Content and Composition of Free Sterols and Free Fatty Alcohols in Jojoba Oil

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A method was developed for the isolation and identification of phytosterols and fatty alcohols in jojoba oil. The method consists in the separation of these compounds from wax esters in the oil by means of an aluminum oxide column followed by further fractionation of the minor components by column chromatography on silica gel. The 4-demethylsterols, 4-methylsterols, triterpene alcohols (4,4-dimethylsterols), and fatty alcohols are identified by means of their gas chromatographic and mass spectrometric data. The present paper includes a method for the quantitation of the free sterols in jojoba oil.

Keywords: *Plant; alcohol; sterol; lipid; Simmondsia*

INTRODUCTION

Jojoba oil is obtained from the seeds of the evergreen shrub, *Simmondsia chinensis* (Link) Schneider. The seeds contain about 50% of a light yellow, odorless wax ester commonly referred to as jojoba oil. The oil is composed of straight chain monoesters of alcohols and fatty acids. As described by Miva (1971, 1984), Hamilton et al. (1975), and Bhatia et al. (1981), both the acids and alcohols are cis-monounsaturated at the 9-position. The alcohols are mainly a mixture of eicos-11-enol and docos-13-enol; the acids, mainly eicos-11-enoic acid and docos-13-enoic acid. These are combined in the wax ester to give long molecules with 38, 40, 42, and 44 carbon atoms, with the C-40 and C-42 homologues being the most abundant.

The oil is extensively used in the cosmetic industry for its dermatological properties. Those properties are partially due to the present wax esters but possibly may result from the presence of phytosterols in the oil, since the phytosterols of avocado and soya oil are widely used in dermatological and medicinal applications as described by Chaze (1972), Robert et al. (1975), Thiers (1971), Rancurel (1985), and F. Gunstone (1990). Nutritionally, phytosterols are important because of their ability to lower serum cholesterol. In crude oils, phytosterols exist as free sterols and as sterol esters, although sterol glycosides and acylated glycosides may also be present. Sterol patterns are used to characterize vegetable oils and to prove authenticity or adulterations as described by Grob et al. (1994); in the present case the determination of sterols can be used for the discrimination between authentic jojoba oil and synthetic substitutes. Triterpenes and 4-methylsterols are accompanying substances of the sterols with very similar chromatographic properties; as described by Homburg and Bielefeld (1990), those sterols can influence in some cases strongly the exact determination of sterols by gas chromatography if no preceding group separation is done. Jojoba oil is actually used instead of sperm oil in the lubrificant industry and as a plasticizer for polymers. It also may be considered as a low-energy replacement for conventional fats and oils as it is poorly digested (Heise et al., 1982; Yaron and Benzioni, 1980; and Verschuren, 1989).

Kitsuwa et al. (1980) describe the isolation of campesterol, stigmasterol, and sitosterol from jojoba oil by a combination of different extraction and separation procedures. Burgos and Donaire (1996) describe the determination of the same 4-demethylsterols in plasma membranes from jojoba roots.

The present paper deals with the isolation and identification of the 4-demethylsterols, 4-methylsterols, and triterpene alcohols in jojoba oil. The determined composition of the different sterols allowed for the elaboration of an easy method for the determination of free sterols in jojoba oil. The same procedure also allows the determination of the free fatty alcohols. The relative importance of the different fatty alcohols is studied by means of gas chromatography and liquid-assisted secondary ion mass spectrometry (L-SIMS).

MATERIALS AND METHODS

Materials. Jojoba oil was prepared by continuous extraction with *n*-hexane from jojoba meal obtained from EMEC Agro Industries (Antwerp, Belgium). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were obtained from Pierce (Rockford, IL); *n*-hexane, analytical grade, from Merck (Darmstadt, Germany); and aluminum oxide, activated, neutral, 150 mesh from Aldrich (Steinheim, Germany). 24-Methylcholesterol, probably a mixture of 24*R*- and 24*S*-isomers of 24-methylcholest-5-en-3β-ol), stigmasterol, and sitosterol were obtained from Applied Science Laboratories (State College, PA); cholesterol and lanosterol from Sigma Chemie (Bornem, Belgium); fucosterol from ICN Biomedicals (Aurora). Reference mixtures of 4-demethylsterols (24-methylcholesterol, stigmasterol, sitosterol and isofucosterol), 4-methylsterols (obtusifoliol, gramisterol, and citrostadienol), and 4,4-dimethylsterols (cycloartenol, 24-methylenecycloartanol, and cyclobranol) were isolated from corn oil in our laboratory (Merckx, 1982).

Nomenclature. The following common names are used: campesterol, 24-methylcholesterol, a mixture of 24*R*- and 24*S*-isomers of 24-methylcholest-5-en-3β-ol; cholesterol, cholest-5-en-3β-ol; citrostadienol, 4α-methyl-24-ethylidene-5α-cholest-7-en-3β-ol; cycloartenol, 4,4,14α-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol; cyclobranol or 24-methylcycloartenol,

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4,4,14α,24-tetramethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol; cycloeucalenol, 4α,14α-dimethyl-24-methylene-9β,19-cyclo-5α-cholestan-3β-ol; fucosterol, 24(*E*)-ethylidenecholest-5-en-3β-ol; gramisterol, 4α-methyl-24-methylene-5α-cholesta-7-en-3β-ol; isofucosterol, 24(*Z*)-ethylidenecholest-5-en-3β-ol; lanosterol, lanosta-8,24-dien-3β-ol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol); 24-methylenecycloartanol, 4,4,14α-trimethyl-24-methylene-9β,19-cyclo-5α-cholestan-3β-ol; obtusifoliol, 4α,14α-dimethyl-24-methylene-5α-cholest-8-en-3β-ol; sitosterol, 24-ethylcholest-5-en-3β-ol; stigmasterol, 24-ethylcholesta-5,22-dien-3β-ol.

Gas Chromatography. A Chrompack 9000 gas chromatograph equipped with a flame ionization detector was used for the analysis of the sterols. Separations were made on a 30 m × 0.32 mm i.d. glass capillary column with a chemically bonded cross-linked methylsilicone gum [Ultra 1, Hewlett-Packard (HP), Brussels, Belgium] 0.17 μm film. Samples of 2 μLwere injected by means of a split injector, 1:100. To protect the column, a special insert glass liner (Chrompack, Antwerp, Belgium) was used. Injector and detector temperatures were 280 °C. Helium was used as carrier gas at linear velocity of 20 cm/s (set at 260 °C). The oven temperature was kept at 180 °C for 2 min, programmed to 260 °C at 10 deg/min, and further kept at 260 °C for another 20 min.

Mass Spectrometry. Liquid surface-assisted ionization mass-spectrometry (L-SIMS) was performed with a Kratos Concept 1H instrument using a 7 keV Cs beam. The spectra, in positive ion mode, were obtained by dissolving the samples in a thioglycerol matrix and placing them on a copper probe tip prior to the bombardment with Cs ions.

Gas Chromatography–Mass Spectrometry (GC–MS). Mass spectra were obtained with a HP 890 Series II gas chromatograph equipped with a HP 5971A mass selective detector and a HP-1 column (cross-linked methylsilicone gum, 30 m × 0.25 mm i.d., and 0.25 μm film thickness). The linear velocity of the carrier gas, He, was 30 cm/s. The column was coupled with the ion source without interface. Extracts were introduced by splitless injection at 70 °C. The injector temperature was kept at 70 °C for 2 min without operation of the amplifier and programmed to 100 °C at 35 deg/min and from 100 to 270 °C at 10 deg/min. The oven temperature was further kept at 270 °C for 30 min.

Thin Layer Chromatography (TLC). Analytical thin layer chromatography was performed on precoated silica gel plates, 40 × 80 mm (Polygram Sil G/UV 254, Machery-Nagel, Germany) using a mixture of *n*-hexane and ethyl acetate (80/20, v/v) as solvent. The spots were localized by spraying the plates with sulfuric acid reagent, a mixture of concentrated sulfuric acid and ethanol (50/50, v/v). After being sprayed, the plates were heated at 100 °C in an oven until appearance of the rose colors of the reference sterols.

Preparative thin layer chromatography was performed on precoated silica gel plates, 20 × 20 cm, 0.25 mm thick (Sil G - 25HR, Machery-Nagel, Germany) using a mixture of hexane and diethyl ether (85/15, v/v). The extracts were applied as strips along with a mixture of cholesterol and lanosterol as a reference. For an optimal separation of the different zones, the plates were run 4 × 15 cm. The zones were detected by spraying a part of the plate with a 0.01% rhodamine 6G solution in ethanol and observing the plates under long-wave UV radiation (365 nm). The different detected zones were scraped off; the silica gel was brought into empty glass columns, and the compounds were eluted from the silica gel with 10 mL of diethyl ether. After evaporation of the solvent, the residues were further examined by GC and GC–MS.

EXPERIMENTAL PROCEDURES

Separation of the Minor Compounds from the Wax Esters. The free sterols in jojoba oil were isolated by means of an aluminum oxide column. A column of 20 cm length and 1 cm diameter was prepared by filling the column with a uniform slurry of 10 g of aluminum oxide and 50 mL of *n*-hexane. Jojoba oil (2.5 g), dissolved in 50 mL of *n*-hexane, was brought on top of the column. After application of the hexane, the column was repeatedly washed with 50 mL fractions of *n*-hexane in order to complete elution of the wax esters. Eluates were examined by TLC for the presence of residual wax esters. After complete removal of jojoba wax esters (200 mL), the column was eluted with 50 mL fractions of acetone. The presence of sterols in the eluates was checked by TLC. The acetone fractions (200 mL) containing sterols were collected and evaporated in vacuum; the residue was examined by gas chromatography and gas chromatography–mass spectrometry before and after separation by column chromatography on silica gel.

Fractionation of the Minor Compounds. A glass column, 50 cm × 2 cm, was filled with a slurry of 50 g of silica gel 60 (230–400 mesh) in *n*-hexane. A residue (100 mg) of the acetone eluates was brought on top of the column. The column was eluted with a mixture of *n*-hexane and ethyl acetate (93/7, v/v). Fractions of 10 mL were collected by means of a fraction collector, concentrated under a stream of nitrogen at 50 °C, and examined by TLC. Four different zones were distinguished with R_f values of 0.32, 0.28, 0.24, and 0.20, respectively. The fractions showing only one spot were collected and examined by gas chromatography and gas chromatography–mass spectrometry.

Quantitative Determinations of Free Sterols. *Calibration.* For establishing the calibration curves, jojoba oil previously stripped of free phytosterols by means of an aluminum oxide column was used.

To 2.5 g of jojoba oil samples were added respectively 24-methylcholesterol, stigmasterol, and sitosterol in hexane, in order to obtain concentrations of 0.05, 0.1, 0.2, and 0.5% (w/v); 7.5 mg of cholesterol in hexane was added to each sample as an internal standard. A calibration graph with fucosterol was established separately in the same way. The different samples were dissolved in 50 mL of *n*-hexane and applied to an aluminum oxide column (see above); the wax esters were eluted with 200 mL of *n*-hexane and the sterols subsequently with 200 mL of acetone. After evaporation of the organic solvent, the residues were dissolved in 2.0 mL of ethyl acetate. Two microliter aliquots were injected in the gas chromatograph. Calibration graphs were constructed by plotting the concentration of the different sterols against the ratios of peak areas between the sterols and the internal standard.

Calibration curves were also constructed following purification of the concentrated ethyl acetate fractions by TLC. The extracts were applied as strips on 10 × 20 cm silica plates along with cholesterol as standard. After development and visualization of the cholesterol, the zones corresponding to cholesterol were scraped off and transferred to empty mini glass columns. The sterols were eluted from the silica gel with 10 mL of ether. After evaporation of the solvent, the residues were dissolved in 2.0 mL of ethyl acetate and used for the construction of the calibration curves.

Silylation. To 0.2 mg of fatty alcohol was added 0.2 mL of BSTFA reagent along with 0.1 mL of TMCS reagent. The reaction was carried out in 1 mL plastic stoppered vials. The vials were put in an oven at 70 °C for 60 min, and the reaction mixture was examined by GC and GC–MS.

RESULTS AND DISCUSSION

Identification of the Minor Compounds. *Identification of Fatty Alcohols.* The spot with an R_f value of 0.28 in the mentioned TLC system corresponds to about 1% of the examined jojoba oil measured on a weight basis and was obtained as a pure fraction by the described column chromatography. When examined by GC (Figure 1), this zone showed two major peaks along with two minor peaks. The identity of the four components was further examined by GC–MS before and after silylation. GC–MS data, before and after silylation, are represented in Table 1. Comparaison of the mass spectra of the derivatized and underivatized compounds elucidated some important features concerning the ions with the highest m/z values. Each silylated product shows an increase of 90 mass units compared to the

Table 1. GC−MS Data for the Free Fatty Alcohols

R_t[a]	fragment ions m/z (percentage abundance relative to base peak)	name
	Before Silylation	
16.61	250 (4),152 (3), 138 (7), 96 (57), 82 (66), 69 (51), 55 (100)	octadec-9-enol
18.56	278 (10), 250 (3), 180 (2), 138 (11), 96 (80), 82 (97), 69 (62), 55 (100)	eicos-11-enol
20.31	306 (9), 278 (1), 208 (1), 138 (11), 96 (82), 82 (95), 69 (64), 55 (100)	docos-13-enol
22.17	334 (5), 306 (1), 236 (1), 138 (9), 96 (71), 82 (83), 69 (64), 55 (100)	tetracos-15-enol
	After Silylation	
17.32	340 (1,M^+), 325 (3), 297 (1), 269, 250 (1), 75 (100), 73 (43)	octadec-9-enol−TMS
19.10	368 (3,M^+), 353 (10), 325 (3), 297 (1), 75 (100), 73 (46)	eicos-11-enol−TMS
20.80	396 (5,M^+), 381 (12), 353 (3), 306 (1), 75 (100), 73 (41)	docos-13-enol−TMS
22.67	424 (4,M^+), 409 (8), 381 (2), 353 (1), 334 (2), 75 (100), 73 (40)	tetracos-15-enol−TMS

[a] R_t: GC retention time.

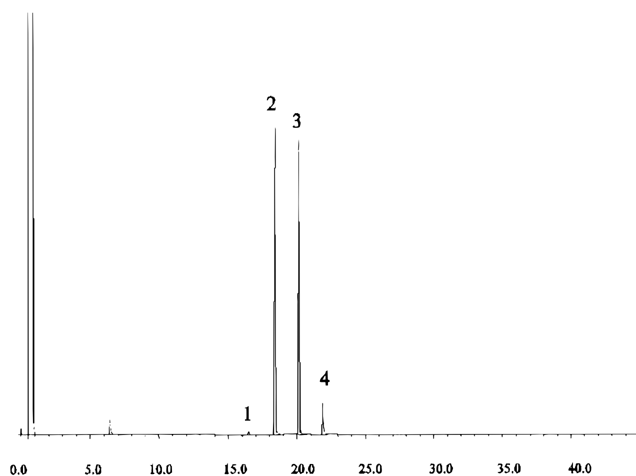

Figure 1. Gas chromatogram from the free fatty alcohols in jojoba oil: (1) octadec-9-enol; (2) eicos-11-enol; (3) docos-13-enol; (4) tetracos-15-enol.

underivatized compound; counting for a difference of one silyl group (72) and a molecule of water (18). This means that the underivatized compounds do not show molecular ions; the ions with the highest m/z values represent M − 18 fragment ions. This feature is characteristic for monoalcohols as is confirmed by the incorporation of one silyl group. The four consecutive eluting compounds in the gas chromatogram all show an increase of 28 mass units, indicating an homologous series of alcohols, each time differing by two carbon atoms.

The molecular ions of the derivatized products also suggest the presence of one double bound; the exact position cannot be proved by mass spectrometry. All the characteristics strongly suggest that the consecutive eluting peaks, 1, 2, 3, and 4, correspond to alken-1-ols with respectively 18, 20, 22, and 24 carbon atoms. The fatty alcohols we isolated from the jojoba esters following a procedure described by Tonnet et al. (1984) showed GC−MS data identical with that of the described free alkenols. Hamilton et al. (1975) proved by chemical means the $\omega 9$ cis unsaturated structure of the fatty alcohols in the jojoba wax esters; this means that the isolated free alcohols are indeed $\omega 9$ cis unsaturated fatty alcohols. The relative abundance of the different fatty alcohols based on the areas of the GC peaks is octadec-9-enol, 0.9%; eicos-11-enol, 47.3%; docos-13-enol, 45.8%; and tetracos-15-enol, 6.0%. The fatty alcohol mixture was further examined by L-SIMS. The spectra obtained with this method are characterised by high pseudomolecular ion sensivity $(M + H)^+$ in the positive ion scan mode as described by Fenselau and Cotter (1987). The present L-SIMS spectra show indeed pronounced pseudomolecular ions along with less pronounced (M − 18)

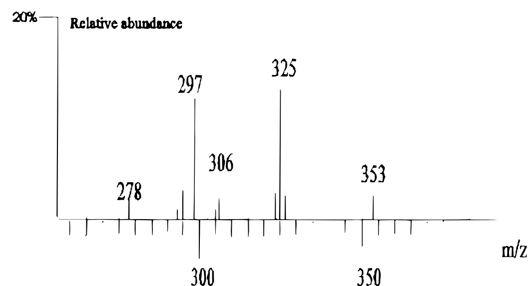

Figure 2. Mass spectrum from the fatty alcohols mixture obtained with L-SIMS.

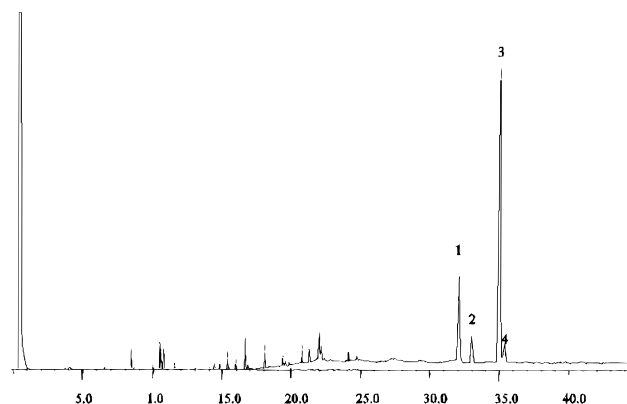

Figure 3. Gas chromatogram of the 4-demethylsterols in jojoba oil: (1) campesterol; (2) stigmasterol; (3) sitosterol; (4) isofucosterol.

fragment ions (Figure 2). However the relative intensities of the pseudomolecular ions (297, 325, 353) do not correspond very well with the relative intensities of the peaks obtained by gas chromatography, and different recordings of the spectrum of the alkenol mixture resulted also in important variations (±3%) of the relative intensities of the molecular ions of two most important fatty alcohols. The M + 1 peak for octadecenol (269) was seen only in the high sensitivity mode and the relative intensity was each time lower than 1%.

Identification of the Sterol Fractions: Identification of 4-Demethylsterols. The fraction with the R_f value of cholesterol (0.20) showed four components following analysis by GC and GC−MS. A typical gas chromatogram is represented in Figure 3. Peaks were identified by comparaison of the obtained gas chromatographic and mass spectrometric data with the corresponding data of reference compounds and data described in the literature (Itoh et al., 1974, 1982; Jeong et al., 1975). The GC−MS results are represented in Table 2. The peak with RR_t of 1.09 was identified as 24-methylcholesterol, the peak with RR_t of 1.12 as stigmasterol, and the peak with RR_t of 1.19 min as sitosterol. The sterol with RR_t of 1.20 showed a fragmentation pattern

Table 2. GC−MS Data for the Free Sterols of Jojoba Oil

sterols	characteristic fragment ions m/z	RR_t[a]
	4-Demethylsterols	
campesterol	400 (M^+, 46), 385 (15), 382 (21), 367 (17), 315 (30), 273 (16), 255 (21), 231 (15), 229 (3), 213 (32)	1.09
stigmasterol	412 (M^+, 35), 397 (3), 394 (3), 379 (6), 327 (3), 273 (35), 255 (40), 231 (5), 229 (10), 213 (18)	1.12
sitosterol	414 (M^+, 62), 399 (20), 396 (25), 381 (18), 329 (32),273 (16), 255 (20), 231 (14),229 (6), 213 (32)	1.19
isofucosterol	412 (M^+, 5), 397 (2), 314 (100), 299 (28), 296 (11), 281 (43), 273 (2), 255 (5), 231 (15), 229 (43), 213 (21)	1.20
	4-Methylsterols	
obtusifoliol	426 (M^+, 19), 411 (43), 393 (4), 327 (9), 283 (2), 281 (8)	1.13
gramisterol	412 (M^+, 19), 397 (15), 379 (3), 328 (21), 313 (5), 285 (96), 269 (11), 267 (6)	1.18
cycloeucalenol	426 (M^+, 4), 411 (6), 408 (9), 393 (10), 300 (6), 299 (1), 283 (8), 267 (4)	1.20
unidentified	426 (M^+, 9), 411 (6), 328 (3), 285 (27), 269 (6), 267 (2)	1.28
citrostadienol	426 (M^+, 4), 411 (6), 393 (1), 328 (41), 285 (100), 269 (7), 267 (8)	1.34
	4,4-Dimethylsterols or Triterpene Alcohols	
cycloartenol	426 (M^+, 7), 411 (10), 408 (12), 393 (15), 365 (7), 339 (7), 286 (14), 271 (8)	1.24
24-methylenecycloartenol	440 (M^+, 6), 425 (7), 422 (11), 407 (15), 379 (5), 353 (6), 300 (14), 285 (4)	1.32
cyclobranol	440 (M^+, 9), 425 (13), 422 (15), 407 (17), 379 (10), 353 (8), 300 (18), 285 (4)	1.42

[a] RR_t = retention times relative to cholesterol.

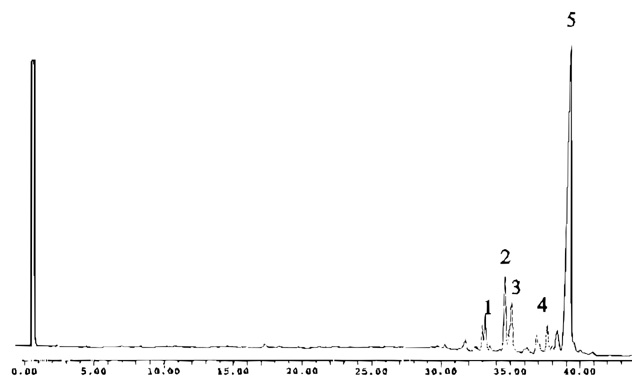

Figure 4. Gas chromatogram of the 4-methylsterols in jojoba oil: (1) obtusifoliol; (2) gramisterol; (3) cycloeucalenol; (4) unidentified; (5) citristadienol.

compatible with both fucosterol and isofucosterol. The mass spectra of the isomers 24(*E*)-ethylcholesta-5,24(28)-dien-3β-ol or fucosterol and 24(*Z*)-ethylcholesta-5,24(28)-dien-3β-ol or isofucosterol are identical as already described by Brooks et al. (1972). However it was possible to identify the component as isofucosterol on the basis of RR_t from the reference isloated from corn oil and the data mentioned in the literature by Itoh et al. (1982).

Identification of 4-Methylsterols. The fraction eluting from the silica column after the fatty alcohols and with an R_f value of 0.25 on TLC was examined in the same way. Peaks were identified by comparison of the obtained gas chromatographic and mass spectrometric data with the corresponding data of reference compounds isolated from corn oil or data described in the literature. A gas chromatogram is represented in Figure 4 with only one major compound along with minor peaks. The GC−MS results are represented in Table 2. The major compound was definitively identified as citrostadienol (RR_t = 1.34). In the same way two minor peaks in the chromatogram could be identified as obtusifoliol (RR_t = 1.13) and gramisterol (RR_t = 1.18). A third minor 4-methylsterol, RR_t of 1.20, could be identified by means of the gas chromatographic data (Itoh et al., 1982) and the mass spectrum mentioned in the literature (Itoh et al., 1974) as cycloeucalenol. The product shows a fragment ion at m/z 300, typical for 4-methylsterols containing the 9,19 cyclopropane ring. The component with RR_t = 1.28 and with mass spectrum similar to the spectrum of citrostadienol (Table 2) could not be identified.

Identification of Triterpene Alcohols (4,4-Dimethylsterols). The fraction eluting in front of the fatty alcohols on the silica column and with R_f = 0.32 corresponds to the triterpene alcohol fraction was first purified by preparative TLC. The gas chromatogram (Figure 5) of this fraction showed one major and two minor peaks with characteristic ion fragments from triterpene alcohols. The obtained GC−MS data are represented in Table 2. The three mass spectra show fragment ions at either m/z 300 or 286. Those fragments are typical for the fragmentation of 9,19-cyclosterols as described by Aplin and Hornby (1966). The positions of those peaks are independant of the C_4 substitution but are shifted in accordance with the substituents present in the side chain. These data correspond with those of 24-methylenecycloartanol (major compound, RR_t = 1.34), cycloartenol (RR_t = 1.24), and cyclobranol (RR_t = 1.42).

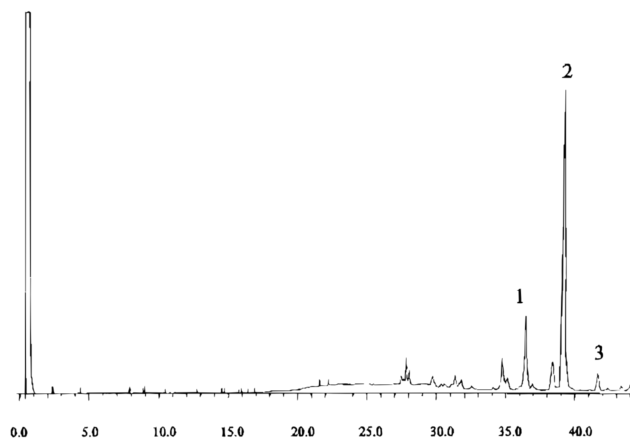

Figure 5. Gas chromatogram of the triterpene alcohols in jojoba oil: (1) cycloartenol; (2) 24-methylenecycloartenol; (3) cyclobranol.

Quantitative Results. The calibration curves for the quantitative determination of the free 4-demethylsterols were linear in the described concentration range, and no difference was observed between the calibration curves obtained for the extracts before (r = 0.997) or after purification by TLC (r = 0.997). The calibration curve constructed for fucosterol was used for the quantitative determination of isofucosterol, because pure isofucosterol was not available. The results obtained for the concentration of the free sterols in jojoba oil with and without TLC purification were not significantly different as proved by the mentioned correlation coefficients for both calibration curves.

Free sterols accounted for 0.4% of the examined jojoba oil. The total free sterol pattern (w/w) of jojoba oil was

as follows: 7% isofucosterol, 18% 24-methylcholesterol, 7% stigmasterol, and 63% sitosterol. The relative quantity of 4-methylsterols and triterpenes measured on the basis of the areas of the gas chromatographic peaks correspond to 5% of the free sterols. This composition is of no importance for the determination of the free 4-demethylsterols.

Direct gas chromatography of the fraction absorbed on the aluminum oxide column allows for an easy determination of free sterols and fatty alcohols in jojoba oil. This procedure allows for a discrimination between natural jojoba oil and synthetic substitutes. Only for the determination of minor 4-methylsterols and triterpenes do the aluminum oxide extracts have to be separated by TLC or by column chromatography prior to the gas chromatography.

CONCLUSIONS

Column chromatography on aluminum oxide resulted in the isolation of the free phytosterols and free fatty alcohols from jojoba oil. Subsequent column chromatography on silica gel allows us to separate the minor constituents in four groups. Only the resolution of capillary gas chromatography is sufficient for the separation of the different compounds of the four groups. In this way it was possible to identify the following components: (A) the fatty alcohols (octadec-9-enol, eicos-11-enol, docos-13-enol, and tetracos-15-enol); (B) the 4-demethylsterols (24-methylcholesterol, stigmasterol, sitosterol, and isofucosterol); (C) the 4-methylsterols (citrostadienol, cycloeucalenol, grammisterol, and obtusifoliol); and (D) the triterpene alcohols (cycloartenol, 24-methylenecycloartanol, and cyclobranol).

This composition allows for an easy method for the determination of phytosterols in jojoba oil following extraction of the oil, spiked with internal standard, by means of an aluminum oxide column and direct gas chromatographic analysis of the extract.

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