Influence of Kernel Maturity, Milling Degree, and Milling Quality on Rice Bran Phytochemical Concentrations

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Rice bran is a rich source of phytochemicals including tocopherols (T), tocotrienols (T3), and γ-oryzanol that have purported positive effects on human health. The screening of germplasm to determine the genetic diversity influencing contents of these compounds requires knowledge of how sample preparation influences concentrations of the phytochemicals in rice bran. Obtaining this knowledge was the objective of this study. Cultivars with different milling qualities were all milled to different degrees. The differences in bran removal among cultivars decreased as the milling time increased. Samples that were milled for 30 and 40 sec (milled to the degree of 0.23-0.44% surface lipid content [SLC]) showed no significant differences in T and T3 concentrations in the bran within cultivars. Bran starch concentration affected the rankings of cultivars based on phytochemical contents. Expression of the γ-oryzanol concentration in bran after subtracting starch reduced the concentration differences resulting from differences in degree of milling (DOM). Bran from the mature thin kernels had phytochemical contents similar to that of the mature thick kernels milled for 30 sec. The immature thin kernels had significantly lower contents of most of the bran phytochemicals than did the mature kernel fractions.

However, thinner kernels tended to have higher breakage during the milling process than the thicker kernels (Mathews and Spadaro 1976; Sun and Siebenmorgen 1993). This suggested that the collected bran from thinner kernels would likely have a higher proportion of starchy endosperm than that of thicker kernels milled to the same degree. Thus, the questions that need to be answered are 1) whether the phytochemical concentrations in the brans collected from well-milled or overmilled kernels at a slightly different degree of bran removal would be affected by their distributions in the bran layer; and 2) how to mill rice cultivars of different kernel thickness or milling quality to similar degree. If well-milled or overmilled is the choice, then would the endosperm starch contents be a factor affecting the concentrations of phytochemicals in the collected bran? Rohrer and Siebenmorgen (2004) reported that the bran from thinner kernels had fewer phytochemicals than the bran from thicker kernel fractions. However, the endosperm starch content in collected bran was not analyzed, and whether the immature, green-colored kernels were included in the thinner fraction was not specified in their study. In this study, the thinner kernel fractions were further separated into mature and immature fractions based on the seed coat color and the bran phytochemical concentrations were measured taking into account the starch content in the bran.

The objectives were 1) to examine how factors that affect rice milling influence the concentrations of phytochemicals in bran; 2) to determine whether the bran contents of phytochemicals in thin, mature, and immature kernels differ from those of mature, thicker kernels; and 3) to develop criteria for sample preparation to minimize the influences of these factors on rice bran antioxidant content measurement.

MATERIALS AND METHODS

Samples of two medium-grain cultivars Oryza sativa L. ‘Brazos’ (BRAZ) and ‘Orion’ (ORIN), and two long-grain cultivars Oryza sativa L. ‘Cypress’ (CPRS) and ‘LaGrue’ (LGRU) were studied. These four cultivars were chosen based on milling quality data collected using the standard milling procedure for the Uniform Regional Nursery, Beaumont, TX. The objective was to choose one good milling quality and one poor milling quality cultivar for both the long- and medium-grain types. The milling qualities (or head rice yield) calculated on a percentage basis of the mass of milled head rice divided by the rough rice of BRAZ, ORIN, CPRS, and LGRU were 61.6, 39.1, 60.1, and 42.1%, respectively. The cultivars were grown under field conditions in Beaumont, TX, in 2000, using cultural management practices common for the region.
Sample Preparation
Samples were harvested at ≥20% moisture, dried to 12% moisture, and dehulled using a rice huller (model THO35A, Satake, Tokyo, Japan). The dehulled kernels were then size-fractionated by kernel thickness using a Carter precision sizer (Philip Rahm International, Houston, TX). The thickness ranges of the thicker kernel (mature thick) fractions of BRAZ, ORIN, CPRS, and LGRU were 1.79–1.98, 1.94–2.08, 1.69–1.89, and 1.79–1.94 mm. Immature (green) and diseased kernels were removed from these mature thick fractions and discarded. Four 50-g subsamples of each cultivar of the mature thick kernels were milled for 20, 30, 40, and 60 sec (mill #1, HT McGill, Houston, TX) with a 858-g weight in positions 12 or 6 for long- or medium-grain cultivars, respectively (Chen and Bergman 2005). Thinner kernels that had a thickness less than the mature thick fraction were separated manually into mature thin (brown colored seed coat) and immature thin (green colored seed coat) kernels, and ≥50 g of each were milled for 30 sec using the milling procedure just mentioned. The bran (the word bran is used to mean bran and germ collectively) was collected, sieved through a 28-mesh screen, flushed with nitrogen, and stored at −20°C until further analysis.

Chemical Analysis
The DOM was assessed by measuring the amount of lipid content in the outer layers (surface lipid content) of milled head rice. Milled head rice (5 g) was refluxed with petroleum ether (Fisher, ACS grade) in a Goldfisch extraction apparatus for 30 min. The solvent was collected and evaporated at 100°C for 30 min, and the percent surface lipid content (SLC %) was calculated as the mass of the extracted lipid divided by the beginning total milled head rice mass × 100. Analysis was performed with two replicates; results were expressed on a dry weight basis (Goffman and Bergman 2003). Bran moisture concentration was determined by drying 0.2 g of bran at 105°C for 2 hr, cooling in a desiccator for 45 min, and weighing the dry bran. The percent bran moisture was calculated by taking the difference of fresh bran weight and the dry bran weight then dividing it by the fresh bran weight.

The starch concentration in the bran (w/w, dry weight basis) was determined using a total starch assay kit (AA/AMG 11/01, Megazyme Int. Ireland Ltd. Co., Wicklow, Ireland) following procedure B using dimethyl sulfoxide (DMSO) (Sigma) for starch solubilization and the procedure A, alternative step 6, by adjusting the final volume to 10 mL (McClarey et al 1997). In brief, 100 mg of rice bran was weighed into a 15-mL polypropylene tube, and wetted with 0.2 mL of aqueous ethanol (80%, v/v). Immediately after, 2 mL of DMSO were added. The contents of the tube were mixed with a vortex mixer and placed in a boiling water bath for 5 min. Immediately after, 3 mL of thermostable α-amylase (300 units) in MOPS buffer (50 mM, pH 7.0) were added and the tube contents were mixed with a vortex mixer. The tube was incubated in a boiling water bath for 6 min (stirring with a vortex mixer after 2 and 4 min). Next, the tube was placed in a 50°C water bath and 4 mL of sodium acetate buffer (200 mM, pH 4.5) was added, followed by amyloglucosidase (0.1 mL, 20 units), with mixing, then the tube was incubated for 1 hr. At the end of the digest, the volume was brought to 10 mL with deionized water. An aliquot of the supernatant was used for glucose determination (following the total starch assay kit manual). The analyses were performed with three replicates; data was expressed on a dry weight basis.

Analysis of Phytochemicals
The T, T3, and γ-oryzanol in the bran were extracted with methanol using an equilibrium extraction method and quantified by reversed-phase HPLC (Chen and Bergman 2004). Briefly, 50 mg of rice bran was vortexed in 3 mL of methanol for 3 min at room temperature. The mixture was centrifuged for 10 min at 825 × g, filtered, and the filtrate analyzed by reversed-phase HPLC. The HPLC system consisted of a Waters 2690 Alliance separa-

tions module, a Waters 2487 Dual Wavelength UV/Vis absorbance detector, and a Waters 474 scanning fluorescence detector (Millipore, Bedford, MA). Chromatograms were recorded and processed by Millennium II v. 3.20 chromatography software (Waters). The sample extract was injected through a guard-column (Waters, Nova-Pak C18, 4 μm, 3.9 × 20 mm) and separated on a Nova-Pak C18 column (3.9 × 150 mm, 4 μm, Waters) (Chen and Bergman 2005). The extraction studies were performed using the initial mobile phase conditions of 45% acetonitrile, 45% methanol, 5% isopropanol, and 5% acetic acid (1%) at a flow rate of 0.8 mL/min for 6 min. The mobile phase was changed linearly to acetonitrile-methanol-isopropanol at 25:70:5 (v/v) over the next 10 min and then held there for 12 min before being returned to the initial conditions. The T and T3 (α, β-, and δ forms) were detected by fluorescence at the excitation and emission wavelengths of 298 and 328 nm, respectively, and the γ-oryzanol by absorbance at 325 nm. The phytochemicals were quantified against the corresponding standard curves or response factor values as described in Chen and Bergman (2005). The standards of T were purchased from Matreya, Inc. (Pleasant Gap, PA). The T3 standards were purchased from CalBiochem (San Diego, CA). Z. Xu and J. S. Godler of Louisiana State University generously provided the standard of the total γ-oryzanol.

The phytochemical concentrations (w/w) were expressed in two ways: 1) “with starch” expressed on an as-is bran dry weight basis; and 2) “zero starch” expressed on zero starch bran weight basis. The formula for this was: Phytochemical concentration, zero starch (w/w) = phytochemical concentration, with starch (w/w)/(1− proportion of starch in bran).

Analyses were performed with three replicates; phytochemical concentrations (w/w) were expressed on a dry weight basis.

Statistical Analysis
All statistical analyses were performed using statistical software (v. 7. SAS Institute, Cary, NC). PROC GLM was used for analysis of variance determination, and the mean separation analysis was conducted using the Student-Newman-Keuls tests. PROC CORR was used to perform the Pearson’s Product Moment Correlation analysis.

RESULTS AND DISCUSSION

Degree of Milling
The higher the degree of milling (DOM), the lower the surface lipid content (SLC %) in the outer layers of milled head rice. The SLC % was negatively correlated to milling duration at r = -0.86 (P < 0.0001) across all cultivars (Fig. 1). As the milling time increased, the DOM increased and the SLC decreased. The range of SLC (highest to lowest) at the milling time of 20 sec among the cultivars was 0.242%, with a mean SLC of 0.5% 0.112% (1 SD). The range in SLC among cultivars decreased with longer milling times. At milling times of 30, 40, and 60 sec, the SLC ranges were 0.12, 0.16, and 0.12%, with a mean SLC of 0.40 ± 0.056% (1 SD), 0.31 ± 0.065% (1 SD), and 0.20 ± 0.052% (1 SD), respectively. Thus, a longer milling time decreased the difference in DOM for cultivars with different milling qualities, grain types (medium or long), and thicknesses.

Concentration of Starch
The concentration of starch in the bran was correlated to milling time (r = 0.58) (Fig. 2) and negatively correlated to SLC (r = -0.80) (Fig. 3). Both long-grain cultivars (CPRS and LGRU) had higher starch content in the bran than the medium-grain cultivars (BRAZ and ORIN). Within each grain type, the cultivar with superior milling quality (long-grain CPRS; medium-grain BRAZ) had a lower starch content in the bran at each milling time. The long-grain cultivar LGRU with poor milling quality had significantly higher starch content in the bran than the other sam-

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Concentration of Phytochemicals

The mean comparisons of the phytochemical concentrations in the bran collected from different milling times are presented in Table I. The δ-T3 and δ-T were eliminated for the comparisons in this study because the quantities of δ-T3 were ≈3% (w/w) of total T3, and the quantities of δ-T were 2% of total T, in the bran. The concentrations of (β+γ)-T3 and α-T3 decreased as milling time increased in the bran with starch content. Subtracting starch content from the bran lessened the differences in the concentration of these two homologs due to increased DOM. This resulted in no significant differences in the concentrations of (β+γ)-T3 and α-T3 between the milling times of 30 and 40 sec in all four cultivars (α = 0.1). The concentrations of (β+γ)-T and α-T in the bran, either with starch or zero starch, were not significantly different (α = 0.1) for milling times of 30, 40, and 60 sec for BRAZ, ORIN, and CPRS, nor for milling times of 20, 30, and 40 sec for LGRU. The concentrations of these compounds were lower at the 20-sec milling time for the two medium-grain cultivars. These results suggest that (β+γ)-T and α-T are distributed throughout the bran layer, although in an increasing concentration from pericarp to endosperm for the medium-grain cultivars. The bran γ-oryzanol concentration was strongly related with the percent of SLC because of the γ-oryzanol concentration gradient in the bran layer (Table II). Subtracting the starch content reduces the bran γ-oryzanol concentration dependence on DOM because it removes the influence due to bran starch content. Taking BRAZ as an example, a 0.1 percentage point reduction in SLC reflected a 0.194 percentage point reduction of zero starch bran γ-oryzanol versus a 0.272 percentage point reduction with starch. The starch subtraction also reduced the differences in the coefficient of slope of SLC versus γ-oryzanol among the cultivars. There was a range difference (per 1 percentage point change of SLC) of 0.75 (2.72–1.97) with starch versus 0.52 (1.94–1.42) zero starch.

The subtraction of the starch content also affected the ranking of the samples based on concentrations of T3 homologs and γ-oryzanol. Both the T3 homologs and γ-oryzanol showed more of a concentration gradient through the bran layer than did the T homologs. The cultivars that will be most affected by starch concentration when ranking concentrations of T3 homologs and γ-oryzanol among cultivars with all kernel shapes are those that are of poor milling quality or that have a long grain shape. Both LGRU and CPRS had higher starch concentration in the bran than the two medium-grain cultivars, BRAZ and ORIN. Both LGRU and CPRS had (β+γ)-T3 concentrations at the same level as ORIN before starch content subtraction, but after starch content subtraction, the (β+γ)-T3 concentrations were comparable to that of BRAZ. LGRU, the long-grain cultivar with poor milling quality, had a higher concentration of γ-oryzanol than CPRS, the long-grain cultivar with good milling quality, after starch content subtraction; whereas both were ranked the same in γ-oryzanol concentration without starch content subtraction.

Fig. 1. Surface lipid content (SLC) of whole, milled rice kernels milled for four different milling times of four cultivars of Oryza sativa L. 'Brazos' (BRAZ, medium grain); 'Orion' (ORIN, medium grain); 'Cypress' (CPRS, long grain); 'LaGrue' (LGRU, long grain). Four 50-g portions of brown rice of each cultivar were milled for 20, 30, 40, and 60 sec. Degree of milling (DOM) of rice kernels at each milling time was determined by measuring the SLC of whole (nonbroken) milled rice kernels. Each data point represents mean ± 1 SD (n = 2).

Fig. 2. Starch concentration in rice bran collected from milling brown rice kernels at four different milling times for four cultivars of Oryza sativa L. 'Brazos' (BRAZ, medium grain); 'Orion' (ORIN, medium grain); 'Cypress' (CPRS, long grain); 'LaGrue' (LGRU, long grain). Five 50-g portions of brown rice of each cultivar were milled for 20, 30, 40, and 60 sec. Collected bran was passed through a 28-mesh screen and the starch concentration in the bran determined. Each data point represents mean ± 1 SD (n = 3).

Fig. 3. Starch concentration in rice bran associated with degree of milling (DOM) or surface lipid content (SLC) of milled rice at four different milling times (right to left) of 20, 30, 40, and 60 sec for four cultivars of Oryza sativa L. 'Brazos' (BRAZ, medium grain); 'Orion' (ORIN, medium grain); 'Cypress' (CPRS, long grain); 'LaGrue' (LGRU, long grain). Each data point represents mean ± 1 SD (n = 3).
The above results suggest that starch content subtraction is necessary when samples with a wide range of grain characteristics such as milling quality and grain shape are compared for phytochemical concentrations. There is a DOM range of 0.23-0.44% SLC (milling time of 30 and 40 sec), in which the bran concentrations of T3 and T homologs after starch subtraction are not significantly different within the cultivars. Within this range, the differences in γ-oryzanol concentration resulting from differences in DOM both between and within cultivars were also minimized. This suggests that diverse samples could be milled to this range of SLC for comparative studies of these phytochemicals in bran.

**Kernel Size, Maturity, and Phytochemical Content**

The bran phytochemical concentrations collected from mature thin and immature thin kernels were analyzed and compared with those of the mature thick kernel fractions. Mature thin and immature (green) thin kernels of BRAZ and LGRU (thickness <1.69 mm) were tested for bran phytochemical contents (Table I). A milling time of 30 sec was chosen because it had the smallest range of SLC and phytochemical contents for the mature thick kernel fraction under different DOM. The mature thick kernels of BRAZ were milled to a DOM of 20-30 sec of the mature thick kernel fractions. The bran phytochemical concentrations of the T3 and T homologs and γ-oryzanol of mature thin kernels of BRAZ fell between those of 20-30 sec of the mature thick kernel fractions. The mature thick kernels of LGRU were milled to a DOM slightly less than that of the 20-sec mature thick kernel fraction. The bran phytochemical concentrations of T3 and T homologs (except α-T) and γ-oryzanol of the mature thick kernels were comparable to those of the 20-sec mature thick kernel fraction. These results suggest that the mature thick and mature thin kernels are different in grain filling, but they both progressed to the same phytochemical biosynthetic stages. In contrast, the bran collected from the milling of immature thin kernels of both cultivars had twice as much starch as that of the mature thin kernels, and the concentrations of phytochemicals, all but (β+γ)-T, were significantly lower than those of both the thick and thin mature kernels.

Chen and Siebenmorgen (1997) demonstrated that a higher DOM (0.3-0.6% SLC) of three long-grain cultivars (unfractionated samples) resulted in significantly smaller differences in bran removal between the thicker and thinner kernel fractions (kernels were fractionated after milling). Using SLC as a comparison, the high DOM is similar to the level of our milling time of 30 sec or longer. We have extended their findings to demonstrate that milling to a high DOM reduces the difference in bran removal across samples with different milling qualities and grain

### TABLE I

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Milling Time (sec)</th>
<th>DOM% (SLC %)</th>
<th>(β+γ)-T3</th>
<th>α-T3</th>
<th>(β+γ)-T8</th>
<th>α-T8</th>
<th>γ-Oryzanol</th>
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<tr>
<td></td>
<td>With Starch</td>
<td>Zero Starch</td>
<td>With Starch</td>
<td>Zero Starch</td>
<td>With Starch</td>
<td>Zero Starch</td>
<td>With Starch</td>
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<td>0.617</td>
<td>190.3a-d</td>
<td>196.6a</td>
<td>62.0e-h</td>
<td>64.1e-g</td>
<td>34.5j-h</td>
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<td>186.6d-e</td>
<td>192.3ab</td>
<td>57.2j-i</td>
<td>61.1e-i</td>
<td>39.9ed</td>
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<td>0.509</td>
<td>181.7e-g</td>
<td>189.8d</td>
<td>60.8i-e</td>
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<td>171.2h-j</td>
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<td>180.5g</td>
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<td>30</td>
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<td>161.8k</td>
<td>176.2l</td>
<td>69.9d</td>
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<td>180.3c-e</td>
<td>190.9a</td>
<td>66.1d-e</td>
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<td>27.4j-m</td>
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<td>0.301</td>
<td>169.2g-j</td>
<td>184.1e</td>
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### TABLE II

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<th>R²</th>
<th>A</th>
<th>B</th>
<th>R²</th>
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<td>0.89</td>
<td>4.08</td>
<td>1.42</td>
<td>0.68</td>
</tr>
</tbody>
</table>

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a. Student-Newman-Keuls mean separation comparison test was performed (α = 0.1).
b. With starch indicates that antioxidant concentration (w/w) is expressed on a zero starch dry weight basis, whereas “zero starch” indicates that the antioxidant concentration (w/w) is expressed on a zero starch dry weight basis.
c. *Oryza sativa* L. 'Brazos' (BRAZ, medium grain); 'Orion' (ORIN, medium grain); 'Cypress' (CPRS, long grain); 'LaGrue' (LGRU, long grain). DOM, degree of milling; SLC, surface lipid content; T, tocopherols; T3, tocotrienols.

d. Concentration unit is mg/kg of bran, dry weight basis. Mean value is the average of three replicates. Values followed by the same letter within each homolog are not significantly different at α = 0.1.

e. Concentration unit is g/kg of bran, dry weight basis. Mean value is the average of three replicates. Values followed by the same letter within each homolog are not significantly different at α = 0.1.

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Regression equation: Bran γ-oryzanol concentration = A + B × SLC, where SLC was expressed as a percentage.

b. Samples with degree of milling (DOM) of 0.2-0.5% surface lipid content (SLC) were analyzed by linear regression.

c. *Oryza sativa* L. 'Brazos' (BRAZ, medium grain); 'Orion' (ORIN, medium grain); 'Cypress' (CPRS, long grain); 'LaGrue' (LGRU, long grain).
shapes. The starch concentration in the bran reflected milling quality, DOM, and possibly other factors. Thus, putting all bran trait data on a zero starch content basis should remove variability resulting from differences in the kernel characteristics of different cultivars. The phytochemical concentrations, after starch subtraction, were not significantly different for most of the vitamin-E homologs (except γ-oryzanol) in the bran resulting from milling to different degrees, especially at the higher DOM times. We have further demonstrated that the thinnest kernel fraction, which falls below the 2-mm thickness range of the thick kernels when milled for 30 sec, had bran phytochemical concentrations similar to those of the thick kernel fractions. Thus, fractionation of kernels by thickness did not appear to be necessary before milling for bran phytochemical studies.

CONCLUSIONS

Based on these results, the following conclusions have been drawn concerning how samples should be prepared before comparing rice bran phytochemical contents of different cultivars: 1) immature green kernels should be removed before milling; 2) unfractionated rice samples should be milled to a high DOM (0.23-0.44% SLC); and 3) the starch content in the bran should be analyzed and all data expressed on a zero starch content basis.

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LITERATURE CITED


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