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LipidSearch 5.0: A New Software for Processing Data from Direct Infusion and LC-MS High Resolution Mass Spectrometry Based Lipidomics Workflows

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ABSTRACT

Thermo Scientific™ LipidSearch™ 5.0 software is designed to identify lipid species analyzed by infusion MS and MS/MS data from total lipid extracts.

Mass spectrometric analysis of a 33-component standard mixture was performed on a Thermo Scientific™ Orbitrap Fusion Lumos™ Tribrid™ mass spectrometer operated at a resolution of 120,000 to 500,000 (FWHM at *m/z* 200) for the MS and MS/MS experiments.

First, "sum-composition" MS level lipid identification was performed by searching against an in-silico database of user-defined lipid species.

Then, MS/MS data was processed to identify molecular lipid species by searching against the predicted mass spectral product ions based upon the results obtained in the MS level identification.

Results from both positive and negative ion MS or MS/MS experiments were then merged together to give a comprehensive and correct level of lipid annotation by correlating individual lipid identifications.

INTRODUCTION

Direct infusion and LC-MS high resolution accurate mass (HRAM) spectrometry based workflows are typically processed with disparate and often multiple software tools. One of the main bottlenecks limiting the wide-spread application of these workflows for high-throughput untargeted lipidomics analysis has been the lack of integrated software tools for automated data processing, database searching and visualization of complex lipidomics data sets. In addition, the lack of clear standards for reporting MS-based lipidomics makes it challenging to compare the lipid identifications obtained from these different approaches. We report here LipidSearch 5.0 software designed specifically for searching data from HRAM workflows for untargeted lipidomics analysis.

MATERIALS AND METHODS

Sample Preparation

Lipid standards (Table 1) were obtained from Avanti Polar Lipids (Alabaster, AL) as powders or stock solutions in chloroform. A mixture of 21 different lipid standards was prepared by combining the stock solutions. The lipid mixture (500 µL) was mixed with 100 µL of SPLASH™ (Avanti 330707) labeled internal standard mixture in methanol and diluted with 4:2:1 isopropanol/methanol/chloroform with 20mM ammonium formate to give an estimated range of final concentrations from 0.1 ~ 100 µg/mL.

Mass Spectrometry

Mass spectrometric analysis was performed on an Orbitrap Fusion Lumos MS. The Orbitrap Fusion Lumos MS was operated in a full MS scan mode (resolution 120,000 at *m/z* 200) followed by ddMS² (120K resolution). The AGC target value was set at 4E5 and 1E5 for the MS and MS/MS scans, respectively. The maximum injection time was 50 ms for MS and 450 ms for MS/MS. HCD was performed with a stepped collision energy of 30 ± 10% for neg. and 30 ± 5% for pos. ion mode with an isolation window of 1.0 Da. The lipid mixture was infused at a flow rate of 3 µL/min. Additional experiments were performed on a Thermo Scientific™ Q Exactive™ HF mass spectrometer operating at 240K res. for MS and 120K res. for MS/MS scans from 280.25 – 1000.90 Da in 1.0009 Da steps.

Data Analysis

Lipid identification was performed with LipidSearch 5.0 pre-release software. First, the MS data was searched for monoisotopic precursor ions (Figure 1) specified in a user-editable database using the parameters given in Table 2. Next, a product ion search (Figure 2) was performed using parameters given in Table 3. Finally, MS and MS/MS search results were merged (Figure 3) into sum composition (precursor) and molecular (product ion) results, respectively (Table 4).

Figure 1. LipidSearch 5.0 software Precursor Ion Workflow

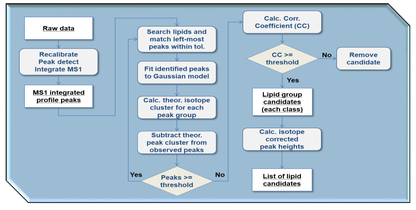


Figure 2. LipidSearch 5.0 software Product Ion Workflow

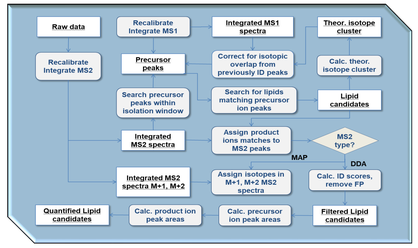


Table 1. Elemental Composition and Calc. *m/z* Values of the Lipid Standard Adducts

#	Lipid Species	Elemental Comp.	m/z				
			$[M+H-H_2O]^+$	$[M-H]^-$	$[M+H]^+$	$[M+NH_4]^+$	$[M+HCO_2]^-$
1	Sphingosine d17:1	$C_{17}H_{35}NO_2$	268.26349	284.25950	286.27406		330.26498
2	Sphingosine d18:1	$C_{18}H_{37}NO_2$	282.27914	298.27515	300.28971		344.28063
3	$d_3$18:1 MG	$C_{21}H_{33}D_3O_4$			364.34387	381.37042	
4	Cholesterol (d7)	$C_{27}H_{39}D_7O$	376.39552		393.39825	411.43263	
5	17:1 Lyso PA	$C_{20}H_{39}O_7P$		421.23606	423.25062	440.27717	
6	Lyso SM d17:1	$C_{22}H_{47}N_2O_5P$			451.32954		495.32046
7	$d_3$18:1 LPE	$C_{23}H_{39}D_3NO_7P$		485.33785	487.35240		
8	18:1 Lyso PC	$C_{26}H_{52}NO_7P$			522.35542		566.34634
9	$d_3$18:1 LPC	$C_{26}H_{49}D_3NO_7P$			529.39935		573.39028
10	d18:1/16:0 Cer	$C_{34}H_{67}NO_3$	520.50881	536.50482	538.51937		582.51030
11	d18:1/18:0 Cer	$C_{36}H_{71}NO_3$	548.54011	564.53612	566.55067		610.54160
12	d18:0/18:1 Cer	$C_{36}H_{71}NO_3$	548.54011	564.53612	566.55067		610.54160
13	15:0/$d_3$18:1 DG	$C_{36}H_{63}D_3O_5$			588.55789	605.58444	
14	17:0/14:1 PA	$C_{34}H_{65}O_8P$		631.43443		650.47553	
15	$d_3$18:1 ChE	$C_{45}H_{71}D_3O_2$			658.65140	675.67794	
16	19:0 ChE	$C_{46}H_{82}O_2$			667.63876	684.66531	
17	15:0/$d_3$18:1 PA	$C_{36}H_{65}D_3O_8P$		666.50967		685.55077	
18	17:0/14:1 PE	$C_{36}H_{70}NO_8P$		674.47663	676.49118		
19	17:0/14:1 PG	$C_{37}H_{71}O_{10}P$		705.47121	707.48576		724.51231
20	15:0/$d_3$18:1 PE	$C_{38}H_{72}D_3NO_8P$		709.55187	711.56642		
21	17:0/14:1 PC	$C_{39}H_{76}NO_8P$			718.53813		762.52906
22	d18:1/18:1(9Z) SM	$C_{41}H_{83}N_2O_6P$			729.59050		773.58143
23	15:0/$d_3$18:1 PG	$C_{39}H_{68}D_3O_{10}P$		740.54645	742.56100	759.58755	
24	15:0/$d_3$18:1 PC	$C_{41}H_{73}D_3NO_8P$			753.61337		797.60429
25	15:0/$d_3$18:1 PS	$C_{39}H_{67}D_3NO_{10}P$		753.541694	755.556248		
26	d18:1/$d_3$18:1 SM	$C_{41}H_{73}D_3N_2O_6P$			782.637918		760.628937
27	18:1(9Z) PC	$C_{44}H_{84}NO_8P$			786.600732		830.591658
28	17:0/14:1 PI	$C_{40}H_{75}O_{13}P$		793.487253	795.501806	812.528355	
29	15:0/$d_3$18:1/15:0 TG	$C_{51}H_{89}D_3O_6$			812.771904	829.798453	
30	15:0/$d_3$18:1 PI	$C_{42}H_{72}D_3O_{13}P$		828.562489	830.577043	847.603592	
31	18:0/16:0/18:1 TG	$C_{55}H_{102}O_6$			859.774917	876.801466	
32	18:1(6Z,9Z,6Z) TG	$C_{57}H_{104}O_6$			885.790567	902.817116	

Figure 3. LipidSearch 5.0 software Merging Group Annotations and Quantitation Workflow

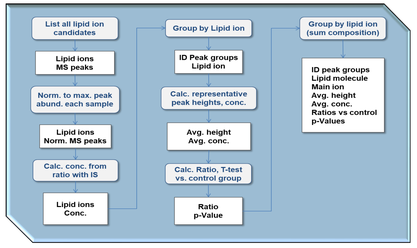


Table 2. Internal Standard Masses Used for Positive and Negative Ion Mass Correction

m/z	Polarity	Lipid Standard	Adduct
282.279141	pos	d18:1-Sphingosine	M+H-H_2O
300.289706	pos	d18:1-Sphingosine	M+H
376.395515	pos	d7-Cholesterol	M+H-H_2O
485.337850	neg	18:1(d7) Lyso Phosphatidylethanolamine	M-H
487.352403	pos	18:1(d7) Lyso Phosphatidylethanolamine	M+H
529.399353	pos	18:1(d7) Lyso Phosphatidylcholine	M+H
573.390279	neg	18:1(d7) Lyso Phosphatidylcholine	M+HCOO
666.509666	neg	18:1(d7) Phosphatidic Acid	M-H
675.677944	pos	18:1(d7) Cholesterol Ester	M+NH_4
709.551865	neg	18:1(d7) Phosphatidylethanolamine	M-H
753.541694	neg	18:1(d7) Phosphatidylserine	M-H
753.613369	pos	15:0-18:1(d7) Phosphatidylcholine	M+H
755.556248	pos	15:0-18:1(d7) Phosphatidylserine	M+H
797.604294	neg	15:0-18:1(d7) Phosphatidylcholine	M+HCOO
829.798453	pos	15:0-18:1(d7)-15:0 Triacylglycerol	M+NH_4

Table 3. Parameters for Precursor Ion and Product Ion Database Search

Search Parameter	MS	MS/MS
Search type	precursor IFW	product IFW
Data type	profile	profile
Merge mode	average	average
Merge range (min)	0.0 – 5.0	0.0 – 5.0
Recalibration mass tolerance (ppm)	5.0	5.0
Precursor threshold (counts)	1,000	10,000
Precursor mass tolerance (ppm)	1.0	3.0
Isotope correlation threshold	0.3	0.2
Isotope threshold (%)	0.1	1.0
Recalc. Intensity threshold (counts)	10	10
Maximum isotope number	3	3
Isotope Weight M0, M1, Mn	1.0, 1.0, 0.3	1.0, 1.0, 0.3
IFW Product Method		DDA or MAP, Isolation = 1.0 Da
Product ion threshold (%)		5.0
Product ion mass tolerance (ppm)		5.0

DATA PROCESSING

Data analysis with LipidSearch 5.0 software starts with the user configuring the database for the desired lipid species. Database entries for glycerolipids (Figure 4) serve to illustrate how the database is organized. Lipids are named by category, class and subclass[1]. However, given the nature of MS-based identification, partial structures are specified using SMILES notation. Substituents are specified at the sum composition (MS) or molecular species (MS/MS) level. Exact lipid structures are not recommended unless further experiments are performed to provide the positional isomer, cis/trans configuration and double bond locations[2]. Adducts, modifications such as labeling, the range of carbons and double bonds for each lipid subclass, and product ions are specified for pos. and neg. ion adducts as neutral loss (NL), head group, backbone or FA specific fragmentations.

Figure 4. Database Editor Details for Glycerolipids

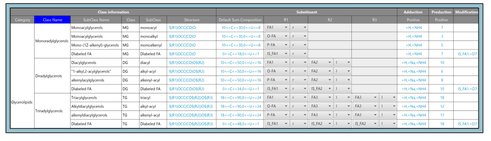


Figure 5. Example Parent Ion Search Results – Positive Ion MS Analysis of Standard Mixture

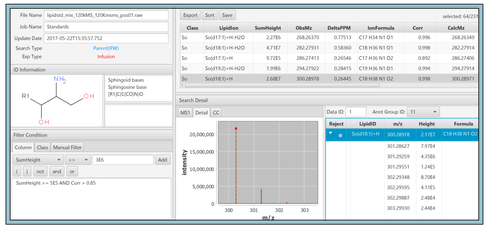
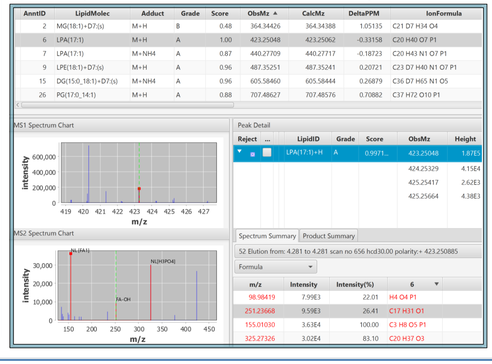


Figure 6. Example Product Ion Search Results – Positive Ion MS/MS Analysis of Standard Mixture



RESULTS

Search results obtained from MS (Figure 5) and MS/MS (Figure 6) experiments were merged at the sum composition and molecular lipid species levels, respectively. The merged results were manually compared for the 31 lipid species matching the standard mixture of 32 standards.

Two isomeric d36:1 ceramide species were detected as a mixture. A false positive was obtained for cholesterol (M+H-H_2O) due to in-source fragmentation of 19:0 ChE. Impurities and false positives were detected but were filtered out by peak height and lack of MS/MS confirmation. There were 5 lipid standards species that were detected at sum composition level due to the lack of MS/MS data. Due to incomplete database entries, 7 species were not confirmed at the MS/MS level.

Table 4. Merged Results Summary: Sum Composition and Molecular Levels – Standard Mixture

#	Lipid Standard	Confirmed SumComp	Confirmed Molec.	#	Deuterated Standard	Confirmed SumComp	Confirmed Molec.
1	Sph d17:1	☑	DB entry	3	MG $d_7$18:1	☑	☑
2	Sph d18:1	☑	DB entry	4	Cholesterol d7	☑	DB entry
X	Cholesterol (FP)	☑	DB entry	7	Lyso PE $d_7$18:1	☑	☑
5	Lyso PA 17:1	☑	☑	9	Lyso PC $d_7$18:1	☑	no MS2
6	Lyso SM d17:1	☑	DB entry	12	DG 15:0/ $d_7$18:1	☑	☑
8	Lyso PC 18:1	☑	☑	14	ChE $d_7$18:1	☑	DB entry
10	Cer d18:1_16:0	☑	☑	16	PA 15:0/ $d_7$18:1	☑	no MS2
11	Cer d36:1 (2 iso)	☑	☑	19	PE 15:0/ $d_7$18:1	☑	☑
13	PA 17:0/ 14:1	☑	☑	22	SM d18:1/$d_9$18:1	☑	☑
15	ChE 19:0	☑	DB entry	23	PG 15:0/ $d_7$18:1	☑	☑
17	PE 17:0/ 14:1	☑	☑	24	PC 15:0/ $d_7$18:1	☑	☑
18	PG 17:0/ 14:1	☑	☑	25	PS 15:0/ $d_7$18:1	☑	no MS2
20	PC 17:0/ 14:1	☑	☑	28	TG $d_7$18:1-15:0	☑	no MS2
21	SM d18:1/18:1	☑	☑	29	PI 15:0/$d_7$18:1	☑	no MS2
26	PC 18:1/18:1	☑	☑				
27	PI 17:0/ 14:1	☑	☑				
30	TG 18:1_16:0_18:1	☑	☑				
31	TG 18:1_18:1_18:1	☑	☑				

CONCLUSIONS

- LipidSearch 5.0 software is a new software enabling data processing for UHRAM infusion workflows from Orbitrap-based mass spectrometers leading to exciting new possibilities in lipidomics research.
- No false negatives were observed when analyzing mixture of 32 known standards that varied in response by at least 4 orders of magnitude in ion abundance (3 orders in concentration).
- MS/MS confirmation is being repeated to fully identify all of the standards in the mixture and the m/z 369 from cholesterol can be eliminated by properly adjusting ion source conditions. Missing sphingolipid and cholesterol ester DB product ion entries are being added.
- Final validation is being conducted on the merge step and calculation of normalized and lipid quantitation. Beta testing is expected soon followed by release of the software.

REFERENCES

1. Fahy E, et al., *J. Lipid Res.* **2005,** 46: 839-861.
2. Ryan and Reid. *Acc. Chem. Res.* **2016**, 49, 1596−1604.

TRADEMARKS/LICENSING