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Effect of degree of milling on phenolic profiles and cellular antioxidant activity of whole brown rice

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ABSTRACT

The impact of increasing degree of milling (DOM) on free and bound phenolics and flavonoids and on cellular antioxidant activity (CAA) of *japonica* and *indica* brown rice was investigated. As the average DOM increased from 0 to 2.67, 7.25 and 9.60%, the average total phenolic content decreased by 21.1, 42.6 and 55.6%, and the average total CAA value decreased by 37.4, 84.0 and 92.8%, respectively. Furthermore, the percentage contributions of bound forms to total phenolics and flavonoids decreased with increasing DOM. The contents of nine phenolic compounds significantly decreased with increasing DOM, including quercetin, ferulic and coumaric acids. Interestingly, as the DOM increased to 9.6%, free ferulic and coumaric acids were undetectable in *japonica* rice, while neither free nor bound caffeic acid was detectable in *indica* rice. These findings indicate that DOM should be carefully controlled for acceptable sensory quality and retention of phytochemicals during brown rice milling.

1. Introduction

Rice (*Oryza sativa* L.) is the staple food of more than half of the world's population. Whole brown rice is the unpolished whole grain, which are comprised of bran, embryo, and endosperm layers. Traditionally, whole brown rice is rarely eaten because of its poor sensory quality. Instead, it is milled into white rice by stripping away the embryo and bran layers. The degree of milling (DOM) is a critical parameter affecting both the sensory quality and nutrient content of rice, and the grading of rice in many countries is frequently based on DOM (Wang et al., 2011). In general, a higher DOM reflects superior sensory quality and consequently commands a higher market value (Lamberts et al., 2007).

It has been reported that the exclusive consumption of white rice in developing countries may lead to deficiencies in essential minerals, vitamins, protein, dietary fiber, and other nutrients (Bouis, Chassy, & Ochanda, 2003; Radhika, Van Dam, Sudha, Ganesan, & Mohan, 2009), as most of the nutritional components of brown rice are discarded during the milling process (Itani, Tamaki, Arai, & Horino, 2002; Lamberts et al., 2007). Recent research has focused on phytochemical contents in rice with different degrees of milling (DOMs). Shobana et al. (2011) evaluated phytochemicals including dietary fiber, γ-oryzanol, vitamin E, and total phenolics in two varieties of Indian rice with different DOMs, and a recent study investigated the effect of DOM on total phenolics, flavonoids, anthocyanins and proanthocyanidins in pigmented rice with different DOMs (Paiva et al., 2014). In addition, rice grains contain high amounts of several phenolic acids, such as ferulic and coumaric acids which are not present in significant quantities in fruits and vegetables (Bunzel, Ralph, Martia, Hatfield, & Steinhart, 2001; Liu, 2007). Therefore, further studies are required to investigate the effect of DOM on phenolic acids and individual flavonoids in brown rice.

With the increasing popularity of whole foods, whole brown rice and lightly milled rice have been used to produce whole

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grains-related products (Chung, Cho, & Lim, 2014). Increasing evidence indicates that whole grain consumption is associated with the prevention of chronic diseases, such as cancer and cardiovascular disease (Okarter & Liu, 2010), and these beneficial effects have been attributed in part to unique phytochemicals such as tocopherols, tocotrienols, vitamin B, γ-oryzanols, and phenolic compounds (Liu, 2007). Whole brown rice is an important source of two types of phenolic compounds that are beneficial to overall health (Friedman, 2013). These types include free phenolics, which can be extracted into solution, and significant amounts of insoluble phenolics which are often covalently conjugated to cell wall components via ester bonds (Tian, Nakamura, & Kayahara, 2004). Previous studies investigated the free and bound phenolics including flavonoids in rice grain of different bran colors (Irakli, Samanidou, Biliaderis, & Papadoyannis, 2012; Min, Gu, McClung, Bergman, & Chen, 2012). A recent study found that the contents of the free and bound phenolics in whole rice were significantly decreased after hydrothermal treatment (Scaglioni, Souza, Schmidt, & Badiale-Furlong, 2014). The bound phenolics are the major form found in rice bran, which is lost during the milling process (Shao, Xu, Sun, Bao, & Beta, 2014). Currently, the study of phenolics is an active area of research on rice, however, few studies have addressed changes in free and bound phenolics in brown rice that occur during the milling process.

Phenolic compounds in whole grains have potent antioxidant activity, which can be analyzed using various chemical methodologies. Finocchiaro et al. (2007) first reported the effects of DOM on the antioxidant activity of brown rice using the 2,2′-azinobis 3-ethylbenzothiazoline-6-sulphonate (ABTS) method. Recent studies have reported antioxidant activity in rice husks, bran and polished rice measured using various chemical assays (ABTS; ferric reducing antioxidant power, FRAP; and oxygen radical absorbance capacity, ORAC) (Butsat & Siriamornpun, 2010; Shao et al., 2014; Ti et al., 2014). However, these chemical methods are not suitable for accurate determination of antioxidant activity *in vivo*. For this purpose, the cellular antioxidant activity (CAA) assay is regarded as a more biologically relevant method than other widely used chemical methods, and has been used to investigate antioxidant activity in numerous fruits and vegetables (Song et al., 2010; Wolfe et al., 2008). More recently, Hirawan, Diehl-Jones, and Beta (2011) reported the use of CAA to determine antioxidant activity of purple wheat and red rice in the small intestine-derived cell line FHs 74 Int. However, further studies are necessary to assess the impact of increasing DOMs on the CAA of brown rice.

Polished rice is the most common type of rice consumed by humans (Deng et al., 2013). Of the two types of rice cultivated in China, *O. sativa* L. *japonica* is primarily consumed in the north, and *O. sativa* L. *indica* is mainly consumed in the south. In the present study, these two types of brown rice were successively milled for 10, 20, 30 s to obtain rice with three different DOMs, which was clarified as 3 grade rice, respectively, based on a grading standard of rice quality established by the China Department of Standardization (CDS, 2009). The objectives of this study were (1) to determine the total phenolic content, total flavonoid content, and phenolic composition of brown rice with different DOMs; (2) to investigate the effect of DOM on the percentage contribution of free and bound fractions to total phenolics; and (3) to evaluate the antioxidant activity of brown rice with different DOMs using the CAA assay.

2. Materials and methods

2.1. Chemicals and reagents

Methanol (MeOH), acetone, hexane, ethyl acetate, hydrochloric acid (HCl), sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), and sodium hydroxide (NaOH) of analytical grade were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). We purchased 2′,7′-dichlorofluorescein diacetate (DCFH-DA), phosphate-buffered saline (PBS), Folin–Ciocalteu reagent, quercetin dehydrate, acetonitrile (chromatographic grade), acetic acid (HAC, chromatographic grade), and catechin hydrate from Sigma–Aldrich, Inc. (St. Louis, MO, USA). Aluminum chloride ($AlCl_3{\cdot}6H_2O$, analytical grade) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Gallic acid was purchased from ICN Biomedicals, Inc. (Aurora, OH, USA). We purchased 2,2′-azobis(2-amidinopropane)dihydrochloride (ABAP) from Wako Chemicals, Inc. (Richmond, VA, USA). HepG2 human liver cancer cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Dulbecco's modified eagle's medium (DMEM) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), and Hanks' Balanced Salt Solution (HBSS) were purchased from Gibco Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Ferulic acid, coumaric acid, protocatechuic acid, vanillic acid, quercetin, (+)-catechin, isoferulic acid, caffeic acid and chlorogenic acid were purchased from Aladdin (Shanghai, China).

2.2. Grain samples and sample preparation

Grains of the *indica* cultivar Yinfengxue and the *japonica* cultivar Wujingyun 27, which are consumed primarily in southern and northern China, respectively, were obtained from the experimental farm of Guangdong Academy of Agricultural Sciences, China. The rice varieties used in this research were grown in the 2013 season, harvested directly from fields in July 2013, air-dried until their moisture content was reduced to approximately 12%, and stored at room temperature for 3 months. Brown rice samples were obtained by removing husks with a rice dehulling machine (Taizhou Grain Instrument Co. Ltd., Taizhou, China). Brown rice samples (100 g) were successively milled for 5, 10, 15, 20, 25, and 30 s using a Satake Rice Test Mill (Satake Co., Hiroshima, Japan) to obtain brown rice samples of increasing DOM. DOM was calculated based on the weight of rice bran and whole brown rice using the following equation: DOM = [1 − (weight of milled rice/weight of brown rice)] × 100%. Milled rice percentage (MRP) was calculated based on the weight of milled rice and rough rice as follows: MRP = (weight of milled rice/weight of rough rice) × 100%. Increasing milling times (0, 10, 20, 30 s) corresponded to increasing average DOM values (0, 2.67, 7.25 and 9.6%), respectively (Table 1). Brown rice samples with four different DOMs were ground to a powder to enable passage through a 60-screen mesh, and stored at −40 °C until analysis. Milling experiments and sample preparations were performed in triplicate.

2.3. Extraction of free phenolics

Free phenolic compounds were extracted according to a previously reported method, with slight modifications (Paiva et al., 2014). Briefly, 2 g of sample flour was blended with 50 mL chilled 80% acetone. Then, the supernatant was separated by centrifugation at 2500*g* for 10 min, and the extraction procedure was repeated once. The two supernatants were pooled and evaporated under vacuum. The residual was recovered by adding 10 mL chilled MeOH, and then stored at −40 °C until use.

2.4. Extraction of bound phenolics

Bound phenolics were extracted from the residue remaining after extracting free phenolic compounds using the methods described by Finocchiaro et al. (2007). Briefly, the residue was hydrolyzed with 40 mL 2 M NaOH at room temperature for 1 h

Table 1
The relationship between DOM, MRP and milling time.

Rice type	Milling time (s)						Linear equation	*R*
	5	10	15	20	25	30		
DOM (%)								
Japonica rice	1.07	2.59	5.20	7.44	8.40	9.49	$y = 0.3427x - 0.2562$	0.9901
Indica rice	1.15	2.70	4.90	7.10	8.50	9.71	$y = 0.3436x - 0.2901$	0.9957
MRP (%)								
Japonica rice	79.15	77.93	75.84	74.05	73.28	72.41	$y = -0.2741x + 80.205$	0.9901
Indica rice	79.08	77.8	76.09	74.35	73.19	72.25	$y = -0.2749x + 80.232$	0.9957

DOM = [1 − (weight of milled rice/weight of brown rice)] × 100%;
MRP = (weight of milled rice/weight of rough rice) × 100%.
DOM: degree of milling; MRP: milled rice percentage.

with shaking under nitrogen gas. The pH was then adjusted to 1.0 with 6 mol/L HCl and the solution was extracted with hexane to remove lipids. The remaining mixture was extracted 5 times with ethyl acetate. The pooled ethyl acetate fractions were evaporated to dryness. The extract containing the bound phenolics was reconstituted with MeOH to a final volume of 10 mL and then stored at −40 °C until analysis.

2.5. Determination of total phenolic content

The total phenolic content was measured using the Folin–Ciocalteu colorimetric method as described previously by Dewanto, Wu, Adom, and Liu (2002). Briefly, a 125 μL aliquot of the extract prepared as described above was diluted with 0.5 mL distilled water, and subsequently reacted with 125 μL Folin–Ciocalteu reagent for 6 min. Then, 1.25 mL 7% aqueous sodium carbonate solution was added, and the solution was diluted to a final volume of 3 mL. The mixture was incubated in the dark for 90 min, and the absorbance at 760 nm was determined using a Shimadzu UV-1800 spectrometer (Shimadzu Inc., Kyoto, Japan). Gallic acid was used as the standard, and the total phenolic content is expressed as mg gallic acid equivalents (GAE) per 100 g dry weight (DW) of sample.

2.6. Determination of total flavonoid content

The total flavonoid content was measured using a colorimetric method described previously (Dewanto et al., 2002). A 300-μL aliquot of the extract described above was added to 1.5 mL distilled water. Then, 90 μL 5% $NaNO_2$ solution were added and the mixture was incubated for 6 min. Subsequently, 180 μL 10% $AlCl_3{\cdot}6H_2O$ solution were added, and the mixture was incubated for 5 min before adding 0.6 mL 1 M NaOH solution. The mixture was diluted to a final volume of 3 mL, and the absorbance was measured immediately at 510 nm using a Shimadzu UV-1800 spectrometer. (+)-catechin was used as the standard, and the total flavonoid content is expressed as mg (+)-catechin equivalents (CE) per 100 g DW of sample.

2.7. Determination of phenolic composition

Quantitative analysis of phenolic compounds was performed by HPLC using an Agilent 1200 HPLC system (Waldbronn, Germany) equipped with an Agilent 1200 series VWD detector, autosampler, and a Zorbox SB-C_{18} column (250 × 4.6 mm i.d., 5 μm; Agilent Technologies, Palo Alto, CA, USA); column temperature was maintained at 30 °C. The mobile phase consisted of a 0.4% aqueous solution of acetic acid (solution A) and acetonitrile (solution B), with the following gradient program: 0–40 min, solution B 5–25%; 40–45 min, solution B 25–35%; 45–50 min, solution B 35–50%, with a flow rate of 1.0 mL/min, injection volume of 20 μL, and a run time of 50 min. Eluted compounds were detected at 280 nm. All samples were filtered through a 0.25-μm membrane filter (Millipore, Billerica, MA, USA) prior to analysis. Individual compounds were identified by comparing their retention times with those of authentic standards. The concentrations of each compound were calculated based on a standard curve. The percentage recovery of these phenolics was greater than 94%.

2.8. Cellular antioxidant activity assay

The CAA assay was performed according to the method of Wolfe and Liu (2007). Briefly, human hepatocellular carcinoma HepG2 cells were seeded at a density of 6×10^4/well in a 96-well microplate in 100 μL growth medium (DMEM containing 10% fetal bovine serum), and maintained in a CO_2 incubator at 37 °C for 24 h. After rinsing with PBS, the inoculated wells were treated for 1 h with DMEM medium containing 25 μM DCFH-DA in the presence or absence of rice phenolic extracts (sample wells or control wells, respectively). Then, 600 μM ABAP in 100 μL of HBSS were added to each well except for blank wells, which contained cells treated with DCFH-DA and HBSS. The 96-well microplate was placed into a Fluoroskan Ascent FL plate reader (Thermo Labsystems, Franklin, MA, USA) at 37 °C. The fluorescence values (emission at 538 nm, excitation at 485 nm) of all wells were measured every 5 min for a total of 12 cycles. Each sample was analyzed in triplicate, including control and blank wells. The CAA results are expressed as μmol quercetin equivalents (QE) per 100 g DW of sample.

2.9. Statistical analyses

All experiments were repeated 3 times and data are expressed as mean ± standard deviation. Data were analyzed by one-way analysis of variance using SPSS13.0 software (SPSS Inc. Chicago, IL, USA). The level of significance was set at $p < 0.05$.

3. Results

3.1. DOM and MRP

Whole brown rice was successively milled (5–30 s) to obtain rice with various DOM and MRP. The relationships among milling time, DOM, and MRP are shown in Table 1. DOM and MRP were the linear function of milling time, respectively. Brown rice samples with the three different DOMs were classified as grades 3, 2, and 1, respectively, according to the grading standard for rice quality in China (CDS, 2009). As the average DOM increased from 0% to 9.6%, the average MRP decreased from 80.21% to 72.33%.

3.2. Total phenolic content

Table 2 shows the content of free, bound, and total phenolics in two types of brown rice with different DOMs, and their percentage contributions to total phenolics. In both types of brown rice, the free, bound, and total phenolic contents differed significantly among the four different DOMs ($p < 0.05$), and decreased with increasing DOM ($p < 0.05$). For *japonica* rice, as the DOM increased from 0, to 2.59, 7.44 and 9.49%, the total phenolic contents significantly decreased by 18.0, 44.2 and 57.3%, respectively. The free phenolic content significantly decreased by 15.9, 40.9 and 51.7%, and the bound phenolic content significantly decreased by 21.8, 50.6 and 67.8%, respectively. In addition, as the DOM increased, the percentage contribution of the free fraction to total phenolics increased from 65.4% to 73.9%, and the percentage contribution of the bound fraction to total phenolics decreased from 34.6% to 26.1%, respectively. The effect of DOM on the free, bound, and total phenolics of *indica* rice was similar with that of *japonica* rice.

3.3. Total flavonoid content

The contents of free, bound, and total flavonoids in brown rice samples with different DOMs, and their percentage contributions to total flavonoids, are shown in Table 2. The trend of the flavonoids of two types of brown rice was the same with their phenolics. For *japonica* rice, as the DOM increased from 0% to 9.49%, the total flavonoid content decreased by 20.2, 53.6 and 73.4%, respectively. The free flavonoid contents decreased by 22.5, 49.1 and 72.3%, and the bound flavonoid content decreased by 20.2, 58.9 and 74.6%, respectively. As the DOM increased, the percentage contribution of the free fraction to total flavonoids increased from 55.3% to 57.5%, and the percentage contribution of the bound fraction to total flavonoids decreased from 44.7% to 42.5%, respectively.

3.4. Phenolic composition

We next analyzed nine phenolic compounds, including ferulic acid, coumaric acid, protocatechuic acid, vanillic acid, quercetin, (+)-catechin, isoferulic acid, caffeic acid, and chlorogenic acid, in both their free and bound forms in the two types of brown rice with different DOMs. The individual phenolic content in *indica* and *japonica* rice, as well as the percentage contribution of the free and bound forms to the total content of each phenolic compound, are shown in Table 3. The composition and the forms of phenolic compounds were similar between the two types of brown rice. Protocatechuic acid, caffeic acid, and chlorogenic acid were present in free forms, while vanillic acid, quercetin, and isoferulic acid were present in bound forms. Ferulic acid, and (+)-catechin were present in both free and bound forms in the two types of brown rice; however, coumaric acid was present mainly in the bound form and, to a much lesser extent, in free form in *japonica* rice, while in *indica* rice coumaric acid was present almost exclusively in the bound form.

As the DOM increased, the contents of nine individual phenolics in the two types of brown rice significantly decreased ($p < 0.05$). Concretely, take the *japonica* rice for example, the decreases in the total amount of each phenolic compound were as follows: ferulic acid, 70.8%; coumaric acid, 89.8%; protocatechuic acid, 56.7%; vanillic acid, 94.0%; quercetin, 62.5%; (+)-catechin, 81.2%; isoferulic acid, 59.0%; caffeic acid, 61.3%; and chlorogenic acid, 80.0% (Table 3). The decrease in the free form of each phenolic compound was as follows: ferulic acid, 100%; coumaric acid, 100%; (+)-catechin, 83.6%; the decrease in the bound form of each phenolic compound was as follows: ferulic acid, 70.0%; coumaric acid, 89.6%; (+)-catechin, 70.7%.

Table 2
Total phenolic and flavonoid content of brown rice with different DOMs and percentage contributions of free and bound fractions to the total content.

Rice type	Milling time (s)	Free	Bound	Total
Phenolics (mg GAE/100 gDW)				
Japonica rice	0	65.6 ± 0.6a[a](65.4)[b]	34.8 ± 1.3a(34.6)	100.4 ± 1.3a
	10	55.2 ± 0.3b(67.0)	27.2 ± 0.2b(33.0)	82.3 ± 0.2b
	20	38.8 ± 0.3c(69.3)	17.2 ± 0.7c(30.7)	56.0 ± 0.4c
	30	31.7 ± 0.3d(73.9)	11.2 ± 0.7d(26.1)	42.9 ± 1.0d
Indica rice	0	62.0 ± 1.9a(62.5)	37.3 ± 2.7a(37.5)	99.3 ± 1.2a
	10	53.1 ± 1.6b(70.5)	22.2 ± 0.6b(29.5)	75.3 ± 1.0b
	20	44.2 ± 0.5c(75.4)	14.4 ± 0.3c(24.6)	58.6 ± 0.2c
	30	34.5 ± 2.2d(75.2)	11.4 ± 0.1d(24.8)	46.0 ± 2.2d
Flavonoids (mg CE/100 gDW)				
Japonica rice	0	42.6 ± 0.8a[a](55.3)[b]	34.3 ± 1.1a(44.7)	76.9 ± 0.6a
	10	33.0 ± 0.2b(53.7)	28.4 ± 1.8b(46.3)	61.4 ± 2.0b
	20	21.7 ± 1.0c(60.7)	14.1 ± 0.8c(39.3)	35.7 ± 1.1c
	30	11.8 ± 0.2d(57.5)	8.7 ± 0.7d(42.5)	20.4 ± 0.5d
Indica rice	0	56.3 ± 0.9a(50.3)	55.7 ± 4.4a(49.7)	112.1 ± 5.3a
	10	42.3 ± 0.3b(51.8)	39.3 ± 1.3b(48.2)	81.6 ± 1.6b
	20	32.5 ± 0.5c(53.6)	28.1 ± 1.9c(46.4)	60.6 ± 1.9c
	30	24.1 ± 0.3d(57.9)	17.5 ± 1.0d(42.1)	41.6 ± 1.1d

DOMs: degrees of milling; GAE: gallic acid equivalents; CE: (+)-catechin equivalents.
[a] Values with different letters in each column are significantly different with respect to different milling time in a given rice type ($p < 0.05$).
[b] Values in parentheses indicate the percentage contribution to the total content.

3.5. Cellular antioxidant activity

The free, bound, and total CAA of brown rice with different DOMs and their percentage contributions to total CAA are shown in Table 4. In both *indica* and *japonica* brown rice, the free, bound, and total CAA values differed significantly among the four different DOMs, exhibiting a sharp decrease with increasing DOM. Simply, the free, bound and total CAA values of the *japonica* rice decreased by 95.7, 91.2 and 92.6%, respectively. The free, bound, and total CAA values of the *indica* rice decreased by 97.4, 91.3 and 93.1%, respectively. In addition, Increasing DOM was also associated with changes in the percentage contribution of the free form to total CAA of *japonica* rice, which changed from 30.9 to 19.4, 43.9 and 18.0%, while the percentage contribution of the bound form to total CAA changed from 69.1 to 80.6, 56.1 and 82.0%, respectively. The trend of *indica* rice was a slight different with the *japonica* rice, but also showed an overall drop in the percentage contribution of the free form and increase in the percentage contribution of the bound form.

4. Discussion

In the present study, we investigated the effects of DOM on the phenolics profile and CAA in two types rice (*japonica* and *indica*), which are widely consumed in northern and southern China. As the average DOM increased from 0% to 9.6%, the average total phenolic content of the two types of brown rice significantly decreased by 21.1–55.6%, and the average total flavonoid content significantly decreased by 24.3–67.2%, respectively (Table 5). These results indicate that the total phenolic and total flavonoid contents are lower in grade 1 rice than in grades 2 and 3 rice. The progressive loss of phytochemicals with increasing DOM is related to phytochemical distribution in different milled fractions. It has been reported that most of nutrients including phenolics, dietary fiber, γ-oryzanols, tocopherols and minerals is focused on bran layer, while starch is primarily distributed on endosperm of brown rice (Lamberts et al., 2007; Shobana et al., 2011). Furthermore, rice bran is composed of pericarp, aleurone and embryo. At the tissue level,

Table 3
Phenolic profiles of brown rice with different DOMs, and the percentage contributions of the free and bound fractions to the total content of each phenolic compound.

Phenolics	Milling time (s)	Free (μg/g)	Bound (μg/g)	Total (μg/g)
Japonica rice				
Quercetin	0	nd	25.6 ± 3.2a[a](100)[b]	25.6 ± 3.2a(0)[c]
	10	nd	21.7 ± 0.5a(100)	21.7 ± 0.5a(15.2)
	20	nd	16.2 ± 1b(100)	16.2 ± 1b(36.7)
	30	nd	9.6 ± 1.7c(100)	9.6 ± 1.7c(62.5)
(+)-Catechin	0	17.7 ± 0.7a(81.3)	4.1 ± 0.3a(18.7)	21.8 ± 1a(0)
	10	10.2 ± 1.3b(72.6)	3.9 ± 0.3a(27.4)	14.1 ± 1.2b(35.3)
	20	4.7 ± 1.3c(71.6)	1.9 ± 0.2b(28.4)	6.6 ± 1.5c(69.7)
	30	2.9 ± 0.5c(71.3)	1.2 ± 0.4c(28.7)	4.1 ± 0.5d(81.2)
Ferulic acid	0	3.3 ± 0.2a (1.6)	207 ± 20.3a(98.4)	210.3 ± 20.2a(0)
	10	3 ± 0.8a(1.9)	152.2 ± 10b(98.1)	155.2 ± 29.2b(26.2)
	20	1.4 ± 0.2b(2.0)	69.5 ± 3.3c(98)	70.9 ± 3.2c(66.3)
	30	Tr	61.4 ± 7.6c(100)	61.4 ± 7.6c(70.8)
Coumaric acid	0	1.4 ± 0.2a(2.1)	65.4 ± 6.3a(97.9)	66.8 ± 6.2a(0)
	10	0.8 ± 0.4a(2.4)	34.3 ± 8.7b(97.6)	35.1 ± 8.8b(47.4)
	20	1 ± 0.3a(8.3)	11.1 ± 0.3c(91.7)	12.1 ± 0.6c(81.9)
	30	Tr	6.8 ± 0.7d(100)	6.8 ± 0.7d(89.8)
Protocatechuic acid	0	32.3 ± 2.6a(100)	nd	32.3 ± 2.6a(0)
	10	33 ± 1.1a(100)	nd	33 ± 1.1a(-2.1)
	20	13.2 ± 2.7b(100)	nd	13.2 ± 2.7b(59.1)
	30	14 ± 2.4b(100)	nd	14 ± 2.4b(56.7)
Vanillic acid	0	nd	28.7 ± 2.9a(100)	28.7 ± 2.9a(0)
	10	nd	27.2 ± 4.2a(100)	27.2 ± 4.2a(5.3)
	20	nd	1.5 ± 0.2b(100)	1.5 ± 0.2b(94.9)
	30	nd	1.7 ± 0.4b(100)	1.7 ± 0.4b(94)
Isoferulic acid	0	Tr	8.3 ± 0.6a(100)	8.3 ± 0.6a(0)
	10	Tr	5.9 ± 0.1b(100)	5.9 ± 0.1b(28.9)
	20	Tr	4.7 ± 0.5c(100)	4.7 ± 0.5c(43.4)
	30	Tr	3.4 ± 0.7d(100)	3.4 ± 0.7d(59.0)
Caffeic acid	0	7.6 ± 1.2a(100)	nd	7.6 ± 1.2a(0)
	10	7.1 ± 1a(100)	nd	7.1 ± 1a(33.3)
	20	4.4 ± 0.9b(100)	nd	4.4 ± 0.9b (58)
	30	4.7 ± 0.8b(100)	nd	4.7 ± 0.8b(61.3)
Chlorogenic acid	0	6 ± 1.7a(100)	nd	6 ± 1.7a(0)
	10	5.4 ± 1.5a(100)	nd	5.4 ± 1.5a(10)
	20	1.8 ± 0.8b(100)	nd	1.8 ± 0.8b(70)
	30	1.2 ± 0.3b(100)	nd	1.2 ± 0.3b(80)
Indica rice				
Quercetin	0	nd	15.9 ± 1.7a(100)	15.9 ± 1.7a(0)
	10	nd	12.4 ± 1.2b(100)	12.4 ± 1.2b(22.0)
	20	nd	7.9 ± 1c(100)	7.9 ± 1c(50.3)
	30	nd	7.9 ± 1.1c(100)	7.9 ± 1.1c(50.3)
(+)-Catechin	0	4.5 ± 0.3a(31)	10 ± 0.5a(69)	14.5 ± 0.3a(0)
	10	2.8 ± 0.5b(34.6)	5.2 ± 0.7b(65.4)	8.0 ± 0.8b(45.2)
	20	2.0 ± 0.9b(57.7)	1.5 ± 0.3c(42.3)	3.5 ± 1c(76.0)
	30	Tr	1.1 ± 0.2c(100)	1.1 ± 0.2d(92.5)
Ferulic acid	0	1.7 ± 0.3a(2.5)	65.5 ± 6.3a(97.5)	67.2 ± 6.1a(0)
	10	1 ± 0.4b(1.5)	64.7 ± 3a(98.5)	65.7 ± 2.6a(2.1)
	20	0.5 ± 0.2b(0.9)	50.4 ± 8.2b(99.1)	50.9 ± 8.3b(24.1)
	30	Tr	36.6 ± 4.3c(100)	36.6 ± 4.3c(45.5)
Coumaric acid	0	Tr	24.3 ± 2a(100)	24.3 ± 2a(0)
	10	Tr	18.8 ± 2.1b(100)	18.8 ± 2.1b(22.5)
	20	Tr	10.8 ± 3.4c(100)	10.8 ± 3.4c(55.5)
	30	Tr	4.1 ± 0.4d(100)	4.1 ± 0.4d(83.1)
Protocatechuic acid	0	47.8 ± 7.7a(100)	nd	47.8 ± 7.7a(0)
	10	41.4 ± 6.2a(100)	nd	41.4 ± 6.2a(13.4)
	20	42.3 ± 3.5a(100)	nd	42.3 ± 3.5a(11.6)
	30	21.9 ± 5.2b(100)	nd	21.9 ± 5.2b(54.2)
Vanillic acid	0	nd	199 ± 12.2a(100)	199 ± 12.2a(0)
	10	nd	98.3 ± 5b(100)	98.3 ± 5b(50.6)
	20	nd	42.6 ± 13.5c(100)	42.6 ± 13.5c(78.6)
	30	nd	26.9 ± 4c(100)	26.9 ± 4c(86.5)
Isoferulic acid	0	Tr	8.4 ± 0.7a(100)	8.4 ± 0.7a(0)
	10	Tr	8.1 ± 0.6a(100)	8.1 ± 0.6ab(3.6)
	20	Tr	7.4 ± 0.5a(100)	7.4 ± 0.5b(8.6)
	30	Tr	5.6 ± 0.4b(100)	5.6 ± 0.4c(30.9)
Caffeic acid	0	5.2 ± 0.4a(100)	nd	5.2 ± 0.4a(0)

Table 3 (*continued*)

Phenolics	Milling time (s)	Free (μg/g)	Bound (μg/g)	Total (μg/g)
	10	4.7 ± 0.6a(100)	nd	4.7 ± 0.6a(9.6)
	20	0.6 ± 0.3b(100)	nd	0.6 ± 0.3b(88.5)
	30	Tr	nd	Tr(100)
Chlorogenic acid	0	5.2 ± 0.4a(100)	nd	5.2 ± 0.4a(0)
	10	4.8 ± 0.3a(100)	nd	4.8 ± 0.3a(7.7)
	20	1.7 ± 0.3b(100)	nd	1.7 ± 0.3b(67.3)
	30	0.5 ± 0.2c(100)	nd	0.5 ± 0.2c(90.4)

Tr: Trace; nd: not detectable.
[a] Values with different letters in each column are significantly different with respect to different milling time in a given rice type ($p < 0.05$).
[b] Values in parentheses indicate percentage contribution to the total content.
[c] Values in parentheses indicate percentage loss of total phenolic compounds compared to those of brown rice (DOM = 0).

Table 4
Cellular antioxidant activity of brown rice with different DOMs and percentage contributions of free and bound fractions to the total cellular antioxidant activity.

Rice type	Milling time (s)	Cellular antioxidant activity (μmol QE/100 g DW)		
		Free	Bound	Total
Japonica rice	0	74.0 ± 2.5a[a](30.9)[b]	165.2 ± 4.2a(69.1)	239.2 ± 4.1a
	10	33.4 ± 4.7b(19.4)	139.4 ± 7.3b(80.6)	172.8 ± 4.8b
	20	19.0 ± 1.4c(43.9)	24.3 ± 3.6c(56.1)	43.3 ± 3.9c
	30	3.2 ± 0.2d(18.0)	14.6 ± 0.6d(82.0)	17.8 ± 0.8d
Indica rice	0	53.7 ± 8.6a(30.2)	124.2 ± 8.1a(69.8)	177.9 ± 8.9a
	10	29.2 ± 6.7b(33.1)	59.1 ± 7.2b(66.9)	88.3 ± 3.8b
	20	9.1 ± 1.4c(39.0)	14.3 ± 2.6c(61.0)	23.4 ± 2.3c
	30	1.4 ± 0.2d(11.5)	10.8 ± 0.8d(88.5)	12.2 ± 0.6d

QE: quercetin equivalents.
[a] Values with different letters in each column are significantly different with respect to different milling time in a given rice type ($p < 0.05$).
[b] Values in parentheses indicate percentage contribution to the total CAA.

higher concentrations of phenolic compounds are found in the outer layers of plants, e.g., in the epidermis, than in the inner layers (Naczk & Shahidi, 2004). Previous study indicated that the concentrations of phenolic acids decreased from the aleurone layer to endosperm in brown rice (Butsat & Siriamornpun, 2010). Shao et al. (2014) also found that bran and embryo exhibit higher free and bound phenolic content than endosperm. As the DOM gradually increased to 9.6%, the bran layer including pericarp, aleurone and embryo, was totally removed, and so the total phenolic contents of brown rice significantly decreased. Obviously, even though milling improves the sensory qualities of the grains, several phytochemicals in brown rice that are beneficial for human health are lost as the DOM increases.

Our results show that the percentage contributions of bound forms to total phenolics and total flavonoids decreased with increasing DOM (Table 2). The decreasing level of bound phenolics is primarily attributable to the removal of rice bran, which contains a significant amount of bound phenolics, during the milling process (Shao et al., 2014; Wang et al., 2015). The bound phenolics in rice bran are covalently linked to cell wall polymer, such as cellulose, hemicelluloses, lignin, pectin and rod-shaped structural proteins (Acosta-Estrada, Gutierrez-Uribe, & Serna-Saldivar, 2014). Moreover, phytochemicals in bound form cannot be digested by human enzymes, they remain intact during passage through both the stomach and small intestine to reach the colon, where they exert their bioactive activities (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001; Liu, 2007). On the other hand, the phenolic compounds in the endosperm exist mainly in free forms, and therefore a greater proportion of free phenolics exists in rice with higher DOMs, because the endosperm represents a greater proportion of those samples (Shao et al., 2014). Interestingly, the percentage contributions of bound coumaric acid, ferulic acid and (+)-catechin to their total content increased concomitant with increasing DOM, presumably due to their uneven distribution among milling fractions in different types of brown rice.

Table 5
Percentage loss of total phenolic, flavonoid, and cellular antioxidant activity of brown rice with different DOMs.

Rice type	Milling time (s)	Percentage loss (%)		
		Total phenolic	Total flavonoid	CAA
Japonica rice	10	18.0	20.1	27.8
	20	44.3	53.5	81.9
	30	57.3	73.4	92.6
Indica rice	10	24.1	27.2	50.4
	20	41.0	45.9	86.8
	30	53.7	62.9	93.1

DOMs: degrees of milling.

We also observed effects of DOM on the phenolic composition of *indica* and *japonica* brown rice. Increasing DOM resulted in loss of phenolic compounds, although the percentage loss differed significantly not only among the various phenolic acids and flavonoids but also between the two rice types. As the DOM of *japonica* increased from 0% to 2.59% (milling time of 10 s), the total contents of quercetin, protocatechuic acid, vanillic acid, caffeic acid and chlorogenic acid did not change significantly ($p > 0.05$), while the total contents of (+)-catechin, ferulic acid, coumaric acid and isoferulic acid decreased by 35.3, 26.2, 47.4 and 28.9%, respectively ($p < 0.05$). As the DOM further increased to 9.49% (milling time of 30 s), the total contents of quercetin, (+)-catechin, ferulic acid, coumaric acid, protocatechuic acid, vanillic acid, isoferulic acid, caffeic acid and chlorogenic acid decreased by 62.5, 81.2, 70.8, 89.8, 56.7, 94, 59.0, 61.3 and 80%, respectively ($p < 0.05$) (Table 3). These patterns of loss during *japonica* rice milling suggest that coumaric acid exists mainly in the outer layer of rice bran (pericarp), while vanillic acid, chlorogenic acid, and ferulic acid are primarily distributed the inner layer (aleurone layer), and protocatechuic acid is largely found in the endosperm. Summarily, individual phenolics decreased to different degrees during milling process, which is similar for both types rice, but for specific phenolic acids, e.g. ferulic and vanillic acids, the pattern of loss was different between the two types of brown rice.

The patterns of loss are also closely related to the known distribution of phenolic compounds in brown rice. Zhou, Robards, Helliwell, and Blanchard (2004) reported that the contents of total phenolic acids (sum of ferulic acid, coumaric acid, gallic acid, vanillic acid, caffeic acid, and syringic acid) in the bran comprised 70–90% of total phenolic acids in brown rice, depending on cultivar and the specific phenolic acid. In our recent work, we found that the contents of ferulic acid and coumaric acid are comparatively higher than those of other phenolic acids in both rice bran and

polished rice (Ti et al., 2014). Also, significant differences exist in the contents of phenolic compounds between the bran layer and the embryo of brown rice (Moongngarm, Daomukda, & Khumpika, 2012; Shao et al., 2014). Taken together, these results indicate an uneven distribution of individual phenolic compounds in brown rice, providing an explanation for the different degrees to which the contents of individual phenolics decreased during milling.

CAA assay takes into account the phenolic bioactivity within cells (Huang, Sun, Lou, Li, & Ye, 2014; Wolfe & Liu, 2007), which can more accurately reflect the antioxidant activity of phenolics *in vivo*. In this study, increasing DOM also impacted CAA of *indica* and *japonica* whole brown rice. As the average DOM increased, the average total CAA value of whole brown rice decreased from 208.6 to 15.0 μmol QE/100 g DW (Table 4). The results of our quantitative analysis are consistent with previous studies in which antioxidant activity was determined by chemical methods such as DPPH or ABTS. Finocchiaro et al. (2007) also reported a decrease of antioxidant activity from 10.17 to 4.48 μmol TE/g in brown rice following an increase in DOM from 0% to 6%, while an additional study of black and red rice varieties indicate that antioxidant activity decreased by more than 85% at a DOM of 10% (Paiva et al., 2014). Previous study pointed out that antioxidant activity of whole grain rice is closely correlated with free and bound phenolics contents (Min et al., 2012). Therefore, it is easily deduced that the decrease of CAA was mainly attributed to the loss of phenolics in brown rice during the milling process. In addition, Hirawan et al. (2011) reported CAA values of red rice and purple wheat ranging from 15.3 to 30.4 CAA units using the small intestine-derived cell line FHs 74 Int, but it is not possible to directly compare these values with our findings due to differences in cell lines and methods of CAA quantitation.

Importantly, as the DOM of *japonica* rice increased, the percentage contributions of the bound fraction to the total CAA value changed from 69.1 to 80.6, 56.1 and 82.0%, which differed from observed changes in phenolic fractions. The similar phenomenon was also observed in *indica* rice. This is largely attributable to alterations in percentages of individual phenolic compounds during milling of brown rice. Previous studies have observed differences in CAA values among quercetin, caffeic acid, catechin and ferulic acid (Liu, 2007), as well as increases in the percentage of individual phenolic compounds with higher CAAs. Another possibility is that the presence of as yet unidentified phenolic compounds contributes to the CAA of brown rice samples of different DOMs, thus further studies are required to comprehensively evaluate the CAA of phytochemicals in brown rice.

Whole brown rice is used in the manufacturing of numerous food products, including rice flour, cookies, bread, and noodles. Koyama and Kitamura (2014) have reported that whole brown rice can also be used to produce beverages following wet stone milling. Whole brown rice is not widely consumed as a staple food, (i.e. as steamed rice) in part due to its hard texture and extended time required for cooking. Previous work has shown that the cooking time of brown rice significantly decreased after milling (Billiris, Siebenmorgen, Meullenet, & Mauromoustakos, 2012), and brown rice with a low DOM is consumed in China as an alternative to whole brown rice, due to its acceptable cooking qualities.

5. Conclusion

Milling is a traditional method for rice processing, and serves to improve the stability and sensory quality of rice during storage. The presented research describes a systematic evaluation of the effects of the DOM on both free and bound phenolic profiles as well as CAA of whole brown rice. Our results indicate that as the DOM increases, loss of phytochemicals beneficial to health occurs, and CAA decreases. Moreover, the contents of nine phenolic compounds decreased, and the percentage loss differed significantly among the various compounds. Thus, brown rice products with higher DOMs may have better sensory quality but lower nutritional quality. Based on these findings, we recommend that during rice processing, the DOM should be carefully controlled to optimize both the sensory quality and nutritional composition (amount of phytochemicals). Compared with brown rice of 9.6% DOM (polished rice), brown rice with a low DOM (<2.67%) exhibits a more ideal balance between sensory quality and retention of beneficial phytochemicals. This research provides important evidence in support of health benefits of consuming brown rice, particularly lightly milled rice.

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