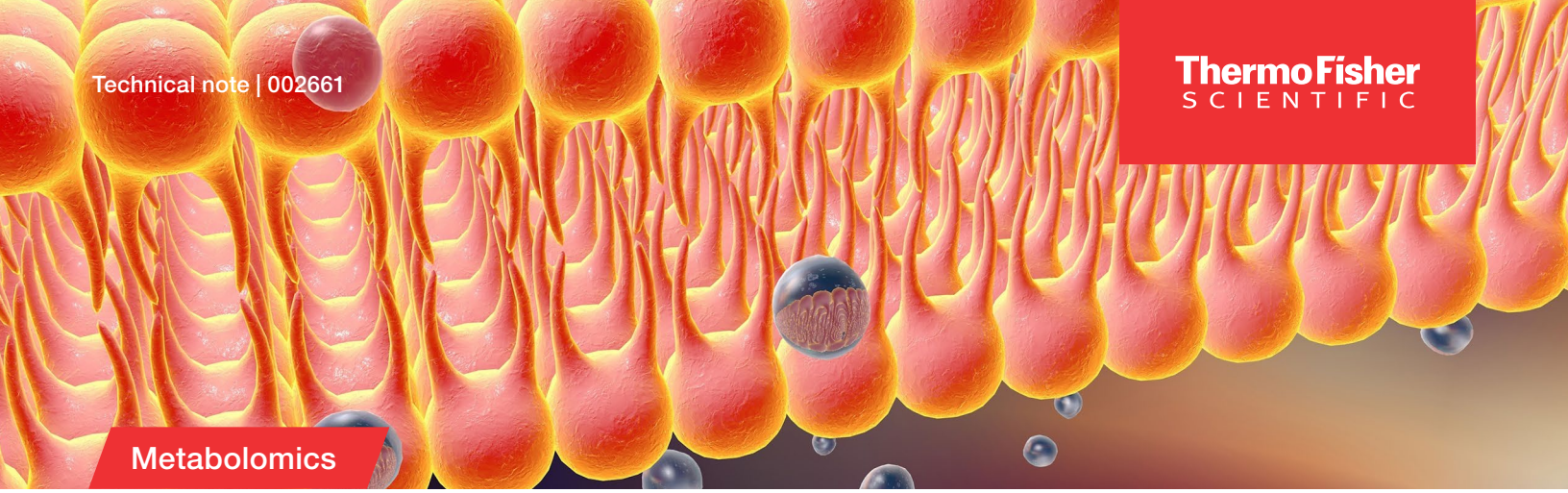

General workflow for untargeted plasma lipidomics on Orbitrap mass spectrometers

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Keywords

Lipid, lipidomics, AcquireX workflow, Orbitrap Exploris mass spectrometer, Orbitrap IQ-X mass spectrometer, Vanquish Horizon UHPLC, triglycerides, QA/QC, plasma

Introduction to lipidomics

Lipids play a key role in cell, tissue, and organ physiology as key components of cell membrane structure, cellular signaling, and energy storage. Many diseases, such as cancer and diabetes, are directly related to the disruption of lipid metabolic enzymes and pathways. The identification of unique lipid biomarkers that distinguish health from disease can have an impact on overall disease phenotyping, the design of therapies, and early disease detection in the pursuit of personalized medicine. Due to the complexity of the lipidome, which includes eight major categories of lipids, over 80 major classes, 300 sub-classes, and thousands of lipid species over a large dynamic range in concentration, UHPLC separation followed by high resolution accurate mass-mass spectrometry (HRAM MS) and MS^2 fragmentation methods are often used to separate and detect the many lipid species with optimal sensitivity, specificity, and quantitative accuracy.

Discovery lipidomics experiments analyze the entire biological lipid extract with the goal of broad lipid coverage. The capability to separate and identify the many isobars and isomers from a lipid extract requires key considerations for the analytical method, including chromatographic separation, the resolution and accuracy of the mass spectrometer, and the diagnostic fragmentations found via MS^2 or MS^n experiments.

Orbitrap-based lipidomics

Thermo Scientific™ Orbitrap Exploris™ series mass spectrometers are benchtop quadrupole-Orbitrap instruments designed for exceptional reliability and ease-of-use, resulting in improved data quality and increased productivity. The Orbitrap mass analyzer provides high resolving powers, up to 1 million resolution, to differentiate ions of similar mass, such as isobaric species, background ions, or isotopologues, providing accurate isotope fine structure to confirm elemental composition prediction.

Consistent sub-ppm accurate mass measurements (achieved using the Easy-IC internal calibrant) allow for narrowing database search tolerances for both full scan and higher-energy collisional dissociation (HCD) fragmentation spectra, providing confident structural characterization. Additionally, fast polarity switching increases the throughput.

To address the dynamic range complexity of lipidomics experiments, the Thermo Scientific™ AcquireX™ intelligent data acquisition workflow can be used to generate comprehensive fragmentation coverage of unique sample-relevant compounds by automated exclusion of non-biological and redundant features, providing improved identification of lipids and higher-quality annotations. As shown in Figure 1, by first injecting a study blank, a comprehensive exclusion list is generated from all unique features found. A sample is then run in full scan to create a full inclusion list of compounds. The workflow then begins iterative data-dependent injections, where the exclusion list is automatically updated to include the ions fragmented in the previous runs, while the inclusion list is automatically reduced by removing the ions already fragmented. The iterative process continues until all the ions in the inclusion list are fragmented or the number of injections programmed is reached. The number of injections is dependent on the complexity of the sample, with the recommended number for plasma lipids being five. The AcquireX workflow ensures that fragmentation data are obtained for even the low abundant ions, which might have been missed when doing regular data-dependent MS^2/MS^n acquisition that is solely based on peak intensity in the spectrum. Key parameters for lipidomics that have to be modified include indicating the preferred ions formed during ionization, the protonated, sodium, and ammonium adducts for the positive mode, with deprotonated ions and the formate adduct for the negative mode to ensure the most information-rich fragmentations are achieved.

Along with the earlier-described advantages of the Orbitrap technology, the Thermo Scientific™ Orbitrap™ IQ-X™ Tribrid™ mass spectrometer includes both an Orbitrap and ion trap mass analyzer. This architecture enables multi-stage fragmentation (MS^n) and multiple dissociation techniques, including HCD and collision-induced dissociation (CID), to be performed, followed by detection in either the linear ion trap or Orbitrap mass analyzer. This unique ability provides complementary diagnostic fragment ions that are used to confidently annotate co-eluting isomeric lipids, such as phospholipids and triglycerides.

Here we will describe an optimized workflow for doing untargeted lipidomics in plasma using the Thermo Scientific™ Accucore™ C30 HPLC column connected to a Thermo Scientific™ Vanquish™ Horizon UHPLC system and either an Orbitrap Exploris series mass spectrometer or an Orbitrap IQ-X Tribrid mass spectrometer. This technical note includes considerations for overall experimental design, the extraction of lipids from plasma, experimental LC-MS conditions, and lipid annotation examples. These are the recommended settings and conditions to start doing lipidomics in your own laboratory, and a few examples of how our Orbitrap mass spectrometer-based workflows can help increase your depth of lipid coverage and the specificity of each annotation.

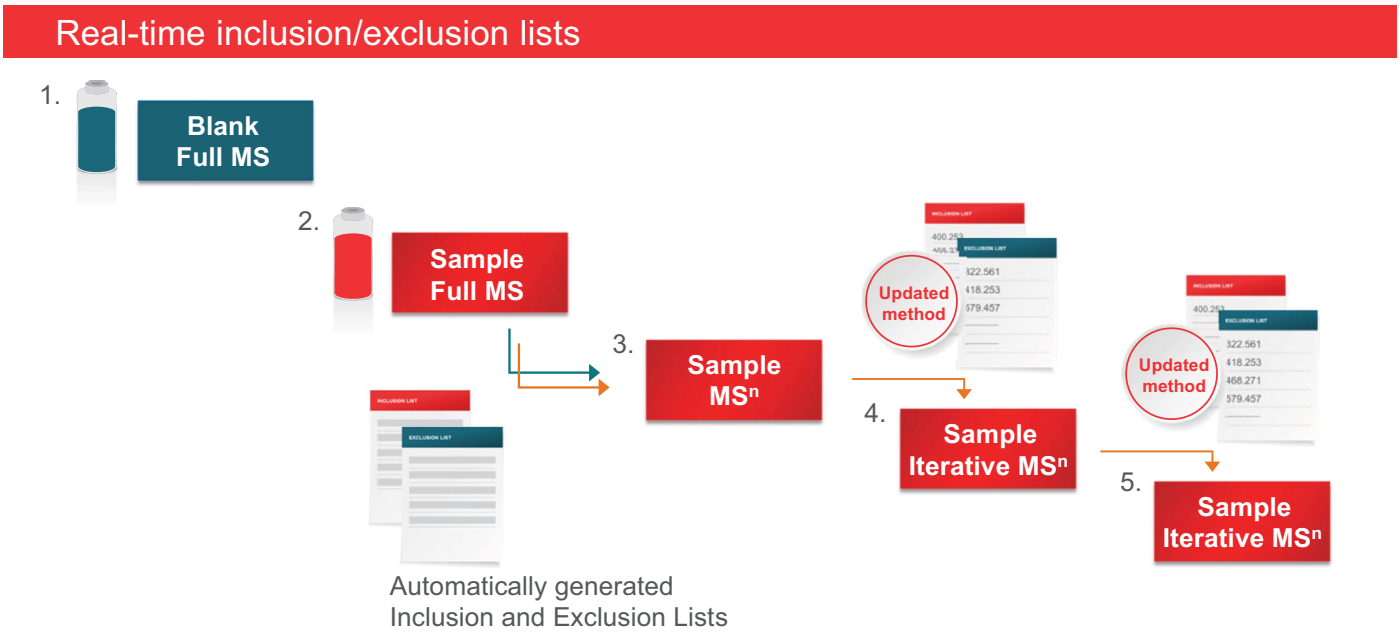

Figure 1. Deep scan AcquireX workflow on an Orbitrap mass spectrometer

Example study design (QA/QC)

To account for inherent analytical variability throughout the study, quality control (QC) practices are employed to identify any outlying biological results. Before extraction, plasma samples are spiked with stable-labeled internal standards and randomized prior to LC-MS analysis (Figure 2). This standard can be used to assess variations in extraction efficiency, while additional labeled standards are incorporated post-extraction prior to analysis to measure any analytical variability. Finally, a study sample pool QC is created by combining equal volumes from each biological study sample. This pooled sample is used to condition the UHPLC column and is injected into every 10 study samples to monitor for instrument variability such as mass drift, chromatographic drift, and internal standard sensitivity. The pool is also used for AcquireX data acquisition. A more detailed description of how these practices are directly related to overall data quality can be found in the technical note titled "Quality assurance and quality control in metabolomics: achieving high-quality data for high-quality results."[1]

Extraction of lipids from plasma

Before extraction, 20 µL of the UltimateSPLASH™ ONE Mass Spec Standard (330820) mixture obtained from Avanti Polar Lipids, Inc. (https://avantilipids.com) is added to 100 µL of plasma. Next, 400 µL of methanol is added to precipitate the proteins, and then 200 µL of chloroform is added along with 100 µL of water for phase separation. The mixture is kept at 4 °C for 1 h before centrifugation, and then 150 µL of the lower organic phase is carefully removed, dried down under nitrogen, and resuspended in 75 µL of acetonitrile: isopropanol (1:1). The lipid extracts are stored in the organic solvent at -20 °C before analysis by LC-MS. Please note that when analyzing other sample types, tissues, cells, etc., the same ratios of sample to solvent can be utilized for efficient extraction of lipids.

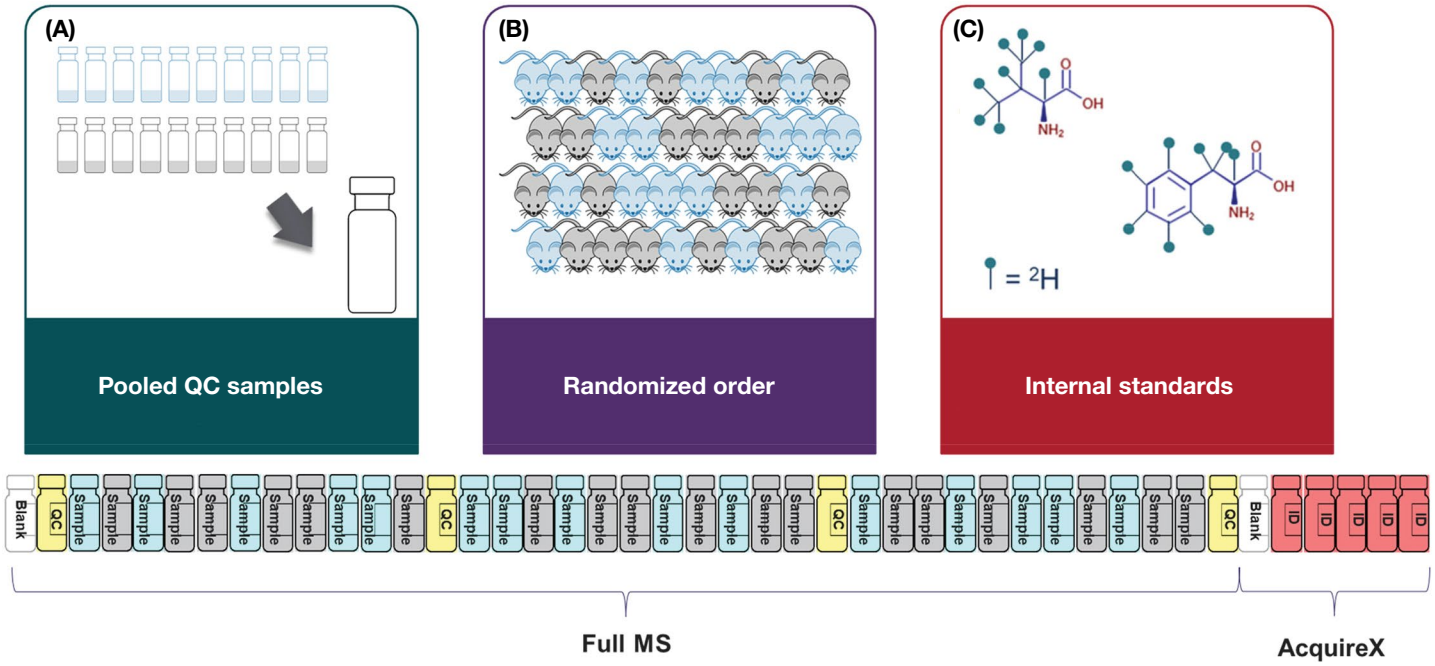

Figure 2. The analytical steps for acquiring data from samples. (A) Pooled QC samples are created by pooling equal volumes of all experimental samples. QC samples are integrated into the experimental sample analysis queue every 10 to 15 samples to assess reproducibility. (B) The sample injection order is randomized to remove sample-related bias. (C) Lipid-specific internal standards, added to each sample, provide instrument monitoring over the course of data acquisition. (D) Samples are analyzed with a full scan LC-MS and lipids are identified by pool samples with data-dependent MS^2 using the AcquireX intelligent acquisition method with the deep scan workflow.

Chromatography

Chromatographic separation is performed using a Vanquish Horizon UHPLC system and an Accucore C30 HPLC column (2.1 × 150 mm, 2.6 µm). The column temperature is maintained at 45 °C and the autosampler temperature at 15 °C. The operating pressure for the UHPLC gradient method ranges from 200 to 350 bar.

Solvent A is 60:40 acetonitrile/water and solvent B is 88:10:2 IPA/acetonitrile/water and both A and B contain 10 mM ammonium formate with 0.1% formic acid. The flow rate is 260 µL/min, and the injection volume is 2 µL.

Table 1. HPLC gradient conditions

Time, min	Flow, mL/min	% B
-3	0.26	30
0	0.26	30
2	0.26	43
2.1	0.26	55
12	0.26	65
18	0.26	85
20	0.26	100
25	0.26	100
25.1	0.26	30
28	0.26	30

Mass spectrometry

Regardless of the Orbitrap mass spectrometer used (Thermo Scientific™ Orbitrap Exploris™ 120 mass spectrometer, Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer, and Orbitrap IQ-X Tribrid mass spectrometer), all study samples are analyzed by full scan MS at 120,000 resolution (FWHM) and collected in positive and negative mode using polarity switching. Sub-ppm mass accuracy is maintained throughout the analysis using the Thermo Scientific™ EASY-IC™ runstart found in the instrument method template. The global source and instrument parameters for the Orbitrap Exploris series of mass spectrometers can be found in Table 2.

Fragmentation workflow on the Orbitrap Exploris 120 mass spectrometer

For lipid annotation, pooled samples are analyzed by MS^2 (30,000 resolution setting) using a data-dependent strategy with the top 4 ions for fragmentation (Figure 3). The dynamic exclusion window is generally kept at 3 s. This is less than what one would use for a metabolomics workflow to facilitate fragmentation of potential coeluting isobaric or isomeric analytes. Data are acquired using both positive and negative modes, as the fragmentation from both modes offers complimentary information, which leads to confident annotation. These settings can be used in the AcquireX workflow if it is available on the system.

Table 2. Orbitrap Exploris 120 mass spectrometer and Orbitrap Exploris 240 mass spectrometer full MS and MS^2 instrument parameters

Parameter	Value
Ion source	
H-ESI spray voltage	+3,500 V / -3,000 V
Sheath gas	40
Aux. gas	10
Sweep gas	1
Vaporizer temperature	275 °C
ITT temperature	300 °C
MS^1 profiling	
MS resolution	120,000
Scan range	*m/z* 200–1,700
Max. injection time	50 ms
AGC target	Standard
Easy-IC	On
Mild trapping	Off
RF lens	40
MS^2 identification	
MS^2 resolution	30,000
First mass	*m/z* 75
Cycle time	1.5 s
Isolation width	1.0 *m/z*
Intensity threshold	20,000
Max. injection time	54 ms
Normalized collision energy 25, 30% (stepped)	
Dynamic exclusion	3 s
Exclusion mass tolerance	5 ppm
Mass list targets	Determined by AcquireX acquisition

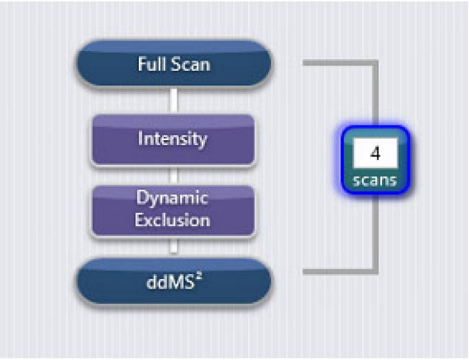

Figure 3. Data-dependent acquisition on the Orbitrap Exploris 120 mass spectrometer

Fragmentation workflow on the Orbitrap Exploris 240 mass spectrometer

Pools are analyzed similar to the Orbitrap Exploris 120 mass spectrometer with the data-dependent strategy of the top 4 being replaced by a cycle time of 1.5 s (Figure 4). These parameters need to be adjusted based on the peak width if different chromatography or solvent systems are used. Narrower peak widths will require shorter cycle times.

Figure 4. Data acquisition on the Orbitrap Exploris 240 Mass spectrometer using HCD MS². This is the method referenced in the AcquireX workflow.

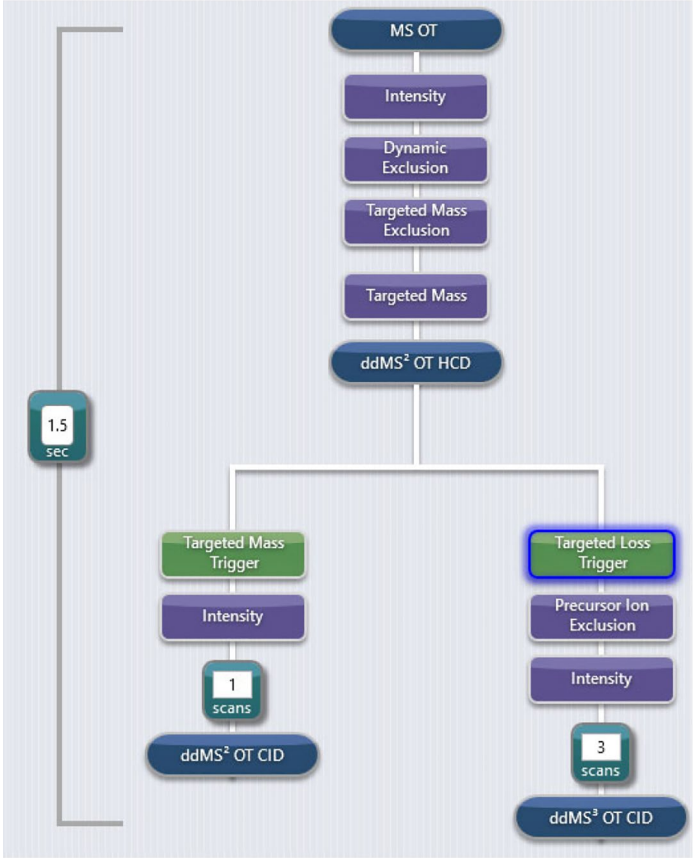

Figure 5. Lipidomics template for positive mode fragmentation on the Orbitrap IQ-X Tribrid mass spectrometer

Fragmentation workflow on the Orbitrap IQ-X Tribrid mass spectrometer

The instrument control software lipidomics template labeled "AcquireX lipid characterization HCD-CID-MS³" is used for positive mode identification runs (Figure 5). The template includes a comprehensive data-dependent HCD MS² experiment, run at top-speed using a 1.5 s cycle, with conditional CID MS² and MS³ data acquisition for more in-depth characterization of the lipids present in the lipid extracts. Additional CID MS² scans are triggered on the same precursor ion for PC lipids with a diagnostic fragment ion (*m/z* 184.0733) detected from the positive HCD MS² data. This second scan event will produce greater diagnostic fragments to complement the head group ion. For triglycerides (TG) and other lipids, additional CID MS³ scans are subsequently triggered on the three most intense HCD product ions that have lost a neutral fatty acid plus ammonia. The specificity of this MS³ scan allows unambiguous identification of all fatty acids on the glycerol backbone.

Lipid identifications performed in negative ion mode utilize both HCD (30,000 resolution setting) and CID using the AcquireX data-dependent intelligent acquisition with the deep scan workflow (Figure 6). The incorporation of both fragmentation modes yields complementary diagnostic ions for increased confidence.

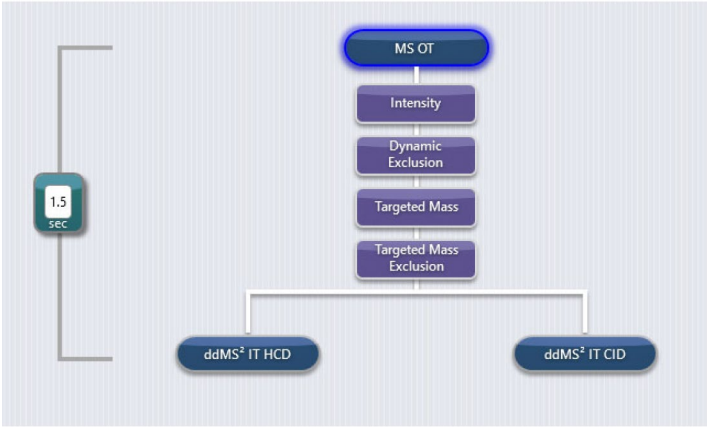

Figure 6. Data acquisition in negative mode on Orbitrap IQ-X Tribrid mass spectrometer

Default optimized parameters such as RF value, AGC target, dynamic exclusion, etc. on the template are used. Due to the multiple branches in the method, some of these parameters vary based on the type of experiment being run at the time. The default settings are the best parameters to start with and adjust as needed for additional sensitivity. For example, when loading lower amounts of lipids, injection times can be increased. The global source parameters are given in Table 3.

Table 3. Global source parameters for Orbitrap IQ-X Tribrid mass spectrometer

Parameter	Value
Ion source	
H-ESI spray voltage	+3,500 V / -3,000 V
RF lens	50
Sheath gas	50
Aux. gas	10
Sweep gas	2
Vaporizer temperature	300 °C
ITT temperature	320 °C

Data processing

Analysis of the study data is performed in parallel using the Thermo Scientific™ Compound Discoverer™ 3.3 software and the Thermo Scientific™ LipidSearch™ 5.1 software.

Results

Liquid chromatography

It is challenging to identify and quantify thousands of lipid molecular species from a biologically complex sample due to the inherent complexity of sample extracts. One of the main challenges is that lipids are diverse in structure, but many have similar physical and chemical properties. Within each lipid category, there can be additional structural complexity. For example, although TGs are composed of one glycerol molecule and three fatty acid molecules, they differ in fatty acid chain length, degree of saturation, position of double bonds, and position of the fatty acid chain on the glycerol backbone, resulting in numerous isomeric and isobaric triglyceride molecular species. It is difficult to distinguish all these isomers solely based on MS due to their isobaric overlap. Chromatographic separation is generally needed prior to MS analysis. C18 HPLC columns are commonly used for the separation of the lipid mixture. However, it is inefficient to use a C18 HPLC column to separate large numbers of isomeric lipid species. It has been demonstrated that the C30 HPLC column provides higher resolution for the separation of hydrophobic structurally related isomers compared to the C18 HPLC columns.[3]

Mass spectrometer

Figure 7 shows both positive and negative base peak chromatograms observed from a plasma lipid extract run in positive and negative polarity switching at 120K. The inset indicates 18 scans per peak in each polarity for PC 36:2, showing enough points across the peak to achieve accurate quantitation. Over the duration of the run, high lipid separation efficiency for major lipid classes is achieved by using the UHPLC C30 column, while the ultra-high resolution of 120K used for full-scan MS enables high mass accuracy over a wide inter-scan dynamic range.

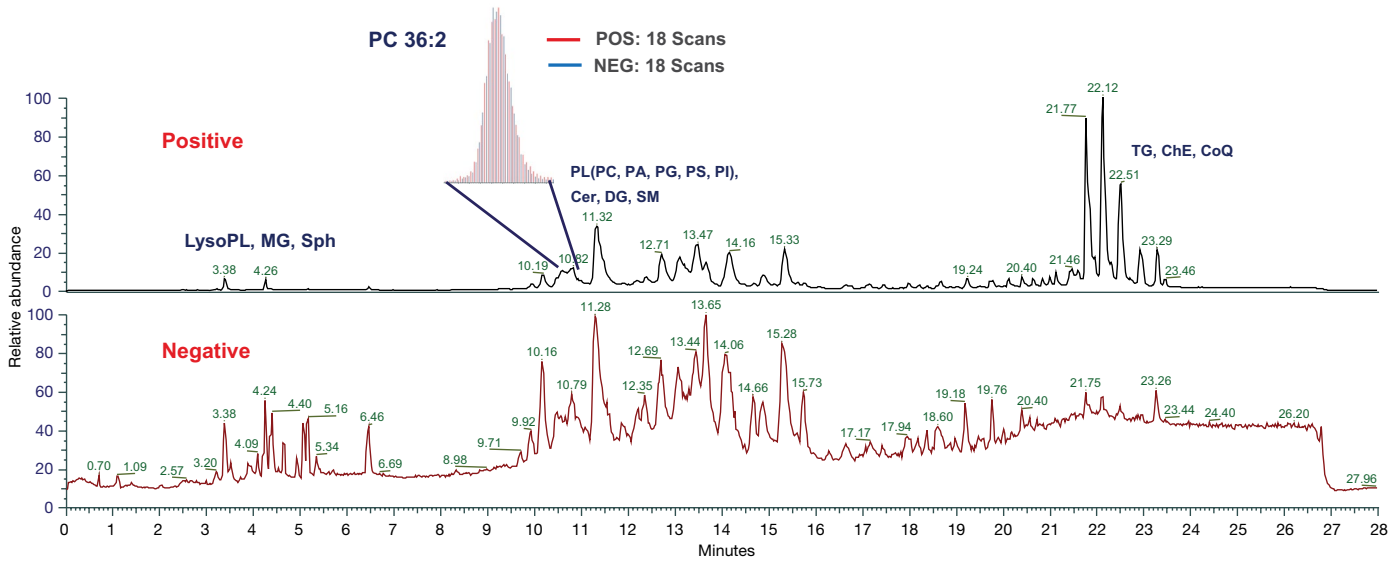

Figure 7. Base peak chromatograms of an example lipid extract in positive and negative mode ionization

Quality control

The pooled QC samples integrated into the experimental sequence are analyzed to determine the quality of the data. Some of the parameters to monitor are the reproducibility of the base peak, peak areas of the internal standards, and mass accuracy.[2] For more information on these technical details, please refer to the application note titled "Reproducible measurement and confident identification of the plasma lipidome from obese mice using the Orbitrap Exploris 240 mass spectrometer."[2]

Annotation of lipids

The use of the AcquireX workflow for generating MS^2/MS^n data results in the identification of more lipid species compared to the traditional data-dependent workflow (Figure 8). This is due to both the implementation of automatic background subtraction and the automated and iterative expansion of the exclusion and inclusion lists based on prior injections. Although the traditional data-dependent acquisition (DDA) subsequent injections also increase the rate of identification due to the inherent complexity and overlap of lipids in a complex sample, the AcquireX workflow shows an enhanced capability to drive unknown lipid identifications.

HCD fragmentation examples

Orbitrap Exploris series mass spectrometers are equipped with HCD fragmentation, which yields rich spectra for confident lipid annotations. Figure 9 shows that the fragment ion information observed from the precursor ion of *m/z* 822.7545 is clear and abundant enough to determine the molecular composition of this triglyceride lipid species. The spectra are collected from an Orbitrap Exploris 240 mass spectrometer.

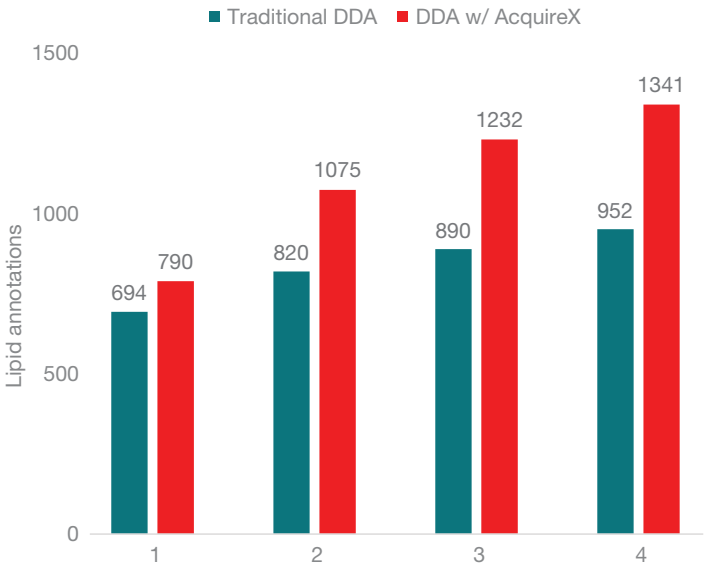

Figure 8. Number of lipid molecule annotations in sample matrix (NIST SRM 1950 Plasma) using LipidSearch 5.1 software by number of injections on the Orbitrap IQ-X Tribrid mass spectrometer in positive ion mode

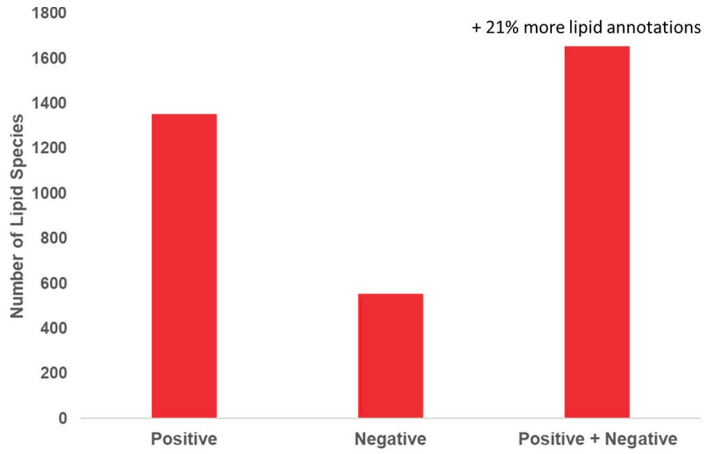

Figure 10. Increase in number of lipid species identified using LipidSearch 5.0 software with negative ion fragmentation in a sample matrix (NIST SRM 1950 Plasma)

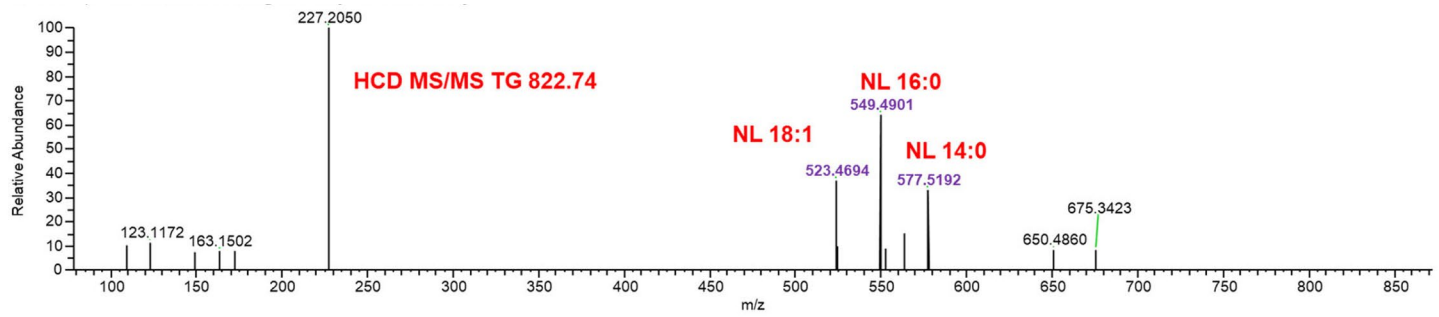

Figure 9. Confident TG molecular composition determination using HCD MS^2 of TG (48:1) species

By incorporating both negative and positive ion fragmentation experiments, complementary fragments can then be used to gain greater annotation specificity and quality in addition to more lipid identifications, as shown in Figure 10, where 21% more lipids are identified through running both polarities. For example, some lipids, like cardiolipins, do not ionize well in positive mode and hence require negative ionization for both detection and annotation. Whereas, as shown in Figure 11, the negative fragmentation information in combination with the positive fragmentation leads to the confident annotation of phosphatidylcholine 18:0_20:4. The fatty acyl chains in the PC molecule are annotated using the negative ion mode. To confidently annotate PC, both positive and negative ion fragmentation data is required on an Orbitrap mass spectrometer, and LipidSearch software can be used to combine both polarity runs in the identification process, automatically creating a streamlined workflow.

HCD and CID fragmentation on the Orbitrap IQ-X Tribrid mass spectrometer

Incorporation of multiple fragmentation modes on the Orbitrap IQ-X Tribrid mass spectrometer allows for a streamlined single polarity annotation of PCs in comparison to the multi-polarity method described above for the Orbitrap Exploris mass spectrometers. 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 the lipid class but does not give information on the fatty acyl chains. The built-in template on an Orbitrap Tribrid mass spectrometer enables the collection of a single CID MS^2 spectrum only when the diagnostic product ion (184.0733) is observed, generating information for the annotation of the fatty acyl chains, all in the positive ion mode. Figure 12 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. Orbitrap Tribrid mass spectrometers can identify the fatty acyl chains on phospholipids without the use of negative ion fragmentation.

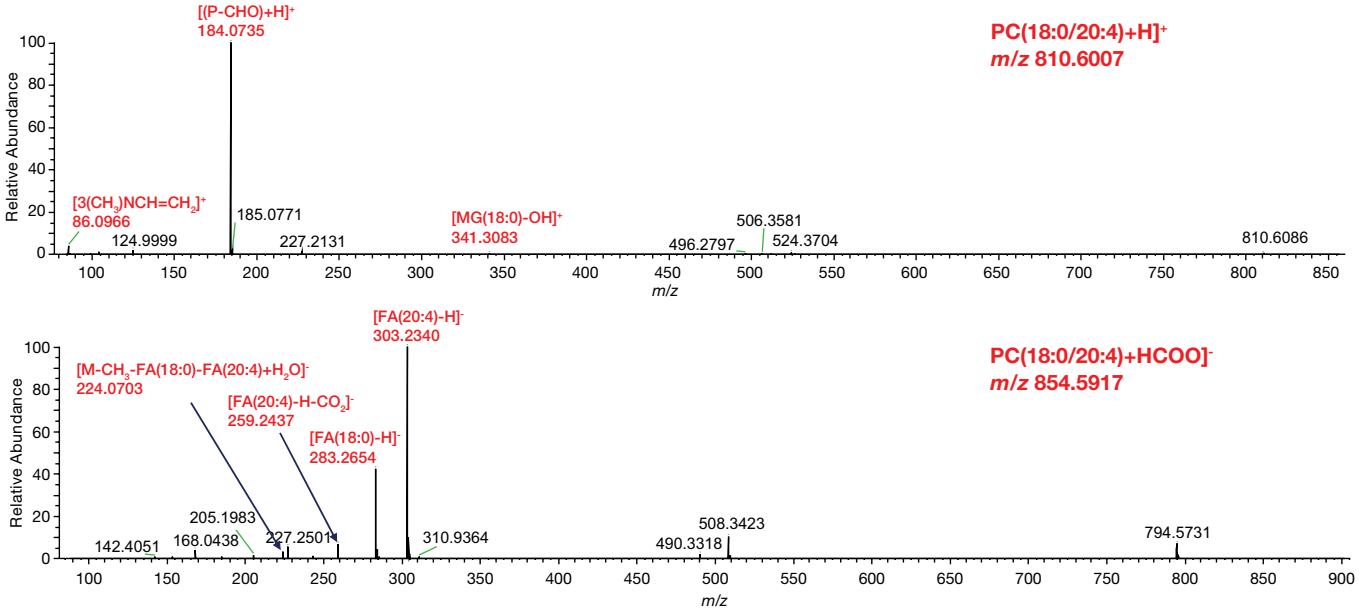

Figure 11. Confident PC molecular composition determination using MS^2 of PC 18.0_20:4 and a combination of positive and negative mode HCD MS^2 fragmentation

Identification of co-eluting TGs on an Orbitrap Tribrid mass spectrometer

For chromatographically well-resolved TG isomers, the HCD MS^2 spectrum provides sufficient product ion information for characterizing the molecular species, as shown in Figure 9. However, for co-eluting TG isomers, the HCD MS^2 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. Full characterization of the co-eluting TG isomers can be achieved by the acquisition of CID MS^3 spectra when a neutral loss of a fatty acid plus ammonia is detected from the HCD MS^2 spectrum. The HCD fragmentation spectra of *m/z* of 792.7070 produce fragments corresponding to the loss of multiple fatty acyl chains, indicating the presence of co-eluting TG isomers (Figure 13C). CID MS^3 fragmentation of these MS^2 fragments helps to annotate these multiple isomers. For example, in Figure 13D, the MS^3 fragmentation of the MS^2 HCD fragment with a *m/z* of 547.4722, indicating a neutral loss of the fatty acyl chain of 14:0, results in the confident annotation of one of the isomers present in the sample. Using the data obtained from Orbitrap Exploris instruments would lead to low-confidence annotations and putative identifications of these coeluting isomers that include the total carbon and double bond values only for the TG species. For true annotation specificity, an Orbitrap Tribrid mass spectrometer with HCD, CID, and MS^n capabilities are necessary.

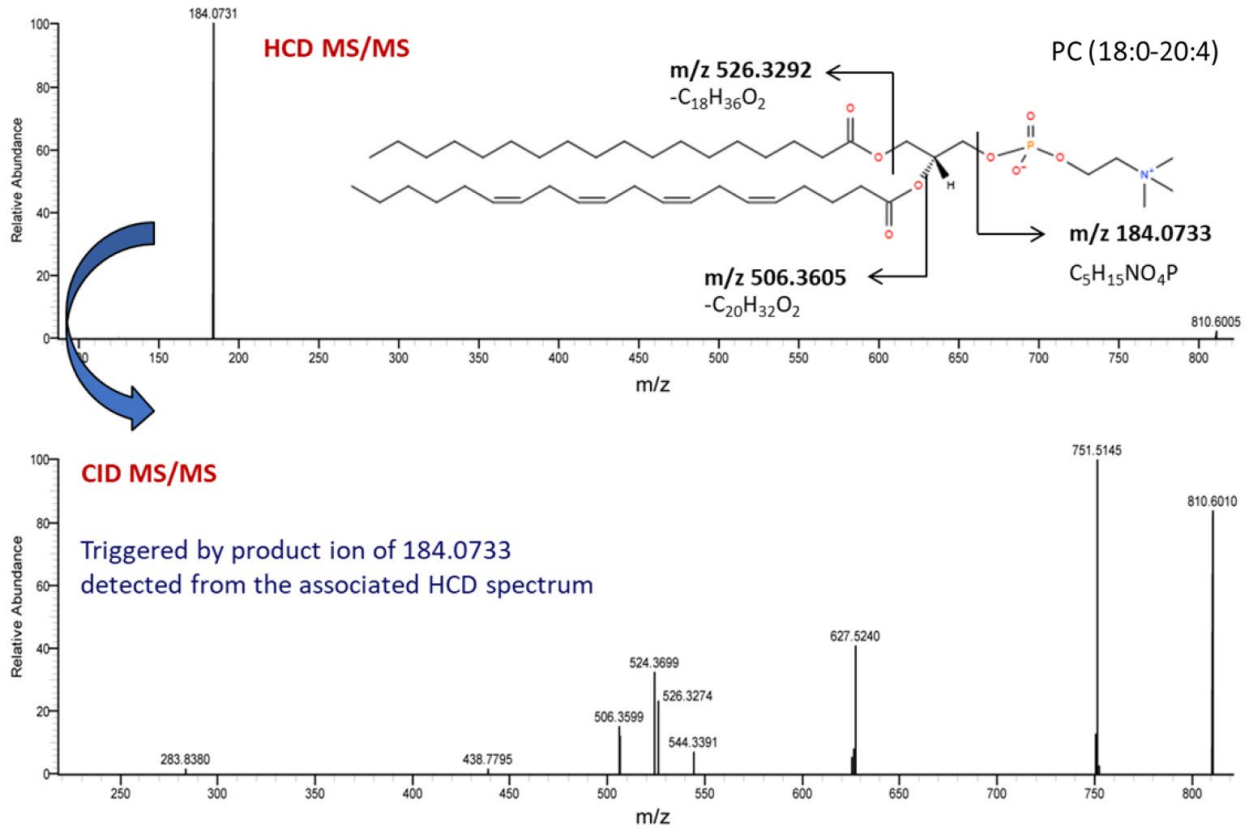

Figure 12. Comprehensive positive fragment ion information from the HCD and CID MS^2 identifying the PC species as PC (18:0_20:4)

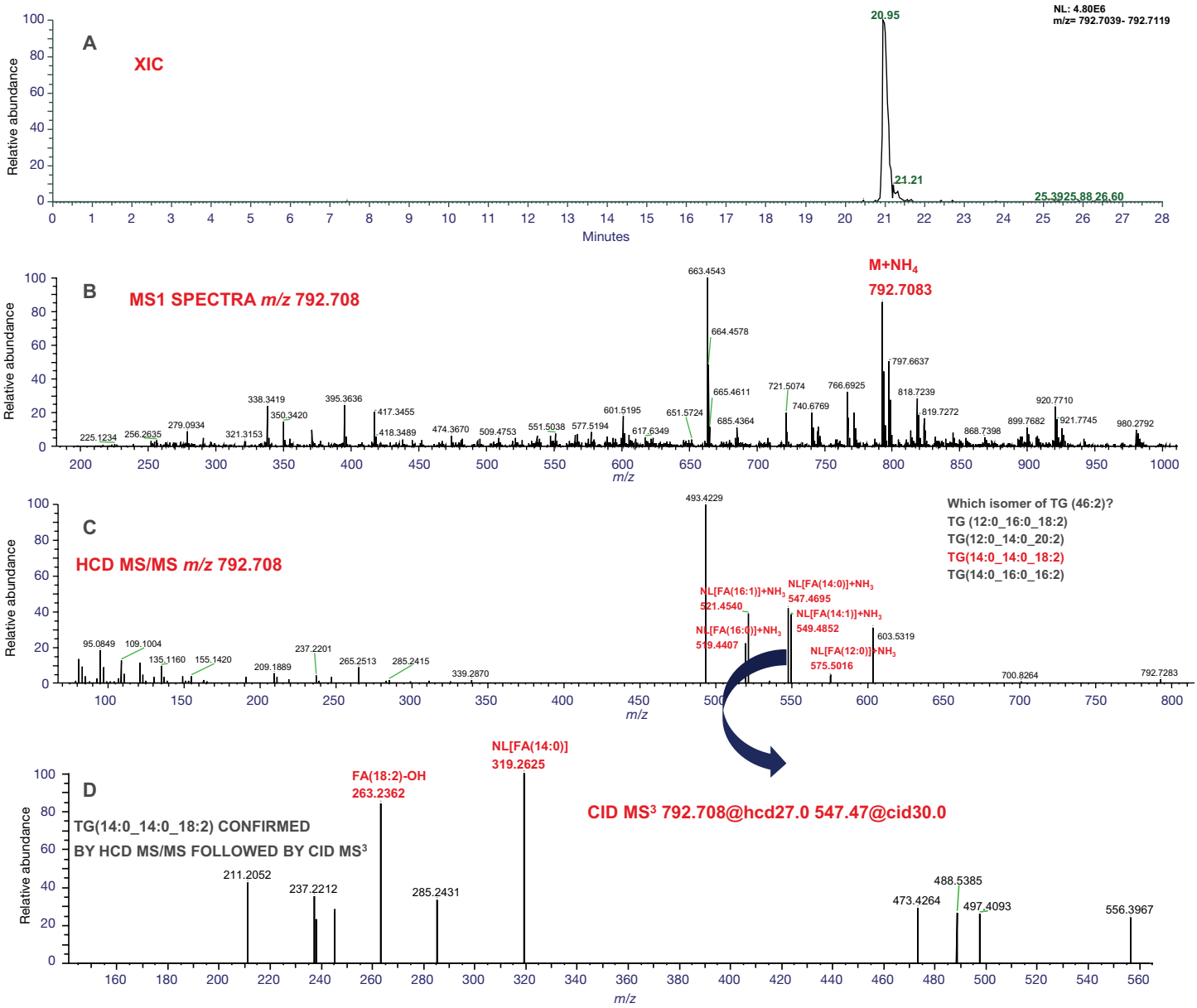

Figure 13. Characterizing co-eluting TGs using HCD MS² and CID MS³. (A) Extracted ion chromatogram of TG with mass of 792.7079. (B) MS^1 spectrum showing a highly complex region full of co-eluting species. (C) The HCD MS^2 fragmentation spectrum of the isolated species, with many potential isomers of TG (46:2) being possible. (D) Confident identification of TG (14:0_14:0_18:2) through MS^3 CID fragmentation triggered by the NL of FA (14:0 + NH_3) from the associated spectrum in panel B.

Conclusion

The use of HRAM Orbitrap technology provides high-quality lipidomics profiling with the ability to separate, detect, and annotate lipids in a complex mixture. The use of the Accucore C30 HPLC column offers unmatched separation of lipids, especially the very polar TGs, which tend to overlap significantly using C18 separation. The AcquireX acquisition allows full annotation of lipid species while excluding background and redundant signals, maximizing the quality of the LC-MS^2 identification and the depth of the analysis. High-resolution Orbitrap Exploris mass spectrometers with MS^2 and positive and negative ionization switching help in the resolution of isobaric lipid species and the throughput of individual study samples. The use of MS^2 and MS^n fragmentation data on the Orbitrap Tribrid mass spectrometers aids in the resolution of coeluting isobaric species as well as coeluting isomeric species of the same lipid class. Both platforms offer enhanced performance in the elimination of false positives and confident structural annotation of lipid species. The LipidSeach 5.1 software streamlines the process through combining both positive and negative ion mode, annotating HCD and CID fragments, as well as MS^n experiments. Through the use of highly reproducible and accurate HRAM full scan and fragmentation data, Orbitrap-based lipidomics methods are unparalleled in their ability to detect, identify, and quantify lipid species in a complex matrix.

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