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Large-scale isolation of flavan-3-ol phloroglucinol adducts by high-speed counter-current chromatography

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Abstract

Flavan-3-ol phloroglucinol adducts were synthesised through acid catalysed degradation of a procyanidins-rich grape seed extract in the presence of phloroglucinol. The reaction mixture (3.3 g) was fractionated without further sample preparation using the all-liquid chromatographic technique of high-speed counter-current chromatography (HSCCC). Selected solvent systems were hexane–ethyl acetate–methanol–water (0.1:5:0.1:5, v/v/v/v) and (1.5:10:1.5:10, v/v/v/v). The fractions obtained were found to contain almost pure compounds, in some cases final purification was achieved by preparative HPLC. The so-obtained pure standards of (+)catechin-(4α→2)-phloroglucinol, (−)epicatechin-(4β→2)-phloroglucinol, (+)catechine, (−)epicatechin-3-*O*-galloyl-(4β→2)-phloroglucinol, (−)epicatechin, and (−)epicatechin gallate are required for quantification of acid-catalysed phloroglucinol degradation products of procyanidins.

Keywords: Procyanidins; Proanthocyanidins; Grape seed extracts; Phloroglucinol; High-speed counter-current chromatography

1. Introduction

The acid catalysed-degradation with phloroglucinol or other nucleophilic reagents (e.g. benzyl mercaptan in case of a thiolytic degradation) is one important step in the analysis of polymeric compounds like proanthocyanidins. Through quantification of the degradation products the mean degree of polymerisation (mDP) is obtained, which is calculated by dividing the sum of all degradation products (terminal monomeric flavan-3-ols and 4-arylflavan-3-ols) by the amount of acid released terminal flavan-3-ols. Several studies describe the practice of this analytical method in detail [1–4].

The thiolytic degradation with benzyl mercaptan doesn't necessarily require the respective benzylthioether standards for quantification, because catechin and epicatechin exhibit the same response factor as their benzyl thioethers [3]. In the case of phloroglucinol adducts, however, standard calibration is inevitable. Although the phloroglucinol method has several advantages (e.g. easy handling of the chemicals, no unpleasant odour), authentic standards of flavan-3-ol phloroglucinol adducts must be synthesised [1]. This may explain the fact that an easy and fast method was developed for the preparation of the required standard compounds. CCC has been shown to be a fast and gentle separation method, which enables a high sample load [5–7]. The aim of this study was to show the application of CCC for the rapid separation of the flavan-3-ol phloroglucinol adducts.

2. Experimental

2.1. Chemicals

The grape seed extract (P100) was a gift from Breko (Bremen, Germany). Phloroglucinol was obtained from Fluka Chemika (Buchs, Switzerland). Hexane, ethyl acetate, and acetonitril were of p.a. grade and purchased from Acros Organics (Geel, Belgium). Methanol was distillated from industrial quality. Glacial acetic acid was obtained from J.T. Baker (Deventer, The Netherlands) and hydrochloric acid was pur-

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chased from Riedel-de-Haën (Seelze, Germany). Water was double ion-exchanged water.

2.2. Degradation of grape seed extract (P100)

The grape seed extract (500 mg) and phloroglucinol (3.0 g) were dissolved in 50 mL of 0.1N methanolic HCl (0.84 mL 37% aqueuos HCl in 100 mL methanol). The reaction was carried out in a stirring water bath at 50 °C for 20 min. Then the reaction was stopped by adding 10 mL of 0.5 N $NaHCO_3$ solution. Methanol was removed using a rotary evaporator. The residue was freeze-dried and directly separated by HSCCC.

2.3. HSCCC-conditions

2.3.1. HSCCC-apparatus

The HSCCC apparatus was a triple coil centrifuge (PTR-1000, Baltimore, MD, USA) with a total coil capacity of 850 mL driven by a speed control. The solvent was pumped with a Biotronic HPLC pump. The effluent was monitored by a UV detector (K-2501, Knauer, Berlin, Germany) connected to a plotter (Servogor 120, Metrawatt GmbH, Germany). The fractions were collected with a fraction collector (SuperFrac, Pharmacia LKB, Uppsala, Sweden).

Table 1
^{1}H NMR data of (+)catechin-(4α→2)-phloroglucinol (**1**), (−)epicatechin-(4β→2)-phloroglucinol (**2**) and (−)epicatechin-3-*O*-galloyl-(4β→2)-phloroglucinol (**4**); chemical shifts δ in ppm and coupling constant in Hz (0.75 mL d_6-acetone + 0.05 mL D_2O)

H	1	2	4	
2	4.38 (d 9.2)	5.03 (s)	5.45 (br s)	C ring
3	4.48 (dd 9.2, 7.8)	3.98 (dd 2.0, 1.0)	5.24 (m)	
4	4.43 (d 7.8)	4.54 (d 2.0)	4.62 (d 1.5)	
4a				A ring
5				
6	5.89 (d 2.3)[a]	6.00 (d 2,3)[a]	6.02 (d 2.3)	
7				
8	5,91 (d 2.3)[a]	6.02 (d 2.3)[a]	6.10 (d 2.3)	
8a				
1′				B ring
2′	7.00 (d 1.8)	6.97 (d 1.8)	6.95 (d 1.8)	
3′				
4′				
5′	6.79 (d 8.1)	6.76 (d 8.1)	6.73 (d 8.2)	
6′	6.85 (dd 8.1, 1.8)	6.69 (dd 8.1, 1.8)	6.79 (dd 8.2, 1.8)	
1″				D ring
2″				
3″	5.96 (br s)	5.91 (br s)	5.97 (br s)	
4″				
5″	6.00 (br s)	5.91 (br s)	5.97 (br s)	
6″				
1‴				
2‴			7.05 (s)	
3‴				
4‴				
5‴				
6‴			7.05 (s)	
7‴				

d: doublet, br s: broad singulett, m: multiplett.
[a] Assignments with the same footnote are interchangeable.

Table 2
^{13}C NMR data of (+)catechin-(4α→2)-phloroglucinol (**1**), (−)epicatechin-(4β→2)-phloroglucinol (**2**) and (−)epicatechin-3-*O*-galloyl-(4β→2)-phloroglucinol (**4**); chemical shifts δ in ppm (d_6-acetone)

C	1	2	4	
2	84.7	77.8	76.2	C ring
3	74.3	73.3	75.8	
4	39.0	37.6	35.0	
4a	106.4	101.4	101.6	A ring
5	158.9[d]	159.3[d]	159.1	
6	98.1[a]	96.7[a]	97.3	
7	158.5[d]	158.7[d]	158.4	
8	97.1[a]	97.2[a]	96.5	
8a	158.1[d]	158.5[d]	158.1	
1′	133.0	133.2	132.1	B ring
2′	116.6	115.9[b]	115.5	
3′	146.5[b]	146.1[c]	146.2	
4′	146.2[b]	146.0[c]	146.3	
5′	116.3	116.3[b]	116.5	
6′	121.5	120.0	119.9	
1″	108.0	107.6	106.7	D ring
2″	158.6[d]	158.6[d]	158.9	
3″	97.9[c]	97.2[a]	97.6	
4″	159.2[d]	159.4[d]	159.2	
5″	96.8[c]	97.2[a]	97.6	
6″	158.6[d]	158.6[d]	158.9	
1‴			122.1	
2‴			110.9	
3‴			146.7	
4‴			139.9	
5‴			146.7	
6‴			110.9	
7‴			168.0	

Assignments with the same footnote (a–d) are interchangeable.

2.3.2. HSCCC solvent systems

The solvent system hexane–ethyl acetate–methanol–water (0.1:5:0.1:5, v/v/v/v) was applied to the separation of

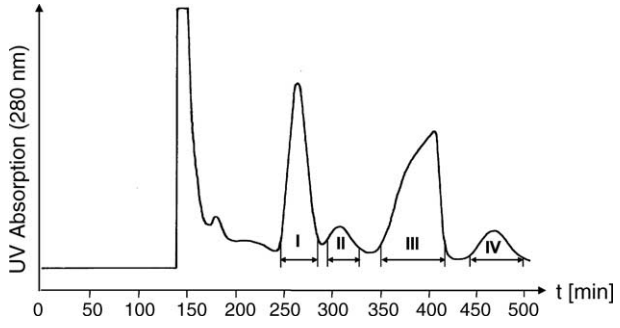

Fig. 1. HSCCC-separation 1: hexane–ethyl acetate–methanol–water (0.1:5:0.1:5, v/v/v/v); flow rate 3.0 mL/min, 3.3 g sample: (−)epicatechin-(4β→2)-phloroglucinol (**I**): (+)catechin-(4α→2)-phloroglucinol (**II**), phloroglucinol (**III**), (−)epicatechin (**IV**).

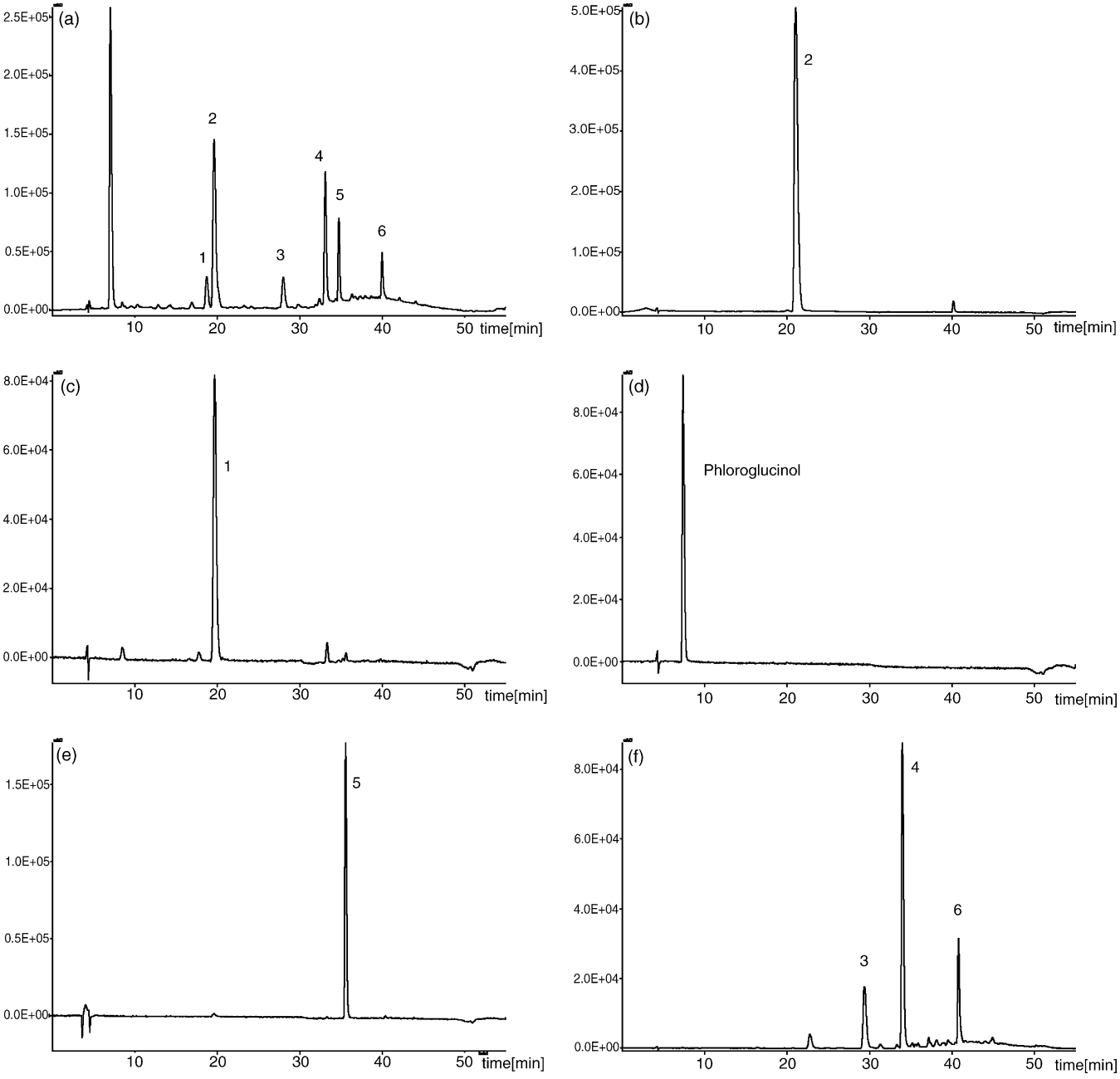

Fig. 2. HPLC–UV-280 nm chromatograms of HSCCC-separation 1: (a) degradation products after acid-catalysed hydrolysis (sample); (b) fraction I; (c) fraction II; (d) fraction III; (e) fraction IV and (f) coil fraction (for peak assignment of Fig. 5.).

the degradation products. The compounds which remained on the coil after the first fractionation were rechromatographed with the modified solvent system: hexane–ethyl acetate–methanol–water (1.5:10:1.5:10, v/v/v/v).

2.3.3. HSCCC separation

First, the multilayer coil was entirely filled with the upper organic phase as the stationary phase. Then the lower aqueous phase was pumped into the head end of the coil system at a flow rate of 3.0 mL/min, while the centrifuge was rotating at 800 rpm. The sample was dissolved in 10 mL of each phase and injected with a sample loop directly after applying the mobile phase flow. The effluent from the tail side was monitored with a UV detector at 280 nm and collected with a fraction collector, while a chromatogram was recorded. Fractions of the same peak were pooled.

2.4. HPLC-DAD (analytical)

The fractions were analysed with a Jasco HPLC system consisting of a PU-980 Intelligent HPLC Pump, a ternary gradient unit LG-980-02 with degasser DG-980-50, and MD-910 multiwavelength detector driven by BORWIN chromatography software. A RP-18 Prontosil column (4.6 mm × 250 mm,

5 μm, Knauer) was applied with a binary linear gradient of 2% aqueous acetic acid (A) and acetonitrile (B): from 97% A and 3% B to 90% A and 10% B in 25 min, to 65% A and 35% B in 20 min, to 25% A and 75% B in 5 min and back to initial conditions in 5 min.

2.5. HPLC-UV (280 nm) for preparative isolation

The collected fractions were purified by preparative HPLC with a Knauer pump, a Rheodyne injection assembly, and a Knauer UV detector. The column was a RP-18 (Hypersil from Phenomenex, 5 μm, 20 mm × 250 mm, with a guard column). The elution was isocratic with the binary solvent system (water–acetonitrile): 8% acetonitrile for fraction II and III of HSCCC-1, 18% acetonitrile for fraction V of HSCCC-2, and 27% acetonitrile for the coil fraction of HSCCC-2.

2.6. NMR

The experiments were carried out with a Bruker AMX-300 spectrometer (Karlsruhe, Germany) with 300 MHz for ^{1}H and 75 MHz for ^{13}C measurements, respectively. The calibration was applied to the solvent signal and all data were compared with those of references [1,8,9].

2.6.1. *(2R,3S,4R)-2,3-trans-3,4-trans-4-(2,4,6-trihydroxyphenyl)-3′,4′,5,7-tetrahydroxyflavan-3-ol [(+)catechin-(4α→2)-phloroglucinol] (**1**)*

^{1}H NMR (0.75 mL acetone-d_6 + 0.05 mL D_2O): c.f. Table 1.

^{13}C NMR (acetone-d_6): c.f. Table 2.

2.6.2. *(2R,3R,4S)-2,3-cis-3,4-trans-4-(2,4,6-trihydroxyphenyl)-3′,4′,5,7-tetrahydroxyflavan-3-ol [(−)epicatechin-(4β→2)-phloroglucinol] (**2**)*

^{1}H NMR (0.75 mL acetone-d_6 + 0.05 mL D_2O): c.f. Table 1.

^{13}C NMR (acetone-d_6): c.f. Table 2.

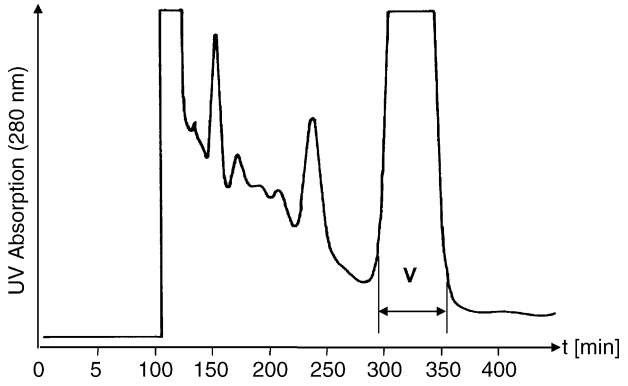

Fig. 3. HSCCC-separation 2: hexane–ethyl acetate–methanol–water (1.5:10:1.5:10, v/v/v/v); flow rate 3.0 mL/min, 150 mg of coil fraction; fraction V; (+)catechin (**3**) and (−)epicatechin-3-*O*-galloyl-(4β→2)-phloroglucinol (**4**), while (−)epicatechin gallate (**6**) remained on the coil.

2.6.3. *(2R,3S,4R)-2,3-trans-3,4-trans-3-O-galloyl-4-(2,4,6-trihydroxyphenyl)-3′,4′,5,7-tetrahydroxyflavan-3-ol [(−)epicatechin-3-O-galloyl-(4β→2)-phloroglucinol] (**4**)*

^{1}H NMR (0.75 mL acetone-d_6 + 0.05 mL D_2O): c.f. Table 1.

^{13}C NMR (acetone-d_6): c.f. Table 2.

3. Results and discussion

Reaction conditions for the acid-catalysed degradation of polymeric proanthocyanidins were the same as reported by Kennedy and Jones [1], except for the use of buffer solution for termination of the reaction. Instead of sodium acetate an aqueous $NaHCO_3$ solution was chosen for neutralisation of the acid, which was added in equal molar amounts. Replacement of the buffer by $NaHCO_3$ solutions reduced the amount (mass) of side compounds like salts (702 mg salt, which comprised 410 mg sodium acetate and 292 mg sodium chloride, is reduced to 292 mg sodium chloride), which would

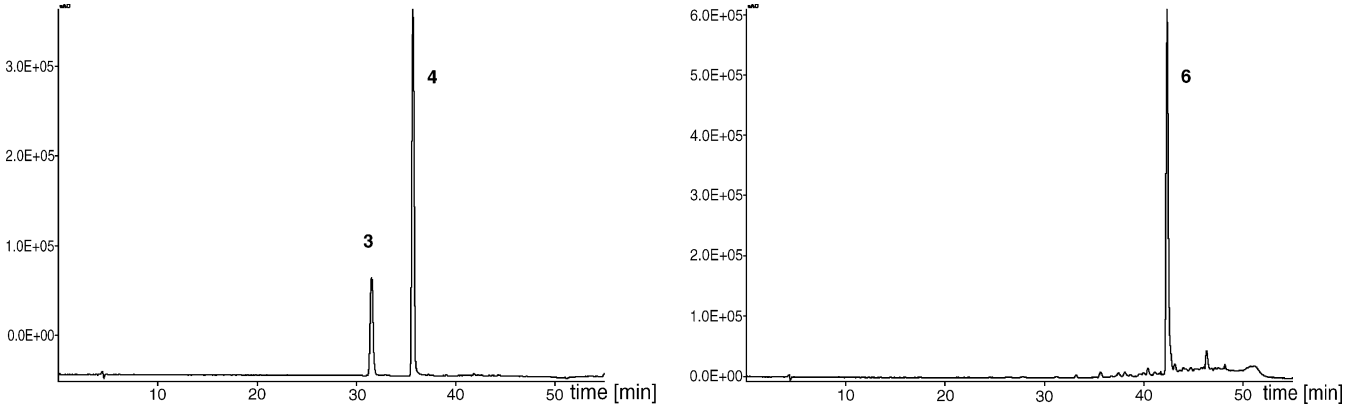

Fig. 4. HPLC–UV-280 nm chromatograms of HSCCC-separation 2: (left: fraction V, (+)catechin (**3**), (−)epicatechin-3-*O*-galloyl-(4β→2)-phloroglucinol (**4**); right: coil fraction, (−)epicatechin gallate (**6**)).

impair the counter-current chromatographic conditions. Reaction conditions were easy scaled-up to preparative conditions without any loss in yield. The freeze-dried reaction mixture was directly separated by HSCCC using hexane–ethyl acetate–methanol–water (0.1:5:0.1:5, v/v/v/v) as the solvent system (Fig. 1). Fig. 2 shows the HPLC–UV (280 nm) chromatograms of the isolated fractions. Fraction I which eluted after 270 min contained 149 mg of nearly pure (−)epicatechin-(4β→2)-phloroglucinol (**2**) (purity >98%). Fraction II contained 44 mg of approximately 90% pure (+)catechin-(4α→2)-phloroglucinol (**1**), which was further purified with preparative HPLC. Fraction III consisted of unreacted phloroglucinol showing an asymmetrical shaped peak. This was caused by the high local concentration of phloroglucinol, which produced a local emulsion that could be seen in the corresponding collected fractions, and included some of the stationary phase. The fraction IV contained 33 mg of pure (−)epicatechin (**5**). (+)Catechin (**3**), (−)epicatechin gallate (**6**) and (−)epicatechin-3-*O*-galloyl-(4β→2)-phloroglucinol (**4**) remained on the coil (approx. 150 mg) and were separated with the modified solvent system hexane–ethyl acetate–methanol–water (1.5:10:1.5:10, v/v/v/v) after freeze-drying. The results are shown in Figs. 3 and 4. The chromatogram of the HSCCC separation shows one big peak, which elutes between 300 and 360 min (fraction V). This peak contained 24 mg (44%) of (+)catechin and 31 mg (56%) of (−)epicatechin-3-*O*-galloyl-(4β→2)-phloroglucinol, which were separated by preparative HPLC. (−)Epicatechin gallate (**6**) remained on the coil of the second separation (approx. 40% purity, 22 mg) with other not characterised polymeric material and was also purified by preparative HPLC.

3.1. NMR

The ^{1}H, and ^{13}C NMR data unambiguously confirmed the structures of compound **1**, **2**, and **4** (Tables 1 and 2). In contrast to reference data [1,8,9], ^{13}C spectra were analysed solely in d_6-acetone, while ^{1}H data were recorded in a mix-

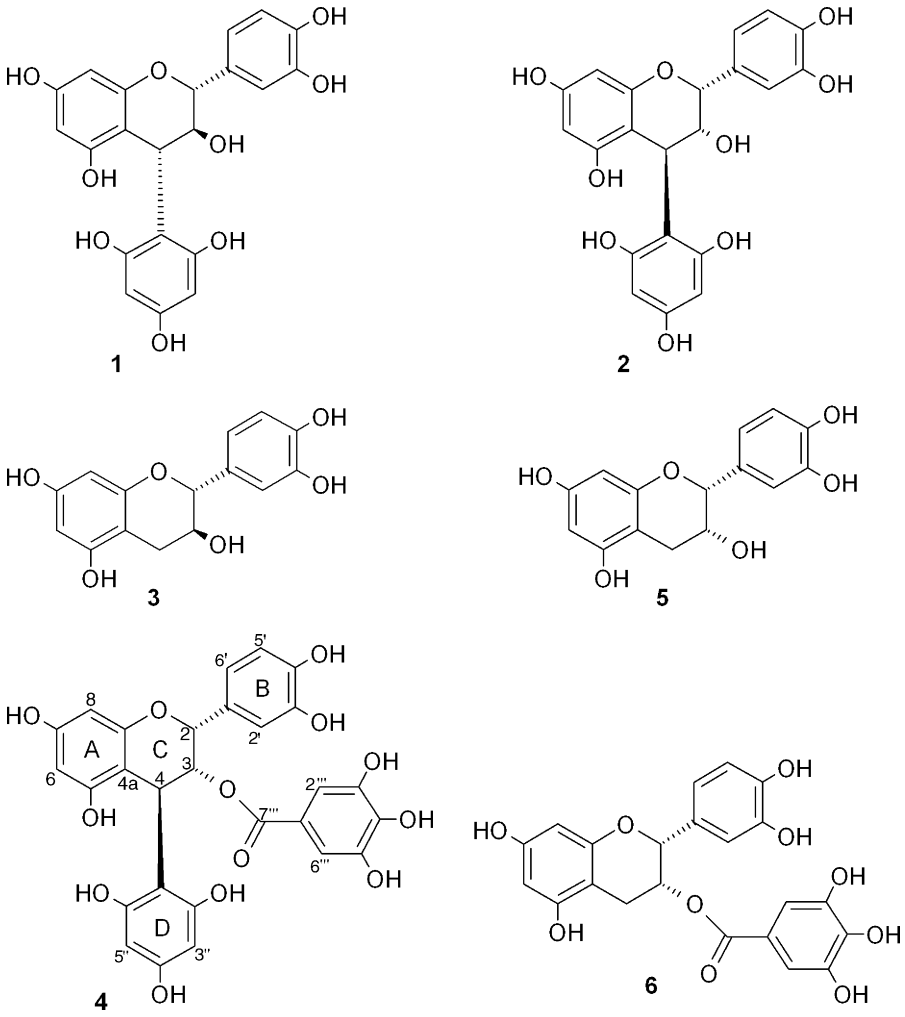

Fig. 5. Flavan-3-ol cleavage products of acid catalysed hydrolysis of procyanidins: (+)catechin-(4α→2)-phloroglucinol (**1**), (−)epicatechin-(4β→2)-phloroglucinol (**2**), (+) catechin (**3**), (−)epicatechin-3-*O*-galloyl-(4β→2)-phloroglucinol (**4**), (−)epicatechin (**5**), (−)epicatechin gallate (**6**).

ture of d_6-acetone and D_2O (0.75:0.05 mL) enabling the D/H exchange. Therefore, ^1H- and ^{13}C-signals slightly differed to the δ values given in literature.

Significant δ value shift differences occurred for all carbon atoms in ring C, i.e. C-2, C-3, C-4, and C-4a (c.f. table) clearly distinguishing all the synthesized diastereomeric phloroglucinol-flavonol adducts **1**, **2**, and **4**.

Relative stereochemistry of the catechin adduct **1** with a 2,3-*trans*-3,4-*trans* configuration was indicated in the ^1H by two large diaxial coupling constants (J_1 9.2 and J_2 7.8 Hz).

The 2,3-*cis*-3,4-*trans* configurations in the C-ring of compounds **2** and **4** were elucidated by smaller coupling constants ($J \sim 1.5$–2.0 Hz). In the ^1H spectrum, substance **4** showed a strong downfield shift for H-3 due to galloylation.

4. Conclusion

HSCCC enables a fast separation of phloroglucinol degradation products in large quantities, which are required for the analysis of proanthocyanidins. The advantage of this method compared to classical gel or solid phase chromatography is that no irreversible adsorption occurs. Therefore, a 100% recovery of the sample is obtained.

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