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1. Title page

Title: Comprehensive characterization and simultaneous analysis of overall lipids in Reconstructed Human Epidermis using NPLC/HR-MSⁿ: 1-O-E (EO) Cer, a new ceramide subclass

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Abbreviations:

APCI: Atmospheric Pressure Chemical Ionization

APPI: Atmospheric Pressure Photo-Ionization

CER: Ceramide

ESI: Electrospray Ionization

FFA: Free Fatty Acid

GlcCER: Glucosylceramide

HPLC: High Performance Liquid Chromatography

HR-MS: High Resolution Mass Spectrometry

LA: Linoleic acid

NPLC: Normal Phase Liquid Chromatography

RHE: Reconstructed Human Epidermis

RP-LC: Reversed Phase Liquid Chromatography

SC: *Stratum Corneum*

SM: Sphingomyelin

Sph: sphingoid base moiety

ULC: Ultra Long Chain

1. Abstract and keywords

Abstract:

Stratum Corneum lipids are responsible for the skin's barrier function. They are the final product of epidermis lipid biosynthesis. During this process, lipids evolve from simple to complex structures in three main levels respectively, (*Stratum basal level, Stratum Granulosum level and Stratum Corneum level*).

Our aim was to simultaneously analyze and characterize the structure of total epidermis lipids. A powerful analytical method (Normal Phase Liquid Chromatography coupled with High-Resolution Mass Spectrometry NPLC/HR-MSⁿ) was developed in order to separate, in a single run, lipid classes with a wide polarity range. Chromatographic conditions were particularly designed to analyze lipids of intermediate polarity such as ceramides. Rich information was obtained about the molecular structure of keratinocyte differentiation biomarkers such as: ceramides, glucosylceramides, sphingomyelins and the microstructures of Reconstructed Human Epidermis lipids using HR-MSⁿ. A new subclass of ceramides, 1-O-Acyl Omega-linoleoyloxy ceramides [1-O-E (EO) Cer] has been highlighted. This class is double esterified : on the 1-O-position of sphingoid base with long to very long chain acyl residues (1-O-E) and on the position of ω -hydroxyl group of fatty acid with the linolenic acid (EO).

Considering its chemical structure and hydrophobicity, this subclass can contribute to the skin barrier. In addition, we detected a new epidermis sphingomyelins. Our lipidomic approach offers a direct access to epidermis biomarkers.

Keywords: 1-O-E (EO) Cer, Ceramides, Glucosylceramides, Sphingomyelins, Reconstructed Human Epidermis, NPLC/HR-MSⁿ.

2. Introduction:

The epidermis is the upper layer of the three main skin layers: hypodermis, dermis and epidermis [1]. It acts as a protective barrier against water loss and eliminates foreign substances and micro-organisms [2]. The barrier function strongly relies on the outermost layer of the epidermis the *Stratum Corneum* (SC) [3]. The SC is composed of three major components: corneocytes, corneodesmosomes, and an intercellular lipid matrix [4] which is responsible for the formation and maintenance of the skin barrier [5-7]. The major lipid classes in human's SC are cholesterol, free fatty acids, and ceramides (CERs). Ceramides are essential to maintain the integrity of the skin barrier and the epidermis hydration [8].

Since various ceramide species and metabolites have well-recognized roles in signaling different cellular outcomes, such as proliferation, differentiation and/or apoptosis, therefore, it is increasingly important to understand how the production and the function of ceramides are controlled in the epidermis. Reduced levels of epidermal ceramides have been linked to water loss and barrier dysfunction [9]. Conversely, inappropriate accumulation of some ceramides leads to keratinocytes death and alteration of barrier function [10, 11]. These observations indicate that the balance of ceramide metabolism plays a major role in the state of the skin barrier. Ceramides are synthesized from keratinocytes by De Novo pathway. At the level of germinative cells, newly synthesized CERs are immediately converted to glucosylceramides (GlcCERs) and sphingomyelins (SM). These molecules are then packaged in single secretory lamellar bodies (LB), which their quantities increase in the later stages of keratinocyte differentiation. GlcCERs and SMs are transported and secreted at the interface of *Stratum Granulosum* and *Stratum Corneum*, where they are respectively hydrolyzed by beta-glucocerebrosidase and sphingomyelinase and then converted to CERs [9]. These elements are mostly known at the level of lipid classes but remain unclear at their structural microheterogeneity. However, the variations in compactness of intercorneocyte lipids observed during ageing and in several skin diseases are correlated with the structural microheterogeneity of ceramides [12-14].

Our aim is to develop an analytical method to separate the epidermis lipid classes in a single run and to characterize three main lipid classes : ceramides, glucosylceramides and sphingomyelins at a molecular level, in order to follow - in further studies- the evolution of these lipids during the keratinocyte differentiation through all epidermis layers. In fact, these three classes are considered to be the biomarkers of the principal steps of the epidermis lipid biosynthesis. Numerous analytical advances [15-17] have been made in the last few years allowing the separation and the characterization of these epidermal lipids but often they are limited to lipid classes and subclasses, which differed by

the number and / or position of the hydroxylation, the length and / or number of fatty acid chain, the number and position of the unsaturation. In addition to the large difference in the polarity between the classes and the large number of molecules, the structural microheterogeneity within each class or subclass, can be considered as a source of complexity in the characterization of such a lipid mixture.

Although, several studies have established an analytical method of total lipids [18], our analytical method is specific to the epidermis lipidome: free fatty acids, cholesterol, glycerides, ceramides, glucosylceramides, sphingomyelins and phospholipids. The particularity of this lipidome is that the majority of the lipid classes are ceramides. This class can be composed by fifteen subclasses based on the polarity of the polar head. In contrast of ceramides found in other body regions, ceramides in the *Stratum Corneum* have a wide variety of structures [19, 20]. A ceramide is composed of a sphingoid base and a fatty acid [21, 22]. In human SC, the CERs differ from each other in the architecture of the polar head group, the length of the acyl chain and their unsaturations. They can be subdivided into five groups based on sphingoid base: dihydrosphingosine (DS), sphingosine (S), phytosphingosine (P), 6-hydroxysphingosine (H) or the dihydroxy-dihydro sphingosine (T) [23, 24]. Regarding the part of fatty acids, the acyl chain is a non-hydroxy (N), α -hydroxy (A), or ω -hydroxy (O) [25]. The latter class, esterified ULC-ceramides, represents a distinctive class of skin ceramides, and derived from esterification (E) of the ω -hydroxyl group of the ULC fatty acid moiety (O) [21, 26-28]. These ceramides could be either ester bound to another FA (predominantly linoleic acid (C18:2)) (EO) or to glutamate residues of the proteins (mostly involucrin) at the surface of a corneocyte (PO). The letter **E** precedes for ceramide esterified with another FA, and **P** for ceramides bound to proteins. In addition, Rabionet et al [29], have identified the class of 1-O-acylceramides (1-O-AcylCers) with three hydrophobic chains, the third chain ester-linked to the primary hydroxyl in position 1 of the sphingoid base (1-O-E) , as 1-O-ENS and 1-O-EAS ceramides. Noteworthy, this complexity can be encountered also in ceramide precursors; glucosylceramides and sphingomyelins classes, contributing to complex molecular heterogeneity of epidermal lipids.

Lipid epidermis profiling and molecular characterization represents a real analytical challenge and require the use of a highly efficient chromatographic separation coupled to mass spectrometric detection.

-In RPLC, the retention of lipid molecular species is mainly governed by the extent of interaction between the hydrocarbonaceous moiety of the molecule and the stationary phase where the nature of the polar group is playing a

minor role [30]. NPLC remains interesting to the analysis of a total lipid extract and the objective to a complete separation of lipid classes [31, 32, 15, 33-35]. The mobile phases are usually made of solvent mixtures to ensure miscibility and to encompass a wide range of polarity. The main advantage of NPLC is the elution of lipids according to their polar moieties or “head group” which also defines their family or “lipid class”. In complex lipid mixtures analysis, NPLC produces easily interpreted chromatograms in which molecular species of the same class co-elute within a single peak. Mass spectrometry is normally used to access the molecular species identification and relative distribution [32].

In this configuration, the selection of the most appropriate interface with the mass spectrometry represents an important element in the development of the analytical method, because the ionization mode could have a significant impact on the detection of molecules. According to the literature, ESI does not allow the detection of the whole lipid classes especially the non-polar lipids [30, 32, 36]. In addition, important ceramides with a ω -ester linkage, such as Cer [EOS], Cer [EOP] and Cer [EOH], were not accessible [13]. Coupled with APCI, provided satisfactory results, but APPI is generally more sensitive and does not require any dopant make-up in NPLC chromatographic conditions [32].

In this publication, we suggest an analytical method for the structural identification and the overall evaluation of all molecules in epidermis lipid classes and subclasses (cited above). The developed approach has been used to characterize the lipids of Reconstructed Human Epidermis especially the biomarkers of the keratinocyte differentiation. In addition, we describe a new class of epidermal ceramides. These ceramides contain an ultra-long chain esterified with a linoleic acid in the N- position and a long to very long acyl chains in the 1-O-position of the sphingoid base. We suggest naming these compounds 1-O-E (EO) Cer. Considering their chemical structure and hydrophobicity, we expect that this new identified subclass to contribute to the skin barrier. Moreover, up till now, sphingomyelins were considered precursors exclusively for sphingosine containing NS- and AS-ceramides. In this work, we characterized several sphingomyelins subclasses which could be precursors of other ceramides subclasses such as NdS, NP, NH, NT, AH, AdS, AP, EOS, EOH. This new chemical information leads to biological interpretations that could be very interesting in understanding the barrier function.

3. Material and method:

Chemicals:

All analytical standards: tripalmitin (TG (16:0/16:0/16:0)), dipalmitin (DG (16:0/16:0)), monostearin (MG (18:0)), cholesterol (Chol), palmitic acid (FFA (16:0)), ceramide 1 (EOS (d18:1/26:0/18:1)), ceramide 9 (EOP (t18:0/26:0/18:1)), ceramide 3 (NP (t18:0/18:0)), ceramide 3A (NP (t18:0/18:2)), ceramide 3B (NP (t18:0/18:1)) and ceramide 6 (AP (t18:0/18:0)) Glucosylceramides (GlcCER), phospholipids (PL): phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelins (SM), were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Glucosylceramides (GlcCER (d18:2/16:0)) and sphingomyelins (SM (d18:1/12:0)) mono-compounds were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) for fragmentation investigative.

Solvents for extraction and for NPLC/APPI-HR-MSⁿ were of HPLC grade and were all purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Lipid mixture:

Test mixture is composed by 14 lipids standards : tripalmitin (TG), dipalmitine (DG), cholesterol (Chol), palmitic acid (FFA), monostearin (MG), ceramide 1 EOS, ceramide 9 EOP, ceramide 3 NP, ceramide 6 AP, glucosylceramides (GlcCER), phospholipids (PL): phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelins (SM), at an individual concentration of 0.5 g/L in chloroform.

Instrumentation and analytical method NPLC/APPI-MS:

Samples were analyzed in HPLC (Dionex Ultimate 3000, ThermoFisher Scientific, San Jose, CA). The separation of the lipids was carried out in normal phase liquid chromatography using a polyvinyl alcohol (PVA)-Sil column (PVA-bonded column; 5 µm particle size, 150 × 4.6 mm) purchased from YMC (Kyoto, Japan). NPLC was performed using a solvent gradient of Heptane/ethyl acetate (99.8: 0.2 v/v) (Solvent A), acetone and ethyl acetate (2: 1 v/v) with acetic acid (0.02% v/v) (Solvent B) and 2-propanol/ water (85:15, v/v) (with acetic acid and ethanolamine each at 0.025% v/v) (solvent C). The mobile phases and the solvent program are presented in table 1. The flow rate was set at 1 mL.min⁻¹. The injected volume was 10 µL.

The outgoing LC flow rate was split in post-column into two detectors: a Corona CAD® and an hybrid mass spectrometer LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific, San Jose, CA) equipped with an Atmospheric Pressure Photoionization APPI. A negative APPI were used for analysis of free fatty acids and a positive APPI for the identification of the other lipid classes . The APPI heater was set to 400°C and the heated capillary to 350°C. Sheath gas and auxiliary gas flow rates were set at 40 and 5 L/minute respectively. The mass scan range was 220-1800 atomic mass units (amu). A full scan detection mode and fragmentation was performed by the orbital trap at a resolution of 100 000. The MS²/MS³ spectra were obtained simultaneously in data dependent acquisition (DDA) mode. Fragmentation was performed in CID (Collision-induced dissociation) at 35 eV of collision energy in positive ionization mode in the double linear trap with high resolution. For MS², CID was performed on the 5 most abundant precursor ions with a signal threshold 10⁺³. For MS³, CID was performed on the 3 most abundant ions with a signal threshold 10⁺³. Dynamic exclusion parameters were : repeat count: 3 of each parent ion, repeat duration: 30 seconds, exclusion duration: 15 s and exclusion mass with 1.5 uma. Analyses were performed using Thermo Fisher Xcalibur software. Data were searched at +/- 10 ppm between theoretical masses experimental values.

Artificial skin:

Episkin® (Episkin, France) is a Reconstructed Human Epidermis (RHE) model obtained from the culture of normal human derived epidermal keratinocytes (NHEK) on polycarbonate filters in the interface air-liquid. Culture diameter is 0.5 cm² for each sample. Over 11 days of culture, RHE represents a multilayered model of the human epidermis. Samples were received at age of 11 days, then transferred into the medium supplied by the provider and incubated until D22 in CO₂ at 37°C.

Extraction procedure:

Total lipids of cell cultures were extracted by the modified methods of Folch [37, 38] and Bligh and Dyer [39]. The epidermis was separated from the polycarbonate filters using pliers, placed into 1.5 mL of methanol, then vortexed for 2 min. 3 mL of chloroform was added and the agitation of the whole volume was done at room temperature during 1 hour in the dark. For the separation phase, 1.5 mL of water was added and mixed using a magnetic agitator for 10 minutes at room temperature. Centrifugation at 3000 rpm for 10 minutes at 20° C was done. Recovery for the organic phase was achieved. 2 mL of chloroform was added on the aqueous phase (H₂O + methanol + skin) as a washing phase

and the whole mixture was vortexed and centrifuged for 10 minutes at 3000 rpm and at 20 ° C. The organic phase was collected and evaporated under a stream of nitrogen until dryness. The lipid residue was taken up with 20 μ L of chloroform.

4. Results:

Chromatographic separation:

Our objective was to achieve the highest degree of separation allowing the characterization of epidermis lipidome. For this purpose, a developed analytical method was tested on a mixture composed of 14 lipid standards representing all lipid classes in the epidermis. Separation of lipids was based on the NPLC method developed by Graeve et al.[15]. The elution gradient was the increasing polarity to increase eluting force. The initial eluent n-heptane modified with 0.2% ethyl acetate (solvent A) was selected because of its low polarity to separate non-polar lipids. Separation of triglycerides, diglycerides and monoglycerides was achieved with a slow increase of solvent B from 3% to 6% during 5.0 min after the initial period of 5 min of the gradient program. The final proportion of 50% solvent B was found to be optimal for the separation of the remaining neutral lipids such as free fatty acids and especially ceramide subclasses. All neutral lipids were fully eluted before 38 min. With increasing proportions of eluent C, the polar lipids were eluted such as phospholipids and sphingomyelins (*Table 2*). As final step a progressive return to initial conditions was achieved, using first solvent B (5min) followed by solvent A (12 min) at 1mL/min. The total run time between injections and elution of all lipid classes was 51 min.

Therefore, we optimized new analytical conditions allowing a good separation of the overall lipid classes. Moreover, an ideal elution window of the ceramide subclasses in the area of medium polarity lipids with retention times intermediate between Monoglycerides (MG) and Glucosylceramides (GlcCER) was obtained. In addition, this gradient profile offers the possibility to separate Sphingomyelins from the other phospholipids which represent a close degree of polarity. In summary, we were able in a single run to analyse all epidermis lipids with a good resolution, and especially, to separate the 3 classes (and sub classes) of interest for our biological objective: Ceramides, Glucosylceramides and Sphingomyelins (*Figure 1*).

Identification of MS fragments

The study of fragmentation was carried out on analytical standards representing the overall epidermis lipid classes. In full scan mass spectra, one or two major ions of each lipid have been identified: $[M-H]^-$ for Free Fatty acid, $[M+H-FA]^+$ for TriGlycerides, $[M+H-H_2O]^+$ for MonoGlycerides, Diglycerides and Cholesterol. Regarding Phospholipids

class we observed systematically $[M+H\text{-polar head}]^+$ while $[M+H]^+$ or $[M+Na]^+$ were detected depending of phospholipids type (PI, PC or PE).

For non-esterified ceramides (such as NP, AP), two groups of interest were obtained in full scan and MS²: on the first hand, the molecular peak and the losses of several water molecules corresponding to the total number of hydroxyl groups on the ceramide polar head, on the other hand, the fragments of the sphingoid base part and the fatty acid part. However, for esterified ceramides (such as EOS, EOP) a third additional group is obtained which gives information on the acid part combined to the ester moiety or the acid part combined to the base without the ester moiety (Figure 2).

Regarding glucosylceramide and sphingomyelin, they were fragmented in the ionization source. Thus, we collected all information from the full scan mass spectra.

1- Non-esterified ceramides :

Ceramide 3 (NP, C₃₆H₇₃NO₄, t18:0/18:0), 3A (NP, C₃₆H₆₉NO₄, t18:0/18:2) and 3B (NP, C₃₆H₇₁NO₄, t18:0/18:1):

The ceramide 3 carries 3 hydroxyls on its base moiety. In full scan mass spectrum, we detected the molecular peak $[M+H]^+$ at m/z 584.56 and the losses of three water molecules corresponding to m/z 566.55, 548.54 and 530.53.

Fragmentation was achieved for the two most intense peaks of m/z 584.56 and 566.55.

Fragments exposed below could be obtained from the fragmentation of m/z 584.56 and/or m/z 566.55 using MS² mode. Thus, we observed the peak of the sphingoid base part $[\text{CH}_3\text{-(CH}_2\text{)}_{13}\text{-(CH(OH))}_2\text{-CH(CH}_2\text{OH)-NH}_3]^+$ at m/z 318.29 $[\text{C}_{18}\text{H}_{40}\text{NO}_3]^+$ and fragments corresponding to three water losses at m/z 300.29 $[\text{C}_{18}\text{H}_{38}\text{NO}_2]^+$, 282.28 $[\text{C}_{18}\text{H}_{36}\text{NO}]^+$ and 264.27 $[\text{C}_{18}\text{H}_{34}\text{N}]^+$.

In addition, the fragment $[\text{CH}_3\text{-(CH}_2\text{)}_{16}\text{-CO-NH}_3]^+$ m/z 300.29 $[\text{C}_{18}\text{H}_{38}\text{NO}]^+$ corresponds to the fatty acid part $[\text{FA}+\text{NH}_3]^+$.

For ceramide 3A and 3B, the fragmentation is the same as ceramide 3 but with one unsaturation on the fatty acid part of CER 3B and two unsaturations on the fatty acid part of CER 3A. Then in MS² spectra, the fatty acid part of CER 3B and CER 3A were observed at m/z 280.26 $[\text{C}_{18}\text{H}_{34}\text{NO}]^+$ and m/z 282.28 $[\text{C}_{18}\text{H}_{36}\text{NO}]^+$ respectively. (Fig. 3 C)

Thus, in the case of CER 3A, $[\text{C}_{18}\text{H}_{36}\text{NO}]^+$ is obtained from two fragmentation ways, one from the sphingoid base part and the second from the fatty acid part.

The characteristic fragments of the acid part as well as the base part are very useful in the determination of the carbon chain length and especially the number of unsaturation and hydroxyl groups that could be carried by each chain. This information reinforces the hypothesis on the corresponding ceramide subclass.

Ceramide 6 (AP, C₃₆H₇₃NO₅, (t18:0/18:0)):

This ceramide contains 4 hydroxyl groups on its polar head of which one is on the fatty acid part. Similarly, in full scan spectrum, we observed the molecular peak [M+H]⁺ at *m/z* 600.55 with the losses of four water molecules at *m/z* 582.54, 564.53, 546.52 and 528.51.

Fragments exposed below could be obtained from the fragmentation of *m/z* 600.55 and/or *m/z* 582.54 using MS² mode. Thus, we observed the peak of the sphingoid base part [CH₃-(CH₂)₁₃-(CH(OH))₂-CH(CH₂OH)-NH₃]⁺ at *m/z* 318.29 [C₁₈H₄₀NO₃]⁺ and fragments corresponding to three water losses at *m/z* 300.29 [C₁₈H₃₈NO₂]⁺, 282.28 [C₁₈H₃₆NO]⁺ and 264.27 [C₁₈H₃₄N]⁺.

In addition, the fragment [CH₃-(CH₂)₁₅-CH(OH)-CO-NH₃]⁺ *m/z* 300.29 [C₁₈H₃₈NO₂]⁺ corresponds to the fatty acid part [FA+NH₃]⁺. (Fig. 3 D)

Thus, [C₁₈H₃₈NO₂]⁺ is obtained from two fragmentation ways, one from the sphingoid base part and the second from the fatty acid part.

2- Esterified ceramides :

Ceramide 1 (EOS, C₆₂H₁₁₉NO₅ (d18:1/26:0/18:1)):

This ceramide is esterified on the ω position of its acid part and contains 2 hydroxyl groups on its base part moiety. In the full scan spectrum, we observed the molecular peak [M+H]⁺ *m/z* 958.92 with two water losses of *m/z* 940.9 and *m/z* 922.89 corresponding to the number of hydroxyl groups.

Fragments exposed below could be obtained from the fragmentation of *m/z* 958.91 and/or *m/z* 940.9 using MS² mode. Therefore, we observed *m/z* 300.29 corresponding to the sphingoid base part [C₁₈H₃₅O₂-NH₃]⁺ with fragments resulting from the losses of two water molecules at *m/z* 282.28 and *m/z* 264.27. In addition we detected *m/z* 676.66 related to the acid part with the ester [CH₃-(CH₂)₆-CH=CH-(CH₂)₈-COO-(CH₂)₂₅-CO-NH₃]⁺, the *m/z* 394.4 corresponding to the fatty acid part [FA+NH₃]⁺ only without the ester part [CH₂=CH-(CH₂)₂₃-CO-NH₃]⁺ was observed. Finally, the *m/z*

694.67 corresponding to the ceramide after the loss of the ester of the fatty acid part $[\text{CH}_3-(\text{CH}_2)_{12}-\text{CH}=\text{CH}-\text{CH}(\text{OH})-\text{CH}(\text{CH}_2\text{OH})-\text{NH}-\text{CO}-(\text{CH}_2)_{24}-\text{CH}_2-\text{OH},\text{H}]^+$. (Fig. 3 A)(Fig. 2)

Ceramide 9 (EOP, $\text{C}_{62}\text{H}_{121}\text{NO}_6$, (t18:0/26:0/18:1)):

This ceramide is esterified on the ω position of its fatty acid part and contains 3 hydroxyl groups on its sphingoid base part.

In the full scan spectrum, we observe the molecular peak $[\text{M}+\text{H}]^+$ at m/z 976.92 with the losses of three water molecules at m/z 958.91, 940.91 and 922.9.

The fragmentation of m/z 976.92 and/or m/z 958.91 using MS² mode allowed the detection of: m/z 318.3 corresponding to the sphingoid base part $[\text{C}_{18}\text{H}_{37}\text{O}_3-\text{NH}_3]^+$ and all the losses of three water molecules at m/z 300.29, 282.28 and 264.27. Moreover, m/z 676.66 corresponding to the acid part with the ester $[\text{CH}_3-(\text{CH}_2)_6-\text{CH}=\text{CH}-(\text{CH}_2)_8-\text{COO}-(\text{CH}_2)_{25}-\text{CO}-\text{NH}_3]^+$, and the m/z 394.4 matching the fatty acid part $[\text{FA}+\text{NH}_3]^+$ only without the ester part $[\text{CH}_2=\text{CH}-(\text{CH}_2)_{23}-\text{CO}-\text{NH}_3]^+$ were evidenced. Finally, the m/z 712.68 related to the ceramide after the loss of the ester of the fatty acid part $[\text{CH}_3-(\text{CH}_2)_{13}-\text{CH}(\text{OH})-\text{CH}(\text{OH})-\text{CH}(\text{CH}_2\text{OH})-\text{NH}-\text{CO}-(\text{CH}_2)_{24}-\text{CH}_2-\text{OH},\text{H}]^+$ was observed (Fig. 3 B)

3- Glucosylceramide ($\text{C}_{40}\text{H}_{75}\text{NO}_9$, (d18:2/16:0)):

In the full scan mass spectrum, we observed the molecular peak $[\text{M}+\text{H}]^+$ at m/z 714.55 which was associated with the fragment representing water molecule losses at m/z 696.54 and 678.53, that correspond to the hydroxyl groups carried by the fatty acid moiety and the base moiety.

However, glucosylceramide has lost the glucose moiety in the ionization source; thus, we detected $[\text{M}+\text{H}-180]^+$ at m/z 534.49, which was associated with m/z 516.48 and 498.47 due to the losses of two water molecules.

Finally, we observed m/z 280.26 matching the sphingoid base part after fatty acid part cleavage $[\text{CH}_3-(\text{CH}_2)_8-\text{CH}=\text{CH}-(\text{CH}_2)_2-\text{CH}=\text{CH}-\text{CH}(\text{OH})-\text{C}(\text{CH}_2)-\text{NH}_3]^+$ and one water molecule loss at m/z 262.25. (Fig. 3 E)

4- Sphingomyelin ($\text{C}_{35}\text{H}_{71}\text{N}_2\text{O}_6\text{P}$, (d18:1/12:0)):

Similarly, the sphingomyelin was fragmented in the ionization source. Then, in the full scan spectrum we observed the molecular peak $[\text{M}+\text{H}]^+$ m/z 647.51 with one water molecule loss at m/z 629.51 corresponding to the hydroxyl group carried by the base moiety.

However, Sphingomyelin has mainly lost the phosphocholine group in the ionization source, thus, we detected $[M+H-183]^+$ at m/z 464.45, which was more intense than the molecular peak, and we observed one water molecule loss at m/z 446.44.

Finally, we characterized the fragment corresponding to the sphingoid base part after the fatty acid part cleavage $[\text{CH}_3-(\text{CH}_2)_{12}-\text{CH}=\text{CH}-\text{CH}(\text{OH})-\text{C}(\text{CH}_2)-\text{NH}_3]^+$ at m/z 282.28 and one water molecule loss at m/z 264.27. (*Fig. 3 F*)

Characterization of lipid epidermis:

After the development of the separation protocol and the characterization method of ceramides as well as glucosylceramides and sphingomyelins, this method was used to study a biological sample: the Reconstructed Human Epidermis at the 22nd day (D22) of differentiation. The lipids were extracted and analyzed using our approach, in which the totality of different lipid classes was detected: triglycerides, cholesterol, diglycerides, free fatty acids and ceramides were eluted between 20 and 30 mins, glucosylceramides detected at 36 mins and finally phospholipids and sphingomyelins at 40 and 43 mins respectively (Figure 1). The identification of ceramide subclasses, as well as glucosylceramides and sphingomyelins, was then achieved based on characteristic fragments defined above. For example, the most intense ceramide molecules in each class were exposed in table 3. Thus, 15 subclasses of ceramides were determined. Based on a sphingoid base we resume the identified ceramides and we indicate the range of the total carbon number of each subclass : Sphingosine S: NS (C41-C48), AS (C34-C56), OS (C52-C56), EOS (C64-C72) dihydrosphingosine dS: NdS (C41-C48), Ads (C34-C56), phytosphingosine P: NP (C34-C56), AP (C42 -C52), EAP (C58-60), and 6-hydroxysphingosine H: NH (C33-C48), AH (C44-C54), OH (C50-57), ENH (C61-66), EAH (C58-C64), EOH (C64-C70), , tetrahydroxylated sphingoid base T: NT (C44-C54), OT (C50-56), ENT (C57-60) (tables 4 and 5). In all subclasses, ceramides were saturated or unsaturated with one or two degree of unsaturation on fatty acids moiety, and double unsaturation for ester chain when the ceramides are linked to linoleic acid

In addition, we identified the glucosylceramides and sphingomyelins.

Interestingly, comparing the molecular composition of ceramides, glucosylceramides and sphingomyelins, we are seeing the complete similitude of the ceramide moieties overall these three lipid classes in terms of the total numbers of carbons, unsaturation and hydroxyl groups on the polar head. The table 3 summarizes all identified classes and subclasses.

Identification of a novel ceramide subclass:

A new ceramide subclass was detected in RHE sample; it was eluted at retention time between 19.0 and 20.0 min (Table 6). The particularity of this subclass is that the ceramide is double esterified : on the 1-O-position of sphingoid base with a long to very long chain acyl residues (1-O-E) and on the position of ω -hydroxyl group of fatty acid with the linolenic acid (EO).

Following the nomenclature developed by Motta et al. [40] and expanded by Robson et al. [41], Masukawa et al.[42] and Rabionet [21], we suggest the following name to this subclass 1-O-E (EO) Cer.

In this new identified subclass 1-O-E (EO) Cer, three sphingoid bases were characterized: sphingosine (S), 6-hydroxysphingosine (H) or the dihydroxy-dihydro sphingosine (T). As for the fatty acid part, it is derived from the esterification of the ω -hydroxyl group of the ULC fatty acid moiety with the linoleic acid (C18:2) (EO).

In figure (4), we represent the characteristic fragments of 3 molecules from 3 different subclasses as explained below:

Double esterified ceramide 1-O-E (EO)-T, (C₈₆H₁₆₅NO₈) (1-O-E (18:0) EO (18:2/32 :0) T (te18:0))

In the full scan spectrum, the most intense peak was $[M+H-2H_2O]^+$ corresponding to m/z 1305.24. The ion $[M+H]^+$ at m/z 1341.26 was less intense.

In the MS² fragmentation of the parent ion m/z 1305.24 $[M+H-2H_2O]^+$, we observed a m/z 1020.97 indicating the loss of third water molecule and the acyl chain (18:0) at the 1-O-position of sphingoid $[M-2H_2O-C_{18}H_{36}O_2]^+$.

Then, in MS³ of m/z 1020.97, we observed the m/z 756.72 related to the cleavage of the linoleic acid (18:2) esterified on the ω -hydroxyl group of ceramide ultra-long chain fatty acid of ceramide $[M-2H_2O-C_{18}H_{37}O_2-C_{18}H_{32}O]^+$ and the m/z 738.71 due to one water molecule loss.

Finally, we observed the peak of the fatty acid moiety at m/z 494.49 $[CHO-C_{30}H_{60}CO-NH_3]^+$ and the sphingoid base moiety without its hydroxyl groups at m/z 262.28 $[C_{11}H_{23}-CH=CH-CH=C=CH-C(CH_2)-NH_3]^+$ (Fig. 4 a).

Double esterified ceramide 1-O-E (EO)-S, (C₈₄H₁₅₇NO₆), 1-O-E (16:1) EO(18:2/30 :0) S (d20:1):

In the full scan spectrum, the most intense peak was $[M+H]^+$ at m/z 1277.21.

In the MS² fragmentation of the parent ion m/z 1277.21 $[M+H]^+$, we observed a m/z 1004.97 indicating the loss of one water molecule and the acyl chain (16:1) at the 1-O-position of sphingoid $[M-H_2O-C_{16}H_{30}O_2]^+$.

Then, in MS³ of m/z 1004.97, we observed the m/z 740.73 corresponding to the cleavage of the linoleic acid (18:2) esterified on the ω -hydroxyl group of ceramide ultra-long chain fatty acid of ceramide $[M-H_2O-C_{16}H_{30}O_2-C_{18}H_{32}O]^+$.

Finally, we observed the peak of the fatty acid moiety $[\text{CHO-C}_{28}\text{H}_{56}\text{CO-NH}_3]^+$ and the sphingoid base moiety after one water molecule loss $[\text{C}_{15}\text{H}_{31}\text{-CH=C=CH-C(CH}_2\text{)-NH}_3]^+$ at m/z 466.46 and 292.30 respectively (Fig. 4 b).

Double esterified ceramide 1-O-E (EO)-H, (C₉₂H₁₇₅NO₇), 1-O-E (24:0) EO (18:2/32:0) H (t18:1) :

In the full scan spectrum, the most intense peak was $[\text{M+H-H}_2\text{O}]^+$ matching m/z 1389.33. The ion $[\text{M+H}]^+$ at m/z 1407.34 was less intense.

In the MS² fragmentation of the parent ion m/z 1389.33 $[\text{M+H-H}_2\text{O}]^+$, we observed a m/z 1020.97 indicating the loss of one water molecule and the acyl chain (24:0) at the 1-O-position of sphingoid $[\text{M-H}_2\text{O-C}_{24}\text{H}_{48}\text{O}_2]^+$.

Then, in MS³ of m/z 1020.97, we observed the m/z 756.72 corresponding to the cleavage of the linoleic acid (18:2) esterified on the ω -hydroxyl group of ceramide ultra-long chain fatty acid of ceramide $[\text{M-H}_2\text{O-C}_{24}\text{H}_{48}\text{O}_2\text{-C}_{18}\text{H}_{32}\text{O}]^+$ and the m/z 738.71 due to the loss of the second water molecule.

Finally, we observed the peak of the fatty acid moiety $[\text{CHO-C}_{30}\text{H}_{60}\text{CO-NH}_3]^+$ at m/z 494.49 and the sphingoid base moiety $[\text{C}_{11}\text{H}_{23}\text{-CH=CH-CH=C=CH-C(CH}_2\text{)-NH}_3]^+$ at m/z 262.28 after two water molecule loss (Fig. 4 c).

5. Discussion:

The aim of this study was to investigate at the molecular level, the overall epidermis lipidome; especially the biomarkers of keratinocyte differentiation: Ceramides, Glucosylceramides and Sphingomyelins. Indeed, studying lipid biosynthesis as well as the lipids from the *Stratum Corneum* help us to explain the impaired barrier function induced by metabolic pathways disruption.

To this end, a new analytical approach in NPLC/HR-MSⁿ was developed herein to permit the simultaneous characterization of epidermis lipids.

The complexity of lipids mixture represents a real analytical challenge. Epidermis lipids consist of 9 lipid classes (TG, DG, MG, Chol, FFA, Cer, GlcCer, SM and PL) and 18 subclasses of ceramides, with a large polarity range. In addition, within each class or subclass, the structures are very diversified.

Thus, our analytical protocol allowed us to separate correctly all epidermis lipid classes according to the polar head, while preserving the structural integrity of molecules, by starting from the less polar molecules the triglycerides until the more polar the sphingomyelins in one single run. In fact, the ceramide class is divided into different subclasses in form of several peaks between 18.3 and 30 min, which differ by the structural microheterogeneity, due to the number of hydroxylation, the number of instauration, the chain-length of amide-linked fatty acid and the isomeric. However,

for glucosylceramides and sphingomyelins, all the lipids of each class are eluted in a single chromatographic peak at 36.9 min and 43.1 minutes respectively, because the polarities of the glucosyl and phosphocholine groups are greater than the polarity differences induced by their under-class structural microheterogeneity

Additionally, the use of high-resolution mass spectrometry to determine the exact mass of molecules and the optimization of the fragmentation parameters enabled us to identify and to determine the microstructure within the ceramide subclasses in the Reconstructed Human Epidermis (RHE). Full scan and MS² spectra were explained and illustrated for the three biomarkers of interest: ceramides, glucosylceramides and sphingomyelins. Thus, we were able to determine the subclass, the number of water losses corresponding to the numbers of the hydroxyl groups of the polar head and to obtain precise information on the lengths of the alkyl chains as well as the number of unsaturation of the acid part and the sphingoid part.

In the literature, several methods were developed to analyze ceramides in detail in order to study only the *Stratum Corneum*, the upper layer of epidermis, but they do not take into consideration the totality of epidermis layers [43, 44, 7, 8]. Other studies reported the analysis of lipid mixtures using the separation by class in normal phase liquid chromatography coupled with high-resolution mass spectrometry. Nevertheless, the elution gradient was not adapted to epidermis lipids especially to discriminate ceramide subclasses following the increasing polarity and to separate sphingomyelins from phospholipids [32, 30, 15]. In addition, the epidermal lipids are subjected of wide isomeric phenomenon i.e. different molecules can have the same molecular formula but belong to two lipid subclasses coming from two different metabolic pathways. Thus, the characterization of the fine structure of biomarkers, is crucial to be able to follow and to study the metabolic pathways. In summary, using a unique analysis, we have gathered the advantages of two types of methods: those able to analyze a wide range of compounds and those offering a precise characterization by molecular species [29, 45, 43, 46]. This lipidomic approach will permit the comparison of epidermis lipid profiles between several samples in order to identify the eventual markers of a physio-pathologic status. The identified compounds within the RHE represent the overall classes of skin CERs: long, very long and ultra-long chain CERs (NS, NdS, NP, NH, NT, AS, AdS, AP, AH), esterified ultra-long chain CERs, which are esterified either with linoleic acid (EOH, EOS) or with cornified envelope proteins and are required for the water permeability barrier (OS, OH, OT) and 1-O-acylceramides (EAH, ENT, EAP). Thus, we would be able to characterize overall ceramide subclasses in the epidermis according to the literature, including the ceramide subclasses which are present at low

proportions in the *Stratum Corneum*, such as the NT, OT. This requires a significant sensitivity of the analytical method to detect these molecules and to demonstrate the advantage of our analysis protocol.

In the present study, a new ceramide subclass was characterized in the RHE sample. We suggest the following nomenclature for it: 1-O-E (EO) Cer. These ceramides are double esterified : on the 1-O-position of sphingoid base with a long to a very long chain acyl residues (1-O-E) and on the position of ω -hydroxyl group of fatty acid with the linolenic acid (EO). To our knowledge, so far it has been shown that during epidermis ceramides biosynthesis, the 1-O-acylceramides (1-O-AcylCers) subclass is generated by the 1-O-Acyl esterification of an alkyl chain to the primary hydroxyl in position 1 of the sphingoid base (1-O-E). Only two 1-O-acylceramides (1-O-AcylCers) subclasses has been identified in the literature: 1-O-ENS and 1-O-EAS ceramides. In these cases, the sphingoid base is the sphingosine (S), and on the fatty acid part, the acyl chain is a non-hydroxy (N) or α -hydroxy (A). [21]. In the new identified subclass 1-O-E (EO) Cer, three sphingoid bases were characterized: sphingosine (S), 6-hydroxysphingosine (H) or the dihydroxy-dihydro sphingosine (T), and fatty acid part is derived from the esterification of the ω -hydroxyl group of the ULC fatty acid moiety with the linoleic acid (C18:2)) (EO) (*table 6*). The identification of this new class leads to suggest a novel biosynthetic pathway in which 1-O-Acyl esterification could be done on ω -hydroxy-ultra long chain-ceramide before the step of glycosylation on Golgi apparatus. Indeed, regarding the chemical structure and the hydrophobicity of this class, its role in the barrier function seems to be very important to explore. Moreover, the mechanism already illustrated of ω -hydroxy-ultra long chain-ceramide ester bound to glutamate residues of the proteins (mostly involucrin) at the surface of a corneocyte [47] to establish the barrier function could be possible to transpose on the 1-O-E (EO) Cer. In addition, the chain length could have an impact on lipid organization if they are present at *Stratum Corneum* level and also participate in the barrier function.

At the level of biomarkers of keratinocytes differentiation, the same ceramides subclasses identified within RHE sample have been characterized in glucosylceramide and sphingomyelin classes. A previous study mentioned that GlcCER species are predominant in epidermis ceramides, being precursors for non-h-ceramides (N: NS, NH, NP), α -h-ceramides (A: AS, AH, AP), as well all ω -esterified (EO: EOS, EOH, EOP) and protein-bound ceramide species (O: OS, OH) [21, 48]. SM are precursors for exclusively sphingosine-containing NS- and AS-ceramides [21, 20]. Hence, the lack of sphingomyelinase results in a milder compromised barrier as compared to barrier disruption in the case of β -glucocerebrosidase deficiency [21, 49]. Interestingly, in this work and for the first time, studying the totality of epidermis lipids allowed us to characterize several new sphingomyelins subclasses: NS, NdS, NP, NH, NT, AH, AdS,

AS, AP, EOS, EOH(*table 3*). Thus, we assume that the lack of sphingomyelinase could cause significant disruption to the barrier function. These observations show the possibility and the importance of following lipids biosynthesis simultaneously in each epidermis layer and highlight the relevance of our method at different levels: sample preparation, chromatographic separation, detection and molecular characterization.

Finally, the analytical method described herein is adapted to the analysis of epidermis lipids in particular. We have established an approach which is well adapted for the complexity of these lipids and can be widely exploited in lipidomic studies with a direct access to the biomarkers of interest within the cosmetic and dermato-pharmaceutical investigations.

6. Conflict of Interest :

All authors State that they have no conflict of interest to declare.

7. Acknowledgements

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9. **Tables :**

Temps (min)	Phase A%	Phase B%	Phase C%
0	100	0	0
3	100	0	0
5	97	3	0
10	94	6	0
15	80	20	0
20	70	30	0
25	60	40	0
30	50	50	0
35	46	39	15
40	40	30	30
50	40	0	60
51	40	0	60
56	0	100	0
61	0	100	0
62	100	0	0
74	100	0	0

Table 1: Composition of the ternary gradient of the mobile phase in NPLC/HRMSⁿ. Phase A: heptane/ethyl acetate (99.8: 0.2, v/v), B: acetone/ethyl acetate (2: 1 v/v) with acetic acid (0.02% v/v) and C: 2-propanol/water (85:15, v/v) (with acetic acid and ethanolamine each at 0.025% v/v).

Analytical standards	Retention time (min)
Tripalmitin (TG)	10.36
Dipalmitin (DG)	14.59
Cholesterol (Chol)	15.12
Palmitic acid (FFA)	15.6
Monostearin (MG)	20.43
Ceramide 1 EOS	21.11
Ceramide 9 EOP	21.75
Ceramide 3 NP	23.74
Ceramide 6 AP	24.84 and 27.91
Glucosylceramides (GlcCER)	36.96
Phospholipids (PL : PI, PC, PE)	40-43
Sphingomyelins (SM)	44.16

Table 2 : Retention time of analytical standards lipids in NPLC/HRMSⁿ

Identified ceramides subclasses			Ceramides		GlucosylCeramides		Sphingomyelins	
			Molecular formula	RT (min)	Molecular formula	RT (min)	Molecular formula	RT (min)
E(18:0) N(28:0) H(20:1)			C ₆₆ H ₁₂₉ NO ₅	19.32				
E(18:0) N(26:0) H(20:1)			C ₆₄ H ₁₂₅ NO ₅	19.39				
E(18:0) N(26:0) H(18:1)			C ₆₂ H ₁₂₁ NO ₅	19.46				
E(18:0) N(27:0) H(18:1)			C ₆₃ H ₁₂₃ NO ₅	19.48				
E(18:0) N(24:0) T(18:0)			C ₆₀ H ₁₁₉ NO ₆	19.53				
E(18:0) A(24:0) P(18:0)								
E(18:0) N(25:0) H(18:1)			C ₆₁ H ₁₁₉ NO ₅	19.54				
E(18:0) N(23:0) T(18:0)			C ₅₉ H ₁₁₇ NO ₆	19.68				
E(18:0) A(24:0) P(17:0)								
E(18:0) N(22:0) T(18:0)			C ₅₈ H ₁₁₅ NO ₆	19.75				
E(18:0) A(24:0) P(16:0)								
E(18:0) N(22:0) T(17:0)			C ₅₇ H ₁₁₃ NO ₆	19.84				
E(18:2) O(30:1) S(18:0)	E(18:2) O(28:1) S(20:0)		C ₆₆ H ₁₂₇ NO ₅	19.99	C ₇₂ H ₁₃₇ NO ₁₀	35.29		
E(18:2) O(28:2) S(18:1)	E(18:2) O(26:2) S(20:0)		C ₆₄ H ₁₂₃ NO ₅	20.06				
E(18:2) A(24:0) H(18:1)			C ₆₀ H ₁₁₇ NO ₆	20.26				
E(18:2) A(24:0) H(16:1)			C ₅₈ H ₁₁₃ NO ₆	20.5				
E(18:2) O(32:2) S(22:0)			C ₇₂ H ₁₃₇ NO ₅	20.62	C ₇₈ H ₁₄₇ NO ₁₀	36.03	C ₇₇ H ₁₄₉ N ₂ PO ₈	41.82
E(18:2) O(31:2) S(22:0)			C ₇₁ H ₁₃₅ NO ₅	20.74	C ₇₇ H ₁₄₅ NO ₁₀	35.88	C ₇₆ H ₁₄₇ N ₂ PO ₈	41.96
E(18:2) O(30:2) S(20:0)			C ₆₈ H ₁₂₉ NO ₅	20.9	C ₇₄ H ₁₃₉ NO ₁₀	35.29	C ₇₃ H ₁₄₁ N ₂ PO ₈	41.82
N(30:0) S(18:1) N(29:1) dS(19:0)	N(28:0) S(20:0) N(28:1) dS(20:0)	N(26:0) S(22:0) N(26:1) dS(22:0)	C ₄₈ H ₉₅ NO ₃	21.15	C ₅₄ H ₁₀₅ NO ₈	35.88	C ₅₃ H ₁₀₇ N ₂ PO ₆	42.62
N(28:0) S(18:1) N(28:1) dS(18:0)	N(26:0) S(20:0) N(26:1) dS(20:0)	N(24:0) S(22:0) N(27:1) dS(19:0)	C ₄₆ H ₉₁ NO ₃	21.28	C ₅₂ H ₁₀₁ NO ₈	36.18	C ₅₁ H ₁₀₃ N ₂ PO ₆	42.81
N(28:0) S(19:1) N(28:1) dS(19:0)	N(27:0) S(20:0) N(27:1) dS(20:0)	N(26:0) S(21:0) N(26:1) dS(21:0)	C ₄₇ H ₉₃ NO ₃	21.3	C ₅₃ H ₁₀₃ NO ₈	35.96	Not found	
N(26:0) S(18:1) N(27:1) dS(17:0)	N(24:0) S(20:0) N(26:1) dS(18:0)	N(23:1) dS(21:0)	C ₄₄ H ₈₇ NO ₃	21.36	C ₅₀ H ₉₇ NO ₈	36.18	C ₄₉ H ₉₉ N ₂ PO ₆	43
N(27:0) S(18:1) N(27:1) dS(18:0)	N(26:0) S(19:0) N(25:1) dS(20:0)	N(25:0) S(20:0) N(24:1) dS(21:0)	C ₄₅ H ₈₉ NO ₃	21.37	C ₅₁ H ₉₉ NO ₈	36.18	C ₅₀ H ₁₀₁ N ₂ PO ₆	42.55
N(25:0) S(18:1) N(25:1) dS(18:0)	N(24:0) S(19:0) N(24:1) dS(19:0)	N(23:0) S(20:0) N(23:1) dS(20:0)	C ₄₃ H ₈₅ NO ₃	21.52	C ₄₉ H ₉₅ NO ₈	36.33	C ₄₈ H ₉₇ N ₂ PO ₆	43.06
N(26:0) S(16:1) N(26:1) dS(16:0)	N(24:0) S(18:0) N(24:1) dS(18:0)	N(22:0) S(20:0) N(23:1) dS(19:0)	C ₄₂ H ₈₃ NO ₃	21.59	C ₄₈ H ₉₃ NO ₈	36.4	C ₄₇ H ₉₅ N ₂ PO ₆	43.19
N(25:0) S(16:1) N(25:1) dS(16:0)	N(24:0) S(17:0) N(24:1) dS(17:0)	N(23:0) S(18:0) N(23:1) dS(18:0)	C ₄₁ H ₈₁ NO ₃	21.66	C ₄₇ H ₉₁ NO ₈	36.55	C ₄₆ H ₉₃ N ₂ PO ₆	43.3
N(20:0) P(16:0) A(20:0) dS(16:0)	N(18:0) P(18:0) A(18:0) dS(18:0)	N(16:0) P(20:0)	C ₃₆ H ₇₃ NO ₄	22.12	C ₄₂ H ₈₃ NO ₉	37.07	C ₄₁ H ₈₅ N ₂ PO ₇	43.93

Table 3 : The identified ceramides, glucosylceramides and sphingomyelins in a biological sample (RHE at D22). The first 3 columns show the identified subclasses of ceramides then their molecular formula and their retention time in column 4 and 5 respectively. Column 6 and 7 represent respectively the molecular formula and the retention time of identified glucosylceramides (if present) for each ceramide subclasses. Finally, column 8 and 9 show respectively the molecular formula and the retention time of identified sphingomyelins (if present) for each ceramide subclasses. Different molecules could be co-eluted at the same retention time.

Identified ceramides subclasses			Ceramides		Glucosylceramides		Sphingomyelins	
			Molecular formula	RT (min)	Molecular formula	RT (min)	Molecular formula	RT (min)
N(18:0) P(16:0) A(18:0) dS(16:0)	N(17:0) P(17:0) A(17:0) dS(17:0)	N(16:0) P(18:0) A(16:0) dS(18:0)	C ₃₄ H ₆₉ NO ₄	22.26	C ₄₀ H ₇₉ NO ₉	37.14	C ₃₉ H ₈₁ N ₂ PO ₇	42.81
N(26:0) P(22:0) A(28:0) dS(20:0)	N(24:0) P(24:0) A(27:0) dS(21:0)	A(26:0) dS(22:0)	C ₄₈ H ₉₇ NO ₄	22.78	C ₅₄ H ₁₀₇ NO ₉	36.55	C ₅₃ H ₁₀₉ N ₂ PO ₇	42.62
N(24:0) P(22:0) A(28:0) dS(18:0)	N(28:0) P(18:0) A(27:0) dS(19:0)	N(26:0) P(20:0) A(26:0) dS(20:0)	C ₄₆ H ₉₃ NO ₄	22.98	C ₅₂ H ₁₀₃ NO ₉	36.93	Not found	
N(26:0) P(18:0) A(26:0) dS(18:0)	N(24:0) P(20:0) A(25:0) dS(19:0)	A(23:0) dS(21:0)	C ₄₄ H ₈₉ NO ₄	22.99	C ₅₀ H ₉₉ NO ₉	36.93	C ₄₉ H ₁₀₁ N ₂ PO ₇	43.3
N(24:0) P(18:0) A(26:0) dS(16:0)	N(26:0) P(16:0) A(24:0) dS(18:0)	A(23:0) dS(19:0)	C ₄₂ H ₈₅ NO ₄	23.14	C ₄₈ H ₉₅ NO ₉	37	C ₄₇ H ₉₇ N ₂ PO ₇	43.49
N(25:0) P(18:0) A(18:0) dS(25:0)	N(26:0) P(17:0) A(17:0) dS(26:0)		C ₄₃ H ₈₇ NO ₄	23.2	C ₄₉ H ₉₇ NO ₉	36.63	C ₄₈ H ₉₉ N ₂ PO ₇	43.36
N(24:0) P(17:0) A(23:0) dS(16:0)	N(23:0) P(18:0) A(22:0) dS(18:0)	A(25:0) dS(19:0)	C ₄₁ H ₈₃ NO ₄	23.44	C ₄₇ H ₉₃ NO ₉	36.93	C ₄₆ H ₉₅ N ₂ PO ₇	43.56
N(24:0) P(16:0)	N(23:0) P(17:0)	N(22:0) P(18:0)	C ₄₀ H ₈₁ NO ₄	23.57	C ₄₆ H ₉₁ NO ₉	37.07	C ₄₅ H ₉₃ N ₂ PO ₇	43.72
N(34:2) P(22:0) A(34:1) S(22:1) A(35:2) dS(21:0)	N(32:2) P(24:0) A(32:1) S(24:0) A(34:2) dS(22:0)		C ₅₆ H ₁₀₉ NO ₄	23.95	C ₆₂ H ₁₁₉ NO ₉	36.82	Not found	
N(30:1) P(22:0) A(30:0) S(22:1)	N(32:1) P(20:0) A(32:0) S(20:0)		C ₅₂ H ₁₀₃ NO ₄	24.19	C ₅₈ H ₁₁₃ NO ₉	36.88	C ₅₇ H ₁₁₅ N ₂ PO ₇	42.55
N(34:2) P(18:0) A(34:1) S(18:1) A(31:2) dS(21:0)	N(32:2) P(20:0) A(32:1) S(20:0) A(34:2) dS(18:0)	N(30:2) P(22:0) A(30:1) S(22:0)	C ₅₂ H ₁₀₁ NO ₄	24.34	C ₅₈ H ₁₁₁ NO ₉	36.88	C ₅₇ H ₁₁₃ N ₂ PO ₇	43.56
N(32:1) P(18:0) A(32:0) S(18:1)	N(30:1) P(20:0) A(30:0) S(20:0)	N(28:1) P(22:0) A(28:0) S(22:0)	C ₅₀ H ₉₉ NO ₄	24.42	C ₅₆ H ₁₀₉ NO ₉	36.93	C ₅₅ H ₁₁₁ N ₂ PO ₇	43.64
N(34:2) P(20:0) A(34:1) S(20:1) A(35:1) dS(19:0)	N(34:2) P(22:0) A(32:1) S(22:0) A(33:1) dS(21:0)		C ₅₄ H ₁₀₅ NO ₄	24.43	C ₆₀ H ₁₁₅ NO ₉	36.88	Not found	
N(30:1) P(18:0) A(30:0) S(18:1) A(31:1) dS(17:0)	N(28:1) P(20:0) A(28:0) S(20:0)	N(26:1) P(22:0) A(26:0) S(22:0)	C ₄₈ H ₉₅ NO ₄	24.51	C ₅₄ H ₁₀₅ NO ₉	36.93	C ₅₃ H ₁₀₇ N ₂ PO ₇	43.85
N(32:2) P(18:0) A(32:1) S(18:1) A(32:2) dS(19:0)	N(30:2) P(20:0) A(30:1) S(20:0) A(31:2) dS(19:0)	A(29:2) dS(21:0)	C ₅₀ H ₉₇ NO ₄	24.6	C ₅₆ H ₁₀₇ NO ₉	36.93	C ₅₅ H ₁₀₉ N ₂ PO ₇	43.72
A(32:1) P(18:0)	A(30:1) P(20:0)	A(28:1) P(22:0)	C ₅₀ H ₉₉ NO ₅	24.6	C ₅₆ H ₁₀₉ NO ₁₀	37.14	C ₅₅ H ₁₁₁ N ₂ PO ₈	43.93
A(34:1) P(18:0)	A(32:1) P(20:0)	A(30:1) P(22:0)	C ₅₂ H ₁₀₃ NO ₅	24.68	C ₅₈ H ₁₁₃ NO ₁₀	37.22	C ₅₇ H ₁₁₅ N ₂ PO ₈	43.79

Table 3-continued 1 : The identified ceramides, glucosylceramides and sphingomyelins in a biological sample (RHE at D22). The first 3 columns show the identified subclasses of ceramides then their molecular formula and their retention time in column 4 and 5 respectively. Column 6 and 7 represent respectively the molecular formula and the retention time of identified glucosylceramides (if present) for each ceramide subclasses. Finally, column 8 and 9 show respectively the molecular formula and the retention time of identified sphingomyelins (if present) for each ceramide subclasses. Different molecules could be co-eluted at the same retention time.

Identified ceramides subclasses			Ceramides		GlucosylCeramides		Sphingomyelins	
			Molecular formula	RT (min)	Molecular formula	RT (min)	Molecular formula	RT (min)
N(30:1) P (17:0) A(30:0) S (17:1) A(30:1) dS(17:0)	N(29:1) P(18:0) A(29:0) S(18:0) A(29:1) dS(18:0)	N(26:1) P(21:0) A(26:0) S(21:0) A(26:1) dS(21:0)	C ₄₇ H ₉₃ NO ₄	24.9	C ₅₃ H ₁₀₃ NO ₉	36.93	C ₅₂ H ₁₀₅ N ₂ PO ₇	43.93
N(27:1) P(18:0) A(27:0) S(18:1) A(27:1) dS(18:0)	N(25:1) P(20:0) A(25:0) S(20:0) A(25:1) dS(20:0)	N(24:1) P(21:0) A(24:0) S(21:0) A(24:1) dS(21:0)	C ₄₅ H ₈₉ NO ₄	24.96	C ₅₁ H ₉₉ NO ₉	36.93	C ₅₀ H ₁₀₁ N ₂ PO ₇	43.19
N(28:1) P(18:0) A(28:0) S(18:1) A(28:1) dS(18:0)	N(26:1) P(20:0) A(26:0) S(20:0) A(24:1) dS(21:0)	N(24:1) P(22:0) A(24:0) S(22:0)	C ₄₆ H ₉₁ NO ₄	25.1	C ₅₂ H ₁₀₁ NO ₉	36.93	C ₅₁ H ₁₀₃ N ₂ PO ₇	43.06
N(25:1) P(17:0) A(25:0) S(17:1) A(25:1) dS(16:0)	N(24:1) P(18:0) A(24:0) S(18:0) A(24:1) dS(17:0)	N(20:1) P(22:0) A(20:0) S(22:0) A(20:1) dS(19:0)	C ₄₂ H ₈₃ NO ₄	25.15	C ₄₈ H ₉₃ NO ₉	36.93	C ₄₇ H ₉₅ N ₂ PO ₇	43.49
N(26:1) P(18:0) A(26:0) S(18:1) A(26:1) dS(18:0)	N(24:1) P(20:0) A(24:0) S(20:0) A(27:1) dS(18:0)		C ₄₄ H ₈₇ NO ₄	25.22	C ₅₀ H ₉₇ NO ₉	36.93	C ₄₉ H ₉₉ N ₂ PO ₇	43.24
E(18:2) O(32:0) H(20:1)			C ₇₀ H ₁₃₃ NO ₆	25.29	C ₇₆ H ₁₄₃ NO ₁₁	36.76	C ₇₅ H ₁₄₅ N ₂ PO ₉	42.11
E(18:2) O(30:0) H(20:1)			C ₆₈ H ₁₃₁ NO ₆	25.41	C ₇₄ H ₁₄₁ NO ₁₁	36.88	C ₇₃ H ₁₄₃ N ₂ PO ₉	42.11
A(26:0) P(16:0)	A(25:0) P(17:0)	A(24:0) P(18:0)	C ₄₂ H ₈₅ NO ₅	25.43	C ₄₈ H ₉₅ NO ₁₀	36.93	C ₄₇ H ₉₇ N ₂ PO ₈	43.06
E(18:2) O(31:0) H(20:1)			C ₆₉ H ₁₃₁ NO ₆	25.44	C ₇₅ H ₁₄₁ NO ₁₁	36.76	C ₇₄ H ₁₄₃ N ₂ PO ₉	42.18
E(18:2) O(30:0) H(20:1)			C ₆₈ H ₁₂₉ NO ₆	25.5	C ₇₄ H ₁₃₉ NO ₁₁	36.88	C ₇₃ H ₁₄₁ N ₂ PO ₉	42.18
E(18:2) O(29:0) H(20:1)			C ₆₇ H ₁₂₇ NO ₆	25.55	C ₇₃ H ₁₃₇ NO ₁₁	36.88	C ₇₂ H ₁₃₉ N ₂ PO ₉	42.26
E(18:2) O(28:0) H(18:0)			C ₆₄ H ₁₂₃ NO ₆	25.61	C ₇₀ H ₁₃₃ NO ₁₁	36.88	C ₆₉ H ₁₃₅ N ₂ PO ₉	42.33
N(24:1) P(16:0) A(24:0) S(16:1) A(24:1) dS(17:0)	N(20:1) P(20:0) A(20:0) S(20:0)	N(18:1) P(22:0) A(18:0) S(22:0)	C ₄₀ H ₇₉ NO ₄	25.69	C ₄₆ H ₈₉ NO ₉	36.93	C ₄₅ H ₉₁ N ₂ PO ₇	43.72
E(18:2) O(28:0) H(20:1)			C ₆₆ H ₁₂₅ NO ₆	25.73	C ₇₂ H ₁₃₅ NO ₁₁	36.88	C ₇₁ H ₁₃₇ N ₂ PO ₉	42.26
N(18:1) P(16:0) A(18:0) S(16:1) A(17:1) dS(17:0)	N(17:1) P(17:0) A(17:0) S(17:0)	N(16:1) P(18:0) N(16:0) S(18:0)	C ₃₄ H ₆₇ N O ₄	26.31	C ₄₀ H ₇₇ NO ₉	37.14	C ₃₉ H ₇₉ N ₂ PO ₇	44.62
N(30:0) H(18:1)	N(28:0) H(20:0)	N(26:0) H(22:0)	C ₄₈ H ₉₅ NO ₄	26.32	C ₅₄ H ₁₀₅ NO ₉	36.93	C ₅₃ H ₁₀₇ N ₂ PO ₇	43.85
N(28:0) H(19:1)	N(27:0) H(20:0)	N(26:0) H(21:0)	C ₄₇ H ₉₃ NO ₄	26.48	C ₅₃ H ₁₀₃ NO ₉	36.93	C ₅₂ H ₁₀₅ N ₂ PO ₇	43.93
N(27:0) H(18:1)	N(28:0) H(17:0)	N(25:0) H(20:0)	C ₄₅ H ₈₉ NO ₄	26.55	C ₅₁ H ₉₉ NO ₉	36.93	C ₅₀ H ₁₀₁ N ₂ PO ₇	43.19
N(28:0) H(18:1)	N(27:0) H(19:0)	N(26:0) H(20:0)	C ₄₆ H ₉₁ NO ₄	26.7	C ₅₂ H ₁₀₁ NO ₉	36.93	C ₅₁ H ₁₀₃ N ₂ PO ₇	43.06
N(28:0) H(16:1)	N(26:0) H(18:0)	N(24:0) H(20:0)	C ₄₄ H ₈₇ NO ₄	26.93	C ₅₀ H ₉₇ NO ₉	36.93	C ₄₉ H ₉₉ N ₂ PO ₇	43.24
N(26:0) H(16:1)	N(25:0) H(17:0)	N(24:0) H(18:0)	C ₄₂ H ₈₃ NO ₄	27.09	C ₄₈ H ₉₃ NO ₉	36.93	C ₄₇ H ₉₅ N ₂ PO ₇	43.49
N(24:0) H(16:1)	N(22:0) H(18:0)	N(20:0) H(20:0)	C ₄₀ H ₇₉ NO ₄	27.1	C ₄₆ H ₈₉ NO ₉	36.93	C ₄₅ H ₉₁ N ₂ PO ₇	43.72
N(26:0) T(16:0)	N(24:0) T(18:0)	N(22:0) T(20:0)	C ₄₂ H ₈₅ NO ₅	27.33	C ₄₈ H ₉₅ NO ₁₀	36.93	C ₄₇ H ₉₇ N ₂ PO ₈	43.06

Table 3-continued 2 : The identified ceramides, glucosylceramides and sphingomyelins in a biological sample (RHE at D22). The first 3 columns show the identified subclasses of ceramides then their molecular formula and their retention time in column 4 and 5 respectively. Column 6 and 7 represent respectively the molecular formula and the retention time of identified glucosylceramides (if present) for each ceramide subclasses. Finally, column 8 and 9 show respectively the molecular formula and the retention time of identified sphingomyelins (if present) for each ceramide subclasses. Different molecules could be co-eluted at the same retention time.

Identified ceramides subclasses			Ceramides		GlucosylCeramides		Sphingomyelins	
			Molecular formula	RT (min)	Molecular formula	RT (min)	Molecular formula	RT (min)
N(18:0) H(16:1)	N(16:0) H(18:0)		C ₃₄ H ₆₇ NO ₄	28.16	C ₄₀ H ₇₇ NO ₉	37.14	C ₃₉ H ₇₉ N ₂ PO ₇	44.62
N(17:0) H(16:1)	N(16:0) H(17:0)		C ₃₃ H ₆₅ NO ₄	28.31	C ₃₉ H ₇₅ NO ₉	37.22	C ₃₈ H ₇₇ N ₂ PO ₇	44.62
A(36:1) H(18:1) N(36:2) T(18:0)	A(34:1) H(20:0) N(34:2) T(20:0)	A(32:1) H(22:0) N(32:2) T(22:0)	C ₅₄ H ₁₀₅ NO ₅	29.08	C ₆₀ H ₁₁₅ NO ₁₀	37.14	Not found	
A(27:0) H(20:1) N(27:1) T(20:0)	A(26:0) H(21:0) N(26:1) T(21:0)	A(25:0) H(22:0) N(25:1) T(22:0)	C ₄₇ H ₉₃ NO ₅	29.15	C ₅₃ H ₁₀₃ NO ₁₀	37.14	C ₅₂ H ₁₀₅ N ₂ PO ₈	43.43
A(28:0) H(18:1) N(28:1) T(18:0)	A(26:0) H(20:0) N(26:1) T(20:0)	A(24:0) H(22:0) N(24:1) T(22:0)	C ₄₆ H ₉₁ NO ₅	29.64	C ₅₂ H ₁₀₁ NO ₁₀	37.14	C ₅₁ H ₁₀₃ N ₂ PO ₈	41.67
A(27:0) H(18:1) N(27:1) T(18:0)	A(26:0) H(19:0) N(26:1) T(19:0)	A(25:0) H(20:0) N(25:1) T(20:0)	C ₄₅ H ₈₉ NO ₅	29.78	C ₅₁ H ₉₉ NO ₁₀	37.22	C ₅₀ H ₁₀₁ N ₂ PO ₈	43.56
A(28:0) H(16:1) N(28:1) T(16:0)	A(26:0) H(18:0) N(26:1) T(18:0)	A(24:0) H(20:0) N(24:1) T(20:0)	C ₄₄ H ₈₇ NO ₅	29.84	C ₅₀ H ₉₇ NO ₁₀	37.29	C ₄₉ H ₉₉ N ₂ PO ₈	41.82
A(26:0) H(17:1) N(26:1) T(17:0)	A(25:0) H(18:0) N(25:1) T(18:0)	A(24:0) H(19:0) N(24:1) T(19:0)	C ₄₃ H ₈₅ NO ₅	29.98	C ₄₉ H ₉₅ NO ₁₀	37.36	C ₄₈ H ₉₇ N ₂ PO ₈	41.82
A(26:0) H(16:1) N(26:1) T(16:0)	A(24:0) H(18:0) N(24:1) T(18:0)	A(22:0) H(20:0) N(22:1) T(20:0)	C ₄₂ H ₈₃ NO ₅	30.04	C ₄₈ H ₉₃ NO ₁₀	37.36	C ₄₇ H ₉₅ N ₂ PO ₈	41.82
A(30:0) H(18:1) N(30:1) T(18:0)	A(28:0) H(20:0) N(28:1) T(20:0)	A(26:0) H(22:0) N(26:1) T(22:0)	C ₄₈ H ₉₅ NO ₅	30.05	C ₅₄ H ₁₀₅ NO ₁₀	37.14	C ₅₃ H ₁₀₇ N ₂ PO ₈	44.2
A(25:0) H(16:1) N(25:1) T(16:0)	A(24:0) H(17:0) N(24:1) T(17:0)	A(23:0) H(18:0) N(23:1) T(18:0)	C ₄₁ H ₈₁ NO ₅	30.33	C ₄₇ H ₉₁ NO ₁₀	37.44	C ₄₆ H ₉₃ N ₂ PO ₈	44.13
A(24:0) H(16:1) N(24:1) T(16:0)	A(23:0) H(17:0) N(23:1) T(17:0)	A(22:0) H(18:0) N(22:1) T(18:0)	C ₄₀ H ₇₉ NO ₅	30.53	C ₄₆ H ₈₉ NO ₁₀	37.51	C ₄₅ H ₉₁ N ₂ PO ₈	42.55

Table 3-continued 3 : The identified ceramides, glucosylceramides and sphingomyelins in a biological sample (RHE at D22). The first 3 columns show the identified subclasses of ceramides then their molecular formula and their retention time in column 4 and 5 respectively. Column 6 and 7 represent respectively the molecular formula and the retention time of identified glucosylceramides (if present) for each ceramide subclasses. Finally, column 8 and 9 show respectively the molecular formula and the retention time of identified sphingomyelins (if present) for each ceramide subclasses. Different molecules could be co-eluted at the same retention time.

	S	dS	P	H	T	1-O-E_S	1-O-E_P	1-O-E_H	1-O-E_T
N	C ₄₁ -C ₄₈	C ₄₁ -C ₄₉	C ₃₄ -C ₅₆	C ₃₃ -C ₄₈	C ₄₄ -C ₅₄		-	C ₆₁ -C ₆₆	C ₅₇ -C ₆₀
A	C ₃₄ -C ₅₆	C ₃₄ -C ₅₆	C ₄₂ , C ₅₀ , C ₅₂	C ₄₄ -C ₅₄	-	C ₆₄ -C ₇₂	C ₅₈ -C ₆₀	C ₅₈ -C ₆₄	-
O	C ₅₂ -C ₅₆	-	-	C ₅₀ -C ₅₇	C ₅₀ -C ₅₆	-	-	-	-
EO	C ₆₄ -C ₇₂	-	-	C ₆₄ -C ₇₀	-	C ₈₀ -C ₈₉	-	C ₉₁ -C ₉₅	C ₈₃ -C ₉₀

Table 4: Total carbon numbers of identified ceramide subclasses obtained for the RHE culture at D22. Sphingoid base moiety in columns and fatty acid moiety in lines. The newly identified subclasses of ceramide obtained for the RHE culture at D22 are colored on yellow.

**

RT (min)	Chemical formula	Δ PPM (+/-)	Identified class
19.96	C ₅₂ H ₉₉ NO ₄	2.66	OS (C52:2)
	C ₅₃ H ₁₀₁ NO ₄	2.32	OS (C53:2)
	C ₅₄ H ₁₀₃ NO ₄	2.6	OS (C54:2)
	C ₅₅ H ₁₀₅ NO ₄	3.01	OS (C55:2)
	C ₅₆ H ₁₀₇ NO ₄	3.26	OS (C56:2)
21.73	C ₅₀ H ₉₅ NO ₅	1.97	OH (C50:2)
	C ₅₁ H ₉₇ NO ₅	2.43	OH (C51:2)
	C ₅₂ H ₉₉ NO ₅	2.88	OH (C52:2)
	C ₅₃ H ₁₀₁ NO ₅	3.01	OH (C53:2)
	C ₅₄ H ₁₀₃ NO ₅	1.93	OH (C54:2)
	C ₅₅ H ₁₀₅ NO ₅	2.82	OH (C55:2)
	C ₅₆ H ₁₀₇ NO ₅	2.63	OH (C56:2)
	C ₅₇ H ₁₀₉ NO ₅	2.23	OH (C57:2)
22.48	C ₅₀ H ₉₅ NO ₆	1.68	OT (C50:3)
	C ₅₂ H ₉₉ NO ₆	1.91	OT (C52:3)
	C ₅₄ H ₁₀₃ NO ₆	2.04	OT (C54:3)
	C ₅₅ H ₁₀₅ NO ₆	1.6	OT (C55:3)
	C ₅₆ H ₁₀₇ NO ₆	2.39	OT (C56:3)

Table 5: The identified protein-bound ceramide species obtained for the RHE culture at D22. Column 1 and 2 represent respectively the retention time and the chemical formula of the identified subclasses. The difference between the exact mass and the observed mass is shown in column 3.

RT (min)	Chemical formula	Δ PPM (+/-)	Identified class
18.45-20	C ₈₀ H ₁₄₉ NO ₆	2.365	1-O-E(EO)S
	C ₈₀ H ₁₅₁ NO ₆	2.884	
	C ₈₁ H ₁₅₃ NO ₆	3.943	
	C ₈₁ H ₁₅₁ NO ₆	1.884	
	C ₈₂ H ₁₅₅ NO ₆	4.578	
	C ₈₂ H ₁₅₃ NO ₆	1.863	
	C ₈₄ H ₁₅₇ NO ₆	2.53	
	C ₈₉ H ₁₆₇ NO ₆	3.68	
	C ₉₁ H ₁₇₃ NO ₇	0.766	1-O-E(EO)H
	C ₉₂ H ₁₇₅ NO ₇	3.15	
	C ₉₃ H ₁₇₇ NO ₇	2.78	
	C ₉₄ H ₁₇₉ NO ₇	4.9	
	C ₉₄ H ₁₇₇ NO ₇	3.09	
	C ₉₅ H ₁₇₉ NO ₇	4.04	1-O-E(EO)T
	C ₈₃ H ₁₅₉ NO ₈	5.332	
	C ₈₄ H ₁₆₁ NO ₈	5.321	
	C ₈₅ H ₁₆₃ NO ₈	4.88	
	C ₈₆ H ₁₆₅ NO ₈	3.26	
	C ₈₇ H ₁₆₇ NO ₈	7.05	
C ₈₈ H ₁₆₉ NO ₈	4.76		
C ₉₀ H ₁₇₃ NO ₈	4.9		

Table 6: The newly identified subclasses of ceramide obtained for the RHE culture at D22. Column 1 and 2 represent respectively the retention time and the chemical formula of the identified subclasses. The difference between the exact mass and the observed mass is shown in column 3.

10. **Figures and figure legends**

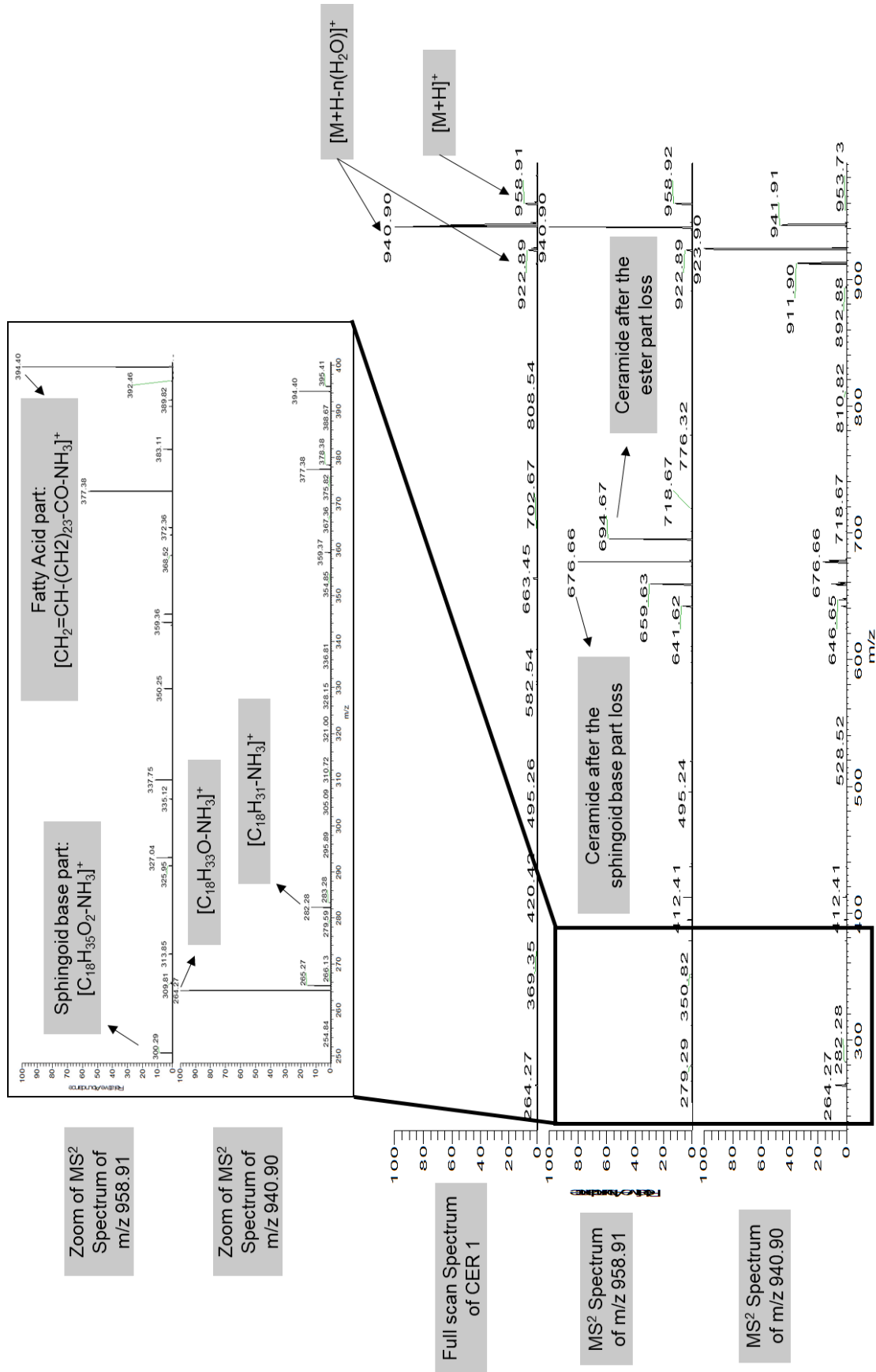


Figure 2: Mass spectra obtained in Full scan and MS² of esterified ceramide: Ceramide 1 (EOS, C₆₂H₁₁₉NO₅ (dl18:1/26:0/18:1))

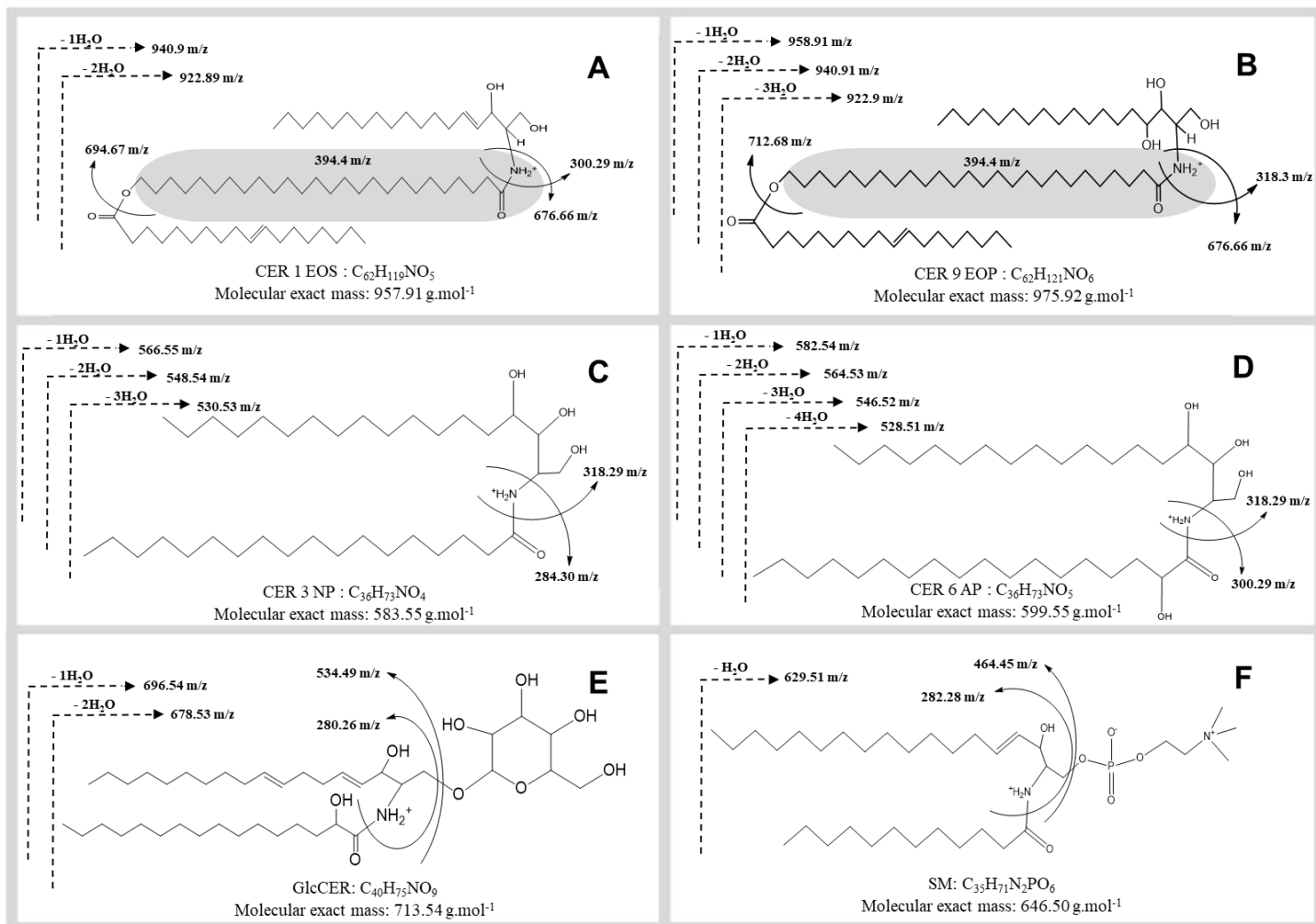


Figure 3: The characteristic fragments observed in full scan and MS² spectra of analytical standards of ceramides: A) CER 1 EOS B) CER 9 EOP C) CER 3 NP D) CER 6 AP E) GlcCER F) SM

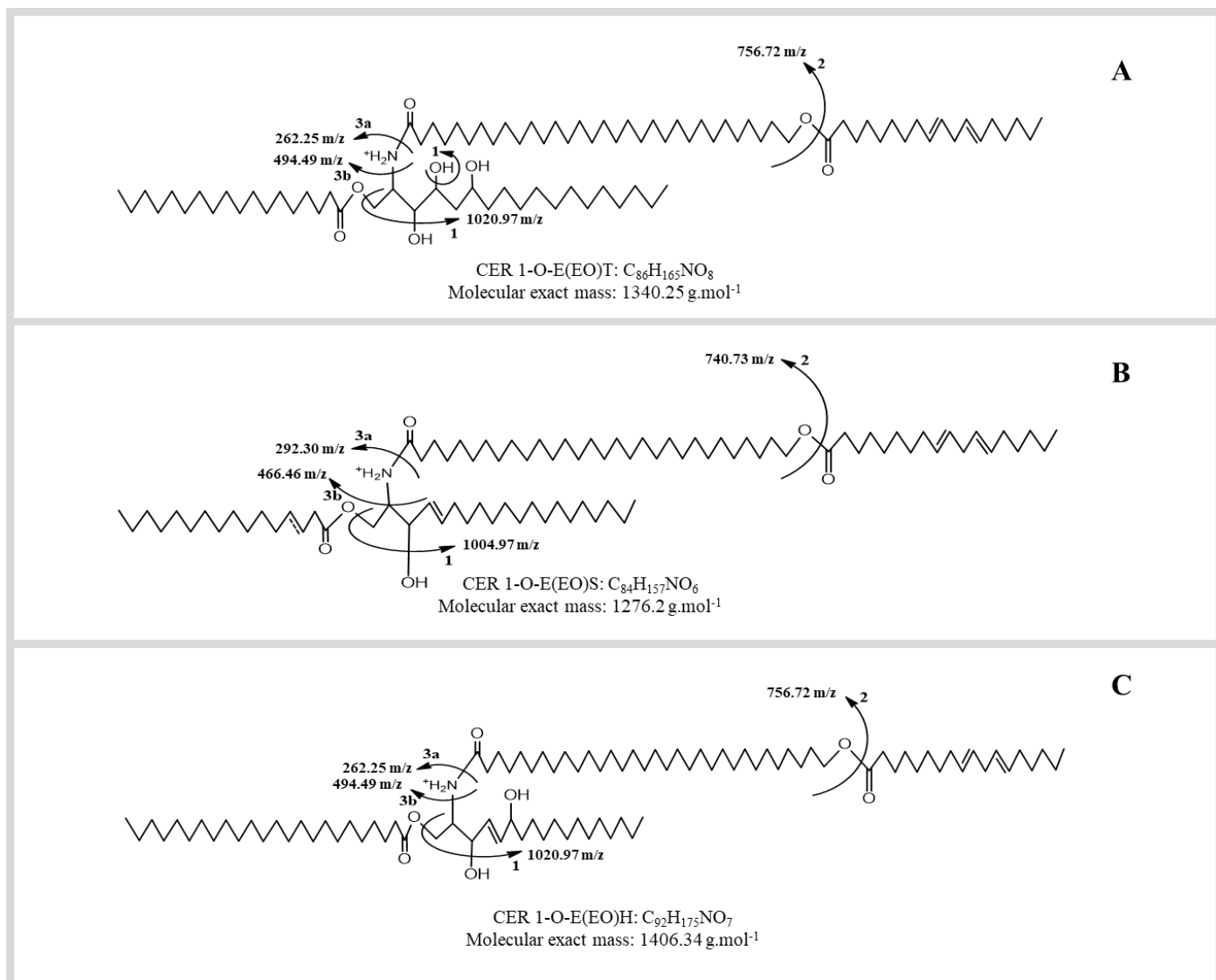


Figure 4: The fragmentation observed in full scan and MS² spectra of the new ceramide subclass 1-O-E(EO) Cer : A) 1-O-E(EO)T, B) 1-O-E(EO)S, C) 1-O-E(EO)H.

Figure legends :

- Figure 1: NPLC/Corona[®] chromatographic profile of lipid classes and subclasses obtained for the RHE culture at D22.
- Figure 2: Mass spectra obtained in Full scan and MS² of esterified ceramide: *Ceramide 1 (EOS, C₆₂H₁₁₉NO₅ (d18:1/26:0/18:1))*.
- Figure 3: The characteristic fragments observed in full scan and MS² spectra of analytical standards of ceramides: A) CER 1 EOS, B) CER 9 EOP, C) CER 3 NP, D) CER 6 AP, E) GlcCER, F) SM.
- Figure 4: The fragmentation observed in full scan and MS² spectra of the new ceramide subclass 1-O-E(EO)Cer : A) 1-O-E(EO)T, B) 1-O-E(EO)S, C) 1-O-E(EO)H.