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CAPILLARY GAS CHROMATOGRAPHIC ANALYSIS OF HUMAN SKIN SURFACE LIPIDS AFTER MICROSAMPLING ON GROUND-GLASS PLATELETS

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SUMMARY

Small amounts of human skin surface lipids, in the 1—20 μg range, sampled on ground-glass platelets are investigated using capillary gas chromatography.

A first system allows the separation of the neutral lipids, up to the triglyceride fraction. A second system reveals the distribution of the free fatty acids or of the free + glyceride fatty acids, after a methylation or transesterification step.

Examination of samples from nine subjects shows that the unsaturation of the free fatty acids increases during a four-day period of accumulation. Comparison of the free fatty acid fraction and the free + glyceride fatty acid fraction shows that the free fraction is more saturated than the latter. It is concluded that the bacterial lipases which cleave the fatty acids from the ester bond favor the liberation of straight-chain saturated fatty acids from sebum triglycerides.

This result is confirmed by comparison of the free fatty acid fraction with the glyceride fatty acid fraction separated from bulk samples of skin surface lipds from hair and scalp.

INTRODUCTION

Human skin surface lipids (SSL) are a very complex and rather unique mixture [1] which has been investigated using various sampling procedures and analytical techniques. The average class composition of the mixture is [2]: squalene 11—13%; wax esters 20—22%; sterol esters 2—3%; free fatty acids 30—33%; sterols 1—2%; glycerides 29—32%.

In the early seventies, Schaefer and Kuhn-Bussius [3, 4] described an original method for the quantitative determination of human SSL: during the application of a small ground-glass platelet upon the skin, lipids were deposited

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on the rough surface. The transparency of the glass, as measured using a photometric device, could be correlated with the amount of lipidic material deposited on it. Later other authors used this procedure to collect quantitative data, such as the casual level of SSL [5, 6] or the sebum excretion rate [7].

If considered as a sampling method for SSL, the ground-glass method has several positive features: the procedure is quick and easy to perform; the sampling site is not attacked by any solvent or surfactant; the sampled lipids are not diluted in a solution or an emulsion.

Therefore, it seemed very attractive to develop adequate methods for closer qualitative and quantitative examination of SSL deposited on ground-glass platelets. A thin-layer chromatographic (TLC) method, which separates the mixture into five basic classes (cholesterol, free fatty acids, triglycerides, wax esters and squalene) has been reported recently [8]. However, this analytical approach does not give any information about the distribution of the individual components within the basic classes, and does not consider the sterol ester fraction. Here we describe chemical derivatization procedures of these microsamples, followed by capillary gas chromatography.

EXPERIMENTAL

Sampling procedures

The ground-glass microsampling procedure was performed according to the description of Saint-Léger and Bague [8]. Bulk sampling of SSL was performed from individual subjects by washing the scalp and hair with a surfactant solution. The lipids were extracted from the emulsion using diethyl ether [9].

Fractionation of the bulk samples

The bulk samples from individual subjects were fractionated into the free fatty acid fraction and the glyceride esterified fatty acid fraction according to the method of Boré et al. [10]. A pool of SSL (from several subjects) was fractionated using preparative TLC and the following basic classes of components were recovered: wax esters; sterol esters; triglycerides.

The separation was performed on silica gel G precoated plates with a thickness of 250 μm (Uniplate® from Anachem, Luton, Great Britain). The plates were cleaned by elution with diethyl ether. Then 150 μl of chloroform containing 23 mg of human SSL were deposited on a 16-cm length with a Linomat III (from Camag, Muttenz, Switzerland). A first elution was performed using hexane, followed, after drying of the plate, by elution with benzene. The eluted strips were revealed at their extremities by carbonisation, scraped out and the lipidic fractions extracted with diethyl ether. Each fraction was then rechromatographed using the same conditons, in order to ensure minimal contamination from its neighbours.

Prechromatographic treatment of microsamples

The loaded ground-glass platelet is placed into a 1-ml Reacti-Vial® (from Piearce Eurochem B.V., Rotterdam, The Netherlands) and rinsed by addition of 400 μl of diethyl ether or dichloromethane. For absolute quantitation of

the chromatogram, a standard solution of an adequate internal standard is used instead of pure solvent.

After homogenisation, the platelet is withdrawn from the vial; half of the solution is transferred into a second vial and both solutions are evaporated to dryness under a nitrogen stream at room temperature.

Direct chromatographic analysis without any derivatization can be performed after redissolution of the residue in 50 μl of diethyl ether or dichoromethane.

For derivatization with diazomethane, 200 μl of the reagent (3 g of diazomethane in 250 ml of diethyl ether prepared according to ref. 11 are added to the evaporation vial. The mixture is allowed to stand at room temperature for a few minutes and then evaporated to dryness at room temperature under a stream of nitrogen. The residue is dissolved in 50 μl of diethyl ether or dichloromethane and injected.

For transesterification of the glycerides, 50 μl of reagent [Meth-Prep II from Applied Science Labs., State College, PA, U.S.A.: 0.2 *N* methanolic solution of (*m*-trifluoromethylphenyl)trimethylammonium hydroxide] are added to the evaporation vial. The mixture is allowed to react at room temperature during 15 min and then injected.

Gas chromatography of the fatty acids

A borosilicate glass capillary (90 m × 0.3 mm I.D.) was preconditioned using the $BaCO_3$ procedure of Grob et al. [12—14]. The tube was then dynamically wall-coated with free fatty acid phase (FFAP) stationary phase.

The operating conditions were:
injector and flame ionization detector temperature, 230°C; column temperature, programmed from 150°C to 210°C at 3°C min^{-1}; carrier gas, helium; inlet pressure, 2 bars; injected volume, 3 μl; split ratio, 1:10.

The internal standard for absolute quantitation was *n*-heptadecanoic acid. Five micrograms of internal standard are a convenient amount for platelets loaded with 1—30 μg of SSL. Possible substitutes to *n*-heptadecanoic acid as an internal standard are *cis*-9-hexadecenoic acid (palmitoleic acid) or 2-hydroxypalmitic acid.

Gas chromatography of the neutral lipids

A borosilicate glass capillary (9 m × 0.3 mm I.D.) was preconditioned using the $BaCO_3$ procedure of Grob et al. [12—14]. The tube was then dynamically coated with OV-1 stationary phase.

The operating conditions were: injector and temperature, 350°C; column temperature, programmed from 200°C to 330°C at 4°C min^{-1}; carrier gas, helium; inlet pressure, exponentially programmed from 0.2 to 2 bars in 50 min using an FP 222 flow programmer from Dani, Monza, Italy. The injected volume was 5 μl in the splitless mode according to the procedure of Grob et al. [15, 16].

Evaluation of the analytical procedure

The free fatty acid and glyceride fatty acid percentages of a bulk sample of SSL were determined by gravimetry after fractionation. The sample was found

to contain 25 ± 2% of free fatty acids and 35 ± 2% of glyceride fatty acids.

Standard solutions of this sample in diethyl ether or dichloromethane (from 50 to 1000 μg/ml) were used to test the reproducibility and linearity of the analytical procedure. Therefore, aliquots (10–20 μl) of the standard solutions were deposited on ground-glass platelets with a microsyringe and, after evaporation of the solvent, the platelets were analyzed using the procedure described above.

RESULTS AND DISCUSSION

Chromatogram of the neutral lipids

The chromatogram of the neutral lipids was obtained after injection of an underivatized sample or after derivatization with diazomethane. The esterified fractions (waxes, sterol esters and triglycrides) which have been isolated by TLC were treated with the diazomethane reagent in the same manner. This showed that no side-reaction such as transmethylation was occurring to a detectable extent during this treatment.

A typical chromatogram of human SSL microsampled from the scalp is shown in Fig. 1. The following compounds or classes of compounds are successively eluted: free fatty acis — these compounds are not properly resolved using these chromatographic conditions; they will be separated on another column; squalene; cholesterol; wax esters, cholesteryl esters and triglycerides — the elution range of these classes of compounds was determined using the fractions isolated from TLC.

No attempt was made to identify single compounds in the wax ester fraction since these species will be further studied by gas chromatography—mass

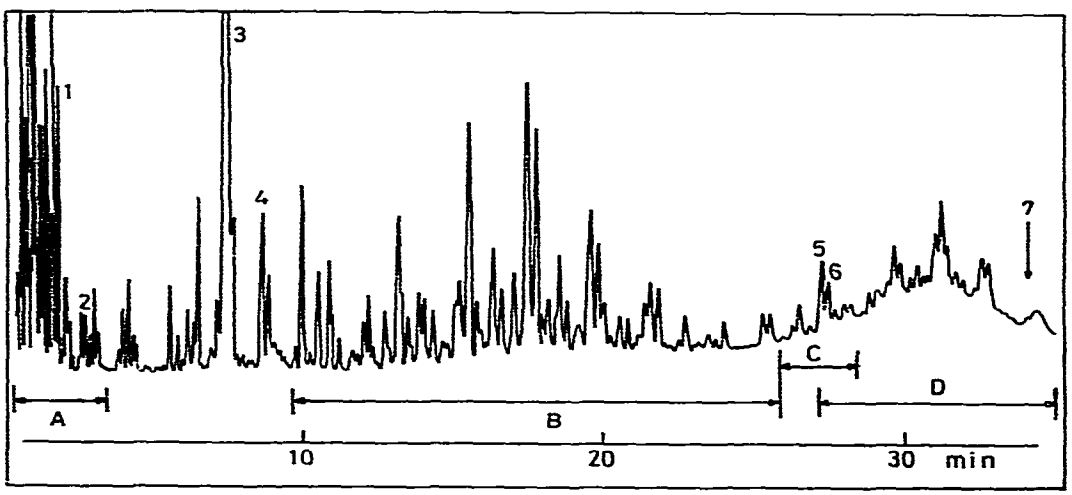

Fig. 1. Chromatogram of the neutral lipids of SSL on an OV-1 capillary column. (A) Elution range of the free fatty acid fraction. (B) Elution range of the wax esters. (C) Elution range of the sterol esters. (D) Elution range of the triglycerides. 1 = Stearic acid, 2 = arachidic acid, 3 = squalene, 4 = cholesterol, 5 = cholesteryl palmitoleate, 6 = cholesteryl palmitate, 7 = elution position of tristearin.

spectrometry. Cholesteryl palmitoleate and palmitate proved to be the main components in the sterol ester fraction.

The elution ranges of the sterol esters and the triglycerides overlap partially. The latter fraction extends up to compounds which are somewhat heavier than tristearin. Using flow programming in conjunction with temperature programming, complete elution of that fraction could be achieved, as shown by the return to baseline at the end of the chromatogram.

Chromatogram of the free fatty acid fraction

The free fatty acids are converted into the corresponding methyl esters with diazomethane. A typical chromatogram is shown in Fig. 2. The chain lengths are within the C_{12} to C_{20} range, with the straight-chain saturated and monounsaturated species being prominent in the mixture. The monounsaturated chains from C_{14} to C_{17} bear their double bond at the 6-position [17] whereas the peak corresponding to the C_{18} species is of composite structure, indicating a mixture of positional *cis*-isomers [17]. The elution level of *cis*-9-octadeconoic acid (oleic acid) has been determined by addition of the reference compound.

Heptadecanoic acid is a minor component in the mixture (1–2%). It is possible to use it as an internal standard provided it is added to the sample in a sufficient amount, the endogenous contribution then being negligible (5 μg of internal standard for SSL samples in the 1—30 μg range).

cis-9-Hexadecenoic acid (palmitoleic acid) is another possible choice as an

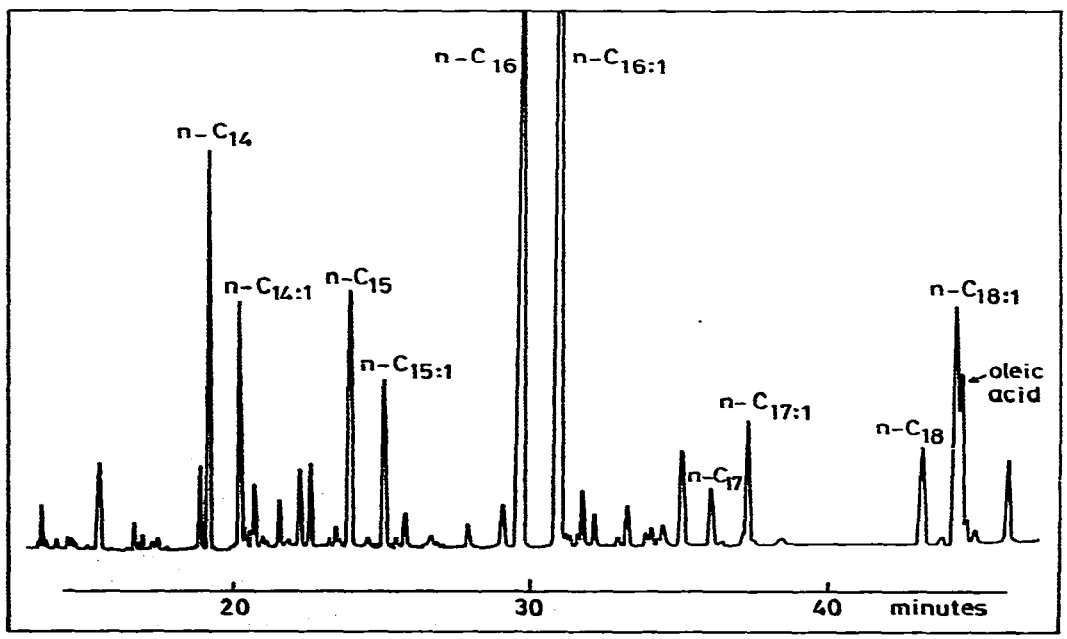

Fig. 2. Chromatogram of the fatty acids of SSL on an FFAP capillary column. The *n*-monoenic acids with chain length from 14 to 17 carbon atoms are 6-unsaturated. A number of positional isomers are found at the C_{18} level, the 8- and 9-unsaturated species being predominant. The other peaks of the chromatogram, between *n*-C_{14} and *n*-C_{18} arise from branched-chain, mainly saturated, species [17].

internal standard, it being eluted just after its positional isomer. The separation of the isomers, however, is very critical, requiring a column of good efficiency.

α-Hydroxy-palmitic acid is also possible internal standard; on our column we found it to be eluted just before heptadecanoic acid, at a level where no compound of endogenous origin interferes. However, since this hydroxy compound is not homologous to the fatty acids, its retention relative to the acids may be not very reproducible from column to column, or may change as a column ages. As a consequence, interferences with endogenous compounds then become possible.

Derivatization with Meth-Prep II®

The effect of this reagent upon the different species found in human SSL was investigated using isolated fractions. The triglycerides proved to be quantitatively transersterified by the reagent, within 15 min at room temperature. No significant transformation of the waxes could be observed after contact with the reagent during 15 min at room temperature. However, after 12 h at room temperature, the waxes were quantitatively cleaved into the corresponding fatty alcohols and methyl esters. The cholesteryl esters proved to behave in a manner comparable to the waxes. The free fatty acids were quantitatively derivatized by the reagent to methyl esters, using the same conditions as for triglyceride transesterification.

As a consequence, using both derivatizing reagents and different conditions, separate classes of fatty acids can be determined in human SSL. Derivatization with diazomethane yields the methyl esters of the free fatty acids from SSL. Derivatization with Meth-Prep II® during 10 min yields the free + glyceride fatty acids methyl esters. Prolonged derivatization with Meth Prep II® results in complete recovery of all fatty acid moieties which are found in SSL as methyl esters.

Evaluation of the analytical procedure

Fifteen ground-glass platelets were loaded with 15 μg of SSL, and the amounts of free and free + glyceride fatty acids were determined. The experimental values were free fatty acids 3.70 μg (standard deviation 0.16), and free + glyceride fatty acids 8.89 μg (standard deviation 0.55). These are in good agreement with the composition determined by gravimetry.

The linearity of the analytical procedure was tested by successive analyses of platelets loaded with 1, 2, 3, 5, 10, 15, 20 and 30 μg of SSL. A linear relationship between amounts of free fatty acids and SSL, and amounts of free + glyceride fatty acids and SSL was found, the correlation coefficient of the regression curve being 0.99.

From each chromatogram we also determined the $C_{16}/C_{16:1}$ ratio. The relative standard deviation was 4% for both the free and the free + glyceride fatty acid fractions. Previous studies [9, 17] showed that this ratio is a good reflection of the balance between saturated and unsaturated species in the considered fraction.

In situ evolution of free fatty acids

The free fatty acids of human SSL are believed to originate through enzymatic hydrolysis of the triglycerides by bacterial lipases [2, 18]. There-

fore, an increase in the amount of free fatty acids is expected during accumulation of SSL, resulting from the cumulative effect of continuous excretion and increasing degree of hydrolysis.

An experimental population of nine subjects was investigated to check if the expected evolution can be observed using the ground-glass platelet sampling method. After a cleaning shampoo, SSL aged one, two, three and four days were recovered from neighbouring sites on the scalp and subsequently analyzed for amount and composition of the free fatty acid fraction.

The results are given in Table I. It can be seen that, in some cases, no determination at all could be performed, the amount of free fatty acids being less than 0.1 μg per platelet. This apparent failure of the sampling procedure is probably a consequence of non-uniform distribution of SSL on the scalp.

For most subjects, the amount of free fatty acids (FFA) seems to increase as SSL accumulate on the scalp; however, this evolution as observed through the microsampling methodology is far from being even over the four-day period.

In Table II, we report the values of $\Delta(\mu g\ FFA) = (\mu g\ FFA)_{4\ days} - (\mu g\ FFA)_{1\ day}$ relative to the eight cases for which these data could be obtained. The mean value is 4.16 μg, with a very wide standard deviation (4.25); so it can hardly be stated from these data that the amount of free fatty acids is actually increasing with time of accumulation.

This observation also suggests that, due to non-uniform distribution of SSL, the amount of material which is collected on a single platelet with a diameter of 6 mm does not reflect the average status on the complete site.

The distribution pattern of the free fatty acids seems to undergo an evolution during accumulation of SSL on the scalp, with the relative proportion of unsaturated compounds being increased. This situation can be characterized by the determination of the ratio $C_{16}/C_{16:1}$ as reported in Table I.

In Table II we also report the values of $\Delta(C_{16}/C_{16:1}) = (C_{16}/C_{16:1})_{4\ days} - (C_{16}/C_{16:1})_{1\ day}$ relative to the eight cases for which these data could be obtained. The mean value is -0.50 with a standard deviation of 0.32, which shows that the relative proportion of unsaturated species in the free fatty acid fraction increases during accumulation of SSL on the scalp.

TABLE I

IN SITU EVOLUTION OF THE FREE FATTY ACIDS (FFA) FRACTION DURING ACCUMULATION OF SSL ON THE SCALP

Subject	1 day		2 days		3 days		4 days	
	μg FFA	$C_{16}/C_{16:1}$	μg FFA	$C_{16}/C_{16:1}$	μg FFA	$C_{16}/C_{16:1}$	μg FFA	$C_{16}/C_{16:1}$
1	5.5	1.66	*	*	13	1.11	*	*
2	3.5	2.66	*	*	3.5	1.50	3.5	1.69
3	1	1.15	2.5	0.99	5	0.87	4.2	0.65
4	4.5	1.43	3	1.53	6	1.32	3	0.92
5	3	1.57	4	1.34	12	0.88	15	1.0
6	8	2.00	3.5	1.50	17	1.81	11.5	1.28
7	1	1.48	0.2	1.37	*	*	3.8	0.96
8	1	1.06	2.5	1.08	10.8	0.95	6.5	1.22
9	1.8	1.36	2.6	1.37	5.7	1.20	9.5	0.97

*No determination could be performed, the amount of FFA being less than 0.1 μg per platelet.

TABLE II

VARIATIONS OF AMOUNT AND COMPOSITION OF THE FREE FATTY ACID (FFA) FRACTION WITHIN THE 1—4-DAY PERIOD

$\Delta(\mu g\ FFA) = (\mu g\ FFA)_{4\ days} - (\mu g\ FFA)_{1\ day}$
$\Delta(C_{16}/C_{16:1}) = (C_{16}/C_{16:1})_{4\ days} - (C_{16}/C_{16:1})_{1\ day}$

Subject	$\Delta(\mu g\ FFA)$	$\Delta(C_{16}/C_{16:1})$
2	0	—0.97
3	3.2	—0.50
4	—1.5	—0.51
5	12	—0.57
6	3.6	—0.72
7	2.8	—0.52
8	5.5	0.16
9	7.7	—0.39
Mean	4.16	—0.50
S.D.	4.28	0.32

This result suggests that the enzymatic hydrolysis which cleaves the fatty acid moieties from the triglycerides is not a random process but is actually affecting first the saturated chains.

Another explanation could, however, be proposed, which relates the increasing unsaturation of the free fatty acids to a 6-dehydrogenase from a microbial source. Biotransformations by the enzymes of the skin surface microflora have already been observed, such as 9-hydroxylation of fatty acids [19]. However, a 6-dehydrogenation was never reported; on the contrary the rather uncommon Δ^6 unsaturation in the acidic chains of the SSL is generally considered to be highly characteristic of the sebaceous production.

Comparison of free fatty acids with free + glycerides fatty acids from a single microsample

If the bacterial lipases which hydrolyse the triglycerides proceed in a structure-dependent manner, then there must be a difference between the composition of the free fatty acid fraction and the triglyceride fatty acid fraction. A difference is also to be expected between the composition of the free fatty acid fraction and the free + glyceride fatty acid fraction, and these two fractions can be investigated starting from a single platelet, using the two derivatization procedures described in this paper.

These determinations were performed over a population of nine subjects, and the results are given in Table III. The degree of hydrolysis of the samples can be calculated considering the respective amounts of the free fatty acids and the free + glyceride fatty acids within the investigated population of Table III, this degree of hydrolysis ranges from 0.32 to 0.65. In all cases, the free fatty acid fraction proved to be more saturated than the corresponding free + glyceride fatty acid fraction; from the first fraction to the other, the $C_{16}/C_{16:1}$ ratio decreases by a mean value of 0.38, with a standard deviation of 0.18.

TABLE III

COMPARISON BETWEEN THE AMOUNT AND THE COMPOSITION OF THE FREE FATTY ACID (FFA) FRACTION AND THE FREE + GLYCERIDE FATTY ACID FRACTION DETERMINED FROM THE SAME MICROSAMPLE

Subject	Fatty acids (μg)		$C_{16}/C_{16:1}$ ratio	
	FFA	Free + glyceride FA	FFA	Free + glyceride FA
1	5.5	9.4	1.82	1.51
2	3.8	6.6	1.94	1.76
3	3.2	10	2.05	1.56
4	6.8	13.7	1.50	0.99
5	6.5	11.8	1.56	0.97
6	7.3	18	1.62	1.02
7	10.3	20.2	1.32	1.10
8	6.2	9.5	1.73	1.39
9	5	9.5	1	0.85

TABLE IV

COMPARISON BETWEEN THE FREE FATTY ACID (FFA) FRACTION AND THE GLYCERIDE FATTY ACID (FA) FRACTION OF BULK SAMPLES OF SSL COLLECTED FROM SCALP AND HAIR

	Subject	$C_{16}/C_{16:1}$	
		FFA	Glyceride FA
1-day-old samples	1	0.81	0.56
	2	1.21	0.60
	3	0.82	0.53
	4	1.29	0.73
	5	1.21	0.68
4-day-old samples	6	0.80	0.49
	7	0.63	0.48
	8	0.63	0.47
	9	1.04	0.68
	10	0.88	0.58

These results show that the enzymatic hydrolysis is structure-discriminative, and cleaves first the saturated moieties. As this point seems of importance, the validity of the conclusion was checked using another sampling procedure and a different prechromatographic approach.

Investigation on SSL from bulk samples

SSL from scalp and hair were sampled by washing with a surfactant solution according to ref. 9. The free fatty acid fraction and the glyceride fatty acid fraction were recovered according to the procedure of Boré et al. [10].

The chromatographic profiles of the two fractions were compared over an experimental population of ten subjects. Both 1-day-old and 4-day-old SSL

were investigated; the results are given in Table IV. Here again it can be seen that the bacterial lipases perform a structure discriminatory hydrolysis which affects more readily the saturated species. This, however, does not necessarily mean that the lipases actually recognize the saturated structure of the acyl moiety to be cleaved. Another explanation could be that the lipases are selective toward the position (α and β) of the acyl group within the triglyceride. In this case, the favored release of saturated acids would be the consequence of a non-random distribution of the saturated and unsaturated chains between the α and β sites. A definitive conclusion on this point needs further experimentation using in vitro methodology.

CONCLUSIONS

The microsamples of human SSL, as obtained using the ground-glass platelet technique, can be very closely examinated by gas chromatographic methods.

The amount and distribution of the fatty acids in the free fatty acid fraction and in the free + glyceride) fatty acid fraction can be determined from a single sample containing 1—20 μg of fatty acid using two derivatizing reagents.

These examinations show that lipolytic activity linked to the presence of bacteria in the sebaceous gland favors the liberation of straight-chain saturated fatty acids from sebum triglycerides.

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