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Analysis of Sterol and Other Components Present in Unsaponifiable Matters of Mahua, Sal and Mango Kernel Oil

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Abstract: The amount and characterization of phytosterol and other minor components present in three Indian minor seed oils, mahua (*Madhuca latifolia*), sal (*Shorea robusta*) and mango kernel (*Mangifera indica*), have been done. Theses oils have shown commercial importance as cocoa-butter substitutes because of their high symmetrical triglycerides content. The conventional thin layer chromatography (TLC), gas chromatography (GC) & gas chromatography-mass spectroscopy (GC-MS) techniques were used to characterize the components and the high performance thin layer chromatography (HPTLC) technique was used to quantify the each group of components. The experimental data showed that the all the three oils are rich in sterol content and among all the sterols, β-sitosterol occupies the highest amount. Sal oil contains appreciable amount of cardenolides, gitoxigenin. Tocopherol is present only in mahua oil and oleyl alcohol is present in mango kernel oil. Hydrocarbon, squalene, is present in all the three oils. The characterization of these minor components will help to detect the presence of the particular oil in specific formulations and to assess its stability as well as nutritional quality of the specific oil.

Key words: mahua oil, sal oil, mango kernel oil, TLC, GC, GC-MS, HPTLC, phytosterol, tocopherol, squalene

1 INTRODUCTION

Characterization of oils and fats has mainly been focused on the principal components, which constitute the saponifiable fraction and comprises over 95% of oils and fats. However, it is now generally recognized that the minor components, which generally constitute the unsaponifiable (unsap) matter, have important bioactive, nutritional, and characteristic compositional properties that affect the quality of individual oils and fats [1–3]. The unsaponifiable matters present in vegetable oils & fats are usually composed of sterols, fatty alcohols, tocopherols, triterpene alcohols, and hydrocarbon (squalene) which have individual biological importance. Some of the above constituents may exert a pronounced effect on stability of the oils and their nutritional properties. Some times characteristic unsap matters help to detect adulteration of the oil. Works in this field, however, also showed that the presence of high amount of some specific unsaponifiables may be toxic for human health[4].

Literature survey reveals a great deal of study related to unsap constituents in case of major edible oils[5–10]. However, such kind of studies did not receive much attention in case of minor oils. Mahua (*Madhuca latifolia*), sal (*Shorea robusta*) and mango kernel (*Mangifera indica*), are three important indigenous minor oils of India and have shown commercial importance as cocoa-butter substitutes because of their high symmetrical triglycerides content. The use of these fats in chocolate as well as in bakery fat formulation made them worth studying in terms of their unsap content. Reversibly, study of unsap components of chocolate will indicate the presence of these particular fats.

The present study aims to determine the composition of the unsap matters of mahua, sal, mango kernel oil, the three important minor oils of India. The conventional thin layer chromatography (TLC), gas chromatography (GC) & gas chromatography-mass spectroscopy (GC-MS) were adopted for composition analysis and the high performance thin layer chromatography (HPTLC) technique was used to quantify the different group of components present in the unsap matter of these oils as a substitute of preparative TLC.

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2 EXPERIMENTAL PROCEDURES

2.1 Materials

All the oils were supplied by Progressive Exim Ltd., Raipur, India, in two consecutive years 2007 & 2008. All solvents & reagents used were of analytical grade and procured from SRL India Ltd.

2.2 Methods

2.2.1 Isolation of unsap matters from oils

Unsaponifibale matters were isolated from the specific oils by standard AOCS method. Oil samples were saponified by refluxing with 0.5 (N) ethanolic potassium hydroxide in presence of pyrogallol. After refluxing for 2 h on a water bath the unsap matters were extracted with petroleum ether, washed with water, dried over anhydrous sodium sulphate and finally flushed with nitrogen for solvent evaporation. The dry residue was dissolved in chromatographic grade hexane and passed through a Chromabond silica SPE cartridge. The filtrate was then dried and re-dissolved in HPLC grade hexane for further analysis.

The unsaponifiables obtained were first separated into sterol and other fractions by preparative TLC & HPTLC. The quantitative analysis was done by HPTLC and the qualitative analysis was done by GC & GCMS after extracting them from TLC plates. Pure β-sitosterol, oleyl alcohol, hydrocarbon and unsap matter obtained from rice bran oil were used as standard for identification of unknown components.

2.2.2 TLC analysis

Unsaponifiable matter was fractionated on 20 × 20 cm plates spread with a 0.2 mm layer of Silica gel G (Merck). The plate was developed with 8:2 hexane: diethyl ether solvent with a continuous flow development[11]. The plate was sprayed with a 0.01% rhodamine 6G solution in ethanol and observed under UV light (3600A). Four separate zones containing highly polar compounds namely sterols & methyl sterols, tri-terpene alcohols, fatty alcohols and less polar compound like hydrocarbons respectively (from lower edge to solvent front) were observed.

2.2.3 Preparative TLC analysis

The same procedure of TLC was followed but with the higher amount of sample and instead of spots, samples were fractionated in bands. The bands were visualized by adsorbing iodine, different fractions were marked, and the each fraction was scrapped off from the TLC plate after the complete evaporation of iodine. The fractions were extracted completely from silica gel by repeated extraction with petroleum-ether and diethyl ether (1:1). Finally the solvent was evaporated off and the pure fractions obtained were weighed and stored for further analysis.

2.2.4 HPTLC Analysis

Hexane solutions of the unsaponifiable matters obtained from sal, mahua and mango kernel fat were prepared by the combined stirring and sonication procedure to get a clear test solution for spotting on the HPTLC plates. Rice bran unsap was used as standard (5 $mgmL^{-1}$) in hexane[12]. Analyses were performed on TLC aluminium sheets 20 cm × 20 cm Silica Gel 60 F_{254} plates (Merck, Germany) with concentrating zone, 19 channels, and fluorescent indicator. Plates were developed to a distance of solvent front 65.0 mm, application position was 8.0mm, vol 10 mL, solvent system used to develop plate was di-ethyl-ether: hexane = 33:66 in a CANMAG-HPTLC twin-trough chamber 10 × 10 cm, temperature was kept at 25℃, lined with a saturation pad (Analtech, Newark, DE, USA) and the chamber was equilibrated with the mobile phase for 15 min before inserting the plate. Approximately 30 mL mobile phase (15 mL in the trough containing the plate and 15 mL in the trough containing the pad) were used for each development, which required approximately 20 min. After development, the plates were air-dried in a fumehood, for 5 min and sample and standard zones were quantified by linear scanning at 200 nm by using a CAMAG-HPTLC Scanner 3" (Scanner 3_ 130214" S/N 130214) with a D_2 source, (5 × 0.45 mm, micro) and a scanning speed of 20 mm/sec, data resolution 100 μm/step. The WINCATS-3 software controlling the densitometer produced a calibration plot by linear regression relating standard zone weights to their scan areas, and the experimental weight of unsaponifiables in samples was automatically interpolated from the calibration curve.

The analysis was validated by standard addition analysis. A test solution (1000 μL) was mixed with stock solution (50.0 μL) to double the unsap concentration. The difference between the mean experimental weights and the weight added was calculated to determine the accuracy of the method. Precision (repeatability) was validated by spotting six 8.00-μL volumes of one sample on the same plate and calculating the relative standard deviation (RSD) of the experimental weights.

2.2.5 Fatty acid composition of oil by GC

Methyl esters of three oils were prepared by the method described by Litchfield[13] and the fatty acid composition was determined by GC analysis using an analytical gas chromatography (Agilent 6890 Series Gas chromatograph) equipped with a Flame Ionization Detector (FID) and HP-5 capillary column (J & W Scientific Columns From Agilent Technologies) of 30 m length with 0.25 mm (i.d) and 0.25 mm (film thickness). The GC inlet temperature and FID detector temperature was maintained at 250℃ and oven temperature was maintained at 250℃ for 2 min, then temp was increased at 10℃ /min, upto 280℃, then 20 min hold at 280℃. The gas flow was 1 mL/min, 300mL/min and 30 mL/min for N_2, H_2 and air respectively.

2.2.6 Sterol analysis by GC

The quantitative analysis of sterol was done by GC. The GC (make: Agilent, model: 6890 N) instrument used was

equipped with FID detector and capillary HP 5 column (30 mL, 0.32mm I.D, 0.25 μm FT). N_2, H_2 and airflow rate was maintained at 1 mL/min, 30 mL/min and 300mL/min respectively. Inlet & detector temperature was kept at 250℃ & 275℃ respectively and the oven temperature was programmed as 65–230–280℃ with 1 min hold at 65℃ and increase rate of 20℃/min and 1 min hold up to 230℃ and 8℃/min with 24 min hold up to 280℃.

2.2.7 GCMS analysis

Gas chromatographic analysis was carried out on a Varian–Chrompack CP–3800 coupled to Varian–Chrompack Saturn 2200 MS under electron impact ionisation (70 eV). The MS scan range was 40–650 atomic mass units (AMU). The chromatographic column for the analysis was a fused silica WCOT capillary column (30 m × 0.25 mm i. d.; VF–5 MS, 0.25 μm). The carrier gas used was helium at a flow rate of 1.0 mL/min. Samples were analysed with the column held initially at 70℃ for 1.5 min and then increased to 200℃ with 10℃/min heating rate. Finally temperature was increased to 280℃ and hold for 20 min. The injection was performed in split less mode at 250°C. The identification of the individual components was done by Wiley and NIST mass spectral library on the basis of the mass fragments and e/Z values of each component.

2.2.8 Data analysis

Unless otherwise stated, experiments for determination of phytosterols and other compounds by HPTLC, GC and GC–MS analyses were carried out in triplicate sets of each oil sample and samples of two consecutive years were analysed and results are expressed as mean values ±SD.

3 RESULTS

3.1 Fatty acid composition of three oils

Fatty acid composition as shown in Table1 indicates that mahua *(Madhuca latifolia)* oil contains fatty acids palmitic (16:0) 21.3%, stearic (18:0) 24.3%, oleic (18:1) 36.7%, linoleic (18:2) 15.2% and arachidic (20:0) 1.3%. Sal oil (*Shorea robusta*) contains fatty acids palmitic (16:0) 6.9%, stearic (18:0) 41.4%, oleic (18:1) 43.2%, linoleic (18:2) 4.2% and arachidic (20:0) 6.8%. Mango kernel oil (*Mangifera indica*) contains fatty acids palmitic (16:0) 8.4%, stearic (18:0) 41.4%, oleic (18:1) 43.2%, linoleic (18:2) 4.2% and arachidic (20:0) 2.3%.

3.2 Analysis of unsap matter present in three oils

Total unsap matter content of mahua oil is 2.4% where as sal and mango kernel contain 2.02% and 2.01% of unsap respectively (Table 2). The unsap matter of each oil contains methyl sterol & sterol, triterpene alcohol, fatty alcohol and hydrocarbon in varying amounts. Only Sal oil contains 12.37% of an unidentified component (Ia) which when further analysed by GCMS, was identified as 'gitoxigenin' by measuring the ion fragmentations obtained from GC–MS which is a cardenolides and usually found in plant origin[14].

3.3 Analysis of sterol fraction

The analysis of the sterol fraction of the three oils was done by GC and identified by measuring the relative retention time of the individual sterols separated and comparing with standards (Tables 3 & 4). Mahua oil contains campesterol (0.97%), stigmasterol (7.47%), β–sitosterol (64.78%), Δ^5–Avenasterol (9.53%), Δ^7–stigmasterol (4.08%), and Δ^{7-}Avenasterol (9.67%); Sal oil contains campesterol (0.97%), stigmasterol (22.7%), β–sitosterol (59.41%), Δ^5–Avenasterol (1.7%), Δ^7–stigmasterol (3.26%), and Δ^{7-}Avenasterol (11.76%); Mango kernel contains campesterol (0.07%), stigmasterol (10.66%), β–sitosterol (58.63%), Δ^5–Avenasterol (10.19%), Δ^7–stigmasterol(4.34%), and Δ^{7-}Avenasterol (19.10%).

3.4 Total composition analysis

The GC–MS analyses of unsap matter of three oils are presented in the Tables 5, 6 & 7 (also vide Figs. 1, 2 and 3). On the basis of the mass fragmentation pattern of each component, the NIST library indicates that Mahua oil (Table 5) contains squalene, tocopherol, campesterol, stigmasterol, β–sitosterol, Δ^5–avenasterol, Δ^7–stigmasterol, Δ^{7-}avenasterol, α–amyrin & β–amyrin etc. Sal oil contains (Table 6) gitoxigenin, phytol, squalene, campesterol, stigmasterol, β–sitosterol, Δ^5–avenasterol, Δ^7–stigmasterol, Δ^{7-}avenasterol, α–amyrin/ β–amyrin, lupeol etc. Mango kernel oil (Table 7) contains oleyl alcohol, lupeol, squalene, campesterol, stigmasterol, β–sitosterol, Δ^5–avenasterol, Δ^7–stigmasterol, and Δ^{7-} avenasterol etc.

Table 1 Fatty Acid Composition of Mahua, Sal and Mango Kernel Oil.

Name of the oil	Fatty acid composition (% w/w)				
	C16:0	C18:0	C18:1	C18:2	C20:0
Mahua oil	21.3 ± 1.01	24.3 ± 0.30	36.7 ± 0.27	15.2 ± 0.64	1.3 ± 0.15
Sal oil	6.9 ± 0.22	41.4 ± 0.37	37.5 ± 0.40	1.9 ± 0.06	6.8 ± 1.34
Mango kernel oil	8.4 ± 0.12	41.4 ± 0.48	43.2 ± 0.16	4.2 ± 0.16	2.3 ± 0.67

Values are averages ± SD from six replicate analyses

4 DISCUSSION

The fatty acid composition of the three oils indicate that these oils are highly suitable for preparing coco butter substitute (CBS) or coco butter equivalents (CBE). The study has clearly demonstrated how analysis of the constituents of the unsaponifiable matter, i.e. the minor components of seed oils, could be done. These analyses are essential for detailed characterisation of oils and fats. As

Table 2 Percent Content & Composition of Total Unsaponifiable Matter of Three Oils.

Name of the oil	Unsap matter present in oil(%, w/w)	Fraction unsaponifiables (%, w/w)				
		Ia	I	II	III	IV
Mahua oil	2.41±0.01	–	30.19±0.15	39.02±0.03	21.40±0.5	9.39±0.6
Sal oil	2.02±0.03	12.84±0.10	31.84±0.06	15.89±0.78	10.65±0.46	28.78±0.08
Mango kernel oil	2.01±0.01	–	51.37±0.03	7.72±0.03	7.72±0.03	33.29±0.15

Fraction Ia: unknown layer; Fraction I: Methyl Sterol & Sterol; Fraction II: Triterpenealcohol; Fraction III: Fatty alcohol; Fraction IV: Hydrocarbon

Values are averages ± SD from six replicate analyses

Table 3 Relative Retention Time of Sterols in Gas Liquid Chromatogram.

Sterols	Relative Retention time[a]
Cholesterol	0.88
Campesterol	0.95
Stigmasterol	0.99
β-sitosterol	1
Δ^5- Avenasterol	1.009
Δ^7- stigmasterol	1.016
Δ^{7-} Avenasterol	1.020

Relative retention time[a] for β-sitosterol (retention time: 37.237) was taken as 1.00

Table 4 Percent Composition (%w/w) of Each Sterol Present in the Three Oils.

	Composition (% w/w) according to relative retention timea of individual sterols						
	I 0.88	II 0.95	III 0.99	IV 1*	V 1.009	VI 1.016	VII 1.02
Sterol of Mahua unsap	tr	0.97±0.02	7.47±0.05	64.78±0.07	9.53±0.13	4.08±0.02	9.67±0.02
Sterol of Sal unsap	tr	1.6±0.03	22.27±0.05	59.41±0.02	1.7±0.04	3.26±0.11	11.76±0.08
Sterol of Mango kernel	tr	0.065	10.66	58.63	7.19	4.34	19.10

Relative retention time for β-sitosterol taken as1.00.*

I-cholesterol; II-campesterol; III-stigmasterol; IV- β-sitosterol; V- Δ^5- Avenasterol; VI- Δ^7- stigmasterol; VII - Δ^{7-} Avenasterol

Values are averages ± SD from six replicate analyses

Table 5 GCMS Analysis of Mahua Oil* Unsaponifiables.

Components identified	Retention time(R_t) min	Molecular Formula	MW	Mass fragments
Squalene	20.274	$C_{30}H_{50}$	410	257,189
Tocopherol	33.240	$C_{29}H_{50}O_2$	430	165
Campesterol	35.127	$C_{28}H_{48}O$	400	315,289,255,213,81
Stigmasterol	37.12	$C_{29}H_{48}$ O	412	300,213,55
β-sitosterol	37.237	C_{29} H_{50} O	414	382,329,303,213,105,81,55
Δ^5-Avenasterol	37.57	C_{29} H_{48} O	412	300,133
Δ^7-stigmasterol	37.83	C_{29} H_{48} O	412	351,300,159
Δ^{7-}Avenasterol	37.98	C_{29} H_{48} O	412	394,300,133
β-amyrin& α-amyrin	38.233	C_{30} H_{50} O	426	218,189,147,122,95

*One sample of each year was analysed by GC-MS

Table 6 GCMS Analysis of Sal Oil* Unsaponifiables.

Components identified	Retention time(R_t) min	Molecular Formula	MW	Mass fragments
Gitoxigenin	17.572	$C_{23}H_{34}O_5$	390	203,147
Phytol	19.23	$C_{20}H_{40}O$	269	123,71,56
Squalene	28.869	$C_{30}H_{50}$	410	231,203,95,70
Campesterol	32.889	$C_{31}H_{52}$ O_2	456	315,289,255,213,81
Stigmasterol	35.482	$C_{29}H_{48}O$	412	300,213,55
β-sitosterol	37.142	C_{29} H_{50} O	414	382,329,303,213,105,81,55
Δ^5-Avenasterol	37.57	C_{29} H_{48} O	412	300,133
Δ^7-stigmasterol	37.83	C_{29} H_{48} O	412	351,300,255,159
Δ^{7-}Avenasterol	37.98	C_{29} H_{48} O	412	394,300,255,133
α-amyrin/ β-amyrin	38.001	C_{30} H_{50} O	426	218,189,147,122
Lupeol	39.08	C_{30} H_{50} O	426	257,207,190,147,121,95,68

*One sample of each year was analysed by GC-MS

Table 7 GCMS Analysis of Mango Kernel Oil* Unsaponifiables.

Components identified	Retention time(R_t) min	Molecular Formula	MW	Mass fragments
Oleyl alcohol	18.567	C_{18} H_{36} O	268	137,109
Squalene	28.913	C_{30} H_{50}	410	231,203,123
Campesterol	35.153	C_{28} H_{44} O	396	367,315,289,255,213,81
Stigmasterol	35.945	C_{29} H_{48} O	412	394,351,300,255,159,133
β- sitosterol	37.204	C_{29} H_{50} O C_{29}	414	382,329,303,213,105,81,55
Δ^5- Avenasterol	37.57	H_{48} O	412	394,300,133
Δ^7- stigmasterol	37.83	C_{29} H_{48} O	412	351,300,255,159
Δ^{7-} Avenasterol	37.98	C_{29} H_{48} O	412	394,300,255,133

*One sample of each year was analysed by GC-MS

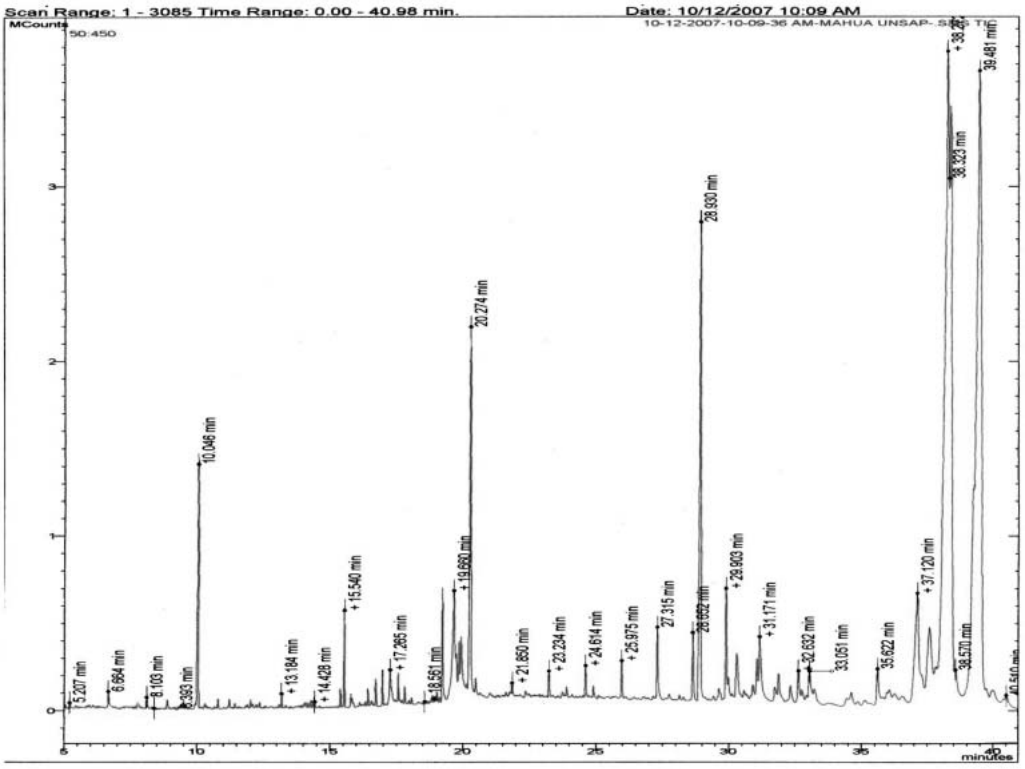

Fig. 1 TIC Profiles of the Unsaponifiable Matter of Mahua Oil by GC–MS in the Full–mode Scan at 70 eV.

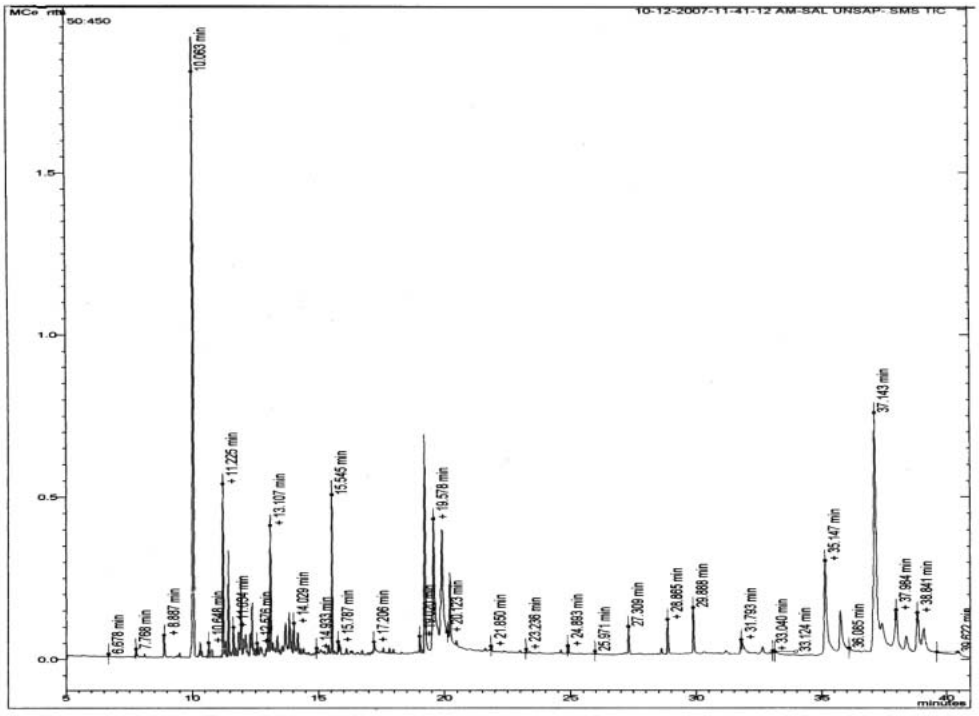

Fig. 2 TIC Profiles of the Unsaponifiable Matter of Sal Oil by GC–MS in the Full–mode Scan at 70 eV.

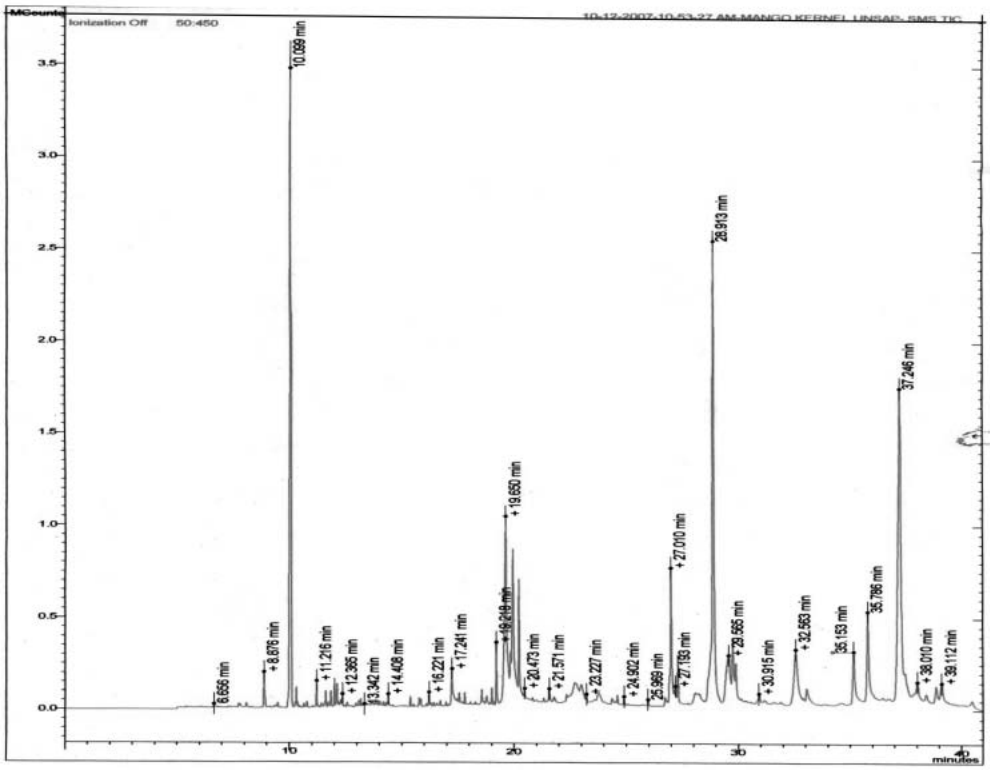

Fig. 3 TIC Profiles of the Unsaponifiable Matter of Mango Kernel Oil by GC–MS in the Full–mode Scan at 70 eV.

shown above, the phytosterol profile of three oils clearly distinguish between the oils, indeed much more than their fatty acid compositions. The mass chromatogram also clearly demonstrate the difference in unsap composition of three oils. The characterization of these minor components will help to detect the presence of the particular oil in specific formulations and to assess its stability as well as nutritional quality of the specific oil.

5 CONCLUSION

This study has revealed qualitatively and quantitatively the particular compounds that make up the bulk of the unsaponifiable matter in the three oils and has further demonstrated the uniqueness of the composition of these compounds as being characteristic of the oil of a particular source. In many cases the composition of the minor fractions, i.e. the profiles of phytosterols and other compounds more distinctly define the genuineness of individual oils and fats along with their respective fatty acid compositions.

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