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Stability and Color Changes of Thermally Treated Betanin, Phyllocactin, and Hylocerenin Solutions

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Thermal degradation of betanin, phyllocactin (malonyl-betanin), and hylocerenin (3″-hydroxy-3″-methyl-glutaryl-betanin) solutions isolated from purple pitaya (*Hylocereus polyrhizus* [Weber] Britton and Rose) was monitored by spectrophotometric and high-performance liquid chromatography−diode array detection (HPLC-DAD) analyses. For betanin and phyllocactin solutions, the color shift upon thermal treatment was found to be nearly identical, while hylocerenin samples exhibited an intelligibly higher chromatic steadiness. Betanin proved to be the most stable individual pigment structure, while the enhanced tinctorial stability of the integral phyllocactin and especially hylocerenin solutions was due to the formation of red degradation products exhibiting improved color retention as opposed to their respective genuine pigments. Individual structure-related stability characteristics can exclusively be assessed by HPLC-DAD analyses and may not be noticed by mere spectrophotometric assessment of color and tinctorial strength.

KEYWORDS: Betacyanins; betanin; phyllocactin; hylocerenin; degradation; thermal treatment; color purple pitaya; *Hylocereus polyrhizus*; red beet; *Beta vulgaris*

INTRODUCTION

Besides anthocyanins, carotenoids, and chlorophylls, betalains represent one of the four plant pigment classes commercially used for natural food coloring, allowing one to substitute synthetic colorants that are increasingly rejected by consumers. Betalain incidence is restricted to a few families of the plant order *Caryophyllales* (*1*), with the red beet root (*Beta vulgaris* L.) hitherto being the only betalainic plant exploited for commercial food coloring (*2*).

First investigations into the stability of red beet juice and its isolated pigments were performed by von Elbe and co-workers (*3*−*6*), focusing on the effects of elevated temperatures on betanin (betanidin 5-*O*-*β*-glucoside), the main betalain pigment of red beet. While quantitative changes during thermal treatment were extensively evaluated, color modification was only marginally considered. Only recently, the yellow shift observed during heating of red beet juice was found to be due to dehydrogenation of betanin to form yellow degradation products, especially neobetanin (*7*).

Purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose) juice has been recognized as a promising source of betalains. Its pigment pattern was described as composed of a mixture of nonacylated (betanin) and acylated [phyllocactin = betanidin 5-*O*-*β*-malonyl-glucoside, hylocerenin = betanidin 5-*O*-*β*-(3″-hydroxy-3″-methyl-glutaryl)glucoside] constituents, together with their respective C_{15} stereoisomers (*8*, *9*). The improved betalain stability of purple pitaya juice as opposed to red beet has been ascribed to the allegedly more stable structure of acylated betacyanins (*10*) but may also be supported by protective matrix effects of purple pitaya juice, rich in pectic substances. However, to get a clearer picture of the structure-dependent degradation characteristics of betanin, phyllocactin, and hylocerenin, heat-induced degradation of betacyanin isolates devoid of food matrix components was required and very recently elucidated by high-performance liquid chromatography−tandem mass spectrometry (HPLC-MS/MS) (*11*). In the present study, the effects of structural characteristics on stability, betacyanin contents, and color evolution of purified betanin, phyllocactin, and hylocerenin solutions were examined. Monitoring of both the entire betacyanin solutions and the individual pigments should be accomplished by combining spectrophotometric measurements with HPLC analyses.

MATERIALS AND METHODS

Plant Material. Fresh *H. polyrhizus* (Weber) Britton and Rose fruits originating from Israel were cut in halves and peeled manually. The fruit flesh was strained through a 0.4 mm sieve of a finisher (Bertuzzi Food Technology, Brugherio, Italy) at a rotational speed of 400 rpm. The strained pulp was centrifuged at 9750 rpm for 20 min, and the supernatant juice was flushed with nitrogen and stored at −30 °C until use.

Solvents and Reagents. All reagents and solvents were purchased from VWR (Darmstadt, Germany) and were of analytical or HPLC grade. Sephadex LH-20 was obtained from Amersham (Uppsala, Sweden). Deionized water was used throughout.

Isolation and Purification of Betanin, Phyllocactin, and Hylocerenin. As previously described (*8*), precipitation of pectic substances

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in purple pitaya juice was carried out by the addition of ethanol. Betanin, phyllocactin, and hylocerenin were isolated from depectinized purple pitaya juice by semipreparative HPLC (*12*). By the addition of ammonium hydroxide (25%), the pH of the resulting isolates was adjusted to pH 5−6. After concentration in vacuo at room temperature, the samples were repeatedly washed with deionized water and reconcentrated to gently remove residual acid.

The samples were further purified at 4 °C on a Sephadex LH-20 column (650 mm × 30 mm i.d.) preequilibrated with purified water. After application of the sample, betacyanins were separated from yellow degradation products generated during sample workup, the latter exhibiting longer retention times upon elution with deionized water. The betacyanin fractions were collected, concentrated in vacuo at room temperature, lyophilized, and stored in a sealed tube at −80 °C until use.

Preparation of Betacyanin Solutions. To adjust identical betacyanin concentrations of 100 mg/L, 5.0 mg of betanin, phyllocactin, and hylocerenin lyophilizate, respectively, was made up separately to 50 mL each with deionized water previously acidified to pH 4.5 using 1 M HCl. After the pH of the resulting solutions was adjusted to 4.0 by adding one or two beads of 0.02 M HCl, aliquots of 4 mL were filled in test tubes with screw caps and immediately stored at −30 °C until further use within 2 weeks.

Thermal Treatment. After they were thawed for 20 min at 20 °C in a water bath, test tubes were fitted with a magnetic stirrer and heated in a water bath at 85 °C. For comparison of thermal stability, heating periods of 10, 20, and 30 min were applied for each betacyanin. Further data points for half-life value determination (see below) were recorded after thermal treatment for 5 and 45 min for betanin, 15 and 40 min for phyllocactin, and 40 and 60 min for hylocerenin solutions, respectively. After heating, the samples were instantly cooled in an ice bath for 1 min and then transferred in a water bath at 20 °C. Exactly 5 min after heating, an aliquot was filled in an HPLC vial and immediately analyzed by HPLC−diode array detection (DAD), and the residual remained in the water bath until it was used for spectrophotometric measurements (see below). After injection, the vial was stored at 4 °C. Twenty-four hours after heating, the vial was placed in the sample table of the HPLC-DAD autosampler thermostated at 20 °C and allowed to stand for 20 min before reinjection. Unheated betanin, phyllocactin, and hylocerenin solutions were treated the same way. All determinations were performed in duplicate.

Spectrophotometric Measurements. All spectrophotometric measurements were performed with a UV−vis spectrometer (Perkin-Elmer, Überlingen, Germany) equipped with a UV−vis (UVWinLab V 2.85.04) and a color (Wincol V 2.05) software (Perkin-Elmer Instruments, Norwalk, CT).

Color Analyses. Maximum absorption of the unheated betanin, phyllocactin, and hylocerenin samples was adjusted to 1.00 ± 0.05 by dilution with McIlvaine buffer (pH 6.5). The same dilutions were subsequently applied for all samples of the respective betacyanin.

Exactly 10 min after heating, the thermally treated betacyanin samples were diluted, and visible spectra (380−780 nm) were recorded in 1 cm path length disposable cuvettes. Chroma [$C^* = (a^{*2} + b^{*2})^{1/2}$] and hue angle {$h^\circ$ = [arctan (b^*/a^*)]} were calculated from CIE a^* and b^* values using illuminant D_{65} and 10° observer angle. The total color difference (ΔE^*) of heated betacyanin samples (a) as compared to the respective not heated betacyanin solutions (b) was estimated according to the equation (*13*): [$\Delta E^* = (\Delta L^{*2} + \Delta C^{*2} + \Delta H^{*2})^{1/2}$] with $\Delta L^* = (L_a^* - L_b^*)$, $\Delta C^* = (C_a^* - C_b^*)$, and $\Delta H^* = 2 \times [\sin (h_a^\circ - h_b^\circ)/2] \times (C_a^* \times C_b^*)^{1/2}$. All determinations were performed in triplicate.

Quantification of Betacyanins. After 30 min of equilibration, quantification of betanin in the diluted betanin samples was carried out applying the equation: betanin (mg/L) = ($A \times F \times$ MW $\times 1000/\epsilon \times l$), where A is the absorption value at the absorption maximum corrected by the absorption at 650 nm, F is the dilution factor, MW is the molecular weight of betanin (MW = 550 g/mol), ϵ is the molar extinction coefficient of betanin (ϵ = 60000 L/mol cm), and l is the path length of the cuvette (*14*−*16*). For quantification of phyllocactin and hylocerenin, molecular weights of 636 and 694 g/mol, respectively, were applied, while keeping the molar extinction coefficient of betanin for all calculations. All determinations were performed in triplicate.

Determination of Half-Life Values ($T_{1/2}$) Based on Spectrophotometric Data. As previously described (*10*), determination of the half-life value ($T_{1/2}$) was carried out by calculating the ratio of the betacyanin content in the unheated betacyanin sample (Bc_b) and in the samples heated at 85 °C (Bc_a). The natural logarithm (ln) of this ratio (Bc_b/Bc_a) was plotted against the heating period. The slope of the line through the origin obtained by connecting the data points was equated with k from which the half-life value {$T_{1/2} = [(\ln 2)/k]$} was calculated.

HPLC-DAD Analyses. *HPLC System.* All samples were analyzed with a Merck Hitachi LaChrom Elite HPLC system (Merck, Darmstadt, Germany) consisting of a pump L-2130, an autosampler L-2200, a JetStream column oven, and a diode array detector L-2450. Separation was achieved on an analytical scale Atlantis dC_{18} column (250 mm × 4.6 mm i.d.) with a particle size of 5 μm (Waters, Wexford, Ireland) fitted with a C_{18} ODS security guard column (4 mm × 3.0 mm i.d.; Phenomenex, Torrance, CA) operated at 30 °C. Solvents were 0.2% (v/v) formic acid in water (A) and MeCN (B). At a flow rate of 1 mL/min, the gradient program was applied as follows: 100% A isocratic for 4 min, 100 to 93% A in 3 min, 93 to 90% A in 17 min, 90% A isocratic for 8 min, 90 to 85% A in 15 min, 85 to 83% A in 15 min, and 83 to 0% A in 8 min. Simultaneous monitoring was performed at 535 (betacyanins and red betacyanin degradation products) and 470 nm (yellow betacyanin degradation products), respectively.

Purity Checks. The purities of the single betacyanins in the respective pH 4 solutions were accomplished after 20 min of thawing in a water bath at 20 °C. The purities at 535 and 280 nm were determined as follows: 94.6 and 89.7% for betanin, 78.9 and 79.0% for phyllocactin, and 98.7 and 97.3% for hylocerenin, respectively.

Applying an HPLC-MS/MS method as described previously (*11*), specific protonated molecular ions $[M + H]^+$ at m/z 551, 637, and 695 were obtained for betanin, phyllocactin, and hylocerenin, respectively, showing identical daughter ions [betanidin + H]$^+$ at m/z 389.

Adjustment of Injection Volumes. To achieve comparable peak area (PA) values for all three betacyanins in unheated samples, injection volumes for betanin, phyllocactin, and hylocerenin solutions were fixed at 25, 40, and 38 μL, respectively. To allow direct comparison of PA units, these volumes were kept constant for all samples of the respective betacyanin type.

Determination of Half-Life Values ($T_{1/2}$) Based on HPLC Data. As described above for spectrophotometric measurements, determination of the half-life value ($T_{1/2}$) based on HPLC data was carried out by calculating the ratio of the respective PA in the unheated betacyanin sample (PA_b) and in the samples heated at 85 °C (PA_a). The natural logarithm (ln) of this ratio (PA_b/PA_a) was plotted against the heating period. The slope of the line through the origin obtained by connecting the data points was equated with k from which the half-life value {$T_{1/2} = [(\ln 2)/k]$} was calculated. All determinations were performed in duplicate.

Calculation of Color Shade and Isomerization Index. The color shade of the betacyanin samples was calculated by dividing the total PAs monitored at 535 and 470 nm, respectively, representing the red and yellow color impression. The isomerization index was calculated by correlating the PAs of a betacyanin and its respective C_{15} stereoisomer, i.e., of betanin and isobetanin, phyllocactin and isophyllocactin, and hylocerenin and isohylocerenin, respectively.

RESULTS AND DISCUSSION

Betanin, phyllocactin, and hylocerenin solutions adjusted to pH 4 were exposed to thermal treatment at 85 °C. During heat exposure, the purified pitaya-derived betacyanin solutions displayed differences in terms of color development, betacyanin concentration, and structural changes, as monitored by spectrophotometric and HPLC-DAD analyses immediately after heat treatment. Alterations of thermally treated samples upon cool storage (CS) at 4 °C for 24 h were monitored solely by HPLC, as indicated by CS in the respective tables and figures.

Table 1. Lightness, Chroma, Hue Angle, and Total Color Difference Values of Betanin, Phyllocactin, and Hylocerenin Solutions after Thermal Treatment at 85 °C (Assessed by Spectrophotometric Analysis)

heating period	betanin	phyllocactin	hylocerenin
	lightness (L^*)[a]		
not heated	64.1 (±0.3)	65.1 (±0.4)	65.1 (±0.2)
10 min	73.8 (±0.2)	76.8 (±0.3)	73.5 (±0.3)
20 min	82.7 (±0.5)	84.2 (±0.4)	75.5 (±0.7)
30 min	88.4 (±0.3)	87.5 (±0.6)	76.9 (±0.2)
	chroma (C^*)[a]		
not heated	72.0 (±0.4)	70.1 (±0.3)	71.1 (±0.3)
10 min	40.8 (±0.2)	33.1 (±0.3)	41.8 (±0.5)
20 min	21.4 (±0.6)	16.5 (±0.2)	37.3 (±1.2)
30 min	10.2 (±0.3)	10.4 (±0.1)	33.3 (±0.5)
	hue angle ($h°$)[a]		
not heated	331.2 (±0.2)	331.5 (±0.1)	331.1 (±0.1)
10 min	339.8 (±0.2)	344.6 (±0.3)	340.7 (±0.2)
20 min	344.7 (±0.4)	2.2 (±0.7)	344.4 (±0.9)
30 min	352.2 (±0.7)	18.7 (±1.3)	347.9 (±0.3)
	total color difference (ΔE^*)		
10 min	33.2	40.4	31.8
20 min	54.7	59.7	37.4
30 min	67.1	67.3	42.1

[a] Values are expressed as means ± SD.

Chromatic Changes of Thermally Treated Betanin, Phyllocactin, and Hylocerenin Solutions. Color characteristics of betanin, phyllocactin, and hylocerenin solutions immediately after thermal treatment are listed in **Table 1**.

Lightness. Lightness (L^*) values increasing with heat exposure could be monitored for all betacyanin solutions, indicating a decline of betacyanin concentrations. Interestingly, during the first 20 min of heating, L^* values of betanin increased slower as compared to phyllocactin, whereas after 30 min at 85 °C the values for betanin and phyllocactin were nearly identical, reflecting higher stability of phyllocactin upon prolonged heating. Hylocerenin solutions exhibited more stable lightness values during thermal treatment, resulting in lightness increments only half as high as observed for betanin and phyllocactin solutions heated for 30 min at 85 °C, respectively.

Chroma. For chroma (C^*) values, corresponding developments were observed (**Table 1**). Betanin solutions yielded higher C^* values as compared to phyllocactin after 10 and 20 min of heating, respectively, indicating greater pigment retention of the former. After 30 min of thermal exposure, however, the C^* of betanin and phyllocactin was identical, while chroma retention was intelligibly higher for hylocerenin solutions throughout the heating period. Additionally, the C^* of betacyanin-based samples has been reported to decline with increasing betaxanthin contents (*17*); that is, incremental amounts of differently colored compounds result in decreasing chroma values. Hence, it was suspected that generation of degradation products in hylocerenin solutions was less pronounced than in betanin and phyllocactin samples, respectively.

Hue Angle. The formation of degradation products is also reflected by the hue angle ($h°$) values of the different betacyanin solutions. Unheated betacyanin samples exhibited values of 331° (**Table 1**), representing a red-violet tonality. During heat treatment, increasing hue angle values were found for all betacyanin solutions. For the phyllocactin sample heated for 30 min at 85 °C, $h°$ exceeded the 0°/360°-axis and reached a value of 19°, resulting in a notable shift to yellow. Hence, the formation of yellow compounds appeared to be most pronounced in phyllocactin solutions, with betanin and hylocerenin samples exhibiting minor and the least color changes, respectively. These findings seem to be in disagreement with chroma evolution upon thermal treatment, where betanin and phyllocactin showed nearly identical performance. This discrepancy may be explained by predominant degradation of betanin through hydrolytic cleavage resulting in the formation of the bright yellow betalamic acid (λ_{max} about 410 nm) and the colorless *cyclo*-dopa 5-*O*-β-glucoside (*11*; **Figure 1**), while dehydrogenation leading to yellow betacyanin degradation products (λ_{max} = 450−490 nm; *7*, *10*, *11*, *18*) is more pronounced for phyllocactin (**Figure 2**) and hylocerenin (**Figure 3**) degradation (*11*). Because of the comparatively low molar extinction coefficient of betalamic acid, exhibiting values only half as high as compared to betanin (*19*),

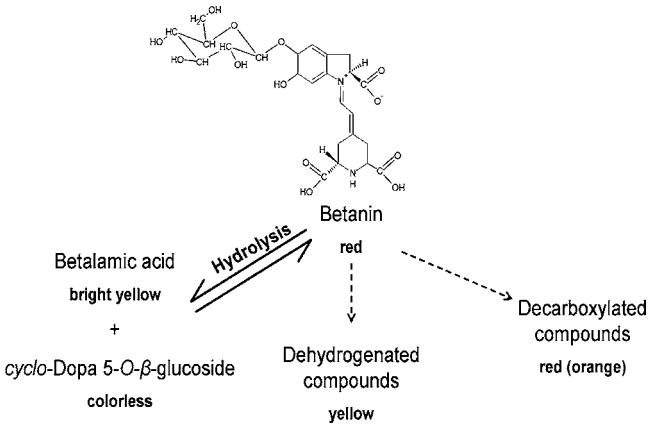

Figure 1. Initial steps of thermal betanin degradation based on HPLC analyses (solid arrow, main pathway; dashed arrows, secondary pathways).

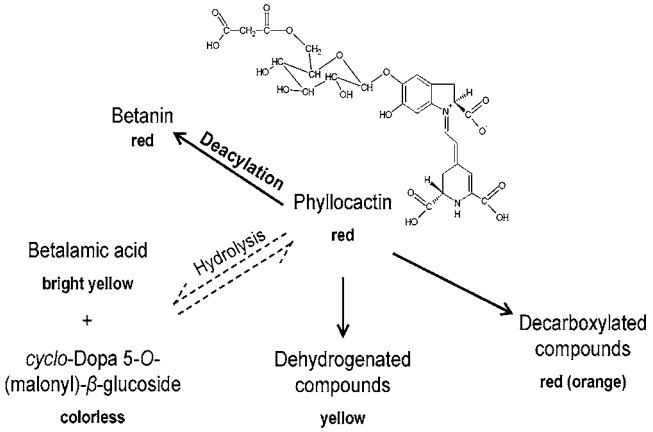

Figure 2. Initial steps of thermal phyllocactin degradation based on HPLC analyses (solid arrows, main pathways; dashed arrow, secondary pathway).

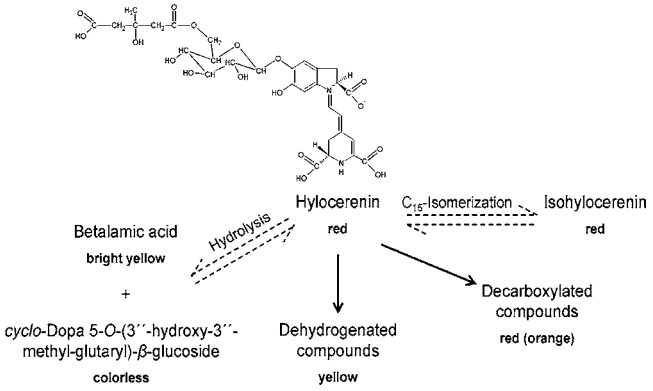

Figure 3. Initial steps of thermal hylocerenin degradation based on HPLC analyses (solid arrows, main pathways; dashed arrows, secondary pathways).

Table 2. Color Shade (Red/Yellow) of Betanin, Phyllocactin, and Hylocerenin Solutions Immediately after Thermal Treatment at 85 °C and after Consecutive CS at 5 °C for 24 h (Assessed by HPLC Analysis at 535 and 470 nm, Respectively)

heating period	betanin	phyllocactin	hylocerenin
not heated	3.7	3.7	3.8
not heated, CS	3.7	3.7	3.7
10 min	3.5	3.2	3.5
10 min, CS	3.4	2.9	3.4
20 min	2.1	2.2	2.8
20 min, CS	2.1	1.8	2.8
30 min	1.8	1.7	2.3
30 min, CS	1.7	1.3	2.1

Table 3. Betacyanin Content and Half-life Value ($T_{1/2}$) of Betanin, Phyllocactin, and Hylocerenin in Samples Heated at 85 °C (Assessed by Spectrophotometric and HPLC Analyses, Respectively)

heating period	betanin	phyllocactin	hylocerenin
	betacyanin content assessed by spectrophotometric analysis (mg/L)[a]		
not heated	79.0 (±0.9)	74.2 (±0.8)	67.8 (±0.8)
10 min	44.1 (±0.2)	35.4 (±0.6)	40.1 (±0.7)
20 min	21.9 (±0.7)	18.2 (±0.2)	35.0 (±0.6)
30 min	10.6 (±0.3)	11.1 (±0.1)	31.0 (±1.4)
	half-life value at 85 °C assessed by spectrophotometric analysis (min)		
$T_{1/2}$ (R^2)[b]	11.1 (0.99)	11.3 (0.98)	27.4 (0.73)
	half-life value at 85 °C assessed by HPLC analysis (min)		
$T_{1/2}$ (R^2)[b] individual PA	10.6 (0.99)	8.0 (0.99)	8.1 (0.99)
$T_{1/2}$ (R^2)[b] total PA	10.7 (0.99)	10.9 (0.94)	32.4 (0.91)

[a] Values are expressed as means ± SD. [b] R^2 = correlation coefficient.

only minor effects on the tonality of heated betanin solutions are to be expected.

Total Color Difference. Interestingly, the total color difference (ΔE^*) corresponded to the results obtained for chroma, yielding nearly identical values for betanin and phyllocactin after 30 min of thermal treatment. The reason for the lacking correlation of hue angle and ΔE^* values of these samples could not be disclosed further.

Color Shade. Additionally, the color shade of the heated pigment solutions was calculated based on HPLC data by dividing the total peak areas (PAs) monitored at 535 (red) and 470 nm (yellow), respectively. Hence, decreasing color shade values indicate a shift toward yellow. As shown in **Table 2**, color shade and hue angle development are consistent, i.e., chromatic changes in heated betacyanin solutions can be monitored both by spectrophotometric measurements and by peak detection at two characteristic wavelengths during HPLC analysis, respectively.

Quantitative Changes of Thermally Treated Betanin, Phyllocactin, and Hylocerenin Solutions. As the betacyanin solutions were reconstituted by weighing out 5 mg of the respective purified and lyophilized betacyanin and making up to 50 mL, the betacyanin concentrations of the unheated solutions were adjusted to 100 mg/L. However, the betacyanin concentrations of the purified pigment solutions calculated from the data obtained by spectrophotometric measurements were lower than expected, reaching values between 67.8 and 79.0 mg/L (**Table 3**). Although the correct molecular masses for the respective betacyanins were applied, the calculated betacyanin contents declined with increasing molecular weight of the respective betacyanin. This may be ascribed to missing specific extinction coefficients for phyllocactin and hylocerenin and the consequential use of the value for betanin. In addition, the betacyanin concentration of the unheated betanin solution was already underestimated by 20%. Hence, the spectrophotometric betacyanin quantification is suspected to yield too low results.

Half-Life Values ($T_{1/2}$) Based on Spectrophotometric Data. Phyllocactin and betanin exhibited virtually the same half-life value ($T_{1/2}$) of 11.3 and 11.1 min, respectively (**Table 3**). In a previous study on betanin solutions performed at similar temperature conditions, a half-life value of 15 min was reported (*20*). However, in the former investigation, pH 5.5 was applied (*20*), whereas the pH has been adjusted to 4.0 in the present study. Consequently, the pH dependency of thermal betalain stability could be confirmed (*5*, *21*). In accordance with the results obtained by color analysis, hylocerenin solutions exhibited a significantly higher pigment stability as compared to betanin and phyllocactin. In general, the decreasing betacyanin concentration was associated with declining C^* and increasing L^* values, respectively (**Table 1**). After 30 min of heating, nearly 50% of the betacyanin content of the unheated hylocerenin solutions was still retained, the content of the betanin sample dropping to an eighth of the initial value upon identical thermal exposure (**Table 3**).

The half-life value of 27.4 min for hylocerenin was nearly 3-fold the values obtained for betanin and phyllocactin and reflected the superior stability of hylocerenin. In contrast to betanin ($R^2 = 0.99$) and phyllocactin ($R^2 = 0.98$), hylocerenin degradation did not ideally follow first-order reaction kinetics, as indicated by a comparatively low correlation coefficient ($R^2 = 0.73$).

Half-Life Values ($T_{1/2}$) Based on HPLC Data. The half-life value determination based on the PA decrease of the respective betacyanin obtained by HPLC analyses yielded intelligibly different results (**Table 3**):

Betanin proved to be the most stable pigment structure during heating with respect to its PA, while the half-life values of the acylated betacyanins phyllocactin and hylocerenin were determined to be 20% lower. On the other hand, calculation of $T_{1/2}$ based on the total PAs monitored at 535 nm yielded values comparable to those obtained by spectrophotometric measurements (**Table 3**), whereas the correlation coefficient of hylocerenin half-life value was noticeably higher for HPLC analyses. Interestingly, for betanin, both the application of the individual betanin PA and the total PA at 535 nm resulted in virtually identical half-life values, indicating that thermally treated betanin solutions were nearly devoid of degradation products absorbing at 535 nm, as already reported in a previous study (*11*). Accordingly, virtually the entire absorption at 535 nm resulted from betanin (**Figure 4**).

In contrast, considerable differences were obtained by calculation of the half-life values of phyllocactin and the total PA at 535 nm in the respective phyllocactin solution (**Table 3**), indicating the formation of red compounds during phyllocactin degradation, as demonstrated in **Figure 5** by the notable discrepancy between the specific phyllocactin area and the total PA at 535 nm. Also, different values were registered even for the unheated phyllocactin solution, possibly due to the instability of the malonic acid moiety, the latter being prone to both decarboxylation and deacylation (*11*, *22*, *23*). Thus, inevitable formation of both red decarboxylated degradation products and betanin (**Figure 2**) even during phyllocactin purification was plausible. Betanin formation upon thermal treatment of phyllocactin solutions is demonstrated in **Table 4**. During the first 20 min of heating, deacylation of phyllocactin caused a betanin

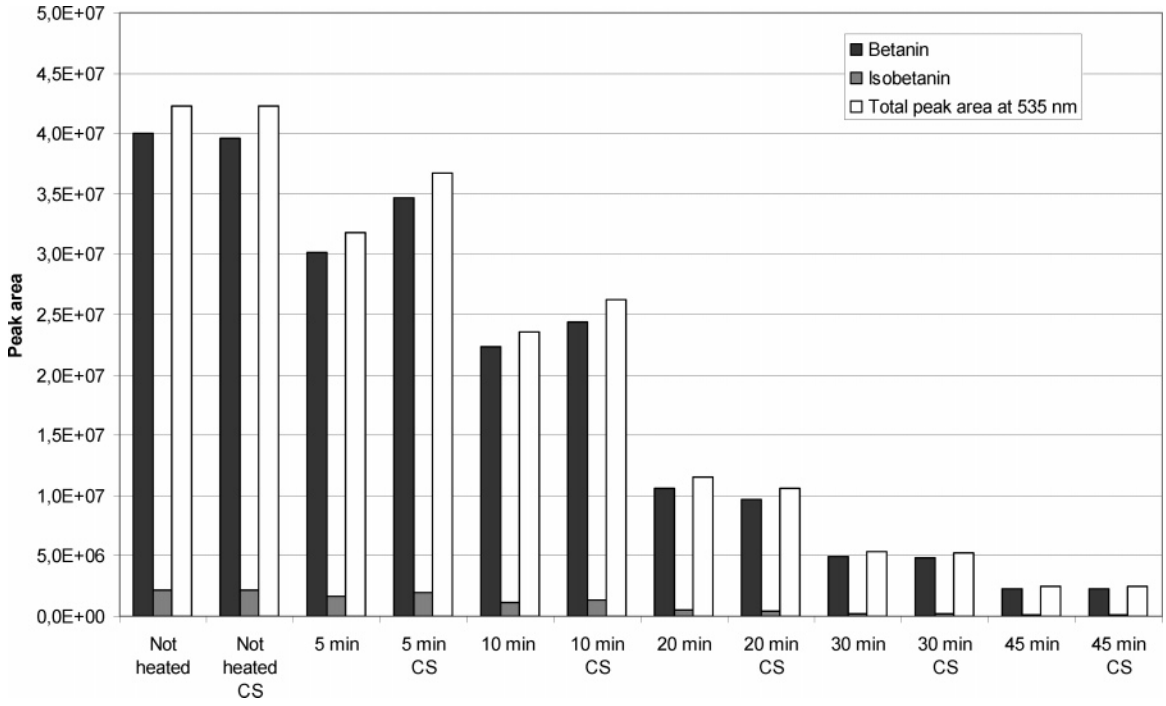

Figure 4. HPLC PAs of betanin and isobetanin together with total PA of betanin solutions monitored at 535 nm immediately after thermal treatment at 85 °C and after consecutive CS at 5 °C for 24 h.

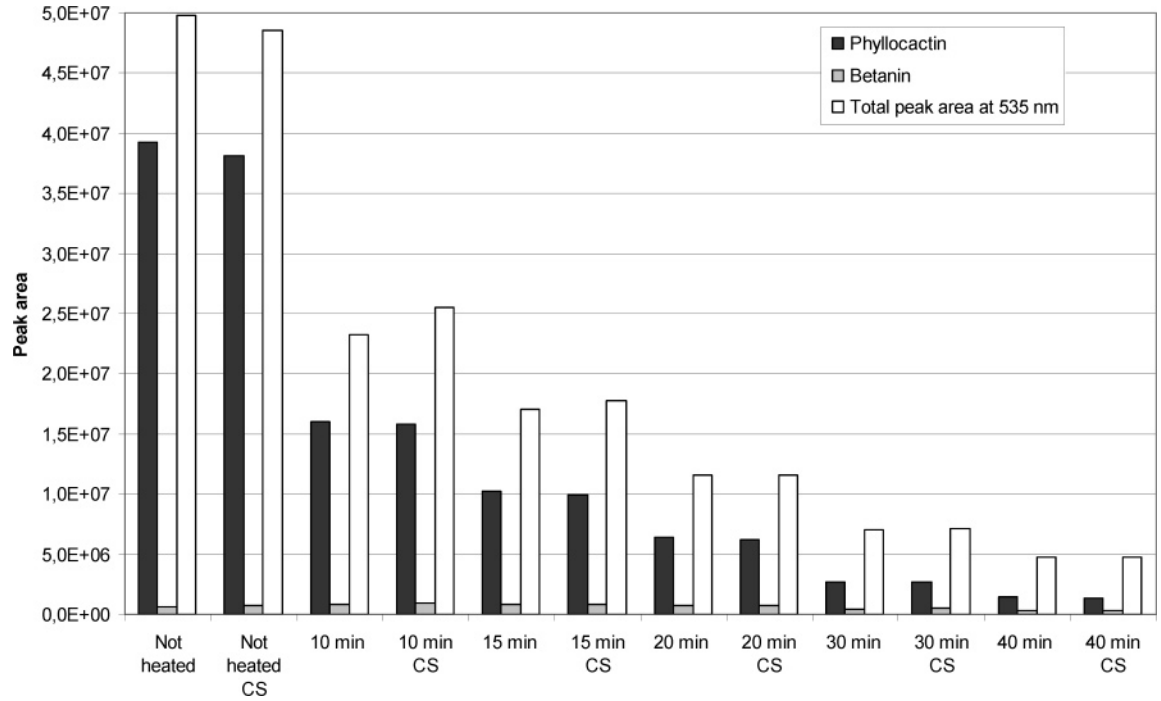

Figure 5. HPLC PAs of phyllocactin and betanin together with total PA of phyllocactin solutions monitored at 535 nm immediately after thermal treatment at 85 °C and after consecutive CS at 5 °C for 24 h.

gain as compared to the unheated phyllocactin solution. Most interestingly, degradation of phyllocactin resulted in a pigment mixture obviously exhibiting thermal stability superior to that of the genuine compound (**Figure 2**).

This effect was found to be even more pronounced in thermally treated hylocerenin solutions. The half-life value calculated from the total PA at 535 nm was four times the value based on the individual hylocerenin area (**Table 3**). Additionally, the unheated hylocerenin solution was nearly devoid of accompanying compounds absorbing at 535 nm (**Figure 6**). Upon thermal treatment, red compounds were formed from hylocerenin exhibiting a considerably higher retention than genuine hylocerenin. As shown in **Figure 6**, basically one compound was generated, preliminarily identified as 2-decarboxy-hylocerenin in a previous study (*11*). Its tremendous concentration gain of reaching 11000% of its initial PA after heating for 20 min is demonstrated in **Table 4**.

In short, phyllocactin, and particularly hylocerenin, were not per se more stable than betanin but were transformed into red products that exhibited considerably higher thermal stability as compared to the reactants, as reflected by their characteristic HPLC degradation profiles (**Figure 7**). Because the half-life

Table 4. Relative PA Changes (%) of Betanin, Phyllocactin, and Hylocerenin in Purified Pigment Solutions Monitored at 535 nm Immediately after Thermal Treatment at 85 °C and after Consecutive CS at 5 °C for 24 h

	betanin solution			phyllocactin solution				hylocerenin solution			
heating period	betanin	isobetanin	total peak area	phyllocactin	isophyllo-cactin	betanin	total peak area	hylocerenin	isohylo-cerenin	2-decarboxy-hylocerenin	total peak area
not heated	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
not heated, CS	98.8	98.4	98.7	97.2	96.6	117.0	97.6	97.2	100.4	148.5	97.1
10 min	55.6	53.0	55.7	40.8	34.3	136.1	46.8	50.2	110.6	4467.0	68.7
10 min, CS	60.9	60.8	62.1	40.2	34.0	145.7	51.3	55.4	133.9	6582.2	83.2
20 min	26.6	21.8	27.2	16.2	11.2	110.6	23.2	21.8	53.4	9350.1	61.9
20 min, CS	24.2	20.0	24.9	15.8	10.8	112.1	23.2	21.8	55.1	11379.0	71.2
30 min	12.3	8.4	12.6	6.9	3.8	73.9	14.1	9.6	[a]	10236.0	54.1
30 min, CS	12.1	8.4	12.4	6.8	3.7	75.1	14.3	9.0	[a]	11087.5	57.7

[a] Isohylocerenin area < 100000.

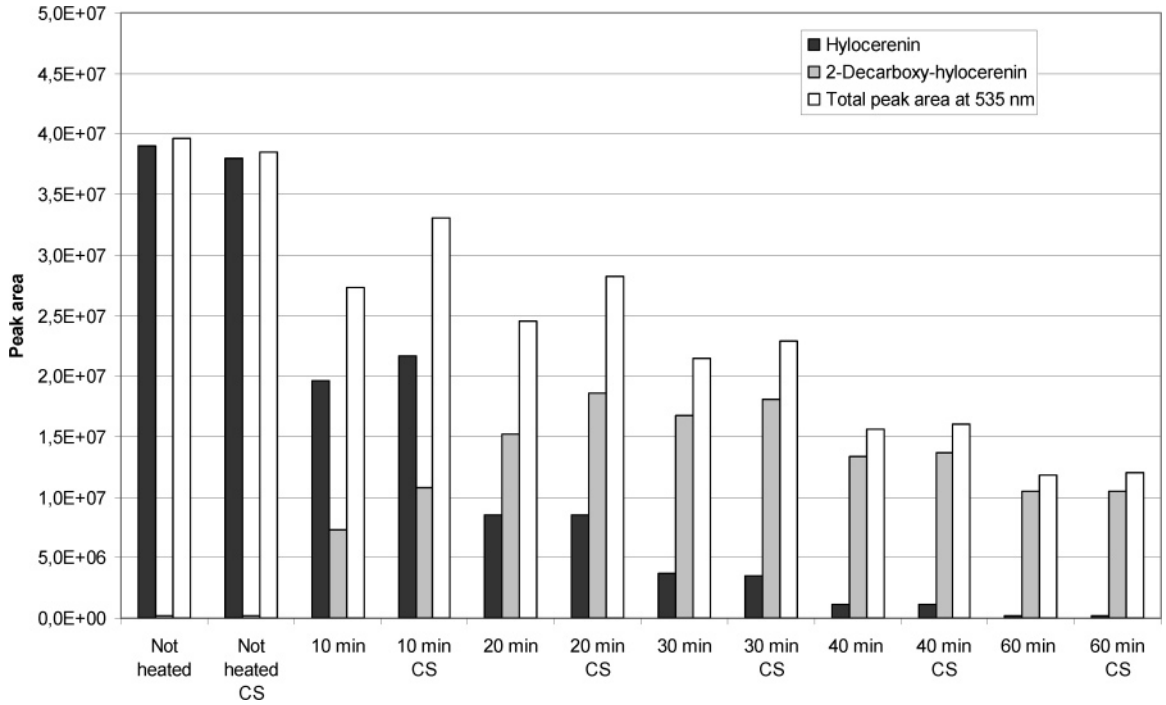

Figure 6. HPLC PAs of hylocerenin and C_2-decarboxy-hylocerenin together with total PA of hylocerenin solutions monitored at 535 nm immediately after thermal treatment at 85 °C and after consecutive CS at 5 °C for 24 h.

values obtained for isolated betacyanins from purple pitaya were significantly lower as compared to the value of betacyanins in purple pitaya juice ($T_{1/2}$ = 2.5 h; *10*), a protective effect of matrix compounds on overall betacyanin stability may be assumed (*5*, *10*, *24*, *25*). Furthermore, the plant matrix may favor specific degradation trails (*11*).

Betacyanin Regeneration in Thermally Treated Betanin, Phyllocactin, and Hylocerenin Solutions. To study betacyanin regeneration from their hydrolysis products (betalamic acid and *cyclo*-dopa 5-*O*-*β*-glucoside derivatives; **Figures 1**−**3**), CS and subsequent analysis of the betacyanin solutions were performed. Recondensation of the fragments has been described to proceed in betanin solutions (*3*), red beet juice (*4*), and purple pitaya juice (*10*).

Betanin. After CS of unheated betanin solution, a decrease in betanin concentration was observed (**Table 4** and **Figure 4**), indicative of the low stability of the purified betacyanin. In the sample heated for 10 min, CS resulted in a marked increase of betanin concentration and the total PA monitored at 535 nm, being even more distinctive in the sample heated for 5 min (**Figure 4**). However, after 20 min of heat exposure, recondensation was not observed. This may be due to the poor retention of *cyclo*-dopa-5-*O*-*β*-glucoside after extended heating, thus precluding betanin regeneration (*11*).

Phyllocactin. After CS of phyllocactin solutions heated for a short period, no phyllocactin regeneration was registered. Instead, a decrease in the phyllocactin PA was found (**Table 4**), whereas the total PA monitored at 535 nm increased during CS after 10 min of heating, possibly due to condensation of betalamic acid and *cyclo*-dopa derivatives [decarboxylated *cyclo*-dopa 5-*O*-*β*-glucoside and decarboxylated *cyclo*-dopa 5-*O*-*β*-(malonyl)glucoside; *11*]. Because of phyllocactin deacylation, betanin concentration in the phyllocactin solution rose not only upon heating but also during CS over the complete heating period (**Table 4** and **Figure 2**).

Hylocerenin. An increment of total PA was monitored after CS of all thermally treated hylocerenin solutions (**Table 4**), while the individual PA of hylocerenin leveled off and even decreased during CS of the samples heated for 20 and 30 min, respectively. This can mainly be ascribed to the formation of 2-decarboxy-hylocerenin (see above), as reflected by its considerable gain in PA upon CS (**Table 4** and **Figure 6**), which

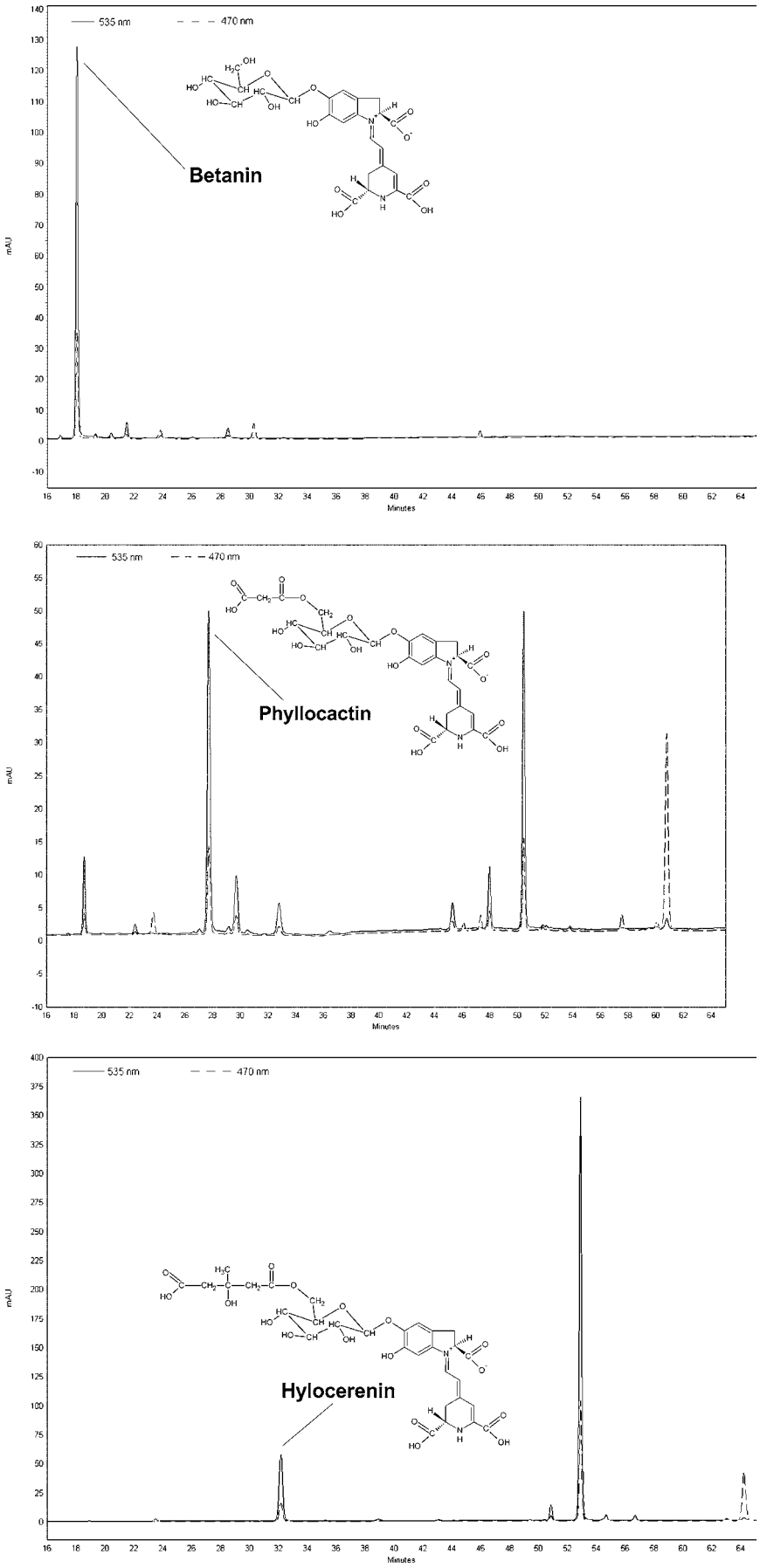

Figure 7. HPLC degradation profiles of betanin (**A**), phyllocactin (**B**), and hylocerenin (**C**) solutions monitored at 535 and 470 nm immediately after thermal treatment at 85 °C for 30 min.

Table 5. Isomerization Index (PA Ratio of 15*S* and 15*R* Stereoisomers Monitored at 535 nm) of Betanin, Phyllocactin, and Hylocerenin in Purified Pigment Solutions Immediately after Thermal Treatment at 85 °C and after Consecutive CS at 5 °C for 24 h

heating period	betanin/ isobetanin	phyllocactin/ isophyllocactin	hylocerenin/ isohylocerenin
not heated	18.3	5.0	164.7
not heated, CS	18.4	5.0	159.5
10 min	19.2	5.9	74.8
10 min, CS	18.3	5.9	68.1
20 min	22.3	7.2	67.4
20 min, CS	22.2	7.2	65.2
30 min	26.8	9.0	[a]
30 min, CS	26.8	9.1	[a]

[a] Isohylocerenin area < 100000.

may partly be due to the condensation of betalamic acid and decarboxylated *cyclo*-dopa 5-*O*-β-(3″-hydroxy-3″-methyl-glutaryl)glucoside (*11*). On the other hand, amplified formation of 2-decarboxy-hylocerenin on the expense of hylocerenin during thermal treatment supports the assumption of hylocerenin being directly decarboxylated to form 2-decarboxy-hylocerenin.

Betacyanin Isomerization in Thermally Treated Betanin, Phyllocactin, and Hylocerenin Solutions. *Betanin.* Betanin C_{15} isomerization, i.e., 15*S*−15*R* racemization, has been described to be induced by thermal treatment of red beet juice (*4*, *7*), reflected by a decreasing PA ratio of betanin and isobetanin. Unexpectedly, in this study, an increase in betanin/isobetanin ratio was found during heating of purified betanin (**Table 5**); that is, isobetanin decay proceeded faster than betanin loss. This may be ascribed to the missing matrix effect in purifed pigment solutions as opposed to juices. Whether the declining ratio during CS after 10 min of heating was due to betanin isomerization or rather to enhanced recondensation of the cleavage products isobetalamic acid and *cyclo*-dopa 5-*O*-β-glucoside (*7*) could not be elucidated.

Phyllocactin. Likewise, thermal treatment of phyllocactin solutions resulted in an increase of the phyllocactin isomerization index, while consecutive CS did not change this value (**Table 5**). Interestingly, the 15*S*−15*R* proportion of phyllocactin was considerably lower as compared to betanin, thus underlining the specific structural feature of malonic acid acylation.

Hylocerenin. In contrast to betanin and phyllocactin, isomerization was observed in hylocerenin samples, where hylocerenin/isohylocerenin ratios decreased both during heat treatment for 20 min and consecutive CS (**Table 5**). Because only trace amounts of isohylocerenin were retained after 30 min of heating, further evolution of this ratio could not be monitored adequately.

The significantly higher isomer ratio in hylocerenin solutions as compared to betanin and phyllocactin samples may be explained by their respective chemical structure, while an increased stability of the 15*S*-acylglucosides, as previously reported for betacyanins substituted with aromatic acid moieties (*26*), could not be confirmed.

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LITERATURE CITED

(1) Steglich, W.; Strack, D. Betalains. In *The Alkaloids—Chemistry and Pharmacology*; Brossi, A., Ed.; Academic Press Inc.: San Diego, California, 1990; pp 1−62.

(2) Stintzing, F. C.; Carle, R. Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends Food Sci. Technol.* **2004**, *15*, 19−38.

(3) Huang, A. S.; von Elbe, J. H. Kinetics of the degradation and regeneration of betanine. *J. Food Sci.* **1985**, *50*, 1115−1120.

(4) von Elbe, J. H.; Schwartz, S. J.; Hildenbrand, B. E. Loss and regeneration of betacyanin pigments during processing of red beets. *J. Food Sci.* **1981**, *46*, 1713−1715.

(5) von Elbe, J. H.; Maing, I.-Y.; Amundson, C. H. Colour stability of betanin. *J. Food Sci.* **1974**, *39*, 334−337.

(6) von Elbe, J. H. Stability of betalaines as food colours. *Food Technol.* **1975**, *29*, 42−46.

(7) 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*, C491−C498.

(8) Stintzing, F. C.; Schieber, A.; Carle, R. Betacyanins in fruits from red-purple pitaya, *Hylocereus polyrhizus* (Weber) Britton & Rose. *Food Chem.* **2002**, *77*, 101−106, 517.

(9) Wybraniec, S.; Platzner, I.; Geresh, S.; Gottlieb, H. E.; Haimberg, M.; Mogilnitzki, M.; Mizrahi, Y. Betacyanins from vine cactus *Hylocereus polyrhizus*. *Phytochemistry* **2001**, *58*, 1209−1212.

(10) Herbach, K. M.; Stintzing, F. C.; Carle, R. Thermal degradation of betacyanins in juices from purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose) monitored by high-performance liquid chromatography-tandem mass spectrometric analyses. *Eur. Food Res. Technol.* **2004**, *219*, 377−385.

(11) Herbach, K. M.; Stintzing, F. C.; Carle, R. Identification of heat-induced degradation products from purified betanin, phyllocactin and hylocerenin by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2603−2616.

(12) Stintzing, F. C.; Conrad, J.; Klaiber, I.; Beifuss, U.; Carle, R. Structural investigations on betacyanin pigments by LC NMR and 2D NMR spectroscopy. *Phytochemistry* **2004**, *65*, 415−422.

(13) Gonnet, J.-F. Colour effects of co-pigmentation of anthocyanins revisited—1. A colorimetric definition using the CIELAB scale. *Food Chem.* **1998**, *63*, 409−415.

(14) Cai, Y.; Corke, H. Amaranthus betacyanin pigments applied in model food systems. *J. Food Sci.* **1999**, *64*, 869−873.

(15) Wyler, H.; Meuer, U. Zur Biogenese der Betacyane: Versuche mit [2-^{14}C]-Dopaxanthin. *Helv. Chim. Acta* **1979**, *62*, 1330−1339.

(16) Stintzing, F. C.; Schieber, A.; Carle, R. Evaluation of colour properties and chemical quality parameters of cactus juices. *Eur. Food Res. Technol.* **2003**, *216*, 303−311.

(17) Mosshammer, M. R.; Stintzing, F. C.; Carle, R. Colour studies on fruit juice blends from *Opuntia* and *Hylocereus* cacti and betalain-containing model solutions derived therefrom. *Food Res. Int.* **2005**, *38*, 975−981.

(18) 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**, *53*, 3483−3487.

(19) Trezzini, G. F.; Zryd, J.-P. Characterization of some natural and semi-synthetic betaxanthins. *Phytochemistry* **1991**, *30*, 1901−1903.

(20) Saguy, I.; Kopelman, I. J.; Mizrahi, S. Thermal kinetic degradation of betanin and betalamic acid. *J. Agric. Food Chem.* **1978**, *26*, 360−362.

(21) Castellar, R.; Obón, J. M.; Alacid, M.; Fernández-López, J. A. Color properties and stability of betacyanins from *Opuntia* fruits. *J. Agric. Food Chem.* **2003**, *51*, 2772−2776.

(22) Määttä, K. R.; Kamal-Edin, A.; Törrönen, A. R. High-performance liquid chromatography (HPLC) analysis of phenolic compounds in berries with diode array and electrospray ionization mass spectrometric (MS) detection: *Ribes* species. *J. Agric. Food Chem.* **2003**, *51*, 6736−6744.
(23) Lo Scalzo, R.; Iannoccari, T.; Summa, C.; Morelli, R.; Rapisarda, P. Effect of thermal treatments on antioxidant and antiradical activity of blood orange juice. *Food Chem.* **2004**, *85*, 41−47.
(24) Singer, J. W.; von Elbe, J. H. Degradation rates of vulgaxanthine I. *J. Food Sci.* **1980**, *45*, 489−491.
(25) Havlíková, L.; Míková, K.; Kyzlink, V. Heat stability of betacyanins. *Z. Lebensm. Unters. Forsch.* **1983**, *177*, 247−250.
(26) Schliemann, W.; Strack, D. Intramolecular stabilization of acylated betacyanins. *Phytochemistry* **1998**, *49*, 585−588.

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