High salt-solvent systems in separation of betanin and its derivatives from red beet (*Beta vulgaris* L.) by high-performance countercurrent chromatography (HPCCC)

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A study on a separation of betanin and its decarboxy- and dehydro-derivatives obtained from red beet roots (*Beta vulgaris* L.) using analytical high-performance countercurrent chromatography (HPCCC — Dynamic Extractions Ltd., UK) was performed. The HPCCC process was accomplished in the 'tail to head' mode with three highly polar solvent systems with high salt concentrations: 1-propanol-acetonitrile-saturated ammonium sulphate-water (v/v/v/v, 1:0.5:1.2:1); ethanol-acetonitrile-1-propanol-saturated ammonium sulphate-water (v/v/v/v/v, 0.5:0.5:0.5:1.2:1) and ethanol-1-butanol-acetonitrile-saturated ammonium sulphate-water (volume ratio), 0.5:0.5:0.5:1.2:1). HPLC analysis was performed in a conventional reversed phase mode with diode-array (DAD) detection to characterize the composition of obtained fractions.

The applied solvent systems enabled the separation of the betalain pigments with high efficiency for the first time. In the mode of separation selected, the more hydrophobic compounds eluted first as expected. Moreover, for the first time, the applied HPCCC solvent systems generated a separation of 2-decarboxy-betanin from 17- and 2,17-bidecarboxy-betanin as well as from neobetanin and betanin.

Keywords and phrases: betanin, betalains, betacyanins, countercurrent chromatography, *Beta vulg*aris L.

Introduction

HPLC is frequently used as an analytical and preparative technique for the analysis and separation of complex mixtures of pigments from their natural sources. This method enables isolation and characterization of currently well-known betalains [1, 2].

Countercurrent chromatography is a liquid chromatography with support-free liquid stationary phase which was invented by Dr. Yoichiro Ito in his laboratory at the National Institutes of Health in Maryland (USA) in the late 1960s. The liquid stationary phase is retained in the coil by the centrifugal field generated from the coil rotation, while the mobile phase is pushed through the coil with a pump. The liquid-liquid nature of countercurrent chromatography eliminates the problem of irreversible adsorption of the sample on supports such as silica gel or organic lipophilic gels.

The application of a liquid stationary phase enables recovery of all the components introduced into the HPCCC apparatus due to a technique known as the elution extrusion (EE) process. One of the advantages of EE process is the complete recovery of compounds which have higher affinity to the stationary phase. Other advantages of HPCCC over conventional column chromatography are that higher amounts of sample that can be introduced into the HPCCC apparatus due to lack of solid support. The application of high-performance countercurrent chromatography (HPCCC) in the separation of the most important natural plant pigment classes (carotenoids, chlorophylls, anthocyanins and betalains) demonstrates the versatility of this preparative and analytical technique [3–6].

Betalains (red-violet betacyanins and yellow-orange betaxanthins) are present in plants belonging mainly to the families of the *Caryophyllales*. Betacyanins, the condensation products of betalamic acid with *cyclo*--DOPA or its *O*-glycosylated derivatives, are water soluble pigments extensively used as food colorants. The variety of betacyanin structures is determined mainly by their C-15 carbon configuration. The *O*-glycosylation of betacyanins provides two main types of derivatives (the 5-*O*- and 6-*O*-glycosylated compounds). So far, the typical sugar moieties in betacyanins are: glucose,

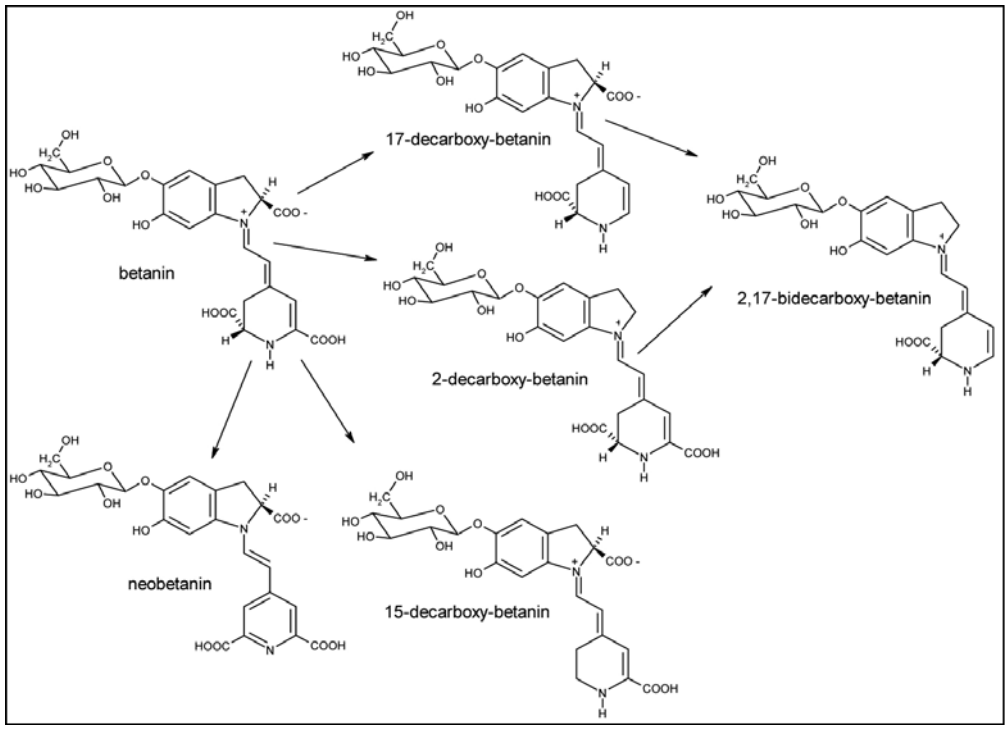

Fig. 1. Products of decarboxylation and dehydrogenation of betanin presented in red beet roots (*Beta vulgaris* L.).

glucuronic acid and apiose. Futhermore, the additional sugar moieties as well as the esterification of the *O*-glycosides with acids increases the number of possible structures. So far, the esterification products of betacyanins with acids: malonic, 3-hydroxy-3-methyl--glutaric, sinapic, caffeic-, p-coumaric and ferulic were observed. Studies on the stability of betalains has revealed new structures and degradation pathways of betalains. The compounds are sensitive to several factors, including elevated temperature, low pH and high water activity and will undergo decarboxylation or dehydrogenation reactions. Decarboxylation can occur at position C-2, C-15 or C-17 and dehydrogenation at C-14,15 or C-2,3. The main product of dehydrogenation is neobetanin **(5)** (14,15-bidehydro-betanin), which is present in red beet (*Beta vulgaris* L.) [Fig. 1].

Red beet (*Beta vulgaris* L.) is increasingly utilized as a source of natural food colorant due to growing interest in potential health benefits (anticarcinogenic) and the non-toxic features of betalains. The main betacyanin in the beet roots is called betanin **(1)** and its diasteroisomer isobetanin. The ratio of betacyanins to betaxanthins depends on beet varieties. Preparative isolation of instable betalains is problematic, therefore the introduction of the HPCCC technique creates a new possibility for obtaining pure pigments on a larger scale, which could be used widely in the food industry and pharmacy [5, 7–12].

Experimental

HPLC-grade acetonitrile, 1-propanol, ethanol, 1-butanol, ammonium sulphate were obtained from

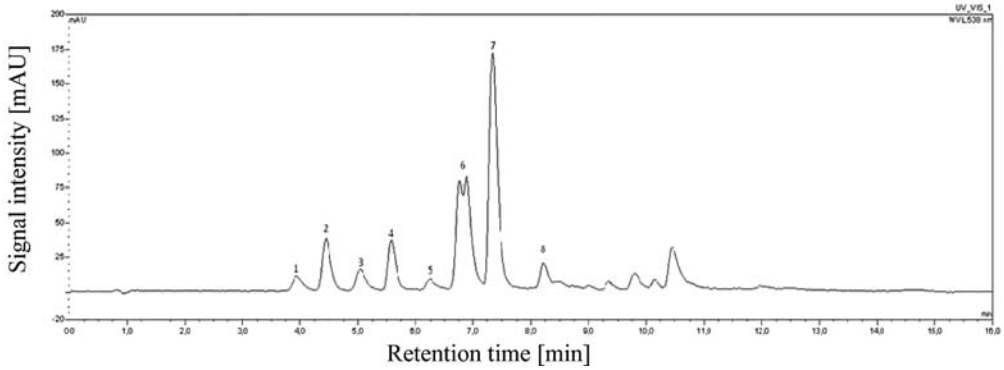

Fig. 2. Chromatogram of betalains analyzed by HPLC-DAD (Numeration of peaks — see Tables 1–3).

Fisher Chemicals (Loughborough, UK). Water was deionised (Purite, Thames, Oxon, UK).

For the study, a freeze-dried mixture of betalains together with their decarboxylated and dehydrogenated derivatives of red beet root (*Beta vulgaris* L.) from the market (Cracow, Poland) was taken. Decarboxylated and dehydrogenated compounds were previously obtained through heating betalains for 30 min at 85°C in a water bath acidified with 20 ml of formic acid. The products of degradation were purified on a preparative solid-phase extraction (SPE) column packed with C-18 reversed phase material (Merck). The extract was then frozen and lyophilized for the analytical HPCCC experiments. A Gynkotek HPLC system with UVD340U, Gynkotek HPLC pump series LPG-3400A, and thermostat (Gynkotek Separations, H.I.Ambacht, TheNetherlands) was used for the chromatographic analysis.For the extract and fraction analysis, a 10 cm × 2.1 mm, 2.7 µm Supelco (18) column was used with system: 6% A in B at 0 min and a gradient to 17% A in B at 15 min (A, methanol; B, 2% formic acid in water). Detection was performed by diode array (DAD. The chromatogram of separated compounds is demonstrated in Fig. 2.

In the HPCCC technique, three polar solvent systems composed of: 1-propanol-acetonitrile-saturated ammonium sulphate-water (v/v/v/v, 1:0.5:1.2:1); ethanol-acetonitrile-1-propanol-saturated ammonium sulphate-water (v/v/v/v/v, 0.5:0.5:0.5:1.2:1) and ethanol--1-butanol-acetonitrile-saturated ammonium sulphate--water (v/v/v/v/v, 0.5:0.5:0.5:1.2:1) were applied. The similar solvent system composed of ethanol--acetonitrile-saturated ammonium sulphate-water (v/v/v/v, 1:0.5:1.2:1) was used to separate and purify betalains by high-speed countercurrent chromatography by Andreas Dagenhardt and Peter Winterhalter [13]. The systems had different polarity where the system II was the most polar and system III was the most non--polar. Individual components of the mixture were separated based on their partitioning properties in applied solvent systems [12]. In the experiment, an HPCCC chromatograph (Dynamic Extractions Ltd., UK) with 18.4 ml capacity, coil length 37 m and i.d. 0.8 mm, running at a flow rate 0.25 ml/min in normal phase mode was used. The sample (0.043 g) was dissolved directly in 1.5 ml of the stationary phase. The column was first entirely filled with the stationary phase. The mobile phase was pumped at a flow rate of 0.25 ml/min in the 'tail to head' direction while rotating the column at 2000 rpm at a constant temperature of 20°C. Then the retention of the stationary phase (S_f) was measured which was 64% (system I), 43% (system II) and 52% (system III). Dissolved in mobile phase the sample was introduced into the coil using a sample injection loop (0.6 ml). The effluent from the outlet of the HPCCC column was monitored using a UV-ViS detector (Gilson) and collected into test tubes with a fraction collector at 8 min intervals.

For the fraction analysis, a 10 cm × 2.1 mm, 2.7 µm Supelco (18) column was used with system: 6% A in B at 0 min and a gradient to 17% A in B at 15 min (A, methanol; B, 2% formic acid in water). Detection was performed by diode array (DAD).

Results and Discussion

During HPLC analysis on reversed phase, the retention times of the decarboxylated and dehydrogenated products are longer in comparison to their corresponding betacyanins due to their lower polarity. In HPCCC, the elution order of betalains is often difficult to predict as it can vary wildly with different solvent systems and run conditions. The present study is a first attempt of the separation of decarboxy- and dehydro-betalains by HPCCC.

The betalains mixture (betalains, decarboxy and dehydro-derivatives) was loaded into the HPCCC apparatus dissolved in the stationary phase and separated using three very polar, high salt-solvent systems in "tail to head" mode.

I. 1-propanol-acetonitrile-saturated ammonium sulphate-water (v/v/v/v, 1:0.5:1.2:1) — S_f = 64% (Table 1);
II. ethanol-acetonitrile-1-propanol-saturated ammonium sulphate-water (v/v/v/v/v, 0.5:0.5:0.5:1.2:1) — S_f = 43% (Table 2);
III. ethanol-1-butanol-acetonitrile-saturated ammonium sulphate-water (v/v/v/v/v, 0.5:0.5:0.5:1.2:1) — S_f = 52% (Table 3).

Stationary phase retention depends on many factors (for example: temperature, density of the solvent system, diameter of the coil, flow rate of the mobile phase, rotation speed of the coil) and influences the efficiency of compound separation. The higher the stationary phase retention, the better the compound separation. Surprisingly, the application of high salt-solvent systems resulted in a not very high stationary phase retention in the HPCCC chromatogaph. This was probably a result of the high density of the solvent systems and the small diameter of the coil. In spite of this, the betalain separations in the applied solvent systems were satisfactory.

Application of the three solvent systems resulted in different chromatographic profiles from that of a preparative HPLC purification. The separation of betalains was carried out at small flow rates in order to enable the HPCCC system to adequately separate

Table 1. Chromatographic profiles of betanin and its decarboxylated and neo-derivatives from red beet (Beta vulgaris L.) separated by HPCCC in system I.

No	Compound	Content of betalains in selected HPCCC fractions as numbered below as analysed by HPLC-DAD (%)																					
		16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	52*	53*	54*	55*
1	Betanin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	50	50
2	17-decarbo-xy-betanin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	70	26	5	–
3	Isobetanin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	41	59
4	17-decar-boxy-isobe-tanin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	53	34	13	–
5	15-decarbo-xy-betanin	–	–	–	–	–	–	–	7	12	15	17	19	16	10	5	–	–	–	–	–	–	–
6	2-decarbo-xy-betanin	–	–	3	15	33	37	11	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	2,17-bide-carboxy-be-tanin	4	25	43	23	5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	Neobetanin	–	–	–	–	–	–	–	–	6	8	8	12	13	14	13	13	10	3	–	–	–	–

* HPCCC fractions obtained during EE process.

Table 2. Chromatographic profiles of betanin and its decarboxylated and neo-derivatives from red beet (Beta vulgaris L.) separated by HPCCC in system II.

No	Compound	Content of betalains in selected HPCCC fractions as numbered below as analysed by HPLC-DAD (%)																		
		12	13	14	15	16	17	18	19	20	21	26	27	28	29	30	31	32	33	34
1	Betanin	–	–	–	–	–	–	–	–	–	–	–	–	–	7	20	31	24	15	–
2	17-decarbo-xy-betanin	–	–	–	–	–	–	–	–	–	–	2	12	24	28	23	10	–	–	–
3	Isobetanin	–	–	–	–	–	–	–	–	–	–	–	–	–	5	14	25	25	22	9
4	17-decarbo-xy-isobeta-nin	–	–	–	–	–	–	–	–	–	–	–	–	14	26	29	22	9	2	–
5	15-decarbo-xy-betanin	–	–	–	–	3	7	17	40	28	6	–	–	–	–	–	–	–	–	–
6	2-decarbo-xy-betanin	3	32	40	15	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	2,17-bide-carboxy-be-tanin	7	48	33	8	4	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	Neobetanin	–	–	–	7	18	19	25	26	3	–	–	–	–	–	–	–	–	–	–

Table 3. Chromatographic profiles of betanin and its decarboxylated and neo-derivatives from red beet (Beta vulgaris L.) separated by HPCCC in system III.

No	Compound	Content of betalains in selected HPCCC fractions as numbered below as analysed by HPLC-DAD (%)																				
		20	21	22	23	24	25	26	27	29	37	38	39	40	41	42	43	44	45	46*	47*	48*
1	Betanin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	11	74	16
2	17-decarbo-xy-betanin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	90	10	–
3	Isobetanin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10	70	20
4	17-decarbo-xy-isobetanin	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	85	15	–
5	15-decarbo-xy-betanin	–	–	–	–	–	–	–	–	–	–	5	7	11	12	16	16	19	16	–	–	–
6	2-decarboxy--betanin	–	–	–	2	12	23	31	24	7	–	–	–	–	–	–	–	–	–	–	–	–
7	2,17-bide-carboxy-be-tanin	3	18	32	31	13	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	Neobetanin	–	–	–	–	–	–	–	–	–	3	6	6	9	11	13	13	29	9	–	–	–

* HPCCC fractions obtained during EE process.

the complex pigment mixtures. The principal, highly polar pigments of red beet root, betanin **(1)** and isobetanin, were more strongly retained than other less polar compounds and were eluted during an "elution extrusion" process in which betanin **(1)** and isobetanin were separated from 17-decarboxy-betanin **(2)** and 17-decarboxy-isobetanin with quite high efficiency. Results observed in system I and III are better than in system II, possibly because of the higher stationary phase retention. 2,17-bidecarboxy-betanin **(6)** eluted first and in all solvent systems its retention time was similar to 2-decarboxy-betanin **(3)**. In system II, these two compounds separated with low efficiency. Moreover, for the first time, the applied HPCCC solvent systems gave a separation of 2-decarboxy-betanin **(3)** from 17- **(2)** and 2,17-bidecarboxy-betanin **(6)** as well as from neobetanin **(5)** and betanin **(1)**. The very small polarity difference between 15-decarboxy-betanin **(4)** and neobetanin **(5)** did not allow these compounds to be completely resolved. However, in system I in fractions 31–33, some small amounts of pure neobetanin **(5)** were observed.

The applied solvent systems differ by polarity and stationary phase retention, therefore, the retention of betalains was significantly different in each case, however, the elution order was similar.

Conclusions

In this study, the separation of betalains and their decarboxy- and dehydro-derivatives was carried out for the first time. The applied solvent systems gave a separation of 2,17-bidecarboxy-betanin from 2-decarboxy-betanin. Moreover, betanin and isobetanin eluted in different fractions to those of 17-decarboxy--betanin and 17-bidecarboxy-isobetanin (systems I and III). The use of system I allows the partial separation of neobetanin from 15-decarboxy-betanin.

In the HPCCC set-up, a relatively low stationary phase retention was achieved; however, in general, the separation results were much better in comparison to earlier CCC experiments.

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