Spiral-coil countercurrent chromatographic separation of betanin and betanidin ethyl-esters monitored by continuous ESI-MS/MS injection and LC-ESI-MS/MS

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Betalains present in *Beta vulgaris* L. (Chenopodiaceae) have a high potential as natural pigments for food applications. They have been used as a substitute of synthetic dyes in processing of gelatine, strawberry yogurts, ice creams, fruits cocktails, candies and biscuits. The stability of betalains is strongly influenced by numerous factors – sugar content, and impact of light, oxygen, water activity, pH and temperature. A mixture of betacyanin mono-, di- and tri-ethyl-esters was separated by a large-volume *spiral-coil countercurrent chromatography* (*spCCC*) prototype which yielded fractions continuously *off*-line injected to an ESI-MS/MS device. This method yielded a reconstituted pigment profile and finally enabled the MS-*target-guided* isolation procedure for the whole s*pCCC*-experiment. The individual fractions were analyzed by LC/ESI-MS/MS. Betanin and betanidin mono-, di- and tri-ethyl-esters as well as other decarboxylated derivatives were detected in the chromatograms. The ethyl-esters of betanin were only partially fractionated, but clearly fractionated from the ethyl-esters of betanidin which will be of great value for biological evaluations.

Key words: *spiral-coil countercurrent chromatography* (*spCCC*), betacyanin ethyl-esters, betalains, betacyanins, betanin, betanidin

Introduction

Betalains from *Beta vulgaris* L. (Chenopodiaceae) have a high potential as natural pigments for food applications. Currently, this pigment class is intensively investigated in respect of stability in food systems. Quite popular is the use of red beet extracts in dairy products such as yoghurts, and ice-creams. So far, there are no issues of toxicity using these natural pigments in food – so they seem to be a very good alternative to replace synthetic dyes which had been frequently discussed for negative side-effects to human health.

Several methods for isolation and purification of betalains have been reported [1,2] including ion exchange chromatography, electrophoresis, HPLC and TLC [3, 4].For the first time, betalain pigments were isolated by *countercurrent chromatography* (CCC), in 2008 [5] *Countercurrent chromatography* is liquid chromatography (LC) technique that uses two immiscible liquid phases without any solid support [6]. One phase (stationary phase) is retained in the column by the planetary movement of the column, while the other phase (mobile phase) is pumped into the column. The sample is separated according to the different distribution coefficients [7].

The first preparative fractionation of betacyanins by means of *ion-pair high-speed countercurrent chromatography* (IP-HSCCC) using the *elution-extrusion* (EE) approach for a complete pigment recovery, was described [5, 8, 9]. Authors used solvent system consisting of 1-butanol-acetonitrile-water with the addition ion-pair forming trifluoroacetic acid (TFA), as well as heptafluorobutyric acid (HFBA). However, these acids are toxic, therefore, in this study, the preparative fractionation of new groups of betacyanin ethyl-esters by the *spiral-coil countercurrent chromatography* (*spCCC*), using the similar solvent system, but containing no toxic acids was performed. It was possible, because the three carboxylic groups of the betanin pigment backbone were the target functional groups for the derivatization. This way, the polarity of the betacyanin ethyl-esters was reduced. However, the polarity of natural betalains is significant and requires the use of ion-pair reagents or other homologue fluorinated additives under standard separation conditions on the *spCCC.* Betalains containing ethyl-ester groups had not been analyzed by modern chromatographic techniques before, therefore, were also not separated by the *spiral-coil countercurrent chromatography*.

The *spiral-coil countercurrent chromatography* (Fig. 1) is a type of CCC separation for large preparative quantities (hundreds of grams) and it can easily be scaled up for in-

Fig. 1. Spiral-coil countercurrent chromatography (spCCC) device

dustrial production through volume enlargement of the convoluted multilayer coil. It is commonly operated at a lower rotation speed (less than 200 rpm) and longer separation time [10].

Experimental

The mixture of violet betacyanin ethyl-esters (15 g) was synthesized by esterification of betanin/isobetanin from red beet root (*Beta vulgaris* L.) with ethanolic hydrogen chloride. The separation of the freeze-dried pigment extract was performed by a large-volume *spCCC*-prototype equipped with a single coil bobbin with PTFE (polytetrafluoroethylene) tubing (8 mm ID). The total *spCCC* volume was 5.7 L (Fig. 1). The solvent was pumped with a preparative HPLC pump (Knauer, Berlin, Germany) and the effluent was monitored with a Vis-detector.

The *spCCC* system was operated in the '*head-to-tail*' mode applying biphasic solvent system TBMe – ACN – H_2O at volume ratio of 2:2:3 (v/v/v). This solvent system was thoroughly equilibrated in a separatory funnel, following which the upper and lower phases were separated and degasser in an ultrasonic bath for 15 min. The column was filled with upper stationary phase at a flow rate of 50 mL/min. Then 120 mL of sample (15 g betacyanins ethyl-esters extract dissolved in 1:1 mixture of lower and upper phases) was injected into the separation column, and then the instrument *spCCC* was revolved at 280 rpm while the lower mobile phase was pumped in at a flow rate of 15 mL/min.

Analysis of CCC fractions were performed by C18-HPLC-DAD (Jasco, Germany) and LC-ESI-MS/MS in the positive ionization mode (HCT-Ultra ETD II, Bruker Daltonics, Germany). For the chromatographic analysis, a column Luna C18(2), 250 x 4.6 mm (Phenomenex, Torrance, CA, USA), particle diameter 5 µm, was used. The injection volume was 10 µL, and the flow rate was 0.5 mL/min. For the separation of the analytes, the following gradient system was used: 10% A in B at 0 min; gradient to 40% A in B at 30 min (A – acetonitrile, 1% formic acid in water). The column was thermostated at 35 °C.

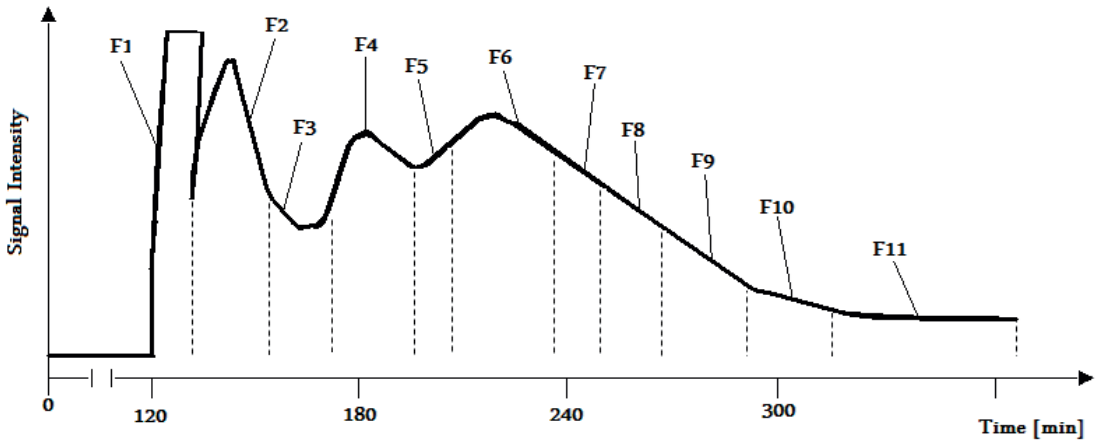

Fig. 2. Spiral-coil CCC chromatogram of betacyanin ethyl-esters (15 g) in the solvent system TBME-ACN-H_2O 2:2:3 (v/v/v); spCCC conditions: flow rate: 15 mL/min, CCC-operation: head-to-tail mode, velocity: 280 rpm and detection wavelength λ 540 nm

Results and Discussion

Fig. 2 shows the *spiral-coil countercurrent chromatogram* of a new group of betalains such as betacyanins ethyl-esters with a solvent system composed of *tert.*-butyl methyl ether/acetonitrile/water at volume ratio of 2:2:3 (v/v/v). The effluent was monitored with a UV-detector at 540 nm. As shown in the chromatogram, as a result of the fractionation of crude pigments extract, 10 fractions from the *elution*-mode were obtained (Fig. 2). In the *extrusion*-mode, the solvent phases in the coil column were ejected with nitrogen gas, and a quite good stationary phase 68% retention was calculated.

After fractionation of betacyanin ethyl-esters, every fourth collected tube of the fraction collector was in sequence *off*-line injected to an ESI-MS/MS device (Bruker HCT Ultra ETD II, Bruker Daltonics, Germany, ion trap-MS/MS). The elution orders and co-elution effects of minor and major concentrated ethyl-ester pigments were clearly monitored by selected ion-traces (pos. mode, scan: *m/z* 100-1500, $[M+H]^+$). This method yielded a reconstituted molecular weight pigment profiles, and finally enabled the MS-*target-guided* isolation procedure for the whole *spCCC*-experiment (Fig. 3). Compounds of the following *m/z:* 579; 607; 635 (mono-, di- and tri-ethyl-esters of betanin, respectively) and 445; 473 (di- and tri-ethyl-esters of betanidin, respectively), and other decarboxylated derivatives were detected in the chromatograms. Mono-, di-, and tri-ethyl-esters of betanin were partly fractionated, but clearly fractionated from di- and tri-ethyl-esters of betanidin.

Using of a combination of two chromatographic techniques preparative *spCCC* and analytical C18-HPLC (DAD and MS/MS detection) was a very effective resolution for pigment analysis and identified new compounds of betacyanin ethyl-esters in the separated CCC fractions (Table 1). In Fig. 4, the C18-HPLC chromatogram of the crude betacyanin ethyl-esters extract at λ 540 nm was depicted.

The chromatograms of individual fractions and Table 1 show mono- (**2**, **3**, **5**), di-(**9**, **10**, **15**, **16**), and tri-ethyl-esters of betanin (**24**/**24'**) (*m/z:* 635, 607, 579, respectively) and mono-(**8**), di-(**18**, **19**, **25**), and tri-ethyl-esters of betanidin (**29**/**29'**) (*m/z:* 417, 445, 473, respectively), as well as their isoforms. The other new betalains reported for the

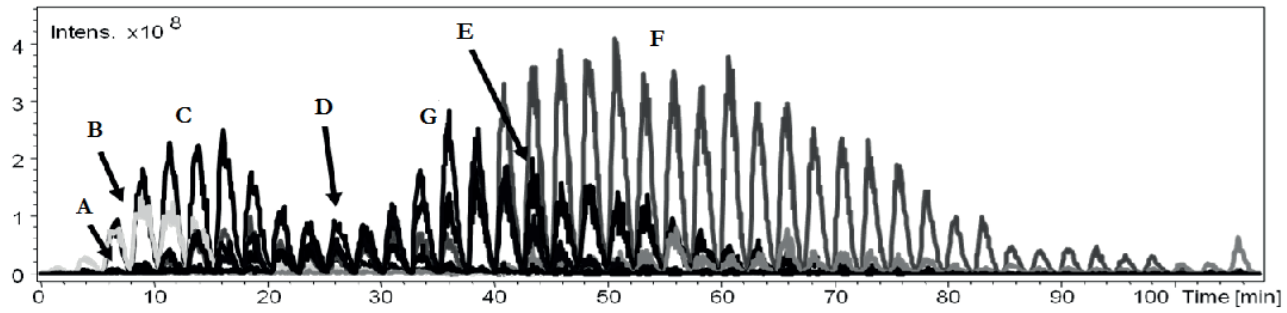

Fig. 3. Elution order of betacyanin ethyl-esters of the spiral-coil CCC run monitored by continuous injections to ESI-MS. Selected ion traces of visualized the separation and co-elution effects of mono- (A: m/z 579), di- (B: m/z 607), tri- (C: m/z 635) ethyl esters of betanin, mono- (D: m/z 417), di- (E: m/z 445), tri- (F: m/z 473) ethyl esters of decarboxy-betanins, and mono-decarboxy betanidin di-ethyl ester (G: m/z 401). Each injection in the reconstituted ESI-MS trace is equivalent to a CCC-elution volume of 90 mL

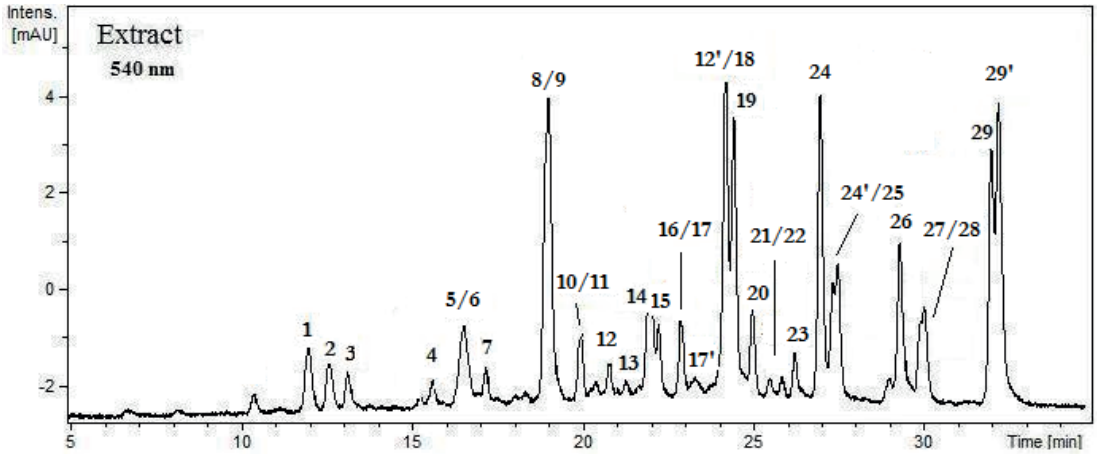

Fig. 4. C18-HPLC profiles of betanin and betanidin ethyl-esters betacyanins analyzed in crude extract of betacyanin ethyl-esters

Table 1. C18-HPLC-chromatographic and ESI-mass spectrometric data of the analyzed betanin- and betanidin ethyl-esters, as well as their decarboxylated derivatives found in the spCCC fractions

Peak no.	Compound [a]	R_f [min]	m/z $[M+H]^+$	Fraction no.
1	n.d.	12.0	222	2-3
2	betanin/isobetanin mono-ethyl ester	12.6	579	2-4
3	betanin/isobetanin mono-ethyl ester	13.2	579	2-4
4	mono-decarboxy-betanin/isobetanin mono-ethyl ester	15.3	535	2-3
5	betanin/isobetanin mono-ethyl ester	15.6	579	2
6	mono-decarboxy-betanin/isobetanin mono-ethyl ester	15.6	535	2-3
7	mono-decarboxy-betanin/isobetanin mono-ethyl ester	16.6	535	2-3
8	betanidin mono-ethyl ester	19.0	417	1-7
9	betanin/isobetanin di-ethyl ester	19.0	607	1-6
10	betanin/isobetanin di-ethyl ester	19.9	607	2-4
11	mono-decarboxy-betanidin/isobetanidin di-ethyl ester	19.9	401	2-3
12	mono-decarboxy-betanin di-ethyl ester	20.5	563	2-4
13	mono-decarboxy-betanidin mono-ethyl ester	20.8	373	4-6
14	n.d.	21.2	413	6-10
15	betanin/isobetanin di-ethyl ester	22.2	607	2-4
16	betanin/isobetanin di-ethyl ester	22.9	607	2-4
17	mono-decarboxy-neobetanin di-ethyl ester	22.9	561	3-5
17'	mono-decarboxy-isoneobetanin di-ethyl ester	23.3	561	5-7
12'	mono-decarboxy-isobetanin di-ethyl ester	24.2	563	2-4
18	betanidin/isobetanindin di-ethyl ester	24.2	445	2-10
19	betanidin/isobetanindin di-ethyl ester	24.4	445	2-10
20	n.d.	25.0	621	1-13
21	n.d.	25.6	399	5-6
22	mono-decarboxy-betanidin/isobetanidin di-ethyl ester	25.8	401	5-9
23	neobetanin di-ethyl ester	26.2	605	2-10
24	betanin tri-ethyl-ester	27.2	635	1-6
24'	isobetanin tri-ethyl-ester	27.5	635	2
25	betanidin/isobetanindin di-ethyl ester	27.5	445	2-6
21'	n.d.	27.9	399	5-8
26	mono-decarboxy-betanidin/isobetanidin di-ethyl ester	29.3	401	5-9
27	n.d.	30.0	459	5-6
28	n.d.	30.8	397	4-10
29	betanidin tri-ethyl-ester	32.3	473	2-10
29'	isobetanidin tri-ethyl-ester	32.5	473	2-10

[a] tentatively identified
n.d. not determined

first time as the betacyanin ethyl-esters were decarboxylated compounds, derivatives of betanin, as well as betanidin. In the individual fractions we could find: mono-decarboxy-betanin/isobetanin mono-(**4**, **6**, **7**) and di-ethyl esters (**12/12'**) (*m/z:* 535, 563, respectively), and also mono-decarboxy-betanidin mono-(**13**) and di-ethyl esters (**11, 22, 26**) (*m/z:* 373, 401, respectively). Another group of ethyl-ester compounds which was detected in the *spCCC* fractions were decarboxylated derivatives of the neobetanin and neobetanidin, such as di-ethyl esters of mono-decarboxy-neobetanin (**17/17'**, *m/z:* 561). After fractionation of betacyanin ethyl-esters by *spCCC,* unidentified compounds also were depicted.

The data obtained from C18-ESI-MS/MS confirm that the mono-, di-, -tri-ethyl-esters of betanin were partly fractionated, but clearly fractionated from mono-, di- and tri-ethyl-esters of betanidin.

All the new compounds described in this study are tentatively identified. Due to the complex mixture of betacyanin ethyl-esters separated by *spiral-coil CCC*, is very difficult to establish their absorption maxima from the C18-HPLC chromatograms. On the basis of HPLC- chromatographic and ESI-mass chromatographic data, we are not able to determine which positions of these compounds are decarboxylated, and we don not know which peaks are isoforms. In order to establish their structures, a further purification of the individual compounds is necessary.

Conclusions

In this study, the preparative fractionation of betalains containing ethyl groups by the *spiral-coil countercurrent chromatography* (*spCCC*) was performed for the first time. In contrast to previous reports on separation by CCC of betacyanins, in this study, a non-toxic solvent system was presented. It was possible, because more hydrophobic derivatives of betalains were separated by *spiral-coil CCC*. These derivatives are much less polar than the natural be-

talains, and therefore they do not require the use of ion-pair reagents. Moreover, the resulting compounds will be further studied for their stability. It is assumed that the stability of betacyanin ethyl-esters will be increased compared to the natural betalains. The *spiral coil-CCC* enabled a recovery of the specific betacyanin esters which will be of great value for biological evaluations.

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References

[1] Bilyk A. "Extractive fractionation of betalains". *Journal of Food Science* 44 (1979): 1249-1251.

[2] Moreno-Alvarez M.J., Viloria-Matos A., and D.M. Hidalgo-Báez "A new method for the isolation of betalaines by HPTLC". *Revista de la Facultad de Agronomía (LUZ)* 21 (2004): 140-144.

[3] Bilyk A. "Thin-Layer Chromatographic Separation of Beet Pigments". *Journal of Food Science* 46 (1981): 298-299.

[4] Delgado-Vargas F., Jiménez R., and O. Paredes-López. "Natural Pigments: Carotenoids, Anthocyanins, and Betalains - Characteristics, Biosynthesis, Processing, and Stability". *Critical Reviews in Food Science and Nutrition* 40(3) (2000): 173-289.

[5] Jerz G., Skotzki T., Fiege K., Winterhalter P., and S. Wybraniec. "Separation of betalains from berries of Phytolacca Americana by ion-pair high-speed counter-current chromatography". *Journal of Chromatography A* 1190 (2008): 63-73.

[6] Berthord A. et al. "Countercurrent chromatography in analytical chemistry". *Pure and Applied Chemistry* 81 (2009): 355-387.

[7] Cao X., Pei H., Huo L., Hu G., and Y. Ito. "Development and evaluation of a spiral tube column for counter-current chromatography". *Journal of Separation Science* 34 (2011): 2611-2617

[8] Wybraniec S, Stalica P., Jerz G., Klose B., Gebert N., Winterhalter P., Spórna A., Szaleniec M., and Y. Mizrahi. "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.

[9] Wybraniec S., Jerz G., Gebers N., and P. Winterhalter. "Ion-pair high-speed countercurrent chromatography in fractionation of a high-molecular weight variation of acyl-oligosaccharide linked betacyanins from purple bracts of *Bougainvillea glabra*". *Journal of Chromatography B* 878 (2010): 538-550.

[10] Yao S., Cao Y., Jia Ch., Wang Y., Song H. "Developments of instruments and methods related with high-speed countercurrent chromatography and their applications in research of natural medicines". *Central European Journal of Chemistry* 10(3) (2012): 417-432.