Targeted metabolomics in rat urine using Zeno MS/MS

Analysis on the SCIEX ZenoTOF 7600 system

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Often researchers in academic labs and those performing clinical trials are only given very small amounts of sample, and need to get the most information from the modest volume. This requirement drives the need for targeted assays with high sensitivity and specificity, to obtain high-quality, quantitative results in complex biological samples and to deliver biological insight. High-resolution accurate mass (HRMS) systems have excellent utility in untargeted metabolomics and compound identification in complex matrices because of their high specificity and full-scan MS/MS quality. However, HRMS instruments typically have not been the chosen instrument to perform accurate quantification of a targeted panel of analytes because of their lower sensitivity. In addition, some high resolution platforms cannot keep high resolution at the scan speeds required for fast analyses.

With the introduction of the SCIEX ZenoTOF 7600 system, scientists can now achieve quantitative results at high speeds with high mass accuracy. The core innovation on the ZenoTOF 7600 system is the Zeno trap that when activated, provides significant improvements in duty cycle due to the optimization of ion transmission from the collision cell into the accelerator.[1] This duty cycle improvement provides a substantial increase in MS/MS sensitivity and thus enables targeted high resolution workflows. Here, a small, targeted assay for metabolites in urine from a diabetic rat model was used to characterize the MRM[HR]

workflow and the impact of Zeno MS/MS on the quality of quantitative results.

Urine samples from a Zucker diabetic rat model were obtained and a targeted assay for 13 metabolites was developed. Sensitivity between the Zeno trap on and Zeno trap off methods was compared and a significant gain in MS/MS signal was observed (for example: cAMP fragment in Figure 1 shows a ~12-fold improvement). In this technical note, the complete workflow from data acquisition through to statistical analysis of results is described.

Key features of the ZenoTOF 7600 system for targeted metabolomics

- Significant increases in MS/MS sensitivity due to Zeno trap, which delivers ≥90%[1] duty cycle on MS/MS fragments while still maintaining fast acquisition rates (up to 133 Hz)
- MS/MS XIC peak area gains of ~13 fold with Zeno trap activated for high-sensitivity quantification of detected metabolites
- Flexibility of a QTOF instrument for additional workflow
  - SWATH acquisition, data dependent acquisition, electron activated dissociation (EAD)
- Powerful data processing tools in SCIEX OS software for accurate quantification, followed by multivariate statistical analysis using MarkerView software
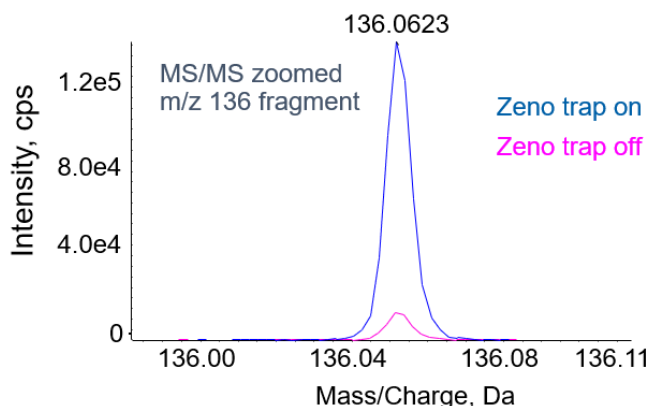
Figure 1. Significant sensitivity gains in MS/MS. Comparison of extraction ion chromatograms (XICs) for cAMP fragments obtained from MS/MS collect with Zeno trap on (blue) vs. Zeno trap off (pink). Signal/noise ratio improved ~12.5 fold when using the Zeno trap. This XIC is from a 2 µL injection of diluted urine.

Methods

Sample preparation: Urine samples were collected from four distinct rat groups: Zucker diabetic fatty (ZDF) rats, male and female; Sprague Dawley (SD) rats, male and female. Urine was collected from N=5 rats per group. 20µL of urine was aliquoted and diluted 10-fold with mobile phase A prior to LC-MS/MS analysis.

Chromatography: An ExionLC AD HPLC system (SCIEX) with a Phenomenex Luna Omega Polar C18, 3 µm 150 x 2.1 mm (00F-4760-AN) was used for sample separation. A simple linear gradient from 0 to 95% B was used with standard reverse phase mobile phases (A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile) with a flow rate of 300 µL/min. Either a 0.2 or 2 µL injection volume was used and the column temperature was maintained at 40 °C throughout the analysis. The total run time was 13.1 min including 2 min of equilibration.

Mass spectrometry: MRM[HR] data was acquired on the SCIEX ZenoTOF 7600 system in positive ESI mode using SCIEX OS software. The ion source conditions were as follows: CUR 35, GS1 55, GS2 55, ISVF 5500, TEM 600 °C. High resolution MS/MS was collected for each metabolite using an accumulation time of 10 msec. A collision energy (CE) of 30 was used for each MS/MS. Methods were built with the Zeno trap both activated and deactivated to enable the sensitivity comparisons. Three replicates were collected on each sample with each method.

Data processing: MS/MS interpretation, peak integration, and quantitative analysis were conducted in SCIEX OS software, then results were imported into MarkerView software for multivariate statistical analysis (Figure 2). To build a processing method for MRM[HR] data, the MS/MS spectrum was first examined in the Explorer module to select the best fragment ion. This was also compared to the library spectrum from LibraryView software using the SCIEX Accurate Mass Metabolite Library
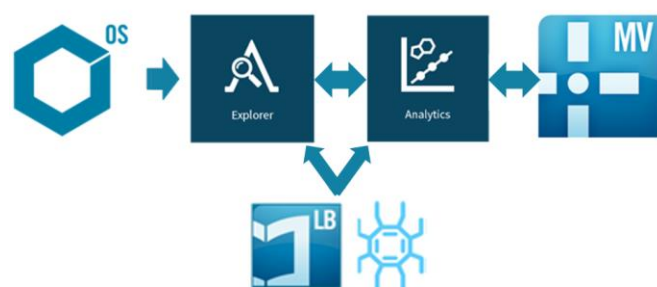
Figure 2. Workflow diagram. Data was both acquired and processed using SCIEX OS software. MS/MS was interpreted using both Explorer and Analytics, library searching was performed using the Library View and ChemSpider.
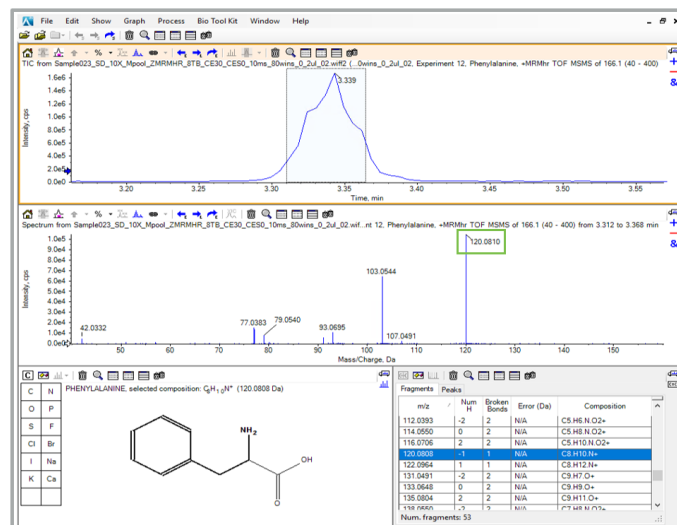

Figure 3. Explorer in SCIEX OS software for metabolite identification. The top pane shows the extracted ion chromatogram (XIC) of phenylalanine, whereas the middle pane shows the experimental accurate mass MS/MS which can be used to select the fragment mass of interest from LibraryView software. The bottom pane shows the structure obtained from ChemSpider that provided in silico (theoretical) fragmentation and accurate mass information that was later used in the calculation of mass error.

(AMMSL 2.0). Structural information from ChemSpider was also used to confirm the identity of the fragment and obtain the theoretical m/z of fragment of interest to be used. This fragment accurate mass information obtained in Explorer mode (Figure 3) was then used to build a final processing method in the Analytics module of SCIEX OS software. Peak areas of the fragment ions were then imported into MarkerView software for statistical analysis.

Zeno MRM[HR] workflow for targeted quantification

When activated, the Zeno trap provides a significant increase in MS/MS signal on the ZenoTOF 7600 system, while maintaining very high acquisition rates, and not sacrificing mass resolution. Using a targeted MRM[HR] assay for 13 metabolites in urine, the sensitivity gains due to the activation of the Zeno trap was explored. Extracted ion chromatograms (XICs) were compared from the data collected with the Zeno trap on and off to determine gains in sensitivity. As shown in Figure 1, cyclic AMP produces a dominant fragment ion at *m/z* 136.0618 with significant signal gains of over 10 fold observed.

To address the concerns of limited sample volume, a comparative experiment was performed using a 0.2 µL injection volume with the Zeno trap on, and a 2 µL injection volume with Zeno trap off (Figure 4). Even with ten-fold less sample injected on column, the peak area for the XIC of the *m/z* 136.0618
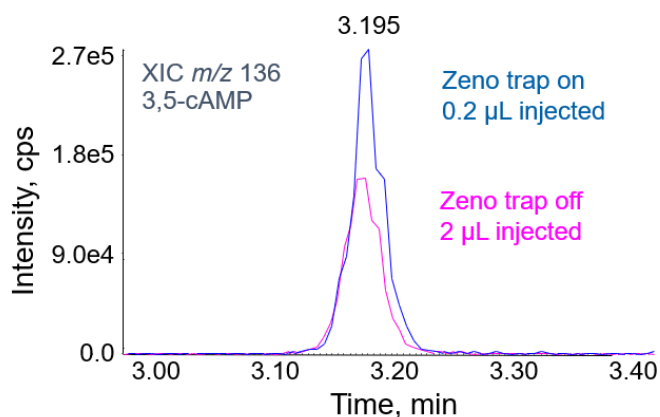
Figure 4. Better sensitivity in MS/MS with 10x less sample.
Comparison of extraction ion chromatograms for cAMP fragments obtained from MS/MS collect with Zeno trap on (0.2 µL injection, blue) vs. Zeno trap off (2 µL injection, pink). Signal/noise ratio improved ~1.5 fold when using the Zeno trap. MS/MS acquisition rates are very fast (10 msec accumulation) providing 15 data points across the peak at base.

fragment mass with the Zeno trap on ~1.5 fold higher. With higher dilution factors or lower loading of complex matrices on column, matrix effects are reduced improving data quality. Also the ability to analyze much lower sample volumes can be an advantages for some researchers with very precious samples.

The comparison between Zeno trap off vs. on was done for each of the 13 metabolites analyzed and the results are summarized in Table 1. The data quality specifications such as fragment mass error, library match and area gains with the Zeno trap on are presented in Table 1. The mass error for the quantifiable fragment ions were <3ppm for 12 out of 13 compounds analyzed in this study. On average, the significant area gains obtained with the Zeno trap on for MS/MS is 14-fold higher compared to the Zeno trap off. It is important to note that the MS/MS acquisition rate was very high (10 msec accumulation time per MS/MS).

MarkerView software for statistical analysis

Here, a small sample set was explored to test the workflow from quantification to statistical analysis (Figure 5, Table 2).[3,4] Note these metabolites were selected based on previous results from a SWATH acquisition study on the same sample set.[2] Metabolites of interest were selected and included in this targeted assay study. Unsupervised principle component analysis (PCA) was used to generate the two-dimensional score plots in MarkerView software (MV). The four categories of mouse models, ZDF-male and female, SD-male and female have clustered differentially, are clearly separated, and 97% of the variance was explained by the PC1 and PC2 (Figure 5, top).

Table 1. Increased quality of MS/MS spectra due to Zeno trap.
Activation of the Zeno trap provided significant MS/MS signal increase and therefore large increases in fragment ion XICs (average of 13.6 fold). High mass accuracy and very good library hits were observed for the resulting Zeno MS/MS spectra.

Metabolite	Fragment ion (m/z)	Library match	MS/MS fragment mass error (ppm)	Area gain with Zeno trap on (on/off)
Acetylglutamate	84.0444	√	-0.14	12.51
Arginine	70.0651	√	3.73	13.18
Carnitine	103.0401	√	-4.52	11.12
Creatine	43.0291	√	3.87	18.11
Cyclic AMP	136.0618	√	2.56	10.00
Glutamine	84.0444	√	1.72	18.08
Histidine	110.0713	√	0.24	26.72*
Leucine	69.0699	√	0.56	15.83
Methyladenosine	150.0778	√	1.28	10.28
Phenylalanine	120.0808	√	2.37	10.38
Tryptophan	118.0651	√	0.26	11.93
Tyrosine	119.0495	√	-3.81	8.89
Uric acid	141.0407	√	4.24	10.29
			Average area gain:	**13.64**

Zeno trap off peak area very low, hard to measure

The loading plots showed four PCV groups (data not shown). Metabolites showing large changes on the loadings plot were selected and displayed as box and whisker plots across the samples (Figure 5 bottom). cAMP and methyladenosine had similar pattern of difference across the samples, while creatine showed a different pattern across the ZDF and SD urine samples.

SWATH acquisition to MRM[HR] workflow

Here, the ability to create a SWATH acquisition to MRM[HR] workflow for metabolomics was also demonstrated, as SWATH acquisition was performed on the same sample set on the same instrument. Metabolites that showed differences in abundance between experiment groups from the SWATH acquisition data were selected along with a few additional metabolites, and used to build a targeted MRM[HR] method. Good correlation was observed in the fold change results between the different diabetic mice vs. the SD male sample for the eight metabolites measured in both datasets ($r^2 \geq 0.92$ for all the group comparisons, Figure 6). This highlights the feasibility of
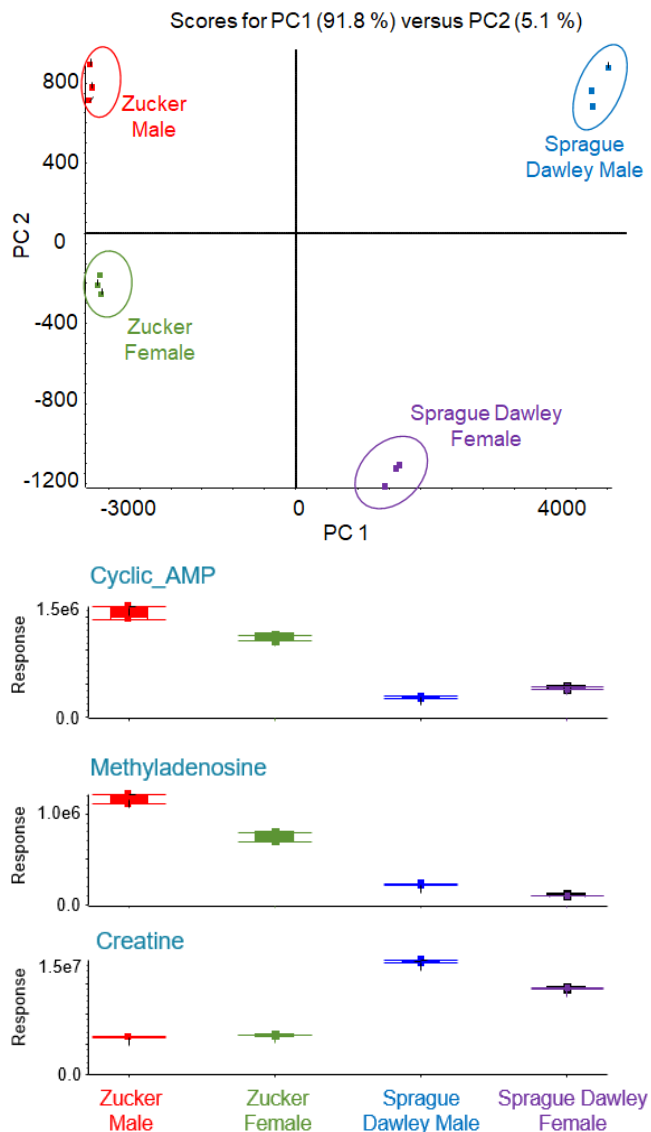
Figure 5. Principal component analysis (PCA) highlights clear differences between samples. Peak areas from MRM^HR data were imported into MarkerView software and PCA-PCVG was performed. (Top) Scores plot shows both good reproducibility between replicate injections and clear separation of the different rat urine samples, even with just these 13 targeted metabolites. (Bottom) Box and whisker plots for selected analytes highlights the differences seen across the samples.

performing the non-targeted screening workflow as well as a targeted quantification assay on a single HRAM system.

With the SWATH acquisition workflow, a large number of metabolites can be quantified from a single run and provide preliminary quantitative results to find differences between samples. When an MRM^HR assay is next developed for the same analytes, a much more narrow Q1 isolation window is used

Table 2. Differences in metabolite abundances between the rat urine samples. Using the male SD rats for comparison, the peak area fold changes (log 2 peak area ratio) were computed.

	Log2 peak area ratios		
Metabolite	**SD female / SD male**	**ZDF female / SD male**	**ZDF male / SD male**
Acetylglutamate	-0.94	0.40	0.36
Arginine	-1.32	-4.42	-1.44
Carnitine	1.77	3.59	2.90
Creatine	-0.38	-1.53	-1.60
Cyclic AMP	0.56	1.97	2.39
Glutamine	3.62	6.34	4.92
Histidine	-0.09	-1.08	-2.33
Leucine	-1.35	-2.06	-2.47
Methyladenosine	-1.11	1.78	2.39
Phenylalanine	-0.76	-2.04	-3.48
Tryptophan	-0.51	-3.50	-3.49
Tyrosine	-0.83	-3.01	-3.94
Uric acid	0.17	1.71	0.73

providing higher specificity of detection, and thus providing an addition confirmation of the screening results. And with the ZenoTOF 7600 system, this can be performed on the same system.
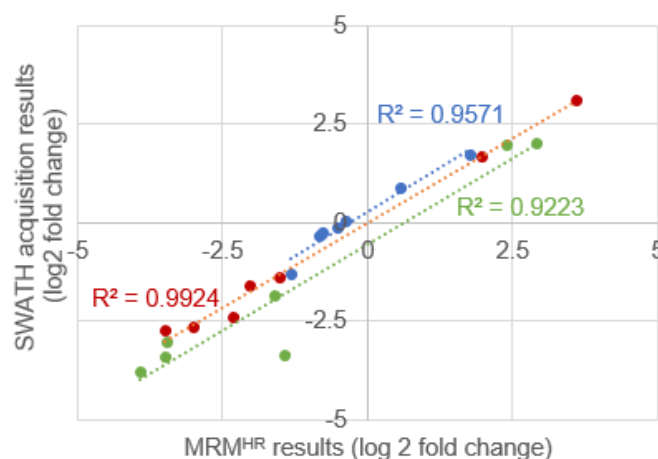
Figure 6. Correlation of quantitative results from SWATH acquisition and MRM^HR workflow. The log2 fold change results were computed for eight compounds across the three groups of comparisons: SD female/SD male, ZDF female/SD male and ZDF male/ SD male. Very good correlation was obtained between the two different quantitative techniques.

Conclusions

Here, the targeted MRM^{HR} workflow on the ZenoTOF 7600 system has been explored for use in quantification of metabolites in biological samples.

- Zeno MS/MS provided a 13-fold average increase in MS/MS sensitivity, thus providing both high-quality, full-scan MS/MS data for each metabolite for confident compound identification as well as large increases in fragment ion XIC areas for higher sensitivity quantification

- The sensitivity improvements with the Zeno trap provides the user with more workflow options; for instance, greater sample dilution to reduce matrix effects, or to perform small injection volumes for the analysis of volume-limited samples

- Raw data processing in SCIEX OS software and multivariate statistical analysis visualization using MarkerView software delivers the complete workflow from identification to quantification

- In addition, the ability to transition from non-targeted SWATH acquisition studies to targeted MRM^{HR} workflow on a single MS instrument was demonstrated.

References

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