



Increased confidence of insect lipidome annotation from high-resolution Orbitrap LC/MSⁿ analysis and LipidSearch software

Authors

David Peake¹, Reiko Kiyonami¹,
Yasuto Yokoi², Yelena Aldefinskaya³,
Jeff Gilbert³, and Daniel Gachotte³

¹Thermo Fisher Scientific,
San Jose, California, USA

²Mitsui Knowledge Industry Co., LTD,
Tokyo, Japan

³CORTEVA agriscience,
Indianapolis, Indiana, USA

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phosphatidylcholine, triglyceride,
insect, larvae, western corn rootworm

Goal

- Demonstrate the utility of LC/MS and data-dependent MSⁿ acquisition for simultaneous lipid profiling and more complete lipid annotation using the Thermo Scientific™ Orbitrap™ ID-X™ Tribrid™ mass spectrometer and Thermo Scientific™ LipidSearch™ software.
- Show that the use of the Thermo Scientific™ AcquireX intelligent acquisition method combined with neutral loss and product ion scan filters increases the overall quality and number of lipid annotations.
- Investigate the utility of combining HCD MS² and CID MSⁿ for structural determination of phosphatidylcholine and triglyceride species in lipid extracts from insect larvae.

Introduction

The application of lipidomics to phenotypical analysis of a wide range of plant tissues¹ and insects is becoming a more important aspect of agricultural research². Insect lipids are highly structurally diverse species that perform many important functions including storage of metabolic energy, contributing to the structure of membranes, protection against dehydration and pathogens, and circulating energy molecules.³ In addition, essential lipids such as sterols (precursors to hormones) and polyunsaturated fatty acids

are only available from the diet. Understanding how lipid molecular species change in response to both diet and age is critical to define nutrient requirements and fitness relationships. However, conducting detailed studies of insect lipid composition has been technically challenging due to the complex nature of insect lipid extracts and the lack of software for automated lipid annotation.

We evaluated the robustness of a new high-resolution LC/MSⁿ approach to perform non-targeted lipid profiling experiments. The western corn rootworm (WCR) larvae (*Diabrotica virgifera virgifera*) was chosen due to its economic impact, estimated at 2 billion US dollars associated with its control and corn production loss in the Americas.^{4,5} In this study, the effects of larval growth and diet were investigated using a Thermo Scientific™ Orbitrap™-based mass spectrometer and LipidSearch software. New software algorithms were introduced specifically to reduce false positives, improve quantitation using labeled internal standards, and automate processing of LC/MSⁿ structural data obtained by higher-energy collisional dissociation (HCD) and linear ion trap collisional induced dissociation (CID) fragmentation methods.

The analytical challenges of identifying isomeric/isobaric lipids using an infusion workflow was recently reviewed.⁶ The choice of the mass spectrometric method, mass resolution used for analysis, and the specific structural information obtained with the optimized MS/MS conditions determines the level of annotation that is reported for every lipid species. A fundamental issue in lipid identification is that lipids dissociate in a very predictable way to give relatively few product ions, and thus structural details are often incomplete. Obtaining more complete lipid structural information can be addressed in several different ways, including the following:

- Selective derivatization to shift the mass of isomeric species or to change fragmentation
- Separation by chromatographic or other means such as differential mobility (FAIMS)
- CID MSⁿ analysis to obtain a series of selective transitions revealing structure
- Alternative dissociation techniques such as UV photodissociation (UVPD)

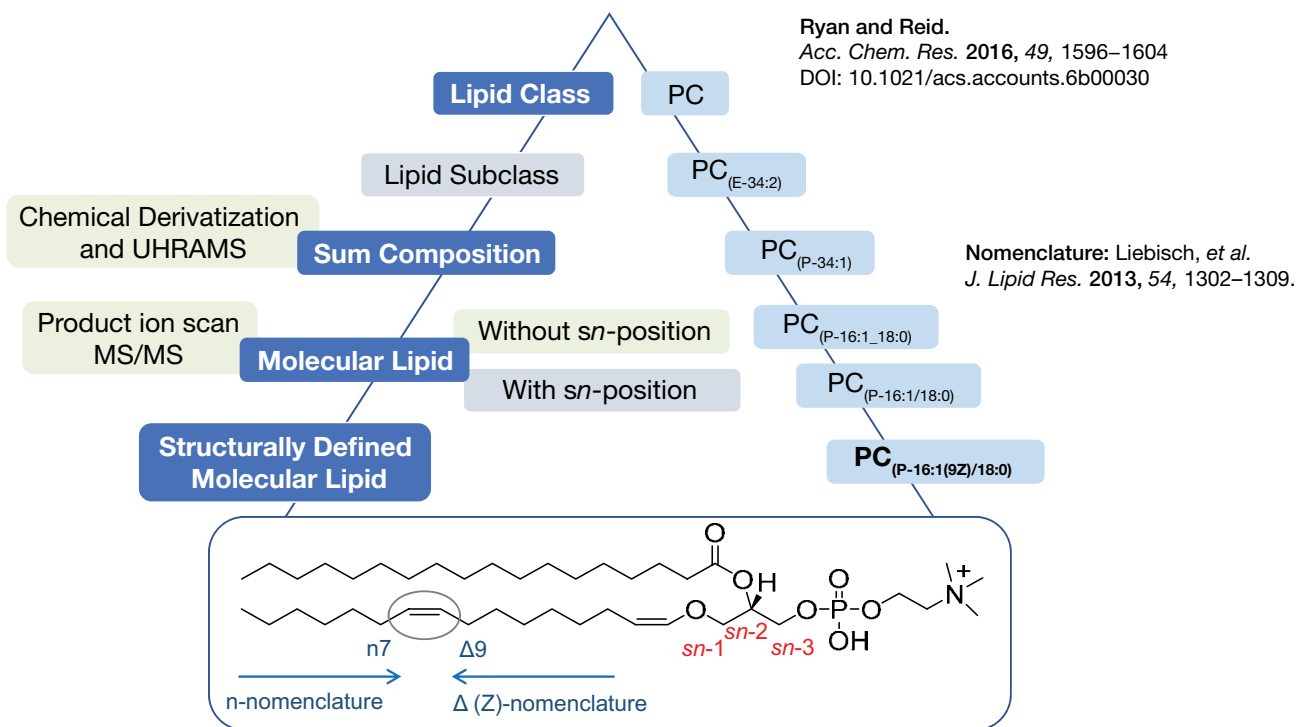
Here, we use a reversed-phase UHPLC separation and lipid class-based LC/MSⁿ analysis in combination with the AcquireX data acquisition strategy⁷ to provide deeper annotation and higher confidence in lipid annotation for isomeric phosphatidylcholine (PC), diglyceride (DG), and triglyceride (TG) lipids.

As shown in Figure 1, ultra-high resolution (more than 100,000 resolution) MS data provides lipid annotation at the sum composition (elemental formula) level. The addition of MS/MS data provides fatty acyl information and allows annotation at the molecular lipid level. However, MS² information is often incomplete or ambiguous due to the presence of isomeric mixtures. CID MSⁿ information is essential when it is not possible to determine the fatty acyl composition from a mixture of isomers, such as in the analysis of triacylglycerol lipids. Determination of structurally defined molecular lipid species (Figure 1) requires additional structural details (e.g., fatty acyl position on the glycerol backbone, double bond location, *cis* vs. *trans*) from chemical reaction/derivatization or MSⁿ in combination with alternative activation methods such as UVPD (ultra-violet photodissociation).

Experimental methods

Sample preparation

Fresh frozen insect larvae (approximately 100 mg) were weighed and softened in hot isopropanol (IPA) for 20 minutes to inhibit lipases.⁸ The samples were then homogenized using a motorized glass rod and re-extracted for an additional 10 minutes. All organic solvent contained 0.01% of butylated hydroxytoluene. After the addition of chloroform and water (1:0.4), the samples were agitated vigorously for 30 minutes at room temperature. For tissue containing a significant amount of neutral lipids, a second extraction with chloroform-methanol (2:1) was performed with 30 minutes agitation at room temperature. The organic phases were pooled and washed with 1 M potassium chloride followed by water, and the organic layer was then filtered (PTFE, 0.45 µm) and evaporated to dryness under nitrogen prior to reconstitution in isopropanol-methanol-chloroform (0.45:0.45:0.1) containing SPLASH® LIPIDOMIX® standards (Avanti Polar Lipids, Inc) at a 1:10 dilution. In this study, four different larvae populations (natural and artificial diet, Instar 1 and Instar 3 developmental stages) were extracted and analyzed in triplicate as shown in Figure 2.



Adapted from: Ekroos, K. In *Lipidomics*, Ch 1. 2012 Wiley-VCH Verlag GmbH & Co

Figure 1. Hierarchal scheme of lipid classification

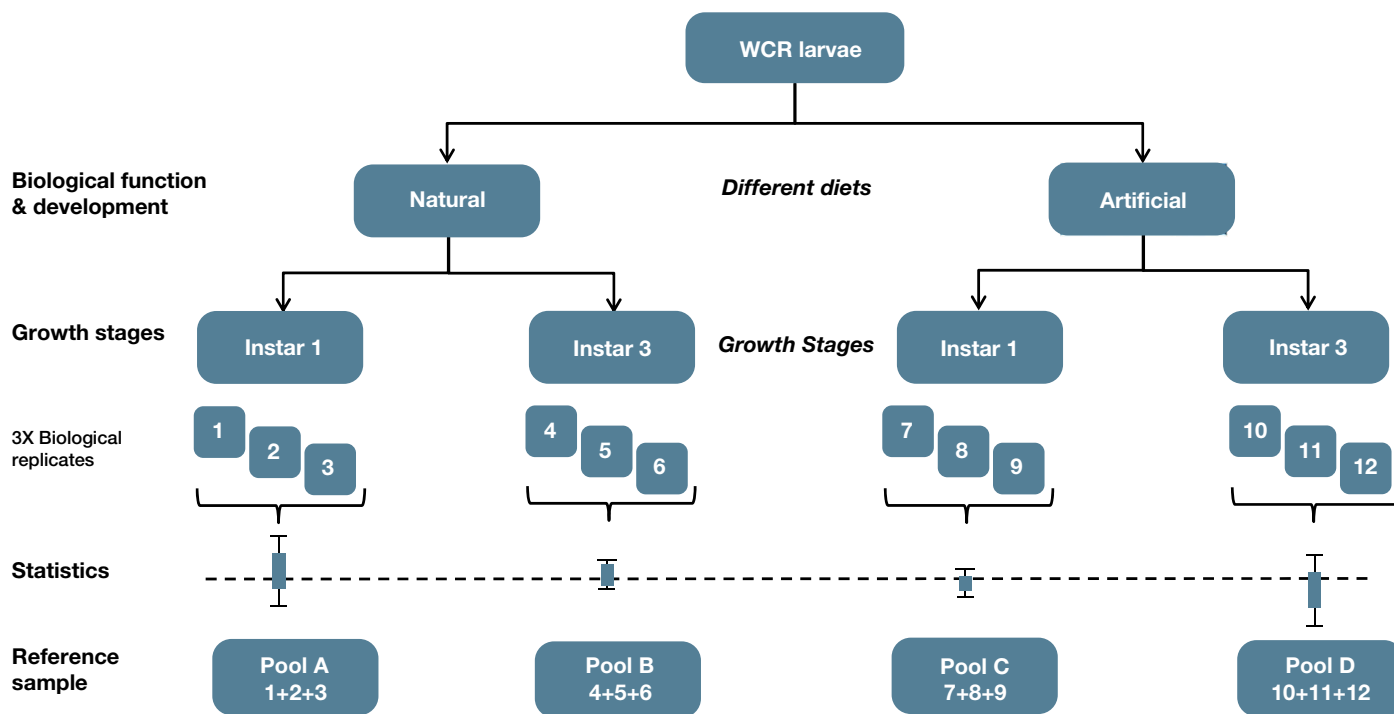


Figure 2. Western corn rootworm lipidomics study: experimental design

Mass spectrometry

Total lipid extracts from western corn rootworm (WCR) larvae were separated using a Thermo Scientific™ Accucore™ 2.1 × 150 mm C30, 2.7 µm column and a Thermo Scientific™ Vanquish™ chromatograph. The chromatograph was operated at a flow rate of 260 µL/min, the 2.1 × 150 mm, 2.7 µm C30 Accucore column maintained at 45 °C, and the injection volume was 2 µL. Chromatographic gradient conditions are shown in Table 1. Mobile phase A was 60:40 acetonitrile/water and mobile phase B was 90:10 isopropanol/acetonitrile. Both mobile phase A and B contained 10 mM ammonium formate and 0.1% formic acid.

LC/MSⁿ analyses were performed using a Thermo Scientific™ Orbitrap ID-X™ Tribrid™ mass spectrometer (Figure 3) using two different methods: 1) a standard HCD data dependent MS² method and 2) an AcquireX experimental workflow⁷ for lipid characterization (Figure 4). LC/MS at 120,000 resolution (FWHM @ *m/z* 200) and data-dependent HCD MS² experiments (15,000 resolution) were performed in positive and negative ion modes with the instrument conditions summarized in Table 2. During each 1.5 s cycle of the AcquireX dd-MSⁿ profiling method, additional targeted product ion (*m/z* 184.0733) or neutral loss (fatty acid

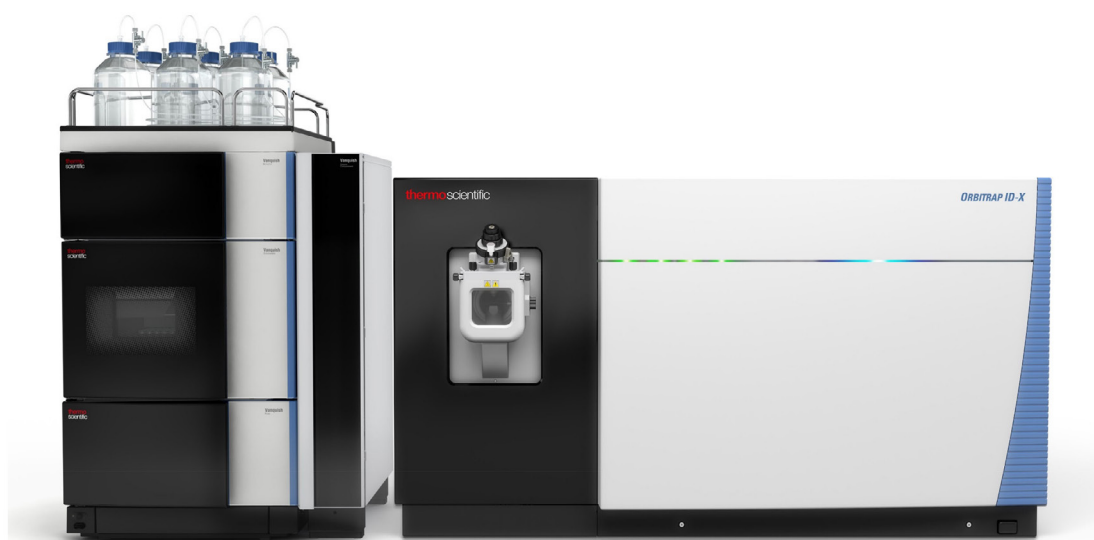


Figure 3. Thermo Scientific Orbitrap ID-X mass spectrometer

Table 1. Chromatographic gradient conditions

Time (min)	% A	% B
0.00	70	30
2.00	57	43
2.10	45	55
12.00	35	65
18.00	15	85
20.00	0	100
25.00	0	100
25.10	70	30
30.00	70	30
31.00	70	30

Table 2. Orbitrap ID-X conditions

Parameter	Value
Spray voltage	3500 V pos, 2400 V neg
Vaporizer temperature	300 °C
Ion transfer temperature	300 °C
RF Lens	45
MS OT resolution	120,000 (FWHM @ <i>m/z</i> 200)
MS ² /MS ³ OT resolution	15,000 (FWHM @ <i>m/z</i> 200)
HCD CE	25-30-35 pos, 20-40-60 neg
CID MS ² CE	32%
CID MS ³ CE	35%
Isolation	1.6 Da HCD, 2.0 Da CID
Dynamic exclusion	5 s
Cycle time	1.5 s

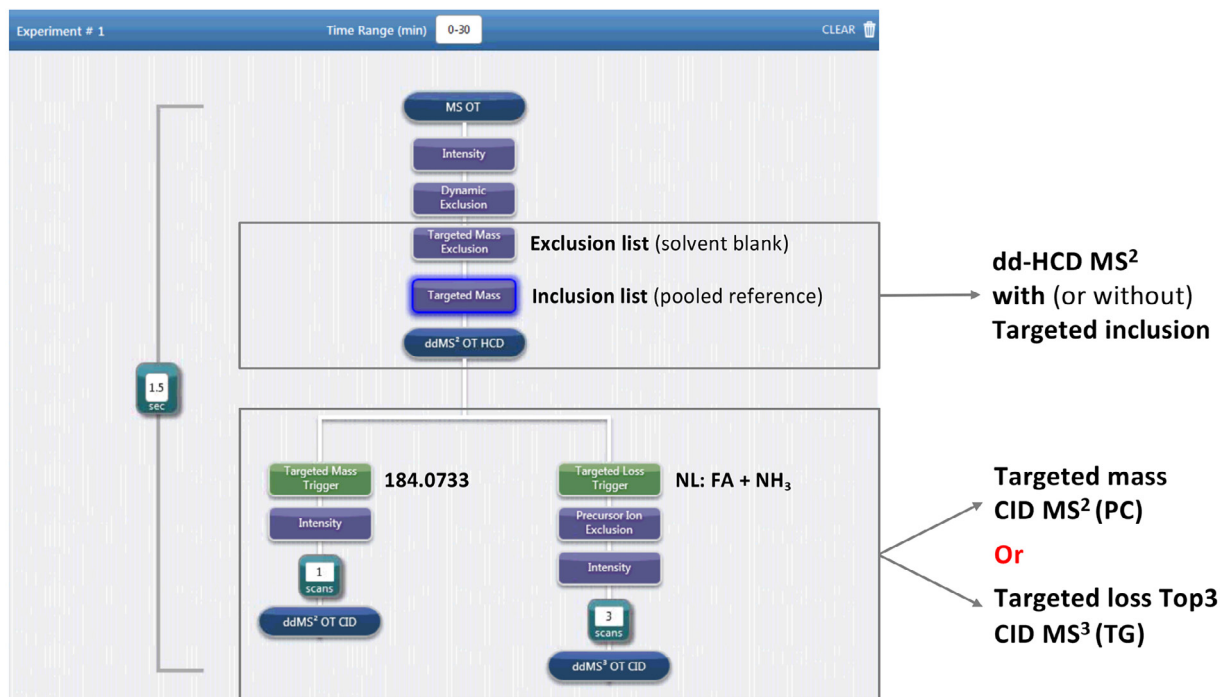


Figure 4. AcquireX instrument acquisition method

plus ammonia, Table 3) CID MS² and MS³ experiments were selectively performed to provide higher quality characterization of phosphatidylcholine (PC) and triglyceride (TG) lipids. Prior to analysis of the biological samples, LC/MS (120,000 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/MSⁿ analyses of the individual biological replicates.

Table 3 (part 1). Neutral loss inclusion list

FA	Formula	NL (FA + NH ₃)
12:0	C ₁₂ H ₂₄ O ₂	217.2042
12:1	C ₁₂ H ₂₂ O ₂	215.1885
14:0	C ₁₄ H ₂₈ O ₂	245.2355
14:1	C ₁₄ H ₂₆ O ₂	243.2198
16:0	C ₁₆ H ₃₂ O ₂	273.2668
16:1	C ₁₆ H ₃₀ O ₂	271.2511
16:2	C ₁₆ H ₂₈ O ₂	269.2355
17:0	C ₁₇ H ₃₄ O ₂	287.2824
17:1	C ₁₇ H ₃₂ O ₂	285.2668
18:0	C ₁₈ H ₃₆ O ₂	301.2981
18:1	C ₁₈ H ₃₄ O ₂	299.2824
18:2	C ₁₈ H ₃₂ O ₂	297.2668
18:3	C ₁₈ H ₃₀ O ₂	295.2511
19:0	C ₁₉ H ₃₈ O ₂	315.3137
20:0	C ₂₀ H ₄₀ O ₂	329.3294
20:1	C ₂₀ H ₃₈ O ₂	327.3137

Table 3 (part 2). Neutral loss inclusion list

FA	Formula	NL (FA + NH ₃)
20:2	C ₂₀ H ₃₆ O ₂	325.2981
20:3	C ₂₀ H ₃₄ O ₂	323.2824
20:4	C ₂₀ H ₃₂ O ₂	321.2668
20:5	C ₂₀ H ₃₀ O ₂	319.2511
21:0	C ₂₁ H ₄₂ O ₂	343.3450
22:0	C ₂₂ H ₄₄ O ₂	357.3607
22:1	C ₂₂ H ₄₂ O ₂	355.3450
22:2	C ₂₂ H ₄₀ O ₂	353.3294
22:3	C ₂₂ H ₃₈ O ₂	351.3137
22:4	C ₂₂ H ₃₆ O ₂	349.2981
22:5	C ₂₂ H ₃₄ O ₂	347.2824
22:6	C ₂₂ H ₃₂ O ₂	345.2668
23:0	C ₂₃ H ₄₆ O ₂	371.3763
24:0	C ₂₄ H ₄₈ O ₂	385.3920
24:1	C ₂₄ H ₄₆ O ₂	383.3763
26:0	C ₂₄ H ₅₂ O ₂	413.4233

Data processing

LC/MSⁿ datasets were processed using Thermo Scientific™ LipidSearch™ 4.2 software (Figure 5) with an expanded lipid database, improved peak detection during alignment of annotated peaks, and better rejection of false positives. The database for TG species was modified to include 2:0, 3:0, 5:0 and 7:0 fatty acids in order to annotate some unusual TG species with one very short acyl chain⁹. The data processing parameters used for the WCR lipid samples are summarized in Table 4.

Multiple HCD MS², CID MS², and MS³ product ion mass spectra for the same precursor ions were automatically combined to provide more comprehensive

annotation for hundreds of lipid molecular species.

The search results from positive/negative ion, HCD MS² and CID MS² and MS³ spectra were aligned by retention time and the combined annotation was obtained from all the lipid adduct ions. In the search results, isomeric lipid species are scored by how well each predicted fatty acyl combination matches the actual MS/MS spectrum. These match scores are used to rank order these results and the isomer with the best ranking is used to represent the lipid species in the alignment results. Thus, LipidSearch reports the main lipid species based on the best match score, which includes the results obtained for CID MS² and MS³ spectral matches at a specified retention time.

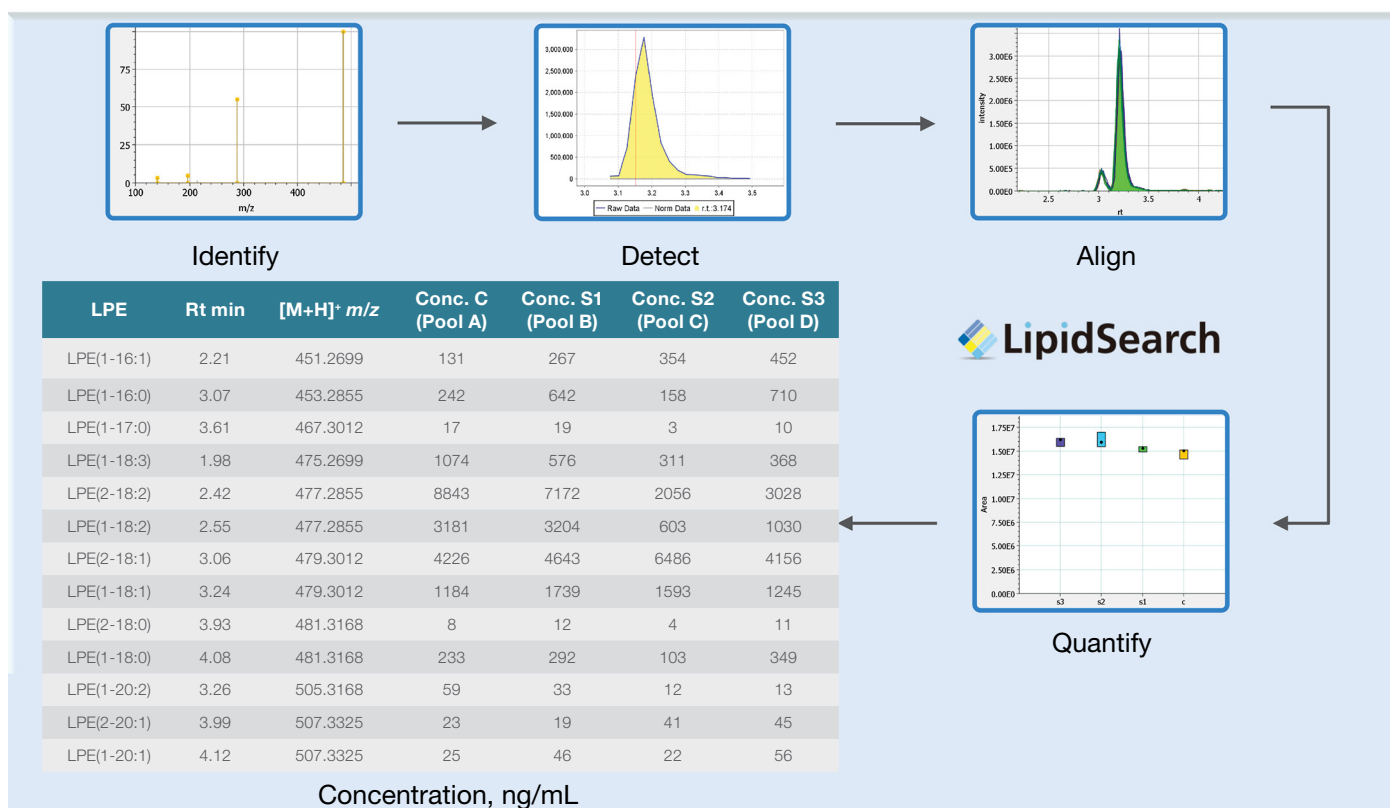


Figure 5. LipidSearch Software Workflow

Table 4. LipidSearch processing conditions

Search Parameter	Setting	Units
Precursor mass tolerance	5	ppm
Product mass tolerance	10	ppm
Product intensity threshold	1.0	%
m-Score threshold/display	2.0/5.0	
Quan <i>m/z</i> 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 ₂ O	
Adducts (neg. ion)	-H, -CH ₃ , +CH ₃ CO 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	

Results

In parallel to the AcquireX method for efficient acquisition of the data-dependent LC/MS², neutral loss and product ion directed CID MS² and MS³ experiments were performed in the same instrument cycle to improve the structural characterization of two of the main lipid sub-classes (phosphatidylcholines and triglycerides) expected in the insect larvae. Ultra-high-resolution LC/MS analysis provides lipid annotation at *sum composition level* (Figure 1). The high-resolution positive ion mass spectrum of the control sample 1 from pool A (Figure 6a) shows the molecular ion region for a lipid species with the elemental composition C₄₂H₈₂NO₈P. The protonated and sodiated species fit PC 34:1 or PE 37:3 lipids.

For annotation at the molecular lipid level (Figure 1), MS² and MS³ information allows determination of lipid headgroup and fatty acyl information. As illustrated in Figure 6b, the HCD MS² spectrum of *m/z* 760.5851 gives a predominant *m/z* 184 product ion, which allows assignment of PC 34:1 as the lipid species. Observation of the *m/z* 184 product ion using the scan filter triggered

the acquisition of the CID MS² mass spectrum shown in Figure 6c. The neutral loss of the phosphocholine lipid headgroup (183) and the neutral losses of fatty acid 16:0 and 18:1, as well as the loss of the corresponding ketenes, provide unequivocal assignment of the lipid annotation: PC 16:0_18:1.

Note that LipidSearch software version 4.2 has been modified to use the shorthand notation suggested by Liebisch, *et al.*¹⁰ and for lipid species with positional isomers, the use of an underscore indicates that the *sn* configuration is unknown. For example, PC 16:0/18:1 indicates 16:0 in the *sn*1 position of the glycerol and 18:1 in the *sn*2 position as shown in Figure 7. PC 16:0_18:1 indicates the *sn* position is 16:0/18:1 or 18:1/16:0.

LipidSearch software was used to search the MS² and MS³ spectra against the predicted product ions and neutral losses for all potential lipid species within 5 ppm precursor and 10 ppm product ion mass tolerances. For each LC/dd-MSⁿ analysis, potential lipid species were identified separately from the positive or negative ion MS² or MS³ spectra. All 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. This approach provides lipid annotations that reflect the appropriate level of MSⁿ information 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, main adduct ion, and ID quality.

The numbers of the highest quality lipid annotations per sub-class after filtering rejected peaks are summarized in Table 5. Analysis of the insect lipid extracts using data dependent LC/MS² gave a total of 866 lipid annotations after filtering, whereas the LC/MSⁿ approach with AcquireX gave a total of 1045 lipid annotations, representing an overall increase of 179 annotations (+17%). Moreover, the average match scores increased from 31.4 for the HCD results to 38.5 (+7.1) for the AcquireX results. The increase in match scores is more apparent when looking at the targeted PC and neutral lipid species for CID MSⁿ (ChE, CoQ, DG, LPC, PC, SiE, TG and WE). The average match score for these sub-classes increased from 33.9 for HCD to 46.5 (+12.6) for the AcquireX results. Thus, both the number of lipid annotations and the quality of the spectral matches improves substantially using the AcquireX approach, leading to higher confidence.

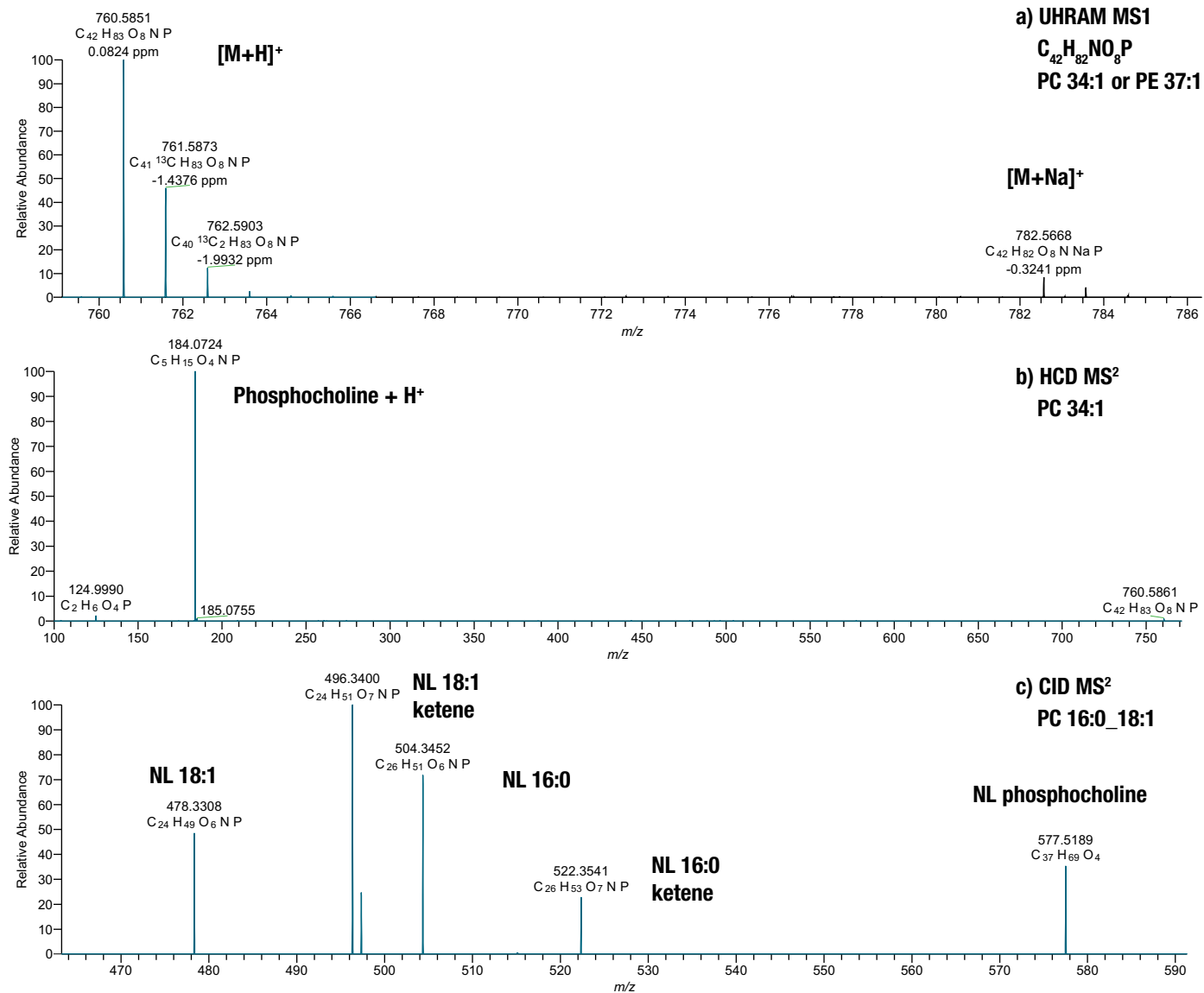


Figure 6. Confident annotation of phosphatidylcholine 34:1

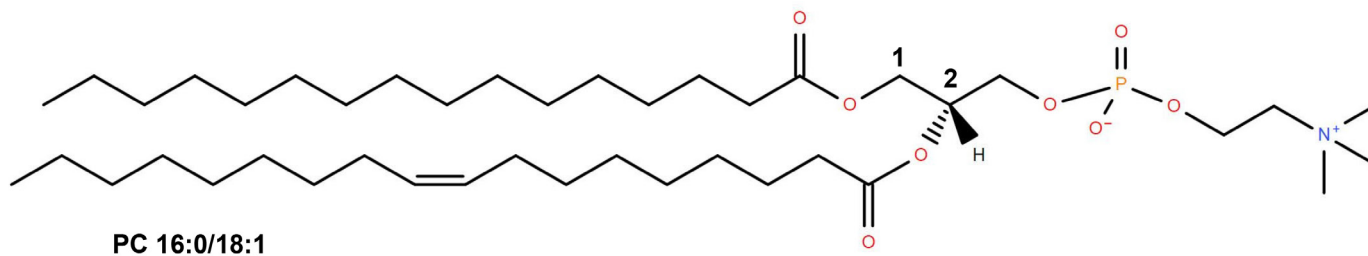


Figure 7. Structure: PC 16:0/18:1

Table 5. Summary of lipid species from WCR extracts

Lipid Species by Sub-class	dd-MS ² (filtered)	AcquireX (filtered)	% Change
Acylcarnitine	5	4	-25
Ceramides (Cer)	66	55	-20
Ceramide PE (CerPE)	1	3	67
Cholesterol ester (ChE)	3	4	25
Co-enzyme Q	0	4	
Diacylglycerol (DG)	38	51	25
Hexosyl ceramide (Hex1Cer)	25	23	-9
Hexosyl ₂ ceramide (Hex2Cer)	2	2	0
Hexosyl ₃ ceramide (Hex3Cer)	5	3	-67
Hexosyl sphingosine (HexSPH)	1	1	0
Lyso PC (LPC)	29	50	42
Lyso PE (LPE)	27	27	0
Lyso PG (LPG)	6	1	-500
Lyso PI (LPI)	15	6	-150
Lyso PS (LPS)	10	11	9
Phosphatidic acid (PA)	16	15	-7
Phosphatidylcholine (PC)	40	68	41
Phosphatidylethanolamine (PE)	83	70	-19
Phosphatidylglycerol (PG)	10	6	-67
Phosphatidylinositol (PI)	59	34	-74
Phosphatidylserine (PS)	36	29	-24
Sphingomyelin (SM)	52	117	56
Sitosterol ester (SiE)	1	2	50
Triacylglycerol (TG)	329	449	27
Wax ester (WE)	7	10	30
TOTAL	866	1045	17

Discussion

LC/MSⁿ

For lipid identification, a standard HRAM workflow is the LC/data-dependent MS² approach along with LipidSearch software for structure annotation (Figure 5). This provides simultaneous untargeted profiling and identification for lipids from cells, plasma, and tissues.

A common issue in lipid software today is the over-reporting of lipid annotations. For example, exact structures are used to represent lipids from LC/MS profiling experiments even though the MS/MS information is often incomplete. Without additional

experiments with reference standards it is not possible to unequivocally confirm assignment of double bond location, stereo-chemistry and sn position on the glycerol backbone. Thus, LipidSearch software reports a shorthand notation as suggested by Liebisch¹⁰ to represent a partial structure. Instead of the exact structure for phosphatidylcholine 1-16:0/2-18:1 (9Z), PC 16:0_18:1 is reported indicating that sn position, double bond location, and stereochemistry are not assigned. LipidSearch software reports the sum composition level (PC 34:1, Figure 6a, 6b) to indicate that the fatty acyl information is not known. If CID MS² data are also present for the protonated molecular ion, the fatty acyl groups are confirmed and then PC 16:0_18:1 is reported (Figure 6c).

The analysis of complex lipid extracts from insect larvae requires a more sophisticated approach to distinguish coeluting isomers. Structure-specific CID LC/MS² or LC/MS³ experiments (Figure 3) were used to selectively characterize specific lipids during a data-dependent LC/MS² run using the Thermo Scientific ID-X Tribrid mass spectrometer and advanced scan filters. WCR larvae contain a high content of triglycerides in addition to phospholipids, sphingolipids, and sterols. In this application, we focused on PC and neutral TG lipids for further LC/MSⁿ characterization. With HCD MS² data, neutral loss of fatty acid and ammonia is the signature fragmentation observed for several classes of neutral lipids including CoQ, 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 structure-based information without wasting time scheduling targeted experiments within predefined retention times. The use of AcquireX helps to increase the efficient use of the full capabilities of the Orbitrap ID-X Tribrid mass spectrometer by getting

more information on relevant ions resulting in an increase in the number of lipid annotations. In addition, targeting the class specific information increased the lipid match scores for the neutral TG and PC lipid sub-classes of interest in this experiment.

Figure 8a shows the mass chromatogram obtained for protonated PC 34:0 at m/z 762.6010 and a retention time of 12.22 min. The combined HCD and CID MS² spectrum shown in Figure 8b identifies the lipid as a phosphatidylcholine (HCD, m/z 184.0733) with 16:0 and 18:0 fatty acyl (FA) chains, information derived from the CID neutral loss of 16:0 ketene (m/z 524.3711) and 18:0 FA (m/z 478.3292), respectively. Since PC 34:0 was lacking fatty acyl information in negative ion mode, it would not be possible to assign the fatty acyl information using HCD MS² alone. By obtaining the positive ion CID MS² spectrum, it is feasible to obtain the information needed for more complete annotation. Figure 8c shows the peak areas obtained for the triplicate biological replicates for the four different experimental conditions. The pattern in the peak areas indicates an “Age” phenotype related to the larval developmental stage.

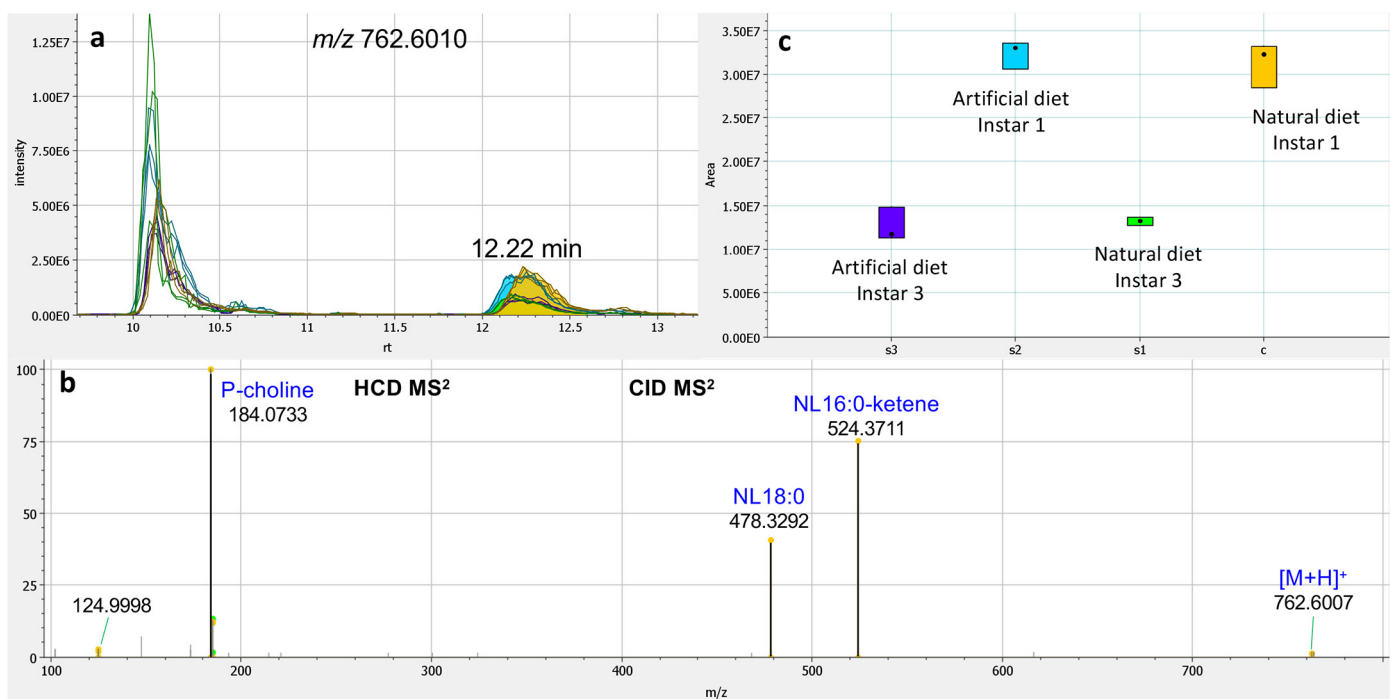
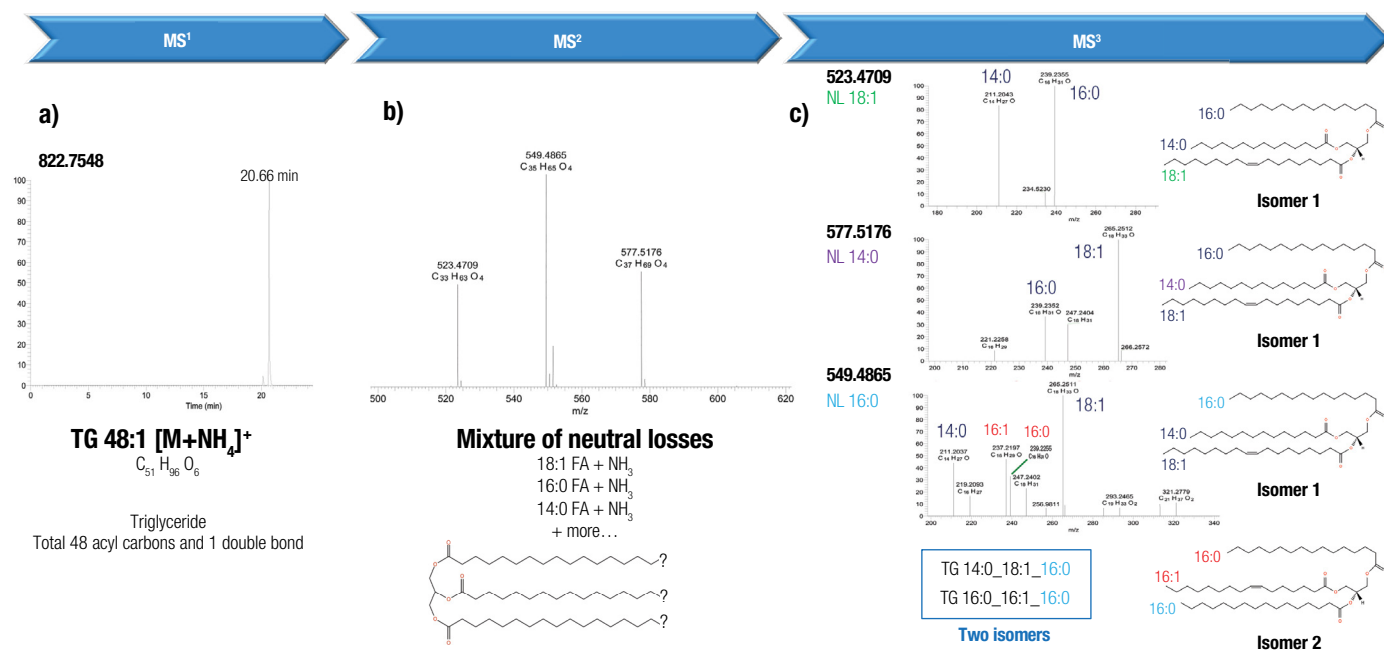


Figure 8. PC 34:0 [M+H]⁺ a) Mass chromatogram m/z 762.6007, b) HCD and CID MS² spectra and c) peak area statistics

Figure 9 illustrates the annotation of triglyceride 48:1 ammonium adduct (m/z 822.7548 at 20.66 min) found in corn rootworm larvae lipid extracts. Three product ions are observed in the HCD MS² spectrum corresponding to neutral loss of 18:1, 16:0, and 14:0 fatty acids. During a single scan cycle, the neutral losses of fatty acid were automatically detected and three additional CID MS³ scans were performed. The MS³ spectrum corresponding to loss of 18:1 fatty acid (Figure 9a) produces 14:0 and 16:0 acyl ions giving the assignment TG 18:1-14:0-16:0 (*isomer 1*). Similarly, the MS³ spectrum corresponding to 14:0 loss (Figure 9b) produces 16:0 and 18:1 acyl ions giving the same assignment (*isomer 1*). However, the MS³ spectrum from 16:0 loss (Figure 9c) is a mixture consisting mainly of *isomer 1* (14:0 and 18:1 acyl ions) and a lesser amount of *isomer 2*, TG 16:0-16:1-16:0, giving rise to 16:0 and 16:1 acyl ions. This example illustrates the power of LC/MSⁿ for elucidating the structure of isomeric mixtures.

Figure 11a shows the chromatogram of 18:2 sitosteryl ester $[M+NH_4]^+$ at m/z 694.6497 and 21.70 minutes. Figure 11b shows the main HCD product ion is the loss of 18:2 FA and ammonia to give the sitosterol carbonium ion (m/z 397.3829). The CID MS^3 spectrum of the m/z 397 ion (Figure 11c) gives the same product ions as those observed in the HCD spectrum, confirming these ions come from the sitosterol hydrocarbon backbone.



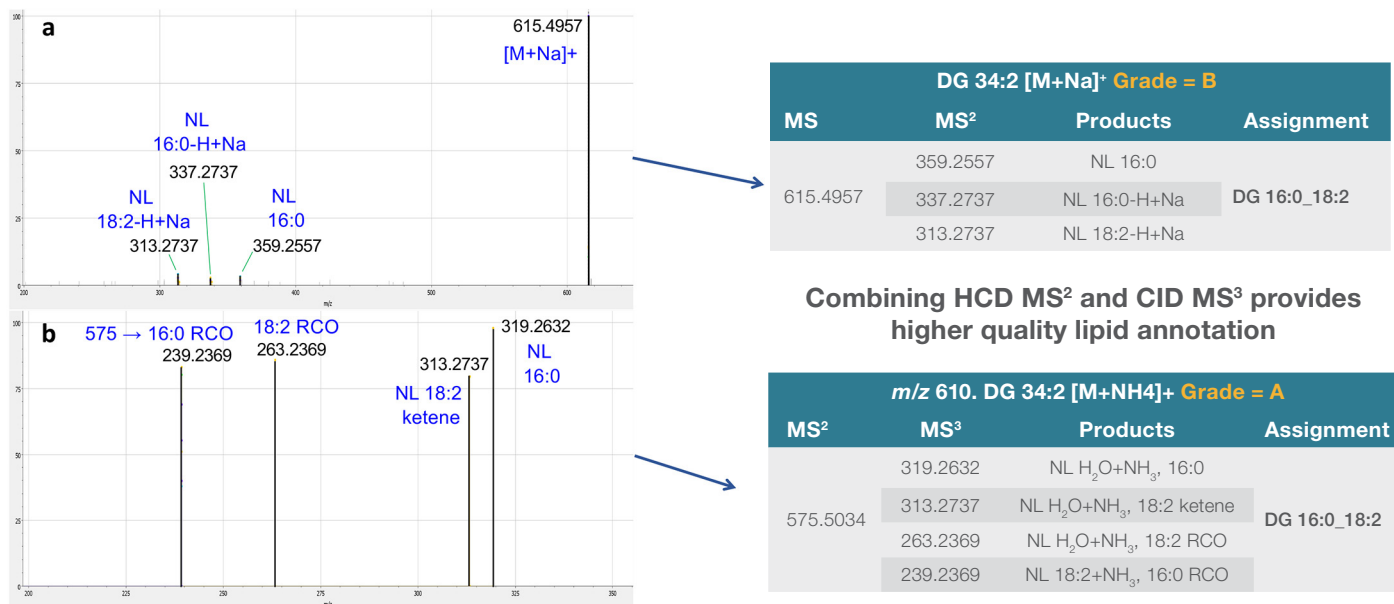


Figure 10. a) HCD MS² of DG 34:2 [M+Na]⁺ at 12.31 min and b) CID MS³ spectra of DG 34:2 [M+NH₄]⁺

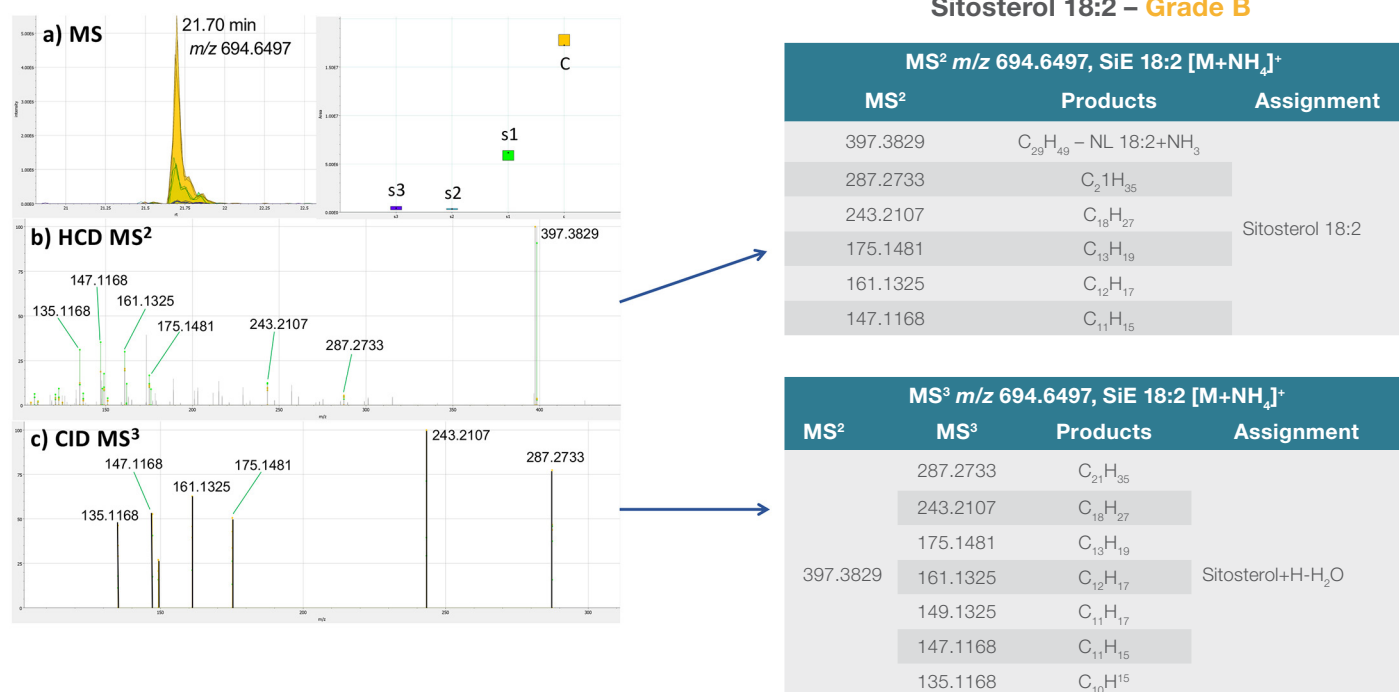
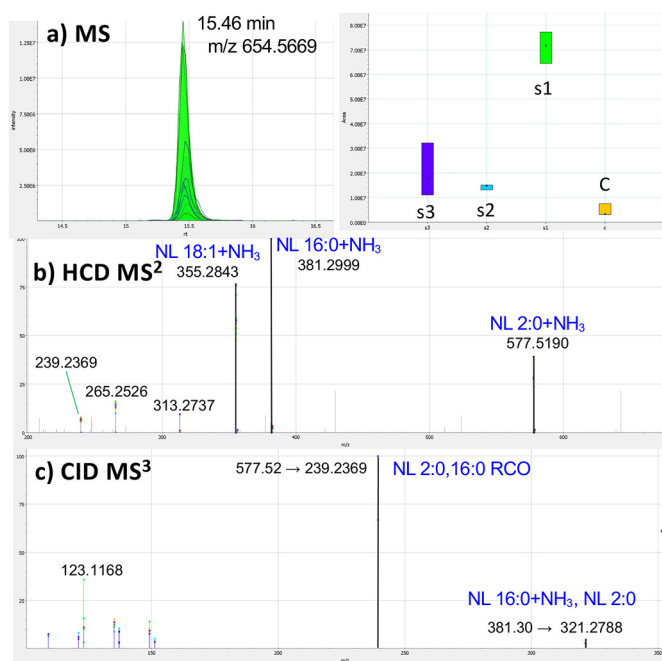


Figure 11. a) Chromatogram of sitosterol 18:2, [M+NH₄]⁺, b) HCD MS² spectrum and c) CID MS³ spectrum

Figure 12 shows the annotation of an unusual 2:0 fatty acid containing triglyceride. The *m/z* 654.5669 chromatogram (Figure 12a) shows a very abundant ammonium adduct for TG 36:1. The HCD MS² spectrum (Figure 12b) could not be explained using the normal range of fatty acids contained in the LipidSearch database for triglycerides. Manual inspection of the first neutral loss (77.0479 Da) suggested that a 2:0 fatty acid

was present. After modifying the database to include 2:0 FA, the assignment of TG 2:0_16:0_18:1 was supported by all the HCD MS² product ions. The MS³ data (Figure 12c) also fully supports this assignment by confirming the neutral loss of 2:0 FA after the initial loss of 16:0 FA and ammonia, adding confidence to the assignment.



TG 36:1 – 2:0-16:0-18:1
All major product ions are accounted for with a single isomer containing 2:0 fatty acid.

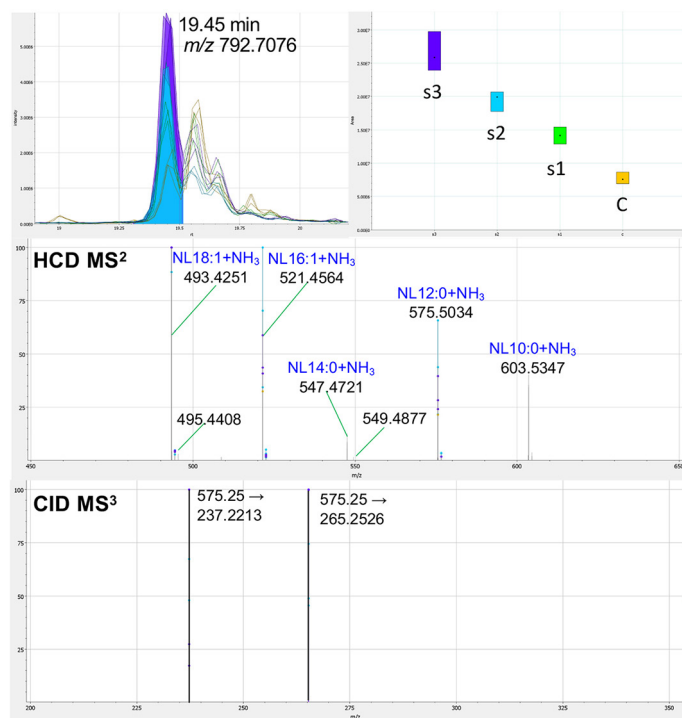
MS ² Results, <i>m/z</i> 654.5669, TG 36:1 M+NH ₄ ⁺			
MS ²	Products	Assignment	
577.5190	NL 2:0+NH ₃	2:0-16:0-18:1	
381.2999	NL 16:0+NH ₃		
355.2843	NL 18:1+NH ₃		
313.2737	NL 2:0+NH ₃ + NL 18:1 ketene		
265.2526	18:1 RCOO		
239.2369	16:0 RCOO		

MS ³ Results, <i>m/z</i> 654.5669, TG 36:1 M+NH ₄ ⁺			
MS ²	MS ³	Products	Assignment
355.2843	239.2369	NL 18:1+NH ₃ , 16:0 RCO	2:0-16:0-18:1
381.2999	321.2788	NL 16:0+NH ₃ , NL 2:0	2:0-16:0-18:1
577.5190	239.2369	NL 2:0+NH ₃ , 16:0 RCO	2:0-16:0-18:1

Figure 12. HCD MS² and CID MS³ of 36:1 triglyceride [M+NH₄]⁺ from insect larvae

Figure 13 illustrates the difficulty of annotating a complex mixture of co-eluting triglyceride species. The information provided by the HCD MS² analysis for TG 46:2 (*m/z* 792.7076) gives 12:0_16:1_18:1 as the predominant

species and this is confirmed by the MS³ results in LipidSearch software as shown by the assignments in blue. However, there are at least 4 other possible combinations based on fatty acid neutral losses.



Mixture of TG 46:2 isomers – 12:0-16:1-18:1 and 10:0-18:1-18:1, 14:0-16:1-16:1, 14:0-14:1-18:1, 14:0-14:0-18:2? MS² Results, *m/z* 792.7076, TG 46:2 [M+NH₄]⁺

MS ² Results, <i>m/z</i> 792.7076, TG 46:2 [M+NH ₄] ⁺		
MS ²	Product Ions	Possible Assignments
603.5347	NL 10:0+NH ₃	10:0-18:1-18:1
575.5034	NL 12:0+NH ₃	12:0-16:1-18:1
549.4877	NL 14:1+NH ₃	14:0-14:1-18:1
547.4721	NL 14:0+NH ₃	14:0-16:1-16:1
521.4564	NL 16:1+NH ₃	12:0-16:1-18:1 or 14:0-16:1-16:1
495.4408	NL 18:2+NH ₃	14:0-14:0-18:2
493.4251	NL 18:1+NH ₃	12:0-16:1-18:1 or 10:0-18:1-18:1

MS ³ Results <i>m/z</i> 792.7076, TG 46:2 [M+NH ₄] ⁺			
MS ²	MS ³	Product Ions	Assignment
575.5034	265.2526	NL 12:0+NH ₃ , 18:1 RCO	12:0-16:1-18:1
	237.2213	NL 12:0+NH ₃ , 16:1 RCO	12:0-16:1-18:1
521.4564	265.2526	NL 16:1+NH ₃ , 18:1 RCO	12:0-16:1-18:1
493.4251	237.2213	NL 18:1+NH ₃ , 16:1 RCO	12:0-16:1-18:1

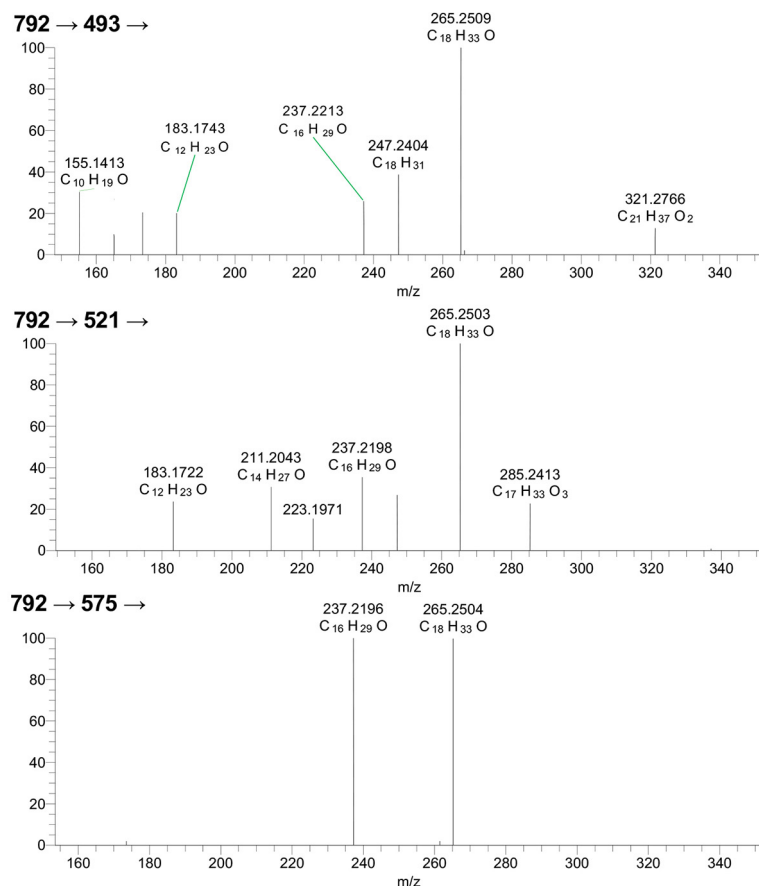
Figure 13. HCD MS² and CID MS³ of 46:2 triglyceride [M+NH₄]⁺ from insect larva

Figure 14 shows the three MS³ spectra obtained for the most abundant species in the MS² spectrum. The MS³ spectra for *m/z* 493 and 521 also confirm there are at least two additional isomers (TG 10:0-18:1-18:1 and 14:0-16:1-16:1, shown in the black assignments) and rule out a substantial amount of the remaining isomers (TG 14:0-14:1-18:1 and 14:0-14:0-18:2). Thus, LC/MS³ analysis is absolutely required to confirm correct annotations when mixtures of isomeric triglycerides are present.

Conclusions

- This LC/MSⁿ 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 lipid structure characterization.
- The AcquireX intelligent LC/MSⁿ workflow provides 17% more lipid annotations compared to using data-dependent MS/MS analysis alone. DG, PC, and TG annotations, targeted for CID MS²/MS³ using product ion or neutral loss scan filters, increased by 25%, 41% and 27%, respectively.
- Furthermore, 275 CID MS² / MS³ spectra were merged with the HCD data to give higher confidence in annotations by an increase in the LipidSearch match score.



Mixture of TG 46:2 isomers – 12:0-16:1-18:1 and 10:0-18:1-18:1, 14:0-16:1-16:1, ~~14:0-14:1-18:1, 14:0-14:0-18:2~~

MS ³ Results <i>m/z</i> 792.7066, TG 46:2 [M+NH ₄] ⁺			
MS ²	MS ³	Product Ions	Assignment
	321.2788	NL 18:1+NH ₃ , NL 10:0	10:0-18:1-18:1
	265.2526	NL 18:1+NH ₃ , 18:1 RCO	10:0-18:1-18:1
493.4251	237.2213	NL 18:1+NH ₃ , 16:1 RCO	12:0-16:1-18:1
	183.1743	NL 18:1+NH ₃ , 12:0 RCO	12:0-16:1-18:1
	155.1430	NL 18:1+NH ₃ , 10:0 RCO	10:0-18:1-18:1
	285.2424	NL 16:1+NH ₃ , NL 16:1 ketene	14:0-16:1-16:1
521.4564	265.2526	NL 16:1+NH ₃ , 18:1 RCO	12:0-16:1-18:1
	237.2213	NL 16:1+NH ₃ , 16:1 RCO	14:0-16:1-16:1
	211.2056	NL 16:1+NH ₃ , 14:0 RCO	14:0-16:1-16:1
	183.1743	NL 16:1+NH ₃ , 12:0 RCO	12:0-16:1-18:1
575.5034	265.2526	NL 12:0+NH ₃ , 18:1 RCO	12:0-16:1-18:1
	237.2213	NL 12:0+NH ₃ , 16:1 RCO	12:0-16:1-18:1

Figure 14. CID MS³ spectra of 46:2 triglyceride [M+NH₄]⁺ from insect larva

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