A Process for the Preparation of Food-Grade Rice Bran Wax and the Determination of Its Composition

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ABSTRACT: A two-step method was developed for the preparation of food-grade wax. The first step involved the solvent-defatting of crude wax, which gave a dark brown, dry, powdered wax with a m.p. of 75–79°C. The major impurity in the defatted wax was the dark brown resinous matter. In the second step, the resinous matter was removed by bleaching with sodium borohydride in isopropanol. This step yielded a pale yellow, odorless wax with purity higher than 99% and with a m.p. of 80–83°C. The resinous matter was a mixture of aliphatic aldehydes, fatty alcohols, and FA. High-temperature GC analysis of the purified rice bran wax indicated that it contained 11 major and 9 minor types of saturated wax esters. The major and minor peaks contained C_{44}–C_{64} and C_{45}–C_{59} wax esters, respectively. Rice bran wax was mainly a mixture of 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 alcohol portion of the wax esters also contained small amounts of branched and odd carbon number fatty alcohols.

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Wax is an ester of a long-chain carboxylic acid and a long-chain alcohol. In the extraction of rice bran oil (RBO) from rice bran, a certain amount of wax is also obtained. The content of wax in RBO varies with extraction conditions, such as the extraction temperature, the solvent used, and the source and history of the bran. On a total lipid basis, RBO with 3–4 wt% wax has been reported (1). Wax in crude oil is removed by a dewaxing step during the refining process. The refining of RBO usually involves dewaxing, degumming, deacidification, bleaching, and deodorization. Dewaxing is accomplished by cooling the oil, followed by separation of the wax by filtration or centrifugation.

Rice bran wax (RBW) has potential applications in the cosmetic, pharmaceutical, food, polymer, and leather industries. A large number of patents and papers have been cited on the use of RBW in cosmetic preparations such as cold creams, drugs, and hair-conditioning compositions (2,3). In such applications, the performance of RBW is reported to be comparable to carnauba and other waxes (2), and the characteristic physical properties of purified RBW are similar to those of carnauba wax (3). In addition, RBW is a rich source of high-M.W. aliphatic alcohols known as policosanols. Earlier investigators demonstrated the multitude of beneficial therapeutic properties associated with policosanol intake, such as blood lipid-lowering effects (4). In studies conducted by Hernandez *et al*. (5), policosanols from beeswax were used in different pharmaceutical formulations for the treatment of gastric and duodenal ulcers and exhibited substantial anti-inflammatory activity. Hence, RBW is a potential versatile raw material with a multitude of food and nonfood applications, provided a suitable method can be found for its purification.

The exact composition of RBW is still not fully established, and only a few studies about the composition of RBW have been reported. However, results on the composition of RBW have been controversial. Iwama and Maruta (6) fractionated RBW into hard wax (38.5%) and soft wax (11.2%) and found the composition of hard wax to be a mixture of esters of C_{22}, C_{24}, and C_{34} FA and C_{18} to C_{34} fatty alcohols (FAL). Yoon and Rhee (7) reported the presence of a substantial amount of dark brown resin-like matter in both soft and hard wax and only C_{22} to C_{30} FAL and C_{16} to C_{26} FA. Belavadi and Bhowmick (8) concluded that some esters exist in polymeric form involving aromatic moieties. Ito *et al.* (9) reported that RBW contains esters of branched-chain aliphatic C_{32}, C_{34}, and C_{36} FAL. Wang *et al*. (10) reported that refined RBW contains C_{22}–C_{38} FA and C_{14}–C_{38} FAL. The inconsistency of the reports on the composition of RBW is likely due to the lack of an efficient purification method and a suitable analytical technique. The lack of a standard procedure for the purification of RBW also limits its commercial application. Therefore, developing an efficient method for removing the resinous matter (RM) from crude RBW is a worthwhile task.

The presence of RM, a dark reddish-brown substance, is the major culprit responsible for the dark color and offensive odor of crude RBW. Earlier researchers have neither developed an efficient method for the removal of RM from RBW nor determined its chemical nature (6–8,11–13). RM could not be removed by physical adsorption onto clay or charcoal. The existing chemical methods for refining and decolorizing crude wax have been successful to varying degrees. Bleaching agents studied in the past include: (i) a mixture of CrO_3

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and 40% H_2SO_4 (1:1.5 w/w) at 95°C for 2 h (11); (ii) treatment of wax with acid and bleaching with $NaClO_2$ followed by H_2O_2 (12); (iii) degumming with 1% H_3PO_4 and decolorization with 2% H_2O_2 followed by 15% NaClO (13); (iv) solar bleaching followed by H_2O_2 treatment (14); and (v) bleaching with concentrated HNO_3 (15). However, none of these methods are commercially acceptable because of their poor bleaching results, the corrosive carcinogenic chemicals involved, and the long reaction times. In this study a simple, efficient method was developed for the removal of RM from crude RBW. Compositions of RM as well as RBW were also determined.

MATERIALS AND METHODS

Materials. Four crude RBW samples were collected from RBO processing industries around Hyderabad, India. The fifth RBW sample was obtained in our laboratory from RBO sediment. Oil was extracted from rice bran by Soxhlet extraction with hexane. Lipozyme IM 60 (*Rhizomucor miehei* lipase immobilized on a macroporous anion exchange resin) was a gift sample from Novo Nordisk A/S (Bagsvaerd, Denmark). Reagent-grade sodium borohydride ($NaBH_4$, 99.9% pure), hexane, and isopropyl alcohols were procured from S.D. Fine-Chem. Ltd. (Mumbai, India). All solvents were distilled prior to use. Glass silica gel TLC plates (general purpose, 20 × 20 cm, particle size of 250 μm) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Standard FA, FAL, and wax ester (WE) were obtained from Sigma Chemical Company (St. Louis, MO).

Methods. Purification of RBW involved defatting followed by chemical bleaching.

(i) Defatting of the crude RBW. The crude wax sludge contained 20–80% oil content. Crude wax (100 g) was dissolved in hexane (700 mL), and the solution was refluxed at 65°C for 30 min. The content was cooled to 20°C, and the insoluble wax was filtered off. The defatted wax contained impurities such as polar lipids, RM, FFA, and residual oil. The defatted wax (50 g) was dissolved in isopropanol (350 mL) and refluxed at 80°C for 30 min. The contents were allowed to cool to ambient temperature, and the insoluble wax crystals were then filtered off using an Ace Buchner funnel (25–50 μm), which on drying yielded a dark brown, powdery material with a m.p. of 75–79°C.

(ii) Bleaching of the defatted RBW. The defatted wax still contained a substantial amount of RM, which could be removed by bleaching. Typically, 25 g of defatted wax was put in a 500-mL two-necked round-bottomed flask fitted with a reflux condenser, with one of the necks closed with a rubber septum. Isopropanol (175 mL) was added and refluxed at 80–82°C for 30 min. During refluxing, 0.5 g of $NaBH_4$ was added dropwise as a 10% aqueous solution through the rubber septum over a period of 15 min. During the addition of $NaBH_4$, a reddish-brown solid (RM) appeared in the solution. Refluxing was continued for another hour after starting the addition of $NaBH_4$. The contents were cooled to 60°C, and the isopropanol-soluble fraction was removed and then cooled to room temperature. Light yellow crystals of wax were separated from the isopropanol-soluble fraction by filtering through a Buchner funnel, and the filter cake was washed with fresh isopropanol followed by water. After vacuum drying, the cake yielded odor-free light yellow wax crystals with a high m.p. (81–83°C).

(iii) Saponification of the defatted, bleached RBW. A mixture of 0.5 g of RBW with 20 mL of 30% KOH in isopropanol was placed in a 100-mL flask. The mixture was refluxed for 4 h in an oil bath at 100°C. High-temperature GC (HTGC) analysis confirmed that hydrolysis of the WE was complete in less than 4 h. The solvent was evaporated under reduced pressure until the residue was completely dry. Fifty milliliters of ethyl acetate was added to this residue under stirring at 50°C for 2 h. The mixture was carefully filtered, giving a filtrate (FAL) and a solid residue (FA potassium salt). The solid residue was then washed with ethyl acetate (3 × 20 mL). All filtrates were collected and dried over anhydrous sodium sulfate. The composition of FAL was analyzed by HTGC, and the results showed that the unsaponifiable matter contained only FAL. The solid residue was further washed with ethyl acetate (3 × 20 mL) and filtered. The filtrate was discarded, and the solid portion was acidified with 30 mL of 30% HCl for 1 h at 50°C. To this acidified mixture, distilled water (20 mL) was added, and the FA were extracted with ethyl acetate (3 × 20 mL). The combined extract was washed with water to neutral pH, and the ethyl acetate layer was then dried over anhydrous sodium sulfate. FA in the liquid phase were recovered by removing the solvent in a vacuum rotary evaporator. For GC analysis, 0.1 g of FA was converted to FAME by heating with 2 mL of 5% methanolic sulfuric acid at 70°C for 2 h.

(iv) Lipase-catalyzed synthesis of WE. Syntheses of WE standards were performed by the esterification of C_{30} FAL (triacontanol) with long-chain FA (C_{20}–C_{30}). Typically, a mixture of FAL (10 mg) and FA with a molar ratio of 1:1.2 was put into a 7-mL flat-bottomed glass vial sealed with a Teflon-lined cap. The contents were dissolved in 1 mL of *n*-hexane at 50°C and the reaction was started by adding Lipase IM 60 (50% based on the weight of the substrate). The reaction mixture was carried out at 50°C with magnetic stirring at 200 rpm for 8 h. The reaction was monitored by withdrawing aliquots of the reaction mixture (20 μL) at regular time intervals and analyzing them by HTGC.

(v) TLC analysis of RM and WE. Twenty milligrams of RM or WE was dissolved in 1 mL of chloroform, and 10 μL of sample was spotted on a TLC plate and developed in a solvent system of hexane/ethyl acetate/acetic acid (80:20:1, by vol). The bands were visualized under UV light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol and/or by spraying with methanolic sulfuric acid (10%), followed by heating at 120°C. The spots were identified by using authentic standards.

(vi) Chemical analyses. Melting points were determined with a PerkinElmer DSC 7 (Norwalk, CT) equipped with 1020 series Thermal Analysis System software. The heating

rate and the temperature range were 1°C/min and 50 to 100°C, respectively. AOCS methods (16) were adopted for the determination of the following properties: acid value (AOCS Cd 3a-63), iodine value (AOCS Cd 1-25), saponification value (AOCS Cd 3b-76), and phosphorus content (AOCS Ca 12-55). For the determination of the ash content of wax, 5 g of purified wax was taken into a 50-mL porcelain crucible, and the ash content (%) was determined according to an AOCS official method (AOCS Ca 11-55) (16). The RM obtained before bleaching was subjected to color reactions with 2,4-dinitrophenylhydrazine and Benedict's test solution (17). A ^{1}H NMR (200 MHz) spectrum of purified RBW was obtained on a Bruker DPX-200 instrument (Spectrospin AG, Sallenden, Switzerland) in $CDCl_3$ solution with Me_4Si as the internal reference.

(vii) Analysis by HTGC. The FA composition of the saponified WE was determined by GC as described elsewhere (18). The peaks in the chromatogram were identified by using authentic FAME standards. The compositions of WE and FAL were identified by HTGC. 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 the injector and detector were both set at 380°C. The temperature of the column was held at 80°C for 0.2 min, then increased to 380°C at a rate of 15°C/min and maintained at 380°C for 10 min. The split ratio was 1:50, and the carrier gas was nitrogen. Samples (10 mg) were dissolved in 1 mL of chloroform, and 1 μL of sample was injected into the high-temperature gas chromatograph. The identification of C_{24}–C_{30} FAL was achieved by using FAL standards. The equivalent chain lengths (ECN) of C_{44}–C_{60} WE were determined by matching peak retention times with those of synthesized WE standards. ECN of FAL and WE beyond C_{30} and C_{60}, respectively, were tentatively estimated by the relationship between the carbon number and retention time.

RESULTS AND DISCUSSION

Defatting of the crude RBW. Crude wax is dark brown or black in color. The oil content of crude wax varies from 20 to 80 wt%, depending on the separation and processing conditions. Defatting was achieved by successive extraction with hexane and isopropanol. Crude wax (100 g) was first refluxed with hexane (700 mL) for 30 min. The contents were cooled to room temperature and the insoluble wax was filtered off. This step removed most of the oil from the crude wax. After hexane extraction, the wax still contained 5–15% oil and a minor amount of polar lipids. Isopropanol was used as the solvent to obtain pure wax from the RBO settlings (8). In this study, a second extraction with isopropanol was used to remove residual oil and other impurities from the crude RBW. More than 98% of oil in the crude RBW could be removed by this defatting step. The defatted wax possessed sharper m.p., as shown in Table 1. Crude RBW had no sharp m.p. and appeared to be semisolid. This second extraction with isopropanol was necessary to bring the FFA and residual oil contents down to an acceptable level. The quality of the defatted

TABLE 1
Physicochemical Characteristics of Four Crude[a], Defatted[a], and Bleached[a,b] Rice Bran Wax (RBW) Samples

	Wax samples											
	RBW 1			RBW 2			RBW 3			RBW 4		
Characteristic	Crude	Defatted	Bleached	Crude	Defatted	Bleached	Crude	Defatted	Bleached	Crude	Defatted	Bleached
Acid value (10, max)[c], (2–10)[d]	10.6	2.2	1.88[d]	34.5	6.8	2.1	26.3	4.2	1.12	8.8	1.34	82
m.p. (°C) (75–80)[c], (82.5–86)[d]	62–70	76–78	81	65–70	75–77	81	62–69	75–77	82	61–68	77–78	ND[e]
Phosphorus content (ppm)	177	41.6	6.6	220.7	55.4	8.2	167	35.6	ND	80	16.4	ND
Iodine value (20, max)[c], (7–14)[d]	44.8	16.3	8.4	62.0	22.4	6.5	52.7	24.6	6.2	38.9	14.5	8.4
Saponification value (75–120)[c], (78–88)[d]	144.5	92.5	78.4	152.6	101.0	84.6	162.1	105.4	82.6	133.9	89.7	85.5

[a]Results are mean values of three independent determinations.
[b]Bleaching conditions: Defatted RBW (25 g) was refluxed in isopropanol (wax-to-solvent ratio 1:7, wt/vol) at 80°C and bleached by adding 2 wt% (based on wax weight) sodium borohydride as a 10% aqueous solution at 80°C for 1 h.
[c]Food and Drug Administration specification, as mentioned in Reference 21.
[d]Properties of refined carnauba wax, as mentioned in Reference 30.
[e]ND, not detected.

TABLE 2
Compositions (wt%)[a] of Defatted RBW Samples After $NaBH_4$ Bleaching[b]

Sample	Bleached wax	Resinous matter	Isopropanol-soluble
RBO-1	77.3 ± 2.4	16.9 ± 1.1	5.8 ± 0.55
RBO-2	76.8 ± 1.2	18.8 ± 1.6	4.4 ± 0.32
RBO-3	79.0 ± 1.5	15.5 ± 0.8	5.5 ± 0.41
RBO-4	80.62 ± 2.0	16.2 ± 1.2	3.2 ± 0.45

[a]Mean absolute deviation of three independent determinations.
[b]Bleaching conditions: Defatted RBW (25 g) was refluxed in isopropanol (wax-to-solvent ratio 1:7, wt/vol) at 80°C and bleached by adding 2 wt% (based on wax weight) aqueous $NaBH_4$ at 80°C for 1 h. RBO, rice bran oil; for other abbreviation, see Table 1.

wax depends on the efficiency of defatting. Residual oil, if present, interferes with subsequent bleaching and results in poor bleaching action. After defatting, the wax was a reddish-brown powder. Crystals were observed with m.p. higher than 75°C. As can be seen from Table 1, the acid value and phosphorus content of crude wax were four to six times those of the defatted wax. The decreases in iodine value and saponification value of the defatted wax indicated a substantial reduction of acylglycerol and unsaturated oil contents. Thus, defatting removed impurities such as acylglycerols, FFA, and phospholipids and improved the physicochemical properties of the wax, i.e., acid value, iodine value, saponification value, and m.p., as can be seen from Table 1.

Bleaching of the defatted RBW. A dark color and objectionable odor are two major obstacles hindering the use of RBW. The material responsible for the color and odor in the defatted wax is primarily RM, as well as a minor amount of pigment. The removal of RM and other minor impurities from the defatted wax can be achieved by using $NaBH_4$ as a bleaching agent in isopropanol. The use of isopropanol as a solvent was dictated by several considerations. It is a good solvent for effectively reducing the action of $NaBH_4$; it is relatively cheap; it is a poor solvent for long-chain WE at room temperature; it boils just above the m.p. of wax; and its b.p. (82–83°C) is low enough to allow its easy removal from the wax and oil. Moreover, isopropanol is less carcinogenic than chlorinated and aromatic solvents.

The use of $NaBH_4$ as the bleaching agent for RBW is novel and unique. As discussed earlier, physical methods are ineffective, and the chemical oxidative conditions used by earlier workers not only are harsh but also can chemically alter the composition of the wax. Furthermore, problems arising from the use of strong acids can be avoided by using $NaBH_4$ as the bleaching agent. Bleaching with $NaBH_4$ is mild and does not cause any chemical transformation of the wax. $NaBH_4$ is an efficient, water-soluble reducing agent with the following features: (i) It has a low equivalent weight (4.75 g/mol e^-), and 1 mol of $NaBH_4$ can supply 8 mol of electrons; (ii) it has a high reducing power; its redox potential is –1.24 V vs. SHE at pH 14, decreasing to –0.48 V at pH 0; and (iii) $NaBH_4$ redox reaction can take place in different media, such as water and other protonic alcoholic solvents, and under acidic, neutral, and alkaline conditions (19). These properties distinguish it favorably from other reducing agents. Hence, it is most widely used as a bleaching and brightening agent in the pulp and paper industry to bleach the reddish-brown pitch in the pulp. The bleaching action of $NaBH_4$ on cellulose or on lignin involves a reduction of the carbonyl group to hydroxyl groups (20). $NaBH_4$ was used as the bleaching agent in this study. The bleaching effect was more pronounced when the reagent was used in isopropanol. The important feature of using $NaBH_4$ as the bleaching agent is that it achieves an effective separation of RM from the defatted wax. RM was obtained as a reddish-brown solid during the addition of $NaBH_4$. At the end of bleaching, the transfer of the isopropanol solution at 60–65°C, followed by cooling to 20°C, crystallized the WE, which on filtration yielded a pale yellow RBW. After evaporation of the solvent from the filtrate, a light yellow, soft waxy substance was obtained. Analysis of the isopropanol solubles by HTGC and TLC using authentic standards showed that it contained residual WE, TAG, partial glycerides, FA, FAL, and sterols. The content and percent composition of various components of the isopropanol-soluble fraction varied from sample to sample (data not shown). The purity of the bleached wax was higher than 99%, as determined by HTGC. Thus, bleaching of the defatted wax gave three distinct fractions: bleached RBW, RM, and isopropanol solubles. The percentages of these fractions obtained from four defatted RBW samples are presented in Table 2.

The physicochemical properties of four different bleached wax samples are shown in Table 1. The m.p. of the purified wax was 80–83°C. Bleaching also removed the "branny" odor of crude wax and yielded a pale yellow wax. The ash content of the bleached RBW was determined according to an AOCS official method (AOCS Ca 11-55) (16). Each sample was determined in triplicate, and the ash content of various bleached RBW samples was less than 0.06%. The results showed that bleached wax met the specifications of the U.S. Federal Food and Drug Administration Act (21). Furthermore, the properties of the refined RBW were similar to those of refined carnauba wax (22) as shown in Table 1. It has been reported (3) that purified RBW can be used as a substitute for carnauba wax. This is based on the close resemblance of RBW to carnauba wax in hardness and m.p.

Analysis of RM. As shown in Table 2, the defatted wax contained about 15–19% RM. Removal of RM from the defatted wax is of utmost importance to obtain a high-quality food-grade wax. Several papers (6–8,22) have reported the presence of RM, but little is known about its chemical nature and how to remove it efficiently from crude wax. In this work, RM was separated from RBW by reductive bleaching with $NaBH_4$. The physical appearance of RM obtained from different RBW samples was slightly different. A preliminary study of RM was undertaken in this study. For this purpose, RM obtained before the bleaching of RBW was purified by refluxing with isopropanol to remove residual WE. A small portion of this purified RM was subjected to $NaBH_4$ treatment

and then analyzed by TLC, HTGC, and chemical color tests as described in the Materials and Methods section. TLC of RM isolated from the defatted wax before bleaching, when carried out in a solvent system of hexane/ethyl acetate/acetic acid (90:10:1, by vol), showed two weak spots at R_f 0.9 and 0.25 and two strong spots at R_f 0.78 and 0.55. Weak spots at R_f 0.9 and 0.25 matched the R_f values of authentic WE and FAL, respectively. On the other hand, R_f values at 0.78 and 0.55 of dark spots matched the R_f values of standard TG and FA, respectively. HTGC analysis showed peaks corresponding to WE, FA, FAL, and the absence of corresponding TAG peaks. This result indicates that the spot at R_f 0.78 was not related to TAG. Furthermore, additional unknown peaks were observed before and after the FA and FAL regions in the chromatogram. A similar TLC pattern was observed with RM after the $NaBH_4$ treatment. However, the unknown spot at R_f 0.78 became a light band, and the spot at 0.25 corresponding to FAL became more intense. These results suggest that the unknown spot at R_f 0.78 could be carbonyl compounds. Several papers have reported the presence of aldehyde compounds in waxes isolated from various plant sources (23). To confirm the functional group of the spot at R_f 0.78, the characteristic color reaction test for the aldehyde functional group was used. Purified RM gave characteristic color reactions with 2,4-dinitrophenylhydrazine (yellow precipitate) and with Benedict's test solution (green precipitate) (17). These results further suggest that the major component of RM in RBW was aldehyde.

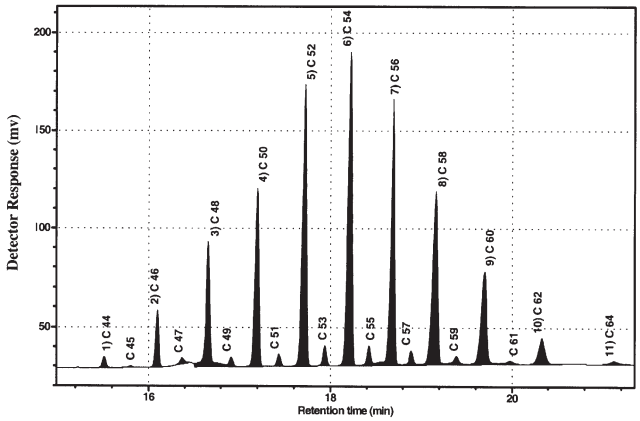

FIG. 1. High-temperature GC (HTGC) analysis of defatted, bleached rice bran wax (RBW) (sample RBW1).

Composition of the defatted, bleached RBW. There are discrepancies in the published reports (6–10) concerning the chemical composition of RBW. These might be due to inefficient purification of the wax, inefficient saponification of RBW, and/or inefficient separation of FAL and FA from the saponified RBW, as well as a lack of suitable analytical methods. An efficient process for the purification and identification

TABLE 3
Wax Ester (WE) Composition of Defatted, Bleached RBW Samples[a,b]

Peak no.	Retention time (min)	WE carbon no.	Composition of WE				
			RBW 1	RBW 2	RBW 3	RBW 4	RBW 5
1	15.51	44	0.557	1.400	0.141	0.361	0.563
2	15.80	45	0.075	0.187	ND	0.0934	0.092
3	16.09	46	2.891	5.389	1.921	2.5077	3.629
4	16.36	47	0.292	0.384	0.117	0.3157	0.390
5	16.65	48	6.583	8.940	3.649	5.5296	7.822
6	16.90	49	0.455	0.586	0.294	0.5507	0.575
7	17.19	50	10.23	11.68	6.978	9.2811	9.975
8	17.42	51	0.636	0.749	0.486	0.8610	0.666
9	17.72	52	16.29	16.21	13.74	15.07	16.04
10	17.93	53	1.046	1.034	0.935	1.087	1.086
11	18.22	54	19.45	18.08	19.96	20.02	19.01
12	18.42	55	0.879	0.952	0.957	1.002	1.073
13	18.69	56	14.99	13.625	17.36	16.15	15.16
14	18.88	57	0.817	0.756	0.874	0.852	0.896
15	19.16	58	12.58	11.12	15.40	13.30	12.39
16	19.38	59	0.731	0.712	0.829	0.851	0.517
17	19.69	60	7.732	6.424	10.26	9.694	7.262
18	19.98	61	0.465	0.340	0.719	0.474	0.229
19	20.36	62	2.840	1.181	4.074	1.503	2.247
20	20.61	63	ND	0.263	0.492	ND	ND
21	21.12	64	0.425	ND	0.806	0.500	0.380
Total even-numbered WE (%)			94.56	94.04	95.10	93.91	94.38
Total odd-numbered WE (%)			5.436	5.964	5.703	6.087	5.524

[a]RBW samples 1–4 were obtained from RBO industries in India, and RBW sample 5 was separated from RBO in our laboratory.
[b]High-temperature GC (HTGC) analysis of each sample was carried out in triplicate, and the results are mean values of three determinations. For other abbreviations see Tables 1 and 2.

of the chemical composition of RBW may help researchers better utilize this RBO by-product. The findings in this study show that development of analytical techniques for the analysis of unhydrolyzed WE is essential to fully understand the composition of RBW. HTGC was used for the analysis of unhydrolyzed WE. A typical HTGC chromatogram of the defatted, bleached RBW (sample 1) is presented in Figure 1. The chromatogram shows 11 major peaks, and in between these peaks are 9 minor peaks. These major peaks account for 93–94 wt% of total WE with even carbon numbers between C_{44} and C_{62}. Saponification of WE resulted in the formation of C_{24}–C_{40} FAL and C_{16}–C_{24} FA. The minor peaks accounted for less than 7 wt% of the total WE with odd carbon numbers of C_{45}–C_{61}, which is the combination of C_{16}–C_{24} FA and C_{25}–C_{39} FAL. Because WE standards higher than C_{44} were not available, the ECN of C_{44}–C_{60} WE were made by matching peak retention times with those of WE standards synthesized in our laboratory. C_{20}–C_{30} FA were esterified with C_{30} FAL (triacontanol) catalyzed by Lipozyme IM 60 lipase using a 1.2:1 molar ratio of FA/FAL in hexane at 50°C. The reaction reached 76.5% conversion in 8 h (data not shown). Under the HTGC conditions described earlier, a good and complete resolution of WE was obtained within 30 min. All RBW sludge samples (four industrial and one laboratory sample), after defatting and bleaching, showed similar chromatographic trends (Fig. 1). The content of WE increased gradually from C_{44} to C_{54} and then decreased to C_{64}, with C_{54} being the most predominant (19–20%) WE, in agreement with that reported by Ito *et al.* (9). The percentage compositions of WE in purified RBW samples are shown in Table 3. The results in Table 3 are similar to the reported WE composition of crude RBO (24), which is a mixture of C_{44}–C_{62} WE, with C_{52} WE being the predominant one. The finding of a high yield of C_{48}–C_{64} esters (>93%) in RBW is significant and has not been reported in other vegetable oils. Furthermore, the low-carbon-chain WE (C_{36}–C_{42}), which are most commonly present in vegetable oil waxes, were not observed in RBW (24). The existence of high-M.W. saturated WE in the RBW is the reason it has a high m.p. and forms a sediment in RBO. The ^{1}H NMR spectrum of purified RBW shows the characteristic peaks of aliphatic saturated esters: δ 0.83–0.9 ppm attributable to a terminal $–CH_3$, δ 1.0–1.75 ppm for methylene $–CH_2–$ protons, δ 2.27 ppm protons for the FA α-methylene, and a triplet at δ 3.9–4.1 ppm for the $–OCH_2–$ of the alcohol residue. The absence of the characteristic sterol moiety signals between δ 0.2 and 1.0 ppm and four methylene protons between δ 4.1 and 4.25 ppm and a methine proton signal between δ 4.9 and 5.1 ppm of the glycerol moiety (25) indicate that the purified wax was free of steryl esters and TAG.

Composition of the saponified RBW. Hydrolysis of RBW with a 30 wt% solution of KOH in isopropanol at 100°C for 4 h resulted in the complete hydrolysis of the WE, as was evidenced by HTGC. The product mixture was evaporated to dryness after saponification. The alcohol portion of the mixture was then extracted with ethyl acetate, while the acid was recovered by subsequent acidification and extraction. Figure 2 is a typical HTGC chromatogram of FAL obtained from saponified RBW (sample 1). Eight major saturated FAL (C_{24}–C_{38}) and a minor amount of odd-numbered FAL in between the even-numbered ones were identified (Fig. 2). In addition, traces of C_{22} and C_{40} FAL were identified in a few samples of RBW FAL. C_{22}–C_{30} FAL were identified using authentic standards. ECN of higher FAL (>C_{30}) were estimated only tentatively by the relationship between carbon number and retention time. Table 4 shows the composition of saponified RBW FAL. Even-numbered FAL accounted for 95–96% of the WE, with triacontanol (C_{30}) being the predominant one (24–27%). Of the saponified RBW FAL, dotriacontanol (C_{32}) ranked second and octacosanol (C_{28}) third, with contents of 17–20 and 12–17 wt%, respectively. Small amounts of branched, long-chain FAL (C_{34}, C_{36}, and C_{38}) were also found. The existence of branched-chain FAL was also reported by earlier authors (9). Thus, the FAL derived from RBW is a mixture of higher aliphatic alcohols (policosanol) between C_{24} and C_{38}, especially with high contents of triacontanol (C_{30}), dotriacontanol (C_{32}), and octacosanol (C_{28}). This natural mixture of FAL has been used, with efficacy, as an active ingredient in various pharmaceutical formulations, with proven anti-inflammatory activity against ulcers and/or as a protector of gastric and duodenal mucosa (5).

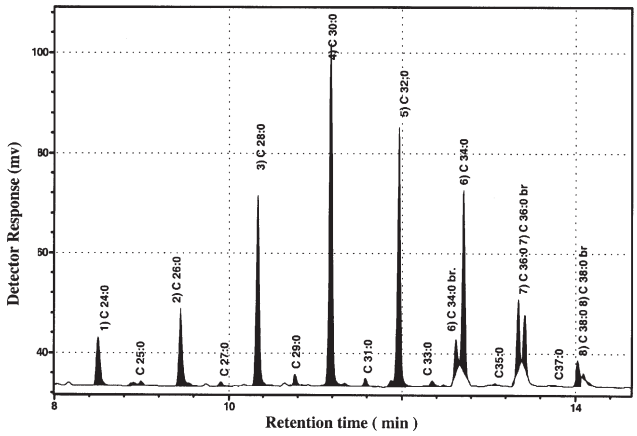

FIG 2. HTGC analysis of saponified RBW fatty alcohols (Sample RBW1). br, branched fatty alcohol; for other abbreviations see Figure 1.

Comparison with authentic reference standards of saturated FA showed that the major composition of RBW was of the long-chain saturated FA, as shown in Table 5. Lignoceric acid (24:0) and behenic acid (22:0) accounted for more than 85% of the total FA. The most abundant was lignoceric acid (62–67%).

In conclusion, an efficient method for the purification of crude RBW was developed in this work, and FA and FAL compositions of the purified wax were determined. The physicochemical characteristics of the purified RBW were comparable to those of carnauba wax, which makes RBW a potential substitute for carnauba wax.

TABLE 4
Fatty Alcohol (FAL) Composition of Saponified RBW[a] Samples[b,c]

Peak no.	Retention time (min)	WE carbon no.	Composition of saponified RBW FAL (%)				
			RBW 1	RBW 2	RBW 3	RBW 4	RBW 5
1	7.397	22	ND	0.338	ND	0.402	ND
2	7.947	23	ND	0.122	ND	0.150	ND
3	8.493	24	4.813	4.087	2.077	1.666	2.263
4	8.987	25	0.425	0.307	0.417	0.450	0.392
5	9.438	26	7.411	6.411	5.552	4.883	6.029
6	9.903	27	0.455	0.546	0.602	0.651	0.590
7	10.33	28	14.37	15.01	14.07	14.38	17.67
8	10.76	29	0.820	0.783	0.911	0.908	0.981
9	11.17	30	23.20	25.58	26.74	24.89	24.08
10	11.57	31	0.705	0.8608	0.730	0.952	0.760
11	11.95	32	18.38	19.58	18.24	18.30	17.04
12	12.34	33	0.650	0.719	0.660	0.700	0.716
13	12.60	34 br[d]	2.750	2.335	4.430	5.770	4.120
14	12.70	34	12.44	12.03	11.41	8.905	10.07
15	13.07	35	0.160	0.131	0.489	0.507	0.502
16	13.34	36	6.075	4.338	3.874	4.555	5.896
17	13.41	36 br	4.008	3.340	6.703	8.047	3.386
18	13.76	37	0.264	0.078	0.333	0.203	0.225
19	14.02	38 br	2.860	3.306	2.263	2.776	4.062
20	14.08	38	0.107	0.097	0.519	0.174	0.150
22	14.75	40 br	ND	ND	ND	0.560	1.069
Total even-numbered FAL (%)			96.41	96.46	95.86	95.30	95.83
Total odd-numbered FAL (%)			3.59	3.54	4.14	4.70	4.17

[a]Samples were defatted and bleached before saponification, as described in the Materials and Methods section.
[b]RBW samples 1–4 were obtained from RBO industries in India, and RBW sample 5 was obtained from RBO in our laboratory.
[c]HTGC analysis of each sample was carried out in triplicate, and the results are mean values of three determinations.
[d]br, branched fatty alcohol; for other abbreviations see Tables 1–3.

TABLE 5
FA Composition of Saponified RBW[a,b] Samples

FA	FA composition (%)				
	RBW 1 FA	RBW 2 FA	RBW 3 FA	RBW 4 FA	RBW 5 FA
14:0	0.342	0.227	0.670	0.305	0.209
16:0	8.021	8.902	10.88	8.694	10.05
16:1	ND	0.400	ND	0.188	ND
18:0	1.882	1.667	2.550	1.877	2.201
18:1	2.044	1.802	1.980	2.099	2.670
20:0	1.255	2.003	3.704	2.572	2.853
22:0	20.25	19.55	20.00	17.12	17.87
24:0	66.20	65.45	60.22	67.14	64.16

[a]Samples were defatted and bleached before saponification, as mentioned in the Materials and Methods section, and values are expressed as mean values of three independent determinations.
[b]RBW samples 1–4 were obtained from RBO industries in India, and RBW sample 5 was obtained from RBO in our laboratory. For abbreviations see Tables 1 and 2.

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