Increased Throughput and Confidence for Lipidomics Profiling Using Comprehensive HCD MS^2 and CID MS^2/MS^3 on a Tribrid Orbitrap Mass Spectrometer

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Key Words
Orbitrap Fusion Lumos MS, high resolution, accurate mass, lipid profiling, intelligent scan functions, triglyceride (TG), phosphatidylcholine (PC), isomeric species, positive/negative switching, fatty acids, comprehensive dissociation techniques, HCD MS^2, CID MS^2, CID MS^3, LipidSearch software

Goal
Develop a high-resolution, accurate-mass LC/MS^n workflow that enables confident, sensitive, and high-throughput characterization and quantitation of lipid species within a single LC/MS run on a Tribrid Orbitrap MS system.

Introduction
Phospholipids play a key role in cell, tissue, and organ physiology and are associated with many diseases, such as cancer, cardiovascular disease, obesity, and diabetes, through disruption of lipid metabolic enzymes and pathways. Triglycerides are normally present in the blood plasma as lipoprotein particles and are naturally stored in fat deposits. In order to understand the biological roles of individual molecular lipid species, it is important to comprehensively characterize the hundreds to thousands of phospholipid and triacylglycerol species in biological samples. This is a very challenging task that requires high-resolution lipid separations, highly-sensitive detection, and accurate lipid identification using dedicated software.

Recent advances in high-resolution, accurate-mass (HRAM) mass spectrometers have allowed for rapid and sensitive detection of a variety of lipid species with minimal sample preparation.[1] However, for certain lipid classes such as triglyceride (TG) species, the combination of HRAM and MS/MS data does not provide sufficient information to confidently characterize co-eluting TG isomers. For mixtures of isomeric species, additional MS^n stages are required for differentiation and characterization. Further adding to the challenges is that certain lipid species, such as phosphatidylcholines (PC), require both positive and negative ion MS/MS fragmentation for complete characterization. Unfortunately, most commercial mass spectrometers are ill-equipped to acquire both ion modes simultaneously and thus require separate analyses for complete characterization. Additionally, the ionization efficiency of PC adduct ions in the negative ion mode is generally lower, leading to incomplete identification for lower abundance species. Therefore, it would be extremely useful to obtain additional characterization information during a single profiling experiment.

The Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer features a new API interface, Advanced Quadrupole Technology, high-field Orbitrap analyzer, and a dual-pressure linear ion trap in a Q-OT-qIT configuration. (Figure 1). Due to the unique configuration of the Orbitrap Fusion Lumos MS, Orbitrap mass analysis can be extensively parallelized with operation of the mass selecting quadrupole, the ion routing multipole (used for both accumulating ions and for HCD fragmentation), and the ion trap. This allows for maximal concurrent ion manipulations and mass analyses, increasing both scan rates and duty cycle of the instrument. Additionally, this unique instrument architecture enables multiple dissociation techniques including HCD and CID to be performed at any fragmentation stage, followed by analysis in either the linear ion trap or Orbitrap mass analyzer.

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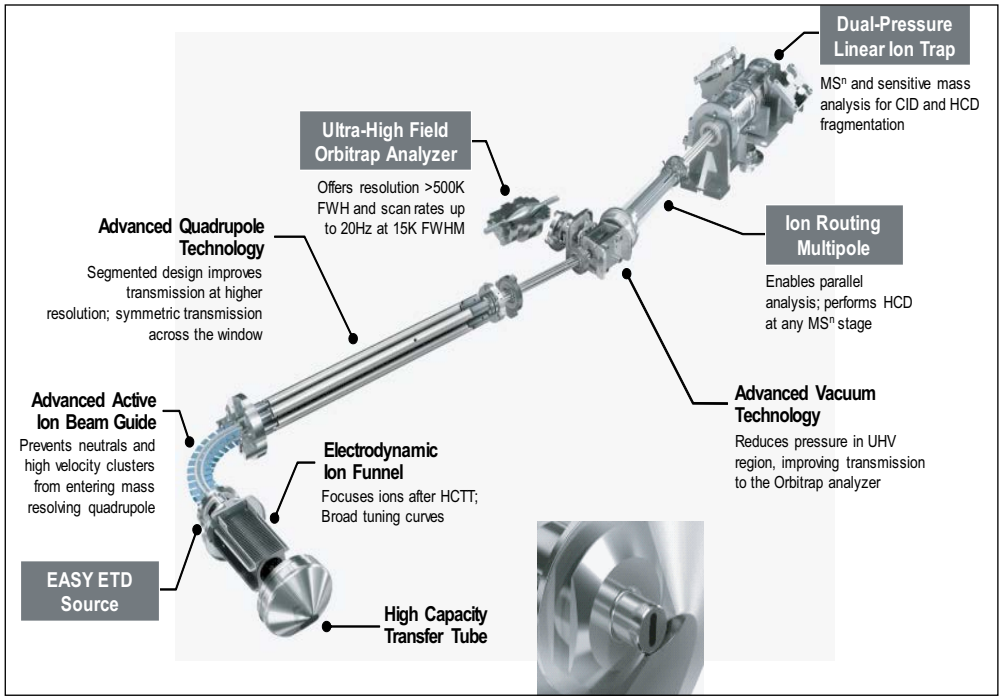

Figure 1. Instrument layout of Orbitrap Fusion Lumos Tribrid mass spectrometer.

Taking advantage of the tribrid architecture and increased sensitivity of the Orbitrap Fusion Lumos MS, we have developed a novel workflow using alternating positive and negative ion mode MS and MS/MS acquisition. In this workflow, HCD MS^2 is acquired for characterization of most lipid classes, and complementary CID MS^2 or MS^3 is acquired to enhance characterization of the PC and TG molecular lipid species. This acquisition works as follows. Data-dependent HCD MS^2 spectra are acquired while the phospholipids are eluting. When a diagnostic product ion of the phosphocholine head group (*m/z* 184.0733) is detected from the positive ion HCD MS^2 spectrum, an additional CID MS^2 experiment is triggered selectively. Furthermore, during the elution of TG species, three separate CID MS^3 experiments are triggered selectively when loss of a neutral fatty acid and ammonia are observed in the positive ion HCD MS^2 spectrum. The primary advantage of this acquisition strategy is the efficient use of the instrument duty cycle to achieve intelligent parallelization of multiple scans. We report here that PC and TG molecular species are fully characterized and quantified within a single LC/MS run using this approach.

Experimental

Samples

Sample 1: Egg PC lipid extract purchased from Avanti Polar Lipids (840051C, 10 mg/mL in chloroform) was diluted ten times using methanol and isopropanol (50:50, v:v), yielding a 1.0 µg/µL final solution.

Sample 2: Bovine heart lipid extract purchased from Avanti Polar Lipids (171201C, 2.5 mg/mL in chloroform) was diluted five times using methanol and isopropanol (50:50, v:v), yielding a 0.50 µg/µL final solution.

Samples 3–5: The total lipid extracts from three food plates (UC Davis, CA, US) were provided by the NIH West Coast Metabolomics Center. From each food plate, three 2 mg aliquots of freeze-dried material (corresponding to approximately 20 mg of homogenized food per aliquot) were used for analysis. The lipids in the freeze-dried sample were extracted using methyl-*tert*-butyl ether (MTBE) following the protocol of V. Matyash.[2] The food total lipid extracts were dissolved in 300 µL of methanol / isopropanol (50:50, v:v) prior to LC/MS analysis.

HPLC Method

All separations were performed on a Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation LC (RSLC) system using the gradient conditions shown in Table 1.[1] Mobile phase A was 60:40 (v:v) acetonitrile / water and mobile phase B was 90:10 (v:v) IPA / acetonitrile; both A and B contained 10 mM ammonium formate and 0.1% formic acid. The column was a Thermo Scientific™ Accucore™ C18 (2.1 x 150 mm, 2.6 µm) operated at 45 °C and a flow rate of 260 µL/min. The injection volume was 2 µL.

Table 1. HPLC gradient.

Time	% A	%B
0	70	30
2	57	43
2.1	45	55
12	35	65
18	15	85
20	0	100
25	0	100
25.1	70	30
30	70	30

Mass Spectrometry

A Top-speed, data-dependent HCD MS/MS data acquisition method with 1.0 or 2.4 s cycle times (Table 2) was employed to perform lipid profiling experiments on bovine heart lipid extract using an Orbitrap Fusion Lumos mass spectrometer or a Thermo Scientific™ Orbitrap Fusion™ mass spectrometer.

Table 2. MS setup for HCD MS^2 lipid profiling.

HESI Source	Orbitrap Fusion MS Orbitrap Fusion Lumos MS
Sheath Gas: 40	Pos Ion (250–1200 amu) Neg Ion (200–1200 amu)
Aux Gas: 3	MS Resolution: R=120K (FWHM at *m/z* 200)
Spray Voltage: 3.5 kV	Top-Speed dd-MS^2: 1.0 s at 15K (FWHM at *m/z* 200) per cycle 2.4 s at 30K (FWHM at *m/z* 200) per cycle
RF-Lens: 50	MS^2 Isolation Width: 1.0 Da
Cap. Temp: 320 °C	Stepped NCE – Pos. 27 ± 3 Stepped NCE – Neg. 30 ± 10
Heater Temp: 350 °C	AGC Target: 4E+5 MS, 50 ms max. 5E+4 MS^2, 80 ms max. for 2.4 s 35 ms max. for 1.0 s

Then, a comprehensive data-dependent HCD MS^2 experiment with conditional CID MS^2 and MS^3 data acquisition was developed for more in-depth characterization of lipids present in the bovine heart and food lipid extracts. Alternating positive and negative ion Top-speed, data-dependent HCD MS^2 experiments were performed using two 1.0 s cycles for early eluting phospholipids including PCs (0–18 min retention time range). Additional CID MS^2 scans were triggered on the same precursor ion for PC lipids with a diagnostic fragment ion (*m/z* 184.0733) detected from the positive HCD MS^2 data. For the later-eluting TG and other lipids (18–30 min retention time range), a positive ion, top-speed, data-dependent MS^2 experiment was performed using a 2 s cycle time. Additional CID MS^3 scans were subsequently triggered on the three largest HCD product ions that lost neutral fatty acid plus ammonia. Figure 2 shows the flow chart for this comprehensive HCD MS^2 and CID MS^2/MS^3 workflow.

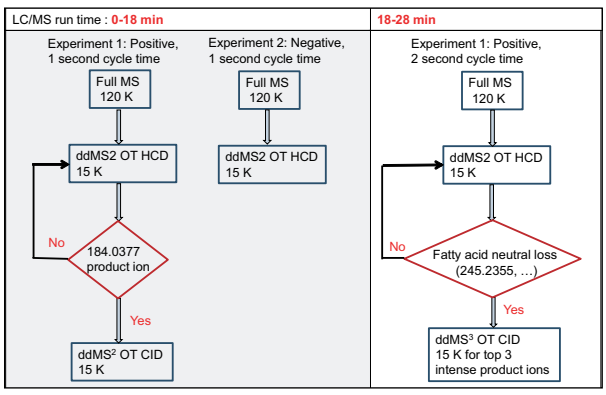

Figure 2. Flow chart of comprehensive HCD MS^2 and CID MS^2/MS^3 workflow.

Data Processing

Thermo Scientific™ LipidSearch™ software version 4.1 SP1 was used for lipid identification.[3] Table 3 shows the parameters used for the lipid database search. First, the individual data files were searched for product ion MS^2 spectra of lipid precursor ions. MS^2 fragment ions were predicted for all precursor adduct ions measured within ±3 ppm. The product ions that matched the predicted fragment ions within a ±5 ppm mass tolerance were used to calculate a match-score and those candidates providing the highest quality match were determined. Next, the search results from the individual positive or negative ion files from each sample group were aligned within a retention time window (±0.1 min) and the data were merged for each annotated lipid. The annotated lipids were then filtered to reduce false positives and the number of annotations for each lipid class were reported.

Table 3. LipidSearch software conditions.

Search Parameter	Settings
Precursor Ion Mass Tol.	3 ppm
Product Ion Mass Tol.	5 ppm
Lipid Sub-classes	**Phospholipids:** LPC, PC, LPE, PE, LPS, PS, LPG, PG, LPI, PI, LPA, PA, CL
	Sphingolipids: So, SM, Cer, CerG1, CerG2, CerG3
	Glycerolipids: MG, DG, TG, DGDG, DGMG, MGMG, MGDG, SQMG, SQDG
	Neutral lipids: ChE, CoQ, SiE, StE, ZyE

Results

Evaluating the Improvements Offered by the Orbitrap Fusion Lumos MS for Lipid Profiling Experiments

For lipid profiling, a faster MS/MS scan speed is required because many lipid species exist in multiple isomeric forms, which leads to closely-spaced or overlapping chromatographic peaks. Since ion accumulation and detection occurs in parallel[4] on Tribrid MS instruments, ion filling and FT detection times should be equal in order to maximize the instrument duty cycle. An overall 20 Hz MS/MS scan speed can be attained by using a 35 ms ion accumulation time on Tribrid Orbitrap mass spectrometers. However, a shorter ion filling time may cause poor MS^2 data quality for low abundance lipid species if an insufficient number of ions is transferred into the c-trap, leading to fewer lipid IDs. In order to take advantage of faster scan speed, it is important to increase the ion transfer efficiency from the ion source to the c-trap, thereby filling the trap within a short accumulation time.

We first investigated whether the Orbitrap Fusion Lumos system can provide the same number of lipid IDs for lipid profiling using a faster cycle time, thus enabling time for additional characterization. Figure 3 shows that for 1 µg bovine heart lipid extract, there is no loss in lipid identifications observed between the maximum ion injection time of 84 ms (2.4 s cycle) and 35 ms (1.0 s cycle) on the Orbitrap Fusion Lumos system. However, fewer lipid IDs are observed for the same sample when comparing the 2.4 s and 1.0 s cycle times on the original Orbitrap Fusion system. These results demonstrate that a 1.0 s cycle time can be routinely applied to dd-MS^2 experiments on the Orbitrap Fusion Lumos system without loss of IDs. In addition, a 1.0 s cycle time is fast enough to allow alternating positive and negative ion dd-MS^2 in a single LC/MS run while maintaining enough scans across the chromatographic peak for precise quantification.

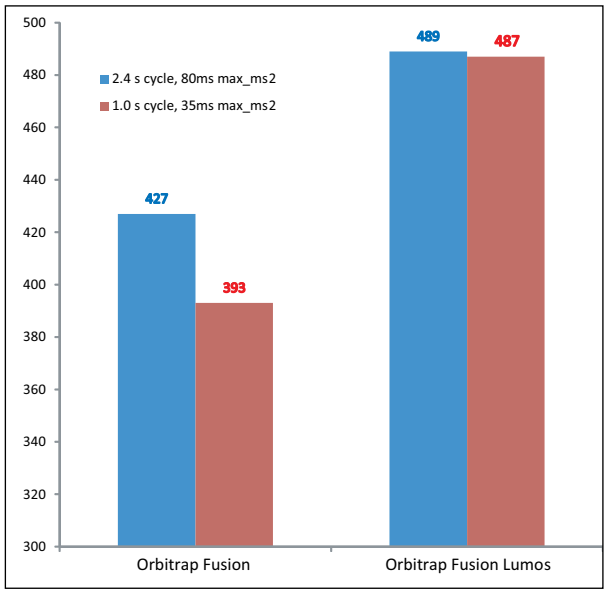

Figure 3. Comparison of Lipid IDs in 1 µg of bovine heart lipid extract with the Orbitrap Fusion MS and Orbitrap Fusion Lumos MS Instruments with 1.0 second and 2.4 second cycle times. The number of lipid IDs were determined using an average of duplicate positive data dependent HCD MS^2 runs.

Evaluating the Characterization of Phosphatidylcholine (PC) Using Combined HCD and CID MS/MS

The characterization of PCs by mass spectrometry is influenced by their ionization efficiency and fragmentation patterns. Different ion polarities and activation modes are required for successful structural elucidation. For example, a typical HCD MS^2 spectrum of a protonated PC molecular ion is dominated by the *m/z* 184.0733 (phosphocholine) product ion which is used to specify lipid class and determine the number of fatty acyl carbons and double bonds or "sum composition". However, there is often insufficient information in the positive ion HCD MS^2 spectra to assign the fatty acyl chains. In order to characterize PC at the molecular composition level, negative ion HCD MS^2 data is usually performed to generate fatty acyl fragment ions. The main drawback to this approach is that negatively-charged PC adducts suffer from lower ionization efficiency than protonated species and, thus, many PC species are not detected in the negative ion mode, yielding a lower number of molecular identifications. Alternatively, one can acquire CID MS^2 data to fill in the missing information that is lacking in positive ion HCD MS^2. Ideally, complete characterization requires data acquisition in both polarity modes along with HCD and CID MS^2 data. Most commercial mass spectrometers require separate LC/MS runs for obtaining positive and negative mode data, which doubles the LC/MS analysis time.

The Orbitrap Fusion Lumos system is the ideal platform to overcome these challenges: it can perform polarity switching and acquire the corresponding MS^2 spectra, both HCD and CID MS^2, during the same LC/MS experiment. In addition, the built-in intelligence enables collection of a single CID MS^2 spectrum only when the diagnostic product ion (184.0733) is observed, thereby maximizing the instrument duty cycle. Figure 4 shows that when HCD and CID fragments are formed from 38:4 PC, one can confidently assign the molecular species as 18:0_20:4 PC (underscore is used to indicate that the fatty acyl position on the glycerol is not assigned).[5]

In order to evaluate the best approach for molecular PC characterization, we compared the number of PCs that were fully identified with the fatty acyl composition from egg PC extract using three different experiments:

1. Positive ion data-dependent (dd) HCD MS^2-CID MS^2
2. Separate positive and negative ion dd-HCD MS^2
3. Alternating positive ion HCD MS^2-CID MS^2 and negative ion HCD MS^2

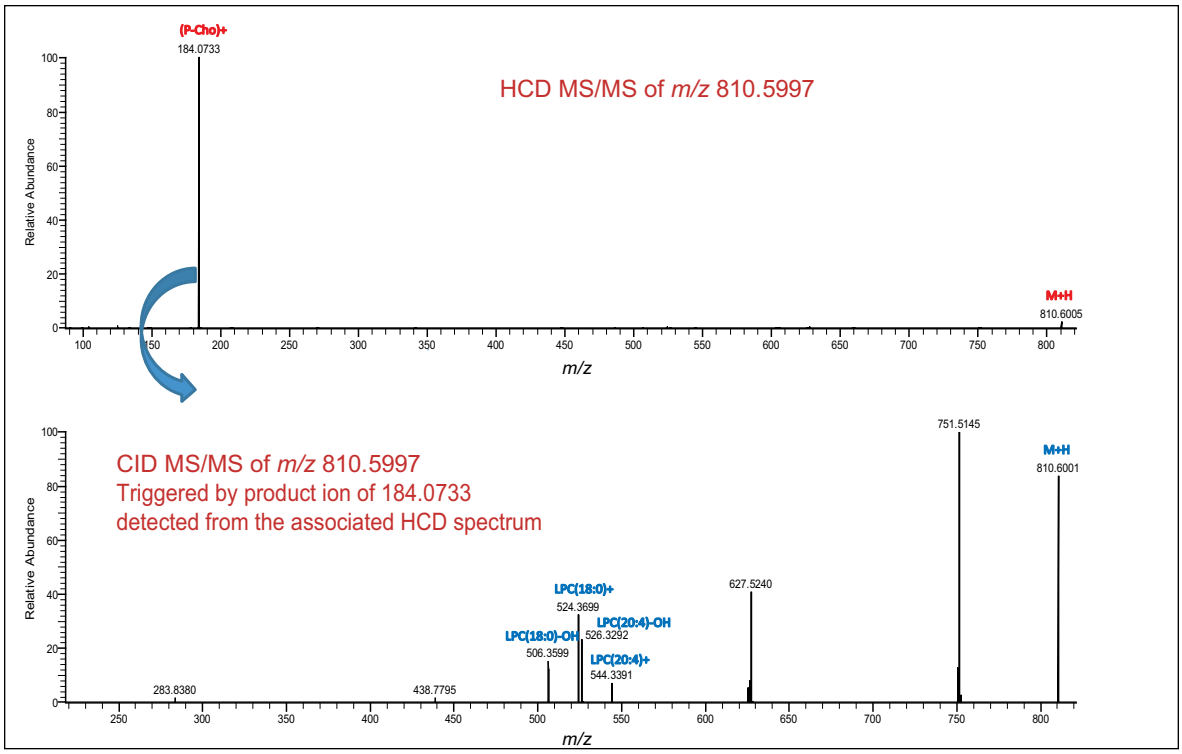

Figure 4. Comprehensive positive fragment ion information from the HCD and CID MS² identified the PC species as PC (18:0_20:4).

Data Processing with LipidSearch Software

All raw files were processed using LipidSearch 4.1 SP1 software. The search results from each experiment were combined, and the total number of PC species identified at the molecular composition level and quantified with peak areas less than 20% CV were reported. Figure 5 shows the PC ID results for the three experiments. As expected, a positive ion LC/MS run with the dd-HCD MS²-CID MS² approach provided more extensive characterization, yielding a total of 158 PC species with complete fatty acyl chain identification. By combining search results from separate positive and negative ion dd-HCD MS² runs, the number of PC IDs increased to 166, which is comparable to the positive ion dd HCD MS²-CID MS² approach, but with twice the number of LC/MS runs. Because of the short cycle time (1.0 s), not all PC species get complementary CID MS² fragment ion information in the positive ionization mode. In fact, the most comprehensive characterization was achieved using positive ion HCD-CID MS² and alternating negative ion HCD MS², which yielded complete information on 208 different molecular species.

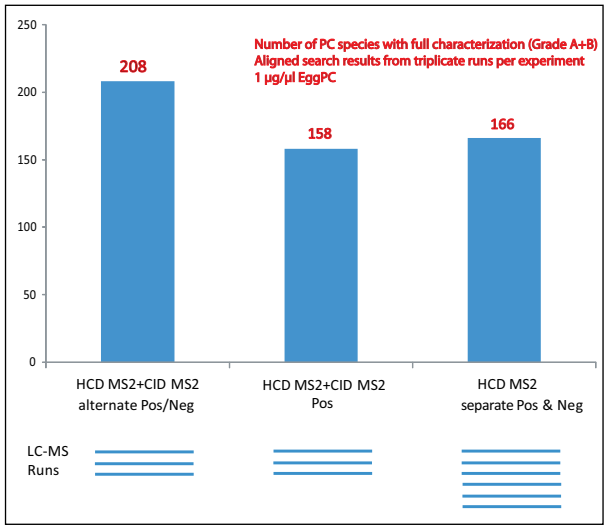

Figure 5. Comparison of PC characterization coverage with data-dependent HCD MS² runs vs data-dependent HCD MS² and comprehensive data-dependent HCD MS² with complementary CID MS² runs.

Evaluating Triglyceride (TG) Characterization Using HCD MS² Combined with CID MS³

Although TGs are composed of one glycerol and three fatty acid molecules, they vary in fatty acid chain length, degree of saturation, double bond position, and position of particular fatty acid chains on the glycerol backbone. These differences can result in the presence of numerous isomeric triglyceride molecular species. Using a chromatographic approach, it is not currently possible to separate all of the isomeric TG species and enable successful MS structural elucidation.

For well-resolved TG isomers, the HCD MS² spectrum provides sufficient product ion information for characterizing the molecular species. However, for co-eluting TG isomers, the HCD MS² spectrum includes a mixture of fragment ion information from multiple isomeric TG species, thereby requiring an additional stage of MS^n data to determine each isomer's unique molecular composition. One of the unique capabilities of the Orbitrap Fusion Lumos MS is the ability to acquire any type of MS^n information at any stage of acquisition. By tapping into this feature, we can automate the acquisition of CID MS³ spectra when a neutral loss of a fatty acid plus ammonia is detected from the HCD MS² spectrum. By combining the fragment ion information from the HCD MS² and CID MS²/MS³ spectra, full characterization of the co-eluting TG isomers can be achieved (Figure 6).

A Novel Workflow that Uses HCD MS² for General Lipid Profiling and CID MS² and MS³ for Further Characterization of PC and TG Lipid Molecular Species

Through evaluation of the results described above, we have demonstrated the following advanced capabilities for lipid analysis with the Orbitrap Fusion Lumos mass spectrometer:

1. The brighter Orbitrap Fusion Lumos ion source allows data-dependent HCD MS² experiments with a very short cycle time (1.0 s) without a loss in sensitivity.
2. Additional CID MS² and MS³ data provide complementary fatty acyl fragment ion information for further characterization of PC and TG lipid molecular species.
3. The built-in intelligence in the Orbitrap Fusion Lumos MS enables targeted CID MS² and MS³ data acquisition only when specific fragment ions/neutral loss are detected from HCD MS² data.

Taking advantage of these advanced capabilities, we developed a novel, intelligent, automated high-throughput acquisition strategy for lipidomics profiling that provides complete characterization of PC and TG molecular species (Figure 2). For early eluting lipids, including glycerophospholipids and sphingolipids, an alternating positive and negative ion data-dependent HCD MS² data acquisition is performed using Top-speed mode (1.0 s cycle time per each polarity mode) to cover most lipid species. However, when a diagnostic fragment ion for PC (*m/z* 184.0733) is detected within the positive HCD MS² spectrum, an additional high resolution CID

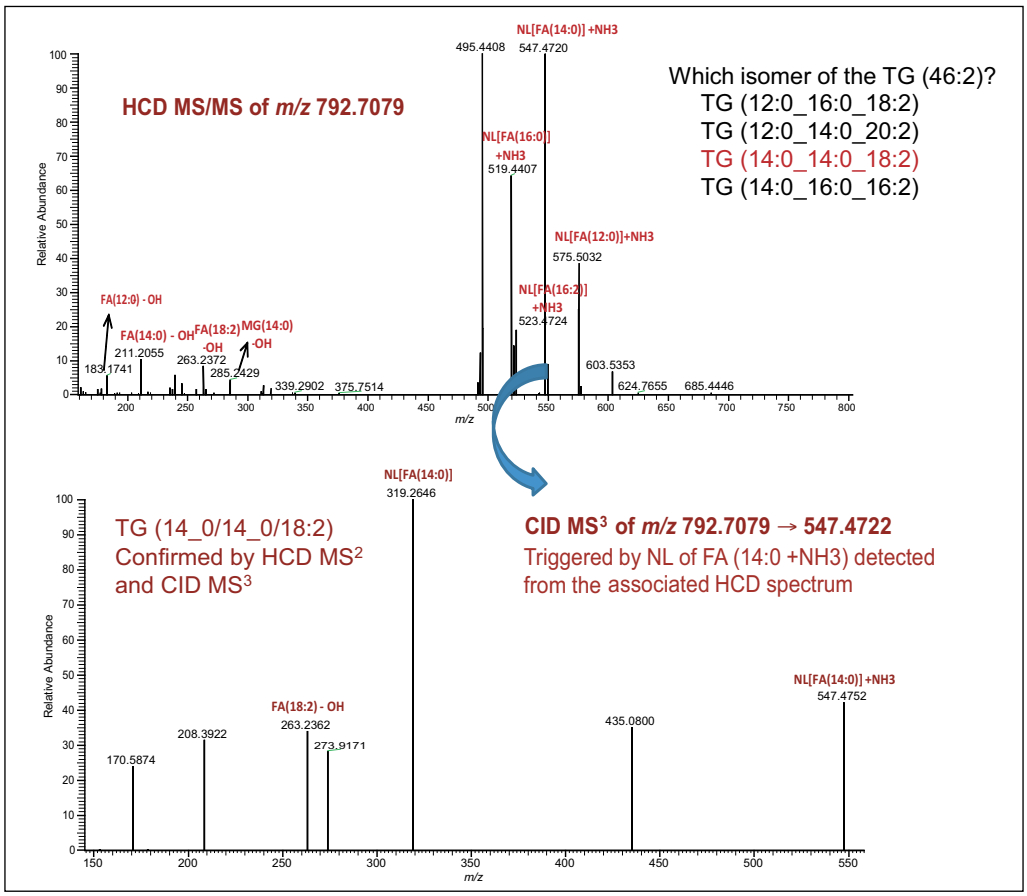

Figure 6. Characterizing co-eluting TGs using HCD MS² and CID MS³.

MS^2 spectrum is acquired for the same precursor. For later-eluting lipids, including TGs, positive–ion-only HCD MS^2 acquisition is performed using the Top-speed mode. Additional CID high-resolution MS^3 acquisitions are performed on the three most intense HCD MS^2 fragment ions formed via fatty acid plus ammonia neutral loss (Table 4) with an overall cycle time of 2.0 s.

Figure 7 shows the extracted base peak ion chromatograms of the bovine heart sample from both ion modes using the comprehensive workflow. Using a 1.0 s dd-HCD MS^2 cycle time for each polarity provided enough full-scan MS data points across each chromatographic peak to perform relative quantitation with good precision (Figure 7, insert).

Table 4. Fatty Acid Neutral Loss Inclusion List Used for Triggering CID MS^3.

FA	Formula	NL ($FA+NH_3$)	FA	Formula	NL ($FA+NH_3$)
12:0	C12 H24 02	217.2042	20:3	C20 H34 02	323.2824
14:0	C14 H28 02	245.2355	20:4	C20 H32 02	321.2668
14:1	C14 H26 02	243.2198	20:5	C20 H30 02	319.2511
16:0	C16 H32 02	273.2668	21:0	C21 H42 02	343.3450
16:1	C16 H30 02	271.2511	22:0	C22 H44 02	357.3607
16:2	C16 H28 02	269.2355	22:1	C22 H42 02	355.3450
17:0	C17 H34 02	287.2824	22:2	C22 H40 02	353.3294
18:0	C18 H36 02	301.2981	22:3	C22 H38 02	351.3137
18:1	C18 H34 02	299.2824	22:4	C22 H36 02	349.2981
18:2	C18 H32 02	297.2668	22:5	C22 H34 02	347.2824
18:3	C18 H30 02	295.2511	22:6	C22 H32 02	345.2668
19:0	C19 H38 02	315.3137	23:0	C23 H46 02	371.3763
20:0	C20 H40 02	329.3294	24:0	C24 H48 02	385.3920
20:1	C20 H38 02	327.3137	24:1	C24 H46 02	383.3763
20:2	C20 H36 02	325.2981	26:0	C26 H52 02	413.4233

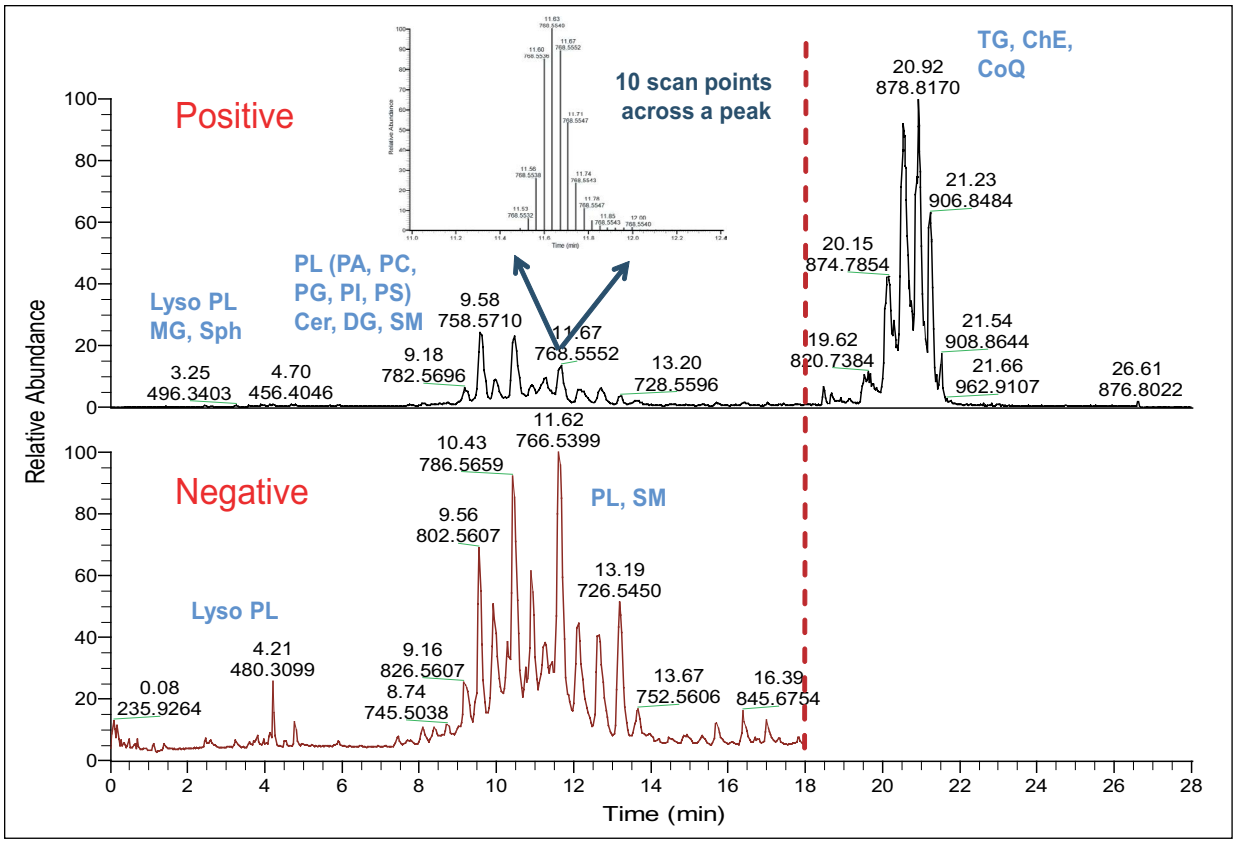

Figure 7. Base peak chromatograms of positive and negative ion modes of bovine heart lipid extracts in a single run using the novel intelligent comprehensive workflow.

LipidSearch software automatically combines the fragment ion information from the HCD MS^2, CID MS^2, and MS^3 spectra for characterization of individual lipid species (Figure 8). Figure 9 shows the numbers of identified lipids from 1.0 µg of bovine heart lipid extract using the new workflow compared to the standard HCD MS^2 workflow in which data were collected in positive and negative ionization mode, respectively. The comprehensive workflow was able to identify a comparable number of molecular species with twice the throughput and also offers higher confidence for PC and TG characterization by using the combined HCD/CID, MS^2, and MS^3 fragment ion information.

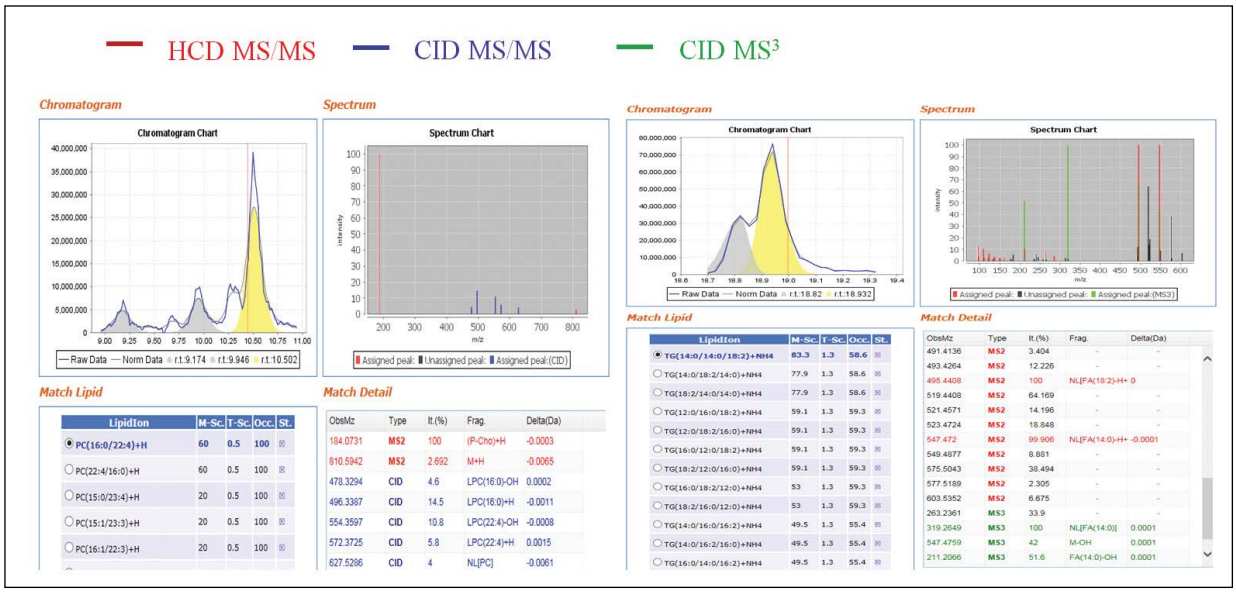

Figure 8. LipidSearch software automatically uses comprehensive fragment ion formation from multiple fragmentation techniques for molecular lipid IDs.

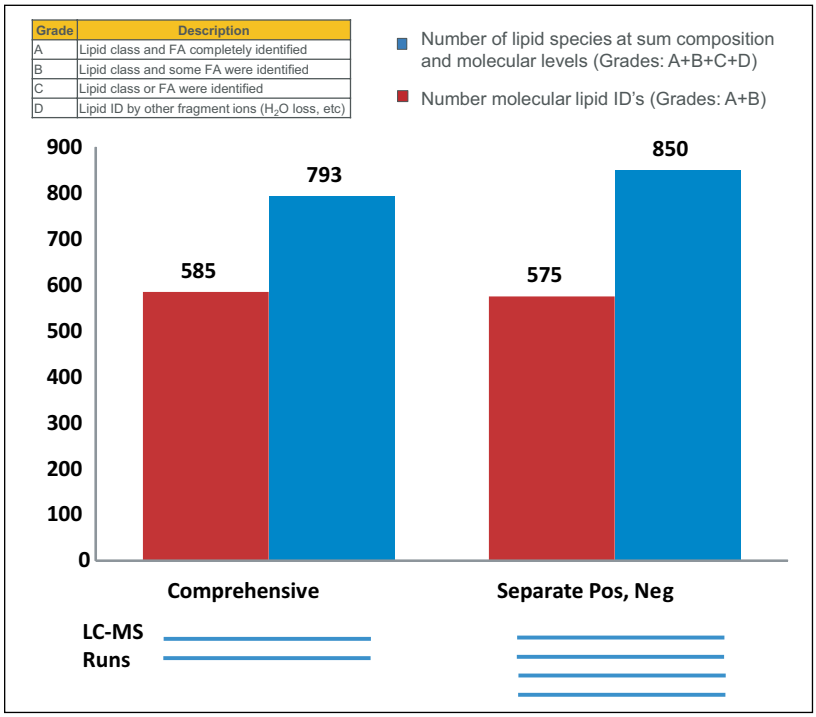

Figure 9. Comparison of identified lipid species using the comprehensive LC-MS^n workflow and general HCD MS^2 workflow.

Applying the High-Throughput Workflow to Lipidomics Profiling of Three Different Food Samples

The new lipidomics profiling workflow can be applied not only to biological samples (such as plasma/serum, tissue, cells), but also to food and plant tissue samples. As proof of concept, the new workflow was applied to lipidomics profiling of three food plates provided by NIH West Coast Metabolomics Center. These food plates were selected on large differences in dietary components: USA plate (fast food meal), California plate (based on USDA My Plate dietary recommendations), and Davis plate (inspired by Korean cuisine) (Table 5).[6] All food items on each plate were photographed, homogenized, and then lyophilized; the "CA plate" and "USA plate" were also carefully balanced with respect to dietary parameters.[6] From each of the food plates, three 2 mg aliquots of freeze-dried material (20 mg of homogenized food per aliquot) were used for total lipid extraction as described in the sample section.

Table 5. Description of food plate samples.

Food	Plate Description
Davis	Fried egg, white rice, sesame seeds, tofu, spinach, bean and soy sprouts, carrots, radish, zucchini, rice punch
US	Hamburger (beef, bacon, cheese, pickle, lettuce, tomato), French fries, baked beans, chocolate chip cookie, regular coke
CA	Salmon, brown rice, almonds, lemon, broccoli, cabbage, carrots, onions, red bell pepper, grapes, yogurt, blueberries, green tea

A PC Internal Standard (17:0/14:1) purchased from Avanti Polar Lipids was spiked into each food plate extract for evaluating peak area reproducibility. All raw files were processed using Lipid Search 4.1 SP1 software. More than 700 lipid species were identified and quantified from the three food samples with high confidence and analytical precision (Table 6, Figure 10). The Orbitrap Fusion Lumos MS was able to quantify the different classes of lipids over six orders of dynamic range, from most abundant triacylglycerols ($1E^{12}$ peak areas) to least abundant mono-galactosyl diacylglyerols (MGDG) ($1E^{6}$ peak areas) using the comprehensive workflow (Figure 11). Significant relative concentration differences were observed for many lipids among the three food plates reflecting the very different diets (Figure 12).

Table 6. Summary of identified lipid classes from three food samples.

Glycerol Lipids	IDs	Phospholipids	IDs	Sphingolipids	IDs
DG	10	**PA**	16	**So**	3
TG	404	**LPC/PC**	116	**Cer**	37
MGDG	1	**LPE/PE**	101	**CerG1**	20
DGMG	1	**PG**	7	**SM**	46
DGDG	6	**PI**	10		
		PS	9		

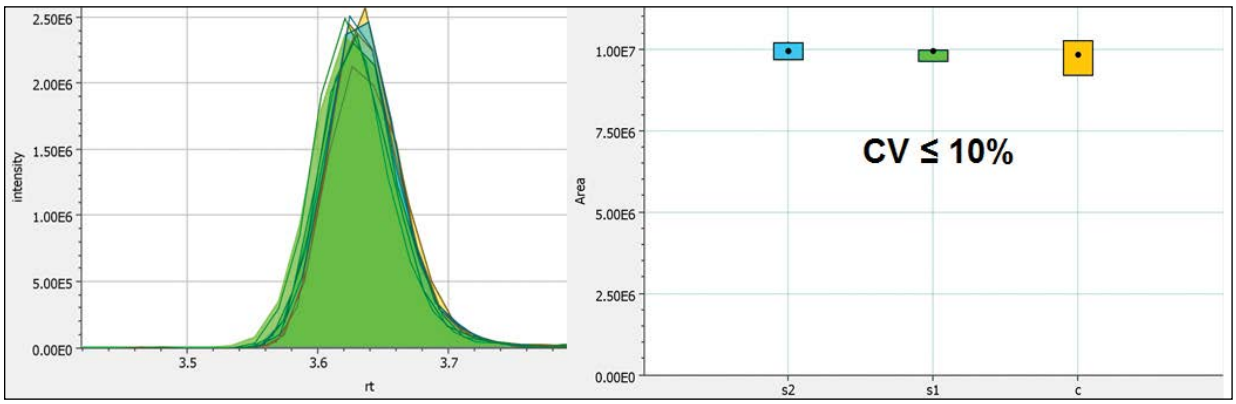

Figure 10. Aligned peak areas for PC Internal standard 17:0/14:1 spiked into three food samples.

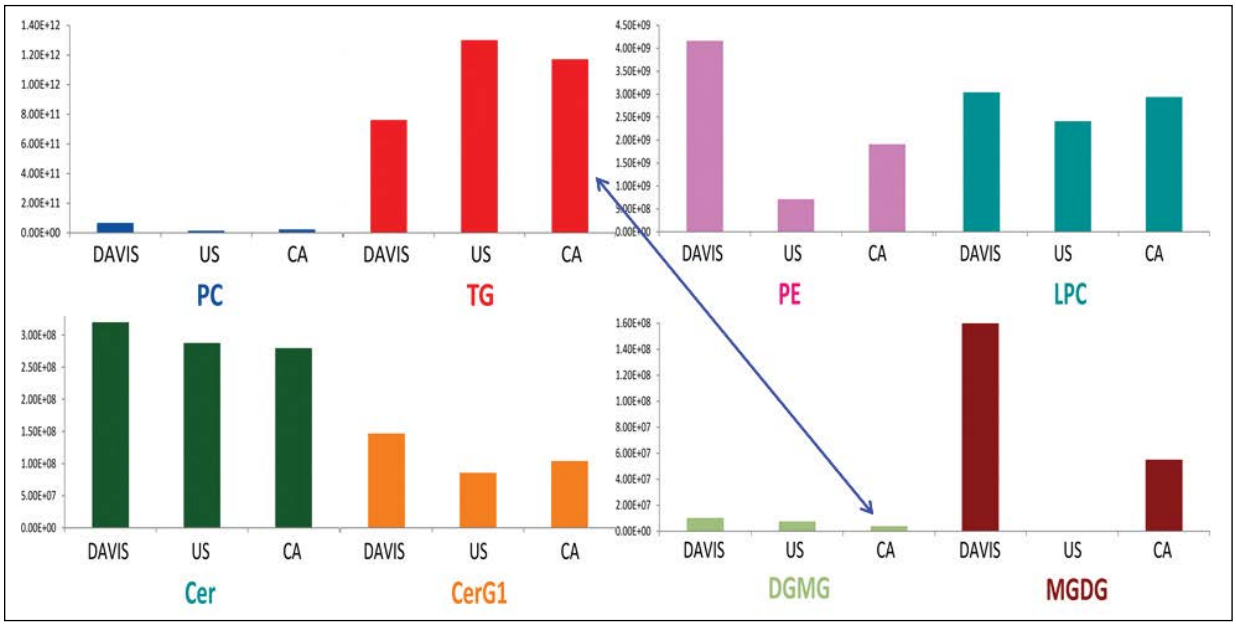

Figure 11. Representative lipid classes profile for the three food samples. Six orders of dynamic ranges were observed for the quantitation.

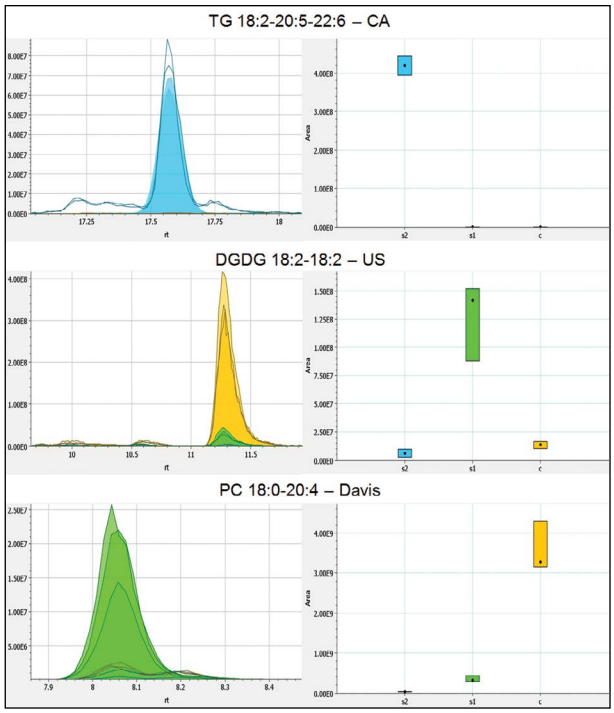

Figure 12. Significant abundance differences were observed in representative lipids.

Conclusion

- A high-throughput comprehensive lipidomics profiling LC/MS^n workflow was developed by taking advantage of the new capabilities of Orbitrap Fusion Lumos MS platform including increased sensitivity, multiple dissociation technologies, and fast duty cycle obtained using intelligent data acquisition.
- The comprehensive LC/MS^n workflow requires only a single combined positive and negative ion LC/MS analysis, providing twice the throughput compared to separate runs for each ion mode.
- The combination of HCD MS^2 and CID MS^2 enables higher confidence for characterizing PC molecular species.
- The combination of HCD MS^2 and CID MS^3 enables higher confidence for identifying TG molecular isomers.
- The comprehensive LC/MS^n workflow was successfully applied to bovine heart and food lipid extracts. Improved lipid identification with higher confidence and simultaneous relative quantitation were achieved.

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