

SWATH[®] Acquisition Allows a Deeper Level of Comprehensive Metabolite Quantitation

A Data Independent Acquisition (DIA) Technique on the TripleTOF[®] 6600 System

Zuzana Demianova
SCIEX, Germany

The field of metabolomics and metabolic profiling faces a large challenge, in accurately identifying and quantifying hundreds to thousands of metabolites in a single run. Generally, quantitative metabolomics is performed on triple quadrupole or QTRAP[®] systems in a targeted manner by multiple reaction monitoring (MRM) for enhanced sensitivity and selectivity. Internal standards are often used to enhance quantitative accuracy. SWATH acquisition, a data independent acquisition (DIA) technique, is well adopted in quantitative proteomics¹, but still not commonly used in quantitative profiling of metabolome.

Variable window SWATH acquisition has been shown to identify a higher number of metabolites compared to the traditional data dependent acquisition (DDA) approach, thus enabling broader metabolome coverage². Here, SWATH acquisition is used for quantitation of selected metabolites using the MS/MS data, for reduced interferences, improved signal-to-noise and deeper

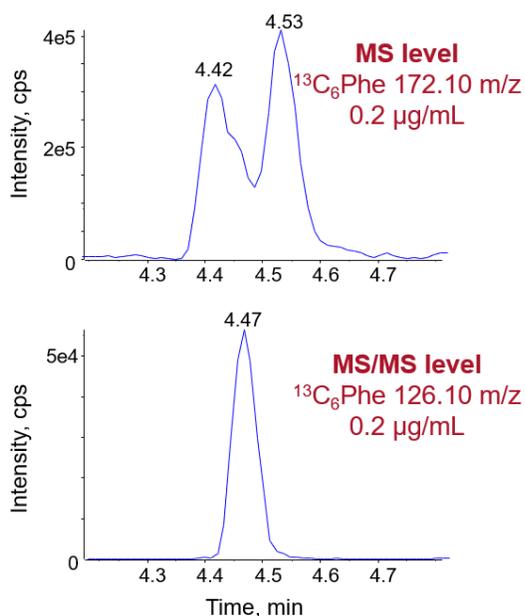


Figure 1. Significant Reduction in Interferences / Background using MS/MS-Based Metabolite Quantitation. Extracted ion chromatograms for the L-[¹³C₆]-Phe precursor ion (top) and its fragment ion (bottom) at 0.2 $\mu\text{g/mL}$ concentration in urine matrix. Interference in the MS data is not observed in the MS/MS data, providing a clean peak and more accurate quantitation, especially at low concentrations.

metabolite quantitation (Figure 1). The use of MS/MS fragments for metabolite quantitation provides better selectivity, and ultimately increased sensitivity compared to simply relying on the precursor ion for quantitation. A SWATH acquisition map contains MS and MS/MS information of every detectable metabolite in the sample and is therefore a digital archive of the sample. This reduces the need to go back and re-run samples; data can just be re-mined as the hypothesis evolves.

Benefits of SWATH Acquisition for Quantitative Metabolomics

- No prior MS method development
- Increased specificity with variable Q1 isolation windows for SWATH acquisition
- Use of MS/MS fragment data for quantitation provides
 - Less interference from co-eluting metabolites
 - Better Signal to Noise (S/N) and improved quantitation
- Structural information of metabolites from their fragmentation pattern for confirming identity
- Permanent digital map of the metabolome of each sample

Methods

Sample Preparation: Human urine was processed according to standard extraction protocols³. Urine was diluted with water at a ratio of 1:4 (v/v) and centrifuged for prior analysis. For accurate quantitation of amino acids in urine sample, the Metabolomics QC kit with mixture of a stable isotope-labeled standard metabolites (Cambridge Isotope Laboratories Inc., USA) were spiked into the matrix. To create the calibration curve, 50 μL of urine matrix was spiked with various concentrations of [^{13}C]-metabolites; their concentrations are listed Table 1.

Table 1. Various Concentration Levels [^{13}C]-Metabolites Were Spiked into the Urine Matrix. Metabolite QC kit was spiked at four different concentration levels into 50 μL of urine and diluted 1:4 with water.

Amino Acid	Concentration ($\mu\text{g/mL}$)				
	Blank	1	2	3	4
L- $^{13}\text{C}_6$ -Leu	0	0.2	0.4	2	4
L- $^{13}\text{C}_6$ -Phe	0	0.2	0.4	2	4
L- $^{13}\text{C}_6$ -Tyr	0	0.2	0.4	2	4
L- $^{13}\text{C}_{11}$ -Trp*	0	2	4	20	40

*Note the standard mixture was premixed with 10x higher [Tryptophan], resulting in some signal saturation.

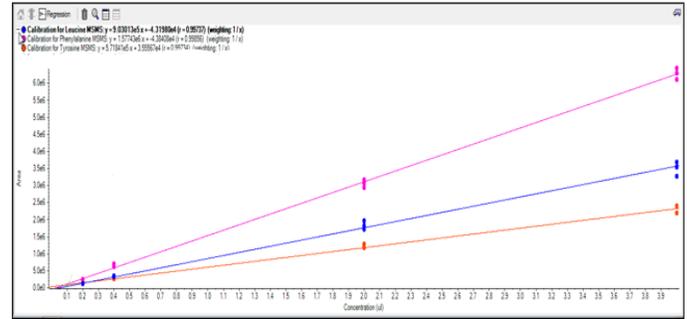
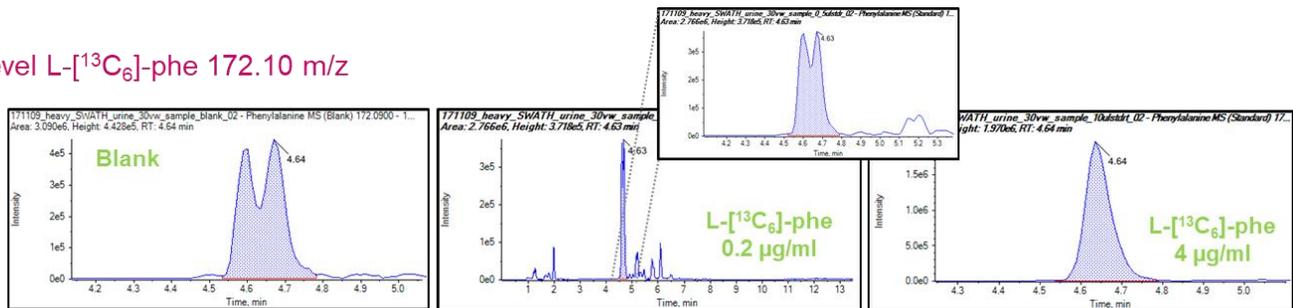


Figure 3: Calibration Curves of [^{13}C]-Metabolites Spiked into Urine Matrix for Accurate Quantitation of Metabolites. Orange curve: L- $^{13}\text{C}_6$ -tyrosine, blue curve: L- $^{13}\text{C}_6$ -leucine and pink curve: L- $^{13}\text{C}_6$ -phenylalanine. All [^{13}C]-metabolite calibration curves were measured with correlation coefficients of >0.98 (at the MS level) and >0.99 (at the MS/MS level).

Chromatography: Separation was performed on an ExionLCTM AD system using an Acquity UPLC BEH C18 column (100mm x 2.1 mm ID, 1.7 μm - Waters, USA) using a flow rate 200 $\mu\text{L}/\text{min}$. A gradient was employed from 1-10 minutes from 2-98% of 0.1% formic acid in acetonitrile, total length of LC separation was 14 minutes and column oven was set to 40 $^\circ\text{C}$. Injection volume was 5 μL , measurements were run in triplicates.

MS level L- $^{13}\text{C}_6$ -phe 172.10 m/z



MS/MS level L- $^{13}\text{C}_6$ -phe 126.10 m/z

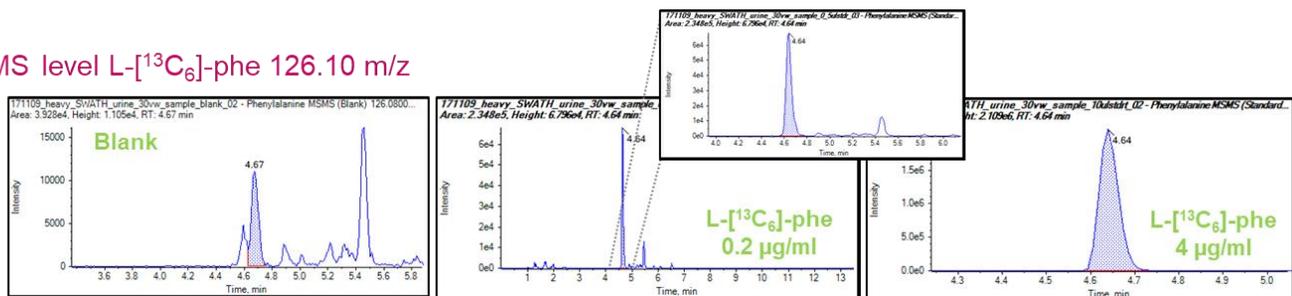


Figure 2: Interferences from Metabolites of the Same Feature can Compromise Accurate Quantitation. From the top panel is clearly visible that urine sample has a contaminant in the blank (left) that interferes with the same features as L- $^{13}\text{C}_6$ -phenylalanine ($[\text{MH}^+]$ 172.10 m/z). Increasing the amount of the analyte results in peaks merging (top right). Interfering masses do not have the same fragmentation masses and therefore the L- $^{13}\text{C}_6$ -phenylalanine fragment (MS/MS fragment 126.10 m/z) is a good candidate to quantify endogenous phenylalanine in the complex urine matrix with no interferences.

Mass Spectrometry: Data was collected using a TripleTOF® 6600 system using the follow source conditions: Curtain Gas 35, GS1 40, GS2 40, ISVF 5500 V, Source temp. 600°C, Declustering Potential 80 V. SWATH acquisition method has 30 variable windows⁵ and windows sizes were calculated from the MS information from urine sample using SWATH Acquisition Variable Window Calculator⁴. The data was collected in positive mode in MS range 70-500 m/z and in MS/MS 50-500 m/z with accumulation time of 50 msec and 18 msec in MS/MS for a cycle time of 0.64 sec. High sensitivity MS/MS mode was used with a collision energy for 30 V with 15 V spread.

Data Processing: Data were processed using SCIEX OS Software 1.4. with the following settings: MQ4 with 0.5 point as smooth width, minimum peak width 30 msec and minimum heights 100 cps; integration parameters: noise percentage 95%; baseline subtraction of 0.5 min and peak splitting with 2 points. Linear regression with 1/x weighting was used to create external calibration based on areas of heavy-labelled amino acids. This calibration was used to obtain concentration of selected amino acids in the urine matrix.

Results

First, the quantitation quality obtained from SWATH acquisition using the 30-variable windows approach was assessed in the urine matrix, using both the precursor masses of the spiked L-¹³C-metabolites as well as the fragments for accurate metabolite quantitation. Figure 2 shows the extracted ion chromatograms for the stable isotope-labeled phenylalanine. The top panel shows an XIC of L-¹³C-phenylalanine precursor at the MS level, the panels show that the urine matrix contains an interference at the same mass that make peak assignment

difficult. These interfering peaks were merging with the desired peak at every concentration of L-¹³C-phenylalanine standard, which resulted an incorrect quantification of amino acid in the urine matrix. To correctly assign a peak to the metabolite of interest, the MS/MS fragment of L-¹³C-phenylalanine precursor was used for an accurate quantitation of metabolite. Identification and quantification of metabolites on the MS/MS level significantly improves the signal to noise ratio and limits the interferences from other analytes in the matrix (Figure 2, bottom).

Next, accurate quantification of selected amino acids within a complex matrix was evaluated. Figure 3 presents calibration curves for select stable isotope-labeled amino acids were acquired within a complex matrix; correlation coefficients of >0.98 (at the MS level) and >0.99 (at the MS/MS level) were observed. In the MS/MS data, the blank shows very low noise, at the higher concentration the signal/noise ratio for L-¹³C-leucine peak is significantly increased vs the MS data (Figure 4). It is clear, that using the MS/MS spectrum for relative or accurate quantification of metabolites will have a lower noise background and thus lower limits of quantitation could be reached. Another example of enhanced S/N in the MS/MS XICs as compared to the MS data is shown in Figure 5 for L-¹³C-leucine.

The different benefits of SWATH acquisition have been highlighted here. The MS/MS information can be used not only for identification, but also for accurate quantification of metabolites in complex sample matrices. Having the MS/MS information of all detectable metabolites gives a better chance to also identify unknown metabolites and their structures. SWATH acquisition can be also used for relative quantitation of untargeted metabolomics.

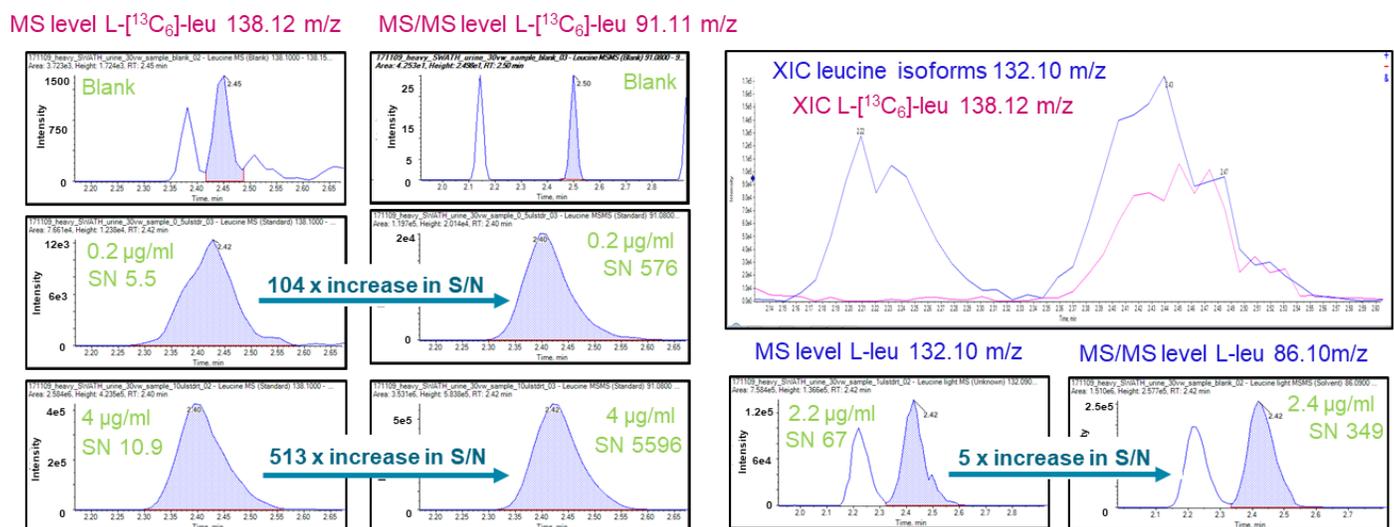


Figure 4: Accurate Quantitation of L-Leucine Based on L-¹³C₆-Leucine Measured Calibration Curve on MS and MS/MS Level. (Left) L-¹³C₆-leucine peaks from different calibration points are shown for the MS and MS/MS data, providing higher S/N ratios for the MS/MS data. (Top right) XIC of L-leucine and L-¹³C₆-leucine in urine. (Bottom right) Accurate quantitative of L-leucine shows improved signal to noise ratio on the MS/MS level.

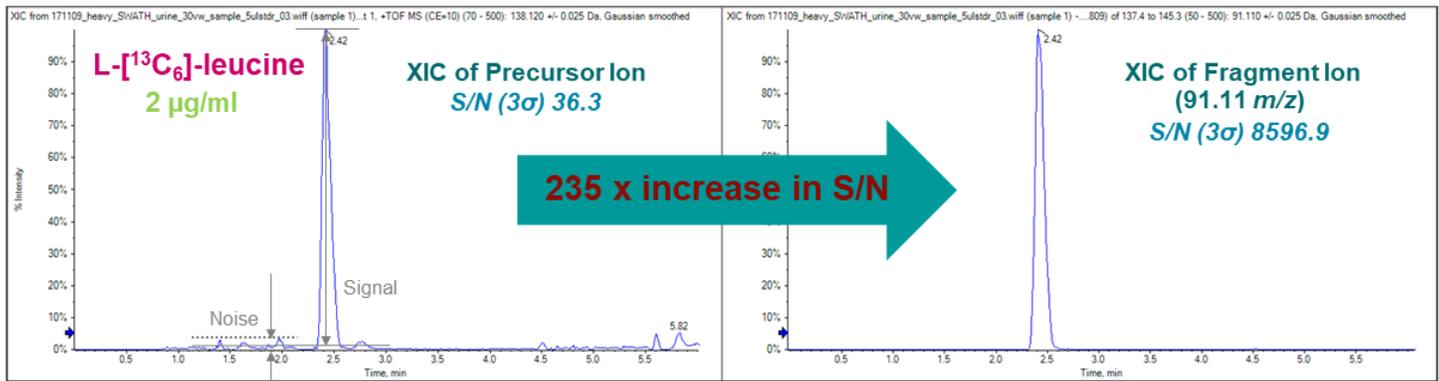


Figure 5. Significant Increase in Signal-to-Noise Improvement for Metabolite Quantitation. Shows the extracted ion chromatograms of the L-[¹³C₆]-leucine precursor ion and its fragment. Quantitative results show a significant increase of signal to noise ratio (>200) versus the precursor ion (left).

Note this same SWATH acquisition dataset was processed using the Accurate Mass Metabolite Spectral Library (AMMSL) and a non-targeted workflow². This further highlights that one can re-mine the SWATH acquisition data in different ways, as the project/study hypothesis changes.

Conclusions

Variable window SWATH Acquisition provides good quality quantitative data for metabolites in complex matrices. Using the full scan MS/MS data provides both confidence in identification and quantitation data that is less prone to issues with interferences.

- SWATH acquisition measures MS and MS/MS spectra of every detectable metabolite in the sample, providing a digital archive of the sample that can be easily re-mined.
- Due to many coeluting species in complex matrices, using only the MS spectrum and retention time is often not sufficient for metabolite identification. MS/MS information is necessary to obtain further structural knowledge about the metabolite.
- MS/MS quantitation of metabolites often leads to lower detection limits due to significantly improved signal to noise ratios vs MS data. Measuring the whole MS/MS spectrum allows selection of the best fragments for metabolite quantitation.
- SCIEX OS software combines comprehensive qualitative and quantitative data analysis, making data processing easier and more efficient.

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