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# Preparative Isolation and Purification of Flavonoids and Protocatechuic Acid from Sea Buckthorn Juice Concentrate (*Hippophaë rhamnoides* L. ssp. *rhamnoides*) by High-Speed Counter-Current Chromatography

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## Abstract

High-speed counter-current chromatography (HSCCC) - a support free all liquid-liquid chromatography technique - has been successfully used for the preparative isolation of isorhamnetin 3-O-*ß*-D-glucoside, isorhamnetin 3-O-*ß*-rutinoside, quercetin 3-O-*ß*-D-glucoside, syringetin 3-O-*ß*-D-glucoside and protocatechuic acid from sea buckthorn juice concentrate (*Hippophaë rhamnoides* L. ssp. *rhamnoides, Elaeagnaceae*). The preparative HSCCC instrument was a multilayer coil planet centrifuge equipped with three preparative coils. Separation was performed with a two phase solvent system (*n*-hexane - *n*-butanol - water, 1:1:2 *v/v/v*) in '*head-to-tail*' mode. Each injection of 4.1 g crude ethyl acetate extract yielded isorhamnetin 3-O-*ß*-D-glucoside (95 mg), isorhamnetin 3-O-*ß*-rutinoside (10 mg), quercetin 3-O-*ß*-D-glucoside (5 mg), and protocatechuic acid (34 mg) with purities > 98 %. The flavonoid syringetin 3-O-*ß*-D-glucoside (2 mg) was a novel compound for *Hippophaë rhamnoides*. Chemical structures of all compounds were determined by HPLC-ESI-MS-MS, 1D-NMR ($^1$H,

$^{13}C$, DEPT 135) spectroscopy and for elucidation of glycosidic linkages 2D-NMR (HMBC) spectroscopy was used.

Keywords:

Column liquid chromatography

High-speed counter-current chromatography

LC-ESI-MS

Nuclear magnetic resonance

Sea buckthorn

*Hippophaë rhamnoides* L. ssp. *rhamnoides*

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## Introduction

The berries of *Hippophaë rhamnoides (Elaeagnaceae)* are rich in flavonol-glycosides and are traditionally used for ethnomedicinal remedies in Tibet, Mongolia, China and Central Asia [1]. In 1977 sea buckthorn was officially listed for the first time in the Chinese Pharmacopoeia by the Ministry of Public Health [2]. In medical studies, total flavones prevented *in vivo* thrombogenesis [3]. Flavonols have antioxidant properties and thus they are able to reduce free radical formation [4-7]. This suggests the flavonoids of *Hippophaë rhamnoides* may provide beneficial effects in prevention of coronary heart disease and arteriosclerosis [8-9]. Furthermore sea buckthorn flavones promote wound healing activity and anti-tumor activity has been reported for isorhamnetin [10-11].

Flavonols constitute the main group of flavonoids present in sea buckthorn plant material [12-15]. Many studies have concentrated on the identification of flavonol aglycons and some of their glycosides by means of HPLC-ESI-MS and DAD-UV, HPLC with chemiluminescence detection, capillary zone electrophoresis and NMR spectroscopy [16-24]. The preparative isolation and purification of the flavonoids were performed by conventional separation methods such as size-exclusion chromatography on the lipophilic organic resin Sephadex LH 20 ® [25].

High-speed counter-current chromatography (HSCCC) is a support-free all liquid-liquid chromatography technique which eliminates irreversible adsorption effects of the sample on the solid support. Injection of high sample amounts and total recovery are the principal advantages compared to conventional preparative methods such as normal phase and reversed phase column chromatography [26]. This method has been successfully employed in the analytical and semi-preparative separation of the flavonol aglycones isorhamnetin, quercetin and kaempferol of crude sea buckthorn extracts. However flavonol glycosides of *Hippophaë rhamnoides* have not been separated preparatively by means of high-speed counter-current chromatography (HSCCC) to date [27-31].

The aim of this study was to develop an efficient preparative HSCCC method using a two phase solvent system with retention capabilities optimized for the isolation and purification of a complex mixture of flavonol glycosides from industrially processed sea buckthorn juice concentrate (*Hippophaë rhamnoides* L. ssp. *rhamnoides*).

## Experimental

### High-Speed Counter-Current Chromatography (HSCCC)

The preparative HSCCC instrument used in the present study was a multilayer coil planet centrifuge model CCC 1000 (Pharma-Tech Research Corp., U.S.A.), equipped with three preparative coils connected in series (polytetrafluorethylene tubing: 2.6 mm i.d. x 165 m, 850 mL total volume). A manual sample injection valve with a 25 mL loop was used to introduce the sample into the coil system. The mobile phase was delivered with a Biotronik BT 3020 HPLC pump (Jasco, Grossumstadt, Germany). The effluent stream was monitored by UV-detection at λ 280 nm with a Knauer K-2501 UV detector (Berlin, Germany) equipped with a preparative cell (0.5 mm layer thickness).

### Reagents

Organic solvents, *n*-hexane, *n*-butanol used for HSCCC separations were of analytical grade (Merck, Darmstadt, Germany). For LC-ESI-MS analysis, HPLC grade acetonitrile (Merck, Darmstadt, Germany) and Nanopure ® (Barnstead, U.S.A.) water were used.

### Processing of Sea Buckthorn Juice Concentrate

The applied sea buckthorn fruits were harvested in Romania in September 2005. The frozen berries were preheated to 8 – 12 °C before mashing. The mash was subjected to a treatment with pectolytic enzymes 1 – 2 h on 52 °C and separated into juice and pomace

by a decanter machine. The turbid juice product, high concentrated in pulp and oil was clarified by a plate separator. Further, sea buckthorn juice was clarified with fining agents, i.e., bentonite (8–12 h, 10–12 °C). After filtration with diatomeous earth under vacuum, the clear juice was concentrated by thermovacuum evaporation (five stage evaporator, 80–85 °C). The °Brix value was 65 for clear juice concentrates. Before aseptic filling the juice was treated in a HTST process (high-temperature-short-time treatment: 90°C, 45 s) and rechilled, immediately.

Preparation of Hippophaë Juice Concentrate Fractions for HSCCC and Sephadex LH 20®

For removing lipophilic natural products such as sterols and other amphiphilic lipids with emulsifying properties, the concentrated juice of *Hippophaë rhamnoides* L. ssp. *rhamnoides* (1015 g, Bayernwald Früchteverwertung GmbH, Germany) was exhaustively defatted with *n*-hexane (total volume: 2000 mL), and was treated similarly with dichloromethane. The polar residue was extracted six times with 500 mL ethyl acetate. After filtration, the ethyl acetate extract was evaporated to dryness by rotary evaporation at 40 °C and 40 mbar to yield 31.6 g of crude extract enriched in flavonoids for subsequent preparative HSCCC.

Preparation of Two-Phase Solvent System and Sample Solution for HSCCC

The HSCCC experiments were performed with a two-phase solvent system composed of *n*-hexane - *n*-butanol - water (1:1:2, *v/v/v*). After thoroughly equilibrating the solvent mixtures in a separatory funnel at room temperature, two phases were separated shortly before use and degassed by ultrasonication. The upper organic phase was used as stationary phase and the lower aqueous phase as mobile phase.

Separation Procedure of HSCCC

The multilayer coiled column was initially completely filled with the upper organic phase. After rotation at 800 rpm, the sample solution (4.1 g) was introduced in 14 mL of a mixture of upper and lower phase (1:1, *v/v*) into the separation column through an injection loop. The lower phase was pumped into the head end of the HSCCC coil column at a flow rate of 3 mL $min^{-1}$. The effluent from the tail outlet of the column was monitored by absorbance at λ 280 nm and collected into test tubes with a fraction collector at 4-min intervals. After separation, the solvent in the coil column was ejected with nitrogen gas and 90 % of the stationary phase was retained.

Evaluation of purity of the HSCCC fractions

Evaluation of purity of the HSCCC fractions was performed by thin-layer-chromatography on normal-phase silica gel plates 60 $F_{254}$ Merck (Darmstadt, Germany) with the solvent system (S1) dichloromethane-methanol-water (75:25:1, *v/v/v*), and on reversed phase plates RP-18W (Macherey-Nagel, Düren, Germany) with system (S2) methanol-water (45:55, *v/v*). Visualization was performed with anisaldehyde -

concentrated sulfuric acid - acetic acid (1:2:97), and flash heating (110 °C) on a hot plate [32].

Further sample purification of fraction 1 and 2 was performed with RP-18 cartridges (Waters, Milford, USA). RP-18 phase material was rinsed with 10 mL methanol and then conditioned with methanol/water (30/70, *v/v*). In the following step, HSCCC fraction (1 mL) was injected onto the cartridge. The elution of single compounds occured with methanol/water 10 mL (30/70, *v/v*). Final purity of fractions 1 - 5 was verified by thin-layer-chromatography on reversed phase plates RP-18W (Macherey-Nagel, Düren, Germany) with system (S2) methanol-water (45:55, *v/v*), by NMR spectroscopy, furthermore flavonoid fractions 1 – 4 by HPLC-electrospray ionization (ESI) MS analyses.

Separation Procedure of Sephadex LH 20 ®

The crude ethyl acetate extract (4 g) was dissolved in methanol and was separated by size-exclusion chromatography on the lipophilic organic resin Sephadex LH 20 ® column, 77 cm x 5 cm i.d. (Amersham Pharmacia Biotech, Piscataway, U.S.A) with methanol as mobile phase at a flow rate of 0.5 mL $min^{-1}$ at ambient temperature. The effluent of the column was collected into test tubes with a Pharmacia Superfrac fraction collector (Uppsala, Sweden) at 60-min intervals.

Analytical Control and Structure Elucidation

Electrospray-MS (Syringe-Pump), and HPLC-ESI-MS

All ESI-MS-MS experiments were performed on a Bruker Esquire LC-MS ion trap multiple mass spectrometer (Bremen, Germany) in negative ionization mode analysing ions up to *m/z* 2200. During ESI-MS, and MS-MS fragmentation studies, the purified samples were introduced via a syringe pump at a flow-rate of 240 µL $h^{-1}$. Drying gas was nitrogen (flow 5.0 L $min^{-1}$, 330 °C), and nebulizer pressure was set to 5 psi. ESI-MS parameters (neg. mode): capillary +3500 V, end plate +3000 V, capillary exit -90 V, capillary exit offset -60 V, skim 1 -30 V, skim 2 -10 V; MS-MS experiments afforded fragmentation amplitude values between 0.8 and 1.2. For HPLC-ESI-MS-MS analysis, 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 nitrogen (flow 9.0 L $min^{-1}$, 310 °C), and nebulizer pressure was set to 40 psi. ESI-MS parameters (neg. mode): capillary +3500 V, end plate +3000 V, capillary exit – 95 V, skim 1 -25 V, skim 2 -10 V.

HPLC-ESI-MS was performed on a Prontosil $C_{18}$ Aqua column, 5 µm, 250 x 2.0 mm (Bischoff, Leonberg, Germany), flow-rate was 0.25 mL $min^{-1}$, and eluents were water (solvent A) and acetonitrile (solvent B). Initial gradient conditions were 97 % A and 3 % B, hold over 10 min, starting a linear gradient in 30 min to 40 % A and 60 % B, in 15 min to 0 % A and 100 % B, hold for 10 min and back to initial conditions.

Nuclear Magnetic Resonance (NMR) Analysis

$^{1}H$, $^{13}C$, and DEPT 135-NMR spectra were recorded in MeOH-$d_4$ at 25 °C on a Bruker AMX 300 spectrometer (Karlsruhe, Germany; $^{1}H$ 300 MHz; $^{13}C$ 75.5 MHz). 2D-NMR experiments (HMBC: heteronuclear multiple bond correlation) were performed on a Bruker DMX 600 spectrometer. Chemical shifts ($\delta$) are reported in ppm relative to the residual solvent signals ($\delta_H$ 3.31 and $\delta_C$ 49.0 ppm) and coupling constants (J) in Hz.

Isorhamnetin 3-O-*ß*-D-rutinoside (narcissin) **(1)**, 10 mg: ESI-MS (negative) *m/z*: 623 $[M-H]^-$, MS/MS *m/z*: 315 $[M\text{-glc-rha-H}]^-$; $^{1}H$-NMR (300 MHz, $CD_3OD$) $\delta$ [ppm]: 1.11 (3H, *d*, *J* = 6 Hz, Me-6```) 3.20 – 3.90 (~14H, *m*, sugar protons), 3.94 (3H, s, $OCH_3$ at C-3`), 4.53 (1H, *d*, *J* = 1.5 Hz, H-1```), 5.22 (1H, *d*, *J* = 8 Hz, H-1``), 6.20 (1H, *d*, *J* = 2 Hz, H-6), 6.41 (1H, *d*, *J* = 2 Hz, H-8), 6.90 (1H, *d*, *J* = 8 Hz, H-5`), 7.63 (1H, *dd*, $J_1$ = 8 Hz, $J_2$ = 2 Hz, H-6`), 7.94 (1H, d, *J* = 2 Hz, H-2`); $^{13}C$-NMR (75.5 MHz, $CD_3OD$), $\delta$ [ppm]: 17.86 (C-6```), 56.85 ($OCH_3$ at C-3`), 68.57 (C-6``), 69.78 (C-5```), 71.67 (C-4``), 72.10 (C-2```), 72.34 (C-3```), 73.89 (C-4```), 75.94 (C-2``), 77.41 (C-5``), 78.24 (C-3``), 94.93 (C-8), 99.98 (C-6), 102.52 (C-1```), 104.46 (C-1``), 105.76 (C-10), 114.68 (C-2`), 116.14 (C-5`), 123.06 (C-1`), 124.06 (C-6`), 135.52 (C-3), 148.37 (C-4`), 150.88 (C-3`), 158.52[a] (C-9), 158.91[a] (C-2), 163.02 (C-5), 166.00 (C-7), 179.38 (C-4).

[a] assignments with same superscript may be interchanged.

Retention time (HPLC-ESI-MS): 29.9 min.

Elution time (HSCCC): 116 – 138 min.

Syringetin 3-O-*ß*-D-glucoside **(2)**, 2 mg: ESI-MS (negative) *m/z*: 507 $[M-H]^-$, MS/MS *m/z*: 345 $[M\text{-glc-H}]^-$; $^{1}H$-NMR (300 MHz, $CD_3OD$) $\delta$ [ppm]: 3.22 (1H, *m*, H-5``), 3.35 (1H, *m*, H-4´´, under $CD_3OD$ solvent signal), 3.44-3.49 (2H, *m*, H-3``, H-2``), 3.58 (1H,

*dd*, $J_1$ = 12 Hz, $J_2$ = 5 Hz, $H_A$-6``), 3.71 (1H, *dd*, $J_1$ = 12 Hz, $J_2$ = 2.5 Hz, $H_B$-6``), 3.94 (6H, *s*, $OCH_3$ at C-3`and at C-5`) 5.44 (1H, *d*, *J* = 8 Hz, H-1``), 6.22 (1H, *d*, *J* = 2 Hz, H-6), 6.43 (1H, *d*, *J* = 2 Hz, H-8), 7.55 (2H, *s*, H-6`, H-2`); $^{13}$C-NMR (75.5 MHz, $CD_3OD$), $\delta$ [ppm]: 57.22 ($OCH_3$ at C-3` and at C-5`), 62.62 (C-6``), 71.30 (C-4``), 76.01 (C-2``), 78.15[a] (C-3``), 78.60[a] (C-5``), 94.79 (C-8), 99.93 (C-6), 103.55 (C-1``), 106.11 (C-10), 108.42 (C-2`, C-6`), 122.04 (C-1`), 135.68 (C-3), 140.21 (C-4`), 148.91 (C-3`, C-5`), 158.50[b] (C-9), 158.56[b] (C-2), 163.15 (C-5), 166.00 (C-7), 179.45 (C-4).

[a, b] assignments with same superscript may be interchanged.

Retention time (HPLC-ESI-MS): 30.9 min.

Elution time (HSCCC): 172 – 206 min.

Quercetin 3-O-*ß*-D-glucoside (isoquercitrin) **(3)**, 5 mg: ESI-MS (negative) *m/z*: 463 [M-H]$^-$, MS/MS *m/z*: 301 [M-glc-H]$^-$; $^1$H-NMR (300 MHz, $CD_3OD$) $\delta$ [ppm]: 3.22 (1H, *ddd*, $J_1$ = 10 Hz, $J_2$ = 5 Hz, $J_3$ = 2 Hz, H-5``), 3.35 (1H, *m*, H-4´´, under $CD_3OD$ solvent signal), 3.42 (1H, *t*, *J* = 8,5 Hz, H-3``), 3.48 (1H, *t*, *J* = 8 Hz, H-2``), 3.58 (1H, *dd*, $J_1$ = 12 Hz, $J_2$ = 5 Hz, $H_A$-6``), 3.71 (1H, *dd*, $J_1$ = 12 Hz, $J_2$ = 2.5 Hz, $H_B$-6``), 5.21 (1H, *d*, J = 8 Hz, H-1``), 6.20 (1H, *d*, J = 2 Hz, H-6), 6.38 (1H, *d*, *J* = 2 Hz, H-8), 6.87 (1H, *d*, *J* = 8 Hz, H-5`), 7.59 (1H, *dd*, $J_1$ = 8 Hz, $J_2$ = 2 Hz, H-6`), 7.71 (1H, *d*, *J* = 2 Hz, H-2`); $^{13}$C-NMR (75.5 MHz, $CD_3OD$), $\delta$ [ppm]: 62.64 (C-6``), 71.30 (C-4``), 75.76 (C-2``), 78.18[a] (C-3``), 78.39[a] (C-5``), 94.90 (C-8), 100.16 (C-6), 104.53 (C-1``), 105.51 (C-10), 116.04 (C-2`), 117.59 (C-5`), 123.14[b] (C-1`), 123.20[b] (C-6`), 135.68 (C-3), 145.95 (C-3`), 149.91 (C-4`), 158.58[c] (C-9), 159.03[c] (C-2), 163.04 (C-5), 166.74 (C-7), 179.46 (C-4).

[a, b,c] assignments with same superscript may be interchanged.

Retention time (HPLC-ESI-MS): 29.6 min.

Elution time (HSCCC): 218 – 258 min.

Isorhamnetin 3-O-*ß*-D-glucoside **(4)**, 95 mg: ESI-MS (negative) *m/z*: 477 $[M-H]^-$, MS/MS *m/z*: 315 $[M\text{-}glc\text{-}H]^-$; $^1$H-NMR (300 MHz, $CD_3OD$) $\delta$ [ppm]: 3.22 (1H, *ddd*, $J_1$ = 10 Hz, $J_2$ = 5 Hz, $J_3$ = 2 Hz, H-5``), 3.35 (1H, *m*, H-4´´, under $CD_3OD$ solvent signal), 3.45-3.48 (2H, *m*, H-3``, H-2``), 3.55 (1H, *dd*, $J_1$ = 12 Hz, $J_2$ = 5 Hz, $H_A$-6``), 3.72 (1H, *dd*, $J_1$ = 12 Hz, $J_2$ = 2.5 Hz, $H_B$-6``), 3.94 (3H, *s*, $OCH_3$ at C-3`), 5.38 (1H, *d*, *J* = 8 Hz, H-1``), 6.20 (1H, *d*, *J* = 2 Hz, H-6), 6.38 (1H, *d*, *J* = 2 Hz, H-8), 6.89 (1H, *d*, *J* = 8 Hz, H-5`), 7.59 (1H, *dd*, $J_1$ = 8 Hz, $J_2$ = 2 Hz, H-6`), 7.91 (1H, *d*, *J* = 2 Hz, H-2`); $^{13}$C-NMR (75.5 MHz, $CD_3OD$), $\delta$ [ppm]: 56.85 ($OCH_3$ at C-3`), 62.64 (C-6``), 71.57 (C-4``), 75.94 (C-2``), 78.14[a] (C-3``), 78.52[a] (C-5``), 94.94 (C-8), 100.16 (C-6), 103.91 (C-1``), 105.62 (C-10), 114.50 (C-2`), 116.04 (C-5`), 123.15[b] (C-1`), 123.90[b] (C-6`), 135.41 (C-3), 148.45 (C-4`), 150.92 (C-3`), 158.55[c] (C-9), 158.64[c] (C-2), 163.05 (C-5), 166.70 (C-7), 179.37 (C-4).

[a, b,c] assignments with same superscript may be interchanged.

Retention time (HPLC-ESI-MS): 30.8 min.

Elution time (HSCCC): 258 – 440 min.

Protocatechuic acid (3,4-dihydroxybenzoic acid) **(5)**, 34 mg: ESI-MS (negative) *m/z*: 153 $[M-H]^-$, MS/MS *m/z*: 109 $[M\text{-}H\text{-}CO_2]^-$; $^1$H-NMR (300 MHz, $CD_3OD$) $\delta$ [ppm]: 6.78 (1H, *d*, *J* = 8.0 Hz, H-5), 7.41 (1H, *dd*, $J_1$ = 8 Hz, $J_2$ = 1,5 Hz, H-6), 7.44 (1H, *d*, *J* = 1.5 Hz, H-2); $^{13}$C-NMR (75.5 MHz, $CD_3OD$), $\delta$ [ppm]: 115.75 (C-5), 117.78 (C-2), 123.72 (C-1), 123.84 (C-6), 146.00 (C-3), 151.34 (C-4), 170.59 (C-7).

Retention time (HPLC-ESI-MS): 17.00 min.

Elution time (HSCCC): 460 – 600 min.

## Results and Discussion

### HSCCC Separation

The aim of the present study was to assess the applicability of high-speed counter-current chromatography (HSCCC) for the isolation of polar constituents from the ethyl acetate extract of sea buckthorn juice concentrate. In an initial step, the crude material was investigated by silica gel thin-layer chromatography (cf. Experimental) where characteristic colorization using anisaldehyde spray reagent [32] indicated the presence of flavonoid glycosides. While a subsequent HPLC-ESI-MS-MS (neg. mode) analysis of the ethyl acetate extract detected nine flavonoids, isorhamnetin 3-O-*ß*-rutinoside (**1**), syringetin 3-O-*ß*-D-glucoside (**2**), quercetin 3-O-*ß*-D-glucoside (**3**), and isorhamnetin 3-O-*ß*-D-glucoside (**4**), quercetin rhamnoside **(6)**, isorhamnetin acetyl-glucoside **(7)**, isorhamnetin rhamnoside **(8)**, quercetin (**9**), isorhamnetin (**10**) (cf. Fig. 1).

For the preparative isolation by HSCCC different biphasic solvent systems of high polarity were evaluated. The best results for equal partitioning of *Hippophaë* constituents between the two non-miscible phases were obtained with a system composed of *n*-hexane - *n*-butanol - water (1:1:2, *v/v/v*). The separation was performed in the '*head-to-tail*' mode using the lower aqueous solvent layer as mobile phase. The

selected solvent system exhibited favorable retention capabilities of the organic stationary phase (90 %) in the HSCCC coil-system during the complete separation even though large sample amounts were injected.

In a single-step HSCCC run (cf. Fig. 2), 4.1 g of the ethyl acetate extract of *Hippophaë* juice concentrate were separated, leading to the isolation of five polyphenols whose structures were elucidated by HPLC-ESI-MS-MS, 1D-NMR ($^{1}H$, $^{13}C$, DEPT 135), and 2D-NMR (HMBC) spectroscopy (structures cf. Fig 3): 10 mg of isorhamnetin 3-O-*ß*-rutinoside (**1**), 2 mg of syringetin 3-O-*ß*-D-glucoside (**2**), 5 mg of quercetin 3-O-*ß*-D-glucoside (**3**), 95 mg of isorhamnetin 3-O-*ß*-D-glucoside (**4**), and 34 mg of protocatechuic acid (**5**).

Only the HSCCC peaks of fraction **1** and **2** showed minor impurities after investigation by TLC (cf. Experimental) and were purified prior to HPLC-ESI-MS-MS and NMR-spectroscopy using short RP-18 cartridges (cf. Experimental) to give in addition to fractions **3** and **4,** the pure flavonoid glycosides **1** and **2** (cf. Fig. 4). Repetition of the separation applying identical HSCCC conditions and injection of the same amount of sample confirmed the reproducibility of the preparative-scale chromatography.

As a result of the preparative HSCCC procedure, syringetin 3-O-*ß*-D-glucoside (**2**) - a minor concentrated flavonoid glycoside - was substantially enriched in the corresponding HSCCC-peak 2 and hence was recovered for the first time from the crude ethyl acetate extract of *Hippophaë* material.

The observed elution order for $RP_{18}$-HPLC is not necessarily identical with that for HSCCC operating in the '*head to tail*' mode using a lipophilic organic stationary phase. In this arrangement HSCCC theory predicts that more polar components are eluted initially and the increasing lipophilicity of components causes a higher affinity to the stationary phase leading to longer retention times. Interestingly, protocatechuic acid (**5**) with the highest polarity of all separated components eluted towards the end of the HSCCC run at 460 – 600 min. For large-scale countercurrent separations on a 850 ml volume HSCCC - as performed in our study with the *Hippophaë* constituents - intermolecular associations between polar components need to be considered. The shifted retention times under preparative counter-current conditions of polar substances might be the result of `hydrophobic` cluster formation and hence stronger affinity to the stationary phase causing retarded HSCCC retention times. This physico-chemical effect has already been demonstrated by Nernst [33] during the partitioning of benzoic acid between two immiscible solvents (water and benzene) that afford an equilibrium between monomeric benzoic acid molecules and dimer formation in the organic phase [33, 34]. The observed intense shift of the retention times of protocatechuic acid under preparative counter-current conditions might be the result of an increasing concentration of dimers in the stationary phase. Further investigations to evaluate the change of partitioning of natural products during large-scale HSCCC experiments are in progress.

In order to compare the efficiency of preparative HSCCC with conventional size-exclusion chromatography, 4 g of crude ethyl acetate extract were also separated using the lipophilic organic resin Sephadex LH 20®. Principal advantages of the HSCCC methodology resulted in a relatively short separation time of 10 hours compared to 18

days using the Sephadex LH 20 ® material, and the immediate isolation of pure compounds. The HSCCC technique shows superior chromatographic properties compared to conventional preparative $RP_{18}$-HPLC for separating and resolving components with slightly differing polarities in large preparative scale procedures (1 - 4 g).

Determination of Chemical Structures

Structural elucidation of substances **1**, **3**, and **4** was performed by heteronuclear NMR correlation techniques (HMBC), and corroborated the flavonoid aglycone units as well as the glycosidic linkages. The relevant $^{2,3}J$-CH correlations for isorhamnetin 3-O-*ß*-rutinoside (**1**) are summarized in Fig. 5. Correlations of the singlet proton resonance at $\delta$ 3.94 ppm (3H) identified the position of the C-3′ methoxyl group and the $^{2,3}J$-CH correlation from the anomeric H-1'' of glucose at $\delta$ 5.22 ppm to C-3 $\delta$ 135.5 ppm identified the attachment of the sugar unit at the isorhamnetin backbone. The disaccharide linkage glucose (6→1) rhamnose was confirmed by the correlation peak of H-1''' of the rhamnose $\delta$ 4.53 ppm to the glucose carbon at $\delta$ 68.6 ppm. Characteristic coupling constants afforded the configuration of the glycosidic bonds: $J$ 8 Hz for the anomer H-1'', together with characteristic $^{13}$C chemical shifts, indicated the *ß*-glucopyranosidic linkage to the aglycone moiety, and a value of $J$ 1.5 Hz for the anomer H-1'''and relevant chemical shift data identified the $\alpha$-rhamnopyranoside unit. Similar data unambiguously identified isorhamnetin 3-*O*-*ß*-glucoside (**4**). $^{1}$H and $^{13}$C NMR data of **1** and **4** are in agreement with literature data [37].

Structure relevant $^{2,3}J$-CH correlations for quercetin 3-O-*ß*-D-glucopyranoside (**3**), are presented in Fig. 5 and confirmed also a 3-O-glucosidation. $^{13}$C-NMR data were in good accordance with literature data [35].

The $^{1}$H NMR data of syringetin 3-O-*ß*-D-glucoside (**2**), in comparison to **1** and **4**, indicated a symmetrically substituted B-ring system with a singlet-signal (6H) of two methoxyl groups at δ 3.94. All NMR data of **2** [36] and also for protocatechuic acid (**5**) were in excellent accordance to published reference data [37-38].

For sensitive detection of flavonoid glycosides, HPLC-ESI-MS is operated in the negative ionization mode resulting in abundant $[M-H]^-$ ions. Further $MS^2$-experiments showed intense fragment ions for the flavonoid aglycones. The observed molecular weight differences (Δamu) provide information about the sugar type, i.e. the neutral loss of a glucose or galactose (Δ*m/z* 162), rhamnose (Δ*m/z* 146), or if disaccharide units are present in the structure. In our HPLC-ESI-MS-MS experiments the isorhamnetin 3-O-*ß*-D-rutinoside (**1**) cleaved the complete rutinoside unit (glucose-rhamnose, Δ*m/z* 308) in one step leading to the abundant fragment ion at *m/z* 315 of the isorhamnetin backbone, which is also observed in **4**. Quercetin 3-O-*ß*-D-glucoside with $[M-H]^-$ at m/z 463 showed the typical cleavage of Δ*m/z* 162 resulting in the characteristic fragment ion $[M-H]^-$ at m/z 301.

Interestingly, negative ionization of protocatechuic acid (**5**) resulted in an ion yield of relative low abundance with $[M-H]^-$ at *m/z* 153. Despite this we were able to isolate significant amounts (34 mg) of **5** by HSCCC, even though the analytical HPLC-ESI-MS analysis did not show an intense peak (cf. Fig. 1). ESI-MS-MS of *m/z* 153 yielded a

fragment ion at *m/z* 109 through cleavage of the carboxylic function. HPLC-ESI-MS data of the coil residue revealed that all constituents with higher retention times than peak 4 – isorhamnetin 3-O-*ß*-D-glucoside (**4**) - did not elute from the coil system in the present HSCCC experiment (Fig. 1, 2). All the ESI-MS-MS data of compounds **1** – **5** confirmed the structure elucidations performed by NMR spectroscopy.

ESI-MS analysis of the coil-residue indicated the presence of the typical *Hippophaë* flavonoid aglycons quercetin (**9**) $[M-H]^-$ at *m/z* 301 amu (35.5 min), and also isorhamnetin (**10**) $[M-H]^-$ at *m/z* 315 amu (38.6 min). Clearly the absence of the sugar moieties resulted in an increased affinity for the stationary RP18-HPLC-phase. We postulate that peak **6** in the HPLC-MS arises from quercetin rhamnoside $[M-H]^-$ at *m/z* 447 amu (32.1 min) and peak **8** from isorhamnetin rhamnoside $[M-H]^-$ at *m/z* 461 (34.4 min) derived from the molecular weight and the fragmentation patterns $[M-146-H]^-$ indicating the cleavage of a rhamnose sugar units. On the basis of MS-MS fragmentation compound **7** ($[M-H]^-$ at *m/z* 519) was tentatively assigned to an acylated flavonol glycoside, isorhamnetin acetyl-glucoside from the loss of a glucose and a complete acetyl-glucose unit producing ions at *m/z* 357 $[M-162-H]^-$ and at *m/z* 315 $[M-162-42-H]^-$, respectively, as the strongest intensity fragment ions.

## Conclusion

Our HSCCC study on the isolation of pure flavonoid glycosides from *Hippophaë rhamnoides* demonstrates the high effectiveness of the methodology for selective

preparations, and indicates its advantage for providing significant amounts of natural products that are then available for further biological '*in-vitro'* and '*in-vivo*' studies.

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