Profiling and characterizing skin ceramides using reversed-phase liquid chromatography – quadrupole time-of-flight mass spectrometry

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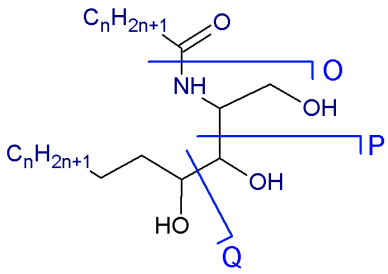
S-1. Nomenclature of CER species.

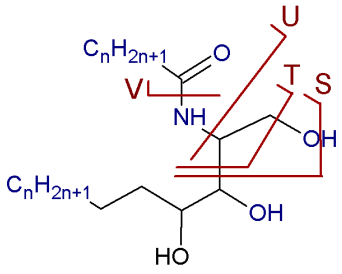
Throughout the manuscript, the nomenclature originally proposed by Motta *et al.* and subsequently extended by Masukawa *et al.* is used.[1,2] The different CER classes are termed according to their combination of SB and FA chain. The SB building blocks can be dihydrosphingosine (DS), sphingosine (S), phytosphingosine (P) or 6-hydroxy-sphingosine (H). The newly identified SB contains, compared to sphinganine, two additional hydroxyl groups on the SB and can be appointed as dihydroxy dihydrosphingosine or dihydroxysphinganine (T). The FA part can be a non-hydroxy FA (N), an α-hydroxy FA (A), an *ω*-hydroxy FA (O) or an esterified *ω*-hydroxy FA (EO). Based on the already described FAs and SBs, 16 CER classes can be listed (see also Scheme 1 in manuscript): CER[NDS], CER[NS], CER[NP], CER[NH], CER[ADS], CER[AS], CER[AP], CER[AH], CER[ODS], CER[OS], CER[OP], CER[OH], CER[EODS], CER[EOS], CER[EOP], CER[EOH]. Based on this terminology, each CER molecule is individually termed as follows: the number of FA moiety carbon atoms and unsaturation (if present) is expressed after the letter N, A, O or E, whereas the number of SB carbon atoms is expressed after the letter(s) DS, S, P, H or T (e.g. CER[N(24)S(18)] or CER[E(18:2)O(30)DS(18)]). Nomenclature of the CER fragments generated following collision induced dissociation is based on Ann *et al.* and Lee *et al.* [3,4]

Main fragments after cleavage of CERs (phytosphingosine based)[3,4]:

Containing sphingoid chain



Containing N-acyl chain

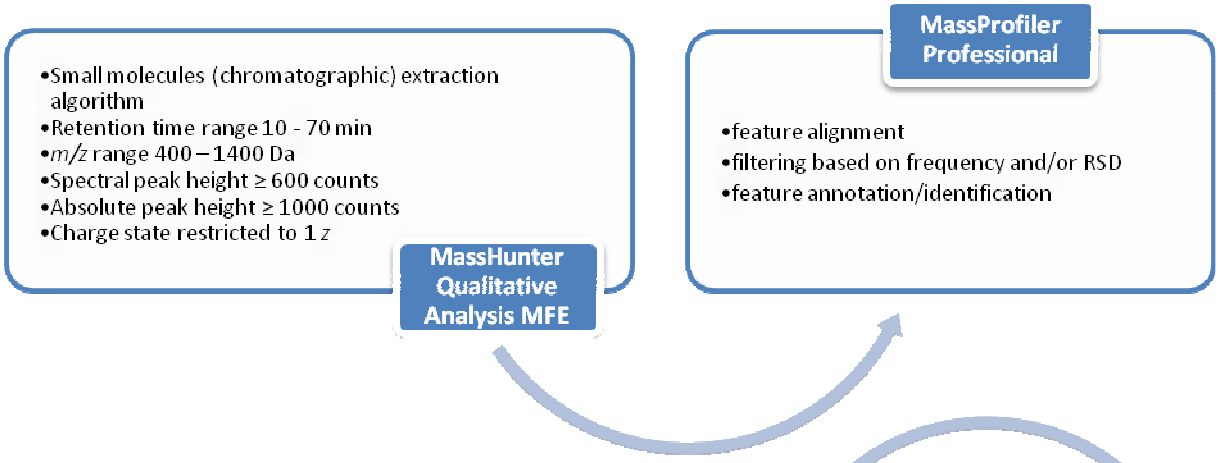


(O = loss of fatty acyl group, O' = loss of fatty acyl group and 1 mole of water, O'' = loss of fatty acyl group and 2 moles of water, O''' = loss of fatty acyl group and 3 moles of water)

S-2. Data processing.

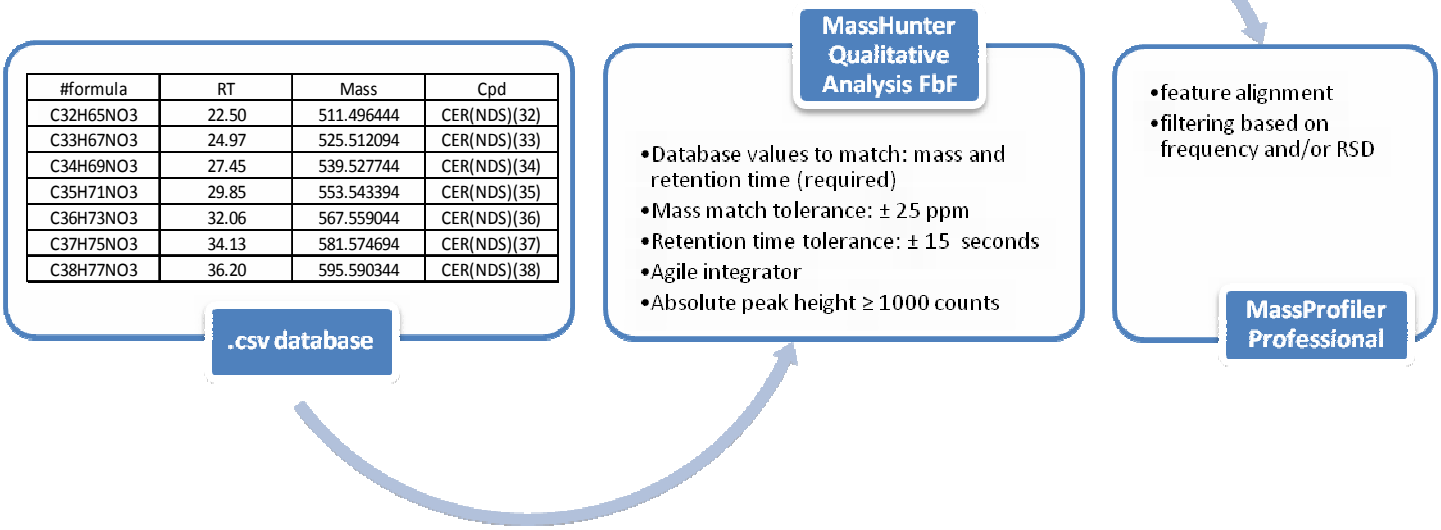
Raw LC-MS data files were processed in an untargeted fashion (a) using the Molecular Feature Extraction (MFE) algorithm incorporated in the MassHunter Qualitative Analysis software package (Agilent Technologies). The resulting feature files were subsequently imported in MassProfiler Professional (Agilent Technologies) which aligned, visualized and filtered the features. CER identification was based on accurate mass-based annotation, making use of the Molecular Formula Generation algorithm (MassHunter Qualitative Analysis) and a preliminary MassHunter compatible CER database containing all species described in the literature, followed by MS/MS fragmentation interpretation. The unique within class elution pattern furthermore facilitated identification. On top, some species were confirmed using commercially available standards. An in-house accurate mass retention time (AMRT) library was subsequently built with formula, exact mass and retention time of all identified skin CERs in comma-separated values (.csv) format (compatible with the MassHunter software), providing an automated and targeted data-processing of LC-MS CER profiles.[5] For targeted CER analysis (b), the Find By Formula application in the MassHunter Qualitative Analysis software was used, which searched for known CERs from the AMRT library. Mass and retention time had to match in a window of ± 25 ppm and ± 15 seconds, respectively. The Agile integrator was used, with an absolute peak height cut-off of 1000 counts. After targeted extraction of CERs, datafiles were imported in MassProfiler Professional for further processing. The two-dimensional plot of the raw data file (Figure 2a in manuscript) was created with MSight (http://web.expasy.org/MSight).[6]

(a) Untargeted data processing



(b) Targeted data processing

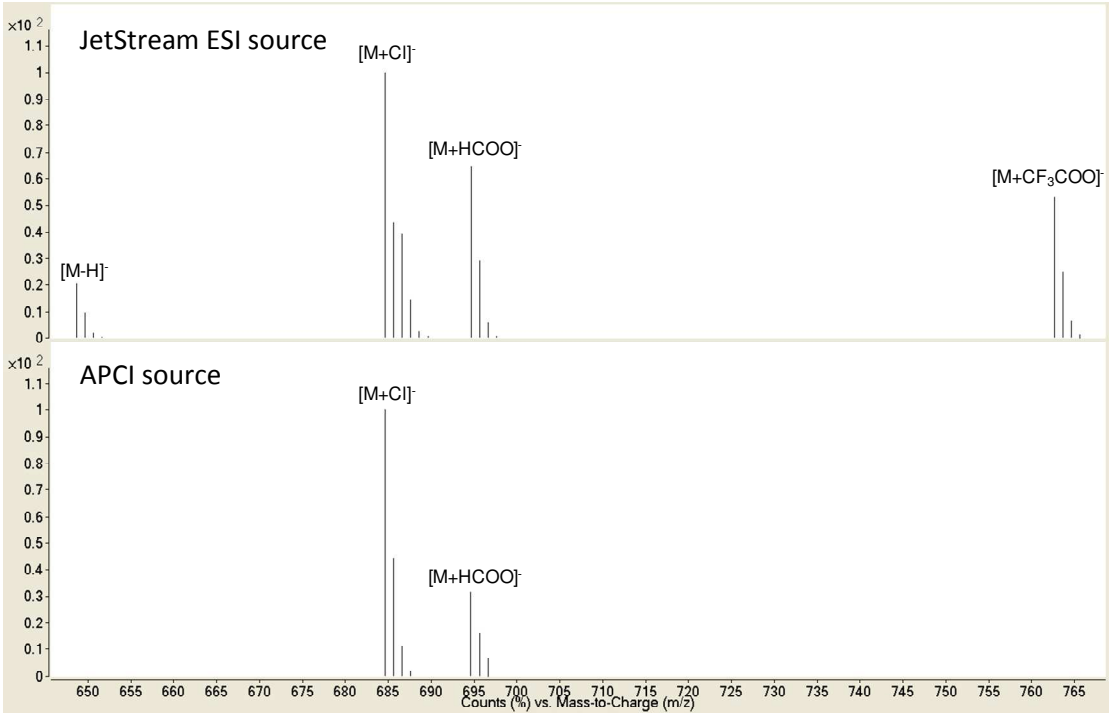
#formula	RT	Mass	Cpd
C32H65NO3	22.50	511.496444	CER(NDS)(32)
C33H67NO3	24.97	525.512094	CER(NDS)(33)
C34H69NO3	27.45	539.527744	CER(NDS)(34)
C35H71NO3	29.85	553.543394	CER(NDS)(35)
C36H73NO3	32.06	567.559044	CER(NDS)(36)
C37H75NO3	34.13	581.574694	CER(NDS)(37)
C38H77NO3	36.20	595.590344	CER(NDS)(38)



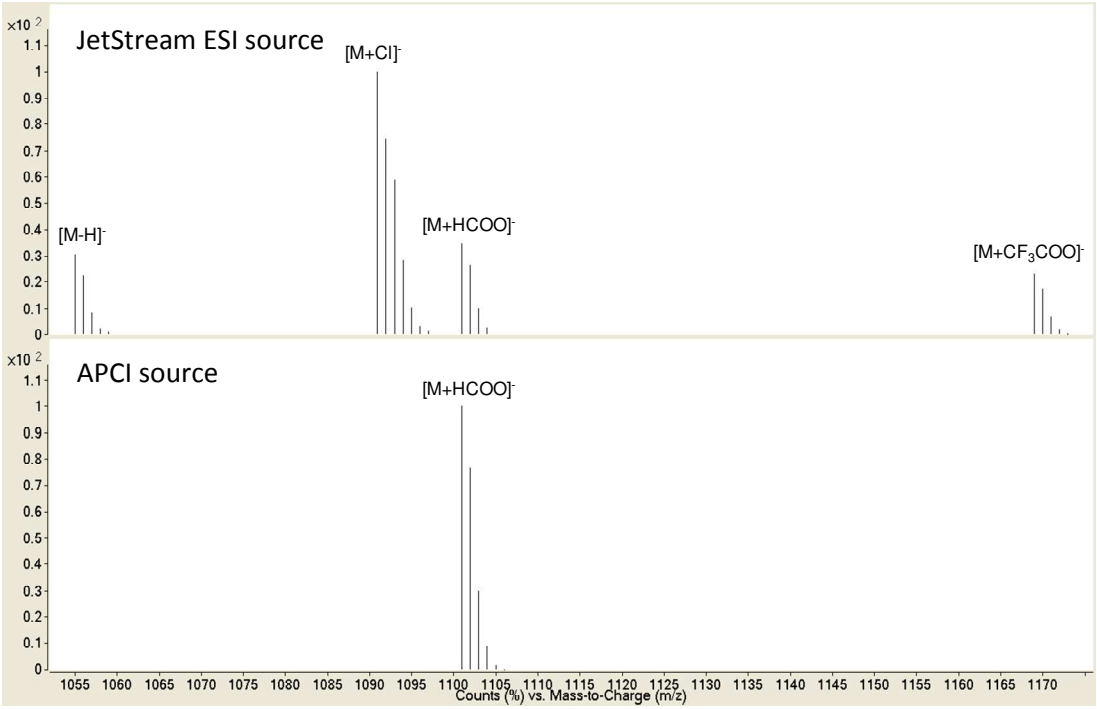
S-3. Typical adduct formation of CERs in negative ESI and APCI mode.

Extracted Compound Spectra (ECC) are shown for (a) CER[NS]C_{42} and (b) CER[EOH]C_{68} using APCI, displaying increasing signal intensity of $[M-H]^-$ <<< $[M-Cl]^-$ and $[M+HCOO]^-$, and JetStream ESI, showing an increasing signal intensity from $[M-H]^-$ < $[M+HCOO]^-$ ≈ $[M+CF_3COO]^-$ < $[M+Cl]^-$.

(a) CER[NS]C_{42}

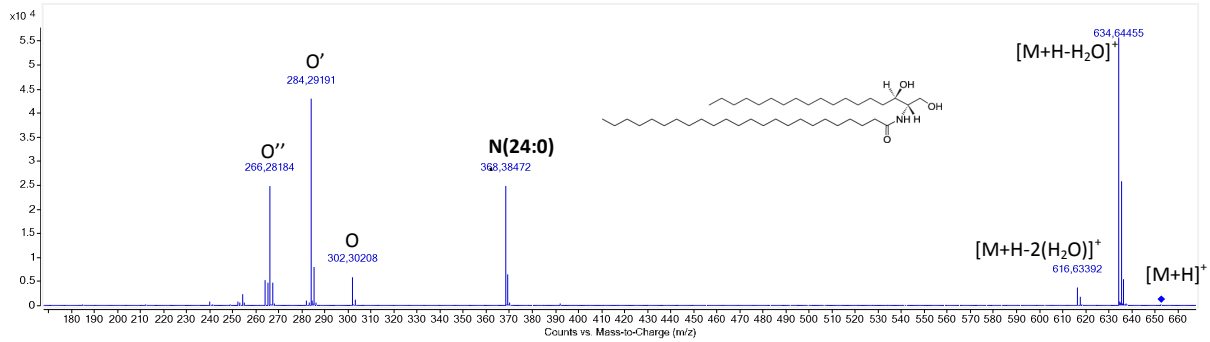


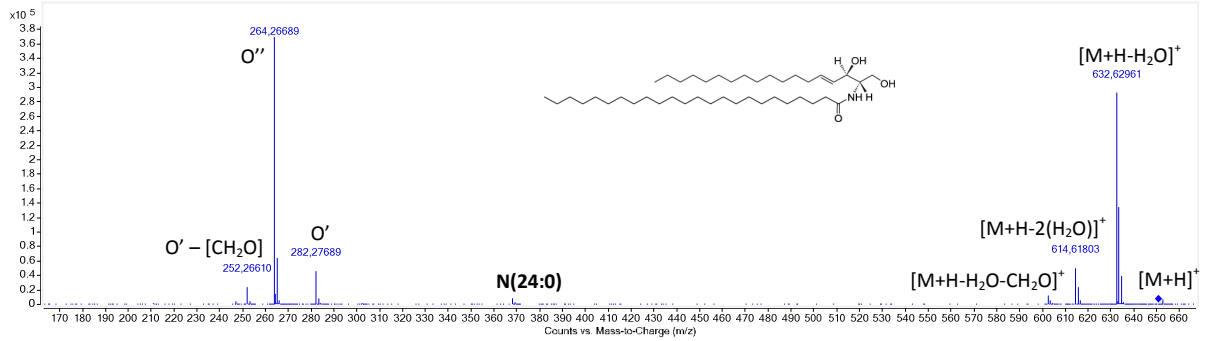
(b) CER[EOH]C_{68}

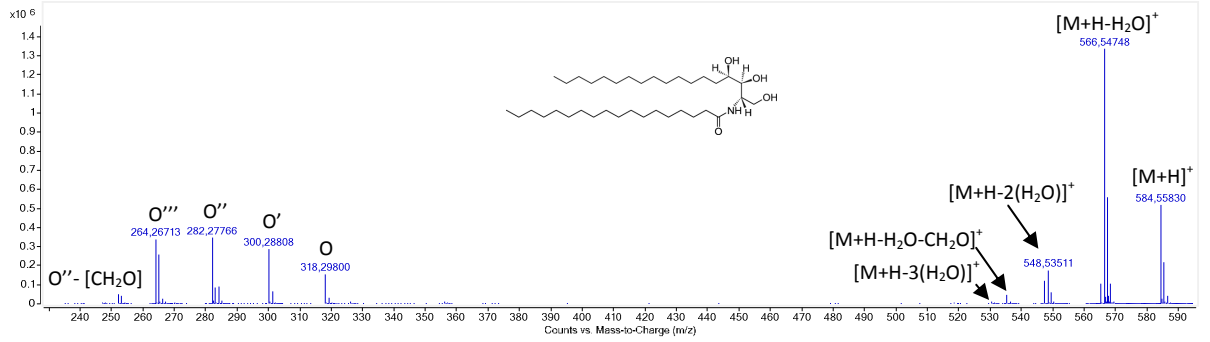


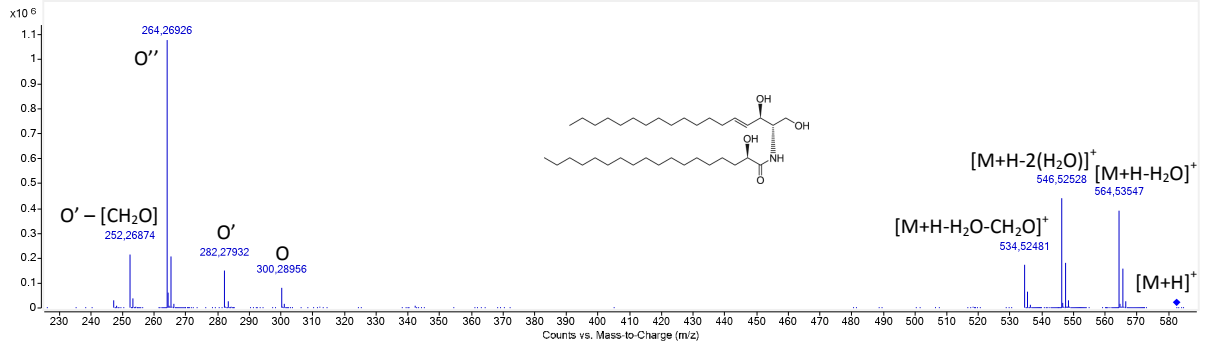
S-4. MS/MS fragmentation patterns of standard CERs.

MS/MS fragmentation pattern of standard CERs in positive ESI mode at a collision energy of 35 eV: (a) N-lignoceroyl-D-*erythro*-sphinganine or CER[N(24)DS(18)], (b) N-lignoceroyl-D-*erythro*-sphingosine or CER[N(24)S(18)], (c) N-stearoyl 4-hydroxysphinganine or CER[N(18)P(18)] and (d) N-(2'-(R)-hydroxystearoyl)-D-erythro-sphingosine or CER[A(18)S(18)]. All fragments related to the fatty acid part are printed in bold, while all fragments related to bases are printed in regular fonts.





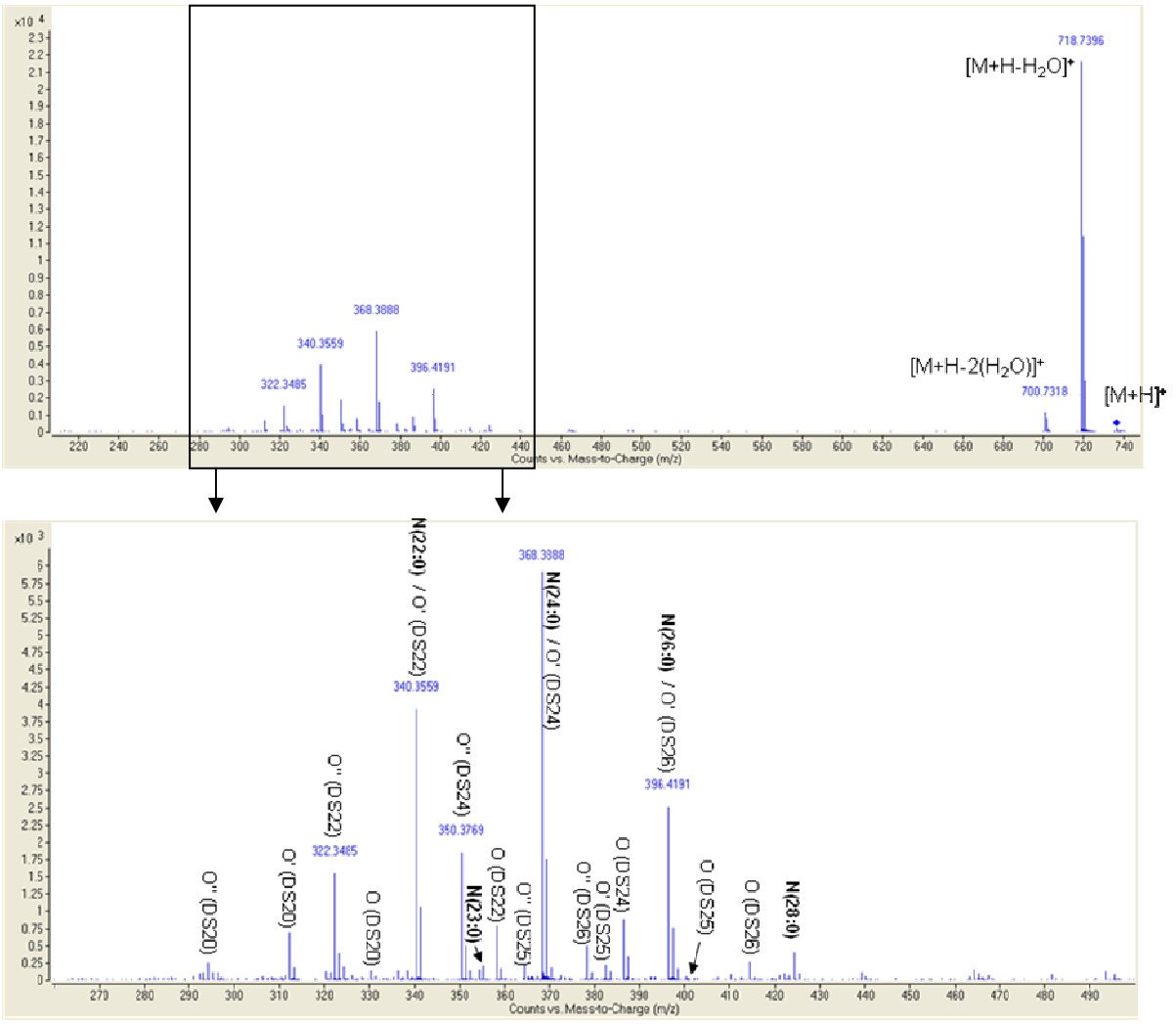




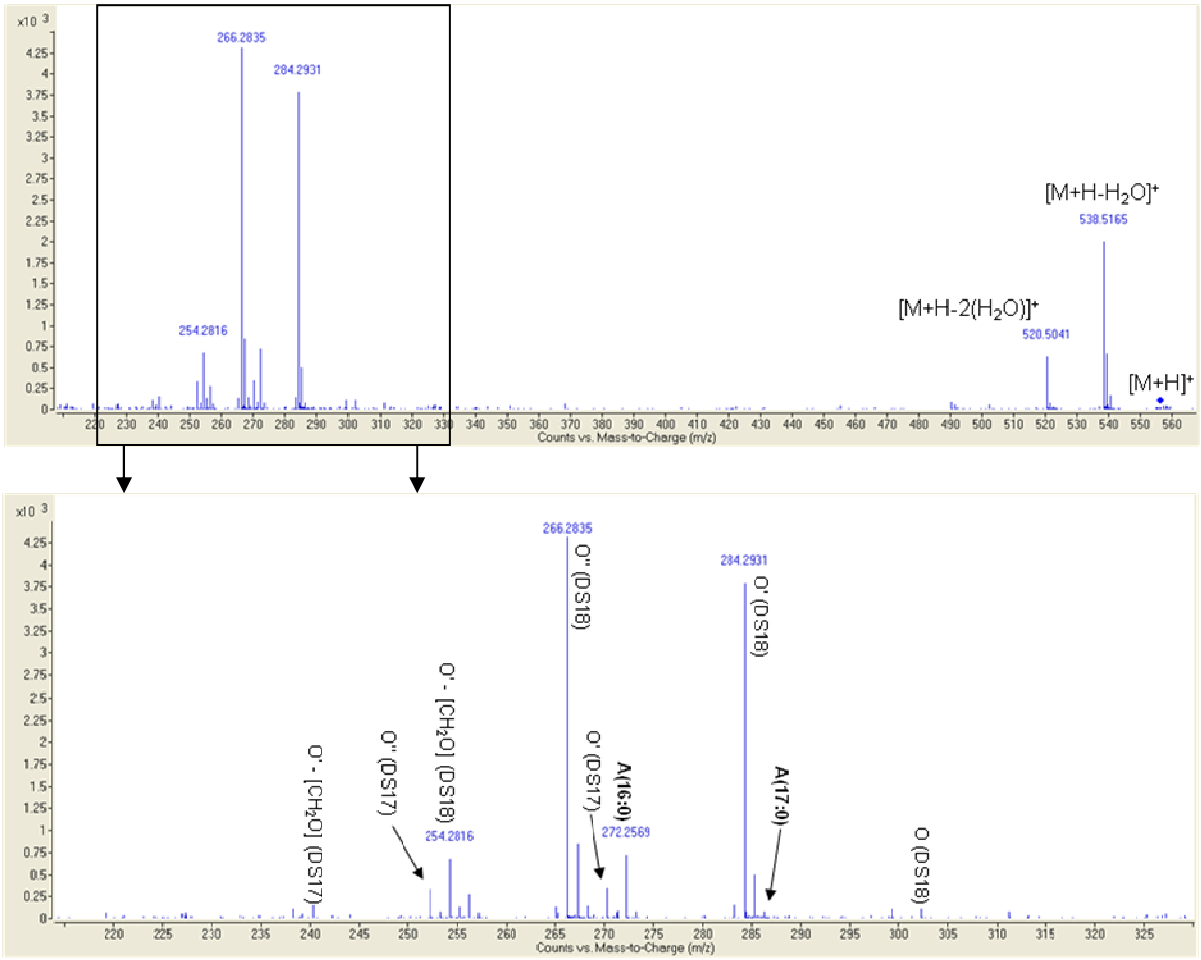
S-5. MS/MS fragmentation patterns of different identified CER species in skin.

MS/MS fragmentation patterns of different identified CER species (mostly positive ESI $[M+H]^+$), where each species can be defined as the result of various constitutional isomers of CERs varying in carbon length of SB and FA building blocks. After the full MS/MS spectrum, a zoomed spectrum is given where fragmentation of SB and/or fragments of FA constituents are present: (a) CER[NDS]C_{48}, (b) CER[ADS]C_{34}, (c) CER[EODS]C_{72}, (d) CER[NS]C_{49}, (e) CER[AS]C_{34}, (f) CER[OS]C_{52}, (g) CER[EOS]C_{66}, (h) CER[AP]C_{42}, (i) CER[EOP]C_{70}, (j) CER[NH]C_{42} $[M+H-H_2O]^+$, (k) CER[NH]C_{42} $[M-H]^-$, (l) CER[AH]C_{42} $[M+H-H_2O]^+$, (m) CER[EOH]C_{70}, (n) CER[EODS]C_{68} $[M-H]^-$. All fragments related to the fatty acid part are printed in bold, while all fragments related to bases are printed in regular fonts.

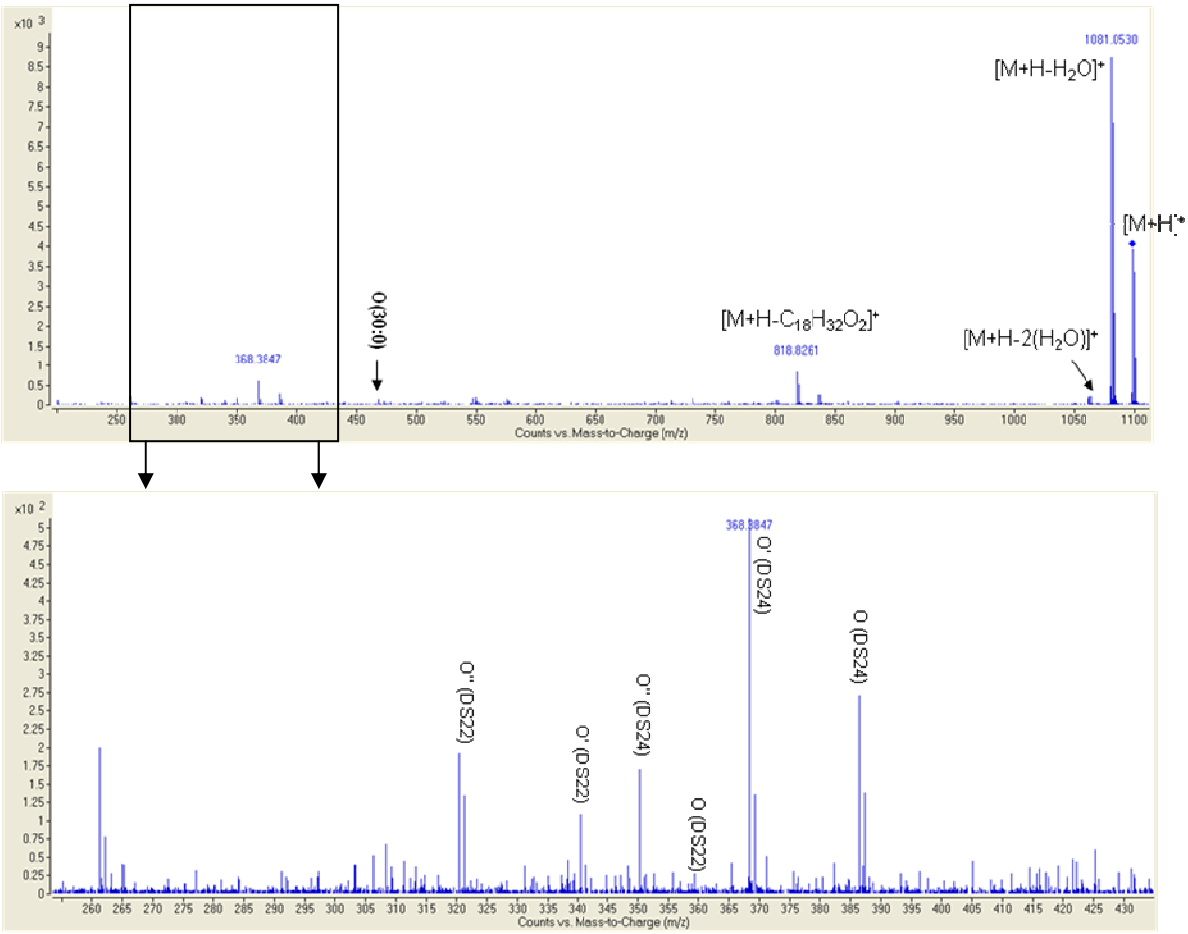
(a) **CER[NDS]C_{48} $[M+H]^+$** – consists mainly of constitutional isomers CER[N(28:0)DS(20)], CER[N(26:0)DS(22)], CER[N(24:0)DS(24)], CER[N(23:0)DS(25)] and CER[N(22:0)DS(26)].



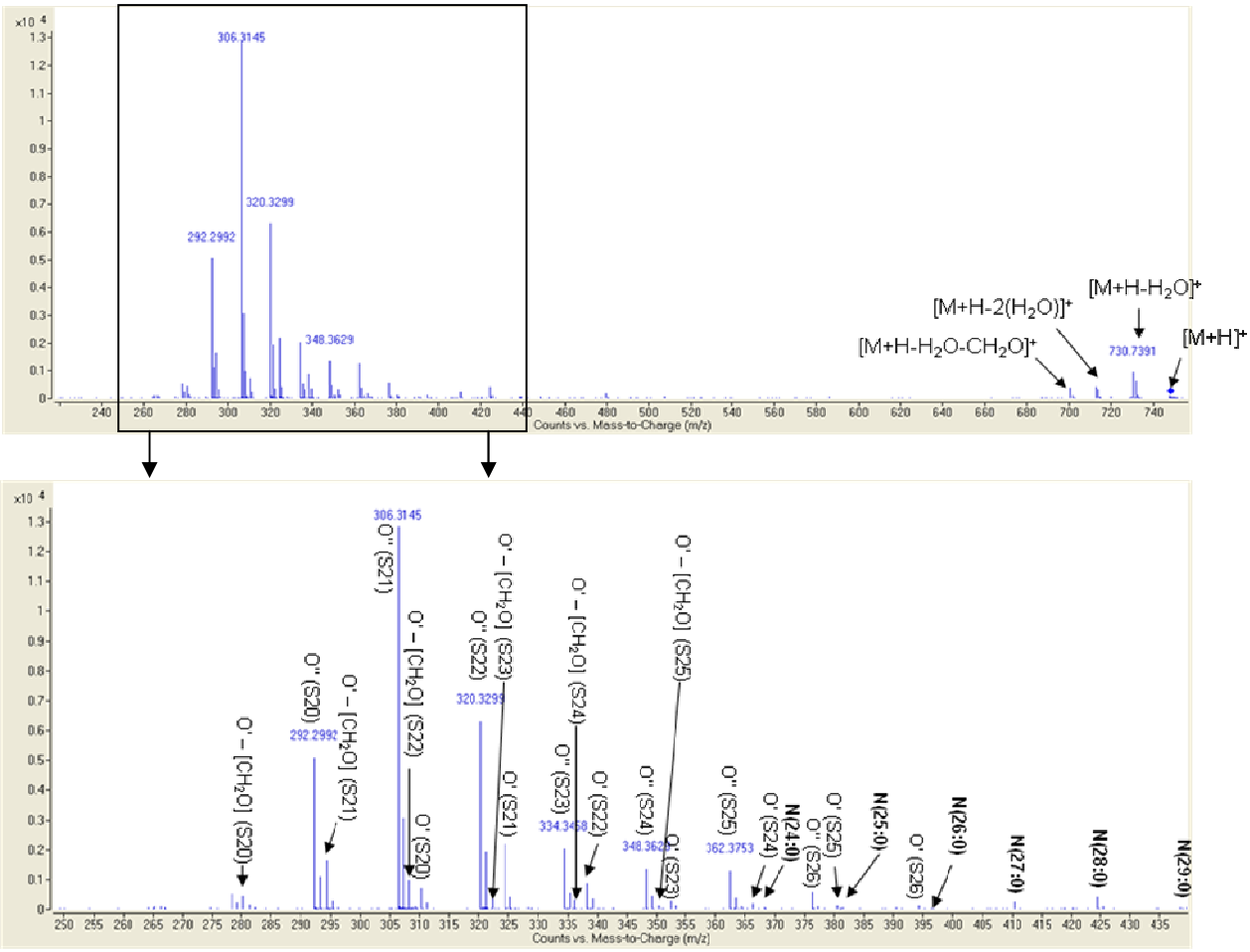
(b) **CER[ADS]C_{34} $[M+H]^+$** - consists mainly of constitutional isomers CER[A(17:0)DS(17)] and CER[A(16:0)DS(18)].



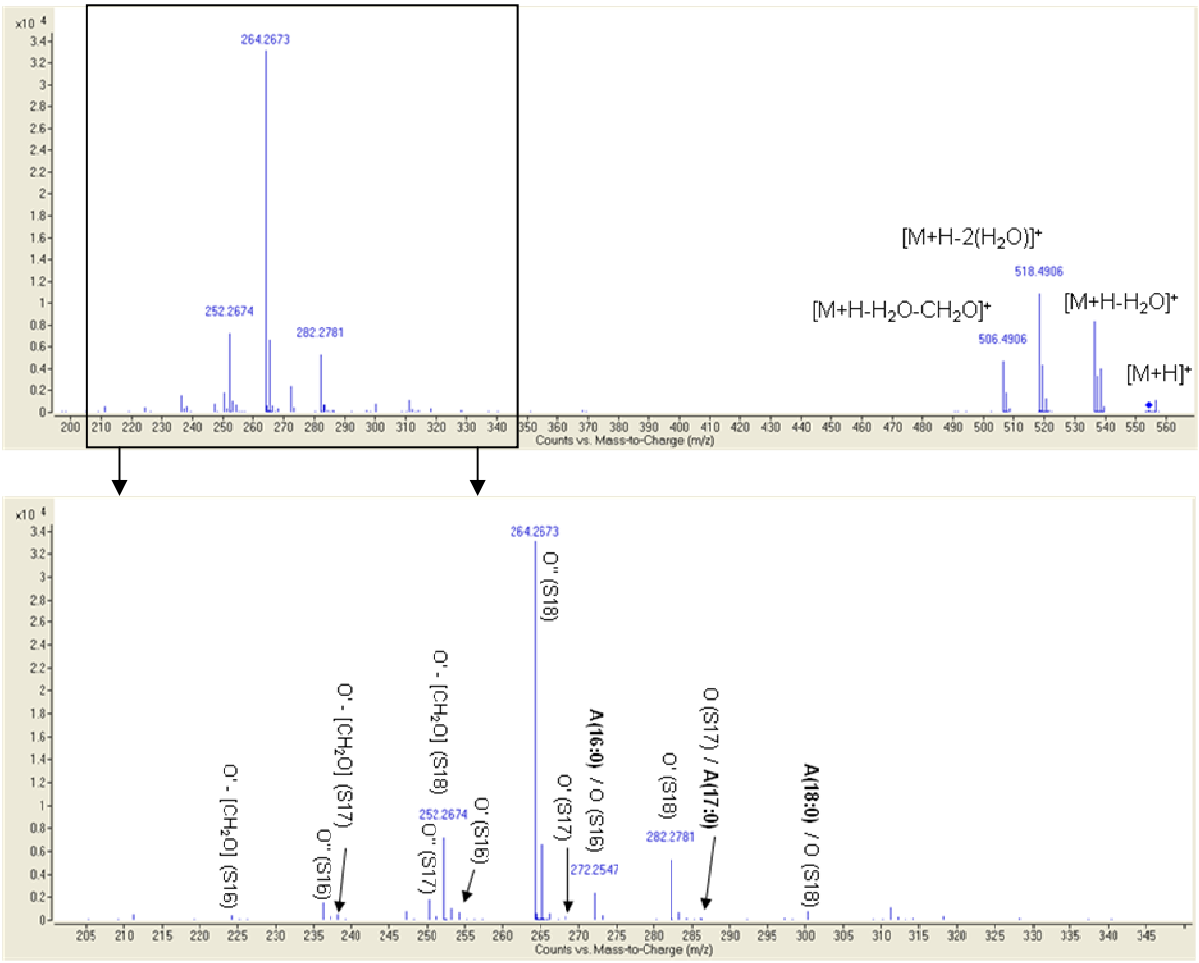
(c) **CER[EODS]C_{72} $[M+H]^+$** - consists mainly of constitutional isomers CER[E(18:2)O(30:0)DS(24)] and CER[E(18:2)O(32:0)DS(22)].



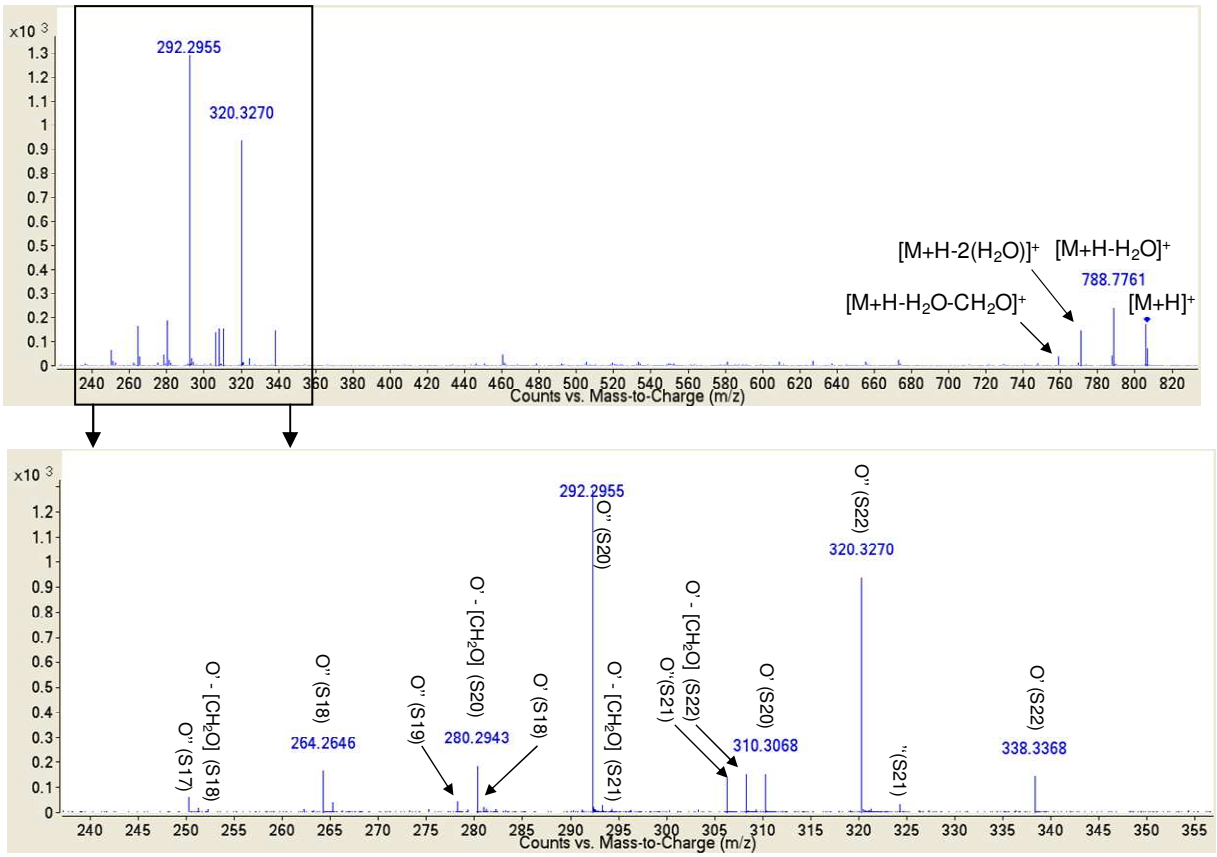
(d) **CER[NS]C_{49} [M+H]$^+$** – consists mainly of constitutional isomers CER[N(29:0)S(20)], CER[N(28:0)S(21)], CER[N(27:0)S(22)], CER[N(26:0)S(23)], CER[N(25:0)S(24)], CER[N(24:0)S(25)] and CER[N(23:0)S(26)].



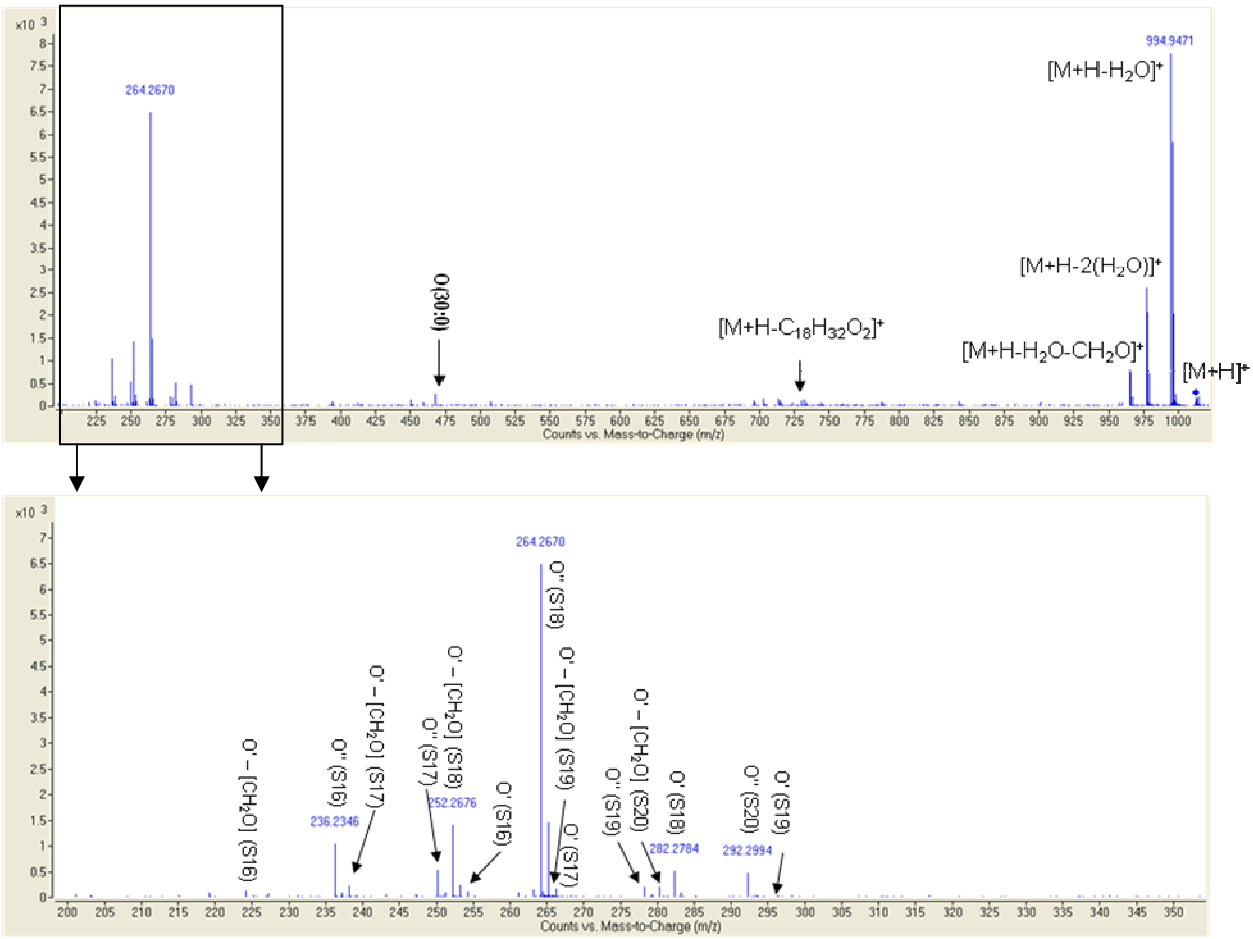
(e) **CER[AS]C_{34} [M+H]$^+$** – consists mainly of constitutional isomers CER[A(18:0)S(16)], CER[A(17:0)S(17)] and CER[A(16:0)S(18)].



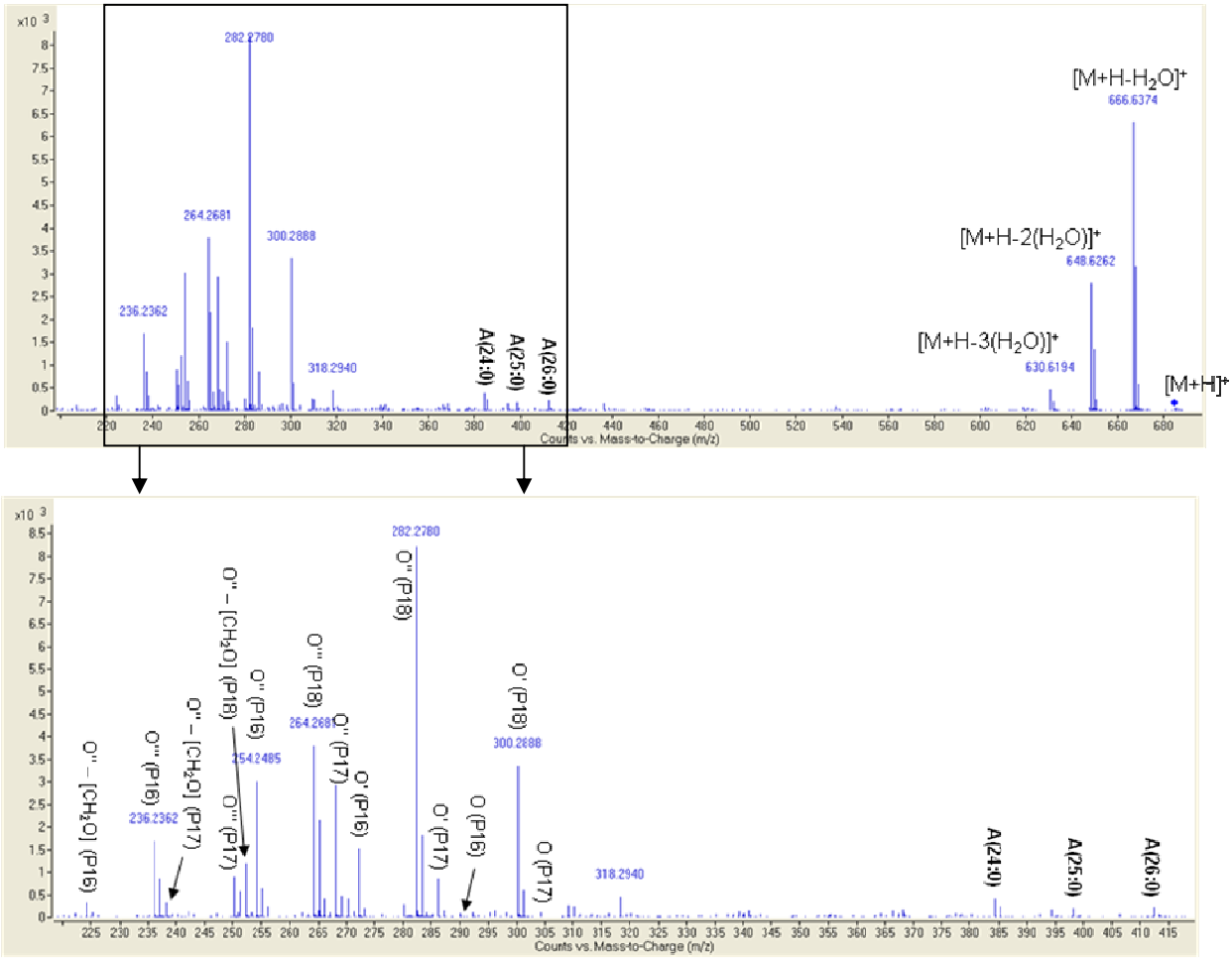
(f) **CER[OS]C_{52} [M+H]$^+$** – consists mainly of constitutional isomers CER[O(35:0)S(17)], CER[O(34:0)S(18)], CER[O(33:0)S(19)], CER[O(32:0)S(20)], CER[O(31:0)S(21)] and CER[O(30:0)S(22)].



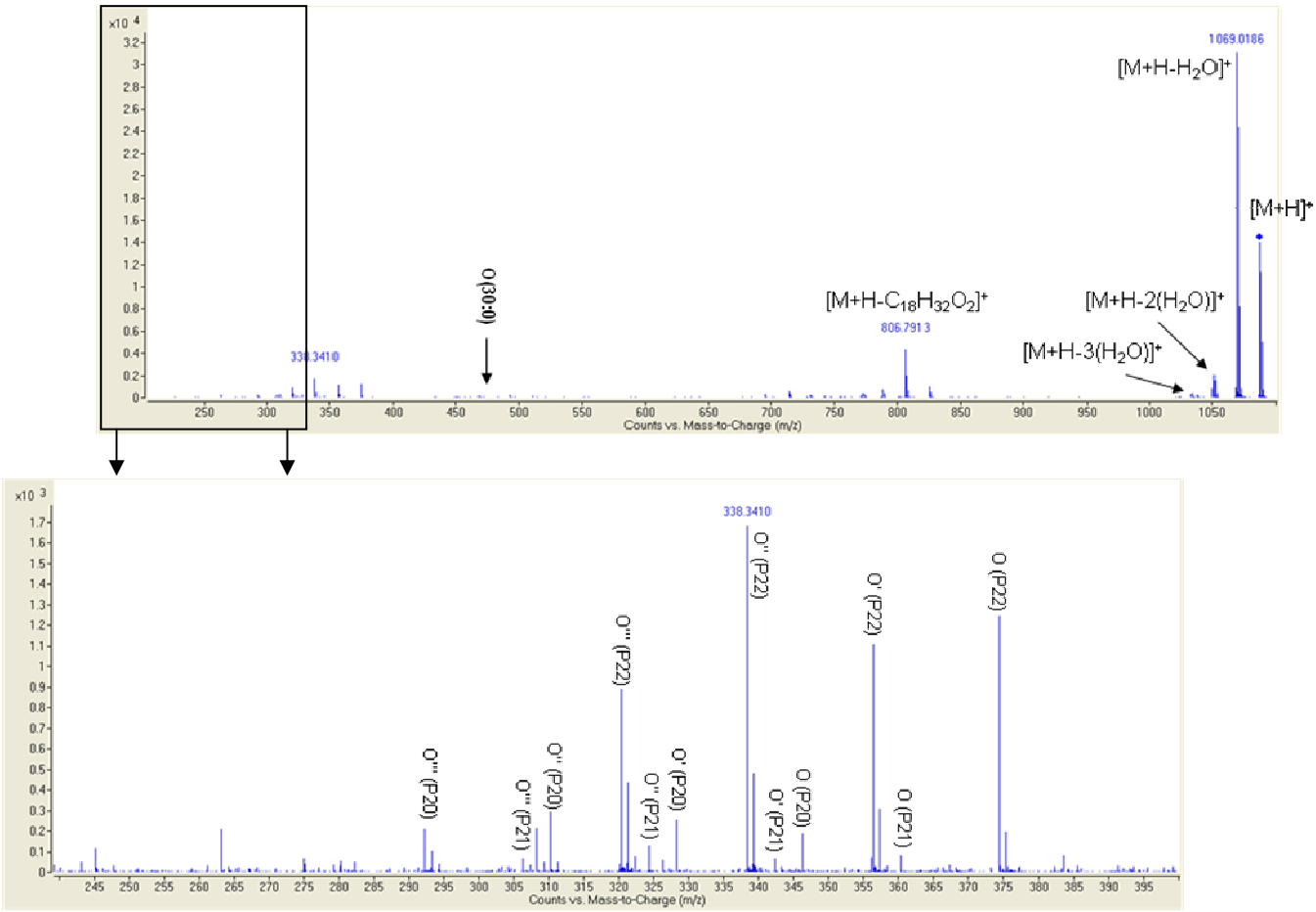
(g) **CER[EOS]C_{66} $[M+H]^+$** – consists mainly of constitutional isomers CER[E(18:2)O(28:0)S(20)], CER[E(18:2)O(29:0)S(19)], CER[E(18:2)O(30:0)S(18)], CER[E(18:2)O(31:0)S(17)] and CER[E(18:2)O(32:0)S(16)].



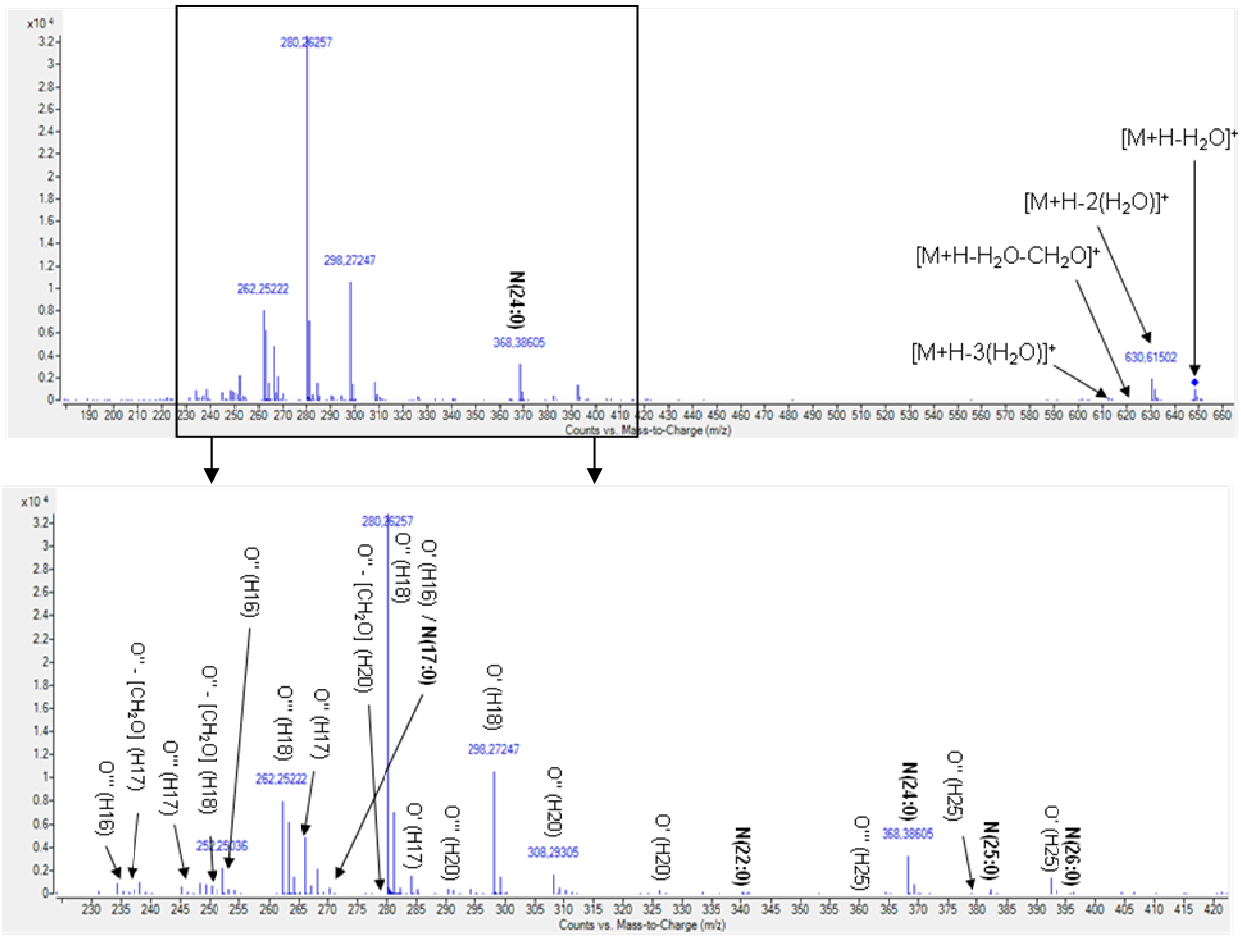
(h) **CER[AP]C_{42} $[M+H]^+$** – consists mainly of constitutional isomers CER[A(26:0)P(16)], CER[A(25:0)P(17)] and CER[A(24:0)P(18)].



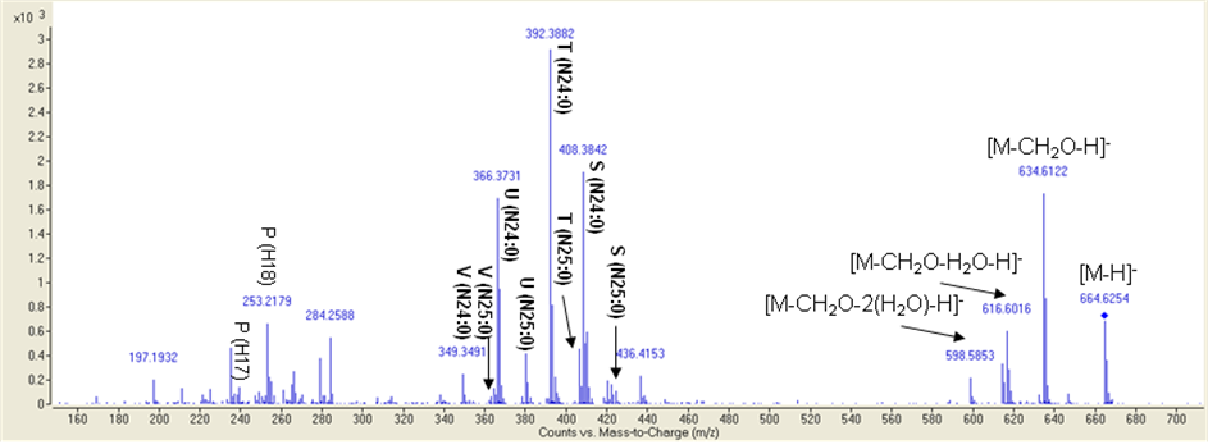
(i) **CER[EOP]C_{70} $[M+H]^+$** – consists mainly of constitutional isomers CER[E(18:2)O(30:0)P(22)], CER[E(18:2)O(31:0)P(21)] and CER[E(18:2)O(32:0)P(20)]



(j) **CER[NH]C_{42} $[M+H-H_2O]^+$** – consists mainly of constitutional isomers CER[N(17:0)H(25)], CER[N(22:0)H(20)], CER[N(24:0)H(18)], CER[N(25:0)H(17)] and CER[N(26:0)H(16)]



(k) **CER[NH]C_{42} [M-H]$^-$** – consists mainly of constitutional isomers CER[N(17:0)H(25)], CER[N(22:0)H(20)], CER[N(24:0)H(18)], CER[N(25:0)H(17)] and CER[N(26:0)H(16)]. (here focus on CER[N(17:0)H(25)] and CER[N(24:0)H(18)])



P (H18) = $[C_{16}H_{29}O_2]^-$ (-2.35ppm) P (H17) = $[C_{15}H_{27}O_2]^-$

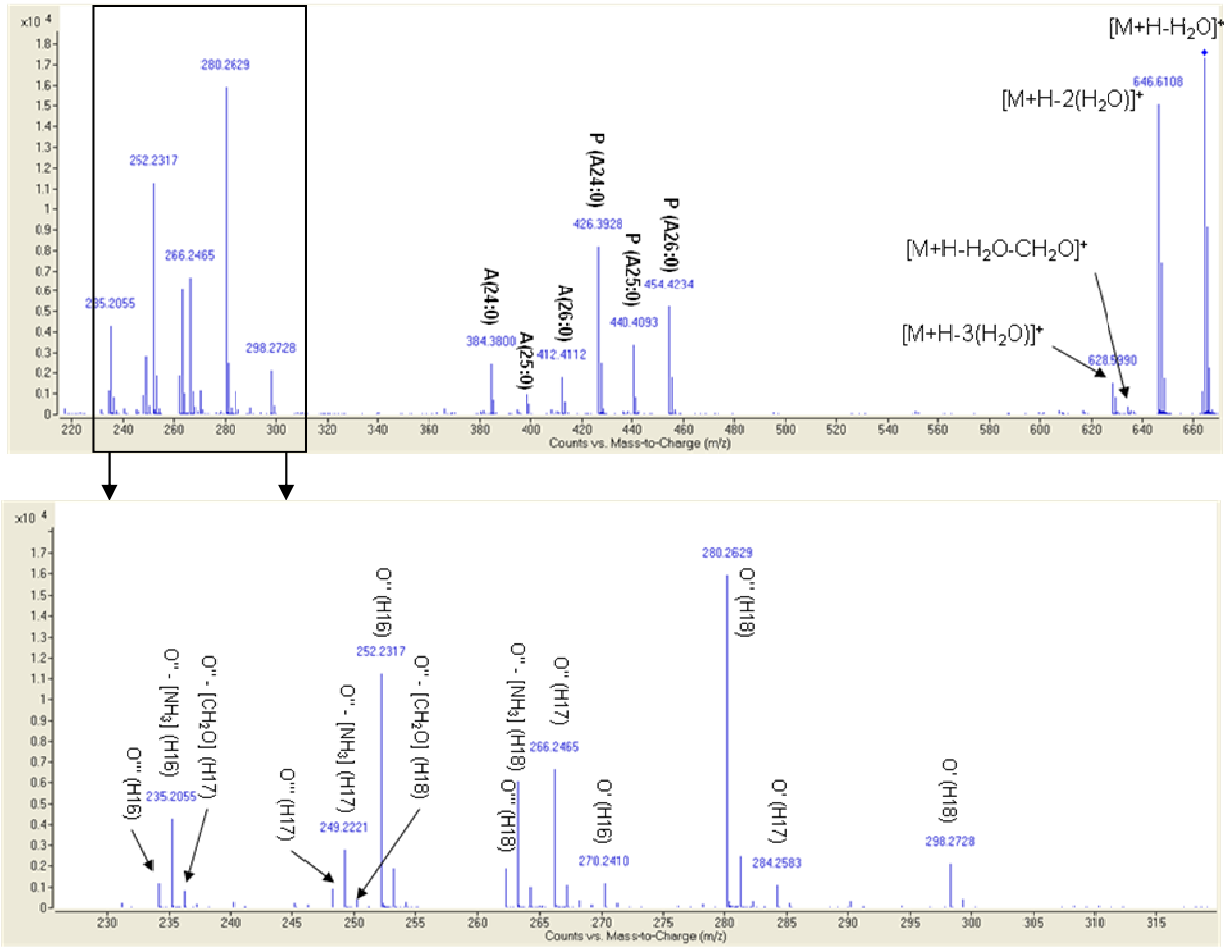
S (N24:0) = $[C_{26}H_{50}NO_2]^-$ (1.23 ppm) S (N25:0) = $[C_{27}H_{52}NO_2]^-$

T (N24:0) = $[C_{26}H_{50}NO]^-$ (4.04 ppm) T (N25:0) = $[C_{27}H_{52}NO]^-$

U (N24:0) = $[C_{24}H_{48}NO]^-$ (2.83 ppm) U (N25:0) = $[C_{25}H_{50}NO]^-$

V (N24:0) = $[C_{24}H_{45}O]^-$ (-4.31 ppm) V (N25:0) = $[C_{25}H_{47}O]^-$

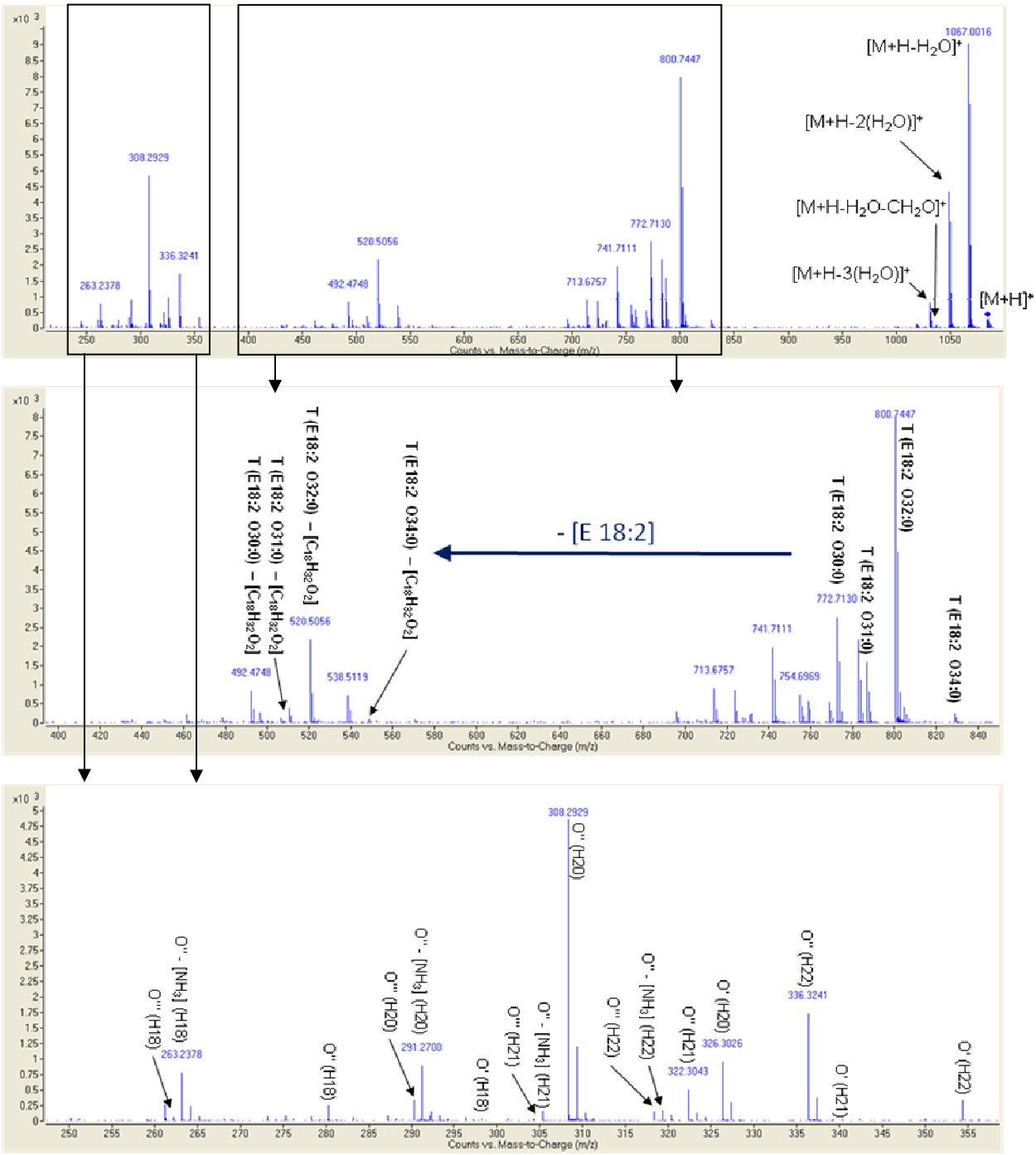
(l) **CER[AH]C_{42} [M+H-H_2O]$^+$** – consists mainly of constitutional isomers CER[A(24:0)H(18)], CER[N(25:0)H(17)] and CER[N(26:0)H(16)]



α-hydroxy group on the FA induces other fragmentation mechanisms:

α-hydroxy FA part of P fragmentation:	P (A24:0) = $[C_{26}H_{52}NO_3]^+$ (3.22 ppm)
	P (A25:0) = $[C_{27}H_{54}NO_3]^+$ (1.19 ppm)
	P (A26:0) = $[C_{28}H_{56}NO_3]^+$ (4.57 ppm)
Loss of NH_3 in 6-hydroxy-sphingosine base:	O'' - [NH_3] (H16) = $[C_{16}H_{27}O]^+$ (0.61 ppm)
	O'' (H16) = $[C_{16}H_{30}NO]^+$ (1.95 ppm)

(m) **CER[EOH]C_{70} $[M+H]^+$** – consists mainly of constitutional isomers CER[E(18:2)O(30:0)H(22)], CER[E(18:2)O(31:0)H(21)], CER[E(18:2)O(32:0)H(20)] and CER[E(18:2)O(34:0)H(18)]



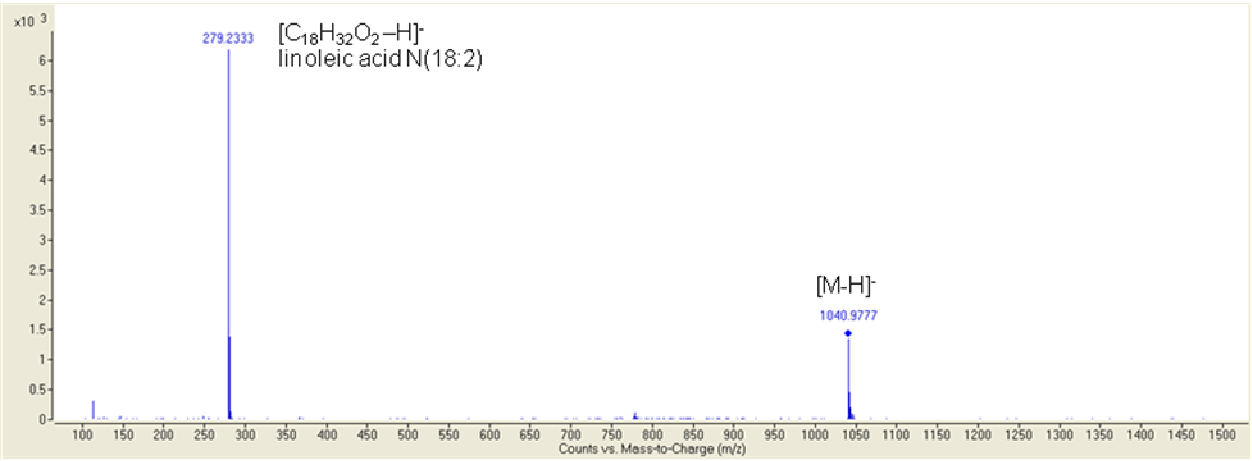
T (E18:2 O34:0) = $[C_{54}H_{102}NO_4]^+$

T (E18:2 O32:0) = $[C_{52}H_{98}NO_4]^+$

T (E18:2 O31:0) = $[C_{51}H_{96}NO_4]^+$

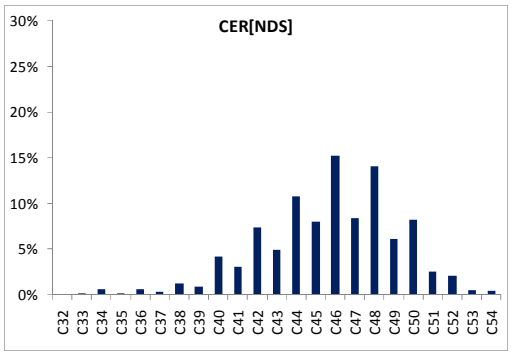
T (E18:2 O30:0) = $[C_{50}H_{94}NO_4]^+$

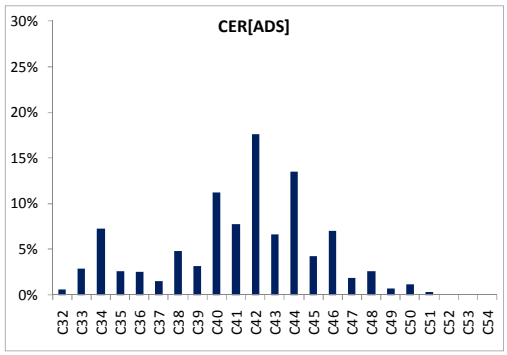
(n) **CER[EODS]C_{68} [M-H]$^-$** – showing linoleic acid as the ester-linked FA.

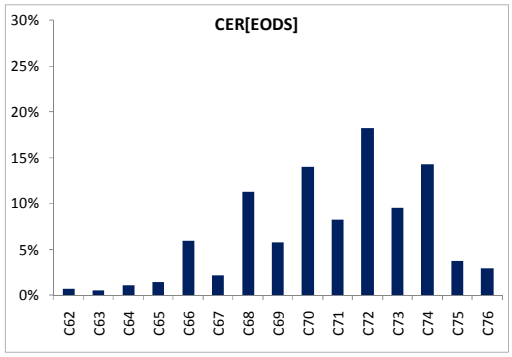


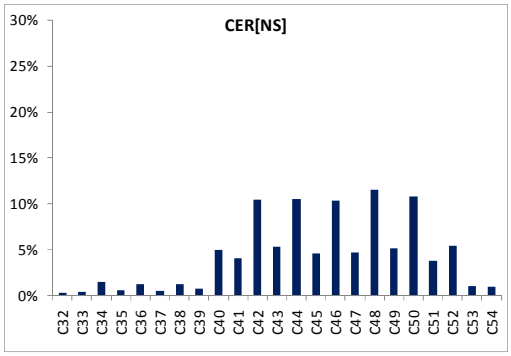
S-6. Relative distribution of CER species within each class.

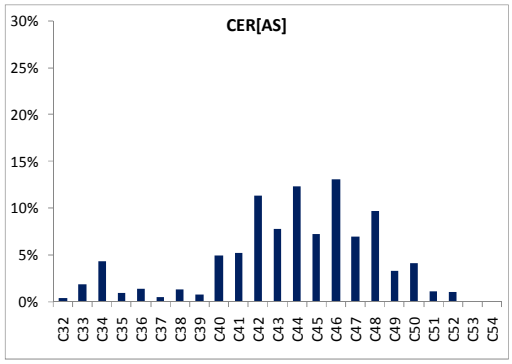
The X-axis defines the total carbon atom number, while the Y-axis shows the percentage of each CER within its class. The total carbon atom number is shown from C_{32} to C_{54}, except for EO CER, where the range is from C_{62} up to C_{76}. All distributions contain the same scale on the Y-axis scale (a maximum of 30%).

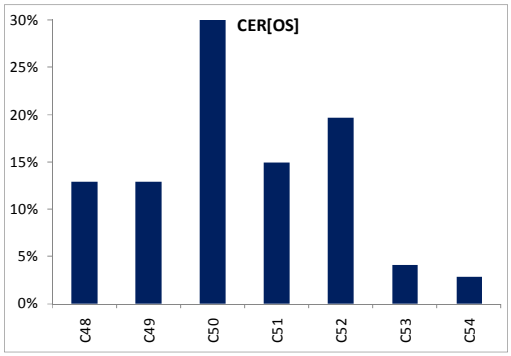


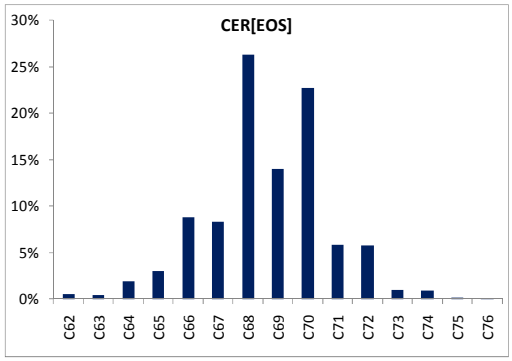


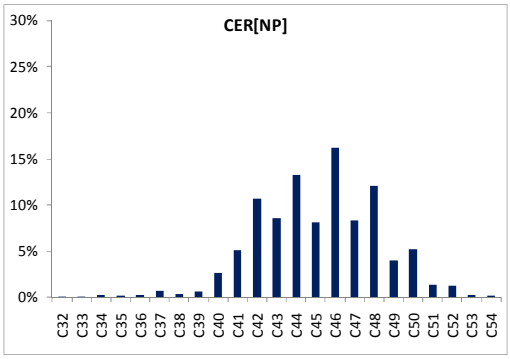











CER[NP]
30%
25%
20%
15%
10%
5%
0%
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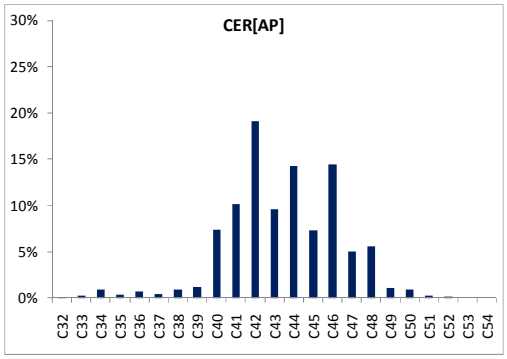
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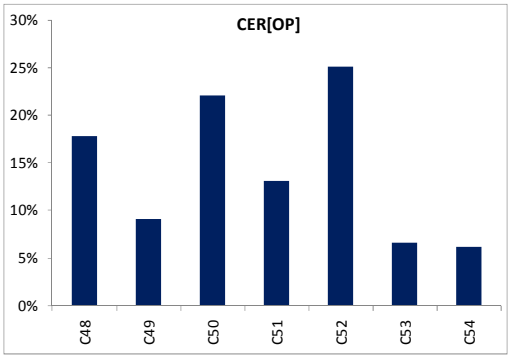
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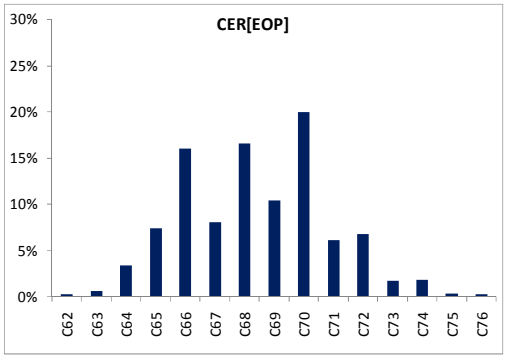
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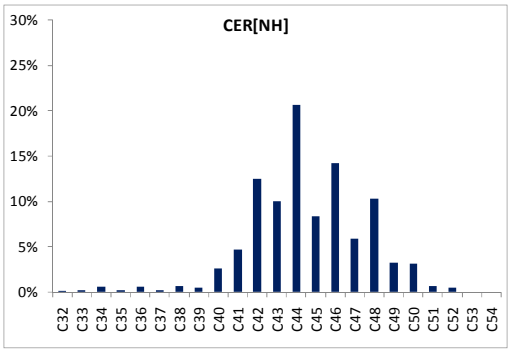
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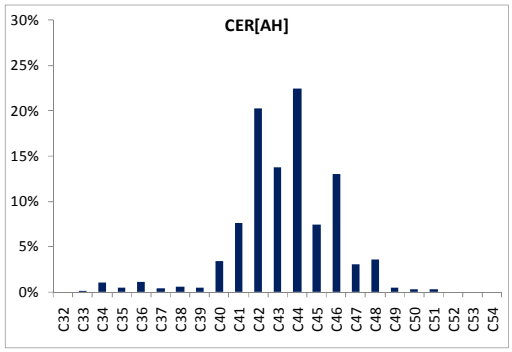
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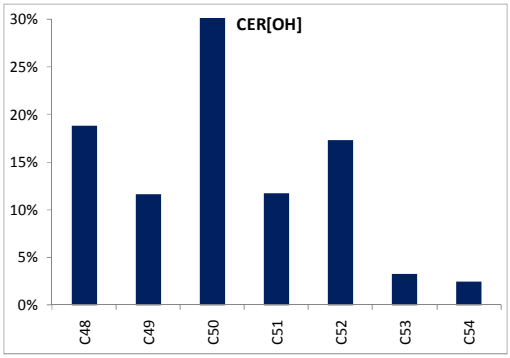
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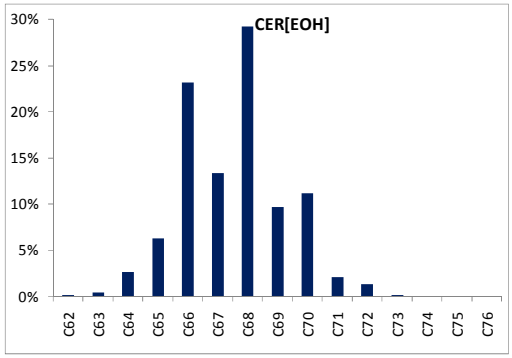
CER[EOH]
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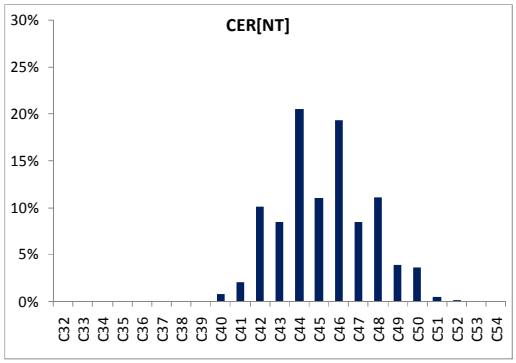
CER[NT]
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S-7. Annotation of fragments of CER[NT]C_{46}, based on mass accuracy after MS/MS.

Main fragments are annotated in accordance with the cleavage of phytosphingosine CERs[3,4]. (O = loss of fatty acyl group, O' = loss of fatty acyl group and 1 mole of water, O'' = loss of fatty acyl group and 2 moles of water, O''' = loss of fatty acyl group and 3 moles of water, O'''' = loss of fatty acyl group and 4 moles of water, U = loss of sphingoid base while maintaining the amino group)

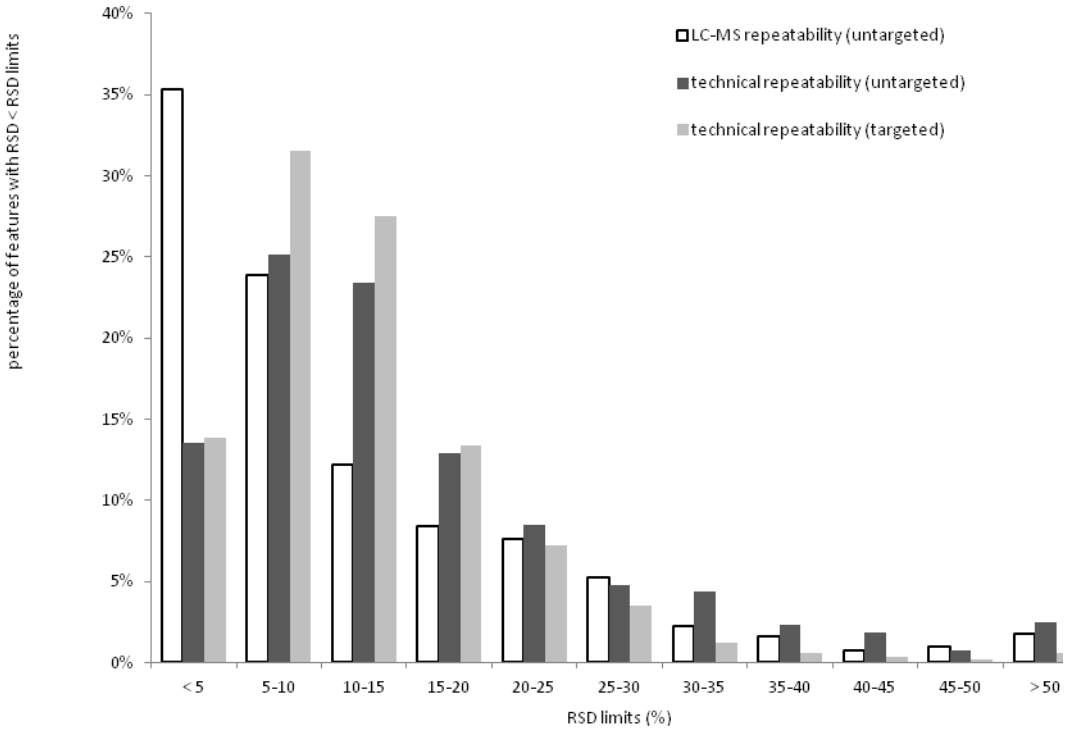
CER fragment	Measured *m/z*	Elemental composition	Mass accuracy
$[M+H]^+$	740.7126	$[C_{46}H_{94}NO_5]^+$	0.07 ppm
$[M+H-H_2O]^+$	722.6979	$[C_{46}H_{92}NO_4]^+$	5.80 ppm
$[M+H-2(H_2O)]^+$	704.6878	$[C_{46}H_{90}NO_3]^+$	5.29 ppm
$[M+H-3(H_2O)]^+$	686.6798	$[C_{46}H_{88}NO_2]^+$	1.69 ppm
$[M+H-4(H_2O)]^+$	668.6748	$[C_{46}H_{86}NO]^+$	-6.68 ppm
O (T20)	362.3257	$[C_{20}H_{44}NO_4]^+$	2.17 ppm
O' (T20)	344.3139	$[C_{20}H_{42}NO_3]^+$	5.89 ppm
O'' (T20)	326.3034	$[C_{20}H_{40}NO_2]^+$	6.01 ppm
O''' (T20)	308.2920	$[C_{20}H_{38}NO]^+$	9.08 ppm
O'''' (T20)	290.2826	$[C_{20}H_{36}N]^+$	5.62 ppm
U(N26:0)	395.4127	$[C_{26}H_{54}NO]^+$	-0.8 ppm
O (T18)	334.2952	$[C_{18}H_{40}NO_4]^+$	-0.04 ppm
O' (T18)	316.2841	$[C_{18}H_{38}NO_3]^+$	1.49 ppm
O'' (T18)	298.2723	$[C_{18}H_{36}NO_2]^+$	5.91 ppm
O''' (T18)	280.2632	$[C_{18}H_{34}NO]^+$	1.04 ppm
O'''' (T18)	262.2502	$[C_{18}H_{32}N]^+$	10.44 ppm
U(N28:0)	424.4491	$[C_{28}H_{58}NO]^+$	5.06 ppm
O (T22)	Too low	$[C_{22}H_{48}NO_4]^+$	-
O' (T22)	372.3455	$[C_{22}H_{46}NO_3]^+$	4.63 ppm
O'' (T22)	354.3363	$[C_{22}H_{44}NO_2]^+$	0.87 ppm
O''' (T22)	336.3236	$[C_{22}H_{42}NO]^+$	7.43 ppm
O'''' (T22)	318.3129	$[C_{22}H_{40}N]^+$	8.28 ppm
U(N24:0)	368.3887	$[C_{24}H_{50}NO]^+$	0.03 ppm

S-8. Ceramide hydrolysis.

CER hydrolysis was based on the procedure described by Johnson and Brown.[7] In short, skin SC extracts were transferred in reaction vials after SPE fractionation (Reacti-Vial, Thermo Scientific, Rockford, IL, USA) and dried under nitrogen. Samples were hydrolyzed for 1 h at 100°C under nitrogen using 0.5 M HCl in 1 mL of 90/10 acetonitrile/water (v/v). After the acid hydrolysis, samples were dried under nitrogen, and as such dissolved in 300 µL of 50/50 acetonitrile/water (v/v) for SB analysis or in 300 µL of isopropanol/chloroform 50/50 (v/v) for FA analysis.

S-9. LC-MS repeatability and technical repeatability of the CER analysis.

RSD% is calculated for features with 100% frequency after untargeted or targeted data processing.



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