High Throughput, Data Independent Acquisition for Qualitative and Quantitative Shotgun Lipidomics

*Automating the Infusion MS/MS\textsuperscript{ALL} Workflow*

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Lipidomics has emerged as an essential component of global metabolic profiling and the biological importance of lipid metabolism in energy storage, cell membrane structure and signaling is well-documented. Changes in the abundance and distribution of lipids can closely correlate to progression of numerous diseases such as cancer, neurodegeneration, diabetes and other metabolic disorders. Mass spectrometry has emerged as the ideal approach for direct interrogation of the lipidome due to recent advances in software and hardware technology. The Infusion MS/MS\textsuperscript{ALL} workflow, a data independent acquisition (DIA) strategy, enables in-depth analysis of lipid molecular species in a high throughput qualitative and quantitative manner. Combined with automated sample introduction and dedicated data processing strategies, large sample sets can be analyzed in a high throughput, streamlined fashion. Post-acquisition data processing software such as LipidView™, MarkerView™ and MultiQuant™ Software, allows the user to identify and quantitate lipid species in complex samples to derive important biological conclusions.

**Key Features of Automated Infusion Workflow coupled with Data Independent Acquisition**

- Infusion MS/MS\textsuperscript{ALL} workflow acquires product ion spectra of all precursors within a specified mass range, providing a digital record of your sample (Figure 1).
- Complete digitization of your sample allows for retrospective data mining, removing the need to re-run samples in the future as your research needs change.
- Minimal set up is required to automate infusion using an HPLC system and a flow injection strategy.
- Automated infusion gives improved accuracy and %CV compared to manual infusion by a syringe pump, generating data comparable in quality to standard MRM quantitation methods.
- Identification and semi-quantitation from a discovery experiment using LipidView™ and MarkerView™ Software.
- Accurate quantitation for targeted analysis using MultiQuant™ Software.

**Figure 1. Infusion MSMS\textsuperscript{ALL} Workflow for Shotgun Lipidomics.** The precursor ions within the mass range are sequentially fragmented and all product ions are detected simultaneously within the 1 Da Q1 isolation window. This generates a 3D data set as shown in the contour plot (middle). The ordered collection of product ions from Q1 stepping across m/z 200 to 1200 (bottom left) can be visualized using PeakView® software and produces a high resolution MS/MS at every step.
Experimental Design

**Materials:** All solvents, methanol, acetonitrile, 2-propanol, dichloromethane and water were purchased as HPLC grade from EMD Millipore (Billerica, MA). Acetic acid and ammonium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO).

**Sample Preparation:** A total of 80 samples with variable treatments were collected. Lipids were extracted by following a standard Bligh and Dyer protocol with minor modifications. Lipid extracts were diluted 50 fold with a spray solvent containing 45% dichloromethane, 45% methanol, 10% water and 2 mM ammonium acetate.

**Automated Flow Injection Workflow (FIA):** Lipid extracts were analyzed using a microLC 200 system (SCIEX) and a flow injection analysis approach (Figure 2). The mobile phase consisted of dichloromethane/methanol (50/50 v/v) and 2 mM ammonium acetate and was initially pumped at a flow rate of 30 µL/min for 1.5 minutes for sample delivery. The flow rate was then reduced to 4 µL/min for data acquisition (4.5 min). Once data acquisition was complete the flow rate was increased to 30 µL/min for a fast wash of the sample loop and transfer line to reduce any carry-over. All post sample loop transfer lines were 50 µm ID PEEKsil tubing (IDEX). A 25 µm ID hybrid electrode was used in the DuoSpray™ Source for better spray stability and sensitivity (Figure 2). The total time required to obtain a comprehensive profile of the lipidome was approximately 7.5 minute per sample.

**Mass Spectrometry:** Lipid extracts were analyzed in both positive and negative ion modes for complete lipidome coverage using the TripleTOF® 5600+ System. Infusion MS/MS\textsuperscript{ALL} workflow experiments consisted of a TOF MS scan from m/z 200-1500 followed by a sequential acquisition of 1001 MS/MS spectra acquired from m/z 200.015 to 1200.051, with a step size of 1 Da.

**Data Analysis:** Batch processing of lipid identification, quantitation, and normalization with internal standards was performed using LipidView™ software 1.2. Multivariate statistical analyses including principal component analysis (PCA) and T-tests were conducted in MarkerView™ software 1.2 to find any differential features between the sample groups.

**Infusion MSMS\textsuperscript{ALL} Workflow**

A fully automated workflow was developed using the Infusion MS/MS\textsuperscript{ALL} data acquisition strategy on the TripleTOF® 5600+ system. The overall workflow provided a broad range of lipid identification and relative quantitation with high throughput and reproducibility. This workflow is a powerful approach for discovery quantitation. During the MS/MS\textsuperscript{ALL} experiment, a complete record of all precursors, product ions, and neutral losses within a sample was collected.

The data was acquired with high resolution (>30 000) and high mass accuracy (~2 ppm RMS). Data processing using LipidView™ Software identified 650-900 lipid species, covering diverse lipids classes (Figure 3), which included glycerophospholipids, cardiolipins, glycerolipids, sphingolipids and steroid lipids. The peak intensities for each identified lipid, across all samples were normalized against an internal standard from same lipid class. Both the identification and quantitation results were exported to MarkerView™ Software for multivariate data analysis.
Principal Component Analysis

The output of the normalized peak intensities for each lipid species from LipidView™ Software can be directly imported into MarkerView™ software for data visualization and statistical analysis. Principal component analysis (PCA) highlights the largest variation in the dataset and aids the identification of any features of interest (Figure 4, left). This indicates how much variability there is between samples within the same group and how much difference there is between different groups. Principal component variable grouping (PCVG) clusters ions sharing similar trends, which are color coded in the Loadings plot (Figure 4, right). There the key features that distinguish the sample groups can be found.

Relative Quantitation

Once data analysis is complete in MarkerView Software, a targeted list of lipid species can be built in MultiQuant™ Software for accurate quantitation. Here the lipid molecular species that had statistically significant concentration differences between samples (p < 0.01) were quantitated. MultiQuant Software was used for targeted quantitation based on the fragment ion intensities from the lipid species identified from their MS/MS spectra (Figure 4). For a complete study, the lipid identification needs only to be processed once and the targeted list in MultiQuant can be used repeatedly for numerous samples of the same matrix.

The quantitation results calculated for the samples correspond well to the results expected as a consequence of treatment. The quantitation method can be routinely applied to a set of targeted lipid species for a large number of clinical samples (n >5000). The powerful reporting features in MultiQuant Software provides a simple way to review, transfer, and manage results.
Conclusions

The Infusion MS/MS\textsuperscript{ALL} workflow on the TripleTOF\textsuperscript{®} 5600+ system is a novel data independent acquisition strategy for qualitative and quantitative molecular characterization of complex lipid samples. The 3D nature of the dataset enables post acquisition data analysis to determine molecular compositions, confident identifications and relative quantitation in a fast and simplified workflow. Coupled with the automated flow injection strategy described here, a high-throughput quantitative profiling for lipids is straightforward.

Figure 4. MarkerView\textsuperscript{™} Software for Data Review and Statistical Analysis. Identified, normalized quantitative lipid data can be exported from LipidView\textsuperscript{™} Software for further analysis. Importing into MarkerView Software retains individual lipid identification tags, facilitating data interpretation. (Left) The Scores plot generated after PCA analysis highlights any differences between groups of samples. (Right) The Loadings plot highlights the variables responsible for the differences between the groups in the scores plot. Here each variable is an individual lipid species that has been quantified across all the samples and labeled by the lipid class in this view.

Figure 5. Rapid, High-Throughput Lipid Profiling. The MS/MS fragment ion intensities of selected lipids from 80 samples were integrated and normalized against their class specific internal standards using MultiQuant\textsuperscript{™} Software. Each infusion takes 7.5 minutes, meaning the total analysis time for the sample set was 8-9 hours to perform 80 injections for each MS polarity.