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ARTICLE

Thermal and pH Stability of Betacyanin Pigment of Djulis (*Chenopodium formosanum*) in Taiwan and Their Relation to Antioxidant Activity

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The betacyanin pattern of Djulis (*Chenopodium fromosanum*), a native cereal plant in Taiwan, was reported for the first time. The determination of characteristics of the pigment, including pH and thermal stability and their relation to antioxidant activities, indicated that betacyanin of Djulis exhibited the maximum absorbance at 530 nm and their thermal stability was dependent on the pH. Antioxidant capacities as well as red color of that pigment were significantly positively related with A_{530}. Therefore, A_{530} can be used as a simple index of the antioxidant capacity of that pigment. Four peaks including betanin (47.8%), isobetanin (30.0%) with *m*/*z* 551.0, and armaranthin (13.6%), isoamaranthinee (8.6%) with *m*/*z* 727.0 were purified from the pigment extract by HPLC and identified through LC/MS. Among them, betanin and isobetanin totally accounted for more than 70% of FRAP reducing power or DPPH scavenging capacity and were a major source of the antioxidant capacities. Our findings of this pigment confirmed that Djulis can be used as a novel source of betanin antioxidants and may provide a basis for its sustainable utilization in the food industry.

KEYWORDS: Djulis (*Chenopodium fromosanum*); betacyanin; antioxidant capacity

INTRODUCTION

Djulis (*Chenopodium formosanum*) is a native cereal plant in Taiwan. In addition to be an additive of wine making, the colorful grains are traditionally used as insect repellant. The pigment of Djulis is considered not only to have good coloring potential but also positive physiological benefits for human health. These effects are believed to be attributable to its antioxidant capacity. However, we could find no information about its pigment or antioxidant capacity. In preliminary testing, the pigment of Djulis exhibited characteristics similar to those of betalain, known as both a natural colorant and an antioxidant.

Betalains belong to the nitrogenous pigments (*1*) with a wide pH range of application (*2*). Two categories of betalains include yellow betaxanthins (absorbance ranging from 475 to 485 nm) and violet−red betacyanins (absorbance ranging from 530 to 545 nm) (*3*, *4*). Numerous studies have dealt with the identification of betalain structure or color stability during processing. For example, more than 50 betacyanins and 20 betaxanthins have been reported. Ten betacyanins, including betanin, phyllocatin, and hyllocactin, were purified from red−purple pitaya (*5*), while in amaranthaceae, eight amarnarthine-type, two betanin-type, and six gomphrenin-type pigments have been reported (*6*). The betalain profile varied in different varieties or genotypes (*7*). In addition, betalain is quite sensitive to light, heat, and oxygen due to the unstable resonance of the coloring structure. They are not stable under most storage conditions. The stability of the pigment was found to be highest in the pH range between 4.0 and 5.0, and the activation energies in degradation kinetics were pH dependent (*1*). Furthermore, heating may lead to structural alterations of pigment (*8*).

Further, betalain has been reported to decrease the sensitivity to inflammatory disease (*4*), protect the liver (*9*), and inhibit the growth of B16F10 melanoma cells (*10*). Betanin was found to perform better than catechin in inhibiting linoleate peroxidation and H_2O_2 activated LDL oxidation (*11*). However, we could find no data related to changes of the antioxidant capacity of betalain after being subjected to heat and pH treatments. On the basis of possible similarities to betalain, the aim of this study was to evaluate the characteristics of Djulis pigments and their role in antioxidant activities.

MATERIALS AND METHODS

Preparation of Samples. The Djulis for this study were harvested on campus of NPUST. After air-drying (Aw = 0.6), the grains were stored at −20 °C. Preliminary experiments were carried out to establish the optimal extraction procedures. The pigment extract was prepared by immersing 1 g of red Djulis grains in 100 mL of water at 4 °C for 12 h and then frozen dried (FD). Then 1 g of the FD pigment extract was dissolved in 100 mL of buffer solutions with different pHs (2−11) and subjected to heating at different temperatures (50−90 °C) for 30 min.

Color Measurement (*12*). The wavelength of the pigment samples was measured by a Hitachi spectrophotometer U-2001 (Hitachi, Tokyo, Japan).

A Nippon Denshoku color meter (ZE2000, Japan) was used to obtain Hunter *L*, *a*, *b*, and color differences of the samples. The higher *L*, *a*, and *b*,

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the higher lightness, red color, and yellow color shows.

$$\text{hue : hue angle}_{ab} = \arctan(b/a)$$

$$\text{chroma : chroma}_{ab} = (a^2 + b^2)^{1/2}$$

$$\Delta E = ((L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2)^{1/2}$$

Betacyanin Separation and Quantity Estimation by HPLC. The procedures were modified from Cai et al. (*16*). The pigment extract was separated by HPLC. After the identification of betanin peak and its peak area percentage by reference to an external betanin standard, the concentration of betanin was estimated by using the extinction coefficient (ε) as 61600 through the formula: $A_{\lambda max.} = 6.16 \times 10^4 \times$ concentration (M). Then, the total betacyanin pigment was estimated by calculation from the concentration and peak area percentage. All samples were filtered by 0.45 μm PTFE minipore.

Following are the parameters for HPLC:

Column: Lichrosorb RP-18 (250 mm × 4.6 mm)
Detector: Hitachi UV−vis detector L-7420
Pump: Hitachi L-7100
Detector wavelength: 530 nm
Mobile phase was achieved by gradients: The A (formic acid) was decreased from 92% to 84% and B (acetonitrile) was increased from 8% to 16% within 15 min. From the time points 15−25 min, A and B were restored to 92% and 8%, respectively. Flow rate: 1.0 mL/min.

Betacyanin Analysis by LC-MS (*6*). The LC/MS/MS is LCQ series LC-MS system (LCQ Advantage mass spectrometer system, Thermo Finnigan). HPLC parameters are the same as above; PDA was adjusted to be 400−700 nm.

Analyzer: ion trap
Scan mode: full scan (m/z 300−2000)
Nebulizer gas: N_2
Spray voltage (KV): 4.50
Capillary temp (°C): 275
Capillary voltage (V): 19
Settings for ion detection: m/z 551 (betanin, isobetanin) and m/z 727 (amaranthine, isoamaranthine)

Concentration Determination of Purified Pigment Fractions and Their Antioxidant Capacity Analysis. To evaluate the concentration and antioxidant capacity in each fraction purified, the pigment of each fraction was collected, concentrated to dryness, and the volume of each pigment was made into 10 mL by adding distilled water before concentration determination and antioxidant capacity analysis. The concentration of the individual pigment was estimated by using ε as 61600 for betanin/isobetanin and 56600 for amaranthine/isoamaranthine through the formula $A_{\lambda max} = \varepsilon \times$ concentration (M).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity. The radical scavenging activity was measured according to the method of Tsai, Wu, and Cheng (*12*). First, 1 mL of freshly prepared 1 mM DPPH solution was added to the sample (4 mL) and then kept at 25 °C for 30 min. The absorbance was read at 517 nm relative to the control (as 100%), and the percentage of scavenging effect was shown as [1 − (A_{517} of sample/A_{517} of control)] × 100 (*12*).

Ferric Reducing Ability of Plasma (FRAP) (*12*). FRAP assay is a method of measuring the capacity of reductants (antioxidants) to reduce Fe^{3+} to Fe^{2+}. The formation of dark-blue colored Fe^{2+}−TPTZ complex (Fe^{2+} tripyridyltriazine) will increase the absorbance at 593 nm, which is the sign for higher antioxidant capacity. This was described by Tsai et al., (*12*). Freshly prepared FRAP reagent (including acetate buffer, TPTZ, and $FeCl_3$) 1.2 mL was added with 0.12 mL of water and 0.04 mL of sample. The absorbance at 593 nm of the mixture was measured after 6 min of reaction. The reducing power (μmol/L) was calculated from the standard curve constructed using $FeSO_4$ solution.

Activation Energy. Samples were heated at 50, 60, 70, 80, or 90 °C, and their betacyanin components were calculated at time intervals. By determining the changes in betacyanin components versus time, the rate of degradation was calculated. The activation energy was then calculated by the formula $\text{Ln}\, K = -E_a/RT$, K = rate constant, R = 1.986, and T = (°C + 273).

Half-Life of Betacyanin. Betacyanin content was quantified by an HPLC system according to the method described above. The time needed for the betacyanin degraded until 50% residual was recorded as the half-life of betacyanin.

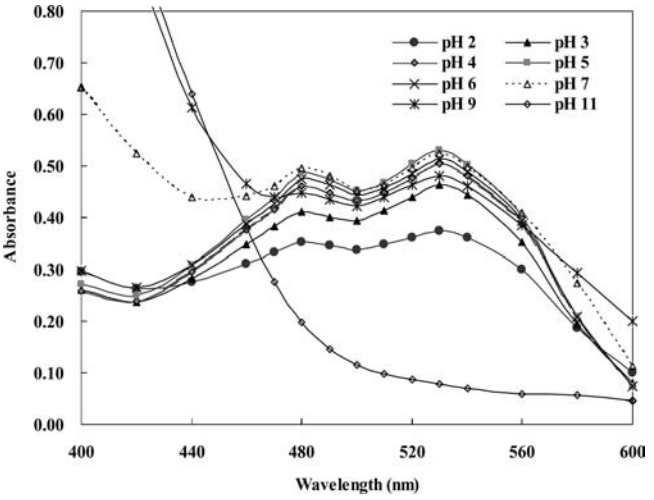

Figure 1. Absorbance spectrum of red Djulis grain pigments extracted with water and adjusted with buffer at pH 2−11.

Table 1. Half-Life ($T_{1/2}$, min) and Activation Energy of Red Djulis Grain Pigments with Different Levels of pH after Heating at Different Temperatures

		pH				
	temperature, °C	3	4	5	6	7
$T_{1/2}$ (min)	50	272.13	285.25	321.04	290.24	297.25
	60	106.53	140.02	282.65	207.27	241.34
	70	36.52	65.00	124.72	82.34	101.69
	80	25.00	45.26	72.40	54.37	64.55
	90	18.33	32.04	63.76	38.95	55.37
activation energy (kcal/mol)		9.25	11.21	15.58	13.16	15.31

Statistical Analysis (*12*). Statistical analysis was conducted using SAS statistical software (SAS, 1988). General linear model procedures were used to determine the effects of treatments. Duncan's multiple range tests were used to compare means. All data were analyzed by triplicates.

RESULTS AND DISCUSSION

Effect of pH on the Color of Djulis Pigments. As illustrated in **Figure 1**, two absorbance peaks were found when pH ranged between 2 and 9, one at A_{530} and the other at A_{480}, which were representative of betacyanin and betaxanthin, respectively. This is similar to the absorption spectrum of freshly extracted red beet pigment (*13*). Therefore, betacyanin of this pigment was characterized by A_{530}. The maximum peak was found at pH 5. A_{530} increased from 0.375 (pH 2) to 0.462 (pH 3) and 0.531 (pH 5) and decreased to 0.512 (pH 6), 0.503 (pH 7), and 0.480 (pH 9). At pH 11, no maximum absorbance could be found. It was speculated that the pigment was degraded by the strong alkaline (*14*). In addition, the original pH of Djulis was 5.5 and its absorbance spectrum at pH 5.5 (data not shown) was almost the same as that at pH 5. It suggested that betacyanin of Djulis may show the highest red color when at its natural pH. As to the appearance, purple−red was found for samples between pH 4−7, while orange or pale yellow was found for the sample with pH over 9 or lower than 3 (data not shown).

Thermal Stability of Djulis Pigment at Different pH. The pigment extract at different pH was heated to various levels of temperature. Then the changes of A_{530} under different pH (3−7) and temperatures (50−90 °C) were investigated. Half-life of the pigment at different levels of pH was further used to assess its thermal stability. As shown in **Table 1**, the higher the heating temperature the higher the degradation rate of the pigments.

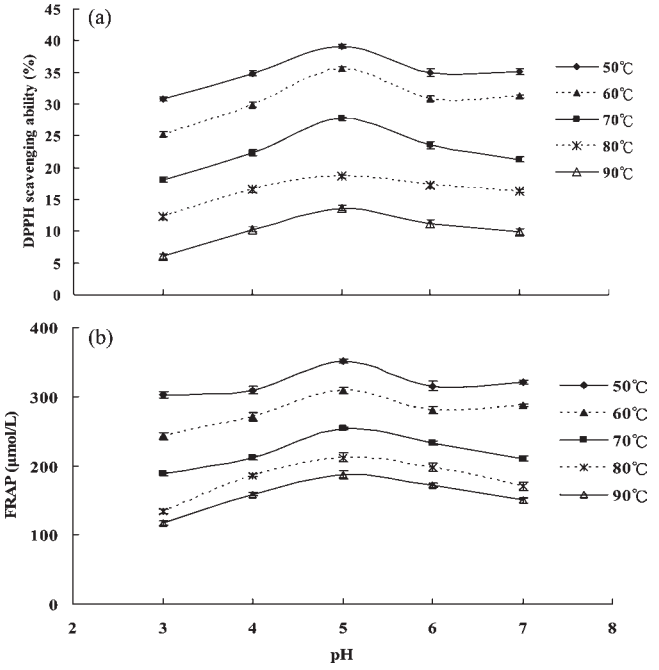

Figure 2. Changes of antioxidant capacities in red Djulis grain pigments after being heated at different temperature and pH.

The half-life was highest at pH 5 when heated at 50 °C (321.04 min) and lowest at pH 3 when heated at 90 °C (18.33 min). We observed a sharp drop in half-life when the temperature was higher than 60 °C, indicating that the temperature used in thermal processing of red Djulis grains should be lower than 60 °C even in an adequate pH range (the half-life of pigment in pH 5, 90 °C was only 63.76 min). Activation energy of pigment degradation was often used to explain the stability of pigment (*15*). The activation energy was the highest at pH 5 (15.58 kcal/mol) and was 15.31, 13.16, 11.21, and 9.25 kcal/mol for samples at pH 7, 6, 4, and 3, respectively. This is in agreement with the half-life (*3*) and activation energy (*1*) of pigment in cactus fruit.

Stability of Antioxidant Activity of Djulis Pigment at Different pH. The stability of the antioxidant activity of the pigments extracted at different pH was assessed by FRAP and DPPH scavenging capacity analysis. As demonstrated in **Figure 2**, both FRAP or radical scavenging ability decreased when temperature increased. The sample at pH 5 showed the highest stability during heating. For example, the original values for FRAP at pH 2, 3, 4, 5, 6, 7, 9, and 11 samples were 365.3, 374.2, 376.3, 388.9, 377.6, 381.6, 373.7, and 357.6 μmol/L, respectively (data not shown). But after being heated at 50, 60, 70, 80, and 90 °C, they drop to 300, 240, 180, 130, and 120 μmol/L for pH 3 samples, while 350, 310, 260, 220, and 180 μmol/L were found in the pH 5 samples. It suggested that the best antioxidant activity and stability was observed at pH 5. The DPPH scavenging capacity showed the similar tendency. They shift from the original 42.34% of pH 5 samples (data not shown) to 39, 36, 27, 18, and 13% after heating at 50, 60, 70, 80, and 90 °C, respectively.

Correlation between Color and Antioxidant Capacity of Djulis Pigment. As listed in **Table 2**, antioxidant capacities were significantly correlated with red color qualities (A_{530} and Hunter *a* value). At pH 5, the correlation coefficient was highest when compared with that of pH 3 or pH 7. For example, the correlation coefficient between A_{530} and FRAP and DPPH scavenging ability were 0.99 and 0.98, while that of samples at pH 3 were 0.85 and 0.8, respectively. The result revealed that the thermal stability of betacyanin in Djulis is pH dependent. Both red color or antioxidant capacity of Djulis pigment were most stable at pH 5. It suggested the important role of betacyanin in antioxidant capacity of Djulis and A_{530} can be used as a good index of biological activity in this study.

Table 2. Correlation Analysis between Color and Antioxidant Activity of Pigments Extracted from Red Djulis Grains[a]

	pH 3		pH 5		pH 7	
	DPPH	FRAP	DPPH	FRAP	DPPH	FRAP
A_{530}	0.80***	0.85***	0.98***	0.99***	0.88***	0.90***
Hunter *L*	−0.87**	−0.91**	−0.93**	−0.96**	−0.89***	−0.82**
Hunter *a*	0.81***	0.79**	0.88***	0.86**	0.84***	0.78**
Hunter *b*	0.53	0.62	0.47	0.64	0.61	0.69
chroma	0.86**	0.88**	0.97**	0.95**	0.90**	0.89**
hue angle	0.86**	0.94**	0.81**	0.87**	0.85**	0.90**

[a] *, **, ***: Significant at 5%, 1% and 0.1% levels, respectively.

Purification of Betacyanin of Djulis by HPLC. The betanin pattern of Djulis was further elucidated by HPLC purification. As shown in **Figure 3**, four peaks with retention time 4.45, 5.97, 7.35, and 9.42 min were purified from Djulis pigment by HPLC. Two of them matched with the standards which occurred at 7.39 and 9.49 min, respectively, and revealed that betanin (peak 3, 47.8%) and isobetanin (peak 4, 30.0%) were the major pigments (totally 77.8%) in water extract of red Djulis grains. This is in accordance with the report that betanin and isobetanin are two most common structures of betacyanin in nature, they usually coexisted, and the content of betanin is higher than that of isobetanin (*2*). The total betacyanin were therefore estimated as 0.94 mg/g fresh weight or 3.78 mg/g dried weight of shell. This agrees with the range of total betacyanin content (0.08−1.36 mg/g fresh weight) in Amaranthaceae (*6*). The two constituents eluted at 4.45 min (peak 1, 13.6%) and 5.97 min (peak 2, 8.6%) were further directly characterized by LC-MS because no standards are available.

Identification of Betacyanin of Djulis by LC-MS. The four peaks mentioned above were further characterized by an ion detector with mass spectrometer (**Figure 4**). Both peak 1 and 2 showed the signal *m*/*z* 727. This is in accordance with the *m*/*z* ratio (727) of amaranthine and isoamaranthine in amaranthaceae (*6*) or *Celosia argentea* (*16*). The signal *m*/*z* 551 from peaks 3 and 4 is consistent with the *m*/*z* ratio of betanin and isobetanin, which was confirmed by the standards with HPLC already (*6*, *17*). Therefore, we concluded that betanin, isobetanin, amaranthine, and isoamaranthine were the four major pigments in the water extract of red Djulis grains. The content was in the order of betanin > isobetanin > amaranthine > isoamaranthine. According to Huang and Von Elbe (*18*), amaranthine showed greater heat sensitivity in solution than betanin due to the fact that the equilibrium constant (*K*) for the ratio of betalamic acid (BA) × cyclo-dopa-5-*O*-[2-*O*-(β-D-glucuronic acid)-β-D-glucoside] (CGG)/amaranthine vs reaction time is twice that for BA × cyclodopa-5-*O*-glycoside (CDG)/betanin during thermal processing. It leads to greater degradation and less regeneration of amaranthine when compared with betanin. This explained the less stability of amaranthine than betanin in this study.

Contribution of Betacyanin Fraction on the Antioxidant Capacity. The antioxidant capacity of betacyanin may depend on their structure. For example, glucose at C6 (−OH group at C5, such as gomphrenin type) was more bioactive than that at C5 (−OH group at C6, such as betanin type). A higher extent of glycosylation of betanin led to lower antioxidant activity (*19*). In this study, we analyzed the four peaks purified from HPLC and determined their antioxidant capacity by FRAP and DPPH scavenging ability (**Table 3**). Results showed that betanin was the predominant contributor (56.82%) of FRAP, followed by isobetanin (15.96%), amaranthine (15.66%), and isoamarnathine (11.55%).

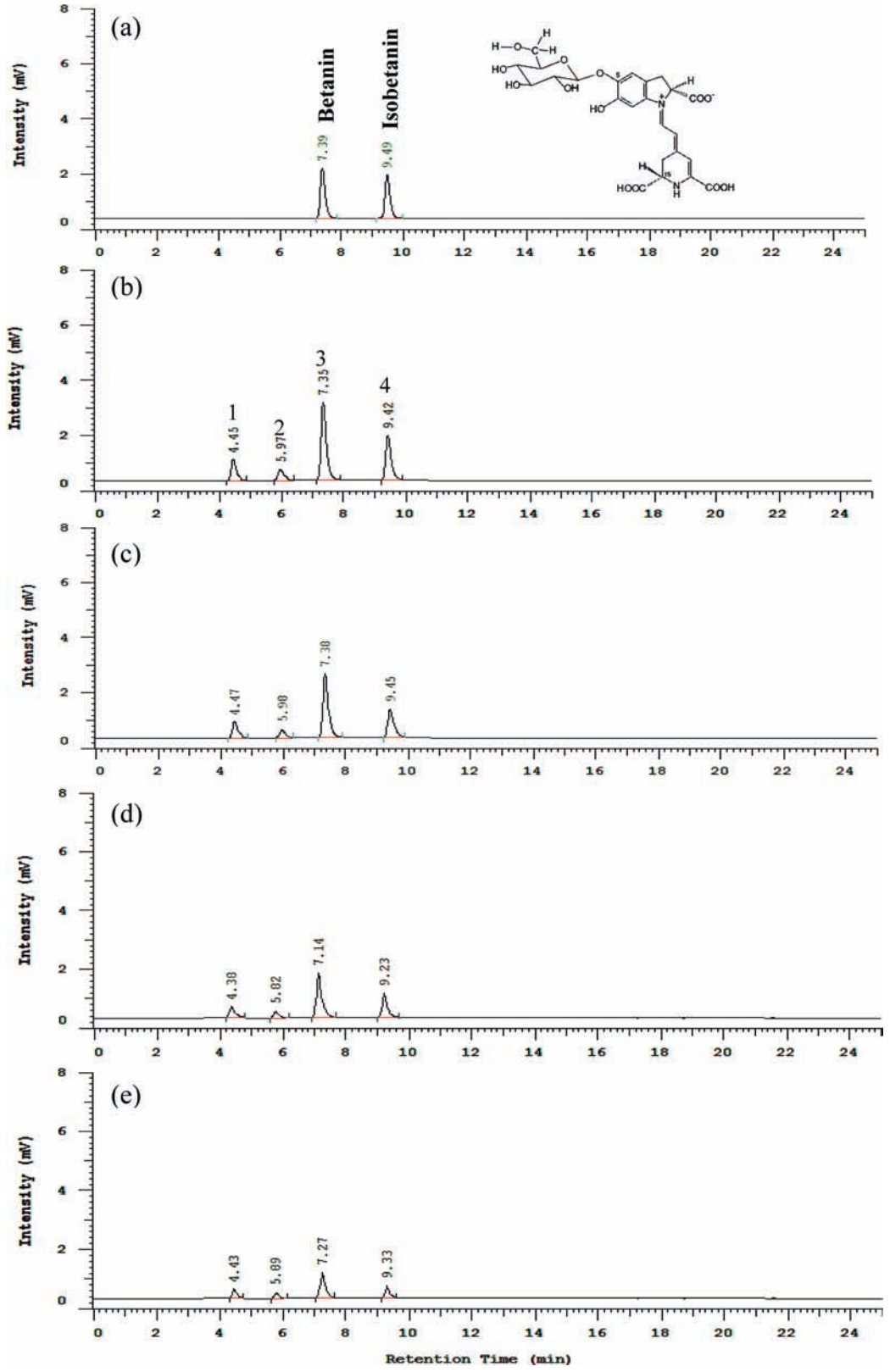

Figure 3. HPLC chromatograms for (**a**) betanin and isobetanin standards, and pigments extract of red Djulis grains at (**b**) pH 3, (**c**) pH 5, or after being heated at 90 °C in (**d**) pH 3 and (**e**) pH 5.

For the DPPH scavenging ability, betanin (51.75%) and isobetanin (22.22%) accounted for totally 73.97% of them, while amaranthine and isoamarnathine accounted for 16.62% and 9.40%, respectively. Apparently, the antioxidant capacity of amaranthine type betacyanins were much less than that of betanin type betacyanin in Djulis. This might be due to the difference of their structures. According to Cai, Sun, and Corke (*19*), the EC_{50} in DPPH inhibition were 8.37 and 8.35 (μM) in amaranthine and isoamaranthine, while that in betanin and isobetanin were only 4.88 and 4.89 (μM), respectively. Because the R group in C5 of betanin was glucose and that in amaranthine was 2′-glucuronic acid-glucose, it was explained by the cause that

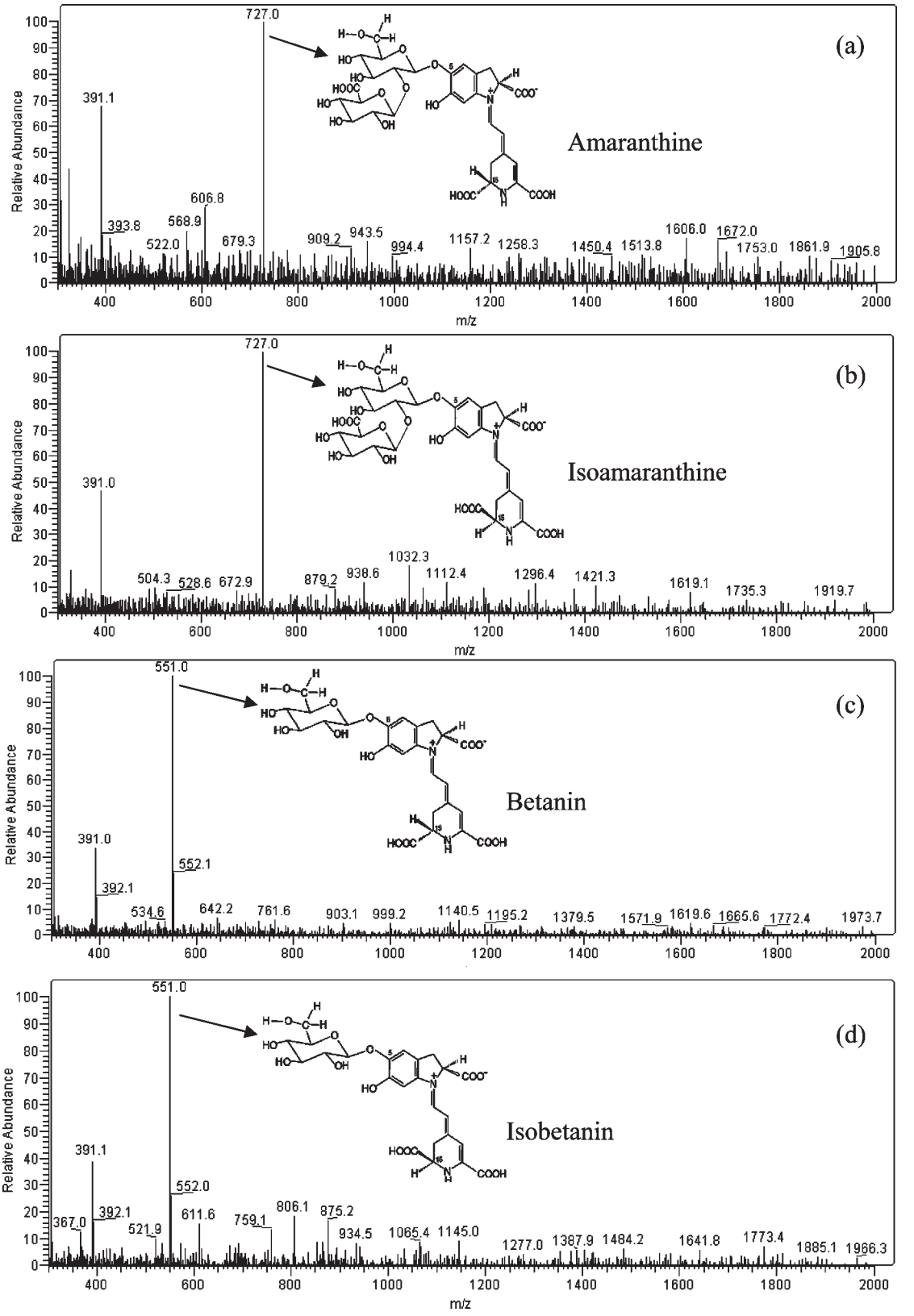

Figure 4. Mass spectra chromatograms of pigment extracted from red Djulis grains (**a**) peak 1 amaranthine, (**b**) peak 2 isoamaranthine, (**c**) peak 3 betanin, and (**d**) peak 4 isobetanin.

higher extent of glycosylation led to lower activity. That is, the glycosylation of amaranthine will reduce their antioxidant capacity while compared with that of betanin. In addition, the predominant contributions of betanin in both FRAP and DPPH scavenging ability might be attributed to its highest concentration (12.6 μM) among the four pigment fractions. For example, betanin, with concentration twice amount that of isobetanin, showed the FRAP 3.6 folds that of isobetanin. The concentration effects on the significant difference in FRAP activity between betanin (56.82%) and isobetanin (15.96%) might not be excluded.

Table 3. Contribution on the Antioxidant Capacities of Each Pigment Fraction from Red Djulis Grains Purified by HPLC[a]

pigment fraction	pigment concentration (μM)	FRAP (Fe^{2+} equivalents, μmol/L)	contribution of FRAP (%)	DPPH scavenging (%)	contribution of DPPH scavenging (%)
amaranthine	4.29	35.53 b	15.66	16.97 c	16.62
isoamaranthine	2.12	26.20 c	11.55	9.60 d	9.40
betanin	12.6	128.87 a	56.82	52.84 a	51.75
isobetanin	5.8	36.20 b	15.96	22.69 b	22.22
total		226.80	100.00	102.09	100.00

[a] Values in columns with different letters a−d indicate significant difference ($p < 0.05$).

In conclusion, our study is the first report of betanin profiles in Djulis and their stability and antioxidant capacity. The data presented here indicate that Djulis can be used as a novel source of betanin antioxidants and may provide a basis for its sustainable utilization in the food industry.

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