# A Direct-Infusion MS/MS$^{ALL}$ Approach for High-Throughput Untargeted Metabolomics

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## ABSTRACT

Powerful analytical tools are required for metabolomics analysis, to study the complexity and the large variety of metabolites from various metabolic pathways in biological systems. One of the main goal of researchers in the field of untargeted metabolomics is to analyze a large number of samples and obtain the most information in the shortest time with limited sample preparation. Mass spectrometry-based untargeted metabolomics requires analytical approaches to analyze metabolites in an unbiased fashion. However current LC approaches create some bias based on the column chemistry, choice of column effects which metabolites can be measured based on which can be retained and separated on the columns.

Here the utility of the direct infusion or "shotgun" approach for high-throughput metabolomic profiling analyses is demonstrated, using the Infusion MS/MS$^{ALL}$ Acquisition[1] approach in conjunction with fast sample introduction by flow injection analysis (FIA) on two groups of human urine samples. This data-independent (DIA/SWATH) technique is already well established in the field of lipidomics[2-4] and allows quantitation and direct structural identification based on MS and MS/MS acquisition of all ionizable and detectable analytes.

## INTRODUCTION

Identifying the changes in metabolic pathways is a great challenge due to the complexity of metabolome in biological samples. Several techniques are used for metabolomics analysis of biological samples which require long analysis time. Infusion by FIA using using an electrospray source is a simple strategy to maximize analytical throughput and yet allow analyte discrimination in complex samples by using an untargeted approach.

By using Infusion MS/MS$^{ALL}$ acquisition, a rapid metabolic fingerprint of each biological sample can be captured. By optimizing a method with optimized DIA mass window ranges, one method was developed to capture all relevant metabolites per ionization mode (positive and negative mode). This method was used for a urine extract case study which highlighted the metabolites responsible for differentiating the different sample groups. Mass ranges were evaluated from 50 to 1000 with different m/z windows sizes to optimize the number of MS/MS per cycle and the time needed to collect the data. Data was also collected for a small case study of a cell line extract. Metabolites were extracted from several biological samples and directly infused by using the developed method. The total ion chromatogram showed the presence of a large number of metabolite peaks. The extracted ion chromatograms showed different metabolites, which are characteristic for each sample. "Shotgun" approach for metabolomics has proved to be a powerful and rapid tool to obtain huge and complementary data of numerous samples even if metabolites present only at very low levels in biological samples. Post-acquisition data processing software allows the user to rapidly profile metabolites occurring in complex samples and perform statistical analysis to derive important biological conclusions.

## MATERIALS AND METHODS

**Sample Preparation:**100µL of urine were mixed with 400µL of acetonitrile followed by vortexing and then centrifugation at 14000 rpm for 20 min at room temperature. After centrifugation, the supernatant was collected and dried, and then dissolved in 150µL of buffer (Isopropanol:Water; 60:40 (v/v), 1mM Ammonium hydroxide pH 9, for negative mode; and Methanol:Water 60:40 (v/v), 0.1% Formic acid pH 3, for positive mode). Samples were further diluted 1:10 and 1:5 (v/v) for positive and negative modes, respectively.

**Methods:** Data were acquired using a TripleTOF® 6600 System (SCIEX) equipped with 65µm ESI probe and coupled to a ExionLC™ system (SCIEX), using Infusion MS/MS$^{ALL}$ Acquisition method to collect one survey scan and then MS/MS at every mass (Unit wide Q1 isolation window is stepped across mass range in 1 Da steps). Autosampler injected full sample loop volume at isocratic gradient. Eluents were: Isopropanol:Water; 60:40 (v/v), 1mM Ammonium hydroxide pH 9, for negative mode; and Methanol:Water 60:40 (v/v), 0.1% Formic acid pH 3, for positive mode. The optimized flow gradient was: 0.25min, 0.02µL; 0.26min, 0.007µL; 5min, 0.007µL; 5.1min, 0.09µL; 7min, 0.09µL (Figure 1). The injection volume was set to 50 µL. Different mass ranges were evaluated to optimize the number of MS/MS and time needed to collect the data. The useful mass range was found to be 50-900 m/z range. Data were processed using MasterView™ Software and the accurate mass metabolomics spectral library were used for fast metabolite identification and confirmation. All statistical analysis were done completed in MarkerView™ Software (SCIEX).

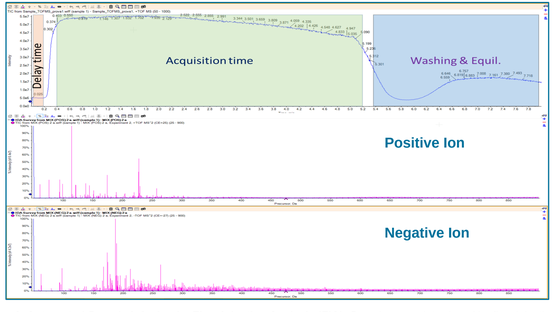


**Figure 1: Automated Sample Infusion by Flow Injection Analysis (FIA):** Samples were introduced by flow injection, the solvent flow profile and tubing configuration was set up such that the elution profile of the sample (top panel) allowed for 5 minutes of constant sample spray after a delay time of 0.4 minutes. The time between 0.5 min and 5 minutes can be used for the collection of MS/MS$^{ALL}$ data. After the acquisition time, the flow rate is increased to flush out remaining sample. The middle and bottom panel show the TICs of the MS/MS scans in positive and in negative mode respectively.

## RESULTS

Infusion MS/MS$^{ALL}$ is a data-independent mode of product ion acquisition from all ionizable precursors in the sample. Here a 1Da Q1 isolation window is stepped across the full precursor mass range and a full scan MS/MS is collected at every mass. This provides a digital map of the sample where the datafile that contains the MS and MS/MS data on every detectable species (Figure 2).

As the window size used in Infusion MS/MS$^{ALL}$ is set to Unit (isolation of 0.7 amu centered at a Q1-mass), the mass defect of the relevant metabolites must also be considered. To do this, the mass defects of a range of relevant metabolites were plotted against the respective precursor masses and a regression equation was determined. The table in Figure 3, lists a range of tested metabolites with their mass defects, which ranges between 0.037 - 0.105 amu.

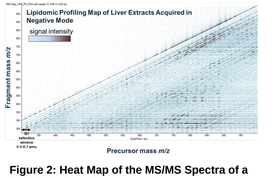


**Figure 2: Heat Map of the MS/MS Spectra of a MS/MS$^{ALL}$ Run.** The fragment masses are plotted agains the precursor masses.

| Metabolites | Exact Mass | Nominal Mass | Defect Mass |
|---|---|---|---|
| Trimethylamine | 60.079 | 60 | 0.079 |
| Urea | 61.037 | 61 | 0.037 |
| Trimethylamine-N-oxide | 76.074 | 76 | 0.074 |
| Alanine | 90.055 | 90 | 0.055 |
| Glycerol | 93.051 | 93 | 0.051 |
| Choline | 104.105 | 104 | 0.105 |
| Serine | 106.054 | 106 | 0.054 |
| Creatinine | 114.064 | 114 | 0.064 |
| Proline | 116.075 | 116 | 0.075 |
| Valine | 118.082 | 118 | 0.082 |
| Threonine | 120.069 | 120 | 0.069 |
| Cysteine | 122.031 | 122 | 0.031 |
| Taurine | 126.025 | 126 | 0.025 |
| Pipecolic acid | 130.098 | 130 | 0.098 |
| Creatine | 132.074 | 132 | 0.074 |

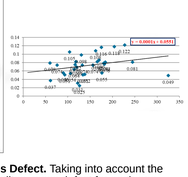


**Figure 3: Calculating the Mass Defect.** Taking into account the most common reference metabolites, mass defect for each one was obtained and a regression equation was calculated and used to develop the list of precursor ion for this MS/MS$^{ALL}$ method.

**Direct Identification of Metabolites from of MS/MS$^{ALL}$ Data in PeakView® Software:** Although accurate mass measurements of the precursor masses may provide formula assignment, this is often not sufficient for compound identification. Isomerism or assignment of multiple formulas despite very high mass accuracy and resolution make compound identification based on the MS accurate mass measurement alone impossible. In contrast, MS/MS$^{ALL}$ data provides MS/MS fragmentation information of the precursor ions, allowing for a more confident identification of analytes. As shown in the Table in Figure 4, each Q1 window used for fragmentation is listed and clicking on the entry shows the associated product ion spectrum., for which three examples are shown in the remaining panels of Figure 4.

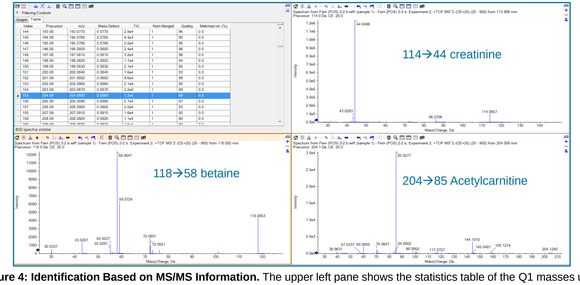


**Figure 4: Identification Based on MS/MS Information.** The upper left pane shows the statistics table of the Q1 masses used for fragmentation in the MS/MS scans. The remaining panels show three example spectra of identified metabolites: creatinine, betaine and acetylcarnitine.

**Screening MSMS$^{ALL}$ Data for Compound Classes:** A common approach for screening of compound classes is the precursor ion/neutral loss scan (PIS/NLS), where the untargeted MS/MS$^{ALL}$ data is probed for common neutral losses or class specific fragments in a semi-targeted fashion[6]. Using the fragment finder tool of PeakView® software (Figure 5), the fragmentation data is easily screened for functional. In this way, metabolite classes occurring in the different samples can be identified on the basis of their diagnostic fragment peaks and/or neutral loss.

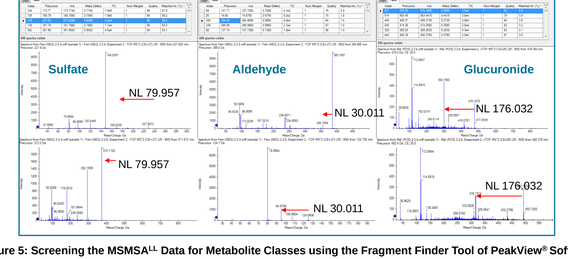


**Figure 5: Screening the MSMS$^{ALL}$ Data for Metabolite Classes using the Fragment Finder Tool of PeakView® Software.** For each neutral loss, two examples are shown. Left panels - sulfates (NL 79.9); Middle panels - aldehydes (NL 39.1); Right panels - glucoronides (NL 176.03).

**Semi-Quantitative Comparison between Sample Groups Analyzed by MS/MS$^{ALL}$:** One of the advantages of the direct infusion approach is the constant signal from the sample as well as the constant composition of the mobile phase. This allows the peak-intensity based quantitation at the MS as well as the MS/MS level with identical solvent composition for all analytes. The metabolic profile of urine from two sample groups was compared. The peak intensities were directly imported into MarkerView™ software for analysis by Partial Least Squares Discriminant Analysis (PLS-DA). The data showed that the urine from the two samples groups (male vs. female) are readily separated along the D1-dimension, with the pooled QC samples located between both groups. Notably, the clustering of the intra-individual technical replicates appears to be tighter than the intra-group clustering of the individual samples. This result shows the utility of this shotgun MS/MS$^{ALL}$ approach for metabolomic profiling.

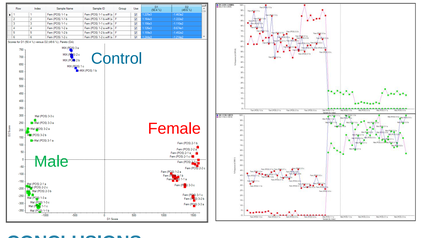


**Figure 6: PLS-DA Plot of the Urine Samples.** (Left) Scores plot showing the clustering of samples; Green - Male; Blue - Mixed; Red – Female. This indicates the very good technical repeatability reproducibility of the method. (Right) Selected peak profiles are plotted showing observed changes.

## CONCLUSIONS

Here the utility of the MS/MS$^{ALL}$ approach for untargeted metabolomic profiling is demonstrated. To our knowledge, prior approaches utilized high resolution MS scans only for FIA based metabolomic profiling[5]. However, isomerism and ambiguity in compound identification based on only the accurate precursor mass is insufficient for successful metabolite identification. Here this shotgun direct infusion approach is combined with the data independent acquisition strategy of acquiring MS/MS from all precursors, which allows for more confident identification of analytes and quantitation at the MS and MS/MS level. The short run times and automated FIA-based sample infusion makes this work flow ideally suited for high-throughput studies where a fast and semi-quantitative approach for metabolomic profiling with the requirement for direct compound identification is called for.

## REFERENCES

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