Purification and Identification of Rice Bran Oil Fatty Acid Steryl and Wax Esters

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ABSTRACT: Fatty acid steryl esters (FASE) and wax esters (WE) of rice bran oil (RBO) have potential applications in cosmetic, nutraceutical, and pharmaceutical formulations. FASE and WE were extracted from RBO by a modified Soxhlet extraction using hexane as the solvent. FASE and WE were then separated by storage in acetone at 10°C for 24 h. The FASE fraction was further purified by silica gel column chromatography. The contents and compositions of FASE and WE, as well as their saponified products, were identified by GC and GC–MS. The identification of FASE and WE was carried out by comparing the retention time of GC peaks and mass spectral analysis with standards synthesized in our laboratory. FASE and WE accounted for ca. 4.0% of crude RBO, of which 2.8–3.2% and 1.2–1.4% are FASE and WE, respectively. GC–MS of FASE showed five major peaks. Major FA in the FASE fraction were linoleic acid and oleic acid, which were esterified with 4-desmethyl, 4-monomethyl, and 4,4-dimethyl sterols. The contents of 4-desmethylsterol, 4-monomethylsterol, and 4,4-dimethylsterol esters in crude RBO were 76.1, 8.7, and 15.1%, respectively. WE of RBO consisted of both even and odd carbon numbers ranging from C_{44} to C_{64}. The major constituents were saturated esters of C_{22} and C_{24} FA and C_{24} to C_{40} aliphatic alcohols, with C_{24} and C_{30} being the predominant FA and fatty alcohol, respectively. The advantages of using a modified Soxhlet extraction over column chromatography are less solvent usage and larger sample size per batch with shorter operation time.

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KEY WORDS: Fatty acid, fatty acid steryl ester, GC–MS, phytosterol, rice bran oil, Soxhlet extraction, TLC, wax ester.

FA steryl esters (FASE) and wax esters (WE) have wide applications in cosmetic, nutraceutical, and pharmaceutical industries (1,2). FASE have water-holding properties and are widely used as ingredients of cosmetics and bath additives (3). Recently, phytosteryl ester was found to be effective in lowering plasma cholesterol concentration by inhibiting the absorption of cholesterol from the small intestine (4). This physiological activity has led to the development of functional foods, such as salad oil, dressing with added sterol, and margarine blended with FASE. Because FASE is completely soluble in TAG, there is a growing interest in the use of FASE in lipid-containing foods (5).

Rice bran wax (RBW) has potential applications in cosmetic, pharmaceutical, food, polymer, and leather industries (2). A large number of patents and papers have been issued on the use of RBW in cosmetic preparations such as cold cream, drugs, and hair-conditioners. In such applications, the performance of RBW is reported to be comparable to carnauba and other waxes (2). RBW is also a rich source of high-MW aliphatic alcohols known as policosanol. Earlier investigators demonstrated the multitude of beneficial therapeutic properties associated with policosanol intake, such as the lowering of blood lipids (6). Policosanol from beeswax, which has been used in different pharmaceutical formulations for the treatment of gastric and duodenal ulcers, exhibits substantial anti-inflammatory activity (7). Hence, RBW can potentially be a raw material for a multitude of applications if there is a method suitable for its purification.

Crude rice bran oil (RBO) contains a significant amount of WE and FASE (8,9). Little information about FASE in RBO is available in the literature. Previous attempts at the characterization of FASE in RBO have achieved only limited success (8,10). A better knowledge of the separation, purification, and chemical composition of FASE in RBO may help researchers find better ways to use this RBO by-product. Ito *et al.* (8) showed that FASE separated from neutral lipid fraction of RBO had 6.9% FASE, which were 4-desmethylsterols esters of FA. GC chromatograms showed that they were FASE of campesterol, stigmasterol, and sitosterol. Fujino (10), however, reported that FASE in RBO contained a mixture of 4-desmethyl, 4-monomethyl, and 4,4-dimethyl sterol esters of FA. Detailed information on GC separation of intact FASE and its saponified product has not been reported previously. The wax contents in crude RBO reported in the literature vary owing to variations in the rice bran source and wax isolation methods. Ito *et al.* (8) reported that the wax content of RBO was about 0.86%, and Saunders (9) reported that there was 3–4% WE on a total lipid basis in RBO. Recent studies by Arumughan *et al.* (11) found a wax content of 2–5% in crude RBO. In these literature sources a wide range of wax content has been recorded, but no information on the FASE content in crude RBO was included. FASE and WE have nearly identical physical and chemical properties and show similar chromatographic behavior. To determine the exact content and composition of FASE and WE in RBO, enrichment followed by separation of these lipid classes prior to analysis is important. The purpose of this work was to separate FASE and WE from crude RBO and determine their content and composition.

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MATERIALS AND METHODS

Materials. Rice bran was donated by a rice mill located in Taoyung County, Taiwan. Analytical TLC silica gel glass plates (general purpose, 20 × 20 cm, particle size: 250 μm) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Lipase from *Candida rugosa* was a product of Sigma Chemical Co. (St. Louis, MO). Lipozyme IM 60 (*Rhizomucor miehei* lipase immobilized on macroporous anion exchange resin) was a gift sample from Novo Nordisk A/S (Bagsværd, Denmark). Silica gel 60M (60–200 mesh) was purchased from Macherey-Nagel GmbH & Co., KG. (Duren, Germany). Standard squalene, FA, fatty alcohol (FAL), and FAME were obtained from Sigma Chemical Company. Standard β-sitosterol practical grade was obtained from MP Biomedicals, LLC (Aurora, OH). All solvents and reagents were either of HPLC grade or analytical reagent grade and were obtained from commercial sources. Fresh rice bran obtained from the rice mill was stored at −50°C before use. Rice bran removed from the refrigerator was passed through a mesh (<0.6 mm) to remove foreign material.

Determination of moisture content of rice bran. Moisture content of rice bran was determined by AOCS Official Method Bc 2-49 (12).

Extraction of RBO. Crude RBO was extracted from rice bran (50 g) by Soxhlet extraction for 3 h using hexane as the solvent.

Determination of oil content. Oil content of rice bran was determined by AOCS Official Method Bc 3-49 (12).

Determination of FFA content in RBO. FFA content of RBO was determined by AOCS Official Method Ca 5a-40 (12).

Extraction of nonpolar lipids from crude RBO. A modified Soxhlet extraction was used in this study. Crude RBO (8 g) was dissolved in 100 mL hexane in a 250-mL round-bottomed flask at 50°C. Silica gel (32 g) was added to this solution. The mixture was magnetically stirred at 300 rpm for 1 h. The sample was adsorbed onto silica gel by the removal of solvent at 60°C. The sample-loaded silica gel was packed into an extraction thimble (9.4 × 3.3 cm i.d.), the top surface was covered with cotton, and then the thimble was introduced into an extractor (145 mL). Nonpolar lipids were extracted by hexane (140 mL) at 54–56°C for 3 h at a rate of 7–8 extractions/h. The extract was separated and concentrated by rotary evaporation and dried under vacuum to give the hexane extractive (3–3.2 g). Hydrocarbons, FASE, and WE were preferentially extracted into the hexane phase. The hexane extractive containing enriched hydrocarbons, FASE, and WE is designated as nonpolar lipid fraction A (NPLF-A). The remaining lipid that was absorbed on the silica gel was then extracted with ethyl acetate for 3 h at 70–72°C, and the extract was designated as polar lipid fraction A (PLF-A). The NPLF-A was subjected to a second, modified Soxhlet extraction to further purify squalene, FASE, and WE. The sample (NPLF-A) was adsorbed onto the silica gel using a sample-to-silica gel ratio of 1:6 w/w, and extractions were repeated using hexane and ethyl acetate as the extracting solvents as described above. The hexane extract was collected and solvent was removed by rotary evaporation; the dried sample (~1.2 g) was designated as the nonpolar lipid fraction B (NPLF-B). The ethyl acetate extract was designated as the polar lipid fraction B (PLF-B).

Separation of WE from NPLF-B. NPLF-B (1.2 g) was dissolved in 40 mL of acetone in a 100-mL glass-stoppered conical flask, and the mixture was stirred at 40°C for 30 min. The solution was cooled to room temperature and stored at 10°C for 24 h. After removal from the cold storage, solid and liquid phases were separated immediately by vacuum filtration using an Ace Büchner funnel (25–50 μm pore size). The conical flask and solid phase were washed with acetone (3 × 10 mL) and filtered. The filtrates were pooled, and lipid in the filtrate was recovered by removing the solvent in a vacuum rotary evaporator at 60°C. This filtrate was designated as nonpolar lipid fraction C (NPLF-C). The solid phase (WE) on the filter paper was dried under vacuum. The filtrate and solid phase were analyzed by high-temperature gas chromatography (HT-GC).

Column chromatography. FASE in NPLF-C was isolated by silica gel column chromatography. Silica gel (25 g, 60–200 mesh; Merck, Darmstadt, Germany) was dispersed in 50 mL hexane, transferred into a glass column (2.4 cm i.d., 35 cm length) in which there was no trapped air. As soon as the silica gel settled to the bottom of the column packing, NPLF-C (1 g, dissolved in 3 mL hexane) was applied onto the column. When the solution had drained to the top of the column packing, the sample was eluted at a flow rate of 1.5 mL/min, and eluate was collected in 10-mL fractions in successive test tubes. Squalene and other hydrocarbons were eluted first with 100 mL *n*-hexane/ethylacetate (99.5:0.5, vol/vol). A second fraction containing FASE was eluted with 150 mL *n*-hexane/ethylacetate (99:1, vol/vol). Finally, TAG and other polar components were obtained by eluting with 150 mL ethyl acetate. The purity of each fraction was analyzed by HT-GC as well as by silica TLC plate (6 × 2 cm, 250 μm) using authentic standards. Each plate was developed with *n*-hexane/ethyl acetate/acetic acid (90:10:1, by vol) as the developing solvent. After air-drying, the spots on each plate were visualized by exposing the chromatogram to iodine vapor. The FASE spot was detected by spraying with a fresh solution of 50 mg ferric chloride in a mixture of 90 mL water, 5 mL acetic acid, and 5 mL sulfuric acid. After heating at 100°C for 3–5 min, the FASE spot was indicated by a red-violet color (13). Fractions containing only FASE were combined, and solvent was removed with a rotary evaporator.

Saponification of FASE. One hundred milligrams of FASE (96% purity) and 25 mL of 1 N potassium hydroxide in 90% ethanol were refluxed at 65°C under a nitrogen atmosphere until the reaction was completed (3–4 h), as verified by analytical TLC (silica gel; eluted with 80:20:0.5 by vol hexane/ethylacetate/acetic acid) and HT-GC. To this saponified mixture, 25 mL distilled water was added and the unsaponifiable matter (sterol) was separated by extraction with diethyl ether (3 × 20 mL). The extracts were collected and dried over anhydrous sodium sulfate. The aqueous phase containing saponified matter (FA potassium salt) was acidified to pH 2 with dilute HCl (50% vol/vol). The mixture was transferred to a separatory funnel and FA were recovered by extraction with hexane (2 × 20

mL). The hexane extract was washed with water to neutral pH and then dried over anhydrous sodium sulfate. Solvent was removed at 50°C to recover FA, which was then converted into their corresponding FAME by heating with BF_3-methanol (20%). The FA composition was analyzed by GC as described elsewhere (14). Peaks in the chromatogram were identified by using authentic FAME standards.

Syntheses of FASE and WE standards. FASE standards were synthesized by the esterification of oleic acid (98%) and linoleic acid (99%) with a sterol mixture of known composition. Typically, FA (0.1 g) and sterols with a molar ratio of 2:1 were placed into a 7 mL flat-bottomed glass vial sealed with a Teflon-lined cap. To this, 30 mg *Candida rugosa* lipase was added, and the mixture was incubated in a water bath at 40°C and magnetically stirred at 200 rpm for 6 h. The content was then dissolved in ethyl acetate and lipase was filtered off. FASE was separated by silica gel column chromatography. Standard WE was synthesized by the esterification of FA with FAL catalyzed by Lipozyme IM 60 as described elsewhere (15).

Analysis by HT-GC. The compositions of FASE and sterol fraction obtained after the saponification of FASE were determined by HT-GC. Chromatographic analysis was performed on a Shimadzu GC-17A (Kyoto, Japan) gas chromatograph equipped with an FID. Separations were carried out on a DB-5HT (5%-phenyl)-methylpolysiloxane nonpolar column (15 m × 0.32 mm i.d.; Agilent Technologies, Palo Alto, CA). The temperatures of injector and detector were both set at 370°C. The initial temperature of the column was 80°C, and was then increased to 305°C at 15°C/min, then to 335°C at 5°C/min and maintained at 335°C for 5 min. Finally, the temperature was raised to 365°C at 15°C/min. The split ratio was 1:50, and the carrier gas was nitrogen. Ten milligrams of sample was dissolved in 1 mL of ethyl acetate, and 1 μL of sample was injected into the high-temperature gas chromatograph. FA composition of saponified WE was determined by GC. The compositions of WE and FAL were identified by HT-GC. Details of the procedures for the determination of WE composition can be found elsewhere (15).

Analysis by GC–MS. The compositions of FASE and sterol were analyzed by a Shimadzu GC-17A (Kyoto, Japan) gas chromatograph equipped with a 5970 mass selective detector and a 5990A MS Chemstation (HP-UX) (Hewlett-Packard, North Hollywood, CA). Separations were carried out on a DB-5HT (5%-phenyl)-methylpolysiloxane nonpolar column using the same temperature program as described in HT-GC analysis. All mass spectra were acquired using the EI mode at 70 eV, with an ion current of 50 μA, and an ion source temperature of 200°C. The mass spectrum was scanned in the range of *m/z* 50–800 at 2.25 scans/s.

Identification of FASE and sterols. The identification of FASE and sterol peaks was achieved by comparing the retention times and the fragmentation patterns in GC–MS spectra of standard sterol and synthesized FASE. Some of the peaks and their structures were identified by MS using the U.S. Department of Commerce National Institute of Standards and Technology (NIST) and Wiley libraries and, when possible, by comparison with the MS of standards. The composition of WE and ECL of C_{44}–C_{60} WE were determined by matching peak retention times with those of synthesized WE standards. ECL of FAL and WE beyond C_{30} and C_{60}, respectively, are estimated tentatively by the relationship between carbon number and retention time. The identification of FA according to their ECL is better done using isothermal conditions (16,17). However, temperature-programmed gas chromatography (TPGC) is more convenient. In addition, peak separation in TPGC is improved, peaks are narrower, and elution is accomplished in a shorter time (18,19).

RESULTS AND DISCUSSION

Extraction of NPLF from crude RBO. Most of the studies for the separation of WE and FASE have used column chromatography or TLC to fractionate and quantify various WE and FASE (8,20–22). The separation and quantification of WE in crude RBO by silica gel column chromatography is particularly difficult because of the high m.p. and saturated nature of WE (15). Wax is not soluble in solvent at room temperature, so it must be liquefied by heating to 45–50°C and dissolved before applying it onto the column at the same temperature. The solvent and column should also be heated to 45–50°C. These difficulties in setting and maintaining uniform conditions have led to inconsistent fractionation results (21). Direct separation and quantification of these compounds in oil by GC or HPLC have proven to be the most difficult task. These compounds co-elute during analysis due to their similarity in MW and nonpolar nature (23,24). Therefore, reported data on the content and composition of WE in RBO were inconsistent.

TLC and HT-GC analyses of ethylacetate extract (polar fractions) show the absence of FASE, WE, squalene, and hydrocarbons. Nonpolar lipids, which contained 28–33% FASE and WE, were extracted using hexane as the solvent. More than 65% of the TAG and other polar components were removed after modified Soxhlet extraction. The hexane extract for the second-stage modified Soxhlet extraction (NPLF-B) had a total FASE and WE content of 47–55%, which was higher than the first extraction. Analysis of the residual lipid extracted with ethylacetate showed that it contained only TAG and other polar lipids. Figure 1 is a flow chart of the extraction and enrichment of nonpolar lipids from crude RBO. Modified Soxhlet extraction is an efficient method for the extraction of WE and FASE from crude RBO using a single solvent. Advantages of the current method are: large sample-to-silica gel ratio, easy recovery and reuse of solvents, and simple operational procedure. Various factors such as sample-to-silica gel ratio, time of extraction, rate of extractions, temperature, solvent amount, and polarity of solvent were systematically studied (data not shown).

Separation of WE and FASE. In general, wax content in vegetable oil is determined using crystallization followed by filtration and weighing of the solid residue. This method is more accurate when applied to oils rich in crystallizable wax, such as crude sunflower oil or RBO (15,23,25). Crude RBO contains crystallizable, high MW, saturated C_{44}–C_{64} wax esters (15,23).

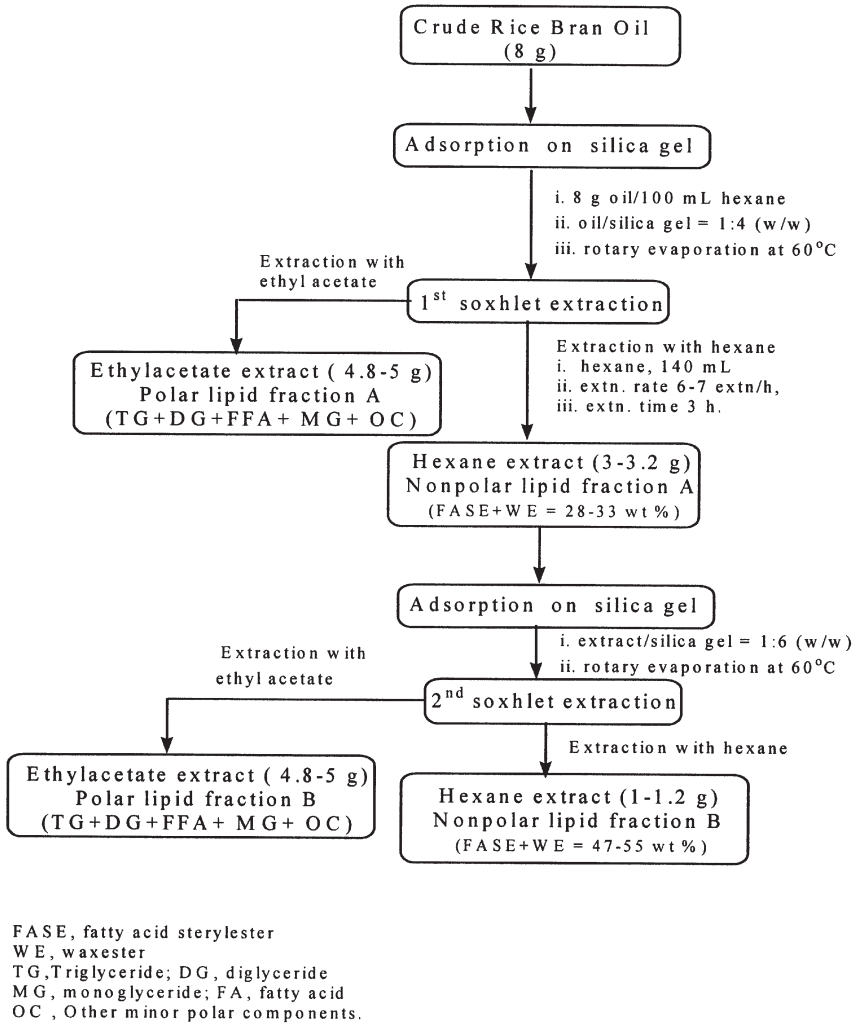


FIG. 1. Extraction and enrichment of WE and FASE from crude rice bran oil (RBO) by modified Soxhlet extraction.

The high m.p. (75–82°C) of these waxes result in the formation of sediment in RBO that can be separated easily by crystallization in a ketonic solvent such as acetone or methyl ethyl ketone (23,26). Significant amounts of phospholipids and saturated compounds were also crystallized with wax such that additional purification steps were required to determine the exact content of WE in crude RBO. In this study, hydrocarbons, FASE, and WE were completely extracted as nonpolar lipid fraction by the modified Soxhlet extraction. The removal of hexane in the nonpolar lipid fraction B followed by crystallization in acetone and washing with acetone yielded a white crystalline wax. The m.p. and purity of the wax were 80–82°C and >99%, respectively, as determined by GC shown in Figure 2. Analysis of the filtrate showed the absence of WE as evidenced by HT-GC. FASE were separated and purified from the filtrate by column chromatography.

WE and FASE content in crude RBO. In rice bran, the moisture content was 10.51 ± 0.89 wt% (mean ± SD, $n = 3$), and the oil content was 16.71 ± 0.86% (oil was extracted by Soxhlet for 3 h with hexane). In RBO, the FFA content was 15.10 ± 2.36 wt%, WE content was 1.33 ± 0.26 wt %, and FASE content was 3.50 ± 0.36 wt % (mean ± SD, $n = 3$). Studies showed a negligible effect of storage on the WE content of rice bran. However, a decrease of about 15–20% in FASE content was observed in RBO extracted from rice bran that had been stored for 3 mon (data not shown), which indicated that FASE were susceptible to deterioration during storage.

Composition of RBO-WE. Figure 2 shows the profile of typical purified RBO-WE, obtained by HT-GC on a DB-5HT (5%-phenyl-95% methylpolysiloxane) stationary phase, which separates major WE on the basis of MW and the degree of unsaturation of WE. The well-resolved peaks shown in Figure 2 represent both even- and odd-numbered WE with carbon number ranging from C_{46} to C_{64}, with C_{54} being the predominant WE. The composition of saponified WE showed that WE consists of long-chain (C_{24}–C_{40}) FAL esterified with long-chain (C_{22}–C_{24}) FA. Triacontanol ($C_{30:0}$) and lignoceric acid ($C_{24:0}$) were the predominant FAL and FA in the saponified RBW,

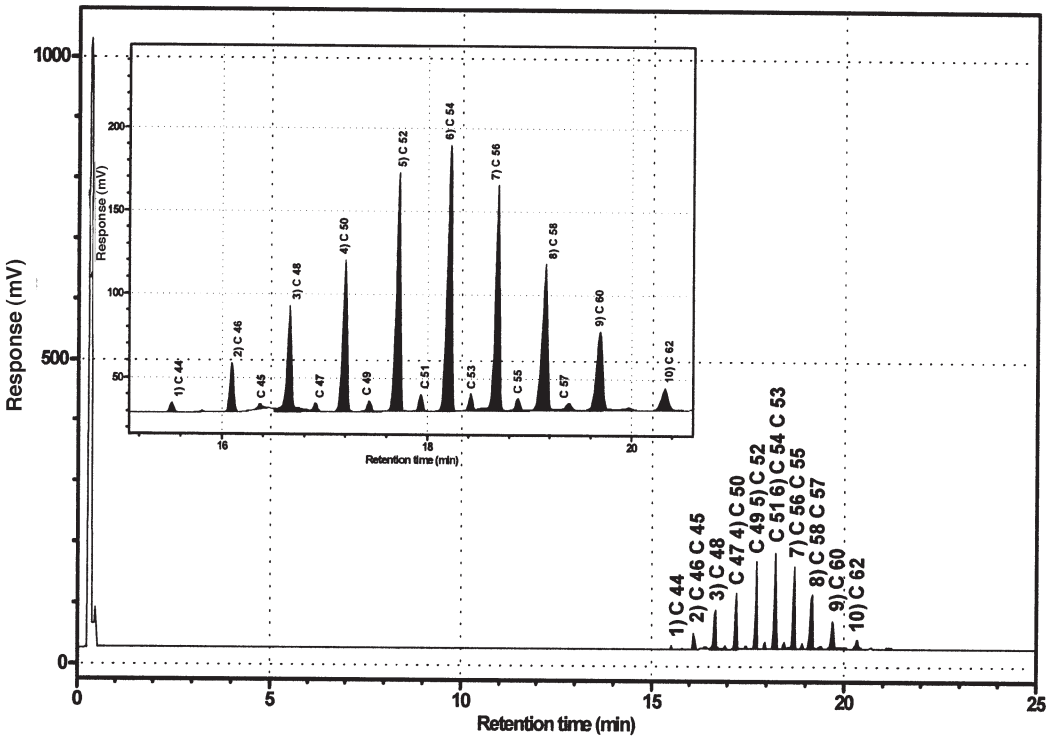


FIG. 2. High-temperature GC (HTGC) analysis of purified WE of RBO. Inset: Enlargement of WE peaks of RBO. For abbreviations see Figure 1.

respectively. The compositions of RBW and its saponified products were similar to that of purified and bleached RBW reported by Vali *et al.* (15).

FA composition of RBO-FASE. TLC of FASE showed a single spot and gave a red-violet color after charring with acidic $FeCl_3$, which is specific for FASE and free sterol (13). The purity of this compound was *ca.* 95% as evidenced by HT-GC. Prior to the determination of the composition of FASE, a small aliquot of the FASE fraction was saponified, and sterol and FA moieties were purified as described in the Materials and Methods section. The major FA of saponified FASE were linoleic acid, oleic acid, and palmitic acid with contents, of 52.8, 38.3, and 4.5%, respectively. The rest were α-linolenic acid (2.3%), stearic acid (0.8%), and 1.2% unknown. These results are in agreement with those reported in the literature (8,10).

Sterol composition of RBO-FASE. The sterol profile as obtained by HT-GC on a DB-5HT (5%-phenyl-95% methylpolysiloxane) stationary column is presented in Figure 3A. Table 1 shows the structure, composition, and mass fragmentation pattern of various sterols in saponified RBO-FASE. All three types of sterol (4-desmethyl, 4-monomethyl, and 4,4-dimethyl sterols) were identified in the sterol fraction of FASE. The major 4-desmethylsterols were identified as campesterol (peak 1), stigmasterol (peak 2), sitosterol (peak 3), and Δ^7 stigmastenol (peak 4). Brassicasterol and cholesterol were not detected in the sterol moiety of FASE. Δ^7 Stigmastenol emerged after sitosterol and appeared as a shoulder on the predominant sitosterol peak. Among these 4-desmethylsterols, the first three sterols (peaks 1–3) had a double bond between carbons 5 and 6 of the ring system and were called Δ^5 phytosterols, whereas Δ^7 stigmastenol (peak 4) had a double bond between carbons 7 and 8 and is called Δ^7 phytosterol (27). The identification of Δ^5 phytosterols was accomplished by comparison with a spectrum from an authentic standard. Other sterols were identified by interpreting the fragmentation pattern of GC–MS data for free phytosterol from the literature (28–31) and also from computerized library matching carried out on the mass spectrum, using the U.S. National Bureau of Standards library, to identify the component. The GC–MS fragmentation of peak 4 (Table 1) showed a molecular ion (M^+) at 414 (relative intensity: 53%) and other ions at 399 ($M^+ - CH_3$), 396 ($M^+ - H_2O$), 381 ($M^+ - [CH_3 + H_2O]$), 273 (M^+ – side chain), and 255 (M^+ – [side chain + H_2O]). Peaks were also observed at *m/z* 231, 229, 95, 81, and 69. These fragmentation patterns were quite similar to those for Δ^7 stigmastenol reported in the literature (28,29). Consequently, peak 4 was recognized as Δ^7 stigmastenol. Peak 5 was identified as citrostadienol, one of the major constituents of 4-monomethyl sterols. The mass spectrum of citrostadienol is shown in Figure 4A. Citrostadienol had a molecular ion (M^+) at *m/z* 426 with other ions at *m/z* 411 ($M^+ - CH_3$) and 408 ($M^+ - H_2O$). The base peak at *m/z* 285 was due to the loss of the side chain (M^+ – SC). Its principal fragmentations were quite simi-

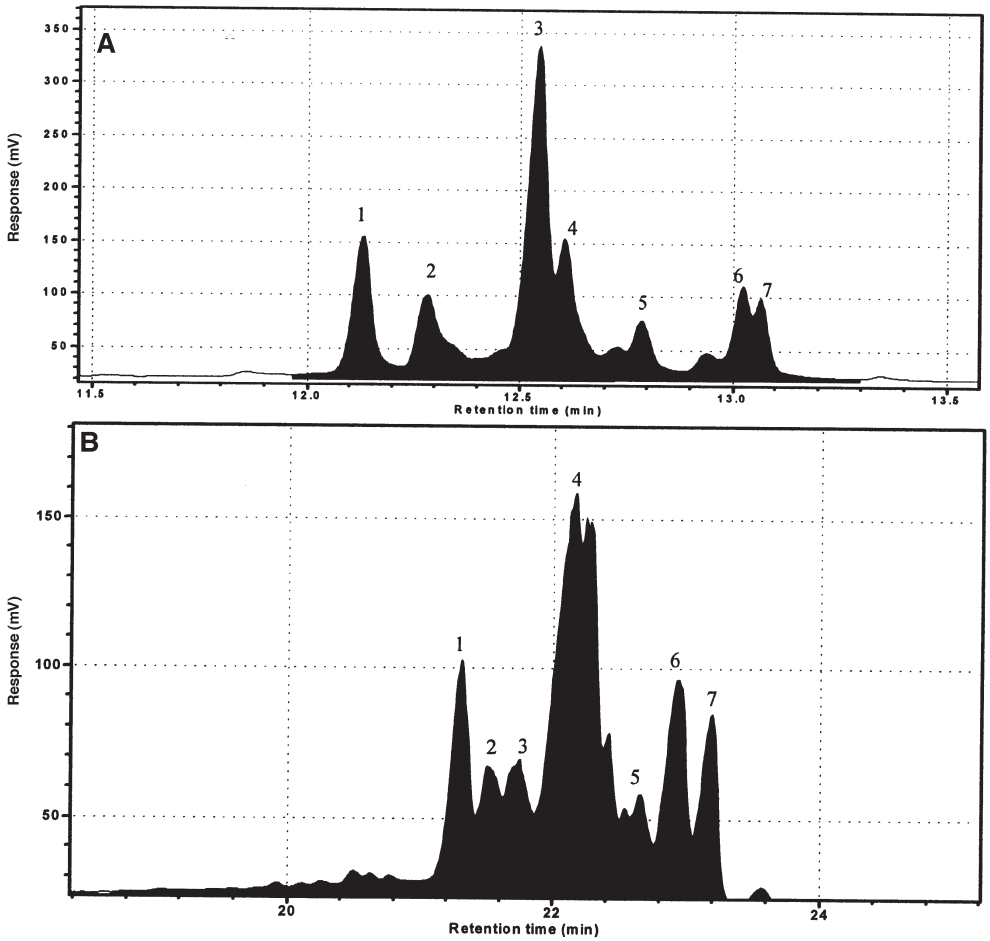


FIG. 3. HTGC analysis of sterol moiety of (A) saponified FASE and (B) unsaponified FASE. For abbreviations see Figures 1 and 2.

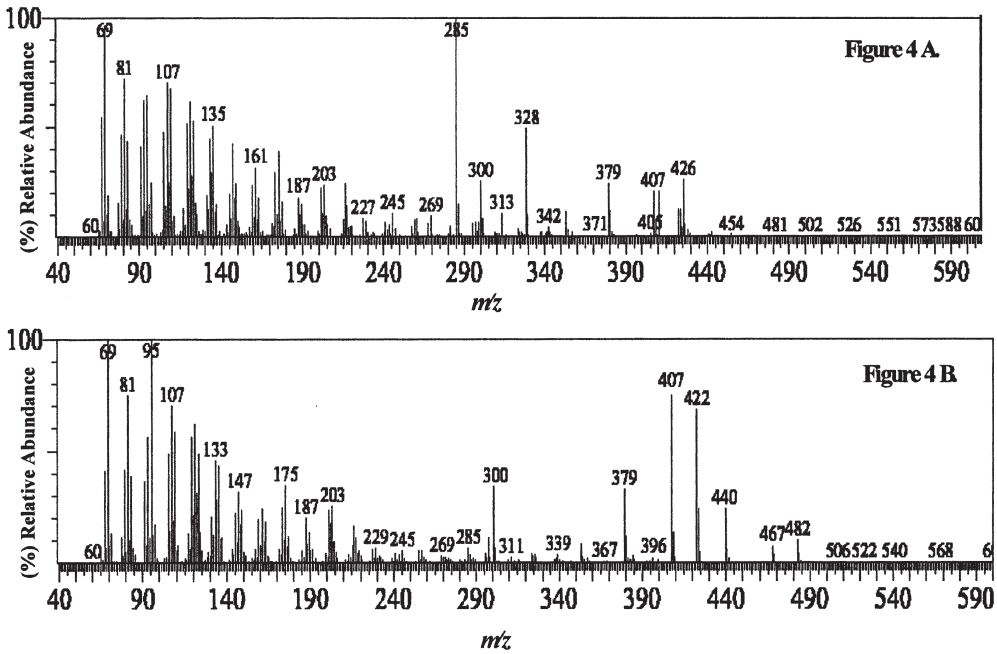
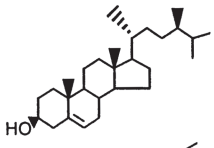
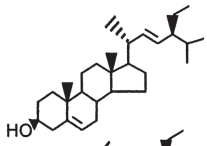
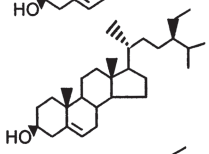
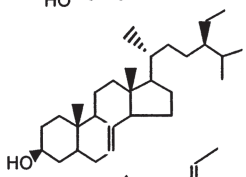
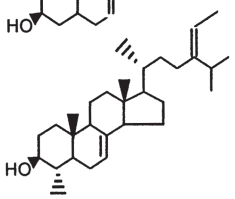
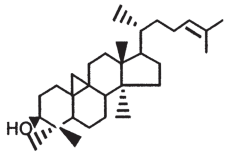
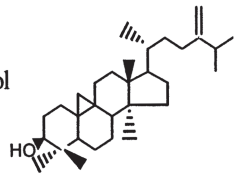


FIG. 4. EI mass spectra of citrostadienol (A) and 24-methylenecycloartanol (B) of saponifed sterol moiety of FASE of RBO. For abbreviations see Figures 1 and 2.

TABLE 1
The Sterol Composition of Saponified FA Steryl Ester of Crude Rice Bran Oil According to GC and GC–MS Analysis

HT-GC Peak no.	Name & M.W	Structure of sterol	Characteristic ions *m/z* (relative intensity (%)
1.	Campesterol (M.W = 400.6)		M^+ 400 (100%), 385 (42%), 382 (52%), 367(45%) 315 (62%), 389 (48%), 274 (7%) 273 (26%), 261 (5%) 255 (16%), 231 (24%), 213 (38%), 145 (25%), 145 (40%), 107 (52%), 95 (48%), 81 (51%).
2.	Stigmasterol (M.W = 412.69)		M^+412 (92%), 397(10%), 394 (10%), 369 (10%), 351 (30%), 300 (45%), 271(67%), 255 (9%), 199 (5%), 151 (11%), 123 (27%), 97 (35%), 83 (100%), 69 (66%).
3.	Sitosterol (M.W = 414.7)		M^+ 414 (100%), 399 (35%), 396 (52%), 381(35%) 329 (55%), 303 (35%), 273 (20%), 231 (14%), 213 (24%), 145 (21%), 107 (28%), 95 (28%), 81 (26%).
4.	Δ-^{7} Stigmastenol (22-dihydrospinasterol) (M.W = 414.7)		M^+ 414 (53%), 399 (43%), 396 (23%), 381 (12%), 360 (14%), 354 (12%) 273 (18%), 255 (37%), 231 (34%), 229 (15%), 95 (81%), 81 (85%) 69 (100%).
5.	Citrostadienol (M.W = 426.7)		M^+ 426 (18%), 411 (17%), 408 (9%), 328 (52%), 342 (3%) 313 (11%), 285 (100%), 269 (9%), 267 (6%), 261 (7%), 245 (10%), 175 (31%), 161 (25%), 147 (35%), 135 (51%),
6.	Cycloartenol (M.W = 426.7)		M^+ 426 (52%), 411 (60%), 393 (43%), 365 (28%), 339 (31%), 339 (10%) 315 (14%), 286 (61%), 203 (15%), 187 (15%), 175 (35%), 95 (62%), 69 (100%).
7.	24-Methylenecycloartanol (Graminasterol) (M.W = 440.7)		M^+ (440, 22%), 425 (5%), 422 (67%), 407 (70%), 379 (33%), 353 (10%), 300 (35%), 216 (17%), 203 (12%), 187 (15%), 175 (31%), 161 (25%), 147 (35%), 135 (51%), 107 (71%), 95 (100%).

lar to those of citrostadienol (MW 426) (28–31). Thus, the 4-monomethylsterol (peak 5) was identified as citrostadienol. The content of 4-monomethylsterols was *ca.* 6–8% of the total sterols. Peaks 6 and 7 were identified as cycloartenol and 24-methylenecycloartanol (graminasterol), respectively; both are 4,4-dimethylsterols. The mass spectrum of 24-methylenecycloartanol is shown in Figure 4B. The characteristic MS fragmentation peaks of 24-methylenecycloartanol were *m/z* 440 (M^+), 425 ($M^+ - CH_3$), 422 ($M^+ - H_2O$), and 407 ($M^+ - [CH_3 + H_2O]$). The peak at *m/z* 315 resulted from the loss of the side chain ($M^+ - 125$) (25,26). The separation between peak pairs such as sitosterol/Δ^7stigmastenol (peaks 3 and 4) and cycloartenol/2-methylenecycloartanol (peaks 6 and 7) in Figure 3A was not improved when other GC temperature programs were tested. The two minor peaks, which eluted before and after citrostadienol (peak 5), were not identified. Analysis of the sterol fraction of FASE from various rice bran samples showed similar chromatographic trends as reflected in Figure 3A with slight variation in their composition. The presence of these sterols, either in free form or esterified with ferulic acid (γ-oryzanol), is common in RBO (10,30).

Composition of intact FASE of RBO. Figure 3B shows a typical HT-GC chromatogram of RBO-FASE. FASE standards were synthesized from the lipase-catalyzed esterification of a

commercially available sterol mixture with FA. These standards were then used to identify the intact FASE (peaks 1–4) through both spiking experiments and comparison of their GC–MS fragmentation patterns. Peaks 5–7 were determined by identifying the sterol and FA moiety in the MS of FASE and when possible, by comparing GC–MS data from the sterol fraction of saponified FASE. The chromatogram shows seven major FASE peaks. The principal FA of saponified FASE were linoleic acid and oleic acid, which accounted for more than 87% of the total FA in FASE. The intense peak (peak 4) was due to sitosterol//Δ^7stigmastenol esters of linoleic and oleic acid. Peak 3 was identified as sitoterol ester of palmitic acid. Peaks 1 and 2 were identified as esters of campesterol and stigmasterol, respectively. The mass spectra of peaks 5, 6, and 7 had the characteristic fragmentation pattern of citrostadienol, cycloartenol, and 24-methylenecycloartanol, respectively. The composition of FASE from RBO was as follows: (i) 4-desmethylsterol esters, peak 1, 12.87 ± 0.86 wt%; peak 2, 5.88 ± 0.63 wt%; peak 3, 7.08 ± 0.77 wt%; peak 4, 50.29 ± 0.95 wt%; (ii) 4-monomethylsterol ester, peak 5, 8.73 ± 0.81 wt%; and (iii) 4,4-dimethylsterol ester, peaks 6 + 7, 15.13 ± 0.8 wt%, where oil was extracted by Soxhlet for 3 h with hexane and the results of HT-GC analyses are mean ± SD (n = 3).

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