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Thermal degradation of anthocyanins and its impact on *in vitro* antioxidant capacity of downy rose-myrtle juice

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Abstract

Downy rose-myrtle, a well-known edible and medicinal plant, has long been used as a folk remedy for various diseases, is a wild berry mainly distributed in Southeast Asia. Anthocyanins in downy rose-myrtle are of great interest to researchers; and commercial entities due to many health benefits of these compounds. Recently, renewed interest has grown in the stability of anthocyanins during the various processing and extraction methods. Anthocyanins easily degrade and form colorless or unacceptable brown-colored compounds during thermal process. In this study, the stability of anthocyanins in downy rose-myrtle juice was investigated undergoing the influenced of temperature (60, 70 and 80°C) at pH 2-4. The impact of various parameters (temperature and heating time) on the antioxidant activity of downy rose-myrtle juice was also evaluated. Additionally, correlation between the total anthocyanin content and total antioxidant activity of the juice based on thermal treatment using ABTS assay was investigated. The results showed that the effect of temperature and pH on anthocyanins of downy rose-myrtle juice was pronounced, and their contents decreased with the increase of the temperature and pH value. During thermal process, total antioxidant activity of downy rose-myrtle juice decreased and similar trends in anthocyanin content were observed. Data analysis showed first-order kinetics for the evolution of anthocyanin content. According to the Arrhenius model, the calculated values of activation energies (Ea) for pH 2-4 were 63.2, 46.1 and 45.0 kJ/mol, respectively. The total antioxidant activity of downy rose-myrtle juice has positive correlation with the total anthocyanins content in it. The stability of anthocyanins in downy rose-myrtle juice may be improved to some extent by an appropriate thermal process.

***Key words*:** Anthocyanins, downy rose-myrtle, degradation kinetics, antioxidant activity, stability.

Introduction

Oxidative damage in the human body plays an important causative role in disease initiation and progression [1]. Natural antioxidants from fruits and vegetables provide a measure of protection that slows the process of oxidative damage [2-4]. Recent studies have shown that many flavonoids and polysaccharides contribute significantly to the total antioxidant activity of many natural plants [5-8]. Anthocyanins are the major subtypes of flavonoids, which are responsible for the attractive colors of many flowers, fruits, vegetables and their derived products [9, 10]. Today, interest in anthocyanin pigments is intensified because of their possible health benefits as dietary antioxidants. Apart from imparting bright color to plant foods, anthocyanins are known to have various health-promoting properties, such as antioxidant, anti-inflammatory and anti-diabetic activities [11-13]. In addition, anthocyanins have little or no known toxicity making them particularly attractive as natural substitutes for synthetic pigments and dietary supplements [14].

Downy rose-myrtle (*Rhodomyrtus tomentosa* (Ait.) Hassk) is a wild berry mainly distributed in Southeast Asia, especially in the southern parts of China, Japan and Thailand [15]. Apart from its appealing flavor, downy rose-myrtle has been recognized as an excellent source of anthocyanins, with the anthocyanin content of its skin being approximately 4.358 g/kg dry weight, indicating that the fruit has great potential as an ingredient for functional beverages [16]. However, anthocyanins degrade easily and form colorless or unacceptable brown-colored compounds that are undesirable in products such as fruit juices, because consumers perceive the color change as an indication of inferior quality [17]. Several factors affect anthocyanin stability, including pH, temperature, light, oxygen, enzymes, ascorbic acid, sugars, sulfur dioxide and metal ions [18, 19]. Thermal treatment (e.g., pasteurization, concentration), which is an extremely common process, has a remarkable influence on the stability of anthocyanins. Hence, there have been many studies on the thermal degradation of anthocyanins, including reports focused on juice of blueberry [20], strawberry [21] and blood orange [22]. Yet, no information is available on the combined effect of temperature, time and pH on the anthocyanin stability and antioxidant activity of downy rose-myrtle juice.

This study investigated the combined effect of temperature/pH/time on anthocyanin stability in downy rose-myrtle juice by using a kinetic reaction model to predict the quality changes that occur during thermal processing. Additionally, ABTS assay was applied to determine the correlation between total anthocyanin content and total antioxidant activity of the juice. It is expected that the present study will increase the understanding of the thermal degradation of anthocyanins in downy rose-myrtle juice.

Materials and Methods

Sample preparation: Fresh downy rose-myrtle fruits were obtained from a local market (Shaoguan, Guangdong Province, China) in September 2011, and frozen at -20°C until processing. Pectolyase Y-23, which has a pectinase activity of 10 U/mg, was purchased from Wako Pure Chemical Industries (Osaka, Japan). About 500 g of frozen fruits were thawed overnight (12 h) at 4°C and mashed with 500 mL of distilled water, using a domestic agitator (Model HR2084, Philips Domestic Appliance & Personal Care, Zhuhai, China). To inactivate the polyphenol oxidase (PPO), the crushed puree was instantly heated to 70±1°C and then immediately cooled down to 40°C in an ice bath. Pectolyase Y-23 (0.01 g) was added to 1 L of puree (pH 4.0 and 40°C). After completely blended, the puree was incubated in a thermostat shaker (COS-100B Shaker, Shanghai Bilon Instruments, China) for 1 h to degrade soluble and insoluble pectin, rapidly heated to 85°C for 5 min, and quickly cooled down to 25°C. After filtering through a double layer of cheesecloth, the juice was centrifuged (12,000 g) at 20°C for 10 min to reduce its turbidity. The obtained juice was immediately frozen and kept at -20°C until analysis. The clarified juice was 6.49°Brix and had a pH of 4.42.

Measurement of anthocyanin content: Total anthocyanin content (TAC) was measured by a pH-differential method [23], using two buffer systems: potassium chloride buffer, pH 1.0 (0.025 M) and sodium acetate buffer, pH 4.5 (0.4 M). Briefly, 1 mL of clarified juice was mixed with 4 mL of each buffer and read at 510 and 700 nm against distilled water as a blank. Absorbance was calculated as $A= (A_{510} – A_{700})$ pH 1.0 – $(A_{510} – A_{700})$ pH 4.5. TAC of samples (mg cyanidin-3-glucoside/L of clarified juice) was calculated using Equation 1:

$$\text{TAC (mg/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times L} \quad (1)$$

where A is the absorbance, MW is the molecular weight of cyanidin-3-glucoside (449.2 Da), DF is the dilution factor, ε is the cyanidin-3-glucoside molar absorbance (26,900), and L is the cell path length (1 cm).

Antioxidant activity analyses: Total antioxidant activity (TAA) of downy rose-myrtle juice was determined using the ABTS•+ scavenging assay method [24]. ABTS•+ was produced by mixing 7 mM ABTS stock solution (prepared in 20 mM sodium acetate buffer, pH 4.5) with 2.45 mM potassium persulfate (final concentration). The solution was held at room temperature in the dark for 12-16 h before use. Once the radical was formed, the absorbance at 734 nm was adjusted to 0.700 ± 0.01 by dilution with 20 mM sodium acetate buffer (pH 4.5). Reaction mixtures containing 20 µL of sample or standard and 3 mL of the ABTS•+ solution were incubated in a water bath at 30°C for 30 min. The percentage inhibition was compared with a Trolox standard curve (0–1.5 mM) and the results were expressed in terms of micromoles Trolox equivalents (mM TE).

Degradation studies: Thermal degradation of anthocyanins in downy rose-myrtle juice was studied at temperatures of 60, 70 and 80°C. Aliquots of 20 mL of juice were transferred into test tubes, which were closed tightly to avoid evaporation, and placed in a thermostatic water bath (Memmert WNE 10, Schwabach, Germany) at a specified temperature, with an accuracy of ± 1°C. At orderly time intervals (0, 1, 2, 3, 4, 5, 6 and 7 h), three tubes were taken from the water bath and quickly cooled down in an ice water bath [25]. The anthocyanin concentrations were determined immediately.

The effect of pH on thermal stability was also studied at three different pHs (2.0, 3.0 and 4.0) at each of the temperatures specified above. Juices with different pHs were prepared by adjusting the pH with 10 M HCl and 10 M NaOH. Thermal degradation of anthocyanins has often been expressed by a reaction 20 (Equation 2 and Equation 3).

$$C_t = C_0 \exp(-kt) \quad (2)$$

$$t_{1/2} = \ln 2/k \quad (3)$$

Dependence of the degradation rate constant on temperature is determined according to the Arrhenius equation (Equation 4):

$$\ln k = \ln k_0 - E_a/RT \quad (4)$$

where t is the time (h), C_0 is the initial anthocyanin concentration (mg/L), C_t is the anthocyanin concentration after time t (mg/L), k is the rate constant (h^{-1}), and $t_{1/2}$ is the half-life, k_0 is the frequency factor (h^{-1}), E_a is the activation energy (J/mol), R is the universal gas constant (8.314 J/mol·k), and T is the absolute temperature (Kelvin).

Data evaluation and statistical analyses: All the analyses were performed in triplicate. Results were expressed as mean ± standard deviation. Linear regression analysis was applied to obtain the degradation rate constants (k) for anthocyanins. Data were analyzed by SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Anthocyanin content and antioxidant activity: Total anthocyanin content of downy rose-myrtle juice (pH 4.42) before thermal treatment was 19.26 ± 0.83 mg/L, while ABTS radical scavenging activity was 1.37 ± 0.24 mM TE, which was comparable to that in Sainsbury's Basic Tomato Juice (1.35 mM TE) and Big Tom Spiced Tomato Juice (1.36 mM TE) [26]. Table 1 shows the anthocyanin content and antioxidant activity of juice after different thermal treatments.

The ABTS radical scavenging activity of juice heated at 60°C, 70°C or 80°C for 1 h was 1.17, 1.05 and 1.02 mM TE, respectively (decreased 14.59%, 23.36% and 25.54%, respectively, compared with the unheated sample). Similarly, after heating at 60°C, 70°C or 80°C for 1 h, the total anthocyanin content of the juices decreased

Table 1. Total anthocyanin content and total antioxidant activity of juice subjected to different treatments.

Thermal treatment		Anthocyanins content	Antioxidant activity
Temperature (°C)	Time (h)	mg/L[a]	mmol/L TE[a]
60	1	16.53 ± 0.45	1.17 ± 0.25
	7	8.34 ± 0.39	0.54 ± 0.28
70	1	13.46 ± 0.61	1.05 ± 0.21
	7	4.12 ± 0.18	0.43 ± 0.11
80	1	12.26 ± 0.32	1.02 ± 0.22
	7	3.62 ± 0.72	0.28 ± 0.19

[a] Values represent means of triplicate determinations ± S.D.

from 19.26 (unheated) to 16.53, 13.46, and 12.26 mg/L, respectively. The total anthocyanin content (TAC) and total antioxidant activity (TAA) of the downy rose-myrtle juice decreased with higher temperature and longer heating time. In particular, juice heated at 80°C from 1 to 7 h experienced a drastic decrease in its antioxidant capacity. These results suggest that the TAA and TAC of downy rose-myrtle juice were susceptible to heat, and TAA was closely associated with TAC. As shown in Fig. 1, a high correlation (R^2 = 0.9623) between total anthocyanins and antioxidant activity was observed, indicating that the TAA of the downy rose-myrtle juice was mainly due to the TAC.

Anthocyanin degradation kinetics: The thermal degradation of anthocyanins in downy rose-myrtle juice subjected to different temperatures, pH values and heating times is shown in Fig. 2. The linear relationship between the logarithm of anthocyanin concentration and time demonstrated that the thermal degradation of anthocyanins in downy rose-myrtle juice fitted a first-order equation with a good regression coefficient ($0.9653 < R^2 < 0.9981$) (Table 2). Our results agree with other previous reports [27-29]. The graphs clearly show that the degradation rate of anthocyanins accelerated when the temperature increased, likewise, anthocyanins degraded faster when the pH value increased. This was reflected in the *k*-values (Table 2).

The isothermal kinetic parameters were calculated according to the first-order reaction kinetics model and the Arrhenius model, and the results are shown in Table 2. At pH 2.0-4.0, with increasing heating temperature there was an increase in the degradation rate constant (*k*) for anthocyanins, and a corresponding decline in the half-life value ($t_{1/2}$). For example, for juice with pH 2.0, heated at 60, 70 or 80°C, the degradation rate constants (*k*) for anthocyanins were 0.0967, 0.1285 and 0.2071 h^{-1}, respectively. When the temperature increased from 60 to 80°C, the $t_{1/2}$ value decreased from 7.17 h to 3.35 h. These results show that the temperature and duration of heating had a great influence on anthocyanin stability,

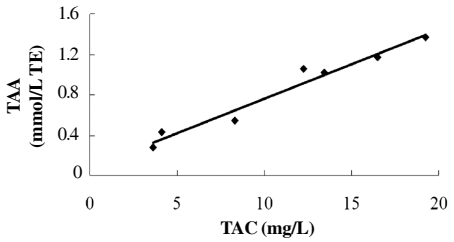

Figure 1. Relationship between total anthocyanin content (TAC) and total antioxidant activity (TAA) of downy rose-myrtle juice.

Table 2. Effect of temperature and pH on the k, $t_{1/2}$ and E_a values of anthocyanin degradation in downy rose-myrtle juice.

pH	Temperature (°C)	k (1/h)[a]	$t_{1/2}$ (h)[a]	Ea (kJ/mol)a
	60	0.0967 (0.9981)	7.17	
2.0	70	0.1285 (0.9690)	5.39	63.2 (0.9756)
	80	0.2071 (0.9878)	3.35	
	60	0.1201 (0.9946)	5.77	
3.0	70	0.1491 (0.9980)	4.65	46.1 (0.9809)
	80	0.2093 (0.9885)	3.31	
	60	0.1321 (0.9653)	5.25	
4.0	70	0.1672 (0.9870)	4.15	45.0 (0.9923)
	80	0.2272 (0.9972)	3.05	

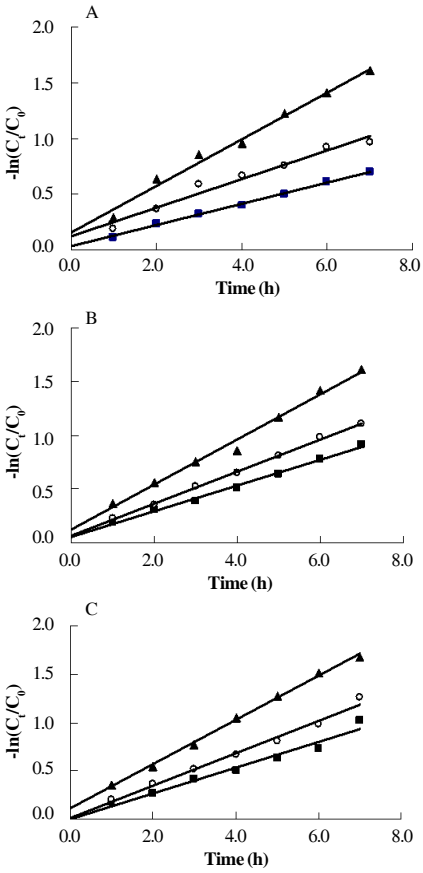

Figure 2. Thermal degradation kinetics of total anthocyanins in downy rose-myrtle juice at different pHs (A) pH 2, (B) pH 3, (C) pH 4. ▲, 60°C, ○, 70°C, ■, 80°C.

which agrees with the results of a previous report on the thermal stability of the anthocyanins of black rice [30]. At a certain temperature (e.g., 60°C), a higher $t_{1/2}$ value was observed at pH 2.0 (7.17 h) than at pH 4.0 (5.25 h), indicating that anthocyanins were more stable at an acidic pH [31, 32]. The $t_{1/2}$ values for anthocyanin degradation in sour cherry juice heated at 60, 70 or 80°C were 54.3, 22.5 and 8.1 h, respectively, while those corresponding to sour cherry concentrate (45°Brix) were 24, 10.9 and 4.4 h, respectively[33]. The $t_{1/2}$ values for anthocyanin degradation in blackberry juice (8.9°Brix) heated at 60, 70 or 80°C were 16.7, 8.8 and 4.7 h, respectively [28]. Compared with sour cherry and blackberry anthocyanins, downy rose-myrtle anthocyanins were more susceptible to high temperatures.

The activation energy (E_a) was determined by plotting against, according to the Arrhenius equation. These plots are presented in Fig. 3. The calculated values at pH 2.0, 3.0 and 4.0 were 63.2, 46.1 and 45.0 kJ/mol, respectively. As expected, the value of E_a decreased when the pH increased, which correlates with the decrease in pigment stability at higher pHs [31]. A value of E_a (73.6 kJ/mol) for the thermal degradation of total anthocyanins in blood orange juice (11.2°Brix) was reported by Kirca and Cemeroglu [22]. These results suggest that anthocyanins in downy rose-myrtle juice were more susceptible to degradation at higher temperatures than those in blood orange juice. Thus, it seems apparent that the different types of anthocyanins in fruit juice have different susceptibilities to heat treatment.

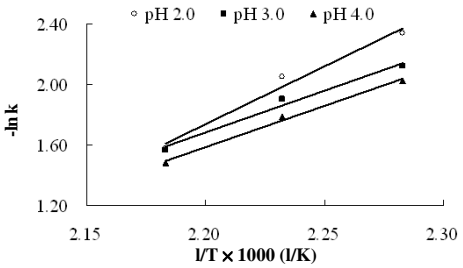

Figure 3. Arrhenius plot for thermal degradation of total anthocyanins in downy.

Conclusions

The antioxidant activity of heated downy rose-myrtle juice was much lower than that of the unheated juice, as determined by the ABTS method. The heated juice also had a lower anthocyanin content. Moreover, TAA was closely associated with TAC. The effects of temperature and pH on the degradation of anthocyanins in downy rose-myrtle juice were investigated in a detailed kinetic study. The results revealed that anthocyanin degradation followed first-order reaction kinetics and the variation in the degradation rate constants was according to the Arrhenius equation. Higher anthocyanin stability was obtained in juice with lower pH values, and heated at lower temperatures for shorter times.

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