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Generation of Decarboxylated and Dehydrogenated Betacyanins in Thermally Treated Purified Fruit Extract from Purple Pitaya (*Hylocereus polyrhizus*) Monitored by LC-MS/MS

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Pigments of purple pitaya [*Hylocereus polyrhizus* (F.A.C. Weber) Britton and Rose] fruits were submitted to extraction and were decarboxylated during heating experiments in acidified ethanolic and aqueous solutions. Groups of betacyanins with different decarboxylation levels were identified in the heating products by LC-DAD and LC-MS/MS. The main decarboxylation products were 2-decarboxy-betacyanins, 17-decarboxy-betacyanins, and 2,17-bidecarboxy-betacyanins. The structures of other compounds were assigned to 2,15,17-tridecarboxy-betacyanins and 14,15-dehydrogenated derivatives (neo-derivatives) of all decarboxylated betacyanins found.

KEYWORDS: Betanin; 2-decarboxy-betacyanins;17-decarboxy-betacyanins; neobetacyanins; betalains; decarboxylation; *Hylocereus polyrhizus*; purple pitaya

INTRODUCTION

The thermolability of natural pigments is usually the most restrictive factor in their widespread application as food colorants. This is also true for betalains, a group of water-soluble, nitrogenous pigments found in botanical species belonging to families of the order Caryophyllales (*1*). From these red-violet or yellow-orange pigments, betanin and its C-15 isoform derived from red beet root (*Beta vulgaris* L.) are extensively used as food colorants in low-temperature products (*2*).

Several studies reported on the structural elucidation and discovery of the new acylated betacyanin, hylocerenin, in fruits of purple pitaya (*Hylocereus polyrhizus)* (*3*, *4*) and other new domesticated species of *Hylocereus* cacti (*5*) which together with betanin and another acylated betacyanin, phyllocactin, are the main pigments in the fruits (**Figure 1**). Until recent reports (*6*, *7*), no studies on stability of purple pitaya betacyanins were performed nor were their degradation products analyzed. Betalains are very sensitive to several factors, including high and low pH and higher temperature or water activity (*8*−*12*). Some studies have already elaborated the conditions under which these pigments (mostly betanin) retain their attractive color and even discovered some of their degradation products (*13*−*15*).

Attempts to analyze degradation products of betanin were done by Schwartz and von Elbe (*15*, *16*), who concluded 15-decarboxylated betanin to constitute the proposed decarboxylation product. Very recent structural studies on thermal

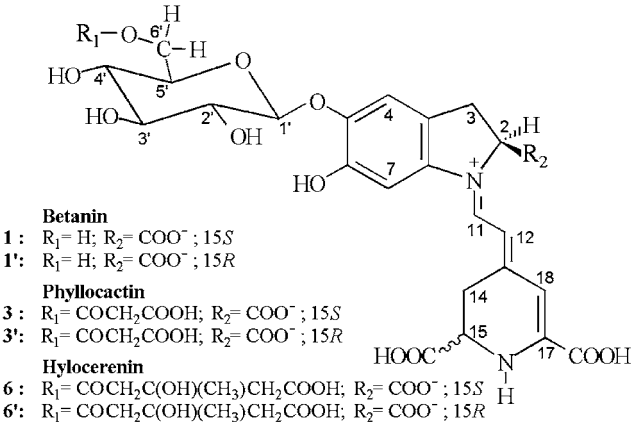

Figure 1. Chemical structures of *Hylocereus polyrhizus* betacyanins and of 2-decarboxy-betacyanins analyzed in this study.

decarboxylation of betacyanins from red beet (*Beta vulgaris* L.) (*17*) and purple pitaya (*Hylocereus polyrhizus*) (*6*) preparations in water solutions revealed several mono- and bidecarboxylated as well as 14,15-dehydrogenated betacyanins. A significant degradation of betacyanins which had been already noticed for ethanolic systems (*8*, *18*, *19*) should be considered

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especially at higher than room temperatures. Fast degradation in ethanolic solutions completing single and double decarboxylation within 10 min at 75 °C has been observed (*19*). Moreover, different initial products of monodecarboxylation in ethanolic and aqueous solutions of betanin/isobetanin were identified (*19*) indicating different decarboxylation mechanisms in these two media. Some of the preliminarily identified compounds (*6*, *17*) were assigned to 15- and 17-decarboxy-betacyanins on the basis of their absorption maxima and LC-MS/MS data. Applying a new generation HPLC column, a recent study on the degradation of betacyanins from red beet juice (*19*) reported contradictory results to the previous findings (*15)* and proved the generation of 2-decarboxy-betanin and its isoform (**Figure 1**). The same study (*19*) also reported the existence of additional new degradation products, namely, bi- and tridecarboxylated betanin and their corresponding 14,15-dehydrogenated derivatives.

Because the identification of betacyanin decarboxylation products is crucial in determination of betacyanin degradation mechanisms in *Hylocereus polyrhizus* and other *Hylocereus* species fruit preparations, the results of decarboxylation experiments performed in betacyanin ethanolic and aqueous solutions obtained from *Hylocereus polyrhizus* fruit extracts are reported.

MATERIALS AND METHODS

Plant Material. Three hundred grams of freeze-dried *Hylocereus polyrhizus* fruit flesh powder was extracted with distilled water and was filtered through a 0.2-μm pore size filter (Millipore, Bedford, MA) and finally through a layer of 0.063−0.200 mm silica gel (J. T.Baker, Deventer, Holland) to obtain a clear solution of betacyanins.

Purification. The purification of the extract was performed on a C18 cartridge according to the procedure of Stintzing et al. (*20*). The C18 cartridge (Merck, Darmstadt, Germany) was activated with 3 volumes of 100% methanol and then was rinsed with 3 volumes of acidified water (pH 3). The sample was applied to the column and was rinsed again with 3 volumes of acidified water (pH 3). The betacyanins were eluted with acidified methanol (methanol/acidified water (pH 2), 95:5, v/v), rotoevaporated under reduced pressure at 30 °C until 50% volume reduction was accomplished, and diluted with water before being freeze-dried. The so-obtained purified betacyanin solution was submitted to semipreparative HPLC or was dissolved in water and ethanol for decarboxylation experiments.

Semipreparative HPLC. A Gynkotek HPLC system with UVD170S detector, HPLC pump Series P580, and thermostat (Gynkotek Separations, H. I. Ambacht, The Netherlands) was used for semipreparative isolation of phyllocactin, hylocerenin, and their isoforms from the betacyanin mixture. For the separation of analytes, a 250 × 10 mm i.d., 10-μm Luna C18(2) column (Phenomenex, Torrance, CA) was used in 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, 0.5% formic acid in water), an injection volume of 100 μL, and a flow rate of 3 mL/min. For semipreparative isolation of 17-decarboxy- and 2-decarboxy-betacyanins from the concentrated betacyanin mixtures submitted to monodecarboxylation in ethanolic and aqueous solutions, a similar gradient system (system 2) was used except for the composition of B that was changed to 7% formic acid in water. Detection was generally performed at λ 538 nm with a UV/vis detector or a DAD (diode array detection) system at 533, 505, and 450 nm, respectively. The column was thermostated at 25 °C.

Decarboxylation. Ten to forty milliliters of aqueous or ethanolic solutions of 5−15 mg purified betacyanin mixture, 2−4 mg purified phyllocactin, hylocerenin, and their isoforms (previously isolated semipreparatively from the betacyanin mixture), 0.1−0.5 mg 17-decarboxy-phyllocactin, 2-decarboxy-phyllocactin, 17-decarboxy-hylocerenin, 2-decarboxy-hylocerenin, and their isoforms (previously isolated semipreparatively from the concentrated betacyanin mixtures submitted to monodecarboxylation in ethanolic and aqueous solutions), always acidified with 100 μL of glacial acetic acid, were heated at 80 °C (aqueous solutions at pH 3) and 75 °C (ethanolic solutions) in a water bath for 60−180 min. 0.2−1 mL aliquots of the heated samples were taken for HPLC analysis every 5−15 min. Ethanolic samples were evaporated in a nitrogen stream and were reconstituted in water before analysis.

LC-DAD Analysis. For chromatographic analysis, the same system was used as in semipreparative HPLC except for the gradient conditions. The analytical column used was a Synergi Hydro-RP 250 × 3 mm i.d., 4 μm (Phenomenex, Torrance, CA). For the separation of analytes, the following gradient system (system 3) was used: 7% A in B at 0 min and gradient to 13% A in B at 40 min and to 30% A in B at 60 min. (A, acetonitrile; B, 0.5% formic acid in water). In each case, the injection volume was 10μL and a flow rate of 0.5 mL/min was applied.

LC-MS/MS Analysis. 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 applied to HPLC gradient system 3. 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 ThermoFinnigan Xcalibur software (San Jose, CA).

RESULTS AND DISCUSSION

A recent analytical report on betanin/isobetanin decarboxylation in *Beta vulgaris* L. juice indicated two basic pathways of decarboxylation influenced by the solvent (water or ethanol) used (*19*). Therefore, both solvents were used in the experiments performed on betacyanins derived from *H. polyrhizus* extracts. Chromatographic separation of complex decomposition mixtures resulted in coelution of some compounds (**Figure 2D** and **2E**), and in some cases their differentiation was possible by changing eluent composition (formic acid concentration) and observation of the respective maximum absorbances or mass spectra. However, the best results were obtained by additional selective decarboxylation experiments which were performed with isolated phyllocactin and hylocerenin (**Figures 3** and **4**, respectively).

Monodecarboxy-betacyanins. Some main groups of compounds arising in the first stages of heating experiments could be observed in the HPLC chromatograms (**Figure 2B** and **2C**) depending on the choice of solvent and heating period. After a 10-min treatment of solutions in ethanol, six main chromatographic peaks arose, of which the spectroscopic data suggested the presence of 17-decarboxy-betacyanins and their isoforms. Compounds **2** and **2′** were identical to 17-decarboxy-betanin and its isoform, recently discovered in *Beta vulgaris* and *H. polyrhizus* heated compositions (*6*, *17*, *19*). The absorption maxima at λ_{max} 505 nm (**Table 1**), characteristic pseudomolecular masses of $[M + H]^+ = 507$ (resulting from CO_2 loss of betanin and isobetanin), and the daughter ion fragments of $[M + H]^+ = 345$ confirmed the conclusion.

Likewise, the other four compounds **5**, **5′**, **9**, and **9′**, possessing the absorption maxima at λ_{max} 505 nm, appeared to be 17-decarboxy-phyllocactin, 17-decarboxy-hylocerenin, and their isoforms, exhibiting pseudomolecular masses of $[M + H]^+ = 593$ and 651, respectively. The daughter ion fragment of $[M + H]^+ = 345$ was an important confirmation that the decarboxylation did not occur at the acyl moiety (in that case the daughter ion fragment would be $[M + H]^+ = 389$), but at the aglycone (betanidin or isobetanidin) part of the molecule. These compounds were identified very recently in ref *6* and *7*.

The six main products (**4**, **4′**, **21**, **21′**, **22**, and **22′**) of the first 30 min of betacyanin decarboxylation in aqueous solutions were assigned to 2-decarboxy-betacyanins and their isoforms (**Figures 1** and **2C**). The formation of 2-decarboxy-betanin and 2-decar-

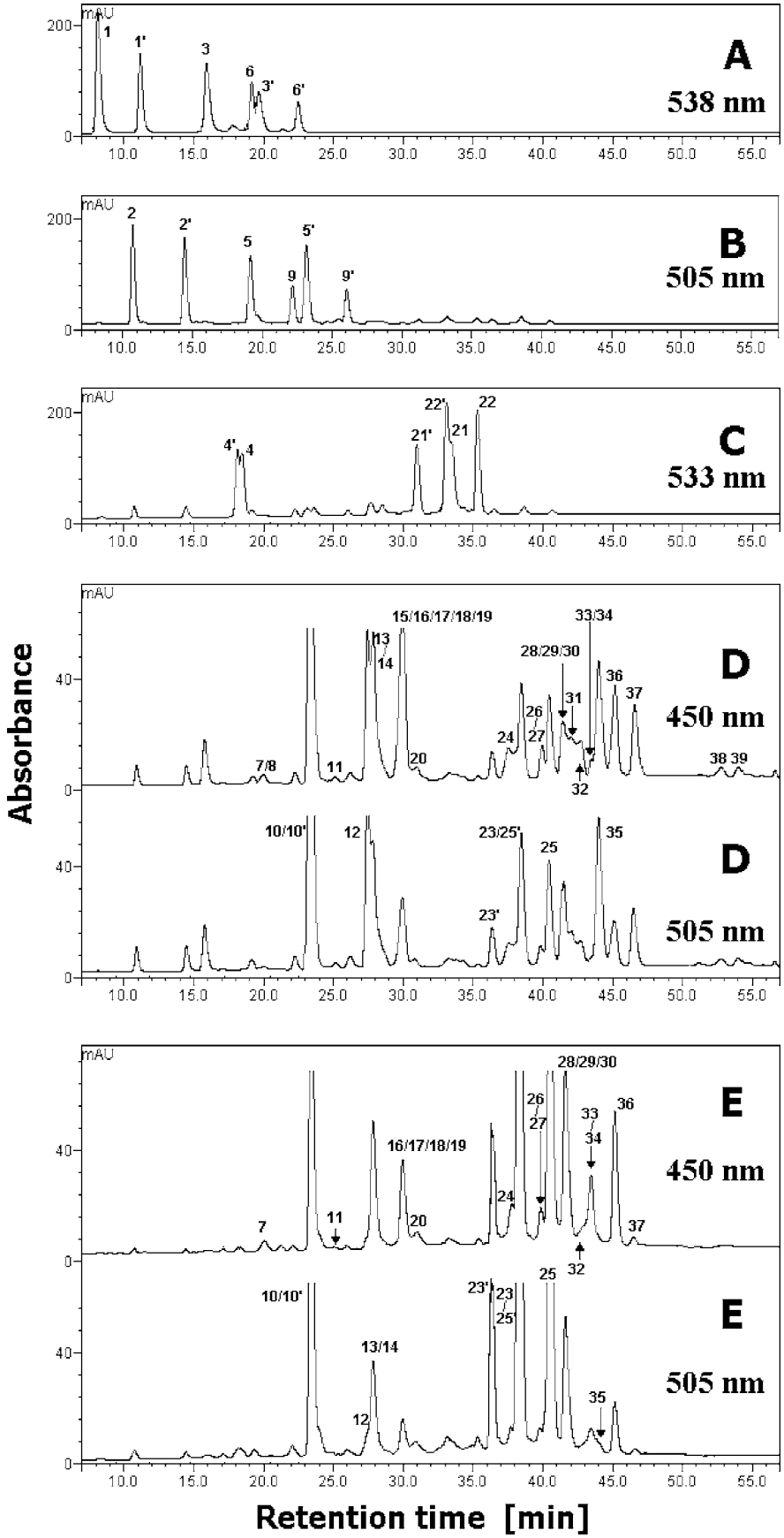

Figure 2. HPLC chromatograms (gradient system 3) of *Hylocereus polyrhizus* fruit betacyanins (**A**) nonheated solution; (**B**) heated in ethanolic solution at 75 °C for 10 min; (**C**) heated in aqueous solution at 80 °C for 30 min, monitored at 533 nm; (**D**) heated in ethanolic solution at 75 °C for 1.5 h, monitored at 505 and 450 nm; (**E**) heated in aqueous solution at 80 °C for 3 h, monitored at 505 and 450 nm.

boxy-isobetanin during heating of *Beta vulgaris* juice has been recently discussed in ref *19*. This excluded the possibility of the formation of 15-decarboxy-betanin (with the loss of the chiral center at C-15) on the basis of the existence of two

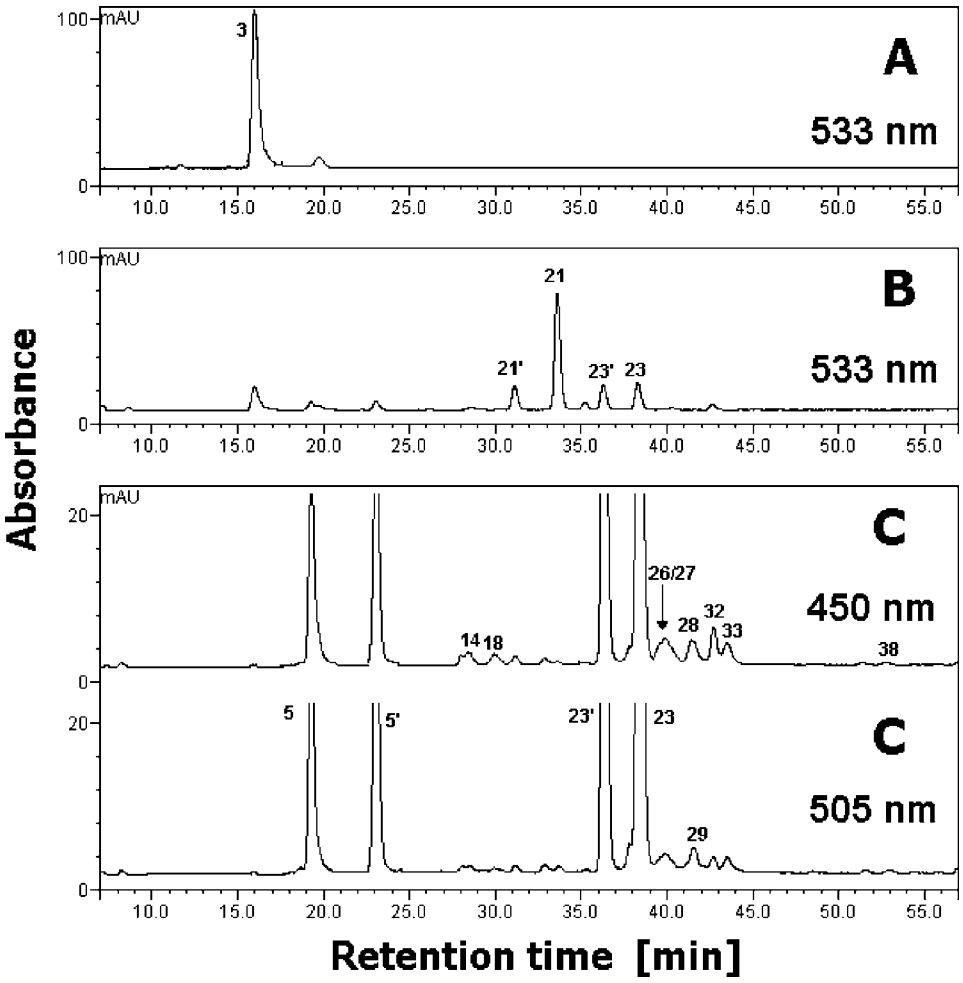

Figure 3. Indication of **21** and **21′** elution order by generation of 2-decarboxy-phyllocactin **21** (**B**) from previously isolated phyllocactin sample (**A**) heated in aqueous solution at 80 °C for 30 min. Additionally, the degradation products of phyllocactin after heating in ethanolic solution at 75 °C for 1.5 h are depicted (**C**). All chromatograms were obtained applying gradient system 3.

diastereoisomers and on another previous mechanistic study (*13*). Additionally, the absorption maxima close to λ_{max} 533 nm suggested the possibility of existence of 2-decarboxy-betacyanins, dopamine-derived compounds, which are endogenous compounds of *Beta vulgaris* hairy roots and *Carpobrotus acinaciformis* flowers (*21*, *22*). On the basis of the same assumptions, the tructures of the six compounds in this study were assigned to 2-decarboxy-betanin **4** ($[M + H]^+ = 507$), 2-decarboxy-phyllocactin **21** ($[M + H]^+ = 593$), and 2-decarboxy-hylocerenin **22** ($[M + H]^+ = 651$) with their isoforms (**Table 1**). The presence of the daughter ion fragment of $[M + H]^+ = 345$ excluded the decarboxylation at the acyl moiety in the case of **21**, **21′**, **22**, and **22′**, indicating the decarboxylation at the aglycone (betanidin or isobetanidin part).

The elution order of 2-decarboxy-betanin and 2-decarboxy-isobetanin on a C18 HPLC column was established by analysis of previously isolated and degraded betanin and isobetanin assuming that isomerization was less strong compared to decarboxylation. This revealed reversed order of elution of **4** and **4′** in comparison to parent betacyanins or 17-decarboxy-betacyanins (*19*). The compounds **4** and **4′** were partially resolved under the analytical conditions applied. Additional studies on decarboxylation of semipreparatively isolated phyllocactin (**Figure 3A** and **3B**), hylocerenin (**Figure 4A** and **4B**), and their isoforms allowed to establish the elution order of arising corresponding 2-decarboxy-betacyanins. The elution order was also reversed as in the case of **4** and **4′**, however, the isoforms were very well separated from their normal forms. Analogous differences in retention times were observed in the case of bidecarboxylated betacyanins as discussed below.

The elution order of the new 17-decarboxy-betacyanins could be unambiguously established by their polarity, which was diminished in comparison to their corresponding parent betacyanins (**Figure 2A** and **2B**). An especially valuable conclusion could be drawn by the observation of the retention times of decarboxylated phyllocactin or hylocerenin (**5** or **9**, respectively) which eluted between the parent betacyanins (**3** or **6**, respectively) and isobetacyanins (**3′** or **6′**, respectively), thus confirming that they were not decarboxylated isoforms.

Bi- and Tridecarboxy-betacyanins. Prolonged heating of the *H. polyrhizus* extracts resulted in very complex decomposition mixtures (**Figure 2D** and **2E**), which were subsequently analyzed by LC-DAD and LC-MS/MS. Because of a very complex nature of the mixtures for identification of the compounds, additional decarboxylation experiments were performed with purified phyllocactin and hylocerenin (**Figures 3C** and **4C**, respectively, for ethanolic solutions). These experiments allowed appropriate spectra of the resulting compounds to be obtained (**Table 1**).

A group of compounds possessing absorption maxima at λ_{max} 507−509 nm appeared in higher quantities after prolonged heating and were identified as bidecarboxylated betacyanins. Compounds **10** and **10′** have been recently identified as 2,17-decarboxy-betanin ($[M + H]^+ = 463$) with its isoform, in heated

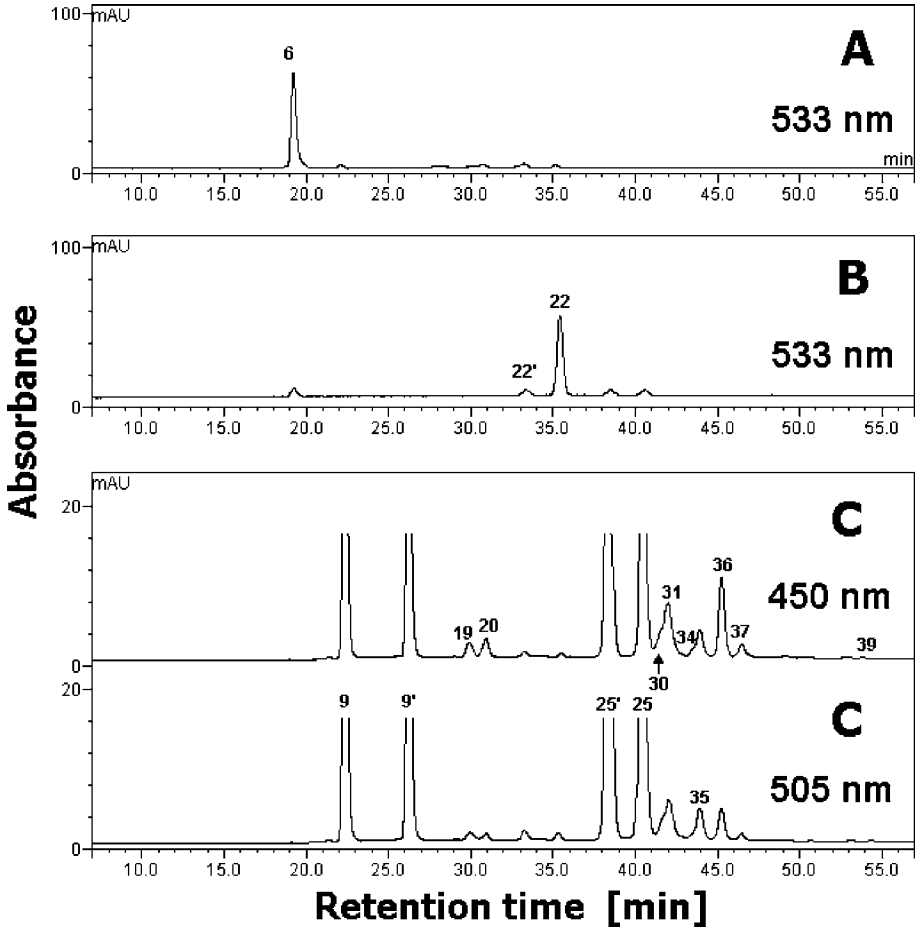

Figure 4. Indication of **22** and **22′** elution order by generation of 2-decarboxy-hylocerenin **22** (**B**) from previously isolated hylocerenin sample (**A**) heated in aqueous solution at 80 °C for 30 min. Additionally, the degradation products of hylocerenin after heating in ethanolic solution at 75 °C for 1.5 h are depicted (**C**). All chromatograms were obtained with gradient system 3.

red beet juice (*19*), and were hardly resolvable on RP-HPLC. Application of ion-pair chromatography allowed partial resolution of the chromatographic peaks confirming the presence of two diastereoisomeric structures at C-15, therefore excluding the possibility of decarboxylation at C-15. By analogy, the structures of the other four bidecarboxylated compounds could be tentatively assigned to 2,17-bidecarboxy-phyllocactin ($[M + H]^+ = 549$) with its isoform (**23** and **23′**) (**Figures 2D**, **2E**, and **3C**) and to 2,17-bidecarboxy-hylocerenin ($[M + H]^+ = 607$) with its isoform (**25** and **25′**) (**Figures 2D**, **2E**, and **4C**). Furthermore, the presence of the daughter ion fragments of $[M + H]^+ = 301$ (instead of 345) excluded the decarboxylation at the acyl moieties in the case of **23**, **23′**, **25**, and **25′** (**Table 1**).

The peaks of both isomeric pairs (**23**, **23′** and **25**, **25′**) were separated very well in contrast to **10** with **10′.** This fact was analogous to the separations in the group of 2-decarboxy-betacyanins as discussed above, however, it was not possible to establish the order of elution of the diastereoisomers. Subsequent experiments on decarboxylation of each semi-preparatively isolated 17-decarboxy-betacyanin or 2-decarboxy-betacyanin (**5**, **5′**, **9**, **9′**, **21**, **21′**, **22**, or **22′**) in aqueous or ethanol solutions resulted in generation of equal quantities of both respective bidecarboxy-betacyanin isomers. The resulting chromatograms of the two experiments with **21** and **22** in ethanolic solutions are depicted in the **Figure 5A-5D**. Generation of equal quantities of the respective bidecarboxy-betacyanin isomers prevented deduction of their elution order.

Three other compounds absorbing at λ_{max} 505−507 nm appeared in lower abundance after prolonged heating of the solutions and were assigned to 2,15,17-tridecarboxy-betacyanins. Compound **12**, less polar than 2,17-bidecarboxy-betanin (**10**) and displaying a pseudomolecular ion of $[M + H]^+ = 419$, has been already determined (*19*) as 2,15,17-tridecarboxy-betanin. Appearance of one chromatographic peak suggested loss of the chiral center at C-15, confirming the lack of the carboxyl at C-15. Similarly, compounds **29** and **35** could be assigned to 2,15,17-tridecarboxy-phyllocactin ($[M + H]^+ = 505$) and 2,15,17-tridecarboxy-hylocerenin ($[M + H]^+ = 563$), respectively. Both compounds exhibited lower polarity than the respective bidecarboxy-betacyanins. In addition, subsequent fragmentations of the pseudomolecular ions to $[M + H]^+ = 257$ proved the presence of tridecarboxylated compounds at the aglycone (betanidin part).

Neoderivatives. Recent publications demonstrated the possibility of generation of neoderivatives (14,15-dehydrobetacyanins and their decarboxylated forms) (**Figure 6**), exhibiting hypsochromic shifts in their absorption maxima to λ_{max} 450−490 nm, during the heating process of red beet (*17*, *19*) and purple pitaya (*6*). Our study confirmed these findings, however, structures of new compounds derived from phyllocactin and hylocerenin are also suggested.

As expected, the appearance of neophyllocactin **14** and neohylocerenin **19** (**Figures 2**−**4** and **6**) was confirmed by their pseudomolecular masses at $[M + H]^+ = 635$ and $[M + H]^+ = 693$, respectively, in addition to the well-known neobetanin **7** ($[M + H]^+ = 549$) (*23*, *24*). All these compounds exhibited lower polarity than their parent betacyanins, the absorption maxima around λ_{max} 490 nm characteristic for neobetanin and

Table 1. Spectroscopic and Mass Spectrometric Data of the Analyzed Pigments

no.	compound	[d]	R_t [min]	λ_{max} [nm]	*m/z* $[M + H]^+$	*m/z* from MS/MS of $[M + H]^+$ (% base peak)
1	betanin		7.1	538	551	389(100)
2	17-decarboxy-betanin	et.	10.7	505	507	345(100)
1′	isobetanin		11.1	538	551	389(100)
2′	17-decarboxy-isobetanin	et.	14.4	505	507	345(100)
3	phyllocactin		16.0	538	637	593(30); 551(42); 389(100)
4′	2-decarboxy-isobetanin	aq.	18.0	533	507	345(100)
4	2-decarboxy-betanin	aq.	18.4	533	507	345(100)
5	17-decarboxy- phyllocactin	et.	19.2	505	593	549(16); 505(9); 345(100)
6	hylocerenin		19.2	538	695	651(4); 551(7); 389(100)
3′	isophyllocactin		19.5	538	637	593(33); 551(41); 389(100)
7	neobetanin	–	20.0	488	549	387(100); 343(9)
8	17-decarboxy-neobetanin[a]	et.	20.0	450	505	343(100); 299(8); 255(3)
9	17-decarboxy-hylocerenin	et.	22.1	505	651	563(20); 345(100)
6′	isohylocerenin		22.5	538	695	651(5); 551(7); 389(100)
5′	17-decarboxy-isophyllocactin	et.	23.1	505	593	549(13); 505(10); 345(100)
10′	2,17-bidecarboxy-isobetanin	–	23.4	507	463	301(100); 257(6)
10	2,17-bidecarboxy-betanin	–	23.4	507	463	301(100); 257(6)
11	dehydrogenated bidecarboxy-neobetanin[b]	–	25.1	412	459	297(100)
9′	17-decarboxy-isohylocerenin	et.	26.1	505	651	563(17); 345(100)
12	2,15,17-tridecarboxy-betanin	–	27.4	505	419	257(100)
13	2-decarboxy-neobetanin[a]	–	27.9	490	505	343(100); 299; 255
14	neophyllocactin[a]	–	28.5	486	635	387(100); 343(11)
15	dehydrogenated tridecarboxy-neobetanin[b]	et.	29.9	408	415	[e]
16	2,15,17-tridecarboxy-neobetanin[a]	–	29.9	451	417	255(100)
17	2,17-bidecarboxy-neobetanin[a]	–	30.0	459	461	299(100); 255(3)
18	17-decarboxy-neophyllocactin[a]	–	30.0	450	591	505(13); 343(100); 299(9); 255(4)
19	neohylocerenin[a]	–	30.0	485	693	387(100); 343(8)
20	17-decarboxy-neohylocerenin[a]	–	30.9	452	649	343(100); 299(3); 255(3)
21′	2-decarboxy-isophyllocactin	aq.	31.0	534	593	505(10); 345(100)
22′	2-decarboxy-isohylocerenin	aq.	33.1	534	651	345(100)
21	2-decarboxy-phyllocactin	aq.	33.6	535	593	505(8); 345(100)
22	2-decarboxy-hylocerenin	aq.	35.3	536	651	345(100)
23′	2,17-bidecarboxy-isophyllocactin[c]	–	36.3	509	549	505(4); 301(100); 257(5)
24	dehydrogenated decarboxy-neobetanin[b]	–	37.8	422	503	341(100)
23	2,17-bidecarboxy- phyllocactin[c]	–	38.3	509	549	505(5); 301(100); 257(7)
25′	2,17-bidecarboxy-isohylocerenin[c]	–	38.3	509	607	563(5); 301(100); 257(3)
26	2-decarboxy-neophyllocactin[a]	–	39.9	489	591	343(100); 299(2); 255(1)
27	dehydrogenated tridecarboxy-neophyllocactin[b]	–	39.9	411	501	[e]
25	2,17-bidecarboxy-hylocerenin[c]	–	40.5	509	607	563(5); 301(100); 257(5)
28	dehydrogenated bidecarboxy-neophyllocactin[b]	–	41.4	409	545	[e]
29	2,15,17-tridecarboxy-phyllocactin	–	41.6	507	505	257(100)
30	2-decarboxy-neohylocerenin[a]	–	41.6	487	649	343(100); 299(1); 255(1)
31	dehydrogenated tridecarboxy-neohylocerenin[b]	et.	42.0	417	559	[e]
32	2,17-bidecarboxy-neophyllocactin[a]	–	42.7	462	547	503(5); 299(100)
33	2,15,17-tridecarboxy-neophyllocactin[a]	–	43.5	451	503	255(100)
34	dehydrogenated bidecarboxy-neohylocerenin[b]	–	43.5	407	603	[e]
35	2,15,17-tridecarboxy-hylocerenin	–	44.0	506	563	257(100)
36	2,17-bidecarboxy-neohylocerenin[a]	–	45.1	460	605	561(7); 299(100); 255(4)
37	2,15,17-tridecarboxy-neohylocerenin[a]	–	46.5	443	561	255(100)
38	dehydrogenated decarboxy-neophyllocactin[b]	et.	52.8	409	589	341(100)
39	dehydrogenated decarboxy-neohylocerenin[b]	et.	54.0	411	647	341(100)

[a] Tentatively identified. [b] Unknown structure of a betacyanin derivative. [c] The stereomer elution order could not be established. [d] Compound primarily generated in ethanolic (et.) or aqueous (aq.) solution as well as in both solutions (–). [e] MS/MS fragments could not be observed.

only one chromatographic peak resulting from the loss of the chiral center at C-15. The subsequent fragmentation ions at $[M + H]^+ = 387$ from the loss of a glucose moiety and 343 from the next loss of CO_2 confirmed the existence of these compounds.

Another group of neoderivatives was assigned to six monodecarboxy-neobetacyanins. Except for 17-decarboxy-neobetanin **8** ($[M + H]^+ = 505$) and 2-decarboxy-neobetanin **13** ($[M + H]^+ = 505$) (**Figure 2**), which were previously reported (*19*), the structures of 17-decarboxy-neophyllocactin **18** ($[M + H]^+ = 591$), 2-decarboxy-neophyllocactin **26** ($[M + H]^+ = 591$) (**Figures 2** and **3**), 17-decarboxy-neohylocerenin **20** ($[M + H]^+ = 649$), and 2-decarboxy-neohylocerenin **30** ($[M + H]^+ = 649$) are suggested (**Figures 2** and **4**). Additionally, subsequent fragmentation ions at $[M + H]^+ = 343$ from the loss of a glucose moiety, with 299 and 255 from the consecutive losses of CO_2, excluded the possibility of decarboxylation at the acyl moieties in the case of **18**, **20**, **26**, and **30,** thus indicating the decarboxylation at the aglycone (betanidin or isobetanidin moiety).

The order of elution of 17-decarboxy-neoderivatives before the corresponding 2-decarboxy-neoderivatives could be deduced from their absorption maxima at λ_{max} around 450 and 490 nm, respectively, as in the case of **8** and **13**, and corroborated previous findings (*19*).

Additionally, three bidecarboxylated neobetacyanins are reported, which were derived by a loss of 2H from the 2,17-bidecarboxy-betacyanins (**10**, **23**, **25**, and their isoforms) present in substantial quantities in the resulting mixtures of the products (**Figure 2D** and **2E**). Thus, it was possible to tentatively determine the structures of these compounds as 2,17-bidecarboxy-neobetanin **17** ($[M + H]^+ = 461$), already preliminarily identified (*6, 19*), 2,17-bidecarboxy-neophyllocactin **32** ($[M + H]^+ = 547$), and 2,17-bidecarboxy-neohylocerenin **36** ([M +

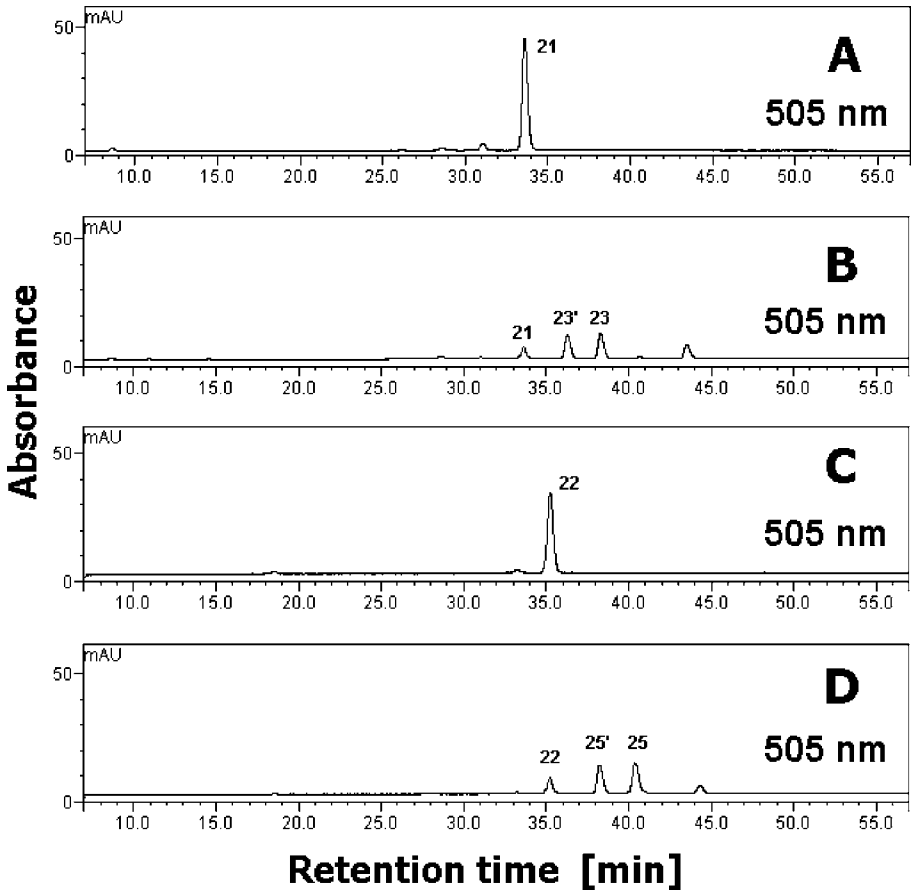

Figure 5. Formation of 2,17-bidecarboxy-betacyanins **23**, **23′** (**B**), **25** and **25′** (**D**) from previously isolated 2-decarboxy-phyllocactin **21** (**A**) and 2-decarboxy-hylocerenin **22** (**C**), respectively, heated in ethanolic solutions at 75 °C for 10 min. In this study, the deduction of the elution order of the respective bidecarboxy-betacyanin diastereoisomers was not feasible because of the generation of similar peak areas for each diastereoisomer. All chromatograms were obtained with gradient system 3.

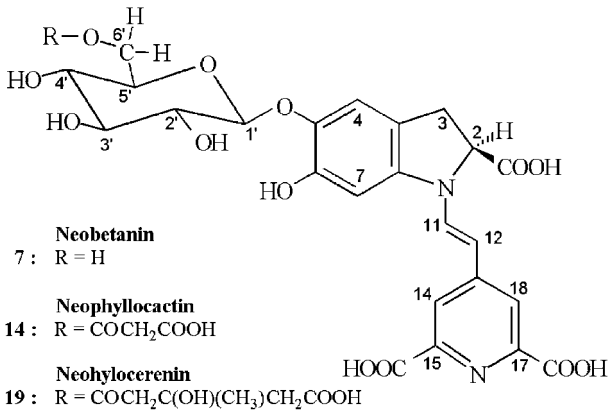

Figure 6. Chemical structures of neobetacyanins analyzed in this study.

$H]^+ = 605$), all exhibiting the absorption maxima at λ_{max} 450−460 nm and longer retention times than their corresponding 2,17-bidecarboxy-betacyanins. A subsequent fragmentation ion at $[M + H]^+ = 299$ from the loss of a glucose moiety and 255 from another loss of CO_2 supported the suggestion of the existence of bidecarboxylated dehydrogenated fragment of betanidin.

The last group of the neoderivatives was assigned to 2,15,17-tridecarboxy-neobetacyanins, appearing at longer retention times than their corresponding 2,15,17-tridecarboxy-betacyanins and exhibiting absorption maxima at λ_{max} 450−460 nm. The compounds displayed pseudomolecular masses of $[M + H]^+ = 417$ (2,15,17-tridecarboxy-neobetanin, **16**) (*19*), $[M + H]^+ = 503$ (2,15,17-tridecarboxy-neophyllocactin, **33**), and $[M + H]^+ = 561$ (2,15,17-tridecarboxy-neohylocerenin, **37**). In each case, a fragmentation ion of $[M + H]^+ = 255$ from the loss of a glucose moiety confimed the presence of the tridecarboxylated aglycone (betanidin or isobetanidin moiety).

Previously, (*19*) some inconsistences were encountered in comparison of the retention times of the decarboxylated neobetacyanins derived from betanin, namely, the order of elution did not increase with the degree of decarboxylation (and the decrease of polarity) for all the compounds. In this study, applying different HPLC conditions, the order of elution of **16** and **17** was still reversed, however, the other compounds eluted in the expected order analogous to the order of decarboxylated betacyanins. In contrast, the order of elution of decarboxylated neobetacyanins derived from phyllocactin and hylocerenin was consistent for all of the compounds.

Other Dehydrogenated Compounds. On closer inspection of the LC-MS/MS spectra, nine compounds were revealed as possibly derived by a loss of 2H from some neoderivatives. These compounds displayed pseudomolecular masses at $[M + H]^+ = 459$ (**11**), 415 (**15**), 503 (**24**), 501 (**27**), 545 (**28**), 559 (**31**), 603 (**34**), 589 (**38**), and 647 (**39**), exhibiting further hypsochromic shift in their absorption maxima to λ_{max} 408−422 nm. Interestingly, the dehydrogenated compounds corresponding to the bi- and tridecarboxy-neobetacyanins always eluted earlier than their parent bi- and tridecarboxy-neobetacyanins. In contrast, the dehydrogenated compounds derived

from the monodecarboxy-neobetacyanins eluted much later than their corresponding monodecarboxy-neobetacyanins (**Table 1**).

The possibility of generation of a 2,3-dehydro-decarboxy-neobetanin as a result of a loss of two additional hydrogen atoms from a decarboxy-neobetanin during a heating process of red beet juice was recently discussed (*17*), reporting that hitherto only a 2,3-dehydrogenation of *cyclo*-Dopa under aerobic conditions in acidic media (*25*−*27*) and its glucoside in red beet peel (*28*) was confirmed. Further studies on the possibility of decarboxy-neobetacyanin derived 2,3-dehydrogenated products formation should be performed. In this report, the possibility of 2-decarboxy-betacyanin generation during the heating of aqueous solutions of acylated betacyanins extracted from fruits of *H. polyrhizus* was confirmed. Also, the fast decarboxylation of acylated betacyanins in ethanolic solutions confirmed the previous results (*19*).

Comparative experiments performed on pasteurization of *H. polyrhizus* and *Beta vulgaris* L. juices at pH 3.5 for 100 min resulted in discoloration of *Beta vulgaris* L. preparations while the color of *H. polyrhizus* juices remained intact (Alex Chechilnitzky, personal communication). In a previous study (*6*), the authors reported that thermal betacyanin degradation in the juices of purple pitaya followed first-order reaction kinetics and that the calculated half-life values for the pigments in the heated pitaya juice preparations were several times higher than formerly reported for betanin and red beet juice solutions. Whether the effect was a result of a different matrix ability to stabilize the pigments or of the acylated pigments from the pitaya exhibiting greater stability is an open question. Moreover, enhanced stability against degradation was confirmed so far only for a betacyanin with feruloylated disaccharide (celosianin II) in contrast to a feruloylated glucoside (lampranthin II); however, acylation with ferulic acid resulted in enhanced C-15 isomerization stability of both compounds (*29*). The experiment was performed at different conditions (pH 2 and room temperature).

In the course of this study, we did not notice any significant differences in the stability of betanin, phyllocactin, and hylocerenin. In fact, all the chromatograms of the main heating products (the groups of mono- or bidecarboxylated betacyanins) showed constant peak area ratios between each of the compounds from a given group, indicating a similar progress of their generation (data not shown). Additional kinetic studies comparing the stability of isolated pigments with the stability of pigments present in the juice matrix should be performed.

Some of the bi- and tridecarboxylated betacyanins and their corresponding neoderivatives were assigned for the first time as degradation products of betacyanins from *H. polyrhizus* fruit extracts. Further confirmational studies on decomposition products of betacyanins from the fruits of *H. polyrhizus* are currently being performed.

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