Semi-synthesis of red beet betacyanin ethyl-esters by esterification

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Red beet pigments recovered from Beta vulgaris L. (Chenopodiaceae), mainly consist of purple betacyanins, such as 15R/15S-betanin. 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. Betacyanins, the glycosidation products of betanidin are very polar and therefore water soluble pigments, which are biosynthetically derived from condensation of cyclo-DOPA and betalamic acid.
The significant polarity of betacyanins requires the use of ion-pair reagents such as trifluoro-acetic acid (TFA) or other homologue fluorinated additives under standard separation conditions on larger scale in spiral coil countercurrent chromatography (spCCC). In our synthetic experiment, the polarity of the natural betacyanins was reduced. This omitted the use of toxic per-fluoro ion-pair reagents. The three carboxylic groups of the betanin pigment backbone were the target functional groups of the derivatisation. In semi-synthesis, fortified red beet pigment extract was esterified with water-free ethanolic hydrogen chloride solution. The reaction was carried out at ambient temperature (7 days) to yield a mixture of betacyanin mono-, di- and tri-ethyl-esters, and their epimeric forms, as well as the respective betanidin-ethyl-esters. The time course of the reaction mixture was monitored by analytical C18-HPLC with ESI-DAD-MS/MS detection. The detected molecular weights of the pigments confirmed the presence of expected products.
To the best of our knowledge, the presence of betalains containing ethyl-ester groups in biological samples have not been studied before.

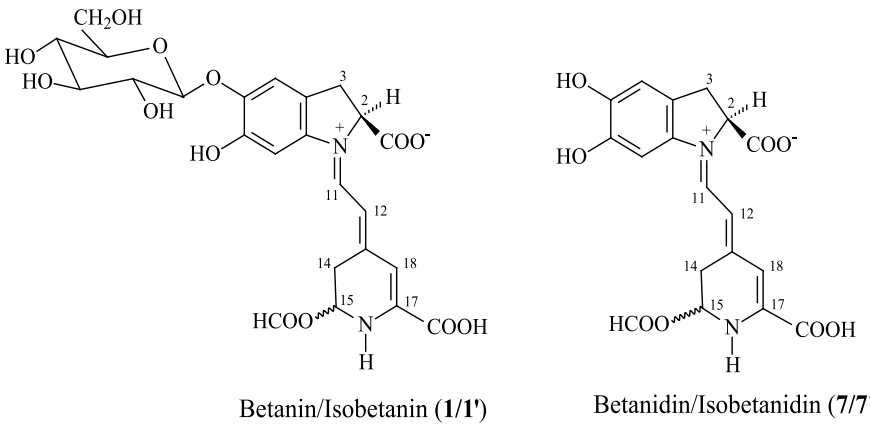
Key words: betacyanin ethyl-esters, betalains, betacyanins, betanin, betanidin, esterification.

Introduction

In recent years, interest in betacyanins as natural pigments has increased considerably, because of their eco-friendliness and apparent lack of toxicity [1]. Betacyanins belong to a group of betalains and are derivatives of glycosylated betanidin (**7/7'**, Fig. 1), an iminium adduct of betalamic acid, and *cyclo*-DOPA [2, 3]. The most popular colorants are betanin and the respective epimeric isoform isobetanin (**1/1'**, Fig. 1).

Betalains are attracting increasing attention because of their use for food coloring and their antioxidant and radical scavenging properties against certain oxidative stress-related disorders, anticancer, antiviral and antiparasitic properties [4, 5].

All betalain pigments can be easily dissolved in water due to their polar character. This property is exploited for the extraction of the pigments. Separation of betalains on



Betanin/Isobetanin (**1/1'**) Betanidin/Isobetanidin (**7/7'**)

Fig. 1. Chemical structures of betanin/isobetanin (15S/15R, 1/1') and betanidin/isobetanidin (15S/15R, 7/7')

a larger scale could be accomplished by *countercurrent chromatography* (CCC). Unfortunately, for this aim, it is necessary to use ion-pair reagent additives, such as trifluoroacetic acid, which are very toxic [6, 7]. In this report, we described a semi-synthesis of derivatized betalains in which the three free carboxylic functions were converted to respective ethyl-esters. These betacyanin ethyl-esters were obtained by esterification of the three carboxylic groups of the pigments, derived from a crude red beet extract, in water free ethanolic hydrogen chloride solution. In this way, the resulting mixture of betacyanin ethyl-esters was isolated by *countercurrent chromatography* using a biphasic solvent system not containing trifluoroacetic acid due to a lower polarity of betalains.

Betalains containing ethyl-ester groups had not been analyzed by modern chromatographic techniques before nor their presence in biological samples have been studied. For the first time, di-*O*-methyl-neobetanidin dimethyl ester was synthesized by Piattelli *et al.* in 1968 [8], and recently, betanidin methyl-esters were analyzed by HPLC [9].

Wiliams et al. [10] used betalains and their ester-derivatives as food quality indicators (FQI). This indicators are sensitive devices for detecting unhealthy levels of food spoilage products in a sealed food product package. A color change of the indicator compound may indicate the presence of an unwanted biological agent, such as bacteria, mold or fungi. For example, certain fungi generate amines when in contact with grains. Smut on unprocessed wheat stored in silos or in cargo holds of ships generates trimethylamine. Ideal indicators are nontoxic and can be used as food additives or dyes, and have a strong color change upon detection of the volatile bases and the color change is apparent even to color blind members of the population.

In our study we synthesized new group of betacyjanin such as ethyl esters of betanin and ethyl esters of betanidin (Fig. 2). It seems that these compounds can be an alternative for betacyanins containing carboxyl groups, and which are sensitive form many factors.

Betacyanins and their ethyl-esters display two absorption maxima – one in the UV-range (270–280 nm) because of cyclo-Dopa input and a second one in the visible range (535–540 nm, depending on the solvent) [10].

Experimental

To a freeze-dried crude pigment extract of betalains recovered from red beet roots (*Beta vulgaris* L.) which was cleaned by solid phase extraction (C_{18}-SPE-cleaned, brand name, 1500 mg), absolute ethanol (50 mL) was added, and their esterification was accomplished by addition of water free ethanolic hydrogen-chloride (60 mL, Sigma Aldrich, Deisenhofen, Germany). The reaction mixture was stirred at room temperature for seven days. During this period, the aliquots of the mixture (0.5 mL) were sampled, dried with nitrogen and dissolved in water (1.5 mL). After seven days, the solution was filtrated. The supernatant liquid was neutralized, while stirring, by drop-wise addition of aqueous ammonia solution (2%, v/v), as long as there was no visible white mist of ammonium chloride from the reaction mixture (40 mL). In the next step, the organic solvent was evaporated to two-thirds of the volume of the flask at 23 °C with a rota-evaporator. The residue of the mixture was dissolved in water (v/v, 1:1) and frozen. Freeze drying led to the crude mixture of betalain mono-, di- and tri-ethyl-esters (1100 mg) which were analyzed by gradient C18-HPLC.

For the chromatographic analysis, a Luna C18(2) column, 250 x 4.6 mm (Phenomenex, Torrance, CA, USA), particle diameter 5 µm, was used. The injection volume was 20 µL, and the flow rate was 0.5 mL/min. The detection of ethyl-esters was performed by HPLC-diode array detection (DAD) (Jasco, Borwin HPLC-software, Germany), and HPLC-ESI-MS/MS in the positive ionization mode (HCT-Ultra ETD II, Bruker Daltonics, Germany).

For the separation of the analytes, the following gradient system was used: flow rate 0.5 mL/min, 10% A in B at 0 min; gradient to 40% A in B at 30 min (A – acetonitrile, 1% formic acid in water).

Results and Discussion

Betacyanin ethyl-esters were prepared by esterification of the three carboxylic groups of a crude red beet pigment extract. Synthesis was performed in two steps: first, betalain pigments in ethanol were dissolved in ethanolic hydrogen chloride, and in the second step the reaction mixture was neutralized with an aqueous solution of ammonia. The synthesis was monitored at defined time sections by analytical C18-HPLC-DAD (Fig. 2), and ESI-DAD-MS/MS detection.

The esterification of betanin/isobetanin (**1/1'**) with HCl/EtOH was stopped after 7 days (Scheme 1). In the C18-HPLC chromatogram of a crude red beet pigment extract (Fig. 2A) the peaks detected are betanin/isobetanin (**1/1'**), and 17-decarboxy-betanin (**2**). The HPLC analysis of the samples obtained after 1 day of reaction (Fig. 3B), showed that there were no peaks of **1/1'** detectable, but new peaks of betanin mono-ethyl ester (**3**), isobetanin mono-ethyl ester (**3'**), double peak of betanidin di-ethyl ester (**4**), and isobetanidin di-ethyl ester (**4'**) were detected. In the chromatogram obtained after 2 days of the reaction (Fig. 3C), the area of the peaks of the compounds **3/3'** is smaller (Table 1), but intensity of the double peak of the compounds **4/4'** (Table 1) is higher than intensity for the chromatogram B (Fig. 3). This is due to the fact that the compounds (**3/3'**) during the reaction lost glucose residue and other carboxylic group has been esterified. Moreover, there are two new peaks 17-decarboxy-betanidin di-ethyl ester (**5**) and betanidin 2,15,17-tri-ethyl ester (**6**) on the chromatogram C. This chromatogram is similar to the chromatograms obtained for the sample after 3, 4 and 7 days of reaction time (Fig. 3D-F), for the sample after am-

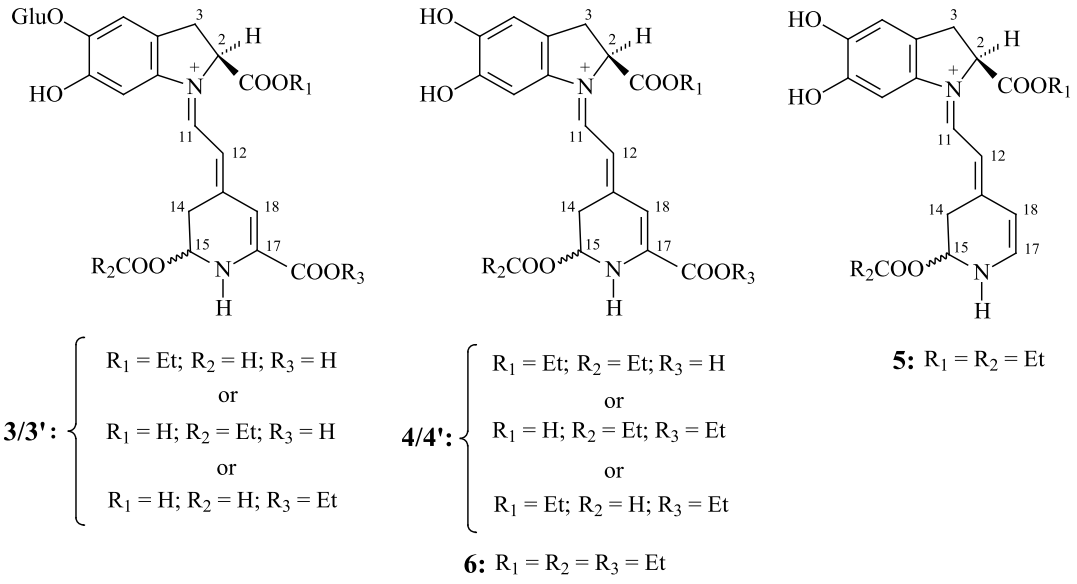
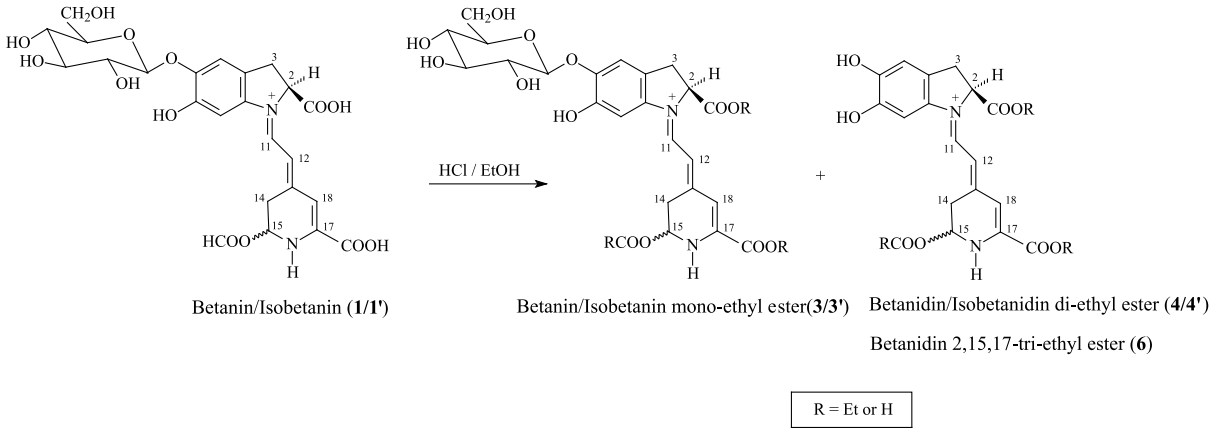


Fig. 2. Chemical structures of betanin/isobetanin mono-ethyl ester (3/3′), 17-decarboxy-betanidin di-ethyl ester (5) betanidin tri-ethyl ester (6)



Scheme 1. Synthesis of betacyanins ethyl-esters from betanin/ isobetanin

monia-neutralization of the betalain ethyl-ester solution (Fig. 3G), and also for the sample after evaporation of two-thirds of the volume of the organic solvents (Fig. 3H).

After seven days of esterification of betanin/isobetanin (**1/1'**) with water free ethanolic hydrogen-chloride, the reaction mixture was filtrated. Obtained precipitate was analyzed by C18-HPLC-DAD at l 540 nm (Fig. 3I). In this chromatogram we could see that compounds **5** and **6** were completely removed. There are the peaks of betanin/isobetanin (**1/1'**), betanin/isobetanin mono-ethyl ester (**3/3'**) and betanidin di-ethyl ester (**4**) as well as its epimeric form **4'**. Betacyanins, the glycosidation products of betanidin are very polar and therefore water soluble pigments. It seems that more hydrophobic compounds such as 2-decarboxy-betanindin di-ethyl ester (**5**) and betanidin 2,15,17-tri-ethyl ester (**6**) are easier soluble in organic solvents. Therefore, the compounds **5** and **6**, as the least polar of the resulting betacyanin ethyl-esters, did not precipitate out during the esterification reaction, while derivatives of betalains **1-4** partly precipitated.

In the crude pigment mixture used for the reaction, mainly betanin and isobetanin were present, but finally also betanidin ester-derivatives were obtained. The reason is the long reaction time of 7 days. Due to the strong acidic reaction conditions for esterification, also the deglycosidation process of betanidin-esters to betanidin ester derivatives occurred. The HPLC analysis showed clearly that neutralization of the reaction mixture with aqueous ammonia and the solvent evaporation were not inducing the glycosidic bond cleavage leading to betanidin esters (Fig. 3G-H). Further studies on the synthesis of betacyanin ethyl-esters are currently conducted.

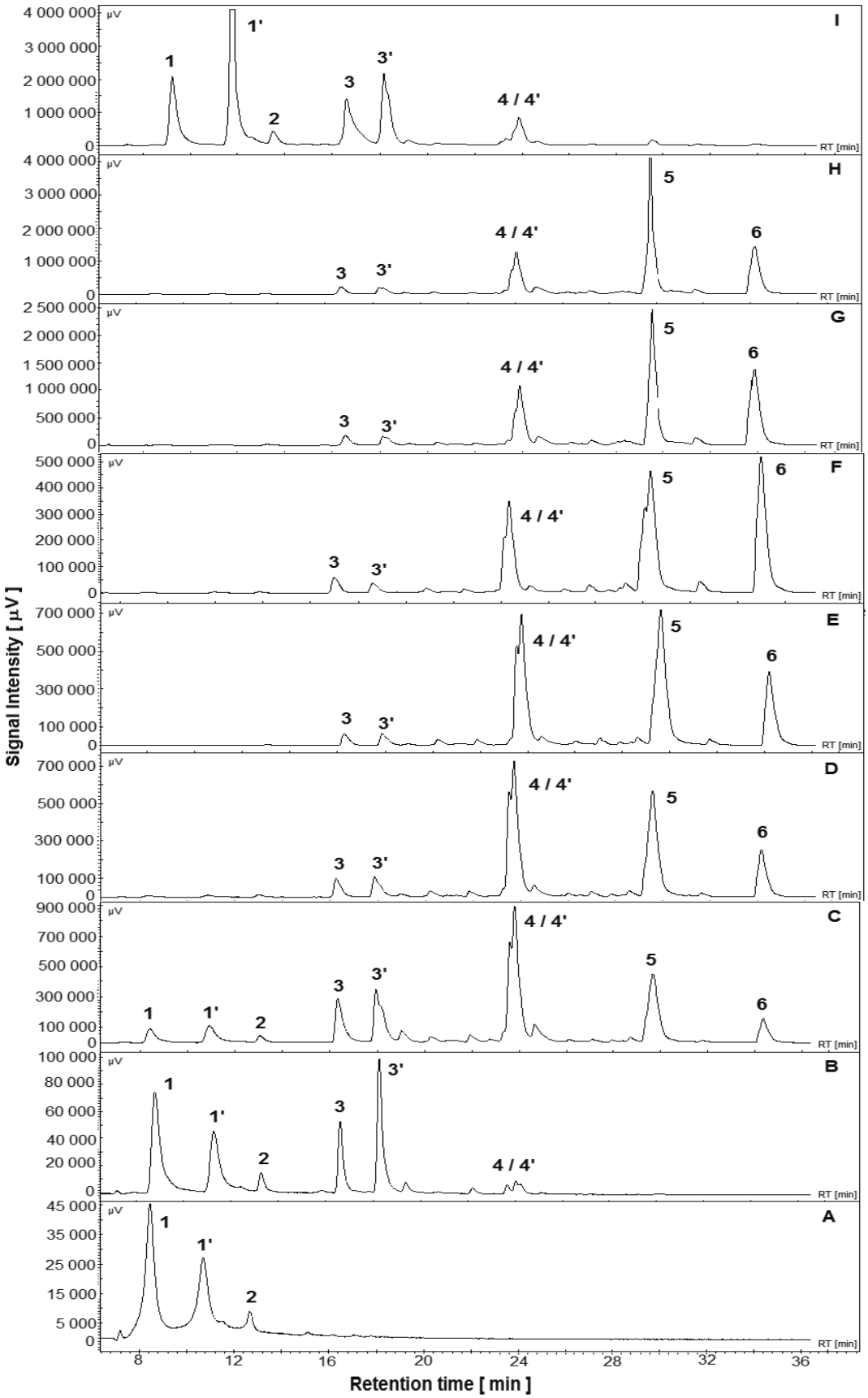


Fig. 3. C18-HPLC chromatograms of betalains at λ 540 nm (A) from crude red beet pigment extract; betacyanin ethyl-esters after: (B) 1 day, (C) 2 days, (D) 3 days, (E) 4 days, (F) 7 days of reaction; (G) neutralized reaction mixture; (H) concentrated solution of betacyanin ethyl-esters; (I) precipitate after filtration of the reaction mixture

Table 1. Chromatographic, spectrophotometric and mass spectrometric data of the analyzed ethylated esters of betanin/isobetanin and betanidin/isobetanidin and 2-decarboxy-betanidin diethyl ester.

No.	Compound	R_f [min]	λ_{max} [nm][a]	m/z $[M+H]^+$	m/z from MS/MS
1	Betanin	8.5	538	551	389
1'	Isobetanin	11.0	538	551	389
2	17-Decarboxy-betanin	12.9	518	507	345
3	Betanin mono-ethyl ester [b]	16.5	528	579	417; 389
3'	Isobetanin mono-ethyl ester [b]	18.5	528	579	417; 389
4	Betanidin di-ethyl ester [b]	24.2	540	445	417; 389
4'	Isobetanidin di-ethyl ester [b]	24.4	540	445	417; 389
5	17-Decarboxy-betanidin di-ethyl ester [b]	29.8	553	401	373; 345
6	Betanidin 2,15,17-tri-ethyl ester [b]	34.5	547	473	445; 417; 389

[a] λ_{max} of betacyanin ethyl esters in the visible range
[b] tentatively identified

As a result of the betanin/isobetanin esterification reaction, three main betanidin-ester products were generated in the mixture confirmed by ESI mass spectrometric detection of $[M+H]^+$ ions at *m/z* 445, *m/z* 401 and *m/z* 473 (Table 1).

Conclusions

In this study, the synthesis of betalains containing ethyl groups was carried out for the first time. Here, particular attention was focused on the analysis of all samples of the synthesized compounds at specific time settings, which are discussed on the basis of the C18-HPLC chromatograms. The application of betacyanin ethyl-esters as natural colorants requires the development of the most efficient methods of isolation of these compounds from the reaction mixture. For their larger scale and gentle separation technique *countercurrent chromatography* could be used. For these betalain derivatives reduced in polarity, solvent systems not containing toxic per-fluoro acids could be used.

Acknowledgement

The research was supported by the European Union through the European Social Fund within „Cracow University of Technology development program - top quality teaching for the prospective Polish engineers; University of the 21st century" project (contract no. UDA-POKL.04.01.01-00-029/10-00).

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