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新興影響精神物質

SCIEX
The Power of Precision

目錄

*SCIEX webinar:
Enhancing high resolution mass spectrometry performance for NPS analysis with improved sensitivity and characterization, Pierre Negri, Global Market Development & Marketing Manager -Forensics

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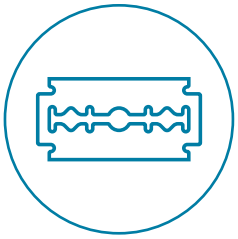
Novel psychoactive substances

In the last decade, there has been a surge in the circulation of, and demand for, novel psychoactive substances (NPS), which are designed to mimic the effects of existing—and illegal—recreational drugs. There is widespread concern about the safety of NPS due to a lack of regulation and knowledge about their constituents. This also makes providing effective treatment, recovery and support a challenge.[1,2]

NPS can be split into the four main categories outlined below.

Stimulants and hallucinogens



Stimulants mimic the effects of amphetamine, cocaine and ecstasy, increasing alertness and producing a sense of euphoria and well-being. Hallucinogens cause altered states of consciousness.

Can cause:
Anxiety | Agitation | Stroke psychosis | Hyperthermia | Depression | Seizures

Examples include:
Bath salts | Amphetamines | Phenethylamines | Cathinones

Can cause:
Hallucinations | Distorted perception of time, direction, distance and reality | Tachycardia | Dilated pupils | Nausea and loss of appetite

Examples include:
LSD | Ketamine | PCP | Mescaline | Psylocybin | Salvia

Opioids

Opioids are a broad group of pain-relieving drugs that block pain signals by interacting with opioid receptors in the cells. These substances are commonly used as adulterants in heroin and counterfeit preparations to mimic the effects of controlled opioids.

Can cause:
Analgesia | Euphoria | Sedation | Respiratory depression | Nausea | Vomiting | Reduced blood pressure and heart rate | Extreme dependence/ tolerance from repeated use

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Examples include:
Fentanyl | Fentanyl analogs | U-47700 | U-48800 | Fluoroisobutyrylfentanyl | Cyclopropylfentanyl | Brorphine | Isotonitazene | Fluorofentanyl | Metonitazene

Synthetic cannabinoids

Synthetic cannabinoids, also referred to as synthetic cannabinoid receptor agonists (SCRAs), are often laced into herbal products and sold as Spice, K2, Kronic, etc.

Can cause:
Agitation | Anxiety | Extreme anxiety | Psychosis | Paranoia | Hallucinations | Seizures | Hypertension | Tachycardia | Psychological dependency | Addictive potential

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Examples include:
APP-BINACA | MDMB-4en-PINACA | 4F-MDMB-BICA | 5F-EDMB-PICA | 4F-ABINACA | ADB-PHETINACA | EDMB-PINACA

Benzodiazepines

Benzodiazepines are one of the most prescribed groups of medications in the world. They are central nervous system depressants that are typically prescribed for their sedative, anxiolytic, hypnotic and anticonvulsant properties.

Can cause:
Delayed responses | Incoordination | Muscle relaxation | Decreased blood pressure and heart rate | Ptosis | Impaired cognitive function and motor skills

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Examples include:
Phenazepam | Etizolam | Diclazepam | Flubromazepam | Fonazepam | Flunitrazolam | Bromazolam | Bromazepam | Deschloroetizolam | Flubromazolam

1. Novel psychoactive substances: types, mechanisms of action, and effects. BMJ 2017; 356: i6848. https://doi.org/10.1136/bmj.i6848
2. Robinson, J. Novel psychoactive substances: what are they and what implications can they have for pharmacists? The Pharmaceutical Journal 2016. https://doi.org/10.1211/PJ.2016.20201674

Enhancing NPS characterization using electron-activated dissociation (EAD)

Using the ZenoTOF 7600 system powered by SCIEX OS software

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Here, electron-activated dissociation (EAD) fragmentation on the ZenoTOF 7600 system was used to confirm the detection of multiple classes of structurally similar and isobaric novel psychoactive substances (NPS), including newly emerging fentanyl opioids, halogenated fentanyl analogs, novel synthetic opioids (NSO) and synthetic cannabinoids.[1] The combination of the Zeno trap with EAD provides the MS/MS sensitivity and selectivity to improve confidence in NPS identification and to differentiate isomeric species otherwise indistinguishable using collision-induced dissociation (CID)-based MS/MS methodologies. EAD is a powerful, reagent-free, tunable orthogonal fragmentation technique that can generate unique diagnostic fragment ions to differentiate between structurally similar compounds (Figure 1) and has the potential to provide in-depth characterization of those substances that do not generate unique fragment ions when subjected to CID.

The growing number of NPS emerging on the recreational drug market continues to pose safety concerns for public health and law enforcement officials. NPS are a diverse group of synthetic substances designed to mimic the action and psychoactive effects of controlled substances and are often used as adulterants in heroin and counterfeit preparations. Newly emerging fentanyl opioids, NSO and fentanyl analogs share similar structure and composition, adding additional complexity.

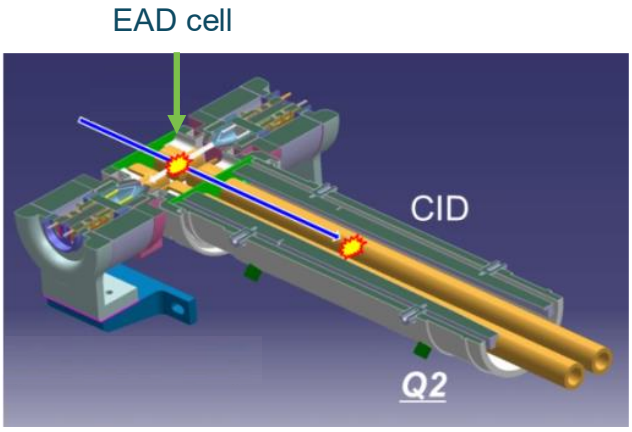


Figure 1. Schematic of the EAD cell on the ZenoTOF 7600 system. EAD provides reproducible and unique fragment ions that enhance the characterization of NPS.

Traditionally, NPS analysis performed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) has used CID for compound fragmentation. In most cases, CID produces unique diagnostic fragment ions that can be used to confidently identify NPS. However, certain classes of NPS, such as isomeric species, do not produce unique fragment ions with CID. Thus, as structurally related NPS have become more prevalent to evade regulations, the challenges to analytically characterize these substances have also increased.

Advantages of EAD on the ZenoTOF 7600 system for NPS characterization

- Zeno data-dependent acquisition (DDA) with EAD provides the specificity and sensitivity required for the characterization of low-level analytes in complex biological matrices, such as discarded postmortem case samples
- Zeno EAD DDA results in increased unique diagnostic fragments, enabling in-depth characterization of NPS and the differentiation of isomeric and structurally related analytes that were previously indistinguishable using CID
- Compatible with drug screening workflows using fast DDA in SCIEX OS software

Experimental details

Target analytes: An NPS panel including newly emerging fentanyl opioids, halogenated fentanyl analogs, synthetic opioids and synthetic cannabinoids was selected for method development. Standards were purchased from Cerilliant Corporation (Round Rock, TX) and Cayman Chemical Company (Ann Arbor, MI). Each standard was injected individually twice to generate custom-built CID and EAD MS/MS spectral libraries of high-quality TOF MS/MS spectra for comparison.

Authentic postmortem case samples: Analytes were extracted from human whole blood using a liquid-liquid extraction (LLE) procedure summarized in Figure 2.

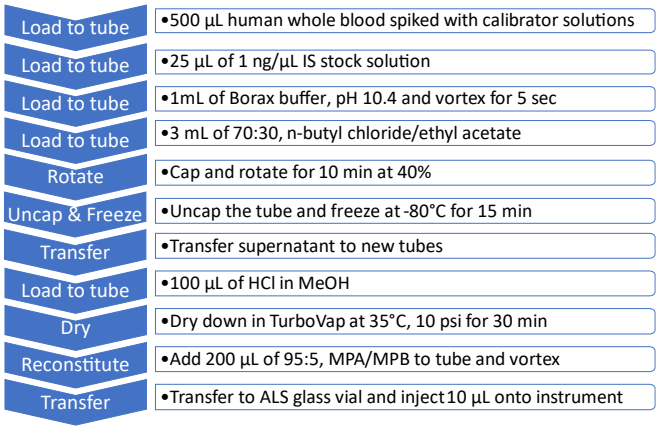


Figure 2. Liquid-liquid extraction (LLE) procedure for human whole blood samples. A 10-step extraction protocol was used to selectively extract drugs from human whole blood samples for analysis with the ZenoTOF 7600 system.

Liquid chromatography: HPLC separation was performed on an ExionLC system using a Phenomenex Kinetex C18 column (50 × 3.0 mm, 2.6 µm, 00B-4462-Y0). Mobile phase A (MPA) and mobile phase B (MPB) were ammonium formate (pH 5) and formic acid in methanol and acetonitrile, respectively. The flow rate was 0.4 mL/min and the total LC runtime was 15.5 minutes. The injection volume was 10 µL.

Mass spectrometry: MS and MS/MS data were collected twice for each sample using Zeno DDA with CID and Zeno DDA with EAD on the ZenoTOF 7600 system. Data acquisition consisted of a TOF MS scan to collect accurate mass precursor ions from 100 to 700 Da, followed by a full scan TOF MS/MS with the Zeno trap activated, with mass range of 25 to 700 Da to ensure all fragments were captured for identification. For each cycle, a maximum of 16 candidate ions were selected for MS/MS. Data were acquired using SCIEX OS software, version 2.0.1.

Data analysis: Data were processed using SCIEX OS software, version 2.0.1. Detection and integration of the peaks from the background were accomplished using the MQ4 algorithm in the Analytics module of the software. Quantitative and qualitative analyses were then performed. Positive analyte identification was accomplished based on confidence criteria, as previously described.[2] The 4 main confidence criteria used included mass error (M), retention time (R), isotope ratio difference (I) and library score (L). Two separate in-house libraries of CID and EAD MS/MS spectra were generated from standards and used to perform spectral library matching and identification of the drugs present in the discarded authentic postmortem case samples.

Optimized EAD conditions for reproducible and comprehensive fragment information

Individual neat standard solutions were injected to optimize the EAD parameters, including electron kinetic energy (KE), electron beam current and reaction time. A series of injections were performed with various parameter combinations to achieve optimal sensitivity, reproducibility and selectivity of the generated fragment ions. The collected TOF MS/MS spectra were reviewed individually to determine the optimized EAD parameter values used for the rest of the experiments. These parameters included 10 eV electron KE, 700 nA electron beam current and 35 ms reaction time. These values were used to collect TOF MS and TOF MS/MS spectra of each of the neat standards. The TOF MS/MS spectra were used to build an in-house EAD spectral library that was compared with that generated using CID data.

Zeno MS/MS for improved sensitivity

Average sensitivity gains of ~9x in the TOF MS/MS data have been reported for drugs and metabolites positively identified in discarded postmortem case samples analyzed using CID with the Zeno trap.[3] Here, the use of the Zeno DDA to improve TOF MS/MS sensitivity was investigated using EAD as the fragmentation mechanism. Figure 3 shows representative TOF MS/MS spectra acquired with and without the Zeno trap activated for 3 drugs positively identified in discarded postmortem case samples, including ADB-PINACA, ortho-chlorofentanyl and norbuprenorphine, which are a synthetic cannabinoid, NSO and synthetic opioid, respectively. Without the Zeno trap activated, analysis of these case samples resulted in low-intensity TOF MS/MS spectra. The use of the Zeno trap increased the TOF MS/MS sensitivity of the low abundance fragments, improving compound identification confidence for low levels of drugs and metabolites. Overall, when the Zeno trap was

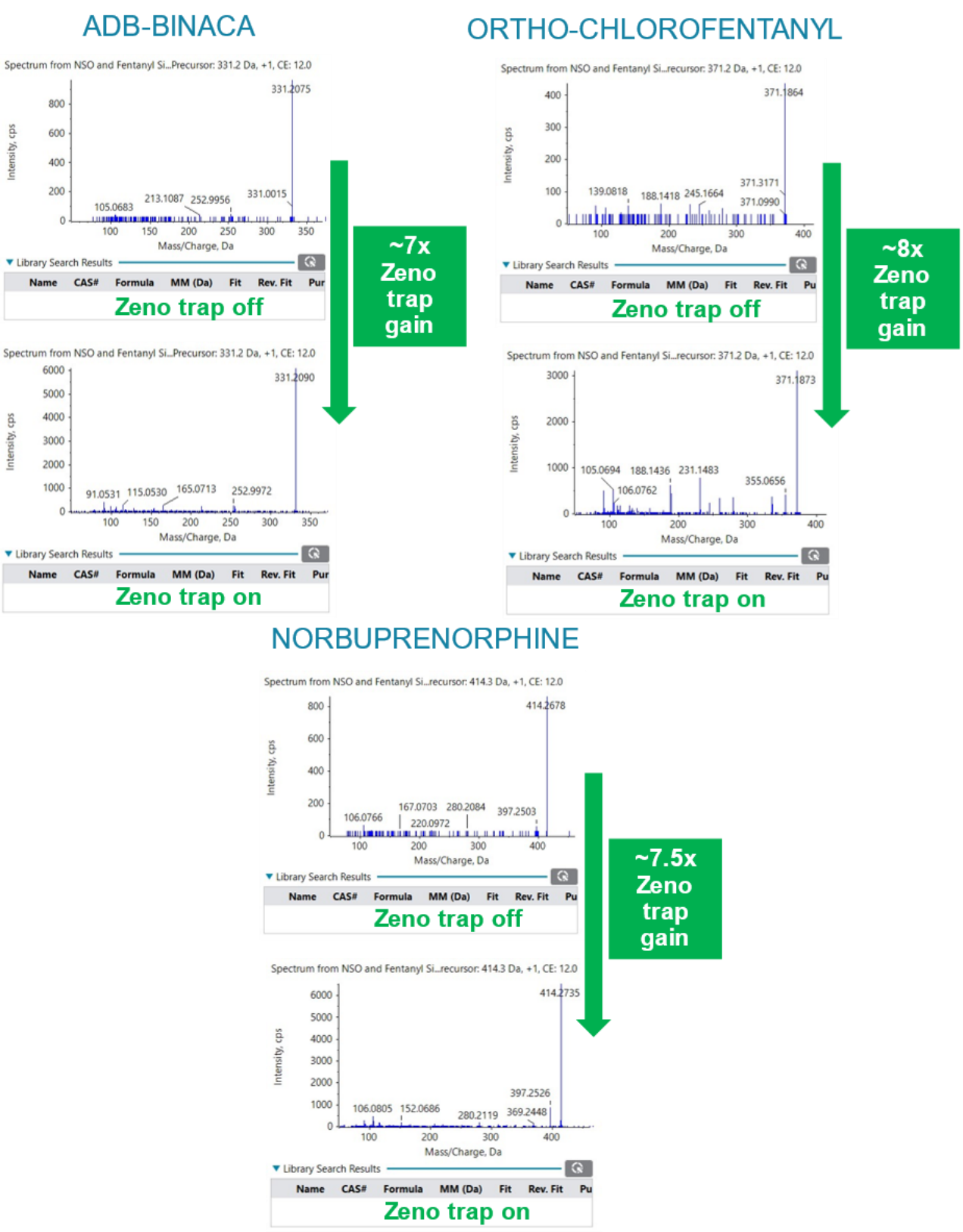


Figure 3. TOF MS/MS sensitivity gains when combining Zeno DDA with EAD for representative NPS. An average of ~8x gain in TOF MS/MS sensitivity was observed across all the analytes positively identified in the discarded postmortem case samples when the Zeno trap was used in combination with EAD.

used, an average 8x improvement was observed in sensitivity across the TOF MS/MS spectra that were positively identified in the discarded postmortem case samples. When combined with EAD, this improvement in sensitivity enabled confident characterization and identification of NPS and metabolites at levels that were not previously achievable.

Comprehensive characterization of synthetic opioids

Some analytes, such as buprenorphine and its main active metabolite norbuprenorphine, are known to fragment poorly when subjected to CID-based fragmentation. As seen in the CID MS/MS spectra shown in Figure 4 (bottom spectra), buprenorphine (A) and norbuprenorphine (B) only produced low-intensity fragment ions that were unreliable for compound characterization and quantification. When EAD was used as the fragmentation technique on the ZenoTOF 7600 system, richer TOF MS/MS spectra containing unique diagnostic fragment ions were generated. As seen in Figure 4, the Zeno EAD MS/MS spectra generated for buprenorphine and norbuprenorphine showed unique diagnostic fragments at m/z 410.2352 and 378.2074 and m/z 356.1843, 338.1772 and 324.1574, respectively. The molecular formulas and the corresponding structures of the identified fragment ions are shown in Figure 4. These results demonstrate that EAD provides richer fragmentation by generating unique diagnostic fragment ions that enable in-depth characterization of these 2 analytes. These identified fragment ions can then be used for downstream development of targeted methods for the sensitive and specific quantification of these analytes.

Differentiation of AP-series NSO

The recent scheduling of fentanyl analogs has sparked the emergence of new classes of NSO. Among those, the cinnamylpiperazine analogs, also known as the AP series isomers, have recently emerged on the recreational drug market. These isomeric species are similar in structure and composition, as they all contain a piperazine core and a cinnamyl moiety. These similarities therefore make their characterization and differentiation analytically challenging. An alternative fragmentation technique such as EAD has the potential to provide additional fragment ions that would enable differentiation of these analogs from one another.

Figure 5 shows the CID MS/MS spectra of AP-238 (top), 2-methyl AP-237 (middle) and para-methyl AP-237 (bottom). The TOF MS/MS spectra for these 3 AP series isomers are similar and share common fragment ions at m/z 131.0848, 117.0692, 115.0536 and 91.0536. The bottom panels in Figure 5 show the EAD MS/MS spectra of the same 3 AP series isomers. Each spectrum contains unique fragments and spectral differences that enable the differentiation of the 3 isobaric synthetic opioids, as circled in red. These unique spectral features highlight the ability of EAD to provide complementary and unique fragment ions for the in-depth characterization of isomeric compounds, such as the cinnamylpiperazine analogs. The use of EAD also enabled the formation of unique lower molecular weight fragments that enabled the differentiation of these analogs.

In-depth characterization of synthetic cannabinoids

Synthetic cannabinoids are a class of NPS that are designed to mimic the active ingredient of cannabis, delta-9-tetrahydrocannabinol (THC). In recent years, these substances have gained popularity and rapidly emerged on the recreational drug market. Most synthetic cannabinoids have an indole or indazole core structure, which makes them challenging to identify since they share similar structures and identical masses to the corresponding indazole analogs. As a result, an alternative fragmentation technique such as EAD can potentially be used to characterize and identify synthetic cannabinoids.

Figure 6 compares the EAD and CID MS/MS spectra as a mirror image for 3 synthetic cannabinoids, including ADB-BINACA, ADB-PHETINACA and 4F-MDMB-BINACA. The Zeno EAD MS/MS (top) and Zeno CID MS/MS (bottom) spectra for each of the 3 cannabinoids share several fragments. However, EAD provides a much richer fragmentation when compared to CID. As circled in red, the EAD spectra show unique diagnostic fragments that enable in-depth characterization of each of the 3 synthetic cannabinoids. For example, EAD generated 4 unique fragments at m/z 274.1458, 257.1173, 131.0598 and 117.0472 in the TOF MS/MS spectrum of ADB-BINACA and 3 unique fragments at m/z 186.0676, 145.0403 and 91.0529 in the TOF MS/MS spectrum of ADB-PHETINACA. Fragments at m/z 275.1089, 131.0612, 117.0470 and 90.0342 were unique fragments in the TOF MS/MS spectrum of 4F-MDMB-BINACA that were not present in its CID MS/MS spectrum. Also shown are the molecular structures for each of these unique fragment ions generated by EAD. These unique spectral features provided complementary structural information that can be leveraged for in-depth characterization synthetic cannabinoids.

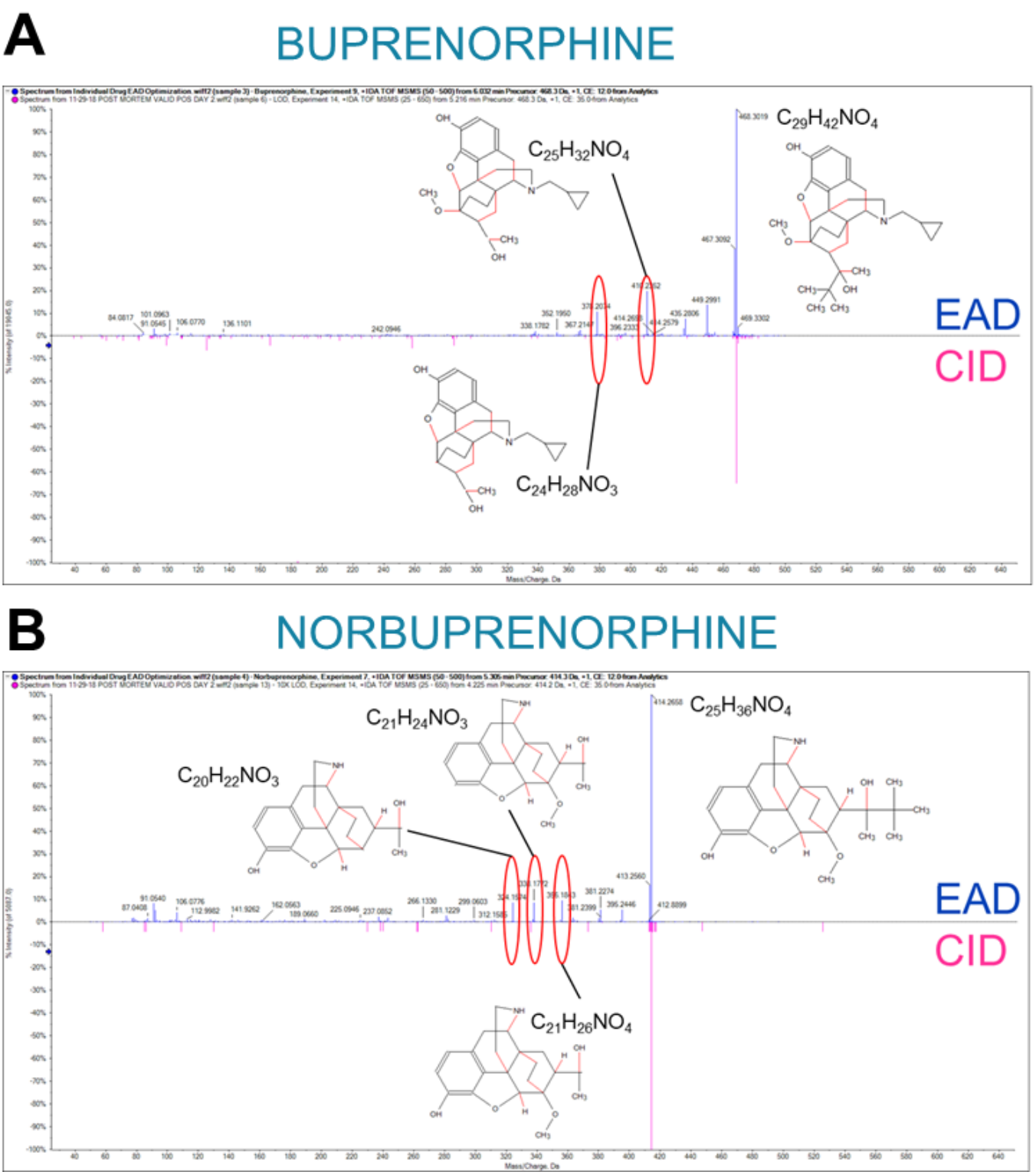


Figure 4. EAD enables in-depth characterization of challenging synthetic opioids. Zeno EAD MS/MS (top) and CID MS/MS (bottom) spectra for A) buprenorphine and B) its main active metabolite norbuprenorphine. The Zeno EAD MS/MS show unique diagnostic fragment ions that enable in-depth characterization of these 2 analytes. Activation of the Zeno trap ensured that high sensitivity was achieved for both MS/MS modes.

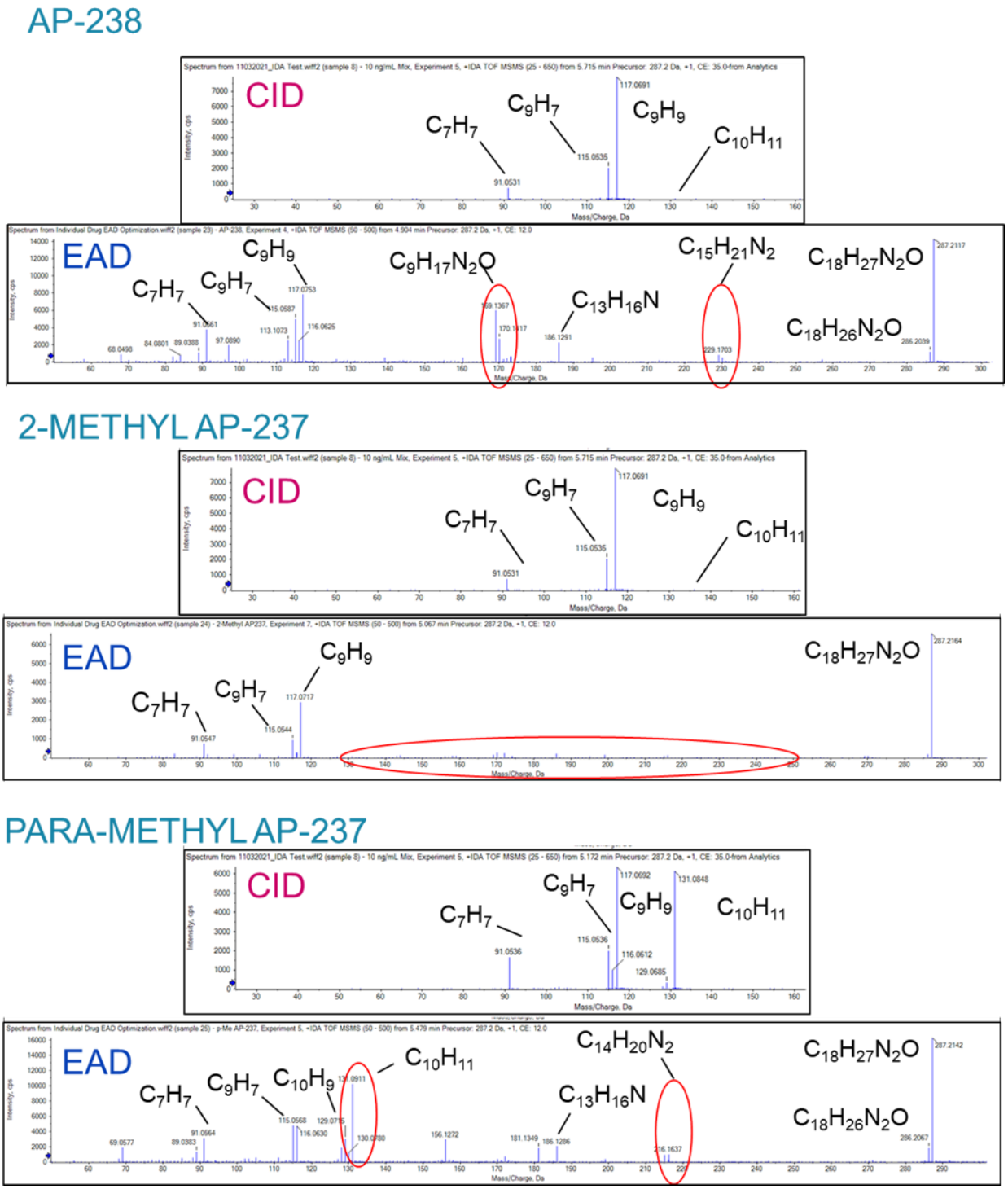
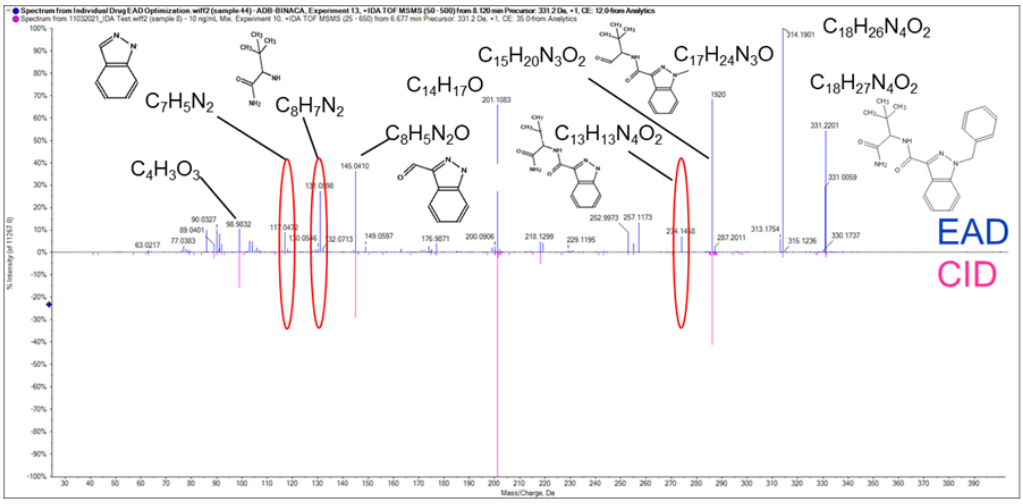
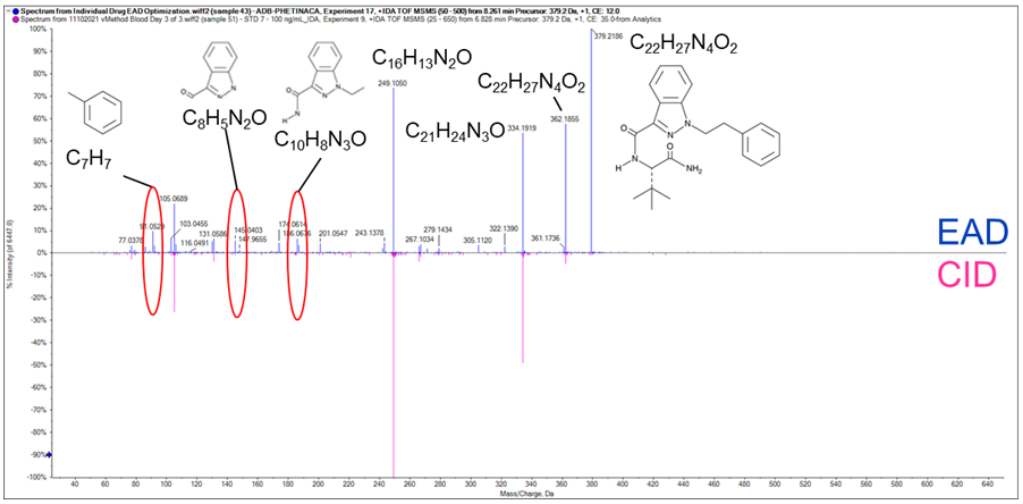


Figure 5. EAD provides rich MS/MS spectral features to enable the differentiation of the isobaric synthetic opioids from the AP series. CID and EAD MS/MS spectra for 3 synthetic opioids: AP-238 (top), 2-methyl AP-237 (middle) and para-methyl AP-237 (bottom). The CID MS/MS spectra are indistinguishable from one another. The Zeno EAD MS/MS spectra have unique spectral features and fragment ions that enable the differentiation of the 3 isobaric species.

A. ADB-BINACA



B. ADB-PHETINACA



C. 4F-MDMB-BINACA

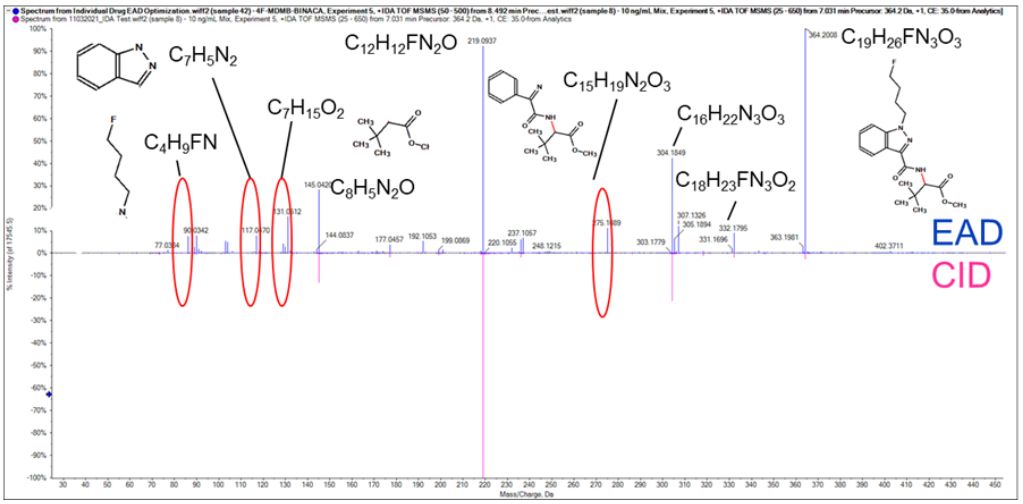


Figure 6. EAD enables in-depth characterization of challenging synthetic cannabinoids. Spectral comparisons between Zeno EAD MS/MS (top) and Zeno CID MS/MS (bottom) for 3 synthetic cannabinoids: A) ADB-BINACA, B) ADB-PHENITACA and C) 4F-MDMB-BINACA. EAD provides richer fragmentation in the form of unique fragment ions that enable structural characterization of challenging NPS.

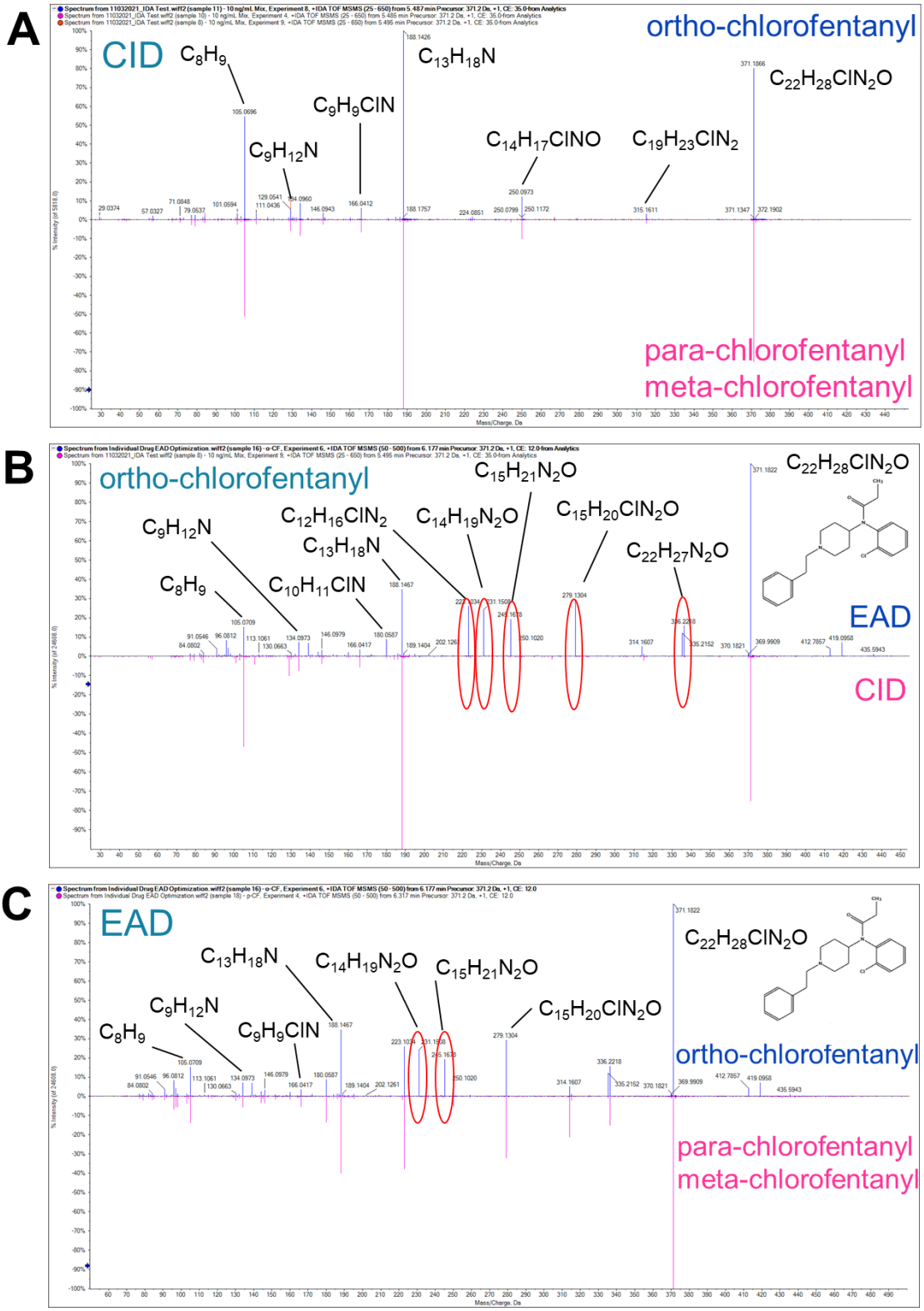


Figure 7. EAD generates unique fragment ions that enable differentiation of isobaric species. A) Zeno CID MS/MS spectra for isobaric NSO ortho-chlorofentanyl (top) and para-chlorofentanyl and meta-chlorofentanyl (bottom) showing no spectral differences, B) spectral comparison between Zeno EAD MS/MS (top) and Zeno CID MS/MS for ortho-chlorofentanyl showing unique fragment ions generated by EAD and C) Zeno EAD MS/MS spectra showing two unique fragments highlighted in red at m/z 231.1034 and 245.1678 in the spectrum of ortho-chlorofentanyl (top) that enable its differentiation from its para- and meta-chlorofentanyl analogs (bottom).

Differentiation of halogenated fentanyl analogs

Fentanyl analogs have been commonly used as adulterants in heroin and counterfeit preparations due to their high potency. In recent years, several different substituents like halogen atoms, methyl or methoxy groups of the aniline or phenethyl ring have emerged on the recreational drug market.[4] More specifically, the addition of a halogen atom to the phenethyl ring has been shown to increase potency and evade substance-specific regulations. Characterization of these designer drugs has been particularly challenging due to their structural similarities.

Figure 7A shows the Zeno CID MS/MS spectra of ortho-chlorofentanyl (top) and para- and meta-chlorofentanyl (bottom), as a mirror image. The 3 chlorofentanyl isobaric species share common fragment ions and are indistinguishable from one another. Figure 7B compares the Zeno EAD (top) and Zeno CID (bottom) MS/MS spectra as a mirror image for ortho-chlorofentanyl. As seen in the top spectrum, EAD contains many additional and unique fragments that can be used for the in-depth characterization of these isobaric species. As circled in red, EAD generated 5 unique fragments at m/z 336.2218, 279.1304, 245.1678, 231.1508 and 223.1034, which were not generated using CID. The molecular formulas of these unique fragment ions are shown with their molecular structures. Figure 7C shows the Zeno EAD MS/MS spectra of ortho-chlorofentanyl (top) and para- and meta-chlorofentanyl (bottom) as a mirror image. The spectrum of ortho-chlorofentanyl (top) contains 2 unique fragment ions highlighted in red at m/z 231.1034 and 245.1678 that are not present in the spectra of para- and meta-chlorofentanyl (bottom). The presence of these unique fragment ions generated by EAD enabled differentiation between ortho-chlorofentanyl from its para- and meta-chlorofentanyl analogs using standard solutions.

The method applicability to differentiate ortho-chlorofentanyl from para- and meta-chlorofentanyl was demonstrated using a discarded postmortem case sample. Figure 8A shows the results table generated in SCIEX OS software, which showed the positive identification of drugs and metabolites in the discarded postmortem case sample when analyzed using CID. The CID results show the positive identification of 3 compounds, which included tramadol, fentanyl and 1 of the 3 isobaric species. Positive identification determination was accomplished using the 4 confidence criteria and sorted out using the traffic light system. The Smart Confirmation algorithm was used for the spectral library, which scores all the spectra that match precursor m/z, collision energy and other filters. The spectra that match known compound names were preferentially selected and therefore each targeted chlorofentanyl isobar matched its corresponding name. This approach did not enable ubiquitous identification of the isobar present in the sample. Figure 8B shows the results table for the same samples analyzed using EAD. The table shows that the algorithm matched ortho-chlorofentanyl as the chlorofentanyl isobar present in the sample for all 3 entries. The presence of the 2 unique fragment ions at m/z 231.1034 and 245.1678 in the EAD spectrum provided unambiguous evidence for the identification of ortho-chlorofentanyl in this discarded postmortem case sample, which was not possible using CID.

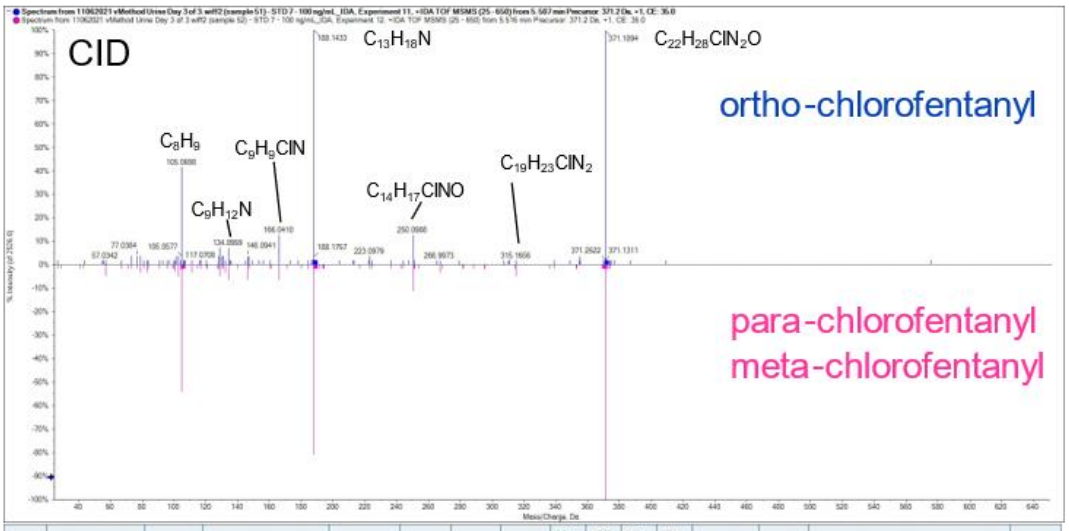
Conclusions

The use of EAD as an alternative fragmentation mechanism to generate unique, diagnostic fragment ions for the in-depth characterization and identification of challenging NPS was demonstrated. The results show that the robustness and reproducibility of EAD can provide forensic toxicologists with a unique tool for the characterization, identification and differentiation of structurally similar and isobaric NPS. The spectra acquired using Zeno EAD MS/MS contained much richer fragmentation with unique spectral features that enabled differentiation of isobaric species that were not previously distinguishable using Zeno CID MS/MS. Combining EAD with Zeno DDA provided the ability to automatically generate high-intensity diagnostic fragment ions that enabled the confident characterization and identification of challenging and low-level NPS in discarded postmortem case samples. Overall, the technological enhancements of the ZenoTOF 7600 system provided a high degree of sensitivity, selectivity and confidence for MS/MS-based characterization experiments for the forensic toxicologist.

References

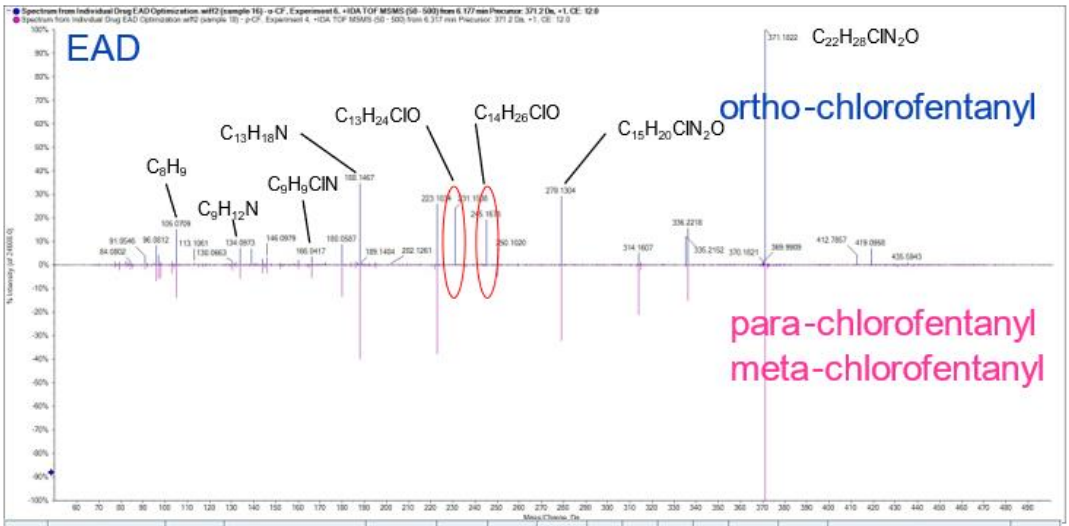
1. Qualitative flexibility combined with quantitative power. SCIEX technical note, RUO-MKT-02-13053-A.
2. vMethod Application – Single-Injection Screening of 664 Forensic Toxicology Compounds on a SCIEX X500R QTOF System.
3. Highly sensitive MS/MS detection for confident identification of potent novel synthetic opioids and their metabolites. SCIEX technical note, RUO-MKT-02-13303-B.
4. UNODC. UNODC Early Warning Advisory (EWA) on New Psychoactive Substances (NPS).

A



Index	Sample Name	Sample Type	Component Name	Compon... Type	Expe... RT	Area	Rete... Time	Mass Err...	RT Con...	Isot... Con...	Libr... Con...	Found At Mass	Mass Error...	Library Hit	Library Score
236	Drug Sample #1...	Unknown	Tramadol	Quantifiers	4.52	2.763e5	4.52	✓	✓	✓	✓	264.1955	-1.0	Tramadol	93.6
502	Drug Sample #1...	Unknown	Fentanyl	Quantifiers	5.69	7.414e8	5.69	✓	✓	✓	✓	337.2274	-0.1	Fentanyl	100.0
788	Drug Sample #1...	Unknown	para-Chlorofentanyl	Quantifiers	6.10	2.551e5	6.11	✓	✓	✓	▲	371.1883	-0.5	para-Chlorofentanyl	53.8
789	Drug Sample #1...	Unknown	ortho-chlorofentanyl	Quantifiers	6.10	2.551e5	6.11	✓	✓	✓	▲	371.1883	-0.5	ortho-chlorofentanyl	43.4
790	Drug Sample #1...	Unknown	meta-Chlorofentanyl	Quantifiers	6.11	2.551e5	6.11	✓	✓	✓	✓	371.1883	-0.5	meta-Chlorofentanyl	65.0

B



Index	Sample Name	Sample Type	Component Name	Compon... Type	Expe... RT	Area	Rete... Time	Mass Err...	RT Con...	Isot... Con...	Libr... Con...	Found At M...	Mass Error...	Library Hit	Libra... Score
249	25 - Drug Sample #1...	Unknown	Fentanyl	Quantifiers	5.02	1.237e8	4.52	✓	✓	✓	✓	337.22...	-0.9	Fentanyl	97.0
252	25 - Drug Sample #1...	Unknown	Tramadol	Quantifiers	4.30	4.805e4	5.69	✓	✓	✓	✓	264.19...	-0.3	Tramadol	83.4
259	25 - Drug Sample #1...	Unknown	ortho-chlorofentanyl	Quantifiers	5.29	4.600e4	6.11	✓	✓	✓	✓	371.18...	-1.2	o-CF [Smart Confirmation]	73.6
260	25 - Drug Sample #1...	Unknown	meta-chlorofentanyl	Quantifiers	5.29	4.604e4	6.11	✓	✓	✓	✓	371.18...	-1.2	o-CF [Smart Confirmation]	73.6
261	25 - Drug Sample #1...	Unknown	para-chlorofentanyl	Quantifiers	5.29	4.604e4	6.11	✓	✓	✓	✓	371.18...	-1.2	o-CF [Smart Confirmation]	73.6

Figure 8. EAD enabled the identification of the correct chlorofentanyl isobar in a discarded postmortem case sample. A) The SCIEX OS software results table showing positive identification of 3 analytes acquired using CID, including a chlorofentanyl isobar. The acquired Zeno CID MS/MS spectra were identical for the 3 possible chlorofentanyl isobars and did not enable correct identification. B) The SCIEX OS software results table for the same sample analyzed using EAD. The results showed the positive identification of ortho-chlorofentanyl as the chlorofentanyl isobar present in the sample for all 3 entries. The acquired Zeno EAD MS/MS spectra showed 2 unique fragment ions that provided unambiguous evidence for the identification of ortho-chlorofentanyl.

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High-sensitivity detection of forensic drug panel in human whole blood

Using the SCIEX Triple Quad™ 7500 LC-MS/MS – QTRAP® Ready System, powered by SCIEX OS Software

Pierre Negri[1] and Ian Moore[2]

[1]SCIEX, USA; [2]SCIEX, CA

Achieving low levels of detection while maintaining reliable quantification is a key performance indicator to any robust bioanalytical workflow. In the forensic laboratory, the ability to accurately quantify large panels of analytes in complex biological matrices over a wide range of concentration is challenging and often requires dilution of the samples to bring them within the calibration range of the instrument. In order to further reduce sample preparation and re-analysis time, sufficient data sampling across the chromatographic peaks and over a wide linear dynamic range is required to ensure comprehensive analyte coverage and rugged quantitative performance.

In this technical note, the SCIEX Triple Quad 7500 System is presented as a robust quantitative platform with exceptional performance in speed, linear dynamic range and sensitivity for the accurate quantification of a panel of 49 drugs in human whole blood. The optimized method maximizes the linear dynamic range capabilities of the assay while retaining the required levels of accuracy and performance. The addition of new hardware features of the OptiFlow® Pro Ion Source, the D Jet™ Ion Guide and the E Lens™ Technology enhance ion sampling and desolvation,[1] resulting in unparalleled sensitivity and quantification limit improvements for the suite of drugs targeted in this study.

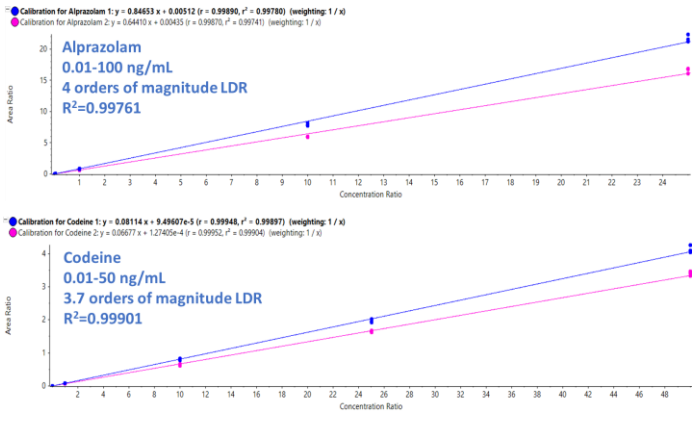


Figure 1. High linearity and linear dynamic range (LDR) demonstrated for chemically diverse panel of drugs. Calibration curves resulting from the calibration series of alprazolam (0.01-100 ng/mL) and codeine (0.01-50 ng/mL) showing excellent linear response even at pg/mL concentrations.

Key advantages of the SCIEX Triple Quad 7500 System for forensic drug panel analysis

- The SCIEX Triple Quad 7500 System is a robust instrument with unparalleled sensitivity and quantitative performance
- SCIEX OS Software provides an easy to use and intuitive platform for both for data acquisition and processing
- Fast (6.5 minutes) chromatographic run time achievable with the Scheduled MRM™ Algorithm Pro in SCIEX OS Software and fast polarity switching during data acquisition
- Robust and easy to use OptiFlow Pro Ion Source provides efficient sample ionization with intelligent source design eliminating the need for physical optimization
- Improved desolvation and ion sampling of system enhanced sensitivity, with LLOQ in the sub ng/mL to pg/mL range, demonstrated in complex biological matrices for the panel of drugs targeted in this study
- Accurate quantification of all the drugs in the panel, over a wide range of concentrations, without any sacrifice to data quality
- Overall performance of the system resulted in excellent precision and reproducibility in the reported data, even at low concentration levels

Experimental details

Target analytes and solutions: A total of 49 drugs and 18 deuterated internal standards were purchased from Cerilliant Corporation (Round Rock, TX). Two solutions were prepared in methanol: a standard mixture containing the 49 target analytes and an internal standard mixture containing the 18 deuterated internal standards. Table 1 lists the name, the calibration range, linear correlation value (R^2), and LLOQ, as well as the accuracy and precision reported at the LLOQ for each of the 49 target analytes used in this panel.

Calibrator preparation: Eight levels of calibrators ranging from 10 µg/mL to 1 pg/mL were prepared in methanol. A 10 ng/mL IS standard stock solution was prepared in MeOH/water (20:80, v/v) for sample reconstitution prior to injection.

Sample preparation: 10 µL of each calibrator solution was spiked into 90 µL of human whole blood. Each spiked human whole blood sample was extracted by using a protein precipitation procedure. In short, 900 µL of methanol/acetonitrile (50:50, v/v) were added into each of the spiked human whole blood samples and vortexed for 1 min then followed by 3 min sonication and another 1 min of vortex mixing. The samples were then centrifuged for 5 min at 8,000 rpm. The supernatant was transferred out to a glass tube and completely dried down under nitrogen gas. The residues were reconstituted with 500 µL of a 10 ng/mL IS standard stock solution in methanol/water (20:80, v/v). The protein precipitation procedure is shown in Figure 2.

Liquid chromatography: HPLC separation was performed on an ExionLC™ System using a Phenomenex Kinetex Phenyl-Hexyl column (50 × 2.1 mm, 2.6µm, 00B-4495-E0). The separation conditions were identical to those previously described in a technical note.[2] Mobile phases were ammonium formate in water (MPA) and formic acid in methanol (MPB). The injection volume was 5 µL and the LC runtime was 6.5 min.

Mix	•10 µL of std mixture with 90 µL of human whole blood
Load to tube	•900 µL of MeOH: MeCN (50:50, v/v)
Vortex	•Vortex vigorously for 1 min
Sonicate	•Sonicate for 3 min
Vortex	•Vortex vigorously for 1 min
Centrifuge	•5 min at 8,000 rpm
Transfer	•Transfer supernatant to glass vial
Evaporate	•Evaporate to dryness under nitrogen
Reconstitute	•Add 500 µL of a 10 ng/mL IS std mixture in MeOH: water (20:80, v/v)

Figure 2. Protein precipitation procedure for human whole blood samples. A 9-step protein precipitation protocol was used for selectively extracting drugs from human whole blood samples for analysis with the SCIEX Triple Quad 7500 System.

Mass spectrometry: A SCIEX Triple Quad 7500 System equipped with an OptiFlow Pro Ion Source with an OptiFlow analytical probe and E Lens™ Technology was used. The ionization source was operated in electrospray ionization (ESI) mode in both positive and negative polarities. A single acquisition method consisting of 134 MRM transitions (98 for the drugs and 36 for the internal standards) was created using the Scheduled MRM Algorithm Pro in SCIEX OS Software 2.0. Two MRM transitions were monitored for each of the targeted drugs and each sample was injected in triplicate to build a data analysis processing method.

Data analysis: Data processing was performed using SCIEX OS Software. Detection and integration of the peaks from the background was achieved within the viewing window using the AutoPeak algorithm. Quantitative analysis was performed in the Analytics module of the software where calibration curves, concentration calculations, assay precision and accuracy statistics were generated.

Method development and optimization

A diluted, neat standard mixture containing the 49 target analytes was used for initial method development. The Scheduled MRM Algorithm Pro in SCIEX OS Software was used to optimize data sampling across each peak while maintaining optimal dwell times for each MRM transition to ensure reliable integration, quantification and confirmation of the peak for each target analyte. In addition, fast polarity switching was used to provide maximum analyte coverage. Most MRM transitions had 15 or more data points across each of the LC peaks, with 10 being the minimum number of data points across each peak for each of the 49 target analytes used in this study. Figure 3 shows the elution profile for the 49 targeted drugs resulting from the optimized data acquisition method.

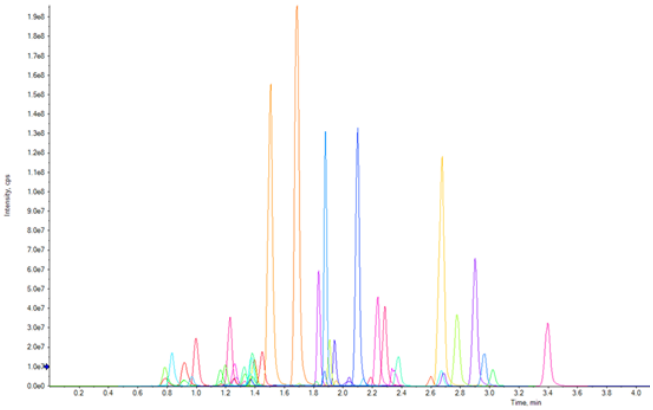


Figure 3. Chromatographic profile of the 49 drugs targeted in this study. Extracted ion chromatograms (XICs) resulting from the optimized data acquisition using a neat standard mixture. Method optimization using the Scheduled MRM™ Algorithm Pro in SCIEX OS Software enabled collection of optimal data quality even during regions of the chromatogram when MRM concurrency was very high.

Optimized detection method enables accurate and reliable drug quantification

Control human whole blood samples spiked with the 49 target analytes were prepared at concentrations ranging from 1 µg/mL down to 0.1 pg/mL. Detection and integration of the peaks was performed automatically using the AutoPeak Algorithm in the Analytics module of SCIEX OS Software. Analyte concentration and ion ratio were calculated automatically in the software.

The potential for drastic variation in detected drug levels in toxicology case samples necessitates the use of an instrument with high range of linear response. Figure 1 shows calibration curves for two of the drugs targeted in this study. Concentration range of 4 (from 0.01-100 ng/mL) and 3.7 (from 0.01-50 ng/mL) orders linear dynamic range was demonstrated for alprazolam and codeine, respectively. Excellent linearity was observed across the concentration ranges analyzed with R^2 average values of 0.99761 and 0.99901 for alprazolam and codeine, respectively. Similar trends were observed for the other analytes used in this study.

OptiFlow Pro Ion Source and E Lens Technology leads to enhanced sensitivity

Developing robust workflows that can deliver high levels of sensitivity is critical to any toxicology laboratory needing to quantify a wide concentration of drugs extracted from biological specimens. To this end, the sensitivity of the SCIEX Triple Quad 7500 System was assessed by determining the lower limit of quantification (LLOQ) values for the 49 targeted drugs in the panel. LLOQ values were determined as the lowest concentration calibration level fitting the following standard performance requirements: signal-to-noise ratio (S/N) of at least 10, calculated concentration accuracy within 20% of 100%, precision (%bias) below 25%, and falling on a linear calibration curve with an R^2 value of at least 0.98.

Figure 4 shows the extracted ion chromatogram (XIC) traces and resulting calibration curves of the two MRM transitions monitored for 7-hydroxymitragynine (Figure 4A) and acetyl fentanyl (Figure 4B). The two series of XIC traces for both the quantifier and qualifier ions of each of the two detected drugs showed a high level of sensitivity and precision across the calibration series for concentrations ranging from 0.1 to 100 ng/mL for 7-hydroxymitragynine and 0.02-50 ng/mL for acetyl fentanyl, respectively. Six levels of calibrators were used to determine the ion ratio criteria for the quantifier and qualifier ions of these two targeted drugs. The results demonstrated excellent correlation of the generated regression curves covering concentration ranges far exceeding typical bioanalytical requirements.

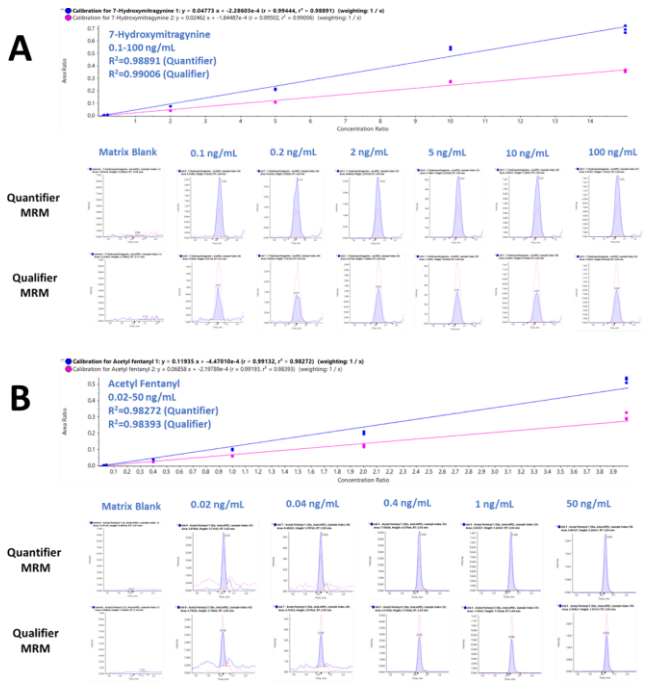


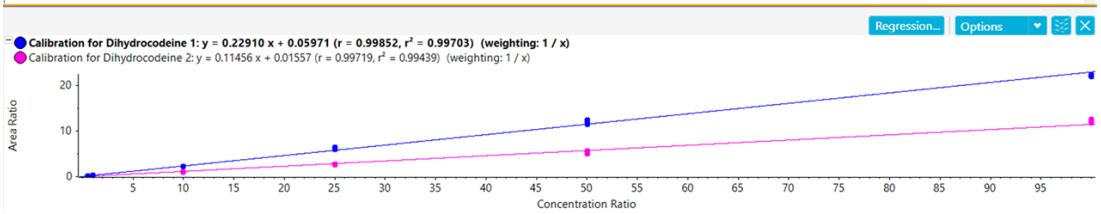
Figure 4: High sensitivity and linearity for selected drugs in the forensic panel. A) Calibration curves and XIC traces resulting from the calibration series for the two transitions of 7-hydroxymitragynine from 0.1 to 100 ng/mL. B) Calibration curves and XIC traces resulting from the calibration series for the two transitions of acetyl fentanyl from 0.02 to 50 ng/mL. The calibration curves and XIC traces demonstrate excellent linearity and sensitivity.

Table 1 summarizes the statistical results obtained for the 49 analytes spiked in human whole blood. The table includes calibration range, linear correlation coefficient (R^2 Value), and LLOQ, as well as the accuracy and precision for each of the two MRM transitions monitored for each drug. Overall, the assay showed excellent reproducibility, precision, accuracy, and linearity, proving the robustness and performance of the developed method.

Figure 5 shows the statistical results and the calibration curves resulting from the peak area integration of dihydrocodeine (Figure 5A) and noroxycodone (Figure 5B) from 0.5 to 100 ng/mL. Excellent linearity, reproducibility, accuracy and precision was observed across the six calibration levels covering the concentration range. The assay showed excellent precision and accuracy, and the averaged R^2 values for the quantifier and qualifier ions were 0.99571 and 0.99323, respectively. Full quantification was achieved with SCIEX OS Software, designed for quick, intuitive and streamlined data processing with accurate and reliable results.

A

Row	Component Na...	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3
13	Dihydrocodeine 1	0.50000000	3 of 3	5.282e-1	2.343e-2	4.44	105.64	5.311e-1	5.500e-1	5.035e-1
32	Dihydrocodeine 2	0.50000000	3 of 3	6.042e-1	2.105e-2	3.48	120.84	5.972e-1	6.279e-1	5.876e-1
14	Dihydrocodeine 1	1.00000000	3 of 3	9.053e-1	6.657e-2	7.35	90.53	9.788e-1	8.880e-1	8.491e-1
33	Dihydrocodeine 2	1.00000000	3 of 3	9.628e-1	4.862e-2	5.05	96.28	9.715e-1	1.007e0	9.105e-1
15	Dihydrocodeine 1	10.00000000	3 of 3	9.515e0	2.840e-1	2.98	95.15	9.380e0	9.842e0	9.324e0
34	Dihydrocodeine 2	10.00000000	3 of 3	9.019e0	5.630e-1	6.24	90.19	9.347e0	8.369e0	9.342e0
16	Dihydrocodeine 1	25.00000000	3 of 3	2.708e1	1.222e0	4.51	108.34	2.784e1	2.774e1	2.567e1
35	Dihydrocodeine 2	25.00000000	3 of 3	2.332e1	9.771e-1	4.19	93.30	2.436e1	2.320e1	2.242e1
17	Dihydrocodeine 1	50.00000000	3 of 3	5.187e1	2.320e0	4.47	103.74	4.942e1	5.216e1	5.403e1
36	Dihydrocodeine 2	50.00000000	3 of 3	4.680e1	2.492e0	5.33	93.59	4.892e1	4.405e1	4.742e1
19	Dihydrocodeine 1	100.00000000	3 of 3	9.660e1	9.927e-1	1.03	96.60	9.639e1	9.572e1	9.767e1
38	Dihydrocodeine 2	100.00000000	3 of 3	1.058e2	3.533e0	3.34	105.79	1.093e2	1.058e2	1.023e2



B

Row	Component Na...	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3
13	Noroxycodone 1	0.50000000	3 of 3	5.526e-1	3.100e-2	5.61	110.52	5.879e-1	5.298e-1	5.402e-1
32	Noroxycodone 2	0.50000000	3 of 3	5.618e-1	3.159e-2	5.62	112.35	5.951e-1	5.578e-1	5.323e-1
33	Noroxycodone 2	1.00000000	3 of 3	9.259e-1	5.623e-2	6.07	92.59	9.588e-1	8.610e-1	9.580e-1
14	Noroxycodone 1	1.00000000	3 of 3	9.381e-1	7.866e-2	8.39	93.81	9.768e-1	8.475e-1	9.898e-1
15	Noroxycodone 1	10.00000000	3 of 3	9.629e0	8.139e-1	8.45	96.29	9.549e0	1.048e1	8.857e0
34	Noroxycodone 2	10.00000000	3 of 3	9.634e0	7.583e-1	7.87	96.34	9.674e0	1.037e1	8.857e0
35	Noroxycodone 2	25.00000000	3 of 3	2.614e1	1.026e0	3.92	104.57	2.732e1	2.569e1	2.542e1
16	Noroxycodone 1	25.00000000	3 of 3	2.635e1	9.865e-1	3.74	105.41	2.740e1	2.622e1	2.544e1
36	Noroxycodone 2	50.00000000	3 of 3	4.491e1	5.527e0	12.31	89.82	5.036e1	4.507e1	3.931e1
17	Noroxycodone 1	50.00000000	3 of 3	4.494e1	3.441e0	7.66	89.88	4.870e1	4.419e1	4.194e1
19	Noroxycodone 1	100.00000000	3 of 3	1.041e2	2.128e0	2.04	104.08	1.064e2	1.036e2	1.023e2
38	Noroxycodone 2	100.00000000	3 of 3	1.043e2	3.668e0	3.52	104.33	1.086e2	1.023e2	1.021e2

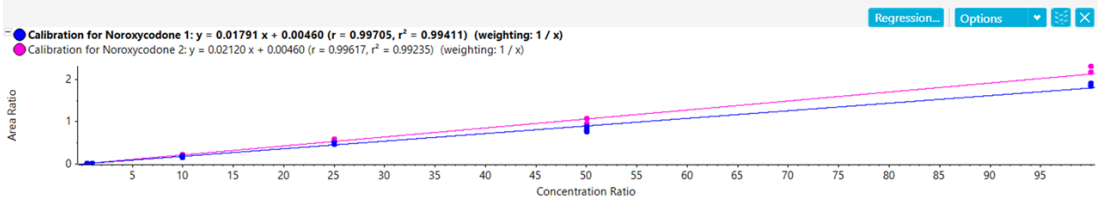


Figure 5. Statistical results and calibration curves for selected drugs in the forensic panel. Statistics pane and calibration curves for dihydrocodeine (A) and noroxycodone (B) from 0.5 to 100 ng/mL. Both analytes showed excellent linearity, reproducibility, accuracy and precision across the six calibration levels, proving the overall robustness of the method.

Conclusions

An optimized and sensitive method for the detection of a panel of 49 drugs in human whole blood is described using the SCIEX Triple Quad 7500 System. The use of the Scheduled MRM Algorithm Pro in SCIEX OS Software enabled optimization of data sampling. The addition of new hardware features, the OptiFlow Pro Ion Source, the D Jet Ion Guide and the E Lens, provided sensitive quantification of all the targeted drugs in the sub ng/mL range, with some down to the pg/mL levels, while maintaining linearity, precision and accuracy of measurement. This exceptional sensitivity was achieved without any sacrifice or compromise to data quality, as demonstrated by the excellent precision and accuracy observed at the LLOQ. Overall, the combination of the features on the SCIEX Triple Quad 7500 System results in unparalleled sensitivity improvement for the suite of drugs targeted in this study.

References

1. Enabling new levels of quantification - using the SCIEX Triple Quad™ 7500 LC-MS/MS System – QTRAP® Ready, powered by SCIEX OS Software. SCIEX Technical Note RUO-MKT-02-11886-A.
2. High Sensitivity and Dynamic Range for 93-Compound Forensic Panel Analysis in Urine. SCIEX Technical Note RUO-MKT-02-9914-A.

Table 1. Statistical results for the 49 drugs targeted in this workflow. The table includes calibration range, linear correlation coefficient (R^2 Value), and LLOQ, as well as the accuracy and precision at the LLOQ for each of the two MRM transitions monitored for each drug.

Compound	Calibration Range (ng/mL)	Linear Correlation (R^2)	LLOQ (ng/mL)	Accuracy at LLOQ (%)	Precision at LLOQ (%)
6-MAM 1	*0.2-200*	*0.99191*	*0.2*	*82.62*	*2.74*
6-MAM 2	*0.2-200*	*0.99020*	*0.2*	*83.02*	*4.91*
7-Aminoclonazepam 1	*0.5-100*	*0.99910*	*0.5*	*117.72*	*2.66*
7-Aminoclonazepam 2	*0.5-100*	*0.99936*	*0.5*	*112.71*	*7.54*
7-Hydroxymitragynine 1	*0.1-100*	*0.98891*	*0.1*	*84.76*	*1.72*
7-Hydroxymitragynine 2	*0.1-100*	*0.99006*	*0.1*	*90.58*	*4.33*
Acetyl fentanyl 1	*0.02-50*	*0.98272*	*0.02*	*113.43*	*6.33*
Acetyl fentanyl 2	*0.02-50*	*0.98393*	*0.02*	*112.01*	*9.49*
Alpha-Hydroxyalprazolam 1	*1-100*	*0.99918*	*1*	*93.38*	*2.57*
Alpha-Hydroxyalprazolam 2	*1-100*	*0.99817*	*1*	*93.67*	*7.67*
Alprazolam 1	*0.01-100*	*0.99780*	*0.01*	*84.15*	*5.96*
Alprazolam 2	*0.01-100*	*0.99741*	*0.01*	*80.98*	*1.33*
Amphetamine 1	*0.2-200*	*0.98925*	*0.2*	*117.13*	*22.79*
Amphetamine 2	*0.2-200*	*0.98784*	*0.2*	*86.02*	*8.95*
Benzoylecgonine 1	*0.5-100*	*0.9777*	*0.5*	*100.00*	*5.82*
Benzoylecgonine 2	*0.5-100*	*0.99776*	*0.5*	*103.70*	*3.07*
Buprenorphine 1	*0.4-400*	*0.99018*	*0.4*	*98.61*	*24.21*
Buprenorphine 2	*0.4-400*	*0.98312*	*0.4*	*96.09*	*5.49*
Carisoprodol 1	*2-200*	*0.95267*	*2*	*95.22*	*13.59*
Carisoprodol 2	*2-200*	*0.98049*	*2*	*117.40*	*12.85*
Codeine 1	*0.01-50*	*0.99897*	*0.01*	*80.29*	*13.48*
Codeine 2	*0.01-50*	*0.99904*	*0.01*	*93.47*	*17.80*
Dextromethorphan 1	*0.5-100*	*0.99598*	*0.5*	*119.17*	*10.95*
Dextromethorphan 2	*0.5-100*	*0.99772*	*0.5*	*101.42*	*5.86*
Diazepam 1	*0.5-100*	*0.99916*	*0.5*	*118.51*	*1.07*
Diazepam 2	*0.5-100*	*0.99522*	*0.5*	*118.30*	*0.96*
Dihydrocodeine 1	*0.5-100*	*0.98889*	*0.5*	*98.40*	*5.04*
Dihydrocodeine 2	*0.5-100*	*0.98006*	*0.5*	*117.31*	*4.45*
EDDP 1	*0.1-200*	*0.98973*	*0.1*	*90.93*	*8.74*
EDDP 2	*0.1-200*	*0.99128*	*0.1*	*109.63*	*13.35*
Fentanyl 1	*0.4-400*	*0.98210*	*0.4*	*112.74*	*3.66*
Fentanyl 2	*0.4-400*	*0.98615*	*0.4*	*110.68*	*2.30*

Compound	Calibration Range (ng/mL)	Linear Correlation (R^2)	LLOQ (ng/mL)	Accuracy at LLOQ (%)	Precision at LLOQ (%)
Gabapentin 1	2-200	0.98159	2	115.14	5.53
Gabapentin 2	2-200	0.98326	2	113.60	3.94
Hydrocodone 1	1-100	0.97652	1	99.30	5.59
Hydrocodone 2	1-100	0.99070	1	103.96	7.82
Hydromorphone 1	1-100	0.98541	1	110.69	0.35
Hydromorphone 2	1-100	0.98618	1	97.29	1.25
Lorazepam 1	0.5-100	0.99699	0.5	0.6.76	2.89
Lorazepam 2	0.5-100	0.99680	0.5	119.49	5.71
MDA 1	10-100	0.92279	10	109.46	19.09
MDA 2	10-100	0.99617	10	108.54	10.94
MDEA 1	2-200	0.99567	2	100.00	7.62
MDEA 2	2-200	0.99682	2	100.00	5.95
MDMA 1	5-200	0.9855	5	108.39	1.94
MDMA 2	5-200	0.99562	5	102.96	5.53
Methadone 1	1-100	0.99040	1	97.53	4.64
Methadone 2	1-100	0.99330	1	115.54	5.37
Methamphetamine 1	2-200	0.99361	2	112.10	324
Methamphetamine 2	2-200	0.99550	2	105.70	1.71
Methylphenidate 1	1-100	0.99276	1	96.82	0.65
Methylphenidate 2	1-100	0.98423	1	87.52	4.34
Midazolam 1	1-100	0.99861	1	92.81	3.91
Midazolam 2	1-100	0.99484	1	114.07	3.21
Mitragynine 1	0.2-200	0.99572	0.2	97.85	16.06
Mitragynine 2	0.2-200	0.99422	0.2	96.69	6.71
Morphine 1	1-100	0.98897	0.2	116.82	0.73
Morphine 2	1-100	0.98830	0.2	116.50	1.46
Naloxone 1	1-100	0.98323	1	100.71	0.90
Naloxone 2	1-100	0.98563	1	97.88	4.11
Naltrexone 1	0.5-100	0.99273	0.5	119.45	4.17
Naltrexone 2	0.5-100	0.98868	0.5	119.72	18.77
Norbuprenorphine 1	0.4-200	0.97745	0.4	98.35	4.73
Norbuprenorphine 2	0.4-200	0.97920	0.4	98.05	0.50
Norcodeine 1	0.5-100	0.98892	0.5	119.46	2.73
Norcodeine 2	0.5-100	0.99030	0.5	117.30	1.03
Nordiazepam 1	1-100	0.99073	1	107.85	0.48
Nordiazepam 2	1-100	0.99087	1	107.46	1.11

Compound	Calibration Range (ng/mL)	Linear Correlation (R^2)	LLOQ (ng/mL)	Accuracy at LLOQ (%)	Precision at LLOQ (%)
Norfentanyl 1	*0.4-100*	*0.99366*	*0.4*	*100.28*	*4.03*
Norfentanyl 2	*0.4-100*	*0.99601*	*0.4*	*99.91*	*4.13*
Norhydrocodone 1	*1-100*	*0.98777*	*1*	*93.83*	*6.16*
Norhydrocodone 2	*1-100*	*0.99486*	*1*	*94.06*	*7.91*
Noroxycodone 1	*0.5-100*	*0.99411*	*0.5*	*110.52*	*5.61*
Noroxycodone 2	*0.5-100*	*0.99235*	*0.5*	*112.35*	*5.62*
Norpropoxyphene 1	*1-200*	*0.97989*	*1*	*105.35*	*9.60*
Norpropoxyphene 2	*1-200*	*0.98323*	*1*	*104.65*	*10.70*
O-Desmethyltramadol 1	*1-100*	*0.96310*	*1*	*93.34*	*13.91*
O-Desmethyltramadol 2	*1-100*	*0.96164*	*1*	*90.77*	*4.82*
Oxazepam 1	*0.5-100*	*0.99704*	*0.5*	*85.31*	*7.74*
Oxazepam 2	*0.5-100*	*0.99195*	*0.5*	*86.37*	*14.15*
Oxycodone 1	*0.5-100*	*0.98486*	*0.5*	*113.73*	*11.84*
Oxycodone 2	*0.5-100*	*0.98492*	*0.5*	*115.65*	*10.38*
Oxymorphone 1	*1-100*	*0.98986*	*0.5*	*114.44*	*2.78*
Oxymorphone 2	*1-100*	*0.99246*	*0.5*	*116.20*	*1.98*
PCP 1	*0.5-100*	*0.99167*	*0.5*	*103.36*	*10.99*
PCP 2	*0.-100*	*0.98672*	*0.5*	*92.84*	*14.21*
Pregabalin 1	*1-200*	*0.98380*	*1*	*94.53*	*16.48*
Pregabalin 2	*1-200*	*0.98277*	*1*	*94.79*	*16.12*
Tapentadol 1	*0.5-100*	*0.98018*	*0.5*	*94.01*	*13.36*
Tapentadol 2	*0.5-100*	*0.99810*	*0.5*	*103.41*	*7.72*
Temazepam 1	*0.05-100*	*0.99217*	*0.05*	*113.97*	*4.89*
Temazepam 2	*0.05-100*	*0.99249*	*0.05*	*119.15*	*4.38*
Tramadol 1	*0.5-100*	*0.99674*	*0.5*	*102.19*	*10.33*
Tramadol 2	*0.5-100*	*0.99779*	*0.5*	*110.82*	*3.63*
Zolpidem 1	*1-100*	*0.98577*	*1*	*93.87*	*4.83*
Zolpidem 2	*1-100*	*0.99810*	*1*	*103.41*	*7.81*
THC-COOH 1	*1-100*	*0.9537*	*1*	*116.92*	*5.86*
THC-COOH 2	*1-100*	*0.9846*	*1*	*118.37*	*7.68*

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Quantitation of novel psychoactive substances in wastewater by direct injection analysis

Using the SCIEX 7500 system powered by SCIEX OS software

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Introduction

This technical note describes a direct injection analysis method for the simultaneous quantitation of 32 novel psychoactive substances (NPS) in wastewater samples. The high sensitivity of the SCIEX 7500 system enabled the development of a simple direct injection method that achieved ng/L limits of quantitation (LOQs) for multiple drug classes. Application of this method to the analysis of Australian wastewater resulted in the detection of 3 NPS (eutylone, clonazolam and etizolam) for the first time by direct injection (Figure 1).[1]

Wastewater surveillance of illicit drugs has been routinely used to study recreational drug consumption trends in many countries.[2] There has been an emerging interest in monitoring NPS at hotspots, such as music festivals at which drug usage might be increased.[1,3] Direct injection analysis minimizes contamination and irreproducible results but is challenged by low concentrations of NPS due to their infrequent consumption and their dilution in wastewater. Solid phase extraction (SPE) and liquid-liquid extraction (LLE) are typically required for the quantitation of low-level NPS, but these methods are laborious and time-consuming.[4,5] In addition, extraction conditions must be optimized to cover the wide range of physicochemical properties of different classes of NPS, such as synthetic cannabinoids, synthetic cathinones, synthetic opioids, benzodiazepines and phenylethylamines. In this work, the sensitivity of the SCIEX 7500 system allowed direct injection analysis of low-level NPS present in wastewater while enabling matrix dilution of interferences that would have otherwise been concentrated during SPE and LLE. High-throughput direct injection analysis can rapidly provide wastewater surveillance data to inform public health alerts and proactive drug education programs.

Key features of the SCIEX 7500 system for direct injection analysis of wastewater

- High sensitivity of the SCIEX 7500 system achieved low- to mid-ppt (0.5–195.3 ng/L) LOQs, resulting in the detection of eutylone, etizolam and clonazolam in Australian wastewater for the first time by direct injection
- A rapid direct injection LC-MS/MS method for the analysis of 32 NPS in wastewater was developed and validated
- Reduced matrix interferences from direct injection enabled the use of solvent-based calibration for quantitation
- Custom calculations and flagging in SCIEX OS software expedited the review of method validation acceptance criteria

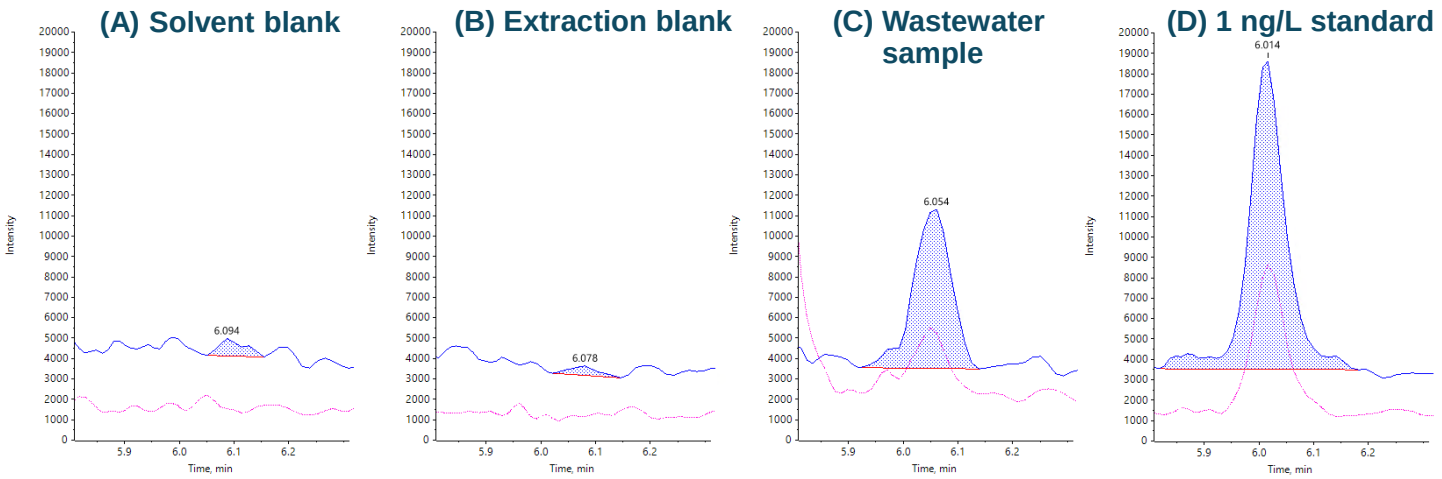

Figure 1. Detection of eutylone in wastewater compared to quality control samples. A) MilliQ water solvent blank. B) Extraction blank comprised of acidified MilliQ water prepared in the same manner as the wastewater samples. C) Wastewater sample. D) 1 ng/L standard. The blue trace represents the quantifier transition (*m/z* 236.0 > 188.1) and the pink trace represents the qualifier transition (*m/z* 236.0 >174.0).

Experimental methods

Chemicals and samples: The target analyte list included 32 NPS and deuterated surrogate internal standards that were assigned to the analytes based on retention time (RT) and chemical class. Individual neat standards were mixed to prepare stock solutions in methanol from which calibration standards (0.02–1000 ng/L) were prepared on the day of analysis.

Influent wastewater samples (250 mL) were collected from various sites in Eastern Australia during the summer holiday period of 2021. Upon collection, all samples were acidified to pH 2 with hydrochloric acid and stored at -20°C until analysis.

Direct injection preparation: Thawed wastewater from each site was filtered through a 0.2 µm filter into a glass tube. A 1 mL aliquot was transferred to a glass vial and spiked with an internal standard mix for LC-MS/MS analysis. Laboratory blanks comprised of acidified MilliQ water were prepared in the same manner for quality control.

Chromatography: LC separation was performed on a Shimadzu Nexera LC40 system using a Phenomenex Kinetex Biphenyl column (50 x 2.1 mm, 2.6 µm) fitted with a SecurityGuard ULTRA Biphenyl cartridge. A flow rate of 0.35 mL/min, an injection volume of 10 µL and a column temperature of 40ºC were used. The gradient used is shown in Table 1.

Table 1. Chromatographic gradient.

Time (min)	%A	%B
0.0	*95*	*5*
2.0	*95*	*5*
13.0	*0*	*100*
15.0	*0*	*100*
15.1	*95*	*5*
18.5	*95*	*5*

Mobile phase A: 95:5 (v/v), MilliQ water/methanol with 0.1% formic acid
Mobile phase B: 95:5 (v/v), methanol/MilliQ water with 0.1% formic acid

Mass spectrometry: Analysis was performed using the SCIEX 7500 system with an OptiFlow Pro ion source in positive electrospray ionization mode. Table 2 shows the method parameters used for the mass spectrometer. Data were acquired in scheduled multiple reaction monitoring (sMRM) mode with a 1-minute window around the RT of each analyte. Figure 2 shows the distribution of RTs for 136 MRM transitions and their corresponding dwell times calculated by the software.

Table 2. Source, gas and temperature conditions.

Parameter	Value
Curtain gas (CUR)	*40 psi*
Collision gas (CAD)	*10 psi*
IonSpray voltage (ISV)	*2600 V*
Temperature (TEM)	*450°C*
Nebulizer gas (GS1)	*60 psi*
Heater gas (GS2)	*60 psi*

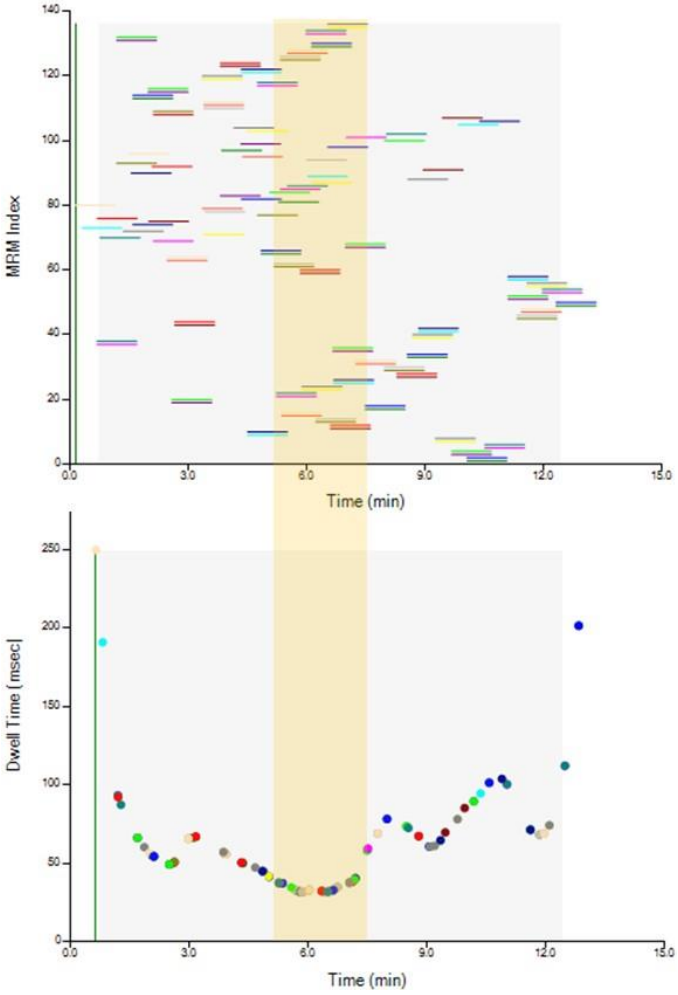

Figure 2. sMRM summary plots of the RT distribution of analytes (top) and their dwell times (bottom). The yellow bar indicates regions of high MRM concurrency, as shown by the lower dwell times for those transitions.

These sMRM summary plots can be used to visually assess the acquisition method during method development. For example, the user might want to manually increase the software-calculated dwell times to optimize the acquisition signal of transitions eluting in regions of high concurrency, as shown in the yellow shaded region in Figure 2.

Data processing: All data were acquired and processed using SCIEX OS software, version 2.1.6. Analyte peak areas were normalized to their corresponding surrogate internal standards. Table 3 lists the quantifier and confirmation MRM transitions and the surrogate internal standard assigned to each analyte. Custom calculations and flagging rules were used to expedite the review of data. These rules considered the tolerance range for method validation parameters, such as a ±20% ion ratio, RT within 2% of the standard and ±30% matrix effects.

Smaller injection load to reduce matrix effects

The sensitivity of the SCIEX 7500 system enabled the use of a small 10 µL injection volume to reduce the matrix load on the LC column. The matrix effect was calculated as the quotient of the peak area in spiked wastewater replicates (*n* = 5) and spiked solvent and displayed with their respective precision (%RSD) at low (5 ng/L), medium (50 ng/L) and high spiking (500 ng/L) levels (Figure 3). In general, matrix effects improved as the spiking level

Sample Name	Sample ID	Sample Type	Inj... Vo...	Compo... Name	Ret... Time	Area	Ion Ratio	Ion Ratio...	RT Co...	*Solve...	*ME Low	*ME Med	*ME High	*RSD Low	*RSD Med	*RSD High
5 ppt	Solvent	Standard	10.00	Eutylone 1	6.08	2.543e5	0.4173	✓	✓							
50 ppt	Solvent	Standard	10.00	Eutylone 1	6.07	1.735e6	0.4147	✓	✓							
500 ppt	Solvent	Standard	10.00	Eutylone 1	6.07	2.409e7	0.3868	✓	✓							
WW 5 ppt replicate 1	Low	Quality Control	10.00	Eutylone 1	6.06	2.177e5	0.3882	✓	✓	2.543e5	86			2.0		
WW 5 ppt replicate 2	Low	Quality Control	10.00	Eutylone 1	6.08	2.179e5	0.4435	✓	✓	2.543e5	86			2.0		
WW 5 ppt replicate 3	Low	Quality Control	10.00	Eutylone 1	6.07	2.171e5	0.4525	✓	✓	2.543e5	85			2.0		
WW 5 ppt replicate 4	Low	Quality Control	10.00	Eutylone 1	6.06	2.206e5	0.4493	✓	✓	2.543e5	87			2.0		
WW 5 ppt replicate 5	Low	Quality Control	10.00	Eutylone 1	6.06	2.275e5	0.4362	✓	✓	2.543e5	89			2.0		
WW 50 ppt replicate 1	Med	Quality Control	10.00	Eutylone 1	6.07	2.182e6	0.4075	✓	✓	1.735e6		126			3.3	
WW 50 ppt replicate 2	Med	Quality Control	10.00	Eutylone 1	6.06	2.355e6	0.4035	✓	✓	1.735e6		136			3.3	
WW 50 ppt replicate 3	Med	Quality Control	10.00	Eutylone 1	6.07	2.255e6	0.3952	✓	✓	1.735e6		130			3.3	
WW 50 ppt replicate 4	Med	Quality Control	10.00	Eutylone 1	6.07	2.321e6	0.4138	✓	✓	1.735e6		134			3.3	
WW 50 ppt replicate 5	Med	Quality Control	10.00	Eutylone 1	6.07	2.364e6	0.4013	✓	✓	1.735e6		136			3.3	
WW 500 ppt replicate 1	High	Quality Control	10.00	Eutylone 1	6.06	2.600e7	0.3984	✓	✓	2.409e7			108			3.8
WW 500 ppt replicate 2	High	Quality Control	10.00	Eutylone 1	6.05	2.679e7	0.3949	✓	✓	2.409e7			111			3.8
WW 500 ppt replicate 3	High	Quality Control	10.00	Eutylone 1	6.06	2.660e7	0.3927	✓	✓	2.409e7			110			3.8
WW 500 ppt replicate 4	High	Quality Control	10.00	Eutylone 1	6.07	2.499e7	0.4018	✓	✓	2.409e7			104			3.8
WW 500 ppt replicate 5	High	Quality Control	10.00	Eutylone 1	6.08	2.456e7	0.4055	✓	✓	2.409e7			102			3.8

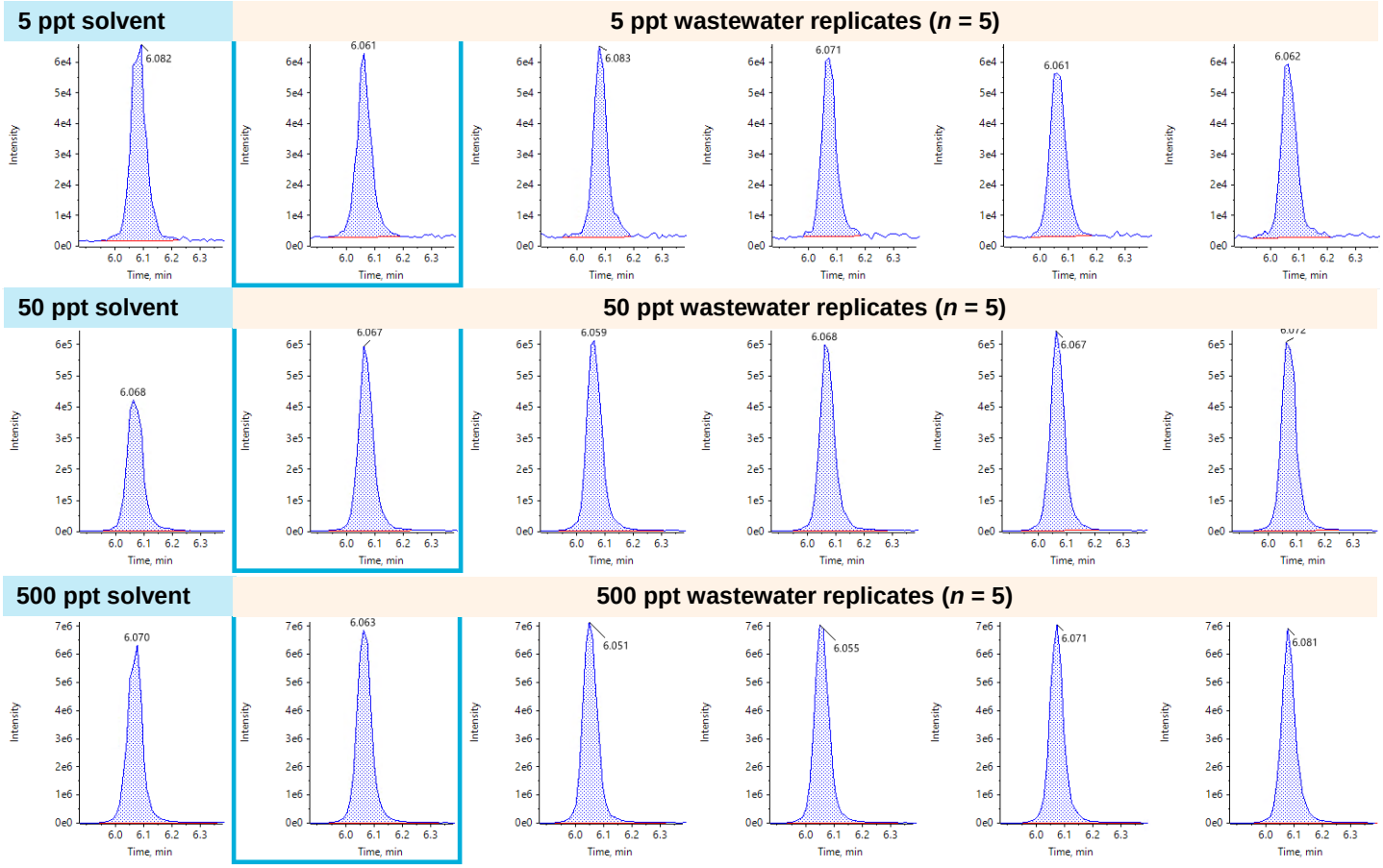

Figure 3. Matrix effects (%) for the quantifier transition of eutylone. Top) Results table showing parameters calculated for solvent spikes (highlighted in blue) and wastewater spikes (highlighted in brown). Bottom) Extracted ion chromatograms (XICs) demonstrating the peak intensity of eutylone in the spiked samples. The matrix effects were determined by calculating the percent quotient of the peak areas between wastewater (brown) and solvent (blue) spikes at low, medium and high concentration levels.

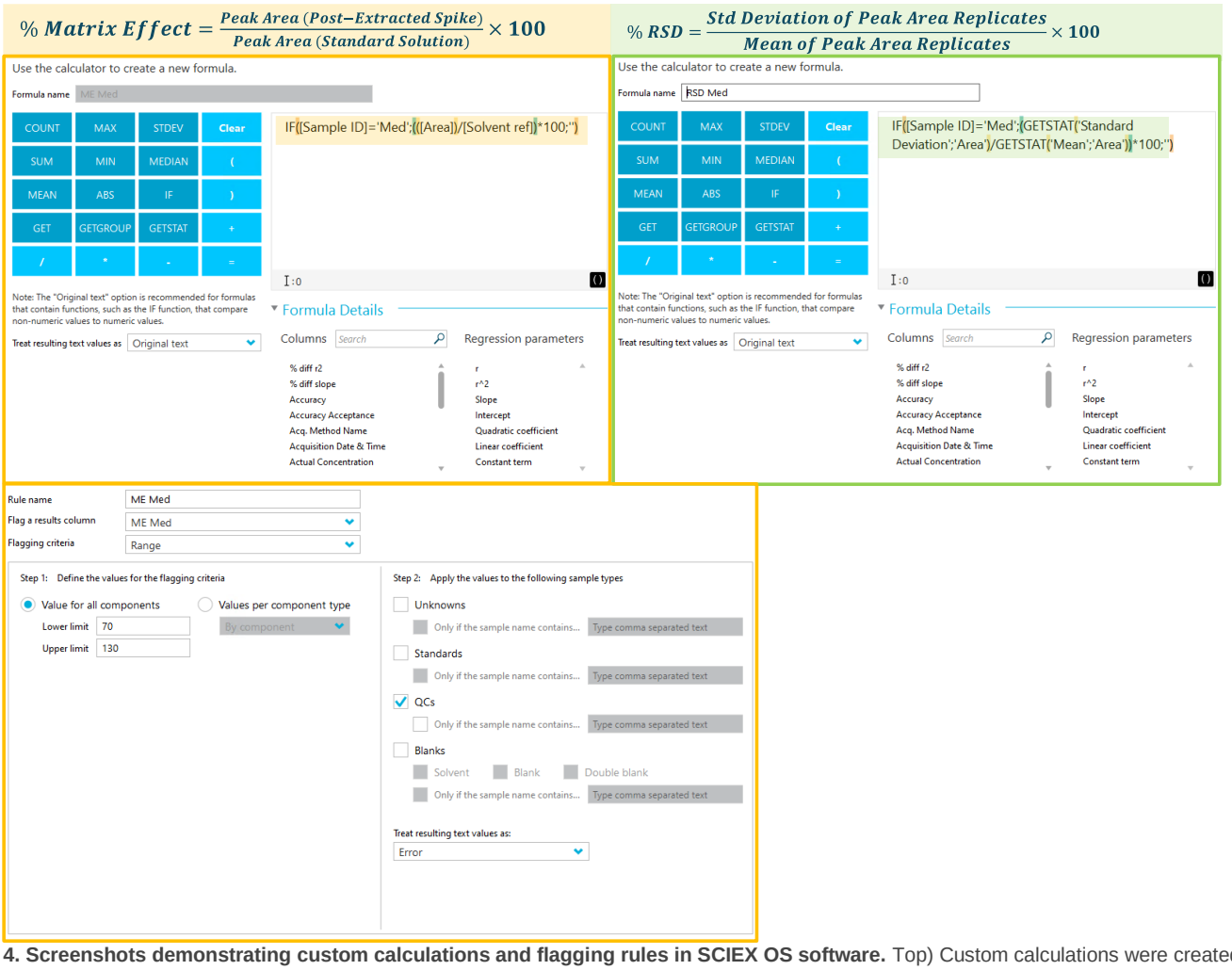

Figure 4. Screenshots demonstrating custom calculations and flagging rules in SCIEX OS software. Top) Custom calculations were created for matrix effects (orange) and replicate precision (green). Bottom) Flagging rules were created in the Analytics processing method of SCIEX OS software.

increased, with most of the target analytes achieving acceptable levels between 70% and 130% at the highest concentration (Table 3). Deuterated surrogate internal standards encompassing a variety of illicit drugs were used to correct for matrix effects.

Rather than transferring the data and performing the calculations elsewhere, the calculated columns feature in SCIEX OS software allows the user to calculate the matrix effects directly in the results table. In addition, the user can selectively apply the calculations to specific samples. For example, as shown in Figure 4, an *IF* condition can be used to apply the formulas for matrix effects (orange) and %RSD (green) only to the medium level wastewater spikes (Sample ID = Med) so that these calculations would not be propagated to other irrelevant samples.

Out-of-bound validation results were identified using flagging rules to highlight values that did not meet user-specified tolerance thresholds. For example, the calculated matrix effects for some of the 50 ppt wastewater spikes were flagged (red) in Figure 3 because they exceeded the upper limit of 130%, as defined in Figure 4.

Two calibration curves were prepared using 10:90 (v/v), methanol/MilliQ water and filtered wastewater (acidified to pH 2). Comparison of their regression parameters, including linearity (r^2) and slope, showed minimal differences (<20%) for most of the analytes (see Figure 5), which indicated that solvent-based calibration was appropriate for analysis. Table 3 summarizes the method performance data for all 32 target analytes, including the LOQs, range, r^2, matrix effects and precision at 3 spiking levels.

The combined approach of direct injection and solvent-based calibration in this method reduced the time, labor and consumables required, while achieving LOQs comparable to those previously published in SPE and LLE methods.[4,5] In addition, the method performance achieved here for different NPS classes suggests that the method can be extended in the future to include emerging drug compounds with similar physicochemical properties.

Table 3. MRM transitions and internal standards assigned to and method validation parameters used for each target analyte. All calculations of precision (%RSD) were based on *n* = 5 replicates at each spiking level.

Compound	MRM transition (Q1 > $Q3_{quant}$, $Q3_{conf}$)	Internal standard	LOD (ng/L)	LOQ (ng/L)	Range (ng/L) (r^2)	Matrix Effects (%, %RSD)			Precision (%RSD)		
						Low	Med	High	Low	Med	High
25C-NBOMe	*337.1 > 121.0, 91.0*	*EDDP-d3*	*8.7*	*28.9*	*10–500 (0.9980)*		*155 (4)*	*116 (2)*		*8.9*	*6.9*
2F-Deschloroketamine	*222.1 > 109.0, 163.1*	*Norketamine-d4*	*7.3*	*24.5*	*10–1000 (0.9979)*		*139 (3)*	*96 (2)*		*4.3*	*1.9*
2-Methyl AP-237	*287.2 > 117.1, 91.1*	*Fentanyl-d5*	*1.8*	*6.0*	*1.5–300 (0.9960)*	*84 (16)*	*104 (14)*	*90 (17)*		*4.7*	*1.9*
2-Oxo-PCE	*218.2 > 173.1, 145.1*	*Ketamine-d4*	*4.0*	*13.3*	*5–1000 (0.9985)*	*62 (3)*	*114 (1)*	*96 (1)*	*2.7*	*5.0*	*4.0*
3-MMC	*178.1 > 144.8, 160.0*	*Mephedrone-d3*	*3.7*	*12.2*	*5–500 (0.9989)*	*95 (3)*	*178 (2)*	*131 (1)*	*2.7*	*5.8*	*4.1*
4-Fluoroamphetamine	*154.1 > 109.0, 137.0*	*Oxycodone-d3*	*27.3*	*90.9*	*10–500 (0.9924)*	*N/A*	*135 (4)*	*122 (5)*		*5.6*	*5.9*
5F-EMB-PICA	*377.2 > 232.1, 144.0*	*Diazepam-d5*	*0.2*	*0.6*	*0.3–300 (0.9993)*	*59 (3)*	*117 (4)*	*97 (2)*	*2.4*	*4.1*	*2.8*
5F-MDMB-PICA	*377.2 > 232.1, 144.0*	*Diazepam-d5*	*0.2*	*0.7*	*0.5–1000 (0.9995)*	*63 (4)*	*124 (4)*	*105 (3)*	*2.8*	*5.2*	*2.1*
5F-MDMB-PINACA	*378.0 > 233.0, 318.0*	*Diazepam-d5*	*0.5*	*1.7*	*1–1000 (0.9990)*	*61 (3)*	*118 (2)*	*92 (3)*	*4.8*	*3.0*	*1.9*
7-Hydroxymitragynine	*415.3 > 190.0, 238.1*	*Cocaine-d3*	*4.5*	*15.2*	*5–1000 (0.9993)*	*90 (2)*	*152 (3)*	*125 (4)*	*2.2*	*3.7*	*5.4*
AMB FUBINCA	*384.2 > 109.0, 253.0*	*Diazepam-d5*	*5.5*	*18.2*	*5–1000 (0.9992)*	*60 (14)*	*134 2)*	*102 (1)*		*4.5*	*5.0*
AP-238	*287.2 > 117.1, 169.1*	*Oxycodone-d3*	*5.2*	*17.2*	*5–212 (0.9968)*	*107 (21)*	*83 (7)*	*69 (4)*	*9.7*	*4.2*	*2.5*
Brorphine	*400.1 > 218.1, 104.0*	*Fentanyl-d5*	*4.1*	*13.7*	*1–200 (0.9985)*	*N/A*	*N/A*	*118 (3)*			*9.5*
Butylone	*222.1 > 174.0, 131.0*	*Benzoylecgonine-d3*	*0.2*	*0.8*	*0.5–1000 (0.9987)*	*74 (3)*	*145 (4)*	*115 (4)*	*3.8*	*2.9*	*5.2*
Clonazolam	*354.0 > 308.0, 326.0*	*Diazepam-d5*	*2.7*	*8.9*	*5–500 (0.9978)*	*94 (3)*	*75 (3)*		*3.8*	*3.3*	*2.0*
Cumyl pegaclone	*373.2 > 255.1, 185.1*	*Diazepam-d5*	*2.4*	*8.1*	*1–1000 (0.9974)*	*45 (2)*	*109 (6)*	*100 (5)*	*3.7*	*6.0*	*5.9*
Cumyl-5F-pegaclone	*391.2 > 273.1, 119.1*	*Diazepam-d5*	*2.1*	*7.1*	*5–1000 (0.9984)*	*45 (5)*	*96 (5)*	*66 (5)*	*3.5*	*3.1*	*4.2*
Dibutylone	*236.1 > 161.0, 86.0*	*Benzoylecgonine-d3*	*1.5*	*5.2*	*5–1000 (0.9981)*	*101 (3)*	*144 (2)*	*104 (4)*	*6.5*	*5.2*	*4.2*
Etizolam	*343.0 > 314.0, 289.1*	*Diazepam-d5*	*0.9*	*2.9*	*1–1000 (0.9957)*	*64 (4)*	*96 (4)*	*81 (1)*	*6.8*	*1.9*	*5.4*
Eutylone	*236.0 > 188.1, 174.0*	*Benzoylecgonine-d3*	*0.2*	*0.6*	*0.5–1000 (0.9996)*	*87 (2)*	*132 (4)*	*107 (4)*	*2.9*	*2.5*	*3.8*
Flualprazolam	*327.2 > 292.2, 223.0*	*Temazepam-d5*	*0.1*	*0.5*	*0.5–1000 (0.9976)*	*50 (3)*	*96 (2)*	*80 (3)*	*1.9*	*6.0*	*4.9*
Flubromazolam	*371.0 > 223.0, 292.0*	*Temazepam-d5*	*15.8*	*52.6*	*10–1000 (0.9942)*		*101 (3)*	*76 (2)*		*5.5*	*3.6*
Isotonitazene	*411.2 > 100.0, 106.9*	*EDDP-d3*	*22.1*	*73.5*	*1–1000 (0.9983)*	*101 (4)*	*215 (4)*	*155 (3)*	*9.4*	*3.5*	*3.6*
MDMB-4en-PINACA	*358.2 > 298.2, 213.1*	*Diazepam-d5*	*0.2*	*0.5*	*0.5–1000 (0.9988)*	*53 (3)*	*107 (3)*	*89 (3)*	*4.7*	*3.3*	*3.7*
Methcathinone	*164.3 > 130.2, 146.2*	*Amphetamine-d6*	*3.2*	*10.6*	*10–500 (0.9965)*	*119 (6)*	*215 (2)*	*161 (1)*		*2.9*	*4.1*
Metonitazene	*383.2 > 100.0, 121.0*	*Fentanyl-d5*	*7.5*	*24.9*	*1–1000 (0.9984)*		*220 (1)*	*144 (2)*	*2.6*	*4.6*	*3.6*
Mitragynine	*399.2 > 174.1, 159.0*	*EDDP-d3*	*44.8*	*149.3*	*50–1000 (0.9804)*		*187 (4)*	*125 (3)*		*5.5*	*3.9*
N-ethylheptedrone	*234.1 > 146.1, 91.1*	*Cocaine-d3*	*5.4*	*18.0*	*5–1000 (0.9993)*		*130 (4)*	*101 (1)*		*2.0*	*3.9*
N-ethylhexedrone	*220.2 > 130.0, 91.1*	*MDMA-d5*	*58.6*	*195.3*	*50–1000 (0.9983)*			*103 (5)*			*4.7*
N-ethylpentylone	*250.2 > 202.0, 175.3*	*MDMA-d5*	*4.5*	*15.2*	*5–1000 (0.9969)*	*82 (4)*	*134 (3)*	*103 (5)*	*5.6*	*3.3*	*3.3*
Pentylone	*236.0 > 188.0, 205.0*	*MDMA-d5*	*0.2*	*0.8*	*0.5–1000 (0.9996)*	*65 (5)*	*127 (5)*	*112 (3)*	*5.6*	*4.6*	*2.4*
Protonitazene	*411.2 > 100.0, 72.1*	*EDDP-d3*	*0.5*	*1.7*	*1–1000 (0.9969)*	*104 (4)*	*229 (3)*	*159 (2)*	*3.7*	*1.3*	*3.0*

**Limit of detection (LOD); Limit of quantitation (LOQ)*
Low = 5 ng/L, Med = 50 ng/L, High = 500 ng/L

Analysis of influent wastewater collected during the holiday period of 2021–2022

Direct injection analysis of influent wastewater samples collected over the summer of 2021–2022 detected eutylone, clonazolam and etizolam at several sites. Solvent and extraction blanks, duplicates and wastewater samples spiked before and after sample preparation were included to assess quality control. Acceptable matrix recoveries ranging from 95% to 119% were calculated for the 3 detected analytes based on their peak areas in wastewater spiked before and after sample preparation (Figure 6).

As shown in Figure 7, both the quantifier and confirmation transitions of eutylone were observed in 2 wastewater samples, while only the quantifier transitions were observed for etizolam and clonazolam. No significant contamination was observed in the solvent and extraction blanks. Here, the sensitivity of the SCIEX 7500 system enabled the detection of eutylone, etizolam and clonazolam in wastewater for the first time by a direct injection LC-MS/MS approach.

Index	U...	Sample Name	Sample...	Sample Type	Compo... Name	Ret... Time	Area	Actual Conc...	Ion Ratio	Ion Ratio...	RT Co...	*Wastewater r2	*Solvent r2-	*% diff...	*Waste... slope	*Solvent slope-	*% diff...
627	☑	WW 1 ppt	Wastewater	Standard	Eutylone 1	6.06	7.100e4	1.00	0.4824	✓	✓	0.9930	0.9996	0.7	44762.000	47221.600	5.2
695	☑	WW 5 ppt	Wastewater	Standard	Eutylone 1	6.07	2.046e5	5.00	0.4072	✓	✓	0.9930	0.9996	0.7	44762.000	47221.600	5.2
763	☑	WW 10 ppt	Wastewater	Standard	Eutylone 1	6.07	4.429e5	10.00	0.4041	✓	✓	0.9930	0.9996	0.7	44762.000	47221.600	5.2
831	☑	WW 50 ppt	Wastewater	Standard	Eutylone 1	6.08	2.096e6	50.00	0.4118	✓	✓	0.9930	0.9996	0.7	44762.000	47221.600	5.2
899	☑	WW 100 ppt	Wastewater	Standard	Eutylone 1	6.07	4.498e6	100.00	0.4029	✓	✓	0.9930	0.9996	0.7	44762.000	47221.600	5.2
967	☑	WW 500 ppt	Wastewater	Standard	Eutylone 1	6.06	2.575e7	500.00	0.3968	✓	✓	0.9930	0.9996	0.7	44762.000	47221.600	5.2
1035	☑	WW 1000 ppt	Wastewater	Standard	Eutylone 1	6.07	4.357e7	1000.00	0.4112	✓	✓	0.9930	0.9996	0.7	44762.000	47221.600	5.2
83	☐	1ppt	Solvent	Standard	Eutylone 1	6.07	4.675e4	1.00	0.3972	✓	✓			N/A			N/A
151	☐	5 ppt	Solvent	Standard	Eutylone 1	6.08	2.543e5	5.00	0.4173	✓	✓			N/A			N/A
219	☐	10 ppt	Solvent	Standard	Eutylone 1	6.07	4.858e5	10.00	0.4156	✓	✓			N/A			N/A
287	☐	50 ppt	Solvent	Standard	Eutylone 1	6.07	1.735e6	50.00	0.4147	✓	✓			N/A			N/A
355	☐	100 ppt	Solvent	Standard	Eutylone 1	6.07	4.367e6	100.00	0.4160	✓	✓			N/A			N/A
423	☐	500 ppt	Solvent	Standard	Eutylone 1	6.07	2.409e7	500.00	0.3868	✓	✓			N/A			N/A
491	☐	1000 ppt	Solvent	Standard	Eutylone 1	6.07	4.685e7	1000.00	0.3907	✓	✓			N/A			N/A

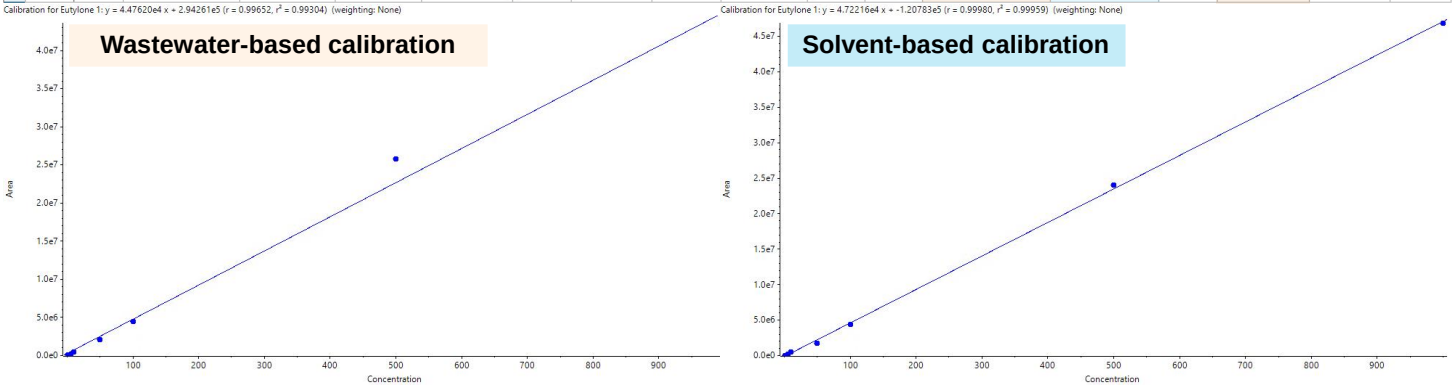

Figure 5. Comparison of regression parameters derived from wastewater- and solvent-based calibration curves. Top) Results table showing parameters calculated for wastewater- (highlighted in brown) and solvent-based (highlighted in blue) calibration samples. Custom formulas were used to calculate the r^2 and slope values from both calibration curves using linear fitting and no weighting. Comparisons of these values were shown in newly generated % difference columns in the results table. Bottom) Calibration curves generated across the concentration ranges tested.

Figure 6. Screenshot demonstrating how matrix recoveries of the direct injection approach were calculated at 3 different spiking concentrations (5 ng/L, 50 ng/L, 500 ng/L). Matrix recoveries in wastewater were calculated as the quotient of the peak areas in wastewater spiked before and after sample preparation and displayed in a newly generated results table column entitled 'WW Matrix Rec'.

Sample Name	Component Name	Area	*WW Prespk	*WW Matrix Rec
WW post spk 5 ppt	Eutylone 1	2.481e5	2.940e5	118.50
WW post spk 50 ppt	Eutylone 1	2.181e6	2.066e6	94.72
WW post spk 500 ppt	Eutylone 1	1.863e7	2.090e7	112.16
WW post spk 5 ppt	Etizolam 1	4.158e4	4.779e4	114.94
WW post spk 50 ppt	Etizolam 1	3.298e5	3.411e5	103.44
WW post spk 500 ppt	Etizolam 1	3.253e6	3.425e6	105.30
WW post spk 5 ppt	Clonazolam 1	5.237e4	5.132e4	98.00
WW post spk 50 ppt	Clonazolam 1	2.904e5	2.854e5	98.27
WW post spk 500 ppt	Clonazolam 1	2.867e6	2.832e6	98.76

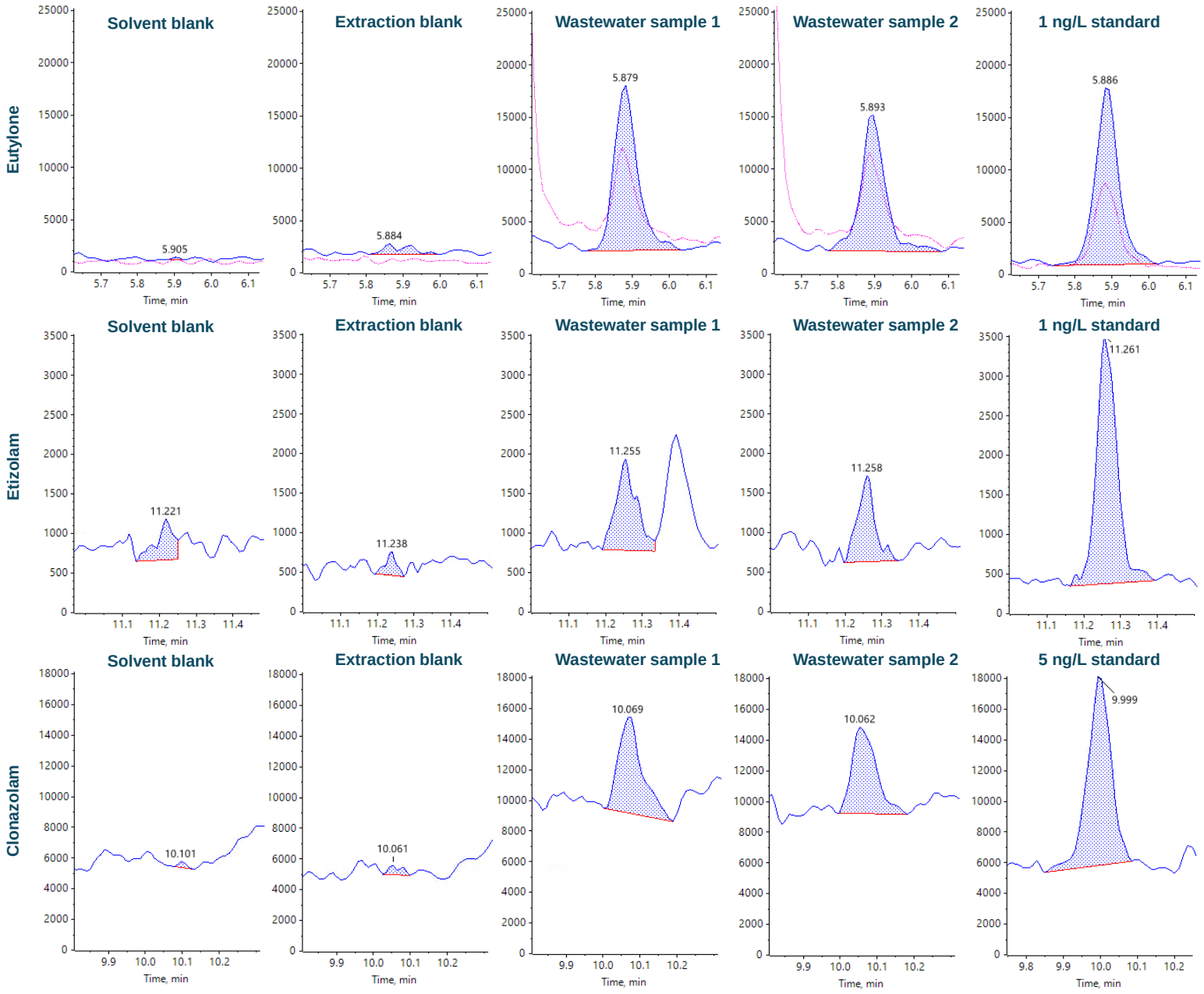

Figure 7. Detection of eutylone, etizolam and clonazolam in 2 wastewater samples compared to quality control samples. Top) Results for eutylone are shown. The blue trace represents the quantifier transition (*m/z* 236.0 > 188.1) and the pink trace represents the confirmation transition (*m/z* 236.0 > 174.0). Middle, Bottom) For both etizolam (middle) and clonazolam (bottom), only the quantifier transitions (*m/z* 343.0 > 314.0, etizolam and *m/z* 354.0 > 308.0, clonazolam) demonstrated peaks with matching RTs compared to the calibration standards. Neither of the confirmation transitions (*m/z* 343.0 > 289.1, etizolam and *m/z* 354.0 > 326.0, clonazolam) demonstrated any observable peaks. The solvent blank was MilliQ water and the extraction blank was acidified MilliQ water prepared in the same manner as the wastewater samples. The calibration standard was prepared at 1 ng/L for eutylone, 1 ng/L for etizolam and 5 ng/L for clonazolam.

Conclusions

- A rapid and sensitive direct injection method was developed for the quantitation of 32 NPS in wastewater with LOQs ranging from 0.5 to 195.3 ng/L
- Application of the method to Australian wastewater samples collected during the holiday period of 2021–2022 revealed the detection of eutylone, etizolam and clonazolam for the first time by a direct injection LC-MS/MS approach
- The high sensitivity of the SCIEX 7500 system enabled the use of a small injection volume (10 µL), which reduced matrix interferences without the need for SPE, LLE or any other pre-concentration steps
- Linearity performance was comparable between wastewater-based calibration and solvent-based calibration, which enabled the simpler solvent-based approach to be used for quantitation
- Acceptable method validation performance was achieved for a diverse range of NPS classes, allowing for easy expansion to include new and emerging substances in future analysis
- The speed and simplicity of the direct injection approach allowed for high-throughput and rapid turnaround time of wastewater surveillance of recreational drug use
- Visualization software tools, such as sMRM summary plots, provide real-time updates of large and complex acquisition methods while editing parameters that propagate across many MRM transitions
- Custom calculations enable the user to perform direct calculations in SCIEX OS software without the need to export the data elsewhere. Flagging rules expedite the review of outlier data in SCIEX OS software.

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Nontargeted acquisition with targeted and suspect screening of pharmaceutical drugs and their metabolites in wastewater

Simultaneous quantitation and screening by the SCIEX X500R QTOF system and SCIEX OS software

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Introduction

This technical note describes the identification of pharmaceutical drugs and their metabolites in wastewater using nontargeted acquisition coupled with suspect screening. A solid-phase extraction (SPE) LC-MS/MS method was developed for the semi-quantitative screening of 105 pharmaceutical drugs. The X500R QTOF system was used to collect MS/MS data by SWATH data-independent acquisition (DIA). These data were used to identify targeted drug compounds and retrospectively detect previously untargeted metabolites from a combined approach of spectral library matching and diagnostic fragment confirmation. Molecule Profiler software provided a complementary workflow for metabolite identification by matching common fragments against those from *in silico* fragmentation.

Wastewater monitoring has been increasingly adopted to assess community drug exposure due to its low costs, non-invasive sample collection and comprehensive analytical coverage.[1] In contrast, drug epidemiological data derived from self-reported surveys and toxicological reports can be expensive and be biased from the lack of or skewed responses from the sampled populations. SWATH DIA produces high-resolution MS/MS spectra that are composites of all analytes present in the sample and can be retrospectively mined.

Here, an end-to-end workflow using the X500R QTOF system and integrated modules within the SCIEX OS software provided high-resolution MS and MS/MS data for targeted and non-targeted screening of drugs and their metabolites in wastewater environments. Figure 1 shows the identification of carbamazepine and its metabolites based on complementary approaches of MS/MS library matching and *in silico* fragment confirmation in the Molecule Profiler software module of SCIEX OS software.

Key features of SWATH DIA on the X500R QTOF system coupled with targeted and nontargeted screening with SCIEX OS software

- SWATH DIA acquisition on the X500R QTOF system provided comprehensive MS/MS coverage for both targeted and nontargeted screening of all compounds
- Integration of the Analytics module and Molecule Profiler software within SCIEX OS software enabled a seamless transition between spectral library matching and *in silico* fragmentation predictions for compound identification in a single software platform
- A SPE LC-MS/MS workflow enabled the simultaneous semi-quantitation and identification of 105 pharmaceutical drugs in small volumes of wastewater samples

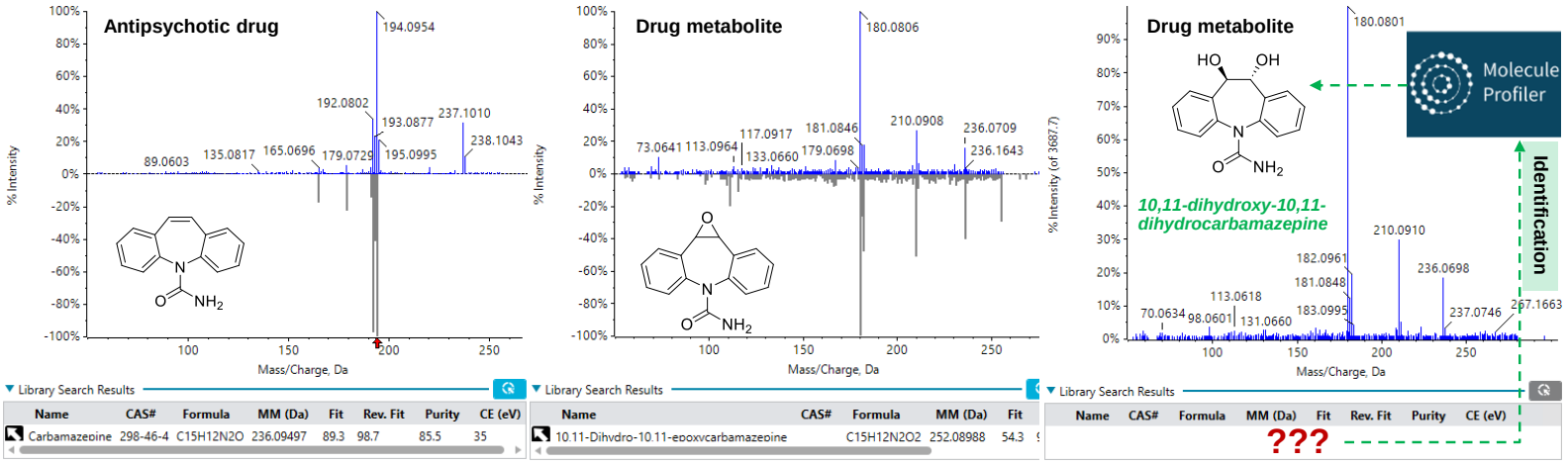


Figure 1. Identification of different targeted pharmaceutical drugs and drug metabolites that were not initially targeted for acquisition. Retrospective analysis of SWATH DIA MS/MS data revealed the detection of several metabolites of carbamazepine via a combined approach of spectral library matching and *in silico* structural elucidation in the integrated Molecule Profiler software and Analytics module of SCIEX OS software.

Experimental methods

Chemicals and samples: The target analyte list included 105 pharmaceutical drugs and 3 surrogate internal standards. Individual neat standards were mixed to prepare stock solutions in methanol from which calibration standards (5–1000 ng/L) were prepared in MilliQ water for semi-quantitation. Influent wastewater samples were collected as 24-hour composites from 4 sites in the northwestern region of Italy. Upon collection, a 1 L aliquot of composite wastewater was transferred to refrigerated glass bottles and stored at -20°C until analysis.

Sample preparation: A 100 mL sample of wastewater was centrifuged at 4000 rpm for 5 minutes and vacuum-filtered through a 0.22 µm filter. A 30 mL aliquot of filtered wastewater was spiked with the surrogate internal standards and extracted using an Oasis HLB SPE cartridge (200 mg, 6 cm^3, Waters, Milford, MA). Each cartridge was preconditioned with 5 mL of methanol and 5 mL of MilliQ water before loading the sample, then was vacuum dried and eluted with 10 mL of methanol. Upon evaporation to dryness, the residue was reconstituted with 50 µL of methanol for LC-MS/MS analysis. Spiked MilliQ water was prepared in the same manner for semi-quantitative assessment of limits of detection (LOD) and extraction recoveries.

Chromatography: LC separation was performed on a SCIEX ExionLC AC system using a Phenomenex Kinetex C18 column (100 x 2.1 mm, 1.7 µm, P/N: 00D-4475-AN). A flow rate of 0.5 mL/min, an injection volume of 5 µL and a column temperature of 45°C were used. The LC conditions used are shown in Table 1.

Mass spectrometry: Analysis was performed using the X500R QTOF system in both positive and negative electrospray ionization mode. Table 2 shows the method parameters used for the mass spectrometer. The SWATH DIA method consisted of 16 variable windows covering a mass range of *m/z* 130–520.

Table 1. Chromatographic gradient.

Time (min)	%A	%B
0.0	*95*	*5*
0.5	*95*	*5*
8.0	*5*	*95*
8.5	*5*	*95*
8.51	*95*	*5*
11	*95*	*5*

Mobile phase A: MilliQ water with 5mM formic acid
Mobile phase B: Acetonitrile with 5mM formic acid

Table 2. Source, gas and temperature conditions.

Parameter	MS	MS/MS
Polarity	*Positive and negative*	
Ion spray voltage	*2500 V*	
Ion source gas 1 (GS1)	*50 psi*	
Ion source gas 2 (GS2)	*45 psi*	
Curtain gas (CUR)	*35 psi*	
Collision gas (CAD)	*8 psi*	
Source temperature (TEM)	*600°C*	
Declustering potential (DP)	*65 V*	
Total scan time	*0.836 s*	
Scan mode	*TOF MS*	*SWATH DIA*
Start/stop mass range	*130 – 520 Da*	*50 – 800 Da*
Accumulation time	*0.25 s*	*0.03 s*
Collision energy (CE)	*10 V*	*35 V*
Collision energy spread (CES)	*0 V*	*15 V*

Data processing: Data were acquired and processed using SCIEX OS software, versions 2.2 and 3.1. A custom library of previously acquired MS/MS spectra was used for library searching. The Molecule Profiler software was used to screen for drug metabolites. Figure 2 shows the overall workflow.

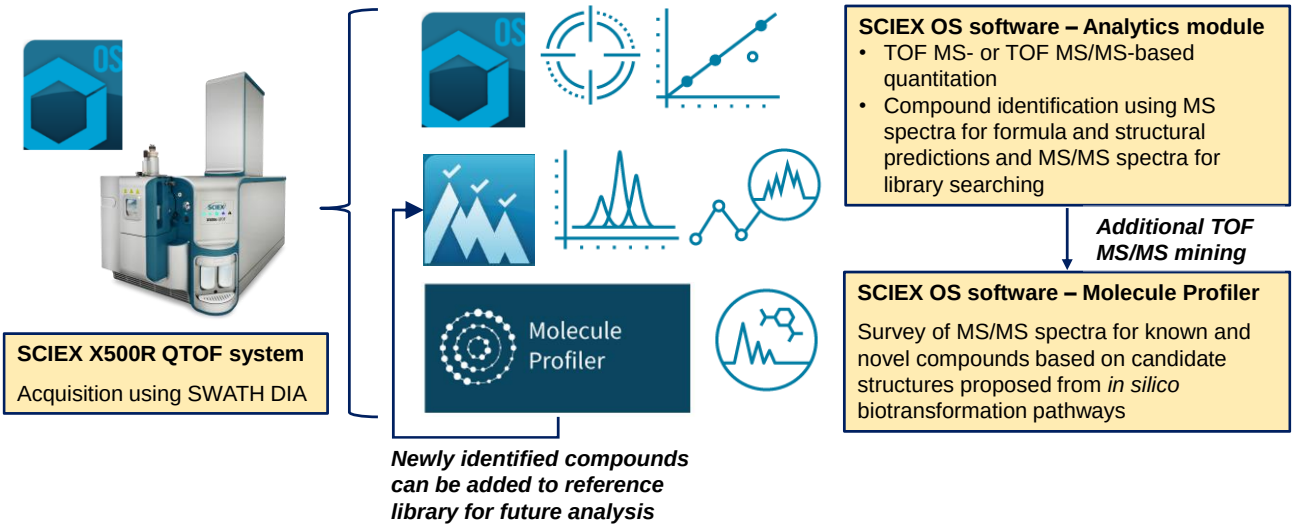


Figure 2. Streamlined acquisition and data analysis workflow using the X500R QTOF system and SCIEX OS software. The Analytics module was used for quantitation and spectral library matching, while Molecule Profiler software was used for metabolite identification.

Targeted analysis of drugs in wastewater

In contrast to the 100–250 mL samples typically used in SPE methods for wastewater analysis, only 30 mL was extracted here, which reduced solvent consumption and the matrix load. The SPE LC-MS/MS method achieved recoveries of ≥70% for 60% of the 105 targeted drugs and 50–70% for most of the remaining analytes based on comparisons of pre- and post-extracted aqueous spikes. Based on aqueous spikes exhibiting signal-to-noise (S/N) ratios ≥3 above the background, the instrumental LODs were estimated to be ≤5 ng/L for 72% of the analytes and 5–15 ng/L for the remainder, consistent with the typical concentration ranges of drugs observed in wastewater. As such, the developed SPE LC-MS/MS method provided acceptable performance based on fit-for-purpose criteria for the semi-quantitation of a large panel of targeted analytes in wastewater influents (Table 3). Table 3 shows the average concentration range for a subset of the 105 targeted drugs detected in wastewater whereby compound identification in each sample was confirmed by retention time (RT) matches against authentic standards, mass error of <5 ppm for the exact precursor and fragment *m/z* peaks, and spectral MS/MS matching against a custom library of previously acquired MS/MS spectra using

Table 3. Compound information for a subset of the 105 targeted pharmaceutical drugs detected in wastewater influents. Chemical formula, adduct, assigned internal standard, precursor and fragment ion *m/z*, retention times (RT), limits of detection (LOD), extraction recovery (RE) and the range of average concentrations reported from the 4 wastewater treatment plants (WWTPs) are included.

Compound	Formula	Adduct	Internal standard	Precursor *m/z*	Fragment *m/z*	RT (min)	LOD (ng/L)	RE (%)	Avg conc range (ng/L) from 4 sites
Antidepressants									
Citalopram	$C_{20}H_{21}FN_2O$	$[M+H]^+$	*Cocaine-d3*	*325.1711*	*109.0453*	*3.84*	*5*	*101*	*nd – 220*
Mirtazapine	$C_{17}H_{19}N_3$	$[M+H]^+$	*Nitrazepam-d5*	*266.1652*	*195.0915*	*2.90*	*5*	*63*	*nd – 23*
Trazodone	$C_{19}H_{22}ClN_5O$	$[M+H]^+$	*Nitrazepam-d5*	*372.1586*	*176.0804*	*3.30*	*5*	*74*	*5 – 19*
Benzodiazepine									
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	$[M+H]^+$	*Nitrazepam-d5*	*321.0192*	*275.0144*	*4.20*	*5*	*91*	*24 – 160*
Lormetazepam	$C_{16}H_{12}Cl_2N_2O_2$	$[M+H]^+$	*Nitrazepam-d5*	*335.0349*	*289.0286*	*4.62*	*5*	*82*	*9 – 160*
Oxazepam	$C_{15}H_{11}ClN_2O_2$	$[M+H]^+$	*Nitrazepam-d5*	*287.0582*	*241.0528*	*4.09*	*5*	*96*	*nd – 36*
Temazepam	$C_{16}H_{13}ClN_2O_2$	$[M+H]^+$	*Nitrazepam-d5*	*301.0738*	*255.0679*	*4.47*	*5*	*90*	*nd – 8*
Antipsychotic									
Amisulpride	$C_{17}H_{27}N_3O_4S$	$[M+H]^+$	*Cocaine-d3*	*370.1795*	*242.0477*	*2.55*	*5*	*67*	*nd – 120*
Carbamazepine	$C_{15}H_{12}N_2O$	$[M+H]^+$	*Nitrazepam-d5*	*237.1022*	*194.0949*	*3.90*	*5*	*82*	*100 – 600*
Quetiapine	$C_{21}H_{25}N_3O_2S$	$[M+H]^+$	*Nitrazepam-d5*	*384.1740*	*253.0795*	*3.63*	*5*	*70*	*nd – 39*
Tiapride	$C_{15}H_{24}N_2O_4S$	$[M+H]^+$	*Cocaine-d3*	*329.1530*	*256.0615*	*1.98*	*5*	*97*	*nd – 5*
Venlafaxine	$C_{17}H_{27}NO_2$	$[M+H]^+$	*Cocaine-d3*	*278.2115*	*58.0656*	*3.25*	*5*	*67*	*nd – >1000*
Antiepileptic									
Lamotrigine	$C_9H_7Cl_2N_5$	$[M+H]^+$	*Nitrazepam-d5*	*256.0151*	*210.9820*	*2.73*	*15*	*69*	*nd – 860*
Oxcarbazepine	$C_{15}H_{12}N_2O_2$	$[M+H]^+$	*Nitrazepam-d5*	*253.0972*	*180.0810*	*3.58*	*5*	*91*	*nd – 380*
Cardiovascular drugs									
Atenolol	$C_{14}H_{22}N_2O_3$	$[M+H]^+$	*Cocaine-d3*	*267.1703*	*145.0638*	*1.60*	*5*	*46*	*nd – 500*
Bisoprolol	$C_{18}H_{31}NO_4$	$[M+H]^+$	*Nitrazepam-d5*	*326.2326*	*116.1068*	*3.38*	*5*	*57*	*25 – 77*
Nebivolol	$C_{22}H_{25}F_2NO_4$	$[M+H]^+$	*Nitrazepam-d5*	*406.1824*	*151.0561*	*4.24*	*5*	*56*	*nd – 68*
Propafenone	$C_{21}H_{27}NO_3$	$[M+H]^+$	*Nitrazepam-d5*	*342.2064*	*116.1067*	*4.12*	*5*	*77*	*30 – 220*
Ramipril	$C_{23}H_{32}N_2O_5$	$[M+H]^+$	*Cocaine-d3*	*417.2384*	*234.1497*	*3.97*	*5*	*67*	*nd – 26*
Telmisartan	$C_{33}H_{30}N_4O_2$	$[M+H]^+$	*Nitrazepam-d5*	*515.2442*	*497.2324*	*4.54*	*5*	*86*	*nd – 350*
Non-steroidal anti-inflammatory drugs									
Ketoprofen	$C_{16}H_{14}O_3$	$[M+H]^+$	*Coumachlor*	*255.1016*	*105.0328*	*4.58*	*5*	*76*	*48 – 900*
Analgesic/opioids									
Paracetamol	$C_8H_9NO_2$	$[M+H]^+$	*Coumachlor*	*152.0706*	*110.0604*	*1.53*	*10*	*80*	*nd – >1000*
Tapentadol	$C_{14}H_{23}NO$	$[M+H]^+$	*Cocaine-d3*	*222.1852*	*107.0488*	*2.90*	*5*	*90*	*44 – 380*
Others									
Dextromethorphan	$C_{18}H_{25}NO$	$[M+H]^+$	*Cocaine-d3*	*272.2009*	*215.1416*	*3.59*	*5*	*83*	*nd – 260*
Gliclazide	$C_{15}H_{21}N_3O_3S$	$[M+H]^+$	*Cocaine-d3*	*324.1376*	*127.1225*	*4.86*	*5*	*83*	*nd – 180*
Lidocaine	$C_{14}H_{22}N_2O$	$[M+H]^+$	*Cocaine-d3*	*235.1805*	*86.0965*	*2.49*	*5*	*77*	*43 – >1000*
Metoclopramide	$C_{14}H_{22}ClN_3O_2$	$[M+H]^+$	*Cocaine-d3*	*300.1473*	*227.0586*	*2.72*	*5*	*75*	*nd – 19*

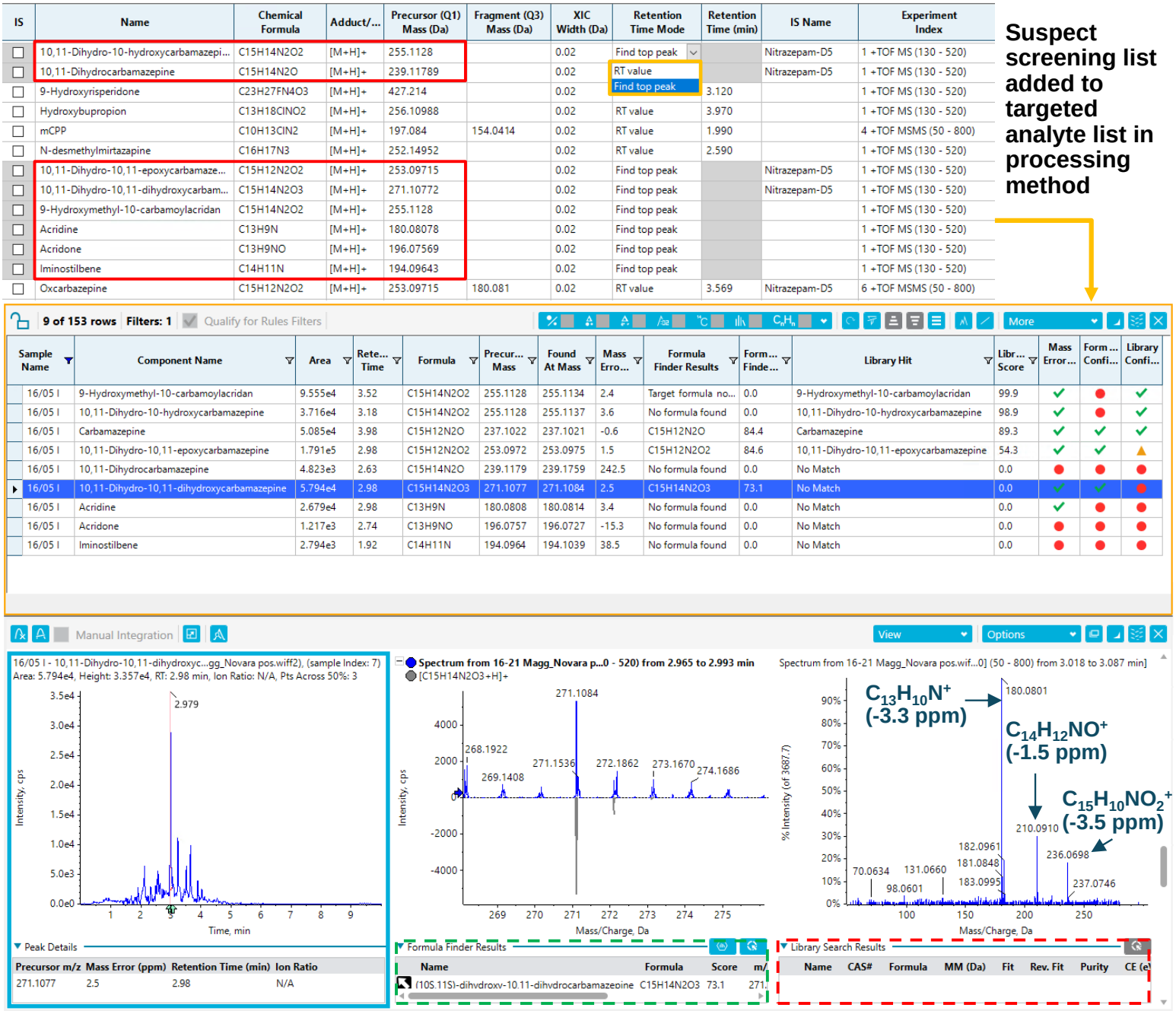
nd = not detected

reference standards. The traffic light system in the SCIEX OS software expedites data review by enabling the user to filter and display only the results passing predefined confidence thresholds for identification, such as mass error and matches in RT, isotope ratio pattern and MS/MS spectra against a library, as shown by some example positive hits in Figure 3.

In addition to confirming positive detection of the parent drugs, monitoring their metabolites has become increasingly prevalent, since specific metabolites have demonstrated toxicity comparable to their parent drugs.

Suspect screening for previously untargeted metabolites using SCIEX OS software

Non-targeted acquisition by SWATH DIA enabled retrospective analysis of TOF MS/MS to screen for previously untargeted compounds, such as the metabolites of positive drug hits in wastewater. Due to its well-documented metabolic pathways,[2-4] carbamazepine (CBZ) was used as the model parent drug to screen for metabolites that were not initially targeted. The molecular formula and exact precursor masses of 8 known CBZ metabolites were determined *a priori* from the literature to



IS	Name	Chemical Formula	Adduct/...	Precursor (Q1) Mass (Da)	Fragment (Q3) Mass (Da)	XIC Width (Da)	Retention Time Mode	Retention Time (min)	IS Name	Experiment Index
☐	10,11-Dihydro-10-hydroxycarbamazepi...	C15H14N2O2	[M+H]+	255.1128		0.02	Find top peak		Nitrazepam-D5	1 +TOF MS (130 - 520)
☐	10,11-Dihydrocarbamazepine	C15H14N2O	[M+H]+	239.11789		0.02	RT value / Find top peak		Nitrazepam-D5	1 +TOF MS (130 - 520)
☐	9-Hydroxyrisperidone	C23H27FN4O3	[M+H]+	427.214		0.02		3.120		1 +TOF MS (130 - 520)
☐	Hydroxybupropion	C13H18ClNO2	[M+H]+	256.10988		0.02	RT value	3.970		1 +TOF MS (130 - 520)
☐	mCPP	C10H13ClN2	[M+H]+	197.084	154.0414	0.02	RT value	1.990		4 +TOF MSMS (50 - 800)
☐	N-desmethylmirtazapine	C16H17N3	[M+H]+	252.14952		0.02	RT value	2.590		1 +TOF MS (130 - 520)
☐	10,11-Dihydro-10,11-epoxycarbamaze...	C15H12N2O2	[M+H]+	253.09715		0.02	Find top peak		Nitrazepam-D5	1 +TOF MS (130 - 520)
☐	10,11-Dihydro-10,11-dihydroxycarbam...	C15H14N2O3	[M+H]+	271.10772		0.02	Find top peak		Nitrazepam-D5	1 +TOF MS (130 - 520)
☐	9-Hydroxymethyl-10-carbamoylacridan	C15H14N2O2	[M+H]+	255.1128		0.02	Find top peak			1 +TOF MS (130 - 520)
☐	Acridine	C13H9N	[M+H]+	180.08078		0.02	Find top peak			1 +TOF MS (130 - 520)
☐	Acridone	C13H9NO	[M+H]+	196.07569		0.02	Find top peak			1 +TOF MS (130 - 520)
☐	Iminostilbene	C14H11N	[M+H]+	194.09643		0.02	Find top peak			1 +TOF MS (130 - 520)
☐	Oxcarbazepine	C15H12N2O2	[M+H]+	253.09715	180.081	0.02	RT value	3.569	Nitrazepam-D5	6 +TOF MSMS (50 - 800)

Sample Name	Component Name	Area	Rete... Time	Formula	Precur... Mass	Found At Mass	Mass Erro...	Formula Finder Results	Form... Finde...	Library Hit	Libr... Score	Mass Error...	Form... Confi...	Library Confi...
16/05 I	9-Hydroxymethyl-10-carbamoylacridan	9.555e4	3.52	C15H14N2O2	255.1128	255.1134	2.4	Target formula no...	0.0	9-Hydroxymethyl-10-carbamoylacridan	99.9	✓	●	✓
16/05 I	10,11-Dihydro-10-hydroxycarbamazepine	3.716e4	3.18	C15H14N2O2	255.1128	255.1137	3.6	No formula found	0.0	10,11-Dihydro-10-hydroxycarbamazepine	98.9	✓	●	✓
16/05 I	Carbamazepine	5.085e4	3.98	C15H12N2O	237.1022	237.1021	-0.6	C15H12N2O	84.4	Carbamazepine	89.3	✓	✓	✓
16/05 I	10,11-Dihydro-10,11-epoxycarbamazepine	1.791e5	2.98	C15H12N2O2	253.0972	253.0975	1.5	C15H12N2O2	84.6	10,11-Dihydro-10,11-epoxycarbamazepine	54.3	✓	✓	▲
16/05 I	10,11-Dihydrocarbamazepine	4.823e3	2.63	C15H14N2O	239.1179	239.1759	242.5	No formula found	0.0	No Match	0.0	●	●	●
16/05 I	10,11-Dihydro-10,11-dihydroxycarbamazepine	5.794e4	2.98	C15H14N2O3	271.1077	271.1084	2.5	C15H14N2O3	73.1	No Match	0.0	✓	✓	●
16/05 I	Acridine	2.679e4	2.98	C13H9N	180.0808	180.0814	3.4	No formula found	0.0	No Match	0.0	✓	●	●
16/05 I	Acridone	1.217e3	2.74	C13H9NO	196.0757	196.0727	-15.3	No formula found	0.0	No Match	0.0	●	●	●
16/05 I	Iminostilbene	2.794e3	1.92	C14H11N	194.0964	194.1039	38.5	No formula found	0.0	No Match	0.0	●	●	●

Figure 3. Suspect screening for the metabolites of carbamazepine (CBZ) in a wastewater sample. The top panel shows the targeted components list in the processing method with the suspect CBZ metabolites added. The bottom panel shows the results table filtered to display CBZ and its suspect metabolites. Formula Finder identified 10,11-dihydro-10,11-dihydroxycarbamazepine (DiOH-CBZ) (highlighted in green), but further confirmation by library searching was not possible due to the absence of this compound in the reference library (highlighted in red). Instead, DiOH-CBZ was identified based on diagnostic fragment comparisons against published MS/MS spectra.

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generate a suspect screening list in the processing method (Figure 3). The RT mode was selected for these suspect compounds with unknown RTs to "Find top peak" to identify the most intense peak eluting at a specific RT within the extracted ion chromatogram (XIC). Three metabolites, 10,11- dihydro-10-hydroxycarbamazepine (10-OH-CBZ), 10,11-dihydro-10,11-dihydroxycarbamazepine (DiOH-CBZ) and carbamazepine 10,11-epoxide (EP-CBZ) were identified based on mass error (<5 ppm), isotope ratio and spectral matching against a custom library (Figure 3). Although DiOH-CBZ was not present in the custom library, its predominant fragments of $[C_{13}H_{10}N]^+$, $[C_{14}H_{12}NO]^+$ and $[C_{15}H_{10}NO_2]^+$ were present with good mass error (<5 ppm), which is consistent with MS/MS spectra reported in published databases.[5] In addition, Formula Finder predicted several candidate formulas based on the MS and MS/MS spectra, one of which matched the structure of DiOH-CBZ found in the ChemSpider database (Figure 4) .

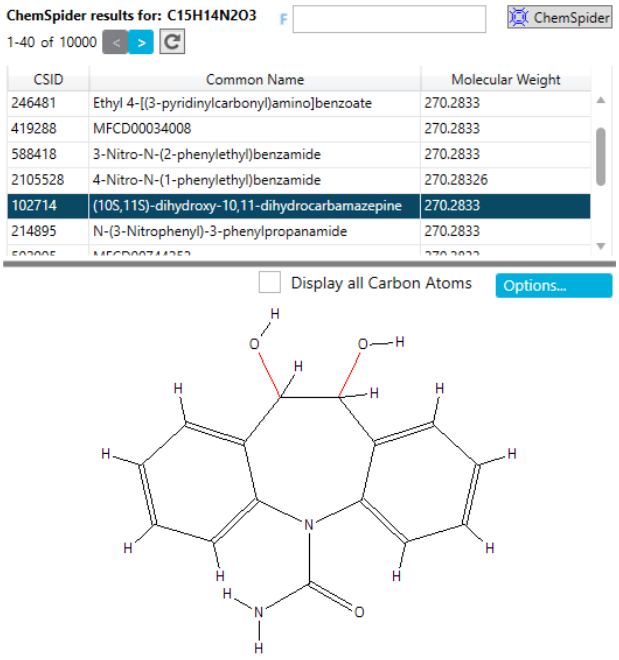

Figure 4. Identification of DiOH-CBZ using Formula Finder and ChemSpider in the Analytics module of SCIEX OS software. Based on the experimental MS and MS/MS spectra, Formula Finder generated a list of candidate formulas and searched them against structures in the ChemSpider database. The experimental MS/MS spectrum matched the *in silico* predicted fragmentation of the candidate structure of DiOH-CBZ.

A limitation of this workflow is that it required *a priori* knowledge of the molecular formula and/or exact precursor mass *m/z* of the compounds to be targeted for suspect screening. This demands an exhaustive search in the literature to produce a comprehensive list of suspect metabolites, which can be time-consuming and labor-intensive. As such, some of the wastewater samples were reinterrogated using the Molecule Profiler module to corroborate these findings here and screen for additional metabolites that may have been missed from suspect screening.

Detection of additional CBZ metabolites using Molecule Profiler software

The Molecule Profiler software in SCIEX OS software provided an orthogonal workflow for detecting metabolites by searching for precursor compounds that also share characteristic fragments from the parent CBZ structure such as *m/z* 194.0941, 192.0795 and 179.0725. These fragments are commonly observed in the MS/MS spectra of CBZ metabolites in the published literature.[1-4] As shown in Figure 5, the software used *in silico* biotransformation pathways to predict a list of expected cleavage metabolites, such as DiOH-CBZ, which could not be previously confirmed by MS/MS library matching due to its absence in the reference spectral library. Table 4 shows a list of metabolites identified based on good mass error (<5 ppm) and comparison between *in silico* fragmentation of the predicted candidate structure and the MS/MS spectra. In addition to the same metabolites found by the Analytics module, Molecule Profiler software tentatively identified additional metabolites such as $C_{14}H_{13}NO_3$ and $C_{15}H_{12}N_2O_2$ that were not previously targeted.

For example, a monohydroxycarbamazepine structure was predicted for the candidate compound $C_{15}H_{12}N_2O_2$, observed at *m/z* 253.0979 at a RT of 3.31 minutes. This peak was separate from its other structural isomers, EP-CBZ and oxcarbazepine (OX-CBZ), which elute at 2.98 and 3.98 minutes, respectively. All 3 isomers lose the carboxamide group ($CONH_3$) to produce the fragment pairs at *m/z* 210.091 and 208.076. EP-CBZ and OX-CBZ have been reported to produce additional major fragments at *m/z* 236.071 and 180.081, which were not observed in the experimental MS/MS spectrum here.[2,4] The lack of a reference MS/MS spectrum for library confirmation precluded further confirmation of the exact positional isomer of the monohydroxycarbamazepine.

Overall, the Molecule Profiler software identified similar metabolites found by suspect screening in the Analytics module of SCIEX OS software and tentatively identified others, all without *a priori* knowledge of the analyte details. Both modules provide complementary approaches such as MS/MS library matching and *in silico*-based fragmentation pattern prediction to aid in the discovery of known and novel metabolites. The integration of Molecule Profiler software with SCIEX OS software enables the user to seamlessly transport their metabolite findings to the Analytics module for further library confirmation and updates of their spectral library with any novel metabolites identified, as shown in Figure 2.

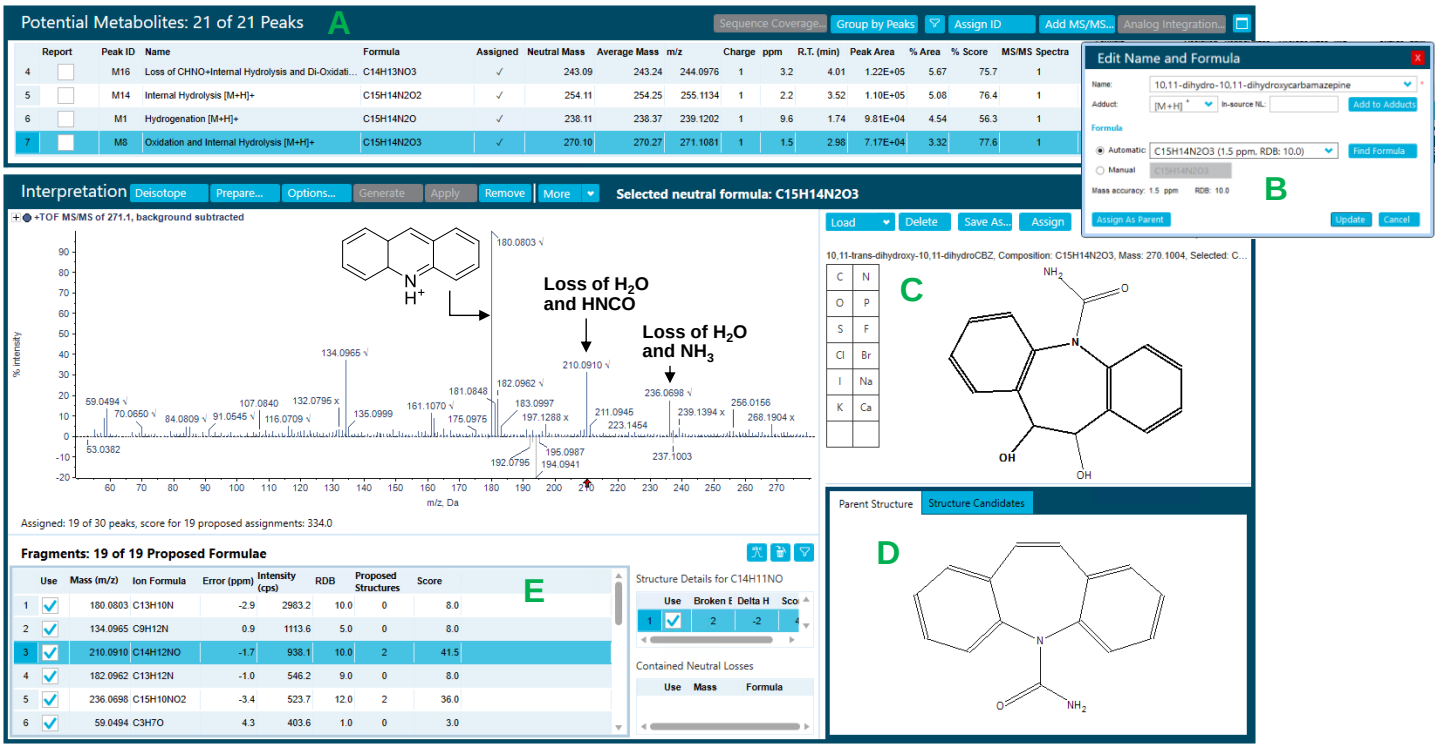

Figure 5. Identification of DiOH-CBZ, a CBZ metabolite, using Molecule Profiler software. The software displays a list of potential metabolites with their formula, *m/z* and scoring (A) with the ability to edit compound details (B). The Interpretation panel enables the user to review and compare candidate structures for the metabolite (C) and parent (D). The software also allows users to edit and assign new structures based on annotated fragment peaks in the TOF MS/MS spectrum (E).

Conclusions

- SWATH DIA of MS/MS spectra enables retrospective mining of previously acquired data for drug metabolites that were not targeted during the initial pharmaceutical drug screen
- Accuracies of ≥70% and LODs of ≤5 ng/L for the majority of the targeted drugs were achieved based on solvent spikes and were deemed adequate for the semi-quantitative screening of 105 pharmaceutical drugs in wastewater using SPE LC-MS/MS
- Molecule Profiler software provided an automated workflow for metabolite identification without *a priori* knowledge of the analyte details for processing
- Integration of Molecule Profiler software with SCIEX OS software enabled a streamlined workflow for transferring metabolite findings to be orthogonally confirmed by interrogation of the MS/MS spectra using diagnostic fragment ions and library searching in the Analytics module of SCIEX OS software

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1. Massano, M.; Salomone, A.; Gerace, E.; Alladio, E.; Vincenti, M.; Minella, M. (2023) Wastewater surveillance of 105 pharmaceutical drugs and metabolites by means of ultra-high-performance liquid-chromatography-tandem high resolution mass spectrometry. *J. Chrom. A.* ***1693***, *463896.*

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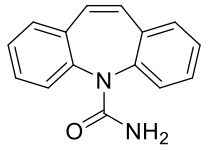
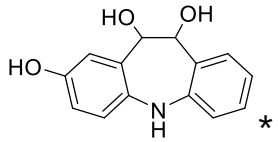
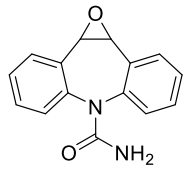
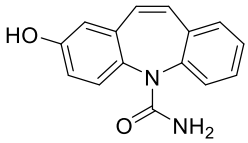
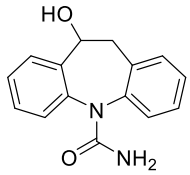
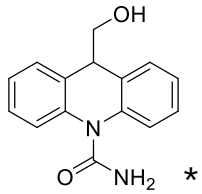
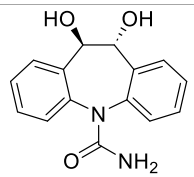
5. National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 83852, Dihydroxycarbazepine. Retrieved April 18, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Dihydroxycarbazepine.

Table 4. List of parent CBZ and proposed metabolites identified in a wastewater sample using Molecule Profiler software. Each proposed metabolite predicted by a biotransformation pathway is highlighted in orange based on identification from the molecular formula of the precursor and fragment ions, the precursor and fragment mass error (<5 ppm), the software-assigned structure, RT and % score that indicates the likelihood that the peak found is a metabolite.

Biotransformation pathway (Compound)	Molecular formula	Structure	Precursor *m/z* (Error, ppm)	Fragment *m/z* (Error, ppm)	Fragment formula	RT (min)	% Score	Found in Analytics module via suspect screening
Parent $[M+H]^+$ *(Carbamazepine)*	$C_{15}H_{12}N_2O$		*237.1020 (-0.9)*	*194.0956 (-4.2)* *179.0730 (0.3)*	$[C_{14}H_{12}N]^+$ $[C_{13}H_9N]^+$	*3.99*	*82.5*	*Yes*
Loss of CHNO+ internal hydrolysis and di-oxidation $[M+H]^+$ *(Unknown)*	$C_{14}H_{13}NO_3$	 *	*244.0976 (3.2)*	*194.0967 (1.7)* *192.0807 (-0.2)*	$[C_{14}H_{12}N]^+$ $[C_{14}H_{10}N]^+$	*4.01*	*75.7*	*No*
Oxidation $[M+H]^+$ *(Carbamazepine-10,11-epoxide)*	$C_{15}H_{12}N_2O_2$		*253.0975 (1.4)*	*180.0806 (-0.9)* *210.0909 (-2.2)*	$[C_{13}H_{10}N]^+$ $[C_{14}H_{12}NO]^+$	*2.98*	*76.8*	*Yes*
Oxidation $[M+H]^+$ *(1-hydroxycarbamazepine, 2-hydroxycarbamazepine, 3-hydroxycarbamazepine)*	$C_{15}H_{12}N_2O_2$		*253.0979 (2.9)*	*210.0916 (1.2)* *208.0756 (-0.2)*	$[C_{14}H_{12}NO]^+$ $[C_{14}H_{10}NO]^+$	*3.31*	*74.3*	*No*
Internal hydrolysis $[M+H]^+$ *(10,11-dihydro-10-hydroxycarbamazepine)*	$C_{15}H_{14}N_2O_2$		*255.1136 (3.1)*	*194.0960 (-2.4)* *192.0809 (0.8)* *237.1022 (-0.2)*	$[C_{14}H_{12}N]^+$ $[C_{14}H_{10}N]^+$ $[C_{15}H_{13}N_2O]^+$	*3.17*	*74.7*	*Yes*
Internal hydrolysis $[M+H]^+$ *(9-hydroxymethyl-10-carbamoyl acridan)*	$C_{15}H_{14}N_2O_2$	 *	*255.1134 (2.2)*	*194.0962 (-1.2)* *180.0805 (-1.3)* *238.0869 (2.6)*	$[C_{14}H_{12}N]^+$ $[C_{13}H_{10}N]^+$ $[C_{15}H_{12}NO_2]^+$	*3.52*	*76.4*	*Yes*
Oxidation and internal hydrolysis $[M+H]^+$ *(10,11-dihydro-10,11-dihydroxycarbamazepine)*	$C_{15}H_{14}N_2O_3$		*271.1081 (1.5)*	*180.0803 (-2.9)* *210.0910 (-1.7)* *236.0698 (-3.4)*	$[C_{13}H_{10}N]^+$ $[C_{14}H_{12}NO]^+$ $[C_{15}H_{10}NO_2]^+$	*2.98*	*77.6*	*Yes*

*Structure was not predicted by Molecule Profiler software. The structure was instead drawn based on manual comparison between the experimental MS/MS and published MS/MS from the literature or inferred from the proposed biotransformation pathway used to predict that metabolite.

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Targeted and non-targeted analysis of fentanyl analogs and their potential metabolites using LC-QTOF

Using the SCIEX X500B QTOF system and SCIEX OS software

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This technical note describes a high-throughput method that enabled the simultaneous quantitation and both targeted and non-targeted screening of fentanyl and its analogs in a single injection of urine samples. Using the X500B QTOF system, SWATH data-independent acquisition (DIA) collected MS/MS data for spectral matching against the SCIEX NIST 2017 MS/MS library and metabolite identification using the Molecule Profiler module of SCIEX OS software.

The Centers for Disease Control and Prevention (CDC) estimates that approximately 150 overdose deaths occur daily due to synthetic opiates, such as fentanyl.[1] The structural diversity of fentanyl complicates this public health crisis because as many as 1400 analogs are known to date.[2] Forensic toxicology laboratories must perform immunoassay tests to screen for different drug classes in urine. However, their lack of specificity often requires LC-MS/MS analysis to quantify and identify specific compounds. This combined approach is lengthy and laborious and it often excludes new designer analogs that are illicitly produced. Under these less optimal synthesis and storage conditions, the resulting product might be an analytical cocktail of the known drugs and their analogs and impurities, all of which necessitate a non-targeted approach to capture as much information as possible for confident identification.

SWATH DIA enables the acquisition of high-resolution MS/MS spectra during each cycle, resulting in the collection of a MS/MS spectrum that is a composite of all the analytes within each isolation window. The resulting spectra can be retrospectively mined for further identification. Here, common MS/MS fragments of fentanyl and their structurally similar analogs were used to search for novel analogs and biomarkers of drug exposure in urine samples using the Molecule Profiler module (Figure 1).

Key features of SWATH DIA using the X500B QTOF system and SCIEX OS software

- High-throughput workflow combining quantitation based on TOF MS peak areas and both targeted and non-targeted screening based on TOF MS/MS spectra in a single injection
- SCIEX OS software offers a single platform for data acquisition, processing and rapid review of complex data
- Molecule Profiler module delivers an intuitive workflow for identifying fentanyl and its analogs in urine samples

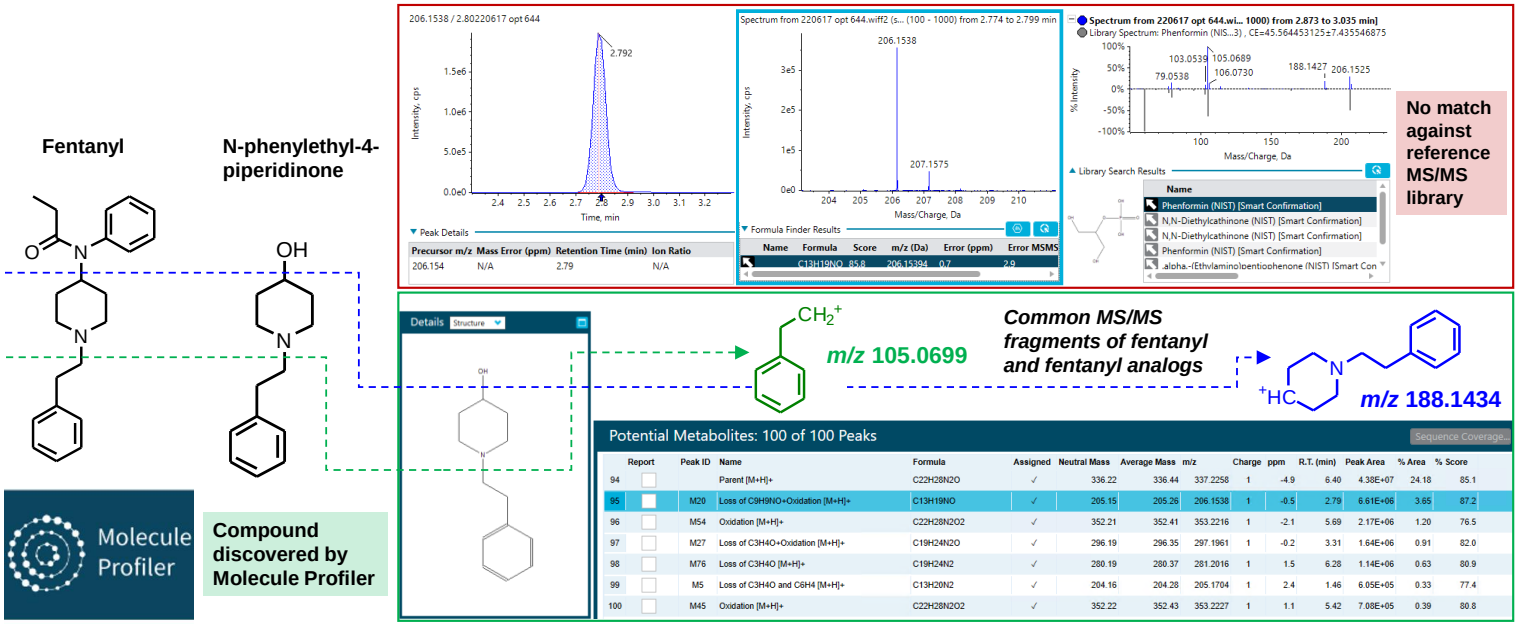

Figure 1. Discovery of *N*-phenylethyl-4-piperidinone, a novel fentanyl analog, in Molecule Profiler. Identification was accomplished by surveying TOF MS/MS data for precursors that share the same product ions at *m/z* 105.0695 and 188.1425 as fentanyl due to common fragmentation of C–N or C–O bonds in their structures. *N*-phenylethyl-4-piperidinone was not found by spectral library matching in the Analytics module of SCIEX OS software.

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Methods

Sample preparation: Frozen urine samples were thawed, vortex mixed, allowed to settle and then diluted 5-fold with 1:1 (v/v), methanol/water for LC-MS/MS analysis.

Standards for 27 fentanyl analogs, including fentanyl and non-fentanyl analog opiates, were purchased from Cerilliant. A solvent-based calibration curve was prepared in 1:1 (v/v), methanol/water, at concentrations ranging from 1 to 100 ng/mL.

Chromatography: LC separation was performed on an ExionLC system using a Phenomenex Luna Omega Polar C18 column (150 × 3.0 mm, 3.0 µm). A flow rate of 0.8 mL/min, an injection volume of 2 µL and a column temperature of 40°C were used. The 15-minute gradient used is shown in Table 1.

Table 1. Chromatographic gradient.

Time (min)	%A	%B
0.0	*90*	*10*
3.0	*82*	*18*
5.0	*58*	*42*
11.0	*53*	*47*
12.0	*35*	*65*
12.5	*5*	*95*
13.5	*5*	*95*
13.6	*90*	*10*
15.0	*90*	*10*

Mobile Phase A: *Water with 0.1% formic acid and 5mM ammonium formate*
Mobile Phase B: *Acetonitrile with 0.1% formic acid and 5mM ammonium formate*

Mass spectrometry: The X500B QTOF system was used in positive electrospray ionization mode to acquire TOF MS and TOF MS/MS data with SWATH DIA. Table 2 shows the method parameters used for the mass spectrometer. The SWATH DIA method consisted of 8 windows, each 35 Da wide, over a start and stop precursor mass range of 200 to 450 Da.

Data processing: All data were acquired and processed using SCIEX OS software, version 2.2. The SCIEX NIST 2017 MS/MS library was used to perform library searching in the Analytics module of SCIEX OS software. Screening for drug impurities, fentanyl analogs and candidate metabolites was performed using the Molecule Profiler module of SCIEX OS software. Candidate precursors were surveyed in the TOF MS/MS data based on the common fragments at *m/z* 188.1434 and 105.0699 for fentanyl and fentanyl analogs. Figure 2 shows the overall workflow.

Table 2. MS and MS/MS conditions.

Parameter	MS	MS/MS
Polarity	*Positive*	
Ion spray voltage	*2500 V*	
Ion source gas 1 (GS1)	*50 psi*	
Ion source gas 2 (GS2)	*50 psi*	
Curtain gas (CUR)	*35 psi*	
Collision gas (CAD)	*9*	
Source temperature (TEM)	*550°C*	
Declustering potential (DP)	*80 V*	
Total scan time	*0.731 s*	
Scan mode	*TOF MS*	*SWATH DIA*
Start/stop mass range	*100 – 1000 Da*	*50 – 1000 Da*
Accumulation time	*0.25 s*	*0.05 s*
Collision energy (CE)	*10 V*	*35 V*
Collision energy spread (CES)	*0 V*	*15 V*

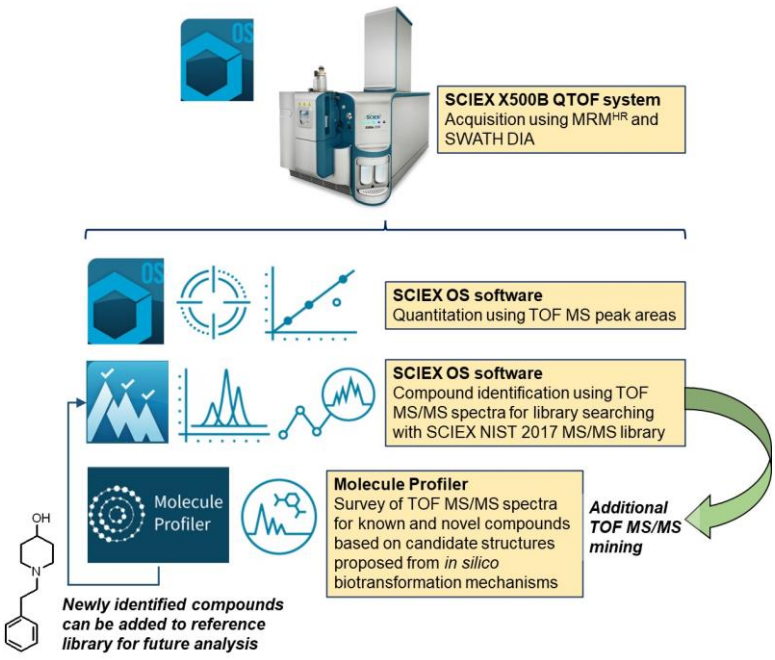

Figure 2. Targeted and non-targeted acquisition and data analysis approach using a streamlined workflow in SCIEX OS software. The Analytics module of SCIEX OS software was used for quantitation and spectral library matching against the SCIEX NIST 2017 MS/MS library. The Molecule Profiler module of SCIEX OS software was used for impurity and metabolite identification.

Sample Na...	Comp... Name	Precu... Mass	Ret... Time	Area	Calculated Concent...	*Conc in urine ng/mL	Accuracy	*Regre... linearity	Mass Error...	Libr ... Score	Library Hit	RT Confi...	Mass Error...	Isotope Confi...	Library Confi...
1 ng/mL std	Fentanyl...	337.227	6.40	5.810e4	0.95		95.26	0.9995	0.1	97.3	Fentanyl (NIST) [Smart Conf...	✓	✓	✓	✓
2.5 ng/mL std	Fentanyl...	337.227	6.39	1.388e5	2.46		98.34	0.9995	0.6	93.9	Fentanyl (NIST) [Smart Conf...	✓	✓	✓	✓
5 ng/mL std	Fentanyl...	337.227	6.39	2.971e5	5.41		108.22	0.9995	-0.1	94.5	Fentanyl (NIST) [Smart Conf...	✓	✓	✓	✓
10 ng/mL std	Fentanyl...	337.227	6.40	5.420e5	9.98		99.79	0.9995	-0.8	90.1	Fentanyl (NIST) [Smart Conf...	✓	✓	✓	✓
25 ng/mL std	Fentanyl...	337.227	6.40	1.307e6	24.24		96.97	0.9995	-1.5	84.8	Fentanyl (NIST) [Smart Conf...	✓	✓	✓	✓
50 ng/mL std	Fentanyl...	337.227	6.39	2.739e6	50.96		101.92	0.9995	-0.6	76.8	Fentanyl (NIST) [Smart Conf...	✓	✓	✓	✓
100 ng/mL std	Fentanyl...	337.227	6.40	5.341e6	99.50		99.50	0.9995	-0.2	88.3	Fentanyl (NIST) [Smart Conf...	✓	✓	✓	✓
Urine sample 2	Fentanyl...	337.227	6.39	2.524e6	46.95	234.746	N/A	0.9995	-0.8	89.5	Fentanyl (NIST) [Smart Conf...	✓	✓	✓	✓
Urine sample 3	Fentanyl...	337.227	6.40	6.231e6	116.11	580.556	N/A	0.9995	-0.9	81.5	Fentanyl (NIST) [Smart Conf...	✓	✓	✓	✓

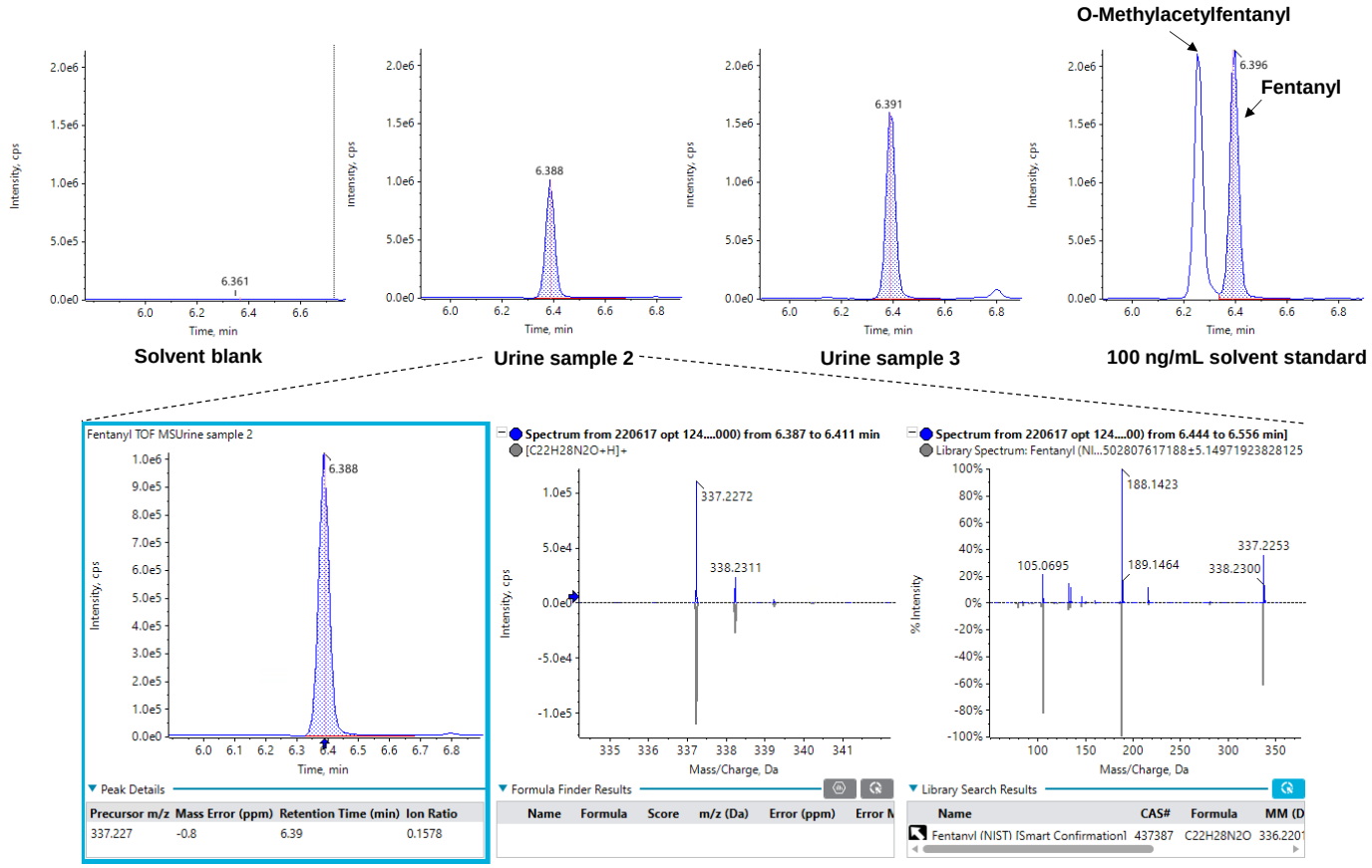

Figure 3. Quantitative and qualitative results in SCIEX OS software. The top table shows the calculations of accuracy and concentration based on the TOF MS peak area of fentanyl and the qualitative points of compound identification, such as retention time error, mass error, isotope ratio and library hits, in a solvent standard and 2 urine samples. The middle row of plots compares the TOF MS XICs of fentanyl in the solvent blank, 2 urine samples and the 100 ng/mL standard. The bottom row of plots shows the same XIC for urine sample #2 and its TOF MS and TOF MS/MS spectra and library search results.

Preliminary analysis of fentanyl and analog composition in urine samples

Urine samples were pre-screened to determine the composition of fentanyl, fentanyl analogs and other drug compounds using a solvent-based calibration to estimate their relative concentrations in each sample. Quantitation was performed based on the TOF MS peak areas. Fentanyl and acetyl norfentanyl were detected in most urine samples at approximately 8–4300 ng/mL and 26–470 ng/mL, respectively, while most other analytes were not observed. Figure 3 shows the results table containing the regression quality and concentrations of fentanyl in 2 urine samples, in addition to their extracted ion chromatograms (XICs) compared against a solvent blank and a standard. The calculated columns feature in SCIEX OS software was used to calculate the original concentrations in the urine samples based on the in-vial concentrations and 5-fold dilution from the dilute-and-shoot protocol. Out-of-bound results were flagged, including, for example, the in-vial concentration of urine sample #3 that exceeded the upper limit of quantitation and would require dilution for re-analysis. The relative distribution of drugs and their estimated concentrations reported here informed the selection of representative urine samples for the subsequent analysis using library matching and Molecule Profiler.

Library matching

The TOF MS/MS data acquired for all urine samples were compared against the SCIEX NIST 2017 MS/MS library using the unknown screening workflow to confirm the known presence of fentanyl and its analogs from the pre-screening and to screen for other drug candidates, metabolites and impurities. By sorting on peak areas and library scores, a shortlist of candidates was used to build a new processing method using their known formula and the $[M+H]^+$ adduct. The new processing method was then used to re-process the data with additional flagging for mass error and isotope ratio to bolster the confidence in any authentic findings.

By defining confidence thresholds on retention time (RT) error, mass error, isotopic ratio matches and library hits, compound identification was rapidly achieved based on data that were flagged and filtered to pass pre-defined thresholds (Figure 3). Table 3 lists representative library findings that exhibited RT error of ≤5%, TOF MS mass error of ≤5 ppm, reasonable isotopic ratio matches and library scores of ≥75%. Most of the urine samples revealed the presence of other drug classes beyond fentanyl and fentanyl analogs, such as cocaine and opioids. Some of these samples were further interrogated with the Molecule Profiler module to corroborate the predicted compounds against the library results and identify additional biomarkers of drug use that were missed by library searching.

Detection of novel fentanyl analogs using Molecule Profiler module in SCIEX OS

The Molecule Profiler module of SCIEX OS software was used to search the dataset for potential precursors that shared the common fentanyl fragments, m/z 105.0695 and m/z 188.1425 (Figure 4). This analysis assumed that additional, novel fentanyl compounds also shared these common fragments. As shown in Figure 5, the Molecule Profiler module produced a list of proposed candidates that included fentanyl, fentanyl analogs, metabolites and impurities based on *in silico* biotransformation mechanisms in the software. For example, structural prediction of the selected peak at *m/z* 281.2016 corresponded to despropionylfentanyl (or 4-aminophenyl-1-phenethylpiperidine (4-ANPP)), which is often present as an impurity in drugs containing fentanyl and other analogs because it is a synthetic precursor of fentanyl. The identity of this compound was further corroborated by the similar RT, isotopic distribution and library hit observed in the Analytics module of SCIEX OS software.

In 3 urine samples, the Molecule Profiler module found a fentanyl analog with an *m/z* gain of 17.99 compared to the parent fentanyl, which corresponded to the replacement of hydrogen with fluorine (Table 3). By examining the same precursor *m/z* and retention time in the Analytics module of SCIEX OS software, this compound was confirmed to be *o*-fluorofentanyl based on the isotopic distribution and library hit.

The Molecule Profiler module also identified several novel fentanyl biomarkers that were not found during library searching due to their absence from the SCIEX NIST 2017 MS/MS library (Figure 6). An oxidation product of fentanyl with *m/z* 353.2224 was consistently observed in many of the urine samples at 2 retention times (5.41 and 7.11 minutes). Using the structural *.mol file saved in Molecule Profiler to search on PubChem, these 2 biomarkers were identified as ω-hydroxy fentanyl and β-hydroxyfentanyl. Both compounds were listed as "fentanyl-related substances with no known legitimate uses" in the International Narcotics Control Board (INCB).[3] β-hydroxyfentanyl

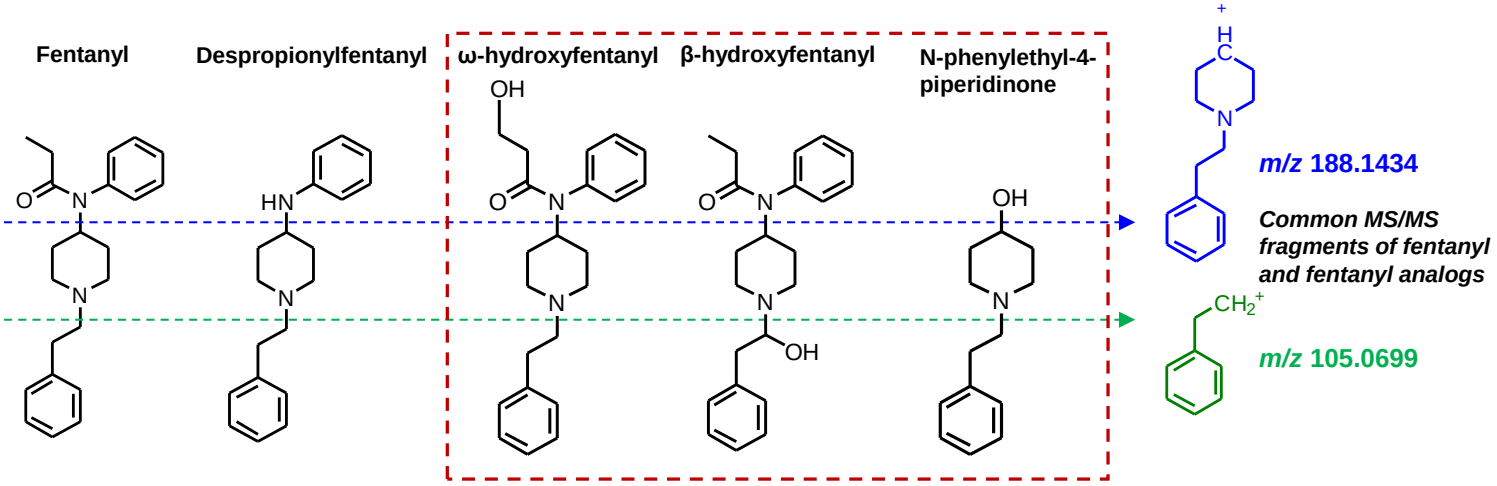

Figure 4. Fragmentation of representative fentanyl analogs and impurities. Fentanyl, despropionylfentanyl, ω-hydroxy fentanyl, β-hydroxyfentanyl and *N*-phenylethyl-4-piperidinone all fragment through C–N or C–O bonds to common fragments with *m/z* 105.0699 and *m/z* 188.1434. Compounds highlighted in red box were uniquely discovered in the Molecule Profiler module of SCIEX OS software.

is a fentanyl analog and was sold illicitly before being listed on the US Drug Enforcement Administration (DEA) National Forensic Laboratory Information System (NFLIS) drug substance list,[4] while ω-hydroxy fentanyl is a known metabolite of fentanyl.[5] Only β-hydroxyfentanyl was identified by a ChemSpider search on its molecular formula.

Another novel biomarker identified was *N*-phenylethyl-4-piperidinone (NPP)) (Figure 6), a compound also listed in the NFLIS drug substance list.[4] Like 4-ANPP, NPP can be used to synthesize fentanyl and thus might be present as a drug impurity.

Here, the Molecule Profiler module of SCIEX OS software provided an alternative way of processing TOF MS/MS data that enabled the discovery of novel compounds, which could be retroactively added to a reference library to improve future spectral matching, as shown in Figure 2.

Conclusions

- SWATH DIA of MS/MS spectra for all analytes during each cycle enables retrospective mining of previously acquired data for new substances that have emerged on the recreational drug market, without the need to re-acquire samples
- Simultaneous quantitation and library searching against >15,000 spectra from the SCIEX NIST 2017 MS/MS library
- Discovery of novel fentanyl biomarkers in the Molecule Profiler module of SCIEX OS software using common fragments of fentanyl and other analogs to screen for additional candidate precursors
- Integration of the Molecule Profiler software with SCIEX OS software enabled streamlined workflow for corroborating fentanyl analog and metabolite identification results against those found by targeted and non-targeted screening in the Analytics module of SCIEX OS software

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Table 3. List of proposed putative metabolites, analogs and impurities identified in the Molecule Profiler module of SCIEX OS software (left) and candidate drug and drug metabolites identified by library searching in the Analytics module of SCIEX OS software (right). From Molecule Profiler, potential candidate metabolites, analogs and impurities with significant peak areas and expected distribution of common fragments at *m/z* 105.0695 and 188.1425 are listed on the left. From Analytics, representative library results of drug and drug metabolites with passing mass accuracy, isotope ratios and library scores are listed on the right.

Putative fentanyl metabolites, analogs and impurities identified in Molecule Profiler					SCIEX NIST 2017 MS/MS library hits	
Sample	***m/z***	**RT**	**Area**	**Predicted biotransformation**	**Compound (purity score)**	**Applicable component class**
126	*367.2023*	*5.33*	*9.03e4*	*Demethylation to COOH*	*Benzoylecgonine (97.7)* *Quinine (91.9)*	*Cocaine metabolite* *Antimalarial*
128	*205.1701* *353.2228* *353.2227* *281.2020*	*1.47* *5.42* *5.51* *6.28*	*6.06e5* *9.83e4* *2.10e5* *1.99e5*	*Loss of C_3H_4O and C_6H_4* *Oxidation* *Oxidation* *Loss of C_3H_4O*	*Despropionylfentanyl (100)* *p-Hydroxybenzoylecgonine (98.6)* *Cocaine (97.0)* *Benzoylnorecgonine (95.5)* *m-Hydroxycocaine (86.4)*	*Fentanyl metabolite* *Cocaine metabolite* *Stimulant* *Cocaine metabolite* *Cocaine metabolite*
130	*353.2227* *337.1926*	*5.50* *6.40*	*1.58e5* *4.83e4*	*Oxidation* *Demethylation and methylene to ketone*	*Trazodone (97.9)* *Cyclobenzaprine (94.8)* *Hydrocodone (91.9)* *Norhydrocodone (94.6)*	*Serotonin modulator* *Muscle relaxant* *Opiate* *Hydrocodone metabolite*
633	*ND*	*ND*	*ND*	*ND*	*Ropinirole (98.9)* *Trazodone (96.0)* *Oxycodone (94.9)* *Noroxycodone (93.6)*	*Dopamine promotor* *Serotonin modulator* *Opiate* *Oxycodone metabolite*
634	*353.2228* *353.2217* *355.2182** *281.2013*	*5.43* *5.52* *6.57* *6.29*	*1.72e5* *1.51e6* *1.62e6* *1.88e6*	*Oxidation* *Oxidation* *Gain of 17.9908* *Loss of C_3H_4O*	*Despropionylfentanyl (99.6)* *Fluorofentanyl (95.4)*	*Fentanyl metabolite* *Fentanyl analog*
636	*205.1701* *353.2226* *355.2187**	*1.47* *5.51* *6.56*	*1.16e5* *2.40e5* *2.10e5*	*Loss of C_3H_4O and C_6H_4* *Oxidation* *Gain of 17.9913*	*Benzoylecgonine (98.4)* *Levamisole (91.1)* *Fluorofentanyl (89.7)*	*Cocaine metabolite* *Cocaine cutting agent* *Fentanyl analog*
637	*297.1960* *281.2015*	*3.30* *6.26*	*6.78e5* *5.27e5*	*Loss of C_3H_4O + oxidation* *Loss of C_3H_4O*	*Despropionylfentanyl (100)*	*Fentanyl metabolite*
638	*281.2011* *353.2218* *377.1534* *353.2226*	*6.28* *5.50* *4.41* *5.42*	*3.64e6* *1.26e6* *6.88e5* *2.66e5*	*Loss of C_3H_4O* *Oxidation* *Gain of 39.9260* *Oxidation*	*Despropionylfentanyl (96.5)* *Oxycodone (81.8)* *Noroxycodone (95.9)*	*Fentanyl metabolite* *Opiate* *Oxycodone metabolite*
639	*205.1701* *353.2227* *353.2227*	*1.48* *5.52* *5.43*	*1.92e5* *1.64e5* *1.18e5*	*Loss of C_3H_4O and C_6H_4* *Oxidation* *Oxidation*	*Quinine (93.6)*	*Antimalarial*
644	*353.2216* *206.1538*** *355.2190**	*5.69* *2.79* *6.56*	*2.17e6* *6.61e6* *4.07e5*	*Oxidation* *Loss of C_9H_9NO + oxidation* *Gain of 17.9916*	*Despropionylfentanyl (100)* *Acetyl norfentanyl (77.8)* *Fluorofentanyl (81.2)*	*Fentanyl metabolite* *Acetyl fentanyl metabolite* *Fentanyl analog*
647	*297.1961*	*3.31*	*4.96e5*	*Loss of C_3H_4O + oxidation*	*Despropionylfentanyl (100)*	*Fentanyl metabolite*
1092	*353.2224*	*5.50*	*5.29e5*	*Oxidation*	*Xylazine (96.6)*	*Veterinarian sedative*

ND = Not detected
*Fluorinated fentanyl analog with *m/z* gain of 17.9908, which corresponded to the replacement of H with F
**Fentanyl impurity with *m/z* 206.1538

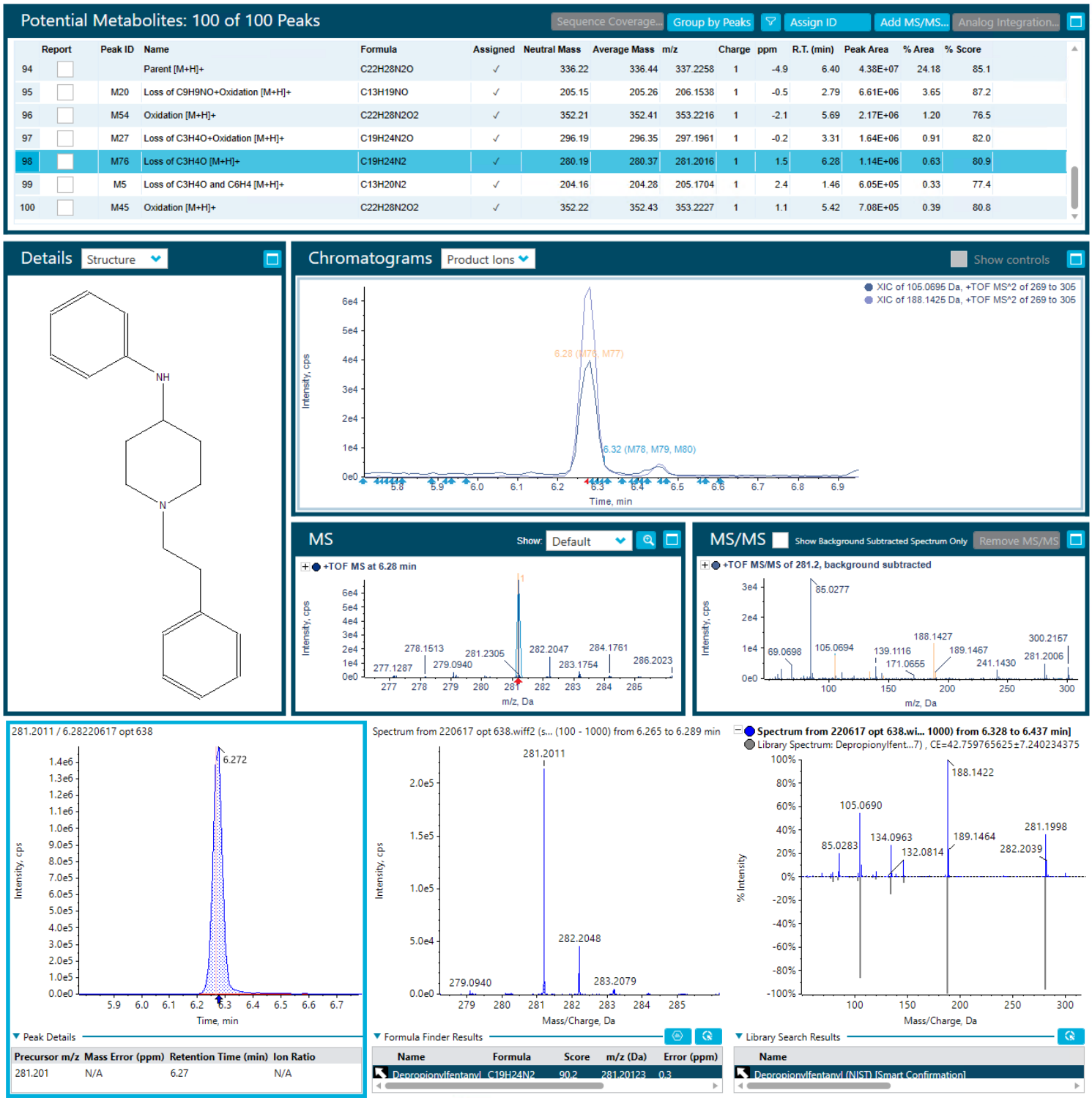

Figure 5. Putative metabolites, analogs and impurities with the same product ions at *m/z* 105.0695 and 188.1425 as parent fentanyl found in the Molecule Profiler module of SCIEX OS software (top) and library search results for 1 proposed candidate in the Analytics module of SCIEX OS software (bottom). In this example, the selected peak found by Molecule Profiler corresponded to despropionylfentanyl (or 4-aminophenyl-1-phenethylpiperidine (4-ANPP)) based on the predicted structure, retention time, TOF MS XIC extracted from the 2 common fragments (*m/z* 105.0695 and 188.1425) and TOF MS/MS spectra (top). Identification of this compound was corroborated by the corresponding library hit found at a similar retention time in the TOF MS XIC, isotopic distribution and TOF MS/MS spectra in the Analytics module of SCIEX OS software (bottom).

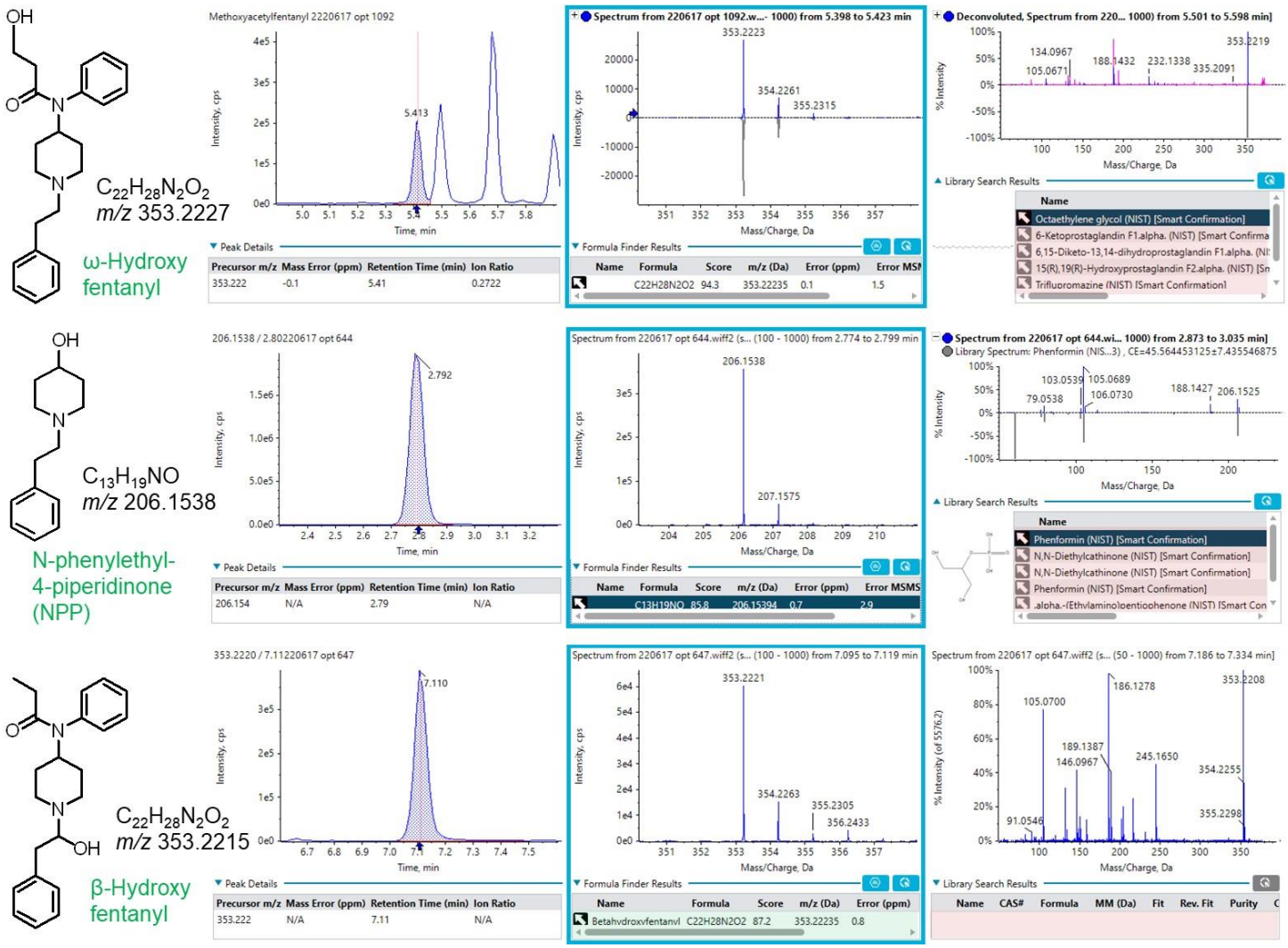

Figure 6. Fentanyl biomarkers found in the Molecule Profiler module of SCIEX OS software with corresponding incorrect or missing library results in the Analytics module of SCIEX OS software. The predicted structures of the 3 fentanyl biomarkers were identified by PubChem and literature search. Only β-hydroxyfentanyl was identified as the 7th-ranked hit with a score of 87.2 with a ChemSpider search on its molecular formula (shaded green). None of the 3 fentanyl biomarkers were correctly identified by library searching (shaded pink) due to their absence from the SCIEX NIST 2017 MS/MS library.

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Expand your NPS screening capabilities using the vMethod application for forensic toxicology screening

The vMethod application provides a comprehensive solution for new psychoactive substances (NPS) screening, including:

- Sample preparation procedures for both human whole blood and urine
- Detailed LC conditions
- MS/MS detection methods
- Robust data processing tailored to deliver concise and confident results

The vMethod application was recently updated with **130 of the newest and most prevalent NPS** on the recreational drug market

This new update to the vMethod application enables laboratories to monitor and accurately identify 900 compounds in biological matrices in a single injection

The vMethod application is compatible with the X500R QTOF system and the ZenoTOF 7600 system, **the SCIEX accurate mass systems that use SCIEX OS software for data acquisition and processing.**
These systems are best used in combination with the Forensic HR-MS/MS spectral library for confident spectral library matching.

Comprehensive MS/MS detection methods

The 2 non-targeted data acquisition methods—data dependent acquisition (DDA) and data independent acquisition (DIA) with SWATH DIA—enable you to:

- Generate comprehensive and high-quality MS/MS spectra
- Create a digital archive of the NPS present in the biological samples at the time of sample collection

Straightforward sample preparation

The detailed sample preparation procedures for both human whole blood and urine enable quick and efficient NPS extraction without compromise in analyte recovery.

Robust data processing

The processing method uses stringent confidence criteria for confident analyte identification, including compound fragmentation comparison to library spectra. Targeted data processing is accomplished in SCIEX OS software using the components tab, which includes the name, molecular formula, precursor mass and retention time of each of the 130 NPS included in this panel. This allows datasets to be re-processed when newly identified forensic targets are discovered without the need to re-inject samples.

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