Separation of betalains by gradient high-speed counter-current chromatography

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A study on separation of betalain mixture obtained from red beet juice (*Beta vulgaris* L.) by analytical high-speed counter-current chromatography (HSCCC) was performed. The extract was obtained by thermal treatment of acidified red beet juice for 30 min in 85 °C. The pigment mixture consisted of betanin/isobetanin as well as their decarboxy- and dehydro-derivatives.

The HSCCC process was accomplished in the 'tail to head' mode with two polar solvent systems containing salt: BuOH-EtOH-$NaCl_{solution}$-H_2O-H_3PO_4 (1300:700-1000:1300:700:2.5-5.5 (system I), 1300:200-400:1300:700:2.5-4.5 (system II); v/v/v/v/v). The retention of the stationary phase was 73% (system I) and 79% (system II). The mobile phase was pumped at 2 ml/min flow rate. HPLC-DAD-ESI-MS analyses were performed in reversed phase mode for the obtained HSCCC fractions and crude extract. The solvent systems enabled separation of betanin and decarboxy-betanins (system I and II) as well as neobetanin (system II). Additionally, some pure fractions of 17-decarboxy-betanin and 2,17-bidecarboxy-betanin were obtained in system II.

Keywords: betanin, betalains, betacyanins, high-speed counter-current chromatography, *Beta vulgaris* L.

Introduction

Betalains – betacyanins and betaxanthins – are a group of plant pigments which are present in *Caryophyllaes* family. Interest in betalains is growing due to their colouring, antioxidant as well as potential chemopreventive properties. Betalains are considered as non-toxic and harmless compounds in comparison to artificial dyes [1-2].

Red beet root (*Beta vulgaris* L.) is a popular source of betalains due to its low cost and high betalains concentration. Epimers – betanin and isobetanin – are the main betalains in *Beta vulgaris* L (Figure 1) [3]. The problem with wider application of betalains is associated with their lower stability in some physico-chemical conditions, e.g. higher temperature, light and oxygen atmosphere. The products of betalains degradation are mainly decarboxy- and dehydro-derivatives. Previous studies demonstrated degradation products as more stable compounds [3-5]. HPLC is a dominant technique for analysis and separation of betalains [6], however, irreversible adsorption of sample which may occur in HPLC is always problematic, therefore, application of counter-current chromatography (CCC) with liquid stationary phase creates a possibility of obtaining pure pigments [3, 7].

Dr. Yoichiro Ito invented a completely new liquid chromatography in the late 1960s where the mobile and the stationary phase are two immiscible liquids. The CCC enables creation of different stationary phase through mixing of different solvents without buying a new, expensive column. Moreover, CCC run can be realize in normal or reversed modes without changing column. The most important and time-consuming stage in CCC is selection of suitable solvent systems. The selection is associated with the measurements of the partition coefficients (K_D) of betalains, settling time of two phases as well as retention of the stationary phase (S_f) on the coil column [8-11].

So far, separation of betalains has been realized in two types of solvent systems: highly polar solvent systems containing highly concentrated salt (ammonium sulphate and sodium chloride) as well as solvent systems containing perfluorinated carboxylic acid (heptafluorobutyric acid – HFBA, trifluoroacetic acid – TFA) acting as anionic ion pair reagents. The addition of TFA and HFBA to the CCC systems changes K_D of ionisable betalains shifting them to less polar organic phase. Creation of ion-pairs by betalains enables their separation in less polar solvent systems in CCC [7-8]. Separation of betalains in systems with salts was realized only in high-performance counter-current chromatograph (HPCCC) which generates 240g. High-speed counter-current chromatography (HSCCC) generates 80g. The increase in g-level enable to obtain higher retention of the stationary phase with the same mobile phase flow rates. The retention of the stationary phase is also depended on internal coil diameter. The larger coil diameter is associated with the higher retention of the stationary phase [3, 7].

In this contribution, the research on betalains separation by HSCCC in solvent systems with polarity and pH gradient was described. For this aim, an influence of pH and systems polarity on betalains separation was studied. Moreover, comparison of separation effectiveness of betalains by HSCCC and HPCCC was discussed. The apparatus were differed in g-level and internal column tube diameter.

Experimental

The betalains mixture was obtained by thermal treatment of beetroot juice at 85 °C for 30 min acidified with 0.2% (v/v) formic acid (Table 1). The decarboxy- and dehydrobetalains were purified by solid phase extraction (SPE) using C-18 as a bed in the column. The conditioning of the column was done with acetonitrile and then water. The aqueous-acetonitrilic solution (20:80, v/v) was used for the pigments elution. The fractions were concentrated in a rotary evaporator and then freeze-dried. Compounds in the extract were identified by LC-DAD-ESI-MS according to their reference materials.

The betalains in the extract were analysed by LC-DAD-ESI-MS according to their retention times, Vis absorption maxima λ_{max} (538/538, 505/505, 533/533, 507/507, 488 nm for betanin/-isobetanin, 17-decarboxy-betanin/-isobetanin, 2-decarboxy/isobetanin, 2,17-bidecarboxy-betanin/-isobetanin and neobetanin, respectively) and protonated molecular ions (Table 1, Figures 1-2).

The HSCCC solvent systems were prepared by dissolving sodium chloride (25 g salt in 100 ml of water, 70 °C) as well as addition and mixing with the remaining solvents (Tables 2-4). The settling time as well as K_D were studied for selected solvent systems consisted of BuOH-EtOH-$NaCl_{solution}$-H_2O-H_3PO_4 in a volume ratio: (1300:0-1000:1300:700:0 and 1300:1000:1300:700:0-200, v/v/v/v/v) (Table 2).

The separations were performed on a HSCCC (Quattro chromatograph, AECS-QuikPrep Ltd., Bristol, UK).

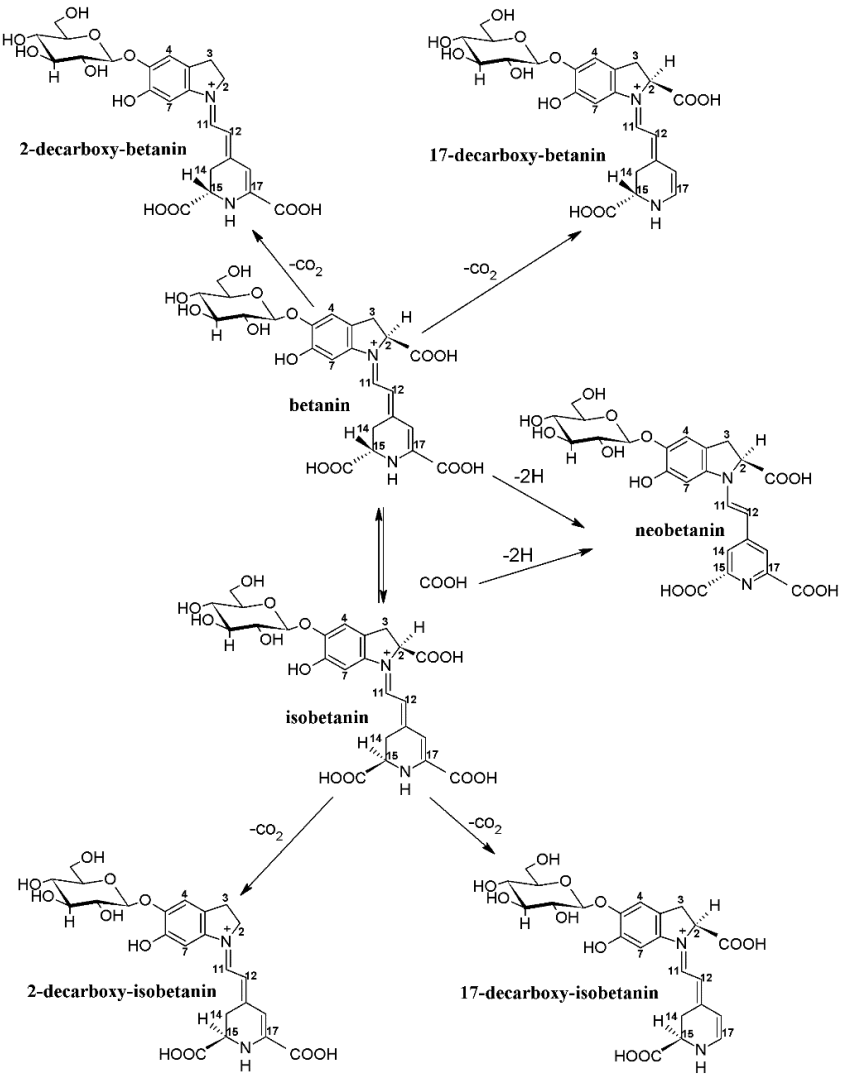

Figure 1. Chemical structures of betanin/isobetanin and its decarboxy-, dehydro-derivatives

Table 1. Chromatographic, spectrophotometric and mass spectrometric data of the analysed pigments

Peak no.	Compound	Abbreviation	R_t [min]	λ_{max} [nm]	*m/z* $[M+H]^+$
1/1'	Betanin/Isobetanin	Bt/IBt	11.2/12.4	538	551
2/2'	17-Decarboxy-betanin/-isobetanin	17-dBt/17-dIBt	12.2/13.5	505	507
3/3'	2-Decarboxy-betanin/-isobetanin	2-dBt/2-dIBt	15.8/15.8	533	507
4/4'	2,17-Bidecarboxy-betanin/-isobetanin	2,17-dBt/2,17-dIBt	17.2/17.2	507	463
5	Neobetanin	NBt	17.5	488	549

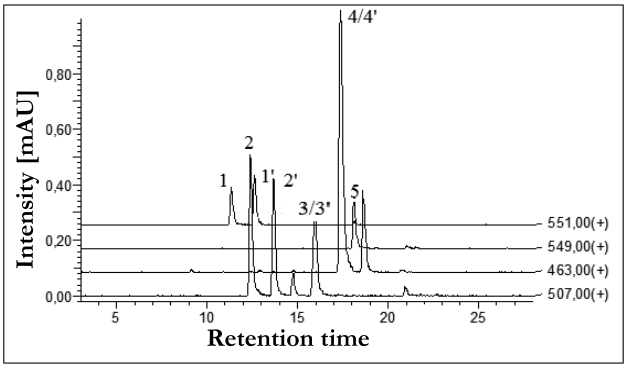

Figure 2. Chromatogram of betalains mixture identified by LC-DAD-ESI-MS

For HSCCC run, the column was first entirely filled with the stationary phase (lower phase). The mobile phase (upper phase) was pumped at a flow rate 2 ml/min in the 'tail-to-head' direction after started rotation of the coil column. The separation was carried out in solvent systems BuOH-EtOH-$NaCl_{solution}$-H_2O-H_3PO_4 (1300:700-1000:1300:700:2.5-5.5 (system I); 1300:200-400:1300:700:2.5-4.5(system II), v/v/v/v/v). The volume of phosphoric acid and ethanol was modified in every run proportionally to the volume of the coil (0.15 V_c, 1.15 V_c, 2.15 V_c, (system I and II) and 3.15 V_c (system II). The fractions were collected into test tubes with a fraction collector at 1.5 min intervals (Tables 3-4).

HPLC-DAD-ESI-MS analyses were carried out using LCMS-8030 (Shimadzu) with Kinetex column (C18 - 100 mm x 4.6 mm x 5 µm) in gradient elution mode at 40°C with 2% aqueous formic acid (A) and methanol (B) system: 95% A and 5% B - 0 min; 80% A and 20% B - 13 min; 70% A and 30% B - 18 min; 40% A and 60% B – 21.9 min. The injection volume was 20 µl and the flow rate was 0.5 ml/min. The ion chromatograms and mass spectra (electrospray voltage 4.5 kV; capillary 250°C; sheath gas: N_2) were recorded by LabSolutions software (Schimadzu). Argon was used to improve trapping efficiency and as the collision gas for CID experiments.

Results and discussion

Shorter settling time in HSCCC is usually related to higher retention of the stationary phase (the percentage of the sta-

Table 2. The settling time of systems with different amount of ethanol and phosphoric acid

System no	Composition [v/v/v/v/v]					Settling time [s]
	$NaCl_{r-r}$ [ml]	H_2O [ml]	EtOH [ml]	BuOH [ml]	H_3PO_4 [ml]	
1	1300	700	**0**	1300	**0**	11.8
2	1300	700	**100**	1300	**0**	10.7
3	1300	700	**200**	1300	**0**	11.4
4	1300	700	**300**	1300	**0**	11.9
5	1300	700	**400**	1300	**0**	12.1
6	1300	700	**500**	1300	**0**	12.3
7	1300	700	**600**	1300	**0**	12.5
8	1300	700	**700**	1300	**0**	13.2
9	1300	700	**800**	1300	**0**	14.4
10	1300	700	**900**	1300	**0**	14.6
11	1300	700	**1000**	1300	**0**	15.2
12	1300	700	**1000**	1300	**2**	16.4
13	1300	700	**1000**	1300	**10**	17.0
14	1300	700	**1000**	1300	**50**	16.1
15	1300	700	**1000**	1300	**100**	18.2
16	1300	700	**1000**	1300	**200**	20.8

Table 3. Composition of the solvent systems (I) and their retention of the stationary phase (S_f) during HSCCC experiments

System no	Composition [v/v/v/v/v]					S_f [%]
	$NaCl_{sol}$ [ml]	H_2O [ml]	EtOH [ml]	BuOH [ml]	H_3PO_4 [ml]	
I	1300	700	**700**	1300	**2.5**	**73**
	1300	700	**800**	1300	**3.5**	
	1300	700	**900**	1300	**4.5**	
	1300	700	**1000**	1300	**5.5**	

Table 4. Composition of the solvent systems (II) and their retention of the stationary phase (S_f) during HSCCC experiments

System no	Composition [v/v/v/v/v]					S_f [%]
	$NaCl_{sol}$ [ml]	H_2O [ml]	EtOH [ml]	BuOH [ml]	H_3PO_4 [ml]	
II	1300	700	**200**	1300	**2.5**	**79**
	1300	700	**250**	1300	**3.0**	
	1300	700	**300**	1300	**3.5**	
	1300	700	**350**	1300	**4.0**	
	1300	700	**400**	1300	**4.5**	

tionary phase left in the column after a separation). The higher retention of the stationary phase the better separation of the compounds [10].

The measurement of the settling time was performed in systems with varying ethanol (0-1000 ml) and phosphoric acid (0-200 ml) amounts (Table 2). Changes of the phosphoric acid amounts influence retention of ionisable betalains which are less polar at lower pH.

The settling time of the solvent systems increased as the ethanol amount increased (0-1000 ml) but insignificantly which result from the presence of high quantity of sodium chloride. Thus, the retention of stationary phase should decrease with the increasing amounts of ethanol in the solvent systems. The salt increases the density differences between the two phases. Previous studies showed that retention of the stationary phase increases with the density difference between the two phases [3] (Table 2, systems 1-11).

In systems containing a constant amount of ethanol (1000 ml) and different amount of phosphoric acid (0-200 ml), the increasing of settling time with increasing of acid was also noticed (Table 2, systems 11-16).

Success in the CCC separation depends on the selection of solvent systems with suitable partition coefficients (K_D) for separated compounds. The studied betalains can be separated only when K_D have different values [10-11].

The studies on K_D were carried out in systems with constant ethanol value (1000 ml) and different amounts of phosphoric acid value (0-50 ml). K_D is a ratio of analyte concentration in the stationary phase and the mobile phase. The experiments demonstrate that K_D decrease with the increasing of phosphoric acid amount in the systems with ethanol. Lower K_D suggest much higher concentration of the analytes in the organic phase.

The system with no acid resulted in high K_D, therefore, this system was not selected for HSCCC run. Systems with 0-50 ml phosphoric acid had lower K_D, thus modifications of these systems were applied in the next HSCCC runs.

Separation of betalains by HSCCC was carried out in gradient systems I and II for better separation of ionisable compounds with similar polarity.

Retention of the stationary phase for system I was significant (S_f = 73%) (ethanol gradient - 700-1000 ml, phos-

Table 5. The partition coefficient of analytes for systems with ethanol (1000 ml) and phosphoric acid (0-50 ml)

System no	Compound						
	Bt	17-dBt	IBt	17-dIBt	2-dBt/2-dIBt	NBt	2,17-dBt/2,17-dIBt
	K_D						
11	91.8	37.4	121	185	34.6	145.9	5.6
12	3.4	1.6	4.3	1.9	1.9	2.5	1.1
13	3.0	0.9	3.6	1.1	2.0	2.2	0.8
14	0.9	0.3	0.9	0.4	0.6	0.3	0.7

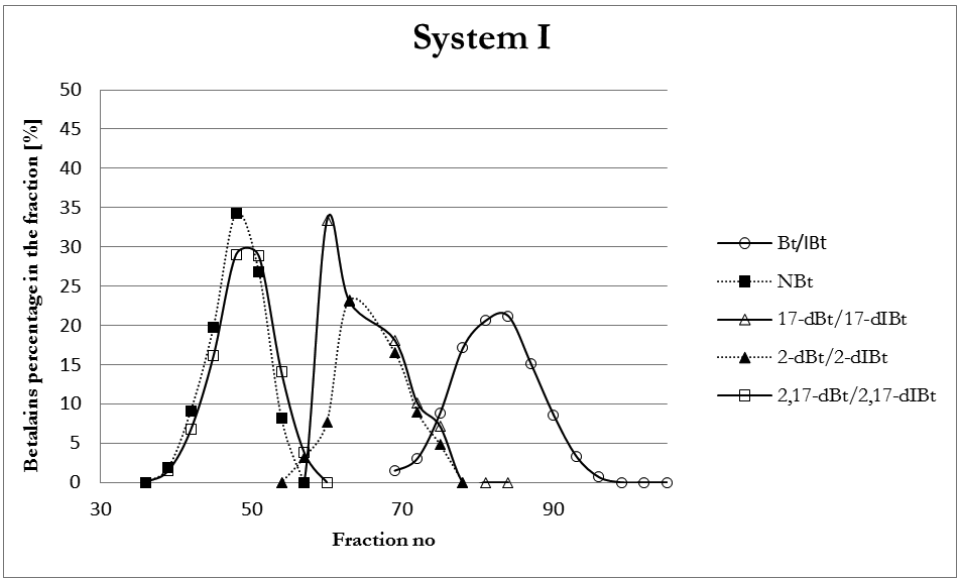

Figure 3. Reconstructed HPLC chromatograms of betalains separated by HSCCC in system I (composition of the solvent systems, see Table 3)

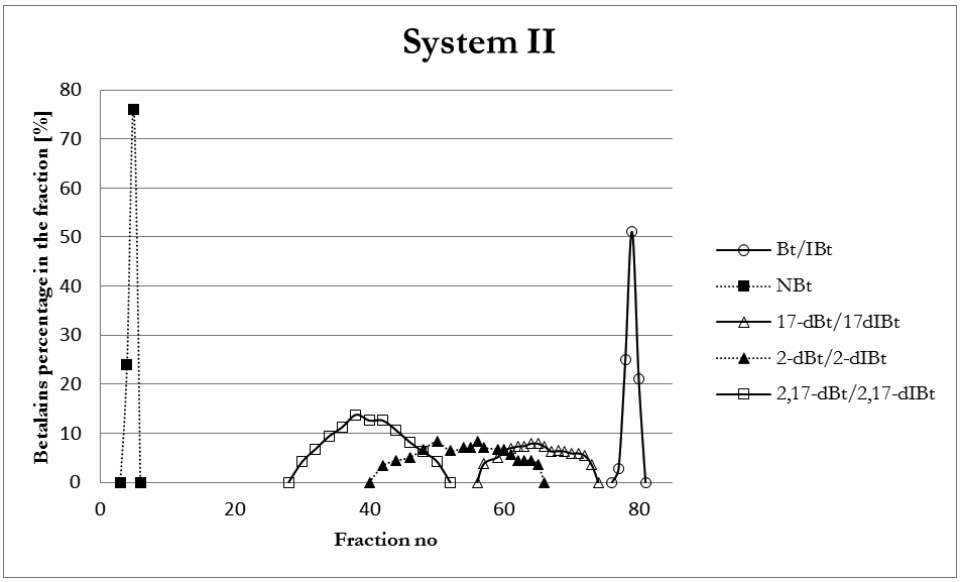

Figure 4. Reconstructed HPLC chromatograms of betalains separated by HSCCC in system II (composition of the solvent systems, see Table 4)

phoric acid gradient - 2.5-5.5 ml) during the chromatographic separation. The elution order for betalains was: NBt, 2,17-dBt/2,17-dIBt, 2-dBt/2-dIBt, 17-dBt/17-dIBt, Bt/IBt. In applied system I, betanin/isobetanin was not co-eluted with the remaining betalains (Figure 3, Table 3).

The highest retention of the stationary phase was observed for system II with lower ethanol (200-400 ml) and phosphoric acid (2.5-4.5 ml) amounts. The elution order for system II was the same as in system I. The applied solvent system enabled separation of NBt and Bt/IBt. Remaining compounds were co-eluted but their resolution was better than in system I. Applied system II enabled obtaining pure fractions of all betalains (Figure 4, Table 4).

Comparison of betalains separation effectiveness in applied system I and II by HSCCC with results obtained during their separation by HPCCC described in previous article [3] demonstrates that the retention of the stationary phases as well as separation effectiveness are similar in different types of machines (HSCCC and HPCCC). The retention of the stationary phases for systems I and II in HPCCC was 75.9 and 85.8% [3] while in HSCCC was 73 and 79%. The different g-levels (240g for HPCCC and 80g for HSCCC) and similar internal coil diameters (1.6 mm – HPCCC and 2.0 mm - HSCCC) suggest that retention of the stationary phase may depends on coil diameter and g-level because it is known that higher g-level guarantees higher retention of the stationary phase.

Conclusions

The results demonstrate that settling time is longer with increasing of acid as well as ethanol amounts in studied systems. Longer settling time is associated with lower retention of the stationary phase. The lower retention of the stationary phase usually results in worst separation of the mixture, therefore, measuring of settling time for new solvent systems is important.

The study on the partition coefficients of betalains showed that application of acid in systems for separation of ionisable compounds is necessary due to modification of their polarity.

The systems: BuOH-EtOH-H_2O-NaCl-H_3PO_4 (1300:700-1000:700:1300:2.5-5.5; 1300:200-400:700:1300:2.5-4.5, v/v/v/ v/v) enable separation of betanin/isobetanin and neobetanin. The retention of the stationary phase as well as separation effectiveness was better for second system with low ethanol and acid gradient. Separation effectiveness of betalains by counter-current chromatography was similar for HSCCC and HPCCC instruments with similar coil diameters (1.6 mm – HPCCC, 2.0 mm - HSCCC) and different g-level (80g and 240g).

Creation of new solvent systems in CCC should be associated with measurement of their settling time because its lower value usually is associated with higher retention of the stationary phase as well as better separation effectiveness.

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