Expanding NPS screening capabilities in the forensic toxicology laboratory

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ABSTRACT

The workflow highlighted in the vMethod was used for the full characterization of 130 new and prevalent NPS that have recently emerged on the recreational drug market. Parameters such as retention times, linear correlation, inter-and intra-day precision and accuracy were determined in human urine and whole blood matrices. In addition, detailed structural information in the form of fragment-rich, TOF-MS/MS spectra was collected for each of the analytes using neat standards and matched against the spectra collected to confirm identification of structurally related analytes (including isomeric species) in matrix. The presented workflow enhances the screening capabilities of relevant NPS on the SCIEX X500R QTOF system and provides an updated list of relevant NPS that can be used for both targeted and untargeted data processing, while also providing the ability to retrospective analyze previously-acquired MS and MS/MS data sets and screen for the presence of these new substances without having to re-inject samples.

INTRODUCTION

The emergence and rising number of novel psychoactive substances (NPS) on the recreational drug market continues to pose health and safety challenges worldwide. NPS are a class of intoxicating substances that are designed to mimic the effects of controlled drugs. They are usually classified into 4 major groups: synthetic hallucinogens, synthetic cannabinoids, synthetic stimulants and synthetic depressants, which include synthetic opioids and benzodiazepines. This simplistic classification, however, does not take into consideration their wide variety of chemical, metabolic, toxicity and potency properties, nor does it address their overall risk profiles and the complexity of their combined effects. In addition, the frequency at which new NPS enter the drug market, the lack of knowledge about their composition, purity and potency, combined with the limited availability of reference analytical standards to provide confirmatory testing in the forensic toxicological laboratories makes it increasingly more challenging to screen and monitor NPS. As more of these NPS emerge on the illegal drug market, timely screening approaches for the accurate detection and identification of these substances are needed.

MATERIALS AND METHODS

Target analytes and solutions:
A list of 130 NPS, including 22 stimulants, 35 benzodiazepines, dissociatives and hallucinogens, 34 synthetic opioids and 28 synthetic cannabinoids was curated based on NPS trends and monitoring information. Reference and internal standards were purchased from Cerilliant Corporation (Round Rock, TX) and Cayman Chemical Company (Ann Arbor, MI). A sample solution was prepared in water with a 1 µg/mL of standard mixture containing the 130 target analytes. A 10 ng/mL standard solution was prepared in water with a mixture containing the 10 internal standards. The neat standard mixture was used to determine the retention time of the 130 components and generate a custom-built spectral library of high-quality TOF MS/MS spectra.

Calibrator preparation:
90 µL samples of human urine or whole blood were fortified with 10 µL of the 1 µg/mL standard mixture containing the 130 NPS. These freshly spiked biological matrix solutions were used to prepare 2 sets of 6 calibrator solutions in each of the 2 biological matrices covering concentrations ranging from 0.1 to 100 ng/mL.

Sample preparation:
The 2 sets of biological calibrator solutions were prepared as follows:

Human whole blood samples:
The 130 NPS were extracted from human whole blood samples using a protein precipitation procedure.

Human urine samples:
The 130 NPS were extracted from human urine samples using a dilute-and-shoot preparation procedure.

Liquid chromatography:
HPLC separation was performed on a Phenomenex Kinetex Phenyl-Hexyl column (50 × 4.6 mm, 2.6 µm, 00B-4495-E0) on an ExionLC AC system using the LC conditions highlighted in the vMethod.2 Mobile phases used were ammonium formate in water and methanol with appropriate additives. The flow rate was 0.7 mL/min. The injection volume was 10 µL and the total LC runtime was 9.5 minutes.

Mass spectrometry:
Two non-targeted data acquisition methods were used and compared. Both data dependent acquisition (DDA) and SWATH data independent acquisition (DIA) generated data that could be analyzed retrospectively. Both experiments started with a TOF MS scan to collect accurate mass precursor ions from 100 to 650 Da followed by a TOF MS/MS full scan ranging from 25 to 650 Da to ensure all fragments were captured for identification.

Data analysis:
Data processing was performed using SCIEX OS software, version 2.0 for positive analyte identification based on confidence criteria, as previously described.[2,] The 4 main confidence criteria used are outlined in Figure 1 and included mass error (M), retention time (R), isotope ratio difference (I) and library score (L). A new processing method was created in the Analytics workspace of SCIEX OS software. The components tab was populated by entering the name, molecular formula, precursor mass and retention time of the 130 NPS that were determined following the neat standard mixture injection.

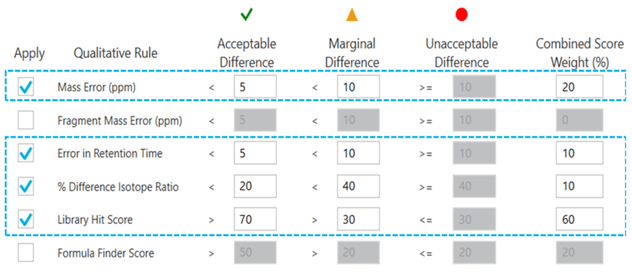

Figure 1. Confidence criteria used for data processing in SCIEX OS software. Qualitative rules including mass error (20%), retention time (10%), isotope ratio difference (10%) and library score (60%) used to assess positive analyte identification using the traffic light system.

RESULTS

Optimized LC conditions to separate isomeric species

The separation conditions of the vMethod application for forensic toxicology screening on the X500R QTOF system were used for these experiments. A diluted 10 ng/mL neat standard mixture containing the 130 NPS was used to determine the retention times of the analytes. Figure 2 shows the chromatographic profile of the 130 NPS. Baseline separation was achieved for most of the analytes, except for a few isomeric species that shared the same precursor mass and chemical formula and therefore had similar structures and MS/MS fragmentation patterns. Although the 9.5-minute LC method gradient and conditions enabled baseline separation of most analytes, the introduction of challenging isobaric substances in this panel might prevent chromatographic resolution and thus identification of those species based only on retention time.

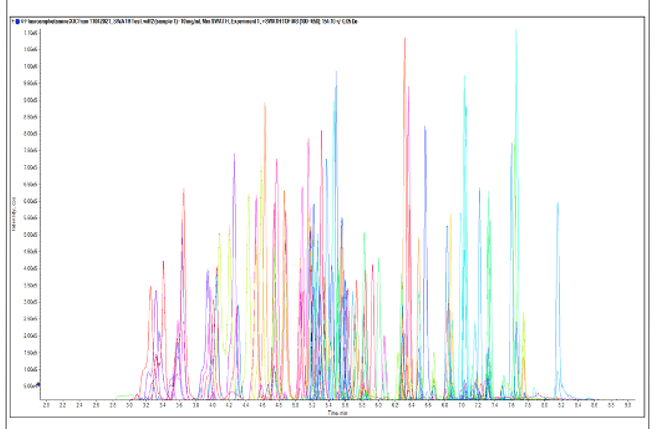

Figure 2. Chromatographic profile of the 130 NPS targeted in this study using the LC conditions of the SCIEX vMethod™ application for forensic toxicology screening on the X500R QTOF system. Extracted ion chromatogram (XIC) resulting from the near baseline separation of the 130 NPS in a 9.5-minute gradient.

Figure 3 shows the extraction ion chromatogram (XIC) traces for 3 groups of isobaric species separated using LC runtimes with 3.5-, 9.5- (vMethod conditions) or 15.5-minute gradients. Figure 3A shows that the 9.5-minute vMethod condition was unable to achieve baseline separation of 5-MDMB-PICA and 5F-EMB-PICA, 2 isobaric synthetic cannabinoids. The use of the longer, 15.5-minute gradient enabled baseline separation of these 2 isobaric species. The use of a shorter, 3.5-minute gradient resulted in co-elution of these 2 species, demonstrating that distinguishing these 2 isobaric synthetic cannabinoids is not possible by only LC separation with such a short gradient. Figure 3B shows that baseline separation of 3 synthetic cathinone isobaric species, eutylone, N-methylone and pentylone, was achieved using the generic, 9.5-minute vMethod conditions.

However, near-baseline resolution of these isobaric species was achieved using the 3.5-minute gradient (Figure 3B). Full baseline separation of 2 potent novel synthetic opioids, isotonitazene and protonitazene, was achieved using the 3.5-minute gradient (Figure 3C).

The results shown in Figure 3 demonstrate that the LC separation conditions of the vMethod provide a good generic method to separate challenging isobaric species. LC conditions such as the runtime and gradient can be modified based on the nature of the analytes screened using this workflow. Good judgement and caution are recommended when making these changes to ensure the method is fit for purpose and tailored based on the screening requirements.

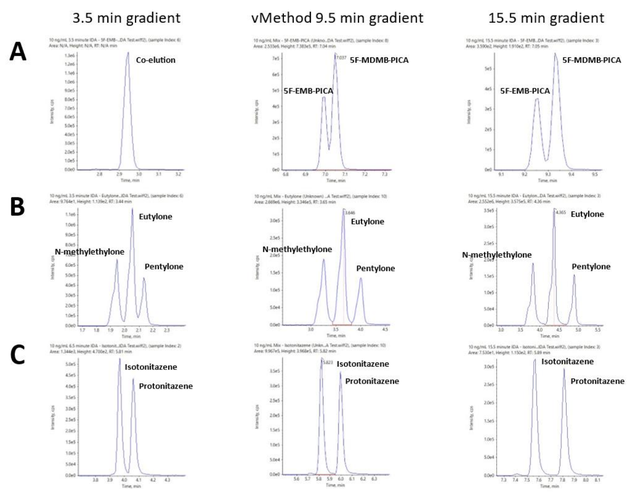

Figure 3. High sensitivity detection of drugs and metabolites extracted from DBS. Extracted ion chromatogram (XIC) traces for methylone (top) and methylphenidate (bottom) showing the quantifier ion traces for the matrix blank (left) and at the LLOQ at 50 pg/mL (right).

Streamlined analyte identification

The data analysis component of SCIEX OS software provides an integrated data processing and management platform that allows streamlined data review based on the scoring and reporting criteria set by the user. This step is systematically performed as part of the data review process to ensure correct drug identification while minimizing false positives and/or false negatives. Identification of the analytes was performed by displaying the XIC and TOF MS and MS/MS spectra of the sample with a library search match for each of the NPS included in the screening workflow. Figure 4 (bottom) shows examples of XICs and TOF MS and TOF MS/MS spectra with library matches for 4-Trifluoromethyl-U-47700 and metonitazene, 2 synthetic opioids included in the NPS panel. The retention time error, mass error, isotope ratio difference and MS/MS library score were automatically calculated in SCIEX OS software. Figure 4 (top) shows the parameters and metrics associated with the identification of the analytes. SCIEX OS software provides a simplified interface for streamlined data review based on a robust and reliable scoring system, enabling confident identification of the NPS.

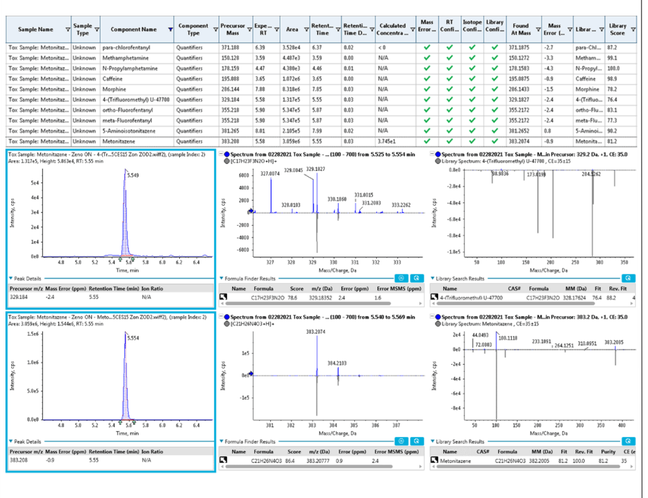

Figure 4. Streamlined and confident NPS identification using SCIEX OS software. (Top) Results table in SCIEX OS software showing the analytes positively identified in an authentic case sample. Mass, library match and library score were assessed using the confidence criteria. (Bottom) XICs, TOF MS and TOF MS/MS spectra collected provided detailed and confident identification of 2 of the positively identified analytes, 4-(trifluoromethyl) U-47700 and metonitazene, 2 synthetic opioids included in the NPS panel.

Improved analyte identification capabilities

Figure 5 shows the results of the retrospective analysis of a postmortem case sample that was initially analyzed in 2019. The results table generated in SCIEX OS software shows the positive identification of 7 compounds. Retrospective data analysis was performed on the same data using the updated list of NPS presented in this workflow. Figure 5 shows the detection of the same 6 compounds that were originally identified in 2019 with the addition of Alpha-PPP, one of the stimulants included in this panel. The same confidence criteria were used for confident identification of Alpha-PPP in the re-interrogated sample. Figure 5 (bottom) shows the XICs and TOF MS and MS/MS spectra of Alpha-PPP. A precursor mass error of -0.9 ppm, retention time error of 0.10% and MS/MS fit score of 98.2% provided excellent measures for the confident identification of this analyte in the postmortem case sample.

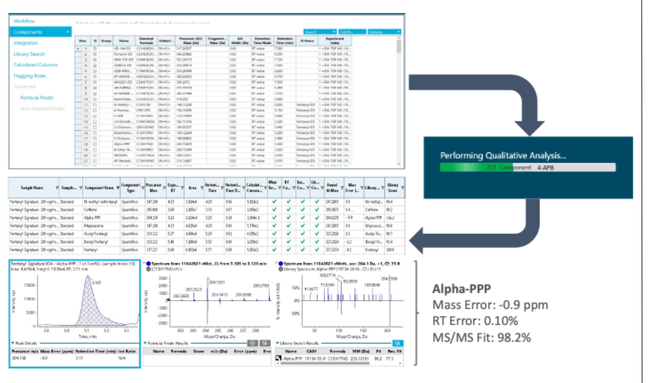

Figure 5. Retrospective analysis performed on a previously acquired dataset using SCIEX OS software. Data acquired using an authentic case sample was reprocessed after modifying the processing method window by adding the 130 NPS included in this panel (top). The stimulant Alpha-PPP was retrospectively identified in the authentic case sample from the SWATH DIA data. A precursor mass error of -0.9 ppm, retention time error of 0.10% and an MS/MS fit score of 98.2% provided excellent measures for the confident identification of Alpha-PPP in the postmortem case sample.

Flexible quantitation

Another advantage of the use of untargeted data acquisition for NPS screening is that it provides flexibility for quantification of the analytes. Since both TOF MS and TOF MS/MS scans are acquired, users can perform quantification using either precursor or fragment ion information. The use of precursor ion information is usually sufficient but the use of fragment ion information is helpful for complex sample matrices. Figure 6 shows representative XICs for 4F-MDMB-BINACA 3,3-dimethylbutanoic acid and its quantification using the precursor ion (206.1534 ±0.005 m/z; Figure 6A) and the fragment ion (206.1534 -->130.0629 m/z; Figure 6B). The series of XICs show the resulting signal for a blank injection (left) and at concentrations ranging from 0.5 to 100 ng/mL. The use of the fragment ion information significantly reduced the background signal. A few other analytes, including most synthetic cannabinoids, benefited from this approach.

The mass errors (less than or equal to 1 ppm), mass spectra library scores (above 97%) and the combined scores (above 96%) provided excellent measures of the confident identification of these two compounds in spiked urine samples.

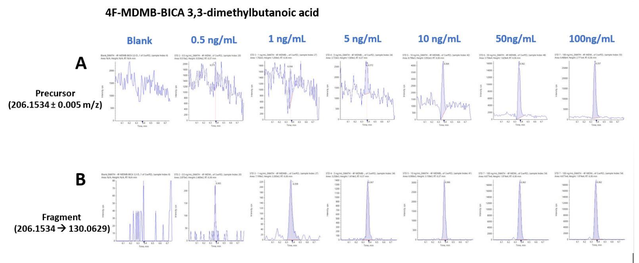

Figure 6. SWATH DIA enables flexible and reliable quantification using precursor (top) and fragment (bottom) ion information. Quantification of 4F-MDMB-BICA 3,3-dimethylbutanoic acid at concentrations ranging from 0.5 to 100 ng/mL using A) precursor ion information (206.1534 ±0.01 m/z) and B) fragment ion information (206.1534 --> 130.0629 ±0.01 m/z). The use of the fragment ion significantly reduced the background signal.

Robust and precise quantitation

Calibration curves were generated for each of the 130 NPS included in the panel. Figure 7 shows the resulting regression lines for A) 22 stimulants; B) 35 benzodiazepines, dissociatives and hallucinogens; C) 34 synthetic opioids and D) 28 synthetic cannabinoids, including the 2 cannabinoids, delta-8 THC and delta-8 carboxy THC. Each of the calibrator solutions was injected in triplicate on 3 consecutive days to yield 9 total injections. These calibration curves demonstrated excellent correlation of the generated regression curves, with R^2 values >0.99 for all NPS targeted in the panel, regardless of the acquisition method used.
The performance of the SWATH DIA and DDA workflows was compared for each of the 2 sets of biological samples. The series of calibrator solutions was injected in triplicate over the course of 3 consecutive days to accurately measure the precision of measurements. Overall, the assay showed great reproducibility over the course of 3 consecutive days with intra- and inter-day precision %CV values below 10% for all the calibrator solutions. These results demonstrate the quantitative robustness of the 2 untargeted acquisition methods in both human urine and whole blood samples.

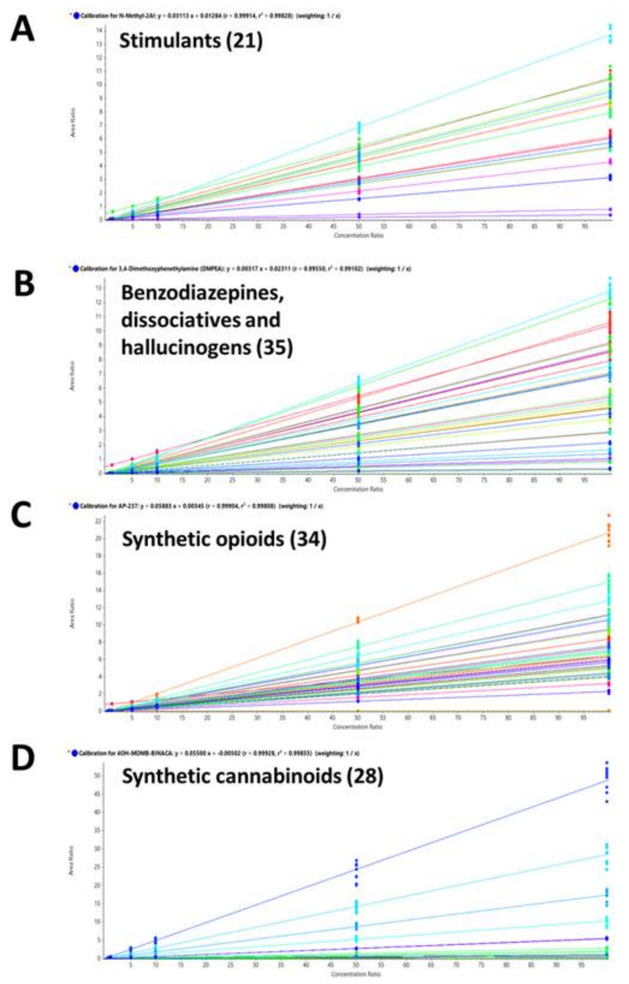

Figure 7. Excellent linearity for the 130 NPS included in the panel. Calibration curves resulting from the calibration series for A) 22 stimulants; B) 35 benzodiazepines, dissociatives and hallucinogens; C) 34 synthetic opioids and D) 28 synthetic cannabinoids.

CONCLUSIONS

The workflow highlighted in the Forensic vMethod was used for the analysis of 130 NPS extracted from human urine and blood samples. The use of the X500R QTOF system enabled comprehensive characterization of each of the 130 NPS using 2 non-targeted acquisition methods, DDA and SWATH DIA. This workflow enabled the generation of fragment-rich TOF MS/MS spectra that allowed accurate compound identification using confidence criteria. Parameters such as calibration range, linear correlation coefficient (R^2) and intra- and inter-day precision at 10 ng/mL in human urine and whole blood were determined. The results demonstrate that the DDA and SWATH DIA data acquisition methods yielded comparable performance.

The results also showcase that this workflow can be used to create a digital archive of the NPS present in the biological samples at the time of sample collection. The flexibility of the processing method provides the ability to screen against a list of targeted compounds and can be quickly adjusted for unknown compound identification using untargeted data processing. Previously acquired datasets can be retrospectively analyzed to look for the presence of newly identified NPS without having to re-inject samples. Overall, the information presented here provides the ability to screen for more than 900 compounds in complex biological specimens a single injection method.

REFERENCES

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2. Single-Injection Screening of 664 Forensic Toxicology Compounds on a SCIEX X500R QTOF System. SCIEX technical note, RUO-MKT-08-6395-A.

3. Intelligently designed SWATH Acquisition for novel psychoactive substances (NPS) detection in whole blood. SCIEX technical note, RUO-MKT-02-9780-A.

TRADEMARKS/LICENSING

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