JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

# New Pathways of Betanidin and Betanin Enzymatic Oxidation

Sławomir Wybraniec* and Tadeusz Michałowski

Department of Analytical Chemistry, Institute C-1, Faculty of Chemical Engineering and Technology, Cracow University of Technology, ul. Warszawska 24, Cracow 31-155, Poland

**ABSTRACT:** Betanidin is a basic betacyanin with a 5,6-dihydroxyl moiety which causes its high antioxidant activity. For the purpose of structural study, the enzymatic oxidation of betanidin and betanin (5-*O*-glucosylated betanidin), followed by chromatographic separation of the oxidation products with spectrophotometric and mass spectrometric detection (LC-DAD-MS/MS) was performed. Within the pH 4−8 range, two main oxidation peaks of betanidin were observed, betanidin quinonoid (possibly betanidin *o*-quinone) and 2-decarboxy-2,3-dehydrobetanidin, whereas at pH 3 only dehydrogenated and decarboxylated derivatives were detected, suggesting different stabilities of the products at different pH values. The presence of two prominent oxidation products, 2-decarboxy-2,3-dehydrobetanidin and 2,17-bidecarboxy-2,3-dehydrobetanidin, at pH 3 indicates their generation via two possible reaction routes with two different quinonoid intermediates: dopachrome derivative and quinone methide. Both reaction paths lead to the decarboxylative dehydrogenation of betanidin. Subsequent oxidation and rearrangement of the conjugated chromophoric system results in the formation of 14,15-dehydrogenated derivatives. Betanin is oxidized with generation of a quinone methide intermediate, which rearranges to 2,3-dehydro- or neoderivatives. The products of enzymatic oxidation of betacyanins thus formed are derivatives of 5,6-dihydroxyindole and related structures known as the key intermediates in melanogenesis.

**KEYWORDS:** betanidin, betanin, betalains, betacyanins, melanogenesis, antioxidation activity, 5,6-dihydroxyindole, dopachrome, aminochrome, quinone methide

## INTRODUCTION

Betalains, water-soluble plant pigments, belonging to most families of the Caryophyllales,[1] are extensively used in the food industry as colorants.[2] In addition, their chemopreventive and strong antioxidant properties [3−6] stimulate research on their new structures, derivatives, and especially their influence on health. Betalains are immonium conjugates of betalamic acid with cyclo-DOPA (forming betanidin) or glycosylated cyclo-DOPA (forming other betacyanins) as well as amino acids or amines (forming betaxanthins).[7] These pigments are present mostly in plant fruits, flowers, and roots as well as in tissues exposed to stress.[1,7,8]

Despite the scientific potential of betalains, systematic research on their activities is still insufficient. In particular, isolated pure pigments were tested in few studies. Betanin, the most known principal pigment of red beet root (*Beta vulgaris* L.), was the first and most frequently studied betalain for its antioxidant activity.[3,5,6,9−13] The influence of pH and other physicochemical conditions on the antioxidant activity of betalains was also investigated.[13,14] A series of differently substituted betacyanins and a few betaxanthins from plants of the Amaranthaceae were researched by DPPH tests,[12] indicating that the most active was 6-*O*-glucosylated betanidin (gomphrenin I) isomeric to betanin. The inhibition of the peroxidation of linoleic acid and the oxidation of LDL by betalains was also investigated.[15] The reducing activity of betanin and betanidin versus (lipo)peroxyl radicals in homogeneous systems as well as peroxyl radical-scavenging assays in liposomal suspensions was recently studied with tentative chromatographic detection of betanidin *o*-quinone.[16]

Extensive studies on the structural implications of semisynthetic natural or artificial betalains done recently confirmed the fact that the high antioxidant activity is affected not only by the presence of one or two phenolic groups but also by other structural factors, not researched as yet.[14] It is of utmost interest to discover this influence for further estimation of the impact of these pigments on human health.

Oxidation pathways were extensively studied for such amino acids as DOPA (precursor of cyclo-DOPA) and dopamine (precursor of decarboxylated cyclo-DOPA) or their precursors (tyrosine and tyramine), in relation to the biosynthesis of melanins, important mammalian pigments formed from indoles, especially 5,6-dihydroxyindoles, which are the products of oxidation of these amino acids.[17−20] Indeed, cyclo-DOPA (leucodopachrome) can be formed by direct chemical or enzymatic oxidation of tyrosine via DOPA and dopaquinone with its subsequent cyclization.[17−20] Further oxidation is accomplished by dehydrogenation or decarboxylative dehydrogenation of cyclo-DOPA at position C-2,3, leading finally to the 5,6-dihydroxyindoles.[17−20] At this stage, the key intermediate is dopachrome, an important compound from a group of aminochromes,[17−20] which rearranges to the 5,6-dihydroxyindolic structure.[17−20] Moreover, further oxidation leading to 2,3-dehydrodopachrome is also possible.[17−20]

Other investigations on melanogenesis revealed two possible mechanisms (via dopachrome or quinone methide) for the conversion of dopachrome to dihydroxyindoles.[21,22] It is probable, therefore, that similar reactions are possible at the cyclo-DOPA moiety of betanidin, with further consequences resulting from its conjugation with betalamic acid.

**Received:** May 20, 2011
**Revised:** August 1, 2011
**Accepted:** August 2, 2011
**Published:** August 02, 2011





dx.doi.org/10.1021/jf2020107 | *J. Agric. Food Chem.* 2011, 59, 9612–9622

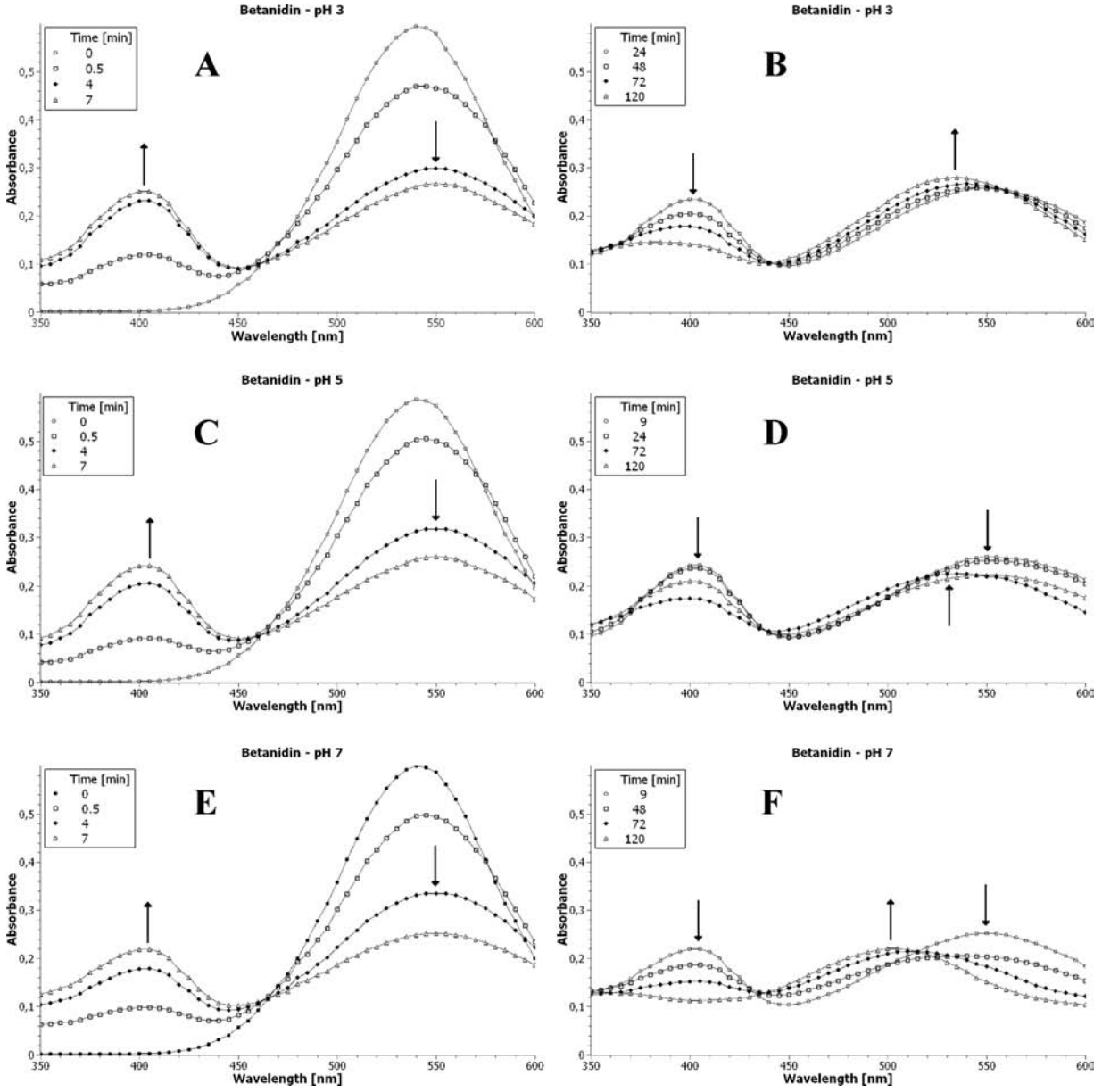


**Figure 1.** Spectrophotometric results of the enzymatic oxidation of 25 $\mu$M betanidin **2** by 0.01 EU/mL horseradish peroxidase II in the presence of 1 mM $H_2O_2$ at pH 3 (A, B), pH 5 (C, D), and pH 7 (E, F).

Recently, betanidin was subjected to oxidation by enzyme tyrosinase, which plays a key role in the betalain biosynthetic scheme.[23] In addition, the oxidation of betalains by peroxidase enzymes was also investigated.[4,24] The tyrosinase-mediated oxidation of betanidin was characterized by high-performance liquid chromatography (HPLC) and spectrophotometry. Addition of ascorbic acid reversed the reaction product, which was apparently betanidin *o*-quinone, to the original pigment. Therefore, no further rearrangement by dopachrome or quinone methide stages was considered.[23] The authors also demonstrated the direct action of tyrosinase on betaxanthins and characterized the reaction products.[25,26] They documented very well the enzymatic hydroxylation of tyramine- and tyrosine-based betaxanthins to the dopamine- and DOPA-based betaxanthins, respectively, which is equivalent to the tyrosinase-mediated oxidation of tyrosine to DOPA and is considered to be the first step in the biogenesis of betalamic acid and betacyanins.[25,26]

Further enzymatic oxidation of dopamine and DOPA, leading to the cyclic structures of aminochromes, is possible.[25,26] However, the tyrosinase-catalyzed oxidation of the dopamine- and DOPA-based betaxanthins does not result in the formation of known cyclic structures such as 2-decarboxybetanidin and betanidin, respectively, but other unknown complex isomeric structures of various intramolecular cyclization patterns are formed.[25,26] Therefore, the complete reaction of tyrosinase-catalyzed oxidation of the starting amino acid-based betaxanthins through betanidin and subsequent betanidin oxidized products was not accomplished.

Recent comprehensive studies established the basic directions of thermal decarboxylation and dehydrogenation of betalains in aqueous[27−29] and alcoholic[27,28] media. It is probable that these

**Table 1. Chromatographic, Spectrophotometric, and Mass Spectrometric Data of the Analyzed Products of Betanidin and Betanin Enzymatic Oxidation**

| no. | compound | $t_R$ (min) (HPLC system 2) | $\lambda_{max}$ (nm) | $m/z$ $[M + H]^+$ | $m/z$ from MS/MS of $[M + H]^+$ |
|---|---|---|---|---|---|
| | | **Betanidin Oxidation** | | | |
| **1** | betanidin quinonoid[a] | 7.3[b] | 440; 550 | 387 | 343, 299 |
| **1′** | betanidin isoquinonoid[a] | 9.2[b] | 440; 550 | 387 | 343, 299 |
| **2** | betanidin | 17.6 | 541 | 389 | 345 |
| **2′** | isobetanidin | 19.2 | 541 | 389 | 345 |
| **3** | 2,17-bidecarboxy-2,3-dehydrobetanidin[a] | 23.2 | 472 | 299 | 255 |
| **4** | 2-decarboxy-2,3-dehydrobetanidin[a] | 24.0 | 498 | 343 | 299; 255 |
| **5** | 2,17-bidecarboxy-2,3-dehydroneobetanidin[a] | 26.9 | 415 | 297 | 253 |
| | | **Betanin Oxidation** | | | |
| **6a** | dihydroxylated **9**[a] | 11.3 | 529 | 539 | 377; 333; 289 |
| **6b** | dihydroxylated **9**[a] | 12.1 | 529 | 539 | 377; 333; 289 |
| **7** | betanin | 13.2 | 538 | 551 | 389 |
| **7′** | isobetanin | 14.5 | 538 | 551 | 389 |
| **8** | 2,17-bidecarboxyneobetanin[a] | 16.1 | 459 | 461 | 299; 255 |
| **9** | 2-decarboxy-2,3-dehydrobetanin[a] | 17.1 | 446 | 505 | 343; 299; 255 |
| **10** | 2,15,17-tridecarboxy-2,3-dehydroneobetanin[a] | 19.3 | 394 | 415 | 253 |
| **11** | 2,17-bidecarboxyneobetanin[a] | 20.0 | 407 | 459 | 297; 253 |
| **12** | 2-decarboxy-2,3-dehydroneobetanin[a] | 25.4 | 422 | 503 | 341; 297; 253 |

[a] Tentatively identified. [b] Retention time obtained in HPLC system 1.

processes are also affected by oxidation, but the mechanism is still unknown. In this study, we investigated the horseradish peroxidase oxidation of betanidin and betanin and attempted to identify the oxidation products by mass spectrometry (LC-DAD-ESI-MS/MS).

## MATERIALS AND METHODS

**Reagents.** Formic acid, ammonium formate, LC-MS grade methanol, acetonitrile, and water, peroxidase from horseradish type II (150−250 units/mg solid (using pyrogallol), Rz = $A_{403}/A_{275} \geq 1.8$, MW = 40 kDa), and almond $\beta$-glycosidase were obtained from Sigma Chemical Co. (St. Louis, MO).

**Betanin and Betanidin Isolation.** Juice from red beetroots was obtained in a juice extractor (Zelmer, Rzeszów, Poland) and was submitted to cleanup on Sephadex DEAE A-25 gel and by solid phase extraction on C18 cartridges before HPLC preparative fractionation.[30] Purified betanin was subjected to enzymatic hydrolysis catalyzed by almond $\beta$-glycosidase[23] and cleanup on Sephadex DEAE A-25 gel and by solid phase extraction on C18 cartridges. The eluates were concentrated under reduced pressure at 25 °C and submitted to HPLC preparative fractionation.

**Semipreparative HPLC.** For the semipreparative isolation of betacyanins from the purified extracts a Gynkotek HPLC system with UVD170S, Gynkotek HPLC pump Series P580, and thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) was used. The semipreparative column used was a 250 mm × 10 mm i.d., 10 $\mu$m, Luna C18(2), with a 10 mm × 10 mm i.d. guard column of the same material (Phenomenex, Torrance, CA) under the following gradient system (system 1): 6% A in B at 0 min; gradient to 10% A in B at 30 min (A, acetonitrile; B, 4% (v/v) HCOOH in $H_2O$). In each case, the injection volume was 100 $\mu$L and the flow rate was 3 mL/min. Detection was generally performed at 538, 505, 480, and 310 nm with a DAD UV−vis detector. The columns were thermostated at 30 °C. All fractions obtained were diluted with water and submitted to freeze-drying and analysis.

**Peroxidase Assay.** The oxidation of betanidin and betanin was performed in 25 mM acetate (pH 3−5.5) and phosphate (pH 6−8) buffers in 96-well plates of a Tecan infinite 200 microplate reader (Tecan Austria GmbH, Grödig/Salzburg, Austria). The action of 0.001−0.01 EU/mL peroxidase II on the 25 $\mu$M pigment solutions in the presence of 1 mM $H_2O_2$ was monitored during a period of 120 min at a temperature of 25 °C by spectrophotometric detection at the wavelength range of 350−600 nm. For the chromatographic analysis, typically 50 $\mu$L reaction mixtures were sampled from the wells and injected directly to the HPLC column, without further purification.

**Chromatographic System LC-DAD.** A Gynkotek HPLC system with UVD170S, Gynkotek HPLC pump series P580, and thermostat (Gynkotek Separations) was used for the chromatographic analysis. For the data acquisition, the software package Chromeleon 4.32 (Gynkotek Separations) was used. For the online UV−vis spectra acquisition, the detection was performed in the diode array detection (DAD) mode. The column used was a 250 mm × 3 mm i.d., 5 $\mu$m, Luna C18(2), with a 4 mm × 2 mm i.d. guard column of the same material (Phenomenex). The injection volume was 10 $\mu$L, and the flow rate was 0.5 mL/min. The detection of analytes was performed typically at 538, 505, 480, and 440 nm. The column was thermostated at 35 °C. For the separation of the analytes, two gradient systems were used: system 1, 90% (v/v) A with 10% (v/v) B at 0 min; gradient to 70% (v/v) A with 30% (v/v) B at 35 min, with (A) 20 mM ammonium formate (pH 4) and (B) methanol; system 2, 93% A with 7% B at 0 min; gradient to 80% A with 20% B at 35 min, with (A) 2% formic acid and (B) methanol. The same chromatographic conditions were applied for the HPLC-ESI-MS/MS analyses.

**LC-ESI-MS/MS Analysis.** The positive ion electrospray mass spectra were recorded on a ThermoFinnigan LCQ Advantage (electrospray voltage, 4.5 kV; capillary, 250 °C; sheath gas, $N_2$) coupled to a ThermoFinnigan LC Surveyor pump utilizing the HPLC gradient systems 1 and 2. The MS was controlled, and total ion chromatograms and mass spectra were recorded using ThermoFinnigan Xcalibur software (San Jose, CA). Helium was used to improve the trapping efficiency and as the collision gas for CID experiments. The relative collision energies for MS/MS analyses were set at 30% (according to relative energy scale).

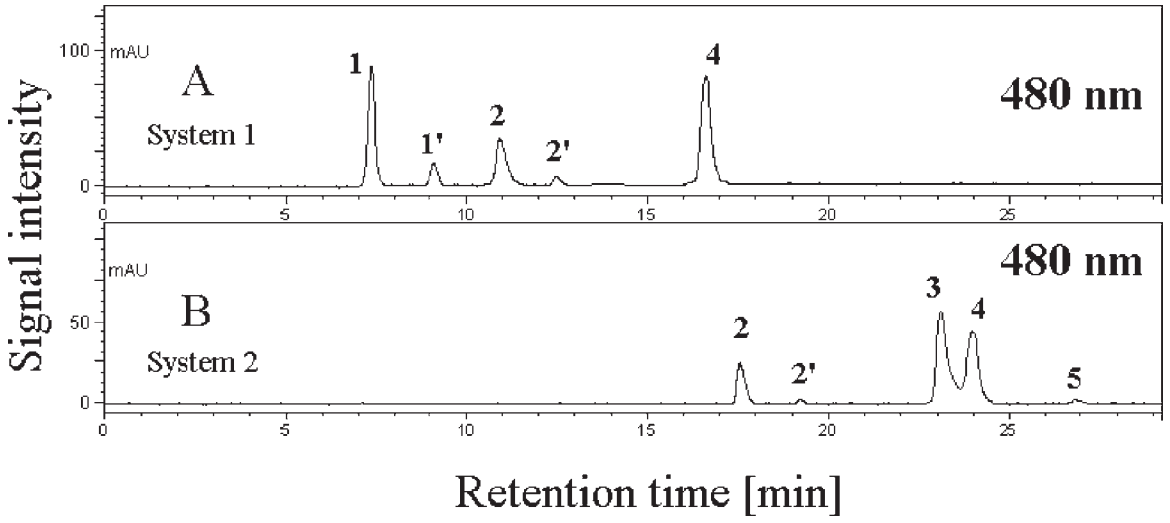


**Figure 2.** Chromatographic traces of the products of enzymatic oxidation of betanidin **2** in solvent systems 1 (A) and 2 (B) after 10 min of reaction.

## RESULTS AND DISCUSSION

**Peroxidase Oxidation of Betanidin.** Betanidin is the basic structure for all betacyanins and the only one with the 5,6-dihydroxyl moiety (catechol moiety) and, therefore, its higher antioxidant activity, strongly dependent on pH value, is expected.[13] Therefore, a series of oxidation experiments at pH 3−8 was performed to compare the kinetics of the reactions as well as the profiles of generated products.

The results of spectrophotometric tests on the enzymatic betanidin oxidation (2*S*/*S*) by horseradish peroxidase II at pH 3, 5, and 7 are depicted in Figure 1. In the initial (up to 10 min) course of the reaction, the main absorbance band diminishes with a shift of $\lambda_{max}$ from 540 to 550 nm, and the second band increases at $\lambda_{max}$ 400 nm (Figure 1A,C,E). This confirms the previous results[16,23] in which the presence of betanidin *o*-quinone was postulated.

In further course of the reaction, a hypsochromic shift of $\lambda_{max}$ of the main absorbance band from 550 nm and a decrease of the second band at $\lambda_{max}$ 400 nm with a bathochromic shift toward 410 nm were noted (Figure 1B,D,F). Interestingly, no meaningful differences in the reaction rate at different pH values were noted. However, a growth in pH results in a stronger hypsochromic shift of $\lambda_{max}$ of the main absorbance band toward 490 nm at pH 7−8 (Figure 1E,F).

The spectrophotometric and mass spectrometric data for all pigments analyzed in this study are presented in Table 1. Typical HPLC-DAD traces of oxidized betanidin **2** mixture (with isobetanidin **2′** at lower concentration) monitored at 480 nm during analysis in solvent systems 1 and 2 are presented in Figure 2, panels A and B, respectively. The analyses were performed after 10 min of each reaction. Chromatographic analyses of oxidized betanidin confirmed the presence of a quinonoid derivative of betanidin as the most polar compound. In agreement with earlier studies,[16,23] this is presumably the betanidin *o*-quinone **1** formed on the first oxidation stage with its characteristic absorption spectrum ($\lambda_{max}$ at 550 and 400 nm) (Table 1). In addition, oxidation of isobetanidin (2*S*/*R*) results in the appearance of a second chromatographic peak **1′** behind the peak of **1** with the same absorption spectrum (Table 1) and suggests the formation of isobetanidin *o*-quinone **1′**. For the HPLC analysis, the solvent systems containing aqueous ammonium acetate at pH ≥4, for example, system 1, were generally used. From the mass spectrum viewpoint, the lowest possible pH (ca. 4.0) in system 1 was compromised between the ionization efficiency in the ESI interface and the stability of the quinone thus formed. A decrease in pH results evidently in the degradation/fast rearrangement of **1**, which completely vanishes in the formic acid solutions (system 2).

The LC-DAD-MS/MS analysis of **1** in solvent system 1 supported the presence of a quinonoid derivative as a result of the loss of 2H from betanidin **2**, based on detection of a protonated molecular ion $[M + H]^+$ at *m*/*z* 387 and subsequent fragmentation ions at *m*/*z* 343 and 299 resulting from the loss of one and two $CO_2$, respectively.

Further inspection of the LC-DAD-MS/MS data proved the presence of additional compounds in the reaction mixtures (Table 1). Two main compounds characterized by $\lambda_{max}$ 498 nm and *m*/*z* 343 (**4**) as well as $\lambda_{max}$ 472 nm and *m*/*z* 299 (**3**) were detected in solvent system 2 (Figure 2B), but only one compound (**4**) was detected in solvent system 1 ($\lambda_{max}$ 498 nm and *m*/*z* 343, Figure 2A).

In any case, the results suggest that the originated betanidin *o*-quinone (Figure 3) undergoes further rearrangement, involved with the generation of dehydrogenated and decarboxylated derivatives (Figure 4). In both cases, these products are formed in effect by slow and very fast decomposition of betanidin quinone **1** in solvent systems 1 and 2, respectively.

In addition, compound **5**, characterized by $\lambda_{max}$ 415 nm and *m*/*z* 297, was detected at lower abundance after a prolonged time of the oxidation experiment, whereas compounds **3** and **4** gradually decayed, suggesting that this pigment can be a final product of the decomposition of **3** or **4** detected in the samples.

Addition of ascorbic acid reverses the reaction of betanidin quinonoid formation (unpublished data), which is in accordance with the previous studies.[23] However, the other oxidation products, that is, **3**−**5**, cannot be reduced and their HPLC peaks do not disappear from the chromatograms; this proves that their generation is a result of irreversible reactions. Interconversions between three tautomeric quinonoid forms of oxidized betanidin **1a**−**c** (Figure 3) are based on the proven pathways of DOPA and dopamine oxidations.[17−22] The initial oxidation of betanidin

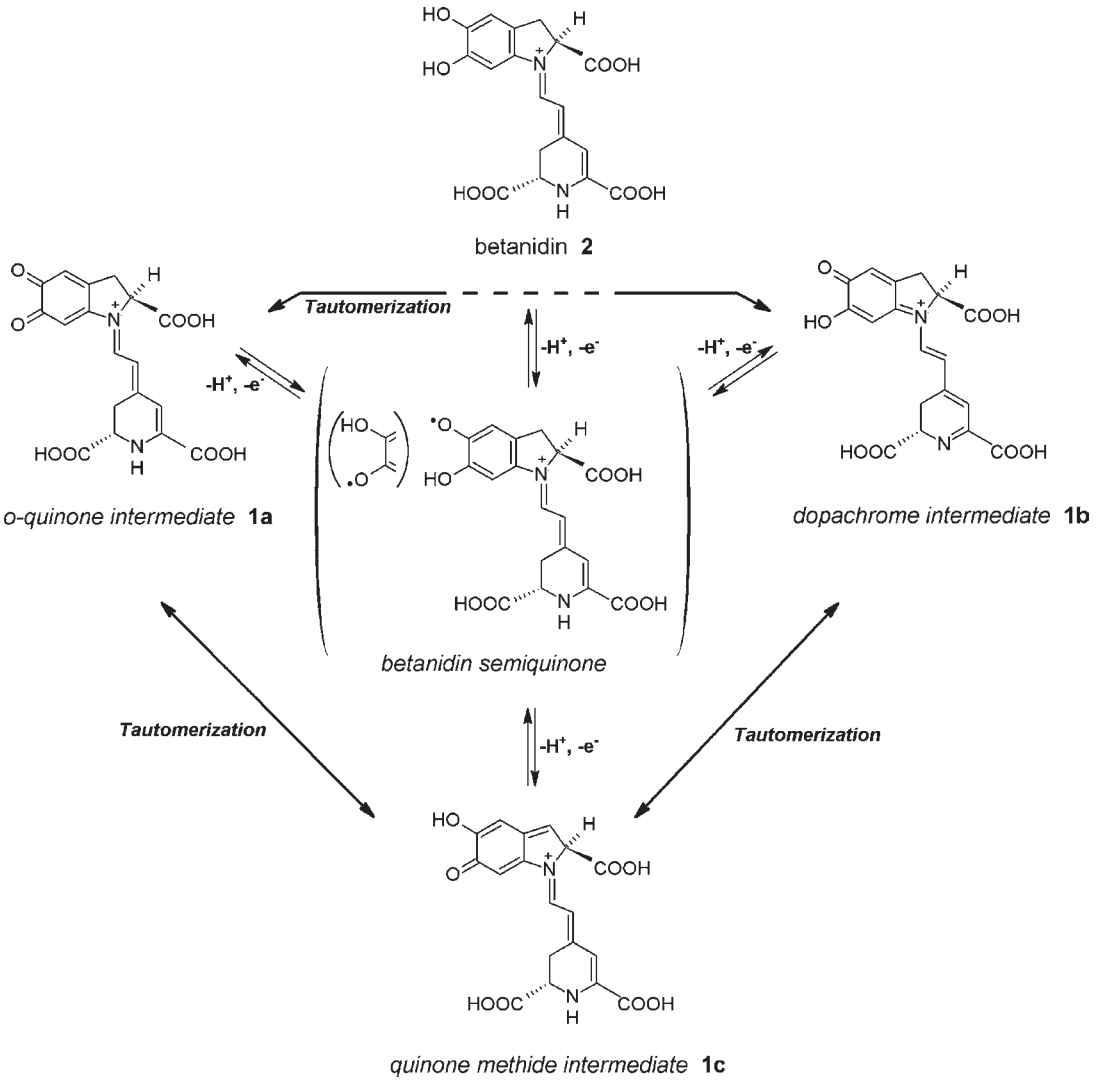


**Figure 3.** Schematic presentation of the initial oxidation of betanidin **2** by horseradish peroxidase II with interconversions between three tautomeric quinonoid forms of oxidized betanidin: betanidin *o*-quinone **1a**; aminochrome (dopachrome derivative) **1b**; quinone methide **1c**.

should generate the transient radical cation, which rapidly loses a proton and forms a neutral phenoxy radical; this is equivalent to donation of a hydrogen atom by betanidin or to a loss of a proton with further electron transfer. In the next step, the loss of a second proton and electron results in the formation of a two-electron oxidized form, that is, one of the three quinonoid tautomers **1a**−**c**.

Investigations on melanogenesis, starting from amino acids, revealed two possible mechanisms for the conversion of dopachrome to dihydroxyindoles.[17−22] The first one is the conversion of dopachrome to indolenine and its subsequent aromatization.[17−22] Alternatively, the stable dopachrome rearranges enzymatically or nonenzymatically to an unstable quinone methide, which can also aromatize and generate dihydroxyindoles.[21,22] The second route is of our particular interest, because it can be considered also for the 5-*O*-glycosylated betanidin (betanin) systems, where it is the only possible mechanism of the oxidation of the semiquinone formed at carbon C-6.

It is possible that the main form existing in the analyzed samples is betanidin *o*-quinone **1a**, as was postulated previously.[16,23] However, other two tautomers, **1b** and **1c**, can also exist in the reaction mixtures (Figure 3) and cannot be completely excluded.[17−22] There is a question as to how easily **1a** can tautomerize to other quinonoid forms (aminochrome **1b** or quinone methide **1c**) as well as whether the latter forms are more easily generated than the typical *o*-quinone (Figure 3).

The possibility of formation of the dopachromic form **1b** is supported by the fair stability of aminochromes, previously studied as oxidation products of amino acids or amines.[31] In addition, it is not excluded that the presence of the conjugated chromophoric system with betalamic acid may stabilize also the quinone methide form **1c**. In any case, the pH and other physicochemical conditions should influence strongly the presence of all three forms considered.

On the basis of the reaction scheme (Figure 4) supported by optical and MS/MS data, the structures of 2,17-bidecarboxy-2,3-dehydrobetanidin (**3**) and 2-decarboxy-2,3-dehydrobetanidin (**4**) detected in system 2 are postulated as the principal oxidation products formed. The decarboxylation at positions C-2 and C-17 is the most often detected reaction pattern during the thermal degradation of betacyanins,[32] which is also influenced by the presence of oxygen dissolved in the solution tested.[29] The

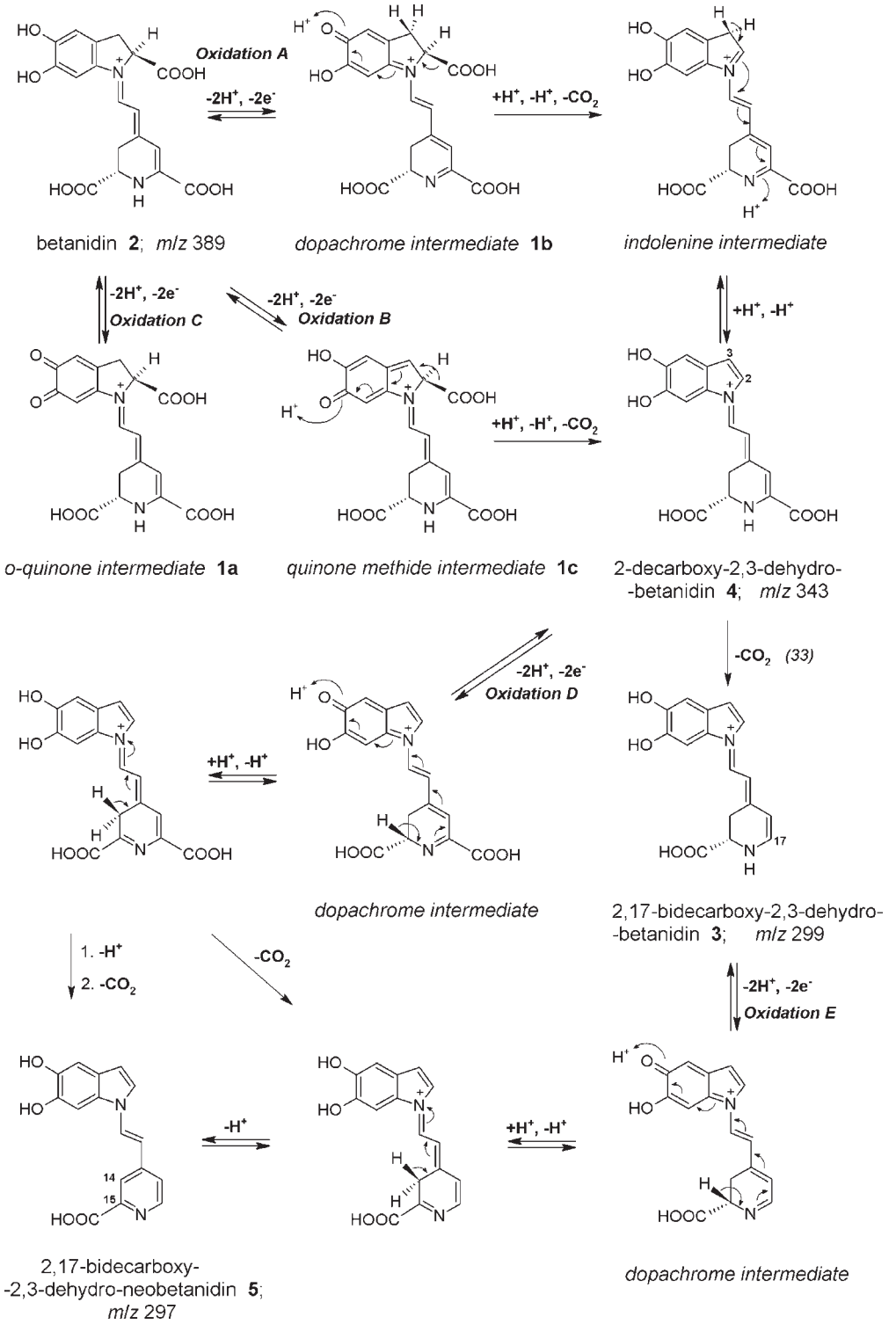


**Figure 4.** Proposed mechanism of betanidin **2** oxidation via two intermediates, aminochrome (dopachrome derivative) **1b** and quinone methide **1c**, based on the data obtained from spectrophotometric, chromatographic, and MS/MS tests. The nonoxidative decarboxylation in **4** proceeds presumably at carbon C-17.[33].

mechanism of decarboxylation resulting in the formation of 17-decarboxybetanidin was studied.[33] One of the oxidation paths would be the formation of betanidin *o*-quinone involved with its rearrangement through the dopachrome or quinone methide derivatives, followed by dehydrogenation and decarboxylation (Figure 4). This results in the formation of 2-decarboxy-2,3-dehydrobetanidin **4** as the principal degradation product. Another product formed from **4** on the nonoxidative decarboxylation path is 2,17-bidecarboxy-2,3-dehydrobetanidin **3**. Further oxidation of **3** involves the formation of one of the two

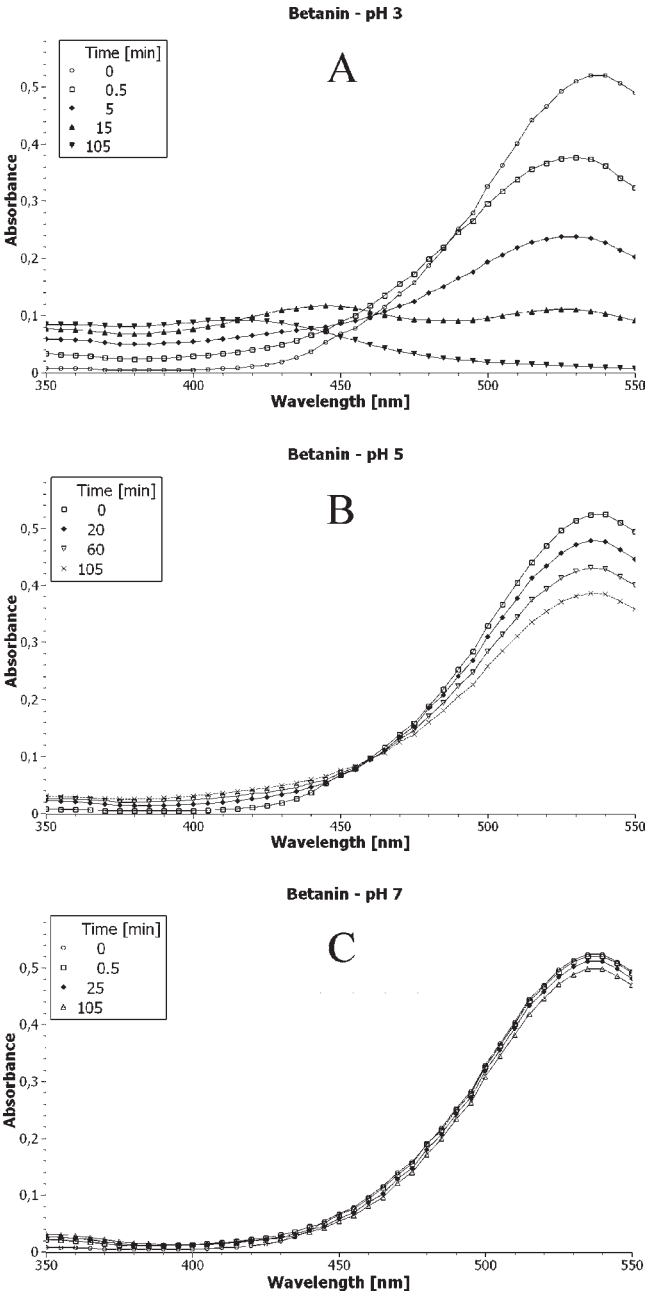


**Figure 5.** Spectrophotometric results of the enzymatic oxidation of 25 $\mu$M betanin **7** by 0.01 EU/mL horseradish peroxidase II in the presence of 1 mM $H_2O_2$ at pH 3 (A), pH 5 (B), and pH 7 (C).

quinonoid forms as the oxidized intermediates, for example, the dopachrome derivative (Figure 4) of the indolic system and subsequent rearrangement of the conjugated system of originated structures into the 14,15-dehydrogenated derivatives, leading finally to 2,17-bidecarboxy-2,3-dehydroneobetanidin **5**, that is, the compound doubly dehydrogenated at positions C-2,3 and C-14,15 (the "neo" derivative). This conclusion was supported by inspection of the MS chromatographic data and detection of a doubly dehydrogenated pigment **5** with $\lambda_{max}$ 415 nm and $m/z$ 297 (Table 1). Therefore, it seems to be another, relatively stable, derivative of betanidin.

No traces of neobetanidin (14,15-dehydrobetanidin) were observed in the chromatograms. Neobetanidin can also be regarded as another tautomeric form of **1**. However, its presence can readily be excluded, owing to its much higher retention time relative to that of **1** and **2** (as in the case of betanin and neobetanin[27−29]). In fact, the pigments **3** and **4** might also tautomerize to their corresponding neoderivatives. However, the time scale of the experiment is presumably too short for such rearrangements.

Overall, the reaction scheme (Figure 4) indicates the initial steps of the general mechanism of betanidin oxidation; further reactions are also possible, however. One of them can be the hydrolytic decomposition of any reaction product by splitting of the aldimine bond[16,29] and subsequent polymerization of the derivatives of betalamic acid or cyclo-DOPA.

**Peroxidase Oxidation of Betanin.** Enzymatic oxidation of betanin (2*S*) **7**, monitored spectrophotometrically at pH 3−8, revealed a strong dependence of the reaction rate on pH. In Figure 5 the consecutive visible spectra registered during the course of reaction at pH 3, 5, and 7 are presented. The fastest changes were observed in the most acidic medium (pH 3), whereas at pH 7−8 the reaction rate was negligibly small. This contrasts with the high reactivity of betanidin in the whole acidity range. Evaluation of the visible spectra, obtained at pH 3 during the first 15 min of reaction, showed an increase in the absorbance band at ca. 445 nm, followed by a peaking at 414 nm during further reaction steps (Figure 5A). The spectroscopic changes were accompanied by a decay of absorption at 538 nm characteristic for betanin, with a small shift of $\lambda_{max}$ to ca. 530 nm. In recent spectrophotometric and chromatographic studies,[16] the absorbance band at 420 nm was assigned to the chromophore of betalamic acid, by its comparison with the spectra of betanidin or betanin hydrolysis products and confirmed by HPLC analysis. The nature of the absorbing products with $\lambda_{max}$ at 531 nm, accumulated after degradation of betanin, was not known.[16]

The presence of betalamic acid was not confirmed in our studies, presumably because of different experimental conditions. In addition, betalamic acid is noted for its high instability.[1] Instead, other oxidation products were detected (Figure 6). After 10 min of reaction, the main chromatographic peak **9** was generated at $\lambda_{max}$ 446 nm. The LC-MS/MS experiment on the corresponding compound resulted in the formation of a protonated molecular ion $[M + H]^+$ at $m/z$ 505. Subsequent fragmentation ion at $m/z$ 343 from the loss of a glucose moiety as well as at $m/z$ 299 and 255 Da from the consecutive losses of $CO_2$ confirmed the generation of a decarboxylated and dehydrogenated betanidin. Therefore, a monodecarboxylated and monodehydrogenated betanin is formed during the oxidation of betanin. Because the dehydrogenation is presumably a result of the oxidation of the only free phenolic moiety at C-6, the position of decarboxylation must take place at C-2 as in the case of the oxidation of betanidin, indicating the presence of 2-decarboxy-2,3-dehydrobetanin **9**. As a result, the most probable mechanism of betanin oxidation is postulated in Figure 7. If the oxidation of betanin results in the formation of a semiquinonoid radical, it should undergo the subsequent rearrangement (Figure 7) following the reaction route involving the quinone methide intermediate,[21,22] taking into account that the formation of the aminochrome intermediate is impossible because the hydroxyl is blocked at C-5. It is not excluded, however, that the generated 2-decarboxy-2,3-dehydrobetanin **9** rearranges to the more stable structure[1] of 2-decarboxy-14,15-dehydrobetanin (synonym = 2-decarboxyneobetanin). Whether these two structures exist in equilibrium will be the subject of another study. However, the presence of 2-decarboxyneobetanin would be contradictory

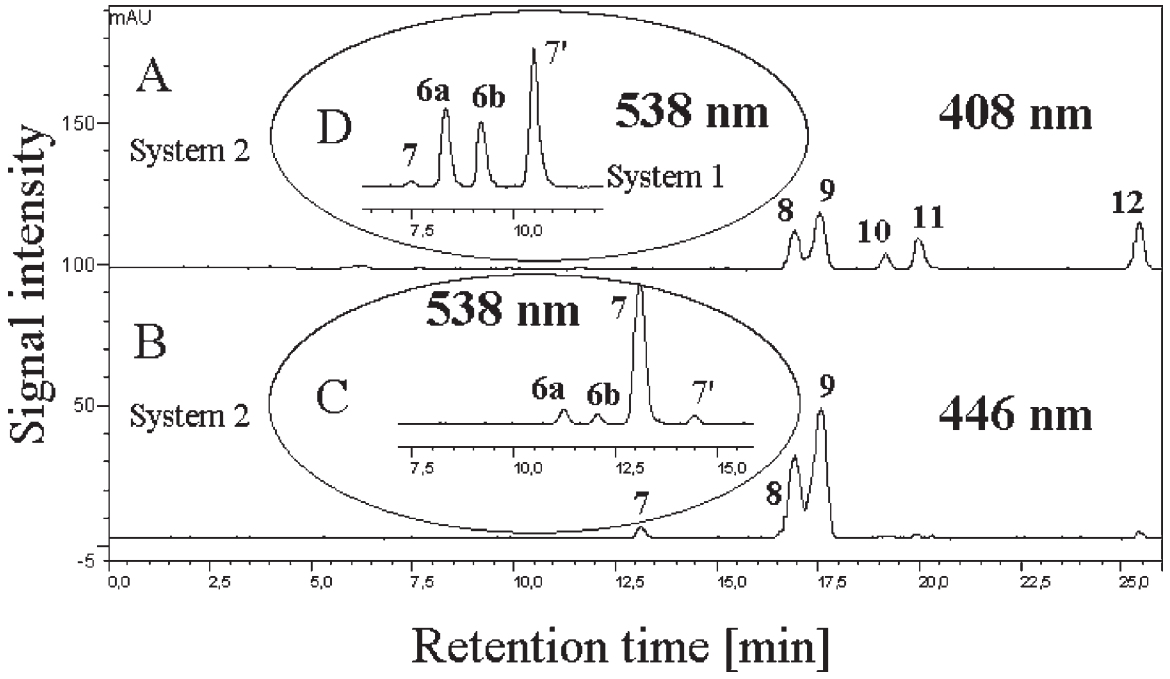


**Figure 6.** Chromatographic traces of the products of enzymatic oxidation of betanin **7** after 10 min of reaction, obtained in solvent system 2 (A–C, monitored at 408, 446, and 538 nm), and of isobetanin **7′** obtained in solvent system 1 (D) (monitored at 538 nm).

to previous indications on account of betanin thermal degradation,[27] because 2-decarboxyneobetanin should be eluted much later in the HPLC system.[27]

In contrast to betanidin oxidation, no compound that could be regarded as a betanin oxidation intermediate (quinone methide) was found in the resulting chromatograms. This should be a consequence of its instability and fast rearrangement, combined with decarboxylation.[21,22] In addition, neobetanin (14,15-dehydrobetanin), as a possible tautomeric form of quinone methide, was also not observed in the samples. Instead, two polar compounds, **6a** and **6b**, were revealed in the chromatograms, with absorption maxima at $\lambda_{max}$ 529 nm, confirmed by the spectrophotometric shift of absorption spectra of the reaction mixtures in the assay wells, from 538 nm to ca. 530 nm. This confirms the previous results of the first chromatographic tests on peroxidase action on betanin,[24] when a prominent (unknown) reaction product was eluted before betanin.

The LC-MS/MS analysis of **6a** and **6b** revealed protonated molecular ions $[M + H]^+$ at $m/z$ 539. Subsequent fragmentation of **6a** and **6b** revealed ions at $m/z$ 377, originating from the loss of a glucose moiety, and further ions, at 333 and 289, resulting from the next losses of $CO_2$, suggesting the presence of a monodecarboxylated pigment. However, all of the ions detected are not typical for any of the possible decarboxy and dehydro derivatives of betanin that would fit into the reaction scheme (Figure 7). The lower $m/z$ value of the protonated molecular ion (539) than the $m/z$ of betanin (551) indicates that **6a** and **6b** can be the result of betanin degradation and further derivatization of this product, for example, **9**, which is a compound originating at the first stage of the reactions. Indeed, the $m/z$ difference between **9** and **6a** (539 − 505 = 34) indicates the possibility of subsequent hydroxylation of **9** by $H_2O_2$. Whether this reaction proceeds with peroxidase assistance or spontaneously, it was not possible to follow. Formation of **6a** and **6b** would confirm the conclusion that some oxidation reactions of betanin initiated by peroxidases could proceed further, without any action of the enzyme.[24]

Interestingly, enzymatic oxidation of isobetanin (2*S*/*R*) **7′** results in the formation of the same pair of HPLC peaks (Figure 6D) as in the case of betanin (2*S*/*S*) **7**. The ratio (1.2:1) of peak areas for **6a** and **6b** is similar after each reaction; this suggests isomerization of the products of hydroxylation of **9**, involved with the formation of a pair of diastereomers **6a** and **6b**. Increasing the pH of the eluent results in an increase of retention time of **6a** and **6b** in relation to **7** and **7′** (Figure 6C,D).

Another betanin derivative, **8**, was detected as a protonated molecular ion $[M + H]^+$ at $m/z$ 461 and absorption maximum at $\lambda_{max}$ 459 nm. Subsequent fragmentation ions at $m/z$ 299, resulting from the loss of a glucose moiety, and $m/z$ 255, from the next loss of $CO_2$, confirms the suggestion of bidecarboxylated dehydrogenated fragment of betanidin. These results indicate the next loss of $CO_2$ from **9** and the formation of bidecarboxylated and dehydrogenated betanin **8**, presumably 2,17-decarboxy-2,3-dehydrobetanin. However, the presence of another isomeric compound (2,15-decarboxy-2,3-dehydrobetanin) cannot be excluded. In addition, the possible rearrangements of the pigments to their corresponding 14,15-dehydro derivatives (neoderivatives) should be taken into account.

The mechanism of the most frequent betanidin decarboxylation, leading to the formation of 17-decarboxybetanidin, studied by Dunkelblum et al.[33] in ethanolic solutions may be also possible for betanin. As a result, the generation of 15-decarboxybetanin was not predicted here. However, the possible presence of 15-decarboxybetanin in red beetroot dried extracts[34] and different reaction conditions (aqueous solutions) as well as different starting structure (**9**) suggest that decarboxylation of **9** at C-15 is also possible. In addition, relatively high absorption maximum of **8** at $\lambda_{max}$ 460 nm when compared with the one for **9** at $\lambda_{max}$ 446 nm would suggest decarboxylation at C-15, because the analogous monodecarboxylated betanin at C-15 is characterized

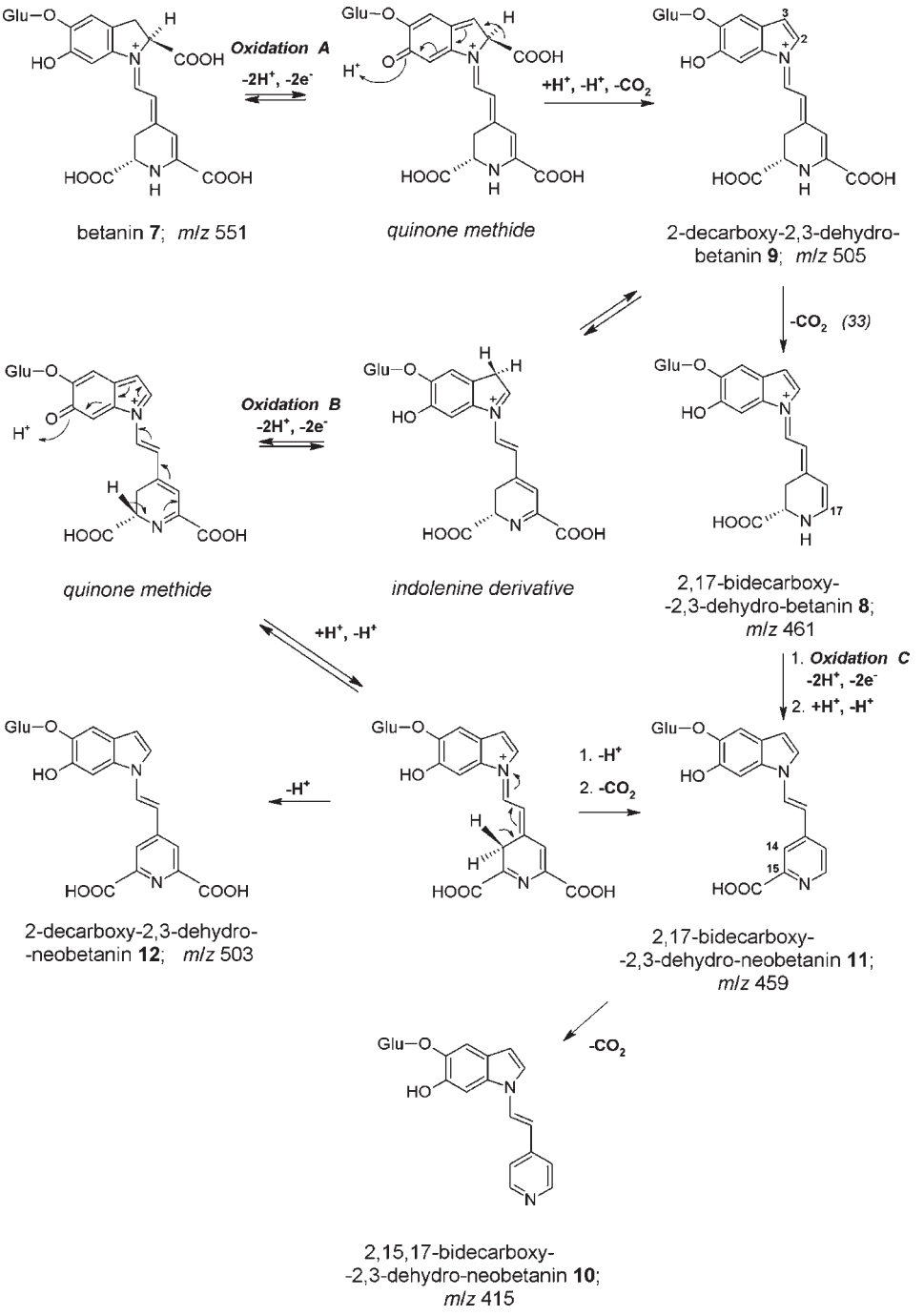


**Figure 7.** Proposed oxidation mechanism of betanin **7** via quinone methide, based on the data obtained from spectrophotometric, chromatographic, and MS/MS tests. The nonoxidative decarboxylation in **9** proceeds presumably at carbon C-17.[33].

by a higher $\lambda_{max}$ (528 nm) than for 17-decarboxybetanin ($\lambda_{max}$ 505 nm). Further inspection of the chromatograms revealed the most hydrophobic reaction product **12** as judged from its high retention time. This compound displayed a protonated molecular ion $[M + H]^+$ at $m/z$ 503 exhibiting further hypsochromic shift of its absorption maximum to $\lambda_{max}$ 422 nm and confirming the oxidation of **9** with further dehydrogenation. Subsequent fragmentation of **12** revealed ions at $m/z$ 341 resulting from the loss of a glucose moiety as well as at $m/z$ 297 and 253 from the next losses of $CO_2$, which confirmed the presence of monodecarboxylated and doubly dehydrogenated derivatives of betanin. Therefore, on the basis of the structure of **9** as 2-decarboxy-2,3-dehydrobetanin, its oxidation results in the formation of 2-decarboxy-2,3-dehydroneobetanin **12**.

Likewise, subsequent oxidation of **8** results evidently in the formation of doubly dehydrogenated derivatives **10** and **11**, which confirms the proposed general mechanism of betanin oxidation. Compound **11** appearing at $t_R$ 20.0 min displays a protonated molecular ion at $m/z$ 459, fragmentation ions at $m/z$ 297 and 253, and an absorption maximum at $\lambda_{max}$ 407 nm, which suggests the formation of a doubly decarboxylated and doubly dehydrogenated betanin derivative. Interestingly, **11** can be

formed through two reaction routes: (i) the oxidation of **8** or (ii) the next decarboxylation of **12** at positions C-15 or (most probably) C-17. Therefore, there are two possible structures of **11**: 2,15-decarboxy-2,3-dehydroneobetanin or 2,17-decarboxy-2,3-dehydroneobetanin. Compound **10**, displaying a protonated molecular ion at $m/z$ 415 and a fragmentation ion at $m/z$ 253, obtained from the loss of a glucose moiety, but no fragmentation ions derived from the loss of $CO_2$, should be a completely decarboxylated and dehydrogenated derivative of betanin. This compound can be an effect of a final decarboxylation of **11**; therefore, the structure of **10** should be 2,15,17-tridecarboxy-2,3-dehydroneobetanin.

As in the case of betanidin oxidation, the reaction scheme (Figure 7) may be used to construct an improved, detailed description of the oxidation pathways of betalains. Likewise, it does not exclude further reactions: different dehydrogenation and decarboxylation variations and hydrolysis of any reaction product as well as subsequent polymerization of the derivatives of betalamic acid or cyclo-DOPA. The presence of the cyclo-DOPA moiety in the structure of betanidin as well as the glucosylated cyclo-DOPA in the structure of betanin chromophore determines the amino acid-like oxidation pathways, modified by the conjugation with betalamic acid. Regardless of which actual quinonoid tautomers are formed in the case of betanidin enzymatic oxidation, the present study provides evidence for the generation of betanidin 5,6-dihydroxyindolic and related structures in acidic and basic media. In the case of betanin, similar 2,3-dehydrogenated structures differing from betanidin derivatives by the presence of the glucose moiety were detected in acidic media.

The mechanism of betalain oxidation is of significant interest because of the recent emergence of these pigments as highly active natural compounds with antioxidative properties and potential benefits to human health. The natural colorant properties of betalains and the absence of their toxicity suggest the wide use of betacyanins as additives in the food industry. This contribution initiates a new approach to the chemistry of betacyanins, especially in the field of their antioxidation properties.

## AUTHOR INFORMATION

### Corresponding Author

*Phone: +48-12-628-3074. Fax: +48-12-628-2036. E-mail: swybran@chemia.pk.edu.pl.

### Funding Sources

This research was financed by the Polish Ministry of Science and Higher Education for the years 2007−2010 (Project N312 3268 33).

## REFERENCES

(1) Strack, D.; Steglich, W.; Wray, V. Betalains. In *Methods in Plant Biochemistry*; Dey, P. M., Harborne, J. B., Waterman, P. G., Eds.; Academic Press: London, U.K., 1993; Vol. 8, pp 421−450.

(2) Henry, B. S. Natural food colours. In *Natural Food Colorants*; Hendry, G. A. F., Houghton, J. D., Eds.; Blackie Chapman & Hall: London, U.K., 1996; pp 40−79.

(3) Tesoriere, L.; Butera, D.; Allegra, M.; Fazzari, M.; Livrea, M. A. Distribution of betalain pigments in red blood cells after consumption of cactus pear fruits and increased resistance of the cells to ex vivo induced oxidative hemolysis in humans. *J. Agric. Food Chem.* **2005**, *53*, 1266–1270.

(4) Martinez-Parra, J.; Munoz, R. Characterization of betacyanin oxidation catalyzed by a peroxidase from *Beta vulgaris* L. roots. *J. Agric. Food Chem.* **2001**, *49*, 4064–4068.

(5) Kapadia, G. J.; Azuine, M. A.; Sridhar, R.; Okuda, Y.; Tsuruta, A.; Ichiishi, E.; Mukainake, T.; Takasaki, M.; Konoshima, T.; Nishino, H.; Tokuda, H. Chemoprevention of DMBA-induced UV-B promoted, NOR-1-induced TPA promoted skin carcinogenesis, and DEN-induced phenobarbital promoted liver tumors in mice by extract of beetroot. *Pharmacol. Res.* **2003**, *47*, 141–148.

(6) Kanner, J.; Harel, S.; Granit, R. Betalains − a new class of dietary cationized antioxidants. *J. Agric. Food Chem.* **2001**, *49*, 5178–5185.

(7) Strack, D.; Vogt, T.; Schliemann, W. Recent advances in betalain research. *Phytochemistry* **2003**, *62*, 247–269.

(8) Wybraniec, S.; Stalica, P.; Spórna, A.; Mizrahi, Y. Profiles of betacyanins in epidermal layers of grafted and light-stressed cacti studied by LC-DAD-ESI-MS/MS. *J. Agric. Food Chem.* **2010**, *58*, 5347–5354.

(9) Escribano, J.; Pedreño, M. A.; García-Carmona, F.; Muñoz, R. Characterization of the antiradical activity of betalains from *Beta vulgaris* L. roots. *Phytochem. Anal.* **1998**, *9*, 124–127.

(10) Butera, D.; Tesoriere, L.; Di Gaudio, F.; Bongiorno, A.; Allegra, M.; Pintaudi, A. M.; Kohen, R.; Livrea, M. A. Antioxidant activities of Sicilian prickly pear (*Opuntia ficus-indica*) fruit extracts and reducing properties of its betalains: betanin and indicaxanthin. *J. Agric. Food Chem.* **2002**, *50*, 6895–6901.

(11) Pavlov, A.; Kovatcheva, P.; Georgiev, V.; Koleva, I.; Ilieva, M. Biosynthesis and radical scavenging activity of betalains during the cultivation of red beet (*Beta vulgaris*) hairy root cultures. *Z. Naturforsch. C* **2002**, *57*, 640–644.

(12) Cai, Y.; Sun, M.; Corke, H. Antioxidant activity of betalains from plants of the Amaranthaceae. *J. Agric. Food Chem.* **2003**, *51*, 2288–2294.

(13) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. Structural implications on color, fluorescence, and antiradical activity in betalains. *Planta* **2010**, *232*, 449–460.

(14) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. The role of phenolic hydroxy groups in the free radical scavenging activity of betalains. *J. Nat. Prod.* **2009**, *72*, 1142–1146.

(15) Tesoriere, L.; Butera, D.; D'Arpa, D.; Di Gaudio, F.; Allegra, M.; Gentile, C.; Livrea, M. A. Increased resistance to oxidation of betalain enriched human low density lipoproteins. *Free Radical Res.* **2003**, *37*, 689–696.

(16) Tesoriere, L.; Allegra, M.; Gentile, C.; Livrea, M. A. Betacyanins as phenol antioxidants. Chemistry and mechanistic aspects of the lipoperoxyl radical-scavenging activity in solution and liposomes. *Free Radical Res.* **2009**, *43*, 706–717.

(17) Prota, G.; Ischia, M. D.; Napolitano, A. The chemistry of melanins and related metabolites. In *The Pigmentary System*; Nordlund, J. J., Boissy, R. E., Hearing, V. J., King, R. A., Ortonne, J. P., Eds.; Oxford University Press: New York, 1998; pp 307−332.

(18) Ito, S. A chemist's view of melanogenesis. *Pigment Cell Res.* **2003**, *16*, 230–236.

(19) Meredith, P.; Sarna, T. The physical and chemical properties of eumelanin. *Pigment Cell Res.* **2006**, *19*, 572–594.

(20) Katritzky, A. R., Ed. *Advances in Heterocyclic Chemistry*; Academic Press: London, U.K., 2005; Vol. 89.

(21) Sugumaran, M. Molecular mechanisms for mammalian melanogenesis. Comparison with insect cuticular sclerotization. *FEBS Lett.* **1991**, *293*, 4–10.

(22) Sugumaran, M.; Semensi, V. Quinone methide as a new intermediate in eumelanin biosynthesis. *J. Biol. Chem.* **1991**, *266*, 6073–6078.

(23) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. Characterization of the activity of tyrosinase on betanidin. *J. Agric. Food Chem.* **2007**, *55*, 1546–1551.

(24) Martínez-Parra, J.; Muñoz, R. An approach to the characterization of betanine oxidation catalyzed by horseradish peroxidase. *J. Agric. Food Chem.* **1997**, *45*, 2984–2988.

(25) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. Betaxanthins as substrates for tyrosinase. An approach to the role of tyrosinase in

the biosynthetic pathway of betalains. *Plant Physiol.* **2005**, *138*, 421–432.

(26) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. Characterization of the monophenolase activity of tyrosinase on betaxanthins: the tyramine-betaxanthin/dopamine-betaxanthin pair. *Planta* **2005**, *222*, 307–318.

(27) Wybraniec, S. Formation of decarboxylated betacyanins in heated purified betacyanin fractions from red beetroot (*Beta vulgaris* L.) monitored by LC-MS/MS. *J. Agric. Food Chem.* **2005**, *59*, 3483–3487.

(28) Wybraniec, S.; Mizrahi, Y. Generation of decarboxylated and dehydrogenated betacyanins in thermally treated purified fruit extract from purple pitaya (*Hylocereus polyrhizus*) monitored by LC-MS/MS. *J. Agric. Food Chem.* **2005**, *53*, 6704–6712.

(29) Herbach, K. M.; Stintzing, F. C.; Carle, R. Betalain stability and degradation — structural and chromatic aspects. *J. Food Sci.* **2006**, *71*, R41–R50.

(30) Stintzing, F. C.; Schrieber, A.; Carle, R. Identification of betalains from yellow beet (*Beta vulgaris* L.) and cactus pear [*Opuntia ficus-indica* (L.) Mill.] by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J. Agric. Food Chem.* **2002**, *50*, 2302–2307.

(31) Remião, F.; Milhazes, N.; Borges, F.; Carvalho, F.; Bastos, M. L.; Lemos-Amado, F.; Domingues, P.; Ferrer-Correia, A. Synthesis and analysis of aminochromes by HPLC-photodiode array. Adrenochrome evaluation in rat blood. *Biomed. Chromatogr.* **2003**, *17*, 6–13.

(32) Wybraniec, S.; Nowak-Wydra, B.; Mizrahi, Y. $^{1}H$ and $^{13}C$ NMR spectroscopic structural elucidation of new decarboxylated betacyanins. *Tetrahedron Lett.* **2006**, *47*, 1725–1728.

(33) Dunkelblum, E.; Miller, H. E.; Dreiding, A. S. On the mechanism of decarboxylation of betanidine. A contribution to the interpretation of the biosynthesis of betalaines. *Helv. Chim. Acta* **1972**, *55*, 642–648.

(34) Nemzer, B.; Pietrzkowski, Z.; Spórna, A.; Stalica, P.; Thresher, W.; Michałowski, T.; Wybraniec, S. Betalainic and nutritional profiles of pigment-enriched red beetroot (*Beta vulgaris* L.) dried extracts. *Food Chem.* **2011**, *127*, 42–53.