# Impact of increased MS/MS sensitivity on the untargeted metabolomics workflow

***Using Zeno IDA on the ZenoTOF 7600 system***

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Data dependent acquisition (DDA or IDA) is a widely used analytical workflow for untargeted metabolomics using mass spectrometry. Typically, DDA consists of two steps. In the first step, an MS survey scan is collected for the specified mass range, and this data can be later used for quantification. In the second step, MS/MS is performed on the most abundant features observed in the survey scan, according to user defined filtering criteria. Maximizing the number of features sent for MS/MS and then identifying those features with high confidence across different samples is paramount for an efficient and effective DDA workflow.

The acquisition rates for MS/MS on QTOF systems are very high, often allowing this feature identification workflow to be performed in the same sample injection. However, higher acquisition rates could significantly lower MS/MS data quality due to lower accumulation times used, which could consequently impact the identification of the compounds during the library search step. Ideally, both spectral quality and resolution are maintained when acquiring MS/MS at very high rates.

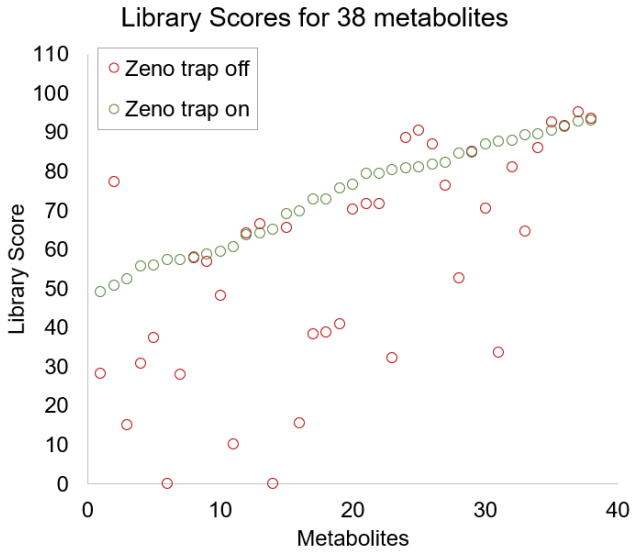


**Figure 1. Large sensitivity gains observed in Zeno MS/MS resulted in higher library scores**. Urine metabolites with library scores between 50 and 90 in the Zeno trap on data (green) were aligned with the Zeno trap off data (red). Library scores were typically higher in the Zeno trap on data, see Figure 4 for more details.

Many orthogonal injection QTOF systems suffer from duty cycle losses (duty cycle typically between 5 to 25%) in the MS region where the continuous ion beam is pulsed into the orthogonal TOF region of the system. However, on the ZenoTOF 7600 system, implementation of the Zeno trap has improved the duty cycle in this region to ≥90% providing significant increases in MS/MS sensitivity while maintaining high spectral resolution.[1] Here, the impact of this sensitivity increase on the ability to identify urine metabolites using a high MS/MS acquisition rate in a single injection Zeno IDA workflow was explored.

## Key feature of ZenoTOF 7600 system for untargeted metabolomics

- The ZenoTOF 7600 system acquires high resolution, high quality spectra at very fast acquisition rates (up to 133Hz), enabling a single injection workflow for untargeted metabolomics
- The Zeno trap significantly increases MS/MS sensitivity on low abundant precursors during Zeno IDA
- The number of MS/MS acquired with spectral quality above 95 has doubled when the Zeno trap is activated during DDA
- Spectral quality increase provides an increase in library matches of 15%
- Two untargeted workflows explored using SCIEX OS software for library searching and MarkerView software for statistical interpretation

## Methods

***Sample preparation:*** Urine samples were collected from four distinct rat groups studied: Zucker diabetic fatty (ZDF) rats, male and female; Sprague Dawley (SD) rats, male and female (n=5 rats per group). 20 µL of urine sample was aliquoted and diluted 10-fold with mobile phase A prior to LC-MS/MS analysis. 2 µL of sample was injected for analysis.

***Chromatography:*** Analyte separations were performed using an ExionLC AD HPLC system (SCIEX) with a Phenomenex Luna Omega Polar C18, 3 µm 150 x 2.1 mm (00F-4760-AN). Using standard reversed phase mobile phases (A = 0.1% formic acid in water and B = 0.1% formic acid in acetonitrile), a linear gradient from 0 to 95% B over 13.1 mins was run with a flow rate of 300 µL/min. The column temperature was maintained at 40 °C.

***Mass spectrometry:*** Data dependent acquisition workflow adopted on the SCIEX ZenoTOF 7600 system in positive ESI mode using SCIEX OS software. The ion source conditions for full scan MS were as follows: CUR 35, GS1 55, GS2 55, ISVF 5500, TEM 600 °C. High resolution MS/MS per cycle were collected with an accumulation time of 10 msec and 60 MS/MS per cycle. A DP of 50 V, collision energy (CE) of 30V was used for each MS/MS with start mass of 10 Da and stop mass of 1000 Da were used in this method. Methods were built using both Zeno trap activated and deactivated to perform the sensitivity comparisons. Three replicates were collected on each sample with each method.

***Data processing:*** Data processing was done using both SCIEX OS software and MarkerView software. In one approach, the IDA data was subjected to a library search using SCIEX OS software and the AMMSL[2] and NIST libraries. In the second approach, feature detection is performed using MarkerView software, then features with significant differences were subjected to library searching in SCIEX OS software.

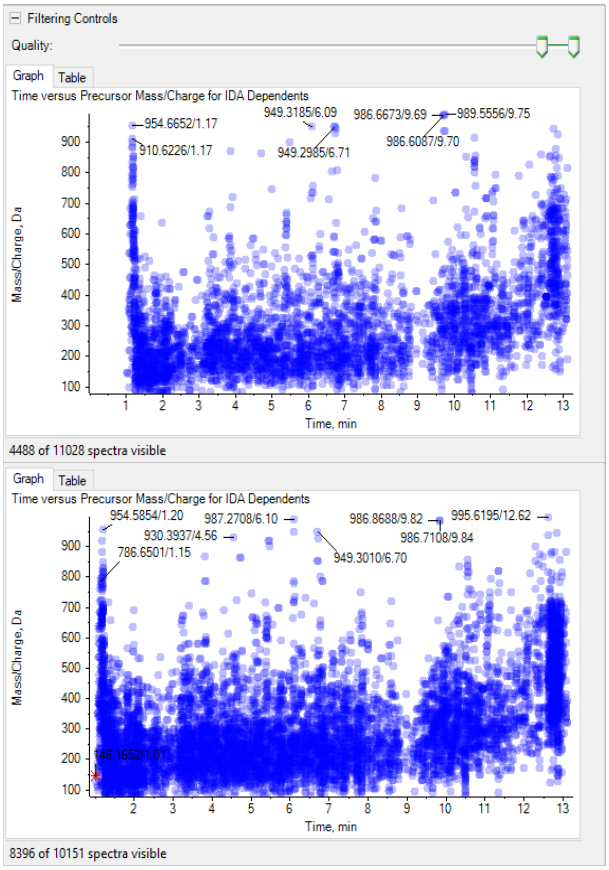


**Figure 2. Use of the Zeno trap greatly increases the spectral quality during data dependent acquisition.** MS/MS spectrum obtained with Zeno trap off (top panel) showed 40% of spectra (4488/11028) had a quality score ≥ 95. With Zeno trap turned on, (bottom panel) 83% of spectra 8395/10151) had a quality score ≥ 95. Data shown is for DDA collected on the ZDF male pooled sample.

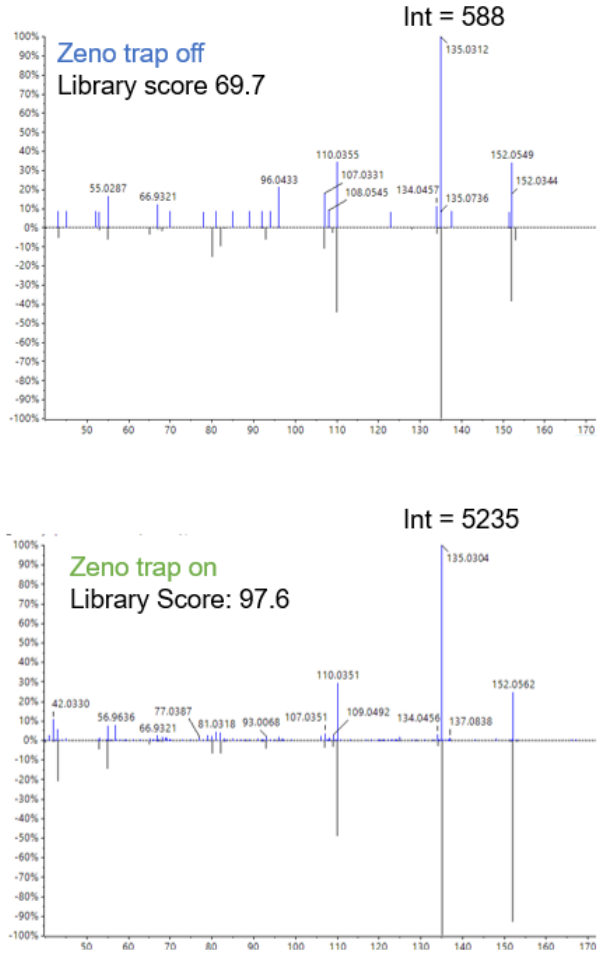


**Figure 3. Improved spectral quality yields higher library scores.** Improved spectral quality in turn provides improved library matching, as highlighted here for guanine. (Top) library score 69.7 for guanine for MS/MS collected with Zeno trap off (Bottom) library score of 97.6 for to Zeno trap on. Peak intensity of the m/z = 135.03 ion is shown on the top of each spectrum highlighting the much higher sensitivity with Zeno trap on.

## Zeno trap improved spectral quality

In addition to high resolution and high mass accuracy, MS/MS data quality (signal to noise) is key for feature Identification using library search. Here, when Zeno trap was activated for MS/MS data acquisition, a significant increase in MS/MS signal and spectral quality was observed (Figure 2). This in turn led to an increase in library scores as highlighted in Figure 3 for guanine.

The first data processing workflow tested involved a library search of the DDA data from pooled samples using the AMMSL library from SCIEX to identify the core biological metabolites in the samples. With the Zeno trap on, 74 metabolites were identified on average in SD male and female rats, ~15% higher than the number identified with the Zeno trap off.

The Zeno trap is automatically activated for MS/MS acquisition when spectral intensity of the precursor is less than the Zeno threshold[3]. Therefore, the increase in MS/MS quality and library score is expected only for the low intensity analytes.

A total of 153 metabolites were identified using library search from a pooled sample of the ZDF male rat urine samples analyzed with the Zeno trap on and off. The results were segregated into three distinct groups for more detailed assessment based on the Zeno trap on library scores (Figure 4).

1. Group one included metabolites with lowest library scores (<50) in the Zeno trap on data. The Zeno trap off library scores varied widely from the Zeno trap on data due to poorer quality MS/MS spectra. A large number of metabolites had no matching MS/MS as seen by the red dots at zero.
2. Group two included metabolites with Zeno trap on library scores between 50 and 90 and largely reflected results obtained on the lower abundant metabolites. This group yielded the majority of the identification gains with Zeno trap on due to significant MS/MS spectral quality improvements and hence improved library scores (Figure 1).
3. The compounds in group three had the highest library scores for the most abundant metabolites. Because the MS/MS spectra was already of good quality, the added sensitivity of Zeno MS/MS did not improve the library scores.

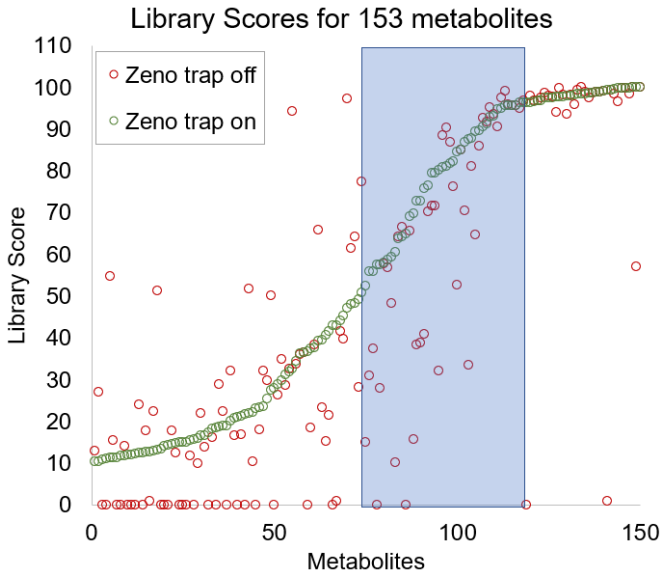


**Figure 4. A total of 153 metabolites were identified with Zeno trap turned on and off.** These metabolites were separated into three groups. Group one had poor library scores, <50 in Zeno trap on data set. Group two has library scores between 50 and 90 with Zeno trap on compared to Zeno trap off, and with greater confidence in library match. Group three had the highest library scores, >90.

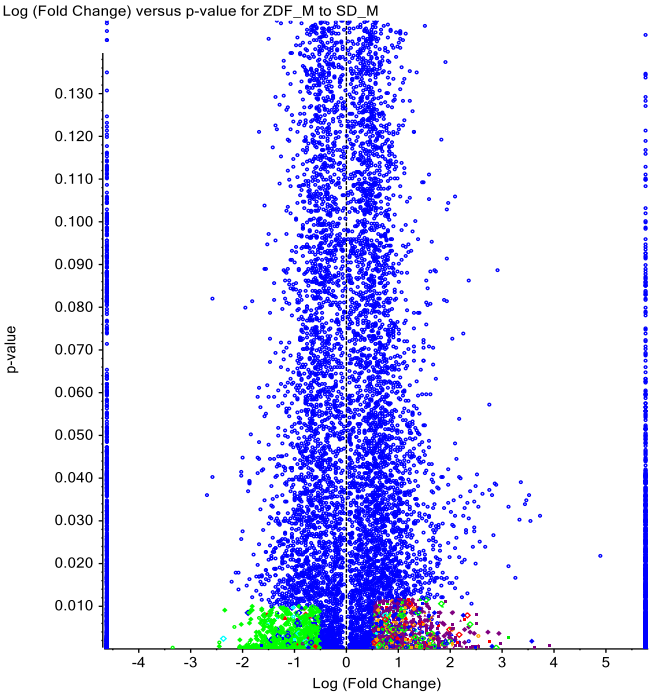


**Figure 5. Detection of significant features from MS1 data.** Significant features (p-value ≤ 0.01, ± Log fold change ≥ 0.5) were selected from the MS1 peak processing in MarkerView software. An interest list of 4222 features was extracted and then processed using SCIEX OS software to identify the features.

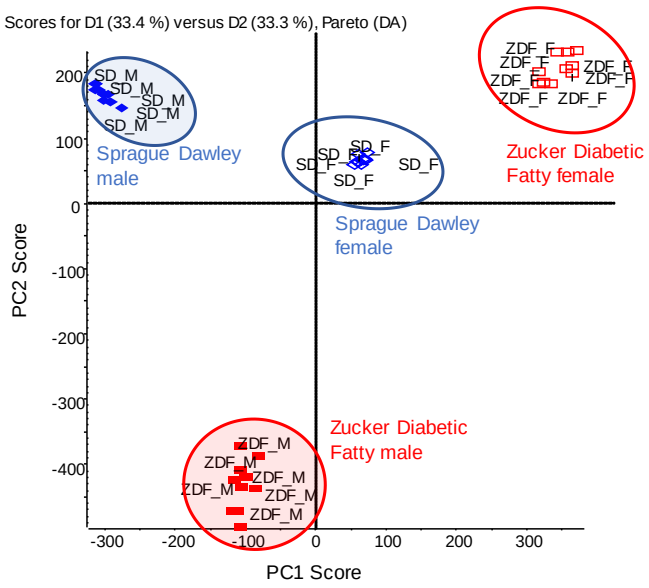


**Figure 6. Principal component analysis clearly separates groups.** The interest list of 4222 significant features (p-value ≤ 0.01, ± Log fold change ≥ 0.5) was used for principle component analysis. Very good separation was observed between the experimental groups and very reproducible clustering is seen within the 10 individual rats in each group.

## Differential feature detection

Data dependent acquisition uses MS1 data for quantification, so this can actually be processed independent of library searching to find differential features just based on MS1 differences. Then only a subset of features need to be identified with library searching. This statistically driven non-targeted analysis can simplify data processing. Here, this was done using MarkerView software and the DDA data collected on the individual rats across the experimental groups (n=10 for ZDF male, ZDF female, SD male and SD female).

Initial processing found a total of 29270 features. A series of pairwise t-test were performed across all the treatments (Figure 5) and significant features were then placed on an interest list. . A total of 4222 features were significant with the chosen criteria (p-value ≤ 0.01, ± Log fold change ≥ 0.5) and put in the interest list. These features displayed very good separation by PCA as well as very high reproducibility between individuals within an experimental group (Figure 6).

Next, the 4222 features needed to be identified, so the interest list was imported into SCIEX OS software in order to use library searching for identification. Fifty features were identified with high confidence using AMMSL and NIST library search from this interest list of differential features.

## Conclusions

Here, a data dependent acquisition (DDA) was performed on the ZenoTOF 7600 system and MS/MS acquisition was performed both with and without the Zeno trap activated. This data was then processed using two untargeted data processing workflows, to explore the impact of the Zeno trap on the ability to identify significant metabolic differences across biological samples.

- With the MS/MS sensitivity gains observed when the Zeno trap is activated, the MS/MS spectral quality was improved, yielding a 15% increase in identified metabolites
- High spectral quality was achieved with very fast MS/MS (10 msec accumulation times) enabling a single injection workflow for each polarity
- Zeno MS/MS has a larger impact on library scoring when the analyte abundance is lower and yields weaker MS/MS spectra
  - Also great impact would also be expected when analyzing lower sample loads
- Very fast MS/MS acquisition rates were used (10 msec) which provides more data points across the peak for good quantification
- With SCIEX OS software and MarkerView software, multiple approaches to data processing can be used for interrogation of DDA sample sets.

## References

1. Qualitative flexibility combined with quantitative power. SCIEX technical note, RUO-MKT-02-13053-A.
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3. What is Zeno-On-Demand and what does it do? SCIEX community post, RUO-MKT-18-13325-A.



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