

# Identification of Procyanidins in Cocoa (*Theobroma cacao*) and Chocolate Using High-Performance Liquid Chromatography/Mass Spectrometry

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Monomeric and oligomeric procyanidins present in cocoa and chocolate were separated 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. The chromatographic separation was achieved using a silica stationary phase in combination with a gradient ascending in polarity. This qualitative report confirms the presence of a complex series of procyanidins in raw cocoa and certain chocolates using HPLC/MS techniques. Although both cocoa and chocolate contained monomeric and oligomeric procyanidin units 2–10, only use of negative mode provided MS data for the higher oligomers (i.e., >pentamer). Application of this method for qualitative analysis of proanthocyanidins in other food products and confirmation of this method as a reliable quantitative tool for determining levels of procyanidins in cocoa, chocolate, and other food products are currently being investigated.

**Keywords:** *Theobroma cacao*; cocoa; chocolate; procyanidin; HPLC/MS

## INTRODUCTION

Procyanidins (Figure 1) are a class of polyphenolic compounds found in several plant species and may be present as individual monomers or, in some cases, as oligomeric units (Porter, 1988). Certain members of this class of compounds can be found in several foods commonly consumed in the diet, including apples, almonds, barley, grapes, tea, maize, cinnamon, cocoa, peanuts, wine, and strawberries (Thompson et al., 1972; Chiavari et al., 1987; Karchesy and Hemingway, 1986; Morimoto et al., 1986; McMurrough and Baert, 1994; Brieskorn and Betz, 1988). Of special interest is the recent attention these compounds have attracted in the fields of nutrition, health, and medicine as a result of the rapidly growing body of evidence suggesting they may act as potent antioxidants and/or modulate key biological pathways in vivo in mammals (Rice-Evans and Packer, 1997; Lunder, 1992). Unfortunately, detailed information on the procyanidin profiles present in many food products is lacking, especially with regard to the more complex oligomeric profiles (Peterson and Dwyer, 1998).

Numerous methods have been developed for routine qualitative analysis of the flavan-3-ols and their corresponding dimers through tetramers, including paper chromatography, thin-layer chromatography (TLC), counter-current chromatography, centrifugal partition chromatography (Okuda et al., 1995), and high-performance liquid chromatography (HPLC). Most of these tech-

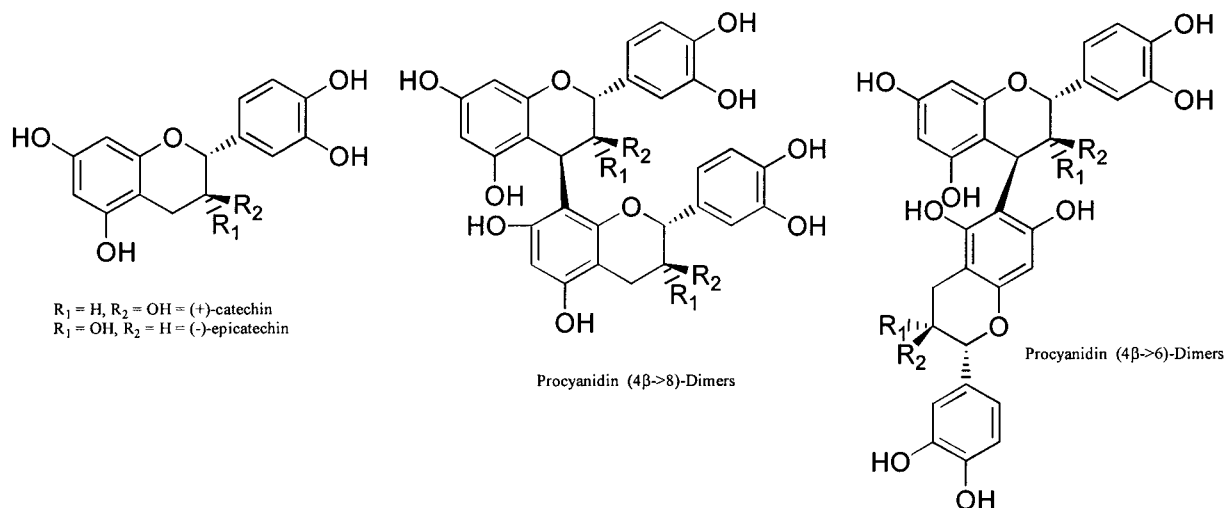
niques have demonstrated the ability to adequately separate the abundant monomers, dimers, and trimers but are unable to resolve the more structurally diverse higher oligomers (Okuda et al., 1989). Traditionally, these larger proanthocyanidin oligomers (i.e., tetramer and higher) were laboriously isolated on the basis of the degree of polymerization using multiple passes through several gel permeation columns. Moreover, TLC and HPLC methods have been described that use silica to effect separation by degree of polymerization and have demonstrated the ability to separate monomeric through heptameric proanthocyanidins as distinct oligomeric classes (Lea, 1978; Rigaud et al., 1993).

Several analytical techniques have been used to obtain structural information necessary to identify proanthocyanidin oligomers, including nuclear magnetic resonance (NMR), mass spectrometry (MS), chemical hydrolysis, circular dichroism, and colorimetric assays (Porter, 1988, 1989). The MS techniques most often used for characterization of proanthocyanidins have been either fast atom bombardment MS (FAB-MS) or liquid secondary ion MS (LSIMS) with fragment ion methods for additional structural information (Porter, 1989). Additionally, electron impact techniques have been utilized for derivatives of low molecular weight compounds. Recently, atmospheric pressure ionization electrospray (API-ES) has been used with fragment ion techniques to aid in structural elucidation of proanthocyanidins in bark from *Byrsonima crassifolia* (Geiss et al., 1995). In general, techniques for structural identification have been used independently of the method used to separate the proanthocyanidins of interest and have not been interfaced with a chromatographic technique that would allow for rapid separation and identification. Two exceptions to this include the use of

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**Figure 1.** Structures of common procyanidins found in cocoa and other foods.

reversed-phase HPLC in tandem with an API-ES quadrupole MS by Baldi et al. (1995) to identify anthocyanidins in red wine and the use of reversed-phase HPLC/MS with a particle beam interface by Facino et al. (1997) to obtain electron impact spectra of rare phenolics in *Krameria triandra* roots.

In cocoa, identification of procyanidins has predominantly been achieved using a variety of chromatographic techniques for separation of oligomers and then using independent methods for structural characterization. Quesnel (1968) and Jalal and Collin (1977) identified procyanidins in cocoa using paper chromatography and TLC methods of analysis, respectively. However, although these papers acknowledged the presence of procyanidins in cocoa, the stereospecific structures of the procyanidins were not identified. More recently, Porter et al. (1991) published a rigorous investigation of procyanidins in cocoa using column chromatography, TLC, HPLC, and negative ion FAB/MS to establish the presence of procyanidin oligomers through heptamers. In addition, the paper confirmed the structures of procyanidin oligomers through tetramers using NMR and found them to consist primarily of (-)-epicatechin. Evidence of cocoa procyanidin oligomers through octamers was reported by Clapperton et al. (1992), who used a combination of column chromatography, reversed-phase HPLC, and positive ion LSIMS to characterize the procyanidin oligomers. This paper demonstrated the utility of positive ion LSIMS and the use of sodium adducts as a means of identifying larger procyanidin oligomers. Later, Rigaud et al. (1993) published a normal-phase HPLC method for the separation of procyanidin oligomers in cocoa and used thiolysis and authentic standards to identify monomers through pentamers. Unfortunately, all of these methods have proven to be laborious and require lengthy preparation times to obtain structural information.

In the present study, the normal-phase HPLC method reported by Rigaud et al. (1993) has been modified and subsequently used to analyze the procyanidin composition by degree of polymerization of the seeds from *Theobroma cacao* and chocolate. More importantly, we report for the first time the coupling of this chromatographic method with API-ES mass spectrometry to simultaneously separate and identify groups of procyanidin oligomers on the basis of their molecular characteristics.

## MATERIALS AND METHODS

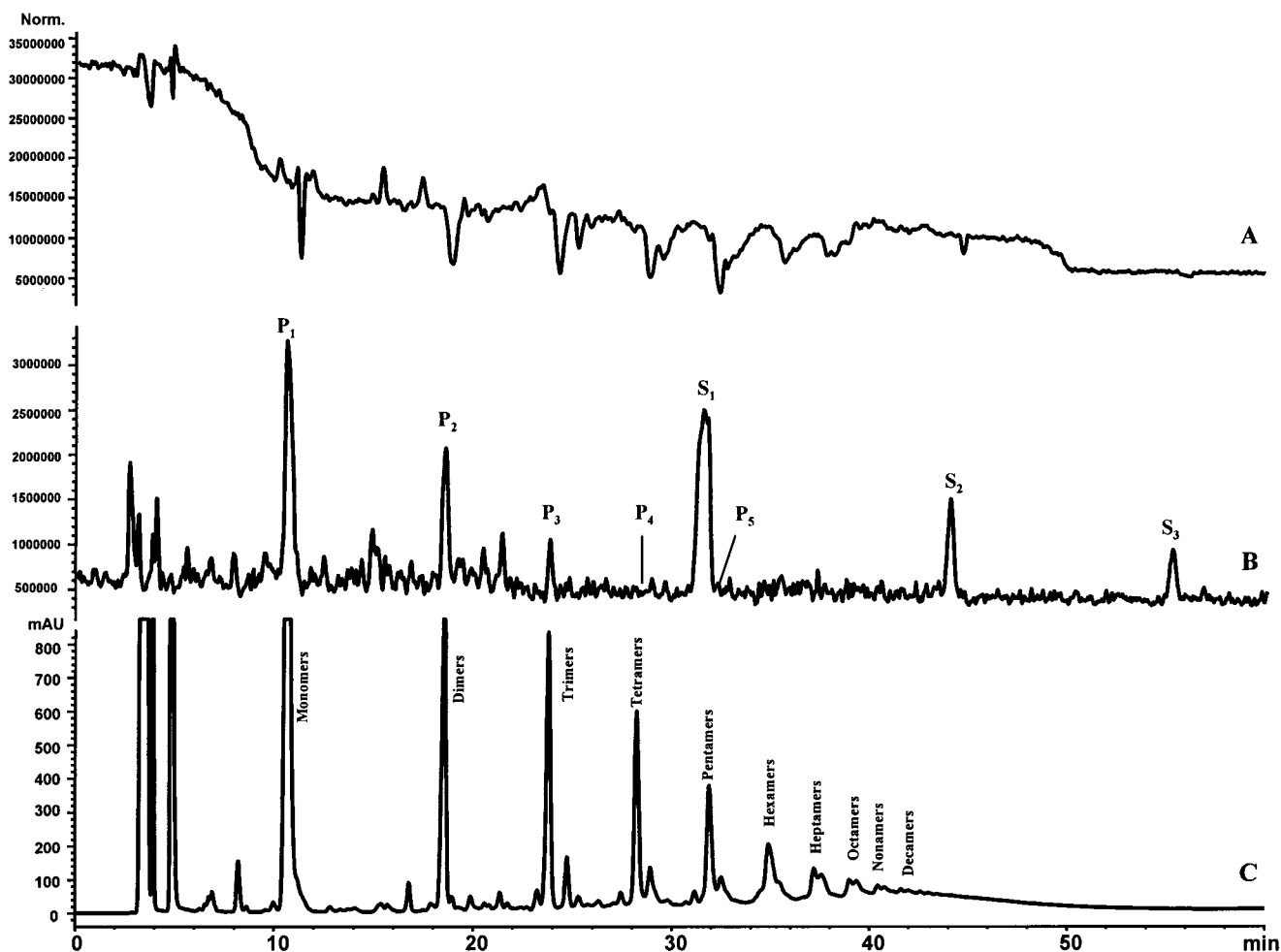
**Samples.** Fresh seeds of *T. cacao* (cocoa) were obtained from the Almirante Center for Cocoa Studies in Itajuípe, Brazil. A dark chocolate was provided by M&M/MARS (Elizabethtown, PA).

**Reference Compounds.** (+)-Maltose monohydrate was purchased from Fluka Chemical (Milwaukee, WI); maltotriose, stachyose hydrate, (-)-epicatechin, quercetin, caffeine, and theobromine were obtained from Sigma Chemical Co. (St. Louis, MO).

**Extraction of Polyphenols from Cocoa and Chocolate.** For extraction of polyphenols from cocoa, the fresh seeds were frozen with liquid  $N_2$  and freeze-dried. The freeze-dried seeds were ground in a high-speed laboratory mill with liquid nitrogen until the particle size was reduced to  $\sim 90 \mu\text{m}$ . Lipids were removed from 100 g of the ground seeds by extracting three times with 450 mL of hexane. The lipid-free solids were air-dried to yield  $\sim 45$  g of fat-free material. A fraction containing procyanidins was obtained by extracting twice with 150 mL of 70 vol % acetone in water followed by two additional extractions with 150 mL of 70 vol % methanol in water. The extracts were combined, and the organic solvent was removed by rotary evaporation under partial vacuum at  $40^\circ\text{C}$ . The aqueous extract was freeze-dried to yield  $\sim 8.5$  g of material.

To obtain procyanidins from chocolate, the lipids were extracted from 45 g of the chocolate as described above using the same ratio of solvent to solids, yielding  $\sim 28$  g of lipid-free material. Ten grams of the fat-free solids was extracted with 50 mL of acetone, water, and acetic acid in a ratio by volume of 70:29.5:0.5, respectively. The solids were pelleted by centrifuging for 10 min at 1500g, and then the supernatant was decanted and the solvent removed by rotary evaporation under the same conditions as above; the resulting aqueous extract was freeze-dried to yield  $\sim 3.5$  g of material.

**Solid-Phase Extraction of Cocoa and Chocolate Polyphenol Extracts.** A Pasteur pipet was packed with Baker (Phillipsburg, NJ) octadecyl packing for flash chromatography to form a bed  $20 \text{ mm} \times 8 \text{ mm}$ . The column was first wetted with methanol and then conditioned with deionized water. Freeze-dried cocoa extract (100 mg) was suspended in 1 mL of water with the aid of sonication. A  $250 \mu\text{L}$  aliquot of the suspension was loaded onto the prewetted column, and the sugars were eluted with 20 mL of water. The phenolics were eluted from the column with 5 mL of a mixture of acetone, water, and acetic acid in a ratio by volume of 70:29.5:0.5, respectively. The column was regenerated and the procedure repeated until the entire 100 mg sample had been passed through the column. The eluates containing the sugars were discarded, whereas the phenolic-containing fractions were combined, concentrated by rotary evaporation, and freeze-dried.



**Figure 2.** HPLC separation of cocoa procyanidins. Comparison of the TIC from API-ES/MS in the positive mode (A), the positive ion TIC of a cocoa extract using NaCl to assist ionization (B), and the UV trace at 280 nm (C). P<sub>1</sub>–P<sub>5</sub> correspond to procyanidin monomers through pentamers, respectively; S<sub>1</sub>–S<sub>3</sub> correspond to di-, tri-, and tetrasaccharides, respectively.

**Table 1.** Timetable Used for Selected Ion Monitoring

time (min)	ions monitored ( <i>m/z</i> )	time (min)	ions monitored ( <i>m/z</i> )
0	289, 290, 291	33	864, 865, 866, 1729, 1730
14	577, 578, 579	36	1008, 1009, 2017, 2018
20	865, 866, 867	38	768, 1152, 1153, 1154, 2305
25	576, 1153, 1154, 1155	40	864, 865, 1296, 1297, 2593
30	720, 1441, 1442, 1443	41	960, 961, 1440, 2881

Similarly, the procedure was repeated using 3 g of freeze-dried chocolate extract suspended in 100 mL of water. Five milliliter aliquots were eluted through 6 mL Supelcosil Envi-18 solid-phase extraction columns (Supelco, Inc., Bellefonte, PA). The carbohydrate-containing fractions were eluted and discarded, and then the procyanidin fractions were eluted, combined, concentrated, and freeze-dried.

**HPLC 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, and HP ChemStation for data collection and manipulation. Normal-phase separations of the procyanidin oligomers were performed on a Phenomenex (Torrance, CA) 5 $\mu$  Luna silica column (25  $\times$  4.6 mm) at 37  $^{\circ}$ C. UV detection was recorded at  $\lambda = 280$  nm. The ternary mobile phase consisted of (A) dichloromethane, (B) methanol, and (C) acetic acid and water (1:1 v/v). Separations were effected 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.

**HPLC/MS Analyses.** HPLC/MS analyses of cocoa and chocolate extracts were performed using an HP 1100 series HPLC as described above and interfaced to an HP series 1100 mass selective detector (model G1946A) equipped with an API-ES ionization chamber. Ionization reagents were 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. For the ammonium hydroxide assisted ionization, the degasser was bypassed on the HPLC pump. Conditions for analysis in the positive ion mode include introduction of 0.05 M sodium chloride at a flow rate of 0.05 mL/min to assist ionization, a capillary voltage of 3.5 kV, the fragmentor voltage at 100 V, the nebulizing pressure of 25 psig, and the drying gas temperature at 350  $^{\circ}$ C. Conditions for analysis in the negative ion mode include 1.5 M ammonium hydroxide as a buffering reagent at a flow rate of 0.09 mL/min for 29 min and then to 0.05 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  $^{\circ}$ C. Data were collected on an HP ChemStation using both scan mode and selected ion monitoring (SIM) with the times and ions given in Table 1. Spectra were scanned over a mass range of *m/z* 100–3000 at 1.96 s per cycle.

## RESULTS AND DISCUSSION

In light of the report by Clapperton et al. (1992), which demonstrated the use of positive ion LSIMS for identifying procyanidin oligomers through octamers in cocoa, attempts were made to optimize the method for positive ions. Using this approach, ionization parameters were optimized using 5  $\mu$ L injections of a 5 mg/

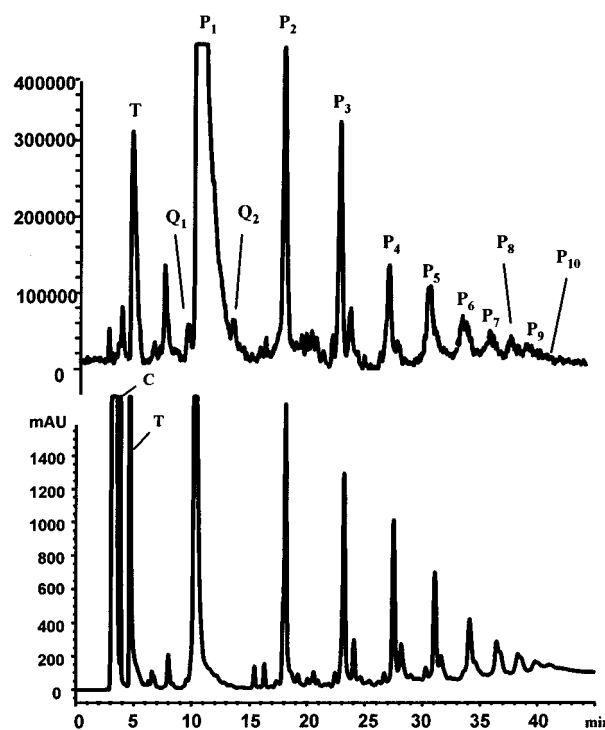
mL solution of (–)-epicatechin (i.e., no analytical HPLC column was in line between the HPLC and MS). Both the molecular ion  $(M + H)^+$   $m/z$  291 and the sodium adduct  $(M + Na)^+$   $m/z$  313 were observed during the ionization optimization process; however, the sodium adduct was by far the predominant ion.

Next, the analytical column was installed in line and the (–)-epicatechin standard injected again. In contrast to the previous observation, only the molecular ion  $m/z$  291 was present and the sodium adduct notably absent, suggesting that sodium was stripped from the molecule as it eluted from the column. As a result, the sensitivity of the system decreased and only a slight peak was observed for (–)-epicatechin in the total ion chromatogram (TIC).

With the ionization parameters optimized for positive ion mode, freeze-dried cocoa extract was reconstituted in acidified aqueous acetone and injected onto the HPLC with both UV and MS (scan mode) data collected. The TIC gave negative peaks (Figure 2A) corresponding to the elution of the procyanidins as recorded by the diode array detector at 280 nm (Figure 2C), up to what was believed to be heptamers. Mass spectral data at these retention times showed molecular ions for monomers through tetramers, with an  $(M + H)^+$  for each oligomer at  $m/z$  291, 579, 867, and 1155, respectively. Negative peaks were believed to be the result of the procyanidins acting as ionization suppressors due to the loss of sodium.

Because the compounds of interest appeared to ionize best in the positive mode in the presence of salt, a system consisting of an auxiliary pump was added in line to the effluent of the HPLC to deliver 0.05 M NaCl just prior to entering the API-ES interface. With the exception of matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS techniques (Chen and Guo, 1997; Ohnishi-Kameyama et al., 1997), addition of salt to the sample matrix is not a common practice in mass spectrometry. This is attributed to the fact that most API-ES sources spray directly at, or slightly off-angle to, the entrance of the mass analyzer, and the addition of a nonvolatile salt would quickly clog the entrance capillary. However, the spray vector for the API-ES source on the HP instrument is at a right angle (orthogonal) to the entrance of the mass analyzer, and only vaporized compound is pulled by the electric field into the capillary, thus allowing for the use of nonvolatile salts.

Ionization parameters were reoptimized for the addition of salt, and the procyanidin extract from unfermented cocoa was reanalyzed. Again, UV data suggested the separation of oligomers up to at least decamers (Figure 2C); however, the TIC indicated the presence of the procyanidin oligomers only through pentamer as shown in Figure 2B.  $(M + Na)^+$  ions for the major peaks labeled  $P_1$ – $P_5$  were  $m/z$  313, 601, 889, 1177, and 1465, respectively. Unexpectedly, ions with  $m/z$  365, 527, and 689 for peaks  $S_1$ – $S_3$  were confirmed using authentic standards as being di-, tri-, and tetrasaccharides, respectively, with the large disaccharide peak almost completely obscuring the peak for pentamers in the TIC. Because the small quantities of sugars that are present in unfermented cocoa (Rohan and Stewart, 1967) gave interfering signals in the mass spectral trace, it was concluded that future analyses, especially those of chocolate extracts, would benefit from a cleanup step to remove or reduce high levels of sugar. Accordingly, a



**Figure 3.** (Top) HPLC/MS trace from a negative ion TIC of a cocoa extract (20 mg/mL; 10  $\mu$ L injection) using  $NH_4OH$  to assist ionization; (bottom) UV trace at 280 nm.  $P_1$ – $P_{10}$  correspond to procyanidin monomers through decamers, respectively;  $Q_1$  and  $Q_2$  correspond to quercetin-3-*O*- $\beta$ -D-glucopyranoside and quercetin-3-*O*- $\beta$ -D-arabinopyranoside, respectively. T represents the peak for theobromine, and C corresponds to caffeine.

solid-phase extraction method using  $C_{18}$  was developed to reduce excess levels of sugars from all samples prior to analysis (see Materials and Methods).

Although the method employing MS detection in the positive mode was successful in detecting procyanidin oligomers through pentamers, it was evident from the decreased ionization efficiency of the higher oligomers that pentamers would be the limit of detection. It had previously been noted by Self et al. (1986) that most polyphenols give superior ionization using the negative ion mode for FAB/MS. Therefore, to develop a mass spectral method that was capable of analyzing all of the procyanidin oligomers eluting from the HPLC column, the classical method of analyzing procyanidins in the negative ion mode was attempted. Using this approach, optimization of MS parameters was performed for negative ionization. However, the injection of a (–)-epicatechin standard resulted in little or no ionization in the negative ion mode regardless of ionization conditions. It was believed that negative ionization was suppressed due to the high concentration of acid in the mobile phase. Therefore, an auxiliary pump was set up in which ammonium hydroxide was utilized as a titrant to adjust the eluant pH to near neutrality. As previously with the salt addition, the strong base (1.5 M  $NH_4OH$ ) was mixed with the eluant just prior to entering the mass spectrometer. Neutralization of the mobile phase was successful in allowing for negative ionization of the (–)-epicatechin standard. Consequently, ionization parameters were optimized for negative ion mode with the (–)-epicatechin standard prior to analysis of sample extracts.

The TIC for cocoa extract is given in Figure 3 and compares favorably with the UV trace at 280 nm. Mass

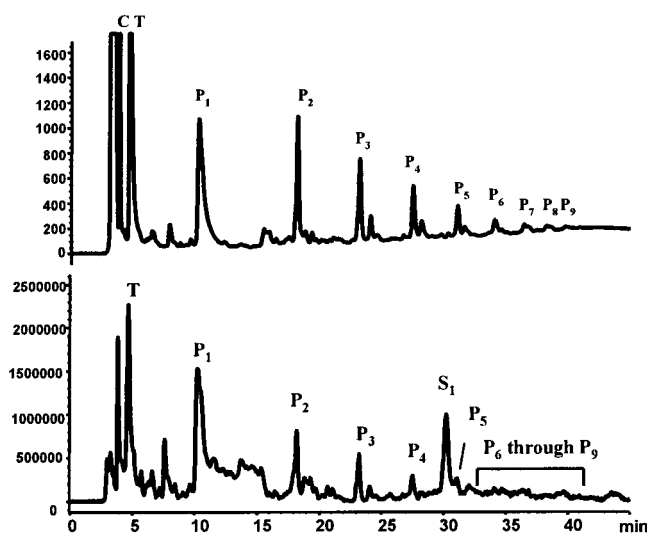
**Table 2. Ions Corresponding to Procyanidins Identified by HPLC/MS Analysis of a Cocoa Extract**

oligomer	peak	molecular ion		
		ion	multiple charges	
		$(M - H)^-$	$(M - 2H)^{2-}/2$	$(M - 3H)^{3-}/3$
monomers	P1	289 (100) <sup>a</sup>		
dimers	P2	577 (100)		
trimers	P3	865 (100)		
tetramers	P4	1153 (100)	576 (33)	
pentamers	P5	1441 (50)	720 (100)	
hexamers	P6	1729 (21)	864 (100)	
heptamers	P7		1008 (100)	672 (33)
octamers	P8		1152 (100)	768 (67)
nonamers	P9		1296 (33)	864 (100)
decamers	P10		1440 (25)	960 (100)

<sup>a</sup> Normalized relative abundance.

spectral data were used to identify the procyanidins, and they have been labeled P<sub>1</sub>–P<sub>10</sub> to correspond to monomers through decamers, respectively. The ions observed for each procyanidin oligomer are shown in Table 2. Molecular ions  $(M - H)^-$  were obtained for the monomers through hexamers with the ions for each oligomer at  $m/z$  289, 577, 865, 1153, 1441, and 1729, respectively. Also, mass spectral data indicated that the higher oligomers ( $\geq$  tetramers) were prone to form multiple charges. For example, doubly charged ions with  $m/z$  576, 720, 864, 1008, 1152, 1296, and 1440 were observed for the peaks corresponding to tetramers through decamers, respectively. In addition, triply charged species were observed for heptamers through decamers at  $m/z$  672, 768, 864, and 960, respectively. The formation of multiply charged species as the degree of polymerization increases has been previously discussed by Guyot et al. (1997). It is interesting to note that for hexamers, while the molecular ion was present, the doubly charged species was the most abundant ion. In addition, although no molecular ions were observed for heptamers through decamers, the doubly charged species was the dominant ion for only the heptamers through octamers. For nonamers and decamers, the triply charged species were the major ions, whereas the doubly charged species were relatively minor in abundance. The normalized relative abundance of each ion corresponding to an oligomer is also indicated in Table 2.

It is important to note that some of these procyanidins have previously been reported by Porter et al. (1991) to exist in the form of glycosides in cocoa. Although minor in comparison to the aglycon forms, ions with  $m/z$  451, 737, and 707 believed to be monomeric glycosides and doubly linked dimeric glycosides from hexose and pentose sugars, respectively, were observed in the TIC of the cocoa extract. However, further analysis with increased voltage for fragmentation would be necessary to confirm the sugar moiety. The monomeric glycosides coeluted with the procyanidin dimers while the dimeric glycosides coeluted with the procyanidin trimers. In addition to the procyanidins, other polyphenolic compounds were observed in the TIC in low abundance. These include ions at  $m/z$  326 and 358, which were found to coelute with the monomers and are believed to be dideoxyclovamide and clovamide, respectively, as reported by Sanbongi et al. (1998). Moreover, ions corresponding to those of quercetin glycosides as first reported by Jalal and Collin (1977) with  $m/z$  433 (Q<sub>1</sub>) and 463 (Q<sub>2</sub>) were observed in the peaks eluting just prior to and after the elution of monomers (Figure 3); however, no evidence for the aglycon was found. In addition to those associated with polyphenols, two other

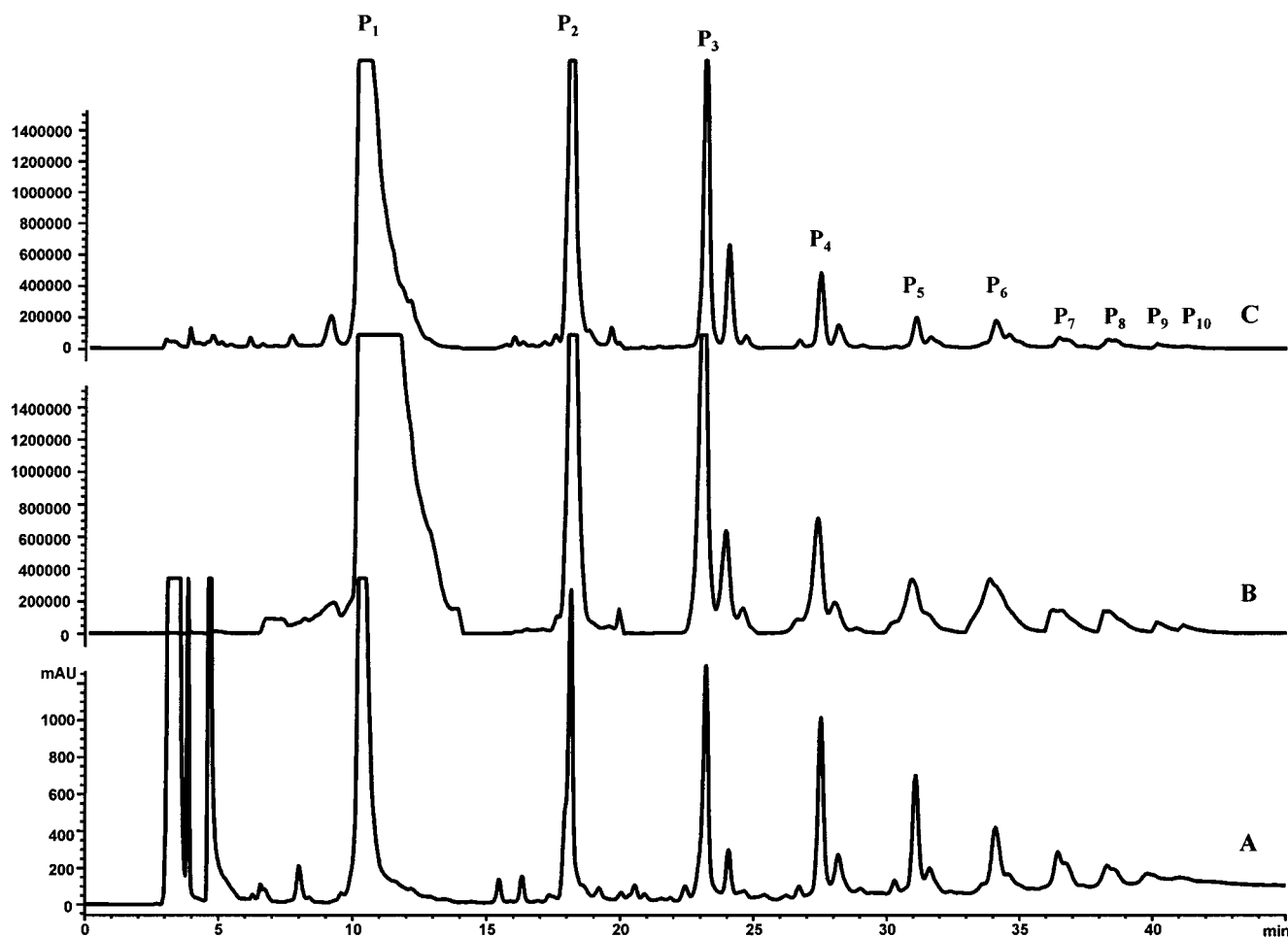


**Figure 4.** (Top) UV trace at 280 nm for chocolate extract; (bottom) HPLC/MS trace from a negative ion TIC of a dark chocolate extract (50 mg/mL; 10  $\mu$ L injection) using  $\text{NH}_4\text{OH}$  to assist ionization. P<sub>1</sub>–P<sub>9</sub> correspond to procyanidin monomers through nonamers, respectively; S<sub>1</sub> is a disaccharide. T is theobromine, and C is caffeine.

peaks were observed in the UV trace of cocoa extract eluting prior to the monomers and were confirmed using authentic standards as being theobromine (T) and caffeine (C). Although no mass spectral data were obtained for the caffeine standard as it did not ionize under these conditions, an ion with  $m/z$  179 was observed in both the cocoa sample and the standard for theobromine.

Next, the dark chocolate extract was analyzed in the same manner as the cocoa extract in the negative ion mode, and, similar to cocoa, this analysis revealed the presence of a complex series of procyanidin oligomers. Once again, the UV trace (Figure 4) indicated the presence of procyanidin oligomers through nonamers and is similar to the UV trace of the cocoa extract (Figure 3). As expected, the relative abundance of the procyanidin oligomers in the chocolate extract was much lower than in the cocoa extract. As shown in Figure 4, the TIC reveals the presence of a large number of compounds not detected by UV at 280 nm due to the use of processed cocoa in combination with other complex ingredients used to make chocolate. Nevertheless, mass spectral data indicated the presence of procyanidins through nonamers. Additionally, it was observed that although the majority of sugar was successfully removed from the chocolate extract prior to analysis, there still remained a small level of sucrose (S<sub>1</sub>) in the TIC. Similar to the cocoa extract, the ions for the procyanidin glycosides were observed in addition to the ions associated with the clovamides, quercetin glycosides, and theobromine. Moreover, although caffeine is not present in the TIC, its presence is indicated on the UV trace for the chocolate extract in Figure 4.

Finally, both the cocoa and chocolate extracts were reanalyzed with the mass spectrometer in the selected ion monitoring (SIM) mode. SIM has the advantage of increased sensitivity in addition to reduced noise from interfering compounds, such as those seen in the TIC of the chocolate extract in Figure 4. The resulting SIM trace for both the cocoa and chocolate extracts are given in Figure 5, and in both cases the SIM trace closely matches the UV trace at 280 nm and eliminates non-



**Figure 5.** Comparison of procyanidins as detected by UV at 280 nm for a cocoa extract (A) to a negative ion SIM trace of a cocoa extract (B) and a negative ion SIM trace of a dark chocolate extract (C). P<sub>1</sub>–P<sub>10</sub> correspond to procyanidin monomers through decamers, respectively.

procyanidin peaks. In contrast to the scan data, use of SIM allowed for the detection of decamers in the chocolate.

In conclusion, we report here for the first time a rapid HPLC/MS method capable of separating and identifying procyanidins present in cocoa and chocolate on the basis of degree of polymerization. Additionally, this HPLC/MS method exploits the novel use of ionization reagents, which was possible due to the orthogonal interface of the MS. Furthermore, using this method, we have confirmed previous data reporting procyanidin oligomers through octamers in cocoa and have reported for the first time a positive identification of nonamers and decamers in raw cocoa and monomers through decamers in chocolate. We are currently investigating the potential for this method to be applied to both qualitative analysis of proanthocyanidins in other food products and quantitative analysis of procyanidins in cocoa and chocolate. Successful application of this method to the analysis of foods such as chocolate will provide compositional data required for accurate characterization of the health benefits, if any, of procyanidins commonly consumed in the diet.

#### ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; MS, mass spectrometry; API-ES, atmospheric pressure ionization electrospray; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; FAB-MS, fast atom

bombardment mass spectrometry; LSIMS, liquid secondary ion mass spectrometry; HP, Hewlett-Packard; SIM, selected ion monitoring; TIC, total ion chromatogram; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight.

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