JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Studies on Nonenzymatic Oxidation Mechanisms in Neobetanin, Betanin, and Decarboxylated Betanins

Sławomir Wybraniec,*[,†] Karolina Starzak,[†] Anna Skopińska,[†] Boris Nemzer,[‡] Zbigniew Pietrzkowski,[§] 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

[‡]Chemistry Research, FutureCeuticals, Inc., 2692 North State Route 1-17, Momence, Illinois 60954, United States

[§]Applied BioClinical Inc., 16259 Laguna Canyon Road, Irvine, California 92618, United States

ABSTRACT: A comprehensive nonenzymatic oxidation mechanism in betanin plant pigment as well as its derivatives, 2-decarboxybetanin, 17-decarboxybetanin, 2,17-bidecarboxybetanin, and neobetanin, in the presence of ABTS cation radicals was investigated by LC-DAD-ESI-MS/MS. The main compounds formed during the first step of betanin and 2-decarboxybetanin oxidation are 2-decarboxy-2,3-dehydrobetanin and 2-decarboxyneobetanin, respectively. In contrast to betanin, the reaction mechanism for 2-decarboxybetanin includes more oxidation pathways. Parallel transformation of 2-decarboxybetanin quinone methide produces neoderivatives according to an alternative reaction that omits the presumably more stabile intermediate 2-decarboxy-2,3-dehydrobetanin. The main oxidation product after the first reaction step for both 17-decarboxybetanin and 2,17-bidecarboxybetanin is 2,17-decarboxy-2,3-dehydrobetanin. This product is formed through irreversible decarboxylation of the 17-decarboxybetanin quinone methide or by oxidation of 2,17-bidecarboxybetanin. Oxidation of neobetanin results primarily in a formation of 2-decarboxy-2,3-dehydroneobetanin by a decarboxylative transformation of the formed neobetanin quinone methide. The elucidated reaction scheme will be useful in interpretation of redox activities of betalains in biological tissues and food preparations.

KEYWORDS: *betanin, neobetanin, betacyanins, dopachrome, aminochrome, quinone methide, antioxidation, 5,6-dihydroxyindole*

INTRODUCTION

Betalains are a group of water-soluble plant pigments that are used in industry as food colorants[1,2] and possess chemopreventive and strong antioxidant properties.[3−6] The betalain subgroup includes betacyanins (Figure 1), which are primarily immonium conjugates of betalamic acid with glycosylated *cyclo*-DOPA .[1,2,7] Betanin **1**, the principal pigment of red beet root (*Beta vulgaris* L.), was the first and most frequently studied betalain for its antioxidant activity.[3,5,6,8−13] Among others, the isolated betacyanins were tested with 1,1-diphenyl-2-picrylhydrazyl (DPPH)[11] and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)[12,13] radicals.

The antioxidant activity of purified betalains against ABTS has been tested under the influence of pH and other physicochemical conditions to explore structure activity relationships in betalains[12,13] and in a particularly important betalain structure constituent, betalamic acid.[14] Although recent enzymatic studies[15] have shed some light on the oxidation pathways of betacyanins, the nonenzymatic structural data are still lacking. Recent studies on the structural implications of semisynthetic natural or artificial betalains confirmed the fact that the high antioxidant activity of these molecules is affected by the presence of one or two phenolic groups, but raised a possibility that other unknown structural factors should also be taken into account.[12−14] With the exception of the enzymatic[15] and electrochemical[16] research on betalains, the mechanism by which ABTS radicals oxidize betalains is also unknown. Understanding the structural features of these pigments that are responsible for their antioxidant effect has great bearing on future development in this area.

ABTS radicals are very commonly used organic probes for evaluating the antioxidant activity of natural compounds. The kinetics of the reactions between the ABTS radical cations and several groups of compounds (e.g., polyphenols) are quite complex, and the lack of a relationship between the rate and stoichiometric factors has been reported.[17] In addition, some of the degradation products of ABTS radicals were identified, suggesting a lability of ABTS radicals at certain conditions.[18]

In recent studies on the enzymatic oxidation of betanidin (deglucosylated betanin),[15] the presence of prominent oxidation products at pH 3, 2-decarboxy-2,3-dehydrobetanidin and 2,17-bidecarboxy-2,3-dehydrobetanidin, indicated their generation via two possible reaction paths with two different quinonoid intermediates: dopachrome and quinone methide derivatives. Both reaction pathways lead to the decarboxylative dehydrogenation of betanidin. Subsequent oxidation and rearrangement of the conjugated chromophoric system results in formation of 14,15-dehydrogenated derivatives. At higher pH (4−8), two main oxidation peaks of betanidin are observed: betanidin quinonoid (presumably betanidin *o*-quinone) and 2-decarboxy-2,3-dehydrobetanidin.[15] In contrast, betanin (5-*O*-glucosylated

Received: February 23, 2013
Revised: May 21, 2013
Accepted: May 22, 2013
Published: June 24, 2013

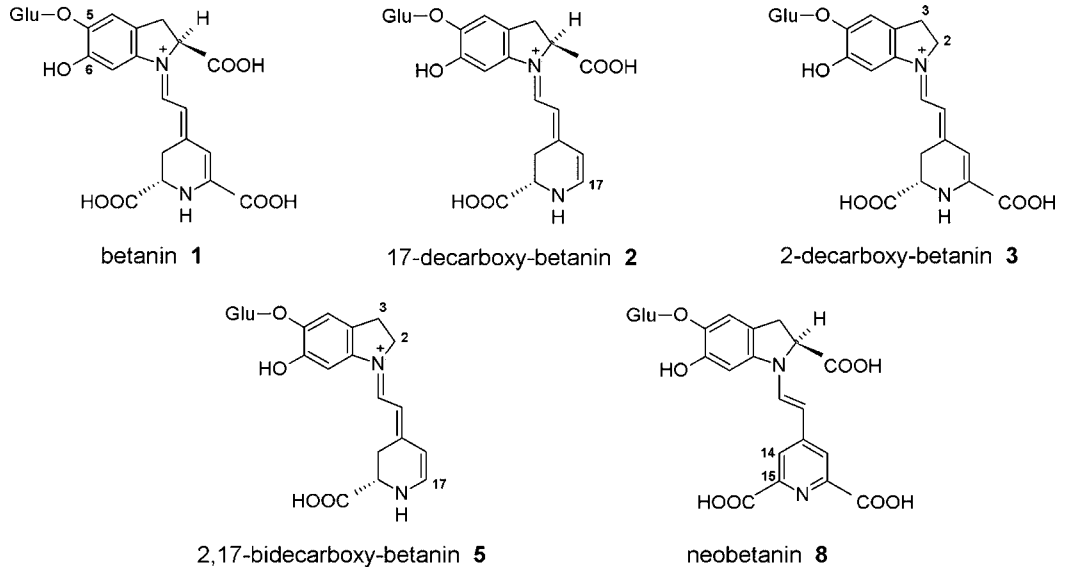

Figure 1. Chemical structures of betalains subjected to the oxidation study.

Table 1. Chromatographic, Spectrophotometric, and Mass Spectrometric Data of the Analyzed Products of Betanin and 2-Decarboxybetanin Oxidation by ABTS Cation Radicals

no.	compound	retention time (min)	λ_{max} (nm)	m/z $[M + H]^+$	m/z from MS/MS of $[M + H]^+$
		Betanin Oxidation			
H-2a	dihydroxylated **6**[a]	11.2	525	539	377
H-3a	dihydroxylated **9**[a]	11.3	432	493	331
H-2b	dihydroxylated **6**[a]	11.9	525	539	377
H-3b	dihydroxylated **9**[a]	12.1	432	493	331
1	betanin	14.4	538	551	389
4	2,17-bidecarboxy-2,3-dehydrobetanin[a]	20.3	462	461	299; 255
6	2-decarboxy-2,3-dehydrobetanin[a]	20.8	446	505	343; 299; 255
7	2,15,17-tridecarboxy-2,3-dehydroneobetanin[a]	21.8	394	415	253
9	2,17-bidecarboxy-2,3-dehydroneobetanin[a]	24.5	407	459	297; 253
12	2-decarboxy-2,3-dehydroneobetanin[a]	28.1	422	503	341; 297; 253
		2-Decarboxybetanin Oxidation			
H-2a	dihydroxylated **6**[a]	11.2	525	539	377
H-2b	dihydroxylated **6**[a]	11.9	525	539	377
3	2-decarboxybetanin	19.1	533	507	345
4	2,17-bidecarboxy-2,3-dehydrobetanin[a]	20.3	462	461	299; 255
6	2-decarboxy-2,3-dehydrobetanin[a]	20.8	446	505	343; 299; 255
7	2,15,17-tridecarboxy-2,3-dehydroneobetanin[a]	21.8	394	415	253
9	2,17-bidecarboxy-2,3-dehydroneobetanin[a]	24.5	407	459	297; 253
10	2,17-bidecarboxyneobetanin[a]	25.8	460	461	299; 255
11	2-decarboxyneobetanin[a]	26.5	482	505	343; 299; 255
12	2-decarboxy-2,3-dehydroneobetanin[a]	28.1	422	503	341; 297; 253

[a]Tentatively identified.

betanidin) is presumably oxidized solely via generation of a quinone methide intermediate that rearranges to 2,3-dehydro- or neoderivatives.[15] The products of enzymatic oxidation of betacyanins thus formed are therefore derivatives of 5,6-dihydroxyindole and related structures known as the key intermediates in melanogenesis.[19,20]

Further research on the oxidation of betanin decarboxylated/dehydrogenated derivatives (Figure 1) should clarify the reaction pathways. Recent comprehensive studies established the basic directions of thermal decarboxylation and dehydrogenation of betalains in aqueous[21−25] and alcoholic[21,22] media. It is probable that these processes are also affected by oxidation, but the mechanism is still unknown.

During the chain of the transformations following the initial quinonoid generation, certain decarboxylation steps are evident. Taking into account the whole scheme of the betanin oxidation pathways,[15] the decarboxylation at carbon C-2 can be explained as a result of the tautomerization of the quinone methide intermediate to the indolic derivative.

Decarboxylation at carbon C-17 can proceed presumably even without any oxidation (e.g., by heating of the pigment alcoholic solutions;[26] however, this reaction is also observed in some of the oxidized semiproducts, and the mechanism can be at least partially explained according to the previous paper.[15] Because betanin oxidation can proceed through many pathways, the initial detachment of the carboxyl from this substrate should

Table 2. Chromatographic, Spectrophotometric, and Mass Spectrometric Data of the Analyzed ABTS Cation Radical Products of 17-Decarboxy- and 2,17-Bidecarboxybetanins as well as the Only Dehydrogenated Substrate, Neobetanin, Oxidation

no.	compound	retention time (min)	λ_{max} (nm)	m/z $[M + H]^+$	m/z from MS/MS of $[M + H]^+$
	17-Decarboxybetanin Oxidation				
H1	dihydroxylated **4**[a]	11.0	494	495	333
2	17-decarboxybetanin	15.5	505	507	345
2′	17-decarboxyisobetanin	16.8	505	507	345
4	2,17-bidecarboxy-2,3-dehydrobetanin[a]	20.3	462	461	299; 255
7	2,15,17-tridecarboxy-2,3-dehydroneobetanin[a]	21.8	394	415	253
9	2,17-bidecarboxy-2,3-dehydroneobetanin[a]	24.5	407	459	297; 253
	2,17-Bidecarboxybetanin Oxidation				
H1	dihydroxylated **4**[a]	11.0	494	495	333
5/5′	2,17-bidecarboxybetanin/-isobetanin	20.5	507	463	301
4	2,17-bidecarboxy-2,3-dehydrobetanin[a]	20.3	462	461	299; 255
7	2,15,17-tridecarboxy-2,3-dehydroneobetanin[a]	21.8	394	415	253
9	2,17-bidecarboxy-2,3-dehydro-neobetanin[a]	24.5	407	459	297; 253
	Neobetanin Oxidation				
7	2,15,17-tridecarboxy-2,3-dehydroneobetanin[a]	21.8	394	415	253
8	neobetanin	21.9	466	549	387; 343; 299
9	2,17-bidecarboxy-2,3-dehydroneobetanin[a]	24.5	407	459	297; 253
12	2-decarboxy-2,3-dehydroneobetanin[a]	28.1	422	503	341; 297; 253

[a]Tentatively identified.

simplify the observed oxidation scheme and give a more definite picture of the whole process.

Decarboxylated betacyanins (Figure 1) have been characterized in heated preparations of red beet root (*B. vulgaris* L.) and purple pitaya fruit extracts and juices.[21−25] The studies on thermal decarboxylation of betacyanins reported derivatives of different decarboxylation levels: 2-decarboxybetacyanins **3**, 17-decarboxybetacyanins **2**, 2,17-bidecarboxybetacyanins **5** (Figure 1), and 2,15,17-tridecarboxybetacyanins. Therefore, for the structural studies, oxidation mechanisms of the well-defined decarboxylated betanin structures should be determined more easily.

In this study, we investigated the possible nonenzymatic oxidation pathways of betacyanins that occur in the presence of ABTS radicals. For a comprehensive insight into possible oxidation structures, betanin **1** and a series of decarboxylated betanins **2**, **3**, and **5** as well as neobetanin **8** (the only dehydrogenated derivative of betanin (Figure 1)) were subjected to a comparative evaluation of oxidation pathways based on the LC-DAD-MS/MS results.

MATERIALS AND METHODS

Reagents. Formic acid, LC-MS grade methanol, acetonitrile, and water, the diammonium salt of ABTS, potassium persulfate, and peroxidase from horseradish type II (150−250 units/mg solid (using pyrogallol)) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of Natural Analytes. Betanin and neobetanin were isolated from a betalain-rich red beet root extract, a novel and proprietary food-based extract prepared from red beet roots, obtained from FutureCeuticals, Inc., USA, where it was produced using a patent-pending technology.[27] The extract was dissolved in water and was separated on Sephadex DEAE A-25 gel and by solid phase extraction on C18 cartridges before HPLC preparative fractionation.[28] The eluates were concentrated under reduced pressure at 25 °C.

Semisynthesis of Decarboxylated Betanins. Thermal decarboxylation of betanin **1** was performed for generation of decarboxylated betanins according to previously published procedures.[21,22,25] Heating betanin in its ethanolic and aqueous solutions produced three pigments differing in decarboxylation position (Tables 1 and 2) and absorption maximum λ_{max} (533, 505, and 507 nm for 2-mono-, 17-mono-, and 2,17-bidecarboxybetanin, respectively) (Figure 1). The diastereomers of 2,17-bidecarboxylated derivatives of betanin/isobetanin are not well separated in various chromatographic systems[21,29] and, therefore, for the experiments, both the pairs were taken.

Semipreparative HPLC. For the semipreparative isolation of betanin, neobetanin, and decarboxylated betanins from the purified red beet root extracts, an HPLC system with a UVD170S detector, an HPLC pump series P580, and a thermostat (Gynkotek Separations, H. I. Ambacht, The Netherlands) was used. The semipreparative column used was a 250 mm × 10 mm i.d., 10 μm, Luna C18(2), with a 10 mm × 10 mm i.d. guard column of the same material (Phenomenex, Torrance, CA, USA) under the following gradient system: 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 μL 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.

Spectrophotometric Monitoring of Oxidation Kinetics. The main betalain oxidation experiments were performed by their reaction with ABTS cation radicals in 25 mM acetate (pH 3−5.5) and phosphate (pH 6−8) buffers in 96-well plates of a microplate reader Infinite 200 (Tecan Austria GmbH, Grödig/Salzburg, Austria). ABTS cation radicals were generated from ABTS salt by reaction of 2.45 mM potassium persulfate with 7 mM ABTS salt in 0.002 M phosphate-buffered saline, pH 7.4, for 16 h at room temperature in the dark. The resultant ABTS cation radical solution was diluted with water to give a 1.2 mM solution for the experiments. Just before the measuring step, 20 μL of ABTS radicals was dispensed to each well containing dissolved 20 μM pigment, bringing the volume to 200 μL. The mixture was then shaken for 20 s by a shaker within the reader. Spectra were collected over 120 min at a temperature of 25 °C by spectrophotometric detection at the wavelength range of 380−650 nm. The wavelength maximum was extended to 650 nm to monitor one of the absorption bands of the ABTS radicals closer to the IR range. For a comparison, the action of 0.01 EU/mL peroxidase II on the 20 μM pigment solutions in the presence of 1 mM H_2O_2 was monitored in the above experimental setup.[15] For the chromatographic analysis, 50 μL samples of each well were injected directly to the HPLC column without further purification.

Chromatographic Analysis. For the chromatographic analysis, the same system as for the semipreparative HPLC was used. Data were acquired with the Chromeleon 4.32 (Gynkotek Separations) software package. Samples were eluted through a 250 mm × 3 mm i.d., 5 μm,

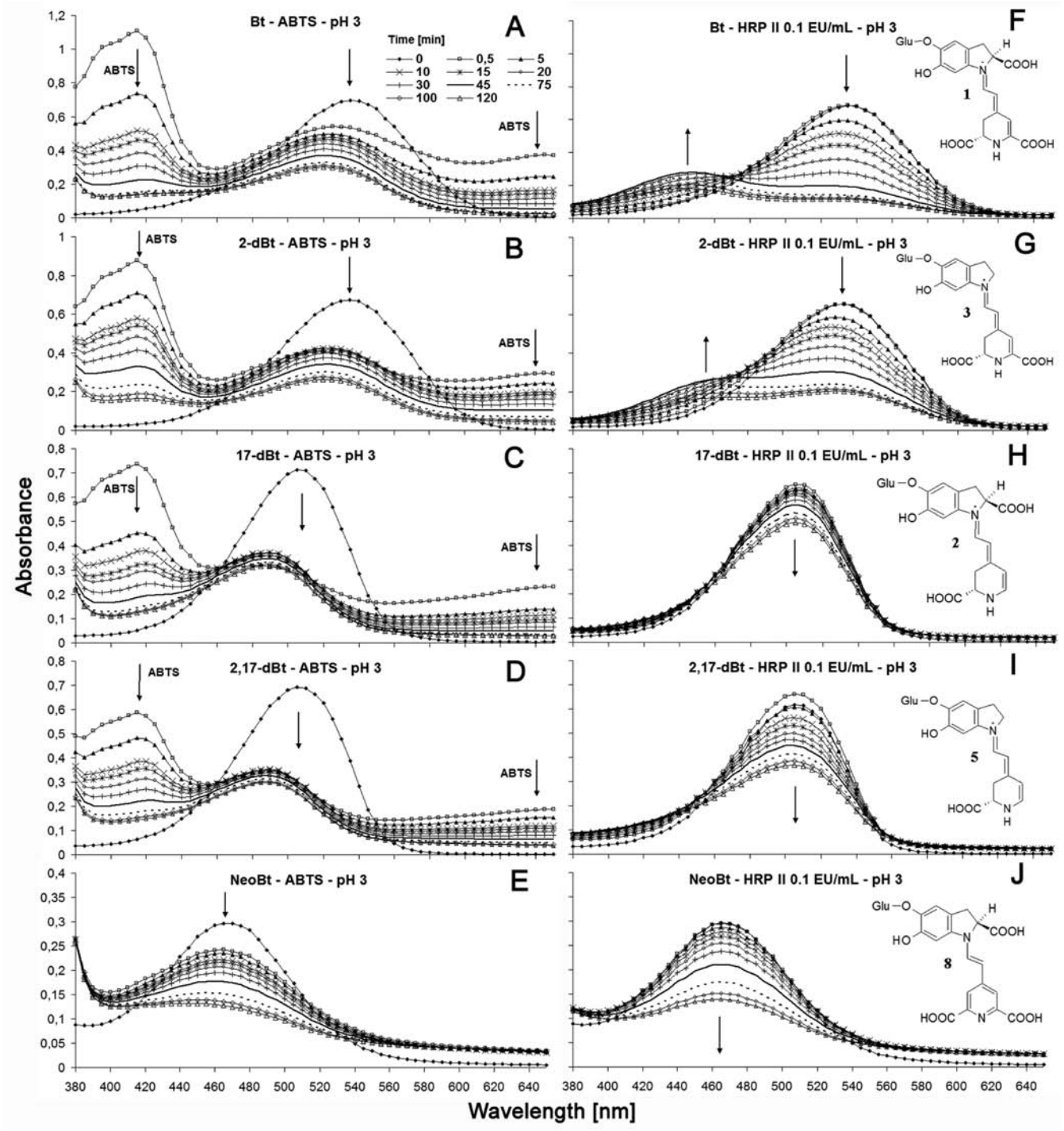

Figure 2. Visible spectra recorded at 25 °C during oxidation of 20 μM betanin (A, F), 2-decarboxybetanin (B, G), 17-decarboxybetanin (C, H), 2,17-bidecarboxybetanin (D, I), and neobetanin (E, J) by 120 μM ABTS radicals at pH 3 (A–E) and 0.01 EU/mL horseradish peroxidase II in the presence of 1 mM H_2O_2 at pH 3 (F–J).

Luna C18(2) column preceded by a 4 mm × 2 mm i.d. guard column of the same material (Phenomenex). The injection volume was 10 μL, and the flow rate was 0.5 mL/min. The column was thermostated at 35 °C. For the separation of the analytes, a gradient system was used: 7% A in B at 0 min; gradient to 20% A in B at 35 mi, (A, methanol; B, 2% (v/v) HCOOH in H_2O). Online UV–vis spectra acquisition was performed using the diode array detection (DAD) mode typically at 538, 505, 480, and 440 nm. The same chromatographic conditions were applied to 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 HPLC gradient systems 1 and 2. The MS was controlled with ThermoFinnigan Xcalibur software (San Jose, CA, USA), which recorded total ion chromatograms and mass spectra. Helium was used to improve 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).

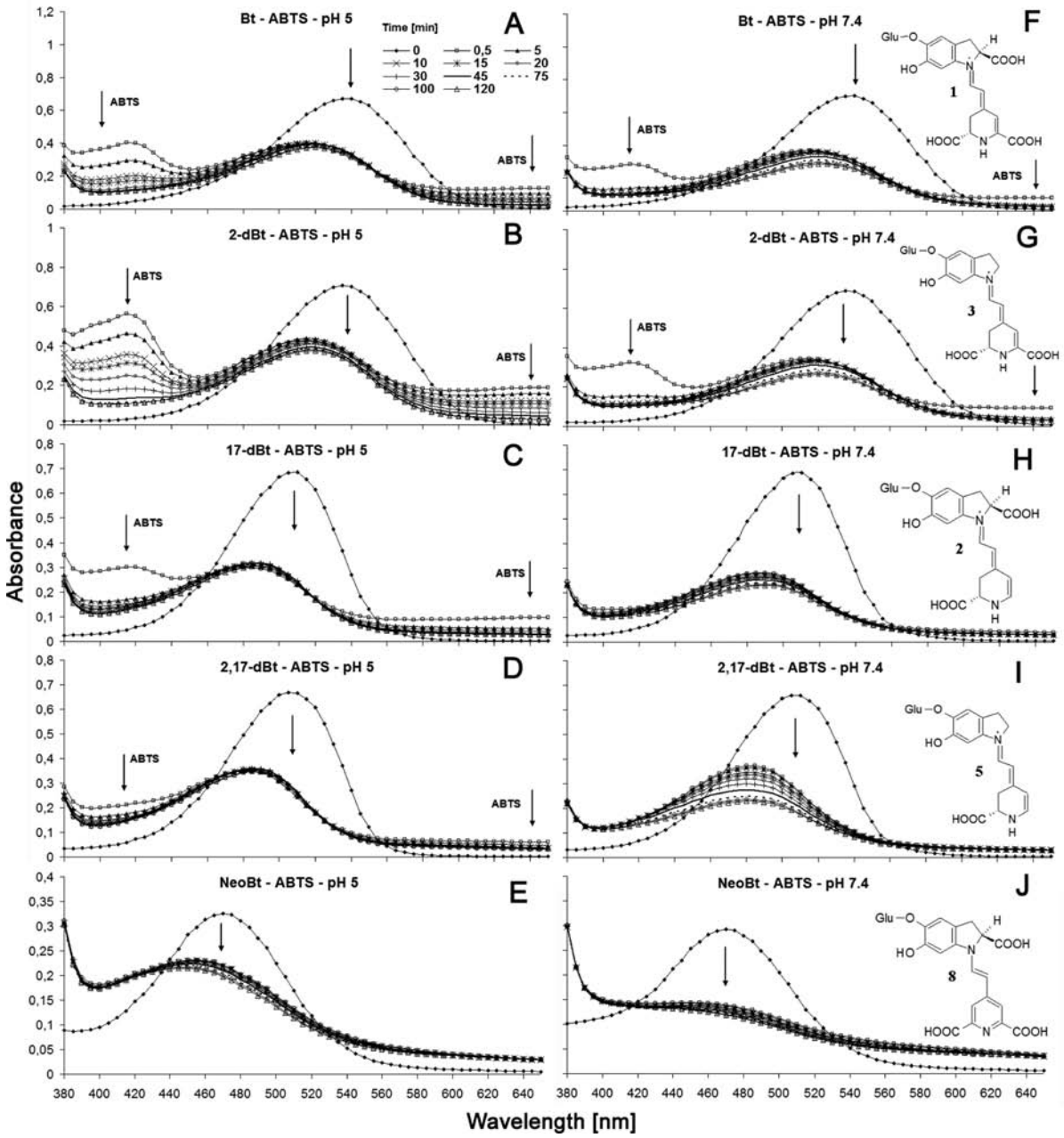

Figure 3. Visible spectra recorded at 25 °C during oxidation of 20 μM betanin (A, F), 2-decarboxybetanin (B, G), 17-decarboxybetanin (C, H), 2,17-bidecarboxybetanin (D, I), and neobetanin (E, J) by 120 μM ABTS radicals at pH 5 (A–E) and pH 7.4 (F–J).

RESULTS AND DISCUSSION

Monitoring of Betalain Oxidation by ABTS Radicals. According to the results of the recent enzymatic study, the oxidation of betanin proceeds solely through a quinone methide intermediate.[15] The mechanism of nonenzymatic oxidation of betacyanins, however, had not been explored, and for a more comprehensive examination of this issue, a group of decarboxylated and dehydrogenated derivatives of betanin as oxidation substrates was also studied. The simplified structures of the derivatives, as well as their different reactivities, were expected to give additional information about the mechanism.

Betanin **1** and decarboxylated betanins **2**, **3**, and **5** are the glucosylated derivatives of the chromophoric structures of betanidin or decarboxylated betanidins. The chromophoric units of betanidin are the only ones with the 5,6-dihydroxyl moiety (catechol moiety) and, consequently, possess higher observed antioxidant activity.[8,12,13] Recent enzymatic oxidation experiments on betanidin at pH 3–8 confirmed the prevailing effect of the *o*-diphenol group on the oxidation pathway.[15]

The spectrophotometric analysis results of ABTS radical-induced oxidation of pigments performed at pH 3, 5, and 7.4 for 2 h are presented in Figures 2 and 3. In addition, the spectra taken during the enzymatic oxidation of the pigments by horseradish peroxidase II at pH 3 are shown for a comparison (Figure 2F–J). The highest enzymatic oxidation activity observed at pH 3 not only for betanin but also for the tested derivatives of betanin (data not shown) is in contrast to the results obtained for betanidin.[15]

Because of the absorption characteristics of the ABTS radicals (especially the absorption at λ_{max} 415 nm), the spectra of the oxidized pigments cannot be well observed at the initial stages of

dx.doi.org/10.1021/jf400818s | J. Agric. Food Chem. 2013, 61, 6465–6476

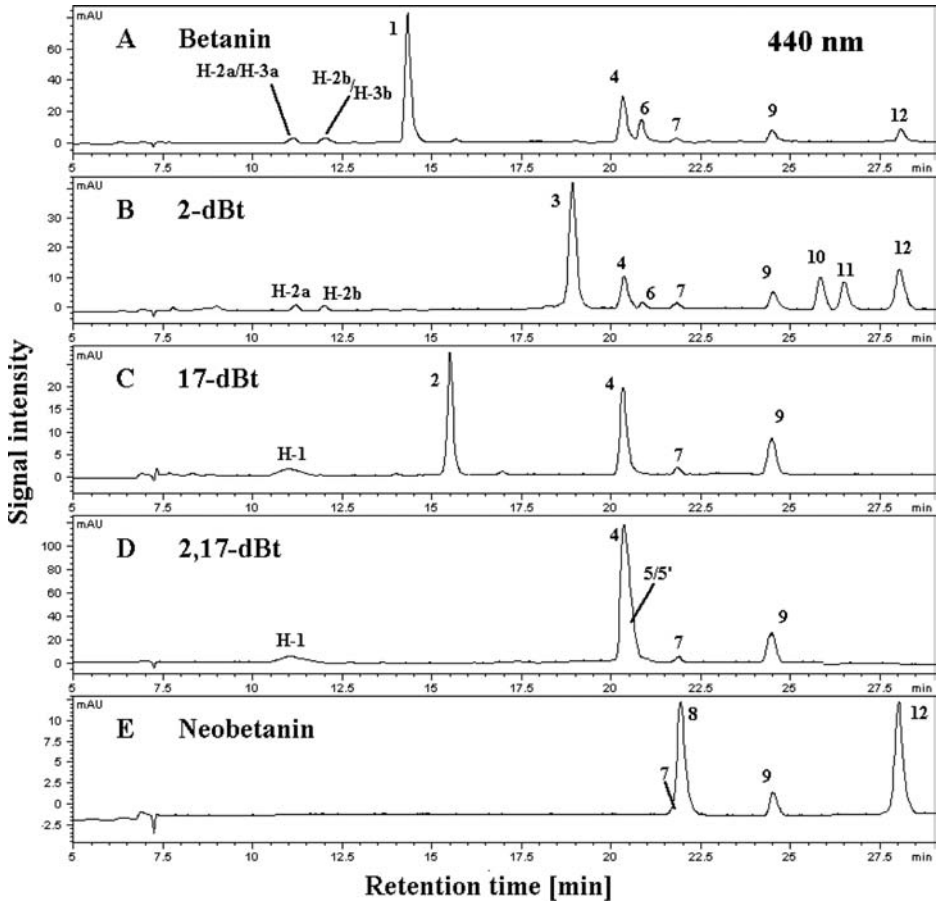

Figure 4. Chromatographic traces of the products of 20 μM (A) betanin, (B) 2-decarboxybetanin, (C) 17-decarboxybetanin, (D) 2,17-bidecarboxybetanin, and (E) neobetanin oxidation by 120 μM ABTS radicals at pH 3 and 25 °C.

the oxidation. Nevertheless, the ABTS overlapping spectra are diminished practically to the baseline during the process because of the excess of the analyzed pigments. Therefore, it is possible to observe the final result of the oxidation without the interfering presence of the ABTS radicals.

The first inspection of the consecutive visible spectra registered during the course of oxidation reveals a fast initial decrease of each of the main absorption bands of the pigment. After this point, further changes are much slower or negligible, such as the reaction at pH 5. The ABTS radical bands continuously decrease throughout the reaction. This may indicate a reaction of ABTS radicals with already formed oxidized pigment products and the presence of multiple pigment oxidations steps based on the stoichiometry of the reaction. However, possible degradation reactions of ABTS radicals induced by the substrates or the intermediary oxidized products must be also considered.[18]

In the case of betanin and 2-decarboxybetanin, the λ_{max} main absorption band is moved from the starting value to 520−525 nm within the first few minutes of the experiment, but is not further changed for the remaining 2 h (Figures 2 and 3). For 17-decarboxy- and 2,17-decarboxybetanin, a shift of the λ_{max} from 505−507 to 480−490 nm is observed. For neobetanin, a shift of λ_{max} from 470 to 445 nm is observed throughout the entire reaction time at pH 5−7.4, whereas the λ_{max} is shifted to 425 nm at pH 3 (Figures 2 and 3).

The highest rate of the reactions is observed at pH 7.4, at which the fastest decrease of ABTS concentration is evident. For betanin and decarboxybetanins, the decrease of the pigment absorption at pH 3 is almost as fast as at pH 7.4; however, the decrease of the ABTS absorption is much slower, reaching the baseline after 2 h. This is dissimilar to the results of a previous study on betanin enzymatic oxidation,[15] when the fastest oxidation was observed at pH 3 and almost no progress was observed at pH 7. For neobetanin, a fast decrease of the ABTS absorption bands is visible for all of the tested pH values; there is a slower decrease rate of pigment absorption at pH 3.

At pH 3, the shift of λ_{max} during HRP oxidation is much smaller than in ABTS oxidation (Figure 2). Furthermore, the enzymatic oxidation of betanin and 2-decarboxybetanin results in a formation of additional absorption bands at λ_{max} 445 and 460 nm, respectively. The above data suggest that the prevailing products of the pigment ABTS oxidation are characterized by λ_{max} ca. 520−525 nm, in contrast to the enzymatic oxidation, when the λ_{max} 445−460 nm is distinctive.

LC-DAD-ESI-MS/MS Analytical Results of the Oxidation of Betanin and Its Derivatives by ABTS Cation Radicals. The LC-MS/MS analysis of betanin oxidized products by ABTS cation radicals (Table 1) indicated mainly the presence of betanin derivatives observed previously during the enzymatic oxidation.[15] The chromatographic traces (λ 440 nm) of the tested mixtures generated during the first 1 h of the pigment oxidation experiments are presented in Figure 4. The presence of betanin oxidized products confirms the initial radical scavenging activity of betanin (Figure 5) as well as the main oxidation pathway of betanin shown in Figure 6. Two consecutive ABTS radical scavenging steps result in the generation of a quinonoid derivative of betanin. If oxidation of betanin results in a formation

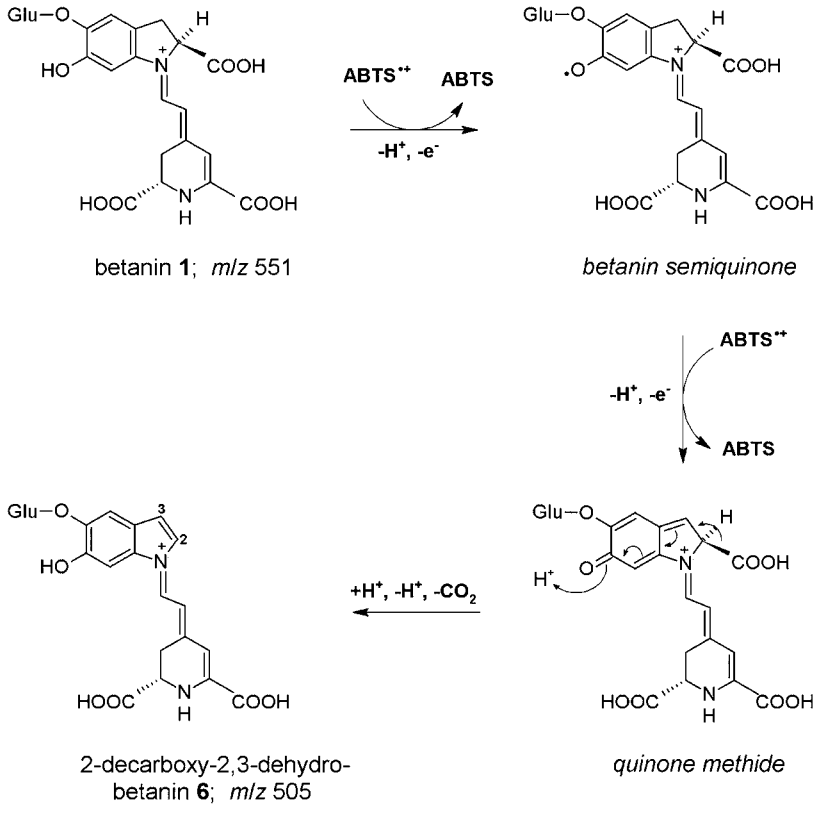

Figure 5. ABTS radical scavenging action of betanin resulting in a formation of betanin semiquinone and quinone methide as well as rearrangement of quinone methide to stable oxidation product 2-decarboxy-2,3-dehydrobetanin **6**.

of betanin semiquinone radical, it should undergo the subsequent oxidation, resulting in the formation of the quinone methide intermediate and rearrangement according to the reaction path depicted in Figure 6,[15] because the formation of the aminochrome intermediate is impossible, resulting from the blocking of the hydroxyl at C-5.

One of the main chromatographic peaks of the oxidation products **6** thus formed is observed with absorption maximum λ_{max} 446 nm. Comparison of **6** with the corresponding compound obtained during the enzymatic oxidation of betanin[15] resulted in coelution of these two compounds, suggesting the presence of 2-decarboxy-2,3-dehydrobetanin. This finding is supported by the LC-MS/MS detection of a protonated molecular ion $[M + H]^+$ at m/z 505 and the subsequent fragmentation ions at m/z 343 (loss of glucose moiety) as well as m/z 299 and 255 (losses of CO_2). As in the case of the enzymatic oxidation,[15] the dehydrogenation leading to **6** is presumably a result of the oxidation of the only one free phenolic moiety at C-6 and, therefore, the position of the resulting decarboxylation must take place at C-2. The presence of **6** in the reaction mixture is crucial for further discussions on the oxidation of betanin and decarboxylated betanins, which determine the main oxidation pathway.

In the course of decarboxylation of **6** (at the C-17 position[26]), another betanin derivative can be detected. Presumably, this product is 2,17-decarboxy-2,3-dehydrobetanin **4** (Table 1). This compound is characterized by a protonated molecular ion $[M + H]^+$ at m/z 461 and absorption maximum at λ_{max} 462 nm, similar to one indicated in the previous papers.[1] The fragmentation ions at m/z 299 and 255 confirm the presence of bidecarboxylated and dehydrogenated fragments of betanidin in the molecule (Figure 6). The mechanism of the most frequent decarboxylation in the betalainic chromophore leading to the formation of 17-decarboxylated derivative was studied by Dunkelblum et al.[26] in ethanolic solutions; therefore, it was taken into account in the case of **6** and other derivatives, favoring the decarboxylation at carbon C-17 instead of C-15.

Even more informative, however, is the presence of 2,17-bidecarboxy-2,3-dehydrobetanin **4** in the oxidation products of 17-decarboxybetanin and 2,17-bidecarboxybetanin (Figure 6). In these cases, compound **4** is the main dehydrogenated derivative that is formed in the course of 17-decarboxybetanin oxidation with subsequent irreversible decarboxylation or by oxidation of 2,17-bidecarboxybetanin. The lack of **6** in the analyzed products of 17-decarboxybetanin oxidation suggests that the generated quinone methide intermediate rearranges to the dihydroxyindolic form by removing CO_2 (instead of proton) at the C-2 according to the previously described mechanism.[15]

In the case of betanin, neobetanin (Figure 6), and 2-decarboxybetanin (Figures 7 and 8), the most hydrophobic reaction product **12** is detected in the chromatograms at λ_{max} 422 nm and has the highest retention time (Tables 1 and 2). The identity of **12** was tentatively assigned to 2-decarboxy-2,3-dehydroneobetanin because of the presence of a protonated molecular ion $[M + H]^+$ at m/z 503 as well as the fragmentation ions at m/z 341, 297, and 253.[15] The doubly oxidized derivative of decarboxylated betanin is a result of the oxidation of **6** and a chain of rearrangements leading to the formation of the 14,15-dehydro derivative (neobetacyanin) according to the recent study.[15] However, as discussed below, we postulate also an alternative mechanism of **12** formation during the oxidation of 2-decarboxybetanin (Figures 7 and 8). In addition, compounds **7**

dx.doi.org/10.1021/jf400818s | J. Agric. Food Chem. 2013, 61, 6465−6476

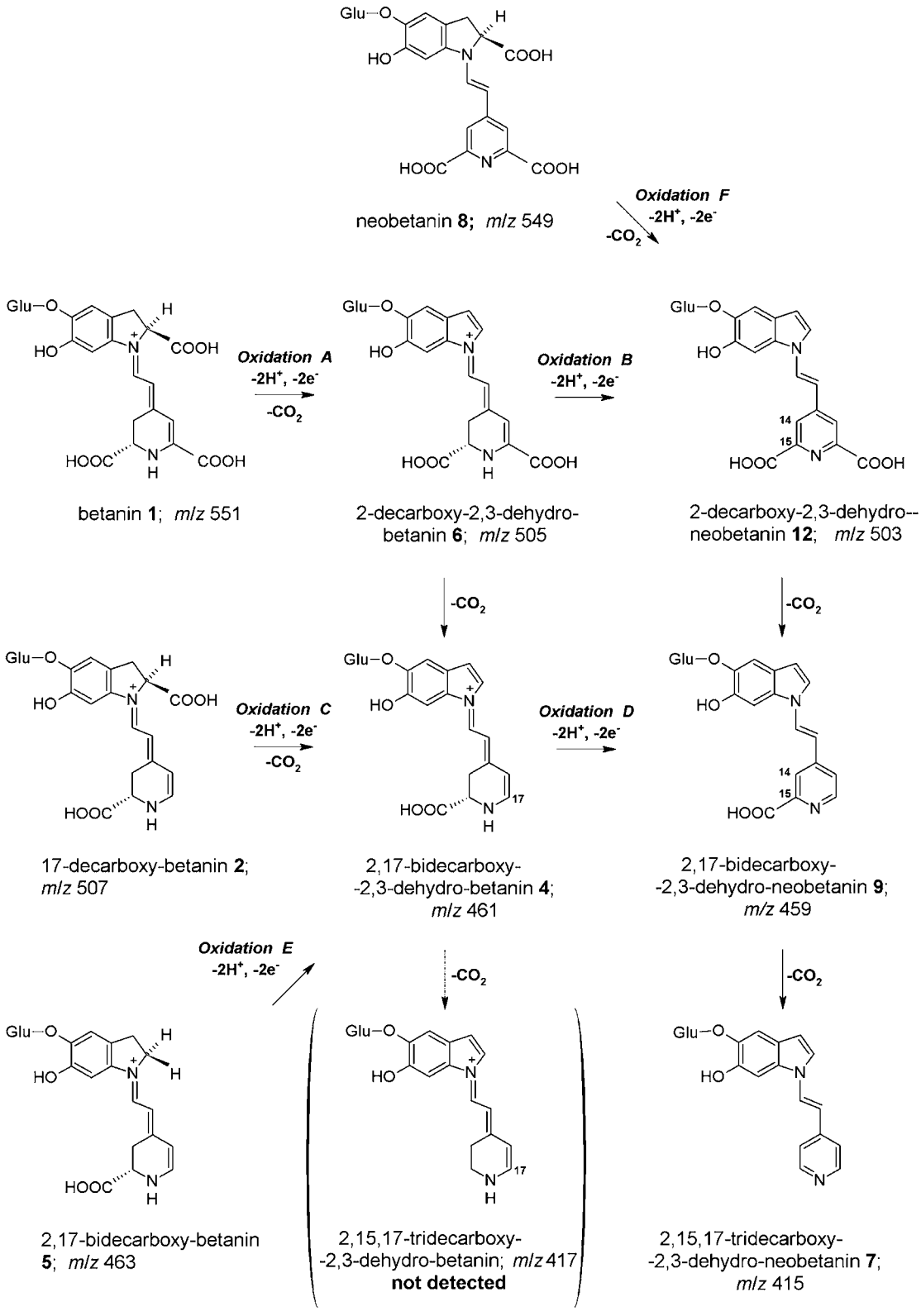

Figure 6. General scheme of betanin, 17-decarboxybetanin, 2,17-bidecarboxybetanin, and neobetanin oxidation by ABTS radicals.

and **9** were detected in all of the tested oxidation mixtures, indicating their generation from **4** and **12** (Figures 6 and 8).

Reaction Pathways for 2-Decarboxybetanin Oxidation by ABTS Cation Radicals. Nonenzymatic oxidation of 2-decarboxybetanin **3** results in generation of the most interesting profile of reaction products; therefore, the reaction mechanism for the first stages of the oxidation is presented in more detail in Figure 7. Except for the reaction products identified after betanin oxidation (**4**, **6**, **7**, **9**, and **12**), several additional compounds (**10** and **11**) were also detected (Figures 4B, 7, and 8). On the basis of the results, more oxidation pathways can be proposed.

dx.doi.org/10.1021/jf400818s | J. Agric. Food Chem. 2013, 61, 6465−6476

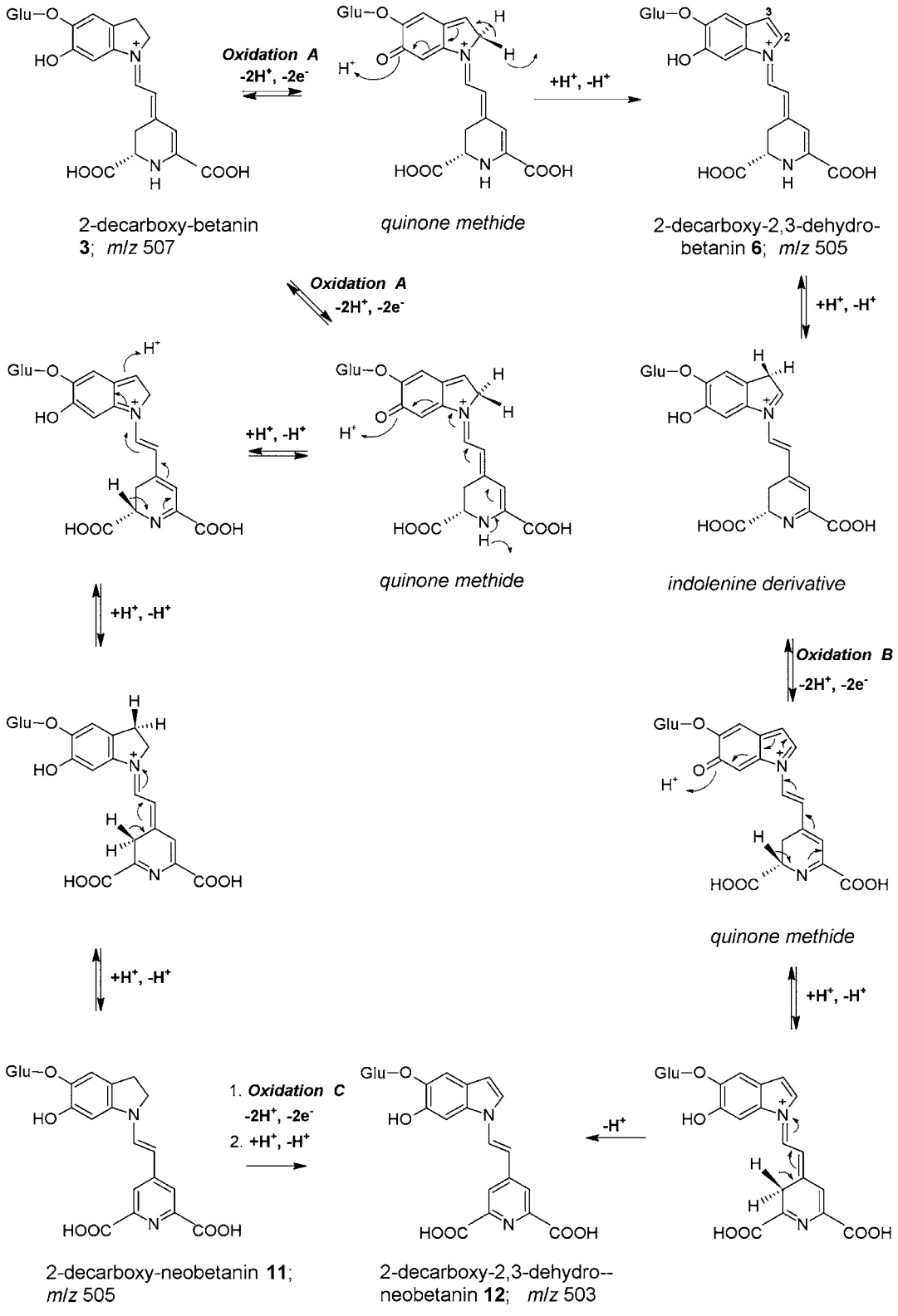

Figure 7. Proposed mechanism of 2-decarboxybetanin **3** oxidation by ABTS radicals via transformations of the formed quinone methide to two isomeric products **6** and **11** of the first step of the reactions (Oxidation A) and to **12** in the second step of the reactions (Oxidations B and C).

Initially, the formation of two isomeric compounds characterized by a protonated molecular ion $[M + H]^+$ at m/z 505 is suggested (Figure 7). Except for product **6**, an isomeric derivative **11** is observed in each chromatogram (Figure 4B). The presence of this compound was confirmed by LC-MS/MS detection of a protonated molecular ion $[M + H]^+$ at m/z 505 with fragmentation ions at m/z 343, 299, and 255. The visible absorption maximum for **11** was observed at λ_{max} 480 nm.

On the basis of the results, we postulate that the formation of 2-decarboxyneobetanin **11** results by a parallel transformation of

dx.doi.org/10.1021/jf400818s | J. Agric. Food Chem. 2013, 61, 6465−6476

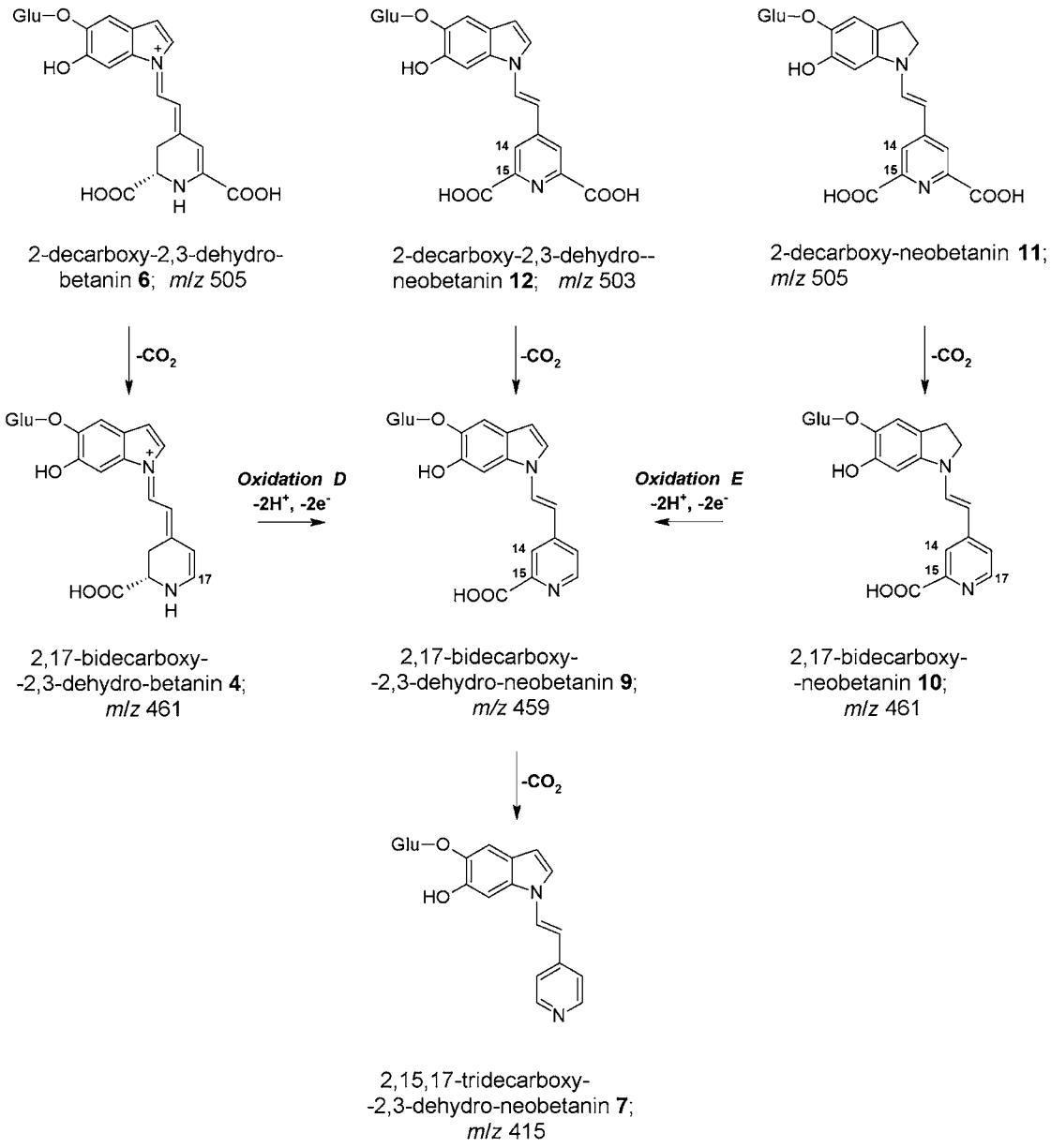

Figure 8. General scheme of the decarboxylation of **6**, **10**, and **12** formed during the first two steps of 2-decarboxybetanin **3** oxidation by ABTS radicals. The nonoxidative decarboxylation at the carbon C-17 proceeds presumably according to the mechanism reported previously.[26]

the quinone methide generated from 2-decarboxybetanin according to the alternate pathway (Figure 7). This alternate rearrangement does not include the presumably more stabile product 2-decarboxy-2,3-dehydrobetanin **6**, which is stabile as shown by the fact that it is present in most of the oxidation product mixtures. In addition, both the pathways lead, in the next oxidation steps, to the formation of the 2-decarboxy-2,3-dehydroneobetanin **12** (Figure 7) mentioned above.

The chromatographic analysis revealed compound **10**, a product that is isomeric with 2,17-bidecarboxy-2,3-dehydrobetanin **4** based on the detection of its protonated molecular ion $[M + H]^+$ at m/z 461 (fragmentation ions at m/z 299 and 255), with the same absorption maximum at λ_{max} 460 nm. In conjunction with the presence of **11** in the reaction mixtures, compound **10** is most probably formed by decarboxylation of **11** (Figure 8).

All of the decarboxylation steps starting from the formed oxidation products **6**, **11**, and **12** are schematically presented in Figure 8. These pathways lead to the formation of **4**, **9**, and **10**, respectively.[26] Subsequent oxidation of **4** and **10** results in the formation of 2,17-bidecarboxy-2,3-dehydroneobetanin **9** as shown by a protonated molecular ion at m/z 459, fragmentation ions at m/z 297 and 253, and absorption maximum at λ_{max} 407 nm. These are the same molecular properties as reported in the betanin enzymatic oxidation experiments.[15] Finally, the subsequent decarboxylation of **9** leads to the formation of 2,15,17-tridecarboxy-2,3-dehydroneobetanin **7**.

Formation of Dihydroxylated Derivatives of the Oxidized Products. The earlier study[15] concluded that the formation of dihydroxylated derivatives of some betanin oxidation products could be a result of a hydroxylation by H_2O_2.[15] In the current study, the hydroxylation of generated

dx.doi.org/10.1021/jf400818s | J. Agric. Food Chem. 2013, 61, 6465−6476

dehydrogenated derivatives can also be discussed (Figure 4; Tables 1 and 2).

The LC-MS/MS analysis of betanin **1** and 2-decarboxybetanin **3** oxidation product **H-2** yielded a protonated molecular ion $[M + H]^+$ at m/z 539 as well as subsequent fragmentation ions at m/z 377, 333, and 289, suggesting the presence of a dihydroxylated 2-decarboxy-2,3-dehydrobetanin **H-2**. This is presumably a result of 2-decarboxy-2,3-dehydrobetanin **6** generation as the first oxidation product and its subsequent reaction with hydroxyl radicals formed during the action of ABTS radicals. This suggestion is supported by chromatographic monitoring of the reaction mixtures in which the initial generation of **6** during the first 30−60 min was followed by a subsequent formation of **H-2a** and its isomer **H-2b** with a parallel decrease of **6** concentration (data not shown). The λ_{max} of **H-2a**/**H-2b** (520 nm) confirms that this compound is presumably the prevailing ABTS oxidation product as observed during the spectrophotometric experiments.

In addition, oxidation of betanin presumably results in a formation of another dihydroxylated derivative, **H-3a**/**H-3b**, which is characterized by a protonated molecular ion $[M + H]^+$ at m/z 493 as well as fragmentation ions at m/z 331 and 289 (Figure 4; Table 1). This suggests a presence of dihydroxylated 2,17-bidecarboxy-2,3-dehydroneobetanin **H-3a**/**H-3b** on the basis of the difference between **H-3a**/**H-3b** and **9** (m/z 493 − 459 = 34). As mentioned above, the presence of **9** is a result of the second betanin oxidation step (Figure 6). Interestingly, no dihydroxylated derivative of 2,17-bidecarboxy-2,3-dehydrobetanin **4** could be detected, which would be characterized by a protonated molecular ion $[M + H]^+$ at m/z 495. However, this compound (**H-1**) was generated during the oxidation of 17-decarboxybetanin **2** and 2,17-bidecarboxybetanin **5** (Figure 4; Table 2). In these cases, the presence of **H-1** with the absorption band at λ_{max} ca. 490 nm suggests that it is a prevailing ABTS oxidation product, taking into account the spectrophotometric experiments. The detection of the protonated molecular ion $[M + H]^+$ at m/z 495 and the fragmentation ions at m/z 333 and 291 supports the conclusion of a formation of dihydroxylated 2,17-bidecarboxy-2,3-dehydrobetanin **H-1** represented by a corresponding broad chromatographic peak (Figure 4). In both cases, 2,17-bidecarboxy-2,3-dehydrobetanin **4** is the most abundant oxidation product and probably reacts with hydroxyl radicals.

All of these dihydroxylated derivatives exhibit higher polarity than the decarboxylated/dehydrogenated betanins, as shown by low retention times in comparison to betanin (Figure 4; Tables 1 and 2). The position of the attachment of the two hydroxyl groups is yet to be elucidated. Considering that there is a double bond at C-2,3 in the structures of **4**, **6**, and **9**, it can be assumed that the hydroxyl groups are bound to the C-2,3 carbons. Furthermore, no diagnostic compounds that would indicate a dihydroxylation of the substrate pigments were detected, suggesting that the pigments saturated at the C-2,3 position are not hydroxylated under these conditions.

In conclusion, we present a detailed description of the oxidation pathways of various betacyanins under nonenzymatic conditions with ABTS radicals, as a commonly used diagnostic oxidation agent. Varying the starting substrates enables generation of a comprehensive survey of the possible reaction pathways. The results of 2-decarboxybetanin oxidation indicate the possibility of two main reaction pathways. For the other substrates, the rearrangement of the quinone methide proceeds only to the 2,3-dehydrogenated derivatives as the initial products.

The mechanism of betacyanins oxidation is of significant interest because of recent appreciation of these pigments as highly active natural compounds with antioxidative properties and their potential benefits to human health as well as a lack of toxicity, which suggest them as candidates for use in the food industry. The current work provides a comprehensive description of the betacyanin oxidation mechanism and may serve as a useful tool needed in further studies on the activity of these natural products.

AUTHOR INFORMATION

Corresponding Author

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

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Moreno, D. A.; Garcia-Viguera, C.; Gil, J. I.; Gil-Izquierdo, A. Betalains in the era of global agri-food science, technology and nutritional health. *Phytochem. Rev.* **2008**, *7*, 261−280.

(2) Henry, B. S. Natural food colours. In *Natural Food Colorants*; Hendry, G. A. F., Houghton, J. D., Eds.; Blackie Chapman and Hall: London, UK, 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) 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.

(9) 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.

(10) 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.

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

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

(13) 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.

(14) Gandía-Herrero, F.; Escribano, J.; García-Carmona, F. Purification and antiradical properties of the structural unit of betalains. *J. Nat. Prod.* **2012**, *75*, 1030−1036.

(15) Wybraniec, S.; Michałowski, T. New pathways of betanidin and betanin enzymatic oxidation. *J. Agric. Food Chem.* **2011**, *59*, 9612−9622.

(16) Wybraniec, S.; Stalica, P.; Spórna, A.; Nemzer, B.; Pietrzkowski, Z.; Michałowski, T. Antioxidant activity of betanidin: electrochemical study in aqueous media. *J. Agric. Food Chem.* **2011**, *59*, 12163−12170.
(17) Osman, A. M.; Wong, K. K. Y.; Fernyhough, A. ABTS radical-driven oxidation of polyphenols: isolation and structural elucidation of covalent adducts. *Biochem. Biophys. Res. Commun.* **2006**, *346*, 321−329.
(18) Osman, A. M.; Wong, K. K. Y.; Hill, S. J.; Fernyhough, A. Isolation and the characterization of the degradation products of the mediator ABTS-derived radicals formed upon reaction with polyphenols. *Biochem. Biophys. Res. Commun.* **2006**, *340*, 597−603.
(19) Ito, S. A chemist's view of melanogenesis. *Pigment Cell Res.* **2003**, *16*, 230−236.
(20) Sugumaran, M. Molecular mechanisms for mammalian melanogenesis. Comparison with insect cuticular sclerotization. *FEBS Lett.* **1991**, *293*, 4−10.
(21) Wybraniec, S. Formation of decarboxylated betacyanins in heated purified betacyanin fractions from red beet root (*Beta vulgaris* L.) monitored by LC-MS/MS. *J. Agric. Food Chem.* **2005**, *59*, 3483−3487.
(22) 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.
(23) Herbach, K. M.; Stintzing, F. C.; Carle, R. Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris* L.) preparations. *J. Food Sci.* **2004**, *69*, 491−498.
(24) Herbach, K. M.; Stintzing, F. C.; Carle, R. Betalain stability and degradation-structural and chromatic aspects. *J. Food Sci.* **2006**, *71*, R41−R50.
(25) 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.
(26) 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.
(27) Nemzer, B.; Pietrzkowski, Z.; Spórna, A.; Stalica, P.; Thresher, W.; Michałowski, T.; Wybraniec, S. Betalainic and nutritional profiles of pigment-enriched red beet root (*Beta vulgaris* L.) dried extracts. *Food Chem.* **2011**, *127*, 42−53.
(28) 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.
(29) Wybraniec, S. A method for identification of diastereomers of 2-decarboxy-betacyanins and 2,17-bidecarboxy-betacyanins in reversed phase HPLC. *Anal. Bioanal. Chem.* **2007**, *389*, 1611−1621.