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A Comparison of Two Determination Methods for Studying Degradation Kinetics of the Major Anthocyanins from Blood Orange

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The thermal degradation of cyanidin 3-(6″-malonyl) glucoside and cyanidin 3-glucoside, which are the two major anthocyanins in blood orange juice, have been investigated using the pH-differential method and RP-HPLC method, respectively. Similar results could be obtained when the degradation kinetics of cyanidin 3-glucoside were studied by either the HPLC method or the pH-differential method. However, contrary results were obtained in the case of cyanidin 3-(6″-malonyl) glucoside. The HPLC analysis indicated that cyanidin 3-(6″-malonyl) glucoside was labile to eliminate a malonyl moiety to form cyanidin 3-glucoside before disappearing during thermal treatment and showed a lower thermal stability than cyanidin 3-glucoside, whereas the result obtained by the pH-differential method actually reflected the content of both cyanidin 3-(6″-malonyl) glucoside and its degradation intermediate. This caused an overestimate of the stability of cyanidin 3-(6″-malonyl) glucoside.

KEYWORDS: Blood orange; anthocyanins; degradation kinetics; RP-HPLC; pH-differential method

INTRODUCTION

The blood orange, a variety of *Citrus sinensis*, has a smooth peel, a soft pulp, and exceptional color. This cultivar is characterized by its unique flesh and rind color due to the phenolic pigment belonging to the anthocyanin class. The low stability of blood orange anthocyanins is a problem during the thermal processing of juice. Preventing anthocyanin degradation is an important aspect that can benefit both processors and consumers.

Until now, many researchers have concentrated on the stability of anthocyanins (*1*−*8*). The degradation of anthocyanins in blood orange juice was investigated during storage by Krifi et al. (*9*). Kirca and Cemeroglu (*10*) further studied the degradation kinetics of blood orange anthocyanins in both juice and concentrates during heating and storage at various temperatures. However, the complexity of blood orange juice anthocyanins used in the previous studies did not allow one to determine the respective kinds of anthocyanin degradation. To gain further understanding of the thermal degradation of anthocyanins in blood orange juice, it is necessary to investigate the degradation kinetics of each major anthocyanin in this juice.

Previous studies have shown that cyanidin 3-glucoside and cyanidin 3-(6″-malonyl) glucoside were the two major pigments in blood orange juice (*11*−*15*). As far as we know, there is no information available in the literature about the thermal degradation kinetics of these two anthocyanins. Moreover, most studies about the thermal degradation kinetics of anthocyanins were carried out by a spectrophotometric method, which could not accurately reflect the fact of the compound's degradation, and the influence of different determination methods on kinetics data was ambiguous. Therefore, the objective of this study was to investigate the thermal degradation kinetics of purified cyanidin 3-glucoside and cyanidin 3-(6″-malonyl) glucoside by both the pH-differential method and the RP-HPLC method, and to compare the difference between these two methods.

MATERIALS AND METHODS

Materials. Blood oranges (Tarocco) were obtained from a commercial orchard in Zigui, Hubei Province, China. The fruits were squeezed using a centrifugal extractor (2000JP, NantongJincheng, China) and the centrifuged juice (4500 rpm) stored at −18 °C before extraction and purification. The yield of blood orange juice was 310 mL per 1000 g of fruit, and the total soluble solids and pH of the juice were 12.5 °Brix and 3.45, respectively.

Extraction and Purification of Anthocyanins. The centrifuged juice was loaded onto an open column (5 cm × 50 cm I.D.) packed with NKA-9 resin (macroporous adsorption resin; matrix structure, cross-linked-polystyrene; polar; 0.3−1.25 mm particle, Chemical Plant of NanKai University), and then washed with distilled water to remove sugars, organic acids, proteins, and salts. Anthocyanins and other flavonoids were retained by the resin. For elution, a mixture of ethanol/water/hydrochloric

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Table 1. λ_{max} and Molar Extinction Coefficients (ε) of Cyanidin 3-Glucoside and Cyanidin 3-(6″-malonyl) Glucoside

pigment	λ_{max} (nm)	ε (L mol^{-1} cm^{-1})
cyanidin 3-glucoside	510[a]	20020[a]
	529[b]	19180[b]
cyanidin 3-(6″-malonyl) glucoside	510[a]	21600[a]
	529[b]	22910[b]

[a] In pH 1.0 buffer. [b] In methnol/HCl (99.99:0.01, v/v).

acid (50:50:0.1, v/v/v) was used. The fractions whose maximal absorbance was at 510 nm were concentrated to a small volume at 40 °C by a rotary evaporator (N-1000, EYELA, Japan). Then, the concentrated fractions were extracted by ethyl acetate (3 × 60 mL). The anthocyanins stayed in aqueous phase, while parts of other flavonoids were in the ethyl acetate phase. The aqueous phase was concentrated under reduced pressure at 40 °C to remove the trace ethyl acetate in aqueous phase.

The above concentrated anthocyanins were purified according to the method described previously (*16*) with some modifications. The anthocyanin extracts were loaded onto a Toyopearl HW-40S column (1.6 cm × 20 cm I.D.) and eluted with 2% formic acid-acidified aqueous methanol (methanol/water, 35:65, v/v) at 0.8 mL/min. Fractions based on the absorbance values at 510 nm were collected using a fraction collector (5 min/tube). Four fractions were obtained from the Toyopearl column during 5 h. According to HPLC analysis, fractions 2 and 3 each contained one of the major anthocyanins in blood orange juice. Fractions 2 and 3 were lyophilized to a dry powder, and the yieldswere 14.4 mg and 8.5 mg/L of orange juice, respectively. Fractions 1 and 4 contained a small quantity of anthocyanins and were not investigated in the following degradation experiments.

Determination of the Purified Anthocyanins by HPLC-ESI/MS Analysis. For structure determination, samples were subjected to mass spectrometry analysis as described previously (*16*) with some modifications. A ZORBAX Eclipse XDB-C_{18} (4.6 mm × 150 mm I.D., Agilent) was used, and 2 μL of sample solution was injected. UV−visible detection was performed at 510 nm. Flow rate was 0.2 mL/min. The elution conditions were as follows: solvent A was water/formic acid (98:2, v/v); solvent B was water/formic acid/acetonitrile (40:2:58, v/v). The percentage of solvent B was increased linearly, from 10 to 35% in 25 min and then to 100% in 10 min. The column was then reconditioned with the initial condition for 10 min. The eluent was subsequently detected by ESI-MS with a positive ion mode. MS conditions were as follows: nebulizer gas (N_2) flow, 4.5 L/min; probe voltage, 4.5 kV; deflector voltage, 40 and 55 V; *m*/*z* acquisition from 100 to 1000 amu.

Molar Extinction Coefficient. The molar extinction coefficient (ε) was determined using solutions of purified anthocyanins over a concentration range between 0.01 and 0.08 mM in pH 1.0 buffer (0.2 N KCl) or in methanol/HCl (99.99:0.01, v/v). The coefficient was calculated from the slope of the graphics of color intensity at the λ_{max} of the anthocyanin, as a function of the concentration of the pigment. Spectral absorbance curves were recorded for all of these solutions from 200 to 600 nm using a 1 cm × 1 cm quartz glass cell in a UV-1700 Shimadzu spectrophotometer.

Thermal Treatment. Thermal degradation kinetics of purified anthocyanins were studied by isothermal heating at different temperatures (70, 80, and 90 °C) in model systems using citrate-phosphate as a pH 3.5 buffer (35.9 mL of 0.1 M citric acid, 14.1 mL of 0.2 M Na_2HPO_4, diluted to 100 mL with distilled water). Purified freeze-dried anthocyanins were dissolved in the buffer to give a final concentration of 0.1 mmol/L. The model systems were periodically agitated to ensure uniform temperature and well capped to avoid evaporation. At regular time intervals, 25 mL samples were taken into tubes and rapidly cooled by plunging into an ice water bath, and the anthocyanin content was determined after cooling for 1 h in the absence of light.

Measurement of Anthocyanins. *pH-Differential Method.* The concentration of anthocyanins was determined by a pH-differential method as described previously (*17*, *18*). It was calculated by the following equation:

$$C_{mg/L} = \frac{(A_{pH1.0} - A_{pH4.5}) \times 484.82 \times 1000}{24825} \times DF$$

The term in parentheses is the difference of absorbance at 510 nm between pH 1.0 and pH 4.5 solutions, 484.82 is the molecular mass of cyanidin 3-glucoside chloride, 24825 is its molar absorptivity at 510 nm in the pH 1.0 solution, and DF is the dilution factor.

RP-HPLC Method. Concentration of anthocyanins was also determined by an RP-HPLC analysis method. RP-HPLC analysis was performed on a waters liquid chromatograph, and detection was carried out using a photodiode array detector. The column used was a Hypersil ODS column (250 mm × 4.6 mm I.D., 5 μm particle size, Thermo). The mobile phases were the same as those of the HPLC-ESI/MS analysis. A 10 μL sample solution was injected. The elution conditions were changed as follows: a linear gradient from 12 to 30% B for 25 min, a linear gradient from 30 to 100% B for 10 min, at a flow rate of 1 mL/min. The column was then reequilibrated with 12% B for 10 min before the next injection. The absorbance of the eluate was monitored at 510 nm. The anthocyanin concentration was calculated by peak area.

Statistical Analysis. For all degradation kinetics experiments in the study, each sample was assayed in triplicate. Means were compared using Tukey's test with a significance level at $P < 0.05$.

RESULTS AND DISCUSSION

Purification and Identification of Cyanidin 3-glucoside and Cyanidin 3-(6″-malonyl) Glucoside from Blood Orange Juice. The two major anthocyanins isolated from blood orange juice were identified by HPLC-ESI-MS. ESI/MS showed that one anthocyanin has a *m*/*z* value of 449 corresponding to $[M]^+$. With higher orifice voltage, a characteristic fragmentation $[M\text{-}162]^+$ at 287 corresponded to the cleavage of a hexosyl moiety from the glycosylated cyanidin. In addition, the UV−visible studies showed that it presented the same spectrum as cyanidin 3-glucoside (*19*). For another anthocyanin, the molecular ion peak was detected at 535 *m*/*z*, 87 amu more than the *m*/*z* value of the first one, which corresponded to a malonyl moiety. The MS data and UV−visible spectrum of this anthocyanins agreed with those reported for cyanidin 3-(6″-malonyl) glucoside (*12*). These data confirmed the anthocyanins purified by Toyopearl HW-40S column from blood orange juice were cyanidin 3- glucoside and cyanidin 3-(6″-malonyl) glucoside.

The purity of the isolated anthocyanins was checked by HPLC analysis, and the absence of additional peaks in the HPLC chromatograms recorded at 510, 350, 310, and 280 nm indicated high sample purity. The molar extinction coefficients (ε) were also employed to measure the purity of anthocyanin samples and are shown in **Table 1**. The ε values of cyanidin 3-glucoside were consistent with the values found in the literature (*20*−*22*). To our best knowledge, no data are available concerning the molar extinction coefficient of cyanidin 3-(6″-malonyl) gluco-

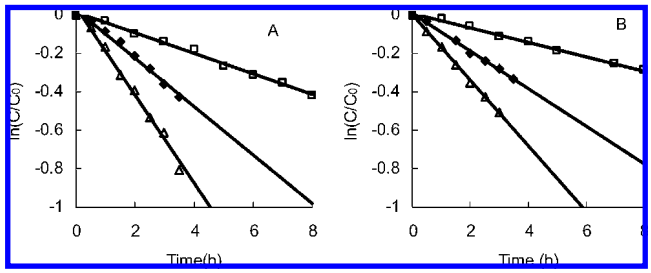

Figure 1. Degradation of cyanidin 3-glucoside (**A**) and cyanidin 3-(6″-malonyl) glucoside (**B**) during thermal treatment determined by the pH-differential method. □, 70 °C; ◆, 80 °C; Δ, 90 °C.

Table 2. Thermal Degradation Parameters of Anthocyanins Extracted from Blood Orange Determined by the pH-Differential Method

anthocyanin	temperature (°C)	*k* (1/h)[a]	$t_{1/2}$ (h)[a]	*Ea* (kJ/mol)[a]
cyanidin 3-glucoside	70	0.0532 (0.9952)	13.03	
	80	0.1257 (0.9894)	5.51	75.4 (0.9922)
	90	0.2278 (0.9892)	3.04	
cyanidin 3-(6″-malonyl) glucoside	70	0.0372 (0.9956)	18.63	
	80	0.0975 (0.9965)	7.1	79.5 (0.9829)
	90	0.1722 (0.9990)	4.02	

[a] Coefficient of determination (R^2) is shown in parentheses. *k*: the first order reaction rate constants. $t_{1/2}$: half-life. *Ea*: the activation energy.

side. However, Giusti et al. (*21*) suggested that acylation with malonic acid did not affect λ_{max} and showed little effect on color characteristics. Our results confirmed their conclusion.

Consequently, the purified anthocyanins were used to investigate the degradation kinetics of cyanidin 3-glucoside and cyanidin 3-(6″-malonyl) glucoside.

Anthocyanin Degradation Kinetics Determined by the pH-Differential Method. According to the result determined by the pH-differential method, the logarithm of the anthocyanin contents (ln C/C_0) was plotted versus time (*t*) (**Figure 1**). The linear relationship indicated that the thermal degradation of cyanidin 3-glucoside and cyanidin 3-(6″-malonyl) glucoside followed first order reaction kinetics.

The kinetic parameters calculated by the first order reaction kinetics model and Arrhenius model are shown in **Table 2**. The *Ea* values for cyanidin 3-glucoside and cyanidin 3-(6″-malonyl) glucoside were 75.4 and 79.5 kJ/mol, respectively. These *Ea* values were similar to the *Ea* values for blood orange juice (*10*).

The *k* values showed that the thermal stability of cyanidin 3-glucoside and cyanidin 3-(6″-malonyl) glucoside decreased with increasing temperature (**Table 2**). At the same temperature, the $t_{1/2}$ value for cyanidin 3-glucoside was lower than that for cyanidin 3-(6″-malonyl) glucoside (**Table 2**), indicating that cyanidin 3-glucoside was less stable than cyanidin 3-(6″-malonyl) glucoside. This result corresponded to the point of view that acylation increased the stability of anthocyanins, which was supported by many researchers (*23*−*27*). In their opinion, this result was probably due to the high steric-hindrance effects caused by intramolecular copigmentation. The high steric-hindrance effects probably have protected the acylated anthocyanin from hydration, and water was incapable of attacking the aglycone, resulting in a stability of the acylated anthocyanin.

Anthocyanin Degradation Kinetics Determined by the RP-HPLC Method. In order to further understand the degradation of those two anthocyanins and make a comparison with the spectrophotometric method, the RP-HPLC method was employed in our study.

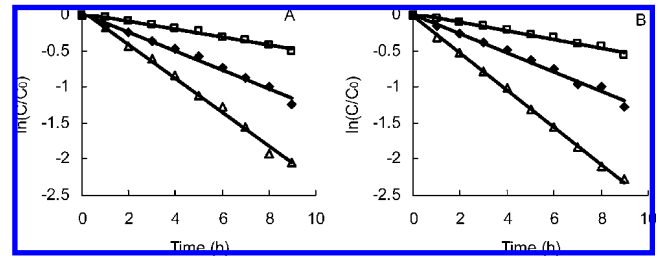

Figure 2. Degradation of cyanidin 3-glucoside (**A**) and cyanidin 3-(6″-malonyl) glucoside (**B**) during thermal treatment determined by the HPLC method. □, 70 °C; ◆, 80 °C; Δ, 90 °C.

Table 3. Thermal Degradation Parameters of Anthocyanins Extracted from Blood Orange Determined by the HPLC Method

anthocyanin	temperature (°C)	*k* (1/h)[a]	$t_{1/2}$ (h)[a]	*Ea* (kJ/mol)[a]
cyanidin 3-glucoside	70	0.0552(0.9902)	12.74	
	80	0.1299 (0.9888)	5.33	74.6 (0.9914)
	90	0.2327 (0.9943)	2.99	
cyanidin 3-(6″-malonyl) glucoside	70	0.0589 (0.9841)	11.77	
	80	0.1335 (0.9899)	5.19	75.8 (0.9973)
	90	0.2545 (0.9984)	2.75	

[a] Coefficient of determination (R^2) is shown in parentheses. *k*: the first order reaction rate constants. $t_{1/2}$: half-life. *Ea*: the activation energy.

According to the result determined by the HPLC method, the degradation of anthocyanins followed first order reaction kinetics (**Figure 2**). In the case of cyanidin 3-glucoside thermal degradation, we found this anthocyanin gradually degraded to colorless compounds. Moreover, the *k* values obtained by RP-HPLC analysis (**Table 3**) were similar to the result obtained by the pH-differential method, confirming the result obtained by Rapisarda et al. (*18*) who reported that the result obtained by the HPLC method consisted of the result obtained by the pH-differential method during the quantitative analysis of anthocyanins in blood orange juices.

However, an interesting result was obtained when using the HPLC method to analyze the thermal degradation of cyanidin 3-(6″-malonyl) glucoside. This acylated anthocyanin eliminated a malonyl moiety to form cyanidin 3-glucoside during thermal treatment (**Figure 3**).

In the degradation system of cyanidin 3-(6″-malonyl) glucoside, we could see that the concentration of cyanidin 3-(6″-malonyl) glucoside decreased obviously in the first 4 h, while that of cyanidin 3-glucoside increased gradually in the degradation system of cyanidin 3-(6″-malonyl) glucoside (**Figure 4**). This result suggested that the formation rate of cyanidin 3-glucoside was faster than the degradation rate of cyanidin 3-glucoside during this period. After 4 h, there was no significant change ($P > 0.05$) in the concentration of cyanidin 3-glucoside (**Figure 4**). This result suggested that the reaction had reached equilibrium and that the degradation rate of cyanidin 3-glucoside was equal to its formation rate. Then, after 7 h of degradation, the concentration of cyanidin 3-glucoside decreased gradually in the system (**Figure 4**). This could be explained by the fact that the formation rate of cyanidin 3-glucoside slowed down with decreasing concentration of cyanidin 3-(6″-malonyl) glucoside. These results also indicated that the formation rate of cyanidin 3-glucoside was related to the concentration of cyanidin 3-(6″-malonyl) glucoside.

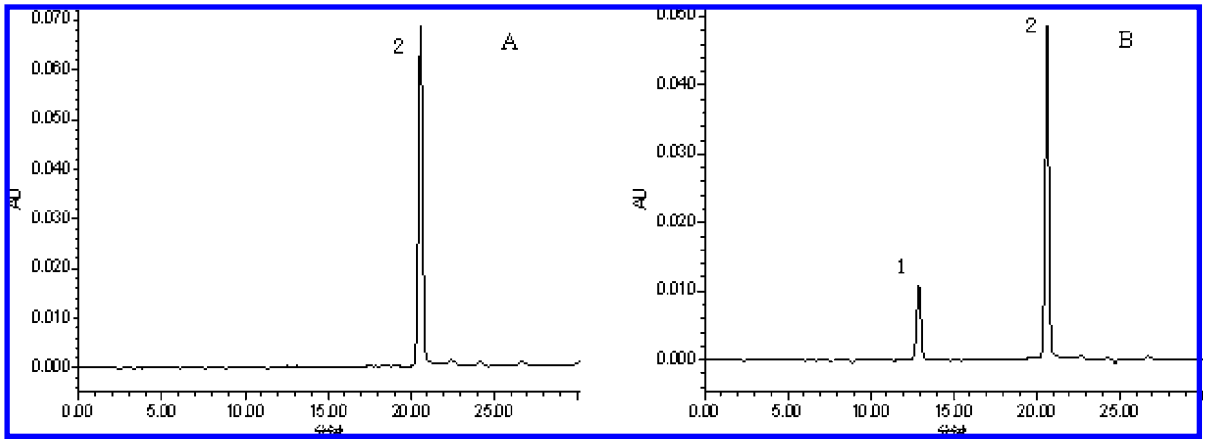

Figure 3. HPLC chromatograms of cyanidin 3-(6″-malonyl) glucoside during thermal treatment, detected at 510 nm. (**A**) No treatment; (**B**) treatment for 1 h.

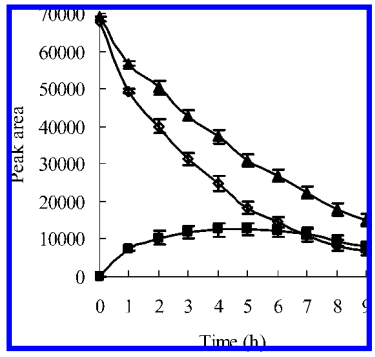

Figure 4. Changes in the contents of total anthocyanins (▲), cyanidin 3-(6″-malonyl) glucoside (◊), and cyanidin 3-glucoside (■) during thermal degradation of cyanidin 3-(6″-malonyl) glucoside at 90 °C.

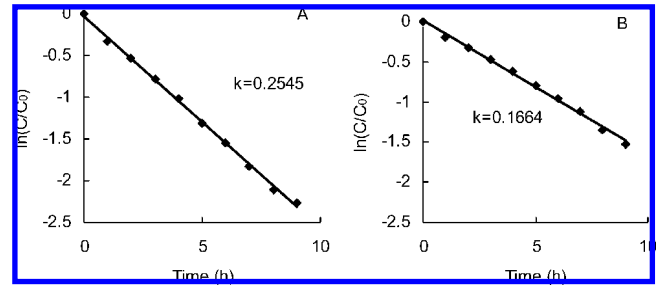

Figure 5. Degradation rate (*k* value) of cyanidin 3-(6″-malonyl) glucoside (**A**) and total anthocyanins (**B**) during the thermal degradation of cyanidin 3-(6″-malonyl) glucoside at 90 °C determined by the RP-HPLC method.

According to the result obtained by the RP-HPLC method (**Table 3**), we observed that in the systems of cyanidin 3-(6″-malonyl) glucoside and cyanidin 3-glucoside, *k* values of the former were higher than that of the latter, indicating that stability of cyanidin 3-(6″-malonyl) glucoside was lower than that of cyanidin 3-glucoside. This result was opposite to the result obtained by the pH-differential method. The contrary results could be explained by the following reason: cyanidin 3-(6″-malonyl) glucoside would degrade to cyanidin 3-glucoside before disappearing. Using the pH-differential method could only determine the total anthocyanins, which contained both cyanidin 3-(6″-malonyl) glucoside and its intermediate cyanidin 3-glucoside, causing the determined concentration of cyanidin 3-(6″-malonyl) glucoside to be higher than its actual concentration. Therefore, the result obtained by the pH-differential method actually reflected the color stability for the system of cyanidin 3-(6″-malonyl) glucoside, and they could not reflect the degradation of cyanidin 3-(6″-malonyl) glucoside itself. This caused an overestimate of the stability of cyanidin 3-(6″-malonyl) glucoside.

Taking into consideration that the previous intramolecular copigmentation studies suggested that acylation increased the stability of anthocyanins involved with anthocyanins acylated with phenolic acids (*23*−*27*), we proposed that anthocyanins acylated with malonic acid might be more labile to eliminate their acidic moiety and show a lower stability than those acylated with phenolic acids. Giusti et al. (*26*) and Rodriguez-Saona et al. (*28*) reported a similar result, which suggested that the malonic acid acylation was less resistant than cinnamic acid acylation to acid hydrolysis.

In addition, from the results discussed above, we could know that the degradation rate of cyanidin 3-(6″-malonyl) glucoside was faster than that of cyanidin 3-glucoside. According to this result and, taking into consideration the theory of multiple reaction, we could draw a theoretical conclusion that the degradation rate of total anthocyanins in the system of cyanidin 3-(6″-malonyl) glucoside should be similar to that of cyanidin 3-glucoside. However, the experimental result presented in this study showed that the degradation rate of total anthocyanins was much slower than either that of cyanidin 3-(6″-malonyl) glucoside itself or that of cyanidin 3-glucoside alone (**Figure 5**). This contrary result indicated that the degradation rate of cyanidin 3-glucoside was different in the two degradation systems of anthocyanins and that the degradation rate of cyanidin 3-glucoside in the degradation system of cyanidin 3-(6″-malonyl) glucoside was much slower than that in the degradation system of cyanidin 3-glucoside alone. This might be due to the fact that the degradation products of cyanidin 3-(6″-malonyl) glucoside could protect cyanidin 3-glucoside from further degradation.

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