Evaluation of the AcquireX workflow for deciphering lipidome analysis of neutral lipids from whole insects using chromatography-based methods with high-resolution Orbitrap MS^n

Daniel Gachotte[1], Yelena Adelfinskaya[1], Jeffrey Gilbert[1], Jonathan Robeson[2], Adane Kassa[2], Reiko Kiyonami[3], Yasuto Yokoi[4] and David Peake[3]

Corteva, Indianapolis, IN, [2]Corteva, Johnston,IA, [3]Thermo Fisher Scientific, San Jose, CA, [4]Mitsui Knowledge Industry, Tokyo, Japan

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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 (precursors of 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 requirement and fitness relationship. Detailed studies of insect lipid composition however, have been technically challenging because of the complexity of lipid profiles. We compared the robustness of a faster LC high resolution LC-MS^n approach to perform non-targeted neutral lipid profiling experiments. Western Corn Rootworm larvae (*Diabrotica virgifera virgifera*) was chosen due to it's economical impact estimated at 2 Billion USD 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 labeled standards. Identification of neutral lipids present in the extracts of whole insects was achieved with two different chromatographic and acquisition methods.

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 $CHCl_3$ and water the samples were agitated vigorously for 30 minutes at room temperature.

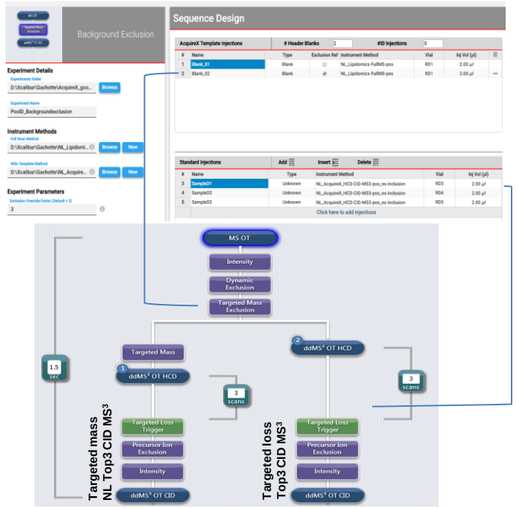
For tissue containing significant amount of neutral lipids a second extraction with $CHCl_3$:MeOH was performed under agitation at room temperature. The organic phase(s) was washed with 1 M KCl followed by water, filtered (PTFE disc 0.45 µm) and evaporated to dryness under N_2 prior to reconstitution in appropriate solvent containing Splash™ mix from Avanti Polar Lipids at 1/10 dilution. All samples were flushed with Argon and kept at -80°C.

Statistical Analysis

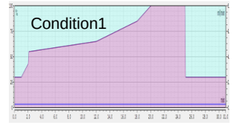
All the neutral lipids (Diacylglycerol, triacylglycerol, cholesterolesters) identifications resulting from ThermoFisher Scientific™ LipidSearch™ 4.2.9 software were determined in positive polarization modes. Only one adduct by lipid species with best quality score was used to compare four groups of samples. 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 (annotation Grade A or B) detected in 2/3 of the injections (biological replicates) were filtered to be compared using the t-test at p<0.01 confidence.

Overview Of The Lipidomic Workflow

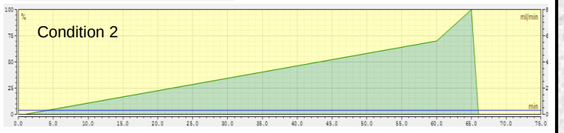
LC: ThermoFisher Scientific™ Dionex Ultimate 3000™ RSLC system
Column: ThermoFisher Scientific™ Accucore™ C30 (2.1 x 150 mm, 2.7 µm, 45ºC)
Condition 1: Mobile phase A: 60:40 AcN/H_2O + 10mM NH_4HCO_2, 0.1% HCO_2H
Mobile phase B: 90:10 IPA/AcN + 10mM NH_4HCO_2, 0.1% HCO_2H
Flow rate: 260 µL/min, Injection volume: 2 µL
Condition 2: Mobile phase A: AcN + 5mM NH_4OAc
Mobile phase B: 75:25 IPA/MTBE + 5mM NH_4OAc
Flow rate: 300 µL/min, Injection volume: 2 µL
Ref: Dionex HPLC-CAD lipid detection application note
Narveaz-Rivas, et al. J. Chrom. A 2016
MS: ThermoFisher Scientific™ *Orbitrap Fusion* MS *with Optamax NG* source
AcquireX™ background exclusion mode described below:



Result: Sample Separation



Eluent profile of two LC separations compared in this study. The first condition could reduce acquisition time by half and eliminate the use of MTBE solvent for neutral lipid determination. Data was not collected during column re-equilibration time.



Splash mix was spiked in the samples at the concentration shown below.
Chromatographic condition 2 was also tested with a Q Exactive Plus.
Better reproducibility was obtained with the Fusion and both neutral lipid LC methods.

Class	Components	Conc.	Condition2			Condition1	
				QE_RSD	Fusion_RSD		
		(µg/ml)	RT (min)	(%)	(%)	RT (min)	RSD (%)
ChE	18:1(d7) Chol Ester	35	47.1	6.2	6.5	23.9	8
DAG	15:0-18:1(d7) DG	1	ND	NA	ND	16.9	5.2
TAG	15:0-18:1(d7)-15:0 TG	5.5	38.1	44	6.75	22.56	7.5
Ch	Cholesterol (d7)	100	ND	NA	NA	NA	NA

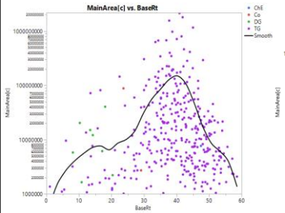
The elution profile of neutral lipids is shown below. A more complete separation of DAG and TAG species is obtained using condition 2. The detection of DAG is better under condition 1.

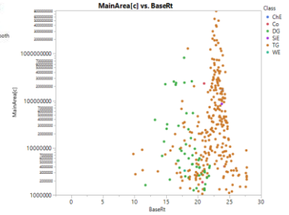
Condition 2

Class	Species
TAG	415
DAG	17

Condition 1

Class	Species
TAG	298
DAG	60

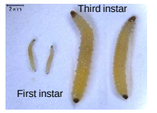




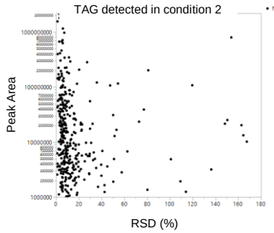
Results: Diet and Age Effects

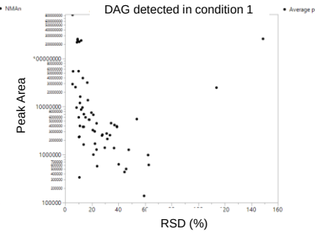
The sample set was divided into 4 classes with two developmental stages (1st and 3rd instar) and 2 diets. The samples were weighed to a target of 100 mg representing different number of larvae between stages (see pictures below). The Acquire-X method allowed Targeted Loss (fatty acid and ammonia) based identification at the MS^3-level for TAGs and DAGs. A maximum of 415 TAG species were detected with chromatographic condition 2 and 60 DAG species with condition 1. Both chromatographic methods were necessary to collect a maximum of information. Peak areas were determined over ~4 orders of magnitude.

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		



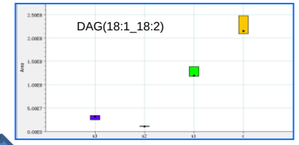
Age \ Diet	Natural	Artificial
1st instar		
3d instar		

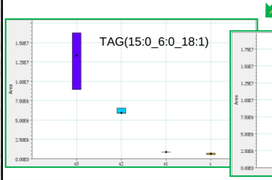


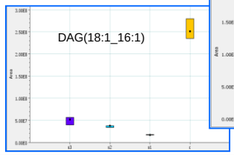


T-test results (p<0.01)

Class	Lipid Species	AGE	DIET
TAG	415	98	295
DAG	60	23	29







Conclusions

Reproducibility measured by the variation of spiked standards representing 4 neutral lipid classes was significantly improved for two classes compared with the analysis performed on a Q Exactive. Chromatographic conditions did not influence the result. The *Optamax*® source and the Fusion instrument reduced variability.

The AcquireX strategy detected more than 358 and 432 neutral lipid species with chromatographic conditions 1 and 2, respectively. Both separations are complementary for DAG and TAG species. Diet conditions influenced the storage lipids levels to a greater extend than age difference.

The same sample set analyzed previously using the Q Exactive under the same separation conditions (Gachotte *et al*, poster 288550, ASMS 2017) gave **163 species** annotated with similar confidence. The same acquisition with the Fusion™ instrument resulted in **415 species** showing the power of this new instrument.

The AcquireX™ strategy allowed us to compile biological variability information (statistical analysis) and provided further annotation with MS^n (HCD and CID). This approach was specifically targeting DAG and TAG, but could accommodate other targeted lipid classes including other moieties after chemical derivatization.