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Preparation of Dimeric Procyanidins B1, B2, B5, and B7 from a Polymeric Procyanidin Fraction of Black Chokeberry (*Aronia melanocarpa*)

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A semisynthetic approach has been used for the preparative formation of dimeric procyanidins B1, B2, B5, and B7. As starting material for the semisynthesis, polymeric procyanidins from black chokeberry were applied. These polymers were found to consist almost exclusively of (−)-epicatechin units. Under acidic conditions the interflavanoid linkages of the polymeric procyanidins are cleaved and the liberated (−)-epicatechin can react with nucleophiles, such as (+)-catechin or (−)-epicatechin. In this way, the polymeric procyanidins are degraded while dimeric procyanidins are formed. During this reaction only dimeric procyanidins are formed that contain (−)-epicatechin in the upper unit, that is, B1 [(−)-EC-4β→8-(+)-C)], B2 [(−)-EC-4β→8-(−)-EC], B5 [(−)-EC-4β→6-(−)-EC], and B7 [(−)-EC-4β→6-(+)-C]. The reaction mixtures of the semisynthesis can be successfully fractionated with high-speed countercurrent chromatography (HSCCC), and it is possible to isolate pure procyanidins B1, B2, B5, and B7 on a preparative scale.

KEYWORDS: Dimeric procyanidins B1, B2, B5, and B7; black chokeberry (*Aronia melanocarpa*); preparation; high-speed countercurrent chromatography

INTRODUCTION

Aronia melanocarpa or black chokeberry is a member of the Rosaceae family. Black chokeberry originates from the eastern parts of North America and was introduced in Europe at the beginning of the 20th century. It is used for the production of, for example, juices, syrups, and soft spreads. Aronia fruits are known to exhibit a wide range of biological and pharmacological properties, such as antioxidative, anti-inflammatory, antiatherogenic, and antidiabetic activities (*1*, *2*). Important phenolic constituents of black chokeberry are polymeric procyanidins (*3*, *4*). Procyanidins consist of the flavan-3-ols (+)-catechin and (−)-epicatechin. In most cases, the linkage is between C_4 of the upper unit and C_8 of the lower unit (*5*). Most important, *A. melanocarpa* was found to contain homogeneous B-type procyanidins (*4*). The concentration of procyanidins in *A. melanocarpa* is among the highest determined in berries (664 mg/100 g of fresh weight) (*6*).

The aim of the present study was the development of a strategy for the preparation of dimeric procyanidins B1, B2, B5, and B7 on a large scale (**Figure 1**). Dimeric procyanidins can be formed directly by the reaction of procyanidin-rich extracts with flavan-3-ols. This approach was first applied to the synthesis of procyanidins with C2 epimers (*7*) using harsh reaction conditions (e.g., 95 °C for 22 h) and more recently modified by Köhler et al. (*8*).

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MATERIALS AND METHODS

Reagents. Black chokeberry pomace was supplied by Walther GmbH (Arnsdorf, Germany). The freeze-dried pomace was separated into seeds and skins using a sieve tower with eight different mesh sizes (2.8 mm−200 μm). The seeds were collected in sieve fraction 0.5−1 mm. Chemicals and solvents were as follows: (−)-epicatechin, p.a. (Sigma, Steinheim, Germany); (+)-catechin-hydrate, ≥98% (Sigma); sodium acetate (anhydrous), p.a. (Merck, Darmstadt, Germany); phloroglucinol, p.a. (Merck); hydrochloric acid, 37% (Riedel-de-Haën, Seelze, Germany); and ascorbic acid, pure (Merck). Solvents for high-performance liquid chromatography (HPLC) analysis were as follows: acetonitrile, HPLC quality (Sigma); acetic acid (Mallinckrodt Baker B.V., Deventer, The Netherlands); and water (deionized, Nanopure). Solvents used for high-speed countercurrent chromatography (HSCCC) and solvent precipitation were as follows: ethyl acetate (Acros Organics, Geel, Belgium); methanol (distilled, industrial quality); *n*-hexane (distilled, industrial quality); water (deionized, Nanopure); 1-butanol, p.a. (Fisher Scientific, Loughborough, U.K.); ethanol (distilled, industrial quality); 2-propanol, p.a. (Sigma); dichloromethane, p.a. (Fisher Scientific); water (deionized, Nanopure); and acetone (distilled, industrial quality).

Solvent Precipitation of a Polymeric Procyanidin Fraction. Approximately 21 g of milled aronia seeds or approximately 23 g of milled aronia pomace was defatted with *n*-hexane (3 × 200 mL) and dichloromethane (3 × 200 mL). Subsequently, the defatted aronia seeds were extracted with 70% aqueous acetone solution (3 × 200 mL). Defatted aronia pomace was extracted first with methanol (3 × 200 mL) and then with 70% aqueous acetone solution (3 × 200 mL). Three grams of the freeze-dried 70% aqueous acetone extracts were stirred for 1 h in 150 mL of ethanol and the insoluble residues were removed by filtration. Then 150 mL of *n*-hexane was dropped into the solution (10 mL/min). After filtration, the obtained precipitate was evaporated with a rotary evaporator and freeze-dried.

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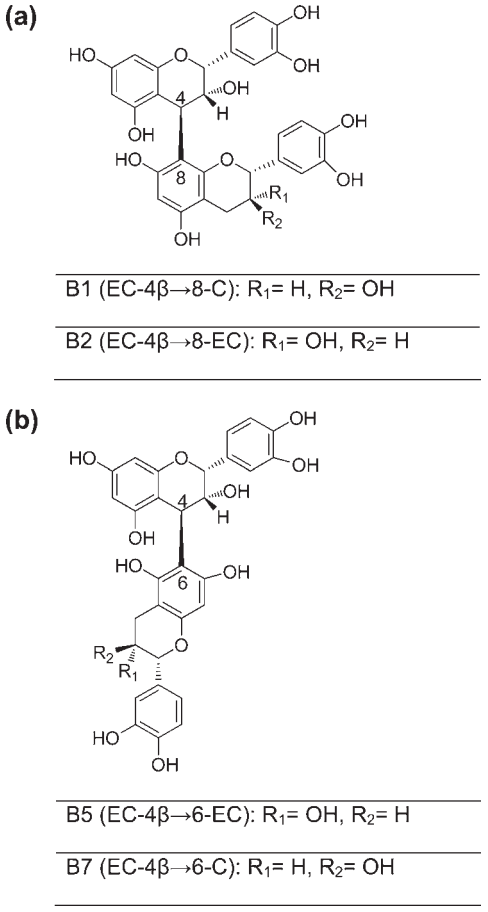

Figure 1. Structures of dimeric procyanidins of the B-type: (**a**) 4β→8 linked dimeric procyanidins B1 and B2; (**b**) 4β→6 linked dimeric procyanidins B5 and B7.

Phloroglucinolysis. Analysis was carried out according to the method of Kennedy et al. (*9*).

Optimization of Reaction Conditions. *Optimization of the Ratio of Substrates*. Aronia seed or aronia pomace precipitate (7.05 mg/mL) was reacted with various ratios of flavan-3-ol [(+)-catechin or (−)-epicatechin)], that is, 7.05, 14.10, and 21.15 mg/mL, corresponding to ratios of 1:0.75 (5.29 mg/mL), 1:1, 2:1, and 3:1 of flavan-3-ol to aronia seed or aronia pomace precipitate, in 1 N methanolic hydrochloric acid (HCl) solution for 20 min at 40 °C.

Optimization of Temperature. Aronia seed or aronia pomace precipitate (7.05 mg/mL) was reacted with flavan-3-ol (14.10 mg/mL) in 1 N methanolic HCl solution for 20 min at 30, 35, 40, and 50 °C, respectively.

Optimization of Reaction Time. Aronia seed or aronia pomace precipitate (7.05 mg/mL) was reacted with flavan-3-ol (14.10 mg/mL) in 1 N methanolic HCl solution at 40 °C for 10, 15, 20, and 30 min. In all cases the reaction was stopped by adding 5 times the volume of 80 mM aqueous sodium acetate solution.

Preparative Formation of Dimeric Procyanidins. Seven hundred milligrams of (+)-catechin or (−)-epicatechin and 350 mg of aronia seed or aronia pomace precipitate were used for semisynthesis. The reactants were dissolved in 50 mL of 0.1 N methanolic HCl and kept at 40 °C for 20 min. The reaction mixture was neutralized with 0.5 N sodium hydrogen carbonate solution. After evaporation with a rotary evaporator, the residual aqueous solution was freeze-dried. Yields of all reaction mixtures were about 1.35 g, but of these only 1 g was applied to HSCCC separation.

HPLC Photodiode Array (PDA) Analysis. A HPLC system from Jasco (Gross-Umstadt, Germany), 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 DAD were used. HPLC separation was achieved on a 250 mm × 4.6 mm i.d., 5 μm, Aqua column (Phenomenex, Aschaffenburg, Germany) protected with a guard column of the same material (4 mm × 4 mm). The mobile phases consisted of 2% aqueous acetic acid (v/v) (A) and acetonitrile (B). The separation was carried out at 25 °C, under the following conditions: 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 (55−60 min), followed by a reequilibration of the column for 10 min. Detection was at 280 nm; flow rate, 0.8 mL/ min. Procyanidins were identified with the help of isolated reference compounds and were quantified as dimeric procyanidin B1 equivalents.

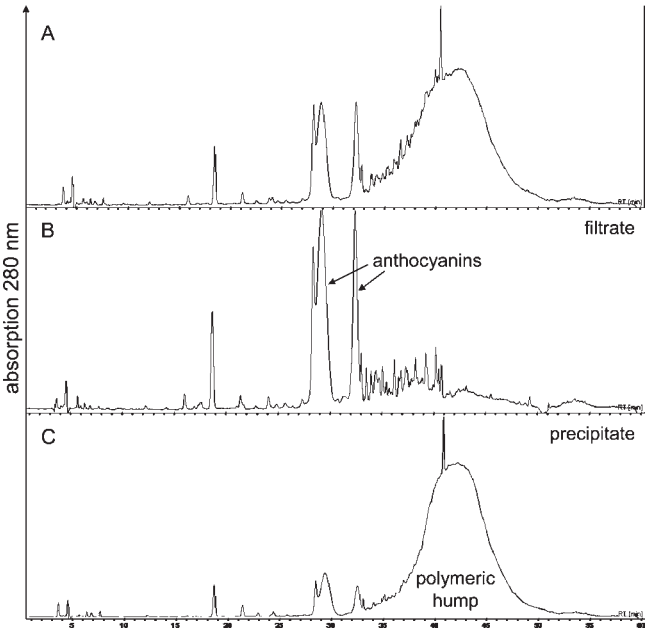

Figure 2. HPLC-PDA chromatograms of *Aronia melanocarpa* before (**A**) and after (**B**, **C**) solvent precipitation.

HPLC-ESI/MS/MS Analysis. Chromatographic analyses were performed on an Agilent 1100 HPLC system (Waldbronn, Germany) equipped with a 1200 autosampler and an 1100 HPLC pump. The HPLC was also interfaced to an Esquire HPLC-MS/MS system from Bruker GmbH (Bremen, Germany). HP ChemStation was used for data collection. MS parameters: negative mode; capillary, 3000 V; end plate, −500 V; capillary exit, −105 V; dry gas, 325 °C; gas flow, 10 L/min; and nebulizer, 40 psi. During the chromatographic run mass spectra of the eluate were recorded from m/z 50 to 2200. HPLC conditions as well as mobile phases were as described above.

HSCCC. A high-speed countercurrent chromatograph model CCC-1000 manufactured by Pharma-Tech Research Corp. (Baltimore, MD) was equipped with three preparative coils, connected in series (total volume = 800 mL). The revolution speed of the apparatus was 1000 rpm. Flow rates between 2.7 and 3.0 mL/min were used. A Biotronik HPLC pump BT 3020 was used to pump the liquids. The aqueous lower phase was applied as the mobile phase in the head to tail elution mode. All samples were dissolved in a 1:1 mixture of upper and lower phase and injected into the system by loop injection (20 mL). The amount of sample injected was 1 g. During each HSCCC separation, UV absorbance of the effluent was monitored by a Knauer UV−vis detector (280 nm) and recorded using a BBC Goerz SE 120 plotter (3 cm/h). Twelve milliliter fractions were collected by using a Super Frac fraction collector (Pharmacia, LKB Super Frac fraction collector). The two-phase solvent system ethyl acetate/*n*-butanol/water (14:1:15, v/v/v) was applied to the separation of dimeric procyanidin B1 (see CCC-1, CCC-3) and ethyl acetate/2-propanol/water (20:1:20, v/v/v) to the separation of dimeric procyanidin B2 (CCC-5, CCC-7). The dimeric procyanidins B5 and B7, which remained on the coil after the first fractionation, were rechromatographed with the more lipophilic solvent system: *n*-hexane/ethyl acetate/methanol/water (1:10:1:10, v/v/v/v) (CCC-2, CCC-4, CCC-6, CCC-8).

RESULTS AND DISCUSSION

Extraction of defatted aronia seeds or pomace with 70% aqueous acetone (v/v) and subsequent freeze-drying yielded a

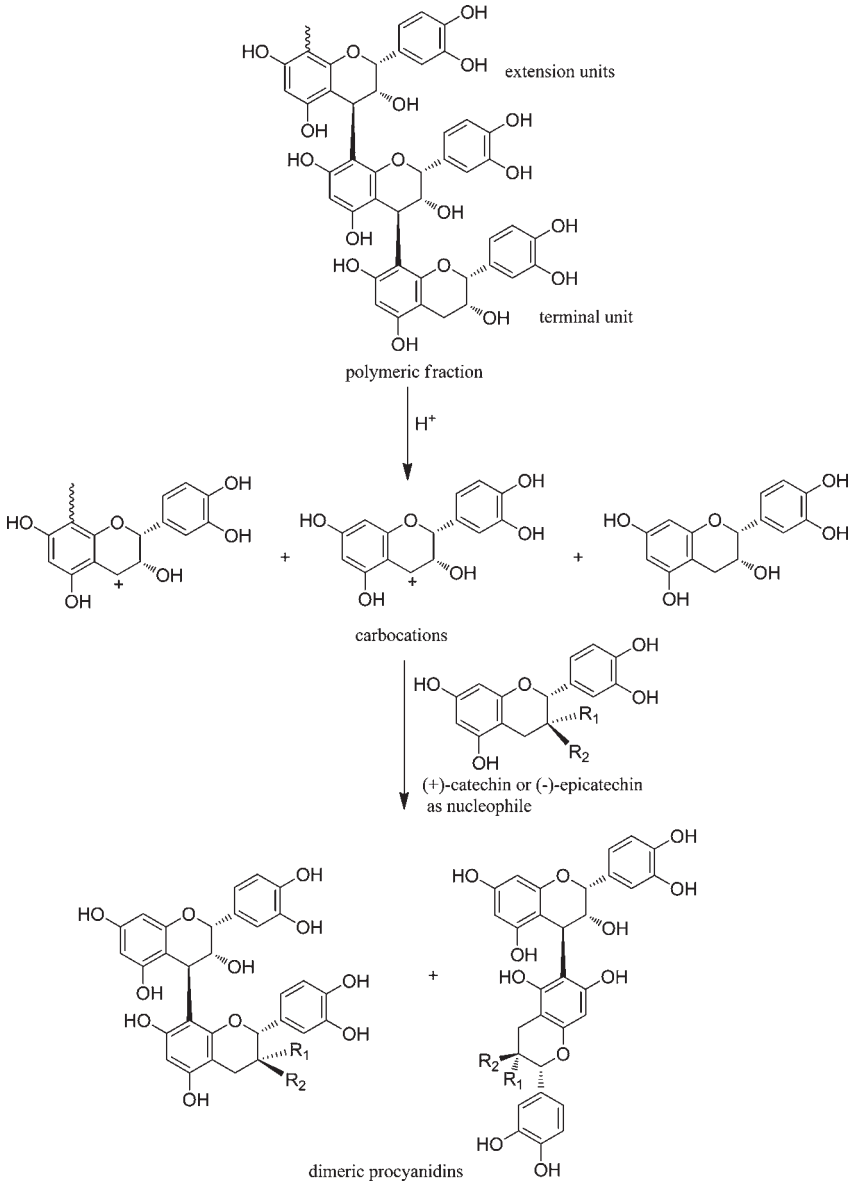

Figure 3. Mechanism of semisynthesis.

polyphenol-rich extract that mainly consisted of anthocyanins and polymeric procyanidins. Enrichment of polymeric procyanidins from this extract is possible by a simple cleanup step using solvent precipitation. For solvent precipitation the extract is redissolved in ethanol, and *n*-hexane is added as a nonpolar solvent. Of the different ratios of ethanol/*n*-hexane tested (0.5:1, 1:1, 1:2, 1:3, and 1:4), the 1:1 mixture was found to give the best yield of polymeric procyanidins in the precipitate (**Figure 2**). Our analysis of the so-obtained polymeric procyanidin fraction by phloroglucinolysis (*9*) revealed that besides a small amount of (+)-catechin (< 1.5%), only (−)-epicatechin units are present in the polymer chain. Galloylated subunits are not present. As mean degree of polymerization (mDP) a value of 25−30 has been determined.

It has recently been shown by Köhler et al. (*8*) that even under gentle reaction conditions, the interflavanoid linkage of polymeric procyanidins can be cleaved with 1 N methanolic HCl by releasing (+)-catechin or (−)-epicatechin as carbocation together with an uncharged terminal unit. The liberated carbocation can immediately react with an excess of added nucleophiles, such as (+)-catechin or (−)-epicatechin, giving rise to the formation of dimeric procyanidins (**Figure 3**). During this process the polymeric procyanidins are cleaved and simultaneously dimeric procyanidins are formed. Crucial conditions for this reaction, for example, the ratio of nucleophile to aronia seed or aronia pomace precipitate, temperature, and reaction time, must be optimized (**Figure 4**) to reduce the formation of so-called gambiriins (chalcane flavan-3-ol dimers, **Figure 5**), which are known by-products of this semisynthesis (*8*). The pathway of chalcane flavan-3-ol adduct formation was described in a previous paper (*8*). Formation of dimeric procyanidins as well as gambiriins was found to increase with higher amounts of added flavan-3-ol. The maximum formation of procyanidin dimers was observed at temperatures between 30 and 40 °C, whereas the yield of gambiriins continued to increase at higher temperature. Optimization of reaction time between 10 and 30 min showed the slightest influence on the formation of procyanidin dimers and gambiriins. Hence, the selected conditions for semisynthesis were a reaction temperature between 35 and 40 °C, a reaction time between 15 and 20 min, and a ratio of reactions partners, nucleophiles (i.e., catechin or epicatechin) and polymeric procyanidins, of 2:1.

Polymeric procyanidins of aronia seeds as well as aronia pomace elute as an unresolved broad peak (polymer hump)

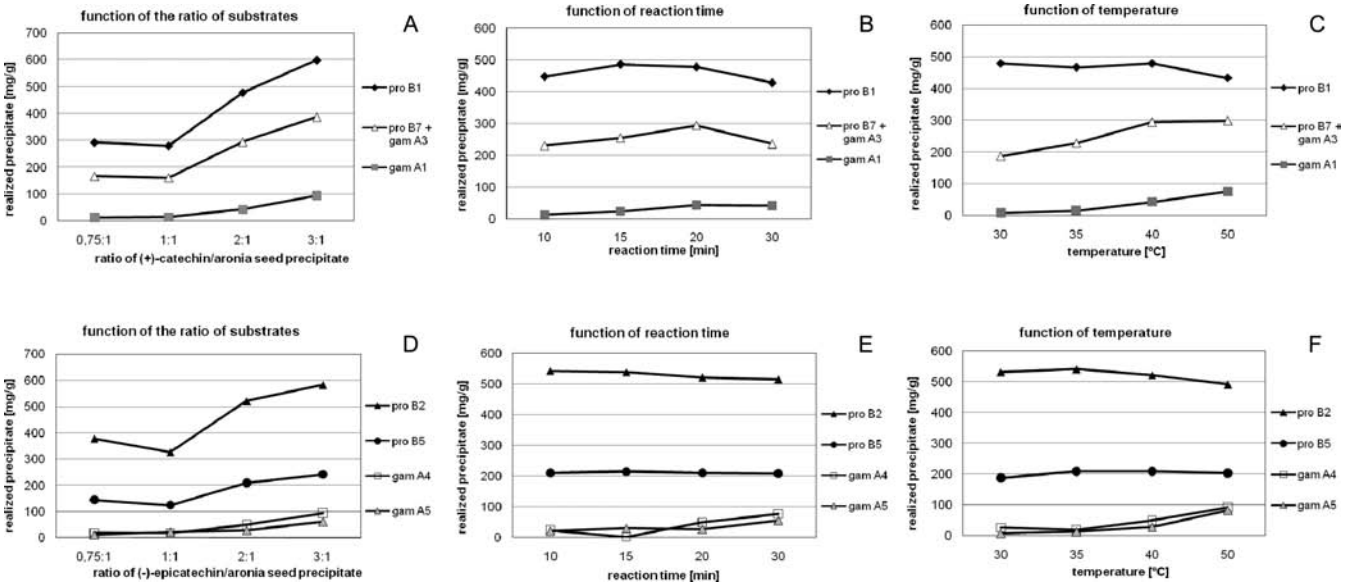

Figure 4. Optimization of reaction conditions: (**A**−**C**) with (+)-catechin; (**D**−**F**) with (−)-epicatechin (pro, procyanidin dimer; gam, gambiriin).

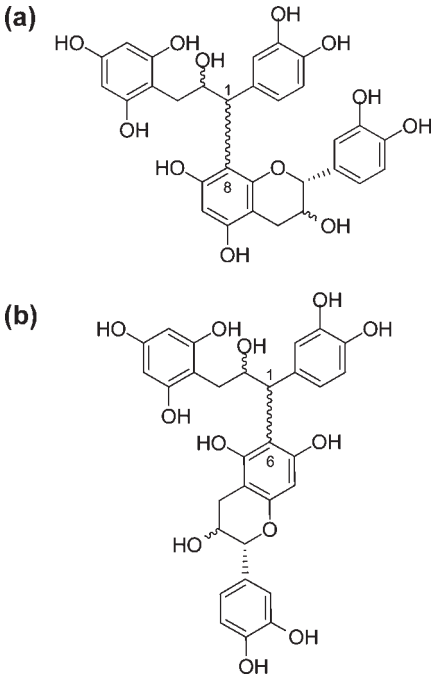

Figure 5. Structures of chalcane flavan-3-ol dimers (gambiriins): (**a**) 1→8 linked gambiriins; (**b**) 1→6 linked gambiriins.

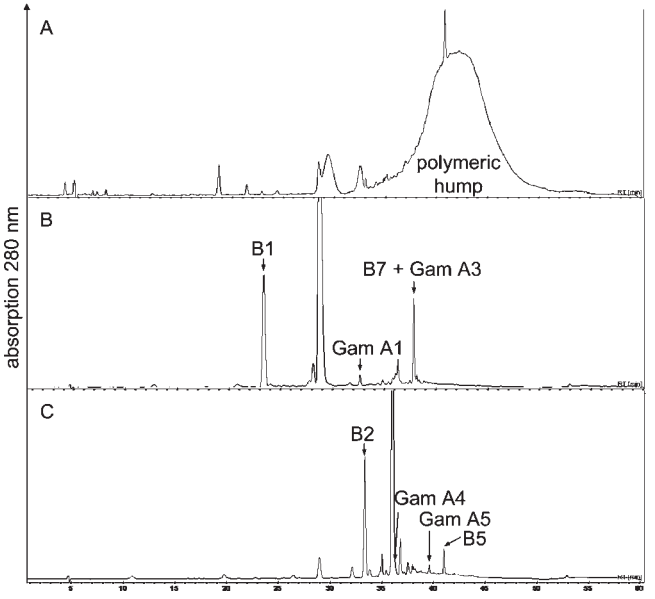

Figure 6. HPLC-PDA chromatograms of the *Aronia melanocarpa* precipitate before semisynthesis (**A**) and after semisynthesis with (+)-catechin (**B**) or (−)-epicatechin (**C**) as nucleophile under optimized conditions (given in the text) (Gam, gambiriin). See also the Supporting Information.

between 30 and 50 min in the HPLC-PDA chromatogram. **Figure 6A** shows the HPLC-PDA analysis of the aronia precipitate. After reaction with (+)-catechin or (−)-epicatechin, the concentration of dimeric procyanidins increased and the polymeric hump nearly disappeared. Consequently, only dimeric procyanidins with (−)-epicatechin in the upper unit, such as B1 (EC-4→8-C), B2 (EC-4→8-EC), B5 (EC-4→6-EC), and B7 (EC-4→6-C), are formed during semisynthesis. This is due to the composition of the polymeric procyanidins of *A. melanocarpa*. Our analyses (phloroglucinolysis) have shown that the polymeric fraction consists almost exclusively of (−)-epicatechin in the upper unit. Hence, with (+)-catechin as nucleophile dimeric procyanidins B1 and B7 are formed (**Figure 6B**), whereas addition of epicatechin produces dimeric procyanidins B2 and B5 (**Figure 6C**). The C4−C8 connected dimeric procyanidins B1 and B2 are formed in a higher yield compared to procyanidins B5 and B7.

HSCCC separations of the reaction mixtures of aronia seed (CCC-1) or aronia pomace precipitate (CCC-3) with (+)-catechin are shown in **Figure 7A**,**C**. As solvent system ethyl acetate/*n*-butanol/water (14:1:15, v/v/v) was selected, which was previously found to be suitable for the isolation of dimeric procyanidin B1 (*10*). From fraction 1 of CCC-1 26.8 mg of nonreacted polymeric procyanidins was recovered. In the case of the aronia pomace precipitate (CCC-3) this fraction was separated into three fractions. Fraction 1 consisted of polymeric procyanidins and fractions 2 and 3 of tetrameric procyanidins. From fraction 2 of CCC-1 129.4 mg of dimeric procyanidin B1 (purity 93.5%) was obtained and 122.5 mg of B1 (86.1%) from fraction 4 of CCC-3. A mixture of two unknown trimeric procyanidins (54.7%; 28.9%) was found in fraction 3 of CCC-1 as well as in fraction 5 of CCC-3

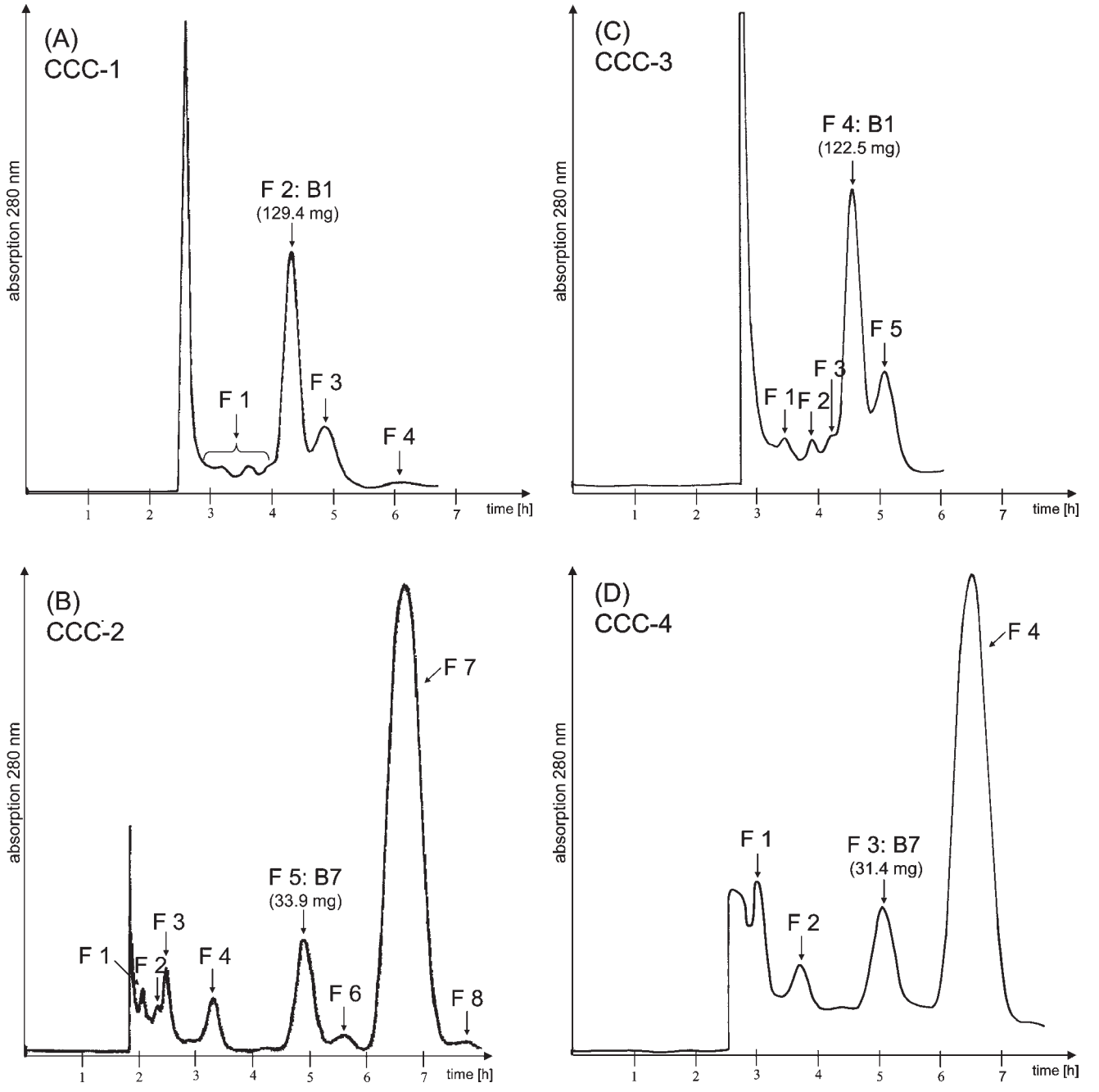

Figure 7. (**A**) CCC-1: HSCCC chromatogram of the reaction products of aronia seed precipitate and (+)-catechin. Injection of sample, 1000 mg; flow rate, 3 mL/min; retention of the stationary phase (Rs), 48.1%. (**B**) CCC-2: HSCCC chromatogram of the coil fraction of CCC-1. 564 mg sample; flow rate, 2.7 mL/min; Rs, 63.5%. (**C**) CCC-3: HSCCC chromatogram of the reaction products of aronia pomace precipitate and (+)-catechin. 1000 mg sample; flow rate, 2.7 mL/min; Rs, 45.8%. (**D**) CCC-4: HSCCC chromatogram of the coil fraction of CCC-3. 507 mg sample; flow rate, 2.7 mL/min; Rs, 49.7%. See also the Supporting Information.

(69.0%; 19.0%). The last fraction 4 of CCC-1 contained two epimerized dimeric procyanidins. The coil fractions of CCC-1 and CCC-3 were afterward separated using the more lipophilic solvent system *n*-hexane/ethyl acetate/methanol/water (1:10:1:10, v/v/v/v), which allowed the isolation of dimeric procyanidin B7 (*10*). **Figure 7B**,**D** displays the results of the HSCCC separations of the coil fractions that were labeled CCC-2 and CCC-4. Oligomeric procyanidins, like tetrameric and pentameric specimens, were found in fraction 1 (2.8 mg) from CCC-2, whereas in fractions 2 (1.8 mg) and 3 (3.6 mg, 75.6%) trimeric procyanidins were enriched. These compounds eluted in fraction 1 of CCC-4 together (15.5%, 52.2%). Fraction 4 of CCC-2 consisted of 9.5 mg of gambiriin A1 and an unknown trimeric procyanidin. Fraction 2 of CCC-4 was composed of 7.3 mg of gambiriin A1 and the unknown trimeric procyanidin in concentrations of 41.6 and 42.0%, respectively. Fraction 5 (CCC-2; 33.9 mg) contained dimeric procyanidin B7 (purity = 92.2%), and fraction 3 (CCC-4) consisted of 31.4 mg of procyanidin B7 (purity = 93.6%). The nonreacted (−)-catechin was recovered in >98% purity from fraction 7 of CCC-2 (368.9 mg) and from fraction 4 of CCC-4 (316.9 mg).

HSCCC separations of the reaction mixtures of the semisyntheses with the precipitates from aronia seed (CCC-5) and aronia pomace (CCC-7) with (−)-epicatechin are displayed in **Figure 8**, panels **A** and **C**, respectively. As solvent system for HSCCC ethyl acetate/2-propanol/water (20:1:20, v/v/v) was employed (*10*).

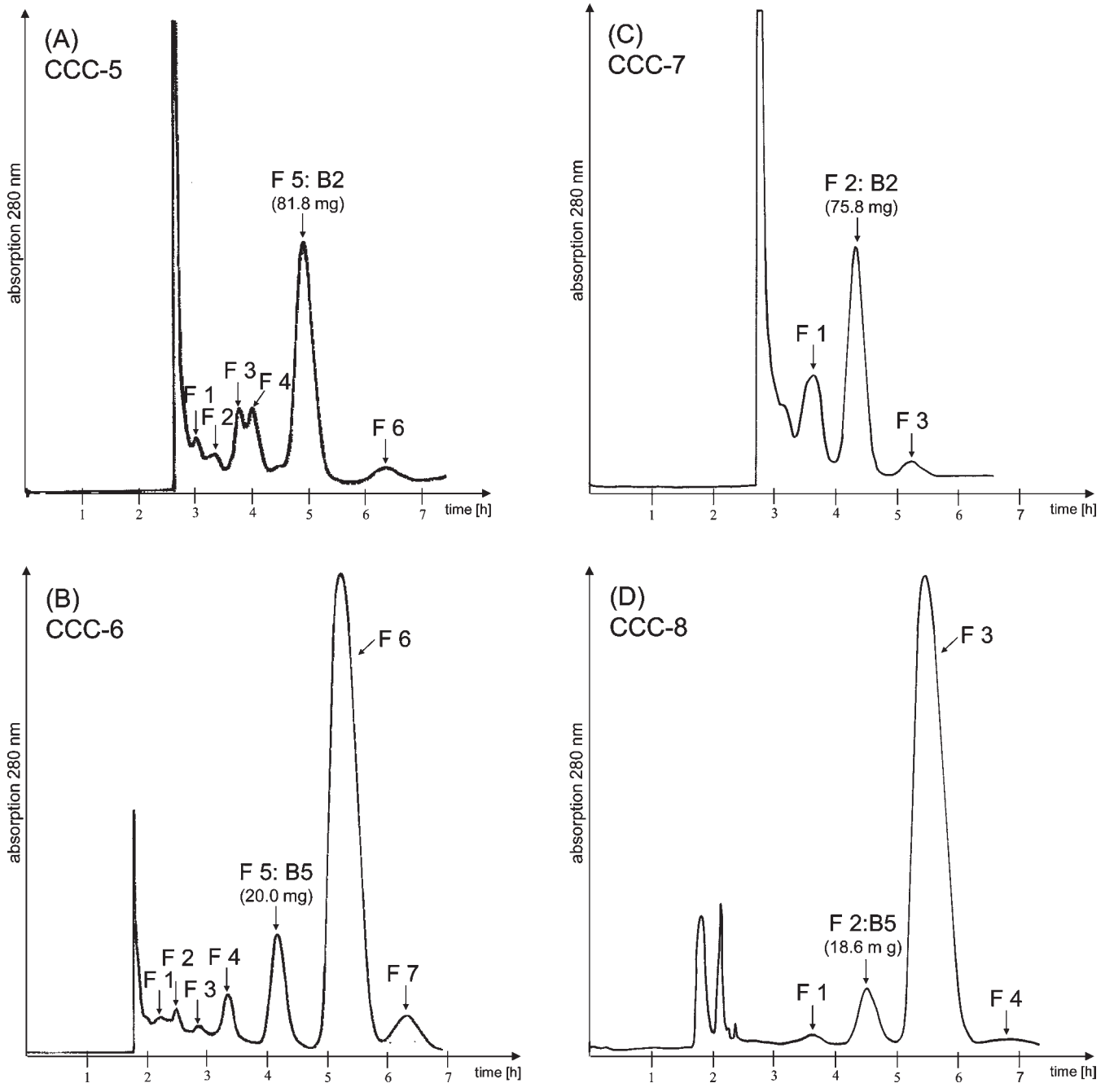

Figure 8. (**A**) CCC-5: HSCCC chromatogram of the reaction products of aronia seed precipitate and (−)-epicatechin. Injection of sample, 1000 mg; flow rate, 2.7 mL/min; Rs, 51.6%. (**B**) CCC-6: HSCCC chromatogram of the coil fraction of CCC-5. 402 mg sample; flow rate, 2.7 mL/min; Rs, 67.1%. (**C**) CCC-7: HSCCC chromatogram of the reaction products of aronia pomace precipitate and (−)-epicatechin. 1000 mg sample; flow rate, 3 mL/min; Rs, 37%. (**D**) CCC-8: HSCCC chromatogram of the coil fraction of CCC-7. 461 mg sample; flow rate, 2.7 mL/min; Rs, 65.8%.

The target compound procyanidin B2 was isolated from fraction 5 of CCC-5 in a purity of 96.4% (81.8 mg) as well as from fraction 2 of CCC-7 (75.8 mg) in a purity of 80.0%. Additional compounds of CCC-5 were in fraction 1 polymeric procyanidins (3.8 mg), in fraction 2 tetrameric procyanidins (3.9 mg), in fraction 3 an unknown trimeric procyanidin (6.4 mg, purity = 66.1%) and an (epi)catechin/(epi)gallocatechin derivative with a molecular ion $[M - H]^-$ at m/z 879 and fragmentations at m/z 861, 757, 727, 709, 633, 591, 547, 467, 439, 377, 301, and 259, and in fraction 4 trimeric procyanidin C1 (10.3 mg, purity = 87.7%). The latter compounds (42.0 mg) were also found in fraction 1 of CCC-7 in purities of 39.0 and 37.9%, respectively.

Compounds that remained on the coil were fractionated with the same solvent as in the case of CCC-2 and CCC-4. The results of the separation are shown in **Figure 8**, panels **B** (CCC-6) and **D** (CCC-8). Fraction 5 of CCC-6 (20.0 mg) contained 88.2% pure dimeric procyanidin B5, which was also found in fraction 2 of CCC-8 (18.6 mg) in a purity of 86.3%. Further compounds of CCC-6 were distributed as follows: polymeric procyanidins (fraction 1, 1.8 mg), unknown trimeric procyanidin (fraction 2, 2.1 mg), an (epi)catechin/(epi)afezelechin derivative (fraction 3; 3.6 mg) with a molecular ion $[M - H]^-$ at m/z 561 and fragmentations at m/z 517, 435, 425, 409, 391, 299, 287, 273, 255, 243, 229, and 161, a complex mixture of the gambiriin A4, propelargonidin, and unknown trimeric procyanidin (fraction 4, 4.3 mg). The excess of nonreacted (−)-epicatechin was isolated from fraction 6 of CCC-6 (284.4 mg) and from fraction 3 of CCC-8 (291.9 mg).

In conclusion, aronia seeds have been applied for the semisynthetic formation of dimeric procyanidins. Equally suitable is aronia pomace, the waste product in the production of chokeberry juices. Both sources represent a good starting material for the formation of dimeric procyanidins B1, B2, B5, and B7. Polymeric procyanidins of black chokeberry are composed almost exclusively of (−)-epicatechin; therefore, during semisynthesis only dimeric procyanidins are formed that contain (−)-epicatechin in the upper unit, such as dimeric procyanidins B1, B2, B5, and B7. HSCCC can be successfully used for the isolation on a preparative scale. In this way, dimeric procyanidins B1, B2, B5, and B7 were obtained in high purity (80.0−98.4%). Dimeric procyanidins B1 and B2 were obtained in highest yields. Otherwise, the C4β→C6 connected procyanidins, such as dimeric procyanidins B5 and B7, are formed in a significantly smaller amount.

ACKNOWLEDGMENT

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Supporting Information Available: Tables 1−8. This information is available free of charge via the Internet at http://pubs.acs.org.

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