Fractionation of Polymeric Procyanidins from Lowbush Blueberry and Quantification of Procyanidins in Selected Foods with an Optimized Normal-Phase HPLC–MS Fluorescent Detection Method

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The polymeric procyanidins were fractionated from lowbush blueberry on a Sephadex LH-20 column. The degree of polymerization (DP) for the polymers was determined by thiolysis to be in a range of 19.9 to 114.1. Normal-phase HPLC analysis indicated that the polymeric procyanidins did not contain oligomeric procyanidins with DP < 10. The polymers eluted as a single peak at the end of the chromatogram. The normal-phase HPLC gradient was modified to improve the separation of procyanidin monomers through decamers and to elute all the polymers beyond those as a distinct peak. Monomers through decamers were quantified individually. All the polymers (DP > 10) were quantified using a mixture of purified polymers as an external standard. Polymers were found to be the dominant procyanidins in brown sorghum bran, cranberry, and blueberry. Thiolysis of the polymer peaks indicated that epicatechin was present as extension units in these foods, however, the composition of terminal units varied considerably between catechin and epicatechin, or an A-type dimer linkage in the case of cranberry.

KEYWORDS: Catechin; procyanidin; cocoa; blueberry; cranberry; sorghum

INTRODUCTION

The procyanidins, one subclass of proanthocyanidins, are mixtures of oligomers and polymers consisting of (+)-catechin and/or (−)-epicatechin units linked mainly through C4→C8 and/or C4→C6 bonds (B-type). These flavan-3-ol units can be doubly linked by a C4→C8 bond and an additional ether bond between O7→C2 (A-type) (*1*). Procyanidins are present in common foods including cereals, fruits, vegetables, and wines (*2*). They have been reported to possess a variety of physiological activities such as antioxidant, antimicrobial, anti-allergy, anti-hypertensive, and inhibition of the activities of some physiological enzymes and receptors (*2*).

It has been reported that the lower molecular weight procyanidins are usually present in plant tissue in relatively low concentrations compared to that of larger oligomers and polymers (*3, 4*). The molecular weight of procyanidins expressed as degree of polymerization (DP) is one of the most important properties. We define procyanidins with DP = 1 as monomers, and those with DP = 2−10 and DP >10 as oligomers and polymers, respectively. Compared with the lower molecular procyanidins with DP < 10, there are very few reports of studies of the polymeric procyanidins, partially due to the difficulties in isolating pure higher polymers and the lack of efficient analytical methods.

Normal-phase HPLC can separate procyanidins according to the degree of polymerization up to decamers (*5*). This method has been used by Adamson et al. (*6*) and Hammerstone et al. (*7*) to quantify procyanidins in cocoa, chocolate, apple, and cranberry cocktail. However, the method is specific for monomers and oligomers and results in a severe underestimation of procyanidins, considering the predominance of the polymers in nature (*3, 4*). The procyanidin monomers through octamers (B-type) in lowbush and highbush blueberries have been identified by Prior et al. (*8*) using a normal-phase HPLC−ESI MS method. In the present study, the oligomeric and polymeric procyanidins covering a large range of DP values (1−141.1) were fractionated

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on a Sephadex LH-20 column. The isolated polymeric procyanidins were used as an external standard to quantify all the polymers in the selected food samples using an optimized normal-phase HPLC method.

MATERIALS AND METHODS

Chemicals. Methanol, methylene chloride, and acetic acid (HPLC grade) were purchased from Fisher Scientific (Boston, MA). Sephadex LH-20 is a product of Sigma Chemical Co. (St. Louis, MO). Benzyl mercaptan was obtained from Fisher-Acros (Pittsburgh, PA).

Samples. Freeze-dried lowbush blueberry, which was used as the quality control sample for the USDA flavonoid and procyanidin database project, was prepared from a composite sample of lowbush blueberries. The composite sample represented lowbush blueberry clones from Maine and south Canada in a proportion approximately the same as that present in the commercial market. The frozen berries were freeze-dried and ground in the USDA Food Composition Laboratory (Beltsville, MD). Freeze-dried cranberry was collected and processed in the Department of Biochemistry of Virginia Polytechnic and State University (Blacksburg, VA). Brown sorghum bran was provided by Dr. Lloyd Rooney (Texas A&M, College Station, TX). Blueberry and cranberry samples were shipped frozen on dry ice and kept at −70 °C before use. Cocoa beans were collected from fresh pods, then pulverized while frozen in liquid nitrogen and also supplied in freeze-dried form by Mars Inc. (Hackettstown, NJ).

Reference Compounds. (−)-Epicatechin, (±)-catechin, (−)-epigallocatechin, (−)-gallocatechin, (−)-epigallocatechin gallate, and (−)-gallocatechin gallate were purchased from Sigma Chemical Co. (St. Louis, MO). Seven partially pure procyanidin oligomers (dimers through octamers) were purified from cocoa. Each oligomer contains procyanidin isomers with the same DP. Their constituents have been characterized by Adamson et al. (6) and described in detail by Lazarus et al. (10). Cyanidin-3-O-β-glucoside, delphinidin-3-O-β-glucoside, and malvidin-3-O-β-glucoside were obtained from Polyphenols Laboratories (Sandnes, Norway).

Fractionation of Procyanidin Polymers from Lowbush Blueberry. Freeze-dried lowbush blueberry powder (120 g) was extracted twice (1.5 L each) in sequence with an extraction solvent containing acetone, water, and acetic acid (70:29.5:0.5, v/v). The extracts were combined, and the acetone was evaporated at 50 °C using a rotary evaporator under partial vacuum. The resultant dark red slurry (approximately 700 mL) was extracted with hexane three times (500 mL each) to remove lipid substances. The water slurry was evaporated again on a rotary evaporator to eliminate the residual hexane. The aqueous fraction was diluted with an equal volume of 20% methanol in water (v/v) and applied to a 50 × 4.5 cm Sephadex LH-20 column. Sephadex LH-20 was hydrated for more than 2 h in 20% methanol in water before the column was manually packed by elution with the same solvent. The column was eluted with 3 L of 20% methanol/water (v/v), followed by 3 L of 60% methanol/water (v/v) to remove all the sugars and phenolic acids, and most of the anthocyanins. The lower molecular procyanidin oligomers, including most of the monomers and a portion of the dimers and trimers, can also be washed out in this step. The column was eluted further with 1.5 L of methanol followed by 1.5 L of 70% acetone/water (v/v). The methanol and 70% acetone/water (v/v) eluents were collected in 500-mL fractions to give F1, F2, F3, F4, F5, and F6. Then the column was washed exhaustively with 1 L of 70% acetone/water (v/v) to obtain the fraction F7. A rotary evaporator was used to remove the organic solvent under partial vacuum at 50 °C. The residual concentrated water solutions were freeze-dried to give seven fluffy powders with approximate weights of 152 mg, 455 mg, 189 mg, 165 mg, 186 mg, 143 mg, and 178 mg for F1−F7, respectively. The color of these fractions decreased from deep red for F1 to light pink for F7.

Extraction of Total Procyanidins from Food Sample. A freeze-dried food sample (1 g) was extracted in a 15-mL screw-cap tube with 10 mL of extraction solvent (acetone/water/acetic acid, 70:29.5:0.5, v/v/v). After solvent was added, the tube was vortexed for 30 s followed by sonication at 37 °C for 10 min. The tube was inverted once in the

middle of sonication to suspend the samples. Then the tube remained at room temperature for 50 min. The tubes were vortexed for 30 s after 25 min. Fifty minutes later the tube was centrifuged at 3500 rpm for 15 min. Eight mL of extract was pipetted out, and the acetone was evaporated at 25 °C in a SpeedVac (SC210A, Thermo, Marietta, OH) under vacuum (1.5 Torr). The residue after evaporation of acetone was dissolved in approximately 6 mL of water and applied to a Sephadex LH-20 column. Three grams of Sephadex LH-20 was equilibrated with water over 4 h and manually packed in the 6 × 1.5 cm column. After loading of the sample, the column was washed with 40 mL of 30% methanol/water to remove the sugars and other phenols. All procyanidins were recovered from the column by eluting with 80 mL of 70% (v/v) aqueous acetone. The eluents were evaporated to dryness under vacuum in a SpeedVac at 43 °C. The dried substance was dissolved in the extraction solvent and transferred to a volumetric flask. The final volume was brought up to 5 mL. The solution was filtered with a polypropylene filter unit (0.45 μm) before being injected for normal-phase HPLC−MS analysis.

Normal-Phase HPLC−MS Analyses of Procyanidins. All the quantitative and qualitative analyses were conducted using an Agilent 1100 HPLC system coupled with a Bruker Esquire-LC ion trap mass spectrometer with the same column and detection parameters as previously described (8). The ternary mobile phase consisted of (A) dichloromethane, (B) methanol, and (C) acetic acid and water (1:1 v/v). The 60-min linear gradient was adapted from that of Hammerstone et al. (7) as follows: 0−30 min, 14.0−28.4% B; 30−45 min, 28.4−39.6% B; 45−50 min, 39.6−86.0% B; 50−55 min, 86.0% isocratic; 55−60 min 86.0−14.0% B, followed by 10 min of reequilibration of the column. The modified 65-min linear gradient based on the above was as follows: 0−20 min, 14.0−23.6% B; 20−50 min, 23.6−35.0% B; 50−55 min, 35.0−86.0% B; 55−60 min 86.0% B isocratic; 60−65 min, 86.0−14.0% B, followed by 10 min of reequilibration of the column before the next run. A constant 4.0% C was kept throughout the gradient. The eluting stream (1 mL/min) coming out of the HPLC apparatus was introduced into a Bruker Esquire-LC ion trap mass spectrometer. Enhancement of the ionization of procyanidins using 10 mmol/L ammonium acetate and data acquisition methods have been described previously (8).

Thiolysis of the Procyanidins. A method based on that of Guyot et al. (10) was adopted to perform the thiolysis. Briefly, in a 250-μL polypropylene insert (Fisher Scientific, Boston, MA), 50 μL of solution of each fraction (2 mg/mL in methanol) was mixed together with 50 μL of methanol acidified with concentrated HCl (3.3%, v/v) and 100 μL of benzyl mercaptan (5% v/v in methanol). The inserts were put into a 1.5-mL vial and sealed with an inert Teflon cap (Agilent Technologies, Wilmington, DE). The reaction was carried out at 40 °C for 30 min and then kept at room temperature for 10 h to ensure complete degradation. To minimize further epimerization and any side reactions, the reaction mixtures were kept in the freezer (−18 °C) until 10 μL was injected directly for reversed-phase HPLC analysis. Pure catechin and epicatechin solutions (1.25 mg/mL in methanol) were thiolyzed in each batch to obtain the epimerization rate to calculate the ratio of catechin and epicatechin in the terminal units.

Hydrolysis of Polymers in HCl/Butanol. Polymeric procyanidin fractions (F5, F6, and F7) were dissolved in methanol (1.0 mg/mL). Each solution (0.5 mL) was mixed with 0.5 mL of 10% HCl in 1-butanol. The mixture was heated at 100 °C in a water bath for 50 min.

Reversed-Phase HPLC−ESI MS Analyses of Thiolysis Media and Hydrolysis Products of Polymers. Separation of procyanidin thiolysis media and the products (anthocyanidins) after hydrolysis in butanol/HCl was performed on the same Agilent-Bruker system used for procyanidin analyses. A 250 × 4.6 mm i.d., 5 μm, Luna C18 column (Phenomenex, Torrance, CA) was used at 25 °C. For thiolysis media, the solvent system consisted of A (2% acetic acid in water, v/v) and B (methanol), which were delivered by an Agilent 1100 binary pump in a linear gradient of B from 15% to 80% (v/v) in 45 min. For hydrolysis products, solvent A was replaced with 5% (v/v) formic acid in water and the linear gradient was 15−55% B in 45 min. The detection wavelength of the diode array detector was set at 280 nm (6 nm breadth) for catechin, epicatechin, and their benzylthioethers, and 520 nm (10

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Table 1. Epimerization of Catechin and Epicatechin in the Thiolysis Procedure and the Total Recovery Rate of the Initial Compounds (Mean ± SD, $n = 3$)

initial compound	proportion after thiolysis (%)		recovery rate (%) (catechin + epicatechin)
	catechin	epicatechin	
catechin	91.0 ± 0.7	9.0 ± 0.7	64.9 ± 8.3
epicatechin	28.7 ± 1.5	71.3 ± 1.5	67.8 ± 5.4
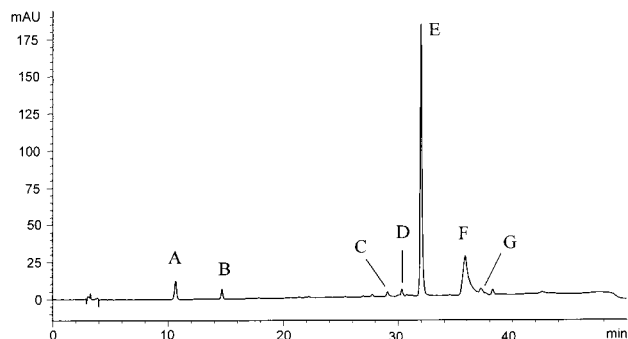
Figure 1. Chromatogram of thiolysis media of blueberry procyanidins (fraction F2) detected at 280 nm (A). Peaks A–G were identified with the aid of MS/MS, and their structures are illustrated in **Figure 2**.

nm breadth) for anthocyanidins. The wavelengths used in the fluorescence detector were the same as those used for procyanidins. Components in the thiolysis media were identified with the mass spectrometer in negative mode using a setting of 50% for compound stability, and 25% for ion trap drive. Positive mode ESI MS was employed to identify anthocyanidins in hydrolysis products with 50% for compound stability, and 80% for ion trap drive level. Target mass was set at 300 m/z.

UV/Vis Spectrophotometry of the Polymers. Solutions of procyanidin fractions (F5−F7) in methanol (2 mg/mL) were diluted with an equal volume of 10% (v/v) formic acid in water. The spectra were immediately determined on a Shimadzu UV-3101PC spectrophotometer (Columbia, MD). The associated anthocyanins were estimated using the molar extinction coefficient of malvidin-3-*O*-β-glucoside at 525 nm under the same conditions.

Elemental Analysis of the Polymers. Carbon, hydrogen, and nitrogen analyses of fraction F5 were performed on a CE-440 Elemental Analyzer (Exeter Analytical, Inc., North Chelmsford, MA). Oxygen was analyzed using the Unterzaucher method as described by Kirsten (*11*).

RESULTS AND DISSCUSSION

Thiolysis of Procyanidins. In thiolysis reactions, all the extension subunits of procyanidins are attacked by benzyl mercaptan to form the corresponding benzylthioether. Only the terminal unit is released as the free flavan-3-ol. The reaction was catalyzed by acid under mild conditions, so that the catechin 3-*O*-gallate units in the procyanidins were preserved. The extension units (benzylthioethers) and terminal units of procyanidins after thiolysis can be distinguished by HPLC analysis (**Figure 1**). The response factors of catechin and catechin benzylthioether were the same using 280 nm detection, as well as for epicatechin and epicatechin benzylthioether (*12*). Thus, catechin and epicatechin can be used as standards to quantify their benzylthioethers, and the mean DP can be calculated by comparing the peak areas based on the following equation.

DP = {total area of catechin benzylthioether and
epicatechin benzlthioether}/{total area of catechin and
epicatechin} + 1

Solutions of (±)-catechin and (−)-epicatechin were thiolyzed according to the thiolysis procedure to evaluate the extent of epimerization, as reported by Prieur et al. (*13*). The results are presented in **Table 1**. No catechin benzylthioether and epicatechin benzylthioether was detected after pure flavan-3-ol was thiolyzed. In the thiolysis media of pure catechin, both epicatechin and catechin were detected with an area ratio of (9.0 ± 0.7):(91.0 ± 0.7) at 280 nm detection, indicating that 9.0 ± 0.7% of the catechin was epimerized into epicatechin under the experimental conditions. The epimerization rate was calculated as 28.7 ± 1.5% for epicatechin. The low overall recovery rate of catechin (64.9%) and epicatechin (67.8%) is due to the heterocyclic ring cleavage induced by benzyl mercaptan, which has been reported by Betts et al. (*14*) and also observed by Matthews et al. (*15*). The epimerization rates of epicatechin and catechin were reported as 15% and 4% by Prieur et al. (*13*), who conducted the thiolysis in a similar system but with higher temperature (90 °C) for a very short time (2 min). Epimerization rates of flavan-3-ols of the same order were also noticed by Matthews et al. (*15*) using acetic acid as the catalyst. Prieur et al. (*13*) suggested that the proportion of catechin in terminal units would be overestimated because of the lower epimerization rate of catechin compared to that of epicatechin. This implied that the estimation of terminal units of procyanidins would never be accurate since the epimerization is inevitable. However, if the epimerization rate of catechin and epicatechin remained stable under specific reaction conditions, the proportion of catechin and epicatechin in the terminal unit could be calculated with precision based upon the hypothesis that the released terminal catechin or epicatechin would behave similarly to pure compounds in the thiolysis system. For example, if the proportion of catechin and epicatechin are α% and (100 − α)%, respectively, in the thiolysis media of a procyanidin sample, while the actual proportion of catechin and epicatechin in terminal units in this sample are X% and (100 − X)%, then the observed α equals the actual amount of catechin (X) minus that which was epimerized into epicatechin, plus the catechin epimerized from the actual amount of epicatechin (100 − X).

The resulting equation is $X − X(9.0\%) + (100 − X)(28.7\%) = α$, in which 9.0% and 28.7% are the epimerization rates of epicatechin and catechin listed in **Table 1**. The equation is simplified to be $X = 1.605α − 46.067$.

Catechin benzylthioether and epicatechin benzylthioether did not epimerize to each other as been reported by Prieur et al. (*13*). This made it easy to characterize the extension unit of procyanidins. Three partially pure oligomers, hexamers through octamers, which were purified from cocoa and contained procyanidin isomers with the same DP, were thiolyzed to test our hypothesis and validate the thiolysis method for procyanidins analysis (**Table 2**). No catechin benzylthioether was detected by HPLC−MS in thiolysis products, indicating that the extension units of these partially pure oligomers consisted exclusively of epicatechin. The actual proportion of epicatechin in terminal units was calculated to be higher than 98%, which was in excellent agreement with the observation that procyanidins in unprocessed cocoa consisted of epicatechin as monomeric units (*6, 16*). In contrast to procyanidins in cocoa, the polymeric procyanidins in brown sorghum bran were found to consist of mostly catechin (88.5%) as the terminal unit based upon our method (**Table 6**). Gupta et al. (*17*) and Schofield et al. (*18*) reported that procyanidins in sorghum consisted of epicatechin as extension units and catechin as terminal units, which was basically consistent with our data considering possible variety differences. The calculated proportion of catechin and epicat-

Table 2. Validation of Thiolysis Method Using the Partially Pure Hexamers, Heptamers, and Octamers Purified from Cocoa (Mean ± SD, $n = 3$)

| | proportion of extension units % | | proportion of terminal units % | | | |
| | catechin benzylthioether | epicatechin benzylthioether | catechin | epicatechin | | |
oligomers					DP value	yield %[b]
hexamers	ND[a]	100	1.3 ± 3.0	98.7 ± 3.0	7.3 ± 0.2	64.5 ± 2.4
heptamers	ND	100	−0.6 ± 2.5	100.6 ± 2.5	8.0 ± 0.3	62.5 ± 13.6
Octamers	ND	100	0.1 ± 1.4	99.9 ± 1.4	9.0 ± 0.4	54.0 ± 15.6

[a] ND, not detected. [b] Yield was based on the weight of each oligomer used for thiolysis.

Table 3. Thiolysis Results of Different Blueberry Procyanidin Fractions Eluted from Sephadex LH-20 Column (Mean ± SD, $n = 3$)

| | proportion of extension units % | | proportion of terminal units % | | | |
| | catechin benzylthioether | epicatechin benzylthioether | catechin | epicatechin | | |
fraction					DP value	yield %[b]
F1	ND[a]	100	67.3 ± 1.2	32.7 ± 1.2	7.2 ± 0.3	28.1 ± 2.9
F2	ND	100	66.9 ± 0.3	33.1 ± 0.3	10.3 ± 0.0	55.6 ± 1.5
F3	ND	100	68.3 ± 0.4	31.7 ± 0.4	13.4 ± 0.1	59.4 ± 10.2
F4	ND	100	67.3 ± 0.5	32.7 ± 0.5	19.9 ± 0.2	49.6 ± 7.5
F5	ND	100	65.3 ± 0.5	34.7 ± 0.5	36.1 ± 0.1	39.9 ± 1.2
F6	ND	100	69.0 ± 0.5	31.0 ± 0.5	58.9 ± 1.6	59.4 ± 3.9
F7	ND	100	67.4 ± 1.0	32.6 ± 1.0	114.1 ± 2.0	55.4 ± 7.8

[a] ND, not detected. [b] Yield was based on the weight of each fraction used for thiolysis.

Table 4. Overall Recovery Rates of Spiked Monomers through Decamers Including the Polymers for the Extraction and Purification Procedures (Mean ± SD, $n = 3$)

constituent	recovery rate (%)	constituent	recovery rate (%)
monomers	96.2 ± 1.9	heptamers	83.3 ± 3.5
dimers	97.6 ± 0.7	octamers	83.2 ± 3.8
trimers	96.1 ± 1.0	nonamers	85.5 ± 2.7
tetramers	93.4 ± 1.1	decamers	87.0 ± 2.4
pentamers	89.7 ± 1.5	polymers	81.2 ± 1.7
hexamers	85.2 ± 3.3		

echin in the terminal units is much closer to the actual value using this method, and the overestimation of catechin was mostly avoided. The epimerization rates of catechin and epicatechin were dependent on time and experimental conditions; thus, it was necessary to thiolyze them in each batch in order to obtain accurate data.

The yield of thiolysis based on weight was in a range of 54.0 to 64.5%, similar to those of other authors (*12*). The calculated DP values were 7.3 ± 0.2, 8.0 ± 0.3, and 9.0 ± 0.4 for hexamers, heptamers, and octamers, respectively, which was one unit higher than the actual values, partially because of the contaminant higher oligomers in the purified oligomers (*9*). Another explanation for this discrepancy, as well as the low yield on thiolysis, is the existence of the side reaction of thiolysis. It was demonstrated by Betts et al. (*14*) that the mercaptoacetic acid analogue of benzyl mercaptan could attack both position 4 and position 2 of flavan-3-ols, which would lead to cleavage of the interflavan bond and heterocyclic ring, respectively. The components in the thiolysis media were separated by HPLC (**Figure 1**) and their structures and characteristic fragment daughter ions were studied using MS/MS (**Figure 2**). Peaks D and G in **Figure 1** were the heterocyclic ring cleavage products of terminal units and the extension units, respectively. They contributed 2.7% and 2.8% of the total area on the chromatogram. It suggested that the side reaction on terminal units was more significant than that on extension units, which may also lead to a calculated DP higher than the actual value.

The validated thiolysis method was employed to characterize all the procyanidin fractions obtained from blueberry, and the data are presented in **Table 3**. The extension units of blueberry procyanidins consisted of only epicatechin, whereas both catechin and epicatechin were found on terminal units with a stable proportion for all fractions (67:33 on average). These data agree with those of Foo et al., who demonstrated that polymeric procyanidins in blueberry consisted of 96% epicatechin and 4% catechin using ^{13}C NMR method (*19*).

Heterogeneity of Fractionated Oligomers and Polymers on Normal-Phase HPLC. The mean DP values determined by thiolysis do not reflect the heterogeneity of the procyanidin mixture. However, an indication of this heterogeneity can be seen in the normal-phase HPLC analysis. The normal-phase HPLC fluorescence profiles of all seven fractions are illustrated in **Figure 3**. The distribution of oligomers in F1 to F3 was consistent with the mean DP obtained by thiolysis (7.2−13.4). No procyanidins below decamers were detected in the polymeric procyanidin fractions (F4, F5, F6, and F7). All the polymers showed a similar profile with a single peak at the end of the run with a retention time of approximately 50.6 min. Those peaks at 50.6 min representing polymers were washed out of the silica column with 5 min (50−55 min) of isocratic elution with solvent containing 86% methanol. This peak of polymeric procyanidins was small for fraction F1, but increased until it became a single peak for fraction F4 and thereafter. The procyanidins up to dodecamers have been resolved on normal-phase HPLC (*20*) in previous studies. However, no procyanidins with DP higher than those can be separated. Given the fact that most of the procyanidins in plants are polymers instead of oligomers (*3*, *4*), the position of procyanidins with DP > 12 on the normal-phase HPLC remains a problem. This study clearly indicated that the polymeric procyanidins with DP > 13.4 could not be resolved by normal-phase HPLC, but were eluted as one single peak at the end of the run. By searching the literature, an extra hump at the end of chromatograms can be observed in most of the papers using normal-phase HPLC to separate procyanidins in cocoa, grape seed (*21*, *22*), and apple (*10*). Except for the hump in the chromatogram of grape seed, which was extrapolated by Waterhouse et al. (*21*) to have a DP of 24

Table 5. Content of Procyanidins in Freeze-Dried Cocoa, Brown Sorghum Bran, Cranberry, and Lowbush Blueberry (mg/g, Mean ± SD, *n* = 2)

constituent	cocoa	brown sorghum bran	cranberry	blueberry
monomers	14.24 ± 0.38	0.30 ± 0.05	0.48 ± 0.05	0.18 ± 0.01
dimers	8.57 ± 0.51	0.83 ± 0.17	1.53 ± 0.21	0.46 ± 0.02
trimers	8.10 ± 0.49	1.34 ± 0.07	1.44 ± 0.06	0.38 ± 0.02
tetramers	8.89 ± 0.54	1.47 ± 0.09	1.46 ± 0.52	0.50 ± 0.01
pentamers	8.86 ± 0.52	1.94 ± 0.07	1.39 ± 0.36	0.47 ± 0.01
hexamers	9.99 ± 0.61	2.30 ± 0.15	2.04 ± 0.20	0.69 ± 0.05
heptamers	6.38 ± 0.38	1.68 ± 0.08	1.56 ± 0.16	0.48 ± 0.02
octamers	5.97 ± 0.31	1.71 ± 0.13	2.17 ± 0.20	0.61 ± 0.03
nonamers	7.36 ± 0.58	2.15 ± 0.22	UI[a]	0.93 ± 0.02
decamers	3.22 ± 0.23	1.19 ± 0.12	UI	UI
polymers	16.17 ± 0.80	32.13 ± 3.57	20.58 ± 0.75	15.28 ± 0.51
total	97.76 ± 5.32	47.05 ± 3.18	32.65 ± 1.49	19.99 ± 0.43

[a] UI: Unable to identify and integrate the specific oligomers due to overlapping peaks. The area was summed into and calculated as polymers. All the data have been corrected by the overall recovery rates listed in **Table 4**.

Table 6. Constituent Units of Polymeric Procyanidins Collected from Normal-Phase HPLC (Mean ± SD, *n* = 2)

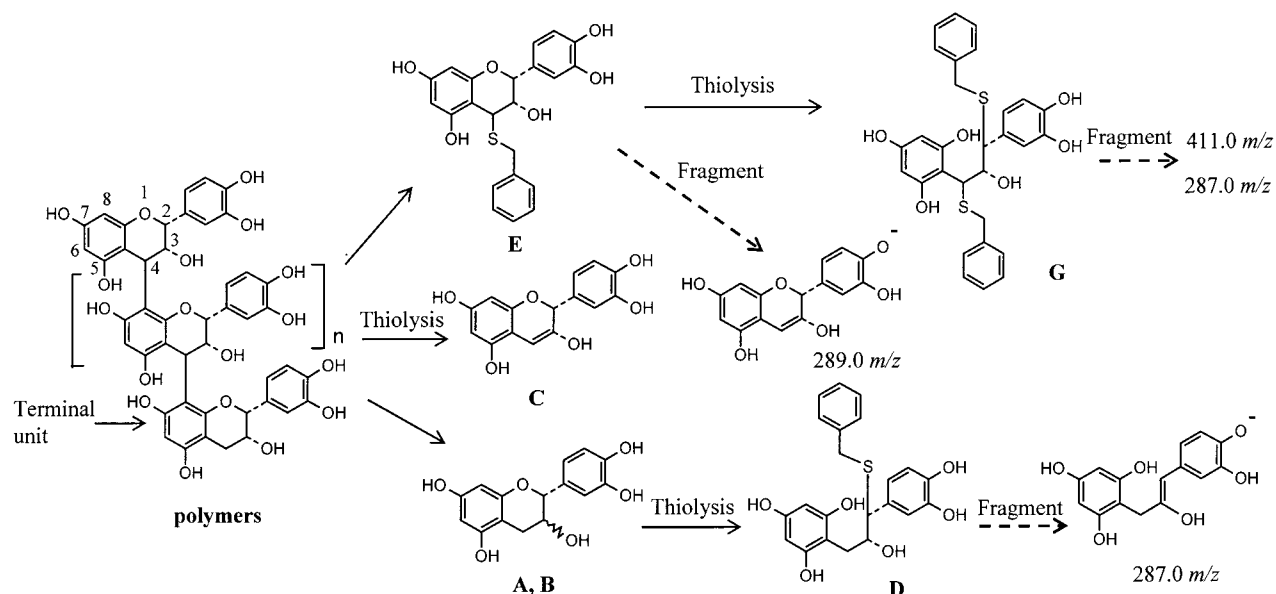|	proportion of extension units %		proportion of terminal units %		A-type	
sample	catechin benzylthioether	epicatechin benzylthioether	catechin	epicatechin	terminals	DP
cocoa	ND[a]	100	−0.2 ± 2.5	100.2 ± 2.5	ND	13.9 ± 0.4
brown sorghum bran	ND	100	88.5 ± 0.2	11.5 ± 0.2	ND	13.5 ± 0.2
lowbush blueberry	ND	100	67.3 ± 1.1	32.7 ± 1.1	ND	38.8 ± 3.5
cranberry	ND	100	7.4 ± 0.1	46.5 ± 0.8	46.1 ± 0.9	15.3 ± 0.2

[a] ND, not detected.

Figure 2. Components in the thiolysis media of procyanidins and their fragment daughter ions on MS/MS. All terminal units in polymers were released as catechin (A) or epicatechin (B). Most of the extension units were degraded into flavan-3-ol benzylthioether (E) while a small amount of flav-3-ene (C) was formed from flavan-3-ol carbocation intermediates. The heterocyclic ring of flavan-3-ols (A, B) and flavan-3-ol benzylthioether (E) could be cleaved by benzyl mercaptan to give rise to (D) and (G). Peak F in **Figure 1** was benzyl mercaptan.

according to the retention times of lower oligomers, they have generally been neglected. Using thiolysis, Cheynier et al. (*23*) located the polymeric procyanidins on the normal-phase HPLC chromatogram. However, the polymers were found to spread all over the chromatogram rather than forming a distinguishable peak, and their chromatograms showed no consistency.

Purity of Polymeric Procyanidins. The purity of procyanidins fractionated from lowbush blueberry need to be tested before use in physiological studies or as standards for quantification. Monitoring of the normal-phase HPLC eluent at different wavelengths did not reveal any peaks corresponding to chlorogenic acids and other flavonoids in fractions F1 through F7. Their absence was also confirmed by the assay of thiolysis media on reversed-phase HPLC.

The red pigments in the fractionated procyanidins suggested the coexistence of anthocyanins as impurities, which could associate with procyanidins through covalent and noncovalent bonding as has been discussed by Kennedy et al. (*24*). The UV/vis spectra of the polymer measured in acidified methanol gave a λ_{uvmax} = 280 nm, λ_{min} = 410 nm, λ_{vismax} = 530 nm, and E_{280}/E_{530} in the range of 7 to 11. This profile largely resembled that of catechin, and the absorption at 530 nm was caused by the

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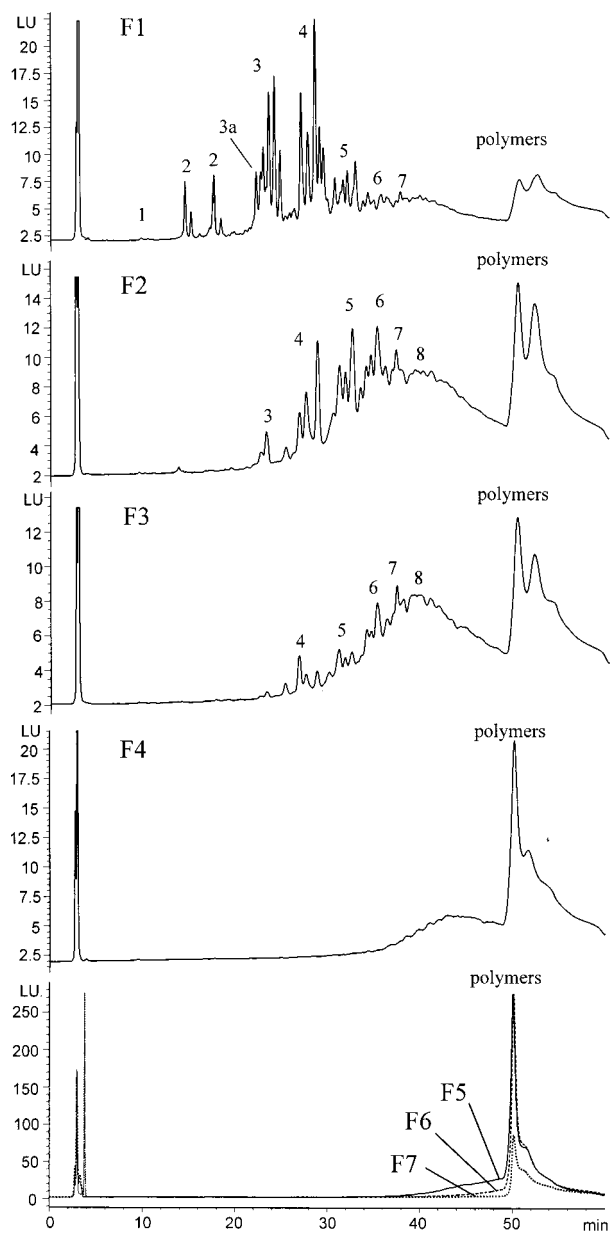*J. Agric. Food Chem.,* Vol. 50, No. 17, 2002   **4857**

Figure 3. Normal-phase HPLC fluorescence trace of seven procyanidin fractions eluted from Sephadex LH-20 column. F1 was the first fraction and F7 was the last. The numbers beside the peaks indicate the degree of polymerization of B-type procyanidins, i.e., 3a is the peak of trimers with one A-type linkage identified by the ESI MS ([M − H]$^{-1}$ 863.3 *m/z*).

associated anthocyanins. The E_{440}/E_{vismax} values have been suggested as being characteristic for anthocyanins, with a value of 30% observed for pure cyanidin-3-*O*-β-glucoside and malvidin-3-*O*-β-glucoside. However, a value around 67% was observed for procyanidin polymers. The difference between them might be caused by impurities other than simple anthocyanins or a co-pigmentation effect (*24*). Cyanidin was detected as the predominant component after the polymers were hydrolyzed in HCl/butanol. Peonidin, delphinidin, petunidin, and malvidin were also detected. The total amount of anthocyanins in procyanidin polymers was calculated in a range of 0.5−2.4% (w/w) using the molecular absorption coefficient of malvidin-3-*O*-β-glucoside. This level of associated anthocyanins was in agreement with that of Kennedy et al. (*24*). The anthocyanins associated with procyanidins polymers should be released from the polymers with intact glycoside structure after thiolysis. However, no signals were detected that correlated to

them in the thiolysis products. There are over 25 anthocyanins in lowbush blueberry (*8*). The level of each may be too low to be detected.

Protein could bond tightly with procyanidins and was detected as a major impurity by Hagerman et al. (*25*). The elemental analysis of one polymer fraction (F5) showed that the weight percentages of carbon, hydrogen, oxygen, and nitrogen were 54.91 ± 0.04%, 4.38 ± 0.05%, 35.98%, and 0.15 ± 0.05% (w/w, mean ± SD, $n = 2$), respectively. This amount of nitrogen corresponded to 0.9% (w/w, calculated using $N \times 6.25$) impurity as proteins. The proportion of atom numbers in F5 was rounded into 15:14.4:7.4 (carbon/hydrogen/oxygen), whereas the theoretical ratios for polymers are 15:12:6. This discrepancy is caused by the moisture content in procyanidins, which has been reported previously (*4*). Kennedy et al. (*24*) demonstrated that pectin could be associated with proanthocyanidins through noncovalent and covalent bonds based on MS data. There were no signals detected corresponding to any kind of carbohydrate in the thiolysis media or in the complete hydrolysis products. It should be noted that the procyanidins in glycoside form are rare in nature, and the covalent linkage between pectin and procyanidins has never been found before. More information is needed to confirm the existence of this novel structure.

Metal ions were not significant impurities in procyanidins according to the inductively coupled plasma (ICP) MS assay of procyanidins extracted from cocoa. Taking all these possibilities into account, over 90% purity of these polymers could be obtained.

Optimization of Normal-Phase HPLC Method. To quantify procyanidins in foods with accuracy and consistency, the normal-phase HPLC method was carefully investigated and optimized.

Extraction and Purification. Aqueous acetone (70% v/v) has proven to be the most potent overall extraction solvent for proanthocyanidins (*26*). Use of acidified aqueous acetone increased the extractability of procyanidins by weakening the hydrogen bond between procyanidins and polar fibrous matrixes (*27*). The pH of our acidified aqueous acetone was determined to be 3.6. Procyanidins have been reported to be stable at this pH (*28*). Incubation of procyanidin extracts in this solvent saturated with nitrogen at 50 °C for 60 min did not result in any significant profile change or degradation (data not presented). The purification procedure on a small Sephadex LH-20 column demonstrated that 40 mL of 30% aqueous methanol (v/v) washed out half of the anthocyanins and chlorogenic acid from blueberry extracts, with bleeding of monomers being less than 4.0%. A one-step elution using 70% aqueous acetone (v/v) was employed to recover all the procyanidins from the LH-20 column. Monitoring the 70% acetone/water eluents in every 10 mL, using normal-phase HPLC, demonstrated that procyanidin monomers, oligomers, and polymers were washed out of the column simultaneously. Most of the procyanidins could be recovered from Sephadex LH-20 with the first 40 mL of 70% aqueous acetone (v/v). Some polymers adsorbed so tightly on the LH-20 that another 40 mL of solvent was needed to elute them from the column and make them undetectable in the eluent by HPLC.

Gradient Modification. The proportion of methanol in the 50-min linear gradient previously applied by Hammerstone et al. (*7*) was 0−30 min 14−28.4%, 45 min 39.2%, and 50 min 86%. This gradient was cut from the original 70 min gradient of Riguard et al. (*22*), and was suitable for the separation of monomers through decamers (*8*). This gradient was adapted into a 60-min gradient in order to wash out the peak of polymers
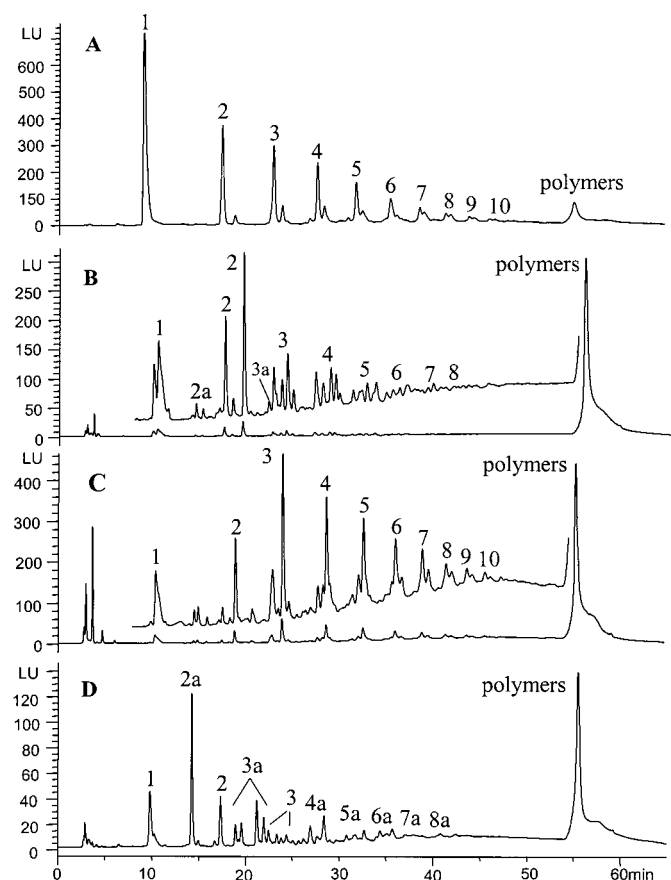
Figure 4. Normal-phase HPLC fluorescence trace of procyanidins extracted and purified from cocoa (A), lowbush blueberry (B), brown sorghum bran (C), and cranberry (D). The numbers beside the peaks indicate the degree of polymerization of B-type procyanidins. 2a–8a Designate the peaks of procyanidin dimers through octamers with one A-type linkage.

by adding a 5-min isocratic elution with solvent containing 86% methanol followed by another 5 min to decrease the methanol level to the initial proportion (14%). The retention times for hexamers and decamers in cocoa were 34.0 and 40.8 min. The peaks were very close to one another, making it difficult to separate samples with complicated constituents, such as those of blueberry and cranberry. The same phenomena were also noticed by Cheynier et al. (*23*). According to their reports, procyanidins in apple were resolved up to pentamers using their regular gradient (identical to that of Hammerstone's in the first 40 min), whereas nonamers could be separated by applying a slower gradient of methanol in the solvent system. To improve the separation of hexamers through decamers, a 65-min gradient was proposed, in which the ramp of methanol was slowed by using a linear increase of methanol from 23.6 to 35% between 20 and 50 min. The gradient increased sharply to 86% methanol at 55 min, followed by 5 min of isocratic elution to wash out all the procyanidins beyond decamers. The retention times of hexamers and decamers were 34.0 and 44.7 min, respectively, in the optimized gradient. The new gradient also resulted in a sharper and higher peak of polymers, suggesting a lower detection limit.

Baseline Shift and Integration Method. A dramatic upward baseline shift can be observed on the chromatograms of all the selected foods (**Figures 3** and **4**). The extent varies among samples. A blank run indicated that an increase of methanol in the solvent system from 14% to 86% led to an upward shift of 2.5 units in the fluorescence reading. The baseline shift caused

by solvent is so small in the actual analysis that the chromatogram of a blank run appears virtually as a straight line. No phenols other than procyanidins produced a fluorescence signal under the given detection method. It can be deduced that the baseline shift is caused by peak broadening and overlapping of procyanidins. ICP−MS assay revealed that the level of aluminum, copper, or iron ions in procyanidins extracted from cocoa were 5.0, 22.8, and 12.6 ppm respectively. Cocoa procyanidins treated with EDTA did not show a significant change in normal phase HPLC chromatogram, suggesting the peak broadening was not caused by metal ions (Mark Kelm, unpublished data). The peak breadth of certain oligomers was solely induced by the number of isomers, which increased exponentially with the degree of polymerization. This can be confirmed by the analysis of the partially pure oligomers. On the basis of this discussion, we proposed a flat baseline integration method to replace the valley-to-valley integration method used previously (*6, 7*). In the valley-to-valley integration method, a straight line was drawn between the lowest points of the valley between adjacent peaks. The enclosed area was integrated. In the flat baseline integration method, a flat baseline was drawn from the beginning of the run until 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 area of the oligomers with the same DP were integrated and quantified individually. The area beyond decamers was integrated and quantified as total polymers. To compare the two integration methods, the solutions of partially pure hexamers and heptamers (1 mg/mL) were spiked into a purified cocoa procyanidin sample and separated on normal-phase HPLC. The area recovery rates of hexamers and heptamers were calculated to be 70.3 ± 2.8% and 61.1 ± 2.6% using the flat baseline integration, in comparison to 46.5 ± 1.2% and 31.9 ± 1.6% (mean ± 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 more remarkable baseline shift due to isomers, like blueberry and cranberry.

The fractionated blueberry procyanidins (fraction F5; DP = 36.1) was used as the polymer standard. The standard curves for monomers through decamers and polymers showed good linearity ($R > 0.98$) and an intercept close to zero. The detection limit of procyanidins using 280 nm recording is in a range of 9.0−11.0 ng (injection on column), in comparison to 2.2 ng reported by Rohr et al. (*29*) and Donovan et al. (*30*) for catechin. The detection limits using fluorescence recording were in a range of 0.023−0.064 ng, nearly one hundred times lower than that with UV detection. A much higher fluorescence detection limit of catechin (3 ng) was reported by Donovan et al. (*30*) using different excitation and emission wavelengths at 280 and 310 nm.

To test the overall recovery rate of our quantification method, the composite procyanidin standard and the standard polymers were spiked into rice powder (1 g) in a 15-mL screw-cap tube. The contents of these tubes were then extracted, purified, and analyzed with the procedure as described above. The overall recovery rates of oligomers and polymers are listed in **Table 4**. Rice was chosen as a representative food sample because it contains a well-defined composition and no procyanidins.

Quantification of Procyanidins in Selected Foods. Procyanidins were analyzed on normal-phase HPLC and the final quantities of each oligomer and polymer were corrected using the overall recovery rates. The chromatograms are illustrated

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in **Figure 4**. The data are presented in **Table 5**. The polymers were found to be the predominant procyanidins in all the selected foods except for cocoa.

The peaks of polymeric procyanidins, which eluted from the normal phase column after 54 min using the 65 min gradient, were collected. They were evaporated to remove the solvent and thiolyzed. Because the amount of polymers that can be collected from the analytical column is too small to be detected with 280 nm UV detection, a more sensitive fluorescence detection was employed to record the chromatogram. The side reaction products in the thiolysis media originating from heterocyclic ring cleavage did not have fluorescence activity. The fluorescence chromatograms were much clearer because only flavan-3-ols and their benzylthioethers were observed. The fluorescence response areas were converted into an equivalent response area at 280 nm using conversion factors so that the constitutive monomer proportions and DP could be calculated (*5*). The data are listed in **Table 6**. A-type interflavan linkages in procyanidins are resistant to thiolysis (*31*). After thiolysis, they would be released as free A-type dimers if present in the terminal unit or as the corresponding benzylthioether if present in the extension unit (*32*). In cranberry, 46.1% of polymers contain A-type linkages as terminal units. A small portion of A-type dimers and trimers were detected in lowbush blueberry. However the A-type linkages were not detected in the higher oligomers and polymers.

In summary, the normal-phase HPLC method was optimized to quantify the individual oligomeric and the total polymeric procyanidins in foods. This method will be used to analyze diverse food samples collected in the United States to establish a procyanidin food database. In so doing, the daily intake of procyanidins can be evaluated and correlated in epidemiological studies with incidence of some chronic diseases. The bioavailability and physiological activities of the polymers have not been studied. Although the polymeric procyanidins are more concentrated than the oliogmeric forms in many foods, their role in the diet of humans remains to be determined.

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