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Lipophilic and Hydrophilic Antioxidants and Their Antioxidant Activities in Purple Rice Bran

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Purple rice bran was separated and collected at two different milling periods, resulting in two bran (outer and inner layer) fractions. The distribution of lipophilic and hydrophilic antioxidants and their antioxidant activities in the two fractions were studied. The bran fractions were extracted with hexane followed by methanol to obtain lipophilic and hydrophilic extracts, respectively. The total phenolic content and free radical scavenging activity of the extracts were determined and compared. The lipophilic extract from the outer bran fraction (OBF) exhibited a lower level of total tocols and γ-tocols, compared with the inner bran fraction (IBF), while the levels of γ-oryzanol in both fractions were not different. However, the lipophilic phenolic content and free radial scavenging activity of the OBF were 6.0 μg catechin equivalent (CE)/g and 5.6 μmol trolox equivalent (TE)/g and higher than those of the IBF, respectively. For the hydrophilic extracts, the level of anthocyanins in the IBF (29.0 mg/g) was 8 times higher than that in the OBF. Also, the hydrophilic phenolic content and free radical scavenging activity of the IBF were 489.1 μg CE/g and 433.6 μmol TE/g, respectively, while they were 113.9 μg CE/g and 78.2 μmol TE/g in the OBF. Both hydrophilic extracts showed significantly higher phenolic content and free radical scavenging activity than any lipophilic extract. The results of this study indicated that the activity of purple rice bran hydrophilic antioxidants was much greater than that of its lipophilic antioxidants and anthocyanins and γ-tocols largely located in the inner portion of purple rice bran.

KEYWORDS: Anthocyanin; rice; antioxidant; bran; mill

INTRODUCTION

The term "rice bran" generally refers to the bran milled off from brown rough rice for producing edible white rice kernel. It is considered as a waste product traditionally, although it makes up to 10% of rough rice and is a rich natural source of the important lipophilic antioxidant vitamin E (*1*). Besides α-tocopherol and γ-tocopherol, the major components of vitamin E, rice bran contains higher levels of α-tocotrienol and γ-tocotrienol, which are not found or are present in much lower amounts in most grains and cereals (*2, 3*). It is also a unique source of γ-oryzanol, which is a mixture of 10 lipophilic phytosterols (*2, 3*). These compounds have been reported to possess the capability of antioxidation and lowering LDL (low density lipoprotein) cholesterol levels (*4*−*6*). Although brown rice dominates the whole world rice production, there are some special varieties of rice that contain other color pigments, such as purple and red rice. They are mainly produced in East and Southeast Asia and are used for food ornamentation purposes.

Anthocyanins, hydrophilic pigments that are responsible for the red and purple color of most fruits and vegetables, contribute to the purple and red color of the colored rice (*7, 8*). The health functions of anthocyanins from berries in prevention of various diseases, such as visual and vascular diseases, obesity, and certain cancers, have been observed by a number of studies (*9*−*12*). Anthocyanins from purple rice were also reported to have the capability of preventing progression of atherosclerosis in a mouse model and a human study (*13, 14*). However, the colored rice or rice bran sample used in those studies was usually from the East Asia region. Information on vitamin E, γ-oryzanol, anthocyanins, and antioxidant activity in the colored rice harvested in the U.S. is limited. In this study, lipophilic and hydrophilic antioxidants including tocols, γ-oryzanol, and anthocyanins in the rice bran obtained from rough purple rice harvested in the Southern U.S. were characterized. Recently, several studies have demonstrated that some major lipophilic antioxidants in brown rice bran were not distributed uniformly in the different bran layers obtained from different milling times (*15, 16*). Their distributions and antioxidant activities in the outer and inner purple rice bran layers were determined and compared in this study. Thus, the study results would provide a more comprehensive understanding of the antioxidants and their distribution in purple rice bran layers. It could be used to obtain the purple rice bran portion containing highly valuable anthocyanins and antioxidants through rice milling optimization and develop value-added utilization methods for purple rice and rice bran. For example, the purple rice bran could become a good material used in health promoting cereal products. As

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consumers become increasingly concerned about the safety of synthetic colorants used in foods, the trend of using natural colorants in food products has increased intensively. The purple rice bran portion with highly concentrated anthocyanins may have the potential to replace purple or red artificial colorants and antioxidants in some food products. Also, the low moisture and cost of purple rice bran could give the bran an advantage over berries to be an inexpensive source of anthocyanins for food applications.

MATERIALS AND METHODS

Chemicals. HPLC grade acetonitrile and methanol were purchased from Fisher Chemicals (Fair Lawn, NJ). Diphenyl-l-picrylhydrazyl (DPPH), trolox, catechin, α-tocopherol and -tocotrienol, γ-tocopherol and -tocotrienol, cyanin chloride, cyanidin chloride, and peonidin chloride were purchased from Sigma-Aldrich (St. Louis, MO). γ-Orzyanol standard was prepared in our laboratory as described by Xu and Godber (*2*).

Preparation of Rice Bran Samples. Rough purple rice was a gift from Louisiana Rice Research Station (Louisiana State University Agricultural Center, Crowley, LA). The rough rice sample of 150 g was milled for 60 s using a McGill No. 2 mill (McGill, Brookshire, TX). Rice bran collected at the milling time between 0 and 40 s was used as the OBF sample. Then, the bran collected between 40 and 60 s was used as the IBF sample. The mill processing was performed in triplicate to get three OBF and IBF samples, respectively.

Extraction of Lipophilic Antioxidants Using Hexane. The extraction for each sample was performed in duplicate. One gram of each bran sample was transferred into a test tube (25 × 150 mm) to which hexane (3 mL) was added, and the mixture was vortex mixed for 30 s. The test tubes were capped and placed in a 60 °C water bath for 20 min. These test tubes were vortex mixed twice during the incubation. Then, the hexane layer in each tube was separated by centrifugation at 2000*g* for 15 min. The solvent supernatant was transferred to a previously weighed clean test tube. The residue was again mixed with 3 mL of hexane. The supernatant was separated as previously described and combined with the previous supernatant. The tube containing supernatant was then placed in a vacuum centrifuge evaporator (CentriVap Mobile System; Labconco, Kansas City, MO) to remove solvent. The dried extract in the test tube was weighed to measure its lipophilic extraction yield. The defatted bran residue was spread on aluminum foil and placed under a laboratory hood to dry.

Extraction of Hydrophilic Antioxidants Using Methanol. Each defatted bran (0.5 g) obtained from the hexane extraction was mixed with methanol (3 mL) to perform hydrophilic antioxidants extraction. The procedure of this extraction was the same as the lipophilic antioxidants extraction, except methanol was used as the extraction solvent. The dried extract in the test tube was weighed to measure its extraction yield of hydrophilic extraction as well.

Determination of Tocopherols, Tocotrienols, and γ-Oryzanol Content Using HPLC. The tocopherols, tocotrienol, and γ-oryzanol concentrations were determined using the method of Bergman and Xu (*17*). The HPLC system consisted of Waters (Milford, MA) 510 pumps, a 715 Ultra WISP injector, and 410 UV and 470 fluorescence detectors. Chromatograms were recorded and processed using Waters Millennium chromatography software. Samples were injected into a 25 cm × 4.6 mm diameter 5-μm Supelcosil LC-Si (Supelco, Bellefonte, PA) column. The column was preceded by a 5 cm × 4.6 mm i.d. guard column packed with 40-μm pellicular silica. The mobile phase consisted of 0.5% ethyl acetate and 0.5% acetic acid in hexane at a flow rate of 1.5 mL/min. The fluorescence detector was set at 290 nm excitation and 330 emission to monitor tocopherols and tocotrienols. The UV detector was set at 330 nm for γ-oryzanol. The dried extract obtained from the lipophilic antioxidants extraction was dissolved in hexane (0.020 g/mL) and vortexed. One hundred microliters of the solution was injected into the HPLC system. Each tocopherol, tocotrienol, and γ-oryzanol concentration was calculated based on the standard curve. The total tocols content was calculated by summing the α- and γ-tocopherol and α- and γ-tocotrienol concentrations and converting to μg/g of bran.

Determination of Anthocyanins Using HPLC. The extract obtained from the hydrophilic antioxidants extraction in the test tube was diluted to be 0.020 g/mL using methanol. Anthocyanins in the extract were quantified using an analytical HPLC system. The HPLC system consisted of a Supelco (Bellefonte, PA) Discovery C18 column (3 mm i.d. × 25 cm), a Waters 2690 separation module, a 996 photodiode array detector, and a Millennium32 chromatography manager. The mobile phase was a mixture of A (0.4% TFA in water) and B (acetonitrile), with the percentage of A = 0.4% TFA in water ramped from 100% to 55% in 45 min with a constant flow rate of 0.8 mL/min. The chromatogram obtained at a wavelength of 520 nm was used to quantify the anthocyanins. The concentration of each anthocyanin was calculated based on its standard curve. The total anthocyanin content was calculated by summing each anthocyanin and converting to μg/g of bran.

Determination of Total Phenolic Content. The Folin−Ciocalteau reagent method was employed to determine the total phenolic content in the rice bran extracts (*18*). The Folin−Ciocalteau reagent was diluted 10 times with deionized water. The bran extracts were redissolved in 6.0 mL of methanol, and 0.1 mL of this solution was placed in the pasteurized test tube, which contained 0.75 mL of diluted Folic−Ciocalteau reagent. The reaction was carried out at 25 °C for 5 min in a dark room. Then 0.75 mL of sodium bicarbonate solution (60 g/L) was added. The mixture was incubated at 25 °C for 90 min and filtered through a 0.45 μm syringe filter (Pall Corp., Ann Arbor, MI). The absorbance of the filtered solution was determined at 750 nm using a UV−visible SpectraMax Plus384 spectrophotometer (Molecular Devices, Sunnyvale, CA). Catechin was used to prepare a standard curve. The total phenolic content of the extract was calculated and expressed as μg catechin equivalent (CE)/g of bran.

Determination of Antioxidant Activity Using the DPPH Free Radical Scavenging Method. The DPPH free radical scavenging capability in the extract was determined using the method of Yue and Xu (*19*). The DPPH reagent (0.025 g) was dissolved in 1000 mL of methanol for preparing the DPPH reagent solution. The bran extract solution which was reconstituted with 6.0 mL of methanol for being employed to measure total phenolic content was used for the DPPH free radical scavenging test. Two milliliters of DPPH solution was mixed with 50, 100, and 150 μL of the extract/methanol solution and transferred to a spectrophotometer cuvette. The reaction solution was carried out at 25 °C for 30 min in a dark room. Then the absorbance of the reaction mixture was monitored at 515 nm using a UV−visible SpectraMax Plus384 spectrophotometer. The inhibition percentage of the absorbance of the DPPH solution was calculated using the following equations:

$$\text{inhibition } \% = [(\text{Abs } t_{0\ \text{min}} - \text{Abs } t_{30\ \text{min}})/\text{Abs } t_{0\ \text{min}}] \times 100$$

where Abs $t_{0\ \text{min}}$ was the absorbance of DPPH at zero time and Abs $t_{30\ \text{min}}$ the absorbance of DPPH after 30 min of incubation for the reaction.

The inhibition percentage of the absorbance of DPPH was plotted against each quantity of the extract solution to obtain a regression line. Trolox (0.5 mM) in methanol was used as a standard to convert the inhibition capability of the extract solution to the trolox equivalent antioxidant activity. The ratio of the slopes of the regression lines of the extract solution and the Trolox solution was defined as the trolox equivalent antioxidant activity. Then, it was converted to μmol of trolox equivalent (TE)/g of bran.

Statistical Analysis. The determination of the tocols, γ-oryzanol, anthocyanins, and total phenolic contents and DPPH free radical scavenging test were duplicated for each extract. The means and standard deviations were calculated and the data were analyzed by one-way ANOVA to evaluate the significant difference at $P < 0.05$ (SAS 9.1.3, Cary, NC).

RESULTS AND DISCUSSIONS

Extraction Yield, Total Phenolic Content, and DPPH Free Radical Scavenging Activity of the Lipophilic Extracts from the OBF and IBF. Table 1 lists the yields of lipophilic

Table 1. Yields, Total Phenolic Content, and DPPH Free Radical Scavenging Capability (TEAC) of the OBF and IBF ($n = 3$)[a]

	lipophilic extract		hydrophilic extract	
	OBF	IBF	OBF	IBF
yield (%)	13.5 ± 0.4[a]	14.1 ± 0.2[a]	10.8 ± 0.1[b]	11.0 ± 0.3[b]
total phenolic content (μg of catechin equiv/g)	6.0 ± 0.1[a]	2.7 ± 0.1[b]	113.9 ± 1.9[c]	489.1 ± 3.8[d]
TEAC (μmol of trolox equiv/g)	5.6 ± 0.2[a]	4.6 ± 0.3[b]	78.2 ± 2.5[c]	433.6 ± 9.4[d]

[a] OBF, outer bran fraction; IBF, inner bran fraction. Significant difference ($P < 0.05$) between two data points in a row is expressed by different letters.

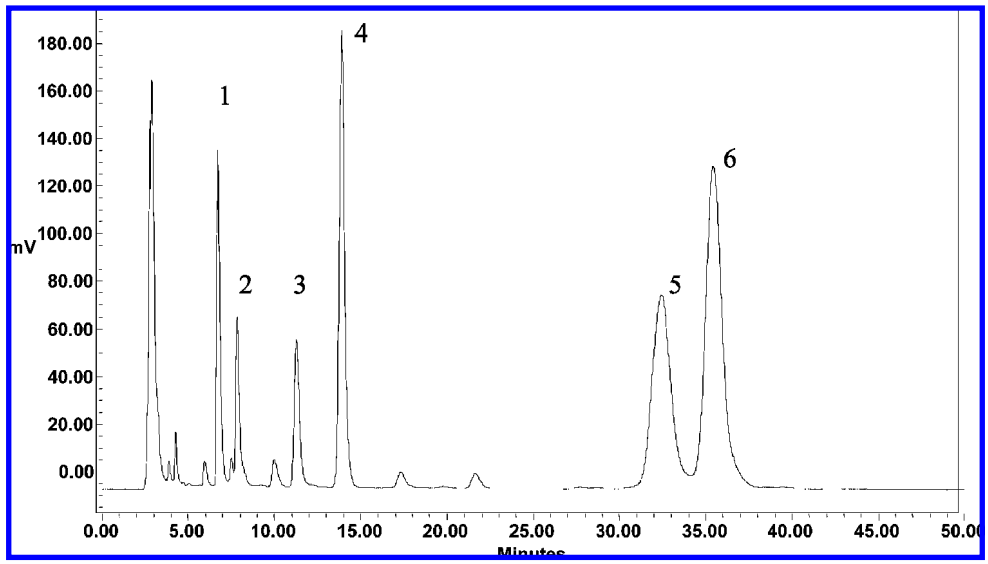

Figure 1. Typical chromatogram of tocols and γ-oryzanol in the hexane extract obtained from purple rice bran: (1) α-tocopherol; (2) α-tocotrienol; (3) γ-tocopherol; (4) γ-tocotrienol; (5 and 6) γ-oryzanol.

extraction from the two bran fractions, which were 13.5% for the OBF and 14.1% for the IBF, and there was no significant difference between them. The yield of lipid content in the two purple bran fractions was in the range of the yields obtained from different varieties of brown rice bran, which was from 12 to 20% (*1*). It was also similar to the case of brown rice bran that the lipid content in the outer and inner layer fractions was not different (*15*). However, the total phenolic content (6.0 μg of catechin equivalent/g) in the OBF was about twice as high as that in the IBF (**Table 1**). Meanwhile, the free radical scavenging capability of the OBF (5.6 μmol of trolox equivalent/g of bran) was slightly higher than that of the IBF (4.6 μmol of trolox equiv/g of bran). This indicated that the outer layer fraction of purple rice bran contained more lipophilic phenolic compounds than the inner layer fraction, although the lipid content of the OBF and IBF did not show a significant difference. In the study of Abdul-Hamid et al., they also found that the level of lipophilic phenolic compounds, carotenoids, in the outer brown rice bran layer was four times higher than that in its inner rice bran layer (*15*). Lipophilic antioxidants concentrated in the outer rice bran layer may be beneficial to enhance the rice grain in defending against UV irradiation and a high moisture growth environment.

Tocopherols, Tocotrienols, and γ-Oryzanol in the OBF and IBF. A typical HPLC chromatogram of lipophilic extract from the purple rice bran is shown in **Figure 1**. Common lipophilic antioxidants in rice bran, α-tocopherol, α-tocotrienol, γ-tocopherol, γ-tocotrienol, and γ-oryazanol were found in the OBF and IBF (**Table 2**). For α-tocopherol and α-tocotrienol, their levels in the OBF and IBF samples were not significantly different, while the level of α-tocopherol was higher than that of α-tocotrienol in both samples. However, the level of γ-tocophenol (66.3 μg/g of bran) or γ-tocotrienol (204.1 μg/g bran) in the IBF was significantly higher than that in the OBF.

Table 2. Tocols and γ-Oryzanol in the OBF and IBF ($n = 3$)[a]

	OBF	IBF
α-tocopherol (μg/g)	116.6 ± 8.5[a]	127.7 ± 4.3[a]
α-tocotrienol (μg/g)	52.4 ± 1.5[a]	56.2 ± 1.9[a]
γ-tocopherol (μg/g)	49.1 ± 1.7[a]	66.3 ± 2.0[b]
γ-tocotrienol (μg/g)	151.2 ± 9.4[a]	204.1 ± 3.2[b]
total tocols (μg/g)	369.3 ± 3.6[a]	454.3 ± 1.5[b]
γ-oryzanol (mg/g)	3.4 ± 0.2[a]	3.4 ± 0.1[a]

[a] OBF, outer bran fraction; IBF, inner bran fraction. Significant difference ($P < 0.05$) between two data points in a row is expressed by different letters.

The higher level of γ-tocopherol and -tocotrienol in the IBF resulted in higher total tocols content in the IBF than that in the OBF (**Table 2**). The total tocols contents in the IBF and OBF were 454.3 and 369.3 μg/g of bran, respectively. Although the total tocols contents in the inner and outer layer purple rice bran were different, both of them were at the median level of a range of total tocols content in brown rice bran, which was widely various from 200 to 900 μg/g in different varieties of brown rice harvested from the southern U.S., Pakistan, Venezuela, and Brazil (*20*−*23*). Chen and Bergman (*20*) reported that γ-tocotrienol was the dominant tocol in the brown rice bran harvested from the southern U.S. areas. Aguilar-Garcia (*22*) also found that total tocotrienols content was higher than total tocopherols content in the brown rice bran and γ-tocotrienol was the highest among all tocols. It is similar to the case for brown rice bran that γ-tocotrienol was the major tocol in both purple rice bran fractions of this study as well. For γ-oryzanol content, both outer and inner layer purple rice bran samples were 3.4 mg/g of bran. The value was also in a range of γ-oryzanol content of brown rice bran, which was reported from 1.5 to 6.9 mg/g (*21*−*23*). Thus, the levels of lipophilic antioxidants in purple and brown rice bran were not different,

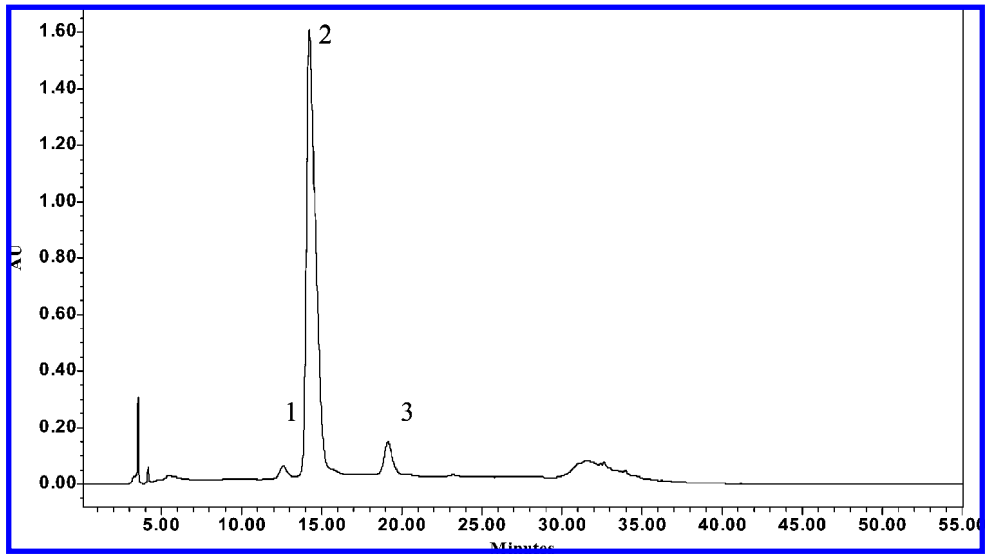

Figure 2. Typical chromatogram of anthocyanins in the methanol extract obtained from purple rice bran: (1) cyanidin-3-galactoside; (2) cyanidin-3-glucoside; (3) peonidin-3-glucoside.

even though they have different types of color.

Extraction Yield, Total Phenolic Content, and DPPH Free Radical Scavenging Activity of Hydrophilic Extracts from the OBF and IBF. The yields of hydrophilic extraction for the two defatted purple rice bran fractions were similar and 10.8% for the OBF and 11.0% for the IBF (**Table 1**). The total extraction or extractable (lipophilic and hydrophilic) content for the OBF or IBF was approximately 25%. Compared with the hexane extracts, methanol extracted larger amounts of phenolic compounds from the purple rice bran. The total phenolic content extracted by methanol was 113.9 (for the OBF) and 489.1 (for the IBF) μg catechin equiv/g of bran (**Table 1**). The OBF and IBF hydrophilic phenolic contents were approximately 20 and 180 times higher than its lipophilic phenolic contents extracted by hexane, respectively. This is in agreement with the fact that methanol was better than hexane in extracting phenolic antioxidants from cereal products (*24*). In that study, the total phenolic content in the extract obtained by methanol was much higher than that of the extract produced by hexane or acetone due to most phenolic compounds having higher polarity and more being extractable in polar solvent (*24*). Also, the DPPH free radical scavenging capability of the bran fraction with methanol extraction was 78.2 (for the OBF) and 433.6 (for the IBF) μmol trolox equiv/g of bran, which were 25 and 100 times higher than that obtained by hexane extraction, respectively. As antioxidant activity is generally correlated with the concentration of phenolic compounds in extracts (*25*), it suggests that the most active antioxidants in purple rice bran were hydrophilic antioxidants in the methanol extract. In contrast to the lipophilic antioxidants, the hydrophilic phenolic content and the antioxidant activity of the inner fraction (IBF) were much higher than those of the outer fraction (OBF). This was also different from the case of brown rice bran which was reported to have higher total phenolic content in the outer layer than in the inner layer (*15*). Thus, the level of total hydrophilic phenolic content and antioxidant activity in purple rice bran may be significantly associated with its unique hydrophilic antioxidants, anthocyanins. Nam et al. also found the extracts from colored rice bran had much higher antioxidant activity than noncolored rice bran (*26*).

Anthocyanins in the OBF and IBF. A typical HPLC chromatogram of the hydrophilic extract from the OBF or IBF bran is shown in **Figure 2**. Three anthocyanins, cyanidin 3-galactoside, cyanidin-3-glucoside, and penoidin 3-glucoside, were found in both extracts. Cyanidin-3-glucoside was the dominant anthocyanin and occupied over 90% of the total anthocyanin content. The total anthocyanin content in the OBF and IBF was 3.5 and 29.0 mg/g of bran, respectively (**Table 3**). Two anthocyanins, penoidin 3-glucoside and cyanidin-3-glucoside, were found in the pigment fraction of colored rice obtained from China in the study of Hu et al. (*7*). Abdel-Aal et al. (*27*) reported that the total anthocyanin content in different varieties of colored rice grain was in the range of 27.2 to 3276 μg/g of grain. The content in the polished purple rice kernel of this study was 1500 μg/g or 1.5 mg/g, which was over twice and twenty times lower than those in the OBF and IBF, respectively. Thus, anthocyanin in the rough purple rice was highly concentrated in the bran, especially in the inner layer bran. The dominant anthocyanin in purple rice bran, cyanidin-3-glucoside, was reported to have higher antioxidant activity in suppressing both reactive oxygen species and nitric oxide in chemical and biological model systems (*7*). The higher total phenolic content and antioxidant activity in the IBF (**Table 2**) may be significantly contributed by its higher anthocyanin content. Also, cyanidin-3-glucoside exhibited the highest bioactivity and bioavailability in anti-inflammatory and reducing obesity and diabetes diseases (*28*, *29*). The inner layer of purple rice bran could be an abundant and inexpensive source of cyanidin-3-glucoside and directly used in some health promoting food products. It could be utilized as a raw source to obtain higher purity anthocyanin powder, which is used as a food colorant or nutrition supplement as well.

In conclusion, the level of lipophilic antioxidants in purple rice bran, tocols and γ-oryzanol, was similar to that in brown rice bran. However, the level of hydrophilic antioxidants and their antioxidant activity in purple rice bran was significantly

Table 3. Anthocyanins in the OBF and IBF ($n = 3$)[a]

	OBF	IBF
cyanidin 3-galactoside (mg/g)	0.1 ± 0.1^a	0.5 ± 0.2^b
cyanidin 3-glucoside (mg/g)	3.2 ± 0.2^a	26.4 ± 0.5^b
peonidin 3-glucoside (mg/g)	0.2 ± 0.1^a	2.1 ± 0.2^b
total anthocyanins (mg/g)	3.5 ± 0.4^a	29.0 ± 0.9^b

[a] OBF, outer bran fraction; IBF, inner bran fraction. Significant difference ($P < 0.05$) between two data points in a row is expressed by different letters.

higher than that of lipophilic antioxidants. Anthocyanins in the purple rice bran contributed to the higher antioxidant activity of the hydrophilic extract than that of the lipophilic extract. Compared with the outer portion of purple rice bran, the inner portion of purple rice bran highly concentrated the health promoting compounds, anthocyanins and γ-tocols. The inner portion of rice bran with high anthocyanins content could be used for functional foods, such as extruded snacks, breakfast cereals, and baked cakes.

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