Journal of Chromatography A, 1216 (2009) 6890–6899

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Separation of polar betalain pigments from cacti fruits of *Hylocereus polyrhizus* by ion-pair high-speed countercurrent chromatography

Sławomir Wybraniec[a,*], Paweł Stalica[a], Gerold Jerz[b], Bettina Klose[b], Nadine Gebers[b], Peter Winterhalter[b], Aneta Spórna[a], Maciej Szaleniec[c], Yosef Mizrahi[d]

[a] *Faculty of Analytical Chemistry, Institute C-1, Department of Chemical Engineering and Technology, Cracow University of Technology, ul. Warszawska 24, 31-155 Cracow, Poland*
[b] *Institute of Food Chemistry, Technische Universität Braunschweig, Schleinitz-Strasse 20, 38106 Braunschweig, Germany*
[c] *Joint Laboratory of Biotechnology and Enzyme Catalysis, Institute of Catalysis and Surface Chemistry, Polish Academy of Sciences, ul. Niezapominajek 8, 30-239 Cracow, Poland*
[d] *Department of Life Sciences, Ben Gurion University of the Negev, POB 653, 84105 Beer-Sheva, Israel*

ARTICLE INFO

Article history:
Received 25 May 2009
Received in revised form 26 July 2009
Accepted 13 August 2009
Available online 18 August 2009

Keywords:
Betanin
Phyllocactin
Hylocerenin
Betalains
Ion-pair countercurrent chromatography
Hylocereus polyrhizus Cactaceae

ABSTRACT

Polar betacyanin pigments together with betaxanthins from ripe cactus fruits of *Hylocereus polyrhizus* (Cactaceae) were fractionated by means of preparative ion-pair high-speed countercurrent chromatography (IP-HSCCC) also using the *elution–extrusion* (EE) approach for a complete pigment recovery. HSCCC separations were operated in the classical '*head-to-tail*' mode with an aqueous mobile phase. Different CCC solvent systems were evaluated in respect of influence and effectiveness of fractionation capabilities to separate the occurring pigment profile of *H. polyrhizus*. For that reason, the additions of two different volatile ion-pair forming perfluorinated carboxylic acids (PFCA) were investigated. For a direct comparison, five samples of *Hylocereus* pigment extract were run on preparative scale (900 mg) in 1-butanol–acetonitrile–aqueous TFA 0.7% (5:1:6, v/v/v) and the modified systems *tert.*-butyl methyl ether–1-butanol–acetonitrile–aqueous PFCA (2:2:1:5, v/v/v/v) using 0.7% and 1.0% trifluoroacetic acid (TFA) or heptafluorobutyric acid (HFBA) in the aqueous phase, respectively. The chemical affinity to the organic stationary CCC solvent phases and in consequence the retention of these highly polar betalain pigments was significantly increased by the use of the more lipophilic fluorinated ion-pair reagent HFBA instead of TFA. The HFBA additions separated more effectively the typical cacti pigments phyllocactin and hylocerenin from betanin as well as their iso-forms. Unfortunately, similar K_D ratios and selectivity factors α around 1.0–1.1 in all tested solvent systems proved that the corresponding diastereomers, 15*S*-type pigments cannot be resolved from the 15*R*-epimers (iso-forms). Surprisingly, additions of the stronger ion-pair reagent (HFBA) resulted in a partial separation of hylocerenin from phyllocactin which were not resolved in the other solvent systems. The pigments were detected by means of HPLC-DAD and HPLC-electrospray ionization–MS using also authentic reference materials.

1. Introduction

The speciality of *countercurrent chromatography* (CCC) is the complete liquid nature of this chromatographic technique. Besides the mobile phase, also the stationary phase is a liquid of relatively low viscosity. As far as the selected solvent mixtures are generating two phases they are potentially suitable to be used in CCC separations. The general basis of separating desired target compounds by countercurrent chromatography are fast and continuous mixing- and demixing-operations of immiscible biphasic solvent systems in strong and rapidly alternating centrifugal force fields. Highly complex mixtures of natural or synthetic origin can be effectively fractionated.

In the last decades, high-speed countercurrent chromatography (HSCCC) has been shown in numerous applications to be a versatile preparative scale separation technique especially used in the field of natural product isolation [1]. Due to liquid–liquid nature of CCC, one can completely avoid the loss of valuable substance material onto solid phase materials such as silica gel or organic lipophilic gels (e.g. Sephadex LH-20) due to chemisorption effects.

Recently, betalains (betacyanin and betaxanthin pigments) were separated for the first time by HSCCC as shown in our study on the pigments from deeply coloured poke-berries (*Phytolacca americana*) [2]. Furthermore, the approach of using the recently introduced *elution–extrusion* protocol is opening access to the fractionation of a full polarity range of the compounds in a sample injected to the CCC system [3–5]. Especially, using ion-pair form-

* Corresponding author. Tel.: +48 12 628 2707; fax: +48 12 628 2036.
E-mail address: swybran@chemia.pk.edu.pl (S. Wybraniec).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.08.035

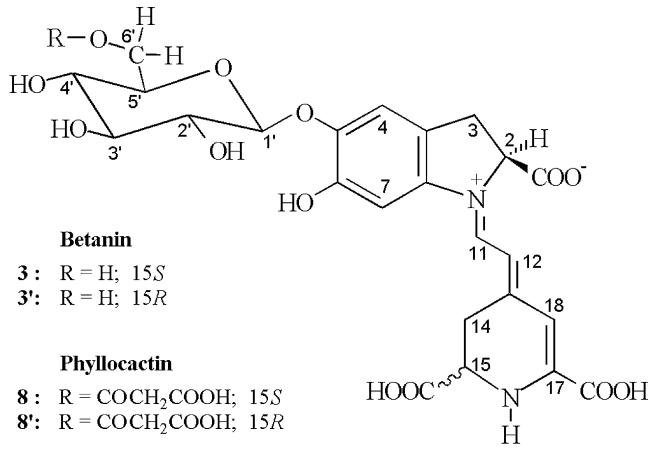

Fig. 1. Chemical structures of betacyanins present in the HSCCC fractions of *H. polyrhizus* fruits.

ing additives had become in our labs a very important direction for large-scale isolation of extremely polar plant pigments which normally do have a very limited thermal and chemical stability [2,6–11].

Furthermore, excellent scale-up possibilities by HPCCC and SRCCC [12,13] provide a lot of potential to recover larger amounts of pure betalains for thorough '*in vitro*' and '*in vivo*' physiological evaluations and other research purposes.

One of the most important physicochemical properties of betalain plant pigments (Fig. 1) are their significant polarity and ionization (dissociation, zwitter-ionic behavior) in aqueous solutions. Depending on the pH-values, the stability could be extremely limited, e.g. alkaline solutions are causing the cleavage into the biosynthetic precursor betalamic acid. The increased polar character of betacyanins and betaxanthins results in insolubilities in any of the popular organic polar or semi-polar solvents except of water and the mixtures with low-molecular alcohols [14,15]. It is known that degradation is advancing in alcoholic solutions [8,9]. Hence, both factors such as high hydrophilicity and low chemical stability of betalain pigments result in strong limitation of finding appropriate solvent systems. Planning the liquid–liquid extraction or solubilization of a betalain sample for HSCCC separations as well as NMR structural analyses requires extended experimental time in solution and enforces degradation reactions such as decarboxylation [6–11].

Our preliminary investigations on the application of cationic and anionic ion-pair agents in betalain analysis proved the effectiveness of transforming ionic betalains into non-charged ion-pairs [16,17]. The ionic and positively charged diazaheptamethinium betacyanin partial structure can easily form strong, much more lipophilic and also stable ion-pairs with the counter-ions of added agents. Resulting effect is a change of chromatographic behavior, e.g. strong retention time shifts to longer elution times in C_{18}-HPLC [16,17]. Likewise, the application of anionic reagents (perfluorocarboxylic acids) resulted in the first successful experiments in preparative HSCCC of betalains using ion-pair forming effects [2]. The significant increase of hydrophobicity of paired betalain ions shifted the partition ratios K_D of most of the analysed pigments to a magnitude that their chromatographic separation by means of HSCCC became possible. However, very polar pigments such as betanin and diglycosidic betacyanins which can be present at high quantities in biological materials (also existent in *Hylocereus* cacti) are, so far, still challenging compounds because of insufficient hydrophobicity of the formed ion-pairs resulting in incomplete separations. Therefore, further investigations with new solvent systems and additives of ion-pair forming capacities have to be performed.

Research focused on separations (C_{18}-HPLC and CCC) of polar pigments requires model compounds that differ slightly by polarity, e.g. caused by the diversity of acyl-group substitution. This condition is fulfilled by betacyanins occurring in large quantities in fruits of *Hylocereus* cacti, which are the subject of our research (including chromatographic one) for several years [18]. Judging from the solubility of betacyanins, they are acting as much more polar than anthocyanins with a flavylium cation partial structure. Hence, this chemotaxonomic pigment class is a valuable and demanding model for chromatographic studies including HSCCC.

Recently, we used a system based on 1-butanol and water with admixture of acetonitrile for the first HSCCC experiments on betalains with increasing additions of TFA. Finally, a solvent system consisting of 1-butanol–acetonitrile–water (5:1:6, v/v/v) with TFA in water (0.7%, v/v) was applied for the separation of lipophilic betalains from betanin present in *P. americana* berries [2].

This report presents further investigation on separation of polar betacyanins present in fruits of *Hylocereus polyrhizus* with the application of new solvent systems containing different concentrations of trifluoroacetic acid (TFA), and heptafluorobutyric acid (HFBA), respectively.

2. Experimental

2.1. Reagents

L-Leucine, L-isoleucine, L-phenylalanine, L-tyrosine, L-γ-aminobutyric acid, L-proline and dopamine were obtained from Aldrich (Milwaukee, WI, USA). Formic acid, trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), HPLC-grade acetonitrile (ACN), *tert.*-butyl methyl ether (TBME), 1-butanol, methanol and HPLC-grade water were obtained from Merck (Darmstadt, Germany).

2.2. Reference compounds

For structure confirmation, completely elucidated reference material (mostly by ESI-MS/MS and 1D/2D-NMR), the betacyanins (betanin, phyllocactin, hylocerenin, 2′-*O*-apiosyl-betanin, 6′-*O*-malonyl-2′-*O*-apiosyl-betanin, 2′-*O*-glucosyl-betanin as well as their C-15 diastereoisomers) were derived from extracts of fruits of *H. polyrhizus* and *Hylocereus ocamponis*. 6′-*O*-malonyl-2′-*O*-glucosyl-betanin (mammillarinin) was isolated from fruits of *Mammillaria coronata* [19]. The betaxanthins were obtained by hydrolysis of betanin/isobetanin and recondensation with appropriate amino acids [20].

2-Decarboxylated and 17-decarboxylated derivatives of betanin, phyllocactin and hylocerenin as well as their diastereomers were generated previously from the pigments derived from fruits of *H. polyrhizus* by preparation procedures described in [9]. The acyl migration products of phyllocactin and hylocerenin and their decarboxylated derivatives were isolated during recent studies [21,22].

2.3. Apparatus

The preparative HSCCC instrument used for the separation of betacyanins and betaxanthins was a multilayer coil planet J-type centrifuge model CCC 1000 (Pharma-Tech Research, Baltimore, MD, USA). The three preparative coils were connected in series equipped with polytetrafluorethylene (PTFE) tubing: 165 m × 2.6 mm i.d. with 876 mL theoretical total volume given by manufacturer,

850 mL measured total volume. The distance (revolution radius = R) of the holder axis of the coils to the central (solar) axis of the instrument was 7.5 cm. The inner β_r-value was measured to be 0.53 at the internal end of the coil and the outer β_r-value was 0.8 (equation: $\beta_r = r/R$; r is defined as the distance from the coil (planetary) axis to the nearest and farthest, respectively, layer of the PTFE tubes wound on the coil system). The HSCCC system's direction of rotation determined the *head* locations at the periphery of the three coils. The system was operated at 1000 rpm in the *elution*-mode '*head-to-tail*'. For the recovery of a complete polarity range of the injected samples the elution–extrusion methodology (EE) introduced by Berthod et al. was applied [3–5]. A velocity of 800 rpm during the *extrusion* procedure was used.

A Gynkotek HPLC system with UVD340U, Gynkotek HPLC Pump Series LPG-3400A and thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) was used for the HPLC analysis. For the data acquisition, the software package Chromeleon 6.0 (Gynkotek Separations) was applied. For the separation of betalains, a Luna C18(2) column 250 mm × 3 mm i.d., protected by a guard column (Phenomenex, Torrance, CA, USA) was used (for gradient conditions cf. Section 2.7).

The positive ion electrospray mass spectra were recorded on Agilent 1100 VL LC–MS (electrospray voltage 3.5 kV; capillary temp. 220 °C; sheath gas: N_2) coupled to an Agilent LC pump and DAD detector (Agilent Technologies, Palo Alto, CA). The MS was controlled and total ion chromatograms and mass spectra were recorded using Chemstation software (Agilent Technologies, Palo Alto, CA).

2.4. Preparation of crude pigment extract

For the study, freeze-dried flesh of fruits of *H. polyrhizus* [(F. A. C. Weber) Britton and Rose] cactus grown on trellis system under greenhouse conditions in Beer-Sheva, Israel, was taken [23]. Pigments were three times extracted with 1000 ml 80% aq. MeOH from the fruits (400 g) and the extract was filtered through a filter cloth and finally through a layer of 0.040 mm silica gel (J.T.Baker, Deventer, Holland) and a 0.2 μm i.d. pore size filter (Millipore, Bedford, MA). The extract for the preparative HSCCC experiments, LC-DAD and LC-ESI–MS analysis was concentrated and cleaned-up according to the previous procedure [2].

2.5. Selection of two-phase ion-pairing solvent systems

For the evaluation of solvent systems with ion-pair forming capacity, the previously published solvent system 1-BuOH–ACN–H_2O (0.7% TFA) 5:1:6 (v/v/v) [2] was compared to new systems based on TBME–1-BuOH–ACN–H_2O 2:2:1:5 (v/v/v/v) with two different additions of the volatile perfluoroacids TFA and HFBA at 20 °C (cf. Table 2). The partition ratios of the principal pigments of *H. polyrhizus* were determined according to the previous procedure [2].

2.6. HSCCC separations of H. polyrhizus extracts

After thoroughly equilibrating the HSCCC solvent mixtures (cf. Table 2) in a separatory funnel at room temperature, the two phases were separated shortly before use and degassed by ultrasonication. The upper organic phase was used in our experimental sequences (five separations: details for the biphasic perfluoroacidified solvent mixtures, cf. Table 2) as stationary phase and the lower aqueous phase as mobile phase in the *head-to-tail* mode. This direction is also defined as *reversed-phase* mode of CCC in direct comparison to C18-HPLC also using the aqueous phase as the eluting phase.

Charging the multilayer coiled column of the HSCCC was done by filling the respective upper organic phase using a Biotronik BT 3020 HPLC pump (Jasco, Grossumstadt, Germany). The HSCCC separation was performed at an ambient temperature.

The freeze-dried pigment samples from fruits of *H. polyrhizus* (C_{18}-SPE-cleaned, 850–900 mg) were dissolved in 10 mL each of upper and lower phase as suggested by Ito and Conway [1]. The filtered sample solution was introduced into the separation column through a manual low-pressure sample injection valve (Rheodyne, Cotati, CA, USA) and a 25 mL loop without prior column equilibration. The crude pigment sample was completely soluble in the two-phase systems, formation of precipitates was not observed in any of the experiments.

The lower phase was pumped at a flow rate of 3.0 mL/min in the '*head-to-tail*' direction with the *head* at the periphery of the coil after start of rotation at 1000 rpm. The effluent stream from the *tail* outlet of the column was monitored by UV-absorbance at λ 540 nm using a Knauer K-2501 UV detector (Berlin, Germany) equipped with a preparative cell (0.5 mm path length) and collected into test tubes with a fraction collector (LKB SupeRac 2211, LKB, Bromma, Sweden) at 4 min intervals.

The *elution*-mode was stopped as the pigment concentrations in the eluates had significantly decreased. Then the *extrusion* process was started by pumping 5.0 mL/min of the upper organic phase and the rotation speed of the system was reduced to the velocity of 800 rpm. This recovered the pigments already separated inside the coil-columns with higher partition ratios in a sequence of rising lipophilicity [3–5]. For the conducted CCC separations we stopped the *elution*-mode later than $K_D \sim 1$ (=elution volume 850 mL). Separating with solvent system **V** we started the *extrusion* approach at a time of 410 min (*switch*-volume 1230 mL = $K_D \sim 1.5$).

Analysis of all recovered HSCCC fractions was performed by HPLC-diode array detection (DAD), and HPLC-electrospray ionization (ESI)–MS.

2.7. Chromatographic system (HPLC-DAD-ESI–MS)

For the separation of the analytes (crude extract, fractions of partitioning experiments and CCC-fraction) the following gradient system was used: 93% A with 7% B at 0 min; gradient to 80% A with 20% B at 35 min (Solvent A: 2% formic acid, Solvent B: acetonitrile). The injection volume was 10 μL, and the flow rate was 0.5 mL/min. The detection of analytes was performed typically at 538, 505, 480, 450 and 310 nm. For the UV–vis spectra aquisition the detection was performed in the DAD mode. The column was thermostated at 35 °C. The same chromatographic conditions were applied for the HPLC-ESI–MS analyses.

2.8. Lyophilization of resulting HSCCC fractions

The HSCCC fractions still contained large amounts of solvents, therefore, they were diluted with nanopure water before being frozen and directly lyophilized (Christ freeze-drier, model Beta 2-8 LD plus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany). The fractions of the *elution*-mode contained in principal aqueous perfluoroacid, with lower amounts of 1-butanol and acetonitrile. More difficult were the resulting fractions of the *extrusion*-mode principally consisting of 1-butanol, acetonitrile and *tert*.-butyl methyl ether and also perfluoroacids. The organic residual solvents had to be condensated in a trap cooled by liquid nitrogen to prevent decomposition of the hybrid vacuum pump (Vacuubrand, Chemistry Hybrid Pump RC 6, Wertheim, Germany). This direct drying of aqueous and organic solvent parts by lyophilization is necessary to omit the fast decomposition of pigments and keep the amount of decarboxylation products as low as possible in the recovered CCC-fractions.

3. Results and discussion

3.1. The pigments of H. polyrhizus

The first structural study on *H. polyrhizus* pigments reported three main betacyanins (betanin **3**, phyllocactin **8** and hylocerenin **10**) with 15*S*-configuration as well as their corresponding C-15 diastereoisomers (15*R*- or so-called iso-forms) (**3′**, **8′** and **10′**) [18] (Fig. 1). That study was supported by research of Stintzing et al. [15] and finally by Wybraniec et al. [21] in which further minor betalain constituents were characterised.

The six principal pigments of fruit flesh of *H. polyrhizus* formed three pairs of compounds of diminishing overall polarity moving from the most polar betanin, then phyllocactin to hylocerenin as observed in HPLC on C_{18} phases [15,18,21]. The other known minor betacyanins were 2′-*O*-glucosyl-betanin **2**, 2′-*O*-apiosyl-betanin **6** and 6′-*O*-malonyl-2′-*O*-apiosyl-betanin **11** as well as the products of acyl group migration in phyllocactin **9** and hylocerenin **12** [21,22]. Recent studies identified also betaxanthins: indicaxanthin **Bx1** and γ-aminobutyric acid-betaxanthin **Bx3** [21] and their respective iso-forms.

The preconcentration effect of betalains in the recovered HSCCC fractions of the different experiments was very effective and resulted in further LC-DAD and LC-ESI–MS detection of not documented minor pigment structures in *H. polyrhizus* fruits as well as already known for other betalain producing plants [19,20]. This study revealed more betaxanthin compounds which were concentrated and separated in the fractions mainly resulting from the *extrusion*-procedure of the HSCCC separations (fractions 19–24, cf. Table 1): portulacaxanthin II **Bx4**, miraxanthin V **Bx5**, isoleucine-bx **Bx6**, leucine-bx **Bx7** and phenylalanine-bx **Bx8** as well as the unknown pigment **Bx2** (m/z 326). In addition, an unknown pair of betacyanins **4**/**4′** (m/z 683) isomeric to known 2′-*O*-apiosyl-betanin **6**/**6′** was detected as well as new pigments **1a**–**1d** (m/z 713) and **5a**–**5d** (m/z 667) (cf. Section 3.3) which were presumably groups of isomeric betacyanins forming clusters of not fully resolved HPLC peaks.

Another group of compounds (cf. Section 3.4) was identified in the HSCCC fractions as decarboxylated and dehydrogenated derivatives of betanin, phyllocactin and hylocerenin which appeared as classical pigment artefacts [7,9,11] (Fig. 1). Interestingly, the products of acyl group migration in decarboxylated phyllocactin and hylocerenin [22] were also detected.

Table 1
Chromatographic, spectrophotometric and mass spectrometric data of betalains in *H. polyrhizus* fruits.

No.	Compound	R_t [min]	λ_{max}[a] [nm]	m/z $[M+H]^+$
	Betacyanins			
1a	Unknown	**11.5**	536	713
1b	Unknown	**11.8**	536	713
2	Betanidin 5-*O*-β-sophoroside	**12.1**	536	713
1c	Unknown	**12.7**	536	713
1d	Unknown	**13.1**	536	713
2′	Isobetanidin 5-*O*-β-sophoroside	**13.3**	536	713
3	Betanidin 5-*O*-β-glucoside (betanin)	**13.6**	536	551
4	Unknown	**14.4**	536	683
3′	Isobetanidin 5-*O*-β-glucoside (isobetanin)	**15.4**	536	551
4′	Unknown	**16.3**	536	683
5a	Unknown	**16.4**	535	667
6	2′-*O*-Apiosyl-betanin	**16.6**	536	683
7	Betanidin 6′-*O*-malonyl-5-*O*-β-sophoroside	**16.9**	534	799
5b	Unknown	**17.4**	535	667
5c	Unknown	**17.5**	535	667
5d	Unknown	**18.2**	535	667
6′	2′-*O*-Apiosyl-isobetanin	**18.6**	536	683
7′	Isobetanidin 6′-*O*-malonyl-5-*O*-β-sophoroside	**18.6**	534	799
8	6′-*O*-Malonyl-betanin (phyllocactin)	**19.2**	535	637
9	4′-*O*-Malonyl-betanin	**20.0**	535	637
8′	6′-*O*-Malonyl-isobetanin (isophyllocactin)	**21.0**	535	637
10	6′-*O*-(3″-Hydroxy-3″-methyl-glutaryl)-betanin (hylocerenin)	**21.3**	535	695
11	2′-*O*-Apiosyl-phyllocactin	**21.8**	534	769
9′	4′-*O*-Malonyl-isobetanin	**22.2**	535	637
12	4′-*O*-(3″-Hydroxy-3″-methyl-glutaryl)-betanin[b]	**22.8**	535	695
10′	6′-*O*-(3″-Hydroxy-3″-methyl-glutaryl)-isobetanin (isohylocerenin)	**23.0**	535	695
11′	2′-*O*-Apiosyl-isophyllocactin	**23.1**	535	769
12′	4′-*O*-(3″-Hydroxy-3″-methyl-glutaryl)-isobetanin[b]	**24.7**	535	695
	Betaxanthins			
Bx1′	Isoindicaxanthin (proline-isobx)	**15.1**	478	309
Bx2	Unknown	**15.7**	472	326
Bx3	γ-Aminobutyric acid-bx	**15.8**	459	297
Bx1	Indicaxanthin (proline-bx)	**15.9**	479	309
Bx4′	Isoportulacaxanthin II (tyrosine-isobx)	**20.7**	473	375
Bx5	Miraxanthin V (dopamine-bx)	**21.1**	462	347
Bx4	Portulacaxanthin II (tyrosine-bx)	**21.6**	472	375
Bx6′	Isoleucine-isoBx	**33.4**	471	325
Bx7′	Leucine-isoBx (isovulgaxanthin IV)	**35.5**	469	325
Bx6	Isoleucine-Bx	**35.6**	472	325
Bx8′	Phenylalanine-isoBx	**36.8**	469	359
Bx7	Leucine-Bx (vulgaxanthin IV)	**37.1**	471	325
Bx8	Phenylalanine-Bx	**38.6**	469	359

[a] λ_{max} of betacyanins in the visible range.
[b] Tentatively identified.

3.2. Calculation of the partition ratios K_D and selectivity factors α

For a better understanding of the separation capabilities of a solvent system separating the major polar betacyanins (**3**/**3**′, **8**/**8**′ and **10**/**10**′), their partition ratios K_D and selectivity factors α were measured in the respective biphasic solvent systems prepared from the *H. polyrhizus* extract which were used later on preparative scale for the HSCCC runs.

The presence of residual non-pigment related compounds such as sugars and fruit acids in the crude pigment extract could potentially alter the equilibrium stages and phase settling times. Therefore, in order to simulate the chromatographic conditions under real CCC-conditions, the crude extract was directly used for partition ratio experiments.

The results of the determination of the K_D partition ratios are presented in Table 3. The highest values for betanin **3**, the most polar of the higher concentrated pigments, were obtained in the system **V** which reflected the higher activity of the HFBA anions and the increased lipophilicity of the resulting ion-pairs in comparison to TFA. This tendency was well seen by comparison of all the measured partition ratios of the solvent systems **II**–**V**, however, in the system **I**, the *K* values were not far from the ratios in the system **V**.

The solvent system **I** with 0.7% TFA was used previously for the successful separation of betanin from much more lipophilic betacyanins and betaxanthins from *P. americana* [2]. However, in our preliminary studies on betanin, phyllocactin and hylocerenin from *H. polyrhizus*, the system **I** seemed not to be suitable enough to resolve the pigments by performing CCC. Despite the rather excellent K_D values for the pigments (cf. Table 3), a critically low retention and stability (30%, cf. Table 3) of the stationary phase of **I** was hampering the CCC-separation. The retention of the stationary phase in the CCC-coil was significantly improved by the addition of TBME (systems **II**–**V**). However, as expected, the presence of lipophilic TBME diminished the partition ratios K_D in the TFA systems (**II**–**III**) (cf. Tables 2 and 3). But the exchange of TFA to the more lipophilic and much higher fluorinated homologue HFBA increased the K_D values (systems **IV**–**V**). Therefore, the K_D values in the system **V** were enlarged to the level of the system **I**, and the retention (cf. Table 3) of the stationary phase was increased which was confirmed in further HSCCC experiments.

Table 2
Composition of the solvent systems used in the HSCCC experiments.

System no.	Composition
I	1-BuOH–ACN–H_2O (0.7% TFA) 5:1:6 (v/v/v)
II	TBME–1-BuOH–ACN–H_2O (0.7% TFA) 2:2:1:5 (v/v/v/v)
III	TBME–1-BuOH–ACN–H_2O (1.0% TFA) 2:2:1:5 (v/v/v/v)
IV	TBME–1-BuOH–ACN–H_2O (0.7% HFBA) 2:2:1:5 (v/v/v/v)
V	TBME–1-BuOH–ACN–H_2O (1.0% HFBA) 2:2:1:5 (v/v/v/v)

Surprisingly, the K_D ratios for hylocerenin (**10**) tended to be shifted to lower values relative to the coefficients for phyllocactin (**8**) in all the tested CCC solvent systems (**I**–**V**). This prediction was confirmed during the HSCCC experiments – especially for systems **IV** and **V** – where hylocerenin was eluted slightly earlier than phyllocactin (Figs. 3 and 4). In general, the results suggested the superiority of the systems **IV** and **V** for the separation of the polar betacyanins.

For evaluation of the separation potential of each solvent system (**I**–**V**), the selectivity factors α for the principal pigments were calculated from the K_D values using the equation $\alpha = K_{D2}/K_{D1}$ (where $K_{D2} > K_{D1}$). It was assumed that a resulting factor $\alpha > 1.5$ would be a clear indication that a separation of components can be achieved [3–5]. The α-values for the pigments measured in system **I** were quite good as we can see for α **8**/**3** (2.13), and α **10**/**3** (1.8). For the pigment pair **8**/**10** the value for α 1.2 was recognized to be not sufficient. The diasteromers 15*S*- and 15*R* structures (iso-form) e.g. **8**′/**8** had very low α-values (1.03).

As mentioned above, the use of the CCC solvent systems containing TBME improved significantly the phase retention. But working just with TFA (0.7% and 1.0%) (systems **II** and **III**) was not sufficient to get a suitable partition of pigments and moved in maximum the K_D ratios close to 0.5.

Working with HFBA addition resulted in the best selectivity values for the system **IV** (0.7% HFBA) in the case of the pigment pairs α **8**/**3** (2.0) and α **10**/**3** (1.9) predicting a very good separation of betanin from phyllocactin (**8**) and hylocerenin (**10**). Here as well the 15*S*-forms couldn't be separated from the 15*R*-iso-forms.

Unexpectedly, the results for system **V** (1.0% HFBA) showed values α for the pigment pairs **8**/**3** (1.7) and **10**/**3** (1.5) with a strong decrease—but still with a separation of betanin/isobetanin (**3**/**3**′)

Table 3
The partition coefficients (K_D) for the most abundant betalains measured in the five different HSCCC solvent systems (cf. Table 2).

No.	Compound	K_D values for ion-pair solvent systems				
		I 0.7% TFA	**II** 0.7% TFA	**III** 1.0% TFA	**IV** 0.7% HFBA	**V** 1.0% HFBA
3	Betanin	0.37	0.21	0.32	0.30	0.43
3′	Isobetanin	0.39	0.23	0.32	0.31	0.45
8	Phyllocactin	0.79	0.38	0.48	0.61	0.73
8′	Isophyllocactin	0.81	0.39	0.49	0.63	0.78
10	Hylocerenin	0.67	0.33	0.46	0.58	0.64
10′	Isohylocerenin	0.69	0.34	0.47	0.59	0.66
		Selectivity factors α[a]				
α 3′/3		1.1	1.1	1.0	1.0	1.0
α 8/3		2.1	1.8	1.5	2.0	1.7
α 8′/3′		2.1	1.7	1.5	2.0	1.7
α 10/3		1.8	1.6	1.4	1.9	1.5
α 10′/3′		1.8	1.5	1.5	1.9	1.5
α 10′/10		1.0	1.0	1.0	1.0	1.0
α 8/10		1.2	1.2	1.0	1.1	1.1
α 8′/10′		1.2	1.2	1.0	1.1	1.2
α 8′/8		1.0	1.0	1.0	1.0	1.1
Retention of stationary phase [%][b]		30.1	55.9	56.0	77.0	57.6

[a] The separation factors α for the principal pigment components were calculated from the equation $\alpha = K_{D2}/K_{D1}$ (where $K_{D2} > K_{D1}$).
[b] Retention of stationary phase [%] was calculated from the break-through volume of mobile phase.

from pigments **8**/**8**′ and **10**/**10**′. Instead, the selectivity values α for the pigment pairs **8**/**10** (1.1) and **8**′/**10**′ (1.2) were getting slightly increased and similar to system **I**.

Increasing the concentration of the acids above the 0.7–1.0% level increased dramatically the degradation rate of the pigments. Therefore, higher concentrations were not further evaluated.

3.3. HSCCC separation of betalains from H. polyrhizus

The principal pigments of *H. polyrhizus* fruits (**3**/**3**′, **8**/**8**′ and **10**/**10**′) form a mixture of highly polar (**3**/**3**′) and medium polar (**8**/**8**′ and **10**/**10**′) betacyanins, which can be well resolved in C18-HPLC systems, however, they are too polar for a successful separation in HSCCC solvent systems used so far [2] because of serious difficulties in finding of appropriate phase systems. Our recent study on *P. americana* pigments proved a possibility of separation of less polar pigments in a solvent system 1-BuOH–ACN–H_2O (0.7% TFA) [2]. These acylated betacyanins had the partition ratios K_D much higher than 0.5, which made their separation possible. In contrast, betanin (**8**) was not separated from the more polar diglucosylated betacyanins.

In this study, we compared five solvent systems (**I**–**V**) (cf. Table 2) containing one of two perfluorinated carboxylic acids, TFA and HFBA at two concentration levels (0.7% and 1.0%). For most of the experiments with *H. polyrhizus* we started using TBME of good polar properties as an admixture to the 1-BuOH phase, because of its lower viscosity allowing for better phase separation and retention of the stationary phase. However, we also compared our previous solvent system 1-BuOH–ACN–H_2O (0.7% TFA) to the newly developed ones.

Two HSCCC chromatograms monitored at λ 540 nm obtained using the solvent systems **IV** and **V** containing 0.7% and 1.0% HFBA, respectively, were shown in Fig. 2. Several partially resolved peaks of the polar betacyanins eluted in the first 8 or 11 fractions, respectively, were visible. As discussed below, detailed HPLC analysis of the resulting HSCCC fractions confirmed advanced betacyanin separation in these systems in comparison to the solvent systems **I**–**III**. The major and minor betalain distribution in the recovered fractions from the HSCCC runs in the solvent system **V** is presented in Table 4.

In Fig. 3, the representative HPLC chromatograms of the HSCCC fractions obtained during the chromatographic runs in the solvent systems **III** (TFA), and **V** (HFBA) containing the highest concentrations of the ion-pairing agents (1.0%) were depicted. Whereas the overlapping of the key betacyanins was noticed for most fractions (i.e. the presence of all the key pigments in most of the HSCCC fractions at a meaningful level) of the system **III**, the separation was much better in the system **V**.

In Fig. 4 the three pigment (**3**, **8** and **10**) profiles in the resulting fractions from the IP-HSCCC experiments in the five solvent systems were compared. The highest ion-pairing properties of HFBA allowed for the best separation of betanin (**3**) from phyllocactin (**8**) and hylocerenin (**10**) in the solvent systems **IV** and **V**. Interestingly, the separation of betanin/isobetanin (**3**/**3**′) from the other pigments **8**–**10** was much better in the system **IV**. One apparent explanation could be that this was a result of the much higher stationary phase retention (20% higher) at lower HFBA concentration and giving a much better chromatographic resolution. But already in the theoretical prediction calculating the K_D values and the selectivity factors α for these pigments we observed this trend (cf. Table 3 and Section 3.2). Therefore, we postulate that there is a perfluoroacid optimum concentration for the highest resolution of the pigments strongly depending of the structural features such as acyl-substitution. There was no meaningful overlapping of betanin (**3**) with phyllocactin (**8**) and hylocerenin (**10**) in comparison to the system **V** where the overlapping was observed in

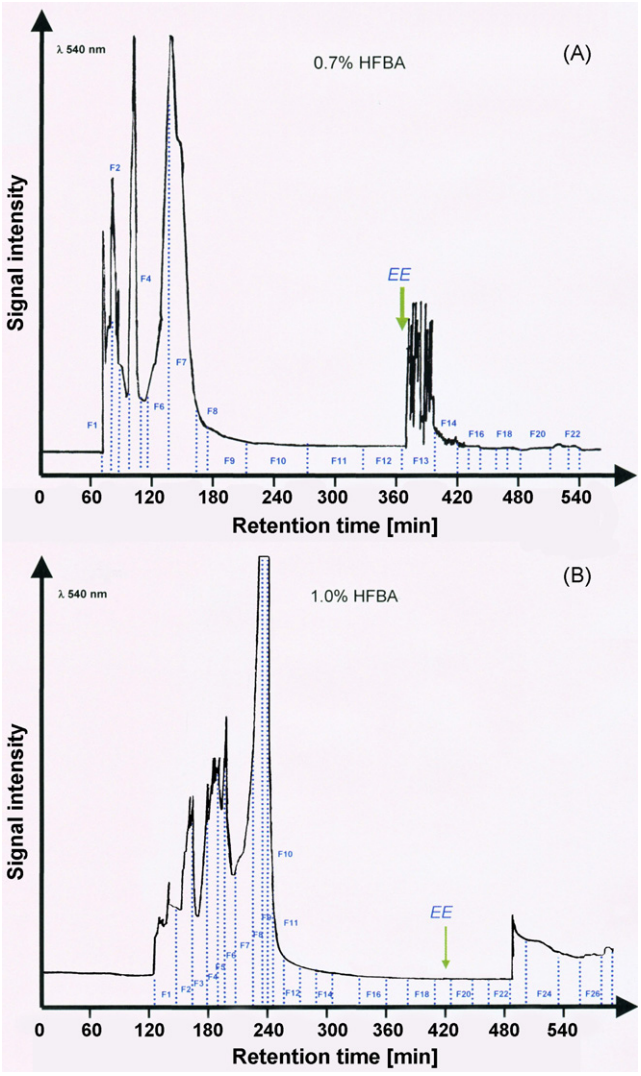

Fig. 2. Ion-pair-HSCCC-chromatograms of C_{18}-enriched pigment extracts (900 mg) of fruits of *H. polyrhizus* in the solvent systems **IV** and **V**. HSCCC conditions: flow rate for the *elution*-mode: 3.0 mL/min; *head-to-tail* mode; flow rate for the *extrusion* stage: 5.0 mL/min; for the composition of the biphasic solvent systems—cf. Table 2.

fraction 6 which contained all the key pigments at high and comparable concentrations. Surprisingly, phyllocactin (**8**) and hylocerenin (**10**) were not resolved in any of the tested solvent systems, even if they were more hydrophobic and better partitioned between the two phases. However, closer inspection of the concentration profiles in the fractions as well as the K_D values revealed that hylocerenin tended to be eluted slightly earlier than phyllocactin. This experimental observation was in accordance to the calculated K_D ratios, where hylocerenin values are always lower than phyllocactin ($\Delta K_D = 0.09$ for system **V**). Therefore, an inversion of elution order in reversed-phase HSCCC (*head-to-tail*) in comparison to reversed phase C18-HPLC could be deduced. Such inversion was also observed between feruloylated and sinapoylated betacyanins from *P. americana* leading to their good separation [2]. The lack of complete separation of phyllocactin and hylocerenin was therefore rather a result of two balanced contradictory effects: the higher hydrophobicity of hylocerenin (the result of the presence of a longer aliphatic chain in the acyl group) and its stronger interaction with the polar phase (presumably caused by the presence of the hydroxyl group in the chain).

It seems that in HPLC on reversed stationary phases the second effect is less pronounced. Further experiments with other

Table 4
Betalain distribution in the recovered fractions obtained from *H. polyrhizus* fruit extract by IP-HSCCC in the solvent system **V**.

No.	*m*/*z*	Relative content [%]of pigment in HSCCC fractions (system V) analysed by HPLC-DAD–MS Betacyanin fraction no.													Total HPLC peak area × 10^{-6}
		1	2	3	4	5	6	7	8	9	10	11	12	13	
1a	713	3.1	38.7	58.2											0.11
1b	713	2.7	47.0	50.3											0.65
1c	713	5.2	38.8	56.0											0.16
1d	713	2.5	48.2	49.3											0.36
2	713	2.4	47.1	50.5											0.30
2′	713		58.6	41.4											0.040
3	551	1.0	5.2	12.9	18.5	22.3	15.7	7.4	6.2	4.8	4.1	1.1	0.8		12.9
3′	551	0.7	8.6	14.3	21.2	22.9	10.1	7.3	6.2	4.9	3.3	0.7			7.41
4	683			43.5	53.2	3.3									0.16
4′	683			46.3	41.7	12.0									0.10
5a	667									19.6	69.2	11.2			0.13
5b	667									17.7	67.8	14.5			0.060
5c	667									27.0	67.1	5.9			0.13
5d	667									0.1	99.8	0.1			0.010
6	683				52.4	27.2	20.4								0.039
6′	683				58.7	35.1	6.2								0.009
7	799		79.3	20.7											0.12
7′	799		77.9	22.1											0.088
8	637			0.5	0.7	1.4	8.1	14.8	22.8	24.0	20.0	5.4	2.1	0.2	14.4
8′	637			0.6	0.6	1.1	7.7	13.2	23.4	25.7	24.4	2.6	0.7		8.53
9	637						5.3	4.2	17.4	25.3	29.9	14.5	3.4		2.58
9′	637						3.4	4.2	10.8	27.5	34.2	14.4	4.9	0.6	1.15
10	695			0.9	1.0	2.1	12.6	23.4	32.7	19.3	6.3	0.9	0.8		10.3
10′	695			0.8	0.9	1.9	12.1	21.5	34.1	17.1	8.7	2.6	0.3		6.67
11	769				7.0	23.5	58.7	10.8							0.21
11′	769				12.9	34.4	43.1	9.6							0.078
12	695											82.8	17.2		0.063
12′	695											64.3	35.7		0.038
Mass [mg][a]		25.1	465.1	87.8	26.7	18.8	25.7	41.0	55.8	23.1	24.9	8.3	6.0	8.0	

No.	*m*/*z*	Relative content [%]of pigment in HSCCC fractions (system V) analysed by HPLC-DAD–MS Betaxanthin fraction no.										Total HPLC peak area × 10^{-6}
		15	16	17	18	19	20 *Ext*[b]	21 *Ext*	22 *Ext*	23 *Ext*	24 *Ext*	
Bx1′	309	4.6	15.4	80.0								0.20
Bx1′	309	1.1	17.3	81.6								2.10
Bx2	326							50.4	21.5	28.1		0.55
Bx3	297							52.1	22.7	14.7	10.5	0.46
Bx4′	375			77.5	22.5							0.036
Bx4′	375			84.4	15.6							0.10
Bx5	347	100										0.041
Bx6′	325					22.6	40.2	37.2				0.036
Bx6′	325					11.8	16.6	71.6				0.069
Bx7′	325							20.1	20.3	59.6		0.050
Bx7′	325								31.7	68.3		0.046
Bx8′	359							4.5	22.2	66.3	7.0	0.98
Bx8	359							7.8	27.8	59.6	4.8	1.80
Mass [mg][a]		6.5[a]	4.8	9.3	8.7	2.7	5.0	6.2	2.6	9.9	8.9	

[a] Mass of fraction after lyophilization.
[b] *Ext* = extrusion process was started after fraction 19 with a flow of organic stationary phase 5.0 mL/min. Fractions 21 and 22 were still containing mobile phase due to *sweeping*-elution [3–5].

ion-pairing agents would answer the question concerning the resolution between phyllocactin (**8**) and hylocerenin (**10**).

Besides betanin, more polar pigments of known and unknown structures were found at lower concentration levels (Table 4). Diglycosylated pigments together with prevailing betanin/isobetanin **3**/**3′** formed a mixture of the most polar betacyanins investigated in chromatography, therefore, their separation in HSCCC was the most challenging task. In the positive ESI-MS mode a group of six diglucosides (**1a**–**1d** and **2**/**2′**), all showing ion-signals at *m*/*z* 713 were detected mainly in the first two fractions of the solvent systems **IV** and **V**, thus, separated from the bulk of betanin (**3**/**3′**). This was the first case when such polar betacyanins were separated in HSCCC. Betacyanins **1a**–**1d** and **2**/**2′** were also well separated from other diglycosides (apiosylated and glucosylated betanidins, **4**/**4′**–**6**/**6′**), malonylated diglucosides (**7**/**7′**) as well as a group of completely unknown betacyanins (**5a**–**5d**).

Recent studies suggested that phyllocactin (**8**) and hylocerenin (**10**) were frequently accompanied by their acyl migration products and the most detectable were the 4′-*O*-isomers [21,22]. The highly concentrated samples always contain some amounts of these isomers as a result of a continuous and reversible acyl migration reaction and they are eluted just behind their precursors in C_{18}-HPLC systems, therefore, their separation in HSCCC was expected to be rather not feasible. Surprisingly, in each case, the 4′-*O*-isomers of phyllocactin (**9**/**9′**) and hylocerenin (**12**/**12′**) were eluted mainly in the peak tails of their precursors (Table 4) giving a chance for their preconcentration during the HSCCC preparative process. In practice, the HPLC separation of the 4′-*O*-isomers from their precursors is a tedious operation because of their continuous interchanging to

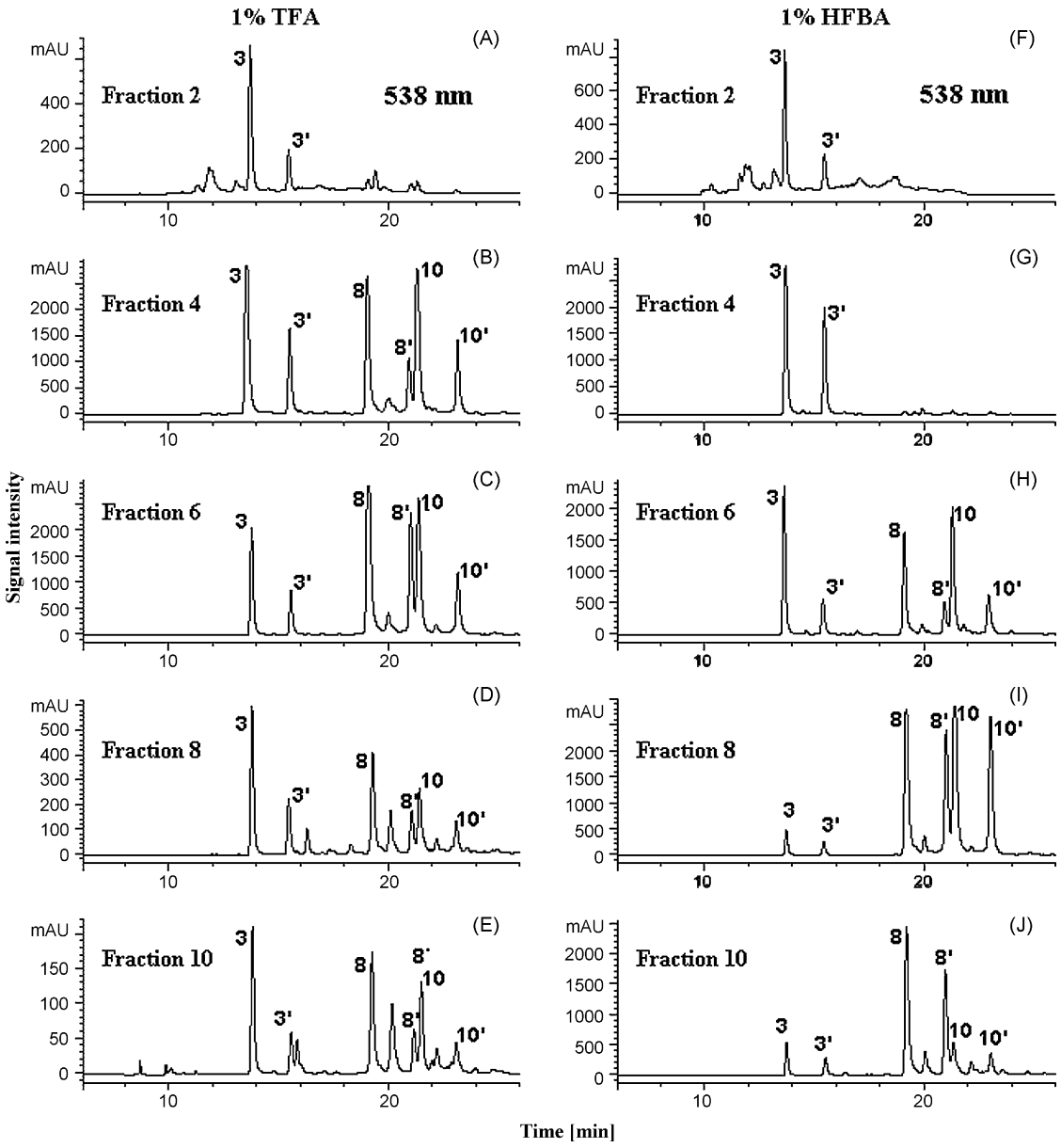

Fig. 3. HPLC profiles of betacyanins (monitored at λ 538 nm) analysed in *H. polyrhizus* fruit extract HSCCC fractions in the solvent systems **III** (A–E) and **V** (F–J). For the composition of the biphasic solvent systems—see Table 2.

the precursor structures, therefore, the application of HSCCC is an interesting alternative for their initial preconcentration on larger scale.

Because betaxanthins were previously identified in *H. polyrhizus* fruits [21], the separation between them was also monitored in the obtained IP-HSCCC fractions. Our previous study reported an interesting separation pattern of betaxanthins from *P. americana* in the 1-BuOH–ACN–H_2O (0.7% TFA) solvent system [2]. Even indicaxanthin eluted after betanin/isobetanin which was in opposite to their elution order in the reversed phase C_{18}-HPLC. This effect was increased in the current study and all the analysed betaxanthins were eluted from the HSCCC column behind all the principal betacyanins in the strongest ion-pair systems **IV** and **V**.

In the case of the separation **V** we stopped the *elution*-mode after fraction F19 and started the *extrusion* approach at a time of 410 min (*switch*-volume 1230 mL = $K_D \sim 1.5$) [4]. All betacyanin components were already eluted and the elution of the stronger ion-pairs formed from betaxanthins was monitored. Berthod and co-workers suggested starting the *extrusion* process after one column volume of pumping mobile phase ($K_D \sim 1$), and then *extrusion* with another column volume of stationary phase for the complete recovery of compounds. This was defined as the two-column volume or 2 V_C-*elution–extrusion* method [4]. Normally the rotation will stay turned on to keep the sharpness of the peak-shape during the *extrusion* process [3–5]. In the first step for a short time, the mobile phase is still moved—this is defined as *sweep*-elution stage [3–5]. Then the 'real' *extrusion* process will start by pushing-out the stationary phase solvent containing all the ion-pairs of the perfluorinated acid and betaxanthin pigments.

All of the betaxanthins formed very lipophilic ion-pairs with TFA or HFBA with extremely large HSCCC retention times. Furthermore, a strong retention of γ-aminobutyric acid-bx (**Bx3**) as well as of unknown betaxanthin (**Bx2**) with *m*/*z* 326 was observed, resulting in their complete separation from indicaxanthin (**Bx1**) (Table 4). All these betaxanthins are eluted close to each other in RP-HPLC. Similarly, phenylalanine-bx (**Bx8**) which was present at higher quantities in the fruits was eluted at latter fractions. The HSCCC preconcentration effect resulted in identification of other minor

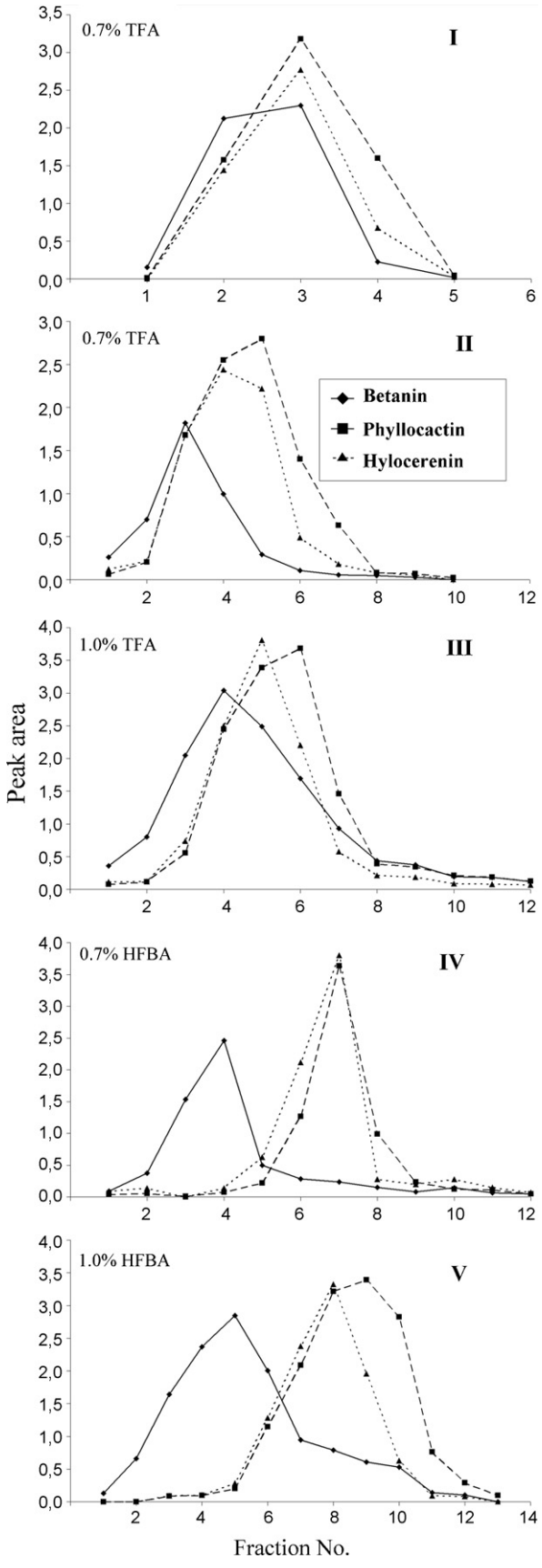

Fig. 4. Comparison of the separation between betanin (**3**), phyllocactin (**8**) and hylocerenin (**10**) in five HSCCC solvent systems (cf. details: Table 2).

betaxanthins eluted in shorter distinctive HSCCC bands which in most cases were resolved from each other: portulacaxanthin II (**Bx4**), miraxanthin V (**Bx5**), isoleucine-bx (**Bx6**) and leucine-bx (**Bx7**) (Table 4). The identification was readily performed after semi-synthesis of betaxanthin standards according to the known procedures [20].

3.4. Artefacts of betacyanins

As the decarboxylation reaction pathways of betacyanins in acidified organic solutions of *Hylocereus* have been studied recently [9], the presence of these artefacts was expected also in the CCC fractions. A further substantial increase of perfluorinated acid concentration in the case of TFA and HFBA (>1.0%) revealed that the amount of generated artefacts was unacceptably high. As a consequence, we were not conducting IP-HSCCC experiments with higher acid concentration levels than 1.0% in the aqueous part. However, the presence of the degradation products was still meaningful. All the 2- and 17-decarboxylated as well as 2,17-bidecarboxylated isomers of betanin, phyllocactin and hylocerenin were present in the fractions (data not shown). Additionally, the dehydrogenated neo-derivatives of the pigments were also noticed. All these products were described in recent reports [7,9,11]. Furthermore, the acyl migration products of decarboxylated phyllocactin and hylocerenin were also recognized in the chromatograms. The phenomenon of acyl migration in decarboxylated betacyanins was studied recently [22].

Betanin, phyllocactin and hylocerenin separated in the HSCCC experiments belong to the more labile group of betacyanins, therefore, their decomposition at these rather drastic acidic conditions is unavoidable—although thermal impact to the pigments was kept as low as possible by performing a complete freeze-drying protocol also during evaporation of the solvents. Finding an alternative for the highly active but strong perfluorinated acids for the separations is still demanding. However, for more stabile betacyanins the tested solvent systems would be gentle enough to perform the separations without substantial degradation. We are currently performing HSCCC experiments with such pigments.

4. Conclusions

The unique advantage of HSCCC using strong perfluorinated carboxylic acids with ion-pair forming activities is the preparative access to highly polar and chemically instable betacyanins and betaxanthins which can be hardly isolated effectively by other techniques on larger scale. Isolation of the pigment class of betalains is greatly hampered by their chemical instability.

In this study we had shown that the *elution–extrusion* approach is able to recover all pigment compounds of *H. polyrhizus* injected to the CCC system on preparative scale. In the case of HFBA, we postulate that there seem to be narrow concentration optima were a CCC-separation under ion-pair conditions is getting to its highest performance—very depending on the compounds (pigments) which need to be separated. The addition of 0.7% of HFBA was extremely effective to separate betanin/isobetanin (**3**/**3′**) from the cacti pigments phyllocactin (**8**) and hylocerenin (**10**). The slight increase of HFBA concentration to 1.0% decreased the chromatographic resolution between the pigments. Interestingly the retention of stationary phase was significantly reduced. As we have seen in the case of solvent system **V**, a lower retention of the stationary phase can decrease the resolution and highly influence the chromatographic results for the pigment separation.

The TFA addition in the solvent systems using TBME was less active and the K_D partition ratios were not at their optimal level.

The selection of ion-pair reagents for IP-HSCCC and their concentrations can be of great influence on stationary phase retention, K_D values and selectivity factors α. The chain length and degree of fluorination are the determinants for transferring the polar pigments to an organic CCC stationary phase. The amphiphilic character of HFBA caused strong emulsifying properties and seemed to highly influence the phase retention/stability of the chosen solvent systems. We had observed that higher homologues – with potentially higher ion-pair capacity – such as undecafluorohexanoic acid strongly hampered phase separation during CCC (data not shown).

The detected betaxanthins (cf. Table 1) developed with the ion-pair reagents such high K_D partition ratios that they occurred at the end of the *elution* section ($K_D > 1.48$, **Bx1**/**Bx1′**, **Bx4**/**Bx4′**) and were finally recovered during the *extrusion*-process (**Bx2**, **Bx3**, **Bx6**/**Bx6′**, **Bx7**/**Bx7′**, **Bx8**/**Bx8′**). Further clean-up by preparative C_{18}-HPLC would easily result in pure substances for 1D/2D-NMR-studies and biological assays.

The increasing demand for pure betalain investigations is clearly justifying the direction to perform ion-pair high-speed countercurrent chromatography. So far, no alternatives for perfluorinated-additives for IP-chromatography are known and will require more research efforts.

Acknowledgement

This research was financed in part by Polish Ministry of Science and Higher Education for years 2007–2010 (Project No. N205 2991 33).

References

[1] Y. Ito, W.D. Conway (Eds.), High-speed Countercurrent Chromatography: Chemical Analysis, vol. 132, Wiley-Interscience, New York, 1996.
[2] G. Jerz, T. Skotzki, K. Fiege, P. Winterhalter, S. Wybraniec, J. Chromatogr. A 1190 (2008) 63.
[3] A. Berthod, M.J. Ruiz-Angel, S. Carda-Broch, Anal. Chem. 75 (2003) 5886.
[4] Y. Lu, R. Liu, A. Berthod, Y. Pan, J. Chromatogr. A 1181 (2008) 33.
[5] A. Berthod, J.B. Friesen, T. Inui, G.F. Pauli, Anal. Chem. 79 (2007) 3371.
[6] K.M. Herbach, F.C. Stintzing, R. Carle, J. Food Sci. 69 (2004) 491.
[7] K.M. Herbach, F.C. Stintzing, R. Carle, Eur. Food Res. Technol. 219 (2004) 377.
[8] S. Wybraniec, J. Agric. Food Chem. 53 (2005) 3483.
[9] S. Wybraniec, Y. Mizrahi, J. Agric. Food Chem. 53 (2005) 6704.
[10] S. Wybraniec, B. Nowak-Wydra, Y. Mizrahi, Tetrahedron Lett. 47 (2006) 1725.
[11] K.M. Herbach, F.C. Stintzing, R. Carle, J. Food Sci. 71 (2006) R41.
[12] I. Sutherland, P. Hewitson, S. Ignatova, J. Chromatogr. A 1216 (2009) 4201.
[13] S. Ignatova, P. Wood, D. Hawes, L. Janaway, D. Keay, I. Sutherland, J. Chromatogr. A 1151 (2007) 20.
[14] D. Strack, W. Steglich, V. Wray, in: P.M. Dey, J.B. Harborne, P.G. Waterman (Eds.), Methods in Plant Biochemistry, vol.8, Academic Press, London, 1993, p. 421.
[15] F.C. Stintzing, J. Conrad, I. Klaiber, U. Beifuss, R. Carle, Phytochemistry 65 (2004) 415.
[16] S. Wybraniec, Y. Mizrahi, J. Chromatogr. A 1029 (2004) 97.
[17] S. Wybraniec, J. Chromatogr. A 1127 (2006) 70.
[18] S. Wybraniec, I. Platzner, S. Geresh, H.E. Gottlieb, M. Haimberg, M. Mogilnitzki, Y. Mizrahi, Phytochemistry 58 (2001) 1209.
[19] S. Wybraniec, B. Nowak-Wydra, J. Agric. Food Chem. 55 (2007) 8138.
[20] F.C. Stintzing, A. Schieber, R. Carle, J. Agric. Food Chem. 50 (2002) 2302.
[21] S. Wybraniec, B. Nowak-Wydra, K. Mitka, P. Kowalski, Y. Mizrahi, Phytochemistry 68 (2007) 251.
[22] S. Wybraniec, J. Chromatogr. B 861 (2008) 40.
[23] Y. Mizrahi, A. Nerd, in: J. Janick (Ed.), Climbing and Columnar Cacti: New Arid Land Fruit Crops. Perspectives on New Crops and New Uses, American Society for Horticultural Sciences Press, Alexandria, 1999, p. 358.