Improved Lipid Annotation Depth using Automatically Generated Inclusion and Exclusion Lists on an Orbitrap-based Mass Spectrometer.

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ABSTRACT

Purpose: To demonstrate the application of an automated background-exclusion and compound-inclusion generation workflow for global lipidome profiling, leading to a greater number and greater confidence of lipid annotations.

Methods: Bovine liver and heart total lipid extracts (Avanti Polar Lipids) were analyzed using a Thermo Scientific™ Vanquish™ UHPLC system coupled with a Thermo Scientific™ Orbitrap ID-X™ Tribrid™ mass spectrometer. The data was processed using Thermo Scientific™ LipidSearch™ 4.2 software.

Results: The use of automated feature exclusion and compound inclusion lists leads to an increase in lipid annotations in the analysis of Bovine liver and heart extracts, in particular for low abundance lipids, from the additional non-redundant fragmentation data that was generated in this experiment .

INTRODUCTION

Lipid profiling provides valuable information to identify disease states and other physiological changes. A common approach for lipidomics profiling is to identify lipid species by their MS/MS spectra prior to extraction of precursor information for relative quantitation. With this approach, collecting MS/MS data on every sample relevant compound is crucial for confident lipid species annotation. The novel intelligent data acquisition strategy, AcquireX, on the Orbitrap ID-X Tribrid mass spectrometer excludes background ions from the MS/MS acquisition and prioritizes MS/MS acquisition on sample relevant compounds automatically, resulting in deeper lipidome coverage. Here we present improved lipid identification coverage for lipid extract samples by using AcquireX on a Orbitrap ID-X Tribrid mass spectrometer.

MATERIALS AND METHODS

Sample Preparation

Bovine liver and heart total lipid extracts (Avanti Polar Lipids, Alabaster, AL) were used as model lipid extracts. The extracts (25 mg/mL) were diluted 1:100 in a mixture of 50:50 acetonitrile/isopropanol containing 1:10 diluted SPLASH™ LipidoMix™ (Avanti Polar Lipids, Alabaster, AL). Samples were placed in low volume glass LC vials using vial caps with PTFE-only septa. The vials were kept at 15 ° C in the autosampler prior to and during analysis.

LC-MS Method

The LC-MS analyses were performed on a Vanquish Horizon Binary UHPLC system coupled to an Orbitrap ID-X Tribrid MS. Chromatographic separation was achieved using the gradient conditions shown in Table 1. The mobile phases consisted of 60:40 acetonitrile/water for mobile phase A and 90:10 isopropanol/acetonitrile for mobile phase B, with both A and B containing 10 mM ammonium formate and 0.1% formic acid. The separation used a Thermo Scientific™ Accucore™ C30 column (2.1X150 mm, 2.6 µm) operated at 45 °C with a flow rate of 260 µL/min. The injection volume was 2 µL. Each sample was analyzed in triplicate.

Data was collected on an Orbitrap ID-X Tribrid mass spectrometer, with the HESI source conditions reproduced in Table 2. For acquisition using the AcquireX DeepScan workflow, the instrument templates “AcquireX lipid characterization HCD-CID-MS3” and “AcquireX lipid characterization Exclusion-Inclusion reference” were used with the key method parameters and modifications as indicated in Table 2.

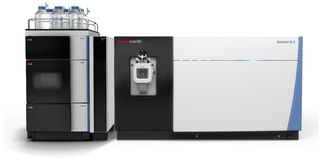


Table 1. HPLC Gradient

Time (min)	%B
0.0	30
2.0	43
2.1	55
12.0	65
18.0	85
20.0	100
25.0	100
25.1	30
31.0	30

Table 2. Source and method parameters used in the analysis on the Orbitrap ID-X Tribrid MS.

HESI Source		Orbitrap ID-X MS	
Sheath gas	40	**MS1: Resolution**	120,000 (FWHM @ *m/z* 200)
Aux gas	10	**Mass range**	200–1700 Da
Sweep gas	1	**AGC Target**	1.0e5
Spray voltage	+3.25/–3.0 kV	**MS2: Resolution**	30,000 (FWHM @ *m/z* 200)
Cap. temp	300 °C	**Cycle time**	1.0 sec
Aux. temp	275 °C	**Isolation width**	1.2 Da
RF Lens	40.0	**HCD Normalized Collision Energies**	24-27-30 (pos) 20-30-40 (neg)
		AGC Target	2.0e4

Data Analysis

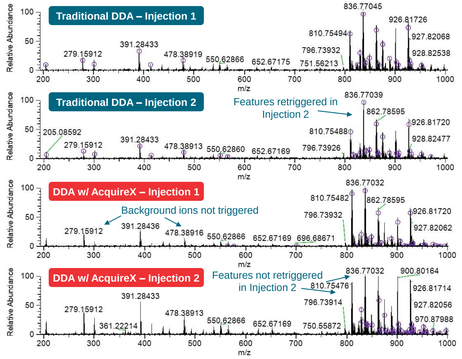
LipidSearch 4.2 software was used for identification and quantitation. The individual RAW files were first searched for MS/MS spectral matches to a database of predicted lipid fragment ions of relevant lipid subclasses for all precursor adduct ions (M+H, M+Na, M+NH_4, M-H_2O+H, M-H, M+HCOO, M-2H, M-CH_3) measured within ±5 ppm. The product ions that matched the predicted fragment ions within a ±5 ppm mass tolerance were used in the calculation of a match-score, with those lipid candidates providing the highest quality match chosen as the annotation. In the next step, search results from the individual files in both positive and negative ion modes were aligned within a retention time window (±0.1 min) and the data merged for each annotated lipid.

RESULTS

Automated Background Inclusion and Compound Inclusion with AcquireX

To prioritize cycle time in data-dependent acquisition (DDA) on sample-related components, MS1-only data was first acquired on both solvent blank and sample, with the instrument automatically performing compound-level detection on each, to create *m/z* and RT-containing exclusion and inclusion lists from the two injections, respectively. In the following DDA MS^n analyses of the sample, these lists were automatically updated between runs to avoid redundant data acquisition, with both outcomes illustrated in Figure 1.

Figure 1. The effect of AcquireX DeepScan – Instead of selecting the same features for fragmentation in subsequent injections, background ions are excluded and lower abundance features triggered. (purple circle denotes features triggered for MS2 within ±6 s of the MS1 spectrum shown



Lipid Annotations with LipidSearch 4.2

As a result of the AcquireX DeepScan data acquisition strategy, a larger number of lipids could be identified based on the acquired MS^n spectral data, when searched against the lipid fragmentation database of LipidSearch 4.2, as shown in Figure 2, with an increase of 41% over a traditional DDA approach after 4 injections. The effect was notably smaller for very confidently identified lipids, shown in Figure 3, in part because the additional lipid annotations are from lower abundance lipids uniquely triggered with AcquireX, as seen in Figure 4.

Figure 2. Number of lipid annotations in LS4.2 by number of injections of Bovine Liver TLE, with background filtered.

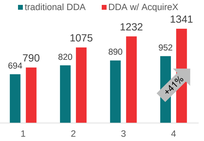


Figure 3. Number of high-quality filtered[1] lipid molecule annotations in LS4.2 by number of injections of Bovine Liver TLE with background filtered.

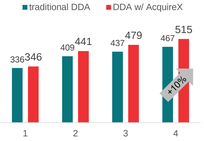


Figure 4. Plot of Peak Intensity over retention time for lipid annotations with (red) and without (black circle) AcquireX DeepScan illustrates the additional low-abundance lipid annotations as a result of low intensity features triggered for MS/MS.

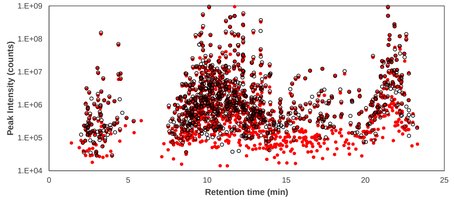
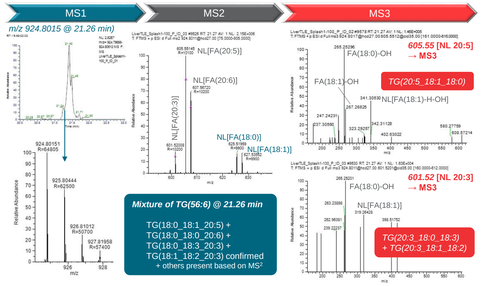


Figure 5. The MS^n capabilities of the Orbitrap ID-X Tribrid MS allow the acquisition of fatty acid neutral-loss triggered MS^3 spectra, to add additional confidence in annotating TGs, particularly in the case of co-eluting isomeric species, as shown here for isomers of TG(56:6).



Dilution Series of deuterium-labeled Internal Standards

To further explore the instrument’s ability to trigger low abundance lipids for identification from MS/MS spectral data using AcquireX DeepScan, a series of experiments were performed by spiking the stable-isotope labeled SPLASH™ Lipidomix™ standard mixture into Bovine liver at varying concentrations and analyzing them with and without AcquireX DeepScan, in turn. Table 3 and Figure 6 illustrate the performance increase due to the intelligent acquisition, which allowed the instrument to trigger and identify the lipid standards at 4–20 fold lower concentration in a complex mixture.

Table 3. Comparing the performance of AcquireX DeepScan and a traditional DDA approach in obtaining MS/MS spectra from three injections, respectively, for stable-isotope labeled standards of Cholesterol, PE, PI and PS in a Bovine Liver Extract at different concentrations.

Cholesterol-d7				15:0-18:1(d7) PE			
Conc. (µg/mL)	Precursor Intensity	MS2 w/ AX	MS2 w/ DDA	Conc. (µg/mL)	Precursor Intensity	MS2 w/ AX	MS2 w/ DDA
100	2.2e6	☑	☑	**5**	5.4e6	☑	☑
10	4.8e5	☑	☒	**0.5**	6.3e5	☑	☑
5	2.4e5	☑	☒	**0.25**	2.8e5	☑	☒
2.5	1.0e5	☒	☒	**0.125**	1.3e5	☑	☒
1	6.4e4	☒	☒	**0.05**	8.3e4	☑	☒
15:0-18:1(d7) PI				**15:0-18:1(d7) PS**			
Conc. (µg/mL)	**Precursor Intensity**	**MS2 w/ AX**	**MS2 w/ DDA**	**Conc. (µg/mL)**	**Precursor Intensity**	**MS2 w/ AX**	**MS2 w/ DDA**
10	2.5e6	☑	☑	**5**	1.5e6	☑	☑
1	2.5e5	☑	☑	**0.5**	1.2e5	☑	☒
0.5	1.4e5	☑	☒	**0.25**	5.2e4	☑	☒
0.25	5.3e4	☑	☒	**0.125**	n.d.	☒	☒
0.1	3.3e4	☒	☒	**0.05**	n.d.	☒	☒

Figure 6. Illustration of a lipid standard (15:0-18:1(d7) PE from Table 3) triggered at low abundance only using AcquireX DeepScan, yielding high-quality MS/MS spectral data to allow identification of the lipid compound, based on the observed PE headgroup neutral loss.

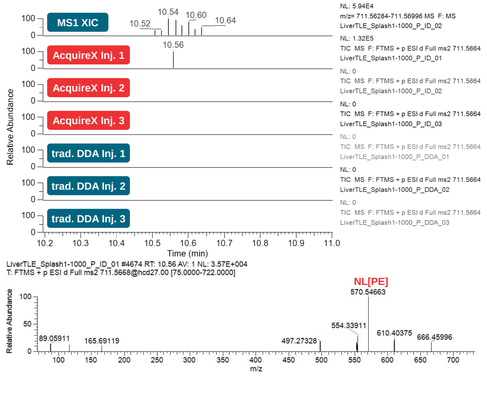


Table 4. Summary of lipids annotated using LipidSearch 4.2 software in both liver and heart total lipid extracts from triplicate injections in both polarities, background lipids removed.

Lipid Subclass	Bovine Liver TLE		Bovine Heart TLE		Combined Processing	
	All Lipids	High Quality ID[1]	All Lipids	High Quality ID[1]	All Lipids	High Quality ID[1]
AcCa	4	4	48	48	49	49
CL	42	2	33	1	47	4
Cer	98	61	93	58	133	94
ChE	13	13	0	0	11	11
Co	2	2	5	5	6	6
DG	44	34	129	66	144	78
DLCL	0	0	1	0	1	0
Hex1Cer	2	2	9	6	10	7
Hex2Cer	2	1	5	2	8	4
Hex3Cer	3	1	5	2	5	2
LPC	42	41	55	52	64	63
LPE	20	20	12	9	18	17
LPI	5	5	1	1	1	1
LPS	1	1	1	0	2	1
LPS	0	0	1	1	2	2
LSM	1	1	0	0	0	0
MLCL	17	0	7	1	11	1
PA	2	2	0	0	0	0
PC	473	111	617	242	854	292
PE	167	109	203	103	280	153
PG	9	8	4	3	11	9
PI	42	27	49	31	74	40
PS	14	10	13	12	22	15
SM	83	83	123	122	144	143
SPH	3	1	2	1	1	1
TG	140	116	495	435	642	569
WE	2	2	3	3	3	3
TOTAL	**1229**	**655**	**1911**	**1201**	**2540**	**1562**

CONCLUSIONS

- The AcquireX DeepScan data acquisition strategy was applied to Bovine-derived lipid extracts using the Orbitrap ID-X Tribrid MS and the increased annotation depth was demonstrated to be a result of additional fragmentation spectra obtained for low abundance lipids.
- In a set of dilution experiments of stable-isotope labeled lipid standards in a Bovine lipid extract, the standards could consistently be triggered for fragmentation at 4–20 fold lower abundance using AcquireX DeepScan, when compared to a traditional approach.
- For the Bovine liver and heart extracts, a total of 2540 lipids were annotated using LipidSearch 4.2, over 60% of which were determined to be high quality annotations[1], based on a combination of observed fragmentation coverage, identity of main adduct ion and chromatographic peak quality.

REFERENCES

1. **AN647.** Reiko Kiyonami, David A Peake, Devin Drew, Xuefei Sun, Xiaodong Liu, and Ken Miller, “Large-Scale Lipid Profiling of a Human Serum Lipidome Using a High-Resolution, Accurate-Mass LC/MS/MS Approach”.

TRADEMARKS/LICENSING

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