Increased Depth and Confidence of Insect Lipidome Analysis using Chromatography Based Methods with High-resolution Orbitrap MS^n

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Introduction

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. In addition, essential lipids such as sterols (precursor of hormone) 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 requirement and fitness relationship. Detailed studies of insect lipid composition however, have been technically challenging because of the complexity of lipid profiles. We evaluated the robustness of a new high resolution LC-MS^n approach to perform non-targeted lipid profiling experiments. Western Corn Rootworm larvae (*Diabrotica virgifera virgifera*) was chosen due to it's economical impact estimated at 2 Billions US$ cost associated with its control and corn production loss in the Americas. Three populations of larvae per condition were extracted and the lipids were mixed with commercially available standards. Several data acquisition modes were tested but only the *AcquireX*™ is presented in this work.

Methods

Sample Preparation

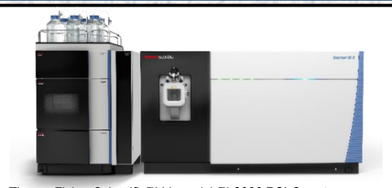
Fresh frozen insect larvae were weighed and softened in hot IPA for 20 minutes to inhibit the lipases (Welti, Li et al. 2002). The samples were then homogenized using motorized glass rod and re-extracted for 10 minutes. After the addition of CHCl3 and water the samples were agitated vigorously for 30 minutes at room temperature.

For tissue containing significant amount of neutral lipids a second extraction with CHCl3:MeOH was performed under agitation at room temperature. The organic phase(s) was washed with 1 M KCl followed by water. The washed organic was then filtered (PTFE disc 0.45 um) and evaporated to dryness under N2 prior reconstitution in appropriate solvent containing Splash™ mix from Avanti at 1/10 dilution.

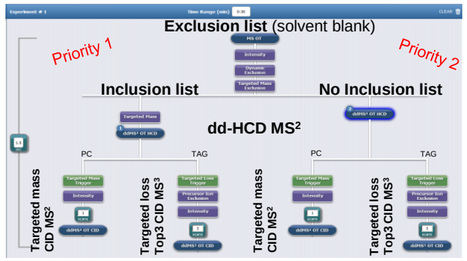
Statistical Analysis

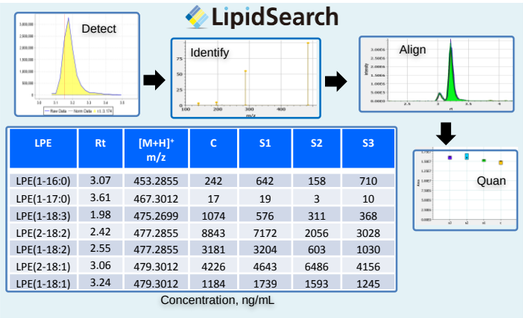
All the lipid identifications resulting from ThermoFisher Scientific™ LipidSearch™ 4.2.9 software were determined in positive and negative polarization modes and combined within the software. Only one adduct by lipid species with best quality score was used to compare four groups of samples. More than 12 measurements for each lipid was tabulated and used for visualization in charts. The tables were exported in Excel then JMP 12.2.0 (SAS Institute). In this poster only the species with highest degree of confidence (rated A or B) detected in 2/3 on the injections (biological replicates) were filtered to be compared with t-test at p<0.01 confidence.

Overview Of The Lipidomic Workflow



LC: ThermoFisher Scientific™ Vanquish™ 3000 RSLC system
Column: ThermoFisher Scientific™ Accucore™ C30 (2.1 x 150 mm, 2.7 µm, 45ºC)
Mobile phase A: 60:40 AcN/H_2O +10mM NH_4HCO_2, 0.1% HCO_2H
Mobile phase B: 90:10 IPA/AcN +10mM NH_4HCO_2, 0.1% HCO_2H
Flow rate: 260 µL/min
Injection volume: 2 µL
MS: *Orbitrap ID-X*™ mass spectrometer with *AcquireX*™ deep scan mode described below





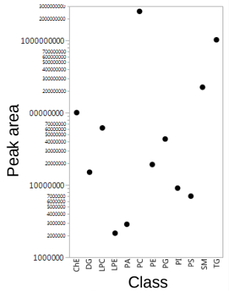
LPE	Rt	[M+H]$^+$ m/z	C	S1	S2	S3
LPE(1-16:0)	3.07	453.2855	242	642	158	710
LPE(1-17:0)	3.61	467.3012	17	19	3	10
LPE(1-18:3)	1.98	475.2699	1074	576	311	368
LPE(2-18:2)	2.42	477.2855	8843	7172	2056	3028
LPE(1-18:2)	2.55	477.2855	3181	3204	603	1030
LPE(2-18:1)	3.06	479.3012	4226	4643	6486	4156
LPE(1-18:1)	3.24	479.3012	1184	1739	1593	1245

Concentration, ng/mL

Result: Sample Preparation

Composition of Splash Mix from Avanti spiked at 10 fold dilution reduced the cost per sample. All but two classes were detected with RSD below 6%.

Class	Components	Conc. (µg/ml)	RT (min.)	RSD (%)
PC	PC15:0-18:1(d7)	16	9.1	4.6
PE	PE15:0-18:1(d7)	0.5	9.6	4.8
PS	PS15:0-18:1(d7)	0.5	8.1	4.9
PG	15:0-18:1(d7) PG	3	8.3	5.7
PI	15:0-18:1(d7) PI	1	7.9	5.4
PA	15:0-18:1(d7) PA	0.7	9.2	6.6
LPC	18:1(d7) LPC	2.5	2.9	4.0
LPE	18:1(d7) LPE	0.5	3.2	5.6
ChE	18:1(d7) Chol Ester	35	21.8	6.2
MG	18:1(d7) MG	0.2	ND	NA
DG	15:0-18:1(d7) DG	1	12.9	5.7
TG	15:0-18:1(d7)-15:0 TG	5.5	20.6	4.9
SM	18:1(d9) SM	3	8.3	5.3
Ch	Cholesterol (d7)	100	ND	NA

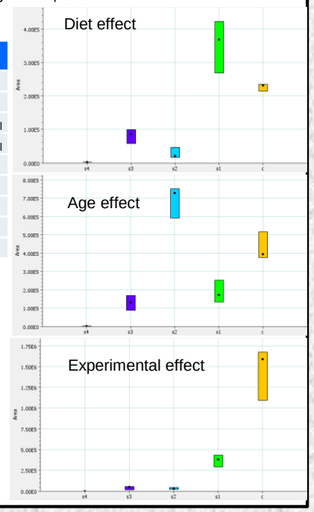


The search for wax esters revealed the presence of (iso)propyl ester. Those esters could be generated during the IPA extraction of whole animals at 80°C with free fatty acids liberated from diet or lipase residual activity products. The T-test on most wax ester reveal an age or diet effect ruling out an experimental flaw due to the slower penetration of IPA in the thicker body of 3rd instar stage development.

Name	RT	Fatty acids	T-test (p<0.01)
WE(21:2)	2.26	18:02	age
WE(21:1)	2.88	18:01	age
WE(23:4)	2	20:04	experimental
WE(23:3)	2.41	20:03	experimental
WE(23:2)	3.02	20:02	age
WE(23:1)	3.9	20:01	age
WE(25:3)	3.2	22:03	diet
WE(25:2)	3.9	22:02	no change
WE(27:2)	4.5	24:02	diet

Below is the color coding for the conditions tested in this experiment

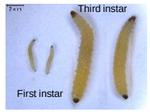
Age \ Diet	Natural	Artificial
1st instar		
3d instar		

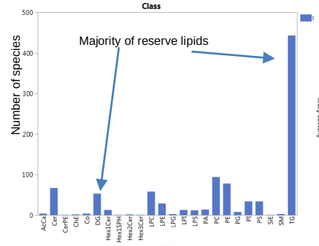


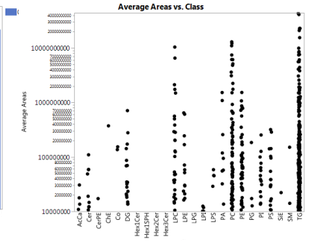
Results: Diet and Age Effects

The sample set was divided in 4 classes with two developmental stages (1st and 3rd instar) and 2 diets. The samples were weighed to a target of 100 mg representing different number of animals between stages (see pictures below). The Acquire-X method allowed the deep scanning for lipid identification and generated three biological replicates per condition. A total of 972 lipid species were compared across all treatments. A large majority of lipids identified were reserved lipids such as TAG and DAG. The peak areas were capture over three orders of magnitude with largest amounts for TAG and PC.

Sample	Stages	Diet	Amount (mg)
1	First	natural	107.1
2	First	natural	110.4
3	First	natural	110.0
4	Third	natural	133.3
5	Third	natural	103.6
6	Third	natural	103.1
7	First	artificial	110.3
8	First	artificial	104.5
9	First	artificial	115.8
10	Third	artificial	111.3
11	Third	artificial	105.5
12	Third	artificial	113.8
13	blank		
14	blank		

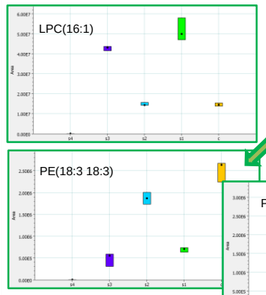


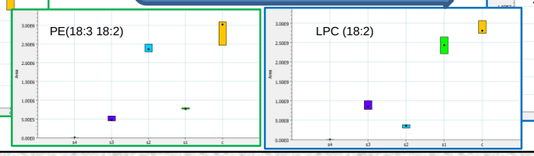


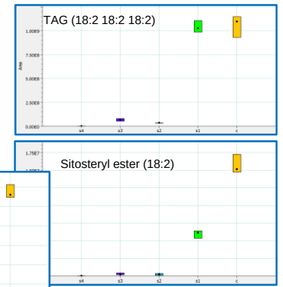


T-test results

Comparison	Lipid Species	Ratio	Ratio	TAG	PE	Cer
		3d <1st	3rd>1st			
Age	313	188	121	145	33	27
		artificial< natural	artificial>natural			
Diet	387	222	159	212	22	19







Conclusions

The performance measured by the RSD of spiked standard representing 12 lipid classes was significantly improved compared with similar analysis performed on a QExactive. This may have been the results of the improved source design incorporated in the Orbitrap ID-X instrument. We still encountered integration issues with PA lipids but it may be resolved by TMSD derivatization.
The IPA extraction step may be producing propyl ester by reacting free fatty acids who origin is still unknown (possibly diet or lipase residual activity).
The Acquire-X strategy detected more than 972 lipid species, which were annotated with high confidence. Diet conditions influenced the storage lipids levels to a greater extend than age difference. More of the functional lipids appeared to correlate with the developmental stages tested in this experiment.
The same sample set was analyzed with QExactive (ddMS2) and processed with LipidSearch under the same separation condition (Gachotte *et al*, poster 288550, ASMS 2017). **226 species** were annotated with similar confidence, while the same acquisition with Orbitrap ID-X™ instrument resulted in **952 species** showing the power of this new instrument for small molecules detection.
The AcquireX™ strategy allowed us to compile biological variability information (enabling statistical analysis) and provided further annotation with MS^n (HCD and CID collisions). This approach was specifically targeting PC and TAG, but could accommodate other lipid classes based on common fragments or targeting added moieties after chemical derivatizations.