



Mass spectrometry

Excel in productivity with the TSQ Altis mass spectrometer

Bridging the gap in large-scale lipid analysis through targeted acquisition

Why are lipids an ideal compound type to leverage for clinical research?

Due to the structural and functional diversity of lipids and lipid metabolism, global lipid analysis presents a gold mine for clinical researchers to evaluate general human physiology in health and disease from one set of data. For example, cholesterol and triglyceride levels measured in plasma are used as clinical biomarkers for cardiovascular disease.^{1,2} Advances in clinical research related to mass spectrometry, data processing algorithms, discovery research, and the availability of commercial standards and reference samples have enabled the community to develop ways to standardize lipid analysis and determine clinically relevant biomarkers. This has led community leaders to develop strategies for determining biological reference intervals for individual lipids.

Research consortia have focused on deriving consensus steps in experimental practices. Workflows utilizing liquid chromatography coupled to mass spectrometry (LC-MS) have demonstrated success at establishing translational pipelines. LC-MS is able to detect and quantify thousands of compounds from a single sample that covers wide dynamic ranges. State-of-the-art mass spectrometers can also perform routine measurements using fast chromatography,

greatly improving the sample throughput to address experimental requirements. Recent development of commercially available labeled standards and reference samples form the basis of extended quality assurance (QA) and quality control (QC) routines, with reliable quantitation to provide high confidence in study results and improved correlation between lab results.

What are the challenges to developing clinical research workflows for large-scale lipid profiling?

To successfully and reliably phenotype donor samples, the LC-MS method must address the overall scale of the experiment. Clinical research experimentation leverages acquisition of large amounts of data to screen for multiple health conditions based on both validated and unvalidated biomarkers. In particular, clinical analysis of lipids requires analytical methods incorporating an extensive lipid panel covering a wide range of lipid classes and sub-classes, each of which are evaluated by measuring multiple lipid species per class. The resulting method must contain appropriate quality assurance (QA) and quality control (QC) measures along with internal standards to ensure reliable quantitation within a sample as well as normalization across all donor samples in a study. Lastly, the analytical method must address throughput concerns for timely analysis of larger sample loads.

The complexity of lipids presents significant challenges to developing a universal workflow. Lipid classes are defined by their polar head groups, and lipid species within a class are further defined by non-polar acyl chain lengths, saturation, and position. Minimal differences in the chemical composition of lipid species can result in isobaric and isomeric compositions that reduce the effectiveness of liquid chromatography for complete separation, relying also on mass spectral differentiation. In addition, lipid composition is dependent on matrix type (e.g., tissue, plasma, urine) containing different lipid classes and species and have concentrations spreading over six orders of magnitude.

To ensure sufficient sample throughput, a balance is made between chromatographic separation and lipid coverage to ensure the resulting analytical method sufficiently meets qualitative and quantitative requirements for a successful study. Ultra-high-performance liquid chromatographic (UHPLC) separation maximizes peak capacity and separation over the analytical gradient, resulting in extremely narrow chromatographic peak widths (2–6 seconds FWHM). The narrower chromatographic peak profile also boosts sensitivity and peak height. While extremely beneficial for throughput, selectivity, and sensitivity, narrow chromatographic peak widths introduce complications associated with mass spectral acquisition speeds that may decrease analytical performance, which is critical for confident data generation.

Can triple quadrupole mass spectrometers effectively perform screening and routine quantitation?

What is needed to perform effective screening using targeted LC-MS/MS?

Screening approaches are designed to characterize the representative sample to determine which lipid species are confidently identified and can be transitioned into the final quantitative panel. This requires a priori knowledge on plausible lipid classes and species from which to evaluate the screening methods and data. The same databases used to search untargeted data can be used to establish the initial targeted data acquisition methods. Previous discovery-based research has determined predominant lipid classes and species for many different biological sample types and disease states.³ In addition, discovery research provides optimum electrospray ionization polarity and fragmentation patterns per polarity to bin lipid species into acquisition modes. Lastly, LC-MS studies have also provided extensive knowledge for chromatographic performance between reverse phase (RP) liquid chromatography and hydrophobic interaction liquid chromatography (HILIC). For example, RP-LC separates lipids based on the acyl chains, and HILIC separates lipids based on the head group. HILIC separation becomes advantageous for improving confidence in building targeted

acquisition methods, evaluating the results, and moving the refined target lists into the quantitative methods as the labeled internal standards (ISTDs) will co-elute with their respective lipid classes.

What are the advantages of triple quadrupole mass spectrometers for effective screening and quantitation?

Targeted data acquisition based on tandem mass spectrometry extends the dynamic range for detection and quantitation as compared to full scan MS analysis. A preferred method to perform targeted LC-MS/MS data acquisition is selected-reaction monitoring (SRM) on triple quadrupole mass spectrometers due to the speed of acquisition and improved quantitation across a wide dynamic range. Tandem mass spectrometry improves the selectivity and sensitivity for the list of study-centric lipids by setting up the triple quadrupole mass spectrometer for Q1 to filter a narrow mass range (1 Da or less) centered on the target precursor m/z , which is transmitted into Q2 for dissociation. All product ions exit Q2 and are transmitted into Q3 where the user defines a second narrow mass filter (1 Da or less) centered on the target product ion m/z value. Selection of unique diagnostic product ions can provide simultaneous lipid identification and quantitation and differentiate co-eluting isomeric lipids to meet fast chromatographic requirements. The improved selectivity translates to more reliable quantitation at lower expression levels, extending the dynamic range of the overall method. State-of-the-art triple quadrupole mass spectrometers can now acquire an SRM transition using <2 millisecond dwell time (citing 550 SRM transitions per second) and with chromatographic scheduling, over 20,000 SRM transitions per method. The fast SRM acquisition speeds are required for LC-MS/MS methods using either RP or HILIC separations with ultra-high performance as the elution peak widths can be 2 to 6 seconds measured at full-width half maximum (FWHM) peak height, and rapid profiling methods may have hundreds of co-eluting targets.

While LC-MS/MS can provide improved low-end quantitation relative to full scan high-resolution accurate mass (HRAM) MS, targeted LC-MS/MS methods have been primarily used to routinely quantitate a few lipid classes per analysis. Establishing LC-MS/MS methods for large lipid panels can be time-consuming due to the need to create SRM transition tables for approximately 2,000 lipid species as well as optimize ion optics and collision energy settings. Global lipid profiling using HILIC chromatography separates lipids by head group, resulting in the co-elution of tens to hundreds of lipid species per unit time that can require dwell time settings ≤ 1 millisecond, which can limit which triple quadrupole mass spectrometers could be used as well as reproducible detection for low-level lipid species. Adding in SRM transitions for labeled internal standards could further reduce the number of lipid species targeted per sample analysis, which lessens the lipidome coverage and phenotyping capabilities.

What advantages does the TSQ Altis triple quadrupole mass spectrometer provide to screening and quantitation of large-scale lipid panels?

With the introduction of the Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer and recently, the Thermo Scientific™ TSQ Altis™ Plus triple quadrupole mass spectrometer, unique capabilities are bridging the gap between targeted and untargeted applications for large-scale lipid analysis. The TSQ Altis and TSQ Altis Plus mass spectrometers have superior acquisition rates specified at 600 SRMs/s operated with effective dwell times below 1 millisecond. The advanced quadrupole mass filters can be set at 0.2 to 0.7 Da resolution for Q1 and/or Q3, improving selectivity as compared to 1.2 Da for most commercially available triple quadrupole mass spectrometers. The source design has demonstrated tremendous robustness to handle the study size for clinical research. The TSQ Altis and TSQ Altis Plus mass spectrometers perform equally well in both positive and negative ESI to maximize lipid coverage, and when operated with the Thermo Scientific™ Vanquish™ Horizon UHPLC system, maximum injection and gradient reproducibility enhance quantitative accuracy. Overall productivity has been increased as direct method transfer is now routine between the existing TSQ Altis mass spectrometer and the TSQ Altis Plus mass spectrometer without the need for re-optimization. In addition, multi-channel Thermo Scientific™ Vanquish™ Duo UHPLC systems for Dual LC-MS or Tandem LC-MS can reduce sample injection cycle times by 50% or greater.

How are the Thermo Scientific Vanquish Horizon UHPLC system and TSQ Altis MS used to perform high-throughput screening and quantitation?

The workflow developed by Drs. Julijana Ivanisevic and Hector Gallart-Ayalla at the University of Lausanne in Switzerland leverages the instrumental performance of the Vanquish Horizon UHPLC system coupled to the TSQ Altis mass spectrometer to maximize lipid coverage and data confidence for the translational lipid analysis workflow. The Avanti™ UltimateSPLASH™ ONE internal standard for lipid analysis is used to perform method optimization, confirmation, QA, QC, and relative/absolute quantitation. The standard mixture consists of 69 deuterium labeled lipid standards that cover 19 different classes. Within the standard mixture, each class contains 3–5 species that cover a range of acyl chain lengths that are mixed at different concentrations ranging from 25 to 100 µg/mL. The original presentation was delivered at the 9th International Singapore Lipid Symposium – iSLS 9 online.⁴

The performance of the Vanquish Horizon UHPLC system has demonstrated tremendous reproducibility and robustness to operate at 600 µL/min with a 100 × 2.1 mm column packed with 1.7 µm particles over the course of large-scale studies, resulting in 2- to 10-second chromatographic peak widths and consistent retention times across large batches. Each sample is injected for +ESI and -ESI using a 12-minute injection cycle and 3.5-minute gradient per polarity. Thus, the 8.5 minutes are used to refresh the column for the next injection. Figure 1 shows the ISTD analysis using this HILIC separation method with the same solvents and gradients in positive and negative mode.

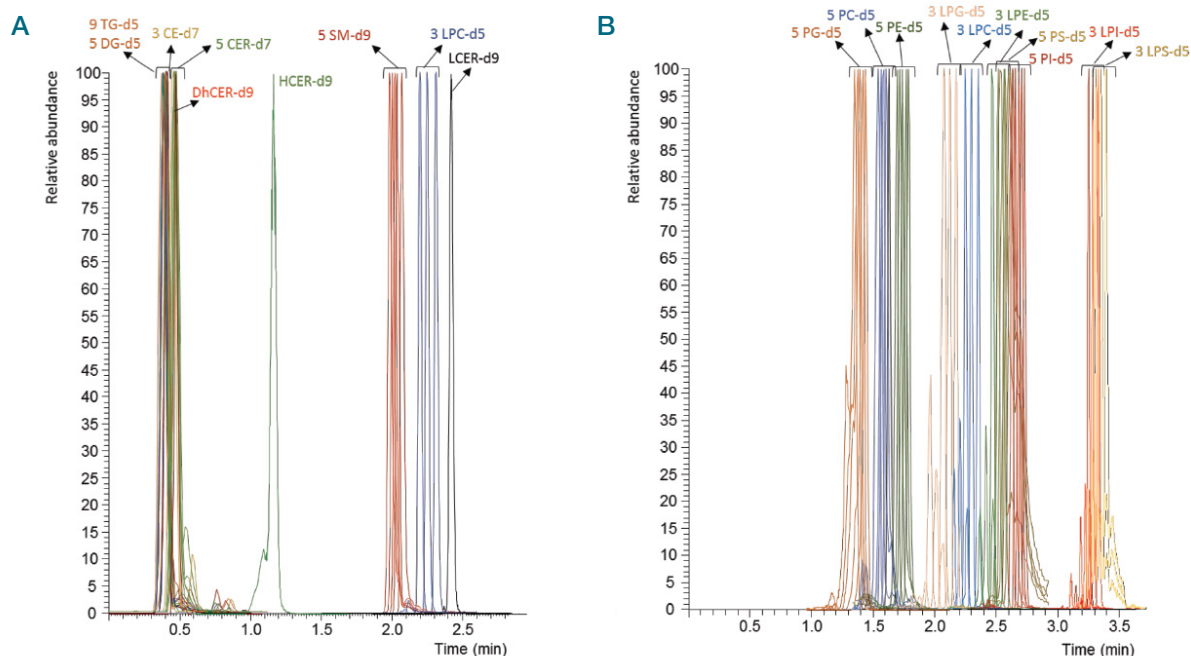


Figure 1. Measured retention time comparison for the 69 labeled lipid standards in the Avanti UltimateSPLASH ONE mixture using HILIC separations. Targeted method analysis separates the lipid classes measured in (A) +ESI mode and (B) -ESI mode using the same gradient conditions. The brackets link the set of lipid standards per class.

Since it is unrealistic to match a labeled standard with each targeted lipid, the Avanti standard provides a set of multiple labeled standards per lipid class.⁵ For example, the UltimateSPLASH ONE mixture contains four cholesteryl-d7 esters with acyl chain length compositions of: 14:1, 16:1, 18:1, 20:3, and 22:4, which provide extensive coverage of experimental parameters described above and can be extended to all endogenous cholesteryl esters targeted in the sample. The Avanti UltimateSPLASH ONE sample provides added benefits compared to other commercially available standards. The Avanti ISTD kit contains more lipid classes and greater diversity in lipid species per class in a prepared solution to minimize preparation steps. Initial method optimization on the different labeled lipid standards showed excellent overlap with optimized ion optics and collision energy settings for endogenous lipids, lending support for the satellite relationship between the ISTDs and endogenous lipids.

The researchers opted for HILIC separation to ensure elution overlap of the ISTDs and endogenous lipids to improve analytical performance of the workflow because it ensures similar class

elution profiles to help mitigate biases due to ion suppression as well as fragmentation efficiencies dictated by acyl chain lengths. A comprehensive review of HILIC performance and separation science as well as support material is found online.⁶ The ISTD mixture was spiked into samples prior to automated extraction and LC-MS/MS analysis using surrogate normalization/quantitation. In addition, the ISTD mixture was spiked into a standard amount of the NIST SRM 1950 sample across expected expression ranges to evaluate the confidence of employing single-point quantitative capabilities of the LC-MS/MS workflow.

Following ISTD optimization, the NIST SRM 1950 sample was used to test the workflow. The NIST SRM 1950 sample has been well characterized with a comparative plasma study of 31 different laboratories to establish consensus concentrations.⁷ The study group utilized similar QA/QC protocols as well as similar internal standards for normalization and quantitation, thus the initial testing of the targeted workflow becomes ideal to evaluate productivity. Figure 2 shows the biological reference intervals determined by the study group.

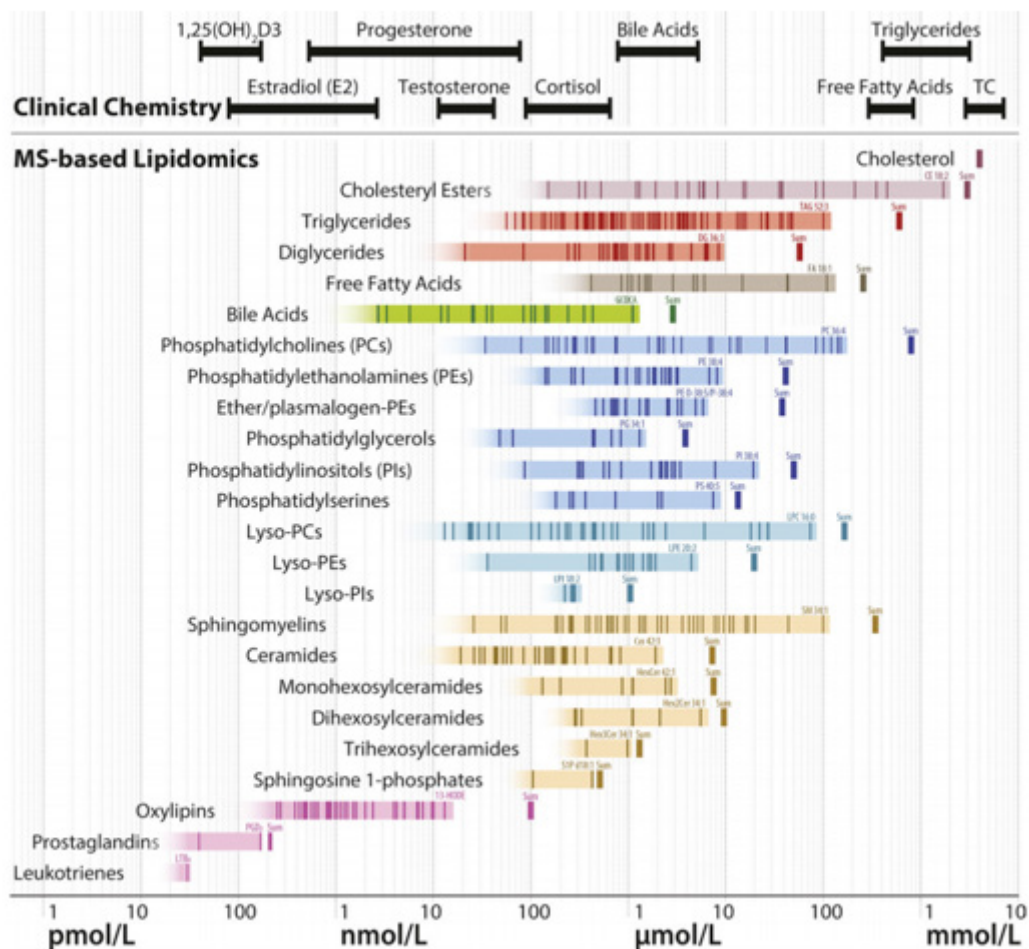


Figure 2. Concentrations of lipid species reported for the NIST SRM 1950 reference plasma sample as determined from participants for the comparative plasma study⁷

The initial screening method performed at the University of Lausanne (UNIL) (using the TSQ Altis mass spectrometer) consists of more than 1,900 lipid species divided between each polarity. The purpose of the screening method is to determine which lipid species are present in a representative study sample for further interrogation. Each lipid species was measured according to a scheduled SRM acquisition window to maximize the cycle time distribution and result in longer dwell times. Even with scheduled SRM acquisition windows, there may be over 400 co-eluting lipid species within a window, requiring very short dwell times to be applied per lipid species to ensure 6–10 data points per peak. The TSQ Altis mass spectrometer offers a substantial benefit for these cases as it can be operated with ≤ 1 millisecond dwell time while maintaining effective screening performance. Figure 3 shows the results for selected endogenous lipid species analysis to demonstrate the TSQ Altis mass spectrometer performance for data acquisition.

The distribution of lipid ISTDs shown in Figure 1 presents the challenges to targeting the extremely large list of possible lipid species across a short gradient. Figure 3A shows chromatographic peak widths of approximately 3.4 seconds (at the base), yet the method is still able to obtain six data points using sub-millisecond dwell times to increase the confidence of determining if the triacylglyceride and ceramide species are present. Later-eluting sphingomyelins do not have the same level of co-elution at 2 minutes as that observed at 0.3 minutes, resulting in 14 data points to be acquired across a similar elution window. Figure 3B shows similar performance in -ESI mode for representative PCs and PEs which elute during the most complex region of the gradient. Each has similar peak width, yet the TSQ Altis mass spectrometer delivers six data points across the peak.

To demonstrate method transferability, the same method and samples were transferred to the Thermo Fisher Scientific research laboratory in San Jose, CA, for direct analysis on the TSQ Altis Plus mass spectrometer. The experimental method originally created at UNIL was used without modification. Figure 4 shows distributions of SRM transitions as a function of the gradient elution time for the screening experiments and quantitation.

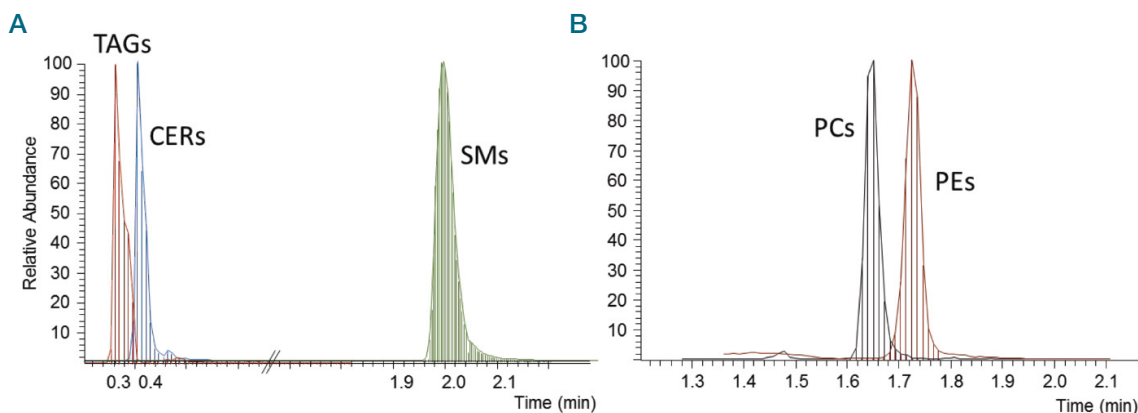


Figure 3. Extraction ion chromatographic response for a select set of targeted lipid species in (A) +ESI and (B) -ESI modes using the 3.5-minute gradient. The lines represent data points acquired during the scheduled acquisition windows for each lipid species based on the initial screening method.

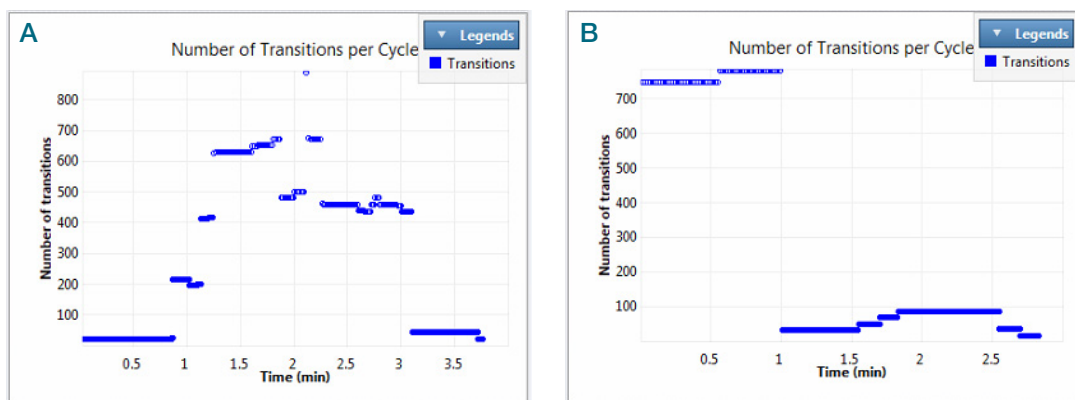


Figure 4. Scheduled SRM acquisition for the screening and quantitative analysis performed on the TSQ Altis Plus mass spectrometer. More than 1,500 SRM transitions were analyzed in -ESI mode (A), while more than 850 SRM transitions were acquired in +ESI mode (B).

The dwell time settings were determined using the estimated peak width and desired number of points for the estimated peak width. The samples provided and analyzed at the Thermo Fisher Scientific laboratory contained the NIST SRM 1950 reference sample spiked with different amounts of the Avanti UltimateSPLASH ONE mix at six different levels ranging from 10 to 100 nmol/μL. Figure 5 shows a quantitation curve measured for the 17:0-16:1(d5) PC internal standard lipid and the points across the peak for 15:0(d5) LysoPC.

The quantitative response shows a linear curve for the six levels analyzed with an unweighted linear regression of 0.998. About 500 concurrent SRM transitions were scheduled during the 2.4-second chromatographic elution profile, requiring a dwell time significantly less than 1 millisecond to collect 14 data points to reproducibly define the chromatographic peak shape.

How is the screening data processed and converted to the final method?

Large, targeted panels can lead to tedious data review prior to converting the screening results to final targeted methods. In this case, the screening workflow starts with data acquisition

on 1,900 lipids followed by data processing to determine which lipids are detected in the sample. To assist in automating this process, the laboratory has been working on a software module to automatically and quickly process screening data to determine which lipids should be kept in the final method based on customizable user-defined “Quality Index” settings consisting of acceptance criteria. In addition to automating lipid selection, the prototype software tool adjusts retention time windows for the final method to optimize dwell times and consequently data quality and reproducibility. In this case, the processed screening data for both polarities to convert the original 1,900 lipids into final targeted methods for 498 species in +ESI and 648 in -ESI.

Figure 6 shows the distribution of lipid species and classes successfully quantified in the final method. All data were processed in Thermo Scientific™ TraceFinder™ version 5.1 software using the ISTDs for normalization and single point quantitation. The replicate samples were processed and evaluated using the entire workflow, and the measured levels were compared to the consensus concentration levels established for the NIST SRM 1950 reference sample.

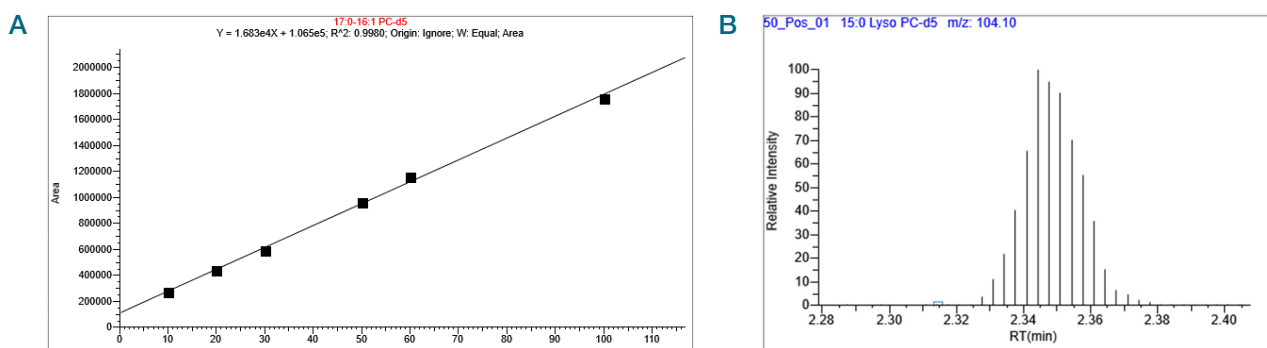


Figure 5. Quantitative response for the Avanti ISTD 17:0_16:1 PC-d5 lipid using the initial screening method. The data was acquired in -ESI mode using one SRM transition. Figure 5B shows the peak shape and number of data points acquired across the chromatographic elution profile at 10 nmol/μL.

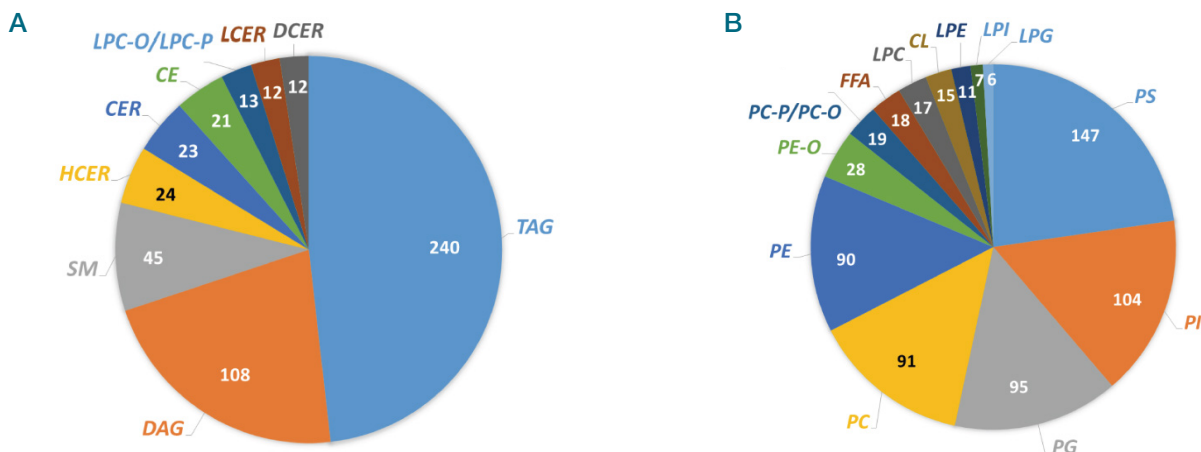


Figure 6. Distribution of targeted lipid species and classes from the NIST SRM 1950 reference sample evaluated using the UNIL LC-MS/MS workflow. Figure 6A shows the distribution measured in +ESI mode and 6B in -ESI.

Delivering an effective LC-MS/MS method for both screening and quantitation to drive clinical lipid profiling

Drs. Ivanisevic and Gallart-Ayalla have developed an effective pipeline for screening and targeted quantitation of lipids that addresses the scope and challenges presented by clinical cohorts using the TSQ Altis mass spectrometer. Leveraging the extensive knowledge accumulated into lipid databases, large-scale SRM transitions can be created for broad screening coverage of lipid species and classes for different studies on various matrices and sample types. Utilizing the commercially available Avanti UltimateSPLASH ONE standard mixture introduces a comprehensive standard to perform workflow optimization, normalization, and single-point quantitation. The availability of universal reference samples, such as NIST SRM 1950, further promotes QA/QC protocols to evaluate workflow performance during the method creation and testing for comparison to accepted biological reference intervals. In addition, the current work is being conducted in concert with the community approach to enable SRM databases for community access to lessen the barriers to entry and productivity. The ability to rapidly transfer methods between two laboratories and across different instrument generations further increases productivity and minimizes the need for new method development or optimization.

The next steps being performed are to extend the sample throughput while maintaining analytical performance. Extension of the Vanquish Horizon UHPLC system from a single channel configuration to a multi-channel Vanquish Duo system adds a second injection and flow path to the UHPLC system.⁸ The benefits of dual channel configuration enable two dedicated HILIC gradients to be performed for +ESI and -ESI as well as reducing dead time between sample loading and column cleaning and

re-equilibration, which is more pronounced using HILIC columns. Using the existing single channel configuration, the sample injection cycle is 12 minutes with only 3.5 minutes devoted to data acquisition and 25 minutes in total for one sample to be fully analyzed. Moving to a dual column approach could improve the effective gradient to 8 minutes per ESI polarity and a total of 16-minute sample analysis cycle time, boosting productivity by about 36%. In addition, the dual channel system could boost signal using dedicated solvent systems per polarity, lengthen the gradient profile supporting a combination of more lipids, increase dwell times per lipid species, or allow more SRM transitions per lipid species, all of which could benefit the workflow.

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Learn more about the instrumentation used to develop the lipid analysis workflow at
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