# Biomarkers and Omics

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# Targeted high resolution metabolite screening workflows

*Maximize your lab's efficiency with the X500R QTOF System and library driven workflows*

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There are two main approaches used in the field of discovery metabolomics today: the untargeted, discovery route to cover a broad range of metabolites and the targeted route whereby one interrogates for a set of known metabolites to quickly generate quantitative results. Untargeted analyses suffer from major challenges in metabolite identification. As the field of metabolomics has rapidly evolved so has the technology and these advancements have allowed for more powerful data acquisition strategies especially in the field of mass spectrometry (MS).  The powerful data dependent workflow of the X500R QTOF System means that the user can collect high resolution, accurate mass MS and MS/MS data in a single injection. Once the data has been collected in an unbiased way (untargeted analyses), one can now search the data using a targeted approach for known metabolites or metabolites, pertaining to a specific pathway or classes – known as targeted metabolite screening. Here, we present a streamlined targeted workflow

using the single injection workflow on the X500R QTOF System followed by data processing using MasterView™ Software with a

high resolution, accurate mass metabolite spectral library. This metabolite library contains over 500 metabolites across a variety of pathways such as TCA cycle, BCAA degradation/synthesis, glycolysis, urea cycle and across many compounds classes such as amino acids, bile acids, sugars, nucleotides, organic acids including many natural and non-natural products (including common drugs).

## Key benefits of targeted metabolomics screening on the X500R QTOF System

- Maximize your lab's efficiency using a robust and integrated system with automated calibration and a benchtop footprint

- Simplified and robust sample analysis using the X500R QTOF System.
  - Simplified data acquisition interface and workflow setup powered by SCIEX OS Software.

- Get to actionable results quickly
  - User-friendly processing software and metabolomics library
  - The Accurate Mass Metabolomics Spectral Library (AMMSL) contains 557 compounds which include endogenous, exogenous, xenobiotics and both natural and non-natural compounds.

- Export results from SCIEX OS Software into MarkerView Software for statistical analysis.
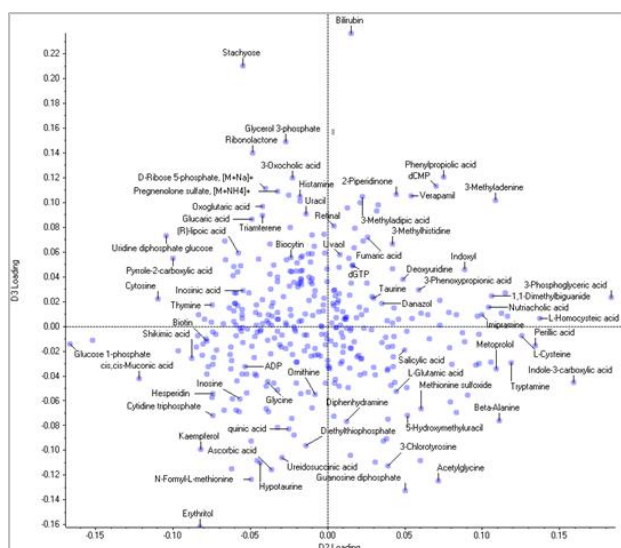


**Figure 1.  Principal component analysis highlighting metabolite targets responsible for changes between samples from a pre-classified prostate cancer study.**  Using a library allows the user to overcome the metabolite identification challenge for untargeted metabolomics studies.   The Loadings plot shows the distribution of the identified metabolites with respect to the principal components (PC). The metabolites are responsible for the differentiation and the degree of change between the sample groups interrogated. Note that the loadings plot above displays metabolite name and not m/z_RT (ion_retention time pairs) as in traditional untargeted metabolomics approaches.

## Methods

**Sample preparation:** A urine sample set from a prostate cancer study was used to study the changes in metabolites between healthy individuals. First the specific gravity of the samples was measured by testing 15 µL of urine samples with a refractometer prism. A 50 µL volume of the thawed urine samples was then transferred to a clean, labeled microcentrfuge-filter tube. An isotopically labeled internal standard mixture (20 µL) was added. The urine sample was then diluted with 400 µL 98:2 acetonitrile/water with 0.1% sodium azide, then vortexed. The sample was then centrifuged, and the supernatant was isolated. This was then dried down and reconstituted in 50 µL of 0.1% formic acid in water. The samples were transferred to glass vial and loaded into the autosampler.

**Chromatography:** The reverse phase HPLC separation was performed using an ExionLC™ System, operating at a flow rate of 350 µL/min. The column used was an Ace Excel C18-PFP column (100 x 1mm, 2 µm) from Ma, maintained at 30 ⁰C. The gradient used is defined in Table 1, using a mobile phase A of 0.1% formic acid in water and mobile phase B of acetonitrile. The injection volume was 3 µL in positive mode and 5 µL in negative mode.

**Table 1: Gradient used for the liquid chromatography.**

| Time (mins) | Mobile phase A (%) | Mobile phase B (%) |
|---|---|---|
| 0 | 99.9 | 0.1 |
| 3 | 99.9 | 0.1 |
| 13 | 20 | 80 |
| 16 | 20 | 80 |
| 16.5 | 99.9 | 0.1 |
| 20 | 99.9 | 0.1 |

**Mass spectrometry:** The data was collected in an untargeted manner using information dependent acquisition (IDA) using the X500R QTOF System (SCIEX). Using optimized source conditions, the MS mass range covered was 70-1000 and the MS/MS was acquired with a mass range of 50-1000 and a 40 msec accumulation time. The collision energy was set to 35 V with a 15 V collision energy spread to ensure high quality MS/MS on most metabolites. The overall workflow is highlighted in Figure 2.
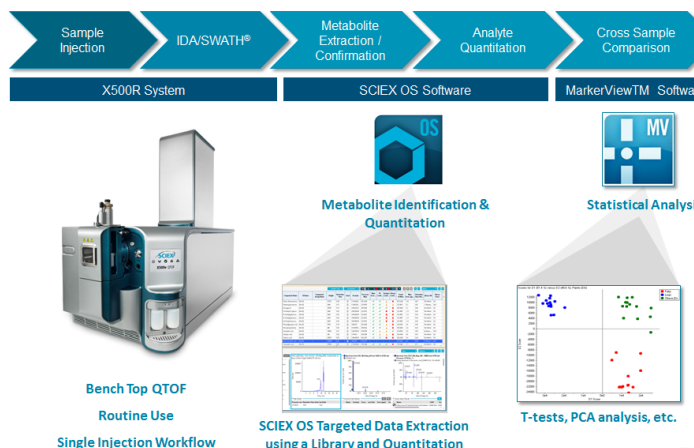


**Figure 2. Targeted screening workflow for metabolomics.** Large numbers of biological samples can be acquired using the X500R QTOF System using the easy to acquisition software and robust design. After data collection, data is processed using the SCIEX OS Software Analytics module.

## Targeted library searching and metabolite identification using SCIEX OS Software

All data was processed in SCIEX OS Software Analytics module (Figure 3). Using the step-by-step guided procedure, a processing method was constructed. The non-targeted acquisition of IDA data allows for both untargeted data analysis to identify features of interest within the dataset as well as the targeted analysis of the data looking for previously identified or known compounds of interest. Here, targeted data analysis was performed using the Accurate Mass Metabolite Spectral Library (AMMSL) for peak integration and compound identification. After processing, a user can review the results as described in Figure 4. The resulting identified and quantified peaks were exported from SCIEX OS Software as a MarkerView™ Peaks List (.mkvw file).



**Figure 3. SCIEX OS Software dashboard.** Easy to use software integrates data acquisition, data processing, data review and reporting. The tile-based dashboard allows intuitive guidance to a user even with minimal MS expertise.
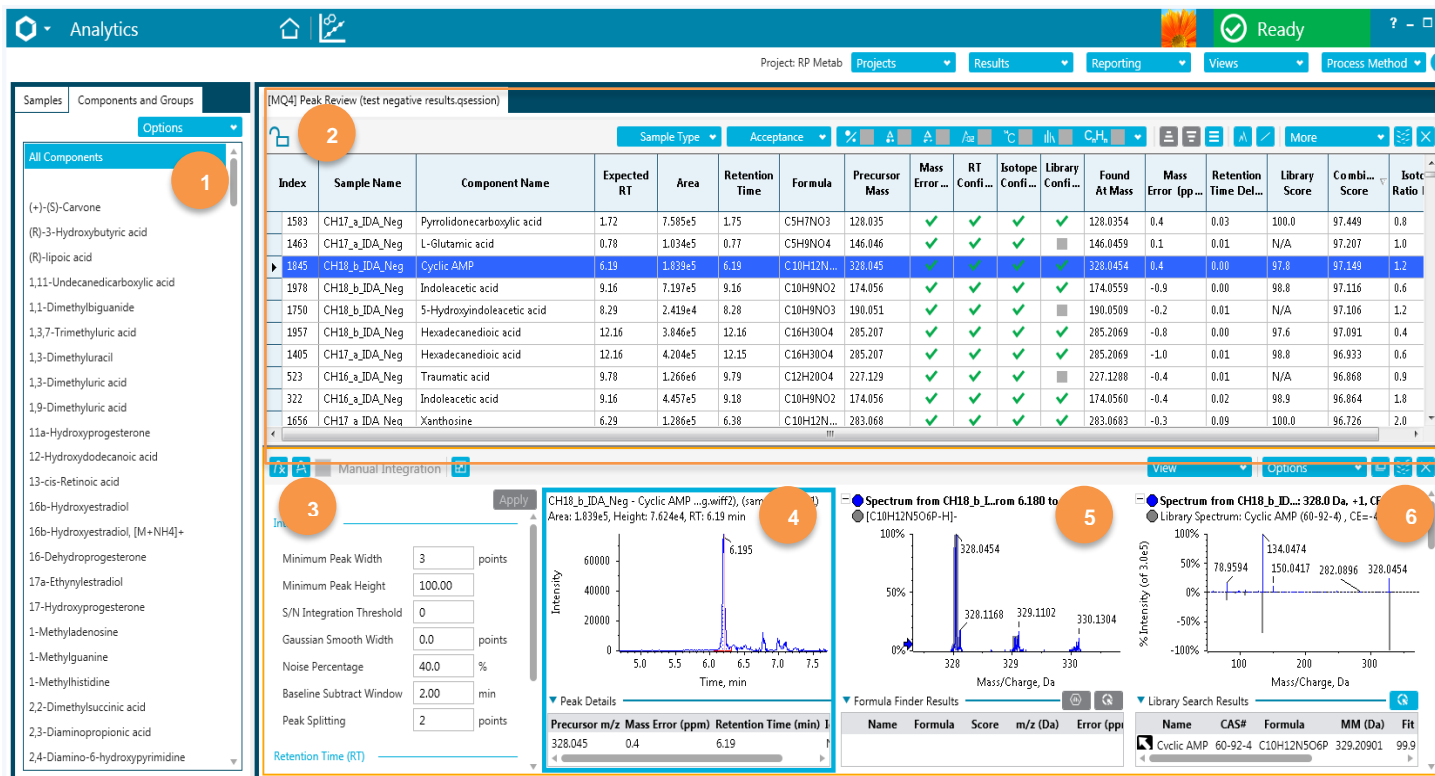
**Figure 4: Data review in SCIEX OS Software analytics module.** The single review pane above allows the user to 1) Review the metabolites contained in the library and or sample review on a second tab. 2) Review any metabolites found in the samples by reviewing the confidence scores for the MS1 mass error, retention time, isotope fidelity and the MS/MS. These are displayed using a traffic light colored system where green mean a confident match, yellow means data must be reviewed and red means no confidence in the match. The data can be swiftly reviewed by filtering only the confident matches (i.e. all green hits only). 3) The integration parameters can be reviewed and adjusted for reporting peak areas. 4) The extracted ion chromatogram is displayed. 5) The MS1 spectrum of the experimental (blue) is overlaid with the library MS1 spectrum (gray). 6) The MS/MS spectrum of the experimental (blue) is displayed with the library MS/MS spectrum (gray) in what is known as a waterfall plot. Here cyclic AMP is identified from the library and match at all criteria levels (MS1 – pane 4, MS/MS – pane 5 and a high library score of 97.8% - pane 2, highlighted row).

## Differentiating compounds and statistical analysis

The peaks list from SCIEX OS Software Analytics module can be directly imported into MarkerView Software. MarkerView Software can be used to visualize any trends or clustering within a dataset, tie them back to the sample groupings, and find the compounds responsible for the differences between the groups. In this pilot prostate cancer study, the compounds in the AMMSL that were identified and quantitated using SCIEX OS Software of the samples with known disease classifications as well as the QC samples (spiked with a mix of known standard compounds) and the Control urine (matrix QC) were analyzed with the MarkerView Software.

In Figure 5, the PCA Scores plot highlights the clustering of the different sample groups and the good reproducibility across the dataset. Repeating the PCA analysis after removing the QC and Control samples shows a clear differentiation in the metabolic profile between the healthy and diseased groups (Figure 6

Scores plot, left). The Loadings plot (Figure 6, right) displays the compounds responsible for the differentiation between the groups, with compounds higher in concentration in the healthy vs disease samples on the left and compounds higher in the diseased vs healthy samples on the right.

Untargeted metabolomics studies often start with the data analysis first meaning the statistical differences drives the metabolite identification. This leads to a large number of features which require a long time to identify. We have removed this identification step using our library of targets whilst still preserving the underlying untargeted data should the user want to go back and re0interrogate the data for any unknowns not in the library. The advantage in reading the loadings plot in MarkerView having done the metabolite identification step first means that the results of any trends and clusters within the data can be directly read with biological context. There is no longer a plot of points with m/z values and retention times but real metabolite names (Figure 1).
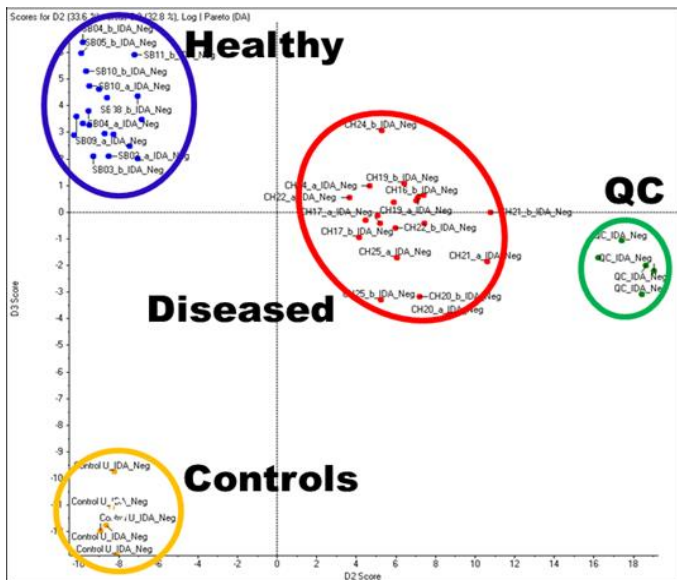
**Figure 5. Principal component analysis with MarkerView Software.**
The peaks list from SCIEX OS with the identification and quantitation of metabolites is imported into MarkerView and PCA is performed. The Scores plot clearly shows the differentiation of the healthy and diseased sample groups in the PCA analysis, confirming the original classification.

## Conclusions

The targeted metabolomics screening workflow employing integrated SCIEX OS Software with the X500 QTOF System enables the following benefits to your lab:

1. **Simplified operation** with new interface and workflow setup powered by SCIEX OS Software

2. **Get to actionable results quickly** with user-friendly processing software and metabolomics library

3. **Maximize your lab's efficiency** using a robust and integrated system with automated calibration and a benchtop footprint

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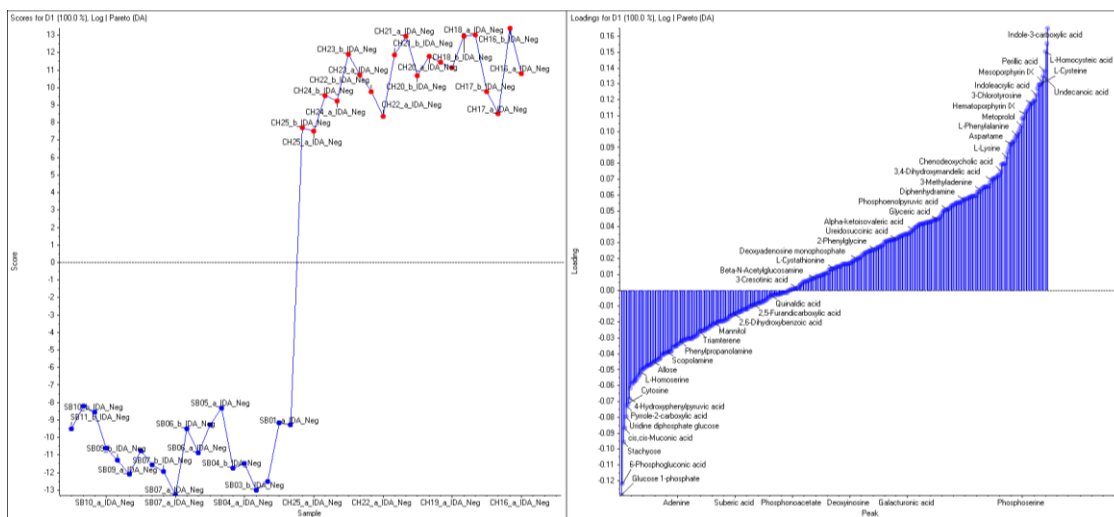


**Figure 6. Scores and loadings plots of the healthy and diseased groups from MarkerView Software.** A direct comparison of the healthy and diseased samples shows a clear differentiation of the groups in the Scores plot (left). The Loadings plot highlights the compounds responsible for the differences in the metabolic profile. The compounds higher in concentration in healthy vs diseases samples are shown on the left and the compounds higher in the diseased vs healthy samples are shown on the right.

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