Impact of High-Pressure Carbon Dioxide Combined with Thermal Treatment on Degradation of Red Beet (*Beta vulgaris* L.) Pigments

XUAN LIU, YANXIANG GAO,* HONGGAO XU, QI WANG, AND BIN YANG

College of Food Science and Nutritional Engineering, China Agricultural University,
Beijing 100083, China

A combined high-pressure carbon dioxide (HP-CO_2) and thermal degradation reaction of betanin and isobetanin in aqueous solution was investigated and can be described by a first-order decay. At 45 °C, the degradation rate constant (k) for each pigment component significantly increased (the half-life ($t_{1/2}$) decreased, $p < 0.05$) with elevated pressure. Furthermore, HP-CO_2 treatment led to lower k values (higher $t_{1/2}$ values) than thermal treatment. However, k and $t_{1/2}$ values approached those of thermal treatment when the pressure was >30 MPa combined with temperatures exceeding 55 °C. Moreover, betanin was more stable than isobetanin under HP-CO_2. E_a values ranged from 94.01 kJ/mol for betanin and 97.16 kJ/mol for isobetanin at atmospheric pressure to 170.83 and 142.69 kJ/mol at 50 MPa, respectively. A higher pressure and temperature as well as longer exposure time resulted in higher values of L^*, b^*, C^*, and $h°$. HP-CO_2 induced more degradation products from betanin and isobetanin than thermal treatment with an identical temperature and exposure time.

KEYWORDS: Betanin; isobetanin; high-pressure carbon dioxide; HP-CO_2; degradation kinetics; color difference; degradation products

INTRODUCTION

To produce safe and shelf stable products is a primary objective of food manufactures and was achieved by multifarious traditional thermal methods in the past decades; food quality was considered of secondary importance (*1*). It is commonly known that thermal treatment, widely used in commercial food production, impacts product quality, including flavor, texture, and nutrients such as vitamins, phenolics, etc. However, with the increasing demand by consumers for fresher, higher quality, minimally processed, and safer food, there is a strong interest in the food industry to develop nonthermal processing techniques to replace traditional thermal methods for food preservation (*2, 3*).

High-pressure carbon dioxide (HP-CO_2) treatment is one nonthermal method that has been the subject of intense scrutiny for potential applications in a number of food processing fields including inactivation of enzymes that can adversely affect the quality of food products (*4*). HP-CO_2 at lower temperatures can inactivate microorganisms and food deteriorative enzymes in fruit juices while only slightly affect nutritional and sensorial food quality aspects (e.g., ascorbic acid content, total acidity, color, flavor, and cloud), most of which are sensitive to heat (*5–9*). With the consideration of extreme pressure stability of some microbial species in low-acid food/vegetable juice (such as carrot, red beet, spinach juice, etc.), HP-CO_2 is required to be combined with proper heat treatment. However, experimental

systematic and quantitative studies are rather scarce concerning the effect of HP-CO_2 and increasing temperature on nutrients in food. Recently, there have been a few reports on HP-CO_2 in commercial use for fruit and vegetable products, but the technology is still under investigation in the laboratory. One of the biggest challenges for the application of HP-CO_2 in fruit juices or nonthermal food products is to evaluate the availability and safety of this processing, including changes of main nutritional components.

Red beet (*Beta vulgaris* L.) is a traditional vegetable distributed in many parts of the world and has been used commercially to produce red beet juice and natural pigment. The essential pigment in red beet is a group of bioactive compounds called betalains (*10, 11*). Ca. 80−90% of the total pigments of red beet is ascribed to betacyanins (violet), mainly betanin (betanidin 5-*O*-β-glucoside), and its C_{15} isomer isobeanin, whereas vulgaxanthin I represents the predominant betaxanthin (yellow) (*12–14*). The red−violet color is regarded as a major quality attribute in determining red beet product acceptability. Like many other natural pigments, betacyanins are very sensitive to heat, light, and oxidation, which especially result from peroxidase (POD) and these are major reasons for discoloration of the pigment (*15–18*). Moreover, enzymatic browning induced by polyphenol oxidase (PPO) in red beet is another frequently encountered problem in the production of red beet juice. HP-CO_2 combined with thermal treatment has been shown to be effective in inactivating POD and PPO in red beet extracts (*19*).

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* Author to whom correspondence should be addressed. Tel.: + 86-10-6273-7034; fax: + 86-10-6273-7986; e-mail: gyxcau@126.com.

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Table 1. Concentrations (mg/L) of Betanin and Isobetanin before and after Different Treatments

temp (°C)	treatment time (h)	atmospheric pressure		10 MPa	
		betanin	isobetanin	betanin	isobetanin
control	0	18.34 ± 0.14	17.04 ± 0.20	18.34 ± 0.14	17.04 ± 0.20
	0.5	17.62 ± 0.04	16.73 ± 0.03	18.18 ± 0.12	16.71 ± 0.15
45	1.5	16.92 ± 0.15	15.90 ± 0.12	17.44 ± 0.08	15.93 ± 0.07
	2.5	15.39 ± 1.56	14.20 ± 1.73	17.40 ± 0.08	15.77 ± 0.08
	0.5	17.14 ± 0.14	15.75 ± 0.12	17.40 ± 0.09	16.00 ± 0.13
55	1.5	14.81 ± 0.05	13.20 ± 0.04	17.39 ± 0.09	15.49 ± 0.07
	2.5	13.49 ± 0.05	11.78 ± 0.04	16.40 ± 0.07	14.39 ± 0.06
	0.25	16.46 ± 0.04	15.14 ± 0.07	17.08 ± 0.06	16.19 ± 0.05
65	0.75	13.01 ± 0.04	11.45 ± 0.04	15.77 ± 0.10	13.61 ± 0.12
	1.3	9.92 ± 0.13	8.36 ± 0.10	12.35 ± 0.09	10.57 ± 0.11

temp (°C)	treatment time (h)	30 MPa		50 MPa	
		betanin	isobetanin	betanin	isobetanin
control	0	18.34 ± 0.14	17.04 ± 0.20	18.34 ± 0.14	17.04 ± 0.20
	0.5	17.54 ± 0.15	15.95 ± 0.18	17.51 ± 0.08	16.66 ± 0.08
45	1.5	17.16 ± 0.06	15.32 ± 0.04	17.13 ± 0.08	15.57 ± 0.06
	2.5	17.00 ± 0.09	14.96 ± 0.11	16.92 ± 0.06	15.00 ± 0.13
	0.5	16.47 ± 0.10	14.75 ± 0.15	17.24 ± 0.05	15.55 ± 0.11
55	1.5	14.22 ± 0.11	12.53 ± 0.10	15.10 ± 0.12	12.97 ± 0.07
	2.5	13.11 ± 0.03	11.44 ± 0.04	11.88 ± 0.03	10.30 ± 0.03
	0.25	17.02 ± 0.40	15.36 ± 0.36	16.26 ± 0.06	14.81 ± 0.02
65	0.75	10.96 ± 0.08	9.68 ± 0.06	7.76 ± 0.16	6.71 ± 0.16
	1.3	4.09 ± 0.02	3.34 ± 0.06	3.90 ± 0.02	3.31 ± 0.04

However, there is little information on the degradation characteristics of betalains under HP-CO₂. The objective of this work was to verify as to whether HP-CO₂ combined with thermal processing can result in improved betalain retention as compared to thermal treatments, to investigate the degradation kinetics of betanin and isobetanin for predicting pigment retention under HP-CO₂, and to describe color changes by CIE parameters and characterize degradation products by spectrophotometry and HPLC-MS. Furthermore, a degradation mechanism for betanin and isobetanin under HP-CO₂ also is proposed and discussed.

MATERIALS AND METHODS

Preparation of Red Beet Pigment (RBP) Solution. RBP powder extracted from red beets, purified, and spray-dried with dextrin (the content of betanin and isobetanin was 98% of total pigments, determined by HPLC) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). The major colored components of the powder were betanin and isobetanin, which were 917 ± 5 and 852 ± 7 μg/g, respectively (determined by HPLC, see Determination of Betanin and Isobetanin). A 2% (w/w) red beet pigment solution (RBPS) was prepared with doubly distilled water.

HP-CO₂ Treatment System. The HP-CO₂ system was manufactured by Huali Pump Co., Ltd. (Hangzhou, China). It consisted of a three-plunger type pump, a 1 L stainless steel treatment vessel, a temperature controller and monitor, two pressure gauges (with an accuracy of ±0.01 MPa), a water bath, and a back pressure regulator. The treatment vessel was enveloped by an electrical water bath heating jacket with a thermocouple attached into the top to monitor the temperature inside the vessel. Another thermocouple, connected to the temperature monitor, was placed in the water bath to control the vessel temperature. The temperature inside the treatment vessel was controlled to an accuracy of ±0.5 °C. The maximum operating pressure of the system was 55 MPa.

**HP-CO₂ Treatment of RBPS.** For each treatment, aliquots of 15 mL of RBPS in a 200 mL stainless steel container (60 mm i.d. × 80 mm) were placed in the treatment vessel, which was preheated to a given temperature and then pressurized by CO₂. A series of trials was carried out with varying values of pressure and temperature (based on the experimental conditions in ref *19* with an extension of pressure up to 50 MPa and temperature to 65 °C): 10, 30, and 50 MPa and 45, 55,

and 65 °C. For each pressure, the samples were treated separately for 0, 0.5, 1, 1.5, 2, and 2.5 h (45 and 55 °C) and 0, 0.25, 0.5, 0.75, 1, and 1.3 h (65 °C). At the end of each treatment, the pressure was slowly released over 15 min, and the samples were removed from the treatment vessel and cooled to room temperature in an ice−water bath. The concentrations of betanin and isobetanin were determined by HPLC at 25 °C. CO₂ (≥99.9%) was purchased from Beijing Analytical Apparatus Co. (Beijing, China).

Thermal Treatment of RBPS. To compare HP-CO₂ treatments with conventional thermal ones, aliquots of 15 mL of RBPS in the treatment vessel (the same volume as before) were placed in a water bath set at 45, 55, and 65 °C for the same times as stated previously. After that, the containers were moved to an ice−water bath to the room temperature, and the concentrations of betanin and isobetanin were determined by HPLC at 25 °C.

Color Measurements of RBPS. The spectrum of RBPS was scanned using a UV−vis spectrophotometer (UV757CRT, Lengguang, Shanghai, China) at ambient temperature; the scanning wavelength ranged from 400 to 700 nm in steps of 1 nm. RBPS was also analyzed with a color difference meter (Xingguang Instrument Co., Beijing, China) using the transmission mode. Samples were poured in 5 cm × 3 cm × 1 cm glass cell and measured. CIE L^*, a^*, and b^* parameters were recorded as L^* (lightness), a^* (redness), and b^* (yellowness). The hue angle ($h°$) and chroma (C^*) were calculated as $h° = \arctan(b^*/a^*)$ and $C^* = (a^{*2} + b^{*2})^{0.5}$.

Determination of Betanin and Isobetanin. The concentrations of betanin and isobetanin in RBPS were analyzed by HPLC following the method defined by Schwartz and Von Elbe (*10*), Pourrat et al. (*20*), and Stintzing et al. (*21*) with minor modifications. HPLC analysis was performed on an Agilent 1100 HPLC system with a diode array detector (DAD). Samples were separated on an Agilent Zorbax SB C₁₈ column (250 mm × 4.6 mm i.d., 5 μm) with a gradient elution system. Solvent A was a mixture of 100% methanol and 0.2% (v/v) formic acid (88%) in water with a ratio of 18: 82 (v/v). Solvent B was 100% methanol. At a flow rate of 1.0 mL/min, the first 6 min was performed isocratically with 100% solvent A, followed by a linear gradient from 0 to 7% B in 6 min, then from 7 to 12% in 5 min, then from 12 to 20% B in 4 min, and finally with isocratic 100% B over 9 min. The injection volume was 20 μL, and peaks were detected at 538 nm. Concentrations of betanin and isobetanin were obtained by comparisons with standard lines. HPLC grade methanol was purchased from Merck Chemical Inc. (Darmstadt, Germany). Other chemicals used were of analytical grade.

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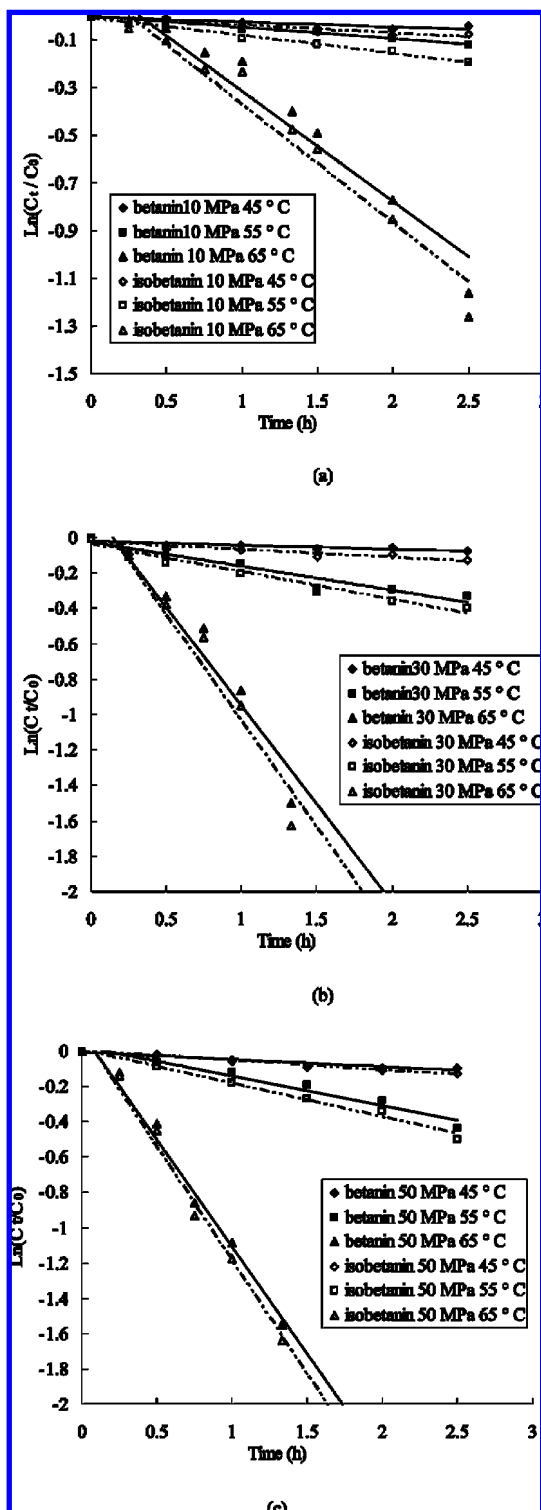
Figure 1. Effect of HP-CO_2 on degradation kinetics of betanin and isobetanin at different pressures and temperatures as compared to traditional thermal treatment.

HPLC-MS Analysis of Pigment Patterns of RBP Treated by HP-CO_2 Combined with Thermal Conditions. Untreated and RBPS treated by combined HP-CO_2 (10−50 MPa) and thermal (55 °C for 2.5 h and 65 °C for 1.3 h) treatments were analyzed by HPLC-MS performed on an Agilent 1100 HPLC-MS system with the same method for HPLC-DAD analysis. The mass spectrometer was equipped with an ESI source operating in the positive ionization mode. Nitrogen was used as the dry gas at a flow rate of 8 L/min and a pressure of 35 psi. The nebulizer temperature was set at 350 °C. Compounds were identified by comparing their protonated molecular ions and derived

daughter ions with those published in the literature (*20, 22–25*). All samples were kept at 4 °C between treatments and analyses to eliminate the degradation effect of room temperature and light.

Data Analysis. The degradation data of betanin and isobetanin were subjected to regression analysis using the following first-order models:

$$\ln\left[\frac{C_t}{C_0}\right] = -kt \qquad (1)$$

where C_t and C_0 are the concentrations of betanin/isobetanin at time t and time 0, and k is the reaction rate constant (h^{-1}).

The Arrhenius equation was applied to estimate the activation energy E_a (kJ/mol) of the degradation reaction

$$\ln\left[\frac{k_1}{k_2}\right] = \frac{E_a}{R}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] \qquad (2)$$

where k_1 and k_2 are the reaction rate constants at temperatures T_1 and T_2, respectively, R is the gas constant (8.3144 J/K mol), and T is the absolute temperature (K).

Statistical Analysis. All treatments were conducted in duplicate, and all measurements were performed in triplicate. Data were subjected to analysis of variance (ANOVA) using the software package SPSS 12.0 for Windows (SPSS Inc., Chicago, IL). Means of degradation data were separated at the 5% significance level using the LSD method.

RESULTS AND DISCUSSION

Thermal/Combined HP-CO_2 and Thermal Degradation Kinetics of Betanin and Isobetanin. Concentrations of betanin and isobetanin in RBPS before and after treatments at different parameters are presented in **Table 1**. As the treatment time extended, the residual contents of betanin and isobetanin in RBPS were remarkably decreased ($p < 0.05$) apart from the treatment at 10 MPa, 45 °C, and 0.5 h where the concentrations of betanin and isobetanin remained largely unchanged ($p > 0.05$). Treatment at 50 MPa, 65 °C, and 1.3 h led to the greatest pigment loss from initial concentrations of 18.34 ± 0.14 and 17.04 ± 0.20 mg/L to only 3.90 ± 0.02 and 3.31 ± 0.04 mg/L for betanin and isobetanin, respectively. As expected, the degradation reaction of the two compounds was obviously accelerated with an elevation of temperature.

From a log linear plot (**Figure 1**) of the relative residual response value [$\ln(C_t/C_0)$] versus treatment time at constant temperature and pressure, it was verified as to whether combined HP-CO_2 and thermal degradation of betanin and isobetanin could be adequately described by a first-order reaction. The kinetic data tabulated in **Table 2** indicated that the degradation reaction of betanin and isobetanin under HP-CO_2 combined with temperature could be described by first-order decays on the basis of regression coefficients >0.81 ($p < 0.05$). As shown in **Figure 1** and **Table 2**, with an elevated temperature, k values for the degradation of betanin and isobetanin increased significantly ($p < 0.05$) at constant pressure. Moreover, the degradation kinetic parameters listed in **Table 2** revealed that the degradation rate of betanin was slightly slower than that of isobetanin at each identical pressure and temperature. This implied that betanin was more stable than isobetanin under HP-CO_2. As compared to treatments at atmospheric pressure and 45 °C, HP-CO_2 treatments led to significant decreases in k values and increases in half-life $t_{1/2}$ values ($p < 0.05$) at the same temperature—the $t_{1/2}$ value was 12.160 and 11.198 h for betanin and isobetanin, respectively, at atmospheric pressure. In contrast, the $t_{1/2}$ value was >16 and 13 h for betanin and isobetanin, respectively, with pressures of 10−50 MPa, indicating that betanin and isobetanin were even more stable under HP-CO_2 than atmospheric pressure at 45 °C. Furthermore, with the elevation of pressure, k values for both pigments dramatically

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Table 2. Degradation Kinetic Parameters of Betanin and Isobetanin

		betanin			isobetanin		
temp (°C)	pressure (MPa)	k (h^{-1})	$t_{1/2}$ (h)	R^2	k (h^{-1})	$t_{1/2}$ (h)	R^2
45	atmospheric pressure	0.057	12.160	0.817	0.062	11.198	0.892
	10	0.022	31.364	0.811	0.034	20.447	0.927
	30	0.025	28.292	0.811	0.045	15.576	0.851
	50	0.027	25.297	0.925	0.053	13.078	0.986
55	atmospheric pressure	0.127	5.475	0.987	0.152	4.554	0.989
	10	0.048	14.321	0.983	0.075	9.267	0.988
	30	0.136	5.108	0.939	0.156	4.435	0.960
	50	0.173	4.018	0.989	0.196	3.536	0.992
65	atmospheric pressure	0.470	1.476	0.997	0.547	1.267	0.997
	10	0.472	1.469	0.939	0.507	1.367	0.949
	30	1.110	0.624	0.939	1.201	0.577	0.943
	50	1.212	0.572	0.986	1.299	0.534	0.988

increased, and $t_{1/2}$ values decreased ($p < 0.05$). However, when the temperature was 55 °C, the degradation parameter values for betanin and isobetanin at 30 and 50 MPa approached those at atmospheric pressure ($p > 0.05$). Meanwhile, when the temperature increased to 65 °C at 10 MPa, the parameters for both pigments were almost the same as those at atmospheric pressure, respectively ($p > 0.05$). Nevertheless, k values for betanin and isobetanin at 30 and 50 MPa were much larger than those at atmospheric pressure, and $t_{1/2}$ values were smaller (p < 0.05). In other words, the influence of HP-CO₂ on the stability of betanin and isobetanin is attributed to both pressure and temperature. This suggests that betanin and isobetanin become less stable at pressures >30 MPa combined with temperatures exceeding 55 °C than atmospheric pressure with accordant temperatures. In conclusion, with HP-CO₂ application in microorganism sterilization or enzyme inactivation for red beet products, the combination of pressure below 30 MPa (if the temperature is >55 °C) or temperature below 55 °C (if the
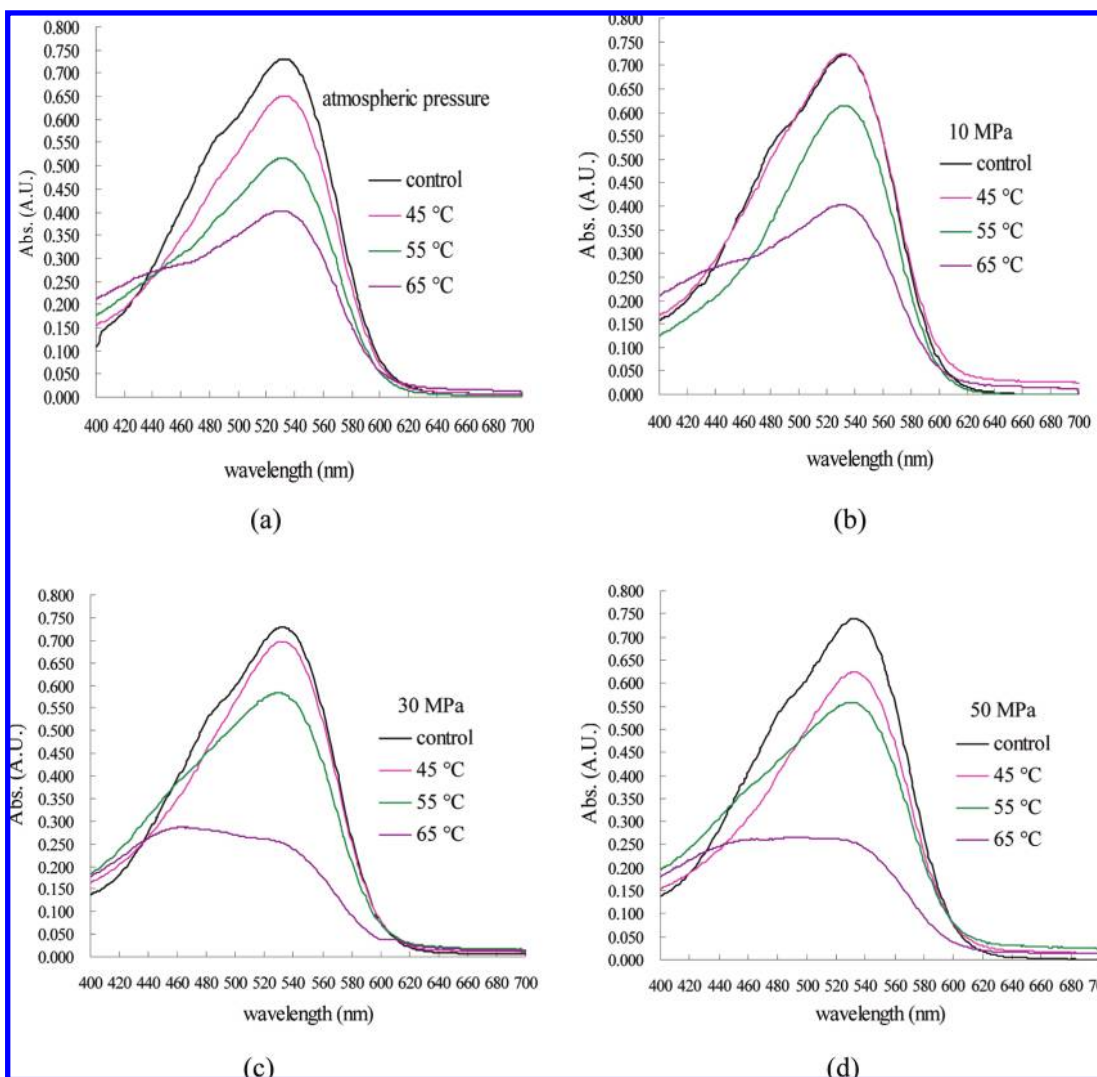
Figure 2. Spectrum of RBPS after HP-CO₂ treatments at different pressures (**a**, atmospheric pressure; **b**, 10 MPa; **c**, 30 MPa; **d**, 50 MPa) and temperatures (treatment time: 2.5 h at 45 and 55 °C and 1.3 h at 65 °C).
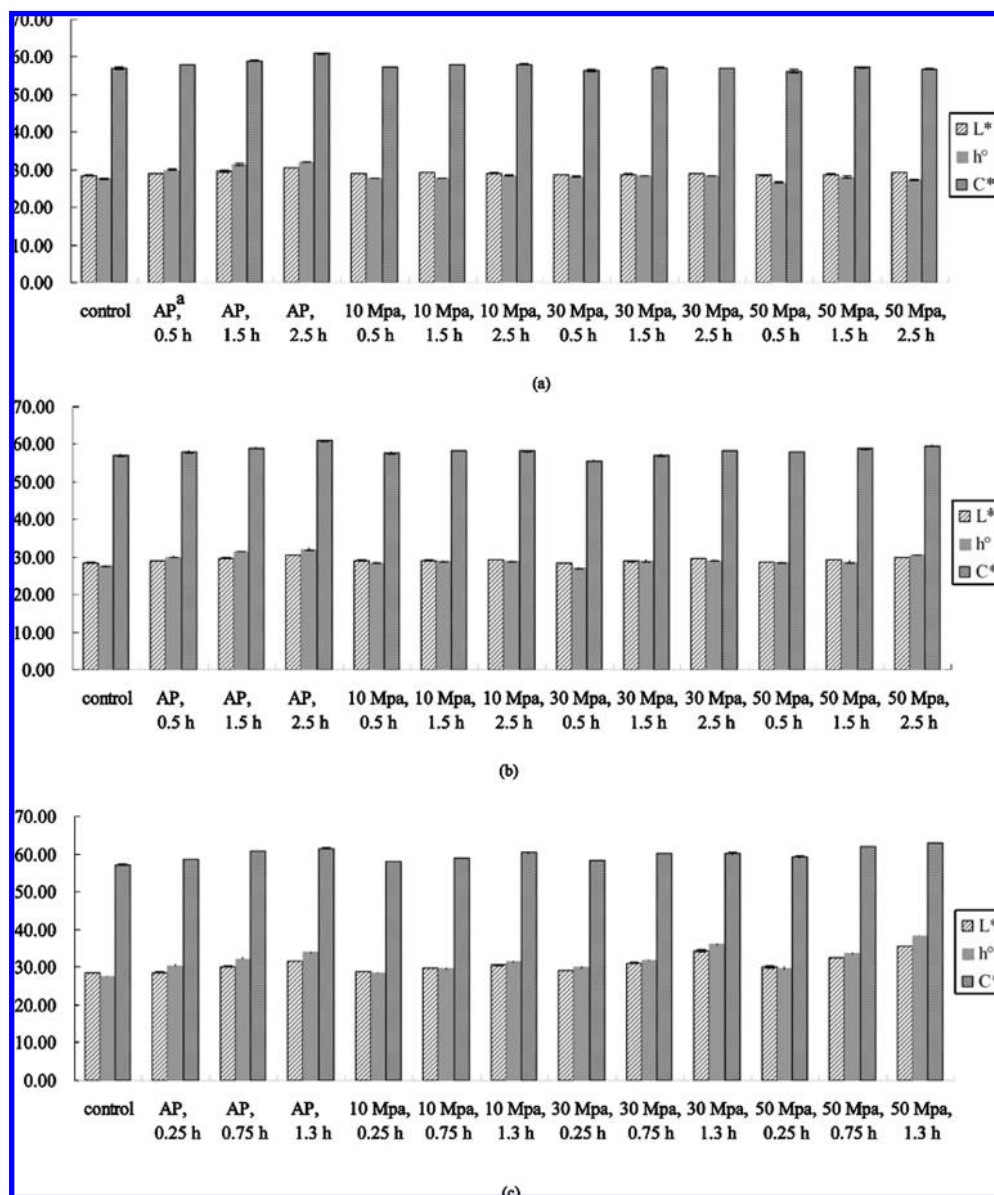
Figure 3. Change in L^*, h°, and C^* values of RBP exposed to HP-CO$_2$ at various conditions (**a**, 45 °C; **b**, 55 °C; **c**, 65 °C).

pressure is >30 MPa) is considered to be effective for a higher retention of betacyanins.

The activation energy (E_a) (energy required for the degradation reaction) is another important parameter for evaluating pigment stability under different treatments. The E_a values for betanin and isobetanin under atmospheric pressure and HP-CO$_2$ were obtained from the Arrhenius equation (eq 2). At atmospheric pressure, E_a values were 94.01 and 97.16 kJ/mol for betanin and isobetanin, respectively, where as compared to the thermal treatment, the degradation reaction under HP-CO$_2$ had considerably higher E_a values: 136.12, 170.25, and 170.83 kJ/mol for betanin and 120.38, 146.89, and 142.69 kJ/mol for isobetanin at pressures of 10, 30, and 50 MPa, respectively. Therefore, this seems to suggest that betanin and isobetanin are more sensitive to heat under atmospheric pressure than HP-CO$_2$, and it also was found that E_a values increased significantly from 10 to 30 MPa ($p < 0.05$) and then remained stagnant between 30 and 50 MPa for betanin and isobetanin ($p > 0.05$). Additionally, the E_a value for isobetanin was higher than that for betanin at atmospheric pressure. In contrast, the E_a value for isobetanin was relatively lower than that for betanin under

HP-CO$_2$. This phenomenon demonstrated that the thermal sensitivities of betanin and isobetanin were changed when exposed to HP-CO$_2$.

Spectra of Betanin and Isobetanin with Thermal/Combined HP-CO$_2$ and Thermal Treatment. The spectra from 400 to 700 nm of RBPS after HP-CO$_2$ treatments at different temperatures are shown in **Figure 2**. The absorbance at 538 nm was reported to be the characteristic peak of betanin and isobetanin (*23*). As the treatment temperature and pressure increased, the absorbance at 538 nm declined. However, the shape of the spectra at different pressures is similar to those under atmospheric pressure except for HP-CO$_2$ treatment at 30−50 MPa and 65 °C for 1.3 h where the maximum absorbance wavelength (λ_{max}) moved to ~445 nm. This might be induced by extra degradation of betanin and isobetanin to some yellow pigments at 50 MPa and 65 °C for 1.3 h.

Color Developments. Color developments of different treatment samples were monitored, and the values are shown in **Figure 3**. With the rise in temperature and pressure and extension of treatment time, C^* and h° values increased significantly ($p < 0.05$), which possibly is attributed to

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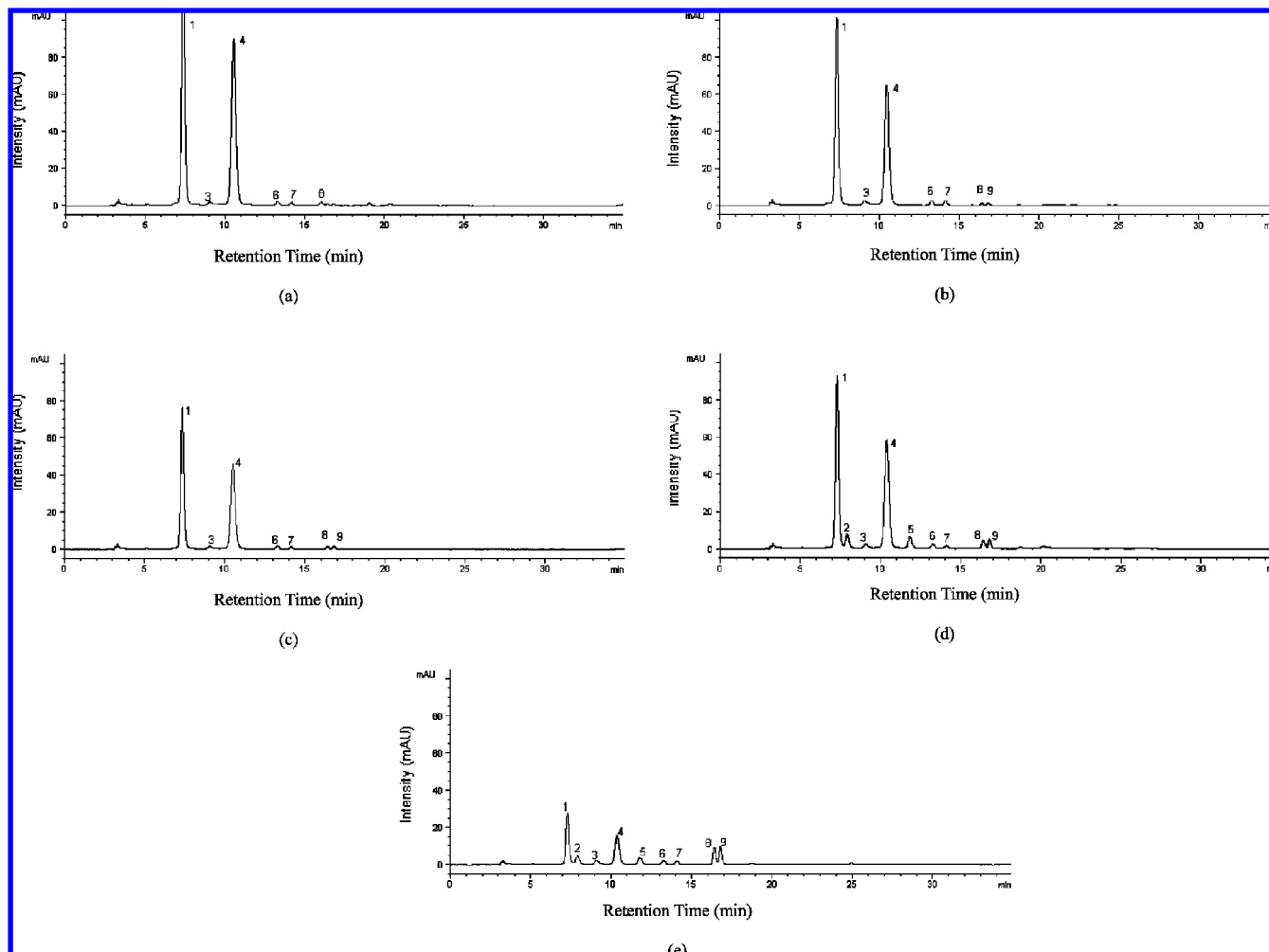
Figure 4. HPLC profiles of RBPS. (**a**, control (untreated sample); **b**, heated for 2.5 h at 55 °C; **c**, heated for 1.3 h at 65 °C; **d**, treated for 2.5 h by HP-CO₂ at 50 MPa and 55 °C; **e**, treated for 1.3 h by HP-CO₂ at 50 MPa and 65 °C).

Table 3. HPLC-DAD and HPLC-MS Data of Betanin and Isobetanin and Their Degradation Products in RBPS

peak number[a]	retention time (min)	HPLC-DAD λ_{max} (nm)	m/z (M + H)$^+$	HPLC-ESI(+)-MS^2 experiment m/z	name/proposal name
1	7.3	538	551	MS^2 [551]:389	betanin
2	7.9	538	551	MS^2 [551]:389	betanin[b]
3	9.1	505	507	MS^2 [507]:345	17-decarboxy-betanin
4	10.4	538	551	MS^2 [551]:389	isobetanin
5	11.8	538	551	MS^2 [551]:389	isobetanin[b]
6	13.3	505	507	MS^2 [507]:345	17-decarboxy-isobetanin
7	14.1	533	507	MS^2 [507]:345	12-decarboxy-betanin
8	16.4	505	507	MS^2 [507]:345	decarboxy-betanin[b]
9	16.8	538	507	MS^2 [507]:345	15-decarboxy-betanin

[a] Peak numbers correspond to **Figure 4**. [b] Proposal name and structures were not determined.

betacyanin loss and creation of orange−red/yellow pigments. As shown in **Figure 2**, a higher temperature and pressure as well as longer exposure time under HP-CO₂ caused a hypsochromic shift of the absorption maxima, resulting in the color changing from purple to orange−red. Moreover, it is known that with an increasing betacyanin concentration, the hue angle shifts from yellow−red to purple (*26*). On the contrary, with decreasing betacyanin content, the hue angle shifts from purple to yellow−red for betanin and isobetanin when exposed to combined HP-CO₂ and thermal treatments. Therefore, the increment of the $h°$ value probably was induced by the larger $b*$ value, which was due to the orange−red degradation products. These consistent results could be disclosed by HPLC-MS analysis. Betanin and isobetanin were degraded to yellow−red

compounds, which resulted in an increase in the $b*$ value and a decrease in the $a*$ value, and then the $h°$ and $C*$ values increased. Besides, higher $C*$ values reflected an increasing color purity through HP-CO₂ combined with thermal treatment.

Pigment Pattern of Betanin and Isobetanin with Thermal/ Combined HP-CO₂ and Thermal Treatment (Peak and Compound Numbers Correspond to Figure 4). As shown in **Figure 4a−e**, two main peaks were identified to be betanin (**1**) and isobetanin (**4**) according to retention times, absorption maxima, protonated molecular ions (m/z 551), and fragments [m/z 389 = 551 − 162 (Glc)] yielded by MS^2 experiments (*22*). As compared to untreated RBPS, thermal treatments at 55 °C (for 2.5 h) and 65 °C (for 1.3 h) could only result in the

Table 4. Stability of Pigments with Different Treatments[a]

compound[b]	control (untreated)	AP[c], 55 °C, 2.5 h	AP, 65 °C, 1.3 h	10 MPa, 55 °C, 2.5 h	10 MPa, 65 °C, 1.3 h	30 MPa, 55 °C, 2.5 h	30 MPa, 65 °C, 1.3 h	50 MPa, 55 °C, 2.5 h	50 MPa, 65 °C, 1.3 h
1	51.16	51.22	52.37	47.21	42.53	47.13	35.41	46.40	34.05
2	0.00	0.00	0.00	1.43	5.54	3.85	6.03	4.15	6.64
3	0.64	1.58	1.40	1.30	1.35	1.06	2.86	1.19	3.11
4	46.18	43.52	42.39	44.78	37.52	39.83	26.55	38.71	25.11
5	0.00	0.00	0.00	1.82	4.56	3.13	5.87	3.64	6.05
6	0.83	1.39	1.24	0.90	1.12	1.03	2.26	1.12	2.83
7	0.49	1.27	0.73	0.53	1.07	0.66	2.06	0.75	2.40
8	0.70	0.54	0.94	0.96	2.97	1.44	9.12	1.97	9.67
9	0.00	0.49	0.92	1.07	3.34	1.87	9.84	2.08	10.15

[a] Nine compounds were quantified by HPLC with peak area (%). [b] Compound numbers correspond to **Figure 4**. [c] AP: atmospheric pressure.

generation of **9**, while **2**, **5**, and **9** were induced by HP-CO$_2$ at 50 MPa (at 65 °C for 1.3 h), and each compound was primarily identified by HPLC-MS analysis. As represented in **Table 3**, **1**, **2**, **4**, and **5** had the same *m/z*, and they might be isomeric compounds since there are two chiral carbon atoms in the molecular structure of beanin; however, **2** and **5** were identified as undescribed structures.

In accordance with thermal treatment in previous findings, HP-CO$_2$ combined with thermal treatment led to a decarboxylated reaction. Peaks 3 and 6−9 with typical fragmentation data in the MS^2 analyses [(*m/z* 345 = 507 − 162 (Glc)] were regarded as decarboxy-betanin with different decarboxylation sites except for **8** (not determined). Moreover, the loss of a carboxy moiety was reported to reduce polarity, leading to longer retention relative to the respective nondecarboxylated structure (*21, 23−25*). On the basis of retention time, structure, and absorption maxima (505 nm, hypsochromic shift of 33 nm relative to betanin and isobetanin), peaks 3 and 6 were identified as 17-decarboxy-betanin and 17-decarboxy-isobetanin, respectively. Similarly, peak 9 was identified as 15-decarboxy-betanin because of the identical absorption maxima with betanin, loss of the chiral center, and absence of a second compound (*22*). By analogy, peak 7 could be assumed to be 2-decarboxy-betanin with a slight hypsochromic shift of 5nm relative to betanin (*25*).

From the chromatograms shown in **Figure 4**, the peak area (%) of each compound is listed and illustrated in **Table 4**. As compared to thermal treatments, HP-CO$_2$ treatments induced high levels of **3** and **6−9** and new isomeric compounds of betanin. In addition, a higher pressure and temperature induced higher levels of **2**, **3**, **5−7**, and **9**. HP-CO$_2$ treatments with higher pressure and temperature accelerate the isomerization and decarboxy reactions of betanin and isobetanin. Therefore, the major degradation products of betanin and isobetanin treated by HP-CO$_2$ and thermal treatments were decarboxy-betanin isomers with different decarboxylation sites (C-15, -17, -2, etc.) and two new isomeric compounds of betanin that were particularly caused by HP-CO$_2$ treatments.

In conclusion, the stability of betanin and isobetanin under HP-CO$_2$ was affected by both pressure and temperature, and betanin was even more stable than isobetanin. Treatments with a pressure above 30 MPa and temperature >55 °C led to a more rapid loss of betanin and isobetanin and a color change from violet to orange−red. At the same treatment temperature and time, more products were generated, and decarboxylation was accelerated by a higher pressure than thermal treatment alone. Therefore, it is necessary to explore proper pressure and temperature ranges for HP-CO$_2$ treatments to achieve not only better pigment retention but also effective microorganism sterilization and enzyme inactivation. However, the safety

evaluation of degradation products as well as the degradation mechanism of betanin and isobetanin under HP-CO$_2$ combined with thermal treatment also need further investigation and should be discussed in a future study.

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