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Formation of Decarboxylated Betacyanins in Heated Purified Betacyanin Fractions from Red Beet Root (*Beta vulgaris* L.) Monitored by LC−MS/MS

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Mixtures of mono-, bi-, and tridecarboxylated betacyanins together with their corresponding neobetacyanins obtained from *Beta vulgaris* L. root juice as heating degradation products of betacyanins were identified by high-performance liquid chromatography with tandem mass spectrometry (LC−MS/MS) and diode-array (LC−DAD) detection. Two monodecarboxy-betacyanin pairs of diastereomers were detected after the decarboxylation in ethanolic and aqueous solutions. Generation of 17-decarboxy-betacyanins and 2-decarboxy-betacyanins was suggested, the latter so far never having been attributed to betacyanin thermal degradation products. Other main products of decarboxylation were 2,17-bidecarboxybetanin, its isoform, and 14,15-dehydrogenated (neobetacyanin) derivatives of all the decarboxylated betacyanins. The results of this research are crucial in determining betacyanin degradation mechanisms in juices or extracts of *B. vulgaris* L. roots and other products containing these pigments.

KEYWORDS: Betanin; 2-decarboxy-betacyanins;17-decarboxy-betacyanins; neobetacyanins; betalains; decarboxylation; red beet root; *Beta vulgaris* L

INTRODUCTION

Red beet (*Beta vulgaris* L.) is an excellent source of nitrogenous pigments, the betalains, mainly being composed of two red-violet betacyanins, betanin and isobetanin (**Figure 1**) and minor yellow betaxanthins (*1*). Betanin is also known for its nontoxic properties, and red beet has been the subject of much experimental interest in using the red pigment in the pharmaceutical and food industries (*1*). Furthermore, its preventive anticarcinogenic properties have recently been reported (*2*). Since the safety and number of permitted synthetic colorants is subject to debate, there is a need to further investigate natural pigments for coloring purposes. However, the lower stability of natural pigments is still an important factor hampering their more widespread use. Betalains are known to be very sensitive to several factors including low pH, elevated temperatures, or high water activity (*3*−*7*). Especially, betalain thermal instability results in their restricted use. Some studies have already elaborated the conditions under which these pigments retain their attractive color and even discovered some of their degradation products (*8*−*10*).

The decarboxylation process of the simplest betacyanin, betanidin, was described by Dunkelblum et al. (*8*) and Minale and Piattelli (*9*). The main studies on decarboxylation of betanidin were performed in ethanol solutions (*8*, *9*). Studies on thermal degradation of betanin in various water/alkohol (ethanol, ethylene glycol, or glycerol) model systems at temperatures ranging between 60 and 86 °C were carried out by Altamirano et al. (*3*) and at 75 °C by Simon et al. (*11*). The lowest stability of betanin was found for ethanol or systems contaning a high fraction of ethanol; however, no structural studies were performed. Whereas the authors discussed the solvolytic splitting of betanin yielding betalamic acid and cyclodopa, they did not exclude any other degradation mechanism that could compete with the suggested one (*11*). Very recent parallel structural studies on thermal decarboxylation of betacyanins from red beet (*B. vulgaris* L.) (*12*) and purple pitaya

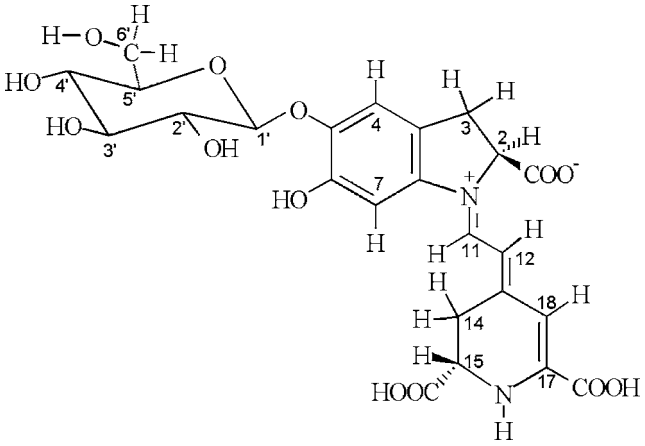

Figure 1. Chemical structure of betanin.

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(*Hylocereus polyrhizus*) (*13*) preparations in water solutions have been performed, and several mono- or bidecarboxylated and 14,15-dehydrogenated betacyanins were discovered. Some of the preliminarily identified compounds were assigned as 15- and 17-decarboxy-betacyanins based on their absorption maximum of λ_{max} 505 nm and HPLC−MS/MS data. Attempts to analyze degradation products of betanin were done by Schwartz and von Elbe (*10*, *14*), which concluded that 15-decarboxylated betanin was the proposed decarboxylation product. However, in our study using a new generation HPLC column we obtained some contradictory results to ref *10*.

During HPLC analysis on reversed phase, the retention times of decarboxylated products should be longer in comparison to their corresponding betacyanins due to the lower polarity of the former. The first attemps to analyze decarboxybetacyanins during HPLC separation on a C18 column with subsequent mass spectrometric detection of some chosen compounds were undertaken only recently (*12*, *13*, *15*). In addition, new decarboxylation products from *H. polyrhizus* fruit betacyanins were considered for the first time (*12*, *13*, *16*). Since according to the earlier reports (*8*, *9*) the main products of betanidin and isobetanidin decarboxylation appeared to be the C-15 stereoisomers, resulting from the carboxyl loss at C-17 and displaying absorption maxima at λ_{max} 505 nm, the structures of the decarboxylated products were deduced as 17-decarboxy-betacyanins and their isoforms. In addition to these compounds, other decarboxylation products were investigated in this study.

EXPERIMENTAL PROCEDURES

Plant Material. A total of 500 g of red beet roots was washed, hand-peeled, and cut into small pieces. The juice from the roots was obtained in a juice extractor (Zelmer, Rzeszów, Poland).

Purification. After filtration, the juice was purified and fractionated on a C18 cartridge according to the procedure of Stintzing et al. (*17*). The C18 cartridge (Merck, Darmstadt, Germany) was activated with 3 volumes of 100% methanol and then rinsed with 3 volumes of acidified water (pH 3). The sample was applied to the column and rinsed again with 3 volumes of acidified water (pH 3). The betaxanthin fraction was eluted with 100% methanol and discarded. Afterward, the betacyanin fraction was eluted with acidified methanol (methanol/acidified water (pH 2), 95:5, v/v) and rotovaporated (not to dryness) under reduced pressure at 30 °C before being freeze-dried. The purified betalains were dissolved in water or ethanol and submitted to decarboxylation experiments.

Decarboxylation. The 40 mL aqueous or ethanolic solutions of the purified betacyanin mixture isolated from *B. vulgaris* L. roots or pure betanin and isobetanin, acidified with 100 μL of glacial acetic acid, were heated at 80 °C (aqueous solution) and 75 °C (ethanolic solution) in a water bath for 60−180 min. The 1 mL aliquots of the heated samples were taken for HPLC analysis every 5−15 min. Ethanolic samples were evaporated in a nitrogen stream and reconstituted in water before analysis.

LC−DAD analysis. A Gynkotek HPLC system with UVD170S, Gynkotek HPLC pump Series P580 and thermostat (Gynkotek Separations, H. I. Ambacht, The Netherlands) was used for chromatographic analysis. The analytical column used was a Synergi Hydro-RP 250 × 3 mm i.d., 4 μm column (Phenomenex, Torrance, CA).

For the separation of analytes, the following gradient system (system 1) was used: 3% A in B at 0 min and a gradient to 17% A in B at 40 min (A, acetonitrile; B, 2% formic acid in water). For the separation of compounds **4** and **4′**, another system (system 2) was applied: 3% A in B at 0 min and a gradient to 17% A in B at 150 min (A, acetonitrile; B, pH 5 sodium formate buffer with 1.5 mM tetrabutylammonium bromide as an ion-pairing agent in water). In each case, the injection volume was 10 μL, and the flow rate of 0.5 mL/min was applied. Detection was generally performed at λ = 538 nm with a UV−vis detector or a DAD (diode array detection) system at 533, 505, and 470 nm, respectively. The columns were thermostated at 35 °C.

LC−MS/MS analysis. Positive ion electrospray mass spectra were recorded on ThermoFinnigan LCQ Advantage (electrospray voltage 4.5 kV; capillary 250 °C; sheath gas: N_2) coupled to a ThermoFinnigan LC Surveyor pump applying HPLC in gradient system 1. Helium was used to improve trapping efficiency and as the collision gas for CID experiments. The relative collision energies for MS/MS analyses ranged from 25 to 30% (according to a relative energy scale) depending upon compounds and fragment ions analyzed. The MS was controlled, and total ion chromatograms and mass spectra were recorded using the ThermoFinnigan Xcalibur software (San Jose, CA).

Semipreparative HPLC. For semipreparative isolation of pure compounds, a 250 × 10 mm i.d., 10 μm Luna C18(2) column (Phenomenex, Torrance, CA) was used in the same chromatographic system as in LC−DAD except for the gradient system (system 3) (from 6% A in B at 0 min to 10% A in B at 30 min), with an injection volume of 100 μL and a flow rate of 3 mL/min.

RESULTS AND DISCUSSION

The resulting HPLC chromatograms from decarboxylation product analyses are depicted in **Figure 2**. Generally, after heating of ethanolic and aqueous solutions for about 10 and 30 min, respectively, some dominant peaks arose in HPLC chromatograms, which after a subsequent heating period disappeared as a result of decomposition. In ethanolic solutions, they corresponded to compounds **2** and **2′** (**Figure 2B** and **Table 1**), or in water solutions, corresponded to **3′** and **3** (**Figure 2D**). Both pairs of compounds exhibited less polar character than their corresponding betacyanins, resulting in longer retention times on the reversed-phase HPLC column. However, much stronger nonpolar character of **3′** and **3** was noted. Prolonged heating of the solutions for 2−3 h resulted in a complex decomposition mixture very similar to the final profile for both ethanol and water (**Figure 2C**,**G**); however, in water, compound **5** was almost absent.

On closer inspection of the LC−DAD and LC−MS/MS spectra, the main products appeared to be monodecarboxylated compounds with absorption maxima at λ_{max} 505 nm for **2** and **2′** or λ_{max} 533 nm for **3′** and **3** (**Table 1**) and with characteristic molecular masses of $[M + H]^+ = 507$, which were the result of CO_2 loss of the parent betacyanins. The daughter ion spectra displayed fragments of $[M + H]^+ = 345$ in each case, indicating the decarboxylated aglycone (betanidin) part of the molecules.

From the previous absorption data, it was possible to conclude that the pair **2** and **2′** should be 17-decarboxybetanin and 17-decarboxyisobetanin because of the characteristic absorption at λ_{max} 505 nm (*8*, *9*). The only known decarboxy-betacyanins absorbing at 533 nm are 2-decarboxy-betacyanins, which have never been attributed to betacyanin thermal degradation products before. Previously, it was assumed that 2-decarboxy-betacyanins were dopamine derived compounds, endogenously appearing in hairy roots of *B. vulgaris* and *Carpobrotus acinaciformis* (*18*, *19*). Furthermore, it was suspected that there was a possibility of forming 15-decarboxybetanin during a decarboxylation process that led to a loss of the chiral center (*10*). The authors provided proof of the decarboxylated product formation by the betanin solution being heated in a closed vessel for headspace gas chromatographic analysis and showed the presence of CO_2 as a result of betanin decarboxylation. They also proved the formation of cyclodopa-5-*O*-glycoside and betalamic acid under heating or hydrolysis of betanin in basic media.

From the study of Dunkelblum et al. (*8*) on the mechanism of decarboxylation and epimerization of betanidin, the generation of a 15-decarboxy-betacyanin must be excluded because the

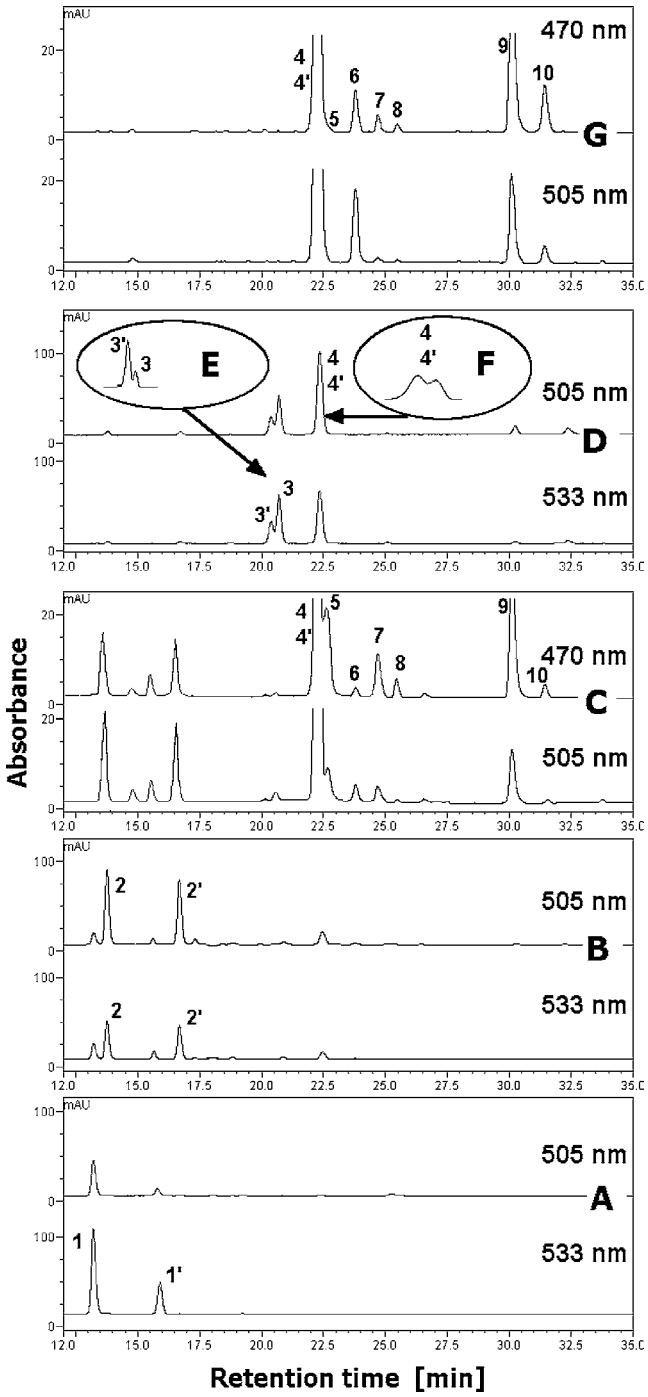

Figure 2. HPLC chromatograms (gradient system 1) of purified *Beta vulgaris* L. root juice, **panel A**: monitored at 533 and 505 nm; **panel B**: heated in ethanolic solution at 75 °C for 10 min, monitored at 533 and 505 nm; **panel D**: heated in aqueous solution at 80 °C for 30 min, monitored at 533 and 505 nm; and **panel C**: heated in ethanolic solution at 75 °C for 2 h, monitored at 505 and 470 nm. **Panel E**: profile of **3′** and **3** mixture obtained by heating of previously isolated isobetanin. **Panel F**: resolution of **4** and **4′** in ion-pair HPLC gradient system (system 2). **Panel G**: sample heated in aqueous solution at 80 °C for 3 h, monitored at 505 and 470 nm.

initial product would transform to a 17-decarboxy-betacyanin as a result of migration of the double bond C(17)═C(18) to C(14)═C(15). From the point of view of our results, the HPLC system applied in ref *10* obviously did not allow for any separation of the two 2-decarboxy-betacyanin epimers (**3′** and **3**). In this study, we applied a new generation HPLC columns

Table 1. Names and Numbers of the Pigments Analyzed in This Study

peak no.	compound	retention time (min)	λ_{max}	*m/z* $[M + H]^+$	*m/z* from MS/MS of $[M + H]^+$
1	betanin	12.7	538	551	389
2	17-decarboxy-betanin	13.3	505	507	345
1′	isobetanin	15.9	538	551	389
2′	17-decarboxy-isobetanin	16.7	505	507	345
3′	2-decarboxy-isobetanin	20.3	533	507	345
3	2-decarboxy-betanin	20.6	533	507	345
4	2,17-bidecarboxy-betanin	22.4	507	463	301
4′	2,17-bidecarboxy-isobetanin	22.4	507	463	301
5	17-decarboxy-neobetanin[a]	22.6	450	505	343; 299; 255
6	2,15,17-tridecarboxy-betanin	23.7	505	419	257
7	neobetanin	24.6	488	549	387; 343
8	2,15,17-tridecarboxy-neobetanin[a]	25.4	451	417	255
9	2,17-bidecarboxy-neobetanin[a]	30.1	459	461	299; 255
10	2-decarboxy-neobetanin[a]	31.4	490	505	343; 299; 255

[a] Tentatively identified.

(Synergi Hydro-RP) based on the polar endcapped stationary phase. The use of endcapped columns is believed to be a prerequisite for the separation of ionized compounds because of reduced silanol activity (*20*). Hence, we were able to resolve the two epimers of monodecarboxy-betacyanins absorbing at λ_{max} 533 nm (**3′** and **3**).

The configuration of the C-15 epimer of betanin [2*S*/15*R*] allows stronger interaction with the stationary phase; therefore, these compounds have greater retention time relative to betanin [2*S*/15*S*] (*14*). The elution order of **2** and **2′** was established based on their lower polarity than **1** and **1′,** respectively, deduced from the chromatograms (**Figure 2A**,**B**). To prove the order of elution of **3′** and **3**, subsequent analyses of previously isolated and degraded betanin and isobetanin from red beet extract were performed. Comparison of chromatograms of degraded betanin (present in higher concentration in *B. vulgaris*) (**Figure 2C**) and isobetanin (**Figure 2E**), where the dominant peak of the pair indicated the resulting decarboxylation product (the other peak was the result of epimerization), established the reversed order of elution of **3′** and **3.** It is not clear why the elution order is reversed in the case of 2-decarboxy-betacyanins, but this fact indicates a different interaction mode of **3′** and **3** molecules with the stationary phase in comparison to betacyanins.

Subsequent inspection of the LC−DAD and LC−MS/MS spectra revealed other decarboxylated betacyanins. The two unresolved compounds **4** and **4′** identified at R_t = 22.4 min displayed absorption maximum of λ_{max} 507 nm and a pseudomolecular mass at $[M + H]^+ = 463$, clearly indicating a loss of two CO_2 moieties. A subsequent fragmentation to $[M + H]^+ = 301$ confirmed the existence of a bidecarboxylated fragment of betanidin and suggested the formation of a bidecarboxy-betanin and its isoform. Proof of the existence of the two epimers of **4** and **4′** was their partial HPLC separation in a long-term gradient system 2 (**Figure 2F**). The addition of tetrabutylammonium bromide as an ion-pairing agent allowed specific interaction with the only carboxylic group left at C-15 in two configurations of the epimers. Thus, the only possible structure of **4** and **4′** was 2,17-bidecarboxy-betanin and its isoform. Interestingly, in both media the bidecarboxybetanin and its isoform were the final double decarboxylation products but formed in two different steps: in ethanol through the 17-decarboxybetacyanins and in water through the 2-decarboxy-betacyanins.

Finally, compound **6,** being slightly less polar than 2,17-bidecarboxy-betanin and displaying a mass of $[M + H]^+ = 419$ and absorption maximum of λ_{max} 505 nm, appeared at R_t = 23.7 min, suggesting formation of a tridecarboxy-betanin. The only tridecarboxylated compound possible could be 2,15,17-tridecarboxy-betanin. Because only one chromatographic peak was observed in our HPLC systems, it confirmed the loss of the chiral center at C-15 for the compound **6**. A subsequent fragmentation of the pseudomolecular ion to $[M + H]^+ = 257$ proved unequivocally the existence of the tridecarboxylated fragment of betanidin.

Additionally, novel compounds revealed in the decarboxylation mixtures appeared to be derivatives of 14,15-dehydrobetanin (neobetanin). The formation of neoderivatives demonstrates the strong tendency for the dihydropyridine ring to aromatize, which can appear during heating of betalains. So far, neobetanin has been discovered as a degradation product of betanin (*12*, *13*, *21*) but also as a genuine compound in extracts from natural products such as recently evaluated *Boerhavia erecta* (*22*), *B. vulgaris* (*23*), and *Opuntia ficus-indica* (*24*). Other decarboxylated neobetacyanins were recently preliminarily identified in refs *12* and *13*. Compound **7** exhibited lower polarity than betanin and isobetanin resulting in a longer retention time. Lower polarity of neobetacyanins than polarity of their parental betacyanins was frequently observed during HPLC analyses (*12*, *13*, *24*). Confirmational data were the absorption maximum at λ_{max} 488 nm and the pseudomolecular mass in the LC−MS/MS system at $[M + H]^+ = 549$, indicating a loss of 2H from betanin/isobetanin during the heating experiment. The subsequent fragmentation ion at $[M + H]^+ = 387$ from the loss of a glucose moiety and 343 from the next loss of CO_2 suggested the existence of a dehydrogenated betanidin structure. The formation of neobetanin results in the loss of the chiral center at C-15, yielding only one chromatographic peak.

All possible decarboxylated derivatives of neobetanin corresponding to the revealed decarboxylated betacyanins were tentatively identified. Namely, two compounds (**5** and **10**) with pseudomolecular masses of $[M + H]^+ = 505$ and absorption maxima at λ_{max} 450 and 490 nm, respectively, were found. A subsequent fragmentation ion at $[M + H]^+ = 343$ from the loss of glucose moiety, 299 and 255 Da from the consecutive losses of CO_2, suggested the generation of decarboxylated and dehydrogenated betanidin. The different absorption maxima suggested that both compounds were not C-15 epimers, but their structure was different in the position of decarboxylation. The hypsochromic shift for **5** and higher polarity (lower retention time), in comparison to the latter compound, suggested that this was 17-decarboxy-neobetanin. In contrast, it could be deduced that **10** was decarboxylated at C-2 because of a much higher retention time than 17-decarboxy-neobetanin and an absorption maximum wavelength at a higher value, similar to the difference in retention time and absorption maxima between 17-decarboxy-betanin and 2-decarboxy-betanin.

Compound **9** displayed a pseudomolecular mass at $[M + H]^+ = 461$, indicating a loss of 2H from the more polar compound (lower retention time) 2,17-bidecarboxy-betanin ($[M + H]^+ = 463$), thus suggesting the appearance of 2,17-bidecarboxy-neobetanin. A subsequent fragmentation ion at $[M + H]^+ = 299$ from the loss of a glucose moiety and 255 from the next loss of CO_2 supported the suggestion of the existence of a bidecarboxylated dehydrogenated fragment of betanidin. Additionally, the absorption maximum found at λ_{max} 459 nm was indicative for a compound being both 14,15-dehydrogenated and decarboxylated.

Compound **8** displaying a mass of $[M + H]^+ = 417$, a fragmentation ion of $[M + H]^+ = 255$, and absorption maximum of λ_{max} 451 nm appeared at R_t 25.4 min, suggesting the formation of 2,15,17-tridecarboxy-neobetanin, exhibiting slightly lower polarity than 2,15,17-tridecarboxy-betanin.

As mentioned previously, all the neoderivatives exhibited higher retention times (lower polarity) than the corresponding decarboxylated betacyanins; however, there were some inconsistences in comparing the retention times of compounds **5** and **7**−**10**. More decarboxylated compounds (less polar) should exhibit higher retention times, which is not the case when comparing compounds **8**−**10** and comparing compound **5** with compound **7**. This fact should be further investigated.

This is the first report on the existence of bi- and tridecarboxylated betanin, their corresponding neoderivatives, and 2-decarboxy-betanin/isobetanin in degradation products of betacyanins from red beet juice, which can be indicative of other food products such as juices or colorant formulations. However, recently some bidecarboxylated betacyanins and neoderivatives were tentatively identified in purple pitaya aqueous preparations (*13*). The results of this research are crucial in determining the betacyanin degradation mechanisms in juices or extracts of *B. vulgaris* roots and other products containing these pigments. Another important aspect that should be taken into account is the environment in which the decarboxylation takes place. A much faster process in ethanolic solutions completing single and double decarboxylation within 10 min was observed. The fast degradation of betacyanins that had been already noticed for ethanolic systems (*3*, *11*) should be considered during performing extraction processes of analytical samples with mixtures containing methanol or ethanol, especially at higher than room temperatures.

This study reports some decarboxylation and dehydrogenation compounds of betacyanins from *B. vulgaris* extract. By applying an endcapped HPLC column in their analysis, it was possible to resolve some of the crucial compounds for subsequent structure interpretation, mainly two C-15 stereoisomers of 2-decarboxy-betacyanins. Further confirmational studies on decomposition products of betacyanins from *B. vulgaris* and other products containing these pigments are currently being performed.

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