

# Non-Targeted Metabolic Profiling of Human Urine Samples

## Discovering Metabolite Markers using the TripleTOF® LC-MS/MS Systems

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The metabolome (Figure 1) is difficult to characterize; there is a wide dynamic range of concentrations of metabolites, which are chemically and structurally diverse with various polarities and sizes. Creating a single analytical method for all these components is challenging. With global profiling techniques, the aim is to detect and quantify as many metabolites as possible. Information dependent acquisition (IDA) provides useful MS/MS information, based on the user selected criteria to prioritize candidates for fragmentation. TripleTOF® systems are capable of delivering high resolution, accurate mass data with high acquisition rates, enabling MS and MS/MS information to be acquired in a single acquisition. Speed is crucial to the workflow, enabling MS/MS information to be collected for as many features as possible, providing a comprehensive interrogation of the samples. Here we present a non-targeted metabolic workflow using the high resolution TripleTOF systems and associated software.

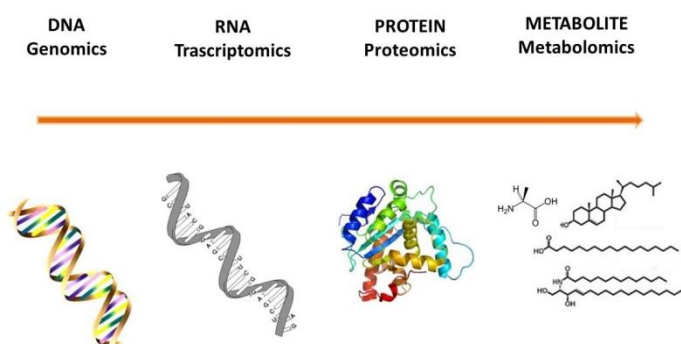
Urine is a non-invasive sample which can be used to determine biomarkers of nutrition and disease. The high resolution TripleTOF 5600+ system was used to profile human urine samples collected from volunteers, to identify and characterize metabolic features of interest.



Statistical data analysis was performed using MarkerView™ Software, to highlight any groups within the data and identify any trends. Using the accurate mass of the full scan and fragment data and the isotope patterns, molecular formulae were generated for features of interest. Structures were proposed from internal and external mass spectral libraries.

## Key Features of the Metabolomics Workflow

- Fast acquisition rates for MS and MS/MS on the TripleTOF® Systems enable more information to be collected in a single acquisition.
- Differential features can be highlighted by MS profiling, then identified using accurate mass MS and MS/MS fragmentation.
- MarkerView™ software allows simple statistical analysis of the data to highlight differences.
- PeakView® software and associated tools allow exploration and identification of these differences.



**Figure 1. Information Flow in Biological Systems.** The “metabolome” describes changes in relative concentration of metabolites that result from the deletion or over expression of a gene<sup>1</sup>.

## Materials and Methods

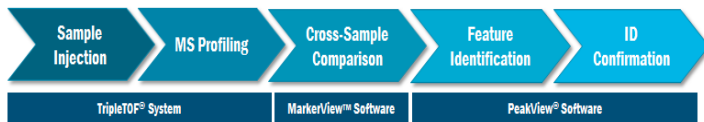


Figure 2. Workflow for Metabolomics using the TripleTOF® System

**Sample Preparation:** Urine samples were collected with consent from eleven human volunteers of varying ages, male and female. The female samples were separated into pre and post-menopausal. A pooled sample was generated by mixing equal volumes of each urine sample. All samples were diluted 1:4 with water before injection.

**Chromatography:** Randomized replicate injections were made on a Shimadzu XR Prominence ultra-high performance liquid chromatography (UHPLC) System, using a Phenomenex Kinetex® C18 column (1.7  $\mu$ m, 50 x 2.1 mm). A gradient elution using water and acetonitrile, containing 0.1 % formic acid was used at a flow rate of 0.5 mL/min over ten minutes.

**Mass Spectrometry:** A TripleTOF® 5600+ system with a DuoSpray™ Source (electrospray ionization) was used for data acquisition in positive mode, over a mass range of 100 – 1000 m/z. Automated calibration was performed using an external calibrant delivery system (CDS) which infuses calibration solution prior to sample introduction. A TOF MS survey scan experiment (100 ms) incorporating IDA set to monitor twelve candidate ions (total cycle time of 400 ms) was performed with a collision energy of 45 eV and a spread of  $\pm 15$ eV.

**Data Processing:** Data were processed using MarkerView™ Software for simultaneous feature finding, alignment, and statistical analysis to highlight metabolites of interest. For features of interest, the MS and MS/MS data were further evaluated using FormulaFinder and the structural elucidation tool within PeakView® Software.

## Results and Discussion

Data analysis was performed using MarkerView software. Figure 3 displays the scores and loadings plots from the principal component analysis (PCA), which is a simple graphical representation of multivariate data within multi-dimensional space. Pooled samples are shown in black (Figure 3, top); they cluster around the centre as they represent an average of all the samples.

One sample clustered away from the others; this can be seen in the top right hand corner of the scores plot. The loadings plot indicates one of the ions contributing to this difference is m/z 655 at a retention time of 0.7 minutes (Figure 3, bottom). PeakView software was used to view the accurate mass of the molecular ion of m/z 655, and the subsequent MS/MS spectra (Figure 4).

Evaluating the MS/MS spectrum of m/z 655 indicated this ion

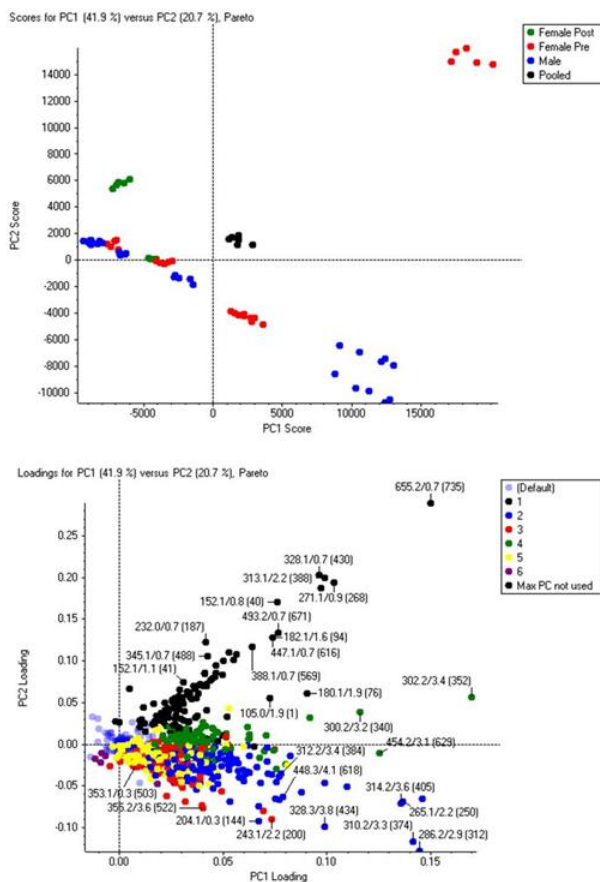


Figure 3. Differential Analysis of Urine Samples. The PCA Scores (top) and Loadings (bottom) plots highlight the differences between the urine samples. Principle Component Variable Grouping (PCVG) is used in the loadings plot to cluster ions with similar profiles (represented here in the same color), accounting for 70% of the variation.

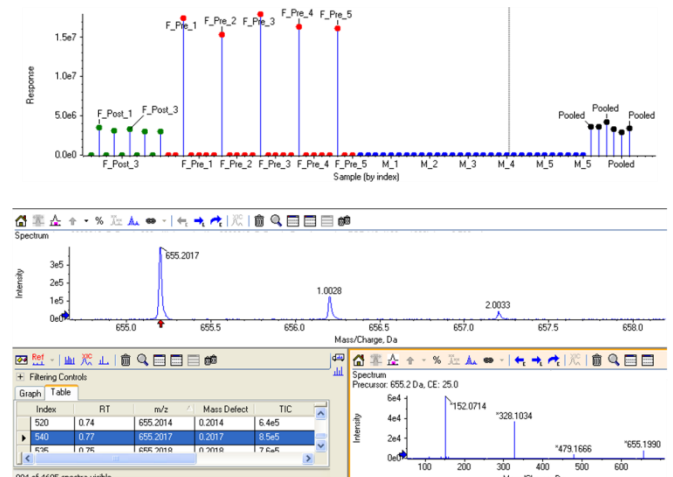
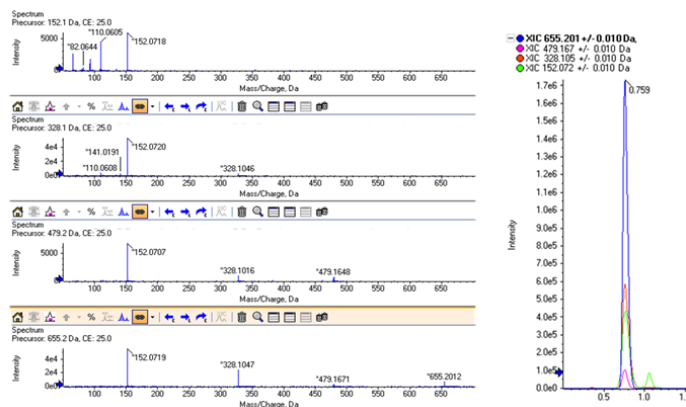


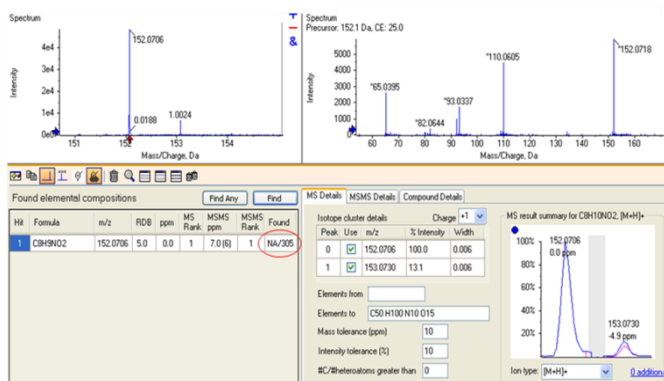
Figure 4. Investigating the Differential Feature m/z 655. (Top) The profile of m/z 655 across all urine samples. The TOF MS spectrum (middle) and the MS/MS spectrum (bottom right) for m/z 655 are shown. The table shows all ions where MS/MS has been acquired; information such as retention time, intensity and mass defect are also given.



**Figure 5. Fragments and Related Features for at m/z 655.** PeakView® software showing MS extracted ion chromatograms of m/z 655 m/z 479, m/z 328 and m/z 152 overlaid (right) and corresponding MS/MS spectra of each (left). The same three ions were observed in the MS/MS spectra of m/z 655 indicating the presence of conjugated species. The parent ion appears to be m/z 152.

could be a conjugate. Major fragments in the spectrum (m/z 479, 328 and 152) were investigated and each m/z was also seen in MS mode with the same elution profiles at a retention time of 0.7 minutes (Figure 5). MS/MS spectra of m/z 479 and 328 also produced the fragment m/z 152. This common ion of m/z 152 could represent the parent ion and it was studied further.

FormulaFinder, a tool within the PeakView Software, calculates the elemental composition / formula using precursor masses, fragment masses and isotope patterns. The software links MS and MS/MS data for confirmation and allows structure files to be imported and manipulated to propose structural information on the formula of interest. Figure 6 shows a screenshot of the FormulaFinder tool. An elemental composition range is defined by the user, and a number of potential formulae are listed, with the associated mass errors. Mass accuracy of the data is generally <2 ppm RMS even in complex matrices. In this case for m/z 152, only a single formula was possible within the



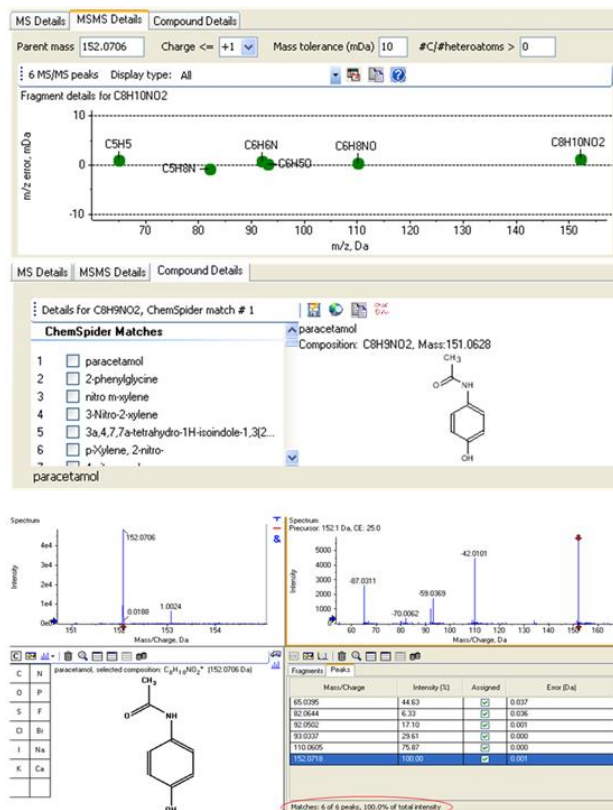
**Figure 6. The FormulaFinder Tool.** The MS and MS/MS information for m/z 152 is shown (top left and right). Elemental composition and number of potential structures for this ion found using an external database, ChemSpider (bottom left). Experimental isotope pattern is overlaid with the theoretical pattern (bottom right).

tolerances set and the mass error is 0 ppm. Internal and external databases can also be searched against the formulae for identification.

Another feature within FormulaFinder is the ability to explore the formulae of the MS/MS fragments generated from the IDA experiments (Figure 7, top). This can be a powerful tool when trying to elucidate structures if no matches are found within databases and for confirmation of the identification. In this example, ChemSpider found 305 possible structures, of which paracetamol was a possibility.

Once a structure has been suggested, or elucidated, it can be imported into PeakView software and directly linked with the MS/MS spectrum for confirmation of a match.

Figure 7 (bottom) shows the imported structure for paracetamol and a list of all the fragments assigned by matching theoretical fragments of the proposed structure to the experimental spectrum. Highlighted in red are the number of peaks that have been matched and a percentage of the total intensity matched.



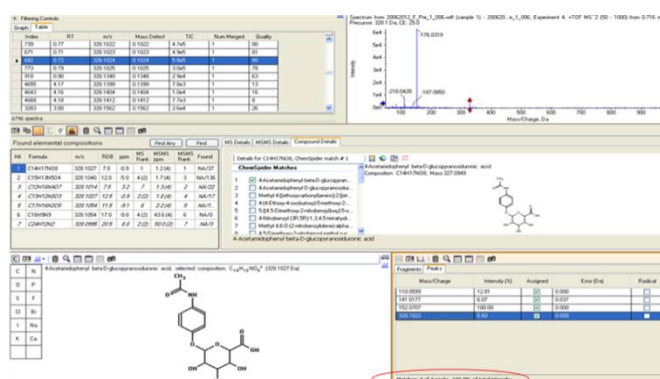
**Figure 7. Elucidating the Structure of the Differential Feature m/z 152.** (Top) FormulaFinder shows the predicted formulae for each fragment and possible structures from a ChemSpider search. (Bottom) Import the structure into PeakView software, manipulate by adding elements or conjugates, removing functional groups or changing bonds and link to experimental MS/MS data to confirm the match.



Using the approach described above, the  $m/z$  328 was also identified and found to be a glucuronide conjugate of paracetamol (Figure 8). Using the MS and MS/MS data, the other two ions of  $m/z$  479 and  $m/z$  655 were tentatively identified as further conjugations and rearrangements of paracetamol, but these require further verification.

## Conclusions

Untargeted metabolomic analysis of human urine, using the TripleTOF® 5600+ system and related software from SCIEX, has enabled the identification of a drug and related metabolites. High resolution, accurate mass data were processed in MarkerView™ software to determine any trends. Differential features of interest were explored in PeakView® software, where molecular formulae and subsequent structures were determined using the FormulaFinder tool. The ability to collect both MS and MS/MS data in a single acquisition helped accelerate the workflow and provided further confidence in the identity of urinary components.



**Figure 8. Identification of a Metabolite of Paracetamol.** Another differential feature at  $m/z$  328, showing the same extraction profile and fragment ions was investigated using the workflow outlined and was determined to be a metabolite of paracetamol – 4-Acetamidophenyl beta-D-glucopyranosiduronic acid).

## References

1. Oliver, S.G., Winson, M.K., Kell, D.B., Baganz, F. (1998) Systematic functional analysis of the yeast genome. Trends in Biotechnology **16**: 373-378.
2. To download a trial version of MarkerView™ Software please visit: <http://sciex.com/software-downloads-x2110>

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