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Betainin, the main pigment of red beet - molecular origin of its exceptionally high free radical scavenging activity

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4 1 **Betainin, the main pigment of red beet - molecular origin of its exceptionally**
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6 2 **high free radical scavenging activity***
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11 6
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13 Footnote

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15 *Analysis' held in Prague, Czech Republic, November 2-4th 2005.*
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1
2
3 **Abstract**
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5 In the present study, the pH-dependent free radical scavenging activity of betanin in the TEAC
6 (Trolox equivalent antioxidant capacity) assay was determined. It was found that at pH > 4
7 betanin is about 1.5-2-fold more active than some anthocyanins considered very good free
8 radical scavengers as determined in the TEAC assay. The increase in the TEAC values of
9 betanin with increasing pH is discussed in terms of its calculated phenolic OH homolytic bond
10 dissociation energy (BDE) and ionization potential (IP). The results suggest that the
11 exceptionally high antioxidant activity of betanin is associated with increasing of its H-
12 donation and electron-donation ability when going from cationic state to mono-, di- and tri-
13 deprotonated states present at basic solutions.
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13 **Keywords:** *Betalains, betanin, pH dependent free radical scavenging activity, TEAC value,*
14 *DFT calculations*
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1 Introduction

Colour is one of the most important factors indicating the quality of food. It plays significant role in consumer acceptance and preference of products. The use of natural and synthetic dyes, as additives for food, cosmetic and drug products, is an ancient practice but currently there is an increasing interest from consumers in the use of naturally derived colourants. This interest is associated with consumer perception of synthetic dyes as harmful, whereas pigments naturally occurring in edible plants are usually considered to be rather safe. A variety of different pigments are produced by nature and a number of them have found practical application in colouring of food e.g. water-soluble anthocyanins and betalains and fat-soluble carotenoids and curcuminoids. Moreover, there is growing evidence suggesting that some natural colourants may be nutritionally important antioxidants and that their presence in the diet may reduce the risk of cardiovascular disease, cancer, and other diseases associated with aging (Cai et al. 2003).

Betalains occur in plants of most families of the plant order Caryophyllales (with the exception of Caryophyllaceae and Moluginaceae) and in some higher fungi (Frank et al. 2005). Among the numerous natural sources of betalains, red and yellow beet, prickly pear, coloured Swiss chard, grain amaranth and cactus fruits are the only foods containing these compounds (Kanner et al. 2001, Stintzing et al. 2004, Frank et al. 2005). Red beet (*Beta vulgaris* L.) is consumed in the form of lactofermented juice, pickled preserves or as a cooked vegetable. The pigment mixture in the form of beet juice concentrate or beet powder are approved additives for use in food, drugs and cosmetic products (Dornenburg et al. 1996). Red beet betalains are composed of two main groups: the red-violet betacyanins (e.g. betanin and isobetanin) and the yellow betaxanthins (e.g. vulgaxanthin I and II). The betacyanins (betanin and isobetanin) are water-soluble immonium conjugates of betalamic acid with 3,4-dihydroxyphenylalanine (cyclo-DOPA), which may be glucosylated. The most important betacyanin in red beet is betanin, which is a betanidin 5-*O*- β -glucoside (Figure 1) containing a phenolic and a cyclic amine groups, both shown to be very good electron donors, acting as antioxidants (Kanner et al. 2001). Betanin makes up 75-95% of the total colouring matter found in the beet. The isobetanin is C15-epimer of betanin (Figure 1) however it is present in fresh beet juice in small amounts. In food processing, betalains are less commonly used than water-soluble anthocyanins, although the colour of betalains is more stable between pH 3 and

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3 1 7 (they retain their tinctorial strength and colour shade). At pH values lower than 3 the colour
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5 2 turns more violet and at pH higher than 7 it becomes more yellowish-brown (Roy et al. 2004).
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7 3 Anthocyanins have greatest colour intensity at pH values less than 4 where they exist in the
8
9 4 form of flavylium cation. At pH 4-5, a colourless carbinol pseudobase is formed upon
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11 5 deprotonation and hydration of flavylium cation (Lapidot et al. 1999). Thus betalains are
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13 6 well suited for colouring acid and slightly acid food whereas anthocyanins are used as a
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15 7 source for food colours in applications, which have an acidic pH such as beverages and
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17 8 dessert products (Strack et al., 2003; Roy et al. 2004).
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10 **Figure 1**

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12 In several studies it was shown that betalains are effective free radical scavengers and that
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14 they prevent active oxygen-induced and free radical-mediated oxidation of biological
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16 molecules (Escribano et al. 1998, Zakharova et al. 1998, Pedreno et al. 2000, 2001, Kanner et
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18 al. 2001, Butera et al. 2002, Pavlov et al. 2002, Wettasinghe et al. 2002, Cai et al. 2003,
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20 Tesoriere et al. 2004, 2005, Allegra et al. 2005, Frank et al. 2005, Stintzing et al. 2005). For
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22 instance, in a study of Kanner et al. (2001) linoleate peroxidation by cytochrome c was
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24 inhibited by betanin, betanidin, catechin, and α -tocopherol with the IC_{50} values of 0.4, 0.8,
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26 1.2, and 5 μ M, respectively. The IC_{50} values for inhibition of soybean lipoxygenase by
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28 betanidin, betanin, and catechin were found to be 0.3, 0.6, and 1.2 μ M, respectively. These
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30 results indicate that betalains can be more potent antioxidants than catechins and other
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32 flavonoids.

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24 Red beet roots contain a large concentration of betanin, 300-600 mg/kg, and lower
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26 concentrations of isobetanin, betanidin, and betaxanthins (Kanner et al. 2001). The prickly
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28 pear (*Opuntia ficus indica*) contains about 50 mg/kg of betanin and 26 mg/kg of indicaxanthin
29
30 (Butera et al. 2002). The bioavailability of betalains is at least as high as flavonoids, which are
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32 well-accepted natural antioxidants. Betalains, as natural antioxidants, may provide protection
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34 against oxidative stress-related disorders (Tesoriere et al. 2005, Kanner et al. 2004).
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36 Therefore, consumers may benefit from regular consumption of products rich in betalains
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38 such as red beet juice and other products made of red beet or foods coloured with betalains as
39
40 safe natural colourants. Betanin, listed as food additive E162, is already used in variety of

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3 1 processed foods, especially in ice creams and frozen desserts because it colours without
4 changing the flavor profile.
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9 4 In the present study betanin – the main red-purple pigment of red beet was investigated in
10 relation to its antioxidant properties. The free radical scavenging activity of betanin at
11 5 different pH (from 2 to 9) in so called TEAC (Trolox equivalent antioxidant capacity) assay is
12 6 discussed in terms of its phenolic OH homolytic bond dissociation energy (BDE) and
13 7 ionization potential (IP). These and some other relevant thermochemical parameters, obtained
14 8 at DFT B3LYP/6-311+G**//B3LYP/6-31G** level of quantum mechanical calculation,
15 9 characterizing betanin, are reported for the first time.
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17 11

12 **Materials and methods**

13 *Chemicals*

14 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-
15 hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and microperoxidase-8
16 (MP8) were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrogen peroxide (30%)
17 and glacial acetic acid were purchased from Merck (Darmstadt, Germany). Acetonitrile was of
18 HPLC grade.
19

20 *Plant material*

21 Beetroots (*Beta vulgaris* L.) were purchased in a local market. They were washed with water,
22 peeled and beetroot juice was obtained. The juice was acidified to pH 3.0 with 1N HCl, left
23 overnight at 4°C and afterwards centrifuged at 12.000 x g for 20 min at 4°C (Nilsson 1970).
24 The supernatant showed two absorption bands at 480 and 535 nm, corresponding to
25 betaxanthins and betacyanins, respectively.
26

27 *Preparation of betanin*

28 Betacyanins and betaxanthins were separated by gel filtration on a Sephadex G-25 column (40
29 x 2.2 cm) essentially as described by Kanner et al. (2001). Briefly, 5 ml of beetroot juice were
30 eluted with 1% acetic acid and 2.5 ml fractions were collected. The absorption spectra of the
31 fractions were measured (Genesys 6, ThermoSpectronic, USA) and evaluated for the presence
32 of betacyanins and betaxanthins. The purity of isolated pigments was analysed using high-
33 performance liquid chromatograph (Waters, Millford, Ma, USA) equipped with Nova-Pak C₁₈

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3 1 column (3.9 x 150 mm, 5 μ m, Waters, Millford, Ma, USA) fitted with μ Bondapak C₁₈
4 cartridge guard column (Waters, Millford, Ma, USA). A mobile phase and elution gradient of
5 2 Tesoriere et al. (2004) was used: a 20 min linear gradient elution from solvent A (1.0% acetic
6 acid in water) to 20% solvent B (1% acetic acid in acetonitrile) with flow rate 1 ml/min. The
7 3 eluate was monitored using the Waters 996 photodiode-array (PDA) detector set at 535 nm
8 and 480 nm for betacyanins and betaxanthins, respectively. Betacyanin fractions were
9 4 combined and HPLC analyses revealed that betanin with small amount of compound
10 5 identified as isobetanin (~10% of the isolated betanin) were the only betacyanin components
11 6 of the betacyanin fraction. The purity of betanin measured at 270 nm was about 86%. The
12 7 purity of betanin and isobetanin was confirmed by UV-VIS spectra, measured by PDA
13 8 detector in the range of 210-700 nm, which were in agreement with those published in
14 9 literature (Cai et al. 2001). Betanin was freeze-dried and kept at -20°C under nitrogen until
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33 15 *TEAC assay*

34 16 The TEAC assay is based on the ability of the antioxidant to scavenge the blue-green coloured
35 17 ABTS^{•+} (2,2'-azinobis(3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt) radical
36 18 cation relative to the ABTS^{•+} scavenging ability of the water-soluble vitamin E analogue,
37 19 Trolox (Miller et al. 1993). The antioxidant activity of betanin was measured by the modified
38 20 TEAC assay performed essentially as described previously (Miller et al. 1993), with some
39 21 modifications (Tyrakowska et al. 1999). In the present study microperoxidase-8 (MP8) instead
40 22 of metmyoglobin, was used to generate the ABTS^{•+} in PBS (0.01 M phosphate buffer, 0.14 M
41 23 NaCl, 0.002 M KCl) pH 7.4. MP8 (final concentration 0.2 μ M) and ABTS (final
42 24 concentration 3.0 mM) in PBS were mixed and the reaction was initiated by the addition of
43 25 hydrogen peroxide (final concentration 0.1 mM). The major advantage of the modified TEAC
44 26 assay is that it permits studies of radical scavenging activity over a wide pH range (2 – 9.5)
45 27 (Tyrakowska et al. 1999).
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55 29 The ABTS^{•+} solution thus obtained was diluted 1:1 (v/v) using 0.2 M potassium phosphate
56 30 buffers of various pH values to give ABTS^{•+} solutions at pH values varying between 2 and 9
57 31 (an absorption was about 0.6 at 734 nm). The ABTS^{•+} solutions thus obtained were used for
58 32 determination of the TEAC values. Antioxidants (Trolox or betanin) were added as 1% (v/v)

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3 of 100 times concentrated stock solutions in methanol (Trolox) or water (betanin) to give the
4 final concentration required. The molar concentration of betanin in aqueous stock solution
5 was determined spectrophotometrically at 536 nm using molar absorption coefficient $\epsilon =$
6 $65.000 \text{ M}^{-1} \text{ cm}^{-1}$ (Kanner et al. 2001, Allegra et al., 2005). The decrease in absorption caused
7 by the antioxidant compound, measured at 6 min, is reflecting the $\text{ABTS}^{\bullet+}$ radical scavenging
8 capacity and was plotted against the concentration of the antioxidant. The TEAC value is
9 defined as the concentration of Trolox solution, used as an antioxidant standard, with
10 equivalent antioxidant potential to a 1 mM concentration of the compound under investigation
11 (Miller et al. 1993, Rice-Evans et al. 1994). The TEAC value was calculated as the ratio of the
12 slope of the plot for scavenging of $\text{ABTS}^{\bullet+}$ by the antioxidant under investigation, to the
13 slope of the plot for $\text{ABTS}^{\bullet+}$ scavenging by Trolox. The free radical scavenging activity of
14 Trolox was previously shown to be unaffected over the whole pH range tested (Tyrakowska et
15 al. 1999).

16 *Quantum mechanical calculations*

17 First, geometry optimization of molecules studied (betanin, cyclo-DOPA-5-O- β -D-glucoside
18 – product of betanin decomposition under high pH) was performed using B3LYP functional
19 with 6-31G** basis set. In order to obtain some useful thermochemical parameters, such as
20 deprotonation energy (DE), phenolic OH bond dissociation energy (BDE) and ionization
21 potentials (IP) values, the compounds were studied in various protonation/deprotonation states
22 as well as in one-electron oxidized state. All thermochemical data given in this work were
23 computed in „single-point” step with more extended 6-311+G** basis set using optimized
24 structures. All theoretical results are expressed in kcal/mol and refer to so-called „gas-phase”
25 calculation. More details on calculation procedure can be found in our previous paper
26 (Borkowski et al. 2005). All calculations were performed using Gaussian 98 computational
27 package (Gaussian Inc., Pittsburg, PA, USA).

28 **Results and discussion**

29
30 Our previous studies have shown that antiradical activity of hydroxyflavones and their
31 metabolites (Lemańska et al. 2001, 2004), and anthocyanins (Borkowski et al. 2005) in so
32 called TEAC assay strongly depends on pH of the medium, in which these antioxidants act.
33 Escribaño et al. (1998) found that free radical scavenging activity of betanin at basic pH is

1 much higher than that at acidic pH. It seemed interesting to find out how free radical
2 scavenging activity of betalains (in the form of mixture of betanin and to lesser extent of
3 isobetanin) isolated from raw beet juice will change in wide range of pH. Figure 2 presents the
4 pH-dependent TEAC values for beet root betanin. From this curve it follows that betanin at
5 $\text{pH} > 4$ is about 1.5-2-fold better radical scavenger than cyanidin-3-*O*-glucoside and cyanidin
6 (Borkowski et al. 2005), the latter one having one of the highest antiradical activities
7 measured in the TEAC assay at pH 7.4 (Rice-Evans et al. 1996). These pH-dependent changes
8 in radical scavenging capacity of natural antioxidants may be of biological relevance because
9 the pH range of different human body fluids is known to vary widely from pH 1 in the
10 stomach, pH 5.3 in the small intestine, pH 6.8 in mouth saliva, pH 7.4 in blood and tissue
11 fluid, pH 8 in the large intestine to pH 7 – 8.7 in the pancreas and pH 8.3 – 9.3 in the
12 duodenum (Grzymiński 2000). pH-dependent changes in antiradical activity of betalains
13 and anthocyanins suggest that possible beneficial health effects of these natural dyes will vary
14 with the tissue under investigation and that betanin at pH higher than 4 could be even better
15 free radical scavenger than extensively studied flavonoids. It is worth noting that free radical
16 scavenging activity of betanin measured in the TEAC assay at pH 7.4 and in the DPPH assay
17 is about 7.5-fold and 3-fold higher, respectively than that of vitamin C, which is commonly
18 accepted as effective natural water soluble antioxidant (Gliszczyńska-Świąło, 2006; Cai et al.,
19 2003). Moreover, in contrast to betanin, the ABTS^{•+} radical cation scavenging activity of
20 vitamin C is not significantly affected over the whole pH range tested (Gliszczyńska-Świąło,
21 unpublished results)

Figure 2

25 In strongly acidic environment, the betanin molecule may exist in cationized form with
26 excessive positive charge localized in proximity of N-1 nitrogen. Because betanin contains
27 three carboxyl groups and potentially ionizable H-N16 and C6-OH protons, in mild acidic
28 solution it can appear in the form of various zwitterionic states as shown in Figure 3. To point
29 out the relevance of the medium acidity in aqueous solution, the charge alteration of betanin
30 and isobetanin upon pH changes were proposed in literature (Nilsson 1970, Frank et al. 2005).
31 It was suggested that at $\text{pH} < 2$ mainly cationic form appears (Figure 1), at $\text{pH} = 2$ zwitterion
32 form (Figure 3A), at $2 < \text{pH} < 3.5$ monoanion with deprotonated C2-COOH and C15-COOH
33 groups, at $3.5 < \text{pH} < 7$ dianion with deprotonated C2-COOH, C15-COOH and C17-COOH

1 groups, and at $7 < \text{pH} < 9.5$ trianion with deprotonated all carboxyl groups and additionally
2 phenolic C6-OH group (Frank et al. 2005). The pKa value for betanin related to its phenolic
3 OH group was measured to be 8.5 (Nilsson 1970). The pKa of two carboxyl groups was
4 suggested to be ~ 3.4 . The third carboxyl group in the C2 position was suggested to have a
5 lower pKa, as the isoelectric point of betanin was found in the range of pH 1.5-2.0 (Nilsson
6 1970). To establish the order of the most easy deprotonating groups, the gas-phase
7 deprotonation energies (DE) were computed by DFT method. Various zwitterionic structures
8 with DE values calculated from betanin cationic state are given in Figure 3. Surprisingly, our
9 calculations predict that H-N16 proton, not C2-COOH (as it was suggested in some papers
10 (Nilsson 1970, Frank et al. 2005) is most easily ionizable group (Figures 3D and A,
11 respectively). It is interesting that, from model theoretical DFT calculation point of view, the
12 C17-COOH carboxyl group (Figure 3C) is also more acidic than the C2-COOH group and the
13 acidity of the C2-COOH group is comparable to that of C15-COOH (Figure 3B) and C6-OH
14 phenolic groups (Figure 3E). In fact, our gas-phase calculations do not take into account
15 solvation effects, which tend to stabilize rather more polar structures.

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Figure 3

To get some more insight in electronic structure of betanin and its consequences for
antiradical activity we have considered two resonance structures for betanin in cationic form
and in 16N^- structure obtained by H-N16 proton dissociation (Figure 4). The cationic state of
betanin may be considered as a mixture of two resonance structures: immonium salt (Figure
4A) with positive charge located on N1 nitrogen atom and ammonium salt (Figure 4B) with
positive charge centered on N16 atom. The existence of these structures is supported by
calculated lengths of double bonds between N1 atom in cyclo-DOPA-5-O- β -D-glucoside and
N16 atom in betalamic acid. It was found, in DFT optimized structures, that the calculated
interatomic distances between N1-C11, C12-C13 and C17-C18 do not correspond to 'pure'
double bonds; their lengths are between double and single bonds. Similarly, the C11-C12,
C13-C18 and N16-C17 bonds are slightly shorter than standard single bonds and slightly
longer than standard double bonds (results not shown). Assuming that betanin in cationic state
exists in two structures of immonium and ammonium salts it seems reasonable that betanin at
H-N16 could be partly dissociated at less acidic conditions than other groups. Analysis of
betanin with H-N16 deprotonated group (Figure 4C) reveals, however that its second

1 resonance structure is neutral amine molecule stabilized by two hydrogen bonds formed by
2 adjacent COOH groups (Figure 4D). However, the existence of such a structure in aqueous
3 solution seems to be less probable, remembering that betanin is very well soluble in water and
4 worse in other solvents. Probably in real solution, depending on pH value, betanin can exist in
5 various deprotonated forms with different contributions. Therefore, we have calculated set of
6 thermochemical parameters describing ability of hydrogen and electron donation by betanin in
7 various possible deprotonation states. These electronic parameters include OH bond
8 dissociation energy (BDE), representing the ease of hydrogen atom donation and IP,
9 representing the ease of electron donation. The calculated BDE values for C6-OH and, if
10 possible, for H-N16 of betanin in cationic and in all mono-deprotonation states are presented
11 in Figure 5. We have found that each mono-deprotonated form of betanin is better H donor
12 (lower BDE values) than betanin in cationic form. In the case of C2-COO⁻ form, the BDE
13 drops by about 10 kcal/mol in comparison to cationic form. Similar decrease in H-N16 BDE
14 value is observed.

Figure 4 and 5

18 Table I shows the calculated thermochemical data extended to various di- and tri-deprotonated
19 forms that may be expected at higher pH. The most stable structures, denoted as "0.0"
20 (calculated as a structure with minimum energy), may be expected in solution in relatively
21 high amounts. It is 16N⁻ for monoanions, 16N⁻/C6O⁻ for dianions and C2-COO⁻/16N⁻/C6O⁻
22 for trianions. The relative stability of other mono-, di- and tri-deprotonated forms is calculated
23 with respect to appropriate the most stable structure. The IP values are generally calculable
24 when C6-OH phenolic group is deprotonated (carboxylate anions themselves have very high
25 ionization potentials that are unrealistic for antioxidant action). From results presented in
26 Table I it follows that each step of betanin deprotonation (mono, di- and tri-deprotonation)
27 leads to the decrease of the BDE values of these forms. The higher degree of betanin
28 deprotonation, the lower BDE values and the more easily betanin donates hydrogen atom. At
29 higher pH, where betanin occurs in tri-deprotonated forms, the BDE of C6-OH reaches the
30 value nearly 60 kcal/mol. The second parameter, i.e. IP, that describes ability of a molecule to
31 donate electron decreases even more dramatically when going from cationic form of betanin
32 (219.3 kcal/mol) through mono-deprotonated (140-160 kcal/mol), and di-deprotonated (84-89
33 kcal/mol) to tri-deprotonated forms (28-35 kcal/mol). However, it should be pointed out that

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3 1 theoretical calculations performed without taking into account the solvation phenomena give
4 overestimated results, in spite of using time lasting extended basis sets. Nevertheless, very
5 careful interpretation of the data presented in Table I still gives some support for explanation
6 of observed extraordinary high antiradical activity of betanin with increasing pH. Both
7 calculated parameters, the BDE and IP values of C6-OH and H-N16, are nicely consistent
8 with pH increasing free radical scavenging activity of betanin observed in the TEAC assay.
9 Clear dependence between decreasing BDE and IP parameters and degree of betanin
10 deprotonation seems to be consequence of strong conjugation between cyclo-DOPA-5-*O*- β -D-
11 glucoside and betalamic acid moieties resulting from two resonance structures shown in
12 Figure 4. Altogether, the calculated molecular parameters clearly show why betanin becomes
13 a very good antioxidant upon subsequent deprotonation at more and more high pH.

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13 It is known that betanin, especially upon heating, decomposes to betalamic acid and cyclo-
14 DOPA-5-*O*- β -D-glycoside and its degradation is greater at basic pH (Pedreño et al. 2001).
15 Although, cyclo-DOPA-5-*O*- β -D-glycoside at basic pH is unstable (Pedreño et al. 2001) it can
16 not be excluded that this moiety, partially, might be responsible for observed high antioxidant
17 activity of betanin in the pH range of 7-9. For this reason, we decided to calculate additionally
18 the BDE and IP parameters for cyclo-DOPA-5-*O*- β -D-glycoside. The results obtained are
19 presented in Figure 6. It was found that anionic forms of cyclo-DOPA-5-*O*- β -D-glycoside are
20 expected to be very good hydrogen and electron donors suggesting high antioxidant activity of
21 this compound.

Figure 6

Conclusions

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In the present study it was found that free radical scavenging activity of betanin, measured in the TEAC assay, is exceptionally high at pH higher than 4. This natural colourant is 1.5-2-fold more active than popular anthocyanins, especially at neutral and basic solutions. The result of the present study indicate that pH dependent increasing of free radical scavenging activity of betanin can be attributed to the formation of its different mono-, di- and tri-deprotonated forms. The contribution of cyclo-DOPA-5-*O*- β -D-glycoside to the antiradical activity of betanin at basic solution can not be also excluded. The calculated OH BDE and IP values of different mono-, di- and tri-deprotonated forms of betanin show that with the increasing degree of deprotonation of betanin molecule, the BDE and IP values significantly decrease. It

1 implies that with higher pH betanin becomes better hydrogen and electron donator what
2 results in the increase in its free radical scavenging activity. Analysis of resonance structures
3 of betanin reveals strong electronic conjugation between betalamic acid and cyclo-DOPA-5-
4 *O*- β -D-glucoside moieties what is an additional factor contributing to the observed high
5 antiradical activity of betanin. Altogether, the calculated electronic parameters give more
6 insight into the mechanism of action of betanin as a exceptionally good free radical scavenger.

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8 The high antiradical activity of betacyanin pigments at wide pH range encourages wider
9 application of these natural dyes in food, cosmetic and pharmaceutical products.

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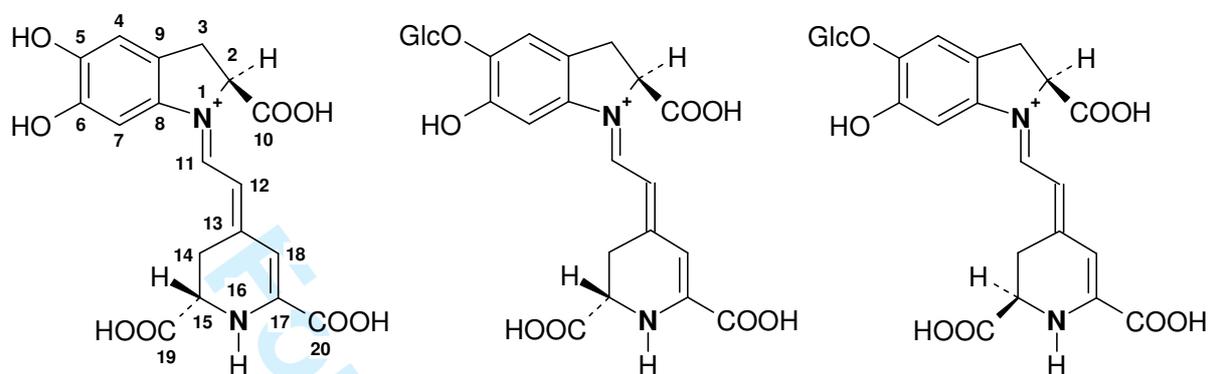
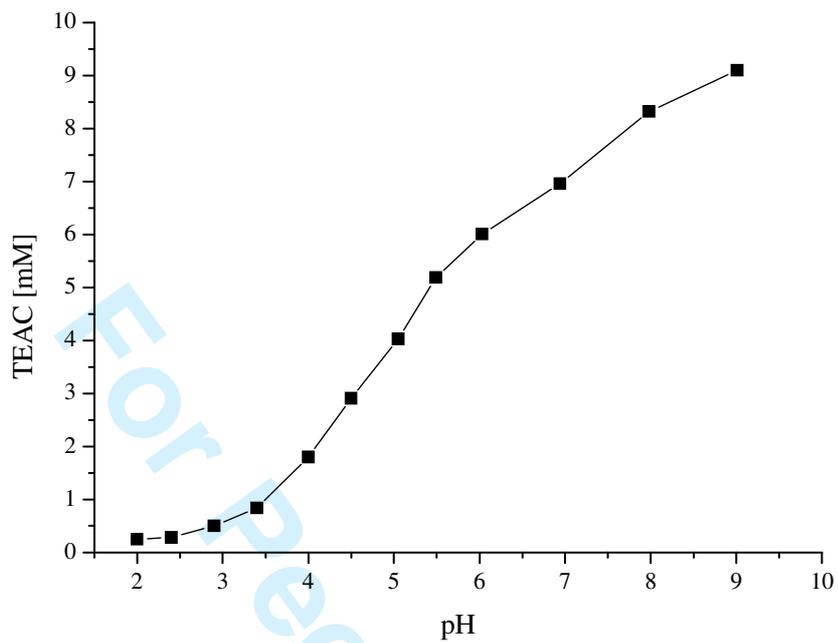


Figure 1. Chemical structures and atom numbering system of betanidin, betanidin 5-O-β-D-glucoside (betanin) and isobetanim.



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Figure 2. pH-dependent antiradical activity of betanin observed in the TEAC assay.

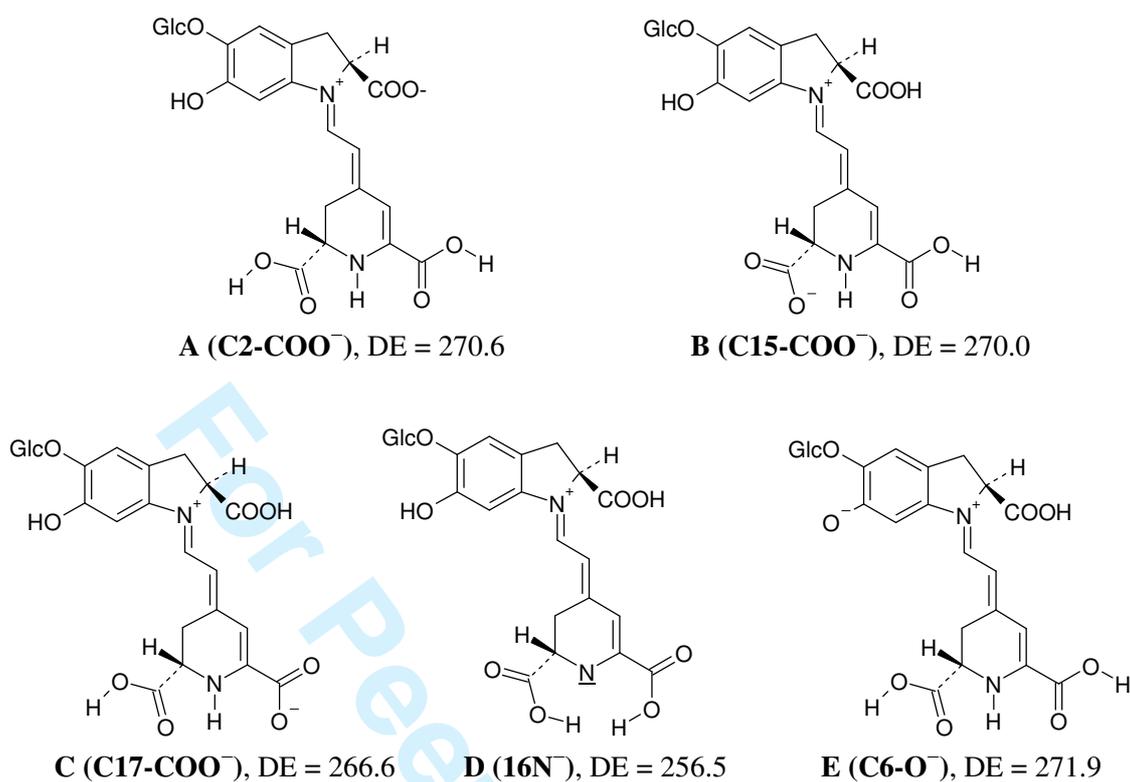


Figure 3. Structures (A, B, C, D, E) of deprotonated betanin molecule and calculated corresponding deprotonation energies (DE, kcal/mol).

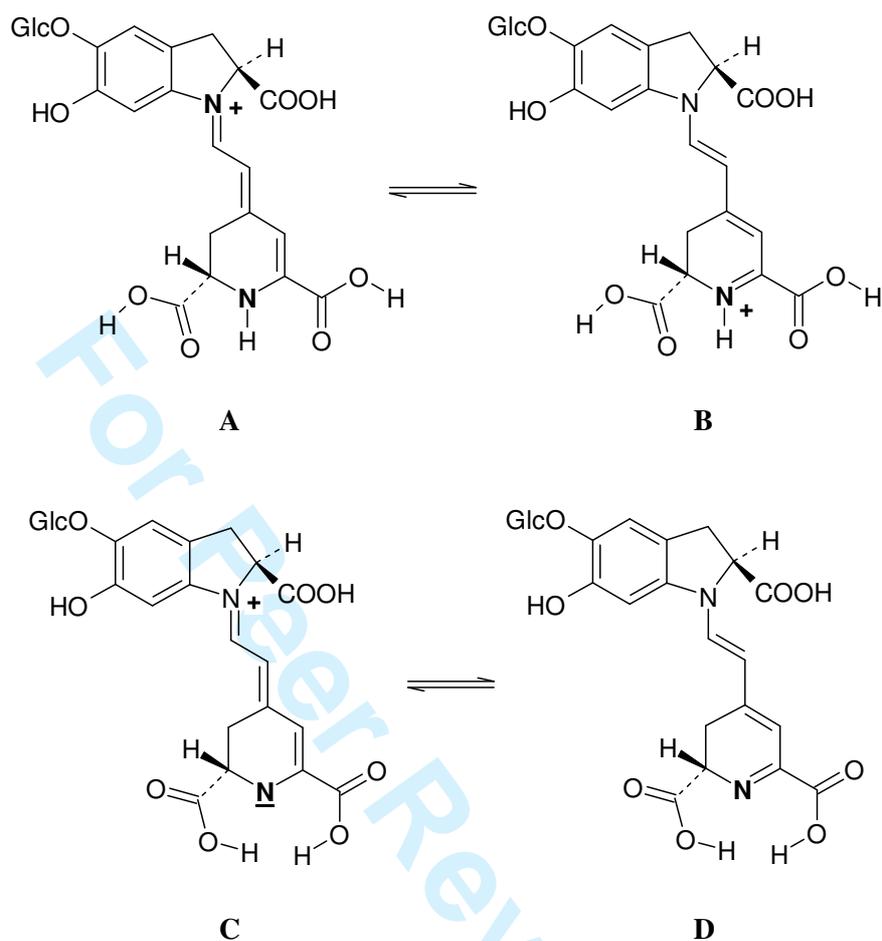


Figure 4. Resonance structures of betanin in cationic state (A, B) and in 16N⁻ deprotonated form (C, D).

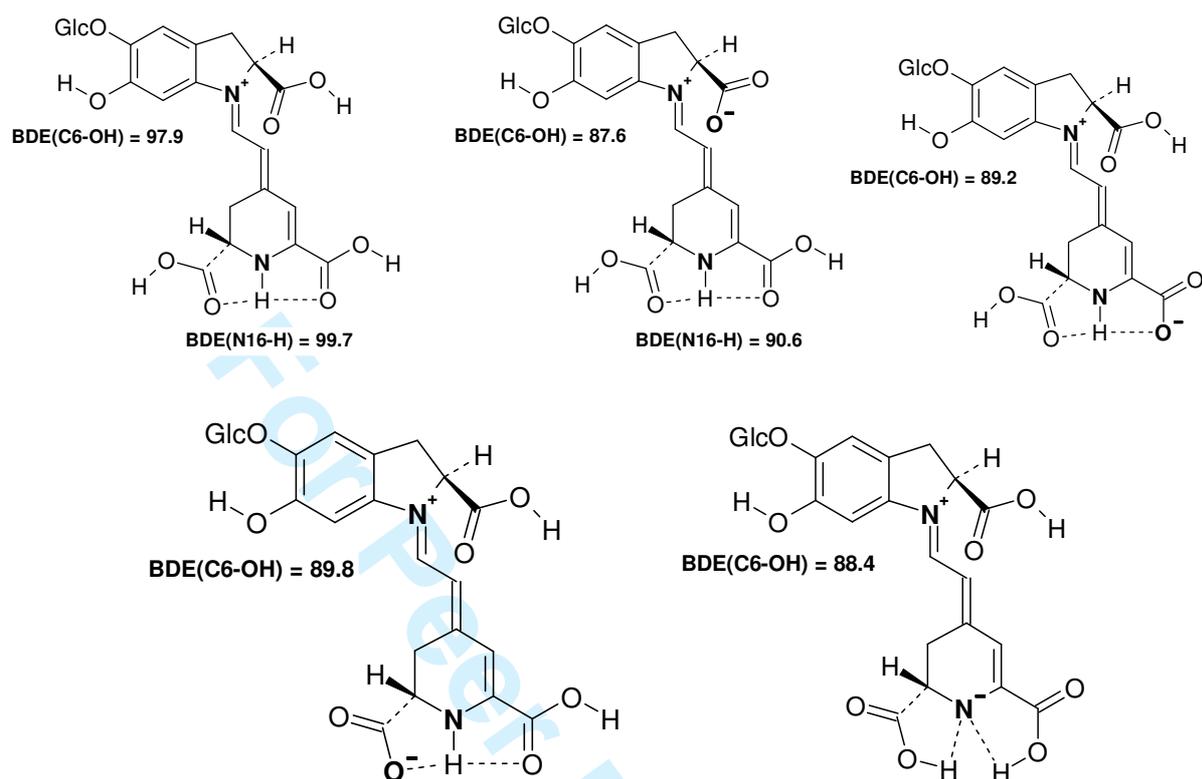


Figure 5. Comparison of bond dissociation energies (BDE, kcal/mol) calculated for betanin in cationic and various mono-deprotonated states.

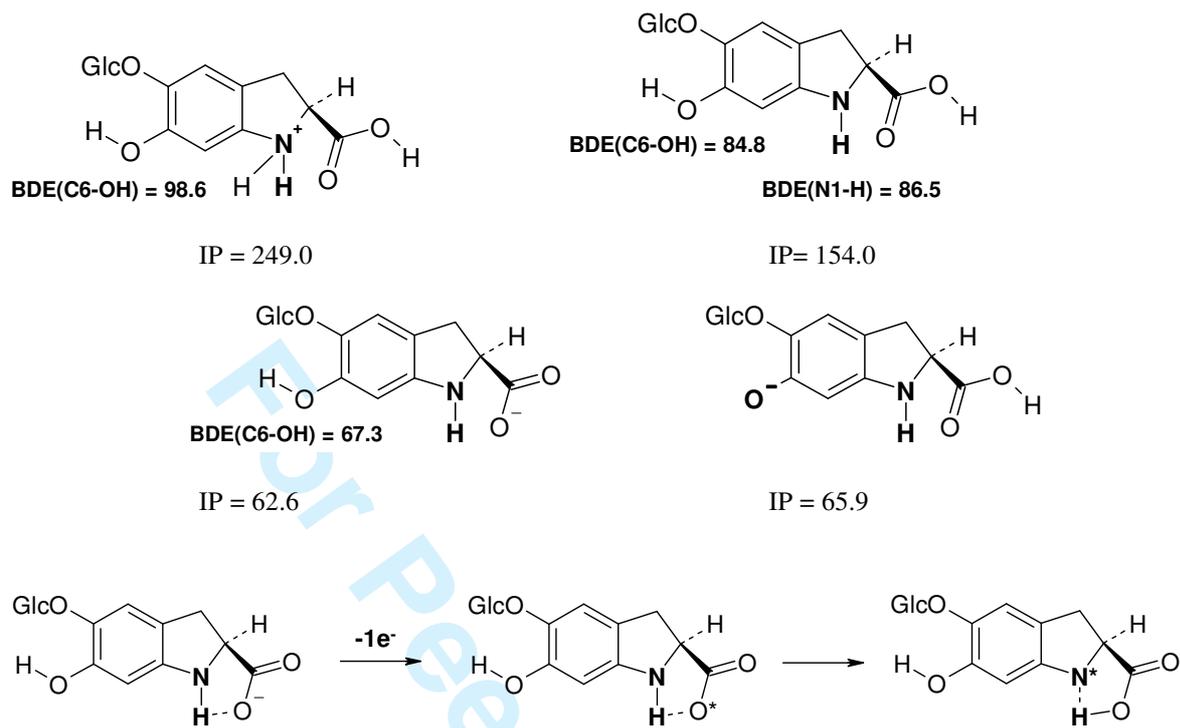


Figure 6. Calculated bond dissociation energies (BDE, kcal/mol) and ionization potentials (IP, kcal/mol) for cyclo-DOPA-5-O- β -D-glucoside in cationic and mono-deprotonated (at C2-COOH) state. Stabilization of deprotonated molecule by proton transfer from N-1 to COO⁻ group upon an electron abstraction is also shown.

Table I. Calculated thermochemical parameters for betanin in different deprotonation states: relative stability with respect to most stable structure assumed as „0.0”, ionization potential (IP, kcal/mol), bond dissociation energy (BDE, kcal/mol)

Pattern of betanin deprotonation	Relative stability	BDE	IP
Cation		97.9 (C6-OH) 99.7 (16N-H)	219.3
Mono-deprotonated			
C2-COO ⁻	14.1	87.6 (C6-OH) 90.6 (16N-H)	-
C15-COO ⁻	13.6	89.8 (C6-OH)	-
C17-COO ⁻	10.1	89.2 (C6-OH)	-
16N ⁻	„0.0”	88.4 (C6-OH)	158.3
C6-O ⁻	15.4	-	141.1
Di-deprotonated			
C2-COO ⁻ , C17-COO ⁻	11.8	78.2 (C6-OH)	-
C2-COO ⁻ , C15-COO ⁻	16.2	79.3 (C6-OH)	-
C2-COO ⁻ , C6-O ⁻	13.2	70.6 (16N-H)	83.9
C2-COO ⁻ , 16N ⁻	7.6	76.2 (C6-OH)	-
16N ⁻ , C6-O ⁻	„0.0”	-	83.9
C15-COO ⁻ , 16N ⁻	18.6	74.9 (C6-OH)	-
C17-COO ⁻ , 16N ⁻	18.8	75.3 (C6-OH)	-
C17-COO ⁻ , C6O ⁻	5.7	88.4 (16N-H)	89.1
C15-COO ⁻ , C6O ⁻	10.5	-	88.3
C15-COO ⁻ , C17-COO ⁻	14.9	79.6 (C6-OH)	-
Tri-deprotonated			
C2-COO ⁻ , C15-COO ⁻ , C17-COO ⁻	15.8	65.0 (C6-OH)	-
C2-COO ⁻ , 16N ⁻ , C6-O ⁻	„0.0”	-	28.0
C2-COO ⁻ , C15-COO ⁻ , C6-O ⁻	5.1	-	34.5
C2-COO ⁻ , C17-COO ⁻ , C6-O ⁻	2.1	81.2 (16N-H)	32.0
C2-COO ⁻ , C15-COO ⁻ , 16N ⁻	26.5	58.1 (C6-OH)	-
C2-COO ⁻ , C17-COO ⁻ , 16N ⁻	22.0	61.4 (C6-OH)	-
C17-COO ⁻ , 16N ⁻ , C6O ⁻	5.5	-	32.7
C15-COO ⁻ , 16N ⁻ , C6O ⁻		-	29.9

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3 **List of Figures:**
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6 3 Figure 1. Chemical structures and atom numbering system of betanidin, betanidin 5-*O*- β -D-
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4 relative stability with respect to most stable structure assumed as „0.0”, ionization potential
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