JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 1571−1576 **1571**

High-Performance Liquid Chromatography Separation and Purification of Cacao (*Theobroma cacao* L.) Procyanidins According to Degree of Polymerization Using a Diol Stationary Phase

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A new chromatographic approach for separating cacao procyanidins according to their degree of polymerization has been developed. It utilizes diol stationary phase columns operating in normal phase mode with a binary gradient of acidified acetonitrile and methanol−water. Performance of the diol stationary phase was evaluated on an analytical scale utilizing classical chromatographic conditions for the normal phase separation of procyanidins according to their degree of polymerization. The new separation approach was developed on an analytical scale but further extended to the preparative scale. These newly developed analytical and preparative high-performance liquid chromatography procedures were successfully applied to the separation, as well as isolation, of cacao procyanidins from unfermented cacao seeds. The degree of polymerization associated with each molecular weight fraction was determined by mass spectrometry.

KEYWORDS: Diol; HPLC; HPLC-MS; normal phase; procyanidin; preparative HPLC; flavanols; cocoa

INTRODUCTION

Proanthocyanidins, the oligomers and polymers of flavan-3-ols $\{(C_6-C_3-C_6)_n\}$, are the second most abundant natural plant phenolics after lignin. The flavan-3-ol subunits are linked primarily through carbon−carbon bonds from the 4 position of one subunit to the 8 position (C4→C8) of another and to a lesser extent through a C4→C6 linkage (**Figure 1**). These single linkages are referred to as B type. Less common are species with A type linkages, which consist of an additional ether bond between C2→O7. The molecular weight of proanthocyanidins is expressed as their degree of polymerization (DP) (*1*), and individual oligomers are commonly referred to as dimers, trimers, etc. Procyanidins represent the largest class of proanthocyanidins and consist of epicatechin and catechin subunits. Out of 41 foods found to contain proanthocyanidins, 27 were found to contain procyanidins (*2*).

Proanthocyanidins play important roles in color stability, astringency, and bitterness in plant foods (*3*). However, interest in flavanols and procyanidins, especially those found in cocoa, has increased substantially due to their potential health benefits (*4*−*6*). Because of the structural complexity and diversity of roles in nature of these compounds, there has been a rich history in their analysis (*7*). Analytical and preparative separations of proanthocyanidins have typically been based upon DP as opposed to the separation of individual compounds. Although unsuccessful, Lea (*8*) was the first to attempt to use normal phase high-performance liquid chromatography (HPLC) for the separation of procyanidins based on DP. However, he observed that using Sephadex LH-20 under isocratic conditions resulted in an elution order where larger molecular weight procyanidins were retained longer than smaller molecular weight procyanidins (*9*). Later, Wilson et al. (*10*) used a gradient mobile phase in combination with tetrahydrofuran−hexane−acetic/formic acid−isopropyl alcohol over a cyano column achieving partial separation based on DP (out to heptamers) of apple juice procyanidins.

Significant improvements in the separation and resolution of the procyanidins were achieved by contributions by Rigaud et al. (*11*), Cheynier et al. (*12*), and Natsume et al. (*13*) on silica stationary phases. In these cases, resolution up to pentamer (DP = 5), on normal phase, was reported. Modifications of these methods by Hammerstone et al. (*14*) further led to improvements in resolution of monomers through decamers in the analysis of unfermented cacao seeds. Further improvements by Gu et al. (*15*) led to the elution of a polymer peak (>10 DP) as well as an enhancement in overall peak shape and resolution. These HPLC methods used gradient mobile phases consisting of methylene chloride−methanol−acetic/formic acid−water to achieve separations of proanthocyanidins based on their DP. Yanagida et al. (*16*) achieved separation up to pentamers with an alternate mobile phase for separation of apple procyanidins using hexane−methanol−ethyl acetate and hexane−acetone over a silica column. Although these methods separate pro-

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10.1021/jf0525941 CCC: $33.50
Published on Web 02/09/2006

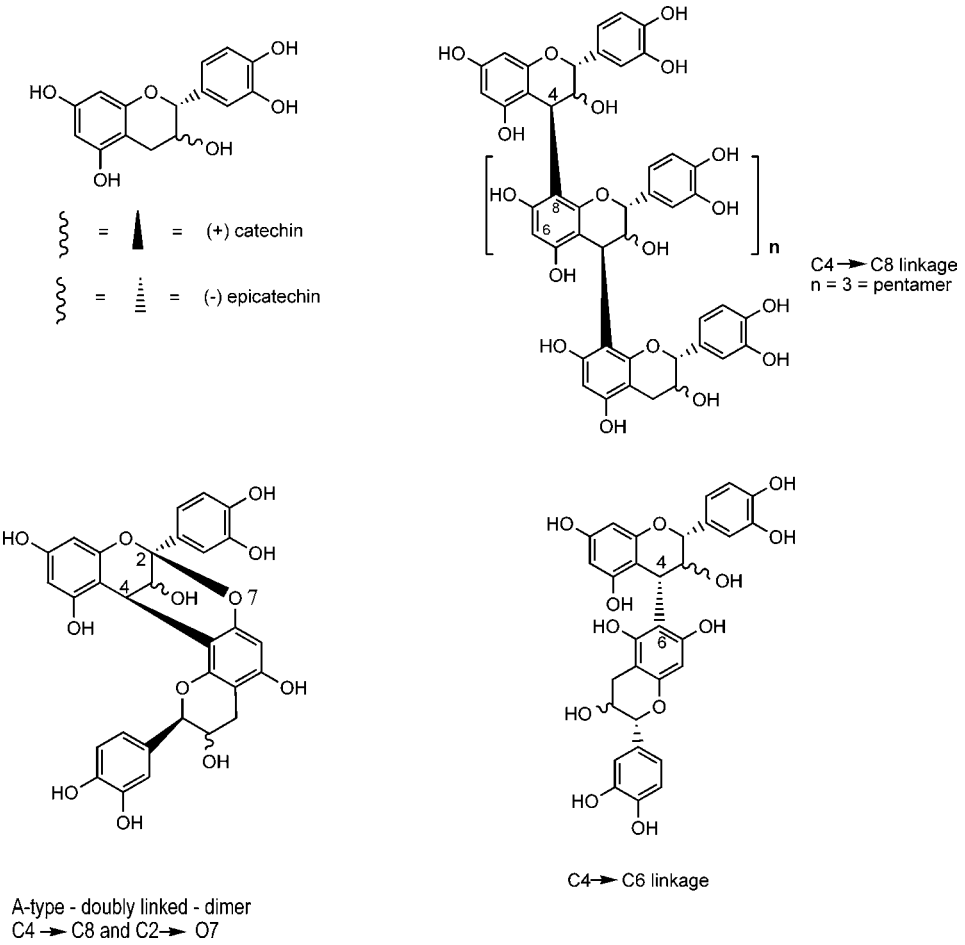

Figure 1. Structure of monomeric flavan-3-ols and procyanidins oligomers linked through a single C4→C8, C4→C6 linkage and double C4→C8, C2→O7 linkages.

cyanidins well, there are several limitations that complicate the routine use of these methods (*11*−*14*). One limitation is that the mobile phase contains chlorinated solvents, such as methylene chloride, raising concerns with respect to laboratory exposure, environmental protection, and disposal costs.

As with analytical methods, preparative methods that fractionate procyanidins based on DP have, to date, relied on normal phase silica chromatography. The separation and physical isolation of procyanidins are important for the generation of purified materials required not only for use as analytical standards but also for investigations aimed at investigating the purported biological effects of specific compounds. When isolated fractions from the larger scale systems are targeted for further in vitro, in vivo, or clinical studies, methylene chloride can be problematic. Concerns regarding the use of a halogenated mobile phase as well as complexity of analysis are present for both currently employed analytical and preparative separations. Hence, the aim of this study was to develop a simpler normal phase chromatographic method for the separation of procyanidins.

MATERIALS AND METHODS

Chemicals. All solvents (methylene chloride, acetonitrile, methanol, and acetic acid) were chromatographic grade and purchased from Fisher Scientific (Fairlawn, NJ). Ethanol was USP food grade, and ammonium acetate was purchased from Sigma-Aldrich (Milwaukee, WI). Water was deionized using a Milli-Q water purification system (Milli-Q, Bedford, MA).

Sample Preparation. The preparation of cacao procyanidin extract involved a multistep process aimed at minimizing the degradation of these oligomeric compounds. Cacao seeds (of Brazilian origin) were harvested, washed free of pulp, and dried. Under ambient conditions, the dried seeds were expeller-pressed to remove cocoa butter and the expeller cake was then ground and extracted with ethanol−water (70:30, v/v). Solids were removed by centrifugation. The liquid extract was then evaporated under reduced pressure to remove the ethanol and finally spray dried.

Analysis of fresh unfermented Amenolado form of Forestero cacao seeds (obtained from the Plant Sciences Department, Rutgers University, NJ) was also performed. To prepare this sample for analysis, fresh unfermented cacao seeds (30 g) were freeze-dried (obtaining 16.9 g). Freeze-dried seeds (10.2 g) were defatted with hexane. A subsample of defatted seeds (6.3 g) was milled and extracted three times with 40 mL of acetone:water:acetic acid (70:29.5:0.5, v/v/v) with sonicating for 10 min at 50 °C. Acetone was removed from the combined extractions by rotary evaporation under reduced pressure. The remaining liquid was freeze-dried to afford a red-purple residue (1.13 g).

Normal Phase HPLC-MS of Cacao Procyanidin Extracts and Purified Procyanidins. The separation, detection, and characterization of procyanidins in cacao extracts and procyanidin fractions obtained from the preparative system described below were performed by normal phase HPLC with fluorescence (FLD) and mass spectrometry (MS) detection. Separations were conducted on an Agilent 1100 HPLC system equipped with an autosampler, quaternary HPLC pump, column heater, and fluorescence detector. The HPLC was also interfaced to a Bruker-Esquire LC ion trap mass spectrometer (model G1946A) equipped with a API-ES ionization chamber. Ionization was enhanced by introducing 10 mM ammonium acetate in methanol via a tee in the HPLC eluent stream at a flow rate of 100 μL/min. The column used was a 250 mm × 4.6 mm i.d., 5 μm Develosil diol (Phenomenex, Torrance, CA). The

binary mobile phase consisted of (A) CH_3CN:HOAc (98:2, v/v) and (B) CH_3OH:H_2O:HOAc (95:3:2, v/v/v). Separations were effected by a linear gradient at 30 °C with a 0.8 mL/min flow rate as follows: 0−35 min, 0−40% B; 35−40 min, 40% B isocratic; 40−45 min, 40−0% B, followed by a 5 min reequilibration time. Eluent was monitored by fluorescence detection with excitation at 276 nm and emission at 316 nm. Extracts and purified fractions were characterized by an MS method adapted from Hammerstone et al. (*14*). Samples were dissolved in acetone:water:acetic acid (70:29.5:0.5, v/v/v) or mobile phase and filtered through 0.45 μm PTFE syringe filters prior to injection.

Comparative Normal Phase HPLC of Cocoa Procyanidin Extracts Conducted on Both Lichrosphere Silica and Develosil Diol Stationary Phases. Separations of cocoa procyanidin fractions were conducted on both Lichrosphere silica and Develosil diol under the chromatographic conditions published by Adamson et al. (*17*). The chromatographic system was an Agilent 1100 Series HPLC system equipped with a temperature-controlled autosampler, quaternary pump, column heater, and fluorescence detector. The columns used were 250 mm × 4.6 mm, 5 μm, 100 Å Lichrosphere Silica and Develosil diol. The chromatographic mobile phase consisted of methylene chloride (CH_2Cl_2), methanol (CH_3OH), and acetic acid:water (1:1, v/v) (HOAc:H_2O). Starting mobile phase conditions were 82% CH_2Cl_2, 14% CH_3OH, and 4% HOAc:H_2O. Subsequently, CH_3OH was ramped to 28.4% after 30 min, 42.8% after 50 min, and 86.0% after 51 min. Throughout the chromatographic run, the HOAc:H_2O ratio was held at a constant 4%. Fluorescence detection was conducted with an excitation wavelength of 276 nm and emission at 316 nm. All samples were prepared by dissolution in acetone:water:acetic acid (70:29.5:0.5, v/v/v) and then filtered through 0.45 μm PTFE syringe filters for subsequent HPLC injection.

Preparative Normal Phase HPLC of Cocoa Procyanidin Extracts. An Agilent 1100 series preparative pump was connected to a HP 1050 UV detector. Preparative separation of procyanidins was achieved using a 300 mm × 50 mm i.d., 100 μm, Develosil diol column (Phenomenex). The mobile phase consisted of solvents A (CH_3CN:HOAc, 99:1 v/v) and B (CH_3OH:H_2O:HOAc, 95:4:1 v/v/v) using a linear gradient of 0−30% B for 35 min, followed by isocratic period for 30 min, finally increasing to 80% B. The reequilibration time was 10 min. The flow rate was set to 55 mL/min, and the column temperature was room temperature (23 ± 2 °C). The detector was set to 280 nm. Column loading was performed with a manual sample injector Rheodyne Valve model 7725 equipped with a 2 mL injection loop. The cacao procyanidin extract was dissolved in mobile phase, centrifuged, and filtered through a 0.45 μm PTFE syringe filter prior to injection. Semipreparative separation of procyanidins was also achieved using a Develosil diol (250 mm × 21.5 mm i.d., 100 μm particle size) column purchased from Phenomenex. The mobile phase consisted of the same composition as discussed above. The linear gradient was 0−30% B for 45 min followed by isocratic period for 20 min and finally increasing to 85% B to wash any remaining residues off the column. The reequilibration time was 10 min. The flow rate for this column was 15 mL/min.

RESULTS AND DISCUSSION

Analytical Method Development. Given the limitations of previously published methods used in the analysis of procyanidins, a new normal phase method for separating procyanidins according to DP was developed. In addition to the use of more environmentally favorable solvents, the diol stationary phase enabled improved oligomeric separation. Diol phases, as well as polar-bonded phases in general, are compatible with a wide range of solvents including water. The selection of running a gradient of methanol into acetonitrile was based on matching physical properties of solvents used in silica column methods. Acetonitrile was selected to replace methylene chloride since both are polar aprotic solvents with similar relative abilities to engage in hydrogen-bonding and dipole interactions (*18*). The addition of acetic acid and water was used to improve peak shape and elution of larger molecular weight procyanidins, respectively. Fluorescent detection was employed since it is more sensitive than UV for procyanidins (*19*). A column temperature of 30 °C was found to provide the best resolution; however, deviations of ±5 °C did not significantly impact separations. **Figure 2** shows the chromatogram, using this methodology, for an unfermented cacao seed extract. Compounds eluted according to their DP and were characterized by LC-MS as flavan-3-ol monomers and procyanidins up to tetradecamer (DP = 14). The flavan-3-ol monomeric and oligomeric composition in unfermented cacao consists exclusively of epicatechin and catechin (*2*). In agreement with our chromatographic separation, Gu et al. (*15*) isolated a procyanidin polymer fraction, following the decamer peak, consisting of procyanidins with an average DP of 14.

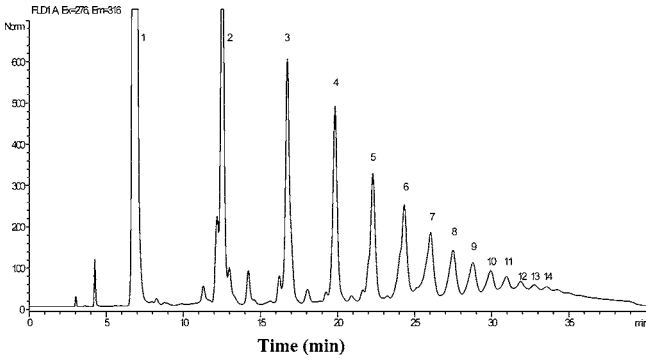

Figure 2. Diol phase HPLC fluorescence trace of procyanidins from unfermented cacao seeds. The labels 1–14 indicate the DP of procyanidins in the peaks.

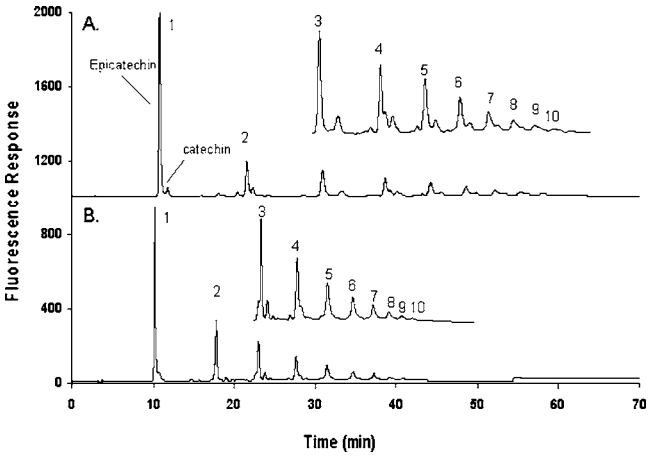

Figure 3. Cacao extract chromatographed on diol (**A**) and silica (**B**) under identical elution conditions.

Comparison with Current Methodology. To assess the chromatographic performance of the Develosil diol phase as compared to Lichrosphere silica, each column was evaluated under the chromatographic conditions described by Adamson et al. (*17*). The new mobile phase and gradient composition described for the diol phase did not separate procyanidins when tested on silica. Typical chromatograms from Lichrosphere silica and Develosil diol are depicted in **Figure 3**. The chromatograms demonstrate a significant difference in retention characteristics under identical chromatographic conditions. The bonded diol phase only showed similar retention characteristics for the procyanidin subunits, epicatechin and catechin. It exhibited stronger retention characteristics for dimer through decamer, with retention increasing with DP. The retention time for the decamer fraction was about 50% longer than that observed for Lichrosphere silica under identical conditions. Consistent with the stronger retention characteristics of diol, an increase in

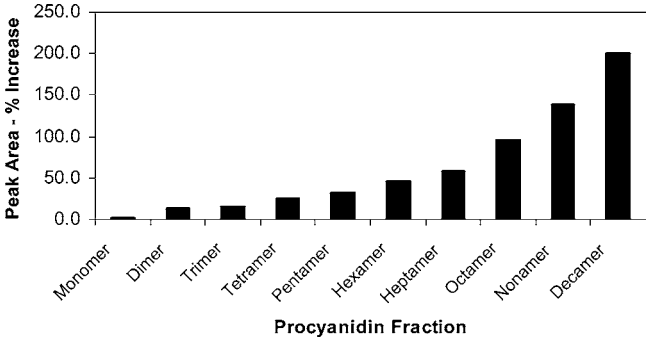

Figure 4. Peak area ratios, diol/silica, as a function of molecular weight through DP = 10. The diol phase yields greater peak area from dimer through decamer. The relative peak area increases with molecular weight.

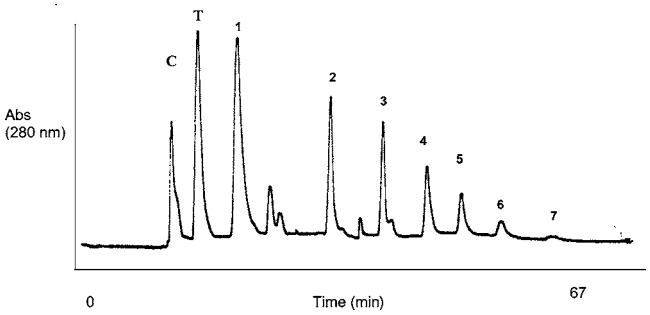

Figure 5. Preparative diol phase HPLC profile of the cacao extract. The fractions are labeled 1–7, according to DP. C and T indicate the xanthines, caffeine and theobromine.

speciation was also observed. This was apparent throughout the entire chromatogram and even impacted the monomer region at 10 min. While silica showed significant coelution, the diol phase yielded almost baseline resolution of epicatechin and catechin. While this enhanced resolution was not a surprising consequence for the more retentive bonded phase, a comparison of peak areas, between diol and silica stationary phases, yielded an unexpected result. The overall peak areas for dimer through decamer increased on the diol phase as compared to the silica phase. The magnitude of increase became greater with increasing molecular weight (DP). This phenomenon is illustrated by the graph in **Figure 4**. The observations were especially striking considering that longer retention, in practice, generates broader peak shapes with smaller area. Typically, the area reduction is a result of increased peak width and reduced signal-to-noise ratio at any given time across a wider peak. The larger peak areas, observed for all oligomers on the diol column is, on the other hand, atypical. A proposed rationale for this observation may be based on fluorescence quantum yields under the two chromatographic conditions. It is known that methylene chloride, the primary eluant at the beginning of the solvent gradient (*17*), can quench fluorescence processes (*20*, *21*). Because of the stronger retention characteristics of diol, increasingly more methanol and hence less methylene chloride are present at the later elution of the procyanidins, dimer to decamer. In effect, the mobile phase composition difference necessary for the elution of dimer and decamer procyanidins on the two stationary phases may be responsible for the increase in sensitivity on diol vs silica. Although this rationale appears to be consistent with the column comparison data and the trend in peak areas, additional investigations are necessary and currently ongoing in our laboratory to better understand this phenomenon.

Preparative Scale. Increasing the scale of this chromatography is of interest for several reasons, most prominently generation of purified material for analytical standards and pure fractions for biological testing. Despite the multitude of investigations on cocoa flavanols and procyanidins, isolating purified fractions for use as standards is still difficult.

The current silica-based analytical methods have been used for the separation of procyanidins, according to DP (*11*, *14*, *15*, *22*); however, the composition of the mobile phase (CH_2Cl_2, CH_3OH, and HOAc) presents challenges when considering scale-up to preparative chromatography. Adamson et al. (*17*) describe the fractionation of cacao procyanidins over a silica column using a gradient of methylene chloride−methanol−acetic acid−water. In a single run (180 min), using a 500 mm × 20 mm Supelcosil LC-Si column, Adamson et al. (*17*) use nearly 4.5 L of methylene chloride as part of the mobile phase. Several runs were employed in order to isolate procyanidin fractions used as reference material in their investigation. With a similar combination of solvents, Labarbe et al. (*23*) used a column packed with inert glass powder to fractionate a proanthocyanidin methyl acetate extract from grape. Alternatively, apple procyanidins were separated over a silica-packed column by gradient elution of acetone into hexane (*16*, *24*). While these preparative methods do produce results, they are labor intensive and, like some of the aforementioned analytical methods, use methylene chloride, which limits the scaling up of these methods to a preparative scale.

Major benefits of the diol method described in this manuscript are the ability to scale the process with no concern for generating large amounts of halogenated solvents, enhanced resolution, and a simplified sample preparation. Gel permeation chromatography (GPC Sephadex LH-20), in many cases, functions as a purification step prior to preparative HPLC (*17*). In GPC, the eluate is not monitored directly off of the column. Instead, fractions of a certain volume are collected and subsequently analyzed before combining and removing solvents. In the present work, we do not employ GPC, and after a simple sample preparation step, the material is directly injected onto the diol column and monitored with UV and collected as it leaves the column. The separation obtained with the preparative Develosil diol column is shown in **Figure 5**. Nine peaks were observed in a 70 min run. These methodological aspects have facilitated the collection of fractions of higher purity (**Figure 6**), reduced the amount of solvent collected per sample (thereby reducing solvent removal efforts), and shortened the time a sample spends on column.

The sample preparation involves dissolution of the cacao procyanidin extract in mobile phase (A:B, 25:75 v/v). Under these conditions, a white precipitate, identified as a mixture of xanthines, and a resinous material form. Increasing the ratio of solvent to solid does not alter these results. After a centrifuge step, the supernatant is isolated and injected onto the column. Addition of ethanol dissolves the resinous material. Analysis of this dissolved material, on the analytical scale method, gives fractions with DP up to 14. The partial insolubility most likely accounts for the fact that on a preparative scale we observe only monomers to heptamers. So as to prevent precipitation on the preparative column, only the material that dissolved in the mobile phase was injected. Currently, it is the solubility of the cacao procyanidin extract in the mobile phase that limits the amount of material in one injection as well as the upper DP at heptamer. Various methodologies for sample preparation to increase injection load per injection volume and enhancing efficiency are currently underway.

Analysis of the isolated peaks (HPLC-FLD) assisted with assessing the purity of the fractions (**Figure 6**). MS-ESI in the negative ion mode was employed for the molecular mass

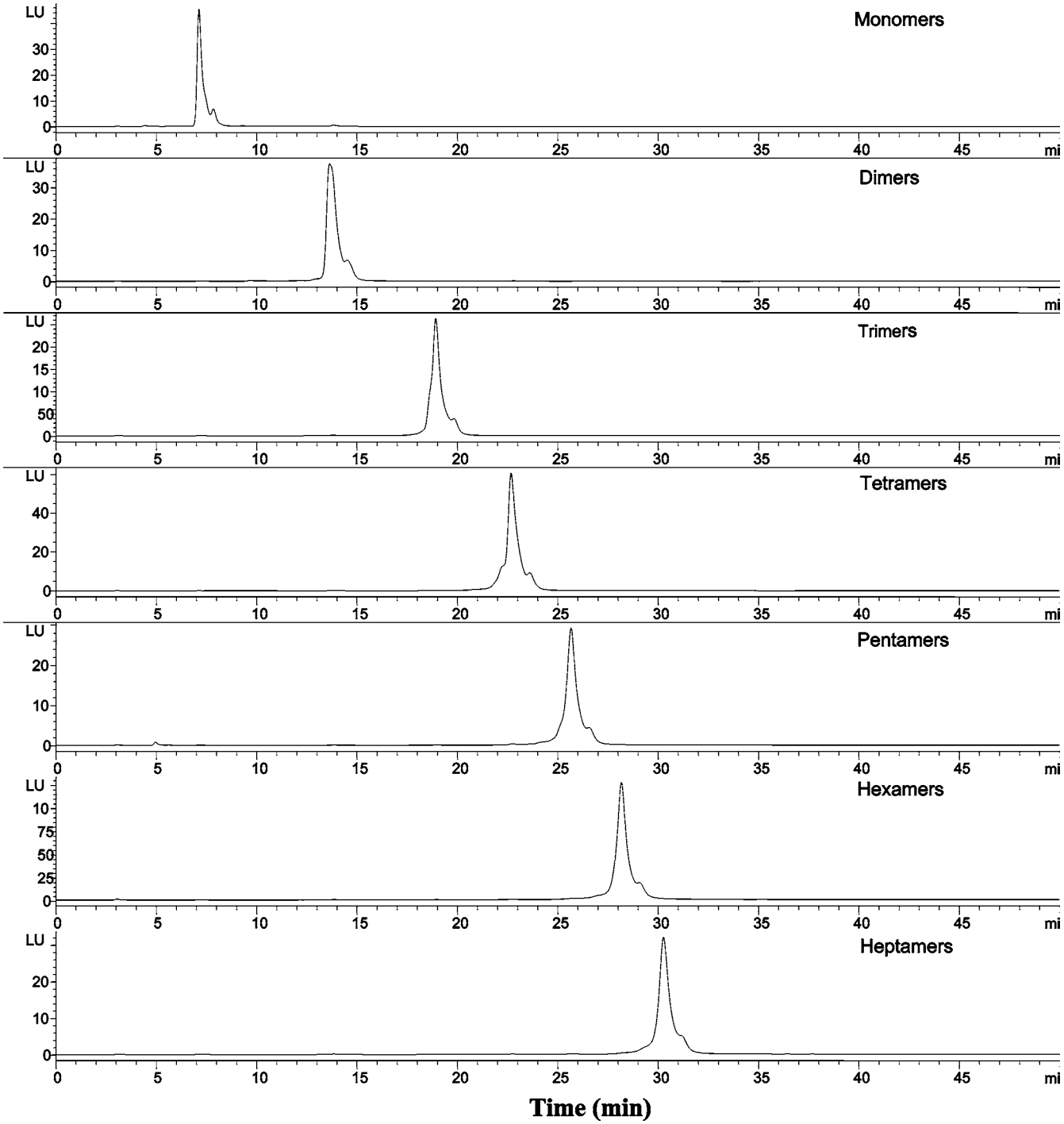

Figure 6. Fluorescence detection traces, on an analytical diol column, of the individual fractions collected from the preparative HPLC separation of cacao extract for oligomeric fractions monomers through heptamers. Fluorescence detection (Ex = 276 nm, Em = 316 nm).

assignments of the procyanidin fractions. These data are consistent with previous literature reports (*14*). The labels given in **Figure 5** (i.e., 1−7) correspond to the DP of the procyanidin fractions. The first two peaks, were identified as the xanthines, caffeine (C) and theobromine (T), using LC-MS ESI in the positive ion mode.

In conclusion, the present work represents the novel use of the diol phase for analytical and a preparative scale separation of procyanidins based on DP. The analytical and corresponding preparative methods detailed herein have several advantages over existing approaches. Our results demonstrate that the diol stationary phase is more robust than the silica phase since it can tolerate a wider range of solvents. The use of a binary mobile phase rather than a tertiary or quaternary gradient makes the current method more readily adaptable to researchers lacking sophisticated quaternary pumps. Moreover, the solvents used in the present method are more environmentally favorable and preliminary evidence indicates that the removal of methylene chloride might enhance sensitivity of the method. Finally, the method enables the physical isolation of fractions of highly purified procyanidins according to DP. This is advantageous with respect to further purifications, such as isolation of pure procyanidin molecules, and generation of standards for further analytical as well as biological research. Development of the quantitative aspect as well as validation of the method described

herein, using the procyanidin fractions isolated as standards, is currently underway.

ACKNOWLEDGMENT

We thank L. Laemmle for her assistance in both data collection and analysis, M. Chimel for his assistance in obtaining cacao extracts, and Dr. T. Gianfagna for providing fresh cacao seeds.

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Received for review October 19, 2005. Revised manuscript received December 21, 2005. Accepted December 21, 2005.

JF0525941