

Research on solvent systems with tetra-*n*-butylammonium bromide for counter-current chromatography of betalains

Aneta Spórna-Kucab, Jolanta Wysoczańska, Anna Bartel,
Tomasz Świergosz, Dominika Szot, Sławomir Wybraniec

Cracow University of Technology, Department of Analytical Chemistry,
Faculty of Chemical Engineering and Technology,
Warszawska 24, 31-155 Cracow, Poland
e-mail: anetasporna@chemia.pk.edu.pl

In this study, new two-phase solvent systems for counter-current chromatography (CCC) consisting of *n*-butanol and water as well as various amounts of acetic acid, acetonitrile, ethanol, acetone or ethyl acetate were tested. Additionally, tetra-*n*-butylammonium bromide (TBAB) was introduced into the system in the form of aqueous solutions or phosphate-citrate buffer (pH 6.7) in order to form ion-pairs with betalains. The selection of buffer pH was based on their ability to create ion pairs by tetraalkylammonium salts, with selected betalains under these conditions.

In this study, it is shown that the settling time of two phases is longer with the increase of acetic acid/acetonitrile/ethanol/acetone/ethyl acetate. For selected solvent systems with high amounts of acetonitrile, ethanol and acetone two phases were not observed. The systems with acetone have the largest increase of settling time. Ethyl acetate systems were characterized by a slow settling time increase.

In systems containing additionally 2% aqueous TBAB, smaller changes in settling time than in similar systems without TBAB were observed. Addition of TBAB in the buffer resulted in a prolongation of settling time.

Solvent systems in which the separation between the aqueous and organic phases was visually best, were selected from among all the tested systems and the betalain partition coefficients were measured by LC-DAD-ESI-MS. The best results were observed for systems: *n*-butanol-water-acetic acid (2:2.5:0.75, v/v/v), *n*-butanol-water-acetic acid (2:2.5:1, v/v/v) and *n*-butanol-TBAB in water-acetonitrile (2:2.5:0.5, v/v/v).

Keywords: betanin; betalains, tetra-*n*-butylammonium bromide, high-speed counter-current chromatography, *Beta vulgaris* L.

Introduction

Betalains are a group of red and yellow water soluble pigments which provide the colours in a wide variety of fruits, vegetables and flowers. Colouring, antioxidant as well as potentially chemopreventive properties contributed to the growth of interest in betalains [1]. In contrary to the artificial dyes, betalains are considered as non-toxic and harmless. Betalains are extracted on a larger scale from beet roots (*Beta vulgaris* L.) which are considered as their cheap and rich source. The main betalains in *Beta vulgaris* L. are be-

tanin and isobetanin (Figure 1), which are epimers [2]. Betalains have low stability in some physicochemical conditions (e.g. higher temperature) in which their transformation to decarboxy- and dehydro-betalains is observed [3].

Previous studies demonstrated higher stability of the transformation products, therefore, further experiments on these compounds will give more information about their applicability as food colorants [3-4].

Preparative isolation of betalains by liquid chromatography on solid stationary phases is always problematic due to their catalytic action in the pigment degradation [1-2,

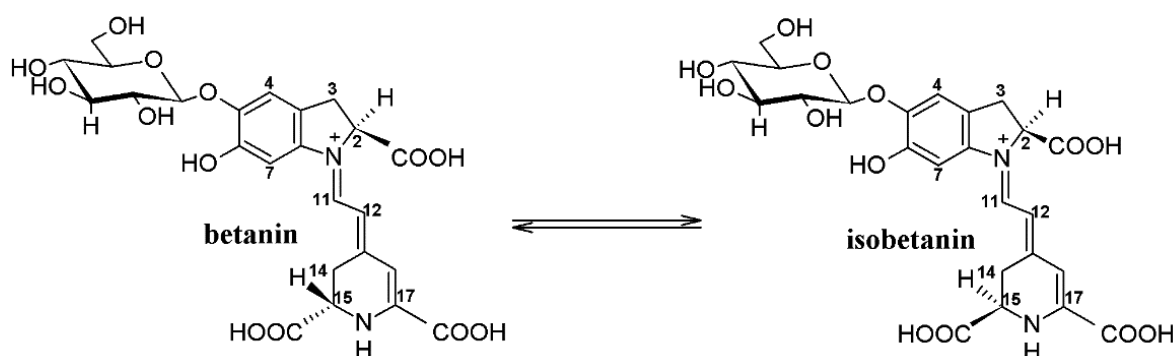


Figure 1. Chemical structures of betanin/isobetanin

5-6]. In addition, application of liquid stationary phase eliminates the problem of irreversible adsorption occurring in HPLC [1-2, 7].

Counter-current chromatography (CCC) is a liquid chromatography technique in which mobile and stationary phases are two immiscible liquids. CCC enables the use of different stationary phases through the application of different solvent systems without buying new, expensive columns. Furthermore, working in normal or reversed modes can be realized even in one run without changing solvent systems. The selection of the solvent systems for CCC is associated with the measurements of the partition coefficient (K_D) of betalains, settling time of two phases as well as retention of the stationary phase (S_f) [7-9].

So far, separation of betalains by CCC has been realized in two types of solvent systems: highly polar solvent systems containing highly concentrated salt as well as solvent systems containing perfluorinated carboxylic acid acting as anionic ion pair reagents [1-2, 5-6, 10-12]. The addition of the perfluorocarboxylic acid (heptafluorobutyric acid – HFBA or trifluoroacetic acid – TFA) to the CCC systems changes K_D of ionisable betalains shifting them to less polar, organic phase. The formation of ion-pairs with betalains enables their separation in less polar solvent systems by CCC [1-2]. The selection of the solvent systems was associated with the betalain profiles.

In this contribution, research on settling time of the solvent phases as well as betalains partition coefficients in completely new solvent systems with/without tetra-n-butylammonium bromide (TBAB) are presented. TBAB has never been applied in counter-current chromatography.

Experimental

The mixture of betalains was obtained by thermal treatment of beetroot juice in 85 °C for 30 min acidified with 0.2% (v/v) formic acid (Table 1, Figure 2).

Betalains with different decarboxylation and dehydrogenation levels were purified by solid phase extraction (SPE) using C-18 as a stationary phase activated with acetonitrile and water. The aqueous-acetonitrilic solution (20:80, v/v) was used to elute betalains from the column. The eluates were concentrated by a rotary evaporator and then freeze-dried. The betalains in the extract were analysed by LC-DAD-ESI-MS according to their retention times, Vis absorption maxima λ_{max} (538, 505, 533, 507, 488

nm for betanin/isobetanin, 17-decarboxy-betanin/-isobetanin, 2-decarboxy-betanin/-isobetanin, 2,17-bidecarboxy-betanin/-isobetanin and neobetainin, respectively) and protonated molecular ions (m/z 551, 507, 507, 463, 549) (Table 1, Figure 2) reported in the previous publication [2]. Pigments differed in their degree of degradation as well as polarity, therefore, more polar betanin/isobetanin were eluted earlier in reversed chromatography than their derivatives.

The CCC systems: BuOH-H₂O (2:2.5; v/v) with different volumes of acetic acid (AA)/acetonitrile (ACN)/ethanol (EtOH)/acetone (AC)/ethyl acetate (EA) without TBAB as well as with 2% TBAB in water or 2% TBAB in phosphate-citrate buffer (pH 6.7) were prepared (Table 2). The phosphate-citrate buffer (pH 6.7) was prepared by mixing 0.2 M sodium dihydrogen phosphate 12-hydrate (Na₂HPO₄ · 12H₂O) with 0.1 M citric acid.

The settling time of system phases was examined through shaking of two phase solvent systems in vials, then observing the separation of the two phases while measuring the time of the process with a stopwatch.

The partition coefficient was measured for selected solvent systems by determination of an analyte concentration ratio in aqueous and organic phases.

LC-DAD-ESI-MS analyses of betalain mixture (Figure 2) as well as their partition coefficient in selected solvent systems (Table 2) were carried out using LCMS-8030 apparatus (Shimadzu, Kyoto, Japan) equipped with Kinetex column (C₁₈, 100 mm x 4.6 mm x 5 μm) and diode-array detector (DAD) in a gradient elution mode at 40 °C with 2% aqueous formic acid (A) and methanol (B) system:

– 95% A and 5% B - 0 min;

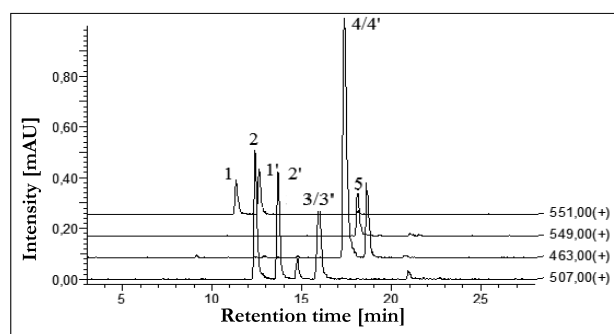


Figure 2. Chromatogram of betalains mixture identified by LC-ESI-MS

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	12.2/12.5	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	Neobetainin	NBt	17.5	488	549

- 80% A and 20% B - 13 min;
- 70% A and 30% B - 18 min;
- 40% A and 60% B – 21.9 min;
- 95% A and 5% B – 22 min;
- 95% A and 5% B - 28 min;

The injection volume was 20 μ l and the flow rate was 0.5 ml/min (LC-DAD and LC-DAD-ESI-MS systems).

Results and discussion

The partition coefficient (K_D) in CCC is the ratio of analyte concentration in the stationary phase and the mobile phase. K_D determination was based on measurements of analyte concentration ratio in aqueous and organic phases in selected solvent systems described in Table 2.

The longer settling time the longer required mixing and separation time of the phases in a chromatographic column. The longer separation time of the phases decreases the retention of the stationary phase (the fraction of stationary phase kept in a column during the CCC run) because the stationary phase is grabbed by the mobile phase. Settling time for CCC solvent systems should be lower than 1 min [7, 12].

In this research, two solvent systems consisted of n-butanol and water as well as different amounts of acetic acid, acetonitrile, ethanol, acetone, ethyl acetate (Table 2) were used in settling time and the partition coefficient measurements. Furthermore, an influence of TBAB concentration in systems with water or phosphate-citrate buffer (pH 6.7) on settling time and the betalain partition coefficient was tested. The selection of pH was associated with ion-pairs creation in these conditions according to literature [13].

In systems containing n-butanol-water and the additional solvents such as acetic acid, acetonitrile, ethanol, acetone or ethyl acetate (Table 2, systems 1-5), a tendency towards longer settling time with increasing amounts of added component was observed. For systems with acetonitrile, ethanol, acetone, no phase separation was observed in samples no. 5 with 1 ml of solvent. The upward tendency in the settling time is not a linear one. Systems with acetic acid, acetonitrile and acetone have much greater increase of phase separation time, than the ethyl acetate system which is characterized by a slow time increase. The sample 3 containing ethanol shows a significantly greater settling time than the sample 4 which is an unexpected deviation (Figure 3a, Table 2, systems 1-5).

In systems containing n-butanol-water and the additional component such as acetic acid, acetonitrile, ethanol, acetone or ethyl acetate with 2% aqueous TBAB (Table 2, systems 6-10, Figure 3b), the lower values of changes in settling time than in the systems of 1-5 (Table 2, systems 1-5, Figure 3a) were observed. The exception is the system comprising ethanol, the sample 9 differs significantly from the rest of the samples. Initially, the phase separation time decreases (Figure 3b, systems 6 and 7) and then, with an increase in the volume of components such as acetic acid, acetonitrile, ethanol, acetone or ethyl acetate, the settling time increases. In the cases of 9 with ethanol and acetone as well as 10 with acetic acid, acetonitrile, ethanol and acetone, a lack of two phases was observed, and no separation occurred (Figure 3b, Table 2).

In systems containing n-butanol-water and the additional component such as acetic acid, acetonitrile, ethanol, acetone or ethyl acetate with 2% solution of TBAB in

Table 2. The composition of the systems applied in determination of the partition coefficient and two phases settling time

Solvent system	Solvent				
	BuOH [ml]	H ₂ O [ml]	AA/ACN/EtOH/AC/EA [ml]	2% TBAB (water) [ml]	2% TBAB (buffer) [ml]
1	2	2.5	0	0	0
2	2	2.5	0.25	0	0
3	2	2.5	0.5	0	0
4	2	2.5	0.75	0	0
5	2	2.5	1	0	0
6	2	0	0	2.5	0
7	2	0	0.25	2.5	0
8	2	0	0.5	2.5	0
9	2	0	0.75	2.5	0
10	2	0	1	2.5	0
11	2	0	0	0	2.5
12	2	0	0.25	0	2.5
13	2	0	0.5	0	2.5
14	2	0	0.75	0	2.5
15	2	0	1	0	2.5

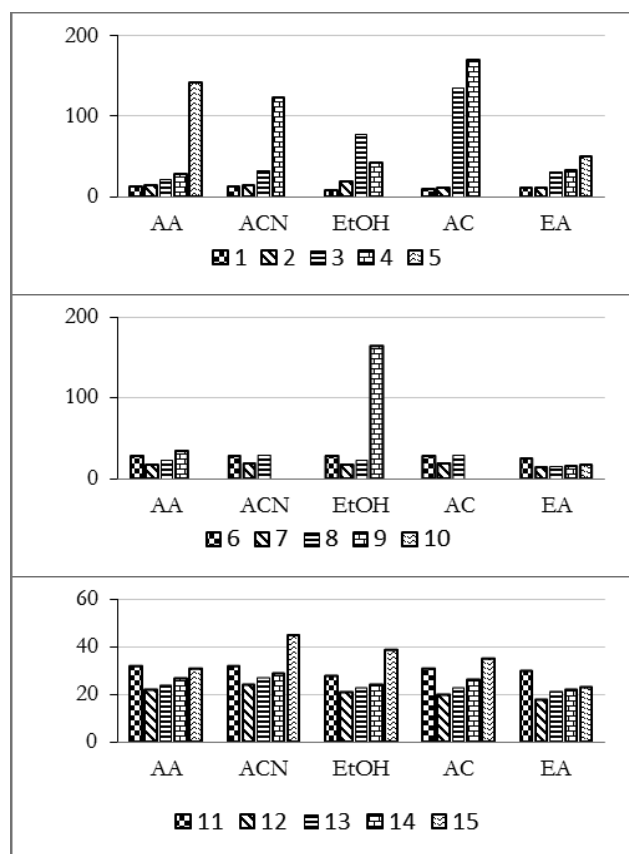


Figure 3. Settling time for solvent systems with different volume of acetic acid/acetonitrile/ethanol/acetone/ethyl acetate (see Table 2)

phosphate-citrate buffer pH 6.7 (Table 2, systems 11-15, Figure 3c) there are some noticeable similarities to systems 6-10 (Figure 3b). Initially, the phase separation time decreases (Figure 3c, system 12), and then rises in the following cases (Figure 3c, systems 13-15).

The partition coefficient (K_D) is a ratio of concentrations of substances in a mixture of two immiscible phases at equilibrium. This study investigated the ratio of betalains concentration in the aqueous phase (stationary phase) to the organic phase (mobile phase). Systems in which the separation between the aqueous and organic phase was evident and the coefficients were visually best (colour observation of the upper and lower phase), were selected among solvent systems prepared according to the Table 2. Long settling time of two phases eliminates them from the application in the CCC. The samples of the upper and lower phases of each selected system were collected into 2 ml vials, followed by their freeze-drying. Further analyses were performed with the HPLC-DAD-ESI-MS. The partition coefficients for selected solvent systems are collected in Table 3.

Among solvents systems with acetic acid, the best partition coefficient characterized systems 4 and 5. For the sample 4, which the phase separation time was short, different values of the partition coefficients of betalains were obtained. Betanin and isobetanin had a high value of partition coefficient, but this is not a problem, as these compounds may be eluted during extrusion elution (by pushing

Table 3. Partition coefficients for selected solvent systems with different volume of acetic acid/acetonitrile/ethanol/acetone/ethyl acetate (see Table 2)

System	Compound						
	Bt	17-dBt	IBt	17-dIBt	2-dBt/2-dIBt	2,17-dBt/2,17-dIBt	NBt
AA	Partition coefficient (K_D)						
4	43.1	4.3	38.5	2.9	2.8	2.3	3.5
5	1.7	1.3	2.5	1.3	1.1	1.0	1.4
9	8.6	0.2	7.1	0.2	2.6	0.2	0.1
14	72.1	25.3	94.6	5.7	33.9	0.5	1.3
15	77.2	20.1	89.9	8.1	6.3	0.5	2.6
ACN	Partition coefficient (K_D)						
4	8.6	3.2	13.2	3.8	2.8	1.2	1.5
8	3.2	1.6	2.6	1.4	3.2	1.4	0.4
14	52.4	47.4	120.8	15.8	157.6	9.3	0.1
15	72.6	372.1	115.9	315.4	160.2	4.5	0.1
EtOH	Partition coefficient (K_D)						
4	38.7	11.1	39.6	12.0	8.1	2.4	3.0
9	1.2	1.5	1.7	1.4	1.9	1.3	0.5
14	48.2	24.8	88.1	14.3	181.2	12.3	0.1
15	64.6	92.3	102.9	28.3	196.5	4.2	0.1
AC	Partition coefficient (K_D)						
4	9.2	3.8	9.1	4.9	3.9	1.6	1.9
8	4.4	6.9	5.6	7.9	6.7	3.7	0.9
14	47.1	380.9	120.9	324.5	192.6	436.4	0.1
15	55.4	11.7	38.7	7.5	0.6	1.4	0.1
EA	Partition coefficient (K_D)						
9	16.7	2.4	17.4	2.2	1.4	13.8	1.8
14	27.7	144.8	82.6	63.3	187.1	20.0	0.1

the stationary phase of the column). The sample 5 with acetic acid had low K_D values, allowing shorter time of the analytes separation in the CCC. The sample 9 with acetic acid was eliminated because of too low value of the partition coefficient for the 17-dBt, 17-dIBt, 2,17-dBt/2,17-dIBt and NBt. Samples 14 and 15 with acetic acid had high K_D value, resulting in a long time separation by CCC (Table 3).

For solvent systems with acetonitrile, partition coefficients were analyzed for 4, 8, 14-15. The sample 4 does not meet the requirements because of too long settling time and was eliminated. The sample 8 is characterized by low K_D values for individual substances and the short settling time, which allows to consider it as potentially a good system for separation of betalains by CCC. Samples 14-15 have too high partition coefficient, which is an adverse factor and excludes their use for further analysis (Table 3).

For solvent systems with ethanol, partition coefficient were analyzed for 4, 9, 14-15. Samples 4 and 14-15 are not suitable for use in further analysis, due to the high value of the partition coefficient, which is quite adverse factor. Although the sample 9 has low K_D values, has also been eliminated due to the long phase separation time (Table 3).

Among partition coefficients for solvent systems with acetone, the sample 4 has adequate partition coefficient, but too long settling time which does not allow it to be used in the analysis by CCC. Subsequently tested samples 8, 14-15 had a K_D values too high which also eliminates them (Table 3).

Based on visual evaluation of solutions, two solvent systems with ethyl acetate were selected in which the partition coefficient was examined. The values obtained for samples 9 and 14 were different for individual compounds, but generally were too high and these systems were rejected (Table 3).

Conclusion

This study demonstrates completely innovative solvent systems consisting of n-butanol and water which contain various amounts of acetic acid, acetonitrile, ethanol, acetone and ethyl acetate. Tetra-n-butylammonium bromide (TBAB) was introduced into the selected systems in the form of aqueous solutions or phosphate-citrate buffer (pH 6.7). The selection of buffer pH was based on the ability to create ion pairs by tetraalkylammonium salts with selected betalains under these conditions. TBAB serves as an ion-pair reagent and complexes charged molecules at different positions of betacyanins and their decarboxy-derivatives, changing them to more hydrophobic structures so that the partition coefficient value is improved.

The settling time of two phases is longer with the increase of acetic acid, acetonitrile, ethanol, acetone, and ethyl acetate concentration. The highest increase of settling time was noticed in solvents with acetone. The ethyl acetate had the lowest change of the settling time. The introduction of the highest amount of acetonitrile, ethanol and acetone contributes to a formation of one phase system.

Comparison of systems with TBAB shows that systems with TBAB in water are characterized by slight settling time change during introduction of acetic acid, acetonitrile, ethanol, acetone and ethyl acetate.

Research of the partition coefficient for visually selected systems enabled selection of the best solvent systems with low partition coefficient value and short settling time: n-butanol-water-acetic acid (2:2.5:0.75; v/v/v), n-butanol-water-acetic acid (2:2.5:1; v/v/v) and n-butanol-TBAB in water-acetonitrile (2:2.5:0.5; v/v/v).

References

- [1] Spórna-Kucab, A., Ignatova, S., Garrard, I., Wybraniec, S. "Versatile solvent systems for the separation of betalains from processed *Beta vulgaris* L. juice using counter-current chromatography". *Journal of chromatography B*, 941 (2013): 54-61.
- [2] Spórna-Kucab, A., Garrard, I., Ignatova, S., Wybraniec, S. „New solvent systems for gradient counter-current chromatography in separation of betanin and its derivatives from processed *Beta vulgaris* L". juice. *Journal of Chromatography A*, 1380, (2015):29-37.
- [3] Wybraniec, S. "A method for identification of diastereomers of 2-decarboxy-betacyanins and 2,17-bidecarboxy-betacyanins in reversed phase HPLC". *Analytical and Bioanalytical Chemistry*, 389, (2007): 1611-1621.
- [4] Strack, D., Vogt, T., Schliemann, W. "Recent advances in betalain research". *Phytochemistry*, 62, (2003): 247-269.
- [5] Wybraniec, S., Stalica, P., Jerz, G., Klose, B., Gebers, N., Winterhalter, P., Spórna, A., Szaleniec, M., Mizrahi Y. "Separation of polar betalain pigments from cacti fruits of *Hylocereus polyrhizus* by ion-pair high-speed countercurrent chromatography". *Journal of Chromatography A*, 1216, (2009): 6890-6899.
- [6] Jerz, G., Skotzki, T., Fiege, K., Winterhalter, P., Wybraniec, S. "Separation of betalains from berries of *Phytolacca americana* by ion-pair high-speed counter-current chromatography". *Journal of Chromatography A*, 1190, (2008): 63-73.
- [7] Berthod, A. "Countercurrent chromatography". *Elsevier*, Amsterdam, (2002): 1-37.
- [8] Ito, Y., Conway, W., D. "High-speed countercurrent chromatography". *Chemical Analysis*, tom 132, (1993): 3-44.
- [9] Degenhardt, A., Winterhalter, P. "Isolation of natural pigments by high speed CCC". *Journal of Liquid Chromatography and Related Technologies*, 24, (2001): 1745-1764.
- [10] Strack, D., Steglich, W., Wray, W. *Methods in plant biochemistry*, the University Press, Cambridge, 11, (1993): 422-447.
- [11] Jerz, G., Skotzki, T., Fiege, K., Winterhalter, P., Wybraniec, S. "Separation of betalains from berries of *Phytolacca americana* by ion-pair high-speed counter-current chromatography". *Journal of Chromatography A*, 1190, (2008): 63-73.
- [12] Ito, Y. "Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography". *Journal of Chromatography A*, 1065, (2005): 145-168.
- [13] Wybraniec, S. "Effect of tetraalkylammonium salts on retention of betacyanins and decarboxylated betacyanins in ion-pair reversed-phase high-performance liquid chromatography". *Journal of Chromatography A*, 1127, (2006), 70-75.

Received: 8 November 2015

Received in revised form: 10 February 2016

Accepted: 28 February 2016