Automated Targeted Screening of Hundreds of Metabolites

Using the TripleTOF® System, an Accurate Mass Metabolite Spectral Library and MasterView™ Software

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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. LC-MS is essential for metabolomics workflows because of its versatility, soft ionization and coverage of metabolites[1] as well as being amenable to automation and high throughput. As the field of metabolomics has rapidly evolved so has the technology and these advancements have allowed for more powerful data acquisition strategies. The powerful data dependent workflow of the TripleTOF® 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 either using a targeted approach for known metabolites or metabolites, pertaining to a specific pathway or classes – known as *targeted metabolite screening*. Or one can search the data more broadly looking for unknowns in an untargeted fashion – known as *untargeted metabolite screening*. As LC-MS data from any discovery metabolomics study can be information rich and quite complex, powerful software tools are needed to extract the relevant information for identification with high confidence.

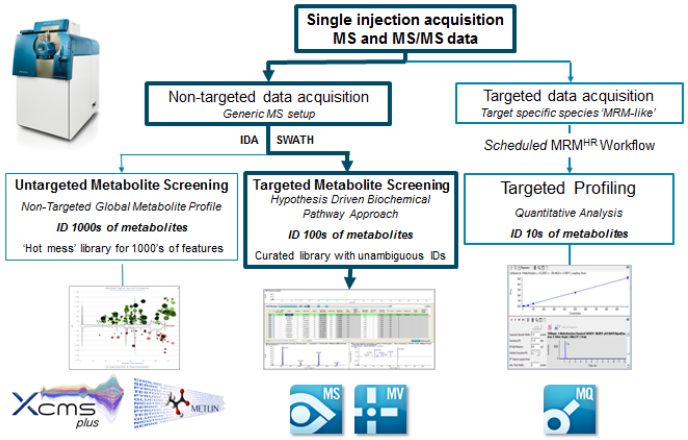
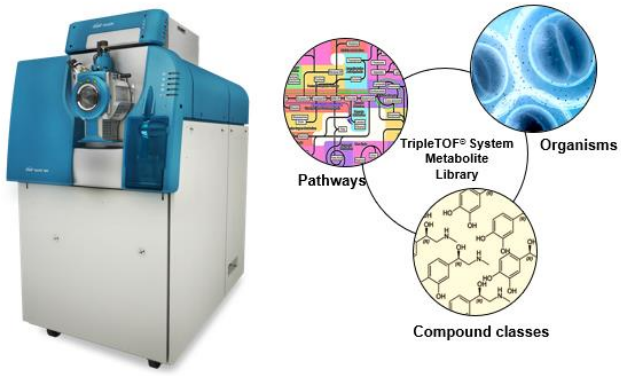


Figure 1. Targeted Metabolite Workflow using the TripleTOF System. Collect data using an untargeted approach and process the data using the accurate mass metabolite spectral library. Any metabolite identification is done upfront and confirmed using MS1, RT, isotope pattern and MS2 criteria. Then data can be transitioned for statistical analyses, quantitation and/or reporting.



Here, a streamlined targeted workflow is presented using the single injection workflow on SCIEX TripleTOF® 5600+ and 6600 Systems followed by data processing using MasterView software with a high resolution, accurate mass metabolite spectral library (Figure 1). 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 Features of Targeted Metabolite Screening

- Fast acquisition of high resolution, accurate mass MS and MS/MS data on the TripleTOF Systems enables a single injection data acquisition strategy
- Easy to use, targeted data processing using MasterView Software enables data to answers in 5 clicks
- Accurate mass metabolite spectral library contains over 500 compounds from most studied biochemical pathways and compound classes across many species/organisms
- Compound identification with high confidence based on automatic evaluation of:
  - Retention time, mass accuracy, isotope pattern and MS/MS
- Reduce data review time, get to biological answers faster
- Easy transition to quantitation & reporting

Materials and Methods

The accurate mass metabolite spectral library[2] contains a library of high resolution, accurate mass MS and MS/MS spectra. The library contains retention time information and all details required to collect the sample data are outlined in *Experimental Conditions* document. This document contains the LC and MS parameters needed to collect the data as well as solvent and column conditions. The workflow for collecting the data and processing for metabolite identification is shown in Figure 2. The data can then be easily transitioned to MarkerView™ for statistical analysis or MultiQuant™ for quantitative analysis such as generating calibration curves and reviewing data integration.

Chemicals: The following standards were used for testing in positive mode analysis: Creatinine, Dimethylglycine, Hippuric acid, Kaempferol, Phenylacetylglycine, Acetylputrescine, Glucose-1-Phosphate, Xanthosine, Taurine and 1-Methylhistidine. For negative mode analysis the following standards were used: Estradiol, Levofloxacin, Lathosterol, Quercetin, Suberic acid, Taurocholic acid, 3-Methylhistidine, Nicotinic acid, Phenylephrine and Omeprazole. Individual stock solutions of each standard were made up at 1 mg/mL.

Sample Preparation: Stock mixtures were diluted and calibrations curves were generated from 1000 ng/mL to 1 ng/mL mixtures.

Urine in Eppendorf tubes was centrifuged for 15 minutes at 20,000 *rcf* (relative centrifugal force) to remove the major particulates to the bottom of the tube. An aliquot of urine was diluted with 3 parts water. Each urine sample was spiked with the appropriate standard mixture to create a final concentration in the urine to be 1000, 100, 10 and 1 ng/mL. A sample of urine was diluted in 3 parts water to use as a blank. All samples were stored at –20°C until ready for injection.

Liquid Chromatography: A Shimadzu Prominence LC system was operated at a flow rate of 300 µL/min using acidic or basic conditions for the chromatography (Table 1).

- Mobile Phase A: 5mM ammonium formate in 1% methanol, adjusted to pH = 3 with formic acid.
- Mobile Phase B: Methanol.
- Mobile Phase C: 5mM ammonium formate in 1% methanol, adjusted to pH = 8 with ammonium hydroxide

An Acquity XSelect HSS T3 column (2.1 x 150 mm, 2.5 µm ID, Waters) was used which retains both polar and non-polar compounds.

Sample and standard mixture injections were made at 1 µL in triplicate to obtain reproducibility data across compounds investigated.

Table 1. Shimadzu LC Method for Both the Acidic and Basic Separation Conditions.

Step	Total Time (min)	Module	Event	Parameter (%)
1	*1.00*	*Pumps*	*Pump B Conc.*	*0*
2	*21.00*	*Pumps*	*Pump B Conc.*	*100*
3	*25.00*	*Pumps*	*Pump B Conc.*	*100*
4	*25.01*	*Pumps*	*Pump B Conc.*	*0*
5	*28.00*	*Controller*	*Stop*	*0*

Note: For acidic LC conditions use mobile phase A, for basic conditions use mobile phase C. Mobile phase B always stays the same.

Mass Spectrometry: Data was collected in IDA mode on the TripleTOF® 5600+ and 6600 Systems using a DuoSpray® Source. The mass range used for data acquisition was 50-800 m/z for MS and 40-800 m/z for MS/MS. Mass Spectrometry settings are shown in Table 2 below:

Table 2. TripleTOF System Acquisition Parameters.

Positive polarity:	***Negative polarity:***
Source/Gas Parameters CUR: 25 psi IS: 5500 V TEM: 500°C GS1: 50 psi GS2: 60 psi CAD: High Compound Parameters: DP: 80 V CE: 35 V CES: 20V	Source/Gas Parameters CUR: 25 psi IS: -4500 V TEM: 500°C GS1: 50 psi GS2: 60 psi CAD: High Compound Parameters DP: -80 V CE: -45 V CES: -25V
IDA criteria: Select 1 to 8 most intense peaks Which exceeds: 300 cps Exclude former target ions: Always After number of Occurrences: 3 For: 3 seconds Dynamic Background Subtraction: ON	

Value of the Metabolite Library

The high resolution accurate mass metabolite spectral library contains 536 metabolites which are found across various biochemical pathways, compound classes and across many species. The library contains compounds from the following classes:

- Amino acids
- Bile acids
- Nucleotides
- Nucleosides
- Carboxylic acids
- Sugars
- Catecholamines
- Organic acids
- Polyamines
- Simple lipids
- Steroids
- Vitamins
- Common drugs
- Common plant metabolites

The library was collected in collaboration with Professor Gerard Hopfgartner at the University of Geneva and his team. The spectra were transferred to SCIEX and the library was built in the LibraryView Software framework. In order to run the library, the MasterView Software add-on to PeakView® Software is required. The workflow for targeted metabolite screening using the TripleTOF is highlighted in the Figure 2. From the total ion chromatogram for a data dependent experiment, the subsequent extracted ion chromatograms can be mined. Both accurate mass of the parent and from the MS/MS are used to identify and then confirm a metabolite.

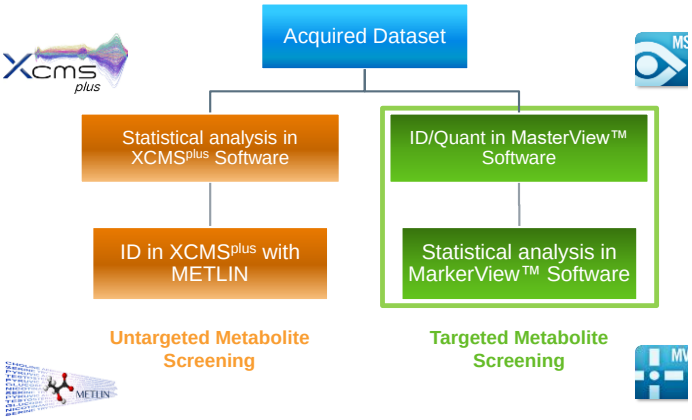


Figure 2. Targeted Metabolite Screening Workflow using the TripleTOF System. Using the approach on the right hand side: 1) Process the data using MasterView Software and the accurate mass metabolite to identify any metabolites in the data. 2) Transition the data to MarkerView Software for statistical analysis of the identified metabolites.

Workflow for Targeted Metabolite Screening using MasterView Software

Extracted Ion Chromatograms (XICs) are generated for all metabolites in the library, based on thresholds set by the user such as formula and expected retention time of all target analytes. The MS and MS/MS information is automatically evaluated if the detected XIC signal exceeds the user defined intensity threshold or signal-to-noise (S/N) (Figure 3). Data processing results are ranked based on 4 selectivity criteria, to provide a high degree of confidence in assigning compound identifications to detected compounds:

- Retention time matching
- Mass accuracy
- Isotope pattern fit
- MS/MS library searching

In addition, the intensity can be compared to a standard sample of known concentration to obtain quantitative information.

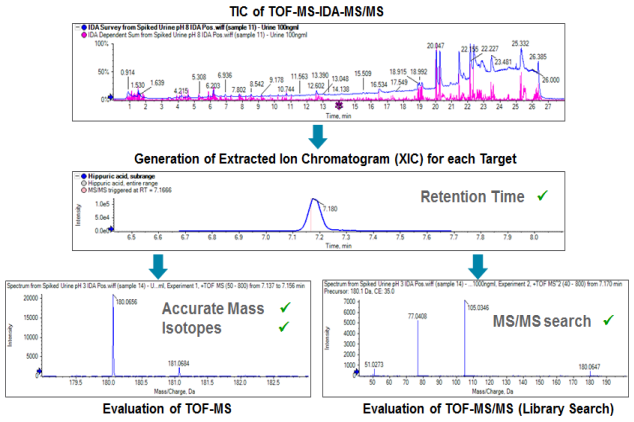


Figure 3. Data Dependent Acquisition of High Resolution MS and MS/MS Data for Identification and Quantification of Metabolites. The top trace shows the sum TIC of the full scan (in blue) and the MS/MS triggered during the IDA experiment (in pink). A compound is identified based on 4 criteria. The first two criteria are retention time and mass accuracy - the middle trace shows an extracted ion chromatogram (XIC) and the match to the retention time to the library compound. Second is mass accuracy and isotope pattern match, the plots on the bottom left highlights the match based on MS1 isotope pattern to the spectral library. The plot to the right highlights the match based on MS2 fragmentation pattern.

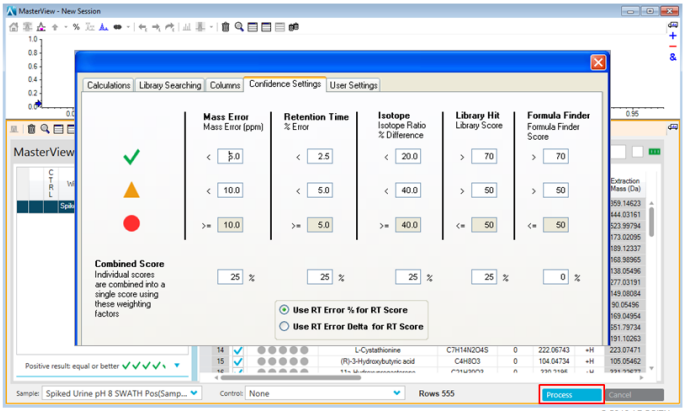


Figure 4. Confidence Settings in MasterView Software. Criteria used to pass, review or fail a parameter for metabolite identification and confirmation. A score is then calculated for the metabolite from the sample. A combined score can also be generated based on weighting to all parameters.

The confidence settings (Figure 4) can be set to define to the criteria which allows for matching the acquired data with either theoretical / library spectra. The criteria can be set for mass accuracy, retention, isotope pattern and MS/MS library matching. Once the data are processed, one can review the results using the “traffic lights” display as well as the MS and MS/MS spectra with library matches (Figure 5).

The results can then be directly reported from the templates available in MasterView software. Or the data can be transitioned for further processing in MultiQuant Software for more in depth quantitative analysis (Figure 6). Save the results with highest confidence as a MQ file by selecting the MQ icon from the toolbar. Import this file in MultiQuant Software as a ‘quantitation method from text” for generating any calibration curves and to review peak integration parameters.

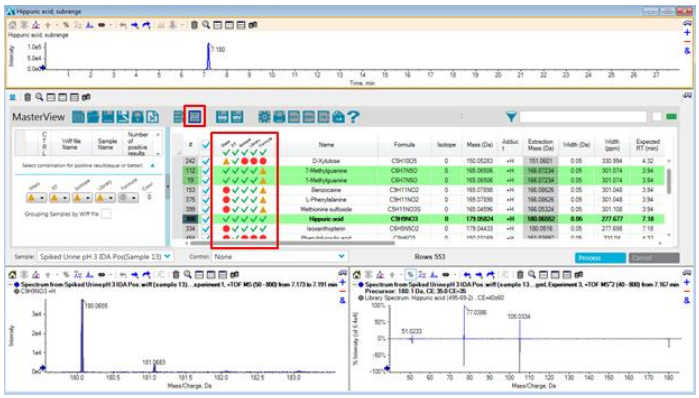


Figure 5. Review Data in MasterView Software. The data can now be reviewed and any matches against the database can be evaluated. The traffic light system allows easy review and a user can filter compounds identified with highest confidence by selecting all the green rows meaning these compounds passed the confidence settings set in Figure 4.

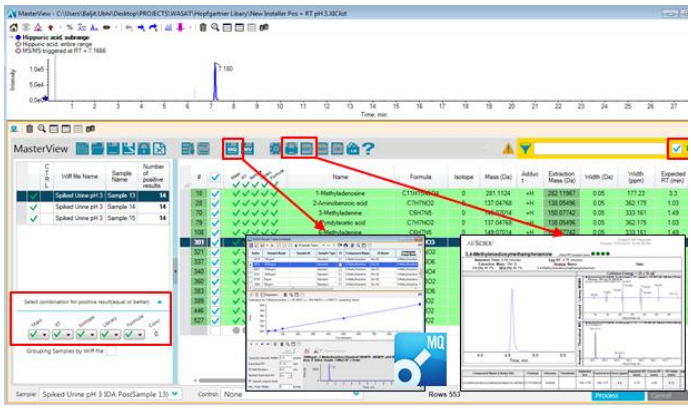


Figure 6. Transition for Quantitation and Report to MultiQuant Software. Generate automatically calibration curves for quantitation without having to build long quantitation methods manually.

The most powerful workflow is to be able to take the identifications found and transition this data for statistical analysis to MarkerView Software (Figure 7). The table can be saved as an MV file by selecting the MV icon from the tool bar. Open this file in MV as a “generic text file” and continue any statistical data analysis. Any principal component analyses can be generated as wells as t-tests and p values. The power here is that every peak in the loading plot has a name as it has been confidently identified from the accurate mass metabolite spectral library in MasterView Software. This drives the biological hypothesis faster than having to now complete an identification step.

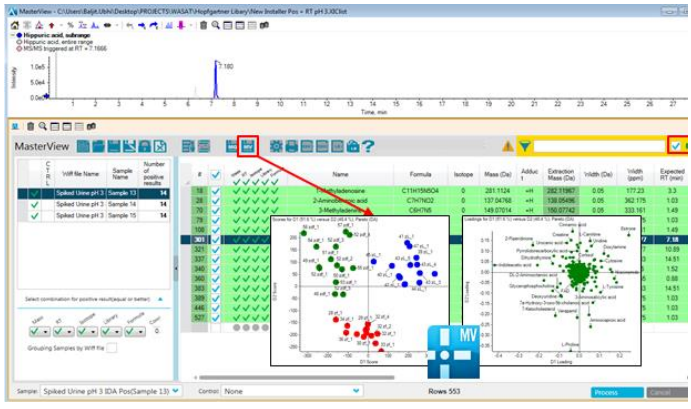


Figure 7. Transition for Statistical Analysis to MasterView Software. Generate any principal component analysis and generate biological interpretation faster as results in the loadings plot (centre right) are already identified. Combine with t-test analysis and rank the significantly differential metabolites by p-value.

Employ the comparative screening tool in MasterView Software to compare all the samples versus a control. After loading the data into MasterView software, specify the control as well as the sample. Metabolites meeting the acceptance criteria as well as threshold (set by the user for comparative results) are indicated as green rows. The threshold can be a factor, i.e. 2 times or 10 times or can be set in terms of intensity and cps (counts per second). This is a quick way to take a snapshot of what is changing on a per sample basis. Although it is not true metabolomics where all samples are compared together, a user can use to screen and quickly capture any major changes compared to a control/baseline sample.

Validation of Library Compounds in a Biological Matrix

A subset of compounds from the total library of compounds was used to analyze metabolites present in a complex biological matrix. Compounds were spiked in at varying concentrations into a diluted urine sample and measured on both the TripleTOF 5600+ and 6600 Systems. Data was collected in both positive and negative ionization modes as well as at acidic and basic conditions. The IDA results are listed in Table 3.

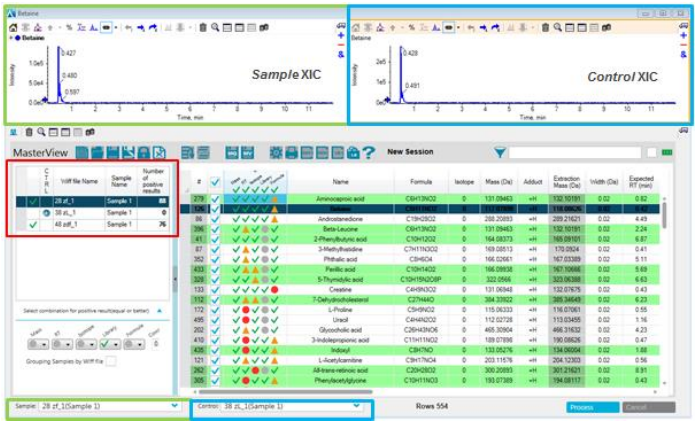


Figure 8. Comparative Screening in MasterView™ Software. A sample is compared to a set control/baseline sample. This can be a healthy/wild-type or blank sample. Setting a threshold means any metabolite highlighted as a green row has passed the acceptance criteria. This quickly gives the user a snapshot of any major changes in metabolite concentrations between their samples. Note this is on a per sample basis.

Conclusions

A new high resolution accurate mass metabolite spectral library was used to verify spiked compounds in a urine matrix and confirm their scoring against MS/MS spectra in MasterView software. Data collected using the single injection approach with IDA means that high resolution MS and MS/MS data are collected simultaneously. For complete metabolite coverage samples should be acquired in both positive and negative ionization modes and at both acidic and basic conditions.

The acquired data can then be reviewed using the “traffic light system” in MasterView Software and then be easily transitioned for reporting, quantitation and statistical data analysis can be acquired simultaneously. The library was validated on both TripleTOF 5600+ and 6600 Systems.

References

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5. MasterView Processing and Review Video: https://na10.salesforce.com/069F0000000tp6V
6. MasterView Software Sales Tool Kit: https://na10.salesforce.com/sfc/#version?selectedDocumentId=069F0000000tu0X

Table 3: Results from the MS/MS Spectral Library Matching, including Purity Scores. Data was collected in both positive and negative ionization modes as well as at acidic and basic conditions for complete metabolite coverage. Data was collected on both the TripleTOF 5600+ and 6600 systems.

Compound	pH	Polarity	MS/MS Purity 5600 (%)	MS/MS Purity 6600 (%)	Comments
Hippuric acid	3	*Positive*	*94.8*	*95.9*	
Kaempferol	3	*Positive*	*99.3*	*97.3*	
N-Acetylputrescine	3	*Positive*	*99.7*	*60.3*	
Phenylacetylglycine	3	*Positive*	*100*	*94.3*	
Xanthosine	3	*Positive*	*97.2*	*88.3*	
1-Methylhistidine	3	*Positive*	*100*	*81.0*	
Suberic acid	3	*Positive*	*94.8*	*95.8*	
Taurocholic acid	3	*Positive*	*100*	*100*	
3-Methylhistidine	3	*Positive*	*99.5*	*80.2*	
Nicotinic acid	3	*Positive*	*100*	*80.3*	
Omeprazole	*3*	*Positive*	*98.3*	*96.3*	
Kaempferol	*3*	*Positive*	*99.8*	*99.4*	
Hippuric acid	*8*	*Positive*	*100*	*94.5*	
Kaempferol	*8*	*Positive*	*100*	*97.4*	
N-Acetylputrescine	*8*	*Positive*	*100*	*67.1*	*Isotope interference for 6600*
Phenylacetylglycine	*8*	*Positive*	*100*	*94.6*	
1-Methylhistidine	*8*	*Positive*	*99.7*	*81.0*	
Suberic acid	*8*	*Negative*	*N/A*	*77.5*	*No MS/MS acquired on 5600 for this precursor*
Taurocholic acid	*8*	*Negative*	*100*	*100*	
3-Methylhistidine	*8*	*Negative*	*94.7*	*81.7*	
Omeprazole	*8*	*Negative*	*N/A*	*97.4*	*No MS/MS acquired on 5600 for this precursor*

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