Application Note

Lipidomics

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Improving Coverage of the Plasma Lipidome Using Iterative MS/MS Data Acquisition Combined with Lipid Annotator Software and 6546 LC/Q-TOF

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Introduction

A major challenge in mass spectrometry-based lipidomics is the comprehensive characterization of a large and diverse set of lipid species, spanning a wide concentration range within a biological sample. While shotgun lipidomics has advanced the field of lipid analysis, it suffers from limitations including the failure to distinguish isobaric species, which may be of biological importance and reduced dynamic range due to ionization suppression. This led to chromatography-based lipid profiling approaches using high-performance liquid chromatography (HPLC) coupled to high resolution mass spectrometry (MS).

To enable product-ion spectral matching against *in silico* generated databases, confident lipid annotation requires data acquisition at the MS/MS level. However, while LC separation helps elucidate isomeric lipid species and reduce complexity, data-dependent high-resolution MS/MS data are limited by the number of precursors that can be selected for fragmentation during chromatographic elution. Therefore, it is not possible to acquire all the MS/MS spectra of interest in a single analysis for complex samples. Due to concentration bias, this strategy often misses important lipid species of low abundance.

This Application Note demonstrates solutions to these challenges. We used reversed-phase (RP) chromatography, which is well suited to resolve many cases of isomeric lipids, and is a popular choice for profiling plasma[1], tissue[2], and cellular lipids in a comprehensive manner. We coupled this LC separation to the Agilent 6546 LC/Q-TOF, a mass spectrometer designed to provide wide dynamic range while simultaneously providing improved resolution independent of acquisition speed. We also evaluated the fully automated Q-TOF Iterative MS/MS acquisition mode, in which a sample is injected multiple times, and precursors previously selected for MS/MS fragmentation are excluded on a rolling basis. These results demonstrate that plasma lipidome coverage can be significantly improved with Iterative MS/MS. Iterative MS/MS data can be used by Agilent Lipid Annotator software as part of a comprehensive lipidomics workflow.

Experimental

Reagents and chemicals

All reagents and solvents were HPLC or LC/MS grade. Acetonitrile, methanol, and isopropanol were purchased from Honeywell (Morristown, NJ, USA). Ultrapure water was produced with a Milli-Q Integral system equipped with a LC-Pak Polisher and a 0.22 µm point-of-use membrane filter cartridge (EMD Millipore, Billerica, MA, USA). Ammonium fluoride and LC/MS grade ammonium acetate were purchased from Millipore Sigma (St. Louis, MO, USA). NIST SRM 1950 human plasma was purchased from Millipore Sigma.

Sample preparation

NIST SRM 1950 plasma was thawed on ice, and plasma lipids were extracted with a modified Folch extraction procedure. Methanol (400 µL) was added to a 50 µL aliquot of thawed plasma in a 2 mL Eppendorf tube, vortexed briefly, then bath-sonicated for five minutes. Chloroform (800 µL) was added, and vortexed for one minute. To induce phase partitioning, 240 µL of water was added. The mixture was then vortexed for one minute, and centrifuged at 16,000 × g for two minutes at 4 °C. The lower layer was carefully removed with a gas-tight glass syringe, and transferred to a 2 mL Agilent A-Line amber glass vial. To re-extract the remaining interphase and upper phase layers, 900 µL chloroform/methanol/water (86:14:1) was added, and the mixture was vortexed for one minute and centrifuged again. The combined lower layers from the two 50 µL extractions were combined and dried by a vacuum concentrator. Dried lipid extracts were reconstituted with 100 µL of a methanol/chloroform mixture (9:1, v/v), vortexed for one minute, and taken to deactivated 250 µL autosampler glass inserts before LC/MS analysis. For positive mode analysis, synthetic rubber septa (p/n 5181-1212) were used, and 2 µL injections were made. For negative mode analysis, PTFE/Silicone/PTFE septa (p/n 5185-5861) were used, and 5 µL injections were made.

Instrumentation

LC system

Agilent 1290 Infinity II LC including:

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Vialsampler with thermostat (G7129B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)

MS system

Agilent 6546 LC/Q-TOF with an Agilent Jet Stream Technology source

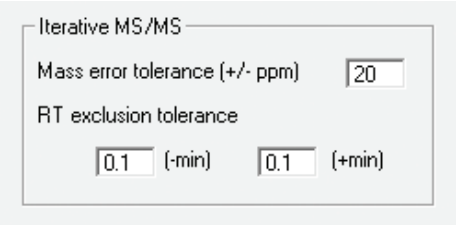
Method

Data were acquired either using a conventional AutoMS/MS method or an Iterative MS/MS method, as indicated. Tables 1 and 2 provide the chromatography and 6546 Q-TOF conditions and parameters.

Table 1. Chromatographic conditions.

Parameter	Agilent 1290 Infinity II LC
Analytical Column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)
Guard Column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 5 mm, 2.7 µm (p/n 823750-911)
Column Temperature	50 °C
Injection Volume	2 µL (positive), 5 µL (negative)
Autosampler Temperature	50 °C
Needle Wash	15 seconds in wash port (50:50 methanol/isopropanol)
Mobile Phase	A) 10 mM ammonium acetate, 0.2 mM ammonium fluoride in 9:1 water/methanol B) 10 mM ammonium acetate, 0.2 mM ammonium fluoride in 2:3:5 acetonitrile/methanol/isopropanol
Flow Rate	0.6 mL/min
Gradient Program	Time (min) %B 0.00 70 1.00 70 3.50 86 10.00 86 11.00 100 17.00 100 17.10 70 19.00 70
Stop Time	19 minutes
Post Time	None
Observed Column Pressure	170 to 330 bar

Iterative MS/MS parameters in the Acquisition method editor were set up as follows:



To invoke Iterative M/MS, the Acquisition worklist was set up as follows:

- Right-click, and select **Add Columns**.
- Select **Iterative** from available columns under **MS Parameter** Column Type (Figure 1).
- Typing **Start** or **Reset** in the Iterative column (Figure 2) indicates the beginning of an iteration set. This resets any prior rolling exclusion list, and begins a new exclusion list.
- Typing **Iterative** or any other word is used to specify the subsequent iterative injections that both use and add to the exclusion list.
- A blank cell indicates the injection neither uses nor adds to the exclusion list, but does not reset the worklist. However, note that a full or partial (time segment) Targeted MS/MS or Scan (MS only) acquisition method will reset the rolling exclusion list.

Inj Vol (µl)	Iterative	Comment
As Method	start	
As Method	iterative	
As Method	iterative	
As Method	iterative	
As Method	iterative	
As Method		

Figure 2. Worklist setup for Iterative MS/MS.

Table 2. 6546 Q-TOF AutoMS/MS parameters.

Parameter	6546 LC/Q-TOF
Gas Temperature	200 °C
Gas Flow	10 L/min
Nebulizer (psig)	50
Sheath Gas Temperature	300 °C
Sheath Gas Flow	12 L/min
VCap	3,500 V (+), 3,000 V (–)
Nozzle Voltage	0 V
Fragmentor	150 V
Skimmer	65 V
Octopole RF Vpp	750 V
Reference Mass	*m/z* 121.050873, *m/z* 1221.990637 (+) *m/z* 119.03632, *m/z* 980.016375 (–)
MS and MS/MS Range	*m/z* 40–1700 (+)
Min MS and MS/MS Acquisition Rate	3 spectra/s
Isolation Width	Narrow (~ 1.3 *m/z*)
Collision Energy	20 eV (+), 25 eV (–)
Max Precursors Per Cycle	3
Precursor Abundance-Based Scan Speed	Yes, target 25,000 counts/spectrum
Use MS/MS Accumulation Time Limit	Yes
Reject Precursors That Cannot Reach Target TIC	No
Threshold for MS/MS	5,000 counts and 0.001%
Active Exclusion Enabled	Yes, one repeat, then exclude for 0.05 minutes
Purity	Stringency 70 %, cut off 0 %
Isotope Model	Common organic molecules
Sort Precursors	1, 2, unknown
Static Exclusion Ranges	*m/z* 40 to 151 (+) *m/z* 40 to 210 (–)

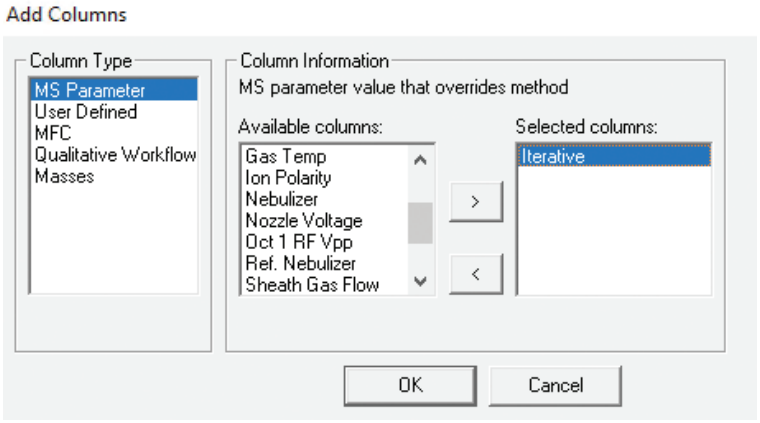


Figure 1. The Add Columns dialog box.

Software

Agilent MassHunter Q-TOF Data Acquisition Version 10 was used to operate the 6546 LC/Q-TOF system. Agilent MassHunter Lipid Annotator Version 1.0 was used for all other data analyses. Default method parameters were used, except only $[M+H]^+$ and $[M+NH_4]^+$ precursors were considered for positive ion mode analysis, and only $[M-H]^-$ and $[M+HAc-H]^-$ precursors were considered for negative ion mode analysis. Agilent MassHunter PCDL Manager Version B.08 SP1 was used to manage and edit the exported annotations.

Results and discussion

Lipid Annotator software analysis of plasma Iterative MS/MS data

Confident lipid annotation requires data acquisition at the MS/MS level to enable product ion spectral matching against *in silico*-generated databases. This study used a novel software tool (Lipid Annotator) with a combination of Bayesian scoring, a probability density algorithm, and non-negative least squares fit to search a theoretical lipid library (modified LipidBlast) developed by Kind; *et al.*[3,4] to annotate the MS/MS spectra. Lipid Annotator takes special care not to over-annotate lipid entities by only providing the level of structural information confidently informed by the MS/MS spectra.

Previously, a Q-TOF Iterative MS/MS acquisition mode was shown to be effective for in-depth peptide mapping of monoclonal antibodies[5]. We applied this mode of Iterative Acquisition on the 6546 LC/Q-TOF to a complex lipid sample. Figure 3 illustrates the strategy of Iterative MS/MS. The first injection is performed as a traditional data-dependent (conventional) Auto MS/MS analysis, where the top N most abundant precursors are selected for fragmentation in consideration of an active exclusion list. In subsequent injections, precursors selected for MS/MS fragmentation in the previous injections are excluded on a rolling basis with customizable mass error tolerance and retention time exclusion tolerance.

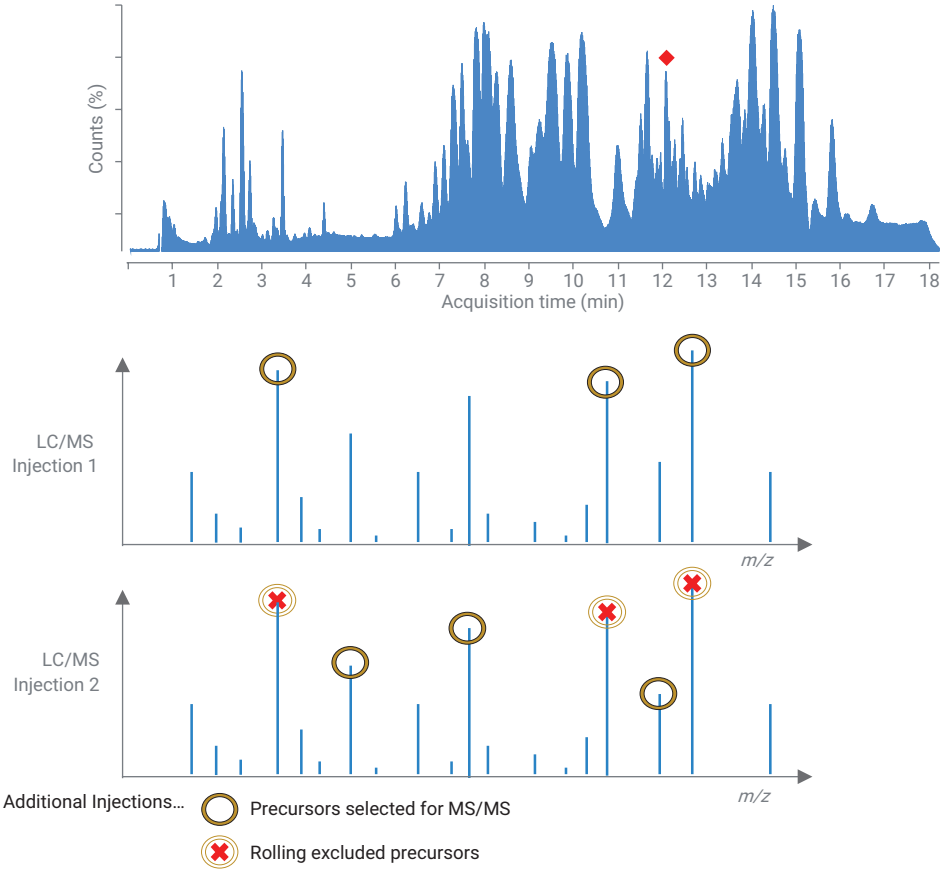


Figure 3. Principle of Iterative MS/MS.

Lipid Annotator provides the ability to analyze multiple MS/MS data files from the same sample origin together as a batch. Figure 4 illustrates an analysis of five plasma Iterative MS/MS data files.

There were 355 specific lipids (including isomers with different RTs) representing 14 classes annotated across the five positive ion mode data files. Separately, a batch of five negative ion mode Iterative MS/MS data files was analyzed, resulting in 326 specific lipids representing 20 lipid classes (not shown).

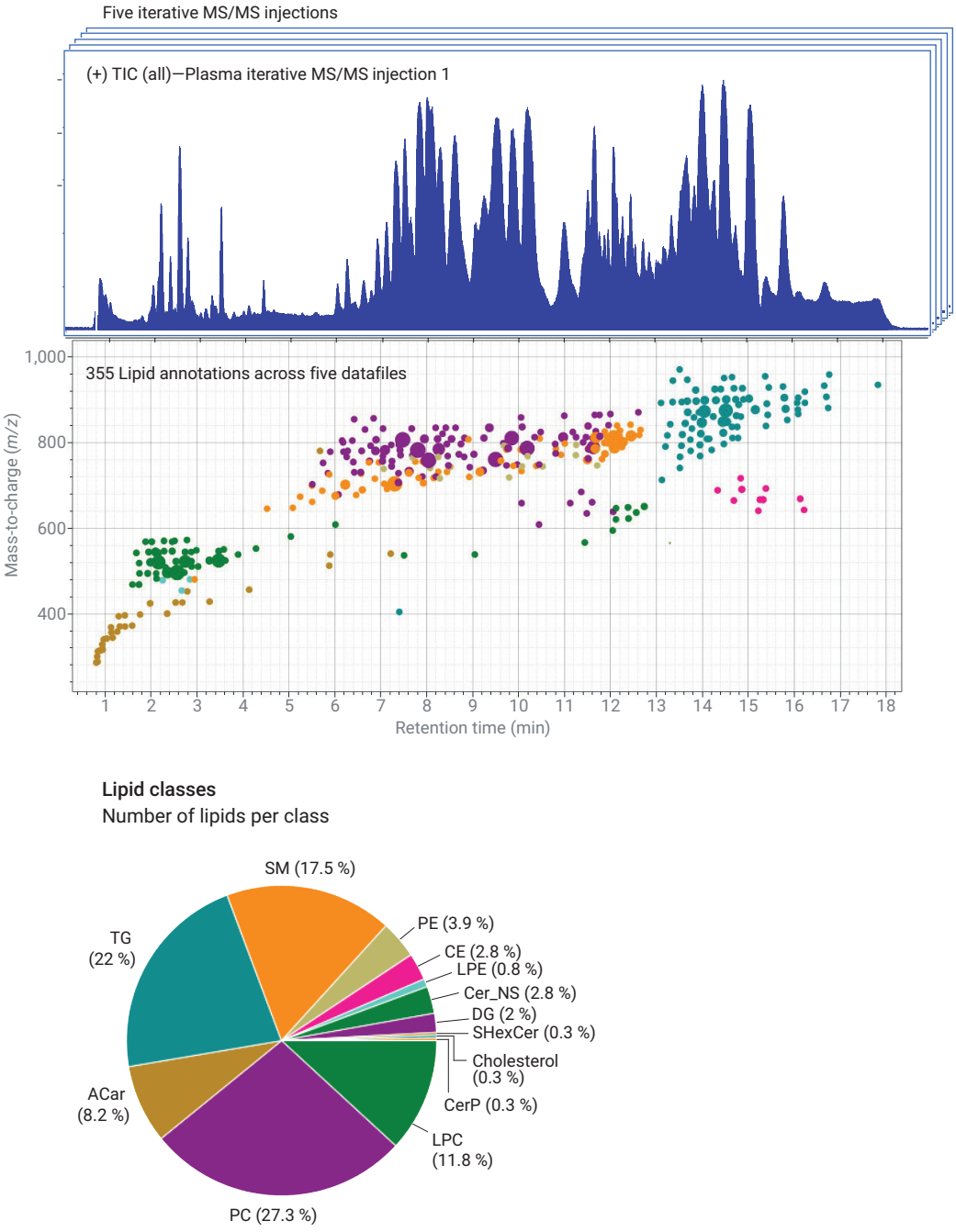


Figure 4. Typical total ion chromatogram (TIC) of plasma in positive ion mode aligned with view of Lipid Annotator software. Results shown are the combined analysis of five Iterative MS/MS data files. Annotated lipid features are plotted as *m/z* versus retention time, and colored by lipid class corresponding to the pie chart, where the numbers of annotated lipids are shown as percentages.

Iterative MS/MS increases lipid annotations

The number of cumulative lipid annotations in plasma across multiple Iterative MS/MS data acquisition files increased compared to conventional AutoMS/MS files (Figure 5). These results suggest that 3 to 5 Iterative MS/MS injections are sufficient for comprehensive lipid annotation in plasma with the method parameters used in this study. While plasma represents a common and complex biological sample, it is important to note that the optimal number of injections may depend on sample complexity and LC/MS acquisition method parameters. For plasma extracts in positive mode, applying Iterative MS/MS to five sequential injections increased the coverage of unique annotated lipids by 69 % (n = 355) compared with conventional AutoMS/MS acquisition (n = 223) across five sequential injections (Figure 5A). Likewise, in negative mode analysis of plasma, 34 % more lipid annotations were obtained with five injections of Iterative MS/MS (n = 326) compared to conventional MS/MS (n = 243) (Figure 5B). Taken together, these results show that due to the spectral density of numerous lipid precursors in a chromatographic run, especially in positive ion mode, a substantial benefit is obtained using Iterative MS/MS for LC/MS/MS-based lipidomics data acquisition.

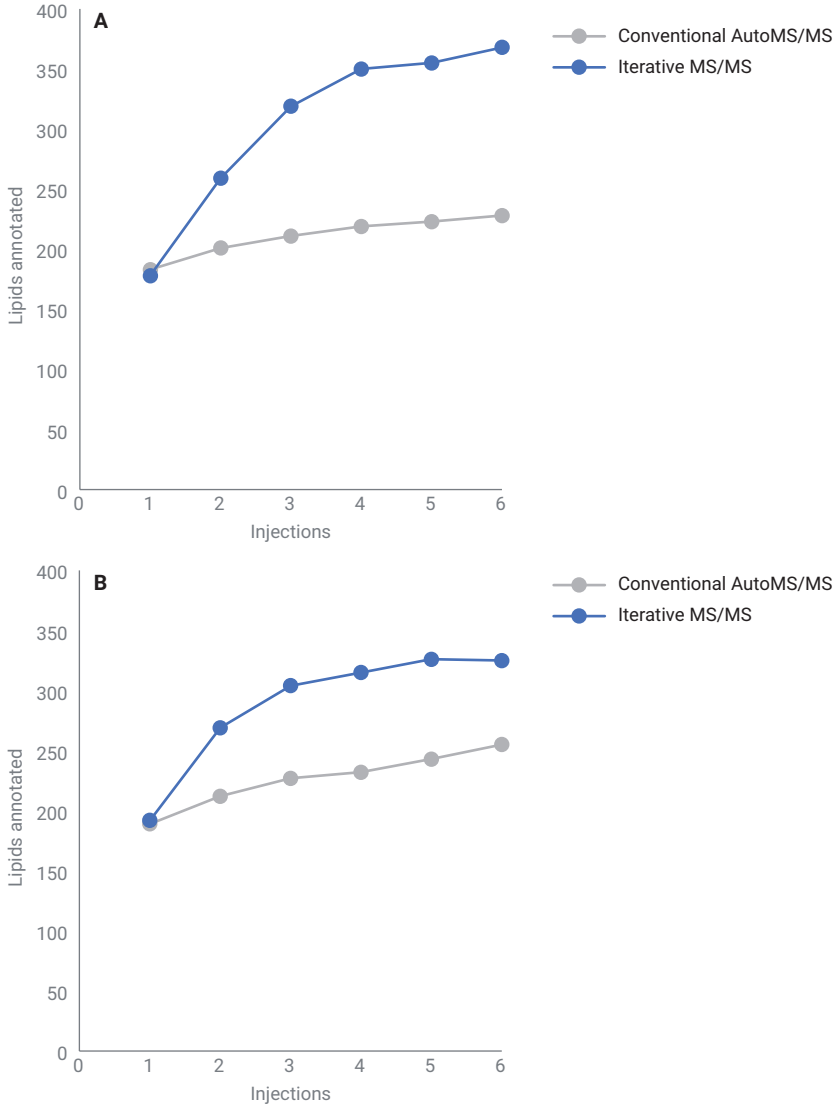


Figure 5. Cumulative unique annotated lipid features from Lipid Annotator software across multiple data acquisition files for positive (A) and negative (B) ionization modes.

Iterative MS/MS enriches lipid ions of low abundance and specific lipid classes

In agreement with the sequential exclusion of highly abundant lipid precursors in Iterative MS/MS mode, we observed that the mean peak abundance of triggered lipid precursors decreased across the first three injections of plasma in both positive (Figure 6A) and negative (Figure 6B) polarity datasets. Additionally, the peak abundances of annotated lipids in the second injection were significantly lower than the initial injection for both positive and negative polarity datasets (t-test p-value <0.001).

Due to the iterative exclusion of abundant precursors, we observed that Iterative MS/MS enriched lipid classes that were of low abundance (for example, diacylglycerols), ionized less efficiently (for example, free cholesterol), or located in spectral-dense regions of the chromatogram (for example, triacylglycerols). Table 3 shows examples of lipid classes that were highly enriched with sequential injections of Iterative MS/MS compared to conventional AutoMS/MS.

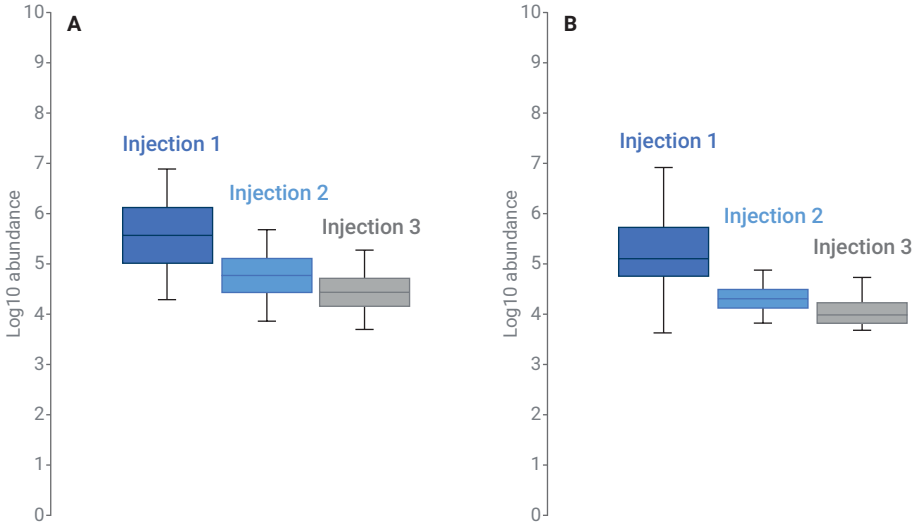


Figure 6. Box plots of feature abundances corresponding to annotated lipids from three sequential Iterative MS/MS injections in positive (A) and negative (B) ionization modes.

Table 3. Lipid classes highly enriched with Iterative MS/MS. Numbers of cumulative annotated lipids are provided for five sequential injections acquired with conventional AutoMS/MS compared to Iterative MS/MS data acquisition.

Lipid Class (polarity)	Number of Annotations AutoMS/MS	Number of Annotations Iterative MS/MS
Ceramide Nonhydroxy Fatty Acid-Sphingosines (+)	2	10
Cholesterol Esters (+)	4	10
Free Cholesterol (+)	0	1
Diacylglycerols (+)	1	7
Phosphatidylethanolamines (+)	2	14
Triacylglycerols (+)	46	78
Ether-linked phosphatidylcholines (–)	17	28
Lysophosphatidylinositols (–)	5	9
Phosphatidylethanolamines (–)	9	20

Figure 7 further demonstrates a specific example of lipid class enrichment, where precursors corresponding to cholesterol esters of low abundance were selected for MS/MS fragmentation in sequential injections.

MS/MS acquisition parameter optimization with consideration of lipid isomers

To ensure optimal coverage of plasma lipids, MS/MS acquisition method parameters were found to be critical. As a first step, Lipid Annotator uses a feature-finding algorithm on the AutoMS/MS or Iterative MS/MS data file. Feature finding is performed at the MS1 level, and MS/MS spectra are associated with each feature in a subsequent step. Only features with associated MS/MS spectra are included in the resulting feature tables. A minimum of four MS1 data points across a chromatographic peak is recommended for feature finding with Lipid Annotator. Therefore, the MS/MS acquisition parameters (acquisition rates and number of precursors per cycle) must be optimized to ensure a cycle time that meets this minimum requirement. With the chromatographic method and plasma sample used in this study, we observed the lipid chromatographic base peak widths to range from ~6 to 14 seconds, with an average peak width of ~8 seconds. Therefore, given the minimum observed peak width of six seconds, the MS/MS parameters were adjusted to yield a cycle time of 1.43 seconds. This ensured a minimum of four points across the narrowest chromatographic peaks.

Iterative MS/MS acquisition parameters were found to be important for lipidome annotation coverage, particularly in the case of lipid isomers. In this context, we define lipid isomers as cases where multiple annotated lipid features have the same sum composition (and same precursor *m/z*), but different retention times. However, in some of these cases, the MS/MS spectra provided further differentiation of isomers at the constituent level, for instance providing information on the esterified fatty acyl groups (for example, PC 18:2_18:2 versus PC 16:0_20:4). Analysis of plasma resulted in significant numbers of lipid isomers, where 164 out of 355 (positive mode), and 143 of 326 (negative mode) annotated lipids represented lipid isomers. To ensure lipid isomers are not missed in the workflow, the active exclusion window and the iterative RT exclusion tolerance must be set low enough so that closely eluting isomers with the same precursor mass have the opportunity to be triggered for MS/MS. Figure 8 demonstrates a typical scenario for a pair of closely eluting lysophosphatidylcholine (LPC) isomers with narrow peak widths.

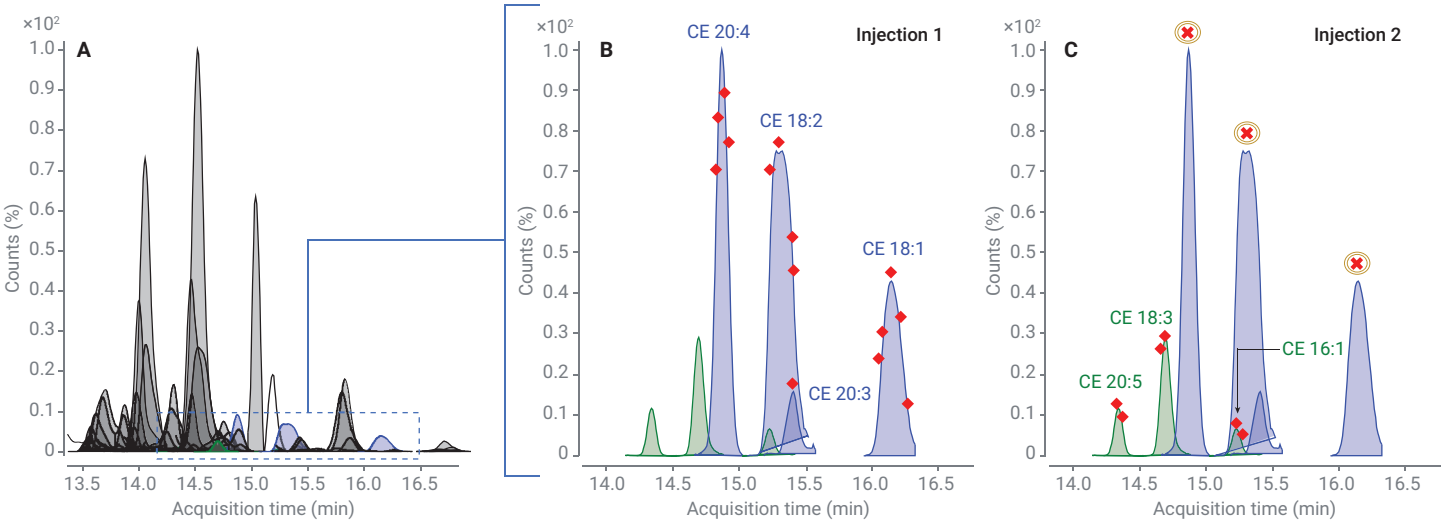


Figure 7. Increased cholesterol ester (CE) precursor selection with Iterative MS/MS from a plasma lipid extract. A) Overlaid extracted MS/MS chromatograms of a retention time region dense with annotated triacylglycerol (TG) lipids (black features). B) Four unique CE lipid precursors (blue features) were selected for fragmentation in the first injection. C) After exclusion of CEs of higher abundance (x symbols) and TGs (not shown), three more unique CE precursors of low abundance (green features) were selected in the subsequent injection. Red diamonds indicate MS1 scans in the chromatograms where the CE $[M+NH_4]^+$ precursors were selected for MS/MS.

Conclusion

This Application Note demonstrates that the Iterative MS/MS mode of LC/Q-TOF data acquisition provides a substantial benefit for improving lipid annotation of complex samples. When applied to a plasma lipid extract, the total number of annotated lipids increased significantly, and lipid classes of low abundance are enriched with Iterative MS/MS.

Lipid Annotator software provides the ability to leverage Iterative MS/MS data to quickly and automatically generate custom PCDL libraries with deep annotation coverage. These libraries with RT information are a critical component of an Agilent lipidomics software workflow that covers targeted and untargeted lipid profiling, from lipid annotation to differential analysis.

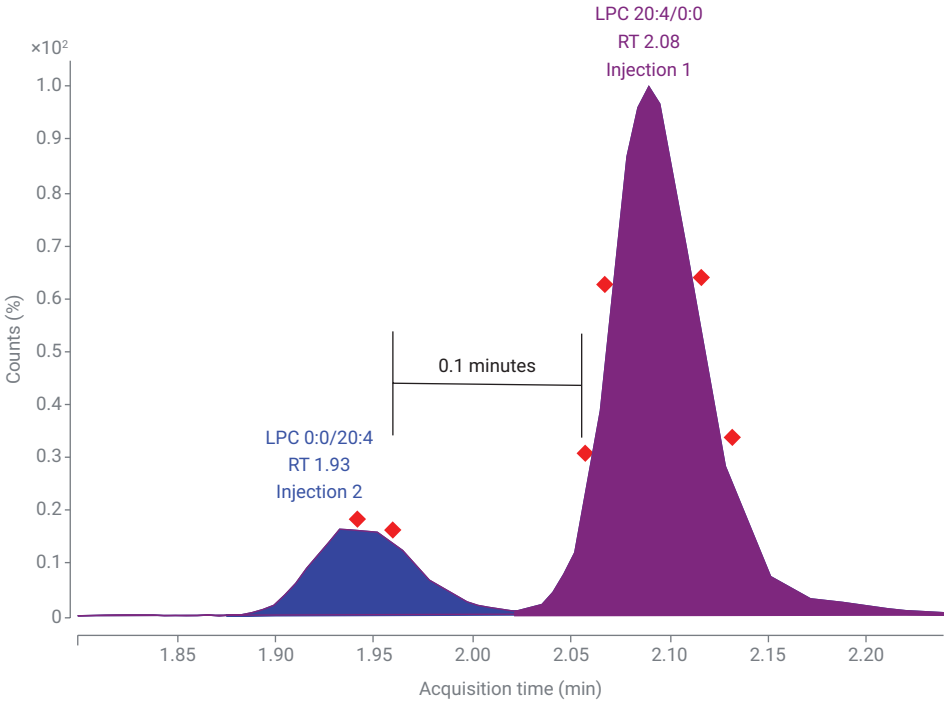


Figure 8. Iterative MS/MS parameters optimized for lipid isomers. Extracted ion chromatograms are shown for two lysophosphatidylcholine (LPC) 20:4 isomers annotated with Lipid Annotator software. The red diamonds indicate hypothetical MS1 scans where the LPC 20:4 $[M+H]^+$ precursor *m/z* 544.3398 is triggered for MS/MS. Setting the Iterative RT exclusion tolerance to ±0.1 minutes ensures that the same precursor *m/z* (±20 ppm) of the neighboring LPC isomer was picked for MS/MS in subsequent injections.

References

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