Software Utilizing Positive and Negative Ion MS^2/MS^3 HCD and CID Spectra for Improved MS^n Lipid Identification

David A Peake[1], Reiko Kiyonami[1], Daniel Gachotte[2], Gavin E Reid[3], Yasuto Yokoi[4], and Andreas Hühmer[1]

[1]Thermo Fisher Scientific, San Jose, CA, United States; [2]CORTEVA Agriscience, Indianapolis, IN, United States; [3]The University of Melbourne, Parkville, Victoria, Australia; [4]Mitsui Knowledge Industry, Atago, Tokyo, Minato-ku, Japan

ABSTRACT

Thermo Scientific™ LipidSearch™ software version 4.2 for untargeted lipidomics with LC dd-MS^n analysis provides enhanced lipid characterization by combining information obtained in HCD and CID MS^2 and MS^3 product ion spectra. This lipid workflow is demonstrated on a new Tribrid™ Orbitrap™ mass spectrometer designed for characterization of small molecules such as metabolites and lipids. Total lipid extracts from western corn rootworm larvae were analyzed giving high confidence in lipid annotations and good reproducibility for relative and estimated quantitation of almost one thousand lipid species. Phosphatidylcholine and triglyceride lipids were automatically targeted by specific product ion or neutral losses, which enabled further CID MS^2/MS^3 characterization during the same dd-MS^2 acquisition cycle. Functional lipids such as PI isomers and sterol esters are readily annotated and quantified at low levels. Different phenotypes are observed between two isomeric fatty acyl combinations: PI 18:1_18:3 and PI 18:2/18:2.

INTRODUCTION

Lipidomics is a rapidly growing field of study that is crucial for understanding the underlying mechanisms for disease progression. The application of untargeted lipidomics profiling to phenotypical analysis of a wide range of plant tissues and organisms is becoming a more important aspect of agricultural research. Identification of lipids by untargeted lipidomics requires sophisticated software with an extensive lipid database. We present here the data processing of western corn rootworm (WCR) lipid extracts using the latest version of LipidSearch software. New algorithms were introduced specifically to reduce false positives, improve quantitation using labeled internal standards, and automate searching of MS^n data obtained by higher-energy collisional dissociation (HCD) and linear ion trap collisional induced dissociation (CID) fragmentation methods.

MATERIALS AND METHODS

Total lipid extracts from western corn rootworm larvae[1] were separated using a Thermo Scientific™ Accucore™ 2.1 x 150 mm C30, 2.7 µm column and Thermo Scientific™ Vanquish™ chromatograph. LC-MS^n analysis was performed using a Thermo Scientific™ Orbitrap ID-X™ Tribrid™ mass spectrometer (**Figure 1**) using Acquire-X™ experimental workflow for lipid characterization (**Figure 2**). Lipid extracts were spiked with SPLASH® LipidoMIX® (Avanti Polar Lipids, Inc.) internal standards to estimate the concentration of annotated lipid species.

LC/MS at 120K resolution (FWHM @ *m/z* 200) and data-dependent HCD MS^2 experiments (15K resolution) were performed in positive and negative ion modes. During each 1.5 s cycle of the untargeted dd-MS^2 profiling method, additional targeted product ion (*m/z* 184.0733) or neutral loss (fatty acid + NH_3) CID MS^2 and MS^3 experiments were selectively triggered to provide higher quality characterization of phosphatidylcholine (PC) and triglyceride (TG) lipids. Prior to analysis of the biological samples, LC/MS (120K resolution) analyses of blank and pooled samples were used to automatically create an exclusion list for background ions and inclusion list for sample-relevant ions. Both lists were dynamically updated following subsequent LC/MS^n analyses of biological replicates.

LC/MS^n datasets were processed using LipidSearch 4.2.9 beta software (**Figure 3**) with an expanded lipid database (**Table 1**), improved peak detection during alignment of annotated peaks, and better rejection of false positives (**Table 2**). Multiple HCD MS^2, CID MS^2, and MS^3 product ion mass spectra for the same precursor ions were automatically combined to provide more comprehensive annotation for hundreds of lipid molecular species. The data processing parameters used for the WCR lipid samples (**Figure 4**) are shown in **Table 3**.

RESULTS

LipidSearch software was used to search MS^2 and MS^3 spectra against the predicted product ions and neutral losses for all potential lipid species within precursor and product ion mass tolerances. For each LC-dd-MS^n analysis, potential lipid species were identified separately from positive or negative ion adducts. The data for each biological replicate were aligned within a chromatographic time window by combining the positive and negative ion annotations and merging these into a single lipid annotation in the results table. This approach provides lipid annotations that reflect the appropriate level of MS^2/MS^3 product ions and neutral losses from the entire dataset giving higher confidence in lipid identifications.

The alignment results were filtered by minimum number of data points, signal-to-noise ratio, intensity ratio, main adduct ion, and ID quality. Compared to results generated only from dd-MS^2 HCD data, the combination of HCD and CID MS^2/MS^3 gives significantly higher quality lipid identifications for PC and TG lipids in the same analysis time. We will continue to refine this approach based on published lipid MS/MS compilations.[2]

LipidSearch software version 4.2 has been modified to use the shorthand notation suggested by Liebisch, *et al.*[3] and sphingolipid annotations now reflect the number of hydroxyl groups (m = monohydroxy, d = dihydroxy, and t = trihydroxy), the number of carbons in the long-chain base, and the number of double bonds. For example, d18:1/16:0 ceramide becomes Cer(d18:1_16:0) and sphinganine as SPH(d18:0). To avoid confusion regarding very long chain ceramides found in stratum corneum lipid extracts with a very different nomenclature,[4] these lipids are now searched using a separate skin lipids target database.

The concentration of lipid species with a deuterated standard were estimated using the internal standard concentration times the peak area ratio of analyte to internal standard (**Table 4**). The number of lipid species per sub-class after filtering rejected peaks (annotation grades A+B) are summarized in **Table 5**.

Figure 1. Untargeted lipidomics high-resolution LC-MS^n workflow using C30 reversed-phase separation, Orbitrap ID-X Tribrid mass spectrometer, and LipidSearch software

Figure 2. Untargeted LC-MS^3 (with/without inclusion) followed by targeted CID MS^2 and MS^3

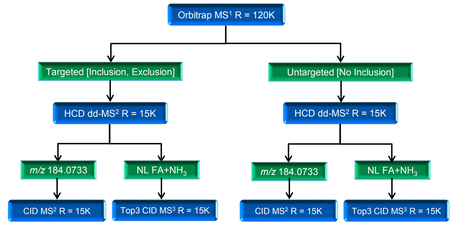
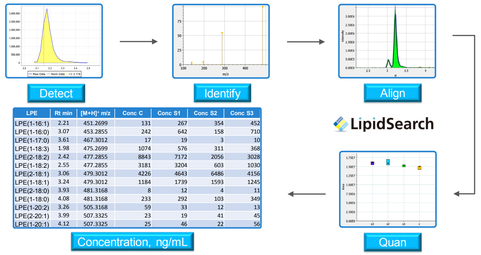


Figure 3. LipidSearch software for lipid annotation and quantitation for LC-MS^n data



LPE	Rt min	[M+H]+ m/z	Conc C	Conc S1	Conc S2	Conc S3
LPE(1-16:1)	2.21	451.2699	131	267	354	452
LPE(1-16:0)	3.07	453.2855	242	642	158	710
LPE(1-17:0)	3.61	467.3012	17	19	3	10
LPE(1-18:3)	1.98	475.2699	1074	576	311	368
LPE(2-18:2)	2.42	477.2855	8843	7172	2056	3028
LPE(1-18:2)	2.55	477.2855	3181	3204	603	1030
LPE(2-18:1)	3.06	479.3012	4226	4643	6486	4156
LPE(1-18:1)	3.24	479.3012	1184	1739	1593	1245
LPE(2-18:0)	3.93	481.3168	8	12	4	11
LPE(1-18:0)	4.08	481.3168	233	292	103	349
LPE(1-20:2)	3.26	505.3168	59	33	12	13
LPE(2-20:1)	3.99	507.3325	23	19	41	45
LPE(1-20:1)	4.12	507.3325	25	46	22	58

Figure 4. Western corn rootworm larvae lipid extract at two different growth stages and diets

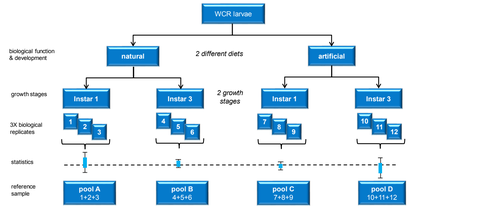
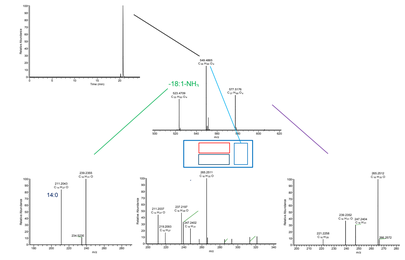


Figure 5. HCD MS^2 and CID MS^3 spectra of Triacylglycerol 48:1 $[M+NH_4]^+$ at 20.66 min



Triglyceride ammonium adducts dissociate in MS^2 to give up to 3 product ions formed by the loss of fatty acids. MS^3 of these product ions may correspond to several different fatty acyl combinations. In **Figure 5**, TG 48:1 gives major product ions corresponding to loss of 18:1 (green), 16:0 (blue), and 14:0 (purple) fatty acids. The MS^3 spectra of 882.7541 → 523.4709 (NL 18:1+NH_3) forms acyl product ions for 14:0 and 16:0, giving a 14:0_18:1_16:0 annotation. Similarly, 882.7541 → 577.5176 NL of 14:0+NH_3 produces 16:0, 18:1 giving the same 14:0_18:1_16:0 annotation. However, 882.7541 → 549.4865 NL of 16:0+NH_3 gives a mixture of product ions: 14:0, 18:1 and 16:0, 16:1 fatty acyl ions. Thus, the TG 48:1 is a mixture of TG 14:0_18:1_16:0 and 16:0_16:1_16:0 isomers. This example demonstrates that MS^3 enables confident annotation of co-eluting triglyceride isomers typically found in complex total lipid extracts of biological origin.

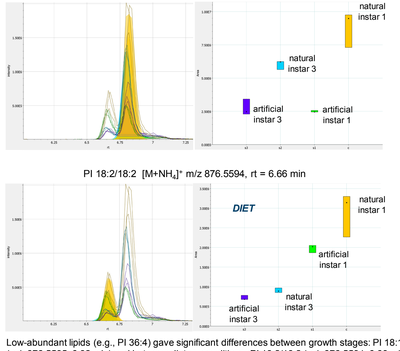
Table 1. Database and lipid nomenclature modifications in LipidSearch 4.2 software

Modification	Description
Added AcHexSiE, AcHexStE, AcHexZyE, AcHexCmE, AcHexChE	Acylhexosyl sterol esters
Added bis-methyl LPA, PA, PC, PE, PG, PS	Methylation of phosphate for quantitation
Added methyl PC	Methylation of phosphate for quantitation
Added MLCL, DLCL	Mono-lyso and di-lyso cardiolipins
Added SL	Seminolipids (glycerol analog of sulfatide)
Added 15:0-18:1(d_7) DG, PA, PC, PE, PG, PI, PS; Chol(d_7) d_7-18:1 LPC, LPE, ChE, MG; 18:1(d_9) SM; 15:0-18:1(d_7)-15:0 TG	SPLASH internal standards for quantitation (Avanti Polar Lipids, Inc.)
Added CerPE	Ceramide phosphatidylethanolamine
Added NAE	N-acylethanolamine
Added WE	Wax esters of fatty acids
Modified sphingolipid nomenclature	As proposed by G. Liebisch, *et al.*[3]
Created skin ceramide database	Very long chain skin ceramides[4]
Added FA chloride adducts	Negative ion APCI for stratum corneum lipids[4]
Added $[M+H-H_2O]^+$ and $[M-CH_3]^-$ adducts	Detection of cholesterol and phosphatidylcholine in-source product ions

Table 2. New features and improvements in LipidSearch 4.2 software

Feature/Improvement	Description
Improved peak detection and peak filtering in alignment of chromatograms and merging of lipid annotations	Minimum number of data points
	Peak intensity ratio threshold
	Improved signal-to-noise ratio threshold
	Filter duplicate IDs at the same retention time
	Automatic filtering of rejected peaks
Improved confidence in annotation by combining HCD and CID MS^n searches	Combines HCD MS^2 with targeted (NL or product ion) CID MS^2/MS^3 spectra for enhancing annotation of PC, TG, and other lipid species
	MS^3Num – reports the number of annotations that include CID MS^3
Estimated quantitation added	Quantification export using SPLASH internal standard concentration
Broader lipid sub-class coverage	Expanded lipid database adds 26 more lipid sub-classes (total 92)

Figure 6. Low-abundant PI isomers show phenotypical differences



Low-abundant lipids (e.g., PI 36:4) gave significant differences between growth stages: PI 18:1_18:3 (*m/z* 876.5595, 6.82 min) and between dietary conditions: PI 18:2/18:2 (*m/z* 876.5594, 6.66 min).

Table 3. LipidSearch 4.2.9 beta software parameters

Search Parameter	Setting	Units
Precursor mass tolerance	5	ppm
Product mass tolerance	10	ppm
Prod. Intensity threshold	1.0	%
m-Score threshold/display	2.0/5.0	
Quan m/z tolerance	+/- 5.0	ppm
Quan range	+/- 0.5	min
Main isomer peak filter	ON	
ID Quality filter	A, B, C, D	
Adducts (pos. ion)	+H, NH_4, Na, +H-H_2O	
Adducts (neg. ion)	-H, -CH_3, +CH_3CO_2	
Lipid Classes	*Lipids	
Alignment Parameter	**Setting**	**Units**
R.T. tolerance	0.10	min
All isomer peak filter	ON	
m-Score threshold	5.0	
ID Quality filter	A, B, C, D	
Configuration	**Setting**	
Number data points threshold	5	
Intensity baseline, %	0.05	
Intensity ratio threshold	3	
S/N ratio threshold	5	

Table 4. Reproducibility of labeled internal standard in biological replicates

Internal Standard (SPLASH)	Calc. m/z	Rt, min	Conc, µg/mL	CV, %
ChE d_7-18:1	657.6441	21.8	35.0	6.2
DG 15:0-18:1(d_7)	587.5506	12.9	1.00	5.7
LPC 18:1(d_7)	528.3921	3.09	2.50	4.0
LPE 18:1(d_7)	486.3451	3.21	0.50	5.6
PA 15:0-18:1(d_7)	667.5169	9.21	0.70	6.6
PC 15:0-18:1(d_7)	752.6061	9.11	16.0	4.6
PE 15:0-18:1(d_7)	710.5591	9.55	0.50	4.8
PG 15:0-18:1(d_7)	741.5537	8.25	3.00	5.7
PI 15:0-18:1(d_7)	829.5698	7.90	1.00	5.4
PS 15:0-18:1(d_7)	754.5490	8.07	0.50	4.9
SM d18:1-18:1(d_9)	737.6397	8.28	3.00	5.3
TG 15:0-18:1(d_7)-15:0	811.7646	20.6	5.50	4.9

***Targeted lipid sub-classes:**
AcCa, AcHexChE, Cer, CerPE, ChE, d7ChE, Co, DG, FA, Hex1SPH, Hex1Cer, Hex2Cer, Hex3Cer, LPA, LPC, LPE, LPG, LPI, LPS, LSM, MG, PA, PC, PE, PG, PI, PS, SiE, SM, SPH, TG, WE; Labeled lipids

Table 5. Total number of annotated lipid species after filtering grades A,B

Lipid Species (LS 4.2.9)	Filtered
Acylcarnitine	4
Ceramides (Cer)	67
CerPE	1
Cholesterol ester (ChE)	2
Co-enzyme Q	4
Diacylglycerols (DG)	53
Hexosyl ceramide (Hex1Cer)	13
Hexosyl sphingosine (Hex1SPH)	1
Hexosyl$_2$ ceramide (Hex2Cer)	2
Hexosyl$_3$ ceramide (Hex2Cer)	1
Lyso PC (LPC)	58
Lyso PE (LPE)	29
Lyso PG (LPG)	3
Lyso PI (LPI)	13
Lyso PS (LPS)	12
Phosphatidic acid (PA)	14
Phosphatidylcholine (PC)	94
Phosphatidylethanolamine (PE)	78
Phosphatidylglycerol (PG)	8
Phosphatidylinositol (PI)	34
Phosphatidylserine (PS)	34
Sphingomyelin (SM)	3
Sitosterol ester (SiE)	1
Triacylglycerols (TG)	443
TOTAL	**972**

DISCUSSION

WCR larvae contain a high content of triglycerides in addition to phospholipids, sphingolipids, and sterol lipids. In this work, we focused on PC and TG lipids for further MS^n characterization. **Figure 5** summarizes the information provided by MS^3 analysis for 48:1 TG and provides unequivocal evidence for two isomeric TG species that co-eluted under the conditions of the analysis. Characterization of TG mixtures is often not possible using MS^2 alone since multiple possible combinations fatty acids may exist.

With HCD MS^2 data, neutral loss of fatty acid and ammonia is the signature fragmentation observed for several classes of neutral lipids including DG, TG, and sterol esters. The Orbitrap ID-X Tribrid mass spectrometer provides an intelligent workflow for monitoring of class-specific product ion or neutral losses and automatically conducting a predefined experiment on the same precursor ion during the instrument cycle. This approach efficiently provides additional information without wasting time scheduling targeted experiments within predefined retention times.

In this study we observed that phenotypical differences related to developmental stage and diet occurred in a wide range of lipids species and concentrations. **Figure 6** shows the changes in PI 36:4 isomer peak areas versus the developmental (PI 18:1_18:3) and diet (PI 18:2/18:2) conditions. A difference in the fatty acyl composition reveals that two distinct phenotypes are observed.

Table 4 summarizes the variation observed for the SPLASH standards spiked into the biological replicates. Very good reproducibility was observed with CVs less than 7% in the extracted ions from the high-resolution MS scan obtained while also performing further lipid characterization using LC-MS dd-MS^n.

Table 5 summarizes the number of lipids per sub-class after filtering. The total number of high-quality lipid annotations submitted for further statistical analysis was 972; in 12 positive and 12 negative ion runs a total of 1131 CID MS^2/MS^3 results were obtained in addition to HCD MS^2 spectra for 1522 different lipid adduct ions.

CONCLUSIONS

- This LC MS^n lipidomics workflow can be applied to any complex biological sample including plasma, plants, tissues, cells, and whole organisms such as insects.
- The Orbitrap ID-X Tribrid mass spectrometer provides a highly sophisticated and customizable workflow for lipidomics and improves the characterization of lipid structures.
- LipidSearch software combines positive and negative ion HCD MS^2 and CID MS^2/MS^3 spectra for database searches providing higher confidence in lipid annotation.
- Almost one thousand lipids were annotated from and quantified with good reproducibility.
- Functional lipids such as PI isomers and sterol esters are readily annotated and quantified at low levels. Different phenotypes are observed between isomeric fatty acyl combinations and congeners with varying numbers of double bonds.
- Methods with insufficient structural characterization miss these meaningful biological differences.

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ACKNOWLEDGEMENTS

The authors would like to thank Elena Sokol for her expert help in making the LipidSearch database definition changes for sphingolipid nomenclature, as well as her feedback regarding usability, and improving the quality of lipid annotations.

TRADEMARKS/LICENSING

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PO65257-EN 0518S