Using MS/MS^{All} with SWATH® Acquisition for Forensic Designer Drug Analysis with SCIEX X500R QTOF System and SCIEX OS Software

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Overview

In this technical note, we investigated the use of MS/MS^{All} with SWATH® acquisition for screening applications in a forensic toxicological setting with particular focus on bath salts. We demonstrate that SWATH® acquisition is a viable tool for identification and quantification in biological samples. A SWATH® (variable window) data acquisition was used that enabled sensitive quantitation of lower concentration species in complex matrices utilizing the more selective MS/MS information as well as using both ion ratio and MS/MS library searching for confident identification purposes.

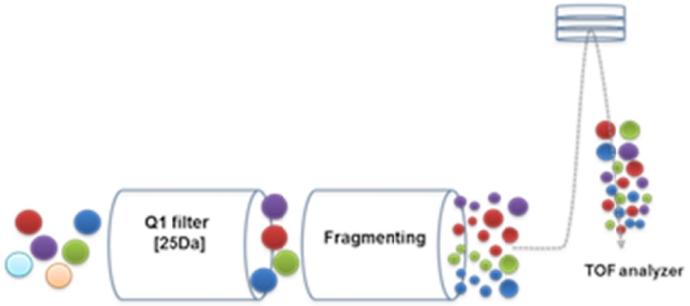

Figure 1: MS/MS^{All} with SWATH® Acquisition

Introduction

Bath Salts refer to a group of drugs containing one or more synthetic chemicals related to cathinone, one of the psychoactive principles naturally found in khat (*Catha edulis* Forsk). Some of the early synthetic cathinones such as mephedrone were first synthesised in the 1920s. However they became popular only in recent years when underground chemists began to use them in designer drugs. Since then, hundreds of other designer drugs or "legal highs" have been reported.

Chemically cathinones are similar to amphetamine and behave as central nervous system stimulants. New synthetic cathinones are constantly emerging, and their widespread availability makes it difficult for regulatory agencies to stay abreast of this major public health threat.

Many techniques have been used for bath salts screening including immunoassay, gas chromatography mass spectrometry (GC-MS), and liquid chromatography tandem mass spectrometry (LC-MS/MS). Limitations in current technologies include insufficient selectivity and sensitivity, cross-reactivity, difficulty in adding new compounds and lack of retrospective analytical capability. Single stage high resolution accurate mass platforms (e.g. Time-of-flight or TOF) solved some of these challenges but have not been able to provide clean and characteristic MS/MS information for high-confidence structural confirmation. Quadrupole TOF (QTOF) mass spectrometers combine quadrupole with TOF analyzer and enable selection of precursor ions within narrow *m/z* window (< 1 a.m.u.) before fragmentation in Q2 and separation of the fragment ions in the TOF, thereby provide high-quality MS/MS information for data analysis.

For screening applications, users often have no prior knowledge about the number and the identities of the drugs in the samples, but need to report possibly all correct identifications (true positives) and not to report erroneous compounds (false positives) or miss correct compounds (false negatives). Constantly emerging synthetic cathinones pose additional challenge since new drugs cannot be detected by existing, targeted analytical methods. Therefore, the collected screening data should contain the necessary information for confident identification of any drug in the sample, dictating a non-targeted data acquisition approach for both MS and MS/MS levels.

Here we introduce a revolutionary new Quadrupole Time-of-Flight (QTOF) mass spectrometer that contains advances in engineering design to bring the high performance TOF-MS and TOF-MS/MS capabilities into a compact benchtop platform. The SCIEX X500R QTOF mass spectrometer is part of a complete workflow from the fully integrated SCIEX ExionLC™ Systems to the freshly designed SCIEX OS software; a new user interface for simultaneous identification and quantification workflows (Figure 2.)

Figure 2: The SCIEX ExionLC™ AC HPLC system (left), the SCIEX X500R QTOF System (middle) and SCIEX OS Software (right).

One of the acquisition methods that can be efficiently set up on the new X500R is MS/MS^{All} with SWATH® acquisition. This MS acquisition method allows recording MS/MS information of everything at all times during the LC gradient. In every data cycle, the instrument acquires TOF-MS information; then it sequentially acquires MS/MS information of all precursor ions across a specified mass range in pre-divided mass windows. SWATH® acquisition significantly improves the MS/MS data quality over traditional MS/MS^{All} techniques by allowing sequentially programed MS/MS experiments, therefore more selective MS/MS data collection (Figure 3).

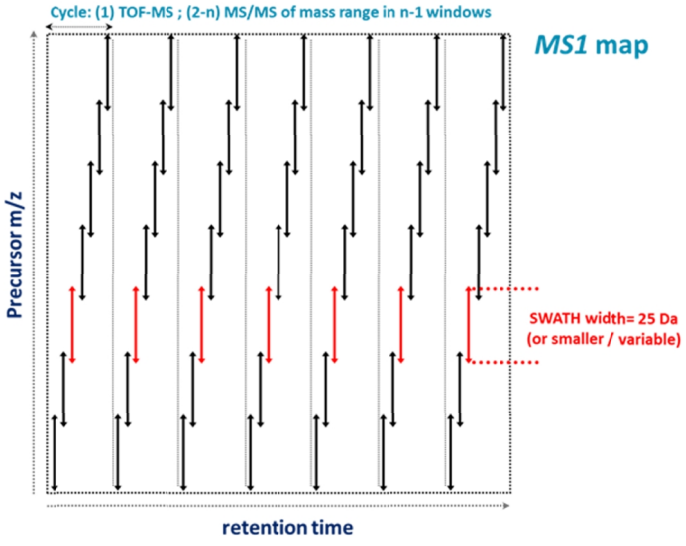

Figure 3: Principle of SWATH™ Acquisition, Demonstrated Using Fixed Window Size of 25 Da.

In this technical note, we investigated the use of SWATH® acquisition for identification and quantification of bath salts. We evaluated improvement on detection sensitivity with MS/MS information for quantitation purposes as well as identification based on unique fragment ions and their ratios and MS/MS library searching.

Experimental

Sample Preparation

Internal Standards. 6-APB-d5, Buphedrone Ephedrine Metabolite –d3 and 4-Fluoro-methamphetamine-d5 were used as internal standards. They were mixed and diluted in methanol at concentration of 1000 ng/mL as IS spiking solution.

Dilute and Shoot. Calibration curve with a mix of known bath salts standards were prepared in human drug free urine. Then 10 µL IS spiking solution was added to 100µL of urine samples which include both the calibration standards and unknown forensic samples. The mix was then diluted 5-fold with 90:10 (v:v), 0.1% formic acid in water : 0.1% formic acid in methanol followed by ultra-centrifugation. Injection volume was 10 µL.

Liquid Chromatography

HPLC separation was performed at 30 °C on a reversed-phase HPLC column (50 × 2.1 mm). Mobile phases used were water and methanol with appropriate additives. The LC flow rate was 0.5 mL/min and the LC runtime was 6.5 minutes.

MS and MS/MS Conditions

MS and MS/MS data were collected using SWATH® acquisition on the new benchtop SCIEX X500R QTOF System with SCIEX OS software, each SWATH® acquisition scan beginning with a TOF-MS experiment. Table 1 lists the data acquisition methods and source conditions. Variable SWATH® acquisition window size was applied to accommodate the application of three internal standards, ensuring that the labeled internal standards were not in the same SWATH® acquisition window as the none-labeled analytes.

Table 1: Data Acquisition Parameters Used for Analyzing Urine Samples

	SWATH® acquisition; variable
TOF-MS	100 to 700 *m/z*, 0.1 sec
Precursors of MS/MS	114 to 500 *m/z*, in 29 windows (variable size)
MS/MS	30 to 500 *m/z* x 29
Collision energy ramp	20 to 50 V
Total cycle time	**0.973 sec**

Table 2: List of Drug targets For Urinary Analysis

Drugs analyzed in positive mode	
2,5-Dimethoxy-4-n-propylthiophenethylamine	*Flephedrone*
2C-B-FLY	*Vigabatrin*
3,4-Dimethylmethcathinone	*Vilazodone*
3-Desmethylprodine	*MDPBP*
4-Ethylmethcathinone	*MDPV*
4-Fluoroamphetamine	*Methedrone*
4-Fluoromethamphetamine	*Methiopropamine*
4-Fluorotropacocaine	*Methoxetamine*
4-Methylephedrine	*Methylhexanamine*
4-Methylethcathinone	*Naloxone-N-Oxide*
4-Methyl-N-ethyl-norephedrine	*Naphyrone*
Cocaine-N-oxide	*Zopiclone-N-oxide*
5-apb	*n-Ethylcathinone*
6-apb	*n-Ethylcathinone ephedrine metabolite*
5-Iodo-2-aminoindane	*Pentedrone*
Codeine-6beta glucuronide	*Salvinorin B*
Alpha-PVP	*Tiagabine*
Alpha-Pyrrolidinopropiophenone	Butylone
n-Desmethylmirtazapine	Desomorphine
Bromo-Dragonfly	Desoxypipradrol
Buphedrone ephedrine metabolite	Etizolam

List of Target Compounds

Data was acquired in a non-targeted fashion but analyzed in a targeted way. A calibration mix was prepared that contained over 50 forensically relevant drugs; thus a targeted list consisting of 56 drugs was constructed for post-acquisition data processing containing the bath salt (detailed in Table 2) and designer drug related compounds.

Data Analysis: Confidence Settings and Screening Criteria

Data was processed in SCIEX OS software version 1.0. Reporting was performed also in SCIEX OS software with customized report templates.

Figure 4 is an example of the confidence setting used for screening. Four main confidence criteria were used for positive identification determination, which were mass error (M), RT error (R), isotope ratio difference (I), and library score (L). Subsequently, a combined score (C) was computed based on these four confidence categories (MRIL) with custom weightings. Finally, when there was no comparison sample (blank sample or sample spiked with drugs at reference level), the absolute peak intensity was used as an additional criteria to help reduce false positive rate.

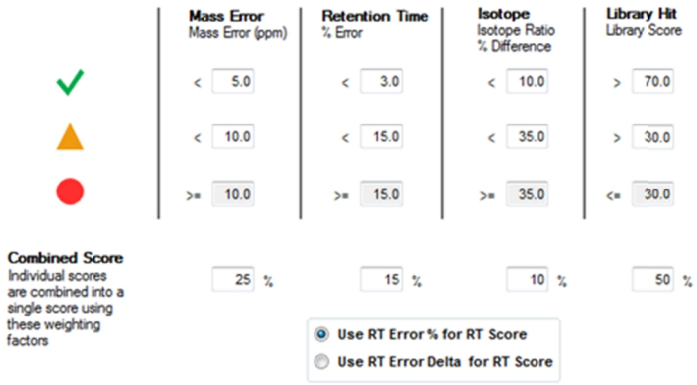

Figure 4. Confidence Settings in SCIEX OS Software

Results and Discussion

Figure 5 shows a representative quantitative linear dynamic range of the SCIEX X500R QTOF System showing 4 orders for the Etizolam compound (0.5 to 1000 ng/mL in urine) in TOF-MS mode. The figure also shows that even at high concentrations the mass error is still below 1.5 ppm. An example from the table in Figure 6 is for the standard at 2000 ng/mL in which the mass

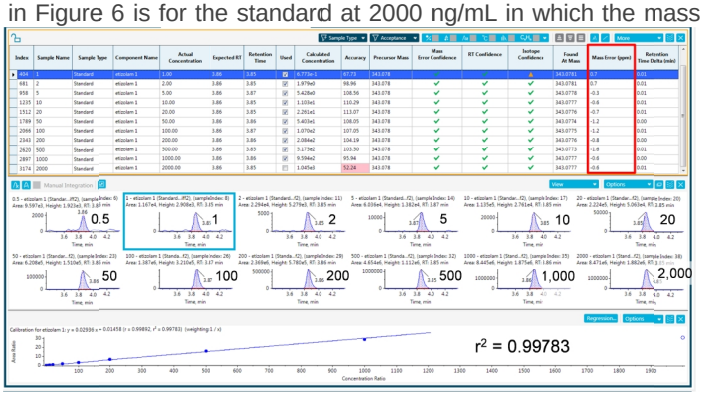

Figure 5. Linearity of the SCIEX X500R QTOF System Shown for Etizolam (0.5 ng/mL to 1000 ng/mL)

error is -0.6 ppm.

MS/MS^{All} with SWATH® Acquisition

Accurate mass TOF MS full scan provides the advantage of a generic methodology. It is a non-targeted method that allows later data re-interrogation to serach for unanticipated drugs. This

is particularly important in the scenario of designer drugs where new drugs emerge on a monthly basis. But full scan TOF MS approach, in a lot of cases, is not selective enough when analyzing biological samples where matrix interference is common. SWATH® acquisition is a non-targeted method providing selective MS/MS detection of every single analyte in addition to the full scan TOF MS data.

SWATH® acquisition allows MS/MS to be acquired all the time and it is therfore possible to use that information to confidently distinquish the presence or absence of structural isomers that fragment to produce unique fragmentation pathways and therefore unique fragment ions.

Although the screening data in the technical note was acquired in a non-targeted fashion, it was anayzed in a targeted way, which means a compound list was pre-assembled to perform the targeted analysis. This list contained the chemical formulas (for extracted ion mass calculation of the precursor molecular ion), mass extraction window, retention times and retention time window. The list also contained the accurate mass of several unique fragment ions for each compound.

Figure 6 shows an example where two structural isomers are barely chromatographically separated and relying on retention time alone may not accurately identify the presence of the correct isomer. Accurate mass of the precursor molecular ion will also not be able to distinguish between the two isomers. SWATH® acquisition however gurantees the collection of MS/MS data which through library searching against the SCIEX High Resolution MS/MS Forensic Spectral Library (Version 2.0) has allowed the confident identification of the 3,4 Dimethylmethcathinone and 4-Ethylmethcathinone (both having an accurate mass of 192.138 with a chemical formula of $C_{12}H_{17}NO+H^+$).

Further to library searching of MS/MS data, acquiring MS/MS all the time, allows the extraction of unique fragment masses which provide a clean extracted ion chromatogram trace for quantifcation purposes; while the TOF-MS equivalent trace of the extracted precursor ion, will in many cases show interference. Figure 7 shows the same example of 3,4 Dimethylmethcathinone and 4-Ethylmethcathinone structural isomers used previously, where the interference in TOF MS trace (pink) is eliminated in the MS/MS traces of the unique fragment ions (blue) *m/z* 159.10425 and 145.0886 for Dimethylmethcathinone and 4-Ethylmethcathinone respectively. Top pane A is the example of 3,4 Dimethylmethcathinone (RT=4.41 min), and the bottom pane (B) refers to 4-Ethylmethcathinone (RT= 4.61min).

Another example of the gain in specificity of choosing a unique fragment ion of a compound over reliance on the accurate mass of the precursor molecular ion is demonstrated in Figure 8. SWATH® acquisition collects all fragment ion data across the whole LC peak and therfore allows the ability to choose the correct ion that is free of interference and high background for more specific detection and quantification. Figure 8 shows an example of n-ethylcathinone ephedrine metabolite; a structural isomer to 4-methylephedrine and buphedrone ephedrine metabolite. The compounds are barely chromatographically separated and extraction of the precursor ion produces high background and interfering peaks. N-ethylcathinone ephedrine metabolite has a unique fragment ion at *m\z* 117 that can be extracted out to remove interfering ions and reduction in the background. The fragment ion at *m/z* 132.080 wouldn't be chosen for quantification purposes as it doesn't provide enough specificity.

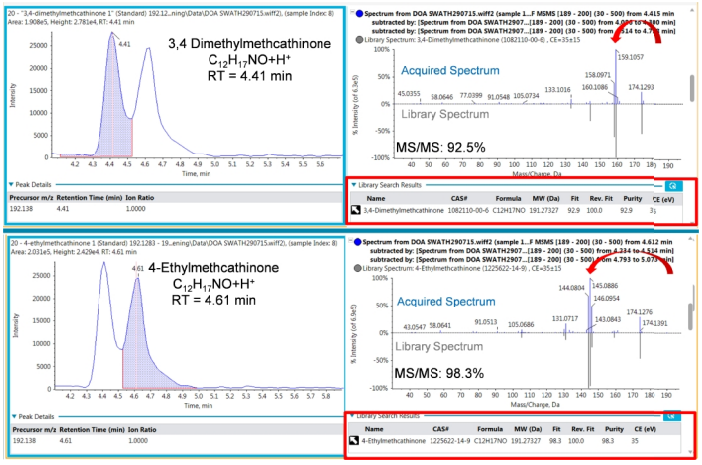

Figure 6. Confident Identification of Structural Isomers using MS/MS Data Collected from a SWATH® Acquisition Experiment

A third example of the specificity advantage gained by the ability to extract fragment ion information from SWATH® acquired data is shown in Figure 9. SWATH® acquisition is a truly non-targeted method providing selective MS/MS detection of every single analyte in addition to the full scan TOF MS data. In the situation when LC is not separating the structural isomer interferences, extraction of unique fragments will provide a clean extracted ion chromatogram, while the TOF MS trace will show interference. Figure 9 shows two examples where the interference in TOF MS trace (green) is eliminated in the MS/MS trace (blue). Top pane A is the example of Cocaine-N-Oxide (RT=6.99), and the bottom pane B refers to the internal standard 6-apb-d5.

The specificity of a fragment ion is not only important in the correct identifcation of a compound but also in accurate and precise quantification. Methoxetamine is given as an example in Figure 10, where the extraction of the unique fragment ion at *m/z* 121.06 (blue trace) has allowed the removal of an interfering

peak that is seen in the precursor ion extracted ion chromatogram for *m/z* 248.1645 (pink trace).

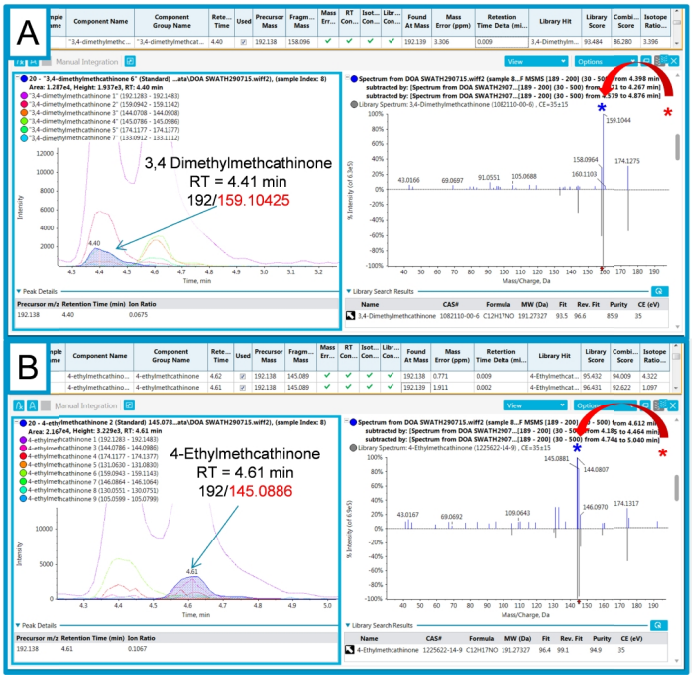

Figure 7. Confident Identification of Structural Isomers using SWATH® Acquisition based on Extraction of Unique Fragment Ions. TOF MS scan pink trace vs. MS/MS blue trace. (A) 3,4 Dimethylmethcathinone; RT=4.41 min, MS/MS extracted using fragment ion at *m/z* 159.10425. (B) 4-Ethylmethcathinone, RT=4.61min. MS/MS extracted using fragment ion at *m/z* 145.0886.

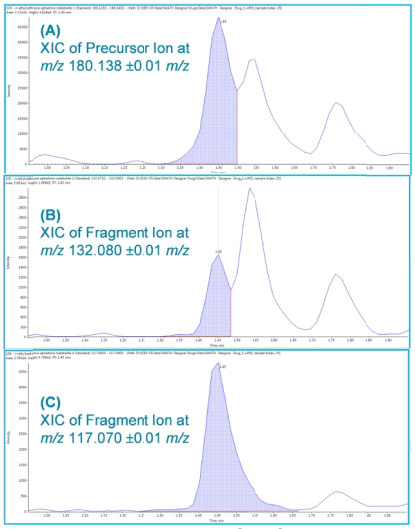

Figure 8. Ability to Extract Compound Unique Fragment Ions from SWATH® Acquired Data (A) XIC of precursor ion for n-ethylcathinone ephedrine metabolite at m/z 180.138; a structural isomer to 4-methylephedrine and buphedrone ephedrine metabolite. (B) XIC of fragment ion at m/z 132.080 (C) XIC of -ethylcathinone ephedrine metabolite unique fragment ion at m/z 117.070.

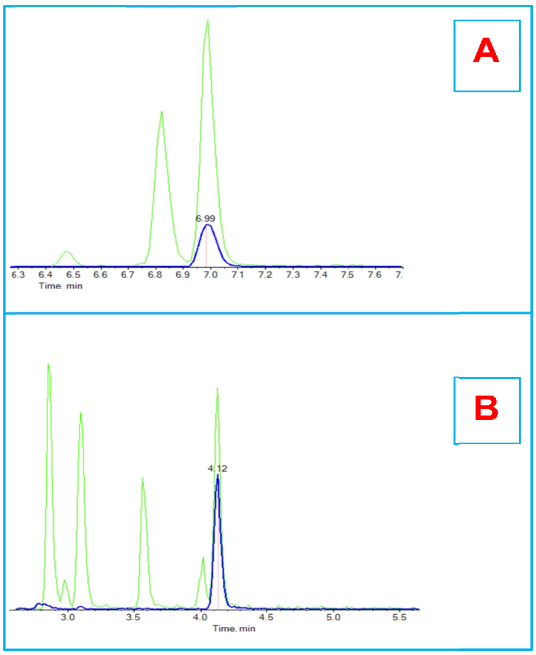

Figure 9. TOF MS Scan (Green Trace) vs. SWATH® Acquired TOF-MS/MS (Blue Trace). (A) Cocaine-N-Oxide, RT=6.99 min, MS/MS extracted using fragment 182.1166. (B)Internal standard, 6-apb-d5, RT=4.12min. MS/MS extracted using fragment 133.0619.

This clean up allows for more efficient integration and reliable quantification with high precision.

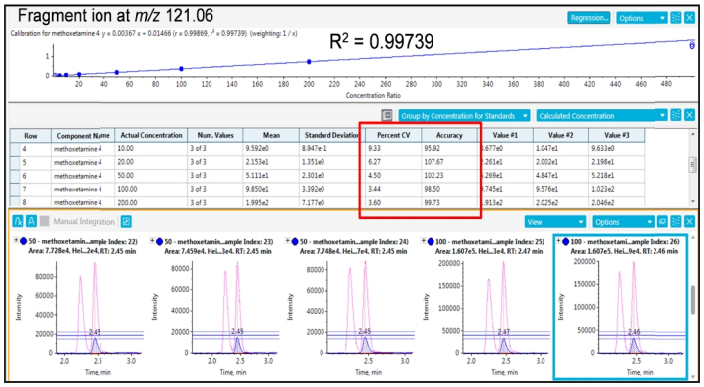

Figure 10. Quantification Statistics, Calibration line (ng/mL) and Extracted Ion Chromatograms (Qualifier, Quantifier ions overlayed) for Methoxetamine Compound from SWATH® Acquired Data.

As already suggested by Figure 10, SCIEX OS software allows for quantification and ion ratio/library searching confirmation to be performed simultaneously and for the results of this processing to be displayed together (Figure 11). Figure 11 shows the result table for a SWATH® acquisition sample set containing calibrators and spiked unknown urine samples. Results can be displayed and mined via sample or by a specific compound/ compound group. The results table has customisable

column displays, one selection being the *Calculated Concentration*. Samples that have compounds above a cutoff concentration can be flagged, as shown by the red highlighted cell for Unknown A, in the *Calculated Concentration* results table column (Figure 11).

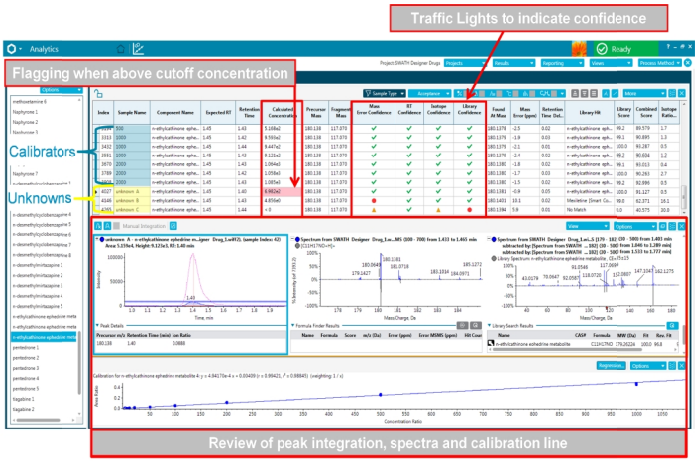

Figure 11.Simultaneous Identification and Quantification in SCIEX OS Software. Reviewing qualitative and quantitative results from SWATH® Acquired Data in the same software window.

As well as determining the amount of compound identified that is present in the sample, the SCIEX OS software also indicates the confidence in the compound identification through the traffic light columns using accurate mass, retention time, iostope pattern and library searching as the criteira.

For a selected compound, the viewer can review the peak integration of the extracted ion chromatogram as well as the TOF-MS and TOF-MS/MS spectra and also the calibration line. As this data was acquired through SWATH® acquisition, post acqusition data processing can also include the extraction of the precursor ion and mutiple accurate mass fragment ions and therefore ion ratio determinations. The extracted ion chromatogram for both the qualifier and quantifier ions can be overlayed with the ion ratio lines displayed.

Figure 12 shows that the results table can be filtered in various ways dependent on sample type and acceptance criteria (based on integration accetance, accuracy and calculated concentation). In the example shown, the software has been set up to only show unknown samples that show a positive finding with a compound above a specified cutoff level of 10 ng/mL.

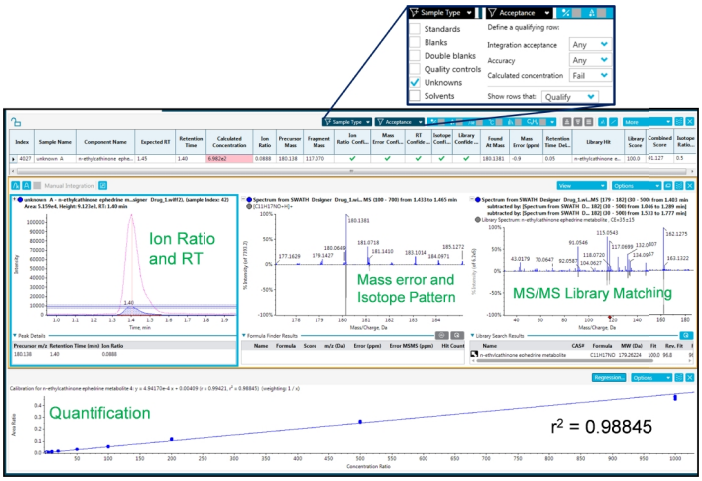

Figure 12. Unknown Sample Analysis. Calculated Concentration (Quant) with Ion Ratio and Library Searching from SWATH® Acquired Data. Filtering the results table to only show the positive findings.

Figure 13 shows an example of a calibration series for 4-methyl-N-ethyl-norephedrine displaying the quantification results as well as the ion ratio confirmation with both the qualifier and quantifier extracted ion chromatograms for each of the calibrators. Library searching confirmation was performed at the same time as ion ratio confirmation and generation of a calibration curve.

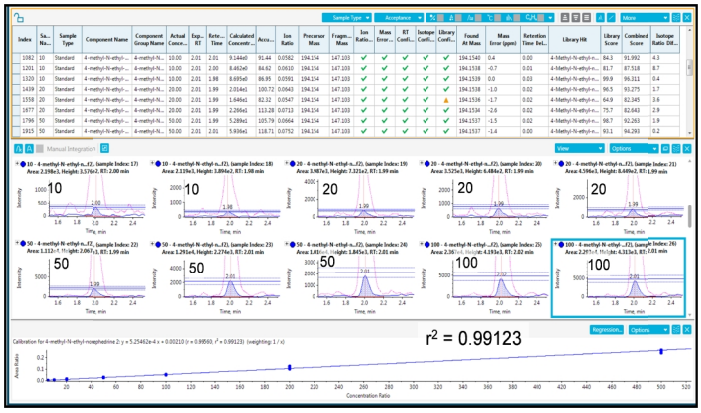

Figure 13. Quantification Results with Ion Ratio/Library Searching Confirmation from SWATH® Acquired Data. Example using calibrators for 4-methly-N-ethyl-norephedrine.

Figure 14 shows that acquiring data via SWATH® acquisition allows multiple ions in which to qant from, retrospectively chosing a different one if a unique interference is found in a one off sample. Figure 14 is also another example showing that even at high concentration, good mass accuracy is obtained.

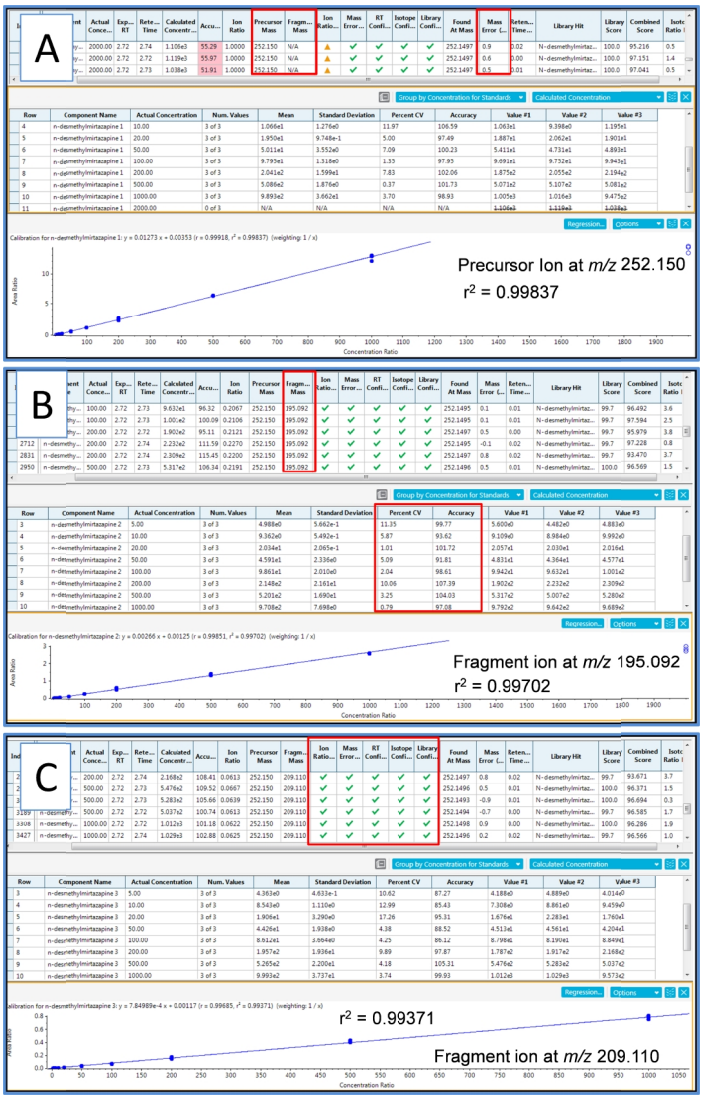

Figure 14. For the Same Compound, Multiple Ions can be Chosen in which to Quantify From After using SWATH® Acquisition. Results are given for the precursor ion at *m/z* 252.150 [A] and two fragment ions, at m/z 195.092 [B] and 209.110 [C] for N-Desmethylmitrapazine.

As an example of the comprehensive information that can be extracted from a SWATH® acqusition sample set, Figures 15,16 and 17 show the qunatification performance (precision and accuracy), ion ratio and library searching results for a calibrator set for the alpha-PVP compound.

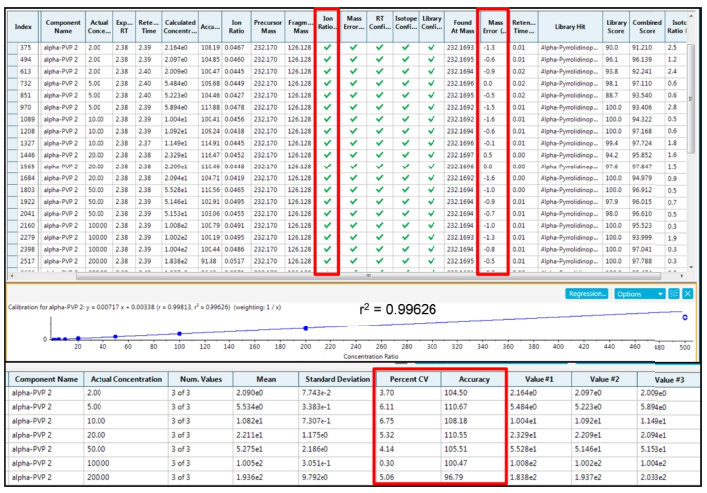

Figure 15. Quantification (Precision and Accuracy), Ion Ratio and Library Matching for an Alpha-PVP Calibration Set from SWATH® Acquired Data.

Figure 16. Extracted Ion Chromatograms for Alpha-PVP (ng/mL) Fragment Ion from SWATH® Acquired Data

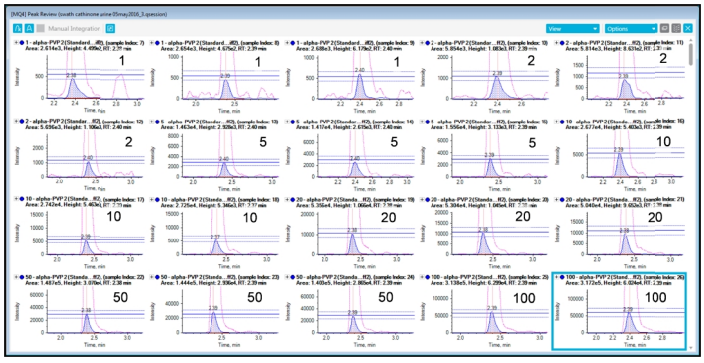

Figure 17. Alpha-PVP; Ion Ratio Chromatograms for Calibrators with Tolerance Lines from SWATH® Acquired Data

Unknown Sample Identification and Quantitation:

Unknown forensic samples were prepared and analyzed the same way as the standards by dilute and shoot. Data from thirteen unknown samples were acquired using SWATH® acquisition and processed in a targeted fashion, mining the data for the targeted list of bath salt compounds. The fact that quantitation can be done with MS informaiton (TOF-MS full scan) or MS/MS information from SWATH® acquisition is demonstrated here using an unknown forensic sample donated by a forensic lab.

In unknown sample 6, two peaks are present at RT=2.9 min and RT=3.1 min, (shown in Figure 18, peak A and B), when extracting out the precursor ion for buphedrone ephedrine metabolite (BEM, formula: C11H17NO) at *m/z* of 179.1301. From the TOF MS scan (dark blue trace) and RT, both peaks can potenticially indicate the presence of buphedrone ephedrine metabolite. Upon closer analysis of the MS/MS extraction, peak A showed overlay of buphedrone ephedrine metabolite fragment ions at m/z 133.09, 162.13 and 91.05. However peak B doesn't have these fragment peaks. Even though BEM has a retention time of 3.1 minutes, the MS/MS data for this peak suggests otherwise. Further analysis on the MS/MS spectra confirms the chromatographic inaccuracy.

In Figure 18, pane C showed acquired MS/MS spectrum (blue) at RT=2.9 min matches well with the grey library spectrum of BEM. On the other hand, pane D acquired spectrum at RT=3.1 doesn't show similiarity to BEM library spectrum, nor does it match up to the library spectrum of Mexiletine (shown in grey in D). Thus we conclude that sample 6 is identified to be positive with BEM. Retention time can be influenced by many factors, incluing column life time, sample matrix (e.g. pH value). In this particular case, even though the standards were prepared with real human urine, it is from a different individual compared to unknown sample 6. It is not surprising that we see slight difference in the retention time.

For BEM, calibration line generation using the precursor ion from the TOF-MS experiment as well as all three fragment ions from the SWATH® acquisition experiment demonstrated good linearity from 1-1000 ng/mL with r values over 0.99. Unknown sample 6 is calculated to contain 108 ng/mL BEM. Figure 19, left, shows the integrated peak of BEM (extracted using fragment ion at *m/z* 162.13). The right pane in Figure 19 shows the linearity of all 4 calibration lines.

Conclusion

In this technical note, we described the use of MS/MS^{All} with SWATH® acquisition for forensic drug screening with the QTOF X500R system and SCIEX OS software. We demonstrated that SWATH® acquisition is a viable tool for screening of bath salts in human urine.

Non-targeted SWATH® acquisition affords retrospective data analysis which is critically important for the fast emerging new designer drugs.

It was shown in this study that, due to the continual and looped MS/MS scan function and better selectivity with the fragment ion information, SWATH® acquisition enabled quantification in MS/MS mode of low concentration species in complex matrix and also allowed for ion ratio confirmation determinations in combination with library searching for confident identifications. We successfully identified and quantified buphedrone ephedrine metabolite in an unknown sample which contained 108 ng/mL BEM.

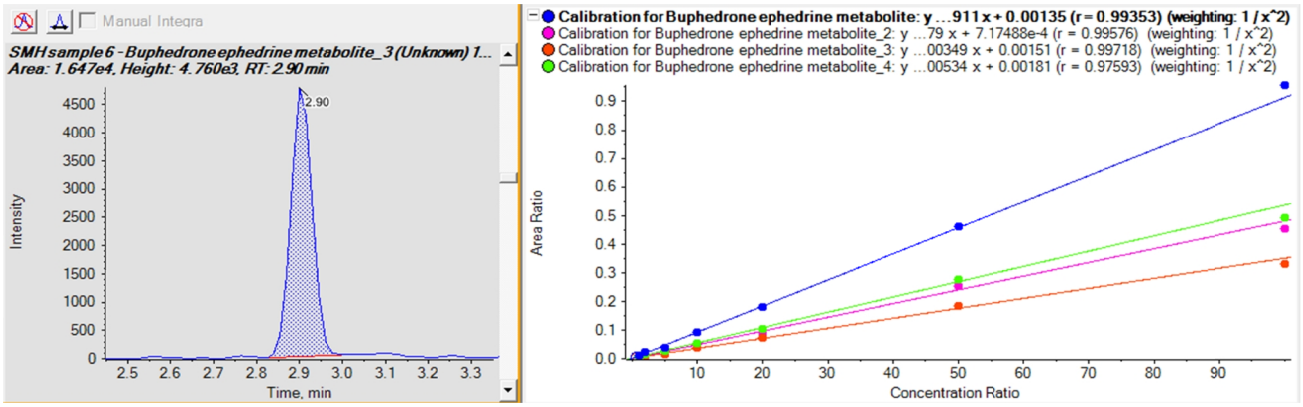

Figure 19. Left, Integrated Extracted Ion Chromatogram for BEM Fragment Ion at *m/z* 162.13 from SWATH® Acquired Data for Unknown Sample 6. Right, Representative Calibration Curves for the BEM Precursor Ion at *m/z* 179.1301 from the TOF MS Experiment and Three Fragment Ions from the SWATH® Acquisition Experiment.

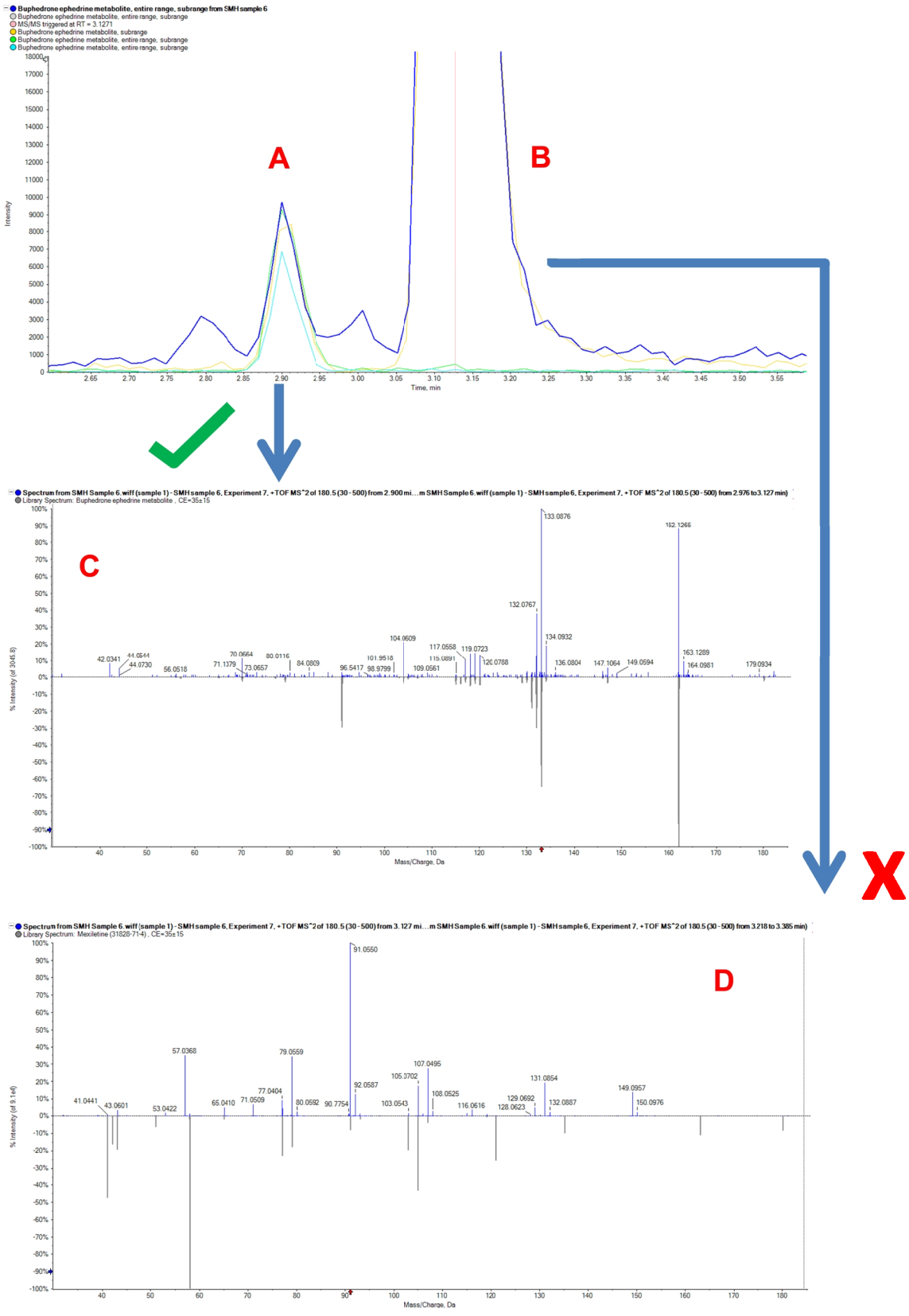

Figure 18. Extracted Ion Chromatograms and MS/MS Data for Unknown Sample Six. Top pane: overlaid extracted ion chromatograms of precursor ion (*m/z* 179.1301; TOF-MS) and fragment ions (at m/z 133.09, 162.13 and 91.05; SWATH® acquisition) for buphedrone ephedrine metabolite from unknown sample #6, containing peaks A (RT=2.9 min) and B (RT=3.1 min). (C) Library matching of buphedrone ephedrine metabolite (peak A), acquired MS/MS spectrum (blue) and library spectrum (grey). (D) Library matching of unknown peak B, acquired MS/MS spectrum (blue) and library spectrum (grey).

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