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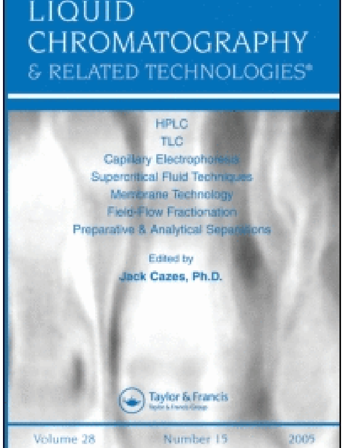

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QUANTITATIVE STUDY OF THE STRATUM CORNEUM LIPID CLASSES BY NORMAL PHASE LIQUID CHROMATOGRAPHY: COMPARISON BETWEEN TWO UNIVERSAL DETECTORS

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□ *The stratum corneum owns a barrier function, which can be altered by a modification of its lipid composition. In this work, a new simple method was developed in normal phase liquid chromatography to separate the three major lipid classes of the stratum Corneum, fatty acids, ceramides, and cholesterol. A quantitative investigation was carried out, comparing an internal normalisation and an external standardisation with two universal detectors: an evaporating light scattering detector and a charged aerosol detector, the Corona. The study points out the fact that the external standardisation is suitable for a quantitative study of the different lipid classes, using a representative standard for each class. Moreover, the use of the Corona is more adapted for further investigation, due to a better repeatability, accuracy, and precision. A test made on a lipid extract from the forearm of volunteers shows that the present method is appropriate for biological extract analysis.*

Keywords CAD, ELSD, liquid chromatography, normal phase separation, quantification, stratum corneum lipids

INTRODUCTION

The barrier properties of the skin reside in the particular lipid composition of the stratum corneum (SC) and on their supramolecular order. Indeed, the lipids, organised in compact lamellar phase, constitute cement which protect our body against external attacks (UV irradiation, pollution, toxics). A modification of the lipid composition can drastically change the barrier function properties.[1,2] For instance, the age can be at the origin of such modifications.[3] It is therefore of interest to develop a simple method to separate the 3 major classes of the SC lipids (cholesterol (CHOL), fatty acids (FAs), and ceramides (CERs)) to enable a quantitative study.

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Several studies have already been carried out using HPLC,[4–8] or HPTLC[9–12] methods to separate the SC lipids. However, most of these studies deal with the separation of the subclasses of CERs or FAs. We have then set up an HPLC method, which permits making a separation and a quantitative study of the FAs, CERs, and CHOL present in the SC.

First, the analytical method was developed, and the separation was optimized considering the mobile phase and the dilution mixture of the lipids.

Different kinds of strategies can be applied for such a quantitative study: the internal normalisation and the external standardisation. To determine which quantitative strategy is the more suitable for our study, a response factor comparison between two universal detectors: evaporating light scattering detector (ELSD) and a charged aerosol detector (CAD) was investigated. On one hand, ELSD is the detector the most used to study lipids because it is compatible with a wide range of solvents,[13] and on the other hand, CAD has recently been introduced for HPLC applications[14] and seem to present several advantages such as wider dynamics and a better sensitivity at the lower end of the calibration curve. These two detectors allow to study every non volatile molecule, and among them lipids.[15,16] To evaluate which of these detectors is better adapted for our study, a comparison was made in terms of response factors, sensitivity and repeatability.

Furthermore, an extract of lipids from the SC of the forearm of a volunteer was tested to confirm the suitability of this method for biological extracts.

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EXPERIMENTAL

Material

HPLC grade heptane (purity of 99.7%), acetone (purity of 99.7%), methanol (MeOH) (purity of 99.8%), and chloroform ($CHCl_3$) (purity of 99.3%) come from VWR (Fontenay-sous-bois, France). Cholesterol, palmitic acid (purity of 99.7) (C_{16}:0), stearic acid (purity of) (C_{18}:0), arachidic acid (purity of 99%) (C_{20}:0), and behenic acid (purity of 99%) (C_{22}:0), CER 2 (purity of 99%) and ethyl acetate (purity of 99.7%) (EA) were obtained from SIGMA-ALDRICH (St Quentin Fallavier, France). CER III, IIIa, and IIIb, VI and I with a purity of 95% were a gift from Cosmoferm (Delft, The Netherlands). The structure of the ceramides are represented Figure 1.

HPLC Instrumentation

A PVA-Sil column (YMC, Kyoto, Japan) 150 × 2.1 mm, 120 A, 5 μm was used and thermostated at 35°C using a Gecko 2000 column oven (CIL

FIGURE 1 Structure of the different ceramides studied.

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Cluzeau, Saint Foy le Grande, France). The solvents were degassed prior to use and the mobile phase was continuously degassed with a Degasys D6-1310 (Eurosep, Cergy Pontoise, France) connected to a Jasco 980 LPG ternary gradient system (Jasco, Kyoto, Japan). A Kontron 360 autosampler equipped with 20 µL sample loop was used and chromatograms were recorded using a Kromasystem 2000 integration package (Biotek Kontron, Milan, Italy). The flow rate was set up at 0.5 mL/min and the injected volume was 20 µL. All gradients were realized with a 20 min. reconditioning time.

The ELSD was a Eurosep DDL 31 (Eurosep Instruments, Cergy, France). Experiments were executed at 1 bar air pressure, the nebulizer was set at 35°C and the drift tube at 50°C. The detection was also performed with a Corona® CAD (ESA Biosciences, Chelmsford, MA, USA). Experiments were executed at 35 psi air pressure with "medium filter" setting. The statistical data and the regression analysis were performed using Matlab software 6.0 R12 (The MathWorks Inc).

The different standards studied were dissolved in a mixture of $CHCl_3$/MeOH (2:1, v/v) at 10^{-4} mol/L. Five concentrations in triplicate were studied for each standard: 3.10^{-5}, 5.10^{-5}, 8.10^{-5}, 1.10^{-4}, 2.10^{-4} mol/L for CAD and 3.10^{-4}, 5.10^{-4}, 1.10^{-3}, 2.10^{-3}, 3.10^{-3} for ELSD.

Extraction Method

The extraction was performed on the inner forearm of a 30 year old woman. The participant did not use cosmetics on the part of skin studied 3 days prior to the experiment, and was judged to be free from skin diseases such as dryness or psoriasis. The forearm is classically used for such experiments because it has less hair follicles and sebaceous glands.

First of all, a paper drenched with ether is applied on the skin to remove the sebum lipids. Four cottons are drenched in a mixture of EA/MeOH (20:80), already used by Weerheim et al.[17] and applied 10 times on a surface of $3 \times 3\,cm^2$. Each cotton is then plunged in 3 mL of a mixture $CHCl_3$/MeOH (2:1, v/v) for one minute. The solutions are centrifuged at 4000 rpm for 10 minutes to eliminate some cotton particles. The homogeneous solution containing the lipids is recuperated. After the evaporation of the extraction solvents with an air flow at room temperature, the dry extract of the lipids is dissolved in 100 μL of $CHCl_3$/MeOH (2:1, v/v) and then in 900 μL of heptane before injection into the HPLC system.

RESULTS AND DISCUSSION

Optimization of the Separation

Optimization of the Mobile Phase Composition

SC lipids can be separated either as a function of the carbon chain volume or depending on their polar headgroup. For a separation by classes, the focus is put on the polar headgroups. The use of the normal phase chromatography is then needed since the reverse phase LC is more suitable for the separation of compounds according to the microheterogeneity of the alkyl chains (carbon chain length and the presence, or not, of unsaturation). The contribution of the different polar headgroups of the classes was calculated using hydrophobic fragmental constant (f) according to Rekker,[18] but with a simplified approach that does not take into account the interactions with the structural environment. Thus, the objective of this calculation is to appreciate the relative polarity of the compounds studied herein.

All FAs have the same polar headgroup ($f_{COOH} = -0.954$), thus, their interactions with the stationary phase are expected to be comparable. Hydroxy-FAs are not taken into account in this study since they are not present in the SC.[7] CERs classes have been established according to their different polar headgroup:[19] phytosphingosine, sphingosine, and α-hydroxyphytosphingosine. The f values of these different polar headgroups are represented in Table 1. These different groups lead to various interactions with the stationary phase and solubility in the mobile phase, responsible for different retention times in normal phase HPLC. Besides, CERs are note single molecular species and may also vary by their carbon chain volume.

According to previous studies,[6,20] heptane and acetone were selected for our experiments as weak and strong solvents, respectively (phase A and B, respectively). The initial gradient Phase A 100%, 0–24 min from 0 to 80% Phase B is tested with different slopes (1%, 2%, and 4% per minute) to

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TABLE 1 Hydrophobic Fragmental Constant of the Polar Headgroup of the Different Molecules Studied Calculated from Rekker Method

Molecule	FAs	CER III and IIIa	CER I and 2	CER VI
Polar headgroup	−COOH	Phytosphingosine	Sphingosine	α-hydroxyphytosphingosine
Hydrophobic fragment constant (f)	−0.954	−5.602	−4.146	−6.858

separate the three lipid classes in one run. C_{22}:0 was selected as the representative of FAs and one CER of each subclass was chosen: CER II, CER III, and CER VI. Some preliminary experiments have shown that CHOL and FA elute closely and are difficult to separate under gradient elution. The gradient slope having no effect on their coelution, 4% per minute, is selected to decrease the time of analysis. To improve the separation, an isocratic step of heptane was applied for four minutes before starting the gradient. Four % of $CHCl_3$ are added to the heptane for a total solubilisation of FAs without changing, in a significant way, the polarity of the mobile phase. The $CHCl_3$ was chosen according to the elution strength scale.[20]

Nevertheless, in these conditions, described in Table 2, CHOL and C_{22}:0 are coeluted at 1.8 min. FAs (C_{16}:0, C_{18}:0, C_{20}:0, and C_{22}:0) are eluted as one peak at 2 min. within a short range of time (around 40 sec). The elution of CER III is observed at a higher time at 18.5 min. Contrary to the FAs, when a mixture of CERs is injected (CER I, 2, III, IIIa, and VI), different peaks are observed between 16.5 and 19.5 minutes. According to their polar headgroup, the elution order is CER I/CER 2/CER III, IIIa/CER VI.

The only other factor, which can be modified to avoid the coelution between CHOL and FAs, playing a role on the retention time of the different compounds, is the dilution mixture of the injected lipid solution.

TABLE 2 Mobile Phase used for the Separation of the Different SC Lipid Classes

Time (min)	Mobile Phase A	Mobile Phase B
	heptane/$CHCl_3$ (96:4, v/v)	acetone
0	100%	0%
4	100%	0%
24	20%	80%

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Optimization of the Dilution Mixture of the Lipids

It was previously observed, that the dilution phase plays a role as important as the mobile phase on the separation when using PAV-Sil stationary phase.[20] Thus, an improvement of CHOL and FAs separation can be expected by using a dilution mixture of the lipids less polar than $CHCl_3$/MeOH (2:1, v/v). The dilution mixture heptane/($CHCl_3$/MeOH) was selected with a fixed ratio $CHCl_3$/MeOH (2:1, v/v) and different percentages of heptane. The results are summed up in Figure 2, and confirm the impact of the dilution mixture polarity on the retention times and the selectivity. The most significant modification is observed for the C_{22}:0, from 1.8 min. for a dilution mixture of 100% $CHCl_3$/MeOH (2:1, v/v) to 11 min. for a dilution mixture of 90% heptane and 10% $CHCl_3$/MeOH (2:1, v/v). Percentages of heptane inferior to 50% in the dilution mixture lead to the coelution of the CHOLE and FAs. Percentages of heptane superior to 90%, the C_{22}:0 peak is asymmetric due to an incomplete solubilisation of the molecules in the dilution mixture. According to the results obtained, 90% of heptane in the dilution mixture is selected for our separation because it provides the best resolution between CHOL and C_{22}:0 (Rs = 3) and permits obtaining a Gaussian peak for FA.

However, C_{18}:0 is eluted as two chromatographic peaks, as well as C_{20}:0; the first one between 8.5 to 9.2 min and the second one from 10 to 10.5 min. We cannot explain why these two FAs show two different retention times. Two solutions can be envisaged to avoid these two peaks: i) the suppression of the isocratic phase heptane/$CHCl_3$ (96:4, v/v), ii) the starting gradient with a mobile phase more polar than heptane/$CHCl_3$ (96:4, v/v).

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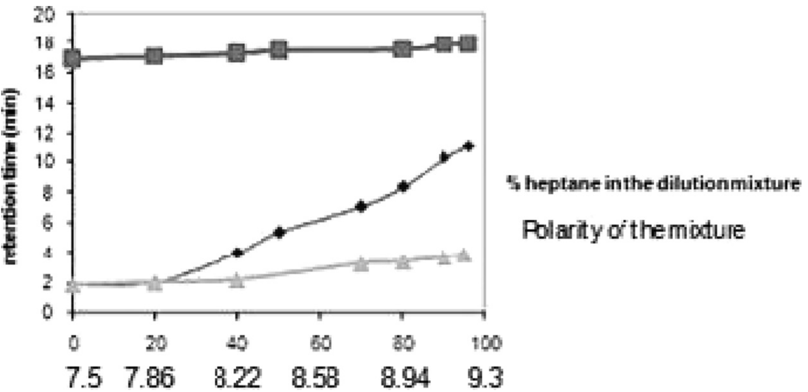

FIGURE 2 Evolution of the retention time of a representative of each lipid class: ▲ CHOL, ◆ C_{22}, ■ Cer III as a function of the % of heptane in the dilution mixture. The polarity of the solvent mixture was calculated from the Hildebrand parameters.

Even without any isocratic step, the signal of the FAs is constituted of two peaks. Despite the presence of these two peaks, the dilution mixture [90% of heptane + 10% of $CHCl_3$/MeOH (2:1, v/v)] is selected because only this method permits separation of CHOL from the FAs.

The starting gradient was then tested with a higher percentage of $CHCl_3$/MeOH (2:1, v/v) to increase its polarity. The best resolution between CHOL and FAs is obtained for a ratio heptane/$CHCl_3$ of 80:20 v/v, which is then selected even if the signal of FAs is still constituted of 2 peaks.

The final HPLC conditions consist of a gradient phase A 100%; 0–20 min 0 to 80% phase B with a slope of 4%/per min; the lipids being first diluted in a mixture of $CHCl_3$/MeOH (2:1, v/v) and then 10 times in heptane.

Figure 3 represents a chromatogram of the different lipid classes, obtained with these conditions using ELSD as detector. For the mixture of CERs (CER I, 2, III, IIIa, and VI) different peaks are observed between 10 and 16 minutes. The elution order is: CER I (10 min) < CER 2 (10.5 min) < CER III and CER IIIa (12.5 min) < CER VI (16.5 min). Similar elution order was found by Farwanah et al.[21] also working in normal phase HPLC on a silica column. This large range of elution time is due to:

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1. The different polar headgroup of the molecules: CER I and CER 2 have a sphingosine headgroup, whereas CER VI, III, and IIIa have a phytosphingosine headgroup. Moreover, the CER VI is more polar due to the presence of an αOH on its fatty moiety. The elution order

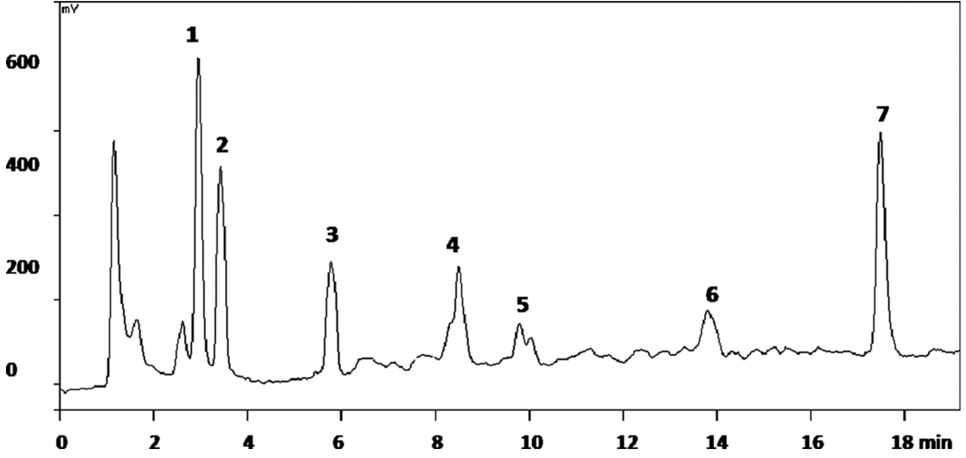

FIGURE 3 Chromatogram of three lipid classes: CHOL, FAs (C_{16}:0, C_{18}:0, C_{20}:0, C_{22}:0), CERs (I, 2, III, IIIa, VI) diluted in heptane/$CHCl_3$/MeOH (90:6.66:3.33, v/v/v) with a mobile phase A heptane/$CHCl_3$ (80:20, v/v) 100%; 0–24 min. 0 to 80% of phase B with a slope of 4%/per min with an ELSD detection. 1: CHOL, 2: FAs, 3: CERs.

is in agreement with the hydrophobic fragmental constant calculated previously for the different polar headgroups.

2. The signals of CER I and 2 are not totally overlapped. However, a total overlapping was expected since they have a similar polar headgroup. This can be attributed to a difference of solubility due to an important difference in their carbon chain length (30 against 16 for CER I and 2, respectively).

The coelution of CER III and IIIa indicates that the presence of a double bound, or not, on the FA moiety does not influence the retention in our experimental conditions.

The peaks obtained for FAs and for CERs with this method are broader than the ones obtained with 100% of $CHCl_3$/MeOH (2:1, v/v) used as the dilution mixture, because of the lower eluotropic strength of the new dilution mixture. However, the resolution ($Rs = 5$) between FAs and CER I is satisfying.

The aim in this first section was to separate the three lipid classes. This will permit to treat each class as a unique entity and then to consider the sum of the peaks obtained for each class.

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Quantitative Approach: Comparison Between the ELSD and the CAD Detectors

The aim of this analysis is to obtain the relative percentage of each class of lipids in sample preparations from natural human SC lipids.

Two kinds of strategies can be applied for this quantitative approach: i) the internal normalisation, which needs similar response factors for all the compounds and all the classes or ii) the external standardisation where one representative of each class has to be selected as standard reference. The interclass response factors are not necessarily similar. However, the response factors have to be comparable for all the molecules within a class. To determine which quantitative strategy has to be applied in our study, a preliminary study was carried out on the response model of ELSD and CAD, followed by a response factor comparison.

Response Model

The response models of both detectors were largely studied. As it was mentioned in several works,[22] the responses are described for both ELSD and CAD by Eqiation (1).

$$Y = Am^b \tag{1}$$

where Y is the output signal from the detector (peak area in our case), m the injected amount and A and b are two numerical coefficients: A the

response intensity and b the response shape. These two coefficients depend on the experimental conditions like flow rate, nature of the solutes, and nature of the mobile phase.[23,24] Both detectors have only a linear response on a restricted concentration range, broader in the case of CAD.

To evaluate the response model and the regression fit, a range of concentrations between 3.10^{-5} mol/L and 2.10^{-4} mol/L was studied in the case of CAD and a range between 2.10^{-4} mol/L and 3.10^{-3} mol/L was selected for ELSD. Different statistical parameters have classically to be taken into account[25,26] to study the regression fit, and are summarised in Table 3. The different results obtained are in favor of the CAD. Indeed, r^2 is closer to 1 and F_{ref} is higher in the case of CAD, whereas F_{lof} is smaller, indicating a better fit of the regression model.

These results allow us to favor the use of CAD compared to ELSD for a quantitative study of the lipids present in the SC.

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Response Factors of Lipids

The response factors were calculated for the smallest concentrations studied (3.10^{-5} mol/L for CAD and 2.10^{-4} mol/L for ELSD (response factor = peak area/concentration). One standard was selected as representing each class: CHOL, C_{22}:0, and CER III. C_{22}:0 was chosen because of its abundance in the SC.[27] CER III was selected because it presents an intermediate behaviour in a retention point of view.

The results, gathered in Table 4, show that the response factors are different from CHOL, FA, and CERs. The strategy of the internal normalization is not usable in this study. For the external standardisation, it was mentioned that the response factors of molecules in a same class have to be similar. To be considered as similar, the relative response factor (response factor for a compound/response factor of the representative standard of the corresponding class) should currently be included between 0.8 and 1.2 as it is defined by the ICH (International Conference of Harmonisation).

Several studies found the response factors to be independent from the nature of the solute.[28,29] However, the response factors of CAD[30] as well as ELSD[23] are known to vary with the solvents chosen and with the use of a gradient.

The three lipid classes are eluted with a percentage of acetone of: 14% for CHOL, 27 to 34% for FAs, 40 to 66% for CERs. These differences of mobile phase compositions would explain why such variations in response factors are observed between lipid families. Several works[31] already observed this phenomenon studying response factors of different sulfonamides. The use of a mobile phase compensation permitted to

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TABLE 3 Comparison of the Different Statistical Parameters of ELSD and CAD. r^2: Correlation Coefficient; F_{reg}: Ratio between the Regression and the Residual Variance; F_{lof}: Lack of Fit

	ELSD									CAD								
	a	σa	b	σb	r^2	F_{reg}	p_{reg}	F_{lof}	p_{lof}	a	σa	b	σb	r^2	F_{reg}	p_{reg}	F_{lof}	p_{lof}
CHOL	70.97×10^5	25.10^5	1.23	0.11	0.955	343	$4.3.10^{-9}$	99.5	3.10^{-12}	11.57×10^5	54.10^3	1.067	0.03	0.993	1830	2.10^{-15}	33.5	$1.6.10^{-5}$
C_{22}:0	1.4×10^{10}	31.10^5	2.34	0.1	0.946	226	0.097	22.8	1.10^{-9}	28.60×10^4	17.10^3	0.95	0.04	0.990	860	1.10^{-3}	12.2	3.10^{-13}
CER III	17×10^5	7.10^4	0.96	0.08	0.965	272	0.054	4.3	1.410^{-8}	31.31×10^3	761	0.65	0.03	0.974	489	1.10^{-11}	3.83	0.05

TABLE 4 Response Factors of the Different Standards studied, with ELSD or CAD as Detector

		ELSD		CAD	
Class	Molecule	Response Factor	Relative Response Factor Compound/Class Standard	Response Factor	Relative Response Factor Compound/Class Standard
CHOLE	CHOLE	556660	1	406000	1
FAs	C_{16}:0	110000	0.2	100600	0.2
	C_{18}:0	242000	0.48	254000	0.53
	C_{20}:0	357000	0.71	345600	0.72
	C_{22}:0	520000	1	482600	1
CERs	CER 1	3210000	0.9	905000	0.82
	CER II	1870000	1.5	980000	0.9
	CER III	2940000	1	1100000	1
	CER IIIa	2105000	1.4	997200	0.9
	CER VI	1605000	1.8	937000	0.85

palliate this problem and to obtain similar factor. Lisà et al.[32] also found a variation of response factor between triacylglycerols as a function on their chain length, corrected by use of a mobile phase compensation.

For the FAs family, the response factors cannot be considered as similar from one molecule to another, their relative response factors being inferior to 0.8. This phenomenon is observed both with CAD and ELSD. However, the highest differences are observed between the standard C_{22}:0 and FAs with shorter carbon chains (18 and 16 carbons). It is known that the major SC FA species owns at least 20 carbons.[17] It can then be considered that the standard chosen is appropriate for our purpose and that the contribution of shorter FA in the signal could be negligible. The phenomenon of an increase of the response factor with the increase of the carbon chain length was also observed by Lisà et al.[32] with triacylglycerols. Moreover, as mentioned before, FAs are coeluted, the difference in the response factors cannot be explained by a difference in mobile phase composition as it was observed for the three lipids classes. In our case, the high variation within the FAs class can be due to a difference of volatility of the different FAs as it was observed by Stolyhwo et al.[33]

In the case of CERs, only the use of CAD show relative response factors between the different compounds and the standard CER III between 0.8 and 1.2 that can be considered as similar. The choice of CER III as intern representative of the CERs class is then appropriate for our study. On the contrary, using ELSD provides relative response factors lower than 0.8, showing that this detector is less suitable for external standardisation.

The CAD seems to be the most adapted detector for further quantitative studies. However, to validate this quantitative strategy of external

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standardization, it is necessary to study deeper the response model of both ELSD and CAD detectors to conclude about the more suitable one.

Validation of the Analysis Method of Lipids by Class with CAD

Several criterions were studied to access the validation of the quantitative approach such as the precision, and the accuracy.

Precision: The results are presented in Table 5. The repeatability was estimated by running each analysis in triplicate. It was estimated by measuring the peak area and calculated for each concentration. For all the concentrations of the three standards, the RSD% are lower with CAD. The recovery is estimated from the comparison of theoretical and experimental value of the concentration.

Accuracy: The accuracy, expressed as recovery %, obtained for each concentration are described in Table 5. For each concentration, the accuracy value corresponds to the average of the three injections. According to the results, this parameter is also in favor of CAD. The recoveries are satisfying for a concentration of 3.10^{-5} mol/L for CHOLE, from 5.10^{-5} mol/L for C_{22}:0, and 3.10^{-5} mol/L for CER III. As observed, the solubility seems to be the limiting factor to obtain a satisfying accuracy, despite the optimization of the dilution phase. Indeed, the accuracy values decrease with the concentrations increase.

To validate the range studied, two criteria have to be considered: the precision has to be inferior to 3% and the accuracy has to be included

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TABLE 5 Comparison of the Accuracy between CAD and ELSD. The Repeatability is Expressed in Relative Standard Deviation of Recovery

	CAD			ELSD		
	Theoretical Concentration mol/L	Recovery (%) (n = 3)	Repeatability (RSD %) (n = 3)	Concentration Injected mol/L	Recovery (%) (n = 3)	Repeatability (RSD %) (n = 3)
CHOL	3.10^{-5}	88	1.55	3.10^{-4}	41	7.78
	5.10^{-5}	70	1.42	5.10^{-4}	74	6.72
	8.10^{-5}	65	0.74	1.10^{-3}	77	4.13
	1.10^{-4}	67	0.72	2.10^{-3}	120	1.65
	2.10^{-4}	68	0.23	3.10^{-3}	95	6.36
C_{22}:0	3.10^{-5}	132	4.57	3.10^{-4}	64	8.56
	5.10^{-5}	103	4.48	5.10^{-4}	60	5.52
	8.10^{-5}	88	2.92	1.10^{-3}	99	5.21
	1.10^{-4}	99	2.74	2.10^{-3}	63	4.53
	2.10^{-4}	94	2.32	3.10^{-3}	64	4.2
CER III	3.10^{-5}	107	3.89	3.10^{-4}	47	5.23
	5.10^{-5}	112	3.54	5.10^{-4}	124	4.25
	8.10^{-5}	102	3.02	1.10^{-3}	93	5.3
	1.10^{-4}	96	1.52	2.10^{-3}	100	3.12
	2.10^{-4}	90	1.46	3.10^{-3}	88	2.56

between 80 and 120%. According to the results, the concentration of 3.10^{-5} mol/L is acceptable for the CHOL, and the ranges studies can be accepted from 8.10^{-5} mol/L to 2.10^{-4} mol/L for C_{22}:0 and for CER III.

Application to Natural Stratum Corneum Lipids

The HPLC method developed with the standard molecules selected previously was applied to human SC lipids extracts obtained by the scrapping method presented above. A chromatogram of the extracted lipids is represented Figure 4. A peak at 2.8 min. from residual by-compounds of the cotton is observed in addition to the different signals of the three lipid classes. Two peaks are observed for the FAs at 5.8 and 8.2 min and three peaks are observed for CERs at 10 (sphingosine), at 13.2 (phytosphingosine), and 17 min. (hydroxyphytosphingosine). For both the FAs and CERs classes, the sum of the different peak area is realized. From the etalon curves realised previously for CHOL, C_{22}:0, and CER III, the concentration of each class can then be expressed in mol/L in the injected solution. Moreover, by treating each standard as representing the class, it is possible to calculate the concentration of each class in g/cm^2 in the skin. The results are presented in Table 6. The relative percentages of the three classes are comparable between the area and the concentration in mol/L or g/cm^2, and correspond approximately to an average ratio of 20/20/60 for CHOL, FAs, and CERs, respectively. The inhomogeneity of the data found in the literature is due to different factors: i) different body sites,[2] ii) the method (stripping, topical application . . .),[17] iii) the nature of the solvent,[34] iv) inter-individual variations. However, despite

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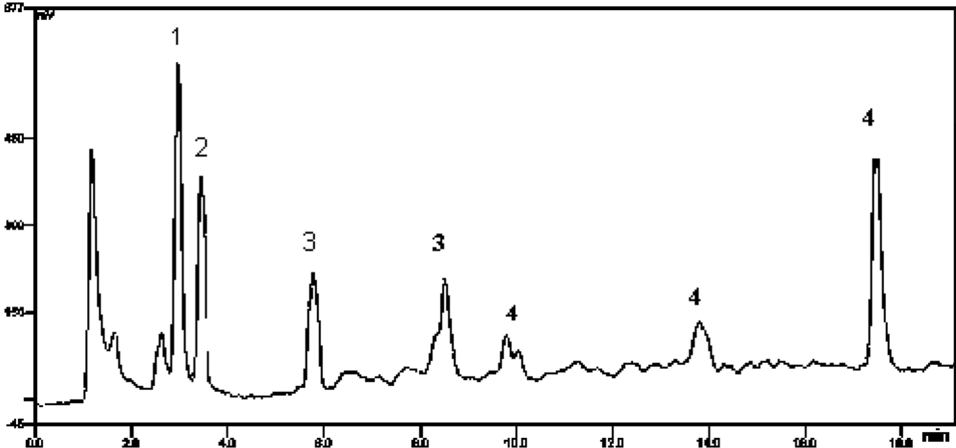

FIGURE 4 Chromatogram of a lipid extract from human SC, diluted in $CHCl_3$/MeOH (2:1, v/v) and then 10 times in heptane/$CHCl_3$ (80:20, v/v) with a mobile phase A heptane/$CHCl_3$ (80:20, v/v) 100%; 0–24 min. 0 to 80% of phase B with a slope of 4%/per min with CAD. 1 coton; 2 CHOL; 3 FAs; 4 CERs.

TABLE 6 Quantitative Datas about the Different SC Lipid Classes Extracted by Scrapping Method. All the Concentrations Correspond to the Concentration of Equivalent Standard: C_{22}:0 for the FAs and CER III for the CERs. For FAs and CERs, the Areas of the Different Peaks are Added

Lipid Class	Total Area	Area Ratio	Concentration 10^{-4} mol/L	Mol/L %	Concentration in the Skin μg/cm^2	g/cm^2 %
CHOL	64	25	1	22	4.3	18
FAs	59	26	1.3	30	5.6	23
CERs	130	51	2.2	48	14.3	59

the inhomogeneity of the data found in the literature, the ratio 20/20/60 is in agreement with most of the works carried out in the inner forearm.[17,35]

Weerheim et al.[17] and Yamamoto et al.[36] also developed a method to separate the SC lipid by class with high performance thin layer chromatography (HPTLC). The relative proportions of the lipid classes are described in weight % and in mol%. However, the molar mass of the different molecules present in human extract are not known due to wide diversities of carbon chain length; the approximation made is then considerable. For this reason, the concentrations of the different classes are expressed in our work in concentration equivalent standards (CHOL, C_{22}:0, CER III) considering the molar mass of the standards. Moreover, HPLC is more precise for quantitative studies than HPTLC where the concentrations are evaluated by densitometry. Our approach is deeper in a term of validation and more detailed about the different notions of accuracy, repeatability, sensibility, than previous published data.

Our first results can only be considered as an example of feasibility of the analytical method here developed. It is difficult to establish if the differences between several studies are due to the extraction mode or to the regression mode used, and to the approximation made due to the lack of information about all the molecular mass. For this reason, it is of great interest to test different extraction modes with the same regression mode. Our quantitative approach will permit easily to compare different extraction modes, but will also be extendable to compare the effects of topical applications of moisturizing formulations.

CONCLUSION

The reasoning made in this work shows the influence not only of the mobile phase but also of the dilution mixture of the entities studied on the separation. The HLPC method developed provides the separation of three classes of the SC lipid classes in less than 20 minutes. CAD has several important advantages compared to ELSD, such as accuracy, precision, and

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allows a study of small lipid concentrations. Besides, an external standardization using one representative molecule for each class permits a quantitative investigation. Studies are on the way in our lab using this separation method to test different extraction modes for a comparison of the lipid composition of the SC as a function of different parameters such as the age, the origin, the color of the skin.

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