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REDUCTION OF SELECTED BETACYANINS

ABSTRACT

Betacyanins are conjugates of betalamic acid and glycosylated cyclo-DOPA which bring about the creation of a specific chromophoric system, the 1,7-diazaheptamethin chain. Betanin, a betacyanin pigment from red beet (*Beta vulgaris* L.), as well as its decarboxylated derivatives were subjected to reduction with the use of NaBH_4 . The pigment structures contain a vinyl linker which is postulated to be protonated during reaction with NaBH_4 . In this study, 17-decarboxybetanin and 2,17-bidecarboxybetanin were obtained from betanin, which had been previously isolated from red beet root (*Beta vulgaris* L.). Purified pigments were tested in the presence of the reduction reagent while looking for the products of the reaction identified by LC-MS.

KEY WORDS

betanin, decarboxybetacyanins, reduction, 1,7-diazaheptamethin, chromophore, LC-MS

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INTRODUCTION

Betanin is a natural pigment approved as a colorant for food. It is obtained from red beet root (*Beta vulgaris* L.)¹, but can be found in many plant species of the *Caryophyllales* order. Natural pigments from betacyanins group have a lot of advantages in comparison with artificial food colorants. They not only are non-toxic but also have a proved antioxidant activity². Therefore, they are prone to degradation leading to creation of decarboxy-derivatives or total decomposition with betalamic acid and cyclo-DOPA release. The decarboxy-derivatives of betanin from red beet were extensively studied. Their formation, initiated by many factors such as oxygen, high temperature and low pH, was reported by Herbach et al.³ Mono-, di- and tridecarboxybetacyanins were obtained, for instance, by heating acidified aqueous as well as ethanolic solutions at a temperature not exceeding 85°C⁴. One of the routes of betanin decarboxylation is presented in Figure 1.

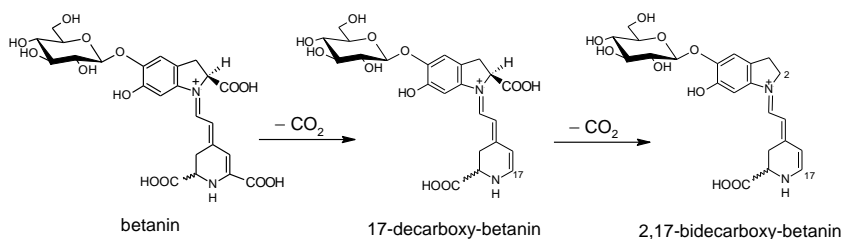


Figure 1. One of betanin decarboxylation routes

A reduction reaction using NaBH₄ has never been performed with betanin nor with its decarboxylated derivatives. However, a protonation of the vinyl linker was reported in previous studies as a presumable result of an inside molecular disproportionation⁵.

¹ B. Nemzer, Z. Pietrkowski, A. Spórna, P. Stalica, W. Thresher, T. Michałowski, S. Wybraniec, Betalainic and Nutritional Profiles of Pigment-enriched Red Beet Root (*Beta vulgaris* L.) dried extracts, *Food Chem.* 2011, 127, 42–53.

² M. R. Castellar, F. Solano, J. M. Obón, Betacyanin and Other Antioxidants Production During Growth of *Opuntia stricta* (Haw.) Fruits, *Plant Foods Hum. Nutr.* 2012, 67, 337.

³ K. M. Herbach, F. C. Stintzing, R. Carle, Betalain Stability and Degradation – Structural and Chromatic Aspects, *J. Food Sci.* 2006, 71, 4, 42–44.

⁴ S. Wybraniec, Formation of Decarboxylated Betacyanins in Heated Purified Betacyanin Fraction from Red Beet Root (*Beta vulgaris* L.) Monitored by LC-MS/MS, *J. Agric. Food Chem.* 2005, 53, 3483–3487.

⁵ T. J. Mabry, H. Wyler, I. Parikh, A. S. Dreiding, The Conversion of Betanidin and Betanin to Neobetainin Derivatives, *Tetrahedron* 1967, 23, 3117.

MATERIAL AND METHODS

Isolation of betanin

Betanin was isolated from juice of red beet root (*Beta vulgaris* L.) by a sequence of chromatographic techniques. Firstly, preliminary purification using a C18 cartridge was carried out. The betanin-rich fractions were eluted by aqueous solution of 30% acetone, 2% formic acid (v/v). Next, a Flash chromatography was used with gradient system: 5% A in B at 0 min; gradient to 35% A in B at 30 min (A, acetone; B, 2% formic acid in water). Afterwards, a preparative HPLC system with HPLC PUMP 64 (Knauer, Germany) was applied. The semi-preparative column was Bischoff C18 (Bischoff Chromatography, Germany) 250 mm x 30 mm i.d., 10 μ m, C₁₈, with a 10 mm x 10 mm i.d. guard column with the same material. An applied gradient system was 6% A in B at 0 min; gradient to 10% A in B at 30 min (A, acetone; B, 1% formic acid in water). In each case, the injection volume was 100 μ L and the flow rate was 3 mL/min. The signal detection was performed at 505 nm by a UV-Vis detector (Knauer, Germany). The temperature during chromatographic isolation was ambient.

Semi-synthesis of betanin derivatives

17-decarboxylated-betanin and 2,17-bidecarboxy-betanin were obtained by heating of betanin aqueous solution acidified with formic acid for 30 min at 85°C. The final isolation of decarboxylated pigments was carried out by the same procedure of semi-preparative HPLC as for betanin.

UV-Vis spectra measurement

The reaction was monitored by collecting UV-Vis spectra for 0.6 h using a microplate reader. For this purpose, 10 μ L of each pigment was dispensed into 6 wells with buffer solutions in a pH range of 3–8. Immediately before measurement, 170 μ L of NaBH₄ aqueous solution (0.85 mM) were added to each well. The spectra were recorded in the range of 350–650 nm at 25°C within a 0,6 h period. The calculated optical path-length for a volume of 200 μ L in a microplate well was 0,53 cm.

LC-DAD-MS analysis

The reaction products were identified by means of positive ion electrospray mass spectra as recorded on ThermoFinnigan LCQ Advantage (electrospray voltage 4,5 kV; capillary 250°C; sheath gas: N₂) with a ThermoFinnigan LC Surveyor pump. An ONYX monolithic column 100 x 4.6 mm i.d. with a guard column (Phenomenex, Torrance, CA, USA) thermostated at 35°C was used. The

injection volume was 10 μL , the flow rate was 0.5 mL/min and the applied gradient system was: 95% A with 5% B at 0 min; gradient to 70% A with 30% B at 40 min (A, 1% (v/v) formic acid; B, acetonitrile). The controlling software, ThermoFinnigan Xcalibur (San Jose, CA, USA), was used for total ion chromatograms and mass spectra recording. The relative collision energies for the CID experiments were set at 30% (according to a relative energy scale). Helium was used as the collision gas for the CID experiments.

RESULTS AND DISCUSSION

UV-VIS spectra of betanin, 17-decarboxy-betanin and 2,17-bidecarboxy-betanin during reaction with NaBH_4 , presented in Figure 2, were measured for 0,6 h at different pH. In acidic solutions (pH 3–5), no significant changes were observed. Meanwhile, at pH 6, a second minor band in the range of 350–370 nm appears for each studied pigment which is more evident at higher pH. An even stronger effect for 17-decarboxy-betanin (17-dBt) is observed at pH 8 (Figure 2H). The new band may suggest the formation of a reduction product.

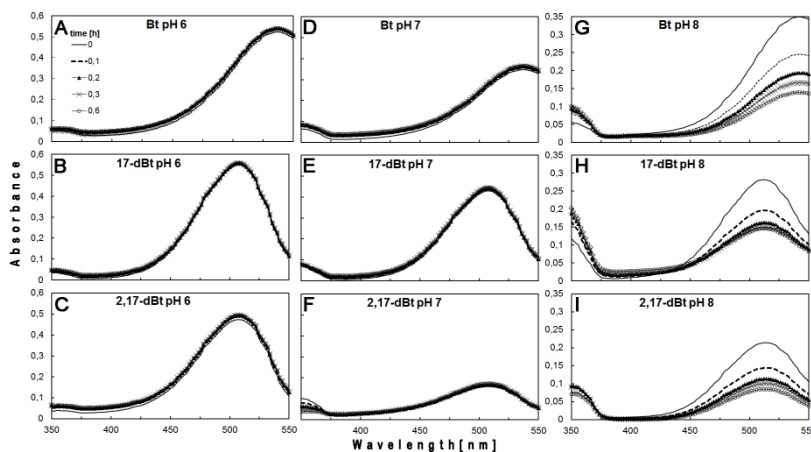


Figure 2. UV-VIS spectra measured during reaction of betanin (Bt), 17-decarboxy-betanin (17-dBt) and 2,17-bidecarboxy-betanin (2,17-dBt) with NaBH_4

The LC-MS data are presented in Figures 3–6 as well as in Table 1 including all measured details. The spectra recorded over LC-DAD-MS analysis prove arising of new absorption maxima in the range of 290–340 nm for each of the pigments (Figure 3, for 17-dBt). After experiment (Figure 3b) a distinct diminishing of absorption maxima at 505 nm of 17-decarboxy-betanin in agreement with an observation of a fading sample colour is shown. In the case of betanin and 2,17-bidecarboxy-betanin, similar results were noticed.

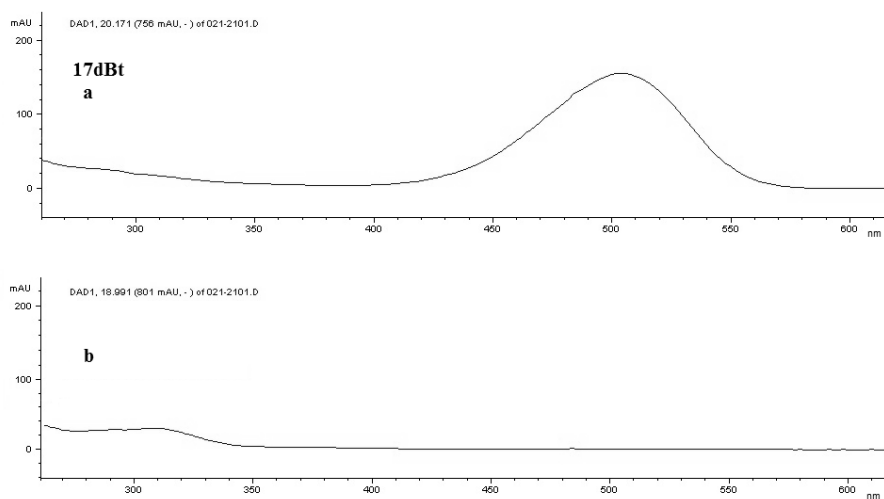


Figure 3. LC-DAD-MS spectra of 17-decarboxy-betanin (a) and 17-decarboxy-11,12,17,18-tetrahydro-betanin, the product of reduction (b)

Table 1. Chromatographic and mass-spectrometric data of analysed pigments subjected to reduction

No.	Compound	R_t [min]	m/z [M+H] ⁺	m/z from MS/MS of [M+H] ⁺
1a	betanin	19,9	551	389
1b	11,12,17,18-tetrahydro-betanin ^a	19,1	555	393
2a	17-decarboxy-betanin	20,3	507	345
2b	17-decarboxy-11,12,17,18-tetrahydro-betanin ^a	19,1	511	349
3a	2,17-bidecarboxy-betanin	22,2	463	301
3b	2,17-bidecarboxy-11,12,17,18-tetrahydro-betanin ^a	10,7	467	305

^a Tentatively identified

In LC-MS data for betanin after reduction by NaBH_4 in comparison to reference betanin (retention time R_t 19,9 min, m/z of protonated ion 551, Figure 4a), there are traces of two coeluting compounds at R_t 19,1 min, m/z of $[\text{M}+\text{H}]^+$ 555 (Figure 4b). This indicates a product of 4 hydrogen atoms addition to betanin. The fragmentation ion at m/z 393 confirms deglycosylation of the compound ($\Delta m/z$: $555 - 393 = 162$), which proves a 4 H addition into aglycone of betanin.

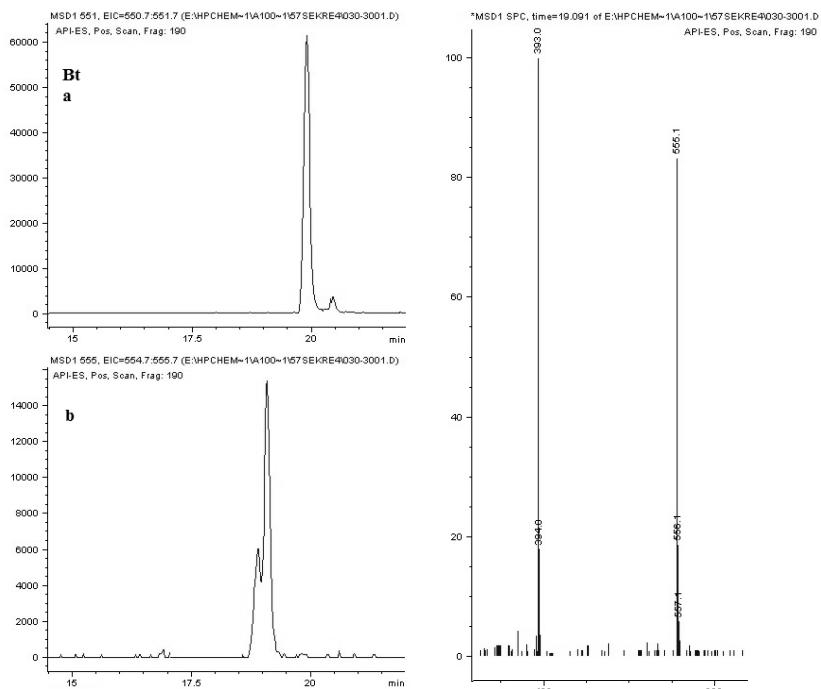


Figure 4. Chromatographic traces of betanin (a), 11,12,17,18-tetrahydro-betanin^a (b) and MS spectrum of 11,12,17,18-tetrahydro-betanin^a (c)

In the case of the product of a 17-decarboxy-betanin reduction, a single peak at R_t 19,1 is present (Figure 5b). Its protonated molecular ion is also increased by about a 4 hydrogen atoms mass. The fragmentation ion at 349,1 in comparison with corresponding ion $m/z = 345.1$ of reference ion is increased as well (Figure 5c). This suggests a similar position of a reduction in the structure of betanin and 17-decarboxy-betanin.

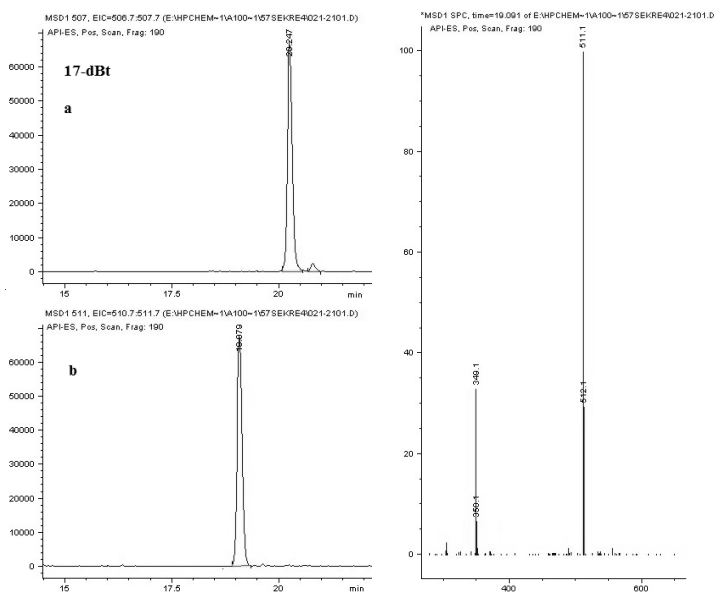


Figure 5. Chromatographic traces of 17-decarboxy-betain (a), 17-decarboxy-11,12,17,18-tetrahydro-betain^a (b) and MS spectrum of 17-decarboxy-11,12,17,18-tetrahydro-betain (c)

For 2,17-bidecarboxy-betain, a similar addition of 4 hydrogens is noted (Table 1). Interestingly, the product of 2,17-bidecarboxy-betain reduction differs from that of betain and 17-decarboxy-betain, because of the shorter retention time, R_t 10.7 min (Figure 6), which may suggest a higher polarity. Further investigation is necessary to explain the fact.

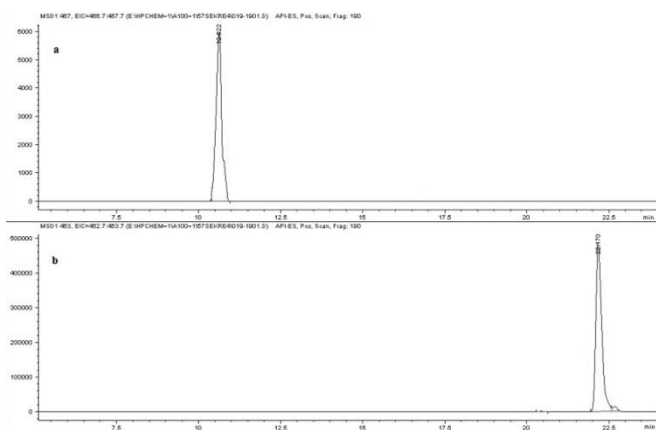


Figure 6. Chromatographic traces of 2,17-bidecarboxy-betain (a) and 2,17-bidecarboxy-11,12,17,18-tetrahydro-betain (b)

CONCLUSIONS

Presumably, the positions of the addition of hydrogen atoms over the reduction by NaBH_4 are C11 and C12 in the vinyl linker as well as C16 and C17. This suggestion, based on the previous study of Mabry et al., is shown in Figure 7⁶. Thus reduction of betacyanin pigments resulted in the destruction of 1,7-diazaheptamethin chromophoric system in their structures, which is proved by decreasing of the main absorption maxima. The proposed products of betanin, 17-decarboxy-betanin and 2,17-bidecarboxy-betanin are: 11,12,17,18-tetrahydro-betanin, 17-decarboxy-11,12,17,18-tetrahydro-betanin and 2,17-bidecarboxy-11,12,17,18-tetrahydro-betanin (Figure 7), respectively.

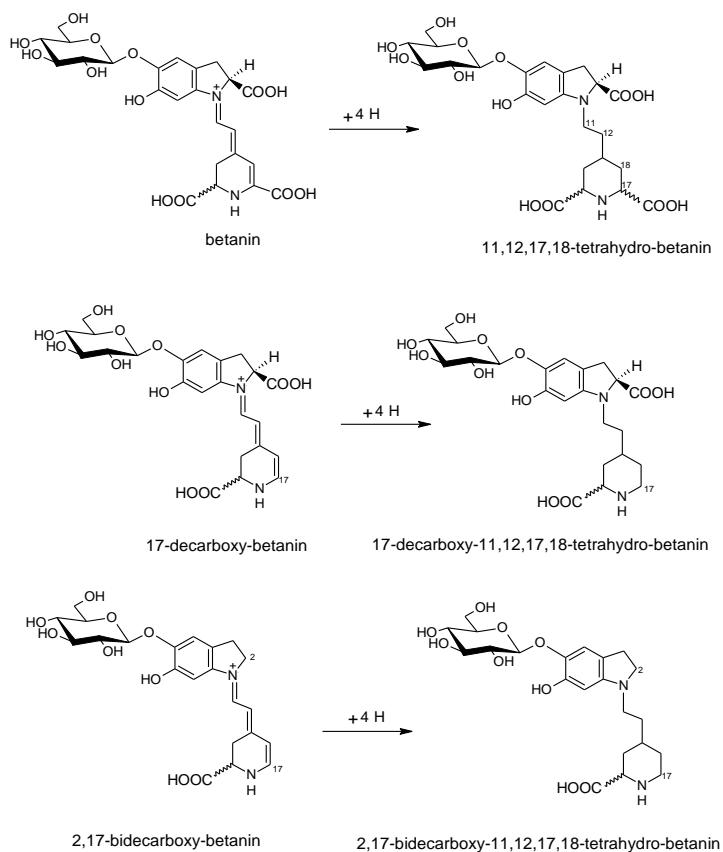


Figure 7. The presumable products of tested pigments reduction by NaBH_4

⁶ Ibidem.

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