labile and not easily volatilized (Ryan et al., 1999). Additionally, APCI is less "soft" than ESI, resulting in greater fragmentation of molecules due to collision-induced dissociation during vaporization and ionization. Since ESI is suitable for the ionization of polar, nonvolatile, and thermally unstable molecules, it finds application in the analysis of several phytochemicals, including anthocyanins (Cho et al., 2004; Wu and Prior, 2005), flavonols (Cho et al., 2005), and isoflavones (Klejdus et al., 2007). ESI in the negative-ion mode was used to identify hydroxycinnamates, procyanidins, flavonols, and

anthocyanins in nectarines, peaches, and plums (Tomas-Barberan et al., 2001). APCI has been used for the ionization of isoflavones (Chen et al., 2005), flavonols, catechins, and phenolic acids (Harris et al., 2007), phytosterols (Lu et al., 2007), and tocopherols (Lanina et al., 2007).

Coupled to the ionization source is a mass analyzer which measures the mass-to-charge (m/z) ratio of the ions. The most common and simplest mass analyzer is the single quadrupole, which has four parallel rods that allow only ions with a given m/z to reach the detector according to the selected voltage and current applied to the rods. These are used for low resolution mass analyses and have a mass range typically up to m/z 4000, which is suitable for the analysis of many phytochemicals (Fossen and Andersen, 2006). Ion trap (IT) mass spectrometers provide more structural information than do quadrupoles by accumulating ions in a chamber according to RF and DC voltages. The stored ions can then be isolated or successively fragmented to provide MS^n spectra (Fossen and Andersen, 2006). IT analyzers have been used for structural characterization of several phytochemicals (de Rijke et al., 2003).

Tandem mass spectrometry or MS/MS is frequently used for structural elucidation of phytochemicals to provide more detailed information about the structure and composition of a molecule. This involves two mass spectral steps and fragmentation of the compound occurs between the steps. The two steps may be separated in space or in time depending on whether two distinct separation elements are used (space) or two different separations occur in the same place over time. As described previously, an IT analyzer can be used to successively fragment a molecule to provide MS^n spectra, an example of separation in time. A triple quadrupole mass spectrometer is a form of tandem mass spectrometry in space in which two quadrupoles serve as mass filters while a third, positioned in the middle, allows for collision-induced dissociation. Triple quadrupoles are common in phytochemical analysis, and have been used to characterize phenolic compounds in fruit juices (Abad-García et al., 2009) and procyanidins and alkaloids in cocoa (Ortega et al., 2010), among others.

Another MS/MS instrument, the Q-TRAP mass spectrometer, merges a triple quadrupole with an IT mass spectrometer, combining high sensitivity with high selectivity (Le Blanc et al., 2003). Q-TRAP instruments have been used to characterize, and in some cases quantify, ellagitannins in red wine (Stark et al., 2010), anthocyanins in red

cabbage (Arapitsas et al., 2008), and phenolics from barley (Klausen et al., 2010). Yet another common tandem mass spectrometer, the Q-TOF, is a hybrid quadrupole and time-of-flight instrument.

In cases where high specificity is required, MS/MS scans can be performed in selected/multiple reaction monitoring mode, a highly sensitive technique used for detection and quantification of specific compounds, which have been well characterized previously. MRM is the plural term and refers to the use of a specific parent compound mass and the mass of a unique fragment ion produced from MS/MS to selectively monitor compounds of interest (Wu et al., 2009). MRM can provide absolute identification of a compound in a matrix, and with the use of external standards, can be used as a highly sensitive quantification tool. MRM has been used for quantification of phenolic acids in black raspberry (Wu et al., 2009) and various beverages (Gruz et al., 2008), procyanidins, phenolic acids, flavones, and alkaloids in cocoa (Ortega et al., 2008), ellagitannins and their transformation products in red wines (Stark et al., 2010), glucosinolates and flavonols in members of the Brassicaceae family (Gratacós-Cubarsí et al., 2010), and phenolic acid metabolites in the GI tract and urine following consumption of cranberries and black raspberries (Wu et al., 2009; Prior et al., 2010).

Recently, a high resolution mass spectrometer (HRMS), known as an Orbitrap mass spectrometer, has been used for applications requiring extremely high sensitivity (Mullen et al., 2010; Vallverdú-Queralt et al., 2010). Mullen et al. (2010) found that the Orbitrap mass spectrometer was 200-fold more sensitive than an mass spectrometer in MRM mode in detecting cyandin-3-*O*-glucoside in brain extracts of birds fed blackberry. Additionally, the Orbitrap was able to distinguish between compounds that differed in mass by as little as 21 mDa. Besides sensitivity, another advantage of the Orbitrap system is its ability to be used in full scan mode, allowing for information to be obtained on all compounds in a mixture without having to select for compounds of interest as in MRM mode (Zhang et al., 2009).

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) is a direct mass spectral technique that has found application for the analysis of various phytochemicals. MALDI represents the ion source, whereas TOF is the mass analyzer. The two techniques may be coupled with other ion sources or mass analyzers, but are most commonly used in tandem. In MALDI, the analytes are mixed with a matrix, which is usually an aromatic organic acid, which aids in

analyte ionization upon absorption of laser energy. MALDI typically generates cationic species such as $[M + Na]^+$ and $[M + K]^+$ (Fulcrand et al., 2008). This technique is particularly useful for large molecules that are not easily ionized, and is considered a "soft" ionization because fragmentation of the ions does not occur (Prasain et al., 2004). TOF analyzers separate ions based on their flight times across a known distance, which is solely dependent upon their *m/z* value (Fossen and Andersen, 2006). This can be performed in either the linear or reflectron configuration. In the linear mode, ions travel in a straight path to the detector at the end of the tube, whereas in the reflectron mode, a series of reflectors bend the flight path of the ions, which lengthens the travel path and corrects for differences in initial kinetic energy among ions with the same *m/z*. This results in increased resolution and mass accuracy. MALDI-TOF has been used to characterize various oligomeric food polyphenols such as polygalloyl-polyflavan-3-ols in grape seed extract, glycosylated polyflavans in sorghum, A-type polyflavan-3-ols and anthocyanin-polyflavan-3-ols in cranberries, and ellagitannins in pome-granate (Reed et al., 2005). Using *trans*-3-indoleacrylic acid as the matrix, researchers were able to differentiate among degrees of poly-merization, intermolecular bonds, patterns of hydroxylation, and de-grees of substitution. Ellagitannins from blackberry were also evaluated using MALDI-TOF in the positive-ion reflectron mode with dihydrox-ybenzoic acid as the matrix (Hager et al., 2008).

2.7　Gas Chromatography

Gas chromatography (GC) is another widely used analytical technique for phytochemical determination. Similar to HPLC, GC requires sample preparation, which may include lipid extraction and/or extraction of phytochemicals. Once the sample is prepared, it enters the inlet system, flows through the column, and then reaches the detector. In the case of phytochemical analysis, the detector is often a flame ionization detector, which is suitable for all organic particles, or more commonly, the sample passes through the column directly to a mass spectrometer, which serves as the detector.

The superior separation capacity of GC comes with one prominent disadvantage in phenolic determination. Namely, since phenolics are not readily volatile, an additional derivatization step is necessary prior

to GC analysis. The hydroxyl groups of phenolic compounds can be converted to ethers or esters and thus be made volatile. Flavonoids are generally derivatized through methylation, trimethylsilyl (TMS) production, or use of N-(*tert*-butyldimethylsilyl)-N-methlytrifuoroacetamide (Stalikas, 2007, 2008).

TMS production involves one specific functional group (–OH, –COOH, =NH, –NH$_2$, or –SH), which loses an activated hydrogen and is replaced by a trimethylsilyl group (Proestos et al., 2006). To achieve silylation, some authors have used BSTFA (N,O-*bis*(trimethylsilyl)trifluoroacetamide) and TMCS (trimethylchlorosilane) successfully in several matrices (e.g. aromatic plants, cranberry fruit) (Zuo et al., 2002; Proestos et al., 2006). Using silylated derivatives is advantageous for several reasons: phenols and carboxylic acids are prone to silylation, these compounds can be derivatized in the same part of the process, and the minor products do not impede analysis and are well documented (Little, 1999; Stalikas, 2008). A two-step methylation procedure was used to analyze catechins and tannins in plant extracts. The first step used trimethylsilyl diazomethane (TMS-diazomethane) to pre-methylate the sample, and the second step used thermally assisted hydrolysis and methylation (THM). The pre-methylation step with TMS-diazomethane stabilized the dimer molecule (*m/z* 540) by minimizing isomerization and reducing reactivity. (Shadkami et al., 2009).

Saraji and Mousavinia (2006) developed an efficient in-syringe derivatization step for fruit juices and fruits. The parameters for in-syringe derivatization optimized with phenolic standards were found to be 10 min at room temperature with 0.7 µL *N*,O-*bis*(trimethylsilyl) acetamide (BSA). Before derivatization, the samples underwent single-drop microextraction, where small volumes of organic solvents were used to extract analytes. The advantage of this approach is that it creates reduced risks for sample loss and contamination.

The speed of the derivatization process can be increased with the assistance of microwaves because microwaves quickly supply energy to the core of the sample, while conventionally the sample is heated slowly via conduction. This method was developed with gallic acid, gentisic acid, vanillic acid, caffeic acid, ferulic acid, and *p*-coumaric acid, which are phenolics commonly found in wine and fruit samples (Chu et al., 2001). The optimized derivatization protocol for compounds found in ginkgo biloba was heating the analyte at 115 °C for 45 min in 50 µL of ethyl acetate with 250 µL of *N,N*-dimethylformamide (DMF)

containing 1% squalane and 250 µL µL of BSTFA with 1% trimethyl-chlorosilane in DMF (1:1). An attempt was made to streamline the procedure through eliminating the drying step and taking the sample from the extraction process directly to the derivatization process (Deng and Zito, 2003).

Generally, GC columns are classified as either packed or capillary. Choosing to use the capillary column, instead of the packed column has many advantages including, vastly improved separations with higher resolution, reduced time of analysis, smaller sample size requirements, and often higher sensitivities (Barry, 2004). The capillary column is able to offer better separation than a packed column because its long, stationary phase coated tube has more theoretical plates. Fused silica is a common choice for capillary column material as it is more inert than metal columns and more flexible than glass columns. Packed columns contain packing material, which is inserted into a tube and held in place with plugs. These columns are shorter than capillary columns and can be used for preparatory functions due to their greater sample capacity (Barry, 2004). Packed columns are also more vigorous and less expensive, though analysis can be slower (Hinshaw, 2004).

The general concept of injecting and vaporizing a liquid-phase sample and then combining it with carrier gas before sending it to the column is applicable to all GC. In the case of the packed column, injection is much less complicated because the entire contents of the syringe enter the column. When using capillary columns, more considerations are necessary. Flavonoids are generally injected in either the split or splitless mode (Stalikas, 2007). These systems were developed in response to challenges that arose when capillary columns were first created. For one, the capillary column was easily overloaded by typical injection volumes; thus, the split inlet was created to divert part of the volume of sample. Second, sample concentration or additional sample preparation became necessary to maintain high detection limits of capillary chromatography. Third, discrimination may leave components of the sample in the injection syringe or inlet (Snow, 2004). Though split and splitless systems are distinct systems, the same inlet is used for both. In the split system, a pre-specified portion of sample is directed away from the column to a waste valve. This method offers quick injection, though it requires concentrated samples and can suffer from potential column overloading. The splitless system uses the same mechanical parts as the split system, however, the waste valve remains

closed for approximately 30 to 60 min and is then opened, and any remaining sample is purged. Meanwhile, the sample vapor is directed entirely to the column. Generally, the majority of the sample enters the column without overloading (Snow, 2004). Cool on-column and programmed-temperature vaporization are also inlet systems, but are not commonly used in phytochemical analysis.

Identification of phenolics in different subvarieties of currants was performed by GC-MS with split–splitless injector set at a split ratio of 1:20. This study was able to determine the profile of the phenolics present, how the sub-varieties differed in polyphenols present (*Vostizza* did not have 3,4-dihydroxy-phenylacetic acid, while the other *Gulf* and *Provincial* did), and the amount of each phenolic present. This study demonstrated the potential of GC-MS to profile subtle differences among similar products (Chiou et al., 2007).

The selection of the carrier gas is another important aspect in GC analysis. Helium and hydrogen are the most common choices. Hydrogen necessitates extra caution due to its high flammability, though it has less variability in plate number as linear velocity raises (Barry, 2004). Helium is nonflammable and is commonly used for analysis of phytochemicals (Määttä et al., 1999; Phillips et al., 1999; Chu et al., 2001; Chiou et al., 2007).

In GC, the part of the system responsible for the response signal of separated chemical moieties is the detector. The detector recognizes a particular physical or chemical characteristic of the separated components through which it can be used to either identify an unknown compound or quantify a known compound (Colon and Baird, 2004).

Many GC detectors exist, but not all are suitable for phytochemicals. The thermal conductivity detector (TCD) is considered a universal detector and is appropriate for most analytes as long as the thermal conductivity of the carrier gas is different from that of the analytes. During the early development phase of GC, TCD was an easy choice because thermal conductivity measuring devices were already in use (Colon and Baird, 2004). Ionization detection arrived with its improved trace determinations and replaced TCD in many applications. While TCD is still used for some food applications (Allegro et al., 1997; Sun et al., 2007) and in the past was used for phenolic acids (Blakely, 1966), currently it is not generally used for phytochemicals. Rather, the flame ionization detector (FID) is better-suited due to its selectivity for organic compounds and superior measuring ability for trace measurements.

These detectors also differ in their primary group classification. TCD is a concentration-sensitive detector, which means it records the proportionate concentration of an analyte in the mobile phase (ng/mL), while the FID is a mass flow detector, which means it records the measured mass of a particular component per unit time (ng/s). Though FID is more suitable than TCD, often, the mass spectrometer is used due its superior detection abilities. Instead of passing through the detector within the GC, separated samples are transported to the mass spectrometer and further analyzed. However, some studies use a combination of methods, where GC-FID is used in conjunction with GC-MS (Määttä et al., 1999; Phillips et al., 1999).

Generally, the FID is appropriate because it is insensitive to some common impurities (e.g. water, carbon dioxide), is not affected by modest operational changes in pressure or temperature, and selects for organic compounds (Colon and Baird, 2004). In FID, the carrier gas forces the organic compounds toward a jet and then a hydrogen-air diffusion flame. An electrode above the flame captures the electrically charged particles that are created. An electrometer measures the amplified current that is proportional to the quantity of carbon in the flame. Single carbon species are created (though the mechanism is not well understood) from this process, and proceed through a series of reactions due to the presence of heat, hydrogen, oxygen, and water in the reaction zone:

$$(1)\ CH\cdot + O\cdot \rightarrow CHO^+ + e^- \quad (2)\ CHO^+ + H_2O \rightarrow H_3O^+ + CO$$
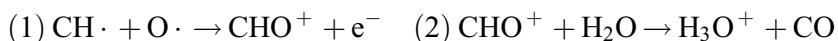
One hydronium ion occurs for approximately every 100,000 carbon atoms produced in the flame; thus, the number of carbon atoms, not the compound weight or moles, determines the FID response (Colon and Baird, 2004). The FID has been applied to flavonoid aglycones in conjunction with a capillary column. Sharp and symmetrical peaks were generated, though the authors suggested that GC could be enhanced by coupling with mass spectrometry. The flavonoids were derivatized with hexamethyldisilazane + trimethylchlorosilane (HMDS + TMCS). In general, an increase in TMS ether groups initiates an increase in retention time, though elution can be affected by the position of the substituents (Koupai-Abyzani et al., 1992). Flavanones from orange and grapefruit juices were extracted and then identified by GC-FID. In orange juice, the predominant flavanones were naringenin and

hesperetin, whereas naringenin was predominant in grapefruit juice (Coffin and Dupont, 1971). FID has also been used to analyze phenolics in propolis, a biologically active glue produced by bees. The primary groups of phenolics identified were flavonoid aglycones, phenolic acids, and their esters. Derivitization was achieved through silylation with BSTFA, and quantitative analysis was performed with internal standard method (Bankova et al., 1992).

The superior separation of the GC can be enhanced with the accurate mass detection of mass spectrometry; thus, the two are often used in tandem. In GC-MS systems, the effluent moves from the GC to the mass spectrometer via a transfer line. Once the effluent enters the mass spectrometer, it first moves through the ion source, which generates ionized fragments. These ions move through a mass analyzer and then a detector. The mass-to-charge ratio (*x*-axis), which is indicative of mass because most formed ions are singly charged, is plotted against relative intensity of the ions (*y*-axis) (Masucci and Caldwell 2004).

Chemical and electron ionization are the most common ion sources for GC-MS. Both are capable of creating the energy needed for ionization. Electron ionization is used more commonly with phyto-chemical analysis with 70 eV being the standard voltage (Stalikas 2008). In order for the mass analyzer to correctly identify *m/z* ratios, the particles that enter the mass analyzer must be charged. However, when analytes first enter the mass spectrometer, they are neutral., In order to make them charged, they are bombarded with electrons in the ion source. This can either result in a radical cation, in which an electron from the ion source projects an electron out of a neutral analyte's orbit, or a radical anion, in which the analyte captures an electron. Some of the radical cations and anions have excess energy, which contributes to bond breakage and decomposition of the analyte (Masucci and Caldwell 2004).

Once analytes pass through the ion source, they move into the mass analyzer. The two common mass analyzers for GC are the magnetic sector and quadrupole. Also available but less common are time-of-flight (TOF), triple quadrupole, and ion trap. The purpose of all of these devices is to separate ions according to their *m/z* ratios. The principles behind the most common mass analyzers have been des-cribed above in the section entitled "HPLC-MS and HPLC-MS/MS". The mass analyzer separates the ions, which are then sent to the detector. The most common detector is the electron multiplier, which involves

ions ricocheting from one dynode at a certain voltage to the next with a higher voltage. This information is sent to the data system, and the compounds in the analyte can then be identified (Masucci and Caldwell 2004).

Within the context of GC, phytochemicals are most often analyzed with a GC-MS combination. This method even allowed for the identification of catechin in plasma samples in human trials (Donovan et al., 1999) after samples were extracted and derivatized with BSTFA. Tocotrienols in annatto were identified and quantified by GC-MS, though the sample preparation was more intensive because of the high lipid content. The extract was first saponified and the sample was then treated with diazomethane, which converted free fatty acids to methyl esters. Next, a 10% benzene solution of unsaponifiable matter was prepared. The sample was then evaporated and a silanizing reagent was used to convert the hydroxylic groups into trimethylsilyl derivatives. The resulting solution was analyzed by GC-FID, and the pre- and post-TMS-reagent-treated samples were analyzed by GC-MS. GC analysis determined that the annatto had a tocotrienol content of 140 mg/100 g dry seeds, which is high compared to other foods that have been tested thus far. It is also unusual that annatto contains no tocopherols, but is rich in tocotrienols (Frega et al., 1998). Bak et al., (2010) used GC-MS and LC-MS to analyze sour cherry seed kernel, the oil of which, similar to annatto, contained tocotrienols and, dissimilar to annatto, also contained tocopherol-like products. The solid fraction contained polyphenols, flavonoids, and pro- and anthocyanidins, which makes sour cherry kernel a good source of many phytochemicals. Since this product contained both lipid-soluble components and water-soluble components, sample preparation was more extensive (similar to the annatto example). The lipids were extracted with hexane, while the solid part was divided into two smaller fractions, one of which was extracted with water–methanol and the other with methanol hydrochloric acid. The oil fraction was analyzed by GC-MS for identification of vitamin E derivatives, and the constituents of the other two fractions – one considered the ester fraction and the other the flavonoid fraction – were analyzed and identified by LC-MS.

Perhaps one of the most useful applications of GC-MS is determining small differences in composition among similar samples. For example, many food products have different cultivars, which may produce different functional benefits depending on individual compositions.

Määttä et al. (1999) identified differences in the phytosterol composition of differing cultivars of oats after samples were derivatized to trimethylsilyl ethers. The Vital and Stork cultivars were shown to be lower in sterols than Elin, Freja, Galopp, Matilda, and Stormogul. Samples were studied from several different locations in Sweden, and no significant differences were found due to location. Acid hydrolysis was shown to be useful in freeing phytosterols in the bound form, which reinforces the importance of sample preparation. The identification of phytosterols also has applications in the study of human diets. Phillips et al. (1999) studied the phytosterol and fatty acid composition of experimental diets that were part of other clinical trials. They found that the total phytosterols were inversely related to saturated fat, but positively related to polyunsaturated fat. This finding implies that making conclusions about the effects of experimental diets based on the fatty acid content alone may be problematic because phytosterols may also affect blood cholesterol levels. In this study, the sterols were converted to trimethylsilyl derivatives before GC analysis, as previously described in the oat study. All of the previously discussed examples demonstrate that GC-MS is versatile in its identification of an array of phytochemicals, as well as in the analyses of varying food matrices.

2.8   Conclusions

The analysis of phytochemicals is a tedious process involving several steps in which care must be taken to avoid degradation and contamination. Recent advancements in extraction, concentration, purification and analytical procedures of phytochemicals have been made, but additional developments are needed to assist in the identification and quantification of the diverse array of phytochemicals present in plants and foods, as well as metabolites in biological samples. Specifically there is a need to: automate sample extraction, clean-up, and concentration steps to facilitate the screening of phytochemicals; develop analytical methods with improved sensitivity, resolution and throughput that utilize less organic solvents; and develop concentration and purification methods to produce analytical standards that are not available commercially. Continued advancements in sample preparation and analytical techniques will assist researchers in their quest to identify and quantify the vast array of phytochemicals present in plants

and foods, and to determine their biological properties associated with health-promotion.

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Chapter 3

Analysis Methods of Phenolic Acids

Zhimin Xu

Abstract

Phenolic acids are broadly distributed in the plant kingdom. Studies have shown that phenolic acids in fruits, vegetables, and grains have health benefits of preventing chronic diseases such as cardiovascular diseases, obesity, diabetes, and various cancers. High-pressure liquid chromatography (HPLC) is widely used for qualitative and quantitative analysis of phenolic acids. Due to the complexity of different sample matrices and bound phenolic acids, different sample preparation methods including acidic, basic and enzymatic hydrolysis, solvent extraction, and solid phase cartridge cleanup step are discussed in this chapter. Also, different mobile phase compositions are introduced for obtaining satisfactory separation and reduced analysis time based on the phenolic acids profile of different samples. In addition ultraviolet (UV) detection, HPLC with florescence (FL), electrochemical (EC), or mass spectrometric (MS) detection used for the analysis are discussed. Gas chromatography (GC) analysis of phenolic acids and sample derivatization are also included in this chapter.

Keywords: Phenolic acid; phenolic; HPLC; GC; antioxidant; analysis

3.1 Introduction

Phenolic acids present in plants are a group of compounds having a single hydroxylated aromatic ring with a directly or indirectly attached carboxyl group. They are synthesized in the plants as secondary
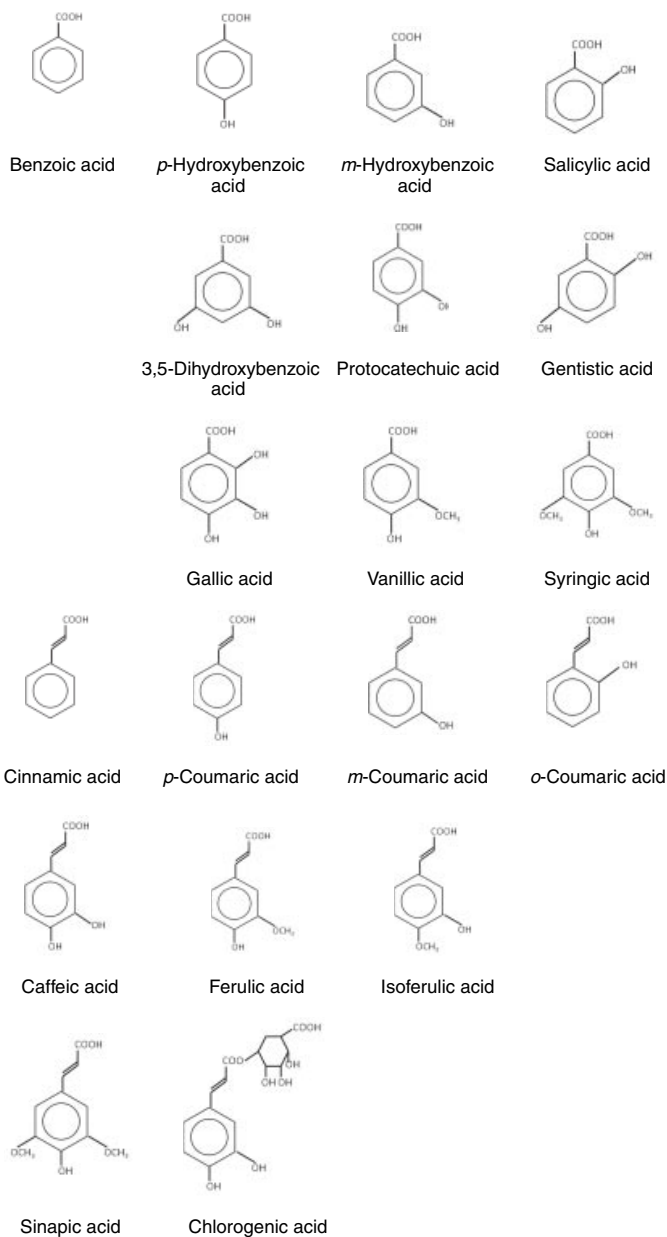
metabolites in response to environmental stress during plant growing, such as presence of insects and pathogens, physical lesion, and UV radiation (Croteau et al., 2000; Hermann, 1989). An essential precursor of phenolic acid synthesis is L-phenylalanine, an amino acid with an aromatic ring, which is produced from shikimate and chorismate biosynthesis (Haslam, 1993; Herrmann, 1995). After a series of reactions involving deamination, enzymatic conversion, and enzymatic hydroxylation, L-phenylalanine is converted to different hydroxylbenzoic acids and other phenolic acids (Haslam, 1993). There are more than a dozen phenolic acids commonly found in fruits, vegetables, and staple commodities. The primary chemical structure of the phenolic acids is either benzoic acid or cinnamic acid (Figure 3.1). There is one position on the aromatic ring of benzoic or cinnamic acid occupied by a hydroxyl group, and there are four positions still available for other groups, such as a methoxyl group or another hydroxyl group. The chemical structures of 17 common phenolic acids are shown in Figure 3.1. Nine of them are derivatives of benzoic acid and the rest are derivatives of cinnamic acid. *p*-Hydroxybenzoic acid, *m*-hydroxybenzoic acid, and salicylic acid are phenolic acids with only one hydroxyl group attached to a benzoic acid. Gentisic, protocatechuic, and 3, 5-dihydroxybenzoic acids have two hydroxyl groups, while benzoic and gallic acids have three. Vanillic and syringic acids have hydroxyl and methoxyl groups on the aromatic ring of benzoic acid. Hydroxycinnamic acid types of phenolic acids include *o*, *m*, and *p*-coumaric acid, caffeic acid, ferulic acid, isoferulic acid, and sinapic acid. Chlorogenic acid is a derivative of cinnamic ester and has a carboxyl group attached to the six-carbon ring (Figure 3.1).

Most phenolic acids are also significantly responsible for the taste and flavor of edible plants. In recent decades, the potential health benefits of plant phenolic acids have been a fascinating subject attracting researchers in different areas, such as food, plant breeding and physiology, medicine, epidemiology, etc. Numerous studies have confirmed that a dietary intake rich in vegetables, fruits, and legumes correlates with reduced risk of cardiovascular diseases and cancers (Johnson and Williamson, 2003; Johnson, 2003). The antioxidant activity of phenolic acids, which is contributed by the hydroxyl group on the aromatic ring, is considered to play an important role in those health benefits (Konishi et al., 2006; Kristinová et al., 2009). Compared with polyphenolics, phenolic acids are more water-soluble and bioavailable. These phenolic acids, such as caffeic and ferulic acid, are directly absorbed and

Benzoic acid   *p*-Hydroxybenzoic acid   *m*-Hydroxybenzoic acid   Salicylic acid

3,5-Dihydroxybenzoic acid   Protocatechuic acid   Gentistic acid

Gallic acid   Vanillic acid   Syringic acid

Cinnamic acid   *p*-Coumaric acid   *m*-Coumaric acid   *o*-Coumaric acid

Caffeic acid   Ferulic acid   Isoferulic acid

Sinapic acid   Chlorogenic acid

Figure 3.1   Chemical structures of benzoic acid, cinnamic acid, and common phenolic acids.

circulated by the bloodstream (Nurmi et al., 2009). Chlorogenic acid was reported to be beneficial to gastrointestinal microflora (Plumb et al., 1999; Azuma et al., 2000). Also, caffeic acid could enhance the body's immune system and inhibit the biosynthesis of leukotrienes, which contribute to asthma, allergic infections, and immune dysfunctional diseases (Koshihara et al., 1984). Some of the cinnamic acid derivatives were reported to possess the capability of inhibiting inflammation, cell differentiation, and cancer cell proliferation (Maggi-Capeyron et al., 2001).

The phenolic acids in plants are in either free or bound form. Free phenolic acids are soluble in the liquid media of plant cell, while bound phenolic acids are largely linked with the plant cell wall fibers and are not solubilized by the cell liquid media (Brett and Waldron, 1996a, 1996b). Bound phenolic acids are also bound with protein, carbohydrates, and other large or small organic compounds (Hartley and Jones, 1976; Xu and Godber, 1999; Andreasen et al., 2000; Lam et al., 2001). Both forms are distributed in all parts of the plant, including the seeds, stems, leaves, and root (Macheix et al., 1999; Wang et al., 2011). The levels and types of phenolic acids are variable at different growing stages (Ellnain-Woitaszek et al., 2001; Kevers et al., 2011). The same plant species may have different content and profile of phenolic acids due to a different growing environment (Zheng and Wang, 2001; Mpofu et al., 2006; Bavec et al., 2010; Fernandez-Orozco et al., 2010). The variability and diversity of phenolic acids increase the difficulty of standardizing an extraction method for the phenolic acids from plant matrix.

To analyze free-form phenolic acids in fruit or vegetable juice, the sample preparation is straightforward and simple. The juice can be directly injected into an HPLC system after it is filtered to remove any insoluble particles. However, for a sample with a solid fraction containing both free and bound phenolic acids, the sample preparation is not as simple. A mechanical method is needed to physically break down the sample and release the free phenolic acids, which are blocked in the inner core of the sample matrix. Chemical (acid or alkaline) or enzymatic hydrolysis must be applied to break down linkages in the bound phenolic acids to release free phenolic acids. However, the recovery of total phenolic acids is significantly affected by hydrolysis and other extraction conditions. An intensive hydrolysis condition may increase the release rate of bound phenolic acids to free phenolic acids; however, it can also cause degradation of some phenolic acids and lower

the recovery in the final extract. Therefore, the stability of phenolic acids should be considered when a chemical hydrolysis method is used in the sample preparation.

In addition to chemical or enzymatic hydrolysis methods, new extraction technologies, such as microwave-assisted solvent extraction and supercritical fluid extraction, can be applied to increase the recovery of phenolic acids during extraction. These technologies effectively break down the sample matrix to release phenolic acids. However, they also increase the possibility that other compounds may be largely extracted with the phenolic acids, and these compounds can interfere in the phenolic acid quantification step. A cleanup step may be needed to remove the irrelevant compounds before quantification. A common cleanup is to use a C18 or another packing polymer cartridge to absorb the phenolic acids and elute out most of interfering compounds. The, phenolic acids fraction is then collected through a desorption method and concentrated for further quantification.

Because the chemical structures of phenolic acids are very analogous, their UV spectra are very similar. This is a major factor in the complexity of individually quantifying each phenolic acid. It is impossible to use a traditional spectrophotometric method to accurately determine each phenolic acid due to other existing phenolic acids in the extract solution that may seriously interfere in the absorbance value and cause an overestimated result. Thus, separating individual phenolic acids from others is a necessary step for performing accurate quantification for each phenolic acid. Chromatography techniques have been widely used to separate and determine individual phenolic acids and eliminate the interferences from other phenolic acids. Although thin-layer chromatography (TLC) is an original chromatography method applied to determine some phenolic acids, it has limitations, such as lower separation resolution, less sensitivity, and longer analysis time. Currently, sophisticated HPLC systems are being used in phenolic acid analysis. The advantages of an HPLC system are speed, sensitivity, and accuracy. Although HPLC systems are more costly than TLC methods, they have become the common analysis instruments equipped in most laboratories. The HPLC method usually can have satisfactory separation for over ten phenolic acids if the run condition is optimized well. The separation of phenolic acids in the extract can also be achieved using a GC method. However, as most phenolic acids are not capable of being vaporized at GC injection temperature, they have to be

derivatized to an ester form using chemical methods in order to lower their vaporization point before being loaded into a GC system. The derivatization reaction is significantly affected by many factors, such as chemical composition in the extract, reaction temperature and time, purity of chemical reagents, etc. The GC method of analyzing phenolic acids is not as straightforward as the HPLC method.

3.2   Sample Preparation for Extraction and Hydrolysis

Because phenolic acids contain hydroxyl and carbonyl groups, they are relatively polar and water-soluble. For fruit and vegetable juice samples, free phenolic acids can be directly used for chromatography quantification after solid particles in the juices are removed by filtration or centrifugation. For solid samples, a grinder is commonly used to break down the sample matrix. Because of the complexity of the plant matrix, which normally contains significant amounts of lipids, proteins, and carbohydrates, an aggressive mechanical method could cause the formation of a water-in-oil emulsion if the sample also contains a large quantity of water. This emulsion could lower the grinding efficiency and prevent the sample matrix from being converted into ideal and uniform small-sized particles. Thus, high moisture samples should be dried before the grinding procedure. Freeze drying is usually used to remove moisture from samples with a smaller chance of activating any possible enzymatic and chemical reactions that may occur with drying. Most components remain intact and are not degraded during drying. The phenolic acids after final identification and quantification are not very different from their original condition. However, a freeze-drying system and its operating cost are fairly expensive. For relatively lower moisture samples, such as cereal and plant leaves, there is an alternative way to remove moisture. They can be dried using a silica absorption gel filled desiccator, which may be equipped with a vacuum vent. The moisture of the sample is absorbed by the silica gel under vacuum at room temperature. Using an oven to dry samples is another choice if the above methods are not available. For high water content samples, the liquid fraction of the sample should be separated as much as possible through centrifugation or filtration to decrease any possible enzymatic or other reactions during oven drying. The residue is then dried under warm air at temperature normally not over 60 °C. The phenolic acid

content in the sample's liquid fraction should be determined and included in the final results.

The dried sample is readily ground to fine particles to increase the contact area with solvent in the extraction step. Because the common high-speed grinder generates heat rapidly during grinding, it should be run at intervals to avoid overheating the sample. Liquid nitrogen is often used to freeze samples to solid form when grinding with a mortar. This is an alternative way to break down solid samples to fine particles while keeping the sample components intact. After grinding, sieve size No. 40 is normally used to collect the ground particles less than 0.42 mm for further extraction.

3.3   Extraction and Hydrolysis for Chromatography Analysis

Although water is an economical and environmentally friendly media, it is not the most popular media for the extraction of phenolic acids due to its boiling point, which is higher than that of other solvents, such as methanol, acetone, etc. The higher boiling point results in longer evaporation time when concentrating the extraction supernatant to obtain a phenolic acid extract. However, water is used to break down bound phenolic acids in extractions requiring enzymatic hydrolysis. Methanol, ethanol, acetonitrile, and acetone are the most common solvents used in phenolic acid extraction. Also, it is recommended that the extraction solution be maintained in an acidic environment. In most studies, methanol acidified with acetic acid is used in the extraction. The acidic environment prevents phenolic acids from being neutralized by compounds potentially released during extraction. However, this acidic condition is too weak to hydrolyze bound phenolic acids from fiber celluloses. Thus, a strong hydrolysis is achieved by adding HCl or NaOH to the extraction, which can efficiently hydrolyze bound phenolic acids, especially in cereals, grains, and berry pomace. Figure 3.2 is an example of acid hydrolysis of bound phenolic acids in raspberry pomace. After acid hydrolysis with 2% HCl, three phenolic acids – protocatechuic, chlorogenic, and ferulic – were significantly released from the raspberry pomace. It was confirmed that hydrolysis is a necessary step to obtain bound phenolic acids in samples.

However, strong pH conditions could cause degradation of some phenolics. For example, in Figure 3.2, two polyphenolics (anthocyanins),
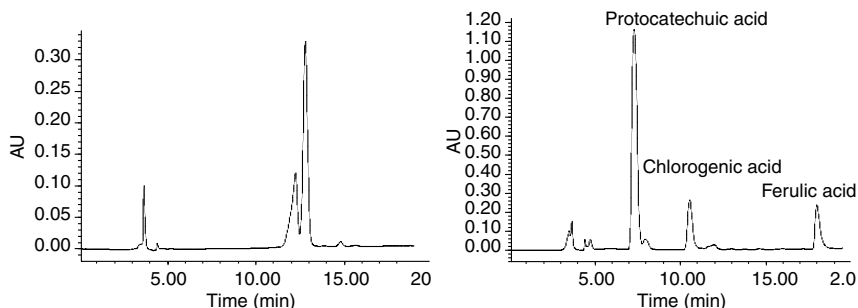
Figure 3.2 HPLC chromatograms of phenolic acids in raspberry pomace before and after acid hydrolysis.

which were the two peaks between 12 and 14 min in the original pomace, were degraded after the acid hydrolysis. Some phenolic acids are also not stable in strong acidic conditions, especially hydroxycinnamic acids. *o*-Coumaric acid and sinapic acid were degraded by 15 and 95% during acid hydrolysis (Krygier et al., 1982). Thus, different acid hydrolysis conditions could give different phenolic acid contents and profiles in the same sample. In addition to acid hydrolysis, alkaline solutions can be used to hydrolyze bound phenolic acids that have ester bonds to other large compounds (Rommel and Wrolstad, 1993; Maillard and Berset, 1995; Kroon et al., 1997; Beveridge et al., 2000). With an alkaline solution treatment, bound ferulic acid with phytosterols in rice bran oil was broken down to be free ferulic acid (Xu and Godber, 1999). Also, amylase and cellulase are two common enzymes used in enzymatic hydrolysis to break down the linkages of bound phenolic acids and sugar moieties or fibers. Enzymatic hydrolysis is generally employed in grains and legumes which are rich in various fibers. The combination of different enzymes in hydrolysis could provide better release rates of bound phenolic acids (Yu et al., 2001).

Generally, extraction is carried out at warm temperatures to increase the recovery of phenolic acids. The incubation temperature should not exceed the boiling point of the extraction solvent. Incubation time is variable from 1 hour to overnight and depends on the characteristics of the sample. During incubation, the extraction solution should be agitated frequently to increase contact between the solvent and sample. After extraction, the next step is to separate the extraction solvent from the sample solid residue. Either a centrifugation or filtration method can be applied in this step. The method selection usually depends on the

quantity of extraction solvent and the number of samples. Centrifugation is an efficient way to handle a dozen samples with a small amount of extraction solvent. After centrifugation or filtration, the extraction solvent is transferred out. The residue needs to be extracted again using the same solvent to increase the recovery. Generally, the residue is extracted up to two times after the first extraction. After the extraction solvents are combined, they are evaporated to obtain a dry extract. A small amount of solvent, can be evaporated using nitrogen flow. With a large amount of solvent, a rotary evaporator under vacuum method is the best way to dry the extract.

Recently, a series of novel technologies have been applied to assist in extraction to increase phenolic acid recovery. For example, ultrasound has been found to be an effective method to break down sample matrix to release free-form compounds, especially for samples containing a large amount of lipid. The power of ultrasound dynamically forces extraction solvent or solution to penetrate the interior of the sample matrix and increase its contact with phenolic acids. Ultrasound-assisted solvent extraction can increase extraction recovery with decreased or no acid or alkaline hydrolysis (Sun et al., 2006; Yue et al., 2006; Xu, 2008). Thus, it prevents the degradation of phenolic acids that may be caused by strong acid or alkaline hydrolysis during extraction. Ultrasound-assisted extraction was developed for extracting phenolic acids from various culinary herbs (Kivilompolo and Hyötyläinen, 2007). Compared with traditional solvent extraction methods, the ultrasound-assisted extraction with water and alcohol mixture significantly increased the recovery of phenolic acids in the herbs. The extraction time and temperature are two critical factors affecting the overall recovery. Higher temperatures may result in the degradation of some phenolic acids. *p*-Coumaric acid, ferulic acid, and *p*-hydroxybenzoic acid in Satsuma mandarin peels were reported to decrease approximately 50% when the extraction temperature increased to 40 °C, although the overall extraction yields using the ultrasound-assisted extraction method at a lower temperature were higher than the traditional solvent extraction method (Ma et al., 2008). Actually, the degradation may not directly result from the temperature and power of ultrasound, but from enzymatic degradation reactions, which were accelerated by ultrasound during the extraction.

Microwave-assisted solvent extraction is another method that uses intensive wave energy to dispense extraction solution to the inner core

of sample matrix. However, the extraction solution can reach much higher temperatures under microwave energy than the solvent boiling point – for example, over $100\,°C$. A special microwave device has been designed to precisely program microwave energy and extraction temperature during the process. Also, the microwave-assisted solvent extraction vessel can withstand the high pressure that is accumulated by solvent vapor. The vessel is made of high-density polymer material and has a thermocouple, which can be connected to the microwave energy controller. The biggest advantage of microwave-assisted extraction is that it can rapidly increase the sample's inner and outer temperatures at the same time, which helps the extraction solvent to reach the interior of the sample matrix. Solvent used in microwave-assisted extraction must be dielectric. Neither hexane nor chloroform is a dielectric solvent and cannot be used in microwave-assisted extraction. Methanol and ethanol are excellent solvents after mixing, with or without water. This extraction method successfully improved the extraction yield of phenolics in oat and wheat brans (Qufnac et al., 2007; Zigoneanu et al., 2008). The extraction temperature and time need to be optimized to achieve the best yield for different types of samples, because relatively higher extraction temperatures could cause degradation of phenolic acids. Although the thermal stabilities of some phenolic acids were reported to be relatively stable up to $100\,°C$ (Liazid et al., 2007), the composition of the solvent used in the extraction can significantly affect phenolic acid stability.

Supercritical fluid extraction has been applied to extract phenolic acids from a variety of plant samples. It uses high-pressure to force carbon dioxide to be a mixture of liquid and gas phases, which is called a supercritical fluid. The liquid and gas phase mixture of carbon dioxide can more readily permeate the sample matrix than only the gas phase of carbon dioxide. The compounds solublized in the liquid phase of carbon dioxide are extracted from the sample matrix and collected after they elute from the outlet of the system. The biggest advantage of supercritical fluid extraction is that there is no, or less, organic solvent involved in the extraction, due to the use of carbon dioxide supercritical fluid as the major solvent.. The carbon dioxide readily evaporates as gas phase at the system outlet. Thus, unlike other solvent extraction methods, there is no evaporation step for the extraction, making this an environmentally friendly method. However, the system is much more expensive and delicate than the other novel technology extraction

methods mentioned previously. The supercritical fluid extraction system is suitable for extracting fewer polar compounds in plant tissues (Xu and Godber, 2000). As most phenolic acids are relatively polar compounds, they are not readily solubilized and extracted by supercritical fluid carbon dioxide. A small amount of solvent, such as methanol or ethanol, is needed as a co-solvent mixed with the supercritical fluid carbon dioxide in the extraction. The co-solvent can increase the polarity of supercritical fluid carbon dioxide to solubilize the phenolic acids in the extraction sample. Several studies compared the yields of supercritical fluid extraction and the traditional method (Braga et al., 2008; Piantino et al., 2008). The polarity of supercritical fluid can be modified by adding different amounts of alcohol, which means that the chemical class of the extracted compounds can be controlled or selected by using different polarities of fluid. For example, the ratio of methanol mixed with carbon dioxide supercritical fluid could be optimized with fluid flow rate, temperature, and pressure conditions to selectively extract phenolic acids with fewer other compounds.

Pressurized fluid extraction is another technology that applies high pressure extraction solution in the sample matrix to perform extraction. The solution is water or water mixed with different polar solvents, and its extraction pressure is lower than 1 atm. The extraction solvent and sample can be heated to 200 °C to weaken the sample matrix and allow solvent to penetrate. Compared to traditional extraction methods, pressurized fluid extraction has short extraction time and requires less solvent. As with other extraction methods, extraction temperature and pressure and solvent composition are very critical to the phenolic acid extraction yield (Palma et al., 2001, 2002; Mukhopadhyay et al., 2006).

As mentioned above, due to the complexity of plant organic compounds, not only are phenolic acids present in the extract, but also other organics, andsome of the contaminated compounds could seriously interfere with phenolic acid analysis. A cleanup step is necessary before the phenolic acid analysis using HPLC or GC. A solid-phase cartridge packed with C18 is widely used in the cleanup step. Solid-phase C18 packing material has a higher affinity for relatively lower polarity compounds than high polarity compounds. Compared to polar compounds –, such as sugars, carbohydrates, amino acids, and peptides –, phenolic acids are low in polarity and selectively absorbed by the C18 packing material. Thus, those polar interfering compounds are excluded by the cartridge and separated from the phenolic acids fraction. Water or

water mixed with 10 or 20% methanol is used to dissolve the crude extract and elute polar contaminating compounds from the cartridge. The absorbed phenolic acids are then desorbed and flushed out from the cartridge using a less polar solvent, such as ethyl acetate or acetone. The eluate is collected and evaporated to obtain a clean extract (Degenhardt et al., 2000; Marinez-Ortega et al., 2004; Svedström et al., 2006). If using a mass spectrometer (MS) as the HPLC detector, this cleanup step is required to remove significant amounts of carbohydrates and proteins from the extract, because these large polar molecules can seriously affect the function of the ionization source of the MS detector. Without the cleanup step, those compounds in the extract result in high noise and cross-contamination, and decrease the detector sensitivity in determining the phenolic acid content.

In general, the sample preparation and extraction steps of phenolic acid analysis are very critical to the final result. Solvent or solution composition, extraction temperature, extraction technology, acid, alkaline, or enzymatic hydrolysis, extraction time, and cleanup conditions are all factors that affect the recovery and profile of phenolic acids. Poor sample preparation and extraction result in unreliable outcomes, regardless of the precision of the chromatography quantification method. Table 3.1 lists various sample extraction methods for different types of samples.

3.4   HPLC Column in Phenolic Acid Analysis

Although many new technologies have been added to the HPLC system since it was invented in the early 1960s, the traditional reversed-phase C18 column is satisfactory for separating major phenolic acids with an optimized isocratic or gradient mobile phase program. As C18 is a nonpolar packing material and the molecular weights of phenolic acids are not very different, the retention time of each phenolic acid is largely dependent on its polarity. Higher polarity phenolic acids have shorter retention times in the reversed-phase C18 column. For example, Figure 3.3 is a group of phenolic acids separated by a C18 column. Based on their retention time, the order of polarity of these acids from high to low is gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and cinnamic acid. The typical size of an analytical C18 column for phenolic acid analysis is 25 cm length

Table 3.1 Extraction methods of phenolic acids from different samples.

Method	Sample	Reference
(1) Without extraction		
Direct use	wine	Roggero et al., 1997
Filtration	wine; grape; juice	López et al., 2001; Spanos and Wrolstad, 1990; Benassi and Cecchi, 1998
Evaporation; filtration	wine	Bétes-Saura et al., 1996
(2) Solvent extraction without hydrolysis		
80% Methanol	prunes	Donovan and Waterhouse, 1998
80% Methanol containing 2 mM NaF	peaches; plums	Tomas-Barberan et al., 2001
Acetone and methanol	mango	Manthey and Perkins-Veazie, 2009
Diethyl ether and ethyl acetate	fruit juice	Bengoechea et al., 1995; Fernández de Simón et al., 1992.
NaCl saturation; diethyl ether	wine	Guillén et al., 1996
Ethyl acetate	berries, olive; olive oil	Azar et al., 1987; Määttä-Riihinen et al., 2004; Medina et al., 1998.
Different polar or nonpolar solvents - comparison of the extraction recovery	olive oil	Montedoro et al., 1992
Hexane, ethyl acetate, dichloromethane, methanol	spicy herbs	Geronthanasis et al., 1998
(3) Acid condition or hydrolysis		
Methanol/water/acetic acid (90:1.5:8.5; v/v/v) containing 2 g/L of butylated hydroxyanisole	Black currant; berries	Tabart et al., 2011
2 M HCl at 100 °C for 1 h; ethyl acetate	sorghum	Svensson et al., 2010
Methanol/water/acetic acid/butylated hydroxyl-toluene (85:15:0.5:0.2, v/v/v/w) room temperature for 4 h	bean flour	Xu and Chang, 2009

(Continued)

Table 3.1 (*Continued*)

Procedure	Sample	Reference
Methanol acidified with trifluoroacetic acid 0.05% mixed with water/acetone (40:60, v/v) acidified with trifluoroacetic acid 0.05% in variable proportions (from 0:100 to 30:70, v/v)	grape	Mané et al., 2007 Optimization of simultaneous flavanol, phenolic acid, and anthocyanin extraction from grapes using an experimental design: application to the characterization of champagne grape varieties
Adjusting to pH 2; ethyl acetate	apple juice	Delage et al., 1991
NaCl Saturation; acidified methanol at pH 2; ethyl acetate	wine	Woodring et al., 1990
Adjusting to pH 2 with 0.1 N HCl; diethyl ether; methanol	wine	Rodriguez-Delgado et al., 2001
1.2 N HCl/methanol (1:1) at 35 °C overnight	berries	Törrönen et al., 1997; Lamuela-Raventós et al., 1994
50% aqueous methanol or HCl in 50% methanol	vegetables	Hertog et al., 1992a; 1992b
10% H_2SO_4 at boiling temperature for 1h.	bee pollen	Negri et al., 2011
(4) Alkaline hydrolysis		
Alkali treatment (2 M NaOH) for 1h; neutralized and acidified with 6 M HCl; ethyl acetate	rice husk; barley	Butsat et al., 2009; Holtekjølen et al., 2006
1.5 N methanolic KOH at 70 °C for 1 h	corn fiber	Yadav et al., 2007
95% ethanol; alkaline hydrolysis; acidified to pH 3	flaxseed flour	Johnsson et al., 2000
2 N NaOH for 4h; neutralized with HCl to pH 1; ethyl acetate	barley and malt	Maillard and Berset, 1995
2 N NaOH for 2h; neutralized with acid; ethyl acetate	oranges; grapefruits	Peleg et al., 1991

| Procedure	Sample	Reference
2 N NaOH for 4 h; neutralized with H_3PO_4; ethyl acetate	orange juice	Rouseff et al., 1992
2 N NaOH for 48 or 62 h; acidified to pH 3.4; ethyl acetate	grapes; cherry juice	Shahrzad and Bitsch, 1996
1 N NaOH at 37 °C; neutralization; ethyl acetate	wheat bran	Kroon et al., 1997
1 N NaOH for 18 h or 4 N NaOH for 2 h; neutralization; ethyl acetate	rice; wheat; rye; barley straws	Sun et al., 2001
Methanol at 80 °C for 1 h and 2 N NaOH for 4 h	coffee	Andrade et al., 1997; Trugo et al., 1991.
(5) Combination of acid and alkaline hydrolysis		
3% H_2SO_4, 15 min, 130 °C; 4–12% NaOH at 50–130 °C during 30–120 min	trimming vine shoots	Max et al., 2010
Methanol containing 2 g/L of butylated hydroxyanisole and 10% acetic acid (85:15) for soluble phenolic acids; 10 M NaOH at 20 °C overnight; acidified to pH 2 with HCl; diethyl ether and ethyl acetate (1:1).	fruits	Mattila et al., 2006
10N NaOH for 16h/6N HCl at 85 °C for 30 min; 1 N HCl at 100 °C for 15 min/1 N NaOH at 100 °C for 15 min	vegetables; fruits; herbs	Mattila and Kumpulainen, 2002; Luczkiewicz et al., 2001
(6) Enzymatic analysis		
0.2 N H_2SO_4 for 1 h at 100 °C; neutralized to pH 6; alpha-amylase at 30 °C for 60 min	grain	Zupfer et al., 1998
Amylase hydrolysis; 2 M NaOH saponification for 1 h at 25 °C; acidified to pH 2; ethyl acetate extraction	rye	Andreason et al., 2000
(7) Solid-phase cartridge cleanup		
Methanol; C18 cartridge cleanup	peaches	Lee et al., 1990

(Continued)

Table 3.1 (*Continued*)

Procedure	Sample	Reference
Methanol; C18 cartridge cleanup; quaternary amine cartridge cleanup	Chinese medicine herb	Glowniak et al., 1996; Zgorka and Glowniak, 2001; Zgorka and Dawka, 2001
Ethyl acetate/ethyl ether at pH 2.0; C18 or PVPP (polyvivylpolypyrroidone) cartridge cleanup	grapes	Oszmianski et al., 1988; Jaworsk and Lee, 1987
Na_2CO_3 at pH 8.3; acidified with 6 N HCl to pH 2; phenyl cartridge cleanup	olive oil	Cartoni et al., 2000
Acidified with acetic acid; C18 cartridge cleanup	beer	Lunte et al., 1988
Filtration; C18 and SAX cartridge cleanup	wine	Cuillén et al., 1996
Acidified with 0.1 N HCl; C18 cartridge cleanup	fruit juices	Amakura et al., 2000; Schieber et al., 2001
85% methanol; acidified to pH; C18 cartridge cleanup	vegetables	Ping et al., 1993
(8) Assisted solvent extraction		
Pressurized liquid extraction with 65% methanol	carrots; potatoes	Søltoft et al., 2010
Pressurized liquid extraction with methanol, ethanol, acetone, and aqueous methanol	eggplant	Luthria and Mukhopadhyay, 2006
Sonication with methanol:water:TFA (100:100:2) for 30 min	medicine herb	Wen et al., 2005
Pressurized liquid extraction with methanol at different temperatures	grape; pomace	Ortuno et al., 1995
Sonication with methanol or 80% methanol	grape; pomace; apples; pears	Escarpa and González, 2001
Supercritical CO2 extraction at 60 °C and 40 MPa with methanol	Brazilian herb	Piantino et al., 2008
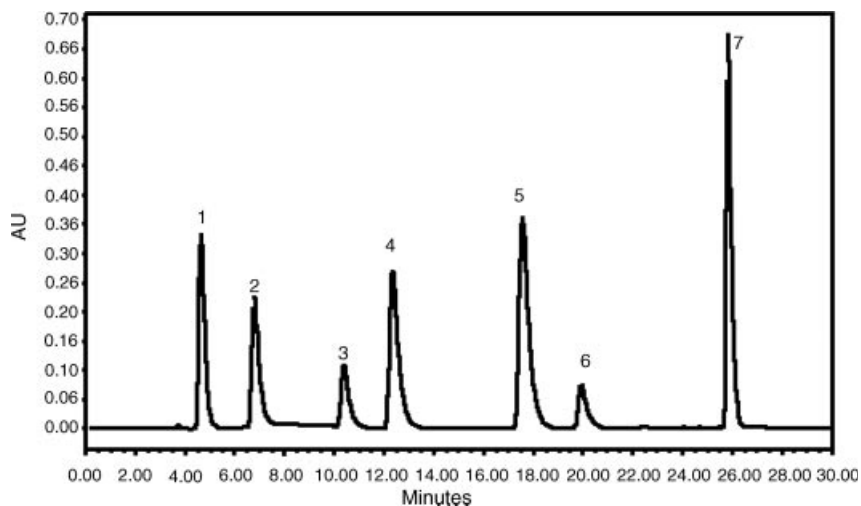
Figure 3.3 Chromatogram of six common phenolic acids and cinnamic acid using C18 reversed-phase HPLC method. 1. Gallic acid; 2. Protocatechuic acid; 3. Chlorogenic acid; 4. Caffeic acid; 5. *p*-Coumaric acid; 6. Ferulic acid; 7. Cinnamic acid.

and 4.6 mm inside diameter, with 5 μm of C18 packing material. Some analysis methods use a relatively smaller size, such as 10 cm length and 3.9 mm inside diameter. Also, a guard column is applied to extend the lifetime of the analytical column. The column separation is carried out at ambient temperature. For direct sample injection, a column temperature maintained at 40 °C is recommended if the sample contains a high level of sugar. Although the total running time with the typical C18 column to separate phenolic acid standards mixture is less than 30 min, it could take up to 45 min to achieve a satisfactory separation and to prevent interferences from other compounds for most sample extracts.

Recently, the ultra-pressure liquid chromatography (UPLC) system has been used in analyzing phenolic acids. Fast flow rate controlled under high pressure significantly increases the separation efficiency and decreases the retention time. The UPLC running time can be 5 times shorter than the normal HPLC method (Spáčila et al., 2008). In addition to fast analysis time, the advantages of UPLC in analysis of phenolic acids are less solvent and smaller sample size. The UPLC method is suitable for dealing with a large number of samples. The column of UPLC is much smaller than the traditional HPLC column and its C18 packing material particle size is smaller than 2.5 μm. Due to the requirement of minimizing the amount of mobile phase for an LC-MS

detector, a small C18 column is used in LC-MS systems. This also provides good separation at a lower flow rate.

3.5   HPLC Mobile Phase in Phenolic Acid Analysis

As the reversed-phase HPLC column is broadly used in phenolic acid analysis, the mobile phase should be strong in ion strength. It usually consists of water, methanol, acetic acid, formic acid, acetonitrile, etc. For the traditional HPLC system, flow rate of mobile phase is between 0.8 - 1.5 ml/min and largely relies on the viscosity and running pressure due to the different compositions of the mobile phase. The mobile phase can run in isocratic or gradient mode to achieve high resolution for each phenolic acid. Table 3.2 shows several successful examples of mobile phase composition, flow rate, and running mode in separating different types of phenolic acids using a C18 column.

From Table 3.2, it can be found that most mobile phases are acidified with acetic acid, formic acid, or phosphoric acid. The pH of the mobile phase is 2 to 4 during running. Although 2-propanol is used as a mobile phase modifier, the amount added should be below 2% due to the high viscosity of 2-propanol, which can lead to high pressure in the system. Inorganic mobile phase modifiers, and ammonium and phosphate salts, are normally used in the HPLC method with an electrochemical detector to increase the detector sensitivity. Also, formic acid is better than acetic acid to be used as a mobile phase modifier in an HPLC system with an MS detector.

3.6   HPLC Detector in Phenolic Acid Analysis

Due to the aromatic ring with hydroxyl and carboxyl groups, colorless phenolic acids have stronger UV absorbance. They present a maximum absorption intensity at a range of 240 to 330 nm. In general, the phenolic acids of benzoic acid derivative have maximum UV absorption between 240 to 280 nm. The phenolic acids of cinnamic acid derivative show high peaks in the UV spectrum between 300 and 330 nm. Figure 3.4 displays several examples of UV spectra of the phenolic acids separated in Figure 3.3. The strong UV absorbance of phenolic acid simplifies the selection of an HPLC detector. A common UV detector is sensitive

Table 3.2 Different reversed-phase HPLC mobile phase gradients used in phenolic acid separation.

Mobile phase	Flow rate (mL/min)	Running mode	Separated phenolic acids	Reference
Methanol/4% acetic acid (27:73, v/v)	0.8	Isocratic	gallic acid; protocatechuic acid; chlorogenic acid; gentisic acid; caffeic acid; syringic acid; sinapic acid; *p*-courmaric acid	Ping et al., 1993
Methanol/acetic acid/water (25:1:75, v/v/v)	1.0	Isocratic	protocatechuic acid; *p*-hydroxybenzoic acid; chlorogenic acid; gentisic acid; syringic acid; caffeic acid; vanillic acid; *p*-coumaric acid; ferulic acid	Zgorka and Glowniak, 2001
KH₂PO₄ (pH 3.0)/acetonitrile (90:10)	1.0	Isocratic	gallic acid; protocatechuic acid; gentisic acid; chlorogenic acid; hydroxybenzoic acid; syringic acid; vanillic acid; caffeic acid; *p*-coumaric acid; ferulic acid; salicylic acid	Jirovský et al., 2003
2-Propanol/acetic acid/ methanol/0.018 M ammonium acetate (2:2:8.7:87.3, v/v/v/v)		Isocratic	gallic acid; gentisic acid; vanillic acid; caffeic acid; salicylic acid; *p*-coumaric acid; ferulic acid	Woodring et al., 1990

(Continued)

Table 3.2 (*Continued*)

Mobile phase	Flow rate (mL/min)	Running mode	Separated phenolic acids	Reference
Methanol/4% acetic acid (27:73, v/v)	0.8	Isocratic	gallic acid; protocatechuic acid; chlorogenic acid; gentisic acid; caffeic acid; syringic acid; sinapic acid; p-coumaric acid	Ping et al., 1993
Methanol/acetic acid/water (25:1:75, v/v/v)	1.0	Isocratic	protocatechuic acid; p-hydroxybenzoic acid; chlorogenic acid; gentisic acid; syringic acid; caffeic acid; vanillic acid; p-coumaric acid; ferulic acid	Zgorka and Glowniak, 2001
KH₂PO₄ (pH 3.0)/acetonitrile (90:10)	1.0	Isocratic	gallic acid; protocatechuic acid; gentisic acid; chlorogenic acid; hydroxybenzoic acid; syringic acid; vanillic acid; caffeic acid; p-coumaric acid; ferulic acid; salicylic acid	Jirovský et al., 2003
2-Propanol/acetic acid/ methanol/0.018 M ammonium acetate (2:2:8.7:87.3, v/v/v/v)		Isocratic	gallic acid; gentisic acid; vanillic acid; caffeic acid; salicylic acid; p-coumaric acid; ferulic acid	Woodring et al., 1990

Mobile phase	Flow rate	Gradient	Compounds	Reference
4% (v/v) THF in ACN and 0.4% (v/v) phosphoric acid in water (35:65)	1.0	Isocratic	chlorogenic acid; caffeic acid; rosmarinic acid	Haghi and Hatami, 2010
A: water containing 0.02% TFA; B: methanol containing 0.02% TFA.	0.5	0 min, 25% B; 5 min, 25% B; 10 min, 30% B; 16 min, 45% B; 18 min, 45% B; 25 min, 80% B; 30 min, 80% B; 40 min, 25% B	gallic acid, chlorogenic acid, gentsic acid, vanillic acid, caffeic acid, syringic acid, sinapic acid, p-coumaric acid, ferulic acid, anisic acid, rosmarinic acid, salicylic acid, and cinnamic acid	Wen et al., 2005
A: acidified water (pH 2.64); B: acidified water/ acetonitrile (20:80, v/v)	1.0	0 min, 100% A; 30 min, 70% A	gallic acid, cinnamic acid, chlorogenic acid, caffeic acid, p-coumaric acid, and ferulic acid	Bétes-Saura et al., 1996
A: formic acid/water (5:95 v/v); B: methanol	1.0	0 min, 10% B; 20 min, 24% B; 32 min, 32% B; 47 min, 45% B; 53 min, 50% B; 54 min, 80% B; 58 min, 80% B; 60 min, 10% B	gallic acid; protocatechuic acid; p-hydroxybenzoic acid; vanillic acid; caffeic acid; syringic acid; p-coumaric acid; ferulic acid; sinapic acid	Donnerb et al., 1997
A: 2% acetic acid B: methanol/acetic acid/water (20:2:68, v/v/v)	0.3 – 0.8	0 min, 100% A 2 min, 100% A; 10 min, 60% A; 15 min, 50% A; 20 min, 40% A; 30 min, 30% A; 40 min, 25% A; 45 min, 15% A; 60 min, 15% A. 65 min 100% A	p-hydroxybenzoic acid; gallic acid; protocatechuic acid; vanillic acid; syringic acid; caffeic acid; ferulic acid; sinapic acid	Fernández de Simón et al., 1992
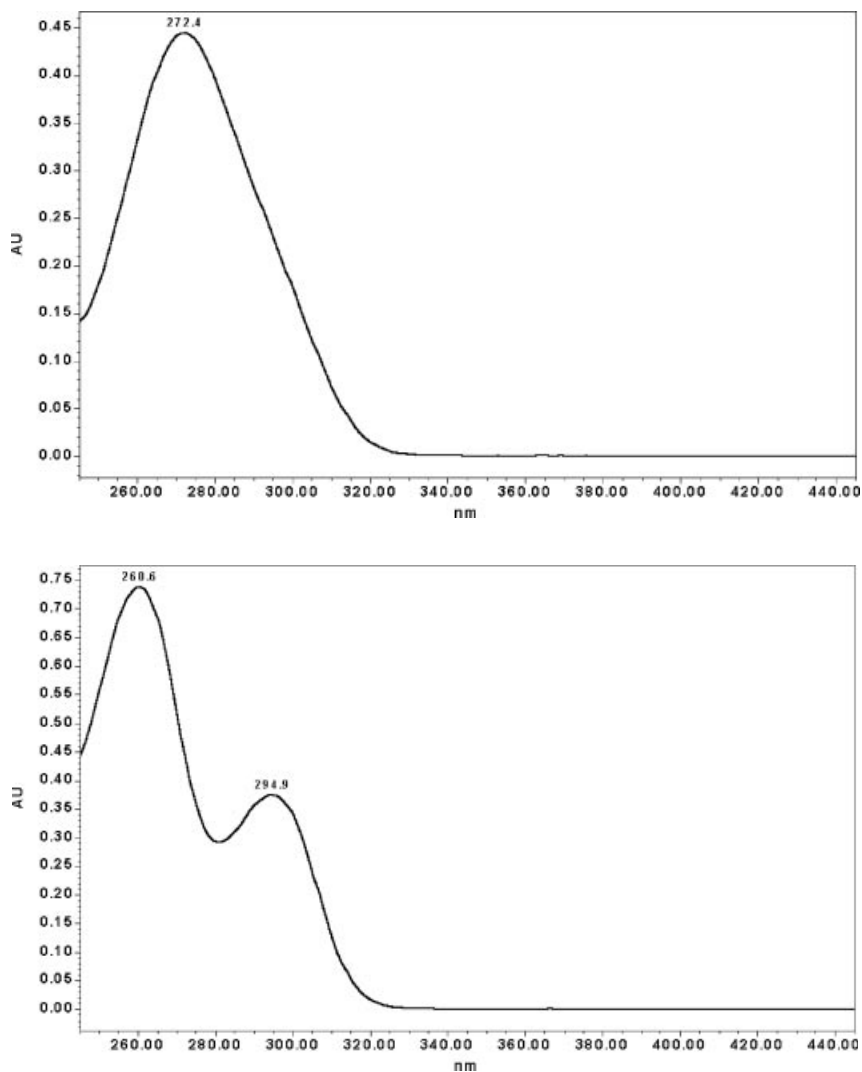
Figure 3.4 UV spectra of common phenolic acids (top: gallic acid; bottom: proto-catechuic acid).

enough in most phenolic acid analyses. However, different phenolic acids have different UV wavelengths for the maximum absorbance. As a fixed wavelength UV detector can only be set at one or two wavelengths to monitor the chromatography elution, it is limited in determining all phenolic acids in an extract simultaneously in a single HPLC run. Another HPLC run has to be started after the UV detector wavelength is
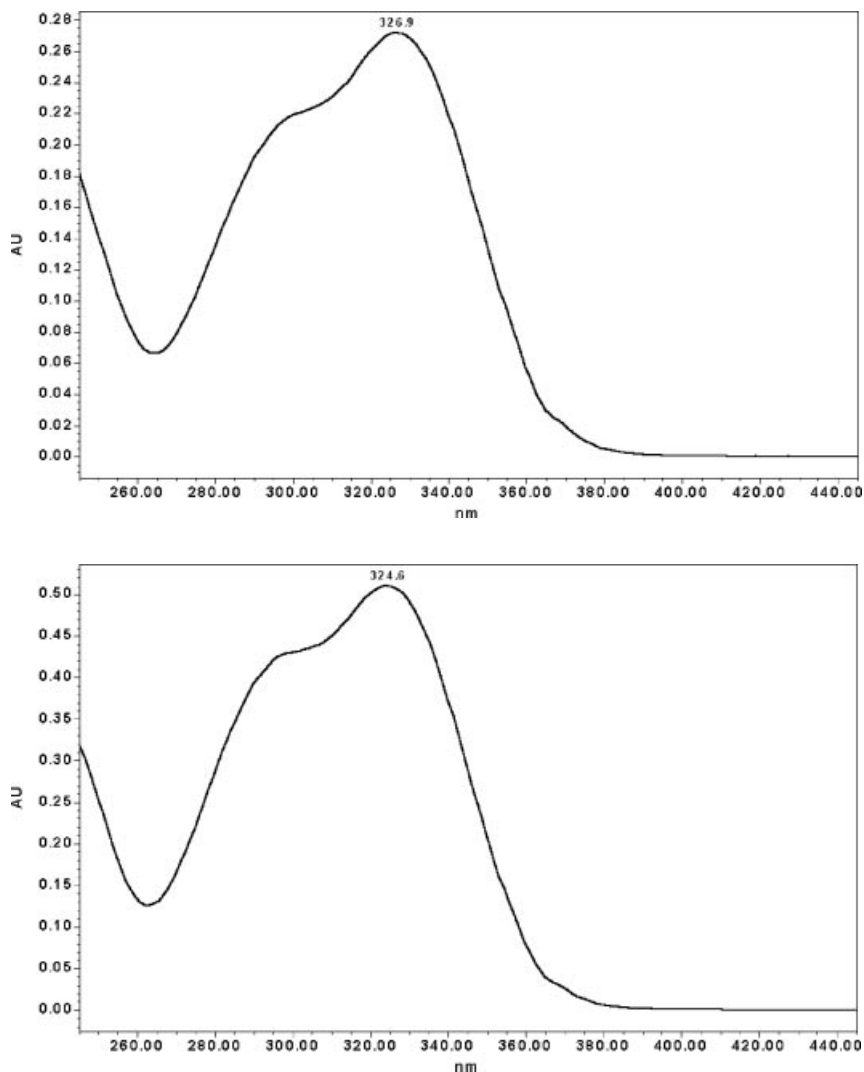
Figure 3.4 (*Continued*) (top: chlorogenic acid; bottom: caffeic acid).

changed to monitor the phenolic acids that were missed under the previous UV setting. This disadvantage has been overcome by a photodiode array detector, which is able to collect all absorbance data at a range of wavelengths from UV to the visible region in a single run. A specific chromatogram for each phenolic acid can be extracted by selecting the wavelength that, matches the maximum absorbance of
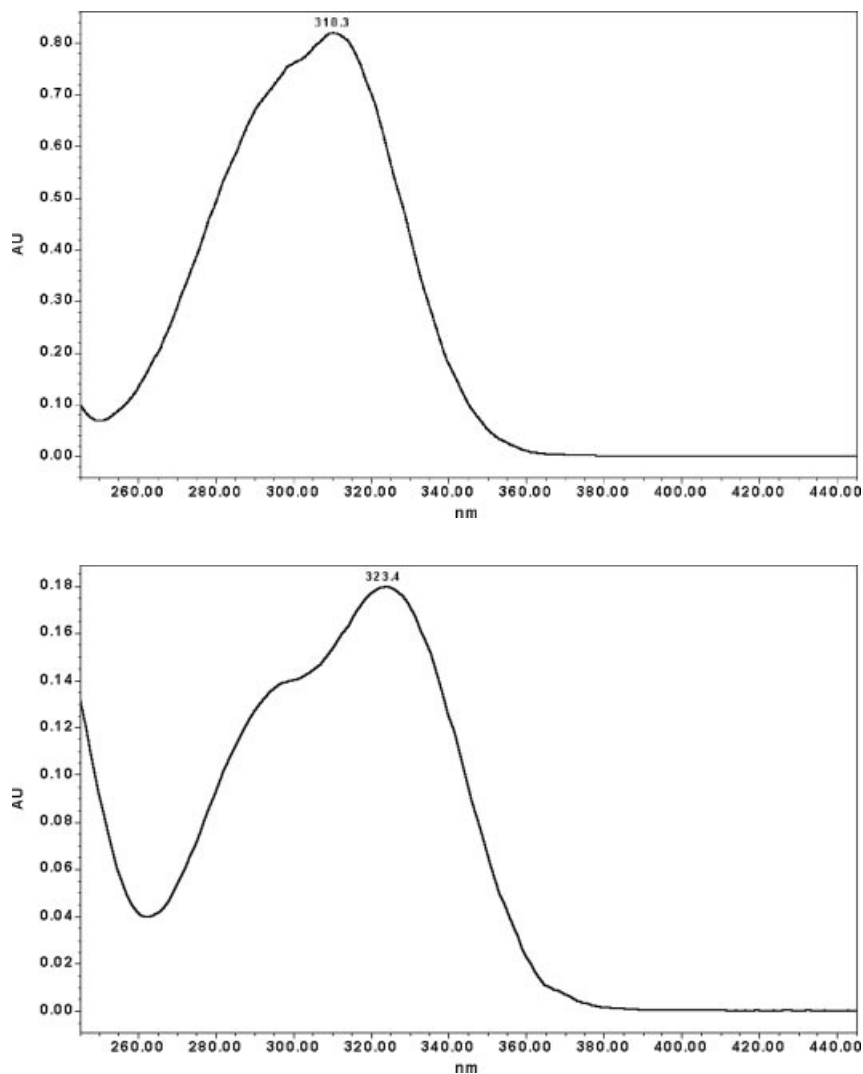
Figure 3.4 (*Continued*) (top: *p*-Coumaric acid; bottom: Ferulic acid).

the phenolic acid during or after running. Also, the spectrum of the phenolic acid at a range of UV wavelengths can be used to confirm the purity of the phenolic acid through comparison with the spectrum of its pure standard. Thus, different phenolic acids can be determined simultaneously with an HPLC system equipped with a photodiode array detector.

In addition to a UV detector, others –, such as fluorescence, electrochemical, and mass spectrometry detectors – are very sensitive in analyzing phenolic acids. Fluorescence detectors have higher sensitivity and selectivity than UV detectors. The high selectivity provides an advantage to fluorescence detectors, eliminating possible interfering compounds that were not completely separated from the phenolic acids, and gives UV responses when using a UV detector. For example, most flavanones in plant tissues have a UV absorption as strong as phenolic acids, but no fluorescence responses. Several studies successfully applied fluorescence detectors in fruit phenolic acid analysis (Torres et al., 1987; Rouseff et al., 1992; Zgorka and Dawka, 2001). Interferences are significantly limited by using a fluorescence detector in HPLC analysis. However, the combination of fluorescence excitation and emission wavelengths of each individual phenolic acid to obtain high sensitivity can vary. Generally, the excitation wavelength for phenolic acids is in a range of 310–350 nm, and the emission wavelength is between 420 and 500 nm. It is possible to overlook other phenolic acids which may not have responses if only one or two combinations of excitation and emission wavelengths are used in a single HPLC run. Thus, HPLC with a fluorescence detector is suitable for monitoring one or two specific phenolic acids which may have been difficult to totally separate from other compounds in the HPLC method.

Electrochemical detectors can only be used in reversed-phase HPLC systems with, for example, formic acid or phosphorus acid in the mobile phase. That condition meets the requirements of most mobile phase compositions used in analyzing phenolic acids. Electrochemical detectors are also very sensitive to compounds having redox reducing-oxidation capability and provide response after the voltage change when a compound flows through the cell applied with a consistent voltage (Hayes et al., 1987a, 1987b; Guo et al., 1997; Nardini and Ghiselli, 2004; Jandera et al., 2005). The disadvantage of using an electrochemical detector is that some sugars in the extract could interfere in the analysis if they are not cleaned out in the sample preparation, or if the HPLC running condition is not optimized to separate the sugars from phenolic acids in the chromatogram. Also, as it is a universal detector, any other existing compounds in the extract could give responses and may overlap with phenolic acids in the chromatogram if the separation resolution is not adequate. Thus, although the electrochemical detector is very sensitive to phenolic

acids analysis, its requirement for separation resolution is much more rigorous than a UV or fluorescence detector.

Recently, the HPLC system with a mass spectrometric detector has been widely used in identifying chemical structures of phenolic acids (Gioacchin et al., 1996; Bianco et al., 2001; Määttä-Riihinen et al., 2003; Yang et al., 2007; Wu et al., 2007; Lin and Harnly, 2008). However, compared to the traditional HPLC system, the HPLC-MS system is not only expensive and delicate, but is also costly to run and maintain. Common HPLC-MS detectors use either an electrospray ionization (ESI), or an atmospheric pressure ionization method to break down molecules to parent and/or fragment ions. The ionization source is easily contaminated if the sample is not well cleaned. The mobile phase of HPLC-MS should consist of acid modifiers and a lower concentration of salt to improve its ionization efficiency. A group of phenolic acids in cereals was determined using HPLC-ESI-MS with acetic acid and acetonitrile or methanol mobile phase (Gioacchin et al., 1996; Ayaz et al., 2005; Sánchez-Patán et al., 2011). Also, phenolic acids in vegetable oil were identified using HPLC-API-MS/MS (Bianco et al., 2001). The MS/MS spectrum is very useful in revealing the chemical structures of phenolic acid isomers.

3.7   Gas Chromatography in Phenolic Acid Analysis

Gas chromatography systems are not widely used to analyze phenolic acids. The hydroxyl and carboxyl groups of phenolic acids provide very strong inter- and intra-molecule hydrogen forces to prevent against vaporization at the temperature of the GC injection port. Thus, a chemical derivatization is required to modify hydroxyl and carboxyl groups to be ester groups, which lower the inter- and intra-molecule affinity force before the GC analysis. The derivatives have lower boiling points and readily become volatile forms in the GC injection port. Diazomethane and methyl chloroformate are the derivative agents used to modify the carboxyl group to the methyl ester group (Husek, 1992; Waksmundzka-Hajnos, 1998). However, the derivatized phenolic acid with methyl ester groups are difficult to distinguish from the phenols naturally containing methyl ester groups in the original samples. It could lead to overestimated phenolic acid concentrations in the original sample.

There are also several other derivative reagents that convert hydroxyl and carboxyl groups into trimethylsilyl groups, which are not present in natural plant samples. The commercially available reagents include *N,O-bis*-(trimethylsilyl)-trifluoroacematide (BSTFA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and *N,O-bis*-(trimethylsilyl)acetamide (BSA) (Husek, 1992; Little, 1999; Ng et al., 2000; Fiamegos et al., 2004; Zhang and Zuo, 2004). As moisture in the sample could reduce the derivatization reaction yield, it should be completely removed before the reaction (Chu et al., 2001). Due to the unpredictable and inconsistent derivatization reaction yield, an internal standard has to be applied as a reference during the sample preparation.

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Chapter 4

Analysis Methods of Carotenoids

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Abstract

This chapter discusses methods of carotenoid analysis, including sample preparation, UV/Visible spectroscopy, and high-performance liquid chromatography with a photodiode array detector (HPLC-PDA), and separation of optical isomers. Additionally, this chapter focuses on methodologies of the last 10 years including separation of *cis*/*trans* geometrical isomers, liquid chromatography–tandem nuclear magnetic resonance (LC-NMR), liquid chromatography–mass spectrometry (LC-MS), separation and identification of carotenoid metabolites, use of stable isotopes of carotenoids, and Raman resonance spectroscopy. This current and comprehensive review emphasizes techniques used for both food and biological samples and focuses on the analysis of β-carotene, α-carotene, lycopene, β-cryptoxanthin, lutein, zeaxanthin, and astaxanthin.

Keywords: Carotenoids; analysis; sample preparation; UV/Visible spectroscopy; geometrical isomers; optical isomers; HPLC; mass spectrometry; nuclear magnetic resonance; metabolites.

4.1 Introduction/Background

Carotenoids are the most widely distributed pigments in nature (Schwartz et al., 2008). Carotenoids are a class of pigments ranging

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from yellow to red in color and are found ubiquitously in plants (both edible and nonedible) and photosynthetic microorganisms. Carotenoids are responsible for the red color in tomatoes, orange in sweet potatoes, and yellow in squashes. They are 40 carbon highly unsaturated hydrocarbons derived from isoprene units and composed either entirely of carbon and hydrogen (carotenes) or carbon, hydrogen, and oxygen (xanthophylls). There are over 750 known carotenoids to date (Eugster, 1995). One function of carotenoids in plants is to aid photosynthesis, mainly by absorbing light and protecting against photosensitization (Demmig-Adams et al., 1996). Additionally, the carotenoid biosynthetic pathway also produces abscisic acid, a critical plant hormone involved in plant growth and response to environmental stress (Seo and Koshiba, 2002). Some carotenoids have provitamin A activity (including β-carotene, α-carotene, and β-cryptoxanthin; those carotenoids with a β-ionone ring) while most do not (lycopene, lutein, zeaxanthin, etc.) (Figure 4.1). Additionally, consumption of carotenoid-rich foods (including those containing carotenoids that cannot be converted to vitamin A) has been associated with a decreased risk of disease (Mayne, 1996), including cancer (Peto et al., 1981), and cardiovascular disease (Sesso and Gaziano, 2004). Carotenoids can act as antioxidants by reacting with free radicals, including singlet oxygen and peroxyl radicals (Sies et al., 1992; Sies and Stahl, 1995). Since carotenoids are highly unsaturated pigments, many possible
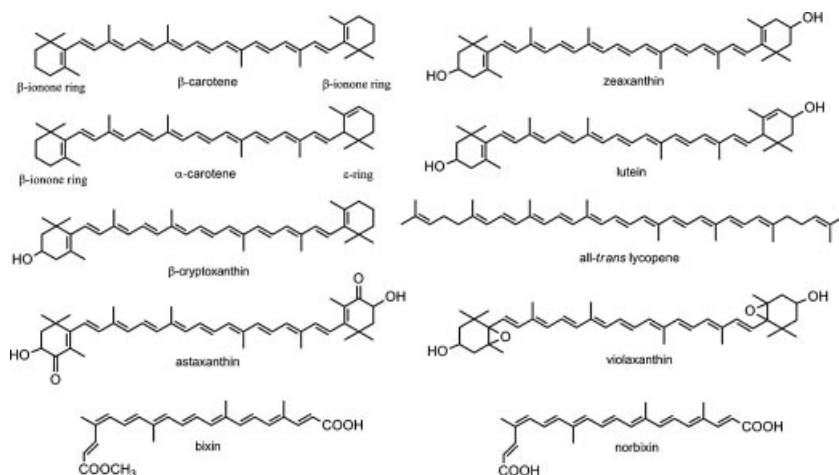
Figure 4.1 Structures of commonly occurring carotenoids in foods.

cis/*trans* isomers are possible, both which have biological implications which will be discussed further. As a result, effective quantitation of carotenoids in both foods and biological samples is necessary.

This review of methods of carotenoid analysis focuses on methodologies of the last 10 years including separation of *cis*/*trans* isomers, liquid chromatography-tandem nuclear magnetic resonance (LC-NMR), liquid chromatography–tandem mass spectrometry (LC-MS/MS) and separation, and identification of carotenoid metabolites. Additionally, methods for sample preparation, UV/visible spectroscopy, and high-performance liquid chromatography with a photodiode array detector (HPLC-PDA) are discussed. This current and comprehensive review emphasizes techniques used both for food and biological samples.

4.2 Sample Preparation

Chromatographic methods are commonly employed to separate, identify, and quantify carotenoids in food, blood, and tissue samples. Prior to analysis, carotenoids must first be extracted from the sample matrix. Due to the inherent instability of these compounds, it is important that this step minimizes isomerization, oxidation, and degradation to ensure accurate analytical results. Carotenoids are highly conjugated, making them susceptible to degradation by heat, light, oxygen, and metal ions (Philip and Francis, 1971; Scita, 1992; Henry et al., 1998). For example, β-carotene is rapidly degraded under ultraviolet and visible light in an aerobic environment (Scita, 1992). Therefore, samples should be handled under dim or red light, solvent evaporated with gas (nitrogen, argon, or helium), and stored in the dark at temperatures below –20 °C. The pH of the solutions used during extraction and analysis should also be considered, as 5,6-epoxides of cyclic carotenoids can rearrange to 5,8-epoxides in the presence of acid (epoxide–furanoid rearrangement) (Schiedt and Liaaen-Jensen, 1995). Carotenoids are generally less stable in extracts than in food or biological matrices, and therefore samples should be analyzed immediately following extraction to limit the formation of artifacts.

4.2.1 *Extraction of Carotenoids from Foods*

As carotenoids are lipophilic compounds, they are usually extracted using a mixture of organic solvents (Rodríguez-Bernaldo de Quirós and Costa, 2006). The solvents used depend on the sample matrix and the

relative polarity of the carotenoids of interest. Due to the high water content of fruits and vegetables, water-miscible solvents, such as acetone and tetrahydrofuran, are needed for complete penetration of the sample (O'Neil and Schwartz, 1992). Acetone, hexane, methanol, ethanol, ethyl ether, tetrahydrofuran, dichloroethane, and their combinations have been used to extract carotenoids from foods (Khachik et al., 1992a; Hart and Scott, 1995; Ferruzzi et al., 1998; Márkus et al., 1999; Hadden et al., 1999; Lee et al., 2001; Oliveira et al., 2003). Homogenization of the sample with the extracting solvents is preferred for complete quantitative analysis. Magnesium carbonate (Khachik et al., 1992a; Lin and Chen, 2003) or calcium carbonate (Ferruzzi et al., 1998) is often added during the extraction process to neutralize organic acids that might be present in the sample. It is generally necessary to repeat the extraction process several times until the filtrate and residue are colorless to ensure complete removal of the carotenoid pigments. During liquid–liquid extractions, carotenoids are partitioned into the organic phase, which is removed and evaporated prior to chromatographic analysis.

The solubility and stability of different carotenoids in the extracting solvent must be considered. Craft and Soares (1992) report the solubility and stability of lutein (as a representative xanthophyll) and β-carotene (as a representative hydrocarbon carotene) in various solvents over time. Polar xanthophylls, such as lutein, are more soluble in alcohols, while nonpolar carotenes, such as β-carotene, are more soluble in hydrophobic solvents. Tetrahydrofuran has been shown to be the best solvent for solubilizing a wide variety of carotenoids, but owing to its tendency to form peroxides, it is often used with antioxidants, such as butylated hydroxytoluene (BHT) (Craft and Soares, 1992). Ethers are also prone to forming peroxides, which can be removed by filtering the solvent through activated alumina (Dasler and Bauer, 1946). Mixtures of hexane and more polar solvents are effective at removing a variety of carotenoids from foods (Heinonen et al., 1989; Ferruzzi et al., 1998). Ferruzzi et al. (1998) successfully extracted carotenoids from raw and processed carrots with methanol, followed by the addition of an acetone/hexane solution (1: 1, v/v). In addition, Lee et al. (2001) used a more complex mixture of hexane, acetone, and ethanol (2:1:1, v/v) to extract carotenoids from oranges. Taungbodhitham et al. (1998) and Lin and Chen (2003) have evaluated various solvent mixtures for the extraction of carotenoids from fruits and vegetables. Both groups

obtained the highest recoveries of lycopene, β-carotene, and lutein with an ethanol/hexane mixture.

While esterified xanthophylls can be extracted using solvent mixtures similar to those used for hydrocarbon carotenes, nonesterified xanthophylls are generally extracted using more polar solvents. For example, xanthophylls have been extracted from spinach using a mixture of methanol and tetrahydrofuran (Kopas-Lane and Warthesen, 1995). Acetone alone has also been used to extract lutein and zeaxanthin from a variety of fresh and processed vegetables (Updike and Schwartz, 2003). However, for the extraction of a wide range of carotenoids, a mixture of polar and nonpolar solvents works best.

4.2.2 Extraction of Carotenoids from Biological Samples

Due to the health-promoting properties associated with the consumption of carotenoids, the analysis of these compounds in plasma, serum, and tissues has become important. The same precautions used when extracting carotenoids from foods must also be employed here to minimize isomerization, oxidation, and degradation.

Sample handing requirements and extraction techniques for blood samples have been extensively reviewed by Su et al. (2002). Carotenoid levels have been found to be equally stable in human serum and plasma during extraction (Su et al., 2002). Extraction of carotenoids from blood plasma and serum generally begins with the precipitation of protein from the sample using a polar solvent (commonly ethanol) (Khachik et al., 1997; Boileau et al., 1999; Gueguen et al., 2002; Unlu et al., 2007). Antioxidants, such as BHT, are often added to the solvent to prevent oxidative degradation (Khachik et al., 1997; Boileau et al., 1999), while ethyl β-apo-8′-carotenoate and echinenone are sometimes added as internal standards for accurate quantitative measurement (Khachik et al., 1997; Unlu et al., 2007). However, it is important that the internal standards chosen effectively mimic the behavior of the carotenoid(s) of interest (similar in structure and polarity) and can be differentiated from the carotenoid(s) of interest during analysis (Franke et al., 1993). Carotenoid extraction from plasma and serum is generally carried out using nonpolar solvents such as hexane (Khachik et al., 1997; Boileau et al., 1999; Lyan et al., 2001; Unlu et al., 2007); however, the use of other organic solvents, such as diethyl ether (Khachik et al., 1992b) and ethyl acetate (Barua, 2001), has been reported.

It has been observed that the storage of newly collected whole blood at 4 °C for 24 hours before freezing reduces carotenoids in the sample by up to 9% (Key et al., 1996); therefore, blood samples should be frozen shortly after collection to avoid any losses. Furthermore, holding blood samples above 35 °C (typical climate conditions in the tropics) can result in the degradation of these compounds (Su et al., 2002). Carotenoids other than lycopene are relatively stable in plasma under fluorescent lighting prior to extraction (Gross et al., 1995; Su et al., 1999). Following extraction in organic solvents, carotenoids have been shown to be more sensitive to light and heat (Su et al., 1999). Significant carotenoid degradation has been reported in serum samples stored at −20 °C (Mathews-Roth and Stampfer, 1984; Comstock et al., 1993) and it is recommended that biological samples be stored at temperatures below −70 °C for the long-term preservation of carotenoids (Comstock et al., 1993; Su et al., 2002). However, repeated freezing and thawing of samples should be limited as a 1.3% decrease in total plasma carotenoids per freeze–thaw cycle has been reported (Comstock et al., 2001).

Extraction of carotenoids from human and animal tissues generally involves tissue breakdown via homogenization or ultrasonication, which is sometimes followed by saponification and extraction with a nonpolar solvent (Schmitz et al., 1991; Clinton et al., 1996; Ferreira et al., 2000). Alternatively, some methods use enzymes to break down the tissue matrix before extraction with a nonpolar solvent (Nierenberg and Nann, 1992). Carotenoids were extracted from human prostate tissue by homogenizing the samples in ethanol (containing 0.1% BHT), saponifying with KOH, and extracting with hexane (Clinton et al., 1996). Ferruzzi et al. (1998) extracted hydrocarbon carotenoids (β- and α-carotene) and xanthophylls (lutein, zeaxanthin, and β-cryptoxanthin) from cervical tissue. Samples were first homogenized in a saline solution (containing collagenase and ascorbic acid), saponified with ethanolic KOH, and then extracted with hexane (containing 0.02% BHT). Nierenberg and Nann (1992) extracted human lung, breast, colon, and skin tissues with hexane, but used enzymatic digestion to break down the tissues due to the recovery losses they observed with saponification. Carotenoids have also been extracted from human and rat tissues using 2-propanol/dichloromethane (2:1, v/v) (Barua and Olson, 1998).

4.2.3   Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) with carbon dioxide (CO_2) has been used as an alternative to the previously mentioned liquid–liquid extraction methods for food samples. One of the main advantages of SFE is that it yields extracts that are free from chemical residues (Spanos et al., 1993). Where other extraction methods require large amounts of organic solvents that can be costly to purchase and dispose of, SFE is low cost, nontoxic, and environmentally friendly (Marsili and Callahan, 1993). Carbon dioxide is the most commonly used super-critical fluid, as its low critical temperature ($31\,°C$) makes it favorable for the extraction of heat-sensitive carotenoids (Vági et al., 2002).

The extraction efficiency of carotenoids with CO_2 has been shown to increase with temperature and pressure (Baysal et al., 2000; Vági et al., 2002; Nakornriab et al., 2008). Baysal et al. (2000) extracted β-carotene and lycopene from tomato waste using CO_2 and studied the effects of temperature (35, 45, 55, and $65\,°C$), pressure (200, 250, and 300 bar), co-solvent (5, 10, and 15% ethanol), extraction time (1, 2, 3 h), and CO_2 flow rate (2, 4, and 8 kg/h) on yield. The maximum recovery of lycopene (54%) and β-carotene (50%) was found at 300 bar with 5% ethanol (2 h with a 4 kg/h flow rate). The optimum extraction temperature was $55\,°C$ for lycopene and $65\,°C$ for β-carotene. The authors hypothesized that temperatures above $65\,°C$ would increase yield, but would also contribute to greater carotenoid degradation. Vagi et al. (2002) used SFE to extract pigments, including carotenoids, from marjoram (*Origanum majorana* L.), with the greatest yield at conditions of $50\,°C$ and 450 bar. The authors found these extracts to have similar amounts of lutein and β-carotene as those from a traditional Soxhlet extraction with hexane.

Solvent modifiers have been shown to increase SFE efficiency by increasing the solubility of the analyte in supercritical CO_2 and decreasing its interaction with the food matrix. The amount of β-carotene extracted using SFE has been found to increase with the addition of 5% ethanol (Marsili and Callahan, 1993) and 5% chloroform (Chandra and Nair, 1997). Conversely, while β-carotene is extremely soluble in meth-ylene chloride, it has been reported that the addition of this modifier enhances degradation of β-carotene during SFE (Marsili and Callahan, 1993). Gamlieli-Bonshtein et al. (2002) were able to separate 9-*cis* and all-*trans* β-carotene isomers from algae using SFE due to their different dissolution rates in CO_2. Gómez-Prieto et al. (2003) studied the effect of

CO_2 density on extraction of all-*trans*-lycopene from tomatoes and found the greatest yield at the highest density (0.90 g/mL) tested. However, while SFE is a relatively mild extraction technique, it is still necessary to consider the effect of extraction conditions on carotenoid stability to minimize the formation of isomer artifacts (Spanos et al., 1993).

4.2.4   Matrix Solid-Phase Dispersion

Matrix solid-phase dispersion (MSPD) has been employed as a novel alternative to traditional solvent extraction methods for the analysis of solid, semi-solid, and viscous samples (Glaser et al., 2003; Putzbach et al., 2005; Rehbein et al., 2007). This technique combines sample preparation, extraction, and fractionation into one process, limiting handling and transfer steps (Barker, 2007). The sample is mechanically ground with a bounded-phase solid support material in a mortar and then packed into a chromatographic column to isolate the carotenoids of interest. The efficiency of MSPD is influenced by such factors as the type of solid support, the nature of the bonded phase, sample modification, the eluting solvent, and the sample matrix. Derivatized solid support material (typically C18) is preferred owing to its ability to completely disrupt and disperse the sample components based on their relative polarities (Barker, 2000). MSPD yields a highly concentrated eluent without the evaporation of large amounts of solvent, making it a preferred method for LC-NMR analysis of carotenoids (Dachtler et al., 2001; Putzbach et al., 2005). Additionally, MSPD has been found to require 95% less solvent and 90% less time than traditional liquid–liquid extraction methods (Barker, 2007). While carotenoids are prone to oxidative degradation, several groups have suggested that MSPD precludes the formation of artifacts (Glaser et al., 2003; Gentili and Caretti, 2011). Gentili and Caretti (2011) used MSPD as an extraction method for the simultaneous analysis of carotenoids and fat-soluble vitamins from maize flour and kiwi. MSPD was also used to extract lutein and zeaxanthin from bovine and chicken retinas (Dachtler et al., 2001). More research still needs to be done to evaluate the effect of this technique on lycopene-containing samples.

4.2.5   Saponification

Alkaline hydrolysis (saponification) can be used to remove interfering lipids and chlorophylls present in plant and food samples, such as

oranges (Lee et al., 2001), marigolds (Hadden et al., 1999), kale (Kurilich et al., 2003), and maize (Howe and Tanumihardjo, 2006). Saponification also hydrolyzes the fatty acid esters of xanthophylls, such as β-cryptoxanthin, lutein, and zeaxanthin, which can simplify chromatographic separation. Saponification is generally carried out using a methanolic, ethanolic, or aqueous solution of potassium or sodium hydroxide (10–90% w/v) prior to extraction (Lee et al., 2001; Kurilich et al., 2003). The AOAC method for analysis of carotenes and xanthophylls in dried plant materials and mixed feeds (970.64) calls for saponification with 40% methanolic potassium hydroxide (KOH) at room temperature for 16 h or in a 56 °C water bath for 20 min (Horwitz, 2006). While the reaction time is reduced at higher temperatures, such conditions could lead to greater carotenoid isomerization (transformation of the all-*trans* form to *cis* configurations) (Kimura et al., 1990). A gentler saponification method using a strongly basic resin (Ambersep 900 OH) has also been reported (Larsen and Christensen, 2005).

The saponification of food samples has been shown to lead to a significant loss of xanthophylls (especially epoxycarotenoids), while having little effect on carotene content (Khachik et al., 1986; Kimura et al., 1990; Oliver et al., 1998). The extent of carotenoid degradation is dependent on the food source and it has been suggested that greater loss occurs in high-fat foods than in nonfat foods (Oliver et al., 1998). Therefore, it is recommended that this step be omitted when accurate quantification of these pigments is necessary (Khachik et al., 1986). However, Kimura et al. (1990) found that saponification was needed for a good separation of carotenoids from papaya due to the presence of carotenol esters. They analyzed five different procedures and concluded that overnight saponification in equal volumes of petroleum ether and 10% methanolic KOH at room temperature (under nitrogen) yielded the best results, but to reduce the time and cost associated with this step, shorter methods requiring less solvent have been explored (Granado et al., 2001).

Saponification has also been shown to degrade carotenoids present in biological samples. Nierenberg and Nann (1992) reported significantly reduced recoveries of lutein, cryptoxanthin, lycopene, α-carotene, and β-carotene when saponifying tissues using 5% methanolic KOH. Therefore, the saponification step should be evaluated to ensure that significant carotenoid loss or isomerization is not taking place during the preparation of biological and food samples.

4.3   UV/Visible Spectophotometric Methods

As a result of a series of conjugated double bonds, most carotenoids absorb in the range of 400–500 nm (although some lycopene precursors, such as phytoene and phytofluene, absorb maximally in the UV region). Spectral information is a useful tool for distinguishing and identifying different carotenoid species; however, it is important to keep in mind that, for common carotenoids, the UV/Vis spectra only provides information about the chromophore of the carotenoid. For example, α-carotene and lutein cannot be identified from each other solely by spectra, and other factors, like retention time, must be considered in distinguishing between these two compounds. Additionally, carotenoid spectra can be influenced by different solvents and carotenoids can interact with proteins and lipids, altering spectral characteristics (Britton, 1995). Carotenoids are unique in that many species usually have three more-or-less distinct peaks instead of a single band (Kohler, 1995). Different carotenoids can vary significantly in their wavelength of maximum absorption as well as in their fine structure. The ratio of peak III compared to peak II can be useful in distinguishing between carotenoids/carotenoid isomers (Figure 4.2). The isomers can also often be tentatively identified by the presence of a "*cis* peak," discussed further in the *cis*/*trans* isomers section of this chapter.
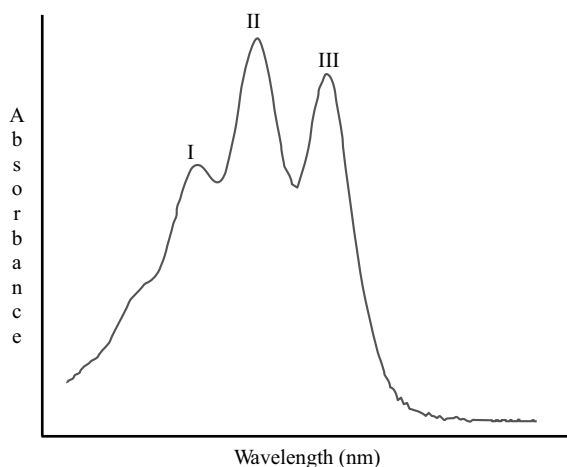
Figure 4.2   Spectral fine features of a typical carotenoid. (Adapted from Britton, 1995).

A compilation of all spectra of known carotenoids can be found in the *Carotenoids Handbook* (Britton et al., 2004).

Carotenoids also have absorbances that are linearly correlated with their concentration (i.e. obey Beer–Lambert's law) allowing spectro-photometric methods to be used to quantify carotenoids in a sample. Colorimetric and spectrophotometric methods have been used for the measurement of carotenoids for almost 100 years (Schertz, 1923), but care must be taken to choose wavelengths without influence from interfering compounds, like chlorophylls or similar caro-tenoids. Saponification is often used to remove chlorophylls, but can cause carotenoid, especially xanthophyll, degradation (Lietz and Henry, 1997). Individual standard curves (e.g. β-carotene) are often used to quantify total carotenoids in a sample. Methods for creating a standard curve and estimating the carotenoid content in food extracts by UV/Vis spectroscopy have been compiled (Scott, 2001). Often, carotenoids have different molar extinction coefficients which can lead to error in quantifying carotenoids using this method for samples with more than one carotenoid species. Although spectrophotometric methods for the determination of carotenoid content have their dis-advantages compared to HPLC in terms of selectivity, they are still extensively used because they cost considerably less and are less time intensive. Biehler and colleagues have compared three spectrophoto-metric methods for determining carotenoids in fruits and vegetables (with and without chlorophylls) to HPLC. They found significant correlation between carotenoid spectrophotometric methods and HPLC, although these methods were less accurate for foods with a mixed carotenoid profile (Biehler et al., 2010).

4.3.1 AOAC Method for Provitamin A Carotenoids

Accurately quantifying the amount of provitamin A carotenoids in a food product is essential for determining nutritional value of foods. The AOAC method for determination of vitamin A (974.29) and carotenoids (941.15 and 970.64) in foods utilizes open column chromatography combined with a colorimeter (vitamin A) or spectrophotometer (car-otenoids) (Horwitz, 2006). It is recommended to extract with acetone-hexane followed by filtration, then remove the acetone by rinsing with water. The extracts in hexane are applied to an activated MgO_2 diatomaceous earth column and eluted using acetone and hexane

(Horwitz, 2006). The carotene fraction elutes first, as it is more nonpolar compared to xanthophylls. Although the method is relatively inexpensive and does not require specialized equipment, it is time consuming and does not always yield the complete separation of carotenoid species. This causes a problem, especially when quantifying provitamin A carotenoids since carotene species (including α- and γ-carotene) are all treated as β-carotene. In addition, all xanthophylls (including provitamin A β-cryptoxanthin) are ignored (Schwartz, 1998).

4.4   High-Performance Liquid Chromatography

Carotenoids, as plant pigments, have been extensively studied for almost 200 years and have helped to facilitate the development of the field of chromatography (Eugster, 1995). The first methods for separating carotenoids and chlorophylls using open column chromatography were developed as early as 1906 (Twsett, 1906). High-performance liquid chromatography (HPLC) analysis for foods has been extensively reviewed (Nollet, 2000) and, for the past few decades has been the preferred method of separating, identifying, and quantifying carotenoids in foods and biological samples. The photodiode array (PDA) is the most commonly used detector for HPLC carotenoid analysis, although other detectors, such as electrochemical detectors (ECD), fluorescence, mass spectrometers (MS), and nuclear magnetic resonance (NMR) can be used. Reviews on PDA detectors in relation to carotenoid analysis can be found elsewhere (Bramley, 1992). Advantages of HPLC include efficiency, short run times, and sensitivity (Pfander and Riesen, 1995). Reversed-phase HPLC is by far the most common, although some normal phase methods have been developed for the separation of mixtures of xanthophylls or carotenoid ketones, like astaxathin (Sedmak et al., 1990), with little affinity for a C18 or C30 "carotenoid" column. Some apocarotenoids like bixin and norbixin are much shorter in chain length and more polar than a typical xanthophyll and require alternative solvent systems (Scotter et al., 1998). C18 columns are often sufficient for separating different carotenoids, although C30 columns tend to yield better separations, especially of very similar compounds (e.g. lutein and zeaxanthin) (Hart and Scott, 1995). Additional methods for separation of carotenoids/carotenoid isomers are discussed in the *cis/trans* isomer portion of this chapter. These methods are sufficient to separate carotenoid species, but often require

longer run times. Ultra high-performance liquid chromatography (UHPLC) has been used to monitor carotenoids, but mostly in conjunction with other fat-soluble vitamins (Chauveau-Duriot et al., 2010; Granado-Lorencio et al., 2010). UHPLC methods for the quantification of a wide range of carotenoids are uncommon. Column temperature should be controlled and maintained above 20 °C to promote a consistent separation and to prevent carotenoids from crystallizing out of the solution (Scott and Hart, 1993; Böhm, 2001).

4.4.1 *Electrochemical Detection*

Often, low levels of carotenoids in biological samples provide significant challenges in quantification by HPLC-PDA alone. Electrochemical detection (ECD) has been successful in quantifying low concentrations of carotenoids (MacCrehan and Schonberger, 1987; Finckh et al., 1995; Yamashita and Yamamoto, 1997). More information about ECD can be found in Chapter 2. ECD has also been successful in quantifying carotenoid isomers in foods, plasma, prostate tissue, cervical tissue, and buccal mucosal cells (Ferruzzi et al., 1998, 2001; Allen et al., 2003; Unlu et al., 2007). Electrochemical array detection for all-*trans*-β-carotene has been reported to be 10 fmol on column, which is approximately 100–1000 times more sensitive than UV/Vis detectors (Ferruzzi et al., 1998).

4.5 *Cis/Trans* Carotenoid Geometrical Isomers

Common carotenoids can have anywhere from 3 to 11 conjugated double bonds (in addition to unconjugated double bonds) and can undergo isomerization to yield mono- or poly-*cis* geometrical isomers. A *cis* isomer has its substituents on the same side of a carbon–carbon double bond, while the *trans* form has its substituents on opposite sides of a carbon–carbon double bond. Geometrical isomers can also be designated using the German *E/Z* system, in which *Z* specifies the two groups of higher priority to be on the same side of the carbon–carbon double bond, while *E* specifies the two higher priority groups to be on opposite sides of the carbon–carbon double bond. Lycopene, for example, has 11 conjugated and 2 unconjugated double bonds and can theoretically yield 1056 *cis/trans* isomers. However, steric hindrance favors the formation of those isomers that exist in the lowest energy state (Zechmeister, 1944). In the majority of foods, the all-*trans* configuration of most carotenoids predominates, although *cis* isomers

have been documented to be biochemically synthesized and found in unprocessed fruits and vegetables, with tangerine tomatoes (*tetra-cis*-lycopene) and annatto (9-*cis*-bixin) as examples (Schwartz et al., 2008).

4.5.1   Biological Significance

The *cis* isomers tend to be more polar, less likely to crystallize, and more oil/hydrocarbon soluble compared to the all-*trans* forms (Castenmiller and West, 1998). It seems that some carotenoid *cis* isomers are more bioavailable compared to the all-*trans* configuration; while for others, the *cis* isomer is less bioavailable. It has been shown that consumption of a tomato sauce high in *cis*-lycopene compared to a sauce high in all-*trans*-lycopene yields significantly higher total, total *cis*-, and all-*trans*-lycopene in a triglyceride rich fraction of plasma (Unlu et al., 2007). Conversely, there is evidence in mice and humans that all-*trans*-β-carotene is preferentially absorbed over *cis* forms, and *cis* forms in a bolus dose can be converted to the all-*trans* form *in vivo* (Gaziano et al., 1995; Tamai et al., 1995; Stahl et al., 1995; You et al., 1996; Ben-Amotz and Levy, 1996; Deming et al., 2002). 9-*cis* and 13-*cis*-β-carotene are converted to vitamin A with 38 and 53% efficiency, respectively, compared to all-*trans*-β-carotene (Zechmeister, 1949).

Cis isomers have also been shown to form as the result of food processing (Chandler and Schwartz, 1987; Lessin et al., 1997; Nguyen and Schwartz, 1998; Updike and Schwartz, 2003; Hackett et al., 2004). *Cis* isomers can also be produced *in vivo*. For example *cis*-lycopene accounts for 9–21% of total lycopene in tomato products, 58–73% of total lycopene in serum, and a surprisingly high 79–88% of total lycopene in benign or malignant prostate tissue (Clinton et al., 1996). This suggests a potential biological relevance for lycopene *cis* isomers. Regardless, methods to quantitate *cis* carotenoids are important because of the presence of the different isomers in food and biological samples.

4.5.2   Development of the C30 "Carotenoid" Column

Most of the early published work on the separation of carotenoids by HPLC employs C18 stationary phases. It has been shown that polymeric surface modified C18 columns are more selective toward isomers compared to monomerically bound columns (Figure 4.3) (Sander et al., 1994). In the 1980s and 1990s, a 30 carbon bound stationary phase (C30 column) was developed and coined the "carotenoid column," because of
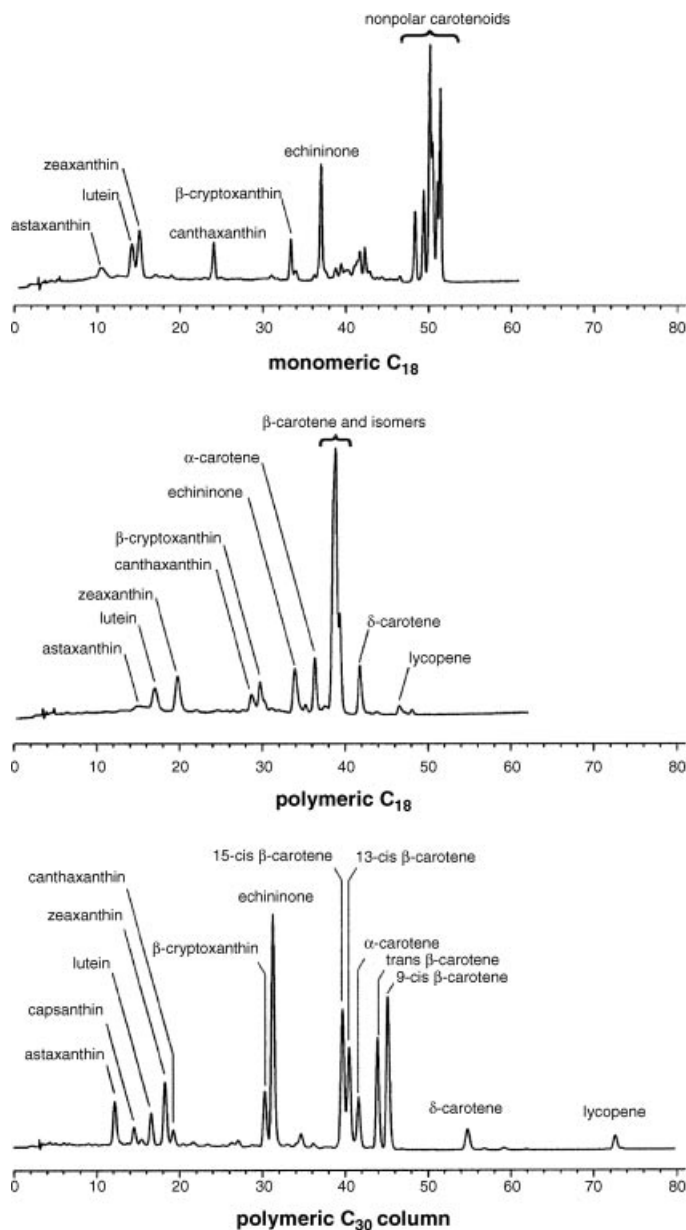
Figure 4.3 Comparison of separation of carotenoid standards on monomeric C18, polymeric C18, and C30 "carotenoid column." Chromatographic conditions are: 81:15:4 to 6:90:4 MeOH/MTBE/water over 90 min at 1 mL/min at 20 °C (Sander et al., 1994).

its shape selectivity and superior ability to separate not only different carotenoid species, but also isomers (Figure 4.3) (Sander et al., 1994). Elution order of lycopene varies greatly between C18 and C30 columns. On a C18 column lycopene elutes before α-carotene and β-carotene. In contrast, on a C30 column lycopene is retained and elutes last, allowing the efficient separation of isomers (Sander et al., 2000).

4.5.3   C30 Analysis Methods

A C30 column can be used to distinguish between all-*trans*-lutein and all-*trans*-zeaxanthin and their *cis* isomers (Updike and Schwartz, 2003), β-carotene and β-carotene *cis* isomers (Emenhiser et al., 1995), and lycopene and *cis*-lycopene isomers (Fröhlich et al., 2007). C30 columns can allow the separation of isomers induced by heat processing (Figure 4.4) and induced *in vivo* (Figure 4.5). Extensive reviews on
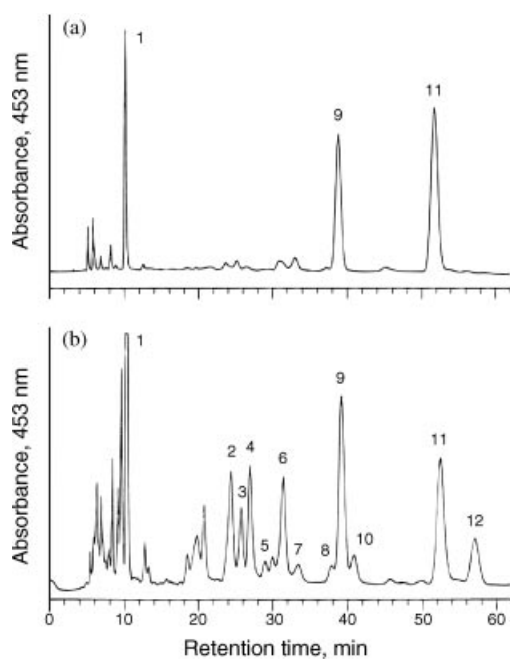
Figure 4.4 Thermal processing induced isomerization of carrots. (a) Raw carrot and (b) thermally processed carrot. Peaks are tentatively identified as (1) all-*trans*-lutein; (2) 13-*cis*-α-carotene; (3) a *cis*-α-carotene isomer; (4) 13′-*cis*-α-carotene; (5) 15-*cis*-β-carotene; (6) 13-*cis*-β-carotene, (7 and 8) *cis*-β-carotene isomers; (9) all-*trans*-α-carotene; (10) 9-*cis*-α-carotene; (11) all-*trans*-β-carotene; and (12) 9-*cis*-β-carotene. (Emenhiser et al., 1996).
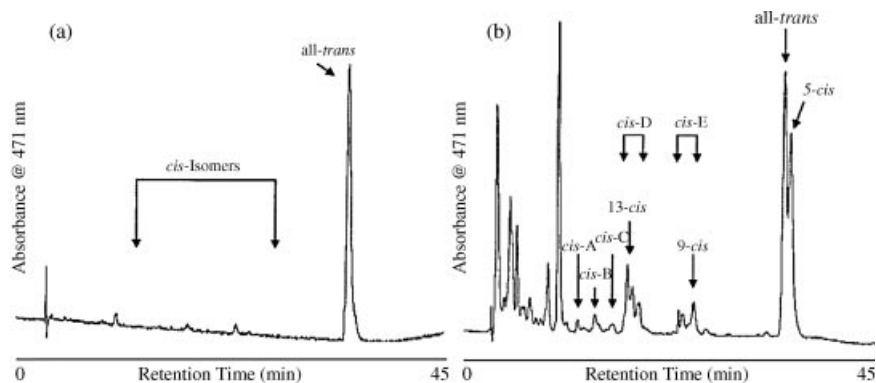
Figure 4.5 Representative HPLC chromatogram of (a) tomato product lycopene isomers in (b) plasma lycopene isomers from healthy individuals consuming different lycopene-containing tomato products for 15 days, demonstrating the presence of isomers in human blood plasma versus the all-*trans* form found in most foods (Hadley et al., 2003).

the development of C30 stationary phases for carotenoid analysis can be found elsewhere (Sander et al., 1994, 2000).

Most C30 methods use some combination of methyl-*tert*-butyl ether (MTBE), methanol, and a small amount of water and employ a gradient for optimum separation of different carotenoid species and their isomers (Yeum et al., 1996; Ferruzzi et al., 1998, 2001). Some C30 methods can also separate carotenoids/carotenoid isomers while simultaneously separating tocopherols and chlorophylls (Puspitasari-Nienaber et al., 2002). Others use a combination of acetonitrile, methanol, isopropyl alcohol, ethyl acetate, dichloromethane or THF, and combinations thereof (Craft, 2001; Hart and Scott, 1995; Krinsky et al., 1990; Epler et al., 1993; Stahl et al., 1992). Ammonium acetate (0.05 M) and/or triethylamine (0.05%) have been shown to improve on-column recovery and buffer acidic uncapped silanol groups of the column backbone (Handelman et al., 1992; Epler et al., 1993; Hart and Scott, 1995). Column temperature can also affect chromatographic separations, generally $23\,°C \pm 1\,°C$ is sufficient to separate different carotenoid species but $30\,°C$ has been found to be ideal for separating isomers (Böhm, 2001). BHT is sometimes added as an antioxidant. A "*cis* peak" (denoted A_B) is often visible in the spectra of a *cis*-carotenoid as an additional peak 142 nm below the wavelength of maximum absorbance (A_{II}) (Britton, 1995;

Liaaen-Jensen, 1995). The intensity of the *cis* peak increases when it is located toward the center of the chromophore. Additionally, the appearance of a double-*cis* peak may indicate an aliphatic or monocyclic chromophore (Britton, 1995; Liaaen-Jensen, 1995). Generally, *cis* isomers tend to absorb maximally at wavelengths approximately 2–6 nm below that of the all-*trans* carotenoid and have a reduction in the fine structure (Zechmeister, 1962). Figure 4.6 shows overlaid spectra of all-*trans*-lycopene and two lycopene *cis* isomers.
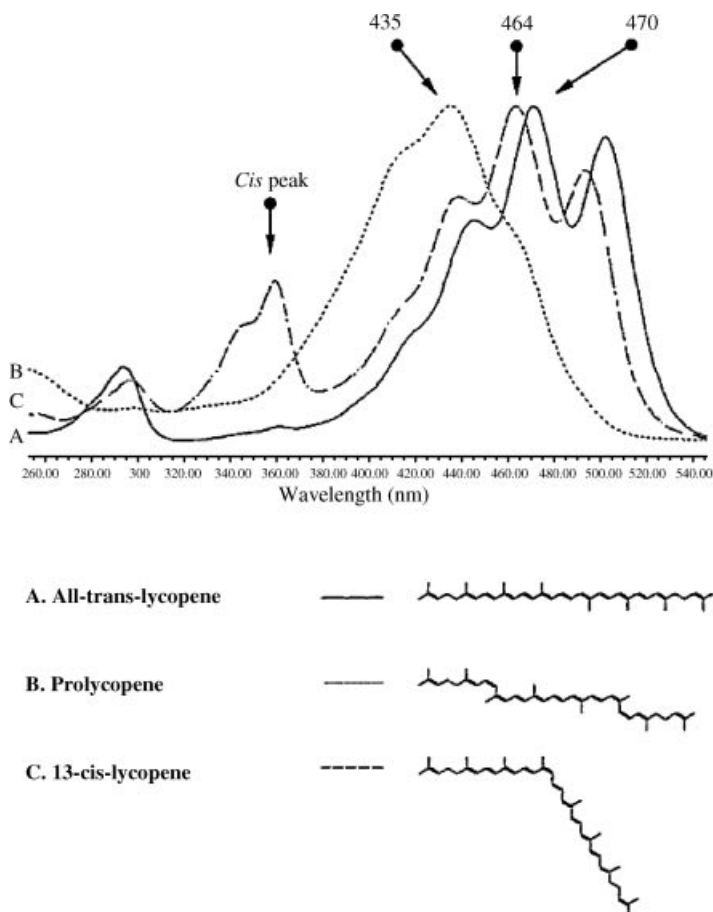
Figure 4.6 Overlaid spectra of all-*trans*-lycopene, prolycopene (also called *tetra-cis*-lycopene) and 13-*cis*-lycopene (Nguyen and Schwartz, 2000).

In order to unequivocally identify the structure of a specific carotenoid geometrical isomer (e.g. 9-*cis*-lycopene) to a peak in a chromatogram, nuclear magnetic resonance (NMR) is used (Hengartner et al., 1992).

4.6 Optical Isomers

In addition to geometrical (*cis*/*trans*) isomers, many carotenoids exist in nature as optical (*R/S*) isomers. Optical isomers differ only in their interaction with polarized light. Lutein and zeaxanthin optical isomers are of importance in the retina, and evidence suggests that they play a role in vision and aid against age-related macular degeneration (Krinsky et al., 2003). Lutein exists as the (3′*R*, 3′*R*, 6′*R*) isomer and zeaxanthin exists primarily in the (3*R*, 3′*R*) and (3*R*, 3′*S*) configurations (termed zeaxanthin and *meso*-zeaxanthin, respectively) with smaller amounts of the (3*S*, 3′*S*) form in the eye (Bone et al., 1993). Interestingly, *meso*-zeaxanthin cannot be detected in serum, which has led to the idea that it is formed in the eye as a conversion product of lutein (Bone et al., 1993). Zeaxanthin can be separated from *meso*-zeaxanthin on a chiral column (Bone et al., 1993, 2007), and lutein and zeaxanthin optical isomers can also be separated using dibenzoate esters (Bone et al., 1993) or by reaction with (*S*)-(+)-α-(1-naphthyl) ethyl isocyanate to create diastereomeric dicarbamates (Pirkle and Hoekstra, 1974; Pirkle and Hauske, 1977; Rüttimann et al., 1983).

Techniques to separate carotenoid optical isomers are also of significant industrial importance. For example, astaxanthin is the carotenoid that imparts the pink color to fish, such as salmon, and is used as a feed ingredient in aquaculture. Different stereoisomers exist depending on the source of the astaxanthin. For example, wild salmon (and salmon consuming *Haematococcus pluvalis*) and lobster accumulate (3*S*, 3′*S*)-astaxanthin. Farmed salmon grown on feed enhanced with an extract of *Xanthophyllomyces dendrohous* (formerly known as *Phaffia rhodozyma*) – a yeast that synthesizes astaxanthin – are found in the (3*R*, 3′*R*) form (Vecchi and Müller, 1979; Turujman et al., 1997). It has been shown that astaxanthin does not convert between the configurational isomers *in vivo* (Schiedt et al., 1981; Foss et al., 1984). As a result of the increased cost of wild salmon compared to

farm-raised salmon, the incentive for misbranding is significant, and analytical techniques are used to separate the two optical isomers. These two forms, in addition to the (3S, 3′R)-*meso* (optically inactive) form, can be separated and quantified using (−)-camphanic acid esters and normal phase-HPLC with a cyano or silica column (Vecchi and Müller, 1979; Schuep and Schierle, 1995; Bowen et al., 2002). Astaxanthin optical isomers can also be separated using chiral columns, including, but not limited to, Pirkle covalent D-phenylglycine (Turujman et al., 1997) and Pirkel covalent L-leucine columns (Turujman, 1993).

4.7   HPLC-NMR

Nuclear magnetic resonance (NMR) has always been a powerful tool for the structural identification of carotenoids (Mercadante et al., 1999; Tiziani et al., 2006), but in the past decade it has been interfaced with high-performance liquid chromatography (HPLC) to enhance carotenoid profiling. Given the sensitivity of carotenoids to oxygen, light, and heat, minimizing the presence of artifacts due to isomerization during analysis is critical. HPLC-NMR allows for carotenoid mixtures to be separated chromatographically and unambiguously identified using NMR in a closed-loop system.

As carotenoids are based on a system of highly conjugated double bonds, they can be found naturally in both the *cis* and all-*trans* conformations. Determining the carotenoid isomer profile in foods and biological samples is important for understanding the functionality of these compounds. LC-MS is a powerful method for the low-level detection of carotenoids in a variety of matrices. However, this technique cannot distinguish between stereoisomers as they yield the same mass spectra and fragmentation patterns. PDA detection can assist in stereoisomer elucidation, but often does not provide enough information for absolute structural determination (Lacker et al., 1999). Therefore, HPLC-NMR can be a preferred method to both separate and unambiguously identify carotenoid stereoisomers.

C30 columns have commonly been used in LC-NMR analyses of carotenoid isomers due to their greater shape selectivity compared to traditional C18 columns (Sander et al., 1994). This feature provides

sharper chromatographic peaks and consequently, a higher concentration of analyte in the NMR flow cell, which enhances sensitivity (Dachtler et al., 2001). Strohschein et al. (1999) used HPLC-NMR for the structural elucidation of β-carotene isomers and found enhanced resolution with a 3 µm C30 column compared to a 5 µm column. However, Tode et al. (2009) were able to identify the major carotenoids in tomato juice, palm oil, and satsuma mandarin orange juice by HPLC-NMR using a C18 column (150 mm × 4.6 mm).

LC-NMR data can be obtained using either a continuous- or stopped-flow method for acquisition of NMR spectra. Dachtler et al. (2001) used both techniques with ^{1}H-NMR to separate and characterize zeaxanthin stereoisomers. Using stopped-flow they were able to identify (13-Z)-zeaxanthin with 800 ng of analyte in the flow cell, compared to 24 µg using continuous-flow. The higher sensitivity with stopped-flow LC-NMR arises because the chromatographic run is paused so that NMR data can be acquired at the peak maximum where the concentration of analyte is greatest (Albert, 1999).

Most LC-NMR analyses of carotenoids have used a binary solvent system of acetone and deuterium oxide. Glaser et al. (2003) analyzed lutein and zeaxanthin stereoisomers in cooked spinach using HPLC coupled with 2D H,H COSY NMR and a solvent system of 84–97% acetone and 3–16% D_2O. The use of deuterated water helps to reduce the intensity of the solvent signal, but does not eliminate the need for solvent suppression prior to data acquisition in HPLC-NMR experiments (fully deuterated solvents are often cost prohibitive) (Albert, 1999). Putzbach et al. (2005) avoided this issue by using capillary HPLC-NMR to unambiguously identify carotenoids in a small-sized spinach sample. With a flow rate of 5 µl/min they were able to use fully deuterated solvents and obtain a high signal to noise ratio in stopped-flow mode (Putzbach et al., 2005). Rehbein et al. (2007) had similar success characterizing small amounts of bixin isomers using capillary HPLC-NMR.

In the past, HPLC-NMR was limited by its sensitivity, but with advancements in NMR magnets and probes, carotenoids can be detected in the upper nanogram range using this technique (Albert, 1999; Dachtler et al., 2001). As chromatography and NMR continue to develop, it is likely that HPLC-NMR will be used more routinely for the structural elucidation of carotenoid stereoisomers in foods.

4.8   Mass Spectrometry and HPLC-MS

Mass spectrometry data is often paired with UV-Visible spectra, NMR, or HPLC retention time for carotenoid identification. In addition, HPLC coupled with MS as a detector has been reported to be 100 times more sensitive than PDA for detection and quantification of some carotenoids (van Breemen, 1995; van Breemen et al., 1996). While mass spectrometry can be a powerful tool, it should be noted that the analysis of carotenoids (which are nonvolatile, thermally labile, and inherently unstable) presents a special challenge to the mass spectrometry analyst. An overview of common MS components and techniques is provided in Chapter 2 (techniques not previously mentioned are briefly described below).

4.8.1   Ionization

The many types of ionization methods that have been tested with carotenoids are discussed below. Because hydrocarbon carotenes do not contain functional groups that can easily gain or lose protons, it has been observed that multiple ionization methods produce a molecular anion $[M^{-\bullet}]$ in negative mode or molecular cation $[M^{+\bullet}]$ in positive mode as the predominant species of carotenes in source. In these instances, an electron is either gained or lost, respectively, to produce a charged species. Since the molecular weight of an electron is negligible and most mass analyzers are not sensitive enough to detect such a small change, the mass of these ions is the same as the mass predicted by the chemical formula. Some compounds, such as peptides, often produce multiply charged species in source, but carotenoids generally do not.

The background matrix often presents a challenge in carotenoid analysis by MS, especially with foods or biological samples in which a significant amount of lipid is present. These lipids (often not visible by PDA) can compete with the carotenoid for ionization in source and increase background noise. If enough background is present, significant decreases in sensitivity and selectivity may be observed. It is often necessary to identify sample preparation steps (as discussed previously) that extract or selectively remove carotenoids from the lipophilic background without degrading carotenoids in the sample. Alternatively, the use of different HPLC methods (to chromatograph background

differently) or the use of stable isotopes as internal standards for quantification (Zhu et al., 2006; Fleshman et al., 2010) can help to reduce or eliminate the problems posed by sample matrix.

Often a mass spectrometer is interfaced with an HPLC-PDA system. This technique is especially useful because isobaric species can be chromatographically separated before entering the MS. Interestingly, there are a large number of isobaric species in the field of carotenoids, such as lycopene, β-carotene, α-carotene, and γ-carotene which all have a parent mass of 536 mu, or β-cryptoxanthin, α-cryptoxanthin, zeinoxanthin, and rubixanthin which all have a parent mass of 552 mu.

Earlier methods of ionization applied to carotenoids, including electron impact (EI), chemical ionization (CI), a particle beam interface with EI or CI, and continuous-flow fast atom bombardment (CF-FAB), have been comprehensively reviewed elsewhere (van Breemen, 1996, 1997; Pajkovic and van Breemen, 2005). These techniques have generally been replaced by "softer" ionization techniques like electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), and more recently atmospheric pressure photoionization (APPI). It should be noted that ESI, APCI, and APPI can be used as ionization methods with a direct infusion of an analyte in solution (i.e. not interfaced with an HPLC system), or as the interface between the HPLC and the MS. In contrast, matrix-assisted laser desorption ionization (MALDI) cannot be used directly with HPLC.

Electrospray ionization (ESI)

Van Berkel and Zhou first tested β-carotene with ESI positive in 1994 (van Berkel and Zhou, 1994). In this study, a doubly charged molecular ion of β-carotene was observed as the primary species when trifluroacetic acid was present in the solution. Van Breemen was the first to utilize ESI as an interface between HPLC and MS to analyze carotenoids (van Breemen, 1995). In this study, ESI operated in negative mode ionized xanthophylls (astaxanthin, β-cryptoxanthin, and lutein), but did not ionize hydrocarbon carotenes (lycopene and β-carotene). In contrast, ESI positive produced only $[M^{+\bullet}]$ for all carotenoids in this study, and the addition of halogenated solvents to the post-column effluent greatly enhanced signal intensity (van Breemen, 1995). A later study by Guarantini et al. demonstrated the ability of ESI positive to produce both $[M^{+\bullet}]$ and $[M+H]^+$ for a number of xanthophylls, and these authors attributed the production of the two species to solvent system

effects (Guaratini et al., 2005). In contrast, the only ion of β-carotene produced was [M$^{+\cdot}$] (Guaratini et al., 2005), further supporting the results of van Breemen (1995).

Novel methods of utilizing ESI have also been developed. LC-ESI-MS has been applied to the detection of silver adducts of tocopherols and carotenoids in various food products including tomato, carrot, infant formula, vegetable juices, and a vitamin drink (Rentel et al., 1998). The addition of silver, a positively charged adduct, creates positively charged species which are easily detected. In this study, an AgClO$_4$ solution was added to effluent post-HPLC before entering the source. The Ag$^+$-carotenoid adducts formed were then monitored, and [Ag-M$^{+\cdot}$] was the primary ionization product for each of the carotenoids studied (Rentel et al., 1998). Similarly, oxime derivatives of zeaxanthin metabolites (Prasain et al., 2005) and the provitamin A carotenoid metabolite retinaldehyde (von Lintig and Vogt, 2000) have been synthesized in biological samples and analyzed via ESI positive. The synthesized oxime derivatives are more stable than the unreacted metabolites, and more easily detected than the parent products.

In terms of quantitation, work by Wang et al. (2000) has demonstrated that the response of β-carotene, ionized by ESI positive and normalized to an internal standard, was nonlinear over a range of concentrations (Wang et al., 2000). This study highlights the importance of testing carotenoid linearity over the anticipated range of analysis on any instrument before quantitative analysis is attempted.

Atmospheric pressure chemical ionization (APCI)
The most popular ionization technique used with carotenoids to date is APCI, which has been reviewed by Pajkovic and van Breemen (2005). A comprehensive table listing studies utilizing APCI for carotenoid analysis can be found in a recent review by Rezanka et al. (2009). In APCI negative, both carotenes and xanthophylls are ionized to produce [M$^{-\cdot}$] and [M − H]$^-$ species. In APCI positive, both carotenes and xanthophylls produce both [M$^{+\cdot}$] and [M+H]$^+$ ions. However, the most abundant species for xanthophylls in APCI positive is often [M + H − 18]$^+$, corresponding to a loss of water (van Breemen et al., 1996; Clarke et al., 1996; Glaser et al., 2003). APCI techniques have been used to ionize carotenoids from plants and plant foods (Clarke et al., 1996; Glaser et al., 2003; Tian et al., 2003), blood plasma (Fang et al., 2003; Kopec et al., 2010), and biological tissues (van Breemen et al., 2002).

APCI with in-source fragmentation has also been utilized to determine the types and substitution locations of fatty acyl moieties of lutein esters (Breithaupt et al., 2002; Tian et al., 2003; Young et al., 2007). In a study of lutein esters from marigold, Breithaupt et al. (2002) observed that the most labile fatty acid or hydroxyl group in-source was covalently bound to the epsilon ring of lutein. These results were confirmed by Young and colleagues (2007) who synthesized various lutein esters, determined structures with NMR, and observed that the epsilon ring preferentially loses water or a fatty acid moiety before the β-ionone ring.

In contrast to ESI, APCI has been found to produce a linear response over a range of concentrations (i.e. has a large dynamic range) for β-carotene (Wang et al., 2000; Hao et al., 2005), lutein, zeaxanthin, β-cryptoxanthin (Hao et al., 2005) and a relatively linear response for lycopene (van Breemen et al., 2002).

Atmospheric pressure photoionization (APPI)
In the APPI method of ionization, the solvent is vaporized in a heated nebulizer and the gaseous analytes are then ionized with photons from a lamp (Rivera et al., 2011). It has been observed that certain solvents, called "dopants," enhance the ionization of analytes via this technique. To date only one study has been published with carotenoids and APPI (Rivera et al., 2011). APPI was compared to ESI and APCI as ionization techniques, and the authors observed that APPI positive produced approximately a 2- to 4-fold greater total ion signal for lycopene and β-carotene as compared to APCI positive and ESI positive. In contrast, APCI positive outperformed APPI positive for a number of xanthophylls and phytoene and phytofluene.

Matrix-Assisted laser desorption ionization (MALDI)
MALDI has been used to ionize multiple carotenoids and carotenoid ester standards (Kaufmann et al., 1996) and carotenoids in a variety of plant tissue samples (Fraser et al., 2007). Fraser et al. (2007) found that the use of a nitrocellulose matrix produced the least variability in analyte detection, and also observed that MALDI was able to detect large differences in carotenoid phenotypes, but not small differences in carotenoid levels. Likewise, MALDI was not able to differentiate between isobaric species (like β-carotene and lycopene, for example).

### *4.8.2 Mass Separation*

Time-of-Flight (TOF)

The accurate mass capabilities of Time-of-Flight (TOF) are useful in determining the composition of new carotenoids or carotenoid metabolites which have not previously been identified (Mercadante et al., 1997; Lakshminarayana et al., 2008; Kopec et al., 2010). In addition, a TOF chamber (interfaced with MALDI or an ESI ion source) is often used with a targeted "metabolomics" approach, where determining the exact mass of a mixture of components is important to successfully differentiate carotenoids from a high level of background ions (Fraser et al., 2007; Chu et al., 2011).

Ion mobility spectrometr y (IMS)

IMS is a relatively new technique in which ions are separated based on size and shape using an electric field. IMS was utilized by Dong et al. (2010) to separate all-*trans*-lycopene from *cis*-lycopene and all-*trans*-β-carotene from *cis*-β-carotene. Unfortunately, the various *cis* isomers could not be separated from each other using IMS alone. The authors provided evidence to suggest that *cis/trans* isomerization of carotenoids occur in-source (ESI positive mode was used in these experiments). Because of this isomerization, it does not appear likely that IMS will replace HPLC as a means of separating geometrical isomers of carotenoids in the near future (Dong et al., 2010).

Tandem mass spectrometry (MS-MS)

Tandem mass spectrometry (MS-MS) has been used to identify key structural components of carotenoids, such as loss of a 69 mu terminal isoprene group from lycopene and γ-carotene (Fang et al., 2003) or the internal loss of a 92 mu toluene group observed for a variety of carotenes (Guaratini et al., 2006). MS-MS is also useful to identify substitutions like fatty acid esters of xanthophylls. In addition, MS-MS (with triple quadrupole instruments) can be used for the quantitation of a compound by selected reaction monitoring (SRM). For example, lycopene and γ-carotene can be distinguished from α-carotene and β-carotene by monitoring the selective daughter ion at 467 produced after CID (Figure 4.7). This approach has been used to previously quantify lycopene (Fang et al., 2003; Kopec et al., 2010).
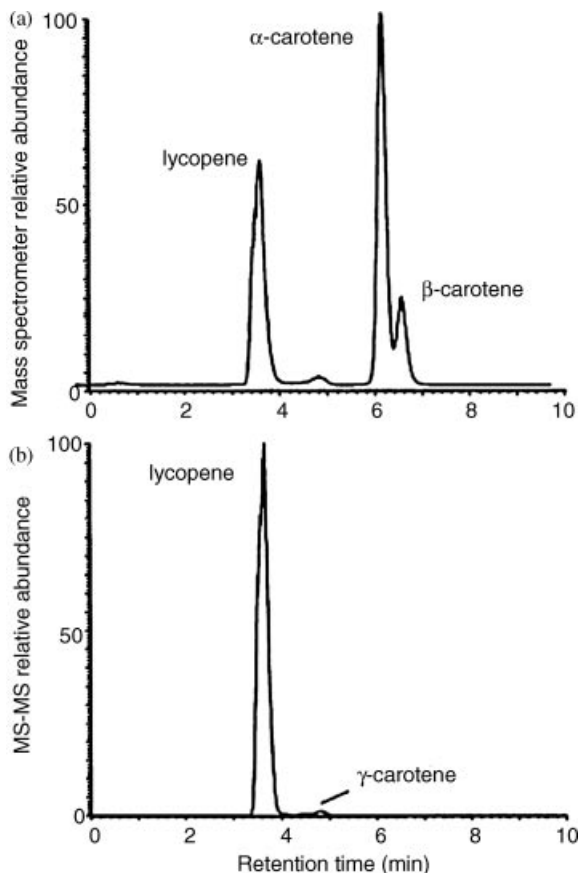
Figure 4.7 (a) LC-MS of lycopene, α-carotene, and β-carotene monitored at 536 *m/z* and (b) LC-MS-MS analysis of the same sample using selected reaction monitoring of *m/z* 536 → 467. (Taken from Fang et al., 2003).

Accelerator mass spectrometry (AMS)

AMS is a special type of MS that has been used to determine various parameters of carotenoid absorption, distribution, and metabolism, and the basics of this technique have been reviewed by Buchholz et al. (2000). A radio-labeled carotenoid (generally labeled with ^{14}C) is fed to a subject, and biological sample or expired air are collected. Samples may be analyzed directly, or first extracted and analyzed by HPLC, where fractions containing the putative isotopically labeled parent carotenoid(s) and/or carotenoid metabolites are collected.

The sample (biological sample or collected fraction) is oxidized to produce carbon dioxide which is then converted to graphite (Getachew et al., 2006). The sample is then ionized, and ions are accelerated to high levels of energy in the MS. The ratio of the rare isotope to the abundant isotope is determined ($^{14}C/^{12}C$, for example). Because of the extreme sensitivity of AMS (in the attomole range), only a small amount of radio-labeled carotenoid is necessary to study absorption and metabolism.

AMS has been used for many years to study the pharmacokinetics of β-carotene, as has been reviewed by van Lieshout et al. (2003). Recent studies have been performed on the absorption of β-carotene and metabolism to vitamin A (Dueker et al., 2000; Hickenbottom et al., 2002a) and other metabolites (Ho et al., 2007) in humans. AMS has also been used to monitor the kinetics of lutein metabolism (Moura et al., 2005) and lycopene bioavailability and metabolism (Ross et al., 2011) in humans.

4.8.3 *Use of Stable Isotopes*

Stable isotopes of carotenoids (usually labeled with ^{13}C or ^2H), or of compounds which react with carotenoids, are often used with MS studies. We previously mentioned the use of stable isotopes as internal standards for carotenoid quantitation (especially important when matrix suppression of signal is observed), but there are also many other applications.

Isotopes can be used to determine mechanism(s) of MS ionization and fragmentation; for example, deuterated chloroform was employed to determine the source of the proton for the molecular ion of β-carotene generated in APCI positive (van Breemen et al., 1996).

Stable isotopes can also be used to demonstrate the mechanisms of enzymatic cleavage; for example, isotopes were used to determine the mechanism of enzymatic cleavage and oxygenation of β-ionone by carotenoid cleavage dioxygenase 1 (CCD1), a plant enzyme (Schmidt et al., 2006). Likewise, stable isotopes have been used by many groups to follow a single dose of β-carotene and observe the enzymatic conversion into vitamin A derivatives in humans (Wang et al., 2000; Burri et al., 2001; Fleshman et al., 2010). The feeding of an isotope allows the dose fed from a specific meal to be differentiated from circulating carotenoid from previous meals. In these studies, samples

are extracted and separated by HPLC which is interfaced with a mass spectrometer. While this technique is not as sensitive as radio-labeled isotopes with AMS, the ability to interface the HPLC with the MS eliminates the need for fraction collection and actually allows the specific compounds to be identified by MS. This also reduces HPLC analysis time, because as long as compounds are not isobaric, they do not need to be baseline separated to be accurately identified and quantified. In contrast, in AMS, fractions are collected for each peak, and any label detected is presumed to be derived from the peak collected.

Stable isotopes of retinal and β-carotene have also been employed to assess liver vitamin A stores in healthy individuals, and the best measure of vitamin A status (Furr et al., 1989). The relative ratio of circulating isotopically labeled retinol is later assessed to estimate liver levels (Wang et al., 2000; Hickenbottom et al., 2002b) and without this technique, a liver biopsy would be necessary to estimate liver levels.

4.9   Analysis of Metabolites

Techniques used to identify new carotenoids are also employed to identify carotenoid metabolites in various photosynthetic organisms, as well as animals and humans. A new metabolite might be identified in a food or biological extract by HPLC-PDA, with the observation of a new peak with a UV/Vis spectrum similar to a carotenoid, or which produces an MS fragment similar to other known carotenoids. Alternatively, the metabolism may be induced in vitro by creating ideal biological conditions for generating metabolites (with intestinal mucosa, for example: dos Anjos Ferreira et al., 2004).

Once a new compound is observed, multiple steps are taken to identify the substance accurately. If it is present in high enough quantities, the compound would be isolated and analyzed by NMR for conclusive identification. Alternatively, if the compound is present in small amounts (as is often the case for metabolites), putative "standards" are synthesized and classified by numerous analytical techniques (including UV/Vis, IR, NMR, HPLC, GC, and MS), and compared against the unknown compound. Standards might be synthesized by building the molecule from smaller intermediates (Khachik and Chang, 2009) or by oxidizing a parent carotenoid to create smaller fragments (Caris-Veyrat et al., 2003).

These types of technique have been employed to identify a number of carotenoids in multiple types of samples. Recently GC-MS and authentic standards were used to identify volatile carotenoid metabolites from plant tissues (Vogel et al., 2008) and numerous studies have identified β-carotene metabolites in animals and humans using a variety of analytical techniques (Hu et al., 2006; Ho et al., 2007). These techniques have also been used to identify lycopene metabolites in both foods and biological samples (Khachik et al., 1997; Bouvier et al., 2003; Kopec et al., 2010) and the metabolism of lutein, zeaxanthin, and β-cryptoxanthin (Bernstein et al., 2001; Prasain et al., 2005; Mein et al., 2011).

It should be noted that extreme care should be taken when attempting to prepare and analyze samples for carotenoid metabolites. As mentioned previously, carotenoids are very labile, and putative "metabolites" can easily be created during sample handling and storage. With increasingly sensitive instrumentation, especially with MS, the ability to detect very small amounts of carotenoid degradation products is of concern. The importance of working with pure solvents, avoiding harsh sample preparation procedures (like saponification), and reducing the time from sample extraction to analysis cannot be overlooked.

4.10   Resonance Raman Spectroscopy

Carotenoids have been shown to be deposited in a number of tissues in the body. However, it is often difficult to safely and noninvasively determine carotenoid levels without sampling tissue. Resonance Raman spectroscopy (RRS) has been shown to be an alternative to assessing carotenoid levels in the macula of the eye (Bernstein et al., 1998). RRS has also been validated as a tool for measuring skin carotenoids, and RRS measurements have been significantly correlated with skin carotenoid levels as determined by HPLC (Ermakov and Gellermann, 2010; Mayne et al., 2010). Skin carotenoids may also provide a better measure of long-term carotenoid consumption as compared to blood carotenoid levels, since the half-life of carotenoids in blood is quite short. Thus, recent work has also suggested that, for large epidemiological studies, RRS measurements of skin carotenoids may serve as a better objective measure of fruit and vegetable consumption than measuring blood levels of carotenoids or administering food frequency questionnaires (Mayne et al., 2010).

4.11 Conclusions and Future Work

If history is any guide, then we foresee great potential for growth in the field of carotenoid analysis in the coming years. Sample preparation methods that quickly and effectively break down and remove sample matrix while preserving carotenoids intact will improve the accuracy of both parent carotenoid and carotenoid metabolite identification and quantitation.

The development of ultra high-pressure liquid chromatography technology has dramatically reduced the analysis time for a number of small molecules. However, we have found that the analysis of carotenes is poor with the currently available column chemistries. The development of C30 columns with backbones that can withstand ultra high pressure would allow carotenoid chemists to take full advantage of this technology.

In addition, the development of even "softer" (i.e. gentler) ionization techniques (beyond those already discussed) will improve the sensitivity of mass spectrometry as a tool for carotenoid identification and quantitation. Interfacing future ionization techniques with other MS tools may further expand opportunities. For example, a softer ionization technique that preserves carotenoid geometrical isomers in-source might allow isomers to be successfully separated via IMS and eliminate the need for prior separation by HPLC.

Finally, the field of metabolomics is still in its early stages of development. With advances in all the areas listed above (sample preparation, HPLC analysis, and MS analysis), the use of metabolomics to analyze metabolic pathways involving carotenoids may evolve from a simple "targeted" approach to a more sophisticated "fingerprinting" approach.

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Chapter 5

Analysis Methods of Anthocyanins

Ronald L. Prior and Xianli Wu

Abstract

Anthocyanins are water-soluble plant pigments responsible for the blue, purple, and red color of many plant tissues. Over 600 naturally occurring anthocyanins have been reported and they vary in: (1) the number and position of hydroxyl and methoxyl groups on the basic anthocyanidin skeleton; (2) the identity, number, and positions at which sugars are attached; and (3) the extent of sugar acylation and the identity of the acylating agent. The analysis of anthocyanins has become particularly important because of the numerous studies dealing with their potential health effects, and the study of absorption, metabolism, and mechanisms of action. The varying complexity of the structure has prompted the need for developing new analytical techniques for their extraction, fractionation, detection, and analysis. This chapter provides an updated review of newer methods that utilize high-performance liquid chromatography, UHPLC, and capillary electrophoresis and their hyphenation with mass spectrometry to study anthocyanins, one of the major subclasses of flavonoids. The many different structures, the complex chemistry, and the instability of anthocyanins have provided challenges in the identification and analysis of anthocyanins. The detection and identification of anthocyanins and their metabolites in blood plasma and body tissues has presented further challenges because of the extremely low levels that are generally present. The lack of commercially available anthocyanin standards has been a limitation in much of the quantitative analysis of anthocyanins.

Keywords: Cyanidin; delphinidin; peonidin; petunidin; pelargonidin; malvidin; polymeric color; HPLC; MS/MS.

5.1   Introduction

Anthocyanins are water-soluble plant pigments responsible for the blue, purple, and red color of many plant tissues. The analysis of anthocyanins has become particularly important because of the numerous studies dealing with their potential health effects (Chen et al., 2009; Galvano et al., 2009; Varadinova et al., 2009; Woodward et al., 2009; de Pascual-Teresa et al., 2010; Knaup et al., 2009; Kokotkiewicz et al., 2010; Krikorian et al., 2010; Takikawa et al., 2010). However, the many different structures of anthocyanins and their complex chemistry have provided challenges in their analysis.

5.2   Chemical Structures, Sources, and Levels Found in Natural Sources

The basic chemical structure of anthocyanidins (aglycone) is shown in Figure 5.1. Over 600 naturally occurring anthocyanins have been

	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3
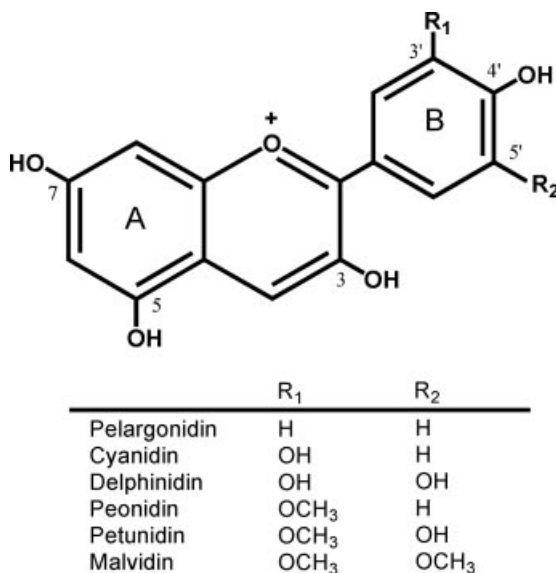
Figure 5.1   Structures of 6 major anthocyanidins found in nature. Sugar moieties are generally on position 3 of the C ring.

reported (Anderson, 2002) and they vary in: (1) the number and position of hydroxyl and methoxyl groups on the basic anthocyanidin skeleton; (2) the identity, number, and positions at which sugars are attached; and (3) the extent of sugar acylation and the identity of the acylating agent (Prior, 2004). Anthocyanins occur primarily as glycosides of their respective aglycone anthocyanidin-chromophores (Figure 5.1), with the sugar moiety mainly attached at the 3-position on the C-ring or, in some cases, at the 5- or 7-position on the A-ring. Differences in the aglycones occur as a result of differing hydroxyl or methoxy substitutions at the 3′- and 5′-positionson the B ring (Figure 5.1). Although about 17 anthocyanidins have been found in nature, only 6 of them – cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg), and malvidin (Mv) –are ubiquitously distributed (Figure 5.1). Glucose (glc), galactose (gal), arabinose (ara), rhamnose (rha), and xylose (xyl) are the most common sugars that are bonded to anthocyanidins as mono-, di-, or tri-saccharide forms (Figure 5.2). The sugar moieties may also be acylated by a range of aromatic or aliphatic acids. Common acylating agents are cinnamic acids (Figure 5.2). Examples of some of the complex structures that can occur in foods are in publications by Wu et al. (2005b, 2005c).

Unlike other subgroups of flavonoids with a similar C6–C3–C6 skeleton, anthocyanins have a positive charge in their structure at acidic pH. In solution, the anthocyanin actually occurs in equilibrium with essentially four forms: the flavylium cation, the quinoidal base, the hemiacetal base, and chalcone (Cooke et al., 2005) (Figure 5.3). The relative amounts of these four forms vary with both pH and structure of the anthocyanins (Brouillard et al., 1977; Brouillard 1982; Mazza and Miniati, 1993). Anthocyanins exist primarily as the stable flavylium cation only when the pH is less than 2.

An estimation of the daily consumption of anthocyanins in the United States was made using NHANES 2001–2002 Nutrition Survey data (NHANES, 2001) and calculated concentrations of anthocyanins in most common foods (Wu et al., 2006). The overall intake was estimated as 12.5 mg/day/person. However, in an earlier paper (Kuhnau, 1976), the average daily intake of total anthocyanins was estimated to be 215 mg during the summer and 180 mg during the winter. In contrast, the daily intake of ACNs in Germany was estimated to average 2.7 mg/person in 2002 and varied between 0 and 76 mg/person (Watzl et al., 2002). This large difference in total anthocyanin daily
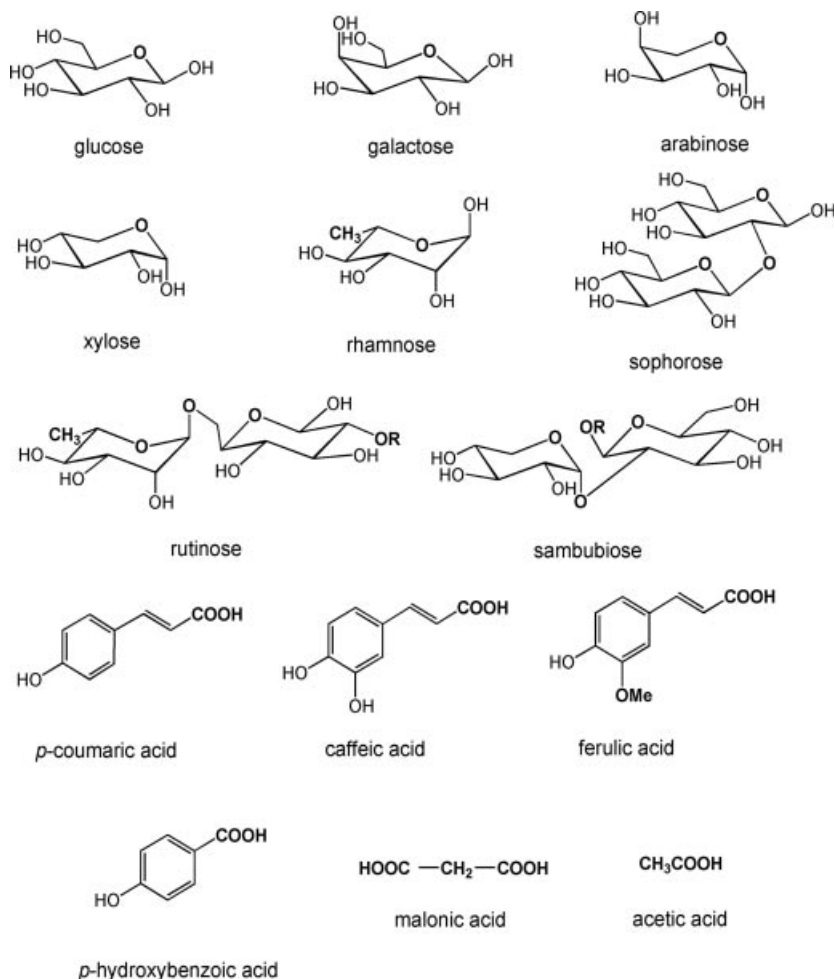
Figure 5.2 Selected sugars and aromatic or aliphatic acids that commonly occur in anthocyanin structures. Sophorose = 2-*O*-b-D-glucopyranosyl-D-glucose; rutinose = 6-*O*-L-rhamnosyl-D-glucose; sambubiose = β-D-xylosyl-(1 → 2)-β-D-glucose.

intake may result from different food intake data. Valid and accurate food intake data as well as complete food anthocyanin concentration data is critical for the estimation of dietary intake. A further complication occurs when the same food is available in a variety of forms that may have distinctly different amounts of anthocyanins (i.e. red or white wine; dark- or light-colored juices, etc.), but the food intake information generally is not detailed enough to differentiate the intakes
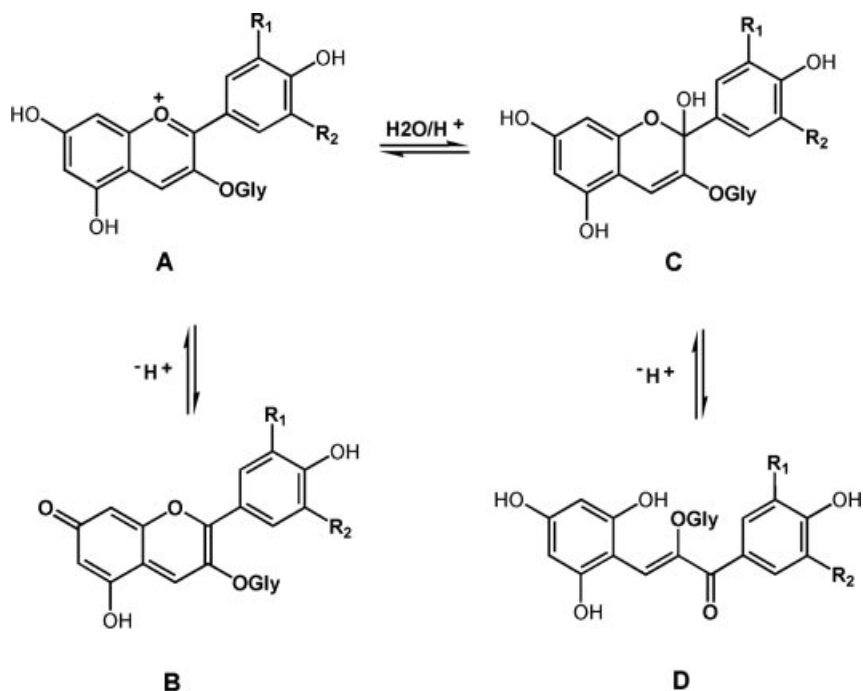
Figure 5.3 Simplified diagram of anthocyanin structural transformation in solution. A: flavylium cation, B: the quinoidal base, C: the hemiacetal base, D: chalcone. (Gly: glycoside) (Modified from Mazza and Miniati, 1993).

of the different sources. Thus, any estimation of anthocyanin intake may be subject to considerable variability.

This review will focus on some of the methods that have been used in the analysis of anthocyanins and will highlight some of the challenges that exist with the different methods.

5.3 Antioxidant and Biological Functions of Anthocyanins

In recent years many studies have shown that anthocyanins display a wide range of biological activities including antioxidant (Lyall et al., 2009; Broncel et al., 2010), anti-inflammatory (Xia et al., 2009; Mauray et al., 2010), antimicrobial (Leitao et al., 2005), and anticarcinogenic (Thomasset et al., 2009; Yun et al., 2010; Zu et al., 2010)

activities. In addition they exhibit a variety of effects on blood vessels, platelets, and lipoproteins and may act to reduce the risk of coronary heart disease (Mazza 2007; de Pascual-Teresa et al., 2010). Hearts from rats fed an anthocyanin-rich diet were more resistant to regional ischemia and reperfusion insult in Langendorff preparations. Moreover, in an *in vivo* model of coronary occlusion and reperfusion, infarct size was reduced in rats that consumed the anthocyanin-rich diet compared to those that consumed the anthocyanin-free diet. Cardioprotection was associated with increased myocardial glutathione levels, suggesting that dietary anthocyanins might modulate cardiac antioxidant defenses (Toufektsian et al., 2008).

Pretreatment of rats with *Aronia melanocarpa* fruit juice (5, 10, and 20 mL/kg), which is rich in anthocyanins, decreased gastric lesions caused by indomethacin (Valcheva-Kuzmanova et al., 2005). Indomethacin-induced gastric mucosal damage in the rat was accompanied by the development of oxidative stress as evidenced by the accumulation of malondialdehyde.

Consumption of anthocyanins (34.5 mg/day), contained in *Aronia melanocarpa* (chokeberry) juice, was found to lower blood parameters of redox status in rowers performing physical exercise during a 1-month training camp. After the supplementation period, the concentrations of TBARS in blood samples collected 1 min after the exercise test, and following a 24-h recovery period, were significantly lower in the subjects receiving chokeberry juice than in the control group. The investigators suggested that the anthocyanins may have enhanced the endogenous antioxidant defense system (Pilaczynska-Szczesniak et al., 2005).

Dietary supplementation with a chokeberry fruit extract (0.2%) decreased the activity of maltase and sucrase as well as increased the activity of lactase in the mucosa of the small intestine (Jurgonski et al., 2008). Its ingestion also led to the improvement of antioxidant status, as indicated by the reduction in the concentration of a lipid peroxidation indicator (TBARS) in organ tissues (liver, kidney and lung); some cholesterol-lowering and distinct hypoglycemic actions were also observed. The mechanism of glucose reduction is likely to be multifactorial, but decreased activity of mucosal disaccharidases may be one of the components of this mechanism (Jurgonski et al., 2008).

Consumption of a blueberry extract by mice was found to ameliorate hyperglycemia and insulin in-sensitivity via activation of AMP-activated protein kinase (AMPK) (Takikawa et al., 2010). Dietary blueberry extract significantly reduced blood glucose concentrations and enhanced insulin sensitivity. AMPK was activated in white adipose tissue, skeletal muscle, and the liver of diabetic mice fed the blueberry extract. This activation was accompanied by the upregulation of glucose transporter 4 in white adipose tissue and skeletal muscle and the suppression of glucose production and lipid content in the liver. At the same time, acetyl-CoA carboxylase was inactivated and PPARα, acyl-CoA oxidase, and carnitinepalmitoyl-transferase–1A were upregulated in the liver. These changes resulted in improved hyperglycemia and insulin sensitivity in type 2 diabetes (Takikawa et al., 2010).

In a double-blind, placebo-controlled, crossover study (Matsumoto et al., 2005), the effects of blackcurrant anthocyanin intake on peripheral circulation in human subjects during rest and during typing work was investigated. The intake of blackcurrant anthocyanins improved shoulder stiffness caused by typing work by increasing the peripheral blood flow and reducing muscle fatigue.

Although anthocyanins have been shown to exert a wide range of health benefits through antioxidant or other mechanisms (Prior 2004; Prior et al., 2006; Crozier et al., 2009), without knowing the rate and extent of their absorption, metabolism, and tissue or cell distribution, the role of anthocyanins in disease prevention will still be an enigma. Furthermore, for anthocyanins and other flavonoid compounds, their primary forms found circulating in blood or in tissues after oral ingestion may not necessarily be the original forms found in the diet, but are the metabolites or breakdown compounds generated during absorption and/or metabolism. These newly generated compounds may have differing, or the same, biological effects compared to their precursors. Thus, knowledge about the absorption, metabolism, and bioavailability of anthocyanins is a crucial step in order to understand their health effects (Prior, 2004). Analytical methods are needed to define levels of consumption from various diet sources as well as the disposition of anthocyanins and their metabolites in body tissues.

5.4   Sample Preparation for Anthocyanin Analysis

5.4.1   *Sample Extraction and Clean-up for Botanical Samples*

It is difficult to find one solvent system that is universal for all of the different polyphenolic or flavonoid compounds found in botanical samples. Normally different solvent systems are chosen on the basis of the specific analytical task. Since the flavyliumcation is the stable form of anthocyanins, extraction solvents should contain a weak acid to maintain the pH at 2 or below. Two commonly used solvent systems for polyphenol analysis and antioxidant capacity measurement are (1) an acetone/water/acid system, which has been demonstrated to be a good solvent system for the extraction of proanthocyanidins (Rohr et al., 2000) or (2) a methanol/water/acid system which has been used to extract anthocyanins. Both acetone and methanol have been used for anthocyanin analysis. However, it has been demonstrated that anthocyanins may undergo unusual facile reactions with acetone to give rise to pyranoanthocyanins (Lu et al., 2001, 2002; Wu et al., 2004a; Leopoldini et al., 2010). Pyranoanthocyanins can further undergo hydrolysis and rearrangement to generate furoanthocyanidins. These findings were observed in blackcurrant and elderberry extracts using acetone/water/acetic acid extractions (Wu et al., 2004a). Six artificial anthocyanins from black currant extracts using acetone/water/acetic acid extractions were observed; four pyranoanthocyanins and two furoanthocyanidins were formed either from cyanidin or delphinidin glycosides (Wu et al., 2004a). From elderberry extracts using acetone/water/acetic acid, in which cyanidin-based anthocyanins are predominant, concentrations of pyranoanthocyanins were much higher and no furoanthocyanidins were detected. Although the quantities of these products formed are small, the data indicate that acetone may not be a good solvent for anthocyanin extraction and analysis (Wu et al., 2004a).

A typical extraction solvent used is a mixture of methanol/water/formic acid in a ratio of 85:15:0.5 (Wu et al., 2004a, 2006). Hager (2008) and Hager et al. (2008b) used methanol/water/formic acid in a ratio of 60:37:3 (v/v/v) to extract pureed blackberry samples. Depending upon the type of sample, the homogenized samples may be filtered, and the filter cakes isolated and the extraction repeated (Hager 2008; Hager et al., 2008b). Barnes et al. (2009) investigated sample homogenization effects, extraction solvent selection, type of acid, and amount in

extraction of blueberry anthocyanins. A mixture of methanol/water/ trifluoroacetic acid (70:30:1, v/v/v) was found to be the best solvent system for blueberry anthocyanin extraction among those tested (Barnes et al., 2009). Trifluoroacetic acid has also been effectively used to stabilize anthocyanins during the extraction of plasma and urine samples (Cao et al., 2001; Wu et al., 2004b, 2005a).

Generally it is not necessary to clean up extracts from plant samples before HPLC analysis. The unique detection wavelength in the visible range by anthocyanins allows them to be selectively detected from other potentially interfering compounds.

5.4.2 Urine Sampling and Sample Clean-up

Due to the fact that anthocyanins only exist as stable flavylium cation at low pH, it is suggested that acid (e.g. TFA) be added into urine to stabilize anthocyanins immediately after the urine is collected. A 20% solution of 0.44 M TFA into urine samples has been used (Wu et al., 2004b, 2005a). Urine should be stored at $-70\,°C$ until analysis. For analysis, urine is normally centrifuged and then subjected to solid-phase extraction (SPE) for further extraction and concentration. In one example, a SepPakC$_{18}$ cartridge was prepared by elution with 10 mL 100% MeOH and subsequently with 10 mL 5% formic acid in water. The urine is then applied to the column and washed with 10 mL 5% formic acid in water. The anthocyanins were eluted from the column with 5% formic acid in MeOH. The acidified methanol fraction was evaporated almost to dryness in a Speed Vac® concentrator (Thermo-Savant, Holbrook, NY) and reconstituted in 1 mL of methanol/water/ formic acid (85:15:0.5 v/v/v) (Hager et al., 2008b).

5.4.3 Plasma and Tissue Sample Preparation for Anthocyanin Analysis

Similar to the procedures of sample preparation for anthocyanin anaylsis of urine samples, acid (e.g. TFA) is recommended to be added into plasma or tissue homogenate to stabilize anthocyanins. The samples should be stored at $-70\,°C$ if they are not to be analyzed immediately. SPE is also the prominent method of cleaning up the sample, mainly to remove proteins and peptides. Nevertheless, different SPE procedures, including the type of SPE cartridge and eluting solvents, varied among different investigators.

In the analysis of biological samples, such as plasma or serum, the quantity of sample available is a critical parameter in most cases. The use of microelution SPE (microSPE) plates as a sample pretreatment technique has been found to be beneficial because the loaded sample volume is low (Marti et al., 2010). An off-line microSPE and ultra-performance LC-ESI-MS/MS (UPLC-ESI-MS/MS) method was developed and validated to determine procyanidins and anthocyanins in spiked plasma samples (Table 5.1; Method 10). The sample pretreatment using microSPE allowed for the simultaneous determination of procyanidins and anthocyanins from plasma by using a small sample volume (350 µL) without an evaporation step prior to the chromatographic analysis. Moreover, the use of the UPLC technique allowed for the determination of the studied compounds at low concentration levels in a short analysis time (approximately 12.5 min). This method was used to determine catechin and epicatechin glucuronide, methyl catechin and epicatechin glucuronide, and methyl catechin and epicatechin sulfate and the parent anthocyanins in rat plasma samples 4 h after consumption of 5 g/kg BW of a grape pomace extract. Compounds were detected in the nM range (Marti et al., 2010).

5.5   Traditional, Nonchromatographic Analysis of Anthocyanins

5.5.1   Quantitation of Anthocyanins by UV/Visible Spectroscopy (pH Differential Method)

Measurement of absorptivity of a solution of a crude extract of anthocyanins from a food or botanical extract at a single wavelength has been used for the measurement of total anthocyanins. This is possible because anthocyanins have a typical absorption band in the 490 to 550 nm region of the visible spectra. This simple method may be inappropriate in some cases because of interferences from anthocyanin degradation products. In an attempt to overcome this deficiency, a subtractive or differential method has been used to quantify anthocyanins separate from their degradation products (Jackman et al., 1996). The differential method measures the absorbance at two different pH values, and relies on the structural transformations of the anthocyanin chromophore as a function of pH (Figure 5.3). This concept was first introduced by Sonheimer and Kertesz in 1948, who

Table 5.1 HPLC conditions for different methods for separation of anthocyanins.

#	Column	Solvents	Detection	Gradient	Sample Type	Reference
1	250 × 4.6 mm Symmetry C_{18} – Waters 1 mL/min	A: Aqueous 5% Formic acid B: 100% methanol	DAD	2% B to 60% B over 60 min	Blackberry	Hager et al., 2008b
2	250 × 4.6 mm, 5 μm, Zorbax Stablebond C_{18} – Agilent 1 mL/min	A: Aqueous 5% Formic acid B: 100% methanol	DAD	5% B, 0–2 min; 5–20% B, 2–10 min; 20% B, 10–15 min; 20–30% B, 15–30 min; 30% B, 30–35 min; 30–45% B, 35–50 min; 45% B, 50–55 min; 45–5% B, 55–65 min; 5% B, 65–68 min	Simple profile for most samples	Wu et al., 2004a; 2005c
3	250 × 4.6 mm, 5 μm, Zorbax Stablebond C_{18} – Agilent 1 mL/min	A: Aqueous 5% Formic acid B: 100% methanol	DAD	5% B, 0–2 min; 5–24% B, 2–10 min; 24% B, 10–15 min; 24–35% B, 15–30 min; 35% B, 30–35 min; 35–45% B, 35–50 min; 45% B, 50–55 min; 45–5% B, 55–65 min; 5% B, 65–68 min	Black raspberry	Wu et al., 2004a, 2005c

(*Continued*)

Table 5.1 (*Continued*)

#	Column	Solvents	Detection	Gradient	Sample Type	Reference
4	250×4.6 mm, 5 μm, Zorbax Stable-bond C_{18} – Agilent 1 mL/min	A: Aqueous 5% Formic acid B: 100% methanol	DAD	5% B, 0–2 min; 5–20% B, 2–10 min; 20% B, 10–15 min; 20–25% B, 15–30 min; 25% B, 30–35 min; 25–33% B, 35–50 min; 33% B, 50–55 min; 33–36% B, 55–65 min; 36–45% B, 65–70 min; 45–53% B, 70–75 min; 53–55% B, 75–80 min; 55–70% B, 80–84 min; 70–5%, 84–88 min; 5% B, 88–90 min	Complex (blueberry, Concord grape, red grape)	Wu et al., 2005c
5	150 mm × 300 μm I.D., 5 μm; Gemini C18 – Phenomenex 5 μL/min	A: 0.12% (v/v) tri-fluoroacetic acid in water + 5% acetonitrile B: 0.12% (v/v) tri-fluoroacetic acid in acetonitrile	Desorption nano-ESI mass MS	90% A, 0–5 min; 80% A, 5–10 min; 70% A, 10–30 min; 50% A, 30–40 min; 50% A, 40–50 min; 30% 50–54 min; 0% A, 54–60 min	Wine	Hartmanova et al., 2010

No.	Column	Mobile phase	Detection	Gradient	Application	Reference
6	250 × 2 mm, 4 μm, Synergi RP-Max, Phenomenex 0.2 mL/min	A: 5% Acetonitrile in 1% formic acid B: 50% acetonitrile in 5% formic acid	DAD	5–50% B, 0–30 min	Raspberry Anthocyanins in brain	Mullen et al., 2010
7	250 × 4.6 mm, 5 μm, Zorbax Stable-bond C$_{18}$ – Agilent 1 mL/min	A: Aqueous 5% Formic acid B: 5% formic acid in methanol	DAD, MS	5% B, 0–2 min; 5–20% B, 2–10 min; 20% B, 10–15 min; 20–30% B, 15–30 min; 30% B, 30–35 min; 30–45% B, 35–50 min; 45% B, 50–55 min; 45–95% B, 55–65 min; 95% B, 65–75 min; 95–5%, 75–78 min;	Urine samples	Hager 2008
8	250 × 4.6 mm, 5 μm, Zorbax Stable-bond C$_{18}$ – Agilent 0.2 mL/min	A: Aqueous 5% Formic acid B: 5% Formic acid in methanol	DAD, MS	8% B, 0–1 min; 8–28% B, 1–25 min; 28–30% B, 25–30 min; 30–45% B, 30–40 min; 45% B, 40–43 min; 45–95% B, 43–48 min; 95% B, 48–50 min; 95–5%, 50–55 min;	Urine samples for increased separation of glucuronide forms	Hager 2008

Table 5.1 (*Continued*)

#	Column	Solvents	Detection	Gradient	Sample Type	Reference
9	50 mm × 2.1 mm i. d., 1.7 µm, Ac-quity UPLC BEH C18 Column, Waters 0.6 mL/min	A: Aqueous 5% Formic acid B: Acetonitrile	MS/MS, MRM mode	2–15% B, 0–3 min; 80% B, 3–4 min; 2% B, 4–5 min	Black currant anthocyanins in plasma	Nakamura et al., 2010
10	250 mm × 2.1 mm, 5 µm, Aquasil C18 column, Thermo Hypersil-Keystone	A: Aqueous 0.1% formic acid B: 0.1% formic acid in acetonitrile	MS – Triple quadrupole	5–30% B, 0–30 min; 5% B, 30 min; Or isocratic 25% B, 0–7 min	Plasma and tissue homgenates of anthocyanins from black raspberry	Ling et al., 2009
11	100 mm × 2.1 mm, 1.8 µm, Acquity high strength sili-ca T3 column	A: Aqueous 10% acetic acid B: Acetonitrile	MS/MS, MRM	5–35% B, 0–10 min; 35–80% B, 10–10.1 min; 80% B, 10.1–11 min; 80–5% B, 11–11.1 min; 5% B, 11.1–12.5	Anthocyanins in plasma	Marti et al., 2010

used pH 2.0 and 3.4. Since then, the use of other pH values (pH 1.0, colored; and pH 4.5, colorless) have been proposed (Fuleki et al., 1968; Wrolstad 1982; Lee et al., 2005). The pH differential method has been described as fast and easy for quantitation of monomeric anthocyanins (Lee et al., 2005). A collaborative study of 11 institutions was conducted to determine the total monomeric anthocyanin concentration by the pH differential method. Sample materials tested represented fruit juices, beverages, natural colorants, and wines. The repeatability relative standard deviation varied from 1.06 to 4.16% and the reproducibility relative standard deviation ranged from 2.69 to 10.12% (Lee et al., 2005). The pH differential spectrophotometric method (AOAC method 2005.02), and a high-performance liquid chromatography (HPLC) method (to be described later) were used on more than 500 samples to determine if a relationship exists between these two frequently utilized analytical methods (Lee et al., 2009). This study demonstrated a high linear correlation ($r > 0.925$, $p < = 0.05$) between the pH differential method and HPLC when determining the amount of anthocyanins within samples. In both methods, total anthocyanins were greater when values were expressed as malvidin–3-glucoside equivalents compared to cyanidin-3-glucoside equivalents. Thus, it is of critical importance to report the standard used to express the values (Lee et al., 2009). This illustrates a continuing challenge in the analysis of anthocyanins which is the availability of certified anthocyanin standards. With the pH differential method, only a single anthocyanin standard is used. Cyanidin-3-glucoside is commonly used since it is the most common anthocyanin found in foods (Francis, 1989).

5.5.2 Anthocyanins and Polymeric Color Analysis

Polymeric color is a term that has been recognized in the wine industries for many years. Condensation of anthocyanins and proanthocyanidins may contribute to the formation of polymeric color, although the structural characterization of the compounds that contribute to "polymeric color" are poorly defined. Studies using electrospray mass spectrometric analysis demonstrated the presence of pigmented material from grape skins that was chiefly composed of direct condensation products of anthocyanins extending up to trimers (Vidal et al., 2004). Recent studies have evaluated the effects of processing of blueberries,

blackberries, and black raspberries on formation of polymeric color (Brownmiller et al., 2008; Hager et al., 2008a, 2008b). Large increases in polymeric color values and a corresponding loss of monomeric anthocyanins with storage in thermally processed products was observed. Upon observing how high the polymeric color values could be in some anthocyanin-containing samples, a number of commercial products were analyzed for monomeric anthocyanins and polymeric color (Prior, unpublished data). Values ranged from 10 to 78% polymeric color. It was particularly surprising to find relatively high values in the lyophilized powders of various berries which ranged from 10 to 53% polymeric color, and the presence of "polymeric color" in samples of "purified" anthocyanins. The presence of "polymeric color" may alter quantitative values for monomeric anthocyanins if the pH differential method is being used for quantitation. A method for the determination of "polymeric color" has been described by Giusti and Wrolstad (2005). Sample extracts were diluted with water in order to have an absorbance reading between 0.5 and 1.0 at 512 nm. For analysis, 0.2 mL of 0.90 M potassium metabisulfite was added to 2.8 mL diluted sample (bisulfite bleached sample) and 0.2 mL of deionized water is added to 2.8 mL of diluted sample (nonbleached, control sample). After equilibrating for 15 min, but not more than 1 h, samples were evaluated at wavelengths of $\lambda = 700$ nm, 512 nm, and 420 nm. Color density was calculated using the control sample according to the following formula:

(1) $\text{Color density} = [(A_{420\ nm} - A_{700\ nm}) + (A_{512\ nm} - A_{700\ nm})]$

$$\times \text{Dilution factor}$$

Polymeric color was determined using the bisulfite-bleached sample using the following formula:

(2) $\text{Polymeric color} = [(A_{420\ nm} - A_{700\ nm}) + (A_{512\ nm} - A_{700\ nm})]$

$$\times \text{Dilution factor}$$

Percent polymeric color was calculated using the formula:

(3) $\text{Polymeric color} (\%) = (\text{Polymeric color}/\text{Color density}) \times 100$

It is critical to recognize the high reactivity and low stability of anthocyanins in the selection of methods of extraction and quantitation in the analysis of anthocyanins. An analysis of processed samples containing anthocyanins for polymeric color as well as monomeric anthocyanins is recommended in nutrition studies until we better understand any health effects (positive or negative) of the compounds that are part of the mix measured as "polymeric color."

5.6 HPLC Analysis of Anthocyanins

In recent years, with the increasing knowledge of the positive health effects of food polyphenols, the need to develop new separation techniques for anthocyanin extraction, fractionation, and analysis has been recognized. An exhaustive review of the application of counter-current chromatography, high-performance liquid chromatography, capillary electrophoresis, and their hyphenation with mass spectrometry to the study of food polyphenols including anthocyanins has been published byValls et al. (2009).

Reversed-phase high-performance liquid chromatography (RP-HPLC) is the usual method of choice for the separation of anthocyanins combined with an ultraviolet-visible (UV-Vis) or diode-array detector (DAD)(Hebrero et al., 1988; Hong et al., 1990). With reversed-phase columns the elution pattern of anthocyanins is mainly dependent on the partition coefficients between the mobile phase and the C_{18} stationary phase, and on the polarity of the analytes. The mobile phase consists normally of an aqueous solvent (water/carboxylic acid) and an organic solvent (methanol or acetonitrile/carboxylic acid). Typically the amount of carboxylic acid has been up to 10%, but with the addition of a mass spectrometer as a detector, the amount of acid has been decreased to as low as 1% with a shift from trifluoroacetic acid to formic acid to prevent quenching of the ionization process that may occur with trifluoroacetic acid. The acidic media allows for the complete displacement of the equilibrium to the flavylium cation, resulting in better resolution and a characteristic absorbance between 515 and 540 nm. HPLC separation methods, combined with electrochemical or DAD, are effective tools for anthocyanin analysis. The weakness of these detection methods is a lack of structural information and some nonspecificity leading to misattribution of peaks, particularly with electrochemical

detectors. With DAD detection, the wavelength monitored can be set to be specific for anthocyanins; however, with the electrochemical detector (ECD), other flavonoids compounds may elute in the same region of the chromatogram as anthocyanins which are detected by the ECD. The peaks cannot easily be assigned without purified anthocyanin standards. Mass spectrometry is currently the most selective analytical technique for the identification and quantification of unknown compounds from crude or partially purified samples of foods or food supplements.

5.6.1   *Identification and Characterization of Anthocyanins*

Analysis and identification of anthocyanins has progressed from thin-layer chromatography (TLC) and paper chromatography (PC) in early times to HPLC with diode array detector (DAD), and then, HPLC with mass spectrometry or with tandem mass spectrometry (Hong et al., 1990; Lee et al., 1992; Da Costa et al., 2000; Mazza et al., 2004). Several different MS technologies have been used for anthocyanin identification, including electron impact mass spectrometer (EI-MS), electrospray ionization mass spectrometer (ESI-MS), atmospheric pressure chemical ionization mass spectrometer (APCI-MS), and matrix-assisted laser desorption/ionization mass spectrometer (MALDI-MS) (Mazza et al., 2004). Among them, ESI-MS has been preferred by many researchers because of its unique advantages (Ryan et al., 1999; Oliveira, 2001). Use of mass spectrometry can reduce the reliance on retention time and UV-visible spectra and provide more structural information based on molecular weight and fragmentation pattern. However, mass spectra alone may not be 100% effective because MS cannot provide complete structural information. For different anthocyanins with the same mass spectra, one has to combine other information that can be obtained for peak identification. Uniquely, MS data can distinguish co-eluting peaks, which is common in samples with a complex anthocyanin composition. For example, in the Concord grape, what appeared to be a single peak in the HPLC chromatogram based upon the DAD signal, was shown to contain three separate anthocyanins when the mass spectra information was analyzed (Wu et al., 2005c).

The identification and peak assignment of anthocyanins can often be based on a comparison of their retention time and MS data with standards and published data. There are some general guidelines that

have been developed (Wu et al., 2005c, 2008) to assist in the identification of anthocyanins from an unknown sample. Retention time (t_R) is very important for the determination of anthocyanins, even if a mass spectrometer data is available. A simple elution order (retention time from short to long) for some common anthocyanidin glycosides using reversed-phase HPLC seems to fit most experimental conditions:

1. For the six common anthocyanidins: delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin.
2. For different glycosides and/or acylated groups with the same anthocyanidin (cyanidin): cyanidin-3,5-diglucoside, cyanidin-3-diglucoside, cyanidin-3-galactoside, cyanidin-3-sambubioside, cyanidin-3-glucoside, cyanidin-3-arabinoside, cyanidin-3-rutinoside, cyanidin-3-(malonyl)glucoside, cyanidin-3-xyloside, cyanidin-3-(6″-malonyl)-glucoside, cyanidin-3-(6″-acetyl)-glucoside, and cyanidin-3-(6″-coumaryl)-glucoside.

The obtained spectra can also give information on the nature of the aglycone, glycosylation, and possibility of acylation (Hong et al., 1990).

It is important to remember that changes in column packings and chromatographic systems may alter the retention of closely eluting compounds, and these elution schemes should only be used in conjunction with other identification tools such as tandem MS fragmentation patterns and high-mass accuracy measurements.

The classical method for anthocyanin analysis works quite well with rather simple mixtures of anthocyanin monoglucosides, but has some limitations for the separation and identification of more complex mixtures. Problems may occur with the fast-moving anthocyanidin-diglucosides, along with considerable overlap between the acetic and coumaric ester series. Multiple ρ-coumaric peaks for a given aglycone are observed which can be attributed to *cis* and *trans* isomers. Caffeoyl esters are difficult to detect because their polarities are very similar to their ρ-coumaroyl derivatives, and the molecular masses for the 3,5-diglucosides are identical with the caffeoylglucosides, which limits absolute identification by HPLC-MS (Valls et al., 2009).

Tandem mass spectrometry (MS-MS) using quadrupole mass analyzers or ion-trap analyzers facilitate the conducting MS-MS experiments and increases the sensitivity of detection. Fragmentation patterns of anthocyanins generally show the loss of a glycoside or

acylated-glycoside giving the corresponding aglycone. Giusti et al. (1999) reported that MS-MS resulted in cleavage of glycosidic bonds only between the flavylium ring and sugars directly attached to it. The only exception to this rule was with the rutinoside. However, there may be some other exceptions to this general rule. Wu et al. (2005c) found from the MS-MS data from cyanidin-3-(6″-malonyl) glucoside of cyanidin in marionberry or pelargonidin 3-(6″-malonyl) glucoside in strawberry two fragment peaks in the MS^2 spectra: the anthocyanidin-3-glucoside and the anthocyanidin. In these anthocyanins, the cleavage did not necessarily just happen between the anthocyanidin and the glycosides. We assume that whether the cleavage happens or not depends on the groups linked to the anthocyanidins. If an unstable acylated group like malonyl is linked to a glycoside, the cleavage between the glycoside and the malonyl group could also occur.

A systematic approach for optimizing the extraction and identification of anthocyanins from blueberries was explored using HPLC-UV and HPLC-ESI-IT-TOF-MS (Barnes et al., 2009). A method was developed for anthocyanin identification without the use of standards. Consideration was given to elution order by chromatographic separation with selective detection at 520 nm, high mass accuracy *m/z* values, tandem MS fragmentation, and previously published literature. Overall, 25 anthocyanins from a wild type highbush blueberry were identified and reported (Barnes et al., 2009).

In the wine industry, anthocyanins can be used as markers to classify wines according to the grape variety. However, this is a complex separation that requires very high chromatographic efficiency, especially in the case of aged red wines, due to the formation of pyranoanthocyanins. A novel mixed-mode ion-exchange reversed-phase column was reported to separate anthocyanins extracted from grapes of *Vitis labrusca* with a different elution order and selectivity for anthocyanins and pyranoanthocyanins, compared with those observed with C_{18} stationary phases (Vergara et al., 2010). Also, the polymeric fraction was found to elute as a clearly separated peak at the chromatogram's end. However, a comparison with a high efficiency C_{18} column with the same dimensions and particle size demonstrated that the tested mixed-mode column had broader peaks with a theoretical plate number below 8000, for malvidin–3-glucoside peak, which can be up to 10 times higher for a high-efficiency C_{18} column, depending on

the column manufacturer. Under the tested conditions, in mixed-mode phase, the analysis time was almost twice that of a C_{18} column with the same dimensions and particle size. The mixed-mode phase may have application for the separation ofanthocyanins in wine (Vergara et al., 2010), due to its improved selectivity, combined with a useful role in a second-dimension separation in preparative anthocyanin chromatography, but would not be a preferred method for the routine quantitative analysis of anthocyanins.

Desorption nano-electrospray (nano-DESI) has also been tested for qualitative analysis of anthocyanins in wine samples (Table 5.1; Method 5) (Hartmanova et al., 2010). Acidifying of the samples and providing an acidic spray liquid (methanol/water; 75:25 with 0.2% HCOOH) were essential for obtaining good quality spectra. From the nano-DESI-MS data, the ratio of two grape cultivars (Neronet and Rubinet) in a mixture could be determined. Detection of the main anthocyanins in slices of wine grapes, chokeberries, and elderberries or in a wine stain on cotton fabric was also possible (Hartmanova et al., 2010).

High-performance liquid chromatography equipped with diode array (HPLC-DAD) and electrospray ionization mass spectrometric detection (ESI-MS(n)) has been used to identify anthocyanins in muscadine grapes (Sandhu et al., 2010). Six different anthocyanin 3,5-diglucosides were identified in muscadine grape skins (Sandhu et al., 2010).

Direct-infusion electrospray ionization ion trap tandem mass spectrometry (ESI-IT-MS/MS) has been used for the qualitative study of anthocyanins in extracts from the skins of Clinton grapes; however, it was impossible to differentiate some compounds with the same nominal mass but with different elemental composition (Flamini et al., 2009). The capabilities of quadrupole time-of-flight mass spectrometry (QTOF-MS) coupled with Chip-liquid chromatography (LC-Chip) was subsequentlyshown to provide the complete sample anthocyanin fingerprint in Clinton grapes in less than 5 min. Multistage mass spectrometry (MS^n; $n > 2$) was not necessary to identify isobaric compounds, nor were deuterium-exchange experiments necessary to distinguish between compounds containing the same aglycone. The fast separation provided by this method bypasses the problem of petunidin–3-O-(6-O-acetyl)monoglucoside and delphinidin–3,5-O-diglucoside quantification, present in the direct-infusion approach, due to overlapping with matrix interferences (Flamini et al., 2009).

5.6.2 Quantitative Analysis of Anthocyanins in Botanicals

In Table 5.1, a number of different conditions are presented for the separation and quantitation of anthocyanins. For simple profiles, the gradient profile is not critical as separations can be achieved relatively easy (Table 5.1; Methods 1–3). However, for samples such as blueberry and Concord grape, which contain complex mixtures with 15 or more anthocyanins or with several acylated anthocyanins, the gradient needed to get resolution can be more complex (Table 5.1; Method 4). Wine samples also fall into this category. Desorption nano-electrospray ionization mass spectrometry has been used to solve some of the resolution and identification issues (Table 5.1; Method 5). However, issues related to quantitation have not been dealt with in this method.

With HPLC methods where individual anthocyanins are separated and the method of detection is DAD, individual anthocyanin standards are preferred for quantitation, but not always available. A standard mix of the glucoside form of the six aglycones of the anthocyanidins has been commercially available. However, obtaining a consistent purity has been a challenge at times. Because of the large numbers of anthocyanins present, it is not reasonable at this point in time to have a standard for every individual anthocyanin. Thus, some assumptions and compromises must be made in any quantitative analysis of anthocyanins. Calculation of the anthocyanins containing the same anthocyanidin based upon the glucoside equivalent has been a workable compromise.

One of the few manuscripts using mass spectrometry for quantitation was recently published (Nagy et al., 2009). A method was developed and validated using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for the quantification of anthocyanin constituents in milk-based food products. Protein precipitation with acidified methanol and ultrafiltration was utilized in sample preparation. Reversed-phase chromatography was optimized to achieve separation of 27 analytes in 10 min in order to reduce suppression effects, achieve a wide dynamic range, and most importantly, to resolve isomeric compounds. Positive-ion electrospray mass spectrometric detection and fragmentation of analytes was optimized, final transitions were selected for maximized selectivity, reliable quantification, and reduction of false positives. The main features of the method include (1) range of lower limits of detection of 0.3–30 ng/mL for glycosylated

analytes, 10–300 ng/mL for aglycones, (2) lower limits of quantification of 1–100 ng/mL for glycosylated analytes, 30–1,000 ng/mL for aglycones, (3) averaged intraday precision 9%, (4) calibrated range 2–180,000 ng/mL for glycosylated analytes, 60–600,000 ng/mL for aglycones, and (5) averaged accuracy 101%. Applications for yogurt and ice cream products are developed. The data presented suggest that this method will be useful to better characterize the polyphenol composition of milk-based food products for quality control, for assessment of dietary intake, and for polyphenol bioavailability/bioefficacy studies (Nagy et al., 2009).

5.6.3 HPLC Analysis of Anthocyanins in Biological (Plasma and Urine) Samples Coupled with MS

The analysis of urine, plasma and tissue samples presents challenges because of the low concentrations of anthocyanins and the formation of metabolites and the lack of appropriate standards. There are few reports of the quantitative analyses of anthocyanins in dietary supplements and biological matrices (Cooke et al., 2006; Tian et al., 2006; Giordano et al., 2007).

During the absorption process, anthocyanins can be conjugated with glucuronide and/or being methylated in the Bring (Wu et al., 2004b, 2005a). Unlike other flavonoids, anthocyanins cannot be treated with enzymes to release the conjugate to enable the primary anthocyanin to be quantitated in two separate chromatographic runs as "Total" and "conjugated", since anthocyanins are unstable to the incubation conditions at pH 7. Thus, methods are needed to detect and quantify the conjugated forms as well as the glycoside forms of the anthocyanins.

The primary methylation reaction that has been observed is the methylation of the 3′ hydroxyl group of cyanidin (Figure 5.1) with the formation of peonidin. A lesser quantity of the methylated 4′ hydroxyl group has been observed forming "isopeonidin." However, both of these compounds can also be conjugated. We have used HPLC-MS-MS analysis of plasma and urine samples to detect these compounds (Table 5.1; Method 6). The urine chromatographic separation was performed on a 250×4.6 mm Zorbax SB-C18 column (5 µm, Agilent, Santa Clara, CA) with 5% formic acid in water (A) and 5% formic acid in MeOH (B) at 1 mL/min with 0.7 mL/min diverted to the diode array detector (DAD) and 0.3 mL/min evaluated in the MS detector. For enhanced glucuronide separation, the

gradient was modified slightly (Table 5.1, Method 7). An MS-MS technique called multiple reaction monitoring (MRM) was used. Common anthocyanin MRM transitions are listed in Table 5.2 and were determined based on previous mass spectrometry work on anthocyanin and anthocyanin metabolites (Wu et al., 2005a, 2005b). Because of the lack of standards for specific metabolites, the quantification of cyanidin and peonidin metabolites was calculated based on cyanidin glucoside (449/287) and peonidin glucoside (463/301) equivalents, respectively (Wu et al., 2004b, 2005a).

In studies using MRM transitions, there was evidence that glucuronidation could occur at more than one site on the anthocyanidin (Hager, 2008). This was indicated by the presence of multiple peaks of both cyanidin and peonidin glucuronide MRM transitions (Figure 5.4). This has been observed in a previous study with cyanidin glucoside metabolism (Ichiyanagi et al., 2005). Approximately 9 peaks were observed for the cyanidin glucoside monoglucuronide transition (625/287). Eight peaks were observed for the peonidin glucoside monoglucuronide transition (639/301) (Figure 5.4). Although there are only 7 free –OH groups on the peonidin glucoside structure and 8 peaks were observed, this discrepancy may be explained by the methylation of the $4'$ OH (as opposed to the $3'$ for peonidin) which results in the formation of isopeonidin. Thus, some of the peaks at 639/301 may be formed from isopeonidin glucoside monoglucuronide. By excluding these additional peaks, the total glucuronide content may be underestimated by approximately 60–85% based on total peak area. Further research is needed with purified metabolites before all of these metabolites can be accurately quantified.

A liquid chromatography tandem mass spectrometry method for simultaneous determination of four anthocyanins from black currant (delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, and cyanidin-3-rutinoside) in human plasma has been developed and validated (Nakamura et al., 2010). Individual purified anthocyanins were used as standards. Samples were prepared using solid phase extraction, followed by chromatographic separation with a reverse phase C_{18} column with gradient elution using mobile phases containing water, acetonitrile, and formic acid. The quantification of four anthocyanins was determined by MRM using electrospray ionization. The method showed good selectivity, sensitivity (limits of quantification for four anthocyanins were 0.2 nmol/L), linearity

Table 5.2 Mass units used to monitor MRM transitions for detection of parent and daughter ions of anthocyanins.

Analyte	Molecular Ion (*m/z*)	Major Fragment Ion (*m/z*)
Native		
Cyanidin	287	137
Cyanidin-3-*O*-arabinoside	419	287
Cyanidin-3-*O*-galactoside	449	287
Cyanidin-3-*O*-glucoside	449	287
Cyanidin-3-*O*-rutinoside	595	287
Cyanidin-3-*O*-sophoroside	611	287
Cyanidin xyloside	419	287
Cyanidin malonylglucoside	535	287
Cyanidin dioxalylglucoside	593	287
Metabolites		
Cyanidin sulfate	367	287
Cyanidin monoglucuronide	463	287
Cyanidin glucoside monoglucuronide	625	287
Native		
Delphinidin	303	303
Delphinidin-3-*O*-glucoside	465	303
Delphinidin-3-*O*-rutinoside	611	303
Malvidin	331	287
Malvidin-3-*O*-glucoside	493	331
Pelargonidin	271	121
Pelargonidin-3-*O*-glucoside	433	271
Native		
Peonidin	301	286
Peonidin-3-*O*-arabinoside	433	301
Peonidin-3-*O*-galactoside	463	301
Peonidin-3-*O*-glucoside	463	301
Metabolites		
Peonidin monoglucuronide	477	301
Peonidin malonylglucoside	549	301
Peonidin dioxalylglucoside	607	301
Peonidin rutinoside	609	301
Peonidin xyloside monoglucuronide	639	301
Peonidin glucoside monoglucuronide	639	301
Native		
Petunidin	317	203
Petunidin-3-*O*-glucoside	479	317
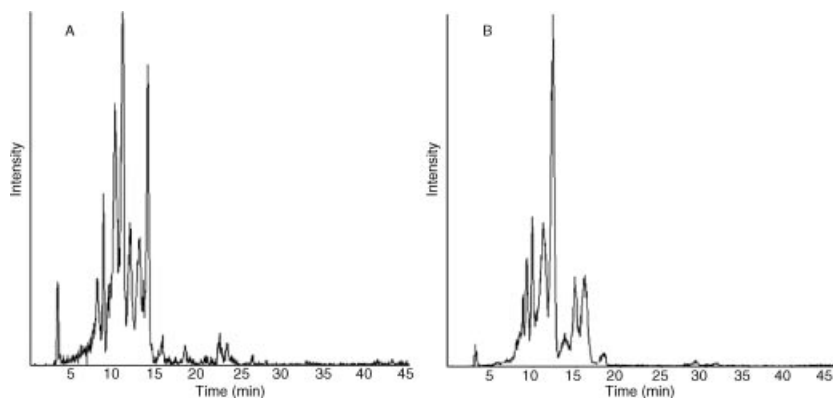
Figure 5.4 Chromatograms with multiple reaction monitoring (MRM) transitions at 625/287 (A) and 639/301 (B) that show the multiple glucuronide forms of cyaniding-3-glucoside and peonidin-3-glucoside, respectively. (From Hager, 2008).

(0.2–20 nmol/L; $r > 0.999$), intra- and interday precision, accuracy ($<14\%$), and recovery was 62.5–85.7%). Analyte stability was investigated in detail. This method was successfully applied to the determination of the four anthocyanins in human plasma after ingestion of a single dose of blackcurrant anthocyanins (87.9 micromol (58.8 mg) total anthocyanins)(Nakamura et al., 2010). No anthocyanin metabolites were studied.

A highly sensitive and specific LC-MS/MS assay has been developed and validated to simultaneously quantify the fouranthocyanins from black raspberry (cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-sambubioside and cyanidin-3-(2(G)-xylosyl) rutinoside) in human saliva, plasma and oral tissue homogenates (Ling et al., 2009). In saliva and plasma, the lower limits of quantification for these anthocyanins were 1.0 ng/mL and up to 5 ng/mL in tissue, depending upon the anthocyanin. The within-run and between-run coefficients of variations at the quality control concentrations (5.0, 50, and 500 ng/mL) were $<12\%$, except for a few cases. This assay was subsequently used in a pilot pharmacology study to evaluate the effects of topical application of a 10% (w/w) freeze-dried black raspberry bioadhesive gel to selected mucosal sites in the posterior mandibular gingiva. Measurable saliva and tissue levels of the freeze-dried black raspberry anthocyanins confirmed that gel-delivered anthocyanins are readily distributed to saliva and easily penetrate human oral mucosa (Ling et al., 2009).

5.7 Conclusion

Considerable advances in methods for the detection, identification and quantification of anthocyanins have been made recently, particularly with the advent of UPLC and improved mass spectroscopy methods. Additional work is needed, particularly with quantitation using MS. However, the biggest limitation in the quantitation of anthocyanins is the commercial availability of anthocyanin standards, which are nonexistent for most anthocyanins, and those that are available are fairly expensive. Some laboratories have been able to isolate and purify the particular anthocyanins of interest (Ling et al., 2009; Nakamura et al., 2010), but most laboratories do not have the equipment, time, and/or expertise to fully validate the anthocyanin standards.

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Chapter 6

Analysis Methods of Ellagitannins

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Abstract

Ellagitannins are a structurally diverse class of polyphenolics found in many fruits, nuts, and woody plants. Plants with high concentrations of ellagitannins have been attributed to a variety of health benefits and they are important contributors to color, flavor, and stability of many foods. These compounds present numerous analytical challenges to identify and characterize based on their structural complexity and difficulty to separate using partition chromatography. This chapter describes the predominant ellagitannins present in fruits, nuts, and oak wood used for the storage of wine and distilled spirits, and describes methods for their extraction, isolation, and analysis using spectrophotometric and chromatographic techniques.

Keywords: Ellagitannin; hydrolysable tannin; ellagic acid; hexahydroxydiphenoyl acid; solid-phase extraction; mass spectroscopy

6.1 Introduction

Ellagitannins are among the most diverse and structurally complex class of polyphenolics in plants. They are important contributors to color, flavor and stability in fruits as well as oaked wine and distilled spirits. Recently, consumption of ellagitannins has also been linked to potential health benefits. The extraction, isolation, and identification

of ellagitannins and other pro-ellagic acid precursors has been the subject of many published works, and present unique analytical challenges not experienced with other polyphenolic compounds. Ellagitannins are found in select fruits, woody plants, and tree nuts, and vary considerably in molecular weight from small monomers to high molecular weight polymers. These differences, coupled with the ability to complex with other phytochemical classes, offer unique analytical challenges to adequately extract, isolate, identify, and quantify ellagitannins. Despite the high molecular weights of many ellagitannins, most are readily water-soluble. However, to adequately extract from plant materials a combination of organic solvents or alcohols are commonly utilized to gain maximal extraction efficiency. After extraction, separating individual ellagitannins by partition chromatography presents physical challenges as ellagitannins often co-elute with other ellagitannins and other classes of polyphenolics. To simplify the extraction matrix, extensive solid-phase extraction (SPE) techniques and liquid-phase partitions are required to isolate or purify ellagitannins for subsequent evaluation, often by NMR to determine structural characteristics. Spectrophotometric assays specific to ellagitannins or hydrolytic products of ellagitannins have been developed for the rapid screening of plants and extracts, but modern high-performance liquid chromatography (HPLC) coupled with normal or high-resolution mass spectroscopy (MS) have become commonplace for evaluating these compounds. Although numerous analytical instruments have been used for ellagitannin analysis, MS dominates as the most common analytical tool and is highly suitable for the step-by-step identification of the various esterified moieties present on a typical ellagitannin. The developed MS methods are rigorous enough for a routine evaluation to screen plants for ellagitannins, yet sensitive enough to characterize novel compounds and ellagitannin complexes. This chapter will describe the predominant ellagitannins present in fruits and oak woods used for the storage of wine and distilled spirits, and describe methods for their extraction, isolation, and analysis.

6.2   Background on Ellagitannins

The presence of ellagic acid derivatives can be used to help to classify plants and determine the wood quality, and is related to antioxidant

capacity and potential health benefits of fruits, nuts, and botanicals. Pro-ellagic acid compounds may exist in many forms, including ellagic acid glycosides and ellagitannins. Ellagic acid glycosides, simple, structurally related compounds to ellagitannins are not true tannins due to their inability to bind protein. Ellagic acid glycosides consist of ellagic acid esterified to varying sugar moieties, mostly at the 4-position. Examples include ellagic acid glucoside, rhamnoside, and xyloside found in muscadine grapes (Lee and Talcott, 2002), ellagic acid arabinoside, acetylarabinoside, and acetylxyloside found in raspberries (Mullen et al., 2003*)*, and ellagic acid pentoside/deoxyhexoside in strawberries (Aaby et al., 2007). Together or independently, plants may also accumulate ellagitannins that consist of a central glucose molecule with one or more biaryl-linked gallic acid units known as hexahydroxydiphenoyl (HHDP) acid. Although the complete biosynthetic route for ellagitannins is still under investigation, it is accepted that monomeric ellagitannins are derived from pentagalloyl-ß-D-glucopyranose (PGG) via oxidative coupling of HHDP and are the basis of larger dimeric or oligomeric ellagitannins (Figure 6.1). Hydrolysis of the HHDP group under mild acid and heat will instantly convert the bislactone to free ellagic acid as in the case of tellimagrandin II, which is among the simplest ellagitannins whose single HHDP unit will form one molecule of free ellagic acid, one molecule of glucose, with three residual molecules of gallic acid.

Some 500 or more ellagitannins have been characterized in plants due to their varying arrangements around PGG and the oxidative coupling that creates increasingly larger and more complex compounds that offer unique analytical challenges. Investigations surrounding the stereochemistry of HHDP linkages have revealed that the most common are 4,6 and 2,3 linkages to the ß-glucosyl unit and generally have (*S*)-configurations, while the less observed 3,6 and 2,4 glucosidic linkages are generally (*R*)-configured (Khanbabaee and Ree, 2001). This work also describes two polyphenolic classes involving ellagitannins, with the first being the classic ellagitannin with at least two biaryl galloyl units (i.e. HHDP) covalently linked and esterified to a polyol. The second class includes ellagitannins that contain a glycosidically-linked catechin. Additional complexity is afforded to ellagitannins when the HHDP groups are synthesized as covalent C–C (*C*-glycosidic) bonds to glucose (vescalagin, castalagin, casuarinin, stachyurin, and casuariin), as gluconic acid (lagerstannin C), to flavonols (camelliatannin A), to a
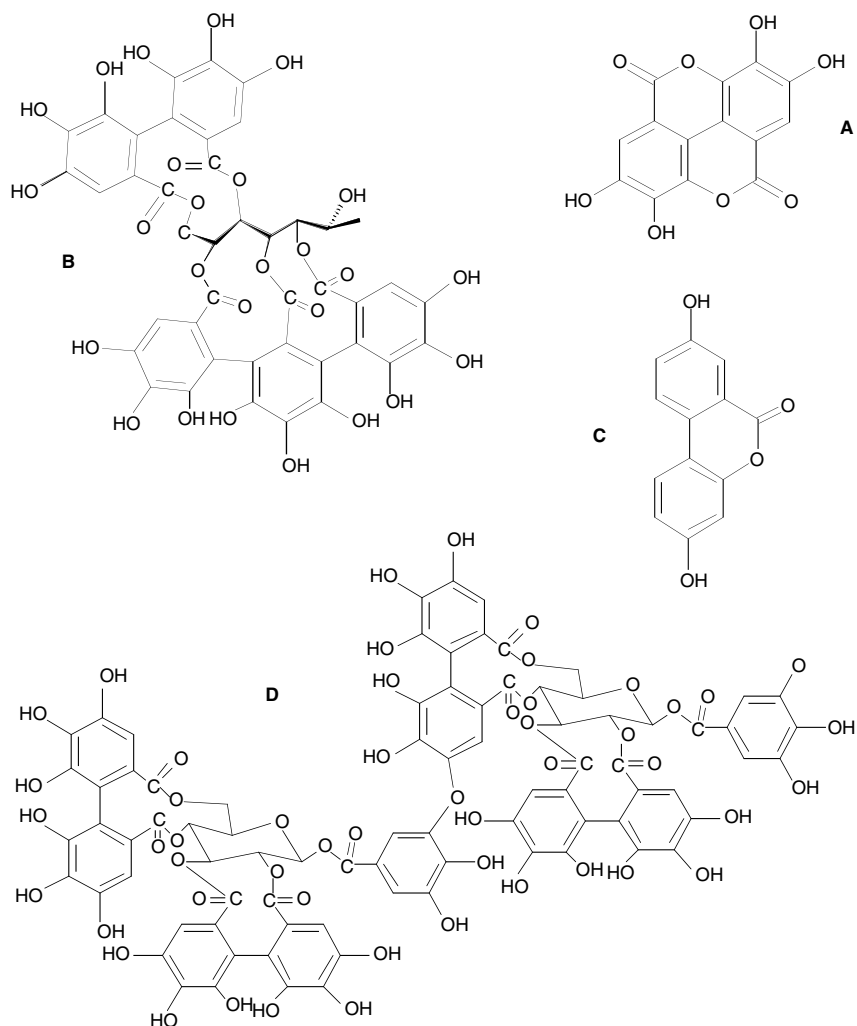
Figure 6.1 Common structures associated with ellagitannins include (A) free ellagic acid (hydrolytic product), (B) castalagin (oak wood), (C) Urolithin A (fecal metabolite), and (D) dimeric Sanguiin H-6 (*Rubus* species).

triterpenoid (castanopsiniin A), or to glucose in the α-glycosidic configuration (agrimoniin and heterophylliin A) (5). Higher molecular weight dimeric or oligomeric ellagitannins are also formed by enzyme-mediated oxidative coupling reactions between galloyl or HHDP groups. Compounds with an HHDP-esterified group are generally classified as ellagitannins, but not all have the ability to bind protein.

In the same manner, not all ellagitannins are hydrolyzable as those with a C-glycosidic bond will not break down with tannin-active enzymes or hot dilute acids to produce free ellagic acid.

Many of the health benefits associated with ellagitannins and free ellagic acid were extensively reviewed (Larrosa et al., 2010) providing evidence for their high antioxidant capacity in augmenting oxidative-related diseases such as cardiovascular disease and overall vascular health. Other benefits of ellagitannins for health include anticarcino-genic, antimutagenic, antimicrobial, and anti-inflammatory activities, as reported in numerous cell culture, animal, and human studies. In animals and humans, the primary metabolites of ellagitannins in blood are derived from intestinal microbiota that produce sulfated or glucur-onidated urolithins (urolithin A glucuronide, urolithin B-glucuronide, urolithin C-methylether glucuronide, and urolithin D-methylether glu-curonide). Urolithins, a class of 6H-dibenzo-pyran–6-one compounds, are the metabolites of free ellagic acid and ellagic acid derived from ellagitannins. They are thought to be the primary bioactive constituent of ellagitannin-rich foods due to their high concentration and residence time in blood (Gonzalez-Barrio et al., 2011).

Ellagitannins were shown to survive the harsh conditions of the stomach with no hydrolysis into free ellagic acid (Tomás-Barberán et al., 2009), and are not broken down to free ellagic acid, a depoly-merized conjugate, until they reach the small intestine. Punicalagin from pomegranates was found absorbed intact *in vivo*, the largest polyphenolic to be found in blood, but most derivatives of ellagitannins are present as conjugated derivatives of ellagic acid and urolithin (Cerdá et al., 2003, Gonzales-Barrio et al., 2010).

6.3   Ellagitannins in Fruits, Nuts, and Oaks

Ellagitannins are present in appreciable quantities in many fruits, nuts, woody plants, and botanicals (Table 6.1). Much of the early work on ellagitannin identification was with oak wood, which may contain up to 10% by dry weight, due to its use in building materials and for storage of red wine and distilled spirits. However, ellagitannins are also found in other wood species such as chestnut, sweetgum, eucalyptus, basswood, birch, and alder. The predominant ellagitannins in all oaks species are castalagin and vescalagin, each containing two HHDP units and

Table 6.1 Common ellagitannins and their LC-MS ions found in fruits, nuts, and oak wood.

Source	Compound	Lambda Max	[M-H]⁻	MS-MS	Reference
American White Oak, Wine Barrels	Vescalagin		933	301, 631	Glabasnia et al., 2007; Saucier et al., 2006
American White Oak, Wine Barrels	Castalagin		933	301, 631	Glabasnia et al., 2007; Saucier et al., 2006
American White Oak	Castalin	229	315		Glabasnia et al., 2007
American White Oak	Vescalin,	229	465		Glabasnia et al., 2007
American White Oak	Deoxyvescalagin,	229	458		Glabasnia et al., 2007
American White Oak	Dehydrocastalin	229	314		Glabasnia et al., 2007
American White Oak	Deoxyvescalin	229	307		Glabasnia et al., 2007
American White Oak	Dehydroroburin D	229	615		Glabasnia et al., 2007
American White Oak	Deoxyroburin A	229	610		Glabasnia et al., 2007
Wine Barrels	Acutissimun A		1205	915, 613, 301	Saucier et al., 2006
Wine Barrels	Acutissimin B		1205	915, 613, 301	Saucier et al., 2006
Wine Barrels	Epiacutissimin A		1205	915, 613, 301	Saucier et al., 2006
Wine Barrels	Epiacutissimin B		1205	915, 613, 301	Saucier et al., 2006
Wine Barrels	Ethylvescalagin		961	915, 480, 301	Saucier et al., 2006
Walnut	Pedunculagin		783.5	481, 301	Fukuda et al., 2003; Anderson et al., 2001
Walnut	Casuarinin	265	935.6	917, 633, 783, 301	Fukuda et al., 2003
Walnut	Valoneic acid dilactone	255,366	470.4	425	Anderson et al., 2001
Walnut	Strictinin		633.5	301	Fukuda et al., 2003

Walnut	Tellimagrandin I	270	437.7	483,301,615	Fukuda et al., 2003
Raspberry, Cloudberry, Boysenberry	Sanguiin H–10		1567.1	1235, 933, 301	Kool et al., 2010; Gasperotti et al., 2010; McDougall et al., 2008
Raspberry	Sanguiin H–6 (–galloyl)		858.1 [2]	1415.1, 200.9	Gasperotti et al., 2010
Raspberry	Lambertianin C (–ellagic acid)		1250.1 [2]	2200.2, 301.0	Gasperotti et al., 2010
Raspberry, Boysenberry	Sanguiin H–2		1103.1	300.9	Kool et al., 2010; Gasperotti et al., 2010
Raspberry, Cloudberry, Boysenberry	Sanguiin H–6		1869.1	1567, 1264, 932, 301	Gasperotti et al., 2010
Strawberry	bis-HHDP-glucose	232	783	481, 301	Aaby et al., 2007
Strawberry, Blackberry, muscadine grape	Galloyl-HHDP-glucose	232, 268	633	481, 301	Aaby et al., 2007; Hager et al., 2008
Strawberry	Galloyl-bis-HHDP-glucose	234	936	633, 391, 301	Aaby et al., 2007
Muscadine grape	HHDP-diglucoside	272	625	464, 303	Lee et al., 2005
Pomegranate	Galloyl-HHDP-Hex	266, 365	633	301, 249, 302, 463	Fischer et al., 2011
Pomegranate	Pedunculagin I	253, 377	783	481, 301, 375	Fischer et al., 2011
Pomegranate	Pedunculagin II	272	785	633, 483, 765	Fischer et al., 2011
Pomegranate	Punicalin	262, 371	781	601, 602, 721	Fischer et al., 2011
Pomegranate	Punicalagin A	258, 378	1083	601, 575, 302, 781	Fischer et al., 2011
Pomegranate	Punicalagin B	378, 258	1083	601, 603, 781	Fischer et al., 2011

(*Continued*)

Table 6.1 (*Continued*)

Source	Compound	Lambda Max	[M-H]⁻	MS-MS	Reference
Pomegranate	Lagerstannin C	250	649	301, 497	Fischer et al., 2011
Blackberry	Pedunculagin		783.2	481, 301, 169	Hager et al., 2008
Blackberry	Castalagin/Vescalagin		933.1	783, 301	Hager et al., 2008
Blackberry	Lambertianin C		1401.2 [2]	1567, 301	Hager et al., 2008
Blackberry	Sanguiin H–6/ Lambertianin A		1870.3	1567, 301	Hager et al., 2008
Blackberry	Lambertianin D		1868.9 [2]	1235, 3010	Hager et al., 2008
Blackberry	Galloyl-bis-HHDP-glucose		935.2	783, 301	Hager et al., 2008
Raspberry, Cloudberry	Lambertianin C	275	2804.2	1235, 935, 633, 301	Hager et al., 2008; McDougall et al., 2008
Boysenberry	Galloyl-sanguiin H–6		2037	1567, 1103, 933	Kool et al., 2010

glucose. The HHDP groups were shown to increase wood durability and prevent fungal/bacterial decay due to interactions among cellular structures in the wood (Vivas and Glories 1996, Zhentian et al., 1999, Helm et al., 1997). Wood ellagitannins readily change as wood ages and also degrade during wood toasting and charring in hydrolytic, pyrolysis, and polymerization reactions associated with barrel manufacture for wine and spirits. The normal oak ellagitannins castalagin and vescalagin and their dimeric forms were shown to thermally degrade in oxidative reactions to form a variety of end-products such as dehydrocastalagin and deoxyvescalagin found in aged spirits after storage (Glabasnia and Hofmann, 2007). These oxidation and thermal changes to oak tannins provide desirable organoleptic properties to wines and spirits.

Ellagitannins are also found in many nuts such as walnut, pecan, hazelnut, and oak acorns. In pecans, ellagitannins were found in the shells, as evidenced by an increase in free ellagic acid after acid hydrolysis (De La Rosa et al., 2011), but limited information is available on pecan ellagitannins found in the edible kernel. Conversely, walnuts have gained significant notoriety as a heart-healthy food due to their complement of unsaturated fatty acids and tocopherols, but its high antioxidant capacity is mostly related to its polyphenolic content, including ellagitannins. Numerous polyphenolic fractions were isolated from walnut using ethyl acetate and *n*-butanol and tertgalloyl ellagi-tannins (glansrins A, B, and C) as well as several previously charac-terized compounds pedunculagin, casuarictin, strictinin, tellimagrandin I and II, rugosin C, casuarinin, and a digalloyl-HHDP-glucoside were identified (Fukuda et al., 2003).

Certain fruits are known to be excellent sources of ellagitannins with a variety of health benefits attributed to these compounds. Identification of individual ellagitannins in context to their bioactivity has led to numerous studies over the past decade with some fruits containing unique ellagitannins that provide health benefits not found in other plants. Ellagitannin monomers are widely reported in fruits as they are readily detected and characterized, but oligomeric forms can constitute more than half of the total ellagitannins present (Karonen at al., 2010). Fruits such as raspberry, blackberry, strawberry, cloudberry, boysen-berry, pomegranate, persimmons, and muscadine grape are among the major fruits that contain appreciable concentrations of ellagitannins and ellagic acid glycosides present in the flesh and seeds. Fruits from the

genus *Rubus* (i.e. raspberry, blackberry, boysenberry, and cloudberry) are known to be excellent sources of ellagitannins, mostly present in their seeds. Hager et al. (2008) characterized 11 ellagitannins in blackberry seeds and pulp using MALDI-TOF including pedunculagin (*bis*-HHDP glucose) and monomeric (casuarictin and potentillin) to tetrameric polymeric forms of galloyl-*bis*-HHDP glucose (sanguiin H–6, lambertianin A, lambertianin C, and lambertianin D respectively). Kool et al. (2010) found sanguiin H–6 and an isomer of sanguiin H–10 as the predominate ellagitannins in boysenberry. Sanguiin H–2 and [galloyl-*bis*-HHDP glucose]$_2$-gallate – a possible degradation product of lambertianin C in the presence of hot water – were present in lower concentrations. The muscadine grape native to the southeastern USA is characterized by its high concentration of pro-ellagic acid compounds including ellagic acid glycosides and ellagitannins (HHDP-diglucoside and HHDP-galloglucose) that are located primarily in the skin (Lee and Talcott, 2004; Lee et al., 2005; Sandhu and Gu, 2010). Pomegranate owes a majority of its total polyphenolic content to the presence of ellagitannins such as punicalagin A and B isomers (Gill et al., 2000; Adams et al., 2006) comprising gallagic acid (ellagic aid with 2 gallic acid moieties) and HHDP-linked glucose that yields gallagic acid and/or punicalin upon hydrolysis.

In many of these fruits, processing operations disrupt the ellagitannin-rich seeds and release higher concentrations of ellagitannins into the fruit pulp or juice, so the severity and type of food process is highly influential on the concentration. Zafrilla et al. (2001) observed an increase in free ellagic acid during raspberry jam processing and storage that may have resulted from ellagitannin hydrolysis under the hot, acidic conditions or from softened, cooked tissues that allowed for better extraction efficiencies. However, only minor changes in blackberry ellagitannins were observed during canning, freezing, and pureeing, and the compounds were stable in the processed products over long-term storage (Hager et al., 2010). However, processing the berries into clarified juices by seed and pulp removal resulted in an 82% overall decrease (Hager et al., 2010). Likewise, pomegranate arils contain low or no punicalagins, but pomegranate husk is an abundant source. Since most commercial pomegranate juices possess moderate to high concentrations of ellagitannins, some indication of the type and severity of juice processing is indicated by its presence. Processing methods for

muscadine grape wines and juices were shown to vary, based on the temperature of juice pressing and the duration of skin-contact time during wine production. Ellagitannins and ellagic acid glycosides were found to be susceptible to degradation during storage, creating sediment that contained free ellagic acid (Lee and Talcott, 2002).

6.4   Analytical Methods for Ellagitannins

6.4.1   Spectrophotometric Methods

Compared to other polyphenolics, ellagitannin analysis is among the most difficult due to their structural diversity and inability to obtain clear chromatographic separations or distinctive UV spectra. The use of UV spectral properties to identify individual ellagitannins from a photodiode array detector can reveal important structural features for some compounds yet no discernible information for others. Most monomeric and oligomeric ellagitannins maximally absorb in the low UV range (<260 nm), but do not have spectral characteristics for compound differentiation. In contrast, punicalagins from pomegranate clearly show spectra similar to free ellagic acid due to the gallagyl unit with a characteristic absorption near 365 nn (Gill et al., 2000). The urolithin metabolites of ellagic acid also have easily discernible UV spectra with dominant bands from 240 to –280 nm and 300 to –380 nm, depending on substitutions (Gonzales-Barrio et al., 2010).

To add to the complex nature of ellagitannins they may be altered during heating, extraction, and storage and are known to readily bind to proteins and other matrix components, are susceptible to oxidation, and can form artifacts, adducts, and multiply-charged species during MS analysis. In a review of methods for hydrolyzable tannins, Mueller-Harvey describes some of the advantages of various ellagitannin testing protocols and methods and emphasizes the need for careful extraction and sample preparation (Mueller-Harvey, 2001). Little is understood regarding the protein–ellagitannin binding properties during extraction and isolation, but such complexes would decrease the ability to solubilize ellagitannins and cause under-represented concentrations in plant extracts (Helm et al., 1997; Klumpers et al., 1994). Likewise, protein binding prevents ellagitannins from being absorbed by human cells *in vitro*, and causes insoluble precipitates in cell culture medium

(Larrosa et al., 2010). Since the general classification of tannins involves their ability to bind or form complexes with proteins under various acidity levels, protein binding and turbidity development can provide qualitative information on the food quality and organoleptic traits such as astringency (Hofmann et al., 2006). It may be difficult to determine if ellagitannins have bound to matrix components during extraction or isolation as complexes can form (without visual clues such as hazing or sedimentation) with DNA (Thulstrup et al., 1999), metal ions (Press and Hardcastle, 1969), or with other large molecules in solution in a pH dependent manner (Okuda et al., 2009). Acidification of plant extracts can help to minimize many of these interactions, potentially inhibiting metal binding and precipitating proteins before they can significantly interact with the ellagitannins. The use of optimized hydrolysis conditions, including time, temperature, and acid type/concentration, are advantageous for ellagitannin analysis. Not only can extracts can be evaluated for free ellagic acid before and after hydrolysis, but the hydrolytic conditions are probably sufficient to break protein-bound ellagitannins. Mild hydrolysis can also break down oligomeric ellagitannins to produce primarily monomers for more accurate identification (Okuda et al., 2009). However, such methods do not distinguish between ellagic acid derived from glycosides or from ellagitannins and cannot be used to determine the degree of polymerization of oligomers. Hydrolysis of ellagitannin extracts is therefore an important analytical tool for analysis using both spectroscopic and chromatographic protocols, as the amount of free ellagic acid or gallic acid released is somewhat proportional to the concentration of ellagitannins present.

Although HPLC methods are widely used for individual ellagitannin determinations, methods that allow for rapid screening of extracts for evidence of hydrolyzable tannins or for guided fractionation of complex mixtures are still useful to researchers. Michel et al. (2011) described a nondestructive near infrared spectroscopy method for ellagitannins in oak staves used for wine storage with good correlation to actual ellagitannin concentration. The first wet chemistry assays that attempted to specifically quantify ellagitannins used sodium nitrite ($NaNO_2$) under acidic conditions, whereby a blue chromophore develops that will slowly degrade into an orange-yellow color that is read at 430 nm (Bate-Smith, 1972). This method has several known deficiencies in that under the acidic conditions free ellagic acid does

not react well with $NaNO_2$, gallic acid can serve as an interference, the method is limited in its sensitivity to some ellagitannins, and the reaction is susceptible to oxidation. Mertz et al. (2007) was able to rapidly identify ellagitannin-rich fractions from SPE partitions using this method following acid hydrolysis in 2M HCl for 2 h at 95 °C. The method was later modified for use with pyridine to determine free ellagic acid in extracts before and/or after hydrolysis of ellagitannins by reacting with $NaNO_2$ to induce an electrophilic aromatic substitution to form the predominant eletrophiles N_2O_3 and NOCl (Wilson and Hagerman, 1990). This reaction under the basic conditions of pyridine results in chromaphore formation with ellagic acid that is measured twice at 538 nm, an initial reading that represents a red, nitrosyated product, followed by a second reading optimized at 36 min later as the product decays to a yellow color. The difference in absorbance values between these values was found to be proportional to the concentration of free ellagic acid. The nitrosylation of other common polyphenolics, including ellagitannins, will either not react or form colored products that do not strongly absorb at 538 nm, providing selectivity for ellagic acid. Another spectrophotometric approach to hydrolyzable tannin (gallotannins or ellagitannins) analysis is reaction with the oxidizer potassium iodate (KIO_3). Although color interference from other polyphenolics can cause inconsistent test results and mild to moderate precipitation can occur, it can be an effective screening tool for mixed tannin classes in plants. The method was modified by Hartzfeld et al. (2002) to specifically target methyl gallate in the presence of acetone or methanol following acid-catalyzed methanolysis to break down the tannin. Similar to the addition of $NaNO_2$ to ellagitannins and ellagic acid, the addition of oxidizers to galloyl esters will form a stable red quinone chromophore that is read at 525 nm, but eventually degrades into a yellow end-product making the concentration of the oxidizer and timing of the experiment critical. Likewise, the use of rhodanine (2-thio–4-ketothiazolidine) can be used under alkaline conditions to test for free gallic acid through a complexation reaction specific to gallic acid (Inoue and Hagerman, 1988). Such methods for determining free or bound gallic acid or methyl gallate after hydrolysis have applications in both gallotannin and ellagitannin research, since both classes will yield a significant amount of gallic acid upon hydrolysis yet are unable to differentiate between the tannin types and cannot detect ellagitannins that do not carry a residual galloyl ester.

6.4.2 Solvent Selection and Solid-Phase Extraction

Extraction and isolation techniques are among the most critical aspects of ellagitannin analysis since many studies report secondary fractionation and isolation to decrease the complexity of the matrix prior to analysis (Table 6.2). However, these steps are performed at the expense of recovery efficiency for target compounds, and are often used as qualitative methods for compound identification. Solvents used for extraction vary based on the starting material and their efficiency to dissolve ellagitannins, as water alone is not a universal solvent for all ellagitannins. Water solubility for most ellagitannins decreases with increased number of galloyl groups present, but increases with additional biaryl HHDP groups. Solubility for most ellagitannins however is usually not a major concern compared to the ability for the solvent to aid in the penetration of the cellular matrix. Organic solvents such as acetone, ethyl acetate, methanol, and ethanol alone or in combination with water are most commonly reported for food and fiber samples and vary in efficiency based on the time, particle size, and density of the sample (i.e. seeds, pulp, roots, leaves). Solvent selection is often based on trial-and-error but acetone is commonly reported for ellagitannin extraction from oak wood or freeze-dried plant materials while hydroalcoholic solutions are generally sufficient for fresh fruits. Zhentian et al. (1999) compared acetone and methanol for their ability to extract the white oak ellagitannins vescalagin and castalagin and found that acetone was more efficient in extracting total phenols. The solubility of ellagitannins is also influenced by oxidative and enzyme-mediated coupling reactions in the plant matrix such as proteins and lignins; therefore, prolonged or acidic conditions are often used to increase extraction efficiency and minimize degradation reactions. In the case of seeds, defatting the sample prior to extraction can prevent analytical interferences and column fouling in subsequent isolations. In most published procedures, extracts are filtered following homogenization and the residual solvent evaporated to concentrate the ellagitannins while monitoring for evidence of protein binding or other complexation reactions as the volume is reduced. For subsequent analytical work, aqueous extracts may be partitioned into ethyl acetate and/or *n*-butanol and further partitioned by various SPE techniques.

For a detailed characterization of ellagitannins, further isolation or fractionation to separate ellagitannins from other polyphenolics and

Table 6.2 Common LC conditions for select oak and fruit samples containing ellagitannins

Source	Solvents	Gradient	Detection	Column	Reference
Oak	A: Aqueous, 0.3% Formic Acid B: 100% Acetonitrile	3 mL/min 5% B; 0–10 min 15% B; 10–25 min 60% B; 25–40 min 60% B; 40–43 min	DAD	RP-ODS-Hypersil 250×10 mm i.d., 5 μm	Glabasnia et al., 2007
Wine aged in oak	A: Aqueous, 0.4% Formic Acid B: 100% MeOH, 0.4% Formic Acid	1 mL/min 3% B; 0–5 min 12% B; 5–35 min 100% B; 35–40 min	DAD MS-ESI	Lichrospher 100 RP18 250×4.6 mm i.d. 5 μm	Michel et al., 2011
White Oak	A: Aqueous B: 100% MeOH, 0.2% TFA	0.75 mL/min 10% B; 0–40 min 100% B; 40–45 min 100% B; 45–50 min 0% B; 50–60 min	DAD	Lichrospher RP–18 250×4 mm i.d. 5 μm	Zhentian et al., 1999
Blackberries	A: Aqueous, 2% Formic Acid B: 80:18:2 Acetonitrile: Water:Formic Acid	0.5 mL/min 5% B; 0 min 25% B; 0–50 min 100% B; 50–60 min 100% B; 60–70 min	DAD MS-ESI	Lichrospher ODS-2 250 mm $\times 4.6$ mm i.d. 5 μm	Mertz et al., 2007

(Continued)

Table 6.2 (*Continued*)

Source	Solvents	Gradient	Detection	Column	Reference
Muscadine	A: Aqueous 0.5% Formic Acid B: 100% MeOH	1 mL/min 5% B; 0–2 min 20%B; 2–10 min 30% B; 10–15 min 35% B; 15–20 min 80% B; 20–60 min 85% B; 60–65 min 5% B; 65–70 min	DAD MS-ESI	Zorbax Stablebond Analytical SB-C18 4.6 mm × 250 mm i.d. 5 µm	Sandhu and Gu 2010
Raspberry	A: Aqueous 1% Formic Acid B: 100% Acetonitrile	1 mL/min 8% B; 0 min 21% B; 0–60 min	DAD MS-ESI	Synergi RP-Max 250 x 4.6 mm. i.d. 4 µm	Mullen et al., 2003
Pomegranate	A: Aqueous 2% Acetic Acid B: Aqueous and Methanol (10:90 v/v), 0.5% Acetic Acid	0.4 mL/min 2% B; 0–13 min 5% B; 13–18 min 10% B; 18–23 min 25% B; 23–43 min 50% B; 43–53 min 100% B; 53–58 min 100% B; 58–63 min 0% B; 63–66 min	DAD MS-ESI	Phenomenex C18 Synergi Hydro-RP 150 × 3.0 mm 4 µm	Fisher et al., 2011

	Mobile Phase	Flow/Gradient	Detection	Column	Reference
Boysenberry	A: Acetonitirile, 0.1% Formic Acid B: Aqueous, 0.1% Formic Acid	0.2 mL/min 5% A; 0–5 min 10% A; 5–10 min 17% A; 10–25 min 23% A; 25–30 min 30% A; 30–40 min 97% A; 40–48 min 96% A; 48–53 min	DAD MS - ESI	Phenomenex Prodigy ODS(3) 5 μm 150 × 2 mm	Kool et al., 2010
Walnut	A: Aqueous, 01.% Acetic Acid B: Acetonitrile, 0.1% Acetic Acid	1.2 mL/min 5% B; 0–1 min 75% B; 1–9.5 min 95% B; 9.5–17.5 min 95% B; 17.5–18.5 min	MS-ESI	Betabasic C18 column 4.6 × 50 mm 5 μm	Anderson et al., 2001

matrix components is required, and may be accomplished with the use of thin-layer chromatography (Mueller-Harvey, 2001), preparative HPLC (Hofmann et al., 2006; Glabasnia and Hofmann, 2007), reversed-phase C18 cartridges (Aaby et al., 2007), gel chromatography using Sephadex LH–20 (Li et al., 2007; Mertz et al., 2007; Reddy et al., 2007; Hager et al., 2008; Karonen et al., 2010), resins such as Amberlite or Toyopearl brands (Li et al., 2007; Reddy et al., 2007; Ito et al., 2007), and liquid–liquid partitioning with ethyl acetate or *n*-butanol (Fukuda et al., 2003; Li et al., 2007; Ito et al., 2007). In most examples, aqueous extracts are loaded onto these supports and ellagitannin-rich fractions are selectively eluted with aqueous alcohol solutions based on trial-and-error. Inefficiencies with the process are common as low molecular weight ellagitannin species may be lost and individual ellagitannins often appear in multiple fractions.

Among the SPE supports available, Sephadex LH–20 is the most widely used for the post-extraction isolation of hydrolyzable tannins. Made from a cross-linked dextran that has been hydroxypropylated, it possesses both hydrophilic and hydrophobic characteristics and can be prepared for adsorption in both water and organic solvents with high affinity for plant polyphenolics. Sorption onto Sephadex LH–20 or in combination with other SPE or size exclusion supports is commonly used for ellagitannins because the extract can be dissolved in low concentrations of alcohol without losing binding efficiency, and extraneous polyphenolics can be sequentially eluted with increasing concentrations of alcohol or acetone. For example, McDougall et al. (2008) removed anthocyanins from crude strawberry and raspberry extract by eluting from a Sephadex LH–20 column with 80% ethanol and then removing the bound ellagitannins with 50% acetone. In a comprehensive extraction and isolation procedure, boysenberry pulp and seed were extracted and ellagitannins were initially isolated by passing through a Dowex 50W-X8 ion exchange column followed by elution with acidified 80% ethanol (Kool et al., 2010). Additional purity was obtained from a C18 column when eluted with 25% methanol with 5% formic acid and four subfractions subsequently collected using preparative HPLC each containing galloyl-sanguiin H–6 and sanguiin H–10, H–6, and H–2 respectively. Compound determinations were made by HPLC-ESI-MS in negative ion mode, higher molecular weight species by MALDI-TOF-MS, and the isolates were pure enough for ^{1}H NMR analysis. In a similar manner, Saucier et al. (2006) isolated oak

ellagitannins and flavo-ellagitannins formed during wine aging by first desorbing from Amberlite XAD7 HP to remove sugars and acids, and then followed by Toyopearl TSK 40 HW gel to isolate and purify prior to HPLC-ESI-MS for which direct identification would not have been possible in such a complex matrix.

6.4.3 HPLC and HPLC-MS Analysis

HPLC/UPLC separations in tandem with a mass spectrometer are significantly more robust than the ambiguous spectrophotometric assays, and allow for more detailed identification of individual ellagitannins. HPLC-MS techniques have only been in use since the late 1990s and are likely the most widely used analytical technique for ellagitannins due to their high polarity and extensive co-elution with other compounds. Sample preparation and subsequent isolation is critical for MS techniques to ensure proper ionization and to take advantage of column resolution during separation. Ionization of ellagitannins is generally efficient in both positive and negative ionization modes given that interfering agents are sufficiently removed. For example, methanol and other extractables from SPE supports are commonly associated with ion suppression in negative ESI mode compared to other solvents such as acetonitrile, yet methanol elutions from SPE cartridges is common for ellagitannin isolation and separation. The determination of a molecular weight and cleavage of constituent esterified moieties is invaluable to determine the composition of ellagitannins. The preponderance of glucosyl, galloyl, and HHDP fragment ions is the hallmark signature of an ellagitannin, coupled with its low UV absorption. Ionization sources vary for the analysis of ellagitannins with electrospray ionization (ESI), matrix-assisted laser desorption ionization/time of flight (MALDI-TOF), fast atom bombardment mass spectrometry (FAB MS), thermal spray ionization (TSI), and atmospheric pressure chemical ionization (APCI), all of which have been successfully used to analyze various polyphenolic classes. Since commercially available standards for most ellagitannins are still not available, the "soft" ionization conditions created by many of these ionization sources are especially suited for the determination of esterified moieties and for breaking polymeric ellagitannins into monomers with predictable adduct formation. For ellagitannin analysis, MALDI and ESI are among the most common ionization probes

reported, probably due to their prevalence in analytical laboratories. A major drawback between ESI and MALDI is the severely limited upper dynamic range for ESI that inhibits the detection of high molecular weight (>2000 amu) ellagitannins, and the formation of multiply-charged species that may result in mischaracterization of compounds (Hager et al., 2008; Gasperotti et al., 2010). However for predicted reactions such as the alcoholic conversion of vescalagin from oak barrel aged red wine to β–1-*O*-ethylvescalagin and suspected flavo-ellagitannins, most ionization sources are suitable, and specific parent or product ions can be evaluated by scan features such as multiple reaction monitoring mode (Stark et al., 2010) or selective ion monitoring (Glabasnia and Hofmann, 2007) to eliminate much of the background noise and interference from other co-eluting polyphenolics. Correct characterization of ellagitannins and other ellagic acid precursors is also important for evaluating organoleptic traits, physicochemical characteristics, and bioactive properties that are highly dependent on the esterified moieties and degree of polymerization.

Analysis of mammalian metabolites of ellagitannins is commonly conducted in blood, urine, and feces to evaluate intestinal microbial transformations as well as nonmetabolized species. Fecal or urine samples are generally extracted with acidified alcoholic solutions to precipitate proteins, filtered, and analyzed without further modification under standard HPLC-MS conditions. Concentration steps may be needed for urine metabolites, and generally employ partitioning from a reversed-phase C18 mini-column and elution with 100% methanol (Gonzales-Barrio et al., 2010). Blood, in the form of isolated, stabilized plasma, or urine samples may be analyzed with or without enzymatic digestions to remove metabolic conjugates using ß-glucuronidase and/ or sulfatase. Analysis of ellagitannin and metabolites from blood plasma require soluble protein removal with acidified alcohol or acetonitrile and subsequent concentration by evaporation (Seeram et al., 2006; Ito, 2011) or by partitioning from a C18 column.

6.5   Analytical Challenges in Ellagitannin Evaluation

Many studies on plants that contain ellagitannins also report numerous unidentified or poorly characterized ellagitannins often in the form of poor peak resolution and detection of isomers that share a common

molecular weight and fragmentation pattern with other known compounds. These isomeric forms are distinctively different compounds, but are difficult to isolate and purify in sufficient quantities for structural characterization by NMR. The inability to completely resolve these ellagitannins by partition chromatography has led many researchers to further fractionate in effort to eliminate the "hump" commonly found in chromatograms as the result of co-elution with ellagitannins and other polyphenolics. When evaluating co-eluting and oligomeric ellagitannins by LC-MS it is often difficult to determine the molecular or parent ion as opposed to a product or fragment ion from a higher molecular weight compound. However, if the molecular ion is known or suspected to be present, analysis by direct flow injection/direct infusion in an ion trap instrument can aid in structural determinations in the absence of chromatography as long as the concentration is low enough as to not cause suppressed ionization (McDougall et al., 2008). Depending on the ellagitannin source, high molecular weight species may exist in multiple isomeric forms and are prone to the formation of multiply-charged species. Although all ion sources are susceptible to this effect, ESI can be particularly susceptible and can lead to confusion when identifying monomers and dimers compared to higher molecular weight species. With increased molecular weight of oligomeric ellagitannins, Gasperotti et al. (2010) and Hagar et al. (2008) both found doubly-charged ellagitannins that made mass interpretations challenging. For example, in the case of trimeric lambertianin C secondary ionization produced singly-charged fragments that were larger than the parent molecular ion, which was doubly charged. In the case of oligomeric ellagitannins, multiple charge states can result from "in-source" fragmentation energy and confuse identification of compounds such as the dimer sanguiin H–6/lambertianin A for the tetramer lambertianin D. High molecular weight ellagitannins are also susceptible to adduct formation and have a greater potential to degrade into smaller products, further complicating their analysis.

6.6   Conclusion

Ellagitannins represent a complex class of polyphenolics present in may fruits, nuts, oaks, and botanicals. A detailed description of many ellagitannins reported in plants is still lacking due to inherent limitations

with isolation, structural characterization, and the lack of authentic standards for comparison. However, ellagitannins are widely recognized to be important contributors toward the health benefits of many foods, as evidenced by the many small berries, fruits, and oak-aged wines that contain these compounds. Future research will further link these compounds to specific disease states as a result of their antioxidant and bioactive properties, and will further elucidate the functional role of ellagitannins and their metabolites for improved human health.

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Chapter 7

Analytical Methods of Flavonols and Flavones

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Abstract

Flavonols and flavones are present in many food products and medicinal plants and show relevant antioxidant activity *in vitro*. In this chapter, classical analytical methods such as thin layer chromatography and two-dimensional paper chromatography together with modern methodologies such as HPLC-MS-MS are reported. Preparative chromatography methods are also reviewed as well as spectroscopic methods used for flavonoid characterization and identification, including UV spectrophotometry and MS spectrometry. Chemical and enzymatic methods used in flavonoid identification are also reviewed.

Keywords: Flavones; flavonols; characterization; identification; chromatography; MS; UV.

7.1 Introduction

Flavones and flavonols are relevant phytochemicals in food products, spices and medicinal plants. They are secondary metabolites belonging to the polyphenols group, and have in common the general structure of two aromatic rings and often more than three phenolic hydroxyls. Flavones and flavonols often show high antioxidant activity *in vitro*, and they complex metal ions and scavenge free radicals. They have a planar structure that allows intermolecular interactions and stacking in

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combination with anthocyanins being considered copigments. They usually occur in nature as *O*-glycosidic combinations with mono- and disaccharides, although combinations with a higher number of sugars are also frequent. *C*-glycosidic combinations, although less common, are presentin different food products including cereals, grains, Swiss chard, and sweet peppers. Glucose, galactose, rhamnose, arabinose, and xylose are the main monosaccharidesin flavone glycosidic conjugations. In addition uronic acids, mainly glucuronic acid, are often found in naturally occurring flavones and flavonols as is the case of strawberries and grapes that contain quercetin and kaempferol 3-*O*-glucuronides and spinach that contains flavonol-methyl ether and methylendioxy glucuronides (spinatoside and jaceidin glucuronide) (Ferreres et al., 1997). The mainflavone disaccharides are sophorosides [glucosyl(1 → 2)glucosides], gentiobiosides [glucosyl(1 → 6)glucosides], rutinosides [rhamnosyl(1 → 6)glucosides] and neohesperidosides [rhamnosyl(1 → 2)glucosides]. Sambubiosides and glucosyl-galactosides are also found in nature although less often. Acylation of the flavonoid-glycosides with aliphatic monocarboxylic (acetic, malic) or dicarboxylic (malonic, tartaric, etc.) acids, or aromatic acids (mainly hydroxycinnamic acids such as caffeic, *p*-coumaric, ferulic and sinapic) is also frequent. In some cases they can also be conjugated with sulfate, as in the case of the flavonoids present in palm dates (Hong et al., 2006). The conjugation with glucuronides and/or sulfates is particularly relevant in the *in vivo* metabolism of flavones, as a general way for detoxification using the Phase II enzymes system. Flavone and flavonol aglycones have also been found in different food products, although they are less frequent as they are more susceptible to oxidative degradation (particularly relevant in polyhydroxylated flavones, the higher the number of phenolic hydroxyls the higher the oxidative degradation). For instance, they are present as polymethoxyflavones in citrus fruits (sinensetin, nobiletin, tangeretin, etc.), and as different flavone and flavonol combinations in honey (quercetin and kaempferol mono-, di-, and trimethyl ethers, luteolin and apigenin methyl ethers, chrysin, and galangin) and propolis, a resinous exudate product collected by bees from trees such as poplars (Tomás-Barberán et al., 1993).

Flavonols are the most common flavonoids in plant-derived food products, and they often occur as glycosidic combinations of myricetin, quercetin, and kaempferol and their methyl ethers (isorhamnetin and tamarixetin). Flavonols are relevant in onions, apples, green tea, grapes,

and wine, although they are also present in many other different food products. Flavones are less frequent in foods, but they occur in sweet and hot peppers, celery, and many herbs and spices. Some plants produce large amounts of specific flavones. This is the case of the methylated flavonol glycosides of spinach (i.e. spinacetin, jaceidin, and patuletin glycosides) (Ferreres et al., 1997). These are nutritionally relevant as they can be ingested in the diet in relatively large amounts and have relevant roles in the sensory properties of food products as they can be bitter (Tomás-Barberán and Espín, 2001).

Flavones show antioxidant activity in different models. They neutralize free radicals, complex iron ions, and can prevent lipid oxidation (Pietta, 2000). This antioxidant activity is related to the phenolic hydroxyls present on the flavone nucleus, and the presence of *ortho*-dihydroxyl grouping (catechol groups) is known to be particularly effective for antioxidant activity. Conjugations with sugars always decrease the antioxidant activity, but can have positive effects on the solubility of the flavonoids in aqueous systems.

7.2 Extraction of Flavonols and Flavones

7.2.1 *Extraction Solvents*

Hydroalcoholic mixtures are generally accepted as the solvents of choice for the extraction of flavones and flavonols from different plants and food products. Both ethanol and methanol can be used. The percentage of water in the hydroalcoholic mixture depends on the nature of the material to be extracted. In general, methanol/water 7:3 (v/v)mixtures are recommended for extraction of dried materials, and methanol/water 8:2(v/v)mixtures for fresh products, as the fresh plant material already provides some water to the extraction mixture. In general, solvent/plant ratios between 5:1 and 10:1 are recommended, and the flavonoid recovery during the first extraction can reach up to 90–95% of the total plant flavonoid content. In some cases, specific solvents, such as acetone, dimethylsulfoxide, or dimethylformamide are needed for the complete recovery of flavonoids. The extraction can be carried out by maceration at room temperature, with or without stirring. This extraction can also be carried out at low temperatures (in an ice bath) when using fresh material in order to decrease the degradation of

phenolics and indirectly the degradation of flavonoids by the action of polyphenol oxidases or other hydrolytic or oxidative enzymes. In some cases the extraction is carried out at higher temperatures (60–100 °C) in order to speed-up the extraction process and enhance the extraction efficiency, and in addition inactivate the degradative enzymes. These high temperatures, however, can have a negative impact on the stability of some sensitive flavonoids, increasing their oxidation or polymerization. In some cases, vacuum or an inert atmosphere (N_2) can be used to increase the stability of the extracted flavonoids and avoid the oxidative degradation. For preparation of flavonoid extracts from fresh plant materials (leaves, fruits, etc.), the extraction can be achieved with an Ultra-Turrax homogenizer, placing the sample in an ice bath, and using pure methanol (or methanol/water 8:2) or acetonitrile. Addition of 0.01 M NaF to the extracting solvent can be used to prevent the action of polyphenol oxidases and the degradation of polyphenols and the production of brown melanin, preserving the flavonoids that naturally occur in the original plant tissues (Gil et al., 1999; Marín et al., 2004). Freeze-drying of the plant material generally enhances flavonoid extraction, as the plant membranous structures are damaged during the freezing process allowing better solvent diffusion and flavonoid extraction. Ultrasound or microwave systems can also be applied to enhance the flavonoid extraction and decrease extraction time.

7.2.2   *Extraction of Plant Surface Lipophilic Flavones*

This requires specific conditions that can lead to quite purified extracts recovering only the lypophilic flavonoids that are excreted to the resins that cover some plant tissues surfaces, as is the case of plants growing in arid habitats. For instance, some flavones occurring in aromatic and medicinal plants [cirsimaritin (5,4′-dihydroxy-6,7,-dimethoxyflavone), xanthomicrol (5,4′-dihydroxy-6,7,8-trimethoxyflavone), nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), etc.] are lypophilic flavones that are excreted to the plant surface together with waxes, resins, and essential oils in many aromatic plants that grow in arid habitats (Tomás-Barberán et al., 1988). These flavonoids can be selectively extracted with apolar solvents using a Soxhlet as the extraction system with dichloromethane or petroleum ether as solvents. In some cases, the whole plant material (preferably fresh, but also dry ungrounded plant material) can be washed with anapolar solvent for a short period of time

(30–50 s) to remove exclusively the surface compounds without the extraction of any intracellular constituent (Wollenweber and Seigler, 1982). With this method, no chlorophyll should be extracted, which demonstrates the preservation of intracellular constituents. This method is useful for the extraction of highly methylated flavonoid aglycones from *Labiatae* and *Compositae* herbs, among others, that have also been introduced in the nutraceuticals and botanicals market.

7.2.3   Preparative Extractions

For subsequent isolation of fractions and flavonoid compounds, it is advisable to use a Soxhlet system for the extraction. This combines high temperature and solvent recirculation for a quantitative extraction with relatively reduced solvent volumes. In this case a series of solvents can be used sequentially in an order of increasing polarity. A good strategy for flavonoids is a first extraction with apolar solvents (*n*-hexane, petrol, etc.) for fat, chlorophyll, and lypophilic compounds extraction. In this extract it is possible to find lypophilic flavones such as isoprenylated, fully methylated flavones, as is the case of nobiletin and sinensetin from citrus fruits, or highly methoxylated flavones (xanthomicrol, cirsimartin, cirsilineol, etc.). A second extraction with methanol will complete the extraction of most flavonoids (Ferreres et al., 1997).

7.2.4   Liquid–Liquid Fractionation

The hydroalcoholic extracts can then be fractionated by liquid–liquid extraction systems. A previous step should involve the removal of alcohol using a vacuum rotary evaporator at low temperatures ($40\,°C$) that allows the distillation of methanol or ethanol under vacuum, leaving a concentrated water extract. This water extract can then be successively extracted using ethyl ether (to extract flavonoid aglycones), ethyl acetate (to recover flavone monoglycosides and the remaining highly hydrolylated aglycones), and *n*-butanol (to extract flavone *C*-glycosides and poly-*O*-glycosylated flavones). This sequential extraction is quite useful as it renders extracts with different flavonoid types that can be directly used for flavonoid analysis, or for the isolation of specific flavonoids. Flavonoid glycosides acylated with hydroxycinnamic acids linked to the hydroxyls of the sugar moieties or the phenolic hydroxyls of the flavonoid residue (less frequent), often behave as aglycones in terms of extracting behavior, and could be present in the lypophilic extracts.

7.3 Isolation and Purification of Flavonols and Flavones

7.3.1 *Purification and Fractionation*

The crude extracts obtained can be used for the extraction and purification of specific flavonoids. In this case the extracts must be submitted to different fractionation processes. The first process can be a liquid–liquid extraction, as described above, which consists of partitioning the extracts components into two immiscible solvents. The different flavonoids are extracted depending on their polarity. The isolation and purification of flavonoids from some sugar-rich matrices (honey, fruit jams, etc.) that also contain other polar compounds is very complicated due to the formation of solvent interphases during the liquid–liquid extraction process. In these cases several non-ionic polymeric resins (Amberlite XAD) can be used to overcome this problem. In a comparative study of different polymeric resins for flavonoid extraction, polystyrene resins (XAD-2, XAD-4, and XAD-16) were found to be more useful for fractionation than the polyacrylic resins (XAD-7 and XAD-8) (Tomás-Barberán et al., 1992). The results suggested that Amberlite XAD-2 was the most appropriate for flavonoid extraction in honey (Tomás-Barberán et al., 1992). Amberlite XAD-2 has been successfully used for the recovery of flavonoids from honey and fruit jams (Ferreres et al., 1991; Tomás-Lorente et al., 1992). The different honey or jam samples were dissolved in five parts of water acidified with HCl (pH 2 in order to favor flavonoid adsorption in the stationary phase) until completely fluid and stirred with a magnetic stirrer to enhance the contact between the resin, the dissolved honey sample, and the flavonoids in solution. The Amberlite resin with the flavonoids adsorbed is then packed into an appropriate glass column and washed with deionized water to remove sugars and other unwanted polar compounds. The flavonoids and other phenolicsare then eluted with methanol and concentrated with a rotary evaporator (40 °C) (Martos et al., 1997).

7.3.2 *Size Exclusion Chromatography*

Other classical methods for the fractionation and purification of flavonoid extracts are filtrations through different Sephadex gels, that separate by a combination of adsorption, partition, and size exclusion using organic solvents (Sephadex LH-20) or aqueous solvents

(Sephadex G-10, G-20, etc.). Sephadex LH-20 is considered the stationary phase of choice for the size exclusion chromatography of flavonoids. This is due to its compatibility with methanol and hydroalcoholic solvents for fractionation. The procedure starts with the treatment of the Sephadex particles with the solvent to be used as mobile phase (methanol). In a beaker, the appropriate amount of Sephadex is stirred with methanol and left to swell for at least 1 hour. The prepared stationary phase is then poured into a glass column and allowed to compact until the column is packed and the solvent on top of the column is clear. The solvent is left to run through the column until the top of the stationary phase begins to dry, and the sample (flavonoid extract dissolved in methanol or in the mobile phase to be used) is then carefully applied with the help of a Pasteur pipette. Once the whole sample has entered the stationary phase, mobile phase is carefully applied on top of the column and the chromatography can start. The separation of different flavonoid fractions can be followed using a UV lamp (360 nm) in a dark room, or with a UV detector placed on line on the outlet of the column. The flavonoids separate primarily by molecular size, therefore the flavonediglycosides elute ahead of the flavonemonoglycosides, and these ahead of the flavone aglycones. In addition, the different compounds are also separated by adsorption, and the higher the number of free hydroxyls, the more flavones are retained. In this case, galangin (3,5,7-trihydroxyflavone) elutes ahead of kaempferol (3,5,7,4′-tetrahydroxyflavone) and this ahead of quercetin (3,5,7,3′,4′-pentahydroxyflavone). The final separation of the extract follows the separation by size, together with the separation by the number of free hydroxyls.

7.3.3   Preparative Chromatography

Preparative column chromatography
Classical separations by open column chromatography with different stationary phases (silica gel, reversed-phase C-18 or C-8, polyamide, cellulose) and elution with appropriate solvent mixtures are also useful for flavonoid fractionation and purification. Different column systems can be used. The classical open column chromatography uses relatively large particle sizes (0.2–6 mm), with limited resolution, and solvent filtration through the column proceeds by the pressure of the solvent column placed on top of the stationary phase. In other cases, smaller

particle sizes (0.030–0.2 mm) can be used for enhanced resolution and flavonoid purification. When using smaller particles different devices have to be applied in order to force a solvent flow through the stationary phase. Vacuum can be applied at the bottom of the column, or pressure on the top of the column. Thus, there are lobar column chromatography and flash chromatography (both low-pressure chromatography), and vacuum chromatography. Different stationary phases can be used for the isolation and separation of different flavonoids. The stationary phase to be used depends on the type of flavonoid to be purified. The different flavonoid fractions can be followed using a UV detector coupled to the outlet of the column, or using a UV lamp (360 nm) to follow the development of the fractionation directly in the stationary phase (this is useful in cellulose and polyamide or Sephadex chromatography, but has some limitations in silica gel and reversed-phase chromatography).

Preparative silica gel chromatography (straight-phase chromatography)

This is used for the separation of apolar flavonoids (aglycones), as the polar compounds can be irreversibly adsorbed to the stationary phase. The separation is carried out using solvent mixtures starting with highly apolar solvents such as *n*-hexane, petrol, etc., and increasing the polarity of the solvent by raising the proportion of ethyl acetate or methanol. The separation takes place following the principles of adsorption chromatography, first eluting the less polar compounds, and then eluting flavonoids with increasing polarity. A typical elution in the separation of a mixture of quercetin methyl ethers would be the following: quercetin 3,7,4′-trimethyl ether, quercetin 3,4′-dimethyl ether, quercetin 3-methyl ether, quercetin 4′-methyl ether, quercetin (3,5,7,3′,4′-pentahydroxyflavone). A key step in silica gel chromatography is the deposit of the extract to be separated on top of the stationary phase of the column. It is recommended that the extract should be dissolved in the mobile phase to be used, but the solubility of the extract to be fractionated is often low in the first solvent used as mobile phase, and this leads to precipitations at the top of the column, a decrease in the solvent flow rate, and poor resolution of the flavonoid bands. An alternative to overcome this problem is to completely dissolve the extract in an appropriate solvent (no water should be present). The dissolved extract is then concentrated in a rotary evaporator (at 40 °C) after the addition of a small amount (a few grams) of the silica gel

stationary phase. This will produce a dried stationary phase mixed with the extract, which can be placed dried on top of the stationary phase of the column before starting the elution. This will help in the separation of different fractions that elute after the application of solvent mixtures with increasing polarity. In this type of chromatography, the stationary phase is used for only one separation and is then discarded (this is not a problem as silica gel is quite inexpensive).

Preparative reversed-phase chromatography
In this case the hydroxyls present in the silica gel stationary phase are blocked by 18 (C-18) or 8 (C-8) carbon hydrocarbon chains. The principle of separation is adsorption, as in the case of silica gel, but in this case the polarity of the stationary phase has been reversed, and the polar compounds elute first and the apolar compounds are more retained in the column. The mobile phase consists of mixtures of methanol or acetonitrile with water. Elution starts with water or mixtures with a small percentage of the organic solvent (5% methanol), to end with 100% organic solvent. Gradient elution is a common strategy although isocratic separations are more appropriate for preparative chromatography. The addition of an organic acid to the solvent mixture (acetic acid) can increase the efficacy of the separation, but the isolated compounds have to be stable under these acidic conditions, particularly during the concentration process of the isolated fractions, as temperature is typically increased to remove the solvents.

Preparative cellulose chromatography
This separates flavonoids by partition chromatography. It can be particularly useful for the separation of flavonoid glucuronides from the rest of flavonoids in complex mixtures. The separation with water as the mobile phase, takes glucuronides with the solvent front, while the other flavonoids are separated by the degree of glycosylation, the more polar eluting first.

Preparative polyamide chromatography
This chromatographic method usually produces very good separations of flavonoids that are difficult to be resolved by other chromatographic methods. The main drawback of this method is the elevated cost of the stationary phase, preventingits use for large scale preparations, but it is quite useful for laboratory scale preparations. Mobile phases like

chloroform/methanol/acetone (1:1:1; v/v/v) for flavonoid aglycones, or chloroform/methanol/ethyl-methyl-ketone/water (11:8:4:2; v/v/v/v) for flavonoid glycosides are useful for the fractionation and purification of flavonoids from crude extracts or purified fractions. In some occasions the compounds isolated by this methodology can be contaminated with some stationary phase and further purification (filtration through a Sephadex LH-20 micro column) may be required, particularly prior to NMR analysis.

Preparative thin-layer chromatography (TLC) and paper chromatography (PC)

For partially purified extracts, TLC and PC have been used for analytical and preparative purposes. TLC using silica gel, polyamide, cellulose or reversed-phase C-18 can be used with success for the separation of specific flavonoids. The type of flavonoid to be separated suggests the type of TLC plate to be used. Thus, reversed-phase, cellulose, or polyamide are recommended for polar flavonoids (flavonoidglycosides, polyhydroxylated aglycones, etc.) while silica gel with organic solvents is recommended for lypophilic flavonoids (polymethoxylated flavones; flavone methyl ether, isoprenylated flavones, etc.). The extract is applied to the TLC plate in the form of a narrow band at the origin of the chromatogram, with the help of a glass capillary or a Pasteur pipette, depending on the amount of extract to be applied. The chromatogram is then developed and, after drying, the different chromatographic bands are visualized under UV light (360 nm). The bands are then scraped out with the help of a stainless steel spatula, and the flavonoids extracted from the silica gel powder with a suitable solvent (i.e. methanol). Paper chromatography with Whatman paper No. 1 or No. 3 can also be used for preparative purposes. The extract is applied to the origin of the chromatographic paper in the form of a narrow band with the help of a glass capillary or a Pasteur pipette, depending on the amount of extract to be applied. After drying, the chromatogram is developed with solvents such as water (for highly polar flavonoids and flavonoid glucuronides), 15% or 30% acetic acid in water (for flavonoid monoglycosides and diglycosides) and *n*-BuOH/acetic acid/water (4:1:5, v/v/v, upper phase) for flavonoid aglycones. The different flavonoid bands can be detected after drying under UVlight (360 nm), and the flavonoids eluted with methanol or methanol/water 7:3 (v/v) from the ground paper bands that are packed into an open glass column.

Preparative HPLC

Finally, a more precise purification obtaining fractions with a higher purity degree can be obtained using preparative or semipreparative HPLC [even analytical HPLC columns (5 μm particle size) can be repeatedly used for the preparation of small amounts of a highly purified compound]. In the case of flavonols and flavones, both aglycones and glycosides, reversed-phase columns are often used (C-18). Preparative columns use a higher particle size, and higher mobile phase rates (10–50 mL/min). A fraction collector and an online UV-detector help with the detection and collection of the different flavonoid bands.

7.4   Chemical and Biochemical Treatments

Once isolated, the flavonoids can be subjected to a number of chemical and biochemical treatments in order to help with the identification of complex molecules through the analysis of more simple derived molecules. Thus, many flavonoid glycosides that are acylated with aliphatic (acetic, malonic, etc.) or aromatic acids (hydroxycinnamics, etc.), can be studied after alkaline hydrolysis that will lead to the deacylated flavonoid glycosides. Acid hydrolysis would then lead to the corresponding aglycones. Enzymatic hydrolysis can also be useful for the identification of glucuronide, sulfate, and glucoside conjugates.

7.4.1   Alkaline Hydrolysis

Basic hydrolysis is applied for removing organic acid esters from the original flavonoid molecules. After this hydrolysis, both the released organic acid, and the flavonoid glycoside can be chemically characterized. This can be applied to isolated flavonoids or even to crude extracts. In this case HPLC-PDA-MS/MS, comparison of the original acylated extract, with the deacylated product is very useful. This is achieved by adding 1 mL 4N NaOH to freeze-dried purified flavonoids (<1 mg), or 1 mL of the hydroalcoholic extract, or flavonoid fraction, and keeping the solutions for 16 h in a stoppered test tube under N_2 atmosphere (to prevent the oxidation of the released compounds). The alkaline hydrolysis products are then acidified with concentrated HCl (color change from yellowish to white, pH ~1) and directly analyzed by HPLC-PDA-MS/MS (Llorach et al., 2003), or other chromatographic or

spectrophotometric methods to identify the acylating organic acids and the remaining flavonoid glycoside.

7.4.2   Acid Hydrolysis

Acid hydrolysis of the naturally occurring flavonoids is used to release the sugar residues linked and the flavonoid aglycone. This is applied to the hydrolysis of flavonoid *O*-glycosides. Flavone-*C*-glycosides are not hydrolysed with acid, but they are isomerized to convert 8-*C*-glycosides partially into 6-*C*-glycosides (it seems that the 6-*C* configuration is more favored). Acid hydrolysis of 8-hydroxyflavone-glycosides produces opening of the flavone ring and isomerization, leading to a mixture of aglycones including the original 8-hydroxyflavone aglycone and the isomeric 6-hydroxyflavone. Acid hydrolysis is achieved by dissolving 1 mg of the isolated flavonoidin 1 mL of MeOH (or 1 mL of the crude extract), and after adding 1 mL of 4N HCl, the solution is heated at 90 °C for 45 min. In the case of flavonoid glucuronides, stronger conditions are needed to complete the hydrolysis [higher temperatures (100 °C) and longer hydrolysis times (4h)]. The aglycones are then extracted from the aqueous solution by liquid-liquid fractionation using diethyl ether, and the unhydrolyzed glycosides (i.e. *C*-glycosides) with ethyl acetate after removal of the MeOH, while the released sugars remain in the aqueous phase.

Partial acid hydrolysis can be used for characterization purposes, including sequential release of sugar residues. In this case mild acid conditions (0.1–0.5 N HCl at 75 °C) are used, and the time is controlled, taking samples after different times of hydrolysis. Samples taken at different times are monitored by TLC or HPLC to follow the hydrolysis process and the production of hydrolysis intermediates. This allows the selection of specific hydrolysis conditions (temperature and time) for isolation of the intermediates of interest.

7.4.3   Enzyme Hydrolysis

Enzymatic hydrolysis can be used for two purposes: for identification of specific sugars and their linkage position, and to preserve the naturally occurring structure of the flavonoid, when the compound is sensitive to the acidic conditions of a classical acid hydrolysis (i.e. 8-hydroxy-flavonoids are converted to 6-hydroxyflavonoids through acid-catalyzed isomerization). As an example, the UV study of the flavonoid *O*-glycosides will show the free phenolic hydroxyls in the naturally

occurring molecule. The UV analysis of the enzyme-hydrolyzed agly-cone will show any additional free hydroxyls released after the removal of the sugar residue by the action of the enzyme, thus indicating the position of the sugar linkage. In addition, a positive hydrolysis with β-D-glucosidase will show that the original flavonoid is a flavonoid-β-D-*O*-glucoside.

β-*Glucosidase hydrolysis*
This is achieved by adding 0.5 mg of flavonoid to 3 mg of β-D-glucosidase in 0.5 mL of 0.1 M citrate-phosphate buffer, pH 5 (37 °C, 24 h). The hydrolysis products are then extracted with ethyl acetate, taken to dryness under reduced pressure (40 °C), and redissolved in methanol for HPLC analysis (Gil et al., 1998).

Hydrolysis with glucuronidase and/or sulfatase
This is useful for the quantification of the phase II conjugate metabolites of flavonoids, present in biological fluids such as plasma and urine. Flavonoid glucuronide and sulfate conjugates can be hydrolyzed by incubation of 100 μL of the sample (urine or plasma) in 50 μL of 0.1 M sodium acetate buffer (pH 5.2) containing an aqueous solution of *Helix pomatia* enzyme extract H-2, G-0876 (EC 3.2.1.31), containing glucuronidase and sulfatase enzymes, at 37 °C for 18 h. The reaction mixture is then extracted with 300 μL of ethyl acetate, vortexed for 3 min and centrifuged at 1000 ×g for 1 min. The supernatant fraction can be dried under N_2 and the residue dissolved in 100 μL of methanol for HPLC analysis (Vallejo et al., 2010). In order to determine if the hydrolysis is complete, it is always recommended to analyze the remaining water phase by HPLC-PDA-MS/MS. The hydrolysis of glucuronides is generally complete while hydrolysis of sulfates with this enzyme preparation is not so efficient. A percentage of the original sulfates (5–30%) can remain unhydrolyzed and this needs to be considered in quantitative studies (Crozier et al., 2009).

7.5   Analysis of Crude Extracts

7.5.1   *Paper Chromatography*

Two-dimensional paper chromatography (2D PC) has been tradition-ally used for direct and complete qualitative analysis of complex

flavonoid extracts. This is usually completed with UV detection following the changes after NH_3 vapor exposure (Mabry et al., 1970). The chromatographic paper used for this purpose is Whatman No. 1. The sample is applied with the help of a glass capillary, and submitted to a first chromatographic run using *n*-butanol/acetic acid/water (4:1:5, v/v/v, upper phase) (BAW). After drying at room temperature, a second run with 15% or 30% acetic acid is completed (depending on the nature of the glycosidic combinations of the flavonoids present in the extract; 15% is recommended for more polar flavonoids) (Figure 7.1). In the first dimension, the flavonoid aglycones run closer to the solvent front, and the more polar glycosides run with shorter Rf values. In the second dimension the more polar compounds run with higher Rf values. This method separates flavonoids mainly by partition chromatography, and is complementary of the reversed-phase HPLC analyses that separate mainly by adsorption chromatography interactions. Both methods are therefore complementary, and could be used in combination for complex extracts.
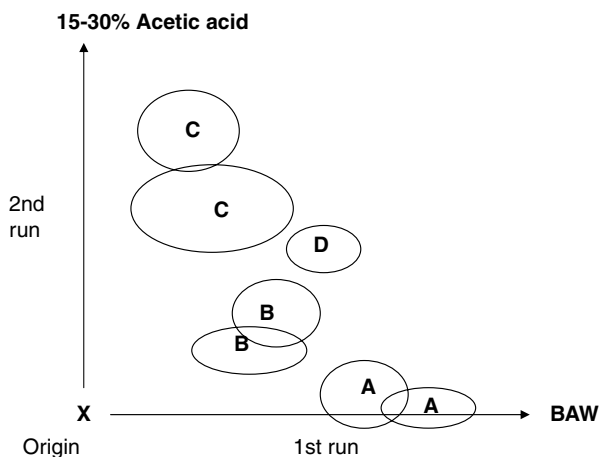
Figure 7.1 Two dimension paper chromatography of plant extracts. The methanol–water extract is deposited in the application point (origin), dried, and developed with BAW (*n*-Butanol/acetic acid/water; 4:1.5, upper phase) and 15% or 30% HOAc (acetic acid). (A) Flavonoid aglycones with different hydroxylation patterns; (B) Flavone monoglycosides; (C) Flavone di- and tri-glycosides; (D) Flavone di- and triglycosides acylated with hydroxycinnamic acids).

Flavonoid glucuronide and sulfate detection by paper electrophoresis
This method is used for the detection of flavonoid glucuronides and
flavonoid sulfates in complex extracts. Both flavonoid glucuronides
and sulfates migrate toward the anode when the extracts are analyzed by
paper electrophoresis using acetate buffer pH 4.5. The nonglucuroni-
dated or sulfated flavonoids do not migrate as they are not charged at this
pH and remain in the application point, while glucuronides and sulfates,
which are acidic, are negatively charged at this pH and migrate toward
the anode (Figure 7.2). The migration of the flavonoid spots can be
followed using a UV lamp (360 nm). After 30 min of migration, the
glucuronides and sulfates are easily spotted and the migration toward
the anode is evaluated. A formic acid solution pH 2.2 can also be used
for the exclusive detection of flavonoid sulfates, as under these con-
ditions only flavonoid sulfates are ionized and migrate (only the sulfate
residue remains ionized at this pH while the glucuronic acid is not
ionized). This methodology could also be used as a preparative method
for the analysis of biological samples (plasma and urine).
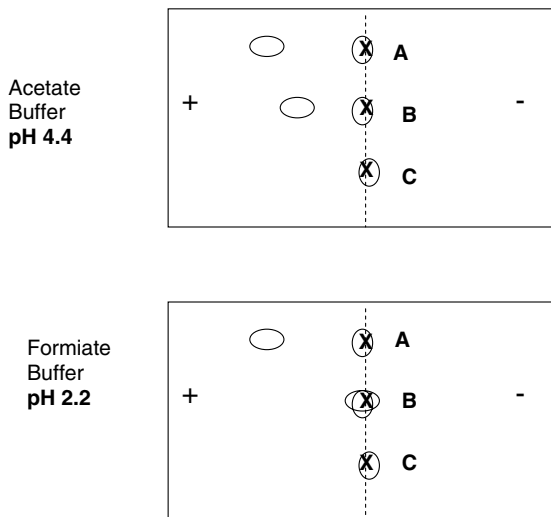
Figure 7.2 Paper electrophoresis for the detection of flavonoid glucuronides (pH 4.4)
and sulfates (pH 2.2). (A) Extract containing flavone sulfates; (B) extract containing
flavone glucuronides; (C) Extract containing flavone glucosides. The glucuronides and
sulfates move toward the anode at pH 4.4. Sulfates keep migrating toward the anode at
pH 2.2, but not glucuronides.

Methodology for paper electrophoresis using acetate buffer pH 4.4
The buffer used is acetate buffer 0.1 M pH 4.4 on Whatman No. 3 paper.
A small amount of the hydroalcoholic extract is inoculated using a glass
capillary (or a Pasteur pipette) in the middle of the paper (Figure 7.2).
After drying, the paper is placed on the electrophoresis cuvette and
the anode and cathode are marked with a pencil on the paper and
moistened with the buffer using a Pasteur pipette. Paper electrophoresis
is carried out at 400 volts; after 30 min the paper is removed and dried
and the flavonoid spots are visualized under UV light (360 nm).

*Methodology for paper electrophoresis using formic/acetic acid
solution pH 2.2*
The methodology is similar to that described above for electrophoresis
at pH 4.4. This methodology is specific for the analysis of sulfates. This
is carried out using a formic acid solution pH 2.2 prepared by adding
12.5 mL formic acid to 38 mL acetic acid in 1 L water. This solution
was used in the place of acetate buffer using the same methodology
described above.

7.6   Analysis of Crude Extracts by High-Performance Liquid Chromatography

7.6.1   Sample Preparation

In the case of flavonoid extracts or other samples that are available in
very small amounts –in which the isolation of flavonoids is not intended
but the study of the crude extract by HPLC is the objective –it is
sometimes recommended to filter the extract through a solid-phase
extraction cartridge (i.e. C-18). In this sense, the aqueous fraction
obtained from the hydroalcoholic crude extract after alcohol removal
using a rotary evaporatoris passed through a C-18 Sep Pak cartridge
previously activated with a volume of methanol (i.e. 5 mL) and then
with water (5 mL) and to end with the same volume of air. The aqueous
flavonoid extract, filtered to remove solid particles, is filtered through
a C-18 cartridge to adsorb the flavonoids, and the cartridge is then
washed with water to remove polar compounds such as sugars and short-
chain aliphatic acids. The water extract should be kept for analysis to
make sure that no flavonoids were eluted (some polar flavonoids, as is
the case of flavonoid glucuronides and sulfates, can be only poorly

adsorbed, and in these cases acidification of the water is recommended to enhance the adsorption on the stationary phase). The adsorbed flavonoids are then eluted with pure methanol, and the extract recovered for HPLC analysis.

7.6.2   *Reversed-Phase HPLC*

This is considered the method of choice for the analysis of flavones and flavonols due the high resolution of the chromatographic separations and the sensitivity of the detection methods that include UV, fluorescence, electrochemical, and MS detectors. The best combination is the detection system that links on-line UV detection with a photodiode array detector (PDA) that allows the registration of the UV spectra of the eluting compounds, with an MS/MS detector as an ESI ion trap that allows the isolation and fractionation of specific ions, even if they co-elute under the same chromatographic peak.

There are in the market different HPLC chromatographic columns with different particles sizes (generally 5 or 3 µm) for regular HPLC analysis. They may offer different resolutions and need to be assayed for the specific extract that is being analysed. The solvents used as mobile phases include mixtures of methanol and water, or acetonitrile and water, usually applying a gradient elution with different solvent rates depending on the extract to be analyzed and the compounds that need to be resolved. The addition of an organic acid (acetic acid or formic acid) to the solvent mixture is necessary to increase the peak resolution. The acidic conditions prevent ionization of phenolic hydroxyls, and therefore a better interaction with the stationary phase leading to less 'tailing,' and a better peak height is obtained.

UPLC (uses smaller columns with smaller particle size and higher resolution and solvent pressure), which has recently been introduced, allows for better resolution of chromatographic peaks, shorter analysis time, and less solvent consumption.

7.6.3   *Capillary Electrophoresis*

Capillary electrophoresis (CE) was first applied to the analysis of flavones in complex extracts in the 1990s (Tomás-Barberán, 1995). There was much expectation on the possibilities of this analytical method, due to the inexpensive columns used (fused silica capillary

columns), the use of buffer solutions that do not consume organic solvents, and the short times of analysis. CE can be coupled to detectors including UV (with PDA detectors that allow the recording of UV spectra of different compounds), and MS detectors. In spite of these expectations, CE has not replaced HPLC as the method of choice for the analysis of flavones. This is mainly due to the lack of reproducibility of the CE conditions for a given analysis. CE has been applied to flavonoid glycosides as capillary zone electrophoresis (CZE), and to flavonoid aglycones by micellar electrokinetic capillary chromatography (MEKCC), whichis based on the addition of micelles and organic solvents to the buffer and separation of flavonoids by a combination of interaction with the micelles and electrophoretic migration.

Capillary zone electrophoresis (CZE)
This is applied for the separation of flavonoid glycosides. It separates the flavonoid molecules following the charge/mass ratio of the different flavonoids. The higher this ratio, the faster the compound migrates in the capillary column. In addition, when borate buffers are used, borate can complex sugar hydroxyls in a different way depending on their stereochemical structure, allowing separation of different flavonoid hexosides, which seldom happens in HPLC separations.

Micellar electrokinetic capillary chromatography (MEKCC)
The addition of micelles that migrate counter-current in the capillary can be used for the separation of apolar compounds, particularly when organic solvents such as methanol are added to the buffer solution. Using this method, flavone and flavonol aglycones present in honey were separated, although no specific advantage with HPLC separation using reversed-phase columns was observed.

7.7   Spectroscopic Methods for Structural Analysis

The main spectroscopic methods used for the structural characterization of isolated flavonoids are ultraviolet spectrophotometry (UV), mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (NMR). UV and NMR methods (both ^{1}H NMR and ^{13}C NMR) have been extensively covered in previous publications, and therefore will only be summarized in this chapter. MS, and particularly HPLC-MS/

MS, has been applied extensively to flavonoid characterization in the last few years, and in this chapter we will review the state of the art of this powerful method, that is particularly useful in the analysis of flavonoid glycosides present in complex extracts.

7.7.1   Ultraviolet-Visible Spectrophotometry

The flavonoid compounds may be easily identified by their characteristic spectra in methanol since the majority exhibit a maximum in the long UV range (band I) between 325 and 400 nm, and a second maximum at shorter wavelengths (band II) between 240 and 295 nm. These two bands can be split into two maxima or a maximum and a shoulder or an inflection (BIa, BIb, BIIa, and BIIb). In some cases it is possible to distinguish a supplementary maximum, shoulder, or inflection (band III) between 295 and 325 nm (Barberán et al., 1985). The BI is associated with absorption of the flavonoid B-ring, which is associated with a cinnamoyl-nucleus, while the BII is associated with A-ring,which corresponds to a benzoyl-nucleus (Mabry et al., 1970) (Figure 7.3).

Thus, the UV spectra of flavones and flavonols (3-hydroxyflavones), disubstituted in B-ring (luteolin, quercetin, etc.) exhibit two maximain BII (usually at $\sim$ 255 and 266 nm) (Figure 7.4). However, the B-ring monosubstituted flavones (apigenin, kaempferol, etc.), show only a single response for this band ($\sim$266 nm). On the other hand, while the BI of flavones and 3-methoxyflavones show an absorption maximum between 325 and 355 nm, the maximum of this band in flavonols with a free hydroxyl at position 3 is shifted to larger wavelengths ($\sim$360–380 nm).
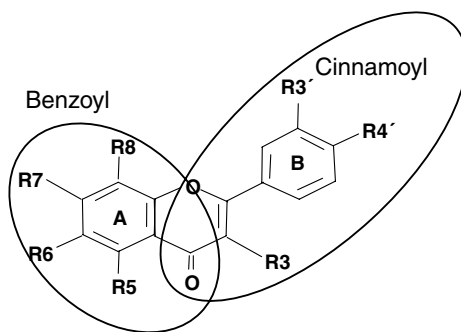
Figure 7.3 General structure of flavones. Contributions of the A- and B-rings to the UV spectrum.
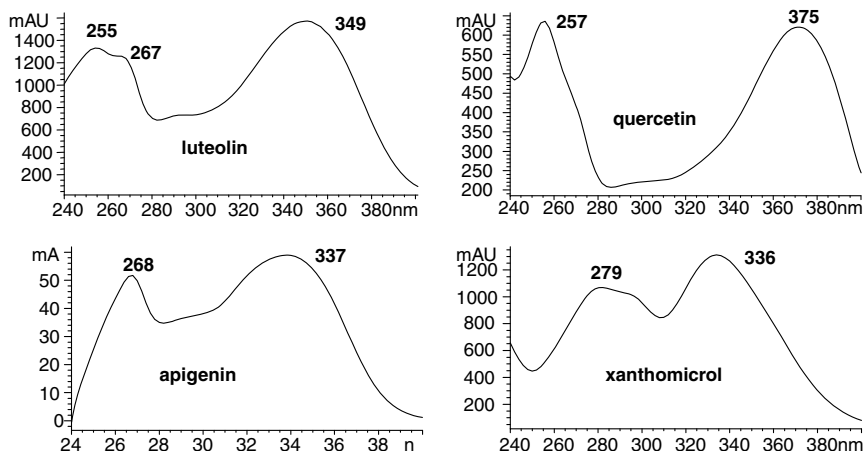
Figure 7.4 UV spectra of flavones, methoxyflavones, and flavonols.

The position of the BII maximum, in flavones, is affected by the introduction of additional substituent(s) at position(s) 6 and/or 8 which, in general, produce a bathochromic shift placing this maximum above 270 nm (Voirin, 1983). This is the case of xanthomicrol (5,4-dihydroxy-6,7,8-trimethoxyflavone) (Figure 7.4).

Hydroxylation at C-6 produces a larger bathochromic shift than hydroxylation at C-8. Moreover, the ratio of absorbance band I/absorbance band II constitutes a criterion for distinguishing the substituted position on the Aring. Thus, 8-substitution particularly decreases this value whereas 6-substitution increasesit (Barberán et al., 1985). Useful information to differentiate among 5,6- and 5,8-dihydroxyflavones, 3-methoxyflavones and flavonols trisubstituted on the Aring, as well as 5,6-dihydroxy-7,8-dimethoxy- and 5,8-dihydroxy-6,7-dimethoxy-flavones can be gained by comparison of their UV spectra in methanol (Barberán et al., 1985).

Flavonoids acylated with hydroxycinnamic acids show a UV spectrum of the flavonoid overlapped with that of the acid, this last being the main one. They are characterized by a maximum with a high absorption ~330 nm (310–335 nm), and eventually can show a small maximum that coincides with the flavonoid band II (~255–268 nm) and a shoulder at higher wavelengths that reflects the flavonoid Band I (Vallejo et al., 2004). In polyacylated flavoneglycosides, in which two or even three hydroxycinnamate molecules are attached to the flavonoid

glycoside, a higher the number of hydroxycinnamic acid residues results in a more predominant absorption of the maximum at 320–330 nm, and in a decreased contribution of the BI and BII of the flavonoid to the whole spectrum. This BI and BII can even become a shoulder or an inflection in the UV spectrum.

The addition of alkaline (NaOMe, NaOAc, and NaOAc/H_3BO_3) or metal reagents ($AlCl_3$ and $AlCl_3$/HCl) to a methanolic solution of isolated flavonoids produce shifts in the UV spectra absorption bands, allowing the detection of the presence or absence of specific hydroxyls in different characteristic positions 5, 7, 3′, and 4′ (Mabry et al., 1970). They studied the effect of these reagents on a large number of flavonoids. The addition of NaOMe to flavonoids with a free hydroxyl in position 4′ induces a bathochromic shift of BI between 40 and 65 nm without a decrease in absorbance intensity. The addition of NaOAc, however, is useful for the determination of the presence of a free hydroxyl in position 7 as it produces a bathochromic shift between 5 and 20 nm in the maximum of BII. Alkaline reagents can also induce a flavonoid decomposition that is reflected in the disappearance of the UV spectrum when the flavonoid structure presents a 3,4′-dihydroxylic or a trihydroxylation (5,6,7-, 5,7,8-, or 3′,4′,5′-), this decrease being more marked when adding NaOMe rather than NaOAc, which is related to the more intense alkaline character of the first reagent. The addition of NaOAc plus H_3BO_3 can be used for the detection of *o*-dihydroxylic groupings, due to the effect of the complex produced between the boric acid and the dihydroxylic grouping, reflected in the UV spectrum as a bathochromic shift in BI between 12 and 30 nm. The $AlCl_3$ reagent forms complexes in the presence of *o*-dihydroxylic groupings (3′,4′; 5,6, or 6,8), and between the hydroxyl in position 5 and the carbonyl in 4 (or the hydroxyl in 3 and the carbonyl in 4) that induce a bathochromic shift in BI. The addition of HCl to a solution that already contains $AlCl_3$ destroys the complex formed with the *ortho*-dihydroxylic groupings, but preserves those complexes involving the carbonyl group, producing a hypsochromic shift of 30–40 nm, and maintaining the bathochromic shift reflected in the UV spectrum in MeOH due to the stable complex between the carbonyl and the hydroxyls at position(s) 5 and/or 3.

Voirin (1983) reported a complete study of the UV spectra of 151 flavonoids in methanol alone and with added $AlCl_3$ and $AlCl_3$/HCl for the differentiation of 5-hydroxy- and 5-hydroxy-3-methoxyflavones with mono-(4′)-, di-(3′,4′)-, or tri-(3′,4′,5′)-substituted B rings, and he

distinguished 20 groups of compounds depending on UV spectra shapes and the position of BI.

7.8   Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy is a powerful analytical method for the determination of flavone structures. It has, however, some limitations as the sensitivity is rather low, and compounds have to be isolated. The assignments of the different proton and carbon signals in ^{1}H and ^{13}C NMR can be based on chemical shifts (δ) and coupling constants (J), and correlations observed in homo- and hetero-nuclear 2D NMR. NMR spectra of flavones have been extensively published previously (Markham and Chari, 1982; Agrawal, 1989; Markham and Geiger, 1993; Fossen and Andersen, 2006).

The most frequently used solvent for flavones is hexadeuterodimethylsulfoxide (DMSO– $\delta 6$) and tetradeuteromethanol (CD$_3$OD). For lypophilic flavone aglycones, solvents such as carbontetrachloride (CCl$_4$) are recommended.

NMR experiments include COSY, TOCSY, ^{1}H-^{13} Cheteronuclear NMR experiments, NOESY (nuclear overhauser enhancement spectroscopy) and ROESY (rotating frame over hauser effect spectroscopy) as well as other two- and three-dimensional methodologies (Fossen and Andersen, 2006).

As comprehensive lists of NMR data for different flavonoids are published in the above-mentioned books and chapters, they will not be repeated here.

7.9   Mass Spectrometry of Flavonoids

The use of mass spectrometry as a tool for the structural analysis of flavonoids has constituted a fundamental step forward, particularly after the development of emerging techniques that allow work to be conductedat atmospheric pressure without the need for compound derivatization before analyzing for volatilization and ionization. On the other hand, coupling these mass detectors to separation systems, particularly HPLC, has permitted the screening and characterization of flavonoids in complex matrices, and a tentative structural identification of complex

flavonoids, without the need for isolation and purification. This is of great importance for research studies in which only very small numbers of samples are available, and with compounds that are present in trace amounts in the extracts as is the case of the study of the flavonoid metabolites present in biological fluids and tissues.

Among the different ionization sources actually used, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) are the ones more generally used for flavonoid analyses, with APCI more appropriate for apolar low molecular weight molecules. ESI is the main system used for the study of flavonoids, particularly for flavonoid glycosides, and the negative mode is the method of choice. The mass detectors with better applications for flavonoid analysis include the quadrupole–time of flight (Q-ToF), triple quadrupole (3Q), and iontrap (IT). The first two are used more for target analyses in which we already know the flavonoid we want to quantify and detect in a complex matrix, while the first one provides the exact mass, which is useful for flavonoid identification. The mass transitions and fragmentation also provide information for flavonoid characterization and identification. The IT-detector is more valuable for structural analysis of unknown compounds, in which a specific ion present in a complex chromatographic peak can be isolated in the ion trap and fragmented –and this can be related to the structural analysis.

Stobiecki (2000) and Cuyckens and Claeys (2004) reported exhaustive studies of the use of mass spectrometry in the structural analysis of flavonoids with a review ofthe available mass spectrometric methodologies currently used in the structural elucidation of flavonoid glycosides.

7.9.1 Flavonoid Aglycones

In general, flavonoid aglycone fragmentation can lead to the loss of small fragments (18 mass units (m.u.) H_2O; 28 m.u. CO; 42 m.u. C_2H_2O, etc). Particularly characteristic is the loss of 15 m.u. (CH_3) in the fragmentation of methoxylated flavonoids, and the relative abundance of the resulting ions can indicate the position of the methoxyl residue on the flavonoid nucleus. For this purpose, Barberán et al. (1986) published a study that differentiated 5,6-dihydroxy-7,8-dimethoxyflavones from 5,8-dihydroxy-6,7-dimethoxyflavones by the relative abundance of the ions produced during fragmentation. Other degradation pathways can
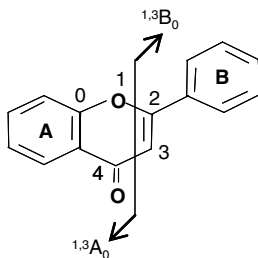
Figure 7.5 Mass Spectrometry Retro Diels–Alder (RDA) cleavage of the flavone nucleus.

lead to fragments providing information of the substituents present in the A- and B-rings. The main one is the retro-Diels–Alder (RDA) fragmentation of the flavone nucleus that results in the cleavage of the C-C bonds at positions 1/3, 0/2, 0/3, 0/4, or 2/4 of the C-ring. The fragmentation pathways depend on the substitution pattern and the class of flavonoid studied, as well as on the collision energy applied (Cuyckens and Claeys 2004). Figure 7.5 represents one of these possible RDA ring cleavages.

7.9.2   *Flavonoid Glycosides*

Most flavonoids are present in food products and biological fluids linked to different sugars by *O*-glycosidic bonds (flavonoid *O*-glycosides), *C*–*C* bonds (flavonoid *C*-glycosides) or both glycosylation types in a single flavonoid molecule (*O*-glycosyl-*C*-glycosyl flavonoids). This last case involves *C*-glycosyl flavonoids in which an additional *O*-glycosylation takes place either on a phenolic hydroxyl or on a hydroxyl group of the *C*-glycosidic sugar, or both. The MS fragmentation of these two types of glycosidic bonds is different due to the difficulty of breaking the *C*–*C* bond. The different theoretical fragmentation patterns of a flavonoid *O*-glycoside are shown in Figure 7.6.

    The classical nomenclature (Domon and Costello, 1988) for glyco-conjugates was adopted to designate the different fragment ions. Ions $^{k,l}X_j, Y_j^n, Z_j^n$ represent those fragments still containing the flavonoid aglycone, where j is the number of interglycosidic bonds broken, counted from the aglycone, n represents the position of the phenolic hydroxyl where the oligosaccharide is attached, and k and l denote the cleavage within the carbohydrate rings (Figure 7.6). In other MS
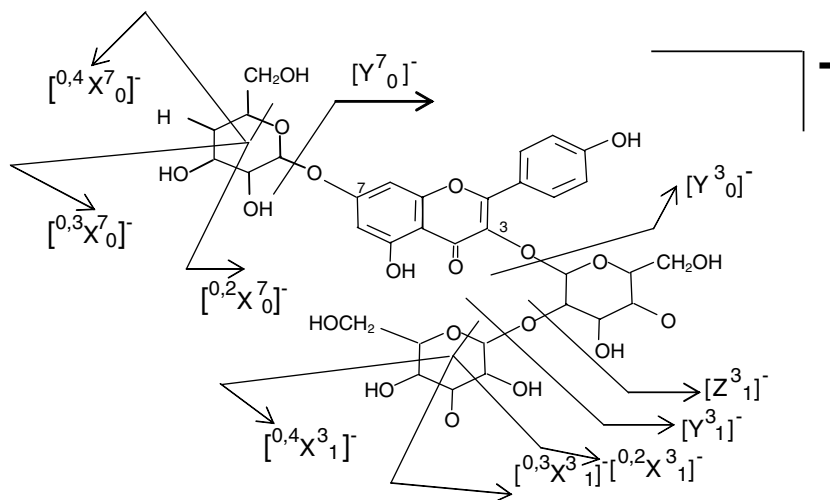
Figure 7.6 General scheme of the MS fragmentations of flavonoid glycosides.

spectra the ions obtained as a consequence of a second oligosaccharide fragmentation have been labeled according to previous reports (Ferreres et al., 2004) (Figure 7.7). Thus, ions obtained from the ion $Y_0^{7-}(-MS3[(M-H) \rightarrow Y_0^7]^-)$ have been labeled starting with the ion Y_0^{7-} and followed by the resultant MS3 ion, e.g. the ion $[Y_0^7 Y_2^3]^-$ and denote the loss of the terminal sugar of the triglucoside in position $[Y_2^3]^-$ from the fragmentation of ion $[Y_0^7]^-$ (total loss of a glycosylation in position 7). The losses indicated in the MS3 scan show that the fragment came from the trapped and fragmented ion $[Y_0^7]^-$ and not from the deprotonated molecule. This terminology completes the nomenclature provided by Domon and Costello (1988) by the addition of specific ways in which ions are obtained from the MS3 event.

In general, it is possible to differentiate the type of sugar by means of the MS losses and the relative abundance of the ions formed, and in some cases the glycosylation position and the interglycosidic linkage. In the following discussion, the results have been obtained using ESI–IT mass spectrometry in the negative mode, coupled to an HPLC.

7.9.3 *O-Glycosyl Flavonoids*

In the MS spectra of flavonoid-*O*-glycosides the deprotonated aglycone ion is always observed as a consequence of the fragmentation of the glycosidic linkage, which is the weakest bond. This aglycone ion is

Intens.
x10⁶

(a) Kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside*O*

771.4
$[Y_0^7]^-$
$[(M-H)-162]^-$

-MS2(933.5)
-MS2[M-H]⁻

x10⁴

284.9
$[Y_0^7Y_0^3]^-$
$[Y_0^7-486]^-$

254.9

348.9

$[Y_0^7Z_1^3]^-$
$[Y_0^7-162-180]^-$

428.8

609.1
$[Y_0^7Y_2^3]^-$
$[Y_0^7-162]^-$

-MS3(933.5 → 771.4)
-MS3 [(M-H) → (M-H-162)]⁻

300   400   500   600   700   800   900   1000 m/z

Intens.
x10⁶

(b) Kaempferol-3-*O*-sophoroside-7-*O*-sophoroside

609.3
$[Y_0^7]^-$
$[(M-H)-324]^-$

$[Y_1^7]^-$
$[(M-H)-162]^-$

771.2

429.2

-MS2(933.5)
-MS2[M-H]⁻

x10⁵

285.1
$[Y_0^7Y_0^3]^-$
$[Y_0^7-324]^-$

257.1

338.9

428.9
$[Y_0^7Z_1^3]^-$
$[Y_0^7-180]^-$

-MS3(933.5 → 609.3)
-MS3[(M-H) → (M-H-324)]⁻

300   400   500   600   700   800   900   1000 m/z

Figure 7.7 MS2$[M-H]^-$ and MS3$[(M-H) \rightarrow Y_0^7]^-$ of isomeric flavonol tetraglucosides: (a) kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside; (b) kaempferol-3-*O*-sophoroside-7-*O*-sophoroside (Ferreres et al., 2004).

generally the base peak (the most abundant peak) when the glycosylation is only on one phenolic hydroxyl of the flavone molecule (Figures 7.8A and 7.8B, ion $[Y_0^3]^-$). In the monoglycosylated flavonoids, the observed loss can be 175/162/146/132 m.u. forhexuronic acid/hexose/desoxyhexose/pentose, respectively. In flavonoid oligosaccharide fragmentation, fragments due to the rupture of the interglycosidic linkage with the losses mentioned above can be observed (Figure 7.8A, ion $[Y_1^3]^-$); and/or the same losses plus18 m.u. (180/164/150 m.u. for hexose/desoxyhexose/pentose, respectively) (Figure 7.8A, ion $[Z_1^3]^-$). This loss of a glycosidic fraction plus water is not observed in the losses of the sugars directly linked to phenolic hydroxyls and can be diagnostically associated with the interglycosidic linkages.

In flavonoids that are glycosylated on more than one phenolic hydroxyl group, the base peak is originated by the loss of one of the glycosidic fractions over one of the phenolic hydroxyls, with the aglycone ion being less abundant or non existent, and the ion $[Z]^-$ that characterizes the interglycosidic linkage not observed (Figures 7.8A and 7.8B). The loss of a glycosidic residue from the hydroxyl at position
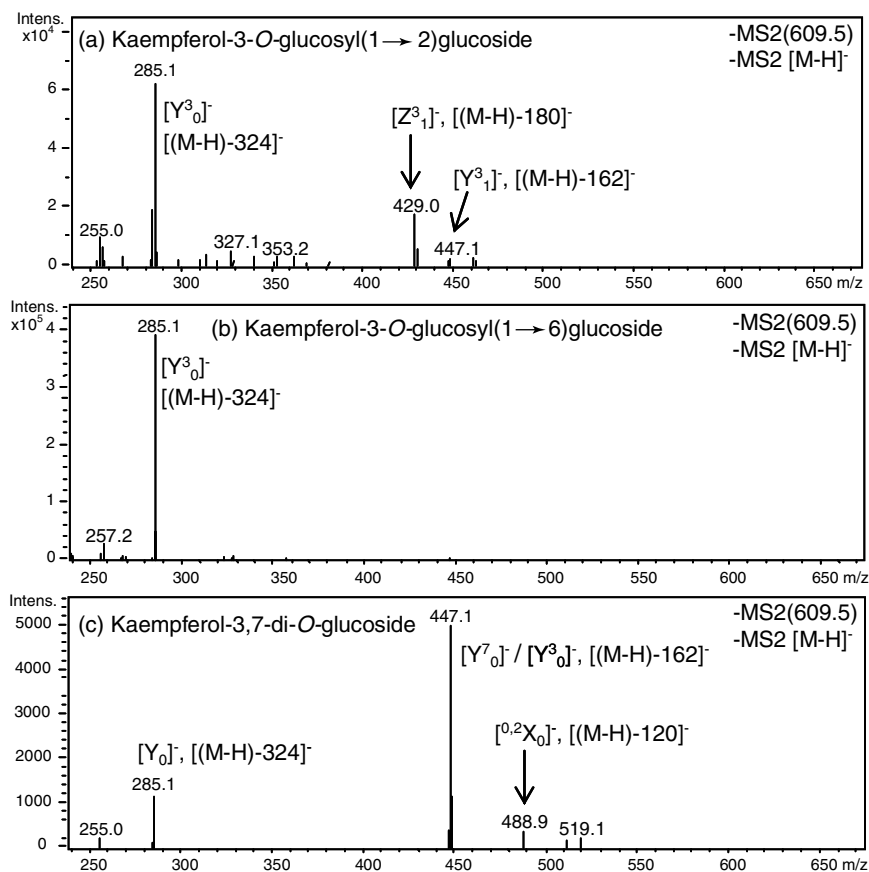
Figure 7.8A HPLC ESI-MS-MS analysis $(MS2[M-H]^-)$ of isomeric flavonol diglucosides: (a) kaempferol-3-*O*-sophoroside (glucosyl(1-2)glucoside); (b) kaempferol-3-*O*-gentiobioside (glucosyl(1-6)glucoside); (c) kaempferol-3,7-di-*O*-glucoside (Ferreres et al., 2004).

7 is always more favored than the loss of the glycosidic residues at position 3, and the former seems to be more stable (Figure 7.9). This can be used for diagnostic purposes.

Claeys and coworkers (Ma et al., 2001; Cuyckens et al., 2001; Cutckens and Claeys, 2002) carried out complete studies of the detection of different interglycosidic linkages of flavonoid glycosides under different analytical conditions. Rhamnosyl-glucosides –either *O*-neohesperidosides [rhamnosyl(1 → 2)glucosides] or *O*-rutinosides [rhamnosyl(1 → 6)-glucosides]– were studied and the authors concluded that differentiation was possible in the negative mode through a
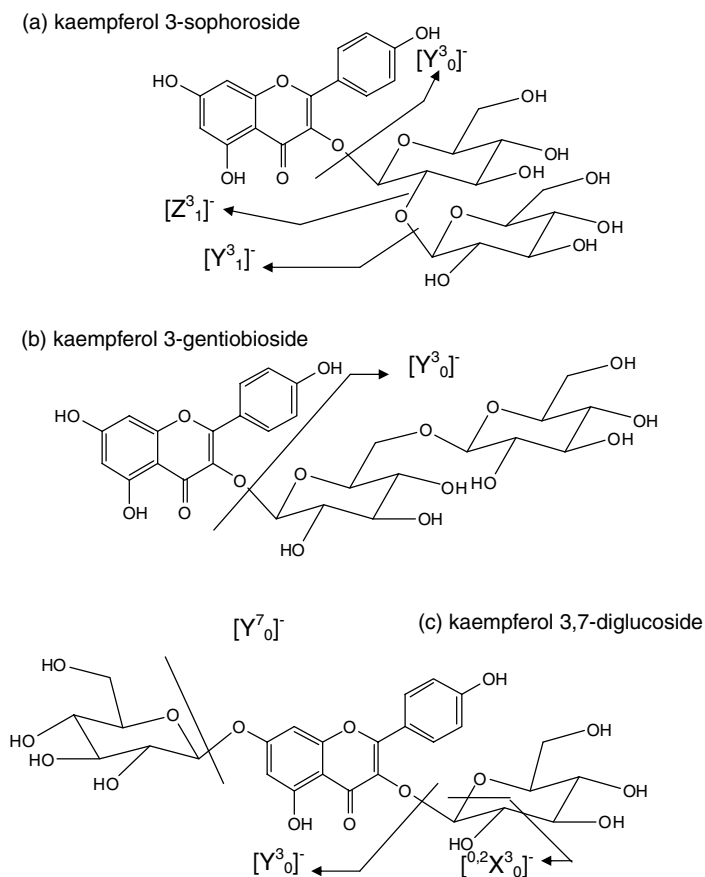
Figure 7.8B MS2[M−H]⁻ fragmentation patterns of isomeric flavonol diglucosides (Ferreres et al., 2004).

comparative study of the relative abundance of the ions produced by fragmentation of the interglycosidic linkage. In the same way, a considerable number of flavonoid diglucosides, including flavonoid sophorosides [glucosyl$(1 \rightarrow 2)$-glucosides] and gentiobiosides [glucosyl $(1 \rightarrow 6)$glucosides] were studied and a similar conclusion was reached (Ferreres et al., 2004). Thus, in the MS spectra of flavonoid sophorosides, the ions produced by the fragmentation of the interglycosidic linkage are always observed (Figures 7.8A and 7.8B), while these fragment ions are not detected in the case of gentiobiosides, or are less abundant, showing that the gentiobioside linkage is generally more stable under the conditions of the MS analysis. In the same study it was also demonstrated
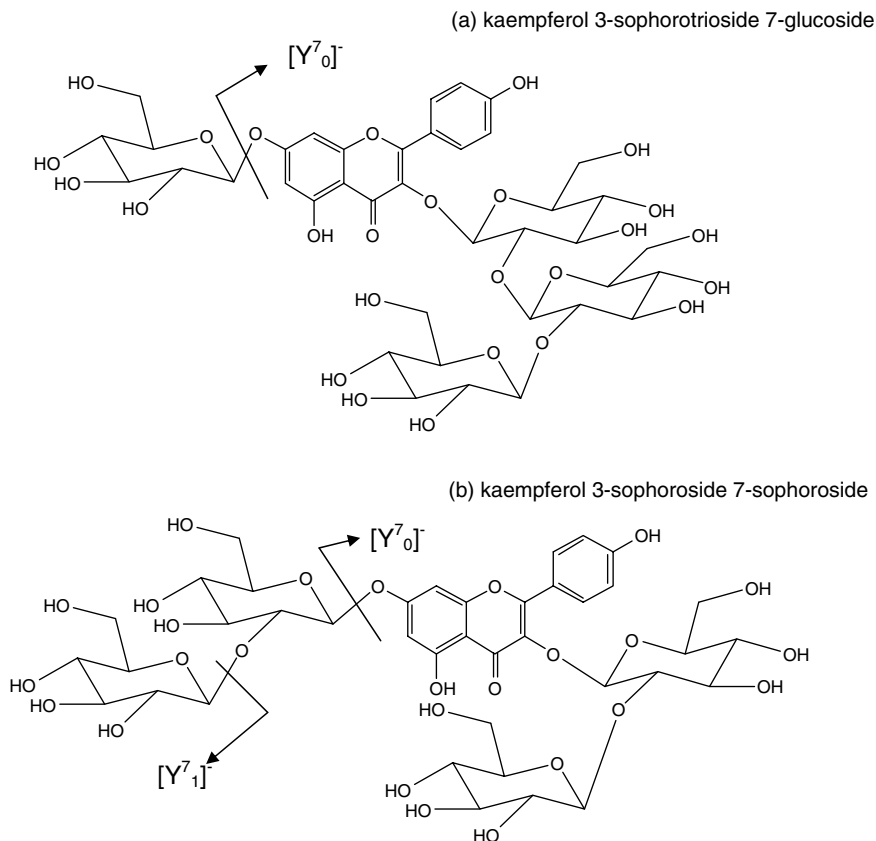
Figure 7.9 $MS2[M-H]^-$ fragmentation patterns of isomeric flavonol tetraglucosides (Ferreres et al., 2004).

that for flavonoid di-*O*-glycosylated in positions 3 and 7, the fragmentation of the sugar linked to position 7 is produced preferably, and therefore, it is possible to determine the type of oligosaccharide linked in each position. Thus, in the $MS2[M-H]^-$ fragmentation of flavonoid isomers with three (–3-*O*-sophorotriosides and -*O*-sophoroside-7-*O*-glucoside) or four (–3-*O*-sophorotriosides-7-*O*-glucoside and -3-*O*-sophoroside-7-*O*-sophoroside) glucoses, the base peak indicated the complete loss of the sugar moieties linked in position 7 (Figure 7.9).

The $MS3[(M-H) \rightarrow Y_0^7]^-$ event (Figure 7.7) revealed the ions resulting from the fragmentation of the glycoside in position 3. The same behavior is also observed in the case of kaempferol 3-sophorotrioside-7-sophoroside (a pentaglycoside) (Ferreres et al., 2004) in which

the main ions produced in MS2 and MS3 events allow the identification of the interglycosidic linkage and the glycosidic residues at positions 7 and 3. The preferential fragmentation $MS2[M-H]^-$ observed is due to the loss from position 7 ($[Y_0^7]^-$ base peak), and other ions due to the fragmentation of the diglucoside in 7 are also observed (($[Y_1^7]^-$), as well as the combined loss of the complete triglucoside linked in position 3 (($[Y_0^3]^-$). In the MS3 events, $[(M-H) \to Y_0^7]^-$ and $MS3[(M-H) \to Y_0^3]^-$ ions are produced by fragmentation of the glycosidic fractions in positions 3 and 7, respectively. Thus, ions at *m/z* 429 ($[Y_0^7 Z_1^3]^-$ and $[Y_0^3 Z_1^7]^-$) are not the same, as they originated by different fragmentation pathways.

7.9.4   *C-Glycosyl Flavones*

In most cases, *C*-glycosylation occurs in the position 6 and/or 8 of the flavone nucleus. They are often additionally *O*-glycosylated. In contrast to flavonoid-*O*-glycosides, a breakdown of the linkage between the sugar and the flavonoid nucleus is not possible, and an internal fragmentation of the sugar is observed instead. Through the analysis of the MS data available, it is possible to know the type of sugar directly linked to the flavonoid. In addition, the comparative study of the relative abundance of the fragment ions allows the detection of the *C*-glycosylation position (positions 6 and 8 of the flavone). The location of the positions of *O*-glycosylation, either on a phenolic hydroxyl oron the *C*-glycosyl sugar, is also feasible, as well asthe tentative differentiation of the position of substitution on the 2 or 6 hydroxyls of the *C*-glycosidic sugar.

In general, the absence of an abundant [aglycone-H]⁻ionin the –MS2 and/or –MS3 events, and the presence of [Aglycone + 41/42]⁻ and/or [aglycone + 71/72]⁻ are indicative of mono-*C*-glycosyl flavones, while for di-*C*-glycosyl flavones [aglycone + 83/84]⁻ and/or [aglycone + 113/114]⁻ are relevant ions.

In mono-*C*-glycosyl flavonoids, internal fragmentation of the sugar in positions 0/2 and 0/3 are the most frequent, leading to the ions $[^{0,2}X]^-$ and $[^{0,3}X]^-$, respectively (Figure 7.10), which is translated into 120 and 90 m.u. losses for hexoses and 90 and 60 m.u. losses for pentoses, and allow the characterization of sugar involved in the *C*-glycosylation. In addition, the abundant ions corresponding to the aglycone + 41/42 and the aglycone + 71/72, respectively, allow the characterization of aglycones, and their relative abundance indicates the position of

C-glycosylation. In mono-*C*-deoxyhexosyl derivatives the ions agly-
cone $+41$ $\{^{0,2}X^-, [(M-H)-104]^-\}$ and aglycone $+83$ $\{[^{0,4}X-18]^-,$
$[(M-H)-62]^-\}$ are observed (Ferreres et al., 2007a).

Thus, in mono-*C*-glycosyl flavones the presence of ion
$[(M-H)-120]^-$, and the simultaneous absence of ion
$[(M-H)-60]^-$, indicate that a hexose is the sugar involved in the *C*-
glycosylation. In these cases the ion $[(M-H)-90]^-$ can also be present,
which is more relevant in the 6-*C*-hexosyl derivatives (being, some-
times, the base peak) than in the 8-*C*-hexosyl derivatives (being,
sometimes, very small). The ion $[(M-H)-18]^-$, which is more frequent
in 6-*C*-hexosyl derivatives than in 8-*C*-hexosyl derivatives, can also be
observed (Ferreres et al., 2003) (Figure 7.10).

In the mono-*C*-glycosyl flavones, the absence of an $[(M-H)-120]^-$
ion, plus the presence of an $[(M-H)-60]^-$ ($[^{0,3}X]^-$) ion, indicate the
occurrence of a pentose as the sugar involved in the *C*-glycosylation;
this ion being also higher than the $[(M-H)-90]^-$ ($[^{0,2}X]^-$) ion in the 6-
C-pentosyl derivative than in the 8-*C*-pentosyl derivative. Both isomers
exhibit the ion $[(M-H)-90]^-$, which can be the base peak.

In asymmetric di-*C*-glycosyl flavones (di-*C*-substituted flavonoids
with different sugars) the preferential fragmentation is that of the sugar
linked to *C*-6 relatively to the one linked to *C*-8 and there is a production
of partial fragments of both sugars that lead to losses of mass from
the molecular ion. The relative abundance of these ions indicates the
C-glycosylation position for both sugars. In all cases, ions A $+83/84$
and A $+113/114$ can be observed, which characterize the aglycone
(Figure 7.10).

In *O*-glycosyl-*C*-glycosyl flavones the study of the relative abun-
dance of the main ions from the MS preferential fragmentation on
–MS2 and/or –MS3 events allows the differentiation of the position of
the *O*-glycosylation, either on phenolic hydroxyl or on the sugar moiety
of *C*-glycosylation. In addition, it is possible to discriminate between
O-glycosylation at positions 2'' and 6'' (Figure 7.11).

The occurrence of an abundant ion Y_0^- ($[(M-H)-132/-146/$
$-162]^-$, mono-*O*-pentosyl/rhamnosyl/hexosyl-*C*-glycosyl derivatives)
after –MS2 fragmentation characterizes the *O*-glycosylation on phe-
nolic hydroxyls (Figure 7.12).

The preferential fragmentation leading to a relevant Z_1^- ($[Y_1-18]^-$)
fragment is characteristic of 2''-*O*-glycosyl-*C*-glycosyl derivatives
(Figures 7.11 and 7.13). The 6''-*O*-glycosyl-*C*-glycosyl derivatives are
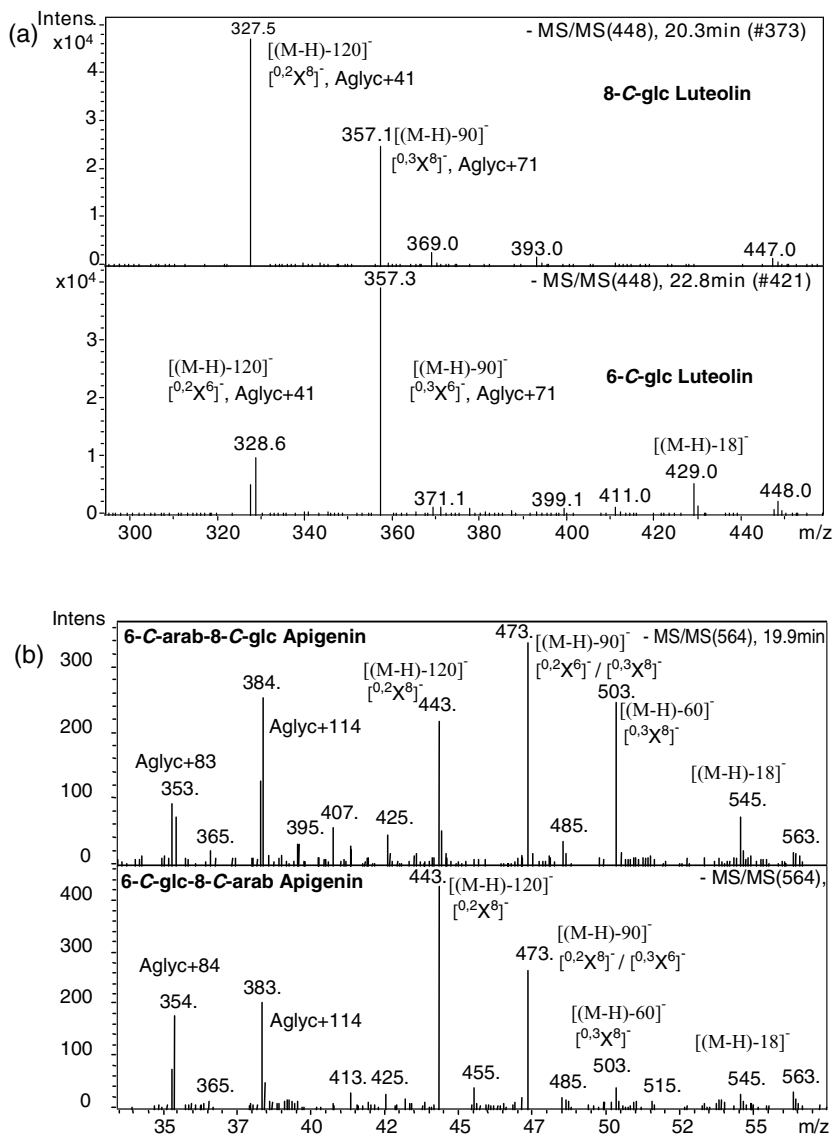
Figure 7.10 MS2[M−H]$^-$ of (a) mono-*C*-glycosyl flavonoid isomers: 8-*C*-glucosyl luteolin and 6-*C*-glucosyl luteolin and (b) asymmetric di-*C*-glycosyl flavone isomers: 6-*C*-arabinosyl-8-*C*-glucosyl apigenin and 6-*C*-glucosyl-8-*C*-arabinosyl apigenin (Ferreres et al., 2003).
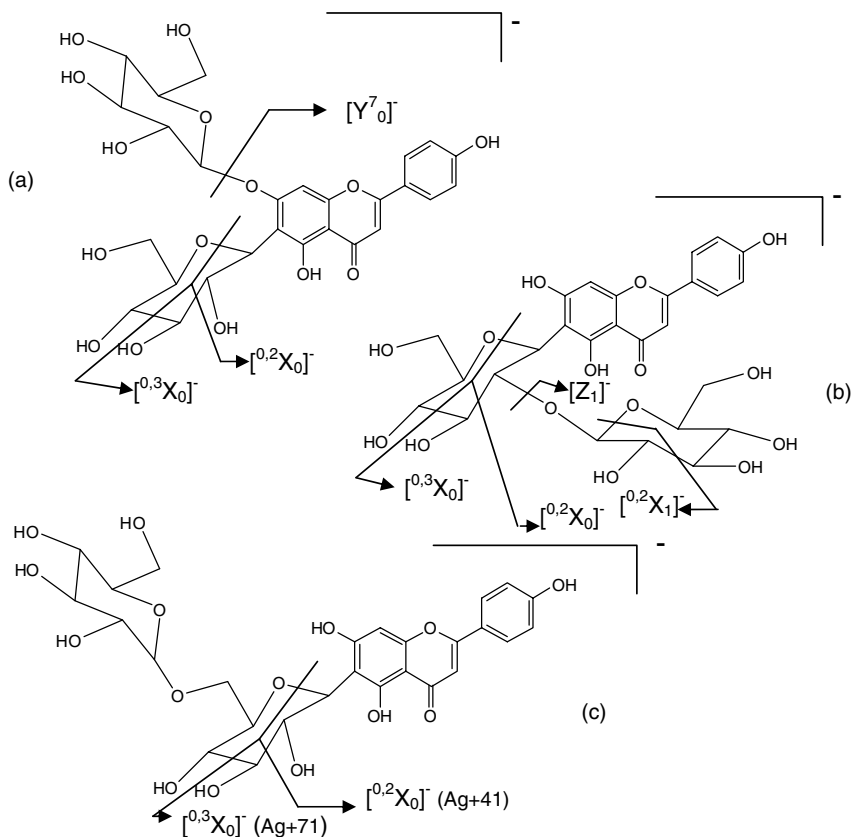
Figure 7.11 General fragmentation of *O*-glycosyl-*C*-glycosyl flavones: (a) *O*-glyco-sylation on phenolic hydroxyls; (b) 2″-*O*-glycosyl-*C*-glycosyl derivatives; (c) 6″-*O*-glycosyl-*C*-glycosyl derivatives.

characterized by $^{0,2}X_0^-$ and $^{0,3}X_0^-$, which is generated by a global loss of the sugar moiety from the *O*-glycosylation at 6″ and the glycosidic fraction that involves the carbons 6″-3″ and 6″-4″ of the *C*-glycosyl residue ($[(M-M)-162-120]^-/[(M-H)-162-90]^-$, in the case of 6″-*O*-hexosyl-*C*-hexosylderivatives) (Figures 7.11 and 7.13).

The simultaneous occurrence of sugars on the hydroxyls in 2″ and 6″ of the *C*-glycosylation sugar produces a fragmentation in which the ions Z_1^- ($[(M-H)-180]^-$, loss of the sugar in 2 with water and $^{0,2}X_0^-$ ($[(M-H)-120-162]^-$, internal fragmentation of the *C*-glycosylation sugar together with the sugar in 6″ are observed, in addition to the ions that characterize the aglycone. In the case of *C*-glycosylation in
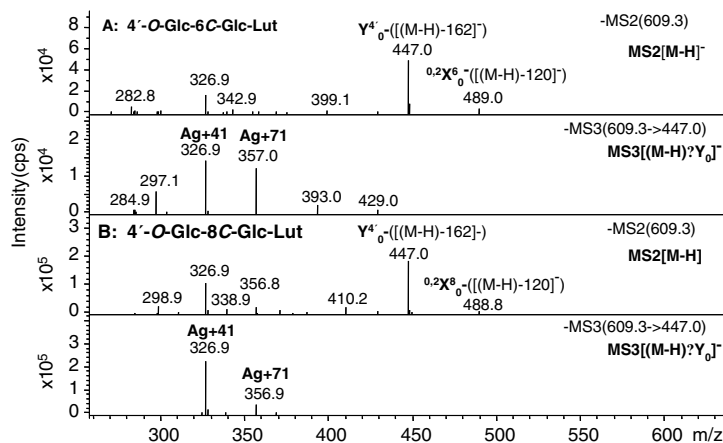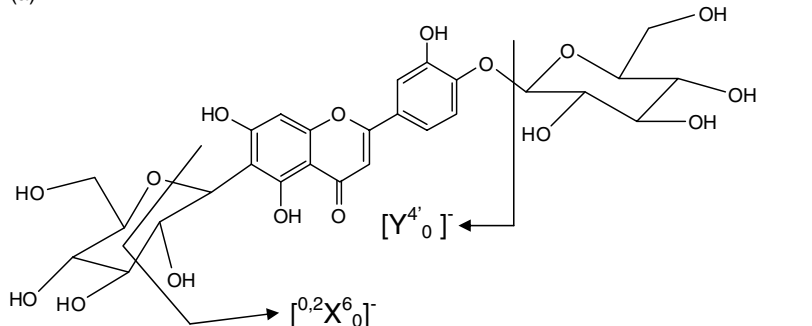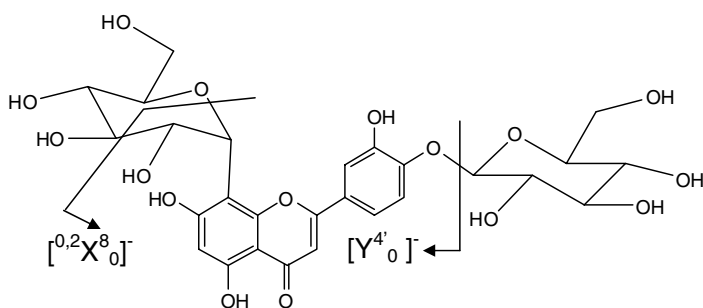
Figure 7.12 MS2[M−H]$^-$ and MS3[(M−H)→Y$_0$]$^-$ of *C*-glycosyl flavones *O*-glycosylated on phenolic hydroxyl: (a) 4′-*O*-glucosyl-6-*C*-glucosyl luteolin; (b) 4′-*O*-glucosyl-8-*C*-glucosyl luteolin (Ferreres et al., 2007b).
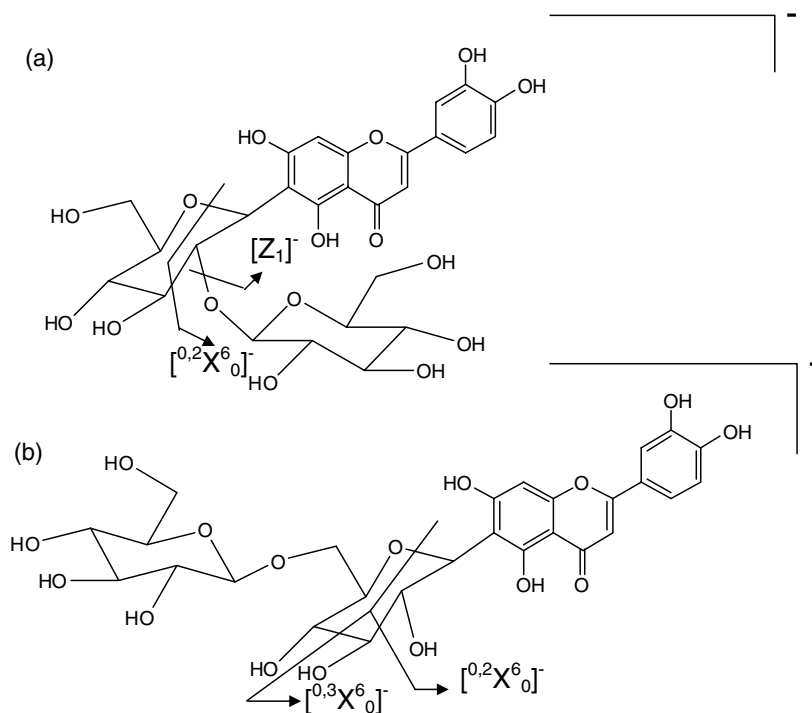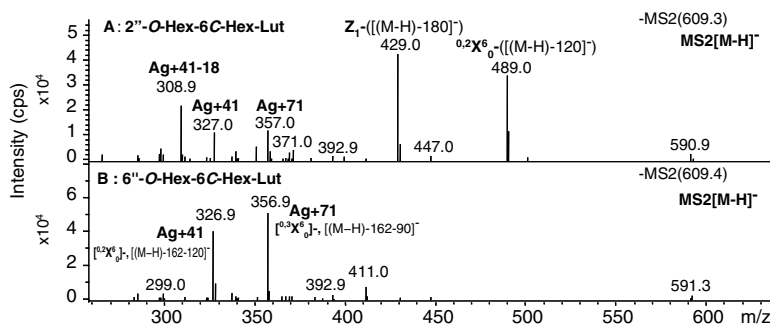
Figure 7.13 Fragmentation and $MS2[M-H]^-$ of X''-O-glycosyl-C-glycosyl flavones: (a) $2''$-O-hexosyl-6-C-hexosyl luteolin; (b) $6''$-O-hexosyl-6-C-hexosyl luteolin (Ferreres et al., 2007b).

position 6, the ion Z_1^- is the base peak, while the ions that characterize the aglycone are more abundant in the 8-C isomers (Figure 7.14) (Ferreres et al., 2007b).

Regarding the combined O-glycosylated compounds (both on phenolic hydroxyl and on sugar moiety at C-glycosylation), the
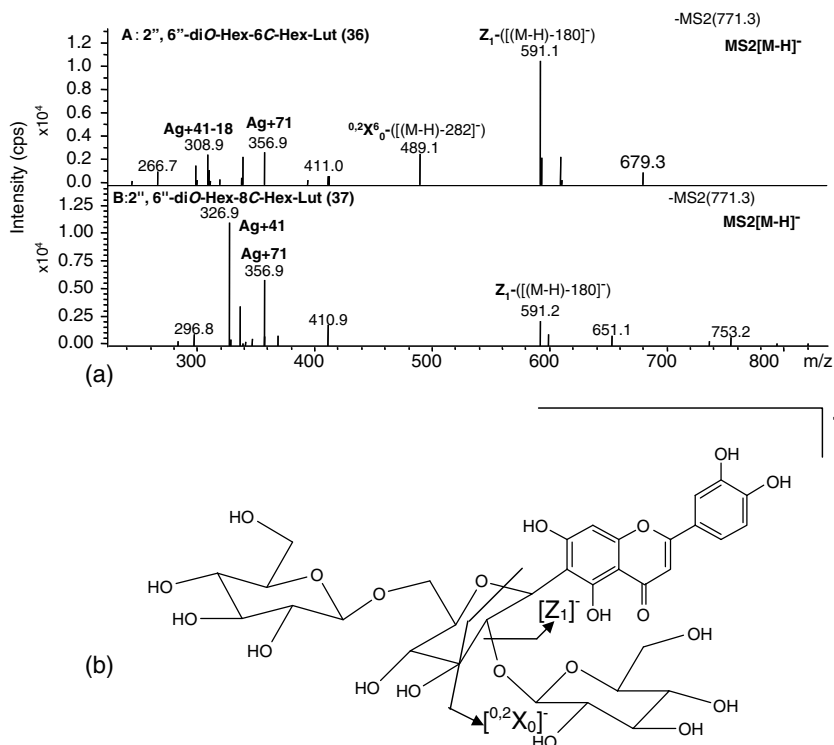
Figure 7.14 Fragmentation and MS2[M−H]⁻ of 2″,6″-di-*O*-glycosyl-*C*-glycosyl flavones: (a) 2″,6″-di-*O*-hexosyl-6-*C*-hexosyl luteolin; (b) 2″,6″-di-*O*-hexosyl-8-*C*-hexosyl luteolin (Ferreres et al., 2007b).

main fragmentation on –MS2 events produces a Y_0^- characterizing the *O*-glycosylation on the phenolic hydroxyl, and the $-MS3[(M−H) \rightarrow Y_0]^-$ fragmentation of the *O*-glycosylation on the *C*-glycosyl residue.

7.9.5   *Acylated Flavone Glycosides*

Flavone glycosides often occur as acylated derivatives either with aliphatic or aromatic acids. Hydroxy-cinnamic acid derivatives are the most frequent in nature and MS fragmentations of these types of compounds show ions produced by the loss of 146, 162, 176, or 206 m.u., corresponding to the losses of the acyl residues *p*-coumaroyl, caffeoyl, feruloyl, or sinapoyl, respectively. Brassicaceae species are rich in flavonoid glycosides acylated with hydroxyl-cinnamic derivatives, and most of them

show the general structure of a flavonol-3-*O*-(hydroxycinnamoyl)-glycoside-7-*O*-glycoside. In their MS2$[M-H]^-$ analysis, the main fragmentation leads to the loss of the sugar linked in position 7. In addition, ions produced by the loss of the acyl residue can also be observed. In the MS3$[(M-H) \rightarrow Y_0^7]^-$ event, representative of the fragmentation of the acylated glycosidic residue on position 3, the fragments due to the loss of the acyl residue are preferentially observed (Vallejoet et al., 2004). *Diplotaxis tenuifolia* (Brassicaceae) shows a glycosylation pattern with conjugations on hydroxyls at positions 3, 3′, and 4′(flavonol-3,3′,4′-tri-*O*-glucosides) and acylation on the glucosyl residue at 3′ (flavonol-3,4′-di-*O*-glucoside-3′-*O*-(acyl)-glucoside) or on the sugar residues at positions 3,3′ (flavonol-3-*O*-(acyl)-glucoside-3′-*O*-(acyl)-glucoside-4′-*O*-glucoside). The MS*n* (*n*, 2–3) fragmentation behavior of the monoacylated derivatives (flavonol-3,4′-di-*O*-glucoside-3′-*O*-(hydroxycinnamoyl)-glucoside) showed sequential losses of hexosyl residues from the $[M–H]^-$ and $[(M–H)-Glc]^-$ ions to give a peak base. In the MS4 event (fragmentation of the ion corresponding to the flavonol-3′-*O*-(acyl)-glucoside) loss of theacyl radical produces an abundant ion that often is the base peak.

In compounds diacylated on the sugar residues at positions 3 and 3′, the MS2 fragmentation showed a first loss of 162 units from the deprotonated molecular ion that must be due to the loss of the glucose that is not linked at position 4′.

In the MS3 event, it was possible to observe a loss of one or both acids, and the loss of glucose with its acyl moiety, where the loss of the acyl-glucosyl radical at position 3 was the base peak in the majority of the ions studied. Finally, the MS4 events of ions that still have both glucose and acyl moieties gave the deprotonated aglycone (base peak) or the ion coming from the corresponding deacylation (Martínez-Sánchez et al., 2007). This means that, in the same manneras occurred in the fragmentation of rutinosides/neohesperidosides and gentiobiosides/sophorosides, if the acyl substitution is on position 6 of the sugar moiety, the linkage is more stable than if the linkage is at position 2, and produces the loss of an acyl-glucosyl residue, while in the 2 substitution there is a breakdown of this bond with subsequent deacylation.

In flavonoids acylated with aliphatic acids, the most common acids are acetic and malonic. In the MS fragmentation of the dicarboxylic acids (as malonic acid), a first loss of 44 mass units is observed (loss of the carboxylic radical, CO_2), and this is due to decarboxylation.

In both cases it is possible to know the acylation position, particularly when they are linked at position 6 of the sugar, due to the fragments produced by the internal fragmentation of the sugar that produces fragments containing the acyl residue. Thus, in *Capsicum annuum* (Marín et al., 2004) it was possible to characterize the acylation position on a di-*C*-glucosyl-luteolin acylated with malonic aciddue to the presence in the MS3 event of ions at *m/z* 489 ($[^{0,2}X^6]^-$, $-(120+42)$) and at m/z 519($[^{0,3}X^6]^-$, $-(90+42)$ showing that the 42 residue was not linked to positions 2 or 3 of the hexose, as these carbons are not involved in these losses. Therefore, the acylation was thought to be at either position 4 or 6 onthe sugar residue. Position 6 is much more frequent in nature, and the compound was tentatively identified as luteolin 6-*C*-(6-malonyl)-hexoside-8-*C*-pentoside (Marín et al., 2004).

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Chapter 8

Analysis Methods of Proanthocyanidins

Liwei Gu

Abstract

Proanthocyanidins are bioactive polyphenols in foods. Quantification of proanthocyanidins has the disadvantage of poor sensitivity and specificity. Normal phase HPLC has a unique capacity to separate proanthocyanidin monomers through decamers according to the degree of polymerization. Proanthocyanidins larger than decamers cannot be separated and they elute as an unresolved peak following decamers. Both silica column and diol-phase columns can be used for proanthocyanidin separation. A diol-phase column offers a better resolution and lower limit of detection for oligomers compared to a silica column. Fluorescent detection using excitation and emission wavelengths at 230 and 321 nm was largely specific to proanthocyanidins. However, the extraction of proanthocyanidins and purification using Sephadex LH-20 is necessary for most food samples in order to obtain good chromatographic results. The identification of proanthocyanidins can be assisted using tandem mass spectrometry according to their established fragmentation pathways. The molecular sizes of proanthocyanidins are determined by depolymerization methods using toluene-α-thiol or phloroglucinol.

Keywords: Proanthocyanidins; procyanidins; catechin; epicatechin; flavan-3-ol.

8.1   Introduction

Proanthocyanidins (PAs) are oligomeric and polymeric flavan-3-ols, better known as condensed tannins. They are ubiquitous and one of the most abundant groups of natural phenols (Porter, 1988). PAs affect the texture, color, and taste of many common foods including cereals, fruits, vegetables, and wines. PAs in foods are also of interest in nutrition and medicine because of their potent antioxidant capacities and beneficial effects on human health in reducing the risk of chronic diseases, such as cardiovascular diseases and cancers (Santos-Buelga and Scalbert, 2000; Prior and Gu, 2005).

8.2   Chemical Structures, Sources, and Levels found in Natural Sources

PAs are mixtures of oligomers and polymers composed of flavan-3-ol units linked mainly through C4→C8 bond and the less frequent C4→C6 bond. Both interflavan bonds are B-type. The flavan-3-ol units can also be doubly linked by an additional ether bond between C2→O7, which is an A-type. The three rings of flavan-3-ols are denoted as A, B, and C (Figure 8.1). They differ structurally according to the number of hydroxyl groups on both aromatic rings and the stereochemistry of the asymmetric carbons of the heterocycle. The three carbons C2, C3, C4 of the flavanol heterocycle are asymmetric and may occur in different configurations. With some rare exceptions, the configuration of C2 is R. Flavan-3-ols with a 2S configuration are distinguished by the prefix enantio (ent-). The stereochemistry of the C2–C3 linkage may be either *trans* (2R, 3S) or *cis* (2R, 3R), as in (+)-catechin and (−)-epicatechin. The interflavan bonds can be α or β. The PAs consisting exclusively of (epi)catechin are procyanidins. Procyanidin dimer has eight possible isomers. Dimer B2 and B5 are epicatechin-(4β→8)-epicatechin and epicatechin-(4β→6)-epicatechin, respectively (Figure 8.2). PAs containing (epi)afzelechin or (epi)gallocatechin as subunits are propelargonidins or prodelphinidins, respectively (Porter, 1988). Propelargonidins and prodelphinidins may also contain catechin or epicatechin as subunits.

   The sizes of the PA molecule are described by their degree of polymerization (DP). PAs with 1, 2, or 3 flavan-3-ol units have a DP
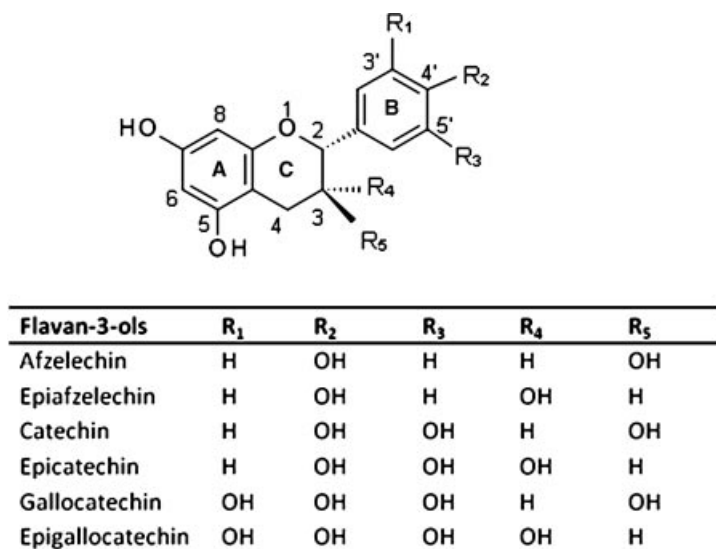
Figure 8.1 Flavan-3-ol monomeric units in proanthocyanidins.

Flavan-3-ols	R_1	R_2	R_3	R_4	R_5
Afzelechin	H	OH	H	H	OH
Epiafzelechin	H	OH	H	OH	H
Catechin	H	OH	OH	H	OH
Epicatechin	H	OH	OH	OH	H
Gallocatechin	OH	OH	OH	H	OH
Epigallocatechin	OH	OH	OH	OH	H

Procyanidin dimer B2
Epicatechin-(4β→8)-epicatechin

Procyanidin dimer A2
Epicatechin-(4β→8, 2β→O7)-epicatechin

Procyanidin dimer B5
Epicatechin-(4β→6)-epicatechin

Terminal unit

Procyanidin trimer C1
Epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin

Figure 8.2 Structures of procyanidin dimers and trimers.

value of 1, 2, or 3. They are monomer, dimer, or trimer, respectively. For procyanidin oligomers, the flavan-3-ol at the lower end is a terminal unit. All flavan-3-ols above the terminal unit are called extension units (Figure 8.2). Proanthocyanidins with a DP value over 10 were defined as polymers (Gu et al., 2002).

An HPLC-MS^n method was employed to test 102 foods and found that 43 foods contained PAs (Gu et al., 2003a; Prior and Gu, 2005). A similar study detected proanthocyanidins in 49 food items out of 99 foods of plant origin (Hellstrom et al., 2009). These foods included fruits, nuts, cereals/beans, beverages, spices, and vegetables (Gu et al., 2003a). Fruits and tree nuts are the major dietary sources of PAs. The majority of the fruits and tree nuts contained PAs, whereas most of the vegetables and roots lacked them completely (Gu et al., 2003a; Hellstrom et al., 2009).

The proanthocyanidin composition and content varied drastically in different foods. The majority of foods contained both oligomers and polymers, but a small number of foods, such as banana and blackberries, contained only oligomers. Many common foods, such as cocoa, pears, apples, peach, and blueberries contained exclusively B-type procyanidins. A few foods that contained A-type proanthocyanidins included cranberries, plums, avocados, peanuts, and cinnamon. Pinto beans, almonds, and strawberries contained primarily procyanidins, in addition to a small amount of propelargonidins (Gu et al., 2003a; Prior and Gu, 2005). The total content of proanthocyanidins varied from 0.04 mg/g in fresh kiwi to 97.8 mg/g in cocoa powder (Gu et al., 2002, 2004). Proanthocyanidin composition and content were also impacted by food processing and storage. Extrusion processing of brown sorghum or blueberry pomace decreased the total PA content, but increased the content of smaller oligomers (Gu et al., 2008; Khanal et al., 2009a). Alkalizing and baking of cocoa significantly decreased its procyanidin content (Gu et al., 2006; Stahl et al., 2009). Blueberry processing and storage caused a significant loss of total procyanidins, whereas mono- and dimers were better retained during processing than larger oligomers (Brownmiller et al., 2009).

8.3   Antioxidant and Biological Functions

Antioxidant and disease prevention capacities of proanthocyanidins from cocoa, grape seed, cranberries, and Pycnogenols™ (French pine

bark extract) have been investigated extensively. Proanthocyanidins were more effective than resveratrol and ascorbic acid in scavenging superoxide anion ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), DPPH, and hypochlorous acid (HOCl) (Maldonado et al., 2005). Grape seed proanthocyanidins directly scavenged free radicals and showed cardio-protective effects against reperfusion-induced injury in rats (Pataki, et al., 2002). Paraoxonase (PON1) is a serum esterase that prevents the oxidation of low-density lipoproteins (LDLs) and acts as a protective enzyme against atherogenesis. A supplementation of grape seed procyanidins increased paraoxonase activities in streptozotocin-induced diabetic rats (Kiyici et al., 2010). Grape seed procyanidins were also found to protect the liver from oxidative damage via the inhibition of lipid peroxidation and the restoration of antioxidant status in tissues (Dulundu et al., 2007). Feeding grape seed procyanidins to rats prevented colorectal cancers by inhibiting proliferation and inducing apoptosis (Nomoto et al., 2004; Velmurugan et al., 2010).

A supplementation of procyanidins from cocoa significantly decreased plasma or liver cholesterol and triglycerides in rats fed a high cholesterol diet. The capacity of procyanidins to inhibit intestinal absorption of cholesterol appeared to be one of the mechanisms (Osakabe and Yamagishi, 2009). Cocoa procyanidins had sustained benefits to reverse vascular dysfunction in diabetic patients (Balzer et al., 2008). Such benefits had been attributed to (−)-epicatechin, which is the constituent unit of cocoa procyanidins (Schroeter et al., 2006). Procyanidins from cacao were found to inhibit growth of human breast cancer and colonic cancer cells (Carnésecchi et al., 2002; Ramljak et al., 2005).

Cranberries have been used traditionally for the treatment and prevention of urinary tract infections. Their effectiveness was demonstrated by a randomized, double-blind placebo-controlled trial (Avorn et al., 1994). *Escherichia coli* are the principal bacterial species responsible for urinary tract infection. The consumption of cranberry juice reduced the adherence of *E. coli* to the uroepithelial bladder cells in healthy human volunteers (Di Martino et al., 2006). A-type procyanidin dimers and trimers that were isolated from cranberries were found to inhibit the adherence of uropathogenic *E. Coli*, whereas (−)-epicatechin and a B-type dimer were not effective (Foo et al., 2000).

8.4   Traditional (Nonchromatography) Analysis Methods and their Limitations

Several colorimetric methods that had been applied to estimate the total proanthocyanidin content included the Folin–Ciocalteu method, hydrochloric acid/butanol assay, vanillin assay, and 4-(dimethylamino)-cinnamaldehyde (DMAC) assay. The Folin–Ciocalteu method – a measure of total phenolic content – is not specific for PAs, and suffers from severe interferences from other phenols, ascorbic acid, ferrous ion, or cysteine (Singleton et al., 1999). In the hydrochloric acid/butanol assay, PAs are depolymerized by acid to form carbocations from their extension units, which are then immediately converted to anthocyanidins that can be measured to estimate PAs. The formation of anthocyanidins from PAs has been reported to be low; the reaction is greatly influenced by the PA structure and the presence of transition metals, and is complicated by side reactions (Porter et al., 1985). The vanillin assay is largely specific for flavanols. In the presence of mineral acid, vanillin condenses with PAs to give a chromophore ($\lambda\max = 500$ nm). The reaction can take place at C6 or C8 on the A-ring of any flavan-3-ol subunit. The absorbance values cannot be correlated either on a weight or a molar basis, and are severely influenced by coexisting anthocyanidins ($\lambda\max = 515$ nm). DMAC reagent reacts with PAs to give a chromophore with molar extinction coefficients ($\varepsilon = 16{,}000$–$19{,}000$) at $\lambda\max = 640$ nm (McMurrough and McDowell, 1978). The advantages of a DMAC assay are that it is simple, fast, and is not subject to interference from coexisting anthocyanidins. This method has been validated to measure the total proanthocyanidins in cranberries and their products (Prior et al., 2010).

Results obtained with colorimetric methods are highly empirical. Estimations of total PAs are often expressed as catechin or epicatechin equivalents, which make the data difficult to interpret and compare across different samples. Qualitative data – such as subunit structures, interflavan bond types, and proportions of oligomers with different degrees of polymerization – is not available with any of these nonchromatographic methods.

8.5   Sample Preparation for Chromatography Analysis Methods

Proanthocyanidins need to be extracted from food samples and undergo purification steps before HPLC analyses, and various extraction

solvents have been used by different authors (Hellstrom and Mattila, 2008). Aqueous acetone is more efficient in extracting proanthocyanidins than is aqueous methanol, especially the polymers. Acetic acid was often added into the extraction solvent, because proanthocyanidins are stable at pH 4–6, and acid also assists to dissociate the bound proanthocyanidins from the plant matrix (Zhu et al., 2002).

A diagram for sample extraction and purification is shown in Figure 8.3. Samples with higher fat content ($>$10%, w/w) should be extracted with hexane to remove the fat. Food samples with higher

Freeze-dried blueberries or defatted cocoa

↓

Acetone:water:acetic acid
(70:29.5:0.5, v/v/v, 10 mL)

↓

8.0 mL supernatant, evaporate acetone at 25ºC

↓

Disperse in 30% methanol

↓

Load on a Sephadex LH-20 column

↓

40 mL 30% methanol elution

↓

Elute with 70 mL of Acetone:water:acetic acid
(70:29.5:0.5, v/v/v)

↓

Evaporate solvent at 25ºC

↓

Dissolve in Acetone:water:acetic acid
(70:29.5:0.5, v/v/v) for HPLC injection

Figure 8.3 Sample extraction and purification steps for proanthocyanidin analysis.

moisture contents, such as fruits, were normally freeze-dried and size-reduced before extraction. Dry samples (1 g) were extracted with 10 mL of acetone: water:acetic acid (70:29.5: 0.5, v/v/v) in a 15-mL screw-cap glass tube at 25 °C. The tube was vortexed for 30 s to disperse the sample. The tubes were sonicated for 5 min, kept at room temperature for 20 min, and sonicated again for another 5 min. The tubes were centrifuged at 1,250 g for 10 min (Wang et al., 2010). Eight milliliters of supernatant were removed and dried in a vacuum concentrator at 25 °C (SpeedVac, Fisher Scientific). The dried extract was then dispersed in 6 mL of 30% methanol before a solid-phase extraction (SPE) step.

Solid-phase extraction used Sephadex LH-20 as an adsorbent. Dry Sephadex LH-20 was soaked in 30% methanol for at least 4 h before it was used to pack a 12-mL empty SPE tube (ID × L, 1.5 × 6 cm). The tubes were filled up to 10 mL. After loading the sample, the tube was eluted with 40 mL of 30% methanol to elute sugars and interfering compounds. This step removes about 50% of the anthocyanins and chlorogenic acid from blueberry extracts. The loss of monomers was less than 4.0%. All proanthocyanidins were recovered from the cartridge by eluting with 70 mL of a 70% aqueous acetone. Sephadex LH-20 separated proanthocyanidins from other phenolic compounds mainly by adsorption mode, instead of by gel permeation. The adsorption affinity between Sephadex LH-20 and proanthocyanidins increases with the degree of polymerization (Gu et al., 2002). The eluents were evaporated to dryness under vacuum in a SpeedVac concentrator at 25 °C. The dried extracts were dissolved in extraction solvent and transferred to a volumetric flask, and the final volume was brought up to 5 mL. Multiple samples can be handled at the same time using a 24-port Visiprep™ SEP manifold (#57250-U, Sigmal-Aldrich) and tube adapters (#57274-U, Sigma-Aldrich).

The extraction and purification methods described here were based on a published method with minor revisions. Overall recovery rates of 96.2%, 93.4%, and 85.5% were achieved for monomers, tetramers, and hexamers, respectively, in the original method (Gu et al., 2002), but minor modifications did not decrease the overall recovery rates. Recovery rates of 91.1—93.4% were also reported in cocoa samples using a similar extraction procedure (Robbins et al., 2009).

An alternative SPE method was reported to purify proanthocyanidins. In this method, proanthocyanidin extracts were loaded on

prepacked Supelco Discovery DPA–6S polyamide cartridges (1 g). Sugars and interfering phenolics were washed off the cartridges using 10 mL of 20% methanol. Proanthocyanidins were eluted with 5 mL of 85% *N,N*-dimethylformamide for HPLC analysis. The recovery rates varied from 69% for decamers to 91% for dimers (Hellstrom and Mattila, 2008). A drawback of this method is that, after SPE, the resultant extracts cannot be concentrated due to the high boiling point of *N,N*-dimethylformamide (153 °C). However, omitting an evaporation step makes this method more efficient. A particular SPE cartridge may be used to purify proanthocyanidins from specific food samples; for example, interfering compounds from processing chocolates, such as **cocoa liquor**, were selectively and consistently removed by cation exchange SPE cartridge (Robbins et al., 2009).

The resultant procyanidin extract can be filtered through polypropylene or polytetrafluoroethylene filter units (0.45 μm) before being injected for HPLC-MS (Gu et al., 2002). There is a concern that polymeric proanthocyanidins may irreversibly bond with filters. A alternative approach is to centrifuge the extract at 14,000 rpm (15,000 g) for 10 min before injecting for HPLC analysis (Gu et al., 2003b).

8.6   Chromatography Analysis Methods

8.6.1   Standards

Procyanidin monomers, a few dimers, and trimers are available from commercial sources, but standards for higher oligomers and polymers are not available. We used two compounds as standards in previous studies. One was a composite standard that was purified from cocoa using preparative HPLC, and contained procyanidin monomers to decamers (Adamson et al., 1999). The polymer standard was purified from low-bush blueberries on a Sephedex LH-20 column. Procyanidins in this standard had a degree of polymerization of 36.1 with no detectable oligomers. Procyanidins purified from cocoa consisted exclusively of epicatechin, whereas polymers from blueberries consisted mainly of epicatechin, with a small amount of catechin as subunits (Gu et al., 2002). Chromatograms for this composite oligomer standard and polymer standard are shown in Figure 8.4. In a similar study, procyanidin oligomers and polymers were isolated from
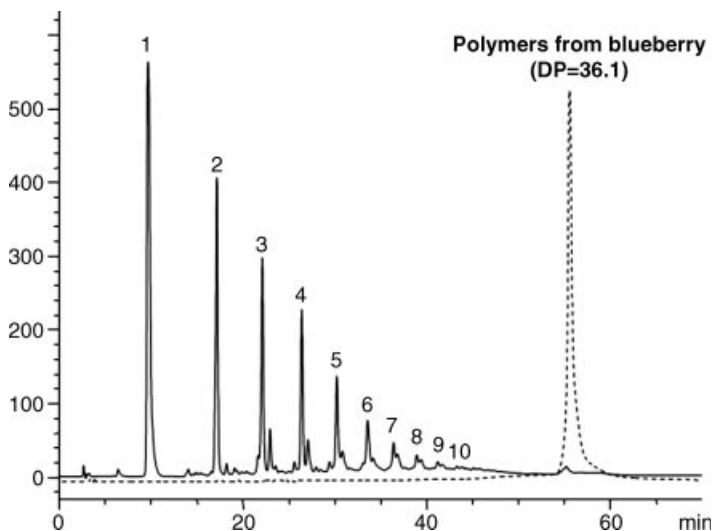
Figure 8.4 Chromatogram of a composite procyanidin standard purified from cocoa (solid line) and polymers purified from blueberries (dashed line). Gradient in Table 8.1 was used. Excitation and emission wavelengths were 276 and 316 nm for fluorescent detection. Numbers above the peak denote the DP values of procyanidins in the peaks.

Saskatoon berries and used as external standards (Hellstrom et al., 2006). Difficulties in obtaining standards remain a major hindrance for chromatographic analysis of proanthocyanidins.

8.6.2   Detection

Catechins and proanthocyanidins can be detected at 280 nm using a UV detector. However, peak intensity at this wavelength is low, and many other phenolic compounds also adsorb light at 280 nm. Fluorescent detection provides better sensitivity and specificity than UV detection. The excitation and emission spectra of procyanidin dimers are shown in Figure 8.5. Excitation at 276 nm and emission at 316 nm had been used in earlier studies; however, an examination of the fluorescent spectra indicated this was not the optimal condition. Excitation and emission wavelengths were set to 230 and 321 nm, respectively, in our most recent study, which caused a 5-fold increase in peak intensity (Robbins et al., 2009).
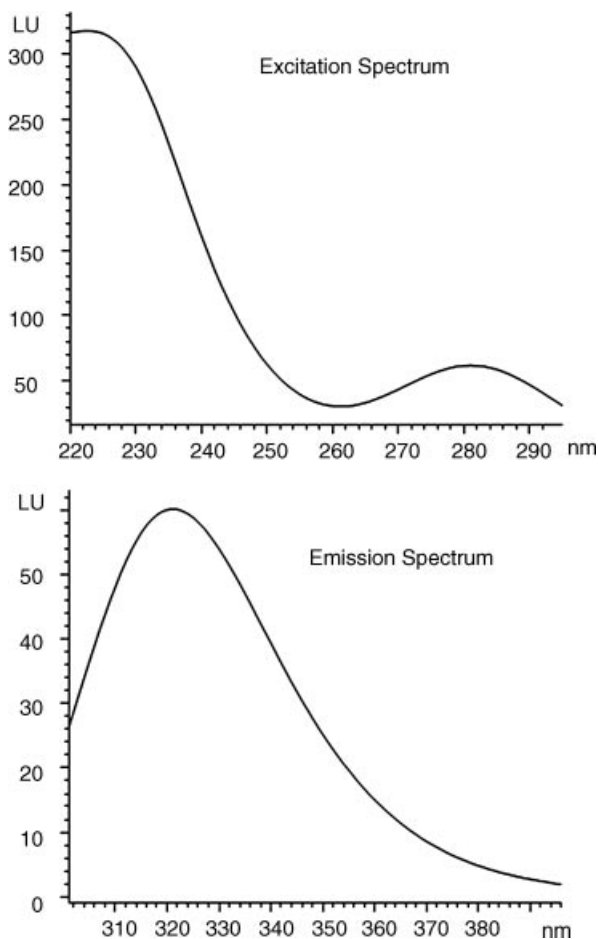
Figure 8.5 Excitation and emission spectrum of procyanidin dimers in cocoa. Scanning was done on an Agilent 1100 fluorescence detector.

8.6.3 Columns and Mobile Phase

Procyanidin monomers through decamers can be separated on normal-phase HPLC according to the degree of polymerization. Procyanidin polymers with DP > 10 elute as a single peak after decamers. No other HPLC method is known to achieve similar or better results. Two types of normal-phase columns may be chosen to separate proanthocyanidins. One option is to use unmodified silica as a stationary phase. Good separation of proanthocyanidins was achieved on a 250×4.6 mm silica column (Luna, 5 μm particle size, part number 00F–4274-E0,

Table 8.1 Ternary HPLC gradient for silica column.*

Time (min)	(A) methylene chloride (%)	(B) methanol (%)	(C) acetic acid/water (1:1, v/v) (%)
0	82.0	14.0	4.0
20	22.4	23.6	4.0
50	51.0	35.0	4.0
55	10.0	86.0	4.0
65	10.0	86.0	4.0
70	82.0	14.0	4.0

*A typical flow rate was 1 mL/min on a 250 × 4.6 mm Phenomenex Luna Silica column (5 μm particle size, part number 00F-4274-E0). Column temperature was 37 °C. Silica pre-column SecurityGuard cartridge was from Phenomenex (part number AJ0-4348).

Phenomenex, Torrance, CA) using a flow rate of 1 mL/min. A ternary mobile phase and gradient is shown in Table 8.1. Such a ternary gradient can be replaced by a binary gradient (Table 8.2) if a binary pump has to be used. These two gradients are identical and produce similar chromatograms. The second option is to use dihydroxypropyl-bonded silica as the stationary phase (diol-phase column). Good separation of proanthocyanidins was achieved on a Develosil Diol 100 Å (250 × 4.6 mm, 5-μm particle size, part number DI11546250W, Phenomenex Torrance, CA) using a flow rate of 1 mL/min. Mobile phases and gradient for the diol-phase column are shown in Table 8.3.

Table 8.2 Binary HPLC gradient for silica normal phase column.*

Time (min)	(A) Methylene/chloride/ methanol/water/acetic acid (82:14:2:2, v/v/v/v) (%)	(B) Methanol/water/acetic acid (96:2:2, v/v/v) (%)
0	100.0	0.0
20	88.3	11.7
50	73.4	25.6
55	12.2	87.8
65	12.2	87.8
70	100.0	0.0

*A typical flow rate was 1 mL/min on a 250 × 4.6 mm Phenomenex Luna Silica column (5 μm particle size, part number 00F-4274-E0). Column temperature was 37 °C. Silica pre-column SecurityGuard cartridge was from Phenomenex (part number AJ0-4348).

Table 8.3 Binary gradient for Diol-phase column.*

Time (min)	(A) Acetonitrile:acetic acid (98:2,v/v) (%)	(B) Methanol:water:acetic acid (95:3:2, v/v/v) (%)
0	93.0	7.0
3	93.0	7.0
60	62.4	37.6
63	0.0	100.0
70	0.0	100.0
76	93.0	7.0

*A typical flow rate is 1 ml/min on a Develosil Diol 100 Å (250 × 4.6 mm, 5 µm particle size, part number DI11546250W, Phenomenex Torrance, CA). Column temperature was 35 °C. Cyano (CN) pre-column SecurityGuard cartridge was from Phenomenex (part number AJ0-4305).

The performance of the silia column and diol-phase column was compared by analyzing the same procyanidin extract from grape seeds. All conditions (injection column, detection method, etc.) remained the same in the analysis except for columns. Chromatograms are shown in Figure 8.6. The chromatogram obtained on the silica column (Figure 8.6(A)) showed monomers through hexamers and a single peak of polymers at 56 min. Monomers at 9.6 min had two peaks, which were catechin and epicatechin. There were a cluster of four peaks at 16—18 min. All of these peaks gave [M–H]⁻ 577 m/z, indicating that they were isomers of procyanidin B-type dimers. Multiple isomer peaks were also observed for dimers, tetramers, and pentamers. Peak for heptamers through decamers were not observed. In the chromatogram obtained on the diol-phase column (Figure 8.6(B)), heptamers, octomers, nonamers, and decamers appeared as separate peaks. The dimer peak appeared as a single peak instead of four isomer peaks. Similar phenomena can be observed in trimer and tetramer peaks. Signal intensity for the dimer peak obtained on the diol-phase column was 8-fold of those obtained on the silica column. There were two reasons for the increases of peak intensity using the diol-phase column. First, methylene chloride is a potent fluorescent quencher. Elimination of this solvent in the diol-phase method resulted in a significant increase in signal intensity of 3- to –10-fold depending on degree of polymerization (Robbins et al., 2009). Second, the merging of the isomer peaks into a single peak increased the peak intensity. The differences between the silica column and the diol-phase column were obvious in chromatograms of blueberry procyanidins (Figure 8.7). Procyanidin trimers
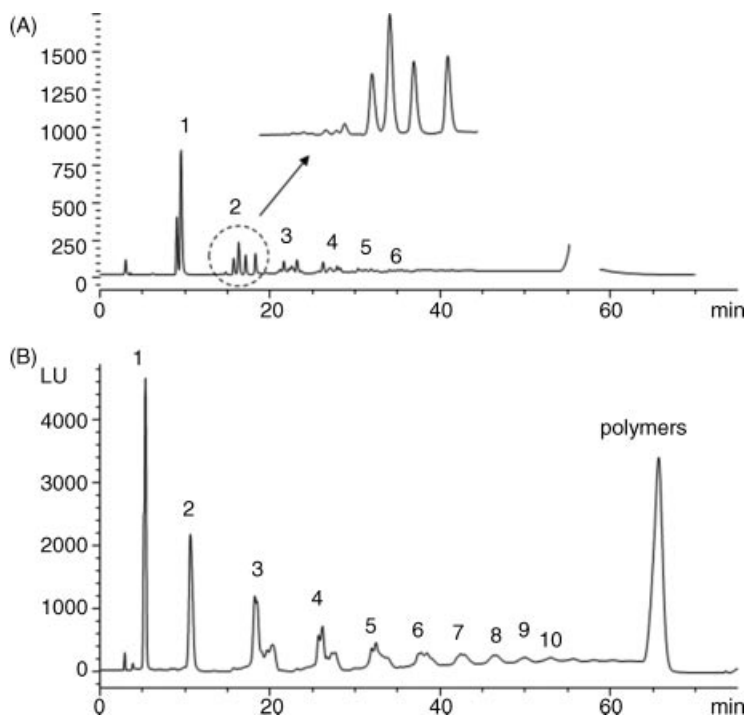
Figure 8.6 Chromatograms of grape seed procyanidins. Excitation and emission wavelength were 230 nm and 321 nm using fluorescence detector. (A) was run on a 250 × 4.6 mm phenomenex Luna silica column using gradient in Table 8.1. (B) was run on a Develosil Diol 100 Å column (250 × 4.6 mm, 5 μm particle size) using gradient in Table 8.3. Same sample and injection volume (10 μL) was used. Numbers above the peaks denote the DP values of procyanidins in the peaks.

had four isomers peaks using the silica column, whereas there was a single peak using the diol-phase column.

The chromatogram of cranberry procyanidins on the diol-phase column is shown in Figure 8.8(A). A-type oligomers elute earlier than B-type oligomers on the diol-phase column; however, separating A- and B-type isomers can no longer be achieved beyond tetramers. The excellent resolution of procyanidin oligomers on the diol-phase column is depicted in the chromatogram of sorghum procyanidins (Figure 8.8(B)).

In summary, the diol-phase column had better separation resolution for procyanidin oligomers than the silica column. Limits of quantitation in the diol-phase method were significantly lower than those in the silica
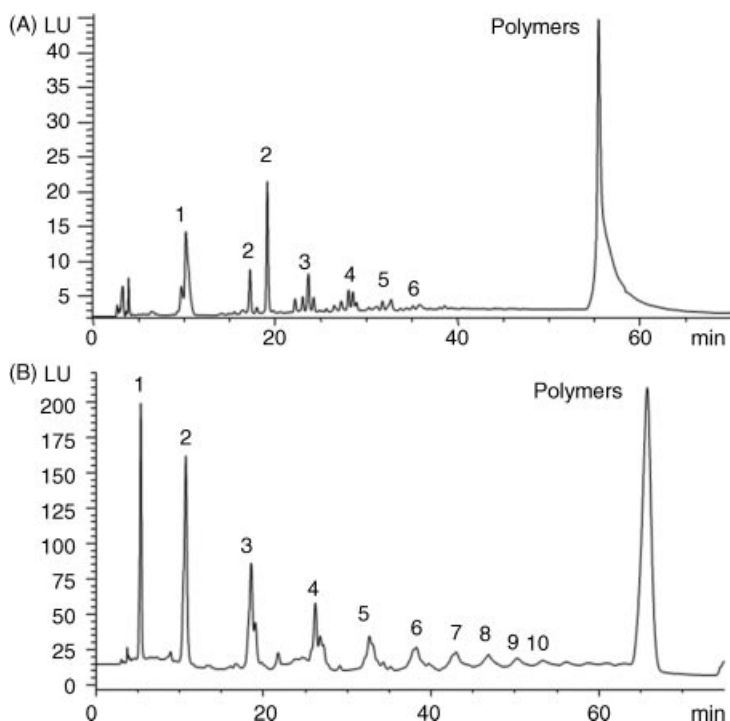
Figure 8.7 (A) Chromatograms of blueberry procyanidin extract. Excitation and emission wavelengths were 276 nm and 316 nm using a fluorescent detector. Sample was run on a 250 × 4.6 mm Phenomenex Luna Silica column using gradient in Table 8.1. (B) Chromatogram of same blueberry procyanidin extract. Excitation and emission wavelength were 210 nm and 321 nm using fluorescent detector. Sample was run on a Develosil Diol 100 Å column (250 × 4.6 mm, 5 μm particle size) using gradient in Table 8.3. Numbers above the peak denote the DP values of procyanidins in the peaks.

column method due to higher peak intensity. However, the silica column is useful for separating the isomers of dimers or trimers. Two special considerations should be noted for anyone opting to use a diol-phase column. First, a new diol-phase column has to be preconditioned before analyzing a sample. To do this, the column is eluted with a mixture of 85% A and 15% B (Table 8.3) at 0.1 mL for at least 8 h, or longer, until the retention time and peak areas are consistent. Second, a cyano phase guard column can be used to protect the diol-phase column if a material matching guard column is not available (Robbins et al., 2009).
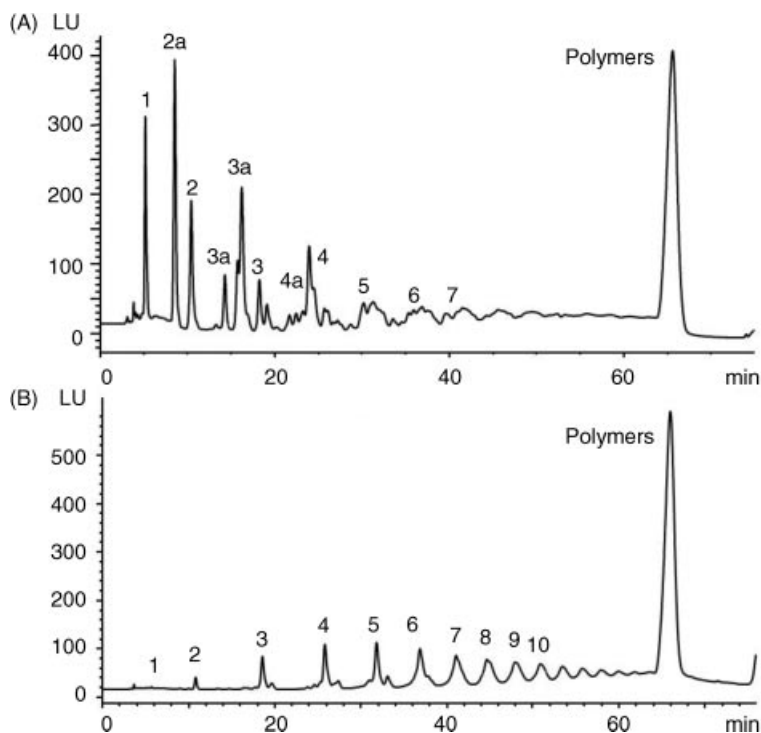
Figure 8.8 Chromatograms of procyanidins extracted from cranberries (A) and Hi-tannin sorghum (B). Excitation and emission wavelength were 210 nm and 321 nm using fluorescent detector. Sample was run on a Develosil Diol 100 Å column (250 × 4.6 mm, 5 μm particle size) using gradient in Table 8.3. Numbers above the peak denote the DP values of procyanidins in the peaks.

Peak identification and interpretation of mass spectra

Mobile phases for proanthocyanidins contain 2% (v/v) of acetic acid, and it suppresses electrospray ionization at negative mode. This can be overcome by adding ammonia acetate as an ionization enhancer. The ammonia acetate (10 mM in methanol, 0.1 mL/min flow rate) can be added into the flow via a three-way micro-splitter (#P-445, Upchurch Scientific, WA) just before the mass spectrometry. It can be delivered by a separate HPLC pump or by a syringe pump.

Procyanidin dimer, trimer, tetramer, and pentamers have molecular masses of 578, 866, 1154, and 1442, respectively. They can be easily identified using a mass spectrometer if [M–H]$^-$ m/z 577, 865, 1155, or 1441 are observed. Proanthocyanidins have three fragmentation
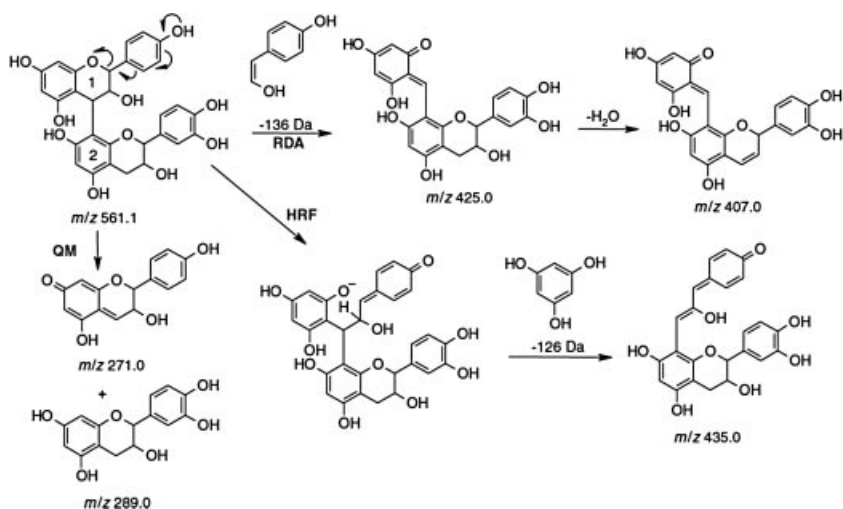
Figure 8.9 Fragmentation pathway of a proanthocyanidin dimer. The fragment mechanisms are RDA (retro-Diels–Alder), HRF (heterocyclic ring fission), and QM (quinone methide).

pathways that can be used for structural identification. The Quinone–Methide cleavage (Figure 8.9) of the interflavan bond provides information on the connection sequence of a heterogeneous proanthocyanidin (Figure 8.10). For a proanthocyanidin trimer that contains one (epi)afzelechin and two (epi)catechin units, the (epi)afzelechin can be at the top, middle, or terminal unit. Their [M–H]⁻ are the same (*m/z* 847); however, fragmentation of these isomers generate fragments with different mass-to-charge ratios (see Figure 8.10: A, B, and C). For example, observation of [M–H]⁻ *m/z* 847 and a fragment *m/z* 273 was consistent with a trimer that has an (epi)afzelechin at the terminal unit (Figure 8.10C). The connection sequence of a heterogeneous proanthocyanidin could be deduced up to pentamers using this method. The position and stereochemistry of the interflavan bond cannot be elucidated using mass spectrometry. For example, procyanidin dimers in grape seeds had at least four stereoisomers, but they gave the same [M–H]⁻ *m/z* 577 and similar product ion spectra (Figure 8.6). The second pathway was retro-Diels–Alder (RDA), which caused the loss of the B-ring of the constituent flavan-3-ols. Afzelechin, catechin, and gallocatchin have one, two, or three hydroxyl groups on the B-ring, respectively. As they will lose 136, 142, or 158 Da after RDA
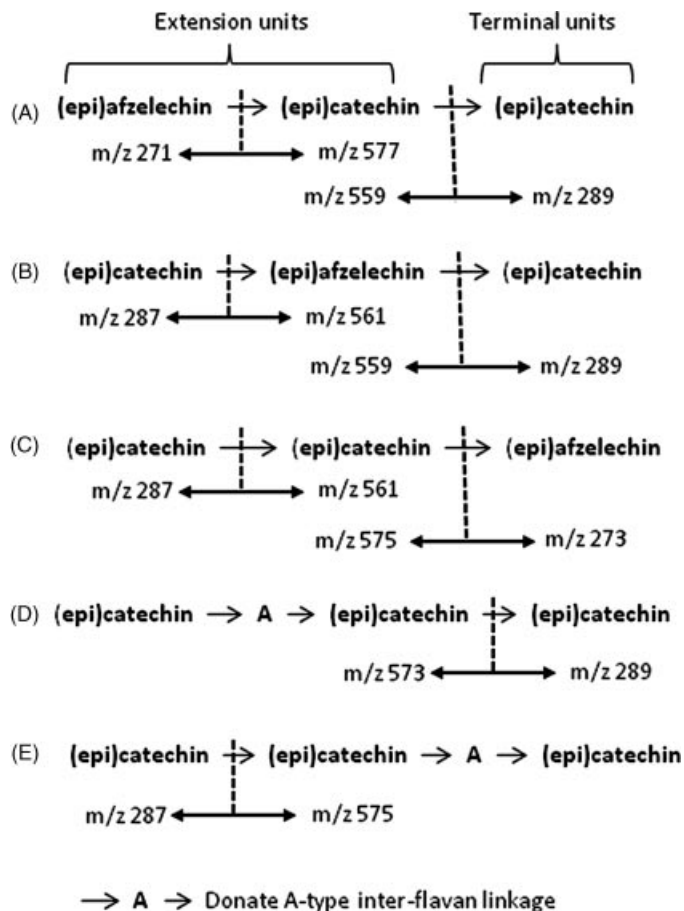
Figure 8.10 Sequencing the heterogeneous proanthocyanidin oligomers according to quinone methide fragment of inter-flavan bonds.

fragmentmentation, this helps to identify the constituent flavan-3-ols. The third typical fragmentation pathway is heterocyclic ring fission (HRF) which causes the loss of the A-ring of flavan-3-ols (Figure 8.9).

Proanthocyanidins with one A-type linkage have two less hydrogen than those of the B-type proanthocyanidins. A procyanidin trimer gave rise to [M–H]⁻ *m/z* 865, whereas a procyanidin trimer with one-type linkage yielded [M–H]⁻ *m/z* 863. A-type interflavan bond differs from B-type bound in that they do not undergo QM cleavage. Thus,

the position of an A-type linkage in a proanthocyanidin oligomer could be identified according to their product ion spectra. For example, A-type linkage in an A-type trimer could be between the top and middle flavan-3-ol units or between middle and terminal flavan-3-ol units. A-type trimers of the first scenario fragmented and gave rise to *m/z* 753 and 289 after cleavage of interflavan bond. A-type trimers of the second scenario yielded *m/z* 287 and 575 (Figure 8.10: D and E).

Peak integration, standard curves, and method performance
There were significant upward baseline shifts in the chromatograms of proanthocyanidins for many foods (Figure 8.6). The extent of these baseline shifts varied among samples. However, a blank run did not cause any significant baseline shift, and the baseline appeared virtually as a straight line. No phenols other than procyanidins produced significant fluorescence signals using the fluorescence detection method. The baseline shift was primarily caused by peak broadening and the overlapping of procyanidins. Cocoa procyanidins treated with EDTA did not show a significant change of HPLC chromatograms, suggesting that the peak broadening was not caused by metal ions (Mark A. Kelm, unpublished data). The major reason for the peak broadening of oligomers was due to the number of isomers, which increased exponentially with the degree of polymerization. On the basis of this discussion, we proposed a flat baseline integration method to replace the valley-to-valley integration method that had been used previously (Gu et al., 2002). In the valley-to-valley integration method, a straight line was drawn between the lowest points of the valley that separate adjacent proanthocyanidin peaks, and the enclosed area was integrated. In the flat baseline integration method, a flat baseline was drawn from the beginning of the run to the end. A perpendicular line was drawn from the lowest point of the valley between peaks of oligomers to the flat baseline. The area enclosed by the curve of peaks, two perpendicular lines, and the flat baseline was integrated. The areas of the oligomers with the same degree of polymerization were integrated and quantified individually. The area beyond decamers was integrated and quantified as polymers. To compare these two integration methods, the solutions of partially purified hexamers and heptamers (1 mg/mL) were spiked into a cocoa procyanidin extract and separated by normal-phase HPLC. The area recovery rates of hexamers and heptamers were calculated to be $70.3 \pm 2.8\%$ and $61.1 \pm 2.6\%$ using the flat baseline integration, in

comparison to $46.5 \pm 1.2\%$ and $31.9 \pm 1.6\%$ (mean $\pm$ SD, $n = 3$) using the valley-to-valley integration method. The content of procyanidins can be severely underestimated if the valley-to-valley integration method is applied, especially for food samples showing a significant baseline shift such as blueberries and cranberries (Gu et al., 2002).

The standard curves for monomers through decamers and polymers showed good linearity (R > 0.98) and an intercept close to zero (Gu et al., 2002; Robbins et al., 2009). The detection limit of procyanidins using a 280 nm recording was in the range of $9.0-12.0$ ng (injection on column). The detection limits using fluorescence recording were in the range of $0.023-0.064$ ng, nearly 100 times lower than that with UV detection (Gu et al., 2002; Prior and Gu, 2005). The transferability and repeatability of the diol-phase column method was assessed by monitoring data over a period of 3 months. The relative standard deviation ranged from 3.2% to 9.8% for seven different sample types, ranging approximately from 1 to 50 mg/g (Robbins et al., 2009).

The content of proanthocyanidin monomers to polymers in cocoa powder, brown sorghum bran, and a grape seed are shown in Table 8.4 (Gu et al., 2002; Khanal et al., **2009a**).

Table 8.4 Proanthocyanidin content of selected foods (mg/g).

Proantho-cyanidins	Cocoa powder*	Brown sorghum bran*	Grape seed (Merlot, dry)**
Monomers	14.2	0.3	0.59
Dimers	8.6	0.8	0.22
Trimers	8.1	1.3	0.24
Tetramers	8.9	1.5	0.24
Pentamers	8.9	1.9	0.18
Hexamers	10.0	2.3	0.13
Heptamers	6.4	1.7	0.12
Octamers	6.0	1.7	0.10
Nomamers	7.4	2.15	0.08
Decamers	3.2	1.19	0.09
Polymers	16.2	32.1	1.12
Total	97.8	47.0	3.13

*Data from (**4**).Samples were analyzed on Phenomenex Luna Silica column (5 μm particle size, part number 00F-4274-E0).

Data from (40**).Samples were analyzed on Phenomenex on a Develosil Diol 100 Å (250 × 4.6 mm, 5 μm particle size, part number DI11546250W) from Phenomenex (Torrance, CA, USA).

8.7 Determination of Molecular Size of Proanthocyanidins

Determination of molecular size and the ratio of constituent flavan-3-ols are of interest because they affect the bioactivity and physiochemical properties of proanthocyanidins. For example, procyanidin dimers and trimers were absorbed and present in blood circulation, whereas proanthocyanidins with higher molecular weight were not (Holt et al., 2002; Tsang et al., 2005; Shoji et al., 2006). The permeability of procyanidin polymers with an average polymerization degree of 6 was 10 times lower than that of (+)-catechin on monolayers of human intestinal epithelial Caco-2 cells (Deprez et al., 2001). In addition, the binding affinity between proanthocyanidins and proteins increased with the degree of polymerization and in the presence of galloyl groups (Soares et al., 2007). Two methods are often used to determine the molecular sizes of proanthocyanidins: (1) depolymerization and (2) MALDI-TOF MS.

8.7.1 Depolymerization Method

Depolymerization of proanthocyanidins can be achieved by mixing proanthocyanidins with a nucleophile in an acidic solution. The most commonly used nucleophiles are toluene-α-thiol and phloroglucinol (Gu et al., 2003a; Koerner et al., 2009). In such an assay, H^+ catalyzes the cleavage of the interflavan bonds in proanthocyanidins to form carbocations at C4. In the absence of a nucleophile, these carbocations undergo structural rearrangement to form anthocyanins. If a nucelophile is present, it reacts with carbocations to form flavan-3-ol adducts. All flavan-3-ol adducts are derived from extension units. Terminal units on proanthocyanidin are released as free flavan-3-ols. The gentle reaction conditions allow the catechin 3-*O*-gallate units in the proanthocyanidins to be preserved. A-type interflavan bonds also remain intact during depolymerization.

In a typical assay using toluene-α-thiol as a nucleophile, a proantho-cyanidin solution (50 μL, in 70% acetone) was placed in a 250-μL polypropylene microtube. The solvent was evaporated at 25 °C under vacuum. Methanol (50 μL) was added to dissolve the residue. Then 50 μL of methanol, acidified with concentrated HCl (3.3%, v/v), and 100 μL of toluene-α-thiol (5% v/v in methanol) were added into this microtube. The microtube was placed into a 1.5-mL vial and sealed with

an inert Teflon cap. The reaction was carried out at room temperature (25 °C) for 10 h. At the end of the reaction, the depolymerization mixture (10 μL) was injected for reversed phase HPLC analysis. A 250 × 4.6 mm i.d., 5 μm, Luna C18 column (Phenomenex, Torrance, CA) was used at 25 °C. The binary mobile phases consisted of: A (2% acetic acid in water, v/v) and B (methanol). The gradient started with 72% of solvent A and 28% of solvent B and ended with 37% of solvent A and 63% of solvent B after 55 min. The detection wavelength of the diode array detector was set at 280 nm (6 nm bandwidth). Typical chromatograms of different depolymerization mixtures were reported by Gu et al. (2003a).

(+)-Catechin extension units of proanthocyanidins react with toluene-α-thiol to yield 3,4-*trans*-(+)-catechin benzylthioether; (−)-epicatechin in the extension units of proanthocyanidins reacts with toluene-α-thiol to form 3,4-*trans*-(−)-epicatechin benzylthioether and 3,4-*cis*-(−)-epicatechin benzylthioether (Gu et al., 2003a). The response factors of catechin and catechin benzylthioether were the same using 280 nm detection, as well as for epicatechin and epicatechin benzylthioether. Thus, catechin and epicatechin can be used as standards to quantify their benzylthioethers. For procyanidins that consisted of catechin and epicatechin, the mean DP (degree of polymerization) can be calculated using the following equation.

$$\text{Mean DP} = \frac{\text{peak area of catechin benzylthioether} + \text{peak area of epicatechin benzylthiother}}{\text{peak area of catechin} + \text{peak area of epicatechin}} + 1$$

There are several caveats associated with this assay that may affect accuracy and precision. (+)-Catechin is the natural form in proanthocyanidins. Part of (+)-catechin epimerizes at the C2 position to form (+)-epicatechin during depolymerization. Similarly, part of (−)-epicatechin epimerizes to form (−)-catechin as an artifact. (+)-Catechin and (+)-epicatechin are an epimer pair in solution (similar to α- and β-glucose in solution), i.e. they are chiral isomers that cannot be separated on a common reversed-phase HPLC column. The degree of epimerization increases with reaction temperature and time. Depolymerization at room temperature for 10 h caused less than 10% of flavan-3-ols to undergo epimerization. Toluene-α-thiol also causes the heterocyclic ring fission of flavan-3-ols to form adducts that

are different from benzylthioether. These side reactions interfere with the determination of the DP value. Toluene-α-thiol is volatile and has an extremely strong odor which resembles that of the thiol compound added to natural gas. Any incidental leaking will almost certainly set off a security alarm designed to detect a natural gas leak. For this reason, many researchers have stopped using toluene-α-thiol and replaced it with phloroglucinol.

In a typical depolymerization assay using phloroglucinol as a nucleophile, proanthocyanidins were dissolved in 0.1 M methanolic HCl that contained 100 mg/mL of phloroglucinol and 10 mg/mL ascorbic acid (Koerner et al., 2009). This mixture was kept at 50 °C for 135 minutes. Aqueous sodium acetate (10.0 mL, 40 mM) was added to stop the reaction. The reaction mixture was centrifuged to remove particulates before the supernatant was injected for HPLC analysis (Koerner et al., 2009). (+)-Catechin and (−)-epicatechin from the extension units of proanthocyanidins react with phloroglucinol to form (+)-catechin-(4α-2)-phloroglucinol and (−)-epicatechin-(4β-2)-phloroglucinol, respectively. These adducts were quantified using purified compounds as external standards. The epimerization or heterocyclic ring fission of flavan-3-ols also occurs when phloroglucinol is used in depolymerization.

8.7.2 MALDI-TOF-MS Analysis

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique used in mass spectrometry. The ionization of biomolecules is caused by a laser beam. A matrix is used to protect the biomolecules from being degraded by the laser beam and to facilitate vaporization and ionization. MALDI is comparable to electrospray ionization in ionization softness. It produces almost exclusively singly-charged ions (Zenobi and Knochenmuss, 1998). Time-of-flight mass spectrometry (TOF-MS) separates ions of different mass-to-charge ratio according to flight time in an electric field. TOF-MS has a higher resolution and can detect ions of larger molecular weight compared with a quadrupole mass spectrometer (Guilhaus, 1995). MALDI-TOF-MS has a unique capacity to reveal the structure complexity for proanthocyanidins from trimers to dodecamers (Monagas et al., 2011). Various matrix compounds differed in their capacity to assist the ionization of proanthocyanidins (Stringano et al., 2011). In a typical

MALDI-TOF-MS assay, purified proanthocyanidins in acetone were mixed with a matrix solution (*trans*-3-indoleacrylic acid, 5 mg/100 μL in 80% aqueous acetone). The mixture (0.2 μL) was applied on a stainless steel target and dried at room temperature. Dried mixtures were subject to MALDI-TOF-MS using an N_2 laser as the ionization and reflection mode for mass separation. Proanthocyanidins trimers to nonomers were detected (Krueger et al., 2003).

8.8   Existing Problems in Current Analysis Methods

For the quantitative analyses of proanthocyanidins, a major problem with the current method is the lack of high purity standards. Pure (+)-catechin, (−)-epicatechin, B-type dimer, and B-type trimer are available commercially, but higher oligomers are not available partly because it is difficult to purify them. Purified cocoa procyanidins can be requested from Mars Inc. (Mar Botanicals, Rockville, MD). Other researchers have used standards purified from different plant sources (Gu et al., 2002; Hellstrom et al., 2009). Use of purified cocoa procyanidins as standards will provide good quantification for cocoa and chocolate samples, but they may not be ideal standards for procyanidins from other foods, due to the structural complexity of procyanidins. Procyanidins consist of (+)-catechin or (−)-epicatechin linked through C4 → C8 or C4 → C6 bonds. These interflavan bonds can be α or β depending on their stereochemistry. A-type procyanidins have an additional ether bond between C2 → O7. Some procyanidins may also contain gallate groups. Procyanidins of different structures may have different responses using fluorescent detection. Using procyanidins as standard to quantify propelargonidins or prodelphinidins will be even less accurate.

For analyzing molecular sizes of proanthocyanidins, depolymerization methods suffer from side reactions, such as epimerization and heterocyclic ring fission of flavan-3-ols. The depolymerization method estimates the average DP of proanthocyanidins in a mixture, but it is not able to determine the ratio of proanthocyanidins of different sizes. MALDI-TOF-MS is not able to analyze proanthocyanidins with a DP value higher than 12. The signal intensity of proanthocyanidins on MALDI-TOF-MS is not proportional to their amount, because proanthocyanidins of different sizes and structures differ in their

ionization efficiency. In general, proanthocyanidins with a large molecular size have a decreased ionization efficiency.

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Chapter 9

Analysis Methods of Flavanones

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Abstract

Flavonoids are a group of polyphenolic compounds present ubiquitously in plants. Flavanones are a class of flavonoids, present abundantly in *Citrus* species. In recent years, flavonoids have received greater attention by different researchers from multidisciplines such as plant biology, phytochemistry, agricultural scientists, and medical scientists. Biological activities of flavonoids specifically their role in plants as well as antioxidant activity, health-promoting properties, and modulation of plant-predator/pathogen interactions have been investigated. However, in order to investigate the biological activities and establish their physiological relevance, it is necessary to develop analytical tools to accurately determine the levels of the flavonoids in various tissues and matrices such as fruit, blood, and plasma. To facilitate such measurement, analytical tools to measure flavonoids were developed throughout the history of flavonoid research. In the present chapter, various tools such as thin-layer chromatography, high performance liquid chromatography, mass spectroscopy, and NMR used in analysis of flavonoids are discussed. In addition, the relevance and application of such tools in the analysis of flavanones, with emphasis on citrus flavanones, is discussed.

Keywords: Citrus; flavonoids; health benefits; phenolics; determination; sample preparation; identification; HPLC; LC-MS; NMR

9.1 Introduction

Flavonoids are prominent secondary metabolites present ubiquitously in plant kingdom. More than 10,000 different flavonoid structures have

been reported to date (Veitch and Grayer, 2008; Williams and Grayer, 2004). In plants, the flavonoids are accumulated in the vacuoles of flowers as colored pigments, and in leaves, stem, and root. Additionally, flavonoids are present in the epidermis of leaves and the skin offruit (Crozier et al., 2009). Flavonoids act as enzyme inhibitors and are part of the plant-defense system against UV radiation and insects. Certain flavonoids act as chelating agents of toxic metals, which are harmful to plants. Furthermore, flavonoids also play a role in photosensitization and energy transfer. Recent research also implicates flavonoids in photosynthesis, the regulation of plant hormones, morphogenesis, and sex determination.

9.2   Chemical Structures and Natural Sources of Flavonoids

Flavonoids are polyphenolic compounds comprising 15 carbons. The base structure of flavonoids comprises two aromatic rings connected by a three-carbon bridge. The C_6–C_3–C_6 base structure of flavonoids is biosynthesized bya series of condensation reactions between hydroxycinnamic acid and malonyl residues (A ring)(Figure 9.1). The hydroxycinnamic acid contributes the B ring and carbon atoms 2, 3, and 4 of the C ring, while manonyl residues produce the A ring. The cyclization of the three-carbon bridge between the A and B rings produces the third C ring.

Flavonoids are classified into several groups based on the cyclization, degree of unsaturation and oxidation of the three-carbon segment. The basic structures of some common flavonoids are shown in Figure 9.2. The major dietary flavonoids can be classified into (a) flavonols, (b) flavones, (c) flavan-3-ols, (d) anthocyanidins, (e) flavanones, and (f) isoflavones. In addition, (g) dihydroflavonols, (h) flavan-3,4-diols, (i) chalcones, (j) dihydrochalcones, and (k) aurones constitute the minor flavonoid component of diet (Crozier et al., 2009). At present, about 523 flavone, 509 flavonol, 438 flavanone, 24 anthocyanidin, and 292 chalcone aglycones have been reported (Wollenweber and Dietz, 1981; Veitch and Grayer, 2008). These basic flavonoid aglycone skeletons have numerous substituents generating a wide variety of different flavonoids. Hydroxyl groups are usually present at the 4′, 5, and 7 positions. Flavonoids may have more than one hydroxyl group attached to the basic structure. In addition to hydroxylation, methylation and glycosylation are the two
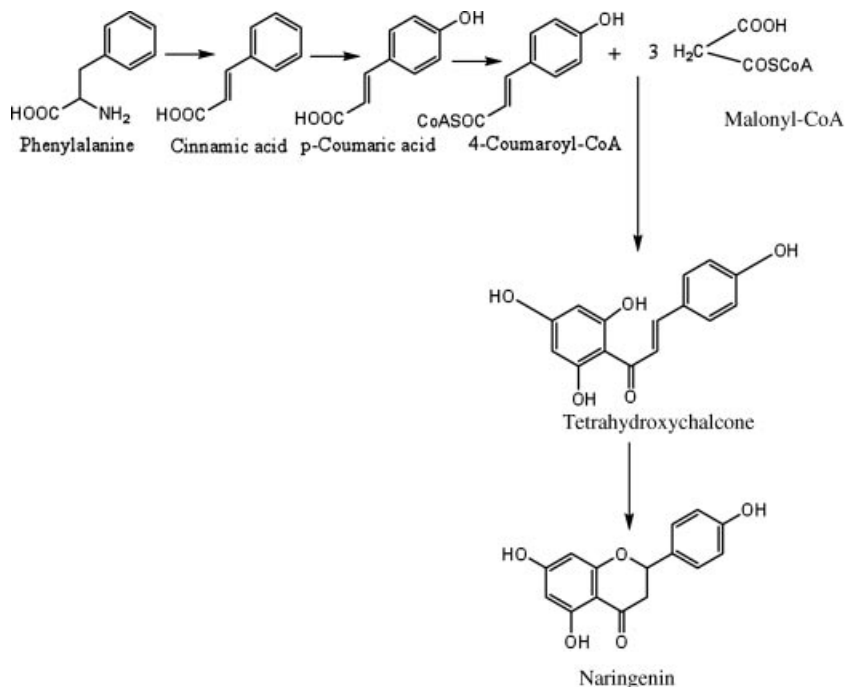
Figure 9.1 Biosynthetic pathway of flavanones. (Adopted from Winkel-Shirley, 2001a).

most common features of naturally occurring flavonoids. Furthermore, flavonoids may also contain prenyl, isoprenyl, sulfate, benzyl, methylenedioxyl, aromatic, and aliphatic acids groups (Harborne and Williams, 2000; Williams and Grayer, 2004). In nature, flavonoids occur as *O*- and *C*-glycosyl flavonoids (Veitch and Grayer, 2008). In *O*-glycosides one or more hydroxyl group of the aglycone are bound to a sugar by a glycosidic O–C bond. The glycosidic bond is an acid-labile hemiacetal bond. As glycosylation renders the flavonoid less reactive and more water-soluble, it is generally regarded to impart a protective function in plants to prevent cytoplasmic damage and to store the flavonoids safely in the cell vacuole (Winkel-Shirley, 2001a). Theoretically, any of the hydroxyl groups can be glycosylated, but certain positions are favored. For example, hydroxylation atthe C7 position in flavones, flavanones, and isoflavones, the C3 and C7 positions in flavonols and flavonol, and the C3 and C5 positions in anthocyanidins are common glycosylation sites. In contrast, 5-*O*-glycosides of flavonoids with a carbonyl group onthe C4 position are rare, possibly because interaction of the 5-hydroxyl group
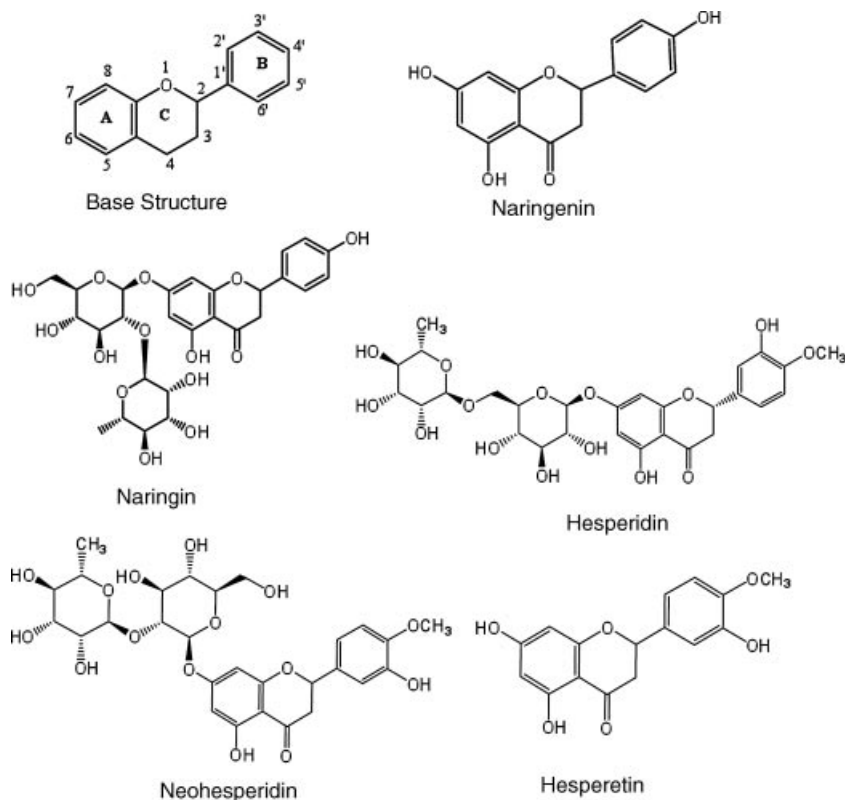
Figure 9.2 Base structure and commonly present flavanones in *Citrus* species.

with the 4-carbonyl group through hydrogen bonding interferes with glycosylation (Harborne and Williams, 2000). Glucose is the most common sugar attached to flavonoids but galactose, rhamnose, xylose, and arabinose are also found attached to the flavonoid nucleus (Di Carlo et al., 1999). In rare cases mannose, fructose, glucuronic, and galacturonic acids were found attached to the flavonoid nucleus. In addition, flavonoid disaccharides were also reported. Rutinose (rhamnosyl-($\alpha 1 \rightarrow 6$)-glucose) and neohesperidose (rhamnosyl-($\alpha 1 \rightarrow 2$)-glucose) are the most common disaccharides, while occasionally tri- and even tetrasaccharides were reported. Acylated glycosides with one or more of the sugar hydroxyls and esterified with an acid also occur in nature.

In *C*-glycosylflavonoids, an acid-resistant C–C bond, attaches the sugar moiety to the flavonoid nucleus, resulting in flavonoid *C*-glycosides. The flavonoid *C*-glycosides are further divided into

mono-*C*-glycosylflavonoids, di-*C*-glycosylflavonoids, and *C*-glycosyl-flavonoid-*O*-glycosides (Crozier et al., 2009). The *C*-glycosylflavo-noid-*O*-glycosides may be *O*-glycosylated either at the C-linked sugar or the aglycone, or both (Veitch and Grayer, 2008).The *C*-glycosylation is most commonly found at the C6 and/or C8 position of the flavonoid nucleus (Veitch and Grayer, 2008).

Flavonols are distributed throughout the plant kingdom, with the exception of algae and fungi (Crozier et al., 2009). The most common dietary flavonols–kaempferol, quercetin, isorhamnetin, and myricetin – are usually present as *O*-glycosides. The glycoside moiety is most frequently conjugated at the 3-position in the C ring, but may also occur at 5-,7-, 4′-, 3′-, and 5′-carbons. Although the aglycones show limited variability, various substitutions result in a large number of reported structures. More than 200 different glycosides are reported for kaemp-ferol alone (Veitch and Grayer, 2008; Williams and Grayer, 2004).

Flavones, such as apigenin and luteolin, lack oxygenation at C3, but have a wide range of substitutions, including hydroxylation, methyl-ation, *O*- and *C*-alkylation, and glycosylation. Most flavones occur as 7-*O*-glycosides (Crozier et al., 2009). In nature, the distribution of flavones is limited to a few plant families. A significant number of structures are reported from plant families such as Umbelliferae, Bignoniaceae, Asteraceae, Labiatae, Verbenaceae, Leguminaceae, Pinaceae, Primulaceae, Rosaceae, Rutaceae, and Salicaceae (Harborne and Williams, 1972; Wollenweber and Dietz, 1981). Citrus species contain polymethoxylated flavones, such as tangeretin and nobiletin.

Flavan-3-ols are nonplanar compounds due to their saturated C3 carbon, and they represent the most complex subclass of flavonoids. The structures vary from simple monomers such as (+)-catechin and (−)-epicatechin to complex structures including oligomeric and poly-meric proanthocyanidins. The monomers can be hydroxylated to form gallocatechins and also undergo esterification with gallic acid to form complex structures. The flavan-3-ols have two chiral centers at C2 and C3, thus producing four isomers for each level of B-ring hydroxylation. Of the four isomers, (+)-catechin and (−)-epicatechin are widely distributed while (−)-catechin and (+)-epicatechin are relatively rare (Porter, 1993). Further, oligomeric and polymeric proanthocyanidins demonstrate chirality at the C4 positionof each additional flavan-3-ol unit. These differences in chirality have a significant effect on the 3-D structure of the proanthocyanidins and, consequently, profoundly affect

binding to the target site. However, altered chirality does not affect the redox properties. Type B proanthocyanindins are formed by the oxidative coupling of (+)-catechin and (−)-epicatechin, at the C4 position of the heterocycle and the C6 or C8 positions of the adjacent unit to create oligomers or polymers. Type A proanthocyanidins have an additional ether bond between C2 and C7. Proanthocyanindins can occur as polymers of up to 50 units. Procyanidins are proanthocyanidins consisting exclusively of (epi)catechin units, and are the most abundant proanthocyanidins in plants. The propelargonidins and prodelphinidins occur less frequently in nature and contain (−)-epiafzelechin and (+)-afzelechin or (epi) gallocatechin. The accumulation of proanthocyanidins and degree of polymer extension varies with species and tissue (Marles et al., 2003).

Naringin, a prominent flavanone in grapefruit, was discovered by DeVry in 1857, from the flowers of grapefruit trees in Java (Poore, 1934). The flavanones are nonplanar due to an asymmetric carbon at C2. In the majority of cases, optically active flavanones have a (2S)-configuration and the C ring is attached to the B ring in an α-configuration (Crozier et al., 2009; Harborne and Williams, 1998). Citrus species contain especially high concentrations of flavanones. The most common flavanone glycosides are hesperidin (hesperetin-7-*O*-rutinoside) and narirutin (naringenin-7-*O*-rutinoside) (Crozier et al., 2009). Flavanone rutinosides are tasteless, whereas neohesperidoses such as neohesperedin (hesperetin-7-*O*-neohesperidoside) are intensely bitter. In addition to common *O*-substitution (OH and Me), *C*-methylation, glycosides, and isoprenylated flavanones, rare 5-methoxy-6,7,8-trihydroxy substitution of the flavanone orebiusin from *Isodonorebius* (Huang et al., 1996) and 2′,3′-dihydroxylated (in the Bring) from *Iristenuifolia* were reported (Kojima et al., 1997). Another rarity observed in the flavanone group is a 2-hydroxy group, and two such structures were reported from *Friesodielsiaenghiana* (Fleischer et al., 1997). Furthermore, laxiflavin, with a rare β-hydroxyethyl substituent at C6 was identified in *Derris laxiflora* (Kim et al., 1995).

9.3   Antioxidant and Biological Functions

In plants, flavonoids are suggested to be involved in various processes including plant–pathogen interactions, pollination, light screening, seed

development, allelopathy, signal transduction, fertility, and sexual reproduction (Brown et al., 2001; Winkel-Shirley, 2001a, 2001b); (Schijlen et al., 2007). The presence of flavonoids in the epidermal cell layers of leaves and tissues in plants coupled with the ultra-violet light absorbing capacity was considered asevidence for the UV-protection role of flavonoids (Winkel-Shirley, 2002). In particular, levels of anthocyanins and flavones were reported to increase in response to high visible light levels (Beggs et al., 1987). In addition, UV-radiation was reported to induce flavonoids and psoralens (Beggs et al., 1985). This hypothesis received further support from studies on Arabidopsis, demonstrating that mutation in chalcone synthase resulted in UV-hypersensitive phenotypes (Li et al., 1993). However, further research demonstrated that UV light induces the synthesis of flavonols such as quercetin and kaempferol, but not the flavones (Ryan et al., 2002). Flavonols are extensively hydroxylated in the B ring, and a high degree of hydroxylation was suggested to decrease UV-B absorption (Lavola et al., 1997). Altogether, the evidence pointed to a conclusion thatthe UV-absorption capacity of the flavonoids was not the primary mode of protection. In contrast, a high degree of hydroxylation correlates well with higher antioxidant activity (Rice-Evans et al., 1996).

Despite the strong evidence ofthe antioxidant properties of flavonoids, demonstrated under *in vitro* conditions, the putative in-planta function remains unclear (Hernández et al., 2009). Experimental data suggests that flavonols and flavan-3-ols, may act as antioxidants in plants, but the chemical diversity and complex biochemistry has hindered efforts to establish an unequivocal correlation between the spatiotemporal localization of flavonoids and oxidative stress reactions (Hernández et al., 2009).

Another line of emerging evidence now links the flavonoids with the control of the polar transport of auxin, a growth regulator (Jacobs and Rubery, 1988; Murphy et al., 2000; Brown et al., 2001). It is suggested that auxin acts in a stress response by controlling thestomatal opening (Dietrich et al., 2001) and by allocating resources under poor growth conditions (Palme and Gälweiler, 1999). Flavonoids do not share a structural similarity with auxin but resemble the synthetic auxin transport inhibitor naphthylphthalamic acid (NPA), which binds to a protein associated with the auxin efflux carrier (Jacobs and Rubery, 1988; Murphy et al., 2000). Although there is some evidence supporting this

hypothesis, more experimental evidence is needed to firmly establish the role of flavonoids in auxin transport regulation.

The health-benefiting properties of flavonoids have been known since the early 20th century. More recently, the health beneficial properties of flavonoids were investigated extensively. Flavonoids demonstrate preventive properties against cancer (Le Marchand, 2002), dementia (Commenges et al., 2000), atherosclerosis (Aviram and Fuhrman, 2002), and coronary heart disease (Hertog et al., 1995; Hertog et al., 1993). The interaction of flavonoids with various enzymatic systems, such as theinhibition of cyclooxygenase and lipoxygenase enzymes (Kim et al., 1998), was reported to decrease platelet activation and aggregation. These properties contribute toward protection against cardiovascular diseases and anti-inflammatory activity. In addition, many other biological activities are attributed to flavonoids, e.g. antiviral, antimicrobial (Jayaprakasha et al., 2000; Negi and Jayaprakasha, 2001), antihepatotoxic, antiosteoporotic, antiallergic, antispasmodic, and antiulcer activities. Several reviews concerning their mechanisms of action and their potential therapeutic uses have been published recently (Nijveldt et al., 2001; Cushnie and Lamb, 2005; Spencer et al., 2008; Crozier et al., 2009; Kay, 2010; Stoner et al., 2010). Furthermore, several different enzymes involved in intracellular signaling can be affected by flavonoids. Of particular interest is the modulation of protein kinase C, which is directly linked to thehost immune system (Middleton Jr and Kandaswami, 1992). Recently, naringenin was demonstrated to interfere with the bacterial cell–cell communication systems in *Escherichia coli* O157:H7 and *Vibrio harveyi* (Vikram et al., 2010). In addition, naringenin was reported to repress type-3 secretion system and flagella, thereby reducing the virulence of *Salmonella typhimurium* (Vikram et al., 2011).

The majority of biological activities of flavonoids have been ascribed toantioxidant properties, either because of their reducing capacities or through their postulated effect on intracellular redox status (Williams et al., 2004; Jayaprakasha et al., 2008a, 2008b). Flavonoids were postulated to demonstrate antioxidant activity through at least four basic mechanisms including (a) scavenging of free radicals, (2) quenching of singlet oxygen, (3) transition metals chelation, and (4) enzyme inhibitory activity (Cos et al., 2000). Owing to their lower redox potential, flavonoids are able to reduce highly oxidizing free radicals (Pietta, 2000). In the process, the flavonoids form more stable flavonoid

radicals and prevent lipid peroxidation (Cos et al., 2001). Lipid peroxidation is one of the most important actions of free radicals, leading to cellular membrane damage, and ultimately cell death. Flavonoids were also reported to scavenge nitric oxide (Vanacker et al., 1995), which form speroxynitrite by reacting with superoxide free radicals. In addition, flavonoids were reported to inhibit xanthine oxidase (Cos et al., 1998), an important biological source of superoxide radicals. Superoxide radicals can react with hydrogen peroxide, and produce-highly toxic hydroxyl radicals in the Fenton reaction. This reaction is catalyzed by iron and can be inhibited by quercetin, which has iron-chelating effects (Ferrali et al., 1997). The antioxidant activity of flavonoids is determined by several factors such as the presence of a catechol group on the B ring (Rice-Evans et al., 1996; Bors and Michel, 1999; Bors et al., 2001), a 2,3 unsaturation in conjugation with a 4-oxo- function in the C ring, and the presence of functional groups capable of binding transition metals such as iron and copper (Rice-Evans et al., 1996). However, it is also important to note that some of the flavonoids, such as quercetin and myricetin, were found to exert a pro-oxidant effect (Cao et al., 1997; Rietjens et al., 2002).

Despite the large number of *invitro* studies on the antioxidant activity of flavonoids, the emerging evidence does not support the classical hydrogen-donating antioxidant activity as the sole explanation for the observed cellular effects. This paradigm shift is based on a number of observations. First, the flavonoids are extensively metabolized in the human gut, resulting in a significant alteration in redox potential. Secondly, the actual concentrations reached by flavonoids in most animal and human tissues following dietary ingestion are frequently insufficient to exhibit any physiological relevance (Galleano et al., 2010).

Absorption and metabolism coupled with excretion are important determinants inthe establishment of health benefits of dietary factors including flavonoids. However, the absorption kinetics and metabolism of dietary flavonoids have not been studied intensively. In addition, the accumulated data issometimes contradictory, leading to a situation-where the prediction and extrapolation of *invitro* and animal studies becomes difficult. Whether flavonoid glycosides or only aglycones (Hollman and Katan, 1997; Walle, 2004) are able to pass the gut wall continues to be a subject of debate. The first step in flavonoid metabolism in the human gut seems to be deglycosylation, which is consistent with the strong β-glycosidase activity within the epithelial cells of the

small intestine (Day et al., 2001; Day and Williamson, 2001; Németh et al., 2003). After deglycosylation, flavonoids are extensively metabolized in the gut. For example, hydroxyl groups may be conjugated with glucuronic acid, a sulfate, or a methyl group (Hollman and Arts, 2000). In addition, colon microflora extensively metabolizes the flavonoids. Owing to the different composition of the intestinal microflora, the extent of flavonoid metabolism varies, resulting in large inter-individual variation (Scalbert and Williamson, 2000). Furthermore, the intake of flavonoids varies greatly between countries (Hollman and Arts, 2000; Nijveldt et al., 2001), depending on the habitual diet. The prominent dietary sources of flavonoids are fruits and beverages such as citrus, onion, red wine, tea, coffee, beer, herbs, and vegetables (Justesen et al., 1997; Scalbert and Williamson, 2000). The flavonoids have been subject to ever-increasing attention due to the irubiquitous presence in the human diet and the health-beneficial properties.

The emerging evidence from the past two decades suggests the potential role of flavonoids in the prevention of coronary heart disease. Hertog et al. (1993, 1995) demonstrated that flavonoid intake was inversely related to the incidence of coronary heart disease. Another cohort study by Knekt et al. (1996) supported the conclusion that flavonoids may have a preventive role against coronary heart disease.

9.4   Traditional Analytical Methods and their Disadvantages

9.4.1   *Spectrophotometric Determination of Flavonoids*

The estimation of flavonoids by measuring the UV absorbance is one of the most common and convenient methods. The spectra of a number of flavonoids was reported by Mabry et al. (1970). The availability and measuring capabilities of flavonoids' UV spectra greatly facilitated their identification in complex mixtures. The spectrophotometric determination of flavonoids facilitated the quantitative and qualitative analysis of samples. In general, UV absorption can be used to determine the concentration of flavonoids in samples, e.g. citrus juices.

Colorimetric methods
Several methods for the colorimetric determination of flavonoids, especially flavanones, were developed during the first half of the 20th

century. A well-known colorimetric method developed by Folin and Ciocalteu (1927) is still very widely used. The method was originally developed for the determination of the amino acids tyrosine and tryptophane, and later adapted for the analysis of flavonoids. Davis et al. (1947) developed a method for measuring flavanones in citrus juices. The method was based on the formation of a yellow color by flavanones in the presence of alkaline diethylene glycol. Another, fast and reliable method was based on the reaction of flavanones with ferric chloride to obtain a quantifiable vinaceous red color (Rygg and Harvey, 1938). A boric acid method developed by Wilson (1939) demonstrated a specificity towards flavones, while the reaction of flavanones yielded no color. Thus the method was useful in the indirect determination of flavanones from flavones. In contrast, Horowitz (1957) observed that only flavanones produced a red to violet color when reacted with sodium borohydride, and subsequently acidified with hydrochloric acid. Thus the method clearly identified flavanones from chalcones, flavones, flavonols, isoflavones, and aurones. In addition to the above-mentioned methods, several other methods were developed based on the colored reaction products of flavanones/flavonoids. Frequently, these methods were used to determine the presence of a specific class of flavonoids. However, the identification of complex mixtures was difficult using these colorimetric methods. In addition, the limit of detection was also high –in most cases up to mg levels.

Filter paper chromatography
The use of flavonoids in the treatment of radiation injury and frostbite during the 1940s–1950s stimulated interest in the identification of new flavonoids and their analytical methods of separation. During the first half of the 20th century, classical chromatographic techniques were developed and employed for the separation of flavonoids from plant extracts. A paper chromatographic method, first reported in 1949, determined the R_f values of 11 flavonoids (Wender and Gage, 1949). The authors used a modified version of a chromatography apparatus reported by Winsten (1948). The apparatus consisted of discarded gasoline pump cylinders, which were closed at each end by a glass plate and troughs made of Pyrex evaporating dishes. Using this crude apparatus the authors measured the mobility and separation of different flavonoids in various solvent systems. The flavonoids were separated on Whatman No. 1 filter paper (47×57 cm) and the development of the

chromatogram took nearly 8 to 22 h (Winsten, 1948). The various flavonoids were identified by UV light.

In addition to UV light, several qualitative tests were also developed around the same time. These qualitative tests were helpful in the tentative identification of flavonoids. Some of the reagents for the colorimetric determination of flavonoids were sodium carbonate, ammonium hydroxide, alcoholic ferric chloride, alcoholic potassium hydroxide, alcoholic aluminum chloride, normal lead acetate, basic lead acetate, ammoniacal silver nitrate, antimony penta-chloride in carbon tetrachloride, zirconium chloride, magnesium acetate, stannous chloride, sodium carbonate, Benedict's reagent, boric acid–citric acid in acetone, and several others (Bate-Smith and Westall, 1950; Casteel and Wender, 1953; Troyer, 1955; Pridham, 1959; Jayaprakasha et al., 2000; Belajová and Suhaja, 2004). The combination of colorimetric assays with chromatographic separation became a very practical (Kivits et al., 1997) and widely adapted tool for flavonoid analysis (Girennavar et al., 2008a, 2008b, 2008c).

Although paper chromatographic separation was faster than the earlier column separation methods, it had several limitations such as a variation inintraday R_f values (Bate-Smith and Westall, 1950). The variation in R_f values of solutes was dependent on the temperature and composition of mobile phases. In addition, some of the low-boiling solvents such as ether, hexane, and dichloromethane tend to evaporate over long period of analysis time. This problem was also more acute in ternary solvent systems containing alcohol, acids, and bases, where it was speculated that the slow formation of esters changes the composition of the solvent system. Furthermore, the complex solvent mixtures were reported to be more temperature sensitive (Bate-Smith and Westall, 1950). In addition, equilibration of the chromatographic chamber with a vapor phase was an important factor, and often caused problems due tothe differential evaporation rates of thevarious solvents present in the system. The vapor tends to escape in the environment; therefore, proper sealing of the chromatographic chamber was another important criterion for reliable and reproducible separation.

Despite several shortcomings, paper chromatography was clearly advantageous to qualitative analytical methods. One of the major advantages was its application in the quantitative analysis of flavonoids. This point is manifested by several reports using paper chromatography

techniques to identify and quantify flavonoids (Bate-Smith and Westall, 1950; Gage et al., 1951; Casteel and Wender, 1953; Troyer, 1955; Pridham, 1959). An important feature of these studies was the combination of chromatographic technique with colorimetric or UV detection of flavonoids. The combined technique provided better resolution for the identification of flavonoids.

Thin-layer chromatography
Historically, the advent of paper chromatography revolutionized the analysis of natural products. The relative mobility data or relative factor (R_f) are available for a large variety of compounds.However, the development of thin-layer chromatography (TLC) supplanted the use of paper chromatography. TLC overcame many of the limitations related to paper chromatography. The major strengths of TLC are its ease and speed of analysis, coupled withitslow operating cost and high sensitivity (Robards and Antolovich, 1997).

The selection of a suitable stationary phase and solvent depends on the nature of the target flavonoids. Polyamide or microcrystalline cellulose layers can be used for theseparation of hydrophilic flavonoids (Robards and Antolovich, 1997). Flavonoids, in particular flavonoid glycosides, can be resolved rapidly on cellulose TLC. On the other hand, silica gel can be used for the separation of less hydrophilic flavonoids.

A good approximation of flavonoid structure can be obtained by the relative mobility, appearance of spots under UV light and use of various reagents (Rowland et al., 1985). Solvent systems such as 15% acetic acid (acetic acid/water, 15:85) and TBA (t-BuOH/acetic acid/water, 3:1:1) can be routinely used for the preliminary analyses of flavonoids (Rowland et al., 1985). Typically, plastic-backed sheets are cut to a suitable size, e.g. 5 cm wide × 7 cm high, the samples are applied as small 1–2 mm spots, allowed to dry, and the sheets are developed in a glass tank (a slide-staining jar is useful for small sheets). The relative mobilities in TBA and 15% acetic acid should give a rough guide to identify the nature of the flavonoid. The chromatographic sheets are dried and viewed under UV light at 366 nm. The spots were identified after spraying with NA reagent (1% solution of diphenylboric acid–ethanolamine complex in methanol), dried, and viewed under UV light. Most flavonoids show color, but most significantly 3′,4′-dihydroxy-flavones or flavonols are orange and the 4′-hydroxy equivalents are yellow-green.

Two-dimensional TLC

Most of the natural products are complex and identification of solutes using one-dimensional chromatography is challenging since the R_f values of the solutes may be similar in a one-solvent system in many cases. Such ambiguities can be resolved by developing the same plate in another solvent system, resulting in the enhanced separation and identification of the solutes. For instance, a two-dimensional chromatogram can be prepared by applying a sample spot in one corner of the TLC plate and developing with TBA in one dimension, while the plate may be developed in the second dimension with 15% acetic acid. Generally, most flavonoids may be separated on two-dimensional TLC and their spot characteristics can be noted. Silica TLC is also a useful screening system for flavonoids. Ethyl acetate/formic acid/acetic acid/ H_2O (100:11:11:27) gives a good range of mobilities for flavone and flavonol glycosides (Rowland et al., 1985). The obtained R_f values generally follow the series diglycosides<monoglycosides<aglycones. Most TLC spots can be visualized with the NA reagent. Anthocyanin-sare best analyzed by TLC using an acidic solvent system. In the majority of TLC and paper chromatographic separations, a small amount of acid or base will be used along with the mobile phase to suppress the dissociation of compounds being analyzed (Jayaprakasha et al., 1998). A useful general eluting solvent for cellulose TLC of these compounds is concentrated HCL/formic acid/H_2O (30.8:7.7:61.5) (Andersen and Francis, 1985). The intensely colored spots (violet for the trihydroxylated Bring, red for dihydroxy, and orange for mono-) do not require spraying, and generally the R_f increases as the degree of glycosylation increases. For citrus flavanones both polyamide and silica-gel-based TLC were prevalent (Schmidtlein and Herrmann, 1976). In the past two decades, precoated silica gel plates were extensively used in the analysis of flavanones from various species, with separation achieved using a number of solvent systems.

Gas chromatography

Gas chromatography was employed in the analysis of flavonoids as early as 1962 (Narasimhachari and Rudloff, 1962). However, the major limitation of GC in flavonoid analysis is the need for derivatization. After the introduction of HPLC, the GC analysis of flavonoids became less common. Typically in GC, the flavonoids are hydrolyzed and

derivatized using trimethylsilyl ether and injected into a nonpolar column (de Rijke et al., 2006).

Capillary electroseparation

In recent years capillary electro separations were found to be useful for the analysis of a variety of small molecules. Several variants of the basic technique– such as capillary zone electrophoresis, micellar electrokinetic chromatography, and capillary gel electrophoresis – are available. However, only a handful of reports are available on flavanone analysis (Cancalon and Bryan, 1993; Desiderio et al., 2005). Typically the separations were performed on silica fused capillaries of 70–75 μm internal diameter packed with RP_{18} or uncoated silica (Cancalon and Bryan, 1993; Desiderio et al., 2005). One of the major strengths of the techniques is the separation of multiclasses of compounds, ranging from amino acids to carotenoids to flavonoids (Cancalon and Bryan, 1993).

9.5 Modern Methods for the Analysis of Flavonones

9.5.1 Sample Preparation

Solvent extraction, solid-phase extraction, and simple filtration have been used as sample preparative techniques for flavonoid analysis (Bronner and Beeher, 1995; Abad-Garcia et al., 2007; Chebrolu et al., 2011). A list of selected sample preparation methods from citrus tissues/juices is presented in Table 9.1. Greiner and Wallrauch (1984) and Ooghe et al. (1994) reported the use of dimethylformamide for the extraction of flavonoids. Later, Schnull (1990) and Wade et al. (1992) suggested the use of water as a solvent at different temperatures for the extraction of compounds from juices. Ultra centifugation and filtration is a common and widely used method because of its simplicity. However, it has the major disadvantage of inefficient extraction of the various flavonoids from citrus fruits and juices. Furthermore, it was recommended against using smaller volumes (<2 ml) for filtration in combination with the type of nylon membrane (pore size 0.45–1.2 μm) (Widmer and Martin, 1992). In smaller volumes, the possibility of adsorptive losses of flavonoids increases, depending upon the type of filter membrane (Widmer and Martin, 1992). The use of PTFE membrane filters was demonstrated to have an advantage over a nylon

Table 9.1 Selected extraction methods for flavanones from citrus species.

Sample matrix	Flavonoids	Extraction conditions	Procedure	Reference
Orange, grapefruit juice	Hesperidin, naringin, naringenin	Vortex mixing, solvent extraction, centrifugation, filtration	1 g sample was extracted with 1 mL × 3 methanol and centrifuged at 25000 × g. Supernatant was filtered.	Bronner and Beeher, 1995
Orange, grapefruit juices	Eriocitrin, narirutin, hesperidin, neoeriocitrin, naringin, neohesperidin	Solvent extraction, heating and centrifugation	5 mL juice was diluted with 10 mL DMF and 10 mL of 0.5 ammonium oxalate solution, heated for 10 min at 90 °C, adjusted to 50 mL and centrifuged at 2500 × g for 10 min	Mouly et al., 1993
Valencia orange, navel orange, grapefruit, mandarin, lemon, lime, tangelo and pummelo	Narirutin, eriocitrin, naringin, hesperidin, neohesperidin, neoponcirin	Ultracentrifugation, heating, pH modulation, solvent extraction,	Method 1: ultracentrifugation. Method 2: heating of juice samples to 55 or 100 °C. Method 3: solvent extraction (Mouly et al., 1993). Method 4: extraction with methanol	Robards et al., 1997
Orange, grapefruit, apple	Phenolics, organic acids, coumarins, hesperidin, naringin, narirutin	Centrifugation and filtration	Samples were diluted to 11.8 brix, centrifuged and filtered through 0.22 µm	Gamache et al., 1993

membrane as the use of a PTFE membrane filter was reported to reduce losses by adsorption in the case of citrus juice samples (Careri et al., 2000). In conjunction with the low solubility of certain flavonoids such as hesperidin, there may be significant error in profiling citrus juices and other matrices. Another major factor affecting the sample preparation is the choice of solvents for the selective extraction of flavonoids for downstream analysis. In a comparative study by Robards et al. (1997), no significant advantage was observed between methanol and dimethylformamide for flavonoid extraction. Even though the extraction of hesperidin was enhanced by demethylforma-mide, no significant increase was noted for other flavonoids. In fact, heat the juice to 100 °C prior to ultracentrifugation and subsequent filtration yielded a similar efficiency as dimethylformamide and methanol ex-tractions. Furthermore, extraction by centrifugation and filtration over the pH range 3.8 to 7 did not demonstrate significant variation in extractability, except for narirutin-4′-glucoside. Altogether, the findings suggest that a careful selection of conditions will yield an efficient extraction of flavonoids. An unusual extraction procedure that was successfully applied for flavanones extraction from citrus juices involves the use of 0.05 M ammonium oxalate in conjunction with dimethylformamide followed by filtration (Mouly et al., 1993; Mouly et al., 1994).

Solid-phase extraction (SPE) is a convenient method for sample clean-up and subsequent analysis. During SPE, target analytes as well as structurally similar compounds are adsorbed onto a solid stationary phase. The solid phase is then washed with a suitable solvent, in order to remove interfering compounds and reduce the complexity of the matrix. Finally the bound target analyte is eluted with the solvent of choice. In this fashion, SPE is a great tool in analyzing compounds in highly complex matrices such as citrus juices. SPE stationary phases are now available in various forms, such as C-8, C-18, silica and diol, among others. In addition, molecularly imprinted polymers are also reported for use in SPE (Weiss et al., 2001).

SPE can be used for both online and offline separation of target analyte in the analysis procedure. However, in citrus juices, especially orange and grapefruit juices, the application of SPE is under debate. The primary reason against its use is that SPE may be ineffective in capturing the flavonoids located in suspended juice-solids, which may represent a large fraction of the total flavonoids (Bronner and

Beeher, 1995). It was further suggested that solvent extraction is a better approach in such complex matrices over SPE and simple filtration.

9.5.2 Chromatographic Methods

HPLC has become the method of choice for flavonoid analysis from complex plants as well as other matrices. Several investigators reported the use of HPLC for flavanone analysis, especially for citrus juices, since the early 1980s. A list of selected HPLC analytical methods used for flavanone analysis in citrus species is presented in Table 9.2. A survey of reported methods suggests that reversed-phase C-18 stainless steel columns were the most preferred for flavanone analysis, especially for citrus species. Both isocratic and gradient solvent systems are reported with equal propensity in the literature, indicating sufficient resolution power of either method. Various researchers have reported the use of different mobile phases; however, a closer look suggests that most of the mobile phases are variants of a common theme. One of the mobile phases is based on a high salt concentration solvent, usually prepared with monobasic sodium phosphate with pH and often adjusted with phosphoric acid. This solvent is then combined usually with acetonitrile or methanol (Gamache et al., 1993; Nogata et al., 1994; Robards et al., 1997; Careri et al., 2000; Belajová and Suhaj, 2004). A second combination of solvents include an aqueous solution of weak organic acid, such as acetic acid or formic acid, usually combined with a medium to high polarity organic solvent such as acetonitrile/methanol (Rouseff et al., 1987; Mouly et al., 1993; Pupin et al., 1998; Justesen et al., 1998; Patil et al., 2009).

   A variety of detectors are used in combination with HPLC systems such as UV-Vis, photodiode array, and refractive index detectors. All the flavonoids contain at least one aromatic ring, and consequently absorb UV light (Mabry et al., 1970). The majority of the flavanones depict an absorption maxima at 280 nm (Table 9.3) (Nogata et al., 1994) due to the presence of an aromatic ring, therefore, UV-Vis or photo-diode array has commonly been employed. Furthermore, simple substitutions such as methyl, methoxy, or non dissociated hydroxyl groups cause minor changes in the absorption maxima. Therefore, the UV detection of flavonoids in general, and flavanones in particular, has become a preferred tool in LC analysis. However, UV-absorption does not discriminate between the aglycone and commonly found glycosidic

Table 9.2 Selected HPLC analysis methods for citrus flavanones.

	Column	Conditions/Method	Detector conditions	Flavanones analyzed	Ref.
1.	(A) Luna C$_{18}$ narrow bore column (150 × 2 mm, 3 μm)	Solvent A: aqueous formic acid, pH 2.4, Solvent B: acetonitrile/ isocratic 80:20 v/v, flow rate 200 μl/min	UV-Vis; 280 nm	Eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin	Careri et al., 2000
	(B) Altech C$_{18}$ (150 × 4.6 mm, 5 μm)	Solvent A: aqueous formic acid, pH 2.4, Solvent B: acetonitrile/ isocratic 80:20 v/v, flow rate 0.8 mL/min	Caulometer (porous graphite electrode);–0.5 V (first cell), +0.7 V (second cell)		
2.	(A) Nova Pak C$_{18}$, (3.9 × 150 mm, 5 μm)	Solvent A: 0.01 M potassium dihydrogen phosphate (pH 3.05), Solvent B: acetonitrile/water (70/30); linear gradient of 100% A to 42% B in 38 min	UV-Vis; 280 nm	Narirutin-4′-glucoside, eriocitrin, narirutin, naringin, hesperidin, neohesperidin, neoponcirin	Robards et al., 1997
	(B) Nova Pak C$_{18}$ (3.9 × 100 mm, 3 μm)	Aqueous acetonitrile; 3 min isocratic run followed by 100% water to 21% acetonitrile in 35 min	UV-Vis; 280 nm		
3.	MS Gel C$_{18}$ (150 × 4.6 mm, 5 μm)	Solvent A: 0.1 M monobasic sodium phosphate+10 mg/L SDS, pH 3.35, Solvent B: acetonitrile- 0.1 M monosodium phosphate+50 mg/l SDS- methanol (60:30:10), PH 3.45; 6% B isocratic for 10 min, increased to 30% B in 20 min, then increased to 100% B in 10 min, hold at 100% B for 5 min; flow rate 1.0 mL/min	Caulometer (Caulochem Electrode Array System; CEAS, Model 5500)	Narirutin, naringin, hesperidin, other phenolics	Gamache et al., 1993

(Continued)

Table 9.2 (*Continued*)

	Column	Conditions/Method	Detector conditions	Flavanones analyzed	Ref.
4.	RP-18 UHS (250 × 4.6 mm, 5 μm)	Mobile Phase: water/acetonitrile/ terahydrofuran/glacial acetic acid (80:16:3:1)	UV-Vis; 280 nm	Naringin, neohesperidin, hesperidin, narirutin, neoeriocitrin, eriocitrin	Mouly et al., 1993
5.	C$_{18}$, Lichrospher 100 RP-18 (250 × 4 mm, 5 μm)	Solvent A: 0.01 M phosphoric acid, Solvent B: methanol; 0–55 min 30% to 45% B linear increment, 55–95 min 45–100% B, 95–100 min 100% B	285 nm	Eriocitrin, neoeriocitrin, robinetin, narirutin, naringin, rutin, hesperidin, neohesperidin, isorhoifolin, rhoifolin, diosmin, neodiosmin, quercetin, poncirin, luteolin, kaempferol, apigenin, isorhamnetin, diosmetin, rhamnetin, isosakuranetin, sinensetin, acacetin, tangeretin	Nogata et al., 1994
6.	Novapak C18 (150 × 3.9 mm, 4 μm)	Solvent A: 0.1 M KH$_2$PHO$_4$ in water + phosphoric acid, Solvent B: 0.1 M KH$_2$PO$_4$ in water + acetonitrile; 100% A for 3 min, 58% A at 38 min, 0% A at 40 min, held at 100% B for 3 min and returned to 100% A in 46 min.	Diode array, 280 nm	Hesperidin, naringin	Ooghe et al., 1994
7.	(A) Phenomenex RP C$_{18}$ (250 × 4.6, 5 μm)	Solvent A: 1% formic acid in methanol/water (30:70), Solvent B: methanol; 25–86% in 50 min; flow rate 1 mL/min	Photodiode array detector, 220–450 nm spectra collected	Aglycones- Myricetin, quercetin, naringein, luteolin, hesperetin, kaempferol, apigenin	Justesen et al., 1998

	Column	Mobile phase	Detection	Compounds	References
	(B) Purospher RP C$_{18}$ (250 × 4.6, 5 μm)	Solvent A: 1% formic acid in water, Solvent B: acetonitrile; 5–60% B in 60 min; flow rate 1 mL/min		Glycosides- Eriodictyol, phloretin, isorhamnetin	
8.	C18 RP (250 × 4.6, 5 μm)	Solvent A: methanol + 0.01 M phosphoric acid, Solvent B: methanol; 100% A to 0% A in 55 min (also used for preparative chromatography)	Photodiode array detector, 282 nm	Naringin, naringenin, prunin	Berhow and Vandercook, 1989
9.	Zorbax ODS	Isocratic system methanol/water (60:40)		Naringin, limonin	Rouseff et al., 1980
10.	Separon SGX C6-Phenyl (150 × 3 mm, 5 μm)	Solvent A- 0.01M phosphoric acid/methanol, Solvent B: methanol; 1 min 100% A, 40 min 0% A, 45 min 100%A, 55 min 100% A; flow rate: 0.8 mL/min	Photodiode array detector, 285 nm	Naringin, hesperidin, neohesperidin, quercetin (quercetin demonstrated 70 % absorbance at 285 nm compared to 260, its absorption maxima)	Belajová and Suhaj, 2004
11.	Nucleosil C18 (250 × 4.6, 5 μm)	Water: acetonitrile/tetrahydrofuran/acetic acid (80:16:3:1); flow rate 1 mL/min	Photodiode array detector, 280 nm	Narinrutin, hesperidin, naringin	Pupin et al., 1998
12.	Zorbax ODS C18 (250 × 4.6 mm, 5 μm)	Mobile phase water/acetonitrile/acetic acid (79.5:20:0.5); flow rate: 1 mL/min	UV-Vis, 280 nm	Naringin, narirutin, hesperidin, neohesperidin	Rouseff et al., 1987
13.	Phenomenex Luna C18 (150 × 3 mm, 5 μm)	Solvent A- aqueous solution of 5 mM KH2PO4, pH 3.05, Solvent B: acetonitile/water/0.25 M KH2PO4 (70:26:4)	Photodiode array detector, 287nm	Eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, didymin, poncirin	Cautela et al., 2008

(Continued)

Table 9.2 (*Continued*)

Column	Conditions/Method	Detector conditions	Flavanones analyzed	Ref.
14. Lichrospher, C18 (250 × 4 mm, 5 μm)	Solvent A: acetonitrile, Solvent B: water; 0–8 min 23%A; 8–15 min 23–65% A linear; 15–20 min 65–70% A linear; 20–21 min 70–23% A linear; 21–22 min 23% A; flow rate: 1 mL/min	Photodiode array detector, 280nm	Naringin, naringenin	Ribeiro and Ribeiro, 2008
15. XBridge C18 (150 × 4.6 mm, 3 μm)	Solvent A: water/acetic acid (96:4), Solvent B: acetonitrile; 0–35 min 85–50% A, 35–40 min 50–85% A; flow rate 1 mL/min	Photodiode array detector, 280nm	Hesperidin, rutin, didymin, hesperetin	Patil et al., 2009

Table 9.3 UV Absorption maxima of selected flavanones.

Common name	λ_{max}nm	Reference
Eriocitrin	285	Nogata et al., 1994
Neoeriocitrin	285	Nogata et al., 1994
Naringin	284	Nogata et al., 1994
Narirutin	282	Nogata et al., 1994
Hesperidin	285	Nogata et al., 1994
Neohesperidin	284	Nogata et al., 1994
Diosmin	253, 268, 345	Nogata et al., 1994
Neodiosmin	255, 268, 345	Nogata et al., 1994
Naringenin	289	Lu et al., 2005

forms of flavonoids, as most glycosides are poor chromophores (Mabry et al., 1970). Though, separation between aglycone and corresponding glycosides can be achieved through HPLC, separation between different glycosidic forms can sometimes be difficult.

In addition to UV analysis, several researchers also employ electrochemical detectors for flavonoid analysis. The use of an electrochemical detector is suggested to provide better selectivity to flavanones and flavonoids. Careri et al. (2000) reported that the LC-UV system detected a higher concentration of naringin compared to the LC-TIS-MS or LC-ED systems. One possibility of such higher anomaly could be interference from the matrix. Gamache et al. (1993) included SDS in the mobile phase as an ion-pairing reagent for the retention of amines. Several researchers have used coulometric array detection of flavonoids and other phenolics to enhance the resolution for HPLC analysis (Gamache et al., 1993; Robards et al., 1997). HPLC with coulometric/amperometric electrochemical detection (ED) provides selectivity and sensitivity for the analysis of electroactive components in complex matrices (Kissinger, 1977) and is particularly well suited for food/juice matrices. The coulometric method differentiates and detects various compounds based on their voltammetric properties. Detailed voltammetric data across a wide potential range may be generated from a single injection and with good precision. The basis for differences in the ease of oxidation among various compounds corresponds with patterns of aromatic substitutions. A comparison of the electrochemical signature across the array may be used qualitatively to provide resolution and structural characterization (Gamache et al., 1993).

Mass spectrometric analysis of flavanones

The mass-spectroscopic instruments combine high resolution with enhanced sensitivity for LC separations. In addition, the use of MS usually provides unambiguous structural data, which is an invaluable tool for the identification of different compounds. Many different ionization tools and methods for mass spectral analysis are available and a variety of them have been applied in flavonoid analysis (Hansen et al., 1999; Li et al., 1992; de Rijke et al., 2003). A selected list of various MS ionization techniques applied in the analysis of flavanones, primarily for citrus species, are presented in Table 9.4. In most cases, single-stage MS is used in combination with the UV detection method for enhanced sensitivity and confirmation of identity. For the identification of unknowns, tandem mass spectrometry (MS/MS) is used. Even though several methods of ionization have been tested, APCI and ESI modes of ionization have gained the upper hand in terms of usage. Both positive and negative ionization are applied. ESI is more frequently encountered in the literature of flavonoid analysis, but APCI is also gaining popularity. Both APCI and ESI in the negative ion mode demonstrate high sensitivity. However, the positive ion mode provides useful complementary information, often in the analysis of unknowns. A good strategy will be to combine the outcomes of both positive and negative ion mode spectra to reach the final conclusion. While interpreting MS spectra, it should be noted that analyte responses vary considerably, dependent on the ionization mode and class of compounds under study. In addition, LC conditions such as the composition of eluent, its pH, and the nature of buffer components may have a distinct influence on the final spectra and should be taken into account while interpreting. In flavonoid analysis the most common additives are acetic acid (Pupin et al., 1998; Patil et al., 2009), formic acid (Careri et al., 2000; Justesen et al., 1998), ammonium acetate, and ammonium formate (Hansen et al., 1999; de Rijke et al., 2003). Some studies have employed trifluoroacetic acid for the MS analysis of flavonoids (da Costa et al., 2000). However, trifluoroacetic acid is known to suppress ionization due to ion-pairing and surface tension effects (de Rijke et al., 2006).

One of the major challenges inthe structural analysis of flavonoids using mass spectrometry is the difficulty of differentiating between isomers. Rutinosides and neohesperidosides are the two most common flavonoid diglycosides. These isomers differ only by the

Table 9.4 Selected LC-MS techniques used in the analysis of citrus flavanones.

Sample/Species	Flavonoids	Ionization mode	Reference
C. paradise, C. aurantium, C. reticulata, C. sinensis	Naringenin, naringin, rutin, quercetin, isoquercitrin	APCI, negative ion mode	de Lourdes Mata Bilbao et al., 2007
Pericarpium citri reticulatae virde, Pericarpium citri reticulatae, Fructus aurantii, Fructus, aurantii, immaturus	Naringin, naringenin, hesperidin, neohesperidin, tangeretin, synephrine	ESI-MS, positive ion mode	Ding et al., 2007
Red Orange juice	Eriocitrin, hesperidin, narirutin, naringin, neoeriocitrin, neohesperidin	Ion spray (IS), Turbo ion spray (TIS); negative ion mode	Careri et al., 1999
Flavone, flavonol and flavanone aglycones	Eriodictyol, naringenin, isosakuranetin, quercetin, fisetin, kaempferol, galengin, kaempferid, luteolin, apigenin, genkwanin, chrysin,	ESI-Trap; negative ion mode	Fabre et al., 2001
Orange juice	Neoeriocitrin, naringin, sinensetin, nobiletin	ESI-TOF; positive and negative ion mode	Tolonen and Uusitalo, 2004

interglycosidic linkage between the two monosaccharides. Citrus fruits contain a very high concentration of these isomers, which are important taxonomic markers for fruit quality. Several studies have been conducted to readily identify the different types of glycosylation pattern. For example, Claey's group used ESI–TOF and fast atom bombardment configurations to differentiate two isomers. Further, it was demonstrated that *O*-, *C*-, and *O-C*-diglycosides can be identified by first-order positive-ion FAB spectra or low energy CID spectra (Li et al., 1992). Due to rearrangement reactions at the interglycosidic bonds, the protonated *O*-glycosides give rise to Y_1^+ and Y_0^+ ions. Under similar conditions, only $[M+H]^+$ ions were detected for *C*-glycosides, while *O-C*-diglycosides gave rise to Y_1^+ ions, which were formed by fragmentation at the interglycosidic linkage (Li et al., 1992). A different fragmentation pattern is usually observed in negative-ion mode FAB experiments. In negative-ion mode, *C*-glycosides are detected as $[M–H]^-$ ions, whereas, in the case of *O-C*-diglycosidesan additional Y_1^- ion was also observed (Cuyckens and Claeys, 2004). A relative abundance >90% of Y_1^- is characteristic of di-*O-C*-glycosides, while *O-C*-glycosides show a weak Y_1^- ion, but reveal a major daughter ion $[Y_1–H_2O]$. In contrast, deprotonated *O*-glycosides give rise to both Y_1^- and Y_0^- ions (Cuyckens and Claeys, 2004). Furthermore, Ferreres et al. (2004) proposed that the two isomers can be differentiated on the basis of the interglycosidic linkage by ion-trap [ESI-ITMS] in negative-ion mode focusing. It was also demonstrated that MS/MS experiments are more suitable for the characterization of flavonoid diglycosides. The main differences between the rutinosides and neohesperidosides mass spectra effects are their $[Y^*]-$ and $[Y^0]+$ intensities and on the basis of their relation, interglycosidic linkage type and glycosylation position can be elucidated (Abad-García et al., 2009). Flavonoid diglycosides present more complex spectra for which the most striking aspect is the $[Y^*]-$ ion, which allows characterization of interglycosidic linkage by a comparison with $[Y^0]+$ ion (Abad-García et al., 2009).

   In addition to ESI and APCI, ionization techniques such as electron ionization (EI) and chemical ionization (CI) have also been used in the analysis of flavanones (Weintraub et al., 1995). As the advancement and refinement in analytical techniques continues, it is expected that these techniques will find an application in flavanone analysis.

Table 9.5 ^{13}C NMR chemical shifts of flavanones, ($\Delta = \delta_{DMSO\text{-}}$ δ_s.state ≥ 1 ppm, in parentheses). Adopted from Wawer et al. (2001).

Atom	Hesperetin		Naringenin	
	DMSO	CP/MAS (Δ)	DMSO	CP/MAS (Δ)
C-2	78.6	76.8	79.2	80.2 (-1.0)
C-3	42.8	42.9	42.7	42.5
C-4	195.5	197.2 (-1.7)	196.4	197
C-5	164	162.5 (1.5)	164.5	164.7
C-6	96.4	97.8 (-1.4)	96.2	99.2 (-3.0)
C-7	166.9	169.5 (-2.6)	166.5	166.2
C-8	95.4	97.8 (-2.4)	95.2	96.3 (-1.1)
C-9	162.9	162.5	163.6	162.3 (1.3)
C-10	102.2	101.2 (1.0)	102.4	102.8
C-1'	131.3	133.1 (-1.8)	130	130
C-2'	113.6	110.7 (2.9)	128.3	129.5 (-1.2)
C-3'	146.6	145.4 (91.2)	115.4	117.0 (-1.6)
C-4'	147.9	146.2 (1.7)	157.8	154.8 (3.0)
C-5'	111.5	110.7	115.4	115.2
C-6'	117.7	114.1 (3.6)	128.3	130.5 (-2.2)
OCH3		54		

NMR analysis of flavanones

Nuclear magnetic resonance spectroscopy is one of the most widely used instrumental methods, with applications ranging from the characterization of pure compounds to the diagnosis of disease (Rabenstein and Guo, 1988). At the molecular level, the electrons shield the nucleus of the atom resulting in a situation where the nucleus experiences a magnetic field of lesser strength compared to the applied magnetic field. The difference between the applied magnetic field and the field at the nucleus is known as nuclear shielding. The chemical shift, which is defined as the nuclear shielding/applied magnetic field, is a function of the nucleus and its environment; it is measured relative to a reference compound. For two bonded or adjacent carbon atoms, the chemical shifts reflect the environment of both atoms and are indicative of the order and thus the strength of the connecting bond.

Despite the advances in mass-spectrometric methods for flavonoid analysis, the NMR technique is essential for an unambiguous structural characterization and has been recognized as the most important tool for the structural determination of flavonoids (Markham and

Table 9.6 ^{1}H NMR chemical shifts of flavanones. Adopted from Maltese et al. (2009).

Atom	Naringenin	Hesperetin	Naringin (2S)	Naringin (2R)	Hesperidin (2S)	Hesperidin (2R)	Neohesperidin (2S)	Neohesperidin (2R)
2	5.47 dd	5.46 dd	5.56 dd	5.52 dd	5.54 dd	5.52 dd	5.55 dd	5.50 dd
3ax	2.71 dd	2.74 dd	2.73 dd	3.43 dd	2.79 dd	3.42 dd	2.80 dd	3.35 dd
3eq	3.30 dd	3.23 dd	3.36 dd	2.77 dd	3.31 dd	2.80 dd	3.31 dd	2.77 dd
6	5.91 s	5.91 d	6.11 d	6.08 d	6.18 d	6.17 d	6.15 d	6.15
8	5.91 s	5.92 d	6.10 s	6.07 d	6.16 d	6.15 s	6.12 d	6.13 d
2′	7.35 d	6.95 d	7.31 d	7.32 d	6.97 d	6.98 d	6.97 d	6.98 d
3′	6.82 d		6.80 d	6.79 d				
5′	6.82 d	6.97 d	6.80 d	6.79 d	6.83 d	6.83 d	6.94 d	6.93
6′	7.35 d	6.90 dd	7.31 d	7.32 d	6.99 dd	6.98i	6.91 dd	6.91
4′-OMe		3.81 s			3.81 s		3.81 s	
Glc-1″			5.17 d		5.01 d		5.17 d	
Rha-1″			5.13 d		4.55 d		5.14 d	
5‴-Me			1.19 d		1.12 d		1.21 d	

Ternai, 1976; Maltese et al., 2009).In addition to the apparent potential for structural elucidation, NMR spectroscopy provides ease and simplicity in a sample preparation, short analysis time, and total sample recovery.

A number of flavonoids have been examined directly or extracted from plants and characterized subsequently by ^{13}C or ^{1}H NMR spectra. ^{13}C and ^{1}H NMR spectra have been reported for a number of citrus flavonoids in dimethyl sulfoxide (Wawer and Zielinska, 2001). Tables 9.5 and 9.6 present the chemical shifts of commonly found citrus flavonoids. In recent years, NMR spectroscopy has been combined with FAB/MS/MS, with HPLC/MS forthe structural characterization of flavonoids and flavonoid-*O*-glycosides, with HPLC-UV-SPE-MS, HPLC/MS, and with HPLC-UV-MS for the identification of flavonoids (March and Brodbelt, 2008). In addition, a predictive model for assessing the ^{13}C spectra of flavonoids has been proposed (Burns et al., 2007). This predictive model will be useful to determine the ^{13}C NMR data for unknown flavonoids or the flavonoids for which the ^{13}C NMR data has not been recorded. For example, ^{13}C NMR spectra of only 11 of 28 commercially available dihydroxy flavonoids have been recorded and analyzed using the proposed strategy, and the ^{13}C NMR data of the remaining 17 can be predicted. In fact, the ^{13}C chemical shifts for 8-hydroxy flavone, which does not occur in nature, have been predicted using this model (March and Brodbelt, 2008). The combination of different techniques such as LC, MS, and NMR has proved to be a much more powerful tool in the structural determination and elucidation of flavonoid properties.

9.6   Summary

Flavonoids gained the attention of medical plant scientists and chemists in the early 20th century after the discovery of the amelioration of capillary fragility by lemon and pepper flavonoids (Rusznyak and Szent-Gyorgi, 1936). Since then tremendous progress in all areas of flavonoid biology and chemistry has occurred. Over the years the improvement in analytical techniques kept pace with the progress in flavonoid biology and biochemistry and was punctuated with the development of several analytical techniques. Specifically, application of HPLC and later LC-MS has revolutionized flavonoid analytical

methods. The major challenges in flavonoid analysis are the complexity of the sample matrix and the diversity of flavonoids. Most often, complex matrices such as plant and food, as well as human and animal serum, contain several hundred to several thousand compounds of differing properties. In order to correctly analyze the flavonoids, several sample preparation steps are required. A greater number of sample preparation steps is generally correlated with higher losses of flavonoids during the process and, consequently, biases the detection and/or quantification. For example, the flavanone glycosides naringin and hesperidin show varying solubility under differing conditions, resulting in a high amount of variability depending upon the sample preparation technique (Table 9.1). Improvements in analytical techniques have ameliorated the situation to some extent. However, sample preparation techniques from complex matrices still have much scope for improvement.

The current efforts in flavanone analysis are directed toward enhanced sensitivity, especially in samples such as human plasma. In addition, advancements in HPLC and LC-MS techniques are another area of focused research. Another area of active research in flavonoid analysis is the capillary electrophoresis/electrochromatography of flavonoids. During the past decade a growing number of publications have addressed the efficient use of capillary electrophoresis. However, there are a limited number of reports on the use of capillary electrochromatography in the analysis of flavanones. This technique needs further consideration to fully elucidate its potential in flavanone analysis.

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Chapter 10

Analysis Methods of Phytosterols

Laura Nyström

Abstract

Recent studies have demonstrated that natural intake levels of plant sterols also have significant health-promoting properties in addition to the intakes from plant sterol-enriched functional foods, which have been on the market for over a decade. Plants contain a wide variety of different molecular species of plant sterols, which further occur in free form or as conjugates (esters of fatty or phenolic acids, glycosides, or acylated glycosides). The levels of plant sterols and their conjugates found in various foods vary significantly, and the selection of the method used for their analysis is dependent on the type of information desired (composition of sterol species and/or conjugation). The main focus of the chapter is on the antioxidant activity and analysis of SFs (ferulic acid esters of sterols, which are characteristic to cereal grains), but other conjugates are also covered.

Keywords: Plant sterol; phytosterol; sterol conjugate; steryl ferulate; gamma-oryzanol; steryl glycoside; acylated steryl glycoside; steryl ester; free sterol.

10.1 Introduction

Phytosterols (also called plant sterols) are secondary plant metabolites found in the cell membranes of all plants. Recent nutritional interest in plant sterols from the diet owes to their capacity to decrease serum cholesterol levels and hence aid in the prevention and control of

cardiovascular diseases. The cholesterol lowering property of plant sterols is very well established, and several foods enriched with phytosterols are commonly available on the market. Phytosterol ingredients have been granted GRAS status (generally recognized as safe) by the US Food and Drug Administration (FDA), and food labels about their cholesterol lowering functionality are accepted in a wide range of foods in the USA and in Europe. The European Food Safety Authority (EFSA, 2010) allows the health claims for the maintenance of healthy cholesterol levels for both free and esterified plant sterols and stanols (saturated sterols) in foods that provide at least 0.8 g per day of sterols/stanols in one or more servings. In the United States Food and Drug Administration (FDA, 2010) is currently working on expanding the health claim to cover free sterols in addition to the sterol esters. It is generally considered that a daily dose of 1–3 grams of phytosterols significantly decreases (10–15% decrease) the total cholesterol level and also the LDL-cholesterol, thus improving the ratio between cholesterol in high-density lipoproteins (HDL-cholesterol, known as "good" cholesterol) and low-density lipoproteins (LDL "bad" cholesterol). For reviews of the cholesterol-lowering functions of phytosterols see, for example, Ostlund (2002) and Trautwein and Demonty (2007).

Recent studies on dietary phytosterols have increasingly focused on the effects of natural intakes from common, nonenriched foods on blood cholesterol levels. The natural daily intake of phytosterols varies from about 170 to 440 mg/day, which is significantly lower than the dose from enriched foods. However, doses as low as 150 mg in one meal have been shown to significantly decrease the intestinal absorption of cholesterol (Ostlund, 2002). Further, a recent epidemiologic study by Klingberg et al. (2008) demonstrated that there is a strong and significant inverse relation between serum cholesterol levels and the natural dietary intake of phytosterols. The association was shown to be a true link between phytosterol intake and serum cholesterol levels, not just decreased cholesterol levels associated with high fiber and low fat diets, which are also rich in phytosterols.

As the vast majority of human studies on the effects of phytosterols on serum cholesterol levels have been done using free sterols and sterol fatty acid esters, there has been a debate about the efficacy of the other natural sterol conjugates in lowering cholesterol. However, recent studies have demonstrated that other conjugates (ferulic acid

esters and glycosides) are also effective in their cholesterol-lowering function in humans (Berger et al., 2005; Most et al., 2005; Lin et al., 2009). In order to confirm these findings as universal, more studies are needed to establish the possible factors that affect the functionality of these conjugates in cholesterol lowering and other biological functions.

These findings, however, highlight the importance of natural dietary sources of phytosterols in maintaining healthy cholesterol levels, and further address the need for comprehensive and concise information about their occurrence in foods. This includes both the total levels of phytosterols in various food sources, as well as the variability caused by varieties, growth locations, agricultural practices, post-harvest, and domestic processing, etc. To be able to effectively gather information about phytosterols in foods, one needs modern analytical methods and special consideration for analytical procedures for variable sample materials, as will be described below. As the focus of this book is on natural antioxidants, the focus of this chapter will be on the antioxidative conjugates of phytosterols and the materials in which they are found, namely steryl ferulates and cereal grain sources; but others will also be covered briefly.

10.2 Chemical Structures, Sources, and Levels in Various Natural Sources

10.2.1 Phytosterols in Natural Sources: General

Phytosterols are found as free sterol alcohols and as conjugates of these (Figure 10.1). The basic ring structure of the phytosterols is identical to cholesterol, but differences are seen in the unsaturation and substitutions of the rings and the side chain (Figure 10.2). The common core sterol structure consists of four carbon rings that may be unsaturated at certain positions, most often between C-5 and C-6 (Δ^5-sterols), or C-7 and C-8 (Δ^7-sterols). Phytosterols may also be structurally classified according to the presence and number of methyl groups on carbon C-4 to desmethylsterols (the most common group), 4-monomethylsterols, and 4,4-dimethylsterols. More than 200 different sterols have been identified in natural sources and the contents of different phytosterols and the ratios between the different conjugates are characteristic of, and
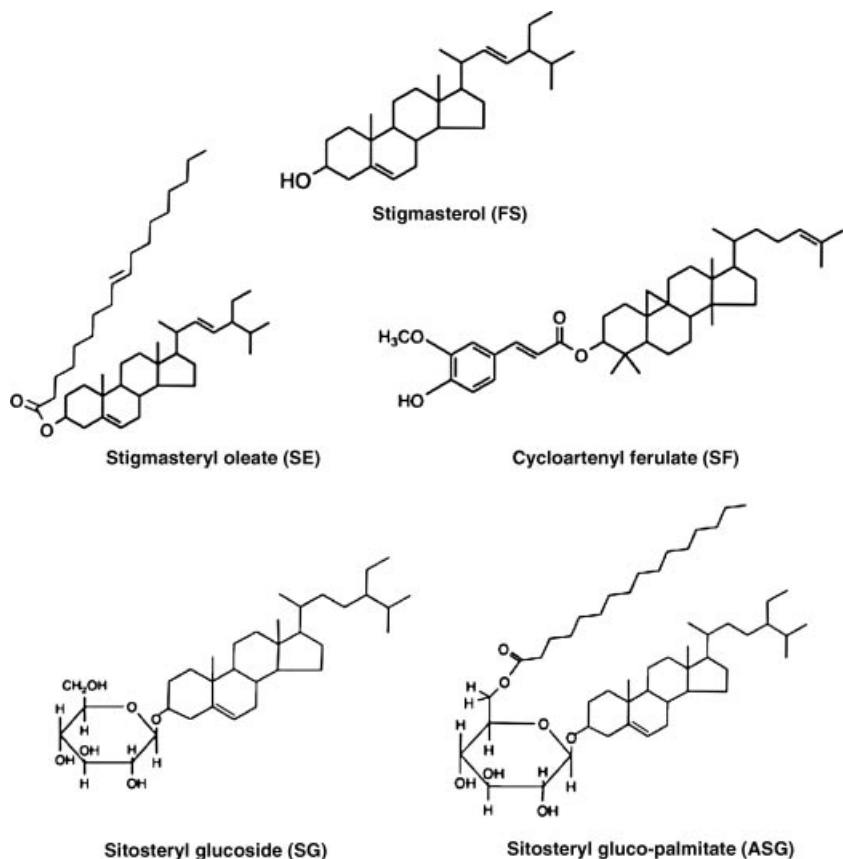
Figure 10.1 Examples of structural formulas of phytosterol and sterol conjugates. FS = free sterol alcohol, SE = sterol fatty acid ester, SF = steryl ferulate, SG = steryl glycoside, ASG = acylated steryl glycoside.

in some cases even unique to, certain plant materials. Sitosterol is the most common sterol, followed by campesterol, stigmasterol, and brassicasterol. In cereal grains a significant proportion of total sterols is also contributed by stanols, i.e. sterols with a saturated ring system without double bonds. Detailed information about the contents of different phytosterols in various foods (total content after hydrolysis) has recently been published in a database eBASIS (BioActive Substances in Food Information System) available at http://ebasis.eurofir .org. In general, vegetable oils and products thereof form the main sources, in addition to cereal grain products. Most vegetable oils
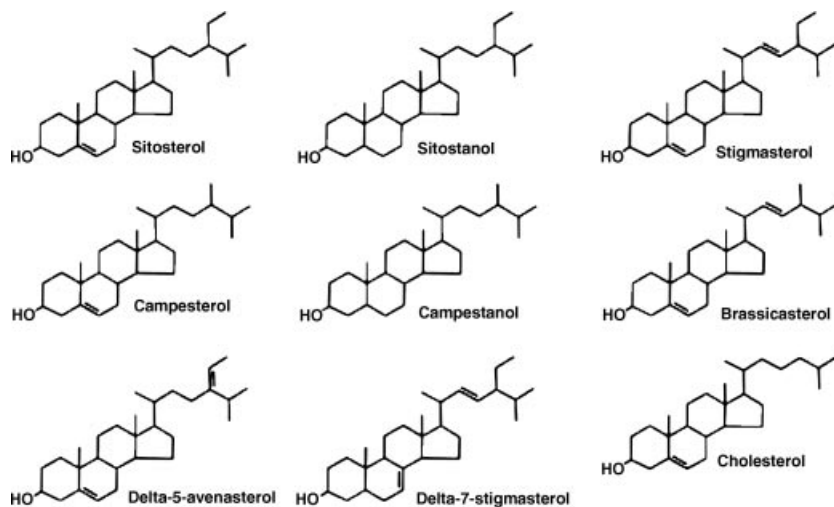
Figure 10.2 Chemical structures of common plant sterols and cholesterol.

and cereal grains contain 1–5 mg/g or 0.35–1.2 mg/g of phytosterols, respectively (Piironen and Lampi, 2004). Even if the content in cereal grains is much lower than in vegetable oils, the larger amounts consumed make grains an important source of natural dietary plant sterols.

The free sterols (FSs) and sterol fatty acid esters (SEs) are the most common forms found in plants, and their levels in natural sources are already well known, whereas the other conjugates, namely steryl glycosides (SGs), acylated steryl glycosides (ASGs), and steryl ferulates (SFs) have been less extensively studied. For a detailed review of the contents of SEs, FSs, SGs, and ASGs – see, for example, Piironen and Lampi (2004). Each of these conjugates is thought to serve a different function in plants, though their roles are still relatively poorly understood (Wojciechowski, 1991). Furthermore, basic information about their levels in various natural sources is available, but more studies are still needed to give a better picture of their occurrence, variability, and functions.

10.2.2 Steryl Ferulates in Natural Sources

Steryl ferulates (SFs) are esters of phytosterols and ferulic acid. Also esters of other phenolic acids, namely *p*-coumaric acid and caffeic acid,

have been reported in the literature (Seitz, 1989; Norton, 1995; Fang et al., 2003). These are often included under the discussion of ferulic acid esters. Steryl ferulates were first reported in 1955, when γ-oryzanol was extracted and characterized by Kaneko and Tsuchiya (1955). γ-Oryzanol, the mixture of sterol and triterpene esters of ferulic acid from rice, is still by far the most extensively studied source of SFs, though in the past decades other grain sources have also gained attention (Table 10.1). Steryl ferulates are almost exclusively found in grains and seeds, though they have also been reported in laminarian seaweeds (Nagasaka et al., 2008).

The sterol composition, i.e. different molecular species of sterols in SFs found in different grains, varies significantly. In rice (γ-oryzanol) the most abundant compounds are cycloartenol (approximately 35–51% of SFs in brown rice), 24-methylenecycloartanol (23–37%), and campesterol (5–18%) esters of ferulic acid (for structures, see Figure 10.3). In addition to these and other common sterols found as ferulic acid esters, a wide range of minor sterols (including hydroxylated sterols) have also been detected in trace amounts (Fang et al., 2003), but information on these SFs is still scarce. Esterification to ferulic acid is selective, as the composition of sterols as ferulic acid esters is significantly different from that of total sterols (Ha et al., 2006). Sitosterol, campesterol, and stigmasterol are the most abundant sterols of total plant sterols in rice; but as ferulic acid esters, 24-methylene-cycloartanol and cycloartenol predominate. In other cereal grains (wheat and rye) the most common sterol moieties in SFs are campestanol and sitostanol, and their unsaturated counterparts campesterol and sitosterol. For a detailed list of studies on γ-oryzanol in rice, see the review of Lerma-Garcia et al. (2009).

10.3   Antioxidant Activities and Biological Functions

10.3.1   Antioxidant Activity of Steryl Ferulates

Of the various plant sterol conjugates, SFs are known to prevent lipid oxidation in various systems. The activity is based on the capability of ferulic acid to donate hydrogen from the phenolic hydroxyl group to a radical. The resulting SF radical formed is resonance stabilized, and the SF radicals may still effectively interfere with the chain reaction of

Table 10.1 Levels of steryl ferulates in natural sources.

Sample		Content of steryl ferulates µg/g	Reference
Rice	Brown rice	200–720	Khatoon and Gopala Krishna, 2004; Miller and Engel, 2006; Aguilar-Garcia et al., 2007
	Rice bran	1550–8400	Bergman and Xu, 2003; Rohrer and Siebenmorgen, 2004; Aguilar-Garcia et al., 2007
	Polished rice	60–120	Khatoon and Gopala Krishna, 2004; Ohtsubo et al., 2005
	Crude rice bran oil	1.5–2.7[a]	Norton 1995; Gopala Krishna et al., 2001; Van Hoed et al., 2006
	Purified rice bran oil	0.3–0.45[a]	Van Hoed et al., 2006
Corn	Corn grains	30–220	Seitz, 1989; Moreau et al., 2001
	Corn bran	200–250	Moreau et al., 1999
	Corn fiber (from wet milling)	440–1800	Moreau et al., 1996, 1999; Wu and Norton, 2000
Wheat			
	Winter wheat	62–126	Seitz, 1989; Hakala et al., 2002; Nurmi et al., 2010
	Spring wheat	66–104	Nurmi et al., 2010
	Wheat bran	297–584	Collins et al., 2002; Hakala et al., 2002; Nyström et al., 2007c
Rye	Rye grains	29–86	Seitz, 1989; Hakala et al., 2002; Nyström et al., 2007c; Nurmi et al., 2010
	Rye bran	150–251	Hakala et al., 2002; Nyström et al., 2007c
Barley	Barley grains	4	Moreau et al., 1998

[a] % of oil

Campesteryl ferulate

Sitostanyl (stigmastanyl) ferulate

Cyclobranyl ferulate

22-Dihydrospinasteryl (schottenyl) ferulate

24-methylenecycloartanyl ferulate

Campestanyl ferulate

Cycloartanyl ferulate

Citrostadienyl ferulate

Cycloartenyl ferulate

Sitosteryl ferulate

Stigmasteryl ferulate

Gramisteryl ferulate
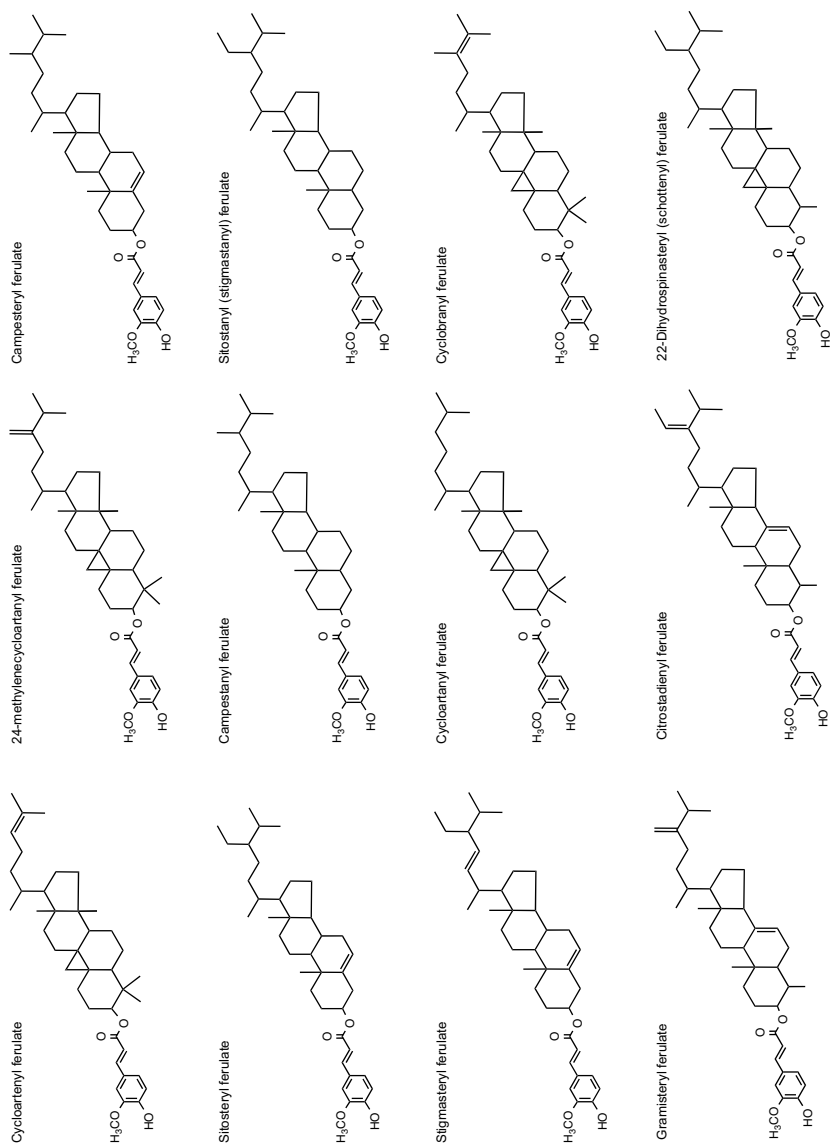
Figure 10.3 Structural formulas of selected steryl ferulates identified in γ-oryzanol.

lipid oxidation as alkyl radicals (Kochhar, 2000). In studies of the antioxidant functions of free ferulic acid, no inhibition with other mechanisms like metal chelation has been observed (Graf, 1992). As it is unlikely that SFs (i.e. when the ferulic acid is esterified to a sterol) would have these activities, their antioxidative effect is based on radical scavenging.

Most of the studies on the antioxidant activity of SF have been performed using γ-oryzanol or the addition of rice bran oil (containing also other components with antioxidant activity), and from those studies it is not possible to evaluate the activities of single compounds. Further, the information obtained on the relative antioxidant activities of the different FSs varies from one study to another (Table 10.2). One reason for this may be that the range of antioxidant tests used in these studies is wide and the results, therefore, are not necessarily comparable. More studies are definitely needed to fully understand the possible differences in the antioxidant activity of different SFs in different oxidation systems.

In addition to the discrepancies in the relative order of antioxidant efficiencies of different SFs, there is inconsistency in the results obtained when the efficacy is compared to α-tocopherol, another common and well-known lipophilic antioxidant. Selected studies have shown that SFs are more effective in their antioxidant activity than α-tocopherol (Gertz et al., 2000; Xu et al., 2001; Huang et al., 2002; Kim et al., 2003), whereas others suggest that the antioxidant activity of SFs is lower than that of α-tocopherol (Tajima et al., 1983; Xu and Godber, 2001; Kikuzaki et al., 2002; Juliano et al., 2005; Nyström et al., 2005, 2007a; Huang et al., 2009). Part of the differences may naturally be a result of the antioxidant model used, but also in this aspect more studies are needed to obtain a better picture on the antioxidant capacity of SFs. Regardless of the differences in the absolute antioxidant activity between SFs and α-tocopherol, the importance of SFs as antioxidants may be emphasized due to their better heat stability. Recent studies have shown that when analyzed alone or in a mixture, SFs are degraded slower than α-tocopherol, and therefore may retain their activity especially at high temperatures longer than α-tocopherol (Nyström et al., 2007a; Huang et al., 2009; Winkler-Moser and Vaughn, 2009). This makes SFs an interesting option to be used as antioxidants in high-temperature applications.

Table 10.2 Antioxidant activities of steryl ferulates in various studies.

Model system (Monitored factor representing oxidation)	Substrate[a]	Temp. (if not ambient)	Steryl ferulates evaluated (in the order of increasing antioxidant activity)[b]	Reference
Diene formation	linoleic acid	30°C	CAM>24-met-CAF>CAF	Yagi and Ohishi, 1979
Scavenging of superoxide radicals (decrease in the superoxide ESR signal)			ORY	Tajima et al., 1983
Formation of lipid hydroperoxides	triacylglycerols of lard*	100°C	CF	Marinova et al., 1998
Formation of lipid dimers and polymers	sunflower oil and rape-seed oil	170°C	ORY	Gertz et al., 2000
Hydroperoxide formation in linoleic acid model	linoleic acid*	37°C	24-met-CAF=CAM=CAF	Xu and Godber, 2001
Formation of cholesterol oxidation products	cholesterol in emulsion*	37°C	24-met-CAF>CAM$\geq$CAF	Xu et al., 2001
Oxidative stability index (OSI) and increase in oil viscosity	1) soybean oil 2) soybean oil FAME	1) 100°C 2) 90°C	SSta>ORY	Wang et al., 2002
Oxidation in multiple different systems	1) Methyl linoleate 2) oxidative stability index	1) 40°C 2) 90°C 3) 40°C 4) 37°C	1) CAF=ORY=24-met-CAF 2) CAF=ORY>24-met-CAF	Kikuzaki et al., 2002

Modified ORAC for lipophilic compounds*	3) ethanol-buffer mixture* 4) PC liposomes*		3) n.a. 4) no inhibition by SF ORY	Huang et al., 2002
Stability of beef patties during storage (formation of TBARS, WOF, hydroperoxides, hexanal and cholesterol 7-oxides)		stored at 4 °C	ORY	Kim et al., 2003
Protein and lipid oxidation (formation of carbonyl groups)	brain cell homogenate*	37 °C	water soluble oryzanol (enzymatic extract from rice bran)	Parrado et al., 2003
Oxidation in multiple different systems	1) Scavenging of OH$^\bullet$ radicals 2) Scavenging of $O_2^{\bullet-}$ radicals 3) Lipid peroxidation in PC liposomes*	3) 41 °C	1) no inhibition by SF 2) no inhibition by SF 3) ORY	Juliano et al., 2005
Hydroperoxide formation in methyl linoleate	1) Methyl linoleate bulk oil	40 °C	1) SF extracts of wheat &rye>SSte = CF> ORY = CAF	Nyström et al., 2005

(*Continued*)

Table 10.2 (*Continued*)

Model system (Monitored factor representing oxidation)	Substrate[a]	Temp. (if not ambient)	Steryl ferulates evaluated (in the order of increasing antioxidant activity)[b]	Reference
	2) Methyl linoleate emulsions		2) SF extracts of wheat&rye = Sste	
Formation of triacylglycerol dimers and polymers	High oleic sunflower oil	100°C and 180°C	SSta	Nyström et al., 2007a
LDL-oxidation	Low density lipoproteins*	37°C	No inhibition by DHCF	Chigorimbo-Murefu et al., 2009
Endothelial cell model	Mouse lymphatic endothelial cells	37°C	24-met-CAF>CAF = CAMF	Huang et al., 2009

[a] In the studies marked with an asterisk an initiator or accelerator of oxidation other than heat (e.g. air flow, metal ions, radical initiator) was used
[b] CAM = campesteryl ferulate, CAF = cycloartenyl ferulate, 24-met-CAF = 24-methylenecycloartanyl ferulate, CF = cholesteryl ferulate, DHCF = dihydrocholesteryl ferulate ORY = γ-oryzanol, SSta = sitostanyl ferulate, SSte = sitosteryl ferulate

10.3.2   Other Biological Functions of Steryl Ferulates/Sterol Conjugates

In addition to antioxidant activity, SFs as well as other sterol conjugates have been demonstrated to have other biological functions. By far the most studied functionality of phytosterols is the ability to reduce serum cholesterol levels by inhibiting the uptake of both dietary cholesterol, as well as the endogenously produced biliary cholesterol. The inhibition of cholesterol uptake has been shown to decrease total and LDL-cholesterol levels, whereas HDL-cholesterol and serum triacylglycerols seem to be unaffected (Jones and AbuMweis, 2009). In addition to free sterols and sterol fatty acid esters, SFs and SGs have also been shown to improve the serum cholesterol status (Berger et al., 2005; Most et al., 2005; Lin et al., 2009). Furthermore, it has been shown that phytosterols are cancer chemopreventive agents, particularly against cancers of the colon, breast, and prostate (Awad and Fink, 2000; Bradford and Awad, 2010).

Some of the additional biological functions of SFs are most likely a result of their antioxidant capacity (radical scavenging activity). Studies reporting the anti-inflammatory, antiviral, cytotoxic, and antitumor properties of SFs are available, but these all require further research to better establish the links between original observations in model systems and the possible effects in humans as a result of dietary intake from foods, dietary supplements, and pharmaceuticals. For example, SFs have been shown to decrease inflammation in mouse ears (Akihisa et al., 2000; Yasukawa et al., 1998), and to inhibit the nuclear factor kappa B (NF-κB) related to inflammation (Nagasaka et al., 2007; Islam et al., 2008). Antiviral activity against a range of viruses has been demonstrated by Galabov et al. (1998), Akihisa et al. (2001), and Iwatsuki et al. (2003), and finally anticancer and antitumor effects have been suggested by, for example, Yasukawa et al. (1998), Luo et al. (2005), and Long et al. (2009).

The glycosylated sterols have also been reported to have additional biological effects in addition to a cholesterol-lowering function, the most studied of which is the inhibition of the benign growth of the prostate gland (Pegel, 1980; Berges et al., 1995). Additionally, SGs in a mixture with free sterols are, for example, suggested to modulate the immune system (van Rensburger et al., 2002), affect the proliferation of cells (Bouic et al., 1996), and enhance the nasal absorption of certain drugs (Ando et al., 1998).

10.4   Traditional (e.g. Nonchromatography) Analysis Methods and their Disadvantages

Analytical methods for plant sterol analysis are commonly based on procedures used for cholesterol analysis. However, a significant shortcoming of these methods is the fact that cholesterol occurs only as free cholesterol and fatty acid esters. Therefore, the analytical methods optimized for cholesterol analysis are not suitable, or only suitable with some restrictions, for the analysis of conjugates found only in plants (SFs, SGs, and ASGs). Further, the methods described below only give the total amount of plant sterols and no information of the different sterol species found in the samples. However, if detailed information about the sterol composition is not required, and the amount of sterols to be analyzed is sufficiently high for these less sensitive but simpler methods, they provide a less laborious alternative for the analysis.

10.4.1   Enzymatic kits

Enzymatic kits are a simple and robust way to analyze cholesterol in foods and serum samples. Enzymatic methods of this type can also be used for the analysis of phytosterols as FSs and SEs, as demonstrated by Moreau et al. (2003a). In enzymatic methods, the sterols are quantified as free sterols (FSs), which require hydrolysis of the conjugates. The enzyme used is a microbial sterol esterase, which hydrolyzes the fatty acid esters, but not the ferulic acid esters. Therefore, in order to use this method to analyze all phytosterols (including SFs, SGs, and ASGs) additional enzymes or chemical hydrolysis would be needed to release all phytosterols from their conjugates. Further, the enzymatic analysis is based on an oxidation reaction by cholesterol oxidase enzyme. This enzyme is known to oxidize also plant sterols but the affinity of the enzyme toward plant sterols is often significantly lower than for cholesterol (Smith and Brooks, 1976). Therefore, the incubation times for the analysis of plant sterols often need to be longer than recommended in the procedures for cholesterol analysis. Furthermore, the specificities of the enzymes from various sources may be different and therefore it is recommended to evaluate enzyme activity using standard sterols before utilizing them in a quantitative analysis on a mixture of phytosterols.

10.4.2 Precipitation with Digitonin

Earlier studies have used precipitation with digitonin as a means of extraction/purification of sterols from a lipid mixture, and also for sterol quantification. Digitonin is a plant saponin that will form a complex with free sterols, meaning that also for this method the conjugates will first need to be hydrolyzed. After precipitation, the complexes can be dissolved and analyzed chromatographically (Goad and Akihisa, 1997). Alternatively, a method that involves dichromate oxidation of the digitonides and titrimetric analysis of the excess dichromate with ferrous solution has been shown to have a good accuracy in determining plant sterols (Waghorne and Ball, 1952). However, digitonin precipitation is perhaps not complete for 4,4′-dimethylsterols and some $\Delta^{5,7}$-sterols, and as digitonin is poisonous this method is no longer commonly used (Goad and Akihisa, 1997).

10.4.3 Spectrophotometric Determination of Steryl Ferulates

Owing to the UV absorbance of ferulic acid, steryl ferulates (SFs) can be analyzed from oils and total lipid extracts using UV-spectrophotometric methods. These methods are robust and easy to perform, and provide a good estimate of the total content of SFs in a sample. The absorption maximum varies between different solvents, being 314 nm, 327 nm, and 328 nm in heptane, isopropanol, and methanol, respectively (Evershed et al., 1988; Seetharamaiah and Prabhakar, 1989; Bucci et al., 2003). Further, the specific extinction coefficients for quantification of SFs are 358.9 $M^{-1} cm^{-1}$ in hexane (314 nm, 25 °C) and 19,500 $L mol^{-1} cm^{-1}$ (328 nm) (Evershed et al., 1988; Seetharamaiah and Prabhakar, 1989).

The oil matrix, i.e. other components present in the oil samples, may naturally interfere with the analysis and quantification. To overcome this, Bucci et al. (2003) suggested using second derivatives or a multicomponent analysis instead of a fixed wavelength. With these it is possible to improve the accuracy of the method, especially with oils with a relatively low content of steryl ferulates.

Another approach for the direct measurement of SFs from total lipid extracts was presented by Lilitchan et al. (2008), who developed a method applying partial extraction for the analysis of SFs and total lipids from rice bran. In this method an adsorption coefficient (K_d) is

determined with several different ratios of solid sample and extraction solvent, and it is defined as the ratio of the solute concentration in the liquid phase to that in the solid sample at equilibrium. Once this coefficient is defined, one can use a simple extraction and a mathematical formula applying the K_d to determine the content of SFs in the sample. The benefit of this method, like the other spectrometric methods when compared to the chromatographic analyses, is that it is rapid to perform, requires less organic solvents, and no special extraction apparatus. The original determination of the K_d value is tedious and very time-consuming to perform, but after the work is done, the analysis works well for rapid determination of the SF content and enables the analysis of a large number of samples.

All in all, the spectrophotometric methods for the measurement of SFs in various samples are a rapid and simple way to get a value for the total SFs. However, they do not provide any information on the sterol composition (molecular species of different sterols) found in a sample, which may be of biological significance. The spectrophotometric detemination works best on oil samples, where the extraction of SFs and the challenges caused by the selection of an extraction solvent are omitted "Extraction".

10.5   Sample Preparations for Chromatography Analysis Methods

There are three main approaches for the chromatographic analyses of phytosterols that may be applied, depending on the level and kind of details of information one wishes to obtain. The method can be chosen to provide information on (1) the total amount of phytosterols and steryl conjugates, (2) the chemical species of phytosterols as various steryl conjugates (including content), or (3) the total phytosterol content without information about the conjugation (for a scheme about the possibilities of chromatographic analyses, see Figure 10.4). Depending on the chosen approach, one then needs to take into account the specific needs of the sample preparation. Further, if information is wanted on, for example, only one group of conjugates, the sample preparation methods may be simplified. Whichever method is applied, a representative and homogeneous sample is naturally a prerequisite for a successful analysis to obtain a reliable result.
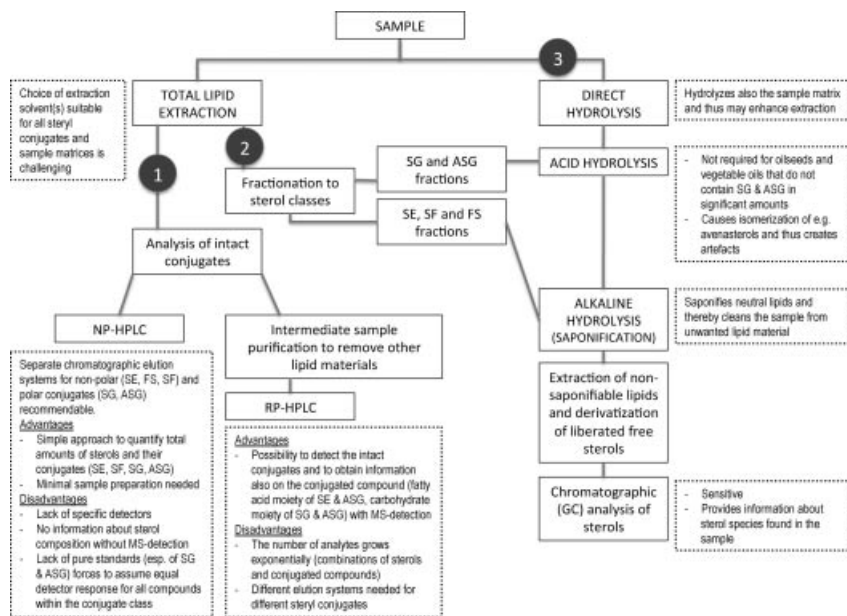
Figure 10.4 Scheme of the three main approaches for the analyses of sterols and steryl conjugates using chromatographic methods.

10.5.1 Extraction

Extraction of the sterols and steryl conjugates is one of the most critical points in the analytical procedure, as insufficient extraction cannot be compensated in later stages of the analysis. In the case of total sterol analysis after direct hydrolysis (Figure 10.4, Scheme 3), a single solvent can be applied as all the phytosterols have been released from their conjugates and are extracted as free sterols. In the procedures applying total lipid extraction of intact conjugates, the choice of the extraction solvent or mixture of solvents is crucial and deserves due consideration. The solvent selection is made complicated by the fact that steryl conjugates have very different polarities, and may be tightly bound in the surrounding plant matrix. For this reason, heating and/or shaking are commonly applied in the extraction procedure (Goad and Akihisa, 1997). Most commonly Soxhlet extraction is used to extract total lipids with selected solvent(s) from the solid sample material. In recent studies accelerated solvent extraction (ASE), which utilizes increased pressure in addition to an elevated temperature to enhance the extraction, has also been applied with success to sterol analyses (Moreau et al., 2003b;

Shen and Shao, 2005). The yield of analytes obtained with ASE may still be somewhat lower than the use of traditional Soxhlet extraction, but ASE is still a promising alternative to Soxhlet as it is faster and uses less solvent, especially with larger samples (Shen and Shao, 2005).

Extraction of free phytosterols and steryl conjugates
In principle, sterols are extracted with common organic solvents like hexane, heptane, methanol, and chloroform, but the more polar conjugates (SFs, SGs, and ASGs) require more polar extraction solvents to be quantitatively extracted from plant sample materials. Therefore, if only selected class(s) of steryl conjugates with similar polarity are analyzed, one solvent extraction can be sufficient. However, for a total lipid extraction for a later fractionation to the individual classes of phytosterol conjugates, one must utilize combinations of extraction solvents to ensure maximum recovery.

In the past, the Bligh–Dyer method using a mixture of chloroform and methanol for extraction has been applied in several studies. However, taking the current recommendations to reduce the use of chlorinated solvents due to environmental and health reasons, an alternative extraction solvent systems should be preferred. Furthermore, despite being one of the most effective extraction solvent mixtures for glyceridic lipids, chloroform–methanol was shown to be among the least effective in extracting SEs from oats (Zhou et al., 1999).

Comparisons of different extraction solvents on various cereal grain samples reveal that the highest yield of phytosterols, and all their conjugates, are often not obtained under the same conditions as the highest yield of total lipids. For example, when comparing a range of solvents (hexane, dichloromethane, isopropanol, ethanol) at two different temperatures (40 °C and 100 °C), Moreau and coworkers (2003b) showed that the highest yield of total lipids from oat and corn was obtained with ethanol at 100 °C, whereas the levels of SEs, FSs, and SFs under these same conditions was the lowest. On the other hand, the yield of the most polar steryl conjugates, SGs and ASGs, was highest with hot alcohol extraction, and significantly lower with such nonpolar solvents as hexane.

To summarize, for a quantitative extraction of phytosterols and steryl conjugates from a plant matrix, a combination of extraction solvents with different polarities, preferably used consecutively in the order of increasing polarity, is highly recommended. However, it

is unfortunately not possible to give a universal recipe of a solvent combination and extraction conditions, as it needs to be optimized for each type of sample material separately.

Extraction of steryl ferulates

For the extraction of the antioxidative SFs for quantitative analysis, the common extraction solvents used in recent studies include, for example, dichloromethane/methanol (2:1) (Miller and Engel, 2006), methanol/ chloroform (4:1) (Ohtsubo et al., 2005), acetone (Nyström et al., 2007c; Nurmi et al., 2010), and hexane (Winkler-Moser et al., 2009). A recent solvent comparison by Kumar et al. (2009), aiming to obtain γ-oryzanol at higher scale to be further used in food applications, showed that the solubility of γ-oryzanol was high in ethyl methyl ketone, dichloro-methane, ethyl acetate, and acetone, but considerably lower in hexane and isopropanol. On the other hand, the study of Lilitchan et al. (2008) suggested that isopropanol was a better solvent for SFs than hexane, showing that not only the extraction solvent, but also other conditions for extraction, have a significant effect on the yield of extraction. Further, the different solvents were also shown to preferentially extract the different species of SFs (Kumar et al., 2009), which also adds a new challenge to the choice of the extraction solvent. For example, 24-methylenecycloartanyl ferulate, sitosteryl ferulate, and cycloartenyl ferulate were most effectively extracted by acetone, ethyl acetate, and ethyl methyl ketone, respectively.

Taken that the yield of SFs is dependent not only on the extraction solvent but also on the sample material, the scene gets even more complicated. Seitz (1989) demonstrated that for the SFs of corn the best extraction solvent was hexane, whereas the SFs of wheat were most effectively extracted by acetone, and that methanol and chloroform were relatively ineffective in extracting the SFs from either of these matrices. Therefore, to be certain of the efficacy of the extraction, it is important to compare different solvents and solvent combinations, and to evaluate their suitability on the sample matrix to be analyzed.

Further purification of the total lipid extract from neutral lipids like triacylglycerols is desirable to increase the separation performance and lifetime of reverse-phase HPLC columns. Several authors have utilized a procedure of base-acid wash for this purpose (Evershed et al., 1988; Seitz, 1989; Hakala et al., 2002), where one takes advantage of the phenolic nature of the steryl ferulates. Total lipid extract in acetone or

methanol is mixed with sodium hydroxide to increase the pH to deprotonate the phenolic hydroxyl group and increase the water solubility of SFs. Neutral lipids are removed from the mixture with a nonpolar solvent (e.g. petroleum ether, hexane, heptane). After this the alkaline aqueous phase is acidified using hydrochloric acid, and the SFs are extracted with diethyl ether, heptane, or hexane, and can be further analyzed with RP-HPLC.

10.5.2   *Fractionation of Intact Sterol Conjugates*

In addition to knowing the total phytosterol content in a sample, it is often desirable to obtain detailed information about the proportions of different phytosterol conjugates, as well as information about the sterol composition and nature of the conjugated moieties (fatty acid, sugar, etc.). For this reason it is often necessary to fractionate the sample containing all intact phytosterols and their conjugates into different classes and analyze them individually (Figure 10.4, Scheme 2). Another option to analyze all steryl conjugates separately is to analyze intact molecules by RP-HPLC, but the number of analytes is so high (at least theoretically with all the possible combinations of sterols and conjugated carbohydrates, fatty acids, etc.) that the amount of data produced is no longer manageable.

The fractionation of phytosterol conjugates can be easily achieved by solid-phase extraction (SPE), which has replaced preparative thin-layer chromatography (TLC) and other column chromatography methods in sample fractionation. SPE cartridges are readily available commercially with a range of different sizes and solid phases. However, none of the methods reported in the literature is able to fully separate all the five sterol conjugate groups (FSs, SEs, SFs, SGs, and ASGs). Using silica SPE cartridges, it is possible to fractionate a total lipid extract into SEs, FSs + SFs, and SGs + ASGs by elution with heptane/diethyl ether 90:10, heptane/diethyl ether 50:50, and acetone, respectively (Nyström et al., 2007c). These fractions can then be hydrolyzed and analyzed individually to obtain the information about the sterol composition found as different conjugates. We have recently developed a method for Diol-SPE columns that separate all the other groups of steryl conjugates apart from free sterols (FSs) and steryl ferulates (SFs) (Nyström et al., unpublished data). However, further studies are needed to either further optimize

the elution for the separation of FSs and SFs, or introduce another step of fractionation.

10.5.3 Hydrolysis of Steryl Conjugates

Hydrolysis of the sample at different stages of the analytical procedure serves multiple functions. When direct hydrolysis of the sample is applied (Figure 10.4, Scheme 3), simultaneous hydrolysis of the sample matrix (enhancing the efficacy of later extraction phases) and release of the sterols from their conjugates are obtained. For the glycosylated steryl conjugates (SGs and ASGs), acid hydrolysis is required to hydrolyze the glycosidic bond. This can, however, be omitted in samples where no glycosylated sterols are found or are found at very low levels (e.g. oilseeds, vegetable oils). Alkaline hydrolysis (saponification), on the other hand, can be recommended for all samples, as in addition to hydrolyzing the steryl esters (SEs, SFs), it will saponify triacylglycerols and other saponifiable lipids, which can then be partitioned to the water phase in a normal liquid–liquid extraction between water and an organic solvent. The nonsaponifiable lipids, including the sterols, will be found in the solvent phase, and are thus effectively purified from saponifiable lipid material that could interfere with the chromatographic separations at a later stage.

Acid hydrolysis is effectively performed using hydrochloric acid (6 M) and heating at 85 °C, followed by dilution with water and extraction of sterols with a mixture of heptane/diethyl ether (1:1). Saponification (alkaline hydrolysis) of the lipids is commonly obtained by hydrolyzing the samples in alkaline alcoholic solutions (treatment with potassium hydroxide under reflux for 30 min to 3 h) (Goad and Akihisa, 1997; Nyström et al., 2009). For a more detailed example of hydrolysis procedures, see Nyström et al. (2009).

When applying acid hydrolysis, one must be aware of the possible isomerization and destruction of some of the less stable sterol species caused by the treatment with acid. The acid treatment is known to cause, for example, the isomerization of Δ^5-avenasterol to stigmastadienol (Kamal-Eldin et al., 1998), which will alter the sterol composition of the sample (artifacts). Furthermore, when acid hydrolysis is performed on an extract of sterols (not direct hydrolysis, where solid material is also present), the treatment must be sensitive enough not to destroy the labile sterols (weaker acid concentration, less heating, shorter reaction time),

or one can add a protective matrix that does not contain phytosterols into the sample (Nyström et al., 2007c).

One possibility to avoid the challenges caused by the acid treatment in liberating the sterols from their glycosides would be to replace the acid hydrolysis with enzymatic hydrolysis. This approach has been applied by Kesselmeier et al. (1985), who used a crude β-glucanase product to hydrolyze steryl glycosides. However, more recent studies using purified enzyme preparations have not been able to reproduce the enzymatic hydrolysis of glycosylated sterols at a rate that would be sufficient for an analytical procedure. To have an alternative to the acid treatment for the release of glycosylated sterols would, however, be most welcome. With the current biotechnological tools, tailoring an enzyme with high specificity toward hydrolyzing the glycosidic bond in steryl glycosides should be possible and deserves more research input.

10.6   Chromatography Analysis Methods

10.6.1   *Standards and Quantification in Chromatographic Analyses*

High purity standards of the phytosterols and different steryl conjugates are essential to obtain the highest possible quality in analytical results (especially quantification). Standards for the main individual sterols (sitosterol, sitostanol, stigmasterol) as free sterols are readily available in analytical purities at reasonable prices from different chemical suppliers. Pure standards of the steryl conjugates, on the other hand, are currently much more difficult to obtain. Phytosterol esters of fatty acids are not abundantly available commercially, but one can, in certain conditions, use cholesteryl fatty acid esters as standards in the analysis. Further, the less common conjugates of single sterols are perhaps even more difficult to find. Again the mixture of rice steryl ferulates, γ-oryzanol, can be purchased at large quantities in high purity, but of single steryl ferulate species only one compound – cycloartenyl ferulate – is available and apparently only from one supplier in the world. Furthermore, steryl glycosides and acylated steryl glycosides are also available from only one supplier, but only as mixtures of several sterols. These mixed standards can be used for calibrating the methods analyzing total contents of the corresponding steryl conjugates with

normal phase-HPLC methods "High-Performance Liquid Chromatographic (HPLC) Methods", but not so well on reverse-phase methods, where the standard is separated into several peaks. In addition to making optimal quantification difficult, the lack of standards makes comparative studies on the chemical and biochemical properties of different molecular species of sterols very difficult, and often the only way to get pure standard compounds is to synthesize them.

The gas chromatographic methods for the analysis of free sterols most often employ quantification with an internal standard. Various nonphytosterols, or compounds that are chemically similar to phytosterols, can be utilized as internal standards, the most common of which are 5α-cholestane and dihydrocholesterol. The major shortcoming of 5α-cholestane is the lack of a hydroxyl group in carbon C-3. As this makes its chemical behavior different from the plant sterols, it does not quantitatively follow the sample through sample preparation steps (Goad and Akihisa, 1997). This means that 5α-cholestane can only be added to the sample immediately before gas chromatographic analysis and serves only the purpose of quantification. Dihydrocholesterol, on the other hand, is chemically similar especially with the other desmethylsterols and can be incorporated into the sample in the first stages of the analytical procedure. In this way, its losses in the intermediate sample preparation steps are in the same proportion as those of the phytosterols, and so these losses are compensated in the calculations of quantification. However, being structurally very similar to cholesterol, dihydrocholesterol might not be separated to the baseline in all chromatographic systems, which may make quantification more difficult (Goad and Akihisa, 1997). The levels of cholesterol in plain plant materials are often so small that this is not a true problem, but might cause difficulties in the analysis and quantification of phytosterols from mixed samples of animal and plant tissues. The detector response of flame ionization detection – still the most commonly used detection method for free sterols – is virtually equal to the different phytosterols, cholesterol and dihydrocholesterol, and so they can all be reliably quantified using same calibration curves. This is a great advantage especially in the quantification of the less common sterols, for which pure standards for constructing standard calibration curves are not available.

Steryl conjugates in liquid chromatographic analyses are most often quantified using external standard methods, which, depending on the

detection system used, have some limitations. Further, as stated above, the lack of pure, single compound standards results in some compromises in the quantification procedures. For steryl ferulates the quantification can be performed using cycloartenyl ferulate (the only single compound standard commercially available) in both RP- and NP-HPLC methods and with γ-oryzanol in NP-HPLC systems. As UV detection, which detects the ferulic acid moiety, is most often used, the response of the detector is equal for all SFs regardless of the sterol species. Other conjugates, namely the fatty acid esters and glycosylated sterols may be somewhat more difficult to quantify using the external standard methods, due to the differences in the detector responses of the different conjugated compounds with universal detectors. For the steryl fatty acid esters, one can use the fatty acid esters of cholesterol, of which a substantial range of different compounds is available commercially. The situation of the glycosylated sterols, on the other hand, is again more complex, as single compound standards are not available and there is some variation in the nature of the conjugated compounds (number of carbohydrate units, type of fatty acid, etc.), which will affect the detector response based solely on the mass of a compound. Therefore, some compromises and assumptions may have to be necessary. With universal detectors it is possible to quantify the overall mass of the compound in question and then, based on some assumptions on the chemistry of the conjugated compound(s), calculate the mass contributed by the sterol moiety (which is perhaps more relevant from a nutritional point of view than the overall mass of the compound, including also the carbohydrate and/or fatty acid). We have used this approach of multiplying factors to determine the sterol content of SFs, SGs, and ASGs after ELSD detection (Nyström et al., 2007b).

10.6.2   *Gas Chromatographic Methods*

The methods used for the analysis of total phytosterol content are often based on gas chromatographic (GC) analysis. Even if gas chromatography can be applied to the intact conjugates – for example, for steryl esters (Evershed and Goad, 1987), steryl ferulates (Evershed et al., 1988), steryl glycosides and acylated steryl glycosides (Gutierrez and del Rio, 2001) – it is by far more common to use analytical procedures where the analysis is done with free sterols. In these procedures all sterols are hydrolyzed from their conjugates and the liberated free sterols are

derivatized prior to GC analysis. The ester bonds in SEs and SFs require alkaline hydrolysis (saponification) and the glycosidic bond of SGs and ASGs require acid "Hydrolysis of Steryl Conjugates". Further, once the phytosterol conjugates are all hydrolyzed to free sterols, they have similar polarity and can be extracted with a single solvent "Extraction". In order to analyze the sterol composition of different conjugates (SEs, SFs, SGs, and ASGs) with GC methods, one needs to fractionate a total lipid extract to different classes of sterol conjugates "Fractionation of Intact Sterol Conjugates", and hydrolyze each group individually prior to GC analysis.

Gas chromatographic analysis of the free sterols is commonly performed as trimethylsilyl (TMS) ethers or as acetates, though relatively good separation has also been obtained with nonderivatized sterols (Moreau et al., 2002). Derivatization often improves the resolution of the method, and so the complex mixtures with a large number of phytosterols can be better separated when derivatized for GC analysis. Further, the thermolabile sterols are stabilized by derivatization, and are thus more reliably included in the analysis if the sample is derivatized. Of the two different derivative types (TMS ethers and acetates), the former is better for quantification and mass spectrometric analysis (Abidi, 2001). TMS derivatization can be simply achieved by dissolving the nonsaponifiables (after saponification, i.e. alkaline hydrolysis) in pyridine and mixed together with a TMS reagent (*bis*-(trimethylsilyl)-trifluoroacetamide, which is readily available from various chemical suppliers). The sample is then heated (60 °C, 15 min) or left at room temperature overnight. After derivatization the sample is diluted and analyzed with gas chromatography (Abidi, 2001).

The columns used for the GC separation of phytosterols are currently almost exclusively capillary columns with 0.1–0.3 mm internal diameter, and fused-silica capillary columns with chemically bonded stationary phases are commonly used (Abidi, 2001). The best separation of structurally very similar sterols, such as sitosterol and its saturated counterpart sitostanol, is obtained with slightly polar stationary phases like 5% diphenyl–95% dimethylpolysiloxane, and they are currently the most used columns for the separation of phytosterols (Lagarda et al., 2006). For detailed lists of different columns used in sterol analysis, see the papers by Abidi (2001) and Lagarda (2006).

The quantification of sterols after GC analysis is commonly (almost exclusively) performed with flame-ionization detectors (FID), and mass

spectrometric detectors are used to confirm peak identities. Full iden-
tification of phytosterols in GC analysis may be achieved by combining
the relative retention times and mass spectrometric identification.
Literature values for relative retention times and mass spectra (main
fragment ions) can be found for most common sterols online at http://
webbook.nist.gov, and also for the less common sterols in various
publications (e.g. Rahier and Benveniste, 1989; Kamal-Eldin et al.,
1992; Goad and Akihisa, 1997).

10.6.3   High-Performance Liquid Chromatographic (HPLC) Methods

Normal-phase HPLC methods for sterol conjugates
The total amount of free phytosterols and their conjugates can be
analyzed relatively easily with normal-phase HPLC methods, as a
diluted lipid extract can be directly injected into a normal-phase liquid
chromatographic system without elaborate purification and derivati-
zation steps. However, these methods do not provide information on
the sterol composition found in each group of conjugates; nor do they
quantify the absolute amount of phytosterols (excluding the conju-
gated compounds) of the samples that would, for example, be of
interest when data is used for the calculations of phytosterol intakes.
Nevertheless, they can be used to analyze and compare the contents of
steryl conjugates in different plant materials. Moreau and coworkers
have reported good separation with a Diol column and elution with
hexane, isopropanol, and acetic acid, but with different elution gra-
dients for neutral lipids including FSs, SEs, and SFs (Moreau et al.,
1996) and polar lipids including SGs and ASGs (Moreau
et al., 2003b). In these chromatographic systems the run times are
moderate for sterol conjugates. It is also possible to separate all the
conjugates in a single run, but this requires a relatively complex
gradient elution system and the run time is quite long (60 min)
(Nyström et al., 2007b).
   The detection of phytosterols after liquid chromatographic separa-
tion can be performed with several different detection methods, such as
ultraviolet (UV), refractive index (RI), and evaporative light-scattering
(ELSD). However, since they all have some limitations, none of them is
necessarily superior to the other. The UV detection of sterols is usually
performed at a wavelength between 200 and 210 nm, at which several

organic compounds absorb UV-light and may therefore interfere with sterol quantification. Further, absorption of the phytosterol molecules is highly dependent on the number and position of the double bonds of the molecule, and so the response of the detector is not universal and would require separate calibration curves for all sterols. An exception to UV-activity are SFs, which have a characteristic UV spectrum and absorption maxima resulting from the ferulic acid moiety.

The most common method used for the detection of steryl conjugates is light scattering, which has a good sensitivity to detect phytosterols. However, as the detector response is unequal between different conjugates and linear only within a relatively narrow range, one must either aim to concentrate or dilute the sample to fit this range, or use polynomial functions for the standard curves (Moreau et al., 1996). However, unlike RI-detectors, ELSD-detectors can be used with gradient elution systems, and hence can be applied for a wider range of analyte polarities within one run.

HPLC methods for steryl ferulates
Steryl ferulates can be analyzed using normal-phase HPLC methods, in which SFs generally elute as one peak; or by reverse-phase HPLC methods, where the different sterol species as ferulates are separated, and so the number of peaks depends on the number of different sterols as ferulates in the sample. Further separation can be obtained between *cis*- and *trans*-ferulates, of which the *trans*- form is the naturally occurring and more abundant one. However, UV-irradiation can cause isomerization of *trans*-ferulates to *cis*-ferulates. These forms can further be identified by their UV-spectra, which for *cis*-ferulates involve a more flat shape (Figure 10.5). Therefore, if one is interested to know the proportions of *cis*- and *trans*- SFs in the original sample, the sample should be protected from light during preparation.

RP-HPLC　The most commonly used methods for the analysis of SFs are reverse-phase liquid chromatography with UV-detection, which allow the separation of SFs with different sterol moieties. The number of components separated is naturally dependent on the type of column used, but most reverse-phase columns provide a full separation of at least five to six components of γ-oryzanol. C18-columns are most abundantly applied for the separation of SFs, but other reverse-phase
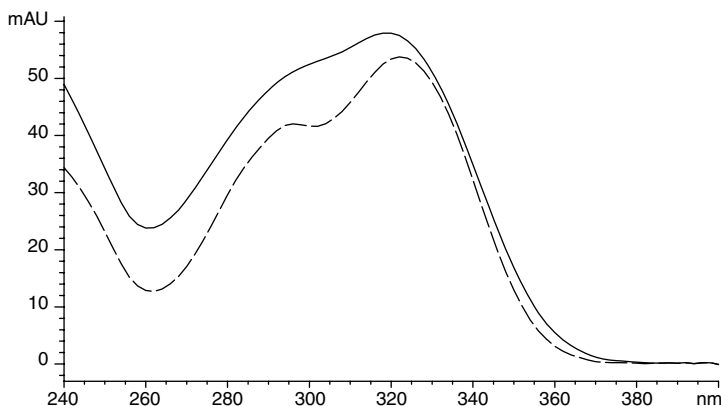
Figure 10.5 UV-spectra of the *cis*-steryl ferulates (upper line) and *trans*-steryl ferulates (lower line).

materials (C8 and C30) have also shown a good separation of γ-oryzanol components. A selection of columns and mobile phases used for the separation of SFs is presented in Table 10.3.

Cis- and *trans*- isomers of SFs are often not separated in reverse-phase systems, but it is sometimes possible to partially separate or see the *cis*-form as a shoulder in the peak eluting after the *trans*-ferulate. The relative retention times of the *cis*-ferulates, obtained with a C18-column and methanol as eluent, have been reported to be about 0.1–0.3 higher than those for the *trans*-ferulates of phytosterols (Akihisa et al., 2000).

NP-HPLC   Normal-phase liquid chromatographic methods applying Diol-columns or common silica columns are well suited for the analysis of the total steryl ferulate content. They require very little sample preparation, as total lipid extracts can frequently be directly injected into the column without purification or fractionation. Run times for SFs are also relatively short, and a good separation from other lipid components can be obtained in less than 10 min in traditional HPLC systems. Depending on the column type and the sample, SFs elute as one or two peaks. Two peaks are obtained from the separation of SFs, which have ferulic acid both in *cis*- and *trans*- configuration (Nyström et al., 2008). The relative retention time (obtained with a silica column and hexane/ethyl acetate 97:3 as eluent) of the *cis*- form is about 0.5 smaller than that of steryl *trans*-ferulates (Akihisa et al., 2000).

Table 10.3 Columns and eluent systems used for the analysis of steryl ferulates in selected studies.

Column type	Column dimensions (mm)	Particle size (μm)	Eluent mixture	Elution type	Temperature (°C)	Detection	Reference
Reverse Phase (RP)-methods							
C18	200 × 2.1	5	ACN-MeOH-IPA-H_2O (45:45:5:5 and 50:45:5:0)			UV 325 nm	Rogers et al., 1993
C18 Microsorb-MV	250 × 4.6		MeOH-ACN-DCM-AcCOOH (50:44:3:3)	isocratic 1.4 ml/min		UV 330 nm	Xu and Godber, 1999
C18 (ODS-2, Waters Spherisorb)	250 × 4.6	5	MeOH-H_2O-AcCOOH (97:2:1)	Isocratic 1.5 ml/min	50	UV 325 nm	Hakala et al., 2002
C8 (Eclipse XDB-C8, Agilent Technologies)	150 × 4.6	5	ACN-H_2O	gradient		UV 320 nm, LC-MS/MS API-ES	Fang et al., 2003
C18 (ERC-ODS-2352)	250 × 10		ACN-BuOH-AcCOOH (47:2:1)	isocratic	25	RI	Iwatsuki et al., 2003

(Continued)

Table 10.3 (*Continued*)

Column type	Column dimensions (mm)	Particle size (μm)	Eluent mixture	Elution type	Temperature (°C)	Detection	Reference
C30 (Prontosil 200-3-C30)	250 × 2	3	MeOH-*tert*-butyl methyl ether (80:20)	isocratic 0.2 ml/min	25	UV 325 nm	Stöggl et al., 2005
Inertsil ODS	250 × 4.6	5	ACN-MeOH 25:75	isocratic 1.5 ml/min		UV 325 nm	Butsat and Siriamornpun, 2010
Normal Phase (NP)-methods							
Si (Nova-Pak, Millipore Waters)	150 × 3.9	4	2.5% ethyl acetate in isooctane	isocratic	Ambient	UV 295 nm	Diack and Saska, 1994
Diol (LiChrosorb)	100 × 3.0	5	hexane-isopropanol-acetic acid	gradient	Ambient	ELSD	Moreau et al., 1996
Diol (LiChrosorb)	150 × 3.0	5	heptane-isopropanol-acetic acid (99:1:0.1)	isocratic	Ambient	UV 315 nm	Nyström et al., 2008

AcCOOH = acetic acid, ACN = acetonitrile, DCM = dichloromethane, MeOH = methanol, IPA = isopropanol, API-ES = atmospheric pressure interface electrospray

Mass spectrometric and NMR analysis of steryl ferulates

Mass spectrometric detection has been used for the identification of SFs in a number of studies. Modern instruments have enabled the identification of ever increasing number of different SFs in a γ-oryzanol mixture from rice bran that also contained, for example, hydroxylated sterols and caffeate esters (Fang et al., 2003). Reports have recently been presented on the mass spectrometric analysis of SFs after liquid chromatographic separation with atmospheric pressure chemical ionization (APCI-MS) (Hakala et al., 2002; Stöggl et al., 2005; Nurmi et al., 2010) and electrospray ionization (ESI-MS) (Fang et al., 2003). Mass spectrometric detection is an invaluable tool for the identification of the compounds that might not be fully separated by the chromatographic systems used. However, it has not yet been systematically used for the quantification of SFs. NMR has also naturally been used for SFs for a final identification of the compounds after separation and purification of different fractions. Data and references on NMR spectral features can be found from, for example, Yoshida et al. (1989), Yasukawa et al. (1998), and Iwatsuki et al. (2003).

10.7 Summary and Comparison of Different Chromatography Methods

The choice of chromatographic system for the analysis of phytosterols and steryl conjugates is very much dependent on the type of information desired. If merely the total phytosterol content is needed, hydrolysis and GC analysis is the method of choice, with its high sensitivity and fewer problems normally caused by the lack of suitable standards, etc. However, as the conjugation may affect the biological activity of the sterol, and since conjugation is often a characteristic feature of the plant source, one may wish to apply a method that separates the conjugates and analyzes them individually. And again, depending on the level of details wanted from the data, one may choose either (i) fractionation, hydrolysis, and gas chromatographic analysis for the content and composition of different sterols as various conjugates, (ii) analysis of intact steryl conjugates with NP-HPLC systems to obtain the total amounts as different conjugates but no information on the sterol composition (without MS detection), or (iii) analysis of intact steryl conjugates with RP-HPLC methods that provide information on the

contents of the different conjugates and, depending on the detector used, on the composition of both sterols and the conjugated compounds. All in all, the method must be chosen to provide data that answers the research question and fits the resources and equipment available.

10.8   Existing Problems in Current Analysis Methods

The main shortcoming of the current analytical methods is the fact that none of them is streamlined to analyze all classes of phytosteryl conjugates separately in one analysis. The methods currently used focus on, and are highly optimized to analyze, one or two groups of the sterol classes but not all of the classes in the same analysis. This multiplies the number of samples to be analyzed and creates some overlaps in the data. On the other hand, analytical procedures for total sterol analysis (without information on the conjugation) are easily available.

Another limitation in the current analytical practices is that the vast majority of the studies focus on the nature of the sterols, but only a few on the characteristics on the conjugated compounds (fatty acids, carbohydrates, etc.). However, as these may have an effect on, for example, the rate of hydrolysis of these compounds in the gastrointestinal tract and other biochemical processes, the chemistry of the conjugated moieties should be analyzed in more detail.

The main problems with the current analytical methods, however, are still very fundamental issues in sample preparation and quantification. When the samples are hydrolyzed and analyzed as free sterols, the current methods for acid hydrolysis in particular cause changes in the sterol composition, and thus do not give a fully correct result on the sterol composition. Therefore, gentler methods for hydrolysis, especially using enzymes, should be developed. On the other hand, if sterols are analyzed as intact conjugates, their quantification should be made more accurate by pure single compound standards that enable reliable quantification of the sterols with different conjugated moieties. Finally, the current chromatographic methods could perhaps be further developed for a better separation of the minor compounds that may not have been detectable earlier, but can be detected bycontemporary detectors of a higher sensitivity. For this, the ultrahigh-performance liquid chromatographic (UPLC) applications seem to be a potential

tool that has until now remained unexplored in the analysis of phytosteryl conjugates.

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Chapter 11

Analysis Methods for Tocopherols and Tocotrienols

Robert A. Moreau and Anna-Maija Lampi

Abstract

Tocopherols and tocotrienols, sometimes called tocochromanols or tocols, are also collectively termed "vitamin E." Vitamins A, D, E, and K are referred to as fat-soluble vitamins. Since the discovery of vitamin E in 1922, many methods have been developed for the analysis of tocopherols and tocotrienols. Early methods were based on derivitization and colorimetric analyses. With the evolution of various chromatographic methods – gas liquid chromatography and then high-performance liquid chromatography with fluorescence – detection has been the most valuable technique for the quantitative analysis of tocopherols and tocotrienols. Although recent advancement in genomics and other "omics" fields have revolutionized many fields of biology and medicine, these methods have not yet been applied to the analysis of tocopherols and tocotrienols. It is likely that rapid metabolomics, or other "omics" methods based on mass spectrometry, will soon be developed for the analysis of tocopherols and tocotrienols.

Keywords: Tocopherols; tocotrienols; antioxidants; chromatography; vitamin E; analysis.

11.1 Introduction

An excellent review entitled "Chromatographic analysis of tocol-derived lipid antioxidants" was published about ten years ago (Abidi, 2000).

This previous review provides a comprehensive overview of the major chromatographic methods for the analysis of tocopherols and tocotrienols developed until about 2000. The current chapter is not a comprehensive overview, but instead is a summary of the major advances in the field that have occurred since 2000. Two other valuable resources that review methods for the analysis of tocopherols and tocotrienols are the websites AOCS Lipid Library (2011) and Cyberlipid (2011).

Tocopherols and tocotrienols, sometimes called tocochromanols or tocols, are also collectively termed vitamin E, which along with vitamins A, D, and K, are referred to as fat-soluble vitamins. Evans and Bishop (1922) conducted rat feeding studies and provided the first experimental evidence for the occurrence of vitamin E, the fifth vitamin, after the discovery of vitamin A in cod liver oil in 1913, vitamin B1 in rice bran in 1910, vitamin C in citrus fruits in 1920, and vitamin D in cod liver oil in 1920 (Wikipedia, 2011). Although Evans and Bishop provided all known nutrients to the rats, they were not fertile. This condition could be overcome, however, by adding wheat germ to the diet. In 1936 an active substance that had vitamin activity was isolated from wheat germ and the formula $C_{29}H_{50}O_2$ was determined. Evans also found that the compound appeared to be an alcohol, and he concluded that one of the oxygen atoms was part of an OH (hydroxyl) group. Evans named the new compound *tocopherol* from the Greek word meaning "to bear young" with the addition of the -ol as an alcohol. The structure was determined in 1938.

Tocotrienols were first reported by Pennock and Whittle in 1964, and published by Dunphy et al. (1965), where they were isolated from rubber. The first biological activity of tocotrienols was reported by Qureshi et al. (1986) when they observed that administering tocotrienols to rats lowered serum cholesterol. Tocotrienols were named by analogy to tocopherols, but with the second half of the name changed to denote the trienes, the three double bonds in their isoprene side chains. During the 1990s several anticancer activities were reported for tocopherols and tocotrienols.

Tocopherols and tocotrienols are considered to be lipids. Lipids are a broad group of natural hydrophobic compounds which includes fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, phospholipids, and others. In recent years the scientific disciplines of genomics and proteomics were developed to attempt to provide systems to quantitatively catalog all of the DNA and protein sequences, respectively, for species and tissues.

Table 11.1 Relationship of tocopherols and tochotrienols to other lipids. Eight categories of lipids according to the Lipid-MAPS classification (Fahy et al., 2005).

Category	Abbrev	Current number of lipids in this Lipid-MAPS category	Examples
Prenols	PR	1177	Tocopherols, tocotrienols, retinol
Glycerolipids	GL	3036	Common fats and oils, including triacylglycerols, diacylglycerols, and monoacylglyerols
Fatty Acids	FA	3562	Oleic acid
Glycerophospholipids	GP	1960	Phosphatidylcholine
Sphingolipids	SP	3909	Sphingomyelin
Sterol Lipids	ST	2134	Cholesterol
Saccharolipids	SL	13	UDP-glucose
Polyketides	PK	6709	Aflatoxin B_1

More recently, a third "omics" field, lipidomics, was developed. Lipidomics was developed to attempt to provide a system in which to quantitatively profile all of the lipid molecules in a given species or in a tissue within that species (Han, 2011; Welti, 2011). Not surprisingly, a new nomenclature system has been developed to help to define and catalog the various lipids in the lipidome of each species. In this system, tocopherols and tocotrienols are part of the category called "prenols" (Table 11.1). Currently, the main database for lipidomics information in the USA is the LIPID-MAPS (2011) and its Prenol category contains the four known tocopherols (α, β, γ, δ), four known tocotrienols (α, β, γ, δ), and a total of 1177 Prenols. Each of the eight individual tocopherols and tocotrienols are sometimes referred to as vitamers.

11.2 Chemical Structures, Sources, and Levels found in Natural Sources

Tocopherols and tocotrienols are sometimes referred to as tocochromanols, or simply abbreviated to tocols. The structures of the four
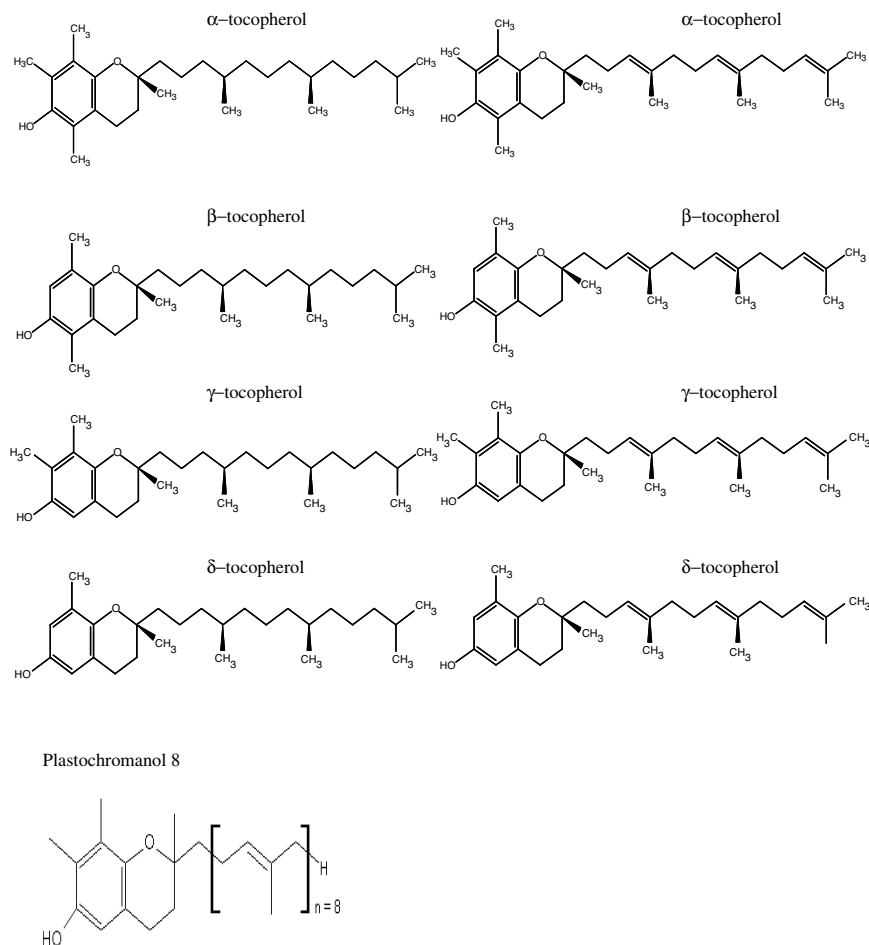
Figure 11.1 Structures of common tocols.

common tocopherols and four common tocotrienols are shown in
Figure 11.1 and the corresponding common name, abbreviation,
Lipid-MAPS number, CAS number, chemical formula, and molecular
mass are presented in Table 11.2. All eight possess a chromanol ring,
with a hydroxyl group that can donate a hydrogen atom to reduce free
radicals, and a hydrophobic side chain which allows for penetration into
biological membranes. Tocopherols and tocotrienols occur in alpha,
beta, gamma and delta forms, determined by the number and position of
methyl groups on the chromanol ring. The three double bonds give

Table 11.2 Major tocopherols and tocotrienols.

Common Name	Abbrev	Lipid-MAPS number[a]	CAS Number	Formula	Mass
α-tocopherol	α-T	LMPR0202001	59-02-9	$C_{29}H_{50}O_2$	430.70
β-tocopherol	β-T	LMPR0202059	148-03-8	$C_{28}H_{48}O_2$	416.68
γ-tocopherol	γ-T	LMPR0202060	54-28-4	$C_{28}H_{48}O_2$	416.68
δ-tocopherol	δ-T	LMPR0202061	119-13-1	$C_{27}H_{46}O_2$	402.65
α-tocotrienol	α-T3	LMPR0202054	1721-51-3	$C_{29}H_{44}O_2$	424.66
β-tocotrienol	β-T3	LMPR02020055	490-23-3	$C_{28}H_{42}O_2$	410.63
γ-tocotrienol	γ-T3	LMPR0202057	14101-61-2	$C_{28}H_{42}O_2$	410.63
δ-tocotrienol	δ-T3	LMPR0202056	25612-59-3	$C_{27}H_{40}O_2$	396.65
Plastochromanol-8	PC-8		4382-43-8	$C_{53}H_{82}O_2$	

[a] From Lipid-MAPS, 2011.

tocotrienols only a single stereoisomeric carbon (and thus two possible isomers per structural formula, one of which occurs naturally), whereas tocopherols have three centers (and eight possible stereoisomers per structural formula, again, only one of which occurs naturally). The d-form is the natural form of tocopherols and tocotrienols. In addition to the eight common tocopherols and tocotrienols, several less common tocochromanols have been reported, including plastochromanol-8, desmethyltocotrienol, γ-tocopherol-9, α-tocomonenol, δ-tocomonenol, and sargochromanol A (Cyberlipid, 2011).

Plastochromanol-8, a homolog of γ-tocotrienol, was first identified in the leaves *of Hevea brassiliensis* by Whittle et al. (1965). It possesses a normal chromanol ring, but unlike common tocopherols and tocotrienols that have three isoprene (five carbon branched chains units) in their side chains, plastochromanol-8 has eight unsaturated isoprene units, so it could also be called "γ-toco-octaenol" (Figure 11.1 and Table 11.2). It has been reported to be present in canola/rapeseed corn, linseed oil, soybeans, *Arabidopsis*, and other plants (Olejnik et al., 1997; Goffman and Mollers, 2000; Schwartz et al., 2008; Mene-Saffrane et al., 2011). It is important to recognize the possible presence of planstochromanol-8 because it is sometimes incorrectly reported as either β-tocopherol or γ-tocotrienol.

Like many other lipids with free hydroxyl groups, tocopherols and tocotrienols can form natural esters with molecules that contain a carboxylic acid group (Panfili et al., 2003; Wang et al., 1998). Although

several reports have provided evidence for the existence of tocol esters via a measurable increase in tocols after alkaline hydrolysis (saponification), nothing is known about the structures of tocol esters. Fratianni et al. (2002) provided evidence that 4–5% of the total tocols in barley were released after saponification and therefore were likely esterified. Hirota et al. (2003) isolated a sterol ester fraction from soybean oil deodorizer distillate.

Manufacturers also commonly convert the phenol form of the vitamins (with a free hydroxyl group) to esters, using acetic or succinic acid. The fortification of foods and feeds with α-tocopherol esters is common because they are more stable and provide vitamin E activity when consumed.

Like other vitamins, tocopherols and tocotrienols are not synthesized in the human body and must be obtained from foods in the diet. Tocopherols and tocotrienols are synthesized in plants and other photosynthetic organisms, mainly in chloroplasts and in oil bodies of seeds (DellaPenna and Pogson, 2006; Hunter and Cahoon, 2007). It is believed that, in plants, their main function is to serve as antioxidants, including protecting lipids and other membrane components by physically quenching and reacting chemically with singlet oxygen (Munne-Bosch and Alegre, 2002). α-Tocopherol has also been shown to increase the rigidity of biological membranes and provide photoprotection for chloroplasts (Munne-Bosch and Alegre, 2002).

γ-Tocopherol is the major form of vitamin E (70%) in the typical US diet but not in the typical European diet (Jiang et al., 2001). In Europe, the γ-tocopherol intake is about half of that of α-tocopherol and is about 25–35% of the US intake (Wagner et al., 2004). As mentioned previously, vitamin E and tocopherols were discovered in wheat germ oil and that is still the richest common source of tocopherols (USDA, 2011). Green leafy vegetables are also a good source of tocopherols, as are most other common vegetable oils (USDA, 2011). The most common commercial source of tocopherols for supplements is in the deodorizer distillate fraction from the refining of vegetable oils, especially soybean oil since international production of soybean oil exceeds all other edible oils and is nearly matched by palm oil. Tocopherols comprise 16.5% of the soybean oil deodorizer distillate (also known as the soybean oil deodorizer distillate tocopherol concentrate or SODDTC) and phytosterols comprise 13.1% (Shimada et al, 2000). Verleyan et al. (2001) reported that γ-tocopherol was the major

tocopherol in the deodorizer distillates of soybean, corn, and rapeseed oils, and α-tocopherol dominated sunflower oil. The deodorizer distillate of soybean also contained a significant amount of δ-tocopherol.

Current commercial sources of tocotrienols include rice, palm, and annatto (Sen et al., 2007a). Other sources of tocotrienols include coconut oil, cocoa butter, barley, and wheat germ. Posada et al. (2007) reported on a molecular distillation process to extract tocotrienols from the distillate (from physical refining) obtained during the refining of palm oil. The levels of tocols and phytosterols were much lower in the palm distillate than in the seed oil distillate described above, because, with seed oils, the fatty acids were removed by alkaline refining before deodorization. However, in palm oil refining, the physical refining step removes fatty acids, tocols, and phytosterols. The palm oil distillate contained 90.0% free fatty acids, 0.12% α-tocopherol, 0.12% α-tocotrienol, 0.19% γ-tocotrienol, 0.09% δ-tocotrienol, and 0.24% phytosterols.

It should also be noted that because α-tocopherol often has a higher value than the other tocopherols, the total tocopherols are sometimes converted to α-tocopherol, using chemical or microbial processes (Chen et al., 2004). This practice has become more popular since 2000, for reasons noted in the next section.

11.3 Antioxidant and Biological Functions

Tocopherols and tocotrienols are often discussed as compounds with separate biological, vitamin, and antioxidant functions, although in a comprehensive review of Traber and Atkinson (2007) it was concluded that all of the biological activities of tocopherols and tocotrienols can be understood and derived from their antioxidant activity to protect polyunsaturated lipids from oxidation in membranes.

Until the year 2000, all tocopherols and tocotrienols were considered to have the same biological activity of α-tocopherol, i.e. vitamin E activity. α-Tocopherol was considered to have the highest activity followed by β- and γ-tocopherol with 50% and 10% of the activity of α-tocopherol. Nutrition data was often reported in α-tocopherol equivalents (α-TE) that were expressed as follows (McLaughlin and Weihrauch, 1979; Schakel et al., 1997): amounts of α-TE were calculated as mg of α-tocopherol, $0.5 \times$ mg of β-tocopherol, $0.1 \times$ mg

of γ-tocopherol, 0.01 × mg of δ-tocopherol, 0.3 × α-tocotrienol, and 0.05 × mg β-tocotrienol. In addition, the following formula was used to convert IU of vitamin E to α-tocopherol equivalents: 1 α-TE = × 1.49 IU vitamin E (Schakel et al., 1997; Eitenmiller and Lee, 2004). These relative values reflect the tocol retention in animals. Although all tocopherols and tocotrienols are believed to be similarly absorbed, the hepatic α-tocopherol transfer protein selectively incorporates α-tocopherol into nascent VLDL and further to other tissues. Other tocols that are discriminated during this process are most likely excreted *via* the bile (Kayden and Traber, 1993; Bramley et al., 2000; Institute of Medicine, 2000; Eitenmiller and Lee, 2004).

Beginning in 2000 in the USA, vitamin E activity was limited to those forms of α-tocopherol retained in human plasma, which is the naturally occurring *RRR*-α-tocopherol and synthetic forms of α-tocopherol with 2*R*-configuration (Institute of Medicine, 2000; Eitenmiller and Lee, 2004). In terms of vitamin E activity, the side chain has to be attached to the chromanol ring with the same stereochemistry. For this reason, α-tocopherol equivalents, which included vitamin E activities from α-, β-, γ-, and δ-tocopherols and α-, β-, and γ-tocotrienols, were dropped from the USDA Nutrient Database starting with SR16 (USDA, 2011). In 2000, the recommended dietary allowance (RDA) for vitamin E was set to 15 mg of 2*R*-α-tocopherol per day, and the estimated average requirement (EAR) set to 12 mg (Institute of Medicine, 2000). A continuing survey of food intake in the USA showed that only 8% of men and 2.4% of women met even the current EAR, indicating that there is a need to increase the intake of 2*R*-α-tocopherol (Maras et al., 2004). Even a diet rich in fruits and vegetables and low in fat probably contains less than 15 mg of 2*R*-α-tocopherol, and those foods rich in oil or fortified with α-tocopherol acetate are the best sources of vitamin E (Eitenmiller and Lee, 2004). It has been suggested that by consuming bread and other cereal products, and especially whole grains, it may be possible to meet the vitamin E intake recommendations without consuming excess oils and fats (Gao et al., 2004; US Department of Health and Human Services, 2005). Yet the current diet in the western world is considered to have enough 2*R*-α-tocopherol to avoid vitamin E deficiency symptoms, and that deficiency is mainly due to fat malabsorption syndromes or defects in α-tocopherol transfer protein (Kayden and Traber, 1993; Eitenmiller and Lee, 2004).

The main physiological effect of α-tocopherol is to avoid vitamin E deficiency symptoms, i.e. neurological abnormalities. Other vitamin E deficiency symptoms observed include spinocerebellar ataxia, skeleton myopathy, and pigmented retinopathy (Institute of Medicine, 2000). Being antioxidants, all tocols reduce oxidative stress and thereby delay the progression of a variety of degenerative diseases such as cardiovascular diseases and cancer. In addition, tocols have been shown to regulate cellular signaling, cell proliferation, and gene expression, and to act as antithrombotic agents (Sen et al., 2007b; Traber et al., 2008). A review of γ-tocopherol emphasized several biological activities – e.g. scavenging of reactive nitrogen species – that were specific for this tocol (Wagner et al., 2004). Tocotrienols are known to contribute to other biological activities, such as the prevention of neurodegeneration, obesity, and osteoporosis, being proapoptotic, and lowering serum cholesterol levels (Khanna et al., 2006; Sen et al., 2006; Nesaretnam et al., 2007; Constantinou et al., 2008). Furthermore, it has been stated that tocotrienols may reduce vitamin E deficiency symptoms (Sen et al., 2007).

Despite the large number of oxidation-related hypotheses on the protective role of vitamin E and epidemiological data supporting them, most clinical studies have failed to demonstrate any positive effects of supplementation with vitamin E. For example, a recent meta-analysis on randomized-controlled trials on vitamin E supplementation in prevention of stroke, was unable to show any statistically significant or clinically important benefits or harms regarding the prevention of any types of strokes (Bin et al., 2010). In another review on randomized, placebo-controlled trials on the effects of antioxidant supplements in the prevention of atherosclerosis, most of the studies did not illustrate protective effects of supplements (Katsiki and Manes, 2009). However, there is a good consensus on the protective role of lifelong optimal dietary vitamin E intake on the incidence of chronic disease (Traber et al., 2008). In the EU, the EFSA panel concluded that a cause-and-effect relationship exists between the dietary intake of vitamin E and the protection of DNA, proteins, and lipids from oxidative damage, but not with other health claims such as maintenance of the normal function of the immune system, normal bone, or normal teeth (EFSA, 2010).

The most important chemical property of tocopherols and tocotrienols is their antioxidant activity. Although the chemical reactions of tocols with oxidizing lipids are fairly well understood, comparisons of

different studies is difficult and even the concept "antioxidant activity" may be confusing. A recent review of Niki (2010) excellently discussed antioxidant capacity.

Tocols are primary antioxidants that scavenge lipid peroxyl radicals (ROO•) by donating hydrogen atoms, and tocopheroxyl radicals (TocO•) may even scavenge another peroxyl radical forming stable products:

$$ROO\bullet + TocOH \rightarrow ROOH + TocO\bullet$$

$$ROO\bullet + TocO\bullet \rightarrow stable\ products$$
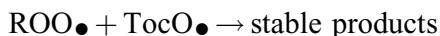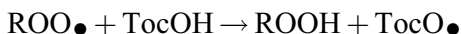
Tocols are also effective quenchers for singlet oxygen, and they react with other reactive oxygen and nitrogen species that are constantly produced during normal metabolism (e.g. Kamal-Eldin and Appelqvist, 1996; Wagner et al., 2004; Eitenmiller and Lee, 2004; Niki, 2010). Although tocols protect lipids from oxidation by similar reactions *in vivo* and *in vitro*, antioxidant activities may differ greatly. *In vivo* α-tocopherol appears to be the major and most efficient lipid-soluble chain-breaking antioxidant. This is partly due to its greater retention in tissues and partly because *in vivo* it works as part of an antioxidant network, which includes, for example, pairs of ascorbate–ascorbate radical, glutathione–glutathione radical, and ubiquinone–ubiquinol radical. Thus *in vivo*, tocopheroxyl radicals may be regenerated to tocopherols, while *in vitro* recycling does not occur. Moreover, many enzymes are also repairing oxidative damage *in vivo* (Niki, 2010).

Kinetic studies have confirmed that α-tocopherol is the most reactive tocol toward peroxyl radicals. The rate constants for hydrogen abstraction had previously been measured in 1981, as 235 ± 50, 166 ± 3, 159 ± 42, and $65 \pm 13 \times 10^{-4}\,\mathrm{M^{-1}\,s^{-1}}$ for α-, β-, γ- and δ-tocopherol, respectively (Burton and Ingold, 1981). The order of reaction rates of hydrogen donation seems to be similar, α- > β- ≈ γ- > δ-tocopherol, under very different conditions such as in homogeneous solutions and membranes (Traber and Atkinson, 2007; Atkinson et al., 2008). Despite this, there are clear differences in the levels and order of antioxidant activities for the various tocols, which might be explained by the numerous methods and experimental setups to evaluate antioxidant activity. Some of the methods measure the capacity for reacting with radicals; some of them the capacity to resist lipid oxidation

(Niki et al., 2010). Moreover, as α-tocopherol is the most reactive tocol it is also the most unstable and is, of all tocols, the first one consumed. This may decrease its activity as a long-term antioxidant in food systems, and a mixture of tocols is often preferred for antioxidant protection. In general, the optimal concentration of α-tocopherol has been lower than those of other tocols (Kamal-Eldin et al., 2006; Seppanen et al., 2010).

Much less is known on the antioxidant activity of tocotrienols than tocopherols. Tocotrienols were shown to have similar reactivities to peroxyl radicals and antioxidant activities than tocopherols in solution and membranes (Yoshida et al., 2003); also, in general, γ-tocotrienol was a better antioxidant than α-tocotrienol, and tocotrienols were better than tocopherols in oil systems (Seppanen et al., 2010). Recently, Muller et al. (2010) conducted a comparative study to investigate the four tocopherols, four tocotrienols, and α-tocopheryl acetate on their antioxidant activities in five different popular *in vitro* assays (FRAP, α-TEAC, DPPH, ORAC, and CL), which were adapted to nonpolar antioxidants. Most notably, they found that α-tocopheryl acetate, a popular ingredient in supplements, had no significant antioxidant activity *in vitro*. However, once ingested, tocol esters are hydrolyzed and antioxidant activities are retained. Overall, the eight tocols performed similarly in the five assays. The authors concluded that *in vitro* antioxidant assays performed in polar solvents are not a good way to predict *in vivo* antioxidant activity.

In addition to antioxidant properties, tocopherols and tocotrienols have been reported to have many other different types of biological activity (Sen et al., 2007a). Some of these major-reported biological activities of tocols are summarized in Table 11.3. As discussed above for antioxidant properties, it is important to remember that individual tocopherols and tocotrienols may have unique biological activities that may differ considerably from those of other tocols, and it is important to be able to analyze each tocol separately.

11.4 Traditional (Nonchromatography) Analysis Methods and their Disadvantages

The traditional colorimetric/spectrophotometric method for the analysis of total tocopherols (calibrated with α-, β-, γ-tocopherols, and

Table 11.3 Medical benefits reported for tocopherols and tocotrienols.

Biological Activity	Tocopherols	Tocotrienols	References
Age Related Macular Degeneration (AMD)	−No effect Vit E only		Moriarty-Craige et al., 2005.
Cataracts	+ Vit E + D + selenium + Vit E + C + β-carotene		Age-Related Eye Disease Study Research Group, 2001
Glaucoma	+		Engin et al., 2007
Cardiovascular Health	+	+	Sen et al., 2007
Stroke Induced Injuries -neuroprotection		+	Sen et al., 2007
Pancreatic Cancer		+	Sen et al., 2007
Breast Cancer		+	Sen et al., 2007
Prostate Cancer	+	+	Helzlsouer et al., 2000 Sen et al., 2007
Skin Cancer		+	Sen et al., 2007
Cholesterol Reduction		+	Sen et al., 2007
Diabetes		+	Sen et al., 2007
Inhibition of LDL Oxidation		+	O'Bryne et al., 2000

hydroquinone and shown to follow Beer's law) involved the use of ferric chloride and measurement of the absorbance at 520 nm (Rawlings, 1944). Fluorescence spectroscopy has also been used to quantify the total tocopherols (Sikorska et al., 2005) and fluorescence detection has become a very sensitive and accurate detection method for the HPLC analysis of tocopherols and tocotrienols (as described in the next section).

11.5   Sample Preparation for Chromatography Analysis

Since tocols are lipid-soluble compounds they are readily soluble in organic solvents. Thus solvent extraction is the most commonly used method to extract tocols from oil seeds, biological fluids, and animal tissues (Abidi, 2000; Bramley et al., 2000; Rupérez et al., 2001). The solvent should be efficient enough to liberate tocols from lipophilic membranes, and to penetrate the water phase to reach tocols. In cereal grains and several processed foods, tocols are associated with other compounds, such as carbohydrates and proteins, and these interactions need to be broken down prior to extraction. Commonly this is done by alkaline hydrolysis, i.e. saponification. Moreover, it is very important that solid samples are finely ground to improve the extractability (Leo et al., 2005). Since tocols are easily oxidized, it is important to avoid conditions that would promote oxidation during sample preparation. It is recommended to work under subdued light and low temperature, and to use an inert atmosphere and antioxidants. The following includes an overview of the traditional extraction methods and some new techniques that have been used in recent years.

11.5.1   Solvent Extraction

Several solvents and solvent mixtures have been used for the extraction of tocols. Hexane and a mixture of ethanol and hexane were used to extract tocols from food samples (Kramer et al., 1997; Sundl et al., 2007), acetone from microwave-oven-dried leaves (Gomez-Coronado et al., 2004), methanol from cereal grains (Michalska et al., 2007), and a mixture of methanol and chloroform from pumpkin seeds (Stevenson et al., 2007). A mixture of chloroform and methanol gave 10–20% greater amounts of tocopherols from fresh tree nuts than hexane, indicating that the mixture was more efficient than hexane

(Miraliakbari and Shahidi, 2008). It has been suggested that the extraction of tocols from plasma samples is improved by deproteinization of the sample with ethanol before solvent extraction (Lee et al., 2003; Chauveau-Durio et al., 2010). Ethanol treatment was also used in α-tocopherol extraction and the analysis of pharmaceutical preparations and milk (Kadioglu et al., 2009).

Extraction with solvents may be enhanced by extensive mixing, e.g. by vortexing, sonication, using ultrasound (Kramer et al., 1997; Gomez-Coronado et al., 2004; Nielsen and Hansen, 2008; Kadioglu et al., 2009), or using hot circulating solvents as in Soxhlet extraction (Fratianni et al., 2002). Although solvent extractions are carried out under relatively mild conditions, antioxidants have been added to solvents to protect tocols from degradation during extraction and storage (Franke et al., 2007; Sundl et al., 2007). Solvent extraction is a simple sample preparation method, because only evaporation of the solvent and dissolving the residue in the mobile phase are needed prior to analysis.

11.5.2   *Alkaline Hydrolysis Assisted Extraction*

Alkaline hydrolysis is commonly used to improve the extractability of tocols from hard tissues and complex foods (Lee et al., 2000; Fratianni et al., 2002; Panfili et al., 2003; Ryynänen et al., 2004; Lampi et al., 2008), although it is not required to hydrolyze ester bonds, because natural tocols occur as nonconjugated free compounds. Food and feed samples may, however, be fortified with esterified tocopherols. When analyzing tocols by normal-phase HPLC, the coextracting neutral lipids do not interfere with the analysis (Kramer et al., 1997), but when analyzing tocols by reversed-phase HPLC, an alkaline hydrolysis is commonly used (Lanina et al., 2007; Diwakar et al., 2010). It may, in any case, be beneficial to perform alkaline hydrolysis with very high-fat samples, to improve the extraction of tocols (Schwartz et al., 2008) or to remove neutral and other saponifiable lipids that might interfere with the chromatographic analysis of tocols (Kramer et al., 1997).

Since tocols are sensitive to alkaline conditions, significant losses may occur during hydrolysis unless the conditions are carefully optimized. During the past years efficient and nondestructive methods, including hot alkaline hydrolysis, have been developed (Lee et al., 2000; Fratianni et al., 2002; Ryynänen et al., 2004). A recent

study showed that tocol yields from oat grains were 25% higher by a method that included hot alkaline hydrolysis than by direct extraction with methanol (Peterson et al., 2007). This provided support for the use of hydrolysis for the liberation of tocols from complex matrices. To avoid oxidation, ascorbic acid (Kramer et al., 1997; Schwartz et al., 2008), pyrogallol (Fratianni et al., 2002; Abidi, 2003; Panfili et al., 2003; Peterson et al., 2007), and butylated hydroxytoluene (Lee et al., 2000) have been added to the saponification mixture, and oxygen has been removed from the vessel by purging the air space with nitrogen. After saponification, tocols are commonly extracted with solvent mixtures such as hexane and ethyl acetate (Panfili et al., 2003; Peterson et al., 2007), heptane and ethyl acetate (Ryynänen et al., 2004), or hexane and ethanol (Lee et al., 2000). The nonsaponifiable lipid extracts are purified from water-soluble impurities, and finally concentrated prior to HPLC analysis.

11.5.3   *Supercritical Fluid Extraction*

As an alternative to traditional solvent extraction methods, the extraction by supercritical (SC) fluids has been used in tocol analysis. This is an environmentally friendly technique as little or no solvents are used. Extraction parameters, e.g. temperature and fluid density, are easily optimized and managed, and as the extraction is fast it is thus suitable for routine work with many samples. SC carbon dioxide has been used to extract tocols from barley (Fratianni et al., 2002), dried bay leaves (Gomez-Coronado et al., 2004), and garden cress seeds (Diwakar et al., 2010). Extractions were carried out in single or multiple steps and with different fluid densities controlled by extraction pressures. Tocol yields from barley were 5% and 14% less than by Soxhlet and chloroform–methanol extractions (Fratianni et al., 2002), yields from garden cress seeds were 26% less than by Soxhlet extraction (Diwakar et al., 2010), and α- and γ-tocopherol yields from dried bay leaves were 22% and 40% less than by acetone extraction (Gomez-Coronado et al., 2004). Despite lower recoveries of tocols, the SC carbon dioxide extraction methods were considered comparable to the classical extraction methods (Fratianni et al., 2002).

Modifiers such as methanol have been investigated to improve the extraction efficiency of SC carbon dioxide. This combination was used to extract fat-soluble vitamins from dairy and meat products, infant

formulas, and canned baby foods (Perretti et al., 2003), and cheese and salami (Perretti et al., 2004). Liquid samples were mixed with an inert drying agent prior to extraction. In the first study, α-tocopherol yields ranged from 17% to 80% and γ-tocopherol yields from 92% to 108% compared to analyses performed by conventional solvent extraction methods with colorimetric measurements. In the latter study on cheese and salami, the repeatability of the extractions was poor and, particularly in the salami extracts, the contents were much smaller than presented in the literature (Perretti et al., 2004). The authors concluded that the protocol needed further development to improve the recovery of α-tocopherol, and to broaden its applicability to moist samples with tocols unevenly distributed in the samples (Perretti et al., 2003; 2004).

11.5.4   *Pressurized Liquid Extraction*

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), is another means to shorten the extraction time, lower the consumption of solvents, and increase the automation in sample preparation that usually is the most labor-intensive step in tocol analysis (Bustamante-Rangel et al., 2007; Delgado-Zamarreño et al., 2009). Prior to PLE, samples are often mixed with drying agents to assist extraction. The main advantage of PLE is that pressurized liquid solvents remain in a liquid state at temperatures above their boiling points, and it is possible to use elevated temperatures to enhance the extraction. In PLE, parameters to be optimized are the solvent (type and volume), extraction time and temperature, number of extractions cycles, and the amount of sample. Of these, the most important is the solvent selection, since the polarity of the solvent should be similar to that of the analytes (Delgado-Zamarreño et al., 2009). The mass of sample being extracted is also critical, especially when the extract is transferred online to the analytical instrument.

Methanol is often used in PLE. It was found to be a better solvent than acetonitrile, or a mixture of methanol and propanol, to extract tocols from cereals (Bustamante-Rangel et al., 2007). Extraction was carried out at 50 °C and a pressure of 110 bar with one extraction cycle of 5 min, because higher extraction temperatures and longer times did not improve analyte recoveries. The same conditions were used to extract tocols from infant formulas (Delgado-Zamarreño et al., 2006). Intra-day and inter-day precisions were very good, being <10%, and the recoveries

of added tocols were 91–109%, indicating that little or no losses of tocols occurred. Moreover, the γ-tocopherol content of a certified reference sample was similar to the labeled value. Post-extraction alkaline hydrolysis was necessary for samples that were fortified with tocopherol esters, such as infant formulas. Later the same authors compared different solvent mixtures to extract tocols from various cereal samples and found that acetonitrile was optimal for oats, a mixture of methanol and propanol for palm oil, and methanol for wheat, rye, barley, and corn (Delgado-Zamarreño et al., 2009).

PLE extraction with hexane (80 °C, 1500 psi, one extraction cycle of 10 min) was used to successfully extract tocols from palm-pressed fiber (Sanagi et al., 2005). Contents and standard deviations of α-tocopherol and α-trienol, and γ- and δ-tocotrienols were comparable to those from Soxhlet extraction; extraction time with the PLE method was reduced to only 25 min and solvent usage to 45 ml, whereas those with Soxhlet extraction were 8 h and 200 ml. Hexane under PLE conditions has also been used to extract tocols from lyophilized nuts (Sivakumar et al., 2005) and grape seeds (Freitas et al., 2008), and always gave higher yields than Soxhlet extraction.

One study compared the efficiency of PLE with ethanol and hexane, and SC extraction with carbon dioxide, to extract tocols from tomato seeds (Eller et al., 2010). The procedures gave comparable total tocol contents in the oils (0.94, 1.08, and 1.11 mg/g), and similar tocol profiles. It was found that tocol yields were inversely proportional to the oil yields, which should be taken into account when evaluating tocol yields from the seeds.

11.5.5   Solid-Phase Extraction

Solid-phase extraction (SPE) has been used to concentrate tocols from extracts, and more recently SPE cartridges have been applied specifically to adsorb tocols from samples. For example, α-tocopherol in a hexane solution of virgin olive oil was concentrated by silica cartridge SPE by first retaining α-tocopherol quantitatively in the cartridge and then releasing it by hexane–diethyl ether elution (Grigoriadou et al., 2007). Online coupling of SPE with HPLC was used to analyze α-tocopherol and δ-tocopherol together with several other fat-soluble vitamins in serum (Mata-Granados et al., 2009). Serum samples were diluted in an acetonitrile–water solution, vortexed and applied

automatically to a solvent delivery unit containing C8-bonded phase cartridges and eluted to a HPLC system. With optimized SPE parameters it was possible to quantitatively and reliably analyze fat-soluble vitamins from serum in 20 min.

Molecularly imprinted (MI) polymers packed in SPE cartridges were used to selectively bind α-tocopherol from filtered suspensions of crushed vegetable samples. Polymers were synthesized using ethylene glycol dimethacrylate, methacrylic acid, 2,2′-azoisobutyronitrile and α-tocopherol (Puoci et al., 2007). After synthesis the polymer was ground, washed to release loosely-associated α-tocopherol, packed in SPE cartridges, and tested for selectivity and affinity. The polymer enabled direct binding and excellent recovery of α-tocopherol and HPLC analysis, with minimal sample preparation of the leaf samples. Only ethanol, water, and acetic acid were used as solvents. In another application, electrospun nanofibers were prepared from polymers to purify and concentrate α-tocopherol and retinol from plasma samples (Liu et al., 2010). Nanofibers of poly(styrene-co-methacrylic acid) were found to give the best extraction efficiency. Together with optimized extraction and elution conditions, direct HPLC analysis gave excellent recoveries and accuracy of plasma samples.

11.5.6   Concluding Remarks on Sample Preparation

Sample preparation is the critical and most labor-intensive part of tocol analysis, and several attempts have been made to simplify it. By using accelerated extraction techniques it has been possible to work up more samples with good repeatability than by manual extraction. This is vital when the number of samples is large and analyses are performed regularly, e.g. in screening and follow-up studies. However, when a complete extraction yield is needed, the extraction should be carefully optimized with careful consideration of the chemical and physical properties of the sample matrix and tocols.

11.6   Chromatography Analysis Methods

Traditionally thin-layer chromatography and column chromatography methods for tocol separation and quantification have been employed (Abidi, 2000); however, most analysts now prefer solid-phase extraction methods (as described in the previous section) for fractionation of a

Table 11.4 GC methods for tocol analysis.

Method (detection)	Column	Elution order	References
1 FID	Dexsil 400, 150 feet × 0.25 mm,	δ-T, γ-T, β-T, δ-T3, γ-T3, α-T3, α-T (TMS ethers)	Slover et al., 1983
2 FID	DG-5, 90 feet × 0.25 mm 0.25 μm	δ-T, γ-T + β-T, α -T	Marks, 1988
3 FID	CP-Sil 8 CB Low Bleed MS, 15 meters × 0.1 μm	δ-T, γ-T, β-T, α-T	Verleyan et al., 2001
4 FID	HP-5 MS (5% phenylsilicon), 30 meters × 0.25 mm,	δ-T, α -T	Du and Ahn, 2002

tocol-enriched fraction and HPLC for quantitative analysis of tocols. Some methods were also developed for the quantitative analysis of tocopherols via capillary supercritical fluid chromatography (SFC). The SFC methods were shown to compare with gas chromatographic (GC) methods, but have not been pursued since HPLC methods have been shown to be more effective (Snyder et al., 1993).

Prior to the 1990s, GC methods that were widely used for the analysis of tocopherols and some tocotrienols, have been reviewed by Abidi (2000). Table 11.4 summarizes two of the best GC methods prior to the year 2000 and two methods that were reported after 2000.

In recent years several normal-phase HPLC methods have been reported for the quantitative analysis of tocopherols and tocotrienols (Table 11.5). The best of these methods have been able to achieve baseline separation of all four tocopherols and all four tocotrienols, as shown in Figures 11.2 and 11.3. Kamal-Eldin et al. (2000) reported the optimal baseline separation of all eight common tocols using a Diol-bonded phase column and an isocratic mobile phase of hexane/methyl tert-butyl ether (MTBE), 96:4, v/v (Figure 11.2). Similar separations were reported by Moreau et al. (2007) using the same type of column and mobile phase. Schwartz et al. (2008) reported that, with a normal-phase silica column, plastochromanol-8 in rapeseed oil eluted between γ-tocopherol and δ-tocopherol.

Table 11.5 A comparison of recent NP-HPLC methods for tocol analysis.

Method (detection)	Stationary/mobile phase	Elution order (run time, min)	References
1 FL, 294 ex, 326 em	Silica, Amino, LiChrosorb Diol Hibar (Merck), 5 μ, 250 × 4 mm Hexane/MTBE, 96/4 (best)	α-T, α-T3, β-T, γ-T, β-T3, γ-T3, δ-T, δ-T3 (30 min)	Kamal-Eldin et al., 2000
2 FL, 290 ex, 330 em	Kromsil Phenomenex Si, 5 μ, 250 × 4.6 mm	α-T, α-T3, β-T, (γ-T + β-T3), γ-T3, δ-T, δ-T3 (25 min)	Panfili et al., 2003
3 FL, 292 ex, 325 em	Varian Inertsil, 5 μ, 250 × 4.6 mm hexane/1,4-dioxane, 97/3	α-T, α-T3, β-T, γ-T, β-T3, γ-T3, δ-T3 (20 min)	Ryyannanen et al., 2004
4 Mass Spectrometry, APCI-MS/MS	Zorbax RX-SIL, 5 μ, 250 × 2.1 mm, hexane/isopropanol.ethyl acetate gradient	β-carotene, α-T, β-cryptoxanthin, Lutein, zeaxanthin (20 min)	Hao et al., 2005
5 UV 295 nm	Hypersil Si, hexane/1,4-dioxane, 96/4	α-T, α-T3, β-T3, γ-T3, δ-T3 (20 min)	Sanagi et al., 2005
6 UV284 + 296 + 326 nm	Pinnacle II silica short bore column, 3 μ, 50 × 2.1 mm, hexane/ethylacetated, 99.5/0.5	Retinol palmitate, retinol acetate, α-T-acetate, α-T, γ-T, δ-T (22 min)	Chavez-Servin et al., 2006
7 FL 290 ex, 330 em, ELSD, UV 295 nm	Inertsil 5 Si column, 3 μ, 250 × 3 mm, hexane/1,4-dioxane, 96.5/3.5	α-T, α-T3, β-T, γ-T, β-T3, γ-T3, δ-T, δ-T3 (25 min)	Cunha et al., 2006
8 FL 294 ex, 326 em CAD	LiChrosorb Diol, 5 μ, 250 × 3 mm, hexane/MTBE, 98/2	α-T, α-T3, β-T, γ-T, β-T3, γ-T3, δ-T, δ-T3 (35 min)	Moreau, 2006,
9 FL, 294 ex, 326 em	LiChrosorb Diol, 5 μ, 250 × 3 mm, hexane/tetrahydrofuran, 98/2	α-T, α-T3, β-T, γ-T, β-T3, γ-T3, δ-T, δ-T3 (35 min)	Moreau et al., 2007
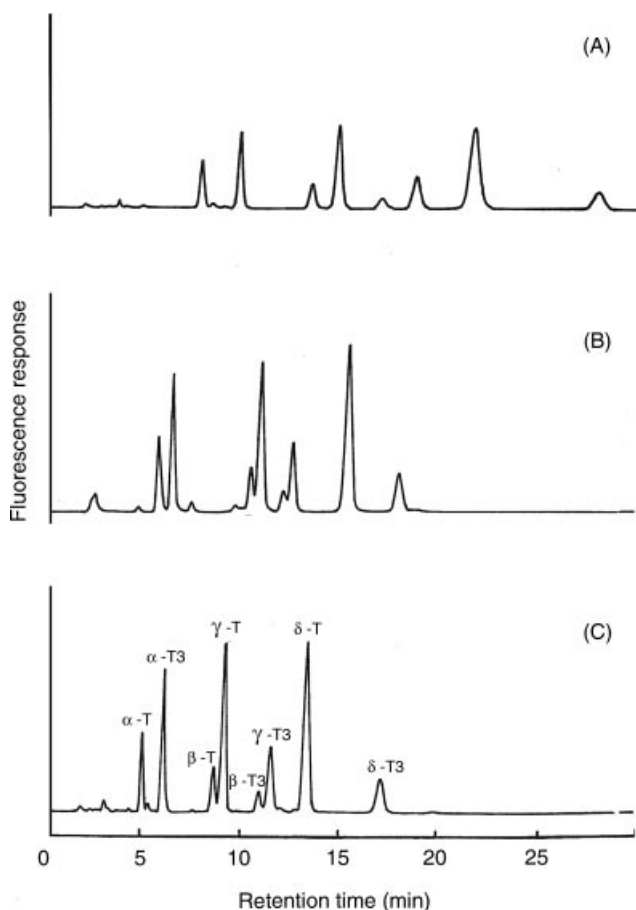
Figure 11.2 Normal-phase separation of Ts and T3 with a Diol column and three different mobile phases. A. hexane/MTBE, 96:4, B. hexane/isopropanol, 99:1, C. hexane/1-4 dioxane, 96:4. (Kamel-Eldin et al., 2000).

Several reversed-phase HPLC methods have also been reported for the quantitative analysis of tocopherols and tocotrienols (Table 11.6). To be able to separate all eight tocols a pentafluorophenylsilica column (Abidi, 2003) was used. A C30-bonded phase silica column separated the three tocotrienols, α-tocopherol, and α-tocomonoenol in palm oil (Ng et al., 2004). C30-bonded silica columns have also been used to simultaneously analyze tocopherols, other fat-soluble vitamins, and carotenoids (Gentili and Caretti, 2011).
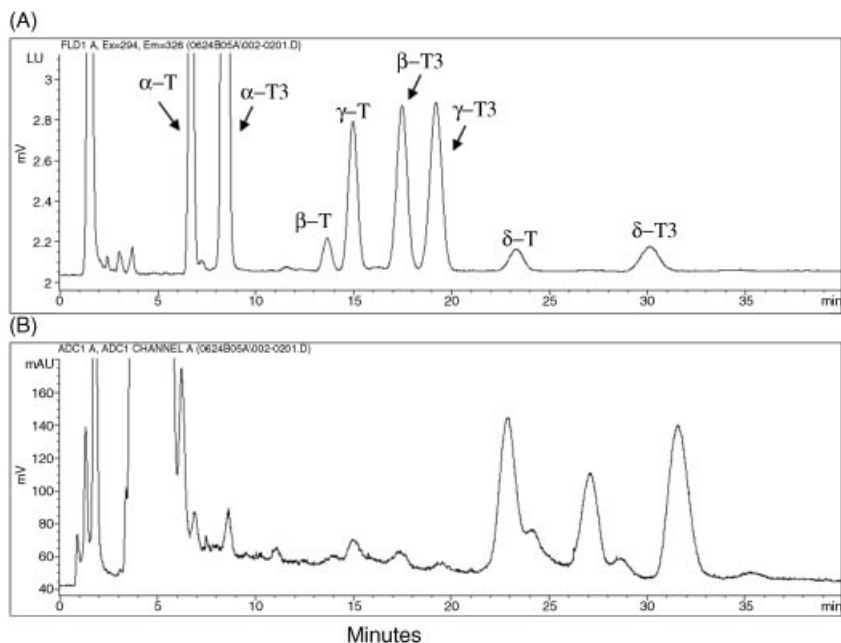
Figure 11.3 Normal-phase HPLC separation of tocopherols (Ts) and tocotrienols (T3s) from barley kernels. A. Detection via fluorescence, ex 294 nm, em 326 nm, B. Detection via a charged aerosol detector. For HPLC parameters see Moreau et al. (2006).

Several capillary electrochromatography (CEC) methods have also been reported for the quantitative analysis of tocopherols and tocotrienols (Table 11.7). Three of these report baseline separation of all four tocopherols, but none has investigated the separation of tocotrienols.

Although tocopherols and tocotrienols can be detected by UV absorbance at 280 nm, fluorescence detection (excitation 294 nm and emission 326 nm), as shown in Figure 11.3, has proven to be a much more sensitive method. Electrochemical detection such as pulsed amperometric and coulometric (Uspitasari-Nienaber, 2002) has also proven to be sensitive and potentially valuable for the quantitative analysis of tocopherols and Tocotrienols (Abidi, 2000), especially for tocol analysis in blood and serum samples. HPLC "mass" detectors such as flame-ionization detectors, evaporative light-scattering detectors, and charged aerosol detectors have proven to be valuable for the quantitative analysis of many types of lipids, but because tocols have

Table 11.6 A comparison of recent RP-HPLC, RP-UPLC, and RP-nano-LC methods for tocol analysis.

Method (detection)	Stationary/mobile phase	Elution order (run time, min)	Ref.
1 electrochemical array, 260 mv	Polymeric YMC™ C30 column, 5 μ, 250 × 4.6 mm, methanol/MTBE/ ammonium acetate/water gradient	α-T3, δ-T, (β-T + γ-T), α-T (16 min)	Puspitasari-Nienaber et al., 2002
2 FL 290 ex, 325 em	Spherisorb ODS-2, 5 μ, 250 × 4.6 mm, methanol/isopropanol, 95/5	Synthetic tocopherols, δ-T, γ-T, α-T (16 min)	Sattler et al., 2003
3 FL 298 ex 345 em	Taxsil (pentafluorophylsilica), 3 μ, 250 × 4.6 mm id) methanol/wa- ter, 9/1, v/v	δ-T3, β-T3, γ-T3, α-T3, δ-T, β-T, γ-T, α-T	Abidi, 2003
4 FL 296 ex, 330 em, and UV 292, 326, 450 nm	Waters NovaPak C18 column, 4 μ, 150 × 3.9 mm, acetonitrile/meth- anol, 65/35, v/v	δ-T, γ-T, α-T, β-cryptoxanthin, lycopene, α-carotene, β-carotene, all trans retinol, δ-T3, γ-T3, α-T3, lutein, zeaxanthin (27 min)	Lee et al., 2003
5 UV at unspecified wavelength	Develosil RP (C30) Aqueous column, 250 × 4.6 mm, 100% methanol	δ-T3, γ-T3, α-T3, α-tocomonoenol, α-tocopherol	Ng et al., 2004
6 FL 290 ex, 330 em	A. Nucleosil C18, 5 μ, 250 × 4.0 mm, methanol/hexane, 85/15 B. same column, methanol/acetoni- trile/water, 72/8/1. C. YMC GmbH C30 column, acetonitrile/ methanol/water, 72/8/1	A. (δ-T3, γ-T3. α-T3, δ-T, γ-T, α-T3), PC-8 (12 min) B. δ-T3, γ-T3. α-T3, δ-T, γ-T, α-T3 (14 min) C. γ-T3, β-T3, γ-T, α-T (12 min)	Gruszka and Kruk, 2007

Table 11.6 (*Continued*)

Method (detection)	Stationary/mobile phase	Elution order (run time, min)	Ref.
7. mass spec electrospray	Eclipse XDB C8 column, 5 μ, 150 × 4.6 mm, formic acid/acetonitrile/water gradient	β-T3, γ-T3, δ-T3, γ-T, α-T, (35 min)	Yu et al., 2007
8 UV 292 nm	Acquity UPLC HSS Tt 1.8 μ, 150 × 2.1 mm, acetonitrile/dichloromethane/methanol/0.05 M aqueous Am Acetate gradient	δ-T, γ-T. α-T. α-T-acetate (30 min)	Chauveau-Duroit et al., 2010
9 UV 295 nm	Chromolith CapRod RP-18 capillary column, 150 × 0.1 mm, acetonitrile/methanol/0.2% aqueous acetic acid	δ-T, γ-T. α-T (30 min)	Cerretani et al., 2009
10 Coulometric, 481 mv	ProtoSIL 200-3-C30 column, 250 × 4.6 mm, methanol/water, 94/4	δ-T, γ-T, β-T, α-T	Mac-Mod, 2010

Table 11.7 A comparison of recent capillary electrochromatography (CEC) methods for tocol analysis.

Method (detection)	Stationary/Mobile Phase	Elution Order (run time, min)	Reference
1 UV	ChromSphere C18, 3 μ, fused silica capillary, 84 mm × 75 μm, id, methanol/acetonitrile, 50:50 with 0.01% ammonium acetate	BHT, δ-T, γ-T. α-T, α-T-acetate (3 min)	Aturki et al., 2005
2 UV 205 nm and 292 nm	ULTIMA C18, 3 μ, fused silica capillary, 24.5 cm × 100 μm, id, methanol/water, 95/5, with 5 mM tris C30 phase, other parameters same as A	δ-T, β-T, γ-T, α-T (30min) δ-T, γ-T. α-T 20min)	Carabias-Martinez, et al., 2006
3 UV 205 nm and 292 nm	Methylacrylate ester-based monolithic capillary column polymerized with 12% 1,3-butanediol, 24.5 cm × 100 μm, methanol/water, 95/5 with 5 mM Tris pH 8.0	Thiourea, BHT, δ-T, γ-T. α-T, α-T-acetate (20 min)×	Lerma-Garcia et al., 2007
4 UV 200 nm	PEDAS-EDMA monolithic column, 32 cm × 100 μm id capillary, 12 mM Tris buffer pH 9.3, MeOH,ACN, 3/10/87, v/v/v.	δ-T, β-T, γ-T, α-T	Chaisuwan et al., 2008

B, baseline resolved
P, incompletely resolved
N, not reported

an excellent fluorophore, fluorescence detection is 10–20-fold more sensitive. Also, as noted in Figure 11.3, when analyzed by detection with both a fluorescence detector and a charged aerosol detector in series, the charged aerosol detector was less sensitive and broad unidentified peaks co-eluted with the tocols (Moreau, 2006).

11.7   Conclusions

11.7.1   Summary and Comparison of Different Chromatography Methods, including Interpretation of Mass Spectral Data

As noted above in our experience, normal-phase HPLC with fluorescence detection is the most convenient and most sensitive method for the analysis of tocopherols and tocotrienols in the majority of samples.

11.7.2   Nonchromatographic Modern Methods

Fourier Transform Infrared (FTIR) spectroscopy methods have been reported for the quantitative analysis of α-tocopherol (Silva et al., 2009) and total tocopherols, tocotrienols, and plastochromanol-8 (Ahmed et al., 2005).

Numerous mass spectra of tocopherols and tocotrienols have been published and many are available in the NIST/EPA/NIH Mass Spectral Library (2011) and MassBank (2011). In addition to the above chromatographic and nonchromatographic methods for tocol analysis, kits are also commercially available for the ELISA (Enzyme-linked immunoabsorbent assay) of vitamin E.

11.7.3   Existing Problems in Current Analysis Methods

Analytical methods for tocol analysis have continued to improve, as noted by Abidi (2000), and in the intervening ten years, as noted in this chapter. We predict that advances will continue to be made in the field of the chromatographic analysis of tocols. Also, we believe that lipidomic methods (quantitative analysis via direct injection tandem electrospray ionization mass spectrometry) will be developed for the rapid analysis of tocols, just as these methods have already been used for the profiling of phospholipids and glycolipids (Han, 2011; Welti, 2011). These methods usually involve the direct injection of lipid samples into a

modern MS/MS instrument, without the need for chromatography. The new field of metabolomics usually includes profiles of molecules of lower molecular weight than tocols (Nikolau and Wurtele, 2007), but it is possible that some of these methods could also be adapted for the rapid analysis of tocols.

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