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Individual variation of human plantar stratum corneum lipids, determined by automated multiple development of high-performance thin-layer chromatography plates

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Abstract

The stratum corneum lipids are unique in composition and have been used frequently as a model system of the skin's lipid barrier. Automated multiple development (AMD) of high-performance thin-layer chromatography plates in combination with a 25-step gradient, based on methanol, diethyl ether and *n*-hexane separated the six major human plantar stratum corneum lipids. Post-chromatographic staining of these lipids with a solution of $MnCl_2$–H_2SO_4 at 130°C or a solution of $CuSO_4$–H_3PO_4 at 140°C allowed visualization of the lipids and quantification. The $MnCl_2$–H_2SO_4 solution stained saturated fatty acids less intensely. Therefore, the $CuSO_4$–H_3PO_4 solution was used for quantification and we found, on average, 2.06% (w/w) cholesterol 3-sulphate, 20.16% (w/w) free fatty acids, 20.25% (w/w) ceramides, 43.53% (w/w) non-esterified sterols, 4.56% (w/w) triacylglycerols and 9.4% (w/w) sterolesters in the human plantar stratum corneum extracts. The concentration of phospholipids was less than 1% (w/w). In addition, the lipid composition of twenty different human plantar stratum corneum extracts was determined. Statistics revealed a correlation between the ratio of free fatty acids and non-esterified sterols ($r=0.832$, $p<0.01$, $n=20$). Several control experiments proved that this correlation is not due to the extraction method, the post-chromatographic staining procedure or bacterial contamination of the stratum corneum.

Keywords: Stratum corneum; Automated multiple development; Lipids; Fatty acids

1. Introduction

The human stratum corneum (SC) consists of corneocytes, embedded in multi-lamellar lipid sheets of unique composition and structure. The major lipid components of the SC are free fatty acids (FA), ceramides (Cer) and sterols (Ch). Cholesterol 3-sulphate (ChS), triacylglycerols (TAG) and sterolesters (ChE) are also present, but at minor concentrations. The concentration of phospholipids is below 5% (w/w) of total lipids [1]. These lipids are important for an intact skin barrier, since lipid depletion by acetone or sodium dodecyl sulphate treatment results in an increased trans-epidermal water loss. There is evidence that the synthesis of individual lipid components is coordinated [2,3]. Inherited or acquired scaling disorders are frequently related to an abnormal lipid composition, due to disturbed lipid metabolism [4,5].

In order to understand the structure of the multi-lamellar lipid sheets of the SC as well as the interaction of SC lipids with applied drugs, different

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model systems were developed [6,7]. We use human SC lipid liposomes as a model to study the physicochemical characteristics of skin lipids [8,9]. In order to characterize the lipid composition of our model system, we used automated multiple development (AMD) of high-performance thin-layer chromatography (HPTLC) plates.

In this article, we describe the separation of human plantar SC lipids by AMD [10] of HPTLC plates. In contrast to a previously described method [11], our AMD gradient separates the FA and the TAG. We show that the post-chromatographic derivatisation with a $CuSO_4$–H_3PO_4 solution is superior to that with the $MnCl_2$–H_2SO_4 solution for the quantification of naturally occurring lipid extracts. Finally, we demonstrate that a linear correlation between the amount of FA and non-esterified Ch in human plantar SC lipids exists.

2. Experimental

2.1. Chemicals

Cholesterol, cholesterol 3-sulphate, cholesteryl oleate, triolein, ceramide types III and IV (both of bovine origin), were products from Sigma (St. Louis, MO, USA). Oleic acid was purchased from Serva (Heidelberg, Germany). Diethyl ether and *n*-hexane were products of Fluka (Neu-Ulm, Germany). CH_3OH, $CHCl_3$, NaCl and Na_2HPO_4 and NaH_2PO_4, all p.a., were from Merck (Darmstadt, Germany). The latter were used to prepare 145 m*M* phosphate buffer, pH 7.3. $MnCl_2 \cdot 4H_2O$, H_2SO_4 (96%), H_3PO_4 (85%), $CuSO_4 \cdot 5H_2O$ and NH_4OH (25%) were from VEB Laborchemie (Apolda, Germany). Silica gel HPTLC plates (60 WRF_{254S} and 60 F_{254}) were purchased from Merck.

2.2. Lipid extraction

Human sole scrapings were obtained at monthly intervals from healthy volunteers. Before sampling, the feet were carefully washed in warm water without any additives. For lipid analysis, a modified Bligh and Dyer [12] extraction was used as follows: Approximately 100 mg of SC was suspended in 10 ml of chloroform–methanol (1:1, v/v) for 24 h in the dark. The extract was filtered through a Filtrak 390 paper filter (VEB Niederschlag, Niederschlag, Germany) to separate cell debris. Subsequently, 3 ml of 145 m*M* phosphate-buffered saline (pH 7.3) was added to salt out ChS into the organic phase. The organic phase was collected, the solvents were removed by a warm stream of nitrogen and the lipids were dried in the dark overnight under reduced pressure. The dry lipids were dissolved in chloroform–methanol (1:1, v/v) at a final concentration of 5 mg/ml and stored at −18°C.

2.3. Application of samples

Each HPTLC plate was prewashed three times with chloroform–methanol–water (16:6:1, v/v) to eliminate interference from material intrinsic to the plate, which otherwise migrated with the cholesterolesters. On each HPTLC plate, seventeen lipid samples were applied with a Linomat IV (Camag, Muttenz, Switzerland) at a distance of 10 mm from the bottom of the plate. Samples were applied in 6 mm long bands, separated by a space of 4 mm. In general, one SC extract was applied to four separate lanes (3 μl on each) and the reference lipid solution (430 μg of lipid in 1 ml), composed of cholesterol 3-sulphate (10 μg), oleic acid (50 μg), ceramide types III and IV (each 50 μg), cholesterol (200 μg), triolein (20 μg) and cholesterololeate (50 μg) was applied on five different lanes, in the range of 1–10 μl. The application sequence was 1abc2abc3a... (numbers indicate the reference lanes and letters the analytical lanes).

2.4. Automatic multiple development (AMD)

AMD allows the sequential development of HPTLC plates with mobile phases of decreasing polarity [10,11]. This enables the separation of lipids within a broad range of polarity to be performed. A 25-step gradient was introduced to develop the HPTLC plates (Fig. 1). During the first twenty steps, the methanol concentration of the mobile phase decreased from 20 to 0% (v/v) and the diethyl ether concentration increased from 80 to 100% (v/v). The last five steps were run with pure *n*-hexane. Between each step, the plate was automatically dried for 3

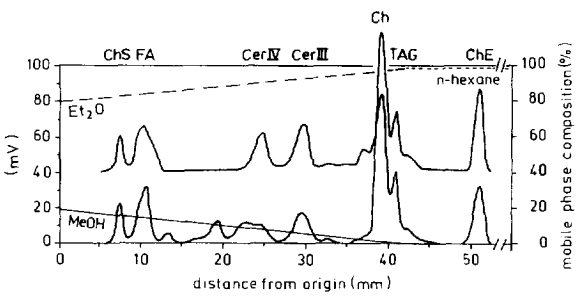

Fig. 1. Densitometric chromatogram of human plantar stratum corneum lipids (lower curve) and of the standard lipid suspension (upper curve). The indicated mobile phase gradient of the AMD, composed of diethyl ether (Et_2O), methanol (MeOH) and *n*-hexane, was used to separate the main stratum corneum lipids: Cholesterol 3-sulphate (ChS), free fatty acid (FA), ceramides (Cer III and Cer IV), sterols (Ch), triacylglycerols (TAG) and sterolesters (ChE). Lipids were stained with a $MnCl_2$–H_2SO_4 solution at 120°C for 30 min.

min in vacuo and thereafter conditioned in a N_2–NH_3 atmosphere, obtained by bubbling N_2 through a 3-*M* NH_3 solution. This resulted in focused FA bands. The use of lower NH_3 concentrations resulted in a broadening of the bands and larger R_f values, as the fatty acids migrated in protonated as well as in deprotonated form.

2.5. Post-chromatographic derivatisation

Remaining ammonia was removed by a stream of warm air at the end of the chromatographic development. In order to visualize the separated lipids, two different staining solutions were used. In some cases, the plate was dipped in a solution of 0.3% (w/v) $MnCl_2$ in methanol–water (1:1, v/v), acidified using 5% H_2SO_4 (v/v) and lipid derivatisation took place at 130°C for 30 min (modified from Ref. [13]). However, this derivatisation procedure stained saturated fatty acids less intensely than unsaturated fatty acids (Fig. 4). Therefore, we used an aqueous solution composed of $CuSO_4$ (10%, w/v), H_3PO_4 (8%, v/v) and 5% methanol, followed by incubation at 140°C for 30 min (modified from Ref. [11]). As a consequence, saturated and unsaturated lipids were stained a brownish colour with almost the same intensity.

2.6. Densitometry

Plates were scanned from the origin to the solvent front, using a Camag TLC II scanning densitometer. The measurement was performed in reflectance mode at λ=550 nm. The beam dimensions were 4×0.2 mm. During the scan, the plate was moved in steps of 0.150 mm and changes in the reflected light intensity were recorded. The overall scan speed was (4 mm/s). Therefore, the spacial resolution of the densitometer could be estimated to be 0.2 mm. Data were stored on-line on a personal computer and integration as well as quantification were performed with the software package CATS (Camag).

2.7. Quantification

Peak heights as well as peak areas were used for quantification. Since each of the seven reference lipids was applied at five different concentrations, a calibration curve was calculated. For this purpose, a non-linear Michaelis-Menten-like equation [$Y=(A_0+(A_1\cdot X)/(A_2+X)$] was chosen for each of the reference lipids, because the photomultiplier response is non-linear, if the substance concentration varies over a broad range (approx. two orders of magnitude). In order to reduce experimental errors, individual calibration curves were obtained for every HPTLC plate. Each sample was applied to four separate lanes and the mean and standard deviation of the lipid concentration was calculated. The total amount of ceramides was calculated according to the standard curve of Cer III (bovine origin). Data were finally analyzed statistically in a Pearson correlation matrix with the aid of SYSTAT.

3. Results

On average, 2.2 mg of lipid were extracted from 100 mg of SC corneum (n=20). The extracted human plantar SC lipids were separated by AMD-supported development of HPTLC plates. A gradient, based on methanol, diethyl ether and *n*-hexane was used (Fig. 1). The polar components, cholesterol 3-sulphate, FA and ceramides were separated using the methanol–diethyl ether gradient. The less polar TAG and sterolesters were separated by the sub-

sequent repetitive development with *n*-hexane. The corresponding R_f values of the main human plantar SC lipids range from 0.13 to 0.93 for cholesterol 3-sulphate and ChE, respectively (Table 1). The same and other mobile phase gradients, based on dichloromethane, methanol and *n*-hexane did not separate the polar lipids (data not shown).

Fig. 2 shows part of a chromatoplate. Lane C contains the reference substances cholesterol 3-sulphate (ChS), free fatty acids (FA), ceramide type IV (Cer IV), ceramide type III (Cer III), sterols (Ch), triacylglycerols (TAG) and sterolester (ChE). Lanes A and B show the analysis of two different human plantar SC lipid extracts. In general, five-to-six additional, unidentified lipid bands occurred between the FA band and the Ch band. Wertz et al. [14] have classified the human SC ceramides according to their chromatographic behaviour. The classification was recently updated by Robson et al. [15]. These naturally occurring SC-derived ceramides differ with respect to chain length, saturation and head group composition. Since our AMD gradient also separates the ceramides according to their polarity, we conclude that these lipid bands represent the six main ceramides, described originally by Wertz et al. [14].

Post-chromatographic derivatisation of the lipids with methanolic $MnCl_2$–H_2SO_4 or with $CuSO_4$–H_3PO_4 stained the lipids a brownish colour (Fig. 2) and allowed quantification. Each lipid was stained at a different intensity and the lower detection limit for the lipids varied between 23 ng (for cholesterol 3-sulphate) and 115 ng (for ceramides, free fatty

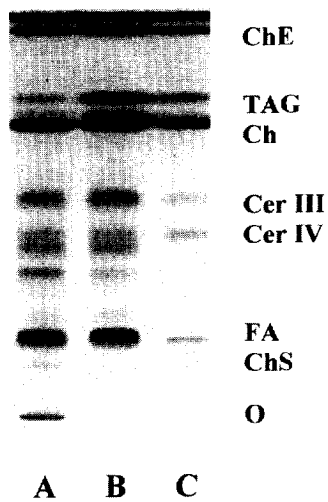

Fig. 2. HPTLC chromatogram of human plantar stratum corneum lipids. Lanes A and B represent the lipid composition of two different human plantar stratum corneum extracts. Lane C shows the separated reference lipids, applied at the origin, O (10 μl; 430 μg/ml): Cholesterol 3-sulphate (ChS), oleic acid (FA), ceramide type IV (Cer IV), ceramide type III (Cer III), cholesterol (Ch), triacylglycerol (TAG) and cholesteryl oleate (ChE), respectively. The plate was developed using the AMD technique with the gradient described in Fig. 1 and stained subsequently with a $MnCl_2$–H_2SO_4 solution at 120°C.

Table 1
Lipid composition (weight.%) of human plantar stratum corneum

Derivatisation procedure	R_f	$CuSO_4$–H_3PO_4 (140°C)				$MnCl_2$–H_2SO_4 (130°C)		
		Mean ($n>100$)	Mean ($n=6$)	Minimum[a]	Maximum[b]	Mean ($n=20$)	Minimum[a]	Maximum[b]
Phospholipids	nd	<1	nd	nd	nd	<1	nd	nd
Cholesterol 3-sulphate	0.13	2.06±0.127	2.14	1.22	2.33	1.6±0.74	0.76	3.55
Free fatty acids	0.18	20.16±1.12	18.75	12.03	25.8	7.5±2.65	3.52	11.52
Ceramides (type IV/III)[c]	0.46/0.53	20.25±0.67	17.06	12.74	20.04	21.9±4.89	16.46	40.24
Sterols	0.69	43.53±3.04	44.23	37.49	54.21	55.8±4.4	46.5	62.6
Triacylglycerols	0.73	4.56±0.54	5.11	3.48	5.98	3.8±2.49	1.03	12.83
Sterolesters	0.93	9.44±0.67	12.69	8.07	18.99	9.9±1.72	6.65	14.04

Peak areas were used for quantification.
Each analysis was performed four times.
Data are expressed in weight percent of total lipid±standard deviation (SD).
[a] Lowest value found in all analyzed samples.
[b] Highest value found in all analyzed samples.
[c] R_f values of bovine ceramide types IV and III, respectively.
nd: not detected.

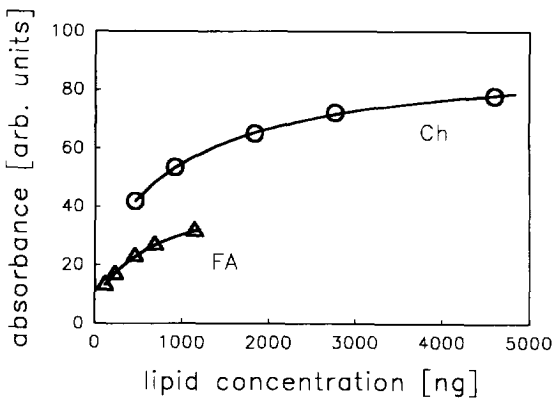

Fig. 3. Standard curves for the free fatty acids (FA) and the sterols (Ch). Curves were calculated from the peak heights of five different concentrations of the reference lipids applied to each HPTLC plate. These curves were used for the quantification of the lipid composition in unknown samples.

Table 2
Parameters of the Michaelis-Menten-like calibration equation $[Y=(A_0+(A_1\cdot X)/(A_2+X)]$, used for lipid quantification

Lipid	A_0	A_1	A_2	SD
Cholesterol 3-sulphate	−3.1	45.3	143	7.4
Free fatty acids	7.73	39.5	745.4	0.9
Ceramide type III	−0.15	49.19	580	2.2
Sterols	20.44	71.09	1064	0.8
Triacylglycerols	4.79	32.09	137.4	5.3
Sterolester	0.16	65.85	309.6	0.8

Standard deviation (SD) of the curve is given in the last column.

acids and sterol esters; applied in a 6-mm long band). Due to the different staining behaviours of the different SC lipids, a calibration curve was calculated for each lipid (Fig. 3). The parameters of these calibration curves are given in Table 2.

In the next step, we tested whether or not the analysis of SC lipids by AMD-supported HPTLC gave reproducible results. Therefore, the same SC extract was quantified in two separate experiments on two separate HPTLC plates. Lipids were visualized after post-chromatographic staining with a $MnCl_2$–H_2SO_4 solution at 120°C. Table 3 shows that the two independent analyses (analysis 1 and analysis 2) gave the same results. Therefore, we conclude that the AMD-supported development of HPTLC plates is reproducible and can be used for the analysis of human plantar SC lipids.

Next, we investigated the variation in the composition of twenty different human plantar SC lipid samples. Again, the post-chromatographic staining was performed with a $MnCl_2$–H_2SO_4 solution. The lipid composition of the twenty extracts differed over a broad range (Table 1); 3.5–11.5% (w/w) for the free fatty acids and 46.5–62.6% (w/w) for the free sterols. Statistical analysis of the lipid composition revealed a correlation ($r=0.823$, $p<0.01$) between the amount of free fatty acids and the non-esterified sterols in the extracts (Fig. 4). Individuals with a high amount of non-esterified sterols in their plantar SC extracts had low amounts of FA. The parameters of the corresponding linear equation were −1.29 for

Table 3
Quantification of the same human plantar stratum corneum lipid extract in two separate experiments, using the $MnCl_2$–H_2SO_4 derivatisation procedure (Analyses 1 and 2) and quantification of the lipid composition of identical stratum corneum samples, using the $CuSO_4$–H_3PO_4 derivatisation solution (Analyses 3 and 4)

Lipid	Analysis 1 ($n=4$)		Analysis 2 ($n=3$)		Analysis 3 ($n=4$)		Analysis 4 ($n=4$)	
	Mean (μg)	C.V. (%)	Mean (μg)	C.V. (%)	Mean (μg)	Weight.%	Mean (μg)	Weight.%
Cholesterol 3-sulphate	10.6	6.7	9.1	4.9	14.1	1.25	6.9	1.15
Free fatty acids	52.5	3.9	44.9	4.2	291.5	25.9	203.3	26.9
Ceramide type III	46.7	4.0	45.7	1.8	95.3	8.5	61.5	8.1
Sterols	232.6	4.2	252.7	13.9	436.4	38.9	306.4	40.5
Triacylglycerols	27.1	5.2	25.34	4.0	88.8	7.9	55.9	7.4
Sterolester	33.84	16.3	31.42	4.5	126.1	11.2	70.9	9.4

Analyses 1 and 2. The amount of lipid (μg) and the coefficient of variation (C.V. in %) for the analyses are given.
Analyses 3 and 4: Both samples were incubated in buffer. However, sodium azide was added to the sample in Analysis 4 in order to prevent bacterial activity. Numbers indicate the absolute amount of lipid extracted (μg) and the relative amount (weight.%).

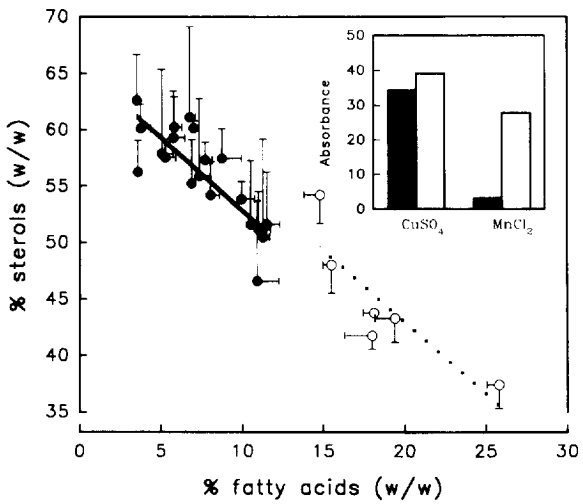

Fig. 4. Correlation between the amounts of free fatty acids and the non-esterified sterols in human plantar stratum corneum lipids. The solid line (●) represents the data of twenty individuals, obtained after post-chromatographic derivatisation with a $MnCl_2$–H_2SO_4 solution. The dashed line (○) represents the data ($n=6$) obtained after derivatisation with a $CuSO_4$–H_3PO_4 solution. Each point represents the mean of four independent measurements and error bars indicate the standard deviation. Inset: Difference in the staining intensity of 115 ng of palmitic acid (black column) and oleic acid (white column) using a $MnCl_2$–H_2SO_4- or a $CuSO_4$–H_3PO_4 solution.

the slope and 65.7 for the y-intercept. This correlation was even higher ($r=0.929$) if peak heights instead of peak areas were used. No correlation between other SC lipids or between lipids and the age or sex of the donor was found.

In order to exclude the possibility that the free fatty acid–sterol correlation was an artifact, due to the selective staining capability of the $MnCl_2$–H_2SO_4 solution, a different post-chromatographic derivatisation was performed. Frequently, a $CuSO_4$–H_3PO_4 solution is used for the visualization of skin lipids on HPTLC plates. The inset in Fig. 4 shows that the two derivatisation procedures differ in their ability to stain saturated and unsaturated fatty acids. The $CuSO_4$–H_3PO_4 solution stained palmitic acid and oleic acid to almost the same intensity. The $MnCl_2$–H_2SO_4 solution, however, stained the saturated palmitic acid less intensely than the unsaturated oleic acid. Since the lipid composition of human plantar SC lipids is heterogeneous (the presence of saturated as well as unsaturated free fatty acids was reported [1]) and our reference lipid solution contained only oleic acid, the $CuSO_4$–H_3PO_4 derivatisation procedure was chosen for further experiments.

The analysis of individual human plantar SC lipid compositions was repeated with six samples, using the $CuSO_4$–H_3PO_4 solution as the derivatisation reagent. The same correlation between the amount of free fatty acids and the sterols in the SC extracts ($r=0.864$, $p<0.01$) was found (Fig. 4). The parameters of the corresponding linear equation were -1.26 for the slope and 68.3 for the y-intercept. Therefore, we conclude that the correlation is not an artifact due to the AMD method or to the post-chromatographic derivatisation procedure.

Since the correlation between the sterols and the free fatty acids of human plantar SC lipids is a novel finding, two additional control experiments were performed. First, we excluded the possibility that the results are based on bacterial activity, located in/on the samples of sole scrapings (Table 3). Two large samples of sole scrapings were divided in two and incubated for 18 h in buffer before the lipid extraction. Sodium azide was added to one sample (analysis 4), to suppress bacterial growth and increase the activity. As a consequence, triacylglycerols may be degraded into glycerol and free fatty acids, thereby increasing the free fatty acid concentration in the sample. Table 3 (analysis 3 and analysis 4) shows that the amount of total lipid extracted varies in both samples due to the different weights of the SC samples. The weights of the SC samples were 56.4 and 74 mg for the SC samples in analyses 3 and 4, respectively. However, the addition of sodium azide (analysis 4) during the buffer preincubation step did not influence the relative lipid composition of the samples. On average, we found 25.9% (w/w) and 26.9% (w/w) free fatty acids, 38.9% (w/w) and 40.5% (w/w) sterols and 7.9% (w/w) and 7.4% (w/w) triacylglycerols for samples in the absence and presence of sodium azide, respectively. Thus, we conclude that the correlation between free fatty acids and sterols was not due to degradation of the SC lipids by bacterial contamination.

We have also excluded the possibility that the correlation was due to enzymic activity in the SC. Lipases, located in the SC, may degrade triacylglycerols (or sterolesters) into free fatty acids and

glycerols (non-esterified sterols), thereby increasing the relative amount of free fatty acids in the extracts. Degradation of lipids by enzymes should occur if the SC is incubated in buffer before the lipid extraction procedure. We have done this and found no increase in the free fatty acid concentration, compared to samples that were extracted directly with chloroform–methanol.

The second control experiment was performed to determine if the extraction method, especially the amount of buffer added, influenced the analysis of the lipid composition. The method of Bligh and Dyer [12] was developed originally for fresh tissues. However, the SC samples are rather dry. Therefore, the method may not be suitable for the extraction of SC lipids. We varied the amount of phosphate buffered saline added to the SC samples and analyzed the composition and the total lipid yield of the extracts. Table 4 shows that the amount of buffer added to the organic phase influenced the yield. Increasing amounts of buffer increased the total amount of lipid extracted. Only 1.16 mg of SC lipid were extracted with a chloroform–methanol (2:1, v/v) solution. In contrast, the addition of 120% (v/v) phosphate buffered saline (PBS) to the organic phase doubled the amount of extracted lipids (2.46 mg). However, the composition of the extracted lipids remained unaffected by the amount of buffer added. Therefore, we conclude that the free fatty acid–sterol correlation that we found was not influenced by the extraction method.

In order to characterize the lipid composition of our human plantar SC model system, approx. 10 g of sole scrapings ($n>100$ individuals) were extracted. After AMD-supported HPTLC, the lipids were visualized using the $CuSO_4$–H_3PO_4 staining procedure (Table 1). The lipid composition of this large sample size was similar to the averaged lipid composition of the six individual analyses (Table 1). It was also within the range of the highest and the lowest amount of lipids, determined in the individual analyses. For further studies on a SC model system, especially physico-chemical characterization, it is remarkable that the amount of all sterols (cholesterol 3-sulphate, free sterols and sterolesters) was 55% (w/w) and the amount of "chain" lipids (free fatty acids, ceramides and triacylglycerols) was 45% (w/w). The phospholipid concentration was less than 1%, determined by the method of Ames [16].

Table 4
Influence of the extraction procedure on lipid yield and composition

	PBS (%)			
	0	30	80	120
Cholesterol 3-sulphate	2.32	1.66	2.63	2.79
Free fatty acids	16.6	15.33	15.32	17.01
Ceramide type III	22.03	21.79	20.96	21.01
Sterols	43.62	44.73	46.02	44.67
Triacylglycerols	6.21	6.15	5.47	4.97
Sterolester	9.17	10.34	9.62	9.56
Yield (mg)	1.16	1.79	2.39	2.46

Human plantar stratum corneum sole scrapings were incubated in chloroform–methanol (2:1, v/v) and the volume of phosphate buffered saline (PBS) added was varied.
The amounts of lipid in the samples are given in weight.%.

4. Discussion

The lipid composition of the SC is crucial for an intact barrier to be maintained. Different HPTLC solvent systems have been invented to separate, quantify and characterize the human SC lipids. Recently, Bonté et al. [11] demonstrated that AMD-supported HPTLC can be used to separate human SC lipids with five different solvent systems. In this article, we show that a simplified gradient (with only three solvents) can be used for the same purpose. Moreover, our gradient is free of chloroform and toluene (used by Bonté et al. [11] in an initial isocratic separation step) and is faster. Most important, however, is the fact that our method separates the free fatty acids and the triacylglycerols, which is not possible with the method of Bonté et al.

The main drawback of our AMD gradient is the fact that it can be used only for palmo–plantar surfaces. Sebaceous lipids (e.g., squalene and wax-esters), which occur in other parts of the body, are not separated and will migrate with the solvent front. However, the palmo–plantar surfaces are free of sebaceous glands [17] and hydrocarbons are usually not present. We proved this by the following HPTLC separation: SC lipids were applied to a silica HPTLC plate and squalene was co-applied on a separate lane. The plate was developed in pure *n*-hexane and squalene was not detected in our lipid extracts.

The total amount of lipids extracted (2.2 weight.%) was similar to the earlier reported value of Lampe et al. [1]. It depended, however, on the amount of phosphate buffered saline added to the organic extraction solution (Table 4). This is in agreement with the data of Bligh and Dyer [12], who pointed out that the methanol–chloroform–water ratio is crucial for the lipid yield. However, we showed that the SC lipids are always extracted in the same ratio (Table 4), independent of the amount of water–buffer added. Therefore, the amount of buffer in the extraction solution determined the total lipid yield, but it had no influence on the ratio of the extracted human plantar SC lipids.

The six major SC lipids were separated in our experiments (Fig. 1) and the concentration of each lipid was determined after derivatisation with a $CuSO_4$–H_3PO_4 solution (Table 1). The $CuSO_4$–H_3PO_4 solution was more suitable for the derivatisation, since it did not discriminate between the saturated and the unsaturated fatty acids (Fig. 4) found in the SC [1]. The composition of the SC lipids was similar to an earlier reported range [17], 20–25% ceramides, 20% free fatty acids, 15% sterolesters and 20% cholesterol. A comparison of our results with those in the literature, however, revealed a broad variation in data. The reported concentration of sterols was varied, i.e. 5.9% [11], 23% [19] and 35% [1,18]. The concentrations of free fatty acids reported are in the range of 9% [1] to 19% [18] and the data of the total concentration of ceramides varied between 17.9% [11] and 35% [1,18,19]. These differences in data might be due to variation of an individual's lipid composition [19]. Other factors such as age [20], seasonal variation [21], differences due to sampling region [1] and the method of extraction [1] influence the comparability of data. We showed that the repetitive analysis of the same sample and the extraction of identical samples gave the same results (Table 3). Therefore, we conclude that our extraction method was reproducible, that the amount of each lipid in different samples is comparable and that the $CuSO_4$–H_3PO_4 solution should be used for the quantification of lipid.

A detailed analysis of the lipid composition of twenty individuals showed that a correlation between the amount of free fatty acids and the non-esterified sterols exists (Fig. 4). Control experiments, however, showed that the $MnCl_2$–H_2SO_4 solution stains unsaturated fatty acids more intensely than saturated ones (Fig. 4). Therefore, six individual lipid extracts were analyzed again, using the $CuSO_4$–H_3PO_4 derivatisation reagent and a new reference lipid solution. The correlation between the amount of free fatty acids and the non-esterified sterols was again reproduced and the parameters of the linear regression are, within experimental error, identical. From these results, we conclude that the free fatty acid–sterol correlation is not an artifact due to the derivatisation procedure.

However, this correlation could have been due to bacterial or enzymic activity. Bacterial or enzymic activity might act on the SC lipids, thereby changing the free fatty acid concentration, if the SC is incubated in buffer before the lipid extraction. We did this in the presence and in the absence of sodium azide (Table 3, analyses 3 and 4) and found no change in the free fatty acid or sterol concentrations. Thus, we conclude that the free fatty acid–sterol correlation is not based on microorganisms or lipases located on/in the SC samples.

Evidence is given in the literature that the sterol and free fatty acid concentrations are somehow linked. Lavrijsen et al. [22] analyzed the fatty acid–sterol ratio in the SC of four healthy volunteers. In their study, the free fatty acid–sterol ratio was in the range of 0.297–0.899. In our experiments, it varied from 0.221 to 0.688 (Table 1, $n=6$; derivatisation with $CuSO_4$–H_3PO_4).

Feingold et al. [3] suggested that "the linoleic acid metabolism and the sterol metabolism (of the skin) are somehow linked". Their studies on rodent skin showed that inhibition of sterol synthesis by a single lovastatin treatment [23] increased the free fatty acid concentration by 61%, while the sterol synthesis remained at 52% of the initial value [2]. Lovastatin is a competitive inhibitor of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in sterol synthesis [24]. This stimulation of fatty acid synthesis occurs also in keratinocytes and fibroblast cultures after HMG-CoA reductase inhibition [25]. In addition, it is known that essential fatty acid-deficient hairless mice exhibit increased cholesterol synthesis [3].

The available experimental data from experiments

performed using cell cultures, rodent skin and human stratum corneum supports the hypothesis that sterol and fatty acid synthesis in viable parts of the skin, eg. the lower epidermis, are linked. A high sterol concentration results in a low free fatty acid concentration. We conclude that linkage of the two biosynthetic pathways results in a linear correlation of the concentrations of free fatty acid and sterol, which can be detected in the outermost layer of the skin, the SC. As a consequence, SC lipid model systems should be prepared from large SC sample sizes, in order to average individual variations in the lipid composition.

5. Abbreviations

AMD	Automated multiple development
Cer III	Bovine ceramide type III
Cer IV	Bovine ceramide type IV
Ch	Sterols (cholesterol)
ChE	Sterolesters
ChS	Cholesterol 3-sulphate
FA	Free fatty acids
HPTLC	High-performance thin-layer chromatography
SC	Stratum corneum
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A

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