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Abstract

In the present study, the pH-dependent free radical scavenging activity of betanin in the TEAC (Trolox equivalent antioxidant capacity) assay was determined. It was found that at pH > 4 betanin is about 1.5-2-fold more active than some anthocyanins considered very good free radical scavengers as determined in the TEAC assay. The increase in the TEAC values of betanin with increasing pH is discussed in terms of its calculated phenolic OH homolytic bond dissociation energy (BDE) and ionization potential (IP). The results suggest that the exceptionally high antioxidant activity of betanin is associated with increasing of its H-donation and electron-donation ability when going from cationic state to mono-, di- and tri-deprotonated states present at basic solutions.

Keywords: *Betalains, betanin, pH dependent free radical scavenging activity, TEAC value, DFT calculations*

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 betaxanhins (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 glucosilated. 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

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

Figure 1

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

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

processed foods, especially in ice creams and frozen desserts because it colours without changing the flavor profile.

In the present study betanin – the main red-purple pigment of red beet was investigated in relation to its antioxidant properties. The free radical scavenging activity of betanin at different pH (from 2 to 9) in so called TEAC (Trolox equivalent antioxidant capacity) assay is discussed in terms of its phenolic OH homolytic bond dissociation energy (BDE) and ionization potential (IP). These and some other relevant thermochemical parameters, obtained at DFT B3LYP/6-311+G**//B3LYP/6-31G** level of quantum mechanical calculation, characterizing betanin, are reported for the first time.

Materials and methods

Chemicals

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

Plant material

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

Preparation of betanin

Betacyanins and betaxanthins were separated by gel filtration on a Sephadex G-25 column (40 x 2.2 cm) essentially as described by Kanner et al. (2001). Briefly, 5 ml of beetroot juice were eluted with 1% acetic acid and 2.5 ml fractions were collected. The absorption spectra of the fractions were measured (Genesys 6, ThermoSpectronic, USA) and evaluated for the presence of betacyanins and betaxanthins. The purity of isolated pigments was analysed using high-performance liquid chromatograph (Waters, Millford, Ma, USA) equipped with Nova-Pak C_{18}

column (3.9 x 150 mm, 5µm, Waters, Millford, Ma, USA) fitted with µBondapak C_{18} cartridge guard column (Waters, Millford, Ma, USA). A mobile phase and elution gradient of Tesoriere et al. (2004) was used: a 20 min linear gradient elution from solvent A (1.0% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile) with flow rate 1 ml/min. The eluate was monitored using the Waters 996 photodiode-array (PDA) detector set at 535 nm and 480 nm for betacyanins and betaxanthins, respectively. Betacyanin fractions were combined and HPLC analyses revealed that betanin with small amount of compound identified as isobetanin (~10% of the isolated betanin) were the only betacyanin components of the betacyanin fraction. The purity of betanin measured at 270 nm was about 86%. The purity of betanin and isobetanin was confirmed by UV-VIS spectra, measured by PDA detector in the range of 210-700 nm, which were in agreement with those published in literature (Cai et al. 2001). Betanin was freeze-dried and kept at –20^{o}C under nitrogen until use.

TEAC assay

The TEAC assay is based on the ability of the antioxidant to scavenge the blue-green coloured $ABTS^{\bullet+}$ (2,2'-azinobis(3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt) radical cation relative to the $ABTS^{\bullet+}$ scavenging ability of the water-soluble vitamin E analogue, Trolox (Miller et al. 1993). The antioxidant activity of betanin was measured by the modified TEAC assay performed essentially as described previously (Miller et al. 1993), with some modifications (Tyrakowska et al. 1999). In the present study microperoxidase-8 (MP8) instead of metmyoglobin, was used to generate the $ABTS^{\bullet+}$ in PBS (0.01 M phosphate buffer, 0.14 M NaCl, 0.002 M KCl) pH 7.4. MP8 (final concentration 0.2 µM) and ABTS (final concentration 3.0 mM) in PBS were mixed and the reaction was initiated by the addition of hydrogen peroxide (final concentration 0.1 mM). The major advantage of the modified TEAC assay is that it permits studies of radical scavenging activity over a wide pH range (2 – 9.5) (Tyrakowska et al. 1999).

The $ABTS^{\bullet+}$ solution thus obtained was diluted 1:1 (v/v) using 0.2 M potassium phosphate buffers of various pH values to give $ABTS^{\bullet+}$ solutions at pH values varying between 2 and 9 (an absorption was about 0.6 at 734 nm). The $ABTS^{\bullet+}$ solutions thus obtained were used for determination of the TEAC values. Antioxidants (Trolox or betanin) were added as 1% (v/v)

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

Quantum mechanical calculations

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

Results and discussion

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

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

Figure 2

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

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

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

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

Figure 4 and 5

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

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

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

Figure 6

Conclusions

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-glucoside 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

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

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

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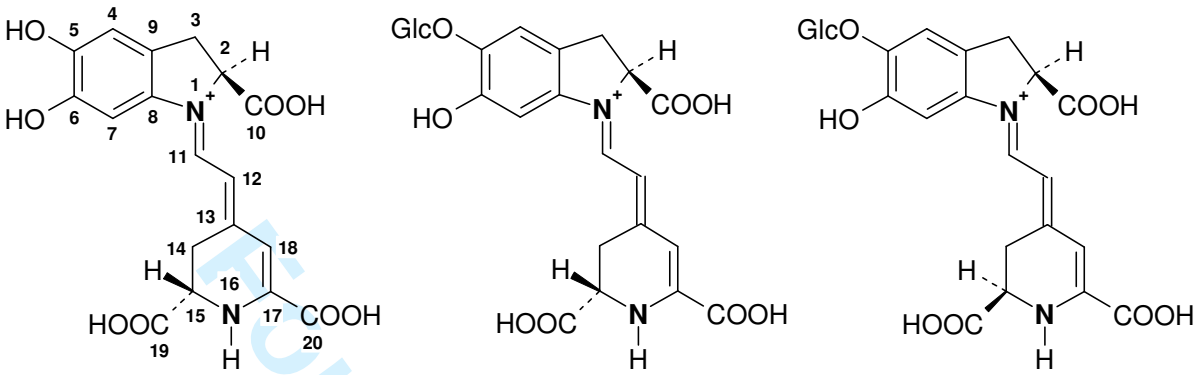

Figure 1. Chemical structures and atom numbering system of betanidin, betanidin 5-*O*-β-D-glucoside (betanin) and isobetanin.

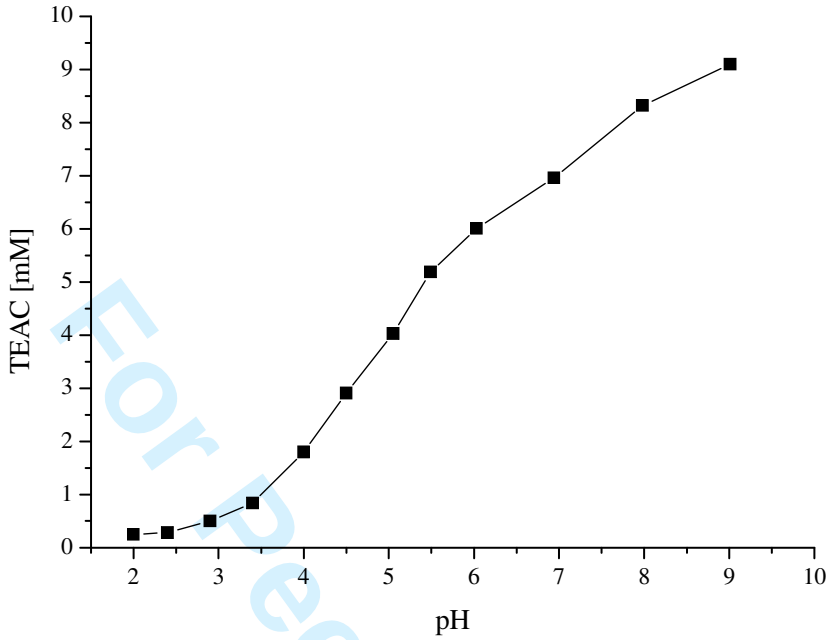

Figure 2. pH-dependent antiradical activity of betanin observed in the TEAC assay.

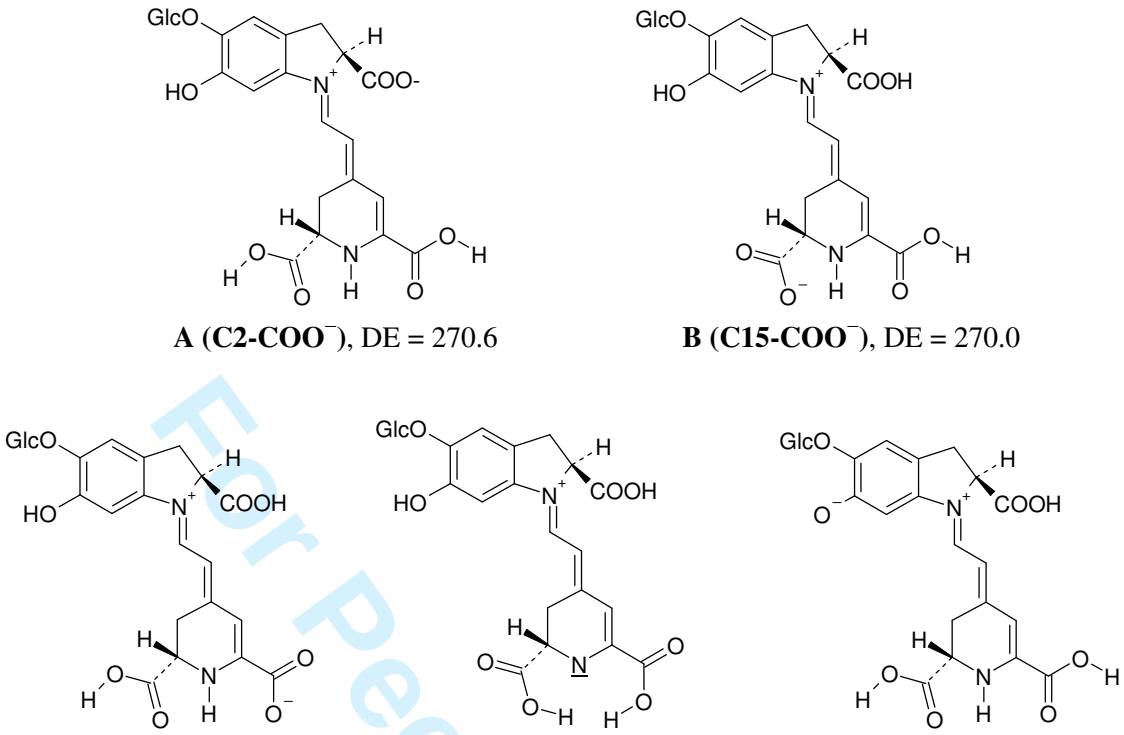

Figure 3. Structures (A, B, C, D, E) of deprotonated betanin molecule and calculated corresponding deprotonation energies (DE, kcal/mol).

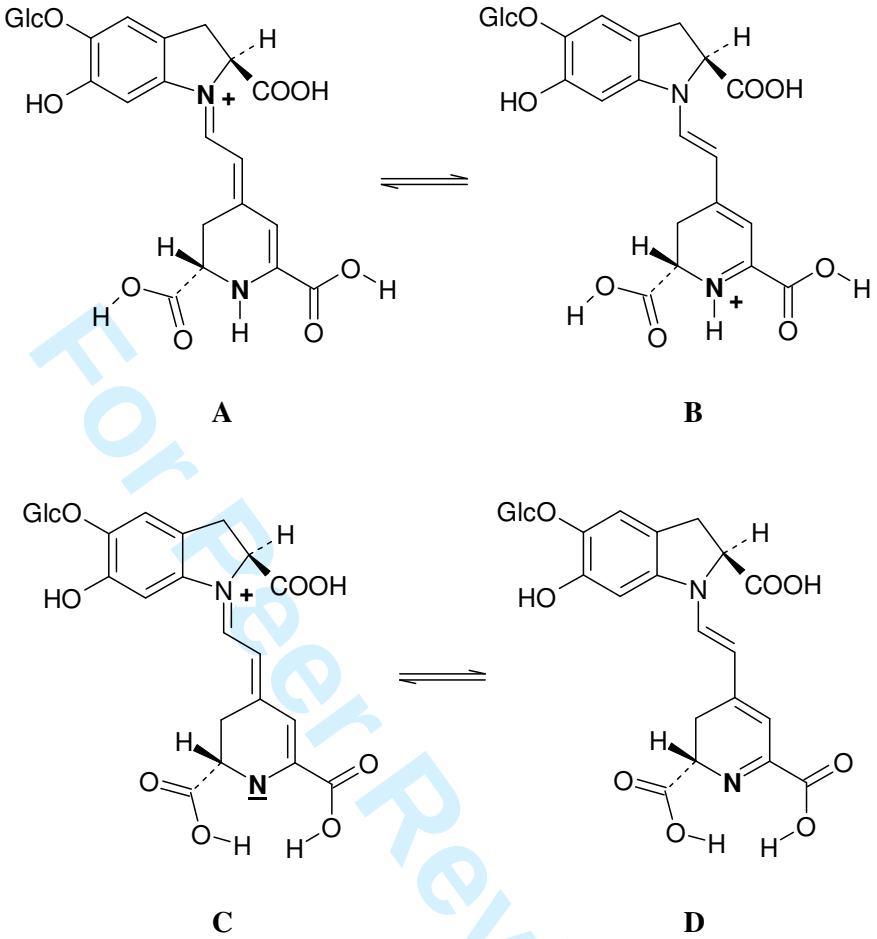

Figure 4. Resonance structures of betanin in cationic state (A, B) and in 16N⁻ deprotonated form (C, D).

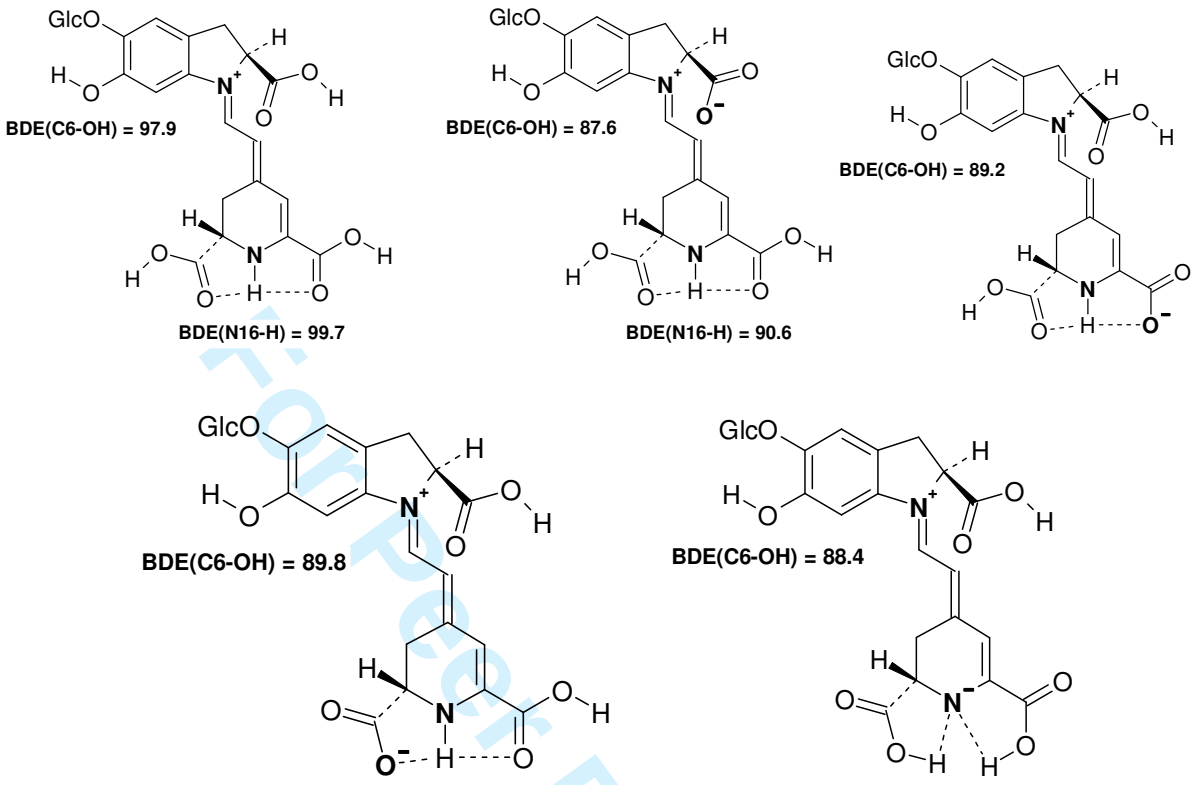

Figure 5. Comparison of bond dissociation energies (BDE, kcal/mol) calculated for betanin in cationic and various mono-deprotonated states.

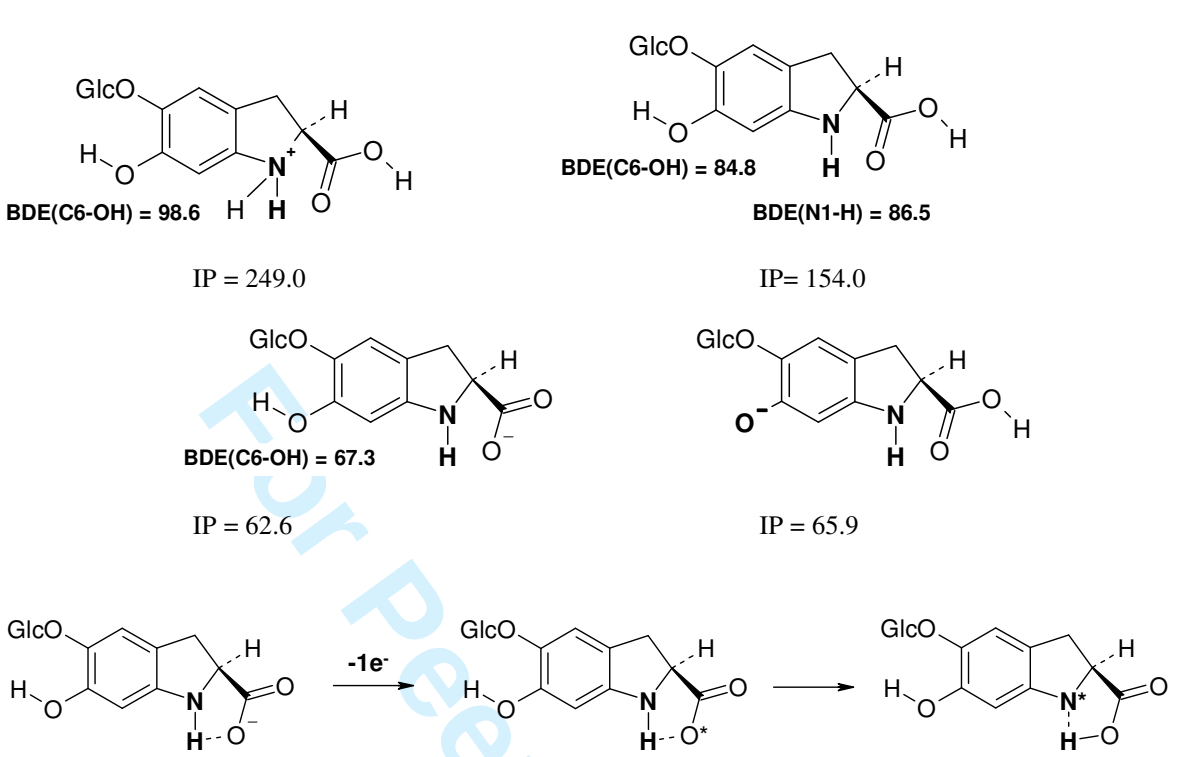

Figure 6. Calculated bond dissociation energies (BDE, kcal/mol) and ionization potentials (IP, kcal/mol) for cyclo-DOPA-5-*O*-β-D-glucoside in cationic and mono-deprotonated (at C2-COOH) state. Stabilization of deprotonated molecule by proton transfer from N-1 to COO^- group upon an electron abstraction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