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Quantification of Vitamin E and γ-Oryzanol Components in Rice Germ and Bran

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Rice bran is a rich natural source of vitamin E and γ-oryzanol, which have been extensively studied and reported to possess important health-promoting properties. However, commercial rice bran is a mixture of rice bran and germ, and profiles of vitamin E and γ-oryzanol components in these two different materials are less well-studied. In the current study, vitamin E and γ-oryzanol components in rice bran and germ were analyzed by liquid chromatography/mass spectrometry/mass spectrometry. The components were identified by electrospray ionization mass spectrometry (ESI−MS) with both positive- and negative-ion modes. Both deprotonated molecular ion $[M - H]^-$ and protonated molecular ion $[M + H]^+$ found as the base peaks in spectra of vitamin E components made ESI−MS a valuable analytic method in detecting vitamin E compounds, especially when they were at very low levels in samples. Ultraviolet absorption was used for quantification of vitamin E and γ-oryzanol components. While the level of vitamin E in rice germ was 5 times greater than in rice bran, the level of γ-oryzanol in rice germ was 5 times lower than in rice bran. Also, the major vitamin E component was α-tocopherol in rice germ and γ-tocotrienol in rice bran. These data suggest that rice bran and germ have significantly different profiles of vitamin E and γ-oryzanol components. The method enables rapid and direct on-line identification and quantification of the vitamin E and γ-oryzanol components in rice bran and germ.

KEYWORDS: Rice bran; rice germ; γ-oryzanol; vitamin E; LC−MS/MS

INTRODUCTION

Free oxygen radicals are reactive and can start chain reactions from oxidation of lipid and protein molecules to generation of mutagens and carcinogens (*1*−*5*), which are involved in the pathogenesis of many diseases, such as atherosclerosis and cancer (*6*). Some studies have centered on the antioxidant nutrition in the control of degenerative diseases through both enzymatic and nonenzymatic systems (*7*−*9*). Rice bran, as the most nutritious part of rice and a storehouse of bioactive phytonutrients, has been the topic of a great deal of research (*10*).

Rice bran is produced as a byproduct during the milling process for the production of white rice (polished rice) from brown rice, and it is a mixture of bran (brown layer) and germ of brown rice. Rice bran has been extensively studied for its antioxidative and disease-fighting properties in disorders such as cancer, hyperlipidemia, fatty liver, hypercalciuria, kidney stones, and heart disease (*10*). Chemical studies indicate that rice bran is rich in vitamin E and γ-oryzanol. A natural form of vitamin E in rice bran consists of eight homologues, four of which are tocopherols and the other four comprising tocotrienols (*10*). A natural form of vitamin E in rice bran has beneficial effects on blood vessels and blood components (*10*). γ-Oryzanol is a mixture of ferulic acid esters of triterpene alcohols and sterols (*11*−*14*). γ-Oryzanol inhibits tumor promotion (*15*, *16*), reduces serum cholesterol levels (*17*−*19*), and can also be used to treat nerve imbalance and disorders of menopause (*20*). Both γ-oryzanol and vitamin E in rice bran have reported significant antioxidant activities, which protect cells from the oxidative damage of plasma very low-density lipoprotein, cellular proteins and DNA and from membrane degeneration (*21*, *22*). Rice bran products have been widely used in agricultural, food, and cosmetic industries, as well as in research studies aimed at unlocking important physiological and pharmacological properties of rice phytochemicals that may be important in health maintenance and disease prevention (*10*, *15*, *23*, *24*).

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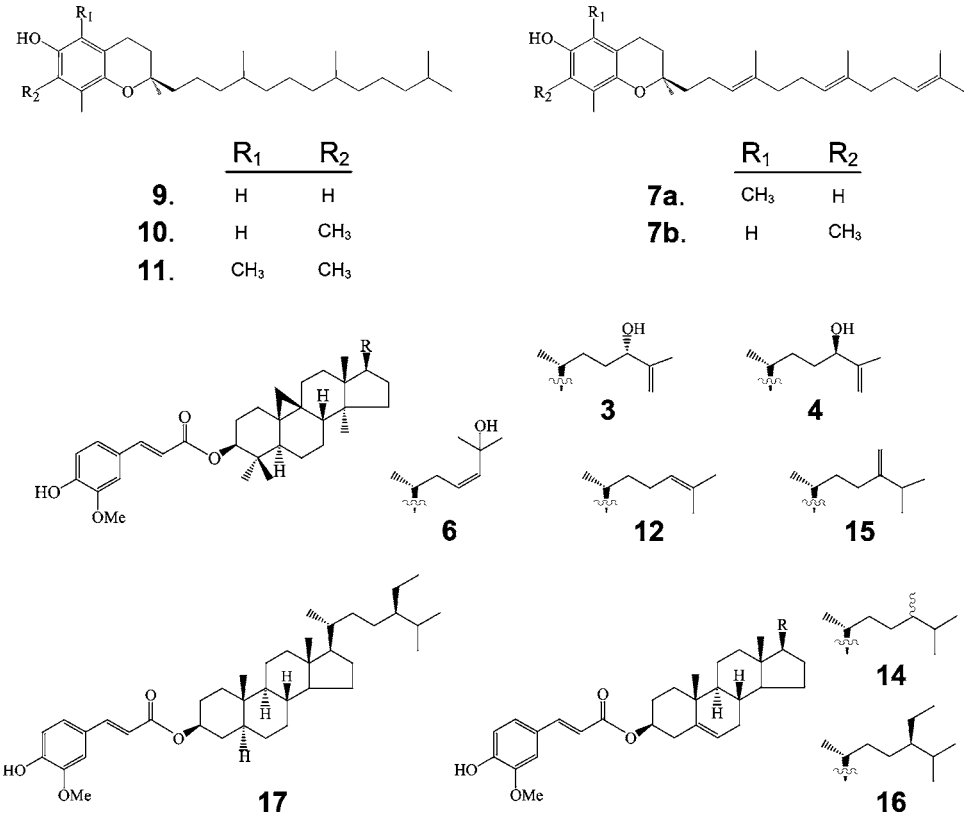

Figure 1. Structures of vitamin E and γ-oryzanol components examined in this study.

However, commercial rice bran is a mixture of rice bran and rice germ (20% of rice bran), and the difference in the chemical composition and nutraceutical effects between rice germ and bran has not been well-studied. The aim of the current study was to quantitatively analyze components of vitamin E and γ-oryzanol in rice germ and chemically distinguish rice germ and bran. We previously identified the γ-oryzanol components in rice bran (*25*, *26*). Here, liquid chromatography/mass spectrometry/mass spectrometry (LC−MS/MS) was used to characterize vitamin E components with their LC retention time and both positive- and negative-ion spectra from electrospray ionization mass spectrometry (ESI−MS). The levels of γ-oryzanol and vitamin E components in rice germ and bran were determined by their peaks in LC−ultraviolet (UV) chromatograms.

MATERIALS AND METHODS

Material. Ethyl 4-hydroxy-3-methoxycinnate (ethyl ferulate), (+)-α-tocopherol, (+)-δ-tocopherol, and (+)-γ-tocopherol were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Internal standard (IS) biochanin A, which was used for monitoring the stability of LC−MS/MS analyses, was purchased from LC Laboratories (Woburn, MA). Rice germ and full-fat raw rice bran were obtained from Riceland Foods, Inc. (Stuttgart, AR). The rice germ and bran were made from Arkansas grown rice, which consisted of 85% long-grain rice from three varieties (Drew, Cypress, and Cocodrie), and the remainder (15%) was made up by 10 varieties, including Alan, Kaybonnet, XL-6, Wells, Jefferson, Lagrue, Lemont, Madison, Millie, and Priscilla.

Preparation of Intact Rice Germ and Bran without Intact Germ Samples. Full-fat raw rice bran as a byproduct during the milling process for the production of white rice was used to obtain two samples: intact rice germs (RGs) and full-fat raw rice bran without intact germ (FFRB). Full-fat raw rice bran was put on clean paper, and RGs were separated from the rice bran one by one using forceps. Separated RGs were about 10% (w/w) of full-fat raw rice bran. This separation was only for research purposes and not involved in the

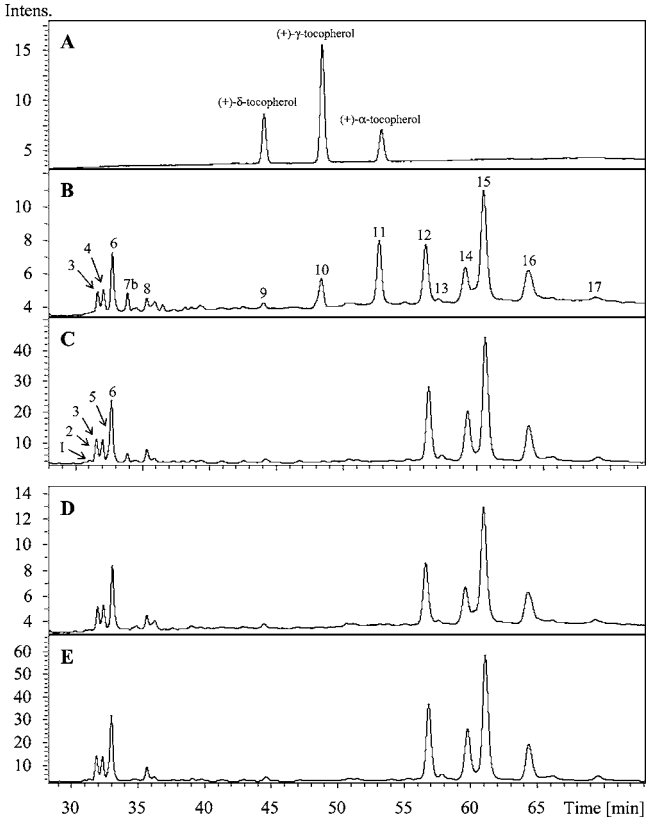

Figure 2. LC–UV chromatograms from LC–MS/MS analyses. (A) LC–UV 290 ± 10 nm chromatogram of vitamin E standards. (B) LC–UV 290 ± 10 nm chromatogram of rice germ extract. (C) LC–UV 290 ± 10 nm chromatogram of rice bran without germ extract. (D) LC–UV 320 ± 10 nm chromatogram of rice germ extract. (E) LC–UV 320 ± 10 nm chromatogram of rice bran without germ extract.

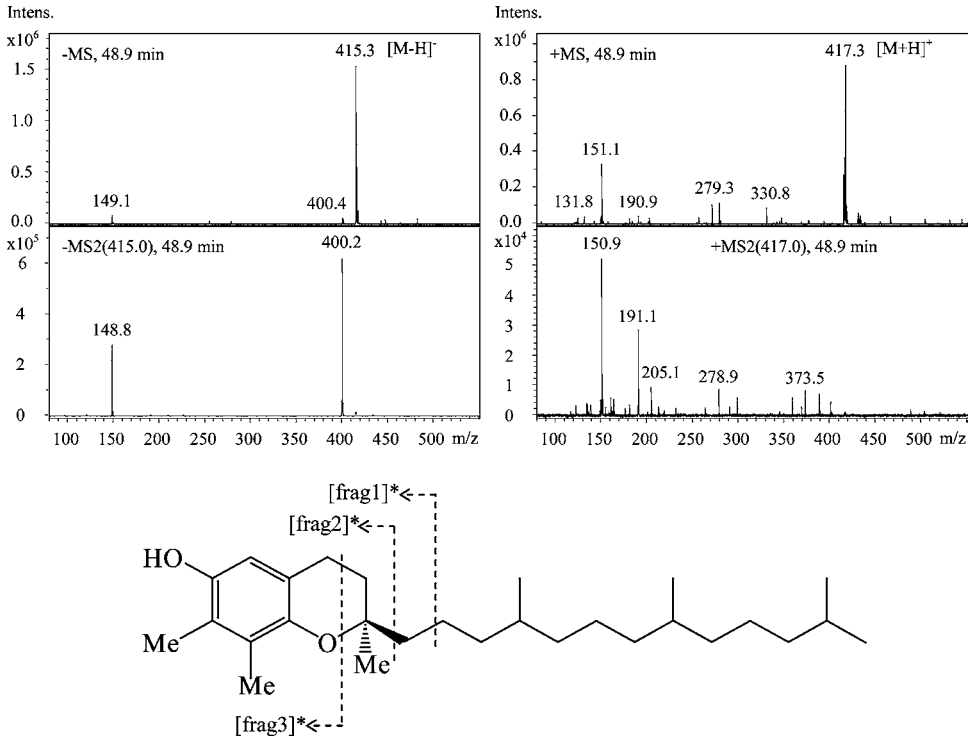

Figure 3. ESI–mass and CID spectra of compound **10** in negative- and positive-ion mode and proposed collision-induced dissociation pathway of vitamin E components. * See **Table 1** for *m*/*z* value.

Table 1. ESI–MS Data for Vitamin E Components in Rice Germ and Bran

compounds	retention time (min)	negative CID spectra *m*/*z* (relative intensity)			positive CID spectra *m*/*z* (relative intensity)			
		parent	$[M-15]^-$	$[frag3-H]^-$	parent	$[frag1+H]^+$	$[frag2+H]^+$	$[frag3+H]^+$
7a, β-tocotrienol	33.2	409 $[M-H]^-$	394 (100%)	149 (25%)	*a*			
7b, γ-tocotrienol	33.8	409 $[M-H]^-$	394 (100%)	149 (25%)	411 $[M+H]^+$	205 (37)	191 (100%)	151 (55%)
9, δ-tocopherol	44.5	401 $[M-H]^-$	386 (100%)	135 (47%)	403 $[M+H]^+$	191 (8%)	177 (38%)	151 (100%)
10, γ-tocopherol	48.9	415 $[M-H]^-$	400 (100%)	149 (45%)	417 $[M+H]^+$	205 (18)	191 (55%)	151 (100%)
11, α-tocopherol	53.4	429 $[M-H]^-$	414 (10%)	149 (100%)	431 $[M+H]^+$	219 (13%)	205 (19%)	165 (100%)

[a] The spectrum of **7a** could not be obtained in LC–MS/MS analysis because of its very low concentration in the dichloromethane fraction.

milling process. According to the information of Riceland Foods, Inc., rice germ is about 20% of full-fat raw rice bran, which suggest that the sample FFRB contains about 10% broken rice germ.

Preparation of a Phytochemical Concentrate of Rice Germ and Bran. RG was ground and used for extraction, and FFRB was directly used for extraction. For a direct comparison, analyses of RG and FFRB were conducted by the same method. Each sample (5.0 g) was treated with 40 mL of 100% ethanol, and the slurry was kept at room temperature for 24 h with occasional stirring. The slurry was filtered through a Buchner funnel with a #4 Whatman filter paper. The extraction process was repeated 3 more times with 100% methanol (40 mL), 80% aqueous methanol (40 mL), and 50% aqueous methanol (40 mL). The four extracts were combined and concentrated on a rotary evaporator under reduced pressure at room temperature until ethanol and methanol were removed followed by drying in a freeze-dryer. The dry extracts (extractable phytochemicals) represented 24.8% of the rice germ and 16.3% of the rice bran, respectively. The extract was partitioned between water (200 mL) and dichloromethane 3 times (200 mL × 3). The aqueous layer and combined dichloromethane extract were rotary evaporated under reduced pressure at room temperature followed by drying in a freeze-dryer. The vitamin-E- and γ-oryzanol-rich fractions (dichloromethane fraction) were 8.0% of the rice germ and 7.1% of the rice bran without intact germ, respectively.

LC−MS/MS Analysis. The dichloromethane fractions were dissolved in 100% methanol (10 μg/μL) and directly analyzed by LC−MS/MS with a 10 μL injection. LC-MS/MS was performed using an Agilent 1100 series liquid chromatograph interfaced to a Bruker Model Esquire-LC multiple ion trap mass spectrometer equipped with an atmospheric pressure interface−electrospray (API−ES) chamber. A HP ChemStation was used for data collection and manipulation. For high-performance liquid chromatography (HPLC), a 150 × 4.6 mm i.d., 5 μm, Eclipse XDB-C8 column (Agilent Technologies, Wilmington, DE) was used at a flow rate of 0.8 mL/min. The HPLC gradient was 0.1% formic acid/acetonitrile (solvent B) in 0.1% formic acid/H_2O (solvent A) as follows: 20−70% B in 15 min, 70−85% B from 15 to 20 min, 85−90% B from 20 to 30 min, 90−100% B from 30 to 70 min, held at 100% B from 70 to 75 min, and finally back to 10% B at 80 min, with diode-array detection set at 200 ± 10, 240 ± 10, 290 ± 10, 320 ± 10, and 355 ± 10 nm. For optimum MS analysis, 10 mM ammonium acetate (for negative-ion mode) or 2% formic acid (for positive-ion mode) in methanol was used as an ionization reagent and added at a flow rate of 0.2 mL/min via a tee in the eluant stream of the HPLC just prior to the mass spectrometer by an auxiliary HP 1100 series HPLC pump. Conditions for ESI−MS analysis of HPLC peaks in both negative- and positive-ion mode included a capillary voltage of 3200 V, a nebulizing pressure of 33.4 psi, a drying gas flow of 8 mL/min, and a temperature of 250 °C. Parameters that control the API and the mass spectrometer were set via the Smart Tune with the compound stability of 50% and trap drive level of 50%. Ion-charge control (ICC) was on, including target, 5000; maximum accumulation time, 50.00 ms; scan, *m*/*z* 80.00−850.00; averages, 10; and rolling averaging, off.

Table 2. Levels of γ-Oryzanol and Vitamin E Components in Rice Germ and Bran

structure (**Figure 1**)	name	RG[a] (mg/kg)	FFRB[a] (mg/kg)
	γ-Oryzanol Components		
1[b]	24-hydroxy-24-methyl-cycloartanol ferulates	nd[c]	17.27 ± 0.51
2[b]	stereoisomers of **1**	nd[c]	10.81 ± 0.12
3	(24*S*)-cycloart-25-ene-3β,24-diol-3β-*trans*-ferulate	20.03 ± 0.47	87.79 ± 0.95
4	(24*R*)-cycloart-25-ene-3β,24-diol-3β-*trans*-ferulate	22.70 ± 0.00	97.97 ± 1.76
5[b]	25-hydroxy-24-methyl-cycloartanol ferulates	nd[c]	30.30 ± 0.70
6	cycloart-23*Z*-ene-3β,25-diol-3β-*trans*-ferulate	60.58 ± 0.30	268.52 ± 6.18
8[b]	hydroxylated cycloartenol ferulate	14.69 ± 0.47	58.84 ± 0.90
12	cycloartenol *trans*-ferulate	95.01 ± 0.29	489.08 ± 10.31
13[b]	stereoisomer of **12**	7.16 ± 0.00	31.28 ± 1.12
14	campesterol *trans*-ferulates	64.42 ± 0.56	404.57 ± 2.96
15	24-methylenecycloartanol *trans*-ferulate	208.92 ± 1.72	941.54 ± 11.44
16	sitosterol *trans*-ferulate	73.76 ± 1.22	326.01 ± 3.83
17	stigmastanol *trans*-ferulate	10.88 ± 0.45	49.62 ± 0.75
	total	578.16 ± 3.83	2813.59 ± 26.01
	Vitamin E		
7[b]	γ-tocotrienol	55.72 ± 1.56	106.01 ± 1.40
9	δ-tocotrienol	30.60 ± 1.28	nd[c]
10	γ-tocotrienol	68.32 ± 1.45	nd[c]
11	α-tocotrienol	457.55 ± 5.62	nd[c]
	total	612.19 ± 4.75	106.01 ± 1.40

[a] RG, rice germ; FFRB, full-fat raw rice bran without germ. [b] Because neither NMR data of these minor compounds nor the corresponding standards were available, identification of **1**, **2**, **5**, **8**, and **13** could not be completed by LC–MS/MS in this study and our previous study (*25*, *26*). [c] nd = not detected.

Samples were analyzed by automatic MS/MS, with the width of the isolation = 4.0, fragmentation amplitude = 1.00 V, and number of parents = 1.

Quantitative Determination. Phytochemicals were separated by LC and monitored by a diode-array detector and MS. Vitamin E components were quantified on the basis of the areas of the UV peak (at 290 ± 10 nm), and γ-oryzanol components were based on the areas of the UV peak (at 320 ± 10 nm). The rice samples (100 μg) or standards were dissolved in 10 μL of 100% methanol, which contained 10 ng of biochanin A as an internal standard (IS). The stability of LC−MS/MS analyses was monitored by comparing the UV and MS peak areas of IS to the LC−MS/MS analyses. Calibration curves of each standard were created from seven concentrations using Microsoft Excel software. The concentrations of individual components in the rice samples were determined using calibration curves of their corresponding standard. The compounds were quantified by calibration curves of their structure-related standards when their corresponding standards were not available. Results are means ± standard deviation (SD) for at least three replicate determinations.

RESULTS AND DISCUSSION

Extraction and Fractionation of Phytochemicals in Rice Germ and Bran. The phytochemicals (24.8%, w/w) obtained from extraction of RG with ethanol, methanol, and aqueous methanol were much higher than those in FFRB (16.3%, w/w). The fractionations of the extracts by the water−dichloromethane partition indicate that RG contains significantly higher levels of polar phytochemicals (16.8 versus 9.2%, w/w) and slightly higher levels of nonpolar phytochemicals (8.0 versus 7.1%, w/w) in comparison with those in FFRB. The vitamin E and γ-oryzanol components discussed in this study are free compounds in RG and FFRB because bound tocotrienols and tocotrienol-like compounds do not release by methanol extraction without prior heating (*27*). The extraction of free vitamin E and γ-oryzanol was considered nearly complete because vitamin E and γ-oryzanol were not detected in a fifth extraction with 100% ethanol. In the water−dichloromethane partition, there were no detectable γ-oryzanols and vitamin E in the water fraction, which suggested that the major part, if not all, of free vitamin E and γ-oryzanol components of RG and FFRB was enriched in the dichloromethane fraction.

Identification of γ-Oryzanol and Vitamin E Components. **Figure 1** shows the basic chemical structures of the compounds investigated in this study. LC−UV chromatograms of the dichloromethane fractions of RG and FFRB from LC−MS/MS analyses are presented in **Figure 2**. A total of 23 components of γ-oryzanol have been identified and characterized in our previous study (*25*, *26*). Compound **8** yielded dominant deprotonated molecular ion $[M - H]^-$ at *m*/*z* 617 in negative mode, and the collision-induced decomposition (CID) pathway of the deprotonated molecular ion is similar to that of major components of γ-oryzanol (cycloartenol *trans*-ferulate) (*25*); therefore, its possible structure is hydroxylated cycloartenol ferulate. Because neither nuclear magnetic resonance (NMR) data of this minor compound nor the corresponding standard was available, identification of **8** could not be completed by the LC−MS/MS in this study. In the present study, three vitamin E components **9**, **10**, and **11** in RG were identified by a direct comparison of their corresponding standards. These compounds were (+)-δ-tocopherol, (+)-γ-tocopherol, and (+)-α-tocopherol, respectively (**Figure 2**). Electrospray ionization of vitamin E compounds yielded mass spectra with predominant base peaks of both deprotonated molecular ion $[M - H]^-$ in negative mode and protonated molecular ion $[M + H]^+$ in positive mode (**Figure 3**). The prominent negative and positive molecular ion species make ESI−MS a valuable analytical method for detecting vitamin E compounds and determining their structures, especially when only very low concentrations of the compounds in the sample or very small quantities of the compounds are available. CID of protonated molecular ions from **7a**, **7b**, **9**, **10**, and **11** produced three diagnostic fragments, $[frag1 + H]^+$, $[frag2 + H]^+$, and $[frag3 + H]^+$, resulting from the cleavage of the side chain accompanied by the breakdown of the chroman structure with a hydrogen rearrangement and the loss of a methyl acetylene ($CH_3-C\equiv CH$) fragment (**Figure 3**). CID of deprotonated molecular ions from **7a**, **7b**, **9**, **10**, and **11** produced abundant ions of $[M - H - Me]^-$, resulting from the loss of a methyl group at the C2 position in chroman moiety and anion $[frag3 - H]^-$. The diagnostic fragmentation patterns for the components of vitamin E in ESI−MS analysis are illustrated in **Table 1**, and α-, β-, γ-, and δ-tocopherols or -tocotrienols

with different numbers of methyl groups in chroman are readily distinguished by the diagnostic fragmentation pattern. However, the fragmentation pattern cannot be employed in the discrimination between β- and γ-tocotrienols, which have the same number of methyl groups with different positions in the chroman (**7a** and **7b**). The elution order of vitamin E components have been reported to be α > β > γ > δ in a reverse-phase system (*28*). Compounds **7a** and **7b** yielded almost identical mass spectra and had a different retention time (**Table 1**). The retention times suggest that **7a** (33.2 min) and **7b** (33.8 min) are β- and γ-tocotrienols, respectively. Compound **7a** was not detected by MS in positive mode and only yielded a very small UV peak, which indicated that a trace amount of **7a** was in the dichloromethane fraction.

Quantification of γ-Oryzanol and Vitamin E Components. The LC−UV chromatograms are shown in **Figure 2**. LC−MS/MS analyses were very reproducible based on the recovery rates of all standards and IS. For example, the mean of the UV peak at the IS (320 nm) was 132.22 ± 0.67 mAU min (mean ± SD, $n = 9$). A wavelength of 320 ± 10 nm was used to quantitate oryzanol components, and a wavelength of 290 ± 10 nm was used for the quantification of vitamin E components. The levels of three tocopherols were calculated on the basis of the standard curves of their corresponding standards. The standard curve of γ-tocopherol was also used to calculate γ-tocotrienol. The UV absorption of γ-oryzanol components results from the ferulic-acid-conjugated system, and the standard curve of standard ethyl ferulate was used for the quantification of γ-oryzanol components. While γ-oryzanol components were detected in LC−UV chromatograms of both 320 and 290 nm (**Figure 2**), they were more sensitive at the 320 nm wavelength. The 320 nm peaks were used to quantitate γ-oryzanol components. The results shown in **Figure 2** and **Table 2** indicate a significant difference between chemical profiles of RG and FFRB. The amounts of vitamin E components (612.19 mg/kg) and γ-oryzanol components (578.16 mg/kg) are almost the same as in RG (**Table 2**). The major vitamin E component in RG is α-tocopherol (457.55 mg/kg). γ-Tocotrienol, which has been reported as the major vitamin E component in commercial rice bran (*14*, *29*), has a lower concentration in RG. Rice bran (FFRB) used in this study was different from commercial rice bran that has been widely used in rice bran studies. In the present study, intact germ was sorted out from rice bran, but the rice bran might contain broken germ generated from the rice milling process. Only one vitamin E component, γ-tocotrienol, was found in FFRB at the level of 106.01 mg/kg. Other vitamin E components were not detected in the dichloromethane fraction of FFRB. The level of γ-oryzanol (2813.59 mg/kg) was 27 times higher than the level of vitamin E (106.01 mg/kg) in FFRB. Because the major vitamin E component in RG was α-tocopherol and the most abundant vitamin E component found in FFRB was γ-tocotrienol, it is reasonable to assume that γ-tocotrienol was extracted from the rice bran but not the broken germ.

In summary, we analyzed free vitamin E and γ-oryzanol components in RG and FFRB. The profiles of free vitamin E and γ-oryzanol in RG and FFRB differ significantly. The levels of free vitamin E and γ-oryzanol are the same as in RG, but the level of free vitamin E is 27 times lower than that of γ-oryzanol in FFRB. The major free vitamin E components are α-tocopherol in RG and γ-tocotrienol in FFRB. The level of γ-oryzanol in RG is much less than that in FFRB, but the profiles of γ-oryzanol components in RG and FFRB are very similar. The order of the levels of γ-oryzanol components are **15** > **12** > **16** > **14** > **6** > **4** > **3** > **17** in RG and **15** > **12** > **14** > **16** > **6** > **4** > **3** > **17** in FFRB. It is important to note that commercial rice bran is the mixture of rice bran and germ and different commercial rice bran may contain different levels of vitamin E and γ-oryzanol components. Also, different extraction methods can result in different levels of these components because some tocotrienols and tocotrienol-like compounds are bound to cellular components in the rice bran (*27*).

ABBREVIATIONS USED

RG, intact rice germ; FFRB, full-fat raw rice bran without intact germ; ESI−MS, electrospray ionization mass spectrometry; API−ES, atmospheric pressure interface−electrospray; ICC, ion-charge control; CID, collision-induced dissociation; frag, fragment.

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