Higher Resolution LC-MS and MS-MS Analysis of Lipid Extracts Using Benchtop Orbitrap-based Mass Spectrometers and LipidSearch Software

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Overview

Purpose: Benchmark the performance of a new LC-MS/MS system and determine the effect of full scan MS resolution vs. the number of identified lipids in total lipid extracts.

Methods: Lipid extracts were profiled using LC-dd-MS/MS analysis using a novel quadrupole-Orbitrap mass spectrometer operated at mass resolution of 30, 60, 120 and 240K.

Results: The data presented here shows that lipidomic profiling at higher full scan MS resolution provides more identified lipids during an LC-MS run. The higher acquisition rate of MS/MS spectra also contributes to significantly more lipids being identified.

Introduction

Lipids play a key role in cell, tissue and organ physiology with diseases such as cancer and diabetes which involve disruption of their metabolic enzymes and pathways. Identification of unique lipid biomarkers to distinguish healthy humans compared to those with a disease can have an impact on the early detection of diseases and personalized medicine.

Because of the complexity of the lipidome, which includes 8 major categories of lipids, over 80 major classes, 300 sub-classes and thousands of lipid species[1], HPLC MS/MS methods are often used to separate many overlapping isomeric or isobaric molecular ions in biological samples for increased lipid identification coverage. By employing a high resolution accurate mass (HR/AM) platform equipped with a ultra-high field Orbitrap mass spectrometer (Thermo Scientific™ Q Exactive HF™ MS) to detect lipids after HPLC separation, ultra-high resolution MS and MS/MS analysis provides unambiguous identification of lipids in biological samples.

However, it is extremely challenging to process all the MS and MS/MS data manually. A sophisticated software with an extensive database is required to identify all detected lipids HPLC MS/MS data. The newly-released Thermo Scientific™ LipidSearch™ software enables automated identification of lipids from biological samples based on its large lipid database which includes >1.5 million lipid ions and predicted fragment ions. In addition, relative quantification of identified lipid precursors can be carried out in the same LC/MS/MS experiment.

In this study, we present that hundreds of lipid species can be simultaneously identified and quantified in a single LC/MS-MS experiment by using optimized HPLC separation and HR/AM MS and data-dependent MS^2 conditions.

Methods

LC-MS Sample Preparation.

Bovine brain, heart, liver and yeast total lipid extracts (2.5 mg/mL in Chloroform) were purchased from Avanti Polar Lipids. Dilution series of each lipid extract was prepared by diluting the stock solutions sequentially into 1.25 µg/µL, 500 ng/µL, 250 ng/µL, 125 ng/µL and 50 ng/µL in 50:50 Methanol and Isopropanol (IPA).

HPLC Method.

A Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation LC (RSLC) system performed separations using the gradient conditions shown in Table 1[2]. Mobile phase A was 60:40 Acetonitrile / Water and mobile phase B was 90:10 IPA / Acetonitrile; both A and B contained 10mM ammonium formate and 0.1% formic acid. The column was an Ascentis Express C18 (Supelco, 2.1 x 100mm, 2.7µm) operated at 55°C, flow rate of 260 µL/min and the injection volume was 2 µL.

MS Conditions.

Thermo Scientific Q Exactive Plus and Q Exactive HF instruments were employed for untargeted lipid profiling experiments using the instrument operating conditions shown in Table 2. Each instrument was operated under optimized conditions providing sufficient scans across the chromatographic peak profile for accurate relative quantification using the HR/AM precursor ion while simultaneously acquiring dd-MS^2 spectra for lipid identification.

Data Analysis Software.

LipidSearch software was used for lipid identification and relative quantification.

TABLE 1. HPLC Gradient

Time, min	% A	% B
0.00	68	32
1.50	68	32
4.00	55	45
5.00	48	52
8.00	42	58
11.00	34	66
14.00	30	70
18.00	25	75
21.00	3	97
25.00	3	97
25.01	68	32
33.00	68	32

TABLE 2. Orbitrap Operating Conditions

HESI Source	Q Exactive Plus	Q Exactive HF
Sheath gas 35	Pos. or Neg. Ion	Pos. or Neg. Ion
Aux gas 3	MS Resolution 17.5K, 35K, 70K, 140K	MS Resolution 30K, 60K, 120K, 240K
Spray volt. 4.2 kV	Top15 dd-MS^2 R = 35K	Top20 dd-MS^2 R = 30K
S-Lens 50	MS^2 Isolation width 1 Da	MS^2 Isolation width 1 Da
Cap. Temp. 320°C	Stepped NCE – Pos. 25, 30 Neg. 20, 24, 28	Stepped NCE – Pos. 25, 30 Neg. 20, 24, 28
Heater temp. 300°C	AGC target 1E+6 MS 1E+5 MS^2	AGC target 1E+6 MS 1E+5 MS^2

Data Processing

LC-MS/MS Data Processing Workflow using LipidSearch Software (Figure 1).

1) Peak Detection. Read raw files, MS^n and precursor ion accurate masses.

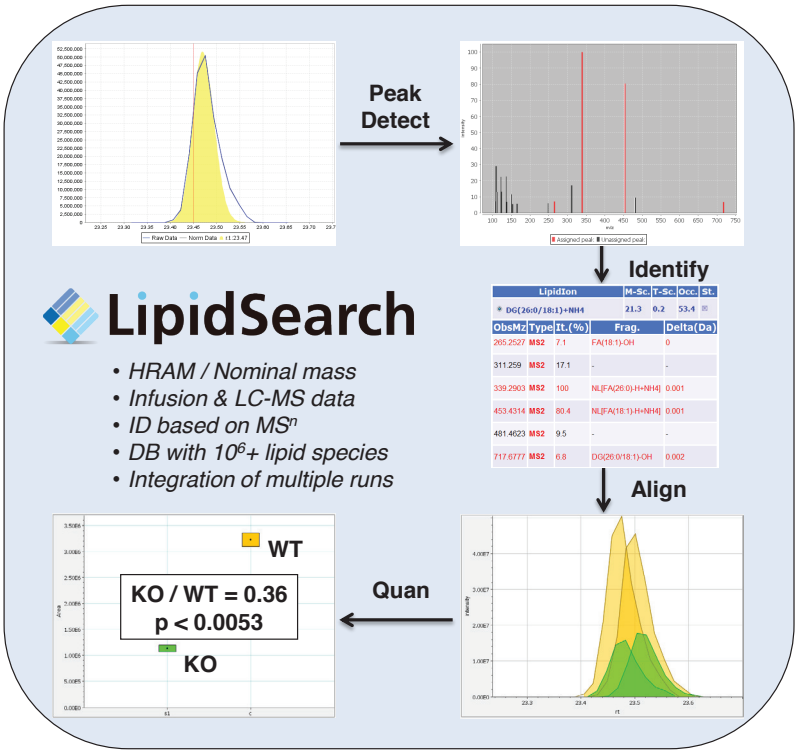
2) Identification. Candidate molecular species are identified by searching a large database >10^6 entries of accurate m/z (lipid precursor and fragment ions) predicted from each potential lipid structure and positive/negative ion adduct.

3) Alignment. The search results for each individual sample are aligned within a time window and the results are combined into a single report.

4) Quantification. The accurate-mass extracted ion chromatograms are integrated for each identified lipid precursor and the peak areas are obtained.

5) Statistical Analysis. t-Tests determine significantly differences between lipids in sample vs. control groups, and results are displayed in a whisker plot.

FIGURE 1. LipidSearch Workflow.



Results

Lipid Identification Results from LC/dd-MS² Data. Bovine heart total lipid extract (1.25 µg/µL) was analyzed by LC/MS and dd-MS/MS in positive and negative ion modes, respectively (Figure 2). LipidSearch software was used to search the LC-MS data for all common lipid classes using a mass tolerance of ±5 ppm for precursor and product ions. For each MS² spectrum matching a predicted fragmentation pattern from the lipid precursor ion m/z stored in the LipidSearch database, search results are summarized for the identified lipid species with m-score indicating the fit to the experimental data (Figure 3).

The speed advantage of Q Exactive HF (120K resolution) and TOP20 dd-MS² is illustrated in Figure 4, compared to Q Exactive Plus (70K) and TOP15 dd-MS². At the higher resolution (120K) the Q Exactive HF obtains 20% more lipid identifications.

FIGURE 2. High Resolution (70,000) Accurate Mass Positive and Negative Total ion Chromatograms of Bovine Heart Total Lipid Extract.

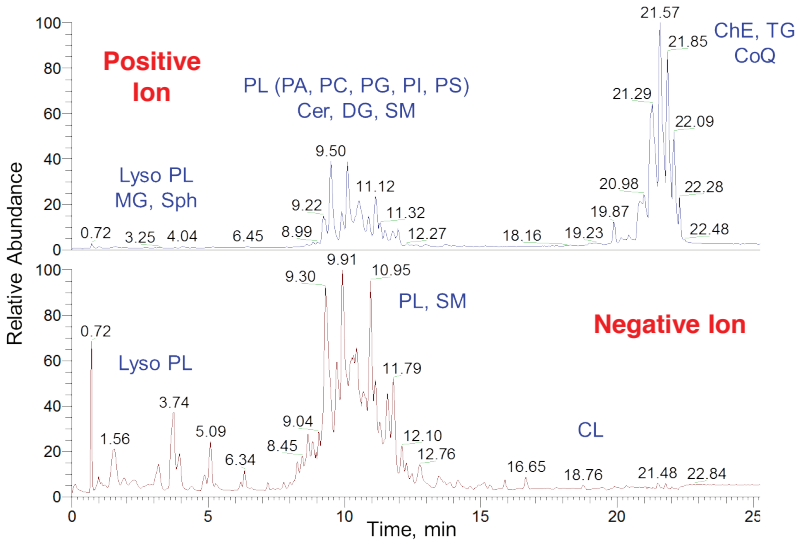


FIGURE 3. Search Results for *m/z* 880.6068, PC(40:5) M+HCO$_2^-$

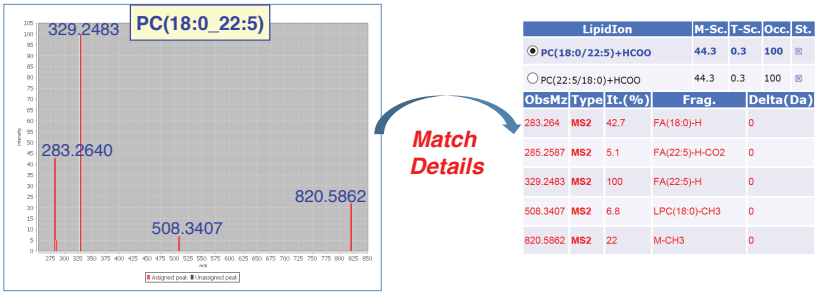
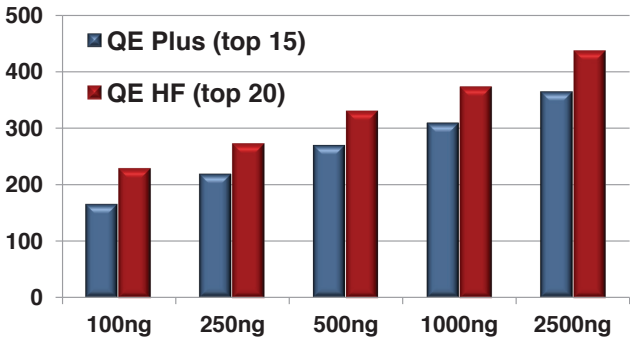


FIGURE 4. Increase in the Number of Lipid Species Identified in Bovine Heart Lipid Extract using dd-LCMS² with Q Exactive HF Compared to Q Exactive Plus



Improved ID and Relative Quantitation Results Obtained with Q Exactive HF

Higher MS resolution lipid profiling translates into improved identification when closely related lipids overlap even under chromatographic conditions. The effect of MS1 resolution for identification of lipid species at the same retention time is illustrated for two Lyso phospholipid species, 18:1 LPE and 16:0p LPC. These two lipids overlap during analysis at 2.2 minutes at m/z in positive ion of 480.3. The mass resolution needed to separate these two lipids is illustrated in Figure 5. At a resolution of 10K, the smaller LPE 18:1 peak (m/z 480.3085) is overlapped by LPC 16:0p (m/z 480.3449) and is only partially resolved at 22K. The 60K setting giving an actual resolution of 42K is required for unequivocal identification of both M+H ions. Thus, identification of minor lipid species is challenging without sufficient mass resolution, leading to fewer ID's.

The number of lipids identified in positive and negative ion, and the aligned (merged) results are shown in Table 3. Note that the number of lipid species after alignment may exceed the sum of positive and negative ion species because of the correlation step where lipids are identified in multiple samples and isomers are present at the MS^2 level.

FIGURE 5. Increased Resolution Improves Identification of Overlapping Lyso PC and PE Lipid Species. Resolution (m/z 200) = 15K, 30K, 60K and 120K

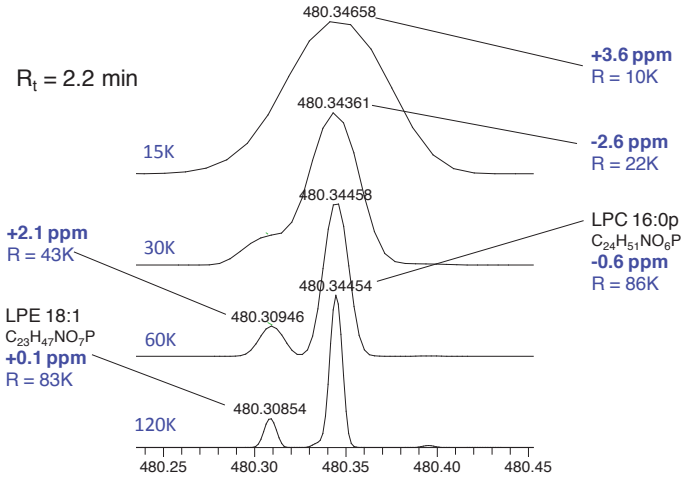


TABLE 3. Comparison of Lipid Species Identified in Total Lipid Extracts of Bovine Brain, Heart, Liver and from Yeast

Lipid Class	Bovine Brain			Bovine Heart			Bovine Liver			Yeast		
	Neg	Pos	Merged	Neg	Pos	Merged	Neg	Pos	Merged	Neg	Pos	Merged
CL				9		**9**	1		**2**	2		**2**
LPC	13	34	**40**	14	28	**30**	18	36	**39**	13	25	**40**
PC	49	152	**242**	78	141	**253**	61	137	**294**	26	58	**92**
LPE	14	15	**24**	14	8	**17**	15	10	**17**	9	9	**12**
PE	88	73	**171**	92	45	**165**	74	51	**248**	19	19	**34**
LPS	6	4	**7**	1	1	**2**	7	1	**7**	3		**3**
PS	35	24	**63**	37	6	**53**	23	1	**65**	24	6	**38**
LPG	2		**2**	4		**4**	5		**5**	1		**1**
PG	8	2	**9**	26	4	**35**	17	5	**48**	10	3	**10**
LPI	4	2	**4**	5	1	**5**	9	3	**9**	4	2	**5**
PI	19	19	**33**	31	25	**55**	34	33	**98**	27	41	**55**
PA	8		**13**	3		**3**	5		**11**	9	3	**18**
SM	34	41	**62**	37	39	**56**	37	39	**91**	6	7	**12**
So		4	**5**		2	**2**		5	**5**		3	**3**
Cer	2	31	**34**	1	17	**18**	5	41	**46**		2	**2**
CerG1	2	33	**37**		1	**1**		9	**9**		2	**3**
CerG2		2	**2**					3	**3**			
ChE		5	**5**					5	**5**			
ZyE											4	**2**
DG		26	**35**		32	**45**		29	**44**		27	**31**
TG		86	**147**		174	**329**		155	**248**		161	**241**
CoQ		1	**1**		5	**5**		3	**3**		4	**3**
Total	284	552	**936**	351	528	**1087**	310	568	**1297**	153	374	**607**

TABLE 4. Typical Composition of Total Lipid Extracts (Avanti Lipids)

Wt %	Brain	Heart	Liver	Yeast
PA	3	1		1
LPC				1
PC	10	5	42	19
LPE				2
PE				1
PG	17	7	22	5
LPI			1	
PI	2	3	8	13
PS	11			4
CL		2		
Chol			7	
Neutrals		50	20	
Unknown	59	32		
Total	**100**	**100**	**100**	**45**

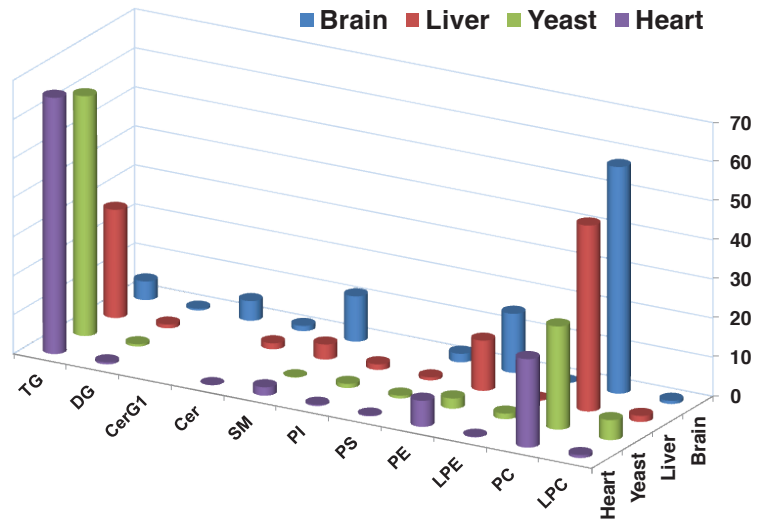
Composition of Total Lipid Extracts

Composition information for the total lipid extracts listed on Avanti Polar Lipids website are shown in Table 4.

The LC-MS^2 results for each lipid extract injected in duplicate were searched using LipidSearch, and positive and negative ion data were aligned within a 0.4 min window (Table 3).

The peak areas for each lipid class were summed and the relative amounts of each lipid class were plotted as % Area from the full scan MS (Figure 6). As expected from the 50 wt % of neutral lipids, the bovine heart extract contained the highest amount of TG (65% by peak area), and the yeast extract contained 61% TG. Brain, liver and yeast extracts contained the most PC.

FIGURE 6. Composition of Lipid Extracts by LC-HRMS Peak Area (%)



Conclusion

- Four different total lipid extracts were profiled and are suitable for benchmarking lipidomics experiments, separation conditions and instrumental optimization.
- Higher resolution and speed of Q Exactive HF provides more confident identification and relative quantification in a single positive and negative ion run.
- At least 20% more lipid ID's were obtained using a ultra-high field benchtop Orbitrap.
- LipidSearch provides automated identification of more than 500 lipid species in a single LC-ddMS^2 experiment, and alignment of multiple samples gives over 1000 lipid species.

References

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2. Lipidomics profiling by high-resolution LC-MS and high-energy collisional dissociation fragmentation: focus on characterization of mitochondrial cardiolipins and monolysocardiolipins. S Bird, et al., Anal. Chem. **2011**, 83, 940–949. dx.doi.org/10.1021/ac102598u.

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