Purification and Identification of Components of γ-Oryzanol in Rice Bran Oil†

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High-purity γ-oryzanol was obtained from crude rice bran oil using a normal-phase preparative scale HPLC. A reverse-phase HPLC method was used for separating the individual components of γ-oryzanol present in rice bran oil. Ten fractions were isolated and collected using the reverse-phase HPLC method, and their structures were identified. Identification was accomplished using GC/MS with an electron impact mass spectrum after components were transformed into trimethylsilyl ether derivatives. The 10 components of γ-oryzanol were identified as Δ^7-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, Δ^7-campestenyl ferulate, campesteryl ferulate, Δ^7-sitostenyl ferulate, sitosteryl ferulate, compestanyl ferulate, and sitostanyl ferulate. Three of these, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate, were major components of γ-oryzanol.

Keywords: *γ-Oryzanol; ferulate; rice bran oil*

INTRODUCTION

γ-Oryzanol from rice bran has been suggested to possess the capability of lowering blood cholesterol (Nicolosi et al., 1993; Rukmini and Raghuram, 1991; Seetharamaiah and Chandrasekhara, 1989) and to have antioxidant functionality (Duve and White, 1991). It was originally considered to be a single component when first extracted from rice bran oil but was later determined to be a mixture of steryl ferulates.

Use of high-performance liquid chromatographic (HPLC) methods has established that γ-oryzanol is a mixture of several components (Diack and Saska, 1994; Norton, 1995; Rogers et al., 1993; Evershed et al., 1988), but depending on the chromatographic approach taken, different numbers of individual components have been identified. γ-Oryzanol was separated into two fractions by Diack and Saska (1994) using normal-phase HPLC and a column packed with spherical silica, but each fraction contained at least two or more constituents and it was not possible to identify and quantify the individual components using this approach. Norton (1995) used a reverse-phase approach and was able to identify five individual components of γ-oryzanol. Six components of γ-oryzanol were identified using reverse-phase HPLC by Evershed et al. (1988) and Rogers et al. (1993). However, in these studies only two of the six components were isolated as single peaks. Incomplete separation of individual components of γ-oryzanol would make quantification difficult.

Separation of individual components of γ-oryzanol in rice bran oil is challenging because of the presence of minor components in the γ-oryzanol fraction. In this study, γ-oryzanol was concentrated using low-pressure chromatography and preparative normal-phase HPLC to reduce interfering substances. Furthermore, it was also possible to isolate and identify minor ferulate components by collecting each fraction of a reverse-phase HPLC separation. The spectra of UV absorbance and fragment ions produced in electron ionization in a gas chromatograph/mass spectrometer (GC/MS) were used to identify the structures of these components.

METHODS

Extraction of Crude Oil. Twenty-five grams of rice bran (Riviana Rice Mill, Abbeville, LA) were placed in a 500-mL round-bottom flask with 1 g of ascorbic acid, 35 mL of hexane, and 15 mL of ethyl acetate. The flask was attached to a rotary evaporator, with vacuum (RE121, Brinkmann Inc., Switzerland), and placed in a 60 °C water bath for 40 min with 180 rpm. Then 25 mL of distilled water was added to the flask. The flask was placed on the rotary evaporator at the same temperature and rotation speed for 10 min. Solvent and rice bran residue were separated by filtration. Rice bran residue was extracted a total of three times using this process. The extracts were pooled and centrifuged at 4000*g* for 10 min. The organic solvent layer was evaporated in a rotary evaporator under vacuum at 60 °C to obtain crude oil.

Semipurification of γ-Oryzanol Using a Low-Pressure Silica Column. A glass column (2.5 cm × 25 cm) packed with 20 g of silica (grade 62) (EM Industry Inc.) was used to remove the triglycerides and other lipids. Initially, the crude oil was solubilized in 50 mL of the solvent (hexane/ethyl acetate = 9:1) for flushing through the column. Then 50 mL of solvent (hexane/ethyl acetate = 7:3) was allowed to flow through the column, and the eluant was collected. The column was then washed with 50 mL of hexane/ethyl acetate (1:1), and the semipurified γ-oryzanol was obtained after the solvent was evaporated.

Purification of γ-Oryzanol Using a Preparative Scale Normal-Phase HPLC. The preparative HPLC system consisted of a Waters (Milford, MA) PrePak RCM base packed with three 25 mm × 10 cm Prep Nova-Pak HR silica (particle size = 6 μm) cartridges and a Guard-Pak insert, a U6K manual injector, a 510 pump, and a 481 LC spectrophotometer detector. A Baseline 810 chromatography workstation (Waters) was used to record the chromatogram and calculate concentrations.

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The mobile phase was 4% (v/v) ethyl acetate in hexane at a flow rate of 21.6 mL/min with detection at λ_{max} = 330 nm (ferulic acid) as indicated by Diack and Saska (1994). The γ-oryzanol fraction was collected and dried under nitrogen flow.

Separation of Individual Components of γ-Oryzanol in an Analytical Reverse-Phase HPLC. The analytical HPLC system consisted of a Dynatech (Baton Rouge, LA) LC-241 autosampler, a Waters 510 pump, a Hewlett-Packard (San Fernando, CA) UV−vis diode array detector (Series 1050), and a 25 cm × 4.6 mm diameter column of Microsorb-MV C_{18} (Rainin Instrument Co., Woburn, MA). The detector was set at 330 and 450 nm as reference. The mobile phase consisted of methanol, acetonitrile, dichloromethane, and acetic acid (50:44:3:3, by volume), and the flow rate was 1.4 mL/min.

Each peak was collected in a 15-mL test tube using a Gilson 202 fraction collector (Beltline, Middleton, WI), and absorbance (330 nm) was measured using a Gilford Response UV−vis spectrophotometer (Oberlin, OH). Mobile phase was evaporated under nitrogen flow. Components of each fraction were derivatized to trimethylsilyl (TMS) ethers for identification by GC/MS.

Hydrolysis and Derivatization. The components obtained from the collected peaks were hydrolyzed with 1 mL of 0.1 N NaOH in methanol at 80 °C. Ultrahigh-purity nitrogen was flushed through the test tube for 15 s during the addition of alkali. Then the test tube was tightly capped as quickly as possible. After 30 min, 1 mL of 0.1 N HCl and 2 mL of ethyl acetate were added to stop hydrolysis. The solution was centrifuged at 1000*g*. Then the upper organic layer was transferred to a 10-mL test tube containing anhydrous sodium sulfate. The aqueous layer was mixed with 2 mL of ethyl acetate and extracted again. The upper organic layer was combined with the previous collection in a 10-mL test tube after centrifugation. The sodium sulfate anhydrous was removed by filtering, and the organic solvent was evaporated under nitrogen flow. The TMS ether derivative of the component was obtained by adding to the test tube 0.1 mL of pyridine and 0.1 mL of bis(trimethylsily)trifluoroacetamide (BSTFA) (Supelco Inc., Bellefonte, PA) that contained 1% trimethylchlorosilane. The reaction was performed at 30 °C (water bath) for 30 min.

GC/MS. Derivatized components were analyzed using a 30-m SPB-5 fused silica column (Supelco Inc.) with 0.25 mm i.d. and 0.1 μm film thickness in a Hewlett-Packard 5790A GC coupled with a 5970B mass selective detector (MSD). Helium was used as the carrier gas and maintained at a flow rate of 1.5 mL/min. Injection was splitless at 250 °C. Oven temperature was programmed from 150 to 280 °C at a ramp of 5 °C/min. The initial and final hold times were 0 and 9 min, respectively. MSD conditions were as follows: capillary direct MS interface temperature, 280 °C; ion source temperature, 280 °C; ionization voltage, 70 eV; mass range, *m*/*z* 30−550 ; scan rate, 1.67 scans/s; and electron multiplier voltage, 1800 V.

RESULTS AND DISCUSSION

Extraction of Crude Oil and Semipurification of γ-Oryzanol Using a Low-Pressure Silica Column. Generally, crude rice bran oil was extracted using a solvent extraction method with saponification. Saponification has been employed in most lipid extractions from plant and animal tissues to remove interfering triglycerides and other hydrolyzable materials and to aid the release of lipid from sample matrix. A saturated aqueous solution of KOH was used in rice bran oil extraction by Diack and Saska (1994). In the present study, the γ-oryzanol concentration was 9.8 mg/g in the crude oil extracted without saponification; however, it was 4.6 mg/g in the crude oil obtained under the saponification described in Diack and Saska (1994). Saponification may hydrolyze the ester bond between triterpenoids and ferulic acid components of γ-oryzanol.

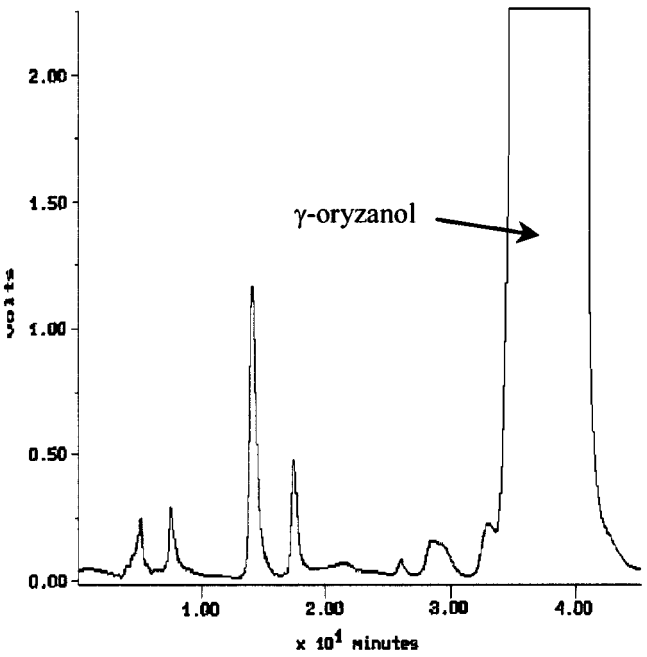

Figure 1. Chromatogram of semipurified γ-oryzanol in the normal-phase preparative HPLC (column, silica; mobile phase, 4% ethyl acetate in hexane; flow rate, 21.6 mL/min; UV detector, 330 nm).

Approximately 3.5 g of crude oil was obtained from 25 g of rice bran under these extraction conditions (14% yield). After the purification using low-pressure silica column, 0.7 g of semipurified γ-oryzanol was obtained (20% yield).

Purification of γ-Oryzanol Using a Preparative Scale Normal-Phase HPLC. Diack and Saska (1994) compared resolution of five commercial silicas in separating the individual components of γ-oryzanol from rice bran oil and found that Nova-Pak (Waters) silica possessed the most desirable attributes. Therefore, in this study, Prep Nova-Pak HR silica cartridges were used as a preparative scale normal-phase column to purify γ-oryzanol from semipurified γ-oryzanol. A chromatogram depicting the preparative chromatography is shown in Figure 1. The γ-oryzanol peak was eluted at 35 to 42 min. The profile of the chromatogram was the same as that of Diack and Saska (1994) in analytical normal-phase HPLC. The occurrence of several fractions before γ-oryzanol eluted from the column may have been due to residual components, such as triglycerides, tocopherols, and tocotrienols, not completely removed during low-pressure column cleanup procedures. After removal of the solvent, ∼36 mg of purified γ-oryzanol was obtained from the 0.7 g of semipurified γ-oryzanol (≈5% yield).

Separation of Individual Components of γ-Oryzanol in an Analytical Reverse-Phase HPLC. The numbered peaks of high-purity γ-oryzanol (Figure 2) were collected for identification of their structures using GC/MS. Components of γ-oryzanol in rice bran oil were separated using reverse-phase HPLC methods by Evershed et al. (1988), Norton (1994, 1995), Rogers et al. (1993), and Seitz (1989). However, the resolution of these separations was not entirely satisfactory, and some peaks could not be identified. Acetonitrile/*n*-butanol/acetic acid/water (94:3:2:1, v/v) was used as the mobile phase in Norton (1995), which produced the highest resolution of separation among these methods.

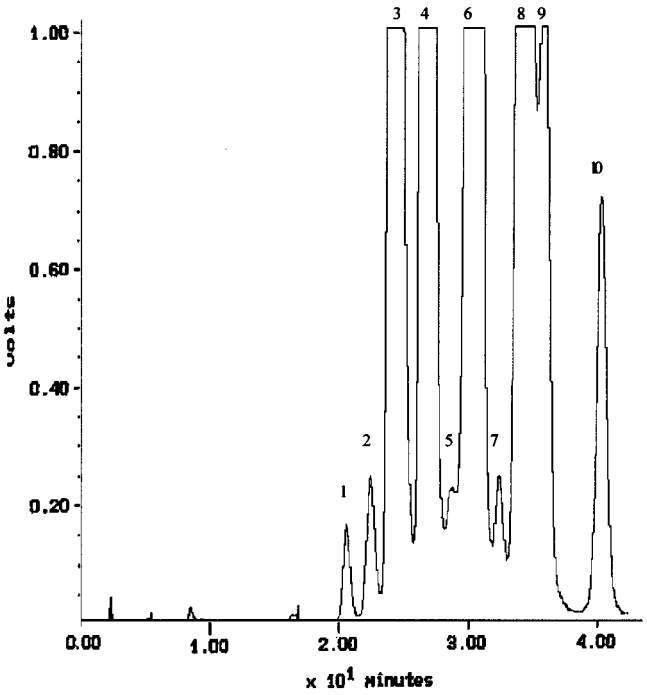

Figure 2. Chromatogram of high-purity γ-oryzanol in the analytical reverse-phase HPLC (column, C_{18}; mobile phase, methanol/acetonitrile/dichloromethane/acetic acid = 50:44:3:3; flow rate, 1.4 mL/min; UV detector, 330 nm).

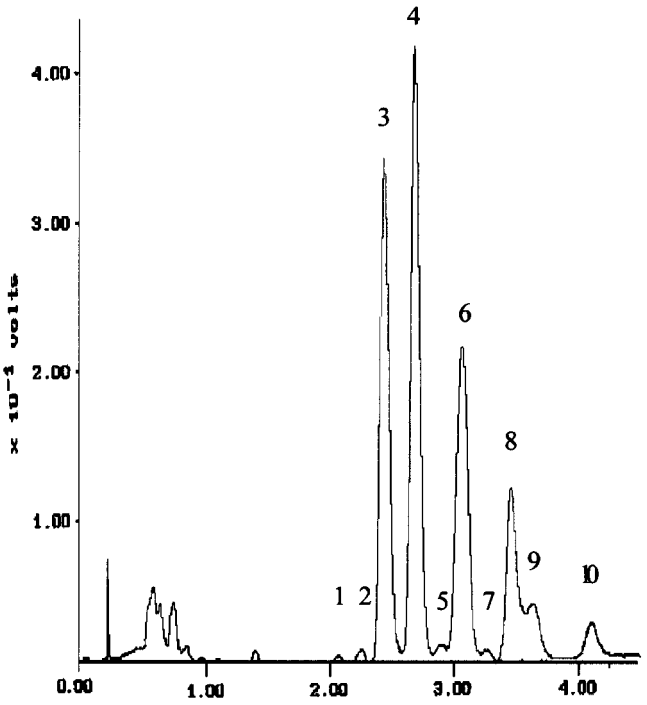

Figure 3. Chromatogram of crude rice bran oil in the analytical reverse-phase HPLC (same conditions as Figure 2).

In this study, the mixture of methanol/acetonitrile/dichloromethane/acetic acid (50:44:3:3, v/v) was used as the mobile phase with a run time of 42 min.

The components in peaks 3, 4, and 6 of a crude oil extract (Figure 3) were the major components of γ-oryzanol from rice bran. This was similar to the results of Norton (1995), Rogers et al. (1993), and Evershed et al. (1988). Also, several minor components were resolved and identified in the present work that had not been previously resolved and/or identified.

UV Absorption Spectra of Components of γ-Oryzanol. All of the individual components had similar absorption curves with highest absorbency at a wavelength of 330 nm. The absorption spectrum profile is comparable to that of Diack and Saska (1994).

Mass Spectra of Components of γ-Oryzanol Obtained Using GC/MS. Table 1 lists molecular ion and major fragment ions in the mass spectrum of each individual TMS derivative. Figure 4 depicts, as an example, the mass spectra of sitostanol-TMS. The structures that were constructed on the basis of the information from mass spectra are depicted in Figure 5. Numerous useful fragment ions were used to interpret the structure of components produced in electron ionization. In the low mass range of every mass spectrum, ions of high abundance were noted at *m/z* 73 and 75. The two ions were explained as $(CH_3)_3Si)^+$ and $HO{=}Si(CH_3)_2)^+$, respectively. Also, the presence of an ion at *m/z* 147 was $(CH_3)_2Si{=}O{-}Si\ (CH_3)_3)^+$ formed from two or more TMS groups in a molecule (Evershed, 1988). The molecular ion of each individual TMS derivative appeared in this study, even though some were of low intensity. The $(M - 15)^+$ was not a major fragment ion. However, $(M - TMS)^+$ and $(M - TMS - CH_3)^+$ were obtained as relatively high abundance ions in each mass spectra. For cycloartenol-TMS and 24-methylenecycloartanol-TMS, $(M - TMS - CH_3 - CH_3 - CH_2 + H)^+$ was an important major fragment ion that did not appear in Δ^7-stenol-TMS, sterol-TMS, and stanol-TMS. It may have occurred because methyl groups in the structure of the triterpene of cycloartenol-TMS and 24-methylenecycloartanol-TMS were readily taken from the molecule during ionization. Many major fragment ions of stanol-TMS in mass spectra were larger than that of Δ^7-stenol-TMS or sterol-TMS by 2 units of *m/z*. The reason for this is that there is no double bond in the triterpene structure of stanol-TMS, which does occur in the structure of Δ^7-stenol-TMS and sterol-TMS.

The most difficult aspect of identification for these components was in distinguishing Δ^7-stenol-TMS and sterol-TMS because they have the same molecular weight and similar structures. The difference in mass spectra between them in this study was *m/z* 129, which was a moderate ion in Δ^7-stenol-TMS but one of the highest ions in sterol-TMS. In contrast, *m/z* 229 was of lower abundance in sterol-TMS but was a higher abundance ion in Δ^7-stenol-TMS. The relatively high intensity *m/z* 229 ion has been found to be characteristic of the Δ^7-stenol compound (Gustafsson et al., 1966). Figure 6 shows the most probable mechanism of formation of *m/z* 129 and 229 in the sterol-TMS and Δ^7-stenol-TMS, respectively. For the sterol-TMS, carbons 1, 2, and 3 together with TMS were readily lost during ionization and formed the *m/z* 129 fragment ion. For the stenol-TMS, the loss of the TMS group, the carbons 16 and 17, and the side chain of carbon 17 often occurs at the same time. The molecular weight of the residual ion was *m/z* 229.

Components of γ-Oryzanol. From mass spectra of TMS derivatives and absorbance curves of components of γ-oryzanol, 10 components were positively identified in this study. They are listed in Table 1 according to the order of retention time in the chromatogram of crude rice bran oil in the analytical reverse-phase HPLC (Figure 3). The molecular size of each component of γ-oryzanol is very close. Therefore, the molecular size was not an important factor that affected the retention

Table 1. Molecular Masses and Major Fragmentation Ion of TMS Ether Derivatives of Each Peak Collected in Reverse-Phase HPLC

peak	molecular ion (*m*/*z*)	major fragment ions (*m*/*z*)	identification
1	484	379, 255, 229, 213, 173, 159, 145, 143, 129, 119, 105, 93, 73	Δ^7-stigmastenol-TMS
2	484	394, 379, 355, 255, 213, 173, 159, 145, 143, 129, 119, 105, 95, 73	stigmasterol-TMS
3	498	408, 393, 365, 339, 297, 255, 203, 187, 159, 149, 131, 95, 81, 69	cycloartenol-TMS
4	512	422, 407, 379, 297, 203, 187, 175, 173, 159, 135, 95, 73, 69	24-methylenecycloartanol-TMS
5	472	382, 367, 255, 229, 213, 173, 159, 145, 129, 121, 107, 95, 85, 75	Δ^7-campestenol-TMS
6	472	382, 367, 234, 289, 261, 255, 213, 173, 159, 129, 121, 107, 95, 85, 75	campesterol-TMS
7	486	471, 396, 381, 255, 229, 213, 173, 159, 145, 129, 121, 105, 95, 75	Δ^7-sitostenol-TMS
8	486	396, 381, 357, 275, 255, 213, 173, 159, 145, 129, 121, 105, 95, 75	sitosterol-TMS
9	474	384, 369, 257, 215, 173, 161, 147, 129, 121, 107, 95, 85, 75	campestanol-TMS
10	488	473, 398, 383, 359, 305, 257, 215, 173, 161, 147, 129, 121, 105, 95, 75	sitostanol-TMS

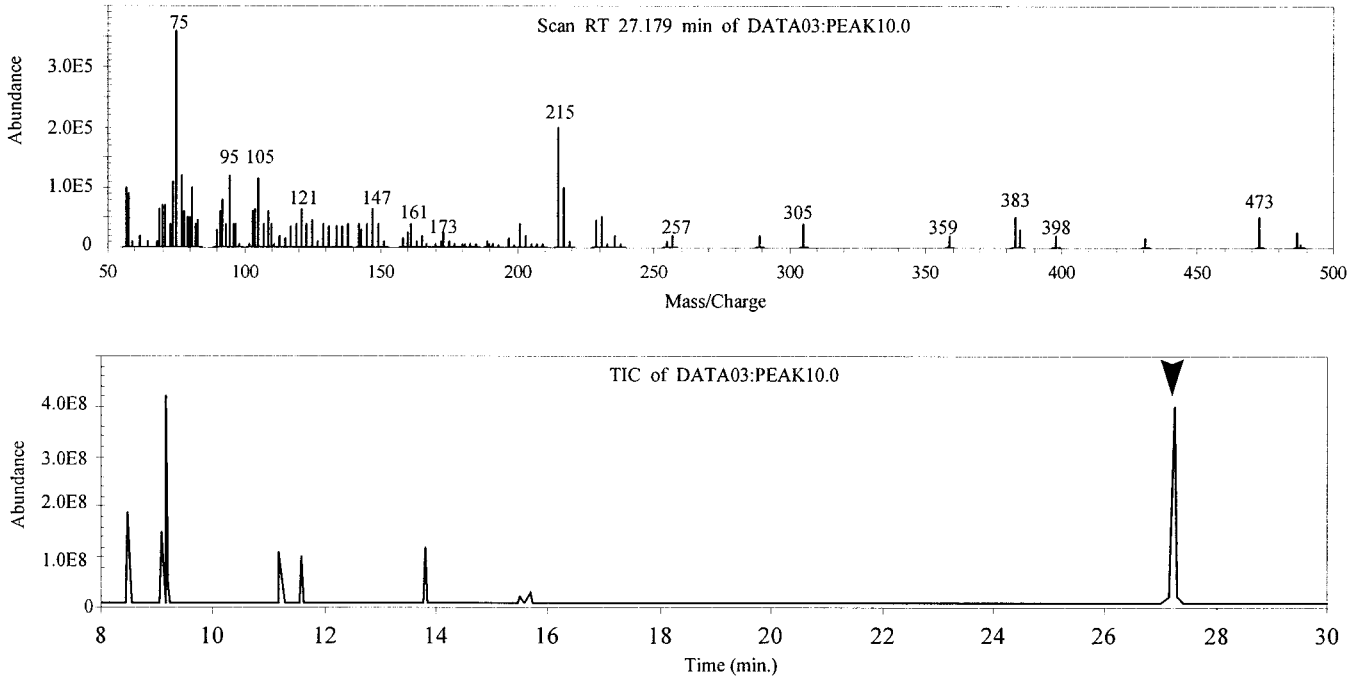

Figure 4. Mass spectra and total ion chromatograph of sitostanol-TMS in GC/MS.

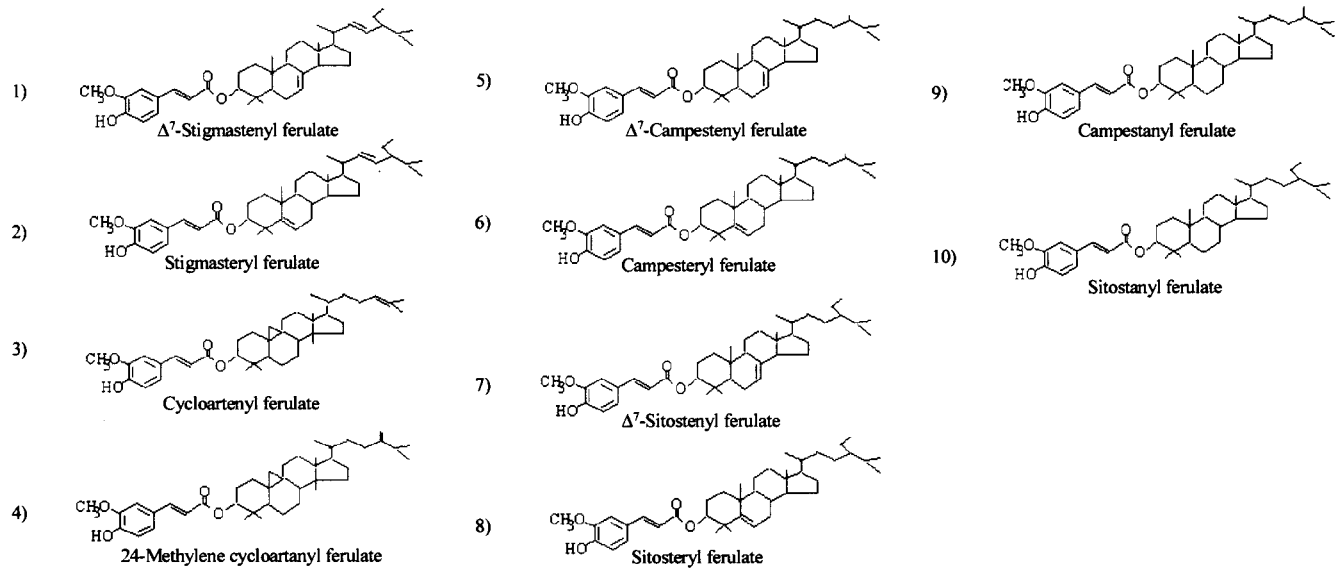

Figure 5. Molecular structures of identified components of γ-oryzanol.

time of each component during chromatography. It is suggested that the peak retention times of components of γ-oryzanol in the analytical reverse-phase HPLC are largely dependent on the number and positions of double bonds, because they are related to the polarity of compounds, although the relationship is not strong. Evidence was found that the retention time of components containing two double bonds was less than that of components having one or no double bond. Components having a double bond on a side chain of the

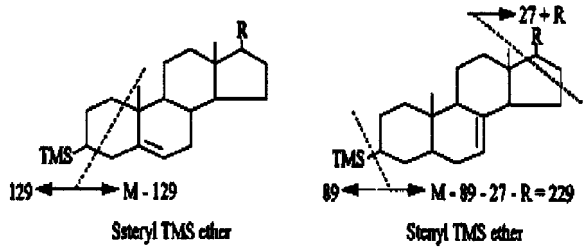

Figure 6. Possible manner of formation of *m/z* 129 in sterol-TMS and *m/z* 229 in Δ^7-stenol-TMS.

triterpene eluted prior to the components having a double bond in the triterpene.

The three largest peaks were cycloartenyl ferulate (peak 3), 24-methylenecycloartanyl ferulate (peak 4), and campesteryl ferulate (peak 6). These are the major components of γ-oryzanol in rice bran oil as has been found by others (Evershed et al., 1988; Norton, 1995; Rogers et al., 1993). Compared with the three major components in rice bran oil, the peak areas of other components were much smaller. This situation usually is the major impediment to the separation and identification of all of the components of γ-oryzanol because minor components are overlooked when they elute with a major component. Peaks 8 and 9 that were identified as sitosteryl ferulate and campestanyl ferulate, respectively, were not separated as two individual peaks in Evershed et al. (1988) and Rogers et al. (1993). They occurred as a single peak in their chromatograms and were considered as sitosteryl ferulate (Evershed et al., 1988) or a mixture of sitosteryl ferulate and cycloartanyl ferulate (Rogers et al., 1993). Although they were separated by Norton (1995), one was identified as sitostanyl ferulate, which differs from this study, and the other was considered an unknown. Stigmasteryl ferulate was reported to elute with campesteryl ferulate in a single peak (Rogers et al., 1993). However, these compounds were successfully separated as two individual peaks in this study. Stigmasteryl ferulate (peak 2) had a shorter retention time, which supports the contention that a component with more double bonds in the triterpene portion or in its side chain has a shorter retention time in reverse-phase HPLC. The last peak in our chromatogram was identified as sitostanyl ferulate. However, cycloartanyl ferulate identified in Rogers et al. (1993) and Norton (1995) was not found in the current study.

In summary, 10 ferulate esters of triterpene alcohol, Δ^7-stigmastenyl ferulate, stigmasteryl ferulate, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campestenyl ferulate, campesteryl ferulate, stigmastenyl ferulate, sitosteryl ferulate, compestanyl ferulate, and sitostanyl ferulate, were identified in γ-oryzanol from rice bran oil. Three of these, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate, are major components. Five of the 10 identified components, Δ^7-stigmastenyl ferulate, Δ^7-campestenyl ferulate, Δ^7-sitostenyl ferulate, campestanyl ferulate, and sitostanyl ferulate, had not been previously reported in rice bran oil. The successful separation and identification of minor components in this study was possible through the purification of γ-oryzanol from crude rice bran oil using low-pressure chromatography and preparative scale normal-phase HPLC.

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