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Isolation of two anthocyanin sambubiosides from bilberry (*Vaccinium myrtillus*) by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography (HSCCC) was used for the separation of anthocyanins on a preparative scale from bilberry fruit crude extract (*Vaccinium myrtillus*, Ericaceae) with a biphasic solvent system composed of methyl *tert*-butyl ether–*n*-butanol–acetonitrile–water–trifluoroacetic acid (1:4:1:5:0.01, v/v). Each injection of 500 mg crude extract yielded 130 mg of pure delphinidin-3-O-sambubioside, and 77 mg of pure cyanidin-3-O-sambubioside. The two anthocyanin disaccharides with a glucose (2 → 1) xylose unit are novel compounds for bilberry fruit, and were elucidated by means of electrospray ionization MS–MS, ^{1}H, ^{13}C, distortion less enhancement by polarization transfer NMR, and two-dimensional HC-correlation experiments (heteronuclear multiple bond correlation and heteronuclear multiple quantum correlation).

Keywords: Counter-current chromatography; *Vaccinium myrtillus*; Anthocyanins; Delphinidin-3-O-sambubioside; Cyanidin-3-O-sambubioside; Flavonoids

1. Introduction

The present study describes the preparative isolation of two pure anthocyanin-3-O-sambubiosides by high-speed counter-current chromatography (HSCCC) from a crude extract of bilberry (*Vaccinium myrtillus*), and their structural identification by electrospray ionization (ESI) MS–MS, one-dimensional (1D) and two-dimensional (2D) NMR experiments.

Anthocyanin components are naturally occurring flavonoid pigments with a flavylium cation structure, widely distributed in fruits, and vegetables showing high antioxidant capacity 'in vitro', as well 'in vivo'. Consumed as part of the human diet they are considered to play an important role as radical scavengers, preventing oxidative stress, and help to maintain physiological functions [1–5]. Dietary intake of natural antioxidants such as flavonoids, including anthocyanins also have been suggested to contribute to the prevention of coronar heart diseases [6,7]. Among fruits and vegetables, bilberry extracts show significant antioxidant activities, which can be correlated to their high content of anthocyanin pigments [8–11], and are currently discussed as dietary supplements in functional food products.

Improvement of blood vessel conditions in the retina and helping to increase night visual abilities of bilberry extracts are still controversially discussed. A recent study shows that bilberry extract reduces epoxidation of the fluorophore A2E, an autofluorescent pigment that accumulate in retinal pigment epithelial cells during aging, a reason for retinal disorders [12].

Anthocyanins also mediate physiological functions related to cancer suppression [5,13–16]. The growth inhibitory effects of anthocyanins in K-562 leukemia, and HCT-15 carcinoma cells are stronger than those of other phenolic components, such as flavonols and flavanons [13]. Among extracts of various anthocyanin containing berries, bilberry extract was found to be most effective for inhibiting HL-60 human leukemia, and HCT-116 human colon carcinoma cells 'in vitro'. Anthocyanins purified from bilberry induced cellular apoptosis in HL-60 cells [16,17]. Bilberry contains the greatest amount of anthocyanins among the different berry varieties, with a ratio of 30:36:13 for cyanidin-,

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delphinidin-, and malvidin-3-O-glycosides. This was shown to be the reason for the strongest inhibitory effects to cancer cell growth [17].

Key problem of isolating polar anthocyanin pigments is that routine conventional column chromatography on silica gel, and reversed phase C18 materials are limited in their separation efficiency. Recently, anthocyanins from red wine were successfully separated by using high-speed counter-current chromatography [18,19]. HSCCC as an all-liquid chromatographic technique, avoids irreversible adsorption on solid support, the separation is solely based on fast partitioning effects of the analytes between two immiscible liquid phases. In the present paper, the isolation and identifcation of anthocyanin-sambubiosides in bilberry extract by means of HSCCC and NMR spectroscopy is reported.

2. Experimental

2.1. Reagents

The organic solvents, *n*-butanol and *n*-hexane used for HSCCC separation were of analytical grade. Water was nanopure quality.

2.2. Anthocyanin extract of bilberry

Anthocyanin extracts of bilberry were industrially prepared by Changsha Kinglong Bio-Produce Co. (Changsha, China). Bilberry fruits (*V. myrtillus*) were extracted with methanol acidified with 0.5% HCl. Routinely, the ratio of the extraction solvent to the dried fruit was 10:1. In general, HSCCC separations of minor concentrated anthocyanins require an adsorbing step on organic resin material, i.e. Amberlite XAD-7 [20] to eliminate polysaccharides, amino acids, proteins and co-pigments. In the case of bilberry, high anthocyanin content encouraged us to perform the separation directly from the crude extract without applying further clean-up steps.

2.3. High-speed counter-current chromatography (HSCCC)

The HSCCC instrument was a multilayer coil counter-current chromatograph, manufactured by P.C. Inc. (Potomac, MD, USA), equipped with a 380 mL coil column made of polytetrafluoroethylene tubing (2.6 mm i.d.). The mobile phase was delivered by a Biotronik HPLC pump BT 3020 (Jasco, Gross-Umstadt, Germany). The HSCCC experiment was performed with a biphasic solvent system composed of methyl *tert*-butyl ether–*n*-butanol–acetonitrile–water–trifluoroacetic acid (1:4:1:5:0.01, v/v). After thorough equilibration of 90 mL methyl *tert*-butyl ether, 350 mL *n*-butanol, 90 mL acetonitrile, 450 mL water and 0.8 mL trifluoroacetic acid in a separatory funnel, the two resulting phases were separated shortly before use. The multilayer coil column was entirely filled with the upper organic phase as the stationary phase, hence the elution mode for the HSCCC separation was '*head to tail*'. Then the apparatus was started to rotate at 650 rpm for equilibration of the system. For a single run, 500 mg of anthocyanin bilberry crude extract was dissolved in 20 mL of mobile phase. Injection to the HSCCC system was done by a teflon sample loop, followed by immediate pumping of the aqueous mobile phase at a flow rate of 1.5 mL/min. Eluates were monitored with a UV–vis detector at 440 nm (LKB UV-Cord, Uppsala, Sweden), and collected with a fraction collector Superfrac (Pharmacia, Uppsala, Sweden). Final purity control of anthocyanin fractions was done by HPLC–ESI-MS analyses in positive ionization mode.

2.4. Analytical controls and structure elucidation

2.4.1. Electrospray MS (syringe-pump) and HPLC–ESI-MS

All ESI-MS experiments were performed on a Bruker Esquire LC–MS ion trap multiple mass spectrometer (Bremen, Germany) in positive and negative ionization mode analyzing ions up to *m*/*z* 2200.

During ESI-MS studies, the pure HSCCC fractions **II** (delphinidin-3-O-sambubioside (**1**)) and **III** (cyanidin-3-O-sambubioside (**2**)) were directly introduced via a syringe pump at a flow rate of 240 μL/min. Drying gas was nitrogen (gas flow 7.0 L/min, 330 °C), and nebulizer pressure was set to 34.5 kPa ESI-MS parameters (negative mode): capillary, +4500 V; end plate, +4000 V; cap exit, −90 V; cap exit offset, −60 V; skim 1, −30 V; skim 2, −10 V; ESI-MS parameters (positive mode): capillary, −4500 V; end plate, −4000 V; cap exit, +90 V; cap exit offset, +60 V; skim 1, +30 V; skim 2, +10 V. MS–MS experiments afforded fragmentation amplitude voltages between 0.8 and 1.2 V.

For HPLC–ESI-MS analysis of all HSCCC fractions **I**–**V**, a binary gradient pump G1312A, series 1100 from Hewlett-Packard (Waldbronn, Germany) was coupled to the Bruker Esquire LC–ESI-MS system. Drying gas was 11.0 L/min, and nebulizer pressure was set to 413.7 kPa ESI-MS parameters (positive mode): capillary, −2500 V; end plate, −3000 V; capillary exit, +95 V; skim 1, +25 V; skim 2, +10 V; fragmentation amplitude, 0.8 V.

HPLC separation was performed on a RP-18 Luna 5 μm, 150 × 4.6 mm column (Phenomenex, Torrance, CA, USA). Gradient elution was carried out with the solvent system A (water–formic acid–acetonitrile, 87:10:3, v/v), and system B (water–formic acid–acetonitrile, 40:10:50, v/v at a flow rate of 0.8 mL/min. The gradient steps were set from 6% B to 20% B in 20 min, to 40% B in 15 min, to 60% B in 5 min, to 70% B in 5 min, at 70% B for 6 min, and back to initial conditions [21].

2.4.2. Nuclear magnetic resonance (NMR) analysis

^{1}H, ^{13}C, distortionless enhancement by polarization transfer (DEPT) 135 NMR, and heteronuclear multiple

bond correlation (HMQC) and heteronuclear multiple quantum correlation (HMBC) experiments were recorded in [$^2H_4$] methanol (MeOH-d_4)–[$^2H_1$] trifluoroacetic acid (TFA-d_1) (19:1) on a Bruker AMX 300 (Karlsruhe, Germany) with 300 MHz for ^{1}H-, and 75.5 MHz for ^{13}C-nuclei, respectively.

3. Results and discussion

3.1. HSCCC separation

Fig. 1 presents the chromatogram of the HSCCC separation of 500 mg bilberry crude extract with the two-phase solvent system composed of methyl *tert*-butyl ether–*n*-butanol–acetonitrile–water–trifluoroacetic acid (1:4:1:5:0.01, v/v). The HSCCC run yielded five baseline separated peaks which were combined to fractions **I**–**V**, respectively. Evaporation of the organic solvents under reduced pressure, and subsequent lyophilization yielded 230 mg of fraction **I**, 130 mg of component **II** (delphinidin-3-O-sambubioside (**1**)), 77 mg of component **III** (cyanidin-3-O-sambubioside (**2**)), and 19 mg each for fractions **IV** and **V**.

Preparative isolation of monomeric anthocyanin-glycosides by HSCCC requires solvent systems of high polarity. By routine separation, the aqueous solvent layer is used as mobile phase, and for stabilization of anthocyanin pigments, addition of 0.1% trifluoroacetic acid is necessary [18,19]. Therefore, elution is in '*head to tail*' mode, and results in a stable detector signal. Principal advantage using HSCCC for anthocyanin separation is that other much more polar matrix constituents, i.e. oligomeric and polymeric proanthocyanidins, also polysaccharides elute immediately from the HSCCC coil system due to a lower stationary phase affinity. LC–ESI-MS analysis of fraction **I** detected polymeric proanthocyanidins visible as a broad peak (25–45 min)—a so-called 'tannin-hump'. Since anthocyanin pigments are often covalently incorporated to polymeric material, this might explain the small UV absorbance of peak **I** at $\lambda = 440$ nm. By means of LC–ESI-MS, HSCCC

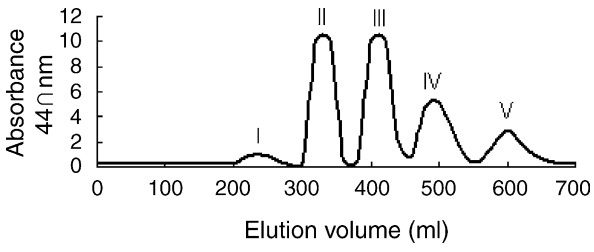

Fig. 1. HSCCC chromatogram of 500 mg crude extract from bilberry fruit. Two-phase solvent system: methyl *tert*-butyl ether–*n*-butanol–acetonitrile–water–trifluoroacetic acid (1:4:1:5:0.01, v/v); stationary phase: upper organic phase; elution mode in the coil system: head to tail; flow-rate: 1.5 mL/min; detection wavelength: $\lambda = 440$ nm, retention of stationary phase: 53%; fraction **II** = substance **1**, fraction **III** = substance **2**.

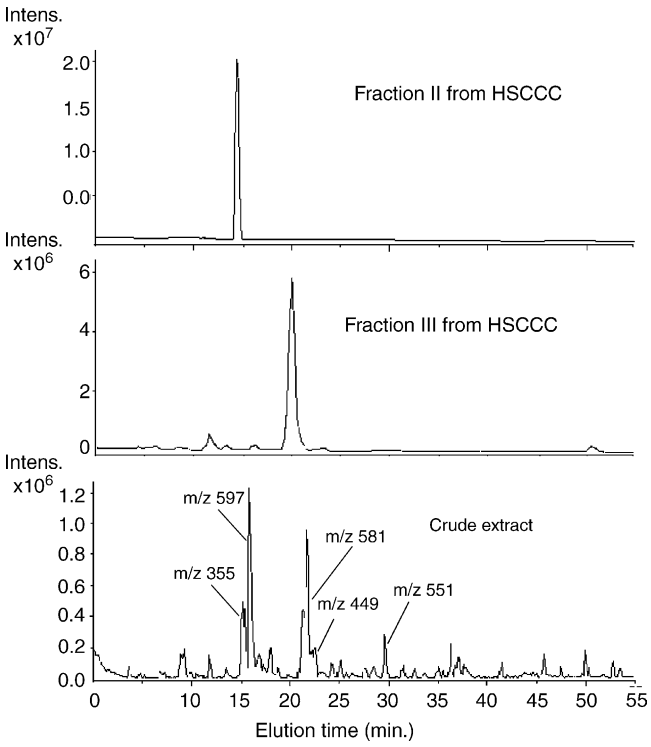

Fig. 2. HPLC–ESI-MS chromatograms (positive ionization) of fractions (**II** and **III**) obtained from the HSCCC separation, and bilberry crude extract. Fraction **II** = component **1**, fraction **III** = component **2**. HPLC–ESI-MS conditions c.f. text.

fractions **II** (delphinidin-3-O-sambubioside (**1**)) and **III** (cyanindin-3-O-sambubioside (**2**)) were detected to be pure components (Fig. 2). Substitution pattern of structure **1** and **2** differ in single position, a trihydroxy-substitution pattern in the flavonoid B-ring system. Here the mayor advantage of HSCCC technique is obvious, slight polarity differences are sufficient to achieve complete baseline separation on preparative scale. Fractions **IV** and **V** were also subjected to LC–ESI-MS (positive mode), and showed a complex mixture of components. For structure elucidation, substances **1** and **2** were directly analyzed by ESI-MS, ^{1}H, ^{13}C, DEPT NMR, HMQC and HMBC.

3.2. Confirmation of chemical structures

The ^{13}C NMR resonances for the two anthocyanin-diglycosides **1** and **2** were in very good accordance to published reference data [20,22,23]. Delphinidin- (**1**), and cyanidin-3-O-sambubioside (**2**) were already described as pigments from blueberry fruits (*Vaccinium padifolium*) [20], but they are novel constituents for bilberry fruits. All NMR spectral data are given in Table 1.

Structural confirmation of **1** was done by heteronuclear correlation experiments (HMQC, HMBC), and corroborated delphinidin as the anthocyanin moiety in the structure.

Relevant long-range HC-correlations are summarized in Fig. 3. The cross signal from the H-1″ of the glucose δ

Table 1
^{1}H, ^{13}C, DEPT 135 NMR spectral data of the anthocyanin-diglycosides, delphinidin-3-O-sambubioside (**1**) and cyanidin-3-O-sambubioside (**2**) (δ values given in ppm, measured in MeOH-d_4–TFA-d_1 (19:1, v/v))

	Delphinidin-3-O-sambubioside (**1**)			Cyanidin-3-O-sambubioside (**2**)		
	^{13}C NMR δ (ppm)	DEPT	^{1}H NMR δ (ppm) J (Hz)	^{13}C NMR	DEPT	^{1}H NMR δ (ppm) J (Hz)
Carbon						
2	164.36	C		164.43	C	
3	145.51	C		145.34	C	
4	135.69	CH	9.00 s	136.41	CH	9.05 s
5	158.70	C		159.28	C	
6	103.3	CH	6.75 d 2	103.40	CH	6.75 d 2
7	170.11	C		170.39	C	
8	94.96	CH	6.97 d 2	95.10	CH	6.99 d 1.5
9	157.49	C		157.66	C	
10	113.08	C		113.20	C	
1′	120.04	C		121.71	C	
2′	112.8	CH	7.87 s	118.20	CH	8.14 d 2.5
3′	147.57	C		147.49	C	
4′	144.73	C		155.80	C	
5′	147.57	C		117.40	CH	7.11 d 9
6′	112.8	CH	7.87 s	128.60	CH	8.37 dd 2, 9
3-O-β-D-glucoside						
1″	101.83	CH	5.55 d 8	101.71	CH	5.54 d 7.5
2″	82.58	CH	4.07 dd 8, 9	81.86	CH	4.05 dd 8, 9
3″	77.95*	CH	3.86 t 9	78.20	CH	3.86 t 9
4″	70.74	CH	3.61 dd 9.5, 9.5	70.99	CH	3.58 dd 9.5, 9.5
5″	77.73*	CH	3.65 m	77.87	CH	3.67 ddd 9.5, 6, 2
6″	62.26	CH_2	4.00 dd 2, 12 3.82 dd 6, 12	62.33	CH_2	4.00 dd 2, 12 3.81 dd 6, 12
2″-O-xylo						
1‴	106.08	CH	4.77 d 7.5	105.78	CH	4.84 d 7.5
2‴	75.77	CH	3.25 dd 8, 9	75.72	CH	3.26 dd 8, 9
3‴	78.71	CH	3.37 t 9	78.78	CH	3.38 m
4‴	70.85	CH	3.43 ddd 9, 10.5, 5	70.84	CH	3.48 ddd 9, 10.5, 5.5
5‴	67.03	CH_2	3.68 ddd 9.5, 6, 2.5 3.04 dd 10.5, 11.5	67.19	CH_2	3.76 dd 5.5, 11.5 3.14 dd 10.5, 11.5

* The assignments might be alternated.

= 5.44 to C-3 δ = 145.5 ppm clearly elucidated the proposed glycosidation to the delphinidin backbone. The disaccharide linkage glucose (2 → 1) xylose was confirmed by the correlation peak H-1‴ of the xylose δ = 4.67 ppm to glucose carbon C-2″ at δ = 81.9 ppm, also having a significant glycosidation shift to the downfield. The ^{13}C NMR data for the disaccharide moieties of **1** and **2** were almost identical (Table 1), confirming also the sambubioside structure for **2**. Typical coupling constants elucidated the glycosidic linkages: J = 8 Hz for the anomer H-1″ at δ = 5.55 ppm determined a β-glucosidic linkage to the aglycone moiety, and a similar value of J = 7.5 Hz observed for the anomer H-1‴, a β-linked xylopyranoside unit.

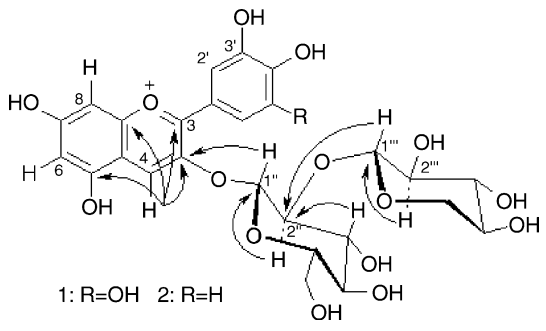

Fig. 3. The chemical structures of delphindin-3-O-sambubioside (**1**) and cyanidin-3-O-sambubioside (**2**), and relevant long-range HC correlations in the HMBC of delphinidin-3-O-sambubioside (**1**).

The LC–ESI-MS spectroscopic data of the two anthocyanin-diglycosides are given as below:

Delphinidin-3-O-sambubioside (**1**), fraction **II**: ESI-MS (positive) m/z: 597 $[M + H]^+$, MS–MS of m/z 597:303 $[M$ − glucose–xylose$]^+$; ESI-MS (negative): m/z 595 $[M - H]^-$, MS–MS of m/z 595:300 $[M$ − glucose–xylose–H$]^-$, 327, 355. R_t: 14.4 min. ^{1}H and ^{13}C NMR data are listed in Table 1.

Cyanidin-3-O-sambubioside (**2**), fraction **III**: ESI-MS (positive) m/z: 581 $[M + H]^+$; MS-MS of m/z 581:287 $[M$ − glucose–xylose$]^+$; ESI-MS (negative), m/z 579 $[M - H]^-$. R_t: 19.8 min. ^{1}H and ^{13}C NMR data are listed in Table 1.

For sensitive detection of anthocyanins, LC–ESI-MS is operated in positive detection mode. For stabilization of

the anthocyanin pigments, LC solvents require addition of strong organic acids, such as formic acid, hence the permanent charge of the flavylium cation enables a favourable ion-formation. Interestingly, anthocyanin-glycosides in acidic solution are detectable in negative mode ESI-mode as well, abundant ions of the nature $[M - H]^-$ confirm the molecular mass of the isolated structures (cf. MS-data below), but further MS–MS fragmentation experiments resulted in no significant structural information.

The full-scan LC–ESI-MS analysis of the bilberry crude extract in positive ionization mode (Fig. 2) displayed for anthocyanin related pigments abundant quasimolecular ion peaks of the nature $[M + H]^+$. Further MS^2-experiments showed intense fragment ions for the anthocyanin aglycones. The observed molecular mass differences (Δu) provides information about the sugar type, i.e. glucose or galactose ($\Delta m/z = 162$), rhamnose ($\Delta m/z = 146$), xylose ($\Delta m/z = 132$), or if disaccharide units are present in the structure.

In our ESI-MS–MS experiments the two anthocyanin-3-O-sambubiosides **1** and **2** cleaved the complete disaccharide unit (glucose–xylose, $\Delta m/z = 294$) in one step. For some anthocyanins single-step cleavage of sugars was observed.

Investigation of substance **1** by LC–ESI-MS–MS (R_t = 14.4 min), and ESI-MS–MS (syringe mode) showed the molecular ion peak at $m/z = 597$ $[M + H]^+$, the diagnostic MS–MS-fragment ion of $m/z = 303$ $[M - glc–xyl]^+$ for the aglycone delphinidin, and indicated the neutral loss of a glucose–xylose unit ($\Delta m/z = 294$ u). ESI-MS in negative mode resulted in ion $\Delta m/z = 595$ $[M - H]^-$ corroborating the molecular mass of **1**.

LC–ESI-MS of substance **2** (R_t = 19.8 min) showed a molecular mass of $m/z = 581$ $[M + H]^+$, and MS–MS experiment resulted in the diagnostic fragment ion $m/z = 287$ for a cyanidin aglycone. The neutral loss $\Delta m/z = 294$ confirmed again the cleavage of the disaccharide unit sambubioside.

4. Conclusion

The present study demonstrates that a single chromatographic separation by HSCCC is able to yield pure anthocyanin-3-O-disaccharides on preparative scale from a complex matrix of natural products (Fig. 1). In this case, for recovery of pigments, time consuming clean-up procedures before HSCCC separation, i.e. done by size-exclusion chromatography on Sephadex LH-20, or adsorbance to Amberlite XAD-7 resin material was not necessary. With our preparative HPLC system, total 18 L of 30% methanol were used for the separation of 500 mg sample, and each separation only 10 mg sample could be loaded. However, only 500 mL of the lower mobile phase was consumed to separate the two compounds. Therefore, valuable attributes of separating anthocyanins by HSCCC compared to preparative HPLC are high sample loading capacity, no irreversible adsorption effects of analytes to solid-phase column material, complete sample recovery and the much lower mobile phase usage.

HSCCC is an effective methodology for selective preparation of pure anthocyanins—done on a laboratory scale, and result of our study is pure delphinidin-, and cyanidin-3-O-sambubiosides which is now available for further biological studies.

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