MS/MS^{ALL} Workflow with optimized automated flow injection platform for lipidomics biomarker discovery

Qualitative and quantitative discovery via un-biased, un-targeted high throughput workflow

Leo Jinyuan Wang[1], Paul R.S. Baker[1], Clementina Mesaros2; Andrew J. Worth[2]; Ian A. Blair[2]
[1]SCIEX, USA, [2]Center of Excellence in Environmental Toxicology, Penn SRP Center, and Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, USA

MS-based lipidomics is mostly conducted either with chromatographic separation or by direct infusion-based shotgun lipidomics.[1,2] The shotgun infusion MSMS^{ALL} approach has several advantages over the commonly used chromatography-based data dependent analysis (DDA) MS method. It is quantitative at the MS/MS level and thus avoids the common problem in MS level quantification of coelution or overlapping isoelemental analytes. It has identical ionization conditions for all analytes and standards and is much less prone to carry-over.

Here, a robust shotgun lipidomics method using infusion MSMS^{ALL} Workflow with automated sample introduction by flow injection analysis (FIA) is described. The automation of the flow injection was realized with a standard HPLC equipment, thus eliminating the need for additional hardware. Excellent reproducibility and low carryover have been confirmed.

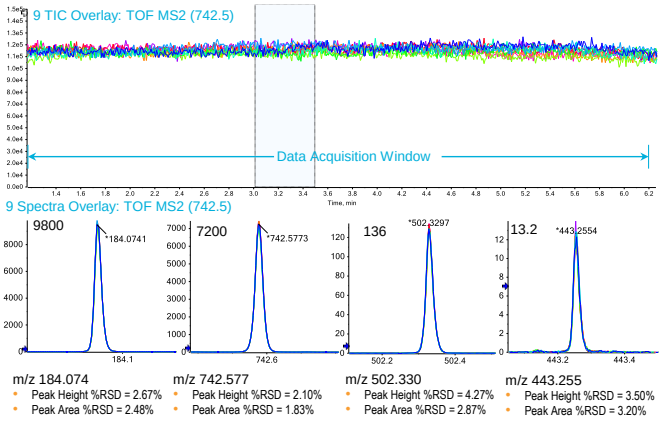

Figure 1. Data reproducibility from the workflow. (Top) TIC overlay of MS/MS m/z 742.5 for 9 repeated injections of BHE sample showed excellent total signal stability and reproducibility with the optimized FIA platform. (Bottom) MS/MS spectra extracted from the middle of the acquisition (zoomed in on 4 different fragment ions) show excellent run-to-run reproducibility for individual spectrum peaks. %CV for peak height was observed in the range from 2.1% to 4.27%. %CV for peak area was observed in the range from 1.83% to 3.20%.

To demonstrate the full workflow, the optimized FIA MS/MS^{ALL} acquisition method was used to quantify serum lipids across multiple samples. Using this quantitative, data-independent approach, lipid profiles were acquired, and lipid differences were identified.

Key features of MS/MS^{ALL} Workflow with optimized flow injection platform

- Optimized automated FIA platform for high throughput shotgun lipidomics
- Infusion MS/MS^{ALL} is a sequential acquisition of TOF MS/MS scans for each of the precursors within the lipidome mass range (200 ~ 2250 m/z)
  - Un-biased, un-targeted workflow for comprehensive coverage of the detectable/accessible lipidome in biological samples
- Quantitative data quality with excellent sensitivity, reproducibility, minimized carryover and long term robustness
- Seamless extraction of lipid profiles with LipidView™ Software followed by statistical analysis in MarkerView™ Software

Methods

Sample preparation: Bovine heart extract (BHE) was purchased from Avanti Polar Lipids and diluted in methylene chloride/methanol (50/50) with 5mM ammonium acetate for workflow development and performance evaluation. Serum samples were extracted with modified Folch method, and the organic phase was concentrated and reconstituted in autosampler vials with methylene chloride/methanol (50/50) and 5mM ammonium acetate for automated MS/MS^{ALL} flow injection analysis.

Automated FIA with MSMS^{ALL} Analysis: The automated FIA with infusion MSMS^{ALL} analysis was performed on a Shimadzu UHPLC system consisting of a binary high pressure mixing gradient pump with degasser, and a thermostated autosampler. The system was re-plumbed with PEEKsil tubing to minimize carryover. Increased flow rate was used during wash step to minimize cross sample contamination.

Table 1. LC method used for automated flow injection sample introduction. The mobile phase used was methylene chloride/methanol (50/50) with 5mM ammonium acetate. An injection volume of 50 µL was used.

Time (min)	Flow rate (µL/min)
0	*7*
8	*7*
8.01	*50*
12	*50*
12.01	*7*
15	*7*

Mass spectrometry: The TripleTOF® 6600 System was equipped with a DuoSpray™ Source plumbed with 50 µm I.D. hybrid electrodes (SCIEX). Data was acquired using the Infusion MS/MS^{ALL} acquisition mode, consisting of a TOF MS scan (5 sec) and series of MS/MS scans (300 msec) stepped across the lipidome mass range (200-2250 m/z). The Q1 isolation windows were optimized for lipid analyte mass defects and each MS/MS is acquired in high sensitivity mode. As seen in Figure 2, a 5.3 minute data acquisition window was established providing a stable and reproducible MS signal. Total run time was 15 mins, including wash and equilibration steps. The calibrant delivery system was used for automated mass calibration. Source conditions were optimized for both positive and negative mode operation (Spray voltage +4500V/-4200V, Temperature - 400 ºC, Curtain Gas - 25, Gas 1 - 18, Gas 2 - 30).

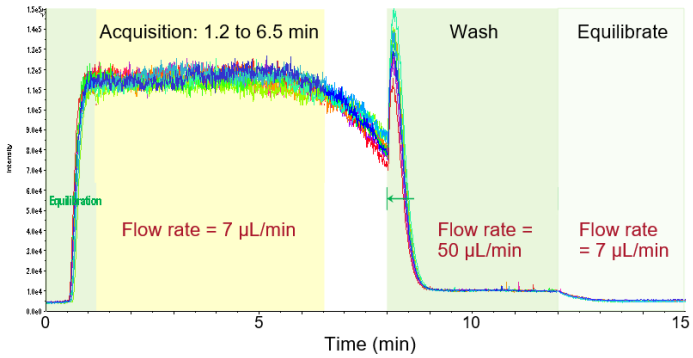

Figure 2. Chromatographic profile of FIA method. Overlaid eluting profiles with automated flow injection analysis shows reproducible stable signal with sufficient acquisition window.

Data Processing: All data was processed using LipidView Software 1.3, and then exported to MarkerView Software for principal component analysis.

Reproducibility of automated FIA workflow

The purpose of this study was to establish a reliable MSMS^{ALL} workflow with automated FIA platform to provide excellent reproducibility and with minimal carryover to ensure that the observed differences in biological samples is not due to analytical variances.

Repeat injections of the BHE sample were performed and the reproducibility of the acquired MS data was evaluated (Figure 1). MS/MS spectral peak heights and peak areas of nine repeated injections were compared across the m/z range and excellent reproducibility was observed even on the lower abundant fragment ions. The observed %RSD for peak height ranged from 2.1% to 4.27%, and the %RSD for peak area ranged from 1.83% to 3.20%.

Excellent reproducibility was observed not only with the acquired raw data, but also for the specific lipid profiles extracted using LipidView Software workflow. The lipid class profile for

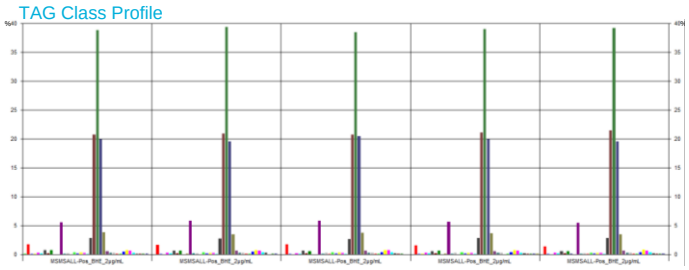

Figure 3. Excellent reproducibility in lipid profiles. Five replicate injections of BHE (2 µg/mL) were acquired with the automated FIA MS/MS^{ALL} workflow and data was processed with LipidView Software. The extracted lipid profiles from each of the replicate showed excellent reproducibility in processed results.

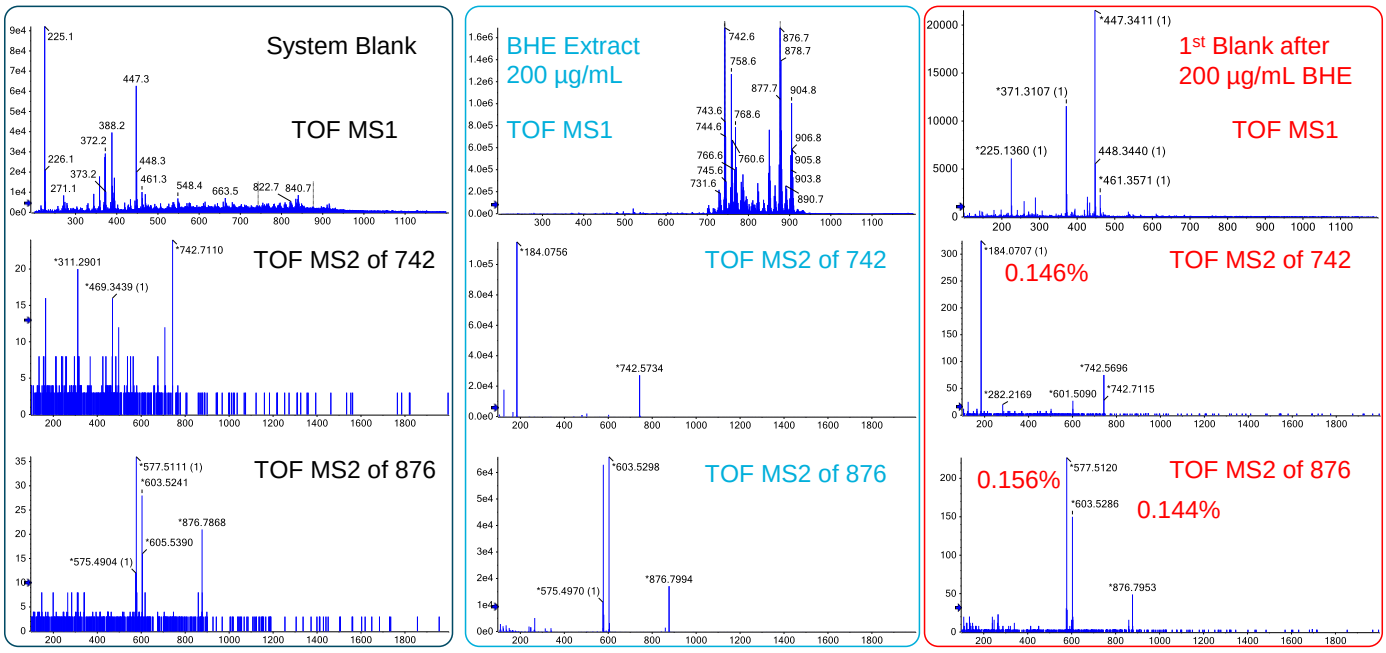

Figure 4. Minimized carryover with the optimized FIA workflow. The system background was first assessed (left) then a high concentration sample was injected (middle) followed by a mobile phase blank injection to calculate the carryover. Low system carryover was observed by comparing fragment ions from different MS/MS scans. Carry over was 0.146% for m/z 742→184; 0.158% for m/z 876 → 577 and 0.144% for m/z 876 → 603, highlighting the effective washing of the sample path.

triacylglycerol class (TAG, Figure 3) from 5 replicated assay of 2 µg/mL BHE showed minimal variation.

Minimizing carry-over

Carryover is one of the main challenges for lipid analysis, especially for lower level quantitation. Polar lipids tend to stick to plumbing components of the HPLC, which can cause false positive data or significantly decreased sensitivity.[3] PEEKsil tubing was used to replace all tubing on the HPLC system after the autosampler, including sample loop. The 50µm ID hybrid electrode was chosen as it is also PEEKsil and provides low backpressure. Carryover evaluation was performed by injecting blank mobile phase to establish system background level, then a high concentration BHE sample at 200 µg/mL, and then repeated injections of mobile phase. By comparing MS/MS spectra of the two most intense peaks from BHE, the carryover was observed at 0.146% ~ 0.156% and the results are shown in Figure 4. The carryover was further reduced to 0.025% and 0.061% on the third mobile phase injection.

Powerful data extraction strategies

MS/MS^{ALL} data is a complete digital record of the samples which has full detailed information for both MS and MS/MS levels (Figure 5, left). All data collected is at high resolution and with high mass accuracy providing additional confidence and specificity. Therefore, it can be interrogated in silico to mimic the classic “shotgun” lipidomics approaches, i.e. multiple precursor ion scan (MPIS) and neutral loss scans (NL). This automated data processing and lipid profiling can be performed with LipidView Software.

As seen in Figure 5 (right), extraction of the m/z 184 fragment ion yields an *in silico* precursor ion scan of the phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyelin (SM) molecular species. Likewise, an in silico neutral loss of m/z 141 highlights the phophatidylethanolamine (PE) molecular species in the sample.

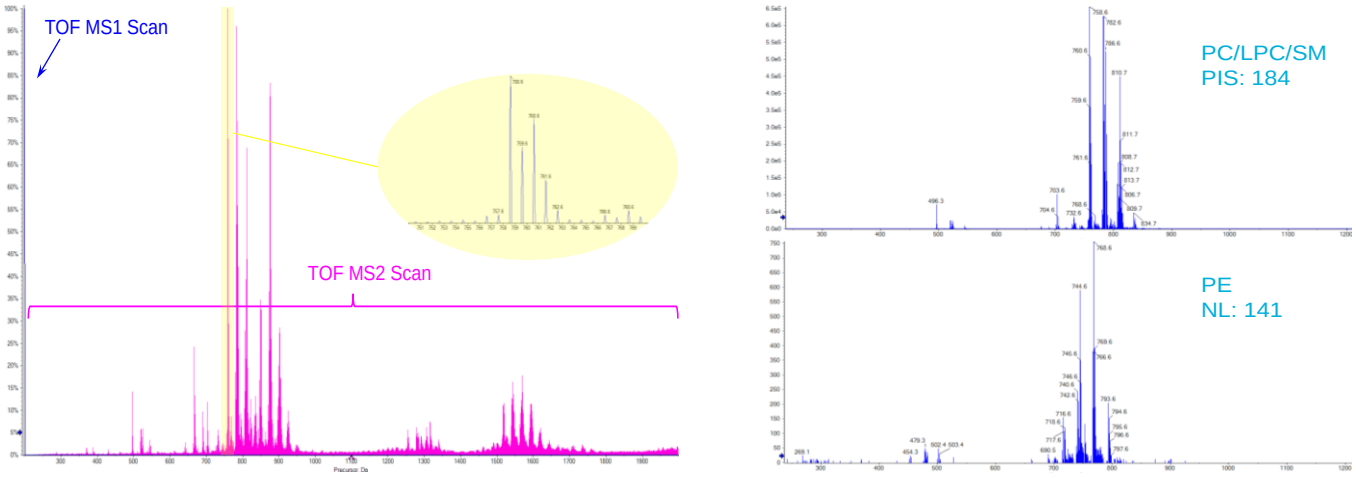

Figure 5. MS/MS^{ALL} Data is a Complete Digital Record of the Lipidome for a Biological Sample. (Left) The blue trace shows the single occurrence of a high resolution TOF MS scan to capture all precursors in the sample. The pink traces are the composite MS/MS fragment ion scans of each precursor m/z. Data can be extracted *in silico* using a variety of approaches. (Right, top) Extraction of data in a precursor ion scan mode using fragment ion m/z 184 m/z shows all PC/LPC/SM species in the sample. (Right, bottom) Extraction of data in a neutral loss scan mode (m/z 141 shows all PE species in the sample.

Automated FIA MS/MS^{ALL} Workflow for biomarker discovery

The MS/MS^{ALL} workflow with automated FIA platform was used to analyze serum lipids in subject and control samples for biomarker discovery (disease classification was performed by accepted clinical techniques). Three groups of samples (disease – 6 replicates, Control – 6 reps, and extraction blanks – 2 reps) were analyzed then the data was processed by LipidView Software to extract the lipid profiles. Then the original or corrected information can be exported to MarkerView Software for principal component analysis to identify differences in the lipid molecular species composition between samples. First, the three different groups were clearly differentiated by principle component analysis (PCA) grouping, as seen in Figure 6 (left) Scores plot. The first principal component (PC1) is the primary differentiating factor to separate extraction blanks and real biological samples and PC2 is the primary factor separating diseased and control samples.

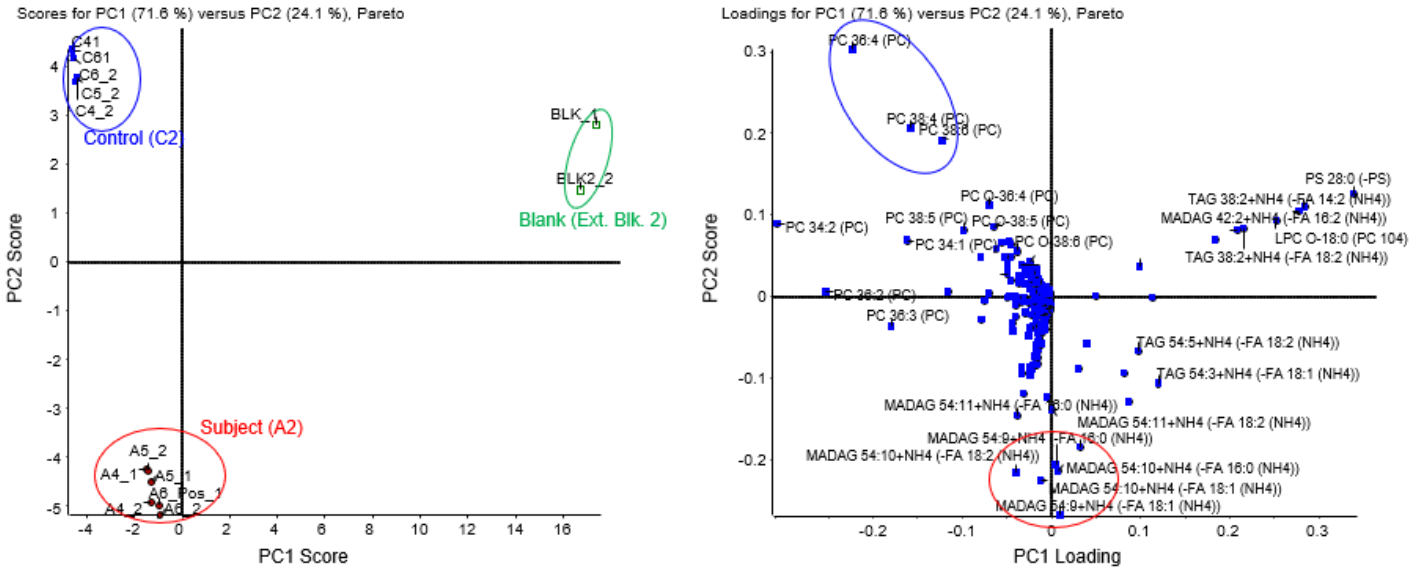

Figure 6. PCA clearly separates sample groups To identify differentiating features. The PCA Scores plot (left) clearly identifies principal component 1 (PC1, x-axis) as the primary differentiating factor between extraction blank and biological samples. Control and subject samples clearly separate by PC2, plotted on the y-axis. Using the Loadings plot, the lipid species responsible for this differentiation between disease and control can be visualized.

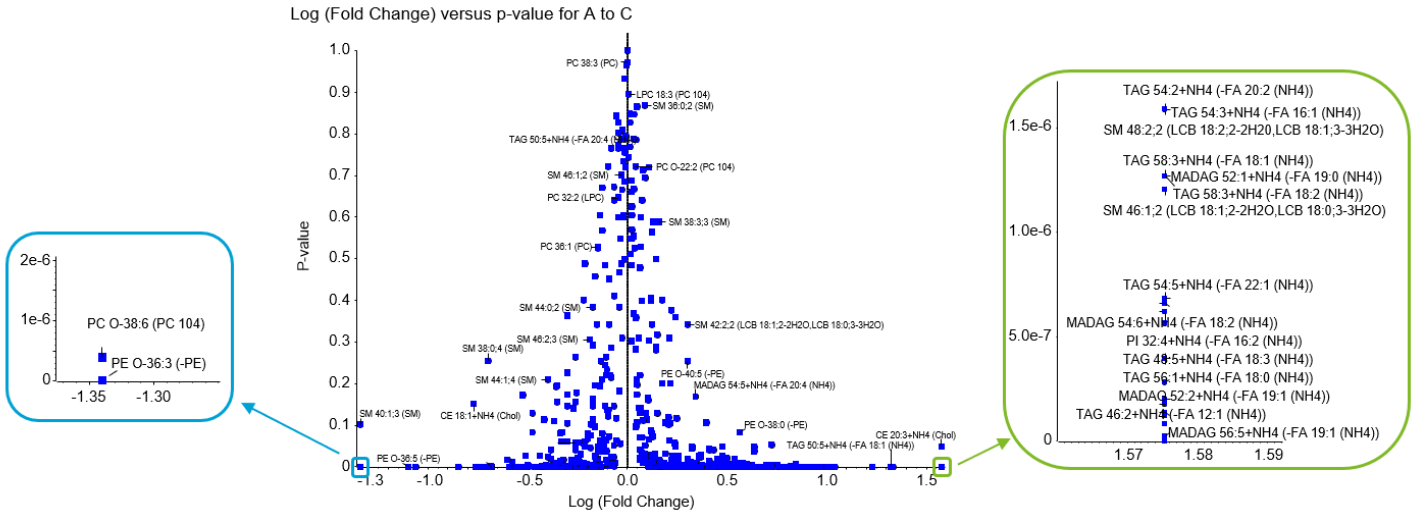

Figure 7. T-test To visualize most significant lipid changes. The t-test plots fold change versus p-value such that the most significant differences between two groups (disease / control) can be easily visualized. The blue and red insets show the lipid species with greatest negative and positive changes respectively.

With the group clearly differentiated, the Loadings plot can be used to determine the lipid species responsible for the differentiation between groups. Knowing that PC2 is the main contributor to separate diseased and control subject, the lipid species with high PC2 scores (Figure 6, right) will be the ones showing the most differences between groups. These species will be the most interesting for further investigation as possible disease biomarkers.

Another statistical analysis tool available in MarkerView Software is the “t test”. Plotting the disease / control fold change of each lipid species vs the p-value, the lipid species with the most significant differences can be easily visualized (Figure 7).

Summary

FIA MS/MS^{ALL} is a seamless workflow for lipidomics biomarker discovery and quantitation providing an automated, untargeted MS acquisition strategy. Here, flow injection analysis was optimized to provide automated infusion with low carry over and excellent reproducibility. Comprehensive data collection provides a sample set that can be processed with LipidView Software, extracting detailed lipid profiles automatically. Data is easily exported to MarkerView Software for statistical analysis and visualization to determine the lipid profiles that are changing across the sample set.

The workflow was applied to the biomarker discovery study with lipid extracts taken from diseased and control samples and significant lipid differences were observed. These will be investigated further in future studies. While this is a small pilot study to demonstrate the workflow, it highlights how this full lipid profiling solution could be used to perform larger biomarker discovery experiments on larger sample sets.

References

1. Han, X.; Gross, R. W., Shotgun lipidomics: Electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrometry Reviews* (2005) **24 (3)**, 367-412
2. Wenk, M. R., The emerging field of lipidomics. *Nat Rev Drug Discov.* (2005) **4 (7)**, 594-610.
3. Karlsson, A. Å., Analysis of intact polar lipids by high-pressure liquid chromatography—mass spectrometry/tandem mass spectrometry with use of thermospray or atmospheric pressure ionization. In Lipid Analysis in Oils and Fats, Hamilton, R. J., Ed. Springer US: (1998), 290-316.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices