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Influence of Cooking on Anthocyanins in Black Rice (*Oryza sativa* L. *japonica* var. SBR)

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The composition and thermal stability of anthocyanins in black rice (*Oryza sativa* L. *japonica* var. SBR) produced in California were investigated. Six anthocyanin pigments were identified and quantified by high performance liquid chromatography using photo diode-array detection (HPLC-PDA) and electrospray ionization mass spectrometry [LC-(ESI)MS/MS]. The predominant anthocyanins are cyanidin-3-glucoside (572.47 μg/g; 91.13% of total) and peonidin-3-glucoside (29.78 μg/g; 4.74% of total). Minor constituents included three cyanidin-dihexoside isomers and one cyanidin hexoside. Thermal stability of anthocyanins was assessed in rice cooked using a rice cooker, pressure cooker, or on a gas range. All cooking methods caused significant ($P < 0.001$) decreases in the anthocyanins identified. Pressure cooking resulted in the greatest loss of cyanidin-3-glucoside (79.8%) followed by the rice cooker (74.2%) and gas range (65.4%). Conversely, levels of protocatechuic acid increased 2.7 to 3.4 times in response to all cooking methods. These findings indicate that cooking black rice results in the thermal degradation of cyanidin-3-glucoside and concomitant production of protocatechuic acid.

KEYWORDS: Black rice; California; anthocyanin; cyanidin-3-glucoside; cooking; protocatechuic acid; HPLC-PDA; LC-(ESI)MS/MS

INTRODUCTION

Rice (*Oryza sativa L.*) is one of the world's most important cereal crops providing a staple food source for more than 50% of the world's population (*1*). Rice has two major subspecies *Oryza sativa* L. *japonica,* primarily consumed in Southeast Asia, northern China, Japan and the U.S., and *Oryza sativa* L. *indica*, which is primarily consumed in India, Southern China, and the lowland areas of Southeast Asia. Rice is typically consumed as white rice with the husk, bran, and germ removed. There are many special cultivars of rice that contain colored pigments and give rise to numerous varieties of red and black rice. The dark purple color of black rice results from the high content of anthocyanins located in the pericarp layers (*2*−*4*). Black rice is a good source of fiber, minerals, and several important amino acids (*5*−*7*). Black rice is primarily produced in Southeastern Asia. However, cultivation in California has grown in recent years because of increased culinary interest and because it is a nutritionally dense source of anthocyanins.

Anthocyanins are a subclass of water soluble flavonoids responsible for the colors of numerous fruits, vegetables, cereals, and flowers. In plants, they function to attract pollinators and seed dispersers, and also act as photoprotectants by scavenging free radicals generated during photosynthesis (*8*). Structurally, anthocyanidins are flavylium (2-phenylchromenylium) cation derivatives that share a common hydroxylation at the C-3, C-5, and C-7 positions but differ in the number and substitution pattern of hydroxyl and/or methoxyl groups on the B-ring (**Figure 1**). Anthocyanins exist as *O*-glycosides and acylglycosides of anthocyanidins in plants. Glycosylation generally occurs at the hydroxyl group located at the C-3 position, although the C-5 and C-7 positions can also undergo glycosylation. Sugars associated with anthocyanidins include rhamnose, glucose, galactose, and arabinose.

Anthocyanins are common constituents in the diet and are of increasing interest to the food industry as natural food colorants. The daily intake is estimated to be 12.5 mg per capita in the United States (*9*). Recent interest in anthocyanins as functional ingredients has also been generated by their potential role in preventing chronic and degenerative diseases due to their antioxidant (*10*−*12*), anti-inflammatory (*12*), antiarteriosclerosis (*13*), anticancer (*14*), hyperlipidemia (*15, 16*), and hypoglycemic activities (*17*). Identifying the disposition of anthocyanidins in foods is important for accurate database development and for understanding the role diet plays in disease prevention. Critical to this objective, is an understanding of how thermal processing and cooking influence anthocyanin stability and distribution in foods, as most foods undergo some type of cooking or thermal processing prior to consumption.

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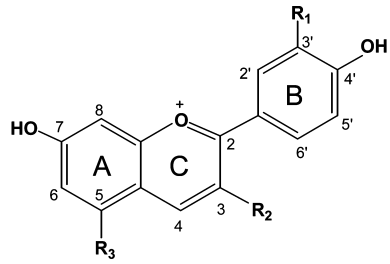

Anthocyanin	R_1	R_2	R_3
Cyanidin	OH	OH	OH
Cyanidin-3-glucoside	OH	O-β-D-glucose	OH
Peonidin-3-glucoside	OCH_3	O-β-D-glucose	OH
Cyanidin-3,5-diglucoside	OH	O-β-D-glucose	O-β-D-glucose

Figure 1. Chemical structures of anthocyanins evaluated in this study.

To date, there are few studies examining the individual anthocyanin content of black rice (*2, 3*), and to our knowledge, no data is available on the anthocyanin and phenolic acid content of black rice cultivars produced in California. Cyanidin-3-glucoside, cyanidin-3-rhamnoside, cyanidin-3,5- diglucoside, and malvidin-3-galactoside have been reported in Japanese and Korean pigmented rice varieties (*3*−*5*). A survey of 10 pigmented *Oryza sativa* L. *indica* varieties demonstrated that cyanidin-3-glucoside (0−470 mg/100 g) and peonidin-3-glucoside (0−40 mg/100 g) are the predominant anthocyanins (*3*). A similar result was shown by Abel-Aal et al. (*2*); however, the rice variety was not specified. Abel-Aal et al. (*2*) also noted the presence of several cyanidin diglucosides and a cyanidin rutinoside in these samples.

The predominant phenolic acids in white and brown rice are ferulic acid and ρ-coumaric acid (*18*). Information on the compliment of phenolic acids in black rice is not available.

The stability of anthocyanins in foods is influenced by the pH, temperature, glycosidic linkages, and food matrix interactions that occur during processing (*19*). To date, little is known about the effects of various thermal cooking methods on the composition and retention of anthocyanins in black rice. Black rice is generally cooked for longer periods than white rice by boiling and/or steaming it. It is commonly cooked in a pot using just enough water to be absorbed into the rice during cooking (absorption method) or using an electric rice cooker as these are increasingly available worldwide and simplify the rice cooking process. Alternatively, pressure cooking can be used to speed the cooking process. In many countries, rice is soaked prior to cooking to soften its texture and help decrease cooking times.

The objectives of the following study were to elucidate the composition of anthocyanins in a predominant cultivar of California black rice and investigate the influence of presoaking and the three predominant methods used to cook rice (i.e., electric rice cooker, pressure cooker, and absorption method using a gas range) on the stability of anthocyanins.

MATERIALS AND METHODS

Sample. Black rice (*Oryza sativa* L. *japonica* var. SBR) produced in California in 2007 was supplied by SunWest Foods, Inc. as three independent composite samples of ∼10 lb each. Raw grains of black rice were kept at room temperature in airtight containers until cooked. The moisture content of the rice was determined as 3.2%.

Chemicals and Standard. Cyanidin-3-glucoside chloride and cyanidin was purchased from Polyphenol Laboratories AS (Sandnes, Norwy), and peonidin-3-glucoside chloride, cyanidin-3,5-diglucoside chloride, and cyanidin-3-galactoside chloride were obtained from Extrasynthese (Genay Cedex, France). Protocatechuic acid (3,4-dihydroxybenzoic acid) and phloroglucinaldehyde (2,4,6-trihydroxybenzaldehyde) were purchased from Sigma (St. Louis, MO). HPLC grade methanol and 30% hydrochloric acid for extraction of anthocyanins, and HPLC grade acetonitrile and 85% *o*-phosphoric acid were from Fisher (Darmstadt, Germany). Nitrogen (desolvation and nebulizer gas) and argon (MS/MS collision gas) were from Airgas Inc. (Radnor, PA).

Cooking. Fresh black rice was cooked using a consistent ratio of 1:1.8 (w/w) using 135 g of nanopure water to 75 g of rice. This rice-to-water ratio resulted in the complete absorption of water by the rice at the end of the cooking time. Rice was cooked using either a commercial Zojirushi NS-LAC05 Micom 3-cup rice cooker (Zojirushi, Osaka, Japan), a Nesco American Harvest PC-6-25-30TPR 3 in 1 digital electric pressure cooker (Nesco American Harvest, Two Rivers, WI), or in a stainless steel pot (18 cm and 2 1/2 qt) on a Viking Professional VGSU161 gas range using a 6000 btus burner on the lowest heat setting (Viking, MI). A series of rice samples were presoaked in 135 g of water for 1 h prior to cooking in the rice cooker to compare the effects of presoaking rice on anthocyanidin retention. Rice cooking time differed depending upon the method used. Rice was cooked for 90 min in the rice cooker, 50 min on the gas range, and 20 min in the pressure cooker. All cooked rice was allowed to steam-cool for 5 min after the heating stopped. Two aliquots of cooked rice, about 20 g each, were randomly selected from each sample and immediately frozen at −80 °C before freeze-drying. Each cooking test was performed three times on independent samples.

Sample Preparation. The rice samples were freeze-dried and ground in an IKA laboratory mill M20 (Janke & Kunkel Co., Staufen, Germany), and the ground material was sieved with 20 mesh (420 m opening) (Tyler Co., Mentor, OH). The milled samples were mixed and kept at −30 °C until extraction. Anthocyanins in the ground grain samples were extracted according to the method described by Abdel-Aal et al. (*2*) with slight modifications. One gram of the ground samples was extracted three times by mixing with 8 mL of methanol acidified with 1.0 N HCl (85:15, v/v) and shaking on an LAB-LINE Orbit Environ-shaker (LAB-LINE Instruments, Melrose Park, IL) at 1,800 rpm for 30 min. The pH of the mixture was checked to be pH 1 during shaking by measuring pH after 15 and 30 min of shaking. The crude extracts were centrifuged at 10,000*g* and 4 °C for 20 min. The extracts were concentrated under a stream of nitrogen. The precipitates formed during the concentration step were separated by centrifugation as described above. The concentrated extracts were adjusted to 5 mL with

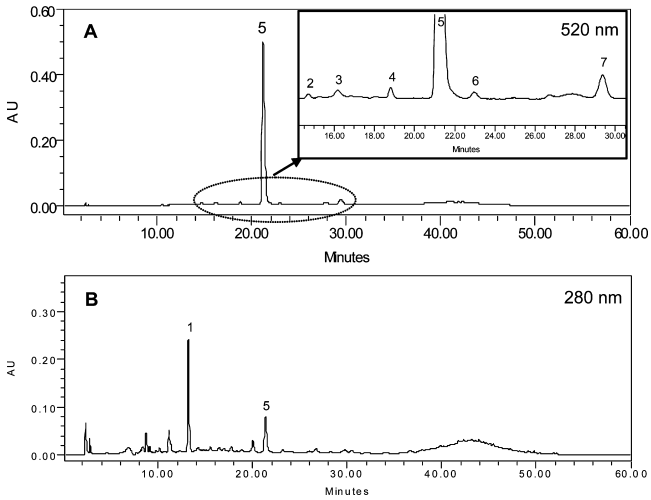

Figure 2. HPLC profiles of anthocyanins and polyphenol compounds in (**A**) raw rice monitored at 520 nm and (**B**) cooked rice monitored at 280 nm. Peak assignments are given in **Table 1**.

acidified methanol and vigorously mixed and filtered through a 0.45 μm Millex-FH hydrophobic fluoropure (PTFE) membrane (Millipore, Milford, MA) and analyzed by HPLC.

HPLC-PDA Analysis. A Waters Alliance 2690 equipped with a 996 photodiode array detector (PDA) and a Millenium v. 3.04 data acquisition system was used for HPLC analyses. Anthocyanins from black rice extracts were separated using reversed-phase HPLC on a Zorbax XiDB-ODS column (5 μm, 250 × 4.6 mm i.d.; Agilent, Palo Alto, CA) at 25 °C. A spectrum was collected over 200−700 nm. Injection volumes of the extracts were 40 μL. Anthocyanins were eluted using a gradient mobile phase consisting of (A) 0.2 M *o*-phosphoric acid (pH 1.5) and (B) 80% acetonitrile/20% 0.2 M *o*-phosphoric acid (pH 1.5) at a flow rate of 1 mL/min. The gradient was programmed as follows: 0−4 min, 100−92% A; 4−10 min, 92−86% A; 10−30 min, 86−83.5% A; 30−35 min, 83.5−75% A; and 35−55 min, 75−20% A. The separated anthocyanins and protocatechuic acid were quantified using 520 and 280 nm, respectively. The identity of major anthocyanins and protocatechuic acid was based on the congruence of retention times and ultraviolet−visible (UV−vis) spectra with those of pure authentic standards. Cyanidin-3,5-diglucoside was used to quantify anthocyanins corresponding to cyanidin-dihexoses (peaks 2−4 in **Figure 2**). Cyanidin-3-glucoside was used to determine recoveries and for quantification of the anthocyanin corresponding to peak 6 in **Figure 2**. Cyanidin-3-glucoside and peonidin-3-glucoside were used for identification and quantification of anthocyanins corresponding to peaks 5 and 7 in **Figure 2**, respectively. External standard solutions were prepared in acidified methanol. The standard solutions ranged from 0 to 6 μg (40 μL injection volume). All anthocyanin and protocatechuic acid standard solutions exhibited linear relationships between area response and amount injected with the coefficients of determination (R^2) ranging from 0.9950 to 0.9999 for the anthocyanins and protocatechuic acid. Peaks corresponding to anthocyanins for which no authentic standard was available were quantified using either cyanidin-3-glucoside (monohexoses) or cyaniding-3,5-diglucoside (dihexoses).

Preparative HPLC. Prior to LC-(ESI)MS/MS analysis, the individual anthocyanins from raw black rice and a polyphenol fraction collected from pressure-cooked rice were isolated using a Phenomenex Prodigy ODS column (5 mm, 250 × 10.0 mm i.d; Phenomenex, Torrance, CA). The composition of the mobile phase was the same as that described as above. The flow rate was 3.0 mL, and the gradient was programmed as follows: 0−4 min, 100−92% A; 4−10 min, 92−86% A; 10−30 min, 86−83.5% A; 30−35 min, 83.5−75% A; and 35−55 min, 75−20% A. Fractions collected were applied to a preconditioned PrepSep C_{18} SPE cartridge (Fisher Scientific, Darmstadt, Germany). After flushing buffer salts from the C_{18} SPE cartridge with water, the phenolic compounds were eluted with 85% methanol. The eluate was concentrated under a stream of nitrogen gas and filtrated as described above.

Table 1. HPLC and MS Data of Anthocyanins and a Phenolic Compound Detected in Raw Black Rice

peak [a]	t_R (min)	λ_{max} (nm)	$[M]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	compound [b,c]
1	13.19	260, 295	155	111, 93	**protocatechuic acid**
2	14.74	262, 525	611	449, 287	cyanidin-dihexoside
3	16.18	279, 525	611	449, 287	cyanidin-dihexoside
4	18.81	278, 514	611	449, 287	cyanidin-dihexoside
5	21.19	279, 515	449	351, 287	**cyanidin-3-glucoside**
6	23.02	279, 525	449	351, 287	cyanidin-hexoside
7	29.43	279, 515	463	365, 301	**peonidin-3-glucoside**

[a] As identified by HPLC elution order in **Figure 2** (peaks 1−7). [b] Compounds in bold were positively identified using authentic standards. [c] Quantification of peaks 2−4 and peak 6 was achieved using the authentic cyanidin-3,5-diglucoside and cyanidin-3-glucoside, respectively.

LC-(ESI)MS/MS Identification. Confirmation of the identity of the components isolated by preparative HPLC was carried out using a Quattro electrospray ionization (ESI) MS/MS (Micromass, Altrincham, UK). Samples (20 μL) were analyzed using direct injection techniques. The mobile phase was 50% methanol in water, and the flow rate was 0.1 mL/min. The ESI interface was operated in positive ion mode using a cone voltage of 20 V, capillary voltage of 3.0 kV, and a collision energy of 12 eV. The ion source temperature was maintained at 140 °C and the desolvation gas temperature at 340 °C. The flow rates of nitrogen and cone gas were 460 L/h and 67 L/h.

Degradation of Cyanidin-3-glucoside. Cyanidin-3-glucoside was dissolved in distilled water with a content of 50 μg/mL. Aliquots of this solution were placed in vials and heated on a block heater at 100 °C. The heating was stopped at 15, 30, 60, 90, and 120 min, and the heated solution was dried under a steam of nitrogen. Products were resolved with HPLC using the mobile phase (A/B, 1:1, v/v) as described above.

Statistical Analysis. Statistical analysis was performed with SPSS 16.0 software (SPSS Inc., Chicago, IL). Data were subjected to analysis of variance using a general linear model to determine the difference among samples cooked by rice cooker and pressure cooker, or gas range cooking. A *t*-test was used to determine the differences between soaked and nonsoaked black rice.

RESULTS AND DISCUSSION

Identification of Anthocyanins in Black Rice. Spike and recovery experiments indicate that the recovery of cyanidin-3-glucoside was 97.3%, when 0.5 mg of authentic cyanidin-3-glucoside was added to 1 g of rice powder. Anthocyanins in the raw black rice extracts were analyzed with HPLC-PDA as shown in **Figure 2A**. Seven peaks were numbered corresponding to their order of elution. Peaks were identified by comparison of retention time (t_R), UV−vis absorption, and mass spectra with authentic anthocyanin standards. These results are summarized in **Table 1**.

Peak 5 (t_R 21.19) was the most abundant peak. MS/MS spectra indicate that this peak is cyandin-3-glucoside having a $[M+H]^+$ at *m*/*z* 449 and predominant fragment ion at *m*/*z* 287 resulting from the loss of hexose (162 Da). Additionally, the UV−vis spectrum gave identical absorption maxima at 279 and 515 nm and t_R as the authentic standard for cyandin-3-glucoside. Upon the basis of these results, peak 5 was positively identified as cyanidin-3-glucoside.

Peak 7 (t_R of 29.43) was the second most abundant peak in the extracts of raw black rice (**Figure 2A**). MS/MS spectra of

Table 2. Content (μg/g Dry Weight) of Anthocyanins and Protocatechuic Acid in Cooked Black Rice[a]

anthocyanin	raw	rice cooker	pressure cooker	gas range
protocatechuic acid	120.44 ± 2.81 c	395.64 ± 22.17 a	405.40 ± 26.90 a	321.87 ± 11.24 b
cyanidin-dihexoside	4.06 ± 0.12 a	3.03 ± 0.46 b	2.43 ± 0.25 c	2.98 ± 0.18 b
cyanidin-dihexoside	9.02 ± 0.41 a	0.91 ± 0.14 c	0.93 ± 0.14 c	1.84 ± 0.84 b
cyanidin-dihexoside	8.82 ± 0.46 a	4.56 ± 0.13 b	3.07 ± 0.31 c	4.50 ± 0.24 b
cyanidin-3-glucoside	572.47 ± 20.70 a	147.62 ± 10.01 c	115.68 ± 14.09 d	198.24 ± 12.66 b
cyanidin-hexoside	4.02 ± 0.11 a	0.50 ± 0.07 c	0.25 ± 0.34 c	0.76 ± 0.27 b
peonidin-3-glucoside	29.78 ± 2.21 a	10.93 ± 0.52 d	8.31 ± 0.89 c	13.18 ± 1.06 b

[a] Content of each anthocyanin and protocatechuic acid are reported as the mean ± SD ($n = 6$). Means ± SD followed by different letters in a row are significantly different in the Duncan's test at $P < 0.001$.

peak 7 indicated it was peonidin-3-glucoside with a $[M + H]^+$ at *m/z* 463 and fragment ions at *m/z* 301, formed by the loss of hexose (162 Da). The absorption maxima (279 and 515 nm) and t_R were consistent with the authentic standard for peonidin-3-glucoside. Upon the basis of these results, peak 7 was positively identified as peonidin-3-glucoside.

Peaks 2−4 were minor constituents that displayed a *m/z* of 611 $[M + H]^+$ and a predominant daughter ion at *m/z* 287 resulting from the consecutive losses of 2 hexose units. Of the cyanidin diglycosides found in foods, only cyanidin-3,5-diglucoside is currently available as an authentic standard. LC-(ESI)MS/MS analysis of the cyanidin-3,5-diglucoside standard indicated that this compound had a different t_R (16.81 min) than peaks 2, 3, and 4; however, it eluted near this group of peaks and gave the same pseudomolecular ion and fragment ions as peaks 2−4. Anthocyanins are usually monoglycosides, diglycosides, or triglycosides linked at C-3 and to a lesser extent at C-3,5 and C-3,7 positions (*20*). Our results suggest that peaks 2−4 are structural isomers of either cyanidin 3,3-, 3,5- or 3,7-diglycosides that may also differ in the composition of sugar moieties attached. UV−vis spectra indicated that peaks 2 and 3 had the same λ_{max} of 525 nm, whereas the λ_{max} for peak 4 was 514 nm. The λ_{max} of cyanidin-3,5-diglucoside is 515 nm. These results suggest that peak 4 may be structurally closer to cyanidin-3,5-diglucoside as compared to peaks 2 and 3.

UV−vis and mass spectral analysis of peak 6 (t_R 23.02 min, **Figure 2A**) indicated that it is a cyanidin-monohexoside. This peak eluted between cyanidin-3-glucoside and peonidin-3-glucoside and did not coelute with any of the authentic standards available to us. Therefore, we assume that peak 6 is an isomer of cyanidin-hexoside (glucose or galactose) with a different glycan binding site (i.e., C-5 or C-7). In an earlier study, Abdel-Aal et al. (*2*) reported the presence of cyanidin-3-galactoside, which eluted just before cyanidin-3-glucoside in black rice. However, in the current study we found no peak corresponding to the retention time of an authentic standard for cyanidin-3-galactoside. This discrepancy may have resulted from the analysis of different black rice varieties.

Quantitative values for the content of the anthocyanins corresponding to peaks 2−7 in raw and cooked black rice are given in **Table 2**. In raw rice, cyanidin-3-glucoside (572.47 μg/g; 91.13%) and peonidin-3-glucoside (29.78 μg/g; 4.74%) were the predominant anthocyanins. Anthocyanidin dihexosides (peaks 2−4 in **Figure 2A**) were quantified using cyanidin-3,5-diglucoside. Peak 6 (cyanidin-hexoside) was quantified using cyanidin-3-glucoside. The sum of peaks 2−4 and 6 amounted to less than 5% of the total anthocyanin content of rice. The total anthocyanin content of the raw black rice was approximately 630 μg/g dry weight. To date, there are five published studies investigating the content of anthocyanins in black rice (*2, 3, 21*−*23*). Abdel-Aal et al. (*2*) reported that the total anthocyanin content of black rice (variety not reported)

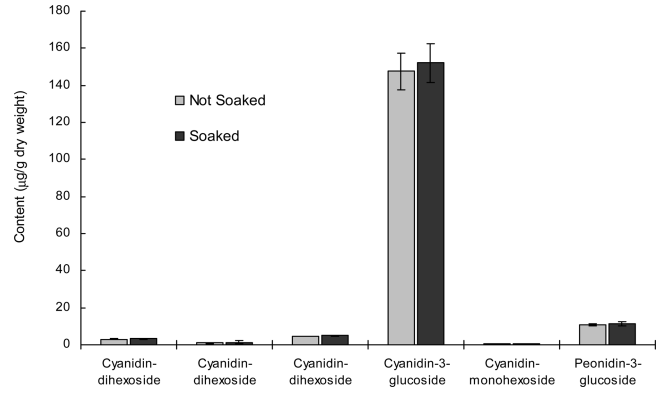

Figure 3. Effect of presoaking rice on the content of anthocyanins in cooked rice. Black rice was presoaked (soaked) in distilled water for 1 h prior to cooking in an electric rice cooker. Values are expressed as the mean ± SD ($n = 6$) on a dry weight basis.

was 2,284 μg/g and that it was composed of cyanidin-3-glucoside (2,013 μg/g; 88%), peonindin-3-glucoside (162 μg/g; 7.1%), two cyanidin-diglucosides (3.1% and 0.7%), and a cyanindin-3-rutinoside (0.9%) (*2*). Ryu et al. (*3*) screened 10 varieties of black rice and reported that the two predominant anthocyanins were cyanidin-3-glucoside (0.0−4700 μg/g) and peonindin-3-glucoside (0.0−400 μg/g); however, the moisture content was not shown. Cyanindin-3-glucoside (85%) and peonindin-3-glucoside (15%) were also identified in *Oryza sativa* L. *japonica* (*22*) and in *Oryza sativa* L. *indica* (*12*). Taken together, these data indicate that cyanindin-3-glucoside and to a lesser extent peonindin-3-glucoside are the predominant anthocyanins in Asian and Californian black rice and that the total amount of anthocyanins varies significantly across varieties. Our results indicated that the black rice variety grown in California had significantly lower levels of total anthocyanins as compared with the values reported for most of the Asian grown black rice varieties.

Effects of Cooking on Anthocyanins in Black Rice. To investigate the influence of presoaking rice on anthocyanin retention and stability, rice samples were presoaked in 135 g of water for 1 h prior to cooking in the rice cooker. Quantitative comparisons of anthocyanins in presoaked and nonsoaked rice indicate that soaking has no significant impact on anthocyanin stability or retention (**Figure 3**).

The six anthocyanins identified were quantified in extracts of rice thermally heated using either a commercial rice cooker, pressure cooker, or gas range (**Table 2**). The content of cyanidin-3-glucoside decreased significantly across all cooking methods (**Figures 4** and **5**). Pressure cooking resulted in the greatest decreases (79.8%), followed by the rice cooker (74.2%) and gas range (65.4%). Peonidin-3-glucoside decreased in these samples in the same manner; however, total losses were lower as compared with cyanidin-3-glucoside. This is contrary to the results of Xu et al. (*24*) who indicated that 100% of peonidin-

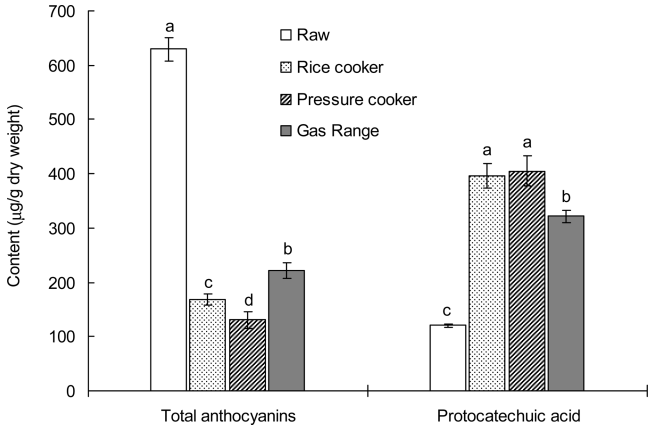

Figure 4. Content (μg/g dry weight) of total anthocyanins and protocatechuic acid in rice cooked with a rice cooker, pressure cooker, or on a gas range. Values are expressed as the mean ± SD ($n = 6$) on a dry weight basis. Significant differences ($P < 0.001$) were determined using Duncan's test and are noted by letters.

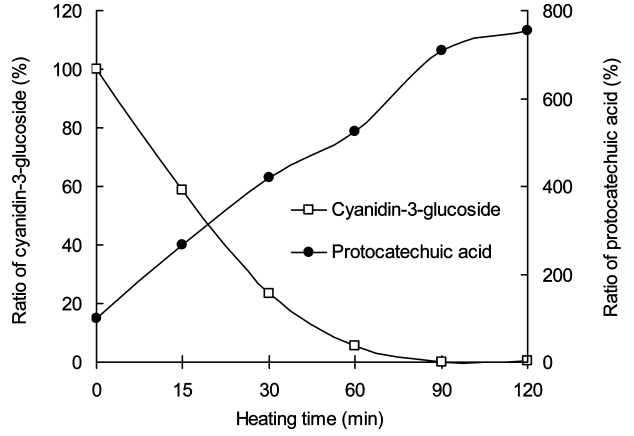

Figure 5. Changes in cyanidin-3-glucoside and protocatechuic acid during thermal treatment. Cyanidin-3-glucoside dissolved in distilled water (50 μg/mL) was heated at 100 °C for 0, 15, 30, 60, 90, and 120 min. Values represent the percentage of retention as compared to starting material.

3-glucoside was lost in black soybean during processing, although the processing conditions used were harsher than those used in the current study. The anthocyanidin dihexoside corresponding to peak 2 showed the highest thermal stability of all anthocyanidins. It is likely because anthocyanin stability increases with increasing number of methoxyl groups in the B-ring and decreases as the number of free hydroxyl groups in the B-ring increase. Glycosylation and acylation increases anthocyanidin stability, and correspondingly, disaccharides are more stable than their monosaccharide counterparts (*25*).

The retention of natural pigments after thermal treatment is an important quality parameter. The content of total anthocyanins in raw rice was approximately 630 μg/g dry weight, whereas the total content in cooked rice ranged from 130.67−221.50 μg/g dry weight (21−35% of raw rice anthocyanins), indicating that there was significant loss of anthocyanins during thermal processing (**Figure 4**). In the study of the effect of cooking on anthocyanins in violet cauliflower (*26*), microwave heating as a cooking method retained 95.4% of total anthocyanins in the raw sample, whereas blanching for 12 min in 0.5% NaCl resulted in only 19.8% retention of anthocyanins in the raw sample (*26*). Therefore, we attempted to control the cooking of black rice in 3 different commercially available microwave-specific rice cookers but found none to be suitable for replicate analytical analyses. Boiling rice on the gas range at low temperatures resulted in the highest retention of total anthocyanins as compared to cooking with either the rice cooker or a higher pressure cooker. These results were also apparent for the individual anthocyanins cyanidin-3-glucoside and peonidin-3-glucoside (**Table 2**). These results are consistent with other reports demonstrating that stability of anthocyanins in cooked foods is predominantly dependent on the temperature at which the food is cooked (*27, 28*). These findings suggest that the loss of anthocyanins in black rice may be attributed to the degradation or decomposition of anthocyanins arising from thermal processing.

During the course of the cooking trial, we noticed that the peak corresponding to cyanidin-3-glucoside (peak 5; **Figure 2A**) decreased dramatically in the cooked rice and that there was a concomitant increase in peak 1, which eluted at 13.19 min (**Figure 2B**). LC-(ESI)MS/MS was used to elucidate the structure of this compound, which demonstrated a pseudomolecular ion of m/z 155 $[M + H]^+$ and gave two predominant fragment ions at m/z 111 and m/z 93 (**Table 1**). Sadilova et al. (*29*) demonstrated that cyanidin-glycosides undergo deglycosylation during heating and produce cyanidin, which is then further degraded into phloroglucinaldehyde and protocatechuic acid. Although phloroglucinaldehyde and protocatechuic acid have the same molecular weight (154 Da), HPLC t_R and MS/MS fragmentation patterns can be used to distinguish the two compounds. The t_R and fragment ions observed in this study corresponded to protocatechuic acid. Correspondingly, the content of total anthocyanins in the cooked rice were almost one-third lower than that in raw rice, whereas the levels of protocatechuic acid increased about three times after cooking (**Figure 4**). These results suggest that the cyanidin-3-glucoside in black rice is degraded into protocatechuic acid (B ring) during cooking. To confirm this observation, in a separate study cyanidin-3-glucoside was heated at 100 °C and the degradation products were monitored by HPLC. Our results indicate that protocatechuic acid was the primary product formed and that its formation increased with heating time (**Figure 5**). Interestingly, phloroglucinaldehyde was not detected in cooked black rice, although it may have been present below the limit of detection. Similarly, Sadilova et al. (*29*) found that phloroglucinaldehyde content was lower than protocatechuic acid when black carrot extract was heated, although the reason for this has not yet been determined.

The aglycone cyanidin was not detected in the present study. However, the pH of the cooking solution was likely involved in the rapid conversion of cyanidin into protocatechuic acid. In earlier studies, Sadilova et al. demonstrated that heating anthocyanins at pH 1 stabilizes the flavylium cation and that cyanidin could be measured in samples heated for 6 h at 95 °C (*30*). In our study, samples were cooked in neutral water. Under these conditions the flavylium cation is likely not stable and is rapidly transformed into the scission product protocatechuic acid (**Figure 6**). Although protocatechuic acid is an in vitro antioxidant (*31*), data on its biological activity are contradictory as it is reported to have both antitumor and tumor-promoting activity (*32, 33*).

Our results indicate that the cyanidin-3-glucoside and peonidin-3-glucoside are the predominant anthocyanins in California black rice and that this rice contains minor amounts of cyanidin-dihexosides. Of the cooking methods evaluated, pressure cooking resulted in the highest loss of total anthocyanins, followed by the rice cooker and gas range methods. Levels of cyanidin-3-glucoside decreased with concomitant increases in protocatechuic acid across all cooking methods. These results

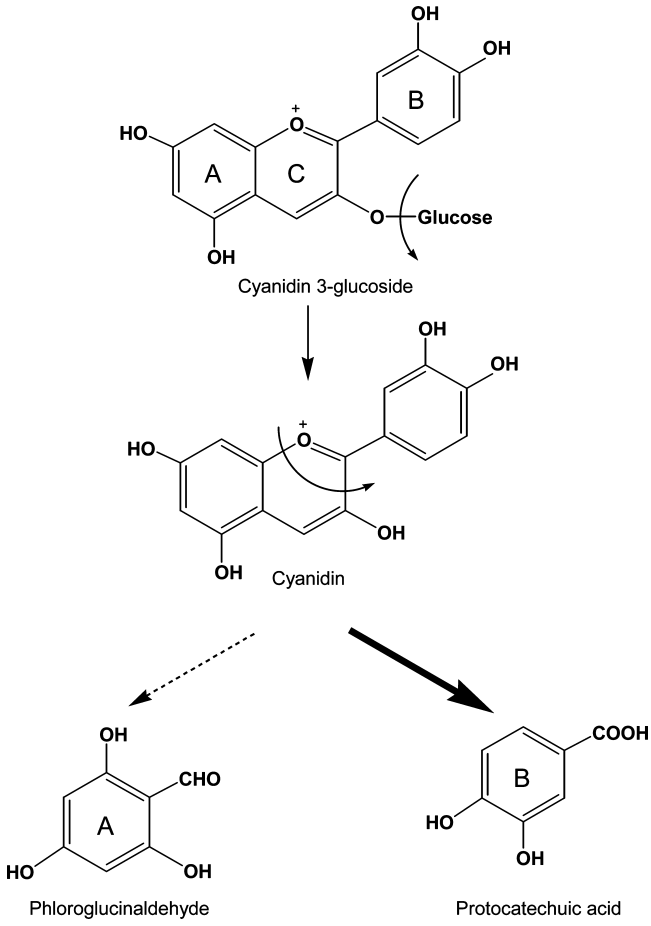

Figure 6. Schematic of the thermal degradation of cyanidin-3-glucoside in black rice.

suggest that cyanidin-3-glucoside in black rice is degraded predominantly into protocatechuic acid during cooking.

ABBREVIATIONS USED

HPLC-PDA, high-performance liquid chromatograph-photo diode array detector; LC-(ESI)MS/MS, electrospray ionization−tandem mass spectrometry; SD, standard deviation; UV−vis, ultraviolet−visible; t_R, retention time; λ_{max}, maximum absorption.

LITERATURE CITED

(1) Clampett, W. S.; Nguyen, V. N.; Tran, D. V. *The Development and Use of Integrated Crop Management for Rice Production*; International Rice Commission, FAO: Bangkok, Thailand, July 23−26, 2002.

(2) Abdel-Aal, E.-S. M.; Young, J. C.; Rabalski, I. Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *J. Agric. Food Chem.* **2006**, *54*, 4696–4704.

(3) Ryu, S. N.; Park, S. Z.; Ho, C.-T. High performance liquid chromatographic determination of anthocyanin pigments in some varieties of black rice. *J. Food Drug Anal.* **1998**, *6*, 729–736.

(4) Cho, M. H.; Paik, Y. S.; Yoon, H. H.; Hahn, T. R. Chemical structure of the major color component from a Korean pigmented rice variety. *Agric. Chem. Biotech.* **1996**, *39*, 304–308.

(5) Ha, T. Y.; Park, S. H.; Lee, C. H.; Lee, S. H. Chemical composition of pigmented rice varieties. *Korean J. Food Sci. Technol.* **1999**, *31*, 336–341.

(6) Zhang, M. W.; Guo, B. J.; Peng, Z. M. Genetic effects on grain characteristics of *indica* black rice and their uses on indirect selections for some mineral element contents in grains. *Genet. Resour. Crop Evol.* **2005**, *52*, 1121–1128.

(7) Itani, T.; Tamaki, M.; Arai, E.; Horino, T. Distribution of amylase, nitrogen, and minerals in rice kernels with various characters. *J. Agric. Food Chem.* **2002**, *50*, 5326–5332.

(8) Stintzing, F. C.; Carle, R. Functional properties of anthocyanidins and beatalins in plants, foods, and in human nutrition. *Trends in Food Sci. Technol.* **2004**, *15*, 19–38.

(9) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Concentration of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agric. Food Chem.* **2006**, *54*, 4069–4075.

(10) Ichikawa, H.; Ichiyanagi, T.; Xu, B.; Yoshii, Y.; Nakajima, M.; Konishi, T. Antioxidant activity of anthocyanin extract from purple black rice. *J. Med. Food.* **2001**, *4*, 211–218.

(11) Kim, M. J.; Hyun, J. N.; Kim, J. A.; Park, J. C.; Kim, M. Y.; Kim, J. G.; Lee, S. J.; Chun, S. C.; Chung, I. M. Relationship between phenolic components, anthocyanins content and antioxidant activity in colored barley germplasm. *J. Agric. Food Chem.* **2007**, *55*, 4802–4809.

(12) Hu, C.; Zawistowski, J.; Ling, W.; Kitts, D. D. Black rice (*Oryza sativa* L. *indica*) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems. *J. Agric. Food Chem.* **2003**, *51*, 5271–5277.

(13) Xia, X.; Ling, W.; Ma, J.; Xia, M.; Hou, M.; Wang, Q.; Zhu, H,; Tang, Z. An anthocyanin-rich extract from black rice enhances atherosclerotic plaque stabilization in apolipoprotein E-deficient mice. *J. Nutr.* **2006**, *136*, 2220–2225.

(14) Chen, P. N.; Kuo, W. H.; Chiang, C. L.; Chiou, H. L.; Hsieh, Y. S.; Chu, S. C. Black rice anthocyanins inhibit cancer cells invasion via repressions of MMPs and u-PA expression. *Chem.-Biol. Interact.* **2006**, *163*, 218–229.

(15) Guo, H.; Ling, W.; Wang, Q.; Liu, C.; Hu, Y.; Xia, M. Effect of anthocyanin-rich extract from black rice (*Oryza sativa* L. *indica*) on hyperlipidemia and insulin resistance in fructose-fed rats. *Plant Foods Hum. Nutr.* **2007**, *62*, 1–6.

(16) Kwon, S. H.; Ahn, I. S.; Kim, S. O.; Kong, C. S.; Chung, H. Y.; Do, M. S.; Park, K. Y. Anti-obesity and hypolipidemic effects of black soybean anthocyanins. *J. Med. Food* **2007**, *10*, 552–556.

(17) Sasaki, R.; Nishimura, N.; Hoshino, H.; Isa, Y.; Kadowaki, M.; Ichi, T.; Tanaka, A.; Nishiumi, S.; Fukuda, I.; Ashida, H.; Horio, F.; Tsuda, T. Cyanidin 3-glucoside ameliorates hyperglycemia and insulin sensitivity due to down regulation of retinol binding protein 4 expression in diabetic mice. *Biochem. Pharmacol.* **2007**, *74*, 1619–1627.

(18) Tian, S.; Nakamura, K.; Kayahara, H. Analysis of phenolic compounds in white rice, brown rice, and germinated brown rice. *J. Agric. Food Chem.* **2004**, *52*, 4808–4813.

(19) Delgado-Vargas, F.; Jimenez, A. R.; Paredes-Lopez, O. Natural pigments: carotenoids, anthocyanins, and betalains-characteristics, biosynthesis, processing, and stability. *Crit. Rev. Food Sci. Nutr.* **2000**, *40*, 173–289.

(20) Košir, I. J.; Lapornik, B.; Andrenšek, S.; Wondra, A. G.; Vrhovšek, U.; Kidrič, J. Identification of anthocyanins in wines by liquid chromatography, liquid chromatography-mass spectrometry and nuclear magnetic resonance. *Anal. Chim. Acta* **2004**, *513*, 277–282.

(21) Ono, K.; Huang, S. A. Identification of anthocyanins in Japanese black rice. *Gifu City Women's Res. Bull.* **2001**, *51*, 135–138.

(22) Robert, Y.; Shinji, T.; Naofumi, M. Identification of phenolic components isolated from pigmented rices and their aldose reductase inhibitory activities. *Food Chem.* **2007**, *101*, 1616–1625.

(23) Abdel-Aal, E.-S. M.; Hucl, P. A rapid method for quantifying total anthocyanins in blue aleurone and purple pericarp wheats. *Cereal Chem.* **2003**, *51*, 2174–2184.

(24) Xu, B.; Chang, S. K. C. Total phenolics, phenolics acids, isoflavones, and anthocyanins and antioxidant properties of yellow and black soybeans as affected by thermal processing. *J. Agric. Food Chem.* **2008**, *56*, 7165–7175.

(25) Francis, F. J. Food colorants: anthocyanins. *Crit. Rev. Food Sci. Nutr.* **1989**, *28*, 273–314.

(26) Scalzo, R. L.; Genna, A.; Branca, F.; Chedin, M.; Chassaigne, H. Anthocyanin composition of cauliflower (*Brassica oleracea* L. var. botrytis and cabbage (*B. oleracea* L. var. capitata) and its stability in relation to thermal treatments. *Food Chem.* **2008**, *107*, 136–144.

(27) Abdel-Aal, E.-S. M.; Hucl, P. Composition and stability of anthocyanins in blue-grained wheat. *J. Agric. Food Chem.* **2003**, *51*, 2174–2180.

(28) Cabrita, L.; Fossen, T.; Andersen, O. M. Color and stability of the six common anthocyanidin 3-glucosides in aqueous solution. *Food Chem.* **2000**, *68*, 101–107.

(29) Sadilova, E.; Stintzing, F. C.; Carle, R. Thermal degradation of acylated and nonacylated anthocyanins. *J. Food Sci* **2006**, *71*, 504–512.

(30) Wende, L.; Mark, D. P.; Trust, B. Effect of thermal processing on antioxidant properties of purple wheat bran. *Food Chem.* **2007**, *104*, 1080–1086.

(31) Masella, R.; Modesti, C. D.; Gennaro, C. L.; Coni, B. E. Antioxidant activity of 3,4-DHPEA-EA and protocatechuic acid: a comparative assessment with other olive oil biophenols. *Redox Rep.* **1999**, *4*, 113–121.

(32) Babich, H.; Sedletcaia, A.; Kenigsberg, B. In vitro cytotoxicity of protocatechuic acid to cultured human cells from oral tissue: involvement in oxidative stress. *Pharmacol. Toxicol.* **2002**, *91*, 245–53.

(33) Nakamura, Y.; Torikai, K.; Ohto, Y.; Murakami, A.; Tanaka, T.; Ohigashi, H. A simple phenolic antioxidant protocatechuic acid enhances tumor promotion and oxidative stress in female ICR mouse skin: dose-and timing-dependent enhancement and involvement of bioactivation by tyrosinase. *Carcinogenesis* **2000**, *21*, 1899–907.

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