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1- Title page

Full title: *Stratum Corneum* ceramide profiles *In vitro*, *Ex vivo* and *In vivo*: characterization of the α -Hydroxy double esterified ceramides.

Joudi Bakar, Rime Michael-Jubeli*, Danielle Libong, Arlette Baillet-Guffroy, Ali Tfayli.

Lipides : systèmes analytiques et biologiques, Université Paris-Saclay, 92296, Châtenay-Malabry, France.

Authors:

- 1- Joudi Bakar : joudi.bakar@u-psud.fr, Université Paris-Saclay, Lipides : systèmes analytiques et biologiques, 92296, Châtenay-Malabry, France.
- 2- Rime Michael-Jubeli : **This author accept correspondance and proofs**, rime.michael-jubeli@u-psud.fr, Université Paris-Saclay, Lipides : systèmes analytiques et biologiques, 92296, Châtenay-Malabry, France. . Orcid ID : 0000-0002-2944-5295.
- 3- Danielle Libong : danielle.libong@u-psud.fr, Université Paris-Saclay, Lipides : systèmes analytiques et biologiques, 92296, Châtenay-Malabry, France.
- 4- Arlette Baillet-Guffroy : arlette.baillet-guffroy@u-psud.fr, Université Paris-Saclay, Lipides : systèmes analytiques et biologiques, 92296, Châtenay-Malabry, France.
- 5- Ali Tfayli : ali.tfayli@u-psud.fr Université Paris-Saclay, Lipides : systèmes analytiques et biologiques, 92296, Châtenay-Malabry, France.

***Corresponding author** : Rime Michael-Jubeli, 5 rue Jean-Baptiste Clément - 92296 Châtenay-Malabry – France, +33146835904, fax: +33146835458, rime.michael-jubeli@u-psud.fr

2- Abbreviations

APPI: Atmospheric Pressure PhotoIonization

Cer: Ceramide

FFAs: Free Fatty Acids

HR-MSⁿ : High Resolution Mass Spectrometry

LC: Liquid Chromatography

NP-HPLC: Normal Phase - High Performance Liquid Chromatography

RHE: Reconstructed Human Epidermis

RT: Retention Time

SC: *Stratum Corneum*

3- Abstract

The presence of a new ceramide subclass, the 1-O-Acyl Omega-linoleoyloxy ceramides [1-O-E (EO) Cer], has been previously highlighted in Reconstructed Human Epidermis (RHE). These ceramides are double esterified on the 1-O-position of sphingoid base moiety with a long to very long chain acyl residues (1-O-E) and on the position of ω -hydroxyl group of fatty acid moiety with the linoleic acid (EO). Considering its chemical structure and hydrophobicity, this subclass can contribute to the skin barrier. Thus, it is important to verify if this subclass is also present in the native human *stratum corneum* (SC). In this work, ceramides structures of this novel subclass were compared between RHE (*in vitro*) and two sources of human SC (*in vivo* and *ex vivo*) using a Normal Phase – High-Performance Liquid Chromatography coupled to High-Resolution Mass Spectrometry (NP-HPLC/HR-MSⁿ). Results confirm the presence of this double esterified ceramide subclass [1-O-E (EO) Cer] in human SC. The molecular profile obtained from the RHE was very close to that found into the human SC (*in vivo* and *ex vivo*). In addition, thanks to the targeted analysis MS²/MS³, a new ceramide subclass has been discovered and characterized in the three studied samples. We propose to name it (A-1-O-E (EO) Cer) because in these ceramides species, the fatty acid -esterified with the sphingoid base on the 1-O-position- is hydroxylated on the α position. These results highlight the potential of the analytical method and the characterization approach employed in this study.

Key words: A-1-O-E (EO) Cer, 1-O-E (EO) Cer, ceramides, Reconstructed Human Epidermis, Human *Stratum Corneum*, NP-HPLC/HR-MSⁿ

4- Introduction

The skin barrier function strongly relies on the outermost layer, the *Stratum Corneum* (SC), which consists of corneocytes embedded in a lipid matrix [1,2]. Ceramides (Cers), cholesterol, and free fatty acids (FFAs) are the main lipid classes in the SC [3]. In human SC, FFAs are predominantly saturated, but mono-unsaturated and trace amounts of poly-unsaturated are present as well [4-6]. Since a long time, the α -Hydroxy Free Fatty Acids (α -OH FFAs) has been detected in the living layer and *Stratum Corneum* lipid of human Epidermis [4,7]. The species with a total carbon number (TCN) below C20 (particularly C16 and C18) have the greatest proportion in the α -OH FFAs of *Stratum Corneum* [4]. These fatty acids participate afterwards for the formation of ceramides classes [8].

The Ceramide is composed of a Fatty Acid (FA) moiety linked to a sphingoid base moiety via an amide bond [9]. Human Cer class can be divided into different subclasses according to their fatty acid moiety (non-hydroxy [N], α -hydroxy [A], or ω -hydroxy [O]) and to their sphingoid base moiety (sphingosine [S], dihydrosphingosine [dS], phytosphingosine [P], 6-hydroxy-sphingosine [H], or the dihydroxy-dihydro sphingosine [T]) [8]. Within these Cer subclasses, a structural microheterogeneity is observed due to the carbon chain length, the number of unsaturation, number of hydroxyl groups and isomers [8,10]. In addition, the Cer can be esterified [E] predominantly with linoleic acid C (18:2) on the position of ω -hydroxyl group of the ultra-long chain (C28–C32) of fatty acid moiety [O] [9]. Also the ω -hydroxyl group of Cer can be esterified to glutamate residues of the cornified envelope (CE) proteins (mostly involucrin) [PO][8]. In 2013, Rabionet et al [8,11] have identified the ceramide subclass [1-O-E], in which the Cer can be esterified [E] to the primary hydroxyl in the position 1 of the sphingoid base [8,11]. Recently, our group has identified in the Reconstructed Human Epidermis model (RHE) the ceramide subclass [1-O-E-(EO) Cer][12]. It is a double esterified ceramide, 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 linoleic acid [EO][12]. The length of the alkyl chain of ceramides play an important role in the maintenance of skin barrier properties [13]. Esterified ceramides are considered as highly hydrophobic molecules, they have a very long and an ultra-long chain of fatty acid [14]. Thus, several studies demonstrate that the ceramides and particularly those having on the fatty acid moiety a terminal ω -hydroxyl group and their esterified derivatives (acyl-ceramide) play a major role in the skin barrier function and are involved in the Corneocyte Lipid Envelope (CLE) formation [9,15,16]. It is worth noting that the ω -O-acyl Cers and especially the Cer [EOS] which are esterified to the linoleic acid [17] are significantly reduced in impaired skin barrier [18] such as in the Atopic Dermatitis (AD) [19] and Psoriasis [20].

Since, this newly discovered ceramide subclass: the double esterified ceramide 1-O-E(EO) Cer have a very long alkyl chain, its role in the skin barrier function seems to be very important to explore. The aim of this study is to investigate if this subclass is also present in the human *SC* and not only in the RHE model. In this order, the lipids of two sources of human *SC* (*in vivo* and *ex vivo*) have been analyzed in parallel to the RHE lipids using NP-HPLC/HR-MSⁿ. Then ceramide structures have been identified and characterized using MS²/MS³ targeted analysis.

5- Materials and methods

Chemicals:

The HPLC grade solvents for lipid extraction and analysis of RHE (*in vitro*) and isolated SC (*in vivo*) and the internal standard (1-Eicosanol) were all purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Solvents for (*ex vivo*) lipid extracted SC: Ether diéthylique MERCK Seccosolv[®], Ethyl acetate $\geq 99.8\%$ HiPerSolv CHROMANORM[®] for HPLC, Methanol $\geq 99.9\%$ HiPerSolv CHROMANORM[®] for LC-MS were all of HPLC grade or trace analysis.

Collection of skin samples:

1- Isolated human SC (*in vivo*).

Skin tissues were collected from plastic surgery of abdominal skin from 3 healthy donors (3 women aged between 31–54 years). All participants gave written informed consent prior to the beginning of experimentation. After excision, the skin was cleaned with purified water and the fatty tissue was removed with a scalpel. The skin was stored in aluminum foils at -30°C .

For SC isolation, frozen skin was thawed to room temperature on a filter paper and then washed at 37°C in distilled water for 120 seconds. To separate the full-thickness skin in dermis and epidermis we followed the protocol of Kligman et al [21]. The skin sheets were immersed in 60°C warm purified water for 60 seconds. Subsequently, the total epidermis was peeled off using forceps and transferred into a petri dish. To separate the SC from the epidermis, the petri dish was covered with a plastic wrap and the epidermis allowed to incubate at 37°C in an oven in a solution of 0.1% trypsin (weight percent) in 0.05 M Tris Buffer (pH 7.9) for 30 min. After this step, the epidermis was removed in fresh purified water and the remaining SC sheets were placed in a clean tray and stocked at room temperature [22].

2- Extracted human SC (*ex vivo*).

The SC lipids were sampled from the forehead of a healthy skin volunteer on an area of 7cm x 3cm previously cleaned with diethyl ether.

The SC lipids were taken using 4 swabs soaked in a solvent solution (Ethyl acetate: Methanol [2:8]). The first swab was passed 10 times from left to right in order to cover the entire sampling area surface. The other 3 swabs were passed in the

other 3 directions (10 passes from right to left, 10 passes from top to bottom and 10 passes from bottom to top). Lipids were extracted from the thawed swab tissues by 3 successive extractions as described later.

Human samples were obtained following review and approval from an institutional review board. No human tissue sampling was performed. All participants gave written in-formed consent prior to the beginning of experimentation.

3- Artificial skin (RHE) (*in vitro*):

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. The culture diameter is 0.5 cm² for each sample. In this study the RHE samples whose batch number is (16 RHE-040) were used at day 22 of keratinocytes maturation.

Lipid extraction procedure:

1- From isolated human SC (*in vivo*):

Total lipids of isolated SC were extracted by the modified method of Folch [23,24] and Bligh and Dyer [25]. Isolated SC was placed into a tube which contains 2 ml of methanol and 4 ml of chloroform. Then 25 µL of 1-Eicosanol (0.3 mg/mL) was added as an internal standard. The whole mixture was vortexed twice for 2 min. The organic phase has been recovered. The lipid extraction was repeated twice. The whole organic phase was collected and evaporated under a stream of nitrogen until dryness. The lipid residue was taken up with 100 µL of chloroform.

2- From human SC (*ex vivo*):

After adding 25 µl of 1-Eicosanol (0.3 mg/ml) as an internal standard to swab tissue, lipids were extracted by 3 successive extractions with 5ml of Folch solution. Between each extraction, the solution was vortexed for 2 min then evaporated to dryness under a stream of nitrogen. The residue was then taken up in 100 µL of chloroform.

3- From RHE (*in vitro*):

Total lipids of RHE samples were extracted by the modified methods of Folch [23,24]and Bligh and Dyer [25] as described in our latest work [12]. The epidermis was separated from the polycarbonate filters using pliers, placed into 1.5 mL of methanol, then vortexed for 2 min. A 25 µL of 1-Eicosanol (0.3 mg/mL) was used as an internal standard. Three milliliters of chloroform was added and the agitation of the whole volume was done at room temperature during

1 h in the dark. For the separation phase, 1.5 mL of water was added and mixed using a magnetic agitator for 10 min at room temperature. Centrifugation at 3000 rpm for 10 min at 20 °C was done. Recovery for the organic phase was achieved. Two milliliters 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 min 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 µL of chloroform.

Instrumentation and analytical method NP-HPLC/APPI-MSⁿ:

Samples were analyzed in HPLC (Dionex Ultimate 3000, Thermo Fisher 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). NP-HPLC was performed using an elution 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 as described in our previously work[12]. The flow rate was set at 1 mL.min⁻¹. The injected volume was 10 µL.

The outgoing LC flow rate was split post-column in 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 source (APPI). A positive mode APPI was used for the identification of ceramides. 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 were performed by the orbital trap at a resolution of 100 000. The MS²/MS³ spectra were obtained simultaneously in data dependent acquisition mode (DDA). Fragmentation was performed in CID (Collision-induced dissociation) at 35 eV of collision energy in positive ion 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. Additional targeted product ion [M+H]⁺ or water molecule loss [M – n(H₂O) + H]⁺ CID MS² and MS³ experiments were selectively triggered to provide higher quality characterization of the novel ceramide

subclasses [1-O-E-(EO)Cer] and [A-1-O-E (EO)Cer]. Analyses were performed using Thermo Fisher Xcalibur software.

Data were searched at +/- 10 ppm between theoretical masses experimental values.

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 NP-HPLC. 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)

6- Results:

In NP-HPLC/HR-MSⁿ, the lipid elution depends on the polar moieties or “head group” of each lipid class. The analytical method used in this study, previously developed by our team, is able to separate the epidermis lipid classes in one single run. The total run time between injections and elution of all lipid classes was 51 min. Moreover, an ideal elution window of the ceramide subclasses in the area of medium polarity lipids was obtained, thus the method enables to separate ceramide subclasses which are present in the form of several peaks between 15 and 30 min (figure 1 in supplementary data) [12].

Presence of the subclass 1-O-E (EO) Cer in the human *Stratum Corneum*.

The double esterified ceramide subclass 1-O-E (EO) Cer was detected in the RHE model as described in our latest work [12]. These compounds are eluted at RT between 15.4 and 19.5 min. In this study, we detected the molecules of this subclass in the chromatograms of the RHE samples (*in vitro*) and in two types of SC: human isolated SC (*ex vivo*) and human extracted SC lipids (*in vivo*). All ceramides of the subclass 1-O-E (EO) Cer are characterized in the three samples as shown in table 2 using the characteristic fragments highlighted in our previous work [12]. Figure 1 illustrates the similarity of the ions (m/z) profiles obtained by the high-resolution mass spectrometry of the subclass 1-O-E (EO) Cer for the three studied samples.

For RT between 15.4 and 19.5 min, we identified the most intense ions correspond to the molecules of the subclass 1-O-E(EO) Cer, which represents 85 ceramides. These molecules present a total carbon number between C78-C99, saturated or unsaturated fatty acid moieties and they are divided into 3 molecular families according the sphingoid base 1-O-E(EO) S and 1-O-E(EO) dS within the RT area between 15.4 and 15.7 minutes and 1-O-E(EO) H within the RT area between 18.7 and 19.5 minutes (Table 2) and (figure 2 in supplementary data).

The m/z corresponding to the double esterified subclass (1-O-E (EO)) which were detected in the RHE model were also totally present in the human extracted SC (*ex vivo*) and partially in the isolated human SC (*in vivo*). Even if the method can be used for quantitative comparison, only qualitative analyses were developed in the current work.

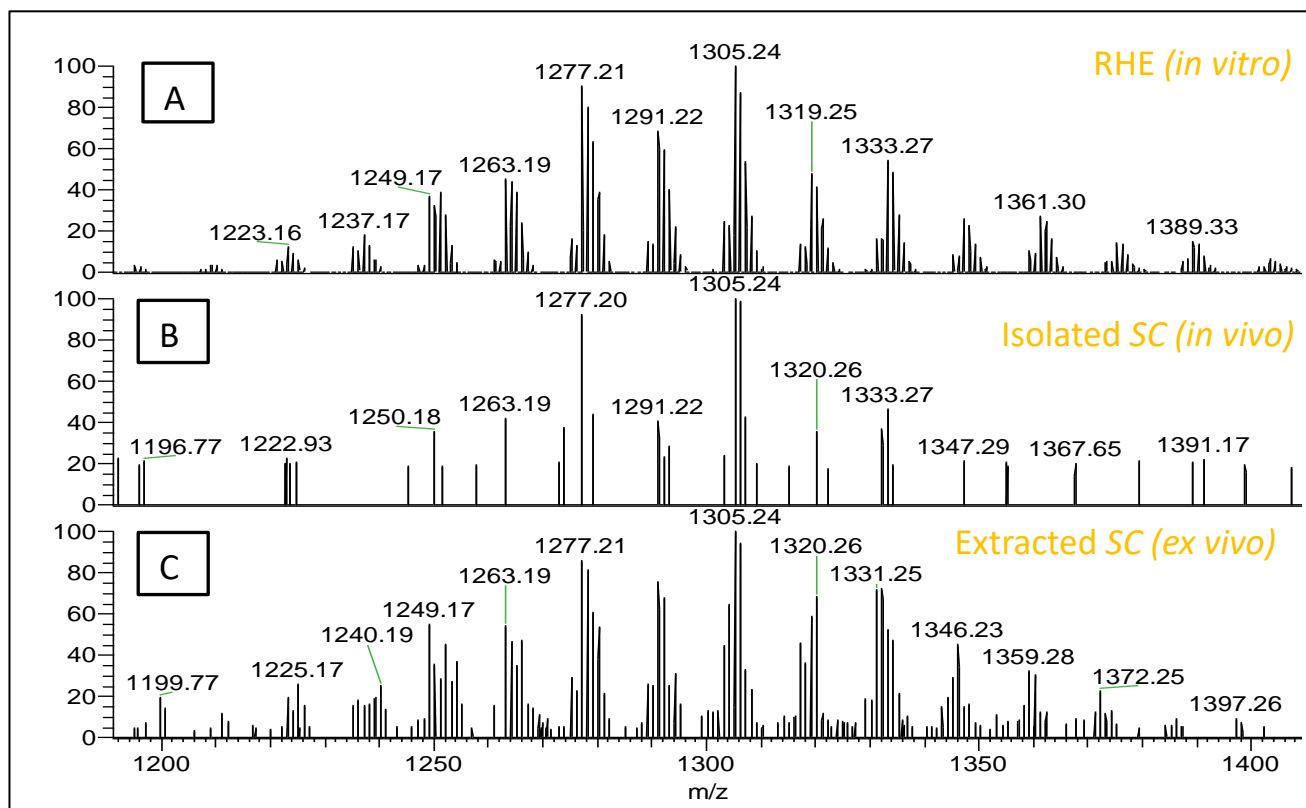


Figure 1: Comparison of the parent ions $[M+H-H_2O]^+$ correspond to the ceramide subclasses 1-O-E (EO) Cer within the RT between 18.7 and 19.5 min obtained for: A) RHE culture (in vitro) at Day 22 of keratinocytes maturation, B) isolated human SC (in vivo), C) extracted human SC (ex vivo).

Novel ceramide subclass A-1-O-E (EO) Cer.

It is interesting to note that the most ions (m/z) detected for RT area 18.7-19.5 min and the RT area 16.6-17.4 min are similar, thus ions at two different Retention Times, present two molecules having the same chemical formula consequently the same mass with different polarity values as shown in figure 2. In addition, thanks to the targeted analysis MS^2/MS^3 we can notice that this ion at same m/z presents two fragmentation paths according to the RT area. Thus, in addition to the ceramide subclass 1-O-E (EO) Cer eluted in RT area 18.7-19.5 min, our analytical method allowed to detect a novel ceramide subclass eluted in RT area 16.6-17.4 min. This is also a double esterified ceramide: 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 linoleic acid (EO). The particularity of this subclass is that the acyl chain residue (1-O-E) is hydroxylated probably on the (α) position. We suggest naming these compounds as (A-1-O-E (EO) Cer).

The MS²/MS³ fragmentation of these novel ceramides subclasses is described below. In this order, the ceramide A-1-E-O(EO)S, C₈₄H₁₅₉NO₇, is taken as example:

In the full scan mass spectrum, the most intense ion peak was [M+H-H₂O]⁺ at *m/z* 1277.21, it corresponds to the molecular mass with a loss of one water molecule and represents the chemical formula of [C₈₄H₁₅₈NO₆]⁺. In fact, the molecular ion [M+H]⁺ at *m/z* 1295.22 was less intense. In the MS² fragmentation of the parent ion at *m/z* 1277.21 [M+H-H₂O]⁺, a major fragment ion at *m/z* 1004.97 [C₆₈H₁₂₆NO₃]⁺ was detected, indicating the loss of the hydroxylated (probably in position α) acyl chain C16:0 at the 1-O-position of sphingoid (C₁₄H₂₉-CH(OH)-C(OH)=O). Then in the MS² fragmentation spectrum of the fragment ion at *m/z* 1004.97, we observed two major fragment ion peaks: the fragment ion at *m/z* 740.73 [C₅₀H₉₄NO₂]⁺ indicating the loss of the linoleic acid C18:2 esterified on the ω-hydroxyl group of ceramide ultra-long chain fatty acid (C₁₈H₃₂O) and the fragment ion at *m/z* 292.30 corresponding to the sphingoid base moiety [C₁₅H₃₁-CH=C=CH-C(CH₂)-NH₃]⁺ and indicating the loss of the ultra-long chain of fatty acid moiety (CHO-C₂₈H₅₄-CHO) as shown in the figure 3 and in the figure 3 of supplementary data. We can conclude that the ceramide is A-1-E-O(EO)S, C₈₄H₁₅₉NO₇, it is composed of: 1) the sphingoid base moiety is an unsaturated chain of 20 carbon atoms with one hydroxyl group, 2) the fatty acid moiety corresponds to C30:0, 3-the acyl chain at the 1-O-position of the sphingoid base is a saturated chain of 16 carbon atoms with one hydroxyl group, 4- the linoleic acid C18:2 esterified on the ω-hydroxyl of fatty acid chain (figure 3).

Thus, within the RT area between 16.6 and 17.4 min, we identified the most intense ions peaks correspond to the most intense molecules of the novel subclass A-1-O-E (EO) Cer, which represents 21 ceramides. These molecules present a total carbon number between C78-C92, a saturated fatty acid moiety on the polar head, and they are divided into 2 molecules family according the sphingoid base A-1-O-E(EO) S and A-1-O-E(EO) dS (Table 3).

In addition, we detected the same ions *i.e.* the same molecules of this novel subclass in the chromatograms of the RHE samples (*in vitro*) and in the two types of SC samples: human isolated SC (*ex vivo*) and human extracted SC (*in vivo*) (Table 3, and figure 4).

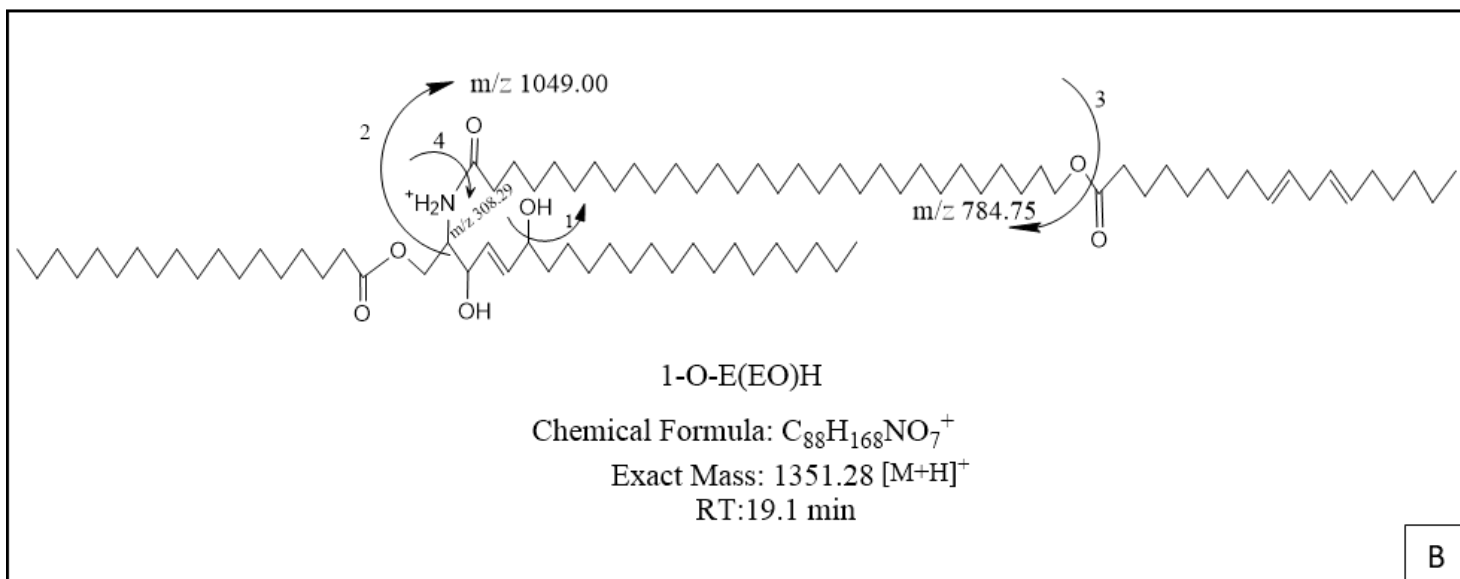
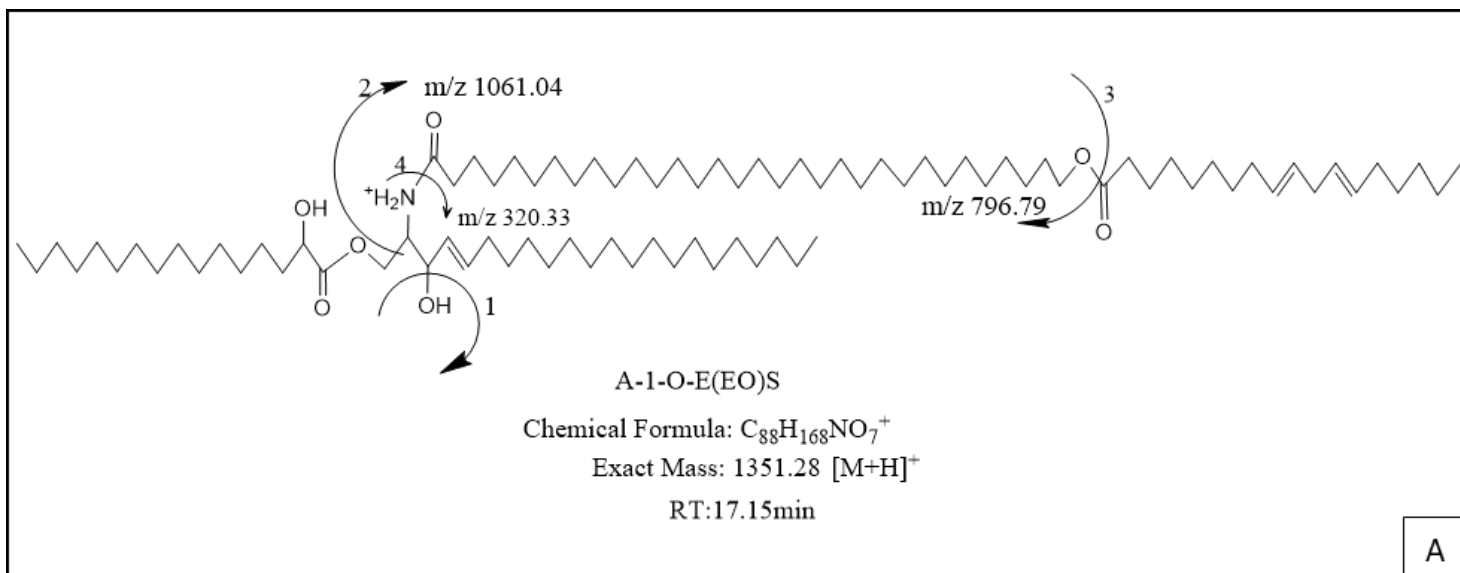


Figure 2: the characteristic fragments ions of two ceramides having the same chemical formula and eluted in two different RT, a) A-1-O-E(EO)S at 17.15 min. B) 1-O-E(EO)H at 19.1 min.

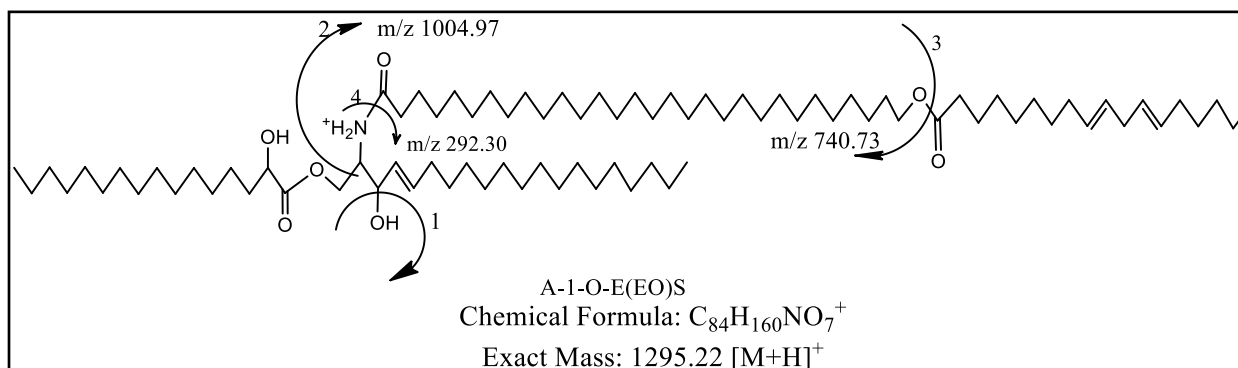


Figure 3: The characteristic fragments ions observed in full scan and MS²/MS³ spectra of the novel ceramide subclass A-1-O-E(EO) Cer in which the acyl chain residue (1-O-E) is hydroxylated probably on the (α) position (A). Ex: (A-1-O-E(EO)S)

RHE (<i>in vitro</i>)					Isolated human SC (<i>in vivo</i>)	Extracted human SC (<i>ex vivo</i>)
<i>m/z</i>	Chemical formula of [M+H-H ₂ O] ⁺	Molecular formula	RT (min)	Ceramide subclass		
1193.1	C ₇₈ H ₁₄₆ NO ₆	C ₇₈ H ₁₄₇ NO ₇	19.52	1-O-E(EO)H		
1207.1	C ₇₉ H ₁₄₈ NO ₆	C ₇₉ H ₁₄₉ NO ₇	19.38	1-O-E(EO)H		x
1221.1	C ₈₀ H ₁₅₀ NO ₆	C ₈₀ H ₁₅₁ NO ₇	19.38	1-O-E(EO)H		x
1233.1	C ₈₁ H ₁₅₀ NO ₆	C ₈₁ H ₁₅₁ NO ₇	19.52	1-O-E(EO)H*		x
1235.2	C ₈₁ H ₁₅₂ NO ₆	C ₈₁ H ₁₅₃ NO ₇	19.38	1-O-E(EO)H		x
1247.2	C ₈₂ H ₁₅₂ NO ₆	C ₈₂ H ₁₅₃ NO ₇	19.52	1-O-E(EO)H*		x
1249.2	C ₈₂ H ₁₅₄ NO ₆	C ₈₂ H ₁₅₅ NO ₇	19.45	1-O-E(EO)H	x	x
1261.2	C ₈₃ H ₁₅₄ NO ₆	C ₈₃ H ₁₅₅ NO ₇	19.38	1-O-E(EO)H*		x
1263.2	C ₈₃ H ₁₅₆ NO ₆	C ₈₃ H ₁₅₇ NO ₇	19.38	1-O-E(EO)H	x	x
1275.2	C ₈₄ H ₁₅₆ NO ₆	C ₈₄ H ₁₅₇ NO ₇	19.38	1-O-E(EO)H*	x	x
1277.2	C ₈₄ H ₁₅₈ NO ₆	C ₈₄ H ₁₅₉ NO ₇	19.31	1-O-E(EO)H	x	x
1289.2	C ₈₅ H ₁₅₈ NO ₆	C ₈₅ H ₁₅₉ NO ₇	19.38	1-O-E(EO)H*	x	x
1291.2	C ₈₅ H ₁₆₀ NO ₆	C ₈₅ H ₁₆₁ NO ₇	19.31	1-O-E(EO)H	x	x
1303.2	C ₈₆ H ₁₆₀ NO ₆	C ₈₆ H ₁₆₁ NO ₇	19.31	1-O-E(EO)H*	x	x
1305.2	C ₈₆ H ₁₆₂ NO ₆	C ₈₆ H ₁₆₃ NO ₇	19.23	1-O-E(EO)H	x	x
1317.2	C ₈₇ H ₁₆₂ NO ₆	C ₈₇ H ₁₆₃ NO ₇	19.31	1-O-E(EO)H*		x
1319.3	C ₈₇ H ₁₆₄ NO ₆	C ₈₇ H ₁₆₅ NO ₇	19.17	1-O-E(EO)H	x	x
1331.3	C ₈₈ H ₁₆₄ NO ₆	C ₈₈ H ₁₆₅ NO ₇	19.17	1-O-E(EO)H*		x
1333.3	C ₈₈ H ₁₆₆ NO ₆	C ₈₈ H ₁₆₇ NO ₇	19.1	1-O-E(EO)H	x	x
1345.3	C ₈₉ H ₁₆₆ NO ₆	C ₈₉ H ₁₆₇ NO ₇	19.17	1-O-E(EO)H*	x	x
1347.3	C ₈₉ H ₁₆₈ NO ₆	C ₈₉ H ₁₆₉ NO ₇	19.1	1-O-E(EO)H	x	x
1361.3	C ₉₀ H ₁₇₀ NO ₆	C ₉₀ H ₁₇₁ NO ₇	19.03	1-O-E(EO)H	x	x
1373.3	C ₉₁ H ₁₇₀ NO ₆	C ₉₁ H ₁₇₁ NO ₇	19.1	1-O-E(EO)H*	x	x
1375.3	C ₉₁ H ₁₇₂ NO ₆	C ₉₁ H ₁₇₃ NO ₇	19.03	1-O-E(EO)H	x	x
1387.3	C ₉₂ H ₁₇₂ NO ₆	C ₉₂ H ₁₇₃ NO ₇	19.03	1-O-E(EO)H*	x	x
1389.3	C ₉₂ H ₁₇₄ NO ₆	C ₉₂ H ₁₇₅ NO ₇	18.96	1-O-E(EO)H	x	x
1403.3	C ₉₃ H ₁₇₆ NO ₆	C ₉₃ H ₁₇₇ NO ₇	18.96	1-O-E(EO)H	x	x
1415.4	C ₉₄ H ₁₇₆ NO ₆	C ₉₄ H ₁₇₇ NO ₇	18.96	1-O-E(EO)H*	x	x
1417.4	C ₉₄ H ₁₇₈ NO ₆	C ₉₄ H ₁₇₉ NO ₇	18.9	1-O-E(EO)H	x	x

1429.4	C ₉₅ H ₁₇₈ NO ₆	C ₉₅ H ₁₇₉ NO ₇	18.96	1-O-E(EO)H*		X
1431.4	C ₉₅ H ₁₈₀ NO ₆	C ₉₅ H ₁₈₁ NO ₇	18.9	1-O-E(EO)H		X
18.9	C ₉₆ H ₁₈₀ NO ₆	C ₉₆ H ₁₈₁ NO ₇	18.9	1-O-E(EO)H*	X	X
1445.4	C ₉₆ H ₁₈₂ NO ₆	C ₉₆ H ₁₈₃ NO ₇	18.9	1-O-E(EO)H	X	X
1457.4	C ₉₇ H ₁₈₂ NO ₆	C ₉₇ H ₁₈₃ NO ₇	18.9	1-O-E(EO)H*		X
1459.4	C ₉₇ H ₁₈₄ NO ₆	C ₉₇ H ₁₈₅ NO ₇	18.76	1-O-E(EO)H	X	X
1471.4	C ₉₈ H ₁₈₄ NO ₆	C ₉₈ H ₁₈₅ NO ₇	18.9	1-O-E(EO)H*	X	X
1177.1	C ₇₈ H ₁₄₆ NO ₅	C ₇₈ H ₁₄₇ NO ₆	15.77	1-O-E(EO)S		X
1179.1	C ₇₈ H ₁₄₈ NO ₅	C ₇₈ H ₁₄₉ NO ₆	15.77	1-O-E(EO)dS		X
1191.1	C ₇₉ H ₁₄₈ NO ₅	C ₇₉ H ₁₄₉ NO ₆	15.85	1-O-E(EO)S		X
1193.2	C ₇₉ H ₁₅₀ NO ₅	C ₇₉ H ₁₅₁ NO ₆	15.77	1-O-E(EO)dS		X
1205.2	C ₈₀ H ₁₅₀ NO ₅	C ₈₀ H ₁₅₁ NO ₆	15.85	1-O-E(EO)S	X	X
1207.2	C ₈₀ H ₁₅₂ NO ₅	C ₈₀ H ₁₅₃ NO ₆	15.77	1-O-E(EO)dS		X
1217.1	C ₈₁ H ₁₅₀ NO ₅	C ₈₁ H ₁₅₁ NO ₆	15.85	1-O-E(EO)S*		X
1219.2	C ₈₁ H ₁₅₂ NO ₅	C ₈₁ H ₁₅₃ NO ₆	15.85	1-O-E(EO)S	X	X
1221.2	C ₈₁ H ₁₅₄ NO ₅	C ₈₁ H ₁₅₅ NO ₆	15.77	1-O-E(EO)dS		X
1231.2	C ₈₂ H ₁₅₂ NO ₅	C ₈₂ H ₁₅₃ NO ₆	15.77	1-O-E(EO)S*		X
1233.2	C ₈₂ H ₁₅₄ NO ₅	C ₈₂ H ₁₅₅ NO ₆	15.77	1-O-E(EO)S	X	X
1245.2	C ₈₃ H ₁₅₄ NO ₅	C ₈₃ H ₁₅₅ NO ₆	15.85	1-O-E(EO)S*		X
1247.2	C ₈₃ H ₁₅₆ NO ₅	C ₈₃ H ₁₅₇ NO ₆	15.77	1-O-E(EO)S	X	X
1249.2	C ₈₃ H ₁₅₈ NO ₅	C ₈₃ H ₁₅₉ NO ₆	15.63	1-O-E(EO)dS		X
1259.2	C ₈₄ H ₁₅₆ NO ₅	C ₈₄ H ₁₅₇ NO ₆	15.77	1-O-E(EO)S*	X	X
1261.2	C ₈₄ H ₁₅₈ NO ₅	C ₈₄ H ₁₅₉ NO ₆	15.77	1-O-E(EO)S	X	X
1263.2	C ₈₄ H ₁₆₀ NO ₅	C ₈₄ H ₁₆₁ NO ₆	15.63	1-O-E(EO)dS		X
1273.2	C ₈₅ H ₁₅₈ NO ₅	C ₈₅ H ₁₅₉ NO ₆	15.77	1-O-E(EO)S*		X
1275.2	C ₈₅ H ₁₆₀ NO ₅	C ₈₅ H ₁₆₁ NO ₆	15.7	1-O-E(EO)S	X	X
1277.2	C ₈₅ H ₁₆₂ NO ₅	C ₈₅ H ₁₆₃ NO ₆	15.63	1-O-E(EO)dS	X	X
1287.2	C ₈₆ H ₁₆₀ NO ₅	C ₈₆ H ₁₆₁ NO ₆	15.7	1-O-E(EO)S*	X	X
1289.2	C ₈₆ H ₁₆₂ NO ₅	C ₈₆ H ₁₆₃ NO ₆	15.63	1-O-E(EO)S	X	X
1291.3	C ₈₆ H ₁₆₄ NO ₅	C ₈₆ H ₁₆₅ NO ₆	15.63	1-O-E(EO)dS		X
1301.2	C ₈₇ H ₁₆₂ NO ₅	C ₈₇ H ₁₆₃ NO ₆	15.63	1-O-E(EO)S*	X	X
1303.3	C ₈₇ H ₁₆₄ NO ₅	C ₈₇ H ₁₆₅ NO ₆	15.63	1-O-E(EO)S	X	X
1305.3	C ₈₇ H ₁₆₆ NO ₅	C ₈₇ H ₁₆₇ NO ₆	15.56	1-O-E(EO)dS	X	X
1315.3	C ₈₈ H ₁₆₄ NO ₅	C ₈₈ H ₁₆₅ NO ₆	15.63	1-O-E(EO)S*	X	X
1317.3	C ₈₈ H ₁₆₆ NO ₅	C ₈₈ H ₁₆₇ NO ₆	15.63	1-O-E(EO)S	X	X
1329.3	C ₈₉ H ₁₆₆ NO ₅	C ₈₉ H ₁₆₇ NO ₆	15.63	1-O-E(EO)S*		X
1331.3	C ₈₉ H ₁₆₈ NO ₅	C ₈₉ H ₁₆₉ NO ₆	15.63	1-O-E(EO)S	X	X
1333.3	C ₈₉ H ₁₇₀ NO ₅	C ₈₉ H ₁₇₁ NO ₆	15.56	1-O-E(EO)dS	X	X
1343.3	C ₉₀ H ₁₆₈ NO ₅	C ₉₀ H ₁₆₉ NO ₆	15.63	1-O-E(EO)S*	X	X
1345.3	C ₉₀ H ₁₇₀ NO ₅	C ₉₀ H ₁₇₁ NO ₆	15.56	1-O-E(EO)S	X	X
1357.3	C ₉₁ H ₁₇₀ NO ₅	C ₉₁ H ₁₇₁ NO ₆	15.63	1-O-E(EO)S*		X
1359.3	C ₉₁ H ₁₇₂ NO ₅	C ₉₁ H ₁₇₃ NO ₆	15.56	1-O-E(EO)S	X	X
1371.3	C ₉₂ H ₁₇₂ NO ₅	C ₉₂ H ₁₇₃ NO ₆	15.56	1-O-E(EO)S*	X	X
1373.3	C ₉₂ H ₁₇₄ NO ₅	C ₉₂ H ₁₇₅ NO ₆	15.56	1-O-E(EO)S	X	X
1385.3	C ₉₃ H ₁₇₄ NO ₅	C ₉₃ H ₁₇₅ NO ₆	15.56	1-O-E(EO)S*		X
1387.4	C ₉₃ H ₁₇₆ NO ₅	C ₉₃ H ₁₇₇ NO ₆	15.49	1-O-E(EO)S	X	X
1399.4	C ₉₄ H ₁₇₆ NO ₅	C ₉₄ H ₁₇₇ NO ₆	15.56	1-O-E(EO)S*	X	X
1401.4	C ₉₄ H ₁₇₈ NO ₅	C ₉₄ H ₁₇₉ NO ₆	15.49	1-O-E(EO)S	X	X
1413.4	C ₉₅ H ₁₇₈ NO ₅	C ₉₅ H ₁₇₉ NO ₆	15.43	1-O-E(EO)S*		X

1415.4	C ₉₅ H ₁₈₀ NO ₅	C ₉₅ H ₁₈₁ NO ₆	15.43	1-O-E(EO)S	x	x
1427.4	C ₉₆ H ₁₈₀ NO ₅	C ₉₆ H ₁₈₁ NO ₆	15.49	1-O-E(EO)S*	x	x
1429.4	C ₉₆ H ₁₈₂ NO ₅	C ₉₆ H ₁₈₃ NO ₆	15.43	1-O-E(EO)S	x	x
1441.4	C ₉₇ H ₁₈₂ NO ₅	C ₉₇ H ₁₈₃ NO ₆	15.49	1-O-E(EO)S*	x	x
1443.4	C ₉₇ H ₁₈₄ NO ₅	C ₉₇ H ₁₈₅ NO ₆	15.43	1-O-E(EO)S	x	x
1457.4	C ₉₈ H ₁₈₆ NO ₅	C ₉₈ H ₁₈₇ NO ₆	15.43	1-O-E(EO)S	x	x
1469.4	C ₉₉ H ₁₈₆ NO ₅	C ₉₉ H ₁₈₇ NO ₆	15.43	1-O-E(EO)S*		x

Table 2: The identified ceramides 1-O-E(EO) Cer in the RHE (in vitro), isolated human SC (in vivo) and extracted human SC (ex vivo). Column (1) presents the list of the parent ions (m/z) detected in the RHE. Column (2) and (3) present the chemical formula of parent ion [M+H-H₂O]⁺ and the molecular formula respectively. Column (4) present the RT of each molecule. The identified ceramide subclass is showed in column (5). The presence of each ceramide was verified in the in vivo and ex vivo SC as shown in column (6 and 7) respectively. *: presence of unsaturation on the fatty acid chain.

RHE (in vitro)					Isolated human SC (in vivo)	Extracted human SC (ex vivo)
m/z	Chemical formula of [M+H-H ₂ O] ⁺	Molecular formula	RT (min)	Ceramide subclass		
1195.13	C ₇₈ H ₁₄₈ NO ₆	C ₇₈ H ₁₄₉ NO ₇	17.43	A-1-O-E(EO) dS		x
1209.14	C ₇₉ H ₁₅₀ NO ₆	C ₇₉ H ₁₅₁ NO ₇	17.43	A-1-O-E(EO) dS		x
1221.14	C ₈₀ H ₁₅₀ NO ₆	C ₈₀ H ₁₅₁ NO ₇	17.43	A-1-O-E(EO) S		x
1223.16	C ₈₀ H ₁₅₂ NO ₆	C ₈₀ H ₁₅₃ NO ₇	17.23	A-1-O-E(EO) dS		x
1235.16	C ₈₁ H ₁₅₂ NO ₆	C ₈₁ H ₁₅₃ NO ₇	17.43	A-1-O-E(EO) S		x
1237.17	C ₈₁ H ₁₅₄ NO ₆	C ₈₁ H ₁₅₅ NO ₇	17.29	A-1-O-E(EO) dS		x
1249.17	C ₈₂ H ₁₅₄ NO ₆	C ₈₂ H ₁₅₅ NO ₇	17.36	A-1-O-E(EO) S		x
1251.19	C ₈₂ H ₁₅₆ NO ₆	C ₈₂ H ₁₅₇ NO ₇	17.29	A-1-O-E(EO) dS		x
1263.19	C ₈₃ H ₁₅₆ NO ₆	C ₈₃ H ₁₅₇ NO ₇	17.36	A-1-O-E(EO) S	x	x
1265.2	C ₈₃ H ₁₅₈ NO ₆	C ₈₃ H ₁₅₉ NO ₇	17.29	A-1-O-E(EO) dS		x
1277.2	C ₈₄ H ₁₅₈ NO ₆	C ₈₄ H ₁₅₉ NO ₇	17.29	A-1-O-E(EO) S	x	x
1279.22	C ₈₅ H ₁₆₀ NO ₆	C ₈₅ H ₁₆₁ NO ₇	17.23	A-1-O-E(EO) dS		x
1291.22	C ₈₅ H ₁₆₀ NO ₆	C ₈₅ H ₁₆₁ NO ₇	17.23	A-1-O-E(EO) S	x	x
1293.23	C ₈₅ H ₁₆₂ NO ₆	C ₈₅ H ₁₆₃ NO ₇	17.23	A-1-O-E(EO) dS		x
1305.24	C ₈₆ H ₁₆₂ NO ₆	C ₈₆ H ₁₆₃ NO ₇	17.23	A-1-O-E(EO) S	x	x
1307.24	C ₈₆ H ₁₆₄ NO ₆	C ₈₆ H ₁₆₅ NO ₇	17.23	A-1-O-E(EO) dS		x
1319.25	C ₈₇ H ₁₆₄ NO ₆	C ₈₇ H ₁₆₅ NO ₇	17.23	A-1-O-E(EO) S		x
1333.3	C ₈₈ H ₁₆₆ NO ₆	C ₈₈ H ₁₆₇ NO ₇	17.15	A-1-O-E(EO) S	x	x
1347.28	C ₈₉ H ₁₆₈ NO ₆	C ₈₉ H ₁₆₉ NO ₇	17.08	A-1-O-E(EO) S		x
1361.3	C ₉₀ H ₁₇₀ NO ₆	C ₉₀ H ₁₇₁ NO ₇	17.08	A-1-O-E(EO) S	x	x
1389.32	C ₉₂ H ₁₇₄ NO ₆	C ₉₂ H ₁₇₅ NO ₇	16.66	A-1-O-E(EO) S		x

Table 3: The identified ceramides A-1-O-E(EO) Cer in the RHE (in vitro), isolated human SC (in vivo) and extracted human SC (ex vivo). Column (1) presents the list of the parent ions (m/z) detected in the RHE. Column (2) and (3) present the chemical formula of the parent ion [M+H-H₂O]⁺ and the molecular formula respectively. Column (4) present the RT of each molecule. The identified ceramide subclass is showed in column (5). The presence of each ceramide was verified in the in vivo and ex vivo SC as shown in column (6 and 7) respectively.

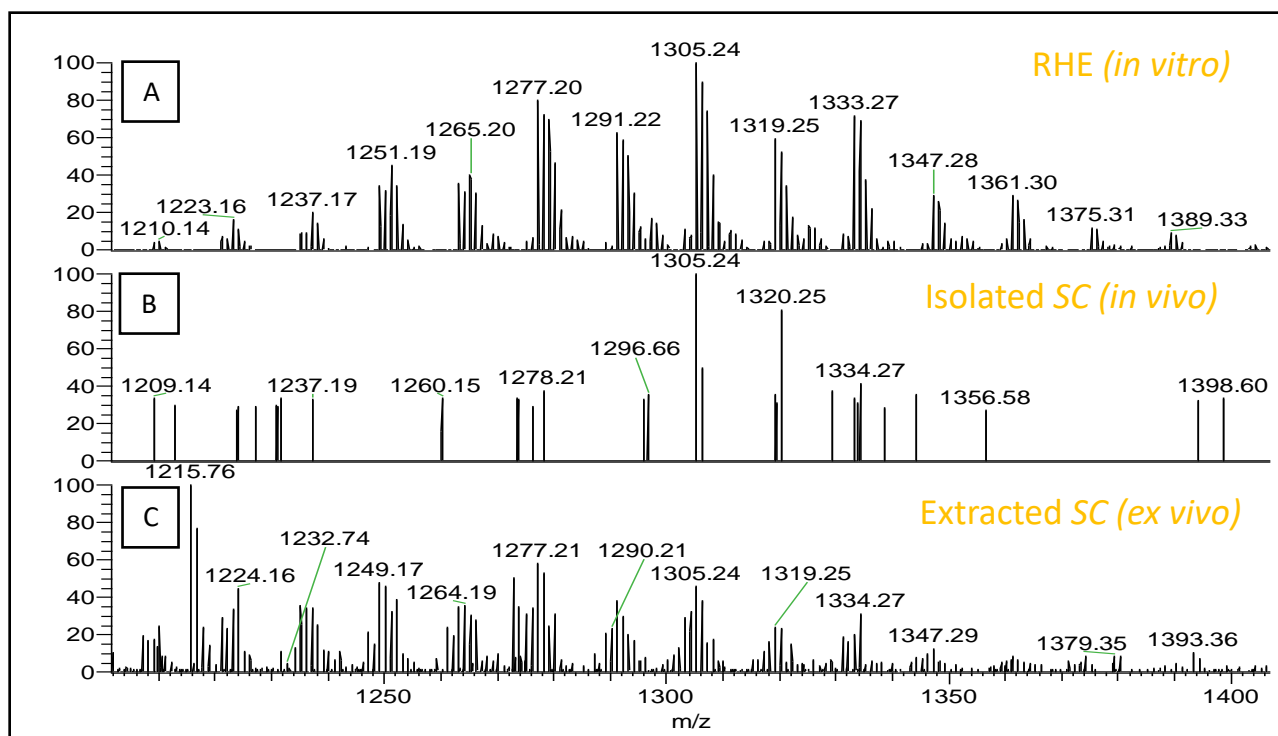


Figure 4: Comparison of the parent ions $[M+H-H_2O]^+$ correspond to the novel ceramide subclasses A-I-O-E (EO) Cer within the RT between 16.6 and 17.4 min obtained for: A) RHE culture (*in vitro*) at Day 22 of keratinocytes maturation, B) isolated human SC (*in vivo*), C) extracted human SC (*ex vivo*).

Discussion

Since 2013, the use of animal testing for cosmetics in the European Union is no longer possible [26]. Different models of *in vitro* Human Skin Equivalent (HSEs) and Reconstructed Human Epidermis model (RHE) are developed to replace the animal test [27]. In our latest publication [12] we characterized a novel ceramide subclass (1-O-E (EO) Cer) in the RHE (Episkin®) samples. In this work, the analysis of human *Stratum corneum* lipids using NP-HPLC / HR-MSⁿ confirmed the presence of this double esterified ceramide subclass 1-O-E (EO) Cer in SC lipids extracted *ex vivo* and *in vivo*. From the overall observation, three families of the 1-O-E(EO) Cer molecules according to the sphingoid moiety were identified: 1-O-E(EO)S, 1-O-E(EO)dS and 1-O-E(EO)H.

Stratum Corneum ceramides represent a highly complex mixture, in which, the number of molecules is very high and the structural micro-heterogeneity present a huge diversity. In addition, ceramide subclasses present important differences in terms of relative intensities. For example the Non-hydroxy Ceramide subclasses such as [NP],[NH],[NdS],and [NS] present the highest relative (%) abundance compared to other Cer subclasses, inversely to molecules which present at a low intensity

such as mono esterified ceramides and particularly the ceramides EOdS which form just 0.4% of total ceramide subclasses [6]. In full scan mass spectrometry, ceramide m/z (corresponding to the ion $[M+H-H_2O]^+$) has a variable intensity according to the type of ceramides subclass. The non-targeted MS² analysis seems to be very useful for characterizing the m/z ions corresponding to the ceramide subclasses which show a very high intensities. However, for the double esterified ceramides 1-O-E (EO) Cer, present at a low content in the SC samples, the use of the targeted analysis MS²/MS³ seems to be very efficient in order to characterize the fine structure of this subclass. The analytical method developed in our latest work [12] and particularly the use of targeted analysis MS²/MS³ allowed us to detect another ceramide subclass in which the fatty acid linked to 1-O-position sphingoid base via ester bond is hydroxylated probably on the α position of this fatty acid (A-1-O-E (EO) Cer) as shown in table 3 and figure 3. As known, the hydroxylation of FFAs in the keratinocytes was referred on two positions according to the alkyl chain length: on the α position when alkyl chain is composed particularly of C16 and less of C18 and C24[4], and on the ω position for ultra-long alkyl chain above C26 [8]. From these bibliographic data, we could suppose that the fatty acid on the 1-O-position sphingoid base of this novel subclass is hydroxylated at α position. In fact, two families of the A-1-O-E(EO) Cer molecules according to their sphingoid moiety were identified: A-1-O-E(EO)S, A-1-O-E(EO)dS. This novel ceramide subclass was detected and confirmed in the three skin samples (RHE, *in vivo* and *ex vivo* SC) . However, the highly hydroxylated ceramides are involved in some skin disturbances such as atopic dermatitis [6,19]. Thus, our results may be of significant interest in dermatology, allowing the complexity of these diseases to be increasingly revealed in order to improve their medical and cosmetic management.

One can conclude the power and the ability of our analytical method to detect all skin lipids even in small amounts in the skin such as the 1-O-E (EO) Cer and A-1-O-E (EO) Cer.

Interestingly, the same 5 ceramides observed families 1-O-E(EO)S, 1-O-E(EO)dS, 1-O-E(EO)H, A-1-O-E(EO)S, and A-1-O-E(EO)dS were detected in the studied samples, which could highlight the similarity in ceramide biosynthesis paths in the three samples *in vitro*, *in vivo* and *ex vivo*. Molecules are completely similar in terms of exact molecular mass, chemical formula, and finally their fine structure notably the lengths of the alkyl chains, the number of the unsaturated bonds and also the number of the hydroxyl groups. Thus, the biosynthesis steps as the alkyl chain elongation, the dehydrogenation and the hydroxylation are closely comparable between the studied samples, RHE and Human SC.

However, in full scan mass spectrometry, the m/z (corresponding to the ion $[M+H-H_2O]^+$) of the double esterified ceramides (1-O-E (EO) Cer) and (A-1-O-E (EO) Cer) detected in RHE models was found in extracted and isolated human SC as shown in

tables 2 and 3. The use of extracted SC (*ex vivo*) is more satisfied compared to isolated one. This observation seems to be very important for dermatological and cosmetic researchers, because the sample preparation from the extracted SC is much easier to carry out compared to the isolated SC, which requires surgical intervention and SC layer separation that may affect the lipid structural integrity.

7- Conclusion

Ceramides are a complex lipid mixture that plays a critical role in skin barrier. Since esterified ceramides have an essential role to maintaining the skin barrier integrity, in this study we investigated the presence in the human SC of double esterified ceramides 1-O-E (EO) Cer previously discovered in RHE model. Our analytical method and characterization approach allowed us to identify a novel subclass of ceramides A-1-O-E (EO) Cer. Thus, we confirm the presence of the two ceramide subclasses 1-O-E (EO) Cer and A-1-O-E (EO) Cer in RHE model as well in the human SC. In addition, these two classes of ceramides are found in the human stratum corneum at low levels, so it is interesting to note that the RHE models can provide information on lipid biosynthesis allowing the production of the less abundant species.

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Compliance with ethical standards Conflict of interest

The authors declare that they have no conflict of interest.

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