Identification and Quantitation of γ-Oryzanol Components and Simultaneous Assessment of Tocols in Rice Bran Oil

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A reverse-phase high-performance liquid chromatography method was developed for the simultaneous separation and quantitation of tocopherols, tocotrienols and oryzanols present in rice bran oil. Tocopherols and tocotrienols were quantitated by fluorescence detection and oryzanols (ferulic acid esters of sterols and triterpene alcohols) by photodiode array detection. Chemical ionization mass spectrometry was used to identify cycloartenyl ferulate, 24-methylene cycloartanyl ferulate, campesteryl ferulate, β-sitosteryl ferulate and cycloartanyl ferulate as the major oryzanols separated by this procedure. The levels of these nutritionally significant components were found to vary in fully processed, edible rice bran oils from different manufacturers.

KEY WORDS: Chemical ionization, cholesterol, diode array, ferulic acid esters, γ-oryzanol, HPLC, mass spectrometry, rice bran oil, tocopherol, tocotrienol.

Previous investigations have demonstrated that the ingestion of rice bran oil causes a cholesterol-lowering effect (1–3) and a reduction in aortic fatty streaks (4). Also, it was determined that the amount of cholesterol lowering occurs to a greater extent than expected from the fatty acid composition of the oil, suggesting that other components in the oil were responsible for this effect.

Fully processed rice bran oil contains a high amount of unsaponifiable components compared to most other vegetable oils. Two groups of components found in the unsaponifiable fraction of rice bran oil have been investigated for possible health benefits. These are the tocotrienols and γ-oryzanol. The tocotrienols are members of the vitamin E family. They are a group of compounds homologous to the tocopherols that differ by the presence of three unsaturated bonds in the phytyl side chain. The four common isomers of tocopherol and tocotrienol that occur in nature are α, β, γ and δ. Like the tocopherols, the tocotrienols possess antioxidant activity (5). Other physiological actions attributed to the tocotrienols are decreasing serum cholesterol (6), decreasing hepatic cholesterol synthesis by suppression of hydroxy methyl glutaryl coenzyme A reductase and anti-tumor activity (7).

The γ-oryzanol was first extracted from rice bran oil and presumed to be a single component (8). Later it was determined to be a fraction containing ferulate (4-hydroxy-3-methoxycinnamic acid) esters of triterpene alcohols and plant sterols. Individual components were identified as ferulate esters of cycloartenol, 24-methylene cycloartanol, campesterol, β-sitosterol and other sterols. Physiological effects that have been shown to be associated with oryzanol intake are decreasing plasma cholesterol (9), decreasing platelet aggregation (10), decreasing hepatic cholesterol biosynthesis (11), increasing fecal bile acid excretion (11) and decreasing cholesterol absorption. Oryzanol has also been used to treat nerve imbalance and disorders of menopause (12). Previous studies that investigated the biological effects of individual oryzanol components did not distinguish whether free sterols and triterpene alcohols or their ferulate esters were used (13). Simple high-performance liquid chromatography (HPLC) methodology for the measurement of tocopherol and tocotrienol isomers in rice bran oil and other oils exist (14), but the separation and quantitation of individual γ-oryzanol components in oils has not been reported.

Here, we present methodology for the simultaneous determination of tocopherols, tocotrienols and γ-oryzanol components of rice bran oil by reverse-phase HPLC with simultaneous fluorescence and photodiode array detection. Identification of the oryzanols separated by this procedure was accomplished by chemical ionization mass spectrometry of individual oryzanols purified by semi-preparative HPLC.

MATERIALS AND METHODS

Reagents. HPLC-grade (distilled in glass) acetonitrile, isopropanol, methanol, methylene chloride, hexane and water were Burdick & Jackson from Baxter Scientific Products (Bedford, MA) and used as received. HPLC-grade *tert*-butyl methyl ether was received from Aldrich Chemical Company (Milwaukee, WI) and redistilled prior to use. Mobile phase was filtered and degassed under vacuum immediately prior to use. The α, γ and δ tocopherol and stripped corn oil were purchased from Eastman Kodak Company (Rochester, NY). Synthetic γ-tocotrienol was a generous gift from Bristol Myers-Squibb (Princeton, NJ). The α- and δ-tocotrienol were isolated in sufficient quantity from palm oil by semi-preparative HPLC. The γ-oryzanol and cycloartenyl ferulate were obtained from Tsuno Rice Fine Chemicals Company (Wakayama, Japan). Muscle Master γ-oryzanol capsules were obtained from Muscle Masters (Farmingdale, NY). Pyridine and N,O-*bis*(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (TMCS), campesterol, stigmasterol and β-sitosterol were purchased from Sigma Chemical Company (St. Louis, MO).

Standard solutions. Nitric acid-washed, low-actinic glassware was used for the preparation of all standard solutions. Individual stock standard solutions of tocols and γ-oryzanol were prepared by weighing each on a Mettler AE 100 microbalance (Highstown, NJ) and by transferring to 100-mL volumetric flasks. Stock solutions of tocols were diluted with hexane, and γ-oryzanol with methylene chloride. Appropriate dilutions from the stocks were made and the ultraviolet (UV) absorbance spectra obtained from a Perkin-Elmer Lambda 4B UV Scanning Dual Beam Spectrophotometer (Norwalk, CT). Absolute concentrations of each tocol were calculated from their respective molar absorptivities (15,16). The purity of each stock was checked by HPLC, and appropriate

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concentration adjustments were made. Standards for calibration curves were prepared by quantitatively transferring the appropriate small volumes of the above stock standard solutions to 100-mL flasks. This was diluted with "stripped" corn oil that contained none of the added components. Corn oil calibration standards were prepared at three different concentrations containing tocopherols, tocotrienols and γ-oryzanol. Butylated hydroxytoluene was added to the standard oil as a preservative. Two-mL aliquots of the standard oils were transferred to brown borosilicate vials and stored at 4°C. Individual vials were discarded after using them one day. These standards were found to be stable for at least one month under these conditions.

HPLC. The LC system consisted of a Hewlett-Packard Model 1090 High-Performance Liquid Chromatograph (Palo Alto, CA) connected to a Rheodyne 7125 six-port injector (Cotati, CA) with a 50-μL sample loop. Oryzanol components were detected at 325 nm with a Hewlett-Packard Model 1040A photodiode array detector (PDA). Tocopherols and tocotrienols were detected with a Hewlett-Packard Model 1046A fluorescence detector (FLD) with excitation set at 290 nm and emission set at 330 nm. The length and internal diameter of the connecting tubing were chosen to allow minimum back pressure across the FLD flow cell while maintaining minimum band dispersion of the oryzanol components in the PDA. Data acquisition, analysis display and system control were accomplished with a Hewlett-Packard Chem Station 9000 with 20-megabyte hard disc drive (Model 9153), HP color Plotter and HP Think Jet Printer. Oryzanol and tocol components were separated on a Hewlett-Packard 200 × 2.1 mm narrow-bore analytical column (Sunnyvale, CA) packed with 5-μm ODS (C18) Hypersil silica. The mobile phase consisted of acetonitrile/methanol/isopropanol/water (45:45:5:5, by vol), which was programmed to acetonitrile/methanol/isopropanol (50:45:5, vol/vol/vol) from six to ten minutes. The final mobile phase conditions were maintained for 15 min before returning to the original conditions.

Sample pretreatments. Samples of rice bran oil were prepared for HPLC analysis by diluting the oil in mobile phase at a 4% concentration (by vol). The sample was vigorously vortexed on a S/P S 8223-1 vortex mixer (Bedford, MA). Slight emulsions that formed were broken by centrifugation at 500 g for 5 min in an IEC Centra-7R centrifuge (Needham Hts., MA). Aliquots were taken for HPLC analysis. These conditions allowed for the extraction of the tocol and oryzanol components from the oil into the mobile phase. The remaining oil droplet at the bottom of the tube contained high concentrations of the more nonpolar triglycerides, which prevented their carryover to the reverse-phase column.

Crude rice bran oil was dissolved in isopropanol at a 10% concentration (by vol) for subsequent isolation of individual oryzanols by semi-preparative HPLC.

Muscle Master capsules (a rich source of γ-oryzanol) were directly extracted with redistilled diethyl ether. The diethyl ether extracts were taken to dryness by first removing the solvent *via* micro Vigreux distillation, followed by evaporation of the remaining solvent under a stream of nitrogen. The extract was then dissolved in acetonitrile/methanol/*tert*-butyl methyl ether (65:30:5, vol/vol/vol) for subsequent semi-preparative HPLC fractionation of individual oryzanols for mass spectral comparison to rice bran oil oryzanols.

Semi-preparative HPLC. Rice bran oil oryzanol components were repeatedly fractionated on a 150 × 4.6 mm Supelcosil LC-18 (3 μm) column (Bellefonte, PA) eluted with methanol/isopropanol (95:5, vol/vol) mobile phase used isocratically at 50°C at a flow rate of 1.2 mL/min. The crude oryzanol fraction was further enriched by thin-layer chromatography (TLC) (17) on Adsorbosil Plus 1, 500 μ TLC plates (Altech Associates, Deerfield, IL) developed with toluene/ethyl acetate (90:10, vol/vol), and the separation was visualized under UV light as blue fluorescent spots. The spots were scraped off the plates and the oryzanols were extracted with redistilled diethyl ether, followed by direct inlet probe (DIP) chemical ionization-mass spectrometry (CI-MS) analysis. One particular spot ($R_f = 0.63$) contained the oryzanols and was subjected to purification by semi-preparative HPLC as described below.

Muscle Master oryzanol components were repeatedly fractionated by semi-preparative HPLC with a Waters Model 6000A solvent delivery system (Milford, MA) equipped with a Rheodyne 7010 injector and Model 440 absorbance detector with 313-nm filter. Separations were effected on a 100 × 8 mm Radial Pak Cartridge packed with 10-μm ODS (C18) held in a Waters Z Module compression holder. The mobile phase consisted of acetonitrile/methanol/*tert*-butyl methyl ether (65:30:5, vol/vol/vol) under isocratic conditions at a flow rate of 3 mL/min. Injection volumes varied from 0.1–0.5 mL, depending on the oryzanol requiring purification. Fractions were collected as front cut, heart cut and end cut which were checked for purity by analytical HPLC. Those fractions requiring additional purification were subjected to repeated fractionation on a Hewlett-Packard 1090 HPLC equipped with a Model 1040A Photodiode Array Detector set at 325 nm. Separations and collections were effected on a 250 × 4.6 mm Supelcosil LC-18 (5 μm) column with acetonitrile/methanol/*tert*-butyl methyl ether (65:30:5, vol/vol/vol) mobile phase used isocratically at 50°C at a flow rate of 1 mL/min. Injection volumes of 25 μL were used. All fractions were investigated by DIP mass spectrometry under CI conditions and by capillary gas chromatography/mass spectrometry (GC/MS) of their trimethylsilyl (TMS) ether derivatives after alkaline hydrolysis.

Mass spectrometry. Analyses were performed on a Hewlett-Packard Model 5987A GC/MS system. Data were acquired from electron impact (EI) and CI by the DIP technique. For CI/MS analysis, the DIP was temperature-programmed from 50 to 250°C at a rate of 10°C/min. Methane was used as the reagent gas at a source pressure of 0.4 Torr, an ionization potential of 200 eV, a multiplier voltage of 3000 V, and a scan range of 100 to 800 amu at a rate of 10 scans/min. EI/MS analysis of the TMS ether derivatives of hydrolyzed oryzanols was performed at 70 eV with a source temperature of 200°C, a scan range of 50 to 600 amu at a rate of 1.2 scans/s. Chromatography conditions were identical to those described below.

Capillary gas chromatography. Capillary gas-chromatographic analysis of the TMS ether derivatives of hydrolyzed oryzanols was performed on a Hewlett-Packard Model 5880A gas chromatograph equipped with a split injector and flame-ionization detectors. Separations were effected

on a 25M × 0.25 mm Quadrex fused silica capillary column (New Haven, CT) coated with 0.1 μ methyl 50% phenyl silicone. Analyses were performed isothermally at 250°C at an inlet pressure of 1 bar. The flame-ionization detector was operated at 300°C, and helium was used as the carrier gas (μ = 30 cm/s). Sample volumes of 1 μL were injected at a split ratio of 50:1.

Oryzanol derivatization. Oryzanol fractions obtained by semi-preparative HPLC were taken to dryness under a stream of nitrogen. After a portion was removed for DIP CI/MS analysis, the remainder was hydrolyzed with ethanol/50% NaOH (80:20, vol/vol) for 30 min at 80°C. After cooling to room temperature, a volume of deionized water was added, and the free sterols were extracted with 2 × 5 mL redistilled hexane. The combined hexane extracts were dried over anhydrous sodium sulfate and then taken to dryness under a stream of nitrogen. A volume of dry pyridine was added, followed by an equal volume of BSTFA with 1% TMCS. After 30 min-reaction, 1-μL injections were taken for capillary GC/MS analysis. TMS ether derivatives of a hydrolyzed cycloartenyl ferulate standard, as well as campesterol, stigmasterol and β-sitosterol were similarly prepared for mass spectral and capillary GC retention time comparisons to individually isolated rice bran oil oryzanols.

RESULTS AND DISCUSSION

Column, mobile phase and detector selection. Two reverse-phase columns and several isocratic, binary and tertiary mobile phase conditions were examined to develop optimum separation conditions for each of the oryzanol components while maintaining maximum separation of tocols. A Hewlett-Packard C-18 Hypersil column was initially used. The composition of the mobile phase was varied by adjusting the proportions of either acetonitrile, methanol or both that contained small amounts of either methylene chloride, isopropanol or both. The use of acetonitrile or methanol alone resulted in poor resolution of oryzanol components. An acetonitrile/methanol ratio of 50:45 (vol/vol) was optimum in regard to this aspect of the mobile phase. However, these two solvents alone did not allow for good separation of the oryzanols. Addition of 5% methylene chloride or isopropanol to the acetonitrile/methanol increased resolution of the components, with isopropanol yielding the best separation. However, the tocopherols and tocotrienols were poorly resolved under these conditions. The incorporation of a small amount of water (5% by vol) in the initial mobile phase, followed by a gradient program to 0% water from six to ten minutes allowed for the best separation conditions for tocol and oryzanol components. The use of a Beckman Ultrasphere Column (San Ramon, CA) resulted in much lower recoveries of oryzanols compared to the Hypersil column.

Separation of tocol and oryzanol components. A typical HPLC chromatogram obtained from a crude rice bran oil extract analyzed by the described conditions is shown in Figure 1. The figure clearly demonstrates the separation of tocopherols and tocotrienols from the oryzanol components. The tocotrienol isomers eluted as a group before the tocopherols. The elution order of each groups' isomers was α > β > γ > δ. The elution order seen here, as well as the inability to separate the β/γ positional isomers, was expected in a reverse-phase system (14). An increase in

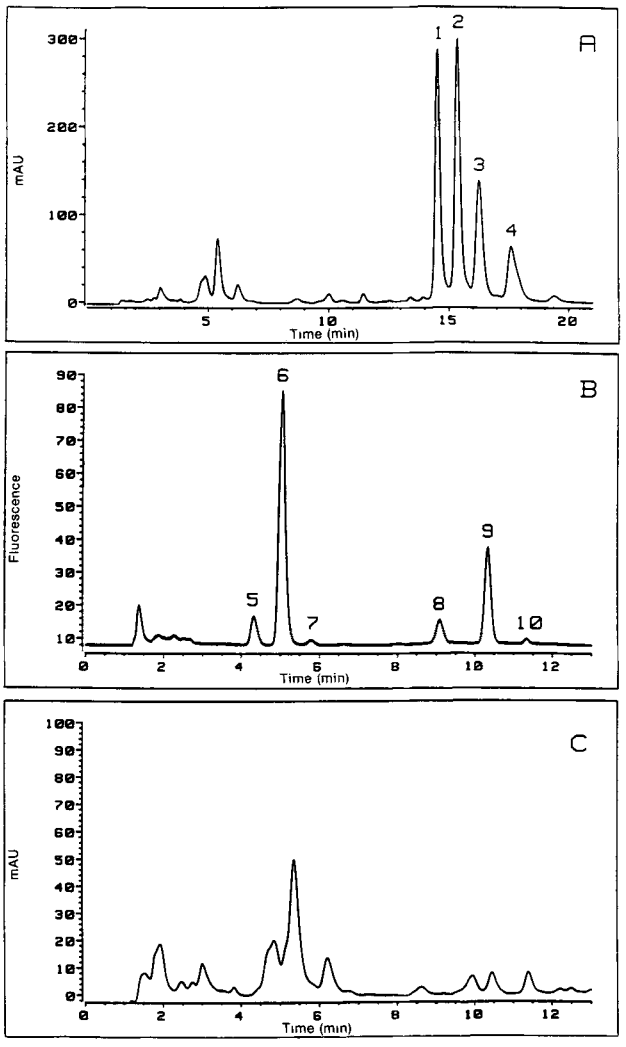

FIG. 1. Simultaneous separation of tocotrienols, tocopherols and γ-oryzanol components from a crude rice bran oil sample. A) Ultraviolet detection of γ-oryzanol components. Peaks: 1 = cycloartenyl ferulate; 2 = 24-methylene cycloartanyl ferulate; 3 = campesteryl ferulate; 4 = β-sitosteryl ferulate and cycloartanyl ferulate. Fluorescent (B) *vs.* ultraviolet (C) detection of tocotrienols and tocopherols. Peaks: 5 = δ-tocotrienol; 6 = β and γ-tocotrienols; 7 = α tocotrienol; 8 = δ-tocopherol; 9 = β and γ-tocopherols; and 10 = α-tocopherol.

the sensitivity for tocols in rice bran oil by using fluorescence detection instead of UV is also demonstrated in Figure 1. A three-dimensional plot was generated by the photodiode array detector of the oryzanol region separated under these conditions and is shown in Figure 2. Absorption spectra for the four oryzanol peaks were also acquired by PDA (Fig. 3). Absorption spectra for all four oryzanol peaks were essentially identical. Maxima were consistent with the literature values of oryzanols (18) and characteristic for a ferulate moiety present in each component. Spectral differences between these two maxima could not be detected through the fourth derivative.

CI mass spectra of oryzanols. The CI mass spectra of the individually isolated rice bran oil oryzanols (HPLC peaks 1–4, Fig. 1) are shown in Figure 4, and their characteristic fragmentation ions are tabulated in Table 1. The resultant data show protonated molecular ions identical

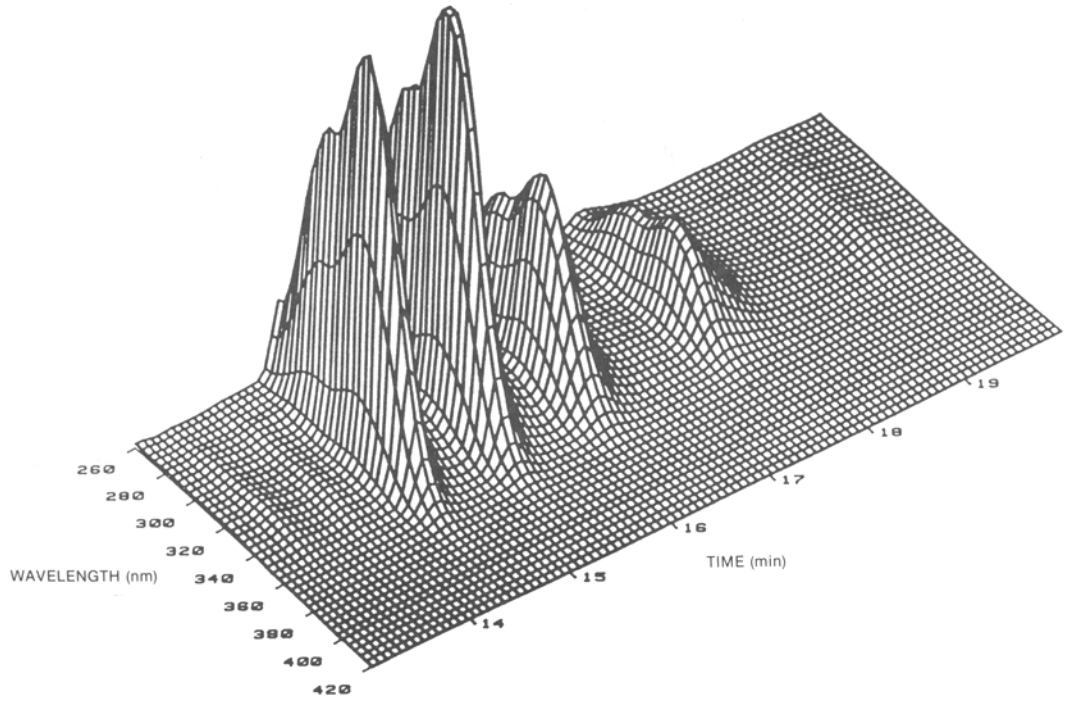

FIG. 2. Three-dimensional chromatogram of the oryzanol fraction of the crude rice bran oil sample from Figure 1.

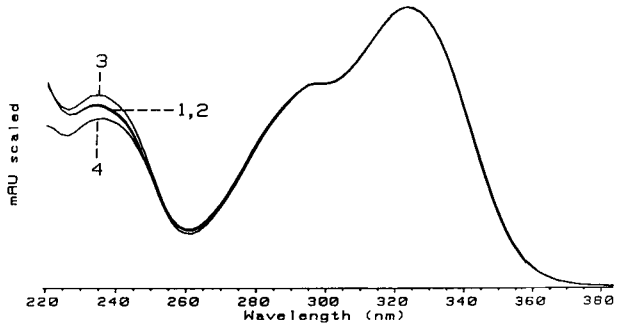

FIG. 3. Overlay of ultraviolet spectra of each of the four γ-oryzanol components in the crude rice bran oil sample in Figure 1, demonstrating similar absorption characteristics.

TABLE 1

DIP CI-MS Predominant Fragmentation Ions for Rice Bran Oil Oryzanols[a]

HPLC peak	$(M + 1)^+$	*(m/z)*	Oryzanol
1	603	409, 195, 177	Cycloartenyl ferulate
2	617	423, 195, 177	24-Methylene cycloartanyl ferulate
3	577	383, 195, 177	Campesteryl ferulate
4	591	397, 195, 177	β-Sitosteryl ferulate
	605	411, 195, 177	Cycloartanyl ferulate

[a]High-performance liquid chromatography (HPLC) peak refers to Figure 1. Abbreviations: DIP, direct inlet probe; CI-MS, chemical ionization-mass spectrometry.

to their calculated molecular weights plus one. Characteristic fragmentation ions were observed for each oryzanol, which included the loss of ferulic acid ($[M + 1] - 194$) from the protonated molecular ion; protonated ferulic acid (*m/z* 195) and protonated ferulic acid minus water (*m/z* 177). Molecular ion adducts for CH_5^+, $C_2H_5^+$ and $C_3H_5^+$ were also observed, but at low abundance. HPLC peaks 1, 2 and 3 were each found to be comprised of one oryzanol, whereas HPLC peak 4 was found to be a mixture of β-sitosteryl and cycloartanyl ferulates (Fig. 4). Identical spectra were also obtained from HPLC fractions prepared from Muscle Master γ-oryzanol capsules. The availability of a cycloartenyl ferulate standard permitted the direct confirmation of HPLC peak 1, obtained semi-preparatively from rice bran oil and Muscle Master γ-oryzanol capsules. The structures of the major γ-oryzanol components found in rice bran oil, as seen in Figure 4, are similar to that of cholesterol. It has been suggested that this similarity may be responsible for the cholesterol-lowering effects of γ-oryzanol (13).

EI mass spectra of oryzanol trimethylsilyl ether derivatives. Structural assignments for the sterol and triterpene alcohol moieties present in the individually separated oryzanols (HPLC peaks 1-4, Fig. 1) were obtained by capillary GC/MS analysis of their TMS ether derivatives, following hydrolysis. The resultant fragmentation ions are tabulated in Table 2. The EI mass spectra for three 4,4-dimethyl sterols (cycloartenol, 24-methylene cycloartanol and cycloartanol) exhibited low molecular ion abundances relative to their $(M - 90)^+$ ions. They also exhibited the characteristic loss of *m/z* 212, as well as abundant $(M - 90 - CH_3)^+$ ions. The EI mass spectra for three

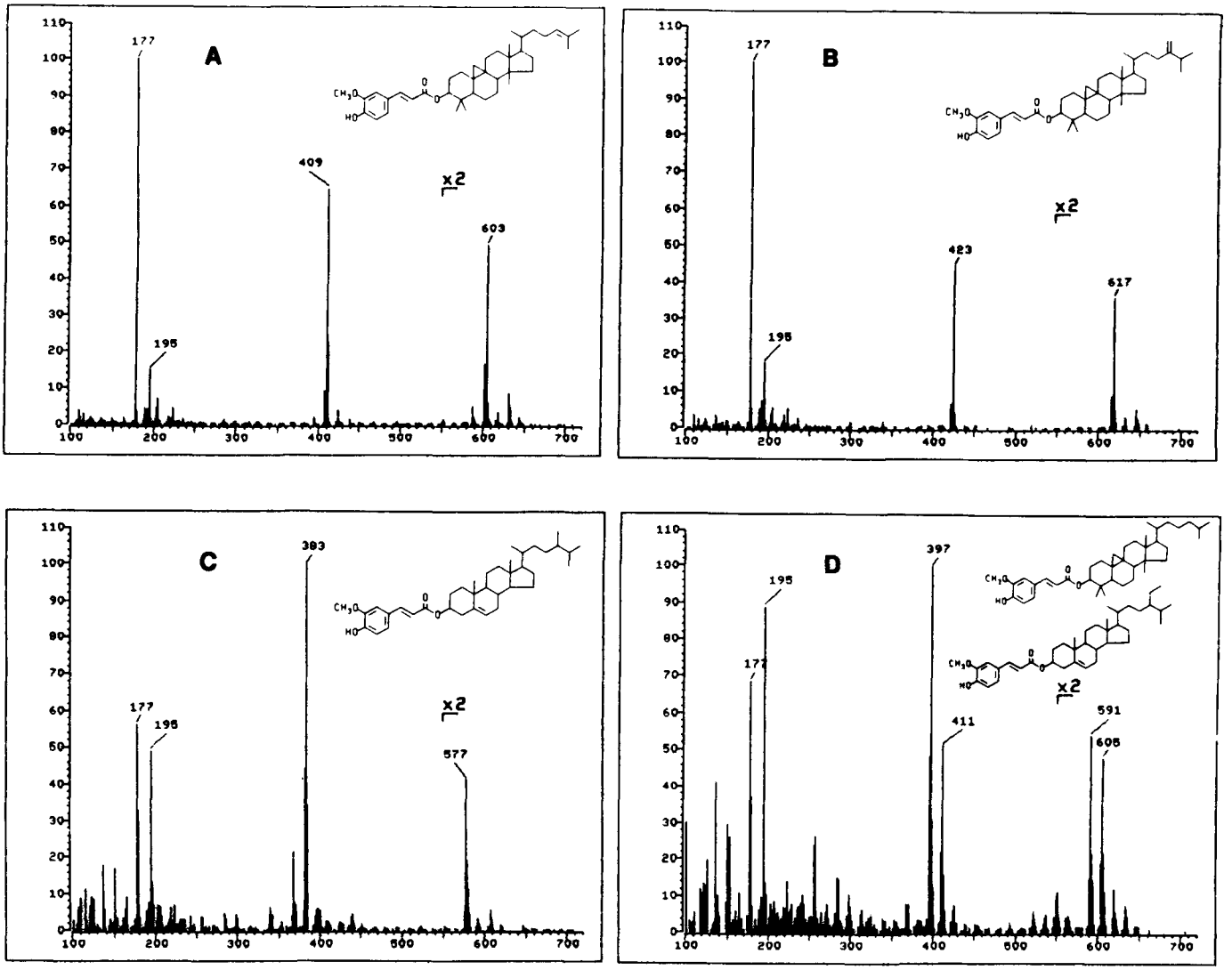

FIG. 4. Chemical ionization-mass spectra for A) cycloartenyl ferulate for HPLC peak 1, Figure 1; B) 24-methylene cycloartanyl ferulate peak 2, Figure 1; C) campesteryl ferulate peak 3, Figure 1 and D) mixture of β-sitosteryl ferulate and cycloartanyl ferulate peak 4, Figure 1.

TABLE 2

EI Mass Spectra for the TMS Ether Derivatives of Hydrolyzed Rice Bran Oil Oryzanols[a]

HPLC peak	TMS ether derivative	*m/z* (relative intensity)
1	Cycloartenol TMS ether	M^+498(0.0), 408(14.6), 393(10.8), 365(10.4), 339(6.4), 286(4.5), 173(16.0). 159(20.6), 95(60.9), 69(100.0)
2	24-Methylene cycloartanol TMS ether	M^+512(1.4), 422(26.7), 407(16.5), 379(19.1), 300(6.2), 203(15.3), 175(22.9), 159(17.7), 95(69.1), 73(100.0)
3	Campesterol TMS ether	M^+472(12.5), 382(20.9), 343(25.1), 255(6.4), 215(6.7), 145(20.5), 129(100.0), 107(32.7), 105(26.2), 75(66.0)
	Stigmasterol TMS ether	M^+484(10.6), 394(9.0), 255(16.1), 213(5.7), 159(13.6), 145(14.4), 129(56.8), 107(20.9), 105(20.3), 83(100.0)
4	β-Sitosterol TMS ether	M^+486(13.7), 396(20.9), 357(23.0), 255(6.8), 213(5.1), 159(14.2), 129(100.0), 107(31.3), 105(25.8), 75(53.8)
	Cycloartanol TMS ether	M^+500(1.5), 410(27.8), 395(27.0), 367(29.6), 341(20.6), 288(12.4), 175(33.2), 161(13.9), 95(100.0), 81(51.7)

[a]High-performance liquid chromatography (HPLC) peak refers to Figure 1. Abbreviations: EI, electron impact; TMS, trimethylsilyl.

TABLE 3

γ-Oryzanol Content of Refined Rice Bran Oil from Different Manufacturers

Sample identification	Peak number[a]				Total
	1	2	3	4	
	(ppm)				
Brand A	232[b]	314	157	84	787 ± 13.5[c]
Brand B	45	45	39	15	144 ± 4.1
Brand C	35	30	50	ND[d]	115 ± 3.8
Brand D	155	61	228	66	510 ± 11.3
Brand E	228	74	342	74	718 ± 12.7

[a]Peak identification: 1 = cycloartenyl ferulate; 2 = 24-methylene cycloartanyl ferulate; 3 = campesteryl ferulate; 4 = β-sitosteryl ferulate and cycloartanyl ferulate.
[b]Mean (n = 3).
[c]Standard deviation (n = 3).
[d]None detected.

TABLE 4

Tocol Content of Refined Rice Bran Oils from Different Manufacturers

Sample identification	Tocotrienols (ppm)				Tocopherols (ppm)				Total tocols (ppm)
	δ	β/γ	α	Total	δ	β/γ	α	Total	
BRAND A	48[a]	478	86	612	17	114	120	251	863 ± 12.2[b]
BRAND B	ND[c]	62	10	72	ND	16	ND	16	88 ± 3.5
BRAND C	16	309	13	338	8	53	16	77	415 ± 7.4
BRAND D	6	322	ND	328	ND	99	218	317	645 ± 9.3
BRAND E	104	975	78	1157	40	358	54	452	1609 ± 18.6

[a]Mean (n = 3). [b]Standard deviation (n = 3). [c]None detected.

Δ5-unsaturated 4-demethylsterols (campesterol, β-sitosterol and stigmasterol) exhibited abundant M^+ and *m/z* 129 ions, as well as the characteristic $(M - 129)^+$ and $(M - 90)^+$ ions. These data were consistent with those reported in the literature (19). Identical mass spectra and capillary retention time data were obtained from the HPLC fractions prepared from Muscle Masters γ-oryzanol capsules, as well as from cycloartenyl ferulate standard, campesterol, β-sitosterol and stigmasterol. Evidence for the TMS ether derivative of stigmasterol was obtained in HPLC peak 3, but at low levels. This suggested the additional presence for stigmasteryl ferulate as part of the rice bran oil oryzanol fraction.

Tocol and oryzanol variations in fully processed, edible rice bran oils. Analytical methodology presented here for the simple and simultaneous measurement of tocopherols, tocotrienols and γ-oryzanol components was used to assess the levels of these nutrients in several brands of available edible rice bran oil products. These results are listed in Tables 3 and 4. The reproducibility of the procedure from triplicate analysis of these oil samples is also listed in these tables. A large variability in the concentration of these components between the oils is evident. Potential causes for these variations include rice varietal differences as well as potential losses of these compounds during specific oil processing steps. Future application of this procedure will permit a more detailed investigation and assessment of the relationship between the concentration of these rice bran oil components and observed biological effects in animal models.

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