JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Triterpene Alcohol and Sterol Ferulates in Rice Bran Using LC-MS/MS

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Ferulic acid esters of triterpene alcohols and sterols in rice bran oil have been extensively studied and reported to possess important pharmacological actions. Inconsistent results on the numbers and structures of ferulates have been reported, primarily because of the analytical procedures employed. Conventional methods for analysis of phytosterol content in oil are carried out by characterization of trimethylsilylated derivatives (TMS) using GC-EI-MS after saponification of oils or individual compound isolated from oils. This study developed an LC-MS/MS method for the direct analysis of triterpene alcohol and sterol esters in rice bran oil. In addition to verifying the results of previous research, nine new relatively polar triterpene alcohol and sterol esters were characterized by their retention behaviors in LC and ESI-MS data from both negative- and positive-ion mode. This is the first evidence for the presence of hydroxylated ferulate esters and caffeate esters as part of γ-oryzanol in rice bran. The method enables rapid and direct on-line characterization of triterpene alcohol and sterol esters in oils. LC-MS/MS equipped with reverse-phase LC and ESI-MS should be well-suited for identification and quantification of the polar metabolites of phytosterols in biological fluids after consumption of rice bran oil or other oils.

KEYWORDS: Rice bran; γ-oryzanol; ferulic acid esters of triterpene alcohols and sterols; hydroxylated ferulate esters; caffeate esters; LC-MS/MS

INTRODUCTION

More than 10 million hundredweight (cwt) of rice bran are produced annually as a byproduct in the United States (Arkansas Agricultural Statistics, 1996). Rice bran contains 18−22% oil and is used in diet preparation in several countries. One of the major bioactive components in rice bran oil, γ-oryzanol, is a mixture of ferulic acid esters of triterpene alcohols and sterols (*1*−*4*). γ-Oryzanol inhibits tumor promotion (*5*, *6*), reduces serum cholesterol levels (*7*−*9*), and can also be used to treat nerve imbalance and disorders of menopause (*10*).

γ-Oryzanol was first reported as a single component in rice bran oil in 1954 (*11*) but later was shown to be a mixture. The majority of the analytical studies of γ-oryzanol in rice bran oil involve separation of components by multistep chromatographies and identification of individual compounds by gas chromatography−electron impact mass spectrometry (GC-EI-MS), positive chemical ionization−mass spectrometry (CI-MS), and nuclear magnetic resonance (NMR) (*2*−*6*). Positive CI-MS by the direct inlet probe (DIP) technique of the separated individual compounds has been used to identify cycloartenol ferulate, 24-methylenecycloartanol ferulate, campesterol ferulate, sitosterol ferulate, and cycloartanol ferulate as the major γ-oryzanols in rice bran oil (*4*). Later, 10 components of γ-oryzanol including three pairs of Δ^7- and Δ^5-isomers were identified in rice bran oil by EI-MS as trimethylsilyl (TMS) derivatives, but the previously reported cycloartanol ferulate was not detected (*2*). Subsequently, 12 triterpene alcohol and sterol ferulates, without Δ^7-isomers but including five pairs of *trans*- and *cis*-ferulate isomers, were identified by NMR and EI-MS after separation of individual compounds (*6*). The multistep separation process provided ample opportunity for the production of *cis*-ferulates as methodological artifacts (*6*), which represents a long-standing problem in oil chemistry. The disadvantage inherent in using EI-MS and positive CI-MS for identification of γ-oryzanol is that triterpene alcohol and sterol ferulates or their TMS derivatives yield very low abundances of molecular ions (as low as 1% of relative intensity in some case) (*2*, *4*, *6*), which would make identification by MS unreliable.

The aim of the current study was to develop a convenient method to directly analyze components of γ-oryzanol in rice bran oil. Using LC-MS/MS, γ-oryzanol components from rice bran were characterized by LC retention time and both positive- and negative-ion spectra from electrospray ionization mass spectrometry (ESI-MS). An automated MS/MS program was employed to get product ion spectra from collision-induced dissociation (CID) of base peaks in mass spectra.

MATERIALS AND METHODS

Material. Full-fat stabilized rice bran was obtained from Arkansas-grown long-grain rice (Riceland Foods, Inc., Stuttgart, AR). According to Riceland Foods, Inc., Arkansas-grown rice consist of 85% long-

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10.1021/jf021162c CCC: $25.00
Published on Web 04/25/2003

grain rice from three varieties (Drew, Cypress, and Cocodrie), and the remainder (15%) is made up by 10 varieties including Alan, Kaybonnet, XL-6, Wells, Jefferson, Lagrue, Lemont, Madison, Millie, and Priscilla.

Sample Preparation for LC-MS/MS. Full-fat stabilized rice bran (200 g) was treated with 300 mL of 80% aqueous methanol, and the slurry was allowed to stand at 5 °C for 24 h with occasional stirring. The slurry was filtered through a Büchner funnel. The extraction process was repeated two more times with the same solvent (300 mL) and twice with 50% aqueous methanol (300 mL). The five extracts were combined and concentrated on a rotary evaporator under reduced pressure at room temperature until the methanol was removed. The concentrated extract was partitioned with hexane (500 mL) three times, and the extracts were combined. The combined extract was rotary evaporated under reduced pressure at room temperature followed by drying in the freeze-dryer to give the hexane extract (3.92%) of full-fat stabilized rice bran.

LC-MS/MS Analysis. The hexane extract of rice bran was dissolved in 100% ethanol (10 μg/μL) and directly analyzed by LC-MS/MS with a 5-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. HP ChemStation was used for data collection and manipulation. For HPLC, a 150 × 4.6 mm, 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 acetonitrile (solvent B) in H_2O (solvent A): 20−70% in 15 min, 70−85% from 15 to 20 min, 85−90% from 20 to 30 min, 90−100% from 30 to 70 min, 100−100% to 75 min, and finally returned to initial concentration from 75 to 80 min, with diode array detection set at 320 ± 10 nm for feruloyl esters in the hexane extract. 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 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 interface 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 the following: target, 5000; maximum accumulation time, 50.00 ms; scan, from *m*/*z* 80.00 to 850.00; averages, 10; and rolling averaging, off. Samples were analyzed by automatic MS/MS with width of the isolation of 4.0, fragmentation amplitude of 1.00 V, and number of parents = 1.

RESULTS AND DISCUSSION

Confirmation of γ-Oryzanol Components in Rice Bran. **Figure 1** shows the basic chemical structures investigated in this study. The composition of γ-oryzanol (triterpene alcohol and sterol ferulates) was monitored at 320 ± 10 nm, and four major peaks (compounds **11**, **14**, **16**, and **19**) eluting at 55−65 min were detected (**Figure 2**). Using negative-ion ESI-MS, **11**, **14**, **16**, and **19** yielded base peaks for the deprotonated molecular ions, and indeed there were no other significant peaks in their mass spectra (**Figure 3** and **Table 1**). The deprotonated molecular ions of **11**, **14**, **16**, and **19** are at *m*/*z* 601, 575, 615, and 589, respectively, in accord with the structures of four major γ-oryzanol components previously identified in rice bran oil (*2*, *4*, *6*). The structures of these four compounds are cycloartenol *trans*-ferulate (**11**), campesterol *trans*-ferulate (**14**), 24-methylenecycloartanol *trans*-ferulate (**16**), and sitosterol *trans*-ferulate (**19**) (**Figure 1**). In contrast to the abundant yield of deprotonated molecular ions, positive-ion ESI-MS generated the base peaks corresponding to cations of triterpene alcohol or sterol moieties ($[M + H - 194]^+$) from neutral loss of ferulic acid. This characteristic fragmentation pathway for these four ferulates has also been reported from positive CI-MS (*4*). Collision-induced

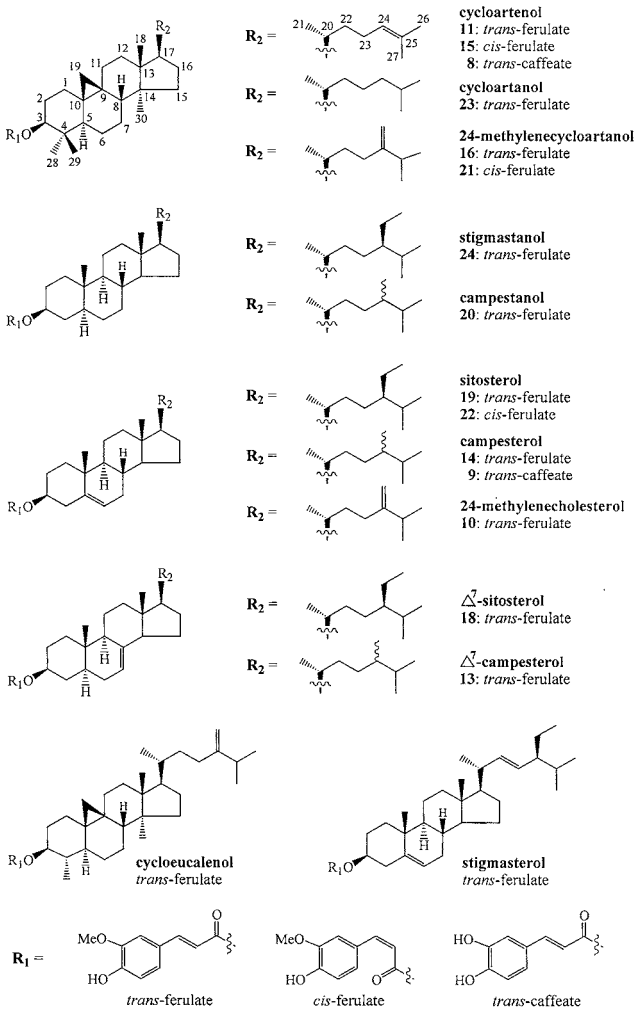

Figure 1. Structures of the triterpene alcohol and sterol esters identified in this study.

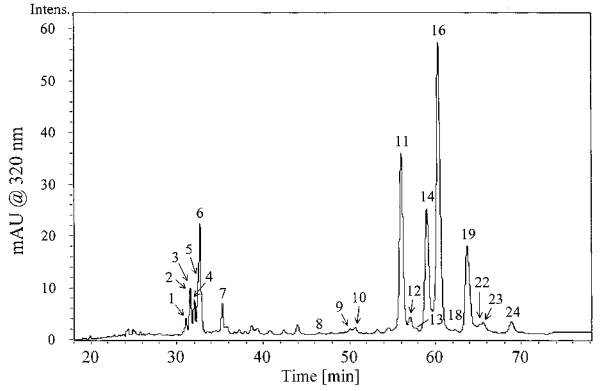

Figure 2. UV (320 nm) chromatogram from LC-MS/MS of a hexane extract of rice bran. The peaks for **15**, **20**, and **21** are shown in **Figure 4**.

decomposition (CID) of deprotonated molecular ions from **11**, **14**, **16**, and **19** produced abundant ions of $[M - H - Me]^-$ resulting from the loss of a methyl group in the ferulic acid moiety. Another anion, $[M - H - 2Me]^-$, was yielded only by **14** and **19**, which are sterol ferulates with a double bond between C-5 and C-6 (**Figure 1**). The mechanism proposed for formation of the ion $[M - H - 2Me]^-$ involved a loss of a methyl group from C-10 to generate a product ion shown in **Figure 3**. Characteristic ions for the feruloyl moiety following the CID of deprotonated molecular ions include deprotonated ferulic acid

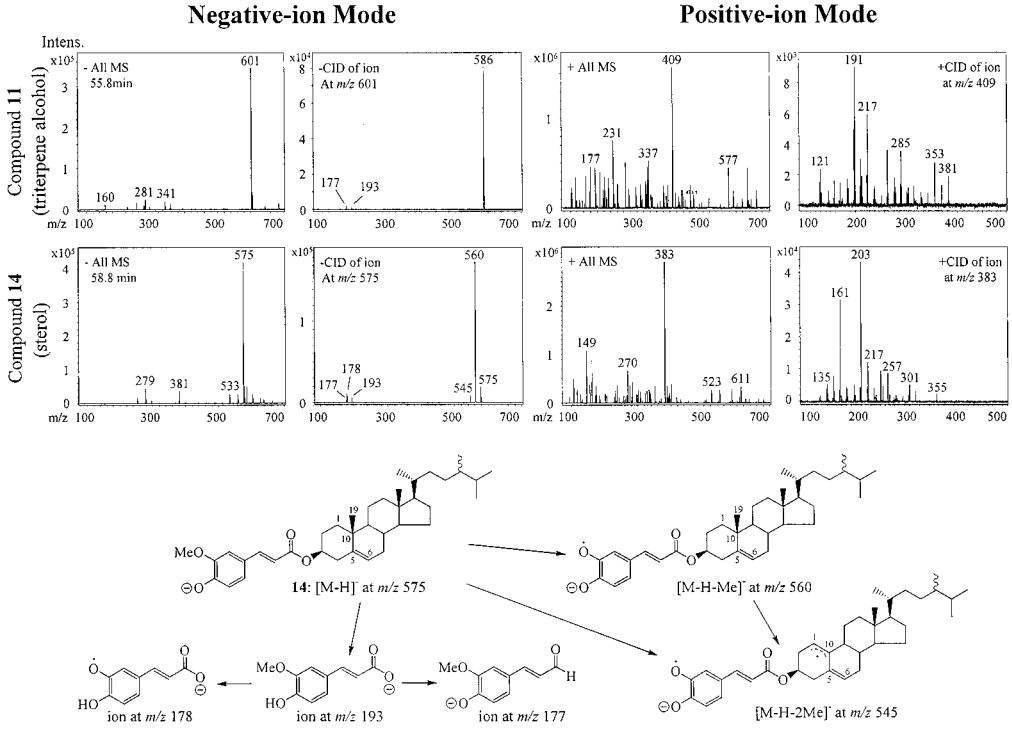

Figure 3. ESI-MS and CID spectra of compounds **11** and **14** in negative- and positive-ion mode and proposed CID pathway of **14**.

at *m*/*z* 193 as well as fragments at *m*/*z* 178, 177, and 175 derived from deprotonated ferulic acid (**Figure 3** and **Table 1**). The deprotonated ferulic acid at *m*/*z* 193 suggests that a diagnostic CID pathway of ferulic acid esters of triterpene alcohols and sterols involves the cleavage of the feruloyl and alcohol moieties. No fragment corresponding to alcohol moieties indicates that triterpene alcohols and sterols are not ionized in the negative-ion mode (**Figure 3**). In positive-ion mass spectra, the base peaks of compounds **11**, **14**, **16**, and **19** are the typical fragments for intact moieties of triterpene alcohols or sterols, and CID of these fragments generated multiple peaks resulting from losses of different numbers of methyl and methylene groups (**Figure 3** and **Table 1**).

The numbers and structures of minor components of γ-oryzanol in rice bran oil reported in previous studies are inconsistent (*2*−*6*). Because the deprotonated molecular ions are the most abundant peaks in the mass spectra of all four major components under these LC-MS/MS conditions, the deprotonated molecular ions of all previously reported γ-oryzanol components were used as extracted ions to trace these components and clarify their presence in rice bran, which include ions at *m*/*z* 573 (24-methylenecholesterol *trans*-ferulate) (*6*), 577 (campestanol *trans*-ferulate) (*2*), 587 (stigmasterol *trans*-ferulate) (*2*), 591 (stigmastanol *trans*-ferulate) (*2*, *6*), and 603 (cycloartanol *trans*-ferulate) (*4*), respectively. Extracted ion chromatograms (EIC) are shown in **Figure 4**. Compounds corresponding to ions at *m*/*z* 573 (**10**), 577 (**20**), 591 (**24**), and 603 (**23**) yielded dominant deprotonated molecular ions during electrospray ionization (**Table 1**), and the CID pathway of their deprotonated molecular ions is similar to that of major components of γ-oryzanol (**11**, **14**, **16**, and **19**) (**Table 1**). All ESI-MS data support previous reports of 24-methylenecholesterol *trans*-ferulate (**10**), campestanol *trans*-ferulate (**20**), stigmastanol *trans*-ferulate (**24**), and cycloartanol *trans*-ferulate (**23**) in rice bran oil (*2*−*6*). The ion at *m*/*z* 587 for stigmasterol *trans*-ferulate exhibited an EIC peak (compound **17**) (**Figure 4J**) but failed to generate the CID spectrum due to its low quantity in the sample. The EIC peak for cycloeucalenol *trans*-ferulate (ion at *m*/*z* 601) might be overlapped with that of **11** because cycloeucalenol *trans*-ferulate has a retention time almost identical to that of **11** on the C18 reverse-phase HPLC (*6*). Therefore, this study could not confirm the presence of these two minor compounds in rice bran reported previously (*2*, *6*).

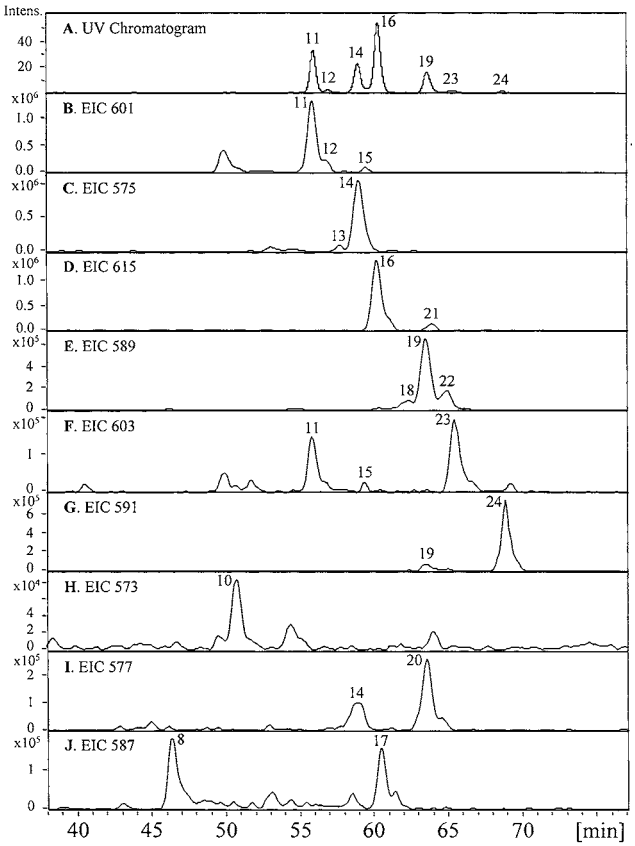

Figure 4. Extracted ion chromatogram (EIC) for confirmation of the triterpene alcohol and sterol ferulates previously reported in rice bran oil.

Table 1. ESI-MS Data for Triterpene Alcohol and Sterol Ferulates Previously Reported in Rice Bran

		negative CID spectra, *m/z* (rel intensity, %)						positive CID spectra, *m/z* (rel intensity, %)			
structure no.	R_t (min)	precursor[a] $[M-H]^-$	$[M-H]^-$	$[M-H-Me]^-$	$[M-H-2Me]^-$	$[feruloyl]^-$	other ions from feruloyl part	precursor[b] $[M+H-194]^+$	product ions (see **Figure 3**)	structure	lit. report
sterol ferulates											
10	50.8	573	573 (6.1)	558 (100)	543 (2.8)	193 (16.9)	178 (2.0), 177 (1.3)			24-methylenecholesterol *trans*-ferulate	*6*
13	57.5	575	575 (10.8)	560 (100)		193 (3.8)	177 (3.6)	383	ions [parent – nCH_3 and/or – nCH_2]$^-$	Δ^7-campesterol *trans*-ferulate	*2*
14	58.8	575	575 (11.5)	560 (100)	545 (5.3)	193 (3.6)	178 (6.2), 177 (2.4)	383	ions [parent – nCH_3 and/or – nCH_2]$^-$	campesterol *trans*-ferulate	*2*, *4–6*
18	62.1	589		574 (100)	559 (3.2)		178 (9.4)			Δ^7-sitosterol *trans*-ferulate	*2*
19	63.5	589		574 (100)	559 (8.2)	193 (5.6)	178 (5.9), 177 (5.8), 175 (2.3)	397	ions [parent – nCH_3 and/or – nCH_2]$^-$	sitosterol *trans*-ferulate	*2*, *4–6*
20	63.6	577	577 (15.1)	562 (100)		193 (1.7)	177 (2.1)			campestanol *trans*-ferulate	*2*
22	65.0	589		574 (100)	559 (6.4)	193 (4.2)	177 (5.1)	397	ions [parent – nCH_3 and/or – nCH_2]$^-$	sitosterol *cis*-ferulate	*6*
24	68.8	591		576 (100)		193 (2.0)	177 (6.5)			stigmastanol *trans*-ferulate	*2*, *6*
triterpene alcohol ferulates											
11	55.8	601		586 (100)		193 (1.8)	177 (2.7)	409	ions [parent – nCH_3 and/or – nCH_2]$^-$	cycloartenol *trans*-ferulate	*2*, *4–6*
12	56.8	601		586 (100)		193 (8.2)	178 (4.1)	409	ions [parent – nCH_3 and/or – nCH_2]$^-$	isomer of **11**	
15	59.5	601		586 (100)			177 (10.8), 175 (1.1)	409	ions [parent – nCH_3 and/or – nCH_2]$^-$	cycloartenol *cis*-ferulate	*6*
16	60.2	615		600 (100)			177 (2.2)	423	ions [parent – nCH_3 and/or – nCH_2]$^-$	24-methylenecycloartanol *trans*-ferulate	*2*, *4–6*
21	63.9	615		600 (100)			177 (2.0), 175 (2.4)	423	ions [parent – nCH_3 and/or – nCH_2]$^-$	24-methylenecycloartanol *cis*-ferulate	*6*
23	65.5	603		588 (100)		193 (7.0)	177 (3.7)	411	ions [parent – nCH_3 and/or – nCH_2]$^-$	cycloartanol *trans*-ferulate	*4*

[a] Deprotonated molecular ions are very abundant peaks in the mass spectra of all ferulates discussed in this table. [b] Ions $[M+H-194]^+$ are base peaks in the mass spectra of all ferulates discussed in this table.

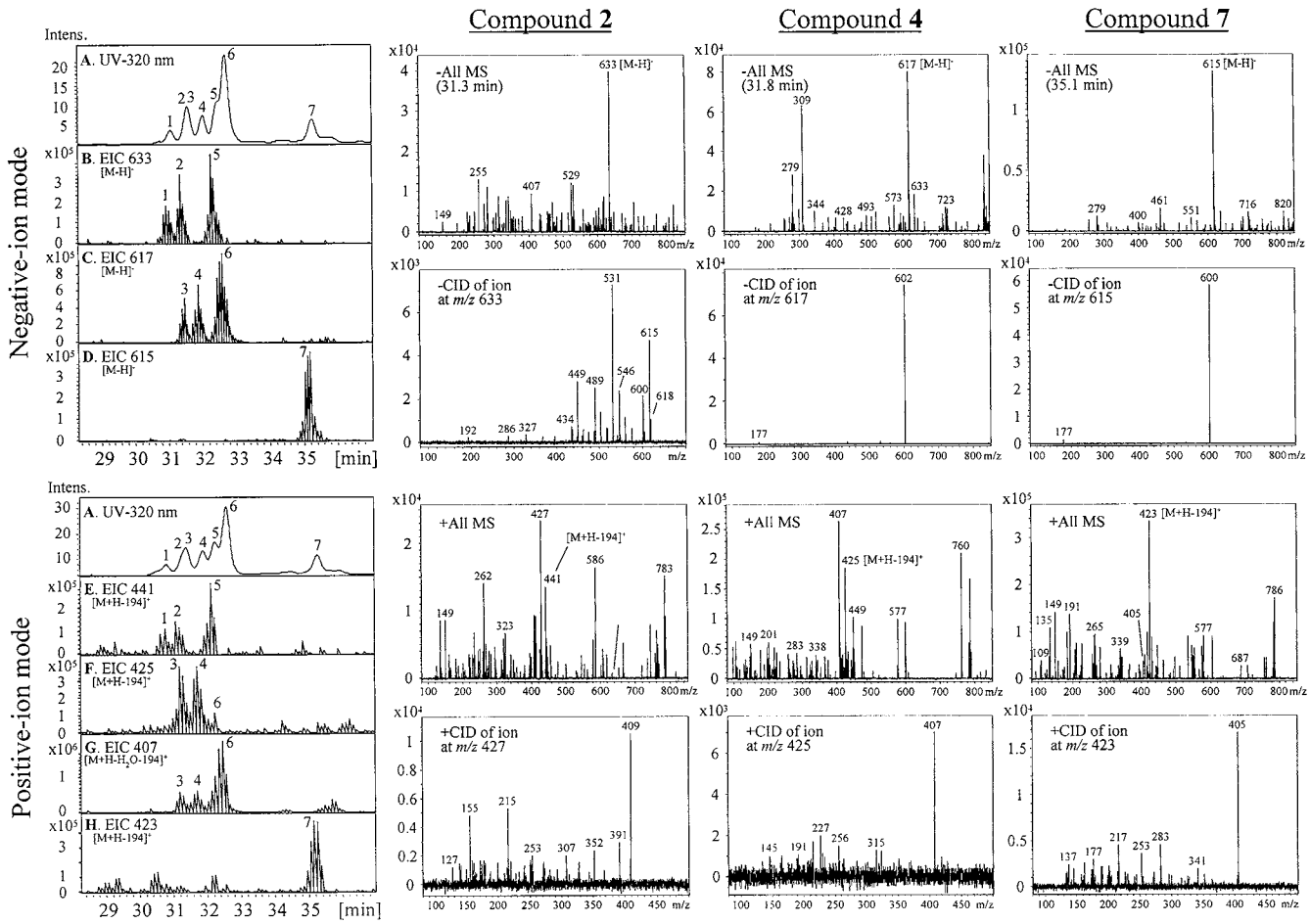

Figure 5. Extracted ion chromatogram (EIC) and mass spectra for characterization of unknown triterpene alcohol and sterol ferulates in rice bran oil.

The Δ^7-isomers of two major γ-oryzanol components, campesterol *trans*-ferulate (**14**) and sitosterol *trans*-ferulate (**19**), have been identified in rice bran oil and reported to have shorter retention times than their Δ^5-isomers on the C18 reverse-phase HPLC (*2*). In the present study, the minor peaks corresponding to **13** and **18** (**Figure 4**) eluted prior to the major components **14** and **19** and gave ESI-MS data similar to those of **14** and **19**, respectively. It is reasonable to assume that **13** and **18** are Δ^7-isomers of campesterol *trans*-ferulate (**14**) and sitosterol *trans*-ferulate (**19**), respectively, which confirms the previous identifications of these two compounds in rice bran oil (*2*).

Column chromatography, TLC, and HPLC have been used to isolate the five pairs of *trans*- and *cis*-ferulate isomers from rice bran (*6*). *trans*- and *cis*-ferulate isomers yield almost identical EI-MS spectra, and *cis*-isomers have longer retention times than their corresponding *trans*-isomers in the C18 reverse-phase HPLC (*6*). In this study, compounds **15**, **21**, and **22** exhibited ESI-MS data very similar to those of cycloartenol *trans*-ferulate (**11**), 24-methylenecycloartanol *trans*-ferulate (**16**), and sitosterol *trans*-ferulate (**19**) and were retained longer on reverse-phase sorbent than **11**, **16**, and **19**, respectively (**Table 1**). Thus, the structures of **15**, **21**, and **22** were assigned as *cis*-isomers of the major γ-oryzanol components **11**, **16**, and **19**, respectively. Because daylight and long-wavelength UV radiation induce *cis*−*trans* isomerization of feruloyl esters (*12*, *13*), artifacts might occur during the manufacture of rice bran. Therefore, the possibility of these three *cis*-ferulates as artifacts could not be ruled out, even though simple extraction and fractionation with direct analysis of hexane extract by LC-MS/MS were employed in the present study. The small peak for compound **12** in the EIC of the ion at *m*/*z* 601 appeared on the side of peak 11. The ESI-MS data from **12** are almost identical to those of **11** (**Table 1**), and it is likely that **12** is a stereoisomer of **11** due to different configuration of the triterpene alcohol.

In summary, components of γ-oryzanol gave most abundant peaks for their deprotonated molecular ions in negative-ion ESI-MS, and CID of deprotonated molecular ions yielded the product ions indicative of the feruloyl moiety and double bond in alcohol moiety. In the positive-ion mode, base ions are the intact alcohol moiety (triterpene alcohols or sterols) derived from the cleavage of feruloyl and alcohol moieties. These base ions yielded the product ion spectra by losses of different numbers of methyl and methylene groups.

Characterization of Unknown Triterpene Alcohol and Sterol Esters. *Ferulate Esters.* There are two clusters of UV peaks in the HPLC chromatograms of rice bran oil (*2*, *4*), and all major known components of γ-oryzanol were identified from the major cluster of UV peaks (*2*−*6*). Another cluster of UV peaks had a much shorter chromatographic run time than that of known compounds in reverse-phase HPLC (*2*, *4*), and the structures of compounds corresponding to this cluster of UV peaks are presently unknown. In the present study, the HPLC profile of the hexane extract of rice bran (**Figure 2**) is comparable to those from the HPLC methods of Rogers et al. (*4*) and Xu and Godber (*2*). This unknown cluster of UV peaks appeared between 30 and 36 min and originated from at least seven components (compounds **1**−**7** in **Figure 5**). The retention

behaviors in reverse-phase HPLC indicated that **1**−**7** are more polar than all known ferulate esters of triterpene alcohols and sterols in rice bran oil. Compounds **3**, **4**, and **6** had the same deprotonated molecular ion at *m*/*z* 617 (**Figure 5C**) and almost identical product ion spectra from CID of their deprotonated molecular ions (**Table 2**), which indicates that **3**, **4**, and **6** are isomers with similar MS fragmentation behaviors. Two prominent cations, $[M + H - 194]^+$ at *m*/*z* 425 and $[M + H - 194 - H_2O]^+$ at *m*/*z* 407, were exhibited in the positive-ion spectra of **3**, **4**, and **6** (**Figure 5F** and **5G**). CID of the cation $[M + H - 194]^+$ yielded the $[M + H - 194 - H_2O]^+$ as the most abundant product ion, and CID of cation $[M + H - 194 - H_2O]^+$ gave a multipeak spectrum similar to those of **11** and **14** (**Figure 3**). Formation of the ion $[M + H - 194 - H_2O]^+$ by a neutral loss of H_2O from the ion of the intact alcohol moiety suggests a hydroxyl group in the triterpene alcohol or sterol moieties. Because cycloartenol *trans*-ferulate (**11**) is the major component and one oxygen (16 u) less than **3**, **4**, and **6**, it is expected that structures of **3**, **4**, and **6** may be three isomers of hydroxycycloartenol ferulate. With the same interpretation of ESI-MS data used for assignments of structures for **3**, **4**, and **6**, the structure of compound **7** with a deprotonated molecular ion at *m*/*z* 615 (**Figure 5D**) was assigned as hydroxydehydrocycloartenol ferulate. Compound **11** and its hydroxylated compounds **3**, **4**, **6**, and **7** yielded very similar product ion spectra. CID of deprotonated molecular ions of **3**, **4**, **6**, and **7** gave a dominant ion $[M - H - Me]^-$ and did not reveal any information about the position of the hydroxyl group in the structures. Deprotonated molecular ions at *m*/*z* 633 and cations $[M + H - 194 - H_2O]^+$ at *m*/*z* 423 produced from CID of $[M + H - 194]^+$ suggest that compounds **1**, **2**, and **5** are hydroxylated and hydrogenated derivatives of 24-methylenecycloartanol ferulate (major component **16)**. In contrast to **3**, **4**, **6**, and **7**, hydroxylation and hydrogenation of 24-methylenecycloartanol *trans*-ferulate would give hydroxylated compounds (**1**, **2**, and **5)** unstable in negative-ion ESI-MS and yielding more complicated product ion spectra for **1**, **2**, and **5** than that from **16** (**Table 2**). Proposed CID pathways of **1**, **2**, and **5** in **Figure 6** led to the assignments of structures 24-hydroxy-24-methylcycloartanol ferulates for **1** and **2** (two stereoisomers) and 25-hydroxy-24-methylcycloartanol ferulates for **5**. As neither NMR data of these minor compounds nor the corresponding standards were available, identification of **1**−**7** could not be completed by the LC-MS/MS in this study.

Caffeate Esters. The mass spectra of compounds **8** and **9** from ESI-MS reveal the same fragmentation behavior as those for known ferulate esters discussed above with most abundant peaks for the deprotonated molecular ion in negative-ion spectra. However, CID of deprotonated molecular ions from MS of **8** and **9** yielded base peaks at $[M - H]^-$ and weak peaks at *m*/*z* 179 (**Table 2**). Because abundant ions of $[M - H - Me]^-$ in CID spectra of $[M - H]^-$ of ferulate esters correspond to loss of a methyl group from the ferulic acid moiety, the absence of the ion $[M - H - Me]^-$ in the product ion spectra of **8** and **9** indicates the absence of a methyl group in the acid moieties. Furthermore, fragments at *m*/*z* 179 in negative CID spectra and cations $[M + H - 180]^+$ for intact alcohol moieties (triterpene alcohols or sterols) in positive mass spectra of **8** and **9** provide strong evidence for caffeoyl moieties and establish that **8** and **9** are caffeate esters. The ions $[M + H - 180]^+$ of **8** (*m*/*z* 409) and **9** (*m*/*z* 383) yielded CID spectra that are essentially identical to those of the major components cycloartenol ferulate (**11**, $[M + H - 194]^+$; *m*/*z* 409) and campesterol ferulate (**14**, $[M + H - 194]^+$; *m*/*z* 383) (**Figure 1** and **Table 2**), respectively.

Table 2. ESI-MS Data for New Triterpene Alcohol and Sterol Esters in Rice Bran

structure no.	R_t (min)	negative-ion mode, CID spectra, *m*/*z* (rel intensity %)						positive-ion mode, *m*/*z* (rel intensity %)			
		precursor[a]						mass spectra		precursor	CID spectra
ferulate esters											
1	30.9	633	618 (17.0)	615 (64.1)	600 (20.6)	546 (15.1)	531 (100)	517 (15.1), 502 (6.4),	441 (100)	423 (26.0)	441	423 (100) and
		$[M - H]^-$	$[M - H - Me]^-$	$[M - H - H_2O]^-$	$[M - H - Me - H_2O]^-$	$[M - H - 87]^-$	$[M - H - 102]^-$	489 (16.4), 449 (15.6)	$[M + H - 194]^+$	$[M + H - 194 - H_2O]^-$		multiple peaks
2	31.3	633	618 (13.7)	615 (56.1)	600 (29.9)	546 (33.0)	531 (100)	517 (7.8), 502 (19.6),	441 (58.1)	427 (100)	427	409 (100) and
		$[M - H]^-$	$[M - H - Me]^-$	$[M - H - H_2O]^-$	$[M - H - Me - H_2O]^-$	$[M - H - 87]^-$	$[M - H - 102]^-$	489 (34.9), 449 (39.2)	$[M + H - 194]^+$	$[M + H - 194 - CH_2]^-$		multiple peaks
3	31.5	617	602 (100)					177 (1.1)	425 (100)	407 (54.8)	425	407 (100) and
		$[M - H]^-$	$[M - H - Me]^-$						$[M + H - 194]^+$	$[M + H - 194 - H_2O]^-$		multiple peaks
4	31.8	617	602 (100)					177 (1.5)	425 (70.3)	407 (100)	425	407 (100) and
		$[M - H]^-$	$[M - H - Me]^-$						$[M + H - 194]^+$	$[M + H - 194 - H_2O]^-$		multiple peaks
5	32.2	633	618 (11.8)	616 (7.6)	601 (100)	599 (14.2)		587 (5.1), 573 (4.9),				
		$[M - H]^-$	$[M - H - Me]^-$	$[M - H - OH]^-$	$[M - H - Me - OH]^-$	$[M - H - 34]^-$		557 (9.6), 475 (8.5)				
6	32.6	617	602 (100)						425 (16.8)	407 (100)	407	multiple peaks
		$[M - H]^-$	$[M - H - Me]^-$						$[M + H - 194]^+$	$[M + H - 194 - H_2O]^-$		(as in **Figure 3**)
7	35.1	615	600 (100)					177 (3.6)	423 (100)	405 (11.0)	423	405 (100) and
		$[M - H]^-$	$[M - H - Me]^-$						$[M + H - 194]^+$	$[M + H - 194 - H_2O]^-$		multiple peaks
caffeate esters												
8	46.3	587	587 (100)					179 (6.1)	409 (100)		409	multiple peaks
		$[M - H]^-$	$[M - H]^-$					[caffeoxyl]$^+$	$[M + H - 180]^+$			(as in **Figure 3**)
9	49.1	561	561 (100)					179 (16.2)	383 (100)		383	multiple peaks
		$[M - H]^-$	$[M - H]^-$					[caffeoxyl]$^+$	$[M + H - 180]^+$			(as in **Figure 3**)

[a] Deprotonated molecular ions are base peaks in the negative mass spectra of all esters discussed in this table.

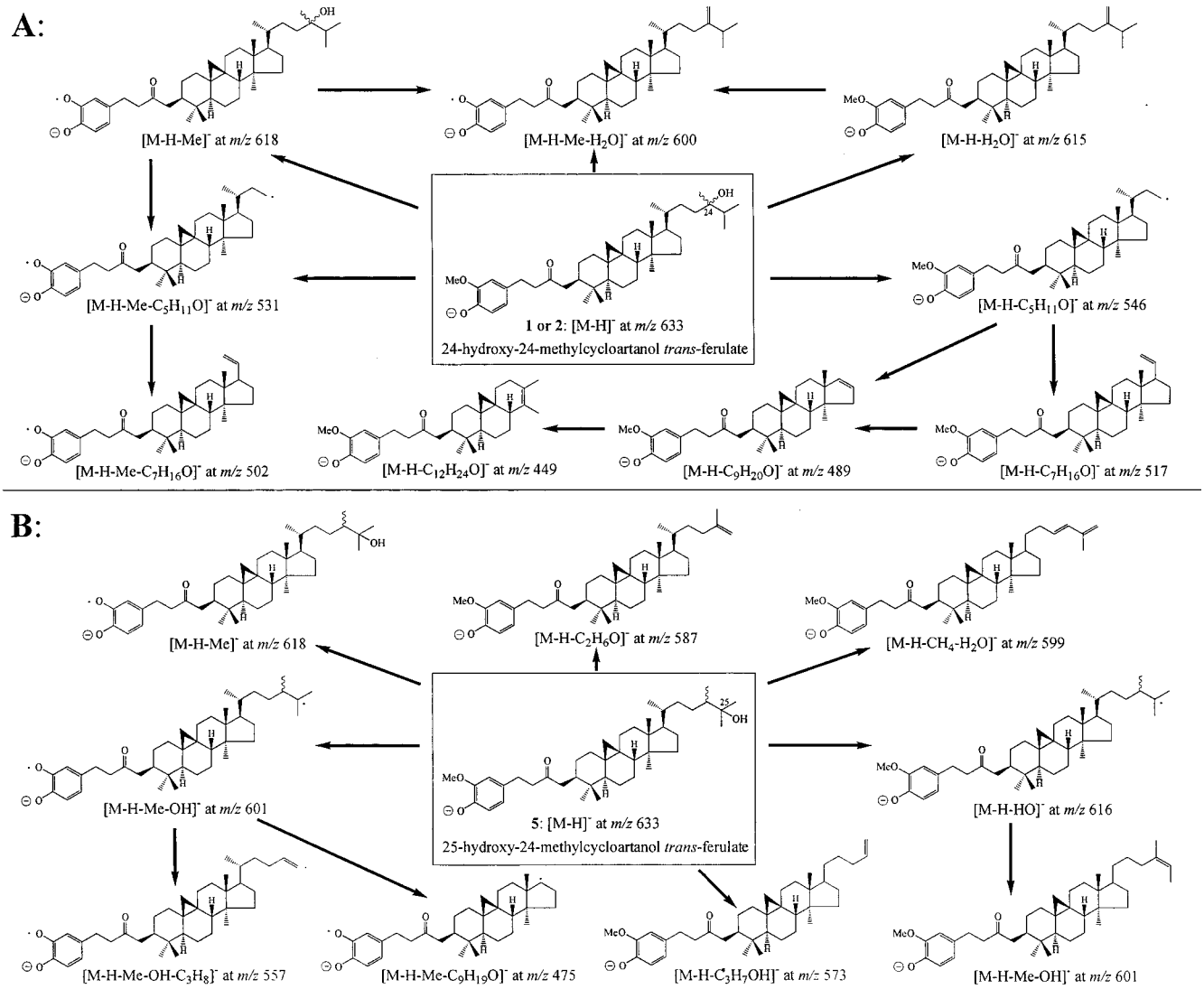

Figure 6. Proposed CID pathways of (A) 24-hydroxy-24-methylcycloartanol ferulates (**1** and **2**, two stereoisomers) and (B) 25-hydroxy-24-methylcycloartanol ferulates (**5**). Structures for **1**, **2**, and **5** were identified tentatively.

Considering that **11** and **14** are the major γ-oryzanol components in rice bran oil, we assigned **8** and **9** as caffeate esters of cycloartenol and campesterol, respectively. It should be noted that this is the first characterization of caffeate esters of triterpene alcohol and sterol in rice bran.

The conventional methods for analysis of phytosterol content in oils are carried out by two procedures: separation by multiple chromatographic steps and identification of individual compounds by GC-EI-MS, positive CI-MS, and NMR (*2*−*6*); and analysis of TMS derivatives by GC-EI-MS after saponification of oils (*14*, *15*). In the present study, an LC-MS/MS technology was developed for direct analysis of γ-oryzanol components in rice bran oil. In contrast to the weak molecular ion in the mass spectra from EI-MS (*2*, *6*) and positive CI-MS (*4*), γ-oryzanol components yielded predominant base peaks for the deprotonated molecular ions in the ESI-MS spectra. Characterization of 23 components of γ-oryzanol suggests that this LC-MS/MS condition is a sensitive method and well-suited for the on-line characterization of phytosterols in oil. Moreover, characterization of nine new, relatively polar, γ-oryzanol components by the LC-MS/MS equipped with reverse-phase LC and ESI-MS suggests that this method could be used for the identification and quantification of the polar metabolites of phytosterols in biological fluids after consumption of rice bran oil or other oils.

ABBREVIATIONS USED

cwt, hundredweight; TMS, trimethylsilylated derivative; EI-MS, electron impact mass spectrometry; CI-MS, chemical ionization mass spectrometry; DIP, direct inlet probe; ESI-MS, electrospray ionization mass spectrometry; API, atmospheric pressure interface; API-ES, atmospheric pressure interface electrospray; ICC, ion charge control; TIC, total ion chromatogram; EIC, extracted ion chromatogram; CID, collision-induced dissociation.

ACKNOWLEDGMENT

We thank Zachary T. Nebus, Don R. McCaskill, and Leo Gingras (Riceland Foods, Inc.) for supplying the rice bran and Drs. Martin J. J. Ronis, Frank A. Simmen, and Rosalia C. M. Simmen for helpful comments.

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Received for review December 10, 2002. Revised manuscript received March 4, 2003. Accepted March 7, 2003. Funding has been provided in part from the U.S. Department of Agriculture, Agricultural Research Service, under Project 0501-00044-001-01S and the Arkansas Rice Research and Promotion Board. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture.

JF021162C