Supporting Information

Automated Annotation of Sphingolipids Including Accurate Identification of Hydroxylation Sites using MS^n Data

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Note S-1. MS-DIAL settings.

For file conversion to abf format, Refycs Analysis Base File Converter version 4.0.0 was used. For the benchmark study, MS-DIAL version 4.0.0 was used. Both applications were downloaded on November 20th, 2019. For MS-DIAL, the following parameters were used:

General settings

Ionization type:	Soft ionization
Separation type:	Chromatography
MS method type:	Conventional LC/MS or data dependent MS/MS
MS1 data type:	Profile
MS2 data type:	Centroid
Ion mode:	Depending on data, negative or positive
Target omics:	Lipidomics

Data Collection:

MS1 tolerance:	0.006 Da
MS2 tolerance:	0.3 Da
Retention time begin:	0 min
Retention time end:	80 min
Mass range begin:	200 Da
Mass range end:	1100 Da
Maximum charged number:	1
Consider Cl and Br elements:	false
Number of Threads:	7
Excecute retention time corrections	false

Peak Detection:

Minimum peak height:	1000 amplitude
Mass slice width:	0.025 Da
Smoothing method:	Linear weighted moving average
Smoothing level	3
Minimum peak width:	4
Exclusion mass list:	none

MS2Dec:

Default parameters were used, except for the following setting:

Keep the isotopic ions w/o MS2Dec:	true

Identification:

MSPFile – Solvent HCOONH4 the following subclasses were selected:

Negative

SM [M+HCOO]-

Cer_NS [M-H]- ; Cer_NS [M+HCOO]- ; Cer_NDS [M-H]- ; Cer_NDS [M+HCOO]- ; Cer_AS [M-H]- ; Cer_AS [M+HCOO]- ; Cer_ADS [M-H]- ; Cer_ADS [M+HCOO]- ; Cer_BS [M-H]- ; Cer_BS [M+HCOO]- ; Cer_BDS [M-H]- ; Cer_BDS [M+HCOO]- ; Cer_NP [M-H]- ; Cer_NP [M+HCOO]- ; Cer_AP [M-H]- ; Cer_AP [M+HCOO]-

CerP [M-H]-

HexCer_NS [M-H]- ; HexCer_NS [M+HCOO]- ; HexCer_NDS [M-H]- ; HexCer_NDS [M+HCOO]- ; HexCer_AP [M-H]- ; HexCer_AP [M+HCOO]- ;

Positive

Sphingosine [M+H]+; Sphinganine [M+H]+; Phytosphingosine [M+H]+;

SM [M+H]+; SM[M+Na]+

Cer_NS [M+H]+; Cer_NDS [M+H]+; Cer_AP [M+H]+;

CerP [M+H]+;

HexCer_NS [M+H]+; HexCer_NDS [M+H]+; HexCer_AP [M+H]+;

Retention time tolerance:	100 min (setting for no retention time selection)
Accurate mass tolerance (MS1):	0.006 Da
Accurate mass tolerance (MS2):	0.3 Da
Identification score cut off:	80% (default)
Use retention time for scoring:	false
Use retention time for filtering:	false

Advance parameters were not changed.

The default settings were as follows:

Text file:	nothing selected
Retention time tolerance:	0.1 min
Accurate mass tolerance:	0.01 Da
Identification score cut off:	85%
Relative abundance cut off (changed):	typically: 0.01%
	murine brain in positive ion mode: 0.001%

Only report the top hit:	control experiment: false
	murine brain: true
Adduct Ion settings (negative ion mode):	[M-H]- ; [M+FA-H]-
Adduct Ion settings (positive ion mode):	[M+H]+; [M+H-H2O]+; [M+Na]+

Alignment

Result name:	different for each search
Reference file:	different for each search
Retention time tolerance:	0.2 min
MS1 tolerance:	0.015 Da
Advanced parameters were not changed	
Retention time factor:	0.5
MS1 factor:	0.5
Peak count filter:	0 %
N% detected in at least one group:	0 %
Remove features based on blank information:	false
Sample max / blank average:	5 fold change
Keep ‘identified’ metabolite features:	true
Keep ‘annotated (wo MS2)’ metabolite features:	false
Keep removable features and assign the tag:	true
Gap filling by compulsion:	true

Isotope tracking

Tracking of isotope labels:	false
Non-labeled reference file:	(first one is automatically selected)
Use target formula library:	false and nothing selected
Set fully-labeled reference file:	false (first one is automatically selected)

Furthermore, the following default value was selected:

Together with Alignment	true

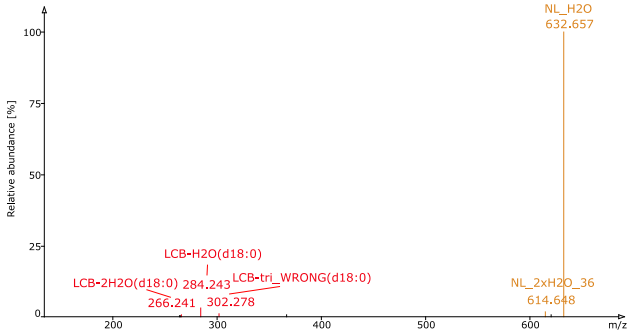
Note S-2. Algorithmic Differences between LDA and MS-DIAL.

The basis of the LDA's decision rule sets is an integrated combination of fragment rules (*m/z* values of fragments) and intensity rules (intensity relations between fragments). Both of these rules are utilized to verify structural annotation levels. Accordingly, the concept is organized hierarchically, i.e., there are rules to verify the lipid subclass/adduct, followed by rules for the fatty acid chain assignment (FA and/or LCB), and if possible, rules to determine the position of the chains.

In comparison, the MS-DIAL[20] hybrid scoring system consists of two consecutive steps (see Extended Data Fig. 1 of the MS-DIAL 4 publication on page 10):

- An MS/MS similarity search by a dot product and a reverse dot product to remove noisy spectra.
- A decision tree algorithm that works on distinct fragments to avoid structural overinterpretation. This decision tree algorithm has some common characteristics with the LDA approach using decision rule sets: i) decisive fragments are defined that must be present to verify the lipid subclass/adduct and derive chain information; ii) for validity, these fragments must exceed a certain intensity threshold (in relation to the base peak).

It is not apparent whether the observed difference in performance is a matter of step one or step two. There are reasons for both of them, which we will explain in the following. The difference cannot be explained by the number of used fragments: In the pages 60-68 of the Supplementary Information of the MS-DIAL 4 publication, it is evident that MS-DIAL utilizes an adequate number of fragments, including neutral loss fragments, for identification. For example, a comparison between the following figure and the example of '(Cer-NDS) Cer 18:0;2O/26:0 as [M+H]+' given at page 61 of Supplementary Fig. 1 in the MS-DIAL supplement[20] shows that for this lipid subclass/adduct almost the same fragments are used for the annotation.



Note S-2 Figure 1. Comparison of the shown LDA annotation of Cer d18:0/n24:1 to the MS-DIAL annotation of Cer d18:0/n26:0 shown at page 61 Supplementary Figure 1 of the MS-DIAL publication[20]. The mass difference of the precursor between the two presented species is 30 Da. Both applications use the NL_H2O (at *m/z* 662 in MS-DIAL and *m/z* 632 in LDA) and the NL_2x2H2O (at *m/z* 614 in MS-DIAL and *m/z* 644 in LDA) as subclass/adduct specific fragments. For LCB identification, both use the fragments at *m/z* 266 and *m/z* 284, and a fragment at *m/z* 254 that was not detected in the LDA example. Additionally, the LDA uses the fragment at *m/z* 302 to distinguish dihydroxylated from trihydroxylated LCBs.

Of note, the 'ideal' MS-DIAL spectrum shows little similarity with the experimental one (both shown on page 61 in Supplementary Fig. 1 of the MS-DIAL publication). Such differences in the spectra might cause removal by the

similarity search used in MS-DIAL. From our experience, it is not essential to exclude noisy spectra entirely from the annotation process, but it is sufficient to remove the noise within the spectra (see Supplementary Note 3 of the LDA 2 publication[10]). As can be seen in the figure of Supplementary Note 3, even in noisy spectra it is possibly to reliably detect the important fragments. The key to do this is to define relations between fragments, a feature that is completely missing in the MS-DIAL decision tree concept. These intensity relations are typically quite stable between platforms, e.g., relations between the produced LCB (or SPB) fragments. In contrast to the missing intensity relations, MS-DIAL uses intensity cutoffs in relation to the base peak. By comparing the intensities of the SPB (LCB) fragments and the NL_H2O fragments of the 'ideal' spectrum to the two experimental ones in the MS-DIAL and the LDA figure, it is obvious that these relations are subject to high variations between MS platforms and collision energies. Such intensity rules can be easily adapted by the LDA decision rule concept to match specific MS instruments, making it platform independent. This is the reason why we implemented a flexible solution that can be readily adjusted by the end user, and not a rigid database that is difficult to modify. Another important feature that is missing in the MS-DIAL concept are rules to differentiate between isobaric/isomeric species. In contrast, the LDA can differentiate between, e.g., di- and trihydroxylated LCBs, and spectra from protonated species and protonated species that lost water.

In summary, we assume that the lower performance of MS-DIAL compared with LDA rests on two limitations: i) MS-DIAL uses a rigid database for the decision tree fragments and intensity thresholds; this global concept ignores any platform specific differences, and provides no means for adaptation; ii) the absence of means to algorithmically differentiate between isobaric/isomeric species, which is particularly problematic when they produce similar fragmentation patterns.

Table S-1. Authentic lipid standards for control experiment.

No.	Lipid subclass	Lipid species	Lipid molecular species	Mix
1	Sphingoid base homologs and variants	SphBase d14:1	SphBase d14:1	2
2	Sphingoid base homologs and variants	SphBase d17:0	SphBase d17:0	1
3	Sphingoid base homologs and variants	SphBase d17:1	SphBase d17:1	1
4	Sphingoid base analogs	SphBase m18:0	SphBase m18:0	2
5	Sphinganines	SphBase d18:0	SphBase d18:0	2
6	4-Hydroxysphinganines (Phytosphingosines)	SphBase t18:0	SphBase t18:0	1
7	Sphingoid base analogs	SphBase m18:1	SphBase m18:1	1
8	Sphingoid base analogs	SphBase d18:2	SphBase d18:2	2
9	Sphingoid base homologs and variants	SphBase d20:0	SphBase d20:0	2
10	Sphingoid base homologs and variants	SphBase d20:1	SphBase d20:1	2
11	N-acylsphingosines (ceramides)	Cer d16:1	Cer d14:1/n2:0	1
12	N-acylsphinganines (dihydroceramides)	Cer d24:0	Cer d18:0/n6:0	2
13	N-acyl-4-hydroxysphinganines (phytoceramides)	Cer t26:0	Cer t18:0/n8:0	1
14	Other Sphingolipids	Cer m30:0	Cer m18:0/n12:0	1
15	N-acylsphinganines (dihydroceramides)	Cer t30:0	Cer d18:0/h12:0	2
16	Other Sphingolipids	Cer m30:1	Cer m18:1/n12:0	2
17	N-acylsphingosines (ceramides)	Cer d30:1	Cer d18:1/n12:0	1
18	N-acylsphingosines (ceramides)	Cer t30:1	Cer d18:1/h12:0	1
19	N-acylsphingosines (ceramides)	Cer d32:1	Cer d16:1/n16:0	1
20	N-acyl-4-hydroxysphinganines (phytoceramides)	Cer t34:0	Cer t18:0/n16:0	1
21	N-acylsphingosines (ceramides)	Cer d34:1	Cer d18:1/n16:0	2
22	N-acylsphingosines (ceramides)	Cer d34:2	Cer d18:2/n16:0	1
23	N-acylsphingosines (ceramides)	Cer d35:1	Cer d18:1/n17:0	2
24	N-acylsphinganines (dihydroceramides)	Cer d36:0	Cer d18:0/n18:0	1
25	N-acylsphinganines (dihydroceramides)	Cer t36:0	Cer d18:0/h18:0	1
26	N-acylsphingosines (ceramides)	Cer d36:1	Cer d18:1/n18:0	2
27	N-acylsphingosines (ceramides)	Cer d36:2	Cer d18:1/n18:1	1
28	N-acylsphingosines (ceramides)	Cer t36:2	Cer d18:1/h18:1	2
29	N-acylsphingosines (ceramides)	Cer d38:1	Cer d18:1/n20:0	2
30	N-acyl-4-hydroxysphinganines (phytoceramides)	Cer t42:0	Cer t18:0/n24:0	1
31	N-acyl-4-hydroxysphinganines (phytoceramides)	Cer q42:0	Cer t18:0/h24:0	1
32	Other Sphingolipids	Cer m42:1	Cer m18:0/n24:1	1
33	N-acylsphinganines (dihydroceramides)	Cer d42:1	Cer d18:0/n24:1	2
34	N-acylsphingosines (ceramides)	Cer t42:1	Cer d18:1/h24:0	2
35	N-acylsphingosines (ceramides)	Cer d43:1	Cer d18:1/n25:0	1
36	N-acylsphingosines (ceramides)	Cer t48:1	Cer d18:1/h30:0	2
37	Ceramide 1-phosphates	Cer1P d25:0	Cer1P d17:1/8:0	2
38	Ceramide 1-phosphates	Cer1P d30:1	Cer1P d18:1/n12:0	1
39	Ceramide 1-phosphates	Cer1P d34:0	Cer1P d18:1/n16:0	1
40	Ceramide 1-phosphates	Cer1P m34:1	Cer1P m18:1/n16:0	2
41	Ceramide 1-phosphates	Cer1P d42:1	Cer1P d18:1/n24:0	2
42	Ceramide phosphocholines (sphingomyelins)	SM d30:0	SM d18:0/n12:0	2

43	Ceramide phosphocholines (sphingomyelins)	SM d30:1	SM d18:1/n12:0	1
44	Ceramide phosphocholines (sphingomyelins)	SM d36:1	SM d18:1/n18:0	2
45	Ceramide phosphocholines (sphingomyelins)	SM d36:2	SM d18:1/n18:1	1
46	Ceramide phosphocholines (sphingomyelins)	SM d42:1	SM d18:1/n24:0	2
47	Ceramide phosphocholines (sphingomyelins)	SM d42:2	SM d18:1/n24:1	1
48	Lysosphingomyelins and lysoglycosphingolipids	LSM d17:1	LSM d17:1	2
49	Lysosphingomyelins and lysoglycosphingolipids	LSM d18:0	LSM d18:0	1
50	Lysosphingomyelins and lysoglycosphingolipids	LSM d18:1	LSM d18:1	2
51	Neutral glycosphingolipids – Simple Glc series	HexCer d24:2	HexCer d18:2/n6:0	2
52	Neutral glycosphingolipids – Simple Glc series	HexCer d26:1	HexCer d18:1/n8:0	1
53	Neutral glycosphingolipids – Simple Glc series	HexCer d30:1	HexCer d18:1/n12:0	1
54	Neutral glycosphingolipids – Simple Glc series	HexCer d34:0	HexCer d18:0/n16:0	1
55	Neutral glycosphingolipids – Simple Glc series	HexCer d36:1	HexCer d18:1/n18:0	1
56	Neutral glycosphingolipids – Simple Glc series	HexCer t36:1	HexCer d18:1/h18:0	1
57	Neutral glycosphingolipids – Simple Glc series	HexCer d36:2	HexCer d18:1/n18:1	2
58	Neutral glycosphingolipids – Simple Glc series	HexCer d42:2	HexCer d18:1/n24:1	2
59	Neutral glycosphingolipids – Simple Glc series	HexCer t44:0	HexCer t18:0/n26:0	2
60	Sphingoid base 1-phosphates	S1P d17:0	S1P d17:0	1
61	Sphingoid base 1-phosphates	S1P d17:1	S1P d17:1	1
62	Sphingoid base 1-phosphates	S1P d18:0	S1P d18:0	2

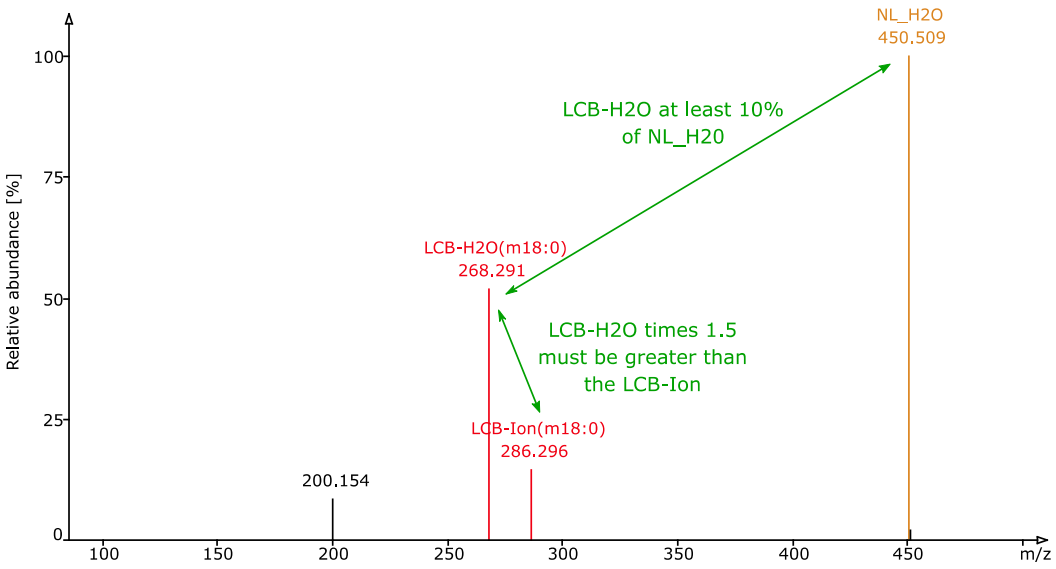
The authentic standards were prepared in two mixes to avoid overlaps. The last column indicates the assigned mix.

Table S-4. Novel lipid species.

No.	Lipid subclass	Lipid species	Lipid molecular species
1	N-acylsphingosines (ceramides)	Cer d38:2	Cer d20:2/n18:0
2	N-acylsphingosines (ceramides)	Cer d41:1	Cer d19:1/n22:0
3	Other Sphingolipids	Cer m43:0	-
4	N-acylsphingosines (ceramides)	Cer d43:2	Cer d18:1/n25:1
5	N-acylsphingosines (ceramides)	Cer d44:2	Cer d20:2/n24:0
6	Neutral glycosphingolipids – Simple Glc series	HexCer t41:0	HexCer d20:0/h21:0
7	Neutral glycosphingolipids – Simple Glc series	HexCer t41:1	HexCer d18:1/h23:0
8	Neutral glycosphingolipids – Simple Glc series	HexCer d41:2	HexCer d18:1/n23:1
9	Neutral glycosphingolipids – Simple Glc series	HexCer t41:2	HexCer d18:1/h23:1
10	”	”	HexCer d18:2/h23:0
11	Neutral glycosphingolipids – Simple Glc series	HexCer d42:2	HexCer d18:2/n24:0
12	Neutral glycosphingolipids – Simple Glc series	HexCer t42:2	HexCer d18:1/h24:1
13	Neutral glycosphingolipids – Simple Glc series	HexCer d42:3	HexCer d18:1/n24:2
14	Neutral glycosphingolipids – Simple Glc series	HexCer t42:3	HexCer d18:1/h24:2
15	”	”	HexCer d18:0/h24:3
16	Neutral glycosphingolipids – Simple Glc series	HexCer t43:1	HexCer d18:1/h25:0
17	Neutral glycosphingolipids – Simple Glc series	HexCer t43:2	HexCer d18:1/h25:1
18	Neutral glycosphingolipids – Simple Glc series	HexCer d43:3	-
19	Neutral glycosphingolipids – Simple Glc series	HexCer t44:2	HexCer d18:1/h26:1
20	Ceramide phosphocholines (sphingomyelins)	SM d33:2	SM d16:1/n17:1
21	Ceramide phosphocholines (sphingomyelins)	SM d35:1	SM d18:0/n17:1
22	Ceramide phosphocholines (sphingomyelins)	SM d35:2	SM d17:2/n18:0
23	Ceramide phosphocholines (sphingomyelins)	SM t36:1	-
24	Ceramide phosphocholines (sphingomyelins)	SM d41:1	SM d19:1/n22:0
25	Ceramide phosphocholines (sphingomyelins)	SM d41:3	SM d17:1/n24:2
26	Ceramide phosphocholines (sphingomyelins)	SM d43:2	SM d20:2/n23:0
27	Ceramide phosphocholines (sphingomyelins)	SM d43:2	SM d19:2/n24:0
28	Ceramide phosphocholines (sphingomyelins)	SM d43:3	SM d19:1/n24:2
29	Ceramide phosphocholines (sphingomyelins)	SM d43:3	SM d20:1/n23:2
30	Ceramide phosphocholines (sphingomyelins)	SM d44:2	SM d20:2/n24:0

Names of lipid species and lipid molecular species that are novel are in green color.

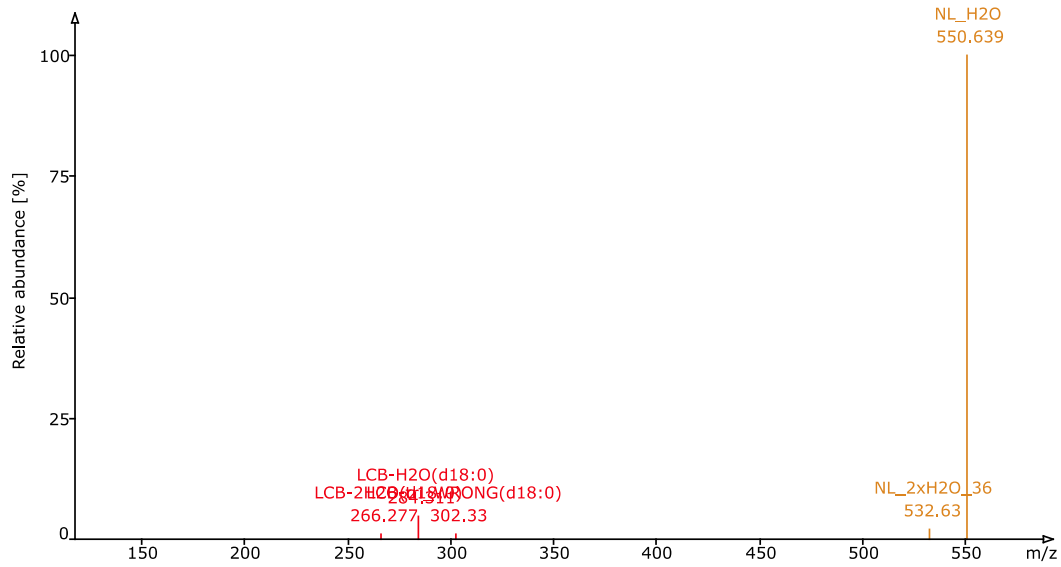
Figure S-1: Visual examples of decision rule sets based on authentic standards acquired on an Orbitrap Velos Pro, CID positive mode, 50%, and CID negative mode, -50%.



Decision rule set: Cer monohydroxylated LCB $[M+H]^+$ Cer m18:0/n12:0

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 468.4788; retention time: 19.63 min; file: Mix1_1).

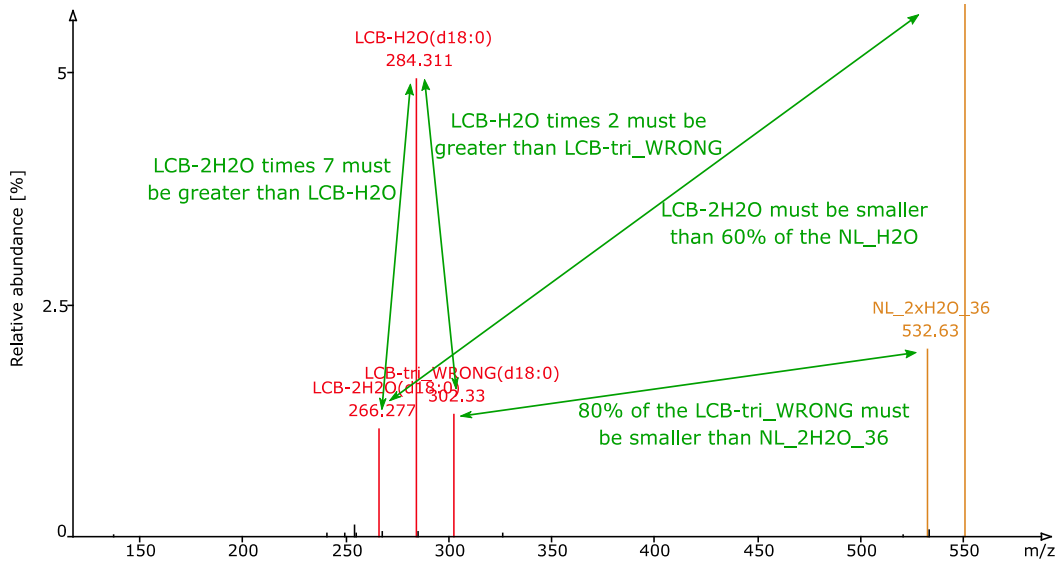
NL_H2O is typically the base peak for all protonated Cer species, and must be observed. For the annotation of a monohydroxylated LCB, the protonated LCB (LCB-Ion) and the protonated LCB that lost water must be found (LCB-H2O). The LCB-H2O is quite strong, and must have at least an intensity of 40% of the base peak, and the intensity of the LCB-H2O times 1.5 must be stronger than the one of the LCB-Ion. To exclude false positive dihydroxylated LCB identifications, a fragment LCB-H2O minus one carbon is defined, that must be smaller than 2% of the LCB-H2O.



Decision rule set: Cer dihydroxylated LCB $[M+H]^+$ Cer d18:0/n18:0

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 568.5665; retention time: 24.65 min; file: Mix1_1).

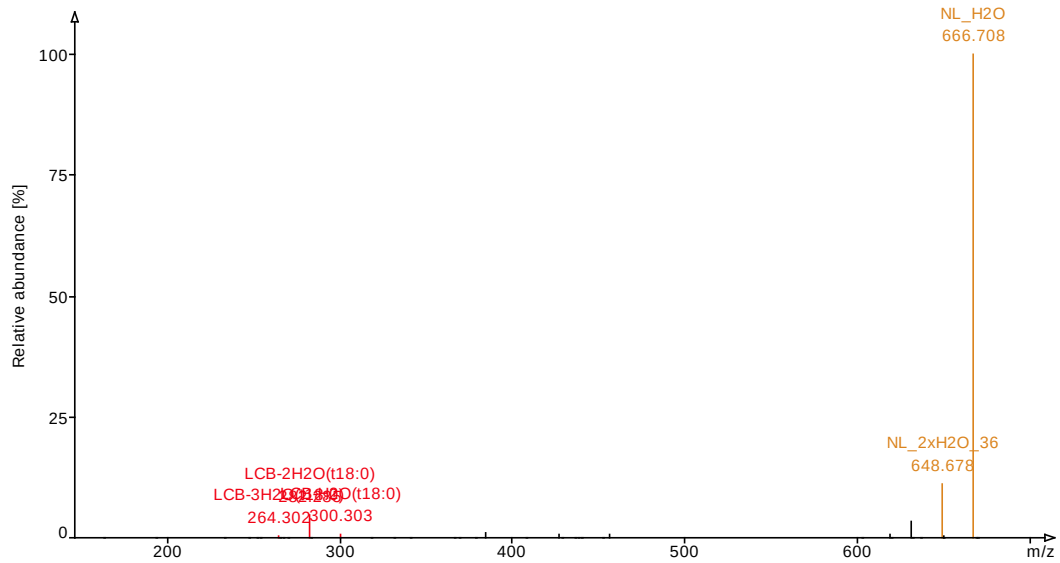
NL_H2O is typically the base peak for all protonated Cer species, and must be observed. Additionally, a fragment with two water losses of the precursor is observed quite often (NL_2xH2O_36). LCB fragments are rather small for protonated dihydroxylated Cer species, and will be explained in the following zoomed figure.



Decision rule set: Cer dihydroxylated LCB $[M+H]^+$ Cer d18:0/n18:0

MS2 spectrum of [M+H]+ zoomed (precursor m/z: 568.5665; retention time: 24.65 min; file: Mix1_1).

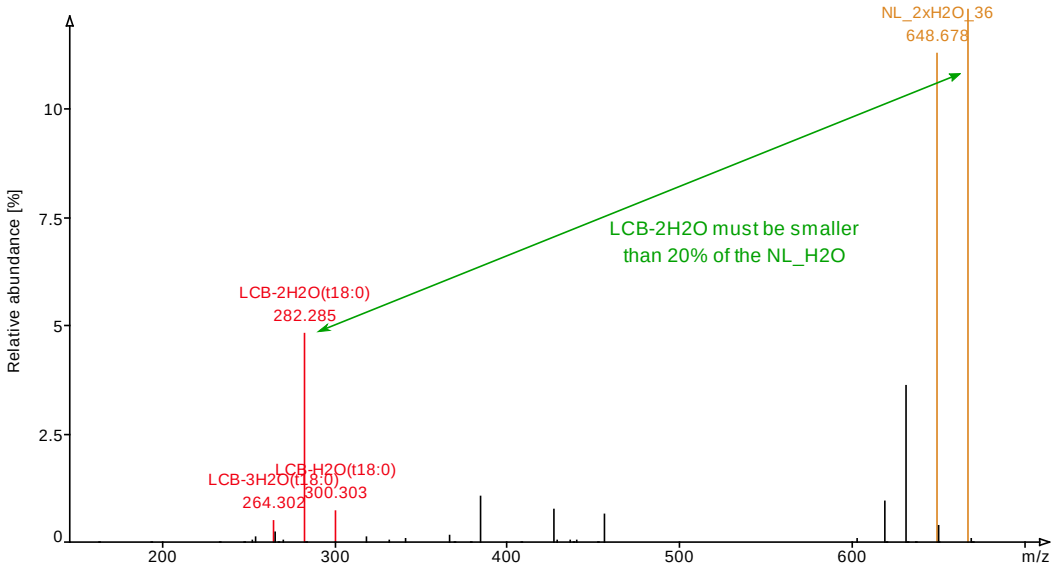
Dihydroxylated LCBs show the protonated LCB minus one water (LCB-H2O) and the LCB minus two water (LCB-2H2O), where the latter one must be observed. For saturated species, typically the LCB-H2O is stronger than the LCB-2H2O; for unsaturated species, it is the other way round. Since the intensity relation between LCB-H2O and LCB-2H2O can change, the intensity relation between both is formulated rather lenient as seven times the intensity of the LCB-2H2O must be greater than the LCB-H2O. Furthermore, the LCB-2H2O fragment must be less intense than 60% the NL_H2O. To remove false positive trihydroxylated identifications, the LCB-tri_WRONG fragment is defined which is essentially the protonated LCB. This fragment must be smaller than two times the LCB-H2O, and 80% of its intensity must be smaller than the NL_2xH2O fragment. Sometimes, an additional fragment is observable that has the mass of LCB-H2O minus one carbon. This fragment must be smaller than the sum of the intensities of LCB-H2O and LCB-2H2O.



Decision rule set: Cer trihydroxylated LCB $[M+H]^+$ Cer t18:0/h24:0

MS2 spectrum of [M+H]+ (precursor m/z: 684.6513; retention time: 27.88 min; file: Mix1_1).

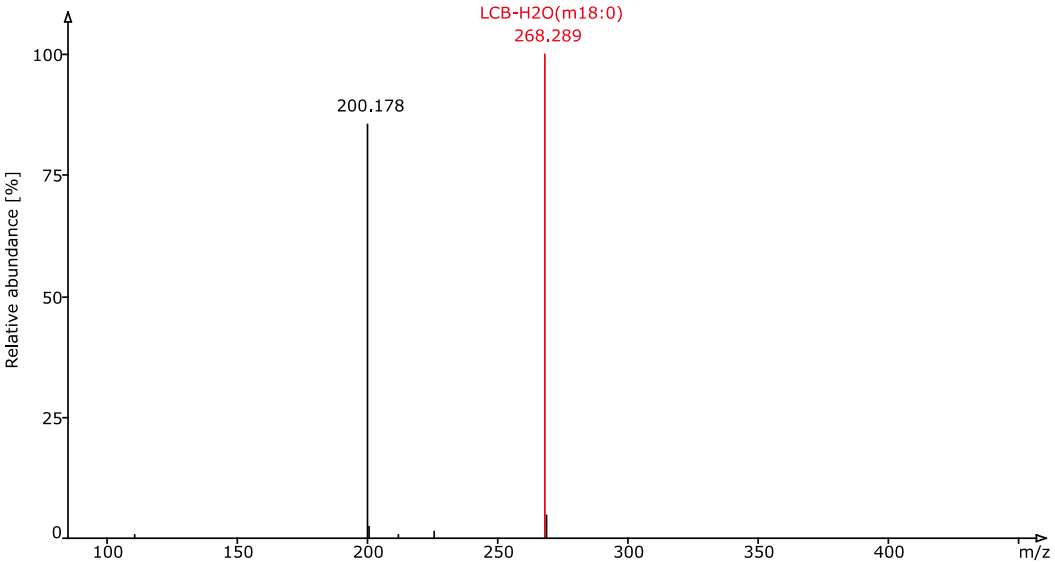
NL_H2O is typically the base peak for all protonated Cer species, and must be observed. Additionally, a fragment with two water losses of the precursor is observed quite often (NL_2xH2O_36), which is typically stronger than for dihydroxylated species. LCB fragments are rather small for protonated di-hydroxylated Cer species, and will be explained in the following zoomed figure.



Decision rule set: Cer trihydroxylated LCB $[M+H]^+$ Cer t18:0/h24:0

MS^2 spectrum of $[M+H]^+$ zoomed (precursor *m/z*: 684.6513; retention time: 27.88 min; file: Mix1_1).

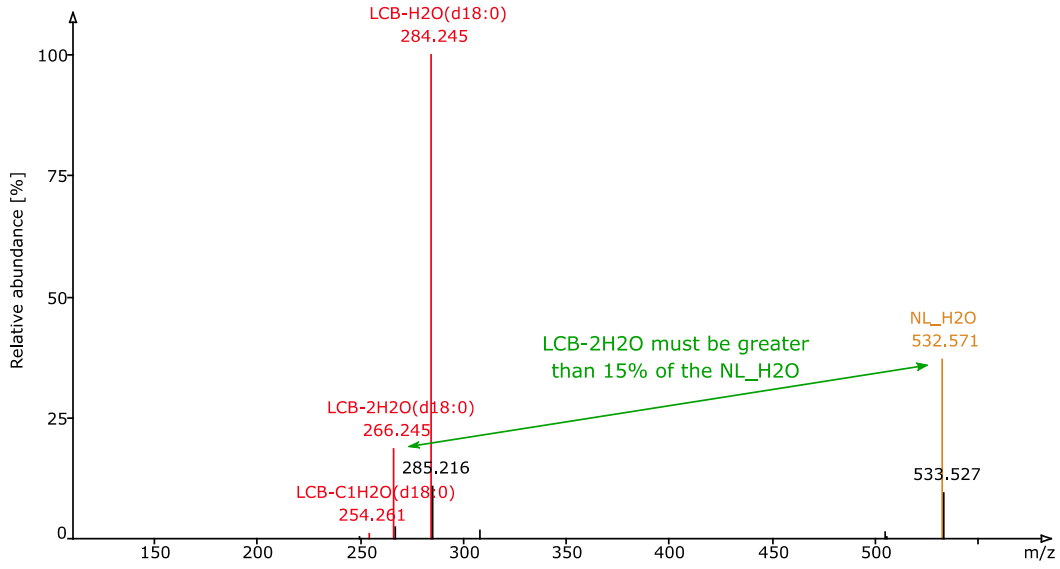
The intensities of the trihydroxylated LCB fragments are typically slightly less intense than the ones of the dihydroxylated forms. For a valid identification, two protonated LCB fragments have to be found, i.e. the one with two neutral losses of water (LCB-2H2O), and the one with three neutral losses of water (LCB-3H2O). Due to the required presence of the unambiguous LCB-3H2O, a single intensity rule is sufficient, i.e. that the LCB-2H2O is smaller than 20% of the NL_H2O. For trihydroxylated species, additionally fragments with one water loss (LCB-H2O), a loss of CH3O2 and a loss of CH5O3 might be observed.



Decision rule set: Cer monohydroxylated LCB $[M+H-H_2O]^+$ Cer m18:0/n12:0

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor m/z: 450.4686; retention time: 19.52 min; file: Mix1_1).

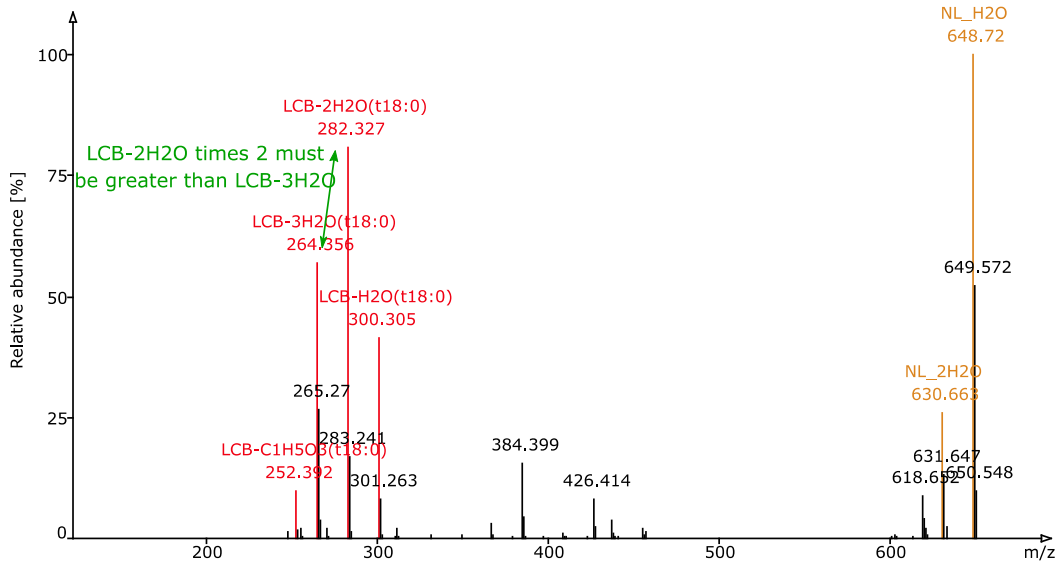
Spectra of precursors from protonated monohydroxylated Cer species that lost one water are primarily dominated by a fragment from the protonated LCB that lost one water (LCB-H2O), which is mandatory. For bigger molecules, neutral losses of the LCB might be observed. Since none of these fragments are reliably present, they are not used in any decision. The intensity rules are the same as for the protonated spectra (see monohydroxylated LCB [M+H-H2O]+) with the addition that LCB-H2O must be greater than 25% of the base peak, and the NL_H2O must be smaller than 5% of LCB-H2O.



Decision rule set: Cer dihydroxylated LCB $[M+H-H_2O]^+$ Cer d18:0/n18:0

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 550.5553; retention time: 24.75 min; file: Mix1_1).

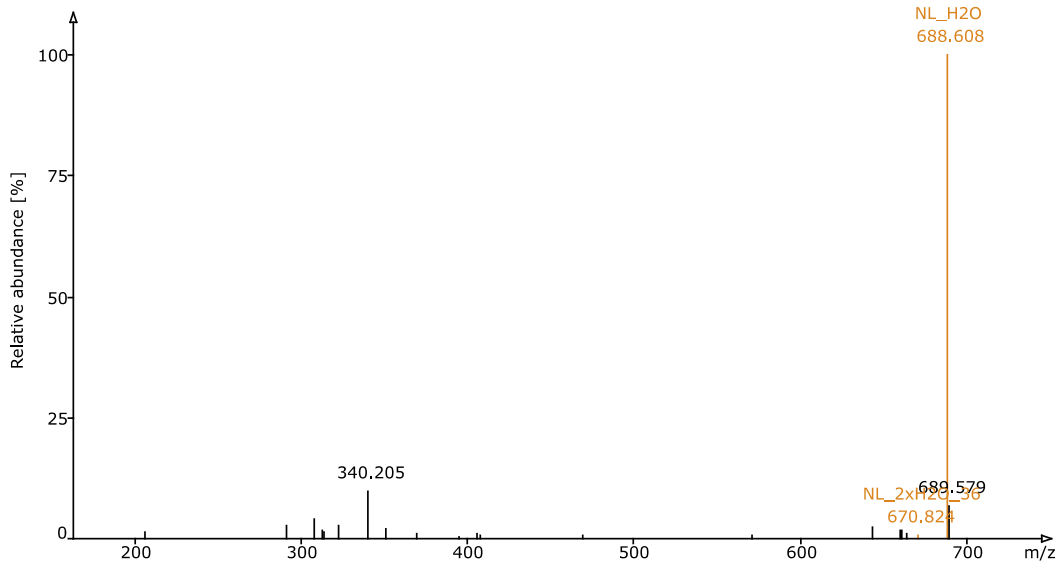
The major difference of dihydroxylated LCB $[M+H-H_2O]^+$ to dihydroxylated LCB $[M+H]^+$ spectra is that the LCB fragments are much stronger in comparison to the NL_H2O fragment. Thus, the intensity rules between the chain fragments are the same as for protonated species (see dihydroxylated LCB [M+H]+). The rules in respect to non-LCB fragments were adapted, i.e. the LCB-2H2O must be at least 15% of the base peak and 15% of the NL_H2O.



Decision rule set: Cer trihydroxylated LCB $[M+H-H_2O]^+$ Cer t18:0/h24:0

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 666.6399; retention time: 27.83 min; file: Mix1_1).

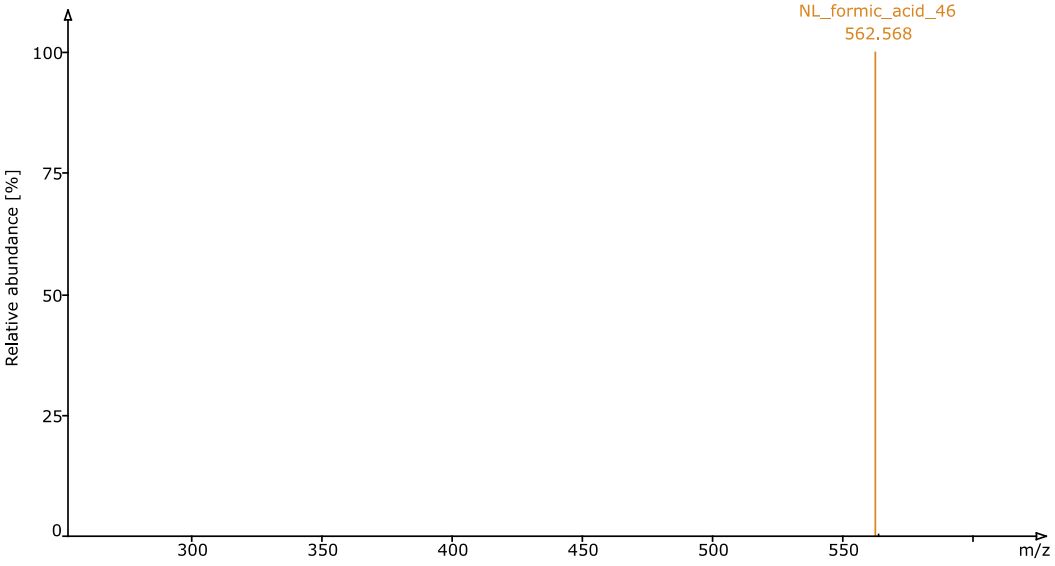
The observed fragments are the same as for the protonated version that did not lose water (see trihydroxylated LCB [M+H]+). The major difference to the protonated only version is that the LCB fragments are much stronger than the NL_H2O fragments. Since LCB-H2O, LCB-2H2O and LCB-3H2O are always present, all three of them were set to mandatory for a valid identification, making the rules highly specific. Due to the specificity of the LCB fragments, only a single lenient intensity rule is present, i.e. the intensity of the LCB-2H2O times two must be greater than the one of the LCB-3H2O.



Decision rule set: Cer $[M+Na]^+$ Cer t18:0/h24:0

MS^2 spectrum of $[M+Na]^+$ (precursor *m/z*: 706.6321; retention time: 27.94 min; file: Mix1_1).

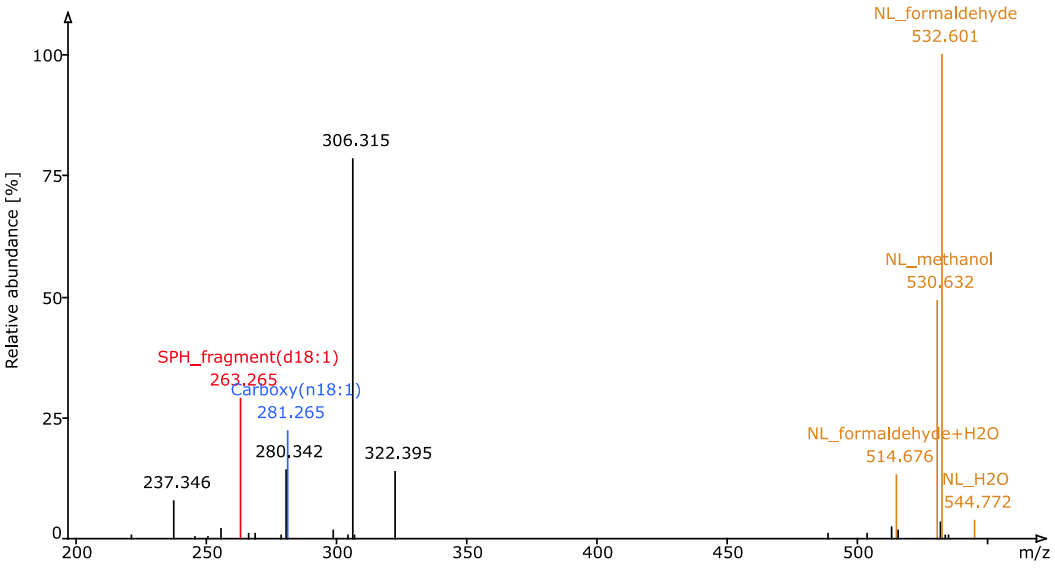
Irrespective of the LCB hydroxylation, all of the Cer $[M+Na]^+$ show a strong neutral loss of water from the precursor (NL_H2O; mandatory), and rarely a fragment where the precursor lost two water molecules. Chain information is not available from sodiated Cer species. Since this is highly unspecific, a rule was added declaring sodiated Cer species only valid if at the same retention time a protonated adduct or protonated adduct with a loss of water is detectable (MS^1 peak is sufficient).



Decision rule set: Cer $[M+HCOO]^-$ – MS^2 Cer d18:1/n18:1

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 608.5267; retention time: 22.71 min; file: Mix1_neg_1).

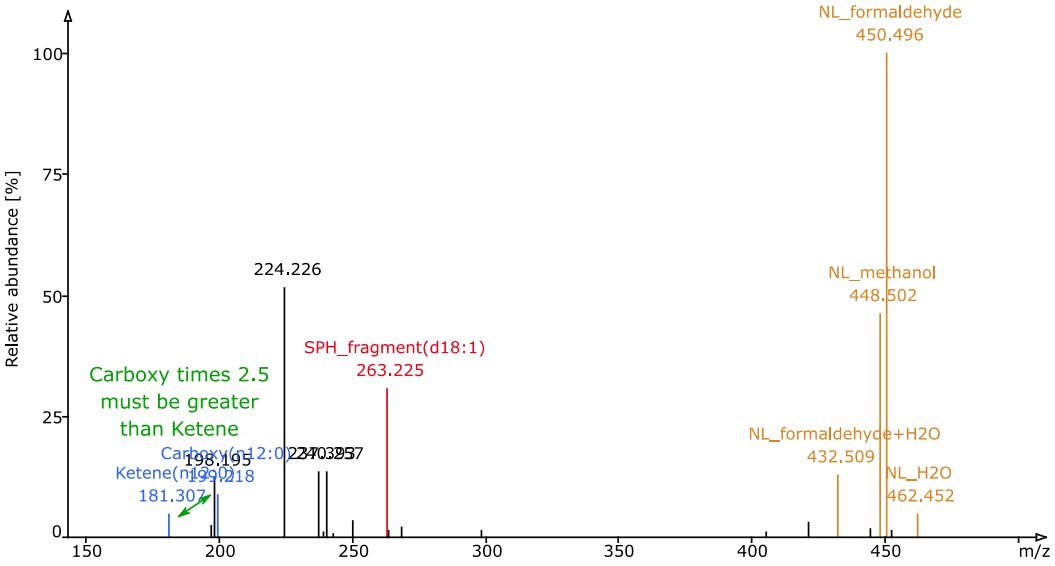
Irrespective of the LCB hydroxylation, all of the Cer $[M+HCOO]^-$ show a strong neutral loss of formic acid (NL_formic_acid_46). No chain information can be derived from MS^2 spectra on this instrument at CID -50%. Chain information is available from MS^3 spectra, except for monohydroxylated species. Additionally, fragment rules and intensity rules were added to differentiate between other isobaric/isomeric species. These fragments include the precursor, a neutral loss of water, a neutral loss of two water, and a neutral loss of sphingosine. These false positives are removed by intensity rules in relation to the NL_formic_acid_46.



Decision rule set: Cer [M+HCOO]$^-$ – MS^3 Cer d18:1/n18:1

MS^3 spectrum of [M+HCOO]$^-$ (precursor *m/z*: 562.5677; retention time: 22.72 min; file: Mix1_neg_1).

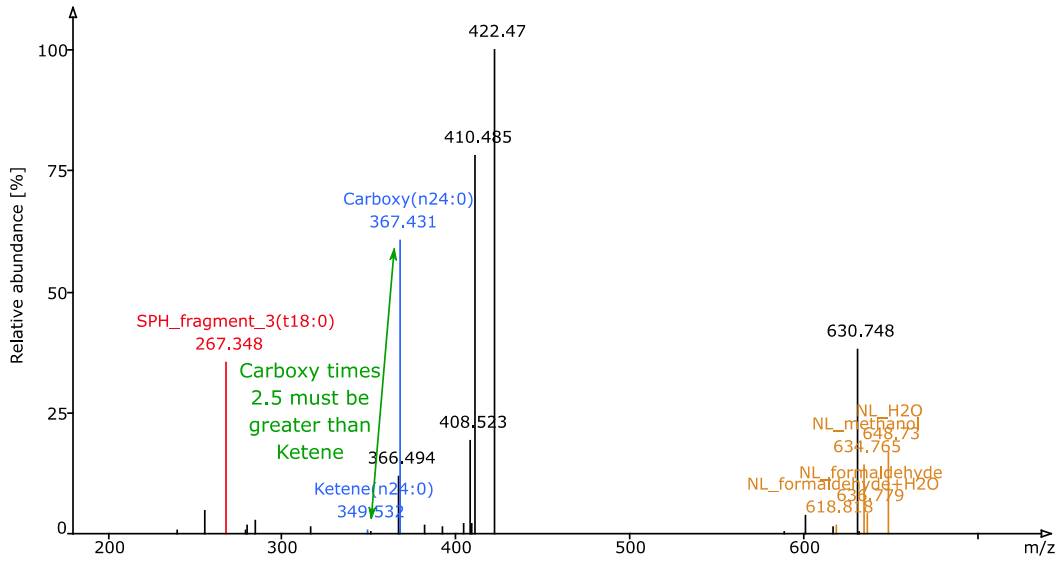
Subsequent to the loss of formic acid, additional class specific fragments are detectable in MS^3 spectra, i.e. neutral losses of water, formaldehyde, formaldehyde and water, and methanol. Di- and trihydroxylated species exhibit reliable chain fragments, but for monohydroxylated species, no reliable rules for the chain identification could be developed.



Decision rule set: Cer dihydroxylated LCB [M+HCOO]$^-$ Cer d18:1/n12:0

MS^3 spectrum of [M+HCOO]$^-$ (precursor *m/z*: 480.4544; retention time: 17.14 min; file: Mix1_neg_1).

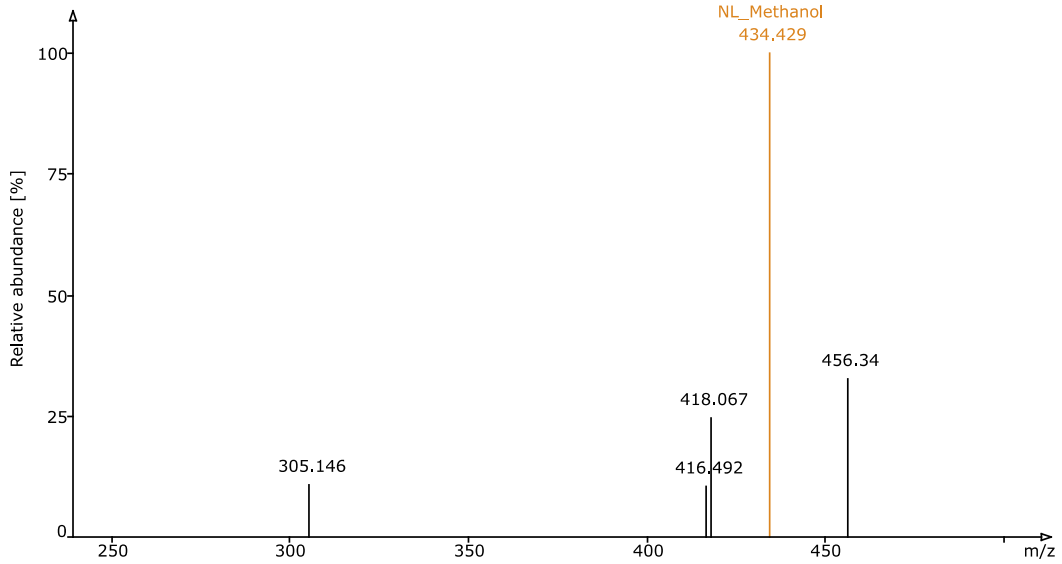
For class specific fragments, please see the previous two spectra. MS^3 spectra show a deprotonated LCB that loses ammonia and water (SPH_fragment) and the FA chain as deprotonated carboxylate (Carboxy) and as ketene (Ketene). The carboxy fragment is typically stronger, thus, there is an intensity rule that Carboxy times 2.5 is stronger than Ketene. All three of these fragments have to be detected for a valid chain identification. In some cases, Ketene cannot be detected, which results in the loss of chain information. However, this was necessary to prevent systematic false positive chain identifications due to isobaric species with different hydroxylation conformation. On a high resolution HCD cell such as the Q-Exactive, it is sufficient that solely the SPH_fragment and Carboxy fragment are obligatory. There is no need for the low abundant Ketene fragment.



Decision rule set: Cer trihydroxylated LCB $[M+HCOO]^-$ Cer t18:0/n24:0

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 666.7313; retention time: 28.78 min; file: Mix1_neg_2).

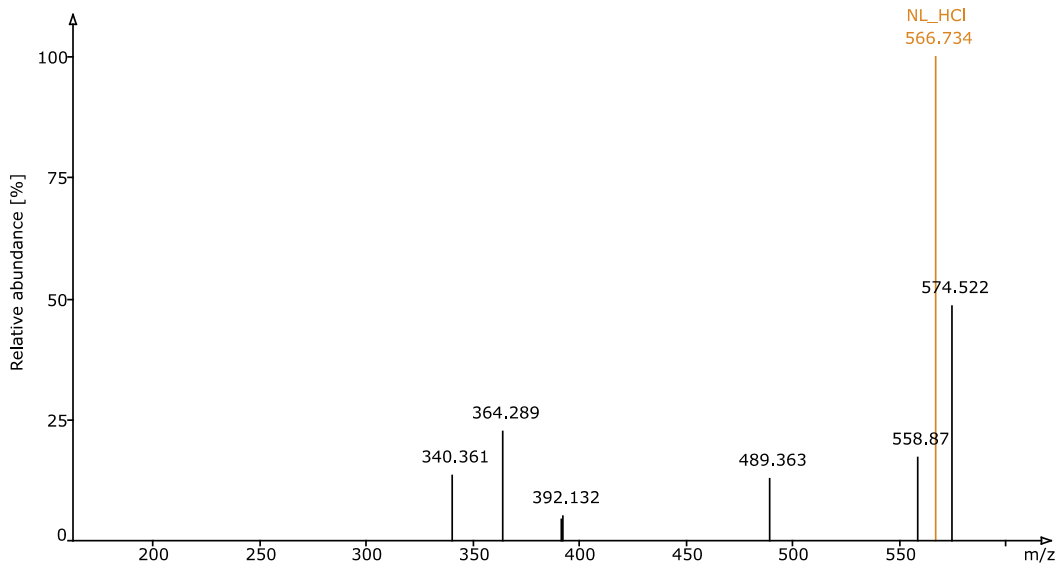
Class specific fragments are described before the previous spectrum. The detectable fragments are essentially the same as for dihydroxylated species, except for the SPH_fragment, which is now the deprotonated LCB that loses CH7NO. For trihydroxylated species, the Ketene fragment of the FA is typically quite small, which makes the obligatoriness questionable (many false negative chain identifications), but for CID spectra, this is the only way to prevent systematic false positive chain assignments (see description of dihydroxylated LCB $[M+HCOO]^-$). On a high resolution HCD cell such as the Q-Exactive, it not a problem to abandon the Ketene fragment.



Decision rule set: monohydroxylated Cer $[M+Cl]^-$ Cer m18:0/n12:0

MS^2 spectrum of $[M+Cl]^-$ (precursor *m/z*: 502.4414; retention time: 19.68 min; file: Mix1_neg_2).

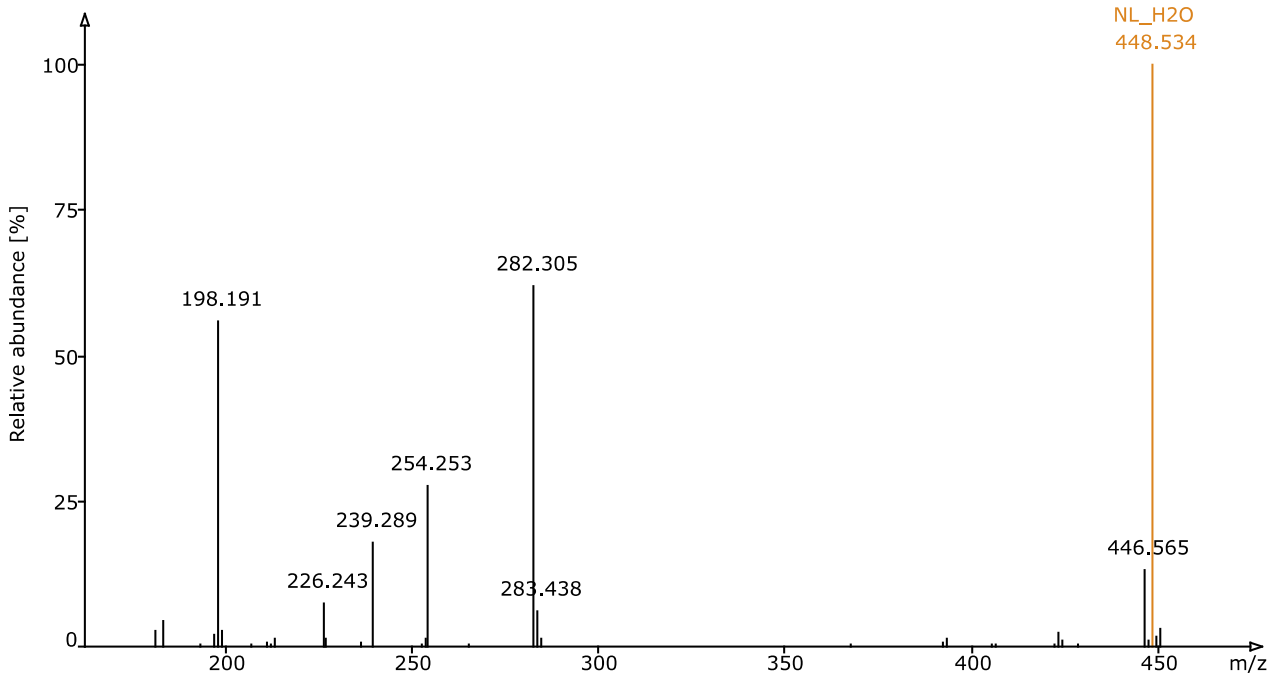
Monohydroxylated chlorated Cer species show primarily a loss of methanol. This neutral loss fragment is unspecific. Thus, a rule was added declaring chlorinated as valid only if at the same retention time a formated adduct is detectable (MS^1 peak is sufficient).



Decision rule set: di- and trihydroxylated Cer $[M+Cl]^-$ Cer d18:0/n18:0

MS^2 spectrum of $[M+Cl]^-$ (precursor *m/z*: 602.5312; retention time: 24.87 min; file: Mix1_neg_2).

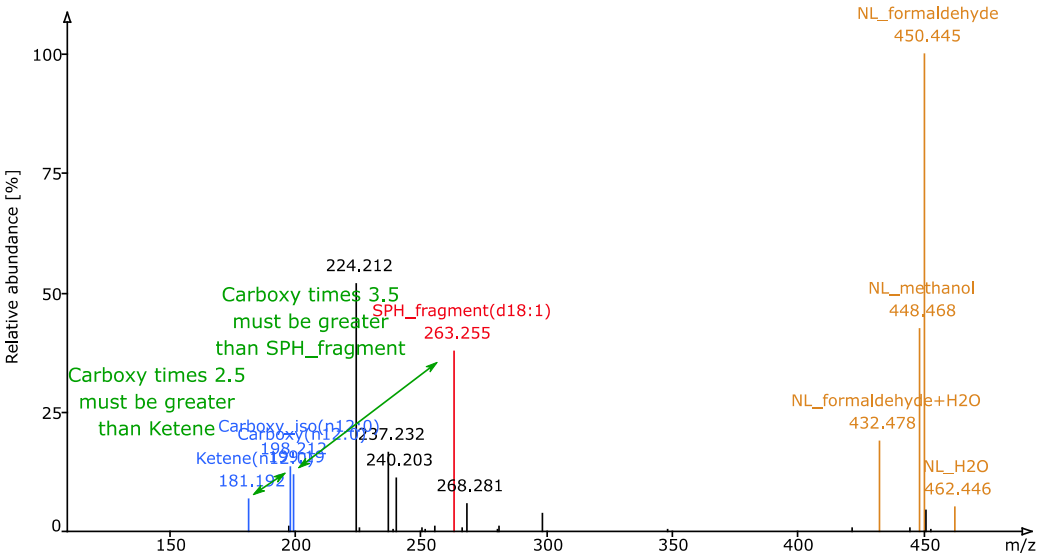
Chlorated Cer with more than one hydroxylation site show primarily a loss of HCl. This neutral loss fragment is unspecific. Thus, a rule was added declaring chlorinated as valid only if at the same retention time a formated adduct is detectable (MS^1 peak is sufficient).



Decision rule set: monohydroxylated Cer $[M-H]^-$Cer m18:0/n12:0

MS^2 spectrum of $[M-H]^-$ (precursor *m/z*: 466.4636; retention time: 19.76 min; file: Mix1_neg_1).

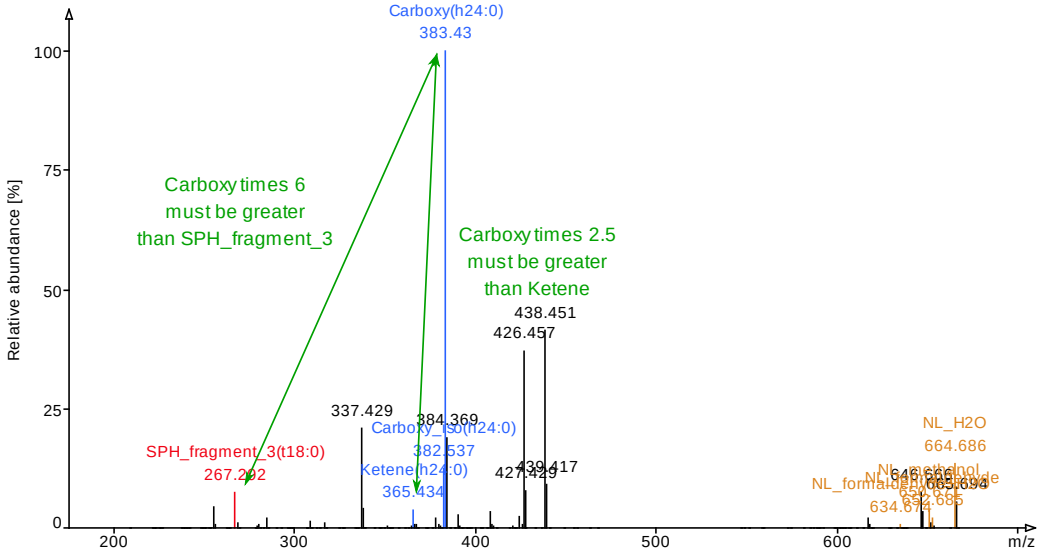
Deprotonated Cer adducts of monohydroxylated species do not ionize well, and the only reliably detectable species fragment is a neutral loss of water, which is highly unspecific. Chain identification is not possible for deprotonated monohydroxylated species.



Decision rule set: Cer dihydroxylated LCB [M-H]$^-$ Cer d18:1/n12:0

MS^2 spectrum of [M-H]$^-$ (precursor *m/z*: 480.4430; retention time: 17.23 min; file: Mix1_neg_1).

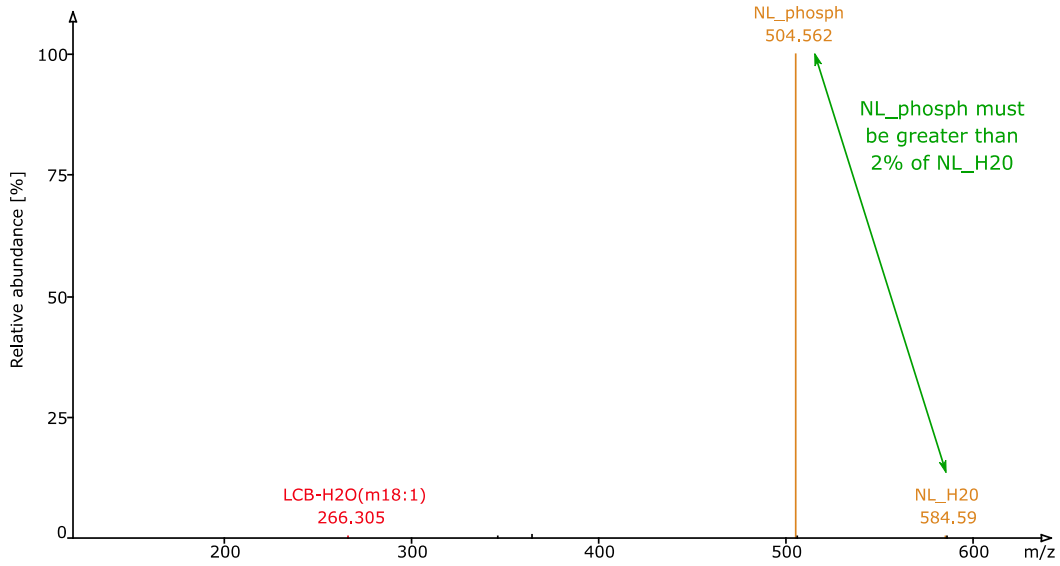
The fragmentation pattern of deprotonated dihydroxylated Cer species is similar to the MS^3 spectra of formate adducts of dihydroxylated Cer species (see Cer dihydroxylated LCB [M+HCOO]$^-$). The class specific fragments are neutral losses of formaldehyde, formaldehyde and water, and methanol, and an unspecific loss of water may be observed. For class verification, any of the specific fragments must be found. The sum of the intensities of those specific fragments is used in various intensity rules. This sum must exceed 90% of the precursor. In order to exclude false positive formate adducts, the neutral loss of formic acid must be smaller than 30% of this sum. This sum must exceed 50%, 15% and 5% of the base peak, for species containing two, three and four hydroxylation sites, respectively. For chain identification, deprotonated LCB that loses ammonia and water (SPH_fragment) and the FA chain as deprotonated carboxylate (Carboxy) and as ketene (Ketene) can be found. The former two are mandatory. There are two intensity rules, i.e. the carboxy fragment times 2.5 must be greater than the Ketene fragment, and the same fragment times 3.5 must be greater than the SPH_fragment. Additionally, an Carboxy_iso fragment is defined to exclude false positive chains. Since this mass would be the same as the plus one isotope of the Carboxy fragment, we called this indicator for false positives 'Carboxy_iso'. The Carboxy fragment times 1.2 must be greater than the Carboxy_iso fragment.



Decision rule set: Cer trihydroxylated LCB [M-H]$^-$ Cer t18:0/h24:0

MS^2 spectrum of [M-H]$^-$ (precursor *m/z*: 682.6363; retention time: 28.12 min; file: Mix1_neg_1).

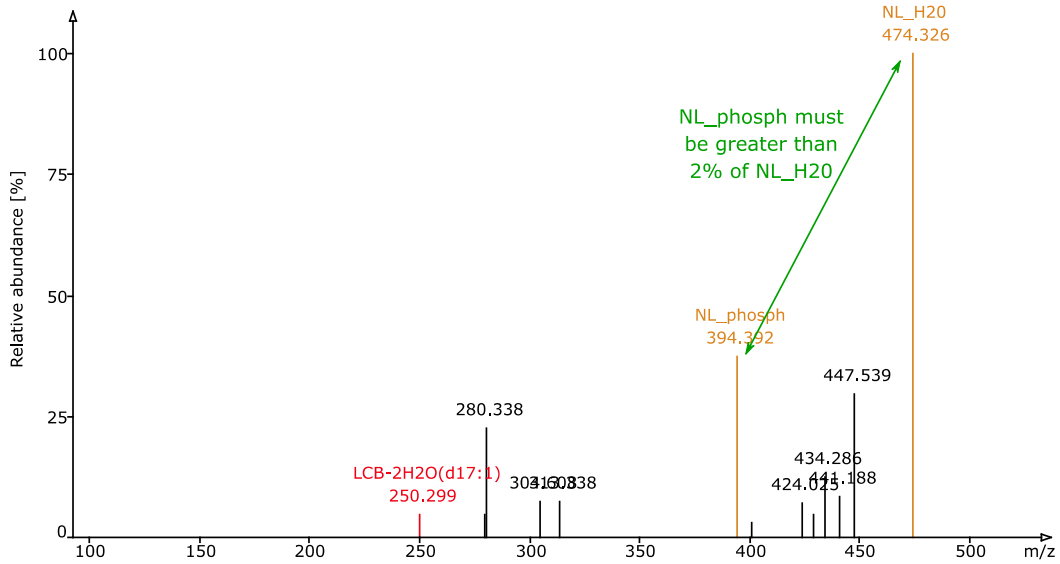
The fragmentation rules for class specific fragments are described in the previous figure (see Cer dihydroxylated LCB $[M-H]^-$). The detectable chain fragments are essentially the same as for dihydroxylated species, except for the SPH_fragment, which is now the deprotonated LCB that loses CH7NO (SPH_fragment_3). For this fragment, the intensity rule is that the Carboxy fragment times six is higher than the SPH_fragment_3.



Decision rule set: Cer1P monohydroxylated LCB $[M+H]^+$ Cer1P m18:1/n16:0

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 602.4909; retention time: 20.28 min; file: Mix2_1).

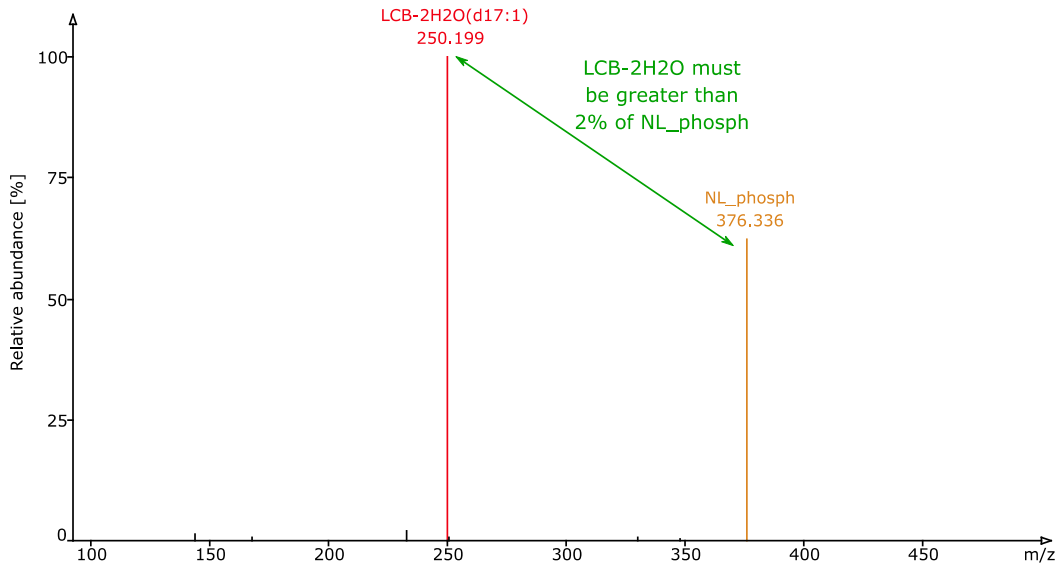
Class specific fragments are the neutral loss of phosphate (NL_phosph) and water (NL_H2O). NL_phosph must be greater than 2% of the NL_H2O. The sum of both intensities must be greater than 40% of the base peak, and greater than a potentially overlapping neutral loss (141) of a PE head group. Furthermore, the NL_phosph must be greater than 2% of the base peak. The fragment rules are not very specific, thus, a rule was added accepting protonated adducts only if at the same retention time a protonated adduct that lost water or a sodiated adduct is detectable (MS^1 peak is sufficient). For chain annotation, only a single fragment is detectable, i.e. the protonoated LCB that lost water (LCB-H2O). This fragment must be smaller than 10% of the base peak.



Decision rule set: Cer1P dihydroxylated LCB $[M+H]^+$ Cer1P d17:1/n8:0

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 492.3450; retention time: 6.96 min; file: Mix2_1).

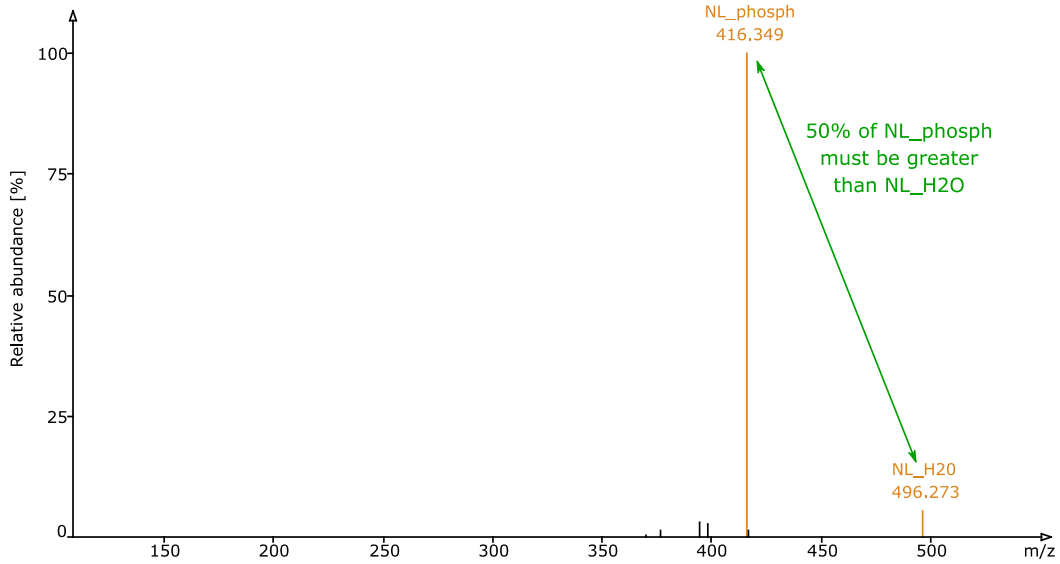
Class specific fragments are the same as for monohydroxylated species. For chain annotation, only a single fragment is detectable, i.e,. the protonoated LCB that lost two water (LCB-2H2O). This fragment must be smaller than 10% of the base peak.



Decision rule set: Cer1P dihydroxylated LCB $[M+H-H_2O]^+$ Cer1P d17:1/n8:0

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 474.3344; retention time: 6.30 min; file: Mix2_1).

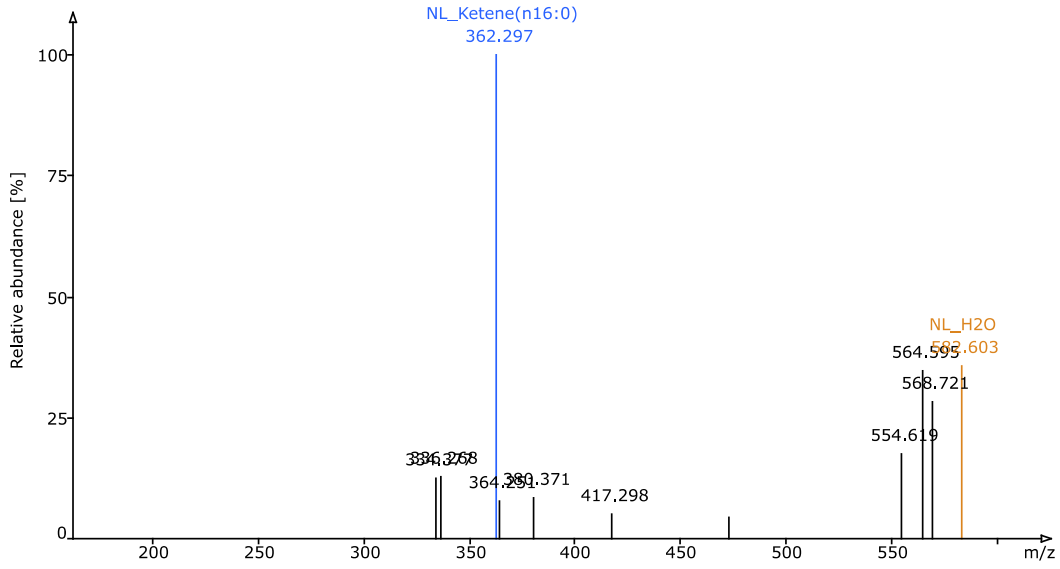
Class specific fragment is the neutral loss of phosphate (NL_phosph). The intensity of this fragment must be greater than 30% of the base peak, and the intensity times two must be greater than a potentially overlapping neutral loss (141) of a PE head group. These rules are not very specific, thus, a rule was added accepting protonated adducts only if at the same retention time a protonated adduct that lost water or a sodiated adduct is detectable (MS^1 peak is sufficient). For chain annotation, only a single fragment is detectable, i.e., the protonoated LCB that lost two water (LCB-2H2O). This fragment must be greater than 2% of the base peak and the NL_posph.



Decision rule set: Cer1P dihydroxylated LCB $[M+Na]^+$ Cer1P d17:1/n8:0

MS^2 spectrum of $[M+Na]^+$ (precursor *m/z*: 514.3266; retention time: 6.36 min; file: Mix2_1).

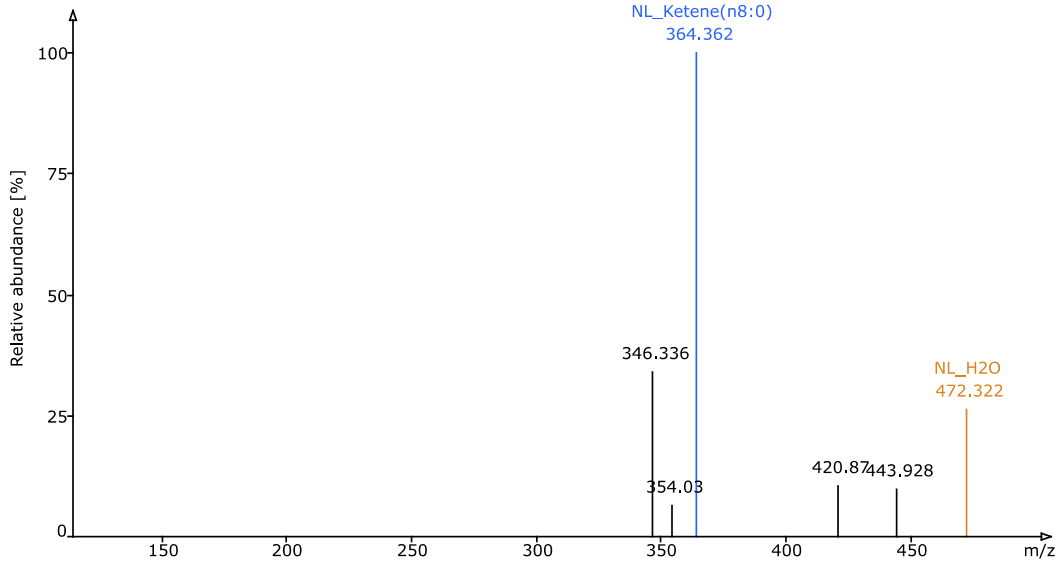
Class specific fragments are the neutral loss of phosphate (NL_phosph) and water (NL_H2O). The former one is mandatory. 50% of the intensity of NL_phosph must be greater than NL_H2O, and the intensity of NL_posph must be greater than 30% of the base peak. Chain annotation is not possible for sodiated adducts.



Decision rule set: Cer1P monohydroxylated LCB $[M-H]^-$ Cer1P m18:1/n16:0

MS^2 spectrum of $[M-H]^-$ (precursor *m/z*: 600.4779; retention time: 20.73 min; file: Mix2_neg_1).

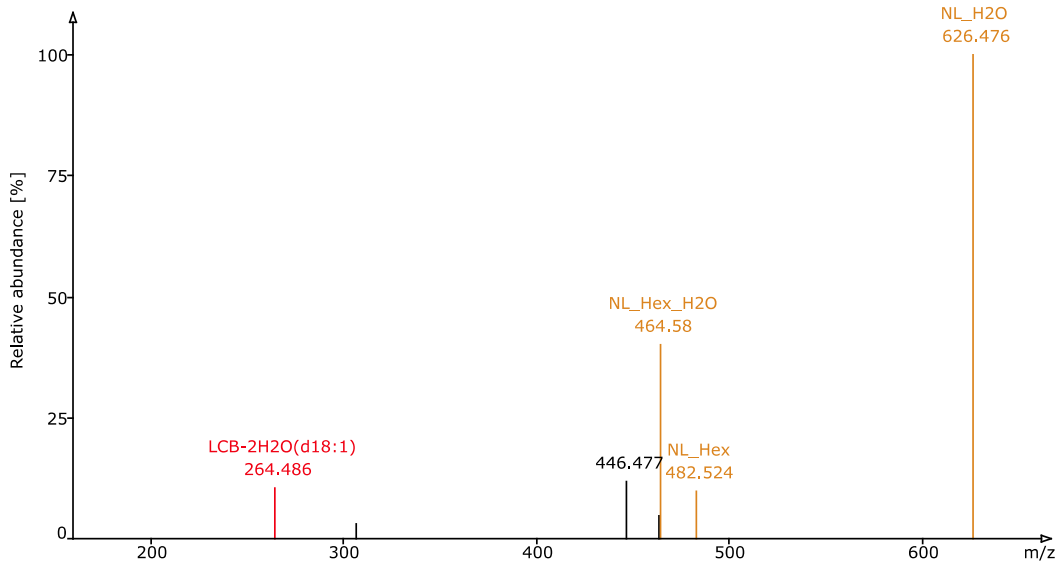
The primary fragment of deprotonated monohydroxylated Cer1P is the neutral loss of the FA as ketene (NL_Ketene). Sometimes, a neutral loss of water from the precursor can be observed (NL_H2O).



Decision rule set: Cer1P dihydroxylated LCB $[M-H]^-$ Cer1P d17:1/n8:0

MS^2 spectrum of $[M-H]^-$ (precursor *m/z*: 490.3319; retention time: 6.70 min; file: Mix2_neg_1).

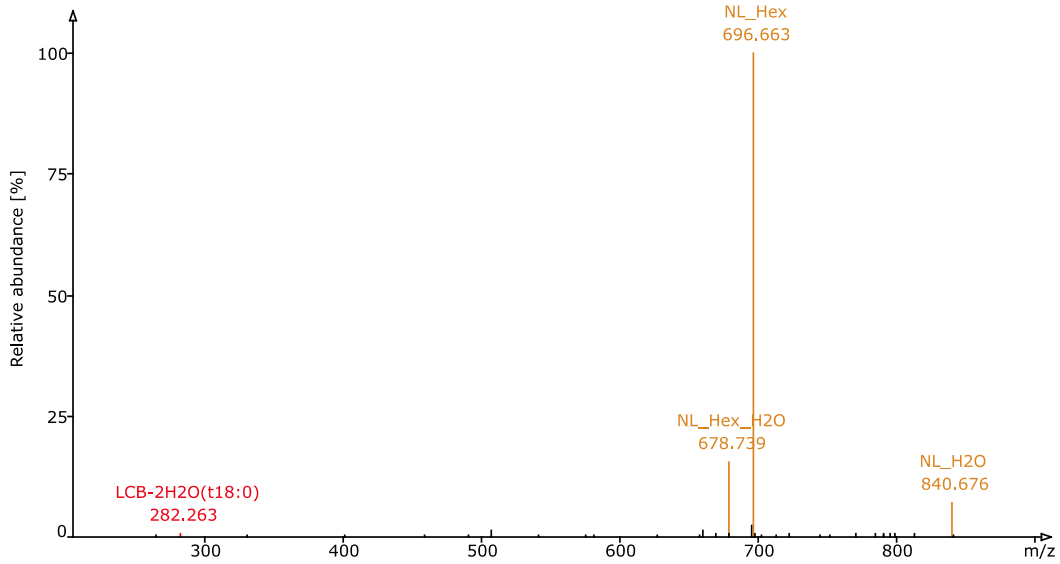
The fragmentation pattern of dihydroxylated Cer1P is essentially the same as for monohydroxylated species.



Decision rule set: HexCer dihydroxylated LCB $[M+H]^+$ HexCer d18:1/n12:0

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 644.5105; retention time: 14.63 min; file: Mix1_1).

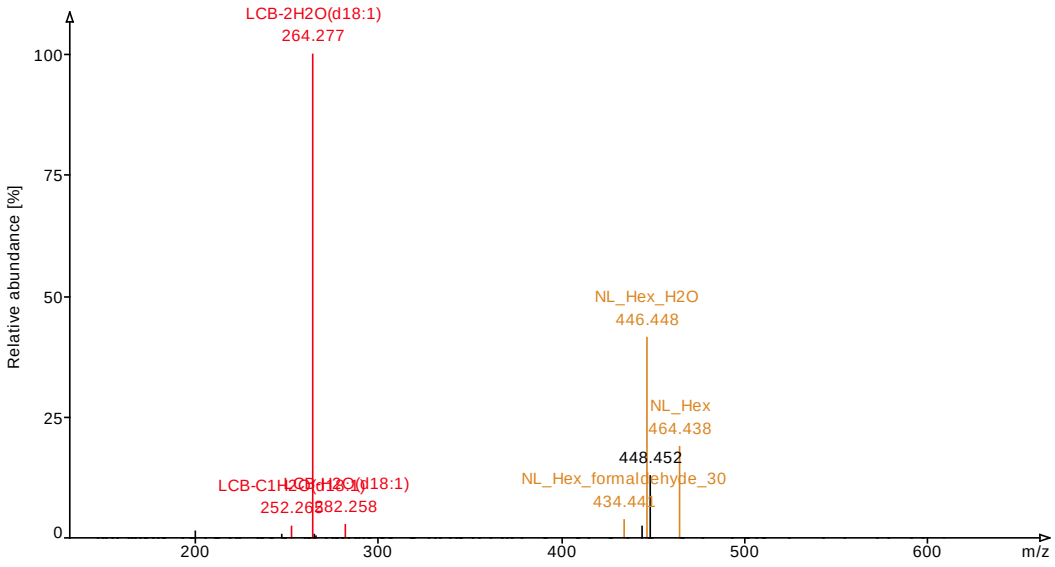
Protonated dihydroxylated HexCer show a neutral loss of water (NL_H2O), which is mandatory. Furthermore, three additional fragments might be observed, i.e. a neutral loss of the hexosyl group (NL_Hex), a neutral loss of the hexosyl group plus water (NL_Hex_H2O), and the neutral loss of C6H6O1 (NL_Prop). For accepting a spectrum as HexCer, one of the three specific fragments have to be found in addition to the unspecific NL_H2O fragment. The intensity of the three fragments changes, and they are hardly ever observed in the same spectrum. For chain annotation, the protonated LCB minus two water molecules (LCB-2H2O) must be observed.



Decision rule set: HexCer trihydroxylated LCB $[M+H]^+$ HexCer t18:0/n26:0

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 858.7382; retention time: 28.75 min; file: Mix2_1).

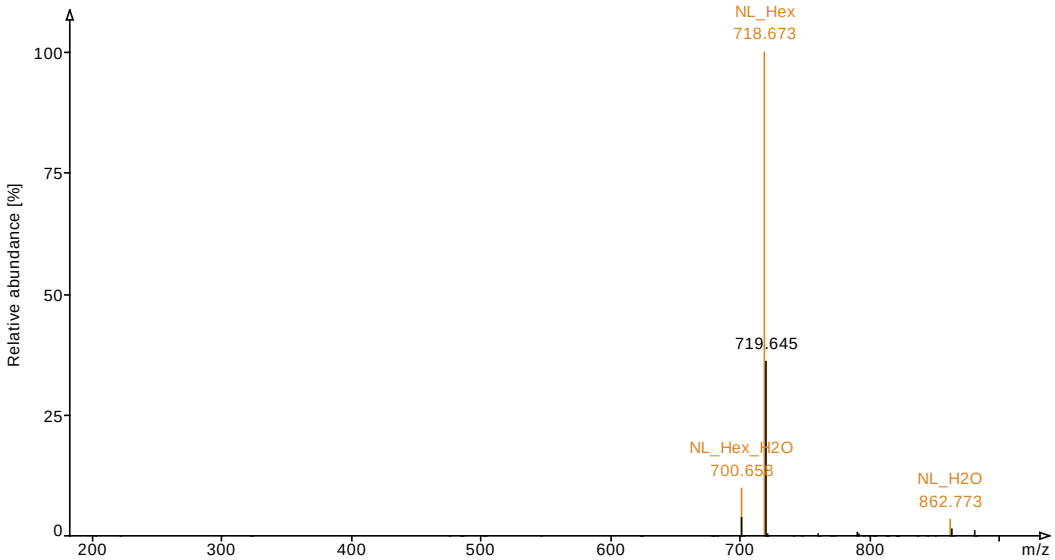
Class specific fragmentation and intensity rules are the same as for protonated dihydroxylated HexCer (see HexCer dihydroxylated LCB $[M+H]^+$). The observed chain fragment is the same too (LCB-2H2O), however, the intensity is much lower. To avoid misidentification of a dihydroxylated LCB, two intensity rules have been added, i.e., LCB-2H2O must be smaller than 5% of the base peak, and LCB-2H2O must be smaller than 3% of the sum of the intensities from NL_Hex and NL_Hex_H2O.



Decision rule set: HexCer dihydroxylated LCB $[M+H-H_2O]^+$ HexCer d18:1/n12:0

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 626.5002; retention time: 14.66 min; file: Mix1_1).

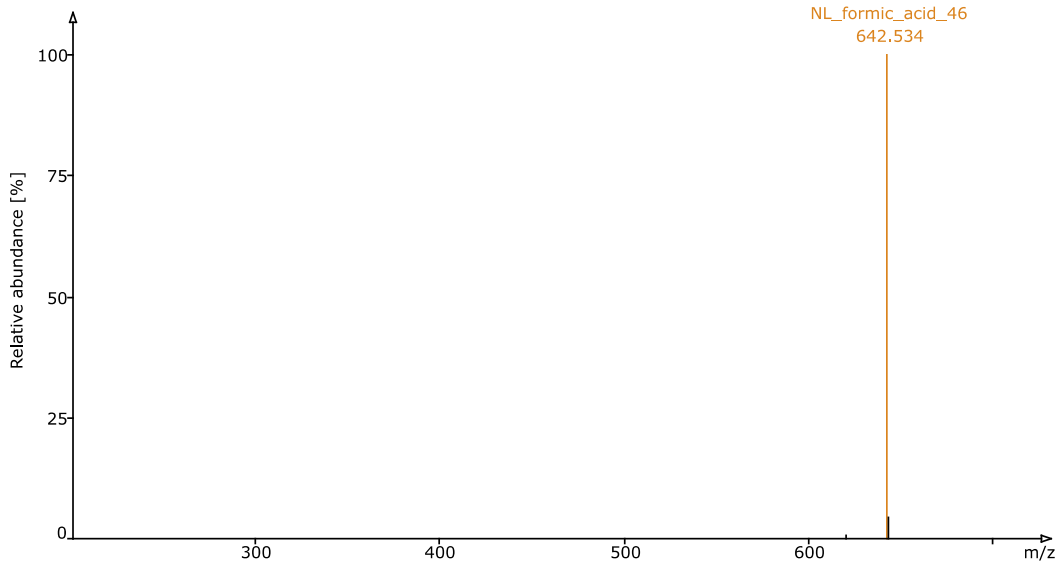
The major class specific fragment is the neutral loss of the hexosyl group plus water (NL_Hex_H2O), which is mandatory. This fragment is often accompanied by a neutral loss of the hexosyl group alone (NL_Hex). Furthermore, the NL_Hex_H2O may further lose formaldehyde (NL_Hex_formaldehyde_30). The sum of the intensities from NL_Hex_H2O and NL_Hex is used in intensity rules, i.e., this sum must exceed 10% of the base peak and, it must be bigger than 5% of a neutral loss of water fragment to distinguish it from other isobaric/isomeric classes. The fragmentation pattern originating from the LCB looks essentially the same as for Cer with dihydroxylated LCB $[M+H-H2O]^+$. Consequently, almost the same chain rules as for protonated Cer with water loss (see 'Cer monohydroxylated LCB $[M+H-H_2O]^+$, 'Cer dihydroxylated LCB $[M+H-H_2O]^+$ and 'Cer dihydroxylated LCB $[M+H-H_2O]^{+}$') were used for HexCer that lost water. One observed phenomenon is that protonated HexCer with trihydroxylated LCB and loss of water hardly ionize, and thus are not selected for MS^2.



Decision rule set: HexCer $[M+Na]^+$ HexCer t18:0/n26:0

MS^2 spectrum of $[M+Na]^+$ (precursor *m/z*: 880.7212; retention time: 26.99 min; file: Mix2_1).

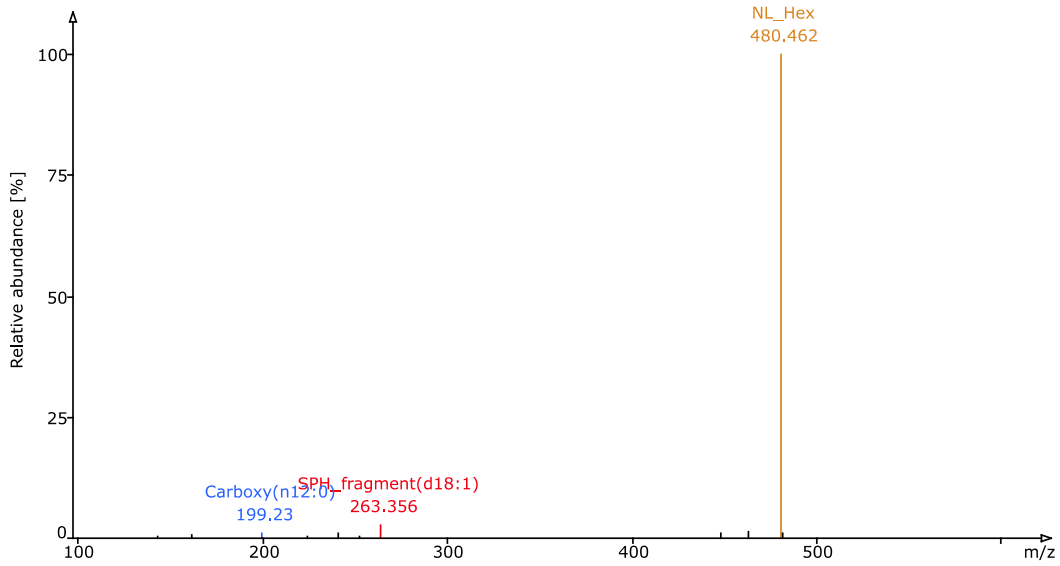
The mandatory class specific fragments are neutral loss of the hexosyl group (NL_Hex), and neutral loss of the hexosyl group plus water (NL_Hex_H2O). A neutral loss of water from the precursor might be observed (NL_H2O). The sum of intensities from NL_Hex and NL_Hex_H2O is used in intensity rules. To avoid false positive identifications, this sum must be greater than a neutral loss of an ethanolamine (NL_Ethanolamine_43_WRONG), and it must be greater than 40% of the base peak.



Decision rule set: HexCer [M+HCOO]$^-$ – MS^2 HexCer d18:1/n12:0

MS^2 spectrum of [M+HCOO]$^-$ (precursor *m/z*: 688.5029; retention time: 14.73 min; file: Mix1_neg_3).

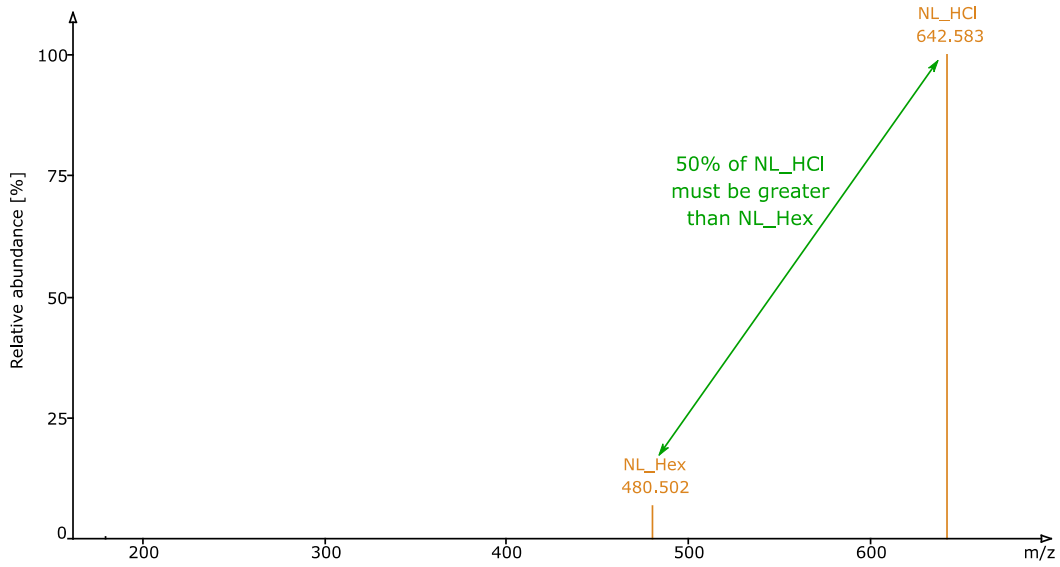
Irrespective of the LCB hydroxylation, as for Cer [M+HCOO]$^-$, all of the HexCer [M+HCOO]$^-$ show a strong neutral loss of formic acid (NL_formic_acid_46), which is mandatory. For trihydroxylated species, a neutral loss of 60 might be observed (NL_60). The sum of the intensities of both fragments must exceed 25% of the base peak, and the NL_formic_acid_46 must be greater than 10% of the intensity of the NL_60 fragment. No chain information can be derived from MS^2 spectra on this instrument at CID -50%. Chain information is available from MS^3 spectra.



Decision rule set: HexCer [M+HCOO]$^-$ – MS^3 HexCer d18:1/n12:0

MS^3 spectrum of [M+HCOO]$^-$ (precursor *m/z*: 642.5344; retention time: 14.74 min; file: Mix1_neg_3).

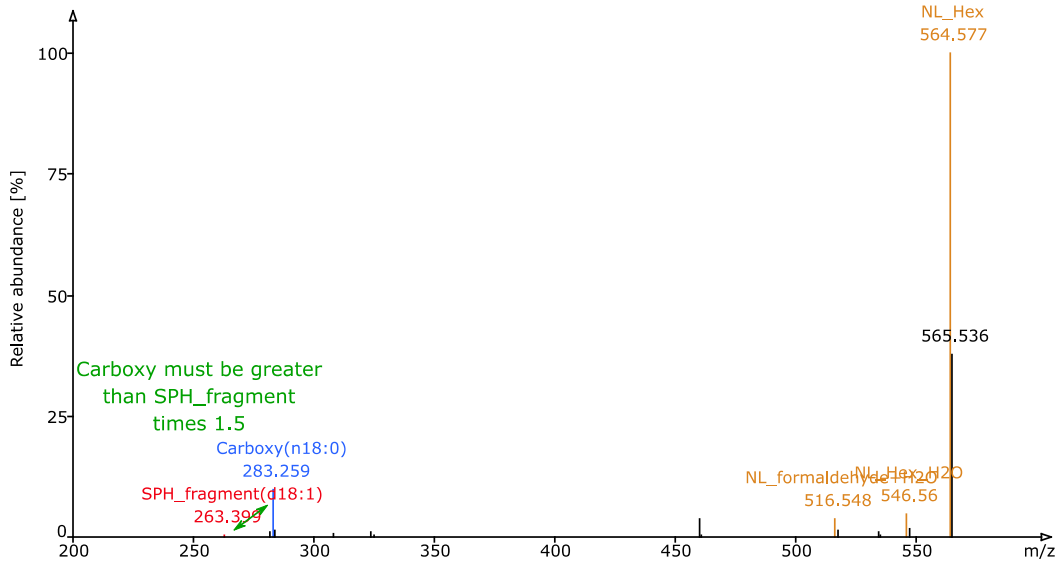
Subsequent to the loss of formic acid, a neutral loss of the hexosyl group (NL_Hex) can be detected in MS^3 spectra. Fragments revealing chain information are of low abundance, and are rarely observed. For chain annotation, the FA chain as deprotonated carboxylate (Carboxy) must be detected. For dihydroxylated LCB and trihydroxylated LCB, the LCB that loses ammonia and water (SPH_fragment) and CH7NO (SPH_fragment_3) are detectable, respectively.



Decision rule set: HexCer $[M+Cl]^-$ HexCer d18:1/n12:0

MS^2 spectrum of $[M+Cl]^-$ (precursor *m/z*: 678.4748; retention time: 14.77 min; file: Mix1_neg_3).

Irrespective of the LCB hydroxylation, the HexCer $[M+Cl]^-$ have the same fragmentation pattern. The two detectable mandatory fragments are the neutral losses of HCl (NL_HCl) and the hexosyl group (NL_Hex). For an identification, 50% of the intensity of the NL_HCl must still be stronger than the intensity of NL_Hex. To remove FPs from another isobaric/isomeric species, the neutral loss of H3Cl (NL_H3Cl – not possible, but the sum formula of an isobaric precursor is different, and it can be assumed that it is not a Cl^- adduct) must be smaller than the NL_HCl. Chain annotation is not possible for chlorinated adducts.

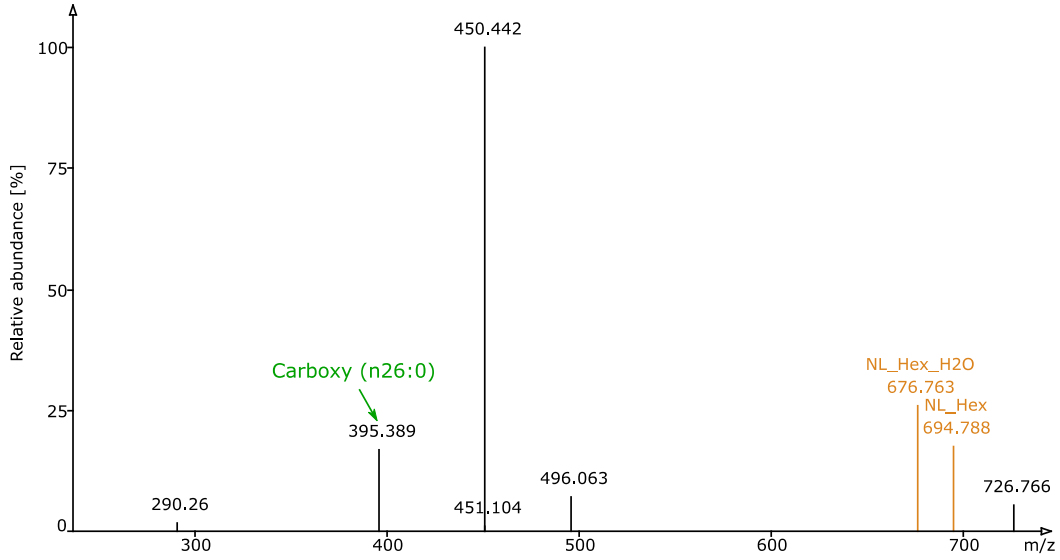


Decision rule set: HexCer dihydroxylated LCB $[M-H]^-$ HexCer d18:1/n18:0

MS^2 spectrum of $[M-H]^-$ (precursor *m/z*: 726.5888; retention time: 22.34 min; file: Mix1_neg_1).

Irrespective of the LCB hydroxylation, HexCer $[M-H]^-$ show three class specific fragments, i.e., a neutral loss of the hexosyl group (NL_Hex), a neutral loss of the hexosyl group plus water (NL_Hex_H2O), and a neutral loss of the hexosyl group plus formaldehyde (NL_formaldehyde+H2O). For an annotation, the detection of NL_Hex or NL_Hex_H2O is required, and the sum of the intensities of both fragments must exceed 40% of the base peak. Deprotonated HexCer adducts frequently show a deprotonated carboxylate (Carboxy). Additionally, the FA chain might be detectable as deprotonated ketene (Ketene), and the deprotonated LCB that loses ammonia and water (SPH_fragment). To avoid false positive chain identification, both the detection of the Carboxy and the

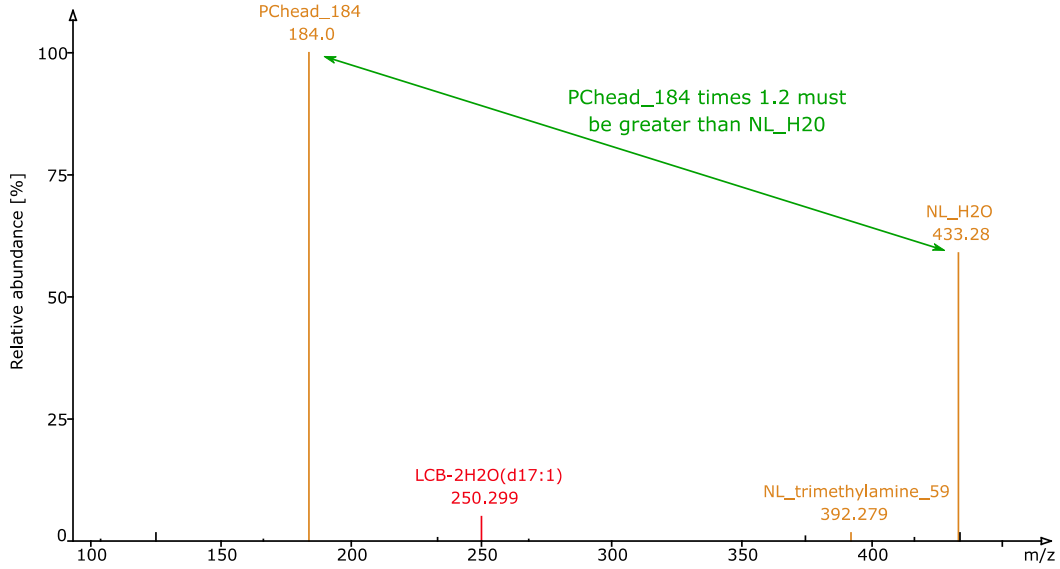
SPH_fragment is mandatory. However, the obligatoriness of the SPH_fragment entails quite some false negatives. Manual re-evaluation might help, since the Carboxy fragments are easily detectable.



Decision rule set: HexCer trihydroxylated LCB $[M-H]^-$ HexCer t18:0/n26:0

MS^2 spectrum of $[M-H]^-$ (precursor *m/z*: 856.7255; retention time: 28.99 min; file: Mix2_neg_1).

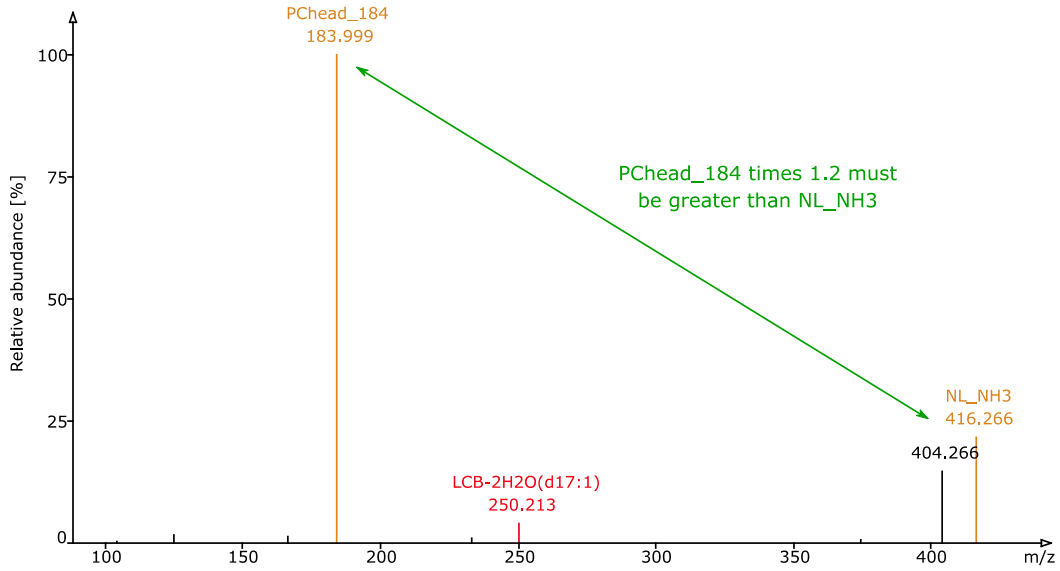
Class specific fragments are the same as for dihydroxylated fragments (see previous figure). The chain fragments are the same too, except that for the SPH_fragment, a neutral loss of CH7NO can be observed from the deprotonated LCB (SPH_fragment_3). Intensity rules are the same as for the dihydroxylated species. However, the SPH_fragment_3 is typically not detectable, which makes an automated annotation of trihydroxylated LCB from deprotonated species unlikely. The Carboxy fragment is frequently present, and can be used for a manual verification. However, deprotonated HexCer species with trihydroxylated LCB do not ionize well, rendering this adduct rather useless in complex samples.



Decision rule set: LSM $[M+H]^+$ LSM d17:1

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 451.3308; retention time: 1.42 min; file: Mix2_1).

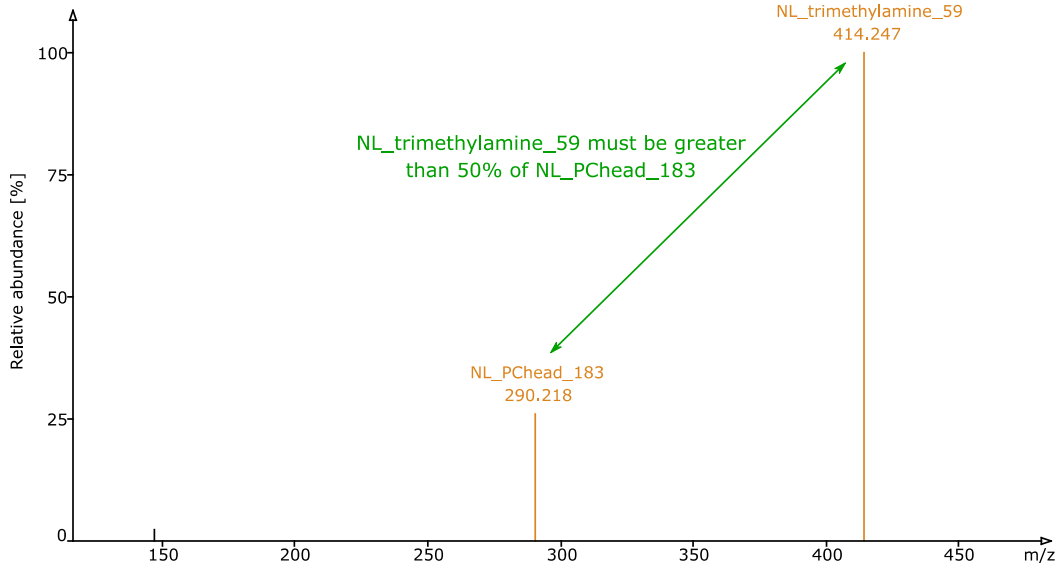
The mandatory fragments for protonated LSM are the phosphocholine head group (PChead_184) and the neutral loss of water (NL_H2O). Lower abundance fragments are the neutral losses of trimethylamine (NL_trimethylamine_59) and the phosphocholine head group (NL_PChead_183), and the LCB that lost two water molecules (LCB-2H2O). All intensity rules relate to PChead_184, whose intensity must be greater than 30% of the base peak, 30% of the precursor, and PChead_184 times 1.2 must be greater than NL_H2O.



Decision rule set: LSM $[M+H-H_2O]^+$ LSM d17:1

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 433.3198; retention time: 1.42 min; file: Mix2_1).

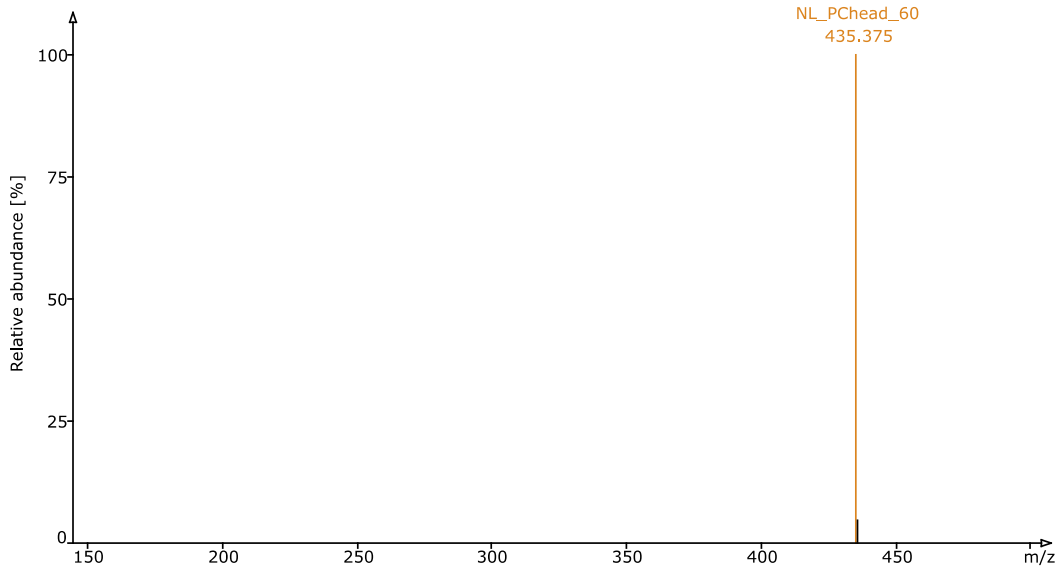
The fragments and the intensity rules are the same as for protonated LSM fragments (see previous figure), except that a neutral loss of ammonia (NL_NH3) can be observed instead of the neutral loss of water, and that the neutral loss fragment of the PC head group is not detectable. The $[M+H-H2O]^+$ is hardly exploitable, because its abundance is lower than 1% of the protonated adduct.



Decision rule set: LSM $[M+Na]^+$ LSM d17:1

MS^2 spectrum of $[M+Na]^+$ (precursor *m/z*: 473.3122; retention time: 1.39 min; file: Mix2_1).

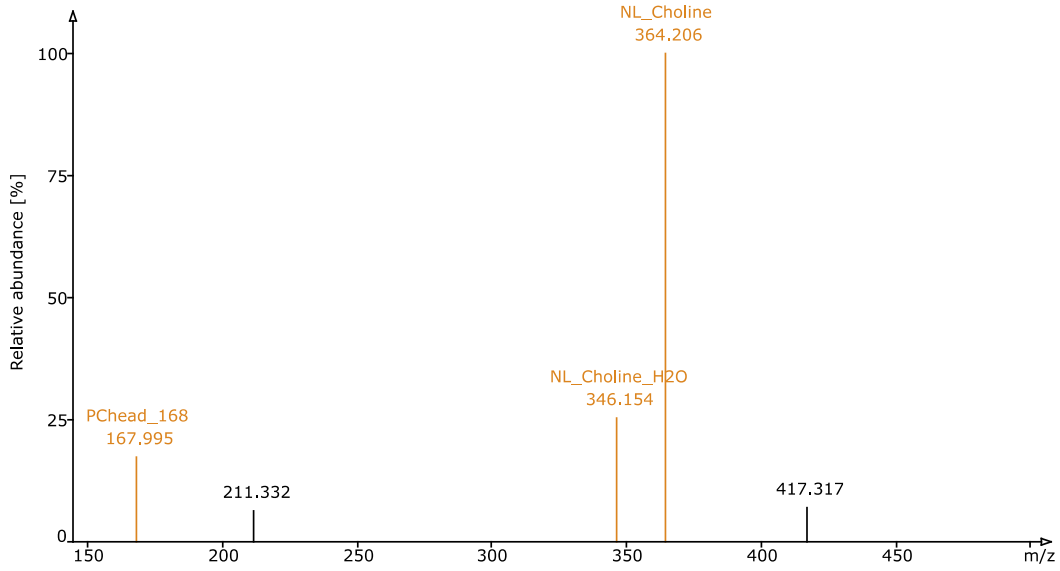
The mandatory fragment of sodiated LSM is the neutral loss of trimethylamine (NL_trimethylamine_59). In addition, the neutral loss of phosphocholine head group (NL_PChead_183) can be observed. All intensity rules relate to NL_trimethylamine_59, whose intensity must be greater than 30% of the base peak, 50% of NL_PChead_183, and 4 times a potential precursor fragment.



Decision rule set: LSM [M+HCOO]$^-$ – MS^2 LSM d17:1

MS^2 spectrum of [M+HCOO]$^-$ (precursor *m/z*: 495.3220; retention time: 1.40 min; file: Mix2_neg_1).

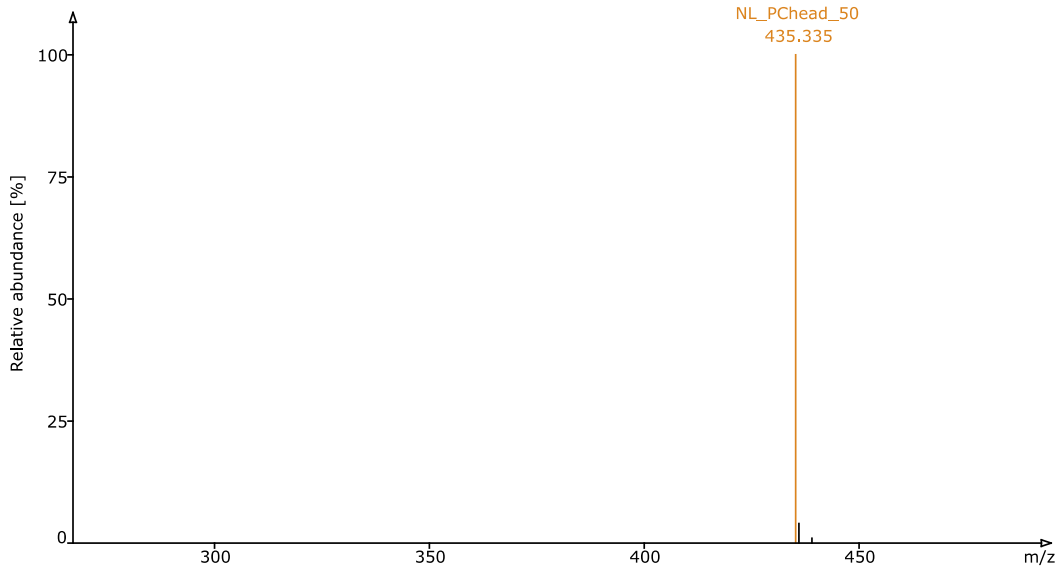
MS^2 spectra of formate adducts of LSM show only a neutral loss of C2H4O2 (NL_PChead_60; mandatory). The intensity of NL_PChead_60 must be greater than 60% of the intensities of the base peak and the precursor. To remove false positives, two additional intensity rules were added, i.e. the NL_PChead_60's intensity must still be greater than 10 times the intensity of a potential loss of formic acid (NL of 46).



Decision rule set: LSM [M+HCOO]$^-$ – MS^3 LSM d17:1

MS^3 spectrum of [M+HCOO]$^-$ (precursor *m/z*: 435.3748; retention time: 1.41 min; file: Mix2_neg_1).

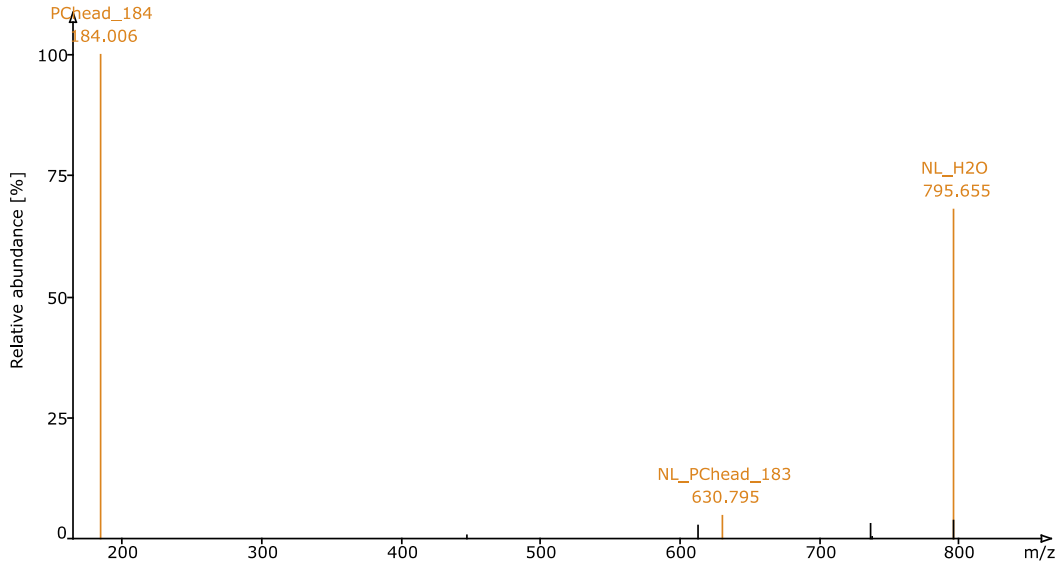
MS^3 spectra of formate adducts of LSM show a head group fragment at 168 (PChead_168), a neutral loss of choline (NL_Choline), and a further loss of water (NL_Choline_H2O). There are no intensity rules for MS^3 spectra, since specificity is already achieved for MS^2 spectra.



Decision rule set: LSM $[M+Cl]^-$ LSM d17:1

MS^2 spectrum of $[M+Cl]^-$ (precursor *m/z*: 485.2942; retention time: 1.39 min; file: Mix2_neg_1).

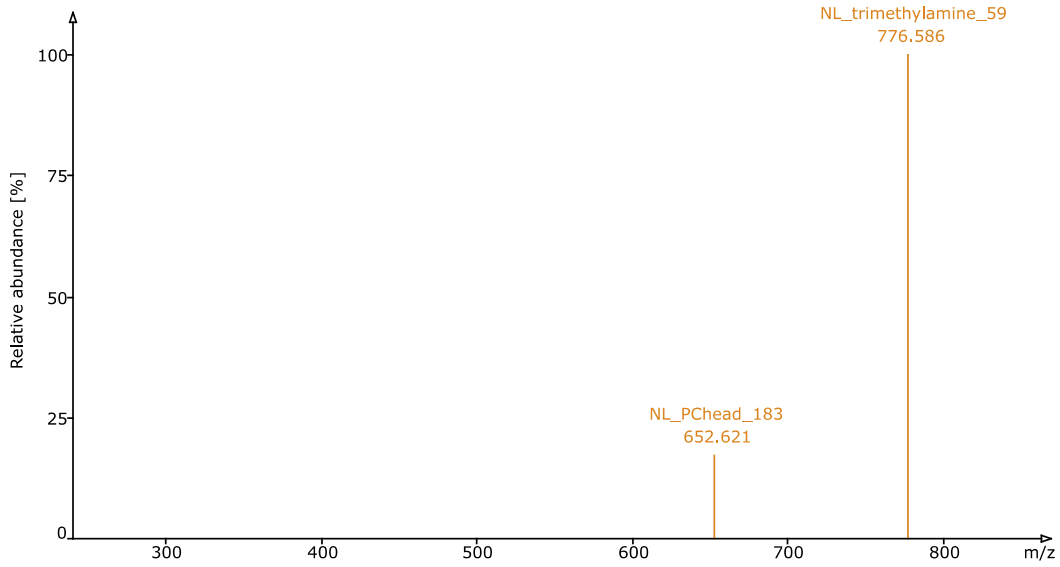
As for the formate adduct, MS^2 spectra of the chlorinated adduct of LSM shows only a single neutral loss, i.e., CH3Cl (NL_PChead_50; mandatory). The intensity of NL_PChead_50 must be greater than 60% of the intensities of the base peak and the precursor.



Decision rule set: SM $[M+H]^+$ SM d18:1/n24:1

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 813.6860; retention time: 25.48 min; file: Mix1_1).

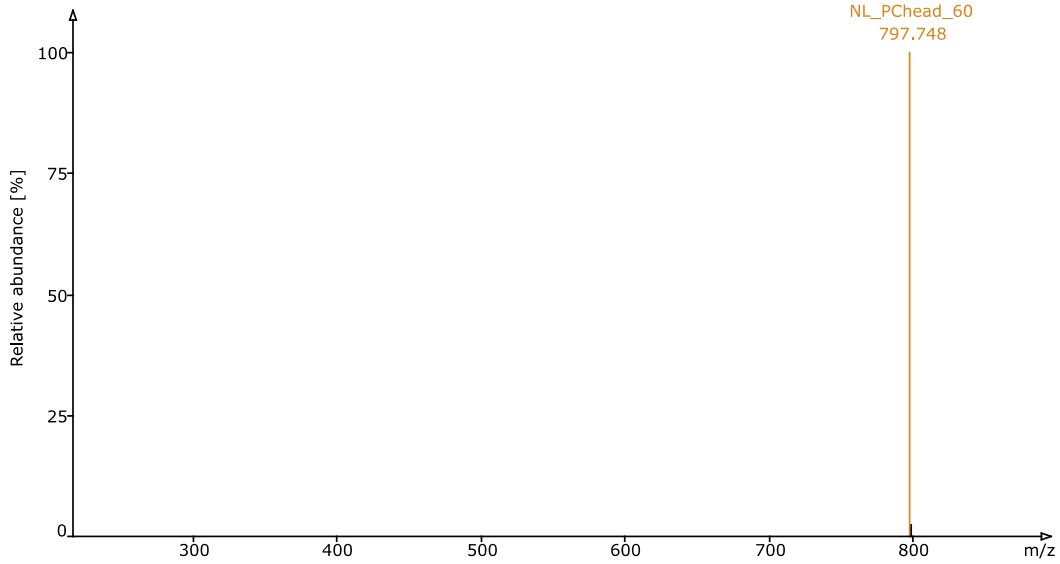
As for LSM, MS^2 spectra of protonated SM species show two prominent fragments, i.e., phosphocholine head group (PChead_184) and the neutral loss of water (NL_H2O). Only the unspecific NL_H2O fragment is set mandatory, since the PChead_184 might fall out of the scan range for heavier molecules due to the dynamic scan range adjustment of ion trap based CID. If present, the intensity of PChead_184 must be greater than 5% of the base peak and 10% of the precursor fragment. To provide the same specificity, the same intensity rules are repeated containing the sum of PChead_184 and NL_H2O instead of using PChead_184 alone. Chain annotation is not possible for protonated adducts.



Decision rule set: SM $[M+Na]^+$ SM d18:1/n24:1

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 835.6655; retention time: 25.54 min; file: Mix1_1).

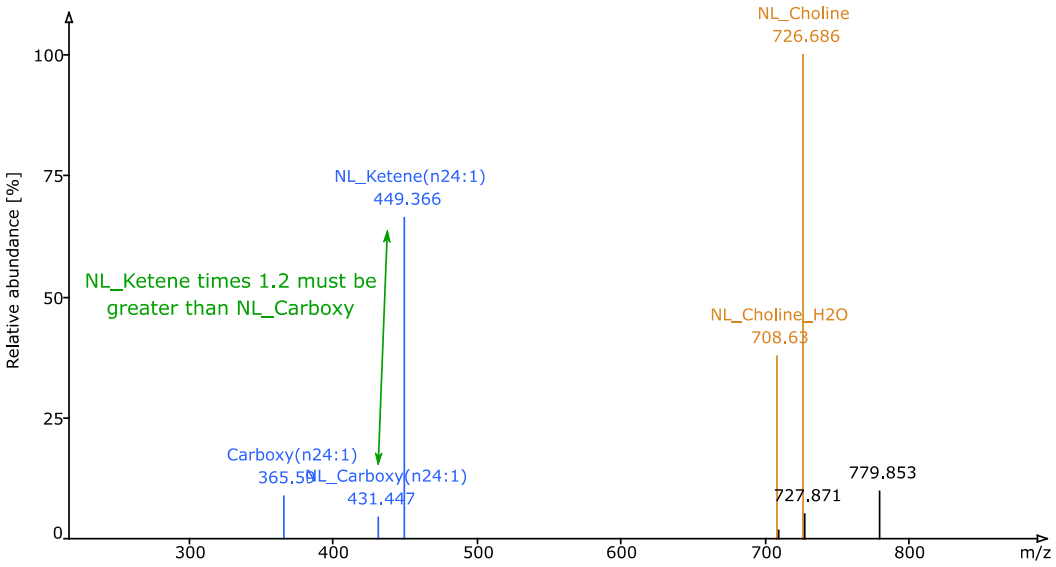
As for LSM, the mandatory fragment for sodiated LSM is the neutral losses of trimethylamine (NL_trimethylamine_59). In addition, the neutral loss of the phosphocholine head group (NL_PChead_183) can be observed. All intensity rules relate to the sum of intensities of NL_trimethylamine_59 and NL_PChead_183. This sum must be greater than the base peak, and 50% of the intensity of the precursor. Chain annotation is not possible for sodiated adducts.



Decision rule set: SM $[M+HCOO]^-$ – MS^2 SM d18:1/n24:1

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 857.6782; retention time: 25.68 min; file: Mix1_neg_1).

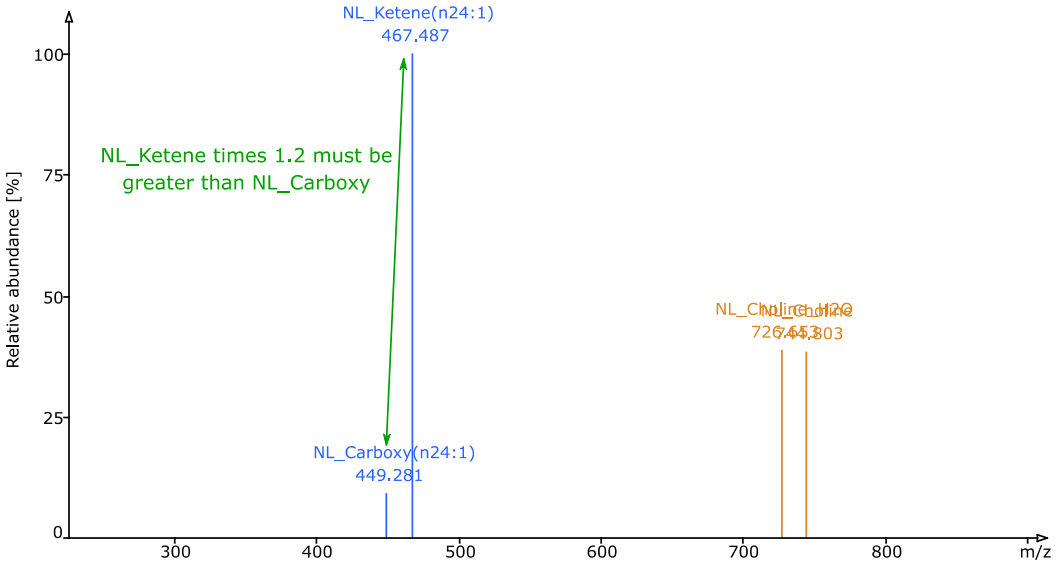
As for LSM species, MS^2 spectra of formate adducts of LSM show only a mandatory neutral loss of C2H4O2 (NL_PChead_60; mandatory). The intensity of NL_PChead_60 must be greater than the precursor, and two times the intensity of NL_PChead_60 must be greater than the neutral loss of 50 to remove isobaric species from chlorinated adducts. Chain annotation is not possible for MS^2 spectra of formate adducts.



Decision rule set: SM dihydroxylated LCB $[M+HCOO]^-$ – MS^3 SM d18:1/n24:1

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 797.7480; retention time: 25.68 min; file: Mix1_neg_1).

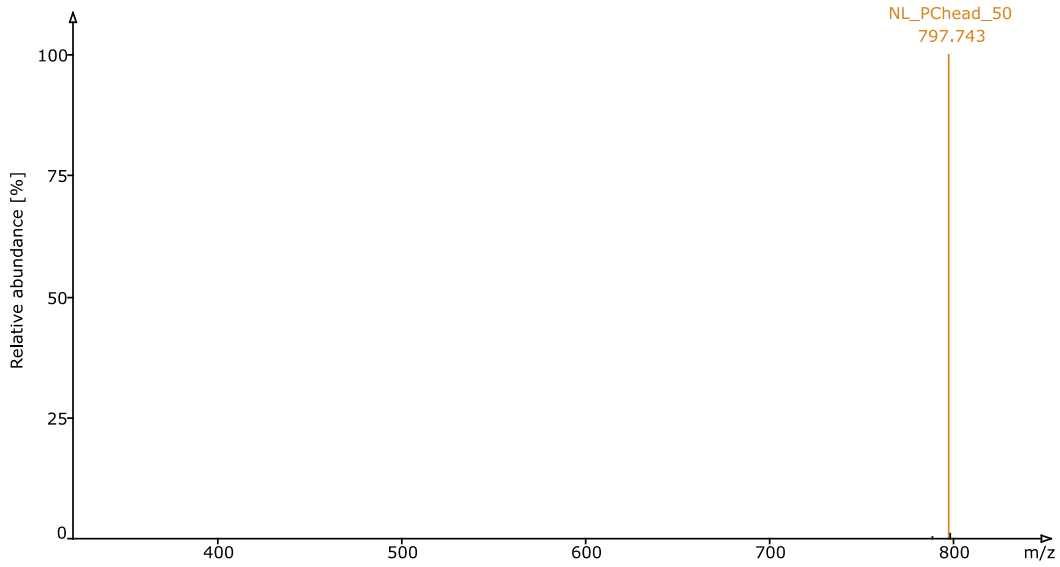
As for MS^3 spectra of LSM adducts, MS^3 spectra of formate adducts of LSM may show a head group fragment at 168 (PChead_168), a neutral loss of choline (NL_Choline), and a further loss of water (NL_Choline_H2O). There are no intensity rules for class specific fragments in MS^3 spectra, since specificity is already achieved by MS^2 spectra. MS^3 spectra of formate adducts reveal chain information. The following fragments are possible: a mandatory neutral loss of the FA as ketene (NL_Ketene), a neutral loss of the FA as carboxylate (NL_Carboxy), and the FA itself as deprotonated carboxylate (Carboxy). The intensity of NL_Ketene times 1.2 must be greater than the intensity of the NL_Carboxy fragment.



Decision rule set: SM trihydroxylated LCB $[M+HCOO]^-$ SM t18:0/n24:1

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 815.7029; retention time: 24.90 min; file: Serum_neg_3).

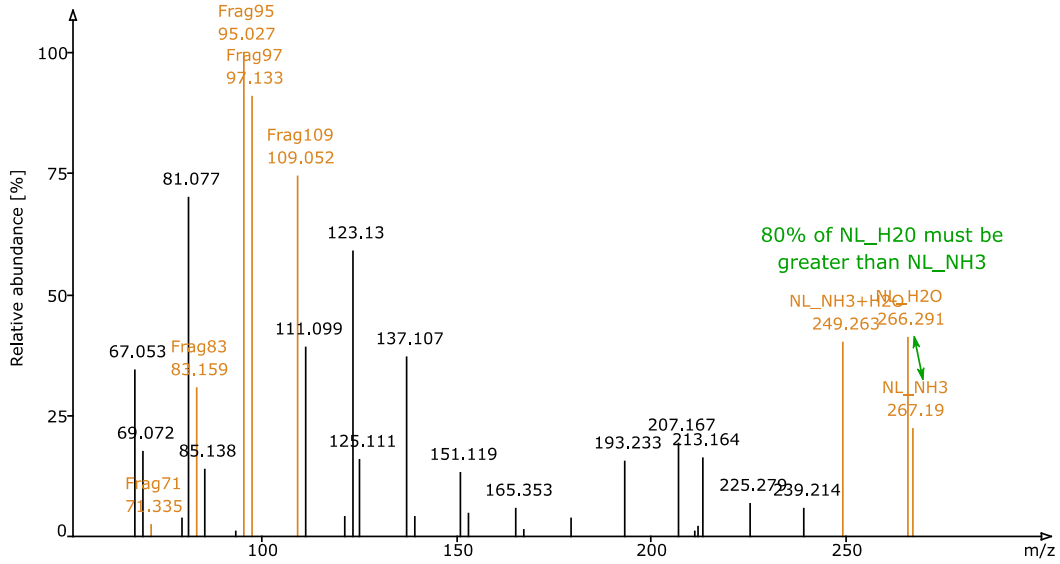
There is no difference in the rules for dihydroxylated and trihydroxylated LCB. For details about the rules, please consult the previous two figures. This figure is only for completeness to show that the rules work for trihydroxylated species too.



Decision rule set: SM $[M+Cl]^-$ SM d18:1/n24:1

MS^2 spectrum of $[M+Cl]^-$ (precursor *m/z*: 847.6511; retention time: 25.60 min; file: Mix1_neg_1).

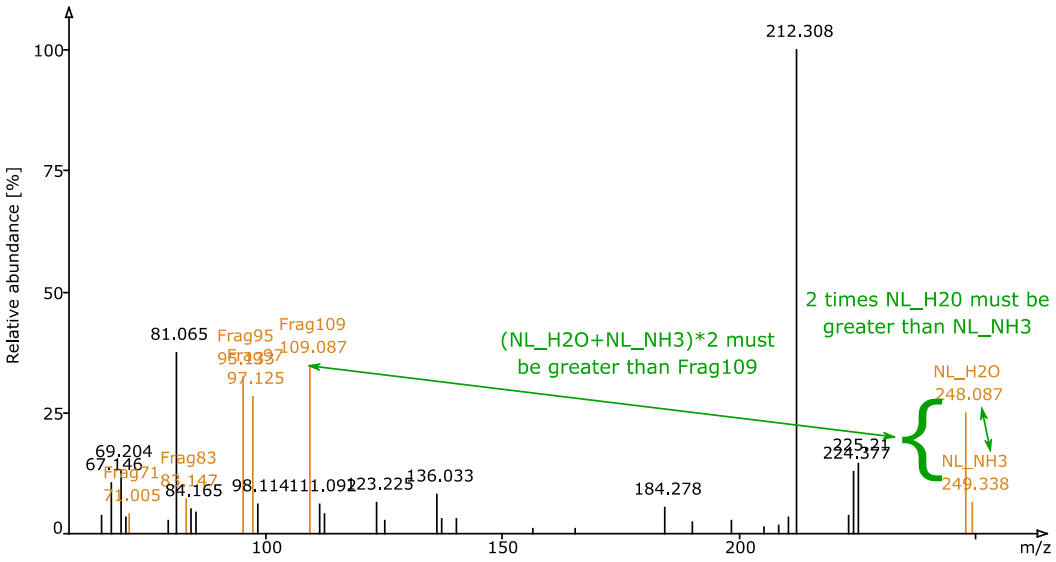
As chlorinated LSM adducts, MS^2 spectra of the chlorinated adducts of SM show only a single neutral loss, i.e. CH3Cl (NL_PChead_50; mandatory). The intensity of NL_PChead_50 must be greater than 20% of the base peak, and two times the intensity of NL_PChead_50 must be greater than the neutral loss of 60 to remove isobaric species from formate adducts. Chain annotation is not possible for MS^2 spectra of chlorinated adducts.



Decision rule set: SphBase $[M+H]^+$ SphBase m18:1

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 284.2956; retention time: 4.03 min; file: Mix1_1).

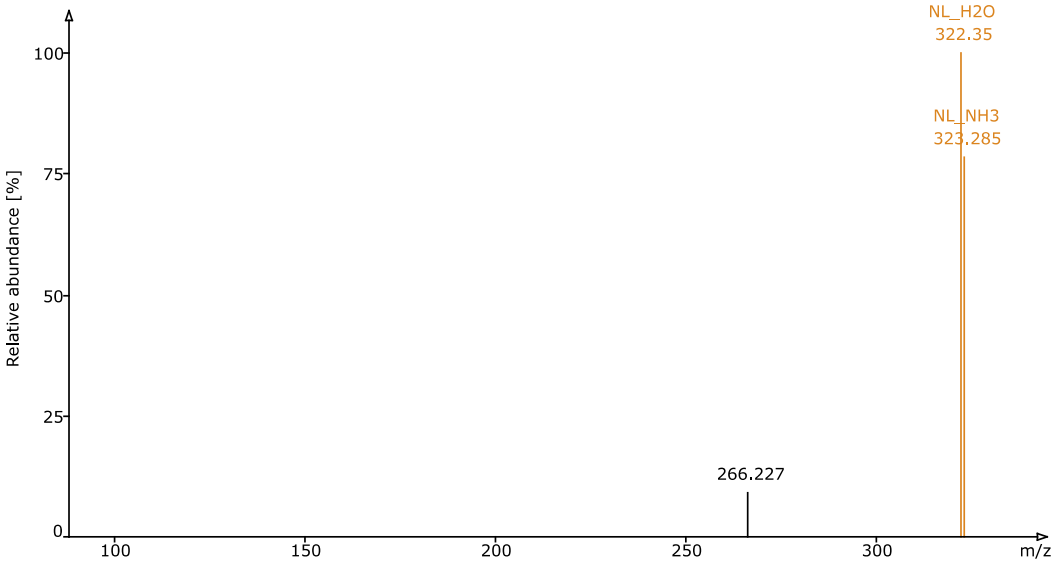
Protonated SphBase adducts show a fragmentation-rich spectrum. However, the detectable fragments change from species to species, and the intensity relations vary heavily. The only fragment which is always present is the unspecific neutral loss of water (NL_H2O; mandatory). Other frequently detectable fragments are at 71 Da (Frag71), 83 Da (Frag83), 95 Da (Frag95), 97 Da (Frag97) and 109 Da (Frag109), a neutral loss of ammonia (NL_NH3) and a neutral loss of ammonia and water (NL_NH3+H2O). For an identification by LDA, one of those fragments have to be detected in addition to the NL_H2O, and 80% of the intensity of the NL_H2O fragment must be greater than the intensity of the NL_NH3 fragment. For trihydroxylated species, a fragment of high abundance with two water losses is detectable (NL_2xH2O_36; mandatory). Since the specificity of this rule set is low, a rule was added accepting protonated SphBase species only if at the same retention time a protonated adduct with a loss of water or a sodiated adduct is detectable (MS^1 peak is sufficient).



Decision rule set: SphBase $[M+H-H_2O]^+$ SphBase m18:1

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 266.2852; retention time: 3.87 min; file: Mix1_1).

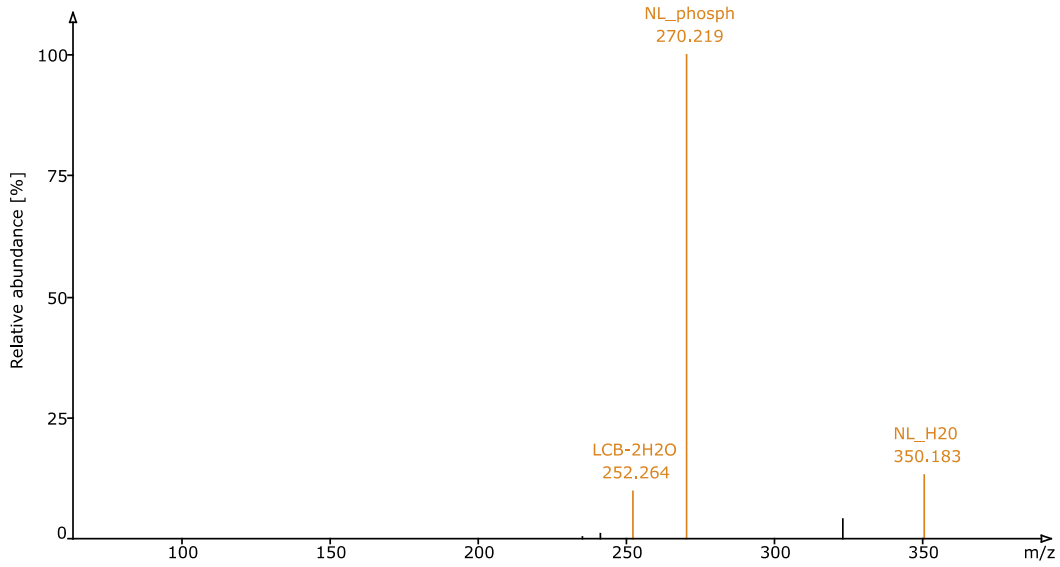
Protonated SphBase precursors that lost water show similar fragments as conventional protonated precursors (see previous figure). The differences are in the intensity rules. For a positive identification, any of the following fragments have to be found in addition to the NL_H2O fragment: Frag71, Frag83, Frag95, Frag97, or Frag109. The intensity of NL_H2O times two must be greater than the intensity of the NL_NH3 fragment. The sum of the intensities of the NL_H2O and the NL_NH3 times 2, times 1.2 and times 0.8 must be higher than the intensity of Frag109 for monohydroxylated, dihydroxylated and trihydroxylated species, respectively. Since the specificity of this rule set is low, a rule was added accepting protonated SphBase species with a loss of water only if at the same retention time a protonated adduct or a sodiated adduct is detectable (MS^1 peak is sufficient).



Decision rule set: SphBase $[M+Na]^+$ SphBase t18:0

MS^2 spectrum of $[M+Na]^+$ (precursor *m/z*: 340.2830; retention time: 2.53 min; file: Mix1_1).

Sodiated SphBase species show either a neutral loss of water (NL_H2O) or a neutral loss of ammonia (NL_NH3), or both. An identification of any of those fragments is sufficient for a positive annotation. Since the specificity of this rule set is low, a rule was added accepting sodiated SphBase species only if at the same retention time a protonated adduct or a protonated adduct with a loss of water is detectable (MS^1 peak is sufficient).



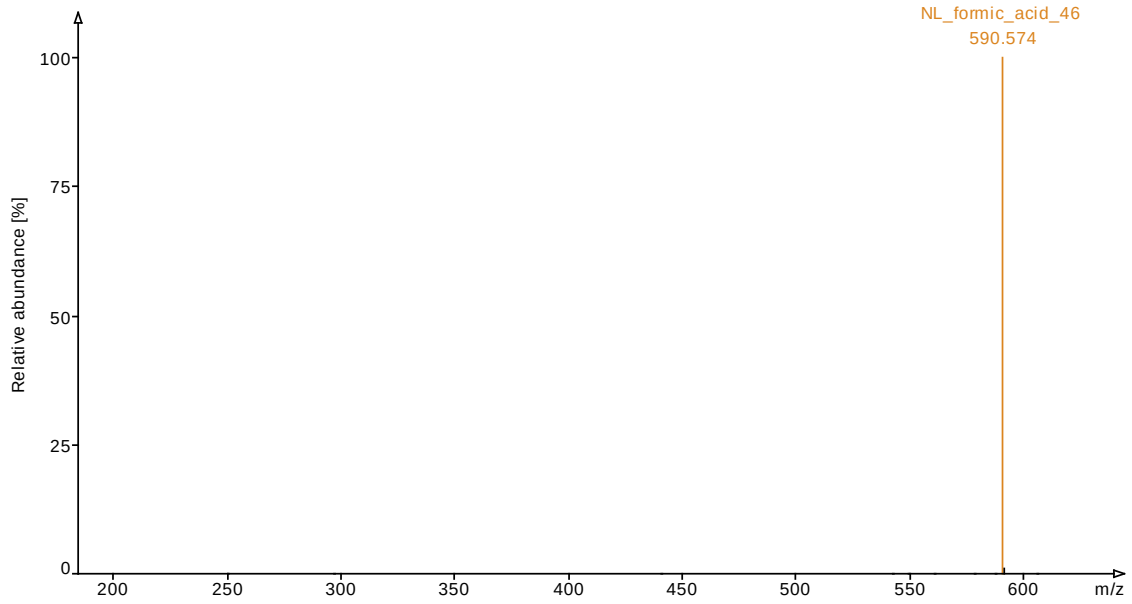
Decision rule set: S1P $[M+H]^+$ S1P d17:0

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 368.2568; retention time: 2.07 min; file: Mix1_1).

Protonated S1P species show a mandatory neutral loss of water (NL_H2O), and two more fragments, i.e. the neutral loss of the phosphate (NL_phosph) and the protonated LCB minus two water (LCB-2H2O). For specificity, any of the latter two fragments have to be found in addition to NL_H2O. Since sometimes the intensity of the one or the other of the latter two fragments is stronger, the sum of both intensities is used in intensity rules. This sum must be greater than 1.2 times the intensity of NL_H2O and 30% of the base peak.

Figure S-2: Spectral evidence for novel lipid species and molecular species identified in murine brain and human plasma.

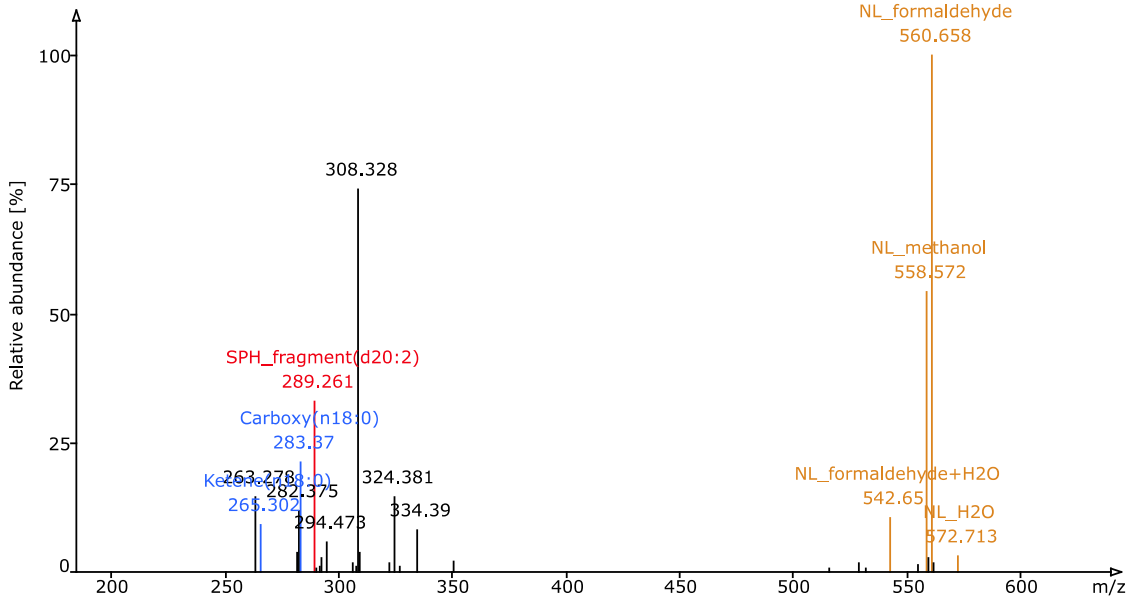
These figures highlight the annotated experimental spectra for novel lipid molecular species identified. Evidence relies on spectra at full mass range and is supported by zoom in spectra where necessary. The spectra are low resolution CID spectra acquired on an Orbitrap Velos Pro. Where available, spectra in positive and negative ion mode are shown. In case the novel species is a lipid molecular species, and the other ion mode verifies the lipid species only, just spectra of the ion mode proofing the existence of the molecular species are shown.



Novel species: Cer d20:2/n18:0

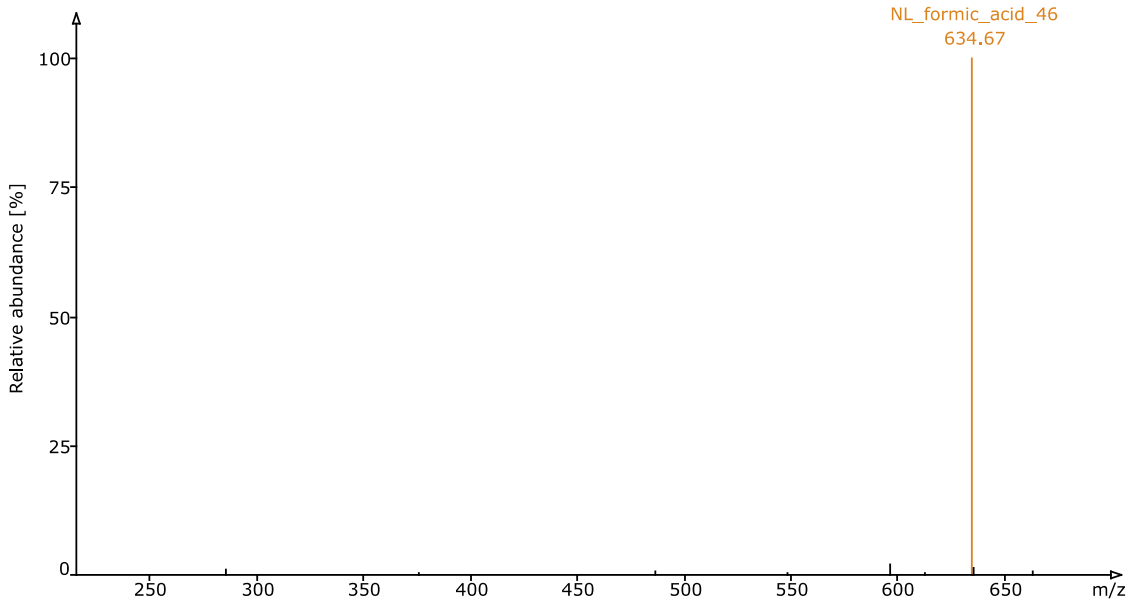
MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 636.5592; retention time: 24.71 min; file: Brain_neg_3).

Comment: MS^2 spectrum does not reveal any chain information.



Novel species: Cer d20:2/n18:0

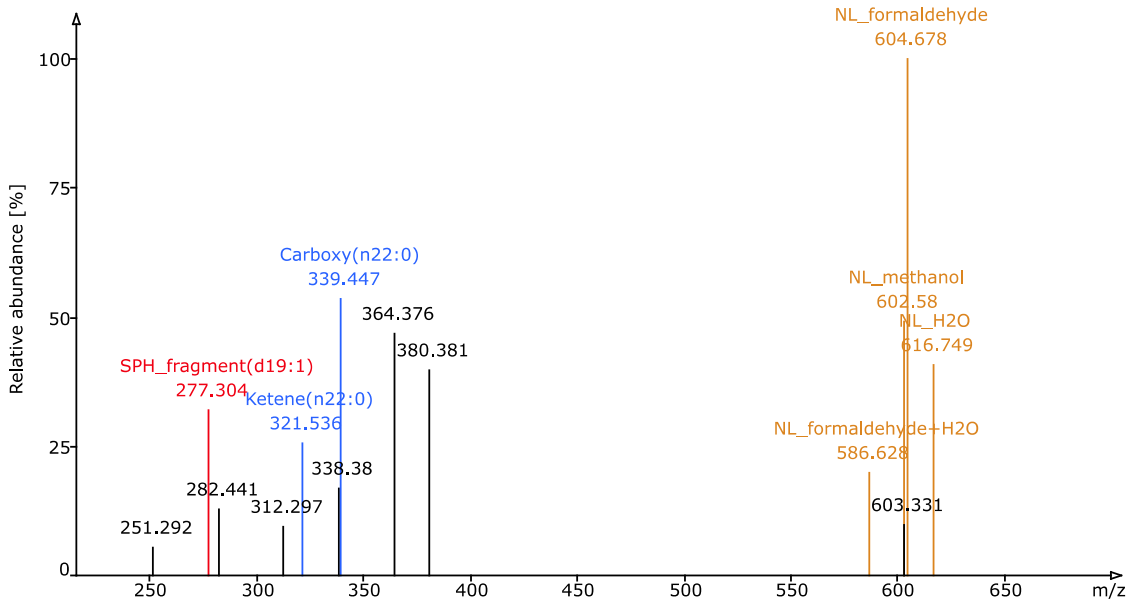
MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 590.5736; retention time: 24.72 min; file: Brain_neg_3).



Novel species: Cer d19:1/n22:0

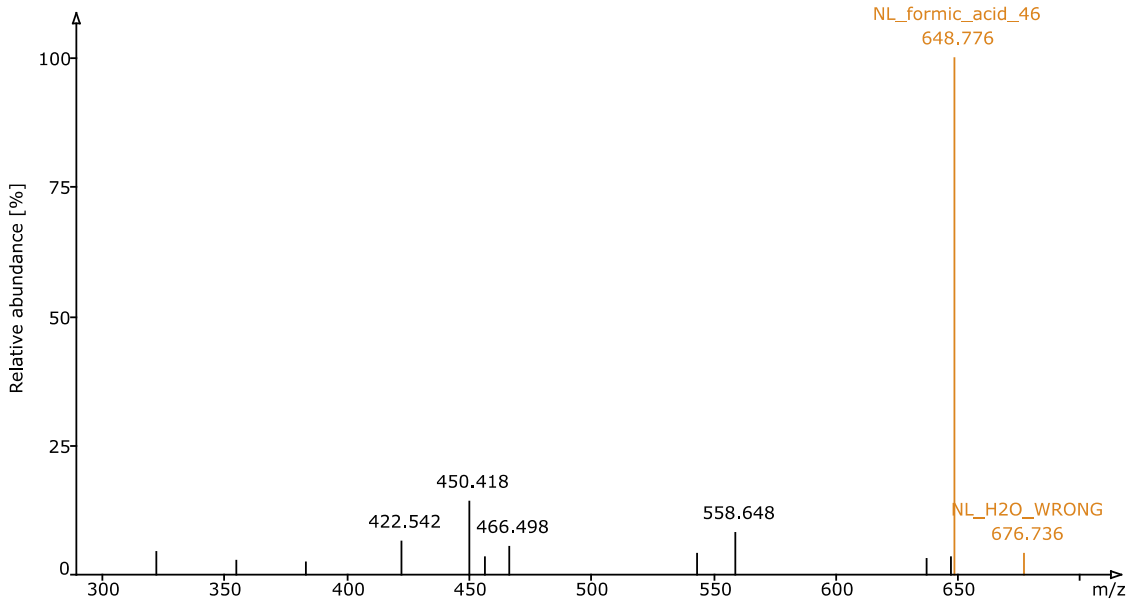
MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 680.6227; retention time: 28.66 min; file: Serum_neg_4).

Comment: MS^2 spectrum does not reveal any chain information.



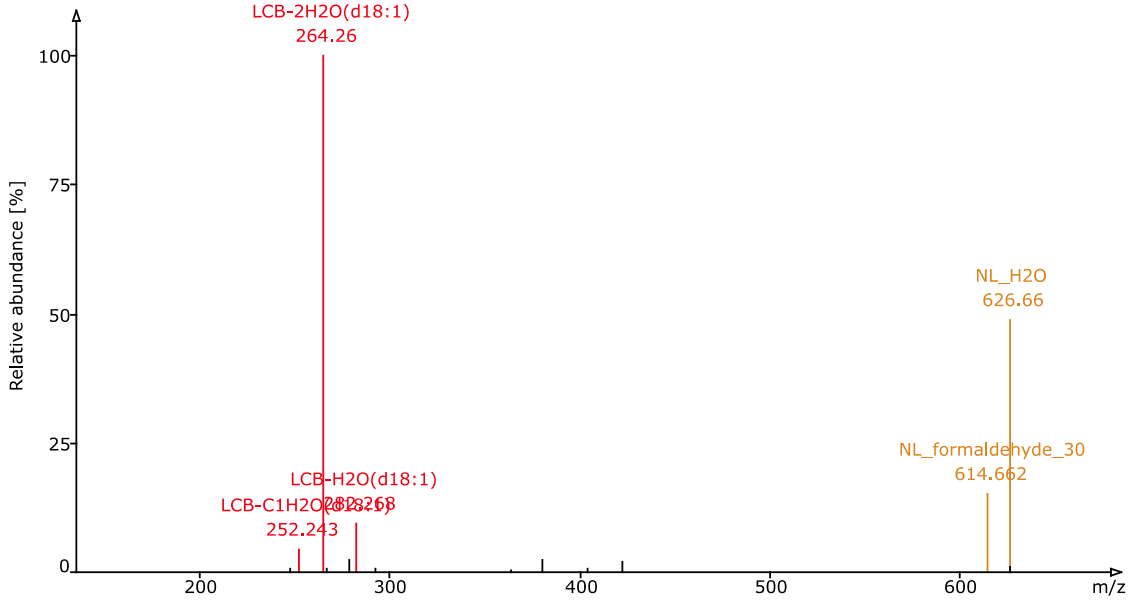
Novel species: Cer d19:1/n22:0

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 634.6698; retention time: 28.66 min; file: Serum_neg_4).



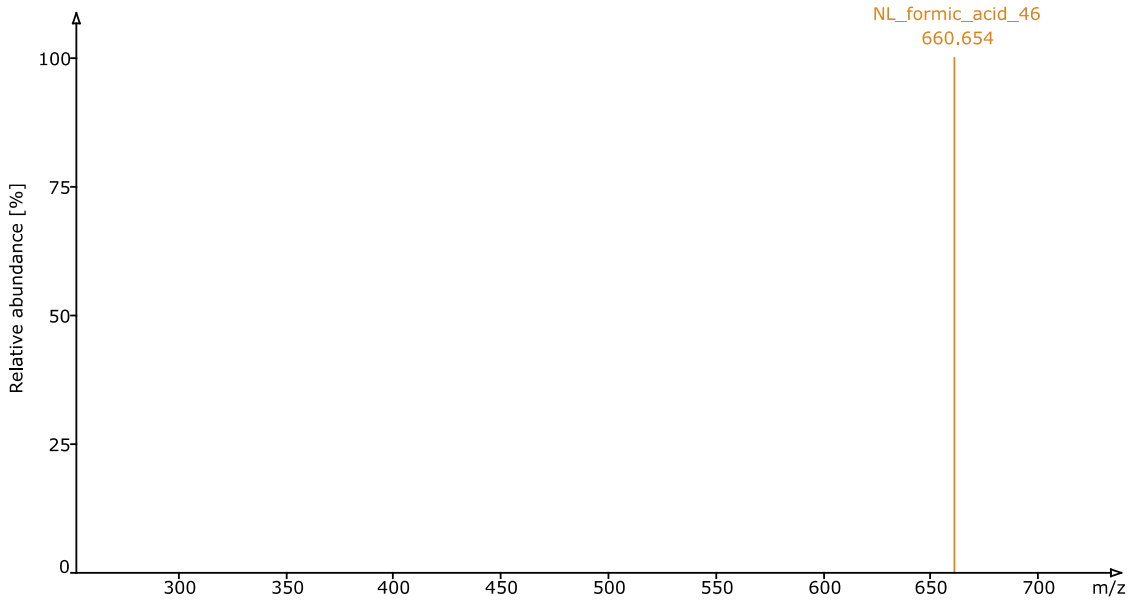
Novel species: Cer m43:0

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 694.6739; retention time: 31.60 min; file: Serum_neg_2).



Novel species: Cer d18:1/n25:1

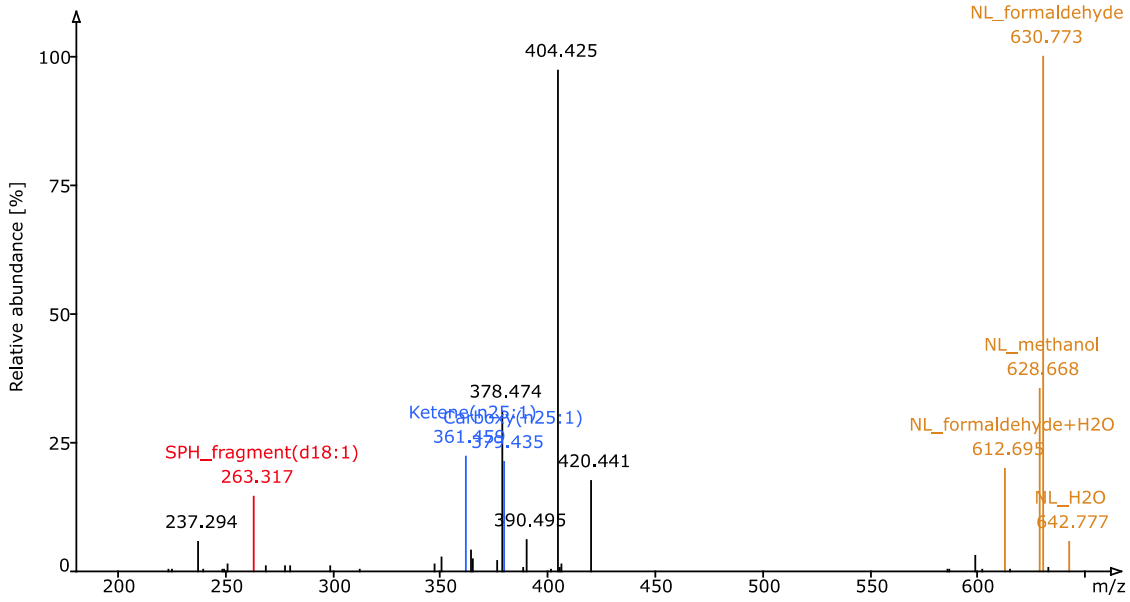
MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 644.6345; retention time: 28.93 min; file: Brain_pos_1).



Novel species: Cer d18:1/n25:1

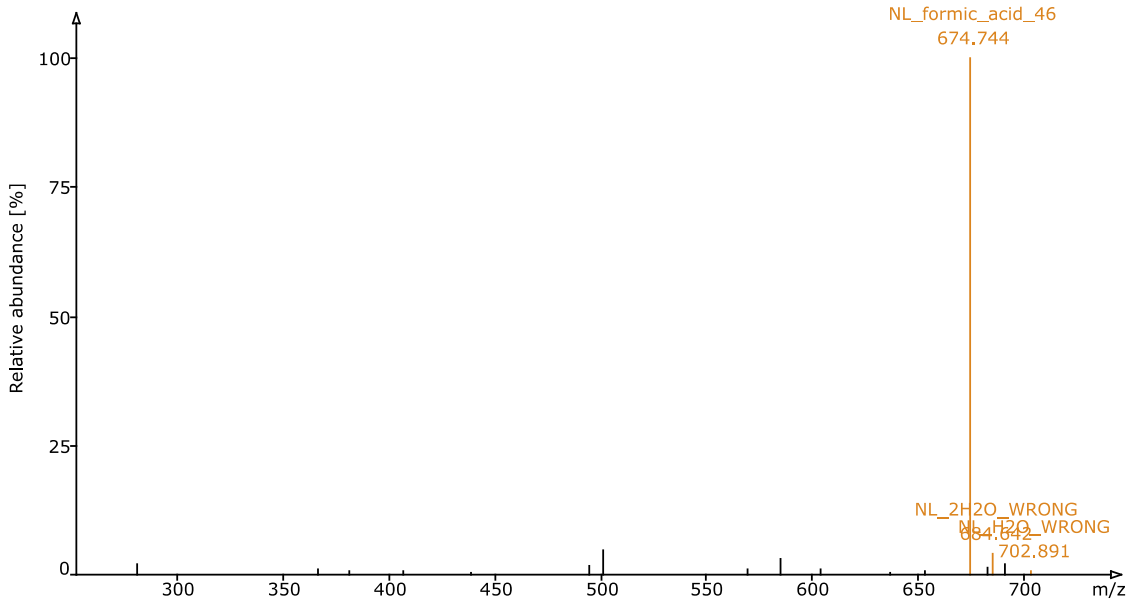
MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 706.6384; retention time: 28.91 min; file: Brain_neg_1).

Comment: MS^2 spectrum does not reveal any chain information.



Novel species: Cer d18:1/n25:1

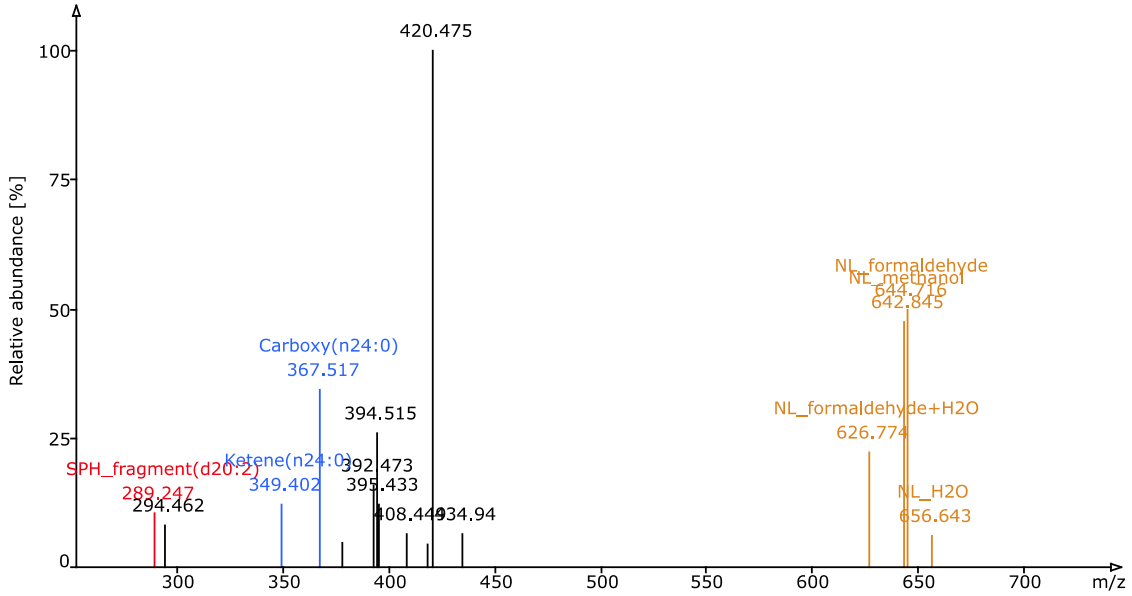
MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 660.6538; retention time: 28.91 min; file: Brain_neg_1).



Novel species: Cer d20:2/n24:0

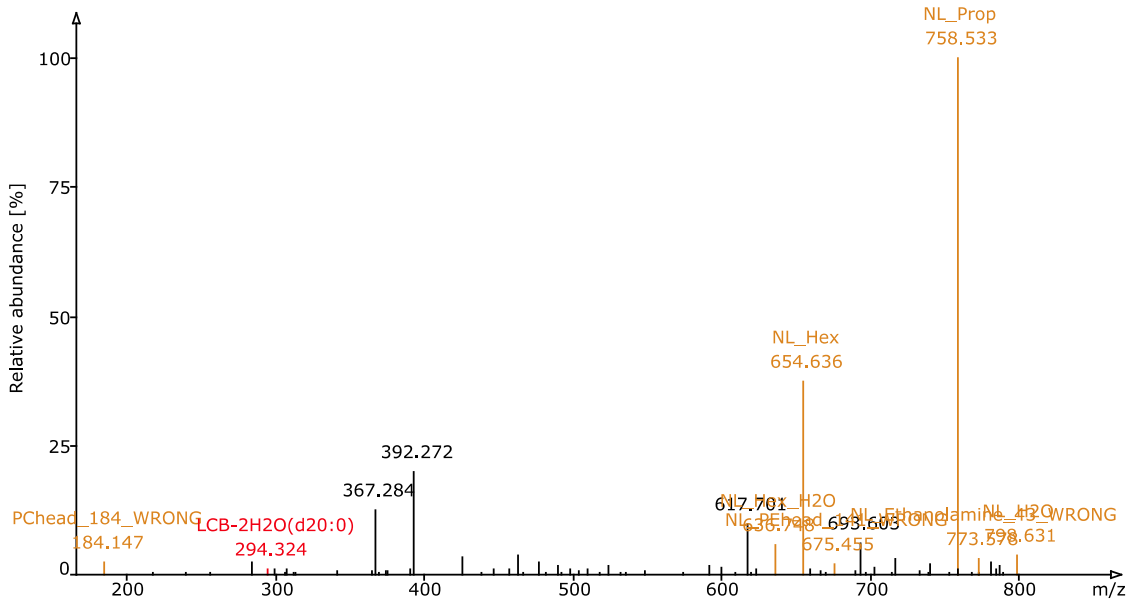
MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 720.6536; retention time: 30.11 min; file: Serum_neg_3).

Comment: MS^2 spectrum does not reveal any chain information.



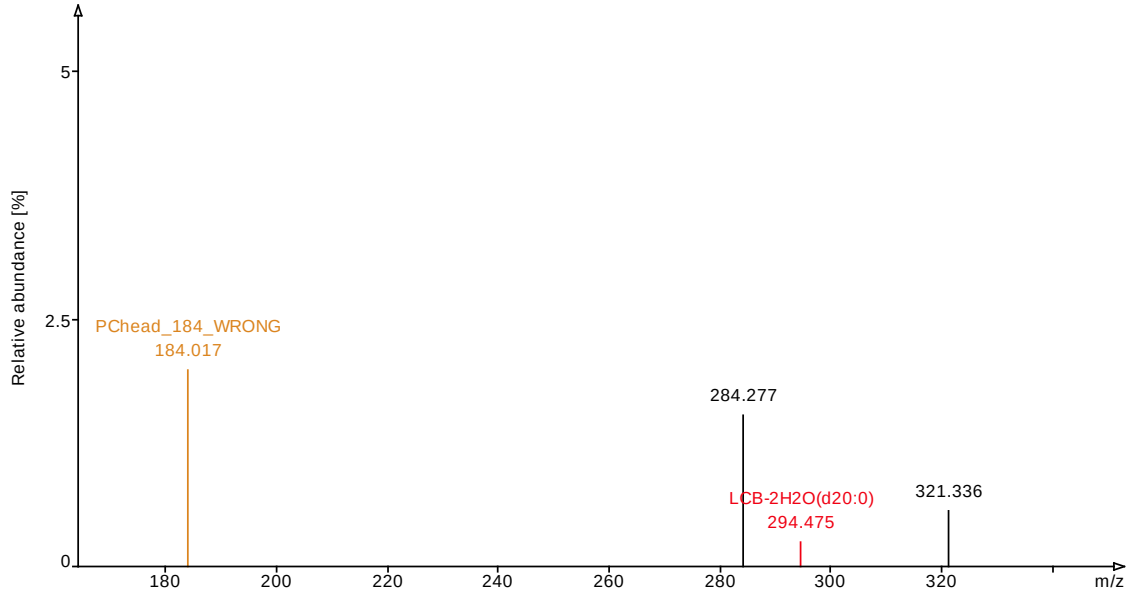
Novel species: Cer d20:2/n24:0

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 674.7439; retention time: 30.12 min; file: Serum_neg_3).



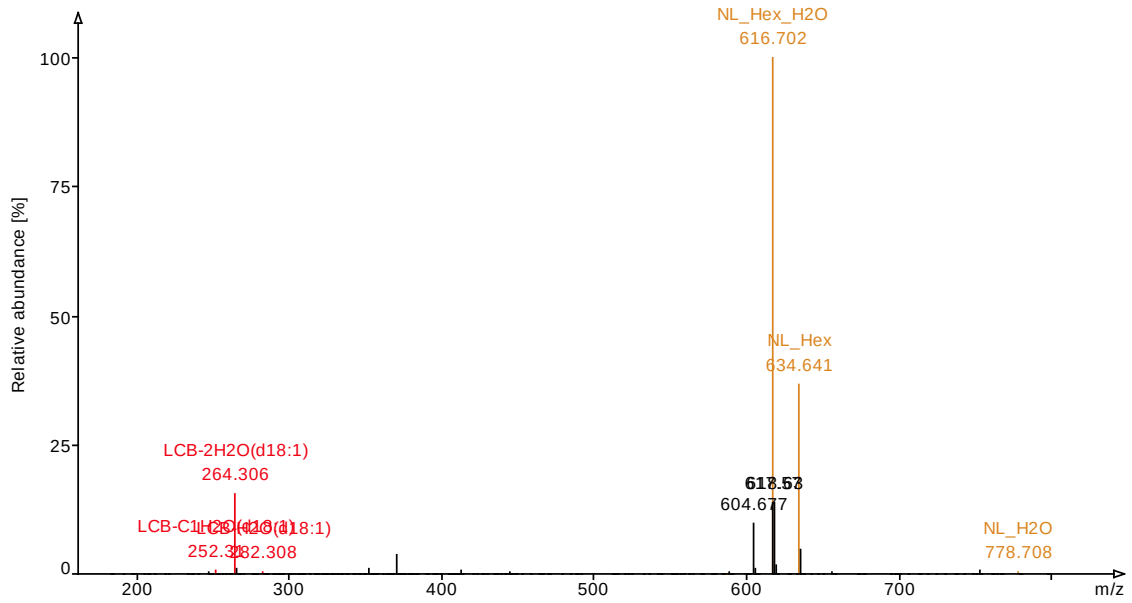
Novel species: HexCer d20:0/h21:0 (and t41:0)

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 816.6959; retention time: 27.33 min; file: Brain_pos_2).



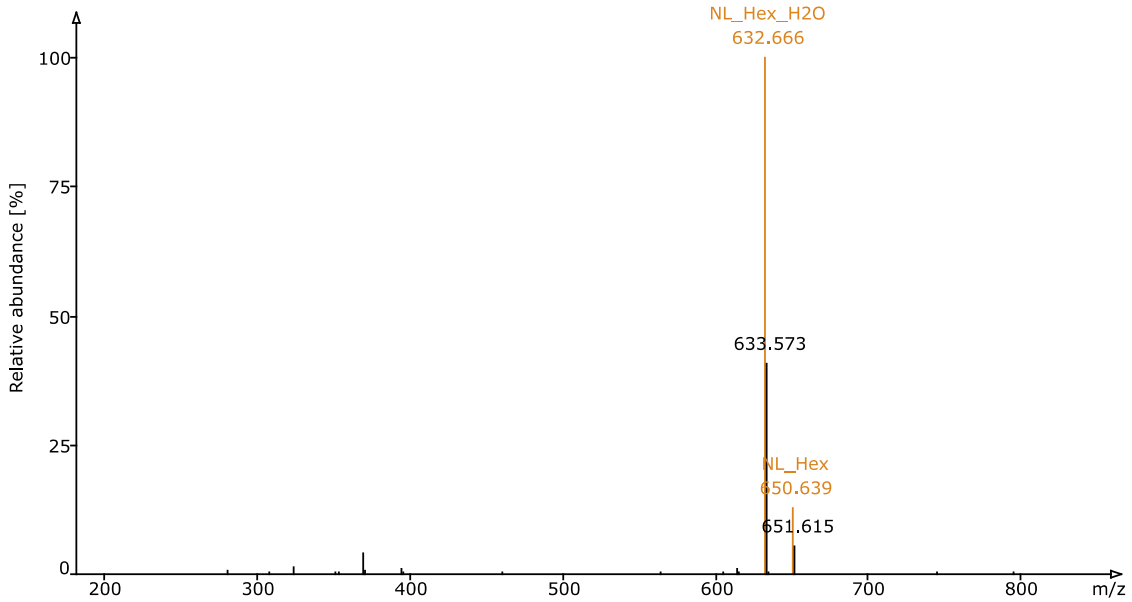
Novel species: HexCer d20:0/h21:0 (and t41:0)

MS^2 spectrum of $[M+H]^+$ zoomed.



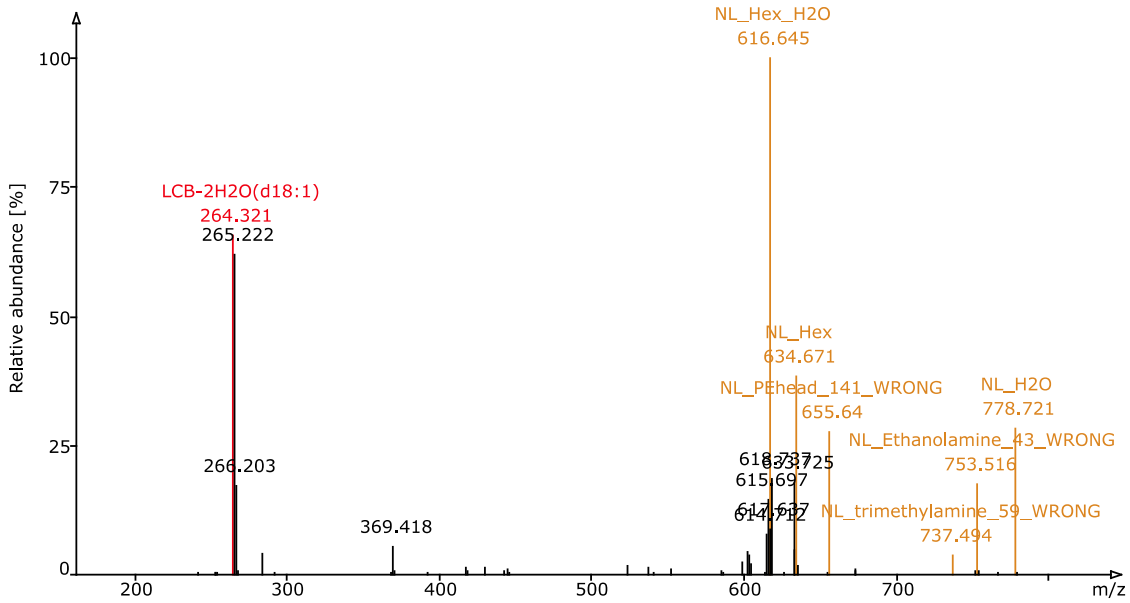
Novel species: HexCer d18:1/h23:0 (and t41:1)

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 796.6673; retention time: 26.74 min; file: Brain_pos_1).



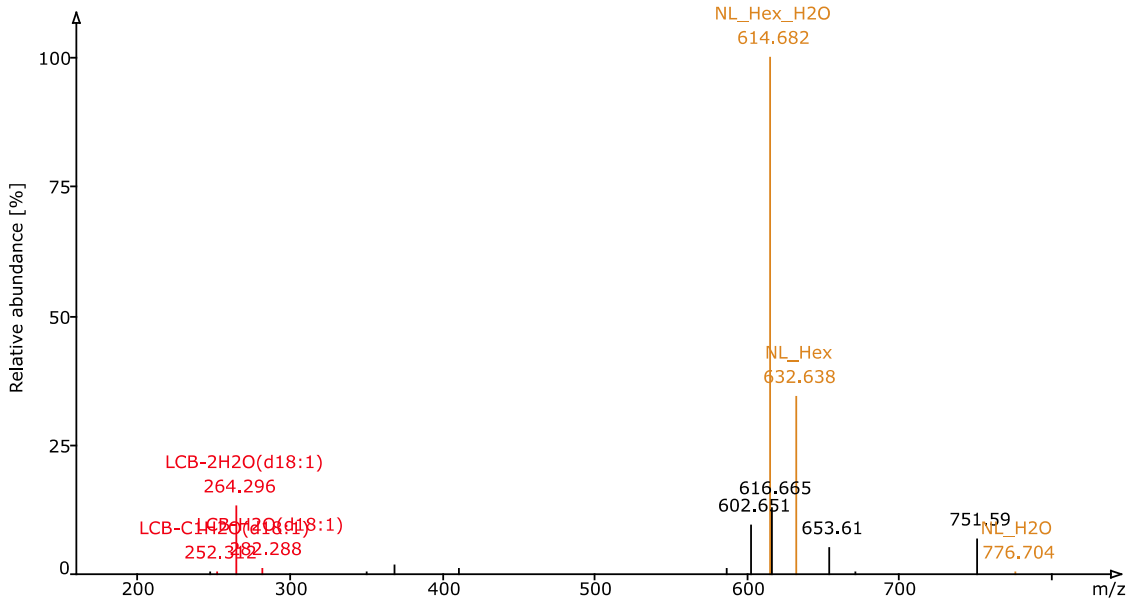
Novel species: HexCer t41:1

MS^2 spectrum of $[M-H]^-$ (precursor *m/z*: 812.6626; retention time: 27.02 min; file: Brain_neg_1).



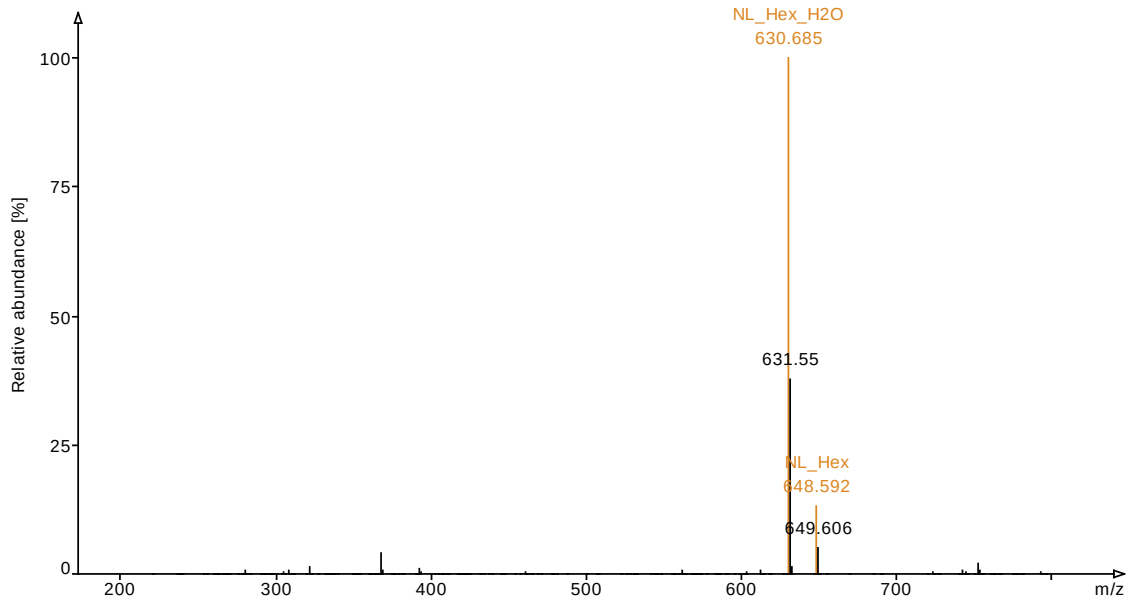
Novel species: HexCer d18:1/n23:1

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 796.6681; retention time: 26.05 min; file: Brain_pos_1).



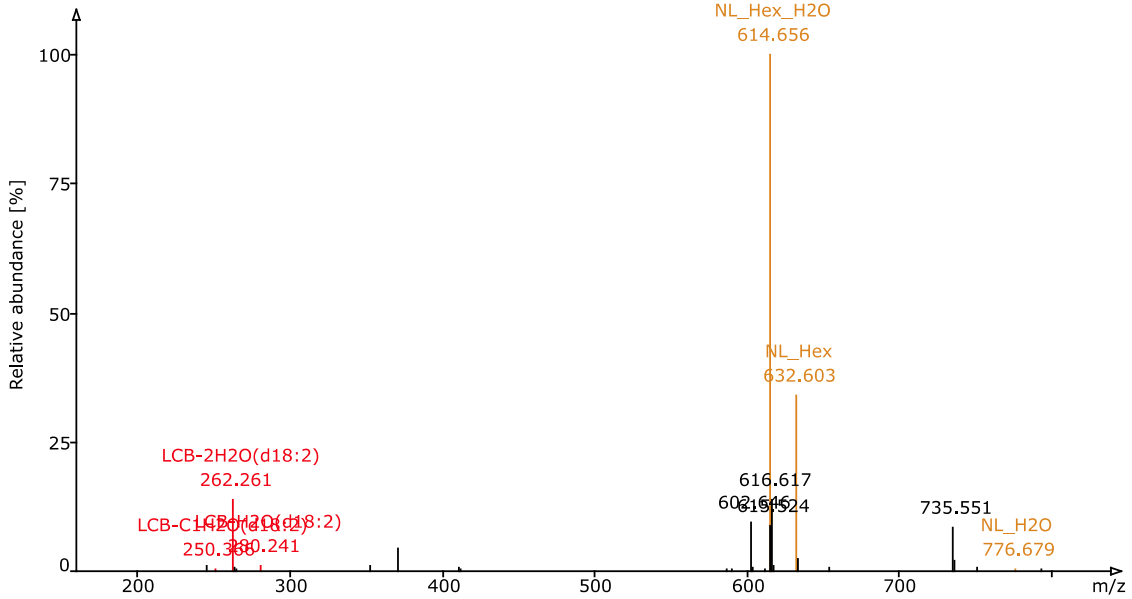
Novel species: HexCer d18:1/h23:1 (and t41:2)

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 794.6507; retention time: 25.01 min; file: Brain_pos_1).



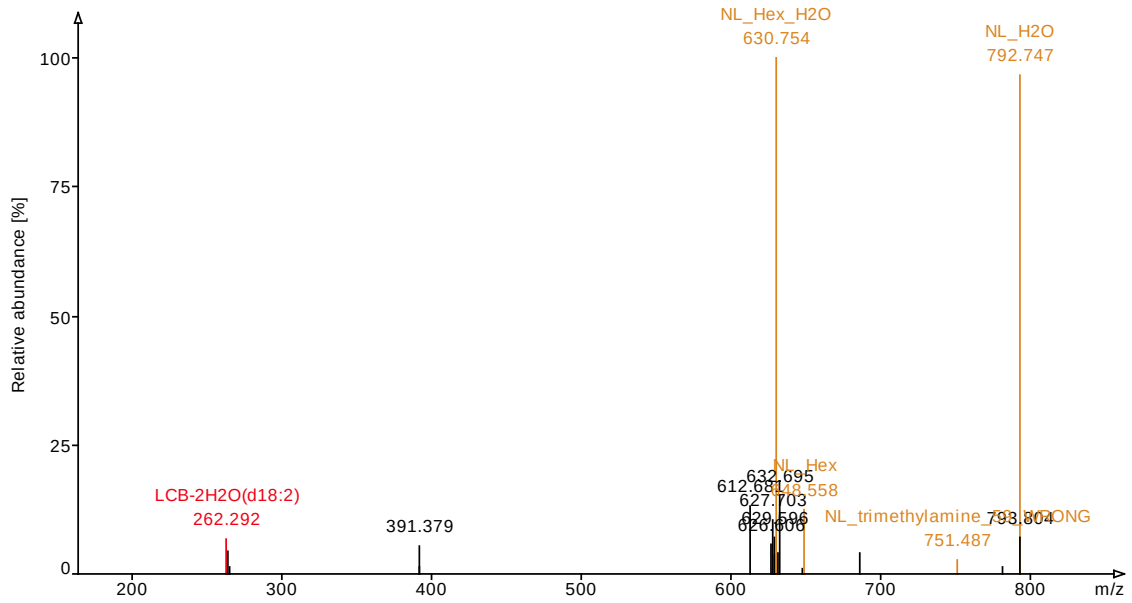
Novel species: HexCer t41:2

MS^2 spectrum of $[M-H]^-$ (precursor *m/z*: 810.6475; retention time: 25.04 min; file: Brain_neg_1).



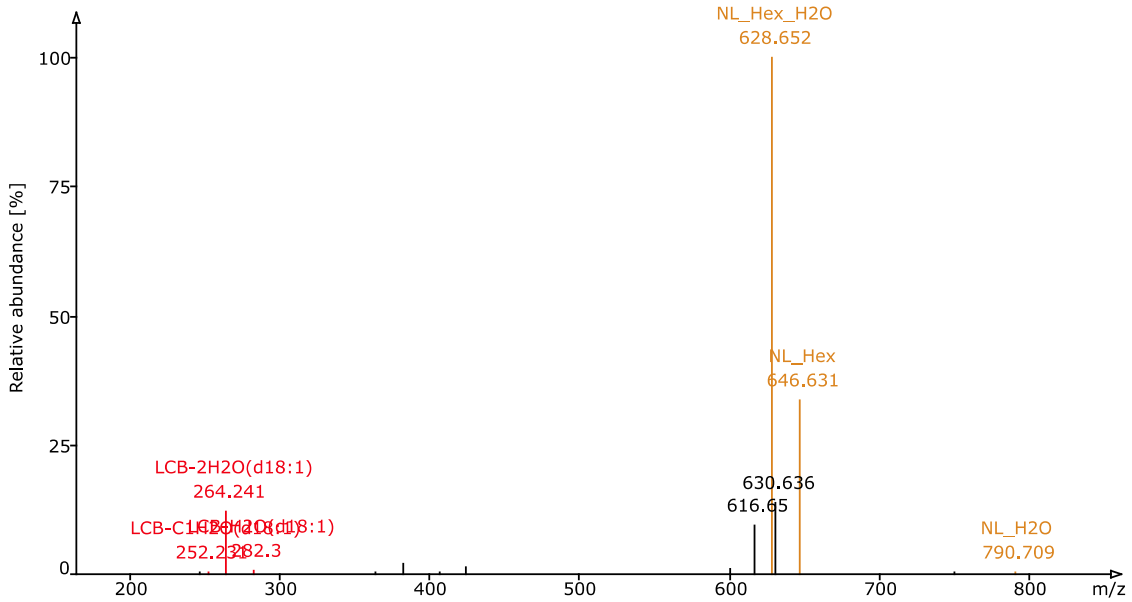
Novel species: HexCer d18:2/h23:0 (and t41:2)

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 794.6511; retention time: 25.45 min; file: Brain_pos_1).



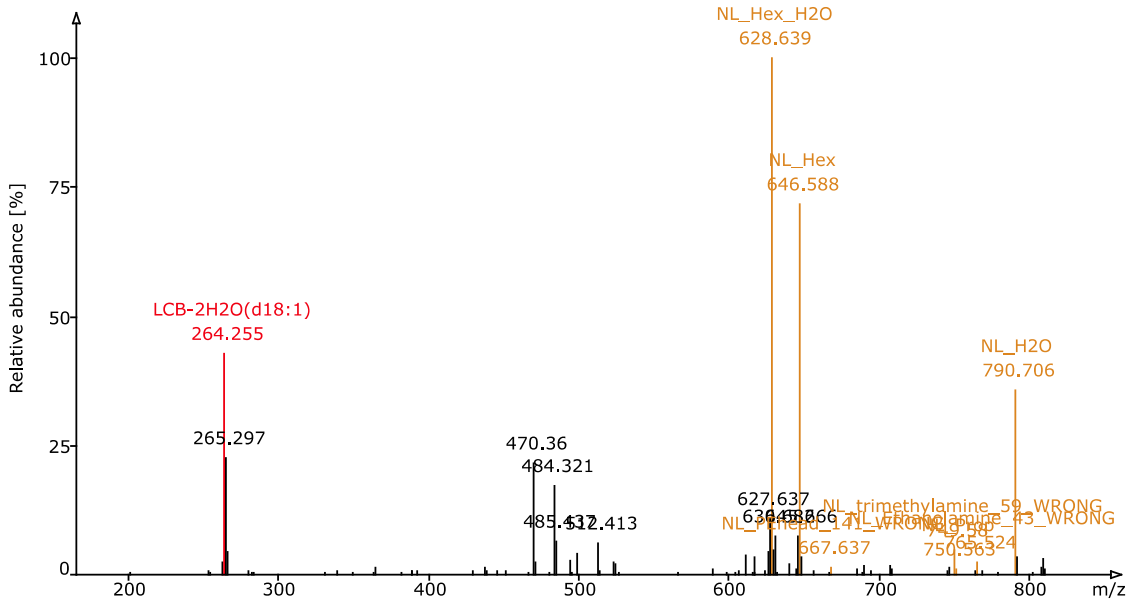
Novel species: HexCer d18:2/n24:0

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 810.6831; retention time: 26.63 min; file: Brain_pos_3).



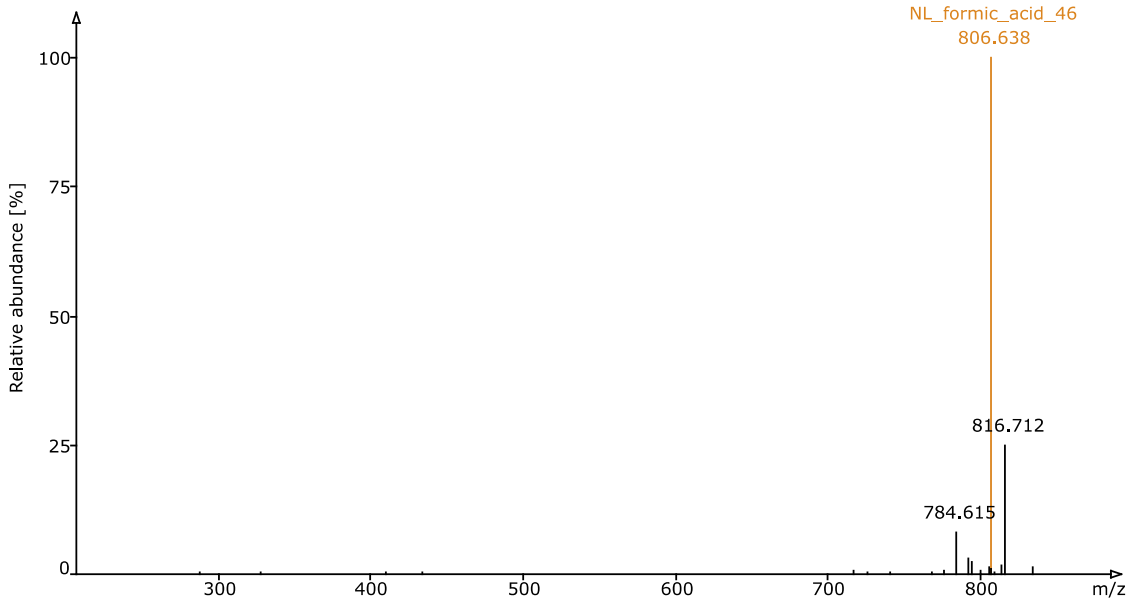
Novel species: HexCer d18:1/h24:1

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 808.6669; retention time: 26.12 min; file: Brain_pos_1).



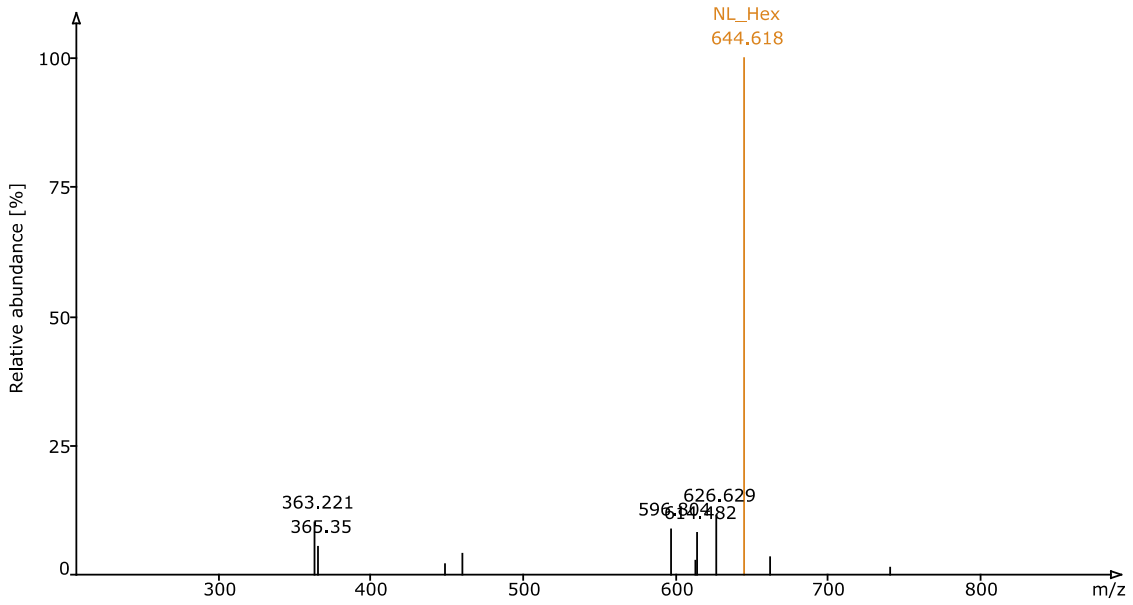
Novel species: HexCer d18:1/n24:2 (and d42:3)

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 808.6601; retention time: 25.07 min; file: Brain_pos_3).



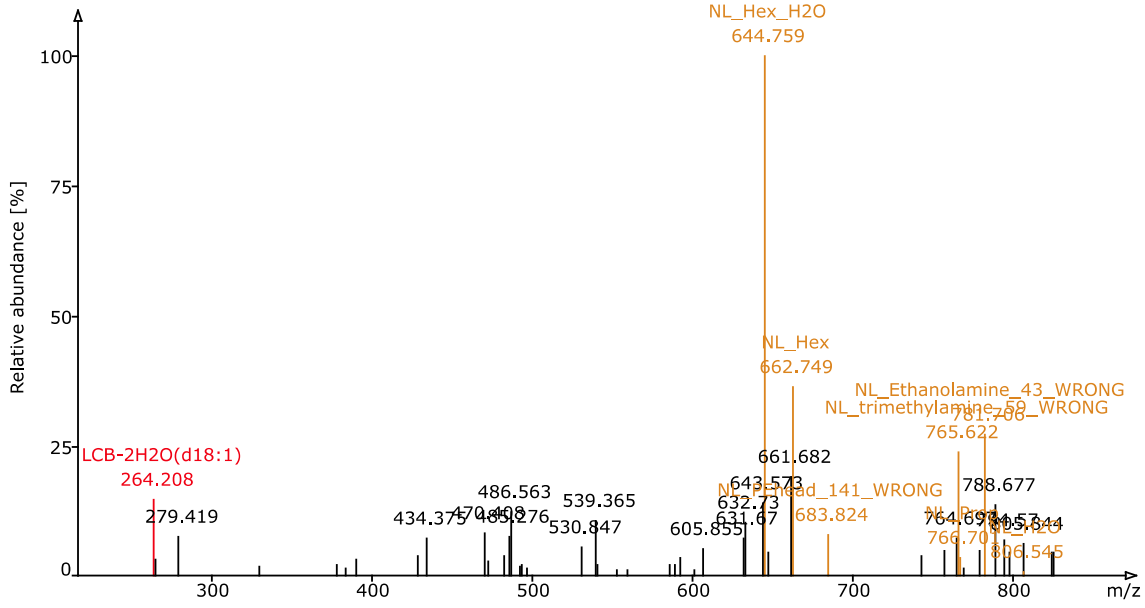
Novel species: HexCer d42:3

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 852.6619; retention time: 25.09 min; file: Brain_neg_1).



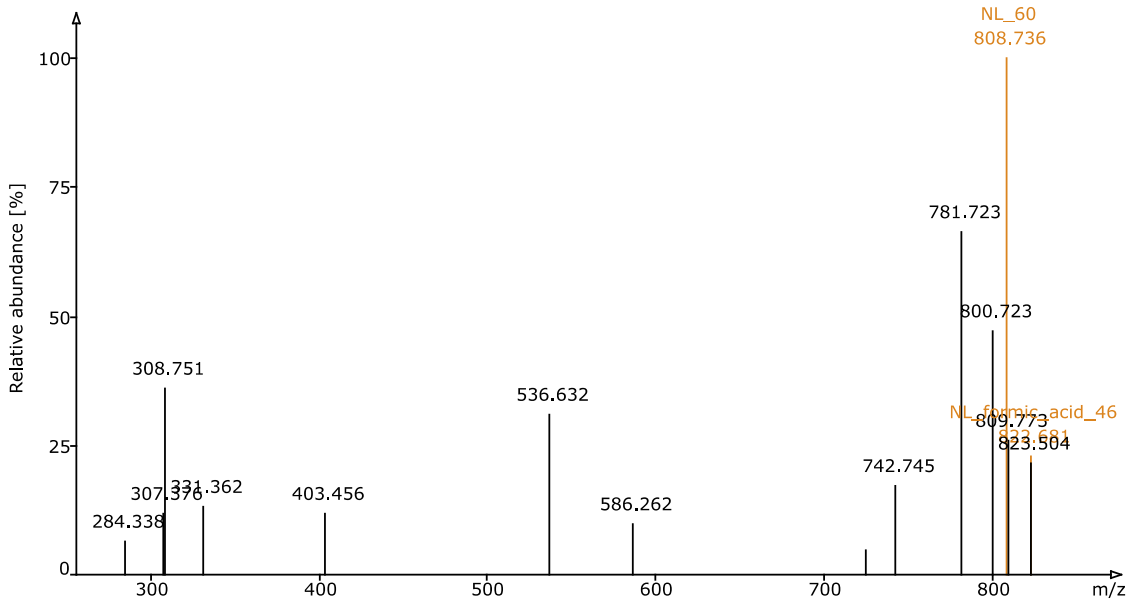
Novel species: HexCer d42:3

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 806.6384; retention time: 25.09 min; file: Brain_neg_1).



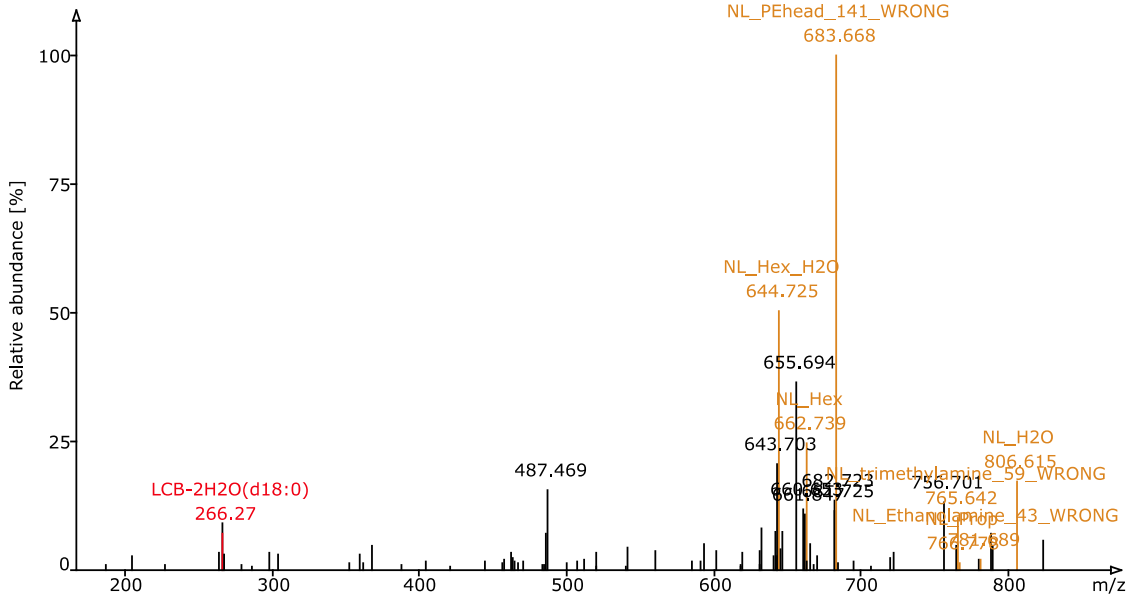
Novel species: HexCer d18:1/h24:2 (and t42:3)

MS^2 spectrum of $[M+H]^+$ (precursor *m/z*: 824.6617; retention time: 24.42 min; file: Brain_pos_1.



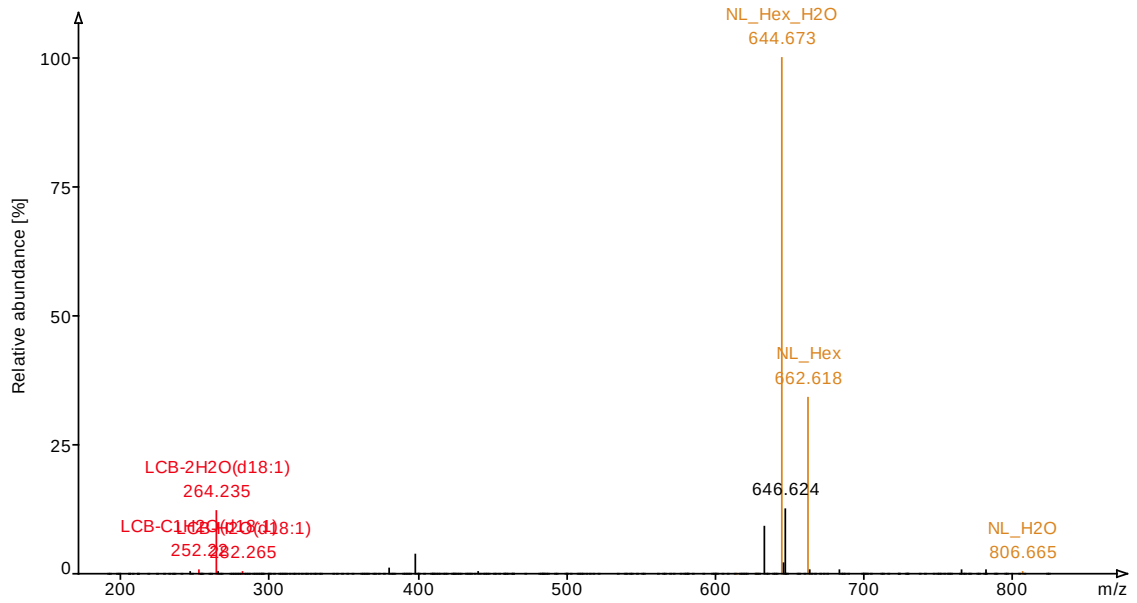
Novel species: HexCer t42:3

MS^2 spectrum of [M+HCOO]$^-$ (precursor *m/z*: 868.6548; retention time: 24.48 min; file: Brain_neg_1).



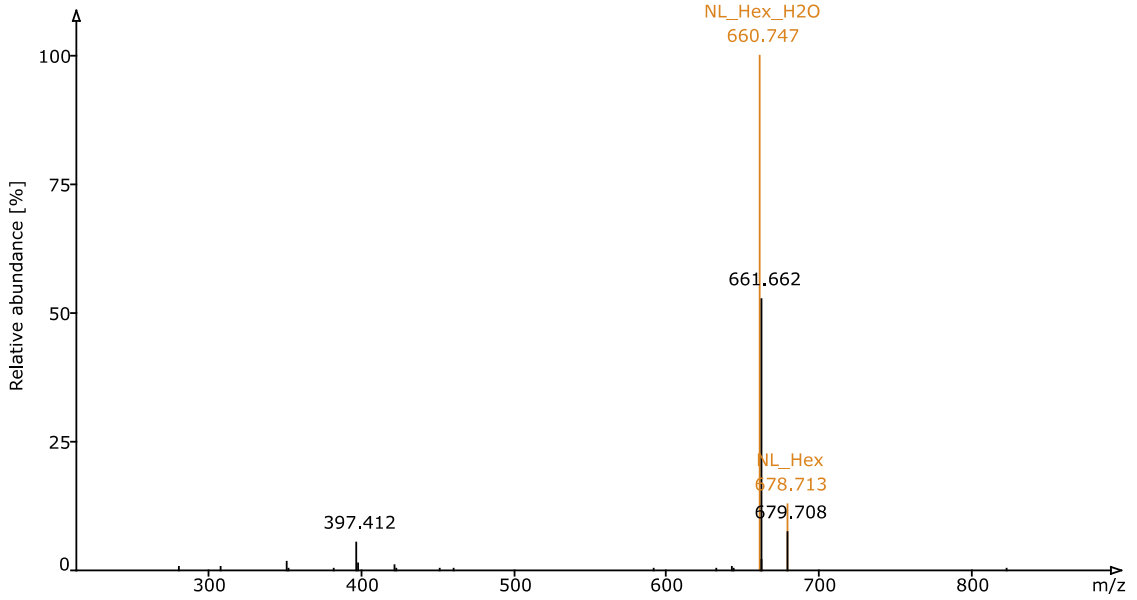
Novel species: HexCer d18:0/h24:3

MS^2 spectrum of [M+H]$^+$ (precursor *m/z*: 824.6614; retention time: 24.65 min; file: Brain_pos_4.



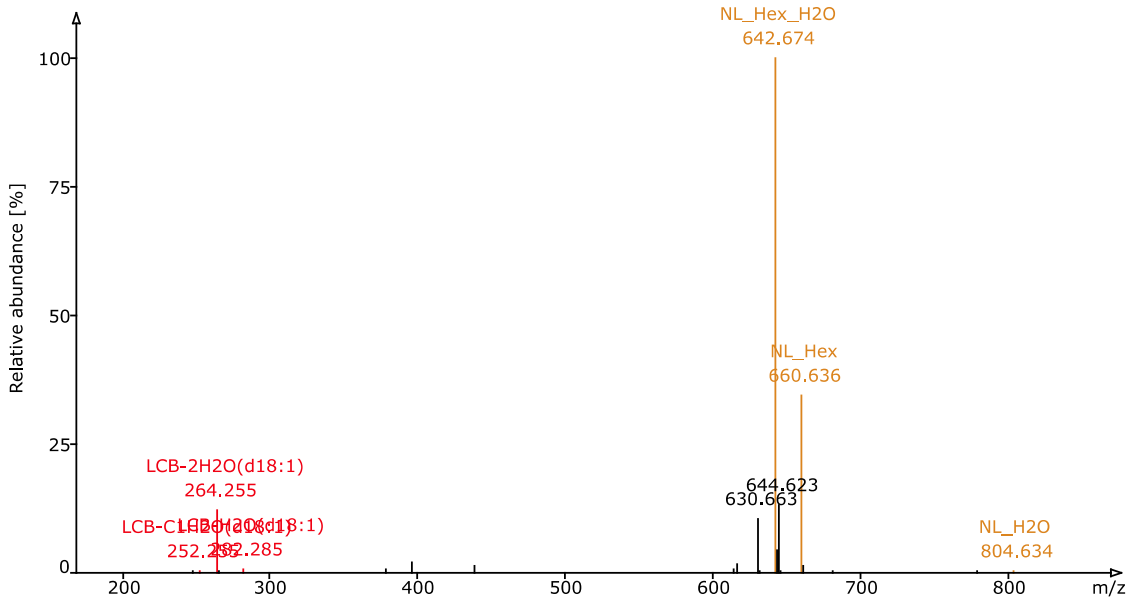
Novel species: HexCer d18:1/h25:0 (and t43:1)

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 824.6979; retention time: 28.52 min; file: Brain_pos_1).



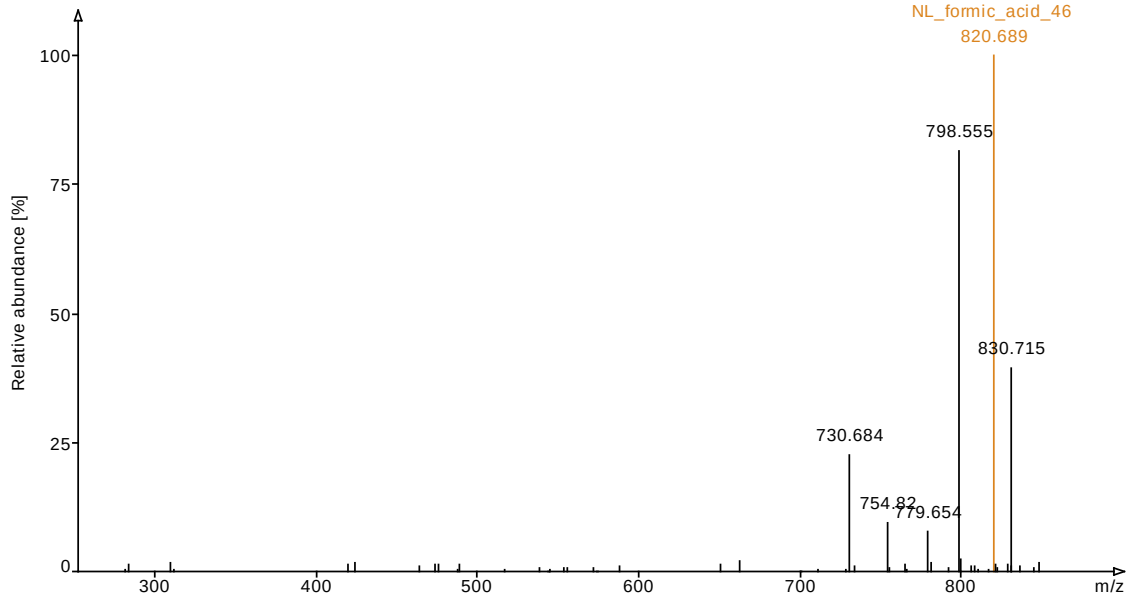
Novel species: HexCer t43:1

MS^2 spectrum of $[M-H]^-$ (precursor *m/z*: 840.6948; retention time: 28.60 min; file: Brain_neg_1).



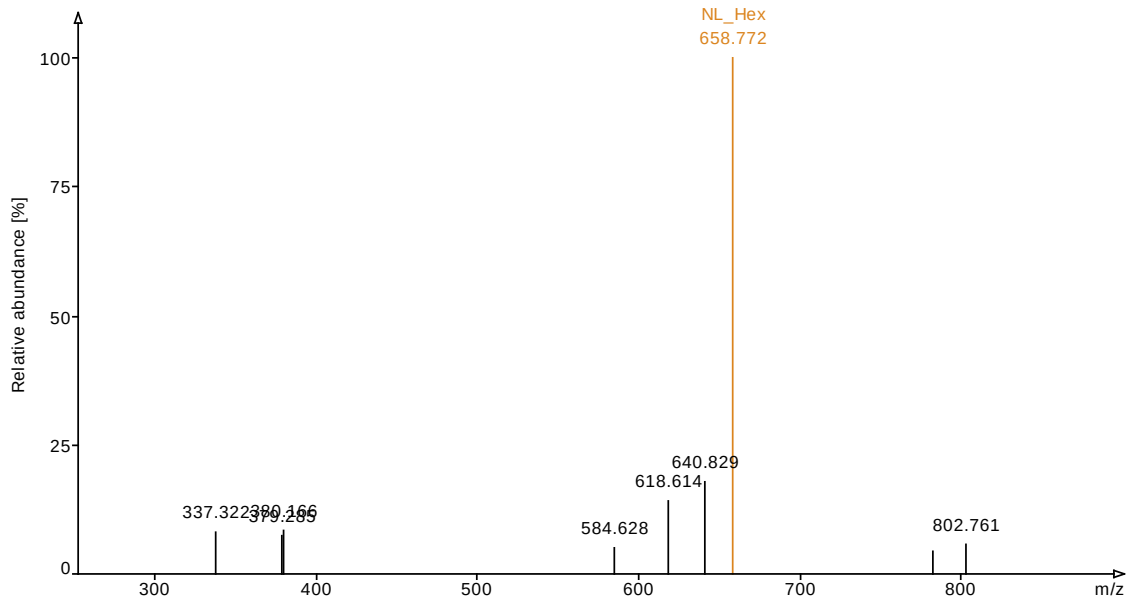
Novel species: HexCer d18:1/h25:1

MS^2 spectrum of $[M+H-H_2O]^+$ (precursor *m/z*: 822.6828; retention time: 26.89 min; file: Brain_pos_2).



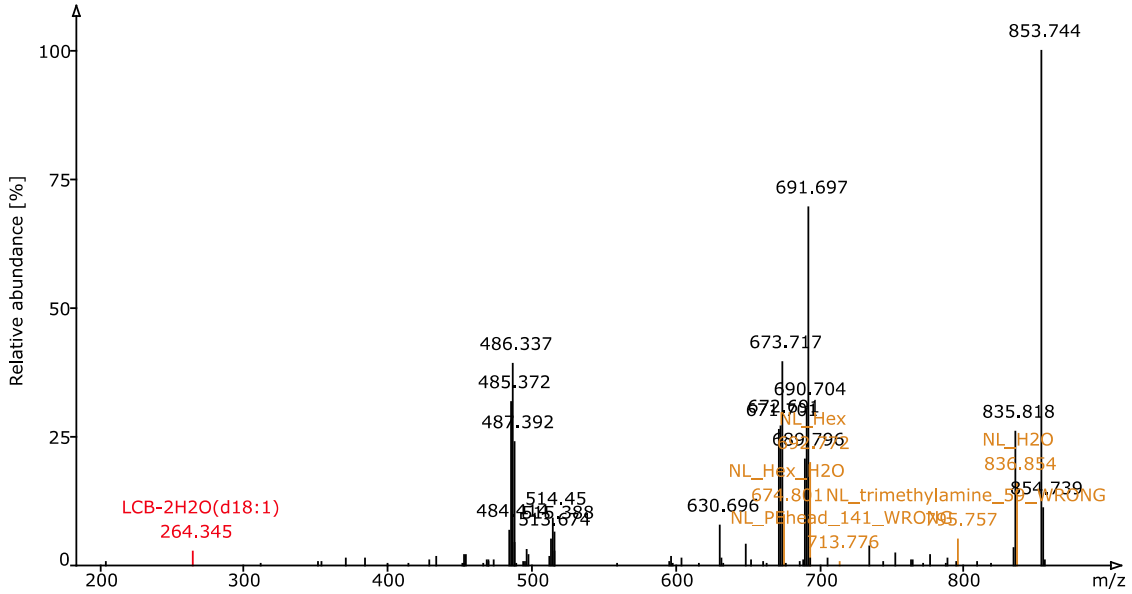
Novel species: HexCer d43:3

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 866.6778; retention time: 26.15 min; file: Brain_neg_3).



Novel species: HexCer d43:3

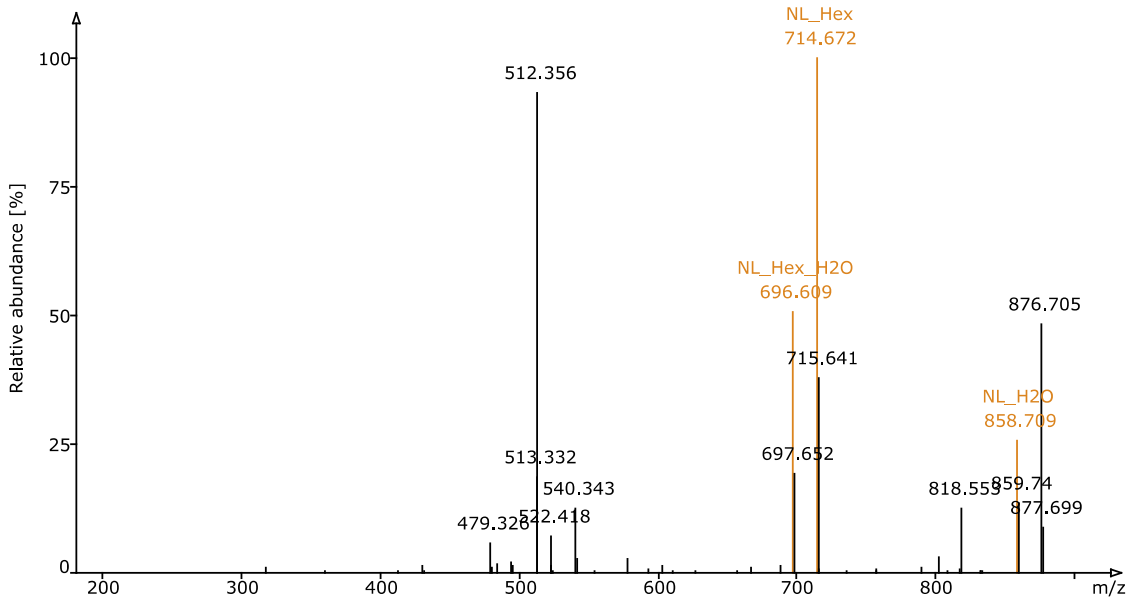
MS^3 spectrum of [M+HCOO]$^-$ (precursor *m/z*: 820.6893; retention time: 26.16 min; file: Brain_neg_3).



Novel species: HexCer d18:1/h26:1 (and t44:2)

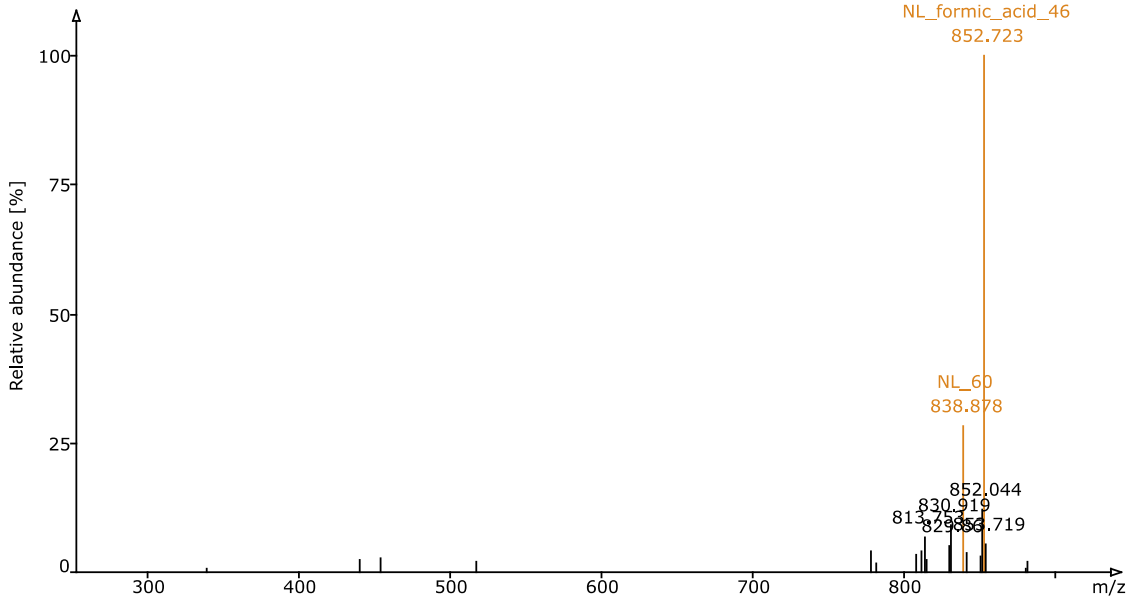
MS^2 spectrum of [M+H]$^+$ (precursor *m/z*: 854.7081; retention time: 27.75 min; file: Brain_pos_1).

Comment: detected in a mixed spectrum together with something else; [M+Na]$^+$ spectra are clean – see next spectrum.



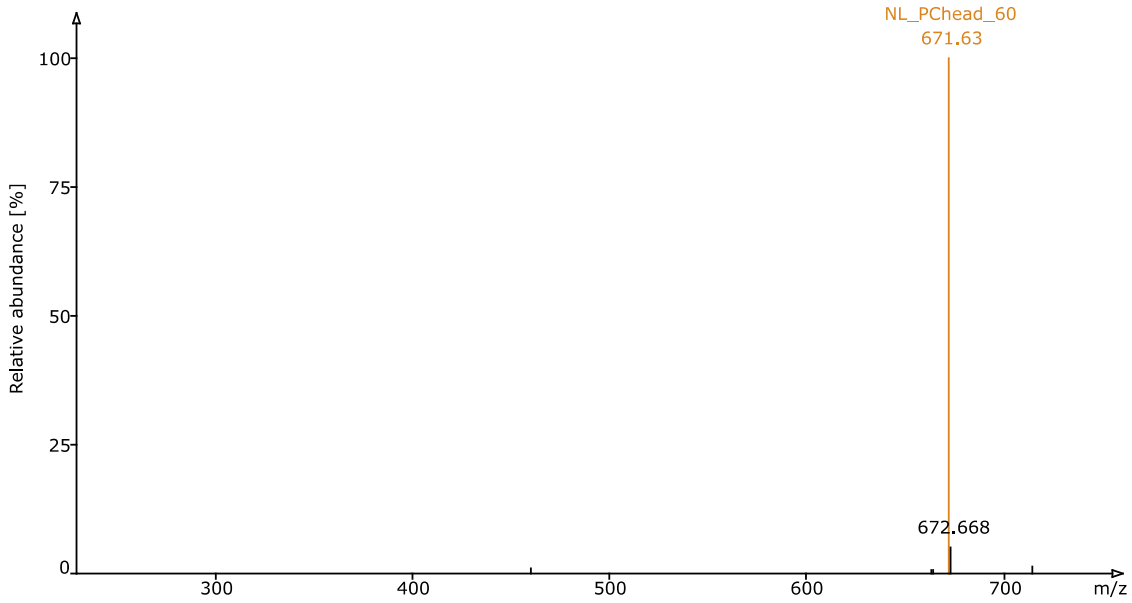
Novel species: HexCer t44:2

MS^2 spectrum of $[M+Na]^+$ (precursor *m/z*: 876.6905; retention time: 27.76 min; file: Brain_pos_1).



Novel species: HexCer t44:2

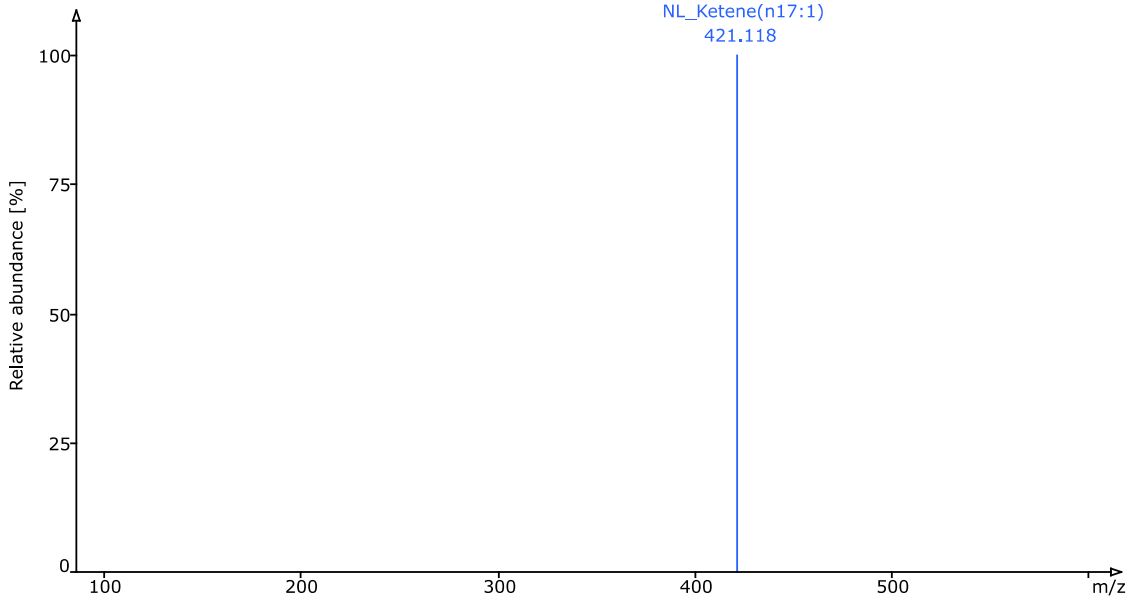
MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 898.6999; retention time: 27.78 min; file: Brain_neg_1).



Novel species: SM d16:1/n17:1

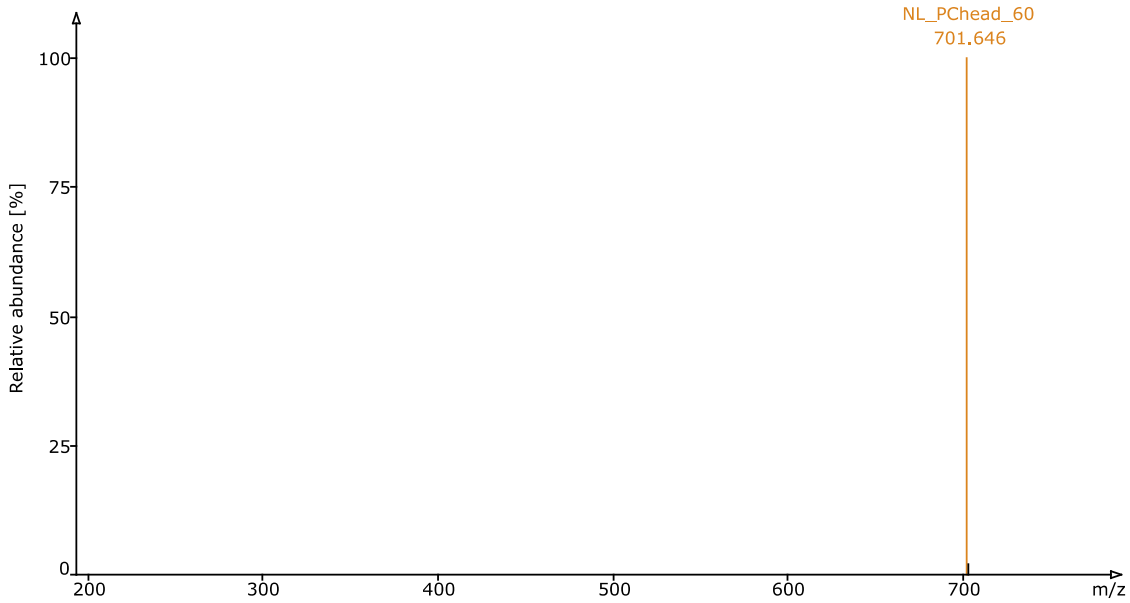
MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 731.5365; retention time: 15.64 min; file: Serum_neg_5).

Comment: MS^2 spectrum does not reveal any chain information.



Novel species: SM d16:1/n17:1

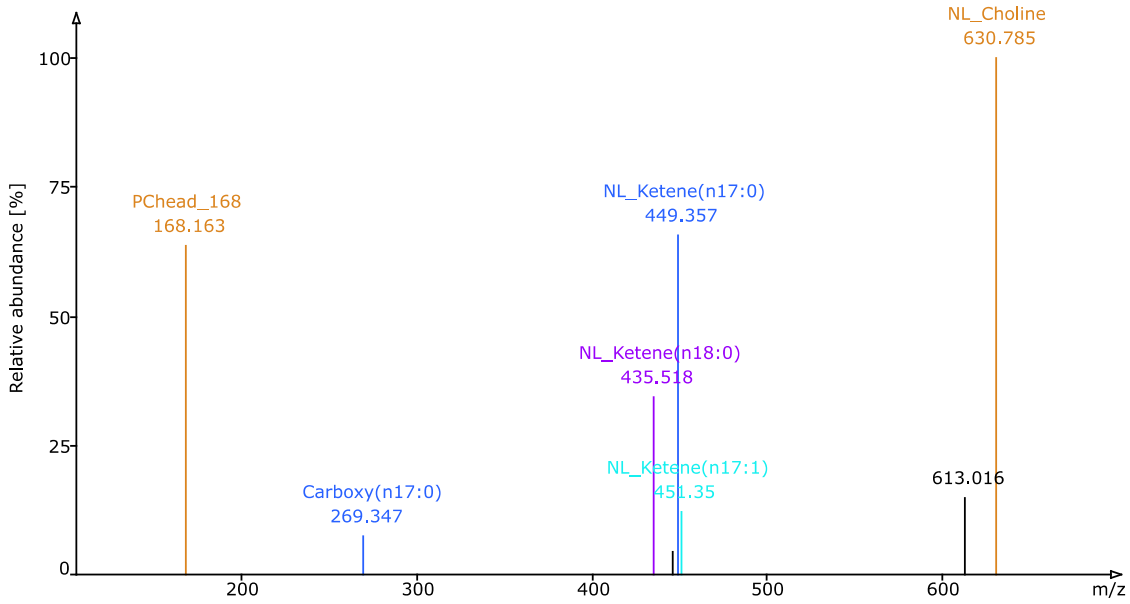
MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 671.6300; retention time: 15.64 min; file: Serum_neg_5).



Novel species: SM d18:0/n17:1

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 761.5852; retention time: 20.11 min; file: Serum_neg_3).

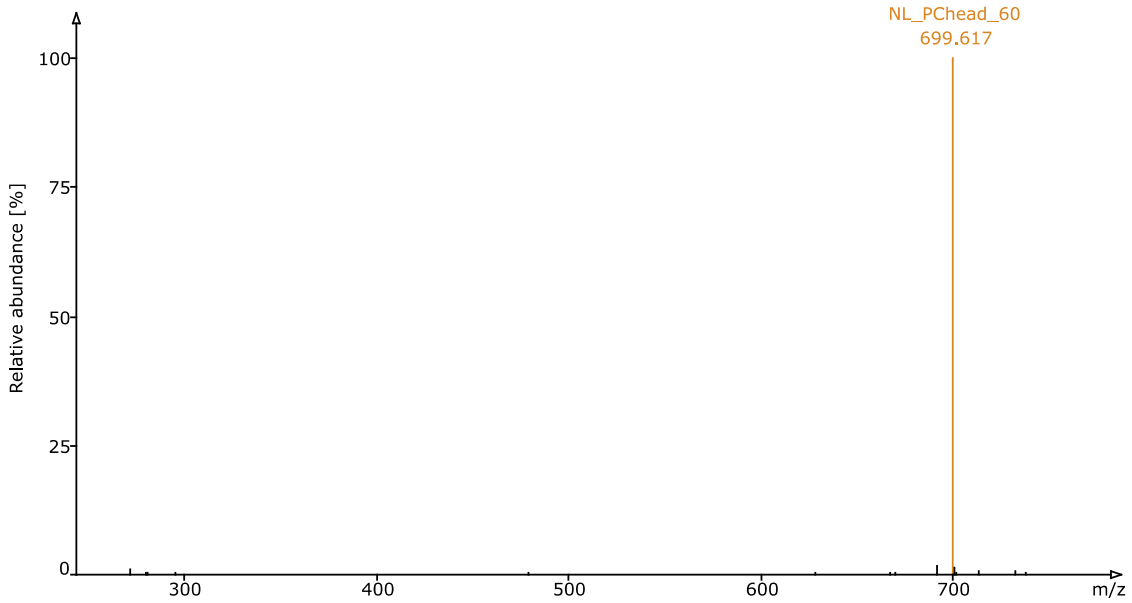
Comment: MS^2 spectrum does not reveal any chain information.



Novel species: SM d18:0/n17:1

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 701.6457; retention time: 20.11 min; file: Serum_neg_3).

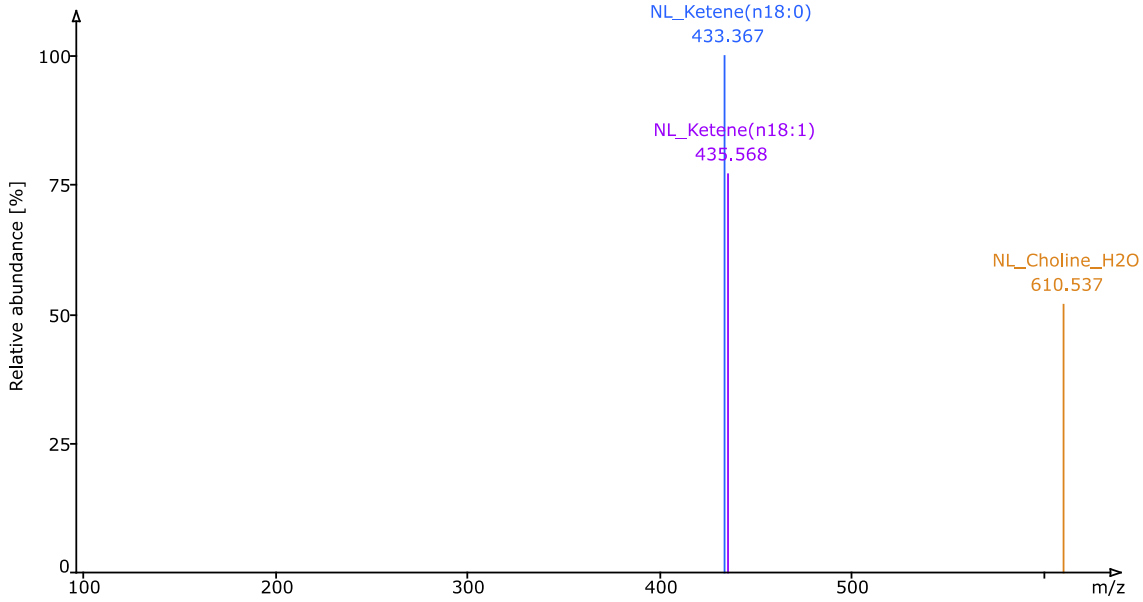
Comment: detected in a mixed spectrum together with the species SM d18:1/n17:0 and SM d17:1/n18:0.



Novel species: SM d17:2/n18:0

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 759.5686; retention time: 18.33 min; file: Serum_neg_5).

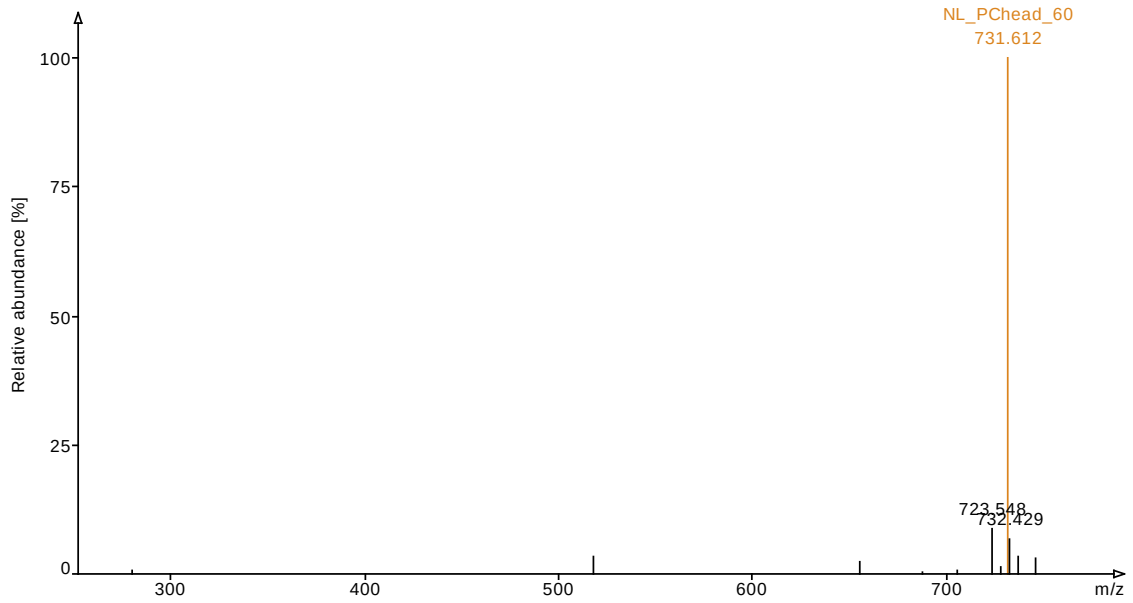
Comment: MS^2 spectrum does not reveal any chain information.



Novel species: SM d17:2/n18:0

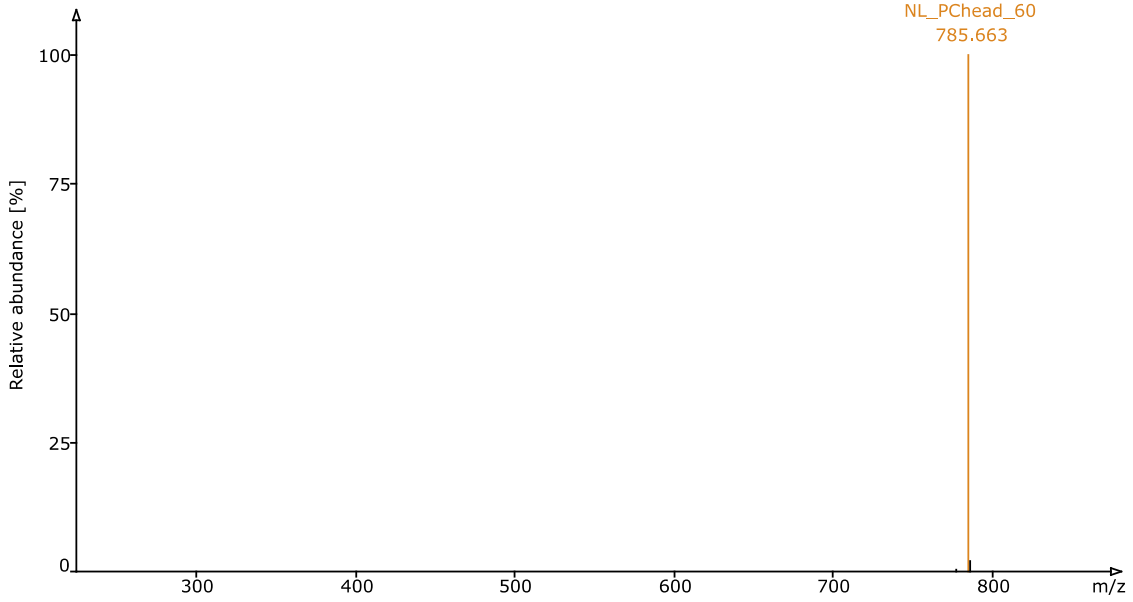
MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 699.6173; retention time: 18.33 min; file: Serum_neg_5).

Comment: detected in a mixed spectrum together with the species SM d17:1/n18:1.



Novel species: SM t36:1

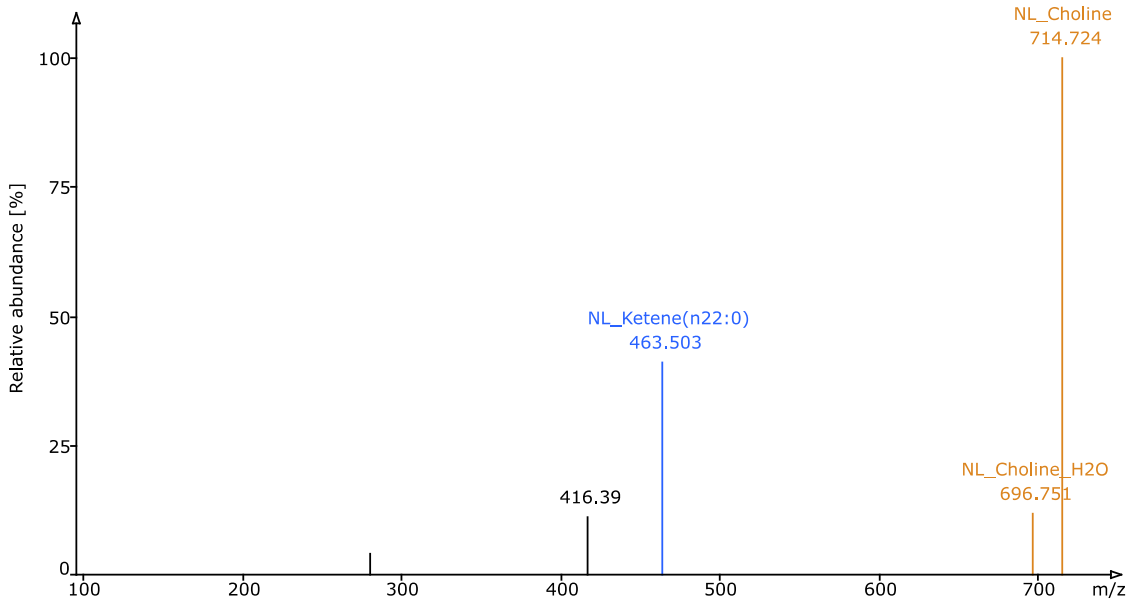
MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 791.5945; retention time: 18.01 min; file: Serum_neg_1).



Novel species: SM d19:1/n22:0

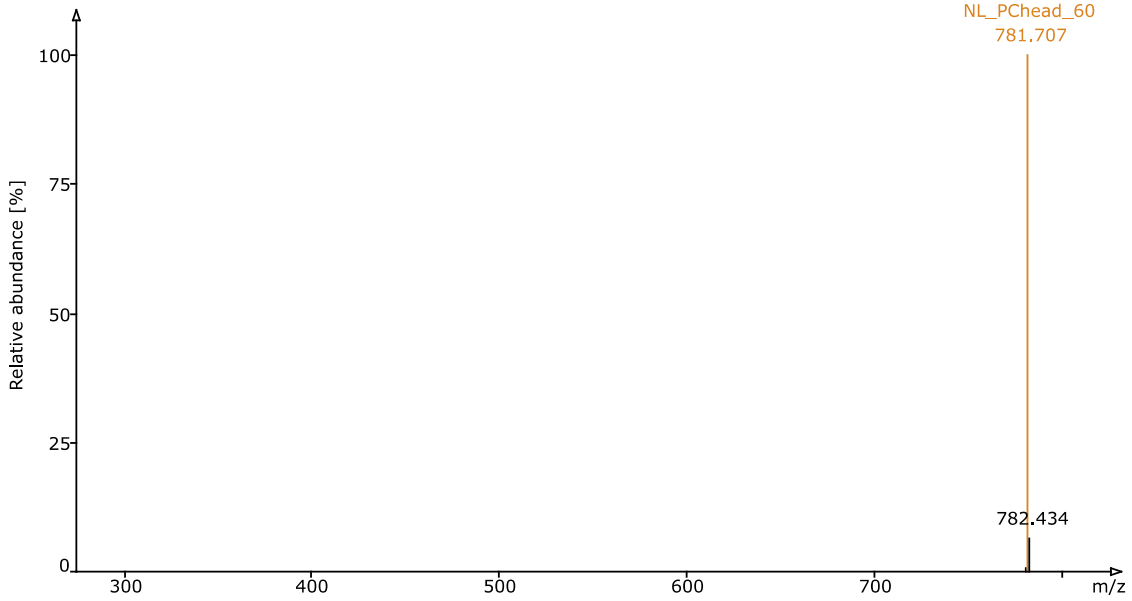
MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 845.6819; retention time: 26.32 min; file: Serum_neg_1).

Comment: MS^2 spectrum does not reveal any chain information.



Novel species: SM d19:1/n22:0

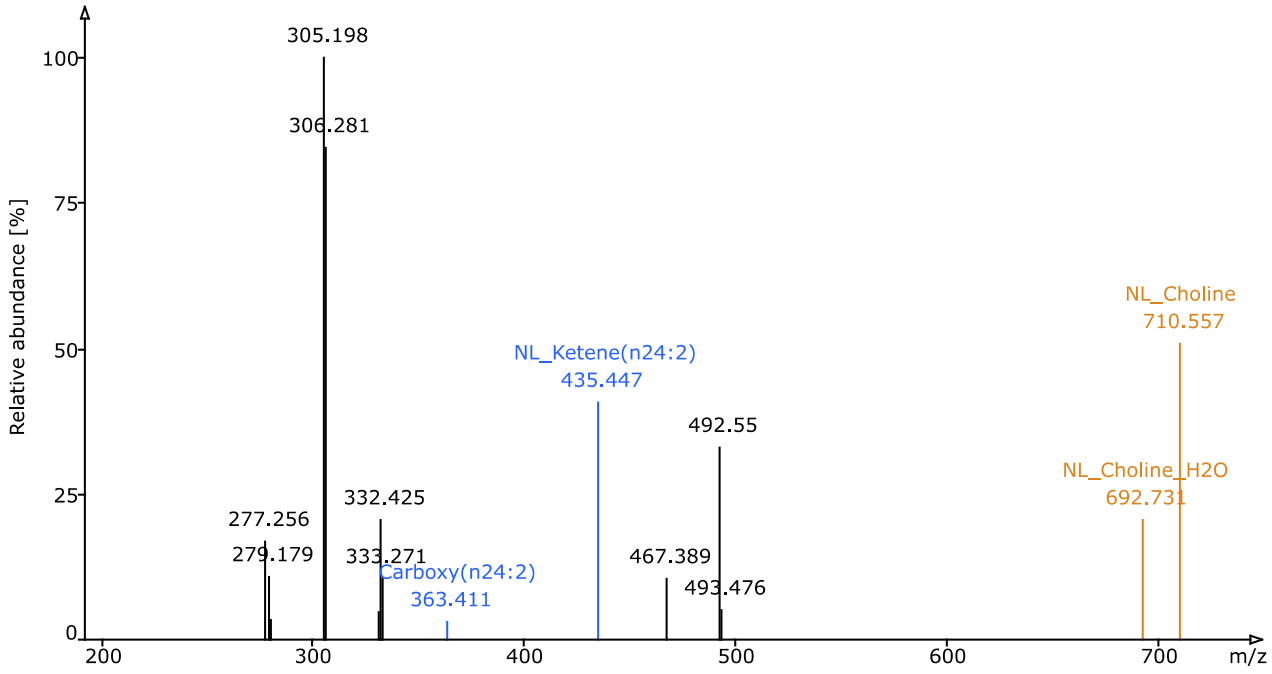
MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 785.6631; retention time: 26.33 min; file: Serum_neg_1).



Novel species: SM d17:1/n24:2

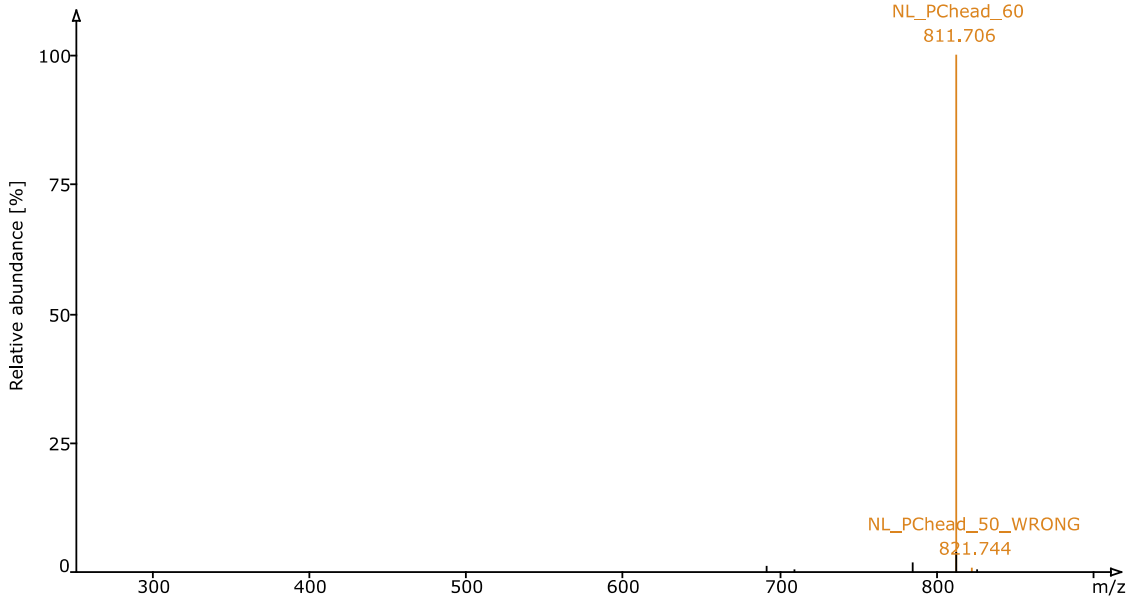
MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 841.6518; retention time: 23.21 min; file: Serum_neg_3).

Comment: MS^2 spectrum does not reveal any chain information.



Novel species: SM d17:1/n24:2

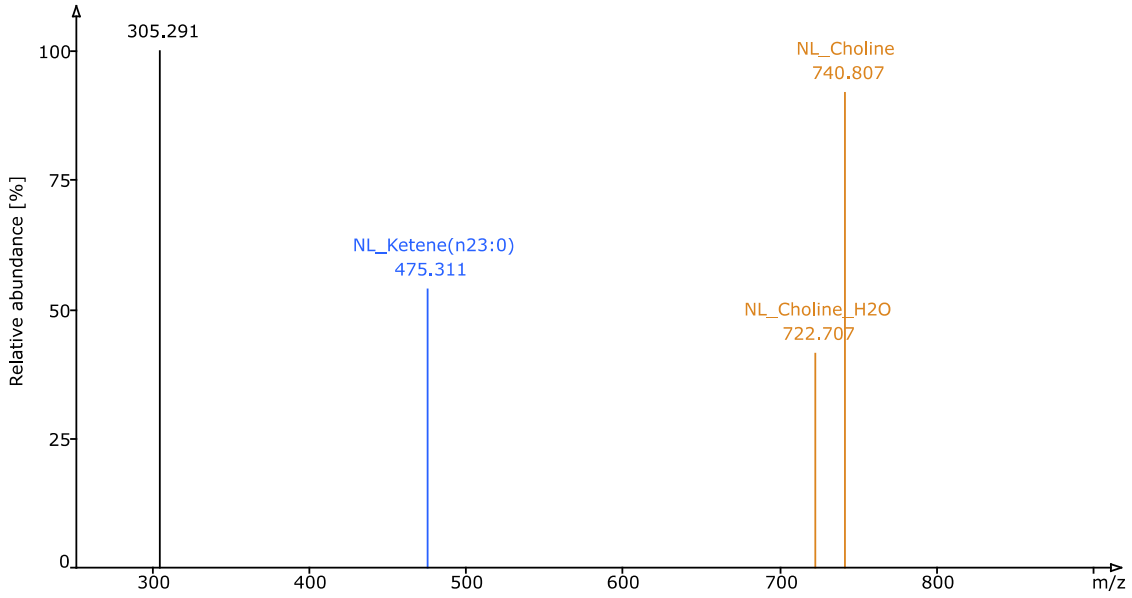
MS^3 spectrum of [M+HCOO]$^-$ (precursor *m/z*: 781.7070; retention time: 23.21 min; file: Serum_neg_3).



Novel species: SM d20:2/n23:0

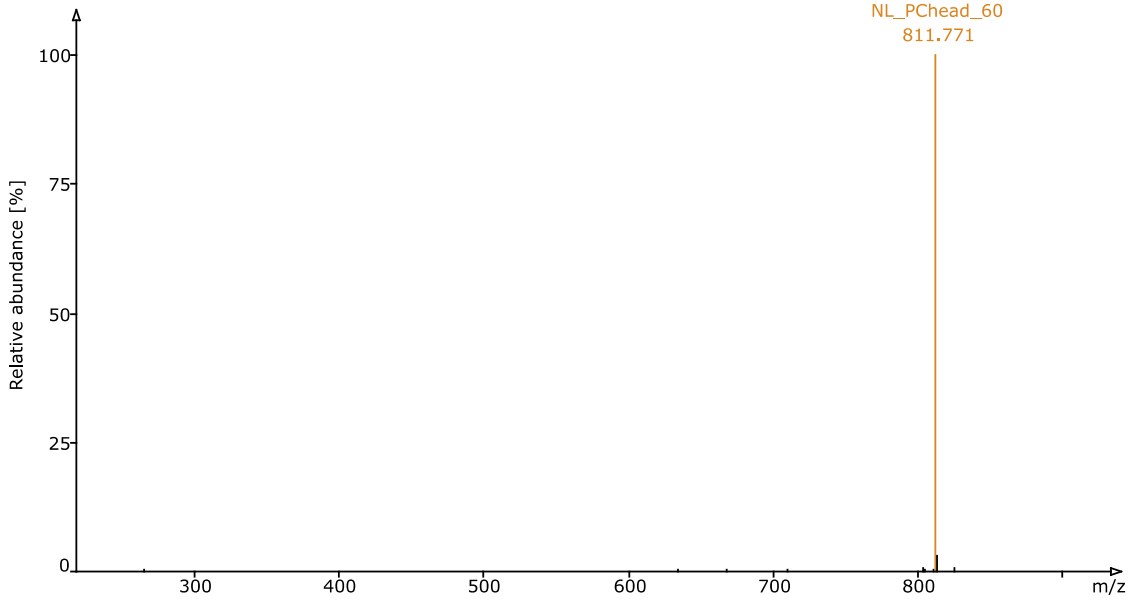
MS^2 spectrum of [M+HCOO]$^-$ (precursor *m/z*: 871.6979; retention time: 27.07 min; file: Serum_neg_1).

Comment: MS^2 spectrum does not reveal any chain information.



Novel species: SM d20:2/n23:0

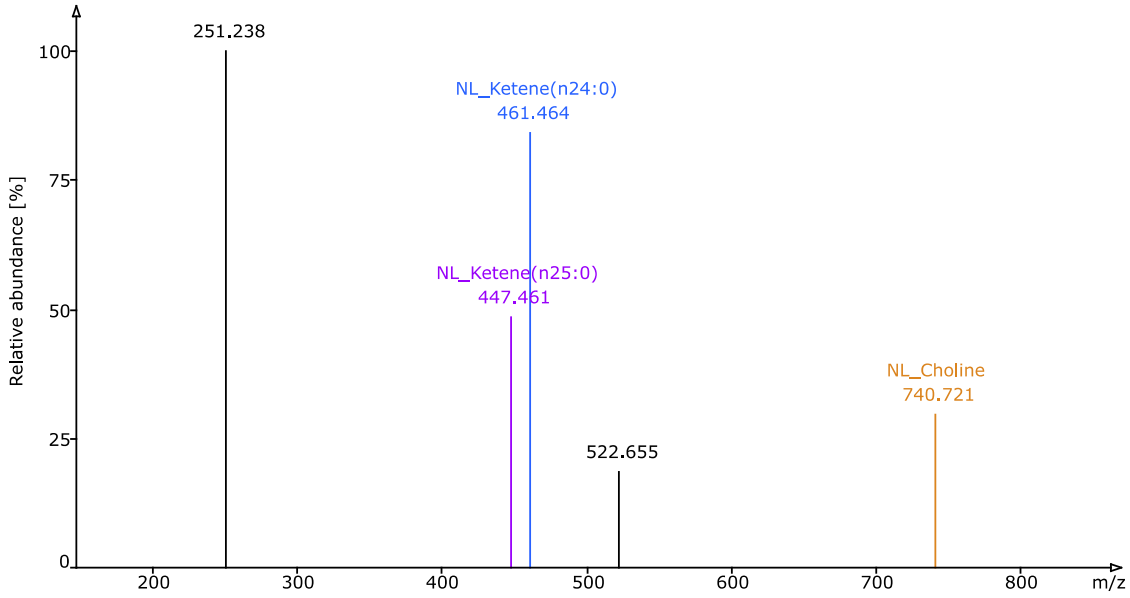
MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 811.7059; retention time: 27.07 min; file: Serum_neg_1).



Novel species: SM d19:2/n24:0

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 871.6974; retention time: 27.04 min; file: Serum_neg_4).

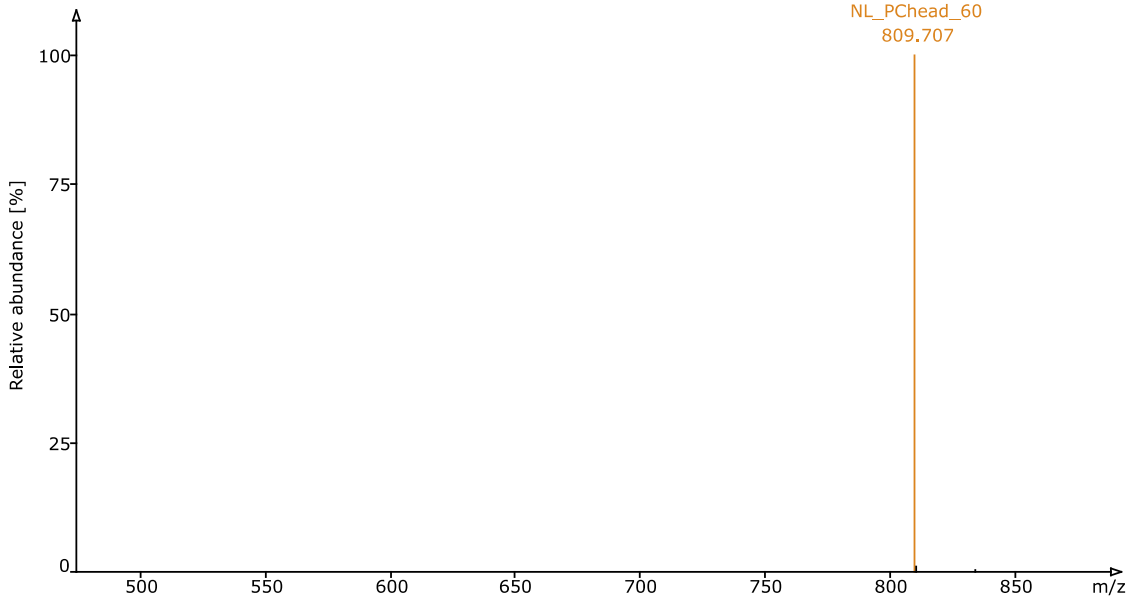
Comment: MS^2 spectrum does not reveal any chain information.



Novel species: SM d19:2/n24:0

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 811.7707; retention time: 27.05 min; file: Serum_neg_4).

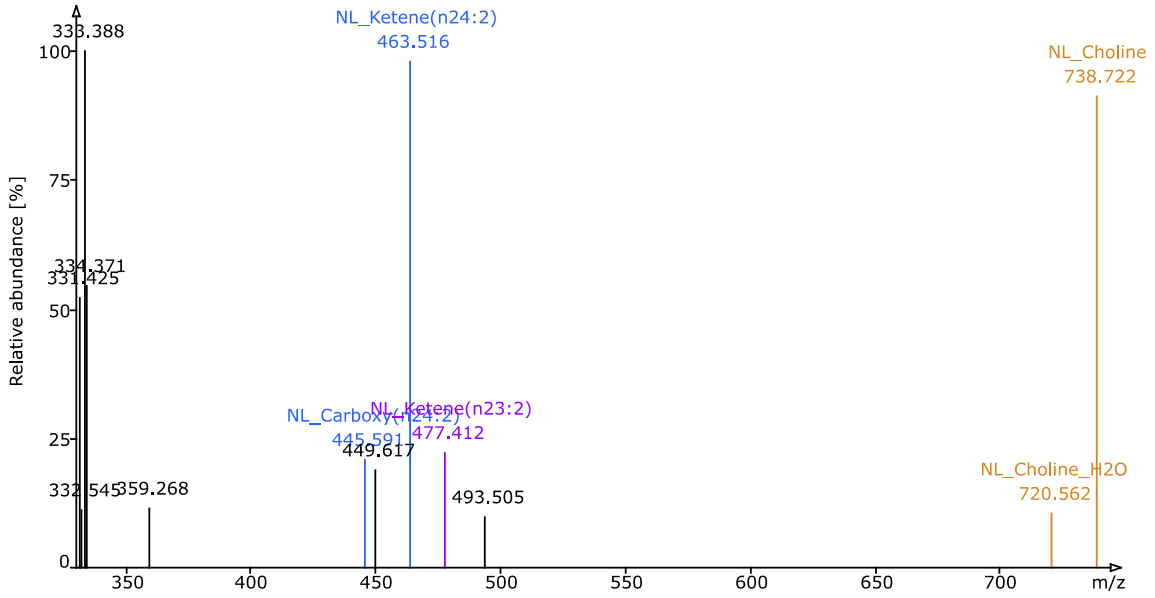
Comment: detected in a mixed spectrum together with the species SM d18:2/n25:0.



Novel species: SM d19:1/n24:2

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 869.6806; retention time: 25.20 min; file: Serum_neg_1).

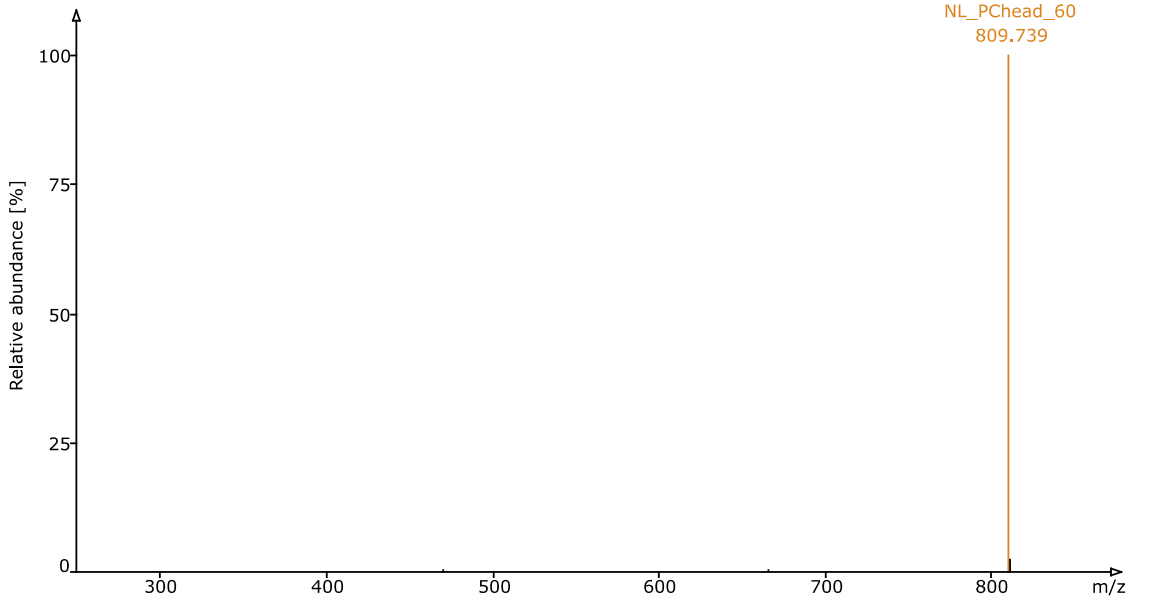
Comment: MS^2 spectrum does not reveal any chain information.



Novel species: SM d19:1/n24:2

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 809.7066; retention time: 25.20 min; file: Serum_neg_1).

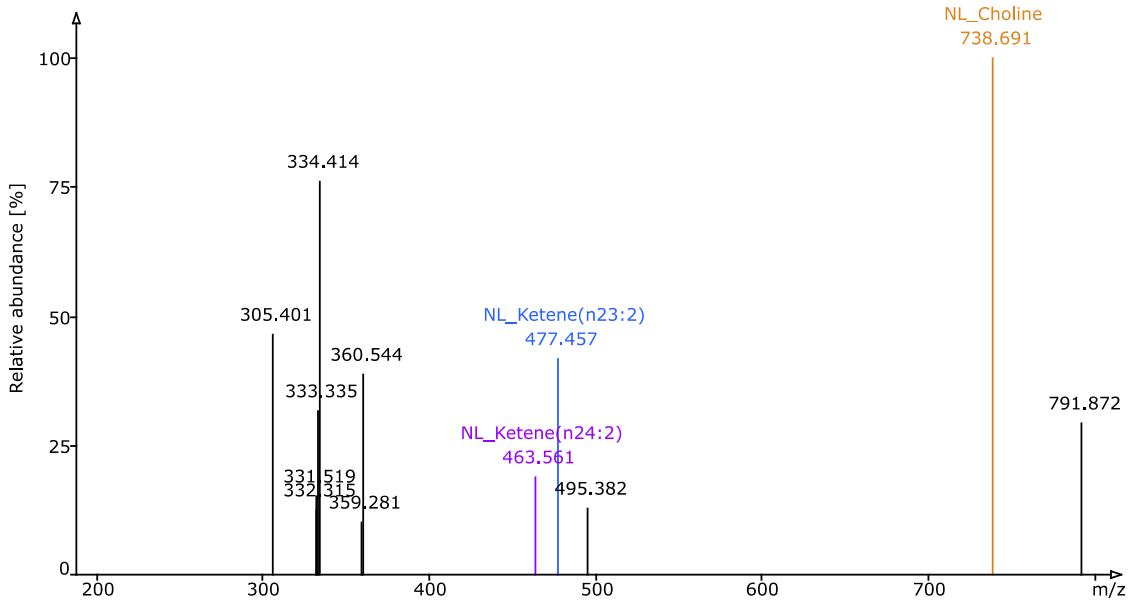
Comment: detected in a mixed spectrum together with the species SM d20:1/n23:2.



Novel species: SM d20:1/n23:2

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 869.6811; retention time: 25.16 min; file: Serum_neg_5).

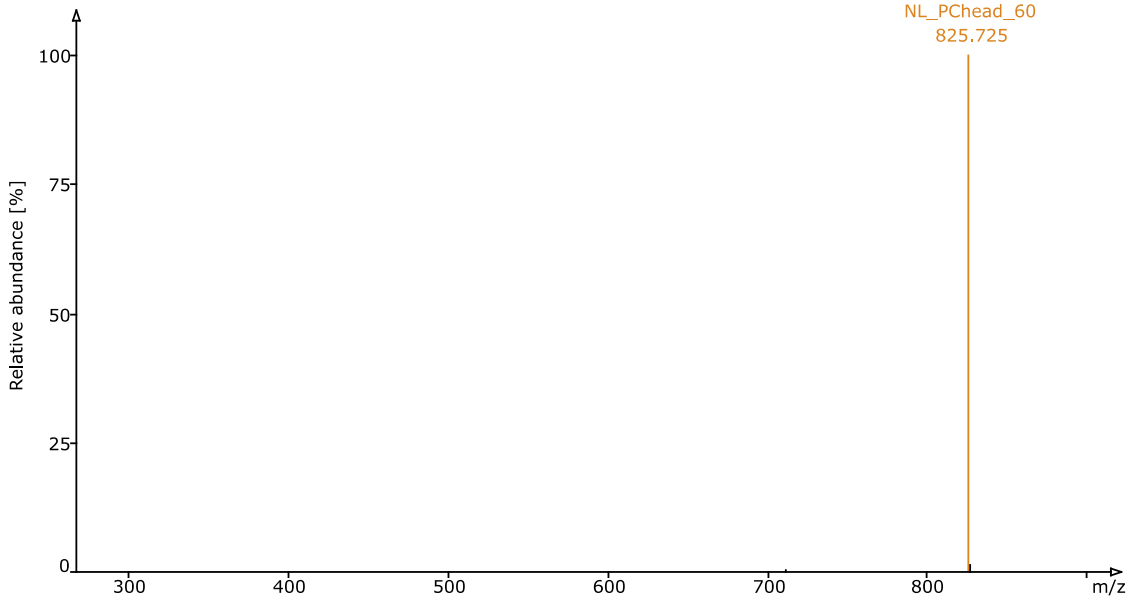
Comment: MS^2 spectrum does not reveal any chain information.



Novel species: SM d20:1/n23:2

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 809.7394; retention time: 25.17 min; file: Serum_neg_5).

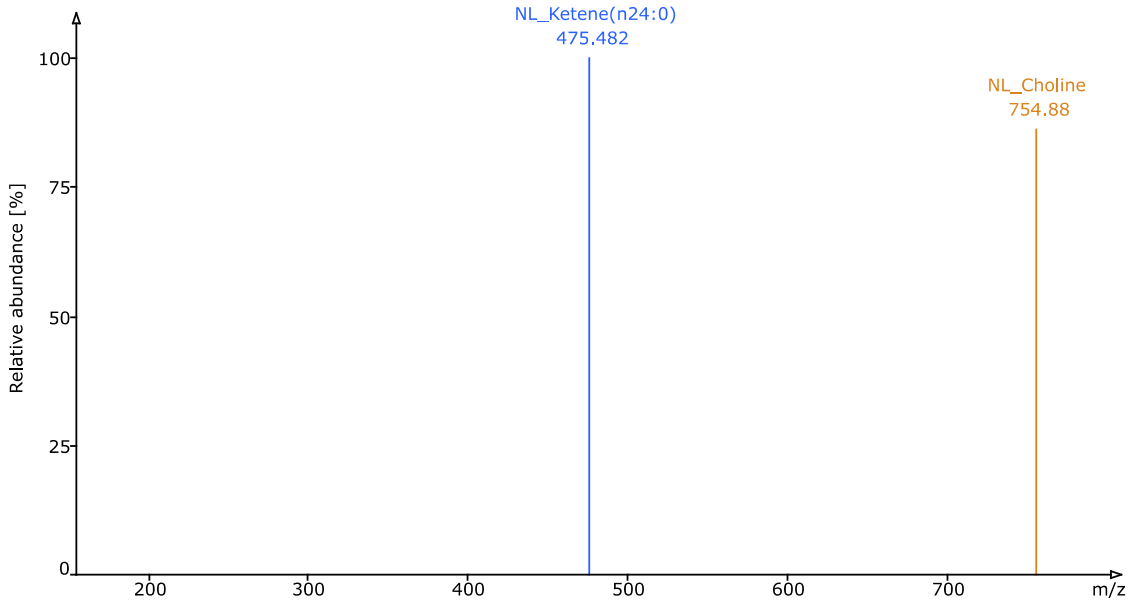
Comment: detected in a mixed spectrum together with the species SM d19:1/n24:2.



Novel species: SM d20:2/n24:0

MS^2 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 885.7090; retention time: 28.10 min; file: Serum_neg_2).

Comment: MS^2 spectrum does not reveal any chain information.



Novel species: SM d20:2/n24:0

MS^3 spectrum of $[M+HCOO]^-$ (precursor *m/z*: 825.7254; retention time: 28.10 min; file: Serum_neg_2).