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# Isolation of ceramide fractions from skin sample by subcritical chromatography with packed silica and evaporative light scattering detection☆

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**Abstract**

Separative method of lipid classes from the *stratum corneum* was developed with packed silica and supercritical $CO_2$ containing 10% of methanol at 15 °C, 15 MPa and 3 ml $min^{-1}$. The elution order of lipid classes was first esterified cholesterol, triglycerides, squalene co-eluted in a single peak, then free fatty acids, free cholesterol, ceramides and finally glycosylceramides. The ceramides were eluted in several fractions which depended on the number of hydroxyl groups in the molecule, i.e. more hydroxyl groups were contained in ceramides, more important was the retention. Moreover, the retention was not altered by the presence of carbon double bond and variation of the alkyl chain length.

The ceramide response with the evaporative light scattering detector was improved by turning the influence of the solvent nature on the response to advantage. Therefore, addition of various solvents with or without triethylamine and formic acid were tested in post-column due to the incompatibility of such modifiers with silica stationary phase. Thereby the solvent conditions for the separation and the detection can be adjusted almost independently. The response was greatly increased by post-column addition of 1% (v/v) triethylamine and its equivalent amount of formic acid in dichloromethane introduced at 0.1 ml $min^{-1}$ into the mobile phase. This device had allowed the detection of 400 ng of ceramide with a S/N = 21, whereas no peak was observed in absence of the post-column addition. Finally, the method was applied to the treatment of skin sample which led to highly enriched ceramide fraction.


*Keywords:* Subcritical fluid chromatography; Evaporative light scattering detection; Lipids; Ceramide

## 1. Introduction

Ceramides are lipids involved in the skin barrier, located in the *stratum corneum* [1–3] and present the highest structural heterogeneity of the whole lipids from this origin [4–6]. In order to determine this heterogeneity, our laboratories had achieved sev-

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eral developments devoted to the separation and the identification of ceramide molecular species using various chromatographic techniques: gas chromatography–mass spectrometry [7], reversed-phase liquid chromatography [8,9] and supercritical fluid chromatography (SFC) [10]. To use these methods for ceramides from skin sample, ceramides should be isolated from the whole lipids present in the *stratum corneum*. Several non-invasive methods for extraction in vivo of lipids from stratum corneum exist, by stripping [11–13], scraping [14,15], skin rinsing [4,16] and extraction chamber [15,17]. Whatever these approaches, the three lipid classes present in the *stratum corneum* were recovered: free fatty acids, cholesterol and ceramides. However, other lipids can contaminate these samples such as squalene, triglycerides and cholesterol ester secreted by sebaceous glands or glycosylceramides from deeper layer of the epidermis [12,15]. Therefore, the separation method of lipids from the *stratum corneum* extraction should be based on the separation of cholesterol, free fatty acids and ceramides and extended to the other lipids potentially present in such samples.

The strategy in order to obtain cutaneous ceramides was the development of a lipid class analysis associated with the collection of ceramide fractions. The main separation methods were performed using thin layer chromatography which led to low amount of lipid recovery. For such purpose, methods in normal phase liquid chromatography were developed [15,18], however, they often required the use of solvent like chloroform and hexane. Therefore, SFC with silica stationary phase was preferentially selected. The high eluotropic strength of carbon dioxide ($CO_2$) avoids the use of toxic modifier, and allows the collection of fractions with low solvent content [19]. Moreover, important features of $CO_2$ based SFC are its possibility of fine tune of eluotropic strength and selectivity by the addition of organic modifier together with pressure and temperature adjustments [20]. Our aim was to develop an analytical scale separation method devoted to the collection of ceramide fractions from skin.

As the stratum corneum is a very thin layer of the skin, the lipid amount per unit surface is very low. Therefore, the method should be sensitive enough for this purpose. The evaporative light scattering detector (ELSD) is an appropriate detection mode for lipids [21–23]. In order to improve its response, the particular property of the triethylamine (TEA) in equimolar amount with formic acid (HCOOH) added in the mobile phase to increase the ELSD response was envisaged [24–27]. As these modifiers are not compatible with all normal phase packings, the addition of the TEA/HCOOH mixture should be performed in post-column. The ELSD response is highly influenced by the mobile phase composition [28,29]. Therefore, a particular study was carried out in order to assess the influence of solvents where TEA and HCOOH were conveyed. Then, the amount of TEA/HCOOH was optimised for this objective.

## 2. Experimental

### 2.1. Chemicals

#### 2.1.1. Ceramide mixtures

Ceramide type III (mixture of non-hydroxy ceramides where the sphingoid base is mainly a sphingosine and the fatty moiety acid can be unsaturated), ceramide type IV (mixture of hydroxy ceramides where the sphingoid base is mainly a sphingosine and the fatty acid moiety can be unsaturated), ceramide standards: *N*-palmitoyl-DL-dihydrosphingosine, *N*-palmitoyl-D-sphingosine, *N*-stearoyl-D-sphingosine, *N*-oleoyl-D-sphingosine, *N*-lignoceroyl-DL-dihydrosphingosine, *N*-nervonoyl-D-sphingosine and cholesterol, cholesterol stearate, free fatty acid, squalene, triglyceride were all purchased from Sigma (St. Quentin Fallavier, France). Ceramide III (stearoyl phytosphingosine), ceramide IIIB (oleoyl phytosphingosine) and ceramide VI (hydroxy-stearoyl phytosphingosine) were a generous gift of Cosmoferm (Delft, The Netherlands).

All of the solvents were HPLC grade: MeOH (methanol; Merck, Darmstadt, Germany), $CHCl_3$ (chloroform; J.T. Baker, Deventer, Holland), $CH_2Cl_2$ (dichloromethane), TEA (SDS, Peypin, France) and HCOOH (Fischer Scientific, Elancourt, France).

### 2.2. SFC apparatus

Subcritical chromatographic separations were carried out using two Jasco 880-PU pumps (Tokyo, Japan): one for the $CO_2$ and the second for the modifier. The pump head used for pumping the $CO_2$ was

cooled to −2 °C by a cryostat (Julabo F10c, Seelbach, Germany) supplied by Touzart et Matignon, Les Ulis, France. After mixing the modifier and the $CO_2$, the fluid was introduced at 3 ml $min^{-1}$ into a dynamic mixing chamber PU 4046 (Pye Unicam, Cambridge, UK), connected to a pulse dampner SEDERE (supplied by Touzart et Matignon, Vitry sur Seine, France). The injection valve was fitted with a 20 μl loop (model 7125 Rheodyne, Cotati, CA, USA). The column was thermostated using a Jetstream 2 temperature controller (Thermotecnics Products GmbH, Austria). The outlet column pressure was controlled by a regulator Jasco 880-81 (Tokyo, Japan). The outlet regulator tube (0.25 mm i.d.) was heated to 60 °C to avoid ice formation during the $CO_2$ depressurisation. Detection was carried out with an ELSD, DDL 31 (Eurosep, Cergy-Pontoise, France). The nebulisation gas was air at 0.17 MPa, the nebulisation temperature and the drift tube were set up at 20 and 35 °C, respectively. The photomultiplier gain was set at 600. Chromatograms were recorded with an Azur acquisition software (Datalys, Saint Martin d'Heres, France). The packed silica was lichrospher Si-60, 250 mm × 4 mm, 5 μm (Merck).

### 2.3. Post-column apparatus

For the post-column addition process of TEA/HCOOH, a Jasco 880-PU pump and a pulse dampner (Touzart and Matignon, Vitry sur Seine, France) was used.

### 2.4. Extraction and collection of skin lipids

Two hundred milligrams of epidermis were obtained by scraping a breast skin biopsy. A liquid extraction by Folch et al.'s method [30] was performed on the epidermis with 5 ml of chloroform:methanol (2:1, v/v) and one-fourth of this volume of water was added. After shaking and decantation, two layers appeared. The lower was composed principally of chloroform and contained most of the extracted lipids. Ten milligrams of material were recovered in the organic fraction and dissolved in 500 μl of chloroform:methanol (2:1, v/v).

This extract was collected with the method developed herein into two fractions after the backpressure regulator. In order to treat the whole extract, the collection was performed 20 times since only 20 μl were injected each time.

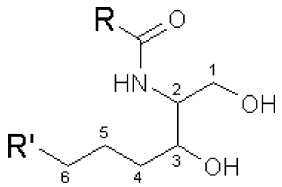


Fig. 1. General structures of ceramides. R and R′ are, respectively, fatty acid and sphingoid base hydrocarbonaceous chain. The represented structure corresponds to ceramide with dihydrosphingosine base. An OH on position 4: structure of ceramides with phytosphingosine base. A double bond between carbons 4 and 5: structure of ceramides with sphingosine base. One double between carbons 4 and 5 and an OH on position 6: structure of ceramide with 6-hydroxy-4-sphingosine base.

## 3. Results and discussion

In the epidermis, eight classes of ceramides were encountered resulting of the combination of three sphingoid bases (sphingosine, phytosphingosine, 6-hydroxy-4-sphingosine) with two types of lipid moieties (α-hydroxy or non-hydroxy fatty acid) and, furthermore, sphingosine and 6-hydroxy-4-sphingosine bases can be also linked with non-hydroxy fatty acid esterified in the ω-position [3–6] (Fig. 1). For the separation development, only six classes were commercially available which were sphingosine, phytosphingosine or sphinganine linked either with α-hydroxy or non-hydroxy fatty acid. Although the latter sphingoid base was not reported in human epidermis, it was considered during this development in order to enrich the retention behaviour understanding.

### 3.1. Separation

Ceramide type III mixture was firstly used for the exploration of the eluotropic strength because this ceramide mixture contains important heterogeneity due to chain length or degree of unsaturation variation. The eventual "backbone" discrimination between molecular species can be thus underscored. MeOH was selected as $CO_2$ modifier in order to elute ceramides. The increase in MeOH content led to a decrease in retention time. For the smaller content of modifier (i.e. 5%), the ceramide type III mixture presented a beginning of discrimination between their molecular species, i.e. two badly resolved peaks appeared. The retention behaviour was also assessed

with temperature and pressure variations. The temperature decrease led to the increase in fluid density which increased the eluotropic strength, and thus decreased retention. This behaviour was already described [31]. The increase in pressure led to the same behaviour. In order to avoid the partial discrimination of type III molecular species, the weakest tested temperature leading to the elution as a single peak was then preferred which was more appropriate for our fractionation objective. The final conditions were: 10% MeOH, 15 °C and 15 MPa (Fig. 2).

*N*-Palmitoyl-D-sphingosine, *N*-stearoyl-D-sphingosine, *N*-nervonoyl-D-sphingosine, *N*-palmitoyl-DL-dihydrosphingosine, *N*-lignoceroyl-DL-dihydrosphingosine and ceramide type III were co-eluted in peak 4 (Fig. 2). The structural differences of these standards are due to the presence of a further carbon double bond on the base moiety (i.e. for ceramides containing

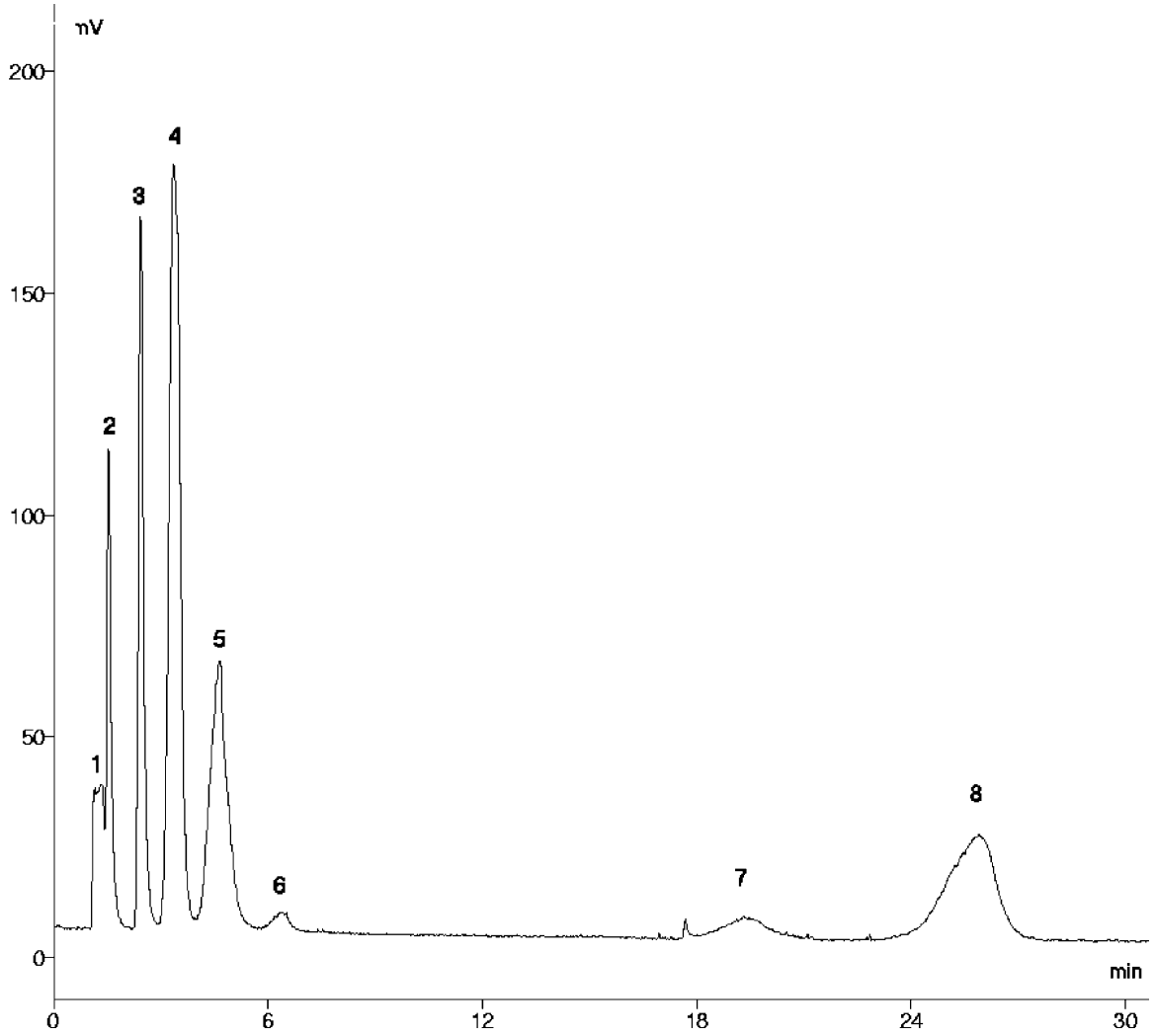


Fig. 2. Ten percent MeOH, 15 °C, 15 MPa. Squalene, triglycerides and cholesterol stearate co-eluted (1); free fatty acid (2); cholesterol (3); ceramides with sphingosine or dihydrosphingosine linked with non-hydroxy fatty acid (4); ceramides with phytosphingosine linked with non-hydroxy fatty acid and ceramides with sphingosine and α-hydroxy fatty acid (5); ceramides with phytosphingosine linked with α-hydroxy fatty acid (6); glucosylceramides (7); galactosylceramides (8).

sphingosine compared to those with dihydrosphingosine) or on the fatty acid moiety (i.e. nervonoyl fatty acid contains one carbon double bond compared to the other fatty acids involved in ceramides). Moreover, these ceramides presented various lengths of their alkyl chains which mean that the hydrocarbonaceous volume of these molecules did not influence the retention on silica stationary phase. These observations were confirmed by the same behaviour in peak 5 which contained co-eluted ceramide III and IIIB. Ceramide IIIB possesses a further carbon double bond in its fatty acid moiety compared to ceramide III.

Difference in retention occurred for ceramides with a different number of hydroxyl groups. Ceramides containing a sphingosine base with non-hydroxy fatty acid (peak 4) were less retained than those containing a phytosphingosine base with non-hydroxy fatty acid (peak 5). This latter ceramide class was co-eluted with ceramides containing a sphingosine base linked with α-hydroxy fatty acid (i.e. ceramide type IV). Then ceramides containing a phytosphingosine with α-hydroxy fatty acid (ceramide VI) were more retained (i.e. eluted in peak 6). Therefore, a pre-fractionation can be obtained between ceramides depending on the number of hydroxyl groups even if this functional group was not located in the same part of the molecule (i.e. base or acid moiety).

The group adsorption energy values are 0.25 and 5.6, respectively, for olefinic carbon and hydroxyl in liquid chromatography with silica [32] which is in accordance with the retention behaviour observed herein. Moreover, the adsorption of these groups requires an accessibility to the adsorption sites of the silica which is influenced by the steric hindrance and the presence of other functional groups in the molecule. The carbon double bond is next to an hydroxyl group in sphingosine base, therefore its contribution to the adsorption phenomenon is of secondary importance compared to the presence of the hydroxyl groups.

This method development did not take into account the totality of ceramide classes encountered in the *stratum corneum*. Four classes were not commercially available: 6-hydroxy-4-sphingosine linked with α-hydroxy or non-hydroxy fatty acid, and sphingosine or 6-hydroxy-4-sphingosine linked with non-hydroxy fatty acid esterified in the ω-position (ceramide I and IV [6]). From the behaviour observed with this method, a retention prediction of some missing classes was possible. 6-Hydroxy-4-sphingosine linked with non-hydroxy or α-hydroxy fatty acid should eluted, respectively, with peaks 5 and 6, since the structural difference consists of a further carbon double in the base moiety in regard to phytosphingosine. Consequently, the behaviour of these two missing classes was expected concomitant with ceramides containing phytosphingosine. For the latter classes (i.e. ceramide I and IV), the retention cannot be predict because the ester group is located in the middle of the alkyl chain.

Other lipids present in *stratum corneum* samples as esterified cholesterol, triglycerides, squalene, free fatty acid and cholesterol, were eluted before ceramides preventing the contamination of ceramide fractions by these other lipids potentially contained in the extracts (Fig. 2). This retention behaviour was similar in liquid chromatography using diol as glyceropropyl bonded silica stationary phase [15,18] or PVA-Sil® as poly(vinyl-alcohol) [26] or silica gel [33].

Glycosylceramides showed an important retention. Therefore, this method remained relevant to prevent contamination of ceramide fractions from this lipid class.

This method provides the resolution between the main lipid classes of the *stratum corneum* with a time analysis shorter than 10 min in isocratic elution with supercritical fluid containing 10% of organic solvent.

### 3.2. Optimisation of the post-column conditions for detection improvement

The amount of ceramides present in the *stratum corneum* is about 20 $\mu g\,cm^{-2}$ and some classes represent about 2% of this amount [13,17]. Therefore, the method should be able to detect, at least, amount about 0.4 $\mu g\,cm^{-2}$ if the extraction was totally achieved [34]. To overcome the moderate sensitivity of ELSD [35], the detection was studied with addition of TEA/HCOOH.

Previously, the addition of this modifier was carried out directly in the mobile phase [8,9,26]. Using octadecyl grafted silica, porous graphitic carbon or PVA-Sil® stationary phases, TEA/HCOOH (1:1, mol/mol) at 0.1% in the mobile phase enhanced the response with only minor effect on the retention of non ionisable lipids. However, the strong interactions

that TEA or HCOOH may develop with the silanols of the silica stationary phase hinder their use as mobile phase additives. Since the mechanism of the response enhancement is explained as a supramolecular assembly of TEA/HCOOH ion pair with the solute [27], the additive could be also introduced after the separation process, i.e. in post-column addition. Two positions of the modifier introduction were tested before the backpressure regulator and between the backpressure regulator and the ELSD. Lower increase in response occurred for position after the backpressure regulator which may be due to a delay of the homogenisation between the compounds solubilised into the bulk of supercritical fluid and the additional detection modifier. Consequently, post-column addition was performed before the depressurisation area.

### 3.2.1. Solvent effect

The phenomenon was found to be solutes, solvents, temperature and flow rate dependent [27]. Fortunately, polar lipids (ceramides or cholesterol) show a higher response increase than squalene or tripalmitin. Thus, for a given solute (ceramide) and with a low drift tube temperature, the post-column solvent and TEA/HCOOH addition must be set up keeping in mind that a compromise must be found between:

(1) the response enhancement due to TEA/HCOOH dissolved in a suitable solvent in order to benefit from the best response enhancement;
(2) the flow rate of the post-column additives since increasing the total flow rate decreases the response amplification.

The total flow rate of the MeOH:$CO_2$ 10:90 effluent was 3 ml min$^{-1}$ resulting in a flow rate at 0.3 ml min$^{-1}$ for methanol reaching the nebuliser outlet after $CO_2$ depressurisation. Various solvents and flow rates in post-column were then tested in the range 0.1–0.7 ml min$^{-1}$ (Table 1). This assessment was carried out with cholesterol which exhibited a similar behaviour than ceramides in regard to the different steps involved in the ELSD [27].

Independent of the nature of the additional solvent, the *N* values show a weak dependence on the variation of linear speed of the effluent entering the nebulisation chamber. No additional molecule dispersion occurred because of the great linear speed of the supercritical mobile phase after depressurisation which seems not modify by the relative low amount of additional solvent added in post-column. The peak area decreased since MeOH addition in post-column occurred. This decrease was less pronounced with $CHCl_3$ until 0.2 ml min$^{-1}$ whereas no response loss was observed with $CH_2Cl_2$ until 0.4 ml min$^{-1}$. Whatever the solvent nature, the increase of solvent flow rate led to a more or less marked decrease in ELSD response. Such decrease was described by Robinson et al. [36] for triglycerides for which the peak area were divided by three when the flow rate was increased from 0.5 to 3 ml min$^{-1}$.

Table 1
Influence of the nature and the flow rate of additional solvent on the response of ELSD

| Post-column flow rate (ml min$^{-1}$) | Solvent | Peak area | *N* |
|---|---|---|---|
| 0.0 | – | 178 | 1000 |
| 0.1 | MeOH | 128 | 950 |
| 0.2 | MeOH | 42 | 1000 |
| 0.1 | $CHCl_3$ | 150 | 1200 |
| 0.2 | $CHCl_3$ | 161 | 1100 |
| 0.4 | $CHCl_3$ | 109 | 1200 |
| 0.7 | $CHCl_3$ | 32 | 1200 |
| 0.1 | $CH_2Cl_2$ | 169 | 1100 |
| 0.2 | $CH_2Cl_2$ | 179 | 1000 |
| 0.4 | $CH_2Cl_2$ | 176 | 1100 |
| 0.7 | $CH_2Cl_2$ | 129 | 1100 |

Cholesterol at 0.2 mg ml$^{-1}$.

The choice of the solvent nature was checked in presence of TEA/HCOOH (Table 2). The concentration of cholesterol was decreased to 0.024 mg ml$^{-1}$ because at 0.2 mg ml$^{-1}$ with the addition of TEA/

Table 2
Influence of the additional solvent nature with 1% (v/v) of TEA and an equimolar amount of HCOOH on the ELSD response

| Nature of the additional solvent | Peak area | Area ratio[a] |
|---|---|---|
| – | 11 | – |
| MeOH | 151 | 14 |
| $CH_2Cl_2$ | 254 | 24 |
| $CHCl_3$ | 214 | 20 |

Cholesterol at 0.024 mg ml$^{-1}$ and flow rate of the additional solvent at 0.1 ml min$^{-1}$.

[a] Ratio between area with post-column addition divided by the area without post-column.

HCOOH, the peak response exceeds the photomultiplier range. These results show that TEA/HCOOH increased the response of the ELSD whatever the solvent, however, the solvent nature was important to exacerbate the influence of TEA/HCOOH. The more important increase occurred with $CH_2Cl_2$, thus the post-column condition was with $CH_2Cl_2$ at $0.1\,ml\,min^{-1}$.

### 3.2.2. Percentage of TEA/HCOOH

In reversed-phase liquid chromatography for ceramide analysis, a low amount of TEA/HCOOH was required to observe the maximum of response increase of the ELSD (i.e. 0.1 and 0.06%, respectively, in HPLC [24,25] and SFC [10]). With the method developed herein at these percentages, an increase was observed, however, other percentages were tested in order to select the optimal percentage (Fig. 3). One percent of TEA/HCOOH in $CH_2Cl_2$ was a sufficient to reach the maximum of the ELSD response. In the total liquid amount entered in the nebulisation chamber, this percentage represented 0.25 and 0.40%, respectively, when the post-column flow rate was set at 0.1 and $0.2\,ml\,min^{-1}$, respectively, since the liquid content in the chromatographic effluent represented $0.3\,ml\,min^{-1}$ after the depressurisation. However, as increasing the post-column flow rate, i.e. increasing the TEA/HCOOH amount, exhibited no further significant improvement of the response, the selected condition was 1% (v/v) of TEA with an equivalent amount of HCOOH carried by $CH_2Cl_2$ at

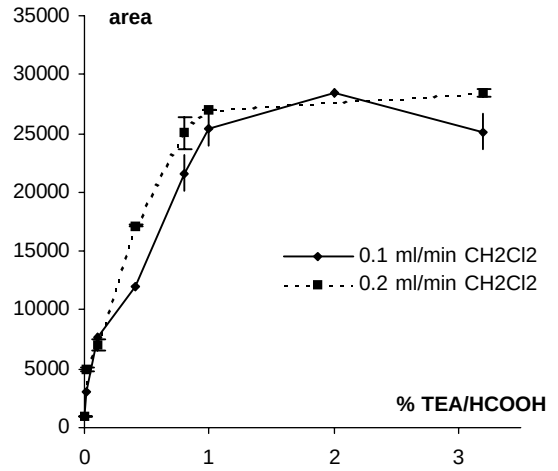


Fig. 3. Response variation of the ELSD with the post-column addition of various amount of TEA/HCOOH. Ten percent MeOH, 15 °C, 15 MPa ($0.024\,mg\,ml^{-1}$ cholesterol).

$0.1\,ml\,min^{-1}$ which was the less solvent consuming condition.

Relatively to the total effluent entering the detector in this condition, the amount of TEA/COOH corresponded to $3.1 \times 10^{-2}$%. The response increase occurred with a lower amount than in HPLC. This difference was already observed in SFC with octadecyl silica stationary phase where $6 \times 10^{-2}$% was sufficient [10]. In SFC, the main part of the effluent (i.e. the $CO_2$) did not have to be nebulised due to its depressurisation. This feature may induce a

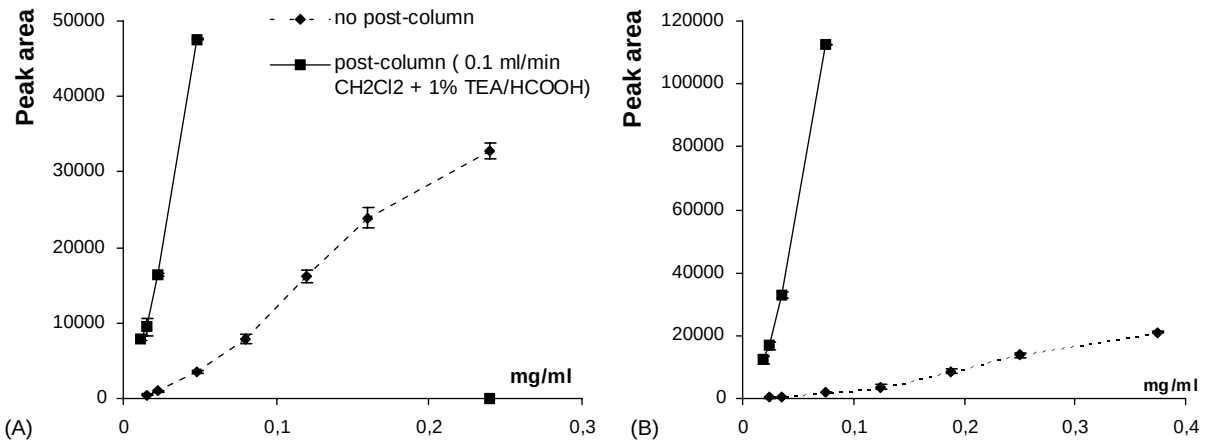


Fig. 4. Calibration curves of cholesterol (A) and ceramide III (B). Injection volume 20 μl, 10% MeOH, 15 °C, 15 MPa.

concentration phenomenon of TEA/HCOOH into the solvent droplets. Therefore, the association between the solute and TEA/HCOOH was encouraged.

### 3.2.3. Nature of the solute

Finally, these conditions were applied to ceramide III. The comparison of calibration curves was performed with cholesterol and ceramide III without and with post-column addition (Fig. 4). As the response is not linear, such curves allowed to show that the importance of the response increase varies with the compound concentration. For the both compounds over the range of the tested concentrations, the response of the ELSD was improved. However, the difference of response was more important with ceramide III than with cholesterol.

A striking increase with the addition in post-column of TEA/HCOOH occurred for ceramide peaks which allowed to clearly observed all ceramide fractions (Fig. 5). When 0.4 μg of ceramide III was injected no peak was observed without post-column addition, whereas with the post-column addition the S/N was equal to 21. This sensitivity was less important than those obtained in previous studies performed in reversed-phase chromatography (i.e. 0.5 ng [37] and 5 ng [38]; S/N = 3). The reasons of this difference were due to the different nature of the solvents used for the mobile phase, the smaller flow rate especially in the case of capillary liquid chromatography [37] and, moreover, the post-column addition generated more important background noise. However, such difference were already observed between normal and reversed-phase conditions without the use of post-column addition [26].

In the context of this work, this approach in post-column had improved the response which allows this method to be relevant for the analysis of reduced skin samples.

## 3.3. Application to a skin sample

The chromatogram of the lipid extract of the skin sample is presented in Fig. 6A. The main peaks were free fatty acid and free cholesterol. Several peaks of ceramides were observed between 3 and 8 min. No glycosylceramides were detected. Two fractions were collected, the first between 0 and 3 min and the second between 3 and 10 min. There were no ceramide in the

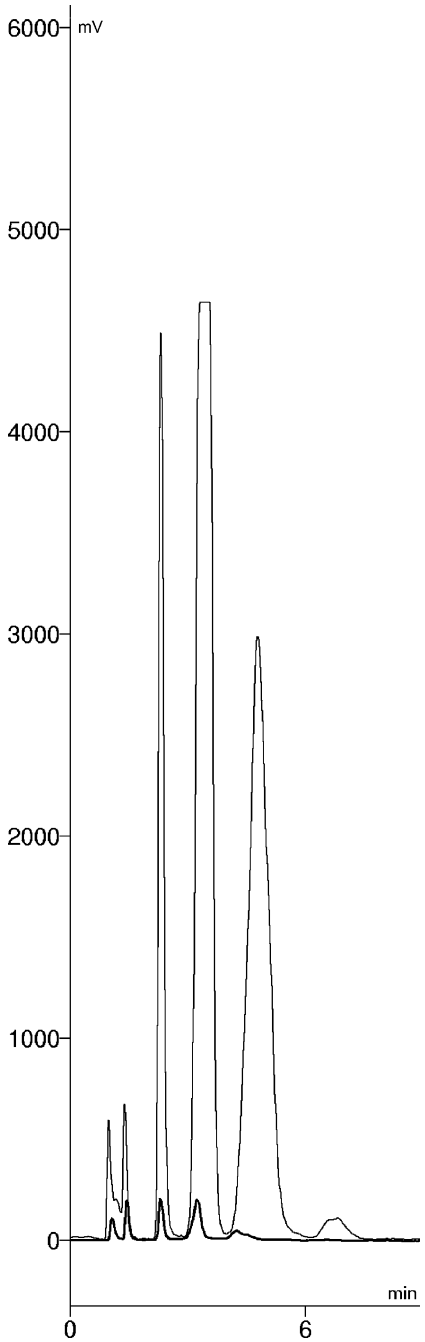


Fig. 5. Mixture of stratum corneum lipids: free fatty acid 0.01 mg $ml^{-1}$; cholesterol 0.015 mg $ml^{-1}$; ceramides 0.035 mg $ml^{-1}$. Ten percent MeOH, 15 °C, 15 MPa. Chromatogram in bold conditions without post-column addition. Thin chromatogram with post-column conditions: 0.1 ml $min^{-1}$ of $CH_2Cl_2$ containing 1% of TEA/HCOOH.

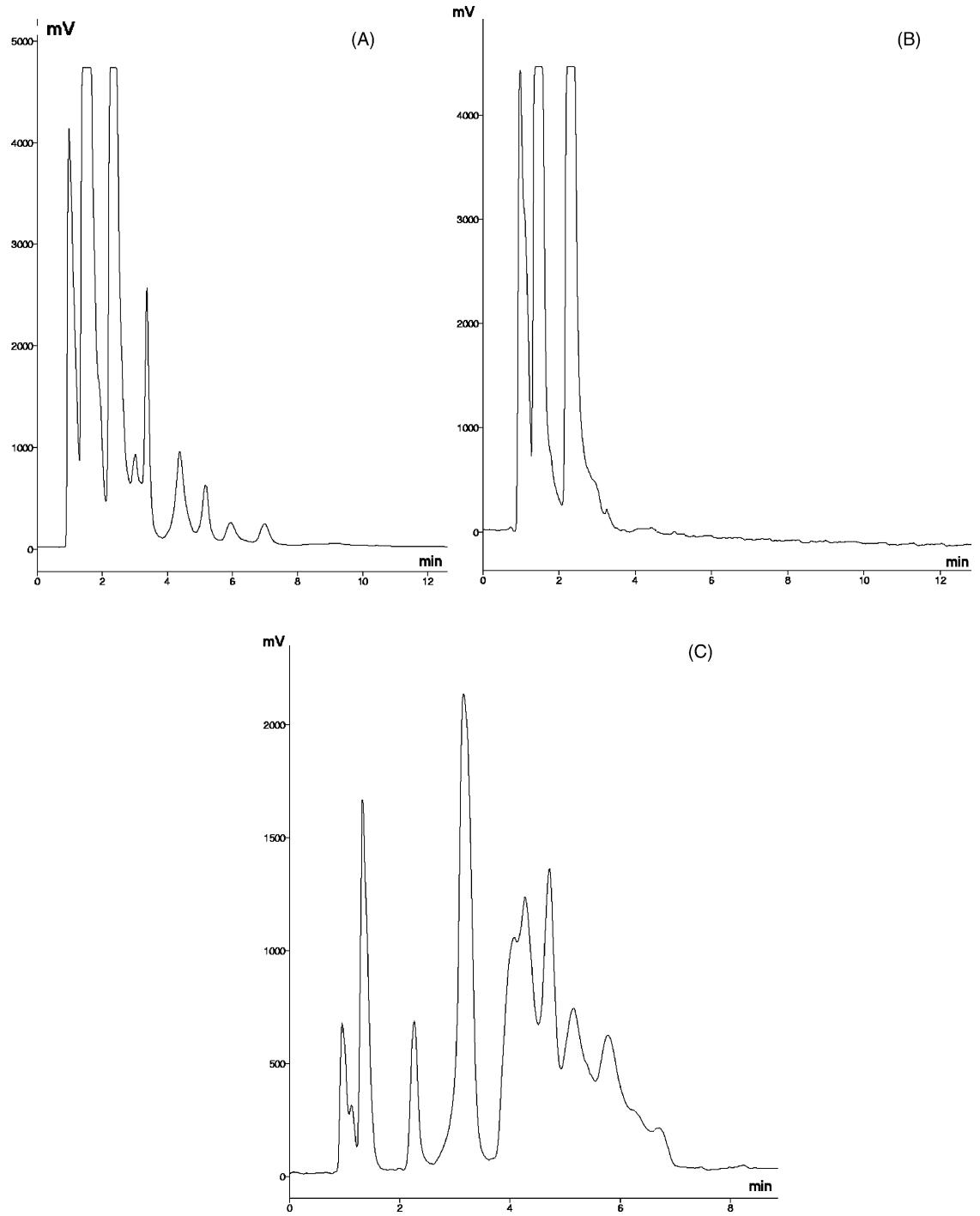


Fig. 6. Ten percent MeOH, 15 °C, 15 MPa with post-column conditions: 0.1 ml $min^{-1}$ of $CH_2Cl_2$ containing 1% of TEA/HCOOH. Chromatogram A: total lipid extract before fractionation; chromatogram B: first fraction enriched of fatty acid and cholesterol; chromatogram C: second fraction enriched of ceramides.

first fraction which mainly contained free fatty acid and free cholesterol (Fig. 6B). The second fraction was highly enriched in ceramides (Fig. 6C).

## 4. Conclusion

This method of lipid class analysis was effective and appropriate for the enrichment of ceramide fractions from extracts of *stratum corneum* with low solvent contents. The feature of TEA/HCOOH was required for the response enhancement of ceramides with the ELSD in order to observe a maximum of ceramide peak when the sample can be reduced in size. However, this modifier addition had to be envisaged in post-column since silica stationary phase was used. Thereby the solvent condition can be optimised independently for the mobile phase and the ELSD detection.

The collection procedure was performed before the ELSD detector, thus before the post-column addition since this detection process induced an important dispersion of the analyte which led to an important loss of the separation [35]. Therefore, TEA/HCOOH and chlorinated solvent were absent in the collected fractions. Moreover, the obtained fractions were thus poor in solvent content as $CO_2$ constitutes 90% of the mobile phase.

It should be noted that the eluent conditions developed herein with silica stationary phase (10% MeOH in $CO_2$) was close to those developed for molecular species analysis of ceramides with octadecyl grafted silica in SFC (6% MeOH in $CO_2$) [10]. Therefore, SFC offers a perspective of column switching for separating in the same analysis the lipid classes and their molecular species.

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