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The Composition of Rat Skin Surface Lipids. Analysis of the Saponifiable and Nonsaponifiable Fractions

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The alkaline hydrolysis products of skin surface fat of normal rat have been analyzed in detail by combination of thin-layer chromatography and gas-liquid chromatography. Homologue series of $C_{14}-C_{34}$ straight and branched chain saturated as well as straight chain unsaturated fatty acids and aliphatic monohydric alcohols with maximum concentrations at $C_{20:1}$ and $C_{24:1}$, respectively, are present. There are also smaller amounts of monohydroxy fatty acids and alkane diols with C_{16}-compounds as the major components. In addition to cholesterol as the chief representant, also other sterols are found and tentatively identified as lanosterol, agnosterol, methosterol, desmosterol and lathosterol.

The skin surface fat consists of a complicated lipid mixture, which is typical for each species of mammals.[1] The *seba* of man [2–7] and sheep [8] have been thoroughly analyzed using modern methods. A comparative study on the *seba* of the common laboratory animals has been made by Wheatley and James.[9] The present paper reports the fractionation of the skin surface lipid of normal rat into saponifiable and nonsaponifiable components and their subsequent analysis with thin layer chromatography (TLC) and gas-liquid chromatography (GLC).

MATERIALS AND METHODS

Male Wistar rats weighing 150–200 g were used in the experiment. The animals were fed *ad libitum* a standard diet, used in this laboratory for years. It consisted of: 100 parts of soybean, 100 parts of corn, 12 parts of defatted milk solids, 5 parts of commercial margarine, and 2 parts of salts (equal amounts of sodium chloride and calcium for animal feeding); once a week vegetables, cod liver oil, yeast and boiled lung were given. Before feeding, the dry mixture was soaked with about 1 ½ of its volume of water. Cages with large-meshed bottoms were used to avoid contamination of the hair of the animals with food and faeces.

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For extraction of lipids the animal was anaesthesized with ether. The feet, the tail and the region of anus were washed with about 10 ml of acetone each. Making sure that the narcosis was sufficient, the rat was grasped with a forceps at the neck and immersed into 350 ml of acetone in a glass beaker about five times during one minute; care was taken to keep the ears and the mouth above the solvent surface. The hair was rinsed with an additional 100 ml of acetone, which was allowed to run into the same beaker. After the above procedure, the hair was washed with bodywarm tap water, dried with a clean tissue, and the rat was replaced in the cage.

The acetone extract was filtered and evaporated in a rotating vacuum evaporator at 40°C. The residue was dissolved in petroleum spirit, dried with anhydrous sodium sulphate, filtered, evaporated as above, and weighed. The extractions were usually done at 1–3 days intervals. The amount of lipid obtained from each individual rat at equal time intervals was fairly constant (from 130 to 170 mg/day/kg of body weight).

Before hydrolysis of the sample, the free fatty acids were extracted with 0.05 N sodium hydroxide in ethanol-water 1:1. The alkaline hydrolysis was carried out in 10 % potassium hydroxide in methanol-toluene-water 9:1:1 under reflux in nitrogen-atmosphere over night. The nonsaponifiable and saponifiable fractions were extracted with ethyl ether-petroleum ether 1:4 (v/v).

The saponifiable material was esterified in 2 % sulphuric acid in methanol under reflux for 4 h. The methyl esters were extracted with petroleum spirit.

Acetylation of the hydroxy compounds was performed in acetic anhydride in sealed tubes at 100°C for 4 h. The compounds were then extracted with petroleum spirit, which was washed with 5 % sodium bicarbonate solution and several times with water.

Hydrogenation was carried out in hexane or methanol in test tubes: a small amount of platinum oxide (Matheson, Coleman & Bell) was added as catalyst and hydrogen was allowed to bubble through a capillary in the solution for 20 min.

The following solvents were used:

acetone *puriss.*, treated with anhydrous calcium chloride and redistilled.
methanol *purum*, redistilled after treatment with magnesium turnings and iodine.
chloroform (*PhF* VII 930/62), treated with calcium chloride, redistilled and mixed with 1 % of methanol.
hexane (pure, Merck).
petroleum spirit (60–80°, Analar).
ethyl ether (G.R., Merck).
benzene cryst. (G.R., Merck).
ethyl acetate (for chromatography, Merck).

The following reference compounds were used:

Fatty acid methyl esters:
Fatty acid methyl ester quantitative mixture (H–104, Applied Science Laboratories, lot No. 458–43).
Methyl esters obtained from commercial corn oil.
Linolenic acid (55 %, Nutritional Biochemicals Corp.) methyl esters.
Hydroxy fatty acids:
α-Hydroxy myristic acid (Applied Science Laboratories, lot No. 335–12).
α-Hydroxy hexacosanoic acid (Calbiochem, Cat. No. 39135).
12-Hydroxy stearic acid (Calbiochem, Cat. No. 39957).
Monohydric alcohols:
Myristyl, cetyl and stearyl alcohols (Applied Science Laboratories, lots Nos. 76–22, 230–70, and 230–72, respectively).
Dihydric alcohols:
α-Hydroxy myristyl alcohol was derived from α-hydroxy myristic acid using the method described by Link *et al.*[10]
12-Hydroxy stearyl alcohol (Calbiochem, cat. No. 39959).
Glyceryl ethers (chimyl, batyl and selachyl alcohols) as well as
Sterols (cholestane, cholesterol, lathosterol and lanosterol) were obtained as a gift from Dr. E. C. Horning, Baylor University College of Medicine, Houston, Texas.

Silicic acid (Mallinckrodt, 100 mesh) was sieved to yield 80–200 mesh material, washed with 3 N hydrochloric acid and water, and dried at 150°C in vacuum for 24 h. Water was added to obtain 10 % moisture.

For TLC, equipment manufactured by Desaga (Heidelberg) was employed. The plates were coated with 0.25 mm layer of Kieselgel G. (Merck) and dried at 120° for one hour. For visualization of the fractions the plates were sprayed either with 30 % sulphuric acid and charred at 120°, or with 0.1 % Rhodamine 6G (BDH) in ethanol.

Isothermal GLC was performed in a Barber-Colman M-10 chromatograph using an argon ionization detector. The following columns and conditions were used:

a 6 ft column of 10 % ethylene glycol adipate polyester (EGA) on 100—140 mesh siliconized Gas-Chrom P (Applied Science Laboratories); argon pressure 1.0 atm.; temperature 189°C.

a 12 ft column of 3 % EGSS-X polyester on 100—140 mesh siliconized Gas-Chrom P (Applied Science Laboratories); argon pressure 1.5 atm.; temperature 189°C.

a 6 ft column of 1 % silicone gum SE-30 on 100—140 mesh siliconized Gas-Chrom P (Applied Science Laboratories); argon pressure 1.0 atm.; temperature 211°C.

The programmed temperature GLC was done in an apparatus described by Haahti.[3] It was equipped with a 6 ft "Cobra" column containing 1 % SE-30 on 100—140 mesh siliconized Gas-Chrom P. Argon ionization detector was used. The pressure of argon was 0.7 atm.

For calculation of the relative amounts of compounds in the gas-chromatograms, each peak of the record was cut off and weighed.

RESULTS

From 67.1 mg of rat skin surface lipid 28.7 mg of saponifiable and 29.2 mg of nonsaponifiable matter were recovered. These were analyzed with TLC (Fig. 1.) It was shown that the saponifiable fraction was not contaminated with the nonsaponifiable lipids.

The free fatty acids of the original sample amounted to 1 % of the total fat. They were not studied further.

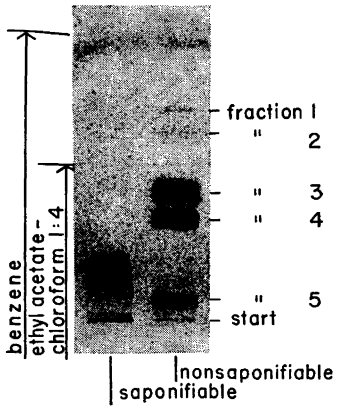

Fig. 1. TLC-pattern of the alkaline hydrolysis products of rat skin surface lipids. *Composition of fractions:* 1—2:unidentified, 3:lanosterol, methosterol, aliphatic monohydric alcohols; 4:cholesterol, lathosterol, desmosterol; 5:dihydric alcohols.

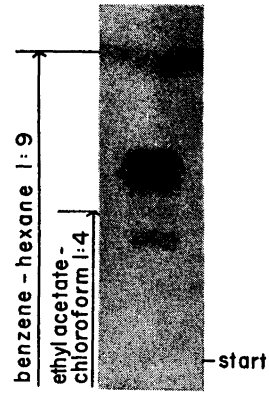

Fig. 2. TLC-pattern of the methyl esters of the fatty acids of rat skin surface lipids.

1. *The saponifiable matter*. The methyl esters of the saponifiable matter were examined with TLC. As seen in Fig. 2, two fractions were resolved: the faster moving one migrated as do the reference methyl esters of aliphatic (nonhydroxylated) fatty acids, the more polar one migrated as do the methyl esters of reference monohydroxy fatty acids. These two fractions were isolated using silicic acid column chromatography: the nonhydroxy methyl esters were eluted with 40 % of benzene in hexane, the hydroxy esters with 10 % of chloroform in benzene; they constituted 89 % and 11 %

Table 1. The percentage composition of the fatty acid methyl esters and acetylated monohydric alcohols calculated from the records of isothermal GLC on EGSS-X at 189°C.

Identification	Fatty acids %	Alcohols %
14:0 iso	1.1	0.4
14:0	0.7	1.1
15:0 anteiso	1.2	0.6
15:0	0.7	0.5
16:0 iso	4.5	3.2
16:0	10.4	2.1
16:1	1.9	0.1
17:0 anteiso	3.8	1.3
17:0	1.2	0.2
18:0 iso	3.8	1.7
18:0	3.5	2.6
18:1	9.3	0.7
18:2	3.0	—
19:0 anteiso	1.4	0.7
19:0	0.3	0.2
20:0 iso	6.2	7.4
20:0	2.0	2.8
20:1	12.5	1.6
20:2	0.2	—
21:0 anteiso	3.6	5.5
21:0	0.4	0.5
22:0 iso	3.3	6.1
22:0	1.9	4.1
22:1	3.7	6.7
23:0 anteiso	2.0	2.1
23:0	0.9	0.5
24:0 iso	2.1	1.5
24:0	3.5	6.6
24:1	2.4	21.6
25:0 anteiso	0.9	0.6
25:0	0.7	1.5
26:0 iso	0.8	—
26:0	0.7	2.9
26:1	0.9	12.6
27	0.4	
28	1.0	
30	1.2 *	
32	1.4	
34	0.5	

* The figures were obtained from the record of programmed temperature GLC.

of the saponifiable matter, respectively. The purity of the isolated fractions was checked with TLC.

The methyl esters of the (nonhydroxy) fatty acids were examined both as such and after hydrogenation with isothermal GLC on EGSS-X. Temperature programmed GLC was also used in order to scan a wider molecular weight range (Fig. 3). The peaks were identified with the aid of standard

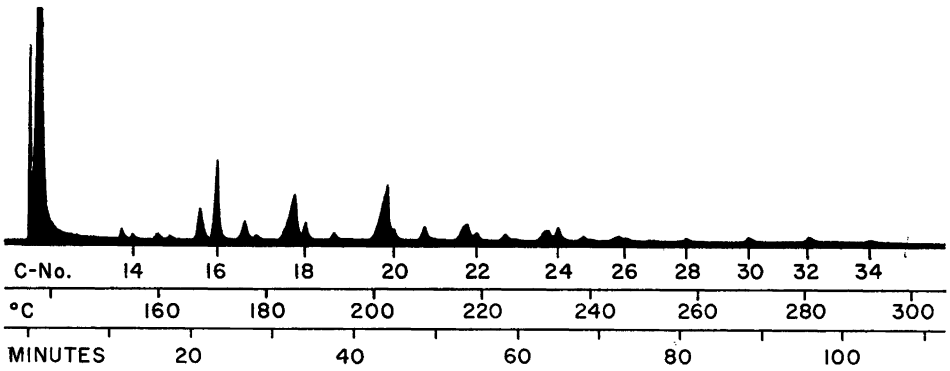

Fig. 3. Programmed temperature GLC-record of the (nonhydroxy) fatty acid methyl esters of rat skin surface lipid on SE-30. For conditions: see text.

mixtures of fatty acid methyl esters and the "carbon number"[11] characterization. The identification and quantitative composition of these fatty acid methyl esters is shown in Table 1.

Correspondingly the methyl esters of monohydroxy fatty acids were examined in isothermal GLC on EGA (Fig. 4). Also here temperature pro-

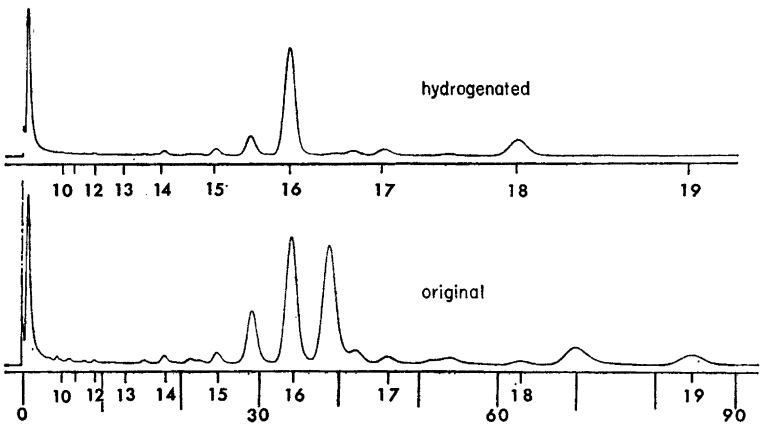

Fig. 4. GLC-record of the monohydroxy fatty acid methyl esters of rat skin surface lipid on EGA. The scales refer to "carbon numbers" and minutes, respectively. For conditions: see text.

Table 2. The percentage composition of the methyl esters of hydroxy fatty acids and the acetylated dihydric alcohols calculated from the records of GLC on EGA (Fig. 4) and EGSS-X (Fig. 6), respectively.

Identification	"Carbon number"	OH-acids %	Diols %
10:0 iso?	9.53		
10:0	10.00		
Unknown	10.44	0.8	—
12:0 iso?	11.53		
12:0	12.00		
14:0 iso?	13.54	0.4	—
14:0	14.00	1.0	1.5
Unknown	14.52	0.6	0.2
15:0 anteiso?	14.68	0.4	—
15:0	15.00	1.6	4.6
16:0 iso?	15.51	10.4	12.1
16:0	16.00	29.0	61.2
16:1	16.41	31.8[a]	—
Unknown	16.51	—	3.1
17:0 anteiso?	16.71	3.5	2.1
17:0	17.00	2.3	4.2
17:1?	17.37	1.2[a]	—
18:0 iso?	17.50	2.5	5.8
18:0	18.00	1.3	5.4
18:1	18.34	8.5[a]	—
18:2	18.97	4.7[a]	—

[a] The peak disappears after hydrogenation.

grammed GLC was applied for analysis of both the native and acetylated esters. The identification of the component monohydroxy acid methyl esters was accomplished comparing their retention times in both the above columns to those of reference (nonhydroxy) methyl esters and of methyl esters of reference α-hydroxy myristic acid and α-hydroxy hexacosanoic acid. Conventional homologue plots were made and the "carbon numbers" were calculated. The tentative identification as well as quantitation is shown in Table 2.

Table 3. The percentage composition of the sterols of the rat skin surface nonsaponifiable lipids calculated from the GLC record on SE-30 (Fig. 5).

Ret. time relative to cholestane on 1 % SE-30 at 211°C	Reference compound with the same relative retention time	Possible constituents according to rel. ret. times	%
2.01	cholesterol	cholesterol	27.1
2.31	lathosterol	lathosterol, desmosterol	39.1
2.52		derivatives of	14.6
2.70		methosterol	8.7
3.22		agnosterol	7.5
3.46	lanosterol	lanosterol	3.0

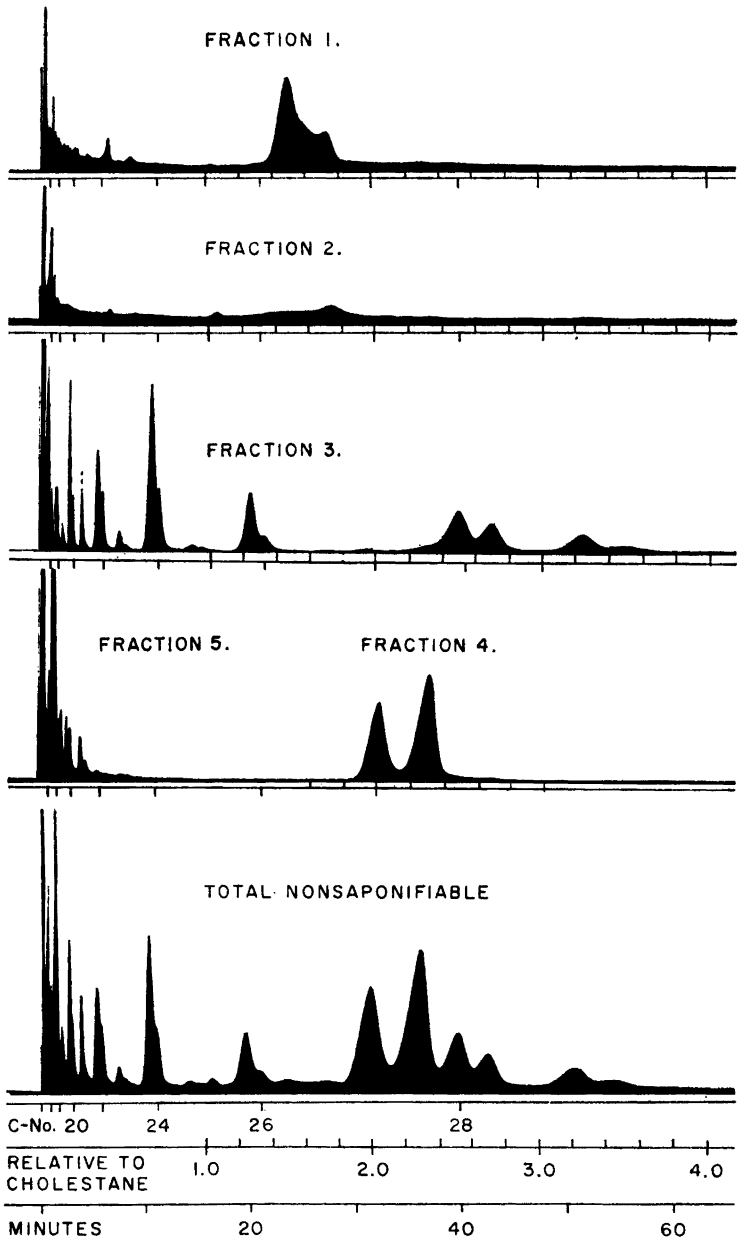

Fig. 5. Isothermal GLC-patterns of the nonsaponifiable matter of the rat skin surface lipids on SE-30. The records of fractions 4 and 5 have been superimposed. "Carbon numbers" refer to the aliphatic alcohols. The fractions correspond to those of Fig. 1. For conditions: see text.

2. *The nonsaponifiable matter.* 20 mg of the nonsaponifiable matter were separated into the five fractions indicated in Fig. 1 on 5 TLC-plates. The fractions were extracted with chloroform from the silica gel powder. 78 % of the material applied on the plates was recovered in the extracts. The relative amounts of the fractions 1, 2, 3, 4 and 5 were 4, 5, 45, 36, and 10 %, respectively.

When the nonsaponifiable matter was acetylated and reanalyzed with TLC, two major fractions were resolved. The less polar band moved slightly faster than the native fraction 1 and corresponded to the acetyl derivatives of fractions 3—4; the more polar band moved between the native fractions 2 and 3 and corresponded to the acetylated fraction 5. It was obvious that the nonsaponifiable matter consisted of monohydric (fractions 3—4) and dihydric alcohols (fraction 5.) The total nonsaponifiable as well as the different fractions were analyzed with both isothermal (Fig. 5) and programmed temperature GLC on SE-30.

The fractions 1 and 2 were not identified, due to the very small amount of material. The fraction 1 stained violet both with $SbCl_3$ and Lieberman-Burchard-reagents; the fraction 2 yielded a brownish-yellow colour with both reagents. They might be degradation products of sterols.

Three reference sterols, cholesterol, lathosterol and lanosterol, were available. Cholesterol moved in the front part of fraction 4 on TLC. The remainder of the fraction 4 had the staining- and chromatographic characteristics of the reference lathosterol. However, desmosterol, when present, would probably move together with lathosterol. The reference lanosterol migrated in the front part of the TLC-spot corresponding to fraction 3. It was followed by other (probably 4-methyl substituted) sterols together with the aliphatic alcohols (Fig. 5). The separation of the aliphatic alcohols from the sterols was not attempted.

Table 3 shows the composition of the sterols calculated from the GLC-records shown in Fig. 5. The sterols were identified using the above reference sterols as well as cholestane as internal standards.[12]

For analysis of the aliphatic alcohols, the fraction 3 was acetylated (and an aliquot hydrogenated) and subjected to GLC in an EGSS-X-column. The identification was done as with the fatty acid methyl esters using myristyl, cetyl and stearyl alcohols as standards. The results are given in Table 1. Because the sterols overlap the C_{28} alcohols, only alcohols up to C_{26} are shown in the Table 1. Analysis of alcohols of waxes which primarily had been separated from sterol esters revealed the presence of small amounts of alcohols with 28, 30, and 32 carbon atoms. Their amount was estimated to be smaller than 5 % of the total alcohols.

Fraction 5 of the nonsaponifiable matter, the dihydric alcohols, was compared both in TLC and GLC (on EGA, EGSS-X and SE-30) with reference alkane diols and with reference glyceryl ethers. A fatty acid methyl ester mixture was used as internal standard in the GLC of the diol sample. A homologue plot was constructed and the "carbon numbers" calculated for each column. The behaviour of the diol fraction and its acetyl derivatives corresponded to that of a homologue series of saturated 1,2-diols with 14 to 18 carbon atoms. Fig. 6 shows the GLC-pattern of the acetylated

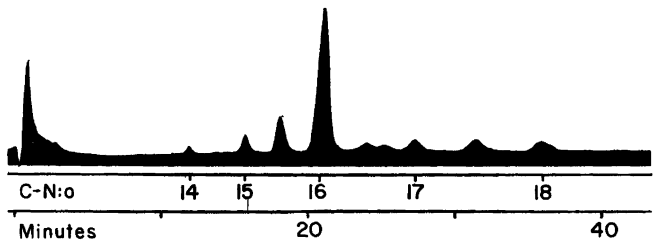

Fig. 6. GLC-pattern of the acetylated alkane-diols of the rat skin surface lipids on EGSS-X. For conditions: see text.

fraction 5 in an EGSS-X-column and Table 2 presents its calculated composition.

On basis of the GLC-data, the relative amounts of different classes of compounds in the nonsaponifiable matter of rat skin surface lipid were calculated. The relative amounts of fractions 1+2; sterols;aliphatic monohydric alcohols; and 1,2-diols was 3.2, 52.2, 35.8 and 8.8 %, respectively. The figure for aliphatic monohydric alcohols is slightly too small because it comprises only the alcohols up to C_{26}.

DISCUSSION

The above results on the fatty acid composition of rat sebum agree well with those of Wheatley and James,[9] but it has now been possible to extend the analysis to include the fatty acids up to C_{34}.

No mention was made by Wheatley and James about the occurrence of hydroxylated fatty acids, which in our material comprise about one tenth of the fatty acids. Recently, Kishimoto and Radin[13] have investigated the α-hydroxy acid composition of the whole skin of rat. They report the presence of α-hydroxy fatty acids up to C_{26} with maximum concentration at C_{16} and C_{24}; no branched chain hydroxy acids were found by them. However, in skin surface fat, we could not detect any monohydroxy acids of chain length longer than C_{18}. The question-marked hydroxy acids in Table 2 might well represent the iso- and anteiso-derivatives of the corresponding straight chain acids, but the presence of acids with the OH-group in a position other than α- is also possible.

The percentage composition of the nonsaponifiable material agrees with that reported by Wheatley and James,[9] except the much higher figure for sterols obtained by us (52 %) than by them (25 %). Their value for "unidentified" material (22 %) might include some sterols. The sterol pattern of our material is fairly similar to that presented quite recently by Horlick and Avigan[14] for the whole skin of rat, except the lower ratio of cholesterol to desmosterol+lathosterol in our material.

The composition of the aliphatic alcohols of rat skin surface lipid has not been studied before. The alcohols display a similar "spectrum" of different

homologues as do the fatty acids. The alcohols exhibit maximum concentration at $C_{24:1}$ and the fatty acids at $C_{16:0}$ and $C_{20:1}$. When randomly combined, the resulting waxes could be expected to show maxima at C_{40}—C_{44}.

As with the hydroxy acids, only straight chain 1,2-diols could be compared to the respective reference compounds; the other peaks of the gas liquid chromatogram might represent the iso- and anteiso-derivatives or diols otherwise substituted. Noteworthy is the entire lack of unsaturated diols as well as the great resemblance of the diol "spectrum" to that of the hydroxy fatty acids (Table 2); both show maximum concentration at C_{16} and are devoid of any appreciable amounts of compounds with carbon atoms more than 18.

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