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Characterization of wax esters, free fatty alcohols and free fatty acids of crude wax from sunflower seed oil refineries ☆

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Abstract

The sunflower seed wax (10–12%) from oil refineries was purified through two steps, namely, extraction using solvents and precipitation with chilled acetone. Fatty esters, free fatty alcohols and free fatty acids were separated by thin-layer chromatography (TLC). The fractions of fatty esters and free fatty alcohols were separated and identified by gas chromatography (GC) using a Dexil-300 column and OV-101 column, respectively, at temperatures from 150–290 °C and were further confirmed by Gas chromatography–mass spectrometry (GC–MS), using a capillary silica column SPB™-1 coated with polydimethyl siloxane. Among the long chain fatty esters (C_{38}–C_{54}), comprised of one unit of fatty alcohol and one unit of fatty acid through an ester linkage, the major esters were C_{40}–$C_{44,}$ of which C_{42} was predominant. These fatty esters, upon hydrolysis, followed by methylation and subsequent GC–MS analysis, showed the presence of C_{16}–C_{30} fatty acids in the bound form, whereas methylation of native wax indicated the presence of C_{18}–C_{30} fatty acids in the free form. Acetylation of hydrolysis product showed the presence of C_{18}–C_{30} fatty alcohols in the bound form, where as acetylation of native wax indicated that the C_{18}–C_{32} fatty alcohols were in the free form. *n*-Triacontanol (C_{30}), a plant growth regulator, was found in both free and bound forms.

Keywords: *Compositae*; *Helianthus annuus*; Sunflower seed oil; Crude wax; Fatty esters; Free fatty alcohols; Free fatty acids; Gas chromatography–mass spectrometry

1. Introduction

Sunflower (*Helianthus annuus*), belonging to the family *Compositae*, is a major oilseed, used for the production of edible oil, with an annual production of 25.1 million tons (FAO, 1996). The varieties that are grown in India have a high oil content (46–51%) while the hull content varies from 24.0% to 32.0% (Earle, Vatten, Clark, & Wolff, 1968). The hull possesses 2.5–3.0% wax, which is composed of long chain fatty esters, free fatty acids and free fatty alcohols. Though the wax is present in all parts of the seed, a high content of wax is found in the hull portion (Morrison, Akin, & Robertson, 1981).

Natural waxes contain a wide variety of simple lipid components, such as hydrocarbons, esters, fatty alcohols, ketones, mono-, di-, tri-acylglycerols and sterol esters (Kolattukudy, 1976). Thin-layer chromatography, using silica gel, has proved to be the method of choice for separating these components (Holloway & Challen, 1966; Hu, Daun, & Scarth, 1993). A few reports are available on composition of sunflower seed wax from the seed hull and tank settlings of the oil extraction industry (Gracian & Arevalo, 1980; Henon, 1986; Kawanishi, Aoki, Hashimoto, & Matsunobu, 1991; Kiosseoglou & Boskou, 1990; Kleimann, Earle, & Woff, 1969). Henon (1986) suggested the separation of wax esters from sunflower oil on a mixed column of silica and silica impregnated with silver nitrate and reported C_{16}–C_{32} fatty acids and C_{16}–C_{32} alcohols in sunflower seed wax.

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Lawrence, Iyengar, Page, and Conacher (1982) reported a method for characterization of wax esters, alcohols and fatty acids of some of the commercial waxes (rice bran, carnauba, sugarcane and yellow bee wax) by gas liquid chromatography (GLC). Rivarola, Anon, and Calvelo (1985) reported that sunflower seed wax contained long chain fatty acids, from $C_{22:0}$ to $C_{30:0}$. Saturated acids of C_{22} and above were found to be present in shell lipids of sunflower seed (Kalistratova, Meerov, & Scherbakav, 1974). Presence of *n*-triacontanol in sunflower seed wax was reported earlier from this laboratory (Sindhu Kanya, Krishna Murthy, & Shamanthaka Sastry, 1993).

A few researchers (Henon, 1986; Liu, Przybylski, & Eskin, 1993) employed thin-layer chromatography, gas chromatography and vapour phase chromatography for studying the components of wax esters. However, there were no reports on the characterization of wax from sunflower seed oil refineries, wherein several treatments are given during the refining process. The present investigation was aimed at isolation and purification of wax obtained from sunflower oil refineries by preparative thin-layer chromatography and characterization of each individual group of compounds, such as long chain fatty esters, free fatty alcohols and fatty acids, by GC and confirmation of components by GC–MS for understanding the structural changes during the refining process.

2. Materials and methods

2.1. Materials

The standards of esters, alcohols, acids and hydrocarbons were procured from Sigma Chemical Co., USA. The crude wax was obtained from sunflower seed oil refineries located at Pochampalli, Tamil Nadu Agro Industries Ltd., Tamil Nadu, India. The hull of sunflower (*Helianthus annuus*) seed var. Morden, was obtained by dehulling of whole seeds using a centrifugal sheller with a standard processing method (Shamanthaka Sastry, 1978). After dehulling, the hull fraction was separated from kernels by an air classifier. The wax isolated from the seed hulls in the laboratory is considered as the control sample.

2.2. Extraction of wax from the crude wax obtained from oil refineries

A known quantity of crude wax (5 kg) was extracted with hexane (1:4 w/v) in the cold, followed by chloroform (1:4 w/v) extraction. The extract was concentrated (4-fold) and precipitated with chilled acetone (1:1). The precipitated wax (520 g) was collected by filtration and air-dried (Sindhu Kanya & Shamanthaka Sastry, 2003; Sindhu Kanya, Udaya Sankar, & Shamanthaka Sastry, 2003).

2.3. Extraction of wax from seed hulls

The hulls were dipped in chloroform for a short period (2–3 min) of time at room temperature (28 °C) and the extract was concentrated and precipitated. The precipitated wax was collected by filtration and air-dried (Sindhu Kanya, 1998).

2.4. Separation of fatty esters, free fatty alcohols and free fatty acids of sunflower seed wax by thin-layer chromatography

The wax extracted and precipitated as above was subjected to TLC to separate it into various fractions, such as fatty esters, free fatty alcohols and free fatty acids. Separations were done on 20 × 20 cm glass plates coated with a silica gel (Glaxo, Mumbai) layer of 0.25 mm thickness. Wax dissolved in chloroform was applied onto the plates using capillary tubes. The plates were developed using the solvent system toluene:chloroform (7:3). Fractionation of these constituents was achieved, based on earlier reported procedures (Holloway & Challen, 1966; Hu et al., 1993). Spots were visualized by placing the plate in a chamber, saturated with iodine for identification of spots. The spots were identified with simultaneous running of authentic standards. The preparative TLC was carried out for the separation and elution of individual fatty esters, fatty alcohols and fatty acids. The separated constituents were eluted with chloroform from adsorbent and dried under a stream of nitrogen.

2.5. Gas chromatography of wax esters

The separation of individual fatty esters from the fatty ester fraction of TLC was carried out on a Shimadzu gas chromatograph equipped with a flame ionization detector, fitted with Dexil-300 (2%) coated with a carborane siloxane S.S column, 2 ft. long (i.d. 1/8 in.). Oven temperature was programmed as 150–290 °C at a rate of 4 °C/min. The injector and detector temperatures were 290 °C and 300 °C, respectively. Identification was based on comparison with authentic standards (Table 1).

2.6. Gas chromatography of free fatty alcohols

The separation and identification of free fatty alcohols fraction was carried out using a 3% OV-101 S.S column, 0.9 m long and 0.3 cm diam. The oven temperature was

Table 1
Composition of wax esters by GC

Compound[a]	Crude wax (%)	Hulls (%)
C38	2.31	2.12
C40	17.0	17.0
C42	22.0	22.9
C44	15.6	15.5
C46	10.9	11.0
C48	9.88	9.85
C50	10.5	10.5
C52	10.4	10.4
C54	Trace	Trace

[a] Identified by authentic standards.

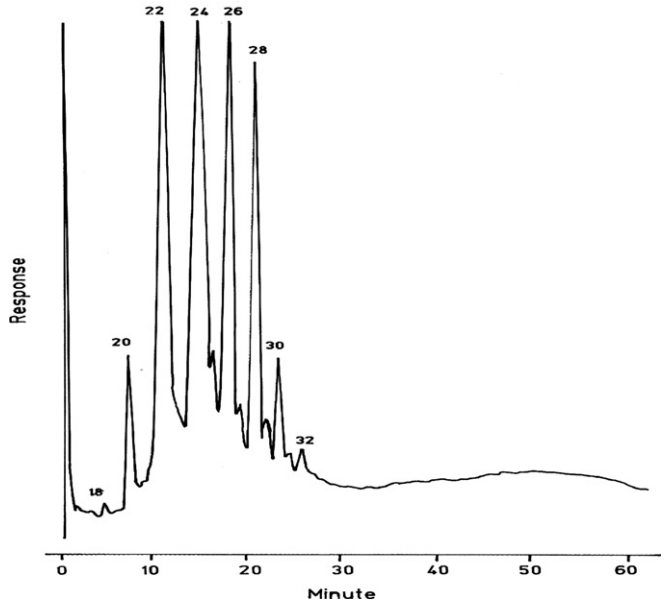

Fig. 1. GC profile of free fatty alcohols.

programmed from 150–290 °C at a rate of 10 °C/min. The injector and detector temperatures were 290 °C and 300 °C, respectively. The nitrogen flow was 30 ml/min. Identification was based on comparison with authentic samples (Fig. 1).

2.7. Preparation of fatty acid methyl esters

Methylation was carried out according to the procedure of Christie (1982). To the free fatty acid fraction from TLC, 0.7 N HCl in methanol (1 ml), hexane (2 ml) and water (2 ml) were added, mixed thoroughly and centrifuged to separate phases. The hexane layer was collected and dried under a stream of nitrogen. To the dried sample, 2 ml of boron trifluoride in methanol were added and kept at 85 °C for 10 min, then cooled to room temperature; hexane and water were added and the hexane layer was collected. Nitrogen was passed to evaporate the solvent and further separation was carried out by gas chromatography.

2.8. Gas chromatographic separation of fatty acids as methyl esters

Separation and identification of methyl esters of free fatty acids was carried out using a 15% DEGS on Chromosorb W-80-100 SS column, 2.4 m long and 0.3 cm diam. The separation was conducted at an isothermal temperature of 190 °C with 30 ml/min nitrogen flow. The injector and detector temperatures were 200 °C and 210 °C, respectively. The identification was based on comparison with authentic standards (Fig. 2).

2.9. Saponification of wax esters

Fatty esters obtained as a TLC fraction were saponified by the modified procedure of Morrison and Smith (1964).

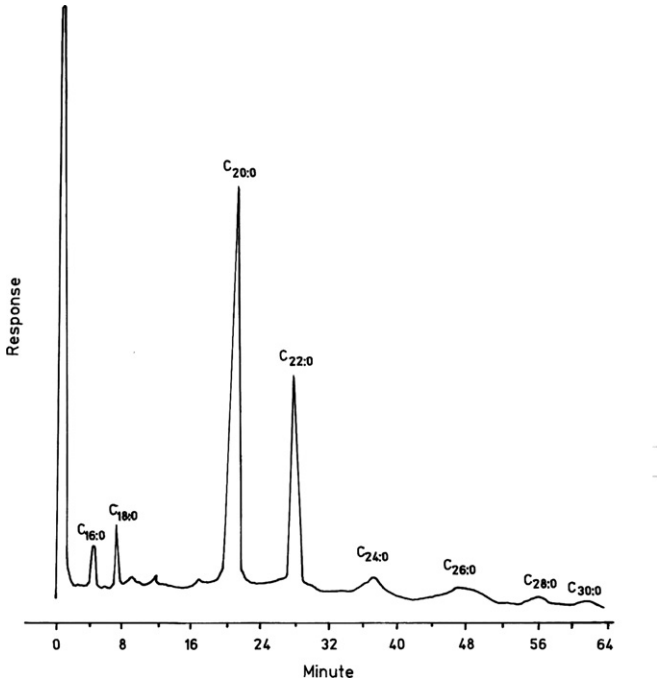

Fig. 2. GC profile of free fatty acid methyl esters.

The fatty ester fraction (0.25 g) was dissolved in benzene:methanol (1:1, 10 ml), followed by addition of 95% ethanol (50 ml) and 60% aqueous potassium hydroxide (3 ml) and refluxed for 6 h. The saponified product of fatty esters was evaporated to dryness.

2.10. Acetylation and esterification

Acetic anhydride (1.0 ml) and anhydrous pyridine (1.0 ml) were added to the saponified residue. The contents were well mixed and kept overnight at room temperature. The product was precipitated with the addition of sufficient water. The acetylated product was filtered and dried. The product was subjected to esterification with boron trifluoride in methanol (2 ml) and kept at 85 °C for 10 min and evaporated to dryness. The residue was dissolved in chloroform for analysis by GC–MS.

2.11. Gas chromatography and mass spectrometry (GC–MS)

Free fatty alcohols (Table 2), free fatty acids as methyl esters (Table 3) and fatty alcohols as acetates, fatty acid methyl esters of saponified wax (Table 4) were analysed and identified using Shimadzu GC-17A Gas chromatograph equipped with QP-5000 (Quadruple) mass spectrometer. A fused Silica Column SPB™-1 (30 M × 0.32-mm i.d., film thickness 0.25 μm), coated with polydimethyl siloxane was used. Helium was the carrier gas at a flow rate of 1 ml/min; injection and detector temperature were 250 °C. The oven temperature was programmed 150–290 °C by raising the temperature at the rate of 3 °C/min. Compounds were identified by comparison of fragmentation patterns in mass

Table 2
Composition of free fatty alcohols by GC–MS

Compound	Molecular weight	Crude wax (%)	Hulls (%)
Stearyl alcohol[a]	270	0.2	0.3
Arachidyl alcohol[b]	298	2.3	1.3
Behenyl alcohol[a]	326	15.6	19.2
Lignoceryl alcohol[b]	354	34.8	33.2
Cerotyl alcohol[a]	382	26.8	25.6
Montanyl alcohol[a]	410	9.2	9.3
Melissyl alcohol[a]	438	3.9	4.1
Domelissyl alcohol[b]	466	2.2	2.5

[a] Identified by authentic standards.
[b] Identified by RT and MS.

Table 3
Composition of methyl esters of free fatty acids by GC–MS

Compound[a]	Molecular weight	Crude wax (%)	Hulls (%)
Methyl palmitate	270	2.6	2.2
Methyl stearate	298	2.3	2.0
Methyl arachidate	326	46.7	50.2
Methyl behenate	354	25.8	28.3
Methyl lignocerate	382	7.5	6.6
Methyl cerotate	410	6.6	4.3
Methyl montanate	438	6.8	3.2
Methyl melissynate	466	3.4	2.4

[a] Identified by authentic standards.

Table 4
Composition of alcohol acetates and methyl esters of acids from saponified wax esters by GC–MS

Compound[a]	RT	Molecular weight	Peak area (%)
Methyl palmitate	13.36	270	0.94
Methyl stearate	19.37	298	0.70
Stearyl acetate	21.37	312	0.27
Methyl arachidate	25.36	326	20.9
Arachidyl acetate	27.25	340	0.45
Methyl forma cosanoate	27.92	340	0.40
Methyl behenate	30.67	354	8.67
Behenyl acetate	32.74	368	5.89
Methyl tricosanoate	33.13	368	0.18
Methyl lignocerate	35.64	382	1.53
Lignoceryl acetate	37.78	396	24.0
Methyl pentacosanoate	38.06	396	0.11
Methyl cereotate	40.39	410	0.73
Cerotyl acetate	42.38	424	18.6
Methyl heptacosanoate	42.66	424	0.10
Methyl montanylate	44.86	438	1.03
Montanyl acetate	46.63	452	6.22
Methyl melissylate	49.08	466	0.47
Melissyl acetate	50.99	480	2.76

[a] Identification based on RT and MS.

spectra with those of the National Institute of Science and Technology Library and published mass spectra.

3. Results and discussion

3.1. Separation of wax constituents by thin-layer chromatography

Wax was extracted from crude wax obtained from oil refineries and sunflower seed hull as reported earlier (Sindhu Kanya & Shamanthaka Sastry, 2003; Sindhu Kanya et al., 2003). The wax fraction of the crude wax from oil refineries (treated sample) consisted of free fatty alcohols (12.6%), free fatty acids (16.2%), fatty esters (66.0%), hydrocarbons (6.0%) and traces of triacyl glycerides. The control sample (wax isolated from seed hull) had a the similar composition of compounds (10.7%, 12.4%, 69.0%, 7.0% and trace, respectively). The results, on comparison, indicated around 5% destruction of wax ester, resulting in more than 25% increase in free fatty acids and 15% increase in free fatty alcohols during the refining process. Fractionation of these constituents was achieved by thin-layer chromatography. Among the solvent systems tried, benzene:chloroform (7:3 v/v) and toluene:chloroform (7:3 v/v) were found to be most suitable. In the present study, the separation was achieved using toluene:chloroform (7:3v/v) as the developing solvent system and the order of movement, of hydrocarbons, fatty esters, fatty alcohols and fatty acids, is indicated by the R_f values of 0.96, 0.92, 0.64 and 0.05, respectively. Henon (1986) suggested separation of fatty esters from sunflower oil on a mixed column of silica and silica impregnated with silver nitrate and reported C_{16}–C_{32} fatty acids and C_{16}–C_{32} alcohols in sunflower seed wax by GC analysis.

3.2. Wax esters

Fatty ester fractions separated from wax were analysed by GC (Table 1). The long chain esters found ranged from C_{38} to C_{54}. The major fatty esters were C_{40}–C_{44}, constituting more than 50% of total fatty esters. Among the fatty esters present, C_{42} was predominant (22.0%) and comparable with that of the control sample (22.9%). This clearly shows that esters are mainly formed from C_{20} to C_{24} acids and C_{20}–C_{24} alcohols. These values are in agreement with the results of Kleimann et al. (1969), for sunflower wax isolated from tank settlings. This indicated that the wax obtained as crude wax was intact, without much destruction during refining and storage.

3.3. Free fatty alcohols

Fatty alcohol fraction separated from wax was subjected to gas chromatographic analysis and the profile shows the presence of eight peaks (Fig. 1). The free alcohols separated were found to contain the even numbered carbon chain, ranging from C_{18} to C_{32} (Table 2) as analysed by GC–MS. The major alcohols were behenyl (C_{22}), lignoceryl (C_{24}) and cerotyl (C_{26}) alcohols, present to the extent of 15.6%, 34.8% and 26.8%, respectively, contributing to 77.2% of the total free alcohols, not much deviation from the control sample (wax obtained from seed hulls contained a total of 78.0%). Montanyl alcohol was the next major free fatty alcohol (~9%). *n*-Triacontanol (melissyl

alcohol), a plant growth regulator was detected and was found to be in the free state up to 4%. Identification of *n*-triacontanol was earlier reported (Sindhu Kanya et al., 1993) and was confirmed in the current study.

3.4. Free fatty acids

The GC profile of methylated free fatty acid fraction is shown in Fig. 1. GC–MS analysis of the free fattyacid methyl ester fraction showed the presence of eight peaks (Table 3). The major esters found were methyl arachidate and methyl behenate, to the extent of 50.2% and 28.3%, respectively, in the free fatty acid fraction of control sample (wax obtained from hulls) and 46.7% and 25.8%, respectively, in wax obtained from crude wax (from oil refineries). This indicates that arachidic and behenic acids are the two major fatty acids present in sunflower wax. The methyl esters of C_{24}, C_{26}, C_{28} and C_{30} are present to the extent of 6.6%, 4.3%, 3.2% and 2.4% in the hull wax and 7.5%, 6.6%, 6.8% and 3.4%, in the crude wax (oil refineries), respectively. These values indicate the dissociation of wax esters of higher order and this resulted in more release of longer chain fatty acids (C_{24}–C_{30}) during the refining process. The identification of methyl esters of C_{16}, C_{18}, and C_{32} indicates the presence of the respective free fatty acids in trace amounts.

3.5. Characterization of fatty alcohols and fatty acids of wax esters as acetates and esters

The wax ester released long chain fatty alcohols and long chain fatty acids upon saponification. Totally, 19 compounds were separated and identified of which 12 were fatty acids and 7 were fatty alcohols (Table 4). The studies revealed that around 62% of long chain alcohols and around 37% of long chain acids were present in sunflower wax. These results are comparable with the reported values (Kleimann et al., 1969; Rivarola et al., 1985). The alcohols present varied from C_{18} to C_{30} and acids from C_{16} to $C_{30.}$ Rivarola et al. (1985) reported that sunflower seed wax contained long chain fatty acids, from $C_{22:0}$ to $C_{30:0.}$ Saturated acids of C_{22} and more were found to be present in shell lipids of sunflower seed (Kalistratova et al., 1974). The presence of *n*-triacontanol in sunflower seed wax was reported earlier from this laboratory (Sindhu Kanya et al., 1993). The results are tabulated (Table 4). The two major fatty acids present in the sunflower wax (arachidic and behenic acids) were identified as methyl arachidate and methyl behenate with the molecular weights of 326 and 354, respectively, and their mass spectra are presented (Fig. 3a and b).

Lawrence et al. (1982) studied the composition of wax esters, alcohols and fatty acids of some of the commercial waxes, such as rice bran, carnauba, sugarcane and yellow

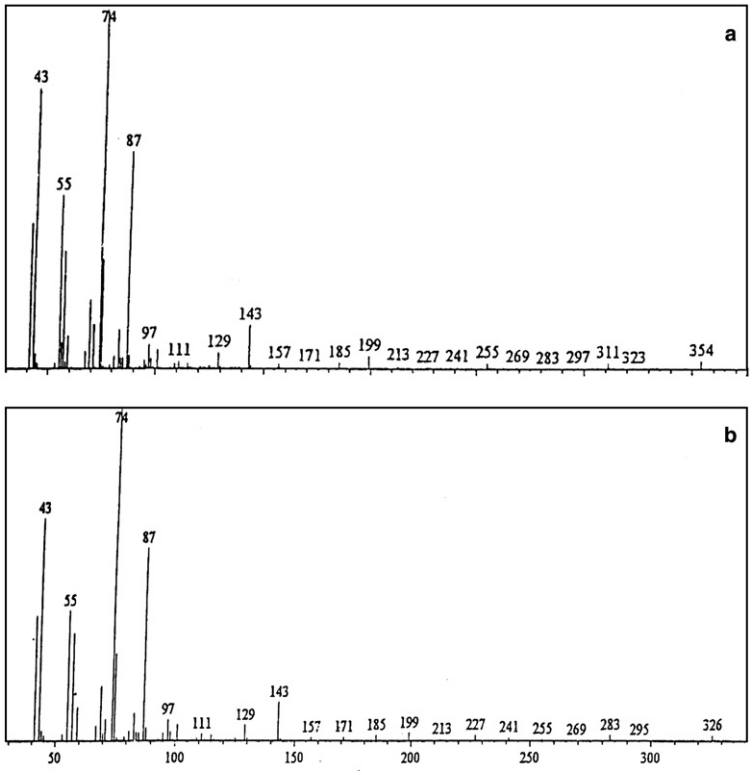

Fig. 3. Mass spectra of major fatty acids (methyl esters) of sunflower wax. (a) Mass spectrum of methyl arachidate. (b) Mass spectrum of methyl behenate.

bee wax, by gas liquid chromatography but gave no mention of sunflower seed wax. The present studies on separation of wax components from crude wax obtained from oil refining industries and their identification by GC–MS, revealed that there was no significant effect of refining on the chemical structure of wax esters or free fatty acids and alcohols.

4. Conclusion

The crude wax obtained from sunflower seed oil refineries contained refined wax (10–12%). Wax esters, free fatty acids and free fatty alcohols were purified by thin-layer chromatography. The composition of wax fractions was: wax esters (66%), free fatty alcohols (10%) and free fatty acids (16%). Methylation of fatty acids indicated the presence of C_{16}–C_{30} fatty acids in the free form. Fatty alcohols present in the free form were C_{18}–C_{32}. Wax esters consisted of 62% alcohol fraction and 37% acid fraction. The major alcohols found were lignoceryl (C_{24}) and cerotyl (C_{26}), whereas the major acids found were arachidic (C_{20}) and behenic (C_{22}) acids, as revealed by GC and GC–MS analyses. Among the long chain wax esters (C_{38}–C_{54}), the major esters were C_{40}–$C_{44,}$ of which C_{42} was predominant. These wax esters, on hydrolysis, followed by methylation and subsequent GC–MS analysis, showed the presence of C_{16}–C_{30} fatty acids in the bound form. Similarly, acetylation showed the presence of C_{18}–C_{30} fatty alcohols in the bound form. A plant growth regulator, *n*-triacontanol (C_{30}), was found in both free and bound forms up to 4%. More release of long chain fatty acids (25%) was observed during the refining process, whereas there was no effective change in the composition of fatty alcohols. The studies on separation of wax components from crude wax obtained from oil refining industries revealed that there was no significant effect of refining, on the structure of wax esters or free fatty acids and alcohols. This indicates that the wax originating from seed hull is intact.

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