Isolation of Dimeric, Trimeric, Tetrameric and Pentameric Procyanidins from Unroasted Cocoa Beans (*Theobroma cacao* L.) Using Countercurrent Chromatography

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ABSTRACT

The main procyanidins, including dimeric B2 and B5, trimeric C1, tetrameric and pentameric procyanidins, were isolated from unroasted cocoa beans (*Theobroma cacao* L.) using various techniques of countercurrent chromatography, such as high-speed countercurrent chromatography (HSCCC), low-speed rotary countercurrent chromatography (LSRCCC) and spiral-coil LSRCCC. Furthermore, dimeric procyanidins B1 and B7 which are not present naturally in the analyzed cocoa beans were obtained after semisynthesis of cocoa bean polymers with (+)-catechin as nucleophile and separated by countercurrent chromatography. In this way, the isolation of dimeric procyanidin B1 in considerable amounts (500 mg, purity >97%) was possible in a single run. This is the first report concerning the isolation and semisynthesis of dimeric to pentameric procyanidins from *Theobroma cacao* by countercurrent chromatography. Additionally, the chemical structures of tetrameric (cinnamtannin A2) and pentameric procyanidins (cinnamtannin A3) were elucidated on the basis of ^{1}H NMR spectroscopy. Interflavanoid linkage was determined by NOE-correlations, for the first time.

Keywords: Cocoa (*Theobroma cacao* L.), procyanidins, isolation, semisynthesis, countercurrent chromatography, NMR spectroscopy

1. Introduction

Procyanidins are products of the secondary metabolism of plants and are composed of the flavan-3-ol monomers (+)-catechin and (–)-epicatechin units linked mainly through C4-C8 and/or C4-C6 (so-called B-type). The structural diversity of proanthocyanidins is due to the type of interflavanoid linkage, and the kind and number of flavan-3-ol units. The number of isomers increases with the mean degree of polymerization (mDP) (Porter, 1994). Procyanidins with 2-10 units are defined as oligomeric and those with >10 units as polymeric procyanidins (Hammerstone, Lazarus, Mitchell, Rucker, & Schmitz, 1999).

Procyanidins are found in various plant-derived foods such as apple, berries, wine, cocoa, and nuts (Gu et al., 2003). *Theobroma cacao* (formerly family Sterculiaceae, newly subfamily Sterculioideae in the family Malvaceae) is native to the tropical regions of America (Rusconi & Conti, 2010; Wollgast & Anklam, 2000) and three varieties of cocoa plants are mainly cultivated worldwide, namely Forastero (bulk grade, low quality), Criollo (fine grade, highest quality) and Trinitario (fine grade, hybrid of Forastero and Criollo). Forastero is the most widely produced and used variety. Its cocoa beans are used for 80% of the overall chocolate production, whereas Trinitario and Criollo beans are only used in 10-15% and 5-10% for chocolate, respectively (Counet, Ouwerx, Rosoux, & Collin, 2004; Rusconi & Conti, 2010).

Cocoa beans contain 53% cocoa butter (Payne, Hurst, Miller, Rank, & Stuart, 2010). Procyanidins, after the flavan-3-ols, are the main classes of polyphenols in cocoa and cocoa products which give them the astringent and bitter taste (Counet et al., 2004; Wollgast & Anklam, 2000). The flavan-3-ol and procyanidin content in various cocoa products ranged from 2 to 500 mg/g (Robbins et al., 2012). The main flavan-3-ol in cocoa beans is (–)-epicatechin which is also the main extension unit of procyanidins (Porter, Ma, & Chan, 1991; Wollgast & Anklam, 2000) i.e. B2, B5 and B1 which are the main dimeric procyanidins (Kothe, Zimmermann, & Galensa, 2013; Rusconi & Conti, 2010). Furthermore, the occurrence of trimeric procyanidin C1 and tetrameric procyanidin (cinnamtannin A2) is well-described (Rusconi & Conti, 2010; Wollgast & Anklam, 2000). Chemical structures of some procyanidins with mDP 2-5 are given in **Fig. 1**. The profile and concentration of

flavan-3-ols/procyanidins in cocoa depend on their variety (genotype) (Counet et al., 2004), origin (Counet et al., 2004) and processing conditions such as fermentation (Di Mattia et al., 2013; Payne et al., 2010; Wollgast & Anklam, 2000), drying (Di Mattia, et al., 2013; Payne et al., 2010; Wollgast & Anklam, 2000) and roasting (Kothe et al., 2013; Payne et al., 2010; Wollgast & Anklam, 2000). Beside fermentation, roasting is the most critical step which leads to lower flavan-3-ol and procyanidin concentrations and structural modifications, especially epimerization (Kothe et al., 2013; Payne et al., 2010). Moreover, *Theobroma cacao* contains alkaloids such as theobromine and caffeine which affect the flavor of cocoa and cocoa products (Sotelo & Alvarez, 1991).

In the European Union the mean intake of flavan-3-ols is 77 mg/d and of proanthocyanidins 123 mg/d. The main food sources of flavan-3-ols and proanthocyanidins in the EU are tea, pome fruits, berries, cocoa beans and cocoa products, stone fruits as well as wine (Vogiatzoglou et al., 2014). Nevertheless, the flavan-3-ol and proanthocyanidin contents in foodstuffs are not well-characterized.

Theobroma cacao has been reported to show several pharmacological activities, such as cardioprotective (Arranz et al., 2013), anti-cancerogenic (Martin, Goya, & Ramos, 2013), anti-inflammatory (Vázquez-Agell et al., 2013) and neuroprotective effects (Nehlig, 2013). It has been shown that health benefits of proanthocyanidins are related to their structures (Caton et al., 2010; Dorenkott et al., 2014).

Proanthocyanidins were isolated from natural sources by extraction, fractionation, and purification (Abe et al., 2008; Köhler, Wray, & Winterhalter, 2008b), or alternatively synthesized (Dennis, Jeffery, Johnston, Perkins, & Smith, 2012; Saito, Mizushina, Tanaka, & Nakajima, 2009) or semisynthesized (Esatbeyoglu & Winterhalter, 2010; Köhler, Wray, & Winterhalter, 2008a).

In the present study, we isolated dimeric procyanidins B2 and B5, trimeric procyanidin C1, tetrameric and pentameric procyanidins and semisynthesized dimeric procyanidins B1 and B7 from fermented and unroasted cocoa beans (*Theobroma cacao*) by various techniques of countercurrent chromatography such as high-speed countercurrent chromatography

(HSCCC) (Ito & Conway, 1996), low-speed rotary countercurrent chromatography (LSRCCC) (Du, Wu, & Ito, 2000) and spiral-coil LSRCCC (Köhler, Chou, Ito, & Winterhalter, 2004) on a large scale and elucidated their structures by ^{1}H NMR spectroscopy.

2. Materials and methods

2.1. Reagents

Water (deionized, Nanopure®, Werner, Leverkusen, Germany), acetic acid, HPLC quality (Mallinckrodt Baker B. V., Deventer, Holland), acetonitrile, HPLC quality (Fisher Scientific, Loughborough, UK), methanol, HPLC quality (Fisher Scientific), dichloromethane (Fisher Scientific), *n*-butanol, p.a. (Fisher Scientific), ethyl acetate, p.a. (Fisher Scientific), (+)-catechin hydrate, ≥98% (Sigma, Steinheim, Germany), (–)-epicatechin, p.a. (Sigma), *p*-anisaldehyde, 98% (Sigma), hydrochloric acid, 37% (Riedel-de-Haën, Seelze, Germany), ethanol, p.a. (Riedel-de-Haën), sulfuric acid, 98% (Riedel-de-Haën), sodium hydrogen carbonate, p.a. (Merck, Darmstadt, Germany), *tert*-butylmethlyether (distilled, industrial quality), methanol (distilled, industrial quality), *n*-hexane (distilled, industrial quality), and acetone-d_6 (Deutero GmbH, Kastellaun, Germany) were used.

2.2. Cocoa beans

Fermented and unroasted cocoa beans were kindly provided from Kraft Foods (München, Germany).

2.3. Preparation of extracts, filtrates and precipitates

Samples were prepared by avoiding heat to prevent epimerization, consequently cocoa beans were frozen in liquid nitrogen before milling (Fritsch Pulverisette, Type: 14.702, No: 720, 1 mm sieve). The cocoa bean powder was defatted three times with *n*-hexane and subsequently extracted by stirring with 70% aqueous acetone (v/v) for 1 h at ambient temperature. Acetone was evaporated in vacuum (<30 °C). Alkaloids were eliminated with dichloromethane by solvent-solvent extraction and the aqueous phase was freeze-dried.

Seventy per cent aqueous acetone extract of the cocoa beans was stirred in ethanol for 1 h at ambient temperature, insoluble residue was filtered off and *n*-hexane was dropped into the

solution (10 mL/min). After filtration, the filtrate was evaporated and freeze-dried. The precipitate was dissolved in Nanopure® and freeze-dried. Ethanol and *n*-hexane were added in a ratio of 1:4, 5:13 or 2:1 (v/v). Cocoa filtrate (5:13, v/v) was used for LSRCCC separation and cocoa filtrate (2:1, v/v) was applied to HSCCC separation.

2.4. Optimization of semisynthetic formation of procyanidins

Optimization of reaction conditions of semisynthesis was performed according to (Esatbeyoglu & Winterhalter, 2010).

2.5. Sample preparation for semisynthetic formation of procyanidins

Preparative formation was performed as described earlier (Esatbeyoglu, Juadjur, Wray, & Winterhalter, 2014; Esatbeyoglu & Winterhalter, 2010; Esatbeyoglu, Wray, & Winterhalter, 2010, 2013).

2.6. HSCCC

Under optimized conditions, 700 mg of (+)-catechin or (–)-epicatechin and 700 mg of cocoa bean precipitate (1:4, v/v) were subjected to semisynthesis with 50 mL of 0.1 N methanolic HCl at 40 °C for 20 min. The semisynthetic preparation was neutralized with about 10 mL 0.5 N sodium hydrogen carbonate solution. After evaporation, the residue was freeze-dried and about 1 g of the reaction mixture was used for HSCCC separation.

2.7. Spiral-coil LSRCCC

Semisynthetic formation of dimeric procyanidins B1 and B7 was performed as follows: 10.1 g of (+)-catechin and 10.1 g of 70% acetone cocoa bean extract were reacted in 500 mL of 0.1 N methanolic HCl by shaking at 40 °C for 30 min, followed by addition of about 45 mL 0.5 N aqueous sodium hydrogen carbonate to stop the reaction, evaporated and freeze-dried. In order to eliminate the unreacted polymeric procyanidins, this reaction mixture was dissolved in 1000 mL ethanol. Insoluble residue was filtered off, and 2600 mL *n*-hexane was added to the solution (10 mL/min). After filtration, the filtrate was evaporated, lyophilized and about 11 g was applied to spiral-coil LSRCCC separation.

2.8. LSRCCC

Ten grams of (+)-catechin and 10 g of cocoa bean precipitate (1:4, v/v) were used for semisynthetic formation of dimeric procyanidins B1 and B7. For further conditions see above. About 10 g of the obtained filtrate was used for LSRCCC separation.

2.9. HSCCC separation

A multilayer coil of the type-J high-speed CCC centrifuge model CCC 1000 (Pharma-Tech Research Corp., Baltimore, MD, USA) was used for the separation of procyanidins. The three preparative coils were connected in series and equipped with PTFE tubing (polytetrafluorethylene; 2.6 mm i.d., 160 m length; total volume: 800 mL). The upper organic phase was used as stationary phase and the lower aqueous phase as mobile phase. Both phases were pumped using a Biotronik HPLC pump BT 3020 (Jasco, Großumstadt, Germany). The freeze-dried samples were dissolved in 10 mL each upper and lower phase and injected into the column using a 25 mL sample loop. The separation was performed in the head to tail elution mode. The effluent stream was monitored at λ 280 nm using a Knauer UV-Vis detector (Berlin, Germany) and recorded by a plotter (BBC Goerz SE 120, Vienna, Austria; 3 cm/h). Fractions were collected by a Super Frac fraction collector (Pharmacia LKB, Bromma, Sweden) at 4 min intervals. The amount of injected sample, revolution speed of the apparatus, flow rate, and the solvent system are given in the discussion section of the respective runs.

2.10. LSRCCC separation

LSRCCC-separations were carried out with a prototype low-speed countercurrent chromatograph (Pharma-Tech Research Corp., Baltimore, MD, USA). A multilayer single coil column was equipped with PTFE tubing (8.2 mm i.d., total volume = 5500 mL). For the solvent delivery, a HPLC Pump 64 (Knauer, Berlin, Germany) was used. For LSRCCC separation of procyanidins the previously studied two-phase solvent system *tert*-butylmethylether/*n*-butanol/water (4.3:0.7:5, v/v/v) was applied (Köhler, 2006). About 8.9 g of cocoa bean filtrate (5:13, v/v) or 10.0 g of the semisynthetic reaction mixture of cocoa bean precipitate (1:4) with (+)-catechin were dissolved in 100 mL each upper and lower phase and introduced into the coil through a sample loop. The LSRCCC separation was performed in the U-H mode with the upper organic phase as mobile phase and in the head to tail direction. The lower phase was pumped at a flow rate of 4 or 5 mL/min. The revolution speed of the apparatus was set to 50 rpm. The effluent stream was recorded at λ 280 nm with a Knauer UV-Vis detector (Berlin, Germany) and collected into test tubes with a fraction collector (Pharmacia LKB Super Frac, Bromma, Sweden) at 10 or 12 min intervals. The separation was recorded using a Servogor 120 plotter (BBC Goerz Metrawatt SE 120, Vienna, Austria).

2.11. Spiral-Coil LSRCCC separation

A spiral tube prototype of LSRCCC (Pharma-Tech Research Corp., Baltimore, MD) was equipped with ten spirals to a single coil of convoluted Teflon tubing (8.5 mm i.d.; total volume: 5600 mL (each spiral 560 mL)). A Knauer HPLC pump 64 (Berlin, Germany) pumped the solvent systems at a flow rate of 5 mL/min. The two-phase solvent system ethyl acetate/*n*-butanol/water (14:1:15, v/v/v) determined by stationary retention studies was applied for the separation of the reaction mixture of 70% acetone cocoa bean extract with (+)-catechin (sample load, 10.8 g). For the separation of the coil fraction (sample load, 8.9 g) the solvent system *n*-hexane/ethyl acetate/methanol/water (1:10:1:10, v/v/v/v) was used. The samples were dissolved in 200 mL of a 1:1 mixture of upper and lower phase of the two-phase solvent systems. The upper organic phase was used as stationary phase and the lower aqueous phase as mobile phase using the elution mode from inside to outside and

head to tail (L-I-H). The rotation was set at 132 rpm. The effluent stream was monitored with a Knauer variable wavelength detector (Berlin, Germany) at 280 nm and collected by a fraction collector (Pharmacia LKB Super Frac, Bromma, Sweden) into 50 mL fractions. The chromatograms were recorded using a Servogor 120 plotter (BBC Goerz Metrawatt SE 120, Vienna, Austria).

2.12. Reversed-phase High-Performance Liquid Chromatography Photodiode Array (RP-HPLC-PDA); HPLC-Electrospray Ionization Multiple Mass Spectrometry (HPLC-ESI-MS^n) and High Resolution ESI-MS (HR-ESI-MS) analysis

RP-HPLC-PDA analysis of proanthocyanidins was performed on a Jasco system (Gross-Umstadt, Germany) equipped with a PU-2080 plus pump combined with a DG-2080053 three-line degasser and an LG 2080-02 ternary gradient unit, and MD-2010 plus photodiode array detector. Proanthocyanidins were separated on a 250 mm x 4.6 mm i.d., 5 µm, Aqua C-18 column (Phenomenex, Aschaffenburg, Germany) protected with a guard column (4 mm x 4 mm) and then eluted in gradient mode with the mobile phases 2% aqueous acetic acid (v/v) (A) and acetonitrile (B): 3-10% acetonitrile (0-25 min), 10-35% acetonitrile (25-45 min), 35-75% acetonitrile (45-50 min), 75% acetonitrile (50-55 min), 75-3% acetonitrile (55-60min), 3% acetonitrile (60-65 min). The flow rate was set at 0.8 mL/min and the injection volume at 20 µL. Proanthocyanidins were analyzed at λ 280 nm.

HPLC-ESI-MS^n analyses were performed on an Agilent 1100 HPLC system (Waldbronn, Germany) equipped with an 1200 autosampler and an 1100 HPLC pump and interfaced to an Esquire HPLC-MS/MS system (Bruker GmbH, Bremen, Germany). The software HP ChemStation was used for data collection. MS parameters are given as follows: negative mode; capillary, 3000 V; end plate, -500 V; capillary exit, -105 V; dry gas, 325 °C; gas flow, 10 L/min; nebulizer, 40 psi and scan range, *m/z* 50 to 2200. HPLC conditions are given above.

High-resolution ESI-MS analyses were performed on a Thermo Science LTQ Orbitrap mass spectrometer in the positive ionization mode (Thermo Fisher Scientific, Bremen, Germany).

2.13. Normal-phase High-Performance Liquid Chromatography Photodiode Array (NP-HPLC-PDA)

A HPLC system from Agilent Technologies Series 1100 (Waldbronn, Germany) consisting of a binary HPLC pump, autosampler, photodiode array detector, column oven, normal-phase column [250 mm × 16 mm i.d., Luna 5µ Silica (2), 100Å (Phenomenex, Aschaffenburg, Germany), equipped with a guard column] and HP ChemStation V 6.0 as software were used. Dichloromethane/methanol/acetic acid/water (82:14:2:2, v/v/v/v) (solvent A) and dichloromethane/methanol/acetic acid/water (10:86:2:2, v/v/v/v) (solvent B) were used as mobile phase. The gradient was as follows: 0 min 0% B, 30 min 20% B, 63 min 75% B, 65 min 100%, 70 min 100% B, 75 min 0% B, 80 min 0% B. The flow rate was set at 1.6 mL/min, the wavelength at λ 280 nm and the column oven temperature at 37 °C. Twenty microliters were injected into the HPLC system.

2.14. Preparative HPLC

A HPLC system from Knauer (Smartline 1000 HPLC pump, Smartline Manager 5000 solvent organizer and degasser, Wellchrom K-2600 UV detector, Berlin, Germany), ChromGate version V3.1.7 software and a preprative HPLC column [Hypersil ODS C-18, 5 µm, 250 x 16 mm i.d. (Phenomenex, Aschaffenburg, Germany)] were used. Conditions: water (solvent A) and acetonitrile (solvent B); gradient 1 for tetrameric procyanidin: 0 min 10% B, 40 min 30% B; gradient 2 for pentameric procyanidin 0 min 11% B, 40 min 25% B. Gradients for dimeric procyanidins B1, B2, B5 and B7 and trimeric procyanidin C1 are given in (Esatbeyoglu et al., 2014). The flow rate was adjusted to 6 mL/min. The eluents were monitored at λ 280 nm.

2.15. Thin layer chromatography

Silica gel 60G F254 20 cm×20 cm plates applied on aluminium (Merck, Darmstadt, Germany) were used. Plates were developed in toluol/acetone/formic acid (3:3:1, v/v/v) as mobile phase. After development and UV detection, the plates were sprayed with *p*-anisaldehyde-sulfuric acid-reagent solution (Stahl, 1967), air-dried at ambient temperature and heated at 100 °C for a few seconds. Proanthocyanidins were visible as orange-red spots.

2.16. Nuclear Magnetic Resonance (NMR) spectroscopy

One-dimensional ^{1}H and two-dimensional ^{1}H-^{1}H correlation spectroscopy (COSY) as well as ^{1}H-^{1}H phase-sensitive NOESY experiments were performed on Bruker Avance ARX 400 NMR spectrometer equipped with a variable temperature unit B VT-2000 (Rheinstetten, Germany) at 240 K. Acetone-d_6 was used as solvent. The chemical shifts were calibrated against the residual solvent signals and are given in ppm. Coupling constants are given in Hz.

(–)-Epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin (cinnamtannin A2)

Amorphous white powder; λ_{max} = 230 and 278 nm; ESI-MS/MS *m/z* 1153 $[M - H]^-$; MS/MS fragments *m/z* 1027, 865, 739, 701, 577, 451, 425, 407, 289, 287, 245; HR-ESI-MS *m/z* 1155.2774 $[M+H]^+$ (calcd for 1155.2765 $C_{60}H_{50}O_{24}$); CD (0.14 mmol/L in methanol): $[\theta]_{202}$ -305238, $[\theta]_{218}$ 322379, $[\theta]_{240}$ 118278, $[\theta]_{280}$ -13547.

^{1}H NMR data see Table 1.

(–)-Epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin (cinnamtannin A3)

Amorphous white powder; λ_{max} = 230 and 278 nm; ESI-MS/MS *m/z* 1441 $[M - H]^-$; MS/MS fragments *m/z* 1153, 865, 721, 575, 451, 425, 407, 289; HR-ESI-MS *m/z* 1465.3223 $[M+Na]^+$ (calcd for 1465.3218 $C_{75}H_{62}O_{30}$); CD (0.08 mmol/L in methanol): $[\theta]_{204}$ -462140, $[\theta]_{220}$ 453753, $[\theta]_{235}$ 216554, $[\theta]_{280}$ -23020.

^{1}H NMR data see Table 1.

For structure elucidation of dimeric procyanidins B1, B2, B5 and B7 as well as trimeric procyanidin C1 see (Köhler et al., 2008b) and (Esatbeyoglu, Jaschok-Kentner, Wray, & Winterhalter, 2011).

2.17. Phloroglucinolysis

Conditions are given in (Esatbeyoglu et al., 2011).

2.18. Circular dichroism

CD spectra were recorded according to (Esatbeyoglu et al., 2013).

3. Results and discussion

Initially the ground cocoa beans were defatted with *n*-hexane and extracted with 70% aqueous acetone. The alkaloids theobromine and caffeine were partly eliminated by solvent-solvent extraction with dichloromethane. The oligomeric procyanidin composition of this 70% acetone extract was analyzed by HPLC on a normal-phase column according to (Hammerstone et al., 1999) with some modifications. Proanthocyanidins were separated according to their mDP (**Fig. 2**), but the absolute characterization of proanthocyanidins is not possible by normal-phase chromatography. Oligomeric procyanidins were quantified as (+)-catechin-equivalents at λ 280 nm. Monomeric flavan-3-ols were present in about 6% and each group of dimers to decamers in 1.29-2.40%.

In fermented and unroasted cocoa beans (unknown origin and variety) dimeric procyanidins B2 and B5 were detected on a reversed-phase column which were isolated by (Köhler, 2006) using high-speed countercurrent chromatography. Moreover, trimeric procyanidin C1 and the tetramer (–)-epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin were verified (Esatbeyoglu, 2011). The aim of this study was on the one hand to isolate the low-molecular oligomeric procyanidins from a cocoa filtrate which were enriched after precipitation of the 70% acetone extract of cocoa beans in the filtrate, and on the other hand to semisynthesize the dimeric procyanidins B1 (EC-4β→8-C) and B7 (EC-4β→6-C) which do not occur naturally in the 70% acetone extract of cocoa beans or cocoa bean precipitate. Semisynthesis allows the cheap formation of dimeric procyanidins from a plant-derived polymeric fraction with flavan-3-ols as nucleophiles (Esatbeyoglu & Winterhalter, 2010; Esatbeyoglu et al., 2010; Köhler et al., 2008a).

3.1. Fractionation of oligomeric procyanidins by LSRCCC

To obtain a procyanidin fraction rich in oligomers the 70% acetone extract was precipitated with ethanol/*n*-hexane in different ratios i.e. 1:4, 5:13, 2:1 (v/v; **Fig. 3**). The lower the ratio of *n*-hexane the higher the high-molecular oligomeric procyanidins in the filtrate (**Fig. 3**). By using a ratio of ethanol/*n*-hexane 1:4 (v/v) only dimeric and trimeric procyanidins could be detected, with no tetrameric procyanidins (**Fig. 3A**).

The main focus of this LSRCCC separation was the enrichment and isolation of trimeric and tetrameric procyanidins. Therefore, about 8.9 g cocoa bean filtrate was applied for LSRCCC separation which was obtained after precipitation with ethanol/*n*-hexane (5:13, v/v) of the 70% acetone extract. The separation was carried out in the U-H elution mode (upper phase as mobile phase; head to tail) with the two-phase solvent system *tert*-butylmethlyether/*n*-butanol/water (4.3:0.7:5, v/v/v). Köhler (Köhler, 2006) used this solvent system for the separation of dimeric procyanidins B1 and B7 from grape seed extracts. After separation, the test tubes were analyzed by thin layer chromatography on a silica gel (visual detection after spraying with *p*-anisaldehyde-sulfuric acid as orange-red spots) (Köhler, 2006; Stahl, 1967) and divided into 8 fractions. Procyanidins were separated according to their mDP. The identification of isomers is not possible because of the identical R_f-values. During the separation in the U-H elution mode, the unpolar compounds eluted first. Therefore, tentatively dimeric procyanidin B5 eluted before B2 and is enriched in the first fractions. **Fig. 4** presents the chromatogram of the LSRCCC separation and the thin layer chromatogram of the selected test tubes. Fraction I was composed of different compounds and the target compounds (–)-epicatechin and dimeric procyanidin B5 were identified. A quercetin-pentoside (*m/z* 433 $[M-H]^-$ with fragment ions *m/z* 301 and 151, tentatively quercetin-3-arabinoside) was also detectable (Stark, Bareuther, & Hofmann, 2005; Wollgast & Anklam, 2000). Fraction II contained the same compounds as fraction I. Additionally, (+)-catechin, an unknown trimeric procyanidin and quercetin-3-glucoside (*m/z* 463 $[M-H]^-$ with fragment ions *m/z* 301 and 151 (Stark et al., 2005)) were enriched. (–)-Epicatechin and dimeric procyanidin B5 were obtained from fraction III. Fraction IV contained mainly (–)-epicatechin. In fraction V dimeric procyanidin B2, (–)-epicatechin, trimeric procyanidin EC-4β→8-EC-4β→6-EC and further an unknown trimeric procyanidin were enriched. Fraction VI was composed of dimeric procyanidin B2 (purity = 64.2%; 194 mg). Fraction VII contained dimeric procyanidin B2 and trimeric procyanidin C1. Using HSCCC (solvent system, ethyl acetate/isopropanol/water (20:1:20, v/v/v); flow rate, 2.7 mL/min; revolution speed, 1000 rpm – data not shown) and subsequent purification by preparative HPLC about 120 mg trimeric

procyanidin C1 and about 160 mg dimeric procyanidin B2 were isolated in standard quality (>95%) from fraction VII. Fraction VIII contained besides caffeine oligomeric procyanidins i.e. trimeric procyanidin C1, tetrameric, pentameric and hexameric procyanidin. The alkaloids theobromine and caffeine were enriched in the coil fraction. The HPLC-chromatograms of the selected LSRCCC fractions are depicted in **Fig. S1**.

To sum up, in addition to the isolation of dimeric (B2 and B5) and trimeric procyanidins (C1), an enrichment of higher oligomeric procyanidins i.e. tetrameric procyanidins using LSRCCC is possible. In the following section, the systematic isolation of tetrameric and pentameric procyanidins is illustrated.

3.2. Fractionation of higher oligomeric procyanidins by HSCCC

The polymeric procyanidins were eliminated by precipitation from the 70% acetone cocoa extract (ethanol:*n*-hexane in a ratio of 2:1). Due to their surface activity they could hamper the phase separation during the CCC isolation. Thereby, polymeric procyanidins were enriched in the precipitate and the oligomeric procyanidins in the filtrate (**Fig. 2**). The cocoa filtrate was fractionated with the two-phase solvent system ethyl acetate/*n*-butanol/water (14:1:15, v/v/v) using HSCCC. Eluted samples were analyzed by thin layer chromatography and combined to 10 fractions. The HSCCC separation is shown in **Fig. S2**. Polymeric procyanidins were enriched in fraction I. The alkaloid theobromine was detected in fraction II, while fraction III was composed of pentameric and hexameric procyanidins. Hexameric procyanidin (11.2 mg; purity ~60%) was enriched in fraction IV and pentameric procyanidin in fraction V. Pentameric procyanidin EC-4β→8-EC-4β→8-EC-4β→8-EC-4β→8-EC was isolated from fraction V (17.2 mg; purity ~60%) by preparative HPLC in standard quality and the structure was elucidated by ^{1}H NMR spectroscopy (see chapter 3.6.). Fraction VI contained tetrameric and pentameric procyanidins and an unknown compound *m/z* 293 [M-H]$^{-}$ with fragment ions *m/z* 276, 193, 179, 132 and 115. Tetrameric procyanidin EC-4β→8-EC-4β→8-EC-4β→8-EC was obtained from fraction VII (20.4 mg; purity 71.5%) and its structure was elucidated by NMR spectroscopy (see chapter 3.6.). Fraction VIII was composed of various trimeric procyanidins, mainly EC-4β→6-EC-4β→8-EC (Esatbeyoglu et

al., 2011) and an unknown A-type trimeric proanthocyanidin tentatively (Epi)C-(Epi)C-(Epi)GC (*m/z* 879 $[M-H]^-$) (Esatbeyoglu et al., 2014). In fraction IX trimeric procyanidin C1 (27.7 mg; purity 86.4%) and in fraction X dimeric procyanidin B2 (30.1 mg; purity 83.5%) was detectable. The coil fraction contained the more unpolar compounds dimeric procyanidin B5 and the flavan-3-ol (–)-epicatechin. These compounds could be isolated with the solvent system *n*-hexane/ethyl acetate/methanol/water (1:10:1:10, v/v/v/v) using HSCCC (Esatbeyoglu & Winterhalter, 2010). To date, gel permeation chromatography with Sephadex G-25, Sephadex LH-20 and Toyopearl TSK HW 40 or normal-phase chromatography were used for fractionation or isolation of proanthocyanidins (Abe, et al., 2008; Hammerstone et al., 1999; Kantz & Singleton, 1991; Rigaud, Escribano-Bailon, Prieur, Souquet, & Cheynier, 1993; Xiao, Liu, Wu, Xie, Yang, & Sun, 2008). **Fig. S2** shows that HSCCC is an improved method for fractionation and isolation of higher oligomeric procyanidins such as tetrameric, pentameric and hexameric procyanidins. This method does not require expensive carrier materials, the separation time is short and the compounds are isolated in high purities. Consequently, the isolation of a tetrameric, pentameric and hexameric procyanidins from cocoa beans in high purities using HSCCC was shown for the first time.

3.3. Semisynthetic preparation of dimeric procyanidins B1, B2, B5 and B7

The yield of the isolated dimeric procyanidins B2 and B5 from cocoa beans (see chapter 3.1.) could be increased via semisynthesis with (–)-epicatechin as nucleophile under optimized conditions (ratio of 70% acetone cocoa extract or cocoa precipitate (1:4) and nucleophile (–)-epicatechin 1:2, reaction temperature 40 °C, reaction time 20 min, data not shown) as documented earlier (Esatbeyoglu & Winterhalter, 2010). During semisynthesis, under acidic conditions the interflavanoid linkage of oligomeric or polymeric procyanidins is cleaved and in the presence of a nucleophile i.e. (+)-catechin or (–)-epicatechin dimeric procyanidins are formed (Esatbeyoglu & Winterhalter, 2010). Moreover, with (+)-catechin as nucleophile the dimeric procyanidins B1 and B7, which do not occur naturally in the analyzed cocoa beans, are formed. Our phloroglucinolysis data showed that cocoa bean procyanidins were composed of 98% (–)-epicatechin in their extension and terminal units. The occurrence

384 of (–)-epicatechin is often-cited (Porter et al., 1991; Wollgast & Anklam, 2000). According to
385 phloroglucinolysis data only dimeric procyanidins B1, B2, B5 and B7 with (-)-epicatechin in
386 the upper unit are formed via semisynthesis from the analyzed cocoa beans. These dimeric
387 procyanidins were isolated by HSCCC as shown earlier (Esatbeyoglu & Winterhalter, 2010).
388 The composition of the HSCCC fractions of the reaction mixture of cocoa beans precipitate
389 with (+)-catechin or (–)-epicatechin was similar to those of the reaction mixtures of *Aronia*
390 *melanocarpa* (Esatbeyoglu & Winterhalter, 2010). Therefore, the HSCCC separations are not
391 described in detail and only the yield and purity of dimeric procyanidins are given here. From
392 1000 mg semisynthetic reaction mixture with (+)-catechin as nucleophile 85.8 mg dimeric
393 procyanidin B1 (purity 89.3%) and 26 mg dimeric procyanidin B7 (purity 88.6%), with (–)-
394 epicatechin 76.6 mg dimeric procyanidin B2 (purity 92.0%) and 23.9 mg dimeric procyanidin
395 B5 (purity 80.7%) were isolated. Because of steric requirements 4→8 linked dimeric
396 procyanidins (B1, B2) are formed in higher amounts compared to 4→6 linked dimeric
397 procyanidins (B5, B7).
398 *3.4. Fractionation of the semisynthetic reaction mixture with (+)-catechin by Spiral-Coil*
399 *LSRCCC*
400 For human intervention studies larger amounts of dimeric procyanidins are required. To date,
401 dimeric procyanidins could not be isolated in large amounts. Recently, Esatbeyoglu et al.
402 (Esatbeyoglu et al., 2014) showed the isolation of dimeric procyanidins B1 to B4 in amounts
403 of 350-740 mg and purities of about 80-90%. The aim of the present study was to show the
404 isolation of the dimeric procyanidin B1 of standard quality on a large scale using a new
405 technique, spiral-coil LSRCCC.
406 Only a few solvent systems were validated for spiral-coil LSRCCC separation (Köhler, 2006;
407 Köhler et al., 2004). According to Köhler (Köhler, 2006) the known solvent system *tert*-
408 butylmethlyether/*n*-butanol/water (4.3:0.7:5, v/v/v) for separation of proanthocyanidins by
409 LSRCCC is not suitable as a solvent system for spiral-coil LSRCCC separation.
410 The known two-phase solvent system ethyl acetate/*n*-butanol/water (14:1:15, v/v/v) for the
411 separation of dimeric procyanidin B1 (Esatbeyoglu & Winterhalter, 2010) was used to

evaluate the stationary phase retention (R_{ST}) and mixing effect between the two phases for fractionation of dimeric procyanidins by spiral-coil LSRCCC. R_{ST} was determined according to Köhler et al. (Köhler et al., 2004). Compared to LSRCCC, spiral-coil LSRCCC allows higher revolution speeds due to the combination of the spiral effect and the Archimedean screw force (Köhler et al., 2004). Different elution modes (**Table S1**) and revolution speeds (20–200 rpm) under a constant flow rate of 10 mL/min were assessed for R_{ST} determination. A high R_{ST} represents good separation efficiency. Only four of eight possible elution modes L-I-T, L-I-H, U-O-H and U-O-T (**Table S1**) showed high R_s, due to the spiral centrifugal force gradient superimposed on the Archimedean screw effect (Köhler et al., 2004). **Fig. S3A** shows the retention curves of the solvent system ethyl acetate/*n*-butanol/water (14:1:15, v/v/v) for four reasonable elution modes. At a revolution speed of 120 rpm the retention curve rose above 50% in the L-I-H elution mode (lower phase as mobile phase, elution from inside to outside and head to tail) (**Fig. S3A**). A revolution speed of 132 rpm and the elution mode L-I-H were chosen for separating proanthocyanidins, because of the nearly constant R_{ST} (about 55%) in the range of 120 to 140 rpm. The reaction mixture of 70% acetone cocoa bean extract with (+)-catechin as nucleophile was applied, after elimination of the unreacted polymeric procyanidins by precipitation (ethanol/*n*-hexane (5:13, v/v)), for isolation of dimeric procyanidins B1 and B7 in large amounts using spiral-coil LSRCCC. After analyzing the collected samples by thin layer chromatography, 5 fractions and the coil fraction were obtained. The spiral-coil LSRCCC separation is shown in **Fig. 5**. The target compound dimeric procyanidin B1 was enriched with caffeine in fraction III (473 mg; purity = 62.2%). The elimination of caffeine was achieved by solvent-solvent extraction with dichloromethane. Subsequently, dimeric procyanidin B1 was isolated using preparative HPLC in amounts of 270 mg in standard quality (>95%). Fraction IV was composed of B-type trimeric procyanidins EC-4β→8-EC-4β→8-C, EC-4β→6-EC-4β→8-C and EC-4β→8-EC-4β→8-EC (C1) (Esatbeyoglu et al., 2011) and an unknown A-type trimeric proanthocyanidin tentatively (Epi)C-(Epi)C-(Epi)GC (*m/z* 879 $[M-H]^-$) (Esatbeyoglu et al., 2014) in addition to the

compounds in fraction III. Fraction V contained the trimeric procyanidins EC-4β→8-EC-4β→8-C and EC-4β→8-EC-4β→8-EC (C1) as well as dimeric procyanidin B2.

It was possible to isolate dimeric procyanidin B1 in large amounts (about 270 mg; purity >95%) by spiral-coil LSRCCC compared to HSCCC (about 72 mg; purity >95%). The elimination of caffeine is recommended before the separation in order to isolate pure dimeric procyanidin B1.

The non-polar compounds dimeric procyanidin B7 (3.3%), (–)-epicatechin (9.0%) and (+)-catechin (81.5%) were enriched in the coil fraction. The two-phase solvent system *n*-hexane/ethyl acetate/methanol/water (1:10:1:10, v/v/v/v) was successfully applied for the isolation of dimeric procyanidin B7 from *Aronia melanocarpa* (Esatbeyoglu & Winterhalter, 2010). The elution mode was determined as described above (**Fig. S3B**). At a revolution speed of 80 rpm the countercurrent flow is very high. Over a revolution speed of 150 rpm there are no huge differences between the four elution modes. A separation of proanthocyanidins is recommended in the elution mode L-I-H or U-O-T because of the sigmoid curves. We decided to separate in the L-I-H elution mode. Here, the lower phase acts as mobile phase. The spiral-coil LSRCCC chromatogram is depicted in **Fig. 6**. Because of the same R_f values of (+)-catechin and (–)-epicatechin the collected samples were analyzed by HPLC-PDA at λ 280 nm. Fraction I contained polymeric procyanidins. The target compound dimeric procyanidin B7 was enriched in fraction II (420 mg) together with (–)-epicatechin. A more polar solvent system such as *n*-hexane/ethyl acetate/methanol/water (0.8:10:0.8:10, v/v/v/v) should be used for the isolation of dimeric procyanidin B7 by spiral-coil LSRCCC. In this case, (–)-epicatechin is expected to elute later due to the larger partition coefficient. Dimeric procyanidin B7 (90 mg; purity >95%) was isolated from fraction II by HSCCC (**Fig. 6**, right side). From fraction III the unreacted nucleophile (+)-catechin was recovered (standard quality, about 4 g).

Spiral-coil LSRCCC is convenient for the isolation of compounds which have a partition coefficient under 1. Compounds with a partition coefficient over 1 lead to a longer separation

time. Compared to HSCCC separation (23 mg B7) about 90 mg dimeric procyanidin B7 was isolated by spiral-coil LSRCCC in standard quality. We reported the choice of solvent system and the isolation of proanthocyanidins i.e. dimeric procyanidins B1 and B7 using spiral-coil LSRCCC here for the first time.

3.5. Fractionation of the semisynthetic reaction mixture with (+)-catechin by LSRCCC

The ethanol/*n*-hexane precipitation of the 70% acetone cocoa extract is a crucial intermediate step before semisynthesis. In this way, it was possible to increase the yield of dimeric procyanidins two-fold if cocoa bean precipitate was applied to semisynthesis instead of a cocoa bean extract. Hence, we repeated the semisynthesis with a cocoa bean precipitate (1:4) and (+)-catechin as reactants. The cocoa precipitate (1:4) was reacted with (+)-catechin in a ratio of 1:1, at a reaction temperature of 40 °C and reaction time of 30 min. The reaction mixture was precipitated with ethanol/*n*-hexane (5:13, v/v) again in order to eliminate the unreacted polymeric procyanidins. Because of their surface activity they would hinder the LSRCCC separation. The separation conditions were nearly the same as described above (**Fig. 4**). **Fig. S4** shows the LSRCCC-chromatogram. After fractionation we obtained 5 fractions and the coil fraction. Fraction I contained the more non-polar compounds (+)-catechin and dimeric procyanidin B7 which would remain on the coil in the L-H elution mode. In fraction II (–)-epicatechin was enriched while (+)-catechin and (–)-epicatechin were enriched in fraction III in the same ratio. Fraction IV was composed of dimeric procyanidin B1 (amount 208 mg, purity 81.6%). From fraction V dimeric procyanidin B1 was isolated in standard quality (>97%) in amounts of 500 mg without the necessity of purification by preparative HPLC. Theobromine was obtained from the coil fraction.

In this investigation, we showed the isolation of dimeric (B2 and B5), trimeric, tetrameric, pentameric and hexameric procyanidins partially of standard quality from cocoa beans by HSCCC as well as LSRCCC. The isolation of dimeric procyanidins B1 and B7 in higher amounts, which do not occur as genuine compounds in the applied cocoa bean extract, were obtained after semisynthesis using spiral-coil LSRCCC and LSRCCC. Only the dimeric

procyanidins B1, B2, B5 and B7 are formed by semisynthesis because of the polymeric procyanidin composition of the upper unit of 98% (–)-epicatechin. Application of a polymer-enriched precipitate for semisynthesis leads to higher yields of dimeric procyanidins.

3.6. Structure elucidation of tetrameric and pentameric procyanidins by NMR, phloroglucinolysis and CD

Tetrameric [(–)-epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin] and pentameric [(–)-epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin-4β→8-(–)-epicatechin] procyanidins were isolated from fractions VII and V of the cocoa filtrate (cf. **Fig. S2**) as amorphous white powders by HSCCC after final purification by preparative HPLC. Their chemical structures are shown in **Fig. 1** with procyanidin units referred to as *A*-*E* units.

The fragmentation of the HPLC-MS/MS analysis is indicating (epi)catechin units in both compounds (cf. 2.16.). HR-ESI-MS analysis with quasi-molecular ions at *m/z* 1155.2774 $[M + H]^+$ (calcd *m/z* 1155.2765) and *m/z* 1465.3223 $[M + Na]^+$ (calcd *m/z* 1465.3218) indicated molecular formulas of $C_{60}H_{50}O_{24}$ and $C_{75}H_{62}O_{30}$ for tetrameric and pentameric procyanidins, respectively.

NMR data were recorded in acetone-d_6 at 240 K to overcome the atropisomerism which caused signal broadening. Chemical shifts and coupling constants are given in Table 1. The ^{1}H NMR spectra of the tetrameric and pentameric procyanidins are shown in **Fig. S5**. These ^{1}H NMR spectra are similar to those of trimeric procyanidin (Esatbeyoglu et al., 2011), extended with signals of one or two more flavan-3-ol units. Tetrameric and pentameric procyanidins were present in one major conformation at low temperature.

In the following section, the exemplary structure elucidation of tetrameric procyanidin cinnamtannin A2 is shown. Only the characteristic points are given here (for further information see (Esatbeyoglu et al., 2011)).

Signals at δ 4.29 were identified as aliphatic hydroxyl groups. At δ 7.17-8.71 16 aromatic hydroxyl groups were observed. The hydroxyl groups at position 7 (A-ring) of the *B*, *C* and *D* units were determined through the correlation with H2 of the next upper unit. A thorough

assignment of the three hydroxyl groups at position 5 (A-ring) of the *B*, *C* and *D* units was not possible, except for OH5*A* δ 8.36. From the comparison with the tetrameric procyanidin, it was possible to assign tentatively the aromatic hydroxyl groups of the B-rings of the pentameric procyanidin.

The comparison of the integration height of the two A-ring protons from the upper unit "*A*" to the three A-ring protons of the other units "*B*, *C* and *D*" allowed determination of the number of flavan-3-ol units.

H6 protons (units *B*, *C* and *D*) showed singlets at δ 5.94. The position of the meta-coupled A-ring protons H6 (δ 6.01) and H8 (δ 5.99) of the upper unit (*A*) were assigned from NOE correlations between H6 and the two aromatic hydroxyl groups (δ 8.36 OH5*A* and δ 8.66 OH7*A*) as well as between H8 and one aromatic hydroxyl group (δ 8.66).

A small value of 2 Hz or a broad singlet for $J_{2,3}$ indicate (–)-epicatechin as flavan-3-ol unit (2,3-*cis* configuration).

COSY-spectra afforded the assignment of the C-ring protons. From the H4A and H4B protons of the terminal unit (*D*) it was possible to determine the H2 and H3 protons of the C-ring of the terminal unit (*D*) from COSY-spectra. NOE correlations allowed the assignment of the order of the flavan-3-ol units and the identification of the interflavanoid linkage. NOE correlations between H2´/H6´ (B-ring, *D*) and H2/H3 of the terminal unit (C-ring, *D*) were observed, as well as the corresponding signals for the *A*, *B* and *C* units. An additional NOE correlation of H2´ and H6´ of the terminal unit (*D*) with H4 of the next upper unit (*C*) allowed the unambiguous assignment of the B- and C-ring protons of unit *C*. Such correlations are characteristic of a 4→8 interflavanoid linkage, as well (Esatbeyoglu et al., 2011; Esatbeyoglu et al., 2010). Due to the absence of these correlations, the upper unit (*A*) was determined readily. The determination of the *B*-unit resulted from correlations of H2´ and H6´ of the *B*-unit to H4 of the *A*-unit. The correlation between H2´/H6´ of the *C*-unit and H4 of the *B*-unit confirmed this assumption. Finally, all units are 4→8 linked. The lack of NOE correlations between H2 and H4, indicated that the flavan-3-ol units are linked quasi-axial (*β*-orientation of the interflavanoid bond).

551 Confirmation of the structures of tetrameric and pentameric procyanidins was conducted by
552 acid-catalyzed degradation (phloroglucinolysis) (Esatbeyoglu et al., 2011). Three
553 (–)-epicatechin-(4*β*→2)-phloroglucinol equivalents for the upper units and one
554 (–)-epicatechin for the terminal unit were observed after complete cleavage of the tetrameric
555 procyanidin. Incomplete cleavage of the tetramer (cinnamtannin A2) yielded 72.8%
556 (–)-epicatechin-(4*β*→2)-phloroglucinol and dimer B2-phloroglucinol, 8.5% dimer B2, 0.9%
557 trimer C1 and 17.8% (–)-epicatechin. For the pentamer (cinnamtannin A3) 70.4%
558 (–)-epicatechin-(4*β*→2)-phloroglucinol and dimer B2-phloroglucinol, 9.4% dimer B2, 3.1%
559 trimer C1, 2.2% tetramer (cinnamtannin A2), 2.4% pentamer (unreacted) and 12.4%
560 (–)-epicatechin were obtained after incomplete cleavage. All these data confirmed 4→8
561 linked cleavage products and complete degradation of the tetramer and pentamer (almost)
562 are characteristic of a 4→8 interflavanoid linkage.
563 To date, the structures of cinnamtannin A2 and cinnamtannin A3 were elaborated on the
564 basis of COSY-, HSQC- and HMBC-spectra and/or acid-catalyzed degradation (i.e. thiolysis,
565 phloroglucinolysis) (Abe, et al., 2008; Bicker, Petereit, & Hensel, 2009; Köhler et al., 2008b;
566 Nakashima, Oda, Masuda, Tagashira, & Kanda, 2012; Porter et al., 1991; Saito et al., 2009;
567 Shoji, Mutsuga, Nakamura, Kanda, Akiyama, & Goda, 2003; Stark et al., 2005). The
568 interflavanoid linkage was determined from HMBC-spectra data (Abe, et al., 2008;
569 Nakashima et al., 2012; Shoji et al., 2003; Stark et al., 2005).
570
571 In this study, we showed for the first time the isolation of oligomers from *Theobroma cacao*
572 with mDP 2 to 5 by different techniques of CCC, partly on a large scale, which could be used
573 to investigate their physiological function in future studies. Moreover, dimeric procyanidin B1
574 was isolated in amounts of 700 mg. All naturally occurring oligomers in *Theobroma cacao*
575 were composed of (–)-epicatechin units which were linked via a C4→C8 bond (B-type). The
576 complete structures of tetrameric and pentameric procyanidins were elucidated using ^{1}H
577 NMR spectroscopy (especially NOE-correlations) without the necessity of ^{13}C NMR spectra
578 and acid-catalyzed derivatization.

Abbreviations used

C, (+)-catechin; EC, (–)-epicatechin; (Epi)C, (–)-epicatechin or (+)-catechin; (Epi)GC, (-)-epigallocatechin or (+)-gallocatechin; HSCCC, high-speed countercurrent chromatography; LSRCCC, low-speed rotary countercurrent chromatography; mDP, mean degree of polymerization; R_{ST}, stationary phase retention

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Appendix A. Supplementary data (Figures S1 to S5, Table S1)

“The authors declare no conflict of interest“

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735 **Figure Captions**

736 **Figure 1.** Chemical structures of dimeric procyanidins B1 (**A**), B2 (**B**), B5 (**C**) and B7 (**D**),
737 trimeric procyanidin C1 (**E**), tetrameric procyanidin Cinnamtannin A2 (**F**) and pentameric
738 procyanidin Cinnamtannin A3 (**G**).

739 **Figure 2.** Normal-phase-HPLC-chromatograms of the precipitate (**A**) and filtrate (**B**) from a
740 70% acetone cocoa extract obtained by precipitation with ethanol:*n*-hexane (2:1) at λ 280 nm
741 (Esatbeyoglu, 2011).

742 **Figure 3.** HPLC-chromatograms of the cocoa filtrate with different ratios of ethanol:*n*-hexane
743 (**A**= 1:4, **B**= 5:13, **C**= 2:1) at λ 280 nm (Esatbeyoglu, 2011).

744 **Figure 4.** Chromatogram of the LSRCCC separation of the cocoa filtrate (5:13) (λ 280 nm)
745 (sample load, 8.9 g; solvent system, *tert*-butylmethlyether/*n*-butanol/water (4.3:0.7:5, v/v/v);
746 flow rate, 4 mL/min; revolution speed, 48 rpm; elution mode, U-H (upper phase as mobile
747 phase, elution from head to tail)) and above the thin layer chromatogram of the selected test
748 tubes (Esatbeyoglu, 2011).

749 **Figure 5.** Chromatogram of the spiral-coil LSRCCC separation from the reaction mixture of
750 the 70% acetone cocoa bean extract with (+)-catechin at λ 280 nm (sample load, 10.8 g;
751 solvent system, ethyl acetate/*n*-butanol/water (14:1:15, v/v/v); flow rate, 5 mL/min; revolution
752 speed, 132 rpm; elution mode, L-I-H (lower phase as mobile phase, elution from inside to
753 outside and head to tail)) (Esatbeyoglu, 2011).

754 **Figure 6.** Chromatogram of the spiral-coil LSRCCC separation of the coil fraction from the
755 reaction mixture of the 70% acetone cocoa bean extract with (+)-catechin (see **Figure 5**) at
756 λ 280 nm (sample load, 8.9 g; solvent system, *n*-hexane/ethyl acetate/methanol/water
757 (1:10:1:10, v/v/v/v); flow rate, 5 mL/min; revolution speed, 132 rpm; elution mode, L-I-H
758 (lower phase as mobile phase, elution from inside to outside and head to tail)). The HSCCC
759 separation of fraction II to isolate dimeric procyanidin B7 is shown on the right side (sample
760 load, 420 mg; solvent system, *n*-hexane/ethyl acetate/methanol/water (1:10:1:10, v/v/v/v);
761 flow rate, 2.7 mL/min; revolution speed, 1000 rpm (Esatbeyoglu, 2011).

762

Figure 1

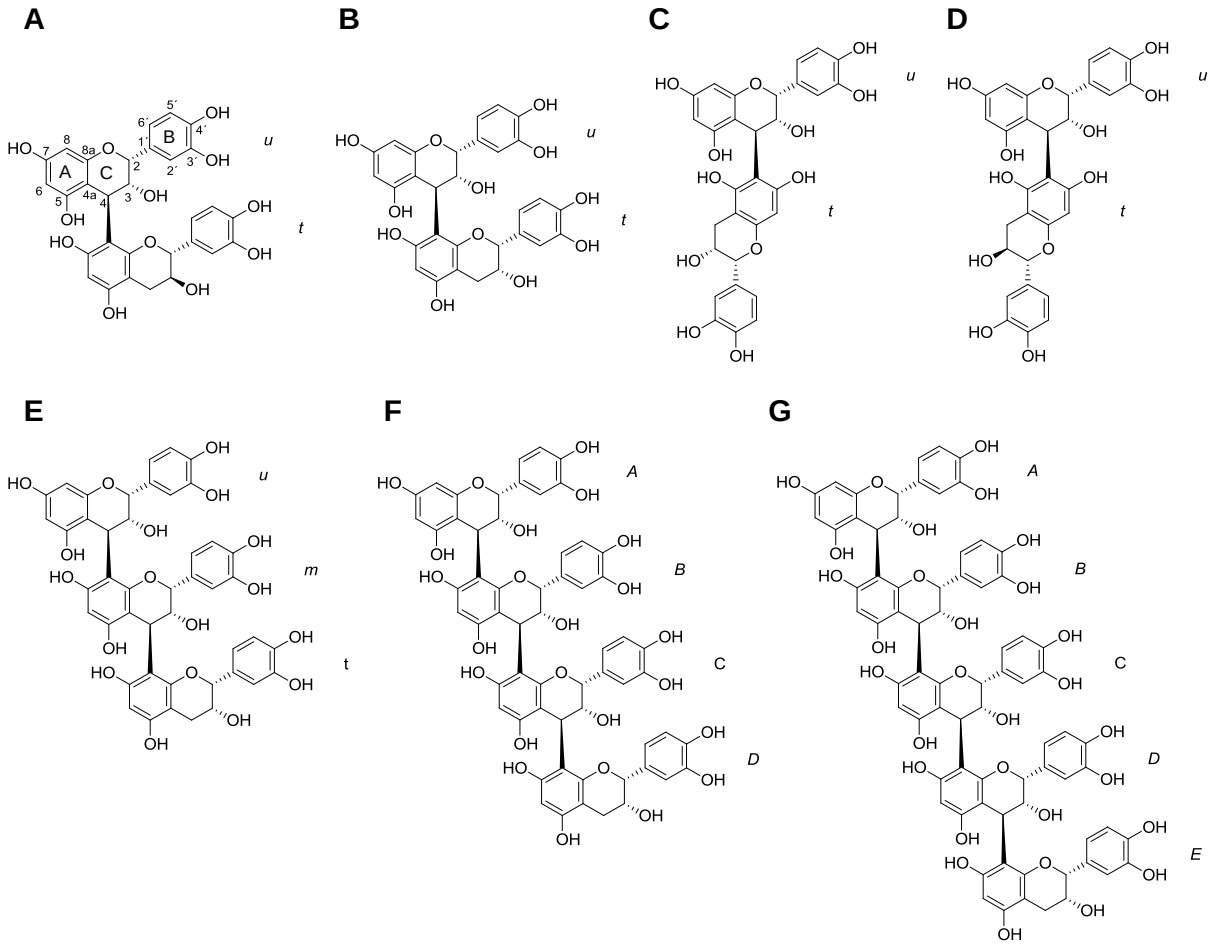

Figure 2

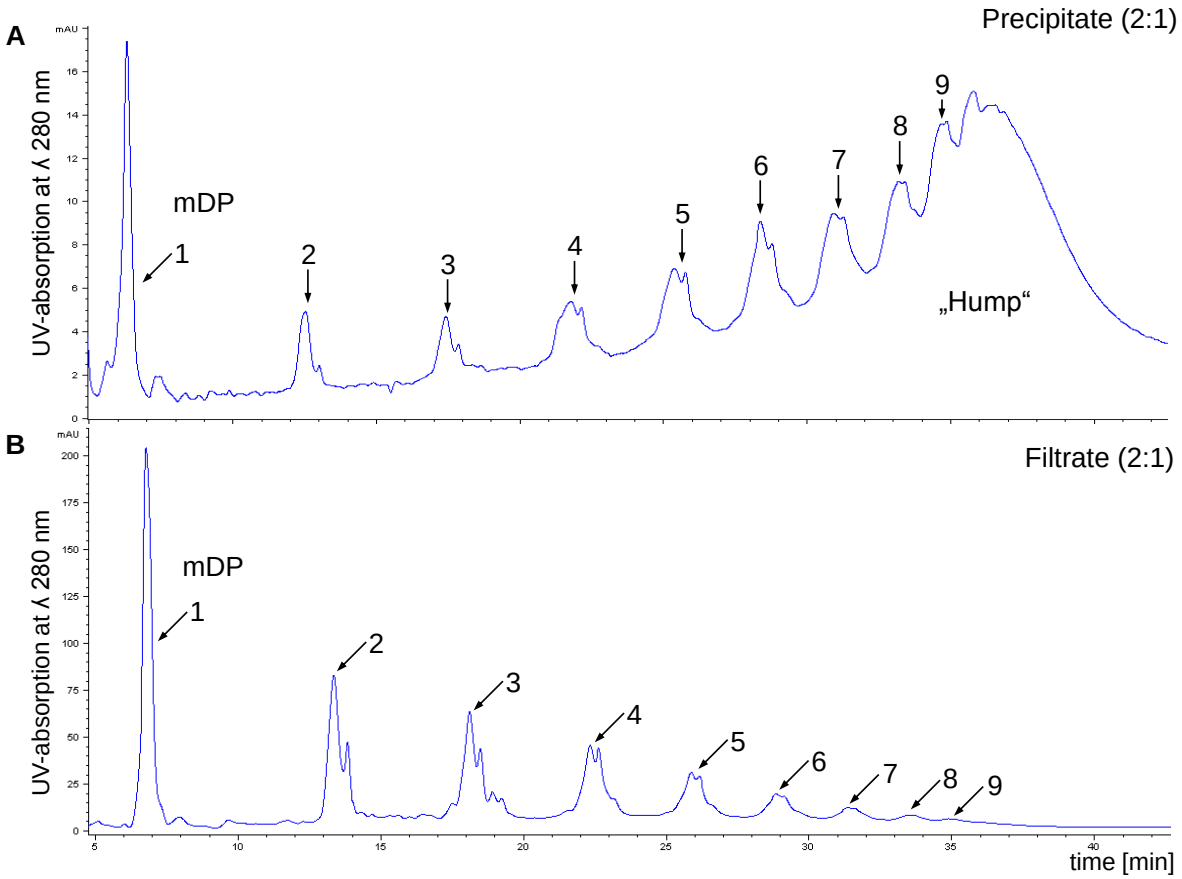
A
Precipitate (2:1)
UV-absorption at λ 280 nm
mAU
mDP
1
2
3
4
5
6
7
8
9
„Hump“
B
Filtrate (2:1)
UV-absorption at λ 280 nm
mAU
mDP
1
2
3
4
5
6
7
8
9
time [min]

Figure 3

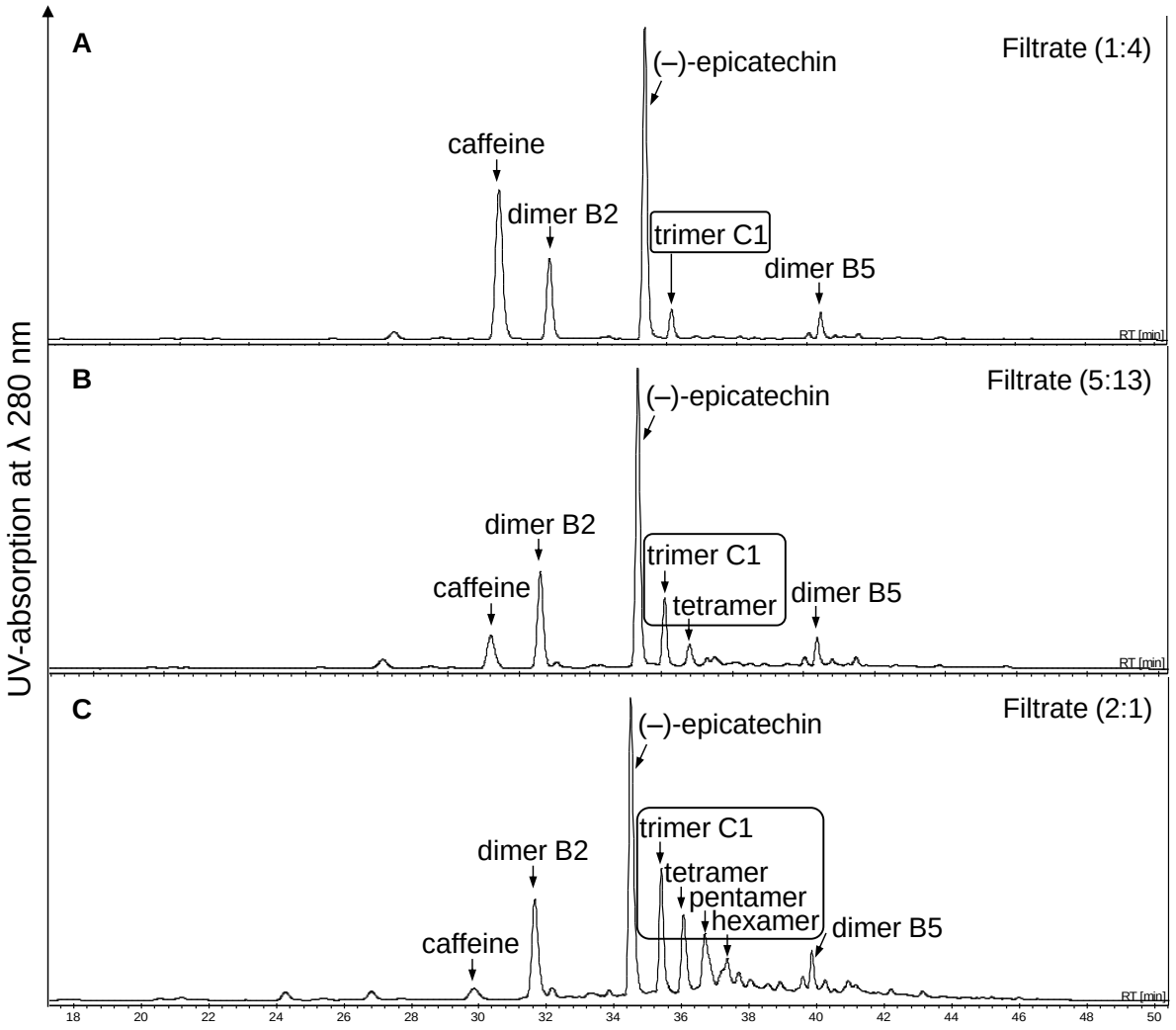
A
Filtrate (1:4)
(–)-epicatechin
caffeine
dimer B2
trimer C1
dimer B5
RT [min]
B
Filtrate (5:13)
(–)-epicatechin
dimer B2
trimer C1
caffeine
tetramer
dimer B5
RT [min]
C
Filtrate (2:1)
(–)-epicatechin
trimer C1
dimer B2
tetramer
pentamer
hexamer
caffeine
dimer B5
RT [min]
UV-absorption at λ 280 nm
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50

Figure 4

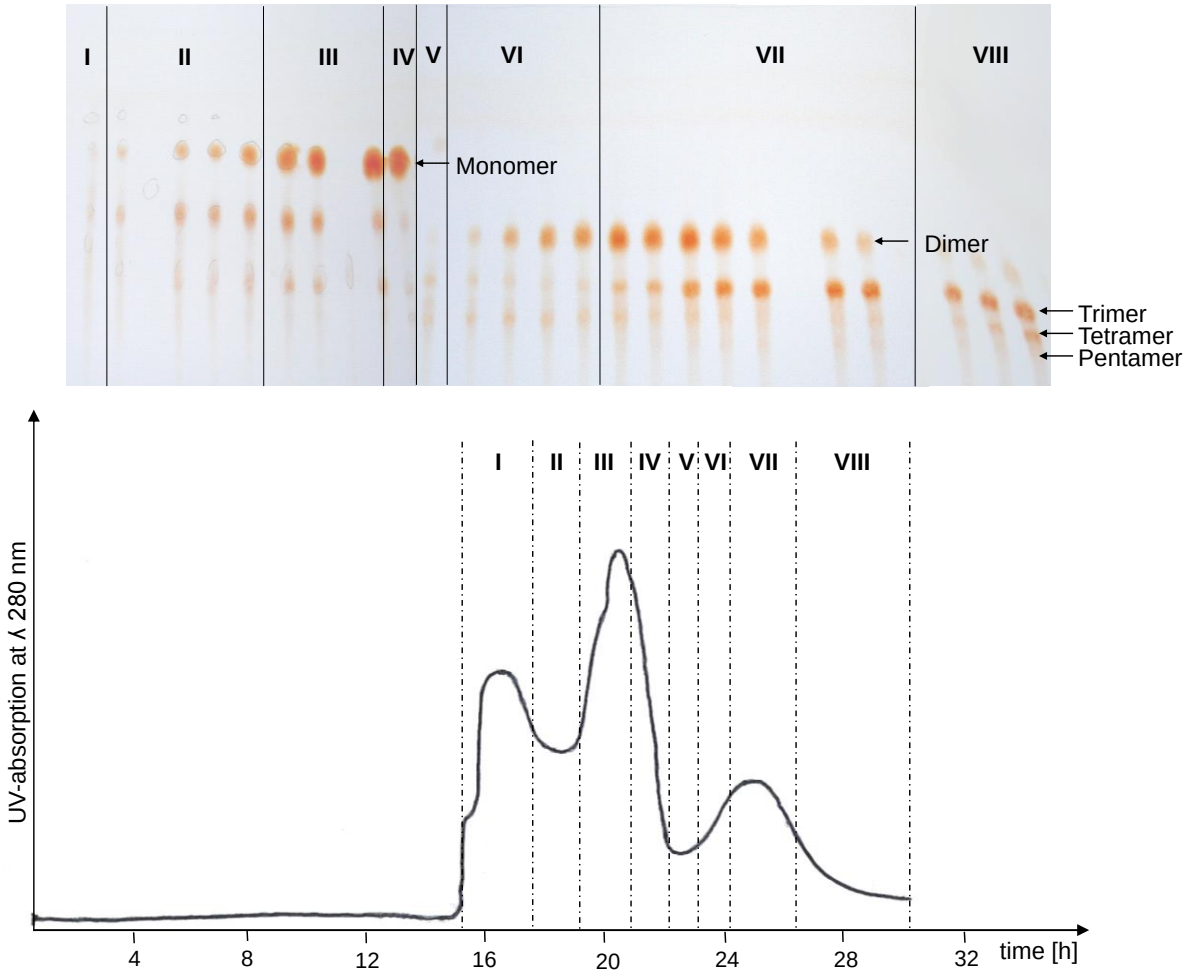
I
II
III
IV
V
VI
VII
VIII
Monomer
Dimer
Trimer
Tetramer
Pentamer
UV-absorption at λ 280 nm
I
II
III
IV
V
VI
VII
VIII
4
8
12
16
20
24
28
32
time [h]

Figure 5

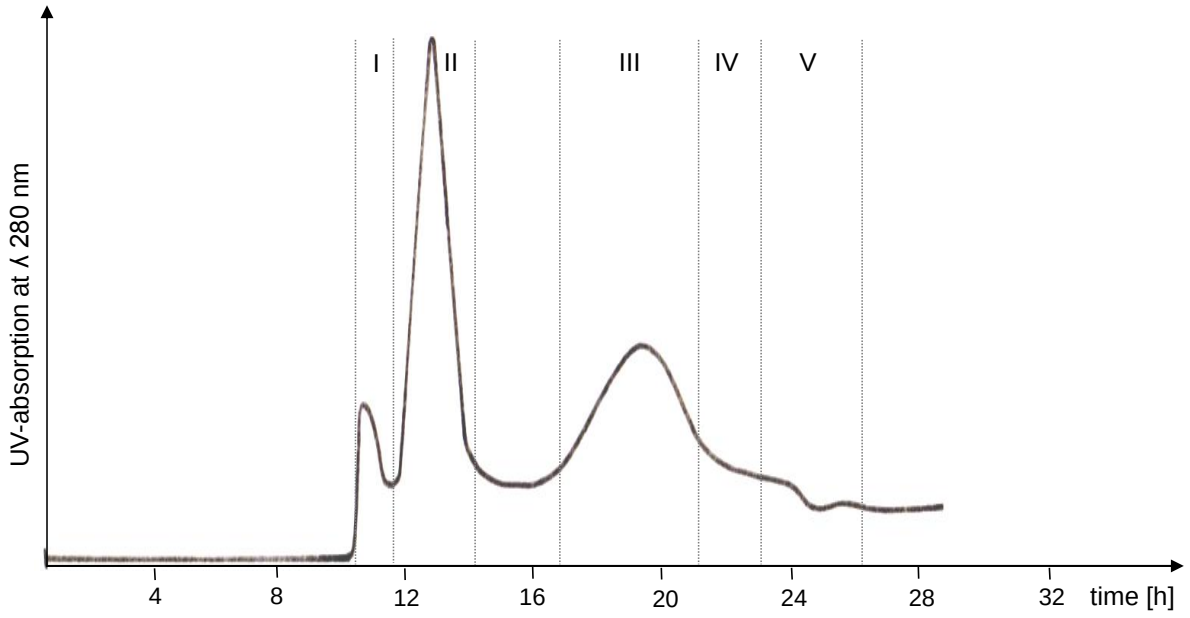

Figure 6

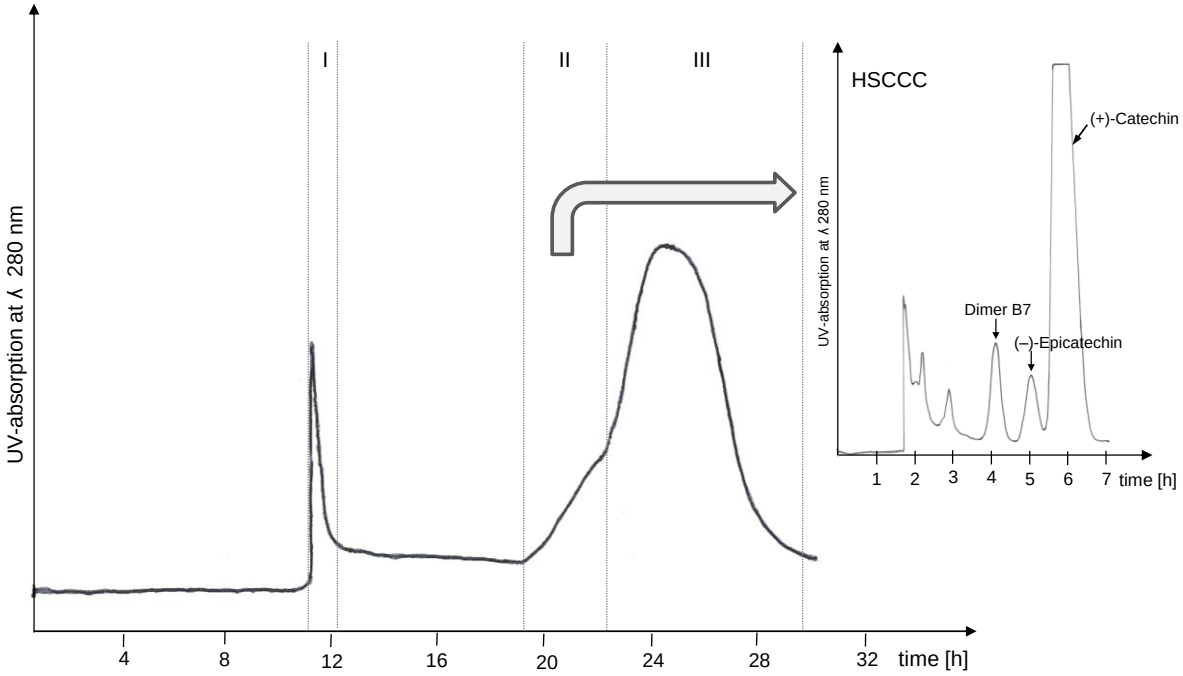
I
II
III
UV-absorption at λ 280 nm
4
8
12
16
20
24
28
32
time [h]
HSCCC
(+)-Catechin
Dimer B7
(–)-Epicatechin
UV-absorption at λ 280 nm
1
2
3
4
5
6
7
time [h]

Table 1. ^{1}H NMR Spectral Data of Tetrameric and Pentameric Procyanidin in Acetone-d_6 at 240 K (400 MHz).

Ring	Position	Tetramer Cinnamtannin A2 δ multiplicity (*J*)	Pentamer Cinnamtannin A3 δ multiplicity(*J*)
Upper unit (*A*)			
C	H2	5.08 s_{br}	5.08 s_{br}
C	H3	4.02 m	4.03 m
C	OH3	4.29	4.28
C	H4	4.80 s_{br}	4.81 s_{br}
A	H6	6.01 d (1.6)	6.02 d (1.6)
A	H8	5.99 d (1.6)	5.99 d (1.6)
A	OH7	8.66	8.70
B	H2´	6.95 d (1.8)	6.95 d (2.0)
B	H5´	6.70 d (8.0)	6.70 d (7.9)
B	H6´	6.62 dd (1.7, 8.1)	6.62 dd (2.1, 7.9)
Upper unit (*B*)			
C	H2	5.24 s_{br}	5.26 s_{br}
C	H3	4.15 m	4.15 m
C	OH3	4.29	4.28
C	H4	4.87 s_{br}	4.89 s_{br}
A	H6	5.94 s	5.95 s
A	OH7	7.17	7.16[b)]
B	H2´	7.08 d (n.d.)	7.07 d (1.8)
B	H5´	6.72 d (8.3)	6.65-6.73 m
B	H6´	6.66 dd (n.d.)	6.64-6.69 m
Upper unit (*C*)			
C	H2	5.26 s_{br}	5.29 s_{br}
C	H3	4.10 m	4.17 m
C	OH3	4.29	4.28
C	H4	4.83 d (2.0)	4.89 s_{br}
A	H6	5.94 s	5.95 s
A	OH7	7.36	7.40
B	H2´	7.12 d (1.9)	7.13 d (n.d.)
B	H5´	6.66 d (8.3)	6.64-6.69 m
B	H6´	6.74 dd (2.0, 8.3)	6.65-6.73 m
			Upper unit (*D*)
C	H2		5.28 s_{br}
C	H3		4.11 m
C	OH3		4.28
C	H4		4.83 s_{br}
A	H6/H8		5.95 s
A	OH7		7.27
B	H2´		7.13 d (n.d.)
B	H5´		6.65-6.73 m
B	H6´		6.75 dd (1.9, 8.2)
Terminal unit (*D*)			Terminal unit (*E*)
C	H2	5.08 s_{br}	5.08 s_{br}
C	H3	4.37 m	4.37 m
C	OH3	4.29	4.28
C	H4A	2.91 dd (4.5, 16.5)	2.92 dd (4.2, 16.2)
C	H4B	2.71 dd (<1, 16.4)	2.72 dd (<1, 16.2)
A	H6	5.94 s	5.95 s
A	OH7	7.25	7.17[b)]
B	H2´	7.19 d (2.0)	7.19 d (2.0)
B	H5´	6.94 d (n.d.)	6.94 d (8.7)
B	H6´	6.93 dd (1.8, n.d.)	6.94 dd (2.1, 8.7)

br = broad; n.d. = not determined

Partial assignment of the aromatic hydroxyl groups for the tetramer: 8.12, 8.18, 8.21, 8.25[a)] (OH5*D*), 8.28, 8.36 (OH5*A*), 8.38[a)] (OH5*C*), 8.40, 8.63[a)] (OH5*B*), 8.66, 8.70, 8.71

Partial assignment of the aromatic hydroxyl groups for the pentamer: 8.12[c)] (OH4´*B*), 8.16[c)] (OH4´*C*), 8.20[c)] (OH4´*D*), 8.20 (OH4´*A*), 8.23[d)] (OH5*B*), 8.35 (OH4´*E*), 8.35 (OH3´*A*), 8.37 (OH5*A*), 8.39[d)] (OH5*C*), 8.41[d)] (OH5*D*), 8.61 (OH3´*E*), 8.64 (OH3´*B*), 8.66[e)] (OH3´*C*), 8.68[e)] (OH5*E*), 8.68[e)] (OH3´*D*)

[a, b, c, d, e] shifts are interchangeable