Application Note 607

Increased Identification Coverage and Throughput for Complex Lipidomes

Reiko Kiyonami, David Peake, Yingying Huang, Thermo Fisher Scientific, San Jose, CA USA

Key Words

Q Exactive HF, high resolution, accurate mass, lipids, LipidSearch, parallel reaction monitoring, untargeted lipidomics, targeted lipidomics

Goal

To achieve improved lipid identification, quantification, and throughput from complex biological samples by employing a hybrid quadrupole-Orbitrap mass spectrometer with greater mass resolution and scan speed.

Introduction

Lipidomics as a branch of metabolomics is a systems-based study of all lipid molecules within a biological system, tissue, or cell. Lipids are the main structural components of biological membranes, a major form of energy storage in cells, but are also well-known mediators of cell signaling. In order to understand cellular physiology and pathophysiology, comprehensive identification and precise quantification of lipids is crucial in lipidomics research.[1]

Recent advances in high-performance liquid chromatography-mass spectrometry (HPLC-MS) platforms allow the rapid and sensitive detection of a variety of lipid species with minimal sample preparation. However, two main challenges remain. First, the lipidome is highly complex. It includes eight major categories, over 80 major classes, 300 sub-classes, and thousands of lipid species[2] spanning a wide range of concentrations. HPLC cannot separate all of the isomeric and isobaric molecular ions in a typical biological sample. In order to obviate interference from the co-eluting species, an ultra-high-resolution, accurate-mass mass spectrometer is required for accurate lipid molecular ion determination. Second, molecular weight information alone is not always sufficient to identify each isomer of an individual lipid species. MS/MS (or sometimes even MS^n) information is often required for unambiguous identification of each individual lipid species in biological samples.

The Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap mass spectrometer features an ultra-high-field Orbitrap analyzer, which doubles data acquisition speed and mass resolution compared to the first generation of Orbitrap analyzer (Figure 1). The ultrahigh resolution (up to 240,000, FWHM at *m/z* 200) of the Q Exactive HF MS allows simultaneous accurate-mass measurement with better than 3 ppm accuracy with external calibration. The faster scan speed (up to 18 Hz) of the Q Exactive HF MS results in a higher number of precursor ions triggered for MS/MS. As a result, more lipid identifications in a single HPLC-MS/MS run can be achieved with improved sensitivity, accuracy, and productivity. The Q Exactive HF MS also allows multiple high-resolution, accurate-mass approaches, including full MS, selected-ion monitoring (SIM), and parallel-reaction monitoring (PRM), for highly sensitive and selective quantification of individual lipid species of interest.

In this study, a Q Exactive HF MS was used to: (i) identify lipids from multiple complex biological lipid extracts (ii) identify large number of lipids and provide simultaneous qualitative and quantitative information in a complex lipidome and (iii) determine linear dynamic range, limit of detection (LOD), and limit of quantitation (LOQ) for specific lipid species of interest.

Thermo SCIENTIFIC

Mass Range	50 < *m/z* < 6,000
Resolution @ *m/z* 200	15,000 at 18 Hz 30,000 at 12 Hz 60,000 at 7 Hz 120,000 at 3 Hz 240,000 at 1.5 Hz
Mass Accuracy	< 1 ppm RMS, Internal Calibration < 3 ppm RMS, External Calibration
Polarity Switching	One full cycle in < 1 sec (one full positive mode scan and one full negative mode scan at a resolution setting of 60,000)

Figure 1. Q Exactive HF MS and its performance specifications

Experimental

Sample Preparation

Sample Series 1 – untargeted lipid identification experiments: Bovine heart total lipid extracts (2.5 mg/mL in chloroform) were purchased from Avanti® Polar Lipids. A dilution series of the bovine lipid extract was prepared by diluting the stock solutions sequentially into 1.25 µg/µL, 250 ng/µL, and 50 ng/µL in 50:50 methanol and isopropyl alcohol.

Sample Series 2 – targeted lipid quantification experiments: Three LIPID MAPS mass spectrometry internal standards (LM-1601, 17:1 LPC; LM-1004, 17:0-14:1 PC; LM-1104, 17:0-14:1 PE) and bovine total lipid extract (2.5 mg/mL in chloroform) were purchased from Avanti Polar Lipids. The stock bovine heart lipid extract was diluted into 500 ng/µL and 250 ng/µL using 50:50 methanol and isopropyl alcohol (Fisher Chemical brand), respectively. Three internal standards were spiked into bovine lipid extract at six different concentrations (0.01 pg/µL, 0.1 pg/µL, 1 pg/µL, 10 pg/µL, 100 pg/µL, and 1000 pg/µL). The final concentration of bovine heart lipid extract for each sample in the dilution series was adjusted to 250 ng/µL.

HPLC Method

A Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation LC (RSLC) system was used for separations with the gradient conditions shown in Table 1.[3] Mobile phase A was 60:40 acetonitrile/water and mobile phase B was 90:10 isopropyl alcohol/acetonitrile; both A and B contained 10 mM ammonium formate and 0.1% formic acid. The column was an Ascentis® Express C18 (Sigma-Aldrich®, 2.1 x 100 mm, 2.7 µm) operated at 55 °C and a flow rate of 260 µL/min. The injection volume was 2 µL.

Table 1. LC separation conditions

Time	% A	% B
0	68	32
1.5	68	32
4	55	45
5	48	52
8	42	58
11	34	66
14	30	70
18	25	75
21	3	97
25	3	97
25.01	68	32
33	68	32

Mass Spectrometer Method

For untargeted lipid identification experiments (sample series 1), a Q Exactive HF MS and a Thermo Scientific™ Q Exactive™ Plus MS with HESI-II probes were employed using the instrument operating conditions shown in Table 2. Each instrument was operated under optimized condition (2.4 s cycle time), providing sufficient scans across the chromatographic peak profile for accurate relative quantification using the high-resolution, accurate-mass (HRAM) precursor ion while simultaneously acquiring data dependant-MS spectra for lipid identification.

For targeted lipid quantification experiments, the Q Exactive HF MS system with HESI-II probe was operated using four scan events (one full MS scan followed by three targeted MS/MS scans) with the instrument operating conditions shown in Table 2. An inclusion list containing precursor ion masses of the spiked lipid standards (17:1 LPC, *m/z* 508.3398; 17:0-14:1 PE, *m/z* 676.4912 and 17:0-14:1 PC, *m/z* 718.5381) was used for the targeted MS/MS scans.

Table 2. Q Exactive HF MS instrument setup

	Lipid Identification Experiment		Targeted Lipid Quantification Experiment
HESI Source	**Q Exactive Plus MS**	**Q Exactive HF MS**	**Q Exactive HF MS**
Sheath gas = 35	Positive ion	Positive ion or Pos/Neg scan-to scan switching	Positive ion
Aux gas = 3	MS resolution R = 70K	MS resolution R = 120K	MS resolution R = 120K
Spray voltage = 4.2 kV	Top15 dd-MS2 R = 35K	Top20 dd-MS^2 for Pos; Top10 dd-MS^2 for Pos/Neg polarity switching R = 35K	Targeted MS/MS (PRM) R = 35K
S-Lens = 50	MS^2 Isolation width 1 Da	MS^2 Isolation width 1 Da	MS^2 Isolation width 1 Da
Capillary temp. = 320 °C	Stepped NCE – Pos. 25, 30	Stepped NCE – Pos. 25, 30	NCE – Pos. 30
	Neg. 20, 24, 28	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	AGC target 1E+6 MS 1E+5 MS^2

Data Processing

Thermo Scientific™ LipidSearch™ software version 4.0[4] was used for lipid identification. Key processing parameters are shown in Table 3.

Table 3. LipidSearch software processing parameters for product ion search.

Product Ion Search	
Precursor tolerance	5 ppm
Product tolerance	5 ppm
Product ion threshold	5%
m-score threshold	5%
Quan *m/z* tolerence	±5 ppm
Quan RT range	±1.0 min
Main isomer filter	On
ID quality	A,B,C,D
Lipid classes	LPA,PA,LPC,PC,LPE,PE,LPG,PG,LPI,PI,LPS, PS,SM,MG,DG,TG,FA,CL,So,Cer,GMSGM1, GM2,CerG1,CerG2,CERG3,ChE,Co
Adduct ions	Positive: +H, $+NH_4$; Negative: -H, +HCOO, -2H

Results

Lipid Identification Coverage and Throughput

In order to benchmark the increased lipid identification coverage on the new Q Exactive HF MS system, a dilution series of bovine heart total lipid extract (1.25 µg/µL, 250 ng/µL, and 50 ng/µL, 2 µL injection) was analyzed by a data-dependent LC MS/MS method in positive mode on the Q Exactive Plus MS system (top 15 MS^2) and the new Q Exactive HF MS system (top 20 MS^2). The cycle time on both systems was 2.4 seconds. The Q Exactive HF MS system delivers 120K (vs. 70K on the Q Exactive Plus system) resolution in full scan and 30K resolution in MS/MS with very fast data acquisition speed, giving significantly more lipid identifications with high confidence. Under the same LC gradient conditions, the Q Exactive HF MS system identified 20% to 27% more individual lipid species than the Q Exactive Plus MS system, with more significant increase in lipid identifications when the amount of total lipid on column is less (Figure 2).

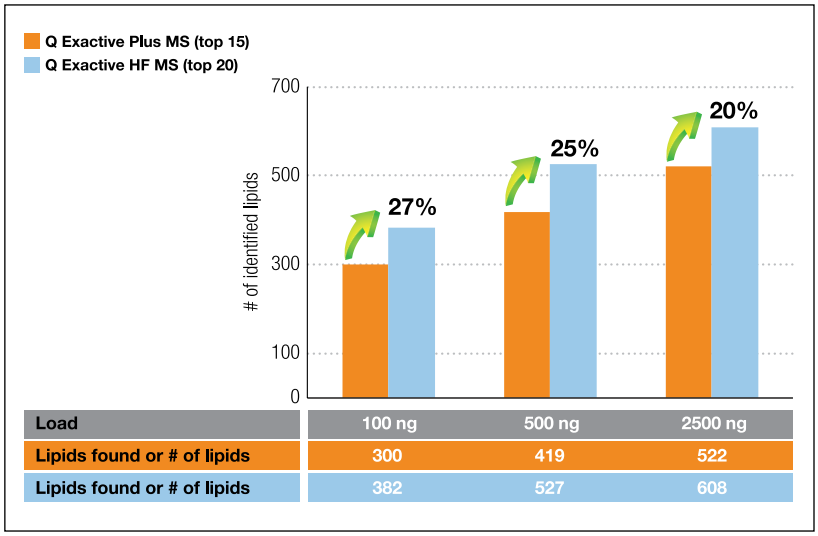

Figure 2. Identification of more lipid species at varying concentrations

In addition, the faster scan speed on the Q Exactive HF MS also allowed a shorter and steeper gradient that was half the original run time, while maintaining approximately the same number of lipid species identified by the Q Exactive Plus MS system (Figure 3).

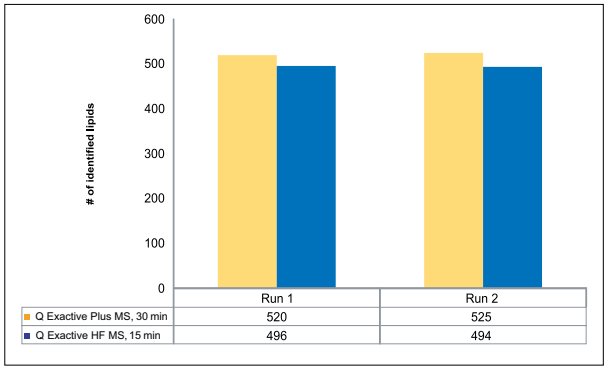

Figure 3. Lipid ID comparison between a 15 min LC-MS run (Q Exactive HF MS system) and a 30 min LC-MS run (Q Exactive Plus MS system). The ID numbers are the average of duplicate HPLC-MS/MS runs. Bovine heart total lipid extract (2500 ng on column).

The faster scan speed of the Q Exactive HF MS system can be combined with the online scan-to-scan polarity switching to trigger MS/MS for a higher number of lipids in both positive and negative modes during the same LC-MS run. It allows broader coverage of lipids and further increases the number of identified lipid species. Figure 4 shows the extracted positive and negative ion chromatograms of the bovine heart lipid extract analyzed using the scan-to-scan polarity switching method. While some lipid precursor ions can be observed in both polarities, many only ionized efficiently in one of the two modes. Under the same HPLC conditions, the online scan-to-scan polarity switching method identified one hundred more lipid species compared to the positive ion mode only method (Figure 5).

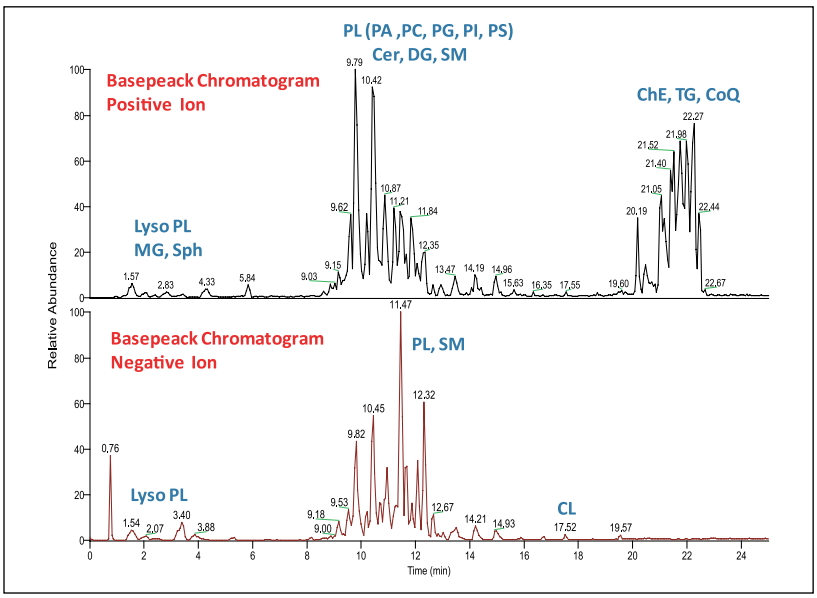

Figure 4. Extracted base peak chromatograms of positive and negative ions from the polarity switching experiment. Bovine heart total lipid extract (2500 ng on column).

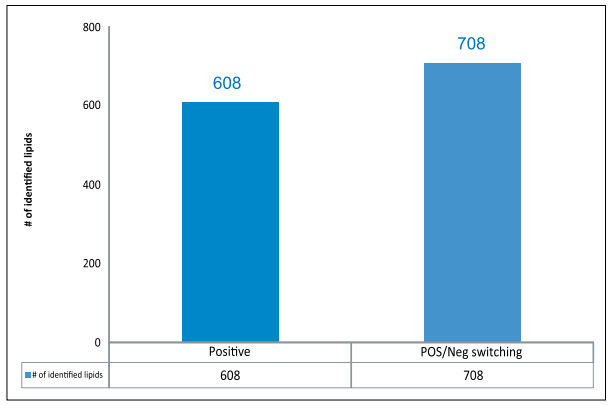

Figure 5. Lipid ID comparison between the positive ion mode only method and scan-to-scan polarity switching method. The ID numbers are the average of duplicate 30 min HPLC-MS/MS runs. Bovine heart total lipid extract (2500 ng on column).

The increased resolving power of Q Exactive HF MS system further enhances the lipid ID coverage of the lipidome by increasing the capability to differentiate lipid species from co-eluting isobaric molecular ions in the biological sample. The effect of MS resolution for identification of lipid species at the same retention time is illustrated for two lysophospholipid species, 18:1 LPE and 16:0p LPC. These two lipids overlap during analysis at 2.2 minutes at *m/z* 480.3 in the positive ion mode. The mass resolution required to separate these two lipids is illustrated in Figure 6. The 60K setting is the minimum resolution required for unequivocal identification of both $[M+H]^+$ ions. Thus, ultra-high resolving power (≥60K) is crucial to differentiate overlapping lipid species and achieve accurate identification and quantification.

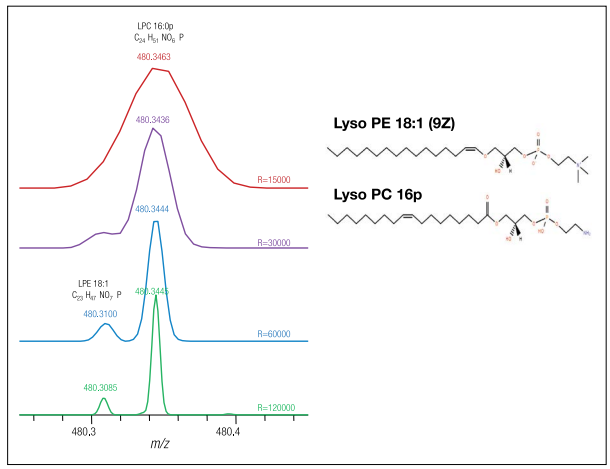

Figure 6. Increased resolution improves identification/quantitation. Identification of minor lipid species is challenging without sufficient mass resolution, leading to fewer identifications. To baseline-separate these two lipid species, a minimum of 60,000 resolving power is required, easily achievable using high-resolution Orbitrap technology.

The advanced quadrupole technology of the Q Exactive HF MS system enables narrow precursor ion isolation width with minimum transmission losses (Figure 7). This is particularly important for lipidomics analysis in which accurate lipid ID can only be obtained via the interpretation of MS/MS. Figure 8 shows two versions of the MS/MS spectra from two co-eluting isomers (LPC(16:0p) and LPE(18:1), whose precursor ions have been shown in Figure 6) collected with 4 amu isolation width (top) and 1 amu isolation width (bottom), respectively. The MS/MS from 4 amu isolation width is dominated with background interference ions from chemical noises, which could make the spectral interpretation very challenging and sometimes even virtually impossible. The MS/MS from 1 amu isolation width is much cleaner and shows much higher selectivity, which enables accurate identification.

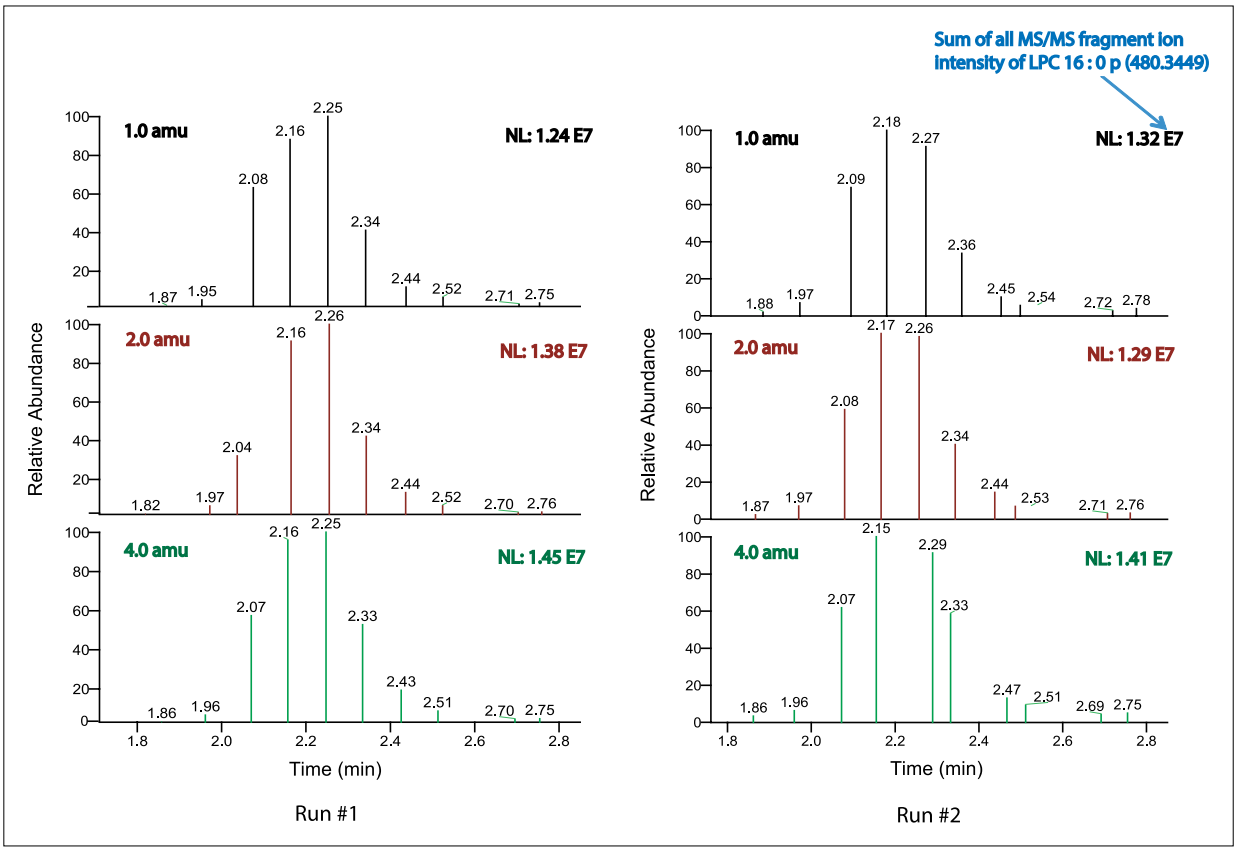

Figure 7. Narrow isolation width with minimum loss of signal. Duplicate 30 min LC-MS/MS runs. Bovine heart total lipid extract (1000 ng on column).

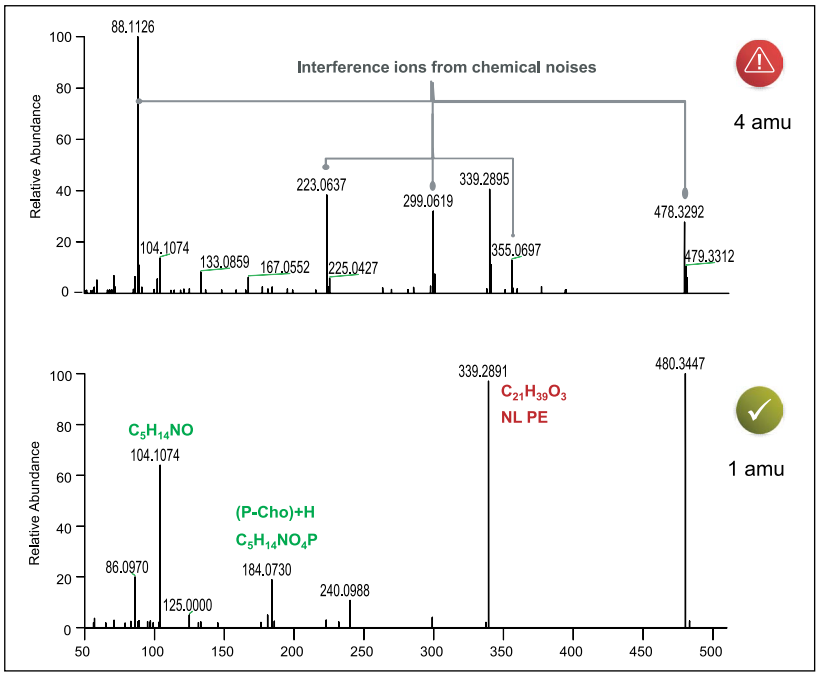

Figure 8. Higher quality MS/MS with enhanced specificity with 1 amu isolation width, 30 min LC-MS/MS run. Bovine heart total lipid extract (1000 ng on column).

Increased Selectivity and Sensitivity for Targeted Lipid Quantification

It is important to monitor changes in the lipidome over time in response to perturbation, because such knowledge increases our understanding of how lipids function in a biological system. Lipidomic studies may then be employed for elucidating the mechanism of lipid-based diseases, performing biomarker screening, and quantitative lipid monitoring in pharmacology studies. The Q Exactive HF system allows simultaneous lipid identification and global lipid profiling using LipidSearch software.[4] The Q Exactive HF MS system also offers several HRAM approaches, including full MS, SIM, and PRM for targeted lipid quantification.

In order to evaluate the performance of the Q Exactive HF MS system for the targeted lipid quantification, a dilution series of three lipid standards were spiked into 500 ng bovine heart total lipid extract at six different concentration ranges (0.01 pg/µL, 0.1 pg/µL, 1 pg/µL, 10 pg/µL, 100 pg/µL, and 1000 pg/µL) and analyzed using the targeted lipid quantification method described in the experimental section. Each spiked lipid sample (2 µL injection) was run in triplicate.

Quantitative data were extracted from both HRAM MS scan data and the PRM scan data. Full-scan HRAM MS data can be used to quantify any detected precursor ions of interest, which enables high-throughput large scale targeted quantification. However, it remains challenging to quantify co-eluting isomers using an HRAM MS approach because it may not be possible to differentiate isomeric lipid species at the precursor ion level. In contrast to the HRAM MS approach, the PRM approach collects all fragment ions of each lipid species and uses one or multiple significant fragment ions for quantification (Figure 9). The MS/MS level selectivity of PRM is capable of separating isomeric lipid species containing different combinations of fatty acid. In general, the LC-PRM method provides lower LOD and LOQ by removing interference from other co-eluting lipids.

There are several additional benefits of using the PRM approach for targeted quantification:

- The identity of the targeted lipid is simultaneously confirmed with high confidence by using the MS/MS spectrum.
- Higher selectivity is achieved by using specific fragment ions, which can significantly decrease the isomer/isobar contaminants observed at the MS level.
- Higher sensitivity is achieved by "enriching" in the C-Trap only the fragment ions from the targeted lipid precursor.

Figure 10 shows the comparison of the full MS data and the PRM data for 17:1 Lyso PC at 20 fg on column. By using only the MS/MS fragment (104.1072 *m/z*) specific to 17:1 LPC, the PRM result eliminates the two false positive peaks at r.t. 1.02 min and 1.48 min found in the full MS result. In addition, it also eliminates the isomeric contamination peak at r.t. 2.27min, also found in the full MS result.

Not only does the targeted PRM approach give higher specificity, it also gives high sensitivity and linear dynamic range. For example, five orders of linear dynamic range (R^2 = 0.9961) were observed for 17:0 – 14:1 PE (Figure 11), demonstrating the excellent analytical precision of the PRM method on the Q Exactive HF MS. For all three lipid standards, a coefficient of variance (CV%) less than 7% was achieved over five orders of magnitude in concentration (Table 4).

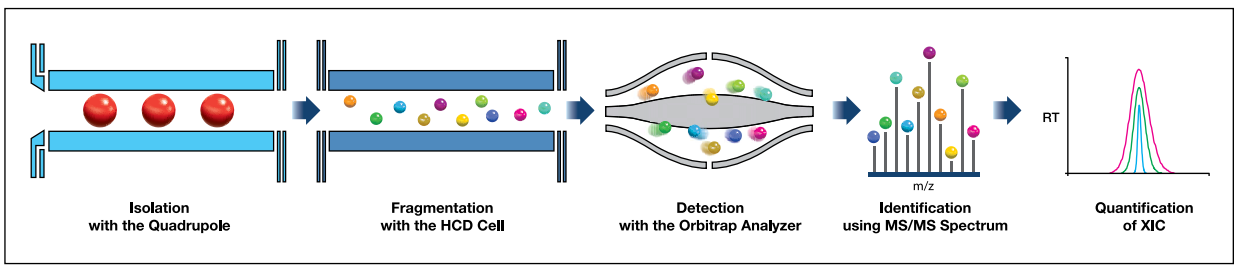

Figure 9. Targeted quantification using parallel reaction monitoring (PRM) on the Q Exactive HF MS

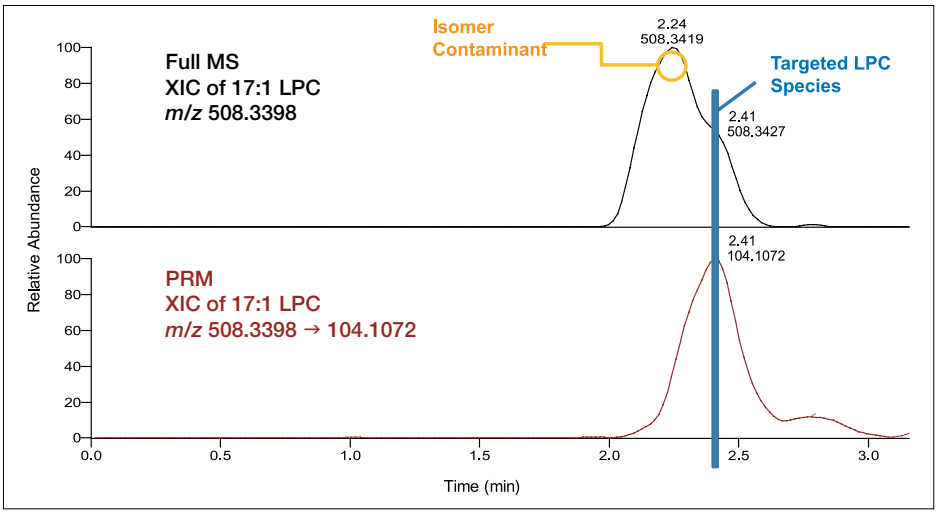

Figure 10. Comparison of the full MS data versus the PRM data for 17:1 Lyso PC at 20 fg on column. PRM was able to precisely detect and quantitate the 17:1 Lyso PC at the lowest concentration level of the dilution series.

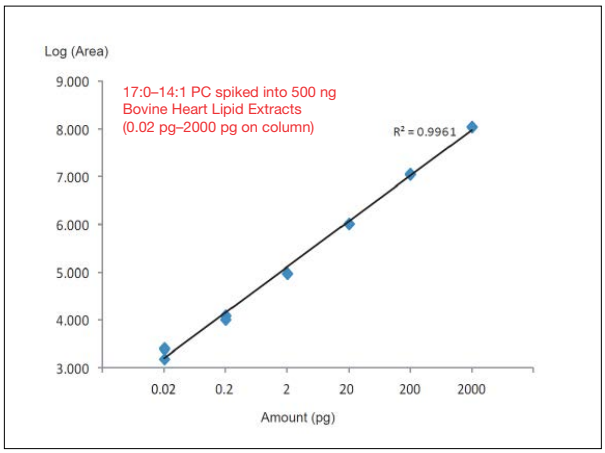

Figure 11. Calibration curve for 17:0–14:1 PE generated using the LC-PRM method. Five orders of linear dynamic range were observed.

Table 4. Coefficients of variation (%CV) for the three lipid standards spiked into a bovine heart total lipid extracts over five orders of magnitude in concentration.

Spiked Internal Lipid Standard	Precursor Ion (*m/z*)	CV% (n=3)					
		0.01 pg/µL	0.1 pg/µL	1 pg/µL	10 pg/µL	100 pg/µL	1000 pg/µL
17:0-14:1 PE (LM-1104)	676.4912	6.9	6.5	1.8	0.8	1.9	1.1
17:0-14:1 PC (LM-1004)	718.5381	3.2	1.7	0.5	3.3	0	7
17:1 LPC (LM-1601)	508.3398	5.9	3.9	2.8	0.6	0.8	0.6

Conclusions

- The Q Exactive HF MS is well suited for the analysis of complex lipid extracts. The faster scan speed and higher resolution enabled increased lipid identification coverage and throughput. Up to 27% more lipid species were identified on the Q Exactive HF MS compared to the Q Exactive Plus MS run under similar conditions. Approximately the same number of lipid species were identified on the Q Exactive HF MS in half the analysis time compared to the Q Exactive Plus MS. Scan-to-scan polarity switching can be utilized to further increase the lipid coverage on the Q Exactive HF MS. Over 700 lipids were confidently identified with supporting MS/MS from a 30 minute LC run of bovine heart total lipid extract (2500 ng on column).
- The higher resolving power of the Q Exactive HF MS provided more confident identification and better relative quantification of a lipidome. Even with LC separation, resolving power at a minimum of 60K is required to resolve lipid isomers.
- Advanced quadrupole technology enables use of narrow isolation windows for MS/MS acquisition without losing signal. This is particular important for lipidomics analysis in which accurate lipid ID can only be obtained via the interpretation of MS/MS. Narrower precursor ion isolation window provides cleaner MS/MS spectra and shows much higher selectivity, which enables accurate identification of individual lipid species.
- For targeted lipid quantification, the HRAM PRM approach provided increased selectivity and sensitivity by using MS^2 fragment ion(s) specific to each associated precursor ion, while simultaneously confirming the lipid identity using the full MS/MS spectrum. Compared to the full MS result, the PRM result showed fewer false positives and higher precision. Down to 20 fg LOD and up to five orders of linear dynamic range were observed for the spiked lipid internal standards. Excellent analytical precision was observed using the PRM method.

References

1 Watson, A.D. Lipidomics: a global approach to lipid analysis in biological systems. *J. Lipid Res.* **2006**, *47*, 2101.

2 Fahy, E. *et al.* LIPID MAPS comprehensive classification system for lipids. *J. Lipid Res.* **2009**, *50*, S9-S14. doi: 10.1194/jlr.R800095-JLR200.

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

4 Peake, D. *et al.* Higher resolution LC-MS and MS-MS analysis of lipid extracts using benchtop Orbitrap-based mass spectrometers and LipidSearch software. ASMS poster. **2014**, WP287.

AN64215-EN 0916S