Isolation and Identification of the Causative Factors Responsible for Color Fixation in Rice Bran Oil

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To understand the chemical nature of the dark coloring constituents responsible for color fixation in rice bran oil, crude and dewaxed rice bran oils of 6.8% free fatty acids were fractionated on a silica gel column to get a dark-colored material (0.57% of the oil). Thin-layer chromatographic analysis of the material showed a spot corresponding to monoglycerides, but there were no spots corresponding to other glycerides. It contained traces of phosphorus (<0.1 ppm, which is equivalent to 2.5 ppm phospholipids) and iron (1.3 ppm) that could not be attributed to phospholipids or to any iron-complex. Upon saponification it yielded 12% nonsaponifiable matter. Gas-liquid chromatographic analysis of the saponifiable matter (after acidification and methylation of fatty acids) showed the presence of palamitic, oleic and linoleic acids. Further, on the basis of comparison with spectroscopic data of synthetic monoglyceride, the constituent was characterized to be a mixture of monoglycerides with side chains of oxidized unsaturated fatty acids.

KEY WORDS: Color fixation in rice bran oil, dark color constituent in RBO, oxidized monoglyceride in RBO, rice bran oil, synergist for increasing viscosity of RBO.

Rice bran oil (RBO) has been described as greenish yellow, dark brown and dark yellowish green (1). Refined RBOs that are colorless can be, and are, produced. However, dark yellow/brown/green colors can get fixed in RBO so that it cannot be bleached. Information is fragmented and incomplete as to how colors are fixed in the bleached oil (2,3). In our experience, compared to refined peanut oil, refined RBO produced in India is five times as colored. RBO contains chlorophylls (0–20 ppm), (4), as well as the usual pigments present in vegetable oils, and these should not be difficult to remove by conventional bleaching processes (2,5).

Apart from chlorophylls, RBO also contains wax, partial glycerides and ferulic acid esters (*trans*-4-hydroxy-3-methoxy cinnamic acid esterified with triterpenyl alcohols chiefly cycloartenol and 24-methylene cycloartanol collectively called oryzanol, which are not normally present in other commercially processed vegetable oils. The problem could arise during multistep processing of the oil, such as degumming, dewaxing, refining, bleaching and deodorization, leading to color fixation. The color thereby produced is carried into the finished oil.

While studying RBO refining, we found that a dark coloring constituent was formed during the degumming step, which, after further processing, fixed color to the bleached oil. However, removal of the dark material by the silica gel column percolation method (6) eliminated the color fixation from the bleached oil. With this background, coloring matter was isolated from unprocessed and processed RBO and then characterized, and the results are presented in this paper.

MATERIALS AND METHODS

Materials. Solvent-extracted RBO was obtained from a local factory near Mysore, India. It had a free fatty acid content of 6.8%. The oil was degummed by using phosphoric acid and dewaxed by chilling at 7–10°C as outlined earlier (7). Other chemicals used were monoolein (Acme Synthetic Chemicals, Bombay, India), *trans*-4-hydroxy-3-methoxy cinnamic acid, commonly called ferulic acid (Sigma Chemical Co., St. Louis, MO) and rice bran wax (obtained from RBO as described previously; Ref. 7).

Methods. Isolation of the colored constituents was done by using a previously reported procedure (6), with a larger sample size. The oil (465 g) in hexane (465 mL) was applied to a glass column (4 × 45 cm) packed with silica gel (100 g) (Glaxo Laboratories, Bombay, India) of 60–120 mesh size and eluted successively with hexane (500 mL × 2), diethyl ether (500 mL × 2) and methanol (200 mL). The methanol fraction contained the coloring constituents, and this fraction (after removal of solvent) was further purified on another fresh silica gel column of similar capacity with solvents in the same order as indicated above. The methanol fraction after solvent removal yielded the purified, dark-colored material (DCM).

Analysis of constituents of the DCM. The lipid class separation was performed by the thin-layer chromatographic (TLC) technique on silica gel G layers of 300 μ thickness coated on glass plates with the solvent system petroleum ether/diethyl ether/glacial acetic acid (60:40:1) (7). Monoglyceride separation was also carried out by TLC on 5% boric acid-impregnated silica gel G layers with chloroform/acetone (96:4), according to Thomas *et al.* (8), but only qualitative analysis was performed. The ultraviolet-visible spectrum of DCM was recorded with a Shimadzu UV-240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) (taking 0.1% solution of the sample in cyclohexane), and the $^{1\%}E_{1cm}$ value was calculated. The infrared spectrum was recorded on a Jasco IR spectrometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan) by smearing about 10 mg sample onto sodium chloride discs. The nuclear magnetic resonance (NMR) spectrum of the compound, dissolved in deuterated chloroform ($CDCl_3$) with tetramethylsilane as internal standard, was recorded with an EM-390 90MHz NMR spectrometer (Varian Instrument Division, Palo Alto, CA).

The fatty acid (FA) composition of DCM was determined by the following gas-liquid chromatographic (GLC) procedure. After saponification of DCM (0.2 g) with 1 N alcoholic potassium hydroxide, the nonsaponfiable matter was isolated (9). The saponifiable matter was acidified, and the FAs were isolated. The FAs were converted into methyl esters (FAME) with diazomethane (10). These were analyzed with a Shimadzu GC-9A instrument equipped with flame-ionization detector (FID) and stainless steel column (152.4 cm × 3.17 mm) packed with 20% diethylene glycol succinate (DEGS) supported on Chromosorb W (80–100 mesh). The column temperature was 180°C, the injection port and FID were 210°C and the nitrogen flow rate was 40 mL/min. The individual FAs were identified by comparing the retention times of sample FAMEs with those of standard FAMEs. The peak area and relative percentage of FAMEs were obtained with a Shimadzu C-R3A Chromatopac printer-plotter system. Standard

methods were followed for the determination of iron (11), phosphorus (12) and peroxide contents (13) in DCM.

RESULTS AND DISCUSSION

The DCM was isolated from crude and dewaxed RBOs of 6.8% free fatty acids (FFA) in a silica gel column. Its characteristics are reported in Table 1. The DCM isolated from dewaxed oil was a viscous semi-solid, whereas the lighter material found in crude oil was less viscous. These DCMs were soluble in chloroform, petroleum ether, cyclohexane and acetone, and were insoluble in methanol. However, they could be eluted out of the silica gel column with methanol. These materials were 97.5% acetone-soluble and only 2.5% acetone-insoluble, indicating that they were neither waxes nor phospholipids. Also, the phosphorus contents of DCMs were low (<0.1 ppm of phosphorus equivalent to 2.5 ppm of phospholipids).

Upon saponification, DCM yielded 12–15% unsaponifiable matter and 73–88% FA from saponifiable matter, indicating that the DCMs are esters of glycerol. GLC analysis of FAME prepared from the saponifiable matter showed palmitic, oleic and linoleic acids in various proportions.

TLC analysis of DCM showed the presence of spots corresponding to monoglycerides, however, no spots were observed that corresponded to other glycerides. Further separation of the monoglycerides by boric acid-impregnated silica gel TLC indicated that the sample was a mixture of *sn*-1 (R_f = 0.05) and *sn*-2 (R_f = 0.1) monoglycerides in the case of crude oil, but the dewaxed oil contained only *sn*-1 monoglycerides [it is probable that the *sn*-2 monoglyceride may have undergone either a chemical change into a compound that has a similar R_f to that of *sn*-1 monoglyceride (see section on oxidation of monoglyceride) or isomerization into an *sn*-1 monoglyceride, or both].

The DCMs isolated from crude and dewaxed oil showed absorption at 258 nm in the ultraviolet region. The spectral characteristics of the compounds are also given in Table 1. The $^{1\%}E_{1cm}$ value for the compounds are low and, therefore, they could not be ferulic acid esters (oryzanol) because the value is 360 at 314 nm for oryzanol (14). The infrared (IR) spectral bands at 3400, 2900 and 1735 cm^{-1} indicate that the molecules contain -OH groups (hydrogen bonded), $-CH_2-$ linkage and >C=O (ester) groups. All these groups are present in a monoglyceride. Therefore, IR spectra for pure monoolein (synthetic) was taken and superimposed on the IR spectra for DCM isolated from RBO (Fig. 1). A small shoulder peak at 3000 cm^{-1} representing stretching vibration of *cis*-double bonds found in monoolein (as well as in the lighter DCM isolated from crude RBO) was absent in DCM isolated from dewaxed RBO. Also, the number of hydroxyl groups seemed to be much greater than in synthetic monoolein, probably due to oxidation. The IR spectra of monolinolein and DCM agreed well (15) except for the absence of *cis*-double bonds in the latter sample. Also, the IR spectra of rice bran wax and ferulic acid did not show similarity with the compound under investigation. Therefore, rice bran wax and ferulic acid esters (oryzanol in RBO) were not responsible for dark color formation in RBO.

The NMR spectra showed a pattern that was similar to that of a glyceride (16) having absorption for -OH (δ = 1.6, 2.1 and 2.4), -CH=CH- (δ = 5.5), $-CH_2-$ (δ = 1.325, 3.75 and 4.25) and $-CH_3$ (δ = 1.00) group protons (15–17) (Fig. 2). It resembled the spectra for synthetic monoolein except for a reduction in peak height for $-CH_2$ and -CH=CH- protons. The NMR spectra of monoglyceride partly agreed with the spectral characteristics reported in the literature for monopalmitin (17) and monolinolein (15). Probably, this could be due to the presence of three monoglycerides (monopalmitin, oxidized monoolein and oxidized monolinolein, as determined by GLC of the FAME from DCM isolated from dewaxed RBO; Table 1). Once again, the NMR spectra gave a clear indication that ferulic acid (or its esters *viz.*, oryzanol in RBO) was not responsible for dark color formation in RBO.

The GLC analysis of FA (as FAME) of DCM isolated from dewaxed RBO showed the presence of only palmitic acid. Oleic and linoleic acids were absent (Table 1) (compared to the fatty acid composition of DCM isolated from crude RBO). This indicates that oleic and linoleic acids containing monoglycerides present in crude RBO are getting oxidized during processing of RBO and, therefore, were not found during GLC analysis. This could be the reason for reduction of peak height for $-CH_2$ and -CH=CH- protons in the NMR spectrum for DCM isolated from dewaxed RBO, as compared to the NMR spectrum of synthetic monoolein. Further proof was obtained by determining the peroxide value of DCM, which was 69 milliequivalents (meq.) of oxygen/kg for material isolated from dewaxed RBO, which is much higher than the 11.4 meq. of oxygen for DCM isolated from starting crude

TABLE 1

Chemical Characteristics of the Dark-Colored Material of Rice Bran Oil (RBO)

Characteristics	Dark-colored material isolated from	
	Crude RBO	Dewaxed RBO
Content in RBO (%)	0.56	0.58
Phosphorus content (ppm)	—	<0.10
Iron content (ppm)	—	1.30
Peroxide value (meq. O_2/kg)	11.4	69.1
Nonsaponifiables (%)	15.0	12.0
Fatty acids from saponifiable matter (%)	85.0	73.0
TLC (qualitative)	MG[a] (*sn*-1, *sn*-2)	MG (*sn*-1) only
Fatty acid composition by GLC of FAME (relative %)		
$C_{16:0}$	36.5	83.1
$C_{18:1}$	25.4	—
$C_{18:2}$	36.6	—
Ultraviolet absorption, $^{1\%}E_{1cm}$ at 258 nm	15.6	28.3
IR absorption (for both samples)	3400, 2900, 1735 cm^{-1}	
NMR chemical shift[b] (δ ppm values for dewaxed RBO)	1.00(*d*), 1.325(*s*), 1.6(*s*), 2.1(*s*), 2.4(*d*), 3.75(*s*), 4.25(*d*), and 5.5(*t*).	

[a]Approximately 1:1 ratio.

[b]The letters *s*, *d* and *t* indicate that the NMR spectral pattern had one (singlet), two (doublet) or three (triplet) peaks for the respective delta value. TLC, thin-layer chromatography; GLC, gas-liquid chromatography; FAME, fatty acid methyl esters; IR, infrared; NMR, nuclear magnetic resonance. MG, monoglycerides.

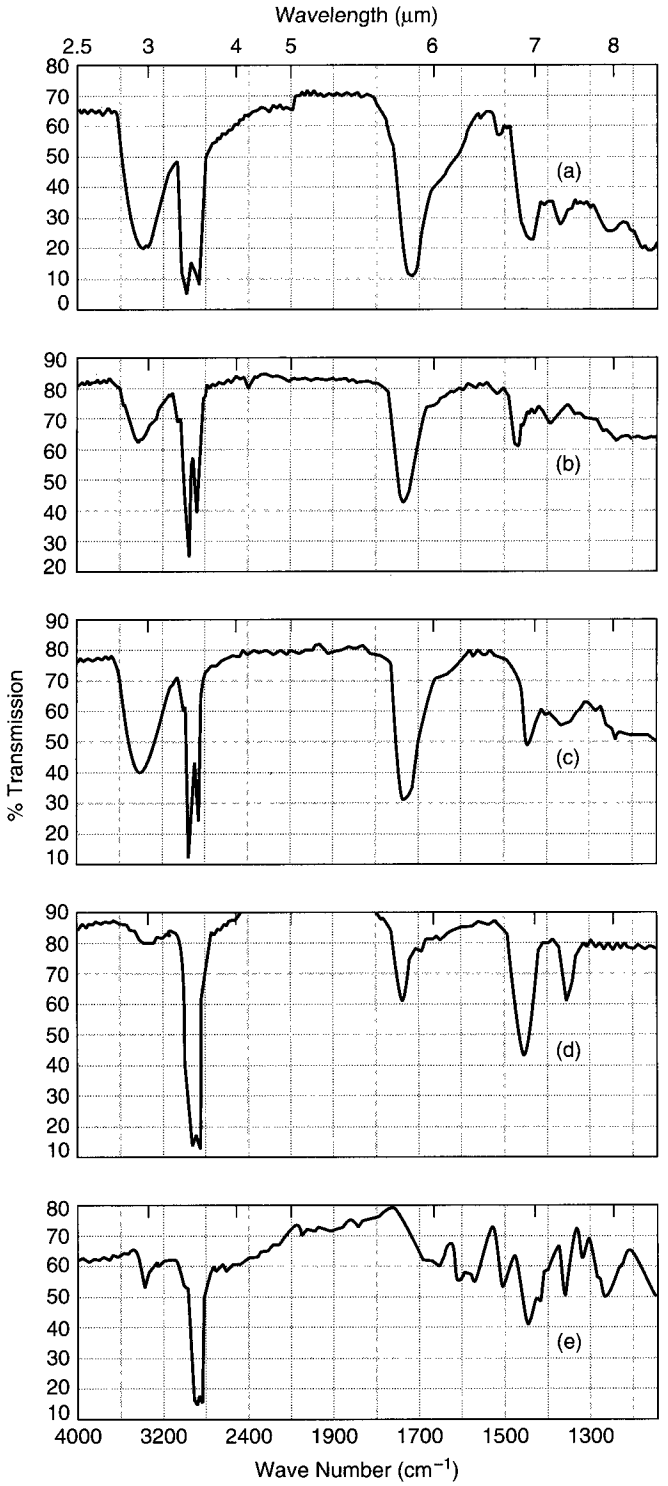

FIG. 1. Infrared spectra of dark-colored constituents of rice bran oil (RBO); (a) dark-colored material from dewaxed RBO, (b) dark-colored material from crude RBO, (c) synthetic monoolein, (d) rice bran wax and (e) synthetic ferulic acid.

RBO. All these results showed that darkening of RBO was a result of oxidation of the monoglycerides present in crude oil during the degumming step, which has not been reported so far in the literature. Further characterization of the oxidized monoglycerides was not attempted.

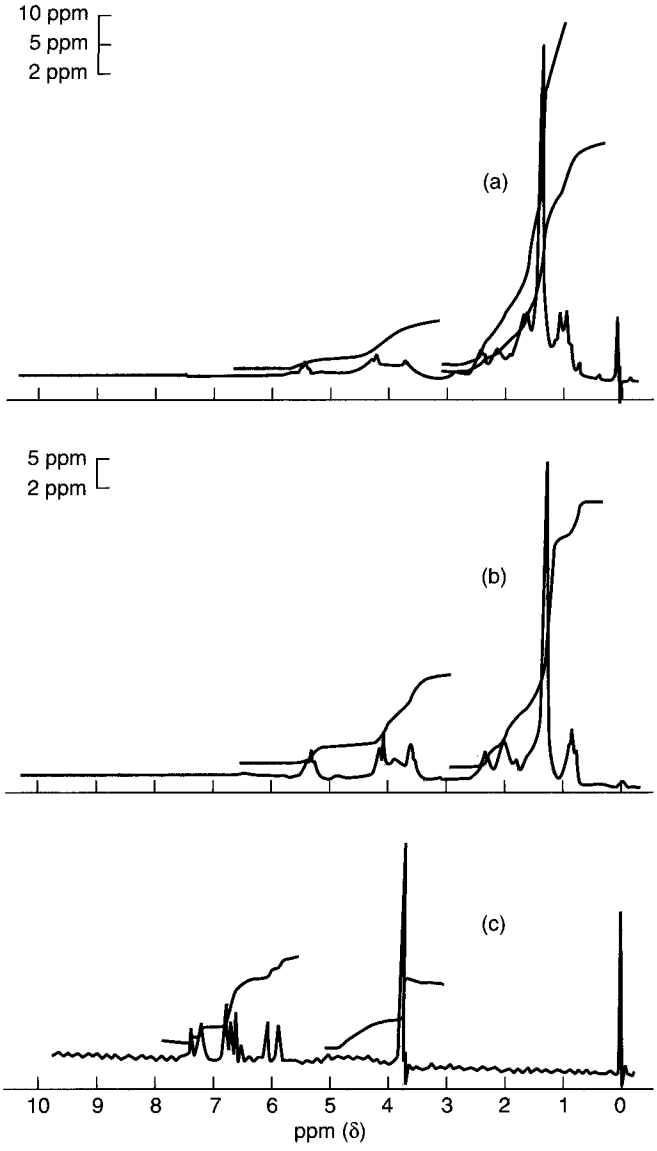

FIG. 2. Nuclear magnetic resonance spectra of dark-colored material from rice bran oil (RBO); (a) dark-colored material from dewaxed RBO, (b) synthetic monoolein and (c) synthetic ferulic acid.

It has been shown by Mistry and Min (15) that monolinolein acts as a prooxidant in soybean oil. The rate of oxidation of monoglyceride in a mixture of other glycerides might be faster than that of triglycerides and diglycerides. The rate of oxidation of *sn*-1 and *sn*-2 monoglycerides of oleic and linoleic acids could also be different. *sn*-2 Monoglycerides are probably more prone to oxidation than *sn*-1 monoglycerides because the DCM isolated from dewaxed RBO contained only *sn*-1 monoglycerides (Table 1), whereas crude RBO contained both *sn*-1 and *sn*-2 monoglycerides. From the oxidation behavior of linoleic and oleic acids, it may be argued that linoleic acid monoglycerides may oxidize more rapidly than the oleic acid monoglycerides in RBO. As both oleic and linoleic acids were absent in the fatty acid composition of DCM isolated from dewaxed RBO, it is not possible to give an explanation based on the oxidation behavior of individual acids alone. Information is lacking on this aspect.

The DCM isolated in this case was not an iron-complex, arising from a reaction of iron with either glycerides/waxes/phospholipids/ferulic acid esters (oryzanol), because the iron content of DCM was just 1.3 ppm, and this amount of iron is generally present in all oils and fats.

The results of these studies suggest: (i) removal of the monoglycerides from crude/dewaxed RBO eliminates the color fixation from the bleached oil (6); (ii) removal of the monoglycerides from crude RBO helps in a faster rate of wax-settling (Gopala Krishna, A.G., unpublished data); (iii) removal of color and wax from RBO may be enhanced by minimizing the formation of monoglycerides or eliminating them by proper processing.

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