Peculiarities of Betacyanins as Antioxidant Colorants in Stability and Analytical Chemistry

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Abstract: Betalains are natural food colorants with high antioxidant activity. But the compounds are characterized by easiness to decondensation to achieve equilibrium of the reaction after dilution in solutions as food colorants. The aim of paper was to find molecular centers responsible for antioxidant activity by quantum chemical calculations. The calculations were performed by PM3 method in HyperChem 8.0 software. It was found that these are alpha C-H bonds to be broken to give radicals with less energy instead of phenolic OH groups commonly referred to as the most active sites of antioxidants. According to results of the calculations, the condensation reaction must not have a great impact upon colorant antioxidant activity. The assumptions made were confirmed by experimental results of the determination of antioxidant activity by Folin-Ciocalteu method with reagent are highly sensitive to reducing agents. Indeed, purified by solid-state extraction betacyanins of Beta vulgaris and of Opuntia joconostle really loosed the color but not an antioxidant activity for first two days of storage. It can be concluded that the trend of betalains for decondendation to reach the equilibrium of condensation-decondensation reaction after colorant solution dilution leads to decrease of color intensity (and betalain concentration) but only slightly affects the antioxidant activity.

Keywords: cálculos químicos quânticos, método semi empírico PM3, betalaínas, atividade antioxidante, reagente de Folin-Ciocalteu

The antioxidant activity of food is becoming one of the most important functions of food in today's urbanized society. It is known that the cause of many diseases is the oxidation of biomolecules, membranes and tissues of living cells by active oxygen-containing particles [1]. As a result of these processes, it became necessary to correct the human diet to achieve increased antioxidant activity and nutritional value, thus one of these directions is "functional food", obtained by enriching ordinary products with various substances important for human health, including antioxidants [2]. Natural water-soluble antioxidants include ascorbic acid, anthocyanins, some other phenolic compounds, and betacyanins. The antioxidant properties of betacyanins have been studied in a number of papers [3,4], and it was found that at high pH, this property of betacyanins is higher even compared to anthocyanins [4]. The detailed report [5] provides information about the use of betacyanins in encapsulated form (containing 0.3 or 1% of betacyanins) or liquid concentrates (containing 60-65% solids) as dyes in the food industry to date. Red beet dyes are used for coloring dairy products such as yogurt and ice cream, salad dressings, lollipops and fruit gum, glazes, cake mixes, gelatin desserts, starch-based puddings, meat substitutes, poultry sausages, sauce mixes, soft drinks and powdered beverage mixes.

Anthocyanins can be used as dyes only in the high possible acidic environments, i.e. in the

area of the exploration of these compounds is restricted by the only flavylium form, betacyanins are not so sensitive to pH and can be used in a wide range of pH (from acidic to alkaline environments), which significantly expands the possibilities of their use as dyes. But one has to take into account the relatively low stability of betacyanins towards to the decondensation reaction [6] – conversion to a mixture of the initial cyclo-dihydroxyphenylalanine-5-β-O-glucoside and betalaimic acid, Fig. 1. In this reaction, the chain of conjugation is broken and the color disappears. It is clear that the higher the concentration of betacyanins leads to the lower the degree of such decondensation.

On the other hand, it was found by ABTS {(2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid))diammonium salt} radical cation decolorization assay [7] that the antioxidant activity of indicaxanthin (a product of betalamic acid condensation with proline) was significantly lower compared to betacyanin (1.76 vs 20 in Trolox equivalents). The monophenol nature of betanin was proposed to be the reason of a higher H-atom or electron donation potential. The latter was found by observed redox potential determined for the two compounds by cyclic voltammetry. In another investigation by the modified DPPH· (1,1-diphenyl-2-picrylhydrazyl) [1] antioxidant activity of the tested betalains decreased in the following order: simple gomphrenins> acylated gomphrenins>dopaminebetaxanthin> (S)-tryptophan-betaxanthin> 3-methoxytyramine-betaxanthin>betanin/isobetanin>celosianins>iresinins> amaranthine/isoamaranthine.

The aim of the research is an investigation of the dependence of the antioxidant activity of betacyanins measured by Folin-Ciocalteu method during decondensation in aqueous solutions and calculation of some quantum-chemical descriptors of the activity.

Materials and Methods
Materials and reagents

Beta vulgaris roots were purchased in local market while *Opuntia joconostle* fruits were brought from Krasnodar region (Tuapse).

Extraction and isolation

Betacyanins were extracted from plant material by infusing in acidified water (pH 1-2) and purified by solid phase extraction on DIAPAK C18 cartridges (BioChemMack ST, RF). Extact was passed through concentrating syringe cartridge after activation with acetone and conditioning with extractant till red colored eluate appiarence. Betacyanins were reextracted by water – ethanol mixtures (1:1).

In the series of antioxidant activity measurement solution of betalains were stored in refrigerator at 4°C.

Determination of betacyanin concentration

Concentration of betacyanins in samles under investigation was determined by spectrophotometric method [14] after a proper dilution by water for measurement of optical density in quatz cuvette by Shimadzu UV 2550 spectrophotometer not exsseeding 1.1.

Determination of anthioxidant activity

Antioxidant activity was determined by Folin-Ciocalteu method [14] and expressed as caffeic acid equivalent. Briefly, to 1 ml of 10 % of water solution of $Na_2CO_3 \cdot 10H_2O$ was edded 1 ml

of Folin-Chiocalteu solution and 50 – 200 mcl of sample/ The mixture was maintained at room temterature for 30 min and after addition of stop-reagent was made up to 5 ml volume with water. Extracts of plant material were diluted by water before the measurement to echiveve optical desity of solutions not exceeding 1.0 at 760 nm in quatz cuvette by Shimadzu UV 2550 spectrophotometer.

Quantum chemical calculations

Quantum chemical geometry optimization and energies calculation were performed in HyperChem8.0softwareby PM3 (UHF) method with Polak-Ribery conjugate gradient algorithm.

Results and Discussion
Quantum chemical calculations

The atom numbering in betacyanins backbone is presented in Figure 1. For betalamic acid for more convenient comparison the numbering was remained as in the product of the acid condensation with cyclo-DOPA component.
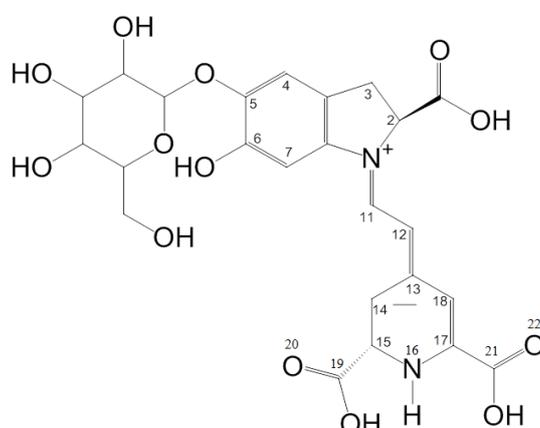

Figure 1. Atoms numbering in betanidin-6-hexoside

It is a common approach to appreciate the antioxidant activity by the easiness of hydrogen atom cleavage form phenolic OH-groups of compounds [10]. Meanwhile it is also well known that oxidation of unsaturated compound often includes C-H bonds at carbon atoms directly connected with unsaturated sp^2-carbon atoms (alpha C-H bond). There are four different alpha C-H bonds in betacyanins structure, in positions 2, 3, 14 and 15. Energies of radicals formed by H atom withdrawal in these positions are presented in Table 1 together with energies for cleavage of H atoms from OH-groups.

According to the data the withdrawal of the hydrogen atom is carried out from alpha C-H bond markedly easily than from phenolic OH-bonds. The most active site is localized at C15 position, requiring energy costs more than 20 kcal/mol less than that for phenolic OH bond in position 6. For phenolic OH-bond in position 5 for gomphrenin type of betacyanins (for betanidin-6-hexoside in Table 1) the required energy in turn increases by more than 20 kcal/mol compared for betanin type (for betanidin-5-hexoside in Table 1), what contradicts to experimentally obtained increase of antioxidant activity form betanin to gomphrein metalains type [8]. But for formation of radicals for gomphrein type at position 15 the amount of energy required is 1.2 kcal/mol less than for betanin type. It is in consistence with experimental data

qualitatively, but for quantitative consistency one need to explore another approach that will be discussed later. For another position of radical formation by cleavage of alpha C-H bond energies are also lower than for phenolic hydrogen atom. The second position according to hydrogen atom easiness of cleavage is at the carbon atom number 2, being also less energy expansive compared to phenolic hydrogen atom.

Table 1
ENERGIES OF CATION-RADICALS OF BETALAINS

No.	Position of hydrogen atom cleavage	Energies of cation-radical, kcal/mol	
		Betanidin-5-hexoside	Betanidin-6-hexoside
Hydrogen atoms of phenolic groups in position			
1	5	-	-6753.447 (101.399)
2	6	-6771.275 (80.150)	-
Hydrogen atoms of α-C-H groups in position			
3	2	-6783.296 (68.129)	-6788.126 (66.720)
4	3	-6778.622 (72.803)	-6781.790 (73.056)
5	14	-6783.0022 (68.403)	-6777.383 (77.463)
6	15	-6792.462 (58.963)	-6797.069 (57.777)
Initial state		-6851.425	-6854.846

Formation of radical at carbon atom No. 15 may be the reason of fast decarboxylation of COOH group in position 17 [11] due to the unpaired electron delocalization in conjugated system that includes this atom as well as 16-nitrogen atom (Fig. 2).

Likewise, the formation of radical at carbon atom No. 2 should results in easy decarboxylation but only at this atom because of the absence of unpaired electron localization for this position and existence of the corresponding COOH group in the state of dissociation at pH higher than 2 [12]. Thus, a role of phenolic OH groups in antioxidant activity of betacyanins is not so pronounced as that of alpha C-H groups. By the way, it explains the higher antioxidant activity of betacyanins compared to anthocyanins [13] that have no such types of groups in flavylium and pseudobase forms.
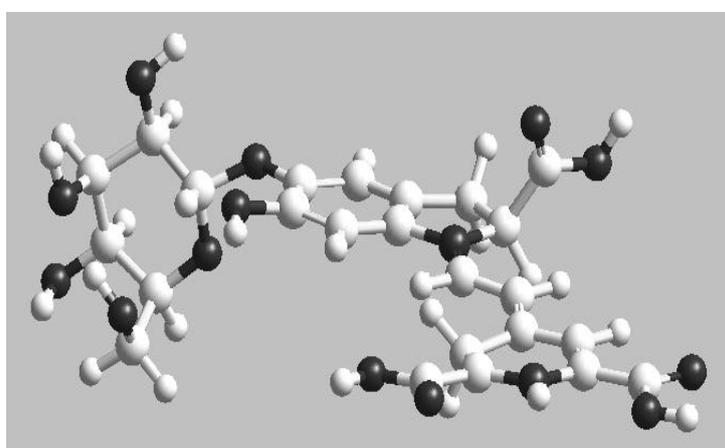

Figure 2. Structure of betanin type radical at carbon No. 15

For elucidation of influence of carbohydrates moieties upon betacyanin antioxidant activity energies of radicals obtained by hydrogen atom cleavage at C-H and O-H groups was also calculated (Table 2). In this case, also expenses of energy were some 20 kcal/mol less for the

former case, exceeding that for alpha C-H bonds of the betacyanin backbone. Thus glycosylation must not have pronounced impact upon betacyanins antioxidant activity unless steric factors will hinder the active cites of betacyanins. May be, this a reason of a sudden drop of antioxidant activity of amaranthin having large glycosidic substituent.

For betacyanins being unstable towards to reaction of decondensation resulting in equilibrium concentrations of starting betacyanin, cyclo-DOPA component and betalamic acid [14] that may be detected even during HPLC separation [6] it is essential to investigate a change of antioxidant activity during this process.

Thus, if the equilibrium of condensation reaction for betacyanin synthesis is not disturbed by oxygen degradation of the three compounds the total antioxidant activity of the sum of compound concentrations must not be markedly affected by the state of equilibrium.

Table 2
ENERGIES OF RADICALS OF CARBOHYDRATE MOIETY OF CYCLO-DOPA-5-HEXOSIDE

No.	Position of hydrogen atom cleavage	Energies of radicals, and difference between the energies of radicals and initial state in parentheses, kcal/mol
Hydrogen atoms of OH-groups in position		
1	2′	-4466.057 (96.290)
2	3′	-4466.017 (96.330)
3	4′	-4464.984 (97.363)
4	6′	-4468.586 (93.761)
Hydrogen atoms of C-H groups in position		
5	1′	-4488.759 (73.588)
6	2′	-4491.551 (70.796)
7	3′	-4491.550 (70.797)
8	4′	-4490.721 (71.626)
9	5′	-4491.177 (71.170)
10	6′	-4489.101 (73.246)
Initial state		-4562.347

Cyclo-DOPA component
The most active position for hydrogen atom cleavage remain the same just as in betalains (Table 3). Moreover there is no essential difference of the requite energies for different direction of glycosylation (positions 5 or 6). C-H bond at position 2 is the most active in the both cases showing a small rise of positon activity compared to condensed betalain.

Betalamic acid component
For betalamic acid position 15 remains more acceptable to hydrogen cleavage if the atom numbering is still as in condensed compound. Moreover the energy requirements are also close: 59.742, 58.963 and 57.777 kcal/mol for betalamic acid, betanin and gomphrenin types of betanidine glycosylation (Table 4).

Table 3
ENERGIES OF RADICALS OF AMINOACID COMPONENT OF BETALAINS

No.	Position of hydrogen atom cleavage	Energies of cation-radical, difference between the energies of radicals and initial state in parentheses, kcal/mol

		Cyclo-DOPA-5-hexoside	Cyclo-DOPA-6-hexoside
Hydrogen atoms of phenolic groups in position			
1	5	-	-4490.518 (71.666)
2	6	-4490.522 (71.826)	-
Hydrogen atoms of α-C-H groups in position			
3	2	-4496.389 (65.958)	-4498.132 (64.052)
4	3	-4489.973 (72.374)	-4489.581 (72.603)
Initial state		-4562.347	-4562.184

Table 4
ENERGIES OF CATION-RADICALS OF BETALAIMIC ACID

No.	Position of hydrogen atom cleavage	Energies of cation-radical, difference between the energies of radicals and initial state in parentheses, kcal/mol
Hydrogen atoms of carboxylic groups in position		
1	15*	-2498.763 (104.202)
2	17*	-2497.809 (105.156)
Hydrogen atoms of α-C-H groups in position		
3	14*	-2533.454 (69.511)
4	15*	-2543.223 (59.742)
Initial state		-2602.965

According to the literature data antioxidant capacity of betalains exceeds that of ascorbic acid by some times. One may take into account that ascorbic acid quickly gives up two electrons converting to inert towards ordinary oxidants product. Hence, betalains exerts some stages of chemical transformations producing successively new antioxidants in series [4]. In this case any descriptors for the starting compound instead of descriptors for all consecutive antioxidants in the series may be senseless.

The obtained results differ from published investigations [5] on quantum chemical calculation not only by simpler and less time-consuming semi empirical method but also by accents in calculation upon alpha C-H bond cleavage. Though results of calculation of ionization potential [5] are interesting for electron-donation mechanism of antioxidant activity estimation, as well as electron-donation possibility to be strengthen by carboxylic group dissociation with creation of negatively charged betalain species at pH of solution increase [5].

Experimental estimation of antioxidant activity
Folin-Ciocalteu method (FCM) [3] is ordinary applied for the determination of the total phenolic compounds content in different samples. Meanwhile from chemical point of view appearance of blue color of solution containing active Folin-Ciocalteucompoundare the consequence of reduction of its heteropolymolybdates and tungstates by any proper reducing substances, e.g. ascorbic acid. Thus, Folin-Ciocalteu reagent acts as mild oxidant and is capable to quantitative estimate the most active antioxidant activity of any samples.

The utilization of betalains as food colorants is accompanied with colorant dilution. But for the substances solutions trend to equilibrate concentrations of compound involved into process of condensation-decondensation must decrease the mole fraction of betalain with dilution at first stage and the further decrease due to oxidation is not unexpected.

The antioxidant activity towards FCM was estimated as caffeic acid equivalent. The acid was

chosen because of existence in its structure of pair of ortho hydroxyl groups, enabling high antioxidant activity to any phenolics [3]. Simultaneously betacyanin content in extracts under investigation were monitored by spectrophotometric method.

The results for beta vulgaris extract are presented in Figure3. It is obvious that decrease of betacyanin concentration at first 2 days of extract storage results due to striving for equilibration of the three substances concentration (cyclo-DOPA component, betalamic acid and betanin) equilibrium in the direction of decondensation. At this period antioxidant activity decreases by substantially lower value (2% vs 20%) as it was predicted by quantum chemical calculations. The subsequent drop of both measured parameters may be the consequence of irreversible oxidation of the three compounds.

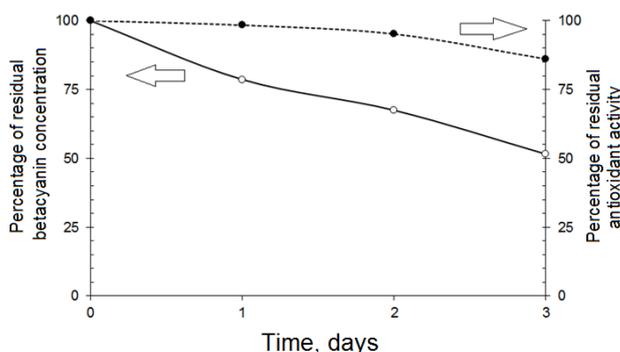The similar results were obtained for Opuntia fruits extract (Fig. 4).

Figure 3. Time of storage influence upon content and antioxidant activity of red beet betacyanins
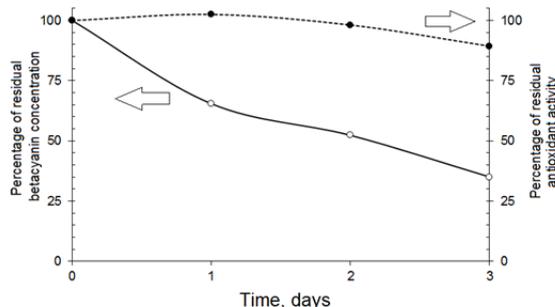

Figure 4. Time of storage influence upon content and antioxidant activity of opuntia betacyanins

High stability of antioxidant activity is accompanied by substantial drop of betacyanins content possibly due to establishing of equilibrium of condensation reaction for solutions storage for 2 days just as in the upper case. Thus, a loss of betacyanins coloration not necessary must lead to loss of antioxidant activity if betacyanins are used as food colorants.

Conclusion

It has been found that betalain antioxidant activity may be determined not by quantity of phenolic OH groups but rather by quantity and energy parameters of hydrogen atom cleavage from alpha C-H bonds. The trend of betalains for decondendation to reach the equilibrium of condensation-decondensation reaction after colorant solution dilution leads to decrease of color intensity (and betalain concentration) but only slightly affects the antioxidant activity.

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