Isolation of γ-Oryzanol through Calcium Ion Induced Precipitation of Anionic Micellar Aggregates†

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A simple method for the isolation of γ-oryzanol from rice bran acid oil has been developed. The basis of the present isolation protocol is calcium ion induced precipitation of anionic micellar aggregates. Rice bran acid oil, a byproduct of rice bran oil processing industries, is subjected to conventional vacuum distillation to remove free fatty acids. Aqueous alkaline hydrolysis of the resultant residue, upon dilution with water, produces anionic micellar aggregates containing solubilized γ-oryzanol. Addition of calcium ions to this aqueous micellar aggregate induces instant coprecipitation of the calcium salts of the fatty acids and the aggregate-associated γ-oryzanol. The dried precipitate is extracted with ethyl acetate. Ethyl acetate is evaporated from the extract, and γ-oryzanol is purified from the residue by silica gel column chromatography. Experimental details for the isolation and characterization procedure are described. The present method will give ready access to the physiologically beneficial and value-added pharmaceutical product γ-oryzanol.

Keywords: *γ-Oryzanol; ferulic acid esters; CAD esters; HPLC; LSIMS*

INTRODUCTION

γ-Oryzanol, first isolated from rice bran oil (Kaneko and Tsuchiya, 1954), is a mixture containing ferulate (4-hydroxy-3-methoxycinnamic acid) esters of triterpene alcohols and plant sterols (Norton, 1995; Rogers et al., 1993; Seitz, 1989; Tanaka et al., 1964, 1977; Tanaka, 1971; Endo et al., 1968; Kato, 1961; Ohta, 1960; Ohta and Shimizu, 1957). Individual components (Chart 1) were identified as ferulate esters of cycloartenol, 24-methylenecycloartanol, campesterol, β-sitosterol, and cycloartanol (Norton, 1995; Rogers et al., 1993; Seitz, 1989). Over the past decade, a number of investigations have demonstrated the beneficial physiological effects associated with the intake of γ-oryzanol (Rong et al., 1997; Nicolosi et al., 1994; Lichtenstein et al., 1994; Rong et al., 1994; Rukmini et al., 1991; Sasaki et al., 1990; Seetharamaiah et al., 1988, 1989; Yoshino et al., 1989a,b; Shinomiya et al., 1983). The blood cholesterol lowering activity of the rice bran oil in some types of hypercholesteromia has been shown to be due to its constituent γ-oryzanol (Rong et al., 1994, 1997; Lichtenstein et al., 1994; Nicolosi et al., 1991, 1992; Rukmini et al., 1991; Raghuram et al., 1989; Seetharamaiah et al., 1989; Sharma et al., 1986). Lipid peroxidation has been shown to be prevented in the retina by γ-oryzanol because of its antioxidant property (Tadahisa et al., 1991). γ-Oryzanol has been shown to be effective in decreasing platelet aggregation (Seetharamaiah et al., 1990) and in treating lipogenic liver cirrhosis in spontaneously hypertensive rats with natural abnormalism in lipid metabolism (Masahiro et al., 1992). Investigations directed toward safety assessment clearly indicate that γ-oryzanol possesses no genotoxic and carcinogenic initiation activities (Tamagawa et al., 1992; Tsushimoto et al., 1991). All of these remarkable beneficial effects of γ-oryzanol on human health have generated global interest in developing (a) commercially viable methods for the isolation of γ-oryzanol components from its various natural sources (Seitz, 1989; Seetharamaiah et al., 1986; Shimizu, 1976; Nishihara et al., 1968; Kato, 1961) and (b) analytical methods for the quantitative estimation of the γ-oryzanol components present in its various natural sources (Norton, 1995; Rogers et al., 1993; Seitz, 1989; Endo et al., 1968). Rice bran oil is one of the most readily accessible natural sources for γ-oryzanol. The γ-oryzanol content of rice bran oil varies within the range of 1.1−2.6% (Rong et al., 1997; Seetharamaiah et al., 1986). During rice bran oil refining, a significant amount of rice bran oil gets trapped within the soap that is formed in the deacidification step. When the soap is removed by centrifugation, the entrapped oil also gets centrifuged with the soap. The soap stock is reacidified to prepare crude dark acid oil. The pure free fatty acids are distilled from the crude dark acid oil under high vacuum. The γ-oryzanol is left in the residue, the so-called pitch, after the removal of free fatty acids from the crude dark acid oil. Herein, we report a simple protocol for the isolation of γ-oryzanol from this inexpensive pitch of crude dark acid oil through calcium ion induced precipitation of anionic micellar aggregates.

MATERIALS AND METHODS

Reagents. Rice bran acid oil was purchased from a local oil mill. HPLC grade acetonitrile, 2-propanol, methanol, chloroform, and water were purchased from E. Merck (India) and were used as received for HPLC analysis. The solvents

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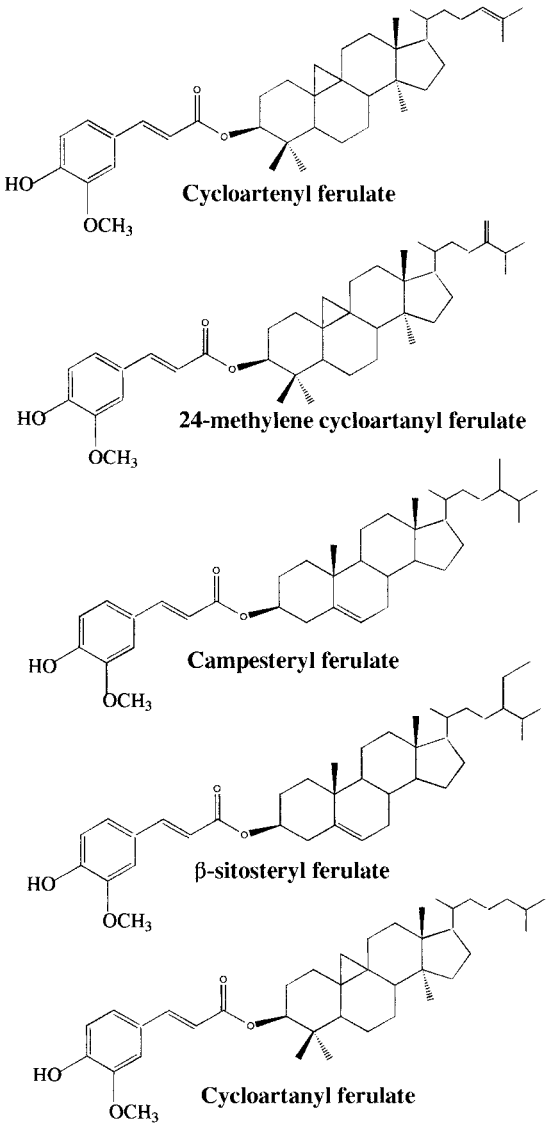
Chart 1



used for isolating γ-oryzanol were prepared by distilling the commercial grade solvents. All other laboratory grade reagents used were purchased from Qualigens Fine Chemicals, India.

HPLC. The LC system consisted of a Shimadzu model LC10A high-performance liquid chromatograph. A 20 μL sample loop was used, and the wavelength of the UV detector was set at 325 nm (Rogers et al., 1993). The separation of the individual components of γ-oryzanol was effected on a 3.9 mm × 150 mm Nova-Pak C18 (Waters) steel cartridge column. The initial mobile phase used (Rogers et al., 1993) was acetonitrile/methanol/2-propanol/water (45:45:5:5, by vol), which was programmed to acetonitrile/methanol/2-propanol (50:45:5, by vol) from 6 to 10 min. This final mobile phase was maintained for 15 min before returning to the initial mobile phase. A 0.8 mL/min constant flow rate was maintained throughout the entire HPLC analysis.

Estimation of γ-Oryzanol. The γ-oryzanol content of the crude dark acid oil and the purity of the isolated γ-oryzanol were estimated by measuring their optical densities in petroleum ether (60−80 °C) at 315 nm and using the literature specific extinction coefficient $E^{1\%}_{1cm}$ = 358.9 for γ-oryzanol (Seetharamaiah et al., 1986). The UV spectra were recorded in a Hitachi U-2000 UV−vis spectrophotometer.

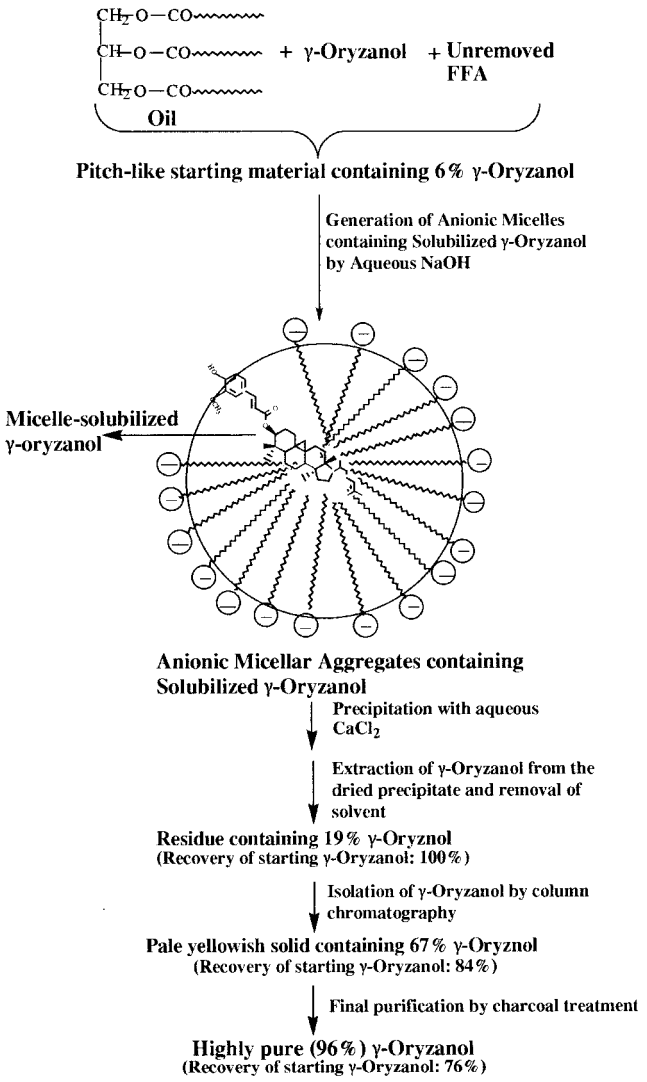
Mass Spectrometry. Analyses were performed on a Micromass Autospec-M mass spectrometer (Manchester, U.K.) with an Opus v3.1X data system. Data were acquired by liquid secondary ion mass spectrometry (LSIMS) technique using *m*-nitrobenzyl alcohol as the matrix. LSIMS analysis of the isolated γ-oryzanol and its separated individual components was performed in the scan range 100−1000 amu at the rate of 3 scans/s.

Isolation of γ-Oryzanol. Commercial acid oil (20.3 g) containing 60% free fatty acid was subjected to vacuum distillation at 250−260 °C (under a pressure of 1 mmHg). The residue (11.3 g) containing 9% free fatty acid was hydrolyzed with 25 mL of 1.8 M aqueous sodium hydroxide at 90 °C for 1 h. The dark brown hydrolyzed product mixture was then diluted with 200 mL of distilled water, and the diluted solution was stirred at room temperature for ∼30 min. An aqueous solution of calcium chloride (2.5 g of $CaCl_2$ dissolved in 30 mL of distilled water) was added dropwise to this stirred solution over a period of ∼15 min. A huge precipitate appeared, and the mixture was stirred for another 15 min. The precipitate was filtered using a water aspirator. The filtrate was discarded and the residue air-dried overnight. The dried solid was extracted with 100 mL of ethyl acetate by stirring at 50 °C (30 min) and centrifuging the resulting mixture at 5000 rpm. The supernatant was collected, and the extraction protocol was repeated twice with the centrifuged precipitate. The combined ethyl acetate extracts were washed with 5% aqueous sodium hydroxide solution (3 × 100 mL) to remove any unhydrolyzed fatty acid. The ethyl acetate extract was then washed with distilled water (3 × 100 mL) and dried over anhydrous sodium sulfate. γ-Oryzanol was finally isolated from the residue left after evaporation of ethyl acetate by silica gel column chromatography using chloroform as the eluent. The γ-oryzanol-containing fractions (R_f = 0.4 using chloroform as the TLC developing solvent) were combined, and chloroform was removed using a rotatory evaporator. The off-white residue (0.85 g) was dissolved in methanol (15 mL) by heating. Addition of 5−6 drops of acetone was necessary to ensure complete dissolution. Activated charcoal (200 mg) was added to this hot methanolic solution and the mixture stirred under boiling condition for 1 min. The methanolic filtrate, obtained after the charcoal was filtered under water aspirator, gave white crystals of γ-oryzanol upon standing overnight at 4 °C. The crystals were filtered and dried under vacuum. The dried γ-oryzanol crystals (510 mg, 76.1% recovery) melted in the range 132−135 °C [reported melting range of pure γ-oryzanol = 137.5−138.5 °C (Kaneko and Tsuchiya, 1954)]. The isolated material was determined to be 96% pure by UV spectrophotometric analysis (as described above under Estimation of γ-Oryzanol).

RESULTS AND DISCUSSION

The principle used in the current extraction protocol is outlined in Scheme 1. The steps involved include (1) removal of the free fatty acids from the acid oil using conventional vacuum distillation, (2) preparation of an aqueous anionic micellar solution containing solubilized γ-oryzanol by aqueous alkaline hydrolysis of the residue obtained in step 1 and dilution of the hydrolyzed product mixture with water, (3) precipitation of the micelle plus the solubilized γ-oryzanol by exchanging the sodium counterion with calcium (calcium salts of fatty acids are insoluble in water), (4) drying of the precipitate and extraction of γ-oryzanol from the dried precipitate using polar organic solvent, and (5) purification of the extracted γ-oryzanol by column chromatography. The residue left after vacuum distillation of the free fatty acids in step 1 contained mostly triglycerides, 6% γ-oryzanol (estimated spectrophotometrically as described under Estimation of γ-Oryzanol), and 9% free fatty acids (estimated following AOCS protocol). Assuming triglycerides to be the remaining 85% of the residue, the equivalent of sodium hydroxide used in the anionic micelle generating aqueous hydrolysis step (step 2) was 1.2 times the equivalent of sodium hydroxide required to hydrolyze the triglycerides and to neutralize

Scheme 1



9% free fatty acids. Triglycerides are rapidly saponified in aqueous alkali compared to the hydrolysis rate of γ-oryzanol. In fact, tocotrienols and γ-oryzanol are the two groups of components found in the unsaponifiable fraction of rice bran oil (Rogers et al., 1993). Thus, most of the sodium hydroxide (~85%) used in step 2 is consumed in saponifying the triglycerides and in neutralizing the free fatty acids.

The products of these fast saponification and neutralization reactions are sodium salts of fatty acids (anionic surfactants), and in aqueous medium, they immediately form the anionic micellar aggregates. γ-Oryzanol, being a hydrophobic material, associates with these micellar aggregates in water (Scheme 1). The concentration of the remaining sodium hydroxide is not strong enough to hydrolyze the micelle-associated γ-oryzanol. The best recovery of γ-oryzanol was obtained when the residue from step 1 was hydrolyzed for 1 h at 90 °C. In step 3, the key step in the current extraction protocol, the added calcium ions induce instant aggregation and precipitation of water insoluble calcium salts of fatty acids. Micelle-solubilized γ-oryzanol is also coprecipitated. Finally, γ-oryzanol is extracted and purified from the dried precipitate in steps 4 and 5 as described under Isolation of γ-Oryzanol.

A typical HPLC chromatogram of the isolated γ-oryzanol is shown in Figure 1. The qualitative nature of this HPLC chromatogram is similar to that published using a Hewlett-Packard 200 × 2.1 mm narrow-bore analytical column packed with 5 μm ODS (C18) Hyper silica (Rogers et al., 1993). The identity of each peak as shown in Figure 1 was established by repeatedly fractionating the isolated γ-oryzanol on 3.9 mm × 150 mm reversed phase Nova-Pak C18 (Waters steel cartridge) column and taking the LSIMS of all the collected fractions 1−4. The purity of all the isolated fractions was further confirmed by checking the HPLC chromatograms of the separated individual fractions 1−4 (Figure 1).

The LSIMS of the isolated γ-oryzanol as well as the LSIMS of the individually isolated components are shown in Figure 2. On the basis of these data, components 1−4 have been identified as cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulates, and β-sitosteryl and cycloartanyl ferulates (Chart 1), respectively. The characteristic fragmentation ions are given in Table 1. The LSIMS data (Figure 2A and Table 1) of γ-oryzanol show the presence of protonated molecular ions identical to their calculated molecular weight plus one. The characteristic [$M^+ + Na^+$] ions were observed (Figures 2D,E and Table 1) for the individually separated campesteryl ferulate (HPLC peak 3, Figure 1), β-sitosteryl ferulate (HPLC peak 4, Figure 1), and cycloartanyl ferulate (HPLC peak 4, Figure 1). In addition, the reported characteristic mass fragmentation peaks at 383 and 177 for campesteryl ferulate, at 397 and 177 for β-sitosteryl ferulate, and at 177 for cycloartanyl ferulate (Rogers et al., 1993) were also observed (Figure 2D,E and Table 1). The peaks at *m/z* 383 and 397 resulted from the loss of ferulic acid

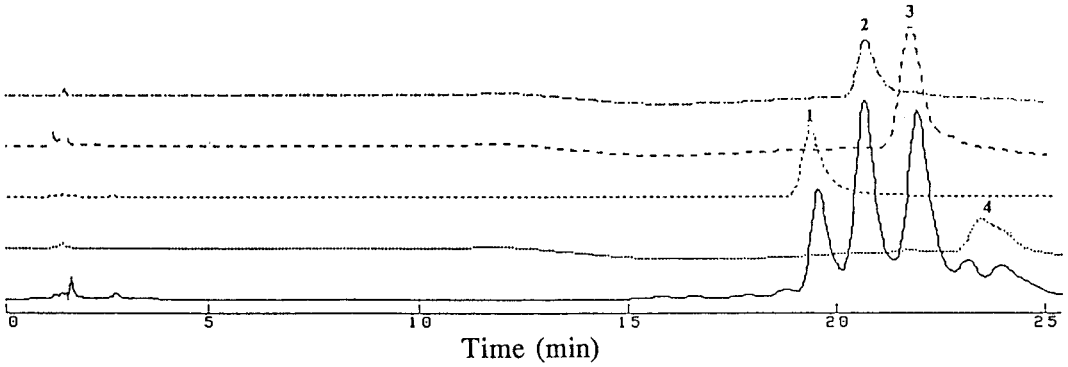


Figure 1. Overlay of HPLC chromatograms of the isolated γ-oryzanol (—) and the individually separated components of γ-oryzanol (- - -). Peaks: 1 = cycloartenyl ferulate; 2 = 24-methylenecycloartanyl ferulate; 3 = campesteryl ferulate; 4 = β-sitosteryl ferulate and cycloartanyl ferulate.

from the protonated molecular ion [(M + 1) − 194]. The peak at *m*/*z* 177 resulted from the loss of water from the protonated ferulic acid (*m*/*z* 195). The molecular ion peaks for the individually separated cycloartenyl ferulate (HPLC peak 1, Figure 1) and 24-methylenecycloartanyl ferulate (HPLC peak 2, Figure 1) were not observed in LSIMS. However, the reported characteristic mass fragmentation peaks at *m*/*z* 409 and 177 for cycloartenyl ferulate and at *m*/*z* 423 and 177 for 24-methylenecycloartanyl ferulate (Rogers et al., 1993) were observed (Figures 2B,C and Table 1). Thus, by comparison with the published mass fragmentation ions (Rogers et al., 1993) for the components of γ-oryzanol, the HPLC peaks 1, 2, and 3 (Figure 1) were found to be cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulates (Chart 1), respectively, whereas HPLC peak 4 was observed to be a mixture of β-sitosteryl and cycloartanyl ferulates.

The UV spectra of the isolated γ-oryzanol components recorded on a Hitachi U-2000 UV−vis spectrophotometer were found to be essentially identical (Figure 3). Such identical absorption maxima for the γ-oryzanol components have already been reported in the literature (Rogers et al., 1993; Seitz, 1989).

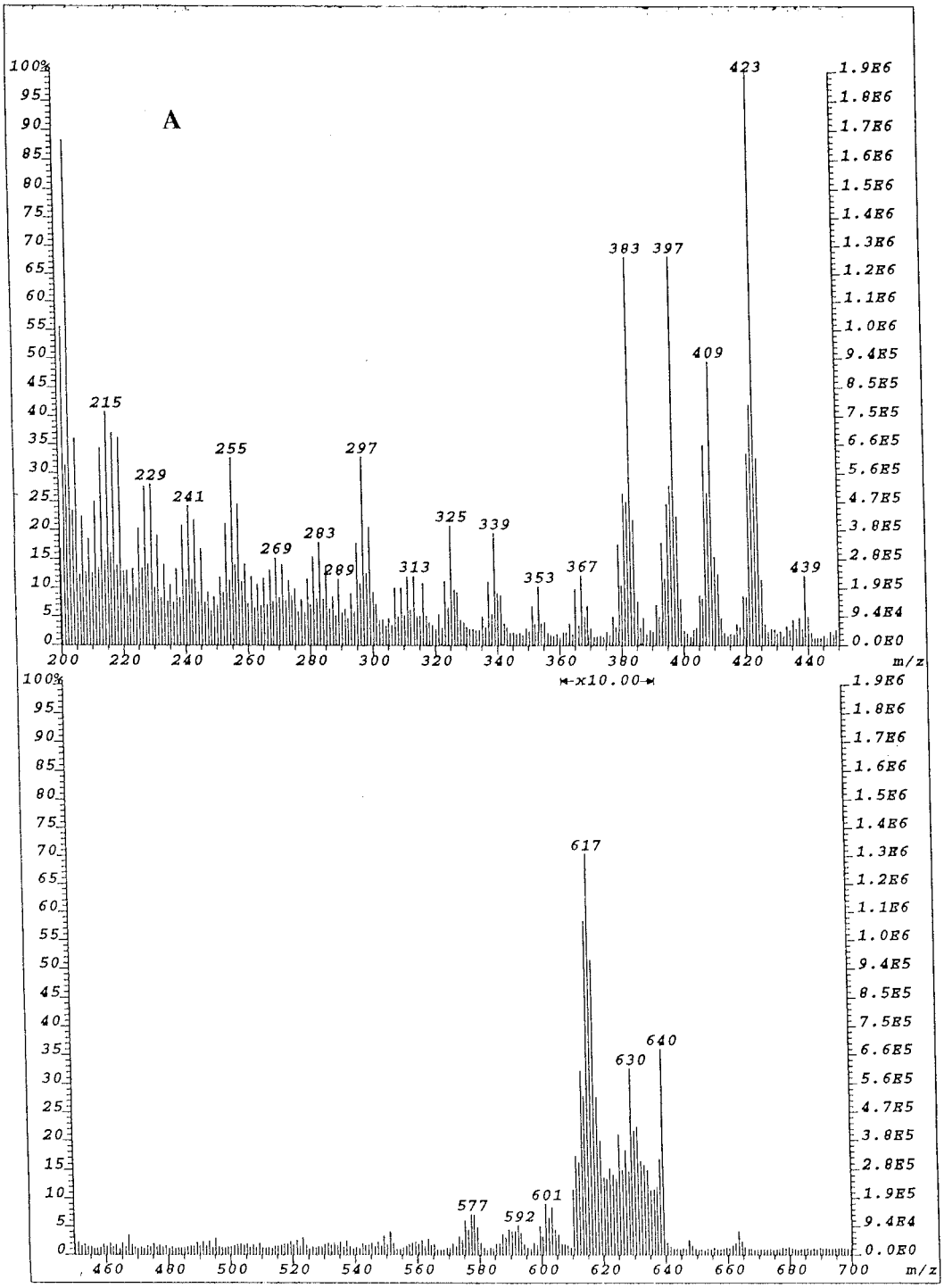
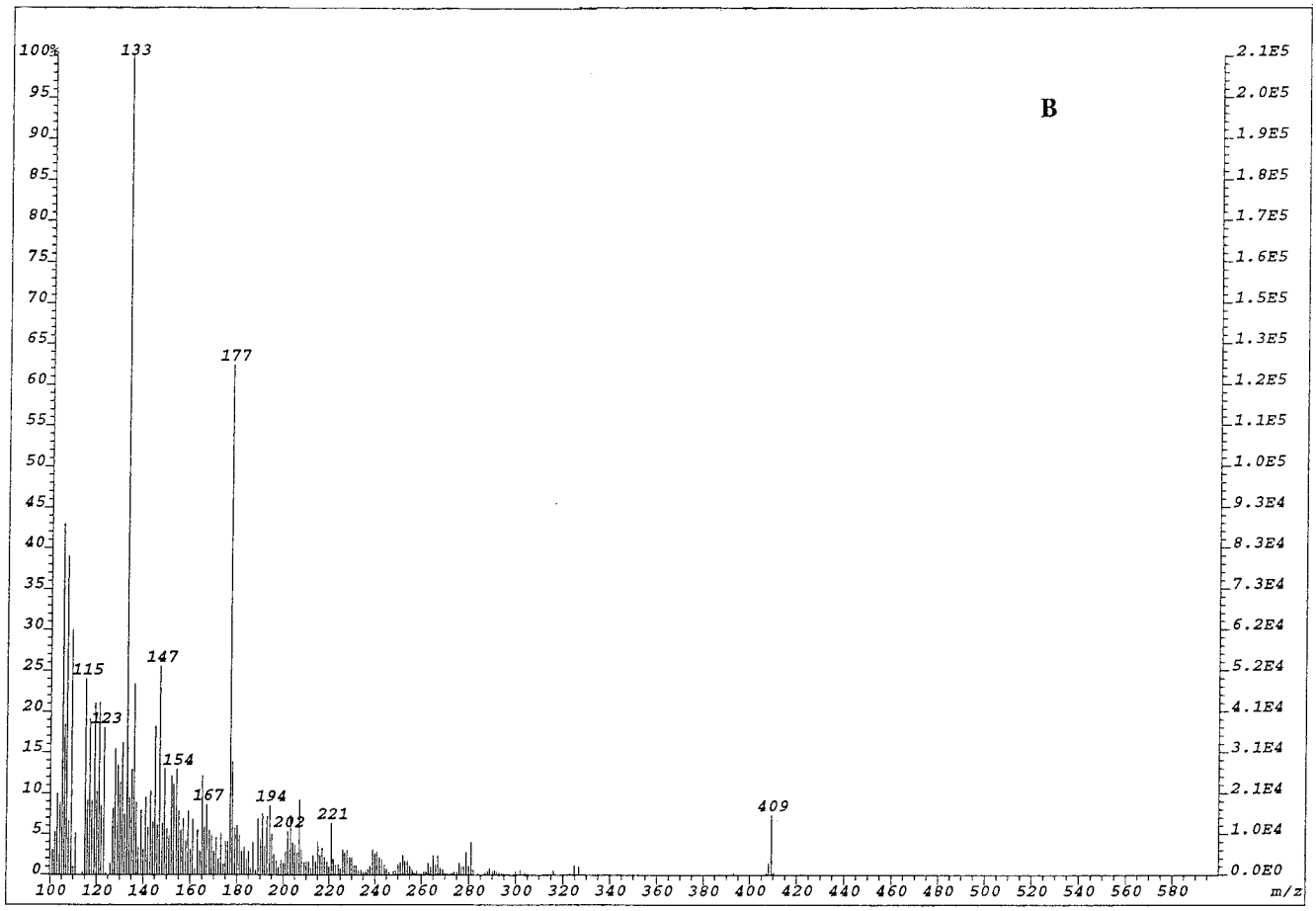


Figure 2. LSIMS spectra of the isolated γ-oryzanol (A) and the individually separated components of γ-oryzanol: (B) cycloartenyl ferulate (peak 1, Figure 1); (C) 24-methylenecycloartanyl ferulate (peak 2, Figure 1); (D) campesteryl ferulate (peak 3, Figure 1); (E) β-sitosteryl ferulate and cycloartanyl ferulate (peak 4, Figure 1).



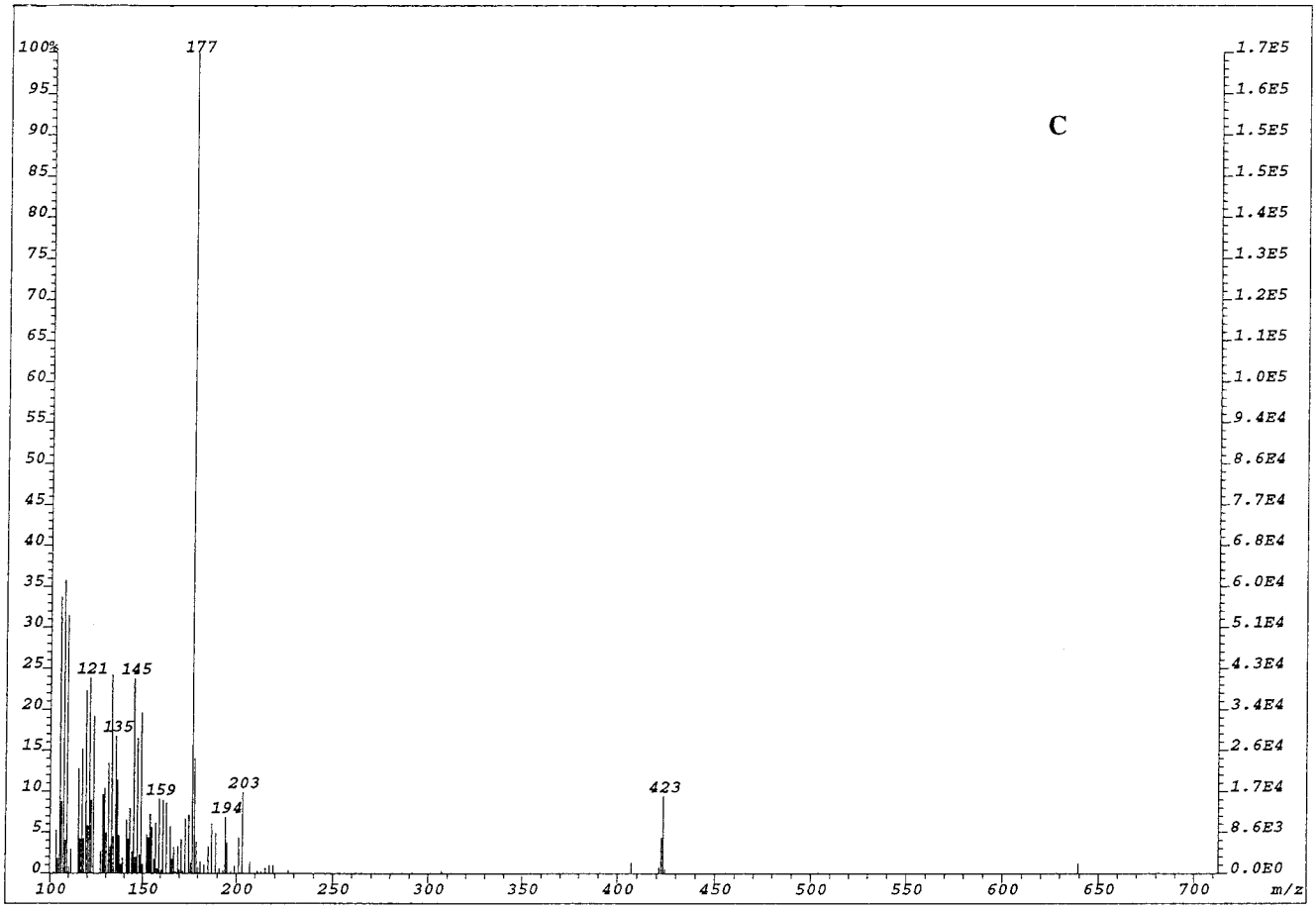
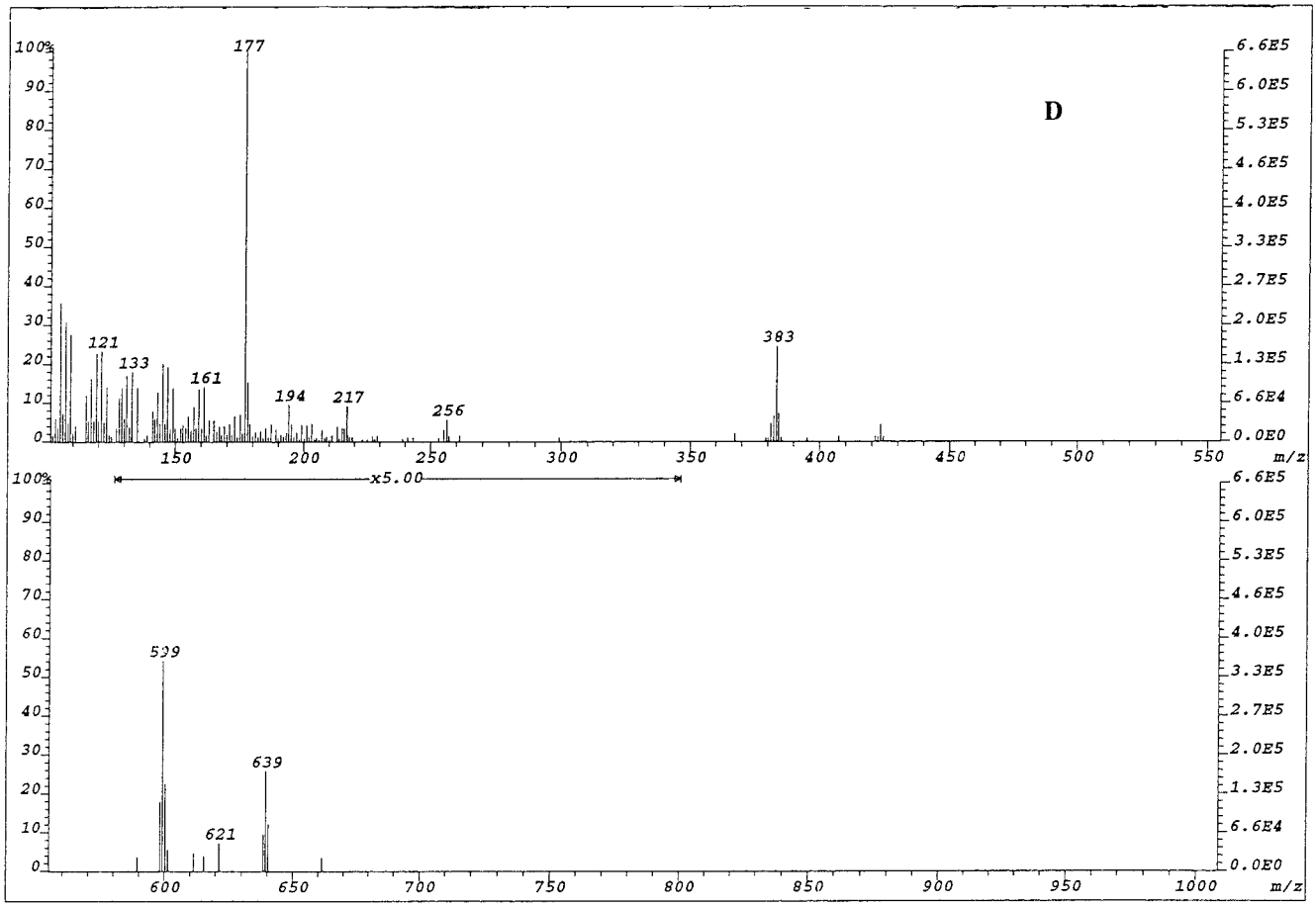


Figure 2 (Continued)



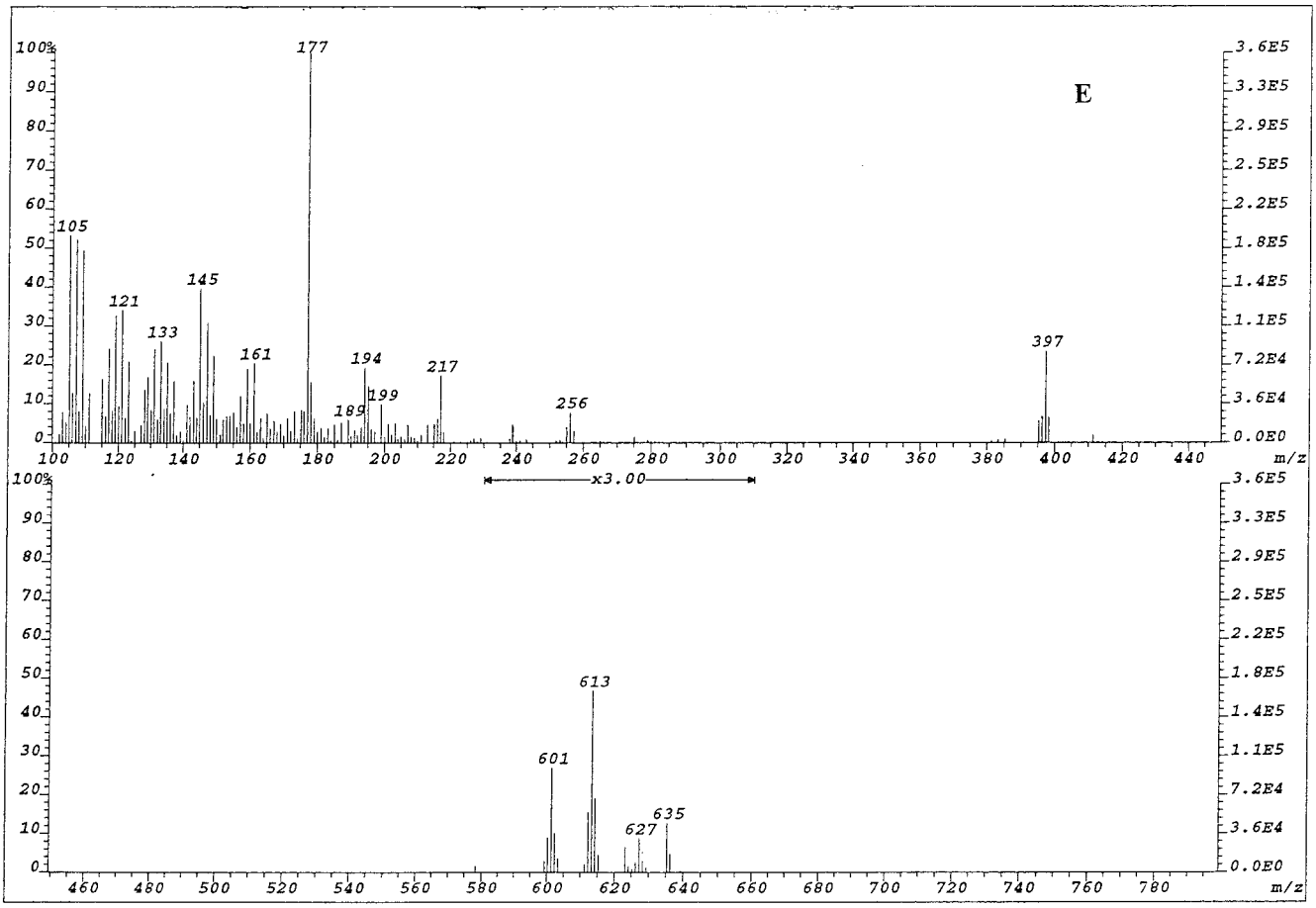


Figure 2 (Continued)

Table 1. LSIMS Predominant Fragmentation Ions of γ-Oryzanol and the Individually Isolated Components

sample[a]	component	calcd (M^+)	obsd (*m*/*z*)
γ-oryzanol (isolated)	mixture of steryl ferulates (Chart 1)	602, 616, 576, 590, 604	603, 617, 590, 577, 591, 604, 423, 409, 397, 383
peak 1	cycloartenyl ferulate	602	409, 177
peak 2	24-methylenecycloartanyl ferulate	616	423, 177
peak 3	campesteryl ferulate	576	576 + Na^+, 383, 177
peak 4	β-sitosteryl ferulate	590	590 + Na^+, 397, 177
	cycloartanyl ferulate	604	604 + Na^+, 177

[a] Peak numbers refer to HPLC peak numbers shown in Figure 1.

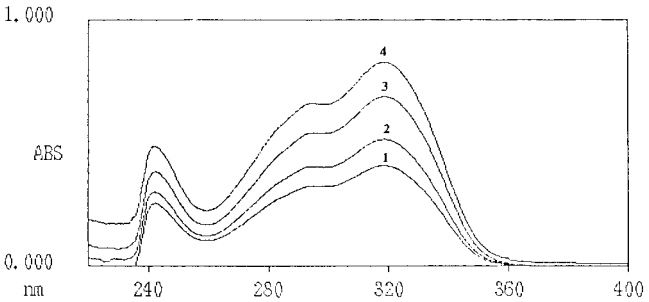


Figure 3. Overlay of UV spectra of the individually separated components of γ-oryzanol demonstrating their similar absorption characteristics. UV spectra: 1 = cycloartenyl ferulate; 2 = 24-methylenecycloartanyl ferulate; 3 = campesteryl ferulate; 4 = β-sitosteryl ferulate and cycloartanyl ferulate.

In conclusion, the present isolation protocol will give ready access to the physiologically beneficial and value-added pharmaceutical product γ-oryzanol from a very low-cost pitch-like byproduct of rice bran oil processing industries. The byproducts of this new method, namely, the calcium salts of fatty acids, can be utilized by industries manufacturing water-proofing fabrics, cements, releasing agents for plastic molding powder, stabilizer for poly(vinyl chloride) resins, lubricants, conditioning agents in pharmaceutical products, etc.

ABBREVIATIONS USED

LSIMS, liquid secondary ion mass spectrometry; CAD, cinnamic acid derivatives; HPLC, high-performance liquid chromatography; UV−vis, ultraviolet−visible.

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