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

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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-

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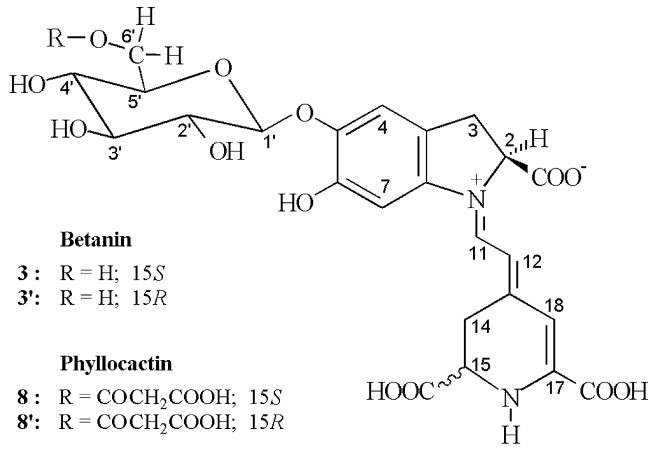

Fig. 1. Chemical structures of betacyanins present in the HSCCC fractions of *H. polyrhizus* fruits.

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,