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Improved procedure for the separation of major stratum corneum lipids by means of automated multiple development thin-layer chromatography

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Abstract

The separation of the major stratum corneum lipids, i.e., ceramides, fatty acids, cholesterol and its esters by means of high-performance thin-layer chromatography is hereby presented. The used automated multiple development technique allows the reproducible development of a 17-step solvent gradient also capable of separating seven ceramide classes in the same run. Reliable quantification has been performed after visualisation and densitometric scanning. The present approach is less time and solvent-consuming than previously described procedures. The application to samples obtained by in vivo skin surface extraction with hexane–ethanol (2:1) demonstrates that the method can be routinely used for diagnostic purposes.

Keywords: Lipids; Ceramides; Fatty acids; Cholesterol

1. Introduction

The stratum corneum lipids are known to play a decisive role in maintaining the barrier function of the skin against transepidermal water loss and penetration of substances from the environment [1,2]. It has been shown that a number of skin disorders, such as psoriasis, atopic dermatitis, ichthyosis or xerosis, entail changes in lipid composition [3–7]. Therefore, the analysis of the major stratum corneum lipid classes, particularly of the ceramides, is a prerequisite to a better understanding of these disorders and to their specific treatment.

For the separation of the major stratum corneum lipid classes, i.e., ceramides, fatty acids, cholesterol and its esters, thin-layer chromatography (TLC) is the method of choice. Normal phase HPLC as a possible alternative is less robust to matrix components from biological lipid extracts. Furthermore, detection problems result from lacking UV absorbance of the lipids and incompatibility of the used mobile phase to electrospray mass spectrometry, respectively. Reversed-phase LC implies a different selectivity due to its susceptibility to chain length influences, therefore not allowing ceramide class separation according to number and position of the hydroxy functions.

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Several TLC approaches have been presented. Most of these rely on laborious manual procedures [8–10], which render a reproducible analysis more difficult. The automated multiple development high-performance thin-layer chromatography (AMD-HPTLC) uses automated procedures for eluent mixing, chamber conditioning, development and drying steps. Furthermore, it is less time and solvent consuming and avoids band broadening due to the reconcentration effect of the bands [11]. The work of Bonté et al. [12] was the first approach to analyse stratum corneum lipids with the help of AMD-HPTLC. The paper of Zellmer and Lasch [13] presents the separation and quantification of the main lipid classes from human plantar stratum corneum using AMD. However, ceramide classes could not be unequivocally identified.

In this article we present an improved method for the separation and subsequent quantification of major stratum corneum lipids, including particularly the seven known ceramide classes in the same run. The method was successfully applied to skin surface extracts obtained in vivo.

2. Experimental

2.1. Chemicals

Cholesterol, cholesterol-3-sulfate, cholesteryl oleate, palmitic acid, triolein, squalene, and Ceramide AS (hydroxy fatty acid ceramide) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Ceramide NP and Ceramide AP were provided by Cosmoferm (now Goldschmidt, Essen, Germany), Ceramide NS by Sederma (Le Perray en Yvelines, France). We use the new ceramide nomenclature throughout, as introduced by Motta et al. [14] and Robson et al. [15], respectively. The last letter indicates the type of sphingoid base (P stands for phytosphingosine, S for sphingosine, H for 6-hydroxysphingosine). The letter next to the last indicates the fatty acid connected via the amide bond to the sphingoid base (A indicating an α-hydroxy fatty acid, N a simple, non-hydroxy fatty acid, O stands for an ω-hydroxy fatty acid). In the latter case, an additional ester-linked fatty acid can be present, indicated by a third letter E. For example, Ceramide EOS indicates a ceramide consisting of a sphingosine base acylated by an ω-hydroxy fatty acid, which bears an additional fatty acid ester-linked to the ω-hydroxy function.

Solvents for extraction and TLC purposes were of analytical grade and purchased from Merck (Darmstadt, Germany), Baker (Deventer, The Netherlands) and Roth (Karlsruhe, Germany). Silica TLC plates (Kieselgel 60 F_{245}, 20×10 cm), were supplied by Merck.

2.2. Extraction procedure

Skin lipid extracts were obtained in vivo from six test persons (caucasian males, age 28±4 years). For surface extraction purposes a cylindrical glass beaker with 4-cm I.D. (contact area 12.56 cm^2) was filled with 8 ml *n*-hexane–ethanol (2:1, v/v). The open side was pressed tightly to a skin area at the inner forearm to prevent lateral leakage. The extraction time was 5 min throughout. The extraction mixture was thereafter evaporated at 50 °C and the residue dried under a stream of argon. The dried lipids were then dissolved in 500 μl chloroform–methanol (1:1).

2.3. Application of the samples

The HPTLC plates were washed three times with chloroform–methanol (65:35, v/v) before use. Sample application has been carried out automatically using an Automatic TLC Sampler 4 (Camag, Muttenz, Switzerland) at a dosage speed of 100 μl/s. Fifteen samples were applied on each plate at a start line 8 mm from the bottom, including six lanes for skin lipid samples (each 6 μl) and nine lanes for reference lipids ranging from blank to 4 μg for each standard lipid. The band length was 8 mm, with 4 mm distance to the neighbouring lane.

2.4. Development of the plates and post chromatographic derivatisation

The development of the plates has been carried out automatically using an AMD-2 apparatus (Camag). The used AMD procedure included a 17-step gradient of decreasing polarity as shown in Fig. 1. After drying, the plates were dipped into an aqueous solution of 10% $CuSO_4$, 8% H_3PO_4 (v/v), and 5%

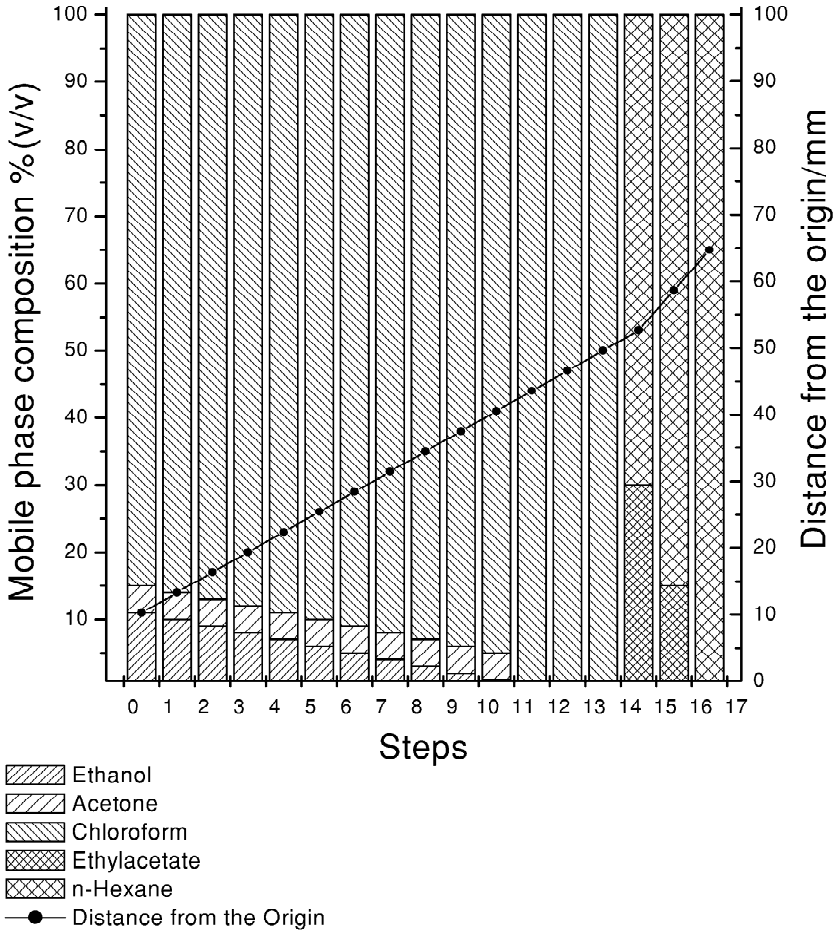

Fig. 1. The AMD-HPTLC elution system.

methanol for 20 s. Afterwards, the plates were charred in a drying oven at 150 °C for 20 min.

2.5. Densitometry

The developed and visualised plates were scanned from 7 cm to front using a TLC Scanner 3 (Camag). The measurement was performed in reflectance mode at a wavelength of 546 nm. The slit dimensions were 4×0.1 mm at a scan speed of 20 mm/s and a data resolution of 25 μm per step. Integration and quantification based on peak areas were performed using CATS software (Camag). To avoid experimental errors, individual curves were set up for each HPTLC plate. Quantitative results for all ceramides were related to Ceramide NP (Fig. 5).

3. Results and discussion

3.1. Chromatographic separation

An improved procedure for the separation of major stratum corneum lipids by means of AMD-HPTLC has been developed (see Fig. 1). The first 11 steps were performed using mixtures of chloroform, ethanol and acetone. Thereafter followed three isocratic steps with chloroform. These steps allowed the separation of cholesterol sulfate, the various ceramide classes and cholesterol. For the separation of cholesterol, fatty acids, triacylglycerol, cholesteryl esters, and squalene, two additional steps were required with a mixture containing *n*-hexane and ethylacetate followed by an isocratic hexane step.

Before each step the plates were automatically dried for 90 s in vacuo and then conditioned in an acetic acid atmosphere, obtained by bubbling air through a 4 *M* acetic acid solution in order to focus the fatty acid bands and to achieve a better resolution in the ceramide fractions.

This gradient enables base line separation of the lipid standards cholesterol-3-sulfate, Ceramide AP, Ceramide AS, Ceramide NP, Ceramide NS, cholesterol, palmitic acid, triacylglycerol, cholesteryl oleate, and squalene (see Figs. 2 and 3). Since the used Ceramide AP is a semisynthetic substance containing a racemic 2-hydroxy fatty acid moiety, the corresponding spot is separated into two bands. Therefore, peak no. 4 (Fig. 3) results from an overlap of Ceramide AS and one of those Ceramide

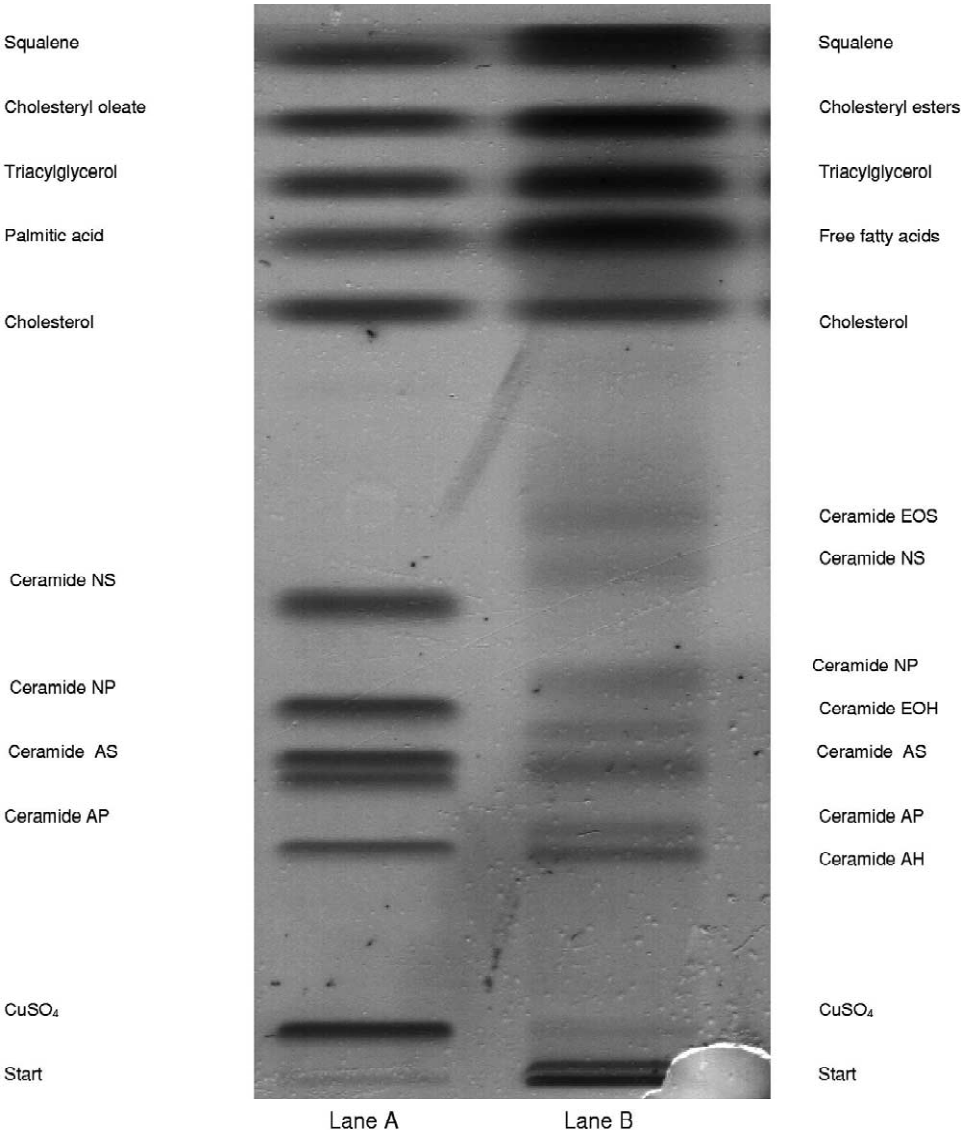

Fig. 2. HPTLC chromatogram of the separated lipids. Lane A represents the used lipid standards. Lane B shows the separation of the lipids from in vivo surface extraction, indicating particularly the seven separated ceramide classes.

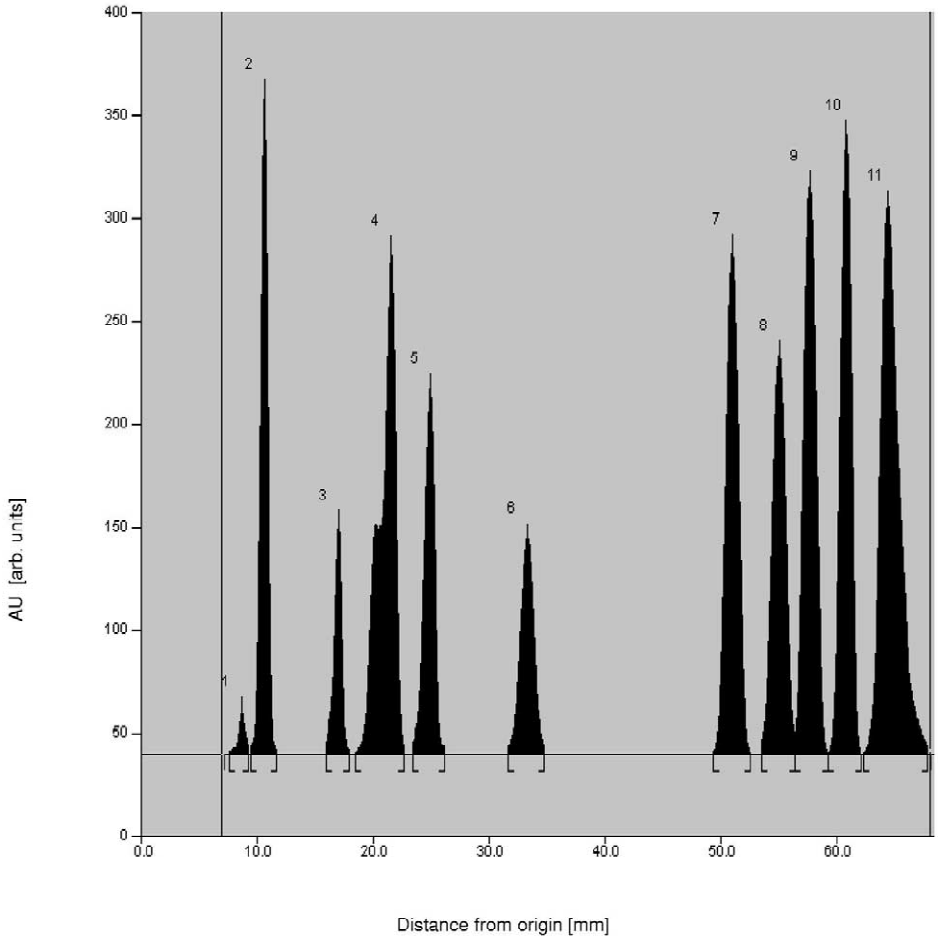

Fig. 3. The densitometric chromatogram of the standard lipids. (1) Start, (2) cholesterol-3-sulfate, (3) first peak of synthetic Ceramide AP, (4) double peak of both Ceramide AS and the second peak of ceramide AP, (5) Ceramide NP, (6) Ceramide NS, (7) cholesterol, (8) palmitic acid, (9) triolein, (10) cholesteryl oleate, (11) squalene.

AP bands. In contrast to the method presented in [12] we were able to separate fatty acids from triacylglycerols. With the apparatus utilized, the use of diethylether as presented in [13] brought about stability problems, since evaporation occurred before plate development was finished. This does not happen with the new solvent system.

3.2. Analysis of skin lipids

The developed AMD-HPTLC method has been applied to surface lipid extracts of human skin. The skin lipids were extracted in vivo as described in Section 2. The general advantage of surface extraction is that it is noninvasive and can be performed easily in vivo. However, one has to bear in mind that sebum lipids consisting mainly of triacylglycerol and wax esters are coextracted with the stratum corneum lipids. Several solvent mixtures have been tested. In contrast to some literature reports [16], mixtures containing diethylether turned out to be impracticable, because the high vapour-pressure caused solvent losses. Furthermore, many solvents including chloroform or acetone should not be used, for they are inducing pain and lasting skin damages. The chosen hexane–ethanol (2:1) mixture was a compromise between extraction efficiency and ethical aspects.

Lipid bands could be unequivocally identified by relating the R_f values to those of the standard lipids as well as profiles from previous studies [16–18] (see Figs. 2–4). In some cases, the R_f values of the skin lipids were slightly larger. This results because the fatty acid as well as sphingoid base moieties in stratum corneum ceramides have longer chain lengths compared to the standards. In addition, the broad spectrum of chain lengths implies a more complex densitometric profile, although the resolution obtainable using AMD is still much better than with conventional manual procedures. The present protocol enables a good separation of all major stratum corneum and sebum lipids in 150 min with less solvent consumption (ethanol (8 ml), acetone and ethylacetate (6 ml each), *n*-hexane (19 ml), and chloroform (81 ml)). It can be emphasised, that particularly the separation of ceramides could be improved in comparison to previously reported AMD protocols [12,13]. Hence, the present procedure is suitable to be used for diagnostic purposes as well as for high-throughput analysis of the major stratum corneum lipids.

The calibration curves for densitometric quantification are usually not linear and were fitted using a saturation function such as the Michaelis–Menten equation as described before (see Fig. 5) [13]. The variation coefficients of the scanned lipids were between 0.2 and 10%. The detection limits were in the same range as described previously [13]. Our study revealed (see Tables 1 and 2) that the lipid class composition shows remarkable interindividual variations, whereas the ceramide composition is relatively stable. This is in good consistency with previously reported results [16]. However, the ceramide profile in our study was slightly different to some literature data [15,18,19]. It is obvious that we found more Ceramide EOS and less Ceramide NS, whereas all other ceramide classes are in a corresponding range. A possible explanation may be the finding of Monteiro-Riviere et al., that the ratio of the extracted lipids depends on the solvent system

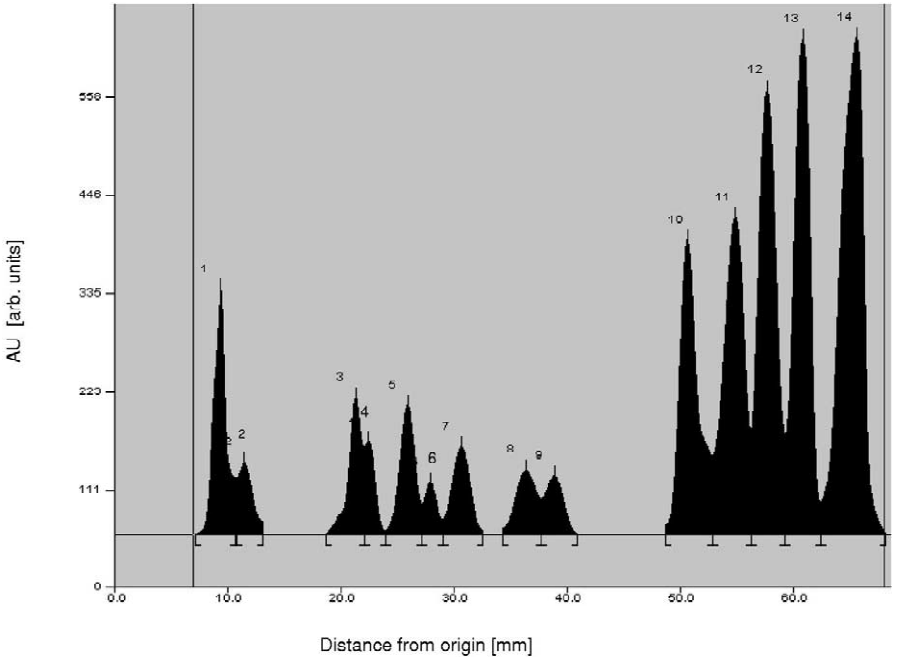

Fig. 4. The densitometric chromatogram of the extracted lipids. (1) start, (2) cholesterol-3-sulfate, (3) Ceramide AH, (4) Ceramide AP, (5) Ceramide AS, (6) Ceramide EOH, (7) Ceramide NP, (8) Ceramide NS, (9) Ceramide EOS, (10) cholesterol, (11) free fatty acids, (12) triacylglycerol, (13) cholesteryl esters, (14) squalene.

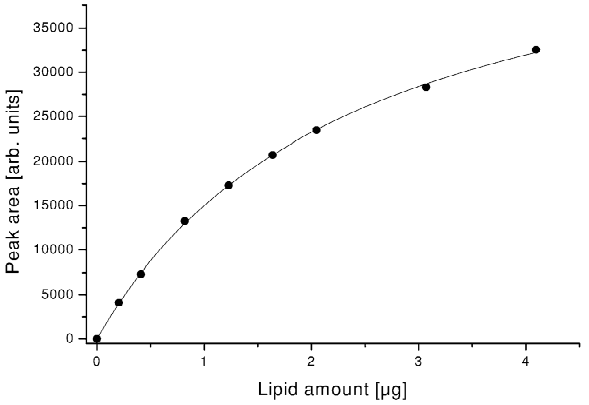

Fig. 5. Example of a calibration curve for densitometry. The curve was calculated from the peak areas of nine different concentrations of the reference ceramide NP applied to each HPTLC plate. The correlation coefficient was 0.9995. The equation used was $y = A1*X/A2 + X$, ($A1 = 51255.4$, $A2 = 2.4$).

Table 1
Profile of the extracted lipids

Lipid	Lipid composition	
	µg/cm^2	% of total lipids
Cholesterol-3-sulfate	1.8±1.7	1.9±1.3
Ceramides	12.0±3.0	15.8±7.2
Cholesterol	8.1±1.0	10.4±4.0
Free fatty acids	30.4±25.2	30.2±15.0
Triglycerides	16.1±5.6	18.7±4.0
Sterol esters/wax esters	19.3±5.7	23.0±6.0
Squalene	ND	ND

The mean values of six determinations are calculated±standard deviation. ND, not determined.

Table 2
Profile of the extracted ceramides

Lipid	Ceramide composition	
	µg/cm^2	% of recovered ceramides
Ceramide EOS	1.9±0.7	15.7±2.3
Ceramide NS	1.7±0.4	14.1±2.3
Ceramide NP	1.5±0.4	12.1±1.6
Ceramide EOH	1.0±0.3	8.1±0.7
Ceramide AS	2.7±0.7	22.3±1.5
Ceramide AP	0.9±0.4	7.9±3.2
Ceramide AH	2.4±0.6	19.8±1.7

The mean values of six determinations are calculated±standard deviation.

used [20]. The relatively soft extraction conditions with respect to test person's compliance may have caused a preference of the easily extractable sebum lipids. Therefore, the detected amounts of cholesteryl esters and triacylglycerol are higher than previously reported [12]. In addition, wax esters comigrate with cholesteryl esters, and alkanes comigrate with squalene. Since the latter elutes with the front, quantification is difficult. However, the focus of dermatological research interest is on the major lipid classes, i.e., ceramides, fatty acids and cholesterol, which makes these limitations acceptable. Another approach to omit the sebum lipids would be to remove them mechanically or chemically before extraction. On the other hand, this may also manipulate the stratum corneum lipid composition. Longer extraction times would open the access to deeper stratum corneum layers which are known to be rich on ceramide [18].

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