High-Performance Liquid Chromatography/Mass Spectrometry Analysis of Proanthocyanidins in Foods and Beverages

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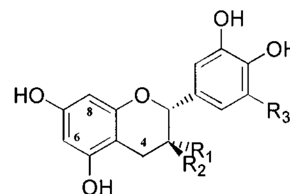
Monomeric and oligomeric proanthocyanidins present in a range of plant-derived foods and beverages were separated by degree of polymerization and identified using a modified normal-phase high-performance liquid chromatography (HPLC) method coupled with on-line mass spectrometry (MS) analysis using an atmospheric pressure ionization electrospray chamber. In addition, ultraviolet (UV) and fluorescence detection were used to monitor the separation of proanthocyanidins, with fluorescence detection demonstrating both increased sensitivity and the ability to reduce interfering signals from other components present in the food and beverage matrices as compared to UV detection. This qualitative study demonstrates the ability of this HPLC/MS technique to separate singly and doubly linked procyanidins, prodelphinidins, and copolymer oligomers, including their galloylated derivatives, present in a range of food and beverage samples.

Keywords: *Procyanidin; prodelphinidin; A-type; HPLC/MS*

INTRODUCTION

Proanthocyanidins are a class of polyphenolic compounds found in several plant species and are oligomers of flavan-3-ol monomer units most frequently linked either 4 → 6 or 4 → 8. The most common classes are the procyanidins, which are chains of catechin, epicatechin, and their gallic acid esters (Figure 1a), and the prodelphinidins (Figure 1b), which consist of gallocatechin, epigallocatechin, and their galloylated derivatives as the monomeric units (Porter, 1989). Structural variations to proanthocyanidin oligomers may also occur with the formation of a second interflavanoid bond by C−O oxidative coupling to form A-type oligomers as shown in Figure 2 (Porter, 1988, 1989). Due to the complexity of this conversion, A-type proanthocyanidins are not as frequently encountered in nature in comparison to the B-type oligomers (Morimoto et al., 1985).

Proanthocyanidins have attracted increasing attention due to the rapidly growing body of evidence associating these compounds with a wide range of potential health benefits. Tea catechins have recently been associated with potent antioxidant activity and with the reduction of tumor multiplicity in laboratory mice (Lunder, 1992; Wang et al., 1992; Chung et al., 1992). Additionally, the proanthocyanidins in grape seed extracts have been shown to have free radical scavenging abilities and to decrease the susceptibility of healthy cells to toxic and carcinogenic agents (Bagchi et al., 1997; Waterhouse and Walzem, 1997; Joshi et al., 1998). Polyphenols in grape juice and red wine have been associated with potential cardiovascular benefits, including the reduction of platelet aggregation, modulation of eicosanoid synthesis, and inhibition of low-density lipoprotein oxidation (Waterhouse and Walzem, 1997; Schramm et al., 1998; Frankel et al., 1995).



a) Procyanidins:
R_1=OH, R_2=H, R_3=H; Epicatechin
R_1=H, R_2=OH, R_3=H; Catechin
R_1=gallic acid ester, R_2=H, R_3=H; Epicatechin gallate
R_1=H, R_2=gallic acid ester, R_3=H; Catechin gallate

b) Prodelphinidins:
R_1=OH, R_2=H, R_3=OH; Epigallocatechin
R_1=H, R_2=OH, R_3=OH; Gallocatechin
R_1=gallic acid ester, R_2=H, R_3=OH; Epigallocatechin gallate
R_1=H, R_2=gallic acid ester, R_3=OH; Gallocatechin gallate

Figure 1. Structures of procyanidin (a) and prodelphinidin (b) monomers and their gallic acid esters.

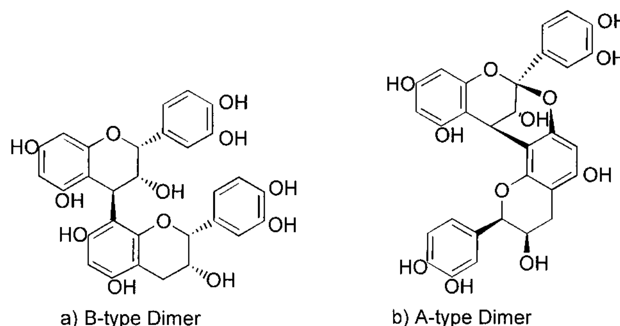


Figure 2. Structure of a B-type dimer (a) compared to an A-type dimer (b).

Recently, it has been suggested that any potential health benefits attributed to these compounds may be affected by the degree of polymerization (Saito et al.,

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1998). Unfortunately, detailed information on the proanthocyanidin profiles present in most foods and beverages is currently lacking, especially with regard to the more complex oligomeric structures (Peterson and Dwyer, 1998).

The most common method employed for the analysis of proanthocyanidins is reversed-phase high-performance liquid chromatography (HPLC). Applications of reversed-phase HPLC analysis of proanthocyanidins can be found in a wide variety of foods. For example, C_{18} columns have been used to separate and identify monomeric and dimeric procyanidins in grape juice and red wine (Jaworski and Lee, 1987; Oszmianski et al., 1988). More recently, reversed-phase HPLC has been used for the analysis of oligomers up to tetramers in apple products (Picinelli et al., 1997). Although reversed-phase C_{18} columns have the ability to separate oligomers of equivalent molecular mass into their isomers, analysis of higher oligomeric proanthocyanidins (i.e., ≥ tetramers) is not feasible due to the fact that, with increasing degrees of polymerization, the number of isomers also increases (Wilson, 1981). This effect results in a retention time overlap of isomers containing differing degrees of polymerization, causing the oligomers to coelute as a large unresolved peak (Guyot et al., 1997).

Degree of polymerization has predominantly been determined by thin-layer chromatography (TLC), thiolysis, or gel permeation chromatography (GPC). Escribano-Bailón et al. (1992) used TLC to hypothesize the existence of oligomers in grape seed extracts through heptamers; however, they isolated and confirmed oligomers only up to the tetramers. Recently, Sun et al. (1998) used thiolysis to determine the average degree of polymerization in fractionated grape seed extract to be as high as 30, while other reports have shown 15 to be the highest (Prieur et al., 1994). Inconsistencies in thiolysis may be attributed to overestimation of the degree of polymerization due to epimerization and disproportionation of its products (Porter, 1988). Furthermore, Rigaud et al. (1993) used GPC on acetylated proanthocyanidins to determine the degree of polymerization of higher oligomers using comparisons with polystyrene standards. However, determinations using GPC may result in overestimation of the molecular masses due to the globularity of oligomers (Prieur et al., 1994).

The most effective HPLC method for the separation of the proanthocyanidin oligomers into their different molecular mass classes employs the use of normal-phase techniques. Wilson (1981) used a cyano column to effect the normal-phase separation of discreet oligomeric groupings through heptamers in apple juice, whereas Rigaud et al. (1993) and Cheynier et al. (1999) used a silica column for the separation of proanthocyanidins in grapes, litchi pericarp, cider apple, and cocoa. This method has recently been modified for the improved separation of monomers through decamers in cocoa and chocolates (Hammerstone et al., 1999).

With HPLC methods dominating proanthocyanidin analysis, various detection techniques have been explored. UV detection is the most common; however, specificity for proanthocyanidins over other polyphenolic compounds is difficult due to the narrow range of UV absorption of many phenolics (Jaworski and Lee, 1987). To circumvent this problem, Treutter (1989) developed a method for the postcolumn reaction of proanthocyanidins with dimethylaminocinnamaldehyde to produce a product with absorbance at 640 nm. Electrochemical detection has also become more widespread due to the smaller number of electroactive substances compared to UV-absorbing compounds (Chiavari et al., 1987; Achilli et al., 1993; McMurrough and Baert, 1994). An interesting approach to polyphenol analysis was reported by Cho et al. (1989), who measured the fluorescent quantum yields of polyphenols at steady state to determine an average molecular mass of procyanidin polymers naturally found in cocoa beans. The optimum excitation wavelength was reported to be 280 nm and the emission wavelength near 320 nm; however, both wavelengths are dependent on the characteristics of the solvent(s) used. Although not frequently employed for proanthocyanidin analysis, fluorescence detection has also demonstrated utility in the HPLC analysis of procyanidins in cocoa (Clapperton et al., 1992).

Apart from the procedures used for the separation of proanthocyanidins and the determination of the degree of polymerization, identification of the monomeric units of oligomers has required tedious purification techniques followed by thiolysis and/or hydrolysis. Escribano-Bailón et al. (1992) used Sephadex LH-20 and preparative reversed-phase HPLC to purify proanthocyanidins in grape seed extract and then hydrolysis and/or thiolysis followed by desulfurization of galloyl esters to identify the structures of dimers and trimers. Additionally, Rigaud et al. (1993) collected fractions from several normal-phase HPLC injections and confirmed monomers through tetramers using microthiolysis and/or enzymatic hydrolysis. Interestingly, A-type proanthocyanidins are resistant to degradation by thiolysis, thus rendering this technique unable to identify their monomeric units (Jacques et al., 1974; Karchesy and Hemingway, 1986).

Although thiolysis, partial acid hydrolysis, and enzymatic hydrolysis are the predominant techniques to determine the monomeric units of oligomers, other methods can be employed, including mass spectrometry (MS). For example, positive ion mode fast atom bombardment mass spectrometry (FAB/MS) has been utilized for wine and apple polymers with the use of the "magic bullet" matrix (Bailey and Nursten, 1994; Ohnishi-Kameyama et al., 1997). Moreover, Ohnishi-Kameyama et al. (1997) used matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS to detect up to pentadecamers in unripe apple in the positive ion mode with the aid of ionization reagents. In addition to FAB/MS and MALDI-TOF, electrospray ionization (ESI) MS has been used for the gentle ionization of procyanidin polymers in cider apples, and multicharged ion signals were used to identify oligomers up to 17 units (Guyot et al., 1997). Likewise, a combination of polyamide column chromatography and reversed-phase HPLC coupled to thermospray MS was used by Kiehne et al. (1997) to identify the composition of dimers and trimers in Chinese green tea. More recently, Le Roux et al. (1998) used a combination of thioacidolysis, nuclear magnetic resonance (NMR), and ESI MS to characterize the A-type procyanidins in Litchi pericarp extracts fractionated by normal-phase HPLC.

Notably, these previous methods are laborious, requiring lengthy preparation and analysis times due to the multistep processes to determine the degree of polymerization followed by structural identification. Recently, a normal-phase HPLC method coupled to an atmospheric pressure ionization electrospray (API-ES)

mass spectrometer was reported by Hammerstone et al. (1999) for separation and identification of oligomeric procyanidins in cocoa and chocolate. In the current study, we explored this method's utility by applying it to numerous food and beverage samples reported to contain various types of proanthocyanidins. Additionally, both ultraviolet (UV) and fluorescence detectors were used to compare the selectivity and sensitivity of each during proanthocyanidin analysis.

MATERIALS AND METHODS

Samples. Green tea bags, grape seed extract supplement pills, premium ground cinnamon, grape juice, and Red Delicious apples were purchased commercially at a local grocery store. Pinot Noir was purchased commercially at a local wine store. Raw peanuts were provided by M&M/MARS (Hackettstown, NJ). Raw almonds were provided by the Almond Board of California (Modesto,CA).

Standards. (−)-Epicatechin, (+)-catechin, (−)-epigallocatechin, and (−)-epigallocatechin gallate were purchased from Sigma Chemical (St. Louis, MO). Epicatechin dimer digallate was generously provided by Georgetown University.

Preparation of Solid-Phase Extraction (SPE) Columns. Supelcosil Envi-18 20 mL SPE columns (Supelco, Inc., Bellefonte, PA) were conditioned with 3 × 5 mL of methanol and then with 3 × 5 mL of water prior to sample loading. After the appropriate sample loading and rinse procedures, the columns were dried under vacuum for 1−2 min. The polyphenols were eluted from the SPE column with 10 mL of acetone, water, and acetic acid in a ratio by volume of 70:29.5:0.5, respectively.

Sample Preparation and Polyphenol Extraction of Various Foods and Beverages. *Green Tea Beverage.* Two tea bags were brewed in ∼460 mL of hot water according to the directions on the package. The tea was cooled to room temperature before 100 mL was loaded onto the conditioned SPE column followed by rinsing with 40 mL of water.

Grape Seed Extract. The contents of one capsule (∼250 mg) was suspended in 20 mL of water and loaded onto the conditioned SPE column, which was then rinsed with 40 mL of water before the proanthocyanidins were eluted as above.

Cinnamon. Two grams of cinnamon was extracted with 5 mL of acetone, water, and acetic acid in a ratio by volume of 70:29.5:0.5, respectively. The resulting suspension was centrifuged for 10 min at 1500*g* and the supernatant decanted and filtered through a 0.45 μm nylon membrane before direct analysis on the HPLC/MS without SPE cleanup.

Peanut Skins. Approximately 3.5 g of peanut skins was ground in a laboratory mill before being extracted in 25 mL of acetone, water, and acetic acid in a ratio by volume of 70:29.5:0.5, respectively. The suspension was centrifuged for 10 min at 1500*g* and the supernatant decanted. Twenty milliliters of water was added to the supernatant before the organic solvent was removed by rotary evaporation under partial vacuum at 45 °C to yield ∼22 mL of aqueous extract. The aqueous extract (22 mL) was loaded onto the conditioned SPE column and rinsed with 40 mL of water, and then the proanthocyanidins were eluted as above.

Peanut Nutmeat. The nutmeat was frozen in liquid nitrogen and then ground into a powder in a laboratory mill. The nutmeat powder (∼10 g) was extracted three times with 45 mL each of hexane to remove lipids. One gram of the resultant defatted nutmeat was extracted with 5 mL of acetone, water, and acetic acid in a ratio by volume of 70:29.5:0.5, respectively. The extract was filtered through a 0.45 μm nylon membrane before direct analysis on the HPLC/MS without SPE cleanup.

Apple. Red Delicious apples were freeze-dried whole. Approximately 19 g of the freeze-dried material was ground in a laboratory mill and extracted with 80 mL of acetone, water, and acetic acid in a ratio by volume of 70:29.5:0.5, respectively. The suspension was centrifuged for 10 min at 1500*g*, and the supernatant was decanted into a round-bottom flask. Fifty milliliters of water was added to the flask before the organic solvent was removed by rotary evaporation, under partial vacuum at 45 °C, to yield ∼66 mL of aqueous suspension. Thirty milliliters of this suspension was loaded onto the conditioned SPE column, which was rinsed with 10 mL of water, and the proanthocyanidins were eluted as above.

Almond Seedcoat. Approximately 24 g of seedcoat was removed from the raw almonds using a razor blade. The seedcoat was then defatted twice with 135 mL of hexane and centrifuged for 10 min at 1500*g* to yield ∼14.6 g of defatted material. The defatted seedcoat was extracted with 90 mL of acetone, water, and acetic acid in a ratio by volume of 70:29.5:0.5, respectively. Thirty milliliters of water was added to the supernatant, and the resulting acidified aqueous acetone was rotary evaporated under partial vacuum at 45 °C to a final volume of 50 mL. The aqueous solution was loaded onto the conditioned SPE column, which was rinsed with ∼10 mL of water, and the proanthocyanidins were eluted as above.

Red Wine. The alcohol was removed from 200 mL of red wine by rotary evaporation under partial vacuum at 45 °C to yield ∼110 mL. Thirty milliliters of the dealcoholized wine was loaded onto the conditioned SPE column and rinsed with 40 mL of water. The proanthocyanidins were then eluted as above.

Grape Juice. Fifty milliliters of grape juice was loaded onto a conditioned SPE column and washed with an additional 40 mL of water before the proanthocyanidins were eluted as described above.

HPLC/MS Analysis of Polyphenols. Chromatographic analyses were performed on an HP 1100 Series HPLC (Hewlett-Packard, Palo Alto, CA) equipped with an autoinjector, quaternary HPLC pump, column heater, diode array detector, HP1046A fluorescence detector, and HP ChemStation for data collection and manipulation. Normal-phase separations of the proanthocyanidin oligomers were performed under the conditions previously described by Hammerstone et al. (1999). Briefly, separations of the procyanidin oligomers were performed on a Phenomenex (Torrance, CA) 5 μm Luna silica column (250 × 4.6 mm) at 37 °C using a 10 μL injection volume. The ternary mobile phase consisted of (A) dichloromethane, (B) methanol, and (C) acetic acid and water (1:1 v/v). Separations were affected by a series of linear gradients of B into A with a constant 4% C at a flow rate of 1 mL/min as follows: elution starting with 14% B in A; 14−28.4% B in A, 0−30 min; 28.4−50% B in A, 30−60 min; 50−86% B in A, 60−65 min; 65−70 min isocratic. Data were collected using both the UV detector at 280 nm and the fluorescence detector (FLD) at an excitation wavelength of 276 nm and an emission wavelength of 316 nm. Other FLD conditions included a photomultiplier tube gain of 12, frequency of 110 Hz, and response time of 2 s.

HPLC/MS analyses of polyphenol extracts were performed using an HP 1100 Series HPLC as described above but interfaced to an HP Series 1100 mass selective detector (Model G1946A) equipped with an API-ES ionization chamber. The buffering reagent was added via a tee in the eluant stream of the HPLC just prior to the mass spectrometer and delivered with an HP 1100 series HPLC pump bypassing the degasser. Conditions for analysis in the negative ion mode include ∼0.75 M ammonium hydroxide as the buffering reagent at a flow rate of 0.04 mL/min, a capillary voltage of 3 kV, the fragmentor at 75 V, a nebulizing pressure of 25 psig, and the drying gas temperature at 350 °C. Data were collected on an HP ChemStation using both scan mode and selected ion monitoring. Spectra were scanned over a mass range of m/z 100−3000 at 1.96 s per cycle.

RESULTS AND DISCUSSION

As previously reported by Hammerstone et al. (1999), a SPE method was required for most sample preparations to remove interfering sugars prior to HPLC/MS analysis. This paper also demonstrated the use of ammonium hydroxide as a buffering reagent to collect data in the negative ion mode on the API-ES MS.

Table 1. Singly Linked Procyanidins in Various Food Samples

	ions *m/z*				
oligomer	(M − H)⁻	(M − 2H)⁻²/2	(M − 3H)⁻³/3	(M − 4H)⁻⁴/4	foods[a]
monomers	289				grape seed[1]; tea[2]; peanuts[3]; almonds[4]; wine[5]; grape juice[6]; apple[7]
dimers	577				grape seed[8]; tea[9]; cinnamon[10]; peanuts[3]; almonds[4]; wine[12,15]; grape juice[16]; apple[7,14]
trimers	865				grape seed[11]; cinnamon[10]; peanuts[3]; almonds[4]; wine[12,15]; grape juice; apple[7,14]
tetramers	1153	576			grape seed[8]; cinnamon[10]; peanuts; almonds[4]; wine[13,15]; grape juice; apple[7,14]
pentamers	1441	720			grape seed; cinnamon[10]; peanuts; almonds; wine[13,15]; apple[7,14]
hexamers	1729	864			grape seed; cinnamon; peanuts; almonds; apple[7,14]
heptamers		1008	672		grape seed; cinnamon; peanuts; almonds; apple[7,14]
octamers		1152	768		grape seed; cinnamon; peanuts; apple[7,14]
nonamers		1296	864		cinnamon; apple[7,14]
decamers		1440	960		apple[7,14]
undecamers		1584	1056	792	apple[7,14]
dodecamers		1728	1152	864	apple[7,14]

[a] Reports in the literature: [1]Prieur et al., 1994; [2]Nonaka et al., 1983b; [3]Karchesy and Hemingway, 1986; [4]de Pascual-Teresa et al., 1998; [5]Oszmianski et al., 1988; [6]Jaworski and Lee, 1987; [7]Ohnishi-Kameyama et al., 1997; [8]Escribano-Bailón et al., 1992; [9]Nonaka et al., 1984; [10]Nonaka et al., 1983a; [11]Rigaud et al., 1993; [12]Ricardo-da-Silva et al., 1992; [13]Remy et al., 1998; [14]Guyot et al., 1997; [15]Lea, 1980; [16]Spanos and Wrolstad, 1990.

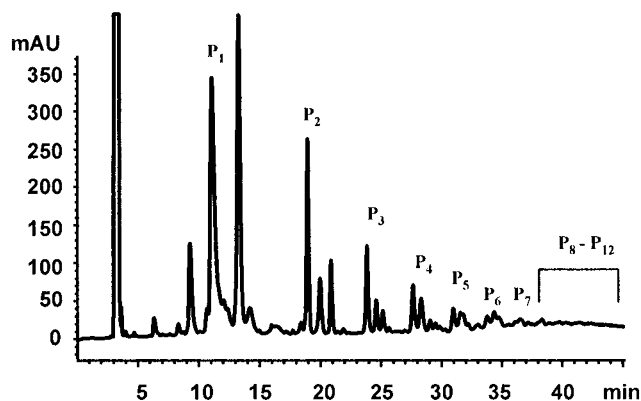

Figure 3. UV trace at 280 nm for a 10 μL injection of whole Red Delicious apple extract: P_1 = monomers; P_2 = dimers; P_3 = trimers; P_4 = tetramers; P_5 = pentamers; P_6 = hexamers; P_7 = heptamers; P_8 = octamers; P_9 = nonamers; P_{10} = decamers; P_{11} = undecamers; P_{12} = dodecamers.

Although those study results are still valid, optimization experiments for the buffering reagent found that a combination of lower concentration and slower flow rate of ammonium hydroxide yields better ionization for a procyanidin extract. Therefore, the concentration of ammonium hydroxide was halved from 1.5 M and the flow rate altered from a step change between 0.09 and 0.05 mL/min to a constant rate of 0.04 mL/min.

Using the newly optimized parameters, the first food sample analyzed was an apple because it has long been known to contain procyanidins (Lea, 1978; Picinelli et al., 1996). It has been reported recently by Guyot et al. (1997) that apple polyphenols comprise ~25% of the pulp extract and ~37% of the apple skin extract. In the current study, the HPLC/MS analysis of an apple extract suggested the presence of a complex series of proanthocyanidin oligomers in a whole Red Delicious apple (Figure 3). Upon review of the mass spectral data seen in Table 1, it can be concluded that oligomers through dodecamers are present and consist entirely of the procyanidin monomers epicatechin and catechin. There have been reports of even higher oligomers present in apples; however, it should be noted that most of these reports are for unripe apples, which may have up to 10 times the polyphenol level as compared to ripened samples (Ohnishi-Kameyama et al., 1997). Furthermore, it can be seen in Figure 3 that other UV-absorbing compounds eluted just prior to (retention time = 9.3 min) and after (13.3 min) the procyanidin monomer peak (11.4 min), and UV data would suggest that they are probably phenolic acids or flavonols.

Given the current results for apple in combination with the previous results for cocoa and chocolate by Hammerstone et al. (1999), this HPLC/MS method has clearly demonstrated its usefulness for the separation and identification of predominantly singly linked procyanidins. To evaluate further the utility of this method, cinnamon and peanuts, which contain a substantial amount of doubly linked procyanidins, were analyzed. The HPLC/MS data for a commercial cinnamon powder provided confirmation for both singly and doubly linked oligomers through pentamers while indicating for the first time the presence of oligomers through nonamers (Tables 1 and 2). It is important to note that the doubly linked oligomers were markedly more abundant and eluted significantly earlier than their singly linked counterparts. Interestingly, the UV data do not clearly indicate the presence of monomers and dimers in the cinnamon extract (Figure 4). However, mass spectral data confirmed the presence of dimers and a notable absence of monomers. Furthermore, trimers (P_3 in Figure 4) through pentamers (P_5 in Figure 4) were in unusually high abundance; this phenomenon was also observed by Nonaka et al. (1983b) for *Cinnamomum zeylanicum*, suggesting that procyanidins constitute the major component in this commercial cinnamon. Additionally, the ions for the doubly linked oligomers were overwhelmingly dominant for trimers through pentamers, whereas singly and doubly linked ions were observed in equal abundance for the higher oligomers.

Next, the procyanidin oligomers present in peanuts were evaluated by analyzing the nutmeat and skins separately to determine the location of the procyanidins. To date, studies in the literature relating specifically to procyanidins in the nutmeat are scarce and have focused mainly on the skins, because they reportedly contain ~17 wt % of procyanidins (Karchesy and Hemingway, 1986).

The extract from the nutmeat was analyzed first, but ionization could not be achieved due to low concentrations of procyanidins. In contrast, the extract from the peanut skins yielded a UV trace at 280 nm and a total ion chromatogram (TIC) suggesting the presence of a

Table 2. Doubly Linked Procyanidins in Various Food Samples

oligomer	ions *m/z* $(M - H)^-$	$(M - 2H)^{-2}/2$	$(M - 3H)^{-3}/3$	foods[a]
dimers	575			peanuts[1]; cinnamon[2]
trimers	863			peanuts[1]; cinnamon[2]
	861			peanuts
tetramers	1151	575		peanuts; cinnamon[2]
	1149	574		peanuts
	1147	573		peanuts
pentamers	1439	719		peanuts; cinnamon[2]
	1437	718		peanuts
	1435	717		peanuts
hexamers	1727	863		peanuts; cinnamon
	1725	862		peanuts
	1723	861		peanuts
heptamers		1007	671	peanuts; cinnamon
		1006	670	peanuts
octamers		1151	767	peanuts; cinnamon
		1150	766	peanuts
nonamers		1295	863	cinnamon

[a] Reports in the literature: [1]Karchesy and Hemingway, 1986; [2]Nonaka et al., 1983b.

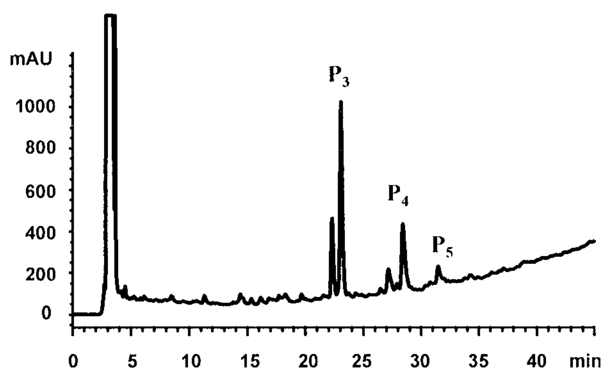

Figure 4. UV trace at 280 nm for a 10 μL injection of cinnamon extract: P_3 = trimers; P_4 = tetramers; P_5 = pentamers. Labels correspond to the predominant A-type oligomers in the extract.

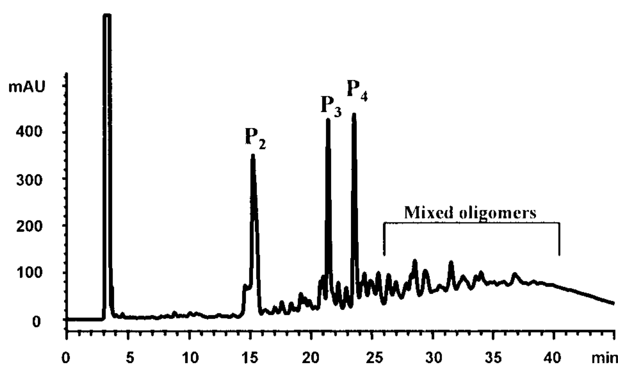

Figure 5. UV trace at 280 nm for a 10 μL injection of peanut skin extract: P_2 = dimers; P_3 = trimers; P_4 = tetramers.

complex series of procyanidin oligomers. Although the UV trace (Figure 5) does not clearly indicate the presence of monomers, mass spectral data show an ion with *m/z* 289 in minor abundance with an elution time previously observed for procyanidin monomers in cocoa and chocolate (Hammerstone et al., 1999). Similar to cinnamon, mass spectral data indicated the presence of both singly and doubly linked oligomers through octamers in peanut skins (Tables 1 and 2). Interestingly, in contrast to the cinnamon extract, oligomers containing more than one A-type linkage were observed in the peanut skins (Table 2). This phenomenon has been reported in other species containing A-type procyanidins such as the litchi pericarp (Le Roux et al., 1998). Another point worth noting is that our mass spectral data indicated that the doubly linked oligomers predominated over their singly linked counterparts, which is in contrast to results reported by Karchesy and Hemingway (1986). For example, the ions corresponding to the doubly linked hexamers were ~5 times more abundant than ions corresponding to singly linked hexamers in the peanut skins analyzed for the current study. The differences may be attributed to the isolation methods employed or result from inherent differences in the peanut genotypes used in the respective studies.

For comparison with other nutmeats, the procyanidin content of almond seedcoats was also evaluated. Although there is little published information, it has been reported by Brieskorn and Betz (1988), that 30% of the almond seedcoat consists of procyanidins of various degrees of polymerization. In our mass spectral data of almond seedcoat, ions were identified that correspond to the procyanidin monomers catechin and epicatechin, on through the heptameric forms of oligomers with the dimers and trimers prevailing in comparison to the other forms. The data further indicated that all observed oligomers were singly linked (Table 1), which is in contrast to peanut skins, in which the doubly linked oligomers were found to account for the majority of the procyanidin content. Additionally, it was found that very few monomeric units remained in peanut skins; in contrast, substantial amounts of monomers were found to still be present in the almond seedcoat.

Next, the application of this HPLC/MS method for the separation of prodelphinidins and galloylated derivatives of both procyanidins and prodelphinidins was explored through the analysis of green tea and grape seed extract. Interestingly, the grape seed extract contained oligomers through heptamers consisting primarily of procyanidins and their galloylated derivatives (Tables 1 and 3), whereas the green tea had a more complex mixture of procyanidins and prodelphinidins with a lower degree of polymerization. As previously observed by Rigaud et al. (1993), who also utilized normal-phase methodology, the galloylated forms in both the green tea and grape seed extract eluted later than their nongalloylated counterparts.

We observed the most abundant tea catechins to be the galloylated derivatives of both the procyanidin (Table 3) and prodelphinidin monomers (Table 4), which is in agreement with the findings of Nonaka et al.

Table 3. Galloylated Procyanidins in Various Food Samples

oligomer	ions m/z			corresponding composition	foods[a]
	$(M-H)^-$	$(M-2H)^{-2}/2$	$(M-3H)^{-3}/3$		
monomers	441			catechin gallates	grape seed[1,2]; tea[3]
dimers	729			dimer gallate	grape seed[4]
	881			dimer digallate	grape seed[5]
trimers	1017			trimer gallate	grape seed[4]
	1169	584		trimer digallate	grape seed[4]
	1321	660		trimer trigallate	grape seed[4]
tetramers	1305	652		tetramer gallate	grape seed
	1457	728		tetramer digallate	grape seed
	1609	804		tetramer trigallate	grape seed
pentamers	1593	796		pentamer gallate	grape seed
	1745	872		pentamer digallate	grape seed
		948		pentamer trigallate	grape seed
hexamers		940		hexamer gallate	grape seed
		1016		hexamer digallate	grape seed
		1092		hexamer trigallate	grape seed
heptamers		1084		heptamer gallate	grape seed
		1160	773	heptamer digallate	grape seed
		1236	824	heptamer trigallate	grape seed
octamers		1228	819	octamer gallate	grape seed

[a] Reports in the literature: [1]Prieur et al., 1994; [2]Sun et al., 1998; [3]Nonaka et al., 1983a; [4]Escribano-Bailon et al., 1992; [5]Rigaud et al., 1993.

Table 4. Prodelphinidins and Copolymer Oligomers Found in Green Tea

oligomer	ions m/z $(M-H)^-$	corresponding composition[a]
monomers	305	gallocatechins[1]
	457	gallocatechin gallates[1]
dimers	609	2 gallocatechins[2]
	745	1 gallocatechin and 1 catechin gallate[3] **or** 1 gallocatechin gallate and 1 catechin[4]
	761	1 gallocatechin gallate and 1 gallocatechin
trimers	897	2 gallocatechins and 1 catechin
	913	3 gallocatechins
	1049	2 gallocatechins and 1 catechin gallate **or** 1 catechin and 1 gallocatechin and 1 gallocatechin gallate

[a] Reports in the literature: [1]Nonaka et al., 1983a; [2]Hashimoto et al., 1992; [3]Porter, 1988; [4]Kiehne et al., 1997.

(1983a). Other minor catechins in fresh green tea leaves have been reported and identified as methylated gallic acid esters (Saijo, 1982); however, the corresponding ions were not detected in our mass spectral data.

Additionally, ions were observed in the mass spectral trace for green tea that suggest the presence of dimers consisting of procyanidin, prodelphinidin, and mixed monomeric units along with their galloylated derivatives as shown in Tables 1 and 4. These findings are in agreement with reports that copolymers of procyanidin and prodelphinidin units are commonly found in nature (Porter, 1989). Other minor dimers containing epiafzelechin gallate as a monomeric unit in green and black tea samples have been reported by Kiehne et al. (1997); however, no unique ions were found that match these compounds. This absence in our data would be expected because the ions corresponding to epiafzelechin monomers were also not observed, which could be attributed to varietal differences and/or processing effects. Furthermore, two other unknown ions in relatively high abundance were observed and coeluted with the trimers. These ions (m/z 755 and 771) are likely to correspond to flavonols because UV data show maximum absorbance around 350 nm. However, further structural characterization will be required for positive identification.

It should be noted that although HPLC/MS is a powerful technique for separating oligomers of proanthocyanidins and identifying monomeric units, caution should be used when copolymers of procyanidins and prodelphinidins are analyzed. Occasionally, copolymer oligomers of different classes will have the same mass. For example, in the grape seed extract, an ion with m/z 881 was observed, which could correspond to a dimer consisting of two epicatechin gallate units or a trimer consisting of two epicatechin units and one epigallocatechin. Because galloylated procyanidins tend to elute late on the normal-phase HPLC trace, it would be easy to confuse these oligomers. Therefore, it may be necessary to confirm the structure of copolymers with an additional analysis on the products of enzymatic hydrolysis using tannase (EC 3.1.1.20). Another alternative is to develop techniques that utilize HPLC/MS/MS to obtain characteristic fragmentation data in the cases where copolymers occur. In the current study, the oligomer is believed to be dimer digallate by comparing its retention time to that of a synthetic standard.

Given the results of the grape seed extract, a California Pinot Noir wine and two commercial grape juices were also analyzed to evaluate the effect of different processing practices on grape proanthocyanidins. First, the California Pinot Noir was analyzed and, as can be seen in the UV trace at 280 nm (Figure 6a), the low concentrations of procyanidins in combination with numerous other UV-absorbing components make interpretation difficult. Therefore, the fluorescence detector (FLD) was explored as an alternate detection system to increase selectivity for procyanidins and reduce the signal from other UV-absorbing compounds. The resulting FLD trace is shown in Figure 6b and, interestingly, the same concentration and injection volume of the red wine gave a saturated signal on the FLD for the procyanidins, thus illustrating its increased sensitivity over the diode array detector for these compounds. As can be seen, the FLD trace clearly indicates the presence of oligomers through pentamers (P_1 through P_5). This is interesting because the grape seed extract contained oligomers through heptamers (Tables 1 and 3). It has been reported that not only do the concentration and forms of phenolic material vary considerably due to factors such as grape variety, environmental conditions, fermentation practices, and processing, but the poly-

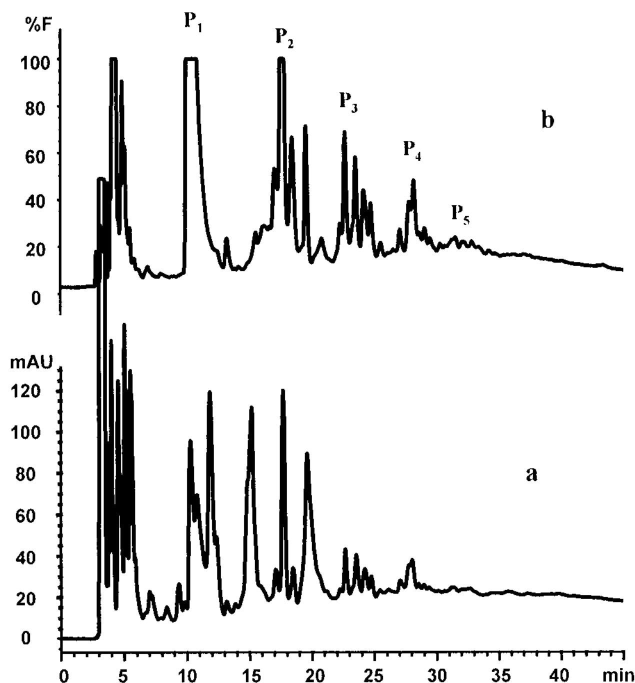

Figure 6. UV trace at 280 nm (a) and FLD chromatogram (b) for a 10 μL injection of red wine extract: P_1 = monomers; P_2 = dimers; P_3 = trimers; P_4 = tetramers; P_5 = pentamers.

merization of procyanidins seems to be regulated by both solution pH and water/ethanol solubility (Frankel et al., 1995; Waterhouse and Walzem, 1997). These processes may result in some depolymerization and the cleavage of interflavan bonds; thus, the sediment that gradually accumulates during the aging of red wine is not a continuous polymerization of catechins but rather an agglomeration of many smaller procyanidin chains (Saucier et al., 1997). This finding is in good agreement with our results, where the predominating oligomers are of a lesser degree of polymerization (i.e., pentamers and lower oligomers). It is also interesting to note that our HPLC/MS data do not indicate the presence of galloylated oligomers in red wine as observed in the grape seed extract (Tables 1 and 3). A possible explanation for this observation may simply be due to differences in processing, wherein the procyanidins released from skins and stems, which are predominantly B1, account for the majority of polyphenols present in wine. Accordingly, there is a poor release from the grape seeds in which the galloylated procyanidins are concentrated (Ricardo-da-Silva et al., 1992).

For the grape juice from one manufacturer, the HPLC/MS data showed a profile similar to that of the red wine with the presence of oligomers through tetramers (Table 1), albeit at much lower concentrations than for a comparable aliquot of red wine. This result is somewhat surprising, because often very small amounts of flavan-3-ols are found in the grape pulp or fresh juice, and, if any procyanidins are commercially present, it is typically the result of the extraction of skins and seeds after crushing the grape. However, these processing conditions are currently achievable commercially, as in vigorous extractors such as the continuous screw press (Waterhouse and Walzem, 1997). Alternately, in juice analyzed from a different manufacturer, there were no detectable procyanidin monomers or higher oligomers. This occurrence can often be expected with traditional juicing methods, such as those using membrane filtration, by which proanthocyanidins have been reported to bind with the proteins that have collected on the filters (Waterhouse and Walzem, 1997). Nonetheless, when procyanidins are present, the primary components in grape juice were the catechin/epicatechin monomers and dimers, with much lower levels of any higher forms.

Notably, two unknown peaks were also present in the mass spectral trace of the juice extract, with primary ions of *m/z* 325 and both *m/z* 733 and 366; the last is likely to be a doubly charged ion. Both of these compounds were found to elute between the monomers and dimers in relatively high abundance. A possible identification of ion *m/z* 325 would be feruloyltartaric acid with a molecular mass of 326, a hydroxycinnamic acid (HCA) known to be present in grapes (Macheix and Fleuriet, 1997). Another hydroxycinnamate phenolic, and the most abundant nonflavonoid present in grapes, is *t*-caffeoyltartaric acid (Jaworski and Lee, 1987; Oszmianski and Lee, 1990; Macheix and Fleuriet, 1997). It is highly probable that this second peak is a derivative of this HCA, such as its ester with tartaric acid. Such components are known to be unique to the grape-bearing species *Vitis.* It is important to note that upon review of the UV data corresponding to these ions, a maximum absorbance was observed around 315 nm, which supports these tentative identifications.

In conclusion, the HPLC/MS method previously described for the separation and identification of procyanidins in cocoa and chocolate by Hammerstone et al. (1999) has been demonstrated to have wide-ranging utility for analysis of multiple types of proanthocyanidins in a large variety of food and beverage matrices. Furthermore, it was concluded that fluorescence detection was superior to UV detection in samples with lower concentrations of procyanidins and more complex matrices. The increased sensitivity and selectivity of the fluorescence detector are likely to prove important when attempts to quantitate procyanidins in food samples are made. Validation of the method described herein as a quantitative tool is currently underway in our laboratory.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; MS, mass spectrometry; API-ES, atmospheric pressure ionization electrospray; TLC, thin layer chromatography; GPC, gel permeation chromatography; FAB, fast atom bombardment; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; ESI, electrospray ionization; SPE, solid-phase extraction; FLD, fluorescence detector; TIC, total ion chromatogram; HCA, hydroxycinnamic acid; NMR, nuclear magnetic resonance; UV, ultraviolet.

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