

Accurate Mass Screening Workflows for the Analysis of Novel Psychoactive Substances

TripleTOF® 5600+ LC/MS/MS System with MasterView™ Software

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Overview

In forensic toxicology testing labs, workflows are trending. Routinely, labs have been tasked with doing targeted analysis, monitoring samples for a standard list of known drug compounds and quantifying any of those targeted compounds that are found. These days with the emergence of many different novel psychoactive substances (NPS), labs are being faced with the challenge to do more surveillance screening. In this workflow, they are required to explore for any compounds that might be present in their samples without any pre-existing knowledge of what those compounds could be.

NPS are structurally and/or pharmacologically related psychoactive drugs. Before or upon emergence, they are not controlled or scheduled and so they can be legally circulated. Some common NPS are derived from the phenethylamine, cathinone ('bath salts') and cannabinoid drug frameworks synthesized in clandestine laboratories and marketed over the internet as 'legal highs'. In response to legislative efforts to these NPS a constant cat and mouse scenario is created as new NPS emerge on a monthly basis in order to overcome the drug laws.

AB SCIEX TripleTOF® LC/MS/MS system provides the acquisition power to screen for and identify compounds in complex matrices. TripleTOF® technology enables the acquisition of high resolution and accurate mass MS and MS/MS information on all peaks observed in any given sample. These information-rich data files can offer insight into the many drugs and metabolites present in a given sample.

The strength of the workflow lies in the software, where PeakView® software with the MasterView™ add-in enables high throughput data analysis, allowing the information rich data files to be quickly screened for both targeted and non-targeted compounds.

The usual workflow in unknown screening for novel psychoactive substances is to analyze the suspicious offenders, discover the unknowns and then add to the routine analysis of targeted drugs. This technical note describes this workflow starting with the



untargeted analysis and adding identified unknowns to the targeted list and the ability to use the TripleTOF® for routine, targeted analysis.

Introduction

Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) is a widely used analytical tool for the screening of compounds and metabolites. Triple quadrupole based mass analyzers operated in Multiple Reaction Monitoring (MRM) mode deliver highly selective and sensitive quantitative results, but are limited to targeted screening only.

With an increasing demand for retrospective and non-targeted analyses of forensic toxicological samples, full scan mass analyzers are gaining popularity. Finding potential NPS in complex samples is hard. Samples contain a mix of healthy/normal components, vitamins, proteins, antioxidants etc. as well as known high-risk targeted compounds. This makes detecting and identifying NPS challenging especially when considering the low therapeutic levels that these NPS generally have and are therefore found in low concentrations. A sensitive method is also required when considering the need to detect the compound sometime after its intake. Urine analysis can be challenging if considering the parent drug only and a high phase metabolism and conjugate cleavage is not performed. The AB SCIEX TripleTOF® systems allow the acquisition of highly sensitive full scan single MS spectra with high resolution and mass accuracy. In addition, Information Dependent Acquisition

(IDA) can be used to collect MS/MS spectra with unmatched speed for accurate and reliable compound identification using MS/MS library searching.

The complexity of such data requires powerful data mining tools. The MasterView™ software can be used for targeted and non-targeted processing of high resolution MS and MS/MS data.

Discovering and Identifying Non-targeted or Unknown Compounds

Non-targeted peak finding algorithms will find thousands of molecular features in a sample, including chemical background, matrix components and unexpected compounds of interest. Figure one shows an example in which a non-targeted extraction of the components of this unknown sample resulted in 1046 extracted peaks. The majority of these peaks will be normal 'healthy' endogenous chemical compounds and the challenge is to identify the significant exogenous components. But how can one find the low level toxic compounds amidst everything else? It would be impossible to investigate every single peak effectively and efficiently, and many of those peaks may be of little interest to a forensic scientist— for example, endogenous components such as sugars, amino acids and nutrients may not be of interest when looking for exogenous, foreign material.

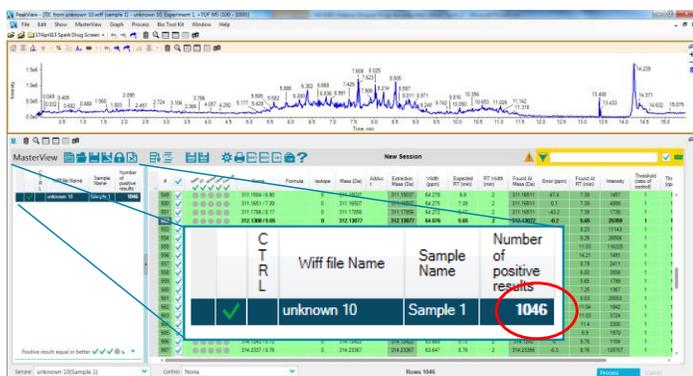


Figure 1. Non-targeted (unknown) screening and identification; Non-target XIC generation using a non-targeted peak finding algorithm will find thousands of molecular features in a sample. Only a handful will be significant components.

The best approach to eliminate the endogenous chemical compounds in a sample is to perform a comparative unknown screening analysis. Comparative screening is simply the comparison of your unknown sample against a known control

sample; comparing a potentially suspect urine sample with a clean urine sample blank for example. The comparative screening process enables the comparison of 2 samples to reduce the list of compounds from thousands to, in this example, 10 – this will eliminate the flagging of compounds that may be 'normal' for that type of sample and will allow the user to get to the answer of what is in the sample, particularly any components that are not 'normal' for that sample, faster and more accurately.

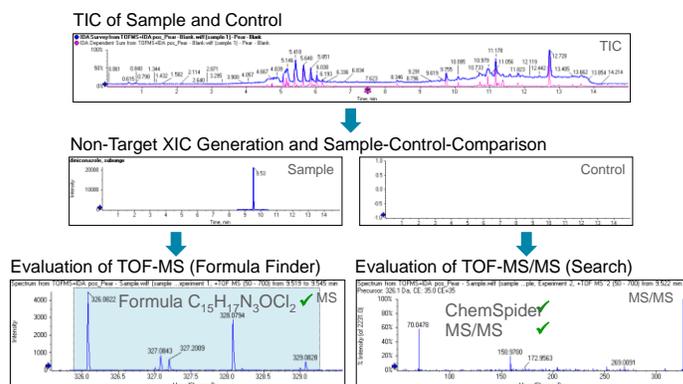


Figure 2. Automatic Non-targeted (Unknown) Screening Workflow

MasterView™ Software has a powerful non-targeted peak finding algorithm that extracts peaks present in the sample. The software can be programmed to automatically perform empirical calculations of potential molecular formula for the extracted mass of the unknown compound using both MS and MS/MS data. Automatic formula finder will produce a suggested molecular formula based on the TOF-MS peak and the isotope pattern. These are the first two clues to help identify what that peak might be. The algorithm for the automatic formula finder uses the MS and MS/MS data to come up with the best possible formulas that match the extracted mass. Once a potential formula has been determined, it can be searched against a ChemSpider database for suggested structures. The structures retrieved from the database are linked to the collected MS/MS spectrum for that extracted mass. A plot of the predicted MS/MS fragmentation pattern can then be made against the collected MS/MS data. This allows you to identify potential structures that match the extracted peak. Since many molecular formulas can give rise to the same accurate mass, it is only through the MS/MS fragmentation data that reliable identification of that peak can be achieved and confirmation that the structure retrieved from the ChemSpider database is the correct one.

The Non-Targeted Screening and Identification Data Analysis Workflow

1. Setting-up your XIC table and processing parameters for unknown screening

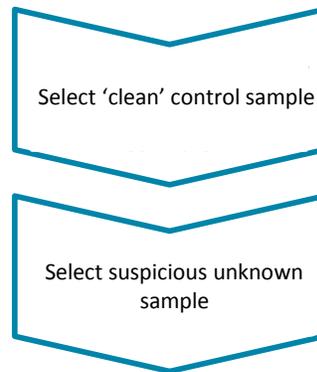
Open one or more data files for unknown (comparative) screening in PeakView® software

Start a new session in the 'MasterView' menu

Select Non-Targeted Peak Finding and 'Peak Detection Sensitivity' level

Figure 3. The MasterView™ Software with two samples loaded and ready for comparative unknown screening. To start the non-targeted peak finding algorithm simply click the highlighted bottom.

- The MasterView™ Software can be used to explore chromatograms using a non-targeted peak finding algorithm to find and identify unexpected drugs.
- Browse for data files anywhere on your computer or network for comparative unknown screening (i.e. sample-control-comparison).
- Select which sample is to be used as the control; the unadulterated sample matrix, blank urine sample in this example.
- Select the suspicious sample using the drop-down menu below the XIC table.
- Note that the XIC table is left blank. No information on expected masses or retention times is required.



2. Performing comparative unknown screening on your data using MasterView™ Software

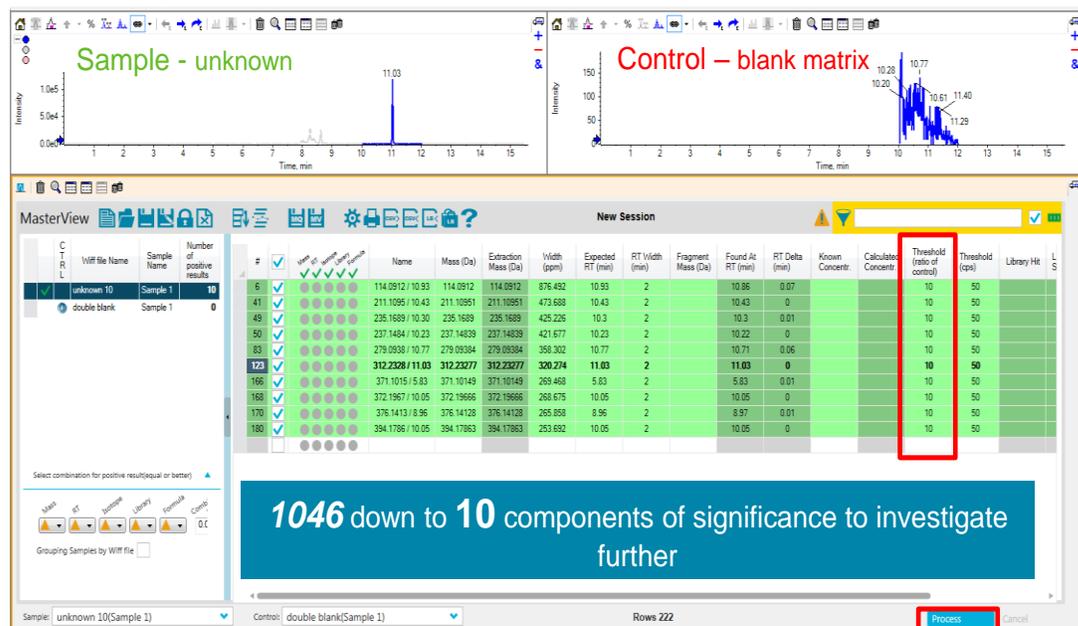
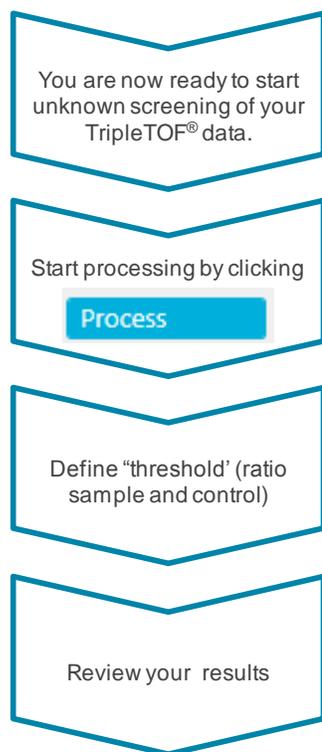


Figure 4. The result display of the MasterView™ Software: Quickly narrowing down to the significant components

- Once the processing is completed, define a threshold for comparison (ratio sample and control).
- A threshold of 10 will highlight all compounds in the XIC table which are present in the suspicious sample with a signal at least 10x higher than in the clean control sample.
- XICs with a higher signal in the unknown sample than in the control are highlighted in green. Click the 'Display Highlighted XIC Only' button in the top right of the XIC table to display only these XICs.
- XICs of all found compounds in the two samples selected are displayed in the chromatogram pane (Figure 2 top).
- Spectra can be reviewed in the MS and MS/MS pane (Figure 3 bottom).
- Spectra of non-identified compounds can be further processed in PeakView® software.

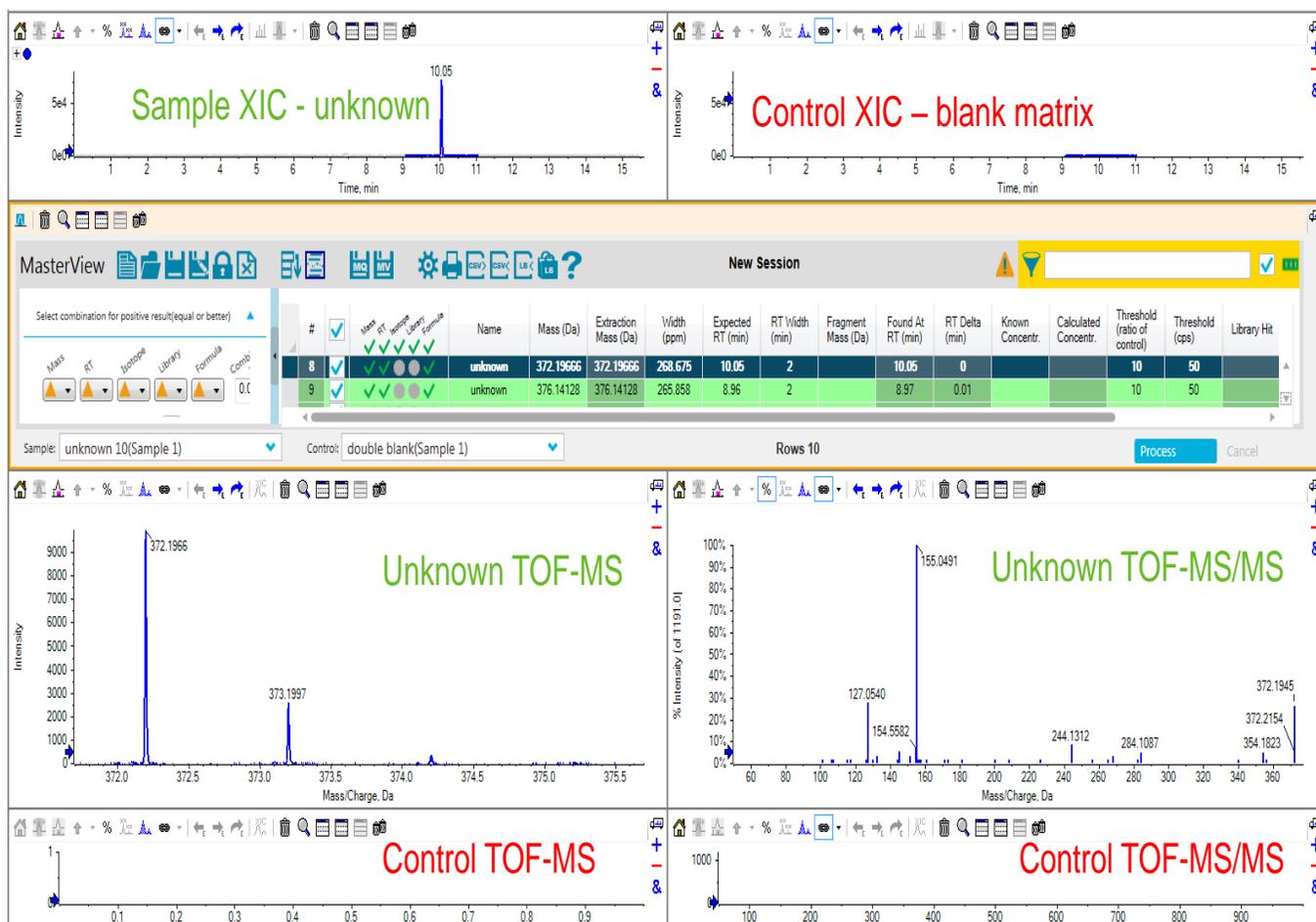


Figure 5. The result display of the MasterView™ Software: Non-targeted peak finding and automatic comparison of an unknown urine with a blank urine control. A number of peaks were identified, the example shown is at m/z 372; RT 10.05 min and an MS/MS was acquired for it. This non-identified compound can be further processed in PeakView® software.

3. Identifying unknown compounds using empirical formula finding and automatic ChemSpider search

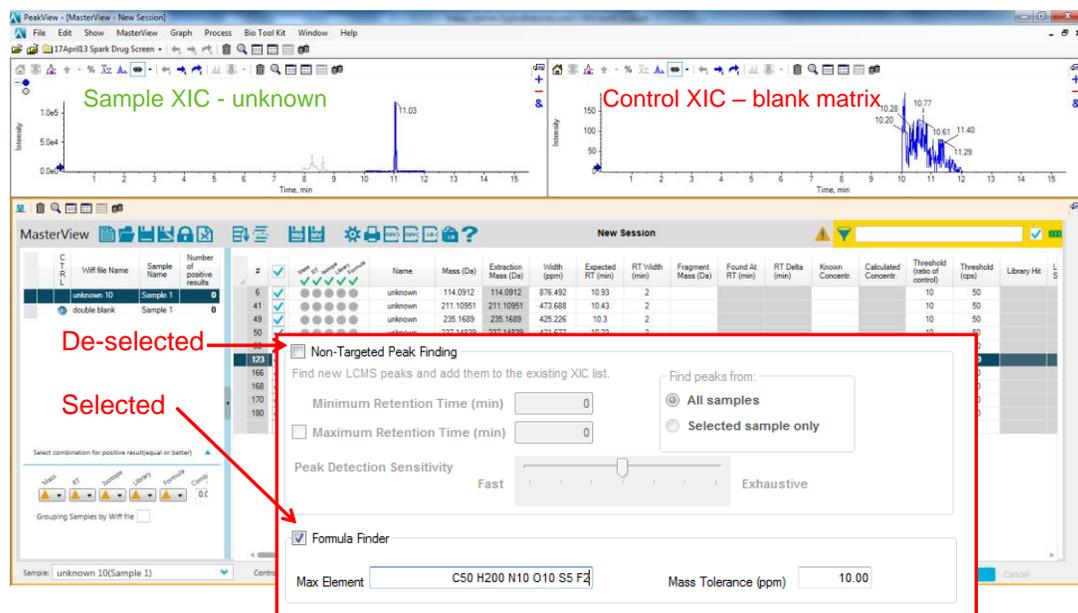


Figure 6. Unknown identification using PeakView® software: The significant extracted components are treated as a targeted list and automatic formula finding operation is performed.

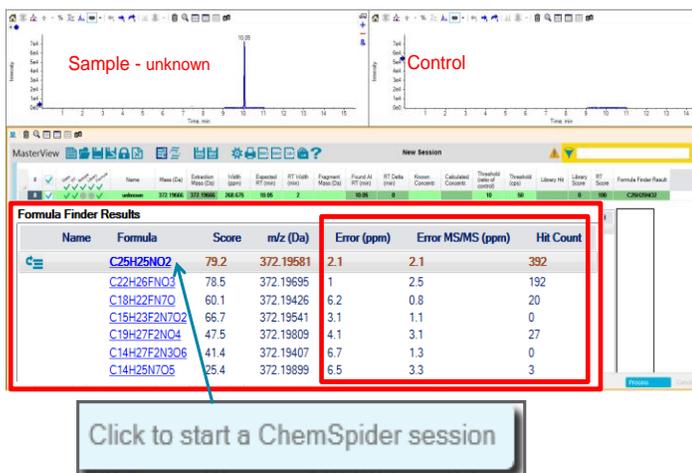


Figure 7: A ChemSpider search session can be started by clicking on a formula.

- MasterView™ software automatically empirically calculates potential molecular formula for the extracted mass of the unknown compounds using both MS and MS/MS data when the 'Formula Finder' check box is selected.

- The Formula Finder automatically uses accurate mass information of the molecular ion, adducts, isotopic pattern, and fragment ion information to empirically calculate potential molecular formulas for the detected compound.
- Click on 'Show XIC Details' to show the 'Formula Finder Results'
- Review the MS and MS/MS ranking. The mass errors for the suggested formula are shown.
- The possible structures hit count from the automatic ChemSpider search for all calculated formulas are recorder in the 'Hit Count' column allowing homing in on which formula to investigate further
- An automatic ChemSpider session can be started by clicking on a highlighted formula
- You can further evaluate structures in the PeakView® ChemSpider session window. This feature links the selected structure to the acquired MS/MS data for the extracted mass and compares the structure with known fragmentation pathways and reports how many fragments of the MS/MS spectrum match.

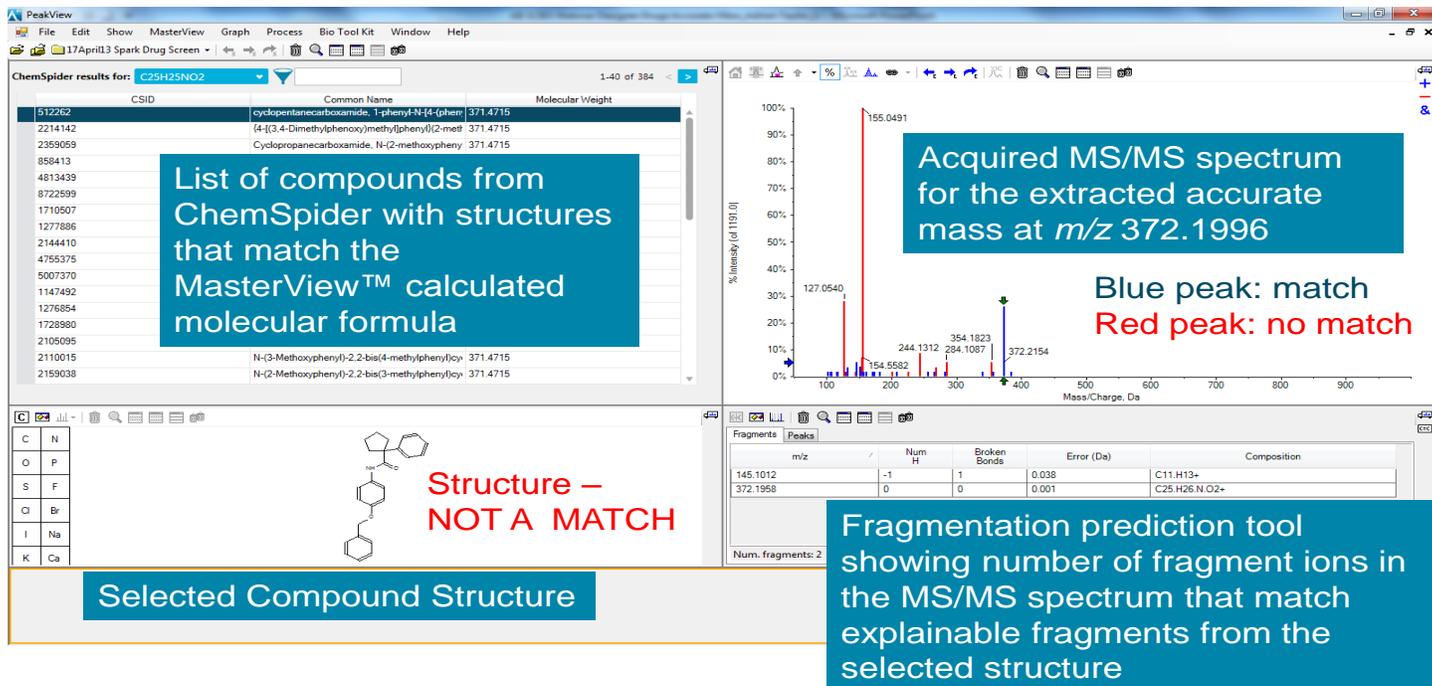


Figure 8. Automatic ChemSpider Search Session; ChemSpider hits are automatically compared against MS/MS spectrum for interpretation

- The ChemSpider session starts by searching ChemSpider for structures that match the calculated formula.
- After the search is complete, a list of matching compounds is displayed which is automatically sorted by the reference count.
- By highlighting a compound in the list, its corresponding structure is displayed underneath.
- The displayed structure is linked to the MS/MS

spectrum displayed in the top right pane.

- Ions in the MS/MS spectrum that are blue match a fragment in the selected structure. Ions that are red do not match any explainable fragment from the selected structure.
- The first structure in the list only has one blue ion in the MS/MS spectrum, the others are all red. This is not the structure for this unknown compound (Figure 8)

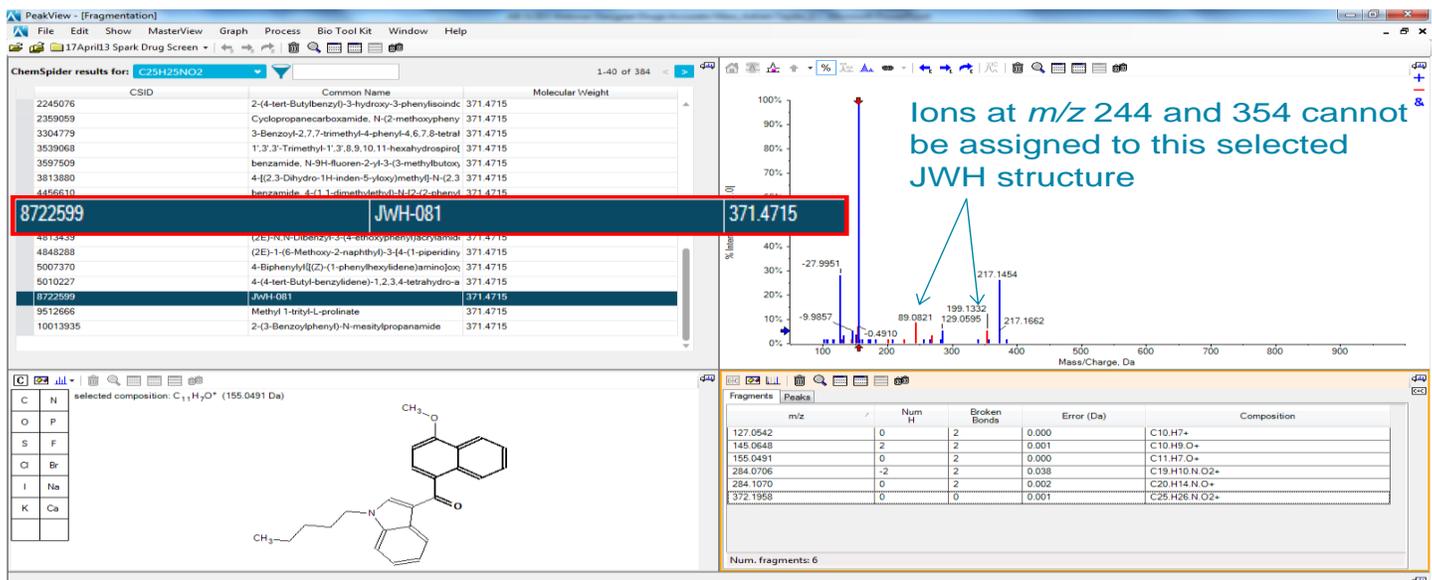


Figure 9. Narrowing the unknown identification to a compound class

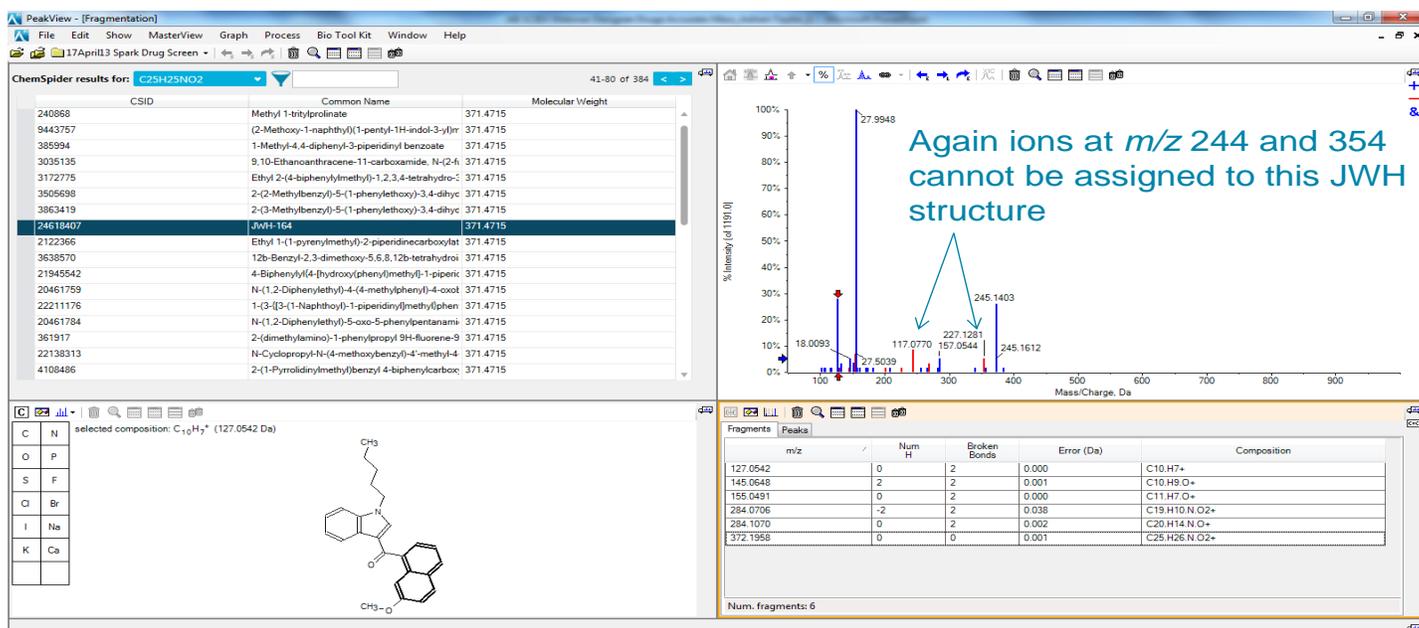


Figure 10. Further evidence to the JWH compound class but again not all MS/MS ions can be assigned to this particular K2 Spice compound

- When continuing down the list the structure for JWH-081 fragments to match the majority of MS/MS fragment ions. However, the ions at m/z 244 and 354 cannot be assigned to this selected JWH structure.
- Continuing further down the list, another JWH structure matched well to the MS/MS spectrum but again not all ions can be assigned to a fragment from this structure.
- At least at this point we can be confident as to the class of compound, but which compound is it?
- With further investigation into the list of compounds a structure is found in which all MS/MS fragment ions in the product ion spectrum are explainable.
- 6 of 6 peaks matched with 100% explainable MS/MS fragment ions for the JWH-019 5-hydroxyhexyl metabolite novel psychoactive substance (Figure 11).
- We have a formula and a retention time for this extracted unknown peak; C₂₅H₂₅NO₂ at retention time 10.05 min. This information can be added to the targeted XIC list for future targeted analysis.

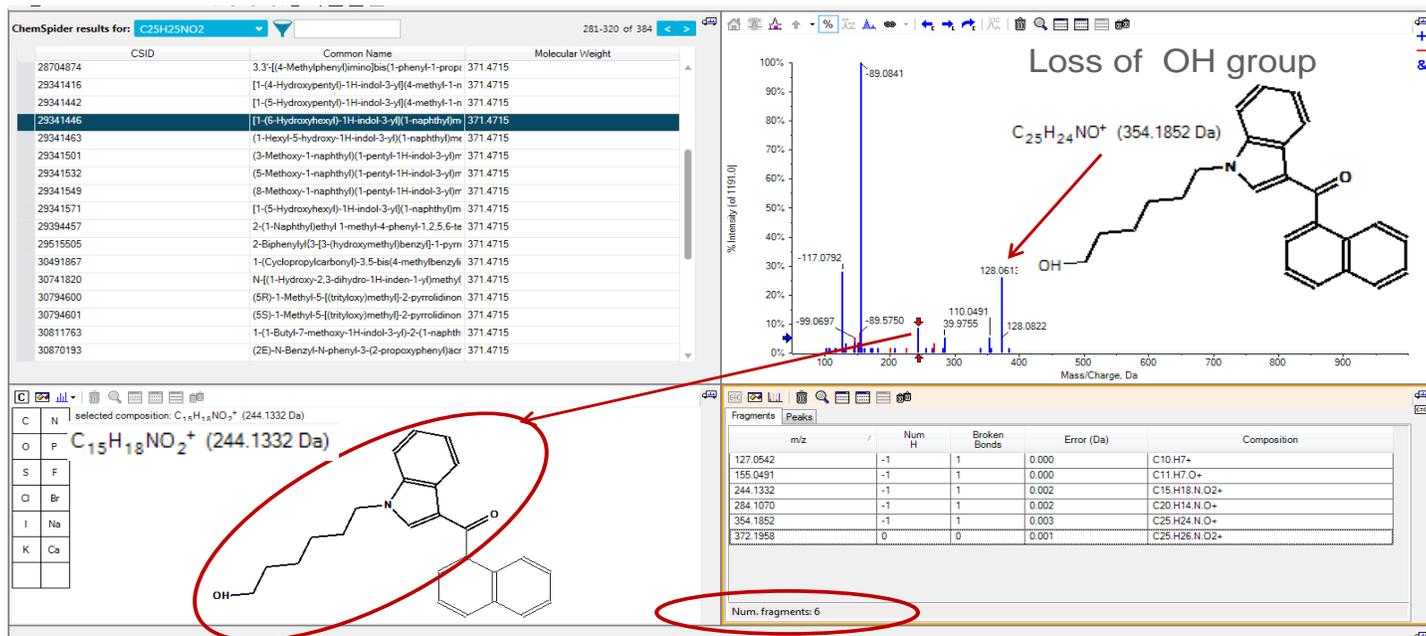


Figure 11. Closest Structural Match; 6 of 6 peaks match with 100% explainable MS/MS fragment ions for the JWH-019 metabolite

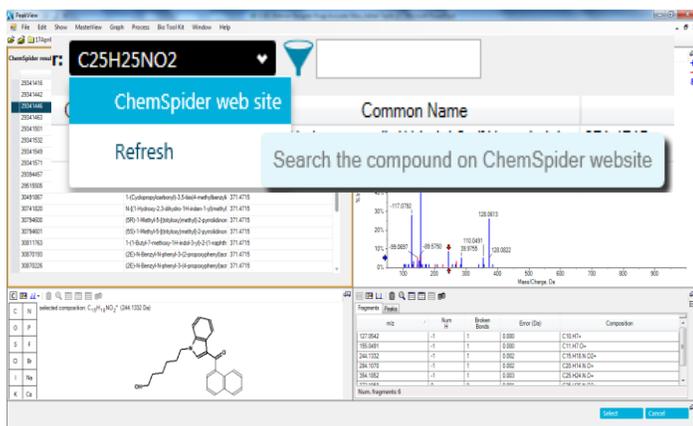


Figure 12. Searching the compound on the ChemSpider Website

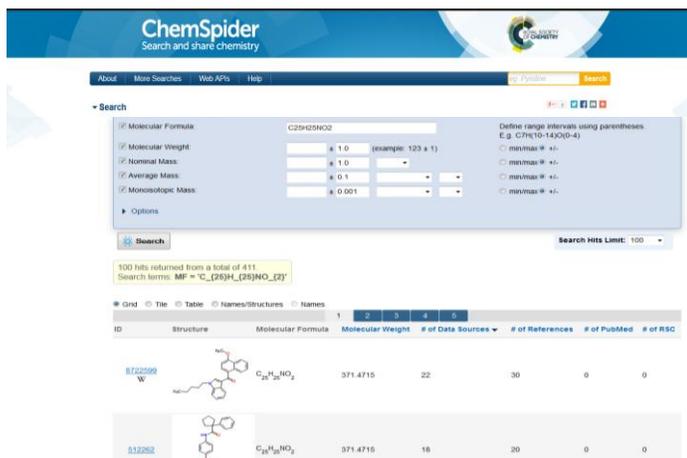


Figure 13. ChemSpider Link; Allows searching based on number of references and data sources

- With a direct link to the ChemSpider website, the compound can be searched regarding references and data sources.

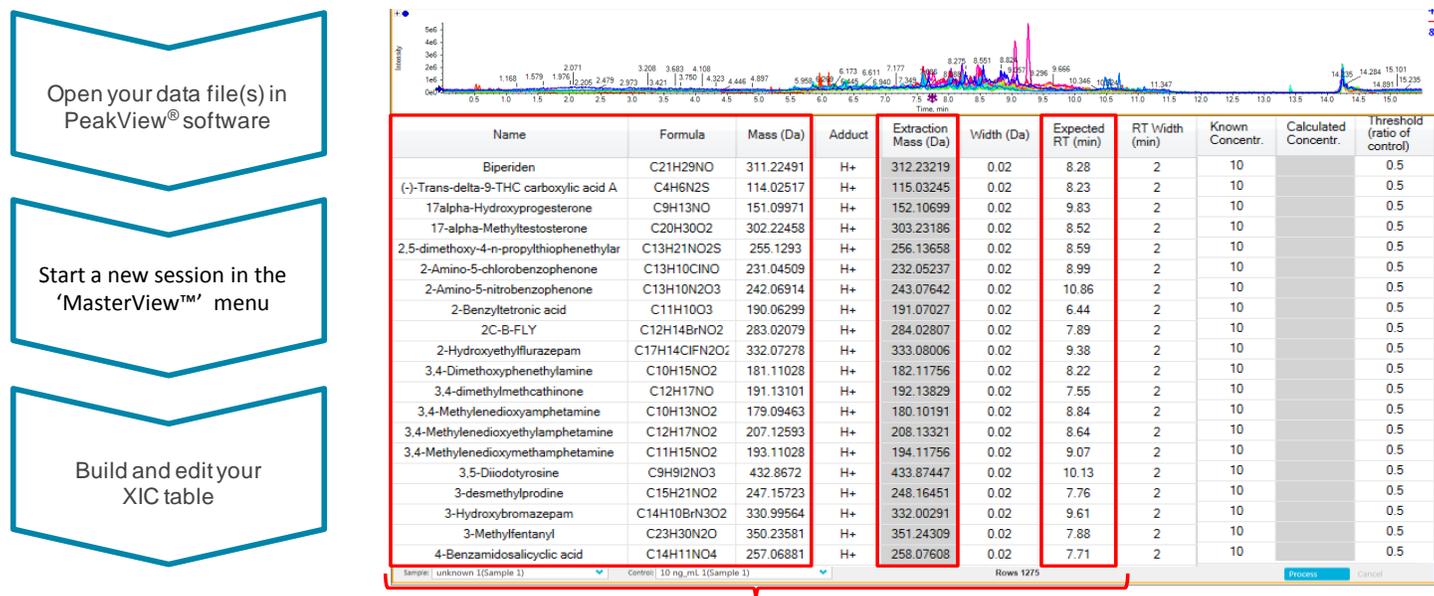
The above results are examples of using the comparative screening function using a non-targeted peak finding algorithm to find unknown compounds in complex forensic samples.

A simple MS/MS library search against the 'All-in-One' High Resolution MS/MS Spectral Library can identify unexpected compounds using this workflow. The Formula Finder and automatic ChemSpider search can be used for identification if the library search does not return a hit for a compound of interest.

Any unknowns that are found can easily be added to the targeted analysis by including the formula and retention time to the targeted XIC list. This now becomes part of the routine analysis workflow which will be demonstrated next.

The Data Analysis Workflow for Targeted Screening and Quantification

4. Setting-up your targeted compound database (XIC table), library and processing parameters



Screening using 1275 extracted ion chromatograms (XIC)

Figure 14. The MasterView™ Software with an XIC table filtered for targeted drug screening and identification

- The XIC table defines a list masses or formulas to generate extracted ion chromatograms (XIC) for targeted compounds.
- The XIC table allows a list of compounds and masses to be modified based on the compounds of interest to your laboratory.
- The columns in the table, including compound name, formula, isotope, adduct, retention time (RT), width, and others, can be edited based on your methods and needs (Figure 14).
- Note that a mass must be entered in the 'Extraction Mass' column in order for the compound to be searchable in your database.
- The 'Extraction Mass' can be entered manually or is calculated automatically based on the formula, isotope, and adduct provided.
- The generated XIC table can be saved for future processing.
- Confidence settings for compound identification based on mass error, retention time error, isotope matching, library searching purity scores, Formula Finder result and combined scoring can be adjusted based on your laboratories' quality control criteria (Figure 15).
- Select your mass spectral library for automatic MS/MS library searching in the 'Options' dialog box.

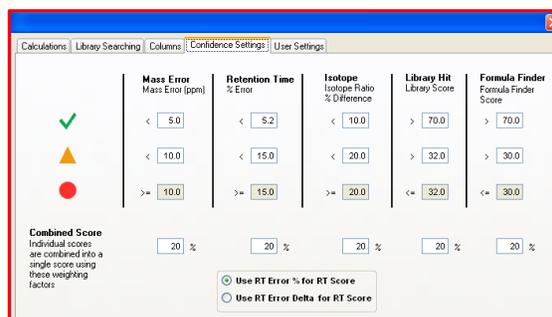


Figure 15. Confidence settings for compound identification

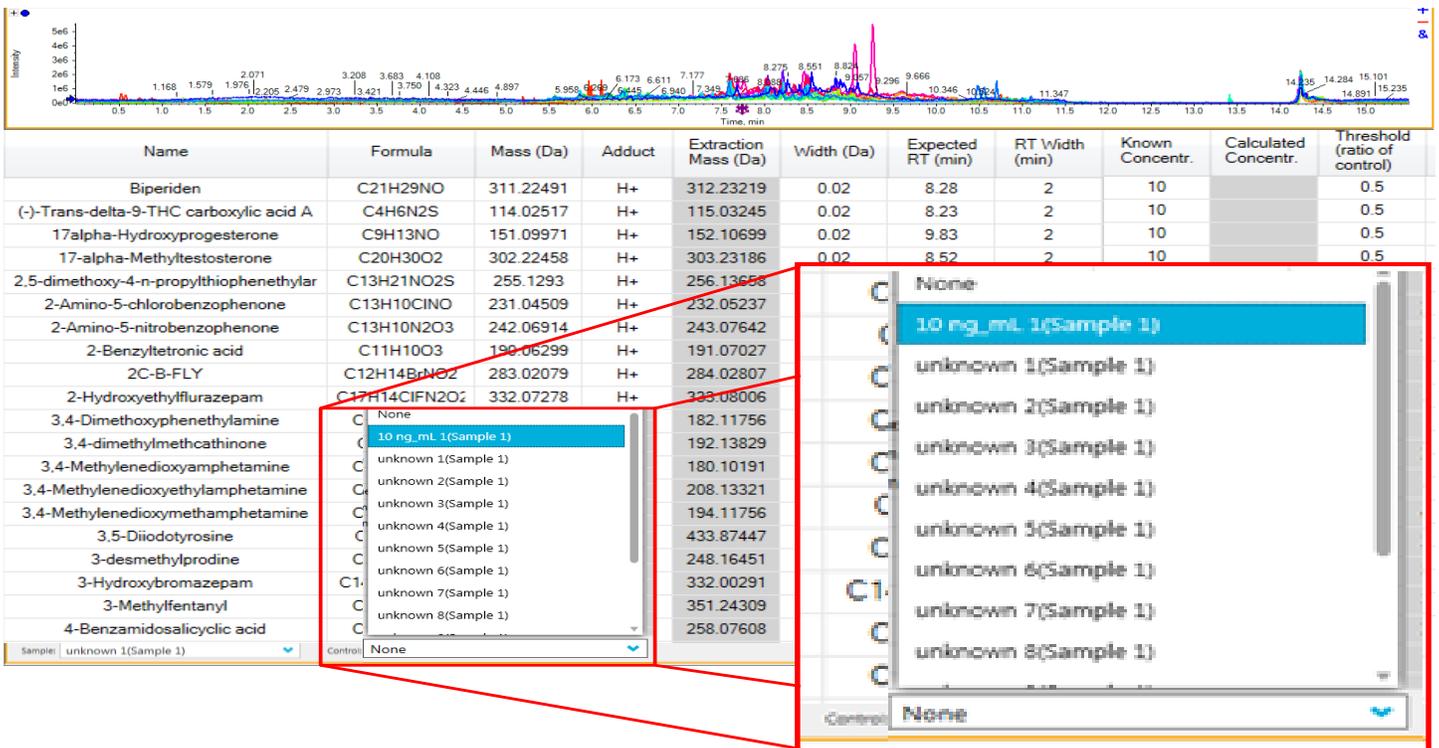
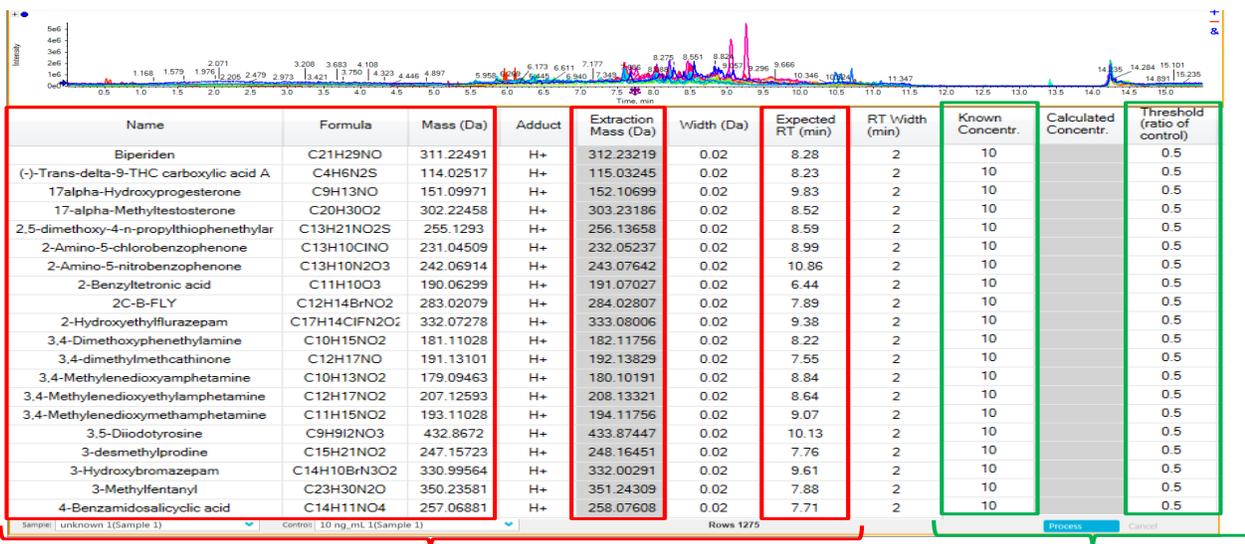


Figure 16. Selecting from the list of samples what is the control; in this case a spiked urine containing all the targeted drugs at a concentration of 10 ng/mL.

Input control concentration and ratio threshold of the control



Screening using 1275 extracted ion chromatograms (XIC) Quantitative comparison

Figure 17. Inputting the control concentration (for single point calibration) and ratio threshold of the control

- MasterView™ Software allows the comparison of an unknown sample to a standard of known concentration that is used as the control. The standard is a blank matrix spiked with all the target compounds at their respective cutoff concentrations.
- MasterView™ Software automatically performs a one point calibration and calculates the concentration of the identified analyte in the unknown sample. By specifying a threshold ratio, all compounds above the cut off threshold will be highlighted in green. This allows a quick way of identifying compounds in the unknown sample that are above the cutoff concentration.

5. Analyzing your data using the MasterView™ Software

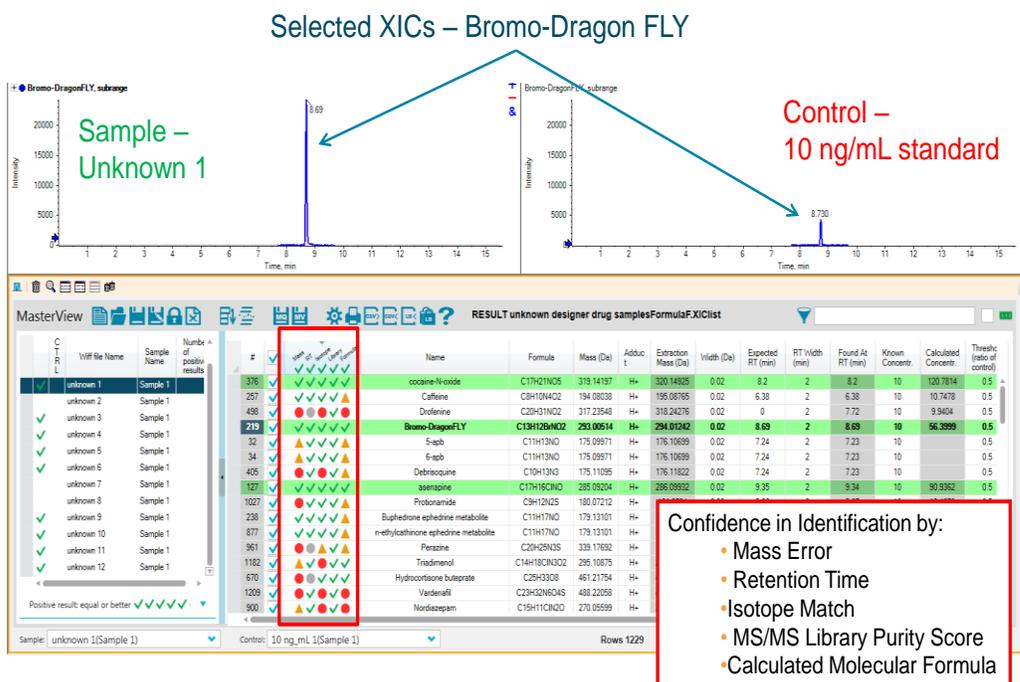
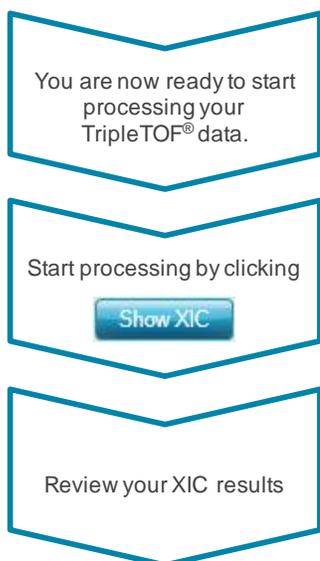
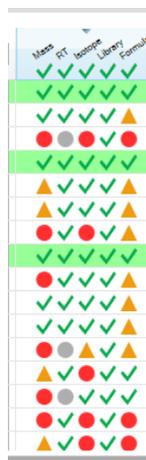


Figure 18. The result display of the MasterView™ Software: the 259 spiked drugs were identified with high confidence in spiked equine urine sample

- XICs of all targeted compounds are displayed in the chromatogram pane (Figure 18 top).
- XICs above a user defined intensity threshold are highlighted in green.
- Compound identification is based on chromatographic and mass spectrometric information, including RT error (min or %), mass error (ppm or mDa), isotope matching, and library search results (Figure 21; middle).
- Identification criteria are visualized using 'traffic lights'.



Review each sample at a glance to see which samples contain positive hits

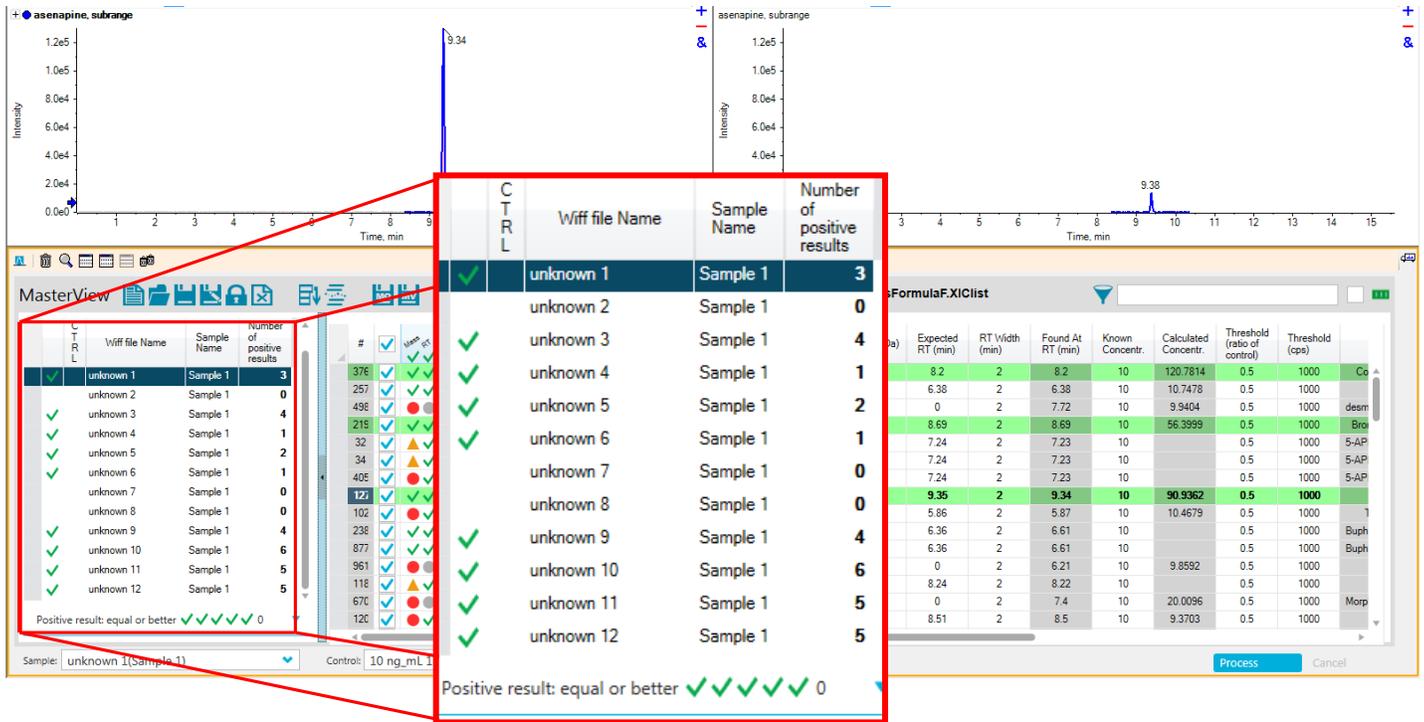


Figure 19. Reviewing results for each sample; identifying quickly which samples contain positive hits

6. Reviewing your results using the 'traffic lights' and the spectra display

- Monitor the 'traffic lights'
- The traffic lights visualize the confidence of your data.
- Review MS and MS/MS spectra by clicking

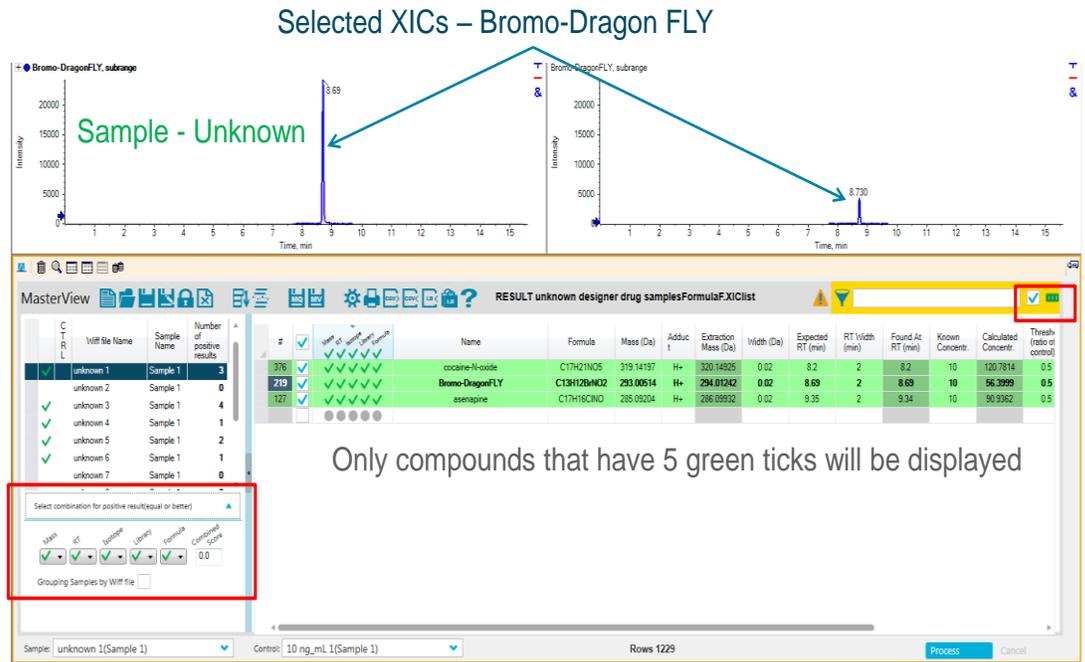
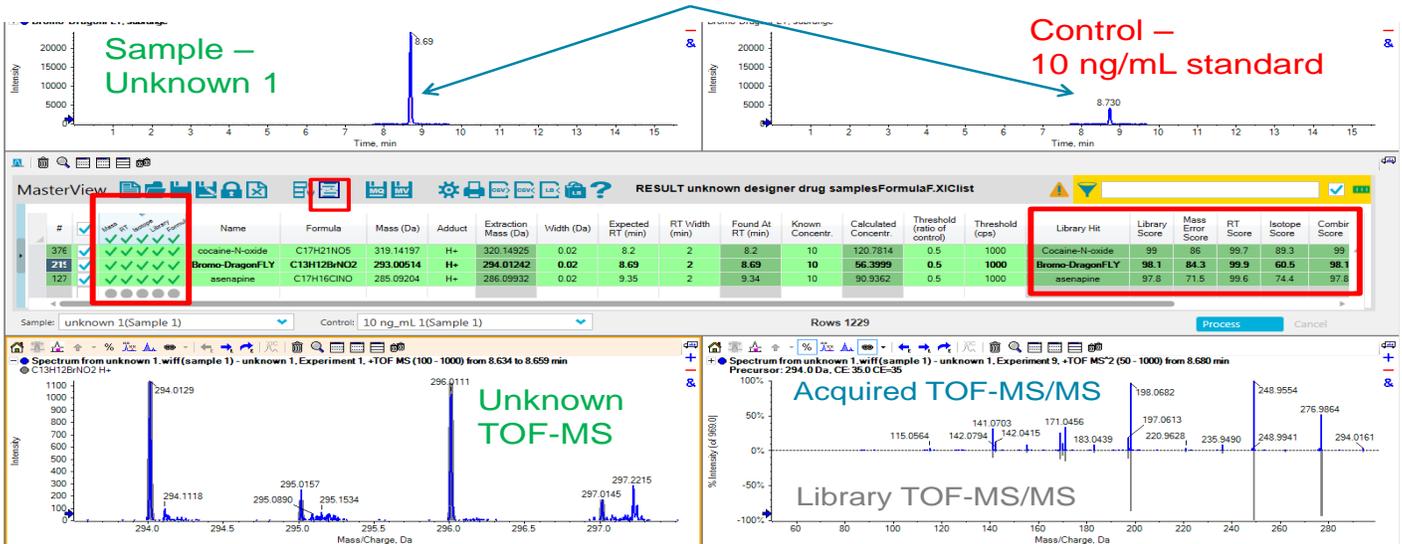


Figure 20. The result display of the XIC Manager: the drugs cocaine-N-oxide, bromo-Dragon FLY and asenapine were identified with high confidence in an unknown urine sample, based on accurate mass, isotope pattern, retention time and MS/MS library match.

Selected XICs – Bromo-Dragon FLY

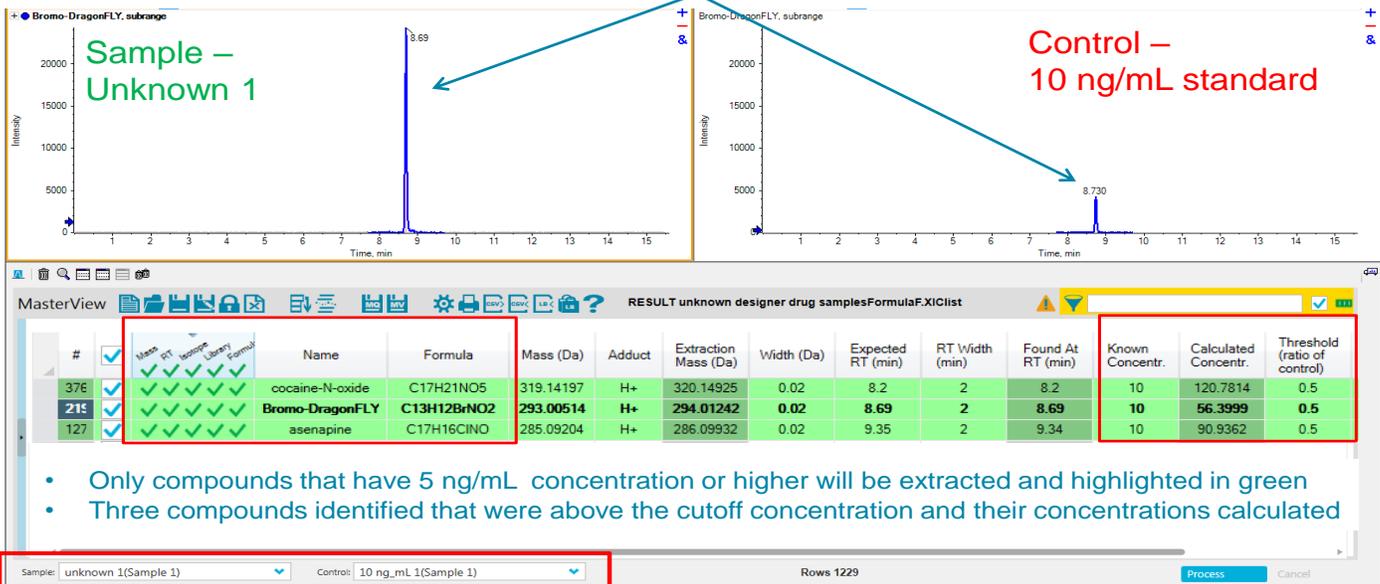


mirror view of spectra for easy comparison

Figure 21. Reviewing MS and MS/MS data

Comparative Screening and Identification - Quantitative Comparison Finding Compounds > cutoff

Selected XICs – Bromo-Dragon FLY



- Only compounds that have 5 ng/mL concentration or higher will be extracted and highlighted in green
- Three compounds identified that were above the cutoff concentration and their concentrations calculated

Figure 22. Comparative Screening and Identification. Single point quantification, quickly identifying compounds above a cutoff concentration

Review other samples in the batch

Selected XICs – JWH-019 5-hydroxyhexyl metabolite

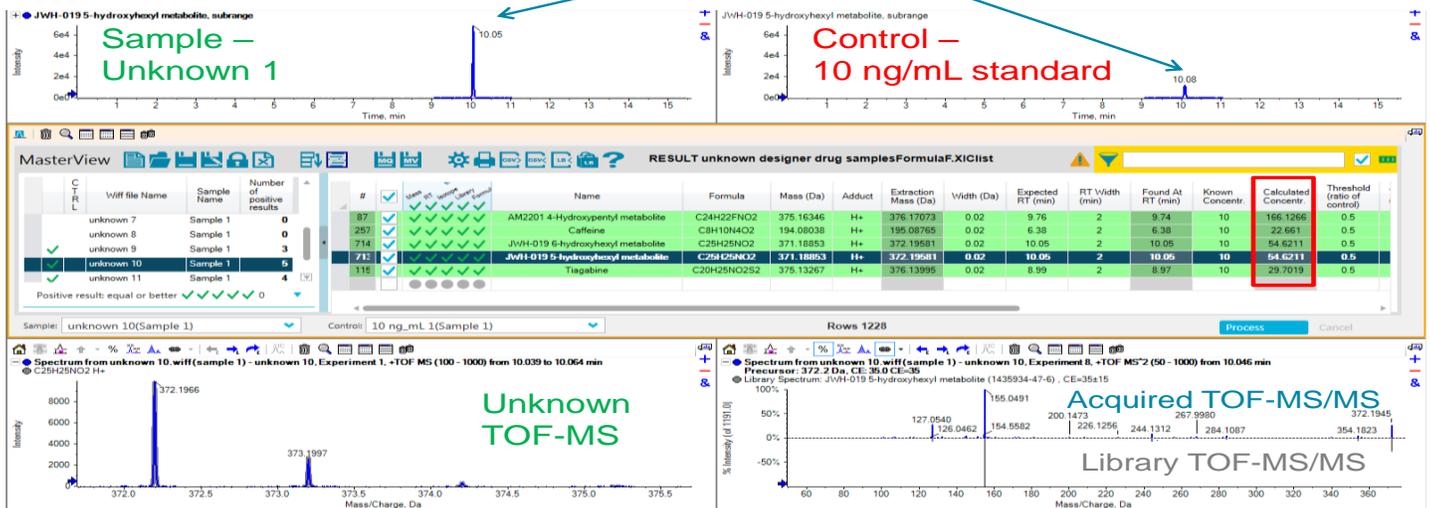


Figure 23. Quickly search other samples and review their results

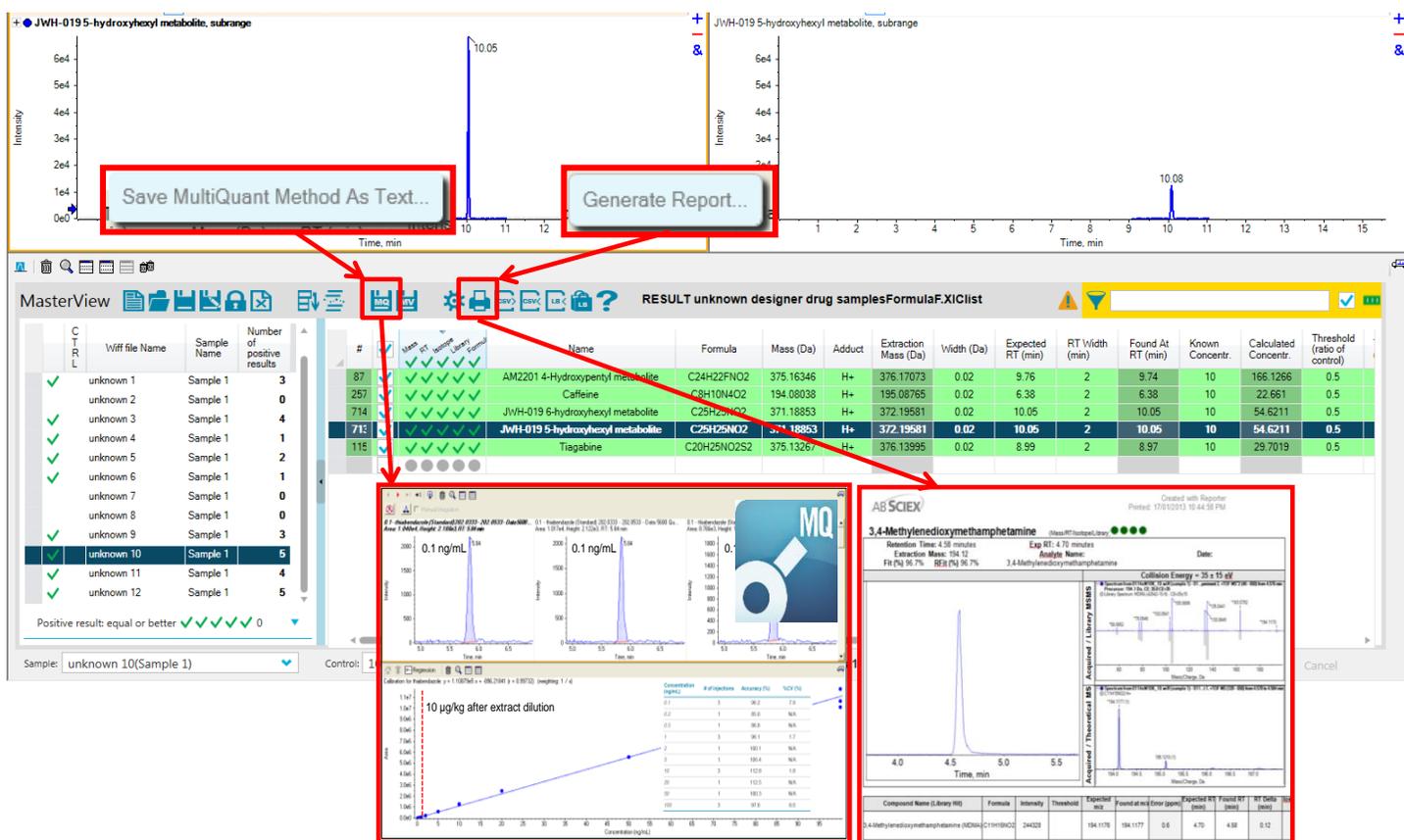


Figure 24. Quantitation through MultiQuant™ Software and Reporting

- The ‘traffic lights’ visualize the confidence of compound identification in your sample(s) for each processing parameter.
- Note that the XIC results are shown relative to the processing options you set-up.
- Review accurate mass MS and MS/MS spectra by clicking the ‘Show MS and MS/MS button’ (Figure 21).
- The acquired MS and MS/MS spectra are displayed in blue (Figure 21).
- The theoretical isotopic pattern of the molecular ion and the library MS/MS spectra for the compound are displayed in gray (Figure 21).
- Combining chromatographic, accurate mass MS and MS/MS information results in highest confidence of compound identification
- The XIC result table can be saved for future review and processing.
- The comparative screening and identification workflow allows a quick semi-quantification finding to identify unknown

- samples that contain targeted compounds that are present above a cutoff level (Figure 22)
- And, of course if you identify a positive, the next step is to confirm the concentration via a full calibration curve. Masterview™ Software has a direct link to create a quantitation method that can be imported into MultiQuant™ software for fast quantitation if you also ran compound standards in your acquisition batch (Figure 24).
- Or the screening results can be quickly generated as a report (Figure 24).

Summary

High resolution and accurate mass LC-MS/MS was used to screen for and identify NPS in urine samples. The AB SCIEX TripleTOF® system was operated in IDA mode to acquire MS and MS/MS information simultaneously.

The MasterView™ add-in of PeakView® software was used to compare samples to a blank control using a non-targeted peak finding algorithm to identify unexpected compounds in suspicious samples. The Formula Finder and automatic ChemSpider search is a useful and easy-to-use tool for identification if the MS/MS library search does not return a hit for a compound of interest.

Any unknowns that are found can easily be added to the targeted analysis by including the identified formula and retention time to the targeted XIC list. This now becomes part of the routine analysis workflow.

The comparative screening and identification workflow allows a quick semi-quantification finding to identify unknown samples that contain targeted compounds that are present above a cutoff concentration level. The workflow combines chromatographic, accurate mass MS and MS/MS information results for highest confidence in compound identification.

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